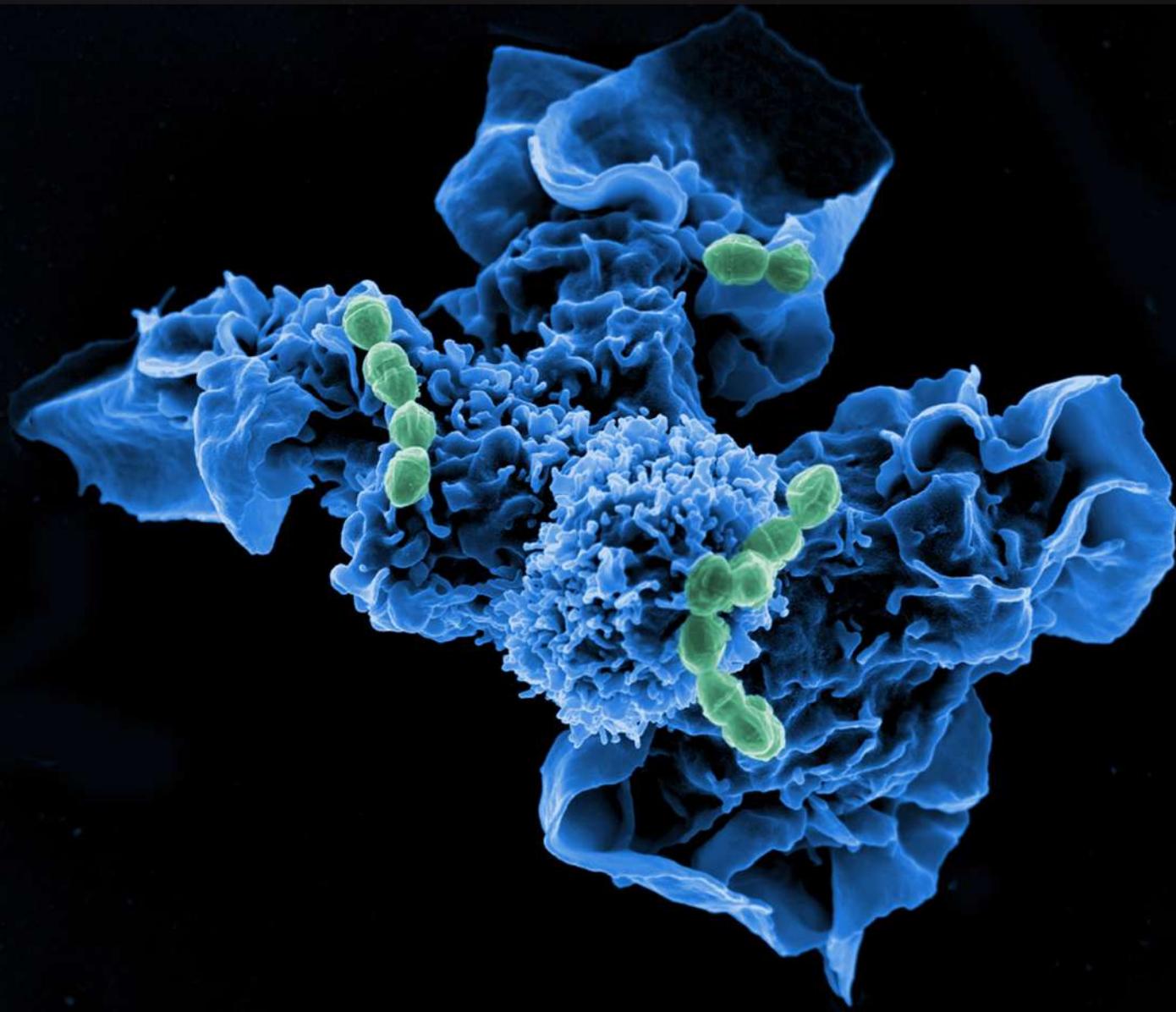
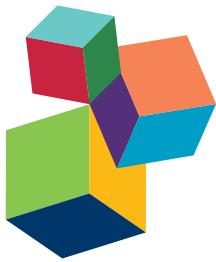


BACTERIAL EXOTOXINS: HOW BACTERIA FIGHT THE IMMUNE SYSTEM

EDITED BY: Inka Sastalla, Denise M. Monack and Katharina F. Kubatzky

PUBLISHED IN: Frontiers in Immunology & Frontiers in Microbiology





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ISSN 1664-8714

ISBN 978-2-88919-991-4

DOI 10.3389/978-2-88919-991-4

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BACTERIAL EXOTOXINS: HOW BACTERIA FIGHT THE IMMUNE SYSTEM

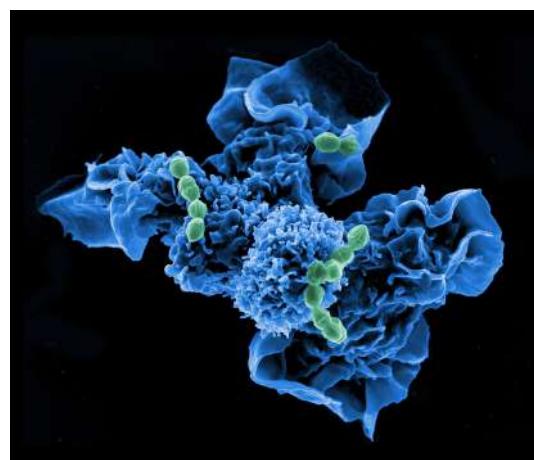
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Murine bone marrow-derived mast cell with *Enterococcus faecalis*. Scanning electron microscopy picture was kindly provided by Dr. Manfred Rohde (HZI Braunschweig).

Bacterial pathogenicity factors are functionally diverse. They may facilitate the adhesion and colonization of bacteria, influence the host immune response, assist spreading of the bacterium by e.g. evading recognition by immune cells, or allow bacteria to dwell within protected niches inside the eukaryotic cell.

Exotoxins can be single polypeptides or heteromeric protein complexes that act on different parts of the cells. At the cell surface, they may insert into the membrane to cause damage; bind to receptors to initiate their uptake; or facilitate the interaction with other cell types. For example, bacterial superantigens specifically bind to major histocompatibility complex (MHC) II molecules on the surface of antigen

presenting cells and the T cell receptor, while cytolsins cause pore formation. For intracellular activity, exotoxins need to be translocated across the eukaryotic membrane. Gram-negative bacteria can directly inject effector proteins in a receptor-independent manner by use of specialized needle apparatus such as bacterial type II, III, or type IV secretion systems. Other methods of translocation include the phagocytic uptake of bacteria followed by toxin secretion, or receptor-mediated endocytosis which allows the targeting of distinct cell types. Receptor-based uptake is initiated by the binding of heteromeric toxin complexes to the cell surface and completed by the translocation of the effector protein(s) across the endosomal membrane.

In the cytosol, toxins interact with specific eukaryotic target proteins to cause post-translational modifications that often result in the manipulation of cellular signalling cascades and inflammatory responses.

It has become evident that the actions of some bacterial toxins may exceed their originally assumed cytotoxic function. For example, pore-forming toxins do not only cause cytolysis, but may also induce autophagy, pyroptosis, or activation of the MAPK pathways, resulting in adjustment of the host immune response to infection and modification of inflammatory responses both locally and systemically. Other recently elucidated examples of the immunomodulatory function of cell death-inducing exotoxins include TcdB of *Clostridium difficile* which activates the inflammasome through modification of cellular Rho GTPases, or the *Staphylococcus* δ-toxin which activates mast cells.

The goal of this research topic was to gather current knowledge on the interaction of bacterial exotoxins and effector proteins with the host immune system. The following 16 research and review articles in this special issue describe mechanisms of immune modification and evasion and provide an overview over the complexity of bacterial toxin interaction with different cells of the immune system.

Citation: Sastalla, I., Monack, D. M., Kubatzky, K. F., eds. (2016). *Bacterial Exotoxins: How Bacteria Fight the Immune System*. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-991-4

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Editorial: Bacterial Exotoxins: How Bacteria Fight the Immune System

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Keywords: bacterial toxins, immune evasion, infectious diseases, innate immune response, adaptive immunity, super antigen, bacterial effector protein, quorum sensing

The Editorial on the Research Topic

Bacterial Exotoxins: How Bacteria Fight the Immune System

Upon infection with a bacterial pathogen, the body initiates both innate and adaptive immune responses with the ultimate goal to eliminate the invader and to return to homeostasis. Occasionally, however, the body may react inadequately, resulting in collateral damage to tissues if the response is too strong or in a failure to eradicate the pathogen if the response is too weak or ephemeral. Functionally diverse toxins released by bacteria during infection can contribute considerably to the outcomes of the immune response. For example, bacterial toxins may mediate bacterial evasion of immune recognition, facilitate dwelling within protected niches of eukaryotic cells, or modulate pro-inflammatory responses. Furthermore, in recent years, it has become evident that beyond their canonical actions, bacterial toxins may initiate other cellular responses. For example, besides inducing cytolysis, pore-forming toxins may also induce autophagy, pyroptosis, or activation of the MAPK pathways, resulting in adjustment of the host immune response to infection and modification of inflammatory responses both locally and systemically (1, 2).

Exotoxins can be single polypeptides or heteromeric protein complexes that act on different parts of the cells. At the cell surface, they may insert into the membrane to cause damage, bind to receptors to initiate their uptake, or facilitate interactions with other cell types. For intracellular activity, exotoxins need to be translocated across the eukaryotic membrane. Gram-negative bacteria can directly inject effector proteins in a receptor-independent manner by use of specialized needle apparatus such as bacterial type II, type III, or type IV secretion systems. Other methods of translocation include the phagocytic uptake of bacteria followed by toxin secretion and receptor-mediated endocytosis. Receptor-based uptake allows the targeting of distinct cell types uniquely expressing the receptor. It is initiated by the binding of heteromeric toxin complexes to cell surfaces and is completed by the translocation of the effector proteins across the endosomal membrane. Once in the cytosol, toxins interact with specific eukaryotic target proteins to cause post-translational modifications of host proteins that often result in the manipulation of cellular signaling cascades and inflammatory responses (3).

The intention of this special issue on bacterial exotoxins is to gather current knowledge on the interaction of these versatile effector proteins with the host immune system and to describe mechanisms of immune modification and evasion. We thank the authors of the following 16 articles for providing diverse overviews, comprehensive reviews, and intriguing new data regarding the effects on, and interactions with, three important groups of immune function: (1) barrier cells such as fibroblasts, and epithelial and endothelial cells that are responsible for mediating local immune responses, (2) innate phagocytic cells, and (3) cells of the adaptive arm of immunity.

OPEN ACCESS

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Immunology

Received: 22 June 2016

Accepted: 25 July 2016

Published: 02 August 2016

Citation:

Sastalla I, Monack DM and Kubatzky KF (2016) Editorial:
Bacterial Exotoxins: How Bacteria Fight the Immune System.
Front. Immunol. 7:300.
doi: 10.3389/fimmu.2016.00300

The first group of manuscripts addresses the interaction of bacterial toxins of both Gram-positive and Gram-negative bacteria with initial barrier cells. Mayer et al. explore the effects of Shiga Toxin (Stx) expressed by enterohemorrhagic *E. coli* on renal endothelial and epithelial cells. In the kidneys, Stx causes hemolytic uremic syndrome, which can result in renal failure. The authors found that Stx-induced damage of renal cells stimulates the release of host-derived damage-associated molecular patterns (DAMPs), such as histones or high-mobility group protein B1 (HMGB1), and that these contribute to the severity of the disease. Ashida et al. review how *Shigella*, a pathogen of the intestinal tract that causes dysentery, uses effector proteins that are injected into host target cells via a type-III secretion system to induce cell death, to modulate protein trafficking and signaling, and to ultimately interfere with both innate and adaptive immunity. Moreover, the poultry pathogen *Salmonella pullorum* expresses an iron-storage protein called bacterioferritin that appears to be a major antigen for the chicken humoral response to *S. pullorum* infection (Xu et al.). Using a chicken fibroblast cell line, the authors furthermore identify the induction of the type I interferon IFN- β as a major consequence of bacterioferritin exposure. The last article on the effects of toxin on host barrier function is by von Hoven et al. who studied the pore-forming α -toxin of *Staphylococcus aureus*. Using mouse embryonic fibroblasts, the authors show that stress-induced basal phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2 α) results in tolerance to the toxin. Macrophages, however, are tolerant to α -toxin independent of such a stress response, underlining the fact that various host cell types can mediate different layers of tolerance and protection.

Phagocytes, such as macrophages, neutrophils, and dendritic cells, provide an immediate response and protection during the early stages of bacterial invasion. Therefore, many bacterial virulence factors and toxins specifically target these innate immune cells. Five articles in this special issue review multiple aspects of macrophage-toxin interactions. While do Vale et al. give a comprehensive overview on how bacteria use toxins as weapons to avoid recognition by phagocytes, Barth et al. focus on the effects of the toxins C3bot of *Clostridium botulinum* and C3lim of *Clostridium limosum*, which exclusively target monocytes and macrophages to ADP-ribosylate cellular Rho GTPases. Because of their cell specificity, the authors propose that engineered inactive variants of these two toxins may potentially be useful as both therapeutic and molecular tools. Greaney et al. review the various interactions between bacterial exotoxins and the inflammasomes, which are intracellular sensors of danger- and pathogen-associated molecular patterns and key to the elicitation of immune responses to a number of pathogens. An additional review by Simon and Hilbi describes how the approximately 300 effector proteins of *Legionella pneumophila*, the causative agent of legionellosis, help to establish a bacterial replication niche in alveolar macrophages. Finally, Zhang et al. investigate the effect of a previously uncharacterized protein of *Streptococcus suis* that signals via activation of TLR2, resulting in the production pro-inflammatory cytokines, such as IL-1, MCP-1, and TNF- α .

Neutrophils are highly motile phagocytes that, in addition to the phagocytic uptake and killing of bacteria, can form neutrophil extracellular traps (NETs) to destroy bacteria in a phagocytosis-independent manner. In a comprehensive mini-review, von Köckritz-Blickwede et al. give an overview on the modulation of NET formation by microbial virulence factors. Similarly, Uchiyama et al. describe how the pore-forming toxin streptolysin O of *Streptococcus pyogenes* manipulates neutrophil activity and renders the bacterium resistant to neutrophil killing by impairing NET formation, extracellular killing, and the oxidative burst reaction of neutrophils. Maurer et al. describe the interesting capacity of a bitter receptor expressed on neutrophils to recognize bacterial quorum sensing (QS) molecules that bacteria employ for bacterial communication. However, the effect of QS molecules on host cell signaling is not restricted to neutrophils; Liu et al. review how secreted QS molecules of *Pseudomonas aeruginosa* modulate and functionally impair host cells.

Finally, various authors address the interactions of toxins with cells of the adaptive immune system. Bacterial superantigens simultaneously bind to the T cell receptor and to the MHC class II molecule on the surface of antigen-presenting cells (APCs) in a non-specific, antigen-independent manner. The crosslinking causes a massive activation of T cells, ultimately resulting in anergy and the activation of regulatory T cells (Tregs). Krakauer et al. review our current knowledge of danger signaling pathways in T cells that are induced by the superantigen staphylococcal enterotoxin B (SEB) of *S. aureus*, and they propose appealing strategies of how these pathways may be exploited to discover novel drug targets. Sähr et al. investigated whether superantigen-triggered signaling cascades in APCs could provide a microenvironment that facilitates the differentiation of Tregs. However, some toxins can also manipulate T cell differentiation directly and in the absence of an APC. Hildebrand et al. show that the *Pasteurella multocida* Toxin (PMT) can modulate T cell signaling through the activation of specific transcription factors, ultimately resulting in the differentiation of naive T cells into Th17.

In summary, the articles in this special issue highlight the complexity of bacterial toxin interaction with different aspects and cells of the immune system. In particular, it becomes apparent that bacteria have developed intricate means to modify immune cell functions in a precise, strain-specific, and targeted manner, emphasizing the fact that during bacterial infections completely different strategies are used by the invading pathogen to escape the immune system. In light of the increasing occurrences of antibiotic resistances in bacteria observed worldwide, research in recent years has therefore focused on more targeted solutions to fight and prevent infections, such as enhancing host immune functions. A more detailed understanding of how bacteria manipulate the immune system may help to better understand the disease and to ultimately find better therapeutic treatments.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was in part supported by the Intramural Research Program of National Institute of Allergy and Infectious Diseases

(NIAID) at the National Institutes of Health (NIH), Bethesda, MD, USA (IS), a grant from the Deutsche Forschungsgemeinschaft, project SPP1468 Immunobone (KK), and by the NIH, grant AI095396 (DM).

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Pro-coagulant endothelial dysfunction results from EHEC Shiga toxins and host damage-associated molecular patterns

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Hemolytic uremic syndrome (HUS) from enterohemorrhagic *Escherichia coli* infection is a leading cause of kidney failure in otherwise healthy U.S. children. The bacterial Shiga toxins (Stx) induce the characteristic coagulopathy of HUS, but the damage to toxin-receptor expressing cells and organ injury due to ischemia likely also releases inflammatory damage-associated molecular patterns (DAMPs), which may exacerbate injury along with the toxins. To examine this, human aortic and renal glomerular cell anti-coagulant and barrier functions were studied after *in vitro* challenge with Stx1, Stx2, and DAMPs. There was significant loss of surface anti-coagulant protein C pathway molecules, increased expression of pro-thrombotic PAR1 and reduced protein C activation capability by 15–27%. Histones nearly completely prevented the activated protein C protection of endothelial cells from thrombin-induced permeability. In mice, lethal Stx2 challenge elevated plasma HMGB1 (day 2, $321 \pm 118\%$; $p < 0.01$) and extracellular histones (day 3, $158 \pm 62\%$; $p < 0.01$). Mice colonized with Stx2-expressing *Citrobacter rodentium* developed increased HMGB1 (day 5, $155 \pm 55\%$; $p < 0.01$) and histones (day 3, $378 \pm 188\%$; $p < 0.01$). Anti-histone antibody reduced both DAMPs to baseline, but was not sufficient to improve survival outcome or kidney function. Together, these data suggest a potential role Stx to produce DAMPs, and DAMPs to produce endothelial injury and a pro-thrombotic environment.

Keywords: hemolytic uremic syndrome, thrombotic microangiopathy, Shiga toxin 1, Shiga toxin 2, protein C, damage-associated molecular patterns, endothelial cells

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) are toxicigenic intestinal bacteria that cause vomiting, diarrhea, edema, and hemorrhagic colitis. In some patients, the disease can progress to a potentially life-threatening syndrome known as diarrhea-associated hemolytic uremic syndrome (D+HUS), characterized by thrombotic microangiopathy, thrombocytopenia, and hemolytic anemia, all of which contribute to acute kidney injury (1). In the U.S., D+HUS is a leading cause of acute kidney failure in otherwise healthy children (2). EHEC produce and secrete Shiga toxin 1 (Stx1), Shiga toxin 2 (Stx2), or both, and serotypes that secrete Stx2 are associated with more clinically severe disease (3). Much of the pathogenesis observed during EHEC infection is attributed to the toxins, which are considered primary virulence factors of EHEC. The toxins bind to globotriaosylceramide (Gb_3 , CD77) receptors whose distribution is particularly high on renal glomerular endothelial cells in humans and on renal tubular epithelium in mice (4–6). The toxins are then internalized and transported to the endoplasmic reticulum, and the A subunit is activated to generate RNA N-glycosidase activity (7). Within the cytosol, the active A subunit cleaves an adenine residue from 28S ribosomal RNA, which prevents protein synthesis (8–10).

and initiates ribotoxic (11, 12) and endoplasmic reticular-stress responses (13), leading to apoptosis and inflammation.

A major clinical feature of D+HUS is thrombotic microangiopathy, in which clots form inappropriately in the microvasculature, resulting in ischemic consequences and organ injury. As endothelial cells are crucial for maintaining blood fluidity and preventing leakage through the vessel walls, it is possible that Stx- and inflammation-induced perturbations in these functions contribute to the observed thrombotic microangiopathy. A critical pathway mediating these functions is the protein C pathway, which provides anti-coagulant, anti-inflammatory, and barrier protection activities (14). The activated form of the protein C zymogen mediates these activities, and activated protein C (APC) is generated by the protease action of thrombin, which is localized to the endothelial cell surface through binding to thrombomodulin (TM). Generation of APC is further facilitated by the endothelial protein C receptor (EPCR), which presents protein C zymogen to the cell surface thrombin/TM complex for more efficient activation (15, 16). During *in vitro* studies using human renal glomerular endothelial cells (HRGEC), Stx2-induced a small decrease in TM antigen expression (17), but resultant functional changes were not determined.

As an anti-coagulant, APC inhibits coagulation cofactors Va and VIIIa (18), but its barrier-protective activity is mediated by its occupation of EPCR and subsequent activation of protease-activated receptor 1 (PAR1). PAR1 is intimately involved in endothelial barrier function, and signaling by this discriminatory receptor is protease-specific depending on whether it is activated by APC, thrombin, or other proteases (19–22). PAR1 activation by thrombin contributes to thrombosis while also increasing endothelial barrier permeability; however, PAR1 activation by APC in concert with EPCR elicits an opposite barrier-protective effect. Although human endothelial cells express the Gb₃ toxin receptor, little is known about how the Shiga toxins impact expression and function of PAR1, EPCR, and TM, and disruption of these molecules can have significant consequences (23, 24).

Enterohemorrhagic *Escherichia coli* are generally non-invasive, but the intestinal damage observed during EHEC infection can be considerable, with inflammation, hemorrhage, edema, and focal necrosis predominating (25). Often released by damaged cells are molecules termed damage-associated molecular patterns (DAMPs) (26): normal, endogenous molecules that can be extruded from the cell into the blood or tissue. Examples of DAMPs include histones, which can circulate or localize in neutrophil extracellular traps (27), or HMGB1 from monocytes (28). DAMPs also are released from necrotic cells, and circulating DAMPs can activate many of the same receptors as pathogen-associated molecular patterns to propagate inflammation and tissue damage (29, 30). Some DAMPs also can cause endothelial dysfunction manifested by increased permeability (31) or increased platelet adhesion (27). Although it has not been repeatedly demonstrated that DAMPs are released in the context of EHEC infection or Shiga toxin release, DAMPs from damaged tissue increase in several patient and animal models of sepsis and trauma (32–35). Given the extent of intestinal and kidney injury after EHEC toxin challenge (36–38), and the relative paucity of Shiga toxins observed in serum during hemolytic uremic syndrome (HUS) (39), we hypothesized that injury to any cell expressing the Gb₃ receptor for Shiga toxins would release DAMPs, and that those DAMPs compromise the antithrombotic and barrier-protective properties of endothelial cells, leading to thrombotic microangiopathy and HUS.

MATERIALS AND METHODS

REAGENTS

Plasma levels of HMGB1 and extracellular histones were measured using ELISAs for HMGB1 (IBL-international, Toronto, ON, Canada, and Chondrex Inc., Redmond, WA, USA) and cell-death detection (Roche, Indianapolis, IN, USA), respectively. Human aortic endothelial cells (Cascade Biologics, Grand Island, NY, USA) or HRGEC (ScienCell, Carlsbad, CA, USA) were purchased and grown in endothelial cell medium (ScienCell) supplemented with 5% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, endothelial cell growth supplements according to the manufacturer's instructions. These cell lines are morphologically similar, but HAEC grow more quickly and form tighter junctions, in addition to not being fenestrated as renal glomerular endothelial cells are. All experiments were performed between passage 2 and 6. Antibodies were purchased against PAR1 (ATAP2, Santa Cruz

Biotechnology, Dallas, TX, USA, un/conjugated to Alexa 488), and TM, clone 1029, conjugated to Oregon green, for flow cytometry, and clone 1A4 for on-cell western (Becton Dickinson, Franklin Lakes, NJ, USA). Anti-EPCR (JRK 1494, un/conjugated to Alexa 488) were available from C.T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). Goat anti-mouse IRDye 800CW for use in on-cell westerns was purchased from Li-COR (Lincoln, NE, USA). Stx1 and Stx2 were purchased from Tufts University and endotoxin was removed by incubation with polymyxin B-agarose (Sigma Aldrich, St. Louis, MO, USA). Residual endotoxin levels were <0.015 ng/mL (LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, Rockford, IL, USA). Hybridomas producing anti-histone IgG clone BWA3 (40) were kindly provided by Dr. Ann Rothstein (Department of Immunology and Virology, University of Massachusetts Medical School, Worcester, MA, USA) and the antibody was purified from conditioned media using HiTrap Protein G columns (GE Healthcare, Piscataway, NJ, USA) and standard methods. Calf thymus histones and TNFα were purchased from Sigma Aldrich. Human plasma-derived protein C was a kind gift from Kaketsukan (Kumamoto, Japan). Spectrozyme PCa was purchased from American Diagnostica (Lexington, MA, USA), human α-thrombin, and anti-thrombin III were from Hematologic Technologies, Inc. (Essex Junction, VT, USA). All other reagents such as bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were laboratory or research grade and purchased from Sigma Aldrich and ThermoFisher Scientific.

MOUSE MODEL OF Stx2 TOXEMIA

Mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) for all experiments. All animal studies were approved by the Boston University Institutional Animal Care and Use Committee. Mice (6-week-old male C57BL/6J) were injected intraperitoneally with 1 ~ 1.2 ng Stx2/20 g bodyweight at day 0 and day 3. Control mice received equal volumes of normal saline by intraperitoneal injection. Blood was collected into 5 mM EDTA by facial vein bleed prior to each Stx2 challenge, and periodically until euthanasia for downstream analyses, consistent with the IACUC-approved protocols. Animals were monitored and weighed daily and plasma blood urea nitrogen (BUN) was quantified as a marker of acute kidney injury using a QuantiChrome Urea Assay Kit (BioAssay Systems, Hayward, CA, USA).

MOUSE MODEL OF EHEC INFECTION

Mice (6-week-old female C57BL/6J) were inoculated with *Citrobacter rodentium*. This is a Gram negative mouse pathogen that either was lysogenized with an Stx2-producing phage (strain DBS770) in order to express the Shiga toxin, or a control strain that does not express the toxin (strain DBS771). *C. rodentium* strains were kindly provided by Dr. John M. Leong (Department of Molecular Biology and Microbiology, Tufts University Medical Center, Boston, MA, USA) and have been described in detail elsewhere (41). Briefly, bacteria were grown in LB broth containing chloramphenicol (final 10 µg/mL) or both chloramphenicol and kanamycin (25 µg/mL) to an OD₆₀₀ of 0.75–0.90. Bacteria were washed, re-suspended in sterile saline, and mice were challenged

with 1×10^9 CFU by oral gavage on day 0. Weight was monitored daily and plasma collected periodically by facial vein bleed. CFU/g feces was determined by mixing feces with 10 volumes of phosphate buffered saline (PBS) (ThermoScientific), homogenizing with a sterile toothpick, centrifuging 30 s at 4000 rpm, and plating serially diluted supernatants on LB agar with appropriate restrictive antibiotics. Plates were grown overnight at 32°C and colonies were counted the next day.

FLOW CYTOMETRY

Human aortic endothelial cells between passage 2 and 6 were seeded on 0.1% gelatin-coated plates at 1.5×10^4 cells/cm². The next day, media was replaced with fresh media containing Stx1 or Stx2 (100 ng/mL), extracellular histones (50 µg/mL), or combinations. Cells maintained $\geq 85\%$ viability under these conditions. Cells were incubated for 24 h, washed with PBS and then detached using Versene (Invitrogen) and passed through a 40 µm nylon mesh. All subsequent steps were done on ice. Cells were washed in PBS with 25 mM HEPES, 0.5% BSA, and 5 mM EDTA, pH 7.4 (flow buffer) before staining with primary conjugated antibodies (2 µg/mL) for 30 min. Cells were washed again and re-suspended in flow buffer. Dead cells were excluded by propidium iodide staining. Cell associated fluorescence was determined using a FACSCalibur flow cytometer (Becton Dickinson) and data analyzed using FlowJo X (Treestar Inc., Ashland, OR, USA) software. Based on peak morphology (one peak vs. bimodal), the geometric mean or an M1 region expressed as a percentage was used for data analysis. One-way ANOVA with a Dunnett posttest was used to determine significant differences between geometric means or M1 regions at $p < 0.05$.

ON-CELL WESTERN

Antigen expression on HRGEC was done by on-cell western due to difficulty in producing large numbers through passaging. On-cell western was performed with aortic endothelial cells as well, specifically to compare surface antigen expression with HRGEC at baseline. Optimal toxin and histone concentrations that impacted surface phenotype while maintaining cell viability were determined in preliminary dose-response studies. Cell viability was maintained at 82–96% under experimental conditions (data not shown). Black-walled, clear-bottomed 96-well tissue culture plates (Corning, Tewksbury, MA, USA), of the type used for fluorescent experiments, were coated with 0.1% gelatin and then seeded with HRGEC (ScienCell) grown in endothelial cell media. When cells were near confluent, media were replaced and challenges were added. After 24 h, cells were washed once with PBS then fixed for 20 min at room temperature in PBS with 3.7% formaldehyde. After fixation, cells were washed twice with 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20 (TBST), and blocked with Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) at room temperature with gentle shaking. Following the blocking step, cells were washed twice with TBST and incubated with primary unconjugated antibodies for EPCR (JRK 1494), PAR1 (ATAP2), or TM (1A4) at 2 µg/mL in Odyssey blocking buffer. Cells were washed twice with TBST and incubated with goat anti-mouse IRDye 800CW (Li-Cor) diluted 1:800 in Odyssey blocking buffer and Draq5 (5 mM stock, Thermo Scientific) diluted 1:10,000. Plates

were washed with TBST, and all liquid aspirated before reading fluorescent values on a Li-Cor Odyssey dual-channel infrared imager at 700 and 800 nm. Images were analyzed with Odyssey 2.1 software (LiCor), and fluorescent intensities at 800 nm were normalized to intact cell number based on Draq5 staining at 700 nm. One-way ANOVA with a Dunnett posttest was used to determine significance.

PROTEIN C ACTIVATION ASSAY

Confluent human aortic endothelial cells (HAEC) were incubated with Stx1 (100 ng/mL), Stx2 (100 ng/mL), calf thymus histones (50 µg/mL), or combinations. Confluent HRGEC were challenged with Stx1 (50 ng/mL), Stx2 (50 ng/mL), or histones (50 µg/mL). For experiments with anti-histone BWA3 monoclonal antibody, endothelial cells were challenged with 30 µg/mL histones with or without BWA3 at 20 µg/mL. All challenges were done for 24 h at 37°C in 5% CO₂. Cells were washed with Hank's balanced salt solution (HBSS; Thermo) and protein C was added at a final concentration of 0.4 µM in 0.1 mM HEPES, pH 7.4, 0.6 mM MgCl₂, and 1 mM CaCl₂. Human α-thrombin (10 nM final) was added and the reaction was stopped after 30 min at 37°C with HBSS, 0.1 mM HEPES, 0.5 mg/mL human ATIII, and 1.4 U/mL heparin. Spectrozyme PCa (American Diagnostica, Lexington, MA) was added (0.2 µM final) and the change in absorbance 405 nm with time was performed on a VersaMax ELISA Microplate Reader and analyzed with SoftMax Pro 5.4 (Molecular Devices, Sunnyvale, CA, USA). APC levels were determined by reference to a standard curve prepared with purified human APC, and normalized to intact cell numbers determined using Draq5 staining of a parallel plate, then normalized to 100% activation by unchallenged cells. One-way ANOVA with a Dunnett posttest was used to determine significant differences.

ENDOTHELIAL CELL PERMEABILITY

Endothelial cell monolayer permeability changes were quantified from changes in electrical resistance across a human aortic endothelial monolayer as monitored by electric cell-substrate impedance sensing using an ECIS® ZO array station (Applied Biophysics, Troy, NY, USA). For each experiment, two 40 electrode/well 8W10E+ electrode chamber slides (Applied Biophysics) were coated with 10 mM L-cysteine/well for 10 min, washed 2 times with HBSS, coated with 5 µM fibronectin in HBSS for 10 min, then washed 2 times more with HBSS and allowed to dry. HAEC between passages 2 and 6 were seeded at a density of 2.5×10^5 cells/chamber and allowed to adhere and form a monolayer as determined by the stabilizing capacitance at 64 kHz. For experiments with pre-treatments, histones (25 µg/mL) or Stx2 (100 ng/mL) were added to the appropriate wells 12 h before thrombin addition. Thrombin was added to 1 U/mL (16.85 nM) and the experiment was followed for at least 12 h after thrombin addition. For experiments using APC, concentrations of thrombin were lowered to 2 nM in order for the protective effect of APC on PAR1 to be observed (19). Pre-incubation with APC (10 nM) was done for 3 h prior to thrombin challenge. The area under the curve for normalized resistance over the selected range of time was determined and one-way ANOVA with a Dunnett posttest was used to determine significance.

RESULTS

LARGE VESSEL ENDOTHELIUM EXPRESSES MORE SURFACE EPCR, PAR1, AND TM ANTIGEN THAN MICROVASCULAR RENAL ENDOTHELIUM

Differential endothelial expression of EPCR and TM is well-recognized, and while TM is widely expressed, surface EPCR expression is known to increase with vessel size (42). Relative quantitation of TM and EPCR levels in different vascular beds is not well established, particularly for renal endothelium. In order to better interpret the impact of Stx and DAMPs on receptor expression, quantitative comparisons of baseline surface EPCR, PAR1, and TM on the surface of normal, unchallenged HAEc, representing large vessel endothelium, and HRGEC, representing small vessel endothelium, were performed. These experiments differed from those involving Stx and DAMP challenges in that they were accomplished by performing on-cell westerns with both cell types. We observed that at baseline HAEc expressed significantly higher antigen amounts of EPCR (7.4-fold), PAR1 (1.8-fold), and TM (3.2-fold) when compared to HRGEC (Figure 1).

Stx AND DAMPs SHIFT ENDOTHELIAL EXPRESSION OF PAR1 AND PROTEIN C PATHWAY MOLECULES

The abilities of the Shiga toxins and histone DAMPs to alter surface expression of endothelial cell molecules have important implications for maintaining blood fluidity and were determined by flow cytometry with HAEc (Figure 2). HAEc challenged with histones demonstrated significantly increased expression of thrombin receptor PAR1 relative to control cells (Figure 2A; $p < 0.001$), whereas challenge with either Stx1 or Stx2 caused a subset of cells (35–40%) to significantly lower their surface expression of PAR-1 (Figure 2B; $p < 0.001$). EPCR surface antigen expression was reduced significantly relative to media alone in cells incubated with histones or Stx (Figures 2C,D; $p < 0.001$). TM expression was

reduced after histone treatment (Figure 2E; $p < 0.001$); however, Shiga toxins did not affect TM levels (Figure 2F).

As the primary target organ of the toxins in humans is the kidney glomerular endothelium, HRGEC were challenged similarly to aortic endothelial cells (Figure 3). The renal cells are more sensitive to the histones and toxins *in vitro* (data not shown), so challenge concentrations were decreased to maintain >80% viability and on-cell western was used to minimize manipulation of the cells. Stx1 and Stx2 challenge reduced PAR1 (Figure 3A), EPCR (Figure 3B), and TM surface expression (Figure 3C). Histones did not appreciably alter PAR1 or TM expression, but did decrease EPCR expression on HRGEC.

HISTONES REDUCE ENDOTHELIAL ACTIVATED PROTEIN C GENERATION

Functional changes resulting from alterations to surface expression of TM or EPCR ought to include changes to the rate of protein C activation. To determine if surface expression level changes of TM or EPCR correlated with functional changes that could be extrapolated to have significance in the development of HUS, the rate of protein C activation was determined on endothelial cells after challenge with Stx or histone DAMPs. Only Stx1 or histones had significant effects on the activation of protein C in aortic endothelial cells (Figure 4A). Histone challenge of HRGEC led to a significant decrease in the rate of protein C activation at 24 h post-challenge (Figure 4B; $p < 0.001$); however, neither Shiga toxin alone caused a significant difference in the amount of protein C activated. The suppression of protein C pathway function by histones was successfully prevented in the presence of anti-histone monoclonal antibody BWA3, which inhibits H4 and H2a (40), and protein C activation was restored on both aortic and glomerular endothelial cells in the presence of the blocking antibody during challenge with histones (Figures 4C,D).

HISTONES BUT NOT Stx ENHANCE THROMBIN-INDUCED LOSS OF ENDOTHELIAL BARRIER FUNCTION

Along with their roles in anticoagulation, both endothelium and APC have important roles in maintaining a selective barrier in the vasculature. Endothelial cell permeability changes in response to Stx and histones were examined by monitoring the resistance of a human aortic endothelial monolayer in a continuous fashion using an electrical cell impedance system. As expected, thrombin (1 U/mL final) stimulation resulted in a rapid decrease in resistance (Figure 5A), which correlates with increased permeability. However, in cells pre-treated for 12 h with histones (25 μ g/mL) prior to thrombin stimulation, the thrombin-induced barrier function decrease was prolonged (Figure 5B). Recovery to pre-treatment levels following thrombin stimulation occurred after ~2 h; recovery from thrombin on histone-treated endothelium had not yet occurred by 18 h post-challenge (data not shown for presentation purposes). Pre-treatment with APC abrogated thrombin's effect on monolayer permeability (Figure 5C), consistent with the known barrier protection properties of APC. APC was not able to rescue the loss of cell permeability due to thrombin when the cells were pre-treated with histones (Figure 5C), and treatment with histones alone did not increase permeability (data not shown). Stx2 did not change the duration of increased permeability of HAEc to thrombin, and APC was able to restore cells from thrombin-induced leak

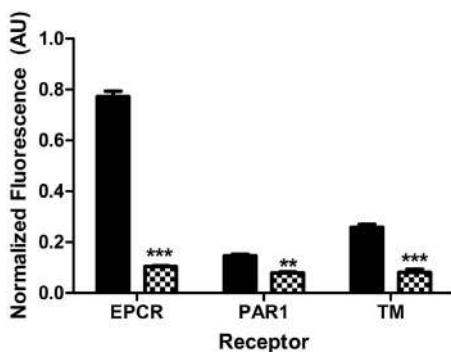
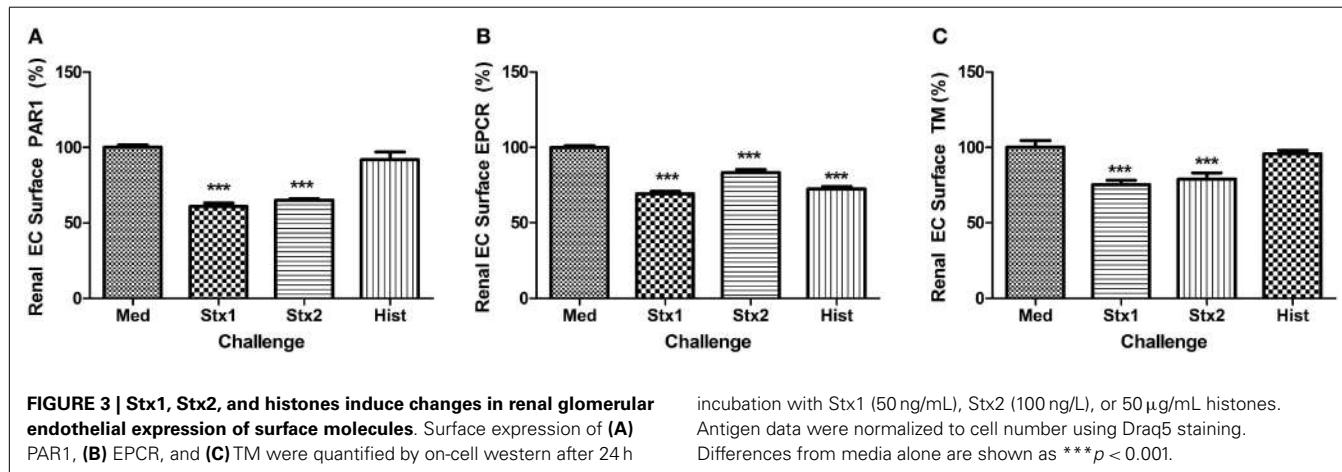
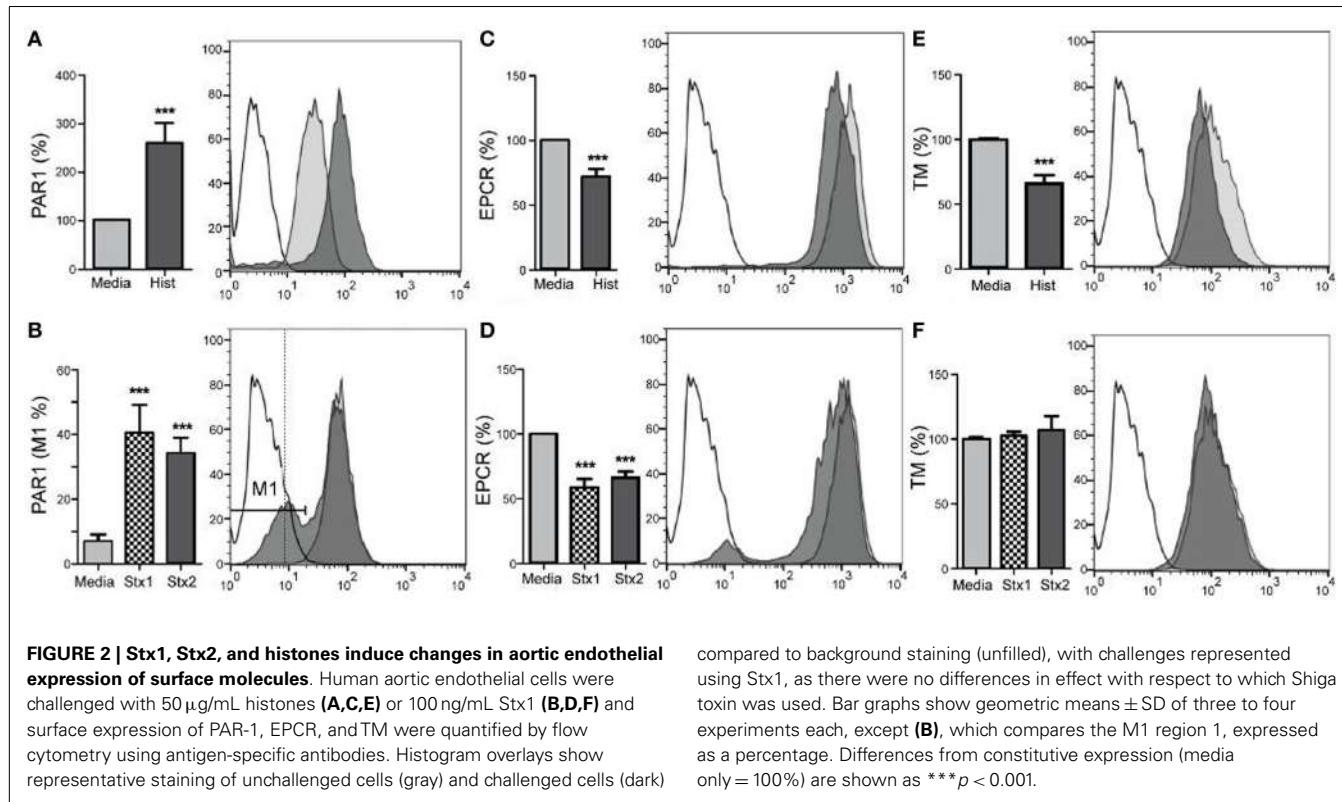


FIGURE 1 | Different endothelium express different amounts of PAR1, EPCR, and TM. Surface expression of EPCR, PAR1, and TM on large vessel human aortic endothelial cells (HAEc, black) and microvascular human renal glomerular endothelial cells (HRGEC, checkered). The significance is determined by comparing the amount of EPCR, PAR1, or TM on each cell type in unchallenged cells at 24 h. Constitutive expression of EPCR, PAR1, and TM on both HAEc and HRGEC for this figure was determined by on-cell western, as described in Section "Materials and Methods." Comparison was done by two-way ANOVA with a Bonferroni posttest. ** $p < 0.01$, *** $p < 0.001$.



regardless of whether or not Stx2 was present (Figure 5D). Challenge with Stx1 did not alter these results. The area under the curves was calculated to quantify the relative effects (Figure 5E). Thus, under these conditions, histone DAMPs greatly exacerbate thrombin's impact and prevent APC from being protective, though the toxins do not appreciably change endothelial barrier function.

DAMPs ARE PRESENT IN THE PLASMA OF Stx2-CHALLENGED MICE

In order to determine whether Stx2 challenge produces DAMPs in mice, we used two different mouse models: a Stx2-toxemia model, and a Stx2-producing bacterial model. Mice challenged with Stx2 (1 ng/20 g body weight; i.p.) on day 0 and day 3 exhibited

decreased survival (Figure 6A, dashed line) and kidney injury evidenced by increasing BUN relative to saline controls (Figure 6B; $p < 0.001$ on day 4). Plasma histone levels in Stx2 challenged mice were significantly elevated on day 3 post-challenge ($258\% \pm 62\%$ $n = 24$) when compared to day 0 (Figure 6C). Plasma levels of HMGB1, another DAMP associated with disease severity in sepsis models, were significantly elevated by 2 days after Stx2 challenge ($421\% \pm 117\%$; $n = 13$) when compared to day 0 (Figure 6E). Pre-treatment with anti-histone BWA3 antibody prevented the significant rise in plasma histones (Figure 6D). Surprisingly, pre-treatment with anti-histone BWA3 antibody also abrogated the rise in HMGB1 (Figure 6F). Despite the reduction in circulating

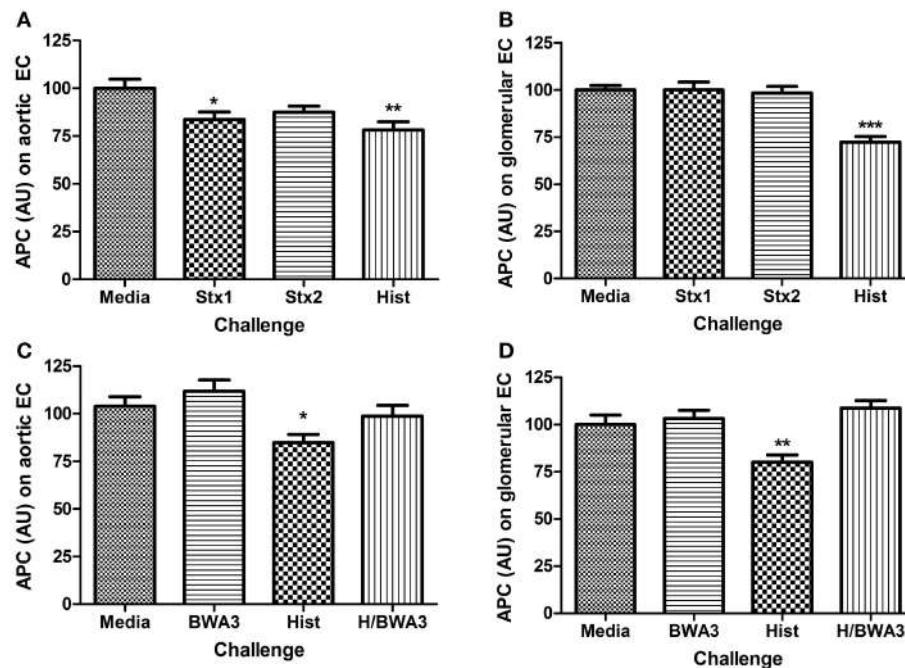


FIGURE 4 | Challenge with Stx or histones decreases activation of protein C. The ability of human aortic endothelial cells (**A,C**) or renal endothelial cells (**B,D**) to activate protein C after 24 h challenge with Stx1, Stx2, or histones was determined on confluent monolayers with 10 nM human alpha-thrombin and 0.2 μ M human protein C at 37°C. Activated

protein C was quantified in cell supernatants with spectrozyme PCa substrate and converted to nM APC using a standard curve, then adjusted for cell number with Draq5 staining. Anti-histone antibody BWA3 (20 μ g/mL) was added before challenge in some wells. Differences relative to media alone are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DAMPs by antibody, there was no alteration in survival outcome after lethal Stx2 challenge (Figure 6A, dotted). This result was not wholly unexpected, as the renal damage in mice is centered more in the tubular epithelium and collecting ducts, as opposed to the renal glomerular endothelium in humans, and the mice do not display thrombotic microangiopathy.

During EHEC infection, Stx exposure occurs over days as the gut is colonized by EHEC that produce and release the Shiga toxins, so we also utilized a murine model in which mice are infected by oral gavage with *C. rodentium* strains that either do or do not express Stx2. The intestinal attaching/effacing lesions and contributions of Stx2 toward organ injury have been described (41), and this model has been shown to produce similar A/E lesions and thus be more relevant than a Stx2-alone model. Inoculating the mice by oral gavage with $\sim 1 \times 10^9$ CFU of *C. rodentium*-Stx2 resulted in decreased survival (Figure 7A; dashed line) compared to mice that received control *C. rodentium*, which does not express the toxin. As expected, kidney injury was toxin-dependent as evidenced by elevated plasma BUN by days 7–9 with the toxin-producing strain (Figure 7B; $p < 0.001$ on day 9). Mice challenged with non-Stx2 producing *C. rodentium* (open circles) did not develop changes in plasma levels of extracellular histones for the duration of the experiment when compared to day 0 (Figure 7C). In contrast, mice challenged with *C. rodentium*-Stx2 (black squares) developed significantly increased plasma levels of histones on day 3. This effect was significant when compared their own day 0 values and was also significant compared to plasma histone levels in

non-Stx2 producing *C. rodentium* challenged mice (Figure 7D). Plasma HMGB1 did not increase significantly in mice challenged with non-Stx2 producing *C. rodentium* (Figure 7F), in contrast to elevated levels of HMGB1 by day 5 in *C. rodentium*-Stx2 mice (Figure 7G). BWA3 anti-histone antibody pre-treatment (dotted line) did not alter survival. Survival was still significantly decreased ($p < 0.001$) compared to animals challenged with *C. rodentium* not expressing Stx2, and there was no significant difference when compared with mice given Stx2-expressing *C. rodentium* and no antibody pre-treatment. The observed increase in plasma histone and HMGB1 levels were abolished by treatment with the anti-histone BWA3 antibody (Figures 7E,H). In conclusion, both murine models demonstrated that DAMPs were consistently elevated when Stx2 was present, and in both cases the rise occurred prior to rise in BUN, confirming that in the presence of Stx2, DAMPs are produced.

DISCUSSION

LOW RELATIVE EXPRESSION OF PROTECTIVE EPCR AND TM ON RENAL GLOMERULAR ENDOTHELIAL CELLS

Differential expression of EPCR and TM between large vessel endothelium and the microvasculature is known from immunohistochemistry studies, particularly for EPCR, which is expressed relatively more on large vessel endothelium (42). Here, we provide quantitative assessment of EPCR, TM, and PAR1 expression on cultured primary endothelial cells using on-cell western. Our results show that there are 3.2-fold lower and 7.4-fold lower

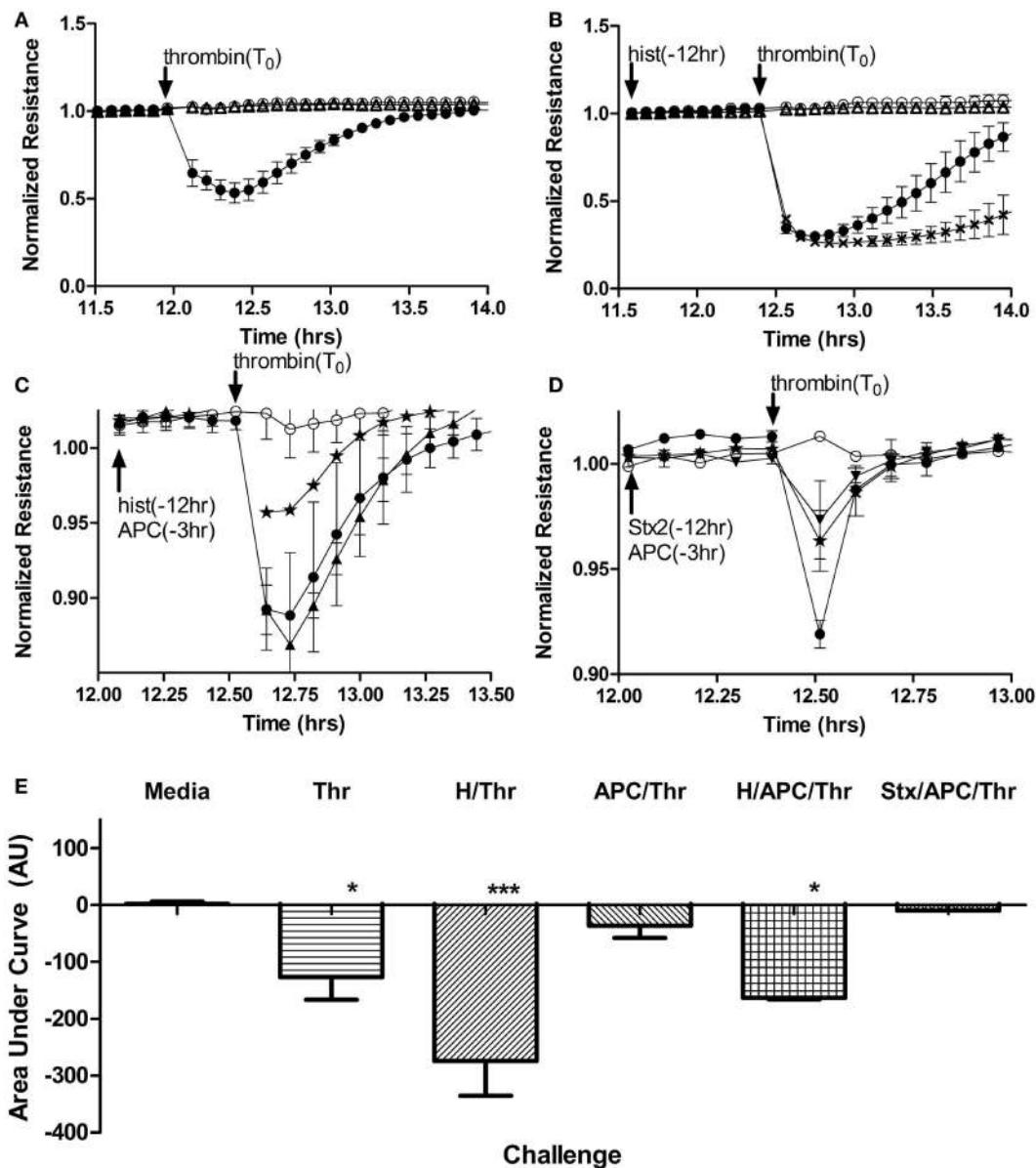


FIGURE 5 | Histones, but not Stx, contribute to increased endothelial cell permeability and block APC rescue. Permeability of human aortic endothelial cells was determined by monitoring changes in electrical resistance across a monolayer as described in Section “Materials and Methods.” **(A)** Steady state resistance observed with media (○) decreases after addition of 1 U/mL (16.85 nM) human thrombin (●), reflecting increased permeability. Challenge with Stx2 (Δ) lead to no change in permeability. **(B)** Increased permeability after thrombin (●) challenge is significantly prolonged if cells are pre-exposed to 50 μg/mL histones (X) for 12 h, but again, Stx2 causes no appreciable change in

permeability (Δ). **(C)** Compared to thrombin (2 nM) alone (●), APC pre-exposure (★) attenuates thrombin effects on permeability, but cannot rescue permeability of the cells if they have also been exposed to histones (▲). **(D)** Thrombin increased permeability (●) and APC rescued cells (★), but adding 100 ng/mL Stx2 (▼) did not change the protective effect of APC. **(E)** Permeability data were quantified as area under the curves; mean ± SD of three to four experiments each. Neither Stx1 nor Stx2 alone altered electrical resistance of the monolayers and so Stx2 is shown on graphs as representative of either Shiga toxin. * $p < 0.05$, *** $p < 0.001$.

expressions of TM and EPCR, respectively, on renal glomerular endothelial cells when compared with aortic endothelial cells in the same experiment. APC is protective in acute kidney injury murine models (43), and the organ system most vulnerable to the sequelae of low EPCR expression is the kidney, which demonstrates enhanced albuminuria and profound

renal hemorrhage after LPS challenge (44). The kidneys are already a toxin target due to glomerular Gb₃ expression, and the current data suggest that further reduction due to toxin and DAMP exposure of already low TM and EPCR expression may provide an environment highly susceptible to thrombus production.

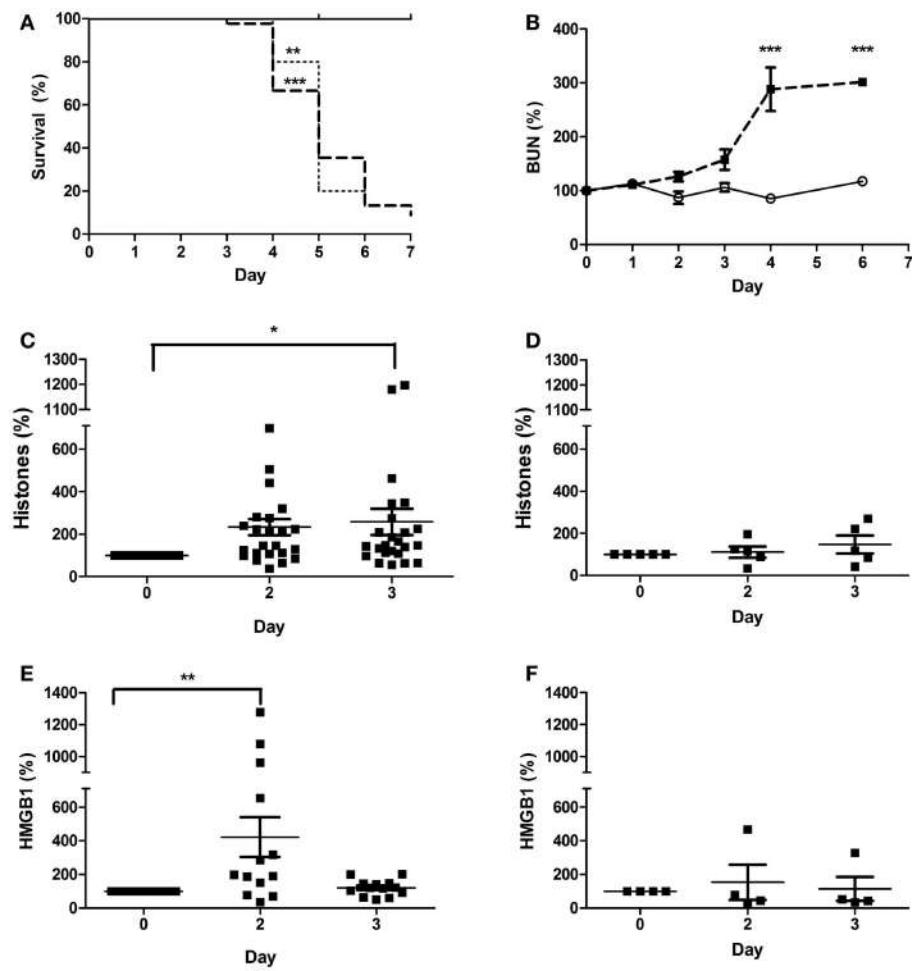


FIGURE 6 | Plasma DAMPs increase after Stx2 challenge in mice. Mice were challenged i.p. with 1 ng/20 g body weight Stx2 [bold dashed line in (A), black squares in (B–F), $n=45$] or saline [solid line in (A), open circle in (B), $n=15$] on Day 0 and Day 3 and monitored for (A) survival and (B) plasma

BUN. Plasma histones (C,D) and HMGB1 (E,F) were measured by ELISA. One group received a bolus dose of anti-histone BWA3 (400 μ g i.p.) before challenge [(D,F); $n=5$]. Survival was not influenced by BWA3 [(A), dotted; $n=5$]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IN VITRO LOSS OF EPCR AND TM SURFACE EXPRESSION ON ENDOTHELIAL CELLS DUE TO Stx AND DAMPs

D + HUS is characterized by thrombotic microangiopathy, or forming of microthrombi in the small vasculature of the glomerulus, suggesting loss of endothelial anti-coagulant capacity. The receptors and enzymes that make up the protein C pathway of endothelial cells are critical contributors to intact anti-coagulant function of the endothelium and loss of these anti-coagulant and cytoprotective molecules could have significant pro-thrombotic consequences. Surface expression levels of protein C pathway molecules are changed in Shiga toxin- or DAMPs-challenged human endothelial cells, as demonstrated here *in vitro*. Shiga toxin and/or DAMP challenge of human endothelial cells resulted in a shift toward a pro-coagulant environment, and this has functional consequences with respect to the generation of APC, an enzyme critical in controlling thrombus formation. We made several significant observations, including reduced APC generation, enhancement of endothelial barrier permeability, and reduction of the ability of

APC to protect barrier function in the context of Stx or histone DAMP challenge. These data provide insights into the mechanism of Stx-induced endothelial dysfunction in D + HUS and strongly suggest a potential role for tissue damage induced DAMPs in this process.

Thrombin bound to TM preferentially cleaves and activates protein C instead of fibrinogen (45), creating APC and ultimately reducing fibrin clot formation. EPCR makes the thrombin/TM cleavage of protein C even more favorable. EPCR also plays a crucial role in endothelial function because APC/EPCR complex activation of PAR1 leads to a cytoprotective signaling cascade (19, 46, 47). We previously demonstrated in a non-human primate model of HUS that Shiga toxins alone can cause HUS and acute renal failure with fibrin deposition and swelling and detachment of glomerular endothelial cells (48, 49). Cellular mechanisms are difficult to study in non-human primates and mouse models do not develop the coagulopathy of HUS, so the current study with human cells extends the *in vivo* observations by demonstrating

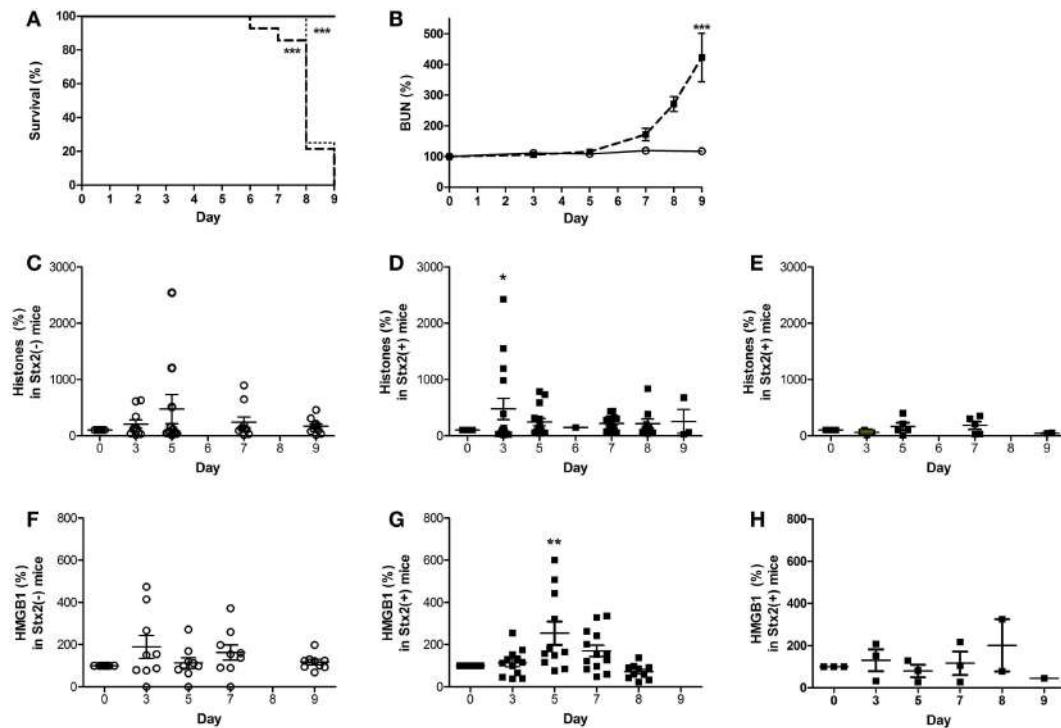


FIGURE 7 | Plasma DAMPs increase during intestinal infection with Stx2+ *Citrobacter rodentium*. Mice were gavaged with 1×10^9 CFU Stx2+ *C. rodentium* [bold dashed line in (A), black square in (B,D,E,G,H), $n = 15$] or Stx2- strain *C. rodentium* [solid line in (A), open circle in (B,C,F), $n = 10$] on Day 0 and monitored for (A) survival and (B) plasma BUN. Plasma DAMPs were measured as histones or HMGB1 in

Stx2- mice (C,F) and Stx2+ mice (D,G). Baseline levels of both DAMPs were observed when anti-histone antibody BWA3 (400 µg; i.p.) was injected 10 min prior to challenge with Stx2+ bacteria [(E,H); $n = 5$ /group]. Pre-treatment with BWA3 did not affect survival after challenge with the Stx2+ strain [(A), dotted line; $n = 5$]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

in vitro loss of EPCR and TM surface expression on endothelial cells due to Stx and DAMPs, particularly histones, and reduced capacity to generate APC. A pro-thrombotic environment is further created by DAMP-induced increased expression of PAR-1, the pro-thrombotic thrombin receptor (50). Both histones and Shiga toxins reduced EPCR expression, and Shiga toxins also lowered TM on HRGEC, with the end result that both Stx1 and histones affected the amount of APC generated by HRGEC in HAEC, with histones alone affecting APC production in HRGEC.

LOSS OF IN VITRO ENDOTHELIAL BARRIER FUNCTION DUE TO HISTONES BUT NOT Stx

D+HUS patients can develop edema, and in our system we observed loss of *in vitro* endothelial barrier function primarily due to histones, rather than through any effect of Stx1 or Stx2 on endothelial cells. Histones increased PAR-1 and lowered EPCR expression and pre-treatment of human aortic endothelial monolayers with histones significantly delayed the ability of the endothelial barrier to recover to baseline after thrombin challenge. Histones also significantly inhibited the barrier-protective effects of APC. It is possible that histones might compete as a substrate for APC (32), but we did not find any effect of histones on APC chromogenic activity even at histone concentrations up to fourfold higher than those used here (data not shown). PAR1 can function in both barrier protective and disruptive capacities,

depending on which protease cleaves it and whether EPCR is occupied by a ligand. Shiga toxins and DAMPs both decrease the surface ratio of EPCR:PAR1 by lowering EPCR; however, only histones also raise PAR1, which could account for the differential effects of histones vs. Shiga toxins with respect to APC inhibition. We hypothesize that the combination of decreased EPCR along with increased PAR1 creates more opportunity for thrombin signaling while dampening the potential protective effects of EPCR occupied with APC, helping to explain the prolongation of the permeability effects of thrombin despite its use.

DAMPs ARE PRODUCED BY THE ACTION OF SHIGA TOXINS IN MICE

The purpose of our mouse models was to demonstrate that DAMPs are produced by the action of Shiga toxins. The finding of significantly elevated DAMPs is to the best of our knowledge a novel finding in mouse models of Shiga toxin challenge and is consistent with prior studies in our non-human primate Stx-HUS models (51). The finding that levels of plasma HMGB1 in both mouse models decreased after an initial significant rise instead of continuing to rise with disease severity could be due to the limitations of current mouse models for this disease, because the distribution of Gb₃ receptors in the mouse differs from that in humans or non-human primates (4, 5, 49). Unlike in human kidneys, Shiga toxins target renal tubule epithelial cells in mice (6) giving rise to the possibility that HMGB1 was excreted as

urination becomes increasingly excessive in the mouse models due to tubular injury. In contrast, humans and baboons express Gb₃ on endothelial cells, particularly in the glomeruli, and disease severity is accompanied by progressive anuria so that the baboon Stx challenge models have and increase in certain plasma DAMPs such as mitochondrial DNA over time (35). However, in contrast, we have previously shown that HMGB1 in non-human primate models also initially rises before dropping (51), so the waxing or waning pattern of different DAMPs in the serum with Shiga toxin challenge likely relies on mechanisms that remain to be understood and fall outside the scope of this discussion.

In our experiments, we introduced the anti-histone antibody BWA3, which targets H4 and H2a, and is derived from an autoimmune mouse model (40). A bolus dose of 400 µg BWA3 prior to Stx2 or *C. rodentium*-Stx2 significantly decreased plasma levels of histones and HMGB1, although in neither case was the outcome altered for the model. These results suggest that these DAMPs may function more as markers of cell injury in these murine models rather than driving pathology as they might in other models; however, it was not our intention to investigate endothelial injury in our murine models, for reasons, which have been stated already. For this purpose, we turned to *in vitro* models of endothelial cell injury. Our human endothelial cell experiments show a significant impact by DAMPs with respect to APC generation and endothelial dysfunction, but this is not possible to replicate in the mice, as these models exhibit Stx-induced tubular epithelial injury (5), with no evidence of glomerular endothelial damage, thrombotic microangiopathy, or thrombocytopenia of HUS as stated before. These are limitations of all mouse models using endotoxin-free Stx challenge. Other investigators have shown that in mice challenged with LPS (a model of endothelial cell injury) BWA3 reduced circulating histones and improved survival (32). The current experiments with human endothelial cells raise the possibility that BWA3 might have more of an effect on outcomes in models of kidney injury driven by endothelial rather than tubular damage, such as a non-human primate model that more closely mimics human pathology (36, 37, 49); however, there are clear limitations to doing the experiments described in this paper in non-human primates, and so we chose to progress as described.

Elevated HMGB1 and histones have been shown experimentally to be involved in SIRS development by the fact that inhibition of either is beneficial in animal models of LPS-induced inflammation (32, 33). Currently, development of HUS in patients during EHEC infection is largely attributed to the bacterially produced Shiga toxin effects on endothelium or platelets (52), but the current data suggest that the host response to injury contributes as well. During active infection, Shiga toxins rapidly become undetectable in the plasma days before development of HUS (39). During this time, cells internalize the toxins, the intestinal bacteria damage the intestinal epithelium, and both processes may lead to release of DAMPs. The observations made here cannot link DAMP activities directly to susceptibility of patients to develop HUS, but they do provide insight into a possible molecular basis for development of increased vascular permeability and a pro-thrombotic environment in the context of Stx2-induced tissue damage. We showed this by demonstrating that DAMPs are produced by the Shiga toxins, and that histone DAMPs cause

demonstrable endothelial dysfunction in two types of human endothelial cells.

LIMITATIONS OF THE CURRENT STUDY AND FUTURE DIRECTIONS

While we have made several important observation in the experiments described here promoting a possible role for Stx2-induced DAMPs in the development of endothelial dysfunction underlying HUS, there are still important questions that remain unanswered. We did not identify cellular mechanisms linking Shiga toxins and extracellular histones to the regulation of EPCR, TM, and PAR1 endothelial expression, as we were more interested in the end consequence of reduced protein C activation; however, this mechanism could also be a source of potential therapeutic targets and warrants investigation. Matrix metalloproteinases and MAPK pathways may contribute to EPCR shedding (53, 54), though how these may be impacted by Stx or DAMPs in this context was not addressed. Histones act through TLR4 (55), which might induce PAR1 through a similar mechanism, though we do not address this here. Finally, Stx1 and Stx2 interact with human neutrophils via TLR4 (56), but how that might impact histone or other DAMP activities is not known. Pilot experiments using TAK-1, JNK, and IRAK1/4 inhibitors demonstrated no significant prevention of Shiga toxin effects on EPCR, PAR1, or TM (data not shown). The largest limitation of our studies was the lack of a mouse model demonstrating full-spectrum HUS, which to date has only been reproducibly done by adding LPS to the Stx challenge (6, 57, 58). Ideally, we would test our hypothesis *in vivo* using animal models that demonstrate kidney injury driven by endothelial dysfunction, and then we could show whether DAMPs are playing a significant role by using therapies such as BWA3 and quantifiable outcomes such as biomarkers and survival. Additionally, quantifying the presence of DAMPs in the blood of patients with HUS would be a good next step prior to experiments with full-spectrum HUS animal models. Previous work has already amply demonstrated the pathology and tissue damage (25, 59), and quantifying the DAMPs that are almost certainly present would provide the evidence needed to investigate whether they function as markers of disease impact or drivers of disease. The data here lay groundwork, giving evidence that this could be a fruitful path for future investigations.

ACKNOWLEDGMENTS

The authors thank Dr. Carl J. Hauser (Beth Israel Deaconess Medical Center, Boston, MA, USA) for the generous use of his ECIS ZΘ system. The authors gratefully acknowledge Priya Jani for technical assistance. CM was supported by NHLBI T32 training grant HL07969 (K. Ravid, PI), U01AI075386 (SK, PI) and R01AI102931 (SK, PI). CP was supported by NIH/NIAID T32AI089673 (C. Genco, PI).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 December 2014; **accepted:** 23 March 2015; **published online:** 07 April 2015.
Citation: Mayer CL, Parella CSL, Lee BC, Itagaki K, Kurosawa S and Stearns-Kurosawa DJ (2015) Pro-coagulant endothelial dysfunction results from EHEC Shiga toxins and host damage-associated molecular patterns. *Front. Immunol.* **6**:155. doi:10.3389/fimmu.2015.00155

This article was submitted to *Microbial Immunology*, a section of the journal *Frontiers in Immunology*.

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***Shigella* manipulates host immune responses by delivering effector proteins with specific roles**

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OPEN ACCESS

Edited by:

Katharina F. Kubatzky,
University Hospital Heidelberg,
Germany

Reviewed by:

Herve Agaisse,
Yale University, USA
Wendy Picking,
University of Kansas, USA

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Specialty section:

This article was submitted to
Microbial Immunology, a section of
the journal Frontiers in Immunology

Received: 07 March 2015

Paper pending published:

27 March 2015

Accepted: 22 April 2015

Published: 07 May 2015

Citation:

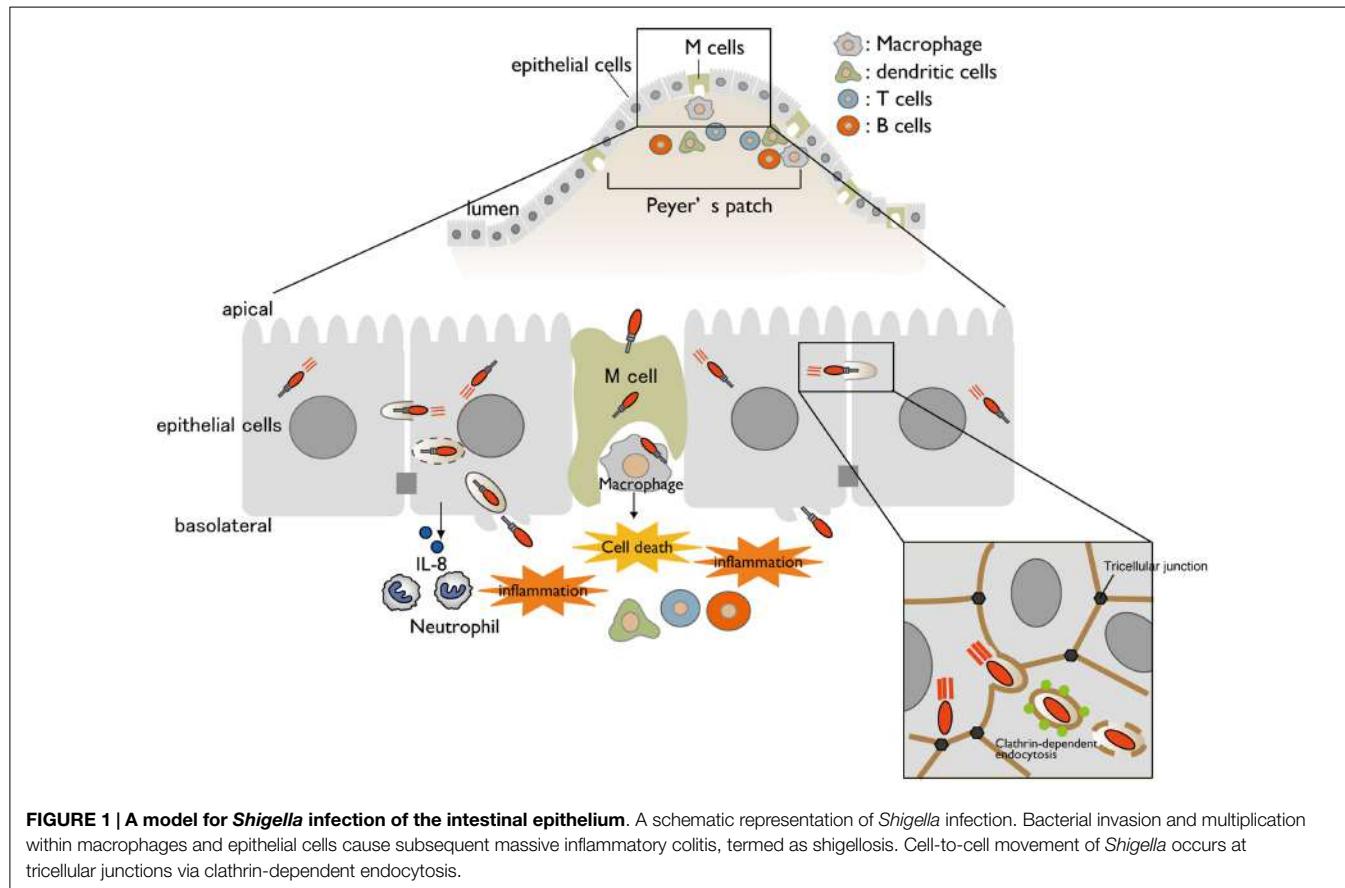
Ashida H, Mimuro H and Sasakawa C (2015) *Shigella* manipulates host immune responses by delivering effector proteins with specific roles. *Front. Immunol.* 6:219.
doi: 10.3389/fimmu.2015.00219

The intestinal epithelium deploys multiple defense systems against microbial infection to sense bacterial components and danger alarms, as well as to induce intracellular signal transduction cascades that trigger both the innate and the adaptive immune systems, which are pivotal for bacterial elimination. However, many enteric bacterial pathogens, including *Shigella*, deliver a subset of virulence proteins (effectors) via the type III secretion system (T3SS) that enable bacterial evasion from host immune systems; consequently, these pathogens are able to efficiently colonize the intestinal epithelium. In this review, we present and select recently discovered examples of interactions between *Shigella* and host immune responses, with particular emphasis on strategies that bacteria use to manipulate inflammatory outputs of host-cell responses such as cell death, membrane trafficking, and innate and adaptive immune responses.

Keywords: *Shigella*, effector, inflammation, innate immunity

Introduction

Shigella is a causative agent of bacillary dysentery, which ultimately leads to severe bloody and mucous diarrhea (shigellosis). Most cases of shigellosis occur in developing countries and affect children under 5 years old. Although antibiotics are the standard care for shigellosis patients, antibiotic-resistant bacterium is becoming common. Therefore, it is urgently necessary to develop a safe and effective *Shigella* vaccine. *Shigella* have neither adherence factor nor flagella, but they are capable of efficiently invading the intestinal epithelium. *Shigella* injects a subset of effectors (secreted virulence proteins) via a type III secretion system (T3SS) (protein delivery system) into host cells, allowing the bacterium to invade, multiply within the intestinal epithelium, and subvert cellular and immune functions during bacterial internalization (1, 2). When *Shigella* cells are ingested via the oral route, the bacteria move down to the colon and rectum, and then preferentially enter the M cells overlying the follicle-associated epithelium of the Peyer's patches (3, 4) (Figure 1). Once the bacteria are endocytosed by the M cells, they are transcytosed toward the M cell pocket, where resident macrophages receive the bacteria. However, *Shigella* can disrupt the vacuolar membranes, disseminate into the cytoplasm, and multiply therein (5). Bacterial multiplication within the macrophages results in massive inflammatory cell death (6) (Figure 1). Meanwhile, *Shigella* cells that are released from dying macrophages subsequently enter the surrounding epithelium via the basolateral surface. Upon epithelial cell contact, the bacteria deliver a subset of T3SS effector proteins that trigger actin rearrangement, promoting bacterial uptake (7). Next, the *Shigella* cells are surrounded by a vacuolar membrane, but the bacteria rapidly disrupt this membrane and



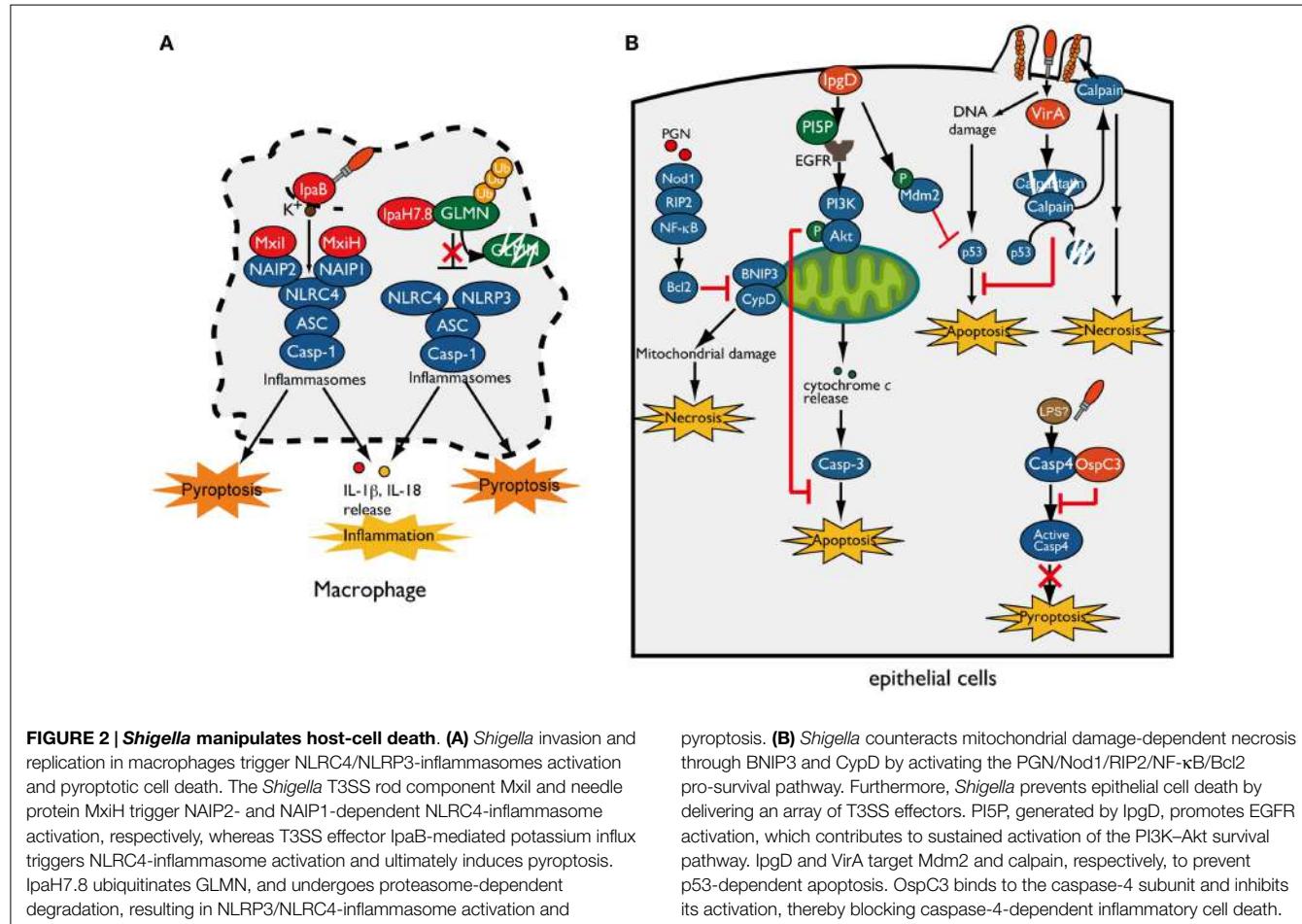
disseminate into the cytoplasm. As *Shigella* proliferates within the cytoplasm, it moves by inducing actin polymerization at one pole of the bacterium, providing the propulsive force required for inter- or intracellular movement (8–10). Intriguingly, *Shigella* cell-to-cell movement preferentially occurs at epithelial tricellular junctions, where three cells meet (Figure 1). At these positions, bacteria-containing pseudopodia are engulfed by neighboring cells via a clathrin-dependent endocytic pathway, resulting in dissemination of *Shigella* (11). By repeating these processes, the bacteria efficiently multiply by constantly renewing their replicative compartment. Thus, multiple infectious events during *Shigella* infection, including macrophage cell death, invasion of and multiplication within epithelial cells, cell-to-cell spreading, demise of the host epithelium, and alteration of the host inflammatory response, are major pathogenic events that lead to shigellosis (2) (Figure 1).

Cell Death

Host-cell death in response to microbial infection is an intrinsic immune defense stratagem against microbial intrusion. The sacrifice of infected cells plays a pivotal role in clearance of damaged cells, elimination of pathogens, local confinement of tissue damage and inflammation, and presentation of bacteria-derived antigens to the adaptive immune system (12). Cell death induced by bacterial infection can be classified into at least three types, depending on the type of cell and stage

of infection: apoptosis, necrosis, and pyroptosis. Apoptosis is a non-inflammatory programmed cell death triggered by the mitochondria-mediated pathway and receptor-mediated pathway, which eventually induce caspase activation (caspase-2, -3, -6, -7, -8, -9, and -10), chromatin condensation, cell shrinkage, plasma membrane blebbing, and cytoplasm retained in apoptotic bodies. On the other hand, necrosis is an inflammatory form of cell death characterized by cell swelling, membrane rupture, and intracellular content leakage. Pyroptosis is pro-inflammatory, lytic, and programmed cell death that is accompanied by activation of caspase-1 or caspase-11 (human homologs: caspase-4/5) inflammasomes, leading to the production of IL-1 β and IL-18.

When bacteria invade and multiply within host cells, they release bacterial components [e.g., lipopolysaccharide (LPS) and peptidoglycan (PGN)] and T3SS components, and further cause infection-associated cellular damage. These are recognized as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) by recognition receptors, such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), AIM2-like receptors (ALRs), and RIG-like receptors, and trigger host immune responses against bacterial infection. Upon recognition of these PAMPs and DAMPs, a subset of NLRs (e.g., NLRP1, NLRP3, and NLRC4) and ALRs (e.g., AIM2) form inflammasomes, which are multi-protein signaling complexes composed of NLR/ALR, the adaptor protein ASC, and inflammatory caspase, such as caspase-1 (canonical inflammasomes) and caspase-11 (non-canonical



inflammasomes) (13, 14). Inflammasome activation ultimately results in release of pro-inflammatory cytokines (IL-1 β and IL-18) and induction of pro-inflammatory lytic cell death (pyroptosis).

When *Shigella* invade and multiply within macrophages, they rapidly induce pyroptotic cell death accompanied by NLRP3- or NLRC4-inflammasome activation, leading to IL-1 β and IL-18 secretion (15–18). T3SS needle or rod components indirectly activate NLRC4 inflammasomes through members of the NAIP subfamily of NLRs, which act as direct pathogen recognition sensors and determine the specificity of the NLRC4-inflammasome for various bacterial ligands. Recent reports have revealed that human NAIP and mouse NAIP1 bind to *Shigella* T3SS needle protein MxiH (19, 20). In addition, NAIP2 binds to the T3SS inner rod component MxiL (16, 21) (Figure 2A). Although *Shigella* lacks flagella, NAIP5 and NAIP6 specifically activate the NLRC4-inflammasome in response to other bacterial flagellins (22–25). Once these NAIP proteins bind to their ligands, they bind to NLRC4 and induce NLRC4-inflammasome activation and pyroptosis. In addition to bacterial T3SS components, cellular damage caused by *Shigella* also triggers inflammasome activation and pyroptosis. As *Shigella* cells multiply within macrophages, the T3SS effector IpaB assembles an ion channel within cell membrane to allow for potassium influx, which is recognized by the

NLRC4-inflammasome and ultimately triggers pyroptosis (26) (Figure 2A).

As described above, because pyroptosis is accompanied by inflammation that would limit bacterial infection, it had not been unclear whether pyroptosis is beneficial for bacterial infection (27). However, a recent study showed that induction of inflammasome activation and pyroptosis in infected macrophages is a *Shigella* strategy that can promote bacterial survival and dissemination. Suzuki et al. showed that *Shigella* induces rapid macrophage pyroptosis via IpaH7.8, an IpaH family effector, mediated NLRP3- and NLRC4-dependent inflammasome activation (28) (Figure 2A). IpaH family effectors, which have a novel E3 ubiquitin ligase activity, are widely conserved among Gram-negative bacterial pathogens, including *Shigella*, *Salmonella*, *Yersinia*, and *Pseudomonas* spp. (29). IpaH7.8 targets GLMN (glomulin/flagellar-associated protein 68), a Cullin-RING E3 ligase inhibitor, for ubiquitination and undergoes proteasome-dependent degradation (Figure 2A) (28). Because GLMN acts as a negative regulator of NLR inflammasomes and pyroptosis, degradation of this factor induces inflammasome activation and pyroptosis. Consistent with results obtained *in vitro*, mice intranasally infected with *Shigella* WT or ΔipaH7.8/WT complement strains induce more severe inflammatory responses and elevated numbers of colonized bacteria relative to ΔipaH7.8 or

ΔipaH7.8/CA E3 ligase-deficient mutant complemented strains (28). Therefore, IpaH7.8-mediated macrophage cell death is prerequisite for allowing bacteria to escape from macrophages, further enter surrounding epithelial cells, and spread to neighboring cells.

Epithelial Cell Death

When *Shigella* invade and multiply within epithelial cells, the cells generate an early genotoxic stress, mitochondrial damage, oxidative stress, and recognize PAMPs and DAMPs, which could induce several types of cell death as part of the host defense system aimed at terminating bacterial infection. However, in contrast to macrophage infection, *Shigella* seems to prevent epithelial cell death until the bacteria have fully multiplied, because it prefers these cells as replicative niche, spread to neighboring cells, and evasion of immune cells (30) (Figure 2B). To support this notion, *Shigella* has several countermeasures that inhibit epithelial cell death: (i) prevention of mitochondrial damage, (ii) activation of cell survival signaling [e.g., phosphoinositide-3 kinase (PI3K)-Akt and transcription factor nuclear factor κB (NF-κB)], and (iii) prevention of caspase activation. For example, *Shigella* prevents necrotic cell death mediated by mitochondrial damage (through BNIP3 and CypD) by activating the Nod1-RIP2-NF-κB-Bcl-2 pro-survival pathway (31) (Figure 2B). When *Shigella* invades epithelial cells, it delivers the T3SS effector IpgD (a homolog of *Salmonella* SopB), a phosphoinositide phosphatase that converts phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 5-phosphate (PI5P), at the bacterial entry site (32). Because PI plays pivotal roles in actin cytoskeleton rearrangement, elevated levels of PI5P at the plasma membrane promotes bacterial invasion. Furthermore, IpgD-mediated PI5P is an important factor involved in cell survival (33). At this step, the PI5P generated by IpgD contributes to epidermal growth factor receptor (EGFR) activation, which sustains the PI3K/Akt pro-survival pathway and thereby contributes indirectly to augmentation of pro-survival signaling (33, 34) (Figure 2B). A recent report showed that the early stage of *Shigella* infection induces genotoxic stress in epithelial cells, followed by p53 pro-apoptotic signaling activation and induction of apoptosis; however, *Shigella* promotes p53 degradation and antagonizes the early stage of cell death by delivering two T3SS effectors, IpgD and VirA (Figure 2B) (35). As described above, IpgD promotes Akt activation, which in turn phosphorylates and stabilizes the downstream E3 ubiquitin ligase Mdm2. Activated Mdm2 targets p53 for ubiquitination and leads to proteasome-dependent degradation, thereby inhibiting pro-apoptosis signaling by p53. Furthermore, VirA promotes further degradation of p53 by activating calpain protease, which is also important for bacterial invasion. VirA binds to the calpain inhibitor calpastatin and promotes its degradation, resulting in degradation of p53 by activated calpain and blocking the p53 pro-apoptotic signaling pathway. Although VirA-mediated calpain activation promotes bacterial entry and prevents the early stage of apoptosis, sustained calpain activation ultimately induces necrosis and restricts bacterial proliferation (35). In addition, *Shigella* exploits calpain activation and mitochondrial function to suppress the innate immune response without inducing host-cell death (see below) (36). These results indicate that *Shigella* deploys

a sophisticated strategy that controls the delicate balance of host-cell death until *Shigella* has succeeded in primary colonization and proliferation within epithelial cells.

Recent reports have shown that non-canonical caspase-4/-11 inflammasome activation is an essential host defense mechanism of epithelial cells against enteric bacterial pathogens such as *Shigella*, *Salmonella*, and enteropathogenic *Escherichia coli* (EPEC). Caspase-4/-11 directly binds to cytoplasmic LPS of Gram-negative bacterial pathogens and activates caspase-4/-11-mediated inflammasomes and pyroptosis, ultimately inducing epithelial cell shedding to eliminate infected cells (37–39). However, *Shigella* antagonizes caspase-4-dependent inflammatory cell death by delivering the T3SS effector OspC3 and promoting epithelial infection (37). OspC3 deploys a unique mechanism that specifically targets and inactivates caspase-4, but not caspase-1 or mouse caspase-11. The C-terminal ankyrin-repeat (ANK) region of OspC3 interacts with the p19 subunit of caspase-4 and prevents its activation by inhibiting p19 and p10 dimerization (Figure 2B). In human epithelial cell lines, the *Shigella ΔospC3* mutant induces early caspase-4-dependent pyroptotic cell death and increased cytokine production relative to that of WT *Shigella*. Of note, the *Shigella ΔospC3* mutant also exhibited severe mucosal cell death and a reduction in the number of colonizing bacteria in a guinea pig rectal infection model, indicating the importance of OspC3-mediated cell death inhibition for bacterial infection (37). Thus, *Shigella* delivers a subset of T3SS effector proteins that delay epithelial cell death until the bacterial cells have fully replicated and further disseminated into surrounding cells.

Autophagy

Autophagy is an essential cellular catabolic process, which targets proteins, organelles, and large protein aggregates by sequestering deleterious cargos within a double-membrane compartment, the autophagosome. Autophagy also plays a pivotal role as a part of the innate immune system, by acting as a cytosolic sensor to recognize DAMPs and PAMPs, and as an “executioner” that engulfs bacteria in autophagosomes that fuse with lysosomes, ultimately destroying bacteria within lysosomal compartments.

