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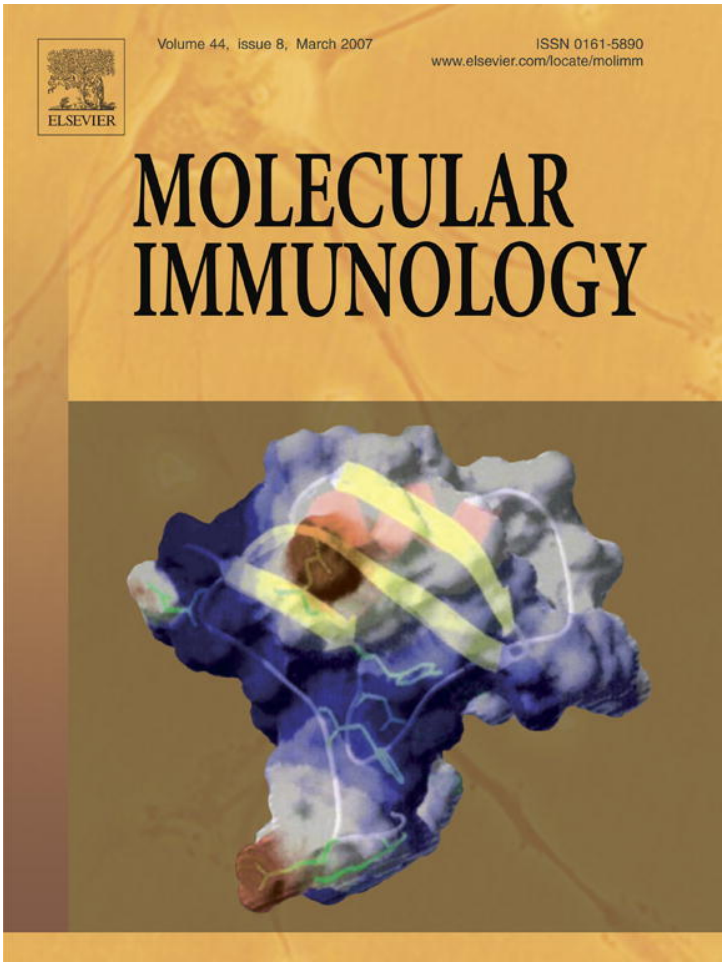
ARTICLE *in* MOLECULAR IMMUNOLOGY · APRIL 2007

Impact Factor: 2.97 · DOI: 10.1016/j.molimm.2006.09.011 · Source: PubMed



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Molecular Immunology 44 (2007) 2097–2104
Molecular Immunology
www.elsevier.com/locate/molimm

Stimulation of mast cells via FcεR1 and TLR2: The type of ligand determines the outcome

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Received 8 August 2006; received in revised form 8 September 2006; accepted 8 September 2006
Available online 13 November 2006

Abstract

Little is known about the interplay between pathophysiological processes of allergy and infection, particularly with respect to mast cell (MC)-mediated responses. The presence and recognition of pathogen-associated molecular patterns (PAMPs) might have broad impact on the development and severity of diseases. In this study, we assessed the influence of toll-like receptor 2 (TLR 2)-dependent synthetic analogs of bacterial lipopeptides (LPs), Pam_3CSK_4 and MALP-2, on Ag (DNP–HSA)-triggered responses in bone marrow-derived MCs (BMMCs). Both LPs strongly synergized with sub-optimal amounts of Ag in the stimulation of cytokine release. Intriguingly, Pam_3CSK_4, but not MALP-2 suppressed Ag-induced degranulation of BMMCs (together with early tyrosine phosphorylation and calcium mobilization) in a TLR2-independent manner. Further analysis revealed that Pam_3CSK_4, most probably by electrostatic forces, reduced the level of active DNP–HSA and that this, in turn, was responsible for the suppression of Ag-induced degranulation. Thus, our work demonstrates that LPs can synergize with IgE + Ag in stimulating the production of IL-6 by BMMCs. As well, our findings with Pam_3CSK_4 indicate that one must be cautious when interpretating results obtained with "model" substances and the combination of ligands must be carefully chosen when functional interactions between the high-affinity receptor for IgE (FcεR1) and TLR2 are examined.

Keywords: FcεR1; MALP-2; Pam_3CSK_4; TLR2; Antigen; Lipopeptides; Calcium mobilization; Degranulation; IL-6

1. Introduction

Lipoproteins (LPs) are constituents of Gram-negative bacteria located in the outer cell wall and are recognized by cells via toll-like receptor 2 (TLR2) (Takeda and Akira, 2004). Depending on the nature of the LP, TLR2 interacts with the LP together with TLR1 or TLR6 (Takeuchi et al., 2001, 2002). A major obstacle in the proper interpretation of experiments with purified lipoprotein is its potential contamination with small amounts of other bacterial products, e.g. lipopolysaccharide (LPS), and its poor solubility in aqueous media (Seifert et al., 1990). These problems are overcome by the use of chemically synthesized lipopeptides as analogues of the biologically active N-terminal moiety of native lipoproteins. Two different model LPs are widely used in immunological research: *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine (Pam_3CSK_4) which is recognized by the combination of TLR2 and TLR1 and Mycoplasma-derived macrophage-activating 2 kDa LP (MALP-2) which activates cells via TLR2 and TLR6 (Takeuchi et al., 2001, 2002). Binding of LPs to target cells via TLRs in general results in the activation of the NF-κB as well as MAPK pathways leading to the transcription of cytokines and pro-survival genes (Takeda and Akira, 2004).

