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Staphylococcus aureus Serine protease-like protein A (SplA) induces IL-8 by keratinocytes and synergizes with IL-17A

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ABSTRACT

Background: Serine protease-like (Spl) proteins produced by *Staphylococcus (S.) aureus* have been associated with allergic inflammation. However, effects of Spls on the epidermal immune response have not been investigated.
Objectives: To assess the epidermal immune response to SplA, SplD and SplE dependent on differentiation of keratinocytes and a Th2 or Th17 cytokine milieu.
Methods: Human keratinocytes of healthy controls and a STAT3-hyper-IgE syndrome (STAT3-HIES) patient were cultured in different calcium concentrations in the presence of Spls and Th2 or Th17 cytokines. Keratinocyte-specific IL-8 production and concomitant migration of neutrophils were assessed.
Results: SplE and more significantly SplA, induced IL-8 in keratinocytes. Suprabasal-like keratinocytes showed a higher Spl-mediated IL-8 production and neutrophil migration compared to basal-like keratinocytes. Th17 cytokines amplified Spl-mediated IL-8 production, which correlated with neutrophil recruitment. Neutrophil recruitment by keratinocytes of the STAT3-HIES patient was similar to healthy control cells.
Conclusion: S. aureus-specific Spl proteases synergized with IL-17A on human keratinocytes with respect to IL-8 release and neutrophil migration, highlighting the importance of keratinocytes and Th17 immunity in barrier function.

1. Background

A tight interplay between epithelial and immune cells is part of an efficient immediate host immunity of our skin barrier. [1–3] One of the most common skin diseases presenting with an alteration in the cellular interplay of epithelial and immune cells and consequently an impaired host immunity is atopic dermatitis. Atopic dermatitis presents as an eczematous, chronic inflammatory skin disease affecting around 20% of children worldwide. [4] Atopic dermatitis is a multifactorial disease with a shift towards Th2 signaling, [5] decreased skin barrier function, [6] and recurrent *Staphylococcus aureus (S. aureus)* skin infections. [7] While the debate is ongoing whether colonialization and recurrent infections with *S. aureus* are triggers or consequences of the initial clinical course of atopic dermatitis, *S. aureus* has frequently been associated with a Th2-biased atopic inflammation and an impaired immune tolerance of the skin barrier. [8–10] Th2 inflammation has been reported to promote *S. aureus* infections in skin lesions, [11] while an intact Th17 immunity is crucial to drive antimicrobial host defense against *S. aureus*. [12–15]

Abbreviations: CCL2, C–C motif chemokine ligand 2; CK, Cytokeratin; HaCaT, Human adult, low calcium, high temperature keratinocytes; HBD2, Human beta-defensin-2; IL, Interleukin; IVL, Involucrin; RTD-PCR, RNA and real-time detection-polymerase chain reaction; *S. aureus*, *Staphylococcus aureus*; SDHA, Succinate dehydrogenase complex subunit A; Spl, Serine Protease-like protein; STAT3, Signal transducer and activator of transcription 3; STAT3-HIES, STAT3-hyper-IgE syndrome.

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However, the interplay between Th2 and Th17 immunity in atopic dermatitis has not been fully elucidated.

The importance of an intact Th17 immunity is also shown by STAT3-hyper IgE syndrome (STAT3-HIES), an inborn error of immunity caused by heterozygous dominant-negative mutations in the *signal transducer and activator of transcription 3 (STAT3)* gene. [16–18] STAT3-HIES patients present with a reduced number of Th17 cells, eczematous skin lesions and recurrent *S. aureus* infections with clinical aspects similar to a Th2-dominated atopic eczema. [18–21] Both Th2-mediated atopic dermatitis and STAT3-HIES show reduced migration of neutrophils in skin lesions associated with *S. aureus* infections. [22–25]

Although 20%–30% healthy individuals in the population are transiently or persistently colonialized by *S. aureus* without adverse effects, [26] *S. aureus* is a major pathogen causing a broad spectrum of human infections ranging from minor superficial skin infections to harmful life-threating systemic conditions. [27–30] An important group of virulence factors contributing to the pathogenicity of *S. aureus* are secretory proteases. Among them are six staphylococcal serine protease-like proteins (Spls), SplA–SplF. [31,32] Spls are unique to *S. aureus* and have been identified to induce lung damage during pneumonia or act as potentially allergenic proteins. [33,34] So far, no association has been described between the presence of *S. aureus*-specific Spls on the skin and the formation of eczematous lesions. Next to driving Th2 or Th17 immunity, another possible link between the effects of *S. aureus* toxins and keratinocyte functions might be the degree of keratinocyte differentiation, which is known to be regulated by complex calcium-dependent processes within the epidermis and tightly regulated by intraepithelial calcium concentrations. [35]

To assess whether *S. aureus*-specific toxins impact epidermal immune responses in atopic-like conditions, we studied the effects of Spls on human keratinocytes dependent on the calcium-induced differentiation and Th2 or Th17 cytokine milieus.

2. Methods

2.1. Subjects

Human keratinocytes were isolated from healthy, non-atopic volunteers and one STAT3-HIES patient. STAT3-HIES was confirmed by genetic testing as described previously. [36] The mutation was reported using the nomenclature of den Dunnen and Antonarakis. [37] Human neutrophils were obtained from one healthy, non-atopic volunteer.

The study was performed according to ethical guidelines approved by the ethical committee of the Technical University of Munich (32/20S). Written informed consent and consent to publish is included in the ethical guidelines signed by all participants or their legal guardians.

