**Staphylococcus δ-toxin promotes mouse allergic skin disease by inducing mast cell degranulation**

Yuumi Nakamura\(^1\), Jon Oscherwitz\(^2\), Kemp B. Cease\(^2\), Susana M. Chan\(^1\), Raul Muñoz-Planillo\(^1\), Mizuho Hasegawa\(^1\), Amer E. Villaruz\(^3\), Gordon Y. C. Cheung\(^3\), Martin J. McGavin\(^4\), Jeffrey B. Travers\(^5\), Michael Otto\(^3\), Naohiro Inohara\(^1\), and Gabriel Núñez\(^1\)

\(^1\)Department of Pathology and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA

\(^2\)Department of Internal Medicine Division of Hematology-Oncology, University of Michigan Medical School, Ann Arbor, MI 48109, and VA Ann Arbor Healthcare System, 2215 Fuller Road, Ann Arbor, MI 48105 USA

\(^3\)Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, US National Institutes of Health, Bethesda, MD 20892, USA

\(^4\)Department of Microbiology and Immunology, and Centre for Human Immunology, Western University, University of Western Ontario, London, Ontario, N6A 5C1, Canada

\(^5\)Department of Dermatology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

**Abstract**

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects 15 to 30% of children and ~5% of adults in industrialized countries\(^1\). Although the pathogenesis of AD is not fully understood, the disease is mediated by an abnormal immunoglobulin E (IgE) immune response in the setting of skin barrier dysfunction\(^2\). Mast cells (MCs) contribute to IgE-mediated allergic disorders including AD\(^3\). Upon activation, MCs release their membrane-bound cytosolic granules leading to the release of multiple molecules that are important in the pathogenesis of AD and host defense\(^4\). More than 90% of AD patients are colonized with *Staphylococcus aureus* in the lesional skin whereas most healthy individuals do not harbor the pathogen\(^5\). Several *Staphylococcus* exotoxins (SEs) can act as superantigens and/or antigens in models of AD\(^6\). However, the role of these SEs in disease pathogenesis remains unclear. Here, we report that culture supernatants of *S. aureus* contain potent MC degranulation activity. Biochemical analysis identified δ-toxin as the MC degranulation-inducing factor produced by *S. aureus*. MC degranulation induced by δ-toxin

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**Correspondence should be addressed to:** Gabriel Núñez, Department of Pathology, University of Michigan Medical School, 4215 CC, 1500 E. Medical Center Drive, Ann Arbor, Michigan 48109, USA. Tel: 734 764-8514; Fax. 734 647-9654; gabriel.nunez@umich.edu.

**Author Contributions**

Y.N., N.I., and G.N. designed the research. Y.N. conducted the experiments and analyzed data with the help of R.M.-P., S. M. C., and M.H. J.O., K.B.C., J. B. T., and M.J.M. generated and provided critical reagents or material. A.E.V, G.Y.C., and M.O. engineered bacterial strains. Y.N and G.N. wrote the manuscript. All authors discussed the results and commented on the manuscript.

**Competing financial interests**

The authors declare no competing financial interests.
depended on phosphoinositide 3-kinase (PI3K) and calcium (Ca\(^{2+}\)) influx, but unlike that mediated by IgE crosslinking, it did not require the spleen tyrosine kinase (Syk). In addition, IgE enhanced δ-toxin-induced MC degranulation in the absence of antigen. Furthermore, *S. aureus* isolates recovered from AD patients produced high levels of δ-toxin. Importantly, skin colonization with *S. aureus*, but not a mutant deficient in δ-toxin, promoted IgE and IL-4 production, as well as inflammatory skin disease. Furthermore, enhancement of IgE production and dermatitis by δ-toxin was abrogated in *Kit^{W-sh/W-sh}* MC-deficient mice and restored by MC reconstitution. These studies identify δ-toxin as a potent inducer of MC degranulation and suggest a mechanistic link between *S. aureus* colonization and allergic skin disease.

