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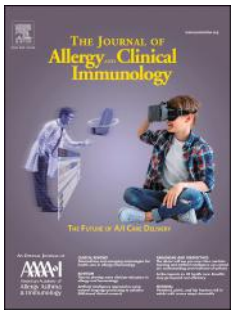

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Staphylococcus aureus Second Immunoglobulin-Binding Protein drives atopic dermatitis via IL-33

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Abstract

Background: *S. aureus* is the dominant infective trigger of atopic dermatitis (AD). How this bacterium drives type 2 allergic pathology in the absence of infection in AD patients is unclear.

Objective: To identify the *S. aureus*-derived virulence factor(s) that initiates the cutaneous type 2-promoting immune response responsible for AD.

Methods: *In vitro* human keratinocyte cell culture, *ex vivo* human skin organ explants and the eczema prone Nc/Tnd mouse were used as model systems to assess type-2 promoting immune responses to *S. aureus*. Identification of the bioactive factor was accomplished using Fast Protein Liquid Chromatography and mass spectrometry. Bioactivity was confirmed by cloning and expression in an *E. coli* vector system, and *S. aureus* Sbi mutant strains confirming loss of activity.

Results: *S. aureus* was unique amongst staphylococcal species in its ability to induce the rapid release of constitutive IL-33 from human keratinocytes independent of the toll-like receptor pathway. Using the eczema-prone NC/Tnd mouse model, we showed that IL-33 was essential in inducing the immune response to *S. aureus in vivo*. By fractionation and candidate testing, we identified the Second Immunoglobulin-Binding Protein (Sbi) as the predominant staphylococcus-derived virulence factor that directly drives IL-33 release from human keratinocytes. Immunohistology of skin demonstrated that corneodesmosin, a component of corneodesmosomes that form key intercellular adhesive structures in the stratum corneum, was disrupted resulting in reduction of skin barrier function.

Conclusion: *S. aureus*-derived Sbi is a unique type 2-promoting virulence factor capable of initiating the type-2 promoting cytokine activity underlying AD.

Clinical Implications: The Second Immunoglobulin-Binding Protein (Sbi) produced solely by *S. aureus* species, is an important factor inducing type-2 promoting cytokine responses and atopic dermatitis.

Capsule summary: Why *S. aureus* is the predominant infective trigger of AD is unclear. This study identifies *S. aureus* derived Sbi as a key virulence factor, capable of triggering type 2 immune responses *in vitro* and *in vivo*.

Key words: *Staphylococcus aureus*, atopic dermatitis, Second Immunoglobulin-Binding Protein, Sbi, virulence factor, skin, keratinocytes, IL-33, TSLP, type 2 immune response.

Abbreviations: AD: atopic dermatitis; CDSN: corneodesmosin; CFU: Colony Forming Units; DAPI: 4′,6-diamidino-2-phenylindole; DSC-1: desmocollin-1; DSG-1: desmoglein-1; DF: *Dermatophagoides farinae*; DLD: DihydroLipoyl Dehydrogenase; DP: *Dermatophagoides pteronyssinus*; DPBS: Dulbecco's Phosphate Buffered Saline; ELISA: Enzyme-Linked Immunosorbent Assay; FITC: Fluorescein isothiocyanate; fnb: fibronectin; FPLC: Fast protein liquid chromatography; *FLG*: filaggrin; FSA: Filtered *S. aureus* supernatant; FSE: Filtered *S. epidermidis* supernatant; HEKa: Human Epithelial Keratinocytes, adult: HKSA: Heat-killed *S. aureus*; IL: interleukin; LAP: Leucine AminoPeptidase; LiSA: live *S. aureus*; LPS: lipopolysaccharide; LTA: lipoteichoic acid; MSM/Ms: *Mus musculus molossinus*/Mishima strain; NC/Tnd: Nishiki-nezumi Cinnamon/Tokyo University of Agriculture and Technology strain; NHEK: Normal Human Epidermal Keratinocytes; PDHa: Pyruvate Dehydrogenase Alpha-subunit; PGN: proteoglycan; Pam_3CSK_4: N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; RT: room temperature; Sbi: Second Immunoglobulin-Binding Protein; SCORAD: scoring AD; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SEM: Standard Error of the Mean; siRNA: small

81 interfering RNA; Spl: Serine-protease-like; TEWL: TransEpidermal Water Loss; TLR: Toll-like

82 receptor; TSLP: thymic stromal lymphopoietin.

Staphylococcus aureus (*S. aureus*) is the dominant pathogen of human skin, causing the majority of skin and soft tissue infections worldwide.[1-3] It is also the most frequent cause of infection-induced flares of atopic dermatitis (AD).[4-6] The bacterium expresses many virulence factors both in its cell wall and secretome.[7-9] Previous studies have focused on recognition of *S. aureus* by host innate immunity, particularly activation of the inflammasome through toll-like receptors (TLR) and subsequent induction of cytokines such as IL-1β.[10] Enzymes such as coagulase, toxins such as enterotoxins, Toxic Shock Syndrome Toxin-1, α-hemolysin, and invasins including pore-forming proteins such as Panton-Valentine Leukocidin induce host cytotoxicity and damage the skin barrier. Defects in skin barrier function, particularly lack of filaggrin expression in the epidermis secondary to pathologic variants in the *FLG* gene, are well recognized to contribute to the pathogenesis of AD.[11] However, patients with *FLG* variants may suffer from ichthyosis without skin inflammation.[12] Thus although skin barrier disruption can predispose patients to AD, the main driving force in AD is the Th2 immune response, as clearly illustrated by the effectiveness of the IL-4/13 receptor antagonist dupilumab in recent clinical trials.[13,14] Delta-toxins and superantigens produced by *S. aureus* can induce mast cell activation and IgE production, but the bacterial trigger of type-2 allergic responses by keratinocytes in the skin is unknown.[15,16]

The current paradigm is that rather than a single factor, a complex array of *S. aureus* virulence factors contribute to the atopic skin response.[4,7] However, the study of immunodeficiency and immune dysregulation disorders have taught us that there is much redundancy in host immunity.[17,18] The hypothesis we set out to explore was whether *S. aureus* expresses a predominant virulence factor that initiates type 2-promoting cytokine release from skin cells and drives the development of AD. If this critical factor could be found, then targeting it therapeutically could negate one of the key advantages *S. aureus* has developed during its evolution and symbiosis with its hosts.

METHODS

Materials and reagents

A list of bacterial strains, materials, reagents, and primer sequences used in this study are listed in the online repository Tables SI and SII.

Staphylococcal species and strains, Group A streptococcus and preparation of filtered supernatants

Methicillin-sensitive *S. aureus* (LiSA) and *Streptococcus pyogenes* were provided by Professor McBain. Other *S. aureus* wild-type strains and staphylococcal species were a gift from Dr Xia, both of University of Manchester. *S. aureus* SH1000 and its isogenic fibronectin (fnb)A^{-} and fnbB^{-} mutant, as well as the Newman strain, Newman Sbi^{-}, Spa^{-}, and pRMC:sbi^{-} mutants were a gift from Professor Geoghegan, Trinity College, Dublin (refer to Online repository Table SI). Both the Newman Sbi^{-} and pRMC:sbi^{-} mutant strains inherently express no Sbi protein, but the pRMC:sbi^{-} strain can be induced to express Sbi with addition of anhydrotetracyclines, although not in these set of experiments.[19] Staphylococcal species were plated overnight (37°C) on nutrient agar to generate colonies. The number of colony-forming units (CFU/ml) were determined by the method of Miles & Misra.

Preparation of filtered supernatant from *S. aureus* and *S. epidermidis*

A single bacterial colony was inoculated with nutrient broth overnight (37°C). 10^8 CFU/species were inoculated in 100ml of Human Keratinocyte Growth Medium 2 (PromoCell) and incubated for 6h at 37°C in a shaking incubator. After incubation, samples were centrifuged (1,600*g*, 5min, RT), supernatants collected and filter sterilized using 0.2µm filters (Millipore, Bedford, USA). The filtered supernatants were then treated with 2% penicillin/streptomycin and stored at –80°C until required for stimulation experiments.

Preparation of modified allergens (ragweed and house dust mites)

Allergens used included modified Ragweed, *Dermatophagoides farinae* (DF) and *Dermatophagoides pteronyssinus* (DP) (gifts from Dr Gianni Mistrello, Lofarma). The allergen extracts were re-suspended in sterile Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich) according to supplier recommendations at a concentration of 6.0mg/ml Ragweed, 1.3mg/ml DF and 2.6mg/ml DP were stored at −20°C until required for stimulation experiments.

