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Tissue microenvironment initiates an immune response to structural components of *Staphylococcus aureus*

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Abstract

Cell-to-cell communication in skin participates to the maintenance of homeostatic responses to foreign substances. Certain strains of *Staphylococcus (S) aureus* are vicious pathogens that cause deleterious effects in host cells and tissues. Both secreted toxins and structural components of *S. aureus* trigger an immune response, though how *S. aureus* stimulates host immune responses is poorly understood. We explored here how keratinocytes and fibroblasts initiate the first steps of an immune response by activating dendritic cells (DCs) through recognition of structural components of *S. aureus*. We treated monocyte-derived Langerhans cells (moLCs) and monocyte-derived DCs (moDCs) with conditioned media from keratinocytes (K-CM) and fibroblasts (F-CM) treated with heat-killed *S. aureus* (HKSA) respectively, or directly with HKSA. Immune and inflammatory responses from keratinocytes, fibroblasts, moLCs and moDCs were assessed by analysis of cell

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AUTHOR CONTRIBUTION

CM designed and performed the experiments, analysed the results and wrote the manuscript. TP helped designing, performing and analysing some experiments. PM and YU assisted the data analyses and the writing of the manuscript. SB, BC, WG reviewed the manuscript. PM and YU reviewed and revised the manuscript. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Phenotypical characterization of in vitro generated monocyte-derived LCs and DCs. (a) moLCs and moDCs were differentiated for 6 days and their morphology was observed under the microscope. moLCs and moDCs displayed dendrites characteristics of dendritic cells. Scale bar = 100 μm. (b) Surface expression of the critical monocyte marker CD14, the DC markers CD1a and CD11c, the DC specific protein CD209 (DC-SIGN), the LC specific protein CD207 (Langerin) and maturation markers HLA-DR and CD86 was examined by flow cytometry. Histograms show surface expression of antigens (unfilled, isotype filled) of 24 000 counted cells of gated scatter plot. Analysis is representative of three independent experiments

Figure S2. Modulation of Langerin expression in moLCs by HKSA and K-CM treatments. Expression of surface markers was analyzed through flow cytometry. (a, c) Percent of moLCs expressing Langerin or not under several treatments. (b, d) Expression of HLA-DR and CD86 characterized activated cells expressing Langerin or not. (e) Intensity of mean fluorescence of Langerin. Data represent mean values ± SEM (n = 3–4). Paired *t* test with statistical data represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S1. Primers used in real time PCR

Table S2. Antibodies used for flow cytometry.

Table S3. Protein expression of pro-inflammatory markers in control-and HKSA-treated keratinocytes and fibroblasts

surface markers and cytokine production using flow cytometry, real-time PCR and ELISA assays. K-CM and F-CM increased the expression of CD86 and HLA-DR on moLCs and moDCs, in association with a specific cytokine profile. K-CM upregulated *TNFA*, *IL-1B* and *GM-CSF* mRNA expression in moLCs, while F-CM upregulated *IL-12* and downregulated *TNFA* and *TGFB* mRNA expression in moDCs. Additionally, F-CM attenuated the induction of an inflammatory profile in monocytes. The recognition of structural components from *S. aureus* by cutaneous microenvironment induces the activation and the expression of specific cytokines from LCs and DCs.

Keywords

dendritic cells; inflammation; microenvironment; *Staphylococcus aureus*; structural components

1 | INTRODUCTION

In the skin, keratinocytes and fibroblasts, which are the dominant cell species in the epidermis and the dermis respectively, are largely responsible for tissue homeostasis and interact with surrounding cells including cutaneous DCs. The establishment of this tissue microenvironment is mediated through a wide biosynthetic repertoire[1,2] that facilitates the crosstalk between cells. Prior studies have shown that epidermal keratinocytes influence the epithelial residence of DCs and memory T cells through transforming growth factor (TGF)-β signaling,[3] while stromal cells and components of the extracellular matrix regulate DC differentiation and maturation.[4,5] Dermal fibroblasts can also regulate DC migration in an interleukin (IL)-6 dependent manner and through IL-6 induced expression of MMP-9.[6]

Langerhans cells (LCs), which are DCs localized in the epidermis, and dermal DCs are the coordinators of the innate and adaptive immune system[7,8] and initiate the immune response upon recognition of foreign antigens. Along with keratinocytes and fibroblasts, DCs recognize pathogens through the expression of receptors of the innate immune system (ie, Toll-like receptors, TLRs) and create a tissue microenvironment that alerts effector immune cells.[9–12] *Staphylococcus (S) aureus* stands as a prominent virulent pathogen that invades and colonizes tissues including skin. Through the secretion of various toxins and the recognition of its structural components,[13–15] *S. aureus* generates a high inflammatory microenvironment and immune responses characterized by the activation of T helper (Th) 1-Th17 polarizing DCs.[11,16,17]

However, it is still not elucidated how *S. aureus* structural components generate an inflammatory microenvironment that leads to DCs activation. We focus here on the initiation of an immune response and especially DCs activation mediated by keratinocytes and fibroblasts secretions in response to structural components of *S. aureus.*

2 | METHODS

2.1 | Generation of heat-killed *S. aureus*

Staphylococcus aureus was heat-killed to avoid the effect of poreforming toxins, exfoliative toxins, superantigens, exoenzymes, etc. and to focus only on the effect of wall components.

Staphylococcus aureus strain USA 300 (a generous gift from Dr. Paul Sullam, UCSF) was cultured and heat-killed as in[18] with the following modifications: addition of 10 μg/mL chloramphenicol (Sigma Aldrich, MilliporeSigma, St. Louis, MO) to culture medium and agar plates, heat inactivation at 68°C and optical density measured at λ = 650 nm.