Because MCs may play a critical role in the pathogenesis of AD\(^{3}\), we asked first whether *S. aureus* can release factors that induce MC degranulation. We found that the culture supernatant of *S. aureus* induced rapid and robust MC degranulation in a dose-dependent manner (Fig. 1a, Supplementary Fig. 1a,b). Analysis of a panel of *Staphylococcus* isolates revealed that the culture supernatant of several *S. aureus* strains as well as of that from *S. epidermidis* and *S. saprophyticus*, but not of several *Staphylococcus* species elicited MC degranulation (Supplementary Fig. 1c). TLR2 stimulation via lipopeptides has been shown by some studies, but not others, to induce MC degranulation\(^{7,8}\). However, neither the culture supernatant of *S. aureus* deficient in lipoproteins (Δlgt), which lacks TLR2-stimulating activity\(^{9}\), nor that from bacteria deficient in α-, β-, and γ-hemolysins (Δαβγ) were impaired in MC degranulation activity (Supplementary Fig. 1c and 3c). The MC degranulation activity was enriched in the culture supernatant of *S. aureus* and was sensitive to heat, phenol/chloroform extraction and protease K treatment (Supplementary Fig. 2a). Furthermore, the MC degranulation-inducing factor bound to both diethylaminoethyl and carboxymethyl cellulose matrices and was present in the void fraction on gel filtration at neutral pH (Supplementary Fig. 2b). Based on these observations, we developed a multiple step strategy for biochemical purification of the MC degranulation-inducing factor (Supplementary Fig. 2c). Liquid chromatography-mass spectrometry analysis revealed that δ-toxin (also called δ-hemolysin, PSMγ), a 2.9 kDa peptide secreted by *S. aureus* that belongs to the peptide toxin family of phenol-soluble modulins (PSMs), was the most abundant and significant protein identified in the purified sample (Supplementary Fig. 2c). Mutant analyses in two *S. aureus* strains revealed that MC degranulation induced by *S. aureus* culture supernatant required expression of δ-toxin whereas deficiency of related PSMα or PSMβ peptides had minimal or no effect on MC degranulation (Fig. 1b and Supplementary Fig. 3a). Importantly, complementation of the Δhld mutant strain with δ-toxin producing plasmid, but not control plasmid, restored the ability of the culture supernatant to induce MC degranulation (Fig. 1b). Stimulation of MCs with 30 µg/ml of synthetic δ-toxin peptide, a concentration of δ-toxin normally found in *S. aureus* culture supernatants (Supplementary Fig. 3b), also induced rapid release of histamine (Fig. 1c). Furthermore, transmission electron microscopy revealed classical features of MC degranulation without loss of plasma membrane integrity upon δ-toxin stimulation (Fig. 1d). These results indicate that δ-toxin is the MC degranulation-inducing factor released by *S. aureus*. 

*Nature. Author manuscript; available in PMC 2014 July 10.*
PSMs, especially PSMα2 and PSMα3 induce cell death and IL-8 release in human neutrophils10,11. In accord with these results10, PSMα2 and PSMα3 induced robust loss of cell viability in MCs (Supplementary Fig. 4a). Non-toxic concentrations of PSMαs did not possess any MC-degranulation activity (Supplementary Fig. 4b). In contrast, stimulation with a concentration of δ-toxin that induces robust MC degranulation did not induce detectable cell death in MCs (Supplementary Fig. 4a,c). Furthermore, formylation of the N-terminus of the δ-toxin peptide was not required for MC degranulation activity, whereas it was essential for the ability of δ-toxin to induce the release of IL-8 from human neutrophils (Supplementary Fig. 4c,d). Consistent with previous results, stimulation of human neutrophils with formylated PSMα2, PSMα3 or δ-toxin induced robust IL-8 release (Supplementary Fig. 4d). Moreover, stimulation of primary mouse macrophages and keratinocytes with PSMα2, but not δ-toxin, triggered robust cell death (Supplementary Fig. 5). Thus, the MC degranulation activity induced by δ-toxin is not associated with cell death and is different from other activities triggered by PSMα2 and PSMα3. Immunoblotting confirmed that the presence of δ-toxin in S. aureus supernatants correlated with MC degranulation activity (Fig. 1e). Notably, the supernatant from S. epidermidis, a bacterium that is present in normal skin, possessed weak MC degranulation which correlated with smaller amounts of δ-toxin when compared to that from S. aureus strains (Fig. 1e and Supplementary Fig. 6). Furthermore, deficiency of δ-toxin had a larger effect on MC degranulation in S. aureus than in S. epidermidis (Supplementary Fig. 6). To assess whether δ-toxin induces MC degranulation in vivo, we injected synthetic δ-toxin into the skin of wild-type mice, but not in MC-deficient KitW-sh/W-sh mice (Fig. 1f,g). Importantly, reconstitution of the skin of KitW-sh/W-sh mice with bone marrow-derived cultured MCs (BMCMCs) restored leaking of the dye upon administration of δ-toxin (Fig. 1g). Moreover, the culture supernatant from the δ-toxin positive LAC strain induced Evan’s blue dye leaking whereas that from δ-toxin negative LAC Δhld and SA113 strains did not (Supplementary Fig. 7). These results indicate that δ-toxin induces MC degranulation in vitro and in vivo.

δ-toxin triggers Ca²⁺ influx through FPR2 in human neutrophils11. Because Ca²⁺ influx is an essential step in MC degranulation, we analyzed whether δ-toxin induces Ca²⁺ influx in MCs. Stimulation of MCs with ionomycin or DNP plus anti-DNP IgE induced rapid Ca²⁺ influx (Fig. 2a). Likewise, δ-toxin triggered Ca²⁺ influx and this was abrogated by treatment with the Ca²⁺ chelator ethylene glycol tetraacetic acid (EGTA) (Fig. 2a). EGTA also blocked MC degranulation induced by ionomycin, DNP plus anti-DNP IgE or δ-toxin (Fig. 2b). Similarly, MC degranulation induced by DNP plus anti-DNP IgE or δ-toxin was inhibited by the PI3 kinase inhibitor, LY294002 (Fig. 2c). However, unlike antigen plus IgE, MC degranulation induced by δ-toxin did not require Syk (Fig. 2d). Fpr1, Fpr2 and related family members were expressed in mouse MCs although their expression was higher in neutrophils (Supplementary Fig. 8). WRW4, a peptide antagonist of formyl peptide receptor 2 (FPR2), blocks human and mouse neutrophil activation induced by PSMs including δ-toxin11. Notably, WRW4 inhibited mouse neutrophil degranulation induced by δ-toxin both in vitro and in vivo (Supplementary Fig. 9a,b). Cyclosporin H, an antagonist of human FPR1,
also partially inhibited mouse MC degranulation induced by δ-toxin (Supplementary Fig. 9c). However, human FPR2 ligands, MMK1 and Lipoxin A4, did not induce mouse MC degranulation (Supplementary Fig. 10a). Furthermore, treatment with pertussis toxin (PTX), an inhibitor of G-protein coupled receptors, reduced partially MC degranulation induced by δ-toxin (Supplementary Fig. 10b). However, MCs from wild-type and Fpr2−/− mice exhibited comparable MC degranulation induced by δ-toxin (Supplementary Fig. 10c). Collectively, these results suggest that δ-toxin induces MC degranulation via a signaling pathway that is different from that induced through antigen and IgE.

