Staphylococcus aureus keratinocyte invasion is mediated by integrin-linked kinase and Rac1

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ABSTRACT **_Staphylococcus aureus_ is a major component of the skin microbiota and causes a large number of serious infections. _S. aureus_ first interacts with epidermal keratinocytes to breach the epidermal barrier through mechanisms not fully understood. By use of primary keratinocytes from mice with epidermis-restricted _Ilk_ gene inactivation and control integrin-linked kinase (ILK)–expressing littermates, we investigated the role of ILK in epidermal _S. aureus_ invasion. Heat-killed, but not live, bacteria were internalized to Rab5- and Rab7-positive phagosomes, and incubation with keratinocyte growth factor increased their uptake 2.5-fold. ILK-deficient mouse keratinocytes internalized bacteria 2- to 4-fold less efficiently than normal cells. The reduced invasion by live _S. aureus_ of ILK-deficient cells was restored in the presence of exogenous, constitutively active Rac1. Thus, Rac1 functions downstream from ILK during invasion. Further, invasion by _S. aureus_ of Rac1-deficient cells was 2.5-fold lower than in normal cells. Paradoxically, staphylococcal cutaneous penetration of mouse skin explants with ILK-deficient epidermis was 35-fold higher than that of normal skin, indicating defects in epidermal barrier function in the absence of ILK. Thus, we identified an ILK-Rac1 pathway essential for bacterial invasion of keratinocytes, and established ILK as a key contributor to prevent invasive staphylococcal cutaneous infection.—Sayedyahossein, S., Xu, S. X., Rudkouskaya, A., McGavin, M. J., McCormick, J. K., Dagnino, L. _Staphylococcus aureus_ keratinocyte invasion is mediated by integrin-linked kinase and Rac1. _FASEB J._ 29, 711–723 (2015). www.fasebj.org**

Key Words: staphylococcal colonization · epidermis · bacterial invasion

EPIDERMAL KERATINOCYTES are responsible for the barrier properties of the skin and are key contributors to the innate immune response to microorganisms. A common component of the skin microbiota is *Staphylococcus aureus* (1). The interactions between *S. aureus* and epidermal keratinocytes are important determinants for the transition of these bacteria from common skin colonizers to invasive pathogens. Given that *S. aureus* is the major causative agent of skin and soft tissue infections (2), understanding such interactions is of critical biologic and clinical importance.

S. aureus initially adheres to keratinocytes through various surface proteins that can interact with pattern recognition receptors and/or with $\alpha\beta1$ integrins on the keratinocyte plasma membrane (3). In response to this initial contact, keratinocytes produce proinflammatory cytokines, chemokines, and antimicrobial peptides, thus initiating early innate immune responses (2). *S. aureus* adhesion to keratinocytes can also be followed by internalization through incompletely understood mechanisms. In particular, virtually nothing is known about the signaling events that bridge bacterial interactions with $\beta1$ integrins and epidermal cell invasion.

Epidermal keratinocytes are nonprofessional phagocytes that can internalize large particles (4). In these cells, phagocytosis requires the formation of membrane extensions, through mechanisms that involve F-actin rearrangements, $\beta1$ integrins, and integrin-linked kinase (ILK) (5). ILK is a pseudokinase that functions as an important adaptor protein in complexes associated with $\beta1$ integrins and with other proteins essential for phagocytosis, including ELMO2 and RhoG (6–8). In the epidermis, ILK is required for a plethora of seemingly unrelated functions, including hair follicle development, keratinocyte stem cell activation and migration to sites of injury, and maintenance of epidermal integrity (9–11). At the cellular level, ILK plays key roles in F-actin dynamics, the development of front-rear polarity, responses to integrin stimulation, cell adhesion, and directional migration in keratinocytes (12, 13). Given that the processes activated during cell invasion by *S. aureus* similarly involve signaling through integrins and changes in the actin cytoskeleton, we investigated the role of ILK in *S. aureus* invasion of primary mouse keratinocytes, as well as in staphylococcal dissemination through

Abbreviations: 7-AAD, 7-aminoactinomycin D; BSA, bovine serum albumin; CFU, colony-forming unit; EMEM, Eagle's minimum essential medium; FACS, fluorescence-activated cell sorting; FnBP, fibronectin-binding protein; FSS, forward scatter; GAPDH, glyceraldehyde 3-phosphodehydrogenase; GFP, green fluorescent protein; ILK, integrin-linked kinase; KGF, keratinocyte growth factor; MOI, multiplicity of infection; SSC, side scatter; TSB, tryptic soy broth

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the skin. We also examined the signaling molecules and pathways involved in *S. aureus* internalization downstream from ILK.

MATERIALS AND METHODS

Mouse strains, reagents, and antibodies

The mouse strains used in this study have been described previously (9, 11, 14–17). All animal experiments were approved by the University of Western Ontario Animal Use Care Committee (Protocol No. 2007-005-02), in accordance with regulations and guidelines from the Canadian Council on Animal Care. The mouse strains used in this study are *Ilktm1Star* (hereafter termed *Ilkf/f*, (14)), *B6;129-Itgb1tm1Efu/J* (hereafter termed *Intb1f/f*, Stock 004605, The Jackson Laboratory, Bar Harbor, ME, USA), *Tg(KRT14-cre)1Amc/J* (hereafter termed *K14Cre*, Stock No. 004782, The Jackson Laboratory), *Rac1tm1Djk* (hereafter termed *Rac1f/f* (15), and *Ilkf/f;mT/mG* (16). The generation and characterization of mice with epidermis-restricted inactivation of *Ilk* has been described previously (9, 11). To generate reporter mice to identify and purify ILK-deficient epidermal keratinocytes, *Ilkf/f; mT/mG* mice were bred with *K14Cre;Ilkf/f* animals to generate *K14Cre;Ilkf/f; mT/mG* or *K14Cre;Ilkf/+; mT/mG* mice (hereafter termed mT/mG-ILK-deficient or mT/mG-expressing mice, respectively). The primer sequences used to genotype mouse strains were: *Cre* forward: 5′-CCATCTGCCACCAGCCAG-3′, reverse: 5′-TCGCCATCTTCCAGCAGG-3′, *Cpxm1* forward: 5′-TCGCCATCTTCCAGCAGG-3′, *Cpxm1* reverse: 5′-GATGTTGGGGCACTGCTCATTCACC-3′, *Ilk* forward: 5′-CTGTTGCAATACAAGGCTGAC-3′, *Ilk* reverse: 5′-CTGGGAGAAGCTCTCTAAGGGG-3′, *Intb1* forward: 5′-CGGCTCAAAGCAGAGTGTCAGTC-3′, *Intb1* reverse: 5′-CCACAACTTTCCCAGTTAGCTCTC-3′. The amplicon sizes were: *Cre*, 281 bp; *Cpxm1*, 420 bp; *Ilk* floxed, 390 bp, *Ilk* wild type, 360 bp, *Intb1* floxed, 280 bp, and *Intb1* wild type, 160 bp. Multiplex polymerase chain reactions were prepared to amplify simultaneously *Cre* and *Cpxm1*.

Reagents and antibodies

Human recombinant KGF (500-P19) was from PeproTech (Rocky Hill, NJ, USA). Cholera toxin (100) and insulin (16634) were from List Biological (Campbell, CA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Polyethyleneimine (PEI), 25 kDa linear (23966), was from Polysciences (Warrington, PA, USA). Chelex 100 resin was purchased from Bio-Rad (Mississauga, ON, Canada). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used were: mouse anti-Keratin 14 from Neomarkers (LL002; Fremont, CA, USA); phospho- and total ERK (9101 and 9102, respectively, Cell Signaling, Beverly, MA, USA); phospho- and total JNK (4668 and 4252, respectively, Cell Signaling); Rac1 (610651) and ILK (6108) were from BD Transduction Laboratories (San Jose, CA, USA); glyceraldehyde 3-phosphodehydrogenase (GAPDH; ADI CSA 335, Enzo Life Sciences, Brockville, ON, Canada); integrin 1 (MAB 1997, Millipore, Billerica, MA, USA); and Alexa Fluor-conjugated phalloidin (A12381), goat anti-mouse IgG, and goat anti-rabbit IgG were purchased from Molecular Probes/Invitrogen (Eugene, OR, USA).

Plasmids

The plasmids encoding green fluorescent protein (GFP)–labeled canine Rab4, Rab5, Rab7, and Rab11 have been described previously (18). Vectors encoding mCherry-tagged Rab proteins were generated by PCR amplification, using the GFP-labeled canine Rab-encoding vectors as templates, and cloning the amplicons into the mCherry-C1 plasmid (Clonetech, Palo Alto, CA, USA), followed by verification using dideoxy sequencing.