Primary human keratinocyte and *ex vivo* organ culture and stimulation

NHEK (PromoCell) and HEKa (ThermoFisher Scientific) were cultured in Keratinocyte Growth Medium 2 plus supplements (PromoCell) and medium 154 supplemented with Human Keratinocyte Growth Supplement without antibiotics respectively for 48 – 72h at 37°C under 5% CO_2 until 70-90% confluent. Cells of passages 3 – 6 were used for experiments. Human keratinocytes were cultured in 24-well tissue culture plates at a density of $5x10^5$ cells/well. Primary human fibroblasts and keratinocytes were isolated from skin biopsies taken from either nonatopic patients with otapostasis undergoing plastic surgery or similarly aged children with moderate-severe AD attending a Regional Paediatric AD outpatient clinic at Royal Manchester Children's Hospital, Manchester, UK.

Human skin was obtained from adult healthy patients following abdominal reduction or liposculpture procedures. Post-excision, subcutaneous fat tissue was removed, biopsies were taken using sterile 4mm biopsy punches (KAI Medical, GP Supplies Ltd, London, UK) and placed in 1ml/biopsy William's E medium (Thermo Fisher Scientific) supplemented with l-glutamine (2mM, Sigma-Aldrich), penicillin (100U/ml)-streptomycin (0.1mg/ml) (Sigma-Aldrich), 0.02% (v/v) hydrocortisone (Sigma-Aldrich) and 0.1% (v/v) insulin (Sigma-Aldrich) in 6-well culture plates containing 0.4µm ThinCert™ cell culture inserts (Griener Bio-One,

Kremsmünster, Austria) dermal side down. Intact epidermal exposed biopsies were treated with 5µl /biopsy of FSA, FSE or allergens including Ragweed, DF, and DP for up to 12h (37°C, 5% CO_2). Following treatments, supernatant was removed and stored at −80°C, and biopsies were snap-frozen in liquid nitrogen and stored at −80°C until use, where they were then embedded in Optimal Cutting Temperature Compound (KP Cryo-Compound) before cryo-sectioning.

Human IL-33 and TSLP ELISA

R&D System ELISA (Abingdon, UK) were used to measure IL-33 (DY3625-05) and TSLP (DY1398-05) release according to the manufacturer's instructions.

Detection of cell death by Annexin-V and DAPI staining

Cells were detached from 24-well plates, harvested into micro-centrifuge tubes, washed with Annexin-V binding buffer and centrifuged (5min, 500*g*, 4°C), then stained with Annexin-V diluted 1:100 in binding buffer for 30min on ice. Following incubation, cells were harvested and re-suspended in Annexin-V binding buffer. DAPI was added just before reading the sample. A total of 10,000 events were acquired on BD LSRFortessa X20 (BD biosciences). Data were analyzed using FlowJo (Treestar© V10).

Histology and Immunohistochemistry

10^5 HEKa seeded in 24-well culture plates containing sterile coverslips were incubated at 37°C until 100% confluent. They were then co-cultured with LiSA for 1h, fixed and permeabilized with 1% triton-X for 15min and stained with mouse anti-human IL-33 monoclonal antibody (Nessy-1 ALX-804-840PF, 1:200 dilution, Enzo Life Sciences, Exeter, UK) and rabbit anti-human cytokeratin 14 polyclonal antibody (PA5-1672, 1:200 dilution, Thermo Fisher Scientific). Cultures were incubated with secondary antibodies (biotinylated anti-mouse, 1:200 dilution,

Vector Laboratories, Burlingame, USA) followed by streptavidin Cy3 (Sigma-Aldrich), and anti-rabbit IgG Alexa Fluor Plus 488 (Invitrogen). Images were acquired at a magnification of 20X using a fluorescent microscope (Olympus, BX51). Histological sections were prepared from snap frozen healthy human skin or NC/Tnd mouse samples embedded in OCT (KP Cryo-Compound, CellPath Services, UK). 10µm sections were stained with anti-human or anti-mouse CDSN, DSC-1 or DSG1 (1:200, Thermo Fisher Scientific, 1:1000 Santa Cruz respectively) and detected using anti-rabbit IgG Texas Red™ (Invitrogen) or Alexa Fluor™ 594-conjugated secondary antibodies. Images were captured at a magnification of 20X using an Eclipse Ci fluorescent microscope (Nikon, Surrey, UK) and a SPOT camera (Image solutions Inc, Preston, UK) or using Olympus BX51 camera.

Densitometry analysis

To quantitatively analyze expression levels, the mean gray intensity of five randomly-selected 5µm square fields from immunofluorescent staining samples and protein bands from Western blots were measured by using Image J software (Ver 1.53a, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Trans-well Assay

Trans-well assays were performed in 24-well trans-well (6.5mm diameter, 0.4µm pore size, Costar). Normal Human Epidermal Keratinocytes (NHEK) were added to the lower chamber and live *S. aureus* (LiSA) was added to the upper chamber. After 6h incubation at 37°C, the supernatant was collected for analysis of type 2 cytokines by ELISA.

Fractionation of 100kDa retained fraction of filtered supernatant from *S. aureus* by Fast-protein liquid chromatography

Filtered supernatant from S. *aureus* (FSA) fractionated by size exclusion centrifugal filter columns (Amicon Ultra 15, 100kDa or 50kDa cut-off membranes) and centrifuged for 15min at 3,000*g* (4°C). 200ml of FSA was fractionated using 100kDa Amicon Ultra 15 as described above. Retained fractions were then pooled and then fractionated further using Superose® 6 Fast Protein Liquid Chromatography (FPLC) column (5 - 5000 kDa). 48 fractions of 0.5ml were collected and stored at −20°C until required.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the molecular weights of FSA proteins using standard methodology. Gels were then stained with Instant Blue Coomassie-based gel stain or silver stain using standard protocols.

Mass spectrometry

Mass spectrometry was used to identify proteins in the bioactive peak derived from the FPLC size fractionation. In-gel digestion and tandem mass spectrometry analysis was performed by the Protein Mass Spectrometry Core Facility, Faculty of Biology, Medicine and Health, University of Manchester. The data was generated against known protein sequences in Swissprot TrEMBL database using Firmicutes phylum (which includes *S. aureus*) as the most optimal taxonomic level available in the database.

Candidate gene cloning and expression

Details of cloning and expression of candidate genes using the *E. coli* vector system are provided in the Methods section of this article's Online Repository.

TLR2 Knockdown/Small interfering RNA (siRNA)

TLR2-specific siRNA (human Hs_TLR2_1 Flexitube siRNA, SI00050015) and a scramble (scr) non-silencing control siRNA (AllStars negative control, 1027281) were purchased from Qiagen. NHEK were seeded at a density of $1x10^4$ one day prior to transfection. After 24h, cells were transfected with 5nm siRNA using 2.5µl of TransIT-TKO transfection reagent (Mirus Bio, UK). Experiments were performed on transfected cells at least two days after siRNA treatments. Knockdown efficiency of TLR2 siRNA mediated gene expression was confirmed by Real-time PCR.

Animals

NC/Tnd mice were generated in the laboratory of comparative animal medicine in Tokyo University of Agriculture and Technology. MSM/Ms mice were purchased from RIKEN Bioresource as wild-type (WT) controls. All animals were kept in a clear acrylic cage and had free access to standard chow and water. They were kept in specific-pathogen free housing to prevent the natural development of eczema that occurs if these mice are housed in conventional housing. Temperature and humidity of the animal room were 22±4°C and 40±15%, respectively. The animal room was maintained on a 12:12-hour light-dark cycle. All animal experiments complied with the guidelines of University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology, as well as with the guidelines of Science Council of Japan for the use of laboratory animals.

Topical application of filtered supernatant from *S. aureus*, S. *epidermidis* or allergen extracts

100µl of filtered supernatant stimulated with *S. aureus* (FSA) (10^7 CFU/ml low dose or 10^8 CFU/ml high does) or *S. epidermidis* (FSE, 10^8CFU/ml) was applied to the back skin of the mice once daily for four weeks. According to the manufacturer's instruction, 100mg of DF ointment

was applied to the back skin of the mice. Application was performed twice/week for 4 weeks. Skin barrier disruption was performed by 4% SDS before the application of FSA, FSE and DF.

Clinical eczema severity and scratching behavior scoring in mice

Clinical eczema scores in mice were assessed as previously described.[20] Briefly, the total clinical severity score was defined in individual mice as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), 3 (severe) for each of five signs and symptoms (itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness).