2.2. Isolation and culture of keratinocytes

The spontaneously transformed aneuploid immortal keratinocyte cell line human adult, low calcium, high temperature keratinocytes (HaCaT) [38] was cultured in RPMI medium (Gibco, Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco).

To investigate the effect of Th2 and Th17 cytokines with SplA, SplD and SplE in a calcium dependent manner, human primary keratinocytes of healthy subjects were isolated by suction blister technology as described previously. [39] To compare the keratinocytes of the STAT3-HIES patient and healthy subjects, skin biopsies were obtained during routine surgery. Keratinocytes from biopsies were isolated by using Dispase I (Sigma Aldrich, Darmstadt, Germany) as previously described [40] and were separated with 0.25% trypsin EDTA (Gibco). Primary keratinocytes were grown in Lifeline medium (Lifeline Cell Technology, Connecticut, USA) to 80% confluence and stored in liquid nitrogen until use.

2.3. Calcium priming of primary keratinocytes

Frozen primary keratinocytes were thawed and cultured with a density of 2.0 x 10^5 cells/ml in 24-well plates over night at 37°C and 5% CO_2 to achieve adherence. Next day, the medium was replaced by Lifeline medium supplemented with either low calcium (0.06 mM) for basal-like, or high calcium (1.50 mM) for suprabasal-like keratinocytes. Calcium-specific treatment occurred 24 h prior to the experiment-specific culture in differentiation experiments, or for 48 h simultaneously with Spls and cytokines.

2.4. Stimulation of keratinocytes

Tag-free Spl proteins were recombinantly expressed in protease-deficient *Bacillus subtilis* and purified by ion-exchange chromatography followed by size exclusion centrifugation as described before. [33] HaCaT cells were cultured at a density of 2.0 x 10^5 cells/ml in 24-wells and stimulated with either native or inactivated Spls (each 1 μg/ml) for 48 h. To inactivate Spls prior to stimulation, Spls were digested with proteinase K (AppliChem GmbH, Darmstadt, Germany) for 30 min at 37°C followed by heat-inactivation at 95°C for 5 min. Calcium-primed primary keratinocytes were left untreated or were stimulated with either Th2 cytokines 20 ng/ml each (IL-4, Promokine, Heidelberg, Germany and IL-13, Peprotech, Hamburg, Germany) or Th17 cytokines 20 ng/ml each (IL-17A and IL-22, both Peprotech) alone or in combination with recombinant SplA, SplD, or SplE (all 1 μg/ml) for 24 h. Cell-free culture supernatants were collected after treatments and stored at −80°C until analysis.

2.5. Enzyme-Linked Immunosorbent assay

Secretion of C–C motif chemokine ligand 2 (CCL2), IL-8 (both BD Bioscience, Franklin Lakes, USA) and human beta-defensin-2 (HBD2) (Peprotech) into the keratinocyte supernatants were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's protocols. The absorbance was measured at 450 nm using a microplate reader (Tecan Spark; Tecan Group AG, Männedorf, Switzerland).

2.6. Isolation of total RNA and real-time detection-polymerase chain reaction (RTD-PCR)

Total RNA from keratinocytes was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, keratinocytes were lysed in RLT buffer (provided in the Mini Kit) supplemented with 1% (v/v) 2-mercaptoethanol (Merck, Darmstadt, Germany), and total lysates were subjected to on-column DNase digestion before eluting the RNA.

RNA was transcribed into cDNA by High Capacity cDNA RT Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Gene expression levels of *IL8* and keratinocyte differentiation markers (*cytokeratin (CK)1, CK5, CK10, CK14, involucrin (IVL)*) were analyzed using an iTaq Universal SYBR Green Supermix (Biorad, Feldkirchen, Germany), and were normalized to *Succinate dehydrogenase complex subunit A (SDHA)* for relative quantification. All RTD-PCR primers were obtained from Metabion (Munich, Germany). Sequences are available upon request.

2.7. Neutrophil migration assay

Neutrophils of the healthy donor were isolated from peripheral blood diluted with 2 mM EDTA in D-PBS (Thermo Fisher Scientific) by a two-step density gradient centrifugation using Histopaque-1077 and -1119 (Sigma Aldrich). Cells were washed twice with D-PBS, and the erythrocytes were lysed for 2 min in 1x RBC Lysis Buffer (Thermo Fisher Scientific). Isolated neutrophils were resuspended in neutrophil

migration medium containing RPMI 1640 (Gibco), 1 mM L-glutamine (Gibco), 1 mM sodium-pyruvate (Gibco), 1x MEM Non-Essential Amino Acids (100x, Gibco), 0.5% bovine serum albumin (Sigma Aldrich), 1% (v/v) penicillin/streptomycin (Gibco), and 10% FBS (Gibco).

For neutrophil migration assay, a 96-well migration plate (ChemoTx disposable chemotaxis system, NeuroProbe, Gaithersburg, USA) was used. Briefly, supernatants were diluted with a patient-specific dilution factor to adjust the IL-8 concentration in the medium control to 50 pg/ml (low dilution) or to 10 pg/ml (high dilution), respectively. 30 μl of keratinocyte supernatants were placed in the lower chamber of the plate and then covered with a polycarbonate membrane of 5 μm pore size provided by the company. 3.0 x 10^5 neutrophils in 50 μl neutrophil migration medium were placed onto the membrane, and the number of neutrophils migrated into the lower chamber was counted after 1 h by flow cytometry (CytoFlex LX analyzer, Beckman Coulter, Pasadena, USA).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). The two-way ANOVA and Sidak's multiple comparisons were used to compare the effects of Spls on keratinocytes and neutrophil migration. Significant p-values are indicated by asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$.