Heat-killed *S. aureus* (HKSA) was stored at 1×10^9 CFU/mL in aliquots in PBS without Ca^{2+}/Mg^{+} (Gibco, Thermo Fisher Scientific, Waltham, MA) at −20°C until experimental use.

2.2 | Isolation and culture of skin cells and generation of keratinocyte-conditioned medium and fibroblast-conditioned medium

Keratinocytes and fibroblasts were isolated from normal human juvenile foreskins as described in Ref. [19] and under an approved protocol by the Committee on Human Research, University of California San Francisco and Veteran Affairs Medical Center, San Francisco.

Keratinocytes were grown in medium 154 supplemented with Human Keratinocyte Growth Supplement, 0.07 mmol/L Ca^{2+} (all Gibco) and 100 U/mL penicillin and 100 μg/mL streptomycin (Corning, Union City, CA). Fibroblasts were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 100 U/mL penicillin and 100 μg/mL streptomycin (Corning).

Keratinocytes (2×10^4 cell/cm^2) and fibroblasts (5×10^4 cell/cm^2) were grown in their respective culture media and treated with 108 HKSA/mL for 4 hours. After three washes, media were refreshed. Keratinocyte-conditioned medium (K-CM) and fibroblast-conditioned medium (F-CM) were collected 24 hours later, centrifuged at 200 ***g*** for 5 minutes and filtered on 0.22 μm PVDF filter membrane (EMD Millipore, Temecula, CA). Fetal bovine serum and calcium concentrations were adjusted to 2% and 1.8 mmol/L, respectively. Conditioned media were aliquoted, labeled as Control or HKSA (from cells without/with HKSA treatment respectively) and stored at −80°C.

2.3 | Generation and stimulation of monocyte-derived LCs and DCs and monocytes

$CD14^+$ monocytes were sorted using the Human Monocyte Isolation Kit II (Miltenyi Biotech Inc., San Diego, CA) from peripheral blood mononuclear cells obtained from buffy-coats of three healthy donors (Blood Centers of the Pacific, San Francisco, CA), under an approved protocol by the Committee on Human Research, University of California San Francisco, and Veteran Affairs Medical Center, San Francisco. The purity of $CD45^+$ $CD14^+$ monocytes was ≥90% as assessed by flow cytometry (data not shown).

The basal medium used for all immune cells was RPMI-1640 (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Gibco) and 100 U/mL penicillin and 100 μg/mL streptomycin (Corning).

Monocyte-derived Langerhans cells (moLCs) and dendritic cells (moDCs) were generated by culturing monocytes with GM-CSF (50 ng/mL for moDCs or 100 ng/mL for moLCs; Shenandoah Biotechnology Inc, Warwick, PA), IL-4 (20 ng/mL for moDCs and 10 ng/mL

for moLCs; Shenandoah Biotechnology Inc) and TGF-β1 (10 ng/mL for moLCs; Shenandoah Biotechnology Inc) as described previously.[20] Cell differentiation and maturation of moLC/moDC were assessed by recording changes in cellular shape under the microscope, and changes in characteristic cell surface markers (Figure S1). For stimulation of moLCs, moDCs and monocytes, 10^6 cells/mL were cultured in 24-well plates for 4 hours (real-time PCR analysis) or 24 hours (FACS analysis) with or without HKSA (10^8/mL), K-CM and F-CM.

2.4 | RNA isolation and Real-time PCR

Total RNA was isolated from cell lysates using Isolate II RNA Mini kit, followed by cDNA synthesis using Tetro cDNA synthesis kit (all Bioline, Taunton, MA), according to manufacturer's instructions. Real-time PCR was performed on a ViiA7 Real-Time PCR system (Thermo Fisher Scientific). Relative gene expression was analysed using SensiMix SYBR kit (Bioline) with the following thermal cycling conditions: 95°C for 12 minutes; 95°C for 15 seconds, 60°C for 1 minute, repeated 40 times. The expression of human genes *IL-1B, IL-6, IL-8, IL-10, IL-12, TNFA, GM-CSF, TGFB*, *TLR2, CD14, CD16 and CD68* were analysed. Human *GAPDH* was used as a reference gene. See Table S1 for primer sequences.

2.5 | Flow cytometry

The cell surface expression of various proteins was analysed by seven to eight colours flow cytometry. Cells were labelled with fluorophore-conjugated monoclonal antibodies and corresponding isotype control (See Table S2) and were examined using a LSR II flow cytometer (BD Biosciences, San Jose, CA) collecting a total of 1×10^4 to 3×10^4 events. Dead cells and debris were excluded by scatter gates and Fixable Viability Dye eFluor™ 450 staining (1:1000 dilution; eBiosciences, Thermo Fisher Scientific). MoLC were gated as live $CD45^+$ $CD14^-$ $CD1a^+$ $CD207^+$ $CD209^-$ HLA-$DR^{+/-}$ $CD86^-$, moDC as live $CD45^+$ $CD14^-$ $CD1a^+$ $CD209^+$ $CD207^-$ HLA-$DR^{+/-}$ $CD86^-$.

2.6 | Multiplexed cytokine assay

Inflammatory secretions were analysed from cell supernatants obtained from three independent experiments and were tested two different times using a VPLEX Proinflammatory Panel 1 (human) Kit (MSD, Rockville, MD), according to the manufacturer's instructions. Samples were diluted at (1:2) or (1:4) and analysed on a MSD Sector Imager 2400 device (MSD). Background was subtracted, and data were adjusted to the standard curve.