Mast cells (MCs) are located at the interface between the host and its environment and are involved in the defense against microbial and parasitic invaders (Marshall, 2004). Stimulation of MCs with LPs results in a variety of responses, like degranulation and cytokine secretion (McCurdy et al., 2003). However,

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doi:10.1016/j.molimm.2006.09.011

in vivo it is very unlikely that MCs encounter LPs or lipoproteins alone, but rather in combination with additional endogeneous or exogeneous stimuli. Amongst these are further bacterial components, like LPS and DNA containing unmethylated CpG motifs, as well as host factors like cytokines, chemokines and extracellular matrix components. MCs are also well known for their pathophysiological role in the initiation and development of allergic hypersensitivity reactions (Costa et al., 1997). In such a context, LPs together with allergen/antigen (Ag) may co-activate MCs and enhance each other's effects. In this respect, several reports have described the influence of bacterial infections on the development of allergic diseases (e.g. asthma) (Kraft et al., 1998; Martin et al., 2001; von Hertzen, 1998) and ligands for TLR2 have been demonstrated to increase inflammatory responses to inhaled Ag in the mouse lung (Redecke et al., 2004).

In this study, we aim at investigating the effect of combined stimulation of murine bone marrow-derived MCs (BMMCs) with LP and Ag. Two different LPs, Pam_3CSK_4 and MALP-2 containing three and two acyl chains, respectively, and two kinds of Ag, DNP–HSA and anti-IgE antibodies are used to analyze various MC effector functions (degranulation and cytokine secretion) as well as underlying signaling events (substrate tyrosine phosphorylation and calcium mobilization). This analysis should provide us with the information whether the described influence of bacterial infections on the development of allergic diseases can be observed already on the cellular level of the MC.

2. Materials and methods

2.1. Cell culture

Bone marrow cells (1×10^6 ml^{-1}) from 6- to 8-week-old male mice (129/Sv) were cultured (37 °C, 5% CO_2) in a single cell suspension in RPMI 1640 medium containing 20% FCS, 1% X63Ag8-653-conditioned medium as a source of IL-3 (Karasuyama and Melchers, 1988), 2 mM L-glutamine, 1×10^{-5} M 2-mercaptoethanol, 50 units ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin. In weekly intervals, the non-adherent cells were reseeded at 1×10^6 cells ml^{-1} in fresh medium. By 4–6 weeks in culture, more than 99% of the cells were c-kit and high-affinity receptor for IgE (FcεR1) positive as assessed by phycoerythrin-labeled anti-c-kit antibodies (Pharmingen, Mississauga, Canada) and FITC-labeled rat anti-mouse IgE (Southern Biotechnology, Birmingham, AL, USA), respectively. SHIP1+/+ and −/− BMMCs were differentiated in vitro using the same protocol but starting from bone marrow cells of 6–8-week-old SHIP1+/+ and −/− littermates (129/Sv × C57BL/6). TLR2+/+ and −/− BMMCs were differentiated from bone marrow cells of 6–8-week-old TLR2+/+ and −/− littermates (C57BL/10).

2.2. Reagents

Monoclonal anti-phosphotyrosine (4G10) and polyclonal anti-p85 (#06–195) antibodies were purchased from Upstate/Biomol (Hamburg, Germany). Rat anti-mouse IgE antibody was purchased from Southern Biotechnology (Birmingham, AL, USA). DNP–HSA containing 30–40 moles dinitrophenyl (DNP) per mole human serum albumin (HSA) and monoclonal IgE with specificity for DNP (SPE-7) were obtained from Sigma (Deisenhofen, Germany). Recombinant murine SF was purchased from Biosource International (Nivelles, Belgium). Lipopeptides Pam_3CSK_4, Pam_2CSK_4, Myr_3CSK_4, and Lau_3CSK_4 were obtained from EMC Microcollection GmbH (Tübingen, Germany) and MALP-2 was a generous gift from Dr. P. Mühlradt, Braunschweig, Germany. The TLR2 stimuli used were verified by the use of wild-type and TLR2-deficient BMMCs (data not shown).

2.3. Degranulation assay

For degranulation studies, cells were pre-loaded with 0.15 μg ml^{-1} IgE anti-DNP overnight. The cells were then washed and resuspended in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% bovine serum albumin (BSA) in 10 mM Hepes, pH 7.4). The cells were adapted to 37 °C for 20 min and treated for 30 min at 37 °C as mentioned in figure legends. The degree of degranulation was determined by measuring release of β-hexosaminidase (Huber et al., 1998b).

