Crosstalk of Keratinocytes with Neutrophils and Neutrophil Extracellular Traps Promotes *Staphylococcus aureus* Skin Colonization

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2023

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	10.04.2024
Dekan:	Prof. Dr. Thilo Stehle
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List of Abbreviations

AD	Atopic dermatitis
AdsA	Adenosine synthase A
Agr	Accessory gene regulatory
AhR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
AP	Activator protein
APC	Antigen-presenting cell
AP-1	Activator protein 1
BCR	B cell receptor
Breg	Regulatory B cell
CA	Community-associated
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CFU	Colony forming units
CHIPs	Chemotaxis inhibiting proteins
CLR	C-type lectin receptor
CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor
Clf	Clumping factor
CLDN	Claudin
DAdo	Deoxyadenosine
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DCF	Dichlordihydrofluorescein
DETC	Dendritic epidermal T cell
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNCB	2,4-Dinitrochlorbenzol

Eap	Extracellular adherence protein
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ELOVL	Elongation of very long chain fatty acids protein
ERK	Extracellular signal-regulated protein kinase
G-CSF	Granulocyte colony-stimulating factor
GlcNac	N-Acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein coupled receptor
FLG	Filaggrin
FPR	Formyl peptide receptor
HBD	Human β-defensin
HMGB1	High mobility group box 1
HOCl	Hypochlorous acid
HSP	Heat shock protein
IFN	Interferon
Ig	Immunglobulin
IκB	NFκB inhibitor
IKK	IκB kinase
IL	Interleukin
ILC	Innate lymphoid cell
IRAK	Interleukin-1 receptor associated kinase
JNK	c-Jun N-terminal kinase
LC	Langerhans cells
LCN	Lipocalin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB4	Leukotriene B4
Luk	Leukocidin
Ly6G	Lymphocyte antigen 6 complex locus G6D
MAPK	Mitogen-activated protein kinase

MDA	Malondialdehyde
MC	Mast cell
MHC	Major histocompatibility complex
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MPRF	Multiple peptide resistance factor
MRSA	Methicillin-resistant Staphylococcus aureus
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
N-Ac	N-Acetylcysteine
NE	Neutrophil elastase
NEMO	NFκB essential modulator
NET	Neutrophil extracellular trap
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	Nod-like receptor
NLRP	Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing
NOD	Nucleotide-binding oligomerization domain
PAD4	Protein-arginine deiminase type-4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PHK	Primary human keratinocyte
PI3K	Phosphoinositide-3-kinase
PMA	Phorbol-myristate-acetate
PMN	Polymorphonuclear Neutrophil
PRR	Pattern-recognition receptor
PSM	Phenol soluble modulin
QS	Quorum sensing
R	Receptor

RA	Receptor antagonist
RAGE	Receptor for advanced glycation endproducts
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
S.	*Staphylococcus*
SAPK	Stress activated protein kinase
SE	Staphylococcal enterotoxin
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
Spa	Protein A
STAT	Signal transducer and activator of transcription protein
TCR	T cell receptor
TEWL	Transepidermal water loss
TEER	Transepithelial electrical resistance
Tfh	T follicular helper
TGFβ	Transforming growth factor-β
TICAM1	TIR domain containing adaptor molecule 1
TIR	Toll/IL1R
TLR	Toll-like-receptor
Tem	Effector memory T cell
Th	T helper
TICAM	TIR domain containing adaptor molecule
TNF	Tumor necrosis factor
TNFAIP	Tumor necrosis factor alpha-induced protein
Treg	Regulatory T cell
TRM	Tissue resident memory T cell
TLSP	Thymic stromal lymphopoietin
WTA	Wall teichoic acid

1 Summary

In the skin, a highly regulated interplay between keratinocytes, immune cells, and the skin microbiome provides a protective barrier against skin infections with *Staphylococcus aureus* (*S. aureus*). A disturbance of this balance can lead to increased *S. aureus* colonization of the skin, which is particularly evident in atopic dermatitis (AD), an inflammatory skin disorder with an impaired barrier function of the skin. The abundant presence of *S. aureus* on AD skin exacerbates inflammation and barrier defects in the skin. In order to develop effective treatments, it is crucial to understand how the immune system interacts in this scenario. Neutrophils, while essential for fighting *S. aureus*, may paradoxically create a favorable environment for its colonization in inflammatory skin conditions, particularly through interactions with keratinocytes and NET formation. However, the underlying mechanisms of this phenomenon remain unknown.