3. Results

3.1. SplA and SplE but not SplD increased IL-8 secretion in keratinocytes in a calcium dependent manner

To test whether human keratinocytes respond to Spls, we stimulated the keratinocyte cell line HaCaT with *S. aureus*-derived Spls and assessed IL-8 secretion after 48 h. We selected SplA and SplE as both proteases

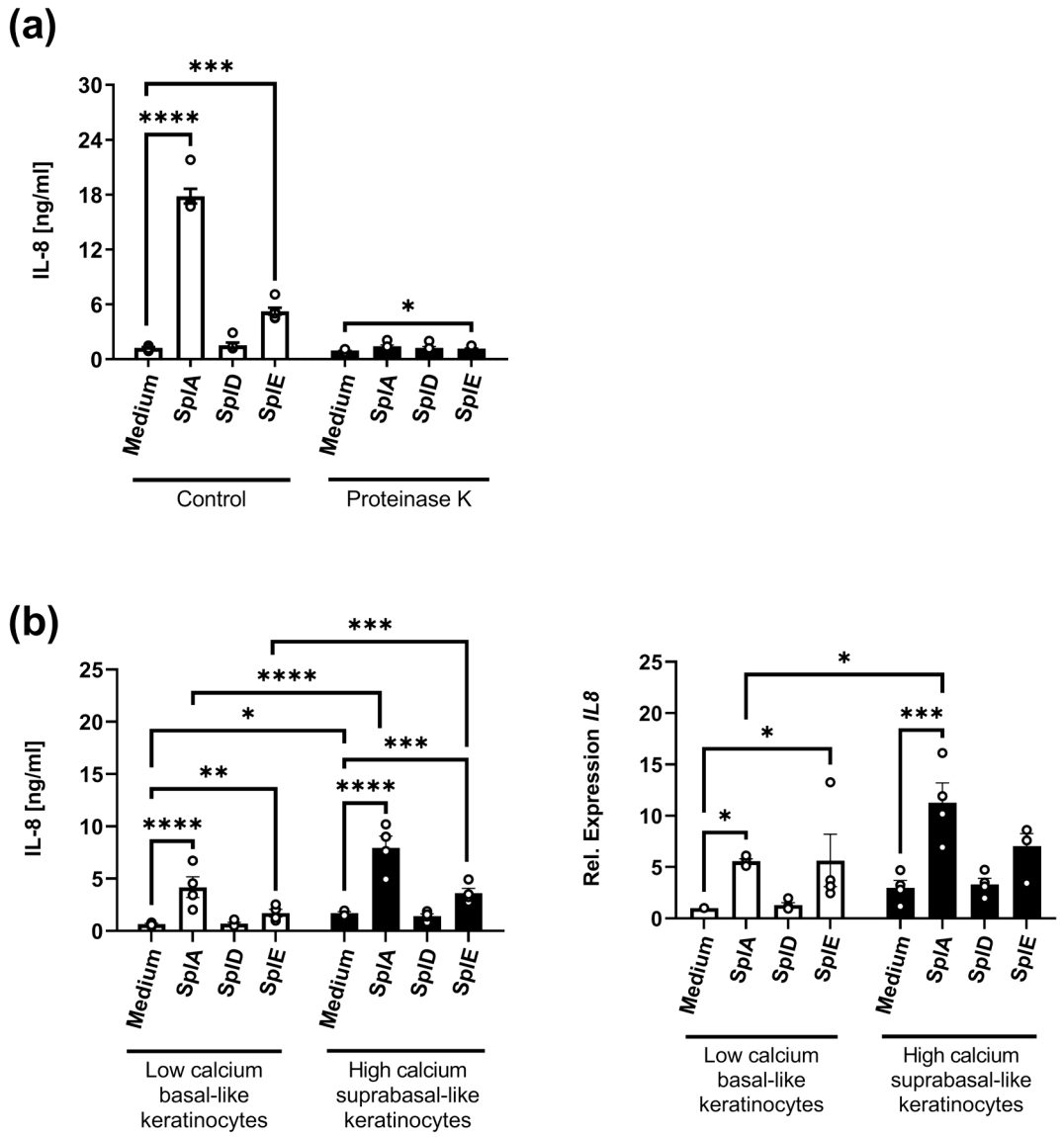

Fig. 1. SplA and SplE but not SplD increased IL-8 expression in a calcium-dependent manner in human keratinocytes. IL-8 production was assessed in (a) HaCaT cells stimulated with either native SplA, SplD or SplE (each 1 μg/ml) (white bars) or with proteinase K inactivated Spls (black bars) by ELISA and in (b) human primary keratinocytes from healthy individuals cultured with 0.06 mM calcium (white bars) for basal-like keratinocytes, and with 1.50 mM calcium for suprabasal-like keratinocytes (black bars) in combination with SplA, SplD or SplE (each 1 μg/ml) by ELISA and RTD-PCR at 48 h. Means ± S.E.M. of six independent experiments with HaCaT cells and four independent experiments with human primary keratinocytes are shown. Statistical analysis was performed by two-way ANOVA and Sidak's multiple comparisons (*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001).