Scratching frequency and duration was measured for 30min each week and analyzed automatically using SCLABA®-Real system (Noveltec, Hyogo, Japan).[20] All mice were kept in an acrylic cage for 30min for the acclimation before each measurement.

Trans-epidermal water loss measurement in mice

Trans-epidermal water loss (TEWL) was measured using Tewameter® TM300 from Courage + Khazaka electronic GmbH (Cologne, Germany), once weekly for four weeks. Temperature and humidity were maintained at 22 ± 0.5°C and 50% ± 10% respectively before the measurements. The measurements for each mouse were taken three times and the mean value was calculated.

Immunoblotting

Whole cell lysates were isolated from the back skin of the mice using radioimmunoprecipitation assay buffer. After SDS-PAGE using a 10% gel, immunoblotting was performed with anti-mouse CDSN monoclonal antibody (1:500; Santa Cruz), anti-mouse DSG-1 monoclonal antibody (4µg/ml; Santa Cruz), anti-mouse DSC-1 monoclonal antibody (1:250; R&D Systems), and horse radish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling). Positive reactions were visualised using Immobilon western chemiluminescent HRP substrate (Millipore).

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284 **Statistical analysis**

285 All *in vitro* and *ex vivo* experiments were carried out with a minimum of three technical and two-
286 three biological replicates as detailed in the figure legends. *In vivo* experiments were performed
287 twice with six mice per group. One-way ANOVA was used for single factor data with 3 or more
288 groups followed by comparisons to a control group using Dunnett's post-hoc test. However, if
289 normality could not be assumed using the QQ residual plot, statistical comparisons between
290 groups was determined using the Kruskal Wallis test with Dunn's multiple comparisons and
291 adjusted P values reported. Single factor data with less than three groups were analysed using the
292 unpaired t-test and the two-tailed P value reported. All analysis was performed using GraphPad
293 Prism 8 Version 8.4.2 (GraphPad Software, Inc. CA, USA). A probability value of less than 0.05
294 was considered statistically significant. Flow cytometry data were analysed using FlowJo
295 (Treestar V10) and represented as mean ± standard error of the mean (SEM).

RESULTS

S. aureus, but no other staphylococcal species, induces type 2-promoting cytokines

Although *S. aureus* is the major bacterial species associated with AD flares,[21,22] the comparative ability of other staphylococcal species and skin flora to stimulate type 2-promoting cytokines by skin keratinocytes has not previously been directly evaluated. Of seven different staphylococcal species we investigated, only live *S. aureus* (LiSA) was able to trigger IL-33 and TSLP release by NHEK (Fig. 1a). *S. epidermidis*, the predominant bacterial skin commensal, was without any type-2 immune potential. Furthermore, Group A streptococci (GAS), the other major bacterial skin pathogen, was also unable to stimulate release of these type 2- promoting cytokines (Fig. 1a). IL-33 and TSLP release was not limited to one strain of *S. aureus* but could be demonstrated in six different strains and mutants tested, including RN4220, RN4220;ΔtagO, RN4220;Δspa SH1000, SH1000;ΔFnBP (Fig. 1b). The unique proinflammatory effect of LiSA was dose-dependent (Fig. 1c) and detected after only 2h, in keeping with release of a prestored cytokine pool rather than *de novo* synthesis (Fig. 1d). To support this supposition, IL-33 demonstrated in the nuclei of unstimulated HEKa,[23] dissipated after stimulation with live *S. aureus* (Fig. 1e). Live *S. aureus*-induced IL-33 release was associated with an increase in DAPI uptake and Annexin V expression, indicating an association with cell death (Fig. 1f-h).

Having shown that live *S. aureus* is unique in its ability to induce Th2-promoting cytokine release by human keratinocytes *in vitro*, we next investigated whether its bioactivity resided in the bacterial cell wall or secretome.

S. aureus secretome induces Th2-promoting cytokines

Heat-killed *S. aureus* (HKSA) was unable to induce cytokine release in primary human keratinocytes, suggesting that the bioactivity did not reside in the cell wall of the bacteria (Fig. 1b). This was explored further by addition of the purified *S. aureus* bacterial wall components PGN and LTA to stimulate TLR2 signalling, as this has previously been reported to drive

immunopathology.[24] Neither exogenous PGN nor LTA induced IL-33 or TSLP release (Fig. 2a, b). Anti-TLR2 blocking antibodies (Fig. 2b) and siRNA knockdown of TLR2 in keratinocytes (Fig. 2c) did not suppress either IL-33 or TSLP release by FSA.

In contrast, experiments where LiSA and NHEK were separated by a 0.4μm pore membrane demonstrated that the type-2 immune promoting bioactivity resided within the bacterial secretome (FSA) (Fig. 3a). The FSA bioactivity was heat-labile, but not affected by addition of antibiotics to remove any contaminating LiSA (Fig. 3b). Interestingly, in contrast to LiSA, co-culture of FSA with NHEK did not induce either DAPI uptake or Annexin V expression, indicating that the bioactivity was independent of cell death (Fig. 3c-e).

Skin barrier disruption and eczema induced by *S. aureus* secretome

We then explored whether the secretome-derived factor was bioactive in *ex vivo* human skin organ culture and in an *in vivo* mouse eczema model. Using human skin organ explants from nonatopic adults undergoing abdominal skin excision for treatment of obesity, we demonstrated that sterile filtered supernatant from *S. aureus* (FSA), but not *S. epidermidis* (FSE), common house dust mite (DP & DF) or pollen aeroallergens (Ragweed) stimulated release of both IL-33 and TSLP (Fig. 4a-b). Examination of corneodesmosome expression from these organ explants suggested that whilst FSA was associated with a more diffuse expression of DSC-1 and DSG-1, it seemed to disrupt CDSN expression when compared with the control and this was confirmed by densitometry (Fig 4c, 4d). CDSN is the only corneodesmosome protein component not covalently linked to the cell membrane.[25]

There has been an ongoing debate as to whether the type-2 immune response in patients with AD is inherently different to that of non-atopic individuals. LiSA induced release of similar quantities of both IL-33 and TSLP by primary keratinocytes derived from skin punch biopsies taken from children with moderate-to-severe AD and non-atopic controls, indicating that the Th2-promoting potential of epidermal keratinocytes is not significantly different in these two

groups (Fig. 4g-h). This suggested that exposure to *S. aureus*-derived bioactive factor(s) through a disrupted skin barrier, rather than an inherent immune hyperactivity, is the important driver of disease.[26]

The NC/Tnd Japanese fancy bred mouse is an excellent animal model of human AD, in that pups from this inbred strain in conventional, but not in specific-pathogen free housing, develop eczema soon after weaning at 6 – 8-weeks old. The immunohistochemistry mirrors that of human AD and the disease responds to topical corticosteroids and tacrolimus ointment.[27,28] Eczema in this mouse model is aggravated by *S. aureus* as topical antibiotics suppress clinical disease and Th2 response.[29] Using this mouse model in specific pathogen free housing, we demonstrate that daily application of FSA and house dust mite allergen (DF), but not FSE for four weeks to SDS primed skin induces a dose-dependent flare in clinical eczema (Fig. 5a-b) associated with increased scratching behaviour (Fig. 5c) and disruption in skin barrier function measured by TEWL (Fig. 5d).

Associated with the clinical eczema flare, skin histology showed marked hyperkeratosis of the epidermis where FSA (or house dust mite allergen) had been applied (Fig. 6a). As with the human explant model, expression of corneodesmosome proteins (particularly corneodesmosin) in the stratum corneum on immunofluorescence appeared to be disrupted (Fig. 6a). Densitometry showed that FSA reduced intensity of expression of CDSN, DSC-1 and CSG-1 compared with the control, with more variable reduction after application of DF and FSE (Fig. 6b-d). Western blot analysis and densitometry from mouse skin illustrated that CDSN was significantly degraded by FSA and DF compared with the control and FSE (Fig. 6e, 6f). There was evidence of degradation of DSC-1 by FSA, DF and FSE (Fig. 6e, 6g), but DF and not FSA degraded DSG-1 (Fig 6e, 6h). In addition to the local cutaneous effects, application of FSA to the skin of these NC/Tnd mice led to significant increases in plasma concentrations of TSLP (Fig. 6i) and IL-33 (Fig 6j). This is in keeping with the known association between AD, circulating IL-33 and TSLP and other non-cutaneous allergies such as food allergies and allergic asthma as part of the “atopic

march".[30] Overall, the *in vivo* mouse data highlight the Th2-promoting and skin barrier disruptive effects of the secretome derived from *S. aureus*.