Stimulation with IgE and antigen, but not monomeric IgE, induces robust MC degranulation\(^4\). Notably, pre-incubation of MCs with anti-DNP or anti-TNP IgE alone increased markedly the degranulation activity of δ-toxin (Fig. 3a). The synergistic effect of monomeric IgE and δ-toxin was abrogated in MCs deficient in Syk (Fig. 3b). To test whether the synergism between monomeric IgE and δ-toxin could be observed \emph{in vivo}, we injected monomeric IgE and δ-toxin (at concentrations that do not induce MC degranulation) into the skin of mice and monitored MC degranulation \emph{in vivo} with the PCA assay. At these inactive concentrations, δ-toxin induced Evans blue dye leaking at the site of injection in mice pretreated with anti-DNP (Fig. 3c). These results indicate that IgE increases the MC degranulation activity of δ-toxin in the absence of antigen.

δ-toxin is encoded by RNAIII, a regulatory RNA that is induced via the \emph{agr} quorum-sensing system of \emph{S. aureus} \(^12\). Notably, supernatants from 26 \emph{S. aureus} strains isolated from the lesional skin of AD patients produced δ-toxin (Supplementary Fig. 11a). Moreover, RNAIII expression was detected in lesional skin colonized with \emph{S. aureus}, but not normal skin, of AD patients (Supplementary Fig. 11b-c). To test whether δ-toxin plays a role in allergic skin disease, we used a modified epicutaneous disease model in which the skin of BALB/c mice was colonized with wild-type or δ-toxin-deficient \emph{S. aureus} and then challenged once with ovalbumin (OVA) to assess antigen-specific IgE production (Fig. 4a). One week after colonization with wild-type \emph{S. aureus}, the mice developed severely inflamed reddened skin at the site of application (Fig. 4b,c). Expression of \emph{hld} was detected in the skin on day 4 after bacterial colonization using a bioluminescent reporter \emph{S. aureus} strain (Supplementary Fig. 12). Histological analysis revealed spongiosis and parakeratosis and marked neutrophil-rich inflammatory infiltrates in the skin of mice colonized with wild-type \emph{S. aureus} (Fig. 4c,d). In contrast, mice colonized with \emph{S. aureus} lacking δ-toxin exhibited a significantly reduced skin inflammatory cell infiltrate and disease score (Fig. 4c, 4b,d). Complementation of the \emph{Δhld} mutant with a plasmid producing δ-toxin restored the disease score to levels comparable to those observed with the wild-type bacterium (Supplementary Fig. 13). The differential ability of wild-type and mutant \emph{S. aureus} to promote inflammatory disease was not explained by differences in skin colonization (Supplementary Fig. 14a, b). Furthermore, mice colonized with wild-type \emph{S. aureus} developed greater amounts of total serum IgE and IgG1, but not IgG2a, as well as IL-4 in the skin than mice inoculated with the δ-toxin mutant bacterium (Fig. 4e and Supplementary Fig. 14c and Supplementary Fig. 15). At three weeks, there was a slight increase in IgG1 production in mice colonized with the δ-toxin mutant bacterium compared to PBS control (Supplementary Fig.15c), suggesting the existence of a minor \emph{S. aureus}-dependent, but δ-toxin-independent pathway for IgG1 production. In
addition, pre-colonization with wild-type, but not the δ-toxin-deficient *S. aureus*, enhanced the production of OVA-specific IgE (Fig. 4f). Colonization with *S. aureus* without disrupting the skin barrier by stripping also induced inflammatory disease and enhanced IgE responses (Supplementary Fig. 16). Pre-colonization with δ-toxin producing *S. aureus* was important to elicit antigen-specific IgE because administration of OVA prior to or concurrent with *S. aureus* colonization did not enhance OVA-specific IgE production (Supplementary Fig. 17). To test whether δ-toxin is sufficient to trigger allergic skin disease, we epicutaneously sensitized the skin of mice with OVA in the presence and absence of δ-toxin and challenged the mice with OVA alone or OVA plus δ-toxin 3 weeks later. We found that δ-toxin triggered inflammatory skin disease including OVA-specific IgE and IgG1 production whereas challenge with OVA alone did not (Supplementary Fig. 18). C57BL/6 mice colonized with wild-type *S. aureus* also developed higher serum IgE levels and more severe inflammatory skin disease than mice inoculated with the bacterium deficient in δ-toxin (Fig. 4g,h). Importantly, MC-deficient *Kit*<sup>W-sh/W-sh</sup> mice inoculated with wild-type *S. aureus* showed reduced IgE serum levels and skin inflammation when compared to wild-type mice (Fig. 4g,h). Adoptive transfer of MCs into the skin of *Kit*<sup>W-sh/W-sh</sup> mice restored skin disease and increase IgE production in mice colonized with wild-type, but not *S. aureus* lacking δ-toxin (Fig. 4g,h and Supplementary Fig. 19). There were increased numbers of *S. aureus* and total bacteria in the skin of *Kit*<sup>W-sh/W-sh</sup> mice (Supplementary Fig. 19), suggesting that mast cells can regulate bacterial colonization under our experimental conditions. Microscopic analysis showed that the dermal MC densities in the skin of *Kit*<sup>W-sh/W-sh</sup> recipient mice were ~50% of those found in age-matched C57BL/6 mice (Supplementary Fig. 19). Furthermore, toluidine-positive granules associated with MC degranulation were present in the skin of mice colonized with wild-type, but not δ-toxin-deficient, *S. aureus* (Supplementary Fig. 19). Taken together, these results indicate that δ-toxin from *S. aureus* promotes allergic skin disease via activation of MCs.