Therefore, this work aimed to provide a detailed mechanistic understanding of how *S. aureus* colonization is enhanced in inflamed skin through the actions of neutrophils and NETs. We have demonstrated that disruption of the skin barrier by tape-stripping enhances *S. aureus* colonization of the skin and the presence of neutrophils in the skin in an *in vivo* epicutaneous colonization model. Neutrophil depletion reduced this enhanced *S. aureus* colonization revealing a functional role for them in this scenario. Further *in vitro* investigations using a human co-culture system of primary human keratinocytes and neutrophils showed that neutrophils co-incubated with keratinocytes are primed for NET formation in response to *S. aureus* infection. We show that the increased presence of neutrophils and NETs causes oxidative stress in the skin, which triggers the secretion of HMGB1. Extracellular HMGB1 induces further oxidative stress in the skin and NET formation in infiltrating neutrophils, leading to the decreased expression of epidermal barrier proteins and increased *S. aureus* colonization of the skin. The clinical evidence of our study is supported by analyses showing increased levels of HMGB1, neutrophils and NETs in the skin of AD patients.

Since neutrophils possess immunomodulatory functions and are associated with the exacerbation of skin inflammation in a variety of skin diseases, we investigated how the interaction between keratinocytes and neutrophils in the skin affects the immune response to *S. aureus* skin infections. Using our *in vitro* co-culture model, we showed

that co-culturing neutrophils with keratinocytes leads to a significant prolongation of neutrophil lifespan, mediated by secreted IL-8, which was associated with an increased activity of neutrophils against *S. aureus*. Furthermore, prolonged co-culture with neutrophils induced inflammation in keratinocytes, which was exacerbated by *S. aureus* infection. Notably, the skin commensal *S. epidermidis* reduced neutrophil-mediated skin inflammation in keratinocytes and induced apoptosis in activated neutrophils, suggesting a beneficial role of the skin microbiome in preventing excessive skin inflammation.

In conclusion, this work provides the intriguing finding that the interaction between neutrophils and keratinocytes plays a critical role in *S. aureus* skin infections in inflamed skin. First, a crosstalk between keratinocytes and neutrophils primes neutrophils for NET formation, which promotes *S. aureus* colonization of the skin by causing skin barrier defects in a ROS-dependent manner. Second, the interplay between neutrophils and keratinocytes exacerbates skin inflammation induced by *S. aureus* infection. Therefore, we propose that the blockade of the interaction between neutrophils, NETs, and keratinocytes could serve as a potential treatment approach for skin disorders associated with *S. aureus* colonization, such as AD.

2 Zusammenfassung

In der Haut sorgt ein hochgradig reguliertes Zusammenspiel zwischen Keratinozyten, Immunzellen und dem Hautmikrobiom für eine schützende Barriere gegen *Staphylococcus aureus* (*S. aureus*) Hautinfektionen. Eine Störung dieses Gleichgewichts kann zu einer verstärkten Besiedlung der Haut mit *S. aureus* führen, was besonders bei atopischer Dermatitis (AD), einer chronisch-entzündlichen Hauterkrankung mit gestörter Hautbarrierefunktion, deutlich wird. Die erhöhte *S. aureus* Kolonisierung in der AD-Haut verschlimmert die Entzündung und die Barriere Störungen in der Haut. Für die Entwicklung wirksamer Behandlungen ist es entscheidend zu verstehen, wie das Immunsystem in diesem Szenario interagiert. Neutrophile, die für die Bekämpfung von *S. aureus* unerlässlich sind, können paradoxerweise bei entzündlichen Hauterkrankungen ein günstiges Umfeld für die Besiedelung der Haut mit *S. aureus* schaffen, insbesondere durch Interaktionen mit Keratinozyten und der Bildung von neutrophilen extrazellulären Fallen (NETs). Die diesem Phänomen zugrunde liegenden Mechanismen sind jedoch unbekannt.