The critical role of IL-33 in inducing both the clinical disease and TEWL was then explored by treating the NC/Tnd mice with a neutralizing anti-IL-33 monoclonal antibody at the time of the first application of FSA. FSA-treated mice, including those given an injection of the isotype antibody developed clinical evidence of eczema and increased TEWL, while administration of the anti-IL-33 monoclonal antibody completely abrogated the disease (Fig. 7a-d). We conclude that, at least in this mouse model, IL-33 is essential for the development of *S. aureus*-induced eczema. This is in keeping with the results of recent phase 2A study of 12 adults with moderate to severe AD, which showed rapid improvement in Eczema Area and Severity Index 50 after a single injection of etokimab (an IgG1 anti-IL-33 monoclonal antibody).[31]

Sbi is the *S. aureus* Th2-promoting factor

Having demonstrated the unique Th2-promoting activity of the *S. aureus* secretome on both human and mouse skin *in vitro* and *in vivo*, as well as the essential role of IL-33 in the pathogenesis of eczema in the NC/Tnd mouse model, we proceeded to purify and characterize the specific bioactive factor. The bioactivity of FSA was retained by both a 50kDa and a 100kDa molecular weight cut-off filter (data not shown). We used a Superose® 6 size exclusion chromatography column with a separation range of 5 – 5,000 kDa to further fractionate the FSA proteins retained by the 100kDa column (Fig. 8a).

Mass spectroscopy of the most bioactive fraction revealed three potential *S. aureus* specific proteins (Fig. 8b). These proteins (DihydroLipoyl Dehydrogenase (DLD), Second Immunoglobulin-Binding Protein (Sbi), and Pyruvate Dehydrogenase Alpha-subunit (PDHa)) were expressed in an *E. coli* system and the purified products screened for Th2-promoting cytokine release by NHEK. Although not identified by mass spectroscopy, SplD, recently considered potentially important in a *S. aureus*-induced asthma mouse model,[32] was also

expressed in the *E. coli* expression system, the protein purified, and its activity assessed. The Th2-promoting bioactivity of a commercially available *S. aureus* protease, Leucine Aminopeptidase 3 (LAP-3), was also tested. Sbi was the only protein to show activity in the NHEK assays comparable with FSA (Fig. 8c). DLD, PDHa, LAP-3 and SplD showed no activity above baseline (Fig 8c). The reduced IL-33 and TSLP release after stimulation with FPLC-purified FSA Sbi fraction is in consistent with loss of protein during the purification process (relative abundance of Sbi in the FPLC-purified fraction compared with the FSA starting material was 2 versus 8 units as assessed using mass spectroscopy). To provide additional evidence that Sbi is indeed the *S. aureus* bioactive factor, *E. coli* pre- and post-transfection with the *S. aureus sbi* gene were added to NHEK. Sbi-expressing *E. coli* induced IL-33 release, while the non-transfected *E. coli* did not (Fig. 8d). Finally, we showed that two loss of function Sbi^{-} *S. aureus* mutants derived from the Newman *S. aureus* strain (Sbi^{-} and pRMC2:Sbi^{-} mutants), the latter added in tetracycline-free medium to ensure non-expression of the Sbi protein) induced little or no IL-33 or TSLP release by NHEK, either using co-culture of the live bacteria when compared with controls including a Spa mutant from the same strain (Fig. 8e), or filtered supernatants (Fig. 8f). Sbi is present in reference proteomes USA300 (methicillin resistant),[33] Newman and NCTC 8325,[34] suggesting that it is a conserved protein within *S. aureus* species.

DISCUSSION

We have identified the Second Immunoglobulin-Binding Protein as a key Th2-promoting bioactive factor of *S. aureus*, responsible for its dominant role as a trigger of cutaneous atopic disease in humans and mouse. Secretome from Sbi$^-$ deficient *S. aureus* mutants induced little or no type 2 immune activity. Co-culture of NHEK with live Sbi$^-$ mutant bacteria resulted in significantly less but detectable IL-33 activity, probably due to nonspecific cytotoxic effects particularly with more prolonged culture. Until now the precise role of *S. aureus* in AD type 2 driven immunopathology has been poorly understood. Using human explants and a mouse model of AD, we demonstrate the critical link between the *S. aureus* secretome and IL-33 release in mediating eczema, in keeping with the effectiveness of an anti-IL-33 monoclonal antibody in a recent phase 2B clinical trial.[33] We also show that the *S. aureus* secretome degrades CDSN. CDSN is a key non-covalently bound component of the stratum corneum corneodesmosome, functioning to maintain skin barrier integrity.[35] Disruption of the skin barrier allows for ingress of microbes and allergens into the epidermis leading to initiation of the type 2 host immune response. This is supported by the comparative ability of both human AD-derived and healthy keratinocytes to release IL-33 in response to the *S. aureus* secretome.

Importantly, neither the predominant skin commensal *S. epidermidis*, nor another skin pathogen Group A streptococcus contains Sbi homologs in their genome. Using Basic Local Alignment Search Tools (BLAST, UniProt.org and NCBI), Sbi was found to be unique to *Staphylococcus aureus* amongst the Bacilli genus and 100% homologous across key USA300 (methicillin resistant), Newman, and NCTC 8325 strains. Homology across other recorded *S. aureus* strains (Uniprot) ranges from 91.7-99.8%. Sbi is known to be present in 16 clinical *S. aureus* strains[36] supporting our conclusion that the bioactivity is not unique to one clinical isolate. Finally, we have excluded other virulence factor candidates such as SplD as contributing to this

bioactivity in the skin, unlike its possible role in the lung, demonstrating the importance of considering the infection niche and responding immune cells.[37]

Sbi was first discovered in 1998 and is a 436-amino acid protein with four globular domains, two of which are homologous to Protein A, allowing binding to the Fc domain of IgG, thus inhibiting neutrophil-mediated phagocytosis.[36,38] The other two domains interfere with complement activity.[39] Unlike the "first" immunoglobulin binding protein (Protein A), Sbi contains a signal peptide and is present in the secretome.[19] The Immunoglobulin binding domains of Sbi have previously been shown to interact with Ig domains of the TNFR1 receptors on murine macrophages *in vitro* and *in vivo* to induce IL-6 and TNF-α.[40] TNFR1 is expressed on human keratinocytes and *S. aureus* can increase its expression further.[41,42] However, the effect of Sbi on the skin and Th2-promoting cytokines has not previously been examined. Immunoglobulin-like domains are common to several different receptor types found on keratinocytes including members of the IL-1R superfamily. It is therefore possible that Sbi binds to a receptor on the surface of keratinocytes, through recognition of an Ig domain, inducing TSLP and IL-33 release. Alternatively, Sbi has been shown to induce epidermal growth factor receptor (EGFR) in macrophages *in vitro* and *in vivo.*[43] As activation of EGFR is known to be involved in the induction of IL-33 and TSLP transcription in keratinocytes,[43,44] Sbi induction of EGFR may be an alternative pathway for the release of IL-33 and TSLP from NHEK. Further studies are required to elucidate the exact mechanism of action of Sbi based on these possibilities.

Regarding the ability of FSA to degrade and disrupt CDSN expression as show in the immunohistology and Western blots, this is likely to be indirect effect of Sbi, as this factor contains no inherent protease activity. Endogenous keratinocyte-derived kallikreins are important mediators of skin barrier disfunction, as exemplified in Netherton syndrome.[45] We have previously shown that kallikrein-5 can be activated by changes in skin pH in NC/Tnd mice leading to disrupted skin barrier function.[20] The complex interplay and exact mechanism by

which Sbi might active these endogenous kallikreins to induce skin barrier dysfunction requires further investigation.

Over the last decade there have been huge advances in our understanding of host pathogen interactions, establishing a link between humans and microbial diversity in health-related outcomes.[46] The prime aim of this study was to address the question as to what makes *S. aureus* the undisputed master of its bacterial class in promoting atopy. Sbi is an important piece of the puzzle. In addition, this is the first time a pathogen-specific molecule has been identified to induce rapid release of IL-33, independently of cell death and TLR2 recognition. Previous studies have suggested a possible role of *S. aureus* TLR2 ligands in the induction of type 2 responses in mouse skin and human keratinocytes. In the study by Brauweiler *et al*,[47] lipoteichoic acid injected intradermally into mouse skin led to an increase in TSLP mRNA and to a lesser extent IL-33, but protein levels were not assessed. Furthermore, it is not possible to conclude if the ligand had a direct effect on keratinocytes or acted indirectly through other resident skin immune cells. Additionally, the relevance of this murine model to human skin is unclear. Vu *et al*[48] found that a synthetic diacylated lipoprotein but not PGN or Pam_3CSK_4 induced small amounts of TSLP in human keratinocytes but the authors did not assay IL-33. SplD induced IL-33 release from airway epithelial cells type II within an hour.[32] The lack of IL-33 release by skin NHEK after addition of SplD in our study suggests that molecular triggers of atopy, including *S. aureus* associated virulence factors, can be tissue-specific.