2.7 | Statistical analysis

Data are shown in column format as mean with SEM. Statistical significance was evaluated by a paired *t* test or unpaired *t* test, as indicated in the text; ns (non-significant), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Statistical analysis was conducted using GraphPad Prism software (La Jolla, CA).

3 | RESULTS

Epidermal keratinocytes, dermal fibroblasts and immune cells respond to the bacterial pathogen *S. aureus*. In the present study, we investigated how keratinocyte and fibroblast inflammatory responses towards *S. aureus* could mediate the initiation of an immune response, focusing on LC/DC/monocyte activation.

3.1 | Monocyte-derived LCs exhibit a mild activation profile under keratinocyte inflammation compared to HKSA

We studied how the microenvironment of keratinocytes challenged by HKSA would affect moLCs activation comparing to a direct HKSA stimulation.

The percentage of moLCs expressing both activation markers HLA-DR$^+$ and CD86$^+$ was significantly increased by treatment with K-CM or HKSA ($P < 0.05$, Figure 1A). HKSA significantly activated moLCs (99.5 ± 0.2% with HKSA compared to 5.7 ± 0.4% with control). K-CM HKSA also activated moLCs (15.2 ± 2.7%) compared to its respective control (8.4 ± 1.6%). The activation status was assessed by an increase in the level of CD86 that was quantified by the geometrical mean of fluorescence intensity (100-fold increase, $P = 0.05$ Figure 1B). Interestingly, we noticed that moLCs activation was accompanied with a decrease of Langerin surface expression (Figure S2, $P < 0.05$).

Cell surface activation was correlated with the expression of pro-inflammatory cytokines. K-CM HKSA treatment induced the significant upregulation of *TNFA* expression (3.1 ± 0.8, $P = 0.04$) in moLCs (Figure 1D). Conversely, HKSA treatment induced the up-regulation of several pro-inflammatory cytokines: *IL-1B* (39.1 ± 6.1, $P = 0.03$), *IL*-8 (25.2 ± 7.3, $P = 0.03$) and *IL*-12 (54.0 ± 16.3, $P = 0.02$) expression compared to Control in moLCs (Figure 1D). These results were confirmed at the protein level in addition to an increase in IL-6, TNF-α and IL-10 secretion under HKSA treatment compared to control (Figure 1E, $P < 0.05$).

Together, these results highlight an effect of keratinocytes secretions in sustaining a low activation profile in moLC in response to HKSA, whereas direct HKSA stress induces an inflammatory response in moLC.

3.2 | Monocyte-derived DCs exhibit a moderate activation profile under fibroblast inflammation compared to HKSA

We next examined the response of moDCs when exposed to HKSA-challenged fibroblasts secretions or direct HKSA stimulation.

Both treatments increased the number of activated HLA-DR$^+$ CD86$^+$ moDCs in our model as depicted on Figure 2A ($P < 0.05$). F-CM HKSA induced a 1.6-fold increase in activated moDC (35.3 ± 5.7%) compared to F-CM Control (22.5 ± 4.6%), whereas HKSA induced a 3.3-fold increase in activated moDCs (69.4 ± 13.1%) compared to Control (21.0 ± 9.6%). The activation status was assessed by an increase in the level of CD86 that was quantified by the geometrical mean of fluorescence intensity (2.8-fold increase $P = 0.05$ Figure 2C).

The upregulation of cell surface markers of activation was associated with the expression of pro-inflammatory cytokines. F-CM HKSA induced the significant upregulation of *IL-12* expression (13.1 ± 2.6, $P = 0.004$) compared to F-CM Control (0.7 ± 0.1) in moDCs (Figure 2D). Conversely, HKSA treatment induced the up-regulation of pro-inflammatory cytokines *IL-1B* (17.3 ± 3.7, $P = 0.01$), *IL-6* (42.0 ± 13.3, $P = 0.03$), *IL-8* (6.0 ± 1.0, $P = 0.009$) and *TNFA* (13.6 ± 1.6, $P = 0.0005$) compared to Control (Figure 2D). Similar results were observed at the protein level, although IL-12p70 was highly secreted under HKSA treatment in moDCs (Figure 2E). *TLR2* expression was not influenced by each of the treatment in moDCs (Figure 2D).

Together, these results highlight a role for the microenvironment of fibroblasts exposed to HKSA in inducing an activation profile in moDCs associated with a specific set of cytokines as opposed to the direct effect of HKSA inducing inflammation without distinction.

3.3 | Monocytes exhibit pro-inflammatory responses towards HKSA, but not with fibroblast secretions

During inflammatory events, immune cells, including blood-derived monocytes that play an essential role in bacterial infections, are recruited into inflamed skin sites and then differentiate into both macrophages and monocyte-derived DCs.[21,22] We tested how the microenvironment of fibroblasts challenged by HKSA would affect monocyte behaviour compared to HKSA stimulation. We first analysed the surface expression of CD68 and HLA-DR, markers expressed by macrophages.[23]

Fibroblast-conditioned medium HKSA decreased the level of fluorescence intensity of CD68 ($P = 0.02$) and of HLA-DR ($P = 0.02$) by a 3-and a 1.6-fold, respectively, compared to F-CM Control, whereas HKSA induced an increase in the level of CD68 by 2.6-fold ($P = 0.051$) compared to Control. No effect was observed on CD1a by neither F-CM HKSA nor HKSA treatment (data not shown).