The *prsA::gfp* bacterial vector was engineered to induce GFP expression in *S. aureus*, using the *prsA* promoter, which directs expression of a membrane lipoprotein required for proper folding of secreted proteins (19) and is a component of the cell wall stress stimulon in *S. aureus* (20). To this end, a 166-bp fragment of the *prsA* promoter was amplified by PCR from *S. aureus* RN6390 genomic DNA (21), using, respectively, the forward and reverse primers 5′-CCCGGATCCACCCCTAAACCTAATGCTC-3′ and 5′-GGGAATTCAGCACTAGCTGTTACCGG-3′, with the *Bam*HI and *Eco*RI restriction sites underlined. Following digestion with those enzymes, the amplicons were cloned upstream from GFP-encoding sequences into the promoterless pCN56 vector, which replicates both in *Escherichia coli* and *S. aureus* (22). The resulting *prsA::gfp* plasmid was propagated in *E. coli* DH5α and verified by dideoxy sequencing. Using *S. aureus* RN4220 (21) as an intermediate host, the *prsA::gfp* vector was electroporated into USA300 FPR3757 (23) that had been cured of endogenous plasmids. This plasmid was maintained in *S. aureus* USA 300 by culture in the presence of erythromycin (10 μg/ml).

Cell culture, transfections, and adenovirus transduction.

Primary mouse keratinocytes were isolated from 2-3-d-old mice and cultured in keratinocyte growth medium as described (7, 12). Keratinocytes were seeded onto cell culture plates or, alternatively, glass coverslips coated with rat tail collagen type I (50 g/ml, BD Biosciences, Bedford, MA, USA) and were transiently transfected using polyethyleneimine (7) or X-tremeGene9 (06365779001, Roche, Mississauga, ON, Canada), using 1 g DNA and 6 L of transfection reagent/2 cm^2 and following the manufacturer's instructions.

To measure changes in MAPK phosphorylation in response to *S. aureus*, freshly isolated *K14Cre;Ilkf/+* and *K14Cre;Ilk f//f* keratinocytes were seeded at a density of 2.5 x 10^6 cells/20 cm^2. Two days after plating, cells were rinsed with phosphate-buffered saline (PBS) and cultured for 4 h in Ca^{2+}-free Eagle's minimum essential medium (EMEM) supplemented with 25 g/ml bovine serum albumin (BSA), followed by the addition of *S. aureus* (MOI 100). To generate 1 integrin- or Rac1-deficient keratinocytes, respectively, *Intb1f/f* or *Rac1f/f* cells were seeded and, 48 h later, were transduced with control Ad-Gal or with Ad-Cre (MOI 50-75). Ninety-six hours (*Intb1f/f*) or 48 h (*Rac1f/f*) after viral transduction, the cells were used for gentamycin protection assays or immunoblot analysis. All experiments were conducted 3 to 5 times, and each experiment contained triplicate samples.

Fluorescence-activated cell sorting

The skin of 2- or 3-d-old mT/mG-ILK-deficient or mT/mG-ILK-expressing mice was harvested and treated with trypsin to obtain the epidermis and prepared for sorting as described previously (24, 25), with minor modifications. In brief, epidermal cells were suspended in sterile PBS containing 1% BSA, 1 mM ethylenediaminetetraacetic acid (EDTA), and 25 mM HEPES, pH 7.0, sorted, and collected in keratinocyte growth medium supplemented with 50% chelex resin-treated fetal bovine serum (FBS). The cells were briefly centrifuged, resuspended in growth medium, and plated. Cells were sorted using a Becton Dickinson FACSAria III cell sorter equipped with a 30 mW Coherent solid state 405 nm violet laser, a 20 mW Coherent Sapphire solid state 488 nm blue laser, a 50mW Coherent Compass solid-state

diode-pumped 561 nm yellow-green laser, a 17 mW JDS Uniphase Helium Neon 633 nm red laser, and FACSDiVa software (version 6.1.2). To sort and purify epidermal cell populations, the blue laser trigon was configured to detect GFP from detector B (530/30 bandpass and 502 longpass filter). The yellow-green laser octagon was configured to detect mTomato from detector D (610/20 band pass and 600 long pass filter), and 7-aminoactinomycin D (7-AAD) from detector C (670/14 bandpass and 630 long pass filter). Cells were sorted at 4°C using a 100 m nozzle at low pressure (20 psi), at a maximum event rate of 6000 events/s. The following gating strategy was used to isolate viable GFP- or mTomato- positive cells: Viable cells were first selected based on 7-AAD exclusion. Forward scatter (FSC) and side scatter (SSC) voltages were adjusted to clearly visualize the epidermal cells as indicated by the "Cells" gate. Doublets were excluded based on consecutive gates on FSC-Height *vs.* FSC-Width, and SSC-Height *vs.* SSC-Width plots. Finally, viable GFP-positive/mTomato-negative and GFP-negative/mTomato-positive cell populations were selected by comparison with an unstained negative control composed of epidermal cells isolated from *K14Cre;Ilkf/+* mice.

Heat-killed bacterial phagocytosis assays

Cells were rinsed with and incubated in Ca2+-free EMEM supplemented with BSA (25 g/ml) for 4 h. The medium was replaced with fresh EMEM containing BSA and heat-killed *S. aureus* (3×10^7 particles/ml; MOI 100) in the presence or absence of KGF (20 ng/ml). Incubation proceeded at 37°C, and at timed intervals, the cultures were extensively washed to remove noninternalized bacteria and processed for microscopy. The number of internalized bacteria was determined from at least 50 cells/sample, and each experiment was conducted in triplicate.

Bacterial strains

Heat-killed Alexa Fluor 594-labeled *S. aureus* (wood strain, S23372) was purchased from Invitrogen (Carlsbad, CA, USA). The community acquired methicillin-resistant *S. aureus* strain USA300-FPR3757 (23) was obtained from the Network on Antimicrobial Resistance in *S. aureus* repository. The strains NE186 and NE728 from the Nebraska Transposon Mutant Library were also obtained from the Network on Antimicrobial Resistance in *S. aureus* repository and carry the *bursa aurealis* mariner-based, erythromycin resistance-expressing transposon within the genes encoding fibronectin-binding protein A or B, respectively. Bacteria were cultured at 37°C in tryptic soy broth (TSB; Difco, Detroit, MI, USA) under shaking conditions (200 rpm) or in TSB agar plates. GFP-expressing bacteria were generated by electroporation of the *prsA::gfp* reporter plasmid, followed by culture in the presence of erythromycin (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA).

To prepare bacterial cultures for keratinocyte invasion assays, GFP-expressing *S. aureus* USA300-FPR3757 was streaked from frozen glycerol stocks onto TSB agar plates containing erythromycin (10 μg/ml) and cultured overnight. A single colony was used as inoculum and cultured for 16 h in TSB containing erythromycin (10 μg/ml). The bacteria were then subcultured to OD_{600} = 0.05 in TSB and cultured for 3–4 h to OD_{600} = 1.0. Bacteria were then harvested by centrifugation (20,800 $\times g$, 22°C, 1 min), washed 3 times with PBS, and resuspended in PBS to yield a suspension with 10^9 *S. aureus*/ml, which was used for experiments with keratinocytes at a multiplicity of infection (MOI) of 100, unless otherwise indicated.

Gentamicin protection/bacterial invasion assays

Keratinocytes cultured in 24-well plates (300,000 cells per well) were rinsed with Ca^{2+}-free Eagle's minimum essential medium (EMEM) containing 25 μg/ml bovine serum albumin (BSA). The cells were infected by culture in 0.5 ml EMEM containing 25 μg/ml BSA and 3×10^7 CFU of *S. aureus* (MOI 100), and culture proceeded at 37°C for intervals indicated in individual experiments. To evaluate the number of intracellular bacteria, cells were rinsed with EMEM with 25 μg/ml BSA and incubated for 1 h in EMEM containing BSA and gentamicin (200 μg/ml). After 3 PBS washes, intracellular bacteria were released by vigorous resuspension in 0.5% Triton X-100 (500 μl/well). Samples of bacteria-containing lysates were diluted in PBS ($1–10^{-7}$ dilutions), plated on TSB agar plates containing erythromycin (10 μg/ml), and cultured at 37°C for 20 h, at which time the number of recovered colony-forming units (CFUs) was determined. The total number of bacteria associated with cells (*i.e.,* bacteria attached, as well as internalized) were quantified in separate samples by omitting the incubation in the presence of gentamicin and washing the cultures 3 times with PBS, prior to lysis in 0.5% Triton X-100 (500 μl/well).

Bacterial attachment assays

Keratinocytes cultured in 24-well plates were incubated at 37°C for 1 h in the presence or absence of cytochalasin D (1.2 mM) and then placed on ice for 30 min. GFP-expressing *S. aureus* was added (MOI 10), and incubation was allowed to proceed for 30 min. The cells were extensively washed to remove unbound bacteria and processed for fluorescence microscopy.