In summary, we believe that our novel results significantly advance the understanding of the etiology of AD, providing compelling evidence that a *S. aureus* protein drives IL-33 release in skin, triggering the allergic-type phenotype associated with AD. Further research is now required to determine the exact mechanism by which Sbi induces IL-33 release, and also how it degrades corneodesmosome proteins critical to skin barrier function.

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Author contributions The impetus and direction of this study were initiated by PDA and JLP, who oversaw and coordinated the experiments in their entirety. AT and HM oversaw and coordinated all mouse experiments. CO'N, along with PDA and JLP supervised the work of AAK. AAL, HW, AMA, SYT, CS and YA conducted the *in vitro* experiments. HW and CS conducted the *ex vivo* skin explant experiments. KM conducted all *in vivo* mouse experiments. HB was instrumental and provided technical advice and support for the *E. coli* cloning experiments. PDA wrote the first draft of the manuscript, which then had input from all other co-authors, who reviewed drafts and the final version of this manuscript.

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631 **Figure legends**

632 **Figure 1 *S. aureus* induces IL-33 and TSLP release by human keratinocytes *in vitro*. a.** Live
633 *S. aureus* (LiSA) (10^7 CFU/ml), but not other *staphylococcus* species, or Group A streptococci
634 (GAS) induced IL-33 and TSLP by NHEK. *S. cap: Staphylococcus capitis; S. car:*
635 *Staphylococcus carnosus; S. coh: Staphylococcus cohnii; S. hem: Staphylococcus hemolyticus; S.*
636 *len: staphylococcus lentis.* **b.** All *S. aureus* strains tested induced IL-33 and TSLP release from
637 NHEK. Cytokine release was **c.** dose- and **d.** time-dependent. **e.** IL-33 was constitutively
638 expressed in nuclei of HEKa (top panel) and released after 1h stimulation with LiSA (bottom
639 panel). Cells stained with anti-human IL-33 monoclonal antibody (red) and anti-human
640 cytokeratin 14 (green). **f.** Dot plots of viable (Annexin V^-/$DAPI^-$), early apoptotic (Annexin
641 V^+DAPI^-), late apoptotic (Annexin V^+/$DAPI^+$) and necrotic (Annexin V^-/$DAPI^+$) cells following
642 stimulation with LiSA (10^7 CFU/ml) for up to 6h. **g. & h.** LiSA cytotoxic activity after 6h
643 stimulation. Data represents three independent experiments performed in triplicate. Images taken
644 at 20X magnification. **P*<0.01, ***P*< 0.001 compared with the control. *P*-values determined by
645 one-way ANOVA with Dunnett's multiple comparisons. Mean ± standard error of the mean.
646
647 **Figure 2 *S. aureus* cell wall components have no bioactivity and its secretome induces IL-33**
648 **and TSLP release independent of TLR2. a.** Exogenous bacterial peptidoglycan (PGN) 0-
649 100µg/ml and lipoteichoic acid (LTA) 0-100µg/ml and did not induce release of IL-33 or TSLP
650 by NHEK at 6h. **b.** Anti-TLR2 antibodies (1 or 10µg/ml) did not inhibit IL-33 or TSLP release
651 induced by LiSA. **c.** Inhibiting TLR2 expression with 20mM of specific siRNA did not suppress
652 FSA-induced release of IL-33 or TSLP by NHEKs at 6h. Scr = scrambled siRNA control. Data
653 represents three independent experiments performed in triplicate. ***P*< 0.001 compared with the
654 control. *P*-values determined by one-way ANOVA with Dunnett's multiple comparisons. Mean ±
655 standard error of the mean.
656

Figure 3 Type 2-promoting bioactivity of *S. aureus* resides in the secretome. **a.** IL-33 release can be induced by NHEK when separated from the LiSA by a 0.4μm transmembrane filter. **b.** Bioactivity of FSA is destroyed by heating to 95°C, but by treatment with penicillin/streptomycin (P/S). Cytokine release (IL-33 and TSLP) was measured by ELISA. **c.** Dot plots of viable (Annexin $V^-/DAPI^-$), early apoptotic (Annexin V^+DAPI^-), late apoptotic (Annexin $V^+/DAPI^+$) and necrotic (Annexin $V^-/DAPI^+$) cells following stimulation with FSA for up to 6h. **d. & e.** Quantification of FSA cytotoxic activity determined by $DAPI^+$ and Annexin V^+ staining of cells. All data are representative of three independent experiments performed in triplicate. **P*<0.05, ***P*<0.001 compared with the control. *P*-values were determined by one-way ANOVA with Dunnett's multiple comparisons. Mean ± standard error of the mean.

Figure 4 *S. aureus* Filtered Supernatant (FSA) induces TSLP and IL-33 and disrupts corneodesmosome expression in human skin organ culture. a. & b. FSA but not filtered supernatant from *S. epidermidis* (FSE), house dust mite allergens (*D. pteronyssinus* (DP), *D. farinae* (DF) or Ragweed allergen induces TSLP and IL-33 after 6h and 12h. Three independent experiments performed in duplicate. **P*<0.05, ***P*<0.001 compared with the control. Mean ± standard error of the mean. **c.** Corneodesmosin *(*CDSN), Desmocollin-1 (DSC-1) and Desmoglein-1 (DSG-1) staining following 6h stimulation with FSA, FSE, and common aeroallergens DP, DF and ragweed. CDSN staining is disrupted following FSA treatment. Images are representative of three independent experiments performed in duplicate. Scale bars in c = 40μm. **df.** Densitometric analysis for CDSN, DSC-1 and DSG-1. Mean of three independent experiments performed in duplicate. Bar represents median and * represents significant difference compared with control. **g. & h**. Primary keratinocytes from 11 children with AD and 11 healthy controls released IL-33 and TSLP in response to LiSA but not heat-killed *S. aureus*

(HKSA) or lipopolysaccharide (LPS). Data represents three independent experiments performed in duplicate. **P<0.001 compared with the control. Bar represents median.

Figure 5 *S. aureus* Filtered Supernatant (FSA) induces eczema and skin barrier disruption in the NC/Tnd mouse *in vivo*. 80-100µl of low (10^7 CFU/ml) and high concentration (10^8 CFU/ml) FSA, FSE, or *D.* farinae (DF) with concomitant 4% SDS were applied topically to the backs of 8-week old NC/Tnd mice each day for four weeks. Mice were housed in specific pathogen free conditions. **a.** Representative images of NC/Tnd mice after application of 4% SDS, or 4% SDS and FSE, DF or FSA had been applied to the back daily for four weeks. **b.** Clinical eczema scores. **c.** Scratching behaviour and **d.** Trans-Epidermal Water Loss (TEWL). All data show results of six individual mice/group. **P<0.01. Mean ± standard error of the mean.

Figure 6 Filtered supernatant from *S. aureus* but not *S. epidermidis* increases plasma IL-33 and TSLP, particularly in NC/Tnd mice and disrupts epidermal corneodesomosme expression. a. Histological sections from eczema-prone NC/Tnd mice. 4% SDS, or 4% SDS and DF, FSE or FSA had been applied daily for four weeks. Images are representative of six individual mice/group performed in duplicate. **b-d.** Densitometric analysis. *P<0.01, **P<0.0001 compared with the SDS control. Bar represents median. **e.** Western blots of CDSN, DSC-1 and DSG-1 native protein and degraded fragments from NC/Tnd mouse skin. Densitometric analysis of Western blots for CDSN **(f)** DSC-1 **(g)** and DSG-1 **(h)**. Data representative of six individual mice/group. *P<0.05, **P<0.001 compared with the naïve control. **i & j.** Plasma IL-33 and TSLP concentrations in NC/Tnd and MSM/Ms. SDS: sodium dodecyl sulphate, FSE: filtered supernatant from *S. epidermidis,* DF: *D. farinae,* FSA: filtered supernatant from *S. aureus.* Data are representative of six mice/group. **P<0.001. Mean ± standard error of the mean.