induced the strongest IgE response in a serum IgE-binding assay in asthmatic patients and healthy adults. [33] SplD was included since it causes a Th2-driven eosinophilic airway inflammation after intratracheal application into mice. [34] While there was no increase of IL-8 after SplD stimulation, SplE and especially SplA increased IL-8 production in keratinocytes compared to unstimulated cells. Since inactivated Spls did not induce IL-8, we concluded that IL-8 secretion was induced by the Spls (Fig. 1a). With the addition of increasing concentrations of extracellular calcium (0.06 to 1.50 mM), we mimicked undifferentiated keratinocytes of the basal-like (0.06 mM calcium) or differentiated keratinocytes of the suprabasal-like (1.50 mM calcium) epidermal layer as shown by the induction of *cytokeratin* (*CK*) 1, *CK10* and *involucrin (IVL)* gene expression (Supplementary Information, Fig. S1). IL-8 production of primary human keratinocytes after stimulation with Spl proteases was comparable to HaCaT cells. SplE and particularly SplA but not SplD increased IL-8 transcription and secretion in HaCaT cells and primary keratinocytes. The response of suprabasal-like keratinocytes to SplA and SplE was higher than that of basal-like keratinocytes (Fig. 1b).

To determine whether higher calcium concentrations increased IL-8 directly or keratinocyte differentiation results in increased IL-8 secretion, we pre-treated keratinocytes with low or high calcium for 24 h to induce keratinocyte differentiation. Then we stimulated the sets of low and high calcium pre-treated keratinocytes with Spls, either in low or high calcium, for additional 24 h to identify calcium-dependent effects (Supplementary Information, Fig. S2a). Suprabasal-like keratinocytes pre-treated in high calcium and stimulated in low calcium showed an increased Spl-mediated IL-8 release compared to basal-like keratinocytes pre-treated and stimulated only in low calcium. IL-8 release was further increased when suprabasal-like keratinocytes were pre-treated and stimulated in high calcium (Supplementary Information, Fig. S2a). The calcium-dependent effect in suprabasal-like keratinocytes affected the responsiveness to SplE stronger than the differentiation effect, whereas calcium-induced keratinocyte differentiation and calcium-dependent effects equally influenced responsiveness to SplA (Supplementary Information, Fig. S2b). Thus, we concluded that both keratinocyte differentiation and extracellular calcium substitution increased responsiveness to Spls inducing IL-8 production.

3.2. SplA mediated IL-8 induction was enhanced by a Th17 cytokine milieu, whereas IL-8 secretion was independent of a Th2 cytokine milieu in human primary keratinocytes

To test if a Th2 or a Th17 cytokine milieu affects the Spl-mediated reactivity of keratinocytes, we assessed the response of basal-like and suprabasal-like keratinocytes to Spls in the presence of Th2 (IL-4 and IL-13) or Th17 (IL-17A and IL-22) cytokines.

First, we showed a successful Th2 cytokine stimulation of keratinocytes by the secretion of CCL2 (Supplementary Information, Fig. S3a) and a Th17 cytokine stimulation by the secretion of HBD2 (Supplementary Information, Fig. S3b).

When assessing keratinocytes primed in a Th2 cytokine milieu, there was no significant difference of Spl-mediated IL-8 secretion to control keratinocytes without Th2 cytokine priming (Fig. 2a). These results under Th2 cytokine milieu were also independent of high or low calcium levels. In contrast, priming of keratinocytes with Th17 cytokines increased the Spl-mediated IL-8 production (Fig. 2b). Here in response to SplE and SplA, respectively, the pattern remained the same as in cytokine-free control with moderate to higher IL-8 release, while SplD did not induce IL-8 production compared to control. The highest IL-8 response to SplA and SplE was observed in medium with high calcium and thus highly differentiated keratinocytes. While there was a clear effect of IL-17A priming of keratinocytes, IL-22 priming had no significant effect on IL-8 secretion in presence of IL-17A. Conclusively, a Th17 cytokine milieu increased IL-8 secretion during Spl stimulation in both basal-like and suprabasal-like keratinocytes while a Th2 cytokine milieu did not alter Spl-induced IL-8 response.

3.3. SplA amplified Th17 cytokine-induced neutrophil migration in healthy keratinocytes

To assess the functional consequences of the SplA- and SplE-induced IL-8 secretion in keratinocytes, we studied their effects on neutrophil migration with the use of a neutrophil migration assay. While increasing recombinant IL-8 protein correlated with the increased migration of neutrophils (Supplementary Information, Fig. S4a) neither Spls, Th2/Th17 cytokines, nor low or high calcium concentrations alone had marked effects on neutrophil migration indicating that the migration of neutrophils is mainly attributed to IL-8 induction (Supplementary Information, Fig. S4b and c).

