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

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citing this paper
Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

1   *Staphylococcus aureus* **Second Immunoglobulin-Binding Protein drives atopic**

2   **dermatitis via IL-33**

3   **Arwa Al Kindi, PhD,[1†] Helen Williams, PhD,[1†] Kenshiro Matsuda, PhD,[2] Abdullah M.**

4   **Alkahtani, PhD,[3] Charis Saville, PhD,[1] Hayley Bennett, PhD,[4] Yasmine Alshammari, MSc,[1]**

5   **Soo Y. Tan, PhD,[5] Catherine O'Neill, PhD,[6] Akane Tanaka, DVM, PhD,[7] Hiroshi Matsuda,**

6   **DVM, PhD,[8] Peter D. Arkwright, MD, PhD,[1*] Joanne L. Pennock, PhD[1*]**

7   *[1]Lydia Becker Institute of Immunology and Inflammation, University of Manchester, United*

8   *Kingdom, [2]Center for Innovative Drug Discovery, University of Tsukuba, Japan, [3]Department of*

9   *Medicine, Microbiology and Parasitology, King Khalid University, Abha, Saudi Arabia,*

10   *[4]Genome Editing Unit, University of Manchester, United Kingdom, [5]National University Health*

11   *System, Singapore, [6]Division of Dermatological and Musculoskeletal Sciences, University of*

12   *Manchester, United Kingdom. [7]Laboratories of Veterinary Molecular Pathology & Therapeutics*

13   *and [8]Comparative Animal Medicine, Tokyo University of Agriculture & Technology, Tokyo,*

14   *Japan*

15   [†,*]authors contributed equally to this work.

16   **Corresponding author:** Dr P D Arkwright, Lydia Becker Institute of Immunology &

17   Inflammation, University of Manchester, Room 2.21, Core Technology Facility, 46 Grafton St.,

18   Manchester, M13 9NT, United Kingdom. Telephone +44 161 306 3771, email

19   peter.arkwright@manchester.ac.uk

27

28 **Conflict of interests** None of the authors declare any competing financial and/or non-financial

29 interests in relation to the work described.

30 **Word count (excluding Abstract, Figures/Table legends): 4,764**

31 **Abstract**

32 **Background:** *S. aureus* **is the dominant infective trigger of atopic dermatitis (AD). How this**

33 **bacterium drives type 2 allergic pathology in the absence of infection in AD patients is**

34 **unclear.**

35 **Objective: To identify the** *S. aureus***-derived virulence factor(s) that initiates the cutaneous**

36 **type 2-promoting immune response responsible for AD.**

37 **Methods:** *In vitro* **human keratinocyte cell culture,** *ex vivo* **human skin organ explants and**

38 **the eczema prone Nc/Tnd mouse were used as model systems to assess type-2 promoting**

39 **immune responses to** *S. aureus***. Identification of the bioactive factor was accomplished**

40 **using Fast Protein Liquid Chromatography and mass spectrometry. Bioactivity was**

41 **confirmed by cloning and expression in an** *E. coli* **vector system, and** *S. aureus* **Sbi mutant**

42 **strains confirming loss of activity.**

43 **Results:** *S. aureus* **was unique amongst staphylococcal species in its ability to induce the**

44 **rapid release of constitutive IL-33 from human keratinocytes independent of the toll-like**

45 **receptor pathway. Using the eczema-prone NC/Tnd mouse model, we showed that IL-33**

46 **was essential in inducing the immune response to** *S. aureus in vivo***. By fractionation and**

47 **candidate testing, we identified the Second Immunoglobulin-Binding Protein (Sbi) as the**

48 **predominant staphylococcus-derived virulence factor that directly drives IL-33 release**

49 **from human keratinocytes. Immunohistology of skin demonstrated that corneodesmosin, a**

50 **component of corneodesmosomes that form key intercellular adhesive structures in the**

51 **stratum corneum, was disrupted resulting in reduction of skin barrier function.**

52 **Conclusion:** *S. aureus***-derived Sbi is a unique type 2-promoting virulence factor capable of**

53 **initiating the type-2 promoting cytokine activity underlying AD.**

54

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$_3$CSK$_4$: 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.

83   *Staphylococcus aureus* (*S. aureus*) is the dominant pathogen of human skin, causing the

84   majority of skin and soft tissue infections worldwide.[1-3] It is also the most frequent cause of

85   infection-induced flares of atopic dermatitis (AD).[4-6] The bacterium expresses many virulence

86   factors both in its cell wall and secretome.[7-9] Previous studies have focused on recognition of *S.*

87   *aureus* by host innate immunity, particularly activation of the inflammasome through toll-like

88   receptors (TLR) and subsequent induction of cytokines such as IL-1β.[10] Enzymes such as

89   coagulase, toxins such as enterotoxins, Toxic Shock Syndrome Toxin-1, α-hemolysin, and invasins

90   including pore-forming proteins such as Panton-Valentine Leukocidin induce host cytotoxicity and

91   damage the skin barrier. Defects in skin barrier function, particularly lack of filaggrin expression

92   in the epidermis secondary to pathologic variants in the *FLG* gene, are well recognized to

93   contribute to the pathogenesis of AD.[11] However, patients with *FLG* variants may suffer from

94   ichthyosis without skin inflammation.[12] Thus although skin barrier disruption can predispose

95   patients to AD, the main driving force in AD is the Th2 immune response, as clearly illustrated by

96   the effectiveness of the IL-4/13 receptor antagonist dupilumab in recent clinical trials.[13,14] Delta-

97   toxins and superantigens produced by *S. aureus* can induce mast cell activation and IgE production,

98   but the bacterial trigger of type-2 allergic responses by keratinocytes in the skin is unknown.[15,16]

99   The current paradigm is that rather than a single factor, a complex array of *S. aureus*

100   virulence factors contribute to the atopic skin response.[4,7] However, the study of immunodeficiency

101   and immune dysregulation disorders have taught us that there is much redundancy in host

102   immunity.[17,18] The hypothesis we set out to explore was whether *S. aureus* expresses a predominant

103   virulence factor that initiates type 2-promoting cytokine release from skin cells and drives the

104   development of AD. If this critical factor could be found, then targeting it therapeutically could

105   negate one of the key advantages *S. aureus* has developed during its evolution and symbiosis with

106   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)

157   dermal side down. Intact epidermal exposed biopsies were treated with 5μl /biopsy of FSA, FSE

158   or allergens including Ragweed, DF, and DP for up to 12h (37°C, 5% CO_2). Following treatments,

159   supernatant was removed and stored at −80°C, and biopsies were snap-frozen in liquid nitrogen

160   and stored at −80°C until use, where they were then embedded in Optimal Cutting Temperature

161   Compound (KP Cryo-Compound) before cryo-sectioning.

162

163   **Human IL-33 and TSLP ELISA**

164   R&D System ELISA (Abingdon, UK) were used to measure IL-33 (DY3625-05) and TSLP

165   (DY1398-05) release according to the manufacturer's instructions.

166

167   **Detection of cell death by Annexin-V and DAPI staining**

168   Cells were detached from 24-well plates, harvested into micro-centrifuge tubes, washed with

169   Annexin-V binding buffer and centrifuged (5min, 500*g*, 4°C), then stained with Annexin-V diluted

170   1:100 in binding buffer for 30min on ice. Following incubation, cells were harvested and re-

171   suspended in Annexin-V binding buffer. DAPI was added just before reading the sample. A total

172   of 10,000 events were acquired on BD LSRFortessa X20 (BD biosciences). Data were analyzed

173   using FlowJo (Treestar[©] V10).

174

175   **Histology and Immunohistochemistry**

176   10^5 HEKa seeded in 24-well culture plates containing sterile coverslips were incubated at 37°C

177   until 100% confluent. They were then co-cultured with LiSA for 1h, fixed and permeabilized with

178   1% triton-X for 15min and stained with mouse anti-human IL-33 monoclonal antibody (Nessy-1

179   ALX-804-840PF, 1:200 dilution, Enzo Life Sciences, Exeter, UK) and rabbit anti-human

180   cytokeratin 14 polyclonal antibody (PA5-1672, 1:200 dilution, Thermo Fisher Scientific). Cultures

181   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

207 Filtered supernatant from S. *aureus* (FSA) fractionated by size exclusion centrifugal filter columns

208 (Amicon Ultra 15, 100kDa or 50kDa cut-off membranes) and centrifuged for 15min at 3,000g

209 (4°C). 200ml of FSA was fractionated using 100kDa Amicon Ultra 15 as described above. Retained

210 fractions were then pooled and then fractionated further using Superose® 6 Fast Protein Liquid

211 Chromatography (FPLC) column (5 - 5000 kDa). 48 fractions of 0.5ml were collected and stored

212 at −20°C until required.

213

214 **Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis**

215 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse

216 the molecular weights of FSA proteins using standard methodology. Gels were then stained with

217 Instant Blue Coomassie-based gel stain or silver stain using standard protocols.

218

219 **Mass spectrometry**

220 Mass spectrometry was used to identify proteins in the bioactive peak derived from the FPLC size

221 fractionation. In-gel digestion and tandem mass spectrometry analysis was performed by the

222 Protein Mass Spectrometry Core Facility, Faculty of Biology, Medicine and Health, University of

223 Manchester. The data was generated against known protein sequences in Swissprot TrEMBL

224 database using Firmicutes phylum (which includes *S. aureus*) as the most optimal taxonomic level

225 available in the database.

226

227 **Candidate gene cloning and expression**

228 Details of cloning and expression of candidate genes using the *E. coli* vector system are provided

229 in the Methods section of this article's Online Repository.