Shigella enter epithelial cells, disseminate into the cytosol by disrupting the surrounding phagosomal membrane, and move into adjacent cells by inducing actin polymerization at one bacterial pole. In *Shigella* invasion of epithelial cells, Nod1 and Atg16L1 are recruited to the plasma membrane beneath the *Shigella* entry site and subsequently trigger autophagy (40). Furthermore, host-cell vacuolar membrane remnants generated by *Shigella* are recognized as DAMPs by galectin-8, and these remnants are also polyubiquitinated, followed by the recruitment of p62 (ubiquitin adaptor protein) and LC3 (autophagosome marker), resulting in autophagic activation (41, 42) (Figure 3). During multiplication within the cytosol, *Shigella* outer membrane protein VirG (IcsA) accumulates at one pole of the bacterial surface. There, VirG recruits and activates N-WASP, which subsequently recruits and activates the Arp2/3 complex, thereby inducing actin polymerization and bacterial motility within the cell (8–10). At this stage, VirG is recognized by the host autophagy protein Atg5, a protein essential for autophagosome maturation, resulting in *Shigella* uptake by autophagosomes. However, *Shigella* prevents

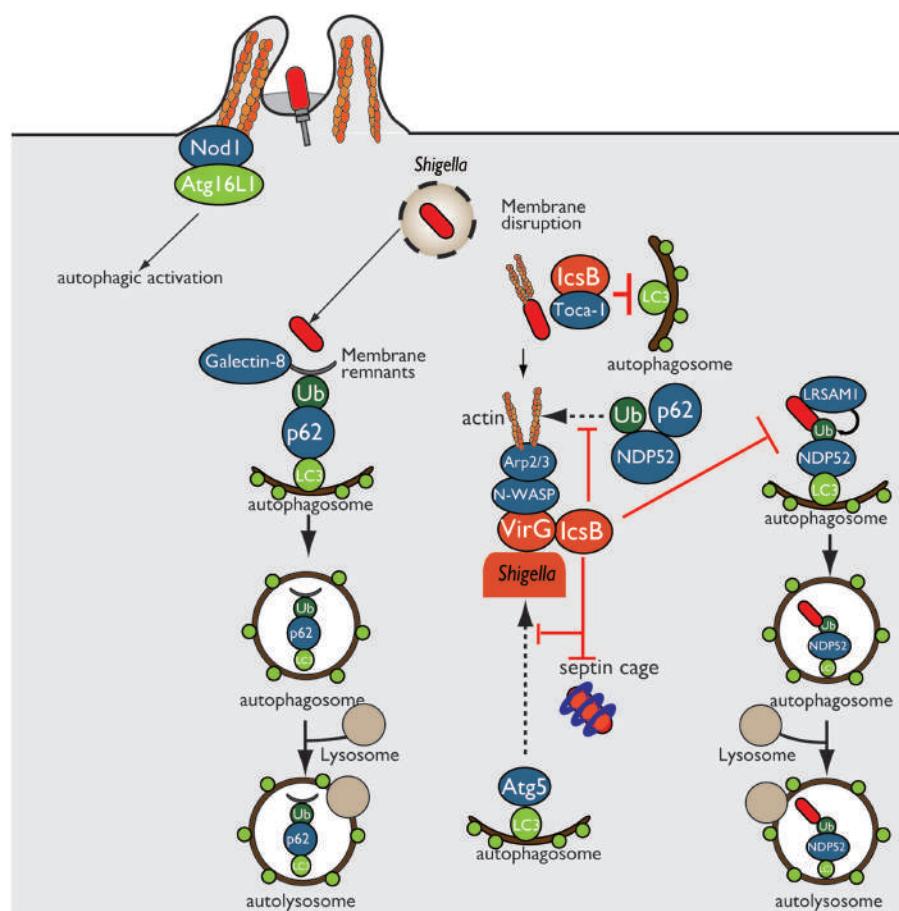


FIGURE 3 | **Shigella prevents autophagic recognition.** *Shigella* prevents autophagic clearance by evading autophagic recognition. *Shigella* delivers the T3SS effector IcsB, which binds to the bacterial outer membrane protein VirG

and prevents Atg5–VirG interaction and ubiquitin recruitment, thereby evading autophagic recognition. IcsB also recruits Toca-1, and prevents the recruitment of LC3 to around intracellular bacteria.

autophagic recognition by delivering the IcsB T3SS effector. At an early stage of infection, IcsB binds to and recruits Toca-1, which is required for efficient formation of actin polymerization, around intracellular bacteria, and IcsB–Toca-1 prevents the recruitment of LC3 (43, 44). Furthermore, at the later stage of infection, IcsB plays a pivotal role in camouflage against autophagic recognition (45). IcsB and Atg5 interact with the same region on VirG, but the affinity of IcsB for VirG is stronger than that of Atg5; therefore, IcsB competitively inhibits the VirG–Atg5 interaction, masking the target VirG protein from autophagic recognition (45) (Figure 3). Consistent with this, the *Shigella ΔicsB* mutant is trapped in the autophagosome and delivered for lysosomal degradation (45). Intriguingly, IcsB has cholesterol binding region, and its ability is involved in evading autophagic recognition without affecting IcsB–VirG binding (46). In *Shigella* infection, ubiquitin-dependent selective autophagy is also triggered, but the bacteria evade ubiquitin recognition. Although *Shigella* WT is not targeted by ubiquitin, LRSAM1 (a mammalian LRR-containing RING E3 ligase), which itself acts as a bacterial recognition molecule, localizes to the *Shigella ΔicsB* mutant, and LRSAM1-mediated bacterial ubiquitylation has been observed *in vitro* (44). LRSAM1 recognition can trigger ubiquitin-dependent selective autophagy,

resulting in restriction of bacterial replication (47). In addition, another host factor septin, which is GTP-binding protein, assembles at sites of VirG-induced actin polymerization and forms cages that surround bacteria, and prevents inter- and intracellular movement, thereby targeting by autophagy and restricting bacterial proliferation (48). A recent report showed that the ubiquitin adaptors p62 and NDP52 target *Shigella* for autophagy in an actin polymerization- and septin-dependent manner, and that the *Shigella ΔvirG* mutant reduces the recruitment of p62 and NDP52 (49). The *Shigella ΔicsB* mutant increases septin-cage formation and the recruitment of ubiquitin, p62, and NDP52. Therefore, although VirG-mediated actin polymerization is targeted by septin- and ubiquitin-dependent selective autophagy, the IcsB–VirG interaction prevents the recruitment of ubiquitin, p62, and NDP52 around bacteria (49) (Figure 3).

Membrane Trafficking

Intracellular trafficking of membranes and proteins is essential for maintenance of epithelial homeostasis and barrier function, and also acts as a host defense system against bacterial pathogens. In eukaryotic cells, intracellular trafficking system can be divided

into two pathways, endocytosis and the secretory pathway. During endocytosis, which internalizes extracellular molecules or delivers plasma membrane proteins to specialized sites, the cargo is sorted in the early endosome and further transported to Golgi, endoplasmic reticulum (ER), or late endosomes and lysosome for degradation, whereas some cargo proteins are transported back to the plasma membrane via recycling endosomes. On the other hand, the secretory pathway, which produces molecules such as antimicrobial peptides, growth factors, cell surface receptors, and cytokine secretion, transport molecules from the ER to the plasma membrane through the Golgi.

Exocytosis, endocytosis, phagocytosis, and cytokine secretion play essential roles as host defense systems for eliminating invading bacterial pathogens. However, many intracellular bacterial pathogens, such as *Shigella*, *Salmonella*, and *Legionella*, hijack and exploit host intracellular trafficking system to enter host cells, evade subsequent phagocytic destruction, and establish their safety replicative niche for their survival and proliferation (7, 50, 51). *Shigella* enter non-phagocytic cells by delivering a coordinated set of T3SS effector proteins that trigger actin cytoskeleton or plasma membrane remodeling, and promote subsequent bacterial uptake by host cells. Soon after internalization, the bacteria are engulfed within a membrane-bound vacuole that is derived from the host plasma membrane. After that, *Shigella* disrupt and escape from this vacuole and replicate within the cytoplasm (7). Vacuolar disruption or modulation by intracellular bacteria is related to the host membrane trafficking system, which is tightly regulated by small GTPases of the Rab and ARF families. The small GTPases act as molecular switches that cycle between the GTP-bound active form and GDP-bound inactive form, catalyzed by two classes of regulatory proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs exchange GDP for GTP to activate GTPases, whereas GAPs inactivate GTPases by promoting hydrolysis of GTP to GDP (52). Rab and ARF GTPases play an important role in resisting bacterial infection, such as secretion of cytokines and antimicrobial peptides, maintenance of epithelial barrier integrity, and maturation of phagosomes into lysosomes to degrade entrapped bacteria. Therefore, *Shigella* target Rab GTPases and interfere with host membrane trafficking by delivering T3SS effector proteins (**Figure 4**).

In *Shigella* infection, the endocytic pathway is essential event for bacterial invasion, vacuolar rupture, and spread to neighboring cells. Using a high-content siRNA screening, Mellouk et al. recently demonstrated that *Shigella* targets and recruits Rab11, a component of host recycling endosomes, to disrupt the vacuole and efficiently escape from it by delivering the IpgD effector (53) (**Figure 4**). As described before, IpgD is a phosphoinositide phosphatase that produces PI5P, which is important for vesicular trafficking. In cells infected with *Shigella ΔipgD* or phosphatase-inactive *ΔipgD/ ipgD C438S* complemented mutant, Rab11 is not recruited to the site of bacterial invasion, and vacuolar rupture is delayed; however, this deficiency is rescued by the complementation of *ΔipgD/ ipgD WT* or *WT Shigella* infection, highlighting the requirement for the phosphoinositide phosphatase activity of IpgD. Furthermore, vacuole disruption by *Shigella* is delayed in Rab11 knockdown epithelial cells without affecting bacterial

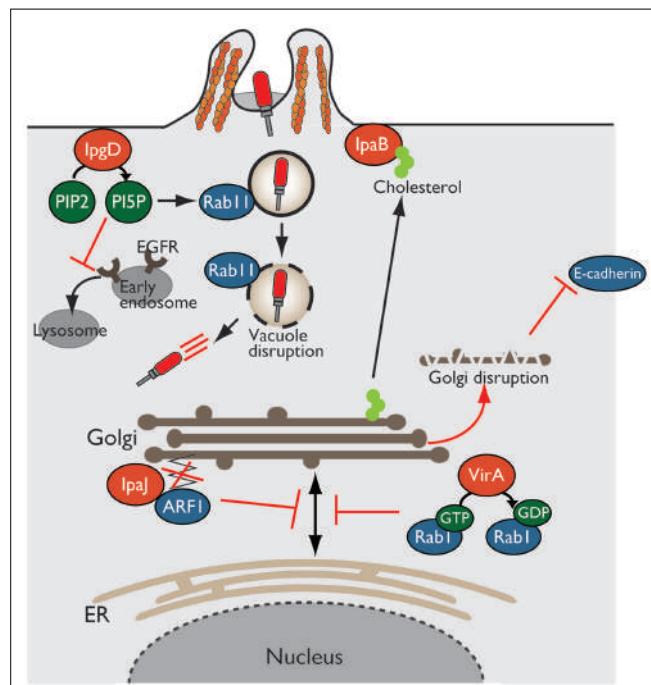


FIGURE 4 | *Shigella* alters host intracellular trafficking. *Shigella* exploits and alters intracellular trafficking to manipulate the host defense system. PI5P produced by IpgD recruits Rab11 to the bacterial entry site and promotes vacuolar membrane disruption. Production of PI5P by IpgD further relocates EGFR in early endosomes, resulting in blocking lysosomal degradation of EGFR and sustaining PI3K–Akt survival pathway. VirA and IpaJ target and inactivate Rab1 and ARF1, respectively, thereby preventing intracellular trafficking and Golgi disruption. IpaB binds to and relocates cholesterol from Golgi to bacterial entry sites, resulting in Golgi fragmentation.

invasion (53). Therefore, *Shigella* targets Rab11 to disrupt the vacuole and allow bacterial escape into the cytoplasm and promote further bacterial replication and dissemination.

In addition, as mentioned above briefly, IpgD subverts host intracellular trafficking to promote host-cell survival for bacterial prolonged colonization (34). The production of PI5P by IpgD at bacterial entry sites recruits active EGFR, which is required for the PI3K–Akt survival signaling pathway. An elevated level of PI5P at the plasma membrane, due to the activity of IpgD, alters EGFR trafficking and relocates it to early endosomes from late endosomes or lysosomes, thereby blocking lysosomal degradation of EGFR and sustaining EGFR–PI3K–Akt survival signaling activation (34) (**Figure 4**).

The Golgi apparatus is a central organelle involved in protein or lipid transport in eukaryotic cells. The Golgi processes and sorts proteins made by the ER, and transport them to various destinations, such as the plasma membrane and lysosome. Therefore, defects in Golgi function result in the loss of intracellular trafficking, including secretion of antimicrobial peptides and cytokines, and transport of epithelial junction proteins that are essential for epithelial barrier function against bacterial infection. Intriguingly, *Shigella* disrupts the Golgi apparatus by inhibiting vesicle trafficking in infected epithelial cells in a T3SS-dependent manner. Of note, three *Shigella* effectors, VirA, IpaJ, and IpaB, play

important roles in disrupting the Golgi apparatus by targeting host intracellular trafficking (**Figure 4**).

Recent reports have shown that VirA and IpaJ, which have specific enzymatic activities targeting Rab and ARF GTPases, respectively, dampen host antibacterial defense systems by inhibiting the host secretory pathway. VirA (a homolog of EPEC EspG), which has TBC (homologous catalytic domain of GAP for Rab GTPase)-like GAP activity, preferentially targets and catalyzes GTP hydrolysis in Rab1, which localizes in the ER and mediates ER-to-Golgi trafficking (54). VirA inactivates Rab1, inhibits ER-to-Golgi transport of Rab1-containing vesicles, and disrupts the Golgi apparatus in host epithelial cells (**Figure 4**). Because Rab1 is important for autophagosome formation, Rab1 inactivation by VirA counteracts antibacterial autophagy. Of note, infection by a *Shigella ΔvirA* mutant or GAP-inactive *ΔvirA/virA-RQ* complemented mutant increases autophagosome formation and reduces the number of colonizing bacteria relative to that of WT *Shigella* in human epithelial cell lines. Thus, TBC-like GAP activity of VirA contributes to bacterial escape from autophagy and intracellular survival by blocking intracellular trafficking through Rab1 GTPase inactivation (54).

A bioinformatics approach revealed that IpaJ belongs to the cysteine protease family, whose members have Cys–His–Asp catalytic triad residues (55, 56). Further yeast genetic screening and mass spectrometry analysis demonstrated that IpaJ specifically cleaves the *N*-myristoylated glycine from ARF1 (**Figure 4**). *N*-myristylation is an essential fatty-acid modification that is involved in protein localization, signal transduction, autophagosome maturation, and organelle function. Because *N*-myristylation of ARF1 is essential for binding to the Golgi membrane, IpaJ-mediated cleavage induces the release of ARF1 from Golgi and its disruption, thereby inhibiting intracellular trafficking, including cytokine secretion and signal transduction (55, 56).

Another effector, IpaB, also disrupts the Golgi apparatus by targeting lipid trafficking, and thereby reduces epithelial barrier function. IpaB binds to and redirects cholesterol at the bacterial entry site from the Golgi apparatus, which depletes the lipid of Golgi and induces its fragmentation (**Figure 4**). The Golgi apparatus fragmentation further induces tubulation of the Rab11-positive compartment and reorganizes the recycling endosome, which is involved in trafficking of E-cadherin to adherence junction, resulting in epithelial junction disruption (57).

As described above, membrane and protein trafficking, such as the phagosome component, epithelial barrier components, antimicrobial peptides, cytokines, and cell surface receptors, is a major host defense system against bacterial infection. Therefore, *Shigella* blocks and alters host intracellular trafficking by delivering subsets of effectors that promote infection.

Manipulation of Host Innate Immunity

Once *Shigella* invades and replicates within host cells, the innate immune system quickly senses PAMPs or DAMPs, and transmits various alarm signals to the rest of the immune system, and ultimately triggers inflammation. Inflammation, which is accompanied by inflammatory cytokine secretion, neutrophil recruitment, and massive tissue destruction, is the hallmark of the host innate

immune response that eventually restricts and eliminates bacterial infection. However, many bacterial pathogens, including *Shigella*, deliver a subset of T3SS effectors that manipulate host innate immune responses, thereby promoting bacterial colonization and survival (2) (**Figure 5**).

ATP Release

During bacterial infection, various molecules are released from damaged or stressed cells as DAMPs (e.g., ATP, uric acid, and membrane remnants), which trigger inflammatory immune responses. For example, infection of intestinal epithelial cells with enteric bacterial pathogens, such as *Shigella*, *Salmonella*, and EPEC, induce connexin hemichannel-dependent ATP release, which acts as an endogenous danger alarm against bacterial infection and triggers inflammatory responses (58). Hemichannel-dependent ATP release is a common host defense system against enteric bacterial infection. To counteract ATP-dependent inflammation, *Shigella* blocks ATP release by delivering IpgD to epithelial cells. IpgD blocks hemichannels and prevents ATP release through production of PI5P, which regulates hemichannel opening, thereby dampening ATP-dependent inflammation (**Figure 5**). Consistent with this, in both *in vitro* and *in vivo* infections, the *Shigella ΔipgD* mutant causes elevated ATP release, severe intestinal inflammation, and mucosal damage, illustrating the key role of IpgD in preventing ATP-dependent inflammation (58).

Manipulation of NF- κ B Signaling

During bacterial infection, the transcription factor NF- κ B, the master regulator of pro-inflammatory cytokines, plays a major role in mediating an inflammatory signaling pathway that triggers a wide range of host inflammatory responses. In response to bacterial infection, several intracellular and extracellular stimuli activate signal transduction cascades, and NF- κ B is translocated into the nucleus, where it promotes the transcription of target genes (59). In particular, ubiquitination of signaling factors is prerequisite for regulation of NF- κ B activity; therefore, many bacterial pathogens, including *Shigella*, inhibit NF- κ B activation by targeting signaling factors and altering signal transduction, thereby dampening inflammation, in order to promote infection (60).

Invasion of epithelial cells by *Shigella* produces membrane ruffles by remodeling the actin cytoskeleton around the bacterial entry site. Aberrant membrane ruffles, which protrude from the bacterial entry site and are accompanied by diacylglycerol (DAG) production, are sensed as DAMPs by the host innate immune system and trigger the activation of the DAG-CBM (CARMA-BCL10-MALT1)-TRAF6-NF- κ B pathway. However, *Shigella* delivers OspI via the T3SS into the host cells; this factor targets and deamidates UBC13 (converts Gln-100 to Glu-100), an E2 required for TRAF6 E3 activity, resulting in abolition of its E2 activity and thereby interfering with the DAG-CBM-TRAF6-NF- κ B pathway (61) (**Figure 5**).

Following membrane ruffling, *Shigella*-mediated vacuolar membrane ruptures are also recognized as DAMPs, triggering an additional alarm-signaling pathway via recruitment and activation of PKC, ultimately leading to the activation of the PKC-NF- κ B pathway. To counteract PKC-NF- κ B activation,

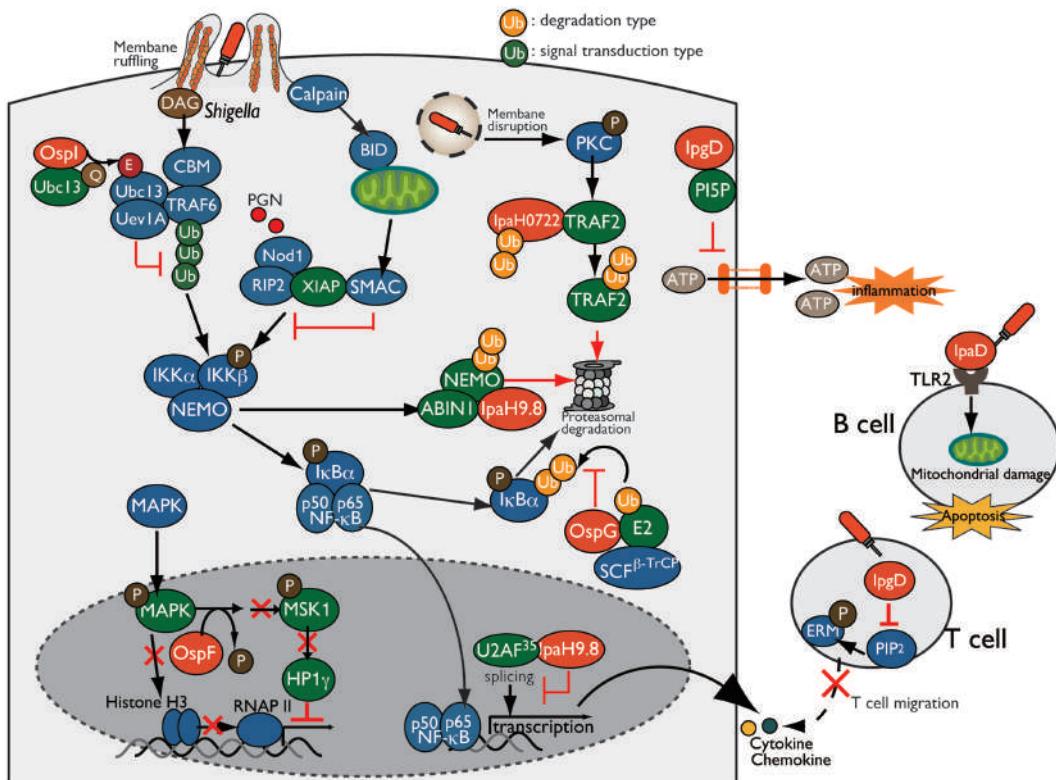


FIGURE 5 | *Shigella* manipulates the host innate and adaptive immune response. *Shigella* manipulates host inflammatory responses by delivering a subset of T3SS effectors, including IpgD, OspL, OspG, OspF, and IpaH. PIP2, generated by IpgD, prevents ATP release-dependent inflammation through hemichannels. OspG binds to ubiquitin and ubiquitinylated E2 proteins and prevents IκBα ubiquitylation, which is required for NF-κB activation. OspL deamidates and inactivates Ubc13, resulting in inhibition of TRAF6 ubiquitination. IpaH0722 and IpaH9.8 target TRAF2 and NEMO, respectively, for ubiquitination, and undergo proteasome degradation. In addition, IpaH9.8

further targets U2AF35, preventing its participation in the splicing reaction via an unidentified bacterial factor. *Shigella* induces calpain-dependent BID activation, which, in turn, releases mitochondrial SMAC to antagonize XIAP-mediated inflammation. IpgD-mediated hydrolysis of PIP2 inactivates ERMs of T cells, which crosslink actin filaments with the plasma membrane, thereby preventing T cell migration. *Shigella* induces B cell death in both invaded and non-invaded cells. In non-invaded cells, the T3SS needle-tip protein IpaD interacts with TLR2 on B cells and triggers mitochondrial damage, which eventually induces apoptosis.

Shigella delivers IpaH0722, one of the IpaH family of E3 ubiquitin ligase effectors. IpaH0722 dampens the acute inflammatory response by ubiquitination of TRAF2, a molecule downstream of PKC, thereby preferentially inhibiting PKC-mediated NF-κB activation (62) (Figure 5).

During *Shigella* invasion of epithelial cells, Nod1 recognizes PGNs released from *Shigella* as PAMPs, triggering the Nod1–RIP2 pathway and activating the downstream mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways. *Shigella* delivers IpaH9.8, an IpaH family E3 ubiquitin ligase effector, which preferentially prevents the Nod1-dependent NF-κB activation. IpaH9.8 interacts with NEMO/IKK γ , an essential component of the IKK kinase complex, and targets NEMO for ubiquitination. IpaH9.8 also interacts with ABIN-1, an ubiquitin-binding adaptor protein, to further promote polyubiquitylation of NEMO. Ubiquitinated NEMO by IpaH9.8 subsequently leads to proteasomal degradation, thereby diminishing NF-κB activation (63) (Figure 5). The activity of this E3 ligase effector during *Shigella* infection contributes to bacterial colonization in a mouse lung infection models (63).

Upon Nod1 activation, the X-linked inhibitor of apoptosis protein (XIAP) plays a crucial role in activating Nod1-dependent inflammatory responses. XIAP interacts with RIP2 and facilitates NF-κB activation, ultimately leading to pro-inflammatory gene transcription. A recent report showed that *Shigella* evades XIAP-mediated inflammation by actively releasing mitochondrial SMAC (36). *Shigella* infection triggers calpain activation and processes and activates BH3-only protein BID, which then translocates to the mitochondria and induces the release of SMAC. Released SMAC binds to XIAP, and inhibits XIAP-mediated NF-κB activation without inducing mitochondrial damage or cell death (Figure 5). Although it remains unknown whether BID-mediated release of SMAC by *Shigella* depends on a T3SS effector, *Shigella* uniquely exploits mitochondrial function to block the host innate immune response while avoiding cell death (36).

In addition, *Shigella* delivers another effector, OspG, which shares sequence similarity with mammalian serine/threonine kinases and inhibits NF-κB activation (64). OspG binds to ubiquitin and ubiquitinylated E2s, which are required for phospho-IκBα ubiquitination by an E3 ligase such as SCF β -TrCP, and formation

of this OspG–E2–ubiquitin complex promotes OspG kinase activity and increases the prevention of NF- κ B activation (64–67) (**Figure 5**).

Epigenetic Regulation of Immune Gene Expression

Because the host transcriptional program plays a pivotal role in triggering inflammation and eliminating bacterial infection, *Shigella* overcomes this defense mechanism by delivering a subset of T3SS effector proteins that regulate gene expression and counteract host immune responses. In addition to interfering with NF- κ B signaling, as described above, *Shigella* hacks the host immune responses by reprogramming the host epigenome to promote infection. The T3SS effector OspF (homolog of *Salmonella* SpvC and *Pseudomonas syringae* HopAl1), which has a unique phosphothreonine lyase activity, translocates into the nucleus of epithelial cells, where it irreversibly dephosphorylates and inactivates MAPKs (Erk and p38) through beta-elimination of the phosphate group (68, 69). Inactivation of MAPK by OspF further blocks downstream phosphorylation of histone H3 at Ser10 at the promoters of a subset of innate immune genes, such as IL-8, and promotes chromatin condensation, resulting in repression of transcription by masking NF- κ B binding sites (68) (**Figure 5**). In addition to histone modification, OspF also alters the activity of the chromatin reader heterochromatin protein 1 (HP1) and represses host gene expression during *Shigella* infection (70). Although phosphorylated HP1 γ at Ser83 proteins positively regulate euchromatin (a transcriptionally active state) and activate transcription, OspF inactivates Erk and consequently reduces the activity of the downstream kinase MSK1, a kinase for HP1 γ at Ser83. As a result of HP1 γ dephosphorylation, HP1 γ dissociates from sites of transcriptional activation at OspF-target genes such as IL-8 (**Figure 5**). Consistent with this, the *Shigella* Δ ospF mutant increased the level of HP1 γ S83 phosphorylation relative to WT *Shigella*-infected cells (70). Therefore, OspF manipulates host transcriptional response via two epigenetic modifications: (i) decreasing the level of phosphorylated histone H3, and (ii) altering the activity of HP1 through dephosphorylation of MAPK, which contributes to downregulation of host inflammatory responses.

In addition to OspF, *Shigella* delivers IpaH9.8 to regulate pro-inflammatory gene expression. IpaH9.8 is translocated into the nucleus of epithelial cells, where it binds to U2AF35, an mRNA splicing factor, and inhibits the U2AF35-dependent splicing reaction, enabling the bacterium to dampen expression of numerous genes, including some that encode pro-inflammatory cytokines and chemokines (71) (**Figure 5**).

Manipulation of Host Adaptive Immunity

Manipulation of the host innate immune response by *Shigella* is a pivotal survival strategy for promoting infection; however, the interactions between *Shigella* and adaptive immune response, such as T and B lymphocytes, have not been thoroughly investigated. One prominent issue is the lack of appropriate animal infection models that mimic human intestinal infection. Until now, however, several studies have proven the importance of adaptive

immunity against *Shigella* infection using a mouse pulmonary infection model that mimics the acute inflammation that occurs during shigellosis. Mice that are genetically deficient in B, T, and NK cells are much more susceptible to *Shigella* infection than WT mice, and T and NK cells play critical roles in clearing *Shigella* (72). Additional studies have shown that CD4 $^{+}$ T helper 17 (Th 17) cells, which are predominantly primed in response to *Shigella*, produce IL-17A and eventually restrict secondary *Shigella* infection, indicating the involvement of Th17 cells in adaptive immunity against *Shigella* infection (73). By contrast, antigen-specific CD8 $^{+}$ T cells, which are usually required for adaptive immunity against cytosolic bacterial infection, are not primed and involved in adaptive immunity against *Shigella* infection (74). As the precise role and the importance of adaptive immunity against *Shigella* infection have been revealed, several recent studies have shown that host adaptive immunity is targeted and subverted by *Shigella* T3SS effectors (**Figure 5**).

T cell migration and activation are key events in the induction of antibody- and cell-mediated immune responses against bacterial infection. Recently, the Phalipon group investigated the interaction between T cells and *Shigella* using *in vitro* and *in vivo* approaches, and demonstrated that *Shigella* interferes with adaptive immune responses by targeting T cells. They also found that *Shigella* invades activated CD4 $^{+}$ T cells and inhibit chemoattractant-mediated T cell migration by delivering IpgD (75). T-lymphocyte migration toward a chemoattractant depends on the membrane cytoskeleton crosslinkers proteins ERM (ezrin, radixin, and moesin), which are tightly converted between the active and inactive conformation by the concentration of PIP2 at the plasma membrane. Because IpgD is phosphoinositide phosphatase, it subsequently hydrolyzes and decreases the concentration of PIP2 at the plasma membrane, thereby inactivating ERM (**Figure 5**). In addition, *Shigella* impairs CD4 $^{+}$ T cell dynamics within lymph nodes, where adaptive immunity is initiated, in the mouse infection model (76). Therefore, *Shigella* targets T-lymphocytes and inhibits their migration by delivering the T3S effector IpgD, thereby interfering with adaptive immunity.

B lymphocytes are key players in antibody-mediated immunity, and they produce and secrete cytokines that contribute to the antibody-independent immune response against bacterial infection. However, massive T- and B-cell deaths are observed in rectal biopsies of *Shigella*-infected humans, indicating that *Shigella* impairs B cell-mediated immunity (77). To support this notion, a recent report showed that *Shigella* targets B cells and induces cell death in both *Shigella*-invaded and non-invaded cells (78). *Shigella* invades B cells and replicates intracellularly, resulting in B cell death. Furthermore, *Shigella* induces B cell apoptosis via the T3SS needle-tip protein IpaD. IpaD binds to TLR2 on B cells and triggers the loss of mitochondrial dysfunction and apoptotic cell death signaling in non-invaded B cells, eventually helping the bacterium to avoid antibody-mediated immune responses (78) (**Figure 5**). Therefore, *Shigella* targets T and B cells and manipulates adaptive immunity against *Shigella* infection, thereby preventing antibody-mediated lasting immunity and promoting bacterial infection.

Conclusion

Although bacterial infection elicits host defense systems that would restrict and eliminate bacteria, many bacterial pathogens have evolved excellent strategies to manipulate host immune responses and enable bacterial colonization. Here, we review the current understanding of how *Shigella* manipulates host immune responses, focusing specifically on T3SS effector-mediated interference with host signaling transduction cascades, alteration of membrane trafficking, and modulation of host-cell death. Our understanding of the molecular basis of the interaction between bacterial effectors and host immune systems has advanced greatly during the past decade. Although accumulating evidence has revealed the importance of manipulation of the host immune system during bacterial infection, our knowledge of bacterial strategy is still in its infancy. Although approximately 50 T3SS effectors of *Shigella* are currently recognized, we have only elucidated the molecular function of one-third of them.

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Acknowledgments

This work was supported by a Grant-in-Aid for Specially Promoted Research (23000012, to CS) and a Grant-in-Aid for Scientific Research (C) (25460527 to HA). Part of this work was supported by grants from the Naito Foundation (HA) and Mochida Memorial Foundation for Medical and Pharmaceutical Research (HA).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A Critical Role of Bacterioferritin in *Salmonella pullorum*-Induced IFN- β Expression in DF-1 Cells

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OPEN ACCESS

Edited by:

Inka Sastalla,
National Institutes of Health, USA

Reviewed by:

Dane Parker,
Columbia University, USA
Kenneth James Genovese,
United States Department
of Agriculture, Agricultural Research
Service, USA

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 26 September 2015

Accepted: 11 January 2016

Published: 03 February 2016

Citation:

Xu Z, Qin Y, Wang Y, Li X, Cao H and Zheng SJ (2016) A Critical Role of Bacterioferritin in *Salmonella pullorum*-Induced IFN- β Expression in DF-1 Cells. *Front. Microbiol.* 7:20.
doi: 10.3389/fmicb.2016.00020

Salmonella enterica serovar Pullorum (*S. pullorum*) causes pullorum disease in poultry and results in great economic losses to the poultry industry. Although an eradication program has been successfully performed in some countries, it remains a major threat to countries with poor poultry disease surveillance. Currently there are no effective control measures for pullorum disease except eradication. In particular, the pathogenesis of *S. pullorum* infection is still largely unknown. Here we identified bacterioferritin (Bfr) as a major antigen of *S. pullorum* to elicit a humoral immune response. Furthermore, we demonstrate that Bfr induces activation of IFN- β promoter and mRNA expression in DF-1 cells, and that the amino acids 1–50 form a critical domain involved in IFN- β expression. Moreover, we found that the p38 MAPK signaling pathway was essential for Bfr-induced IFN- β expression. Importantly, *S. pullorum*-induced IFN- β expression was totally abolished by deficiency of Bfr in the bacteria, indicating that Bfr plays a critical role in *S. pullorum* induced IFN- β expression in DF-1 cells. Our findings provide new insights into the molecular mechanisms of the host response to *S. pullorum* infection.

Keywords: *Salmonella pullorum*, bacterioferritin, interferon, IFN- β

INTRODUCTION

Pullorum disease, an acute systemic disease commonly seen in young birds, is caused by *Salmonella enterica* serovar Pullorum. The clinical signs of pullorum disease are characterized by anorexia, diarrhea, dehydration, weakness and high mortality in young chicks, but this disease usually shows a persistent infection and causes decreased egg production and diarrhea in adult fowls (Shivaprasad, 2000). Pullorum disease is basically controlled in Europe and North America, but it still occurs in many countries such as Brazil, Argentina, India, and China, leading to severe economic losses (Barrow and Freitas, 2011; Barrow et al., 2012). *Salmonella* spp. belongs to the Enterobacteriaceae family. *Salmonella* is a Gram-negative and facultative intracellular pathogen which, depending on the serotype and host, can cause diseases ranging from gastroenteritis to typhoid fever (Marcus et al., 2000). *S. pullorum*, currently belonging to biovars of serovar Gallinarum within serogroup D, has identical somatic antigens (O1, O9, O12) and no flagella due to mutations in flagellar genes while its pathogenicity is restricted only to avian species (Barrow and Freitas, 2011). The relatively high rate of accumulation of mutations in the genome of *S. pullorum* suggests a rapid rate of evolution associated with the host adaptation, particularly in the development of *S. pullorum* (Barrow and Freitas, 2011). During *S. pullorum* infection, the

interaction of this pathogen with the immune system occurs in three main phases, including invasion via the gastrointestinal tract, establishment of systemic infection and induction of cytokine expression (Chappell et al., 2009).

High titers of anti-*Salmonella* IgY were produced by birds infected with *S. pullorum* from 5 weeks onwards and *S. pullorum* was detected in splenic macrophages from 3 days to 10 weeks postinfection (Wigley et al., 2001). It was found that approximate 1 to 2% of macrophages contained fluorescent *Salmonella* bacteria in all birds examined, and dropped to less than 1% at 5 weeks postinfection and even further at 10 weeks to less than 0.5% of cells infected (Wigley et al., 2001), indicating that macrophage plays a critical role in clearance of *S. pullorum*. An antigen-specific T-cell response to *S. pullorum* was found in birds at 5 and 9 weeks postinfection, but dropped to negligible levels at 17 weeks postinfection (Wigley et al., 2005). The numbers of *S. pullorum* bacteria recovered from the spleen, liver, the reproductive tracts and developing eggs increased following the fall in T-cell proliferation activity at 18 weeks postinfection, while T-cells proliferation began to increase at 22 weeks postinfection (Wigley et al., 2005). In contrast to T-cell response, antibody response did not decline (Wigley et al., 2005). Like other pathogens, *Salmonella* infection stimulates cytokine production. The induction of cytokines such as IL-1 β , IL-8, IL-12, IL-17, IL-18, TNF- α , and IFN- γ following *Salmonella* infection of chickens have been previously reported (Withanage et al., 2004; Berndt et al., 2007; Crhanova et al., 2011). One of the most remarkable features of *Salmonella* infection is that IFN- β was induced in fibroblasts and macrophages (Hess et al., 1989; Robinson et al., 2012). The role of IFN- β in the response to bacterial infection is variable, and it contributes to a variety of beneficial and detrimental immune functions (Monroe et al., 2010).

The iron that is acquired by the pathogenic bacterium is used for numerous biochemical activities and any surplus iron that is available is stored within the bacterial cell in the form of Bfr (Ratledge, 2007). Bfr belongs to an outer membrane protein in *S. hadar* as examined by a proteomic approach (Snoussi et al., 2012). Bfr is a major iron storage protein and protects against hydrogen peroxide toxicity, and the haeme-containing Bfr was found exclusively in bacteria (Velayudhan et al., 2007).

Currently it is known that Bfr is a T-cell antigen that induced a strong IFN- γ production and the proliferation of lymphocytes (Denoel et al., 1997; Al-Mariri et al., 2002; Lee et al., 2006). In addition, Bfr induced humoral immune response in mice immunized with DNA vaccine encoding the Bfr or recombinant Bfr proteins (Al-Mariri et al., 2001a,b). The antibodies against Bfr were detected from Crohn's disease, and 53% of Crohn's disease patients were positive, indicating that Bfr was a specific protein antigen of *Mycobacterium paratuberculosis* (Walmsley et al., 1996). However, little is known about the role of Bfr in innate immune responses. DF-1, an immortal chicken embryo fibroblast cell line, is commonly used for the research of *Salmonella* (Li et al., 2006; Szmolka et al., 2015) and type I interferon (Li et al., 2013). To gain a better understanding about the role of Bfr in innate immune responses, we set out to determine if Bfr induces humoral immune response in chickens and

induces type I IFN expression in *S. pullorum* infected DF-1 cells.

In this study, we demonstrate that Bfr is a major antigen of *S. pullorum*, and Bfr induced IFN- β mRNA expression in DF-1 cells. In addition, we show that the amino acids 1–50 form a critical domain involved in activation of the IFN- β promoter. Furthermore, we found that Bfr induced IFN- β expression likely via the p38 mitogen-activated protein (MAP) Kinase signaling transduction pathway. Importantly, we found that *S. pullorum*-induced IFN- β expression was totally abolished by deficiency of Bfr in the bacteria, indicating that Bfr plays a critical role in *S. pullorum*-induced IFN- β expression in DF-1 cells.

MATERIALS AND METHODS

Bacteria and Cells

Salmonella pullorum strain 533 was obtained from China Institute of Veterinary Drug Control (Beijing, China). *Escherichia coli* DH5 α and *E. coli* BL21 (DE3) strains were obtained from TransGen Biotech (Beijing, China). Bacteria were grown in LB medium. DF-1 cells were obtained from ATCC (USA), and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in 5% CO₂ incubator.

Reagents

Protein A/G beads were purchased from GE Healthcare (USA). Protease inhibitor cocktail C was purchased from Yataihengxin Company (Beijing, China). The restriction enzymes *Bam*H I, *Xho* I, and *Sph* I were purchased from TaKaRa (Dalian, China). The Mops, FeCl₂ and H₂O₂ were purchased from Solarbio Company (Beijing, China). The Large Amount Without Endo-Toxin Plasmid Preparation Kits and the monoclonal antibody against GAPDH were purchased from CWBio (Beijing, China). DMEM medium was purchased from Hyclone (USA). The jetPRIME reagent was purchased from Polyplus-transfection (France). The serum against *S. pullorum* was collected from chickens with pullorum disease and the control serum from SPF chickens from Beijing Agricultural University Animal Technology Company (Beijing, China). Monoclonal antibody against His-tag fusion protein was purchased from Abmart (Shanghai, China). Anti-GFP monoclonal antibody, anti-p38 monoclonal antibody, and anti-p-p38 monoclonal antibody were purchased from Santa Cruz Biotechnology (USA). Monoclonal antibody against Bfr (Clone ID: EU-0218), pGL3-chIFN- α -luc and pGL3-chIFN- β -luc were obtained from CAEU Biological Company (Beijing, China). HRP-conjugated goat-anti mouse polyclonal antibodies and HRP-conjugated goat-anti rabbit polyclonal antibodies were purchased from DingGuoShengWu Company (Beijing, China). Rabbit-anti chicken polyclonal antibodies were purchased from Bioss (Beijing, China). pET28a (+) vector was obtained from Novagen (USA). pEGFP-N1 vector was purchased from Clontech (USA). p38 (mitogen-activated protein kinase, MAPK) inhibitor SB203580 and JNK inhibitor SP600125 were purchased from Enzo Life Sciences (USA). Red homologous recombination using the plasmids pKD46, pKD3, and pCP20 were kindly provided by Professor Guo-Qiang Zhu (Yangzhou University, China).

Pull-Down Assay

Salmonella pullorum was grown in LB medium. The bacterial culture were centrifuged at 6000 \times g for 5 min, and the pellet was resuspended and lysed in pH 7.4 PBS buffer by ultrasonic treatment. Then 50 μ L of 25% protein A/G beads were preincubated with 60 μ g rabbit-anti chicken polyclonal antibodies and 200 μ L pullorum-positive serum or negative serum as controls for 8 h at 4°C. The mixture was washed three times with pH7.4 PBS by centrifugation at 825 \times g for 3 min at 4°C and the supernatant was removed after the last wash. The rabbit-anti chicken polyclonal antibody-conjugated beads with anti-*S. pullorum* antibodies mixed with the cell extract from *S. pullorum* and incubated at 4°C for 8 h. The mixture was washed as above described. The immunoprecipitates were suspended with 40 μ L 1x SDS-PAGE loading buffer and boiled for 10 min before resolved on 12% SDS-PAGE gel. Then the gel was stained with Coomassie blue dye for analysis of specific bands.

Mass Spectrometric Identification of Proteins

After separation of proteins on SDS-PAGE gel, the interesting bands were cut out and subjected to liquid chromatography-mass spectrometry. Briefly, the interesting peptides extracted from gel were dissolved in 0.1% formic acid, and then separated by a Nano-LC system (Micro-Tech Scientific, Vista, CA, USA) equipped with a C₁₈ reverse phase column. The peptides were eluted using a 120 min gradients from 0 to 50% acetonitrile in 0.1% formic acid at a constant flow rate of 400 nL/min. Mass spectra were recorded on a 7-T Fourier transform ion-cyclotron resonance (FTICR) mass spectrometer, Apex-Qe (Bruker Daltonics, Bremen, Germany). Data were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics, Bremen, Germany). Three strongest peaks of each MS acquisition were selected for the following MS/MS analysis. The MS/MS spectra were processed by DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany) with S/N \geq 4.0, and automatically searched against IPI.RAT database (version 3.41) using the Mascot 2.1.0 (Matrix Science, London, U.K.). The NCBI database was used in the search.

Construction of Plasmids

The *bfr* gene was amplified from *S. pullorum* genomic DNA by PCR using the specific primers containing *Bam*H I in sense and *Xho* I in antisense (sense: 5'-CGCGGATCCATGA AAGGTGATGTTAAA-3'; antisense: 5'-CCG CTCGAGATC GGTAACCTAATTG-3') that were designed with reference to the published sequence (GenBank, gene ID: 661554730), and the PCR products were cloned into the pET28a (+). The resulting plasmid was named pET28a-*bfr*. The *bfr* gene was then subcloned into pEGFP-N1 vector using primers with *Xho* I in sense and *Bam*H I in antisense (sense: 5'-CCGCTCGAGATGAAAGGTGATGTTAAA-3'; antisense: 5'-CGGGATCCGATCGTAACCTAATT-3') and this plasmid was named pEGFP-*bfr*. Different truncated *bfr* segments were subcloned into pEGFP-N1 vectors and were named Bfr (1–50aa), Bfr (51–158aa), Bfr (101–158aa) accordingly. The

Bfr (1–50aa) plasmid was constructed using the same sense primer as pEGFP-*bfr* plasmid and the antisense primer is 5'-GGATCCGATCAATGGATTGATGGTAC-3' containing *Bam*H I restriction site. The Bfr (51–158aa) and the Bfr (101–158aa) plasmids were constructed using the same antisense primer as pEGFP-*bfr* plasmid. For Bfr (51–158aa) plasmid, the sense primer is 5'-CCGCTCGAGAGATGAAACACGCCGAT A-3'. For Bfr (101–158aa) plasmid, the sense primer is 5'-CTCGAGCTACGTGAGGCAATGCC-3'. All the sense primers contained *Xho* I restriction site. All the primers were synthesized by Sangon Company (Shanghai, China), and all the constructs were confirmed with sequencing analysis by Huada Company (Beijing, China).

Iron Uptake Assays

Iron uptake by rBfr was examined using SpectraMax M5 according to the method described by Timoteo et al. (2012). Briefly, reaction of 0.5 μ M rBfr with 12 μ M Fe²⁺ ions and 72 μ M H₂O₂ in 0.2 M Mops buffer (pH 7) and 0.2 M NaCl, and BSA was used as control. The iron storage capacity was determined by plotting the OD optical density at 200–400 nm in Spectra-Max M5.

Transfection and Reporter Gene Assays

DF-1 cells (1×10^5) were seeded on 24-well plates and cultured overnight before transfection with pEGFP-*bfr* or pEGFP-N1, together with pGL3-chIFN- β -luc (or pGL3-chIFN- α -luc) and pRL-TK using jetPRIME reagents (Polyplus-transfection). Twenty four hours after transfection, cell extracts were harvested, and the luciferase activities were examined with a dual-specific luciferase assay kit (Promega). Firefly luciferase activities were normalized based on Renilla luciferase activities. All reporter gene assays were repeated at least three times. Data are represented as mean \pm SD.

RNA Isolation and RT-PCR Analysis

Total RNA was prepared from DF-1 cells using a RNeasy kit (Aidlab, China) per the manufacturer's instruction, and was treated with DNase I. Two μ g of total RNA was used for cDNA synthesis by reverse transcription using RT-PCR kit (TaKaRa). The specific primers for chicken IFN- α 1 (sense: 5'-CCAGCACCTCGAGCAAT-3'; antisense: 5'-GGCGCTGTAATCGTTGTCT-3'), IFN- β (sense: 5'-GCCTCC AGCTCCTTCAGAACATCG-3'; antisense: 5'-CTGGATCTGGTT GAGGAGGCTGT-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense: 5'-TGCCCCATCACAGCCACA CAGAAC-3'; antisense: 5'-ACTTTCCCCACAGCCTTAGCA G-3') were designed with reference to previous publications (Li et al., 2007; Abdul-Careem et al., 2008; Liu et al., 2010) and synthesized by Sangon Company (Shanghai, China). The real-time PCR assay was carried out with a Light Cycler 480 (Roche, USA). The PCR was performed in a 20- μ L volume containing 1 μ L of cDNA, 10 μ L of 2 \times SYBR green Premix *Ex Taq* (TaKaRa), and a 0.4 μ M of each gene-specific primer. The thermal cycling parameters were referred to the previous study (Li et al., 2013), and they were as follows: 94°C for 2 min; 45 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s;

and 1 cycle of 95°C for 30 s, 60°C for 30 s, and 95°C for 30 s. The final step was to obtain a melt curve for the PCR products to determine the specificity of the amplification. All sample reactions were carried out in triplicate on the same plate, and the GAPDH gene was utilized as the reference gene. Expression levels of genes were calculated relative to the expression of the GAPDH gene and expressed as fold increase or decrease relative to the control samples.

Inhibition of Signal Transduction Pathways

DF-1 cells (4×10^5) were seeded on a 6-well plates and cultured for 24 h before treatment with p38 inhibitor SB203580 (20 μM), JNK inhibitor SP600125 (20 μM), and dimethyl sulfoxide (DMSO) as control for 1 h, and then transfected with pEGFP-*bfr* or pEGFP-N1 as controls using jetPRIME reagents. Twenty four hours after transfection, total RNA was extracted and used for cDNA synthesis. Real-time PCR was performed to examine the expressions of chicken IFN- β and GAPDH at an mRNA level as above described.

Western Blot Analysis

The pET28a-*bfr* recombinant or empty vectors were transformed into *E. coli* BL21 (DE3), and Bfr-his recombinant proteins were expressed by 1 mM IPTG induction at 37°C for 6 h. One mL bacterial cells were centrifuged at $6000 \times g$ for 5 min and the pellets were resuspended with 120 μL 1x SDS-PAGE loading buffer and boiled for 10 min before fractionated by electrophoresis on 12% SDS-PAGE gels, and the resolved proteins were transferred onto PVDF membranes (Millipore, USA). After blocking with 5% skim milk, the membranes were probed with primary antibodies [anti-His-tag (1:10000), pullorum-positive chicken serum (1:500), or pullorum-negative SPF chicken serum (1:500)], followed by incubation with HRP-conjugated goat anti-mouse IgG (1:20000; DingGuoShengWu, China) or HRP-conjugated rabbit anti-chicken IgY (1:5000) secondary antibodies (Bioss, China). The blots were visualized using the ECL reagent according to the manufacturer's instructions (CWBio, China). For GFP-Bfr expression analysis, cells were transfected with pEGFP-*bfr* or pEGFP-N1, cells lysates were prepared and examined with anti-GFP antibodies. For signal transduction pathways analysis, cells were transfected with pEGFP-*bfr* or pEGFP-N1. The whole cell extracts were lysed in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, 10% glycerin, 1% Triton X-100) containing with 1% protease inhibitor cocktail C and 1% 20 mM phosphatase inhibitors (NaF) and examined with Western Blot using anti-p-p38, anti-p38, anti-GFP, and anti-GAPDH antibodies.

Generation of the Bfr-Deficient *S. pullorum*

The Bfr-deficient strain was constructed by λ -Red-mediated recombination system, according to the method described by Datsenko and Wanner (2000). Briefly, the specific primers (ΔBfr1 : 5'-ATGAAAGGTGATGTTAAAATC

ATAAATTATCTCAAT AAACATTGGGAAATGTTAGGCTGG AGCTGCTTCG-3'; ΔBfr2 : 5'-TTAATGGTAACCTTAATTG GATTGCAGATAATTGACATACCAAGTTCATATGA ATAT CCTCCTTAG-3'), including 50-bp homology extension from the 5' and 3' of the *bfr* gene, were designed to amplify the chloramphenicol cassette from the template plasmid pKD3 by PCR. The PCR products were purified and electroporated into *S. pullorum* containing the pKD46 plasmid. Recombinant bacteria *S. pullorum* Bfr::cat was screened and selected on both Cm and Amp resistance LB agar plates. Gene deletion was confirmed by PCR using the specific primers (Bfr1: 5'-ATGAAAGGTGATGTTAAA-3'; Bfr2: 5'-ATCGGTAACCTTAATTG-3'). Then the Cm cassette gene of *S. pullorum* Bfr::cat was excised via introducing the Flp recombinase-expressing vector pCP20 by electroporation. The Bfr-deficiency in parental strain was confirmed by PCR, DNA sequencing and Western Blot.

To generate the ΔBfr -complemented strain, the Bfr open-reading frame was amplified by PCR using *S. pullorum* genomic DNA with the specific primers containing *BamH* I and *Sph* I (PBR-Bfr1: 5'-CGCGGATCCATGAAAGGTGATGTTAAA-3'; PBR-Bfr2: 5'-ACATGCATGCTTAATCGGTAAACCTTAATTG-3'). The expected 477 kb PCR product of *bfr* gene was confirmed by DNA sequencing, and further cloned into the plasmid pBR322. The constructed recombinant plasmid was electroporated into the Bfr-deficient bacteria to obtain the ΔBfr -complemented strain. The restoration of Bfr in parental strains was confirmed by PCR and Western Blot.

Infection of DF-1 Cells with Wild Type (WT), Bfr-Deficient and Complemented *S. pullorum*

DF-1 cells were infected with WT, Bfr-deficient (KO) and ΔBfr -complemented (RS) strains at an MOI of 500 or indicated doses. Eight hours after infection, total RNA was extracted from the infected cells and used for cDNA synthesis. Real-time PCR was performed to examine chicken IFN- β expression as above described.

Statistical Analysis

The significance of the differences between the treatment group and control in the activation of promoters and mRNA expressions (cytokine and transcription factor) was determined by the ANOVA and Mann-Whitney accordingly.

RESULTS

Screening for the Major Antigens of *S. pullorum*

Since *S. pullorum* infection elicits a robust humoral immune response in chickens, we wanted to determine the major antigens of *S. pullorum* responsible for the induction of specific antibodies. We proposed that major antigens of *S. pullorum* could be pulled down by specific IgY in the anti-*S. pullorum* Ab positive serum. To test our hypothesis, we performed a pull-down

assay using anti-*S. pullorum* Ab positive serum of chickens and the bacterial cell extract of *S. pullorum* according to the published method (Cho et al., 2013). We found that there was an extra clear protein band in the immunoprecipitates of the mixture of anti-*S. pullorum* Ab-positive serum with bacterial cell lysate as compared to that of controls as demonstrated by SDS-PAGE (**Figure 1A**), indicating that the antigens of *S. pullorum* could be pulled down by anti-*S. pullorum* antibodies. To analyze the amino acid sequence of this major antigen, we cut-down the interesting protein band and performed a mass spectrometry. As a result, the arrow-pointed band in **Figure 1A** is a protein named bacterioferritin (Bfr) based on online information from GenBank (Gene ID: 661554730; **Figure 1B**). Although Bfr is known as a protein antigen of *M. paratuberculosis* (Walmsley et al., 1996), our data indicate that this protein might be an important major antigen of *S. pullorum*.

To determine the antigenicity of Bfr, we cloned *bfr* gene from genomic DNA of *S. pullorum* and expressed Bfr-his recombinant protein using an *E. coli* expression system. We found that the Bfr-his fusion protein could be detected by anti-*S. pullorum* Ab positive serum of chickens but not by negative chicken serum (**Figure 1C**), indicating that Bfr-his protein is of good antigenicity. These results suggest that Bfr may serve as a major antigen of *S. pullorum*.

To determine the basic function of Bfr-his(rBfr) fusion protein, we performed the iron uptake assays with rBfr according to the published method (Timoteo et al., 2012). We found that the color of Fe³⁺ in the BSA control could be clearly observed. In contrast, Fe³⁺ color was markedly reduced with rBfr treatment (**Figures 2A,B**), indicating that Bfr can rapidly uptake free Fe²⁺ for oxidation in the presence of H₂O₂ as the oxidant. These data suggest that rBfr is a functional iron storage protein.

Bfr Induces IFN- β Expression in DF-1 Cells

Bfr is a major iron storage protein in bacteria (Velayudhan et al., 2007). It was reported that iron could affect innate immune response by influencing IFN- γ mediated pathways in macrophages (Nairz et al., 2014). This information prompted us to examine the effect of *S. pullorum* Bfr on the innate immune response in host cells. We made a pEGFP-*bfr* expression construct, and transfected DF-1 cells with pEGFP-*bfr* or pEGFP-N1 as control. As shown in **Figure 3A**, both GFP-Bfr and GFP were expressed well in the transfected cells as demonstrated by Western Blot using anti-GFP monoclonal antibody. Importantly, transfection of DF-1 cells with pEGFP-*bfr* markedly enhanced activation of IFN- β promoter, but not IFN- α , as compared to that of controls (**Figures 3B,C**). Consistent with this observation, the mRNA expressions of IFN- β in pEGFP-*bfr* transfected cells significantly increased as compared to that of pEGFP-N1 transfected controls, but the mRNA expression of IFN- α was unaffected (**Figures 3D,E**). These results suggest that intracellular Bfr induces IFN- β response in host cells.

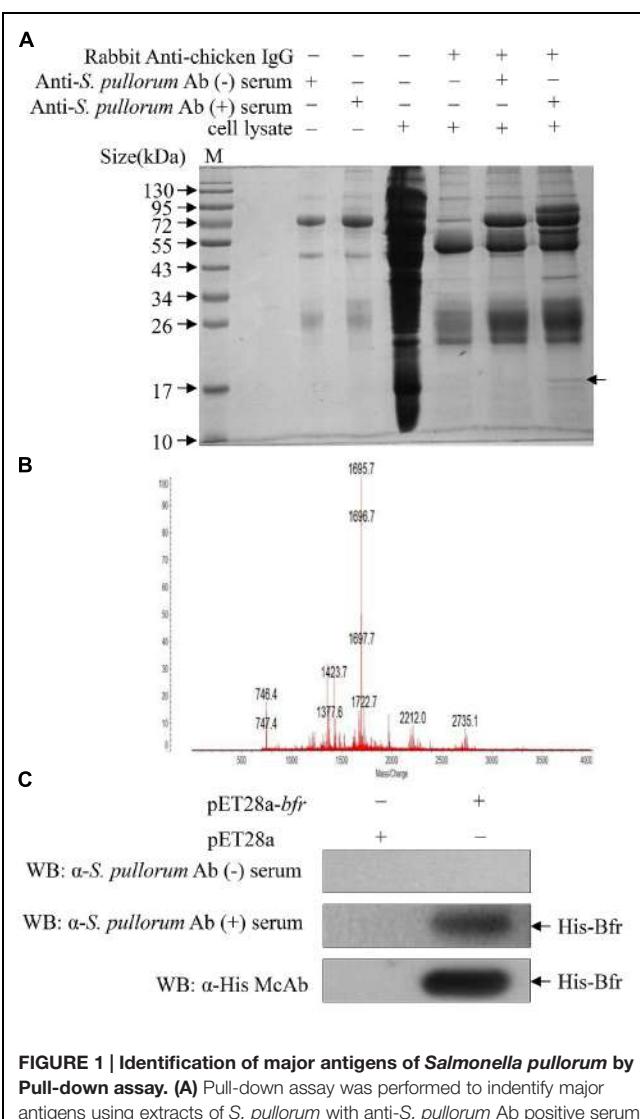
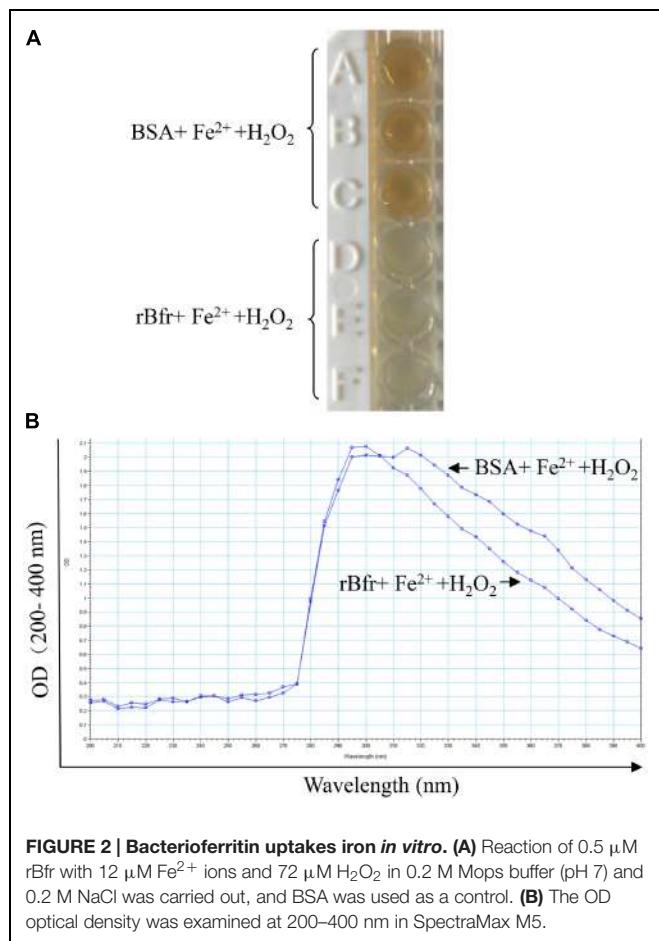


FIGURE 1 | Identification of major antigens of *Salmonella pullorum* by Pull-down assay. (A) Pull-down assay was performed to indentify major antigens using extracts of *S. pullorum* with anti-*S. pullorum* Ab positive serum of chickens and negative serum as controls. The pull-down pellets were examined by SDS-PAGE. (B) The arrow-pointed band was cut down and subjected to Mass Spectrometry analysis. (C) Recognition of recombinant His-bacterioferritin (Bfr) fusion protein by anti-*S. pullorum* Ab positive serum. Expressions of recombinant His-Bfr fusion protein were detected by Western Blot using anti-*S. pullorum* Ab positive serum, negative serum and anti-His McAb as control.

Amino Acids 1–50 of Bfr are Responsible for Inducing IFN- β Expression

To determine the domain of Bfr that is responsible for inducing IFN- β expression, we constructed pEGFP-truncated *bfrs* encoding different lengths of Bfr as indicated in **Figure 4A**. We transfected DF-1 cells with the constructs and performed real-time PCR assay with specific primers. As shown in **Figures 4B,C**, transfection of cells with the vectors carrying the full-length and amino acids 1–50 portion of Bfr significantly induced IFN- β expression as compared to that of pEGFP-N1 transfected controls. In contrast, the construct without the gene encoding the amino acids 1–50 portion of Bfr failed to induce IFN- β



expression. These results indicate that the amino acids 1–50 of Bfr is a critical domain responsible for inducing IFN- β response.

Bfr-Activated p38 MAP Kinase is Involved in IFN- β Expression

It was reported that activation of p38 MAP kinase was required for induction of *ifnb* gene expression in response to bacteria in the cytosol (O’Riordan et al., 2002). To dissect the signaling pathways involved in Bfr-induced IFN- β expression, we treated DF-1 cells for 1 h with the inhibitors of the key signaling molecules including p38 MAPK, JNK MAPK or DMSO as controls before pEGFP-*bfr* transfection. Twenty-four hours after transfection, real-time PCR was performed to examine the mRNA levels of IFN- β . As shown in Figure 5A, p38 MAPK inhibitor, but not JNK MAPK inhibitor, significantly inhibited IFN- β expression in cells with pEGFP-*bfr* transfection ($p < 0.001$). It is well known that the activation of p38 pathway requires phosphorylation of p38 (Wang et al., 1997). We therefore examined whether p38 is phosphorylated during Bfr expression. We transfected DF-1 cells with pEGFP-*bfr*, and examined the phosphorylation of p38 in pEGFP-*bfr* transfected cells with Western Blot using specific antibodies against p-p38, p38, and GAPDH. As a result, p38 phosphorylation was markedly enhanced in pEGFP-*bfr* transfected cells (Figures 5B–D). Taken together, these results

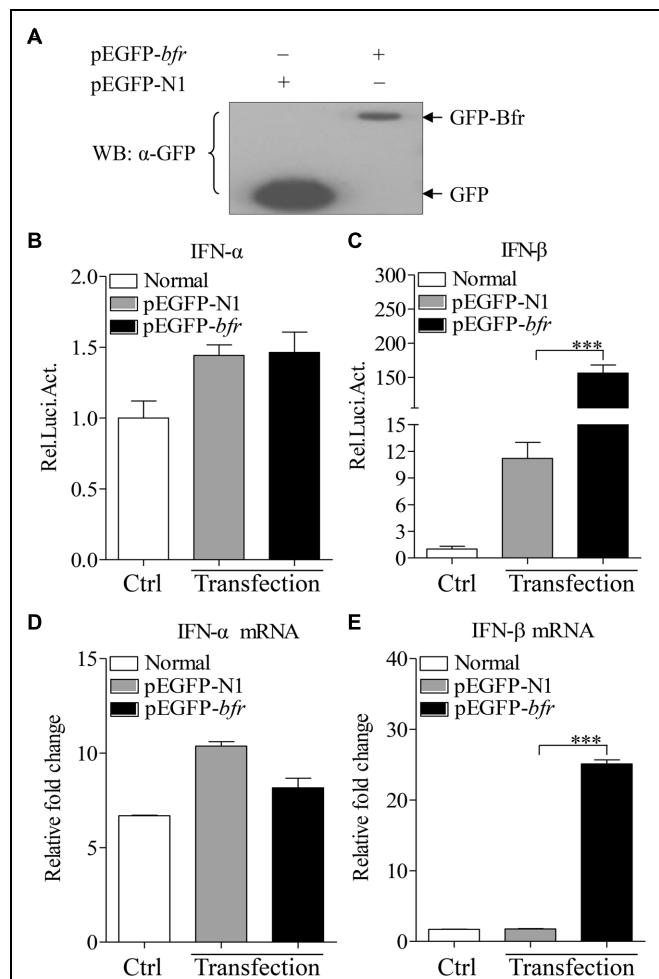


FIGURE 3 | Expression of Bfr induces activation of IFN- β promoter and enhances mRNA expressions of IFN- β in DF-1 cells. (A) DF-1 cells were transfected with pEGFP-*bfr* or pEGFP-N1. Twenty four hours after transfection, cell lysates were prepared and examined with Western Blot using anti-GFP monoclonal antibody. **(B,C)** DF-1 cells were transfected with pEGFP-*bfr* or pEGFP-N1 as controls. Twenty-four hours after transfection, cell lysates were prepared and activation of IFN- α and IFN- β promoters were examined with luciferase reporter gene assays. **(D,E)** DF-1 cells were transfected with pEGFP-*bfr* or pEGFP-N1 as controls. Twenty-four hours after transfection, cells were collected and mRNA expressions of IFN- α and IFN- β were examined with qRT-PCR using specific primers. The expression levels of mRNA were calculated in relation to the expression level of GAPDH. Results are representative of three independent experiments. Data are represented as mean \pm SD, $n = 3$. *** $p < 0.001$.

clearly show that Bfr-induced phosphorylation of p38 is involved in induction of IFN- β expression. Thus the p38 MAPK pathway is essential for Bfr-induced IFN- β expression.

Bfr Plays a Critical Role in *S. pullorum*-Induced IFN- β Expression in Cells

The fact that Bfr induced expression of IFN- β in host cells prompted us to examine the role of Bfr in *S. pullorum*-induced IFN- β response in cells. We generated a Bfr-deficient *S. pullorum*

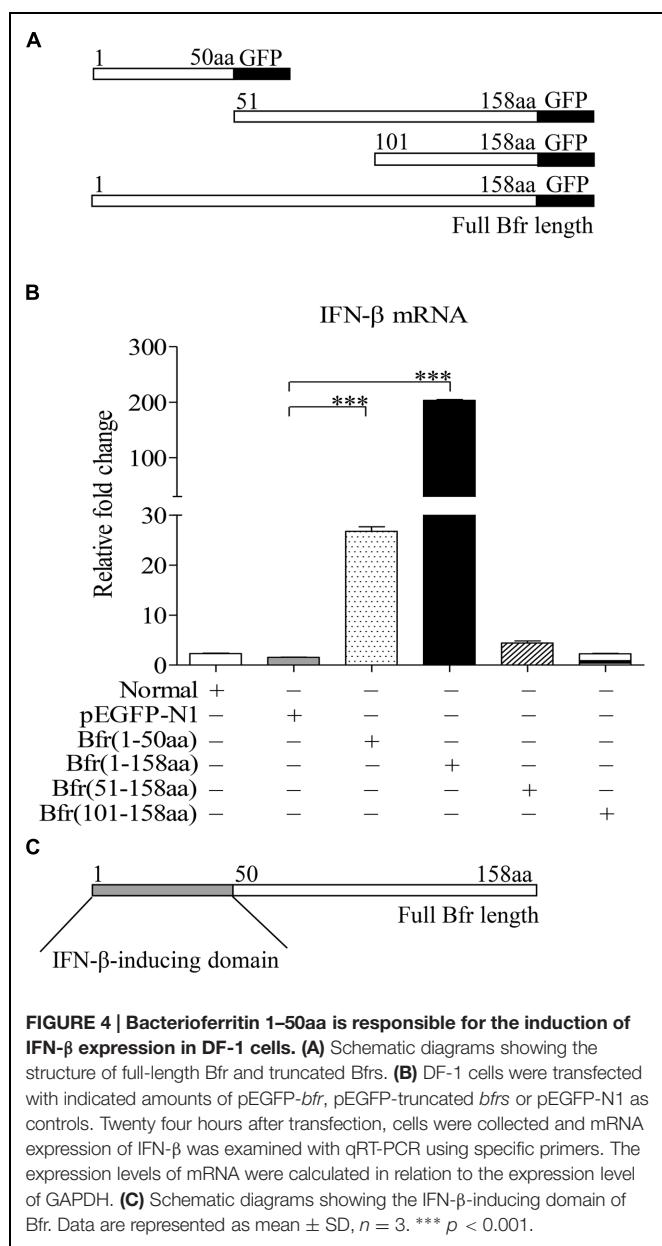


FIGURE 4 | Bacterioferritin 1–50aa is responsible for the induction of IFN- β expression in DF-1 cells. **(A)** Schematic diagrams showing the structure of full-length Bfr and truncated Bfrs. **(B)** DF-1 cells were transfected with indicated amounts of pEGFP-*bfr*, pEGFP-truncated *bfrs* or pEGFP-N1 as controls. Twenty four hours after transfection, cells were collected and mRNA expression of IFN- β was examined with qRT-PCR using specific primers. The expression levels of mRNA were calculated in relation to the expression level of GAPDH. **(C)** Schematic diagrams showing the IFN- β -inducing domain of Bfr. Data are represented as mean \pm SD, $n = 3$. *** $p < 0.001$.

strain using λ -Red-mediated recombination system according to a simple gene disruption strategy (Figure 6A) and also generated the Δ Bfr-complemented strain expressing Bfr by electroporation of Bfr-deficient *S. pullorum* with pBR322-*bfr*. As shown in Figures 6B,C, WT and the complemented *S. pullorum* strains expressed Bfr very well. In contrast, Bfr-deficient *S. pullorum* had no detectable Bfr as examined by PCR and Western Blot. These results indicate that Bfr-deficient *S. pullorum* strain and its complemented strain expressing Bfr were successfully generated.

We cultured WT, Bfr-deficient and the complemented *S. pullorum* strains in culture medium, and compared their growth at 0, 6, 12, and 24 h after culture. As a result, we did not find any difference between WT, Bfr-deficient and the complemented *S. pullorum* strains in terms of their growth (data

not shown), suggesting that deficiency of Bfr in *S. pullorum* does not affect the bacterial replication. We infected DF-1 cells with WT *S. pullorum* at an MOI of 1, 5, 20, 100, or 500. Eight hour after infection, the mRNA expression of IFN- β was examined with real-time PCR assay. As shown in Figure 7A, infection of DF-1 cells with WT *S. pullorum* markedly induced mRNA expression of IFN- β in cells ($p < 0.001$). However, WT *S. pullorum*-induced IFN- β expression was completely abolished by deletion (knock-out) of *bfr* gene from the bacteria (Figure 7B), indicating that Bfr is required for *S. pullorum*-induced IFN- β expression. Thus, Bfr plays a critical role in *S. pullorum*-induced innate response in host cells.

DISCUSSION

Salmonella pullorum is a worldwide distributed poultry pathogen of considerable economic importance to the poultry industry, particularly to that of developing countries. An increasing number of *S. pullorum* strains were isolated in China, and many of these bacteria showed antimicrobial resistance (Pan et al., 2009). This pathogen causes high mortality in young chickens and persistent infection in adult chickens with clinical signs of decreased egg production and diarrhea. Although the humoral immune response against *S. pullorum* cannot clear the pathogens once the bacteria intrude host cells, the specific antibodies still play an important role in mediating phagocytosis of extracellular bacteria by phagocytes. In particular, the examination of anti-*S. pullorum* antibodies is of clinically diagnostic importance. Thus hemagglutination assay with the whole blood of chickens is generally performed to screen for the *S. pullorum*-infected chickens in flocks. Immunoprecipitation of whole bacterial cell lysate by antibodies against *S. pullorum* (Pull-down assay) is an efficient method that allows us to identify major antigens of the microorganism that elicit humoral immune response. The identified antigen would help to develop new diagnostic methods or vaccine in control of the disease (Britton et al., 1988; Sexton et al., 1991). High titers of anti-*Salmonella* IgY were produced by birds infected with *S. pullorum* after 5 weeks (Wigley et al., 2001). However, little information is available regarding the major antigen of *S. pullorum*. In this study, a major antigen Bfr that elicited antibody response was identified with a pull-down assay and Mass spectrometric method. Our results demonstrate that recombinant Bfr can be specifically recognized by pullorum-positive serum. Thus, Bfr-induced antibodies probably play an important role in host response against *S. pullorum* infection.

Bfr, a 17-kDa protein that was previously identified as an outer membrane protein of *S. hadar*, is composed of 24 identical subunits along with the usual ferroxidase sites that have 12 binding sites for heme iron (Wong et al., 2009; Snoussi et al., 2012). Bfr is also the major Fe storage protein in *Salmonella* (Velayudhan et al., 2007). We could observe the color of Fe³⁺ from the purified Bfr-his recombinant protein in our experiments. Inactivation of Bfr induces intracellular free Fe concentration and exhibits increased susceptibility to oxidative stress (Velayudhan et al., 2007). It was found that the

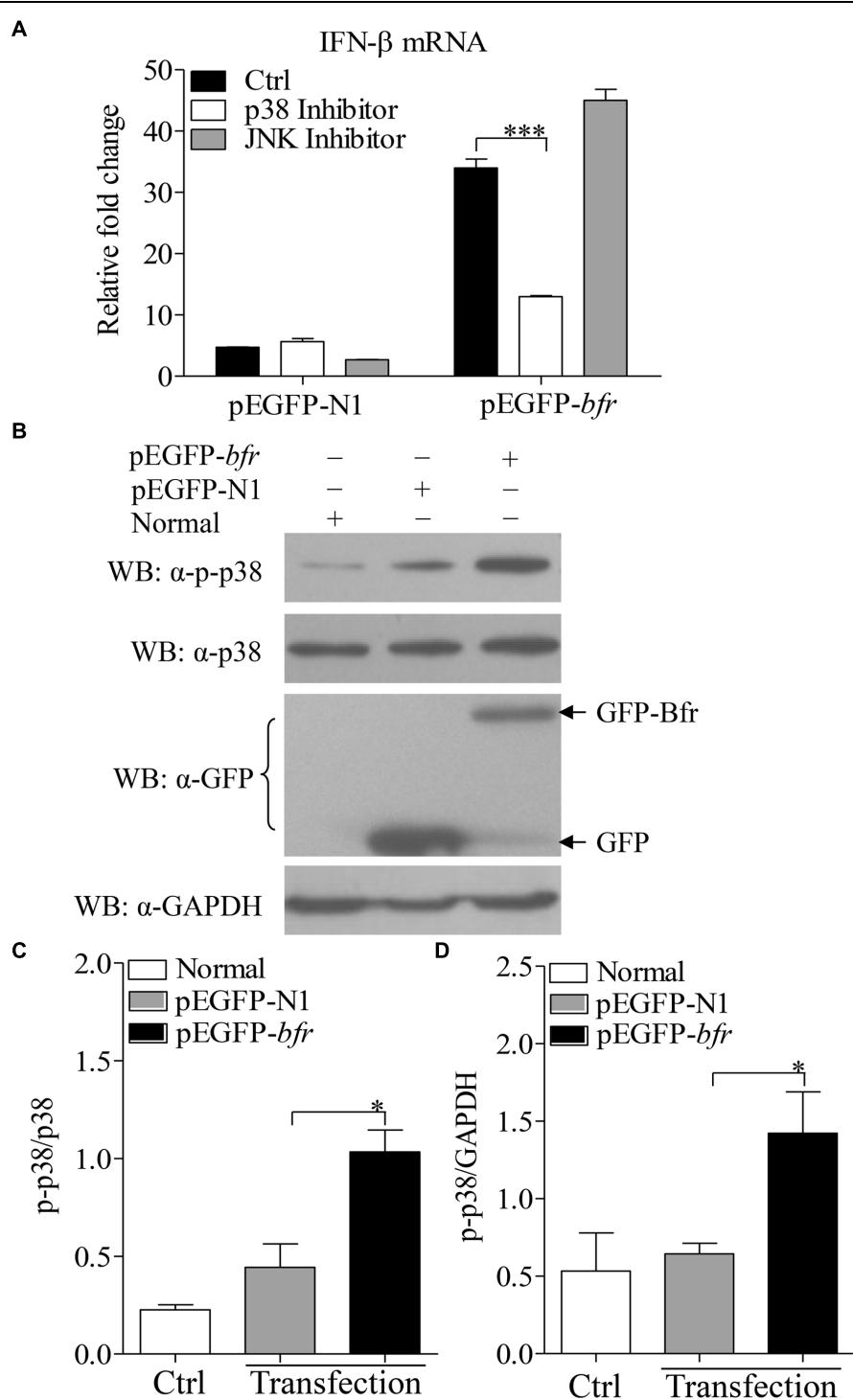


FIGURE 5 | Bacterioferritin induced IFN- β expression via p38 MAP Kinase signaling pathway. **(A)** Effects of p38 and JNK inhibitors on Bfr-induced IFN- β expression. DF-1 cells were transfected with indicated amounts of pEGFP-bfr or pEGFP-N1 as controls, and treated with inhibitors of p38, JNK, or DMSO as controls for 1 h. Twenty four hours after transfection, mRNA expression of IFN- β was examined with real-time PCR. **(B–D)** Effects of Bfr on phosphorylation of p38. DF-1 cells were transfected with pEGFP-bfr or pEGFP-N1 as controls. Twenty four hours after transfection, cell lysates were prepared and examined with Western Blot for the detection of p-p38, p38, GFP, GFP-Bfr, and GAPDH. The band density of p-p38, p38 and GAPDH in normal, pEGFP-bfr or pEGFP-N1 transfected cells in **(B)** was quantitated by densitometry, and the relative levels of p-p38 in **(B)** were calculated as follows: band density of p-p38/band density of p38 **(C)** or GAPDH **(D)**. Results are representative of three independent experiments. Data are represented as mean \pm SD, $n = 3$. *** $p < 0.001$ and * $p < 0.05$.

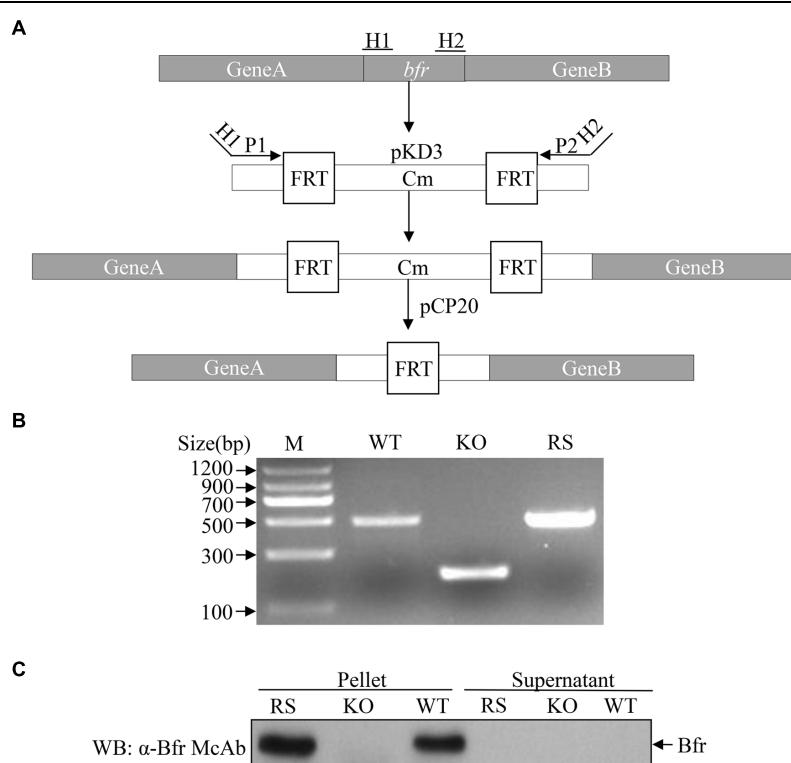


FIGURE 6 | Generation of Bfr-deficient *S. pullorum* strain by Red homologous recombination. (A) Schematic diagrams showing the strategy for the deletion of *bfr* gene. **(B)** Identification of recombinant (KO) and complemented (RS) strains by PCR. PCR was performed to examine *bfr* gene in WT, KO, and RS *S. pullorum* strains. **(C)** Examination of Bfr expressions in WT, KO, and RS *S. pullorum* strains. Cell lysates of WT, KO, and RS *S. pullorum* were prepared, and Bfr expressions were examined with Western Blot using anti-Bfr monoclonal antibody.

transcription factor SsrB controls resistance to reactive oxygen species through Bfr in *Salmonella* (Brown et al., 2014). This information suggests that Bfr is involved in regulation of iron homeostasis and protects against hydrogen peroxide toxicity in bacteria. H₂O₂ can react with Fe²⁺ ions via Fenton and Haber-Weiss reactions, producing ROS (reactive oxygen species) such as hydroxyl radical or superoxide, which are capable of damaging most cellular components, such as nucleic acids, protein or membrane lipids (Timoteo et al., 2012). And Bfr binds to DNA and reduces the damage of ROS by the rapid uptake and oxidation of free Fe²⁺, using H₂O₂ as the oxidant (Timoteo et al., 2012). Our results demonstrate that recombinant Bfr can mediate the rapid uptake and oxidation of free Fe²⁺, using H₂O₂ as the oxidant. Interestingly, we also found that Bfr could induce self-activation in a yeast two-hybrid screening and apoptosis in DF-1 cells in this study (data not shown). This information suggests that Bfr might be a multi-functional protein.

Bacterioferritin is an important factor in bacteria, but few reports are available regarding the cell response to Bfr. Currently it is known that Bfr is a T-cells antigen that induces a strong IFN- γ production and the proliferation of lymphocytes (Denoel et al., 1997; Al-Mariri et al., 2002; Lee et al., 2006). In addition, Bfr induced humoral immune response in mice immunized with DNA vaccine encoding the Bfr or recombinant Bfr proteins (Al-Mariri et al., 2001a,b). The patient sera of *M. leprae* could react

with Bfr, and *M. paratuberculosis* antigen D was identified as Bfr (Brooks et al., 1991; Spencer et al., 2011). These findings suggest that Bfr plays an important role in acquired immunity. However, little is known about the role of Bfr in the innate immune response. Our data show that Bfr not only acts as a potent antigen inducing humoral immune response but also as an inducer for innate immune responses (inducing IFN- β expression), indicating that Bfr is not merely a protein for iron storage and detoxification (Bou-Abdallah et al., 2002). Furthermore, we found that the amino acids 1–50 of Bfr were responsible for induction of IFN- β expression. However, Bfr did not affect the expression of IFN- α in cells. It seems that the role of Bfr is specific, only for induction of IFN- β expression.

Recognition of bacterial products by host surveillance system results in transcription of the *ifnb* gene, and the activation of cytosol-specific signaling is associated with phosphorylation of the p38 mitogen-activated protein (MAP) kinase (O’Riordan et al., 2002). In this study, when DF-1 cells were treated with p38 MAP Kinase inhibitor, Bfr-induced IFN- β expression was markedly inhibited, indicating that Bfr might activate cytosol-specific signaling. In contrast, JNK MAP Kinase inhibitor had no effects on Bfr-induced IFN- β response. These results suggest that Bfr induces IFN- β expression via the p38 signal transduction pathway. As p38 MAP kinases are major players during inflammatory responses, they can be activated by

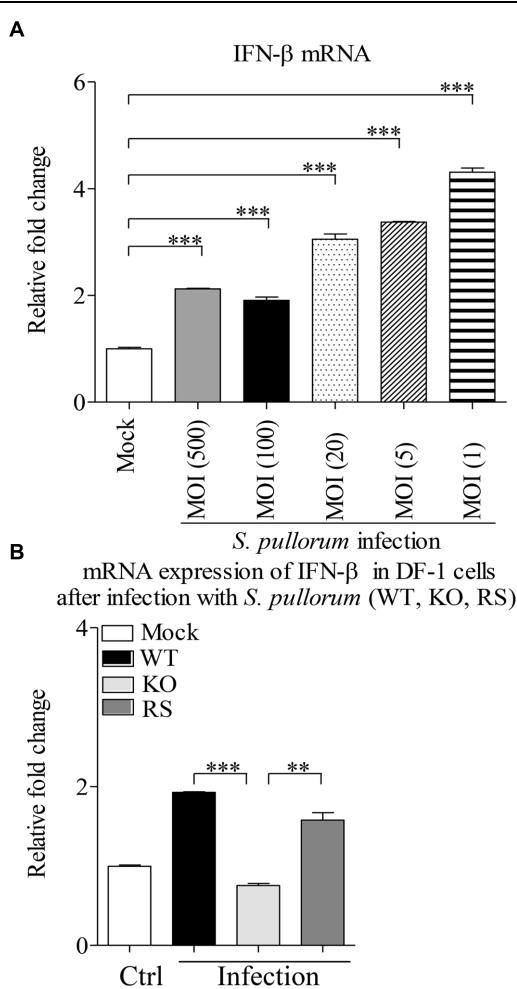


FIGURE 7 | A critical role of Bfr in *S. pullorum*-induced IFN- β expressions. (A) DF-1 cells were infected with WT *S. pullorum* at an indicated MOI, 8 h after infection, mRNA expressions of IFN- β in cells were examined with qRT-PCR using specific primers. The expression levels of mRNA were calculated in relation to the expression level of GAPDH. **(B)** DF-1 cells were infected with WT, KO, and RS *S. pullorum* strains at an MOI of 500. Eight hours after infection, mRNA expressions of IFN- β in cells were examined with qRT-PCR using specific primers. The mRNA expression levels of IFN- β in cells were calculated in relation to the expression level of GAPDH. Results are representative of three independent experiments. Data are represented as mean \pm SD, $n = 3$. *** $p < 0.001$ and ** $p < 0.01$.

environmental and cellular stresses including pathogens, heat shock, growth factors, osmotic shock, ultraviolet irradiation and cytokines (Yang et al., 2014). p38 kinases have two domains: a 135 amino acid N-terminal domain and a 225 amino acid C-terminal domain. The phosphorylation lip of p38 consists of 13 residues, Leu-171-Val-183, and the protein is activated by phosphorylation of a signal threonine (Thr-180) and a single tyrosine residue (Tyr-182) in the lip (Wang et al., 1997). Furthermore, we found that p38 phosphorylation was induced by Bfr, indicating that p38 signal transduction pathway is essential for IFN- β expression in cells.

DF-1, an immortal chicken embryo fibroblast cell line, is commonly used for the research of *Salmonella* (Li et al., 2006; Szmulka et al., 2015) and type I interferon (Li et al., 2013). Our data show that *S. pullorum* infection significantly induces activation of the IFN- β promoter in DF-1 cells, supporting the previous publication by Hess et al. (1989). In contrast, *S. pullorum*-induced IFN- β expression was completely abolished by deficiency of Bfr in the bacteria, indicating that Bfr is required for *S. pullorum*-induced IFN- β expression in cells.

IFN- β is a key cytokine in the innate immune response, mediating expression of hundreds of IFN-stimulated genes (ISGs) that are responsible for the establishment of an antimicrobial state in the infected tissue (Schmolke et al., 2014). It was reported that IFN- β potently represses *S. typhimurium*-dependent induction of IL-1 family cytokines and neutrophil chemokines and IFN- β ^{-/-} mice exhibit greater resistance to oral *S. typhimurium* infection and a slower spread of *S. typhimurium* to distal sterile sites (Perkins et al., 2015). *S. typhimurium* induces the production of IFN- β , which drives necroptosis of macrophages and allows *Salmonella* to evade the immune response that is detrimental to the survival of mice (Robinson et al., 2012). Since Bfr induced IFN- β expression through the p38 MAP Kinase signaling pathway in cells, several important questions need to be addressed. For example, what is the host protein directly targeted by Bfr? What is the role of IFN- β induced by Bfr in host cell response to *Salmonella* infection? Elucidation of these questions will further our understandings of the mechanisms underlying pathogenesis of *Salmonella* infection.

CONCLUSION

Our results demonstrate that Bfr is a major antigen of *S. pullorum*. Our data also show that Bfr induced IFN- β expression via its amino acids 1–50 portion. Furthermore, we found that the p38 MAPK signaling pathway was essential for Bfr-induced IFN- β expression. Importantly, *S. pullorum* induced-IFN- β was totally abolished by deficiency of Bfr in the bacteria, indicating that Bfr plays a critical role in *S. pullorum* induced-IFN- β expression in DF-1 cells. Our findings provide new insights into the molecular mechanisms of the host response to *S. pullorum* infection.

AUTHOR CONTRIBUTIONS

SZ and ZX conceived and designed the experiments; ZX performed the experiments; SZ and ZX analyzed the data; SZ, YQ, YW, XL and HC contributed reagents/materials/analysis tools; SZ and ZX wrote the paper.

ACKNOWLEDGMENTS

We thank Drs. Guoqiang Zhu and Fuyong Chen for their generous assistance. This work was supported by grants from Earmarked Fund for Modern Agro-industry Technology Research System (#NYCYTX-41).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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eIF2 α confers cellular tolerance to *S. aureus* α -toxin

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OPEN ACCESS

Edited by:

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Reviewed by:

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equally to this work.

Specialty section:

This article was submitted to
Microbial Immunology, a section of
the journal Frontiers in Immunology

Received: 28 May 2015

Accepted: 13 July 2015

Published: 27 July 2015

Citation:

von Hoven G, Neukirch C, Meyenburg M, Füser S, Petrivna MB, Rivas AJ, Ryazanov A, Kaufman RJ, Aroian RV and Husmann M (2015) eIF2 α confers cellular tolerance to *S. aureus* α -toxin. *Front. Immunol.* 6:383.
doi: 10.3389/fimmu.2015.00383

We report on the role of conserved stress-response pathways for cellular tolerance to a pore forming toxin. First, we observed that small molecular weight inhibitors including of eIF2 α -phosphatase, jun-N-terminal kinase (JNK), and PI3-kinase sensitized normal mouse embryonal fibroblasts (MEFs) to the small pore forming *S. aureus* α -toxin. Sensitization depended on expression of mADAM10, the murine ortholog of a proposed high-affinity receptor for α -toxin in human cells. Similarly, eIF2 $\alpha^{S51A/S51A}$ MEFs, which harbor an Ala knock-in mutation at the regulated Ser51 phosphorylation site of eukaryotic translation initiation factor 2 α , were hyper-sensitive to α -toxin. Inhibition of translation with cycloheximide did not mimic the tolerogenic effect of eIF2 α -phosphorylation. Notably, eIF2 α -dependent tolerance of MEFs was toxin-selective, as wild-type MEFs and eIF2 $\alpha^{S51A/S51A}$ MEFs exhibited virtually equal sensitivity to *Vibrio cholerae* cytolsin. Binding of *S. aureus* α -toxin to eIF2 $\alpha^{S51A/S51A}$ MEFs and toxicity in these cells were enhanced as compared to wild-type cells. This led to the unexpected finding that the mutant cells carried more ADAM10. Because basal phosphorylation of eIF2 α in MEFs required amino acid deprivation-activated eIF2 α -kinase 4/GCN2, the data reveal that basal activity of this kinase mediates tolerance of MEFs to α -toxin. Further, they suggest that modulation of ADAM10 is involved. During infection, bacterial growth may cause nutrient shortage in tissues, which might activate this response. Tolerance to α -toxin was robust in macrophages and did not depend on GCN2. However, JNKs appeared to play a role, suggesting differential cell type and toxin selectivity of tolerogenic stress responses. Understanding their function or failure will be important to comprehend anti-bacterial immune responses.

Keywords: pore forming toxins, *S. aureus* α -toxin, cellular tolerance, EIF2AK4, MAPK

Introduction

Membrane perforation by pore forming toxins (PFT) is an ancient mode of attack employed by many bacteria, which helps them to establish or sustain infection (1–3). PFT represent a large group of bacterial toxins, which can be divided into various structural families (2). Many PFT have been discovered based on their ability not only to lyse red blood cells but they also affect nucleated cells, with effects ranging from induction of cell death to proliferation, time scales of occurrence from

seconds to days after attack (4). Of particular relevance in the present context, cell autonomous defenses are in place to limit or reverse damage of nucleated target cells of PFT (4–13); they have been discussed as an integral part of the innate immune system, defending against bacteria (14). Work in *C. elegans* identified MAPK as master regulators of defense against PFT (7, 15). Whereas the importance of p38 MAPK is well established (3, 4, 9, 16–18), data on the role of jun-N-terminal kinases (JNKs) are somewhat conflicting (19–21). Large scale analyses of perforated cells have identified multiple additional changes taking place in response to PFT (15, 20, 22, 23), many of which appear to be triggered by the drop of cytosolic potassium (11, 20, 24, 25). Although the contribution of the various pathways to cell autonomous defense against PFT remains to be established in most cases, a basic concept emerges according to which removal of membrane pores (3, 10, 13, 26–32) and metabolic homeostasis (10, 12, 13, 20, 33, 34) are cornerstones of early cell autonomous defense against PFT. Importantly, mechanisms involved in pore removal depend on PFT and cell type (4, 8, 29). It appears that MAPK p38 and autophagy are required if the recovery process is prolonged as with *S. aureus* α -toxin and aerolysin (8, 20).

Phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) is a conserved stress-response activated by various PFT (12, 13, 20, 33–35). How this pathway impacts survival of target cells remains incompletely understood. Eukaryotic translation initiation requires assembly of a 43S ternary pre-initiation complex, consisting of met-t-RNAi(Met), eIF2, and GTP. In mammalian cells, this step is controlled through phosphorylation of eIF2 α at serine 51 by 1 of 4 eIF2 α -kinases (GCN2, PERK, PKR, and HRI), which respond to different types of stress (36). GCN2 serves as nutritional sensor, which is activated by uncharged t-RNAs (37, 38). Several lines of evidence indicate that membrane stress triggers this pathway: first, mutations that affect vesicular transport in yeast trigger phosphorylation of eIF2 α (39). Second, in mammalian cells, plasma membrane perforation by bacterial PFT leads to activation of GCN2 (12, 33), phosphorylation of eIF2 α , transient attenuation of translation, and activation of autophagy (12, 13, 20). Also, membrane damage by chlorpromazine or detergent triggers GCN2 (40). *S. aureus* α -toxin inhibited uptake of leucine by cells, providing an explanation for activation of GCN2 in target cells of PFT (12). In human epithelial cells, eIF2 α , eIF2 α -kinases and the regulatory eIF2 α -phosphatase subunit CReP/Ppp1r15B are all required for efficient recovery from α -toxin-dependent loss of ATP (13). Surprisingly, these proteins served to remove membrane pores, thus, linking control of translation initiation and membrane traffic (13).

Conspicuously, many rodent cell types are not affected even by comparably high concentrations (micromolar range) of α -toxin; but the cause is not known. Receptor density on murine cells might be low, or murine ADAM10 might be an inefficient receptor as compared to its human counterpart. Alternatively, murine cells might be particularly tolerant to the consequences of successful attack. At any rate, to better understand results of *in vivo* experiments with *S. aureus* or α -toxin in mice, it is important to comprehend the mechanisms underlying tolerance of murine cells.

In ecoimmunology, “tolerance” denotes the ability of an organism to cope with high-pathogen load and resulting damage (41–43). To elucidate mechanisms of tolerance, it will be important to investigate the phenomenon at the cellular level using defined noxious agents. Here, we focus on cellular tolerance to PFT. A PFT may fail to cause overt damage to a cell if it cannot bind to, or attack, membranes in the first place. Alternatively, target cells may be able to cope with membrane perforation. In both cases, we consider the target cell “tolerant to the PFT.” The term “susceptibility” shall denote responsiveness of a cell to a PFT as measured by loss of ATP or loss of potassium ions from the cytosol.

In the present work, we have investigated tolerance of mouse cells to *S. aureus* α -toxin. The results support a broader protective function of MAPK and document a cell type- and toxin-selective role of eIF2 α .