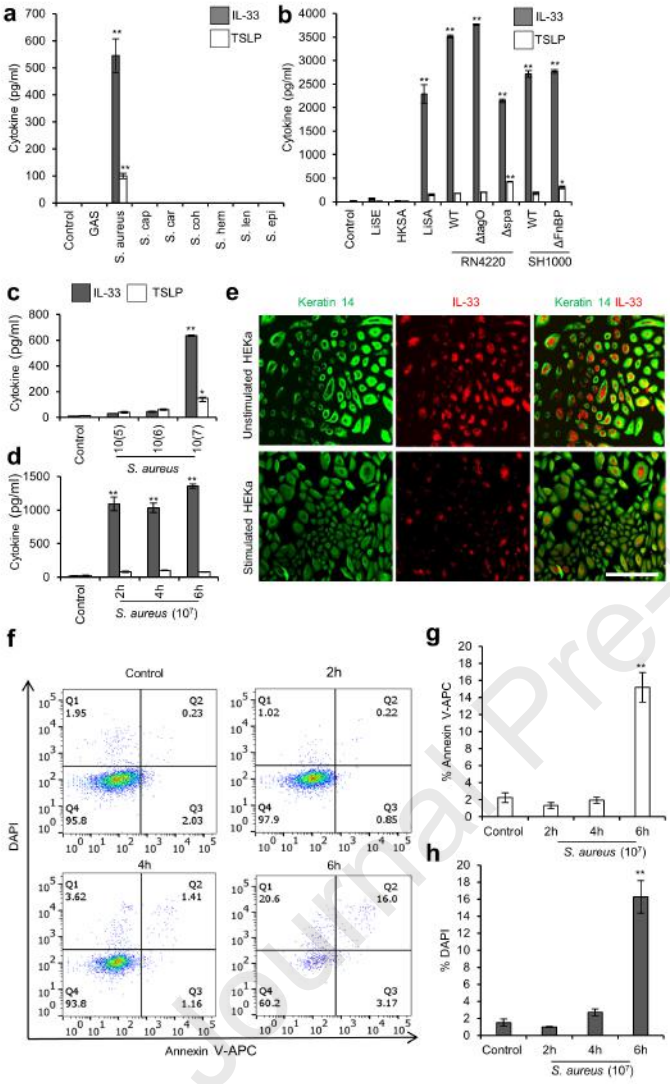
706

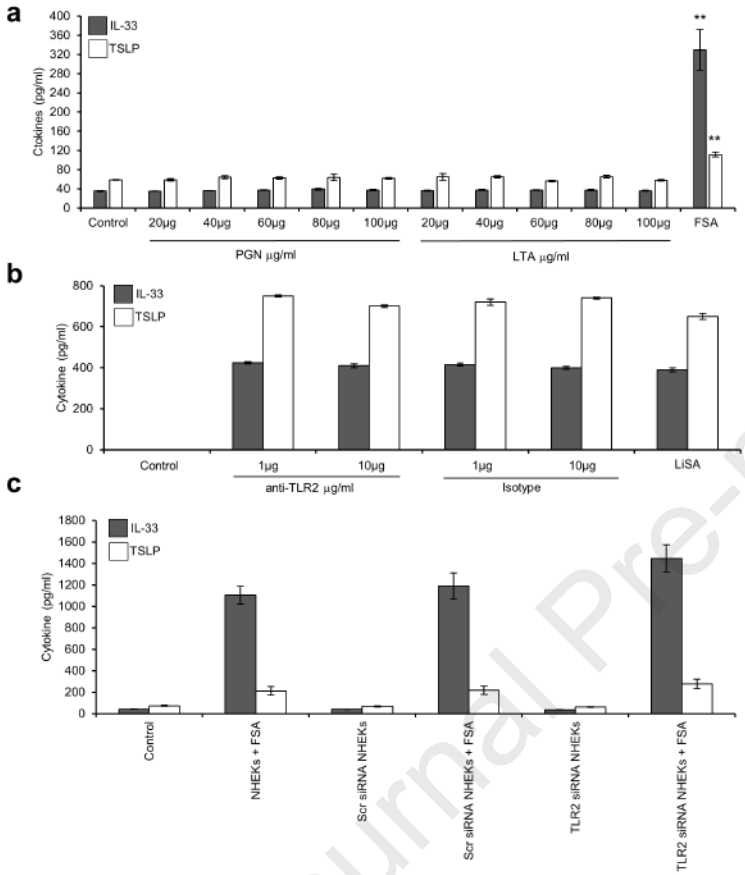
707 **Figure 7 FSA-induced eczema in the NC/Tnd mouse can be completely abrogated by**
708 **neutralizing anti-IL-33 antibodies.** 80-100µl of high (10^8 CFU/ml) concentration FSA with
709 concomitant 4% SDS were applied topically to the backs of 8-week old NC/Tnd mice each day
710 for four weeks. One group also received an intraperitoneal 10µg injection of anti-IL-33
711 monoclonal antibody with the first application of FSA, while a second group received an
712 injection of an isotype control antibody. **a.** Representative images of NC/Tnd mice after
713 application of 4% SDS and FSA, FSA daily for four weeks, with or without isotype or anti-IL-33
714 mAb. **b.** Clinical eczema scores, **c.** scratching frequency and **d.** Trans-Epidermal Water Loss
715 (TEWL) of naïve mice, and mice treated with 4% SDS and FSA, isotype control or anti-IL-33
716 antibody. All data show results of six individual mice/group. $^{**}P<0.01$ compared with pre-
717 treatment or the naïve group. Mean ± standard error of mean.