These results were correlated with the number of cells expressing macrophage marker CD68 (Figure 3B–C). Under both treatments, we observed two populations that were discriminated by their level of CD14 expression: $CD14^{high}$ $CD68^{+}$ and $CD14^{low}$ $CD68^{+}$. We found HKSA treatment to increase the number of cells expressing CD68 especially on cells with low CD14 expression (9.3 ± 2.9% $CD14^{low}$ $CD68^{+}$ for HKSA compared to 1.8 ± 0.8% for Control, $P = 0.04$) and correlated this expression with an increase in cell size and granularity(Figure 3B). Conversely, F-CM HKSA decreased the number of cells expressing CD68 on the population expressing high levels of CD14 (1.3 ± 0.5% $CD14^{high}$ $CD68^{+}$ for F-CM HKSA compared to 9.2 ± 1.0% for F-CM Control, $P = 0.02$, Figure 3C). These populations remained small in size.

Monocytes depicted a low inflammatory profile under F-CM HKSA treatment as depicted by low *IL-8* expression (0.0 ± 0.0 with F-CM HKSA compared to 0.3 ± 0.1 with F-CM Control, $P = 0.02$), the decrease in *TLR2* expression (0.2 ± 0.1 with F-CM HKSA compared to 0.6 ± 0.0 with F-CM Control, $P = 0.02$) and the increase in *IL-10* expression (1.7 ± 0.2 with F-CM HKSA compared to 0.8 ± 0.1 with F-CM Control, $P = 0.02$, Figure 3D). A similar decrease was observed for *IL-1B*, *IL-6*, *TNFA* and *TGFB* although not statistical.

Moreover, we confirmed decreased expression of *CD68* and *CD16* under F-CM HKSA treatment ($P < 0.05$). Conversely, HKSA induced a high inflammatory profile in monocytes/macrophages with an increased expression in *IL-1B*, *IL-6*, *IL-8*, *IL-10* and *TNFA* ($P < 0.05$, Figure 3D). HKSA also induced an increase in *CD68* expression ($P = 0.02$, Figure 3D). HKSA-induced cytokine profile was confirmed at the protein level (Figure 3E).

Together, these results show opposite effects induced by fibroblast microenvironment or bacterial stimulation on monocytes, with an apparent anti-inflammatory effect of fibroblasts.

3.4 | Fibroblasts display increased reactivity to HKSA in comparison with keratinocytes

To further characterize the different inflammatory behaviours induced by keratinocytes and fibroblasts exposed to HKSA on moLCs/moDCs, we analysed the cytokines expressed in each conditioned media (Tables 1 and S3).

The expression of pro-inflammatory cytokines *IL-1B*, *IL-6* and *TNFA* was upregulated by 2.3–2.9-fold ($P < 0.05$) at the gene level in keratinocytes after HKSA stress compared to Control (Table 1). At the protein level, we could find upregulation of chemokine IL-8 by 2.4-fold ($P = 0.01$) in keratinocytes after HKSA stress compared to Control (Table S3).

The gene expression of *IL-6*, *IL-8* and *GM-CSF* (6.9 ± 1.2, 31.6 ± 8.3 and 13.1 ± 2.4, respectively—$P < 0.05$) increased in fibroblasts following HKSA stress compared to Control (Table 1). Consistent with changes in mRNA expression, the protein levels of IL-6 and IL-8 increased by 11.2-and 15.8-fold respectively ($P < 0.05$) following HKSA stress compared to Control (Table S3).

Interestingly, the inflammatory response under HKSA stress was found significantly higher in fibroblasts than keratinocytes ($P < 0.05$), including higher expression of *TLR2* in fibroblasts ($P < 0.05$), receptor responsible for *S. aureus* recognition (Table 1).

Together, these results show that keratinocytes and fibroblasts respond differently to *S. aureus* and that fibroblasts are more prone to induce an inflammatory microenvironment that would activate antigen-presenting cells.

4 | DISCUSSION

The importance of the tissue microenvironment has been highlighted in skin development, skin remodeling and immune responses.[24–27] Yet, studies on the impact of the cutaneous microenvironment and its functional relevance in DCs and LCs are sparse and do not focus on an inflammatory skin microenvironment generated by *S. aureus* wall components. Most studies focus only on the response of keratinocytes or fibroblasts, or effector immune cells as LCs and DCs alone.[11,17,28–30] Here, we studied how an inflammatory cutaneous microenvironment generated under HKSA stress would influence the initiation of an immune response, and in particular key events of LC/DC activation: maturation and cytokine release.

Keratinocytes and LCs, which are constantly exposed to external stressors, are the first lines of defense towards pathogens. In our model, despite modest increases in *IL-1B*, *IL-6* and

TNFA expression, keratinocytes exposed to HKSA displayed a low inflammation profile that was mostly restricted to IL-8 secretion as described elsewhere.[11,30,31] Accordingly, we found that under HKSA stress, keratinocytes secretions induced a mild activation of moLCs along with a basal inflammatory profile characterized by *TNFA* and *GM-CSF* expression, contributing to LCs viability and maturation.[32,33] Similar studies have shown the potential of keratinocytes supernatants to induce moLCs or THP-1 cells maturation markers.[34,35] Others have described a regulatory loop between keratinocytes and LCs, with LCs triggering TNF-α secretion from keratinocytes to sustain their activation and migration.[36] Interestingly, these results point towards a role for keratinocytes in maintaining a basal activation profile in LCs, further arguing for a sentinel role for keratinocytes.[37]