Ex vivo bacterial invasion assays

Following euthanasia, dorsal skins from 3-d-old *K14Cre;Ilk$^{f/+}$* or *K14Cre;Ilk$^{f/f}$* mice were gently stripped 3 times with ordinary adhesive tape (6200; Highland^{MC}, St. Paul, MN, USA), or left without stripping, and then harvested. The tissues were placed epidermis side up on 24 mm tissue culture inserts (8 μm pore size; cat. no. 3428; Transwell, Corning, NY, USA), the tissue edges were sealed with a 1:1 vol/vol mixture of white petroleum jelly and mineral oil, and the inserts were placed in Transwell chambers containing 1.5 ml of Ca^{2+}-free EMEM supplemented with 2% fetal bovine serum in the lower chamber. A 10 μl aliquot of GFP-tagged *S. aureus* (1×10^8 CFUs in PBS) was carefully placed on the epidermis, at the center of the skin, and surrounded by the petroleoum jelly mix to prevent it from spreading. The explants were cultured for 6 h at 37°C. The tissues were embedded in optimal cutting temperature compound (Tissue-Tek 4583; Sakamura Finetechnical Co., Tokyo, Japan) and frozen. Separately, aliquots of the culture medium from the lower chamber were diluted to quantify the CFUs present as described above for bacterial invasion assays.

Confocal and fluorescence microscopy

Cells were fixed in freshly diluted 4% paraformaldehyde and processed for indirect immunofluorescence or epifluorescence microscopy as described previously (5, 7). In experiments using heat killed *S. aureus*, fluorescence from extracellular bacteria was quenched by adding a solution containing 250 g/ml trypan blue to the culture medium for 1 min before processing for microscopy. For immunofluorescence microscopy analysis of skin explants, 7-m cryosections were fixed in 4% paraformaldehyde and incubated with primary antibodies, as

described previously (11). Confocal analysis was conducted with a Zeiss LSM 510 DUO scanning laser confocal microscope, using ZEN 2009 SP1 software (Zeiss, Göttingen, Germany). Fluorescence photomicrographs were obtained with a Leica DMIRBE fluorescence microscope equipped with an Orca-ER digital camera (Hamamatsu Photonics, Hamamatsu City, Japan), using Volocity 6.1.1 software (Improvision, Coventry, United Kingdom).

Immunoblot analysis

Primary keratinocytes were lysed using a modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Na_3VO_4, 1 μg/ml NaF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Proteins in the lysates were resolved by denaturing polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were probed with antibodies indicated in individual experiments. To normalize for protein loading, the blots were also probed for GAPDH.

Measurement of secreted TNF-α

mT/mG-ILK-deficient or mT/mG-ILK-expressing keratinocytes were purified by FACS and plated on 24-well culture dishes at a cell density of 300,000 cells/well. Two days after plating, the cells were cocultured with *S. aureus* as described in the gentamicin protections assays. At timed intervals after bacterial addition, 10^{-1} culture medium aliquots were prepared to measure TNF-α levels, using a mouse TNF-α ELISA Ready-SET-Go! Kits (88-7324-88; eBioscience, San Diego, CA, USA), following the manufacturer's protocol. The TNF-α concentrations determined were normalized to cell numbers in each culture well, determined by quantification of trypan-blue excluding cells, using a haemocytometer.

Data analysis

All experiments were conducted in duplicate or triplicate samples 3 to 8 times. The data were analyzed using ANOVA or Student's *t* test, as described in individual experiments. Significance was set at $P < 0.05$.

RESULTS

Phagocytosis of heat-killed *S. aureus* by primary keratinocytes

To begin to explore the role of ILK in keratinocyte internalization of *S. aureus*, we compared the ability of ILK-expressing (*K14Cre;Ilk$^{f/+}$*) and ILK-deficient (*K14Cre;Ilk$^{f/f}$*) keratinocytes to internalize fluorescently labeled, heat-killed bacteria. The cells were incubated with bacterial particles in serum- and additive-free medium for 4 h at 37°C and were extensively rinsed to remove all non-internalized bacteria associated with the extracellular aspect of the cells. We then determined the number of internalized bacterial particles by confocal microscopy. Under these conditions, we found that ILK-expressing cells engulfed 3-fold more bacteria than ILK-deficient keratinocytes (**Fig. 1*A, B***). We previously showed that the phagocytic capacity of keratinocytes is stimulated by keratinocyte growth factor (KGF) (5). When we conducted phagocytosis assays of heat-killed bacteria in the presence of KGF (20 ng/ml), the number of phagocytosed heat-killed bacteria increased 2-fold in ILK-expressing cells, whereas the impaired phagocytic ability of ILK-deficient cells remained unaltered (Fig. 1*A, B*).

To investigate the association of heat-killed *S. aureus* with Rab GTPases within phagosomes, ILK-expressing keratinocytes were transiently transfected with vectors encoding GFP-tagged Rab4, Rab5, Rab7, or Rab11. The cells were processed for confocal microscopy at timed intervals after addition of bacterial particles, up to 4 h. When we analyzed cells that were incubated with bacteria for 4 h, we found bacteria in Rab5-positive early phagosomes, as well as in Rab7-labeled late phagosomes, which are associated with maturation toward lysosomal compartments (26) (Fig. 1*C*). At all time points analyzed, we did not detect significant bacterial association with the recycling endosomal markers Rab11 or Rab4 (Fig. 1*C* and data not shown). Together, our results suggest the presence of ILK-dependent processes for the uptake of heat killed *S. aureus*, resulting in the formation of phagosomes that mature toward the potential formation of lysosomes.

Invasion of mouse keratinocytes by live *S. aureus*

Cellular invasion by *S. aureus* requires specific interactions of bacterial proteins with the target cell membrane, followed by internalization through poorly understood steps. Thus, we next examined the invasion of epidermal keratinocytes by the pathogenic *S. aureus* USA300 strain. We first assessed the capacity of bacterial internalization by the cells as a function of incubation time, using gentamicin protection assays. In these studies, we incubated keratinocytes with exponentially proliferating bacteria at an MOI of 100 for various times, and added gentamicin to the culture medium 30 min prior to the preparation of cell lysates, to isolate internalized *S. aureus*. Gentamicin does not penetrate eukaryotic cell membranes, thus killing exclusively extracellular bacteria. The latter were then quantified by measuring CFUs in cell lysates. We observed that bacteria were readily internalized in a manner dependent on the cocultivation time. Specifically, the number of internalized bacteria increased from 180 to 12,000 CFUs per 3×10^5 cells after cocultivation times of 30 min and 4 h, respectively (**Fig. 2*A***). These differences do not reflect time-dependent changes in the fraction of cells invaded by *S. aureus*, which had peaked at ≥80% of the cells by 10 min of cocultivation (data not shown).

To address the possibility that the number of CFUs recovered might partially reflect intracellular bacterial replication during the culture time, we cocultured *S. aureus* and keratinocytes for 30 min, followed by incubation in the presence of gentamicin for up to 4 h. We observed that the number of internalized bacteria following 30 min of coculture averaged 170 CFUs per 3×10^5 cells, irrespective of whether keratinocyte lysates were prepared 1, 2, or 4 h after the addition of gentamicin (Fig. 2*B*). This is consistent with the notion that internalized bacteria did not replicate to any appreciable extent within the time frame of these experiments.