2.4. Calcium measurements

IgE-pre-loaded BMMCs were washed with RPMI, resuspended at 5×10^6 cells ml^{-1} in RPMI containing 1% FCS, 30 μM Indo-1 AM (Molecular Probes), and 0.045% pluronic F-127 (Molecular Probes), and incubated for 45 min at 37 °C. Cells were then pelleted, resuspended in RPMI containing 1% FCS and analyzed in a LSR II (Becton Dickinson, Franlin Lakes, NJ, USA) after the indicated stimulation procedures. The FACS profiles were converted to line graph data using the FlowJo application.

2.5. Cell stimulation and Western blotting

IgE-pre-loaded cells (0.2 μg ml^{-1} IgE; overnight) were washed twice in phosphate-buffered saline and resuspended (2×10^7 ml^{-1}) in RPMI 1640 containing 0.1% BSA and 10 mM Hepes, pH 7.4. Cells were adapted to 37 °C for 30 min and stimulated with the indicated concentrations of DNP–HSA or LP, or both. After stimulation, cells were pelleted and solubilized with 0.5% NP-40 in 4 °C phosphorylation solubilization buffer (Liu et al., 1994). After normalizing for protein content, the postnuclear supernatants were separated by SDS-PAGE and subjected to Western blot analysis as described previously (Liu et al., 1994).

2.6. IL-6 measurement

Mouse IL-6 ELISA (BD Pharmingen, Heidelberg, Germany) was performed according to the manufacturer's instructions.

3. Results

3.1. Synthetic LP Pam_3CSK_4 suppresses Ag-triggered degranulation

The initial purpose of this study was to determine whether murine BMMCs could be induced by the LP Pam_3CSK_4 to degranulate in a manner comparable to what has been reported for human MCs (McCurdy et al., 2003). This was carried out with both wild-type (WT) BMMCs and BMMCs deficient for the SH2-containing inositol 5-phosphatase (SHIP1), which have been shown to be more prone to degranulation in response to different stimuli, e.g. IgE, Ag, and Steel Factor (SF) (Gimborn et al., 2005; Huber et al., 1998a,b). In contrast to human MCs, neither WT nor SHIP1−/− BMMCs degranulated in response to Pam_3CSK_4 at concentrations up to 50 μg ml^{-1}, but did so in response to 20 ng ml^{-1} of Ag (DNP–HSA) (Fig. 1A) as reported earlier (Gimborn et al., 2005; Huber et al., 1998a). Furthermore, Pam_3CSK_4 was unable to augment Ag-induced degranulation. In fact, it completely inhibited Ag-triggered degranulation in WT BMMCs when added at 10 μg ml^{-1} immediately before the Ag (Fig. 1B). A comparable inhibition was observed with SHIP1−/− BMMCs (data not shown). In a next set of experiments we examined the effect of Pam_3CSK_4 on calcium mobilization, an upstream signaling event critically involved in Ag-triggered degranulation (Huber et al., 1998a). Pam_3CSK_4 did not trigger a calcium influx and almost completely suppressed Ag-induced calcium mobilization (Fig. 2A). To determine whether Pam_3CSK_4 blocked degranulation induced by a ligand other than Ag, its effect on SF-induced signal transduction was tested. Pam_3CSK_4 neither suppressed SF-triggered degranulation of SHIP1−/− BMMCs (Fig. 2B) nor SF-induced calcium mobilization of WT BMMCs (data not shown). Consistent with these results, Pam_3CSK_4 suppressed Ag-induced, but not SF-induced, tyrosine phosphorylation events in BMMCs (Fig. 2C). These data suggest that the synthetic LP Pam_3CSK_4 specifically suppresses Ag-induced degranulation of murine MCs.

3.2. Pam_3CSK_4-mediated suppression of MC degranulation requires no TLR2 signaling, nor a specific acylation pattern of the LP

Activation of cells by bacterial LPs is TLR2-dependent (Galanos et al., 2000; Takeuchi et al., 2001, 2002). We therefore investigated the involvement of TLR2 in the suppression of degranulation by Pam_3CSK_4. BMMCs were differentiated from the bone marrow of WT and TLR2-deficient mice and subjected to Ag and Pam_3CSK_4 treatment. As shown in Fig. 3A, Ag alone caused a comparable degree of degranulation in WT and TLR2−/− BMMCs, while LP alone had no effect. Intriguingly however, Pam_3CSK_4 suppressed Ag-induced degranulation in WT and TLR2−/− BMMCs to the same extent (Fig. 3A), indicating that this action of the LP was independent of its cellular receptor.

We next assessed whether the LP, MALP-2, could also inhibit Ag-triggered degranulation. However, MALP-2 neither triggered degranulation nor did it influence Ag-induced degranulation in TLR2+/+ or −/− BMMCs (Fig. 3A). One major structural difference between Pam_3CSK_4 and MALP-2 is the degree of acylation. Whereas Pam_3CSK_4 is tri-acylated, MALP-2 contains only two acyl residues (Fig. 3C). However, the use of a Pam_3CSK_4 homologue with only two acyl residues (Pam_2CSK_4) did not abrogate the inhibitory action (Fig. 3B). In addition, shortening the acyl chains from C_{16} (Pam_3CSK_4) to C_{12} (Lau_3CSK_4) did not abrogate the inhibitory capacity of this LP either (Fig. 3B). These results show that the suppressive action of Pam_3CSK_4 is independent of TLR2 signaling and that the nature and number of acyl residues in the LP molecule do not play a specific role in the suppression.