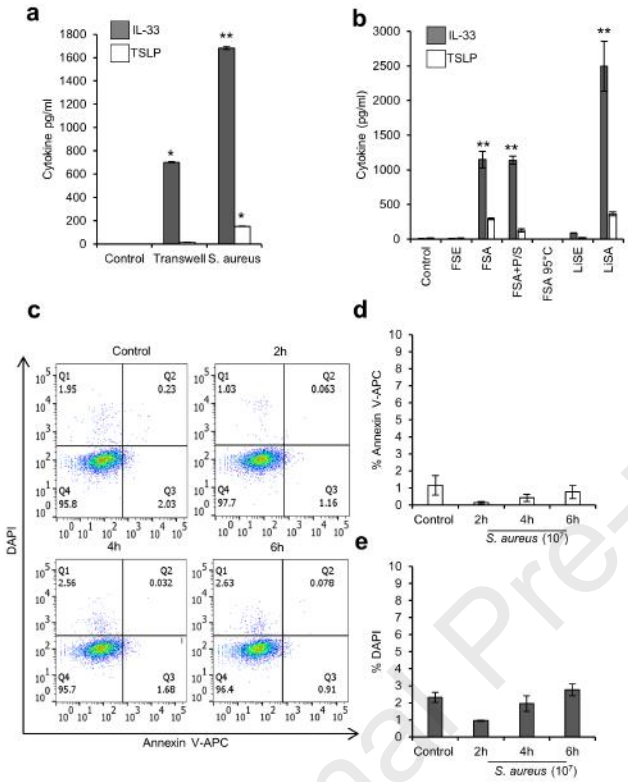
718

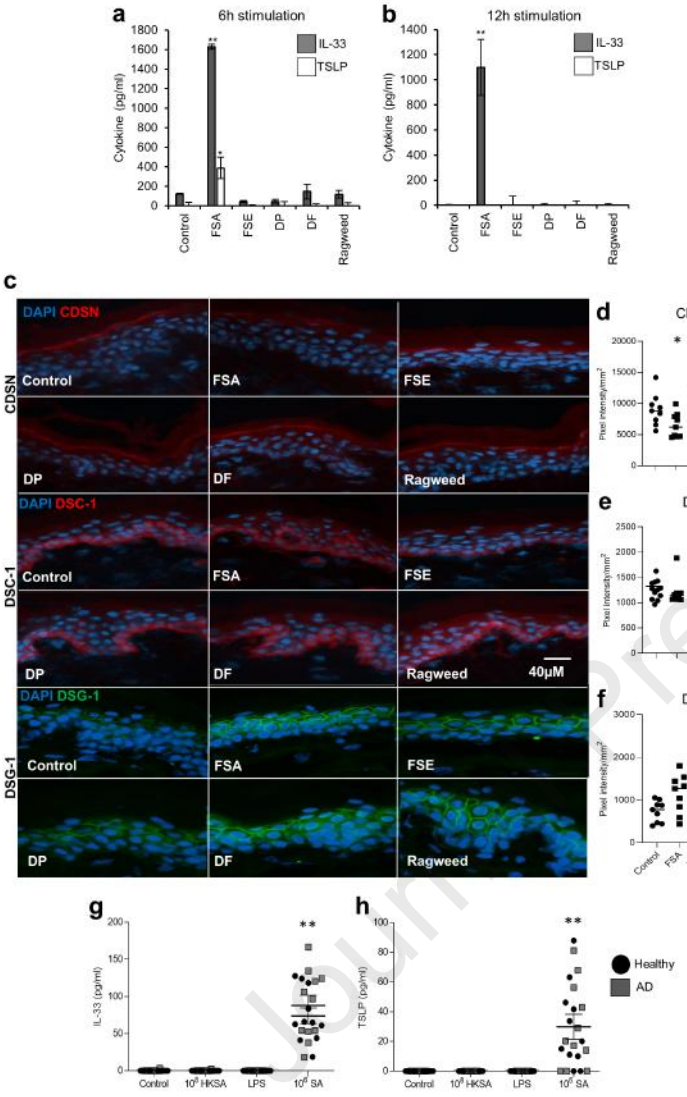
719 **Figure 8 Fractionation of *S. aureus* secretome (FSA) Th2-promoting activity by FPLC and**
720 **identification of the active factor as *S. aureus* Sbi.** **a.** Bioactivity of FSA fractionated using
721 Superose® 6 FPLC of 100kDa retention fraction (insert shows absorbance (280nm); main panel
722 shows IL-33 release by NHEK after addition of specific FPLC fractions). **b.** *S. aureus* proteins
723 identified from bioactive Superose® 6 FPLC fractions by mass spectroscopy. **c.** IL-33 and TSLP
724 release by NHEK after addition of *E. coli* vector-derived DLD, Sbi, PDHa, SplD, or exogenous
725 LAP-3 for 6h. **d.** *E. coli* expressing Sbi, but not untransfected *E. coli* induced IL-33 release by
726 NHEK. **e.** LiSA, and **f.** FSA from Sbi- and pRMC2:Sbi-deficient strains induce less IL-33 and
727 TSLP than parental Newton wild-type *S. aureus*. **e.** Spa-deficient LiSA induces the same IL-33
728 and TSLP as the Newton wide-type LiSA. All data are representative of two to three independent
729 experiments performed in duplicate or triplicate. $^{**}P < 0.0001$ compared with control. Mean ±

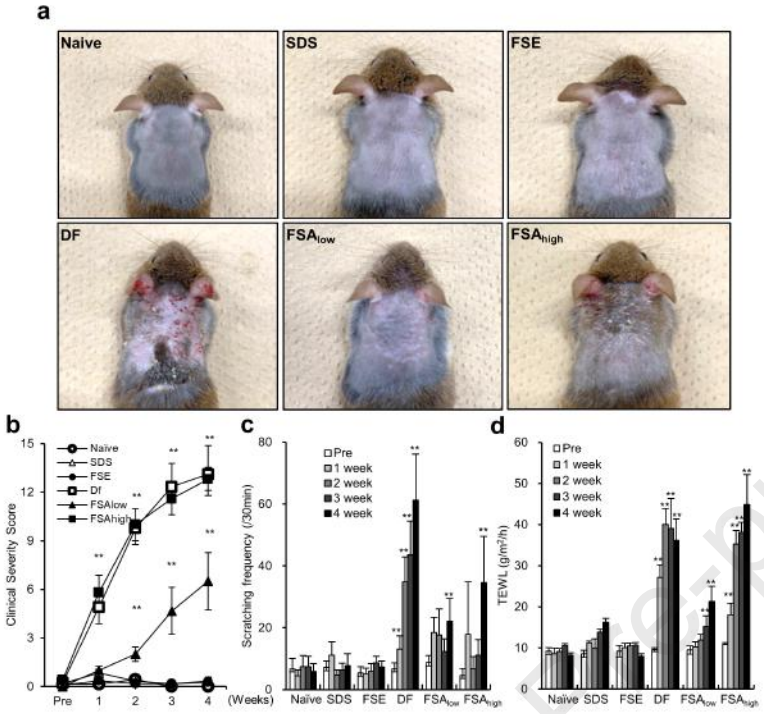
730 standard error of mean. DLD: dihydrolipoyl dehydrogenase, LiSA: live *S. aureus*, Sbi: Second
731 immunoglobulin-binding protein, and PDHa: pyruvate dehydrogenase alpha-subunit.