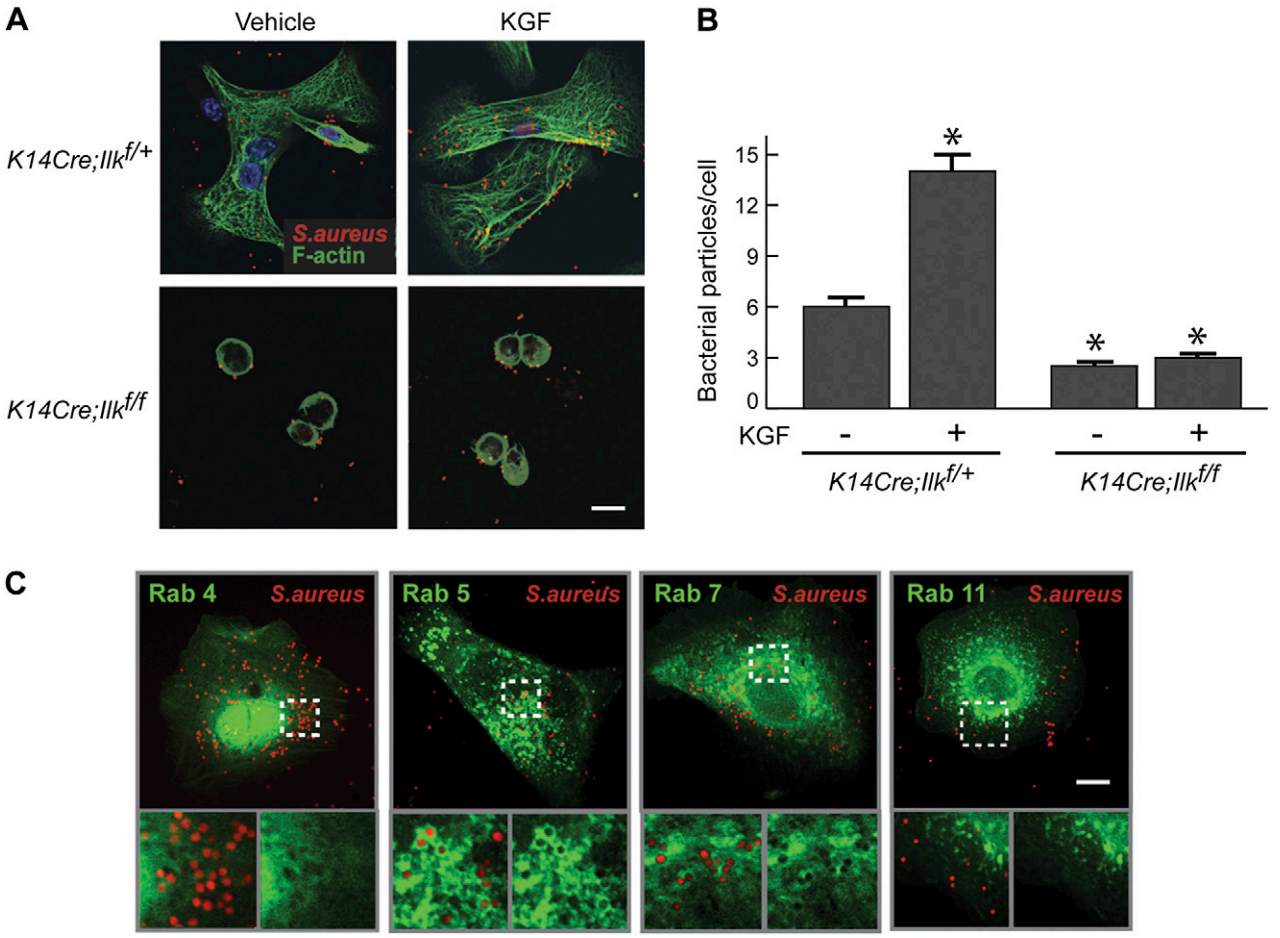

Figure 1. Phagocytosis of heat-killed *S. aureus* by keratinocytes. *A*) Representative micrographs showing the phagocytic uptake of heat-killed *S. aureus*. Primary keratinocytes of the indicated genotypes were cultured in serum- and growth additive-free medium for 4 h prior to the addition of fluorescently labeled *S. aureus* (MOI 100). Culture continued in this medium for 4 h in the presence or absence of KGF (20 ng/ml). The cells were incubated with trypan blue to quench fluorescence of extracellular bacteria and then were processed for confocal microscopy, using phalloidin to visualize the actin cytoskeleton. Bar, 16 μm. *B*) The number of heat-killed *S. aureus* particles internalized in experiments similar to those shown in *A* was determined and is expressed as the average + SEM ($n = 3$). *$P < 0.05$, relative to the number of particles internalized by *K14Cre;Ilk$^{f/+}$* cells in the absence of KGF (ANOVA). *C*) *K14Cre;Ilk$^{f/+}$* keratinocytes were transiently transfected with vectors encoding the indicated GFP-labeled Rab proteins. Forty-eight hours later, the cells were subjected to phagocytosis assays with fluorescently labeled *S. aureus* and visualized by confocal microscopy, described in *A*. Areas indicated in broken lines are shown underneath each micrograph at higher magnification. Bar, 20 μm.

We next investigated the effect of KGF receptor stimulation on keratinocyte internalization of live *S. aureus,* given its positive modulation of heat-killed *S. aureus* uptake in these cells. For these experiments, we cocultured bacteria and cells at an MOI of 10 for either 2 or 4 h, in the presence or absence of KGF (20 ng/ml). Although we observed a 10-fold increase in the number of CFU recovered from the 2 h to the 4 h cocultivation interval, the presence of KGF was without effect on the ability of live *S. aureus* to invade the cells (Fig. 2*C*). Thus, the molecular mechanisms that modulate keratinocyte phagocytosis of heat-killed bacteria appear to be distinct from those implicated in internalization of live bacteria by these cells. To further test this concept, we investigated if internalized live *S. aureus* followed a phagosomal pathway similar to that described for heat-killed bacteria. For these experiments, we transiently transfected keratinocytes with vectors encoding mCherry-tagged Rab proteins, and 48 h later, we cocultured them with GFP-expressing *S. aureus*. Following 5 min to 4 h of coculture, we processed the cells for confocal microscopy analysis. In stark contrast with heat-killed bacteria, we did not detect colocalization of live *S. aureus* with Rab4 or Rab5 at any time point analyzed (Fig. 2*D* and data not shown). A few cells showed partial colocalization with Rab7 or Rab11 in a small fraction of vesicles after 4 h of coculture with the bacteria (Fig. 2*D*). Thus, unlike the uptake of heat-killed bacterial particles, keratinocyte invasion by pathogenic *S. aureus* is not modulated by KGF and appears to follow different endosome/phagosome maturation pathways from those involved in particle phagocytosis.

ILK-deficient cells are resistant to *S. aureus* invasion

Mice with keratinocyte-restricted *Ilk* inactivation develop substantial abnormalities, including epidermal

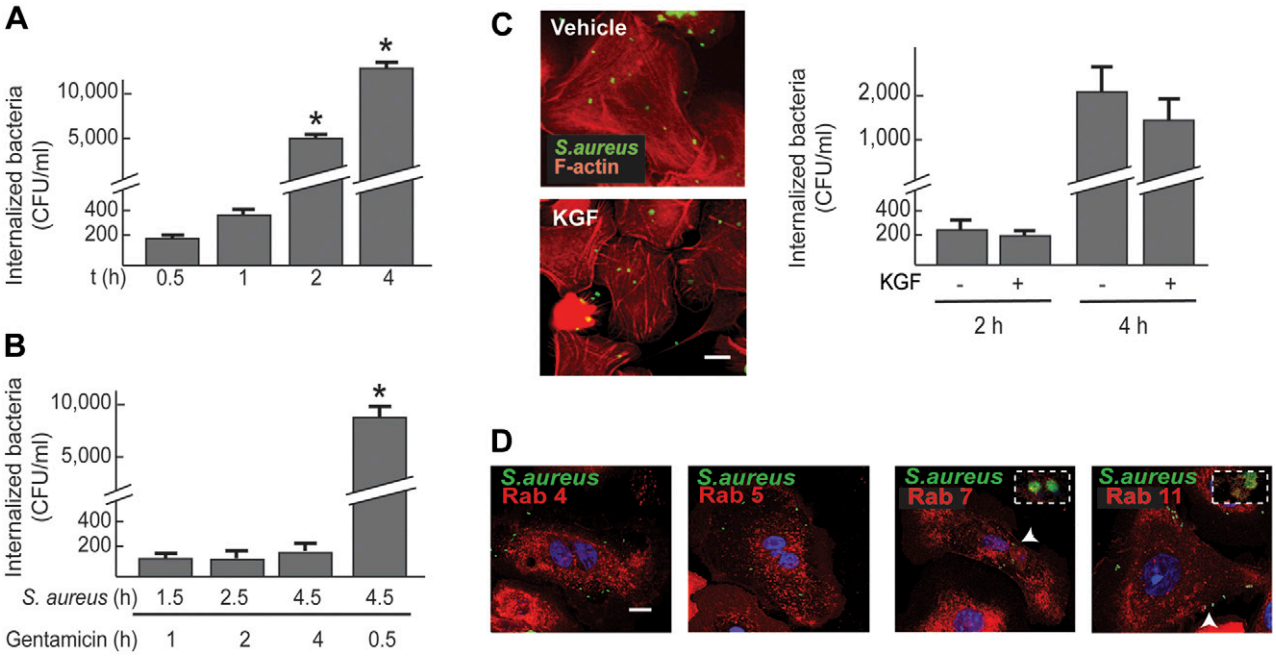

Figure 2. Invasion of keratinocytes by *S. aureus*. *A*) *K14Cre;Ilk*$^{f/+}$ keratinocytes (300,000 cells per sample) were cocultured with live *S. aureus* USA 300 (MOI 100) in gentamycin protection assays. During coculture of keratinocytes and bacteria, gentamicin was added 30 min prior to cell lysis. At the indicated times of coculture, cells were lysed in 500 μl/sample of lysis buffer, as indicated in Materials and Methods. *B*) Keratinocytes and *S. aureus* were cocultured as described in *A*, but in the presence of gentamicin for the indicated intervals prior to cell lysis. *C*) *K14Cre;Ilk*$^{f/+}$ keratinocytes were subjected to gentamycin protection assays, as in *A*, but using an MOI of 10, in the presence or absence of KGF (20 ng/ml). All histograms show the number of CFU obtained from keratinocyte lysates and are expressed as the average + SEM per milliliter cell lysate. *$P < 0.05$ ($n = 3$, ANOVA). *D*) *K14Cre;Ilk*$^{f/+}$ keratinocytes were transiently transfected with vectors encoding the indicated mCherry-labeled Rab proteins. Forty-eight hours later, the cells were subjected to phagocytosis assays with GFP-expressing live *S. aureus* and processed for confocal microscopy. Arrowheads indicate areas shown at higher magnification in the insets. Bar, 16 μm.

detachment at the dermal-epidermal junction, intraepidermal edema, and inflammation (27). To assess the ability of *S. aureus* to invade ILK-deficient keratinocytes without contributions from any inflammatory or immune cells that might be present in mutant skin, we developed a strategy to specifically isolate keratinocytes with Cre recombinase-induced inactivation of the *Ilk* gene. For these experiments, we used *Ilk*$^{f/f}$ mice bred into a Rosa 26-mT/mG reporter background (16, 28). We further bred these mice with *K14Cre;Ilk*$^{f/+}$animals to generate mT/mG-ILK-expressing (*K14Cre;Ilk*$^{f/+}$*;mT/mG*) or mT/mG-ILK-deficient (*K14Cre;Ilk*$^{f/f}$*;mT/mG*) mice. In these animals, expression of Cre recombinase under the control of the keratin-14 promoter results in excision of mTomato-encoding sequences, with the simultaneous induction of GFP expression. This system specifically allows the identification of Cre-expressing keratinocytes through the presence of GFP and the absence of mTomato fluorescence. We isolated epidermal cells from these mice, and used fluorescence-activated cell sorting (FACS) to purify viable populations of GFP-expressing keratinocytes from those nontargeted mTomato-positive cells, which constituted, respectively, ~90% and 10% of the cell isolates (**Fig. 3*A***). We cultured the GFP-positive keratinocytes for 2 d and then used them to measure *S. aureus* invasion, using gentamicin protection assays. We observed a substantial resistance to infection of the mT/mG-ILK-deficient keratinocytes, which gave rise to only ~25–30% of *S. aureus* colonies, relative to mT/mG-ILK-expressing cells (Fig. 3*B*).