230

231 **TLR2 Knockdown/Small interfering RNA (siRNA)**

232 TLR2-specific siRNA (human Hs_TLR2_1 Flexitube siRNA, SI00050015) and a scramble (scr)

233 non-silencing control siRNA (AllStars negative control, 1027281) were purchased from Qiagen.

234 NHEK were seeded at a density of 1×10^4 one day prior to transfection. After 24h, cells were

235 transfected with 5nm siRNA using 2.5µl of TransIT-TKO transfection reagent (Mirus Bio, UK).

236 Experiments were performed on transfected cells at least two days after siRNA treatments.

237 Knockdown efficiency of TLR2 siRNA mediated gene expression was confirmed by Real-time

238 PCR.

239

Animals

241 NC/Tnd mice were generated in the laboratory of comparative animal medicine in Tokyo

242 University of Agriculture and Technology. MSM/Ms mice were purchased from RIKEN

243 Bioresource as wild-type (WT) controls. All animals were kept in a clear acrylic cage and had free

244 access to standard chow and water. They were kept in specific-pathogen free housing to prevent

245 the natural development of eczema that occurs if these mice are housed in conventional housing.

246 Temperature and humidity of the animal room were $22\pm4°C$ and $40\pm15\%$, respectively. The animal

247 room was maintained on a 12:12-hour light-dark cycle. All animal experiments complied with the

248 guidelines of University Animal Care and Use Committee of the Tokyo University of Agriculture

249 and Technology, as well as with the guidelines of Science Council of Japan for the use of laboratory

250 animals.

251

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

253 100µl of filtered supernatant stimulated with *S. aureus* (FSA) (10^7 CFU/ml low dose or 10^8

254 CFU/ml high does) or *S. epidermidis* (FSE, 10^8CFU/ml) was applied to the back skin of the mice

255 once daily for four weeks. According to the manufacturer's instruction, 100mg of DF ointment

256 was applied to the back skin of the mice. Application was performed twice/week for 4 weeks. Skin

257 barrier disruption was performed by 4% SDS before the application of FSA, FSE and DF.

258

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 $22 \pm 0.5°C$ and $50\% \pm 10\%$ 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).

Statistical analysis

283   All *in vitro* and *ex vivo* experiments were carried out with a minimum of three technical and two-

284   three biological replicates as detailed in the figure legends. *In vivo* experiments were performed

285   twice with six mice per group. One-way ANOVA was used for single factor data with 3 or more

286   groups followed by comparisons to a control group using Dunnett's post-hoc test. However, if

287   normality could not be assumed using the QQ residual plot, statistical comparisons between groups

288   was determined using the Kruskal Wallis test with Dunn's multiple comparisons and adjusted P

289   values reported. Single factor data with less than three groups were analysed using the unpaired t-

290   test and the two-tailed P value reported. All analysis was performed using GraphPad Prism 8

291   Version 8.4.2 (GraphPad Software, Inc. CA, USA). A probability value of less than 0.05 was

292   considered statistically significant. Flow cytometry data were analysed using FlowJo (Treestar

293   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

320   immunopathology.[24] Neither exogenous PGN nor LTA induced IL-33 or TSLP release (Fig. 2a, b).

321   Anti-TLR2 blocking antibodies (Fig. 2b) and siRNA knockdown of TLR2 in keratinocytes (Fig.

322   2c) did not suppress either IL-33 or TSLP release by FSA.

323   In contrast, experiments where LiSA and NHEK were separated by a 0.4μm pore

324   membrane demonstrated that the type-2 immune promoting bioactivity resided within the bacterial

325   secretome (FSA) (Fig. 3a). The FSA bioactivity was heat-labile, but not affected by addition of

326   antibiotics to remove any contaminating LiSA (Fig. 3b). Interestingly, in contrast to LiSA, co-

327   culture of FSA with NHEK did not induce either DAPI uptake or Annexin V expression, indicating

328   that the bioactivity was independent of cell death (Fig. 3c-e).

329

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

331   We then explored whether the secretome-derived factor was bioactive in *ex vivo* human skin organ

332   culture and in an *in vivo* mouse eczema model. Using human skin organ explants from nonatopic

333   adults undergoing abdominal skin excision for treatment of obesity, we demonstrated that sterile

334   filtered supernatant from *S. aureus* (FSA), but not *S. epidermidis* (FSE), common house dust mite

335   (DP & DF) or pollen aeroallergens (Ragweed) stimulated release of both IL-33 and TSLP (Fig. 4a-

336   b). Examination of corneodesmosome expression from these organ explants suggested that whilst

337   FSA was associated with a more diffuse expression of DSC-1 and DSG-1, it seemed to disrupt

338   CDSN expression when compared with the control and this was confirmed by densitometry (Fig

339   4c, 4d). CDSN is the only corneodesmosome protein component not covalently linked to the cell

340   membrane.[25]

341   There has been an ongoing debate as to whether the type-2 immune response in patients

342   with AD is inherently different to that of non-atopic individuals. LiSA induced release of similar

343   quantities of both IL-33 and TSLP by primary keratinocytes derived from skin punch biopsies

344   taken from children with moderate-to-severe AD and non-atopic controls, indicating that the Th2-

345   promoting potential of epidermal keratinocytes is not significantly different in these two groups

346   (Fig. 4g-h). This suggested that exposure to *S. aureus*-derived bioactive factor(s) through a

347   disrupted skin barrier, rather than an inherent immune hyperactivity, is the important driver of

348   disease.[26]

349   The NC/Tnd Japanese fancy bred mouse is an excellent animal model of human AD, in that

350   pups from this inbred strain in conventional, but not in specific-pathogen free housing, develop

351   eczema soon after weaning at 6 − 8-weeks old. The immunohistochemistry mirrors that of human

352   AD and the disease responds to topical corticosteroids and tacrolimus ointment.[27,28] Eczema in this

353   mouse model is aggravated by *S. aureus* as topical antibiotics suppress clinical disease and Th2

354   response.[29] Using this mouse model in specific pathogen free housing, we demonstrate that daily

355   application of FSA and house dust mite allergen (DF), but not FSE for four weeks to SDS primed

356   skin induces a dose-dependent flare in clinical eczema (Fig. 5a-b) associated with increased

357   scratching behaviour (Fig. 5c) and disruption in skin barrier function measured by TEWL (Fig.

358   5d).

359   Associated with the clinical eczema flare, skin histology showed marked hyperkeratosis of

360   the epidermis where FSA (or house dust mite allergen) had been applied (Fig. 6a). As with the

361   human explant model, expression of corneodesmosome proteins (particularly corneodesmosin) in

362   the stratum corneum on immunofluorescence appeared to be disrupted (Fig. 6a). Densitometry

363   showed that FSA reduced intensity of expression of CDSN, DSC-1 and CSG-1 compared with the

364   control, with more variable reduction after application of DF and FSE (Fig. 6b-d). Western blot

365   analysis and densitometry from mouse skin illustrated that CDSN was significantly degraded by

366   FSA and DF compared with the control and FSE (Fig. 6e, 6f). There was evidence of degradation

367   of DSC-1 by FSA, DF and FSE (Fig. 6e, 6g), but DF and not FSA degraded DSG-1 (Fig 6e, 6h).

368   In addition to the local cutaneous effects, application of FSA to the skin of these NC/Tnd mice led

369   to significant increases in plasma concentrations of TSLP (Fig. 6i) and IL-33 (Fig 6j). This is in

370   keeping with the known association between AD, circulating IL-33 and TSLP and other non-

371   cutaneous allergies such as food allergies and allergic asthma as part of the "atopic march".[30]

17

372 Overall, the *in vivo* mouse data highlight the Th2-promoting and skin barrier disruptive effects of

373 the secretome derived from *S. aureus*.

374   The critical role of IL-33 in inducing both the clinical disease and TEWL was then explored

375 by treating the NC/Tnd mice with a neutralizing anti-IL-33 monoclonal antibody at the time of the

376 first application of FSA. FSA-treated mice, including those given an injection of the isotype

377 antibody developed clinical evidence of eczema and increased TEWL, while administration of the

378 anti-IL-33 monoclonal antibody completely abrogated the disease (Fig. 7a-d). We conclude that,

379 at least in this mouse model, IL-33 is essential for the development of *S. aureus*-induced eczema.

380 This is in keeping with the results of recent phase 2A study of 12 adults with moderate to severe

381 AD, which showed rapid improvement in Eczema Area and Severity Index 50 after a single

382 injection of etokimab (an IgG1 anti-IL-33 monoclonal antibody).[31]

383

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

385 Having demonstrated the unique Th2-promoting activity of the *S. aureus* secretome on both human

386 and mouse skin *in vitro* and *in vivo*, as well as the essential role of IL-33 in the pathogenesis of

387 eczema in the NC/Tnd mouse model, we proceeded to purify and characterize the specific bioactive

388 factor. The bioactivity of FSA was retained by both a 50kDa and a 100kDa molecular weight cut-

389 off filter (data not shown). We used a Superose® 6 size exclusion chromatography column with a

390 separation range of 5 − 5,000 kDa to further fractionate the FSA proteins retained by the 100kDa

391 column (Fig. 8a).