However, supernatants of Spl-stimulated keratinocytes cultured in presence or absence of Th2 or Th17 cytokines, showed some effects on neutrophil migration dependent on calcium concentration and the specific Spls (Fig. 3a and b). Supernatants of keratinocytes stimulated with Th2 cytokines showed a slight and not significant induction in neutrophil recruitment, when used in a low dilution to 50 pg/ml IL-8 in the medium control. In medium control and a Th2 cytokine milieu there was no calcium-dependent effect on neutrophil migration (Supplementary Information, Fig. S5a). In supernatants of suprabasal-like keratinocytes but not of basal-like keratinocytes, a Th2 cytokine priming of keratinocytes slightly increased the neutrophil migration mediated by SplA and SplE compared to supernatants of unprimed keratinocytes (Fig. 3a). Supernatants of both, basal-like and suprabasal-like keratinocytes primed in a Th17 cytokine milieu without Spls showed increased neutrophil migration, while supernatants of Th17 cytokine primed suprabasal-like keratinocytes showed the highest neutrophil migration (Supplementary Information, Fig. S5b). Furthermore, a direct comparison of neutrophil migration into supernatants of keratinocytes primed only with IL-17A or with IL-17A and IL-22 excludes an inhibitory effect of IL-22 independent of the presence of SplA (Supplementary Information, Fig. S5c and S5d). A neutrophil migration plateau was reached with supernatants of Th17 cytokine primed and Spl stimulated suprabasal-like keratinocytes with a low dilution to 50 pg/ml IL-8 in the medium control (data not shown). Therefore, we diluted all supernatants of Th17 cytokine primed keratinocytes adjusted to a medium control dilution of 10 pg/ml IL-8 to obtain the optimal neutrophil migration range. In accordance to the assessment of IL-8 secretion, supernatants of suprabasal-like keratinocytes primed with Th17 cytokines and stimulated with SplA had the most significant effects on neutrophil migration (Fig. 3b). The supernatant of SplA-stimulated suprabasal-like keratinocytes cultured in presence of Th17 cytokines significantly increased neutrophil migration compared to supernatant from suprabasal-like keratinocytes stimulated without SplA and with Th17 cytokines (Fig. 3b).

To assess whether the response of keratinocytes to Spls and neutrophil migration is different in primary keratinocytes with impaired STAT3 signaling, we compared keratinocytes of a healthy individual with keratinocytes of a STAT3-HIES patient carrying the heterozygous dominant-negative *STAT3* mutation c.1145C > A (p.R382Q). The keratinocytes of the STAT3-HIES patient showed slightly lower SplA and SplE-mediated IL-8 secretion, while neutrophil migration was not obviously different to healthy control keratinocytes (Fig. 4).

4. Discussion

S. aureus is implicated in the pathophysiology of Th2-dominated atopic diseases.[7,41,42] Until now, however, the contribution of recurrent *S. aureus* infections to eczematous skin lesions in patients with an imbalanced Th2 and Th17 immunity is not fully elucidated.

To test the effects of *S. aureus*-specific SplA, SplD and SplE on the epithelial immune response we assessed human keratinocytes in defined cytokine milieus and cellular differentiations. Despite notable sequence similarities between SplA, SplD, and SplE [32], we showed that SplE and to a greater extent SplA and not SplD stimulate IL-8 secretion. The exact