To begin to understand the mechanisms involved in the impaired *S. aureus* invasion of ILK-deficient keratinocytes, we first investigated if bacterial adherence to the cell surface is altered in these cells. Bacterial invasion requires an intact actin cytoskeleton (29). We, therefore, reasoned that incubation of keratinocytes in the presence of low concentrations of cytochalasin D would allow bacterial interaction with the outer aspect of the cell membrane, in the absence of internalization. Using ILK-expressing cells, we found that the number of CFUs obtained in gentamycin protection assays from keratinocytes pretreated with cytochalasin D (2.5 μM) was 10-fold lower than that in vehicle-treated cells (Fig. 3*C*). We next incubated *S. aureus* with ILK-expressing or ILK-deficient cells that had been pretreated with cytochalasin D for 30 min and quantified the number of extracellular bacteria associated with the cells. The number of bacteria associated with the outer aspect of ILK-deficient keratinocytes was 70–80% of that observed in ILK-expressing cells (Fig. 3*D, E*). The magnitude of this decrease does not fully appear to account for the much greater reduction in bacterial invasion observed in ILK-deficient cells, suggesting that ILK modulates the ability of *S. aureus* to both adhere to and invade epidermal keratinocytes.

S. aureus activation of signaling cascades in ILK-deficient keratinocytes

S. aureus infection of human keratinocytes induces production and secretion of proinflammatory cytokines,

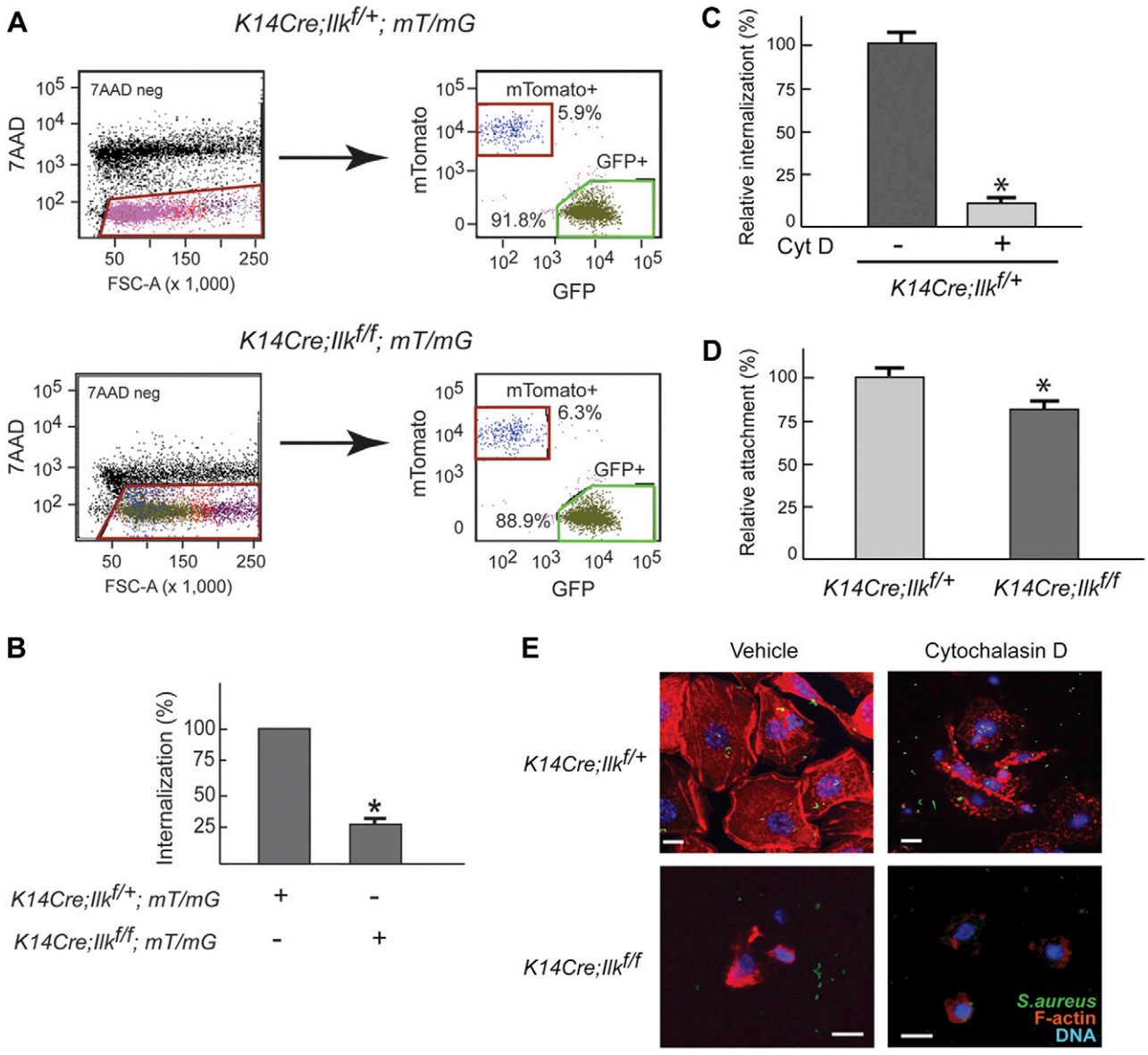

Figure 3. Impaired invasion of ILK-deficient keratinocytes by *S. aureus*. *A*) Epidermal keratinocytes of the indicated genotypes were isolated and FACS purified as described in Materials and Methods to yield targeted, GFP-positive, mCherry-negative 7-AAD-negative viable cells, with a purity of ≥99%. *B*) The cells purified in A were cocultured for 4 h with live *S. aureus* (MOI 100) and subjected to gentamycin protection assays. The results are expressed as the average + SEM of CFU recovered from ILK-deficient keratinocytes, relative to those from ILK-expressing cells, set to 100%. *C*) *K14Cre;Ilk*$^{f/+}$ keratinocytes were cultured in in the presence or absence of cytochalasin D (2.5 μM) for 1 h prior to coculture with *S. aureus* (MOI 10) followed by gentamycin protection assays. The results are expressed as the average CFU number, relative to CFU in vehicle-treated cells, which is set to 100%. *D*, *E*) Keratinocytes with the indicated genotype were cultured in in the presence or absence of cytochalasin D (1.2 mM) for 1 h, incubated on ice for 30 min, and cocultured with *S. aureus* (MOI 10) for 30 min on ice. The cells were processed for fluorescence microscopy, and the number of bacteria attached to the cell surface was determined. The results are expressed as the average number of attached bacteria, relative those in ILK-expressing cells, which is set to 100%. In the micrographs, phalloidin and Hoescht dye 33342 were used to visualize, respectively, F-actin and DNA. Bar, 10 μm. In all histograms, $^*P < 0.05$ ($n = 4$, Student's t test).

including TNF-α (30). We investigated the ability of primary mouse keratinocytes to secrete TNF-α in response to *S. aureus* by measuring levels of this cytokine in the culture medium of FACS-purified mT/mG-ILK-expressing and mT/mG-ILK-deficient keratinocytes in response to incubation with bacteria. Whereas TNF-α was undetectable in bacteria-free cultures, coculture with *S. aureus* for 1 or 4 h was accompanied by accumulation of TNF-α in the culture medium, reaching levels of approximately 10 and 20–25 ng/ml, respectively (**Fig. 4*A***). The secreted TNF-α levels in ILK-expressing and ILK-deficient cultures were indistinguishable from each other, indicating that the production of this cytokine is activated through pathways that do not require ILK.