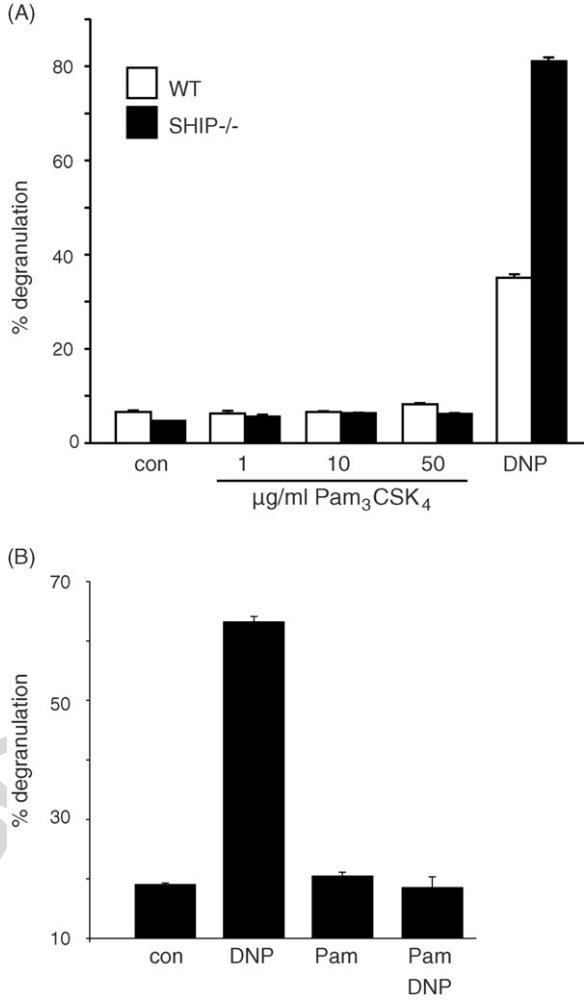

Fig. 1. Lipopeptide Pam_3CSK_4 inhibits Ag-triggered degranulation of BMMCs. (A) IgE-loaded wild-type (WT; white bars) and SHIP−/− BMMCs (black bars) were left untreated (con) or stimulated with the indicated concentrations of Pam_3CSK_4 or Ag (DNP–HSA; 20 ng ml^{-1}) for 30 min. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar represents the mean of duplicates ± S.D. Comparable results were obtained with different BMMC clones. (B) BMMCs were pre-loaded with IgE overnight. Cells were left unstimulated (con) or stimulated with Ag (DNP–HSA; 20 ng ml^{-1}), Pam_3CSK_4 (10 μg ml^{-1}), or Ag after a 15 s pre-stimulation with Pam_3CSK_4. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar is the mean of duplicates ± S.D. Comparable results were obtained with different BMMC clones.