392   Mass spectroscopy of the most bioactive fraction revealed three potential *S. aureus* specific

393 proteins (Fig. 8b). These proteins (DihydroLipoyl Dehydrogenase (DLD), Second

394 Immunoglobulin-Binding Protein (Sbi), and Pyruvate Dehydrogenase Alpha-subunit (PDHa))

395 were expressed in an *E. coli* system and the purified products screened for Th2-promoting cytokine

396 release by NHEK. Although not identified by mass spectroscopy, SplD, recently considered

397 potentially important in a *S. aureus*-induced asthma mouse model,[32] was also expressed in the *E.*

18

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

439   in the skin, unlike its possible role in the lung, demonstrating the importance of considering the

440   infection niche and responding immune cells.[37]

441   Sbi was first discovered in 1998 and is a 436-amino acid protein with four globular

442   domains, two of which are homologous to Protein A, allowing binding to the Fc domain of IgG,

443   thus inhibiting neutrophil-mediated phagocytosis.[36,38] The other two domains interfere with

444   complement activity.[39] Unlike the "first" immunoglobulin binding protein (Protein A), Sbi contains

445   a signal peptide and is present in the secretome.[19] The Immunoglobulin binding domains of Sbi

446   have previously been shown to interact with Ig domains of the TNFR1 receptors on murine

447   macrophages *in vitro* and *in vivo* to induce IL-6 and TNF-α.[40] TNFR1 is expressed on human

448   keratinocytes and *S. aureus* can increase its expression further.[41,42] However, the effect of Sbi on

449   the skin and Th2-promoting cytokines has not previously been examined. Immunoglobulin-like

450   domains are common to several different receptor types found on keratinocytes including members

451   of the IL-1R superfamily. It is therefore possible that Sbi binds to a receptor on the surface of

452   keratinocytes, through recognition of an Ig domain, inducing TSLP and IL-33 release.

453   Alternatively, Sbi has been shown to induce epidermal growth factor receptor (EGFR) in

454   macrophages *in vitro* and *in vivo*.[43] As activation of EGFR is known to be involved in the induction

455   of IL-33 and TSLP transcription in keratinocytes,[43,44] Sbi induction of EGFR may be an alternative

456   pathway for the release of IL-33 and TSLP from NHEK. Further studies are required to elucidate

457   the exact mechanism of action of Sbi based on these possibilities.

458   Regarding the ability of FSA to degrade and disrupt CDSN expression as show in the

459   immunohistology and Western blots, this is likely to be indirect effect of Sbi, as this factor contains

460   no inherent protease activity. Endogenous keratinocyte-derived kallikreins are important mediators

461   of skin barrier disfunction, as exemplified in Netherton syndrome.[45] We have previously shown

462   that kallikrein-5 can be activated by changes in skin pH in NC/Tnd mice leading to disrupted skin

463 barrier function.[20] The complex interplay and exact mechanism by which Sbi might active these

464 endogenous kallikreins to induce skin barrier dysfunction requires further investigation.

465   Over the last decade there have been huge advances in our understanding of host pathogen

466 interactions, establishing a link between humans and microbial diversity in health-related

467 outcomes.[46] The prime aim of this study was to address the question as to what makes *S. aureus*

468 the undisputed master of its bacterial class in promoting atopy. Sbi is an important piece of the

469 puzzle. In addition, this is the first time a pathogen-specific molecule has been identified to induce

470 rapid release of IL-33, independently of cell death and TLR2 recognition. Previous studies have

471 suggested a possible role of *S. aureus* TLR2 ligands in the induction of type 2 responses in mouse

472 skin and human keratinocytes. In the study by Brauweiler *et al*,[47] lipoteichoic acid injected

473 intradermally into mouse skin led to an increase in TSLP mRNA and to a lesser extent IL-33, but

474 protein levels were not assessed. Furthermore, it is not possible to conclude if the ligand had a

475 direct effect on keratinocytes or acted indirectly through other resident skin immune cells.

476 Additionally, the relevance of this murine model to human skin is unclear. Vu *et al*[48] found that a

477 synthetic diacylated lipoprotein but not PGN or Pam_3CSK_4 induced small amounts of TSLP in

478 human keratinocytes but the authors did not assay IL-33. SplD induced IL-33 release from airway

479 epithelial cells type II within an hour.[32] The lack of IL-33 release by skin NHEK after addition of

480 SplD in our study suggests that molecular triggers of atopy, including *S. aureus* associated

481 virulence factors, can be tissue-specific.

482   In summary, we believe that our novel results significantly advance the understanding of

483 the etiology of AD, providing compelling evidence that a *S. aureus* protein drives IL-33 release in

484 skin, triggering the allergic-type phenotype associated with AD. Further research is now required

485 to determine the exact mechanism by which Sbi induces IL-33 release, and also how it degrades

486 corneodesmosome proteins critical to skin barrier function.

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.

REFERENCES

1. Poulakou G, Lagou S, Tsiodras S. What's new in the epidemiology of skin and soft tissue infections in 2018? Curr Opin Infect Dis. 2019;32:77-86.

2. Lunjani N, Hlela C, O'Mahony L. Microbiome and skin biology. Curr Opin Allergy Clin Immunol. 2019;19:328-333.

3. Esposito S, Noviello S, Leone S. Epidemiology and microbiology of skin and soft tissue infections. Curr Opin Infect Dis 2016;29:109-15.

4. Kim J, Kim BE, Ahn K, Leung DYM. Interactions between atopic dermatitis and Staphylococcus aureus infection: Clinical implications. Allergy Asthma Immunol Res 2019;11:593-603.

5. Paller AS, Kong HH, Seed P, Naik S, Scharschmidt TC, Gallo RL, et al. The microbiome in patients with atopic dermatitis. J Allergy Clin Immunol 2019;143:26-35.

6. Weidinger S, Novak N. Atopic dermatitis. Lancet 2016;387:1109-1122.

7. Lacey KA, Geoghegan JA, McLoughlin RM. The role of Staphylococcus aureus virulence factors in skin infection and their potential as vaccine antigens. Pathogens 2016;5:(1).

8. Seiti Yamada Yoshikawa F, Feitosa de Lima J, Notomi Sato M, Álefe Leuzzi Ramos Y, Aoki V, Leao Orfali R. Exploring the Role of Staphylococcus Aureus Toxins in Atopic Dermatitis. Toxins (Basel) 2019:11:(6).

9. Al Kindi A, Alkahtani AM, Nalubega M, El-Chami C, O'Neill C, Arkwright PD, et al. Staphylococcus aureus internalized by skin keratinocytes evade antibiotic killing. Front Microbiol 2019;10:2242.

10. Askarian F, Wagner T, Johannessen M, Nizel V. Staphylococcus aureus modulation of innate immune responses through Toll-like (TLR), (NOD)-like and C-type lectin (CLR) receptors. FEMS Microbiol Rev 2018;42: 656-671.

528 11. Leung DYM, Berdyshev E, Goleva E. Cutaneous Barrier Dysfunction in Allergic Diseases. J

529   Allergy Clin Immunol. 2020;145:1485-1497.

530 12. Smith FJ, Irvine AD, Terron-Kwiatkowski A, Sandilands A, Campbell LE, Zhao Y, et al.

531   Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. Nat

532   Genet. 2006;38:337-42.

533 13. Honda T, Kabashima K. Reconciling innate and acquired immunity in atopic dermatitis. J

534   Allergy Clin Immunol. 2020;Feb 25.

535 14. Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two Phase

536   3 Trials of Dupilumab versus Placebo in Atopic Dermatitis. N Engl J Med. 2016;375:2335-

537   2348.

538 15. Geoghegan JA, Irvine AD, Foster TJ. Staphylococcus aureus and Atopic Dermatitis: A

539   Complex and Evolving Relationship. Trends Microbiol 2018;26:484-497.

540 16. Nakamura Y, Oscherwitz J, Cease KB, Chan SM, Muñoz-Planillo R, Hasegawa M, et al

541   Staphylococcus δ-Toxin Induces Allergic Skin Disease by Activating Mast Cells. Nature

542   2013;503:397-401.

543 17. Casanova JL, Abel L. Human genetics of infectious diseases: Unique insights into

544   immunological redundancy. Semin Immunol 2018;36:1-12.

545 18. Fischer A, Rausell A. What do primary immunodeficiencies tell us about the

546   essentiality/redundancy of immune responses? Semin Immunol 2018;36:13-16.

547 19. Smith EJ, Visai L, Kerrigan SW, Speziale P, Foster TJ. The Sbi protein is a multifunctional

548   immune evasion factor of *Staphylococcus aureus*. Infect Immun 2011;79:3801-3809.

549 20. Jang H, Matsuda A, Jung K, Karasawa K, Matsuda K, Oida K, et al. Skin pH is the master

550   switch of kallikrein 5-mediated skin barrier destruction in a murine atopic dermatitis model.

551   J. Invest. Dermatol. 2016;136:127-135.

552   21. Byrd AL, Deming C, Cassidy SKB, Harrison OJ, Ng WI, Conlan S, et al. Staphylococcus
553   aureus and Staphylococcus epidermidis strain diversity underlying pediatric atopic dermatitis.
554   Sci Transl Med 2017;9:(397).

555   22. Fyhrquist, N., Muirhead G, Prast-Nielsen S, Jeanmougin M, Olah P, Skoog T, et al. Microbe-
556   host interplay in atopic dermatitis and psoriasis. Nat Commun 2019;10:4703.

557   23. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in
558   the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS One
559   2008;3:e3331.

560   24. Lan F, Zhang N, Holtappels G, De Ruyck N, Krysko O, Van Crombruggen K, et al.
561   Staphylococcus aureus induces a mucosal type 2 immune response via epithelial cell-derived
562   cytokines. Am J Respir Crit Care Med 2018;198:452-463.

563   25. Jonca N, Guerrin M, Hadjiolova K, Caubet C, Gallinaro H, Simon M, et al. Corneodesmosin,
564   a component of epidermal corneocyte desmosomes, displays homophilic adhesive properties.
565   J Biol Chem 2002;277:5024-5029.