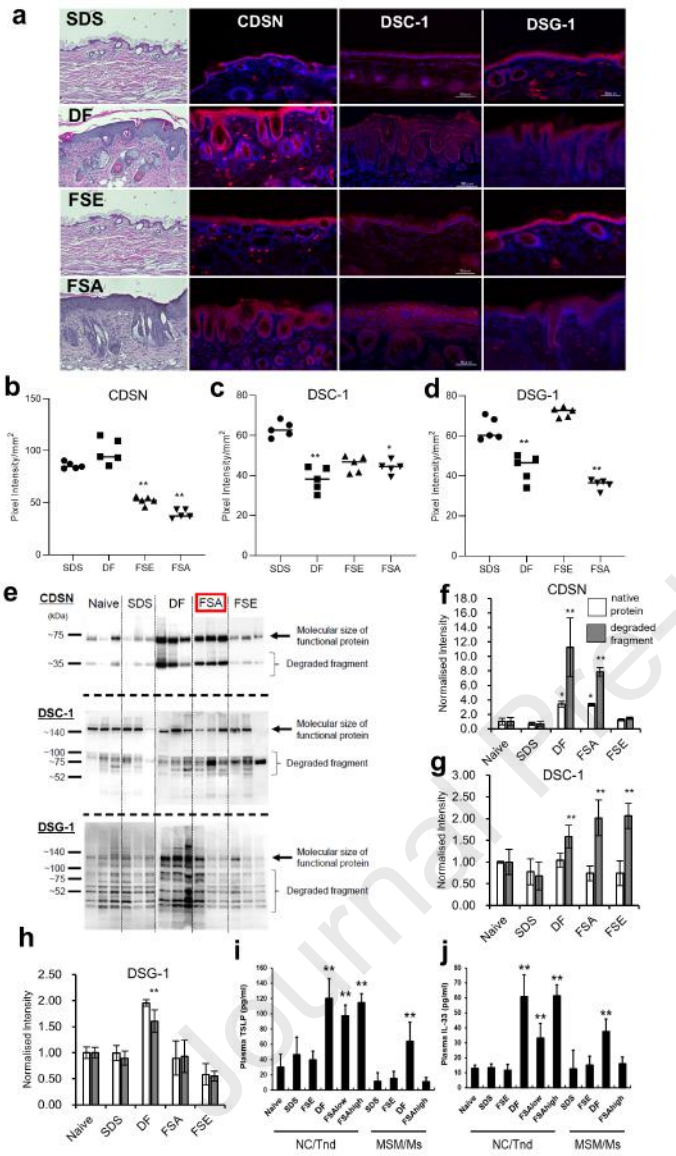


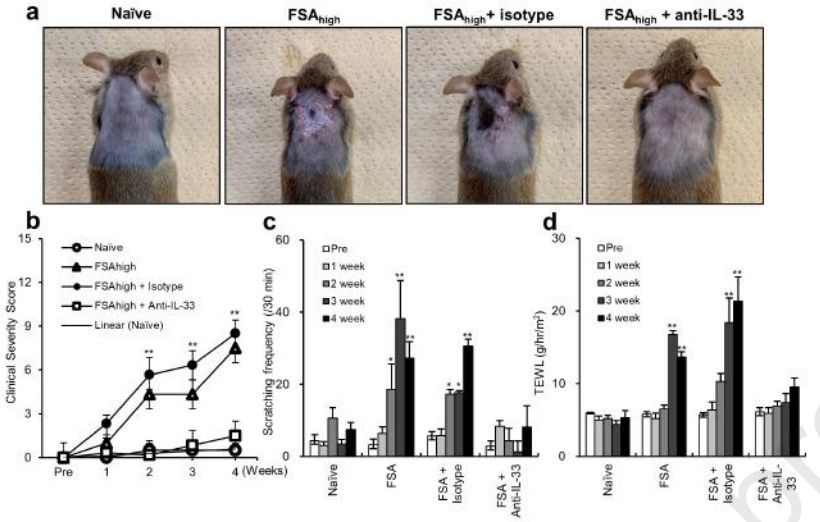


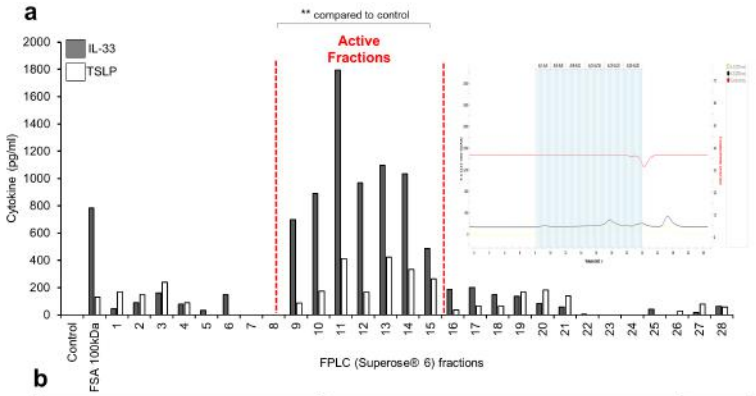






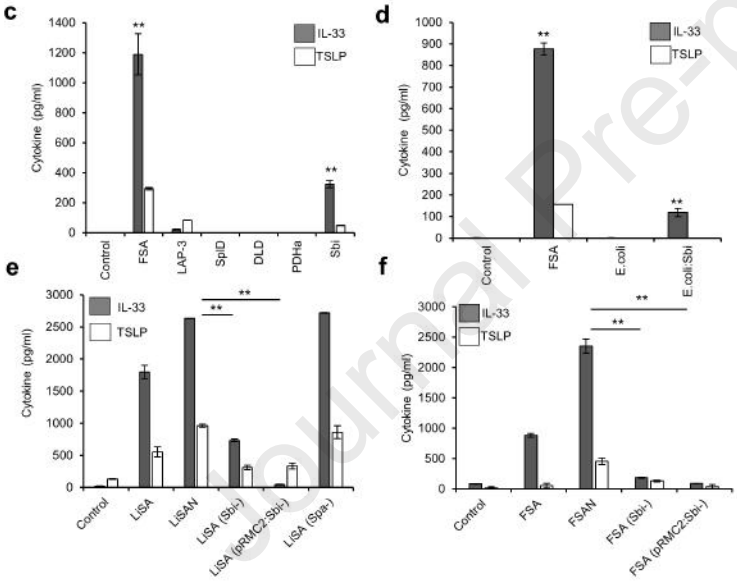






b

Protein	Function	kDa
Dihydrolipoyl dehydrogenase (DLD)	moonlight protein has serine protease activity	54
S. aureus second IgG-binding protein (Sbi)	immunoglobulin binding protein	50
Pyruvate dehydrogenase (PDHa)	catalytic conversion of pyruvate to acetyl-CoA + CO2	41



SUPPLEMENTARY ONLINE INFORMATION

Staphylococcus aureus Second Immunoglobulin-Binding Protein drives atopic dermatitis via IL-33

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Candidate gene cloning and expression

Sequences of *S. aureus* DLD (gene; pdhD), PDHa (gene; pdhA) and Sbi (gene; sbi) were acquired from KEGG (https://www.genome.jp/kegg/). GST-tagged proteins *pdhD*, *pdhA* and *sbi* genes were cloned into a pGEX plasmid using BamHI and EcoRI restriction enzymes, then transformed into NEB® 5-alpha competent *E. coli* cells (New England BioLabs, UK) following manufacturer's instructions. Once cloning was verified, *pdhD*, *pdhA* and *sbi* were expressed in BL21 (DE3) *E-coli* cells (New England BioLabs, UK) for 3h at 37°C or in Arctic Express *E. coli* (Agilent Technologies, USA) for 24h at 12°C. After expression, cell cultures were centrifuged at 3,500g for 20min at 4°C. Supernatant was discarded and cell pellets were frozen in liquid nitrogen and then at −80°C until used for protein purification by Glutathione Sepharose 4B beads.

Cloning and expression of SplD

Full length SplD was amplified from the clinical isolate of *S. aureus* and cloned into an ampicillin-resistant pQE30 vector. The protein was expressed in chemically competent *E. coli* cells BL21 grown at 37°C in TB (Sigma-Aldrich) containing 100µg/ml ampicillin, purified using a HiTrap column (GE Healthcare) and eluted with imidazole.

Table SI List of materials used in this study

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
S. aureus (LiSA)	A. McBain, University of Manchester, UK	MBRG 16.1
S. aureus RN4220 WT	G. Xia, University of Manchester, UK	N/A
S. aureus RN4220;ΔtagO	G. Xia, University of Manchester, UK	N/A
S. aureus RN4220;Δspa	G. Xia, University of Manchester, UK	N/A
S. aureus SH1000 and its isogenic fnbA fnbB mutant	Joan Geoghegan, University of Dublin, Ireland	8325-4
S. aureus Sbi mutant	Joan Geoghegan, University of Dublin, Ireland	**N/A**
S. aureus pRMC2::Sbi mutant	Joan Geoghegan, University of Dublin, Ireland	**N/A**
S. capitis	G. Xia, University of Manchester, UK	ATCC 27840
S. carnosus	G. Xia, University of Manchester, UK	TM300
S. cohnii	G. Xia, University of Manchester, UK	ATCC 29974
S. hemolyticus	G. Xia, University of Manchester, UK	JCSC 1435
S. lentus	G. Xia, University of Manchester, UK	3472
S. epidermidis	G. Xia, University of Manchester, UK	1457
Streptococcus pyogenes (GAS)	A. McBain, University of Manchester, UK	NCTC 12696
E. coli DH5α	Professor Ian Roberts, University of Manchester, UK	N/A
E. coli NEB® 5-alpha	New England BioLabs, UK	C2987H
E. coli BL21(DE3)	New England BioLabs, UK	C2527H
E. coli (DE3) Arctic Express	Agilent Technologies, USA	230192
Human keratinocytes culture		
Primary Normal Human Epidermal Keratinocytes (NHEK)	PromoCell, Heidelberg, Germany	C-12002
DetachKit2 Trypsin, Trypsin neutralizing solution	PromoCell, Heidelberg, Germany	C-41212
Human Epidermal Keratinocytes, (HEKa)	ThermoFisher Scientific, UK	C-0055C

Antibodies and fluorescent labelling		
Anti-Human Corneodesmosin (CDSN)	Invitrogen, UK	PA562936
Anti-Human Desmocollin-1 (DSC-1)	Invitrogen, UK	PA550651
Anti-Human Desmoglein-1 (DSG-1)	Invitrogen, UK	326000
Texas red goat anti-rabbit antibody	Life Technologies, USA	T-2767
Anti-Mouse Corneodesmosin (CDSN)	Santa Cruz	sc-514845
Anti-Mouse Desmoglein-1 (DSG-1)	Santa Cruz	sc-23910
Anti-Mouse Desmocollin-1 (DSC-1)	R&D systems	MAB7367
Anti-Mouse IL-33	R&D systems	AF3626
Plasmids		
pGEM-T easy vector	Promega, UK	A3600
pGEM-T;*SplD*	This study	-
pQE30	Qiagen, Crawley, UK	-
pQE30;*SplD*	This study	
pGEX-6P-1	Sigma Aldrich, UK	GE28-9546-48
pGEX;*pdhA*	This study	-
pGEX;*pdhD*	This study	-
pGEX;*Sbi*	This study	-

Table SII List of Primer sequences used in this study

Oligonucleotide	Sequence (5'-3')	Purpose
SplD BamH1 F	CCTGTAGGATCCATGAATAAAAATATAATCATCAAAAGTATTGCGG	Used to amplify *SplD* from Clin1-SA for cloning using BamHI-EcoRI sites of pGEM-T
SplD HindIII R	GCGCGATAAGCTTTTATTATTTATCTAAATTATCTGCAATAAATTTCTTAAT	
M13 F	CGCCAGGGTTTTCCCAGTCACGAC	Used to sequence *SplD* insert in pGEM-T vector
M13 R	AGCGGATAACAATTTCACACAGGA	
PQE30 F	AAGTGCCACCTGACGTCTAAG	Used to sequence *SplD* insert in pQE30 vector
PQE30 R	GGAGTTCTGAGGTCATTACTG	
pGEX F	GGGCTGGCAAGCCACGTTTGGTG	Used to screen for inserts *(pdA, pdhD and Sbi)* cloned in pGEX
pGEX R	CCGGGAGCTGCATGTGTGTCAGAGG	