Fibroblasts also contribute to the immune response by alerting surrounding immune cells as DCs and monocytes of any danger. In our study, fibroblasts demonstrated high reactivity towards HKSAwith high secretions of pro-inflammatory effectors IL-6 and IL-8. Interestingly, this inflammatory microenvironment appeared to tune monocytes and moDCs activation. We found that fibroblast secretions were able to induce moDCs activation with a Th1 cytokine expression profile without the need for cell-cell interaction as described by Saalbach et al,[38] nor the addition of TNF-α and monocytes conditioned media.[39] This latter difference may be explained by the fact that Asadi and colleagues used conditioned media from untreated fibroblasts that was not containing the necessary activation signals required for DC maturation. As opposed to moDCs, fibroblasts secretions appeared to prevent early steps of monocytes differentiation and pro-inflammatory profile in our study. Although fibroblasts are typically described as contributing to monocytes chemotaxis,[40] recent macrophages studies have reported anti-inflammatory activities for fibroblasts on monocytes/macrophages.[41,42] Collectively, these data and our study highlight an interesting role for fibroblasts in fine tuning the proand anti-inflammatory balance.

When examining the generated conditioned media, we found that keratinocytes displayed less reactivity towards HKSA than fibroblasts, as evidenced by the cytokines expression profiles and the level of moLC/moDC activation. Accordingly, we found that the expression of TLR2, receptor specialized in the recognition of bacterial trior di-acyl lipopeptides including *S. aureus* peptidoglycan and lipoteichoic acid, two of its major wall components, [13,43] was significantly lower in keratinocytes than fibroblasts. Moreover, fibroblasts are less exposed to bacterial threats than keratinocytes, due to specific cell location, which could also provide further evidence towards this difference of reactivity.

Together, these results highlight specific functions and levels of reactivity of keratinocytes and fibroblasts towards structural components from *S. aureus*. Our data suggest a fine-tuning role of both keratinocytes and fibroblasts in directing the early steps of the immune response through the creation of a local microenvironment (Figure 4).

This study further highlights the importance to target the microenvironment to improve one’s host defense or potentiate the immune system to respond better to assaults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SILAB

Abbreviations:

DC	dendritic cell
F-CM	fibroblast-conditioned medium
HKSA	heat-killed *Staphylococcus aureus*
K-CM	keratinocyte-conditioned medium
LC	Langerhans cell
moDCs	monocyte-derived dendritic cells
moLCs	monocyte-derived Langerhans cells
S. aureus	*Staphylococcus aureus*

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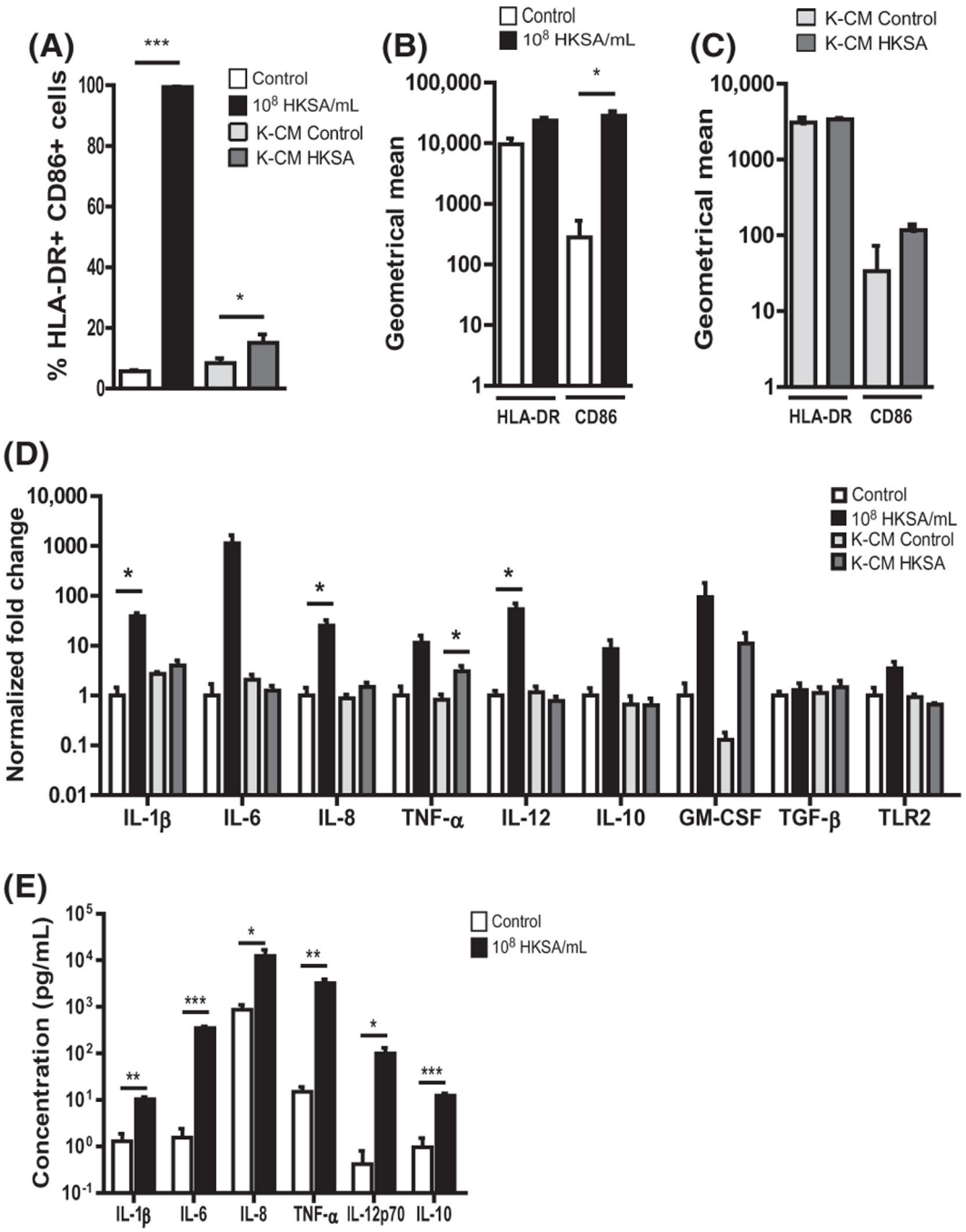

FIGURE 1.