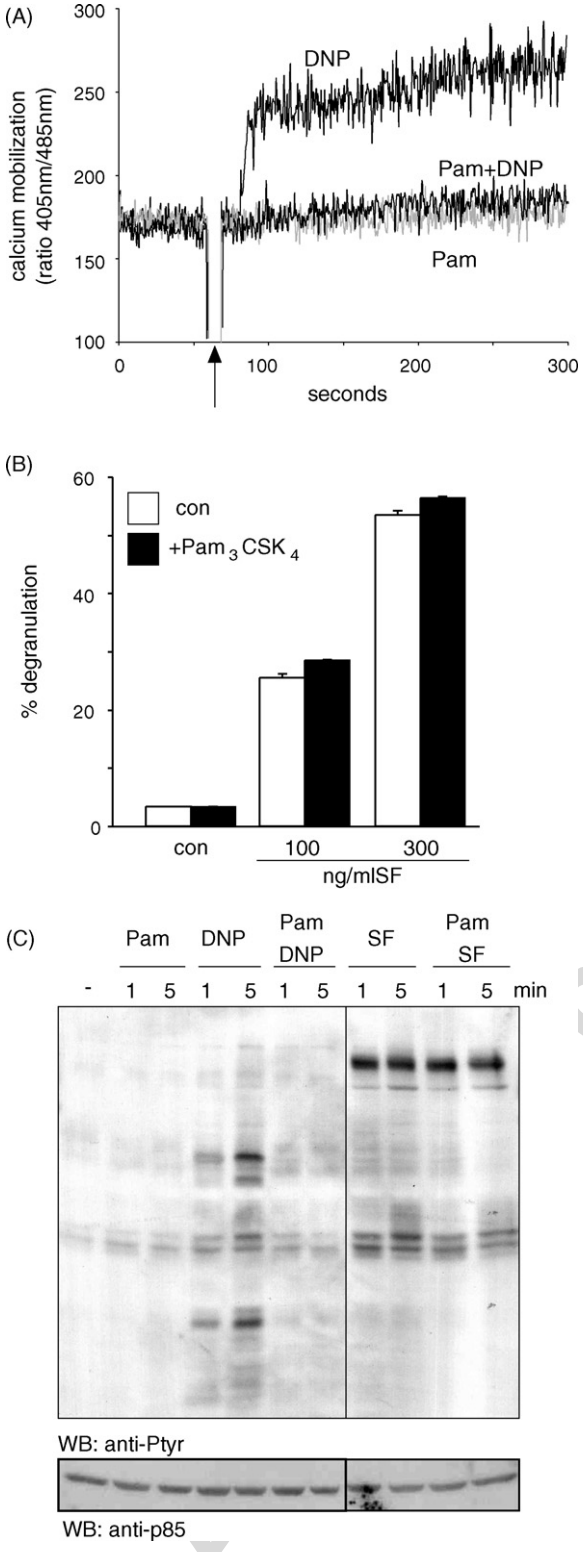

Fig. 2. Lipopeptide Pam_3CSK_4 inhibits Ag-triggered calcium mobilization and substrate tyrosine phosphorylation in BMMCs. (A) Calcium mobilization in IgE-pre-loaded BMMCs stimulated with Ag (DNP–HSA; 20 ng ml^{-1}), Pam_3CSK_4 (10 μg ml^{-1}; grey line), or Ag after a 15 s pre-stimulation with Pam_3CSK_4. The arrow marks the time point of stimulus addition. Comparable results were obtained in separate experiments. (B) IgE-loaded SHIP−/− BMMCs were left untreated (con) or stimulated with the indicated concentrations of SF in the absence (white bars) or presence of Pam_3CSK_4 (10 μg ml^{-1}; black bars) for 30 min. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar represents the mean of duplicates ± S.D. (C) IgE-pre-loaded BMMCs were left untreated (−) or stimulated with Pam_3CSK_4 (10 μg ml^{-1}), Ag (DNP–HSA; 20 ng ml^{-1}), Ag after a 15 s pre-stimulation with Pam_3CSK_4, SF (100 ng ml^{-1}), or SF after a 15 s pre-stimulation with Pam_3CSK_4 for the indicated times. Postnuclear supernatants were analyzed by anti-Ptyr (upper panel) and anti-p85 (lower panel; loading control) immunoblotting.

3.3. Pam_3CSK_4 reduces the amount of Ag (DNP–HSA) and thus suppresses optimal activation

A comparison of the peptide component of Pam_3CSK_4 and MALP-2 reveals a disproportionate content of positively charged amino acids, especially Lys, in Pam_3CSK_4 (Fig. 3C). Since the model Ag (DNP–HSA) used in our study comprises 33–40 negatively charged DNP moieties per HSA molecule, the possibility was investigated that Pam_3CSK_4 competes with the FcεR1-bound anti-DNP IgE for binding to DNP–HSA by electrostatic forces and thus strongly reduces the amount of "active" Ag available. To test this hypothesis, we assessed the effect of short-term pre-incubation with Pam_3CSK_4 on stimulation of IgE-loaded BMMCs with DNP–HSA or anti-IgE antibodies. Indeed, anti-IgE-triggered degranulation was not inhibited by Pam_3CSK_4 (Fig. 4A), suggesting that Pam_3CSK_4 and DNP–HSA interacted directly before binding to the cell. To test this further, we compared the degranulation induced by supra-optimal concentrations of DNP–HSA and of anti-IgE antibodies, both of which are known to trigger only marginal degranulation due to the strong activation of inhibitory pathways (Gimborn et al., 2005). As shown in Fig. 4B, Ag-induced, but not anti-IgE-induced degranulation was increased dramatically by pretreatment with Pam_3CSK_4. This was also true for calcium mobilization triggered by supra-optimal concentrations of Ag (Fig. 4C). We therefore conclude that binding to Pam_3CSK_4 competed with the binding to anti-DNP IgE and reduced thus the "active" concentration of Ag from the supra-optimal to an optimal range.

3.4. Minimal amounts of Ag markedly increase LP-induced cytokine secretion from BMMCs

Having established that Pam_3CSK_4 binds and "neutralizes" the model Ag, DNP–HSA, and thus abrogates DNP–HSA-mediated early signal transduction (tyrosine phosphorylation events and calcium mobilization) as well as degranulation, we stimulated the IgE-bound FcεR1 via anti-IgE antibody-mediated crosslinking rather than DNP–HSA to further examine any synergistic effects with Pam_3CSK_4. Specifically, we investigated whether Pam_3CSK_4 had any effect on IgE plus anti-IgE-induced interleukin (IL)-6 secretion. As shown in Fig. 5A, combined stimulation with Pam_3CSK_4 and anti-IgE antibodies resulted in a synergistic production of IL-6 compared to either stimulus alone. Additional experiments were performed using the LP, MALP-2, which we showed earlier to have no influence on DNP–HSA-triggered degranulation. Stimulation of IgE-loaded BMMCs with MALP-2 in combination with sub-optimal doses of Ag (DNP–HSA) also resulted in synergistic production of IL-6 compared to Ag or MALP-2 stimulation alone (Fig. 5B). This