The δ-toxin transcript is contained within RNAIII, a regulatory RNA that governs *S. aureus* virulence genes<sup>13,14</sup>. The role of δ-toxin in the growth of *S. aureus* is not understood. Because δ-toxin can form pores on the surface of certain bacteria<sup>15</sup>, one possibility is that it promotes pathogen colonization by killing competing bacteria. Our results suggest that the host senses *S. aureus* through the detection of δ-toxin to promote innate and adaptive Th2 immune responses via MC degranulation. Although clinical studies are needed to determine the role of δ-toxin in AD, our results in mouse models suggest that in the setting of genetic defects associated with the disease<sup>2</sup>, δ-toxin may promote allergic immune responses and that strategies to inhibit δ-toxin might be beneficial for the treatment of AD.

**Method Summary**

**Culture of mast cells and degranulation**

Preparations of BMCMCs and fetal skin-derived mast cells (FSMCs) were previously described<sup>16</sup>. The purity of MCs was > 95 % as assessed by surface expression of FcεRI and CD117 (eBioscience). Degranulation of MCs was assessed by β-hexosaminidase assay as described<sup>16</sup>.
Passive cutaneous anaphylaxis (PCA) assay

PCA assay was performed as described with minor modifications\textsuperscript{17}.

Epicutaneous sensitization with S. aureus

The dorsal skin of 6–8 week old female mice was shaven and stripped using a transparent bio-occlusive dressing (Tegaderm\textregistered; 3M). $10^8$ CFU of S. aureus strains were placed on a patch of sterile gauze and attached to the shaved skin with another transparent bio-occlusive dressing (Tegaderm\textregistered; 3M). Each mouse was exposed to S. aureus for 1 week through the patch. After a 2-week interval, each mouse was challenged once with 100 µg ovalbumin epicutaneously for 1 week and the animals were sacrificed for analyses.

Animal study

All animal studies were performed according to approved protocols by the University of Michigan Committee on the Use and Care of Animals.

Statistical analysis

All analyses were performed using GraphPad Prism. Differences were considered significant when p values were less than 0.05.

Methods

Bacterial strains

S. aureus strain 8325-4 and its isogenic toxin mutant ($\Delta\alpha\beta\gamma$) have been previously described\textsuperscript{18}. S. aureus strains SA113 and Newman, and isogenic mutants deficient in lipoprotein diacylglycerol transferase ($\Delta\mathrm{lgt}$) have also been previously described\textsuperscript{19}. S. aureus strains LAC and MW2, their isogenic $\delta$-toxin mutants ($\Delta\mathrm{hld}$), the $\mathrm{psm}$ gene deleted mutants ($\Delta\mathrm{psma}$, $\Delta\mathrm{psm}$), and LAC agr mutant ($\Delta\mathrm{agr}$) have been previously described\textsuperscript{10}. The isogenic $\delta$-toxin mutant of S. epidermidis 1457, a clinical isolate\textsuperscript{20} was produced by an allelic replacement procedure\textsuperscript{21}. This was done in a way analogous to the S. aureus $\Delta\mathrm{hld}$ mutants used herein, abolishing translation by exchanging the third base in the $\mathrm{hld}$ start codon from ATG to ATA (to avoid interfering with the function of RNAIII). LAC P3-$\mathrm{lux}$ was constructed by integration of the S. aureus LAC agr P3 promoter fused to the $\mathrm{lux}$ABCDEF operon of Photorhabdus luminescens with codon usage optimized for staphylococci\textsuperscript{22} into the $\Phi11\ attB$ site of the S. aureus genome, using a procedure described by Luong and Lee\textsuperscript{23}. Plasmid pTX$_{\Delta\mathrm{hld}}$ was constructed by cloning the $\mathrm{hld}$ coding sequence containing the ribosomal binding site region in the BamH1/Mlu1 sites of plasmid pTX$_{\Delta\mathrm{10}}$. The $\mathrm{hld}$ gene was amplified from the genomic DNA of the respective strain, because the $\delta$-toxin sequence differs in one amino acid in position 10 (serine or glycine) in these two strains. The $\delta$-toxin is constitutively expressed in these plasmids. See Supplementary Table 1 for all oligonucleotides used in generation of the strains. Clinical isolates of S. aureus from children diagnosed with AD were obtained originally from the Department of Laboratory Medicine and Pathobiology at the University of Toronto\textsuperscript{24}. S. epidermidis (NI335), S. cohnii (NI446), S. saprophyticus (NI488), S. xylosus (NI987), S. sciuri (NI981), S. succinus (NI534), S. lentus (NI487) and S. fleuretti (NI533) were isolated by plating on BHI after
culturing at 37°C for two days under aerobic conditions. Identification of bacterial species was verified by 16S rRNA gene sequencing as described. Bacterial supernatants were produced by overnight culture with shaking in tryptic soy broth (TSB) followed by filtration through a 0.2 µm filter.