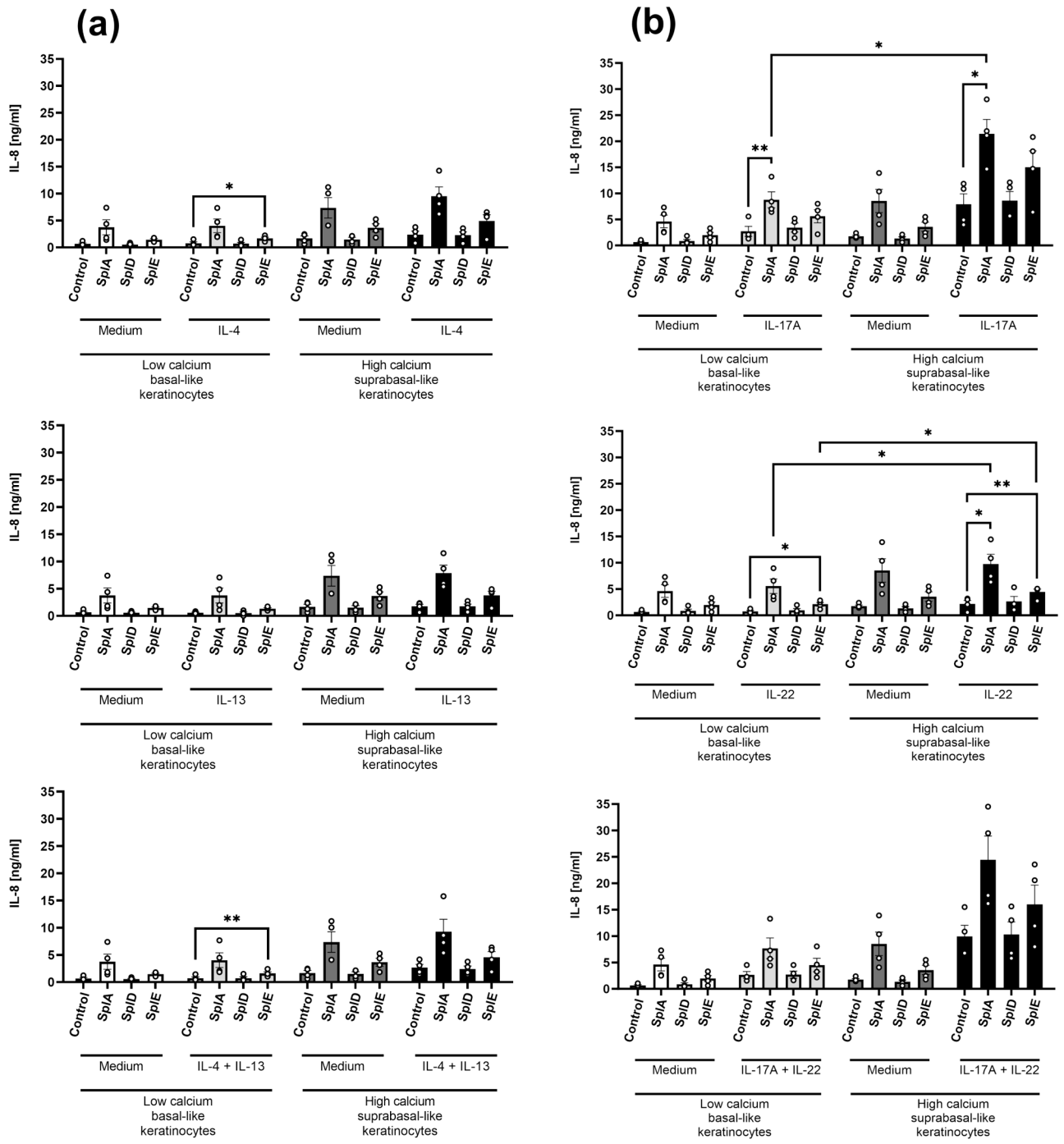

Fig. 2. Th17 but not Th2 cytokine milieu enhances Spl-mediated induction of IL-8 by primary keratinocytes. IL-8 secretion was assessed by ELISA in human primary keratinocytes from healthy individuals stimulated with SplA, SplD or SplE (each 1 μg/ml) in medium with low calcium (0.06 mM) for basal-like keratinocytes or with high calcium (1.50 mM) for suprabasal-like keratinocytes for 48 h. (a) Th2 cytokines (IL-4 and IL-13) or (b) Th17 cytokines (IL-17A and IL-22) (each 20 ng/ml) were added for 48 h. Means ± S.E.M. of four independent experiments are shown. Statistical analysis was performed by two-way ANOVA and Sidak's multiple comparisons (*$p < 0.05$ and **$p < 0.01$).

mechanisms how Spls recognize and cleave their substrates are not yet fully known. However, the importance of Spl protease activity on keratinocytes was emphasized by the fact, that inactivation of Spls significantly reduced their effects on IL-8 release in our hands. In a next step using irreversible serine protease inhibitors or Spl-mutant proteins might provide additional insights if there are unique substrate specificities of the three Spls. [43–45].

Spls are considered as bacterial allergens and known to trigger a Th2-biased immune response in both humans and mice. Recently it has been identified that SplB cleaves and activates the proteinase-activated receptor-2 (PAR2) in mice and mediates endothelial inflammation. [46] In addition, SplD has been reported to attract PAR2-positive cells into the airways triggering a Th2-biased reaction. [47] Keratinocytes express both, Toll-like receptors (TLRs) and PAR2 [48,49] and there is a direct and indirect cross-talk [50,51] between PAR2 and TLRs. Therefore, it is tempting to speculate that the here observed Spl effects in human keratinocytes are mediated by PAR2 and/or TLRs. The detailed interplay between Spls, PAR2 and TLRs in keratinocytes, however, needs further