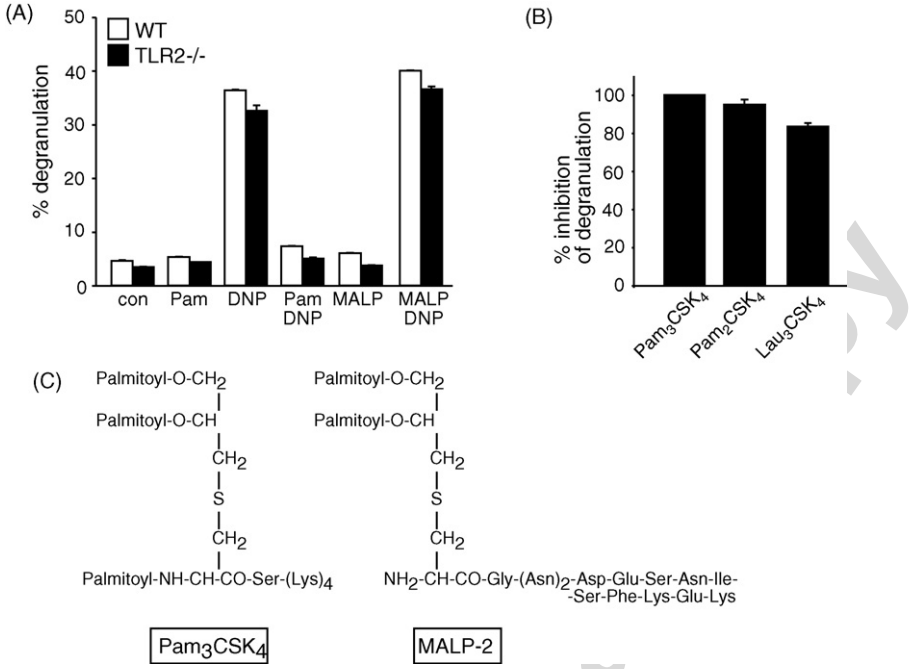

Fig. 3. Inhibitory activity of Pam_3CSK_4 is independent of TLR2 and its lipid moieties. (A) IgE-loaded wild-type (WT; white bars) and TLR2−/− BMMCs (black bars) were left untreated (con) or stimulated with Pam_3CSK_4 (10 μg ml^{-1}), Ag (DNP–HSA; 20 ng ml^{-1}), Ag after a 15 s pre-stimulation with Pam_3CSK_4, MALP-2 (10 μg ml^{-1}), or Ag after a 15 s pre-stimulation with MALP-2 for 30 min. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar is the mean of duplicates ± S.D. (B) IgE-loaded BMMCs were stimulated with Ag (20 ng ml^{-1}) alone or with Ag after a 15 s pretreatment with 10 μg ml^{-1} of the indicated LPs. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar is the mean of triplicates ± S.D. (C) The structures of Pam_3CSK_4 and MALP-2 are depicted. Amino acids are abbreviated using the three-letter code.

suggests that LPs in general are able to synergize with FcεR1 activation to induce cytokines in BMMCs.

4. Discussion

An impressive wealth of information on cellular signal transduction has been gathered by studying cells stimulated via one specific ligand (e.g. Ag, growth factor, etc.). However, in vivo cells are surrounded by a complex mixture of soluble and cell-bound ligands. Thus, one of the challenges for researchers in the field of signal transduction will be to learn how cells integrate input from different receptor systems to decide how to respond appropriately to a given situation. This is also one of the goals of the Alliance for Cellular Signaling (AfCS), i.e., to define the cellular flow of information in response to combinations of stimuli (Gilman et al., 2002). Therefore, it is important to know whether two stimuli functionally interact after binding to their receptors (what is requested) or whether they structurally interact before binding to their receptors (as is the case for DNP–HSA and Pam_3CSK_4; what is not favored). Since the latter interaction is likely not confined to DNP–HSA and Pam_3CSK_4, but might occur with other nitrophenyl-containing compounds and positively charged co-stimuli (e.g. CSK_4-containing LPs), caution must be heeded when carrying out such experiments. In this respect, in an extended analysis of the functional consequences of co-stimulation of mast cells with Ag (DNP–HSA) and different TLR stimuli (Pam_3CSK_4, MALP-2, and LPS) only Pam_3CSK_4 significantly decreased the Ag-triggered calcium mobilization, however, the mechanism was not determined (Qiao et al., 2006).