566   26. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-
567   of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor
568   for atopic dermatitis. Nat Genet 2006;38:441-446.

569   27. Noguchi A, Tominaga M, Takahashi N, Matsuda H, Kamata Y, Umehara Y, et al. Differences
570   in therapeutic effects of topically applied corticosteroid and tacrolimus on atopic dermatitis-
571   like symptoms in NC/Nga mice. J Dermatol Sci 2017;86:54-62.

572   28. Amagai Y, Matsuda H, Tanaka A. Abnormalities in itch sensation and skin barrier function
573   in atopic NC/Tnd mice. Biol Pharm Bull 2013;36:1248-1252.

574   29. Matsui K, Nojima Y, Kajiwara Y, Busujima K, Mori Y. Topical application of doxycycline
575   inhibits Th2 cell development mediated by Langerhans cells and exerts a therapeutic effect on
576   atopic dermatitis. J Pharm Pharm Sci 2020;23:86-99.

577   30. Lowe AJ, Leung DYM, Tang MLK, Su JC, Allen KJ. The skin as a target for prevention of

578   the atopic march. Ann Allergy Asthma Immunol 2018;120:145-151.

579   31. Chen YL, Gutowska-Owsiak D, Hardman CS, Westmoreland M, MacKenzie T, Cifuentes

580   L, et al. Proof-of-concept clinical trial of etokimab shows a key role for IL-33 in atopic

581   dermatitis pathogenesis. Sci Transl Med 2019;11:(515).

582   32. Teufelberger AR, Nordengrün M, Braun H, Maes T, De Grove K, Holtappels G, et al. The

583   IL-33/ST2 axis is crucial in type 2 airway responses induced by Staphylococcus aureus-

584   derived serine protease-like protein D. J Allergy Clin Immunol 2018;141:549-559.e7.

585   33. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete genome

586   sequence of USA300, an epidemic clone of community-acquired methicillin-resistant

587   Staphylococcus aureus. Lancet 2006;367:731-739.

588   34. Berscheid A, Sass P, Weber-Lassalle K, Cheung AL, Bierbaum G. Revisiting the genomes of

589   the Staphylococcus aureus strains NCTC 8325 and RN4220. Int J Med Microbiol

590   2012;302:84-87.

591   35. Jonca N, Caubet C, Guerrin M, Simon M, Serre G. Corenodesmosin: Structure, function and

592   involvement in pathophysiology. Open Dermatol J 2010;4:36-45.

593   36. Smith EJ, Visai L, Kerrigan SW, Speziale P, Foster TJ. The Sbi protein is a multifunctional

594   immune evasion factor of Staphylococcus aureus. Infect Immun 2011;79:3801-3809.

595   37. Stentzel S, Teufelberger A, Nordengrün M, Kolata J, Schmidt F, van Crombruggen K, et al.

596   Staphylococcal serine protease-like proteins are pacemakers of allergic airway reactions to

597   Staphylococcus aureus. J Allergy Clin Immunol 2017;139:492-500.

598   38. Zhang L, Jacobsson K, Vasi J, Lindberg M, Frykberg L. A second IgG-binding protein in

599   Staphylococcus aureus. Microbiology 1998;144:985-91.

600   39. Koch TK, Reuter M, Barthel D, Böhm S, van den Elsen J, Kraiczy P, et al. Staphylococcus

601   aureus proteins Sbi and Efb recruit human plasmin to degrade complement C3 and C3b. PLoS

602   One 2012;7:e47638.

603   40. Gonzalez CD, Ledo C, Giai C, Garófalo A, Gómez MI. The Sbi Protein Contributes to

604   Staphylococcus aureus Inflammatory Response during Systemic Infection. PLoS

605   One 2015;10:e0131879.

606   41. Wang X, Cheng D, Hu G, Liang L, Tan F, Xiao T, et al. Tumor Necrosis Factor (TNF)

607   Receptor Expression Determines Keratinocyte Fate upon Stimulation with TNF-Like Weak

608   Inducer of Apoptosis. Mediators Inflamm 2019;2019:2945083.

609   42. Aufiero B, Guo M, Young C, Duanmu Z, Talwar H, Lee HK, et al. Staphylococcus Aureus

610   Induces the Expression of Tumor Necrosis Factor-Alpha in Primary Human Keratinocytes. Int

611   J Dermatol 2007;46:687-94.

612   43. Meephansan J, Komine M, Tsuda H, Karakawa M, Tominaga S, Ohtsuki M. Expression of IL-

613   33 in the epidermis: The mechanism of induction by IL-17. J Dermatol Sci 2013;71:107-114.

614   44. Segawa R, Shigeeda K, Hatayama T, Dong J, Mizuno N, Moriya T, et al. EGFR

615   transactivation is involved in TNF-α-induced expression of thymic stromal lymphopoietin in

616   human keratinocyte cell line. J Dermatol Sci 2018;89:290-298.

617   45. Kishibe M. Physiological and pathological roles of kallikrein-related peptidases in the

618   epidermis. J Dermatol Sci 2019;95:50-55.

619   46. Integrative HMP (iHMP) Research Network Consortium. The Integrative Human Microbiome

620   Project. Nature 2019;569:641-648.

621   47. Bauweiler AM, Goleva E, Leung DYM. Staphylococcus aureus lipoteichoic acid initiates a

622   TSLP-basophil-IL-4 axis in the skin. J Invest Dermatol 2020;140:915-917.

623   48. Vu AT, Baba T, Chen X, Le TA, Kinoshita H, Xie Y, et al. Staphylococcus aureus membrane

624   and diacylated lipopeptide induce thymic stromal lymphopoietin in keratinocytes through the

625   toll-like receptor 2-toll-like receptor 6 pathway. J Allergy Clin Immunol 2010;126:985-93,

626   993.e1-3.

Figure 1 *S. aureus* induces IL-33 and TSLP release by human keratinocytes *in vitro*. a. Live *S. aureus* (LiSA) (10^7 CFU/ml), but not other *staphylococcus* species, or Group A streptococci (GAS) induced IL-33 and TSLP by NHEK. *S. cap: Staphylococcus capitis; S. car: Staphylococcus carnosus; S. coh: Staphylococcus cohnii; S. hem: Staphylococcus hemolyticus; S. len: staphylococcus lentis.* **b.** All *S. aureus* strains tested induced IL-33 and TSLP release from NHEK. Cytokine release was **c.** dose- and **d.** time-dependent. **e.** IL-33 was constitutively expressed in nuclei of HEKa (top panel) and released after 1h stimulation with LiSA (bottom panel). Cells stained with anti-human IL-33 monoclonal antibody (red) and anti-human cytokeratin 14 (green). **f.** 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 LiSA (10^7 CFU/ml) for up to 6h. **g. & h.** LiSA cytotoxic activity after 6h stimulation. Data represents three independent experiments performed in triplicate. Images taken at 20X magnification. **P<0.01*, ***P< 0.001* compared with the control. *P*-values determined by one-way ANOVA with Dunnett's multiple comparisons. Mean ± standard error of the mean.

Figure 2 *S. aureus* cell wall components have no bioactivity and its secretome induces IL-33 and TSLP release independent of TLR2. a. Exogenous bacterial peptidoglycan (PGN) 0-100µg/ml and lipoteichoic acid (LTA) 0-100µg/ml and did not induce release of IL-33 or TSLP by NHEK at 6h. **b.** Anti-TLR2 antibodies (1 or 10µg/ml) did not inhibit IL-33 or TSLP release induced by LiSA. **c.** Inhibiting TLR2 expression with 20mM of specific siRNA did not suppress FSA-induced release of IL-33 or TSLP by NHEKs at 6h. Scr = scrambled siRNA control. Data represents three independent experiments performed in triplicate. ***P< 0.001* compared with the control. *P*-values determined by one-way ANOVA with Dunnett's multiple comparisons. Mean ± standard error of the mean.

653   **Figure 3 Type 2-promoting bioactivity of *S. aureus* resides in the secretome. a.** IL-33 release

654   can be induced by NHEK when separated from the LiSA by a 0.4μm transmembrane filter. **b.**

655   Bioactivity of FSA is destroyed by heating to 95°C, but by treatment with penicillin/streptomycin

656   (P/S). Cytokine release (IL-33 and TSLP) was measured by ELISA. **c.** Dot plots of viable

657   (Annexin V^-/DAPI$^-$), early apoptotic (Annexin V^+DAPI$^-$), late apoptotic (Annexin V^+/DAPI$^+$)

658   and necrotic (Annexin V^-/DAPI$^+$) cells following stimulation with FSA for up to 6h. **d. & e.**

659   Quantification of FSA cytotoxic activity determined by DAPI$^+$ and Annexin V^+ staining of cells.

660   All data are representative of three independent experiments performed in triplicate. *$P<0.05$,

661   **$P<0.001$ compared with the control. *P*-values were determined by one-way ANOVA with

662   Dunnett's multiple comparisons. Mean ± standard error of the mean.

663

664   **Figure 4 *S. aureus* Filtered Supernatant (FSA) induces TSLP and IL-33 and disrupts**

665   **corneodesmosome expression in human skin organ culture. a. & b.** FSA but not filtered

666   supernatant from *S. epidermidis* (FSE), house dust mite allergens (*D. pteronyssinus* (DP),

667   *D. farinae* (DF) or Ragweed allergen induces TSLP and IL-33 after 6h and 12h. Three

668   independent experiments performed in duplicate. *$P<0.05$, **$P<0.001$ compared with the

669   control. Mean ± standard error of the mean. **c.** Corneodesmosin *(*CDSN), Desmocollin-1 (DSC-

670   1) and Desmoglein-1 (DSG-1) staining following 6h stimulation with FSA, FSE, and common

671   aeroallergens DP, DF and ragweed. CDSN staining is disrupted following FSA treatment. Images

672   are representative of three independent experiments performed in duplicate. Scale bars in c =

673   40μm. **df.** Densitometric analysis for CDSN, DSC-1 and DSG-1. Mean of three independent

674   experiments performed in duplicate. Bar represents median and * represents significant

675   difference compared with control. **g. & h**. Primary keratinocytes from 11 children with AD and

676   11 healthy controls released IL-33 and TSLP in response to LiSA but not heat-killed *S. aureus*

677 (HKSA) or lipopolysaccharide (LPS). Data represents three independent experiments performed

678 in duplicate. **$P<0.001$ compared with the control. Bar represents median.