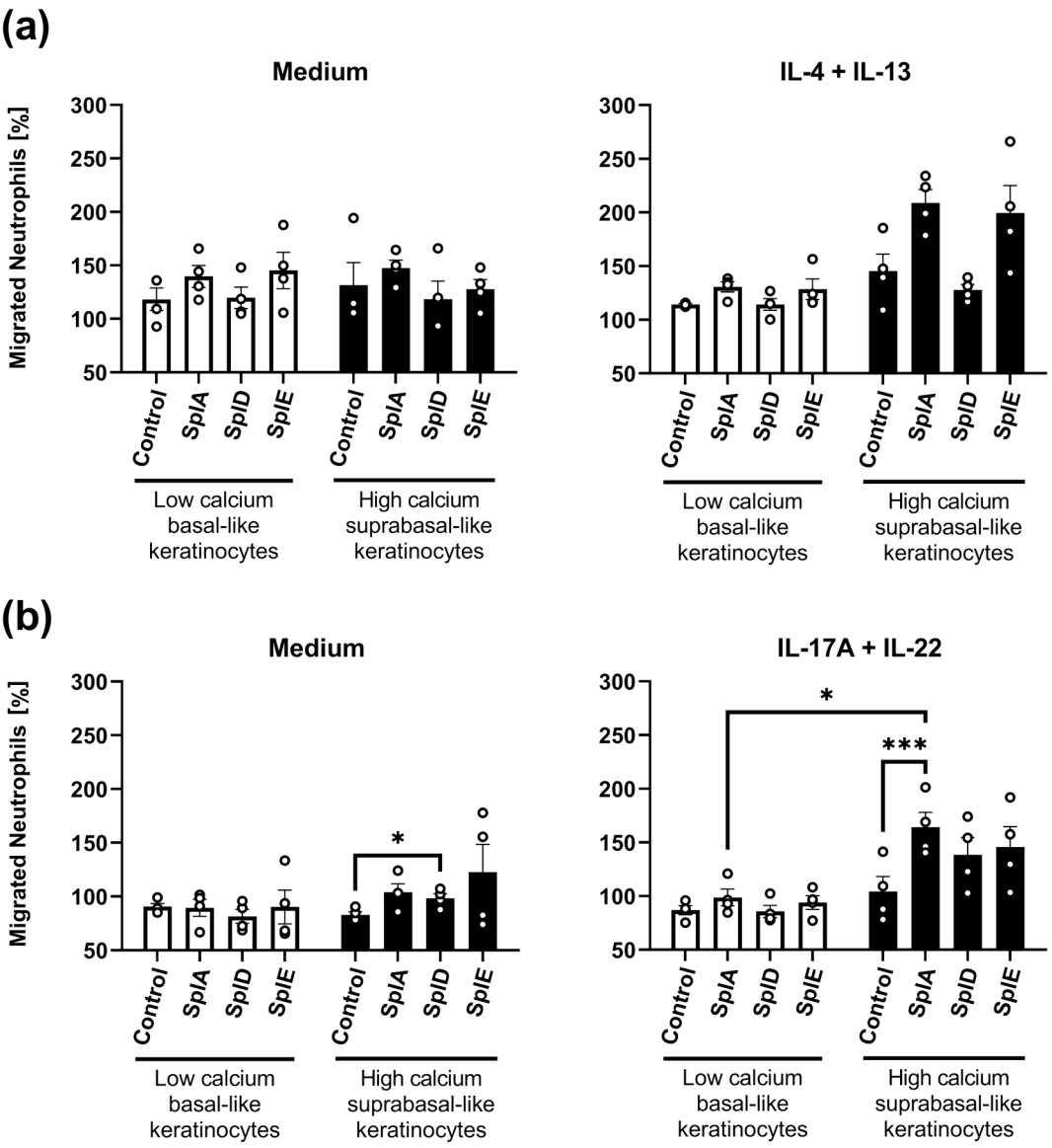

Fig. 3. Spl-mediated neutrophil migration into supernatants of Th2 or Th17 cytokine primed keratinocytes. Migration of neutrophils from a healthy donor into supernatants of Spl- and cytokine-treated basal-like and suprabasal-like keratinocytes. (a) Migration of neutrophils in absence (medium) and presence of Th2 cytokines (IL-4 + IL-13) relative to passive migration into Lifeline medium. Supernatants were diluted relative to 50 pg/ml IL-8 in low calcium medium control. (b) Migration of neutrophils in absence (medium) and presence of Th17 cytokines (IL-17A + IL-22) relative to passive migration into Lifeline medium. Supernatants were diluted relative to 10 pg/ml IL-8 in low calcium medium control. Neutrophils were counted after 1 h migration by flow cytometry. Means ± S.E.M. of four independent experiments are shown. Statistical analysis of neutrophil migration was performed by using two-way ANOVA and Sidak's multiple comparisons (*$p < 0.05$ and ***$p < 0.001$).

assessment.

The six *spl* genes are encompassed on the spl operon. [32] The spl operon is present in most clinical *S. aureus* isolates but the composition of the *spl* genes varies between *S. aureus* strains and is currently not attributable to specific *S. aureus* infections. [52] To our knowledge, there is no detailed information on the *spl* genes in *S. aureus* strains from patients with atopic dermatitis. Data from whole genome sequencing may provide more information. However, due to the large heterogeneity of *S. aureus* strains in atopic dermatitis [53] likely a huge number of *S. aureus* isolates will be required.

We observed a higher responsiveness of differentiated compared to less differentiated keratinocytes to Spls, implying that the outer skin layers may be more involved in activating an inflammatory immune response to *S. aureus* than skin layers near the basal lamina. The latter is reflected by studies that outlined that the amount of IL-8 in the outer epidermal skin layers is related to the severity of local skin inflammation in atopic dermatitis. [54,55].

An epidermal calcium gradient is known to promote keratinocyte differentiation and barrier function. In atopic dermatitis impairments of both epithelial barrier and calcium gradient have been reported. [56–58] In addition, markers for terminal differentiation of keratinocytes are less expressed in atopic dermatitis. [59] Since next to calcium, Th2 cytokines also attenuate differentiation of keratinocytes, [60] we speculate that the interaction of cytokines and calcium significantly influences the antimicrobial defense due to impaired keratinocyte differentiation. Furthermore, a balanced Th2 and Th17 milieu has been reported to be crucial for the epithelial immunity to *S. aureus*, antimicrobial peptide expression and severity of atopic dermatitis. [61–63] Keratinocytes exposed to Th2 cytokines are known to be less differentiated, [64] express lower levels of defensins, [65] and have a higher ability to recruit eosinophils. [66] In contrast to these reported Th2 effects, the IL-8 secretion after Spl stimulation was not significantly