Results

Normal Mouse Embryonal Fibroblasts are Tolerant to *S. aureus* α -Toxin

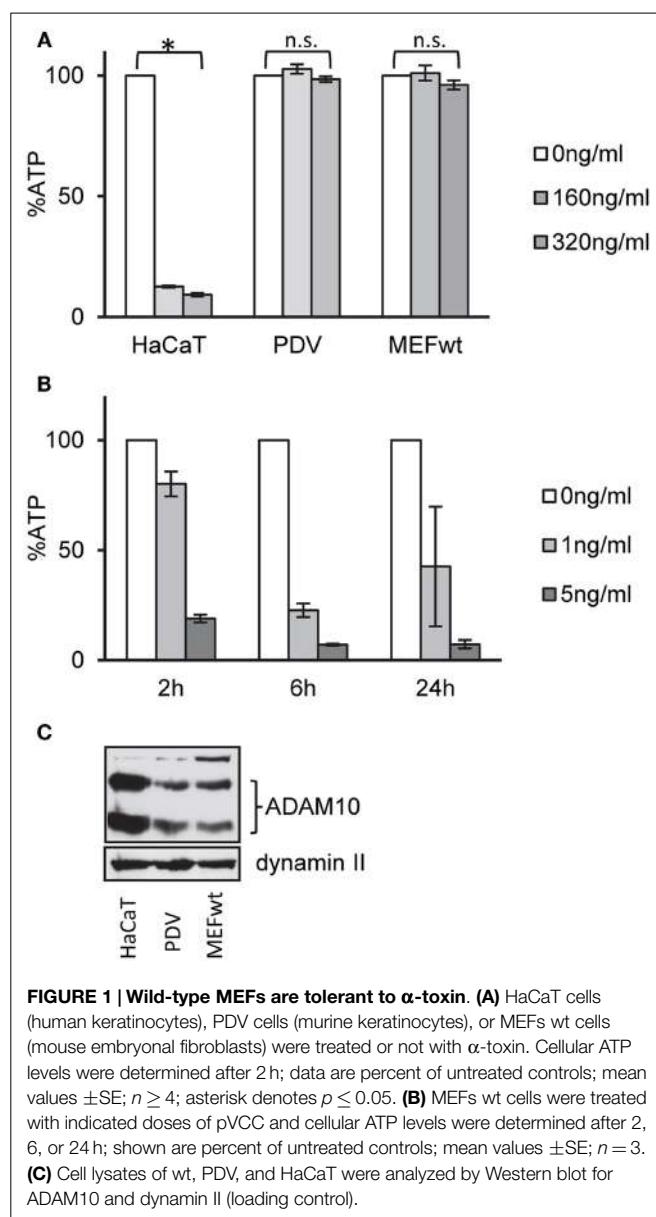
Exposure of human keratinocytes (HaCaT) to nanomolar concentrations of α -toxin for 2 h leads to significant loss of ATP. In contrast, murine keratinocytes (PDV) or mouse embryonal fibroblasts (MEFs) appeared to be not susceptible to α -toxin (Figure 1A). In contrast, MEFs were exquisitely susceptible to PVCC (Figure 1B), another PFT of the small β -barrel family (44).

Inhibitors of Various Signaling Pathways Sensitize MEFs for α -Toxin

One potential explanation for selective tolerance of MEF to α -toxin could be the lack of an appropriate receptor, or lower expression levels of receptor. Amino acid sequences of human and murine ADAM10, the proposed high-affinity receptor of α -toxin, are not identical. Western blots with an antibody against ADAM10 yielded weaker bands with the murine cells (Figure 1C). Although it cannot be excluded that the antibody binds human and murine cells with different efficiency, it is therefore possible that qualitative or quantitative differences in receptor expression could explain lower susceptibility of murine vs. human cells to α -toxin. Alternatively, however, efficient ongoing repair of membrane damage and fitness to cope with metabolic stress could also play a role. Therefore, we tested a panel of small molecular weight compounds that we knew to inhibit recovery from α -toxin-dependent ATP-depletion in HaCaT cells. Several (combinations of) inhibitors sensitized MEFs for α -toxin (Figure 2A). The effect depended on the concentration of inhibitor, as exemplified for JNK3XIISR3576 (Figure 2B). Although JNK3XIISR3576 is selective for JNK3 if applied at nanomolar concentrations, MAPK other than JNK3 seem to be involved here because MEFs do not express JNK3 (45). As shown for Sal/Dyn, inhibitors sensitized wild-type MEF for α -toxin, provided they expressed ADAM10 (Figure 2C). Thus, inhibitors did not sensitize cells by increasing unspecific toxicity of α -toxin.

Lack of Phosphorylatable eIF2 α , GCN2, or Ppp1r15B Sensitizes MEFs for α -Toxin

That Salubrinal, an inhibitor of eIF2 α -phosphatases (46) sensitized MEFs to α -toxin, and similar observations in



keratinocytes (13), prompted us to investigate the response of MEFs with defined genetic modifications affecting expression or function of proteins involved in regulation of translation: first, in eIF2 $\alpha^{S51A/S51A}$ cells, the eIF2 α locus is replaced with a non-phosphorylatable version, thus precluding regulated attenuation of translation via P-eIF2 α . Second, GCN2 $^{-/-}$ MEFs lack nutrient sensitive eIF2 α -kinase GCN2/EIF2AK4, thereby blunting phosphorylation of eIF2 α in response to amino acid deprivation. Third, Ppp1r15B $^{-/-}$ MEFs are devoid of the sole constitutive regulatory subunits of eIF2 α -phosphatase (47); this defect leads to constitutively higher eIF2 α -phosphorylation levels. Fourth, EeF2K $^{-/-}$ MEFs lack eukaryotic elongation factor 2 kinase (EeF2K), which functions downstream of mTOR to control protein synthesis (48).

First, we compared ATP levels in these MEFs to assess metabolic perturbation after treatment with α -toxin. EeF2K $^{-/-}$

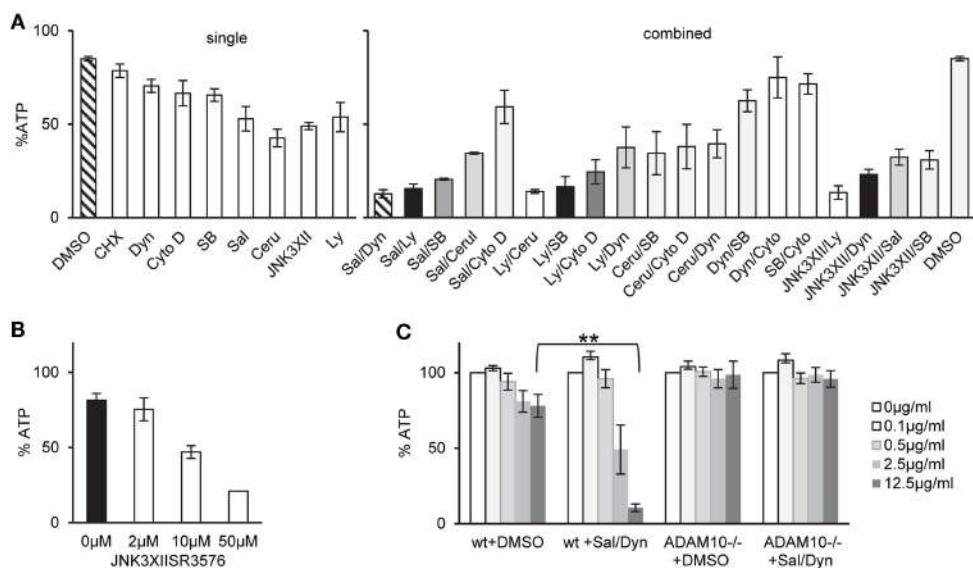
cells were not susceptible to 10 μ g/ml of α -toxin and thus behaved like wild-type MEFs. In contrast, lack of GCN2 or Ppp1r15B both sensitized cells to α -toxin. The strongest effect was observed with eIF2 $\alpha^{S51A/S51A}$ MEFs, which lost up to ~80% of ATP (Figure 3A). Although α -toxin led to dose-dependent loss of ATP in eIF2 $\alpha^{S51A/S51A}$ cells, there was barely any effect on wild-type cells (Figure 3B). Notably, pVCC-dependent loss of ATP was similar in wild-type or mutant MEFs (Figure 3C). Next, we determined the frequency of sub-G1 events, a measure of DNA fragmentation, in cells treated with α -toxin for 48 h. No significant difference was observed between wild-type and eIF2 $\alpha^{S51A/S51A}$ cells in the presence of β -ME and non-essential amino acids (NEAA). However, without these supplements, the number of sub-G1 events was doubled in eIF2 $\alpha^{S51A/S51A}$ cells (Figure 3D), although toxin-dependent loss of ATP and phosphorylation of eIF2 α were equal in media with or without additives (data not shown). Additives thus appear to protect cells from secondary damage.

GCN2/EIF2AK4 Contains α -Toxin-Dependent Stress

Next, we assessed basal or toxin-dependent eIF2 α -phosphorylation in the various MEFs lines. In wild-type cells, some basal phosphorylation of eIF2 α was noted, which was moderately increased by α -toxin; after normalization for eIF2 α , the effect was, however, statistically insignificant. As expected, P-eIF2 α was not detected in eIF2 $\alpha^{S51A/S51A}$ MEFs (Figure 4A). No P-eIF2 α was also discerned with samples of untreated GCN2 $^{-/-}$ cells; this indicated that basal phosphorylation of eIF2 α in cultured MEFs depends on GCN2. Paradoxically, however, α -toxin-dependent phosphorylation of eIF2 α was enhanced in cells lacking this eIF2 α -kinase. This showed that increased susceptibility of GCN2 $^{-/-}$ MEFs to α -toxin (Figure 3A) cannot be accounted for by diminished toxin-dependent eIF2 α -phosphorylation. Membranes were re-probed with antibodies against p38, which becomes phosphorylated in response to PFT. Strikingly, α -toxin caused robust phosphorylation of stress activated protein kinase p38 in GCN2 $^{-/-}$ MEFs, Ppp1r15B MEFs, and eIF2 $\alpha^{S51A/S51A}$ MEFs (Figure 4A), but not in wild-type MEFs, supporting the notion that cells with imbalanced eIF2 α -phosphorylation experienced more severe toxin-dependent stress. p70S6K, substrate of mTORC1 became de-phosphorylated (Figure 4A), indicating that α -toxin inhibits mTORC1, master regulator of translation and autophagy.

CHX Does Not Tolerize eIF2 $\alpha^{S51A/S51A}$ MEFs to α -Toxin

Results from Western blots (Figure 4A) raised the question whether α -toxin impacts translation differentially in eIF2 $\alpha^{S51A/S51A}$ vs. wild-type MEFs. Paradoxically, treatment with α -toxin caused dose-dependent attenuation of translation in eIF2 $\alpha^{S51A/S51A}$ MEFs, but had no such effect on wild-type MEFs (Figure 4B). This suggested that translation was inhibited in response to α -toxin through a mechanism that was independent of eIF2 α -phosphorylation. Together with results from the foregoing ATP-assays, this also revealed that attenuation of translation *per se* is insufficient to maintain

**FIGURE 2 | Inhibitors of various pathways sensitize MEFs to α-toxin.**

(A) wt cells were treated as indicated either with single inhibitors or combinations of two inhibitors listed below and treated with 10 μg/ml α-toxin. Cellular ATP levels were determined after 2 h; data are percent of untreated controls; shown are mean values ± SE with $n \geq 6$, or mean values of $n \geq 2$ for combinations of inhibitors, SE was <30% throughout. Inhibitor concentrations: Salubrinal (40 μM), Dynasore (80 μM), Ly29400L (100 μM), SB203580 (20 μM), Cerulenin (20 μM), Cytochalasin D (20 μM), and JNK3XIIISR3576 (10 μM). **(B)** MEFs wt were treated with

indicated doses of JNK3XIIISR3576 and incubated with 10 μg/ml α-toxin. Cellular ATP levels were determined after 2 h; data are percent of controls treated with similar concentrations of JNK3XIIISR3576 but not treated with α-toxin; mean values ± SE; $n \geq 3$. **(C)** MEFs wt or MEFs ADAM10^{-/-} (mouse embryonal fibroblasts) were pretreated with 40 μM Salubrinal and 80 μM Dynasore or solvent alone for 30 min and treated or not with indicated doses of α-toxin. ATP levels were determined after 2 h; shown are percent of untreated controls; mean values ± SE; $n = 3$; two asterisks denote p -values ≤ 0.001 .

metabolic homeostasis upon attack by α-toxin. Conversely, toxin-dependent ATP-loss in eIF2α^{S51A/S51A} MEFs is not a consequence of translational arrest, because treatment of wild-type cells with CHX stops translation (Figure 4B), but does not hyper-sensitize MEFs for α-toxin (Figure 4C).

Wild-Type eIF2α Modulates Binding and Action of α-Toxin

That inhibition of translation did not protect eIF2α^{S51A/S51A} MEFs provoked the question how phosphorylation of eIF2α tolerizes MEF. So, we investigated a primary event underlying many of the rapid molecular changes induced by PFT, i.e., disturbance of natural ion gradients (4). Loss of potassium appears to be one major trigger (11, 20, 24, 49). We measured loss of intracellular potassium by flame photometry. In line with results from ATP-assays, net loss of potassium was enhanced in GCN2^{-/-} or Ppp1r15B^{-/-} cells and even more so in eIF2α^{S51A/S51A} MEFs (Figure 5A), suggesting that membrane damage was more severe in cells with deficiencies in regulation of eIF2α-phosphorylation.

Pore formation by α-toxin depends on oligomerization and insertion into the plasma membrane. Because oligomers resist SDS, they can be detected by SDS-PAGE. Using cell surface labeling after incubation with internally radio-labeled α-toxin, we compared the amount of α-toxin on the surface of wild-type MEFs and eIF2α^{S51A/S51A} MEFs. The fluorographic analysis shown in Figure 5B reveals that more α-toxin is present at the surface of eIF2α^{S51A/S51A} MEFs as compared to wild-type cells, providing a

straightforward explanation for enhanced loss of potassium and ATP from these cells.

Wild-Type eIF2α Modulates Expression of ADAM10

Increased amounts of toxin associated with eIF2α^{S51A/S51A} MEFs could be due to higher abundance of α-toxin receptors. Therefore, we compared ADAM10 expression in eIF2α^{S51A/S51A} MEFs and wild-type MEFs. Western-blot analysis revealed that ADAM10 is over-expressed in eIF2α^{S51A/S51A} MEFs; and more ADAM10 is exposed on the cell surface of eIF2α^{S51A/S51A} MEFs as compared to wild-type cells (Figure 5C). No significant differences between wild-type cells and eIF2α^{S51A/S51A} MEFs were found in a lipidomics analysis (data not shown). Treatment with α-toxin led to down-regulation of ADAM10 at the cell surface of both wild-type and mutant cells. Because basal eIF2α-phosphorylation in MEFs depends on GCN2 (Figure 4A), the collective data indicate that nutrient stress or basal activity of GCN2 modulates levels of ADAM10 in MEFs.

Role of GCN2 for Tolerance to α-Toxin is Not Conserved in BMDM

Like MEFs, BMDM proved to be highly tolerant to α-toxin although they were susceptible to *Vibrio cholera* cytolsin (VCC) (Figure 6A). However, lack of GCN2-expression in BMDM did not significantly alter susceptibility to α-toxin (Figures 6A,D); α-toxin-dependent phosphorylation of eIF2α appeared slightly reduced (Figure 6B). Further, infection of BMDM by *S. aureus*

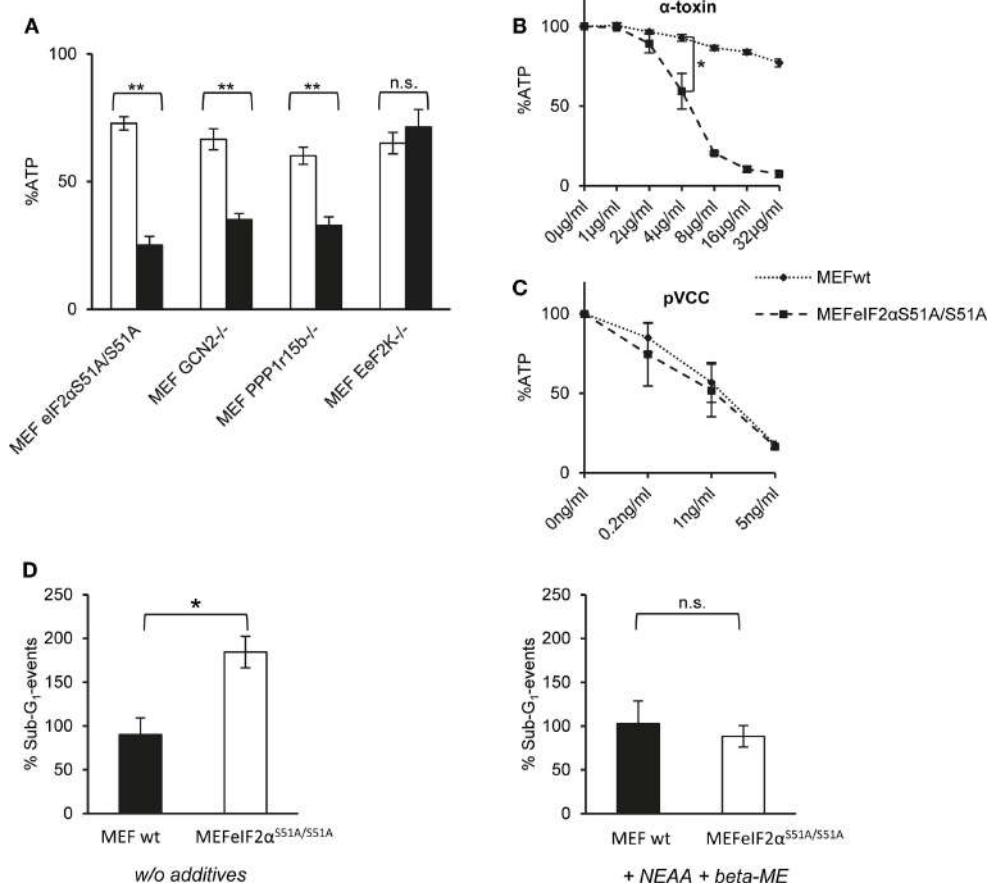


FIGURE 3 | Dysregulation of eIF2α-phosphorylation increases sensitivity for α-toxin. **(A)** MEFs eIF2α^{S51A/S51A}, MEFs GCN2^{-/-}, MEFs Ppp1r15b^{-/-}, or MEFs EeF2K^{-/-} were treated or not with 10 μg/ml α-toxin. ATP levels were determined after 2 h; shown are percent of untreated controls; mean values ± SE; n ≥ 4. Black bars show data with MEFs cell variants as indicated; white bars corresponding control cells; two asterisks denote p-values ≤ 0.001. **(B)** MEFs wt or eIF2α^{S51A/S51A} were treated, or not with indicated doses of α-toxin. Cellular ATP levels were determined after 2 h; data are percent of

untreated control; mean values ± SE; n = 5; asterisk: p = 0.026. **(C)** MEFs wt or MEFs eIF2α^{S51A/S51A} treated or not with indicated doses of pVCC. Cellular ATP levels were determined after 2 h; data are percent of untreated control; mean values ± SE; n = 3. **(D)** MEFs wt or MEFs eIF2α^{S51A/S51A} were cultured in standard media or in presence of β-ME and additional non-essential amino acids and treated with 10 μg/ml α-toxin for 48 h, stained with Propidium iodide and subsequently frequency of Sub-G₁-DNA was determined; data show percent of untreated controls; mean values ± SE; n = 3; asterisk denotes p ≤ 0.05.

was equally efficient with both strains whether or not bacteria produced toxin or not (Figure 6C). Therefore, GCN2 seemed to play no major role for resistance or tolerance to α-toxin of BMDMs.

Small MW inhibitors of JNK and dynamin, a cocktail, which efficiently breaks tolerance to α-toxin in MEFs, moderately sensitized BMDM to purified α-toxin (Figure 6D). Inhibitors did not enhance ADAM10 expression (Figure 6E). Thus, MAPK enhance natural tolerance of BMDM to α-toxin, but modulation of ADAM10 expression appears not to be involved.

Discussion

One conclusion of this work is that regulators of translation initiation (eIF2α, GCN2, and Ppp1r15B) render murine embryonic fibroblasts tolerant to *S. aureus* α-toxin. This is consistent with our previous finding in human epithelial cells that these proteins

promote recovery from successful attack (13). Although it remains to be investigated whether *a priori* tolerance of MEFs to α-toxin is likewise based on efficient endocytic removal of α-toxin pores from the cell surface, the present results document that the tolerogenic effect of eIF2α, GCN2, and Ppp1r15B is conserved in mice and man, and that it is observable in fibroblasts. That both lack of an eIF2α-kinase and of constitutive eIF2α-phosphatase reduce tolerance to α-toxin could be interpreted to show that balanced phosphorylation of eIF2α, or cycling of eIF2α between phosphorylated and unphosphorylated state is required for the tolerogenic effect.

A role of translational regulation for various aspects of innate immunity, and a protective function against PFT has been discussed in the recent literature (34, 50, 51). One established function of eIF2α-phosphorylation and the integrated stress-response is to reprogram expression of genes, including of genes that regulate immunity. Translational attenuation in host cells might

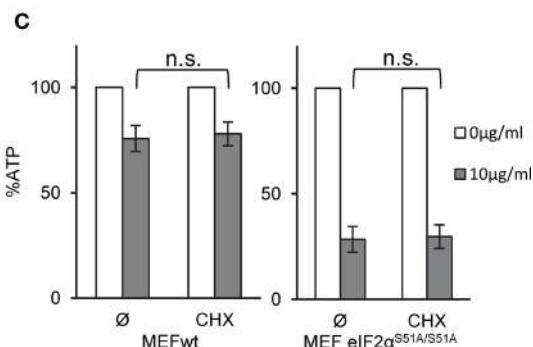
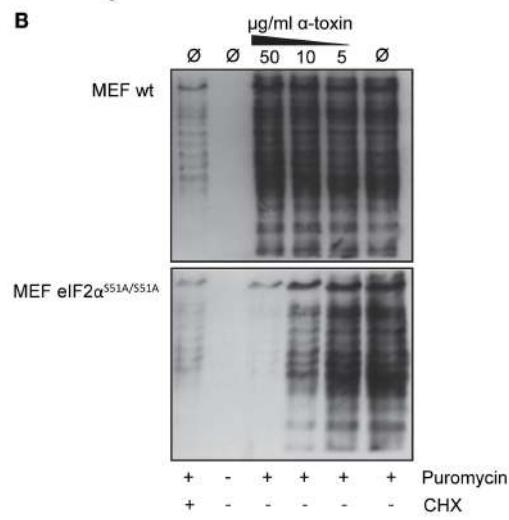
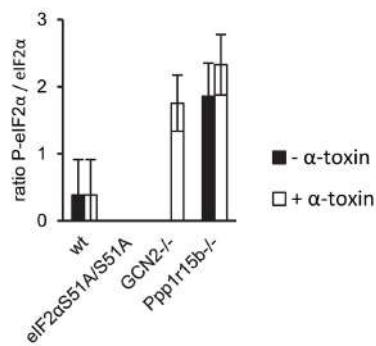
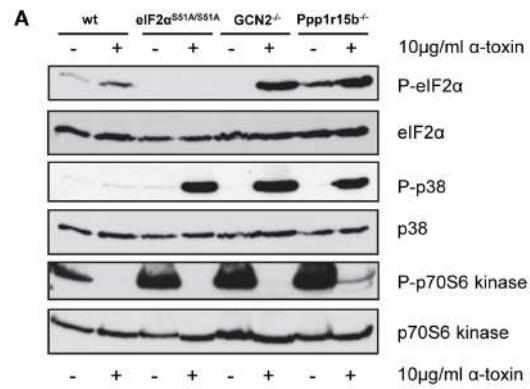


FIGURE 4 | α-toxin causes translational arrest in MEFs eIF2α^{S51A/S51A}. (Continued)

FIGURE 4 | Continued

Cycloheximide does not sensitize wild-type MEFs (A) MEFs wt, MEFs eIF2α^{S51A/S51A}, MEFs GCN2^{-/-}, or MEFs Ppp1r15b^{-/-} were treated or not with 10 µg/ml α-toxin for 2 h. Cell lysates were analyzed by Western blot for P-eIF2α, eIF2α, P-p70S6 kinase, p70S6 kinase P-p38, and p38. One of three similar blots is shown; lower panel summarizes densitometric data for (P)-eIF2α, mean ± SE; n = 3 (B) MEFs wt or MEFs eIF2α^{S51A/S51A} cells were treated or not with indicated concentrations of α-toxin and incubated for 1 h at 37°C. Treatment with CHX served as positive control for translational arrest. Subsequently, the cells were incubated for 1 h at 37°C with 10 µg/ml puromycin, which incorporates during ongoing synthesis into nascent proteins. Eventually, cells were analyzed by Western blot for puromycin. (C) MEFs wt or MEFs eIF2α^{S51A/S51A} cells were treated or not with 10 µg/ml α-toxin and incubated with or without CHX at 37°C. Cellular ATP levels were determined after 2 h; data are percent of untreated controls; mean values ± SE; n = 5.

also help them to conserve energy, as proposed by some authors (20). However, (P)-eIF2α-dependent tolerance of MEF could not be explained by toxin-dependent translational attenuation *per se*: actually, α-toxin caused inhibition of translation in eIF2α^{S51A/S51A} MEFs, but not in wild-type MEFs, and CHX did neither protect nor hyper-sensitize wild-type cells from/for α-toxin. Yet, eIF2α^{S51A/S51A} MEFs proved to be significantly less tolerant to α-toxin. Probably as a consequence, protein synthesis was halted through alternative pathways. This could occur, for instance, by deactivation of mTOR, as indeed suggested by α-toxin-dependent dephosphorylation of p70S6K, a target of mTOR. The data may reflect a hierarchy of stress responses: Activation of eIF2α via nutritional sensor GCN2 contains α-toxin-dependent damage and stress, which would otherwise lead to exaggerated hyperphosphorylation of eIF2α by (an)other eIF2α-kinase(s), possibly PERK and PKR. If eIF2α-phosphorylation fails, translation would be halted via robust deactivation of mTOR. Sustained inhibition of translation is obviously not tolerated by cells (35), but marked α-toxin-dependent inhibition of translation, observed in eIF2α^{S51A/S51A} MEFs, does not explain the increased loss of ATP and potassium from these cells, because CHX did not affect these parameters in wild-type cells.

The fact that lack of phosphorylatable eIF2α was associated with increased toxin-dependent loss of potassium and ATP prompted us to measure the binding of α-toxin and expression of ADAM10, the proposed high-affinity receptor for α-toxin (52). This led to the unexpected finding that ADAM10 levels were significantly higher in eIF2α^{S51A/S51A} cells. Therefore, tolerance to α-toxin in MEFs may be well due to GCN2/P-eIF2α-dependent modulation of its receptor, ADAM10. Whether or not the apparent link between basal nutrient stress and expression of ADAM10 was shaped by co-evolution of *S. aureus* and humans, it may also bear on functions of ADAM10 that are not related to infection.

How basal phosphorylation of eIF2α-levels modulates ADAM10-expression remains to be elucidated. Because P-eIF2α is required for starvation-dependent autophagy (53), a possible role of eIF2α in this context could be to maintain basal autophagic flux, which in turn could impact ADAM10 levels. Recently, Atg16L1, a protein essential for autophagy, has been shown to confer tolerance to α-toxin; the authors proposed that autophagy constitutively dampens ADAM10 levels in a cell-type selective manner (54). Similarly, eIF2α-dependent modulation

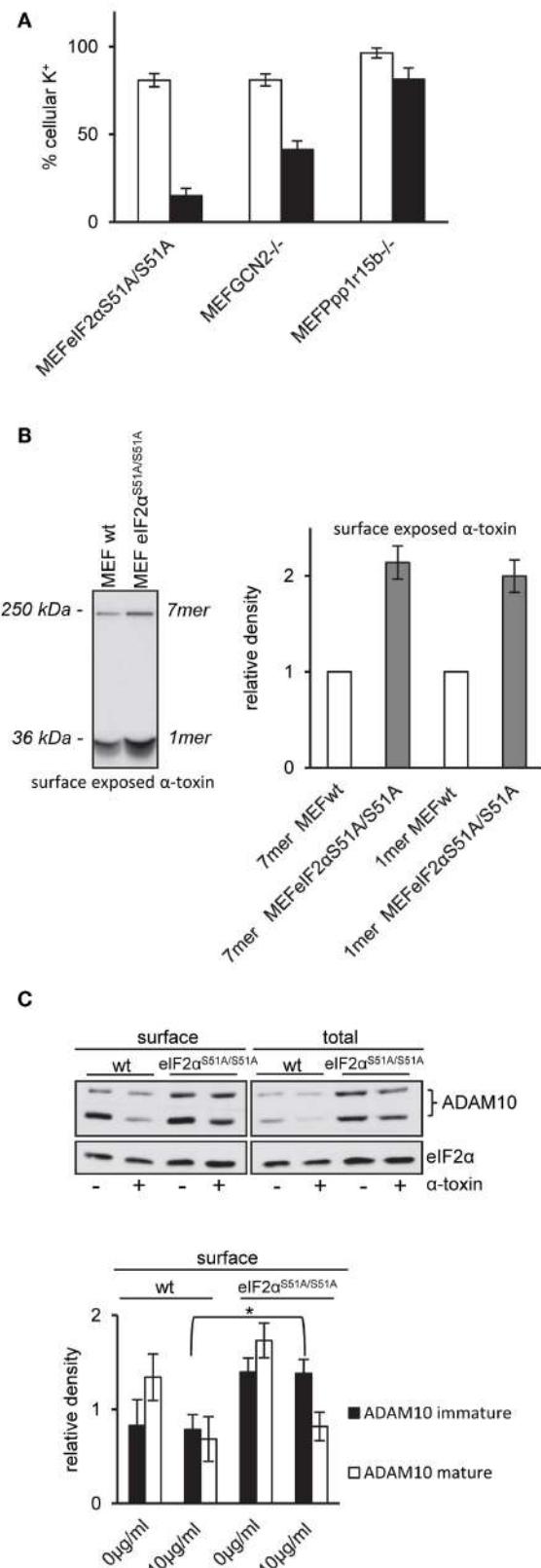


FIGURE 5 | MEFs eIF2α^{S51A/S51A} over-express ADAM10 and bind higher amounts of α-toxin.

(Continued)

FIGURE 5 | Continued

(A) MEFs eIF2α^{S51A/S51A}, MEFs GCN2^{-/-}, MEFs Ppp1r15b^{-/-}, or corresponding control cells were treated or not with 10 µg/ml α-toxin. Potassium levels were determined after 2 h, shown are percent of untreated controls; mean values ± SE; n ≥ 4. Black bars show data of MEFs cell variants as indicated; white bars corresponding control cells. **(B)** Right: MEFs wt or MEFs eIF2α^{S51A/S51A} cells were incubated with radio-labeled α-toxin 2 and 8 µg/ml α-toxin at 37°C for 15 min. Subsequently, cells were surface biotinylated, lysates were obtained and subjected to sequential neutravidin-pulldown (NP) and immunoprecipitation (IP) followed by PAGE/fluorography, as described in Kloft et al. (13). Left: band intensities were measured by densitometry using ImageJ software. Shown are mean values ± SE; n = 4. Variations of loading with toxin and plating of cells were <1 and <10%, respectively. **(C)** MEFs wt or MEFs eIF2α^{S51A/S51A} were treated or not with 10 µg/ml α-toxin for 2 h. Subsequently, cells were surface biotinylated or not and lysed. Lysates were subjected to NP; both lysates and precipitation were analyzed by Western blot for ADAM10 and eIF2α (loading control); upper panel: one of four similar blots; lower panel: bar chart summarizing data (mean ± SE; n = 4).

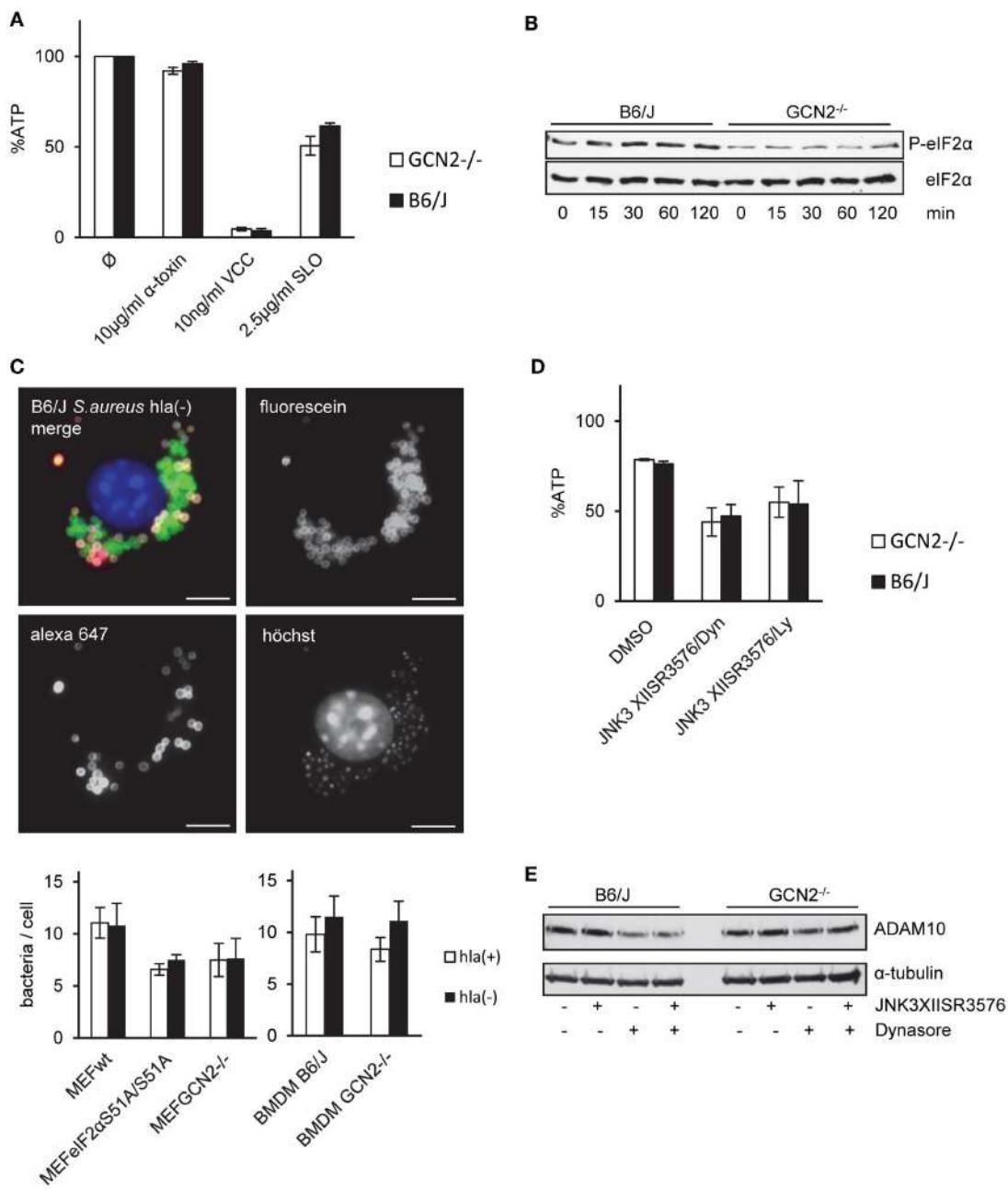
of ADAM10 shown in the present work is constitutive and cell-type selective. Together this seems to suggest that basal eIF2α-phosphorylation functions upstream of autophagy to mediate tolerance. Alternatively, P-eIF2α, eIF2α-kinases, and -phosphatases could function through mechanisms acting in parallel to autophagy, for instance, by regulating endocytosis of membrane pores, as has been shown in epithelial cells (13). Whatever the effector mechanism(s) downstream of eIF2α are, our data reveal that GCN2-dependent basal phosphorylation of eIF2α in MEF modulates ADAM10 levels as well as binding and action of α-toxin. Because GCN2 is activated by low levels of amino acids in cells, basal nutrient stress might be the driving force; the potential links discussed here are summarized in a model (Figure 7).

Inhibitors of various pathways hyper-sensitized MEFs to *S. aureus* α-toxin, which supports the notion that multiple signaling pathways are required to confer cellular tolerance to α-toxin. This raises the possibility that drugs used in pharmaco-therapy may have potential tolerance-modulating effects, an issue that warrants further investigation. The present data support a tolerogenic role of JNKs in both MEFs and BMDM. More work is required to understand the underlying mechanisms, but they seem to be distinct from eIF2α-dependent cellular tolerance to α-toxin. Conserved stress responses may fail to protect against some PFT, as exemplified here by *V. cholerae* cytolysin. Whether cells of the immune system tolerate attack of a given PFT will co-determine the ability of an organism to mount an effective “classic” immune response to infection with corresponding bacteria.

Materials and Methods

Antibodies

Antibodies against P-eIF2α (phosphorylated at Ser51), eIF2α, P-p38 (phosphorylated at Thr180/Tyr182), p-38, P-p70S6K (phosphorylated at Thr389), p70S6K, ADAM10, and α-tubulin were from Cell Signaling Technology. Antibodies against dynamin II were purchased from Santa-Cruz Biotechnology. Anti-Puromycin antibody was from Merck Millipore. Antibodies against LC3 were bought from Sigma. HRP-conjugated secondary

**FIGURE 6 | GCN2 does not render BMDMs tolerant to α-toxin.**

(A) BMDMs isolated from GCN2^{-/-} mice or control mice (B6/J) were treated or not with indicated doses of α-toxin, VCC, or SLO. Cellular ATP levels were determined after 2 h. White bars show BMDMs from GCN2^{-/-} mice and black bars show BMDMs from control mice; mean values ± SE, $n \geq 3$. **(B)** BMDMs of GCN2^{-/-} mice or control mice were incubated with 10 µg/ml α-toxin for indicated times. Cell lysates were analyzed by Western blot for P-eIF2α and eIF2α. **(C)** MEFs variants (right graph) or BMDMs of GCN2^{-/-} and control mice (left graph) were incubated with fluorescein/biotin labeled *S. aureus* hla(–) or hla(+) strains (MOI 1:30) for 1 h, washed and incubated for an additional hour at 37°C. After fixation, extracellular bacteria were stained with streptavidin-coupled Alexa 647. Upper: exemplary picture of solely fluorescein-stained *S. aureus*,

representing intracellular bacteria, and extracellular *S. aureus* that were accessible for Alexa 647-Streptavidin. Lower: graphs show counts of solely fluorescein-stained (intracellular) bacteria per cell; mean values ± SE; BMDMs $n = 2$; MEFs $n = 3$. **(D)** BMDMs of GCN2^{-/-} mice or control mice were incubated with combinations of JNK3 XIIISR3576 (10 µM), Dynasore (80 µM), JNK3 XIIISR3576 (10 µM), and Ly29400L (100 µM) or solvent alone and treated with 10 µg/ml α-toxin. Cellular ATP levels were determined after 2 h (percentage of controls). (Mean values ± SE; $n = 3$). **(E)** BMDMs of GCN2^{-/-} and control mice were treated with the combination of Dynasore (80 µM) and JNK3 XIIISR3576 (10 µM), Dynasore (80 µM) alone, JNK3 XIIISR3576 (10 µM) alone, or solvent alone (DMSO) for 2.5 h. Cell lysates were analyzed by Western blot for ADAM10 and α-tubulin (loading control).

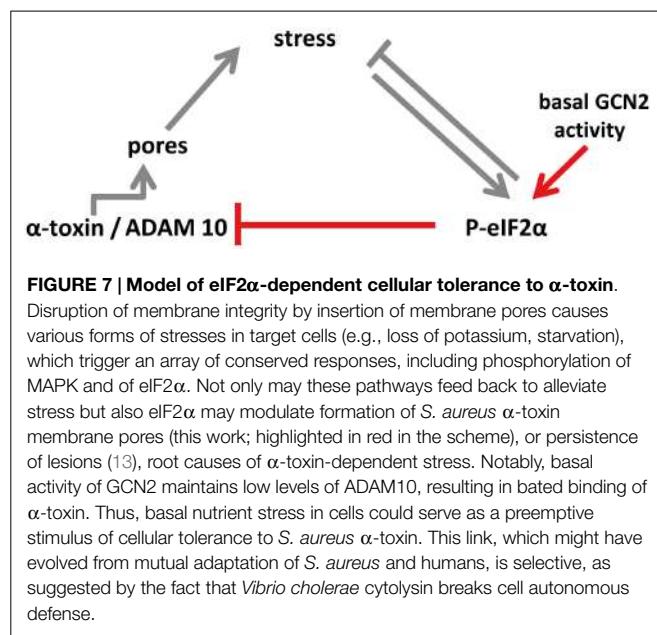


FIGURE 7 | Model of eIF2 α -dependent cellular tolerance to α -toxin.

Disruption of membrane integrity by insertion of membrane pores causes various forms of stresses in target cells (e.g., loss of potassium, starvation), which trigger an array of conserved responses, including phosphorylation of MAPK and of eIF2 α . Not only may these pathways feed back to alleviate stress but also eIF2 α may modulate formation of *S. aureus* α -toxin membrane pores (this work; highlighted in red in the scheme), or persistence of lesions (13), root causes of α -toxin-dependent stress. Notably, basal activity of GCN2 maintains low levels of ADAM10, resulting in bated binding of α -toxin. Thus, basal nutrient stress in cells could serve as a preemptive stimulus of cellular tolerance to *S. aureus* α -toxin. This link, which might have evolved from mutual adaptation of *S. aureus* and humans, is selective, as suggested by the fact that *Vibrio cholerae* cytotoxin breaks cell autonomous defense.

antibodies were from Santa-Cruz Biotechnology (mouse) and Cell Signaling Technology (rabbit).

Inhibitors

Salubrinal (SAL), Cycloheximid (CHX), JNK3XIISR3576 (JNK3XII), and SB203580 (SB) were obtained from Calbiochem. Ly29400L (Ly) was from Cell Signaling. Baflomycin, Cytochalasin D (Cyto D), and Cerulenin (Ceru) were from Sigma and Dynasore (Dyn) was from Tocris bioscience.

Chemicals

RNase, propidium iodide, and puromycin were purchased from Sigma. NHS-fluorescein and EZ-Link Sulfo-NHS-LC-biotin were from Thermo Fisher Scientific and Streptavidin-Alexa 647 was obtained from Molecular Probes. Rapamycin was from Calbiochem.

Toxins

α -toxin, internally radio-labeled α -toxin, streptolysin (SLO), and VCC were made as published elsewhere (10, 12).

S. aureus

In this study, *S. aureus* strain DU1090 (55) and α -toxin producing *S. aureus* strain, plasmid transformed derivative of DU1090 (55), were employed and referred to as hla(–) and hla(+), respectively.

Cells and Culture

Mouse embryonal fibroblasts GCN2 $^{−/−}$ (56) were purchased from ATCC and PDV from CLS Cell Lines Service GmbH. MEFs eIF2 $\alpha^{S51A/S51A}$ and corresponding control cells MEFs eIF2 $\alpha^{S51/S51}$ (57), MEFs Ppp1r15b $^{−/−}$ (58), MEFs EeF2K $^{−/−}$ (59), and MEFs ADAM10 $^{−/−}$ (60) were kindly provided by Heather Harding and David Ron, Randal Kaufman, Alexey Ryazanov, and Paul Saftig, respectively. All MEFs cell lines were

cultured in DMEM GlutaMAX™-I medium with 10% fetal calf serum, 1% HEPES buffer, 1% penicillin/streptomycin, 1% MEM NEAA, and 55 mM 2-mercaptoethanol. Under these conditions, we did not note significant differences in morphology, viability, or growth rate. PDV (murine keratinocyte cell line) were grown in DMEM GlutaMAX™-I medium with 10% fetal calf serum, 1% HEPES buffer, 1% penicillin/streptomycin without MEM NEAA, and 2-mercaptoethanol. HaCaT (non-virally transformed HaCaT) (61) were cultured in DMEM/F-12 GlutaMAX™-I medium with 10% fetal calf serum, 1% HEPES buffer, and 1% penicillin/streptomycin. All media and medium additives were obtained from Gibco by life technologies™. BMDMs were isolated from C57BL/6J (B6/J) or B6.129S6-EIF2 α k4 $^{tm1.2Dron/j}$ (GCN2 $^{−/−}$) mice (Charles River laboratories) and cultured in DMEM GlutaMAX™-I supplemented with 20% fetal calf serum and M-CSF by adding 10% supernatant of L929 cells.

Western Blot

For Western blots, cell lysates were mixed with 2× SDS-loading buffer [65 mM Tris, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, and bromophenol blue] and boiled for 5 min at 95°C. Proteins were separated by SDS-PAGE (10%) and electroblotted onto nitrocellulose membrane. After blocking for 1 h at room temperature in BSA or skim milk in TBST [Tris 50 μM, NaCl 0.15M, Tween 0.1% (v/v)], membrane was incubated with a primal antibody in BSA or skim milk in TBST, washed three times in TBST and incubated with HRP-conjugated second antibody for 1 h at room temperature. After three washing steps, bound antibody was detected by ECL (Roche Applied Science).

Puromycin Incorporation

The assay has been described elsewhere (62). MEFs eIF2 $\alpha^{S51A/S51A}$ cells were seeded at a density of 2×10^5 cells/well into six-well plates. Cells were incubated as indicated with several concentrations of pVCC or α -toxin for 1 h at 37°C. Thereafter, 10 μg/ml puromycin was added and cells were incubated for an additional hour at 37°C. Subsequently, medium was removed; cells were washed with PBS and analyzed by Western blot using anti-Puromycin-antibody in 2.5% (w/v) skim milk in TBST.

ATP-Measurements

Measurements of intracellular ATP were performed, as described elsewhere (22).

Potassium Efflux Measurements

For measurements of intracellular potassium, cells were seeded at a density of 3×10^5 cells/well into six-well plates. Cells were incubated or not with 8 μM Rapamycin for 3 h before adding 10 μg/ml α -toxin. After 2 h incubation at 37°C, cells were washed three times on ice with cold Choline-chlorid-buffer and solubilized in 2 ml Choline-chlorid-buffer containing 0.5% Triton X-100 (63) for 30 min at room temperature under constant shaking. Potassium content of supernatants of centrifuged cell lysates was determined using Sherwood single channel flame photometer M401.

Quantitation of Surface-Exposed or Internalized α -Toxin/ADAM10

Surface labeling, neutravidin pull-down, and fluorometric detection of α -toxin have been described previously (13). Variation of input (labeled S35-Met- α -toxin) was <1%; equal amounts of total protein were loaded.

Quantification of Internalized Bacteria

The assay has been performed, as described elsewhere (64).

Sub-G1 Analysis

Cells were seeded at a density of 1×10^5 cells/well into six-well plates and treated or not with 10 μ g/ml α -toxin permanently for 48 h at 37°C. Cells were harvested including the detached cells in the supernatant, washed in PBS and centrifuged. Cells were resuspended and fixed in cold 70% ethanol in PBS/EDTA for 2 h at 4°C. Subsequently, the cells were washed and treated with RNase for 10 min at 37°C. Then, propidium iodide was added and cells were incubated for 5 min at room temperature. Eventually, cells

were analyzed using a FACScan flow cytometer (BD Biosciences) and CellQuest software.

Statistical Analysis

Two-sided, unpaired Student's *t*-test was employed to assess statistical significance of differences between mean values. Significance was assumed at $p < 0.05$.

Acknowledgments

Research reported in this publication was supported by the National Institutes of Health NIH/NIFMS under award number 7-R01-GM071603-09 to RA and MH (subrecipient); DK042394, DK088227, and HL052173 to RK; MH and GH received support by University Medical Center Mainz. We like to thank Dr. H. Harding and Dr. D. Ron for generously providing Ppp1r15b^{-/-} MEFs, Dr. P. Saftig for ADAM10^{-/-} MEFs, and Dr. B. Bruegger for a large scale lipid analysis. Further, we gratefully acknowledge support by Dr. A. Diefenbach.

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Bacterial Toxins as Pathogen Weapons Against Phagocytes

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OPEN ACCESS

Edited by:

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University Hospital Heidelberg,
Germany

Reviewed by:

Charles C. Caldwell,
University of Cincinnati, USA
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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 16 October 2015

Accepted: 11 January 2016

Published: 01 February 2016

Citation:

do Vale A, Cabanes D and Sousa S (2016) Bacterial Toxins as Pathogen Weapons Against Phagocytes. *Front. Microbiol.* 7:42.
doi: 10.3389/fmicb.2016.00042

INTRODUCTION

Macrophages and neutrophils are central mediators of the innate immune system that act at early stages of bacterial infection and have the ability to clear the pathogen through phagocytosis and subsequent digestion (Mantovani et al., 2011; Wynn et al., 2013). Besides their overlapping functional properties, macrophages, and neutrophils also display distinct and specialized features allowing their concerted and cooperative action against pathogens (Appelberg, 2007; Silva, 2010; Silva and Correia-Neves, 2012). Accordingly to their antimicrobial capacity, toxicity, and lifespan, macrophages and neutrophils have different localizations. Long-lived macrophages are distributed in tissues throughout the body where they perform local immune surveillance activities, including recognition, phagocytosis, and rapid signaling of invading pathogens. The number of resident macrophages in resting tissues is rather low. Upon infection resident macrophages secrete chemokines, rapidly recruiting monocytes, and quiescent neutrophils from blood and bone marrow pools to the infectious foci. Monocytes differentiate to short-lived mature macrophages and neutrophils are activated to produce powerful antimicrobial molecules and release proinflammatory cytokines and chemokines amplifying the initial chemotactic role of resident macrophages and sustaining antimicrobial activities (Mantovani et al., 2011; Wynn et al., 2013).

The outcome of an infection is dictated by the nature of host-pathogen interactions and greatly depends on the efficacy of phagocytes. When, through the action of virulence determinants, the pathogen takes the control of the interaction in detriment of the host, the infection establishes. By the contrary, if the host immune defenses dominate over the pathogen, the infection is controlled and the pathogen is eliminated. In this scenario, several human pathogens evolved an arsenal of sophisticated mechanisms to evade host defenses. In particular, given the central role of macrophages and neutrophils as primary phagocytes,

several pathogens deploy strategies to either survive or annihilate phagocytes antimicrobial functions (Flannagan et al., 2009). To avoid macrophage antimicrobial properties, some intracellular pathogens interfere with the classical maturation of the phagosome, blocking fusion with endosomes, and lysosomes thereby preventing destruction. Others escape the phagosome to multiply in the cytosol and modulate host gene expression to limit production of cytokines that would be deleterious for pathogen progression. Despite the mechanisms of evasion of neutrophil-killing are yet poorly defined, it is known that some pathogens survive and multiply inside neutrophils which may act as Trojan horses for microbial dissemination (Appelberg, 2007). From the extracellular milieu, some pathogens target macrophage and neutrophils mainly through the secretion of toxins that: (1) cause irreversible damage leading to phagocyte death or (2) heavily perturb intracellular signaling pathways, blocking phagocytosis or modulating inflammation (e.g., through the control of expression of chemokines and cytokines; Lemichez and Barbieri, 2013).

Toxins are potent molecules produced by a large variety of bacterial pathogens that target host cells and play key roles in the host-pathogen dialog. They are major virulence factors often sufficient to determine the outcome of the infection. Indeed, attesting their importance in pathogenesis, the injection of small amounts of some purified toxins can recapitulate many key symptoms of the disease. Bacterial toxins can be divided in several groups regarding their nature and mode of action (Lemichez and Barbieri, 2013). In this review we focus on bacterial exotoxins, which are secreted by the pathogen and act on the host cells from the extracellular milieu. Although exotoxins may target different cell types, some specifically target macrophages and neutrophils thus taking the control of innate immune response, providing the pathogen a suitable environment for active proliferation. Interestingly, while the initial steps of phagocytes intoxication are specific for different exotoxins, the ultimate cellular effects leading to the loss of host cell function are often the same. Taking this into account we review here the mechanisms of phagocyte targeting by archetypal exotoxins such as pertussis toxin (PT) and adenylate cyclase toxin (ACT) secreted by *Bordetella pertussis*, anthrax toxin from *Bacillus anthracis* and *Staphylococcus aureus* leukotoxins. We also include here AIP56, a recently described toxin from *Photobacterium damselaе piscicida* (Phdp). In addition, the mode of action of mycolactone, a polyketide molecule produced by *Mycobacterium ulcerans*, and other bacterial secreted products not formally termed as toxins, such as *S. aureus* superantigens-like proteins (SSPs) and phenol-soluble modulins (PSMs), are also reviewed here. Clostridial C3 toxins, which target and modulate macrophage functions, are the focus of another review in this Topic (Barth et al., 2015).

***Bordetella pertussis*: TWO TOXINS ARE BETTER THAN ONE**

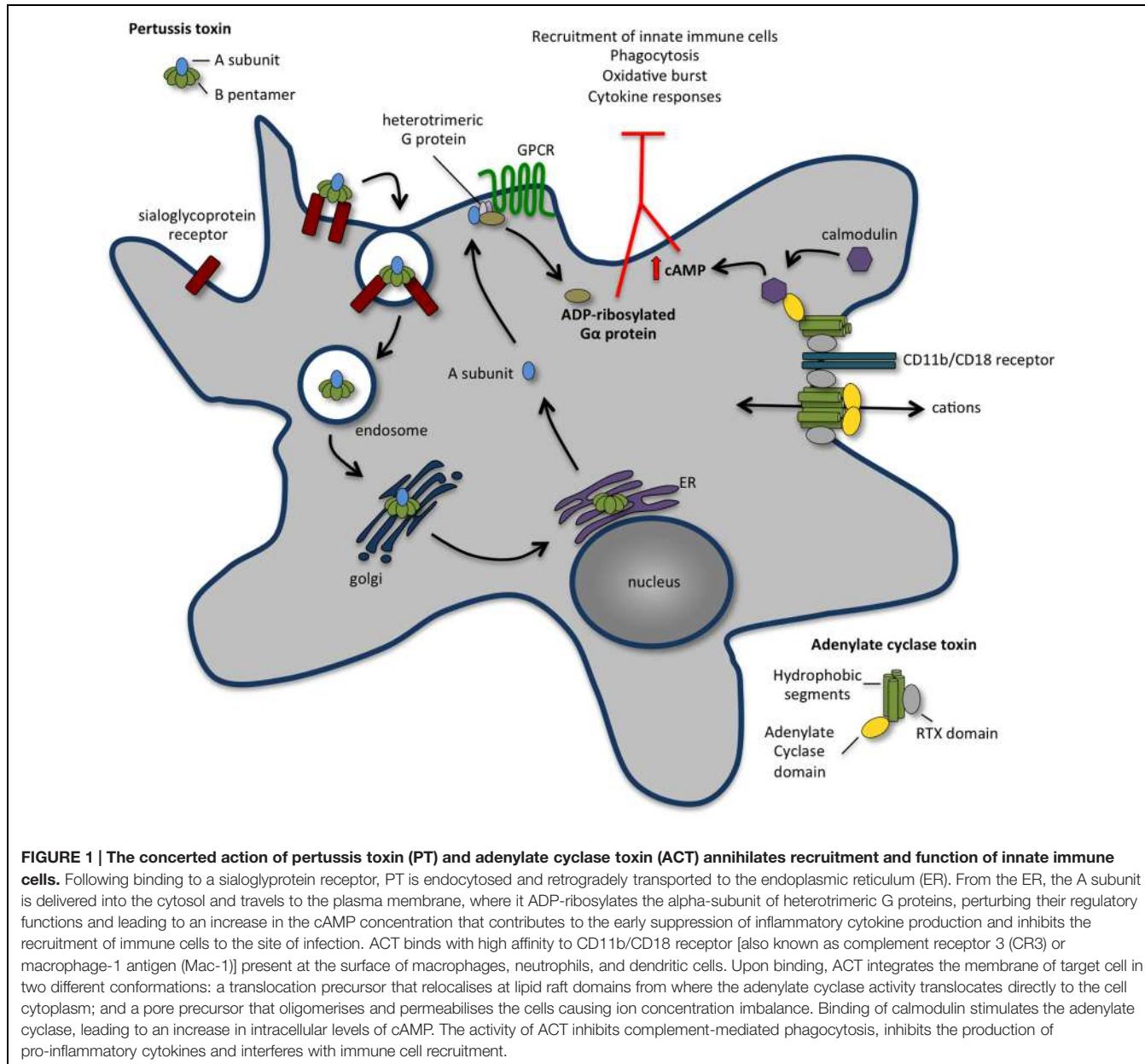
Bordetella pertussis is a Gram-negative pathogen that infects the human respiratory tract causing whooping cough, an acute and highly contagious infection (Mattoo and Cherry, 2005; Melvin

et al., 2014). Initially thought to be a toxin-mediated disease (Pittman, 1984), such as cholera and diphtheria, pertussis disease is instead the result of the coordinated action of a variety of bacterial factors that allow bacterial adherence to ciliated respiratory epithelium, survival to host innate immune defense, multiplication, and resistance to inflammatory cells (Carbonetti, 2007). Two of the major virulence factors of *B. pertussis* are the secreted toxins, PT and ACT, which emerged as key elements for suppression/modulation of the host immune and inflammatory responses (Carbonetti, 2010; Higgs et al., 2012; Melvin et al., 2014). Interestingly, mouse infections with different mutants suggested that these two toxins have complementary functions in pathogenesis assaulting the innate immune cells at different times and from different angles. PT would act at early stages of infection mainly inhibiting the recruitment of immune cells, while ACT would later intoxicate macrophages and neutrophils blocking bacterial engulfment and destruction (Carbonetti et al., 2005).

First Round: PT Inflicts First Blow to the Host

Pertussis toxin is a multisubunit AB-toxin exclusively produced by *B. pertussis*. Through B-subunits, PT binds to any sialic acid-containing glycoprotein at the cellular surface (Witvliet et al., 1989; Saukkonen et al., 1992; Stein et al., 1994), is internalized by endocytosis and follows a retrograde transport pathway through the Golgi complex to the endoplasmic reticulum (Plaut and Carbonetti, 2008; **Figure 1**), from which the A-subunit translocate to the cytoplasm of host cells (Locht et al., 2011). In the host cell cytosol, the A-subunit exhibits ADP-ribosyltransferase activity, hydrolysing NAD and ADP-ribosylating heteromeric G-proteins of the G_i family (Katada et al., 1983). This modification prevents the interaction of G_i proteins with their cognate G-protein coupled receptors (GPCRs) causing the disruption of downstream cell signaling transduction (Mangmool and Kurose, 2011). Besides the activity of the A domain, B-subunits also display signaling function by activating intracellular signaling cascades in a G_i-protein independent manner (Mangmool and Kurose, 2011).

Pertussis toxin has long been considered as a major *B. pertussis* virulence factor. Indeed, PT was reported to be the cause of systemic symptoms of pertussis disease such as lymphocytosis and leukocytosis (Morse and Morse, 1976) and was associated with lethal infection by *B. pertussis* in a neonatal mouse model (Goodwin and Weiss, 1990). However, evidences supporting its role in respiratory infection have only emerged in the last decade. Experiments in the mouse model revealed that the lack of PT confers a defect in *B. pertussis* colonization at the early stages of infection (Carbonetti et al., 2003, 2005). Interestingly, a PT-deficient strain reaches wild type levels of colonization whenever co-infections with both strains are performed or intranasal inoculation of purified PT precedes infection (Carbonetti et al., 2003). Further studies have shown that depletion of resident airway macrophages leads to exacerbated *B. pertussis* infection in a PT-independent manner (Carbonetti et al., 2007), indicating that PT targets



airway macrophages disrupting their protective activity at early steps of infection (Carbonetti et al., 2007). Intranasal administration of PT resulted in ADP-ribosylation of airway macrophages G_i-proteins (Carbonetti et al., 2007), suggesting that its inhibitory function on macrophages *in vivo* results from the immunosuppressive activities ascribed to PT *in vitro*. In particular, *in vitro* PT was shown to inhibit macrophage and neutrophil migration (Meade et al., 1984), phagocytosis (Mork and Hancock, 1993) and cytokine response (He et al., 1988; Hume and Denkins, 1989).

In vivo, PT plays a dual role in the establishment of the disease. Whereas it has immunosuppressive functions at early stages of infection, later it potentiates inflammatory responses, likely prolonging healing and promoting bacterial dissemination

(Eby et al., 2015). Early in infection, PT targets airway macrophages and inhibits neutrophil recruitment to the infection site (Carbonetti et al., 2003, 2005; Kirimanjeswara et al., 2005; Andreasen and Carbonetti, 2008). Neutrophils were reported to play a protective role against *B. pertussis* only in previously infected mice and in the presence of anti-*B. pertussis* antibodies (Kirimanjeswara et al., 2005; Andreasen and Carbonetti, 2009), which may suggest that PT delays neutrophil recruitment to the airways avoiding rapid antibody-mediated clearance of the pathogen. Later, at the peak of infection, high numbers of neutrophils are recruited to the lungs of mice infected with wild type strain, but not to those infected with PT-deficient strain (Carbonetti et al., 2005; Andreasen et al., 2009). This recruitment of neutrophils to the lungs correlates with an increase of

proinflammatory cytokines such as IL-17, TNF α and IFN γ that appears to be dependent on PT activity (Andreasen et al., 2009). Recently, global transcriptional profiles of mice lungs infected with wild type or PT-deficient *B. pertussis* revealed that, at the peak of infection, the ADP-ribosylation activity of PT correlates with upregulation of immune and inflammatory response genes (Connelly et al., 2012). *In vitro* studies suggested that PT directly impairs neutrophil migration through ADP-ribosylation the G i -proteins associated with surface chemokine receptors (Spangrude et al., 1985; Scott et al., 1988; **Figure 1**). However, *in vivo* data showed that besides acting directly on chemokine receptors signaling (Kirimanjeswara et al., 2005), PT also suppresses early neutrophil recruitment by inhibiting the production of neutrophil-attracting chemokines by airway macrophages and lung epithelial cells (Andreasen and Carbonetti, 2008). Transcription of genes expressing CXCL1, CXCL2, and CXCL5 is inhibited in the lungs of mice intranasally infected with wild type *B. pertussis*, as compared to animals infected with strains deficient for PT production or producing a PT variant devoid of ADP-ribosylation activity (Andreasen and Carbonetti, 2008). Interestingly, PT also blocks chemokine gene expression and early neutrophil recruitment to the airways following intranasal administration of LPS (Andreasen and Carbonetti, 2008), which occurs presumably through modulation of TLR-4 signaling, a G i -protein independent pathway.

Altogether, these studies show that PT suppresses early inflammation in the respiratory tract and inhibit microbicidal function of inflammatory cells, potentially providing advantage to the pathogen by allowing its rapid growth and establishment within the host at early phases of infection.

Second Round: ACT Perpetuates the Pathogen Gain

Adenylate cyclase toxin is a bi-functional toxin produced by all species of *Bordetella* that infect mammals. While its N-terminal domain contains an adenylate cyclase activity that converts ATP in cAMP, the C-terminus possesses RTX motifs that bind mammalian cells and form cation-selective pores in the host plasma membrane. Through its combined adenylate cyclase and pore-forming activities, ACT manipulates host cell physiology in two different ways: it interferes with intracellular signaling by increasing the levels of cAMP, and disturbs ion homeostasis by disrupting the permeability barrier of the plasma membrane (**Figure 1**). ACT specifically binds with high affinity CD11b/CD18 (also known as CR3 or Mac-1) present at the surface of macrophages, neutrophils and dendritic cells (DCs; Guermonprez et al., 2001). Upon binding, ACT integrates the membrane of target cells in two different conformations: a translocation precursor that re-localizes at lipid raft domains from where the adenylate cyclase activity translocates directly to the cell cytoplasm; and a pore precursor that oligomerises and permeabilises the cells causing ion concentration imbalance (Fiser et al., 2007, 2012; Bumba et al., 2010; **Figure 1**). While the rapid translocation of adenylate cyclase activity across the host plasma membrane does not depend on endocytosis (Gordon et al., 1988), the clathrin-dependent endocytosis of ACT together

with its receptor CD11b/CD18 was reported (Khelef et al., 2001; Martin et al., 2011). Classical mechanisms of membrane repair upon toxin-induced pore formation include the removal of the pores through endocytosis. Interestingly, ACT-translocated molecules control the rate of ACT-pore removal delaying their endocytic uptake, thus exacerbating the permeabilisation of phagocytes and maximizing the cytotoxic action (Fiser et al., 2012). Despite its interaction with a specific receptor, ACT was reported to promiscuously bind and intoxicate many cell types, including CD11b/CD18-negative cells (Ladant and Ullmann, 1999; Paccani et al., 2008; Eby et al., 2012), although the biological relevance of these findings remains unclear. Indeed, while ACT triggers macrophage apoptosis *in vitro* (Khelef et al., 1993; Khelef and Guiso, 1995) and *in vivo* (Gueirard et al., 1998), the viability of cells from non-haematopoietic origin remains unaffected (Gueirard et al., 1998; Bassinet et al., 2000). *In vivo* studies support that ACT primarily targets phagocytes such as alveolar macrophages and neutrophils, disrupting the early innate antibacterial host immune response (Harvill et al., 1999; **Figure 1**).

cAMP is a key second messenger with pleiotropic effects. Increased levels of cAMP severely compromise cellular functions such as the migration of neutrophils, the plasticity of DC responses, the release of cytokines by macrophages and the homeostasis of actin cytoskeleton. Thus, through the uncontrolled production of intracellular cAMP, ACT subverts phagocytic, and bactericidal function of macrophages and neutrophils in a variety of ways (**Figure 1**). In human alveolar macrophages and neutrophils, as in mouse macrophages, the ACT-mediated cAMP production blocks phagocytosis, chemotaxis, and oxidative burst (Confer and Eaton, 1982; Friedman et al., 1987; Weingart and Weiss, 2000; Kamanova et al., 2008; Eby et al., 2014). High levels of cAMP cause a transient inactivation of RhoA inducing massive actin rearrangements that dramatically decrease macropinocytosis, block complement-mediated phagocytosis (Kamanova et al., 2008) and possibly impair the chemotactic properties of primary monocytes. Recently, ACT-induced cAMP synthesis was shown to trigger pro-apoptotic signaling in phagocytes through the activity of tyrosine phosphatase SHP-1, the accumulation of cytosolic BimEL and the consequent activation of Bax, permeabilisation of the outer mitochondrial membrane and activation of programmed cell death (Ahmad et al., 2015; Cerny et al., 2015). Besides the immediate action of ACT on the ablation of bactericidal functions of phagocytes, ACT activity was also reported to block the release of TNF α and the production of ROS in human monocytes (Njamkepo et al., 2000), to promote incomplete or aberrant maturation of DCs (Skinner et al., 2004; Boyd et al., 2005) and to impair T-cell activation by interfering with immunological synapse signaling (Paccani et al., 2011). ACT was described to suppress the secretion of pro-inflammatory cytokines such as IL-12 and TNF α and favor the production of anti-inflammatory IL-10 molecules (Spensieri et al., 2006; Hickey et al., 2008).

Despite the fact that studies in mouse models established ACT as an important virulence factor for *B. pertussis* infection (Khelef et al., 1992), the effects described above were obtained from

in vitro studies, and their significance during *in vivo* infection requires further investigation. Importantly, recent studies using baboon infection model and clinical samples from humans showed that the concentration of ACT in tissues is much lower than the amount used for *in vitro* experiments (Eby et al., 2013), which may compromise the relevance of some effects reported *in vitro*.

Pertussis toxin and ACT have undeniable roles during *B. pertussis* infection and certainly play key functions in the pathophysiology of pertussis disease. Numerous and wide-ranging effects of the purified toxins on cultured cell lines have been reported, however, establishing the correlation of such effects with the human pathology appeared as an incredible difficult task. In addition, the mouse model only provides limited possibilities to address this issue. Together with analysis of clinical samples from humans, the use of baboons as non-human primate model is expected to shed new light on the mechanisms of action of PT and ACT and on pertussis disease.

***Bacillus anthracis*: ARMED TO ANNIHILATE THE HOST INNATE IMMUNE DEFENSES**

Bacillus anthracis is a Gram-positive, spore-forming rod that causes anthrax, an acute and fast progressing disease that affects humans and other animals, and results from a combination of bacterial infection and toxemia (Moayeri et al., 2015). The spores, which are the infectious form of the pathogen, are able to resist harsh environmental conditions and to infect new hosts when inhaled, ingested, or exposed to skin breaks. Upon entering a potential host, spores germinate into vegetative bacilli that replicate and disseminate through the bloodstream, leading to a systemic infection. Whereas in experimental inhalational anthrax, spore germination requires engulfment by macrophages/DCs (Guidi-Rontani et al., 1999; Hanna and Ireland, 1999), in experimental cutaneous infections it has been shown that most of the spore germination occurs extracellularly (Bischof et al., 2007; Corre et al., 2013). Soon after spore germination, vegetative *B. anthracis* start producing two potent exotoxins – anthrax lethal toxin (LT) and edema toxin (ET) – that along with a poly-D-glutamic acid capsule, are its major virulence factors (Moayeri et al., 2015). *B. anthracis* also secretes anthrolysin O (ALO), a member of the cholesterol-dependent cytolysin family (Shannon et al., 2003). ALO is cytotoxic for several mono- and polymorphonuclear cells (Shannon et al., 2003; Cocklin et al., 2006; Mosser and Rest, 2006) and together with LT, induces apoptosis of macrophages (Shannon et al., 2003). However, ALO-deficient and wild-type strains revealed no differences in virulence in an inhalation infection model (Heffernan et al., 2007). Consistent with these observations, ALO-based vaccines, although conferring protection against lethal intravenous challenge with ALO, do not protect mice against peritoneal infection by *B. anthracis* (Cowan et al., 2007). Altogether, available data indicate that ALO is not essential for *B. anthracis* virulence and thus further studies are needed to determine if ALO contributes to *B. anthracis* pathogenicity.

Anthrax toxins originate from the association of three different protein components: a host cell receptor binding protein named protective antigen (PA) and two enzymatic proteins, edema factor (EF) and lethal factor (LF) (Young and Collier, 2007; **Figure 2**). EF or LF associated to PA is referred as ET or LT, respectively. Host cell intoxication begins when PA binds to either tumor endothelium marker 8 (TEM8, also known as anthrax toxin receptor 1, ANTXR1) or capillary morphogenesis protein 2 (CMG2, also known as anthrax toxin receptor 2, ANTXR2), which are expressed by different cell types, including macrophages and neutrophils (Bradley et al., 2001; Scobie et al., 2003). Although both TEM8 and CMG2 can function as anthrax toxin receptors, studies with TEM8- and CMG2-null mice have shown that CMG2 is the major anthrax toxin receptor mediating toxin lethality *in vivo* and that TEM8 plays only a minor role in anthrax toxin-associated pathogenesis (Liu et al., 2009). Upon binding to its cell surface receptor, PA is proteolytically processed at its N-terminus by a furin-like protease and self-assembles forming an oligomeric prepore able to bind EF and LF. The EF and/or LF-prepore-receptor complex undergoes receptor-mediated endocytosis (Abrami et al., 2003) and the acidic conditions in endosomes induce conversion of the prepore to a pore, allowing translocation of EF and LF into the cell cytosol (Collier, 2009) to exert their cytotoxic effects (**Figure 2**). In endosomes, the toxin complex can also be sorted into intraluminal vesicles that undergo back fusion with the endosomal membrane allowing sequestered EF and LF to reach the cytosol (Abrami et al., 2004).

The toxicity of LT and ET results from the catalytic activities of LF and EF. LF is a zinc-dependent metalloprotease that cleaves mitogen activate protein kinase (MAPK) kinases (MKK) 1-4 and 6-7, preventing the activation of the ERK1/2, p38 and JNK-pathways (Duesbery et al., 1998; Pellizzari et al., 1999; Vitale et al., 2000; Tournier et al., 2005) and thus interfering with critical signaling pathways involved in host defense (**Figure 2**). EF is a calmodulin- and Ca^{2+} -dependent adenylate cyclase that causes a sustained increase of cAMP (Leppla, 1982) by converting ATP to cAMP. As discussed above in the context of the ACT from *B. pertussis*, increased cAMP levels perturb key cellular functions, leading to severe consequences for the host (Leppla, 1982; Firoved et al., 2005).

Anthrax toxins: Weapons to Cripple Host Macrophages and Neutrophils

Anthrax has for long been described as a toxin-mediated disease, mainly because LT and ET can be lethal for experimental animals and anti-toxin immunization is effective in protecting against infection (Kaur et al., 2013). However, it has recently been proposed that extreme bacteraemia and severe sepsis rather than the anthrax toxins *per se* are the cause of anthrax-induced lethality (Coggeshall et al., 2013). Indeed, the fact that anthrax toxins act on multiple tissues simultaneously, due to the ubiquitous expression of the anthrax toxin receptors, complicate untangling their effects on the host and delayed the identification of the key tissue targets responsible for its lethal effects. Recently, using cell-type specific CMG2-null mice and the correspondent

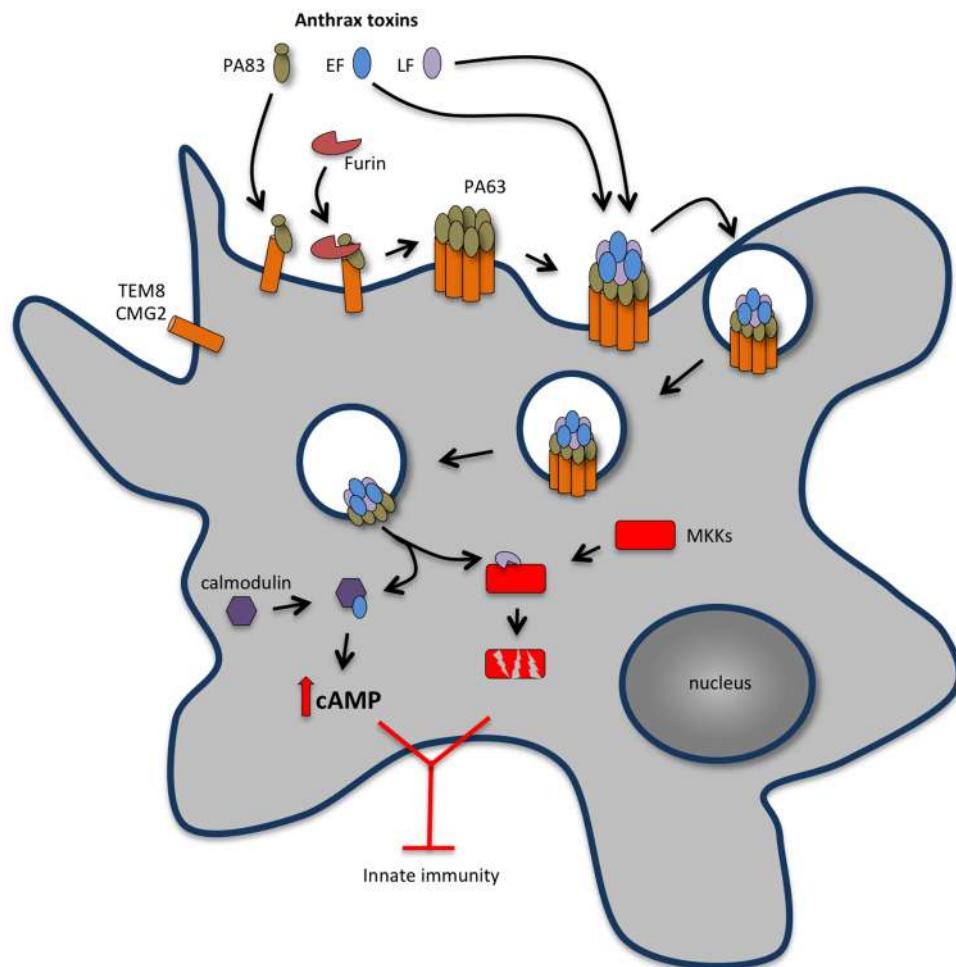


FIGURE 2 | Anthrax toxins cooperatively disable host innate immune response. Host cell intoxication by anthrax toxins involves interaction of protective antigen (PA, 83 kDa) with two cellular receptors [tumor endothelium marker 8 (TEM8), also known as anthrax toxin receptor 1 (ANTXR1) and capillary morphogenesis protein 2 (CMG2) also known as anthrax toxin receptor 2 (ANTXR2)], which are expressed by different cell types, including macrophages and neutrophils. Upon binding to the cell surface receptor, PA83 is proteolytically processed at its N-terminus by a furin-like protease yielding the C-terminal fragment PA63 that oligomerises into a heptameric prepore able to bind edema factor (EF) and lethal factor (LF). The EF and/or LF-prepore-receptor complex undergoes receptor-mediated endocytosis and the acidic conditions in endosomes induce conversion of the prepore to a pore, allowing translocation of EF and LF into the cell cytosol to exert their cytotoxic effects. LT is a zinc-dependent metalloprotease that inhibits activation of neutrophils and macrophages, expression of inflammatory cytokines and cell motility by disrupting mitogen activated protein kinase kinases (MAPKKs)-regulated pathways. LT activity also promotes macrophage apoptosis by interfering with pro-survival MAPKK dependent pathways. ET is a calcium and calmodulin-dependent adenylate cyclase that increases intracellular cAMP concentration, leading to the suppression of the expression of inflammatory cytokines and cell chemotaxis through protein kinase A (PKA)-dependent pathways. The concerted action of LT and ET blocks the function of phagocytic cells.

cell-type specific CMG2-expressing mice, Liu and colleagues have shown that LT-induced mortality requires targeting of cardiomyocytes and vascular smooth muscle cells, whereas ET-induced lethality relies mainly on targeting hepatocytes (Liu et al., 2013). Using myeloid-specific CMG2-null mice, in which both macrophages and neutrophils are insensitive to LT and ET due to their inability to bind and internalize the toxins, the same authors have also clarified the role of macrophages and other myeloid cells in anthrax toxins induced lethality and in *B. anthracis* infection (Liu et al., 2010). Myeloid-specific CMG2-null mice are fully sensitive to both LT and ET, indicating that lethality does not depend on the targeting of macrophages, neutrophils,

and other myeloid cells (Liu et al., 2010). However, they are completely resistant to infection by *B. anthracis*, indicating that the targeting of myeloid cells by anthrax toxins is required for the establishment of a successful infection (Liu et al., 2010). In what concerns the relative contributions of ET and LT to the establishment of *B. anthracis* infection, trials with Sterne strains deleted of PA, LF, or EF suggest that LT plays a more prominent role (Liu et al., 2010).

Available data indicate that LT and ET act in concert to inhibit macrophage activation as well as the activation and recruitment of other immune cells, such as neutrophils, early in infection (Baldari et al., 2006; Tournier et al., 2009; Liu

et al., 2014). This favors bacterial escape and multiplication, and contributes to the severe bacteraemia observed in terminal disease. Macrophage activation requires signaling through MAPK cascades, including JNK and p38 pathways, which are central for induction of inflammatory molecules, including cytokines and chemoattractants, as well as Cox-2 and iNOS. LT interrupts MAPK cascades by directly cleaving MAPKK, whereas ET inhibits MAPK-dependent gene expression by interfering with multiple PKA-related pathways (Baldari et al., 2006). Several reports show that LT inhibits the secretion of pro-inflammatory cytokines by macrophages as well as by DCs *in vitro* and *in vivo* (Pellizzari et al., 1999; Erwin et al., 2001; Agrawal et al., 2003; Alileche et al., 2005; Bergman et al., 2005; Brittingham et al., 2005; Tournier et al., 2005; Ribot et al., 2006). ET has been shown to suppress secretion of inflammatory mediators by DCs (Tournier et al., 2005). Whereas LT inhibits IL-10 secretion by these cells, ET inhibits IL12p70 production (Tournier et al., 2005). Interestingly, LT and ET have a cumulative suppressive effect upon TNF α secretion (Tournier et al., 2005). In addition to suppress pro-inflammatory cytokine secretion by macrophages, LT is also able to trigger programmed cell death in these cells *in vitro*. Indeed, it has been reported that LT induced apoptosis of RAW264.7 cells (Popov et al., 2002). Furthermore, although treatment of three different human monocytic cell lines (HL-60, THP-1, and U937) did not result in cell death, upon differentiation into macrophage-like phenotypes, the cells become susceptible to a cell death program that has features of apoptosis but apparently does not require the activity of effector caspases (Kassam et al., 2005). The apoptogenic activity of LT toward macrophages likely relates to LT-dependent disruption of survival signals triggered by TLR4 and mediated by p38 MAPK (Park et al., 2002, 2005; Hsu et al., 2004) that activates the NF- κ B-dependent expression of pro-survival genes. By cleaving the upstream MAPKK MKK3, LT blocks p38 MAPK and NF- κ B activation, leading to macrophage apoptosis (Huang et al., 2004; Park et al., 2005).

Lethal toxin has also been shown to induce a rapid and lytic form of caspase-1-dependent cell death, called pyroptosis, in macrophages from specific rat and mice strains. The susceptibility of macrophages to pyroptosis has been linked to polymorphisms of the *Nlrp1b* gene in mice (Boyden and Dietrich, 2006) and of the orthologous *Nlrp1* gene in rat (Newman et al., 2010). The ability of LT to induce macrophage pyroptosis was initially interpreted as a virulence mechanism of *B. anthracis* (Muehlbauer et al., 2007). It was speculated that the destruction of macrophages by LT compromised their role in restricting *B. anthracis* infection and that the cytokine burst associated to LT-induced macrophage lysis contributed to LT-dependent pathological effects by aggravating the vascular damage occurring in anthrax (Muehlbauer et al., 2007). More recently, it has been shown that LT is able to directly cleave mouse *Nlrp1b* and rat *Nlrp1* close to their N-terminus (Hellmich et al., 2012; Levinsohn et al., 2012; Chavarria-Smith and Vance, 2013) resulting in the activation of *Nlrp1* inflammasomes in rat and in mice with LT-sensitive macrophages. Ultimately, this leads to caspase-1 activation and pyroptosis accompanied by the release of the inflammatory cytokines IL-1 β and IL-18, which induces a strong innate immune response that is protective

against *B. anthracis* infection (Moayeri et al., 2010; Terra et al., 2010). Therefore, the current view is that LT-mediated activation of *Nlrp1* that leads to inflammasome activation and macrophage pyroptosis is not a virulence mechanism used by *B. anthracis* to promote infection, but rather a protective host-response against anthrax (Chavarria-Smith and Vance, 2015).

Neutrophils play a major role in controlling *B. anthracis* infection and anthrax-toxin mediated neutralization of neutrophil functions is essential for successful infection (Liu et al., 2010). *In vitro* studies suggest that by reducing F-actin formation, LT and ET cooperate to inhibit neutrophil chemotaxis, chemokinesis, and ability to polarize (During et al., 2005, 2007; Szarowicz et al., 2009). It was also reported that neutrophils intoxicated with ET have reduced phagocytic activity (O'Brien et al., 1985). Additionally, LT has been shown to suppress cytokine production by neutrophils *in vitro* (Barson et al., 2008) and LT and ET were found to block neutrophil priming by LPS or muramyl dipeptide, thereby dampening the oxidative burst normally elicited by bacterial products and required for full antimicrobial activity (Wright and Mandell, 1986). In the case of ET, it has been shown that the inhibition of superoxide production results from an impairment of the activation of the neutrophil NADPH oxidase, an effect that likely results from the activity of ET as an adenylate cyclase (Crawford et al., 2006). Although it has been proposed that these effects are due to the ET-induced rise in cAMP leading to phosphorylation (activation) of PKA (Szarowicz et al., 2009), the downstream targets remain to be identified.

It is now unquestionable that anthrax toxins are crucial in anthrax pathogenesis, but their precise roles during human anthrax infections remain to be further clarified. Following the discovery of the anthrax toxins more than half a century ago, a myriad of studies were developed aiming at defining their role in anthrax disease and lethality. However, most of the infection studies were performed in animal models that may not completely reflect the events occurring during human infections. Concerning the data documenting the immunomodulatory effects of the anthrax toxins, most were obtained *in vitro*, often in experimental set-ups that involve the use of purified toxins and do not allow examining intoxication in the context of infection. Therefore, the available data need to be validated in the context of relevant animal models of infections before being extrapolated to the human disease scenario.

***Photobacterium damselae piscicida:* KILLING TWO BIRDS WITH ONE STONE**

Photobacterium damselae piscicida is a Gram-negative extracellular bacterium that causes a systemic and deadly infection with a rapid course and very high mortalities in both wild and cultured marine fish (Romalde, 2002; Barnes et al., 2005). Phdp infections are characterized by the occurrence of generalized bacteraemia and extensive cytopathology with abundant tissue necrosis (do Vale et al., 2007). Infected fish often present whitish tubercle-like lesions of about 0.5 to

3.5 mm in diameter in several internal organs (Tung et al., 1985; Hawke et al., 1987; Noya et al., 1995; Magariños et al., 1996; do Vale et al., 2007), leading to the coining of the disease as fish pseudotuberculosis (Kubota et al., 1970). The lesions consist of accumulations of bacteria and apoptotic and necrotic cell debris (Kubota et al., 1970; Hawke et al., 1987; do Vale et al., 2007).

Photobacterium damselae piscicida-associated pathology is triggered by AIP56 (apoptosis inducing protein of 56 kDa), a plasmid-encoded toxin secreted by virulent Phdp strains (do Vale et al., 2005). The toxin is systemically disseminated in infected animals and induces selective apoptotic destruction of macrophages and neutrophils (do Vale et al., 2003, 2005, 2007). The simultaneous destruction of these cell types by AIP56 has two dramatic consequences for the host. On one hand, the drastic reduction of the number of phagocytes impairs the phagocytic defense, favoring pathogen dissemination (do Vale et al., 2007). On the other hand, it compromises the host capacity to clear apoptosing cells, leading to the lysis of the phagocytes by post-apoptotic secondary necrosis with consequent release of their highly cytotoxic tissue-damaging contents (do Vale et al., 2007; Silva et al., 2008).

AIP56: Spreading the Misery by Killing the Soldiers and Preventing their Burial

AIP56 is the founding and the only characterized member of a continuously growing family of bacterial proteins identified in different organisms, mainly marine *Vibrio* species and *Arsenophonus nasoniae*. It is an AB-type toxin, possessing a catalytic A domain at its N-terminal region and a B domain involved in binding/internalization into target cells at its C-terminal region (Silva et al., 2013; Figure 3). The catalytic domain of AIP56 is a zinc-dependent metalloprotease that cleaves the p65 subunit of NF-κB (Silva et al., 2013), an evolutionarily conserved transcription factor that regulates the expression of inflammatory and anti-apoptotic genes, playing a key role in host responses to microbial pathogen invasion. AIP56 has likely originated from a fusion of two components: its A domain is related to NleC, a type III secreted effector present in several enteric pathogenic bacteria (Yen et al., 2010; Baruch et al., 2011; Pearson et al., 2011; Sham et al., 2011; Hodgson et al., 2015) that are associated with severe human illness and death worldwide, whereas its B domain is related to a protein of unknown function from the lambda-like bacteriophage APSE2, a phage that infects *Hamiltonella defensa* (Degnan et al., 2009).

Although mammals are not susceptible to Phdp infection, likely due to temperature and osmolality restrictions, AIP56 is able to intoxicate mouse bone marrow derived macrophages (mBMDM; Pereira et al., 2014), through a mechanism similar to the one operating during intoxication of fish cells. Upon encountering susceptible cells, AIP56 binds to a still unidentified cell-surface receptor and is internalized through clathrin-mediated endocytosis (Pereira et al., 2014; Figure 3). Once in early endosomes, the toxin either follows the recycling pathway back to the extracellular medium or undergoes low pH-induced translocation across the endosomal membrane into the cytosol

to display its toxic activity (Pereira et al., 2014). AIP56 cleaves an evolutionarily conserved peptide bond of the Rel homology domain of NF-κB p65, removing residues crucial for p65-DNA interaction and compromising NF-κB activity (Silva et al., 2013; Figure 3). During intoxication, the proteolytic activity of AIP56 results in a complete depletion of p65 and leads to the apoptotic death of cells (Silva et al., 2013; Pereira et al., 2014) through a process involving quick activation of caspases-8, -9 and -3, loss of mitochondrial membrane potential, translocation of cytochrome c to the cytosol and overproduction of ROS (do Vale et al., 2007; Reis et al., 2007a,b, 2010; Costa-Ramos et al., 2011).

AIP56 plays a pivotal role in the establishment of Phdp infection and in the development of the infection-associated pathology. In the initial phase of Phdp infection, when local multiplication of Phdp becomes detectable in infected tissues, extensive infiltration of macrophages and neutrophils occurs (do Vale et al., 2007). As the infection progresses, the pathogen extensively multiplies and disseminates systemically, which leads to a septicemic situation paralleled by the occurrence of AIP56 in the systemic circulation (do Vale et al., 2007). The presence of circulating toxin correlates with the appearance of high numbers of apoptotic macrophages and neutrophils in the peripheral blood, in the spleen, liver, and head-kidney vasculature, as well as in the splenic and head-kidney parenchyma and gut lamina propria (do Vale et al., 2007). This systemic apoptotic destruction of macrophages and neutrophils triggered by AIP56 explains the extensive phagocyte depletion observed in advanced Phdp infections (do Vale et al., 2007). The ability of the toxin to neutralize the main players responsible for the phagocytic defense of the host is a very effective pathogenicity strategy that contributes to the severity of Phdp infections by promoting survival of the pathogen and its unrestricted extracellular multiplication. Concomitantly, the AIP56-induced apoptosis of both professional phagocytes leads to tissue damage with deleterious consequences for the host. In fact, the destruction of macrophages, the cells with the crucial role of eliminating apoptotic cells (Parnaik et al., 2000; Wood et al., 2000), compromises the efficient clearance of apoptotic phagocytes and leads to their lysis by secondary necrosis (do Vale et al., 2003) with release of their cytotoxic intracellular contents (do Vale et al., 2007). This has particularly serious consequences in the case of neutrophils, due to their richness in highly cytotoxic molecules, which damage many cell types and produce tissue injury, thus contributing to the genesis of the Phdp-associated cytopathology.

Staphylococcus aureus: A BENCH OF SECRETED MOLECULES TO SHOOT NEUTROPHILS

Staphylococcus aureus is a Gram-positive bacterium that often colonizes the human nares and the skin. Besides being a commensal, *S. aureus* is also a redoubtable human pathogen that causes a variety of severe diseases. To set up a successful infection, *S. aureus* evolved an amazing variety of immune evasive strategies wiping out both innate and adaptive immune responses. *S. aureus* infections involve the invasion of host tissues, replication in

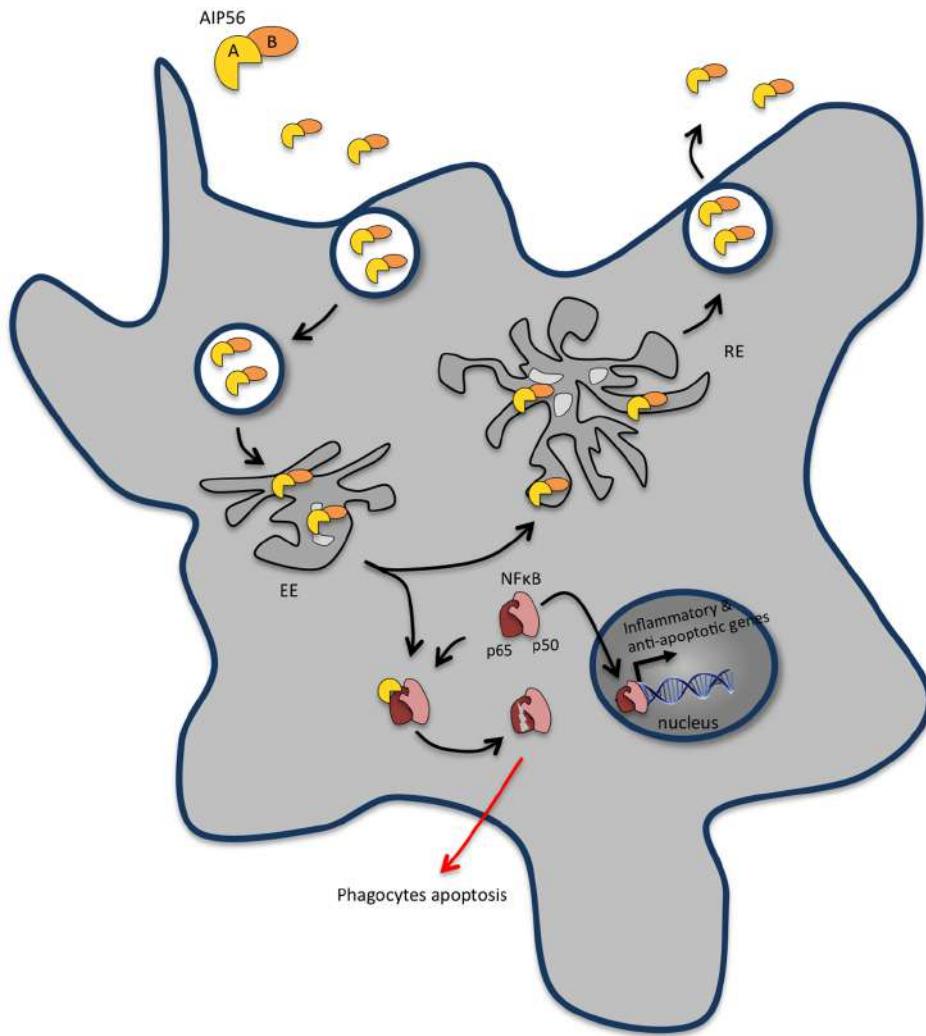


FIGURE 3 | AIP56 blocks innate immunity by inducing massive apoptosis of host macrophages and neutrophils. Upon encountering susceptible cells, apoptosis-inducing protein of 56 kDa (AIP56) binds to a still unidentified cell-surface receptor and undergoes clathrin-mediated endocytosis. Once in early endosomes, the toxin either follows the recycling pathway back to the extracellular medium or suffers low pH-induced translocation across the endosomal membrane into the cytosol to display its toxic activity. AIP56 is a zinc-dependent metalloprotease that cleaves the p65 subunit of nuclear factor- κ B (NF- κ B), an evolutionarily conserved transcription factor that regulates the expression of inflammatory and anti-apoptotic genes and plays a key role in host responses to microbial pathogen invasion. During infection, AIP56 disseminates systemically and its activity leads to depletion of macrophages and neutrophils by post-apoptotic secondary necrosis, thereby blocking the phagocytic defense of the host and contributing to the occurrence of tissue damage.

abscess lesions and dissemination through purulent drainage of these lesions and require the recruitment of immune cells to the site of infection. Such infiltrated immune cells would usually eliminate the bacteria. However, to counter their action, *S. aureus* secretes soluble molecules targeting multiple pathways to manipulate the capacity of neutrophils for chemotaxis, phagocytosis, and bacterial killing, thus enabling pathogen replication and ensuring the success of the infection. Several recent and comprehensive reviews highlight how *S. aureus* virulence factors manipulate the host immune response (Spaan et al., 2013b; Otto, 2014; Thammavongsa et al., 2015). Here we will focus on its secreted molecules mainly targeting neutrophils, modulating their function, or inducing cell killing.