679

680 **Figure 5 *S. aureus* Filtered Supernatant (FSA) induces eczema and skin barrier disruption**

681 **in the NC/Tnd mouse *in vivo*.** 80-100μl of low (10^7 CFU/ml) and high concentration (10^8

682 CFU/ml) FSA, FSE, or *D*. farinae (DF) with concomitant 4% SDS were applied topically to the

683 backs of 8-week old NC/Tnd mice each day for four weeks. Mice were housed in specific

684 pathogen free conditions. **a.** Representative images of NC/Tnd mice after application of 4% SDS,

685 or 4% SDS and FSE, DF or FSA had been applied to the back daily for four weeks. **b.** Clinical

686 eczema scores. **c.** Scratching behaviour and **d.** Trans-Epidermal Water Loss (TEWL). All data

687 show results of six individual mice/group. **$P<0.01$. Mean ± standard error of the mean.

688

689 **Figure 6 Filtered supernatant from *S. aureus* but not *S. epidermidis* increases plasma IL-33**

690 **and TSLP, particularly in NC/Tnd mice and disrupts epidermal corneodesomosme**

691 **expression. a.** Histological sections from eczema-prone NC/Tnd mice. 4% SDS, or 4% SDS and

692 DF, FSE or FSA had been applied daily for four weeks. Images are representative of six individual

693 mice/group performed in duplicate. **b-d.** Densitometric analysis. *$P<0.01$, **$P<0.0001$ compared

694 with the SDS control. Bar represents median. **e.** Western blots of CDSN, DSC-1 and DSG-1 native

695 protein and degraded fragments from NC/Tnd mouse skin. Densitometric analysis of Western blots

696 for CDSN **(f)** DSC-1 **(g)** and DSG-1 **(h)**. Data representative of six individual mice/group.

697 *$P<0.05$, **$P<0.001$ compared with the naïve control. **i & j.** Plasma IL-33 and TSLP

698 concentrations in NC/Tnd and MSM/Ms. SDS: sodium dodecyl sulphate, FSE: filtered supernatant

699 from *S. epidermidis*, DF: *D. farinae*, FSA: filtered supernatant from *S. aureus*. Data are

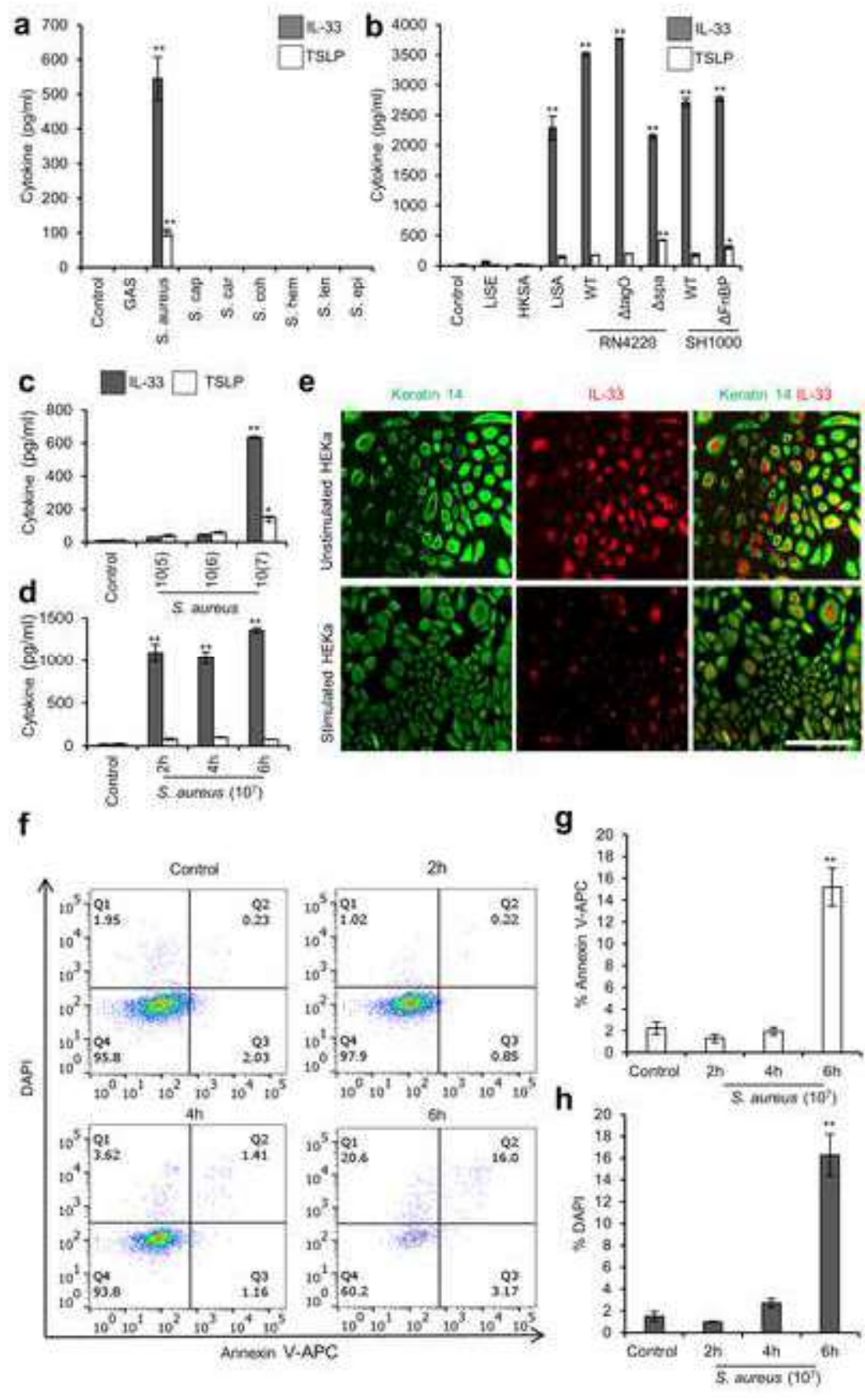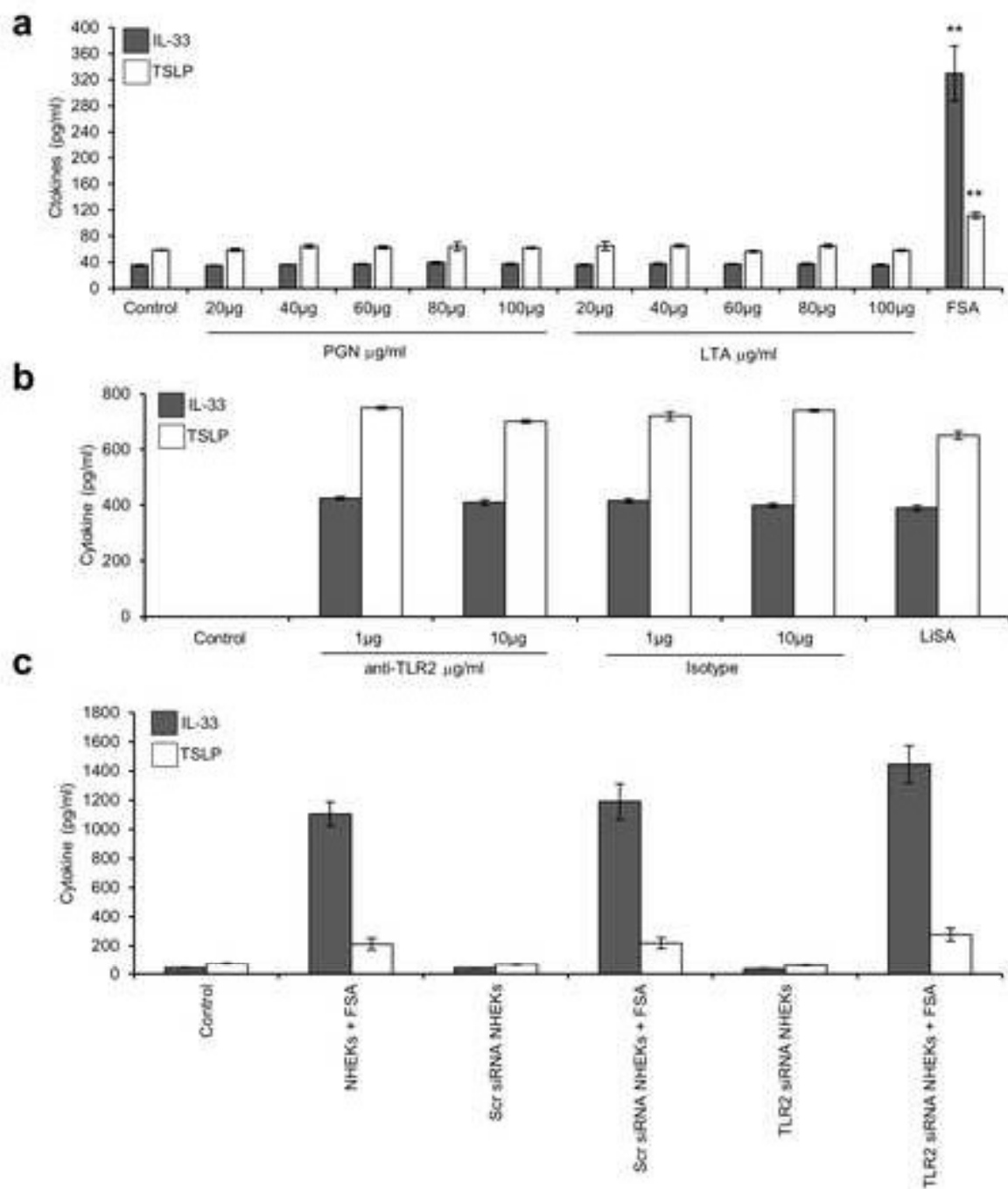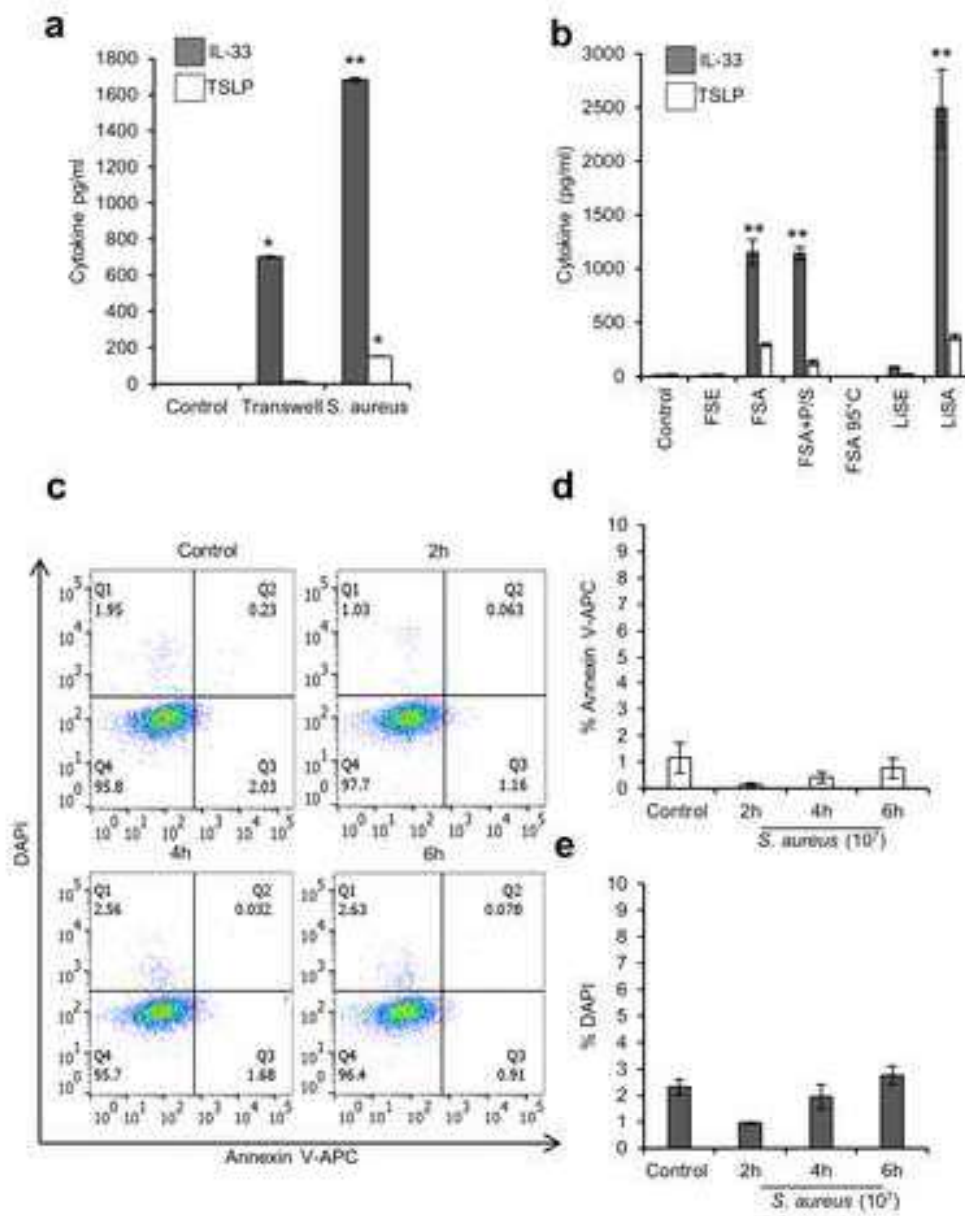700 representative of six mice/group. **$P<0.001$. Mean ± standard error of the mean.