**Mice**

C57BL/6, C57BL/6-Kit^W-sh/Kit^W-sh (B6.CG-Kit^W-sh/HNihrJaeBsmJ), and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Syk^{+/−} mouse breeders were a gift of Dr. Steven Teitelbaum (Washington University School of Medicine, St. Louis, MO) and Syk^{−/−} embryos were generated by intercrossing. We used 4–12 week old age-matched female mice for in vivo experiments. Mice were allocated randomly into experimental groups. All mouse strains were housed under pathogen-free conditions. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

**Materials**

The synthetic peptides fPSMα2 (fMGIIAGIIKVIKSLIEQFTGK), fPSMα3 (fMGIIAGIIKFIKGLIEKFTGK), fδ-toxin (fMAQDIISTIGDLVKWIIDTVNKFTKK), (WRWWWW-CONH2) and MMK-1 (LESIFRSLLFRVM) were purchased from American Peptide. Unformylated δ-toxin (MAQDIISTIGDLVKWIIDTVNKFTKK) was synthesized at The University of Michigan Protein Structure Facility. Polyclonal anti-δ-toxin antibody was produced in rabbits by immunization with a synthetic multiple antigenic peptide displaying an 18 amino acid peptide (IGDLVKWIIDTVNKFTKK) (Sigma-Genosys) from the full length δ-toxin sequence. Rabbit IgG was purified from rabbit serum on Protein A (Pierce) according to the manufacturer’s protocol.

**Protein purification from S. aureus culture supernatant**

_S. aureus_ was cultured in 700 ml chemical defined medium supplemented with 2% yeast extract. Filtrated cultured supernatant was incubated with carboxymethyl cellulose equilibrated with 10 mM sodium citrate (pH 5.5), and eluted with a linear gradient of 0–1 M NaCl. Fractions containing β-hexosaminidase activity were collected and adjusted at pH 7.4, 100 mM HEPES. The sample was concentrated using Amicon Ultra-15, 5 kDa filter (Millipore). Concentrated sample was further fractionated with a Superdex 200 10/300 GL column (GE). Final positive fractions were pooled and concentrated using an Amicon Ultra-15 filter (Supplementary Fig. 2b).

**Protein identification by LC-tandem MS**

Purified sample was denatured in 8 M urea, reduced by incubation with 10 mM DTT at 37 °C for 30 min and alkylated using 50 mM iodoacetamide at room temperature for 30 min. The protein sample was digested with sequencing grade trypsin (Promega) overnight at 37 °C. The reaction was terminated by acidification with trifluoroacetic acid (0.1% v/v) and peptides were purified using a SepPak C18 cartridge following the manufacturer’s protocol (Waters Corporation). Eluted peptides were directly introduced into an ion-trap mass spectrometer (LTQ-XL, Thermo Fisher) equipped with a nano-spray source. The mass
spectrometer was operated in data-dependent MS/MS mode to acquire a full MS scan (400–2000 m/z) followed by MS/MS on the top 6 ions from the full MS scan. Dynamic exclusion was set to collect 2 MS/MS spectra on each ion and exclude it for a further 2 min. Raw files were converted to mzXML format and searched against S. aureus NCTC 8325 database appended with decoy (reverse) database using X! Tandem with k-score plug-in, an open-source search engine developed by the Global Proteome Machine (www.thegpm.org).

Search parameters included a precursor peptide mass tolerance window of 1 Da and fragment mass tolerance of 0.5 Da. Oxidation of methionine (+16 Da), and carbamidomethylation of cysteines (+57 Da) were considered as variable modifications. Search was restricted to tryptic peptides with one missed cleavage. Results of the X! Tandem search were then subjected to Trans-Proteomic Pipeline (TPP) analysis, a suite of software including PeptideProphet and ProteinProphet. All proteins with a ProteinProphet probability of >0.9 were considered positive and verified manually.

**Culture of mast cells and degranulation**

Preparations of BMCMCs and fetal skin-derived mast cells (FSMCs) were previously described\(^\text{16}\). Bone marrow cells from Fpr2\(^{−/−}\) mice were generously provided by Dr. Ji Ming Wang (Center for Cancer Research, National Cancer Institute, US). The purity of MCs was >95% as determined by surface expression of FcεRI and CD117 (eBioscience).