Avoiding Neutrophil Extravasation, Chemotaxis, and Activation

In general, upon pathogen recognition, pro-inflammatory signals released by resident macrophages promote the adhesion of circulating neutrophils and further extravasation across capillary endothelium to the site of infection. This process relies on interactions between the endothelial surface receptors (e.g., selectins and ICAM1) and their respective ligands on the surface of neutrophils (e.g., PSGL1 and β 2-integrin) (Spaan et al., 2013b). To inhibit neutrophil recruitment to the infected tissues, *S. aureus* secretes two anti-inflammatory factors that prevent neutrophil adhesion to the blood vessels and further transmigration (Figure 4A). The staphylococcal SSL5 binds

PSGL1 in a glycan dependent manner at the surface of neutrophils, blocking its interaction with P-selectin expressed by endothelial cells and abrogating the early steps of neutrophil attachment (Bestebroer et al., 2007). In addition, SSL5 was shown to inactivate matrix metalloproteinase from human neutrophils, accounting for the limited capacity of neutrophils to transmigrate into infected tissues (Itoh et al., 2010). The extracellular adherence protein (Eap) recognizes endothelial ICAM1, preventing its interaction with $\beta 2$ -integrins at the surface of neutrophils and further inhibiting extravasation (Chavakis et al., 2002).

Staphylococcus aureus also secretes a number of antagonists of neutrophil receptors interfering with chemokine signaling and limiting neutrophil recruitment (**Figure 4A**). In particular, SSL5 directly binds the N-terminus of G-protein coupled chemokine receptors (GPCRs) inhibiting calcium mobilization and actin polymerization, thus impairing neutrophil responses to a huge diversity of chemokines (e.g., CXCL8, CXCL1, CCL2, and CCL5) and to complement fragments C3a and C5a (Bestebroer et al., 2009). Similarly to the recognition of PSGL1 by SSL5, binding to GPCRs relies on the presence of sialic acid residues (Baker et al., 2007; Bestebroer et al., 2007, 2009). In addition, SSL5 was shown to bind to platelet glycoproteins, inducing platelet activation and aggregation, which could be important for colonization and immune evasion by *S. aureus* (de Haas et al., 2009). SSL10 inhibits CXCL12-mediated responses by targeting CXCR4 (Walenkamp et al., 2009) and SSL3 binds to TLR2, hindering immune recognition of staphylococcal lipoproteins and peptidoglycan (Yokoyama et al., 2012).

Phenol-soluble modulins are produced by all *S. aureus* strains and have multiple roles in staphylococcal pathogenesis (Cheung et al., 2014a). In particular, PSMs are potent pro-inflammatory molecules that interact with human formyl peptide receptor 2 (FPR2) (Kretschmer et al., 2010), a GPCR involved in the recognition of pathogens. At nanomolar concentrations, PSMs bind to and activate FPRs, with the strongest activation occurring through FPR2, stimulating several FPRs effector functions such as chemotaxis and pro-inflammatory cytokine production (e.g., CXCL8; Fu et al., 2006). However, human isolates of *S. aureus* evolved other strategies to counter neutrophil chemotaxis by directly interfering with FPRs signaling and limiting cytotoxicity while promoting bacterial replication (McCarthy and Lindsay, 2013; Cheung et al., 2014b). Inhibition of FPRs-mediated pro-inflammatory signaling occurs via the secretion of the chemotaxis-inhibitor protein of *S. aureus* (CHIPS) (de Haas et al., 2004) and the FPR2/ALS-inhibitory protein (FLIPr) and its homologue FLIPrL (Prat et al., 2006, 2009; **Figure 4A**). While CHIPS binds and inhibits FPR1 and C5aR, FLIPr and FLIPrL block FPR2-mediated signaling, thus avoiding recognition of PSMs secreted by *S. aureus*, impairing pro-inflammatory response and reducing neutrophil recruitment (Haas et al., 2004, 2005; Prat et al., 2006, 2009). Clearly, CHIPS, FLIPr and FLIPrL, and PSMs have opposite effects on FPRs activation. Thus the production/secretion of these bacterial molecules is likely to be under strict control to allow the establishment of the infection and to evade immune recognition.

The repertoire of secreted molecules by *S. aureus* to evade the early steps of immune response also include Stathopain A (ScpA), a cysteine protease that specifically cleaves the N-terminus of human CXCR2 (Laarman et al., 2012), a GPCR responding to several chemokines (e.g., CXCL1-3 and CXCL5-8). Stathopain A inhibits CXCR2-mediated calcium mobilization, migration, intracellular signaling and activation of neutrophils (Laarman et al., 2012), appearing as an important immunomodulatory molecule causing neutrophil unresponsiveness to several chemokines and blocking their recruitment to the site of infection.

Blocking Complement Activation, Opsonization, and Phagocytosis

Staphylococcus aureus display intrinsic physical features (e.g. thick peptidoglycan layer and capsule) that confer resistance to complement-mediated killing and neutrophil phagocytosis. In addition, *S. aureus* secretes a variety of highly specific proteins with complement-modulating functions (**Figure 4B**), thereby delaying the innate immune attack and generating a window of opportunity to replicate and establish within the host (Spaan et al., 2013b; Thammavongsa et al., 2015). Several of these secreted molecules target C3 or C3 convertases, both central molecules in the complement activation cascade. Aureolysin is a metalloprotease that cleaves C3, generating a modified C3b fragment that is further degraded instead of being covalently linked to the bacterial surface where it would promote the generation of the chemoattractant molecule C5a (Laarman et al., 2011). In addition, this proteinase degrades human antimicrobial peptides highly potent against *S. aureus* (Sieprawska-Lupa et al., 2004). Thus, aureolysin activity promotes infection by blocking the complement cascade impairing bacterial C3b opsonization, phagocytosis, and neutrophil-mediated killing (Sieprawska-Lupa et al., 2004; Laarman et al., 2011). Staphylococcal component inhibitor (SCIN) specifically binds to human C3 convertase and blocks its activity thereby preventing the production of C3a, C3b, and C5a, thus interfering with complement activation and with neutrophil-mediated killing of *S. aureus* (Rooijakkers et al., 2005a, 2009; **Figure 4B**). In addition, *S. aureus* secreted extracellular fibrinogen-binding protein (Efb) together with its homologue extracellular complement-binding protein (Ecb), were shown to inhibit both C3 and C5 convertases (Jongerius et al., 2010a,b, 2007). Other *S. aureus* secreted proteins target both C3 and immunoglobulin binding, thus impairing complement activation and opsonization, this is the case of staphylococcal binder of immunoglobulin (Sbi; Haupt et al., 2008) and staphylokinase (Rooijakkers et al., 2005b).

Besides their role in blocking neutrophil extravasation and chemotaxis (described above), some SSLs also hinder complement activation and phagocytosis. SSL7 binds IgA and complement C5, inhibiting the production of C5a and further phagocytosis and bacterial clearance during *in vivo* infection (Bestebroer et al., 2010). SSL10 binds to IgG1 preventing recognition by Fc Receptors (FcR), thus impairing IgG1 opsonization and phagocytosis (Patel et al., 2010; **Figure 4B**).

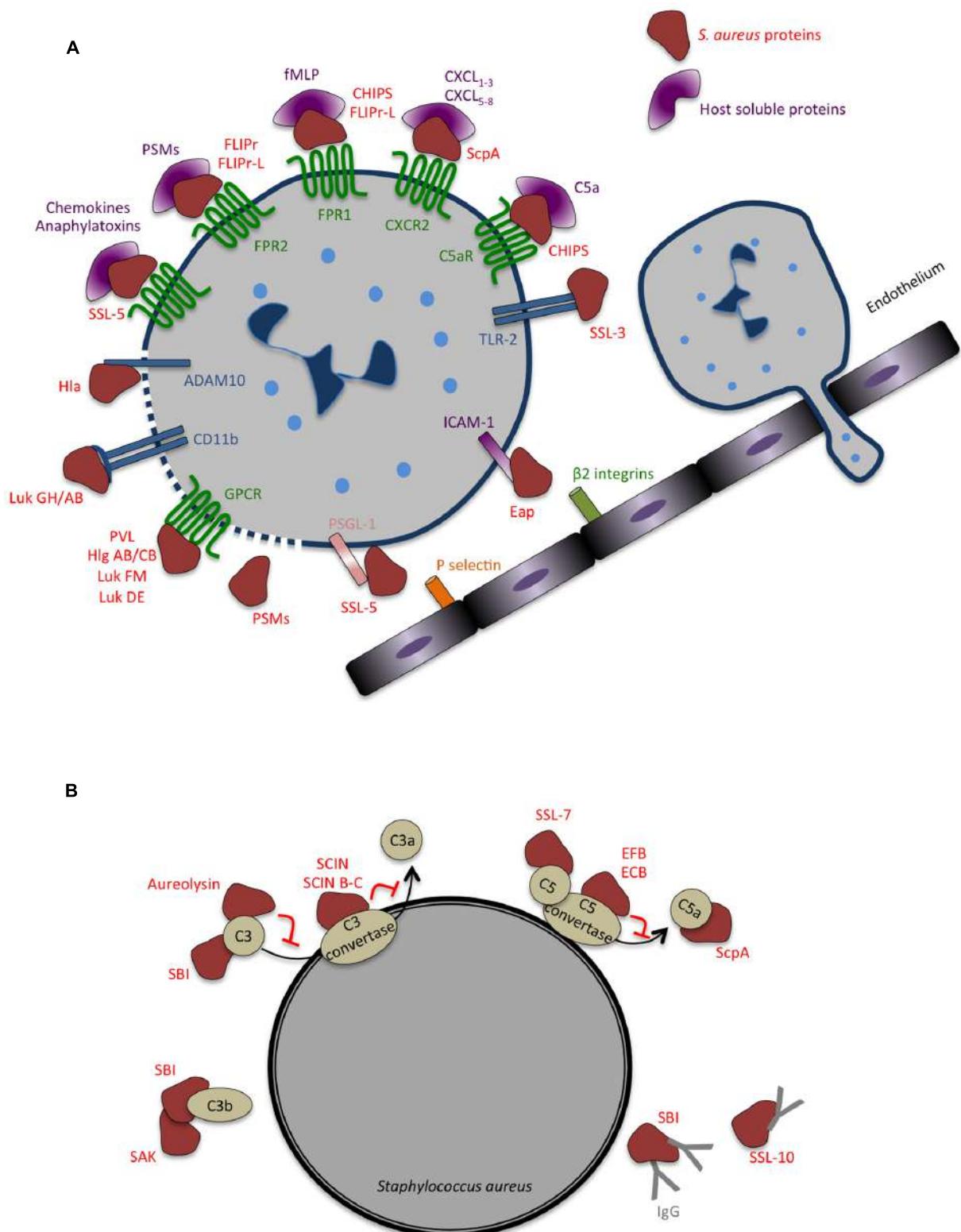


FIGURE 4 | Continued

FIGURE 4 | Continued

Strategies evolved by *Staphylococcus aureus* to counteract innate immune response. **(A)** Secreted bacterial factors that inhibit neutrophils extravasation, chemotaxis and activation. Neutrophil rolling is modulated by staphylococcal superantigen-like 5 (SSL5) that binds P-selectin glycoprotein ligand-1 (PSGL-1), blocking its interaction with P-selectin. The adhesion of neutrophils to the endothelium and consequent transmigration is inhibited by extracellular adherence protein (Eap), which binds to intercellular adhesion molecule 1 (ICAM-1). In addition to inhibiting PSGL-1, SSL5 inhibits neutrophil responses to chemokines and to anaphylatoxins, by binding to different chemokine receptors. Several staphylococcal molecules impair neutrophil chemotaxis and important co-signaling events during migration and phagocytosis: chemotaxis-inhibitor protein of *S. aureus* (CHIPS) binds and inhibits formyl peptide receptor 1 (FPR1) and C5a receptor (C5aR); formyl peptide receptor-like 1 inhibitor (FLIPr)-like inhibit FPR1; FLIPr and FLIPr-like inhibit FPR2; staphopain (ScpA) cleaves Chemokine (C-X-C Motif) Receptor 2 (CXCR2); staphylococcal SSL3 inhibits toll-like receptor 2 (TLR2)-mediated signaling, the bicomponent leukocidins Panton-Valentine leukocidin (PVL), gamma-hemolysin (Hlg) ABC, leukocidin (Luk) FM, Luk GH/AB, and Luk DE interact with chemoattractant receptors of the G-protein-coupled receptor (GPCR) family. Both Hla and Luk GH/AB induce cell lysis by binding ADAM metallopeptidase domain 10 (ADAM10) and CD11b, respectively. The cytolytic peptides phenol-soluble modulins (PSMs) have an amphipathic alpha-helical region that likely contributes to their lytic activity, presumably by membrane insertion and pore formation. **(B)** Secreted bacterial factors that inhibit opsonization and phagocytosis by neutrophils. The secreted metalloprotease aureolysin inhibits phagocytosis and killing of bacteria by neutrophils by cleaving C3. Staphylococcal complement inhibitor (SCIN), SCIN-B, and SCIN-C associate with and inhibits C3 convertase, thereby preventing the production of C3a, C3b, and further C5a and consequently interfering with complement activation. The extracellular fibrinogen binding protein (EFB) and the extracellular complement-binding protein (ECB) also inhibit complement activation by inactivating C5 convertase and staphylococcal SSL7 targets C5. Staphylococcal binder of immunoglobulin (SBI) affect both the function of complement and immunoglobulin binding, blocking the classical complement activation pathway, and associates with C3 inhibiting the alternative pathway. Staphylokinase (SAK) forms enzymatically active complexes with C3b blocking complement activation. Staphylococcal SSL10 binds IgG, affecting Fc receptor (FcR) recognition and complement activation.

Avoiding NET Bactericidal Activities

In addition to phagocytosis and intracellular killing, neutrophils evolved an alternative defense mechanism to trap extracellular pathogens and prevent their dissemination. This strategy relies on the release of nuclear content together with antimicrobial cytosolic and granular proteins to form neutrophil extracellular traps (NETs), which are scaffolds that act as physical barriers to pathogen progression protecting host tissues from damage (Papayannopoulos and Zychlinsky, 2009). A secreted staphylococcal nuclease (Nuc) has the capacity to degrade NETs thereby allowing *S. aureus* to resist their bactericidal activities *in vitro* as well as in a respiratory tract infection model (Berends et al., 2010). NET degradation by Nuc leads to the production of monophosphate nucleotides that are further converted into deoxyadenosine through the activity of adenosine synthase (AdsA), another *S. aureus* secreted protein. Interestingly, the accumulation of deoxyadenosine generated by AdsA activity promotes the autocleavage of pro-caspase-3, triggering caspase-3-induced apoptosis of infiltrating macrophages (Thammavongsa et al., 2013). Together, staphylococcal Nuc and AdsA act in a concerted mode to promote bacterial survival in *S. aureus* abscesses, by excluding macrophages from the infection foci.

Neutrophil Killing: The Ultimate Defense

In line with its ability to evade almost every step of the innate immune response, *S. aureus* induce the death of innate immune cells, through the secretion of PSMs and several other toxins. As mentioned above, PSMs are *S. aureus* secreted molecules with multiple roles in infection (Wang et al., 2007; Cheung et al., 2014a). They trigger inflammatory responses by interacting with FPR2 and display, at higher concentrations, FPR2-independent cytolytic activity likely through membrane insertion and pore formation (Kretschmer et al., 2010; Figure 4A). In particular, PSM α peptides are able to trigger the lysis of the phagosome after neutrophil ingestion allowing intracellular bacterial replication, and ultimately are responsible for lysis of neutrophils promoting

bacterial survival and escape to the extracellular milieu (Wang et al., 2007; Geiger et al., 2012; Chatterjee et al., 2013; Grosz et al., 2014). *In vitro*, expression levels of PSMs correlate with levels of cytotoxicity (Rasigade et al., 2013). Moreover, mutants deficient for PSM α production are perturbed in biofilm formation (Peschel and Otto, 2013) and attenuated in the mouse bloodstream infection model (Wang et al., 2007). Altogether, these observations strongly suggest that PSM α -triggered effects may play a key role in *in vivo* *S. aureus* infection.

In addition to PSMs whose cytolytic activity is receptor-independent, *S. aureus* secretes other cytolytic toxins that interact with specific receptors at the surface of eukaryotic cells, oligomerize and form pores inducing cell leakage and ultimately total lysis. *S. aureus*-produced toxins targeting white blood cells belong to the beta-barrel pore-forming toxins and comprise hemolysin- α (Hla, also called α -toxin) and bicompetent leukocidins (Otto, 2014; Thammavongsa et al., 2015; Figure 4A). *In vivo* infection studies have shown that Hla is required for several *S. aureus*-associated pathologies, such as pneumonia and severe skin infections (Bubeck Wardenburg et al., 2007; Bubeck Wardenburg and Schneewind, 2008; Kennedy et al., 2010). Hla interacts with high affinity with ADAM10 at the surface of host cells (Wilke and Bubeck Wardenburg, 2010) to damage epithelial, endothelial and immune cells (Berube and Bubeck Wardenburg, 2013; Figure 4A). Mice lacking ADAM10 expression in the lung epithelium resist to lethal pneumonia (Becker et al., 2014), whereas animals lacking ADAM10 specifically on myeloid lineage develop exacerbated skin infections (Inoshima et al., 2011). Although these results suggest that the outcome of Hla-mediated effects may depend on the infected tissue, the role of Hla on tissue-specific innate immunity requires further analysis.

Leukocidins are composed by two distinct and independently secreted subunits that form heteromultimeric pores in the membrane of host myeloid cells (Otto, 2014). *S. aureus* produce different arrays of leukocidins with different species and cell type specificities, which are mainly dictated by their

interaction with host GPCRs (Spaan et al., 2013b; Otto, 2014; Thammavongsa et al., 2015; **Figure 4A**). *In vitro* assays with purified proteins, as well as *ex-vivo* infections with *S. aureus*, have shown that leukocidin AB (LukAB, also called LukGH) kills human, but not mouse, neutrophils upon binding to CD11b (DuMont et al., 2013a). In addition, purified LukAB induce the release of NETs (Malachowa et al., 2013) and also promotes the escape from the phagosome in neutrophils, thus enabling *S. aureus* replication (DuMont et al., 2013b). Gamma-hemolysin HlgAB and HlgCB bind chemokine receptors (e.g., CXCR1, CXCR2, and CCR2) and complement receptors (e.g., C5aR), respectively (Spaan et al., 2014), and promote the lysis of human neutrophils and macrophages *in vitro*. LukED, produced by a large majority of clinical isolates of *S. aureus*, triggers the lysis of neutrophils and macrophages of different vertebrates by binding chemokine receptors such as CCR5, CXCR1 and CXCR2 (Alonzo et al., 2013; Reyes-Robles et al., 2013). Importantly, LukED was shown to play a critical role in *S. aureus* systemic infections in mice, by promoting bacterial replication *in vivo* through direct killing of neutrophils (Alonzo et al., 2012). Lastly, Panton-Valentine leukocidin (PVL), which is secreted by a small percentage of *S. aureus* isolates, binds C5aR on neutrophils and macrophages and has a restricted activity toward human and rabbit cells (Spaan et al., 2013a, 2015). Despite the several attempts to evaluate the exact contribution of each of these toxins to *S. aureus*-associated pathologies, their vast species and cell type specificities have rendered this analysis highly challenging. In this context, the data obtained from commonly used animal infection models (e.g., mice and rats) should be interpreted with caution.

***Mycobacterium ulcerans*: SABOTAGE OF THE HOST IMMUNE RESPONSE BY A POLYKETIDE TOXIN**

Mycobacterium ulcerans is the causative agent of Buruli ulcer, a chronic ulcerative skin disease that usually starts as painless nodules on the limbs that then develop into large ulcers. These can lead to severe scars and local deformities, including disabling contractures, if not treated at early stages (WHO Buruli ulcer fact sheet N° 199, Updated July 2014). Buruli ulcer occurs most frequently in children living in tropical environments, near wetlands. The disease is more common in poor and rural areas of Africa but is also found in South America, Asia and Australia. *Mycobacterium ulcerans* is currently recognized as an environmental pathogen, but its reservoirs and mode of transmission remain doubtful (WHO Buruli ulcer fact sheet N° 199, Updated July 2014). Genetically very close to *M. tuberculosis* and *M. marinum*, *M. ulcerans* is unique among human pathogenic mycobacteria due to the secretion of a lipid toxin, the mycolactone (George et al., 1999; Hong et al., 2008). Mycolactone displays cytotoxic and immunosuppressive activities and is considered the major pathogenicity factor in Buruli ulcer, being essential for *M. ulcerans* virulence, immune modulation, and colonization (George et al., 1999;

Coutanceau et al., 2005; Marsollier et al., 2005; Torrado et al., 2007a, 2010; Simmonds et al., 2009; Guenin-Mace et al., 2011).

In animal models, injection of mycolactone alone is sufficient to cause ulcers similar to those found in infected hosts (George et al., 1999, 2000). Concerning the mechanism of cellular intoxication, it has been proposed that, due to its hydrophobic nature, mycolactone passively diffuses through the plasma membrane (Snyder and Small, 2003; **Figure 5**). At micromolar concentrations, mycolactone is highly cytotoxic to a variety of mammalian cells, with variable susceptibility levels depending on the cell type (Hall and Simmonds, 2014). The mycolactone cytotoxicity has been linked to its apoptogenic activity. Apoptosis was observed in several cell types incubated *in vitro* with mycolactone (George et al., 2000; Gama et al., 2014), when primary mouse macrophages were incubated with toxigenic *M. ulcerans* strains (Oliveira et al., 2005), and in guinea pigs (George et al., 2000) and mice (Oliveira et al., 2005; Torrado et al., 2007b) infected with mycolactone-producing *M. ulcerans*. More importantly, massive apoptosis has been observed in Buruli ulcer lesions (Walsh et al., 2005). The mycolactone-induced cell death mechanisms appear to be complex and are not completely understood. It has been shown that mycolactone induces cell cycle arrest at the G1/G0-phase (George et al., 1999), but the connection between this effect of mycolactone and its cytotoxicity remains unclear. Early studies on mycolactone reported the occurrence of early actin cytoskeleton rearrangements, cell rounding, and detachment following incubation with the toxin (George et al., 1998, 1999). More recently, studies with HeLa and Jurkat T cells have shown that mycolactone induces increased actin polymerisation in intoxicated cells, as a consequence of its binding to the GTPase domain of the actin-cytoskeleton regulator Wiskott-Aldrich syndrome protein WASP (Guenin-Mace et al., 2013). This leads to hyper-activation of WASP and re-localization of the Arp2/3 complex and consequently, to major cytoskeletal rearrangements, including the formation of filopodia (Guenin-Mace et al., 2013). In epithelial cells, this causes loss of cell adhesion and E-cadherin-dependent tight junctions, ultimately leading to the death of detached cells by anoikis (Guenin-Mace et al., 2013). Recent studies performed *in vitro* with murine fibroblasts confirmed the cytoskeleton as a main target of mycolactone, by showing that mycolactone causes changes in microtubules and affects several regulators and structural components of microtubules and microfilaments (Gama et al., 2014). These deleterious effects inflicted by mycolactone upon the cytoskeleton likely contribute to the formation of the lesions characteristic of Burulli ulcer. Additionally, given the central role of the cytoskeleton in controlling key cellular functions, such as endocytosis, intracellular trafficking, cell adhesion and migration, it is reasonable to speculate that, by highjacking cytoskeleton functions, mycolactone perturbs the functions of phagocytic cells. Indeed, it is likely that the decreased phagocytic activity of macrophages exposed to mycolactone (Adusumilli et al., 2005; Coutanceau et al., 2005) results from the effect of the toxin upon the cytoskeleton of those cells. Further investigations are required to determine whether

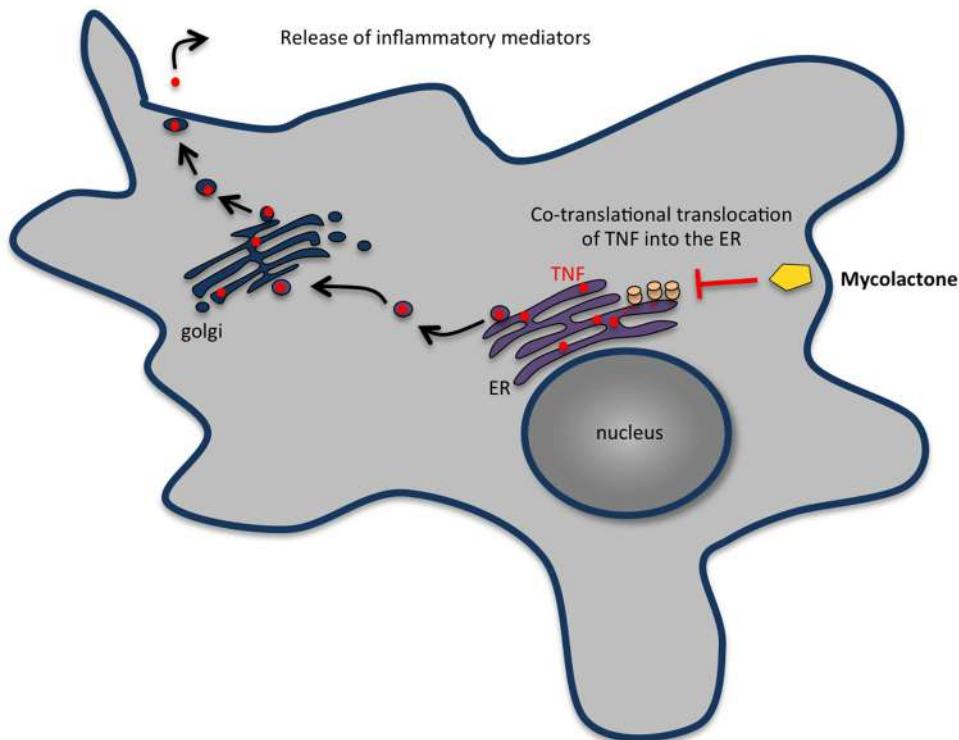


FIGURE 5 | Mycolactone inhibits the secretion of most cytokines, chemokines and other inflammatory mediators by macrophages. In eukaryotic cells, secretory proteins cross the ER membrane before being transported in vesicles to the Golgi complex and then to the plasma membrane. Mycolactone enters cells by passive diffusion through the plasma membrane and inhibits the production of inflammatory mediators by macrophages by blocking the translocation of nascent proteins into the ER. The proteins wrongly accumulated in the cytosol are then degraded by the proteasome.

cytoskeleton is manipulated by mycolactone in macrophages and neutrophils and what are the consequences of this manipulation *in vivo*.

In addition to its known cytotoxic effects toward distinct cell types, at non-cytotoxic concentrations, mycolactone interferes with important functions of immune cells, including monocytes, macrophages, and DCs (Arango Duque and Descoteaux, 2014; Hall and Simmonds, 2014). It is well recognized that macrophages play crucial roles in mycobacterial infections, including in Buruli ulcer. Although Buruli ulcer histopathology is characterized by extensive areas of necrosis with abundant extracellular bacteria, studies on infected humans, and experimental *M. ulcerans* infections revealed that *M. ulcerans* is phagocytosed by macrophages and neutrophils, similarly to the other pathogenic mycobacteria (Torrado et al., 2007b; Silva et al., 2009) and escape the microbicidal activity of the macrophages presumably by mycolactone-dependent interference with the IFN γ -dependent phagosome maturation and NO production required to control *M. ulcerans* infection (Torrado et al., 2010). After an initial phase of intracellular proliferation, varying according to the strain cytotoxicity/virulence, *M. ulcerans* causes apoptosis/necrosis of the host macrophage through a mycolactone-dependent mechanism and becomes extracellular (Torrado et al., 2007b).

Usually, phagocytosis of a microorganism triggers signaling events that rapidly culminate in a controlled inflammatory response involving the secretion of several cytokines and chemokines that recruit other inflammatory cells to the site of infection. However, available evidence suggests that this early response is heavily perturbed by mycolactone. Indeed, cells exposed to mycolactone-producing strains of *M. ulcerans* secrete much less TNF than those infected with mycolactone-negative strains (Torrado et al., 2007a) and purified mycolactone has been shown to suppress the production of several cytokines, chemokines, and other inflammatory mediators by macrophages (Arango Duque and Descoteaux, 2014; Hall and Simmonds, 2014). It has been proposed that this suppression is associated with a mycolactone-induced blockade of co-translational protein translocation into the ER and subsequent degradation of the aberrantly located proteins in the cytosol (Hall et al., 2014; Figure 5). Failure to produce cytokines and chemokines may contribute to the absence of inflammatory infiltrate at the central necrotic areas of the lesion containing high numbers of extracellular bacilli, in addition to the lysis of recruited inflammatory cells induced by the build-up of mycolactone. The inflammatory infiltrates occupy a band at the periphery of the lesion that represents a front that is continuously advancing into healthy tissues in progressive *M. ulcerans* lesions (Silva et al., 2009).

CONCLUDING REMARKS

The clearance of infectious agents greatly depends on the host innate immune responses that take place at early stages of infection and in which macrophages and neutrophils are the central players. To counteract the host defense mechanisms, bacterial pathogens secrete a bench of different toxins that neutralize, at different levels, the host innate immune response and in particular, annihilate the function of macrophages and neutrophils. Despite having different features, secreted toxins targeting the function of innate immune cells often display similar and/or complementary activities and modulate the same central pathways of the host cell (e.g., inflammatory response, cytoskeleton dynamics, and cAMP signaling). Furthermore, a single pathogen may secrete several toxins that act differently to produce the same outcome (e.g., inhibit chemotaxis or induce phagocyte death). These apparently redundant strategies of bacterial attack ensure the multistep impairment of the early host immune responses mounted against the pathogen and guarantee the control of host-pathogen interaction providing a window of time and opportunity for bacterial growth and establishment within the host.

In the past, many studies aiming to uncover the molecular functions of bacterial toxins on host cells were performed *in vitro* in several cultured cell lines, and more recently in primary cells, using a wide range of concentrations of purified toxins. In addition, studies on animal models (mainly in rodents) using either purified toxins, wild type bacteria and toxin-deficient mutants, provided a number of important observations regarding the toxin-mediated pathologies. Together, these studies generated an incredible amount of data that paradoxically poorly contributed to the understanding of the role of toxins in human infections. Whereas in the perspective of using toxins as molecular tools to address cell biology topics there is great value in testing toxin effects in many *in vitro* cell systems, several issues render extremely difficult the interpretation of data from *in vitro* studies in the context of infection. In particular, the concentration of purified toxin used is often much higher than that produced by bacteria during infection and it is highly variable among different studies, and the cell lines tested are often non-relevant for the pathophysiology of the infection. Regarding studies performed in animal models, two major concerns have been pointed out: (1) many toxins display species-specificity and thus routinely used models, specially rodents, are non-relevant for the study of many toxin-mediated human pathologies compromising the extrapolation of data and (2) direct inoculation of purified toxins in the animals only provide limited information that do not necessarily recapitulate the effect of a given toxin in the context of human bacterial infection. Thus, the data generated so far

needs to be cautiously analyzed whenever we aim to better understand the role of toxins in the *in vivo* infectious process. The accurate role of toxins in human infections needs to be analyzed in the context of bacterial infections in different animal species. In this perspective, future efforts should concentrate in the development and use of appropriate animal models, possibly non-human primates, in which available *in vitro* and *in vivo* data can be confirmed and possibly extrapolated to the human pathologies.

AUTHOR CONTRIBUTIONS

AdV and SS wrote the manuscript. DC contributed with figures design. The manuscript content was discussed and decided by all the authors.

FUNDING

This work was supported by FEDER funds through Programa Operacional Factores de Competitividade – COMPETE and by national funds through FCT – Fundação para a Ciência e a Tecnologia (project PTDC/BIA-MIC/3463/2012 FCOMP-01-0124-FEDER-028364; to AV). Research in the groups of Molecular Microbiology and Fish Immunology and Vaccinology is supported by national funds through FCT – Fundação para a Ciência e a Tecnologia/MEC – Ministério da Educação e Ciência and co-funded by FEDER within the partnership agreement PT2020 related with the research unit number 4293. The Group of Molecular Microbiology also receives support from a Research Grant 2014 by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (to SS) and the PT2020 research project Infect-ERA/0001/2013 PROANTILIS. AdV received the FCT fellowship SFRH/BPD/95777/2013 by national funds through FCT – Fundação para a Ciência e a Tecnologia/MEC – Ministério da Educação e Ciência and co-funded by QREN and POPH (Programa Operacional Potencial Humano). SS is supported by FCT-Investigator program.

ACKNOWLEDGMENTS

We apologize to authors whose relevant work could not be cited owing to space limitations. We thank Nuno MS dos Santos (IBMC/I3S, Porto, Portugal) for critical reading of the manuscript and Jorge Pedrosa (ICVS/Universidade do Minho, Braga, Portugal) for reviewing the content concerning mycolactone.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Clostridial C3 toxins target monocytes/macrophages and modulate their functions

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Microbial Immunology, a section of
the journal Frontiers in Immunology

Received: 19 May 2015

Accepted: 17 June 2015

Published: 30 June 2015

Citation:

Barth H, Fischer S, Möglich A and
Förtsch C (2015) Clostridial C3 toxins
target monocytes/macrophages and
modulate their functions.
Front. Immunol. 6:339.
doi: 10.3389/fimmu.2015.00339

The C3 enzymes from *Clostridium (C.) botulinum* (C3bot) and *Clostridium limosum* (C3lim) are single chain protein toxins of about 25 kDa that mono-ADP-ribosylate Rho-A, -B, and -C in the cytosol of mammalian cells. We discovered that both C3 proteins are selectively internalized into the cytosol of monocytes and macrophages by an endocytic mechanism, comparable to bacterial AB-type toxins, while they are not efficiently taken up into the cytosol of other cell types including epithelial cells and fibroblasts. C3-treatment results in disturbed macrophage functions, such as migration and phagocytosis, suggesting a novel function of clostridial C3 toxins as virulence factors, which selectively interfere with these immune cells. Moreover, enzymatic inactive C3 protein serves as a transport system to selectively deliver pharmacologically active molecules into the cytosol of monocytes/macrophages without damaging these cells. This review addresses also the generation of C3-based molecular tools for experimental macrophage pharmacology and cell biology as well as the exploitation of C3 for development of novel therapeutic strategies against monocyte/macrophage-associated diseases.

Keywords: C3, Rho, macrophage, cellular uptake, targeted drug delivery

Introduction: Bacterial C3 Proteins

Various Gram-positive bacteria produce and secrete C3 proteins (~25 kDa, pI > 9) that selectively mono-ADP-ribosylate the small GTPases Rho-A, -B, and -C. C3 ADP-ribosyltransferases catalyze the covalent transfer of the ADP-ribose group from the cosubstrate NAD onto Asn-41 of Rho (1, 2). In 1987, Aktories and co-workers described the first C3 protein (C3bot), which is produced from *Clostridium botulinum* C and D strains (3, 4). It became evident that there are two isoforms, C3bot1 and C3bot2 with 60% sequence identity. Further, C3 proteins were identified in *Clostridium limosum* (C3lim) (5), *Bacillus cereus* (C3cer) (6), as well as *Staphylococcus aureus* (C3stau) (7). C3stau alias epithelial differentiation inhibitor (EDIN) includes three isoforms (A, B, and C), which share ~35% sequence identity to C3bot1 and ADP-ribosylates RhoE in addition to Rho-A, -B, and -C (7).

The pathophysiological role of the clostridial C3 proteins is not clear so far, but it was suggested that C3stau might play a role as virulence factor, since more C3stau-producing *S. aureus* were isolated from patients with impetigo, diabetic foot ulcers, and other skin infections than from healthy carriers (8–11). In contrast to the clostridia, *S. aureus* enters mammalian cells and releases C3stau into the host cell cytosol, where the C3stau-catalyzed ADP-ribosylation of Rho results

in reorganization of the actin cytoskeleton (12). In tissues, this mode of action causes disruption of cell–cell contacts and barrier functions, which allows the dissemination of *S. aureus* through the disturbed tissue barriers into deeper tissues, as already demonstrated in animal models (12, 13). Moreover, in endothelial cells, the C3stau-mediated reorganization of the actin cytoskeleton results in the formation of large transendothelial channels, so-called macroapertures (TEMs) (14–16), which increase the endothelial permeability and facilitate the dissemination of *S. aureus* from blood to deeper tissue layers.

C3 Enzymes Mono-ADP-Ribosylate Rho and Inhibit Rho Signaling

Up to date, C3 enzymes represent the only known specific Rho inhibitors and were widely used in biochemistry, cell biology, and experimental pharmacology as highly valuable molecular tools to investigate the role of Rho-signaling *in vitro* and in living cells (17, 18). However, it soon became evident that C3 proteins are not efficiently taken up into the cytosol of the tested cell types including epithelial cells and fibroblasts and it was suggested that C3 proteins might be exoenzymes rather than typical exotoxins, which become internalized only by non-specific mechanisms, such as pinocytosis, when cultured cells were treated for long incubation periods (>24 h) with high C3 concentrations (>10 µg/ml) in the medium (19). Therefore, C3 proteins were introduced into the cytosol of cells by artificial methods including microinjection, transfection, or by the use of molecular transporters like cell-penetrating peptides, viruses, or portions of bacterial AB-toxins (18, 20).

In line with the observation of their very limited and non-specific uptake in the tested cell types, no binding and translocation subunit was found in the C3 proteins, which could mediate their uptake into the host cell cytosol. Structure analysis of C3bot and C3stau revealed that these proteins consist of a single enzyme domain with a catalytic core containing the conserved NAD binding site and a catalytic pocket with an α-helix bent lying over two antiparallel β-sheets, which form a central cleft (21, 22) and show high structure similarity with the catalytic domains of binary actin-ADP-ribosylating toxins (18, 22, 23). Moreover, like the binary actin-ADP-ribosylating toxins, C3 enzymes contain the highly conserved amino acids, which were also identified in other ADP-ribosyltransferases: the STS-motif 174Ser-Thr-Ser176, which is flanked by Arg128 and Glu214 (22) and the “catalytic glutamate” Glu214, which plays a central role in the covalent transfer of ADP-ribose onto Asn41 of Rho. Furthermore, the binary actin ADP-ribosylating toxins and the C3 enzymes contain the so-called ADP-ribosylating toxin turn-turn (ARTT) motif, two adjacent protruding turns, which play a role for the toxin-specific recognition of actin or Rho: at position 212 in turn 2, the Rho-modifying C3 enzymes have a Gln residue, the actin-ADP-ribosylating toxins a Glu residue (22, 24). The precise molecular mechanisms of the ADP-ribosylation as well as the function of the individual amino acid residues of the ADP-ribosyltransferases is described in more detail in highly acknowledged reviews (18, 25).

Besides the Rho ADP-ribosylation, C3 acts on RalA, a member of the Ras GTPase family, in a non-enzymatic manner independent from its ADP-ribosyltransferase activity. C3 directly binds to RalA (6), which keeps RalA in its inactive GDP-bound conformation and prevents the activation of downstream RalA effectors (26, 27). However, the cellular consequences of this C3–RalA interaction are not known so far.

Molecular and Cellular Consequences of the C3-Catalyzed Rho ADP-Ribosylation

The C3 enzymes specifically mono-ADP-ribosylate the isoforms Rho A, -B, and -C at Asn-41 and prefer Rho-GDP as substrate (28, 29) because in this structure, Asn41 is accessible to C3 (30). As a consequence, the ADP-ribosylated Rho-GDP binds more efficiently to GDI, which traps Rho in the Rho-GDI complexes in the cytosol [Ref. (31), for review on Rho regulation see Ref. (32)]. This prevents the translocation of cytosolic Rho to the cytoplasmic membrane and consequently its activation to Rho-GTP and the subsequent activation of the various cellular Rho-effector molecules (33, 34). This disturbed Rho signaling is the reason underlying most of the cellular effects observed after treatment of cells with C3. However, it should be kept in mind that due to the very limited cellular uptake of C3 proteins, all the experiments with cultured cells or tissues summarized below, were performed either by incubating the cells for a long time with high concentrations of C3 protein, or by introducing C3 into the cytosol via artificial approaches.

In 1989, Chardin and co-workers described that C3-treatment of Vero cells results in a characteristic change of the cell morphology with cell-rounding, formation of long protrusions, and reorganization of the actin filaments (35). Importantly, this was the first evidence that Rho signaling might be associated with the structure of actin filaments in mammalian cells. Due to its substrate specificity, C3 proved to be a very useful tool to unravel the role of Rho for the organization of the actin cytoskeleton and for actin-dependent processes in various eukaryotic cell types. It was found that C3-treatment inhibited endocytosis, exocytosis, cytokinesis, cell cycle progression, modulated the neuronal plasticity, and induced apoptosis (25, 36, 37). In leukocytes, the C3-catalyzed Rho-inhibition inhibited migration (38–40), adhesion (41, 42), and phagocytosis (43), suggesting a pathophysiological role of C3 proteins toward such immune cells. However, the role of C3 proteins remained unclear, mainly because of their limited cellular uptake.

The increasing knowledge on the C3-mediated effects resulted in the pharmacological exploitation of C3 for novel therapeutic strategies. The C3-catalyzed inhibition of Rho signaling has protective effects on neurons of the central nervous system because it prevented the ephrin-A5-induced growth cone collapse of such cells *in vitro* (44). Moreover, independent of its ADP-ribosyltransferase activity, C3bot – but not C3lim – exhibits neurotrophic effects on cells of the central nerve system, as demonstrated for murine hippocampal neurons (45–48), but not on cells of the peripheral nerve system (49). The recombinant cell-permeable C3bot protein BA-210 (Cethrin™), which acts as Rho inhibitor, became a drug for

treatment of acute spinal cord injury in men. This Rho-inhibitor showed promising effects after local application in pre-clinical animal studies (50) and is evaluated by the U.S. National Institutes of Health in a phase IIb clinical trial for its efficacy and safety.

Clostridial C3 Toxins Selectively Enter Monocytes/Macrophages and Allow for Targeted Pharmacological Modulation of Rho-Dependent Processes in these Cells

Based on reported inhibitory effects of C3 on basic leukocyte functions, a potential pathophysiological role of C3 toward immune cells was suggested and in 2010, Barth and co-workers identified monocytes/macrophages as target cells for C3bot and C3lim (19). It was demonstrated that within short incubation periods (e.g., 3 h), comparatively low concentrations of C3bot or C3lim (e.g., 0.5–1 µg/ml) were efficiently taken up into the cytosol of cultured monocytes and macrophages, such as murine (J774A.1, RAW 264.7) and human cell lines, and primary cultured human macrophages after their differentiation from blood monocytes, while under comparable experimental conditions, no relevant uptake of clostridial C3 toxins into the cytosol of cultured epithelial cells and fibroblasts was observed. Most likely, C3bot and C3lim enter the cytosol of monocytes/macrophages by a specific endocytotic mechanism because their uptake was decreased after pretreatment of cells with baflomycin A1 (19, 51), which inhibits endosomal acidification. Taken together, these results suggest that the clostridial C3 proteins might act on monocytes/macrophages like fully functional bacterial exotoxins but the precise molecular mechanisms underlying their cellular uptake and intracellular transport are not known so far. However, Just and co-workers, who confirmed and extended the studies on the interaction of clostridial C3 proteins with macrophages, got experimental evidence that C3 binds to proteinaceous structures on J774A.1 macrophages, which exhibit significantly more C3-binding sites compared to other cell types (52). Moreover, vimentin might be involved in the uptake of C3 protein into macrophages (52).

The C3-treatment of macrophages resulted in characteristic morphological changes due to a reorganization of the actin cytoskeleton (19), as shown in Figures 1A,B. Importantly, this effect strictly depended on the C3-catalyzed ADP-ribosylation of Rho in the cytosol of the macrophages as enzymatically inactive C3botE174Q had no effect on cell morphology but was taken up into macrophages comparable to wild-type C3 (19). The C3-mediated impairment of Rho-signaling inhibits essential macrophage functions, such as phagocytosis (53) and migration [Ref. (54), Figure 1C], suggesting an immunosuppressive mode of action of the clostridial C3 proteins.

Moreover, treatment of RAW264.7 macrophages with recombinant C3bot and C3lim proteins prevented their differentiation to osteoclast-like cells and C3-treatment of already differentiated and fully active osteoclast-like cells, which can be considered as “specialized macrophages,” inhibited their resorbing activity *in vitro* (55). Interestingly, a recombinant C3lim fusion protein (C2IN-C3lim), which contains C2IN, an enzymatically inactive portion of C2I, the enzyme component of the binary actin ADP-ribosylating *C. botulinum* C2 toxin (56), was much more efficient

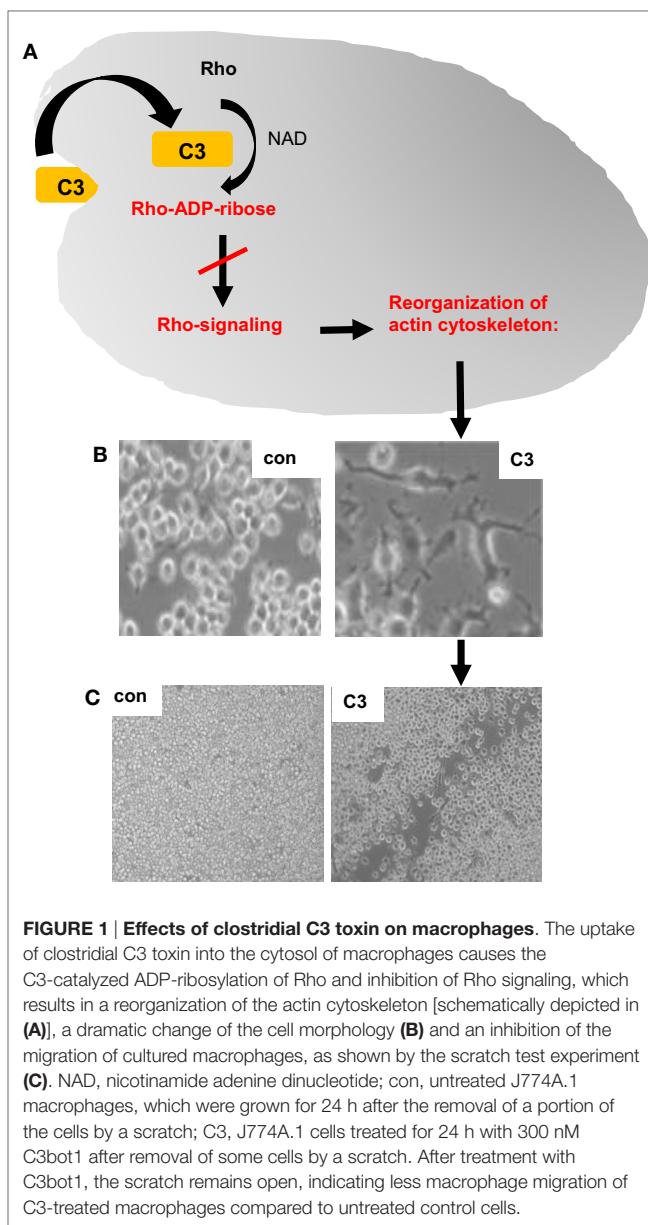


FIGURE 1 | Effects of clostridial C3 toxin on macrophages. The uptake of clostridial C3 toxin into the cytosol of macrophages causes the C3-catalyzed ADP-ribosylation of Rho and inhibition of Rho signaling, which results in a reorganization of the actin cytoskeleton [schematically depicted in (A)], a dramatic change of the cell morphology (B) and an inhibition of the migration of cultured macrophages, as shown by the scratch test experiment (C). NAD, nicotinamide adenine dinucleotide; con, untreated J774A.1 macrophages, which were grown for 24 h after the removal of a portion of the cells by a scratch; C3, J774A.1 cells treated for 24 h with 300 nM C3bot1 after removal of some cells by a scratch. After treatment with C3bot1, the scratch remains open, indicating less macrophage migration of C3-treated macrophages compared to untreated control cells.

then C3bot and C3lim regarding the targeted pharmacological manipulation of osteoclast formation and activity *in vitro* (55); most likely, this fusion protein is more efficiently internalized into the macrophages than the wild-type C3 proteins. Taken together, the recombinant C3 proteins and fusion proteins represent attractive candidates for targeted pharmacological manipulation of osteoclast formation and activity with great potential for the development of novel anti-resorptive therapies in low bone mass diseases, such as osteoporosis.

Clostridial C3 Proteins for Targeted Monocyte/Macrophage-Selective Drug Delivery

Due to the fact that comparatively low concentrations of the clostridial C3 proteins are much more efficiently taken up into monocytes and macrophages than in other cell types, such as

epithelial cells or fibroblasts, enzymatically inactive C3 protein should represent an optimal transporter for the targeted delivery of pharmacologically active (macro)molecules including therapeutic proteins and peptides into the cytosol of monocytes/macrophages. Therefore, Barth and co-workers developed and characterized novel transport systems based on enzymatically inactive C3bot1E174Q (57), which is efficiently taken up into monocytes/macrophages (19), but does not exhibit adverse effects or induces macrophage activation (51). For proof-of-concept, a pharmacologically active enzyme, namely, the actin ADP-ribosylating *C. botulinum* C2I, was genetically fused as a reporter enzyme to C3botE174Q and the uptake of the resulting recombinant C3botE174Q-C2I (see **Figure 2A**) into the cytosol of cultured macrophages was investigated. Indeed, C3bot1E174Q served for delivery of C2I into the cytosol of macrophage-like cell lines and primary human macrophages derived from blood monocytes. Incubation with C3bot1E174Q-C2I in the culture medium resulted in the ADP-ribosylation of actin in the cytosol of the macrophages and consequently in the depolymerization of F-actin (51), a clear indication that enzymatically active C2I reached the cytosol. Importantly, C3bot1E174Q-C2I had no effect on epithelial cells or fibroblasts, indicating the cell-type selectivity of this fusion toxin (51). Moreover, an application of C2I alone into the medium had no effect on the macrophages because C2I is not taken up into cells. In conclusion, enzymatically inactive C3 protein was established as a novel macrophage/monocyte-selective transport system. The generated recombinant actin-inhibitor C3bot1E174Q-C2I can serve for the targeted modulation of actin-dependent processes in monocytes and macrophages, such as the inhibition of migration and phagocytosis in the context of macrophage-associated diseases.

Prompted by this first successful exploitation of C3 for protein delivery, novel modular transporters based on biotin/streptavidin technology were developed (58, 59), as depicted in **Figure 2B**. Here, the C3botE174Q moiety mediates the specific transport of streptavidin into the cytosol of monocytes/macrophages. Streptavidin was either coupled to C3botE174Q by chemical crosslinking (59) or by genetic fusion (58) and serves as a delivery platform for biotin-labeled cargo molecules, which are then released in the host cell cytosol to elicit their pharmacological effects, as demonstrated for the enzymatic domain DTA of diphtheria toxin (58), which triggers cell death by inactivating the elongation factor EF-2, which is essential for protein biosynthesis (60) (see **Figure 2B**). In conclusion, enzymatically inactive C3 protein serves as a transport system for delivery of “foreign” proteins including pharmacologically active enzymes into the cytosol of cultured monocytes/macrophages.

Conclusion

Recombinant C3 toxins and C3 fusion toxins proved to represent valuable tools to modulate Rho- and actin-associated cell functions in monocytes and macrophages and the novel C3-based transport systems are attractive carriers for targeted drug delivery into these cells. The detailed characterization of the cellular uptake and the cellular consequences of the C3-based molecules in clinically

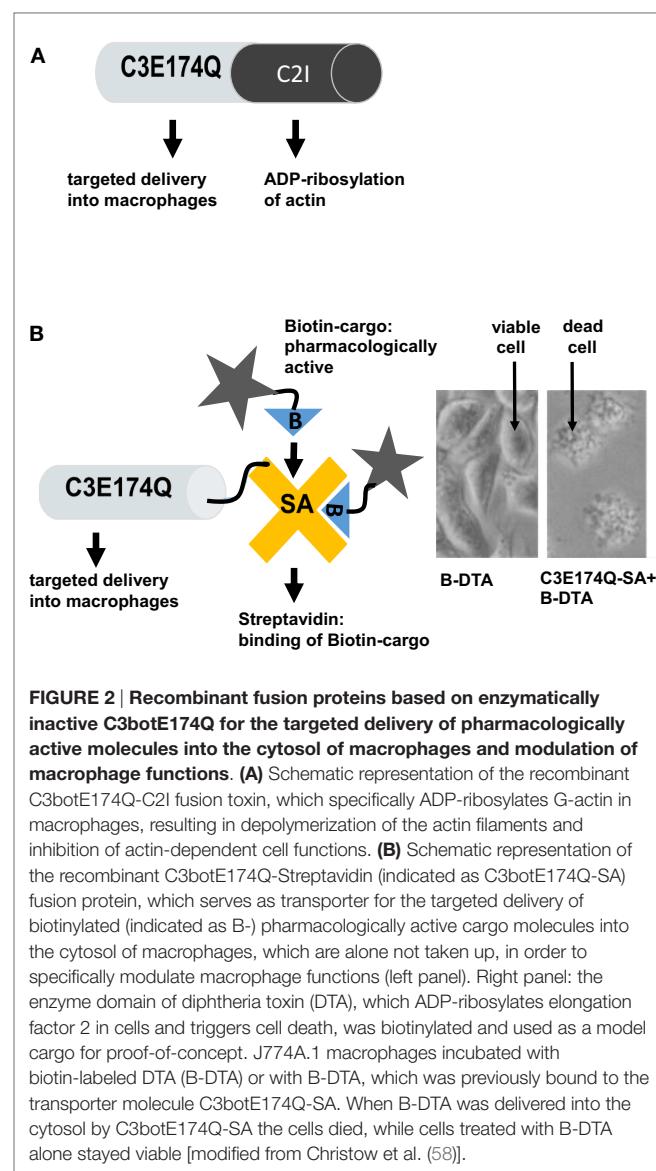


FIGURE 2 | Recombinant fusion proteins based on enzymatically inactive C3botE174Q for the targeted delivery of pharmacologically active molecules into the cytosol of macrophages and modulation of macrophage functions. (A) Schematic representation of the recombinant C3botE174Q-C2I fusion toxin, which specifically ADP-ribosylates G-actin in macrophages, resulting in depolymerization of the actin filaments and inhibition of actin-dependent cell functions. (B) Schematic representation of the recombinant C3botE174Q-Streptavidin (indicated as C3botE174Q-SA) fusion protein, which serves as transporter for the targeted delivery of biotinylated (indicated as B-) pharmacologically active cargo molecules into the cytosol of macrophages, which are alone not taken up, in order to specifically modulate macrophage functions (left panel). Right panel: the enzyme domain of diphtheria toxin (DTA), which ADP-ribosylates elongation factor 2 in cells and triggers cell death, was biotinylated and used as a model cargo for proof-of-concept. J774A.1 macrophages incubated with biotin-labeled DTA (B-DTA) or with B-DTA, which was previously bound to the transporter molecule C3botE174Q-SA. When B-DTA was delivered into the cytosol by C3botE174Q-SA the cells died, while cells treated with B-DTA alone stayed viable [modified from Christow et al. (58)].

relevant animal models, for example, after local application of C3 molecules coupled to tailored supramolecular nanocarriers (61, 62), will show whether the very promising results obtained *in vitro* and *ex vivo* can be exploited for the targeted pharmacological modulation of cellular functions in the context of monocyte/macrophage-associated diseases *in vivo*, such as inflammation, infection, or low bone mass diseases.

Acknowledgments

The research underlying parts of this review, which was performed in the laboratory of HB was supported by the Deutsche Forschungsgemeinschaft (SFB1149, project A04), the International Graduate School in Molecular Medicine Ulm (IGradU) and the Medical Faculty of the University of Ulm. SF and AM are members of IGradU.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bacterial Exotoxins and the Inflammasome

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The inflammasomes are intracellular protein complexes that play an important role in innate immune sensing. Activation of inflammasomes leads to activation of caspase-1 and maturation and secretion of the pro-inflammatory cytokines interleukin (IL)-1 β and IL-18. In certain myeloid cells, this activation can also lead to an inflammatory cell death (pyroptosis). Inflammasome sensor proteins have evolved to detect a range of microbial ligands and bacterial exotoxins either through direct interaction or by detection of host cell changes elicited by these effectors. Bacterial exotoxins activate the inflammasomes through diverse processes, including direct sensor cleavage, modulation of ion fluxes through plasma membrane pore formation, and perturbation of various host cell functions. In this review, we summarize the findings on some of the bacterial exotoxins that activate the inflammasomes.

OPEN ACCESS

Edited by:

Denise Monack,
Stanford University
School of Medicine, USA

Reviewed by:

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Immunology

Received: 27 July 2015

Accepted: 26 October 2015

Published: 10 November 2015

Citation:

Greaney AJ, Leppla SH and
Moayeri M (2015) Bacterial Exotoxins
and the Inflammasome.
Front. Immunol. 6:570.
doi: 10.3389/fimmu.2015.00570

INTRODUCTION

Inflammasomes are multiprotein complexes that form in response to microbial effectors, metabolites, nucleic acids, and other danger signals. These signals are sensed by cytosolic pattern recognition receptors [reviewed in Ref. (1)]. The most well-known inflammasome sensors, the nucleotide-binding domain/leucine rich repeat (NLR) proteins, contain common domains, including a leucine rich repeat (LRR), a nucleotide-binding domain (NACHT), a caspase activation and recruitment domain (CARD), and in some, but not all cases, a pyrin domain (PYD). Other inflammasome sensors are the absent in melanoma 2 domain (AIM2) protein, which will not be further discussed in this review, and a sensor called Pyrin, whose function remains controversial and has been described as both an inflammasome activator and inhibitor (2, 3). Upon activation, the inflammasome sensors initiate assembly of a complex that often includes an adaptor protein [usually the apoptosis-associated speck-like protein containing a CARD (ASC)], and pro-caspase-1. Proximity-based autoproteolysis of pro-caspase-1 then leads to cleavage of its substrates – the pro-inflammatory cytokines interleukin (IL)-1 β and IL-18, and initiation of a rapid lytic cell death called pyroptosis that requires caspase-1 activity and targeting of unknown death substrates [reviewed in Ref. (1)]. IL-1 β and IL-18 are well-studied for their roles in recruiting innate immune cells and promoting adaptive and humoral immunity. Pyroptosis and the accompanying release of cellular contents also act as danger signals, resulting in effects on bystander cells that can impact both innate and adaptive immune responses.

Different sensors activate inflammasome assembly in response to seemingly disparate stimuli. The mechanism of activation of some inflammasome sensors, such as rodent NLRP1, NAIP/NLRC4, and AIM2, are now known [for review see Ref. (1)]. Exactly how the promiscuous NLRP3 inflammasome, however, is activated by a diversity of seemingly disparate stimuli is still a matter of much debate [reviewed in Ref. (4)].

This review focuses on effector bacterial exotoxins and how they activate different inflammasome sensors (**Table 1**; **Figure 1**). Most toxins that have been described to activate the inflammasomes are pore-forming toxins that activate NLRP3. However, toxins with unique enzymatic actions also activate the inflammasomes. There are also many other bacterial proteins that activate inflammasomes, such as flagellin and needle/rod components of the bacterial type III secretion system (T3SS), which activate NAIP/NLRC4 by direct binding. These are reviewed elsewhere (5–7). Furthermore, the “non-canonical” caspase-11 inflammasome, which directly senses bacterial endotoxin Lipid A, has been examined in a series of elegant studies (8–12) and is also reviewed elsewhere (1, 13, 14). We will discuss bacterial exotoxins that directly activate the inflammasome by sensor modification, namely, *Bacillus anthracis* lethal toxin (LT), and other bacterial exotoxins that activate the NLRP3 and Pyrin inflammasomes indirectly by altering host cell function in ways that include plasma membrane pore formation, co-option of the host actin cytoskeleton, and as-of-yet unknown mechanisms.

ANTHRAX LETHAL TOXIN AND DIRECT ACTIVATION OF THE NLRP1 INFLAMMASOME

Anthrax LT is a major virulence factor of *B. anthracis*, inducing vascular collapse during anthrax infection of animals ranging

from rodents to monkeys (15–17). LT is a bipartite toxin made of a receptor-binding moiety, protective antigen (PA), and a zinc-dependent metalloprotease, lethal factor (LF). Upon endosome acidification, PA delivers LF to the host cell cytosol [reviewed in Ref. (18)]. LF activates the NLRP1 inflammasome in macrophages and dendritic cells from certain inbred rodent strains by cleavage of NLRP1 (19, 20). Cleavage occurs in an N-terminal region of unknown function, located in a position corresponding to the PYD of human NLRP1 (19, 21). This cleavage is necessary and sufficient for inflammasome activation (19, 22). In most rat strains, only one of the two NLRP1 paralogs is expressed, and susceptibility to pyroptosis is perfectly correlated with the ability of LT to cleave the expressed NLRP1 sensor (19, 21, 23). Rat strains, such as Fischer and Brown Norway, express NLRP1 variants that are cleaved by LF and have macrophages that pyroptose in response to the toxin. Furthermore, when these rats are challenged with LT, they undergo death in <1 h. Strains such as SHR, Lewis, and Copenhagen have an altered sequence within the LF cleavage site, rendering NLRP1 resistant to cleavage, macrophages resistant to pyroptosis, and the animals highly resistant to toxin challenge (19, 21, 23).

Rat NLRP1 proteins from all tested rat strains are 98% identical (21), pointing to the evolutionary pressure conserving the sensor's sequence outside of the few polymorphisms in the LF cleavage site. In mice, three *Nlrp1* alleles (*Nlrp1a*, *Nlrp1b*, *Nlrp1c*) exist (24, 25). The one most homologous to the expressed rat NLRP1 is NLRP1a, which is, in a manner similar to rat NLRP1, highly

TABLE 1 | Inflammasome-activating bacterial exotoxins.

Toxin	Source	Mechanism	Sensor	Effect sensed by NLR (direct or indirect)	Reference
Lethal toxin (LT)	<i>B. anthracis</i>	Protease	NLRP1	NLRP1 cleavage	(19–24)
Nigericin	<i>S. hygroscopicus</i>	Ionophore	NLRP3	K ⁺ efflux	(40)
α-Hemolysin	<i>S. aureus</i>	Pore former	NLRP3	K ⁺ efflux	(60–62)
Panton-valentine leukocidin	<i>S. aureus</i>	Pore former	NLRP3	K ⁺ efflux	(68)
Leukocidin A/B	<i>S. aureus</i>	Pore former	NLRP3	K ⁺ efflux	(69)
Listeriolysin O	<i>L. monocytogenes</i>	Pore former	NLRP3	K ⁺ efflux	(42, 43, 50)
Aerolysin	<i>A. hydrophila</i>	Pore former	NLRP3	K ⁺ efflux	(44)
Tetanolysin O	<i>C. tetani</i>	Pore former	NLRP3	K ⁺ efflux	(49)
Pneumolysin	<i>S. pneumonia</i>	Pore former	NLRP3	K ⁺ efflux	(51–53)
β-Hemolysin	Group B <i>Streptococcus</i>	Pore former	NLRP3	K ⁺ efflux	(55–57)
Streptolysin O	<i>S. pyogenes</i>	Pore former	NLRP3	K ⁺ efflux	(58, 59)
α-Hemolysin	<i>E. coli</i>	Pore former	NLRP3	K ⁺ efflux	(77)
Enterohemolysin	<i>E. coli</i> O157:H7	Pore former	NLRP3	K ⁺ efflux	(76)
Various hemolysins	<i>Vibrio</i> species	Pore former	NLRP3	K ⁺ efflux	(78–80)
TcdB	<i>C. difficile</i>	Glucosylase	Pyrin	Rho GTPase inactivation	(87, 90)
VopS	<i>V. parahaemolyticus</i>	Adenylyltransferase	Pyrin	Rho GTPase inactivation	(87)
IbpA	<i>H. somni</i>	Adenylyltransferase	Pyrin	Rho GTPase inactivation	(87)
C3 toxin	<i>C. botulinum</i>	ADP-ribosyltransferase	Pyrin	Rho GTPase inactivation	(87)
Unknown	<i>B. cenocepacia</i>	Deamidase	Pyrin	Rho GTPase inactivation	(87)
Pertussis toxin (PTX)	<i>B. pertussis</i>	ADP-ribosyltransferase	Pyrin	Unknown	(87)
CdtB	<i>Aggregatibacter</i> <i>actinomycetemcomitans</i>	Lipid phosphatase	NLRP3	GSK3β-induced P2X7 activation	(92, 93)
IpaH7.8	<i>S. flexneri</i>	E3 ubiquitin ligase	NLRP3, NLRC4	GLMN degradation	(94)
Pertussis adenylate cyclase toxin	<i>B. pertussis</i>	Adenylate cyclase and pore former	NLRP3	Pore formation; K ⁺ efflux	(98)
Heat-labile enterotoxin (LT)	<i>E. coli</i>	ADP-ribosyltransferase	NLRP3	cAMP increase	(100, 101)
Cholera toxin (CT)	<i>V. cholerae</i>	ADP-ribosyltransferase	Unknown	cAMP increase	(101)

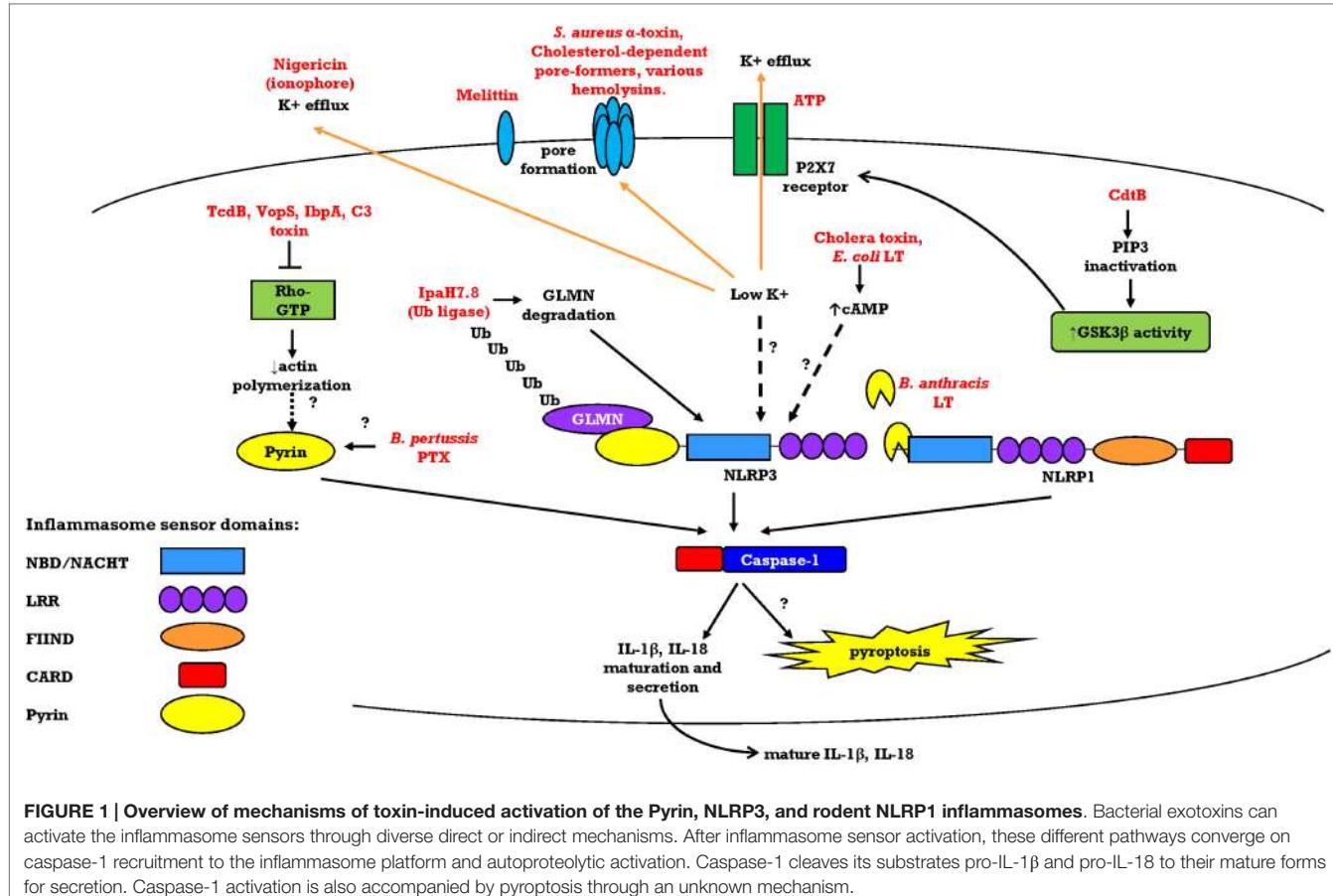


FIGURE 1 | Overview of mechanisms of toxin-induced activation of the Pyrin, NLRP3, and rodent NLRP1 inflammasomes. Bacterial exotoxins can activate the inflammasome sensors through diverse direct or indirect mechanisms. After inflammasome sensor activation, these different pathways converge on caspase-1 recruitment to the inflammasome platform and autoproteolytic activation. Caspase-1 cleaves its substrates pro-IL-1 β and pro-IL-18 to their mature forms for secretion. Caspase-1 activation is also accompanied by pyroptosis through an unknown mechanism.

conserved among all mouse strains in which it is expressed (25). Murine NLRP1a, however, is not cleaved by LF and its activator is currently unknown.

Rather, the highly polymorphic mouse NLRP1b, which has up to 200 polymorphisms between different inbred mouse strains, is cleaved by the toxin (24, 25). NLRP1 activation in rodent macrophages and dendritic cells and the resulting pyroptosis requires proteasome activity (26–28) and can occur independently of ASC (29–31). Activation results in an inflammatory cytokine (16, 32, 33) and eicosanoid response (34) that confers an increased resistance to *B. anthracis* spore infection (32, 33). Thus, among inbred mouse strains, there is an inverse correlation between sensitivity of macrophages and dendritic cells to LT-induced pyroptosis and animal susceptibility to *B. anthracis* infection.

The unique, rapid death induced by LT in rats, which is not replicated in toxin-sensitive mice at even 10-fold higher doses, does not display the inverse relationship observed in mice. Instead, rat death following both toxin and spore challenge is positively correlated to NLRP1 sensitivity to LT cleavage (35). Human NLRP1 proteins sequenced thus far do not contain an LT cleavage site and instead have an N-terminal PYD. Human NLRP1 is not activated by LT, and human macrophages and dendritic cells are resistant to this toxin (35).

In addition, the obligate intracellular parasite, *Toxoplasma gondii*, has also been shown to activate the NLRP1 inflammasome

in select inbred rat strains by an unknown mechanism (36–39). However, it is unknown if *T. gondii* activates NLRP1 in a manner similar to LT, through actions of a protease or toxin. We speculate that NLRP1 has evolved to sense diverse pathogen proteases, and polymorphisms present in different NLRP1 alleles may define responsiveness to different pathogens. Future work is needed to identify other NLRP1 agonists and the pressures driving the evolution of its conserved and polymorphic sequences.

PORE-FORMING TOXINS AND INDIRECT NLRP3 ACTIVATION

Unlike the toll-like receptors (TLRs) and the NAIP/NLRC4 and AIM2 inflammasomes, which directly sense microbial products, some inflammasome sensors indirectly sense the effects of bacterial toxins on host cell function. For example, NLRP3 is believed to be activated by an indirect mechanism. While the precise signals that activate NLRP3 remain unknown, it has been proposed that NLRP3 may be indirectly activated by K $^{+}$ efflux, lysosomal damage and cathepsin B release, mitochondrial damage, or reactive oxygen species production (1). The best-studied example of indirect inflammasome activation by bacterial toxins is the impact of pore formation on cellular potassium levels and subsequent NLRP3 activation.

Early studies linking IL-1 β responses to addition of exogenous ATP or the *Streptomyces hygroscopicus*-derived potassium ionophore, nigericin (40), occurred before inflammasome components were identified and the term introduced. Bhakdi and colleagues confirmed that additional agents that deplete potassium in cells, including the pore-forming *Staphylococcus aureus* alpha toxin, the ionophore valinomycin, and the Na $^+$ /K $^+$ ATPase inhibitor ouabain, can trigger processing of IL-1 β (41). These investigators hypothesized that cellular K $^+$ concentration changes could control the function of caspase-1. Ten years later, studies with the calcium channel activator maitotoxin, nigericin, and ATP showed that induction of IL-1 β and IL-18 secretion in TLR-primed macrophages treated with these K $^+$ efflux-inducing agents occurred in a manner dependent on the inflammasome adaptor ASC and the NLRP3 sensor (42). It was found that IL-1 β secretion induced by *Listeria monocytogenes* infection of macrophages required listeriolysin expression (42, 43), suggesting that pore formation and perturbation of cellular K $^+$ levels could also be the basis for inflammasome activation by this toxin, although the purified toxin itself was not tested.

The first demonstration of NLRP3- and ASC-dependent inflammasome formation in response to a purified pore-forming bacterial toxin was reported with studies using aerolysin purified from *Aeromonas hydrophila* (44). Interestingly, this study identified a novel caspase-1-dependent protective response in cells treated with either aerolysin or *S. aureus* α -hemolysin. In contrast to the pyroptosis usually observed following caspase-1 activation, NLRP3-mediated activation of caspase-1 by sublytic doses of the pore-forming toxins resulted in induction of sterol regulatory element-binding proteins that altered membrane biogenesis and promoted cell survival (44). The link between pore-based lowered intracellular K $^+$ and NLRP3 activation was later confirmed by multiple other groups (45, 46). These early studies led to the testing of a large range of purified bacterial lysins and pore formers for their ability to activate the NLRP3 inflammasome.

Other cholesterol-dependent pore-forming toxins similar to aerolysin were also previously linked to IL-1 β production (47, 48) and have since been tested in cells with inflammasome component deficiencies to link this response directly to caspase-1 activation. Tetanolysin O, from *Clostridium tetani* induces IL-1 β maturation and release from bone marrow-derived macrophages (BMDMs) at low, non-lytic doses in an NLRP3-, cathepsin B-, and caspase-1-dependent manner (49). Listeriolysin, originally suggested to play a role in IL-1 β responses during *Listeria* infection via pore formation (42), directly activates the inflammasome by K $^+$ efflux induction (50).

Streptococcus pneumoniae pneumolysin induces NLRP3-dependent IL-1 β secretion that is linked to a pro-inflammatory cytokine cascade, which includes IL-17 and IFN- γ responses (51). Pneumolysin mutants and bacterial serotypes associated with variable toxin production confirm the requirement for this lysin in NLRP3-, ASC- and caspase-1-dependent IL-1 β and IL-18 production (52). The host cytokine response to pneumolysin has also been shown to increase protection against pneumonia in a mouse model (52). In a murine model of pneumococcal meningitis, the extent of caspase-1 activation via the NLRP3 inflammasome has been linked to clinical disease severity (53). In contrast to the

study described above, in this model, inflammasome activation was associated with pathology rather than bacterial clearance. Pneumolysin was the key inducer of the IL-1 β response associated with disease, and inhibitors of IL-1 β and IL-18 signaling altered pathological responses. Pneumolysin activation of the inflammasome was suggested to require ATP release and lysosomal destabilization associated with cathepsin B cytosolic activity (53). Interestingly, pneumolysin has also been linked to the sensing of *S. pneumoniae* by the cytosolic DNA-sensing molecule STING and the downstream type I interferon responses, likely in an inflammasome-independent manner (54). Other *Streptococcal* proteins can also mediate inflammasome activation through their pore-forming ability. The pigment associated with group B streptococci was recently shown to be hemolytic and capable of pore formation and induction of a K $^+$ efflux linked to NLRP3 activation (55). Previous studies demonstrated that β -hemolysin (56, 57) and other streptococcal lysins (58, 59) also activate the NLRP3 inflammasome.

Staphylococcus aureus is another gram-positive bacterium that has evolved to activate the NLRP3 inflammasome through the actions of multiple pore-forming toxins and hemolysins. Following up on Bhakdi's early observation that *S. aureus* α -hemolysin is associated with the IL-1 β response, this toxin was verified to induce NLRP3-dependent activation of caspase-1 in both human and mouse monocytic cells (60). Activation of the inflammasome by α -hemolysin has been reported to have both protective and detrimental consequences *in vivo*. For example, α -hemolysin-mediated activation of NLRP3 in a mouse pneumonia model has been linked to IL-1 β -independent necrosis, pulmonary damage, and severe pneumonia (61). By contrast, inflammasome activation is protective in other models. Neutrophil-derived IL-1 β in response to α -hemolysin was protective in an abscess model of infection in mice (62). Studies of patients with atopic dermatitis have linked α -hemolysin upregulation of NLRP3 expression and *S. aureus*-mediated caspase-1 activation in monocytes to the control of Th2 responses, which may ameliorate this disease (63). IL-1 β has also been linked to protection against *S. aureus*-induced CNS disease and brain abscesses (64). Hemolysin-dependent induction of IL-1 β was attenuated in NLRP3- and ASC-deficient microglia following bacterial infection. Interestingly, in these studies, IL-18 secretion from microglia occurred in a manner independent of NLRP3, suggesting a different cleavage mechanism for IL-18 (65). Recently, *S. aureus* phenol soluble modulins (PSMs), another class of pore-forming substances that are important virulence factors [reviewed in Ref. (66)], were shown to induce IL-18 secretion from human keratinocytes in a caspase-1-independent manner (67). In a mouse model, PSMs were also shown to be important for *S. aureus*-induced neutrophil recruitment and systemic inflammation. These authors suggest that IL-18 release and neutrophil recruitment induced by PSMs is inflammasome independent, again suggesting novel mechanisms for IL-18 activation (67).

Other pore-forming toxins from *S. aureus* have also been shown to activate the inflammasome. The Panton–Valentine leukocidin associated with tissue necrosis has been shown to induce release of IL-1 β and IL-18 in an NLRP3-dependent manner (68). Leukocidin A/B is another pore-forming toxin that can

activate the NLRP3 inflammasome in human monocytes (69). Interestingly, it has also been suggested that *S. aureus* hemolysins do not activate caspase-1 alone and require *S. aureus* lipoproteins for inflammasome activation, albeit independently of the lipoprotein actions on TLR-mediated inflammasome priming (70).

Certain *S. aureus* toxins have also been described to have effects on necroptosis in a manner that may link to their activation of the inflammasome. For example, it was found that the virulent methicillin-resistant (MRSA) strain USA300 induces receptor-interacting serine-threonine kinase (RIP)1/RIP3/mixed lineage kinase domain-like (MLKL)-dependent necroptosis, thereby contributing to inflammatory lung pathology (71). *S. aureus* treatment of human and murine macrophages leads to phosphorylation of MLKL- and necroptosis-induced cell death. *Rip3^{-/-}* macrophages are protected from *S. aureus*-induced cytotoxicity. Live bacteria or supernatants from mutants lacking either the α -hemolysin, leukocidin A/B, or PSM toxins display reduced induction of macrophage cytotoxicity. Interestingly, α -hemolysin-induced inflammasome activation has been shown to be dependent on MLKL pore formation, supporting the link between the NLRP3 inflammasome and necrosome components RIP1, RIP3, and MLKL (71–75). Necroptosis contributes to lung pathology and reduced bacterial clearance *in vivo* by depleting immunoregulatory resident alveolar macrophages that are essential for bacterial clearance (71).

Gram-negative bacteria also produce a variety of hemolysins that have been implicated in activation of the NLRP3 inflammasome. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, the causative agent of hemorrhagic uremic syndrome, produces a hemolysin that induces IL-1 β from the THP-1 macrophage/monocyte cell line, and RNA-interference experiments indicate a role for NLRP3, ASC, and caspase-1 in the initiation of the cytokine release (76). Uropathogenic *E. coli* also induces IL-1 β , and *E. coli* α -hemolysin was found to be responsible for NLRP3-dependent responses in mouse macrophages infected with certain bacterial strains. Other strains activated an NLRP3-independent cell death pathway and hemolysin-independent IL-1 β secretion from human macrophages (77). *Vibrio vulnificus* and *Vibrio cholerae* were first shown to activate the NLRP3 inflammasome in macrophages through the action of hemolysins (78). *Vibrio parahaemolyticus* also produces thermostable direct hemolysins known as TDHs that can activate the NLRP3 inflammasome (79). *Vibrio fluvialis*, which induces a diarrheal disease in humans, produces a hemolysin that can activate the inflammasome in mouse and human macrophages. Importantly, in a mouse model, the toxin was associated with IL-1 β production, and toxin-containing cell-free culture supernatants induced higher levels of cytokine production. Hemolysin-deficient bacteria and their supernatants had lower levels of response *in vivo* (80).

The study of pore formation-mediated inflammasome activation first started with analysis of marine and fungal ionophores (42) and has now again been extended beyond bacterial toxins. Mold pore-forming mycotoxins (81), viral viraporins (82), melittin, the small cationic pore-forming peptide found in bee venom, which can form a single alpha helix spanning the plasma membrane (83), and the *Bombina maxima* frog derived aerolysin-like protein (84) are examples of unique non-bacterial

toxin pore-forming agents that activate the NLRP3 inflammasome and caspase-1. The utility of these and many of the toxins described in this section as potential adjuvants for amplification of the immune response, or pro-inflammatory therapeutics is an area that awaits investigation.

INDIRECT ACTIVATION OF THE INFLAMMASOMES BY OTHER BACTERIAL EXOTOXINS HAVING ENZYMATIC ACTIONS

In addition to NLRP3 activation by pore-forming bacterial toxins, inflammasome activation can also occur by other mechanisms of indirect sensing of bacterial effectors.

Rho family of GTPases is molecular switches that control the dynamics of the actin cytoskeleton. The actin cytoskeleton can be co-opted by bacterial pathogen effectors targeting Rho GTPases for either hyper-activation or inactivation (85). Several cytosolic innate immune-sensing pathways have evolved to recognize the sequelae of Rho GTPase actions. For example, activation of Rho GTPases, including Rac1 and Cdc42, by bacterial toxins activates the cytosolic NOD1 sensor and leads to NF κ B-dependent expression of pro-inflammatory genes (86). Recently, Shao and colleagues identified Pyrin as an innate immune sensor of bacterial toxin inhibition of Rho GTPases (87). They demonstrated that the *Clostridium difficile* toxin TcdB, which glucosylates and inactivates Rho proteins, activates the Pyrin inflammasome. This discovery provided a link between the previous findings that Pyrin controlled inflammatory familial Mediterranean fever pathogenesis (88, 89) through inflammasome activation (2), and that *C. difficile* toxins TcdA/B activate the ASC-dependent inflammasome (90). Interestingly, Pyrin specifically recognizes Rho subfamily inhibition, as cytochalasin D, an inhibitor of actin polymerization, and the *Clostridium sordellii* lethal toxin TcsL, which modifies Rac/Cdc42 and some Ras-related GTPases, do not activate the Pyrin inflammasome. Furthermore, a variety of bacterial toxins with different enzymatic activities can activate the Pyrin inflammasome. Other Pyrin activators include *Parahaemolyticus* VopS, *Histophilus somni* IbpA, *Clostridium botulinum* C3 toxin, and *Burkholderia cenocepacia*. These toxins inactivate Rho subfamily GTPases by modifying I-switch residues through glucosylation, adenylation, ADP-ribosylation, and deamidation. Together, the diversity of chemical modifications of Rho GTPases, and the inability to co-IP RHOA/B/C and Pyrin suggest that Pyrin does not directly interact with Rho GTPases and detect modifications; rather, it is proposed that Pyrin senses downstream effects on the actin cytoskeleton (87).

Interestingly, the *Bordetella pertussis* toxin PTX, which is an ADP-ribosyltransferase (similar to the botulinum C3 toxin, but targeting a different substrate), has been shown to activate the Pyrin inflammasome *in vivo* (91), as well as to upregulate IL-1 β expression in a TLR4-dependent manner. Both events require on the toxin's ADP-ribosyltransferase activity. While the mechanism of PTX activation of the Pyrin inflammasome is unknown, the intriguing possibility remains that PTX, which ADP-ribosylates the α_i subunit of heterotrimeric G proteins, may activate the

Pyrin inflammasome via an indirect mechanism similar to that of the other bacterial toxins targeting the GTPases, discussed above.

The *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin (Cdt), can act as both signal 1 and signal 2 for inflammasome activation, up-regulating pro-inflammatory cytokine expression (signal 1) and also activating the NLRP3 sensor (signal 2) (92, 93). Cdt is a heterotrimeric protein found in several bacterial species, also including *Campylobacter jejuni*, *Shigella* species, and some *E. coli* isolates. The CdtA and CdtC subunits comprise the receptor-binding moiety, and the CdtB subunit is an lipid phosphatase. CdtB dephosphorylates the signaling lipid phosphatidylinositol-3,4,5-triphosphate (PIP3) and leads to its degradation, decreased phosphorylation of Akt and glycogen synthase kinase 3 β (GSK3 β), decreased Akt kinase activity but increased GSK3 β kinase activity, and inhibition of the PI-3K signaling pathway. GSK3 β activation induced by CdtB in human macrophages and monocytes is believed to lead to NF κ B activation and the expression of pro-inflammatory cytokines, including IL-1 β , dependent on CdtB lipid phosphatase activity (92). Cdt-induced GSK3 β activation leads to the generation of extracellular ATP, activation of the P2X7 purinergic receptor, K $^{+}$ efflux, activation of the NLRP3 inflammasome, and IL-1 β release (93). Inflammasome activation is NLRP3, ASC, and caspase-1 dependent (93). This is a case where a single bacterial toxin, once in the macrophage cytosol, may act as both signal 1 and signal 2 for inflammasome activation. Interestingly, it should be noted that Cdt toxins are also considered to act as DNases that induce DNA damage and cause cell cycle arrest. Which of these activities is more important *in vivo* is not clear, and the possibility remains that the DNase activity could also result in activation of other sensors.

The NAIP/NLRC4 inflammasome has been demonstrated to be activated by bacterial flagellin and T3SS needle and rod proteins [reviewed in Ref. (7)]. In contrast to previously known NAIP/NLRC4 agonists, which activate the inflammasome by direct binding to the NAIP sensors, a novel, indirect activator of the NLRC4 inflammasome has recently been identified (94). A *Shigella flexneri* E3 ubiquitin ligase effector protein secreted into the host cell cytosol via the type III secretion system (T3SS), invasion plasmid antigen H7.8 (IpaH7.8), was found to activate the NLRP3 and NLRC4 inflammasomes (94). This protein was found to be an important virulence factor, as enzymatically inactive mutants were defective in lung colonization following intranasal infection. Although in most cases inflammasome activation is important for controlling bacterial infection, this is a case where the activation is used by the bacterium to promote dissemination. The LRR domain of IpaH7.8 was found to directly interact with the host protein glomulin/flagellar-associated protein 68 (GLMN) and target both GLMN and itself for degradation by the proteasome. GLMN was suggested to inhibit the NLRP3 inflammasome through an unknown mechanism. Future studies may reveal whether GLMN inhibits the inflammasome, or if IpaH7.8 activates this sensor through a different mechanism (94).

Interestingly, ricin, a highly poisonous toxin found in the seeds of the *Ricinus communis* plant, and an inhibitor of protein syntheses, has also been shown to induce a macrophage IL-1 β -mediated pro-inflammatory response in the airways,

contributing to lethality (95). Upregulation of IL-1 β transcription and the pro-inflammatory response is believed to be mediated by activation of the stress-activated protein kinases (SAPKs), including JNK and the p38 MAPK [reviewed in Ref. (96)]. Ricin was also demonstrated to activate the NLRP3 inflammasome in a proteasome-dependent manner (97). Inflammasome activation was independent of the ribotoxic stress response and phosphorylation of p38 and JNK. It was proposed that inflammasome activation was the result of breakdown of an unidentified, labile NLRP3 inhibitor whose synthesis is blocked by ricin. JNK- and p38-independent NLRP3 inflammasome activation may contribute to the previous finding that IL-1 β plays an important role in ricin-induced severe lung inflammation and lethality (95).

BACTERIAL TOXIN INFLAMMASOME ACTIVATORS AND ADAPTIVE IMMUNITY

Some studies demonstrated that toxin-induced production of IL-1 β also influences adaptive immunity, and it is likely that many toxins that activate the inflammasomes will have similar effects. The pertussis RTX adenylate cyclase toxin previously mentioned contains, in addition to its enzymatic domain, a separate pore-forming domain, which activates the NLRP3 inflammasome. This activation leads to IL-1 β production by dendritic cells and induction of antigen-specific Th17 cells that require the cytokine for expansion. Th17 differentiation induced by inflammasome activation is required for control of infection and clearance of bacteria from the lungs in a mouse model (98). Similarly, the IL-17 response associated with NLRP3 inflammasome activation by *S. pneumoniae* pneumolysin is also associated with protective immunity against intranasal infection, and IL-1 β is required for promoting the IL-17 response (51). Interestingly, the non-toxic trehalose-6,6-dibehenate (TBD) adjuvant, which promotes Th1/Th17 responses, also requires inflammasome adaptor ASC-dependent activation of IL-1 β production (99).

The adjuvant role of non-pore-forming bacterial enterotoxins and their enzymatically inactive mutants in inducing inflammasome dependent, Th17-polarized protective immunity has also been studied (100, 101). The *E. coli* heat-labile enterotoxin (HL-LT, commonly termed LT) and cholera toxin (CT) are AB toxins that ADP-ribosylate the Gs component of adenylate cyclase, leading to an increase in cAMP. They have been shown to be potent adjuvants, but their enterotoxicity precludes their use in human oral vaccines. HL-LT along with an enzymatically highly attenuated mutant can both activate the NLRP3/caspase-1 inflammasome and induce mature IL-1 β secretion from LPS-primed dendritic cells (100). Inflammasome activation and the associated IL-1 β response are required for promoting Th17 responses *in vivo*, which in turn protect against *B. pertussis* challenge in a model where the toxin is the primary adjuvant in the pertussis vaccine (100). In related studies, the heat-killed mycobacteria components in Complete Freund's Adjuvant (CFA) have been shown to drive Th17 differentiation through a mechanism that requires NLRP3 inflammasome activation (102).

A recent study examined the ability for double-mutant HL-LT and multiple-mutated CT to induce a Th17-mediated adjuvant

response in human peripheral blood mononuclear cells (PBMCs) (101). These variants have highly reduced enzymatic activity and, unlike the wild type toxins, are non-toxic but still induce very low levels of cAMP and can stimulate IL-17A production from human PBMCs when administered to cells with a polyclonal T cell superantigen (101). This effect is dependent on cAMP/protein kinase A (PKA) signaling, as a PKA inhibitor inhibits IL-17A production, and a cAMP analog recapitulates the toxin effects. Monocytes were demonstrated to have a modest increase in cAMP in response to the mutant toxins, and monocytes pre-incubated with the toxins and then co-cultured with CD4⁺ T cells led to an increase in IL-17A production in a caspase-1-, IL-1 β -dependent manner (101). These studies demonstrate the important role that toxin-induced inflammasome activation may play in vaccine development.

CONCLUDING COMMENTS

The recognition of self from non-self is the foundation of the innate immune response. Pattern recognition receptors such as the TLRs and some of the inflammasome sensors detect highly conserved microbial molecular patterns, including LPS, peptidoglycan, flagellin, and CpG DNA. The inflammasome receptors discussed in this review, however, are able to detect highly diverse bacterial effectors because they sense these toxins through their functional effects rather than their molecular patterns. In the case of NLRP1, the sensor is activated by direct cleavage by a bacterial protease that also cleaves the mitogen-activated protein

kinase kinases (MEKs) and results in cell – and animal – death. The NLRP3 and Pyrin inflammasomes, on the other hand, detect indirect effects on host cell state including ion fluxes and perturbation of the actin cytoskeleton. In this way, the inflammasome sensors have evolved to respond to different bacterial toxins with diverse mechanisms of action to converge on caspase-1 activation and initiation of the immune response.

Toxin activation of the inflammasome in the first-responder cells of the innate immune system plays an important role in pro-inflammatory and pyroptosis events that can have protective or pathogenic consequences in the host. Interestingly, bacterial effectors that inhibit the inflammasome or suppress its activation have also been described in recent years [reviewed in Ref. (103)], suggesting that microbes have also evolved to evade inflammasome detection by the host. With the threat of antimicrobial-resistant bacteria, an understanding of the mechanisms by which microbes modulate the innate immune responses is essential to studies of microbial pathogenesis and therapeutic development.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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Conflict of Interest Statement: The authors declare no commercial or financial relationships that could be construed as a potential conflict of interest.

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Subversion of cell-autonomous immunity and cell migration by *Legionella pneumophila* effectors

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Specialty section:

This article was submitted to
Microbial Immunology, a section of
the journal Frontiers in Immunology

Received: 30 June 2015

Accepted: 17 August 2015

Published: 14 September 2015

Citation:

Simon S and Hilbi H (2015)
Subversion of cell-autonomous
immunity and cell migration by
Legionella pneumophila effectors.
Front. Immunol. 6:447.
doi: 10.3389/fimmu.2015.00447

Bacteria trigger host defense and inflammatory processes, such as cytokine production, pyroptosis, and the chemotactic migration of immune cells toward the source of infection. However, a number of pathogens interfere with these immune functions by producing specific so-called “effector” proteins, which are delivered to host cells via dedicated secretion systems. Air-borne *Legionella pneumophila* bacteria trigger an acute and potential fatal inflammation in the lung termed Legionnaires’ disease. The opportunistic pathogen *L. pneumophila* is a natural parasite of free-living amoebae, but also replicates in alveolar macrophages and accidentally infects humans. The bacteria employ the intracellular multiplication/defective for organelle trafficking (Icm/Dot) type IV secretion system and as many as 300 different effector proteins to govern host–cell interactions and establish in phagocytes an intracellular replication niche, the *Legionella*-containing vacuole. Some Icm/Dot-translocated effector proteins target cell-autonomous immunity or cell migration, i.e., they interfere with (i) endocytic, secretory, or retrograde vesicle trafficking pathways, (ii) organelle or cell motility, (iii) the inflammasome and programmed cell death, or (iv) the transcription factor NF-κB. Here, we review recent mechanistic insights into the subversion of cellular immune functions by *L. pneumophila*.

Keywords: bacterial pathogenesis, *Dictyostelium*, inflammasome, *Legionella*, macrophage, pathogen vacuole, phosphoinositide, small GTPase

Introduction

Phagocytic cells of the innate immune system, such as macrophages, neutrophils, and dendritic cells (DC), produce pathogen recognition receptors (PRRs) comprising families of membrane-bound or cytosolic receptors (1–4). The membrane-bound receptors are toll-like receptors (TLRs) and C-type lectin receptors (CLRs), such as the mannose receptors. TLRs trigger cytokine production by signaling through the adaptor molecules MyD88 or TRIF and activate the transcription factor NF-κB or MAP kinase pathways. Cytosolic receptors are Nod-like receptors (NLRs) involved in inflammatory responses and cell death pathways through the activation of multiprotein complexes termed inflammasomes (5), as well as retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs). Collectively, these receptors recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), thus alerting the infected host cell, if material arising from cell infection or cell damage is present. Subsequent cytokine production promotes the clearance of invading microorganisms. The conserved process of cell-autonomous immunity includes not only

inflammasome activation and interleukin (IL)-1 β production, as well as autophagy, but also NF- κ B- and type I interferon (IFN)-dependent cytokine production (6, 7).

Pathogenic bacteria target eukaryotic cells either as adversaries in the case of potentially bactericidal cells of the metazoan immune system and/or as a rich source of nutrients (8, 9). Accordingly, pathogens developed means to counteract the arsenal of the humoral and cellular components of the innate and acquired immune system and to survive and replicate within eukaryotic cells, including phagocytes (10). To this end, a number of pathogens produce specific so-called “effector” proteins, which are delivered via dedicated secretion systems into host cells, where they interfere with immune functions and cell migration (11).