701

702 **Figure 7 FSA-induced eczema in the NC/Tnd mouse can be completely abrogated by**

703 **neutralizing anti-IL-33 antibodies.** 80-100µl of high (10^8 CFU/ml) concentration FSA with

704 concomitant 4% SDS were applied topically to the backs of 8-week old NC/Tnd mice each day for

705 four weeks. One group also received an intraperitoneal 10µg injection of anti-IL-33 monoclonal

706 antibody with the first application of FSA, while a second group received an injection of an isotype

707 control antibody. **a.** Representative images of NC/Tnd mice after application of 4% SDS and FSA,

708 FSA daily for four weeks, with or without isotype or anti-IL-33 mAb. **b.** Clinical eczema scores,

709 **c.** scratching frequency and **d.** Trans-Epidermal Water Loss (TEWL) of naïve mice, and mice

710 treated with 4% SDS and FSA, isotype control or anti-IL-33 antibody. All data show results of six

711 individual mice/group. **$P<0.01$ compared with pre-treatment or the naïve group. Mean $\pm$
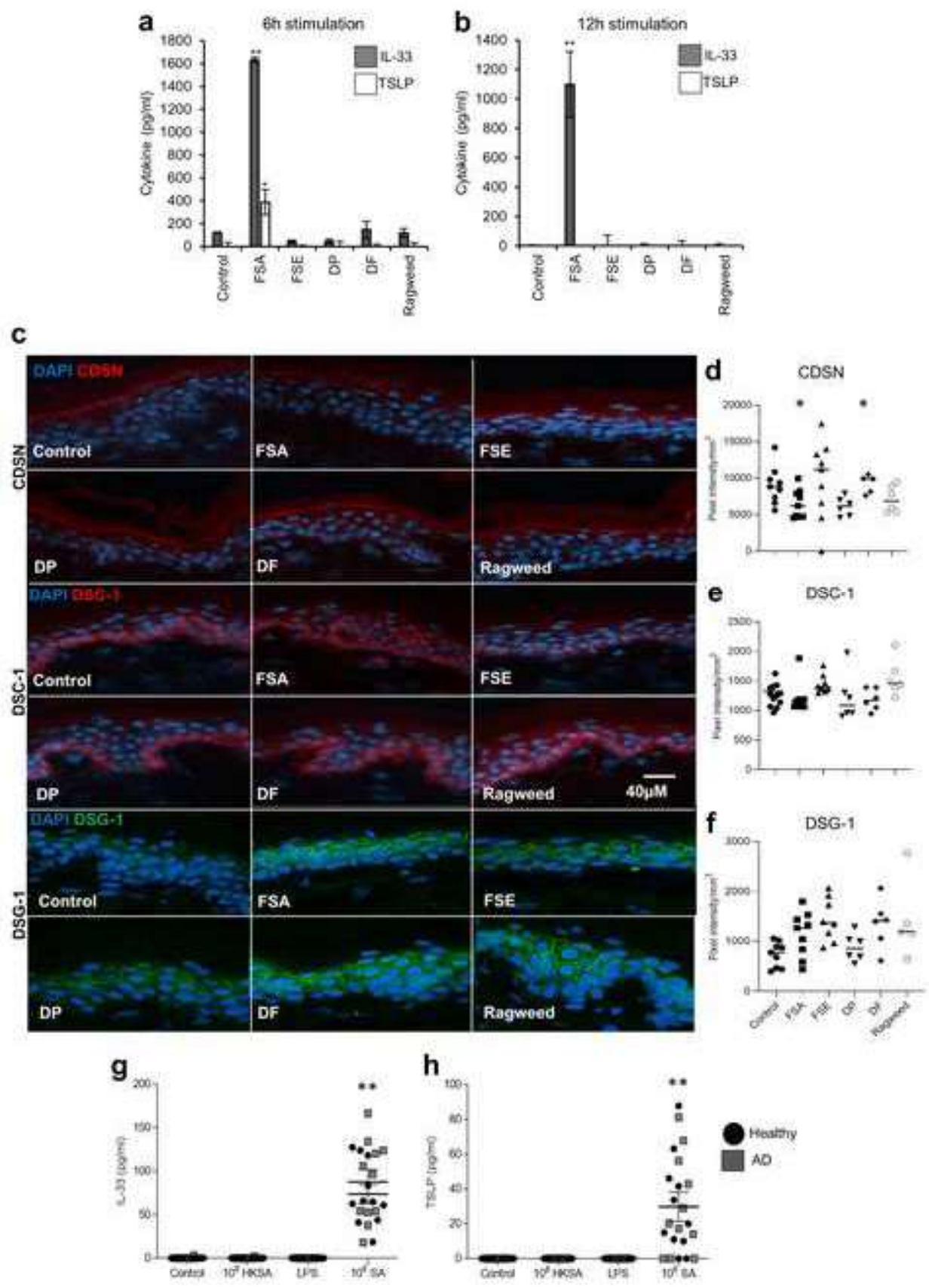
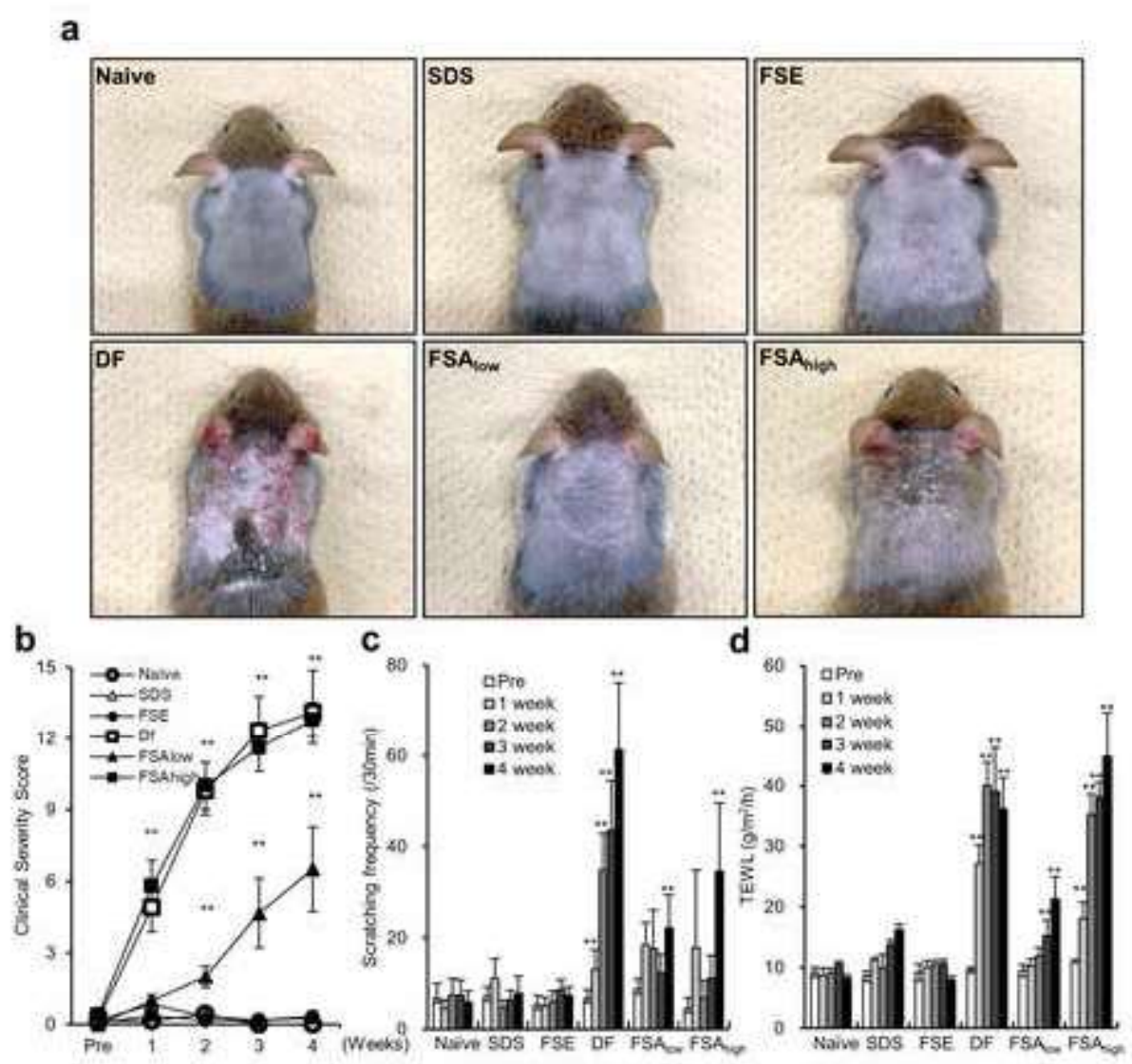712 standard error of mean.

713

714 **Figure 8 Fractionation of *S. aureus* secretome (FSA) Th2-promoting activity by FPLC and**