Ziel dieser Arbeit war es daher, ein detailliertes mechanistisches Verständnis darüber zu erlangen, wie die *S. aureus* Kolonisierung in entzündeter Haut durch die Wirkung von Neutrophilen und NETs gefördert wird. Anhand eines *in vivo* epikutanen Kolonisierungsmodells konnten wir zeigen, dass eine Störung der Hautbarriere durch Tape-stripping die Kolonisierung der Haut mit *S. aureus* und die Anwesenheit von Neutrophilen in der Haut erhöht. Die Depletion von Neutrophilen reduzierte diese erhöhte *S. aureus* Kolonisierung, was auf eine funktionelle Rolle der Neutrophilen in diesem Szenario hindeutet. Weitere *in vitro* Untersuchungen unter Verwendung eines humanen Ko-Kultursystems aus primären humanen Keratinozyten und Neutrophilen zeigten, dass die Ko-Kultur mit Keratinozyten die Neutrophilen zur Bildung von NETs als Reaktion auf eine *S. aureus*-Infektion anregt. Wir postulieren, dass die erhöhte Anwesenheit von Neutrophilen und NETs oxidativen Stress in der Haut induziert und die Sekretion von HMGB1 anregt. Extrazelluläres HMGB1 vermehrt die Induktion von oxidativem Stress in der Haut und die Bildung von NETs in infiltrierenden Neutrophilen, was zu einer Herunterregulierung von epidermalen Barriere Proteinen und einer verstärkten Kolonisierung der Haut mit *S. aureus* führt. Die klinische Relevanz unsere

Studie wird durch Analysen unterstützt, die ein erhöhtes Vorkommen von Neutrophilen, NETs und HMGB1 in der Haut von AD-Patienten zeigen.

Da Neutrophile immunomodulatorische Funktionen besitzen und mit der Verschlimmerung von Entzündungen bei einer Reihe von Hautkrankheiten in Verbindung gebracht werden, haben wir untersucht, wie die Interaktion zwischen Keratinozyten und Neutrophilen in der Haut die Immunantwort auf *S. aureus* Hautinfektionen beeinflusst. Mit unserem *in vitro* Ko-Kultursystem konnten wir zeigen, dass die Ko-Kultivierung von Neutrophilen und Keratinozyten zu einer signifikanten Verlängerung der Lebenszeit der Neutrophilen führt, die durch die Sekretion von Il-8 vermittelt wird und mit einer erhöhten Aktivität der Neutrophile gegen *S. aureus* einhergeht. Außerdem führte eine längere Ko-Kultur mit Neutrophilen zu einer entzündlichen Reaktion der Keratinozyten, die durch eine *S. aureus* Infektion noch verstärkt wurde. Bemerkenswert ist, dass der Kommensale *S. epidermidis* die durch Neutrophile vermittelte Hautentzündung reduzierte, was auf eine positive Rolle des Hautmikrobioms bei der Verhinderung übermäßiger Hautentzündungen hindeutet.

Zusammenfassend liefert diese Arbeit die interessante Erkenntnis, dass die Interaktion zwischen Neutrophilen und Keratinozyten eine entscheidende Rolle bei *S. aureus* Infektionen in entzündeter Haut spielt. Erstens regt eine Wechselwirkung zwischen Keratinozyten und Neutrophilen die Neutrophilen zur Bildung von NETs an, die die Kolonisierung der Haut mit *S. aureus* fördern, indem sie auf ROS-abhängige Weise Defekte der Hautbarriere verursachen. Zweitens verschlimmert das Zusammenspiel von Neutrophilen und Keratinozyten die durch eine *S. aureus* Infektion ausgelöste Hautentzündung. Dies deutet darauf hin, dass eine Blockade der Interaktion zwischen Neutrophilen, NETs und Keratinozyten als potenzieller Behandlungsansatz für Hauterkrankungen dienen könnte, die mit der Kolonisierung durch *S. aureus* assoziiert sind, wie z.B. AD.