Legionella pneumophila employs the intracellular multiplication/defective for organelle trafficking (Icm/Dot) type IV secretion system (T4SS) and as many as 300 different effector proteins to govern host–cell interactions. The role and molecular mode of action of effectors involved in pathogen vacuole formation has recently been reviewed in detail (12). Here, we will review mechanistic insights into the subversion of cell-autonomous immunity and cell migration by *L. pneumophila*.

Pathogenesis of *Legionella pneumophila*

Legionella pneumophila is an opportunistic pathogen and causes a severe pneumonia termed Legionnaires’ disease. The Gram-negative genus *Legionella* comprises more than 55 species with several serogroups; yet, at least 85% of human infections are caused by *L. pneumophila* (13). Evolutionary adaptation allows *L. pneumophila* to persist in a variety of extra- and intracellular niches. The aerobic bacterium can not only replicate in biofilms but also resists degradation by free-living protozoa and replicates within, e.g., *Acanthamoeba*, *Hartmannella*, and *Tetrahymena* species, as well as in *Dictyostelium discoideum*, although the latter amoeba is likely not a natural host (14, 15). *L. pneumophila* is ubiquitously found in natural and technical water systems, including cooling towers, whirlpools, and showers.

Upon inhalation of contaminated aerosols, *L. pneumophila* resists degradation and replicates within alveolar macrophages, which is a precondition for the onset of disease (16). The acquisition of the pathogen from environmental sources is the only infection route; transmission between humans has never been observed. Since *L. pneumophila* probably mainly evolved as a parasite of free-living protozoa, the human host represents a dead-end for this “accidental” pathogen. Thus, *L. pneumophila* likely has not been exposed to a rigorous evolutionary selection to avoid recognition by mammalian PRRs, and accordingly, the bacteria trigger the activation of all PRR families (17).

Most humans and mice are able to clear a *Legionella* infection, and therefore, the development of a suitable small animal model was crucial. Initial studies using guinea pigs exposed to *L. pneumophila*-containing aerosols revealed a high susceptibility of these animals, which developed an illness reminiscent of typical human Legionnaires’ disease (18). While most inbred mouse strains are resistant to *L. pneumophila* infection and disease progression, the A/J mouse strain was found to be susceptible and to present with acute pneumonia that resembled human

disease (19). These results correspond to *in vitro* infections of peritoneal mouse macrophages, which indicated that cells from A/J mice were much more permissive for intracellular replication of *L. pneumophila* than macrophages from other mouse strains, such as C57BL/6 and BALB/c (20, 21). Macrophages from C57BL/6 and BALB/c mice restrict *L. pneumophila* by the activation of a programmed cell death pathway as an ultimate line of defense against the intracellular pathogen (see below). Accordingly, mice lacking components of this pathway fail to restrict *L. pneumophila* and faithfully mimic *Legionella* pathology (17, 22).

In A/J mice, *L. pneumophila* elicits an acute inflammatory reaction, including production of the cytokines tumor necrosis factor (TNF)- α , IFN- γ , IL-12, and IL-18, which restrict pathogen replication (17, 22). IFN- γ is particularly important to inhibit bacterial growth in monocytes and alveolar macrophages, thus contributing to limiting the infection by *L. pneumophila* (23–25). These inflammatory cytokines recruit and activate polymorpho-nuclear neutrophil granulocytes (PMNs) (26–28). PMNs are central innate effector cells that not only resolve *L. pneumophila* infection but – in concert with IFN- γ -producing natural killer (NK) cells – also secrete cytokines, such as IL-18 (29, 30). In a feedback loop, IFN- γ triggers IL-12 production by DC, which activate NK cells. Therefore, DC are also essential to control *L. pneumophila* infection (31). Interestingly, DC restrict the intracellular growth of *L. pneumophila*, despite that the pathogen resides in an apparently non-bactericidal compartment derived from the endoplasmic reticulum (ER) (32).

Formation of the Intracellular Replication Niche

In permissive macrophages as well as in protozoa, *L. pneumophila* employs a complex and apparently evolutionarily conserved mechanism to establish its replication-permissive membrane-bound niche, the *Legionella*-containing vacuole (LCV) (Figure 1). LCV formation is governed by the Icm/Dot T4SS, which translocates approximately 300 different effector proteins into the host cell (12, 33–37). Since *L. pneumophila*–host interactions are defined to a large extent by the Icm/Dot apparatus, the T4SS represents a major virulence factor of *L. pneumophila*.

The Icm/Dot T4SS and translocated effectors control every step in the infection process of *L. pneumophila* (Figure 1), i.e., the uptake (38, 39), inhibition of fusion with lysosomes (40) and acidification (41), subversion of retrograde trafficking (42), as well as interception of secretory vesicle trafficking (43), coalescence with the ER (44–46), and finally, egress from the host cell (47). To interfere with host cell processes, many *L. pneumophila* effector proteins target pivotal components of eukaryotic membrane dynamics, such as phosphoinositide (PI) lipids and small GTPases (12, 33, 37). The PI lipids PtdIns(4)P and PtdIns(3)P control the secretory and the endosomal pathway, respectively, while the small GTPases Arf1 and Rab1 are pivotal regulators of ER-Golgi secretory trafficking.

Some Icm/Dot substrates subvert PI lipids by using PtdIns(4)P and PtdIns(3)P as membrane anchors, both of which are found on LCVs (33, 48–50). Accordingly, the effectors SidC, SdcA, SidM, LidA, Lpg1101, and Lpg2603 bind PtdIns(4)P (51–57), and

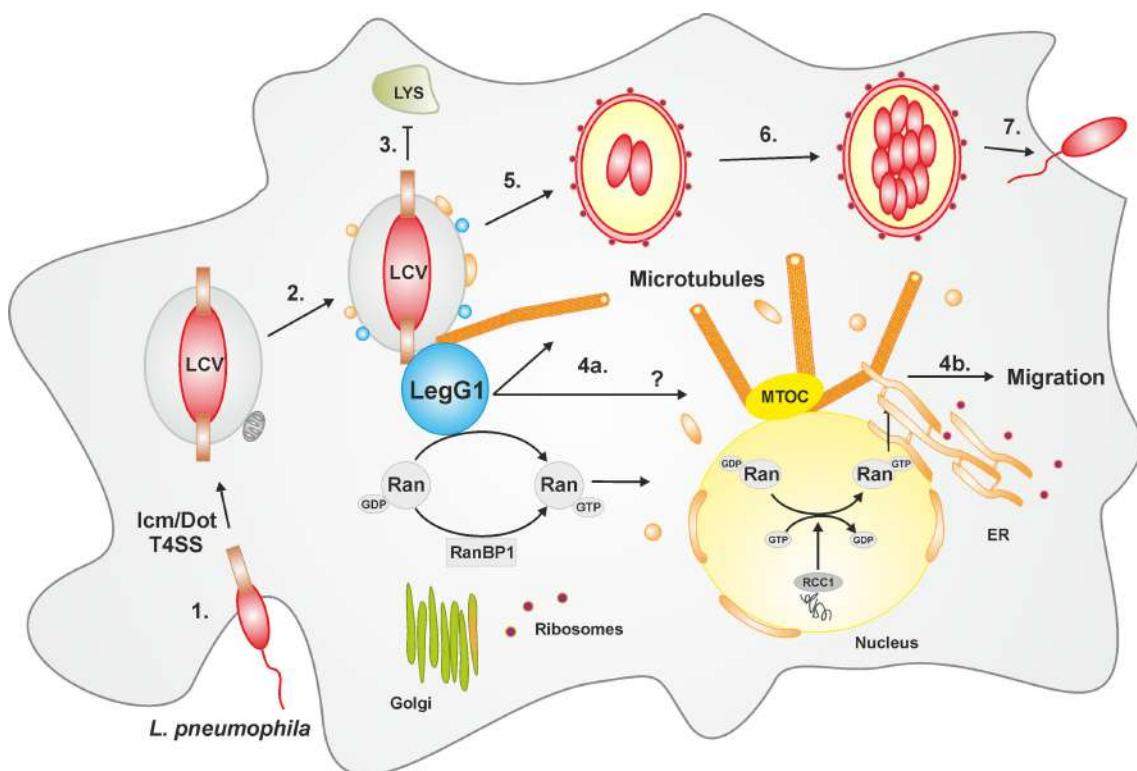


FIGURE 1 | LCV formation and LegG1-dependent modulation of cell migration. The intracellular phases of *L. pneumophila* can be divided in seven main steps: (1) Adhesion and entry into the host cell via macropinocytosis; (2) Formation of the LCV in an Icm/Dot T4SS-dependent manner and recruitment of vesicles from the ER as well as mitochondria; (3) Evasion from the lysosomal trafficking; (4a) The bacterial effector protein LegG1 activates the GTPase Ran, stabilizes microtubules in the vicinity of the LCV or possibly at a distance (?), and (4b) promotes cell migration. In the nucleus, Ran activation is

triggered by the eukaryotic GEF RCC1. (5) The LCV communicates with host vesicle trafficking pathways, and acquires and eventually fuses with the ER; (6) The pathogen compartment turns in a rough-ER-like vacuole, wherein the bacteria replicate; (7) *L. pneumophila* is released and re-infects new host cells or is transmitted to other environmental niches. Abbreviations: ER, endoplasmic reticulum; LCV, *Legionella*-containing vacuole; MTOC, microtubule organizing center; Ran, Ras-related nuclear protein; RCC1, regulator of chromosome condensation 1; T4SS, type IV secretion system.

the effectors LidA, LptD, RidL, SetA, and LpnE bind PtdIns(3)P (42, 52, 58–60). Two *L. pneumophila* “CX₅R” domain PI phosphatases have been identified: SidF, a PI 3-phosphatase that hydrolyzes PtdIns(3,4)P₂ [and also PtdIns(3,4,5)P₃] *in vitro* (61), and SidP, a PI 3-phosphatase that hydrolyzes PtdIns(3)P as well as PtdIns(3,5)P₂ *in vitro* (62). Thus, these PI phosphatases might cause PtdIns(4)P to be formed on and PtdIns(3)P to be removed from LCV membranes. LppA is another Icm/Dot substrate that hydrolyzes polyphosphorylated PIs to mainly produce PtdIns(4)P *in vitro* (49). However, LppA does not affect the LCV PI pattern in infected cells, but rather functions as a translocated hexakisphosphate inositol phosphatase (phytase), which possibly promotes intracellular replication of *L. pneumophila* by removing the intracellular micronutrient chelator phytate.

Intracellular multiplication/defective for organelle trafficking T4SS substrates modulate the activity of the small GTPases Arf1 (RalF) (63) or Rab1 (SidM) (55, 64–67) by acting as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs; LepB) (68), AMPylase/deAMPylyase (SidM, SidD) (69–72), or phosphocholinase/dephosphocholinase (AnkX, Lem3) (73–77). The covalent modifications of Rab1 by AMP

or phosphocholine cause a prolonged activation of the GTPase, by preventing its inhibition by the GAP LepB. Similarly, the Icm/Dot substrate LidA binds to activated Rab1 and prevents inactivation by LepB, as well as covalent modifications by SidM or AnkX (64, 78–80). Finally, the PtdIns(4)P-binding effector SidC and its paralog SdcA promote the monoubiquitination of Rab1, in agreement with their function as an E3 ubiquitin ligase containing a catalytic Cys–His–Asp triad (81–83). In summary, after their translocation a number of *L. pneumophila* Icm/Dot substrates employ PI lipids on the cytoplasmic side of the LCV membrane as membrane anchors. Thus, PI lipids determine at least in part the subcellular localization of the effectors, which show different biochemical activities, including subversion of small GTPases, ubiquitylation, or binding to and modulation of vesicle trafficking machinery.

Inhibition of Retrograde Trafficking and Autophagy

Retrograde trafficking in eukaryotes comprises the transport from early or late endosomes through the *trans*-Golgi

network (TGN) to the ER (84). The cation-independent mannose 6-phosphate receptor (CI-MPR) is a well-documented substrate of the retrograde transport pathway. CI-MPR binds mannose present on hydrolases and transports the cargo destined for the endocytic pathway from the TGN to the endosomal system. After releasing the cargo in the lysosomal lumen, the receptor is recycled back through the retrograde pathway to the TGN (85). Proper recycling requires a protein complex termed the retromer, composed of a cargo recognition subcomplex (Vps26, Vps29, and Vps35) and a membrane-deforming subcomplex, which consists of a dimer of sorting nexins (SNXs) (86). The cargo recognition complex is recruited to the membrane by the activated small GTPases Rab5 and Rab7 (87).

Recent findings indicate that *L. pneumophila* communicates with the retrograde trafficking pathway. The small GTPases Rab5 and Rab7 (88–91), as well as the retromer subunits Vps26, Vps29, and Vps35 (42, 92) localize to the LCV. Interestingly, the Icm/Dot T4SS-translocated substrate RidL functions as a bacterial interactor of the eukaryotic retromer complex (42). RidL, which localizes on LCVs in *D. discoideum* cells and macrophages, binds to the Vps29 subunit of the cargo recognition subcomplex of the retromer. Subunits of the retromer cargo recognition complex localize to the LCV in an Icm/Dot-dependent but RidL-independent manner. RidL competes with SNX1 and SNX2 in its capacity for membrane binding specifically through PtdIns(3)P, thus possibly triggering the removal of SNXs. By altering the retrograde trafficking cascade, RidL might promote the formation of a non-lysosomal replicative vacuole and intracellular replication of *L. pneumophila* in protozoan and metazoan phagocytes (42). Earlier work already indicated that the retrograde pathway might control intracellular replication of *L. pneumophila*. The PtdIns(4,5)P₂ 5-phosphatase OCRL1 and its *D. discoideum* homolog Dd5P4 are implicated in retrograde trafficking (93, 94) and localize to LCVs (60). Depletion of OCRL by RNA interference (42) or deletion of Dd5P4 (60) increased intracellular growth of *L. pneumophila*, indicating that the PI 5-phosphatase OCRL/Dd5P4 indeed restricts pathogen replication.

Autophagy is a major cell-autonomous defense mechanism used by infected cells against intracellular bacteria. The process of macroautophagy is responsible for the degradation of cytoplasmic constituents, such as bacteria or damaged organelles, which are engulfed by autophagosomes and subsequently fuse with lysosomes (95). In the course of microautophagy, the constituents are directly delivered to the lysosomes. Over 30 autophagy-related genes (Atg) have been discovered during the last years (96). An Atg protein essential for autophagy is the microtubule-associated protein light chain 3 (LC3, alias Atg8), which is conjugated to phosphatidylethanolamine and localized on autophagosomal membranes (97). Measurement of LC3/Atg8 during *L. pneumophila* infection revealed an Icm/Dot-dependent inhibition of autophagy by the bacteria. Intriguingly, the bacterial protein RavZ was identified as an effector required for autophagy inhibition, yet further effectors could be involved, since *L. pneumophila* lacking ravZ still evaded the autophagy cascade (98). RavZ is a cysteine protease, which irreversibly deconjugates phosphatidylethanolamine from LC3/Atg8, thus reducing its membrane accumulation and activity. While most autophagy factors are not

necessary for *L. pneumophila* replication in *D. discoideum* (99), amoebae lacking Atg9 do not internalize the pathogen as efficient as wild-type cells, yet allow more effective intracellular growth (100). In summary, these findings suggest that *L. pneumophila* inhibits retrograde vesicle trafficking as well as autophagy to promote intracellular growth.

Modulation of Organelle Motility and Cell Migration

The Icm/Dot T4SS is crucially involved in the formation of the LCV, and many *L. pneumophila* effectors selectively decorate the pathogen vacuole membrane. Intact LCVs can be purified by a straight-forward two-step protocol involving immuno-affinity enrichment using an antibody against the *L. pneumophila* effector SidC selectively decorating the pathogen vacuole and a secondary antibody coupled to magnetic beads, followed by a conventional density centrifugation step. Proteomics analysis of purified preparations of LCVs from infected *D. discoideum* amoebae (91) and RAW 264.7 macrophages (90) revealed the presence of 670 and 1150 host proteins, respectively, including 13 small GTPases of the Rab family, as well as the small GTPase Ran and its effector Ran binding protein 1 (RanBP1).

Ran is a member of the Ras superfamily of small GTPases and is fundamental in numerous cellular processes, such as nuclear pore translocation (101), or mitotic spindle assembly and post-mitotic nuclear envelope formation (102, 103). Ran GTPase also plays an important role in cytoplasmic processes involving non-centrosomal microtubules, e.g., endocytic receptor trafficking and retrograde signaling along microtubules in nerve axons (104). Ran can be activated by a nuclear (or in mitotic cells: chromatin bound) Ran GEF termed regulator of chromosome condensation 1 (RCC1) (105). Ran(GTP) is inactivated by the cytoplasmic Ran GTPase-activating protein 1 (RanGAP1) together with RanBP1, which harbors a Ran(GTP)-binding domain (104).

Intriguingly, *L. pneumophila* produces an Icm/Dot-translocated effector termed LegG1 that harbors eukaryotic-like RCC1 domains (106, 107). The corresponding gene is conserved among the *L. pneumophila* genomes sequenced to date, including the strains Philadelphia-1, Paris, Lens, Corby, Alcoy, and Lorraine. LegG1 (Lpg1976/PieG) is encoded in the plasticity island of effectors (Pie) gene cluster (108). The C-terminal CAAX tetrapeptide motif of LegG1 is lipidated by the host prenylation machinery (109), thus promoting the membrane localization of the effector to small vesicular structures upon ectopic expression in eukaryotic cells (108).

Recent work revealed that LegG1 is indeed an *L. pneumophila* virulence factor, which promotes intracellular bacterial replication but is dispensable for uptake (110) (**Figure 1**). A Δ legG1 mutant strain is not less cytotoxic than the parental strain, but outcompetes by wild-type *L. pneumophila* upon co-infection of *A. castellanii* amoebae. Moreover, Ran, RanBP1, and LegG1 accumulate in an Icm/Dot-dependent manner on the LCV, and the bacterial effector localizes to the cytosolic face of the LCV in *L. pneumophila*-infected phagocytes. *L. pneumophila* wild-type but not the Δ legG1 mutant strain activates Ran on LCVs and in cell lysates; yet, while LegG1 promotes the accumulation

of RanBP1 on LCVs, the effector is dispensable for the recruitment of the small GTPase. Several experimental approaches indicated that in infected phagocytes *L. pneumophila* triggers the polymerization of microtubules in a LegG1-dependent manner (110) (**Figure 1**). “Microbial microinjection” of LegG1 by *Yersinia enterocolitica* confirmed the positive effect of LegG1 on microtubule stabilization. Here, *Y. enterocolitica* harboring a T3SS, but lacking endogenous effectors, translocated LegG1 fused to a type III secretion signal into HeLa cells. Furthermore, while LCVs harboring wild-type *L. pneumophila* vividly move along microtubules in infected *D. discoideum*, the pathogen vacuole harboring Δ legG1 mutant bacteria is stalled (110). In summary, the discovery and characterization of the *L. pneumophila* Ran activator LegG1 revealed an unexpected role of the small GTPase Ran in the formation of pathogen vacuoles.

Microtubule polarization and dynamics represent pivotal determinants of eukaryotic cell migration (111) (**Figure 2**). Given the prominent role of Ran and LegG1 on the dynamics of the microtubule cytoskeleton, we investigated the effect of *L. pneumophila* and LegG1 on host cell motility (112). Studies using *D. discoideum* amoebae or immune cells, such as RAW 264.7 macrophage-like cells or primary PMN, in different migration assays (under-agarose and Boyden chamber assays), revealed an Icm/Dot-dependent inhibition of migration. Phagocytes infected with wild-type *L. pneumophila* or *Legionella longbeachae* showed

a substantially reduced migration when compared to cells lacking a functional Icm/Dot T4SS. Uptake and cytotoxicity assays demonstrated that the observed effect is not due to a defect in infection.

Based on these findings, the modulation of cell migration by LegG1 was assessed in *D. discoideum* as well as in RAW 264.7 macrophages or PMN (112) (**Figure 1**). Interestingly, the Δ legG1 mutant strain hyper-inhibited the directed migration of phagocytes in the under-agarose assay, even to a larger extent than wild-type *L. pneumophila*. Overproduction of LegG1 in the Δ legG1 mutant background re-established the migration range to an extent comparable to cells infected with a Δ icmT mutant strain. Single cell tracking revealed that the forward migration and the velocity of cells infected with wild-type or Δ legG1 *L. pneumophila* was impaired. Similarly, upon “microbial microinjection” by *Y. enterocolitica*, LegG1 was sufficient to stimulate migration of epithelial cells in scratch assays. Moreover, using RNA interference a role of Ran in the LegG1-dependent migration inhibition was demonstrated. Upon depletion of Ran, cells infected with the strain overproducing LegG1 were not able to migrate and to close scratch wounds over time (112). Taken together, the Ran activator LegG1 promotes cell motility by modulating microtubule dynamics and thus antagonizes Icm/Dot-dependent inhibition of cell migration. LegG1 might reverse the otherwise deleterious impact of other *L. pneumophila* effectors on the host cytoskeleton, and thereby

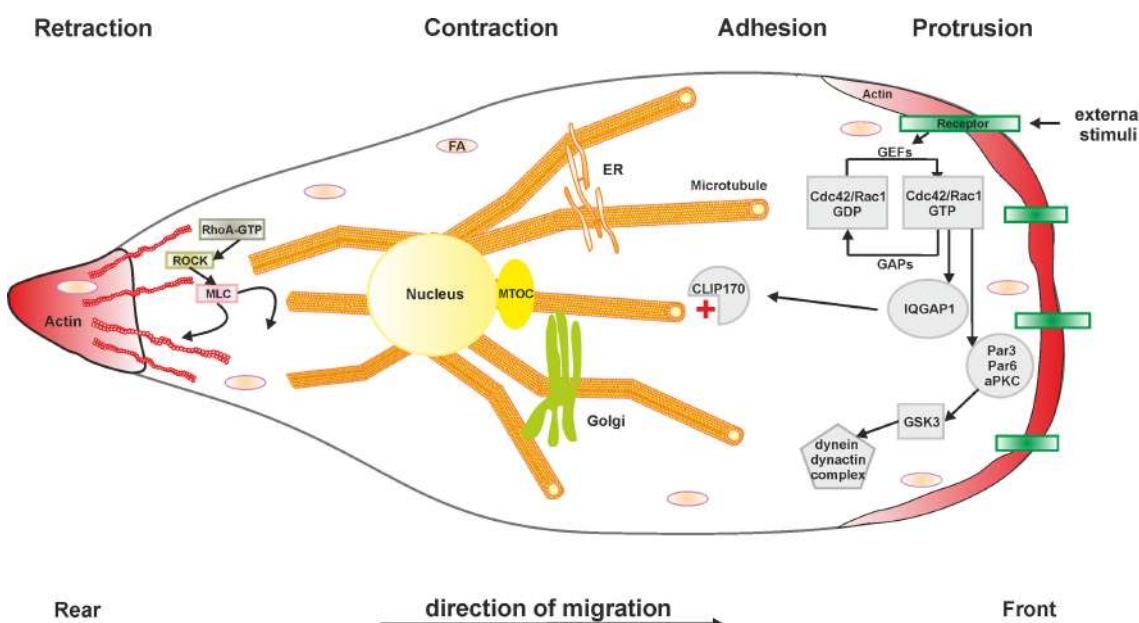


FIGURE 2 | Schematic representation of cell migration. Generally, the cellular migration cycle can be divided into four major steps: (1) protrusion at the leading edge, (2) cell adhesion, (3) contraction to generate the required forces, and (4) retraction at the rear edge. Microtubules and the actin cytoskeleton in concert with Rho GTPases are fundamental components of each phase. Abbreviations: aPKC, atypical protein kinase C; Cdc42, cell division control protein 42; CLIP170, cytoplasmic linker protein 170; FA, focal adhesion; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange

factor; GSK3, glycogen synthase kinase 3; IQGAP1, IQ motif-containing GTPase-activating protein 1; MLC, myosin light chain; MTOC, microtubule organizing center; Par3/6, partitioning defective 3/6 homolog; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog gene family member A; ROCK, Rho-associated protein kinase. The *L. pneumophila* effector protein LegG1 promotes cell migration by activating the small GTPase Ran and stabilizing microtubules. Other *L. pneumophila* factors modulating cell migration are not known.

sustain vesicle trafficking and organelle motility required for the establishment and maintenance of LCVs.

Triggering Inflammasomes and Programed Cell Death

Maintenance of LCV integrity and regulation of programed cell death is critical for preserving the intracellular replication niche of *L. pneumophila* (113). Accordingly, impaired activation of programed cell death turned out to account for the failure of A/J mice to restrict *L. pneumophila*. The allele conferring sensitivity against *L. pneumophila* was mapped to the *NAIP5* gene (114, 115) within the *Lgn1* locus (116). The Naip5 (Birc1e)/Nlrc4 (Ipaf) inflammasome recognizes flagellin and triggers caspase-1 activation, pore formation, and pyroptosis (117–125). Naip5/Nlrc4 inflammasome activation represents a crucial mechanism of *L. pneumophila* restriction. In agreement with this notion, macrophages as well as DC restrict *L. pneumophila* replication through a cell death pathway mediated by Naip5, caspase-1, and caspase-3 (126). Yet, neither inflammasomes nor caspases are conserved in amoebae (127).

In addition to PAMPs, such as flagellin, a number of specific Icm/Dot substrates are implicated in the regulation of programed host cell death. The *L. pneumophila* PI 3-phosphatase SidF inactivates the pro-apoptotic factors BNIP3 and Bcl-rambo by an unknown mechanism, and thereby counteracts cell death induction (128). Furthermore, the effector SdhA plays a role in maintaining the LCV membrane integrity and also contributes to the prevention of cell death (129, 130). Cell death induction in absence of *sdhA* is suppressed by a secreted bacterial phospholipase A through an unknown mechanism. *L. pneumophila* lacking *sdhA* resides in the cytoplasm and triggers caspase-1 activation and IL-1 β secretion, as well as macrophage pyroptosis through the DNA-sensing AIM2 inflammasome (131). The Icm/Dot substrate SdhA is also a key suppressor of the type I IFN (IFN- α/β) response to *L. pneumophila* through nucleic acid-sensing PRRs. Accordingly, RNA from *L. pneumophila* lacking *sdhA* triggers the RIG-I-dependent production of type I IFNs (132).

Legionella pneumophila also promotes programed cell death in an Icm/Dot-dependent manner. The phospholipase VipD destabilizes mitochondrial membranes by means of its phospholipase A activity, thus potentially contributing to the release of cytochrome *c* into the cytosol. This triggers pro-apoptotic caspase-3 activation and subsequently promotes host cell death (133). Analogously, the Icm/Dot-translocated phospholipase PlcC hydrolyzes several lipids, including phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol, which might destabilize target membranes and cause cell toxicity (134). Stimulation of apoptosis by an intracellular pathogen seems counterintuitive, but might reflect a tight spatial and temporal control of LCV maturation, followed by the release of the bacteria from the pathogen compartment and the host cell at the end of an infection cycle. Thus, the elaborate coordination of anti- and pro-apoptotic factors optimally supports intracellular bacterial proliferation.

Finally, caspases not only regulate cell death during *L. pneumophila* infection but also control vesicle trafficking pathways and thus contribute to the formation of LCVs. Dependent on the

Nlrc4 inflammasome and Naip5, *L. pneumophila* activates caspase-7 downstream of caspase-1, and consequently, the pathogen is delivered to lysosomes (117, 135). In turn, upon deletion of the caspases, *L. pneumophila* forms an ER-derived replicative compartment. Similarly (but independently of caspase-1), active caspase-11 restricts the replication of *L. pneumophila* by promoting the fusion of the pathogen compartment with lysosomes (136).

Activation of the Transcription Factor NF- κ B

The transcription factor NF- κ B is a master regulator of the mammalian innate immune response and controls the production of anti-apoptotic and pro-survival factors as well as inflammatory mediators (137). Thus, the activation of NF- κ B (which is not conserved in amoebae) represents another, more indirect way to prevent host cell death. NF- κ B is composed of five Rel family proteins: RelA, RelB, c-Rel, and the precursors p100 and p105, which are processed to their mature forms, p52 and p50. The transcription factor is maintained in its inactive form in the cytoplasm by a family of inhibitors termed I κ Bs (I κ B α , I κ B β , I κ B γ , and Bcl-3).

Bacterial PAMPs, including LPS or flagellin, initiate the NF- κ B cascade through TLR2 or TLR5 and the adaptor MyD88. *L. pneumophila* triggers the production of pro-inflammatory cytokines, such as IL-1 β , and the activation of the inflammasome platform through these receptors (138–141). The deletion of single TLRs does not dramatically alter *L. pneumophila* infection; yet, mice lacking MyD88 fail to produce cytokines, such as NK cell-derived IFN- γ , and are highly susceptible to *L. pneumophila* infection (142–144, 29). Moreover, mice lacking the IL-1 receptor are impaired for PMN recruitment and bacterial clearance (145). Therefore, IL-1R/MyD88-dependant signaling is critical for host resistance to *L. pneumophila* infection.

The NF- κ B pathway can be activated not only by bacterial PAMPs via a TLR-dependent pathway but also by distinct bacterial effectors (146). The Icm/Dot T4SS and its substrates LnaB and LegK1 strongly induce NF- κ B (147–149). While the molecular mechanism of the novel effector LnaB is not understood, LegK1 is a Ser/Thr kinase that phosphorylates the NF- κ B inhibitor I κ B α , leading to a robust NF- κ B activation by triggering the release and nuclear translocation of the transcription factor (150). Since *L. pneumophila* lacking legK1 is not impaired for intracellular replication, other effectors might also modulate the NF- κ B response. Among the five *L. pneumophila* Icm/Dot substrates that show protein kinase activity *in vitro* (LegK1–LegK5), LegK2 is a virulence factor, which promotes intracellular replication and efficient recruitment of the ER to LCVs (151).

Translation inhibition by the Icm/Dot substrates Lgt1-3, SidI, and SidL also specifically decreases the production of I κ B, even though the effectors are overall cytotoxic (152, 153). To shut down translation, the UDP-glucosyltransferases Lgt1-3 modify the elongation factor eEF1A (154, 155), and SidI inactivates eEF1A/eEF1B γ by an unknown mechanism (153). Finally, *L. pneumophila* also modulates host transcription more directly. The Icm/Dot substrate RomA/LegAS4 promotes

intracellular replication as a SET domain-containing histone methyltransferase that modifies (immune) gene expression (156, 157). In summary, *L. pneumophila* modulates protein production and turnover through the activation of NF- κ B and by altering the epigenetic pattern, as well as through the inhibition of translation.

Conclusion and Outlook

The ubiquitous environmental bacterium *L. pneumophila* triggers an acute and potential fatal pneumonia termed Legionnaires' disease. The opportunistic pathogen employs the Icm/Dot T4SS and as many as 300 different effector proteins to govern interactions with phagocytes and form an intracellular replication niche, the LCV. Some Icm/Dot-translocated effector proteins interfere with (i) endocytic, secretory, or retrograde vesicle trafficking

pathways, (ii) organelle or cell motility, (iii) the inflammasome and programmed cell death, or (iv) the transcription factor NF- κ B. Future studies will address the molecular mechanisms of action of the many Icm/Dot substrates, which remain uncharacterized to date. The further analysis of *L. pneumophila* effector proteins will likely continue to provide novel insights into the elaborate pathogen–host interactions between a highly adapted opportunistic pathogen and its phagocytic host cells.

Acknowledgments

This work was supported by the Swiss National Science Foundation (SNF; 31003A_153200), the German Research Foundation (DFG; HI 1511/3-1, SPP 1580, SFB 914), and the Bundesministerium für Bildung und Forschung (BMBF; 031A410A; Infect-ERA project EUGENPATH).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A novel pro-inflammatory protein of *Streptococcus suis* 2 induces the Toll-like receptor 2-dependent expression of pro-inflammatory cytokines in RAW 264.7 macrophages via activation of ERK1/2 pathway

OPEN ACCESS

Edited by:

Denise Monack, Stanford University School of Medicine, USA

Reviewed by:

David Weiss, Emory University, USA
Heinrich Korner, Menzies Research Institute Tasmania, Australia

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Specialty section:

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

Received: 10 November 2014

Paper pending published:

09 January 2015

Accepted: 18 February 2015

Published: 09 March 2015

Citation:

Zhang Q, Yang Y, Yan S, Liu J, Xu Z, Yu J, Song Y, Zhang A and Jin M (2015) A novel pro-inflammatory protein of *Streptococcus suis* 2 induces the Toll-like receptor 2-dependent expression of pro-inflammatory cytokines in RAW 264.7 macrophages via activation of ERK1/2 pathway. *Front. Microbiol.* 6:178.
doi: 10.3389/fmicb.2015.00178

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Streptococcus suis 2 is an important swine pathogen and an emergent zoonotic pathogen. Excessive inflammation caused by *S. suis* is responsible for the high levels of early mortality observed in septic shock-like syndrome cases. However, the mechanisms through which *S. suis* 2 (SS2) causes excessive inflammation remain unclear. Thus, this study aimed to identify novel pro-inflammatory mediators that play important roles in the development of therapies against SS2 infection. In this study, the novel pro-inflammatory protein HP0459, which was encoded by the SSUSC84_0459 gene, was discovered. The stimulation of RAW 264.7 macrophages with recombinant HP0459 protein induced the expression of pro-inflammatory cytokines (IL-1 β , MCP-1 and TNF- α). Compared with the wild-type (WT) strain, the isogenic knockout of HP0459 in SS2 led to reduced production of pro-inflammatory cytokines in RAW264.7 macrophages and *in vivo*. The pro-inflammatory activity of HP0459 was significantly reduced by an antibody against Toll-like receptor 2 (TLR2) in RAW264.7 macrophages and was lower in TLR2-deficient (TLR2 $-/-$) macrophages than in WT macrophages. Furthermore, specific inhibitors of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathways significantly decreased the HP0459-induced pro-inflammatory cytokine production, and a western blot assay showed that HP0459 stimulation induced the activation of the ERK1/2 pathway. Taken together, our data indicate that HP0459 is a novel pro-inflammatory mediator of SS2 and induces TLR2-dependent pro-inflammatory activity in RAW264.7 macrophages through the ERK1/2 pathway.

Keywords: *Streptococcus suis* 2, inflammation, pro-inflammatory protein, Toll-like receptor 2, ERK1/2 pathway

Introduction

Streptococcus suis is a major pathogen responsible for important economic losses to the swine industry worldwide, particularly during the past 20 years (Gottschalk et al., 2010). It causes meningitis, septicemia, pneumonia, endocarditis, arthritis, and other diseases in swine (Wei et al., 2009). Among the 33 serotypes based on capsular antigens that have been described, serotype 2 is the most frequently isolated from diseased pigs, particularly in Europe and Asia (Wisselink et al., 2000). Since the first human case was reported in Denmark in Perch et al. (1968), more *S. suis* infections in humans have been documented in several European and Asian countries as well as in North and South America, Australia, and New Zealand (Wertheim et al., 2009; Gottschalk et al., 2010). For a long time, it has been considered to be a fact that *S. suis* only infects people working with pigs or pork-derived products (Arends and Zanen, 1988); however, *S. suis* infections in the general population were recently reported in Southeast and East Asia (Gottschalk et al., 2010). Although most reports concern sporadic cases of infection, a large series of 151 *S. suis* meningitis cases was recently reported in southern Vietnam (Mai et al., 2008). Furthermore, an important outbreak, which involved 215 cases and 38 deaths, occurred in China in during 2005 (Yu et al., 2006). In addition, *S. suis* is considered one of the most important causes of meningitis in humans in various locations, including Vietnam, Thailand, and Hong Kong (Suankratay et al., 2004; Hui et al., 2005; Ip et al., 2007; Mai et al., 2008). These findings emphasize the importance of *S. suis* as an emerging zoonosis and indicate that *S. suis* represents a significant public health concern (Fittipaldi et al., 2012). The increased severity of *S. suis* infection in humans underscores the critical need to better understand the factors associated with the pathogenesis of *S. suis* infection (Gottschalk and Segura, 2000).

Although several virulence-related molecules have been proposed, only the capsular polysaccharide (CPS) has been proven to play a critical role in the virulence of *S. suis* (Charland et al., 1998; Smith et al., 1999; Segura et al., 2004). Some putative virulence factors have also been reported in *S. suis*, such as suilysin, muramidase-released protein (MRP), subtilisin-like protease (SspA), and LTA D-alanylation (DltA; Smith et al., 1992; Lun et al., 2003; Fittipaldi et al., 2008; Bonifait et al., 2011); however, the current knowledge on the pathogenesis of *S. suis* infection remains limited (Baums and Valentin-Weigand, 2009). To cause disease, *S. suis* must breach epithelial barriers, reach and survive in the bloodstream, invade different organs and cause exaggerated inflammation (Fittipaldi et al., 2012). The upregulated expression of several pro-inflammatory cytokines and chemokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6 and monocyte chemotactic protein-1 (MCP-1), has been reported in *S. suis* infection (Gottschalk and Segura, 2000; Segura et al., 2002; Al-Numani et al., 2003). Furthermore, inflammation is thought to be responsible for most clinical signs of meningitis, septicemia and sudden death (Segura et al., 2006). *S. suis* sequence type (ST) 7 was recognized as the causative agent for the Sichuan outbreak, and streptococcal toxic shock-like syndrome (STSLS)

was observed for the first time in this large outbreak (Ye et al., 2006). A previous study showed that the increased virulence of *S. suis* ST7 is associated with an increased ability to stimulate excessive pro-inflammatory cytokines that may be responsible for the shock syndrome (Zheng et al., 2008). In addition, the most important clinical feature associated with *S. suis* is meningitis in pigs (Gottschalk and Segura, 2000); however, the mechanisms of *S. suis* crossing the blood-brain barrier (BBB) to cause meningitis are poorly understood. Even so, some mechanisms, such as the up-regulation of pro-inflammatory cytokines and increased leukocyte trafficking, have been proposed to contribute to the breakdown of the BBB (Vadeboncoeur et al., 2003; Adam et al., 2004; Jobin et al., 2005; Tenenbaum et al., 2005). The activation of the innate immune response depends on the recognition of pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are critical sensors that activate the innate immune response (Beutler, 2009; Kawai and Akira, 2010). For example, TLR2 can form heterodimers with TLR1 or TLR6 to recognize bacterial lipoprotein, lipoteichoic acid (LTA), peptidoglycans (PGNs) and zymosan and induce the release of many cytokines and chemokines responsible for inflammation (Akira and Hemmi, 2003; Beutler, 2004; Lachance et al., 2013). Many previous studies have reported that TLR2 is the major (but not exclusive) immune receptor involved in *S. suis* recognition (Graveline et al., 2007; Li et al., 2010; Lecours et al., 2012).

As mentioned above, inflammation has been thought to be a hallmark of *S. suis* infection (Gottschalk et al., 2007). However, the research on inflammation induced by *S. suis* remains limited. Therefore, it is important to identify novel pro-inflammatory mediators of *S. suis* in order to improve our understanding of the mechanism of inflammation induced by this pathogen. In our previous study, more than 50 extracellular proteins of *S. suis* were expressed, including membrane proteins, secreted proteins and cell wall proteins. And several pro-inflammatory proteins were identified, of which a novel protein HP0459 displayed rather robust pro-inflammatory activity (data not shown). In this study, through measuring the IL-1 β and MCP-1 levels by relative quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA), the pro-inflammatory ability of HP0459 was examined. We investigated the recognition receptor and signal transduction pathway through which HP0459 induces IL-1 β , TNF- α and MCP-1 in RAW264.7 macrophages. As a result, we elucidated the mechanism through which HP0459 stimulation induces pro-inflammatory cytokine production.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Streptococcus suis serotype 2 strain SC-19, which was isolated from the brain of a dead pig during the epidemic outbreak in Sichuan province of China in 2005, was selected as the wild-type (WT) strain. SC-19 was grown in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA) plates (Difco, MI, USA) with 5% newborn bovine serum (Sijiqing Biological Engineering

Materials Co., Ltd., Hangzhou, China) at 37°C (Li et al., 2013). A temperature-sensitive *S. suis*-*Escherichia coli* shuttle vector (pSET4s) was used to construct the $\Delta hp0459$ mutant, which carries a spectinomycin resistance gene (*spc*^r). pSET2, a *S. suis*-*E. coli* shuttle vector carrying *spc*^r, was used in the construction of the complementary bacterium (Takamatsu et al., 2001).

Cell Culture

RAW 264.7 macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 5% CO₂ atmosphere at 37°C (Kang et al., 2009). The primary mouse macrophages were isolated from TLR2-deficient (TLR2^{-/-}; JAX® Mice) and WT mice. The mice were injected intraperitoneally (i.p.) with 4% thioglycolate, and peritoneal exudate cells were harvested 4 days later (van Lint et al., 2010). More than 90% of the exudate cells were identified as macrophages by microscopic analysis and non-specific esterase staining (Sodhi et al., 2005). The macrophages were plated at a density of 10⁶ cells per well in 12-well plates.

Cloning, Expression, Purification and Endotoxin Removal of HP0459 Protein

The HP0459 protein, which was reported as a secreted protein in a previous study, was encoded by SSUSC84_0459 (Liu et al., 2009). The HP0459 protein was cloned and purified according to published methods (Liu et al., 2012). Briefly, the *hp0459* gene was amplified from the chromosomal DNA of SC-19 by PCR using the primers listed in **Table 1**. The

purified PCR product was inserted into pET-28a and harbored in *E. coli* BL21 (DE3) cells. HP0459 was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and purified by ultrasonication and Ni-NTA agarose chromatography. The endotoxin in the purified recombinant protein was removed using an Endotoxin Removal Kit (Genmed Scientifics Inc. USA), and the endotoxin level was tested using a Quantitative Chromogenic Tachypleus Amebocyte Lysate For Endotoxin Detection kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China; Liu et al., 2011). The protein was then treated using a 0.22-μm filter. After the above-mentioned treatment, the HP0459 protein was stored at -80°C.

RNA Extraction and qPCR Assay

After the treatment of RAW 264.7 cells with HP0459 at 10 μg ml⁻¹ for 10 h, the total RNA of the cells was extracted with the TRIzol® reagent (Invitrogen, Paisley, UK), according to the manufacturer's guidelines. The RNA pellets were suspended in RNase-Free water, and the DNA contamination of the RNA was removed by DNase treatment (Promega, Madison, WI, USA). cDNA was obtained from 4 μg of RNA by reverse transcription using AMV reverse transcriptase (TAKARA, Japan) and the oligo-dT primer (300 pmol) in a total reaction volume of 40 μl (Moore et al., 2005). Relative quantitative PCR (qPCR) was performed to measure the mRNA levels of pro-inflammatory cytokines (IL-1β, MCP-1 and TNF-α) using a SYBR green PCR Kit (Roche) and the ABI ViiA7 instrument. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene, and all of the primers used in the

TABLE 1 | Oligonucleotide primers used in this study.

Primers	Primers sequence (5'-3') ^a	Functions
hp0459-F	CCCG <u>AATT</u> CACGGAGGTAGCTAACGAAC	For amplification of the <i>hp0459</i> ORF gene
hp0459-R	CCC <u>CTC</u> GAGTTATCCGGTGTTGAAATAG	
hp0459-L1	AAT <u>GAAT</u> CTTCCTGATAAGAAGGTGGCTAAC	Upstream border of <i>hp0459</i>
hp0459-L2	AAC <u>GTC</u> GACCCTGAAGGATCCTAATGAAGTT	
hp0459-R1	TTAG <u>TGAC</u> GCACTGATAAACTCGAAGTAG	Downstream border of <i>hp0459</i>
hp0459-R2	CTCA <u>AGCT</u> TTGACTAAACCATTAGCCA	
chp0459-1	CGCG <u>CAT</u> GCCCCTTATTATGTCAAGTTCAGAT	To complement <i>hp0459</i> in the PCR assays
chp0459-2	CGCG <u>GAAT</u> CTTATTCCGGTGTTGAAATAG	
MCP1-F	TGGGTCAGACATACATTA	For qPCR assay
MCP1-R	TCAGATTACGGGTCAACT	
TNFα-F	CGATGAGGTCAATCTGCCCA	For qPCR assay
TNFα-R	CCAGGTCACTGTCCCAGCATC	
IL1β-F	CACCTGGTACATCAGCACCTCAC	For qPCR assay
IL1β-R	CATCAGAACAGTCCAGCCCCATAC	
GAPDH-F	CGTCGGTGCTGAGTATGTCGT	For qPCR assay
GAPDH-R	CAGTCTCTGGGTGGCAGTGAT	
P1	TAGTTCTGATAAACTTCATTAGGA	To identify the <i>hp0459</i> gene by PCR
P2	AAATGCGCTCGAAATGA	
P3	TGGAAATGTTCAAGTCAACC	To identify the <i>gdh</i> gene by PCR
P4	CGTTTTCTTGATGTCAC	
P5	GCACAGATGCGTAAGGAG	To identify the pSET4s by PCR
P6	ACTCTGTAGCACCGCTA	

^aThe underlined nucleotides denote enzyme restriction sites.

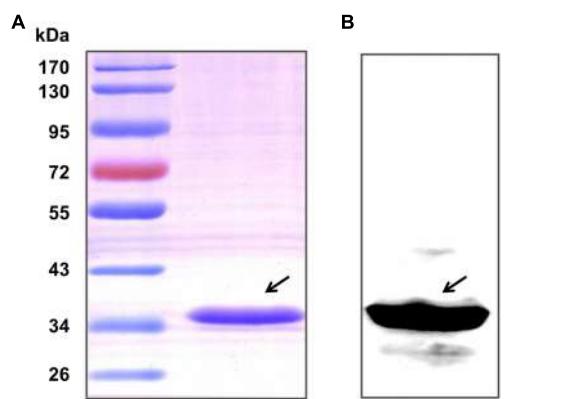


FIGURE 1 | Purification of the recombinant Hp0459 protein.
(A) SDS-PAGE. **(B)** Western blot analysis. The blot was probed with his tag monoclonal antibody (Cali-Bio).

qPCR assay are listed in **Table 1**. The data were analyzed using the ViiA7 software (Applied Biosystems; Zhao et al., 2014).

Enzyme-Linked Immunosorbent Assay for Cytokines

After treatment of RAW 264.7 cells with HP0459 at $10 \mu\text{g ml}^{-1}$ for 10 h, the protein levels of IL-1 β , MCP-1 and TNF- α in the cell culture supernatants were determined using commercially available ELISA kits (Biolegend) according to the manufacturer's instructions.

Generation of an Isogenic *hp0459* Deletion Mutant and Complemented Strains

The construction of the $\Delta hp0459$ -knockout mutant was performed using a previously described procedure (Takamatsu et al., 2001). Briefly, DNA fragments were amplified from the genomic DNA of SC-19 by PCR using two pairs of specific primers, namely *hp0459-L1/hp0459-L2* and *hp0459-R1/hp0459-R2* (**Table 1**), which carry *EcoRI/SalI* and *SalI/HindI* restriction enzyme sites, respectively. The fragments were digested with the corresponding restriction enzymes and sequentially ligated into the temperature-sensitive *S. suis-E. coli* shuttle vector pSET4s to generate the *hp0459*-knockout vector pSET4s $\Delta hp0459$. To obtain the isogenic mutant $\Delta hp0459$, competent cells of SC-19

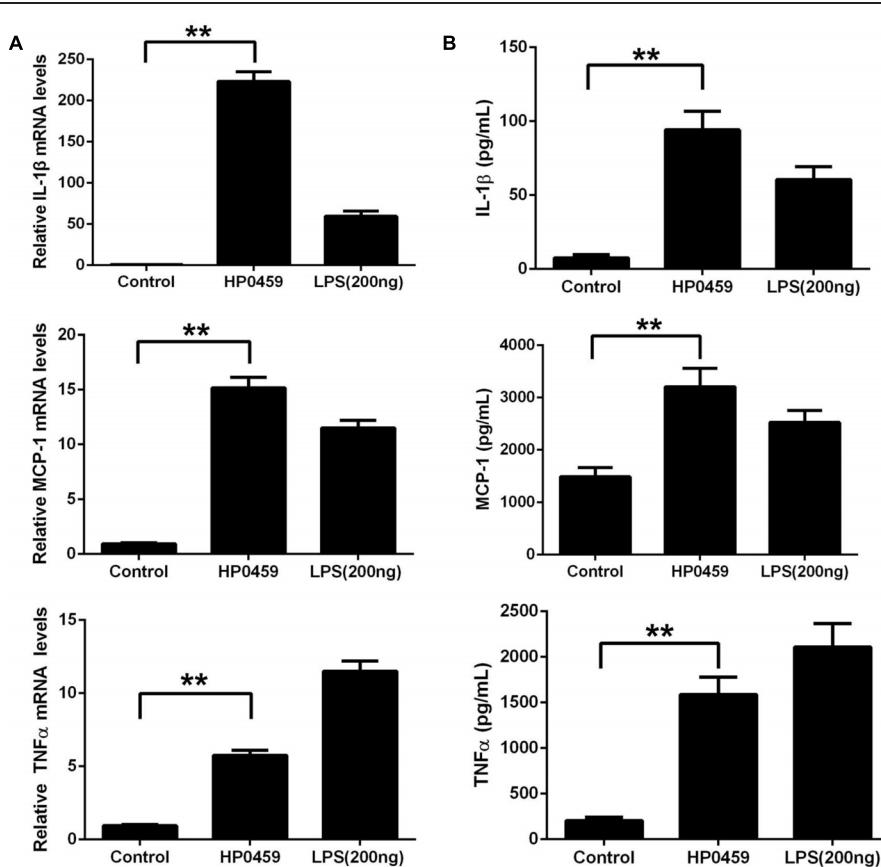
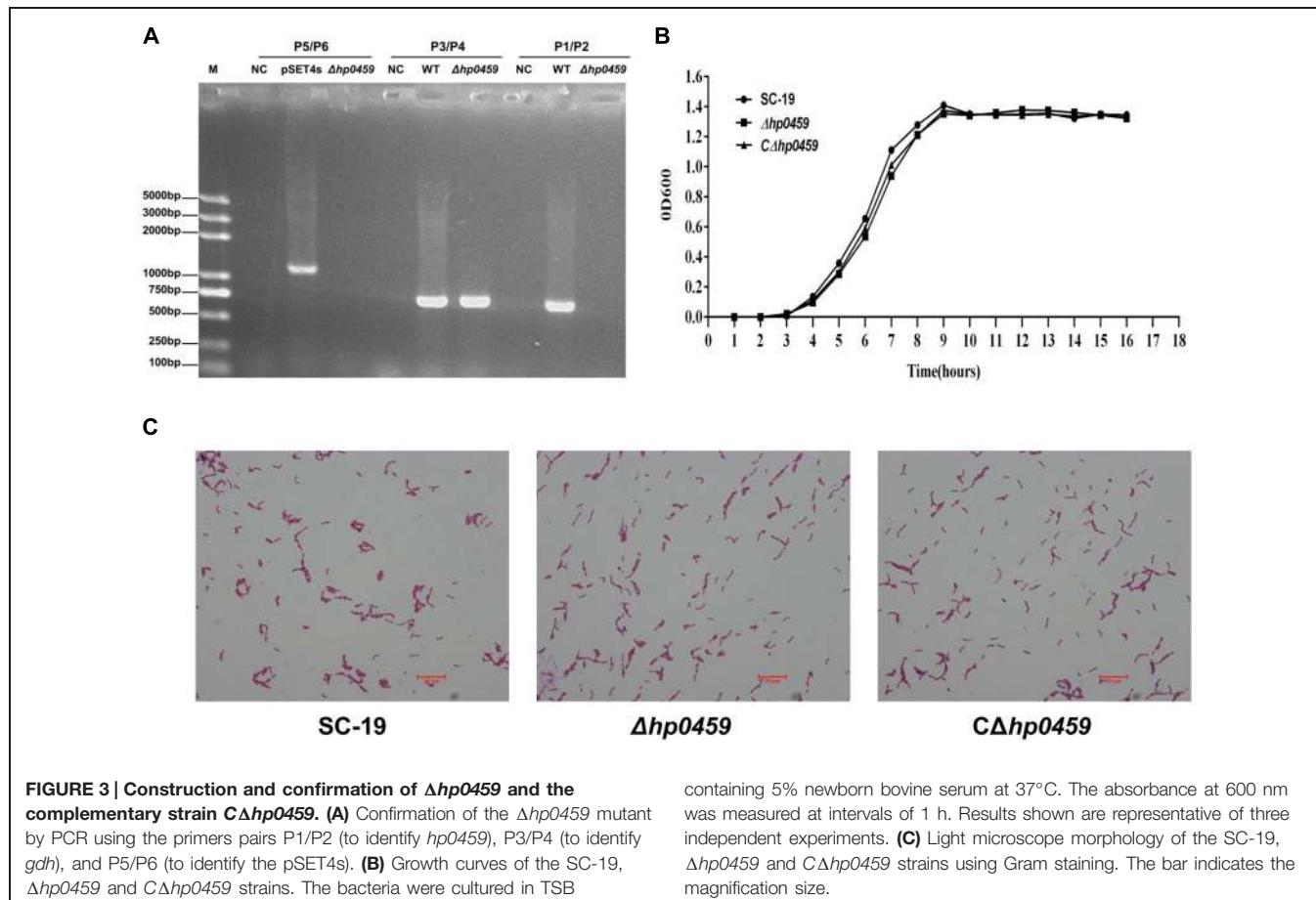


FIGURE 2 | Induction of cytokine mRNA and protein in RAW 264.7 macrophages by recombinant HP0459 stimulation. RAW 264.7 macrophages were incubated with $10 \mu\text{g ml}^{-1}$ HP0459 and 200 ng ml^{-1} LPS (positive control) for 10 h, as well as single culture media (negative control),

(A) the cytokine mRNA levels were then determined by qPCR, (B) and the protein levels of IL-1 β , MCP-1 and TNF- α in the culture supernatants were determined by ELISA. The bars represent the standard errors of the means, based on three independent experiments. ** $P < 0.01$.



were subjected to electrotransformation with pSET4s $\Delta hp0459$ as described previously (Takamatsu et al., 2001). The suspected mutant was verified by PCR using three pairs of primers: P1/P2 (to identify $hp0459$), P3/P4 (to identify gad) and P5/P6 (to identify the pSET4s).

The complemented strain of $hp0459$ was constructed as described previously (Zhang et al., 2012). Briefly, a DNA fragment that contained the $hp0459$ gene and its predicted upstream promoter was amplified by PCR using the primers *chp0459-1/chp0459-2* (Table 1), which carry *SphI/EcoRI* restriction enzyme sites, respectively. To generate the recombinant plasmid pSET2Chp0459, the fragment was digested with the appropriate restriction enzymes and was cloned into the *E. coli*-*S. suis* shuttle vector pSET2 carrying the same cohesive terminus. The plasmid was then electrotransformed into $\Delta hp0459$ to obtain the complemented $C\Delta hp0459$ strain.

Experimental Infections *In Vitro* and *In Vivo*

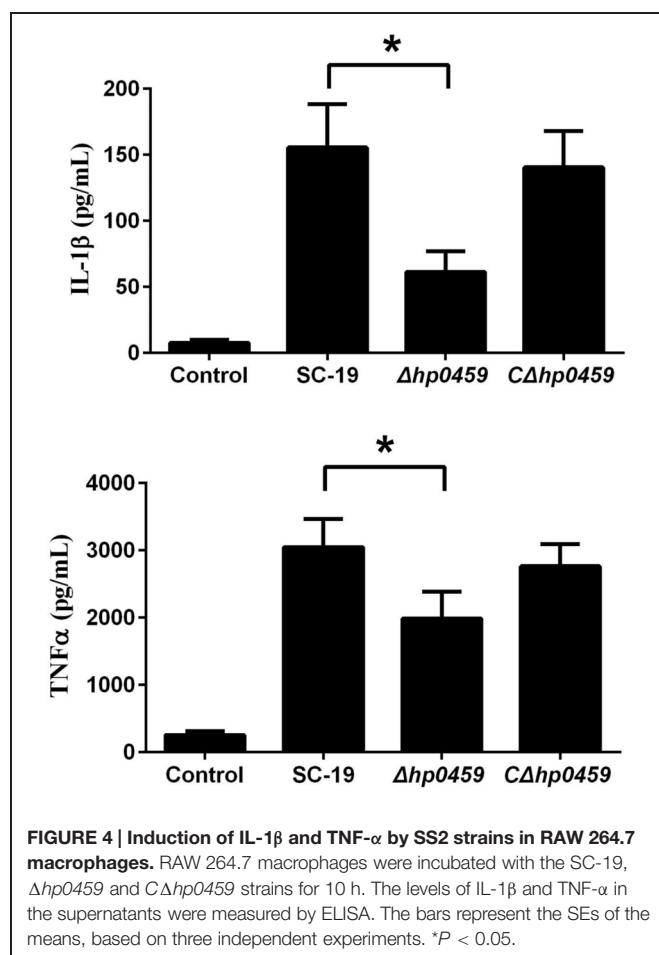
In vitro, RAW 264.7 cells were infected with 5×10^6 CFU of the WT (SC-19), $\Delta hp0459$ or $C\Delta hp0459$ strains in the logarithmic phase of growth. The supernatants were collected for western blot analysis after 10 h at 37°C.

All of the animal studies were performed according to the experimental protocols approved by the Laboratory Animal Monitoring Committee of Hubei Province, China. A total of 125

6-weeks-old female C57BL/6 mice were randomly divided into three groups with 40 mice per group, and the remaining five mice were used as controls. The three groups of mice were challenged intraperitoneally (i.p.) with 5×10^8 CFU log-phase WT (SC-19), $\Delta hp0459$ or $C\Delta hp0459$ strains, respectively. At certain times post infection (3, 6, 9, and 12 h), an equal number of mice in each group were killed. Bacteriological isolation from the blood or part of the spleen was performed essentially as described previously (Zhang et al., 2011), and the lungs or another part of the spleen were used to determine the IL-1 β and TNF- α levels by qPCR.

Investigating the Recognition Receptor of HP0459

Antibody blocking assays were performed to investigate the recognition receptor of HP0459 in RAW264.7 cells using the anti-TLR2 (eBioscience) and anti-TLR4 (BioLegend) antibodies. Briefly, RAW264.7 cells were pretreated using 8 μ g of anti-TLR2 and anti-TLR4 antibody for 30 min respectively and then incubated with 10 μ g ml $^{-1}$ HP0459 for 10 h. The expression levels of various cytokines were determined by ELISA. According to the conditions of cytokine activation, the recognition receptor of the HP0459 was analyzed. In addition, TLR2 $-/$ macrophages were isolated from TLR2 $-/$ mice to verify the results of the blocking assays.

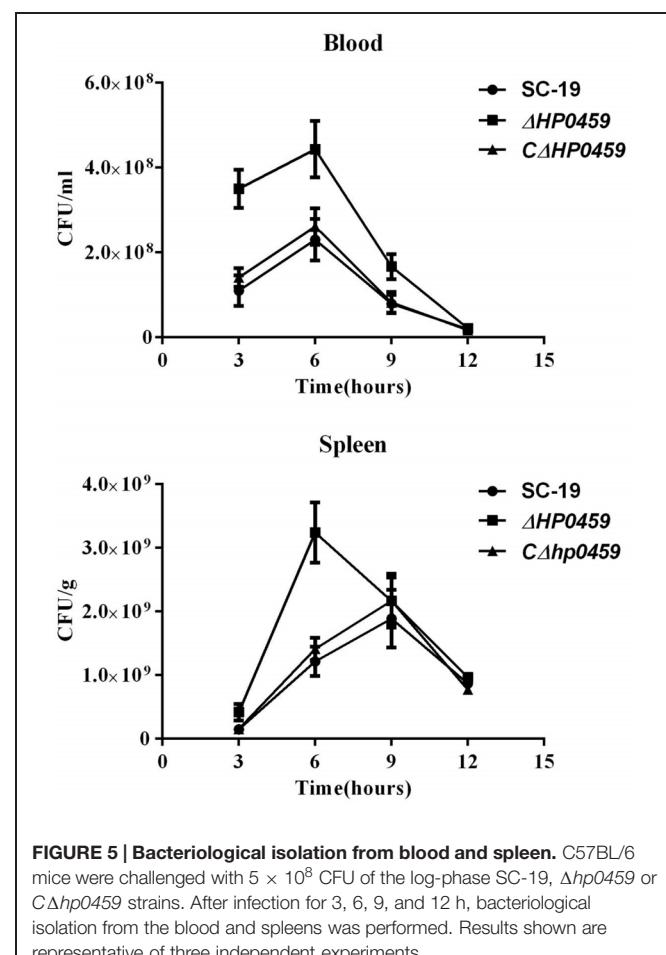


Analysis of HP0459-Induced Cell Signal Transduction Pathways

RAW264.7 macrophages (1×10^6 cells ml^{-1}) were seeded into 12-well tissue culture plates. The cells were pretreated with the following specific inhibitors for 30 min prior to the addition of HP0459 (Liu et al., 2008): U0126 (for ERK1/2; 10 μM), SP600125 (for JNK; 10 μM), pyrrolidine dithiocarbamate (PDTC; for NF- κ B; 20 μM) and LY294002 (for PI3K; 20 μM). All inhibitors were purchased from Cayman Chemical. Culture supernatants were collected at the indicated times and stored at -80°C until assayed.

SDS-PAGE and Western Blot Analysis

To confirm the HP0459-induced phosphorylation of signal transduction molecules, a western blot analysis was performed. To extract the cytosolic protein, after stimulation with HP0459 (10 $\mu\text{g ml}^{-1}$) for 10 h, RAW264.7 cells were washed with cold PBS and harvested by centrifugation. The pellets were then suspended in RIPA lysis buffer with phosphatase inhibitor (Roche) for 15 min on ice. The protein concentrations in the lysates were quantified with the Bradford protein assay, and 40 μg of proteins were subjected to 12% SDS-PAGE and transferred onto a 0.22- μm nitrocellulose membrane. Subsequently, these proteins were probed with specific Abs against the phosphorylated forms of ERK1/2 and NF- κ B p65 (Cell Signaling Technology,



Beverly, MA, USA), and β -actin was assessed as an internal control using anti- β -actin antibody (Wuhan PMK Biotechnology Co., Ltd.). The detection of the bands was performed using HRP-conjugated secondary antibody and an enhanced chemiluminescence (ECL) system (Amersham Life Science, Arlington Heights, IL, USA).

Statistical Analysis

The statistical significance of the data was determined using Student's *t* test with GraphPad Prism software (San Diego, CA, USA), and all the assays were repeated at least three times. For all tests, a value of $P < 0.05$ was considered as the threshold for significance.

Results

Cytokine Secretion from RAW264.7 Cells Stimulated with HP0459

After purification by Ni-NTA agarose chromatography, the SDS-PAGE (Figure 1A) and western blot analysis (Figure 1B) of HP0459 revealed that the HP0459 protein was successfully purified. The average endotoxin level in HP0459 was ~ 0.05 endotoxin

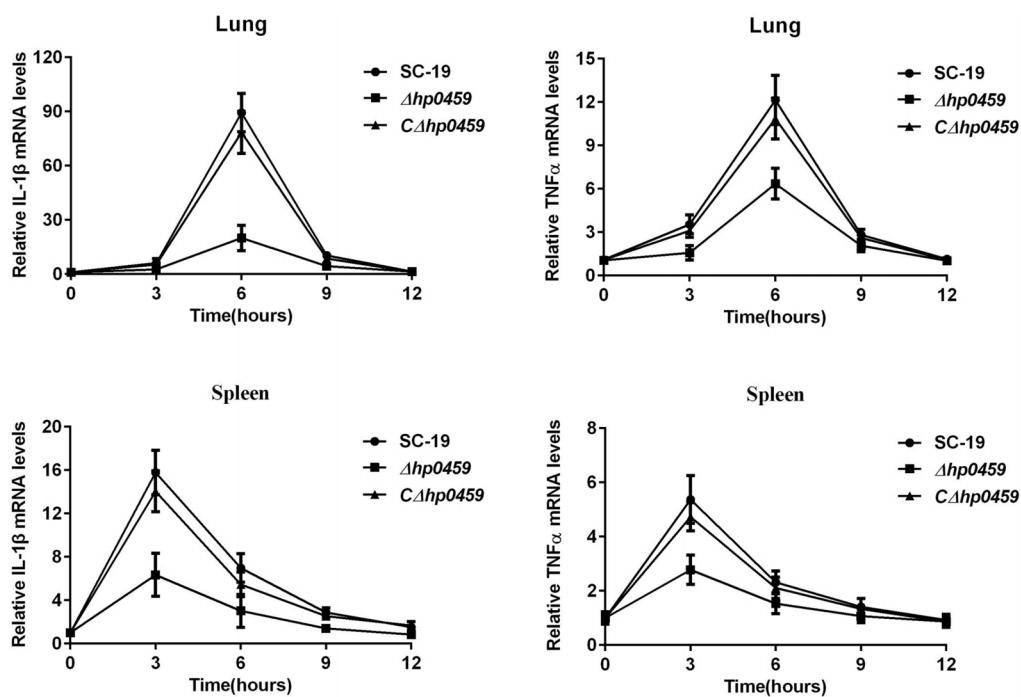


FIGURE 6 | Induction of IL-1 β and TNF- α mRNA *in vivo* by stimulation with SS2 strains. C57BL/6 mice were challenged with 5×10^8 CFU of the log-phase SC-19, Δ hp0459 or C Δ hp0459 strains. After infection for 3, 6, 9, and 12 h, the mRNA levels of IL-1 β and TNF- α in the lungs and spleens were measured by qPCR. Results shown are representative of three independent experiments.

units per milliliter via endotoxin removal. After the above-mentioned treatment, to determine the pro-inflammatory role of HP0459, RAW264.7 cells were stimulated with HP0459 at a concentration of $10 \mu\text{g ml}^{-1}$ for 10 h and analyzed by qPCR and ELISA respectively. As shown in Figure 2, it was confirmed that HP0459 stimulation significantly increased the expression levels of IL-1 β , MCP-1, and TNF- α ($P < 0.01$) by qPCR (Figure 2A) and ELISA (Figure 2B) analyses.

Construction and Characterization of the Mutant Strain Δ hp0459

To study the role of HP0459 in the *S. suis*-induced pro-inflammatory response, the HP0459-knockout mutant Δ hp0459 was constructed by homologous recombination, and the double-crossover event was confirmed by PCR (Figure 3A). To examine the growth characteristics of the mutants *in vitro*, the OD600 values of cultures of the SC-19, Δ hp0459 and C Δ hp0459 strains in TSB containing 10% newborn bovine serum at 37°C were determined (Figure 3B), and Gram-staining of these three strains was also performed (Figure 3C). No significant difference in growth was found between the SC-19, Δ hp0459 and C Δ hp0459 strains.

Pro-inflammatory Attenuation Induced by the Δ hp0459 Strain *In Vitro* and *In Vivo*

After construction of the Δ hp0459 strain, the role of HP0459 in the *S. suis*-induced pro-inflammatory response was assessed in RAW264.7 cells *in vitro*. The culture supernatants of RAW264.7 cells incubated with the SC-19, Δ hp0459 and C Δ hp0459 strains

were harvested, and the levels of IL-1 β and TNF- α in these supernatants were measured by ELISA. The results showed that the pro-inflammatory activity of the Δ hp0459 strain was significantly lower than those of the SC-19 and C Δ hp0459 strains (Figure 4). This finding suggested that HP0459 plays an important role in the *S. suis*-induced pro-inflammatory response *in vitro*.

The role of HP0459 *in vivo* in the *S. suis*-induced pro-inflammatory response was then assessed using an experimental infection model in C57BL/6 mice. Bacteriological isolation from the blood and part of the spleen showed that, compared with the SC-19 and C Δ hp0459 strains, the bacterial content of the Δ hp0459 strain was significantly increased (Figure 5). At the same time, the lungs and another part of the spleen from infected and control mice were separated to extract the total RNA, and the mRNA levels of IL-1 β and TNF- α were measured by qPCR. The results indicated that the mRNA levels of IL-1 β and TNF- α induced by the Δ hp0459 strain *in vivo* were significantly lower than those induced by the SC-19 and C Δ hp0459 strains (Figure 6). Thus, the HP0459 protein plays an important role in the *S. suis*-induced pro-inflammatory response.

HP0459 Protein Induced the Expression of IL-1 β , MCP-1 and TNF- α by TLR2

To determine the recognition receptor responsible for the HP0459-mediated induction of cytokines, antibody blocking assays were performed. Compared with the positive control, anti-TLR2 antibody could significantly reduce the expression of

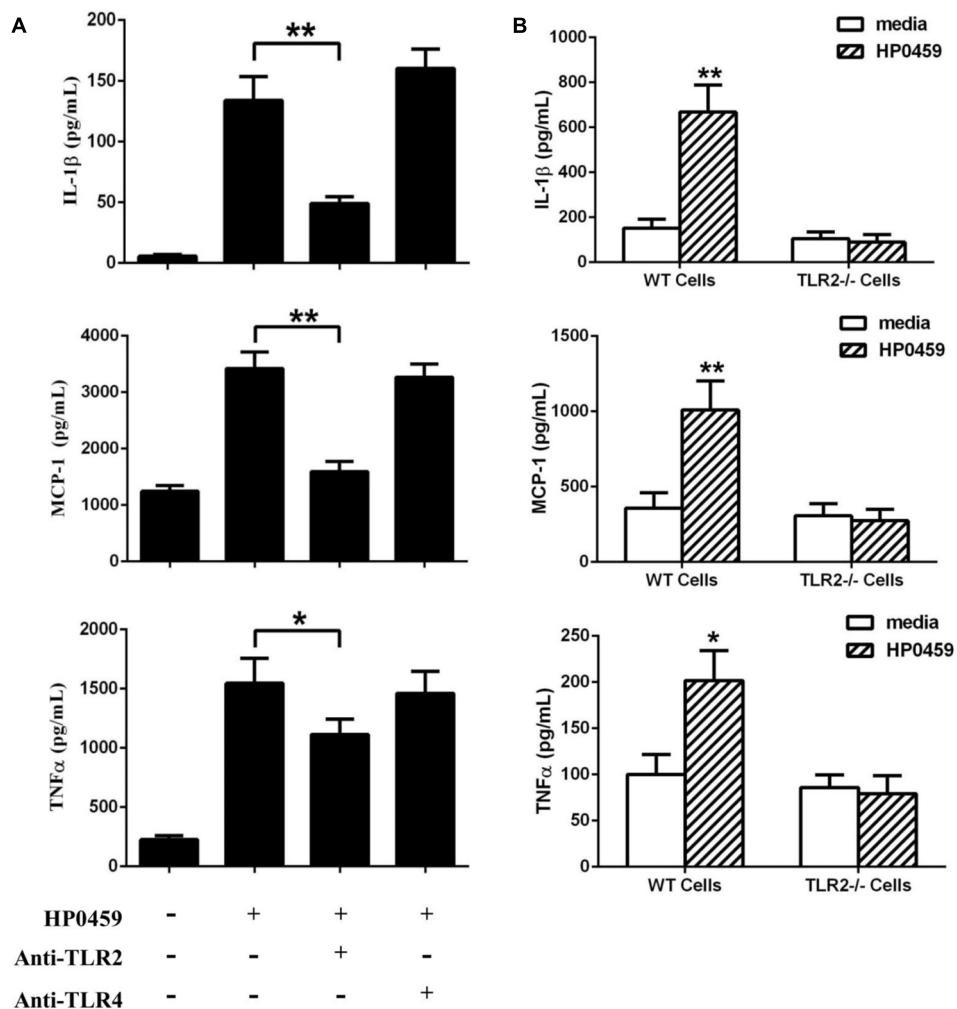


FIGURE 7 | Recognition receptor of the HP0459-stimulated pro-inflammatory response. (A) Antibody blocking assays. After pretreatment with 8 μ g of anti-TLR2 and anti-TLR4 antibody for 30 min, RAW 264.7 macrophages were incubated with 10 μ g ml $^{-1}$ HP0459 for 10 h. The expression levels of various cytokines were then determined by ELISA.

(B) Primary peritoneal macrophages were isolated from TLR2 $^{−/−}$ and WT mice and incubated with 10 μ g ml $^{-1}$ HP0459 for 10 h. The induction of IL-1 β , MCP-1 and TNF- α in the supernatants was then determined by ELISA. The bars represent the SEs of the means, based on three independent experiments. * $P < 0.05$, ** $P < 0.01$.

IL-1 β , MCP-1 and TNF- α induced by HP0459, whereas anti-TLR4 antibody could not (Figure 7A). The results showed that the HP0459-induced cytokine secretion may depend on TLR2. To verify this result, TLR2 $^{−/−}$ and WT macrophages were isolated from TLR2 $^{−/−}$ and WT mice, respectively, and were incubated with HP0459. The results show that HP0459 could significantly induce cytokine secretion in WT macrophages but not in TLR2 $^{−/−}$ cells (Figure 7B), demonstrating that the HP0459 protein can induce a TLR2-dependent pro-inflammatory response.

HP0459-Induced Cell Signal Transduction Pathways in RAW264.7 Cells

Next, to further elucidate the mechanisms through which the HP0459 protein induced cytokine secretion, we investigated the cell signal transduction pathways in HP0459-stimulated

RAW264.7 cells. RAW264.7 cells were pretreated with specific inhibitors of several cell signal pathways for 30 min and were then incubated with HP0459 for 10 h. The levels of IL-1 β , MCP-1, and TNF- α in the supernatants were quantified by ELISA. As shown in Figure 8A, the ERK 1/2 MAPK inhibitor (U0126) significantly decreased the HP0459-induced cytokine production, and the NF- κ B inhibitor (PDTC) induced a lower degree of reduction. This result suggested that the HP0459-induced cytokine production likely primarily depends on the phosphorylation of ERK 1/2 MAPK. To verify this hypothesis, we performed a western blotting analysis to measure the phosphorylation of ERK 1/2 MAPK and NF- κ B in RAW264.7 cells induced by HP0459 stimulation (Figure 8B). The results showed that the phosphorylation of ERK 1/2 MAPK was significantly enhanced, whereas the phosphorylation of NF- κ B was slight. In addition, a western blot analysis with anti-actin antibody was

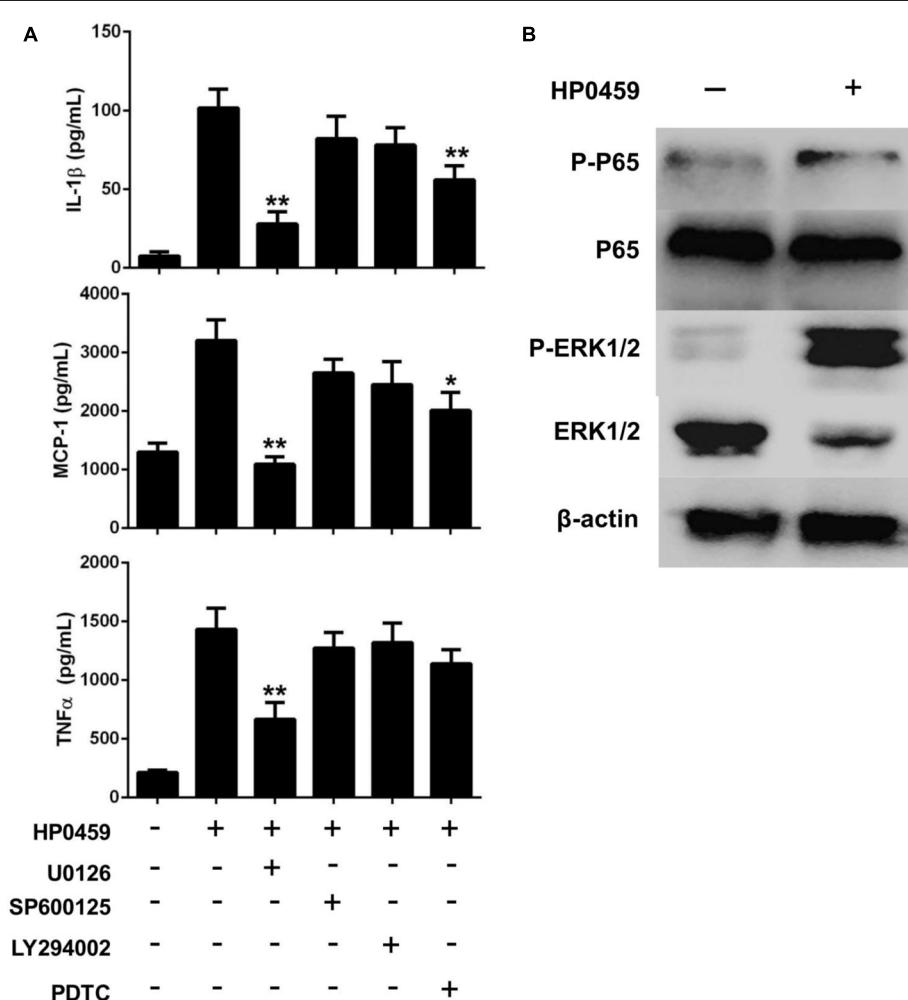


FIGURE 8 | Signal transduction pathways of the HP0459-stimulated pro-inflammatory response in RAW 264.7 macrophages. **(A)** Effect of ERK 1/2 (U0126), JNK (SP600125), PI3K (LY294002) and NF- κ B (PDTc) inhibitors on cytokine production during HP0459 stimulation. After incubation with the inhibitors for 30 min, RAW 264.7 macrophages were stimulated with 10 μ g ml $^{-1}$ HP0459 for 10 h. The cytokine levels were then determined by ELISA. Data are expressed as the mean \pm SD of three independent experiments.

(B) HP0459-induced phosphorylation of ERK 1/2 MAPK in RAW264.7 macrophages. RAW264.7 macrophages were stimulated with HP0459 (10 μ g ml $^{-1}$) for 10 h. The cell lysates were analyzed by western blotting using specific antibodies against ERK 1/2 MAPK, phospho-ERK 1/2 MAPK, NF- κ B and phospho-NF- κ B. At the same time, β -actin was assessed as an internal control using anti- β -actin antibody. Results shown are representative of three independent experiments. * P < 0.05, ** P < 0.01.

used to confirm that equivalent amounts of the samples were loaded into the gels. These results suggested that signal transduction pathway ERK 1/2 MAPK play a primary role in the pro-inflammatory response induced by HP0459 stimulation in RAW264.7 cells.

Discussion

Due to the high prevalence of *S. suis*-induced diseases in humans in Southeast and East Asia, pathogen has been increasingly investigated (Fittipaldi et al., 2012). Although the pathogenesis of *S. suis* infection is not entirely known at present, several viewpoints have been generally recognized, and one of these is that inflammation plays an important role in *S. suis* infection (Segura et al.,

2006). After pathogen invasion into a host, the innate immune system of the host will recognize key molecular signatures borne by PAMPs to activate the inflammatory response in order to clear pathogens (Martinon and Tschoop, 2005). However, during *S. suis* infection, the inflammatory response is excessively activated and thus plays an important role in most clinical signs of *S. suis* disease, including meningitis, septicemia and sudden death (Dominguez-Punaro Mde et al., 2008). Thus, it is significant to identify the pro-inflammatory molecules of *S. suis* in order to understand its pathogenesis. In this study, it was found that HP0459 could significantly activate inflammatory response in RAW264.7 cells, but heat-killed HP0459 couldn't (data not shown). Further, we elucidated the mechanism of HP0459 inducing inflammation. This result contributes to the understanding of the excessive inflammation induced by *S. suis*.

During *S. suis* infection, this pathogen can be recognized by some receptors, including CD14, TLR2, TLR6, and TLR9. CD14 has been considered one of the recognition receptors of LPS, the major component of the outer membrane of Gram-negative bacteria (Wright et al., 1990), and CD14 has been shown to be important in the recognition of cell wall constituents of Gram-positive bacteria (Wright, 1995; Moreillon and Majcherczyk, 2003). A previous study proved that *S. suis* can mediate CD14-dependent cytokine and chemokine production by human monocytes (Segura et al., 2002). Because CD14 lacks transmembrane and intracellular domains, it is not able to transduce the signal by itself. Thus, to activate the CD14-dependent inflammatory response, additional transmembrane receptors, such as TLR2, are required (Manukyan et al., 2005). TLR2, as a major pattern recognition receptor for ligands derived from Gram-positive bacteria (Kawai and Akira, 2005), has been shown to play an important role during *S. suis* ST1 strain interactions with mouse, swine and human cells (Graveline et al., 2007; Zheng et al., 2011, 2012). In addition, it has been indicated that not only TLR2 but also TLR6 and TLR9 play an important role on cell activation through *in vitro* studies carried out with the whole cells of the epidemic ST7 strain and human peripheral blood cells (Zheng et al., 2012). In the present study, using antibody blocking and TLR2^{-/-} mouse macrophages assays, we found that TLR2 plays an important role in the inflammatory response activated by HP0459. This is consistent with *S. suis*-induced pro-inflammatory response, which is primary TLR2-dependent. However, LipoP prediction of HP0459 displayed that the signal peptide of HP0459 belonged to SpI (lipoprotein signal peptide belonged to SpII; Hutchings et al., 2009). This result suggested that the recognition of HP0459 may be different from bacterial lipoproteins. So, the mechanisms of HP0459 recognized by TLR2 need further research. We have proved that HP0459 contributes to pro-inflammatory response during *S. suis* infection. And it is known that there is a close link between excessive inflammation and the development of *Streptococcus* toxic shock syndrome (STSS; Zhao et al., 2011). This implies that HP0459

may be responsible for the pathogenesis of STSS caused by *S. suis* 2.

Compared with the SC-19 strains, the pro-inflammatory activation of the Δ hp0459 strain *in vivo* was found to be significantly reduced in the present study. However, the mouse experiment showed that the lethality of the SC-19 and Δ hp0459 strains were not significant different after challenge with the same CFUs (data not shown). To explain this result, we performed bacteriological isolation from the blood and spleen, and compared with the SC-19 and Δ hp0459 strains, the bacteria content of the Δ hp0459 strain was significantly increased (Figure 5). It is known that higher bacterial counts of pathogenic bacteria *in vivo* may be an important cause leading to disease worsening (Sullivan et al., 1982). Thus, this finding suggests that inflammation is mainly but not exclusively responsible for the pathology of *S. suis* and that the bacteria content also plays an important role.

Finally, our data identified a novel pro-inflammatory protein denoted HP0459 from *S. suis*. Further, we demonstrated that HP0459 induces a TLR2-dependent pro-inflammatory response in RAW 264.7 macrophages via activation of the ERK1/2 pathway. These findings could be important for improving our understanding of the excessive inflammation induced by *S. suis* and may aid the development of therapies against SS2 infection.

Acknowledgments

The authors are grateful to the State Key Laboratory of Agricultural Microbiology (Wuhan, China) for supporting this study. This work was supported by the High-Tech Research and Development Program (863) of China (2011AA10A210), the National Basic Research Program (973) of China (2012CB518805), the Chinese Major Special Science and Technology Project (2012ZX10004214-005) the Special Fund for Public Welfare Industry of Chinese Ministry of Agriculture (201303041), and the Fundamental Research Funds for the Central Universities (2011PY006).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Interaction of Bacterial Exotoxins with Neutrophil Extracellular Traps: Impact for the Infected Host

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OPEN ACCESS

Edited by:

Katharina F. Kubatzky,
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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 29 January 2016

Accepted: 14 March 2016

Published: 30 March 2016

Citation:

von Köckritz-Blickwede M,
Blodkamp S and Nizet V (2016)
*Interaction of Bacterial Exotoxins with
Neutrophil Extracellular Traps: Impact
for the Infected Host.*
Front. Microbiol. 7:402.
doi: 10.3389/fmicb.2016.00402

Since their discovery in 2004, neutrophil extracellular traps (NETs) have been characterized as a fundamental host innate immune defense against various pathogens. Released in response to infectious and pro-inflammatory stimuli, NETs can immobilize invading pathogens within a fibrous matrix consisting of DNA, histones, and antimicrobial peptides. Conversely, excessive or dysregulated NET release may hold a variety of detrimental consequences for the host. A fine balance between NET formation and elimination is necessary to sustain a protective effect during infectious challenge. In recent years, a number of microbial virulence factors have been shown to modulate formation of NETs, thereby facilitating colonization or spread within the host. In this mini-review we summarize the contemporary research on the interaction of bacterial exotoxins with neutrophils that modulate NET production, focusing particular attention on consequences for the host. Understanding host-pathogen dynamics in this extracellular battlefield of innate immunity may provide novel therapeutic approaches for infectious and inflammatory disorders.

Keywords: phagocyte extracellular traps, neutrophils, macrophages, leukocidin, cell death

NEUTROPHIL EXTRACELLULAR TRAPS

The formation of neutrophil extracellular DNA traps (so called NETs) was first recognized as a host innate immune defense mechanism against infections by Brinkmann et al. (2004). This discovery altered the fundamental conception of the innate immune function of phagocytes against pathogenic microbes in a most fascinating way. Whereas it was previously believed that neutrophils kill invading pathogens by intracellular uptake (phagocytosis) and subsequent killing, the discovery of NETs revealed an additional phagocytosis-independent mechanism. The released nuclear material including histones and DNA within the extracellular trap (ET) immobilize and occasionally kill several medically important bacteria, viruses, or parasites (Brinkmann et al., 2004).

Regarding the cellular pathways that lead to NET formation, most studies show that the cell in question undergoes NETosis, a process of death that differs morphologically from necrosis and apoptosis (Fuchs et al., 2007). In contrast to apoptotic or necrotic cells, cells that undergo NETosis show a prelytic decondensation of the chromatin associated with the disruption of the nuclear membrane. This disintegration of the nuclear membrane allows mixing of DNA and histones with granule components, which are then extracellularly released as web-like fibers with a diameter of approximately 15 to 17 nm, associated with globular proteins or antimicrobial peptides.

NET-based antimicrobial activity remains active when cells are treated with the actin microfilament inhibitor cytochalasin D, indicating that this phenomenon is independent of phagocytosis. However, the antimicrobial activity of NETs can be abolished when NETs are treated with DNase, confirming that DNA comprises the functional backbone of the released fibers (Brinkmann et al., 2004; Fuchs et al., 2007).

The formation of NETs was initially thought to be an antimicrobial defense strategy and cell death pathway specific for neutrophils (Brinkmann et al., 2004). However, von Köckritz-Blickwede et al. (2008) mast cells were shown to also deploy mast cell extracellular traps (MCETs) in defense against bacterial pathogens. During recent years, further evidence has accumulated demonstrating ET formation occurs in eosinophils (Yousefi et al., 2008), basophils (Schorn et al., 2012), fibrocytes (Kisseleva et al., 2011), macrophages (Bartneck et al., 2010; Chow et al., 2010), and monocytes (Bartneck et al., 2010; Chow et al., 2010).

It is important to mention that some authors have demonstrated experimentally that eosinophils and neutrophils can release antimicrobial ETs in response to infection while remaining in a viable status (Yousefi et al., 2008, 2009). NET release by viable cells was confirmed *in vivo* in a murine model of *Staphylococcus aureus* skin infection (Yipp et al., 2012). Here, NET formation was seen as a dynamic process, which occurs by vesicular release of nuclear material during migration of neutrophils through the tissue. It is still unclear, however, how such budding and final release of nuclear material is initiated. Thus, based on current knowledge it seems that at least two different mechanisms can lead to NET formation: a viable form involving vesicular release and the more well-understood NETosis form associated with rupture of the nuclear membrane. Research has begun to explore how certain drugs, including statins (Chow et al., 2010) and tamoxifen (Corriden et al., 2015), modulate neutrophil functions to accentuate NET formation, which could be beneficial as an adjunctive therapy for extracellular pathogens such as *S. aureus* that efficiently avoid phagocytic killing by neutrophils.

In contrast to their protective effect against several infections, there is increasing evidence that a dysregulated NET release can provoke autoimmune reactions, tissue damage and impaired cellular functions (Villanueva et al., 2011; Saffarzadeh et al., 2012). Unchecked, aberrant NET formation can result in pathological damage as vascular thrombosis (Fuchs et al., 2010) or chronic lung inflammation in cystic fibrosis (Papayannopoulos et al., 2011). Countermeasures to the excessive NET formation may be of therapeutic utility in such cases as for example DNase treatment in patients with cystic fibrosis (Fuchs et al., 1994) or the usage of anti-histone antibodies to alleviate vascular thrombosis (Semeraro et al., 2011). Since sustained blockade of NET formation may carry a risk of increased susceptibility to certain infections, anti-NET therapy might be targeted to severe autoimmune or inflammatory diseases, when pro-inflammatory activities of NETs outweigh their protective benefits (Saffarzadeh and Preissner, 2013).

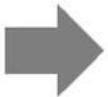
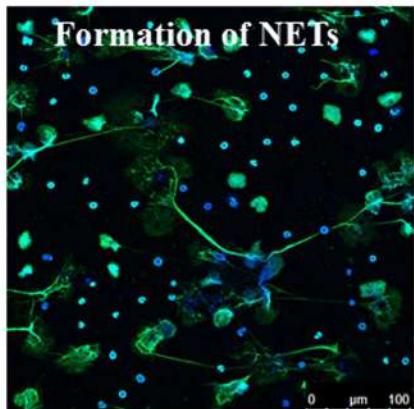
THE ROLE OF NETs AGAINST INFECTION

In several studies, NETs were found to play a protective role against various infecting organisms, often employing mutagenesis of key evasion factors of the respective organisms. It is thought that NETs can act in two ways: (1) pathogen immobilization and (2) growth inhibition or killing of the microbe. The relative importance of killing is a point of some contention in the literature (Menegazzi et al., 2012). However, based on vital immunofluorescent staining of bacteria with DNA intercalating dyes, microbial killing by NETs has been demonstrated definitively by several authors (Lauth et al., 2009; Berends et al., 2010; Halverson et al., 2015). However, since the overall killing potential of NETs is physically restricted, the cumulative effect of NETs may be functionally bacteriostatic (Baums and von Köckritz-Blickwede, 2015).

Because some bacteria have evolved highly efficient resistance strategies against antimicrobial activity of NETs, in some specific circumstances, NETs can help to establish an infectious niche. For example, in the case of a primary influenza A infection of the middle ear which boosts formation of NETs by infiltrating neutrophils, resistant *Streptococcus pneumoniae* can use those NETs to multiply and persist in the middle ear cavity (Short et al., 2014). Additionally, some studies show that *S. pneumoniae* and *Haemophilus influenzae* can incorporate NETs into biofilms that promote their persistence in the host (Hong et al., 2009; Reid et al., 2009). In the end, it depends on the pathogen, its array of immune resistance factors and the anatomical site of infection, as to whether NETs can serve a protective function for the host during an infection (**Figure 1**).

CATEGORIES OF BACTERIAL NET EVASION FACTORS

Neutrophil extracellular trap evasion factors can be classified in three different phenotypes: (1) NET degradation, (2) resistance to the intrinsic antimicrobial effectors within NETs, or (3) the suppression of NET production (von Köckritz-Blickwede and Nizet, 2009). The best-studied NET evasion factor is the activity of microbial nucleases that degrade NETs deployed by innate immune cells to escape entrapment. Membrane-bound or released nucleases have been shown to be expressed by the following microbes as NET evasion factors: *Aeromonas hydrophila* (Brogden et al., 2012), *Escherichia coli* (Möllerherm et al., 2015), *Leptospira* sp. (Scharrig et al., 2015), *Neisseria gonorrhoeae* (Juneau et al., 2015), *S. aureus* (Berends et al., 2010), *S. agalactiae* (Derré-Bobillot et al., 2013), *S. pneumoniae* (Beiter et al., 2006), *S. pyogenes* (Buchanan et al., 2006; Chang et al., 2011), *Streptococcus syringinis* (Morita et al., 2014), *S. suis* (de Buhr et al., 2014), *Vibrio cholera* (Seper et al., 2013), and *Yersinia enterocolitica* (Möllerherm et al., 2015). Whereas, resistance to antimicrobial effectors in ETs is mainly mediated by surface-bound virulence factors, e.g., exopolysaccharide capsules in case of *S. pyogenes* (Cole et al., 2010) or *S. pneumonia* (Wartha et al., 2007), the suppression of NET formation is mainly carried



Protective immune defense of NETs against infections with:

- *Aeromonas hydrophila* (Brogden et al., 2014)
- *Aspergillus fumigatus* (Röhm et al., 2014)
- *Borrelia burgdorferi* (Menten-Dedoyart et al., 2012)
- *Candida albicans* (Urban et al., 2009)
- *Eimeria bovis* (Behrendt et al., 2010)
- *Escherichia coli* (Grinberg et al., 2008)
- *Group B Streptococcus* (Carlin et al., 2009)
- *Human immunodeficiency virus* (Saitoh et al., 2012)
- *Leishmania amazonensis* (Guimarães-Costa et al., 2009)
- *Leishmania donovani* (Gabriel et al., 2010)
- *Mannheimia haemolytica* (Aulik et al., 2010)
- *Mycobacterium tuberculosis* (Ramos-Kichik et al., 2009; Wong and Jacobs, 2013)
- *Myxoma virus (MYXV)* (Jenne et al., 2013)
- *Plasmodium falciparum* (Baker et al., 2008)
- *Pseudomonas aeruginosa* (Young et al., 2011)*
- *Salmonella typhimurium* (Brinkmann et al., 2004)
- *Shigella flexneri* (Brinkmann et al., 2004)
- *Staphylococcus aureus* (Berends et al., 2010)*
- *Streptococcus pneumoniae* (Beiter et al., 2006) *
- *Streptococcus pyogenes* (Lauth et al., 2009)
- *Streptococcus suis* (de Buhr et al., 2014)
- *Toxoplasma gondii* (Abi Abdallah et al., 2012)
- *Vibrio cholerae* (Seper et al., 2013)

Detimental consequences of NETs for the host during infections with:

- *Feline leukemia virus* (Wardini et al., 2010)
- *Haemophilus influenzae* (Hong et al., 2009)
- *Influenza A virus* (Narasaraju et al., 2011)
- *Plasmodium falciparum* (Baker et al., 2008)
- *Pseudomonas aeruginosa* (Fuxman et al., 2010)*
- *Staphylococcus aureus* (Gonzalez et al., 2014)*
- *Streptococcus pneumoniae* (Reid et al., 2009) *

FIGURE 1 | Extracellular trap formation during infections: consequences for the host. The figure summarizes the protective versus detrimental role of NETs for the host during an infection with respective pathogens. In some cases (highlighted with an asterisk), there is benefit and harm depending on site, timing and magnitude of infection. The immunofluorescence micrograph is showing the release of NETs from human blood-derived neutrophils. NETs were stained with antibodies against histone-DNA complexes (green) and DAPI to stain the nuclei and nuclear DNA (blue).

out by released factors including proteases (Zinkernagel et al., 2008).

BACTERIAL EXOTOXINS THAT MODULATE NET FORMATION

During the last 10 years, there is increasing evidence in the literature that different exotoxins released by Gram-positive or Gram-negative bacteria are able to modulate the process of NET formation (**Table 1**): to date, at least 11 different exotoxins have been published to modulate NET formation, with nine of them reported to enhance NET formation while two suppress the process (**Table 1**). First we will set our attention on those toxins that induce NET formation.