715 **identification of the active factor as *S. aureus* Sbi. a.** Bioactivity of FSA fractionated using

716 Superose® 6 FPLC of 100kDa retention fraction (insert shows absorbance (280nm); main panel

717 shows IL-33 release by NHEK after addition of specific FPLC fractions). **b.** *S. aureus* proteins

718 identified from bioactive Superose® 6 FPLC fractions by mass spectroscopy. **c.** IL-33 and TSLP

719 release by NHEK after addition of *E. coli* vector-derived DLD, Sbi, PDHa, SplD, or exogenous

720 LAP-3 for 6h. **d.** *E. coli* expressing Sbi, but not untransfected *E. coli* induced IL-33 release by

721 NHEK. **e.** LiSA, and **f.** FSA from Sbi- and pRMC2:Sbi-deficient strains induce less IL-33 and

722 TSLP than parental Newton wild-type *S. aureus*. **e.** Spa-deficient LiSA induces the same IL-33

723 and TSLP as the Newton wide-type LiSA. All data are representative of two to three independent

724 experiments performed in duplicate or triplicate. **$P < 0.0001$ compared with control. Mean $\pm$

725 standard error of mean. DLD: dihydrolipoyl dehydrogenase, LiSA: live *S. aureus*, Sbi: Second

726 immunoglobulin-binding protein, and PDHa: pyruvate dehydrogenase alpha-subunit.
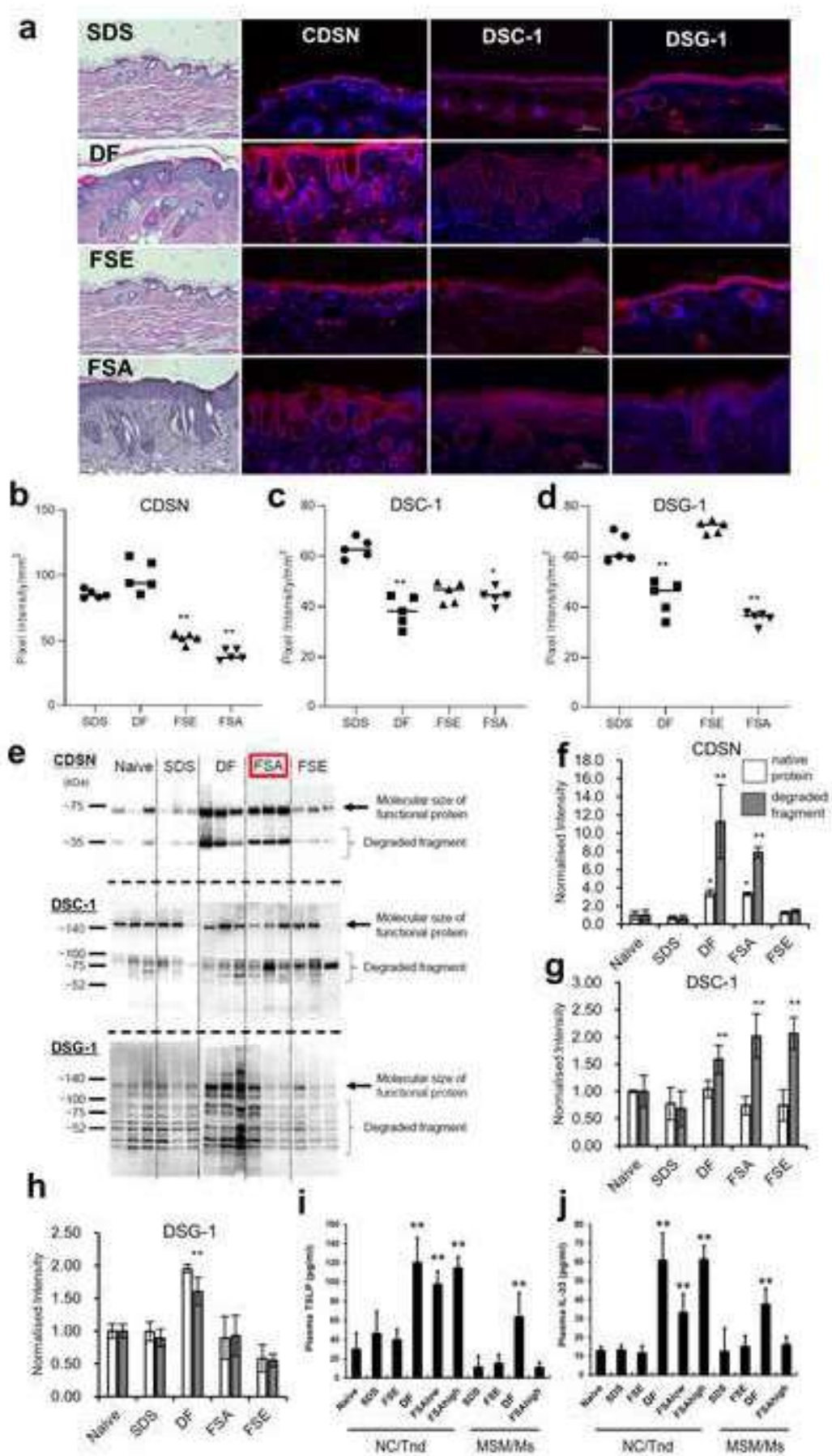
b