Inflammatory response of monocyte-derived Langerhans cells (LCs) towards heat-killed *Staphylococcus aureus* (HKSA) and keratinocyte-conditioned medium (K-CM). Immature cells were incubated in conditioned medium from control or HKSA-treated keratinocytes (A, C, D) or medium with 10^8 HSKA/mL (A, B, D, E). (A) Surface expression of HLA-DR and CD86 was analysed by flow cytometry. (B, C) Geometrical mean of fluorescence intensity for HLA-DR and CD86. (D) Gene expression was normalized to *GAPDH* before ratio on control conditions was calculated (E) Levels of pro-inflammatory cytokines in cell culture

supernatants were measured by Multiplex. Data represent mean values ± SEM (n = 3–9). Paired *t* test with statistical data represented as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

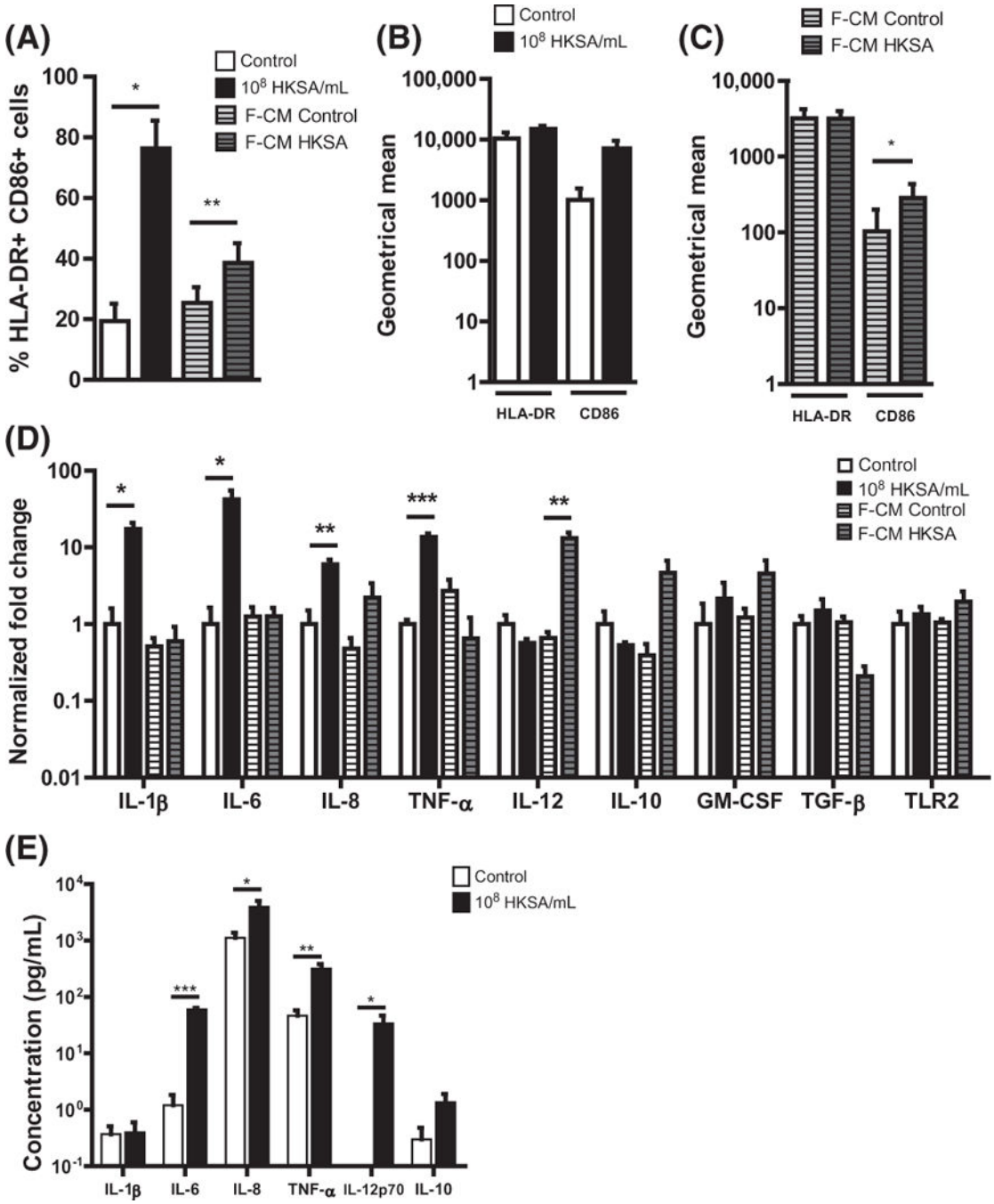

FIGURE 2.