Degranulation of MCs was assessed by β-hexosaminidase assay as previously described\(^\text{16}\). Briefly, MCs (2 × 10\(^6\) ml\(^−1\)) were preloaded with or without IgEs (anti-DNP IgE\{clone; SPE7\}; 0.3 µg ml\(^−1\), anti-TNP IgE\{clone; IgE3 and C48-2\}; 0.5 µg ml\(^−1\)) in RPMI with IL-3 for 15 h. The cells were resuspended in Tyrode’s buffer (Sigma) at 2 × 10\(^4\) cells per 100 µl for FSMCs or 1 × 10\(^5\) cells per 100 µl for BMCMCs and MC/9 cells, aliquoted in triplicate into a 96 well U-bottom plate and incubated with EGTA (1 mM, Sigma), LY294002 (100 µM, Sigma), WRW4 (10 µM) and Cyclosporine H (10 µM, Alexis Biochemicals) for 30 min, and then stimulated DNP-HSA (30 ng ml\(^−1\)) TNP-HSA (30 nM) for 30 min, Ionomycin (1 µM, Sigma), δ-toxin (indicated concentrations), PSMαs (indicated concentrations) or FPR2 ligands for 15 min. Results of various stimuli are given as a relative percentage, where freeze and thaw of total cell culture represents 100%.

**MC-reconstitution in Kit\(^{W-sh/W-sh}\) mice**

For BMCMC reconstitution experiments, 10\(^6\) BMCMCs (cell purity was > 95 %) were injected into the ear skin. 4 × 10\(^6\) BMCMCs in 50 µl × 8 injections were injected into the shaved back skin of non-randomized Kit\(^{W-sh/W-sh}\) mice as described\(^\text{27}\). 4–6 weeks later, the mice were subjected to experimental passive cutaneous anaphylaxis assay or epicutaneous S. aureus sensitization. The number of animals per group (n= 5–8) was chosen as the minimum likely required for conclusions of biological significance, established from prior experience. The reconstitution rate of cutaneous MCs was quantified blindly by an independent observer and scored as number of MCs per low power field in toluidine blue stained tissue slides by microscope. The average rate of reconstituted MCs was ~40% in the ear pina and ~50% in the back skin (Supplementary Fig. 19 and 20).
Passive cutaneous anaphylaxis assay

PCA assay was performed as previously described with minor modifications. Ears of non-randomized mice were injected intradermally with or without αDNP-IgE in 40 µl saline and 15 h later, mice were challenged with 20 µl saline with or without synthetic δ-toxin (100 µg or 5 µg) or TSB bacteria supernatants. The number of animals per group (n= 5–8) was chosen based on previous experience as the minimum likely required for conclusions of biological significance. After inoculation, 0.1 ml of 5 mg ml⁻¹ Evans blue dye was injected intravenously. Extravasation of Evans blue dye was monitored for 30 min, and 4 mm of punched-out biopsies were incubated at 63°C overnight in 200 µl formamide. Quantitative analysis of extracts was determined by measuring the absorbance at 600 nm.

Ca²⁺ influx assay

FSMCs (2 × 10⁶ ml⁻¹) were preloaded with or without anti-DNP-IgE (0.3 µg ml⁻¹) in RPMI with IL-3 for 15 h. Cells were washed and then loaded with Fluo-4AM (5 μM, Life Technologies) for 30 min. Cells were washed again and further incubated in Tyrode’s buffer with or without EGTA (1 mM) for 30 min. DNP-HSA (30 ng ml⁻¹), Ionomycin (1 μM) or δ-toxin (30 µg ml⁻¹) were used to induce calcium flux in these cells. Ca²⁺ flux was measured using a flow cytometer (FACSCalibur, BD Biosciences) to monitor RFU (relative fluorescence units) as described.

Epicutaneous sensitization with S. aureus or OVA

We performed epicutaneous colonization with S. aureus by shaving the dorsal skin of non-randomized 6–8 week old female mice and 3-time stripping using a transparent bio-occlusive dressing (Tegaderm®; 3M). Sample size (n = 5–8 per group) was based on prior experience as the size necessary for conclusions of biological significance and adequate statistical analysis. After overnight culture at 37°C with shaking, S. aureus were cultured in fresh TSB medium for 4 hrs at 37°C with shaking, washed and resuspended in PBS at 10⁸ CFU of S. aureus LAC or LAC (Δhld) strains. 100 µl of the S. aureus suspension was placed on a patch of sterile gauze (1 × 1 cm) and attached to the shaved skin with transparent bio-occlusive dressing. Each mouse was exposed to S. aureus for 1 week through the patch. After a 2-week interval, each mouse was challenged once with 100 µg OVA (Grade V, Sigma) epicutaneously for 1 week and the animals were sacrificed for analyses. For OVA sensitization model, BALB/c mice were sensitized epicutaneously with OVA (100 µg) with or without synthetic δ-toxin (100 µg) for 1 week. After 2 week interval, mice were challenged with OVA (100 µg) with or without synthetic δ-toxin (100 µg) at the same skin site.

Skin disease score

The severity of skin lesions was scored according to defined macroscopic diagnostic criteria in a blind fashion. In brief, the total clinical score of skin lesions was designated as the sum of individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for thickness, erythema, edema, erosion, and scaling.
Histology

Skin tissue was formalin fixed, paraffin embedded and sectioned for H&E and Toluidine blue staining.