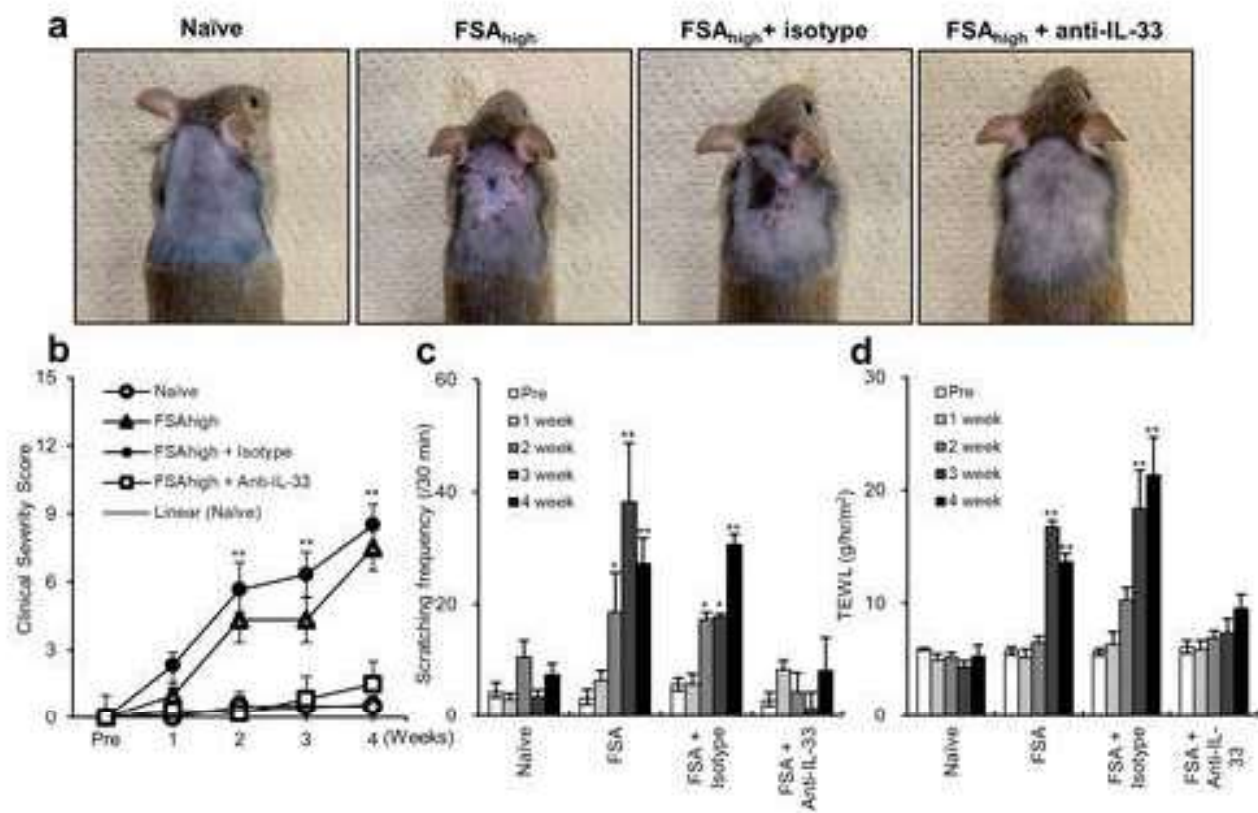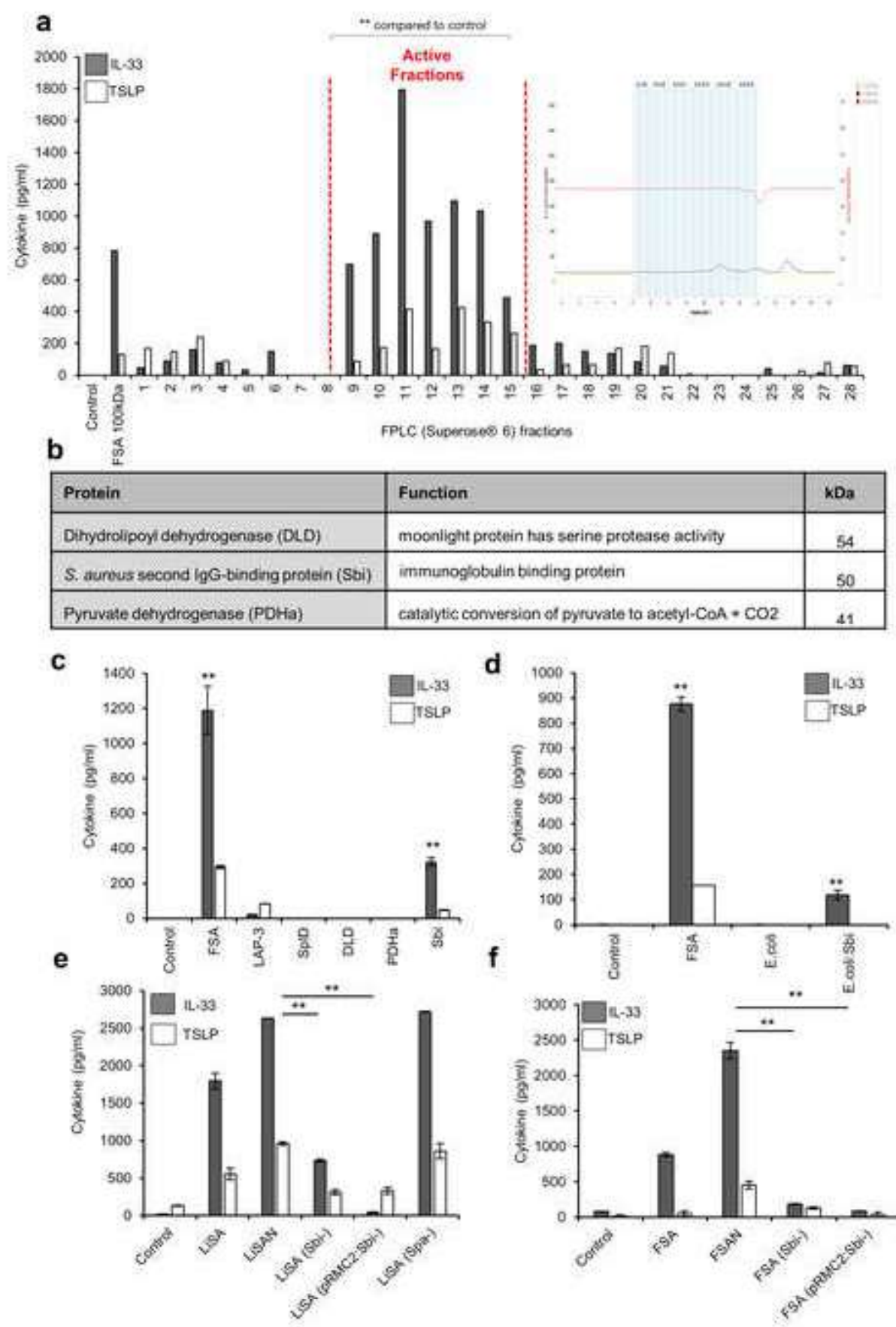| Protein	Function	kDa
Dihydrolipoyl dehydrogenase (DLD)	moonlight protein has serine protease activity	54
S. aureus second IgG-binding protein (Sbi)	immunoglobulin binding protein	50
Pyruvate dehydrogenase (PDHa)	catalytic conversion of pyruvate to acetyl-CoA + CO2	41

SUPPLEMENTARY ONLINE INFORMATION

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

Arwa Al Kindi, PhD,[1†] Helen Williams, PhD,[1†] Kenshiro Matsuda, PhD,[2] Abdullah M. Alkahtani, PhD,[3] Charis Saville, PhD,[1] Hayley Bennett, PhD,[4] Yasmine Alshammari, MSc,[1] Soo Y. Tan, PhD,[5] Catherine O'Neill, PhD,[6] Akane Tanaka, DVM, PhD,[7] Hiroshi Matsuda, DVM, PhD,[8] Peter D. Arkwright, MD, PhD,[1*] Joanne L. Pennock, PhD[1*]

[1]*Lydia Becker Institute of Immunology and Inflammation, University of Manchester, United Kingdom,* [2]*Center for Innovative Drug Discovery, University of Tsukuba, Japan,* [3]*Department of Medicine, Microbiology and Parasitology, King Khalid University, Abha, Saudi Arabia,* [4]*Genome Editing Unit, University of Manchester, United Kingdom,* [5]*National University Health System, Singapore,* [6]*Division of Dermatological and Musculoskeletal Sciences, University of Manchester, United Kingdom.* [7]*Laboratories of Veterinary Molecular Pathology & Therapeutics and* [8]*Comparative Animal Medicine, Tokyo University of Agriculture & Technology, Tokyo, Japan*

[†,*]authors contributed equally to this work.

Corresponding author: Dr P D Arkwright, Lydia Becker Institute of Immunology & Inflammation, University of Manchester, Room 2.21, Core Technology Facility, 46 Grafton St., Manchester, M13 9NT, United Kingdom. Telephone +44 161 306 3771, email peter.arkwright@manchester.ac.uk

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	CCTGTA<u>GGATCC</u>ATGAATAAAAATAT AATCATCAAAAGTATTGCGG	Used to amplify *SplD* from Clin1-SA for cloning using BamHI-EcoRI sites of pGEM-T
SplD HindIII R	GCGCGATAA<u>GCTT</u>TTATTATTTATCTA AATTATCTGCAATAAATTTCTTAAT	
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	