Inflammatory response of monocyte-derived dendritic cells (DCs) towards heat-killed *Staphylococcus aureus* (HKSA) and fibroblast-conditioned medium (F-CM). Immature cells were incubated in conditioned medium from control or HKSA-treated fibroblasts (A, C, D) or medium with 10^8 HSKA/mL (A, B, D, E). (A) Surface expression of HLA-DR and CD86 was analysed by flow cytometry. (B, C) Geometrical mean of fluorescence intensity for HLA-DR and CD86. (D) Gene expression was normalized to *GAPDH* before ratio on control conditions was calculated (E) Levels of pro-inflammatory cytokines in cell culture

supernatants were measured by Multiplex. Data represent mean values ± SEM (n = 3–9). Paired *t* test with statistical data represented as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

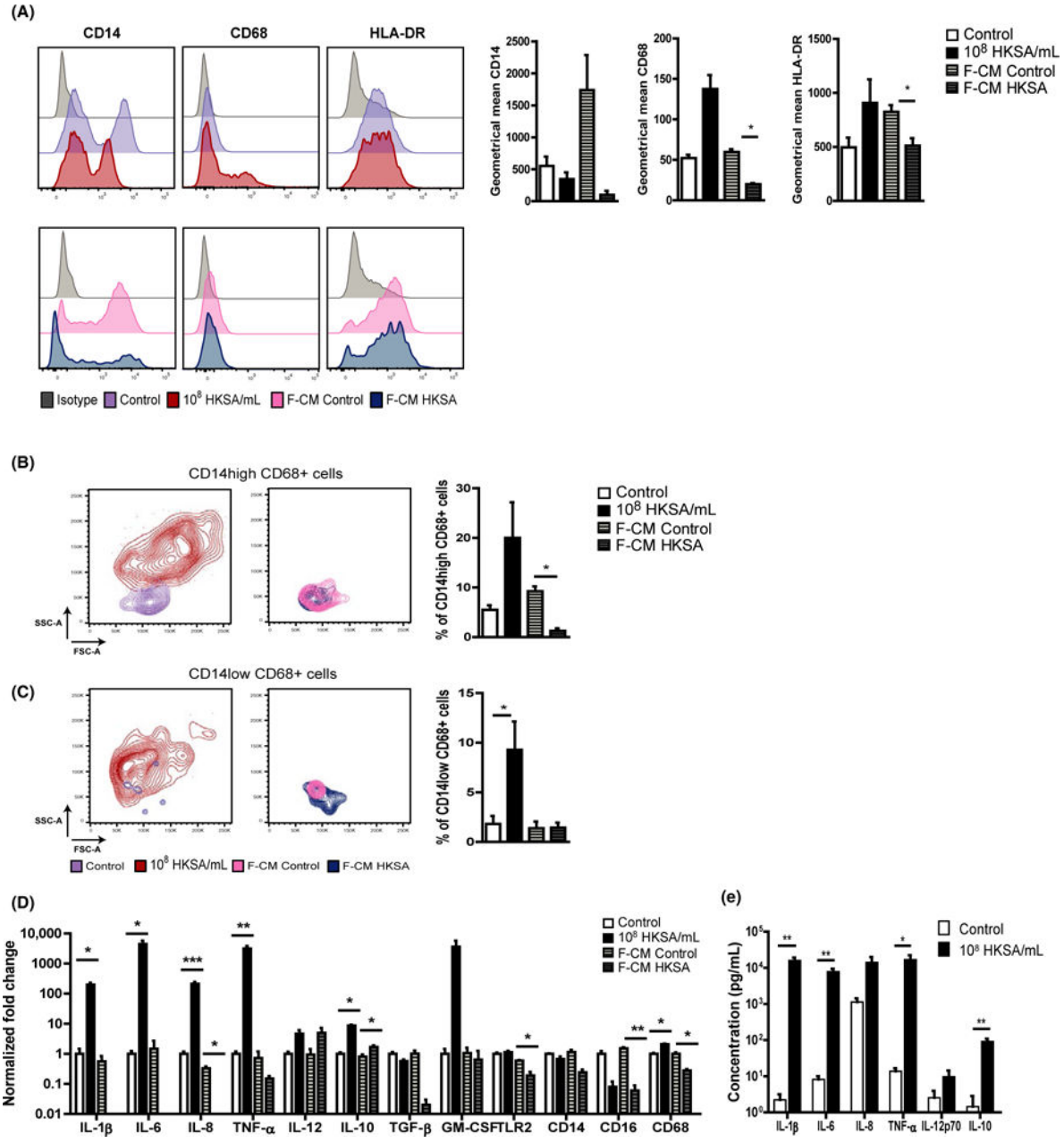

FIGURE 3.

Opposite phenotype of monocytes treated with heat-killed *Staphylococcus aureus* (HKSA) or fibroblast-conditioned medium (F-CM). Monocytes were incubated in conditioned medium from control or HKSA-treated fibroblasts (A-D) or medium with 10^8 HSKA/mL (A-E). (A) The level of expression of CD14, CD68 and HLA-DR was analysed by flow cytometry and is depicted by geometrical fluorescence intensity. (B, C) The per cent of cells expressing CD68 and low or high levels of CD14 was analysed by flow cytometry. Cell size is depicted by SSC/FSC graphics. (D) Gene expression of several pro-inflammatory markers was normalized to *GAPDH*. Ratios towards respective control conditions are depicted here. (E) Levels of pro-inflammatory cytokines in cell culture supernatants were measured by

Multiplex. Data represent mean values ± SEM (n = 3–6). Paired *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