The activation of human keratinocytes in response to bacterial stimuli is also mediated in part by signaling

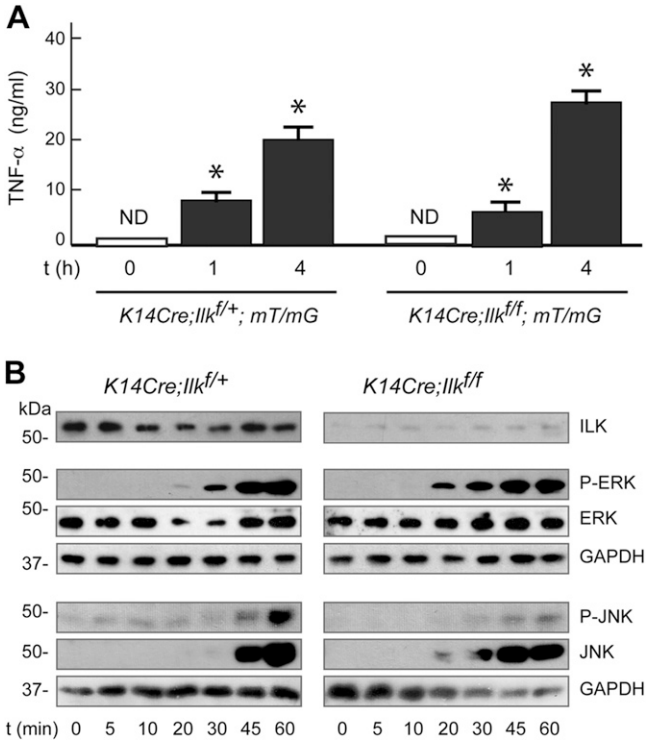

Figure 4. TNF-α secretion and MAPK activation in ILK-deficient keratinocytes cocultured with *S. aureus*. *A*) Epidermal keratinocytes of the indicated genotypes were purified by FACS and cocultured with *S. aureus*. At the indicated times after bacterial addition, samples of the growth medium were processed to measure levels of secreted TNF-α. The results are shown as the average + SEM. $^*P < 0.05$, relative to TNF-α in ILK-expressing cells at $t = 0$ ($n = 3$; ANOVA). ND, not detectable. *B*) Keratinocytes were cocultured with *S. aureus* (MOI 100), and at the indicated times after bacterial addition, cell lysates were prepared. A representative immunoblot is shown, probed with antibodies against the indicated proteins. GAPDH was used to normalize for protein loading. These experiments were repeated 3 times, using 3 different cell isolates.

through MAPKs, such as ERK and JNK (31). Thus, we also investigated if these 2 kinases become activated in response to *S. aureus* in mouse keratinocytes in an ILK-dependent manner. We were able to detect increases in the levels of active, phosphorylated ERK and JNK as early as 20 and 45 min, respectively, of initiating coculture of *S. aureus* and ILK-expressing keratinocytes (Fig. 4*B*). Similar patterns of activation of these 2 kinases were observed in ILK-deficient cells, indicating not only that activation of MAPK signaling cascades is not dependent on ILK, but also that the abnormalities in *S. aureus* internalization in the absence of ILK do not arise from altered ERK or JNK activation.

Role of Rac1 in *S. aureus* invasion of keratinocytes

Given that, in keratinocytes, Rac1 acts downstream from ILK during phagocytosis and in response to various stimuli (5, 12), we next examined the role of this GTPase in *S. aureus* invasion. We began by exogenously expressing a constitutively active Rac1 G12V mutant in ILK-expressing cells and found that this protein increased by 3-fold the number of internalized bacteria (**Fig. 5*A***). Conversely, inactivation of the *Rac1* gene following transduction of keratinocytes containing floxed Rac1 alleles ($Rac1^{f/f}$) with Cre-encoding adenoviruses (Ad-Cre) significantly reduced the number of internalized *S. aureus* by ~50%, relative to Rac1-expressing cells (Fig. 5*B*). Together, these observations implicate Rac1 in staphylococcal invasion of keratinocytes.

We next examined the link between Rac1 and ILK during *S. aureus* invasion. We reasoned that, if Rac1 functions downstream from ILK in this process, expression of Rac1 G12V should restore the susceptibility of these cells to invasion by *S. aureus*. Because $K14Cre;Ilk^{f/f}$ keratinocytes exhibit poor transfection efficiency, we used $Ilk^{f/f}$ cells, which contain floxed *Ilk* alleles that can be inactivated by Cre expression. For these experiments, we initially transduced the cells with a control adenovirus encoding β-galactosidase (Ad-βGal) or with Ad-Cre. Forty-eight hours after transduction, we transfected the cells with vectors encoding GFP, Rac1 G12V, or human ILK. Forty-eight hours later, the cells were cocultured with *S. aureus* in gentamycin protection assays. We observed that exogenous expression of human ILK induced small changes on *S. aureus* invasion in nontransduced or Ad-βGal-transduced keratinocytes, whereas it restored the number of internalized bacteria to normal levels in ILK-deficient cells (Fig. 5*C*). Significantly, the presence of Rac1 G12V increased the number of internalized *S. aureus* between 4- and 18-fold in both ILK-expressing and ILK-deficient keratinocytes (Fig. 5*C*). These results are consistent with the notion that active Rac1 functions downstream from ILK in the pathways used by *S. aureus* during invasion.

Involvement of β1 integrin in *S. aureus* internalization

The interaction of extracellular matrix substrates with β1 integrin triggers a variety of responses mediated by ILK and Rac1 (12). To investigate whether this integrin is necessary for bacterial invasion in mouse cells, we cocultured *S. aureus* with keratinocytes from mice containing floxed *Intb1* alleles, previously transduced with Ad-βGal or Ad-Cre. Whereas the number of internalized bacteria in Ad-βGal-treated cells was indistinguishable from that in nontransduced keratinocytes, the loss of β1 integrin was accompanied by a 5-fold reduction (**Fig. 6*A***). *S. aureus* can interact with cellular integrins through various proteins, including the fibronectin-binding proteins fibronectin-binding protein (FnBP)-A and FnBP-B. However, we observed that the internalization of *S. aureus* strains incapable of expressing FnBP-A or FnBP-B was indistinguishable from that of normal bacteria (Fig. 6*B*). These results suggest the presence of multiple and/or redundant mechanisms in *S. aureus* that, directly or indirectly, interact with β1 integrins and contribute to *S. aureus* invasion of keratinocytes, in agreement with previous studies using human keratinocytes (32).

Invasion of ILK-deficient epidermis by *S. aureus*

Having established the importance of ILK in bacterial invasion of keratinocytes in culture, we next sought to examine whether the absence of ILK also altered interactions with *S. aureus* in the context of whole skin. Because the epidermis-restricted inactivation of the *Ilk*