The M1-protein of the Gram-positive bacterium *S. pyogenes*, also known as group A *Streptococcus* (GAS), was the first exotoxin described to induce formation of ETs by neutrophils and mast cells (Lauth et al., 2009). The surface-anchored M1 protein is a classical virulence factor that promotes resistance to phagocytosis. However, after proteolytic release from the

S. pyogenes surface, the M1 protein forms a proinflammatory supramolecular network with fibrinogen that contributes to the pathophysiology of streptococcal toxic shock-like syndrome (Herwald et al., 2004). Two parallel studies showed in 2009, that the M1 protein is able to mediate formation of NETs as well as MCETs, and thereby contribute to entrapment and killing of the bacteria (Lauth et al., 2009; Oehmcke et al., 2009). Besides induction of ETs, M1 protein promotes resistance to the human cathelicidin antimicrobial peptide LL-37, an important effector of bacterial killing by neutrophils and mast cells (Lauth et al., 2009; LaRock et al., 2015).

Staphylococcus epidermidis is a common colonizer of healthy human skin and is hypothesized to play a mutually beneficial role in the cutaneous niche. Cogen et al. (2010) demonstrated that *S. epidermidis* phenol-soluble-modulin γ (δ-toxin) boosts NET production and further colocalizes with endogenous host antimicrobial peptides within the architecture of the expressed NETs. In antimicrobial assays against the pathogen *S. pyogenes*, δ-toxin cooperated with host-derived antimicrobial peptides cathelicidins (human LL-37 and murine CRAMP) and human beta-defensins (hBD2 and hBD3) to

TABLE 1 | Effect of bacterial exotoxins on formation of extracellular traps.

Microbe	Exoprotein	Effect on cells	Mechanism	Cell death	Putative consequences for the host	Reference
<i>Bordetella pertussis</i>	Adenylate cyclase toxin (ACT)	Suppression of NET formation and apoptosis	Generation of cAMP and inhibition of oxidative burst	Impairment of cell lysis	ACT promotes neutrophil infiltration by inhibiting neutrophil death	Eby et al., 2014
<i>Escherichia coli</i>	Hemolysin	Induction of ETs in murine and human macrophages	Not known	Not known	Not known	Aulik et al., 2012
<i>Mannheimia haemolytica</i>	Leukotoxin	Induction of ETs in bovine neutrophils, as well as bovine, murine, and human macrophages	CD18- and NADPH-oxidase dependent	Delayed LDH release, no necrosis, no apoptosis	Trapping and killing of <i>M. haemolytica</i>	Aulik et al., 2010, 2012
<i>Mycobacterium tuberculosis</i>	Early secretory antigen-6 protein	Induction of secondary necrosis and ET formation of phosphatidylserine externalized neutrophils	Intracellular Ca ²⁺ overload	Cell lysis	Necrotic granulomas during tuberculosis	Francis et al., 2014
<i>Pseudomonas aeruginosa</i>	Pyocyanin	Induction of NETs	NADPH- oxidase- and Jun N-terminal kinase-dependent	Cell lysis	Inflammatory condition during cystic fibrosis	Rada et al., 2013
<i>Staphylococcus aureus</i>	N-terminal ArgD peptides	Induction of NETs	Unknown	Cell lysis	Aggravation of skin lesions	Gonzalez et al., 2014
<i>Staphylococcus aureus</i>	Leukotoxin GH	Induction of NETs	Non-specific cytolysis	Non-specific cytolysis	Entrapment of <i>S. aureus</i>	Malachowa et al., 2013
<i>Staphylococcus aureus</i>	Panton-Valentin-leukocidin	Induction of rapid nuclear NET formation	Vesicular release of nuclear DNA, independent of NADPH- oxidase	No cell lysis	Entrapment of <i>S. aureus</i>	Pilsczek et al., 2010
<i>Staphylococcus epidermidis</i>	Phenol-soluble-modulin γ (δ-toxin)	Induces NET formation and colocalizes with NETs and host antimicrobial peptides	Physically binding to host derived antimicrobial peptides and DNA	Cell lysis	Cooperates with host antimicrobial peptides against bacterial pathogens	Cogen et al., 2010
<i>Streptococcus pyogenes</i>	M1 protein	Induces formation of NETs and MCETs	Unknown	Cell lysis	Trapping and killing of <i>S. pyogenes</i>	Lauth et al., 2009; Oehmcke et al., 2009
<i>Streptococcus pyogenes</i>	Streptolysin O	Suppression of NET formation	Impairment of oxidative burst	Sublytic concentrations	Severe infection	Uchiyama et al., 2015

kill the pathogenic streptococci. Coimmunoprecipitation and tryptophan spectroscopy demonstrated direct binding of δ-toxin to LL-37, CRAMP, hBD2, and hBD3, as well as DNA. These data suggest that commensal *S. epidermidis*-derived δ-toxin cooperates with the host-derived antimicrobial peptides in the innate immune system to reduce survival of an important human bacterial pathogen. This could be corroborated in a mouse wound model where *S. pyogenes* survival was reduced when the wounds were pretreated with δ-toxin. These data described a novel therapeutic application of a bacterial toxin against pathogenic bacteria to enhance formation of NETs and support the host innate immune system.

Mannheimia haemolytica leukotoxin (LKT) induces formation of extracellular traps in bovine neutrophils and macrophages (Aulik et al., 2010, 2012). LKT is a member of the repeats-in-toxin (RTX) family of exoproteins produced by a wider variety of Gram-negative bacteria. In addition to the native fully active LKT, its non-cytolytic pro-LKT precursor also stimulated macrophage extracellular traps (METs) formation. Formation of METs in response to LKT required NADPH oxidase activity, as previously demonstrated for NETosis. METs

induced in response to LKT trapped and killed a portion of the toxin-producing *M. haemolytica* cells. In contrast to NETosis, which is reported to occur between 10 min to 4 h after pathogen exposure, LKT-induced MET formation was extremely rapid, with significant accumulation of extracellular DNA within 2 min after macrophage stimulation.

Interestingly, the LKT-mediated cytotoxic effects are specific to ruminants, since LKT binds to amino acids 5–17 of the signal sequence of CD18, which is not present on mature leukocytes from humans and other mammalian species (Shanthalingam and Srikumaran, 2009). CD18 was confirmed by Aulik et al. (2010) to play a role in LKT-dependent NET formation in bovine phagocytes. Similarly, to the LKT, the related RTX family toxin, uropathogenic *E. coli* (UPEC) hemolysin, induced NET formation in the mouse and human monocyte/macrophage cell lines indicating that NET induction may be a general phenomenon in response to active RTX toxins (Aulik et al., 2012). At this point it remains difficult to assess whether NET production in response to RTX toxins leads to a protective host defense against *M. haemolytica* or other Gram-negative bacteria. In the case of LKT, bacterial cells entrapped in NETs can continue to

secrete LKT, an important virulence determinant, which could exacerbate lung inflammation (Aulik et al., 2010, 2012).

The *Pseudomonas aeruginosa* virulence factor pyocyanin stimulates the release of NETs in a NADPH oxidase-dependent manner (Rada et al., 2013). Pyocyanin is a redox-active pigment associated with diminished lung function in cystic fibrosis. In cystic fibrosis airways, *P. aeruginosa* resides in biofilms protected from neutrophil phagocytic activity or from entrapment by NETs (Rada et al., 2013). The authors speculate that enhanced ROS-dependent NET formation by *P. aeruginosa* pyocyanin contributes to inflammatory conditions observed in chronically infected cystic fibrosis airways. Parenthetically, chronic granulomatous disease (CGD) patients, whose neutrophils are unable to make NETs, are not recognized to be disproportionately susceptible to infections with *Pseudomonas* species (Rada and Leto, 2008).

A specific activity of an exotoxin was shown for the *Mycobacterium tuberculosis* leukocidin ESAT-6, which induced NET formation only in a subpopulation of neutrophils with externalized phosphatidylserine as a marker for apoptosis (Francis et al., 2014). Thus, ESAT-6 induced NET formation was similar to secondary necrosis of pre-activated neutrophils and dependent on Ca^{2+} . In this case, increased NET formation was also speculated to contribute to virulence of tuberculosis, since it was recently observed that the development of necrotic granulomas in a mouse model of progressive tuberculosis were associated with the presence of extracellular bacteria, neutrophil necrosis and NET-like structures (Francis et al., 2014).

In the case of the leading human pathogen *S. aureus*, three different exotoxins enhance the release of NETs: N-terminal AgrD peptides (Gonzalez et al., 2014), leukotoxin GH (Malachowa et al., 2013), also known as LukAB (Dumont et al., 2011), thus named here LukGH/AB, and Panton-Valentin leukocidin (PVL; Pilsczek et al., 2010). AgrD is the precursor for the auto-inducing peptide in a well-known quorum sensing system regulating virulence phenotypes of *S. aureus*. A recent mass spectrometry-based study identified formylated and non-formylated peptide variants derived from AgrD N-terminal leader domain in *S. aureus* cell-free culture supernatant to act cytotoxic, modulate neutrophil chemotaxis and induce formation of NETs. As a consequence a detrimental effect for the host was hypothesized as indicated by aggravation of skin lesions *in vivo* in a murine model. The cellular pathways mediating this process of AgrD induced NET formation remain unclear (Gonzalez et al., 2014).

Staphylococcus aureus LukGH/AB, also promotes formation of NETs in association with death of the neutrophil (Malachowa et al., 2013). LukGH/AB is a pore-forming cytoytic toxin with proinflammatory properties, similar to those established for PVL. But unlike PVL, LukGH/AB did not prime human neutrophils for increased production of reactive oxygen species nor did it enhance binding and/or uptake of *S. aureus*. LukGH/AB promoted the release of NETs, which in turn ensnared but did not achieve killing of *S. aureus*. These authors found that electroporation of human neutrophils, used to create pores in the neutrophil plasma membrane,

induced NET formation in a similar way. This finding indicated that NETs can be generated during non-specific cytosis.

The above phenomenon stands in contrast to NET formation initiated by PVL production in *S. aureus*. Pilsczek et al. (2010) demonstrated a novel rapid (5–60 min) process of NET formation that did not require cell lysis or even breach of the plasma membrane. The authors show that neutrophils treated with *S. aureus* supernatant show rounded and condensed nuclei, followed by the separation of the inner and outer nuclear membrane and budding of vesicles filled with nuclear DNA. The vesicles are extruded intact into extracellular space where they rupture, release the chromatin and form NETs. PVL was identified as one key effector of NET formation present in the *S. aureus* supernatant, but other yet unidentified molecules aside from PVL cannot be excluded as contributors to NET production. This rapid process of NET formation against *S. aureus* was dubbed “dynamic NETosis” and was recently proven to serve as an efficient host defense strategy during *S. aureus* skin infections in man and mice (Yipp et al., 2012). Since, PVL targets a different receptor on the neutrophil compared to the LukGH/AB, namely the C5aR receptor (Spaan et al., 2013) and the CD11b receptor (Dumont et al., 2013), respectively, it still remains to be determined if the receptor-mediated signaling leads to the different NET phenotypes.

Two toxins have been described so far to suppress NET formation, perhaps representing an immune evasion strategy by the respective pathogens: *Bordetella pertussis* adenylate cyclase toxin (Eby et al., 2014) and *S. pyogenes* streptolysin O (SLO; Uchiyama et al., 2015). Both mechanisms are coupled to suppression of neutrophil oxidative burst. In case of *S. pyogenes* SLO, this mechanism plays a key role of SLO in the pathogen’s resistance to immediate neutrophil killing. In case of *B. pertussis*, the relevance of NET inhibition by the toxin is still unclear, since it is not known whether or not NETs are able to entrap and clear *B. pertussis*. This study highlighted how convalescent phase serum from humans following clinical pertussis blocked the ACT-mediated suppression of NET formation (Eby et al., 2014). Based on this finding, the authors mention that their data should alert investigators to the ability for ACT, and antibodies to ACT, to dysregulate neutrophil death mechanisms and potentially influence local tissue damage during infection with *B. pertussis*.

CONCLUDING COMMENTS

With increasing publications in the NET field it is well-known that NETs are on one hand protective against several infections, but may also result in detrimental effects when released in excessive amount. The complexity may be compared with the release of cytokines during infection and inflammation, which serve an essential role in an efficient immune response of the host against infections, but in which overwhelming cytokine storm can lead to septic shock and accelerate death of the host. The cumulative balance between NET formation and NET degradation – similar to cytokine release – defines the

protective versus detrimental effects on the organism. Currently, it is under discussion in the literature if and how NETs can act as a novel therapeutic or prophylactic target for boosting immunity to bacterial infections or mitigating inflammatory diseases associated with detrimental NET formation. To rationally and effectively pharmacologically interfere with the process of NET formation, the cellular processes mediating this phenomenon need to be understood more in detail. Identification of bacterial factors, especially exotoxins, with specific roles in NET modulation, may serve as probes to understand the molecular basis of NET generation and antimicrobial activity. Furthermore, exotoxins might themselves harbor therapeutic potential when their effects against the host cells are fully characterized.

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AUTHOR CONTRIBUTIONS

SB collected the literature, MK-B and VN wrote the manuscript. All authors have proofread the manuscript.

ACKNOWLEDGMENTS

SB was funded by a fellowship of the Ministry of Science and Culture of Lower Saxony (Georg-Christoph-Lichtenberg Scholarship) within the framework of the Ph.D. program ‘EWI-Zoonoses’ of the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology, and Translational Medicine (HGNI).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Streptolysin O Rapidly Impairs Neutrophil Oxidative Burst and Antibacterial Responses to Group A Streptococcus

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the
journal Frontiers in Immunology

Received: 01 September 2015

Accepted: 30 October 2015

Published: 16 November 2015

Citation:

Uchiyama S, Döhrmann S, Timmer AM, Dixit N, Ghochani M, Bhandari T, Timmer JC, Sprague K, Bubeck-Wardenburg J, Simon SI and Nizet V (2015) Streptolysin O Rapidly Impairs Neutrophil Oxidative Burst and Antibacterial Responses to Group A Streptococcus. *Front. Immunol.* 6:581.
doi: 10.3389/fimmu.2015.00581

Group A Streptococcus (GAS) causes a wide range of human infections, ranging from simple pharyngitis to life-threatening necrotizing fasciitis and toxic shock syndrome. A globally disseminated clone of M1T1 GAS has been associated with an increase in severe, invasive GAS infections in recent decades. The secreted GAS pore-forming toxin streptolysin O (SLO), which induces eukaryotic cell lysis in a cholesterol-dependent manner, is highly upregulated in the GAS M1T1 clone during bloodstream dissemination. SLO is known to promote GAS resistance to phagocytic clearance by neutrophils, a critical first element of host defense against invasive bacterial infection. Here, we examine the role of SLO in modulating specific neutrophil functions during their early interaction with GAS. We find that SLO at subcytotoxic concentrations and early time points is necessary and sufficient to suppress neutrophil oxidative burst, in a manner reversed by free cholesterol and anti-SLO blocking antibodies. In addition, SLO at subcytotoxic concentrations blocked neutrophil degranulation, interleukin-8 secretion and responsiveness, and elaboration of DNA-based neutrophil extracellular traps, cumulatively supporting a key role for SLO in GAS resistance to immediate neutrophil killing. A non-toxic SLO derivate elicits protective immunity against lethal GAS challenge in a murine infection model. We conclude that SLO exerts a novel cytotoxic-independent function at early stages of invasive infections (<30 min), contributing to GAS escape from neutrophil clearance.

Keywords: group A Streptococcus, *Streptococcus pyogenes*, pore-forming toxin, streptolysin O, neutrophils, oxidative burst, neutrophil extracellular traps, infection

INTRODUCTION

The Gram-positive bacterium group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a leading human pathogen ranked among the top 10 causes of infection-associated mortality worldwide (1). An estimate of 5–15% of all humans are colonized with GAS, which can cause a wide spectrum of infections ranging from self-limiting pharyngitis and impetigo to invasive and

life-threatening diseases including streptococcal toxic shock syndrome and necrotizing fasciitis (2). Among 700 million GAS infections worldwide annually, an estimated 1.8 million severe infections occur with a mortality rate as high as 25% (1). A commercial vaccine to protect against GAS infections is not yet available (3).

The ability of GAS to produce serious disease, sometimes in previously healthy children and adults, defines a capacity of the organism to resist clearance by frontline elements of the host innate immune system, including neutrophils, the most abundant circulating leukocytes, which are rapidly recruited to sites of bacterial infection (4, 5). GAS express an array of virulence factors that allow the bacterium to counteract neutrophil processes and molecular effectors of bactericidal activity (6), including the anti-phagocytic surface hyaluronic acid capsule and M protein (7, 8), nuclease-mediated degradation of neutrophil extracellular traps (NETs) (9, 10), factors that neutralize reactive oxygen species (ROS) (11), and inhibitors of neutrophil-derived cationic antimicrobial peptides (12). A global increase in invasive GAS infections has been attributed to the rise of a single, globally disseminated GAS M1T1 clone (13, 14). GAS M1T1 strains can undergo a spontaneous mutation in the two component global regulatory system *covR/S* (also known as *csrR/S*) *in vivo* leading to upregulation of capsule, nuclease, and other GAS virulence factors (15, 16), and neutrophil resistance may be an important selection pressure for the survival and bloodstream dissemination of these mutants (17).

In addition to these “defensive” survival properties, GAS is remarkable for its potential to induce the apoptotic cell death of neutrophils (18), a phenotype that was subsequently linked to the bacterium’s production of the potent pore-forming toxin, streptolysin O (SLO) (19). The *slo* gene has universally been found to be present in the genome of all GAS serotypes and strains, and encodes a toxin belonging to the cholesterol-dependent cytolysins (CDCs), a family comprising at least 28 members/bacterial species, including *Streptococcus pneumoniae* pneumolysin and *Listeria monocytogenes* listeriolysin O (20). SLO is a multi-functional protein with pore-dependent and -independent functions (21, 22). SLO is highly expressed by epidemic M1 GAS strains (23), and is further upregulated upon *covR/S* mutation in the GAS M1T1 background (15).

In a general sense, SLO toxicity to neutrophils has been hypothesized to contribute to GAS bloodstream survival and virulence (24, 25). Given the strong bioactivities and high expression of this toxin, as well as its prominence in invasive M1T1 GAS infection, we undertook a mechanistic analysis of its impact on specific neutrophil functions and bactericidal activity. We discovered that SLO has the capacity to suppress neutrophil oxidative burst and other key neutrophil functions including degranulation, directed migration, and formation of NETs, together promoting neutrophil resistance. Immunization with an inactivated form of SLO (rSLOmut) induced high titers of neutralizing IgG and IgM anti-SLO antibodies affording passive protection in a mouse model of systemic GAS infection, confirming its attractiveness as a component of a future multicomponent vaccine against this pathogen.

MATERIALS AND METHODS

Bacterial Strains

Group A *Streptococcus* wild-type (WT) strain M1T1 5448 was originally isolated from a patient with necrotizing fasciitis and toxic shock syndrome (26). The isogenic M1T1 5448 Δ SLO mutant and complemented (Δ SLO + pSLO) strain were described previously (19). The animal-passaged (AP) version of the M1T1 GAS parent strain contains a single inactivating adenine insertion at the 877-bp position of *covS* (16). A panel of 33 GAS isolates of various M serotypes and clinical associations was provided from collections of U.S. Centers for Disease Control and Prevention (CDC). Additional strains used were *S. pneumoniae* D39, *Staphylococcus aureus* Newman strain, and methicillin-resistant *S. aureus* (MRSA) TCH1516 WT and its isogenic Δ Hla mutant. Bacteria were cultivated in Todd-Hewitt broth (THB) at 37°C.

Isolation of Human and Murine Neutrophils

Neutrophils were isolated from freshly collected whole blood of healthy donors under a protocol approved by the University of California San Diego (UCSD) Human Research Protections Program, using PolyMorphPrep Kit (Fresenius Kabi), as previously described (27). Mice were sacrificed and blood was collected via terminal cardiac puncture under a protocol approved by the UCSD Institutional Animal Care and Use Committee. Mouse blood was diluted fivefold in phosphate-buffered saline (PBS, Cellgro) and neutrophils were isolated by layers of Histopaque-1077 and Histopaque-119 (Sigma). Remaining red blood cells (RBCs) were eliminated in lysis buffer (0.15M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA).

Oxidative Burst Assay

Oxidative burst assays were performed as previously described (28). Briefly, 2×10^6 /mL human or 2×10^5 /mL mouse neutrophils were loaded with 20 μ M 2,7-dichlorofluorescein diacetate (DCFH-DA; Fisher) in Hank’s balanced salt solution (HBSS, Cellgro) without Ca²⁺ and Mg²⁺ and incubated with rotation at 37°C for 20 min. Neutrophils were resuspended to a density of 1×10^7 cells/mL and 1×10^6 cells/well were infected at a multiplicity of infection (MOI) = 1 bacteria/cell and incubated for 20 min at 37°C/5% CO₂. Assays were performed using untreated control (control), 25 nM phorbol 12-myristate 13-acetate (PMA, Sigma) as positive control, 10 μ M diphenyleneiodonium (DPI, Sigma) to block generation of ROS, or recombinant SLO protein (rSLO), anti-SLO antibody (Abnova), or water-soluble cholesterol (Sigma) at the stated concentrations. The fluorescence intensity was quantified on a SpectraMax M3 plate reader at 485 nm excitation/520 nm emission using SoftMax Pro software.

Neutrophil Killing Assays

Neutrophils were isolated as described above, and 1×10^6 cells in 100 μ L were challenged with bacteria at MOI = 10 bacteria/cell at 37°C for 30 min with rotation. To pinpoint extracellular killing, cells were treated with 10 μ g/mL cytochalasin D (cyt.

D, Sigma) for 10 min prior to infection to inhibit phagocytosis, in the presence or absence of 500 mU/mL desoxyribonuclease (DNase, Sigma) to evaluate the contribution of DNA-based NETs to bacterial killing. Surviving colony-forming units (CFU) for all killing assays were enumerated by Todd-Hewitt agar (THA) dilution plating. Survival was calculated as the percentage of the initial inoculum.

Red Blood Cell Hemolysis Assay

Red blood cells were isolated from human blood and supernatants from bacterial cultures at various growth phases $OD_{600} = 0.3\text{--}0.7$, and tested for hemolysis activity of secreted SLO. In these assays, supernatant was incubated with 4 mM dithiothreitol (DTT) to stabilize SLO and 0.0004% trypan blue at room temperature (RT) for 10 min to inhibit streptolysin S activity. RBCs were incubated with bacterial culture supernatant with or without anti-SLO antibodies or water-soluble cholesterol (Sigma) as indicated for 30 min at 37°C, with PBS (0% hemolysis) or H₂O (100% hemolysis) as positive and negative controls. Supernatants were collected from assay wells after centrifugation at 3000 $\times g$ for 15 min, and hemolysis determined by absorbance with a SpectraMax M3 plate reader at 541 nm using SoftMax Pro software. Each titer was recorded as the point that the hemolysis reached half of the 100% RBC lysis (H₂O) control.

Neutrophil Elastase and IL-8 Release

Neutrophils were infected with bacteria at an MOI of 1 for 30 min as described earlier. The release of neutrophil elastase in response to bacteria was indirectly determined via the proteolytic activity of elastase. Cell-free supernatant was incubated with 20 μM of peptide substrate *N*-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide (Sigma) for 20 min at RT. The absorbance was measured with SpectraMax M3 plate reader at 405 nm for elastase using SoftMax Pro software. IL-8 release from neutrophils was quantified using the Quantikine ELISA kit (R&D Systems) following the manufacturer's manual. Briefly, 1 $\times 10^6$ neutrophils were infected with live GAS WT and Δ SLO at an MOI of 1 for 60 min and the release of IL-8 was determined via absorbance with SpectraMax M3 plate reader at 450 nm using SoftMax Pro software.

Neutrophil Migration

Migration distance was measured by perfusing neutrophils with GAS WT, Δ SLO mutant bacteria at an MOI of 10 and 50. Five nM IL-8 was used as a chemotactic stimulus. Migration of individual neutrophils was quantified by measuring the distance in micrometer after 3 min compared to starting point by microscopy (Zeiss Axiolab).

Immunostaining of NETs and NET Quantification

Neutrophil extracellular trap visualization was performed as described previously (29). Briefly, 5 $\times 10^6$ neutrophils were infected with GAS WT and Δ SLO mutant was added at MOI = 1 and incubated for 4 h in 37°C/5% CO₂. Cells were fixed with 4% paraformaldehyde and stained with anti-myeloperoxidase (MPO) antibody

(1:300 diluted, Calbiochem) in PBS + 2% bovine serum albumin (BSA, Sigma) for 1 h at RT, followed by incubation with goat anti-rabbit Alexa 488 antibody (1:500 dilution, Life Technologies). Cells were counterstained with ProlongGold + 4',6'-diamidino-2-phenylindole (DAPI, Invitrogen) and imaged on a fluorescent microscope. Representative, randomized images ($n = 5$) were taken for each condition and individual experiment. The ratio of NET-releasing cells to non-NET-releasing cells was determined as % of total cells releasing NETs.

Transmission Electron Microscopy

Neutrophils were infected with GAS WT and Δ SLO mutant for 4 h at an MOI of 1 and cells were prepared for transmission electron microscopy (TEM) as previously described (19, 30). Briefly, cells were fixed with 3% formaldehyde, 1.5% glutaraldehyde, 0.1M sodium cacodylate trihydrate, 5 mM CaCl₂ and 2.5% sucrose at pH 7.4 buffer for 1 h. Cells were washed three times in ice-cold 0.1M sodium cacodylate buffer containing 2.5% sucrose and fixed cells were incubated with Palade's OsO₄ by incubation with 1% osmium tetroxide in acetate/veronal solution for 1 h on ice. After three washing with acetate/veronal solution, the cells were stained and stabilized *en bloc* in 0.5% uranyl acetate with acetate/veronal solution overnight at RT in the dark. After one rinse with ddH₂O and one rinse with ice-cold 50% ethanol, the cells were dehydrated in an ice-cold dehydration series of 70, 95, and 100% ethanol for 15 min followed by three washes in 100% acetone at RT. Cells were then infiltrated in well-mixed Epon-ethanol resin series of three: 3% for 7 h, 66% for 7 h, followed by 100% at least overnight with agitation at RT. Next, the samples were allowed to polymerize in 100% Epon blocks at 60–80°C for a minimum of 48 h. Thin sections (70 nm) were examined with FEI Tecnai 12 transmission electron microscope and images taken with Tietz 214 CCD camera. Images were adjusted in brightness and contrast with Adobe Photoshop CS version 8.

Generation of Inactivated rSLOmut

Based on the SLO crystal structure (31), a recombinant SLO derivate (rSLOmut) with mutated tryptophan residue in the membrane-binding loop W535A was generated similarly as described (32). Loss of hemolytic activity of the rSLOmut protein was determined in J774 murine macrophages by TUNEL assay as described (19). The rSLOmut protein was cloned by overlapping PCR into a pET15b expression vector and expressed in *Escherichia coli* BL21 by induction at OD_{600} 0.6–1.0 with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 h. Protein purification and quantification by A₂₈₀ was performed as described (19).

SLO Immunization Study

Eight-week-old male CD1 mice (Charles River Laboratories) were immunized three times, 2 weeks apart, with recombinant SLOmut protein or PBS control, plus adjuvant. The first immunization was done with Freund's complete adjuvant, and the following two immunizations were done with Freund's incomplete adjuvant. Protein or PBS were mixed with adjuvant 1:1 and emulsified, resulting in a final protein concentration of 500 μ g/

mL. Each mouse was injected intraperitoneally (i.p.) with 100 μ L of the protein/adjuvant emulsified mixture. Mice were infected i.p. with 8×10^7 CFU bacteria 2 weeks after the last immunization. Survival was monitored daily for 14 days. Anti-SLO IgG and IgM antibody titer was measured by ELISA using the serum collected 3 days before challenging the mice with GAS.

Statistical Analysis

All data were collected from at least three independent experiments in triplicate. Experiments using neutrophils were performed with a minimum of three different healthy volunteers. The data were combined and expressed as mean \pm SEM except stated differently. All data were analyzed by unpaired Student's *t*-test using GraphPad Prism version 5.0 (GraphPad Software Inc.). *P* values <0.05 were considered statistically significant.

RESULTS

GAS Production of SLO Suppresses Neutrophil Oxidative Burst

A select group of Gram-positive human pathogens including GAS, *S. aureus*, and *S. pneumoniae* are leading causes of invasive, potentially life-threatening infections worldwide (1, 33, 34). Neutrophils are a critical first line of defense against such invading pathogens, and the rapid production of bactericidal ROS is key element of their effectiveness. Compared to *S. aureus* and *S. pneumoniae*, GAS of the M1 and M3 serotypes elicited a reduced level of oxidative burst from freshly isolated human neutrophils 20 min post infection (Figure 1A). As each bacterial species is replete with pathogen-associated molecular patterns (e.g., lipoteichoic acid, peptidoglycan, and formyl peptides) to stimulate neutrophil activation, we hypothesized that GAS produced factor(s) that suppressed the full host oxidative burst potential. By screening a panel of isogenic M1T1 GAS mutants lacking defined virulence factors, we identified a higher neutrophil oxidative burst in response to a GAS Δ SLO mutant, suggesting that the toxin could play a key role in oxidative burst suppression (Figure 1B).

Streptolysin O is a well-studied, secreted virulence factor of GAS that induces apoptosis in neutrophils and macrophages at later time points (~ 4 h) (19). We, thus, focused the aim of the current study to investigate the role of SLO in the early GAS-neutrophil interaction (<30 min) independent of cell death. Incubation of human neutrophils with GAS WT or Δ SLO mutant at MOI of 1, 10, or 50 bacteria/cell for 30 min did not produce cell death as assessed by LDH release (Figure S1A in Supplementary Material) or Live–Dead staining (Figures S1B,C in Supplementary Material). Complementation of the Δ SLO mutant with the *slo* gene expressed on a plasmid vector (Δ SLO + pSLO) restored the WT phenotype confirming that SLO dampens the neutrophil ROS response (Figure 1C). Additionally, purified recombinant SLO (rSLO) protein was sufficient to reduce the oxidative burst in neutrophils in a dose-dependent manner to the levels of the untreated control (Figure 1D). Oxidative burst suppression was not a universal property of bacterial pore-forming toxins, as live methicillin-resistant *S. aureus* (MRSA) USA300 producing the

pore-forming virulence factor α -hemolysin (Hla) did not differ from an isogenic Δ Hla mutant in elicitation of neutrophil ROS (Figure S2A in Supplementary Material), nor did recombinant Hla toxin at concentrations between 10 and 1000 ng/mL modulate oxidative burst (Figure S2B in Supplementary Material).

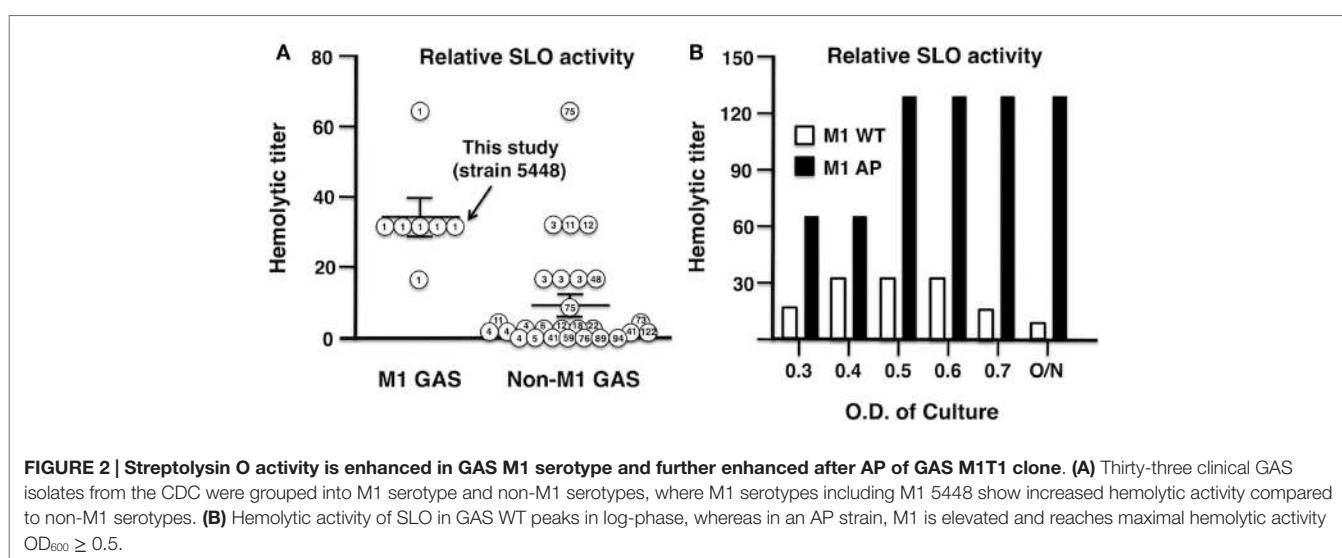
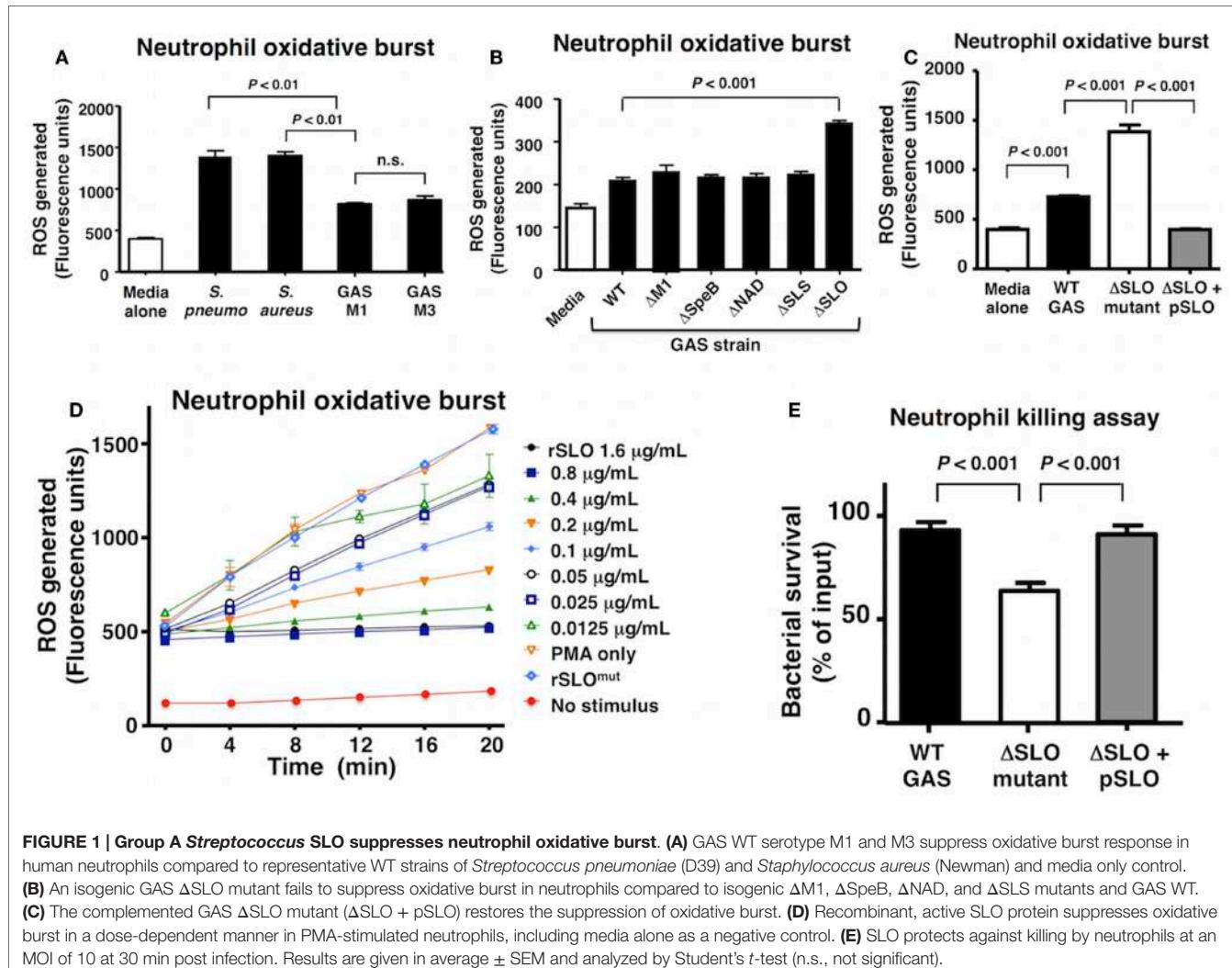
As ROS production is an important bactericidal effector of neutrophils, we confirmed the overall effect of SLO on neutrophil bacterial killing. The Δ SLO mutant was significantly more susceptible to rapid neutrophil killing after 30 min of infection than the M1T1 GAS parent strain, and WT resistance was restored in the complemented mutant (Figure 1E).

GAS M1 Serotype and covRS Mutation Are Associated with Higher SLO Activity

An increase in the production of SLO played a key role in the evolution of the globally disseminated M1T1 epidemic clone responsible for GAS infections of increased frequency and severity (13, 14, 23). Analysis of 33 clinical isolates obtained from the U.S. Center for Disease Control and Prevention (CDC) revealed a higher median hemolytic activity of M1 serotypes compared to non-M1 serotypes (Figure 2A). In this study, we utilized GAS strain 5448, a representative of the globally disseminated M1T1 clone, which is the most frequently isolated clone from patients suffering invasive infections (35, 36). During invasive infections in humans, M1T1 GAS (15); and isolates of certain additional serotypes (37) undergo spontaneous mutations in the CovRS regulator, leading to upregulation of virulence factors including SLO and hyaluronic acid capsule, coupled with loss of cysteine protease SpeB expression, which can degrade SLO during stationary phase (38). This “genetic switch” is recapitulated by AP of M1T1 GAS in mice (15, 38). Consistent with this model, we found that the SLO activity of WT M1T1 GAS is maximal in log-phase (OD₆₀₀ 0.4–0.6) and declines in stationary phase, while its expression is markedly increased in the AP strain bearing a covS mutation, remaining high through late-log phase and stationary phase due to SpeB inactivation (Figure 2B).

Inhibitors of SLO Activity Increase the Oxidative Burst Response of Neutrophils to GAS

To confirm a key role of SLO in suppression of neutrophil oxidative burst, we utilized anti-SLO neutralizing antibodies and water-soluble cholesterol, a known inhibitor of SLO activity (39). Dose-dependent activity of each inhibitor against SLO was confirmed by complete ablation of hemolytic activity (Figure 3A). Both anti-SLO antibodies and cholesterol reversed the suppressive effect of SLO on neutrophil oxidative burst in dose-dependent manners (Figures 3B,C). Interestingly, delayed administration of cholesterol to neutrophils 30 min after exposure to WT GAS restored oxidative burst expression as measured at 60 min (Figure 3D), indicating the effects of the toxin are at least partially reversible upon SLO inhibition. When the pharmacological NADPH oxidase inhibitor, DPI was used to block neutrophil ROS generation (40), the survival difference between the WT and Δ SLO mutant GAS strains was no longer observed (Figure 3E); DPI treatment enhanced survival of both WT and



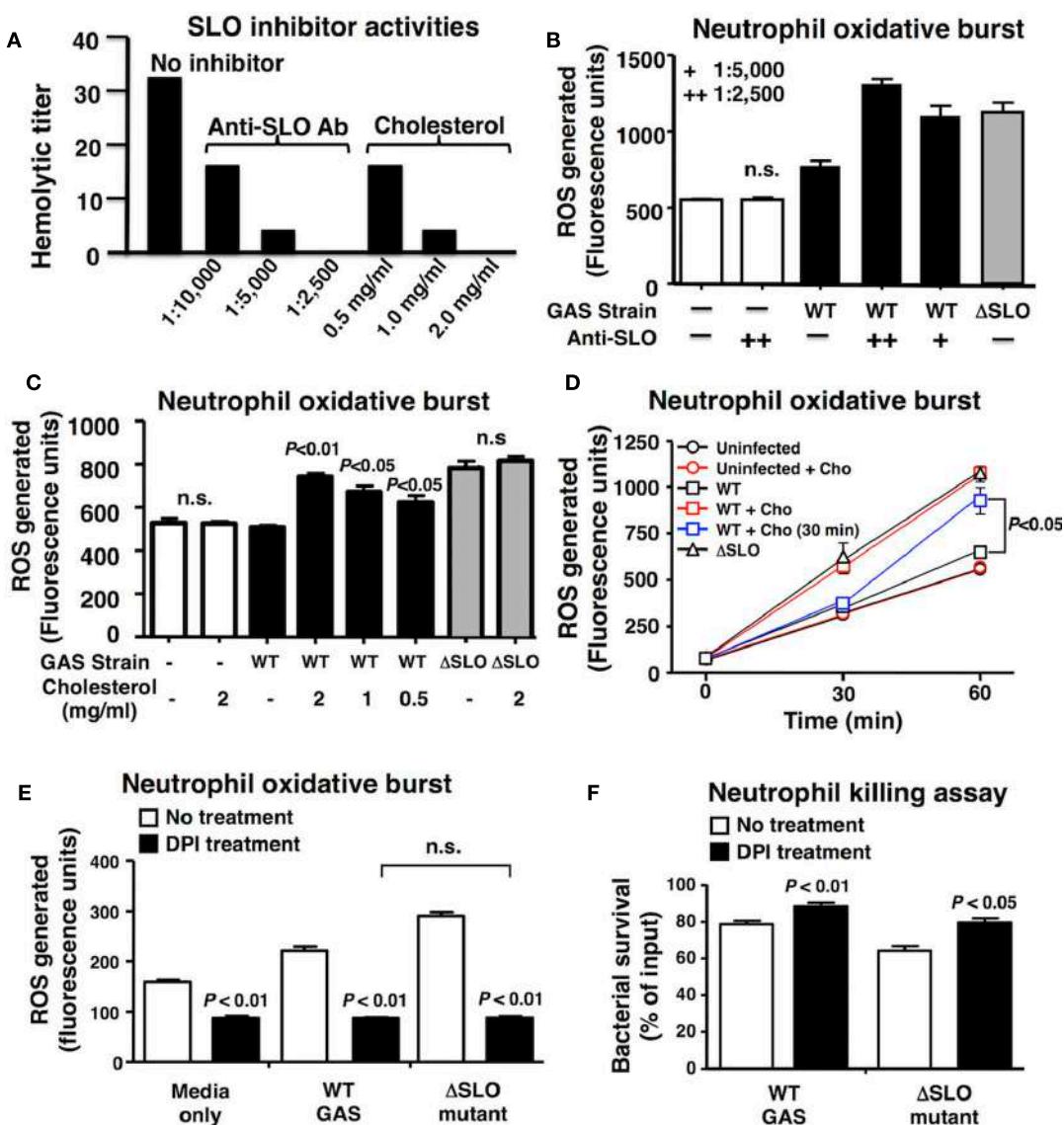


FIGURE 3 | Inhibition of SLO reverses the suppression of oxidative burst. (A) Anti-SLO antibodies and water-soluble cholesterol block hemolytic activity of SLO in a dose-dependent manner compared to control without inhibitor. **(B)** Inhibition of SLO with anti-SLO blocking antibodies or **(C)** cholesterol prevents suppression of oxidative burst in neutrophils in WT GAS with no effect on Δ SLO mutant or media only control 20 min post infection. **(D)** Oxidative burst in neutrophils infected with bacteria was monitored over a 60 min time course and cholesterol (cho) added after 30 min reversed the suppression by SLO. Effect of pharmacological inhibition of NADPH oxidase activity by DPI compared to untreated control in response to live GAS WT and Δ SLO mutant on **(E)** neutrophil oxidative burst and **(F)** neutrophil killing. Results are given in average \pm SEM and analyzed by Student's *t*-test (n.s., not significant).

Δ SLO strains, demonstrating that ROS contributes to neutrophil killing of GAS (Figure 3F).

SLO Suppresses Neutrophil Degranulation and IL-8 Release/Responsiveness

Reactive oxygen species have been implicated in the activation of neutrophil granule proteases including elastase (41, 42). GAS suppressed the degranulation of neutrophils in a SLO-dependent

manner as quantified by elastase release into the supernatant (Figure 4A). Activated neutrophils themselves are an important source of chemokine interleukin-8 (IL-8), the release of which recruits additional neutrophils to the site of inflammation or infection (43). After 60 min bacterial coincubation, we observed a clear reduction in IL-8 secretion by neutrophils exposed to WT GAS compared to the Δ SLO mutant (Figure 4B). Finally, the mean distance of neutrophil migration over 3 min in response to IL-8 as stimulus was significantly reduced in neutrophils exposed to WT GAS compared to those exposed to the Δ SLO mutant

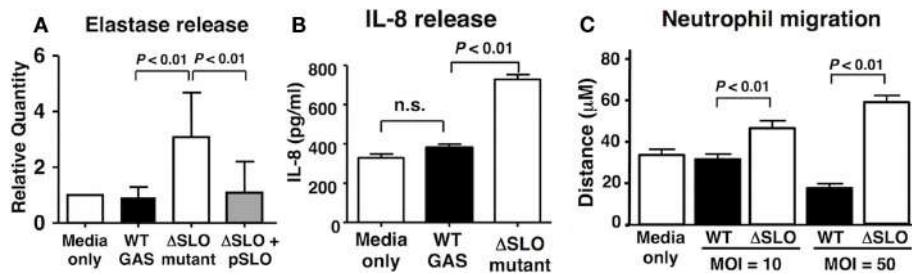


FIGURE 4 | Streptolysin O inhibits neutrophil degranulation and IL-8 release/respondiveness. (A) Neutrophil degranulation was determined by elastase release upon infection with live GAS WT, Δ SLO mutant, complemented bacteria and media only control, as quantified by ELISA. (B) SLO prevents IL-8 release from infected neutrophils compared to Δ SLO mutant bacteria and media only control as quantified by ELISA. (C) SLO inhibits neutrophil migration in MOI-dependent manner in contrast to Δ SLO mutant bacteria and media only control as measured by microscopic analysis. Results are given in average \pm SEM and analyzed by Student's *t*-test (n.s., not significant).

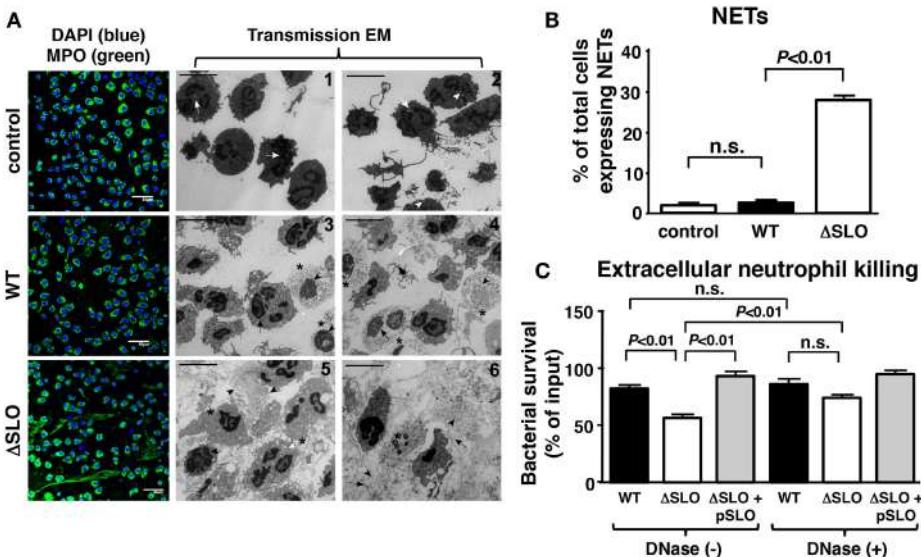


FIGURE 5 | Streptolysin O inhibits NET formation and extracellular killing. (A) Representative images from NET induction with GAS are shown by immunofluorescence (IF) and transmission electron microscopy (TEM) for untreated control [white arrows = multi-lobulated nuclei; white arrowheads = granules; white arrows = multi-lobulated nuclei; white arrow = disrupted nuclear membrane; asterisk = decondensation of chromatin; arrowhead = disintegrated nuclear envelope; arrow = disrupted nuclear membrane; asterisk = decondensation of chromatin; arrowhead = disintegrated nuclear envelope; arrow = disrupted cell membrane] and Δ SLO mutant [arrow = dilated space between inner and outer membrane; arrowhead = cytoplasmic protrusions and vacuolization; white arrowhead = phagocytosed bacteria; asterisk = dissolved membrane and DNA; arrowhead = vesicles of globular proteins; arrow = fibrous DNA strands; asterisk = dissolved membrane], bar = 5 μ m TEM. (B) SLO suppresses NET formation as quantified for GAS WT, Δ SLO mutant and media only control by IF. (C) Extracellular killing in the presence of cytochalasin D of GAS WT, Δ SLO and complemented strain shows that SLO to protect against killing in the presence/absence of DNase control to degrade NETs. Results are given in average \pm SEM and analyzed by Student's *t*-test (n.s., not significant).

(Figure 4C). Thus, SLO production by GAS impairs neutrophil IL-8 release and their activation and migratory responses to the chemokine.

SLO Inhibits Neutrophil Extracellular Trap Formation

Another critical innate immune function from neutrophils dependent on oxidative burst activity is NET formation, a specialized cell death process in which the nuclear DNA, embedded with

antimicrobial histones and proteins, is released to ensnare and kill bacteria at tissue foci of infection (44, 45). Neutrophils were incubated with GAS WT and Δ SLO mutant at MOI of 1 bacteria/cell for 4 h then stained with antibodies against myeloperoxidase (MPO), the most abundant neutrophil granule protein that colocalizes within NETs (46). GAS SLO expression inhibited NETosis indicated by dissolved cell membranes and fibrous, extracellular DNA strands as determined by TEM and immunofluorescence (IF) microscopy (Figure 5A). The percentage of NET-releasing

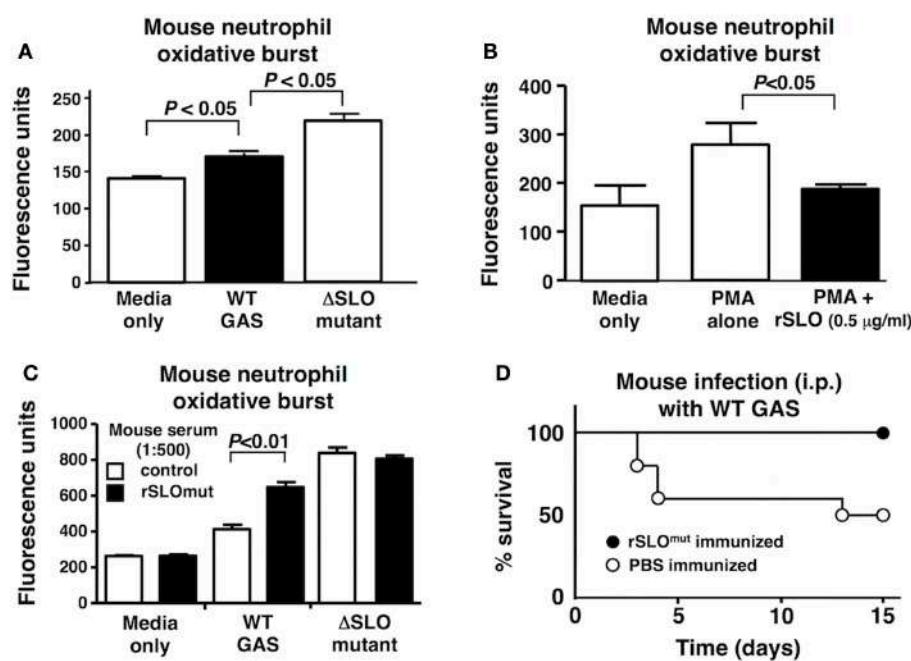


FIGURE 6 | Immunization with inactivated rSLOmut protects against GAS systemic infection. SLO suppresses the oxidative burst in mouse neutrophils with (A) live GAS WT compared to ΔSLO mutant or (B) rSLO protein after PMA-stimulation including media alone as control. Serum from rSLOmut-immunized mice inhibits SLO suppression of neutrophil oxidative burst from live bacteria (C). Immunization of mice with rSLOmut protein Vs mock prevents mortality of mice challenged with GAS WT in systemic infection model (D). Results are shown as \pm SEM from a minimum of three independent experiments. Data were analyzed by Student's *t*-test.

neutrophils was quantified by counting random IF microscopy fields (Figure 5B). M1T1 GAS strains also produce a nuclease (Sda1) that degrades the DNA backbone of NETs (9, 10). We confirmed that the GAS WT and ΔSLO mutant did not differ in their nuclease activity (Figure S3 in Supplementary Material). When neutrophils were treated with cytochalasin D to block phagocytic uptake of bacteria, survival of the GAS WT and complemented strain was greater than the survival of the ΔSLO mutant, differences that were abolished by DNase treatment (Figure 5C). This indicates that a DNA-dependent, extracellular killing process contributes to the neutrophil resistance phenotype provided by SLO expression.

Inactivated SLO as a Protective Vaccine Antigen *In vivo*

Streptolysin O is a potent toxin that triggers cell apoptosis *in vivo* (47), and deletion of SLO from highly hemolytic M1 and M3 GAS strains (Figure 2A) has previously been associated with loss of virulence in murine models of systemic infection (19, 48). Potentially, due to GAS being a human-adapted pathogen, the oxidative burst response of human neutrophils was more robust than that of mouse neutrophils; nevertheless, we found a similar effect wherein murine neutrophil ROS production was significantly reduced upon exposure to WT GAS compared to the ΔSLO mutant (Figure 6A). Recombinant SLO also suppressed the oxidative burst response of PMA-stimulated murine neutrophils (Figure 6B). Native SLO may be a poor vaccine

candidate because of its high cell toxicity, but strategies exist to mutate the cell-binding domain to create a toxoid SLO protein (rSLOmut) (32). Mice were immunized three times 2 weeks apart with rSLOmut, and a significant response in anti-SLO IgG and IgM antibodies was documented in serum by ELISA (Figure S4 in Supplementary Material). The post-immune antisera was found to reverse the inhibitory effect of SLO on the oxidative burst of mouse neutrophils (Figure 6C), demonstrating the capacity of the antibodies to block SLO neutrophil suppression. Mice immunized with rSLOmut were significantly protected against lethal systemic challenge with WT GAS M1 strain (Figure 6D), further supporting the inactivated SLO antigen as an attractive component for future multivalent GAS vaccines.

DISCUSSION

Group A *Streptococcus* remains one of the top 10 causes of infection-related morbidity and mortality worldwide (1). The aim of this study was to investigate the mechanisms GAS employs at early time points of infection (<30 min) to resist innate immune clearance by neutrophils. Generation of ROS upon bacterial encounter is an immediate neutrophil response to kill pathogens and to activate inflammatory signaling to recruit immune cells to the site of infection. As GAS is a non-pigmented and catalase-negative bacterium, it is dependent on alternative strategies to circumvent hostile ROS-rich host environments (11). Here, we identify SLO as the major bacterial factor responsible for

suppressing the oxidative burst in neutrophils, facilitating GAS escape from innate immune killing. Previously, a study using the recombinant Mac-1/IdeS protease from GAS proposed that this virulence factor could be suppressing ROS production (49). However, physiological levels of Mac-1/IdeS from live bacteria had no effect on ROS response or virulence (50). To the best of our knowledge, SLO is the first GAS virulence factor shown to be necessary and sufficient for suppressing production of bactericidal ROS, thereby subverting neutrophil ROS-dependent killing. Our study shows that SLO produced by live bacteria as well as recombinant SLO protein is necessary and sufficient to modulate innate immune cell functions at early time points of infection. A specific suppression of oxidative burst from neutrophils by SLO was further demonstrated by using specific inhibitor of SLO activity, cholesterol, or anti-SLO blocking antibodies.

Streptolysin O, PLY, LLO, and PFO, all belong to the same family of CDCs (20). Interestingly, neither the pore-forming staphylococcal α -hemolysin nor its production from live *S. aureus* had a similar effect on oxidative burst in neutrophils, suggesting a specific unique role of SLO and related toxins that is not a general consequence of membrane perturbation and disruption. Recently, LLO and PFO have been shown to suppress oxidative burst in murine macrophages by preventing the localization of NADPH oxidase with the phagosome (51).

To extend the analysis of SLO in suppressing neutrophil functions, we investigated its role in additional ROS-dependent functions critical in clearance of bacterial pathogens. The production of ROS is linked to degranulation and the elaboration of extracellular traps, which is a critical function for entrapment and extracellular killing of bacteria (44). Our findings show that SLO prevents the release of IL-8 and elastase from neutrophils and

blocks the formation of NETs. In our study, we confirmed that SLO contributes to resistance to an extracellular killing mechanism that is DNA-dependent. The hyperinvasive M1T1 GAS clone expresses a potent phage encoded nuclease (Sda1) that, like SLO, is upregulated on covR/S mutations arising *in vivo* under innate immune selection (15, 16). Thus, GAS has evolved a dual-pronged approach to both reduce the production of NETs, and to further dissolve NETs that are established, effectively neutralizing this arm of innate immunity.

In summary, we described a number of incapacitating effects of SLO on neutrophil function that act rapidly to suppress neutrophil function in advance of the toxin's ultimate and previously established role in triggering cell death pathways in the phagocyte. The importance of these virulence phenotypes is underscored by the protective effect of active immunization with an rSLOmut toxoid vaccine against invasive GAS infection. As SLO is present in all GAS serotypes and strains, our findings support proposals (3, 32) to include inactivated SLO toxoid as a component of a multicomponent vaccine against a leading human pathogen, GAS.

ACKNOWLEDGMENTS

Support for this work was provided by the National Institutes of Health Grants AI077780 and AI096837 to VN, AI047294 to SS, and a German Academic Exchange Service (DAAD) graduate fellowship to SD.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2015.00581>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tasting *Pseudomonas aeruginosa* biofilms: human neutrophils express the bitter receptor T2R38 as sensor for the quorum sensing molecule *N*-(3-oxododecanoyl)-L-homoserine lactone

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Microbial Immunology, a section of
the journal Frontiers in Immunology

Received: 30 April 2015

Accepted: 06 July 2015

Published: 24 July 2015

Citation:

Maurer S, Wabnitz GH, Kahle NA,
Stegmaier S, Prior B, Giese T,
Gaida MM, Samstag Y and
Hänsch GM (2015) Tasting
Pseudomonas aeruginosa biofilms:
human neutrophils express the bitter
receptor T2R38 as sensor for the
quorum sensing molecule
N-(3-oxododecanoyl)-L-homoserine
lactone.
Front. Immunol. 6:369.
doi: 10.3389/fimmu.2015.00369

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Bacteria communicate with one another via specialized signaling molecules, known as quorum sensing molecules or autoinducers. The *Pseudomonas aeruginosa*-derived quorum sensing molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (AHL-12), however, also activates mammalian cells. As shown previously, AHL-12-induced chemotaxis, up-regulated CD11b expression, and enhanced phagocytosis of polymorphonuclear neutrophils. Circumstantial evidence concurred with a receptor for AHL-12, which has been elusive so far. We now investigated the bitter receptor T2R38 as a potential candidate. Although identified as a taste receptor, extragustatory cells express T2R38, for example, epithelial cells in the lung. We now detected T2R38 in peripheral blood neutrophils, monocytes, and lymphocytes. T2R38 is not only found on the cell membrane but also intracellular. In neutrophils, T2R38 was located in vesicles with characteristics of lipid droplets, and super-resolution microscopy showed a co-localization with the lipid droplet membrane. Neutrophils take up AHL-12, and it co-localized with T2R38 as seen by laser scan microscopy. Binding of AHL-12 to T2R38 was confirmed by pull-down assays using biotin-coupled AHL-12 as bait. A commercially available antibody to T2R38 inhibited binding of AHL-12 to neutrophils, and this antibody by itself stimulated neutrophils, similarly to AHL-12. In conclusion, our data provide evidence for expression of functional T2R38 on neutrophils, and are compatible with the notion that T2R38 is the receptor for AHL-12.

Keywords: neutrophils, *N*-(3-oxododecanoyl)-L-homoserine lactone, quorum sensing, bitter receptor T2R38, lipid droplets, *Pseudomonas aeruginosa*

Introduction

The quorum sensing molecule, *N*-(3-oxododecanoyl)-L-homoserine lactone (AHL-12), is produced by *Pseudomonas aeruginosa* and other Gram-negative bacteria. AHL-12 participates in the generation of virulence factors, and is particularly well studied in the context of biofilm formation (1–4). Of note, for AHL-12, a so-called “interkingdom-signaling” was reported, defined as activation of

mammalian cells by bacterial signaling molecules (5, 6). Inhibition of various lymphocyte functions by AHL-12 was reported (7, 8), and was interpreted as a down-regulation of the immune response (9). On the other hand, we and others described activation of phagocytic cells, including enhancement of phagocytosis, increased expression of adhesion receptors, and induction of chemotaxis (10–12), leading to the presumption that recognition of AHL-12 might reinforce the local immune defense by preventing biofilm formation [reviewed in Ref. (13)].

AHL-12 is highly hydrophobic. For T cells and model lipid membranes, free diffusion through membranes was reported (14, 15). A nuclear receptor, PPAR γ , was described in epithelial cells, although physical binding of AHL-12 to PPAR γ has not yet been proven (16). In macrophages and neutrophils, the AHL-12 signaling pathway was compatible with that of a G-protein coupled receptor, whereas participation of toll-like receptors could be excluded (11, 13, 17–20), a receptor, however, has not yet been identified.

More recently, effects of AHL-12 on epithelial cells were described, such as enhanced ciliary movement and NO-production (21), indicating that AHL-12 induces activation of defense-related functions in these cells. On airway epithelial cells, the so-called bitter receptor T2R38 has been indicated as receptor for AHL-12, and there is evidence for a correlation between T2R38 receptor allotypes and susceptibility to airway infections (21–23).

The bitter receptor T2R38 belongs to a family of taste receptors. As the name implies, the receptor is sensing bitter tasting substances, a phenomenon that is thought to prevent the intake of bitter and potentially toxic substances [reviewed in Ref. (24–27)]. Bitter receptors were first described on cells of the taste buds in the oral cavity. Meanwhile, a wider distribution was reported, e.g., expression on epithelial cells of the airways, in the colon, in the brain, and in tumor cells as well (21, 28–30). The role of T2R38 in tissue outside the gustatory systems is still elusive, and so far, AHL-12 appears to be the first natural ligand for T2R38, which leads to the notion that T2R38 participates in “sensing of bacteria.” In that context, we now addressed the question whether T2R38 could also be the receptor for AHL-12 on neutrophils.

Materials and Methods

Human Blood and Human Neutrophils

Blood from volunteers (mainly laboratory personal and students) was drawn into heparin-coated tubes (Sarstedt, Nümbrecht, Germany). Neutrophils were isolated by PolymorphPrep™ (Axis-Shield, Oslo, Norway) and residual erythrocytes were removed by hypotonic lysis. The remaining cells were suspended in Hanks balanced salt solution (HBSS), containing 0.5% bovine serum albumin (BSA), at a final concentration of 5×10^6 cells/ml. This procedure yielded 85–95% neutrophils as judged by cytofluorometry using CD66b as marker for neutrophils. Written informed consent was obtained from the volunteers and the ethic committee of the University of Heidelberg approved the procedure.

Cell Lines

U937 and HL-60 were purchased from ATCC. The cell lines were propagated in RPMI, containing fetal calf serum (10%),

L-glutamine (1%), and penicillin-streptomycin (1%) (all purchased from Gibco, Eggenstein, Germany). The cells were used in their exponential growth phase. Differentiation of U937 was induced by phorbol ester (100 ng/ml); HL-60 was differentiated with DMSO (1.5%).

Antibodies

The following antibodies to T2R38 were used: bs-86508-A488 [rabbit (rb) IgG, labeled with Alexa Fluor 488, Bioss, Woburn, MA, USA]; ab65509 (rb serum); ab 130503 (rb IgG) (both obtained from abcam, Cambridge, UK); sc-76108 (rb IgG), and sc-34294 (goat IgG) (Santa Cruz, Dallas, TX, USA). Isotype controls were rb IgG labeled with Alexa Fluor (bs-0295P-A488, Bioss, Woburn, MA, USA); rabbit IgG (b176094, abcam), or goat IgG (sc-3887, Santa Cruz). Rabbit serum was obtained from our animal facility. Secondary antibodies were FITC-labeled anti-rb IgG from goat (111-096-003, Dianova, Hamburg, Germany) or Alexa Flour 488-labeled anti-goat IgG from life technologies (A11055, Darmstadt, Germany). CD11b expression was determined using a FITC-labeled antibody (IM0530, Beckman Coulter, Germany Krefeld). The antigen peptide, sc-34294P, used to raise the antibody to T2R38 (sc-34294), was purchased from Santa Cruz.

Cytofluorometry

To determine the expression of surface receptors on neutrophils, whole blood (100 μ l) or isolated neutrophils (10^7 /ml) were pre-incubated with an Fc-receptor blocking agent (Biolegend 422302, San Diego, CA, USA), then incubated with the respective antibodies or isotypic antibodies. In the whole blood assays, erythrocytes were lysed using BD Facs Lysing solution (BD Biosciences, NJ, USA). Cells were subjected to cytofluorometry using FACSCalibur and CellQuest Pro software (Becton Dickinson, Heidelberg, Germany). For intracellular staining, the cells were treated with FACS Permeabilizing Solution 2 (BD Biosciences), pre-incubated with the Fc-receptor blocking agent and then incubated with anti-T2R38 (2 μ g), as described above.

Uptake of AHL-12 and Inhibition Experiments

Isolated neutrophils (1×10^6 /ml) in HBSS, containing 1% BSA and 0.1% sodium azide (“FACS buffer”) were incubated with AHL-FITC (Cayman Chemical, Ann Arbor, MI, USA) (50–100 μ M) for 30 min at 4°C, and then subjected to cytofluorometry. For inhibition experiments, isolated neutrophils were washed with FACS buffer and incubated with antibodies to T2R38 for 30 min at 4°C, or with the antigen peptide (2–4 μ g). AHL-FITC (100 μ M) was added and after for 30 min at 4°C, fluorescence associated with the cells was measured.

Detection of T2R38

A test kit (SEF811Hu) was purchased from Cloud Clone Corporation, Houston, TX, USA, and the test was carried out according to the protocol supplied.

Gene Expression Analysis

DNA was isolated from 10^6 purified neutrophils using the Mag-naPure mRNA Isolation Kit I. mRNA was isolated with the

MagnaPure-LC device. An aliquot of mRNA was reversely transcribed using AMV-RT and oligo-dT as primer (First Strand cDNA synthesis kit, Roche) according to the manufactures protocol. For PCR analysis, primer sets optimized for the LightCycler® (RAS, Mannheim, Germany) were developed by and purchased from SEARCH-LC GmbH (www.Search-LC.com). The PCR was performed with the LightCycler® FastStart DNA Sybr GreenI kit (RAS). To correct for differences in the content of mRNA, the calculated transcript numbers were normalized according to the expression of the housekeeping gene peptidylprolyl isomerase B (PPIB). Values represent number of transcripts per 1000 transcripts of PPIB.

Western Blot and Pull-Down Assays

Isolated neutrophils or HL-60 (10^7 cells) were lysed with RIPA buffer (Tris-buffered saline containing 1% Non-ident P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.0004% sodium azide, 0.2M orthovanadate, and 0.5M phenylmethylsulfonyl fluoride). Of each sample, 25 μ l were mixed with 5 μ l 5× Laemmli-buffer and applied to a SDS-Gel (9%). Following blotting, the membrane was incubated with 5% milk powder in TBS, containing 0.1% Tween20. Of the antibody to T2R38 (sc-34294) a 1:1000 dilution was used, of antigen peptide sc-34294P 0.5/10 μ l antibody. Secondary antibody was a POX conjugated mouse anti-goat IgG (#205-035-108, Jackson Immuno Research, Suffolk, UK, 1:20,000). Blots were developed using Amersham Prime Western Blotting detection Reagent (GE Healthcare, Freiburg, Germany).

For the pull-down assay, neutrophils or HL-60 (2×10^7) were lysed in RIPA buffer (2 ml) and incubated overnight at 4°C with or without AHL-12-biotin (300 μ M; Cayman Chemical). Then streptavidin sepharose beads (Cell signaling, Danvers, MA, USA) were added for 2 h at 4°C, then washed with PBS, and incubated with SDS loading buffer containing β -mercaptoethanol. The samples were applied to a 12% SDS-Gel. Silver staining was performed and Western blotting using sc-34294 and the respective antigenic peptide. Alternatively, to whole cell lysate, membrane proteins were extracted using the kit supplied by Calbiochem for transmembrane proteins (#71772-3, Merck, Darmstadt). Extracts of $4\text{--}5 \times 10^7$ cells were used (neutrophils or HL-60, as indicated in the respective experiments).

Isolation of Lipid Droplets

Essentially, the method described by Wan et al. (31) was used. In brief, 3×10^8 U937 cells were suspended in 3 ml ice-cold disruption buffer [25 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 50 μ g/ml N- α -p-tosyl-L-lysine-chloromethyl-ketone (TLCK), 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin] and lysed by nitrogen cavitation (10 min, 800 psi, 4°C). The lysate was mixed with an equal volume of 1.08M sucrose in disruption buffer without TLCK, centrifuged for 30 min, 1000 g at 4°C, to eliminate nuclei and intact cells and then subjected to sucrose density centrifugation. After centrifugation (3.5 h, 34,000 rpm, 4°C), the lipid droplets were in the fraction with 0.54M sucrose. This fraction was diluted to 0.35M sucrose with 0.15M NaCl, overlaid with sucrose (0.27, 0.135 and 0M) for a second ultracentrifugation step (34,000 rpm, 4°C, 3.5 h). Lipid droplets were now in the fraction with 0.35M sucrose.

Droplets could be recognized by fluorescence microscopy after having incorporated Nile red (see below). For Western blotting, the proteins were precipitated with methanol/chloroform, resuspended in Laemmli Buffer (95°C for 10 min) and applied to a 12% SDS-Gel. Silver staining and Western blotting was performed was described above.

Laser Scan Microscopy

To assess binding of AHL-12 to cells and co-localization of T2R38, neutrophils were incubated with AHL-12-FITC (50 μ M, 20 min at 4°C), placed on cover slips, and fixed with 2% PFA for 15 min. Then, anti-T2R38 [ab65509 (diluted 1:300) or sc-34294 (4 μ g)] with the respective secondary antibodies (Alexa Fluor 488-labeled anti-rabbit IgG from donkey (bs-0295D-A488, Bioss); Alexa Fluor 488 Donkey anti-goat IgG (life technologies, Carlsbad, CA, USA) were added, and for comparison rabbit serum or goat IgG. The samples were mounted with Moviol (Sigma-Aldrich, St. Louis, MO, USA) and viewed by Laser Scan microscopy (LSM) (Nikon) using a 40× objective. To detect lipid droplets and the association of T2R38 with the droplets, neutrophils cells were placed on slides by cytocentrifuge, fixed with ice-cold methanol for 5 min and washed with PBS. Nile red (Sigma-Aldrich) was added (10 μ g/ml 20 min, room temperature), and after washing Hoechst 33342 nuclear stain was applied (H3570, life technologies, Darmstadt, Germany) 1:10,000 for 10 min. The samples were embedded with Moviol. To visualize T2R38, 5% goat serum was used as blocking agent and anti-T2R38 (ab65509) in a dilution of 1:300 (for comparison, normal rabbit serum was used). After 30 min, the secondary antibody was applied for 30 min, then Nile red was added (10 μ g/ml, 20 min). In addition, experiments with anti-T2R38 (sc-34294) were performed. In parallel, the antibody was added to the cells together with the peptide sc-34294P. Droplets were visualized by LSM and structured illumination microscopy (SIM) (D-Structured Illumination Microscopy), respectively.

Phagocytosis

A commercially available kit (Glycotope Biotechnology, Heidelberg, Germany) with FITC-labeled bacteria was used according to the instructions supplied.

Migration Assays

Chemotaxis was determined using the BD Falcon HTS Fluoroblok 96-Multiwell Insert System with an insert PET membrane of 3 μ m pore size. Isolated polymorphonuclear neutrophils (PMNs) were labeled with calcein. Cells (10^6 cells/ml) were incubated for 15 min at 37°C with calcein (life technologies; 1 μ g/ml), then washed with HBSS and seeded in a concentration of 2×10^5 /50 μ l into the insert plate. AHL-12, PTU (100 μ M), and for comparison HBSS or fMLP (10^{-7} M) were added to the lower wells. After 2 h at 37°C, the insert plate was removed and cell-associated fluorescence was determined by a Perkin Elmer Wallac Victor 2 Microplate Reader using Wallac Workstation software.

For the “under-agarose” migration assay, cover slips (1.5 × 4 cm) were pretreated with 3M HCl and ethanol (95%). Following washing with water, gelatine (0.5% in RPMI) was applied as a thin film. The cover slips were air dried, then agarose (1% in RPMI) was added (3 ml per cover slip). When the agarose

was solid, two holes were punched in a distance of 5 mm. Cells were placed in one hole (5×10^4 in 50 μ l), in the other the chemokine to be tested. After 2 h at 37°C, the cells were fixed with methanol for 30 min, the agarose was removed and the remaining cells were stained by Giemsa and examined microscopically.

Results

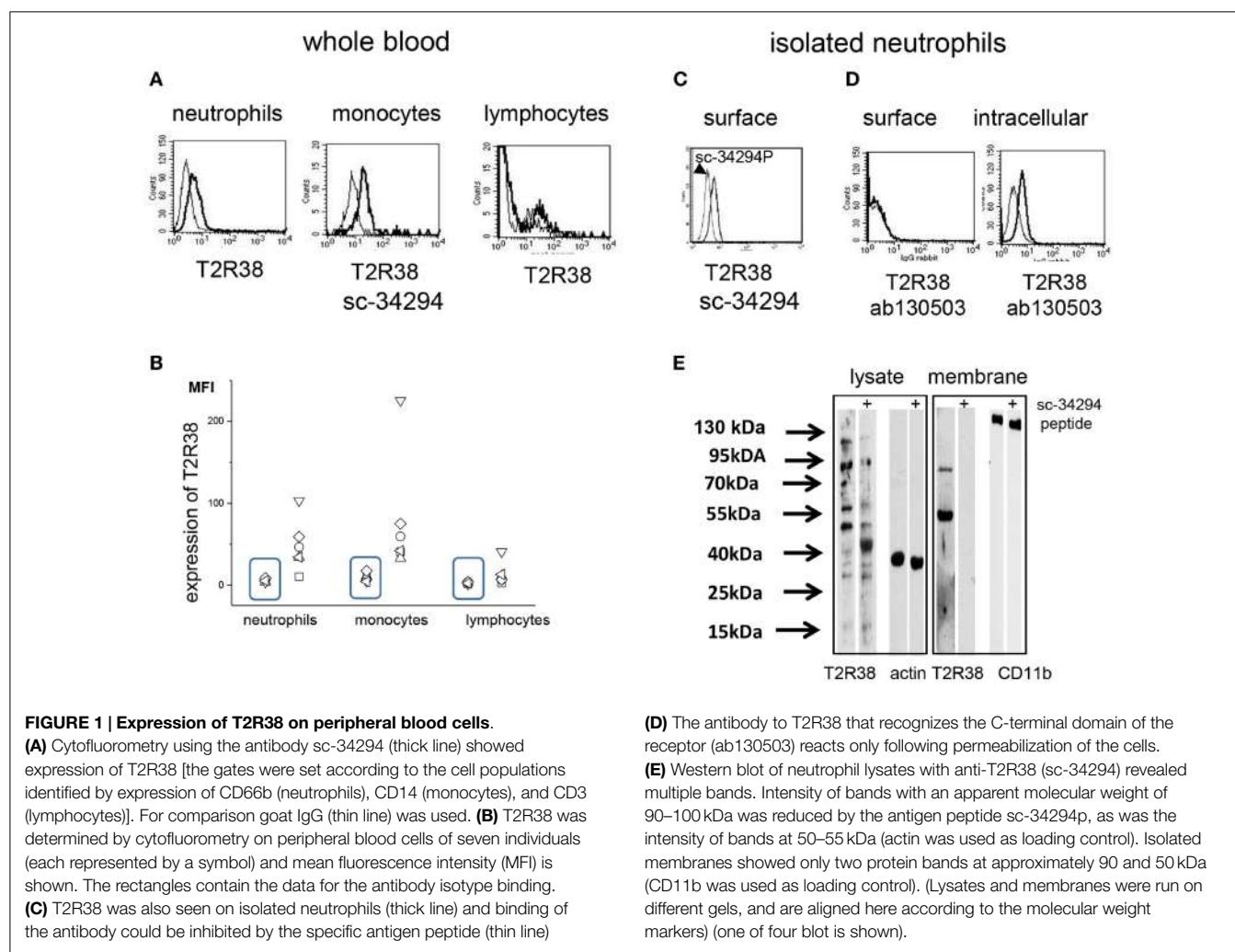
Expression of the Bitter Receptor T2R38 on Neutrophils and Monocytes

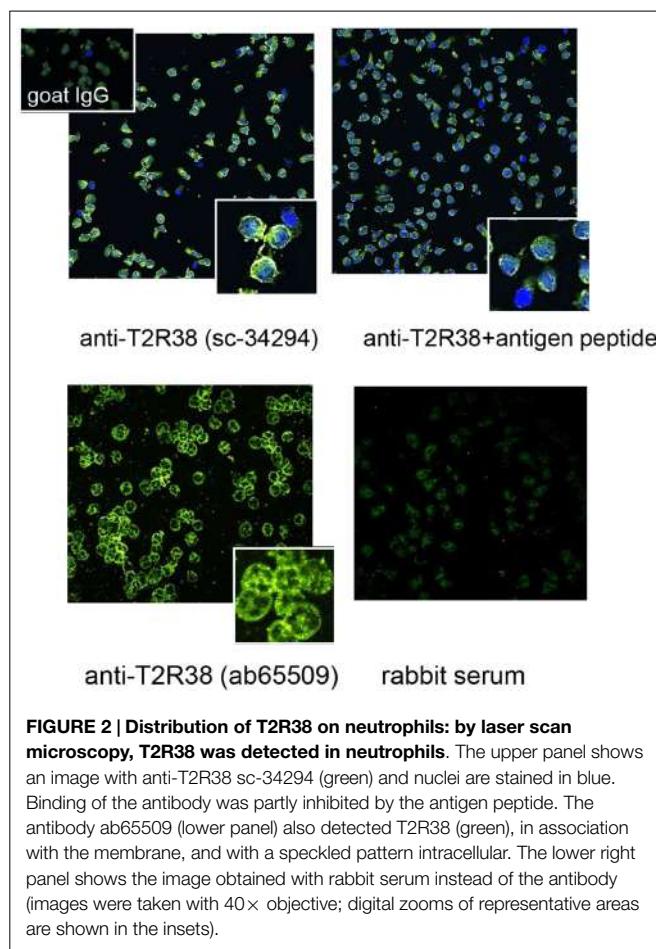
T2R38 was detected on neutrophils, monocytes, and to a lesser extent on lymphocytes in whole blood samples by cytofluorometry. Comparing samples of seven individuals, a considerable variation was seen (Figures 1A,B). Data obtained with the T2R38 antibody sc-34294 are shown, however, antibodies directed against other epitopes within the N-terminus of T2R38 (sc-67108; ab65509) gave essentially similar results (data not shown). For the antibody sc-34294, the antigenic peptide used to raise the antibody was available. This peptide inhibited binding of the antibody to the cells by 50% (Figure 1C). An antibody directed against an amino acid sequence of the C-terminus (ab130503) reacted only with permeabilized cells (Figure 1D). By Western blotting, T2R38

was detected in whole cell lysates and in cell membrane preparations. Specificity of the antibody binding was controlled by the antigenic peptide. In cell membranes, two bands with an apparent molecular weight of 90 and 50 kDa were detected, in the cell lysates multiple bands at 100, 90, 55, and 50 kDa, respectively (Figure 1E). The expected molecular weight of T2R38 is 38 kDa, but post-translational modifications and polymerization are described (for these experiments, sc-34294 was used).

T2R38 was also expressed by U937 and HL-60 cells (data not shown). Following differentiation of HL-60 by DMSO surface expression of T2R38 increased moderately (39.9% increase of the mean fluorescence intensity; average of three experiments).

The distribution of T2R38 in neutrophils was analyzed by confocal LSM. Accumulation at the membrane was seen and a speckled pattern in the cytoplasm (images with two different antibodies are shown in Figure 2). The latter finding was compatible with the notion that intracellular T2R38 was associated with membranous vesicles and/or granules. Stimuli known to induce degranulation, including IL8, f-MLP, C5a, or phorbol ester, did not up-regulate surface expression of T2R38 when applied under conditions causing up-regulation of CD11b. Moreover, T2R38 was not released into the cell supernatant as determined by ELISA





(data not shown). The failure to mobilize T2R38 makes its location in granules unlikely. Hence, we assessed association of T2R38 with lipid droplets. Neutrophils contain numerous lipid droplets visualized by incorporation of Nile red (**Figure 3A**). Confocal LSM indicated an association of T2R38 with droplets, particularly with their membrane (**Figure 3B**). To support this finding, we performed SIM. The circular morphology of the droplets could be resolved by this super-resolution microscopy, and T2R38 clearly was seen on the droplet membrane (**Figure 3C**). To verify the association of T2R38 with lipid droplets further, droplets were isolated by density centrifugation following the protocol by Wan et al. (31). Droplets of different sizes were seen, and again an association of T2R38 with the droplet membrane (**Figures 3D,E**). Lysates of the droplet fraction were positive for T2R38 as tested by blotting (**Figure 3F**).

Effect of Antibodies to T2R38 on AHL-12-Mediated Function

To answer the most pertinent question whether T2R38 might function as a receptor for AHL-12, the effect of antibodies to T2R38 on AHL-12 binding and AHL-12-induced neutrophil activation was tested. For these experiments, FITC-labeled AHL-12 was used. AHL-12-FITC binds to neutrophils, as seen by cytofluorometry and confocal LSM (**Figures 4A,B**). Binding of AHL-12-FITC could be inhibited by anti-T2R38 (sc-34294), on average by 35%, when 4 µg antibody were used per 1×10^6 neutrophils/ml (mean of three experiments with cells of different individuals). With higher antibody concentrations, also the isotype control was inhibitory, precluding the correct interpretation of the data. The other antibodies to T2R38 (sc-67108 and ab65509) did not inhibit AHL-12 binding.

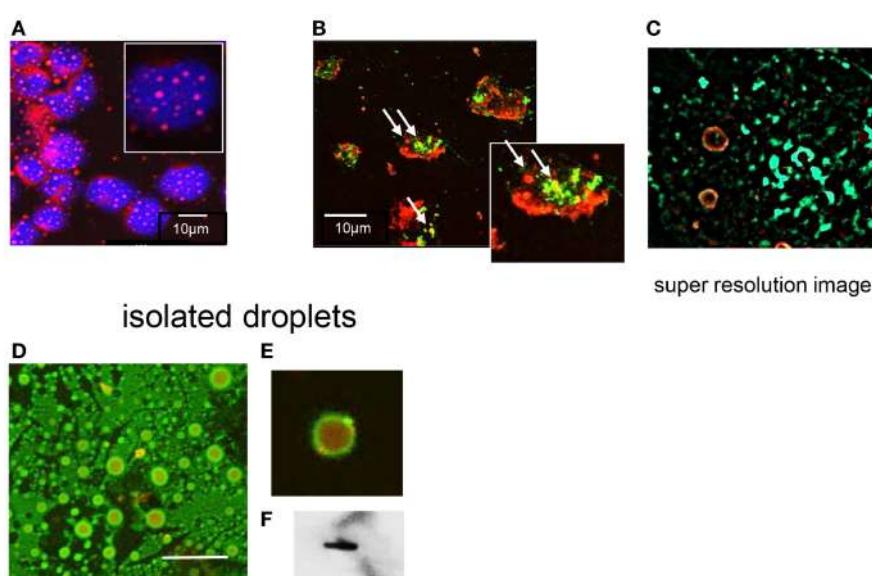


FIGURE 3 | Association of T2R38 with lipid droplets: (A) shows neutrophils stained with Nile red to visualize the lipid droplets; nuclei are in blue. (B) With anti-T2R38 (green) and staining with Nile red, droplets containing T2R38 can be recognized within the cell (marked by arrows). (C) A SIM image of the area in the rectangle shows two lipids droplets with a

distinct yellow staining of their membranes, indicative of a close association of T2R38 with the droplet membrane. (D) The images show isolated lipid droplets stained with Nile red and anti-T2R38; localization of T2R38 on the droplets membrane is seen especially in the zoomed image (E). (F) shows a blot of isolated droplets with ab65509.

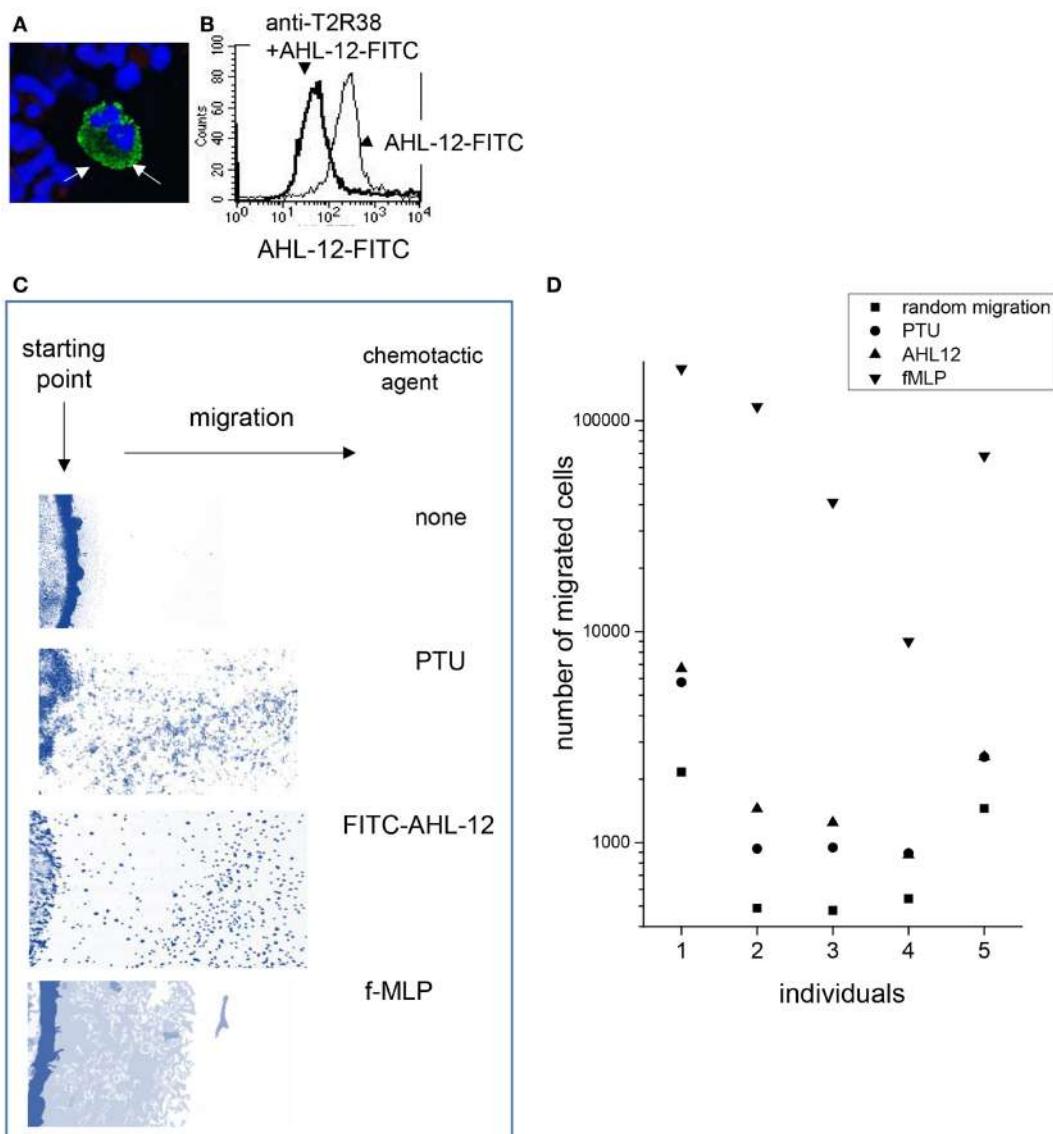


FIGURE 4 | Uptake of AHL-12-FITC by neutrophils and activation of cells: laser scan microscopy (A) and cytofluorometry (B) revealed binding of AHL-12-FITC to neutrophils (thin line). Binding could be inhibited by anti-T2R38 (sc-34294) (thick line) (C) AHL-12-FITC induced migration of neutrophils shown by “under-agarose” migration (cells are stained in blue), as did PTU. In comparison to f-MLP, fewer cells

had migrated but had covered a greater distance. (D) Chemotaxis (here migration through porous membrane is shown; data with neutrophils of seven individuals are summarized) was induced by AHL-12, PTU, f-MLP, and compared to the random migration. Because endpoint was measured, the number of cell migrating toward f-MLP was considerably higher.

Next, the effect of the antibody sc-34294 on AHL-12-mediated activation of neutrophils was assessed. Induction of chemotaxis was measured, enhancement of phagocytosis, and up-regulation of CD11b. Chemotaxis in response to AHL-12 was not inhibited, nor was migration toward PTU, which is widely used as ligand for T2R38, and which also induced chemotaxis of neutrophils (data based on two different methods, under-agarose migration and migration through a filter membrane, are shown in Figures 4C,D). Furthermore, the antibody did not inhibit the AHL-12 mediated up-regulation of CD11b nor the enhancement of phagocytosis. Rather, the antibody sc-34294 by itself activated neutrophils. Following antibody binding surface expression of

CD11b was enhanced (on average by 1.7-fold; mean of three experiments), as was the oxidative burst elicited by phagocytosis of opsonized *E. coli* (on average by 3.5-fold) (examples in Figures 5A,B).

Association of AHL-12 with T2R38 on Neutrophils

When neutrophils were incubated with limited amounts of AHL-12-FITC (50 μ M) at 4°C and then incubated with anti-T2R38, confocal LSM revealed a close proximity of AHL-12 and T2R38 (Figures 6A,B), suggesting a physical association of AHL-12 and T2R38. “Pull-down” assays using biotin-labeled AHL-12 as bait were performed with lysates of neutrophils or HL-60. Cell lysates

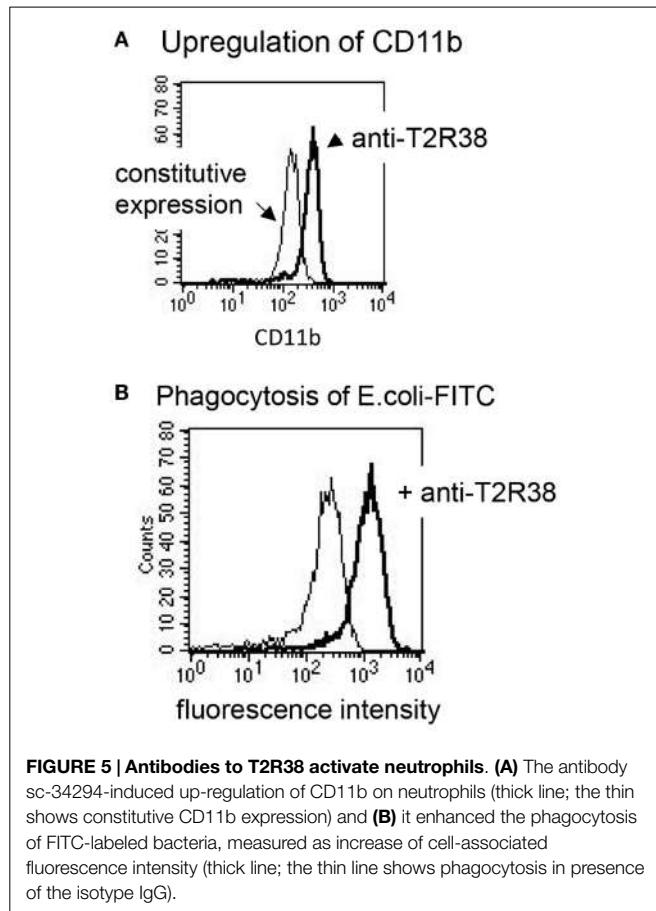


FIGURE 5 | Antibodies to T2R38 activate neutrophils. (A) The antibody sc-34294-induced up-regulation of CD11b on neutrophils (thick line; the thin shows constitutive CD11b expression) and (B) it enhanced the phagocytosis of FITC-labeled bacteria, measured as increase of cell-associated fluorescence intensity (thick line; the thin line shows phagocytosis in presence of the isotype IgG).

were incubated with AHL-12-biotin, and then with streptavidin beads. Eluates from the beads yielded numerous proteins. By Western blotting T2R38 could be identified with an apparent molecular weight of 100 and 60 kDa (data for neutrophils and for HL-60 are shown Figure 6C). When the pull-down assay with AHL-12-biotin was carried with membrane protein fractions, T2R38 could be detected again by Western blotting (Figure 6C).

Discussion

Our data provide evidence for an extragustatory expression of the bitter taste receptor T2R38 on neutrophils, monocytes, and myeloid cell lines. By a variety of methods and with antibodies directed against different epitopes, intracellular expression as well as expression on the cell membrane was seen. At difference with the predicted molecular weight of 38 kDa, proteins with higher apparent molecular weight were detected, presumably the result of post-translational modifications and polymerization of the receptor, as it had been described for T2R38 in epithelial cells (32).

Surface expression of T2R38 varied among individuals, as did the number of gene transcripts. We presume a genetic predisposition, because multiple testing revealed individuals expressing little T2R38 and others expressing moderate levels (own unpublished data). The histograms obtained by cytofluorometry revealed a more or less symmetric peak, indicating that T2R38 expression was not restricted to a subpopulation. The number of

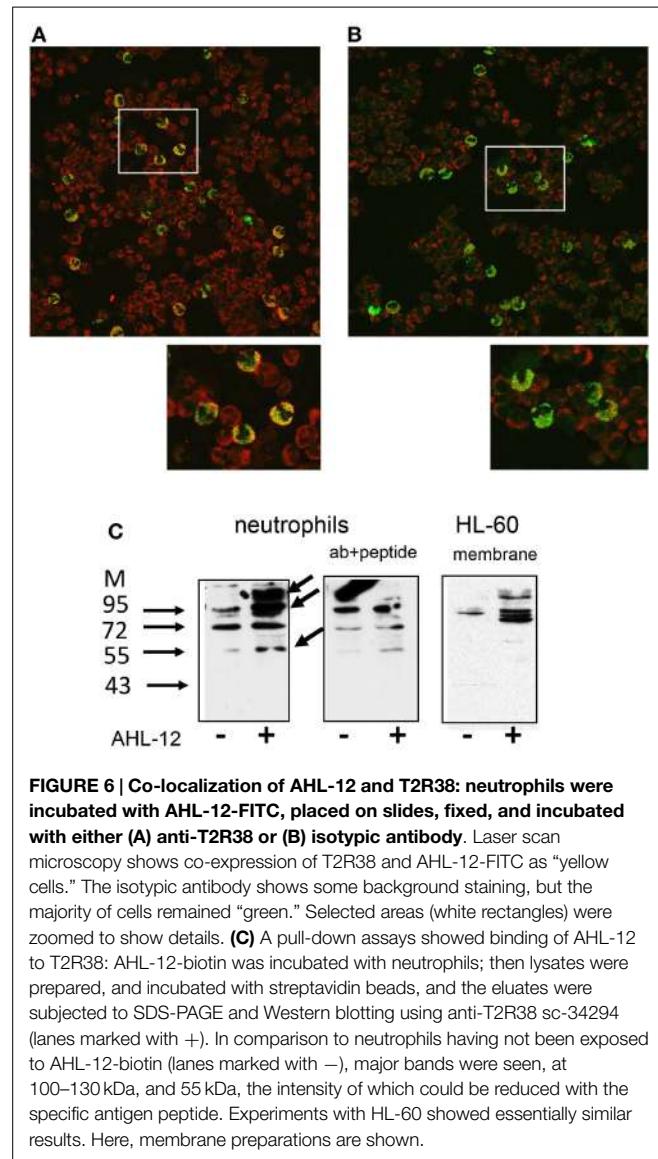


FIGURE 6 | Co-localization of AHL-12 and T2R38: neutrophils were incubated with AHL-12-FITC, placed on slides, fixed, and incubated with either (A) anti-T2R38 or (B) isotypic antibody. Laser scan microscopy shows co-expression of T2R38 and AHL-12-FITC as “yellow cells.” The isotypic antibody shows some background staining, but the majority of cells remained “green.” Selected areas (white rectangles) were zoomed to show details. (C) A pull-down assays showed binding of AHL-12 to T2R38: AHL-12-biotin was incubated with neutrophils; then lysates were prepared, and incubated with streptavidin beads, and the eluates were subjected to SDS-PAGE and Western blotting using anti-T2R38 sc-34294 (lanes marked with +). In comparison to neutrophils having not been exposed to AHL-12-biotin (lanes marked with –), major bands were seen, at 100–130 kDa, and 55 kDa, the intensity of which could be reduced with the specific antigen peptide. Experiments with HL-60 showed essentially similar results. Here, membrane preparations are shown.

gene transcripts was rather low. Absent or marginal gene transcription – even for highly expressed proteins – is not unusual for neutrophils, because in these cells a great majority of proteins is produced during maturation only, and are then stored in intracellular compartments (33, 34).