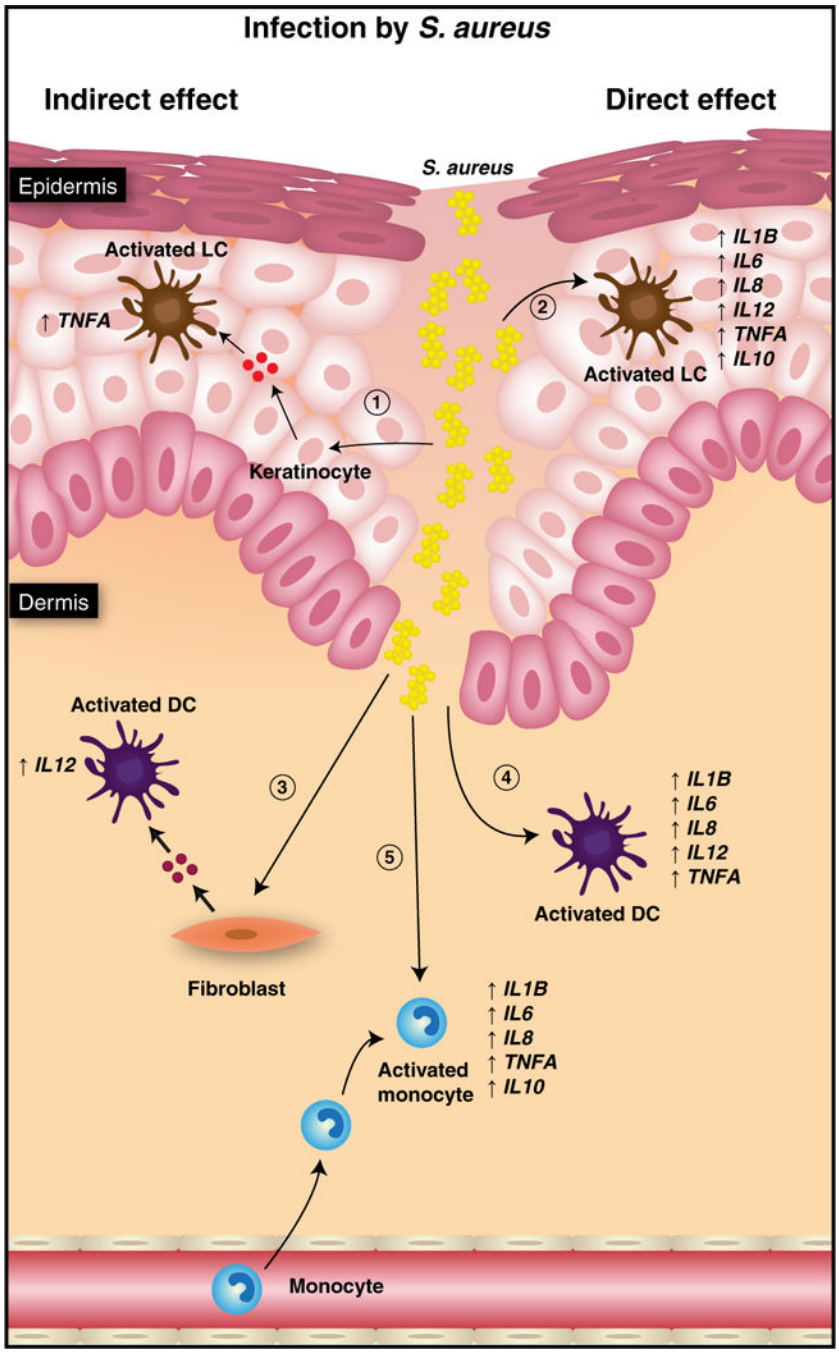

FIGURE 4.

Initiation of a skin immune response triggered by a *Staphylococcus aureus*-challenged tissue microenvironment. When keratinocytes or fibroblasts enter in contact with structural components from *S. aureus*, they create a differential pro-inflammatory microenvironment able to activate Langerhans cells (LCs) (1) and dendritic cells (DCs) (3) and trigger their expression of *TNFA* and *IL12,* respectively. When LCs, DCs and monocytes are in direct

contact with structural components from *S. aureus*, an inflammatory response is triggered with expression of several pro-inflammatory cytokines (2, 4, 5 respectively)

TABLE 1

Pro-inflammatory response of keratinocytes (K) and fibroblasts (F) to HKSA stress

	K HKSA		F HKSA	
IL-1β	**2.91 ± 0.63**	***P* = 0.017**	15.48 ± 6.33	*P* = 0.08
IL-6	**2.56 ± 0.50**	***P* = 0.014**	**6.91 ± 1.15**	***P* = 0.007**
IL-8	7.46 ± 3.50	*P* = 0.10	**31.58 ± 8.33**	***P* = 0.011**
TNF-α	**2.32 ± 0.29**	***P* = 0.002**	2.19 ± 0.67	*P* = 0.14
TGF-β	1.12 ± 0.19	*P* = 0.53	0.10 ± 0.15	*P* = 0.98
IL-10	1.02 ± 0.29	*P* = 0.94	2.42 ± 0.92	*P* = 0.18
GM-CSF	3.94 ± 1.48	*P* = 0.08	**13.06 ± 2.44**	***P* = 0.004**
TLR2	1.04 ± 0.13	*P* = 0.78	2.22 ± 0.62	*P* = 0.1

F, fibroblast; HKSA, heat-killed *Staphylococcus aureus*; K, keratinocytes.

Gene expression of several pro-inflammatory markers was normalized to *GAPDH*. Ratios towards respective control conditions are depicted here. Data represent mean values ± SEM (n = 6–9). Paired *t* test, bold values represent statistical results.