Cytokine and immunoglobulin levels

Chemokines and cytokines were measured with enzyme-linked immunoabsorbent assay (ELISA) kits (R&D Systems). For tissue cytokines, skin tissue (5 × 10 mm area) was removed and homogenized. The skin homogenates were centrifuged and supernatants were collected for cytokine measurements by ELISA. Serum IgG1 and IgG2a were measured with ELISA kit (Cayman chemical). Serum IgE was measured with ELISA kit (Bethyl Laboratories). ELISA for OVA-IgE was described previously.

RNA isolation from human skin samples

Wash fluid derived from lesional and normal skin of AD patients was collected using a 2.5-cm-diameter polypropylene chamber as reported. 100 µl of the samples were mixed with an equal volume of RNAProtect Bacteria Reagent (QIAGEN) and RNA extracted with Bacterial RNA Kit (OMEGA). The human studies were approved by the Indiana University Institutional Review Committee. Informed consent was obtained from all subjects.

Quantitative real time RT-PCR

cDNA was synthesized using High Capacity RNA-to-cDNA Kit (Applied Biosystems), according to the manufacturer’s instructions. Quantitative real time RT-PCR (qPCR) was performed using a SYBR green PCR master mix (Applied Biosystems) and StepOne Real-time PCR system (Applied Biosystems). Primers to amplify mouse Fpr genes and bacterial genes (RNAIII, gyrB, 16S rRNA) have been described. Expression of mouse Fpr genes was normalized to that of Gapdh (F; 5-CCTCGTCCCGTAGACAAAATG-3, R; 5-TCTCCACTTTGACCCTGCAA-3) and expression were analyzed by the 2^−ΔΔCt method. Expression of RNAIII expression in human skin samples was normalized to that of S. aureus gyrB and that of gyrB to universal bacterial 16S rRNA and relative expression calculated by the 2^−ΔCt method. RNAIII and gyrB expression in some human skin samples were below the detection limit and arbitrarily given a value of zero for statistical analysis. LAC wt and LAC Δagr cultured for 24 hrs were used as reference controls.

Measurement of P3-lux expression

For determination of the levels of P3-lux expression in culture, 10^5 ml^-1 LAC P3-lux strain was suspended in TSB and luminescence emitted from P3-lux-expressing bacteria was measured using a LMax luminometer (Molecular Devices). For in vivo bioluminescence imaging (BLI), mice were sacrificed, the skin dressing removed and immediately placed into the light-tight chamber of the CCD camera system (IVIS200, Xenogen). Luminescence emitted from lux-expressing bacteria in the tissue was quantified using the software program living image (Xenogen).
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S. Koonse for animal husbandry, J. Whitfield for ELISA assays, S. Meshinchi for electron microscopy analysis, V. Basrur for mass spectrometry analysis, M.K. Oyoshi and R.S. Geha for experimental advise, Vee Y. Tan for help with constructing the LAC P3-lux strain, and A. Burberry for critical review of the manuscript. Y. Nakamura was supported by fellowships from Chiba University Global COE Program, the Cell Science Research Foundation and Kanae Foundation for the Promotion of Medical Science. J. Oscherwitz and K. Cease were supported by Department of Veterans Affairs Merit Award I01BX000429. A. E. Villaruz, G. Y. C. Cheung and M. Otto were supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), NIH. This work supported by NIH grants R01AR059688 to G. N. and R01HL062996 to J. B. T. and funds to the Michigan Comprehensive Cancer Center Immunology Monitoring Core from the University of Michigan’s Cancer Center Support Grant.

References


Nature. Author manuscript; available in PMC 2014 July 10.
Supplementary References


Figure 1. δ-toxin from S. aureus induces MC degranulation in vitro and in vivo

a, β-Hexosaminidase activity released to the extracellular media of BMCMCs stimulated with medium alone (Control) or indicated stimuli including different concentrations of culture supernatant of S. aureus 8325-4 (S.a sup). b, β-Hexosaminidase activity in supernatants of MC/9 cells stimulated with 10% of culture supernatant from LAC S. aureus wild-type (LAC wt) or isogenic mutants deficient in PSMα peptides (LAC Δpsma), PSMβ peptides (LAC Δpsmb), δ-toxin (LAC Δhld), LAC wild-type expressing vector alone (LAC pTXΔ16), LAC deficient in δ-toxin expressing vector alone (LACΔhld pTXΔ16) and strain Nakamura et al. Page 13 Nature. Author manuscript; available in PMC 2014 July 10.
complemented with δ-toxin plasmid (LACΔhld pTXΔhld). Control represents 10% TSB medium. c, Histamine concentrations in culture supernatant of fetal skin-derived MCs (FSMCs) after stimulation with indicated stimuli including synthetic δ-toxin at 30 µg ml⁻¹ for 15 min. Data represent means ± s.d. of triplicate cultures. Results are representative of at least 3 independent experiments (a-c). P value refers to comparisons between experimental and control groups (a-c). d, Electronmicroscopic images of FSMCs stimulated with synthetic δ-toxin (30 µg ml⁻¹) for 15 min. Images of unstimulated (Cont) and ionomycin-treated FSMCs are also shown. Representative of at least 20 images. e, δ-toxin expression in Staphylococcus culture supernatants (0.5 µl per well). Loading of lanes with synthetic δ-toxin (10 ng, 100 ng) is shown as reference. Representative of three experiments. f, C57BL6 (WT) and MC-deficient (Kitw-sh/w-sh) mice were injected intradermally into the left and right ears with δ-toxin (100 µg) or PBS, respectively. One representative mouse for each group is shown. Representative of 8 mice per group. g, Quantification of Evans blue extracted from skin tissue of WT, Kitw-sh/w-sh, Kitw-sh/w-sh reconstituted with BMCMCs is shown. Dots represent individual ear samples from 2 independent experiments. NS; no significant; *P < 0.05; **P < 0.01; ***P < 0.001, 2-tailed t test.
Figure 2. δ-toxin-induced MC degranulation depends on Ca²⁺ influx/PI3K pathway, but is independent of Syk