Well-characterized storage compartments of neutrophils are granules, which are generated as the precursor cells mature, and which enclose proteins as they are synthesized [reviewed in Ref. (34, 35)]. Release of proteins from those compartments is accomplished by a number of mediators, including IL-8, f-MLP, C5a, or phorbol ester (36). Because these compounds failed to mobilize T2R38, we looked for other storage compartments and found T2R38 in association with vesicles having the properties of lipid droplets. Lipid droplets were originally described as lipid storage sites, but more recent studies described them as functional organelles that actively participate in lipid turnover, cellular signaling, and membrane trafficking (37, 38). As far as we know, storage in or association with vesicles – lipid droplets or

others – has not been investigated for T2R38 in its usual habitat – that is, in cells of the taste buds – or in epithelial cells. Thus, the question remains whether or not storage of T2R38 in vesicles is typical for neutrophils or whether it is a general feature of T2R38 (our own not yet published data point to the latter).

Having established that T2R38 is present in neutrophils, the question was addressed whether it is the receptor for AHL-12 on these cells. LSM showed a co-localization of AHL-12 and T2R38, and the “pull-down-assay” with biotin-labeled AHL-12 indicated a physical interaction of AHL-12 with T2R38, which implies that T2R38 is indeed the receptor for AHL-12 on neutrophils.

To link T2R38 to AHL-12 signaling, the effect of antibodies to T2R38 on AHL-12-induced functions was tested. Only one of the antibodies (sc-34294) inhibited binding of AHL-12 to some extent, but not entirely. A likely explanation is the hydrophobic nature of AHL-12, which allows attachment to cells and probably also partition into the membrane in a receptor-independent manner. That the other antibodies – though binding to the cells – did not block potential AHL-12 binding sites is in line with data reported for ab65509 by others in another setting (32). As anticipated, these antibodies did not inhibit AHL-12-mediated activation of neutrophils.

The antibody sc-34294, which inhibited AHL-12 binding, stimulated neutrophils. The antibody by itself up-regulated CD11b cell surface expression, and it enhanced phagocytosis, two functions that are also induced by AHL-12. Thus, T2R38 is a functional receptor on neutrophils, and according to the co-localization data, AHL-12 is the most likely ligand.

Expression of T2R38 on neutrophils fits the concept that the cells “sense” bacterial infection, and up-regulate defense-relevant actions in consequence. In that, T2R38 qualifies as pathogen-associated molecular pattern recognition receptor. Because bacteria produce and release AHL-12 as an autoinducer, crucially required for biofilm formation, neutrophils will be attracted to the infectious site before or concurrent with biofilm formation.

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Because planktonic bacteria are in general more susceptible to host defense mechanisms compared to bacteria organized as biofilm, the infection can be cleared. Valid objections to this notion are that micromolar concentrations of AHL-12 are required to activate neutrophils – roughly 100- to 10,000-fold more than for established chemokines – and that the weak to moderate surface expression of T2R38 questions the effectiveness of an AHL-12-T2R38 interaction. However, according to our experience, AHL-12 is not very stable under cell culture conditions, meaning that the abundance of active molecules might be lower than the calculated input. In addition, local concentrations of AHL-12 could be rather high, as data using supernatants of biofilm-forming *P. aeruginosa* imply (20). Without question, the biological relevance of our finding is still under scrutiny.

Our data, however, establish for the first time extra gustatory expression of T2R38 on cells of the myeloid lineage, and are compatible with the notion that T2R38 functions as a receptor for AHL-12 on neutrophils as a novel means of recognizing of bacterial components by the host defense.

Author Contributions

All authors approved the final version of the manuscript, and contributed substantially to the study: SM did the cytofluorometry experiments, GW and SS the laser scan microscopy and SIM, NK the initial experiments on neutrophil activation and the uptake studies. BP performed the functional assays (chemotaxis and phagocytosis), TG did the quantitative PCRs, SM, MG, YS, GW, and GH did the data analysis and wrote the manuscript.

Acknowledgments

The discussion with the colleagues from the Karlsruhe Institut für Technology (KIT), Ursula Obst, Gerald Brenner-Weiss, Thomas Schwarz, and Jörg Overhage are gratefully acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Modulation of Host Biology by *Pseudomonas aeruginosa* Quorum Sensing Signal Molecules: Messengers or Traitors

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OPEN ACCESS

Edited by:

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Reviewed by:

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Mexico

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 09 September 2015

Accepted: 20 October 2015

Published: 09 November 2015

Citation:

Liu Y-C, Chan K-G and Chang C-Y (2015) Modulation of Host Biology by *Pseudomonas aeruginosa* Quorum Sensing Signal Molecules: Messengers or Traitors. *Front. Microbiol.* 6:1226.
doi: 10.3389/fmicb.2015.01226

Bacterial cells sense their population density and respond accordingly by producing various signal molecules to the surrounding environments thereby trigger a plethora of gene expression. This regulatory pathway is termed quorum sensing (QS). Plenty of bacterial virulence factors are controlled by QS or QS-mediated regulatory systems and QS signal molecules (QSSMs) play crucial roles in bacterial signaling transduction. Moreover, bacterial QSSMs were shown to interfere with host cell signaling and modulate host immune responses. QSSMs not only regulate the expression of bacterial virulence factors but themselves act in the modulation of host biology that can be potential therapeutic targets.

Keywords: Quorum sensing, *N*-acyl homoserine lactones, *Pseudomonas* quinolone signal, *Pseudomonas aeruginosa*, immunomodulation

INTRODUCTION

Quorum sensing (QS) is coined to describe the phenomenon of an intercellular co-operative behavior of bacteria used to coordinate the activities of individual cells. Diffusible QS signal molecules (QSSMs) play crucial roles in signal transduction of which, when QSSMs reach a threshold concentration, can coordinate multiple gene expression and a change in the behavior of bacterial population through the activation of sensor regulatory proteins (Fuqua et al., 1994; Miller and Bassler, 2001; Williams and Cámara, 2009). Bacteria produce a broad-range of signal molecules. Different types of QSSMs have been identified and characterized (LaSarre and Federle, 2013). Besides prokaryote, bacterial QSSMs also affect the settlement and germination of eukaryotic seaweed zoospores (Joint et al., 2002; Twigg et al., 2013). In several pathogenic bacteria, QS control their virulence determinants and contribute to bacterial pathogenesis. Due to the fact that the population density-dependent regulatory systems used by many bacterial pathogens are not essential for survival under most conditions, the disruption/interference of QS is considered an alternative approach to attenuate bacterial virulence in infections (LaSarre and Federle, 2013). However, this point has recently been argued that the resistance mechanisms against QS inhibitors could be possible and have been identified (Defoirdt et al., 2010, 2013; García-Contreras et al., 2013, 2015a).

Pseudomonas aeruginosa is an ubiquitous Gram-negative bacterium with remarkably large and complex genome and is capable of adapting to versatile environments. In human cystic fibrosis (CF) lungs where *P. aeruginosa* has evolved the ability to form biofilms which are difficult to be eradicated by antibiotics (Heeb et al., 2011; Winsor et al., 2011). QS is responsible for the regulation of a large number of genes, for instance, around 10% of genes in the genome of *P. aeruginosa* are regulated by QS (Williams and Cámara, 2009). Here we review recent advances of *P. aeruginosa* QSSMs focusing on their roles in interference with host cells (**Table 1**) and the development of novel compounds that counteract the QSSMs activities.

N-ACYL HOMOSERINE LACTONES (AHLs) AND THEIR MODULATIONS IN HOST CELLS

Gram-negative bacteria, like *Aliivibrio fischeri* (previous *Vibrio fischeri*; Urbanczyk et al., 2007), have a conserved QS system with two central components, the LuxR-type and LuxI-type proteins, which serve as the signal receptor and signal synthase, respectively. LuxI catalyzes the synthesis of signaling molecules called *N*-acyl homoserine lactones (AHLs). When an AHLs concentration of 10 nM is reached, AHLs interact with LuxR and form a complex which promotes the expression of target genes, *luxICDABE* for bioluminescence production and also the LuxI production (Kaplan and Greenberg, 1985). This forms a positive loop to produce more signal molecules (Fuqua et al., 1994; Cámara et al., 2002; Fuqua and Greenberg, 2002). The *N*-acyl homoserine lactone consists of a homoserine lactone ring from S-adenosylmethionine (SAM) and acyl chain from acyl acyl-carrier-protein (acyl-ACP) linked by an amide bond (Parsek et al., 1999). Based on the acyl-ACP binding site, different LuxI homologs produce different AHLs with various acyl side chains (Watson et al., 2002; Gould et al., 2004). A broad range of AHLs is produced in Gram-negative bacteria and AHL-QS systems control various bacterial behaviors (LaSarre and Federle, 2013). In *A. fischeri* *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) is produced for controlling bioluminescence production. In *P. aeruginosa* two AHL synthases, RhlI and LasI, produce a wide spectrum of AHLs including *N*-butanoyl-homoserine lactone (C4-HSL), *N*-hexanoyl-homoserine lactone (C6-HSL) by RhlI and *N*-(3-oxooctanoyl)-homoserine lactone (3-oxo-C8-HSL), *N*-(3-oxodecanoyl)-homoserine lactone (3-oxo-C10-HSL), *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL) and *N*-(3-oxotetradecanoyl)-homoserine lactone (3-oxo-C14-HSL) by LasI (Ortori et al., 2011). An unusual *N*-(3-oxohexadecanoyl)-homoserine lactone (3-oxo-C16-HSL) secreted by an environmental *Pseudomonas* sp. from a diseased Tilapia fish suggests that 3-oxo-C16-HSL may contribute to the pathogenesis (Chang et al., 2012).

The abundant concentration of 3-oxo-C12-HSL in the culture of *P. aeruginosa* prompted investigations for its role in the pathogenesis with a mechanism potentially distinct from other pathogens. Indeed, 3-oxo-C12-HSL was found to activate

mammalian cells through a mechanism independent of the toll-like receptor (TLR) pathways (Kravchenko et al., 2006). 3-oxo-C12-HSL was shown to activate pro-inflammatory responses in human epithelial and fibroblast cells through the induction the transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) via the phosphorylation of ERK/MARK (Smith et al., 2001). However, this molecule selectively disrupts the NF- κ B signaling pathway in activated macrophages (Kravchenko et al., 2008). Studies indicated that 3-oxo-C12-HSL not only induces apoptosis in haematopoietic cells but is cytotoxic to non-haematopoietic cells including airway epithelial cells, endothelial cells, fibroblasts, and mesenchymal stem cells (Tateda et al., 2003; Shiner et al., 2006; Schwarzer et al., 2010, 2012; Grabiner et al., 2014; Holban et al., 2014). *P. aeruginosa* 3-oxo-C12-HSL also impairs the epithelial barrier integrity through the alternations of calcium signaling and phosphorylation status of junctional proteins in the intestinal epithelial cells (Vikström et al., 2009, 2010).

In addition to its cytotoxicity, the role of 3-oxo-C12-HSL in immunomodulation has been intensively investigated (**Table 1**). Ritchie et al. (2005) reported that 3-oxo-C12-HSL inhibits the differentiation of Th1 and Th2 cells. Human polymorphonuclear neutrophils (PMNs) are attracted by 3-oxo-C12-HSL and increasingly express the adhesion proteins CD11b/CD18 and the immunoglobulin receptors CD16 and CD64 (Zimmermann et al., 2006; Wagner et al., 2007). The downregulation of the immune responses by 3-oxo-C12-HSL was demonstrated in human monocytes and murine macrophage-like cells in the presence of lipopolysaccharides (LPS) that 3-oxo-C12-HSL inhibits the production of pro-inflammatory cytokine tumor necrosis factor α (TNF- α) but promotes the production of anti-inflammatory cytokine interleukin-10 (IL-10; Hooi et al., 2004; Glucksam-Galnoy et al., 2013). Grabiner et al. (2014) noticed that despite the increasing transcriptional expression of the murine interleukin 8 (IL-8) homologs KC and interleukin 6 (IL-6) in murine embryonic fibroblasts (MEFs), KC and IL-6 protein secretion were inhibited by the treatment of 3-oxo-C12-HSL. It was shown that 3-oxo-C12-HSL acts upon the activation of endoplasmic reticulum (ER) stress transducer protein kinase RNA-like ER kinase (PERK) leading to the inhibition of protein synthesis. However, PERK is independent of 3-oxo-C12-HSL induced apoptosis indicating that 3-oxo-C12-HSL interferes with host cell biological activities through different mechanisms (Grabiner et al., 2014). Recent advances on the interactions between 3-oxo-C12-HSL and various types of host cells are highlighted in the review (Holm and Vikström, 2014).

Several host targets of 3-oxo-C12-HSL have been identified (**Figure 1**). In murine fibroblasts and human lung epithelial cells peroxisome proliferator-activated receptor beta/delta (PPAR β/δ) and PPAR γ may be the 3-oxo-C12-HSL receptors for pro-inflammatory responses (Jahoor et al., 2008; Cooley et al., 2010). 3-oxo-C12-HSL interacts and co-localizes with the IQ-motif-containing GTPase-activating protein IQGAP1 in human intestinal epithelial cells that causes the alternation of cell migration in a Rac1 and Cdc42-dependent manner (Karlsson et al., 2012). MEFs in lack of a transcriptional factor X-box binding protein 1 transcription factor (XBPs) are

TABLE 1 | Bacterial quorum sensing molecules and their roles in pathogenesis/immunomodulation.

Quorum sensing (QS) signal molecules (QSSMs)	Mechanism of virulence	Effect concentrations	Reference
3-oxo-C12-HSL			
	Apoptosis and cytotoxicity 3-oxo-C12-HSL is cytotoxic to murine bone-marrow derived macrophages, neutrophils and monocytic cell lines.	12–50 μ M	Tateda et al., 2003
	3-oxo-C12-HSL induces apoptosis in murine fibroblasts and human vascular endothelial cells (HUVEC)	100 μ M	Shiner et al., 2006
	3-oxo-C12-HSL triggers intrinsic apoptotic pathway in airway epithelial cells including depolarization of mitochondrial membrane potential, release of cytochrome C and activation of caspases 3/7, 8, and 9. 3-oxo-C12-HSL-mediated apoptosis is independent of the presence of CFTR in airway epithelial cells	> 10 μ M	Schwarzer et al., 2010
	3-oxo-C12-HSL promotes human mesenchymal stem cells (MSCs) apoptosis	50 μ M	Holban et al., 2014
	Disruption of barrier integrity <i>Pseudomonas aeruginosa</i> 3O-C(12)-HSL causes the loss of epithelial barrier function via calcium signalling and further alteration in the phosphorylation status of junction proteins	20–200 and 10 μ M with slower response	Vikström et al., 2009, 2010
	Immunomodulation and/or signaling		
	3-oxo-C12-HSL promotes the expression and production of IL-8 in human epithelial and fibroblast cells through the induction of NF- κ B via the phosphorylation of ERK/MAPK	100 μ M	Smith et al., 2001
	3-oxo-C12-HSL inhibits ConA-activated PBMCs proliferation and IL-2 secretion	aIC_{50} : 18.24 μ M	Hooi et al., 2004
	3-oxo-C12-HSL inhibits the proliferation of anti-CD3/anti-CD28 antibody activated T cells	IC_{50} : 44.47 μ M	Hooi et al., 2004
	3-oxo-C12-HSL inhibits the differentiation of Th1 and Th2 cells	5 μ M	Ritchie et al., 2005
	3-oxo-C12-HSL increases the cytosolic calcium levels and calcium release through inositol triphosphate (IP3) receptors in the ER.	1 mM	Shiner et al., 2006
	3-oxo-C12-HSL promotes neutrophil chemotaxis, phagocytosis and up-regulates the expression of CD11b/CD18 and CD16/CD64 receptors	100 μ M	Zimmermann et al., 2006; Wagner et al., 2007
	3-oxo-C12-HSL selectively disrupts NF- κ B signaling but not TLR-dependent pathways in activated macrophages	50 μ M	Kravchenko et al., 2006, 2008
	3-oxo-C12-HSL binds to PPAR γ ligand binding domain	25–50 μ M	Jahoor et al., 2008; Cooley et al., 2010
	3-oxo-C12-HSL increases the secretion of IL-1 β in human MSCs	50 μ M	Holban et al., 2014
	3-oxo-C12-HSL activates NF- κ B p65 by preventing the re-synthesis of i κ B, increases transcription of KC and IL-6 but inhibits secretion of KC and IL-6 by MEFs. 3-oxo-C12-HSL activates PERK and inhibits protein synthesis	50 or 100 μ M	Grabiner et al., 2014
Alkylquinolones	Change of bacterial behaviors		
	Bacterial autolysis	Spent culture supernatant 15 μ g on filter discs	Williams and Camara, 2009
	Iron chelation	50 μ M	Diggle et al., 2007
	exDNA release	Genetic and phenotype study	Allesens-Holm et al., 2006
	Oxidative functions	> 100 μ M <i>in vitro</i>	Häussler and Becker, 2008

(Continued)

TABLE 1 | Continued

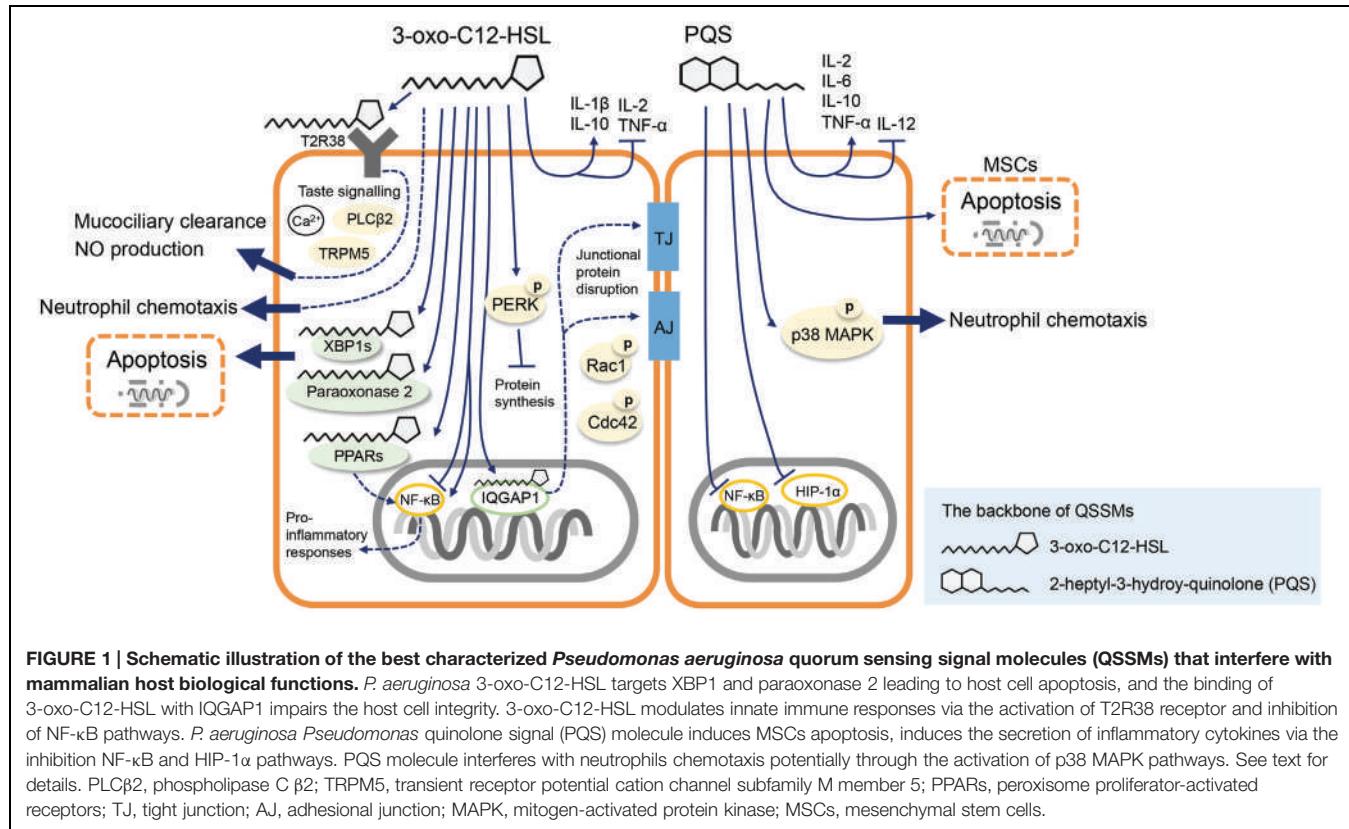
Quorum sensing (QS) signal molecules (QSSMs)	Mechanism of virulence	Effect concentrations	Reference
Apoptosis			
HhQ promotes human MSCs apoptosis		50 μ M	Holban et al., 2014
Immunomodulation and/or signaling			
<i>Pseudomonas</i> quinolone signal (PQS) inhibits the proliferation of ConA-activated PBMCs		IC ₅₀ : 0.90 μ M	Hooi et al., 2004
PQS affects IL-2 secretion of ConA-stimulated PBMCs		IC ₅₀ : 2.03 μ M	Hooi et al., 2004
PQS promotes TNF- α production in LPS-treated monocytes		>25 μ M	Hooi et al., 2004
Inhibition of IL-12 production in dendritic cells resulting in reduction of T-cell proliferation		IC ₅₀ : 17.2 μ M	Skindersoe et al., 2009
Inhibition of NF- κ B and HIF-1 α pathways in murine epithelial cells and murine macrophages		approx. PQS in PA14, 15 μ M approx. PQS in <i>pqsL</i> , 45 μ M	Kim et al., 2010; Legendre et al., 2012
PQS stimulates the secretion of IL-6 and HHQ induces IL-10 secretion by human MSCs		10–100 μ M	Hänsch et al., 2014
		50 μ M	Holban et al., 2014

^a50% inhibitory concentration (IC₅₀).

protective from 3-oxo-C12-HSL and C14-HSL (*N*-tetradecanoyl-homoserine lactone) mediated apoptosis indicating that XBP1s is a critical host target in response of AHLs (Valentine et al., 2013). Paraoxonase 2, in response to 3-oxo-C12-HSL through its lactonase activity, leads to apoptosis in human and murine embryonic epithelial cells (Schwarzer et al., 2015). Interestingly, 3-oxo-C12-HSL activates the expression of a taste receptor T2R38 on the surface of primary human sinonasal cells (Lee et al., 2014) and neutrophils (Maurer et al., 2015). This recognition regulates calcium-dependent NO production thereby stimulates the mucociliary clearance and antibacterial effects suggesting an alternative innate immune defense mechanism distinct from the activation by canonical pattern recognition receptors (PRRs; Lee et al., 2012, 2014). Identification of the host compartments targeted by QSSMs could be the milestone for developing effective therapeutic methods against infections.

PATHOGENIC ROLES OF ALKYL-QUINOLONE SIGNALS

Pseudomonas aeruginosa also employs the alkyl-quinolone (AQ)-based QS system and the signal molecule was termed *Pseudomonas* quinolone signal (PQS; Pesci et al., 1999). The study of the AQs began from their intriguing structures similar to antimicrobial quinolones, although AQs were found no antimicrobial activities. Further studies unveiled that among more than 50 alkyl-quinolones found in *P. aeruginosa*, 2-heptyl-3-hydroxy-4-(1H)-quinolone (PQS) and its precursor molecular 2-heptyl-4(1H)-quinolone (HHQ) are major QSSMs that cooperates with the AHL-QS (Xiao et al., 2006; Heeb et al., 2011). Synthesis of PQS depends on the *pqsABCDE* operon. PqsA, the anthranilate co-enzyme A ligase, catalyzes anthranilate that is produced by PhnAB to anthraniloyl-coenzyme A. PqsD mediates the synthesis of 2-aminobenzoylacetate (2-ABA) from anthraniloyl-coenzyme A and malonyl-CoA, decarboxylating coupling of 2-ABA to an octanoate group of octanoic acid that linked to PqsBC to produce HHQ (Dulcey et al., 2013). A recent study suggested PqsE is involved in the HHQ synthesis through hydrolysing the 2-ABA-CoA to form 2-ABA (Drees and Fetzner, 2015). HHQ can be transformed to PQS by the mono-oxygenase PqsH encoded by *pqsH* located elsewhere on the chromosome (Pesci et al., 1999; Diggle et al., 2006). PqsR, also known as MvfR, is a LysR-type transcriptional regulator, with a conserved N-terminal DNA-binding helix-turn-helix and a C-terminal co-inducer-binding domain. PqsR activates the transcription of *pqsABCDE* and possibly the *phnAB* operon when binding to PQS or HHQ and triggers the typical QS autoinducing response enhancing AQ biosynthesis (Maddocks and Oyston, 2008; Heeb et al., 2011). PQS has been shown to reach the maximal production at late logarithmic phase (Diggle et al., 2003) and its production is promoted by the availability of the substrate anthranilate and the presence of aromatic amino acids (Palmer et al., 2005). AQ- and AHL-QS in *P. aeruginosa* are hierarchical and involved in the regulation of multiple virulence factors including rhamnolipids, pyocyanin, elastases, exotoxin A, and alkaline protease (Xiao



et al., 2006; Dubern and Diggle, 2008; Nadal Jimenez et al., 2012).

Pseudomonas quinolone signal is considered a multifunctional molecule. PQS is involved in bacterial cell autolysis at high population densities in nutrient deprived conditions (Williams and Cámará, 2009). PQS also has iron-chelating properties that contribute to iron transport and facilitates siderophore-mediated iron delivery (Diggle et al., 2007). It has been demonstrated that there is far less extracellular DNA (exDNA) released by a *pqsA* mutant than its wild-type counterpart either in planktonic or biofilm cultures (Allesen-Holm et al., 2006). PQS has dual pro- and anti-oxidative functions for developing different levels of tolerance in *P. aeruginosa* cells to environmental stress (Häussler and Becker, 2008). This may shape the whole population structure, increase the fitness in hostile environments and lead to the development of resistance to host immune systems (García-Contreras et al., 2015a,b).

A new QS molecule, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) encoded by the *ambBCDE* operon was discovered recently (Lee et al., 2013). IQS is induced when *P. aeruginosa* is exposed to a phosphate-deprived environment. Under this unfavorable environment, expression of IQS overcomes the *las*-led QS circuit and promotes the expression of virulence factors. This finding may partially explain how *P. aeruginosa* clinical isolates persist in CF respiratory infections in the absence of a functional *las* system. Despite the fact that the AHL-QS of *P. aeruginosa* have been shown to play central roles

in the regulation of virulence and immune modulation *in vitro*, this situation could differ *in vivo*. Around 50% of strains isolated from lungs of late stage CF patients are deficient in *lasR* function (Winstanley and Fothergill, 2009). Moreover, abolishing the whole AHL-QS by generating a quadruple mutation of *rhlIR* and *lasIR* exerted comparable infectivity to the wild-type strain in a mouse lung infection model (Lazenby et al., 2013), suggesting that the AHL-QS may not be required for full pathogenesis *in vivo* and other regulatory mechanism could be involved.

The contribution of the AQ-QS system to *P. aeruginosa* virulence was firstly described by Cao et al. (2001). *PqsR* positively regulates the expression of *phnAB* operon and the production of elastase, 3-oxo-C12-HSL and PQS that promotes the production of numerous virulence determinants. The *pqsR* mutant was attenuated up to 320-fold in the *Arabidopsis* plant infection model and caused a 65% reduction of mortality in a murine burn wound model (Cao et al., 2001). The interaction of *PqsR* with the AHL-QS was investigated by other groups showed that the effect of *pqsR* deficiency on pathogenesis is independent from *LasR/RhlR* (Déziel et al., 2004; Dubern and Diggle, 2008). Mutations in the multidrug efflux pump, such as *mexI* and *opmD* led to the inhibition of PQS production and the attenuation of *P. aeruginosa* in rat and plant infection models. Provision of exogenous AQs to these mutants restored the virulence on plants (Aendekerk, 2005). Rampioni et al. (2010) found that both *pqsA* and *pqsE* mutants in PAO1 were attenuated in plant, nematode and mouse burn wound infection models. In an acute urinary tract infection model, PQS molecules were

present in the renal and bladder tissue of mice infected with wild-type *P. aeruginosa* but absent in the mice with PQS mutants infections (Bala et al., 2014). Wild-type *P. aeruginosa* caused more severe inflammation and tissue destruction and greater levels of inflammatory cytokines TNF- α , IL-6, and IL-10 at the site of infection in mice infected with wild-type strain than with PQS mutants. The virulence of PQS mutants can be restored by the addition of exogenous PQS molecules (Bala et al., 2014). These findings indicate that PQS participates in the pathogenesis of *P. aeruginosa*.

Pseudomonas quinolone signal has been identified in sputum, bronchoalveolar lavage fluid (BAL) and mucopurulent fluid from distal airways of end-stage CF lungs removed for transplant and at different stages from asymptotic early stage to late progression, suggesting a potential role of PQS in coordinating virulence factors during the course of infections (Collier et al., 2002; Guina et al., 2003). A study involving 60 CF patients with chronic *P. aeruginosa* infection indicated that the AQs were detectable in the sputum, plasma and urine and the concentrations of molecules are positively correlated to the *P. aeruginosa* bacterial cell density. 2-nonyl-4-hydroxy-quinoline (NHQ) in plasma was suggested to be the biomarker for *P. aeruginosa* infection in CF lungs (Barr et al., 2015). An *in vitro* transcriptomic study investigating the physiology of *P. aeruginosa* grown in CF sputum revealed that the genes associated with PQS metabolism, such as those coding for the aromatic amino acid aminotransferase, 4-hydroxyphenylpyruvate dioxygenase (*hpd*) and *pqsABCDE*, were expressed 10-fold greater than the expression when *P. aeruginosa* was cultured in media containing glucose alone as the carbon source (Palmer et al., 2005).

Pseudomonas aeruginosa AQ molecules have been implicated in the immuno-modulation on host cells. PQS was shown to modulate cell proliferation, the production of interleukin-2 (IL-2) and TNF- α in mitogen-stimulated human peripheral blood mononuclear cells (PBMCs; Hooi et al., 2004). PQS inhibited the production of IL-12 by LPS-stimulated bone marrow-derived dendritic cells which led to reduced T-cell proliferation (Skindersoe et al., 2009). Additionally AQ extracts derived from *P. aeruginosa* PA14 supernatants down-regulated host innate immune responses via inhibition of the NF- κ B and hypoxia-inducible factor 1 alpha (HIF-1 α) pathways in murine macrophages and cells obtained from BAL (Kim et al., 2010; Legendre et al., 2012). A recent study addressed the importance of timing in neutrophil infiltration in relation to the role of PQS in interference with neutrophil chemotaxis. Low levels of PQS stimulated the chemotaxis of neutrophils via the MAPK and p38 signaling pathways, whereas high levels of PQS, most likely produced by biofilm-like *P. aeruginosa*, did not interfere with neutrophils phagocytic capability and viability (Hänsch et al., 2014). Massive neutrophil accumulation is commonly seen in CF airways and high levels of neutrophil elastase correlate with poor pulmonary functions (Downey et al., 2008; Gifford and Chalmers, 2014). PQS may thus provide *P. aeruginosa* with another strategy for bacterial survival via the interference in multiple aspects of host biological activities.

THE DEVELOPMENT OF INHIBITORS AND VACCINES AGAINST QSSMs

Due to the fact that QSSMs have been implicated in the involvement of pathogenesis, the search for inhibitors and the development of vaccines that antagonize QSSMs are currently intensively investigated. Chang et al. (2014) suggested a strategy to screen novel anti-QS compounds from plant extraction that potentially could tackle the QS-mediated infections. The antibody 3-oxo-C12-HSL-BSA conjugate was also shown to alleviate the inflammatory responses by *P. aeruginosa* infections in an acute murine lung infection model (Miyairi et al., 2006). In a burn wound infection model, mice immunized with the vaccine 3-oxo-C12-HSL-r-PcrV conjugate before *P. aeruginosa* infection had higher survival than those without immunization (Golpasha et al., 2015). A high-throughput screening approach based on the inhibition of C12-mediated host responses identified triazolo[4,3-a]quinolines as 3-oxo-C12-HSL inhibitors with nanomolar potency that restore NF- κ B activity in 3-oxo-C12-HSL treated cell lines and shown protective using an *in vivo* dermal infection model (Valentine et al., 2014). Since anthranilate (AA) being the precursor of AQs, halogenated AA analogs were found to inhibit the AQ biosynthesis and down-regulate the expression of PqsR controlled genes. Treatment with AA analogs prior to *P. aeruginosa* infection increased mice survival and lowered the bacterial dissemination to the organs (Lesic et al., 2007). MvfR-regulon inhibitors that bind QS transcriptional regulator MvfR (PqsR) were not only protective in murine acute and persistent infections against *P. aeruginosa* but also effectively reduced the formation of antibiotic-tolerant persisters (Starkey et al., 2014). These studies suggest the therapeutic potential of inhibitors and vaccines against QSSMs in both acute and chronic infections.

CONCLUSION

Quorum sensing-based bacterial communication links the individual bacterial cells to behave as multicellular organisms by employing signal molecules and to promote its population survival in the environment or hosts. QSSMs also interact with host cell signal pathways and the modulation of immune cell biology. For more than a decade strategies have been proposed from the use of inhibitors of QS for containing chronic infections (Hentzer et al., 2003) to the application of QSSMs for modulating immune responses to bacterial infections (Hancock et al., 2012). Understanding how QSSMs interact with host cells seems the promising land to tackle bacterial infections. Here we discussed recent advances on the interference of QSSMs with mammalian cells, the recently identified receptors on mammalian cells that target QSSMs and the QSSM inhibitors and their mechanisms. However, contradictory results suggested that many unknown mechanisms in complex bacteria-host interactions are remained. Also concern about bacterial resistance to QS inhibitors (García-Contreras et al., 2015a) and unexpected impact of QS inhibitors to environment (Decho et al., 2010) is increasing. Extending our understanding of the multiple roles of QSSMs would be valuable

in the development of new therapeutic strategies against bacterial infections.

AUTHOR CONTRIBUTIONS

Y-CL, K-GC, C-YC wrote the paper. Y-CL made the figure. Y-CL and C-YC made the table.

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ACKNOWLEDGMENTS

We are grateful for the Open Access Funding from the University of Dundee. This work was also supported by the University of Malaya High Impact Research Grants (UM C/625/1/HIR/MOHE/CHAN/01, A-000001-50001, and UM C/625/1/HIR/MOHE/CHAN/14/1, H-50001-A000027) awarded to K-GC.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Staphylococcal Superantigens Spark Host-Mediated Danger Signals

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OPEN ACCESS

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Immunology

Received: 01 October 2015

Accepted: 18 January 2016

Published: 02 February 2016

Citation:

Krakauer T, Pradhan K and Stiles BG (2016) Staphylococcal Superantigens Spark Host-Mediated Danger Signals. *Front. Immunol.* 7:23.
doi: 10.3389/fimmu.2016.00023

Staphylococcal enterotoxin B (SEB) of *Staphylococcus aureus*, and related superantigenic toxins produced by myriad microbes, are potent stimulators of the immune system causing a variety of human diseases from transient food poisoning to lethal toxic shock. These protein toxins bind directly to specific V β regions of T-cell receptors (TCR) and major histocompatibility complex (MHC) class II on antigen-presenting cells, resulting in hyperactivation of T lymphocytes and monocytes/macrophages. Activated host cells produce excessive amounts of proinflammatory cytokines and chemokines, especially tumor necrosis factor α , interleukin 1 (IL-1), IL-2, interferon γ (IFN γ), and macrophage chemoattractant protein 1 causing clinical symptoms of fever, hypotension, and shock. Because of superantigen-induced T cells skewed toward TH1 helper cells, and the induction of proinflammatory cytokines, superantigens can exacerbate autoimmune diseases. Upon TCR/MHC ligation, pathways induced by superantigens include the mitogen-activated protein kinase cascades and cytokine receptor signaling, resulting in activation of NF κ B and the phosphoinositide 3-kinase/mammalian target of rapamycin pathways. Various mouse models exist to study SEB-induced shock including those with potentiating agents, transgenic mice and an “SEB-only” model. However, therapeutics to treat toxic shock remain elusive as host response genes central to pathogenesis of superantigens have only been identified recently. Gene profiling of a murine model for SEB-induced shock reveals novel molecules upregulated in multiple organs not previously associated with SEB-induced responses. The pivotal genes include intracellular DNA/RNA sensors, apoptosis/DNA damage-related molecules, immunoproteasome components, as well as antiviral and IFN-stimulated genes. The host-wide induction of these, and other, antimicrobial defense genes provide evidence that SEB elicits danger signals resulting in multi-organ damage and toxic shock. Ultimately, these discoveries might lead to novel therapeutics for various superantigen-based diseases.

Keywords: SEB, superantigens, toxic shock, damage response, therapy, animal models

THE BACTERIUM: *Staphylococcus aureus*

Staphylococcus aureus causes many human and animal diseases, some of them are life-threatening and found throughout the world (1–4). Humans are naturally colonized by *S. aureus* that often cause no problems and are considered a “harmless” commensal. However, when given an opportunity (i.e., weakened immune system, compromised epidermis, or mucosa, etc.), this bacterium can cause various diseases that become life threatening.

Staphylococcus aureus is a Gram-positive coccus and member of the Micrococcaceae family, which includes non-pathogenic genera found in soil, water, and on skin. Both planktonic- and biofilm-based versions of *S. aureus* can cause disease in an infected host, subsequently becoming (at times) quite difficult to clear from the body (5, 6). Characteristics of this genus called *Staphylococcus* ("staphyle" in Greek, meaning grape cluster and "coccus" meaning grain or berry) and species called *aureus* ("golden" in Latin, as per colony color on agar media), include Gram-positive, non-spore forming, facultative, β -hemolytic, catalase-positive, mannitol fermentation, and salt tolerant (7).

Staphylococcus aureus is known for producing many different protein toxins involved in pathogenesis. Clearly, the bacterium is very good at surviving harsh conditions in/on a host by using a vast array of virulence factors that promote the many diseases caused by infiltrating *S. aureus* and their toxins. One group of toxins, the staphylococcal enterotoxins (SEs), is the focus of this review. Antibiotic-resistant strains (community-, nursing home-, and hospital-acquired) are a particular problem in health care and its associated economics, requiring effective infection control plans that prevent *S. aureus* spread locally and globally (8).

Staphylococcus aureus was first described in the early 1880s by a Scotsmen, Dr. Alexander Ogston, after microscopy analysis of over a hundred, pus-filled abscesses of human origins (9). *S. aureus* readily colonizes mammalian epidermis/dermis, mucosa, soft tissues, bone, and medical devices such as catheters. Furthermore, medically relevant strains of the bacterium have become variably resistant to many antibiotics, including methicillin and vancomycin, which negatively impacts psychological and economic aspects of human society (10–13). *S. aureus* strains that are methicillin resistant (MRSA) are commonly treated first with vancomycin. Both methicillin and vancomycin target bacterial cell-wall synthesis. Other antimicrobials that have been used for fighting *S. aureus* include (1) linezolid (inhibits protein synthesis at the 50S ribosome); (2) daptomycin (inserts into membranes); (3) fusidic acid (inhibits protein elongation at the ribosome); (4) teicoplanin (like vancomycin, inhibits cell wall synthesis but not used in the United States); and (5) tigecycline (inhibits protein synthesis at the 30S ribosome) (14, 15).

For various reasons, alternative methods for treating antibiotic-resistant strains of *S. aureus* (i.e., MRSA) are being explored by different groups around the world (16–23). Some of the exotic sources for these novel drugs include snake venom, tree bark/stems/leaves, and carnivorous plants. Risk factors for MRSA colonization include antibiotic exposure, admittance into an intensive care unit, surgery, long-term care residency, and exposure to others carrying MRSA (4, 24). In 2011, the United States Centers for Disease Control and Prevention reported >80,000 MRSA cases that were life threatening, with >11,000 fatalities (14%) (22, 25). This is remarkably a number of deaths greater than that attributed to acquired immunodeficiency syndrome (AIDS) for the same time period. Furthermore, the economic burden of MRSA in the United States is upwards to \$13.8 billion/year and represents one of the most costly, acute infectious diseases requiring treatment (10). Invasive forms of MRSA include the following in order of prevalence: bacteremia, pneumonia, cellulitis, osteomyelitis, endocarditis, and septic shock (24). Black males

older than 65 years are the most common patients suffering from invasive MRSA; however, the reasons for this are to date unknown (24). MRSA from hospital/health-care settings are perhaps the origins of community strains now seen throughout the human population (4). The latter types of *S. aureus* are often more susceptible to antibiotics than those from hospital/health-care settings.

From a historical perspective, MRSA was detected within a year after introducing this new beta-lactam antibiotic called methicillin in 1959, further highlighting the rapid adaptability of this microbe (26, 27). Methicillin was meant to overcome the increasingly prevalent, penicillin-resistant strains of *S. aureus* first detected during the mid-1940s in hospitals and throughout the community. These strains were isolated just 2 years after penicillin's introduction into clinical practice (27). This pattern of antibiotic resistance is evident with various pathogens, suggesting furthermore the importance of developing truly novel antimicrobials and embracing a paradigm shift for discovering/creating antimicrobials. Perhaps vaccines will play a bigger role in fighting various pathogens, in the future?

Transmission of antibiotic-resistant strains between livestock and humans working with and/or consuming the former is a real problem, poorly understood to date (28–31). Furthermore, aerosol transmission of MRSA from farm animals or human patients in hospital wards, to humans, is also a scary reality (32–34). As just one known example of *S. aureus* transmission between species, strain CC398 was most commonly associated with asymptomatic colonization of swine, but now causes invasive infections of humans (35). Evidently, farm animals are an important reservoir for disease-causing *S. aureus* that colonize humans. Humans (~30%) can be asymptomatic carriers of *S. aureus* strains that possess virulence factor genes encoding antibiotic resistance, toxins, and other proteins that promote some nasty, potentially fatal diseases (1, 3, 36–40) (Table 1). The risk factors are many for children carrying MRSA nasally, and include (1) <4 years of age; (2) being male; (3) family size >4; (4)

TABLE 1 | *Staphylococcus aureus*: some virulence factors and diseases.

Virulence factors

Antibiotic resistance (multiple mechanisms)
Biofilm/capsule
Coagulase
Exfoliative toxin
Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)
Plasminogen activator
Pore-forming toxins (hemolysins and leukocidins)
Quorum sensing mechanism
Superantigens (enterotoxins and toxic shock syndrome toxin-1)
Toll/interleukin 1 receptor (TIR)-like protein

Diseases

Bacteremia
Endocarditis
Osteomyelitis
Pneumonia
Pyoarthritis
Skin and soft tissue infections (boils, cellulitis, impetigo, scalded-skin syndrome, stye)
Toxic shock syndrome

antibiotic use during previous 3 months; (5) smoking by parents; and (6) sleeping with parents (39). For adults, risk factors that enhance carriage of *S. aureus* include: (1) being male; (2) having diabetes; (3) use of hormonal contraception by women; (4) close physical contact with others (i.e., sports); and interestingly (5) being a non-smoker (40).

Clearly, *Homo sapiens* are not strangers to hosting *S. aureus* on, and in, themselves. This bacterium is rather adaptable and employs various virulence factors seemingly important in its survival. Are we, in the near and distant future, able to co-adapt and effectively counter this microbial medical challenge, which is just one of many? We now focus upon one remarkable virulence factor, a family of protein toxins that vary in structure and biological effects upon mammals: the SEs.

THE STAPHYLOCOCCAL ENTEROTOXINS

From a historical perspective, the SEs (and *S. aureus*) were first described as “ptomaine” (derived from Greek “ptoma,” meaning corpse) in 1914 by Barber (41), after human consumption of cow’s milk contaminated by staphylococci (from the cow) led to vomiting and diarrhea of unsuspecting visitors. Family members were remarkably resistant to this poisoning effect, perhaps due to repeated exposure and development of toxin-specific immunity. Barber included himself as a “human guinea pig” to prove his point that milk from this one farm, during the hot season in the Philippines, was indeed the medium for enteric illness. His final analysis revealed that short-term storage of the milk at ambient temperature enabled staphylococcal growth and toxin production (41).

Dack et al. reported in 1930 an enteric-acting toxin produced by a “yellow hemolytic *Staphylococcus*” isolated from sponge cake involved in food poisoning (42). Upon administering culture filtrate of the isolated bacterium grown in the laboratory to a rabbit (intravenous) or humans ($n = 3$; oral), there was respectively death and uniform nausea/diarrhea. Some of the SEs were first purified by various groups during the 1950s (43–45), further linked to a major cause of food poisoning evident around the world, and found to stimulate specific subsets of T cells leading to shock (1, 46, 47).

Regarding staphylococcal food poisoning and diagnosis, the latter can be done upon suspected food/drink using classic microbiology and agar plates (10^5 viable *S. aureus*/gram food), or by direct detection of toxin (47). SE detection in food is most commonly accomplished by employing immunological techniques (i.e., commercially available enzyme/fluorescent immune assays) that are to date limited in detecting relatively few toxin types. However, next generation assays involving mass spectrometry are now being developed for the future pending higher throughput and cost-effectiveness (47).

The SEs are characteristically stable proteins resistant to high heat, low pH, freezing, drying, and proteases. Partly because of their stable nature and powerful effects upon humans at very low doses, the SEs (particularly SEB) have been studied from a biodefense perspective spanning decades of research by various countries (48, 49). SEB is also considered a Category B select agent by various United States federal agencies. SEB, when inhaled,

can induce several symptoms within 120 min involving an aching feeling (head and muscles), increased heartbeat, coughing, enteric dysfunction (i.e., nausea, vomiting, and diarrhea), as well as eye irritation (49). Nanogram levels of inhaled SEB are incapacitating while microgram levels can be fatal. SEB adversely affects the immune system, and it is plausible that opportunistic viruses and bacteria can cause further harm to the host exposed to any SE (50–52).

Upon ingestion, the SEs (A–V and counting) are responsible for a prevalent form of food poisoning globally (1, 38, 46, 47, 53). There are five, sequence-based groups of SEs generally recognized to date (1). These single-chain proteins (~25 kDa) are generally composed of two domains containing both alpha helices and beta sheets, separated by a groove, as evidenced by one of the first SEs to be crystallized: SEB (54). Since the mid-2000s, the literature has also adopted the designation of SE-like (SEL) (55). This defines a staphylococcal protein that shares amino acid sequence homology with a previously characterized SE, yet has not to date been tested (or at least reported in the literature) for enteric effects in primates (56). Regarding staphylococcal food poisoning, SEA is the most commonly detected culprit in the United States, followed by SED and SEB. Classic poisoning due to food-based SEs can occur after ingesting processed meats or dairy products contaminated by improper handling (i.e., *S. aureus* transfer from the skin of a food handler to food) and subsequent storage of food at an elevated temperature conducive to bacterial growth. Depending upon the *S. aureus* strain, there can be one (or more) SEs released into the tainted food. Following consumption of SEs in food/drink, one may still experience a general malaise 24–72 h later (56). Poisoning by the SEs via many different food types is rarely fatal for healthy individuals, and occurs around the world; however, as is often the case with many diseases, the very young and old represent higher risk groups for severe morbidity and possible death due to SE exposure (57).

Exactly how the SEs cause enteric illness is still remarkably unresolved, but prostaglandins and leukotrienes might mediate the effects (58, 59). Serotonin release in the intestines and vagal nerve stimulation may also be involved in SE-induced emesis (60, 61). The mode of action is seemingly more complex than many other enterotoxins that work directly upon gastric epithelial cells and/or supporting matrix.

The term “superantigen” commonly describes the *S. aureus* SEs and toxic shock syndrome toxin-1 (TSST-1), while related streptococcal pyrogenic exotoxins (SPEs), also known as superantigens, are produced by *Streptococcus pyogenes*. Superantigen was first used in late 1980s literature to describe microbial proteins that activate a large population of specific T-cells at very minute (picogram) concentrations (62, 63). Typical “conventional” antigens stimulate far fewer T-cells at higher concentrations. Superantigens and conventional counterparts also differ by: (1) superantigens bind to the exterior of the peptide-binding groove of major histocompatibility complex class II (MHC II); (2) superantigens bind to different MHC II types; (3) superantigenic effects occur without internalization and antigen processing; and (4) T-cell receptor (TCR) recognition of a superantigen: MHC II complex requires the variable region of a TCR β chain (Vβ), not the Vα–Vβ chain used by conventional antigens (62–67). From a

virulence perspective, superantigens corrupt host immunity that then enables pathogen survival.

Microbial superantigens are reportedly produced by other bacterial genera (*Mycobacterium*, *Mycoplasma*, *Yersinia*), fungi (*Candida*), and even viruses (herpes, rabies), thus suggesting a conserved and successful strategy employed throughout Nature (68). Versus superantigens of *Staphylococcus* and *Streptococcus*, those from the other aforementioned microbes are poorly characterized to date.

RECEPTOR BINDING AND SIGNAL TRANSDUCTION

Superantigens interact with both CD4 and CD8 T-cells and the signaling, post-TCR binding, is similar to conventional antigen binding to TCR; however, binding is to specific V β regions of TCR unlike conventional antigens. In addition, the interaction of superantigen with MHC class II on antigen-presenting cells (APC) is also different, with superantigens binding outside the peptide-binding groove of MHC class II. Structural and binding studies indicate at least two different binding sites on MHC class II for SE and TSST-1. A common overlapping binding region exists on HLA-DR for these toxins, referred to as the generic MHC class II binding site involving the α chain. An additional binding site with higher affinity can be found within the C-terminus of SEA/SED/SEH, which binds to the HLA-DR β chain in a Zn $^{2+}$ -dependent manner (69–71). The presence of two MHC binding sites allows SEA to cross-link MHC class II on APC and activate monocytes, inducing potent proinflammatory mediators. The binding of staphylococcal superantigens to MHC class II, and bridging to TCR, activates a high percentage of T cells. Two decades of intense investigations focused upon superantigen binding to cellular receptors reveal how superantigens activate cells of the immune system (46, 62, 72–76). Interaction of superantigen with TCR transmits the classical first signal for T-cell activation. The binding of superantigen to costimulatory receptors such as CD28 promotes supramolecular clusters, stabilizes cellular interaction, and optimizes protein kinase signal transduction (77). CD28 co-stimulation enhances mRNA stability of IL-2 and T-cell survival by increased expression of anti-apoptotic Bcl-xL (78). Other cell-surface molecules such as CD2, CD11a/ICAM-1, and ELAM facilitate optimal activation of endothelial and T cells by SEB (79). TCR and costimulatory receptors activate protein tyrosine kinases (PTKs), LCK and ZAP-70, resulting in phospholipase C gamma (PLC γ) activation, the release of intracellular second messengers, and subsequent protein kinase C (PKC) activation (67, 80, 81). Accompanying this T-cell activation is F-actin polymerization and increased intracellular Ca $^{2+}$. PTK and PKC activation lead to mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase (ERK), and cJun N-terminal kinase (JNK) pathways that activate transcriptional factors NF κ B, NF-AT, and AP-1 (67, 81, 82). PKC θ activation leads to CARMA1, Bcl10, and MALT1 (CBM) complexes that induce NF κ B transcriptional activation and controls T-cell proliferation (83). Many proinflammatory cytokine genes contain NF κ B-binding sites in their promotor region, and are induced

by NF κ B (84). The NF κ B cascade is a major signal transduction pathway for many pattern recognition receptors (PRR), such as toll-like (TLR) and proinflammatory cytokine receptors (85). The cytokines IL-1, TNF α , IFN γ , IL-2, IL-6 and chemokines, specifically MCP-1, are induced by superantigens (74, 75, 86). IL-1 and TNF α also activate fibroblasts, epithelial and endothelial cells to induce NF κ B and additional mediators, cell adhesion molecules, and tissue proteases (87). Mediators produced by superantigen-activated cells exert profound damaging effects on the immune and cardiovascular system, culminating in multi-organ dysfunction and lethal shock. PTKs and T-cell cytokines also activate phosphoinositide 3 kinase (PI3K), signaling protein kinase B (Akt), and mammalian target of rapamycin complex 1 (mTORC1) downstream (88). Two signaling molecules, NF κ B and mTORC1, are key hubs mediating the major biological responses to SE from TCR, co-stimulator CD28, and cytokine signaling.

PI3K/MTORC1 PATHWAY IN T-CELL ACTIVATION

T-cell activation via the TCR-CD3 complex subsequently activates membrane proximal PTKs that phosphorylate TCR intracellular components, other cellular substrates, as well as adaptors (67, 80–82). PLC γ cleaves phosphatidylinositol 4,5-bisphosphate, generating second messengers to activate PKC and the proto-oncogene Ras. PTK also activates PI3K, generating several inositol phospholipids and ultimately activating Akt and PKC θ (89). A number of receptors besides TCR, including those for CD28, IL-2 (IL-2R), insulin, growth factor, and the G-protein-coupled receptor (GPCR), also transduce activation signals upon ligand binding via the PI3K pathway. The cytosolic signalsome formed by PKC θ and CBM is located at an immunological synapse formed after T-cell activation by anti-CD3 and -CD28 (89, 90). CBM complexes activate the inhibitor of κ B (IkB) kinase complex (IKK) through ubiquitin ligases (91). IKK phosphorylates IkB, releasing NF- κ B for nuclear translocation and gene activation. Nuclear NF κ B binds DNA coding for proinflammatory cytokines and many other NF κ B target genes, activating diverse proinflammatory mediators, as well as pro- and anti-apoptotic molecules.

Toxic shock syndrome toxin-1 induces inositol phospholipid turnover, PKC translocation, and calcium mobilization in human T-cells resembling responses from those of a mitogenic signal (92). SEE uses an alternative LCK-independent pathway by activating PLC β signaling in T cells (93). This alternative pathway also triggers influx of Ca $^{2+}$, activates PKC, ERK1/2, and ultimately nuclear translocation of NF κ B and NF-AT.

Downstream of PI3K is the serine/threonine kinase Akt, which mediates many diverse biological processes upon specific binding to TCR, growth factors, insulin receptor, tyrosine kinase receptor, and GPCR. Two potent cytokines from superantigen-stimulated T cells, IFN γ and IL-2, also activate the PI3K/Akt/mTORC1 pathway via the transducer Janus kinase 1 (JAK1) after binding IFN γ and IL-2 receptors, respectively (94, 95). Site-specific phosphorylation/dephosphorylation modulates the PI3K/Akt signaling to activate mTORC1. Activation of mTORC1 causes phosphorylation and activation of the ribosomal 40S

protein p70S6 kinase (p70S6K) plus eukaryotic initiation factor binding protein 1 (4EBP1) (96–101). Thus mTORC1 controls protein translation essential for G1 to S phase transition (100). An mTORC1-specific inhibitor, rapamycin, blocks SEB-induced T cell proliferation as well as SEB-induced IL-2 and IFN γ *in vitro* and *in vivo* (102). Importantly, inhibition of the SEB-induced PI3K/Akt/mTORC1 pathway by rapamycin prevents lethal toxic shock in a mouse model (102).

INFLAMMATORY MEDIATORS, TCR/COSTIMULATORS, AND MHC CLASS II CROSS LINKING ACTIVATE NF κ B

In vitro and *in vivo* studies reveal that excessive release of proinflammatory cytokines (IL-1, TNF α , IL-6, and IFN γ) mediates the toxic effects of superantigens (49, 51, 52, 76, 79). Proinflammatory cytokines like IL-1 and TNF α activate the transcriptional factor NF κ B in many cell types, thus perpetuating the inflammatory response. IL-1 and TNF α have tissue damaging effects, and together with SEB-induced metalloproteases (MMP) and the pro-coagulant tissue factor (TF), promote inflammation and coagulation (87, 103). IL-2 from superantigen-activated T cells causes vasodilation, vascular leak, and edema.

Many cell types respond directly to staphylococcal superantigens besides T and APC/monocytes, including B, endothelial, synovial fibroblasts, intestinal epithelial, and mast (58, 79, 104–109). Cross-linking of TCR with MHC class II on B cells by superantigen triggers B cell proliferation and differentiation into plasma cells (104). Stimulation of synovial fibroblasts with superantigens induces chemokine gene expression, raising the possibility that superantigens can trigger chemotactic responses and initiate inflammatory arthritis (105). Human T84 colonic cells increase ion flow after incubation with SEB and PBMC, suggesting that superantigens indirectly affect gut mucosa *via* the immune system (106). SEB and TSST-1 transcytose intestinal epithelial cell barriers, unlike SEA (107), and TSST-1 can also induce chemotactic cytokines (IL-8 and MIP-3 α) from human vaginal epithelial cells (109). *S. aureus* frequently colonizes vaginal mucosa, producing TSST-1, which penetrates the mucosa through chemokines from epithelial cells (110). Chemokines recruit neutrophils and other immune cells activated furthermore by superantigens. The potent activation of T cells by superantigen interaction of MHC class II and TCR induces T-cell proliferation *via* cell activation pathways in T cells and APC. The proliferative response as a result of IL-2 induction from superantigen-activated T cells is similar to that induced by mitogens. IL-2 increases vascular permeability, causing edema and multiple organ damage (111). TNF α synergizes with IL-2 to induce pulmonary vascular leak and lymphocyte accumulation (112). The combination of TNF α and IFN γ elaborated by SEB-activated cells disrupt the epithelial barrier, causing edema and vascular leak in gut mucosa (106).

Interleukin 1 interacts with IL-1 receptor 1 (IL-1R1) and an additional accessory protein, triggering downstream signaling molecules like the adaptor myeloid differentiation factor 88 (MyD88), IL-1R-associated protein kinase 1 (IRAK1), and TNF receptor-associated factor 6 (TRAF6) that activate NF κ B

(113). A set of structurally related receptors, the TLRs, signal with similar intracellular adaptors as those used for IL-1R1, but are not used for superantigen signaling (85, 114). The TLRs are conserved, type-1 transmembrane receptors that recognize pathogen-associated molecular patterns (PAMPs), such as lipoprotein, peptidoglycan, LPS, flagellin, dsRNA, and viral RNA, which stimulate host innate immunity and enhance adaptive immunity (85, 114). SEB reportedly increases cellular expression of TLR2 and TLR4, synergistically promoting lethal shock with LPS (115, 116). Activation of NF κ B induces proinflammatory genes as well as pro- and anti-apoptotic genes. An auto-feedback loop exists to downregulate NF κ B, which induces I κ B α . Other cytosolic PRR sense intracellular damage-associated molecular patterns (DAMPs), triggering inflammasome activation and release of caspases plus IL-1 β (117).

TNF α binds to TNF receptor 1 (TNFR1) or TNFR2, activating NF κ B and inducing expression of other cytokines, adhesion, as well as co-stimulatory molecules (118, 119). The cytotoxic functions of TNF α are mostly mediated by binding TNFR1 *via* intracellular death domains that trigger apoptosis through caspase activation. SEB increases expression of CD95 (Fas), a receptor of the TNFR superfamily. Intracellular adaptors TNFR-associated death domain (TRADD), Fas-associated death domain (FADD), and receptor interacting protein kinase (RIP) are used by the TNFR superfamily to activate the caspase 8 cascade, JNK, and NF κ B that subsequently elicit apoptosis, cell activation, coagulation, inflammation, and host defense (119). TNF α and IFN γ act synergistically on epithelial cells to increase ion transport, causing cell damage and epithelial leakage (120). The critical role of TNF α in mediating pathological effects of SEB and lethality was recognized early on as anti-TNF α antibodies confer protection from SEB-induced shock in a D-galactosamine (D-gal)-sensitized mouse model (121). Both IL-1 and TNF α enhance the procoagulant activity of TF and activate neutrophils, accounting for tissue damage and organ injury commonly seen in animal models of septic shock and SEB-induced lethal shock.

Type II interferon (IFN γ) binds to IFN γ R, which belongs to the family of interferon receptors (IFNR), including the structurally different receptors for type I interferons (IFN α , IFN β) (122, 123). The signal transducer and activator of transcription 1 (STAT1) is a common signal transducer for both types of IFNR, and phosphorylation of STAT1 by Janus kinases initiates signal transduction. IFN α and IFN β induce indistinguishable signals, which include anti-proliferative and antiviral activities. Types I and II IFNs have many overlapping activities and stimulate many common interferon-stimulated genes (ISG) (123, 124). However, in addition to host-defense functions, IFN γ also induces immunoproteasomes and expression of MHC class II. IFN γ promotes cell-mediated immunological activities essential for antibacterial defense. The IFN γ -activated JAKs also activate PI3K in a STAT1-independent manner culminating in mTORC1 activation, promoting protein translation (125). IFN γ activates PKC leading to MAPK activation, which is also commonly activated by IL-1, TLR ligands, and TNF α . IFN γ has a critical role in host defense as the induction of immunoproteasome components and antigen-processing peptidases enhance cellular immune responses against pathogens. Both types of IFNs induce apoptosis and many

ISGs have antiviral, anti-angiogenic, and immunomodulatory functions. IFNs induce apoptosis by activating death receptors such as Fas (CD95), which then activates the adaptor FADD, leading to subsequent caspase-8 activation. Activation of the caspase 8 cascade causes cell death plus release of cytochrome c and mitochondrial DNA (mtDNA). SEA-induced hepatotoxicity is mediated by FasL and the hepatocellular damage is independent of leukocyte recruitment (126). IFN γ disrupts barrier function and ion transport in superantigen-activated epithelial cells. *In vitro*, disturbances in epithelial barrier function can be duplicated with IFN γ plus TNF α (106). Recent studies suggest that IFN γ downregulates regulatory T-cells (Treg), accounting for the potent polarizing effects of IFN γ on TH1/TH2 cell differentiation and an inflammatory environment for cell migration and activation (127).

IL-2 binds to IL-2R and activates PI3K and Ras (128). Activation of the PI3K/Akt/mTORC1 pathway and Ras controls proliferation, growth, and differentiation of many cell types. Ras activates the MAPK and ERK cascades, leading to transcriptional activation of AP-1, cJun/Fos, and NFAT. The MAPK cascade induces ER stress and NF κ B (129). IL-2 induces vasodilation and increases microvascular permeability by suppressing endothelin-1, causing perivascular edema seen in SEB-induced acute lung injury (130, 131). IL-2-deficient mice are resistant to SEB-induced toxic shock, providing evidence for the critical roles of PI3K/mTORC1 and NF κ B (132).

The chemokines IL-8, MCP-1, MIP-1 α , and MIP-1 β are induced directly by SEB or TSST-1. These chemoattractant chemokines activate leukocytes and influence migration of neutrophils, dendritic cells, and leukocytes (133, 134). Chemokines bind to seven-transmembrane GPCR, induce early Ca $^{2+}$ flux, activate PLC and signal *via* the PI3K/mTORC1 pathway (133). Cytokine- and chemokine-activated neutrophils, recruited to sites of tissue injury and inflammation, produce reactive oxygen species (ROS) and activate MMPs contributing to organ dysfunction. MMPs cause tissue degradation and change chemokine interactions with the extracellular matrix (ECM), creating a chemokine gradient affecting cell recruitment (133). Exudates from superantigen-injected air pouches are predominantly neutrophils with some macrophages (135). Both systemic and intranasal administration of SEB cause acute lung injury characterized by increased: (1) expression of adhesion molecules ICAM-1 and VCAM; (2) neutrophil and mononuclear cell infiltrate; (3) endothelial cell injury; and (4) vascular permeability (135–137).

The PI3K/mTORC1 pathway plays a dominant role in superantigen-induced cell proliferation and migration as TCR, CD28, IL-2R, IFN γ R, and chemokine receptors all signal through this pathway. IL-1 and TNF α independently activate NF κ B *via* MyD88/TRAF6/IRAK and FADD/TRADD/RIP, respectively (87). TCR and CD28 *via* PKC also activate NF κ B signaling. Other PRR, including surface and cytosolic TLRs, signal *via* MyD88 to activate NF κ B *via* different adaptors. In addition, TLR3 and TLR4 activate TRIF (toll/IL-1 receptor homology domain-containing adaptor inducing interferon β) signaling, inducing interferon regulatory factors (IRFs) (114). The overlap of PRR signaling by NF κ B and IRFs with TCR and co-stimulatory signals by

superantigens to activate NF κ B and mTORC1 cannot be understated. These pathways activate inflammatory genes, as well as antiviral, anti-apoptotic, and pro-apoptotic molecules. Thus the three initial signals provided by TCR, costimulatory receptors, and cytokines converge on NF κ B and mTORC1 to promote host defense against superantigens.

IN VIVO EFFECTS OF SUPERANTIGEN

Injection of SEB into mice has been used to study activation-induced apoptosis and T-cell anergy *in vivo*. This effect may be linked to a rapid (within 1 h) loss of L-selectin on the surface of specific V β -bearing T cells, thus resulting in decreased signal transduction (138). *Via* endocytosis, surface levels of TCR-CD3 decrease ~50% among V β -reactive T cells within 30 min after SEB exposure (139). The rapid hyperactivation and proliferation of T cells in mice following an SEB injection is transient, as within 48 h the majority of proliferating T cells is eliminated by activation-induced cell death (140). CD95 mediates elimination of SEB-activated T cells and the residual V β -specific cells become anergic, or functionally unresponsive. However, controversy still exists regarding the functional ability and fate of these anergic T cells. After injection of SEB into mice, splenic V β 8 $^+$ T cells are deleted or no longer respond to SEB, and produce less IL-2 and IFN γ . In contrast, others report that these anergic cells synthesize less IL-2 but still secrete IFN γ that can mediate toxic shock following a subsequent dose of SEB (141). An evident paradox is that the anti-inflammatory cytokine IL-10, which protects against SE-induced shock (142), is also produced by SEB-primed T cells. Treg cells likely downregulate superantigen activation *in vitro* and *in vivo*.

Immune homeostasis is maintained by Treg cells through a number of mechanisms depending on the “immune environment” (143). Natural and induced Foxp3 $^+$ Treg cells control excessive immune activation deleterious to the host. Treg cells also downregulate autoimmune responses, and superantigens can subvert the functional activity of Treg cells in atopic dermatitis (144). Although the immunosuppressive function of Treg cells is well known, the mechanisms of action and involvement of different cell types are still debated. The markers used in identifying different Treg cells are evolving, but the surface receptor cytotoxic T lymphocyte antigen 4 (CTLA4) likely plays a role particularly in superantigen-related counter regulation (145). CTLA4 interacts with costimulatory molecules CD80 and CD86, preventing costimulatory signals in superantigen-activated T cells (146). Systemic inflammatory responses elicited by SEB actually destabilize Treg cells and additional expansion of them *in vivo* does not protect transgenic mice from SEB-induced toxic shock (147).

SUPERANTIGEN-INDUCED AUTOIMMUNITY

The ability of superantigens to cross-link MHC class II and specific TCR V β enables these microbial toxins to stimulate the immune system and induce autoimmunity by activating APC

and normally quiescent, autoreactive T- and B-cells. Activation results in cytokine and chemokine release, thus mediating a potently acute inflammatory response. Several experimental animal models show that staphylococcal superantigens are arthrogenic (148, 149). TSST-1 exacerbates bacterial cell wall-induced arthritis in rats, possibly linked to accumulating V β 11+ T cells and IFN γ production within arthritic joints (149). TSST-1 also plays a pivotal role in murine septic arthritis, as the frequency and severity of this disease are increased after intravenous administration of TSST-1-secreting *S. aureus* (150). SEA or SEB can also induce relapses of experimental autoimmune encephalomyelitis in a murine model for multiple sclerosis (151). How exogenously administered toxin triggers autoimmune processes like arthritis is unknown, but it is likely that proinflammatory cytokines and chemokines produced in response to superantigens facilitate specific recruitment and migration of autoreactive T-cells into synovial tissue and joints. Thus, proinflammatory cytokines and chemokines have acute toxic effects promoting cell activation and recruitment. Following minor tissue injury or inflammation due to superantigen exposure, an increased presence of immune cells might initiate a destructive autoimmune reaction.

Psoriasis and atopic dermatitis also represent autoimmune diseases linked to staphylococcal and streptococcal colonization of skin and subsequent production of superantigens (152). SEB on healthy human skin induces inflammatory reactions, possibly linked to degranulation of cutaneous mast cells (58). T cells from patients with severe atopic dermatitis are apoptotic, which may lead to chronic infections and subsequent worsening of disease (153). Bacterial density on skin can affect relative sensitivity toward these toxins and development of atopic dermatitis.

PATHWAY ANALYSIS YIELDS POTENTIAL DRUG TARGETS

There are currently no available therapeutics for treating superantigen-induced shock, except for the use of intravenous human immunoglobulin (154, 155). Targeting and neutralizing a superantigen directly is most suitable at early stages of exposure before cell activation and initiation of the proinflammatory cytokine cascade. Therapies targeting superantigens at the receptor level have been extensively reviewed recently (156) and include receptor-blocking peptides derived from toxins, chimeric inhibitors composed of V β and MHC class II domains, as well as synthetic blockers of co-stimulatory receptor CD28. However, preventing toxin-receptor interaction is ineffective post-toxin exposure and some inhibitors must be tailored to target individual toxins. Failed sepsis clinical trials of eritoran (anti-endotoxin), a drug that prevents the early steps of receptor interaction, suggest that blocking superantigen–receptor interactions will likely not protect against SEB-induced shock (157).

An important class of therapeutic compounds blocks signal transduction pathways activated by superantigens, and as these events are post-exposure, they are perhaps more amenable to suppressive manipulation. One example is the NF κ B cascade containing many upstream activators. *In vitro* studies indicate numerous

genes for cell adhesion molecules, cytokines, chemokines, acute phase proteins, and inducible nitric oxide synthase that are implicated in superantigen-induced lethal shock and contain NF κ B binding sites in the promotor/enhancer region (84). Activation of NF κ B leads to the inducible expression of many mediators involved in inflammation and tissue injury seen in SEB-induced lung injury and toxic shock models. Inhibition of NF κ B is beneficial, or has no effect, in preventing SEB-induced shock depending upon the mouse model (136, 158, 159); however, NF κ B inhibitor must be given early and for a long duration to afford any protection (136).

Pathway inhibitors are used for identifying molecules and signaling pathways crucial for cellular responses to a specific stimulus. The superantigenic properties of SEB make it an “ideal” toxin to study cell activation signals and molecular pathways. An obvious step in testing new therapeutic approaches for SEB-induced shock is finding relevant animal models that mimic important aspects of human disease. Mice are much less susceptible to SEB due to lower affinity of superantigen for mouse MHC class II (51, 62, 160). Potentiating agents such as LPS, viruses, d-gal, and actinomycin D are used together with SEB to sensitize mice (50–52). These synergistic agents alone activate similar host signaling pathways as superantigens, confounding the “pure” host response to SEB. An alternative model utilizes transgenic mice, with either human HLA-DR3 or -DQ8, that lethally respond to SEs without a potentiatting agent (161, 162). Another recent, simplified model employs “double-hit,” low dose SEB in C3H/HeJ mice, an LPS-resistant strain (131). Pathological lesions, cytokine response, and time to lethality in this “double-hit” model resemble findings in non-human primates (NHP) and staphylococcal toxic shock syndrome in patients (49). Host signatures arising from “SEB-only” exposure unexpectedly include many IFN-induced genes in multiple organs, not previously linked to SEB pathogenesis (163).

OLIGONUCLEOTIDE MICROARRAY REVEALS SEB-INDUCED DANGER SIGNALS

Oligonucleotide microarray analysis in the “double-hit” SEB mouse model reveals induction of danger signals bearing IFN signatures (163). These genes cover five important molecular hubs signaling danger similar to those activated by IFNs and pathogens. The upregulated transcripts present in PBMC, spleen, lung, liver, kidney, and heart include (1) innate mediators; (2) DNA/RNA sensing system; (3) ER stress; (4) metabolic/oxidative stress; and (5) the apoptosis pathway. Proinflammatory cytokines IL-1, IL-6, TNF α , IFN γ , and IFN γ -induced chemokines (CXCL9, CXCL10, CXCL11) are most prominent in SEB-stimulated PBMC, confirming previous observations *in vitro* and *in vivo* (51, 86, 135, 161, 162, 164). Activation of other innate host-defense genes include the Fc receptor for phagocytosis, MHC class I and II for increased APC function, as well as cell-surface receptors and adhesion molecules to promote cell recruitment. Z-DNA binding protein 1 (ZBP1), a DNA sensor triggering ISGs via DNA binding (165), is surprisingly

upregulated in all tissues from the “double-hit” SEB mouse model (163). T-cell proliferation requires elevated protein translation and increased metabolism, resulting in misfolded proteins and oxidative stress. ER stress response genes such as ubiquitin ligases, immunoproteasome components, proteasome peptidases, and WARS (tryptophanyl-tRNA synthetase) are likely a result of Ca^{2+} flux, unfolded proteins, and activated PKC following cell activation and increased protein synthesis. Unresolved ER stress activates caspases and apoptosis (166, 167). Enhanced activity of the mitochondrial electron transport chain ultimately leads to oxidative stress, evidenced by activated NADPH oxidase in lung (163). MMP, cathepsins, and other proteases from lysosomes are also seen in this mouse model and *in vitro* stimulation of PBMC with SEA or SEB (168, 169). Increased ROS and protease levels are major factors in organ injury. Although the “double-hit” mouse model is not perfect, it mimics many *in vivo* responses of NHP to SEB and there are many similarities of gene expression by mouse and human PBMC incubated with SEB.

The cell death pathway triggered *in vitro* and *in vivo* includes genes associated with apoptosis such as FADD, death receptor ligand TRAIL, caspases, CARD, and phospholipid scramblase 1 (PLSCR1). These genes are activated in PBMC and major organs from the “double-hit” SEB model and human PBMC, following SEA or SEB exposure (163, 168, 169). PLSCR1 is implicated in moving phosphatidyl serine to the outside plasma membrane of apoptotic cells. Other danger signals include K^+ efflux, particulate ligands such as cholesterol crystals and lysosome destabilization that triggers inflammasome activation *via* NLRP3 which converts pro-IL-1 β to IL-1 β through caspase 1 (117). Catabolic enzymes cause destruction of cell matrix, accounting for the liver and lung injury seen in SEB-mediated shock.

Induction of ZBP1 leads to binding of DNA and endosomal TLR9, upregulating antiviral genes *via* the IRF3 and IRF7 (114, 170). TLR9 activation also promotes NF κ B-mediated cytokine gene transcription and inflammasome activation (171). Mitochondria are the most likely source of cytosolic DNA induced by SEB, as ER stress and increased mitochondrial respiration due to T-cell proliferation and activation are known to induce ROS (172). Elevated ROS activates the intrinsic cell death pathway *via* caspase 9, leading to mitochondrial damage that releases cytochrome c and mtDNA (173). mtDNA acts as a direct inducer of ZBP1. In addition, mtDNA has motifs similar to bacterial DNA (CpG), binds cytosolic TLR9, and promotes activation of NF κ B and IRFs. Mitochondrial ROS is also a potent inducer of the inflammasome NLRP3 (117). Both NLRP3 and CARD are identified in microarray studies *in vivo* and *in vitro* with superantigen-stimulated PBMC. Cathepsins, another category of “destroyer” molecules identified by microarray in SEB-activated PBMC (169), are indicative of lysosomal rupture which also activates inflammasomes.

Upon comparing the microarray data from an *in vivo* “SEB-only” mouse model (163), and human PBMC stimulated with SEA (168) or SEB (169), several commonly activated genes importantly induce the pathogenic effects of SEB (**Table 2**). These

TABLE 2 | Common differentially expressed genes induced by superantigens *in vitro* and *in vivo*.

Pathway/network	Gene	Major function
Innate response	IL6, TNF α , LTA, IL17A, IL22	Host defense, inflammation
	IFN γ	Host defense, antimicrobial
	CXCL11, CXXC5, CCL7, XCL1	Host defense, cell migration
	CISH, CIITA, GBP2, TRAF1, RGS16	Signal transduction
	PDE4DIP, PDE4B, PTGER3, P2RY14	Signal transduction
	NEDD9, GNAS, CSF1R	Signal transduction
	STAT1, STAT2, STAT3, IRF7	Transcription factor (TF)
	BATF, BATF2	IFN-inducible TF
	SOCS1, SOCS2, SOCS3	JAK/STAT counter-regulator
	CD69, CD74, ICAM	Immune regulation
	NRP2	Vascular signaling
	Rel A, Rel, NF κ Bia	NF κ B regulator
DNA damage response	RIPK2	DNA sensor interactor
	CTPS, UPP1	Nucleic acid synthesis
	PIM1, PIM2	DNA repair/assembly
	GADD45G	DNA repair adaptor
ER stress/oxidative stress	SIAH2	Ubiquitin E3 ligase
	KCNE4	Membrane integrity
	JunB	Stress response TF
	MGST1	Cell protection
Metabolic stress	IL2, IL2RA, MACF1	Cell proliferation regulator
	FABP4, CD36	Fatty acid metabolism
	HK1, PDK4, PGS1	Cell metabolism
	TARS, NDST2	Synthetase
Apoptosis	PLSCR1, NR4A1	Membrane integrity
	CD40, TNFRSF9	TNFRSF, death receptor
	Casp 4, CFLAR	Caspase regulator
	VCAN, LMNB1	Cell matrix breakdown
	BCL2, BCL6	Anti-apoptotic regulator
	CCND2	Cell cycle regulator
	PLA2G7	Cardiovascular damage
Others	ARID5A, ZBTB32, NDST2	

ARID5A, AT-rich interactive domain 5A (MRF1-like); BATF2, basic leucine zipper transcription factor (TF); CCND2, cAMP specific cyclin D2; CFLAR, caspase 8 and FADD-like apoptosis regulator; CIITA, MHC class II transactivator; CISH, cytokine inducible SH2-containing protein; CTPS, cytidine 5'-triphosphate synthase; FABP4, fatty acid binding protein 4; GADD45G, growth arrest and DNA-damage-inducible 45 gamma; GBP2, IFN-inducible guanylate binding protein 2; GNAS, guanine nucleotide binding protein; HK1, hexokinase 1; KCNE3, potassium voltage-gated channel; LMNB1, lamin B1; MACF1, microtubule-actin crosslinking factor 1; MGST1, microsomal glutathione-S-transferase 1; NDST2, N-deacetylase/N-sulfotransferase; NEDD9 (HEF1), neural precursor cell expressed; NR4A1, nuclear receptor subfamily 4, group A, membrane 1; NRP2, neuropilin transmembrane protein receptor; P2RY14, purinergic receptor P2Y, G protein coupled; PDE4DIP, phosphodiesterase 4D interacting protein; PDE4B, cAMP specific phosphodiesterase 4B; PDK4, pyruvate dehydrogenase kinase isoenzyme 4; PGS1, phosphatidylglycerophosphate synthase 1; PIM1, pro-viral DNA integration; PLA2G7, phospholipase A2 group VII; PLSCR1, phospholipid scramblase; PIM1, proviral integration site 1; PTGER3, prostaglandin E receptor 3; RGS16, regulator of G protein signaling 16; RIPK2 (RIP2), TNFRSF-interacting protein kinase; SIAH2, seven in absentia 2; SLC30A1, solute carrier family 30 (zinc transporter); SOCS1, suppressor of cytokine signaling 1; TARS, threonyl-tRNA synthetase; UPP1, uridine phosphorylase 1; VCAN, verican; ZBTB32, zinc finger and BTB domain containing 32.

genes are activated by IFN and account for the therapeutic effectiveness of rapamycin in preventing SEB-induced shock (102).

DRUG TARGETS: WHAT WORKS AND WHAT DOES NOT

The early induction of three key proinflammatory cytokines, IL-1, TNF α , and IFN γ , work *via* individual receptor-mediated signaling molecules that, respectively, activate distinct pathways: (1) IL-1R/MyD88/NF κ B; (2) TNF α R/FADD/RIP; and (3) IFN γ R/JAK/IRF. IL-1, TNF α , and IFN γ have independent and synergistic effects signaling inflammation, caspase activation/cell death, and an antiviral response. IL-1 from inflammasome activation has pleiotropic effects, while TNF α has an established role that initiates cell death through the adaptor FADD, activating caspases 3 and 8. IFN γ triggers innate host defense responses, antiviral genes, apoptotic programs, immunoproteasomes, and has many immunomodulatory functions. Interruption of these concurrent cascades early after SEB exposure is effective in preventing SEB-induced lethal shock.

Decades of drug development against sepsis and septic shock point to the failure of using anti-inflammatory cytokines alone, and early interruption of cytokine release is perhaps a necessary but insufficient target. Drugs also act on multiple targets, some known and unknown, as exemplified by statins that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A with anti-inflammatory effects (174). Statins, used therapeutically to reduce cholesterol, are under consideration for treating various inflammatory diseases (175). Superantigens trigger multiple pathways that cross-regulate each other positively and negatively, thus targeting downstream effectors might be more specific and perhaps interfere less with normal cell function. Knowledge of immunoregulation within activated pathways by SEB enables a better choice of inhibitors.

Apoptosis plays a critical role in sepsis-induced lethality (176). The two pathways leading to apoptosis are operative in SEB-induced lethal shock, as induction of genes for both pathways occurs in the “double-hit” SEB model and other mouse models employing potentiating agents such as D-gal. The death receptor pathway used by the TNFR superfamily with ligands like TNF α and FasL induces cell death following superantigen exposure (121, 126). A second apoptosis pathway, the intrinsic pathway, is dependent on mitochondria and the Bcl2 family of pro- and anti-apoptotic proteins. ROS and loss of mitochondrial transmembrane potential play important roles, as previous studies implicate both in SEB pathogenesis (177). Other cell-surface receptors like CD44 might also contribute to SEB-induced injury, as CD44-knockout mice have elevated liver damage in the D-gal-sensitized SEB model (178). Since D-gal is hepatotoxic, the induction of pro-apoptotic molecules by SEB likely act synergistically with D-gal to promote cell death.

Microarray gene analysis in an “SEB-only” mouse model implicates both extrinsic and intrinsic pathways in SEB-induced apoptosis. Apoptosis plays a role in down-regulating

immune responses but simultaneously has devastating effects when apoptotic cells or associated molecules are not removed. Autophagy is a cellular mechanism that removes bacteria, protein aggregates, and damaged organelles to maintain homeostasis (176, 179). A recent study indicates that blocking autophagy augments T-cell activation (180). Degradation and removal of Bcl10, which is part of CBM and a critical component for TCR and costimulatory signaling, is dependent on autophagy (176). IFN induces many genes regulating NF κ B and apoptosis. The damage response induced by superantigens likely starts with inflammatory cytokines and apoptotic programs activated by IFN γ and TNF α . DAMPs such as mitochondrial ROS and mtDNA trigger more apoptosis, activate inflammasomes, and induce transcription factors for ISGs. Increasing energy demand and mitochondrial respiratory-chain activity also lead to elevated ROS. Normally, mitochondria damaged by excessive membrane permeability and ROS are removed by a specialized form of autophagy called mitophagy. However, overactivation of PI3K/mTORC1 in superantigen-stimulated cells likely blocks autophagy, resulting in inflammasome activation and accumulation of damaged mitochondria. Rapamycin, a well-known inducer of autophagy, prevents SEB-induced shock by removing damage-inducing molecules and damaged mitochondria. Two other FDA-approved immunosuppressants for organ transplants, cyclosporine A and tacrolimus, do not protect against superantigen-induced shock in NHP and human HLA-DR3 transgenic mice, respectively (181, 182). The calcineurin inhibitor, cyclosporine A, protects D-gal-sensitized mice from SEB-induced shock (121) but does not protect NHP challenged with SEB (181). Tacrolimus suppresses SEB-induced T-cell proliferation *in vitro* but does not confer protection from toxic shock in transgenic mice (182). Tacrolimus also fails to protect mice from lethal pneumonia induced by superantigen-producing *S. aureus* (182).

There is good agreement between the genes significantly induced in mouse PBMC from this “double-hit” SEB model and SEA- or SEG-stimulated human PBMC (163, 168). Many genes of the apoptosis-related cell death pathway account for the damage response initiated by SEB. MtDNA is ancestrally related to bacterial DNA (CpG motifs), inducing a “foreign” DNA sensor (ZBP1) or alternatively binding endosomal TLR9 that triggers host defense including type 1 IFN-mediated responses *via* IRF3 (114). Important clues from animal models, old and new, reveal acute release of proinflammatory cytokines that culminate in damaged organs and lethal shock.

CONCLUSION

Staphylococcus aureus is a toxin-producing pathogen that causes various diseases found throughout the body. Increasingly, *S. aureus* becomes more resistant to various therapeutics (i.e., antibiotics) over time, thus our own immune systems must more effectively clear this pathogen. Further knowledge of our immune system will clearly enable us to better thwart *S. aureus* and other pathogens. Mammals sense invading microbes *via* conserved PRR for detecting molecular patterns on, or released by, various

bacterial, viral, and fungal pathogens. This rapid innate immune response produces proinflammatory mediators, cell activation, and recruitment of inflammatory cells to infection sites. However, some sensors for detecting PAMPs also bind to host DAMPs, confusing the “stranger” versus “danger” signaling. Host response to staphylococcal superantigens typifies the generation of these danger signals as shown in a mouse model of SEB-induced shock. The induction of cell death through apoptotic proteins observed during sepsis or superantigen exposure may provide a common target for therapeutic intervention.

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AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contributions to the work, and approved it for publication.

ACKNOWLEDGMENTS

We thank DTRA for funding. Brad Stiles acknowledges Wilson College for the continuing support of this, and many other, endeavors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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T-cell activation or tolerization: the Yin and Yang of bacterial superantigens

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Bacterial superantigens (SAg) are exotoxins from pathogens which interact with innate and adaptive immune cells. The paradox that SAg cause activation and inactivation/anergy of T-cells was soon recognized. The structural and molecular events following SAg binding to antigen presenting cells (APCs) followed by crosslinking of T-cell receptors were characterized in detail. Activation, cytokine burst and T-cell anergy have been described *in vitro* and *in vivo*. Later it became clear that SAg-induced T-cell anergy is in part caused by SAg-dependent activation of T-regulatory cells (Tregs). Although the main focus of analyses was laid on T-cells, it was also shown that SAg binding to MHC class II molecules on APCs induces a signal, which leads to activation and secretion of pro-inflammatory cytokines. Accordingly APCs are mandatory for T-cell activation. So far it is not known, whether APCs play a role during SAg-triggered activation of Tregs. We therefore tested whether in SAg (Streptococcal pyrogenic exotoxin A) -treated APCs an anti-inflammatory program is triggered in addition. We show here that not only the anti-inflammatory cytokine IL-10 and the co-inhibitory surface molecule PD-L1 (CD274) but also inhibitory effector systems like indoleamine 2,3-dioxygenase (IDO) or intracellular negative feedback loops (suppressor of cytokine signaling molecules, SOCS) are induced by SAg. Moreover, cyclosporine A completely prevented induction of this program. We therefore propose that APCs triggered by SAg play a key role in T-cell activation as well as inactivation and induction of Treg cells.

OPEN ACCESS

Edited by:

Inka Sastalla,
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National Institutes of Health, USA

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 30 July 2015

Accepted: 05 October 2015

Published: 20 October 2015

Citation:

Sährt A, Förmer S, Hildebrand D and Heeg K (2015) T-cell activation or tolerization: the Yin and Yang of bacterial superantigens.
Front. Microbiol. 6:1153.
doi: 10.3389/fmicb.2015.01153

INTRODUCTION

Superantigens (SAg) are bacterial exotoxins which share unique immunological properties. SAg released by staphylococci or streptococci during infection or even colonization induce a strong activation of the immune system. Major hallmark of this activation is the fulminant release of cytokines (Carlsson and Sjögren, 1985) leading to a disastrous cytokine storm (Miethke et al., 1992; Michie et al., 1994) which might lead to an uncontrolled systemic shock with high lethality. The toxic shock syndrome (TSS) was recognized to be mediated by a SAg, the TSS toxin 1, TSST-1 (Miethke et al., 1993). In the meantime, many bacterial exotoxins have been classified as SAGs (Fraser and Proft, 2008), including the erythrogenic toxins of *Streptococcus pyogenes* (SPEA, SPEC) and the enterotoxins from *Staphylococcus aureus* (Lina et al., 2004).

Superantigen behave like bifunctional agents: they bind to conserved regions of MHC class II molecules and to V-beta encoded regions of the T-cell receptor (TCR) (Choi et al., 1990; Dellabona et al., 1990). Crosslinking class II on APC with TCR induces T-cell activation with

subsequent cytokine release. While all SAg bind to class II, the binding to the TCR is V-beta specific, thus single SAg activates a V-beta defined subfraction of the T-cell pool (Kappler et al., 1989). Nevertheless, in the human system a single SAg can activate 1 to 10% of the T-cell pool, leading to a strong oligoclonal T-cell response which exceeds the clone size activated by a protein antigen at least by the factor of 1000 (Herrmann and MacDonald, 1991).

The tracking of SAg reactive T-cells using the V-beta TCR expression has greatly facilitated the analyses of SAg-induced T-cell responses. After initial activation accompanied with secretion of cytokines including TNF, interleukin-2 (IL-2) and IFN-gamma a phase of clonal T-cell expansion follows which is then succeeded by apoptosis and clonal retraction (Herrmann et al., 1992; Lee and Vitetta, 1992; Huang and Crispe, 1993; Miethke et al., 1995). The apoptosis is not completely, roughly 50% of the initial numbers of V-beta bearing T-cells survive. However, these T-cells fail to respond to further stimulation, i.e., display an anergic phenotype (MacDonald et al., 1993; Wahl et al., 1993). Unfolding of unresponsiveness is prevented in the presence of the T-cell immunosuppressive agent cyclosporine A (CsA) but not rapamycin (Vanier and Prud'homme, 1992; Prud'homme et al., 1995). This was taken as an indication that anergy induction is dependent on calcineurin and is triggered primarily in T-cells. Besides anergy induction it was also shown

TABLE 1 | Primers used.

Gene	Forward – primer	Reverse – primer
β-Actin	aga gct acg agc tgc ctg ac	agc act gtg ttg gcg tac ag
calcineurin	aaa cag tga ctg gcg cat c	ccg gct tac agc aaa aga ag
IDO	tta gag tca aat ccc tca gtc c	ttt gca gat ggt agc tcc tc
IL-1 β	agc tga tgg ccc taa aca ga	gca tct tcc tca gct tgt cc
IRF-1	gct ggg aca tca aca agg at	tgg tct ttc acc tcc tcg at
JNK	gca tgg gct aca agg aaa ac	ttc agg aca tgg tgt tcc aa
p38	gac aca aaa acg ggg tta cg	tgg gtc acc aga tac aca tca
p44/42	agt aca tcc act ccc cca ac	cgt agc cac ata ctc cgt ca
CD274	tgc tgt ctt tat att cat gac cta c	tcc tcc att tcc caa tag aca
SOCS1	tcc ccc tca acc ccc t	cat ccc ctc cct cca acc
SOCS3	ggg agt ccc ccc aga aga g	ata gga gtc cag gtg gcc gt
STAT1	cgg ttt tca tga cct cct gt	ggc gtt ttc cag aat ttt cc
STAT3	cag gtt gct ggt caa att cc	tgt gtt tgt gcc cag aat gt
TDO	ggt tcc tca ggc tat cac tac c	cag tgt cgg gga atc agg t

that after stimulation with SAg the fraction of CD4 $^{++}$ CD25 $^{+}$ foxp3 $^{+}$ Treg within the T-cell pool is significantly augmented (Wang et al., 1998; Papiernik, 2001; Feunou et al., 2003; Grundström et al., 2003; Ivars, 2007). The cellular interactions and signaling pathways leading to Treg induction after SAg stimulation are not well understood.

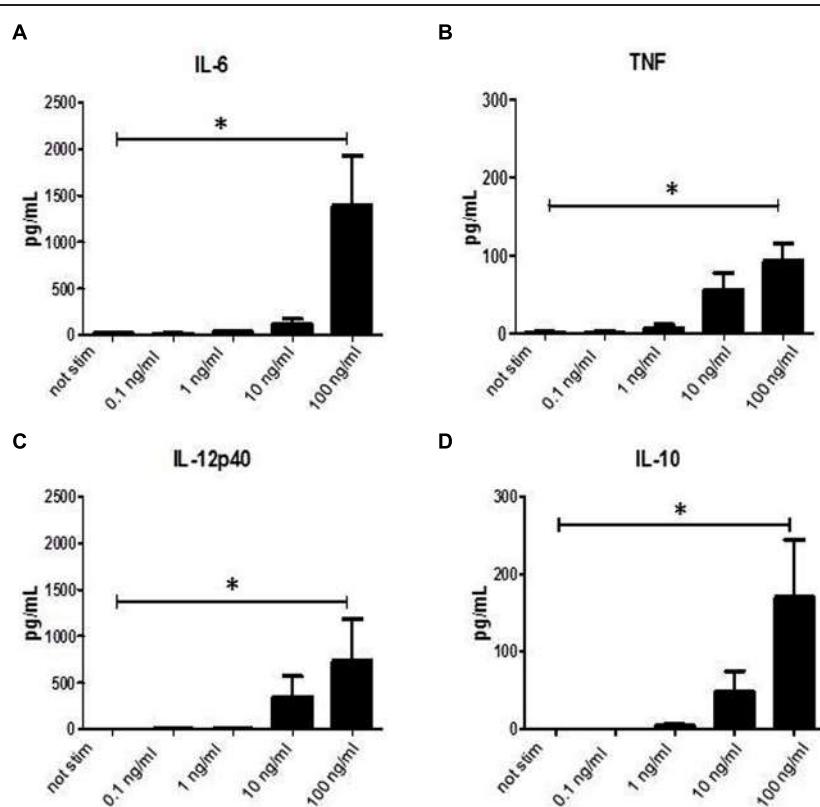


FIGURE 1 | Streptococcal pyrogenic exotoxin A (SPEA)- induced cytokines. CD14⁺ monocytes were stimulated with increasing concentrations of SPEA. 24 h after stimulation supernatants were analyzed by ELISA for (A) IL-6, (B) TNF, (C) IL-12p40 and (D) IL-10 (mean \pm SD, $n = 5$). Statistical analysis was performed using a Multiple Comparison of Means (Tukey Contrasts). Significance code: 0 ****; 0.001 ***; 0.01 **.