Nitrophenyl-containing chemicals have been and are used as reagents in several areas of immunological research. DNP–/TNP-specific IgE molecules are either produced in vivo by immunization or in vitro by hybridoma technique (Eshhar et al., 1980; Malcolm et al., 1979; van Toorenenbergen et al., 1983). Thus, actively or passively immunized animals can be challenged with DNP–/TNP–BSA and their in vivo MC responses monitored. Moreover, MCs in culture can be sensitized with hapten-specific IgE and activated subsequently by the addition of multivalent haptenated BSA to study signal transduction and effector functions (Gimborn et al., 2005). For many studies concerning B cell antigen receptor-mediated B cell activation by specific Ag either B cell lines expressing a 4-hydroxy-3-nitrophenyl-specific B cell antigen receptor (derived from the B1–8 antibody; Reth et al., 1978; Kim and Reth, 1995; Pracht et al., 2002) or splenocytic B cells from mice, where the J_H has been replaced by the $V_HD_HJ_H$ segments of the B1–8 antibody (Minguet et al., 2005; Sonoda et al., 1997), are used. Furthermore, for in vivo immunization studies, TNP-containing T cell-dependent (TNP–BSA) and T cell-independent (TNP–Ficoll) antigens are injected into mice (Jumaa et al., 1999). In case such stimuli are used in combination with positively charged co-stimuli, like Pam_3CSK_4, great caution has to be taken in interpretating the results.

With respect to implications of our results for the human situation, the influence of bacterial infections on the development

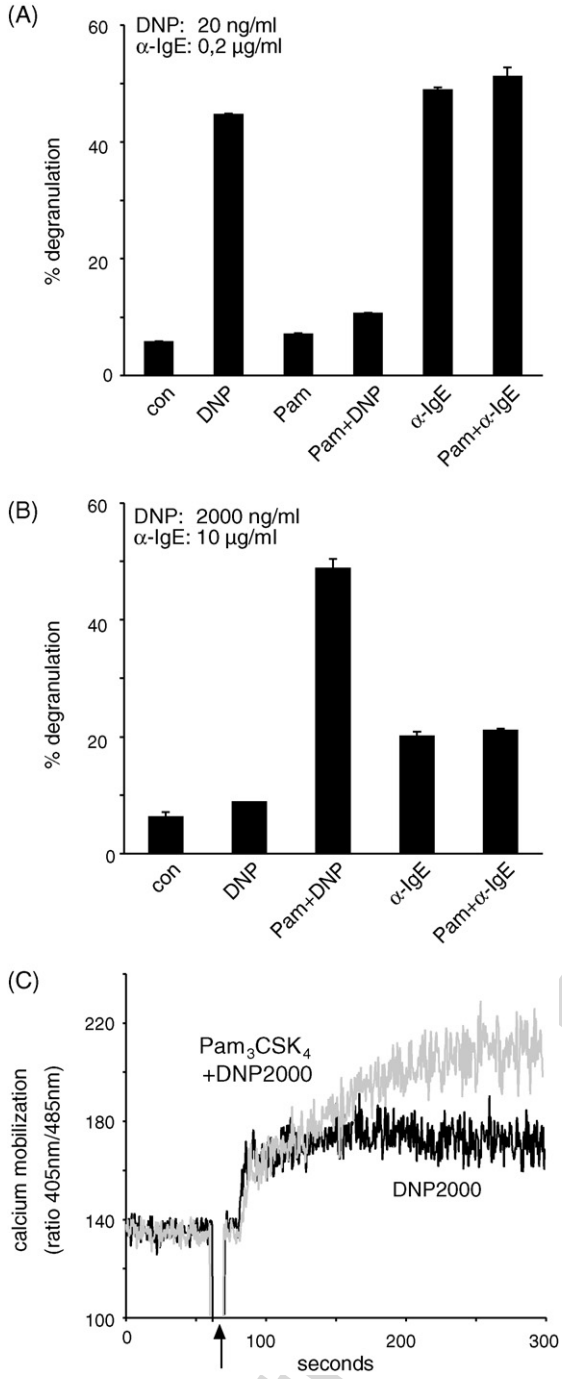

Fig. 4. Pam_3CSK_4 reduces the amount of "active" DNP–HSA. (A) IgE-loaded BMMCs were left untreated (con) or stimulated for 30 min with Ag (DNP–HSA; 20 ng ml^{-1}), Pam_3CSK_4 (10 µg ml^{-1}), Ag after a 15 s pre-stimulation with Pam_3CSK_4, anti-IgE antibodies (0.2 µg ml^{-1}), or anti-IgE-antibodies after a 15 s pre-stimulation with Pam_3CSK_4. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar represents the mean of duplicates ± S.D. (B) IgE-loaded BMMCs were left untreated (con) or stimulated for 30 min with Ag (DNP–HSA; 2000 ng ml^{-1}), Ag after a 15 s pre-stimulation with Pam_3CSK_4 (10 µg ml^{-1}), anti-IgE antibodies (10 µg ml^{-1}), or anti-IgE-antibodies after a 15 s pre-stimulation with Pam_3CSK_4. Subsequently, degranulation was assessed by β-hexosaminidase assays. Each bar is the mean of duplicates ± S.D. (C) IgE-pre-loaded BMMCs were stimulated with Ag (DNP–HSA; 2000 ng ml^{-1}) or Ag after a 15 s pre-stimulation with Pam_3CSK_4 (10 µg ml^{-1}; grey line). The arrow indicates the time point of stimulus addition. Comparable results were obtained in separate experiments.