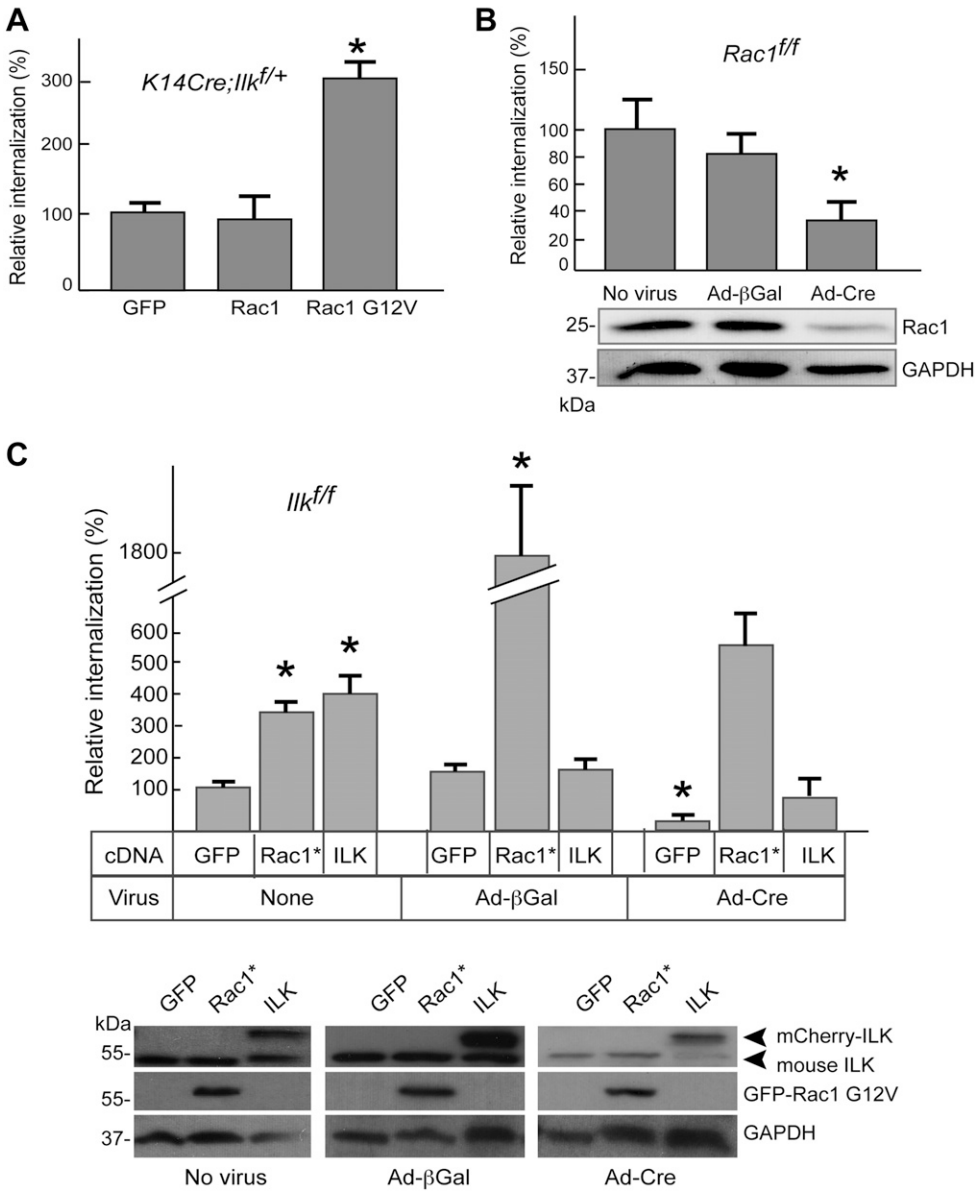

Figure 5. Role of Rac1 in keratinocyte invasion by *S. aureus*. *A*) Keratinocytes were transiently transfected with vectors encoding the indicated proteins, and 48 h later were cocultured with *S. aureus* (MOI 100) for 4 h and subjected to gentamycin protection assays. The number of CFUs recovered from keratinocyte lysates is expressed as the mean + SEM, relative to CFUs in control cells exogenously expressing GFP, which is set to 100%. $^*P < 0.05$ relative to control ($n = 3$, ANOVA). *B*) *Rac1*$^{f/f}$ keratinocytes were transduced with the indicated adenovirus, and 48 h later were cocultured with *S. aureus* (MOI 100) for 4 h and subjected to gentamycin protection assays or processed for immunoblot analysis. The number of CFUs recovered from keratinocyte lysates is expressed as the mean + SEM, relative to CFUs in control untransduced cells, set to 100%. *C*) *Ilk*$^{f/f}$ keratinocytes were transduced with the indicated adenovirus. Twenty-four hours later, the cells were transiently transfected with vectors encoding GFP, Rac1 G12V (denoted as Rac1*), or human ILK. Forty-eight hours after transfection, cells were cocultured with *S. aureus* (MOI 100) for 4 h and subjected to gentamycin protection assays or processed for immunoblot analysis. The number of CFUs recovered from keratinocyte lysates is expressed as the mean + SEM, relative to CFUs in control untransduced, GFP-expressing cells, set to 100%. $^*P < 0.05$ relative to control samples ($n = 3$, ANOVA).

gene results in perinatal lethality (10), we developed a model using *ex vivo* skin explants isolated from 3-d-old mice. In these experiments, dorsal skins were placed epidermis-side-up on 8 μm pore inserts, and a 10 μl aliquot of GFP-tagged *S. aureus* containing 1×10^8 CFUs was applied epicutaneously. These tissues were cultured for 6 h in Transwell chambers, containing growth medium only in the lower chamber. We first analyzed the medium in the lower chamber to investigate whether bacteria had traversed the skin. Although we only found about 2×10^4 CFUs arising from the medium underneath ILK-expressing tissues, the number of colonies obtained in medium from ILK-deficient explants was about 40×10^4, indicating that *S. aureus* was able to penetrate the skin containing ILK-deficient epidermis ~20-fold more efficiently (**Fig. 7**). Consistent with this concept, examination of explant sections showed accumulation of GFP-positive bacteria at the surface of ILK-expressing epidermis, with no detection of GFP-tagged particles through the epidermis or underlying dermis (Fig. 7). In contrast, we observed GFP fluorescence in both the suprabasal and the basal layer of the ILK-deficient epidermis, in and around the few hair follicle pegs and deep into the dermis (Fig. 7). These data suggest that loss of ILK in the epidermis likely compromises its barrier functions, allowing penetration of the skin by *S. aureus* (Fig. 7).

DISCUSSION

Although it is clear that proteins expressed by *S. aureus* allow bacterial interaction with and invasion of epithelial cells, the exact molecular pathways exploited by these organisms are not fully understood. Our study now identifies a nexus between β1 integrins, ILK and Rac1, required for *S. aureus* internalization in keratinocytes.

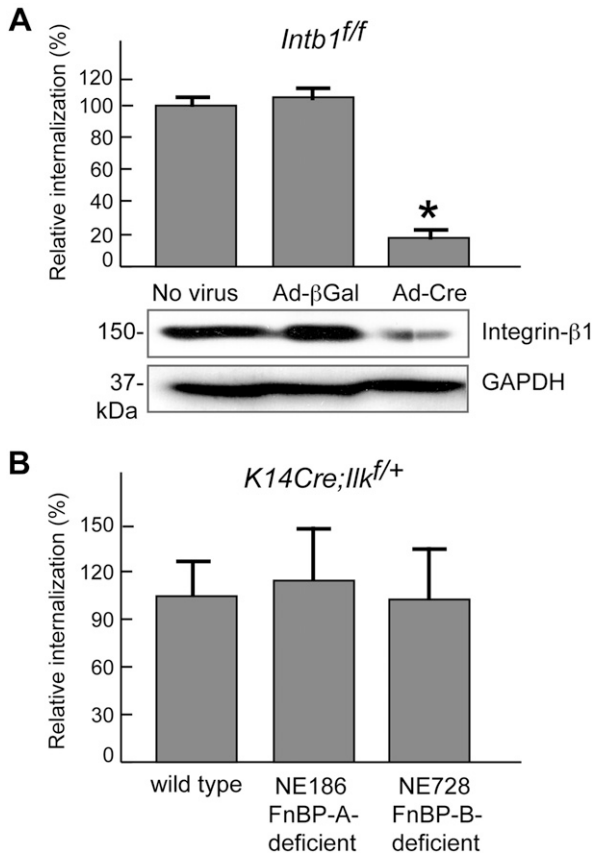

Figure 6. Modulation of *S. aureus* invasion by β1 integrins and ILK in cultured keratinocytes. *A*) *Intb1*$^{f/f}$ keratinocytes were transduced with the indicated adenovirus, and 96 h later were cocultured with *S. aureus* (MOI 100) for 4 h and subjected to gentamycin protection assays or processed for immunoblot analysis. The number of CFUs recovered from keratinocyte lysates is expressed as the mean + SEM, relative to CFUs in control untransduced cells, set to 100%. *$P < 0.05$ relative to control samples (n = 3, ANOVA). *B*) Keratinocytes were cocultured with wild-type or the indicated mutant *S. aureus* USA300 strains (MOI 100) for 4 h and subjected to gentamycin protection assays. The number of CFUs recovered from keratinocyte lysates is expressed as the mean + SEM, relative to CFUs in cells incubated with wild-type bacteria, set to 100%.

The invasion of nonprofessional phagocytic cells by bacterial pathogens involves interaction with cell surface proteins and subversion of actin cytoskeletal dynamics. In particular, *S. aureus* secretes a variety of proteins that bind to host cell integrins, triggering the formation of actin-rich membrane protrusions that aid in bacterial engulfment (29). Our observations show that the requirement for integrins, their effectors, and actin cytoskeletal remodeling during bacterial invasion of keratinocytes is also maintained vis-à-vis the engulfment of various noninfectious particles, including synthetic microspheres (5) and heat-killed *S. aureus*. However, there are also important differences in the uptake mechanisms implicated, as evidenced by the selective KGF-induced enhancement in the uptake of heat-killed *S. aureus*, but not of live bacteria.

The inactivation of the *Ilk* gene in keratinocytes has a small, but significant, effect on the attachment of live *S. aureus* to the cell surface. This defect may be independent of the alterations in the actin cytoskeleton consequent to ILK loss, because disruption of F-actin with cytochalasin D has negligible effects on bacterial attachment in *K14Cre;Ilk*$^{f/+}$ keratinocytes and other cell types (33). However, decreased bacterial attachment in ILK-deficient cells may be associated with abnormalities in the reported reduced abundance in surface β1 integrins in ILK-deficient keratinocyte subpopulations (34), which is significant given that our studies using genetic inactivation of *Intb1* clearly establish a major role for β1 integrin in *S. aureus* keratinocyte invasion.