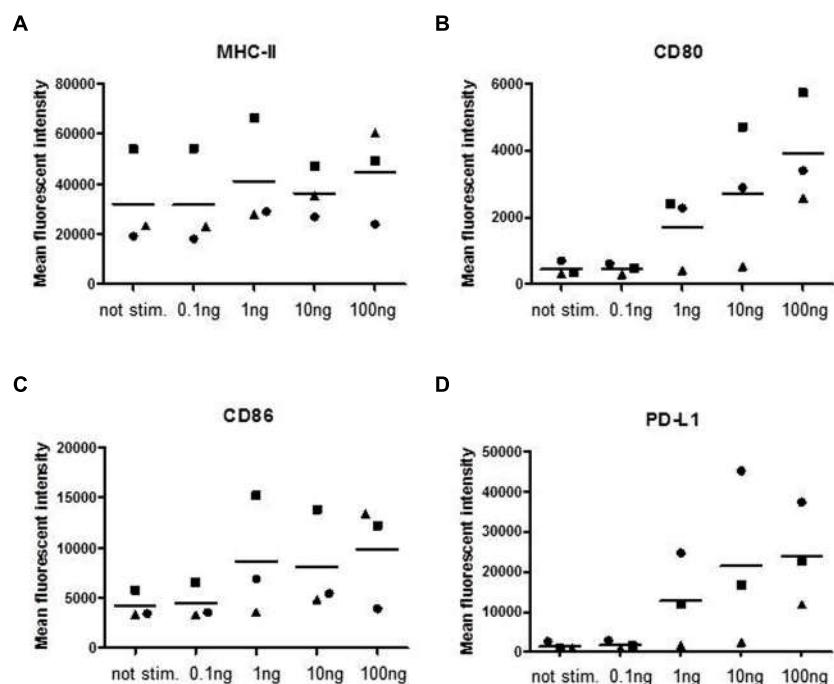


FIGURE 2 | Streptococcal pyrogenic exotoxin A- induced surface molecules. CD14⁺ monocytes were stimulated with increasing concentrations of SPEA as indicated. 24 h after stimulation cells were harvested and analyzed for surface markers by flow cytometry **(A)** MHC-II, **(B)** CD80, **(C)** CD86 and **(D)** PD-L1. The mean fluorescent intensity of three different experiments is shown. The line represents the mean of the experiments.

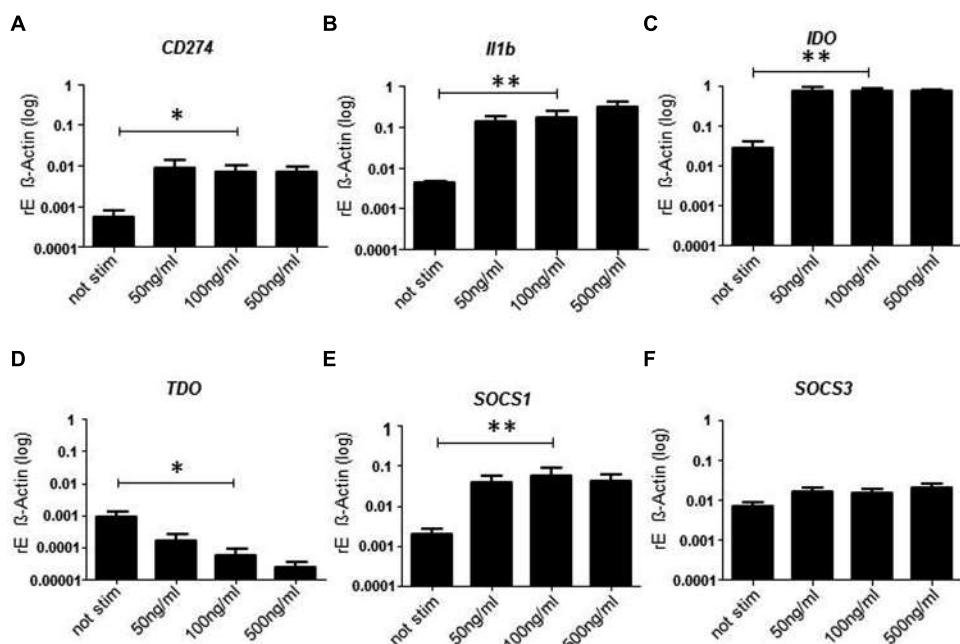


FIGURE 3 | Streptococcal pyrogenic exotoxin A- induced mRNA expression of inhibitory pathways. CD14⁺ monocytes were stimulated 24 h with increasing concentrations of SPEA. Quantitative real-time PCR of cDNA was performed for **(A)** CD274, **(B)** IL1b, **(C)** IDO, **(D)** TDO, **(E)** SOCS1, and **(F)** SOCS3. Shown is the mean of induction compared to β-Actin (\pm SD) of 5 donors. Statistical analysis was performed using a Multiple Comparison of Means (Tukey Contrasts). Significance code: 0 ****; 0.001 ***; 0.01 **.

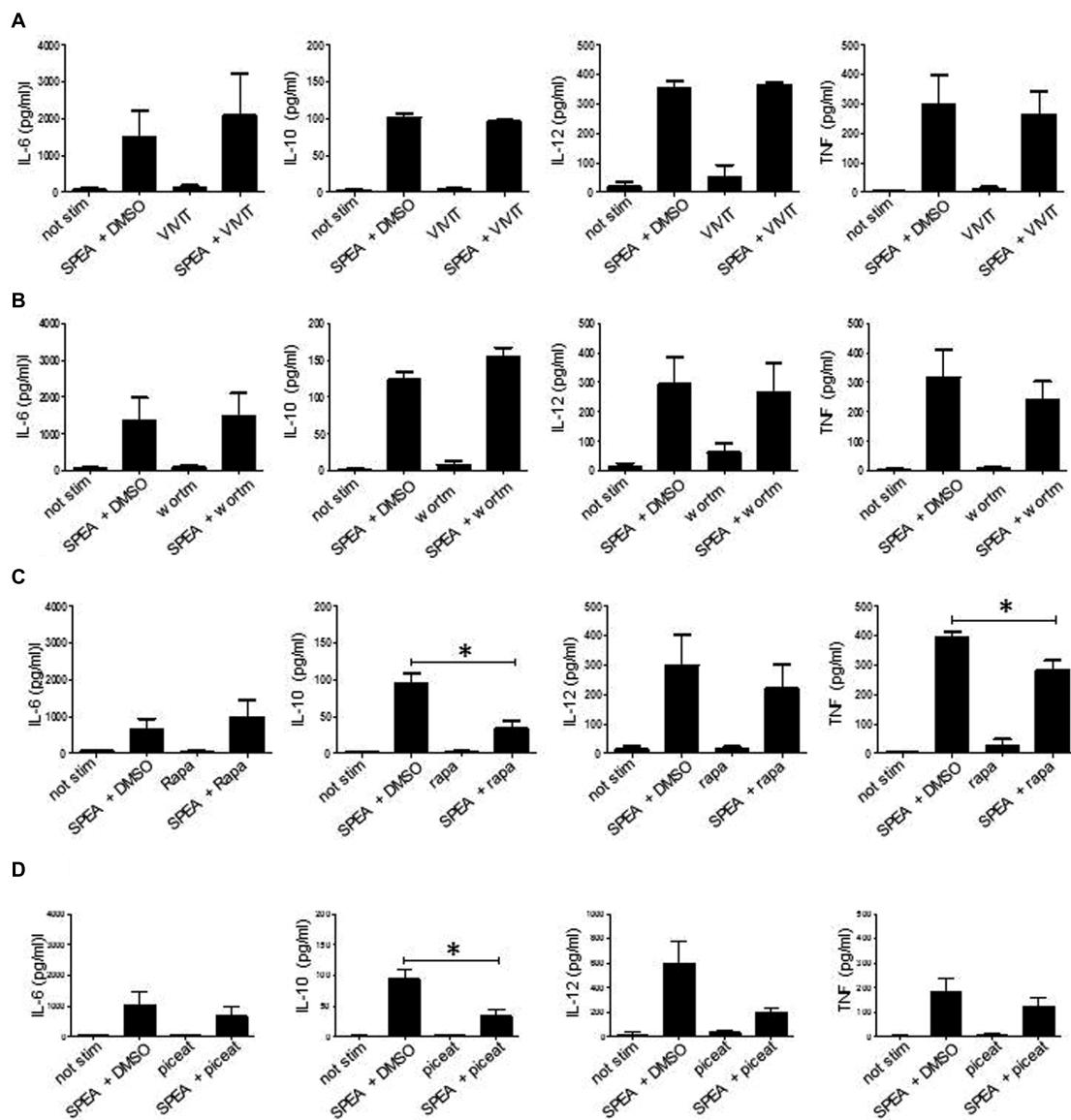


FIGURE 4 | Signaling pathways involved in SPEA-mediated activation of APC. CD14⁺ monocytes were stimulated with SPEA (100 ng/ml) in the presence or absence of (A) VIVIT (1 M), (B) Wortmannin (50 nM), (C) rapamycin (50 nM) and (D) piceatannol (50 μM) or DMSO (equal volume to inhibitor) as solvent control. 24 h after stimulation supernatants were harvested and ELISAs were performed for IL-6, IL-10, IL-12p40 and TNF. Shown is the mean ± SD from 3 different donors, except VIVIT ($n = 2$). Statistical analysis was performed using a Multiple Comparison of Means (Tukey Contrasts). Significance codes: 0 ****; 0.001 ***; 0.01 **.

For T-cell activation, the presence of APC and the binding of SAg to their MHC class II molecules are mandatory (MacDonald et al., 1993; Rink et al., 1997). The binding regions of SAg to MHC class II have been studied and characterized in detail. It became evident that SAg not only binds to class II but also share the ability at different levels to crosslink MHC class II molecules (Hudson et al., 1995; Kozono et al., 1995). This suggested that SAg might confer a signal to the MHC class II expressing APC. Indeed MHC class II signaling after crosslinking has been observed in B-cells and stem cells which was characterized by activation of tyrosine kinases like Syk (Mooney et al., 1990, 1994; Scholl and Geha, 1994; Kanner et al., 1995; Yamaguchi

et al., 1999). In monocytes binding of SAg and thus crosslinking of MHC class II molecules was followed by an intracellular increase of Ca²⁺ which was then succeeded by activation and eventually secretion of pro-inflammatory cytokines like TNF (Palkama and Hurme, 1993; Trede et al., 1993a,b; Mehindate et al., 1995; Espel et al., 1996; Khan et al., 2008). Interestingly Treg seem to utilize class II signaling too. Mature Tregs express LAG-3 (CD223) which has been shown to bind to MHC class II and to crosslink subsequently the molecules (Hemon et al., 2011).

Since SAg-mediated stimulation of T-cells includes activation as well as tolerization processes we hypothesized that both

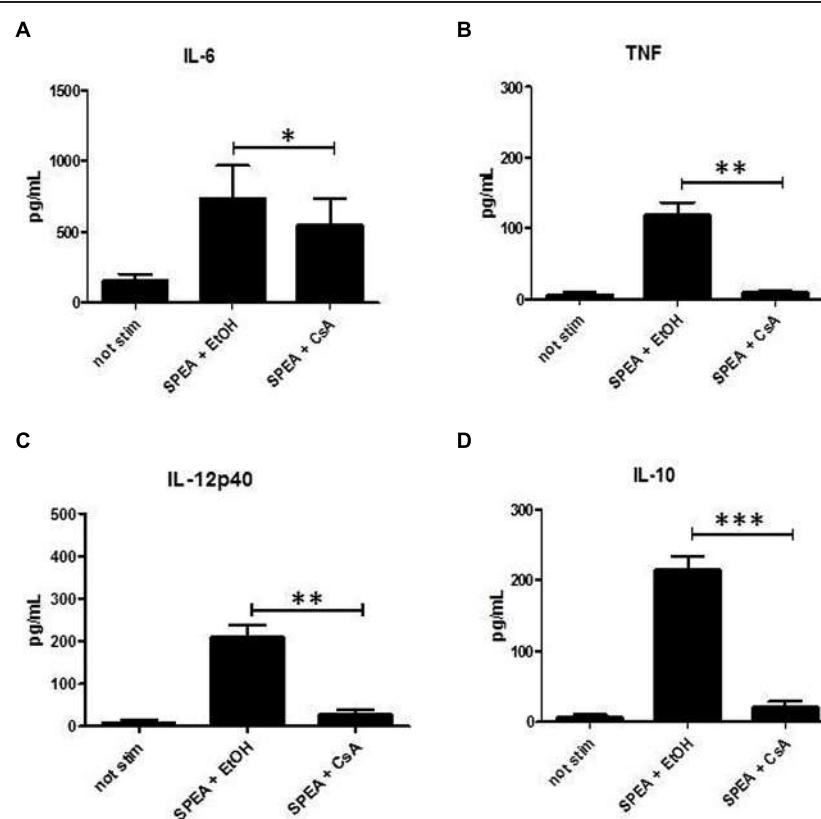


FIGURE 5 | Cyclosporine A (CsA) prevents secretion of cytokines. CD14⁺ monocytes were stimulated with SPEA (100 ng/ml) and CsA (1 μM) or Ethanol (equal volume to CsA) for 24 h. Supernatants were harvested and analyzed by ELISA for (A) IL-6, (B) TNF, (C) IL-12p40 and (D) IL-10. Shown is the mean concentration with standard deviation of 5 different donors. Significance codes: 0 ****; 0.001 ***; 0.01 ** (Tukey Contrasts).

events are determined by respective cellular or humoral events triggered in APC by SAg. We therefore tested whether co-inhibitory molecules (such as PD-L1; Francisco et al., 2009, 2010), negative signaling circuits (such as suppressor of cytokine signaling molecules, SOCS; Alexander, 2002; Strebovsky et al., 2011), or inhibitory effector systems (such as IDO; Lestage et al., 2002; Hill et al., 2007) are induced by SAg in APC.

MATERIALS AND METHODS

Reagents

Streptococcal pyrogenic exotoxin A (SPEA) was purchased from Toxin Technology Inc. (Sarasota, FL, USA). The mTOR-inhibitor rapamycin (50 nM), NFAT-inhibitor VIVIT (1 M), PI3-kinase-inhibitor Wortmannin (50 nM) and piceatannol (50 μM), a Syk-inhibitor were acquired from Calbiochem (Schwalbach, Germany). CsA was purchased from R&D Systems (Wiesbaden, Germany).

Isolation of Primary Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by density gradient centrifugation

(Pancoll 1.077 g/ml; PAN Biotech). Monocytes were isolated via CD14 MicroBeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) with the autoMACS separator. 2 × 10⁶ cells were cultured in RPMI 1640 (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 100 IU/ml of penicillin, 100 μg/ml streptomycin containing 10% heat inactivated fetal calf serum (Promocell, Heidelberg, Germany) at 37°C in a humidified atmosphere in the presence of 5% CO₂ and stimulated for 24 h.

Flow Cytometry

Twenty four hours after stimulation monocytes were analyzed for the surface markers CD14 (clone TÜK4), CD80 (clone L307.4), CD86 (clone IT2.2), PD-L1 (clone MIH1) and MHC-II (clone Tu39). Analyses were performed on a FACS Canto I (BD Biosciences).

The antibodies were purchased from Becton Dickinson (Heidelberg, Germany), except PD-L1 (eBioscience, Frankfurt/Main, Germany).

Western Blotting

8 × 10⁶ cells were lysed 24 h after stimulation in RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail from Roche (Mannheim,

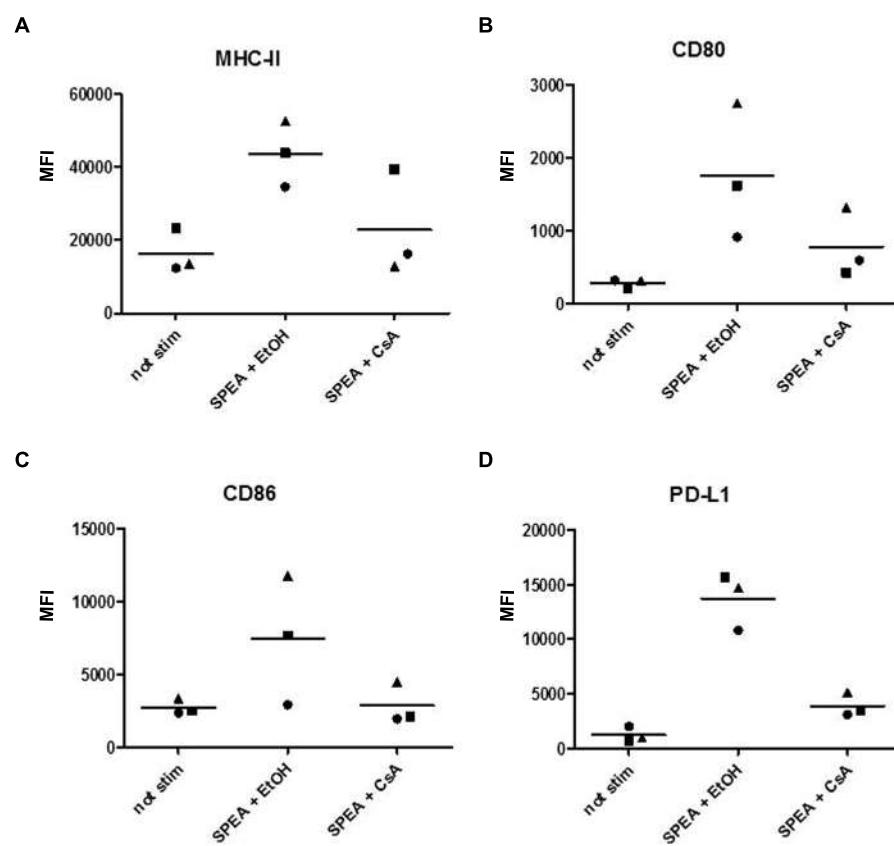


FIGURE 6 | Cyclosporine A prevents the expression of costimulatory and coinhibitory molecules. CD14⁺ monocytes were stimulated with SPEA (100 ng/ml) and CsA (1 μM) or Ethanol (equal volume to CsA) for 24 h. Cells were harvested and analyzed for surface markers by flow cytometry **(A)** MHC-II, **(B)** CD80, **(C)** CD86 and **(D)** PD-L1. The mean fluorescence intensity of three different donors is shown.

Germany). Equal amounts of the lysates were fractionated by SDS-PAGE and electrotransferred to nitrocellulose membranes. After blocking and washing steps the indicated antibodies, purchased from Cell Signaling Technology (Danvers, MA, USA), were incubated for 24 h and detected via chemiluminescence (ECL; Perkin Elmer, Groningen, Netherlands).

ELISA

Cell-free supernatants were harvested 24 h after stimulation and analyzed for IL-6, IL-10, IL-12p40 and TNF by commercial available ELISA kits from Becton Dickinson (OptEIA; Becton Dickinson, Heidelberg, Germany) according to the manufacturer's instructions.

Statistics

Statistical significance was assessed using SPSS statistics software and paired student's *t*-test evaluation with *: $p \leq 0.05$, **: $p \leq 0.005$. Further on we confirmed the results with Multiple Comparisons of Means (Tukey Contrasts), performed with R. Significance codes: 0 *** 0.001 ** 0.01 * 0.05 ' 0.1 ' 1. When the results of the methods differ, significance of Tukey's test is shown.

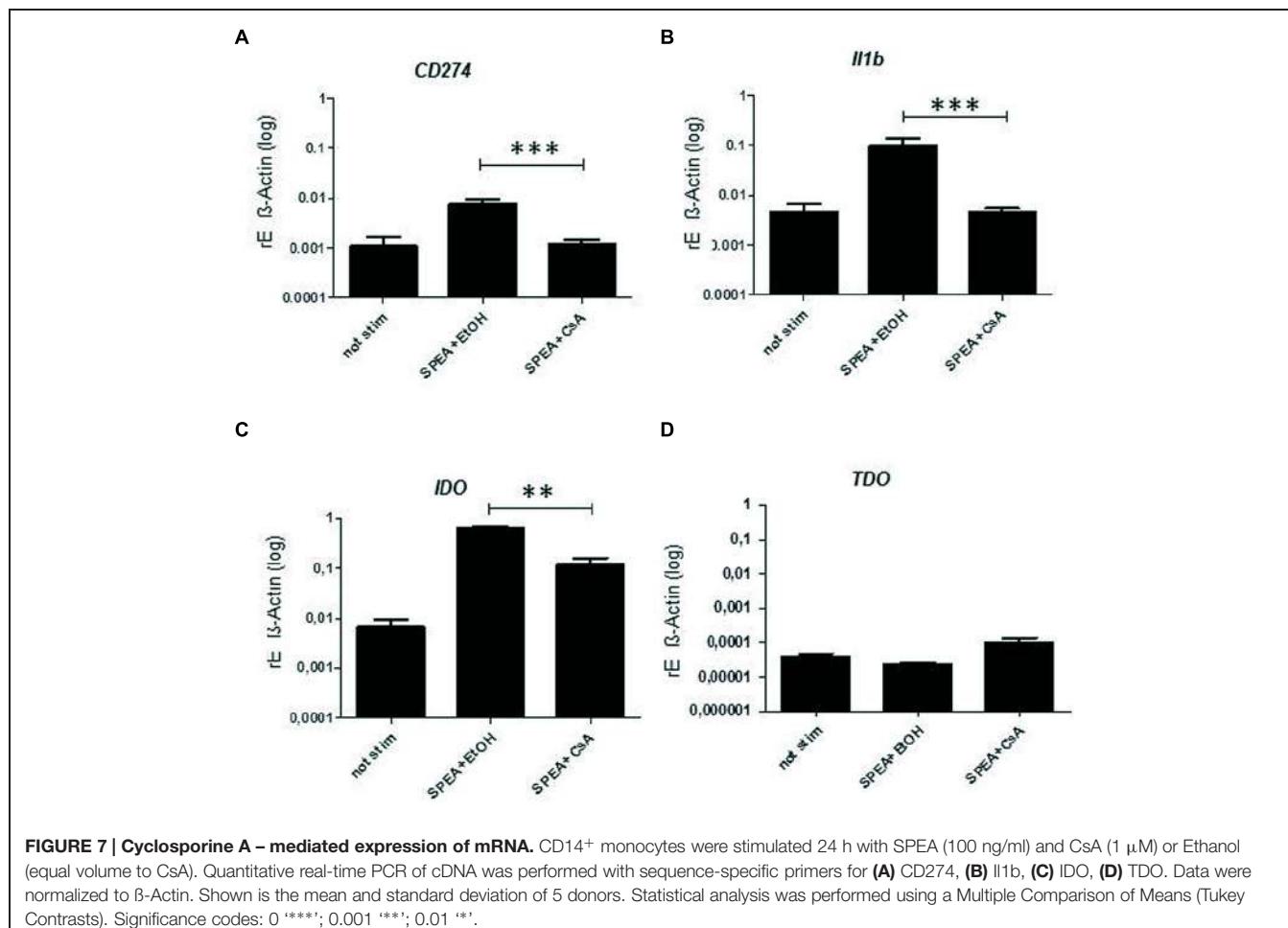
RNA Purification and Quantitative Real – Time PCR

Total RNA from 4×10^6 cells was isolated using the High Pure RNA isolation Kit (Roche, Mannheim, Germany), cDNA was synthesized from equal amounts of RNA using the first strand cDNA kit from Thermo Scientific (Waltham, MA USA). Quantitative real-time RT-PCR was performed using ABsoluteTM QPCR SYBR®Green Low ROX Mix (Thermo Scientific, Waltham, MA, USA). Relative expression was calculated by normalization to β-Actin mRNA expression levels as $2^{-\Delta Ct}$. All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) (Table 1).

RESULTS

Superantigens Induce Cytokine Secretion in APC

It has been shown previously that binding of SAg to MHC class II molecules induces activation and secretion of cytokines. When we incubated monocytes for 24 h with graded doses of SPEA a dose dependent induction of cytokine secretion was found (Figures 1A–C). Not only pro-inflammatory cytokines

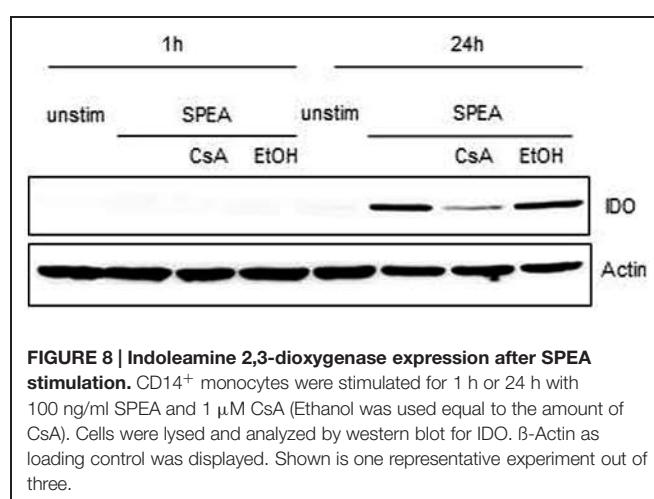


like IL-6, TNF, or IL-12p40 were induced but also secretion of anti-inflammatory IL-10 could be recorded (**Figure 1D**).

Next we assayed for the expression of surface molecules involved in antigen presentation and T-cell interaction (**Figure 2**). The surface expression of MHC class II molecules showed no dependency on SPEA stimulation (**Figure 2A**), while a dose-dependent expression of the co-stimulatory molecules CD80 and CD86 was observed (**Figures 2B,C**). Interestingly the co-inhibitory molecule PD-L1 (CD274) was strongly induced (**Figure 2D**, Supplementary Figure S1).

Superantigens Induce mRNA Expression of Inhibitory Pathways

Since PD-L1 expression indicated that inhibitory pathways might be induced we analyzed the mRNA expression of molecules involved in negative regulatory circuits (**Figure 3**). As expected mRNA expression of PD-L1 was significantly enhanced after treatment with SPEA (**Figure 3A**). The same was true for IL-1beta (**Figure 3B**). As IL-1 beta is produced as inactive precursor we additionally confirmed the elevated release of the active cytokine (Supplementary Figure S2). Surprisingly expression of the inhibitory effector enzyme IDO was observed (**Figure 3C**). In contrast, expression of tryptophan 2,3-dioxygenase (TDO)



was suppressed (**Figure 3D**) suggesting an inverse regulation. Within APCs negative feedback loops have been identified which regulate the response to cytokine stimulation. Molecules of the SOCS family play a pivotal role during these processes. We therefore analyzed the expression of SOCS1 and SOCS3 after

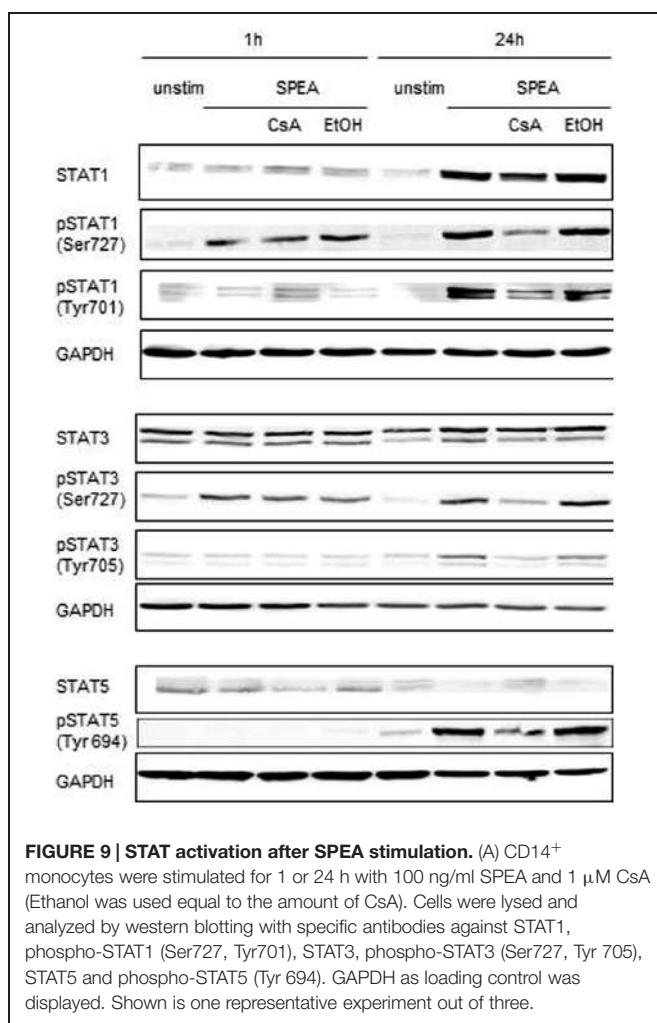


FIGURE 9 | STAT activation after SPEA stimulation. (A) CD14⁺ monocytes were stimulated for 1 or 24 h with 100 ng/ml SPEA and 1 μM CsA (Ethanol was used equal to the amount of CsA). Cells were lysed and analyzed by western blotting with specific antibodies against STAT1, phospho-STAT1 (Ser727, Tyr701), STAT3, phospho-STAT3 (Ser727, Tyr 705), STAT5 and phospho-STAT5 (Tyr 694). GAPDH as loading control was displayed. Shown is one representative experiment out of three.

stimulation of monocytes with SAg. While SOCS1 showed a clear induction, SOCS3 was only slightly induced (Figures 3E,F). mRNA expression of other signaling molecules was unchanged except for STAT1 and IRF-1 which showed a slight increased expression (Supplementary Figure S3).

Signaling Pathways Involved in SAg-Mediated Activation of APC

It was suggested that class II-signaling induces Ca-mobilization (Mooney et al., 1990) and subsequent activation. SAg-mediated signaling in APC could thus be mediated by non-classical signaling pathways. Besides NFAT-dependent or Syk-dependent pathways which have been shown in B-cells (Scholl et al., 1992; Palkama and Hurme, 1993; Morio et al., 1994; Haylett et al., 2009) other non-canonical pathways (mTOR, PI3K) could mediate SAg stimulation of APC. To test this, we resorted to classical inhibition assays (Figure 4). When we tested for inhibition of the induction of pro-inflammatory and anti-inflammatory cytokines we failed to observe an effect with the inhibitors of NFAT (VIVIT) (Figure 4A) and PI3K (Wortmannin) (Figure 4B). Inhibition of mTOR pathway by rapamycin or Syk pathway by piceatannol had

no effect on the induction of pro-inflammatory cytokines (TNF, IL-6, IL-12), however, rapamycin and piceatannol prevented release of IL-10 indicating a differential regulation of pro- and anti-inflammatory cytokines (Figures 4C,D).

Cyclosporine A interacts with cyclophilin and suppresses activation of T-cells and B-cells (Fischer et al., 1989). We thus hypothesized that CsA could interfere with SAg-mediated stimulation of APC. Indeed stimulation of monocytes with SPEA in the presence of CsA prevented the secretion of pro-inflammatory and anti-inflammatory cytokines significantly (Figure 5). Further on CsA modulated the SPEA-induced expression of co-stimulatory and co-inhibitory molecules, although not in a statistically significant mode (Figure 6). A similar pattern was observed for the induction of mRNA: CsA prevented the upregulation of mRNA coding for PD-L1 (Figure 7A), IL-1beta (Figure 7B), and partially reduced induction of IDO (Figure 7C). Accordingly, expression of TDO was not downregulated (Figure 7D).

Indoleamine 2,3-dioxygenase is a rate-limiting enzyme of tryptophan catabolism resulting in kynurenine production. Depletion of tryptophan causes halted growth of T-cells while kynurenine activates the aryl hydrocarbon (AH) receptor system leading to induction of Tregs (Opitz et al., 2011). We therefore tested for the expression of IDO and the subsequent depletion of tryptophan and production of kynurenine after SAg stimulation (Figure 8). In SPEA-stimulated APC IDO protein expression was detected (Figure 8) and the levels of kynurenine were enhanced (data not shown). Both IDO expression and kynurenine production was sensitive to inhibition by CsA corroborating the effects seen with mRNA induction (Figure 7C).

STAT Activation is a Hallmark of SAg-Mediated Stimulation of APCs

To further elucidate the signaling pathways in SAg-mediated stimulation of APC we analyzed the phosphorylation pattern of STAT molecules and MAPK after stimulation. While phosphorylation of MAPK (p38, JNK, P44/42) was not changed (Supplementary Figure S4), STAT1 and STAT3 were immediately phosphorylated, independent on CsA (Figure 9). STAT5 showed no phosphorylation. In contrast, after 24 h STAT1, STAT3, and STAT5 were strongly phosphorylated (Figure 9). The phosphorylation observed was sensitive to inhibition by CsA.

DISCUSSION

Superantigens are bacterial exotoxins that interact with immune cells. It was recognized long ago that activation as well as inactivation/tolerance of T-cells represent an obvious paradox. However, both reaction profiles might contribute to the immune evasion strategy of the pathogens (MacDonald et al., 1993). It is unquestionable that acute release of pro-inflammatory cytokines leads to a dysregulation of the immune and other systemic responses eventually causing septic shock. Staphylococcal or streptococcal TSS are examples of this pathogenesis (Miethke et al., 1993). They resemble cytokine storm events comparable to those observed in humans after accidentally triggering T-cells

with monoclonal antibodies (Suntharalingam et al., 2006). The induction of T-cells requires binding of SAg to MHC class II molecules of APC and the followed crosslinking of T-cells to APCs (Rink et al., 1997). Binding of SAg activates APC, resulting in production of co-stimulatory cytokines and co-stimulatory molecules (Ohnishi et al., 1995).

Concomitantly inhibitory circuits are induced that subsequently dominate. After initial deletion of SAg-reactive T-cells the remaining T-cells become unresponsive and display an anergic phenotype (Kisielow et al., 1991; Wahl et al., 1993). Moreover it was shown that anergy and unresponsiveness are accompanied by the induction of CD4⁺CD25⁺ T regulatory cells (Wang et al., 1998; Noël et al., 2001; Papiernik, 2001). It is quite obvious that cytokine storm, anergy and induction of Treg induce a milieu of dysregulation and suppression that precludes a coordinated immune response and thus allows the pathogen to subvert anti-infective strategies of the host.

We show here that APC function after SAg-binding not only includes the induction of pro-inflammatory responses in terms of cytokine release and costimulation but also the induction of co-inhibitory circuits including anti-inflammatory cytokines (IL-10), co-inhibitory molecules (PD-L1) and also an induction of inhibitory effector programs (IDO). While IDO might result in an unspecific immunosuppression by depleting tryptophan (Taylor and Feng, 1991) PD-L1 and IL-10 suggest an at least bystander activity to induce Tregs (Unger et al., 2009; Wölflé et al., 2011). Moreover kynurenone an intermediate of the tryptophan metabolism produced by IDO, can further screw T-cell differentiation in direction of Tregs (Quintana et al., 2008; Gandhi et al., 2010; Opitz et al., 2011). Altogether an immunosuppressive milieu is induced which clearly foster the generation of Tregs and thus prevents an active immune response. Indeed our experiments with SPEA-treated monocytes confirm their inhibitory influence on CD3-mediated T-cell proliferation (Supplementary Figure S5). Furthermore SPEA-treated co-culture experiments with APCs and T-cells reveal a CD4⁺CD25⁺Foxp3⁺ Treg population (Supplementary Figure S6) that is functionally active and inhibits T-cell proliferation (Supplementary Figure S7).

Interestingly, negative feedback regulators for IFN signaling (SOCS-1) were also induced by SPEA (Song and Shuai, 1998; Dalpke et al., 2008; Masters et al., 2010). This could explain why APCs are refractory to stimulation with proinflammatory cytokines like IFN-gamma and thus maintain their inhibitory phenotype. We also observed a long lasting phosphorylation of STAT1 as well as STAT3 after SAg treatment of APC. Phosphorylated STAT3 induce expression of PD-L1 (Wölflé et al., 2011) and IDO (Litzenburger et al., 2014) and thus contribute to the immunosuppressive milieu. Constitutive STAT1 phosphorylation seems to contradict the immunosuppressive phenotype. Although we have not addressed this here in detail, it was shown that STAT1 action itself is under control of SOCS-1, which could explain why STAT1 phosphorylation does not necessarily induce proinflammatory cascades (Hildebrand et al., 2010).

A critical role during these processes of tolerance induction plays the APC. APC represent the scaffold to present SAg to

T-cells (Dellabona et al., 1990), yet also provide other signals to the T-cells (Mehindate et al., 1995). Therefore class II-binding of SAg has to deliver a signal to the APC. Class II signaling has been described for B-cells (Mooney et al., 1990; Nabavi et al., 1992) and APCs (Chatila and Geha, 1993). Thereby different signal intermediates such as Ca²⁺ (Damaj et al., 1992), PKC (Palkama and Hurme, 1993), NFAT and MAPK (Haylett et al., 2009), and tyrosine kinases (Palkama and Hurme, 1993; Morio et al., 1994; Kanner et al., 1995) were shown. When we analyzed SAg-induced signaling using various inhibitors, we found no indication for an involvement of PI3K (Wortmannin), mTOR (rapamycin) or the tyrosine kinase Syk (piceatannol) (Figure 4). Since Ca²⁺ mobilization can be a consequence of SAg-activation (Damaj et al., 1992) we presumed that NFAT would be involved, yet this was not the case. In contrast all responses induced by SAg could be blocked by CsA. This was not an entirely surprising finding since we have reported recently that in an analogous stimulation model in B-cells signaling was also sensitive to inhibition with CsA but not dependent on NFAT (Ziegler et al., 2014). The sensitivity to CsA of the induction of an immunosuppressive milieu of APC fits well in older reports on the unfolding of tolerance and unresponsiveness. Sakaguchi reported that neonatal administration of CsA induced autoimmune disease in a model which was depended on Tregs (Sakaguchi and Sakaguchi, 1989). It was also shown that CsA inhibits Treg generation (Wang et al., 2006) and that immunosuppressive therapy with CsA might reduce the number of Treg after allogeneic renal transplantation (Korczak-Kowalska et al., 2007).

Cyclosporine A might interfere with the induction of Treg in two ways. Once it inhibits the Treg inducing milieu after class II binding of SAGs as described above. Secondly, CsA might affect the self-stabilizing feedback loop of Treg-APC interaction. Tregs express LAG-3 which interacts with class II molecules (Liang et al., 2008). Interaction could lead to APC activation and subsequent production of inhibitory cytokines and molecules as shown above. That would lead to a self-propagation of Tregs and thus stabilize and expand the Treg pool. Accordingly CsA would prevent this loop by inhibiting the response to class II crosslinking. It has been shown that blockade of PD-L1 and LAG-3 rapidly cleared infection with plasmodia (Butler et al., 2012), indicating that during infection this loop is operative and could be manipulated.

Taken together we show here that APC triggered by SAg are not only responsible for the initial induction of proinflammatory responses but are also crucial for the induction and maintenance of unresponsiveness and anergy. Paradoxically the T-cell immunosuppressive drug CsA prevents in SAg triggered APC the manifestation of the immunosuppressive program, indicating that CsA could be utilized to prevent SAg-induced anergy and unresponsiveness.

FUNDING

This work was supported by a project of the collaborative research center 938 (SFB938) to KH and a postdoctoral fellowship to DH (Medizinische Fakultät Heidelberg).

AUTHOR CONTRIBUTIONS

AS, SF, KH, and DH designed the study. DH and KH wrote the final manuscript. AS, SF performed the experiments. All authors read the manuscript and discussed the results.

ACKNOWLEDGMENTS

We thank Sabrina Klein, Nina Sinke, and Dennis Nurjadi for the fruitful discussion of results and Sébastien

Boutin for statistical analysis. We are grateful to Christiane Opitz, German Cancer Research Center (DKFZ), for the discussions and technical advice (kynurenine assay).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmich.2015.01153>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pasteurella multocida Toxin Manipulates T Cell Differentiation

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Pasteurella multocida causes various diseases in a broad range of wild and domestic animals. Toxigenic strains of the serotypes A and D produce an AB protein toxin named *Pasteurella multocida* toxin (PMT). PMT constitutively activates the heterotrimeric G protein subunits $G\alpha_q$, $G\alpha_{13}$, and $G\alpha_i$ through deamidation of a glutamine residue, which results in cytoskeletal rearrangements as well as increased proliferation and survival of the host cell. In human monocytes, PMT alters the lipopolysaccharide (LPS)-induced activation toward a phenotype that suppresses T cell activation. Here we describe that the toxin also modulates CD4-positive T helper (Th) cells directly. PMT amplifies the expansion of Th cells through enhanced cell cycle progression and suppression of apoptosis and manipulates the differentiation of Th subclasses through activation of Signal Transducers and Activators of Transcription (STAT) family members and induction of subtype-specific master transcription factors. A large population of toxin-treated T cells is double-positive for Foxp3 and ROR γ t, the transcription factors expressed by Treg and Th17 cells, respectively. This suggests that these cells could have the potential to turn into Th17 cells or suppressive Treg cells. However, in terms of function, the PMT-differentiated cells behave as inflammatory Th17 cells that produce IL-17 and trigger T cell proliferation.

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 21 July 2015

Accepted: 30 October 2015

Published: 19 November 2015

Citation:

Hildebrand D, Heeg K
and Kubatzky KF (2015) *Pasteurella
multocida* Toxin Manipulates T Cell
Differentiation.
Front. Microbiol. 6:1273.
doi: 10.3389/fmicb.2015.01273

INTRODUCTION

An effective T cell-driven immune response against microbial pathogens depends on the T cell receptor (TCR)-mediated expansion of antigen-specific T cells as well as the differentiation of specialized T cell subsets. The nature of the invading pathogen determines the resulting CD4-positive Th subtype that is generated. Microbial components are recognized by distinct pattern recognition receptors (PRRs) on innate immune cells. As a consequence, professional antigen-presenting cells (APCs) perform phagocytosis and present foreign antigens on major histocompatibility complexes (MHC) to T cells. Binding of presented antigens to the TCR transmits the activation signal to intracellular molecules, which trigger cellular proliferation. In addition, APCs can express T cell-activating surface molecules that bind the co-receptor CD28. This ligand-receptor binding is required for the full activation and expansion of T cells. Depending on the activated PRR, APCs produce a specific set of cytokines that defines the direction of Th differentiation. The released cytokines bind to their responding receptors on the Th cell and induce signaling cascades that are transmitted through Signal Transducers and Activators of Transcription (STAT) proteins. Depending on the cytokine STAT-3, STAT-4, STAT-5, or STAT-6 then induce the expression of Th subtype-specific master transcription factors. Together with the STAT proteins,

they finally determine the differentiation of effector cells by triggering gene expression of lineage-characteristic cytokines and surface molecules (O'Shea et al., 2011). In this way, pathogen-specific Th effector cells develop to help provide an immune response tailored to recognize and destroy the microorganism. Initially, only two resulting Th subtypes, Th1 and Th2, were known. While Th1 cells that release IFN- γ and TNF- α , stimulate innate and T cell-induced immunity to recognize intracellular bacteria, Th2 cells boost the response against extracellular pathogens in the humoral and mucosal immunity. Today, a much higher variety of effector Th cells such as Th3, Th9, TR1, T follicular helper cells, Th17 and the suppressive regulatory T cells (Tregs) are known (Zhu and Paul, 2010). They can be seen as separate types or as a specific state of a certain main lineage. The plasticity of T cell differentiation is remarkable and allows a quick adaption to the invading microbe. Furthermore, this plasticity allows the control of the sensitive balance of defense activation and suppression, which is a prerequisite for a successful and moderate immune response. Lately it has become clear that the interplay between Th17 cells and Tregs is particularly important to maintain homeostasis (Asty et al., 2015; Chen et al., 2015; Talaat et al., 2015) as these two T cell subtypes have opposite functions in the regulation of the immune system. Th17 cells are named after the IL-17 family of cytokines and activate a broad range of immune cells (Park et al., 2005), hence Th17 cells are considered potent inflammatory cells with a role in autoimmune disorders (reviewed in (Korn et al., 2009)). In contrast, induced Tregs (iTregs) mediate immune suppression and protect from an overactive immune response (Shevach and Thornton, 2014), whereas natural Tregs (nTregs) develop from autoreactive thymocytes in the medulla of the thymus and sustain tolerance to self-antigens (Bettini and Vignali, 2010). The precise division between nTreg and iTreg-mediated modes of suppression however, is still under investigation (Curotto de Lafaille and Lafaille, 2009). Although Th17 cells and Tregs have opposite functions, the differentiation of both lineages is closely connected. Th17 cell development is mediated by TGF- β and IL-6, the activation of STAT-3 and the following induction of RORyt (Ivanov et al., 2006; Tanaka et al., 2014). Induced Tregs can be differentiated from peripheral CD4 $^{+}$ CD25 $^{-}$ T cells through activation of the transcription factors STAT-5 and Foxp3 in the presence of TGF- β and IL-2 (Burchill et al., 2007; Schmitt and Williams, 2013). Thus Th17 differentiation, as well as Treg formation, are dependent on TGF- β . In addition, the transcription factors Foxp3 and RORyt can influence each other and generate intermediate T cell subtypes such as IL-17-releasing Foxp3-positive cells (Voo et al., 2009; Kryczek et al., 2011). Finally, Th17 cells can turn into iTregs and nTregs can be converted into Th17 cells, respectively, under inflammatory conditions (Kong et al., 2012). However, this plasticity of CD4-positive T cells also has disadvantages for the host as it represents a welcome target for pathogens to modulate the immune response. T cell differentiation can either be indirectly modulated through the manipulation of APCs or directly through induction of signaling cascades in the lymphocyte (Miethke et al., 1994; Smith et al., 2004; Ivars, 2007; Sakai et al., 2010; Rodriguez-Garcia et al., 2011). *Pasteurella multocida* toxin (PMT) from *P. multocida*

manipulates the host's signaling cascades through constitutive activation of the alpha subunits of the heterotrimeric G proteins Gq, G13, and Gi through deamidation of glutamine residue (Orth and Aktories, 2010). Downstream of the activated G protein, signaling events such as calcium mobilization, phospholipase C activation, activation of the small GTPase RhoA or mitogen-activated protein (MAP) kinase pathways have been reported (Wilson and Ho, 2011). Furthermore, we could show that PMT mediates long-term activation of the Janus kinase (JAK)-STAT pathway through constitutive activation of Gq. This results in constitutive STAT-3 activation and aberrant expression of its target gene, the kinase Pim-1 which in turn phosphorylates and thus inhibits the negative feedback regulator supressor of cytokine signaling (SOCS)-1 (Orth et al., 2007; Hildebrand et al., 2010). Eventually this causes an enhanced expression of JAK2 and an increase in STAT-3 activity. As a consequence of PMT-induced signaling, the toxin stimulates proliferation of a variety of cells types such as fibroblasts, bladder epithelial cells or osteoclasts, and protects cells efficiently from apoptosis (Preuss et al., 2010; Wilson and Ho, 2012). Recently we have shown that PMT alters the lipopolysaccharide (LPS)-induced activation of human monocytes toward a phenotype which shows decreased T cell activation (Hildebrand et al., 2012). The study presented here aimed to answer the question whether PMT can also directly modulate CD4-positive T cells responses. Our experiments on human blood-derived Th cells demonstrate that the toxin amplifies the CD3/CD28-induced expansion of T cells through enhanced cell cycle progression and survival. Moreover, PMT modulates the differentiation of Th cells into specific subtypes. The toxin mediates activation of STAT family members and the expression of the subtype determining transcription factors RORyt and Foxp3. Our experiments show that the PMT-generated T cell phenotype is able to induce proliferation of naïve T cells as well as the differentiation of Th17 cells.

MATERIALS AND METHODS

Expression of Recombinant Protein

Recombinant PMT and PMT^{C116S} were kindly provided by J. Orth and K. Aktories (Freiburg). The toxin was expressed and purified as described before. Possible endotoxin contaminations were removed by an endotoxin removing gel (Pierce; Hildebrand et al., 2014).

T Cell Isolation

Peripheral blood mononuclear cells were isolated from fresh blood or buffy coats by density gradient centrifugation (Pancoll 1.077 g/mL; PAN Biotech) and washed three times with PBS. CD4-positive T cells were negatively selected (untouched) by magnetic-associated cell sorting (CD4 $^{+}$ T Cell Isolation Kit, Miltenyi Biotec) and AutoMACS technology twice. Sorted cells (93–97% purity) were cultured in RPMI 1640 medium (Biochrom AG) supplemented with 10% FBS and 1% penicillin and streptomycin (PAA laboratories) at 37°C in a humidified atmosphere in the presence of 5% CO₂.

Activation and Stimulation

Cells were seeded at a concentration of 2.5 million per ml and activated with anti-CD3 and anti-CD28-coated beads (T Cell Activation/Expansion Kit, Miltenyi Biotec) at a ratio of 1:2. Cells were stimulated with PMT, PMT^{C1165S} or heat-inactivated dT PMT (65°C, 1 h) at a concentration of 1 nM. Cholera toxin (CT) and nocodazole (Sigma-Aldrich) were used at 100 ng/ml.

T Cell Differentiation *In Vitro*

For the *in vitro* differentiation of Th17 cells, CD3/CD28-activated Th cells were stimulated 5–7 days with 100 ng/ml of recombinant IL-1β, IL-6, IL-23, and TGF-β (Immuno Tools GmbH). Regulatory T cells were generated by treatment with 100 ng/ml rapamycin (Calbiochem) and 200 ng/ml IL-2 (Immuno Tools GmbH).

Cell Viability Assay

T cells were stimulated for 5 days in a 96-well plate. Then the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was performed by following the manufacturer's protocol. By adding the CellTiter-Glo reagent, cells are lysed and a luminescent signal (measured with a microplate reader, Hidex) proportional to the amount of ATP is generated.

Carboxyfluorescein Succinimidyl Ester (CFSE) Staining

Isolated T cells were stained with carboxyfluorescein succinimidyl ester (CFSE; 2.5 million cells/ml, 5 µM CFSE, Biolegend) for 10 min at room temperature (r.t.) in the dark, before the reaction was stopped with cold medium containing 10% FCS. After washing the cells with medium, they were activated and stimulated as described above. Fluorescence intensity of the cells was quantified with a FACSCanto on day one (basis measurement) and day five.

ELISA

Supernatants of cultured T cells were collected at the indicated time points for the subsequent quantification of cytokines. IL-2, IL-17A, and IL-10 ELISAs (Biolegend) were performed following the manufacturer's instructions. Measurements were performed on a Tecan GENios Pro plate reader and analyzed with Magellan5 software.

Cell Cycle Stage Analysis

T cells were synchronized in G0 phase by culturing them overnight in the absence of an activation stimulus. Then the cells (250,000 cells per condition) were activated with anti-CD3/CD28 beads and stimulated with PMT, CT (G0/1 phase), or nocodazole (G2 phase). After 24 h cells were washed and the cell pellet was resolved in 70% ethanol and stored on ice for 15 min for fixation. Washed cells were then resolved in staining-solution (50 µg/ml propidium iodide, 100 µg/ml RNase A, 0.02% Tween 20 in PBS) with constant vortexing. After incubation for 40 min at 37°C cells were analyzed with a FACSCanto (*x*-axis: PE-A, *y*-axis: PE-W).

Immunoblot Analysis

Cells were washed twice and lysed as described before (Hildebrand et al., 2010) in RIPA buffer and analyzed by western blotting. Samples were boiled in Laemmli buffer and separated on a 4–20% gradient polyacrylamide gel (Anamed GmbH) by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. To determine STAT activation, antibodies against p(Y705)STAT-3, p(Y693)STAT-4, STAT-4, p(Y694)STAT-5, STAT-5, p(Y641)STAT-6, STAT-6, actin, and anti-rabbit horseradish peroxidase (HRP) from Cell Signaling Technology and against STAT-3 from Santa Cruz Biotechnology were used. To determine cell cycle-relevant proteins, nuclear extracts were produced as described before (Hildebrand et al., 2010). Antibodies were purchased from Cell Signaling Technology (anti-CDK6, -CDK4, -cyclin D1, -p27, -cyclin B, p(Y15) CDK1), Santa Cruz Biotechnology (anti-cyclin E, C-19), and Sigma-Aldrich (anti-B23). Primary antibodies were used 1:1000, HRP-coupled secondary antibodies 1:5000.

Caspase 3, 7, and 8 Activity Assay

T cells were seeded in 96-well plates, activated with anti-CD3/CD28-coated beads and treated for 1 or 5 days with PMT. Caspase 3, 7, and 8 activities were measured using the Caspase-3/7Glo and Caspase-8Glo Assay (Promega) in accordance to the manufacturer's instructions. Luminescence was measured using the Plate CHAMELEON™V microplate reader (Hidex).

Reverse Transcriptase-PCR

RNA was extracted from T cells with the "High pure RNA Isolation Kit" (Roche), following the manufacturers protocol. Total RNA was quantified with a NanoDrop and cDNA was prepared using "Reversed Aid First Strand cDNA Synthesis Kit" (Fermentas Life Science). The cDNA was then used for quantitative PCR analysis with the "SYBR Green Rox mix" (Thermo Scientific) and sequence-specific primers: *actin*, sense, 5'- AGA GCT ACG AGC TGC CTG AC -3', antisense, 5'- AGC ACT GTG TTG GCG TAC AG-3', human *FOXP3*: sense 5'- GAA ACA GCA CAT TCC CAG AGT TC-3', antisense 5'- ATG GCC CAG CGG ATG AG -3', human *TBET*: sense, 5'- AAG TTT AAT CAG CAC CAG ACA GAG ATG ATC -3', antisense, 5'- AAC AGA TGT GTA CAT GGA CTC AAA GTT CTC -3', human *GATA3*: sense 5'- GCT GTC TGA AGC CAG GAG AGC -3', antisense 5'- ATG CAT CAA ACA ACT GTG GCC AA -3', human *RORC*: sense 5'- CCG CTG AGA GGG CTT CAC -3', antisense 5'- TGC AGG AGT AGG CCA CAT TAC A -3'. The results were analyzed using the Fast Real-Time PCR System (Applied Biosystems). The mean values of the results (mean ± SEM; *n* = 2) were normalized to *actin*.

FACS Staining

T cells were fixed with 4% paraformaldehyde/PBS at r.t. for 15 min. Subsequently, the cells were permeabilized in 0.1% Triton X-100/PBS for 5 min (r.t.). Afterward, one million cells were incubated with 2 µg of antibodies against CD4 (Miltenyi Biotec), RORyt, Foxp3, IL-10, or IL-17 (eBioscience) for 1 h at 4°C. After washing three times, cells were incubated for 30 min at 4°C with

1 µg of the secondary antibody per one million cells (anti-mouse FITC or anti-rat PE, eBioscience). The antibody against CD4 was directly labeled with an APC fluorophore. Washed cells were then analyzed on a FACSCanto.

Co-culture Experiment

Isolated, CD3/CD28-activated T cells were differentiated into Th17 cells or Tregs or stimulated with PMT for 7 days. Then untouched T cells from the same donor were isolated and stained with CFSE (5 µM CFSE/DMSO for 10 min at 37°C, before the staining reaction was stopped with ice cold medium/10% FCS). CFSE-stained cells were activated with CD3/CD28-beads and co-cultured with washed Tregs, Th17 cells, or PMT-generated T cells (ratio 1:1). After 5 days of co-culture the fluorescent signal of the CFSE-labeled cells was quantified on the FACSCanto (FITC channel). Histogram overlays were performed using the Weasel.jar software.

Statistics

Statistical significance was assessed using Prism6 software using a two-sided ANOVA multiple comparison test with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

RESULTS

PMT Augments CD3/CD28-activated Th Cell Proliferation

Our investigation aimed to identify whether Th cell differentiation is targeted by the bacterial toxin PMT. In the experiments, we treated blood-derived CD4-positive T cells with anti-CD3- and anti-CD28-coupled beads to achieve full activation of the cells in the absence of an APC. This activation induces a network of downstream signaling pathways that eventually leads to cell proliferation and subsequently, depending on the cytokine environment, to differentiation into specific effector cells (Coquet et al., 2015). To investigate whether PMT is toxic for T cells, we initially checked its influence on the viability of CD3/CD28-activated T cells. To test this, we quantified the amount of cellular ATP in an assay where the ATP is used to generate a luminescence signal. **Figure 1A** shows that the ATP level of CD3/CD28-activated cells was increased considerably after 7 days in culture which confirmed the activation of cells and suggested an increase of the cell number. The additional stimulation with PMT remarkably enhanced lymphocyte viability. A PMT mutant, where a point mutation within the catalytic domain renders the toxin inactive, as well as heat-inactivated PMT did not alter the CD3/CD28-mediated activation. Therefore we concluded that the observed effect is mediated by the enzymatic function of PMT and that a potential endotoxin contamination can be excluded. To investigate whether the increase in cell viability is due to an increase in Th cell proliferation, stainings with fluorescent CFSE that binds covalently to proteins and other amino groups were performed. During cell division the fluorescent dye is passed on to the dividing cells and thus the fluorescence intensity

decreases with each division. **Figure 1B** shows that PMT treatment leads to an enhanced number of cells that underwent cell division. Additionally, the PMT-stimulated cells divided more frequently than the CD3/CD28-activated cells (mean fluorescence of 4222 for PMT-treated cells vs. 6562 for untreated cells).

The proliferation rate of T cells can be altered by various signals. IL-2 is a potent growth factor that mediates cell cycle entry, increases survival of dividing cells and also guides the differentiation of T cells (Oppenheim, 2007). To investigate whether PMT enhances T cell proliferation through an increased secretion of IL-2, we quantified the production of this cytokine by ELISA. The kinetics revealed that the IL-2 concentration of CD3/CD28-activated cells increased from day one to day seven, as expected. Addition of PMT effectively enhanced the release of the cytokine compared to CD3/CD28-activated cells, at least during the first 3 days of culture, which might be a reason for the observed increase in proliferation (**Figure 1C**).

PMT Mediates Cell Cycle Progression and Survival of Th Cells

In the following experiments we aimed to determine the influence of PMT on cellular proliferation in more detail. Therefore we analyzed the cell cycle stages of Th cells. The cell cycle can be divided into four major phases. In the G0 phase the cell rests. When it enters G1 phase it increases its size and produces proteins as well as other cellular components. DNA replication takes place in the synthesis (S) phase, which is followed by the preparation (G2 phase) for cell division. Finally the division of the nucleus (M Phase, mitosis) and the formation of two daughter cells (cytokinesis) occur. An effective T cell cycle run requires TCR activation to trigger transition from the G0 to G1 phase and subsequently activation of the IL-2 receptor, which mediates the entry into S phase (Cantrell and Smith, 1984; Meuer et al., 1984). To examine the effect of the toxin on the cell cycle, we quantified the DNA content of the cells. Cells were stained with propidium iodide (PI) to determine the amount of cellular DNA by FACS. As a control for G2/M, cells were treated with nocodazole, a substance that interferes with the polymerization of microtubules and thus inhibits division into daughter cells so that nocodazole-treated cells contain the maximum amount of DNA during cell cycle. As a control for G0/1 phase and therefore the minimal amount of DNA, cells were treated with CT from *Vibrio cholerae* which is known to induce cell cycle arrest in the early G1 phase (Zheng et al., 2014). **Figures 2A,B** show that non-activated Th cells paused in G1/0 phase. CD3/CD28-activation stimulated the increase of DNA per cell which is characteristic for the S phase of the cycle. Stimulation of CD3/CD28-activated cells with PMT induced a further increase of the PI signal and revealed a population with a DNA amount that is typical for the G2/M phase. We therefore conclude that the toxin mediates progression of the cell cycle and DNA synthesis. To link this to molecules known to be involved in cell cycle progression, we checked the impact of PMT on the expression and activation level of cyclin-dependent kinases (CDKs), the regulatory cyclins and CDK inhibitors (**Figure 2C**). CDK activity is regulated by its phosphorylation status, association with cyclins

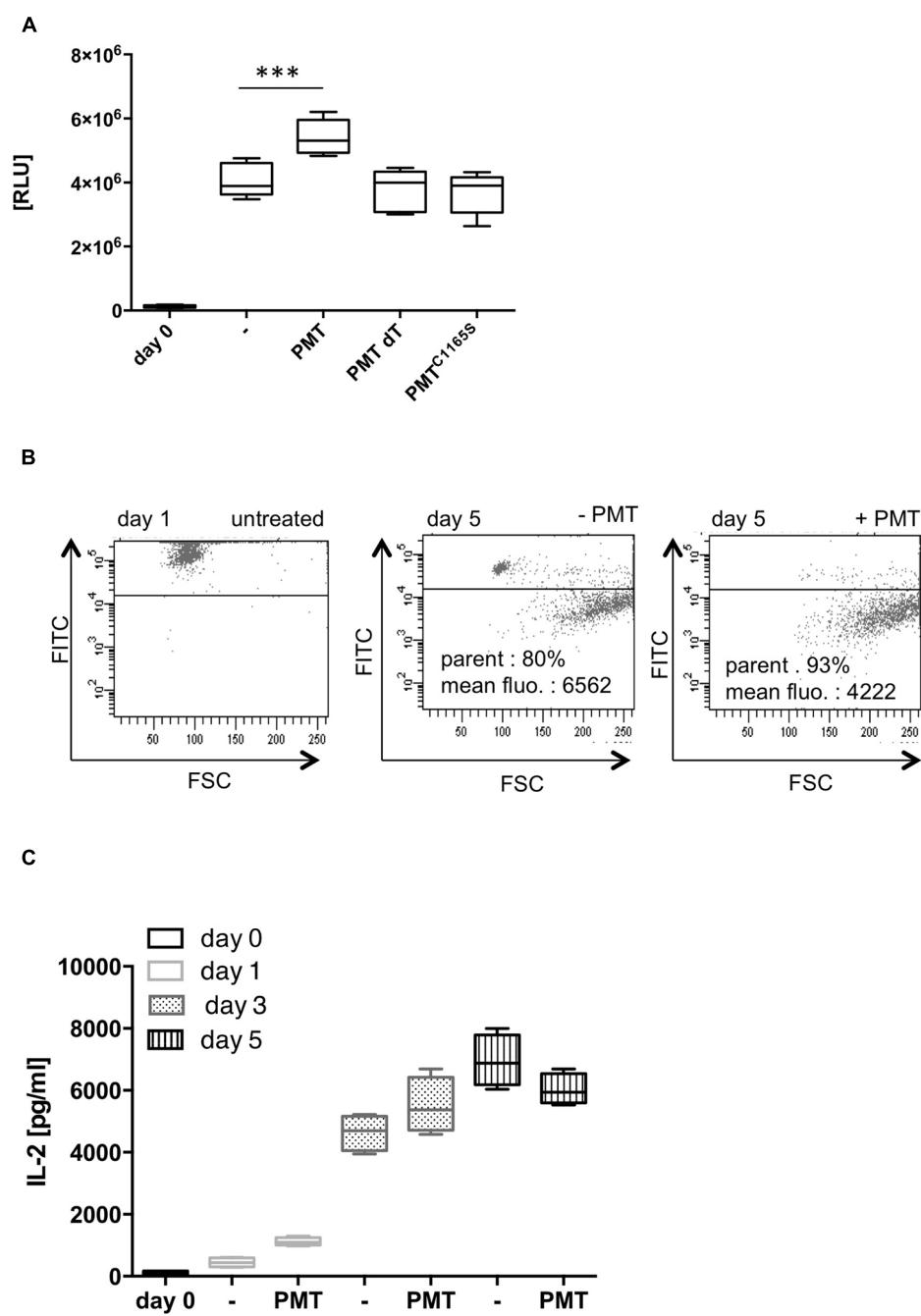


FIGURE 1 | *Pasteurella multocida* toxin (PMT) stimulates CD3/CD28-activated T cell proliferation. **(A)** CD4-positive T cells were activated with anti-CD3/CD28-coated beads (T Cell Activation/Expansion Kit, Miltenyi) and stimulated for 5 days with 1 nM PMT wild-type (wt), heat-inactivated PMT (dT), or catalytically inactive mutant (PMT^{C1165S}). Viability of cells was determined by quantification of ATP using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Shown is the mean \pm SD, $n = 6$ (six donors, each experiment performed in duplicates). **(B)** Th cells were CFSE-labeled, CD3/CD28-activated and stimulated for 5 days with 1 nM PMT. Proliferation cycles were assayed by measuring the decreasing fluorescence by flow cytometry. The mean fluorescence and percentages refer to the gated (dividing) cells. Shown is the result of one representative donor out of three. **(C)** CD3/CD28-activated Th cells were stimulated with PMT for 1, 3, or 5 days. IL-2 ELISAs were performed (mean \pm SD, $n = 4$; four donors, each experiment performed in duplicates). Statistical analysis was performed using two-sided ANOVA for multiple comparisons ($***p \leq 0.001$).

and binding of inhibitory molecules. Entry into S phase is mediated by CDK6/cyclin D and CDK4/cyclin D complexes and their activation requires the degradation of the inhibitor p27.

The active complex then enables transcription factors to activate G1/S-phase gene expression (Bendris et al., 2015). The performed western blot analysis showed that PMT indeed mediates an

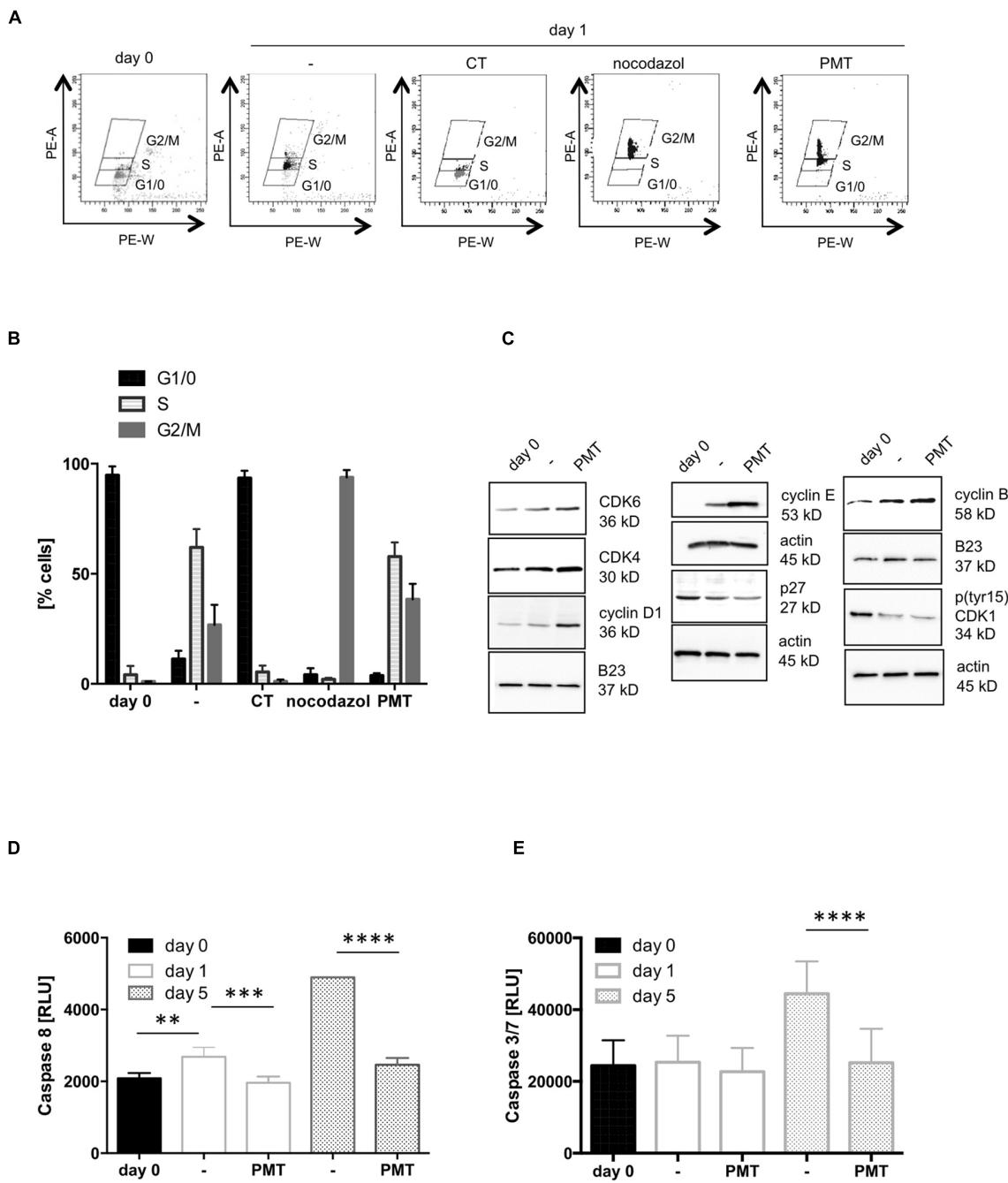


FIGURE 2 | PMT stimulates cell cycle progression. (A) CD4-positive T cells were cultured overnight without activation to synchronize cells in G0 phase. Then cells were CD3/CD28-activated and stimulated with PMT for 24 h. As controls, cells were treated with 100 ng/ml CT (G0/1 phase) or 100 ng/ml nocodazole (G2 phase). Nuclei were stained with propidium iodide (PI) and intensity of the PI signal was quantified by flow cytometry (x-axis: PE-A, y-axis: PE-W). **(B)** Quantification of the percentage of cells in G1/0, S and G2/M phase (mean \pm SD, $n = 3$). **(C)** Cell lysates were used for western blot analysis with specific antibodies against cyclin B, pCDC2 (CDK1), cyclin D1, cyclin E, CDK4, or p27. Equal loading was verified by detection of actin (whole cell lysates) or B23 (nuclear extracts). The results were corroborated with two more donors. **(D,E)** T cells were stimulated with PMT for 1, 3, or 5 days, lysed and the activation of caspase-8 **(D)** or caspases 3 and 7 **(E)** was measured with a Caspase-8Glo[®] Assay or a Caspase 3/7-Glo[®] Assay, respectively (Promega). Shown is the mean \pm SD, $n = 3$ donors. Statistical analysis for this figure **(B,D,E)** was performed using two-sided ANOVA for multiple comparisons ($^{**}p \leq 0.01$, $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$).

increased degradation of p27, an increase in cyclin E expression and additionally induces a stronger accumulation of CDK4, CDK6, and cyclin D in the nucleus than CD3/CD28. Furthermore

PMT increases the expression of cyclin E which in complex with CDK2 promotes degradation of p27 (Bendris et al., 2015). Hence, toxin treatment seems to promote the entry into the S phase of the

cell cycle. The entry of all eukaryotic cells into mitosis is regulated by activation of CDK1 (cdc2) at the G2/M transition. A critical regulatory step is the dephosphorylation of CDK1 at Thr14 and Tyr15 (Atherton-Fessler et al., 1994) and the phosphorylation-regulated translocation of cyclin B1 to the nucleus. Our results show reproducibly that PMT slightly enhances the translocation of cyclin B to the nucleus and a dephosphorylation of Tyr15 of CDK1. These results corroborate earlier findings reporting the upregulation of cell cycle progression by PMT using a fibroblast cell line (Wilson et al., 2000).

From our previous work in HEK 293 cells we knew that PMT is not only able to trigger proliferation, but also protects cells from staurosporine-induced apoptosis through the PI3K-dependent phosphorylation of Akt and the constitutive expression of the survival kinase Pim-1, respectively (Preuss et al., 2010). Programmed cell death is an integral part of the immune response, as the removal of T cells after clearance of the infection restores immune cell homeostasis (Pinkoski and Green, 2005). Taking this into consideration, the increased T cell number under PMT treatment could additionally be caused by the inhibition of apoptosis of activated T cells. To check this possibility, we analyzed caspase-8 and caspase-3/7 activation as central markers of the apoptotic response. The luminescence-based assays show that the activity of caspase-8 (Figure 2D) and caspase-3 and -7 (Figure 2E) increased considerably after 5 days of CD3/CD28-activation. Stimulation with PMT, however, kept the activity of the death caspases at the basal level of freshly isolated, naïve T cells. This supports our hypothesis that the toxin does not only trigger proliferation but also inhibits apoptosis of receptor-activated Th cells. In summary these data demonstrate that PMT expands CD3/CD28-activated Th cells by triggering proliferation and by suppressing apoptosis.

PMT Induces Foxp3 and ROR γ t-Positive T Cells

As we had observed that PMT supports the CD3/CD28-mediated proliferation of Th cells, we next aimed to investigate whether the toxin also modulates the differentiation of naïve T cells into specific T cell subtypes. T cell differentiation is dependent on the cytokine environment. Downstream of cytokine-induced receptor activation, STAT transcription factors play a pivotal role in T cell differentiation through the induction of subtype-specific genes. Hence our first question addressed the PMT-mediated phosphorylation and activation of STAT family members. Western blot analysis of toxin-stimulated cells (Figure 3A) shows a pronounced phosphorylation of STAT-3 in comparison to CD3/CD28-induced cells after 24 h of toxin treatment. As expected, STAT-5 phosphorylation was induced by CD3/CD28 activation, presumably via the production of IL-2, but this activation level could still be enhanced by PMT treatment. Phosphorylation of STAT-4 was not affected by antibody-activation and only slightly induced by PMT. Finally STAT-6 phosphorylation could not be observed under antibody or toxin conditions. As pSTAT-3 mediates differentiation of Th17 cells and pSTAT-5 is important in Treg formation, we generated these T cell subtypes *in vitro* and compared the STAT

activation level of cytokine-differentiated control cells with PMT-treated cells. On day one, PMT treatment caused an intense phosphorylation of both transcription factors, however, on day seven only pSTAT-3 was detectable. Whereas fully differentiated (day 7) Tregs contained activated STAT-5 and only basal level of phosphorylated STAT-3, Th17 cells showed high STAT-3 but no STAT-5 activation. The data show that PMT-stimulated T cells display the same STAT activation pattern like Th17 cells (Figure 3B). When we analyzed the master transcription factors of subtype development after 24 h of treatment (Figure 3C), we found that in line with our previous results, toxin-stimulated CD4-positive cells contained higher copy numbers of *RORC* (STAT-3 target) and *FOXP3* (STAT-5 target) mRNA compared to *GATA3* (induced by STAT-6) and *TBET* (induced by STAT-4). This was confirmed on the protein level by intracellular FACS analysis (Figure 3D) where high expression of Foxp3 and ROR γ t could be found in cells that had been treated with PMT for 7 days. The CD3/CD28-activated population already contained a considerable amount of Foxp3, which is probably due to IL-2-mediated STAT-5 activation. These data raised the question whether PMT stimulates the differentiation of two different T cell subtypes or whether one subtype with two simultaneously expressed master transcription factors was produced. Double stainings for Foxp3 and ROR γ t (Figure 3E) revealed that the antibody-activated T cell population did not contain any double positive cells. In contrast to that the PMT-treated population contained around 30% ROR γ t-positive, 20% Foxp3-positive and almost 46% double positive T cells, even after 10 days of culture.

PMT Mediates Differentiation of an Inflammatory T Cell Subtype

Our results pointed to two different sets of T cell responses toward the bacterial toxin. Th17 cells have an autoinflammatory potential and support the immune response against extracellular bacteria, whereas Foxp3-positive Tregs have a suppressive role and protect the organism from autoimmunity. The different effector T cell types are ultimately defined by the cytokines that they release and by their function. Therefore we investigated which cytokines are actually released from PMT-stimulated cells and whether they support or suppress a T cell-driven immune response. ELISA analysis revealed that PMT-modulated cells release high amounts of IL-17A that is almost comparable to that of Th17 cells (Figure 4A). However, these cells produced nearly no immunosuppressive IL-10 (Figure 4B). FACS data corroborated these findings and additionally showed that there is no defect in the release of cytokines, but that IL-10 is hardly expressed by these lymphocytes (Figure 4C). Some of the CD3/CD28-activated T cells produced IL-10 (Figures 4B,C), which confirmed the differentiation of a few functional Foxp3-positive cells. To investigate whether the PMT-generated T cell subtype would rather have an activated or suppressive phenotype, we measured the ability of PMT-modulated T cells to increase or inhibit proliferation of CD3/CD28-activated CFSE-labeled CD4-positive T cells. Our data show that the CFSE signal of CD4-positive responder cells co-cultured with the PMT-differentiated effector subtype was lower than the signal obtained by CD3/CD28-activated responders. Additionally, the influence

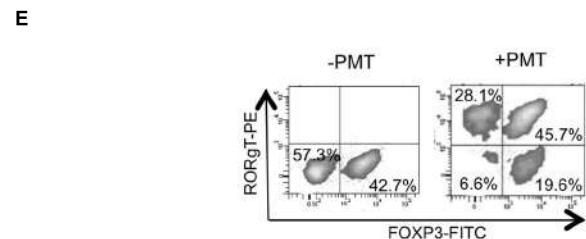
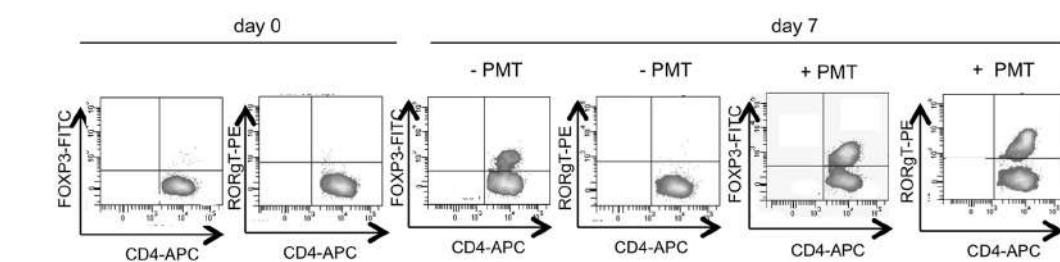
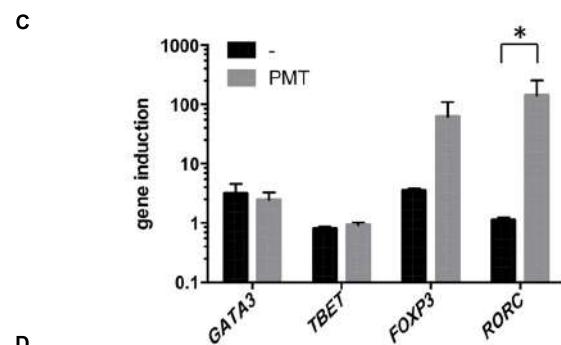
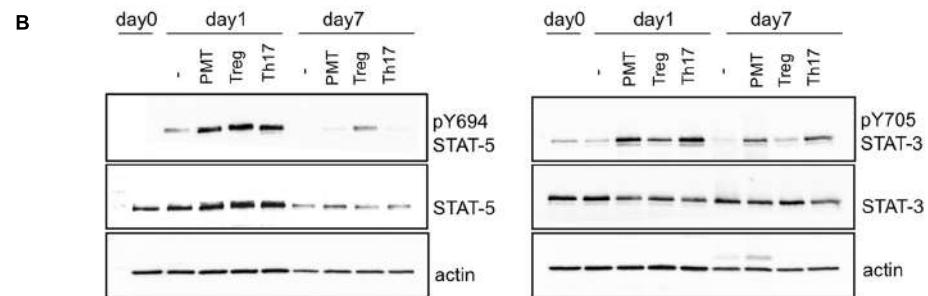
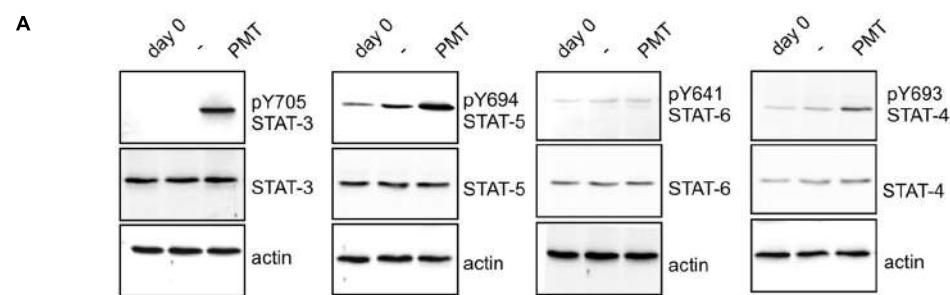


FIGURE 3 | Continued

FIGURE 3 | Continued

PMT induces Foxp3 and ROR γ t-positive T cells. CD3/CD28-activated T cells were stimulated with PMT overnight or for 7 days, respectively. **(A)** Whole cell lysates were produced and antibodies against p(Y705)STAT-3, STAT-3, p(Y694)STAT-5, STAT-5, p(Y641)STAT-6, STAT-6, and p(Y693)STAT-4, STAT-4 were used for immunoblotting. Equal loading of protein was verified by actin detection. **(B)** As a positive control, T cells were treated with 100 ng/ml IL-1 β , IL-23, IL-6, TGF- β (Th17 cells), or 100 ng/ml IL-2 and rapamycin (Tregs). **(C)** Quantitative RT-PCRs were performed using SYBR Green and sequence specific primers for *FOXP3*, *TBET*, *GATA3*, and *RORC*. Results were normalized against the housekeeping gene *actin*. Shown is the mean of induction compared to non-activated cells. SD, $n = 2$ donors, experiment performed in duplicates. Statistical analysis was performed using two-sided ANOVA for multiple comparisons (* $p \leq 0.05$). **(D)** Intracellular FACS analysis. Fixed and permeabilized cells were stained with anti-CD4-APC, purified anti-Foxp3 or anti-ROR γ t and FITC-PE-labeled secondary antibodies. **(E)** Intracellular double stain of Foxp3 or ROR γ t of cells on day ten. Data shown in **(A,C,D)** are representative for three, and **(E)** for two experiments.

of PMT effector cells on proliferation was similar to the impact of Th17 cells. In contrast to this, Tregs showed an inhibitory impact on T cell proliferation (**Figure 4D**). This experiment suggests that the toxin modulates lineage decision of CD3/CD28-activated T helper cells toward an inflammatory subtype.

DISCUSSION

The relationship between the host and microbial pathogens can be quite complex. On the one hand the higher organism uses a highly specialized immune system for the detection and control of invading microorganisms, whereas pathogens have developed various strategies to avoid their recognition and elimination. The host's innate immune system can sense foreign structures through PRRs. In consequence of this recognition, pathogen-induced phagocytosis and presentation on MHC takes place. Presentation of the antigens to the TCR and the resulting association of CD3 with the TCR-peptide-MHC complex transmits the activation signal to intracellular molecules, which trigger a cascade of biochemical events that result in the expansion of antigen-specific T lymphocytes. In addition, the differentiation into effector types is crucial for the course of the immune response. Depending on the microbe and the activated PRRs, a specific set of cytokines is produced by the APC, which mediates the differentiation of appropriate effector types. The plasticity of Th cell differentiation during infection allows the development of pathogen-specific effector T cells. However, also the invading microorganism may profit from this plasticity by directing the differentiation to an ineffective or a suppressive effector type. One example for such a manipulation has been described for *Chlamydia muridarum*. Chlamydia-infected APCs are modulated in terms of the phenotype, cytokine secretion profile and antigen presentation. This eventually shifts the immune response from a protective Th1 to a non-protective Th2 response (Kaiko et al., 2008). Also extracellular bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* disturb the balance between activation and inhibition of immune cells that protects the host from an over-reaction and re-establishes homeostasis after clearing the

infection. Superantigens (SAGs) produced by these bacteria can activate up to 20% of all T cells in an individual, mediate a vast T cell expansion and induce a strong cytokine storm that causes serious harm to the host. Eventually, the majority of activated T cells die and the remaining cells will be anergic or even induce SAg-specific Treg-mediated tolerance to the microbe (Papiernik, 2001).

An excellent target to modulate the differentiation toward an ineffective T cell subtype are STAT transcription factors that transmit the cytokine-encoded differentiation signal. PMT activates the JAK-STAT pathway very efficiently in many types of cells (Kubatzky et al., 2013) and we found that the toxin also induces STAT activity in human T cells. After 1 day of PMT stimulation, the phosphorylation of both, STAT-3 and STAT-5 was very pronounced, whereas after 7 days of stimulation only phosphorylation of STAT-3 was detectable. Compatible with these results, RT-PCRs revealed a high expression of STAT-5-dependent *FOXP3* and STAT-3-promoted *RORC* in PMT-treated cells. This suggested that differentiation of Tregs and Th17 cells had taken place. Although the parallel development of two opposing subtypes appears paradox at first glance, it has been hypothesized that the interplay between iTregs and Th17 cells actually fine-tunes the activation to prevent hyperactivation and to facilitate the return to the steady state. This is supported by the fact that the development of these two subtypes is closely connected. Both subtypes need TGF- β for differentiation and the final direction of differentiation is induced by additional cytokines. TGF- β , in cooperation with IL-2-signaling, promotes STAT-5 activation and induction and maintenance of *FOXP3* via the induction of the Smad pathway. Nevertheless, TGF- β also mediates the maintenance of a functional IL-6 receptor on activated T cells. Under inflammatory conditions, with high concentrations of IL-6, TGF- β enables IL-6/STAT-3 signaling and eventually the differentiation of Th17 cells (Lee et al., 2009). The host's benefit from a high plasticity of Treg and Th17 cells is probably a fast reaction to the course of an infection. After the initial recognition of the pathogen, the inflammatory conditions change from the tolerogenic phenotype of nTregs toward the response-activating Th17 cells. In the process of clearance the cells are able to transdifferentiate again into iTregs to protect the body from hyperactivation (Gagliani et al., 2015). In consideration of this close connection of Th17 cells and Tregs and the complexity of the cytokine environment during infection it is not surprising that naïve, TGF- β -activated T cells can co-express ROR γ t and Foxp3. Our experiments show that after 1 week of culture several CD3/CD28-activated T cells are Foxp3 positive. This is probably due to the CD28-triggered and IL-2 mediated promotion of Foxp3 expression (Burchill et al., 2007; Guo et al., 2008) and may be due to an increased survival of nTregs (Weiss et al., 2011), (**Figure 3D**). Interestingly, 45% of the PMT-stimulated activated T lymphocytes are positive for both transcription factors. This could be caused by the strong activation of STAT-3 and STAT-5 during the first hours of stimulation. Furthermore it suggests that the observed toxin-mediated protection from apoptosis expands primarily Foxp3-positive cells, which later additionally express ROR γ t. The parallel production of ROR γ t and Foxp3 can lead to the

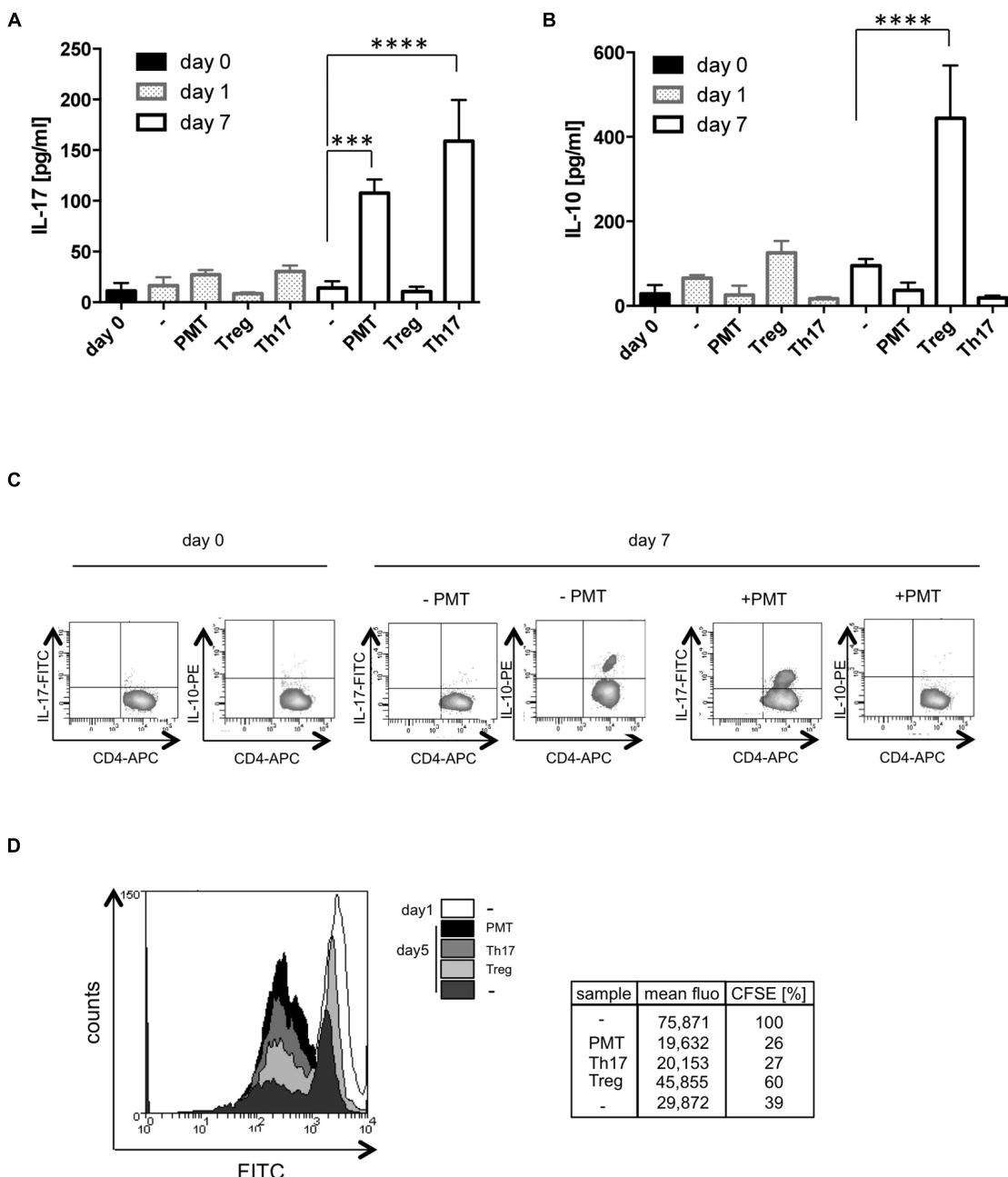


FIGURE 4 | PMT mediates a Th17 phenotype. CD3/CD28-activated Th cells were stimulated with PMT for the indicated time points. In (A) IL-17A and (B) IL-10 ELISAs were performed (mean \pm SD, $n = 3$). Statistical significance of the produced cytokine was compared to the value obtained on day 0 using two-sided ANOVA for multiple comparisons ($^{***}p \leq 0.001$; $^{****}p \leq 0.0001$). (C) For intracellular FACS analysis, fixed and permeabilized cells were double-stained with anti-CD4-APC and purified anti-IL-10 or anti-IL-17 antibodies and the according FITC-/PE-labeled secondary antibodies. (D) Th cells were stimulated with PMT for 7 days. These PMT-generated T cells were then co-cultured with freshly isolated, CFSE-labeled Th cells (ratio 1:1) from the same donor. The fluorescent signal of the FITC-positive cells was quantified on day one and on day five of co-culture. Data shown in (C,D) are representative for three experiments (three donors).

induction of both IL-10 and IL-17 or just one of them (McGeachy et al., 2007; Ichiyama et al., 2008; Zhou et al., 2008) and Foxp3 can directly bind to ROR γ t and prevent its action. Only the additional stimulation with inflammatory cytokines such as IL-6 relieves Foxp3-mediated inhibition through a STAT-3-dependent

suppression of FOXP3 expression (Yao et al., 2007), and promotes differentiation of Th17 effector cells (Ichiyama et al., 2008; Zhou et al., 2008). Our experiments indicate that the release of IL-17 increases from day one to day seven of PMT stimulation whereas the IL-10 concentration did not. This suggests that in

the beginning of PMT-stimulation the activation of STAT-3 and STAT-5 mediates the development of some IL-17 releasing Th17-cells, some IL-10 releasing Tregs, and some RORyt, Foxp3 double positive cells, which probably do not produce IL-17 due to an inhibitory interaction. At later time points, when only STAT-3 but no STAT-5 activation is observable, RORyt is released from Foxp3 inhibition and effectively induces IL-17 expression. An additional explanation for the low IL-10 production of toxin-treated cells could be the expression of the kinase Pim-1. While the expression of Pim-1 is inhibited by TCR activation, inflammatory conditions with high level of IL-6 lead to the induction of Pim-1 in human Tregs (Li et al., 2014). The kinase then phosphorylates Foxp3 at serine 422, which inhibits Foxp3 chromatin binding activity and eventually inhibits IL-10 production. Interestingly, our studies in HEK 293 cells show that PMT is a much more potent inducer of Pim-1 than IL-6 (Hildebrand et al., 2010). Therefore PMT stimulation could overcome the TCR-mediated inhibition of Pim-1 in the Foxp3-positive cells and the following mediated phosphorylation would suppress IL-10 production.

An interesting connection between Th17 cells and the bacterium is the interaction between the bone system and the immune system. IL-17 is involved in the excessive inflammation and activation of osteoclasts that strongly contributes to bone destruction associated with rheumatoid arthritis (Lubberts, 2015). Here, Th17 cells trigger the expression of receptor activator of nuclear factor- κ B ligand (RANKL) in synovial cells, which then, in cooperation with other inflammatory cytokines, stimulate the differentiation and activation of monocytes into osteoclasts (Sato et al., 2006). PMT is known to be a mitogen for osteoclasts (Martineau-Doize et al., 1993; Jutras and Martineau-Doize, 1996) and pigs infected with *P. multocida* suffer from atrophic rhinitis, a pathologic condition characterized by PMT-stimulated osteoclastic bone resorption at the nasal turbinates and an inflammation of the nasal mucosa (Wilkie et al., 2012). We have previously shown that the PMT-triggered differentiation of bone marrow-derived mouse macrophages into osteoclasts is strongly dependent on cytokines released by B cells (Hildebrand et al., 2011). Cytokines produced by Th17-cells could additionally support the PMT-mediated osteoclast formation and the destruction of bone and thereby support the pathogenesis of toxigenic *P. multocida* strains. Nevertheless, the

direction toward a Th17-mediated response is the normal course of an immune response against pulmonary and gastrointestinal pathogens like *P. multocida* (Garrido-Mesa et al., 2013). However, it has been suggested that pathogens that trigger a persistent Th17 response because they have not been sufficiently cleared through the host's immune system, may be more beneficial for the microorganism than the host (van de Veerdonk et al., 2009).

In summary, we show that the bacterial toxin PMT enhances the proliferation and survival of CD3/CD28-activated Th cells and that the toxin manipulates the differentiation toward a Th17-subtype through the activation of STAT transcription factors. This once more illustrates how bacteria are able to target the high plasticity of T cell subtypes to reinforce their pathogenicity and possibly to gain an advantage in terms of survival and reproduction.

AUTHOR CONTRIBUTIONS

KK, KH, and DH designed the study. DH and KK wrote the final manuscript. DH performed the experiments. All authors read the manuscript and discussed the results.

FUNDING

This work was supported by Deutsche Forschungsgemeinschaft, a research grant (SPP1468 IMMUNOBONE, HI1747/4-2) to KK and a postdoctoral fellowship to DH (Medizinische Fakultät Heidelberg).

ACKNOWLEDGMENTS

We thank Ricarda Voigt and Eric Stricker for excellent technical assistance, as well as Sushmita Chakaraborty for the discussion of results. We thank Joachim Orth and Klaus Aktories (Freiburg) for providing us with recombinant PMT and Hilka Rauert-Wunderlich (Würzburg) for the gift of a cyclin E antibody (Santa Cruz).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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