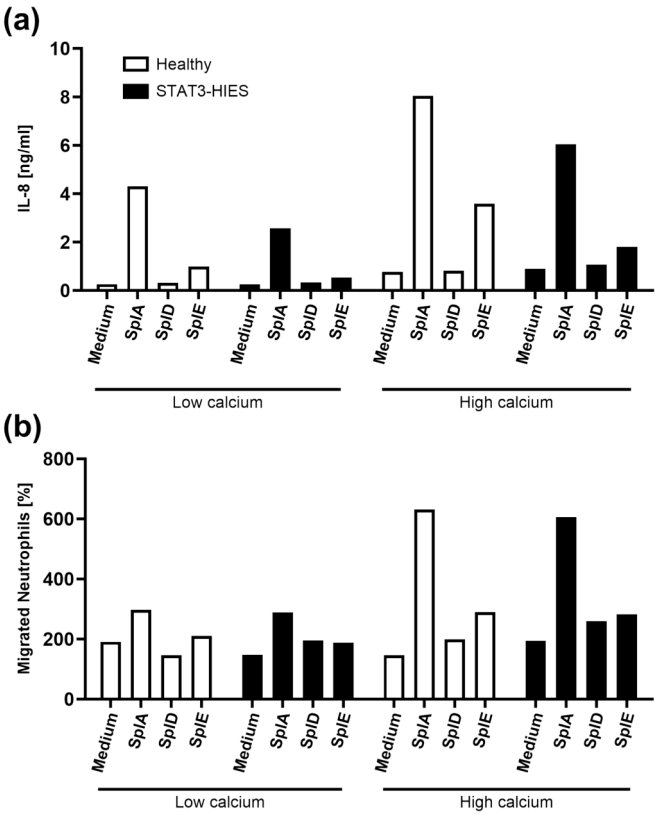

Fig. 4. Spl-mediated IL-8 immune response in STAT3-HIES keratinocytes corresponds to healthy keratinocytes. (a) Secretion of IL-8 by healthy (white) and STAT3-HIES (black) keratinocytes from one donor each stimulated with SplA, SplD or SplE in low or high calcium for 48 h. (b) Migration of neutrophils relative to passive migration into Lifeline medium from one healthy donor into supernatants of healthy (white) or STAT3-HIES (black) keratinocytes. In this representative experiment IL-8 secretion was measured at 48 h by ELISA, and neutrophil migration at 1 h by flow cytometry.

affected in our Th2 primed experiments. It is known that Th2 cytokines promote a proinflammatory response in atopic dermatitis by increasing the production of Th1-associated molecules from keratinocytes and the recruitment of T cells. [67] In our study we focused on the predominant cytokine milieu during the acute phase of atopic dermatitis and showed for the first time a synergizing effect of Spls and a Th17 cytokine milieu on human epidermal immune responses. However, Spls may also emphasize a Th1-effect.

Along with reports that Th17 cytokines strengthen epithelial defense by promoting β-defensins, IL-8 production and neutrophil recruitment, [15,68–70] our IL-17A primed keratinocytes showed increased production of HBD2 and IL-8 with significant differences in suprabasal-like keratinocytes. We studied neutrophil migration mediated by the combination of IL-17A and IL-22 due to our experiments reported for IL-8. The observed differences in the regulation of IL-8 and HBD2 in the IL-17 and IL-22 co-treatment, however, may hint to different signaling pathways regulated by IL-22, which will be of interest but beyond the scope of the here presented work.

That IL-17A in combination with SplA and SplE further increased IL-8 production proposed an amplifying role of Th17 but not Th2 cytokines for Spl-mediated IL-8 secretion in suprabasal skin layers. To assess the extent to which keratinocyte-derived factors including IL-8 support the infiltration of neutrophils into the epidermis, we tested the migration of neutrophils to supernatants of Spl- and cytokine-treated keratinocyte subtypes. IL-8 activates innate immune responses and defense against extracellular pathogens by recruiting neutrophils to the site of infection.[71] In consistence with our IL-8 results, supernatants of SplA and SplE but not SplD stimulated keratinocytes increased migration of neutrophils. Similar to our findings that SplD had negligible effects on neutrophil migration, a previous study has shown that repeated tracheal application of SplD resulted in eosinophilia without neutrophil migration into the lung tissue of mice. [34].

Since STAT3-HIES patients have an impaired production of Th17 cytokines and are like atopic dermatitis patients affected by recurrent, eczematous skin lesions, [72] we took the chance to assess keratinocytes of a single STAT3-HIES patient to address the question if STAT3 activity is essential for IL-8 secretion and neutrophil migration mediated by Spls in keratinocyte. While the investigated STAT3-HIES keratinocytes secreted slightly reduced IL-8 but initiated migration of neutrophils similar to healthy control keratinocytes, we assume that the incomplete clearance of *S. aureus* in the skin of STAT3-HIES patients is less due to an intrinsic epithelial STAT3 response to bacterial components such as Spls and more due to the known lack of IL-17. [19,20,73] To draw final conclusions, however, further studies predominantly focusing on STAT3-HIES are required since only keratinocytes of one STAT3-HIES patient were assessed.

The fact that SplD has been reported to cause an eosinophilic Th2 response in airways of mice, [34] lead us to speculate that *S. aureus*-specific Spls trigger both an eosinophilic Th2- and neutrophilic Th17-like immune response. Since increased eosinophilia and elevated IL-8 levels are associated with worsening of atopic dermatitis, [54,74] it is conceivable that cellular effects of Spls impact severity of atopic dermatitis dependent on keratinocyte differentiation.

Taken together, our results highlight that *S. aureus*-specific SplA, SplD and SplE provoke a distinct host immune response on keratinocytes with SplA and SplE synergizing with IL-17A for IL-8 release and neutrophil migration underscoring the importance of Th17 immunity in barrier function. Our results support the concept that patients with impaired keratinocyte differentiation and skin barrier defects such as patients with atopic dermatitis have an impaired anti-microbial response and favor *S. aureus* infections, even though *S. aureus* elicits a pro-inflammatory immune response.

CRediT authorship contribution statement

D.P. De Donato: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **R. Effner:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **M. Nordengrün:** Methodology, Writing – review & editing. **A. Lechner:** Investigation, Methodology, Writing – review & editing. **M.N. Darisipudi:** Formal analysis, Methodology, Writing – review & editing. **T. Volz:** Resources, Writing – review & editing. **B. Hagl:** Methodology, Supervision, Writing – review & editing. **B.M. Bröker:** Funding acquisition, Methodology, Resources, Writing – review & editing. **E.D. Renner:** Conceptualization, Supervision, Validation, Visualization, Writing – review & editing, Data curation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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