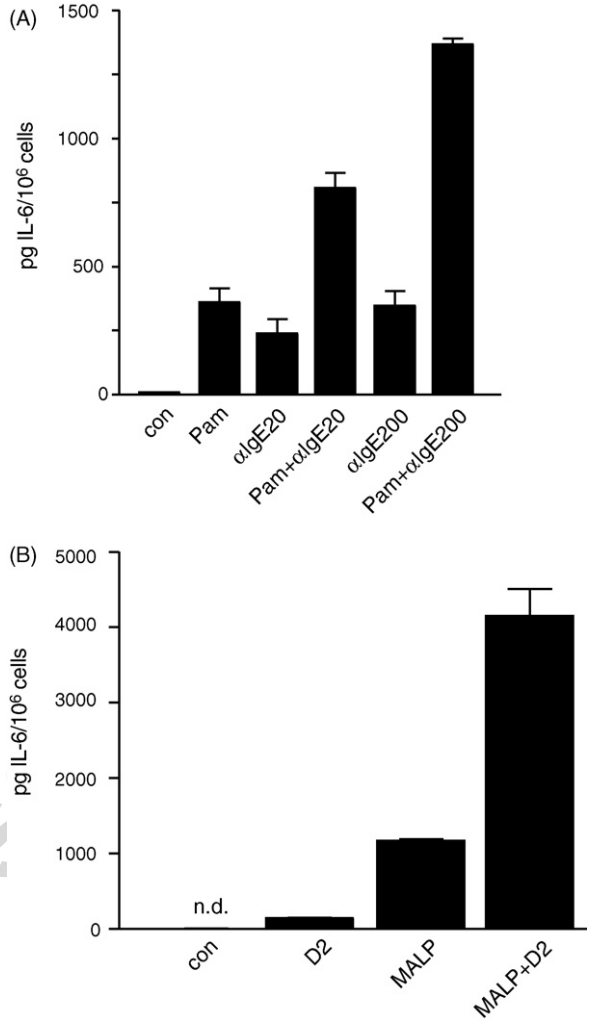

Fig. 5. Synergistic increase of cytokine secretion in BMMCs stimulated with LPs and sub-optimal amounts of Ag. (A) IgE-loaded BMMCs were left untreated (con) or stimulated with Pam_3CSK_4 (1 µg ml^{-1}), anti-IgE antibodies (20 or 200 ng ml^{-1}), or combinations of both. Secreted IL-6 was measured by ELISA. Results shown are the mean of triplicates ± S.D. (B) IgE-loaded BMMCs were left untreated (con) or stimulated with Ag (2 ng ml^{-1}; D2), MALP-2 (1 µg ml^{-1}), or a combination of both and secreted IL-6 was measured by ELISA. Results shown are the mean of triplicates ± S.D. Comparable results were obtained with different BMMC clones. n.d., not detected.

of allergic diseases (like asthma) (Kraft et al., 1998; Martin et al., 2001; von Hertzen, 1998) should be observed already on the cellular level of the MC when taking into account their synergistic effect on MC cytokine secretion (Qiao et al., 2006 and our results). The observed interaction between Pam_3CSK_4 and DNP–HSA, which can be problematic for immunological research using NP-containing substances, cannot be generalized for other LPs and antigens (as shown for MALP-2 and anti-IgE antibodies). In general, the development of pharmacological compounds able to bind to the surface of prominent allergens (e.g. Bet v 1), thereby disabling allergen–IgE interaction might be a way to prevent/reduce allergic diseases.

While it is widely accepted that TLR2 in concert with TLR1 or TLR6 is responsible for signal generation in response to LPs, there is less consensus concerning whether additional help is

required for recognition of LPs. For example, Manukyan et al. (2005) reported that binding of LP to CD14 is the first step in LP recognition, inducing physical proximity of LP and CD14 with TLR2 and thereby promoting the formation of the TLR2 signaling complex. Muroi et al. (2003) on the other hand, reported that the activity of a tri-acylated LP was not dependent on the presence of CD14. It is therefore important to note that BMMCs do not express CD14 on their surface (Huber et al., 2006) and that experiments in the current study were performed without FCS (i.e. in the absence of soluble CD14). Also worthy of note, we found the IL-6 response to LPs to be comparable in BMMCs from wild-type and CD14-deficient mice (data not shown). Thus, BMMCs and most probably other CD14-negative cells as well are able to respond to LPs in the absence of membrane-bound or soluble CD14.

The main result of this study is of technical nature and addresses the immunological community. We demonstrate that the LP Pam_3CSK_4 suppresses Ag (DNP–HSA)-triggered calcium mobilization and degranulation, whereas the LP MALP-2 does not. This is most likely due to positively charged Pam_3CSK_4 complexing with the negatively-charged model Ag DNP–HSA, thereby reducing the amount of available active Ag, and indicates that caution must be taken when negatively charged stimuli are combined with positively charged LPs in studies addressing co-stimulatory effects.

Acknowledgements

We thank Dr. M. Reth for critical discussions. This work was supported by grants from the Landesstiftung Baden-Württemberg (P-LS-AL/3) and the Deutsche Forschungsgemeinschaft (Hu794/4-2).

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