In contrast to its key role during bacterial internalization, ILK is dispensable for the activation of other pathways triggered by bacterial interactions with the cell. Specifically, in epidermal keratinocytes and macrophages, stimulation of the Toll-like receptor TLR2 by synthetic lipopeptides induces production of proinflammatory chemokines (35, 36), whereas TLR2 activation by synthetic ligands induces MAPK activation in corneal epithelial cells (31). Our studies now show a previously unreported effect of live *S. aureus* on primary epidermal keratinocytes, which involved rapid activation of ERK and JNK. The activation of ERK and JNK in response to *S. aureus* coculture occurred in ILK-expressing and ILK-deficient cells, although with slightly accelerated kinetics in the latter cell type. These differences contrast with the similar time course of ERK activation observed following stimulation by KGF in ILK-expressing and ILK-deficient

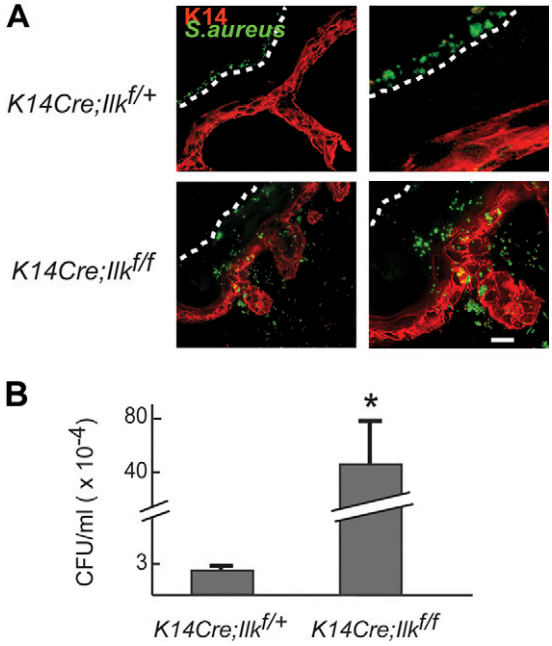

Figure 7. *Ex vivo* invasion of ILK-deficient epidermis by *S. aureus*. *A*) Skin explants with the indicated genotypes were placed epidermis side up on inserts. A 10 μl aliquot containing 1×10^8 GFP-expressing *S. aureus* was added to the center; the tissues were placed in a Transwell chamber containing growth medium and cultured for 6 h. Skin explants were processed for immunofluorescence microscopy. Representative micrographs are shown, and the areas indicated by an arrow are included at higher magnification on the right panels. Dashed lines represent the surface of the epidermis just above the corneocytes; K14, keratin 14. Bar, 32 μm. *B*) The histogram represents the mean + SEM of CFUs recovered from the culture medium in the lower chamber. *$P < 0.05$ (n = 8, Student's t test).

cells (5). The impact of the slightly faster ERK and JNK activation on the biological responses of ILK-deficient keratinocytes to *S. aureus* remains to be determined.

In macrophages, TLR2 activation also results in NF-κB nuclear translocation and production of TNF-α in a MAPK-independent manner (37). Our studies further demonstrate that this pathway is also present in epidermal keratinocytes in response to interactions with live *S. aureus,* although whether these responses are mediated by other receptors in keratinocytes, in addition to TLR2, remains to be established.

The contribution to *S. aureus* invasion of different integrin-interacting proteins appears to differ in various cell types. For example, whereas focal adhesion kinase is essential for *S. aureus* invasion of fibroblasts, vinculin is dispensable (38, 39). Our data now establish that ILK is an indispensable component for staphylococcal internalization in epidermal keratinocytes and that it does not appreciably mediate at least a subset of TLR2-mediated innate immunity responses to microbial pathogens.

The role of Rho GTPase family members in the responses of nonimmune cells to pathogenic microorganisms is poorly understood. In immune cells, the involvement of Rac family members in bacterial phagocytosis has been frequently inferred from studies using expression of dominant-negative Rac1 mutant proteins. However, an important caveat of these approaches is the possibility that dominant-negative proteins can titrate out upstream regulators involved in modulating several small GTPases (40). Using conditional deletion of *Rac1*, we established a specific and key role for this protein in *S. aureus* invasion of keratinocytes. Further, the increase in viable *S. aureus* colonies isolated in gentamycin protection assays from ILK-deficient keratinocytes exogenously expressing the constitutively active Rac1 G12V mutant places Rac1 as a central downstream component in the ILK pathway that permits bacterial invasion of epithelial cells. Rac1 is also involved in a wide variety of phagocytic processes in nonimmune cells, including epidermal cells, retinal pigmented epithelial cells, and neurons (41). In keratinocytes, phagocytic uptake of particles, such as melanosomes, synthetic microspheres, and heat-killed bacteria, requires Rac1 activation and is modulated by receptors such as the KGF and the protease activated-2 receptors (5, 41). In this process, phagocytosed particles traffic through various phagosomes characterized by the presence of Rab4, 5, or 7. In contrast, live *S. aureus* appears to follow different pathways once it has invaded keratinocytes. Significantly, a key step both for bacterial invasion and particle engulfment is the ability of cells to extend protrusions. Our data suggest that F-actin cytoskeletal arrangements triggered by Rac1 activation at sites of engulfment or invasion are a common event in the internalization of heat-killed and live bacteria. It would appear that the signaling pathways that ultimately lead to Rac1 activation at internalization sites involve β1 integrins and ILK. However, important differences appear to exist, probably because of the different ways in which heat-killed and live bacteria interact with keratinocytes. In nonepidermal cells, live *S. aureus* stimulates TLR2 to induce proinflammatory responses, as well as Rac1 activation (42). Whether this pathway of Rac1 stimulation is also active in epidermal keratinocytes remains to be determined.

An effective host defense against microbial skin infections depends on the physical integrity of the cutaneous barrier and requires the coordinate activation of multiple responses. They include the production of antimicrobial peptides and local immunologic events, such as the synthesis of proinflammatory cytokines. In particular, epidermal keratinocytes are the first line of defense against staphylococcal infections (43). Although *S. aureus* had generally been considered to be an extracellular pathogen, increasing lines of evidence indicate that it can also invade keratinocytes and other host cell types. Indeed, viable *S. aureus* can persist within keratinocytes for extended periods of time (43, 44). The precise role of cellular invasion in colonization and infection is not fully understood, although important differences between primary cells and established cell lines have been noted (43). Our studies show that primary mouse keratinocytes are able to interact with *S. aureus* via FnBP-dependent and -independent mechanisms, similar to primary epidermal human keratinocytes (43). The internalization of these pathogens appears to play a role in spreading to various organs, through the ability of bacteria to traverse cellular barriers. Intracellular bacterial pools can also promote pathogen persistence in the organism through evasion of immune surveillance (43, 44). Thus, modulation of the β1 integrin-ILK-Rac1 pathway might provide potential therapeutic avenues to avoid staphylococcal internalization and intracellular bacterial permanence.

Although healthy skin is a formidable barrier against microorganisms, disruption on this barrier promotes skin colonization, which, in turn, may facilitate skin penetration and subsequent invasion of many tissues and organs (45). Indeed, the skin from individuals with atopic dermatitis exhibits abnormal barrier functions and is extremely susceptible to colonization and infection by *S. aureus* (46). Similarly, individuals with genetic blistering diseases frequently present with chronic wounds that become colonized by this pathogen (45). At the cellular level, and with regard to staphylococcal invasion, our data using cultured keratinocyte models show that targeting ILK might potentially be expected to help reduce cellular uptake and persistence of bacteria within the host cell. Paradoxically, we observed that, in the absence of ILK, these bacteria more readily traversed the skin.

S. aureus invasion of the skin requires several events. First, bacteria must breach the corneocyte layer. Our studies show that in ILK-expressing epidermis, this layer offers protection. However, the barrier properties of corneocytes derived from ILK-deficient keratinocytes would appear to be diminished, as evidenced by the ability of *S. aureus* to penetrate inner cell layers in skin explants in which the corneocyte layer was not disrupted by tape stripping. The granular layers of the epidermis, just underneath the corneocytes, function as a second barrier against paracellular movements of chemicals, macromolecules, and pathogens. This is achieved through the assembly of tight junctions (47). ILK-deficient epidermis exhibits decreased mechanical strength, blister formation due to detachment at the dermal-epidermal junction, as well as abnormalities in the actin cytoskeleton, which alter the formation of cell-cell junctions in cultured keratinocytes (5, 9, 10, 13). ILK-deficient epidermis in skin explants also allows penetration of fluorescent tracers (unpublished data), suggesting the

presence of defects in tight junctions. Consequently, at least a fraction of *S. aureus* may reach deeper cutaneous cell layers through paracellular movements. *S. aureus* also secretes virulence factors, such as α-toxin, which disrupts cell membranes and causes host cell lysis, facilitating bacterial transit through tissues (47). The relative contributions of each of these mechanisms to invasion of ILK-deficient epidermis and whether ILK-deficient keratinocytes also have defects in the production and/or secretion of antimicrobial peptides that interfere with pathogen invasion will be important areas for future research.

It has been estimated that up to 35% of the human population is colonized by *S. aureus*, which is equivalent to ~2 billion individuals worldwide (48). Further, infection by this pathogen is the principal cause of hospital-associated infections (48). Therefore, it is crucial to develop a better understanding of the factors that modulate *S. aureus* interactions with host cells. Our study demonstrates that ILK and Rac1 play key roles in the protective function of the epidermis against staphylococcal infections at multiple levels. In future, modulation of one or more of the elements in this pathway may prove useful in the development of therapies for the prevention or treatment of *S. aureus* infections. In summary, our work shows that ILK plays a pivotal role in mediating *S. aureus* invasion of the skin. We also identified a β1 integrin-ILK-Rac1 pathway, which is critically involved in bacterial invasion of keratinocytes. In addition, we established that ILK is essential, at the tissue level, to maintain the epidermal barrier function necessary to prevent invasive cutaneous infection by staphylococci. FJ

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