a, FSMCs loaded with the fluorescent Ca²⁺ indicator Fluo-4AM with or without EGTA were stimulated for 50 sec. Baseline fluorescence (red) was measured, and then the MCs were stimulated with indicated stimuli and fluorescence shift (green) was measured. RFU, relative fluorescence units. b, c, β-Hexosaminidase activity in culture supernatants of FSMCs pretreated with EGTA (b) or LY294002 (c) stimulated with medium alone (Ctrl), ionomycin, DNP-HSA (DNP) plus anti DNP-IgE or δ-toxin (10 µg ml⁻¹). d, β-
Hexosaminidase activity in culture supernatants of FSMCs derived from Syk$^{-/-}$ and wild-type (WT) mice stimulated with indicated concentration of δ-toxin (µg ml$^{-1}$). Data represent means ± s.d. of triplicates cultures and representative of at least 3 independent experiments (b-d). NS: no significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, 2-tailed t test.
Figure 3. Antigen-independent IgE signaling enhances δ-toxin-induced MC activation

a, β-Hexosaminidase activity in culture supernatants of FSMCs stimulated with or without anti DNP-IgE or TNP-IgE and then re-stimulated with δ-toxin (0.01 µg ml⁻¹), DNP-HSA (DNP) or TNP-HSA (TNP). b, β-Hexosaminidase activity in culture supernatants of FSMCs derived from Syk⁻/⁻ and wild-type mice (WT) pretreated with or without anti DNP-IgE, and then stimulated with indicated concentration of δ-toxin (µg ml⁻¹). Representative of at least 3 independent experiments. **P < 0.01; ***P < 0.001, 2-tailed t test (a,b). c, Quantification of Evans blue extracted from skin tissue of C57BL6 mice injected intradermally into the left
and right ears with δ-toxin (5 µg) or PBS, respectively. Data represent means ± s.d. of triplicate cultures and representative of at least 3 independent experiments (a,b). Dots represent individual ear samples. Representative of 2 independent experiments (c). NS: no significant; *P < 0.05, one-way ANOVA with Tukey post-hoc test for multiple comparisons.
Figure 4. *Staphylococcus* δ-toxin promotes IgE production and inflammatory skin disease via mast cells

**a.** *S. aureus* colonization and OVA sensitization protocol. Mice were colonized epicutaneously with $10^8$ CFU *S. aureus* using a gauze patch for 1 week. For OVA sensitization, a patch containing OVA or PBS was applied to the same skin site 2 weeks after *S. aureus* inoculation. **b.** Skin disease score 1 week post colonization with wild-type and δ-toxin mutant (Δhld) *S. aureus* or treated with PBS. **P** < 0.01; **P** < 0.001, Kruskal-Wallis test with post-hoc Dunn’s test for multiple comparisons. **c.** Skin phenotype

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and histopathology of BALB/c mice colonized with *S. aureus* or treated with PBS. Skin sections were stained with H&E. Bar = 100 µm. Inset shows high power image with neutrophil-rich inflammation. Representative of 14 mice per group. 

d, Number of inflammatory cells in skin of BALB/c mice colonized with *S. aureus* or treated with PBS. Results depicted as number of inflammatory cells per high power field (hpf). Error bars represent means ± s. e. m. 

e, Serum levels of IgE in BALB/c mice colonized with *S. aureus* or treated with PBS at 1 and 3 weeks post colonization with *S. aureus*. 

f, Serum levels of OVA-specific IgE after OVA sensitization in BALB/c mice colonized with *S. aureus* or treated with PBS. 

g, Skin disease score in C57BL/6 (B6), MC-deficient (*Kit<sup>W-sh/W-sh</sup>*) and MC-deficient (*Kit<sup>W-sh/W-sh</sup>* ) mice reconstituted with MCs at 1 week after the inoculation with *S. aureus*. 

h, Serum levels of total IgE 1 week after colonization of B6, *Kit<sup>W-sh/W-sh</sup>* and *Kit<sup>W-sh/W-sh</sup>* mice reconstituted with MCs with wild-type and δ-toxin mutant (*Δhld*) *S. aureus* or treated with PBS. Dots represent individual mice pooled from two independent experiments. *P < 0.05; **P < 0.01 ; ***P < 0.001, one-way ANOVA with Tukey post-hoc test for multiple comparisons (e-h)