3 Introduction

3.1 The skin immune system

The skin is a complex organ that acts as a barrier between our body and the environment. As such, it ensures physiological homeostasis by stabilizing body temperature and by preventing water loss as well as the invasion of exogenous substances and microbes [1]. Beyond providing a mechanical barrier, the skin fulfils crucial immunomodulatory functions that help maintain homeostasis and protect the host from inflammatory or infectious conditions [2]. The epidermis, the uppermost layer of the skin, is made up primarily of keratinocytes. Keratinocytes are the first cells to be exposed to exogenous pathogens such as *Staphylococcus aureus* (*S. aureus*), a facultative pathogen that causes most human skin infections [3]. Therefore, they are critical for the initiation and maintenance of inflammation. Besides keratinocytes, several other cell types that either reside in or infiltrate the skin are involved in skin immunity [4,5]. The effective immune function of the skin is mediated by the highly regulated interplay between these different cell types and skin-inhabitant microorganisms, e.g., the skin commensals. This interplay must be tightly controlled, as dysregulation of the skin immune system can lead to infection or chronic inflammation and the development of autoimmune diseases for instance [1,2,4]. One example of an inflammatory skin condition is atopic dermatitis (AD). The skin barrier of AD patients is compromised, resulting from excessive skin inflammation mediated by various cells either residing or infiltrating in the skin [6-8]. Additionally, there is a shift in the skin microbiome, with an overabundance of *S. aureus* [9]. Inflammation-induced impairment of the skin barrier facilitates *S. aureus* colonization of the skin, causing additional skin inflammation and further skin barrier defects [10]. This creates a vicious cycle underscoring the significance of maintaining an intact skin immune system to prevent chronic inflammatory skin conditions.

From a structural view, the skin consists of the epidermal and dermal layer, which are separated by a basement membrane. The epidermis is the uppermost layer of the skin and contains four distinct keratinocyte layers. The stratum basale is the lowest layer of the epidermis and consists of one layer of undifferentiated, proliferating keratinocytes. The main function of this layer is the constant renewal of cells for the epidermis. Starting from the stratum basale, the keratinocytes undergo a 4-week

maturation process during which they migrate to the top of the epidermis before eventually being shed from the skin surface [11]. During this process, keratinocytes differentiate and form the different layers of the epidermis starting with the stratum spinosum, the stratum granulosum, and the stratum corneum. The stratum corneum is the top layer of the epidermis and is composed of dead corneocytes that are integrated in a lipid-rich matrix. This layer acts as a physical barrier to prevent water loss from the skin [12]. An additional barrier is provided by tight junctions, that tightly bind keratinocytes closely together in the stratum granulosum [13]. The epidermis also comprises melanocytes and the antigen-presenting Langerhans cells besides keratinocytes [1]. Beneath the epidermis is the dermis, the second layer of the skin. The primary functions of the dermis are to provide structural support, regulate temperature and facilitate sensory perception. Within the dermis, fibroblasts are responsible for generating proteins of the extracellular matrix like elastin or collagen. In addition, blood vessels, nerves, immune cells, sweat glands and sebaceous glands can be found in the dermis [14]. The hypodermis, forming the deepest layer of the skin's structure, consists mostly of subcutaneous fat and loose connective tissue. It facilitates the connection of the skin to the underlying muscles and bones [14].

The subsequent sections will provide a comprehensive review of the various components of the skin immune system and their roles in homeostasis and skin inflammation. This will cover cellular components, signalling pathways, immunomodulatory mediators, and the skin microbiome.

3.1.1 Cellular components of the skin

The immune system consists of a variety of cell types that contribute to both skin homeostasis and host protection under inflammatory conditions [1]. These cells comprise both skin-resident cells as well as cells migrating to the skin from either the circulation or the lymphatic vessels, as illustrated in Figure 1. In the following section, the various cell types contribute to functional skin immunity in both steady state and inflammatory conditions especially in context of *S. aureus* skin infections will be described in detail.

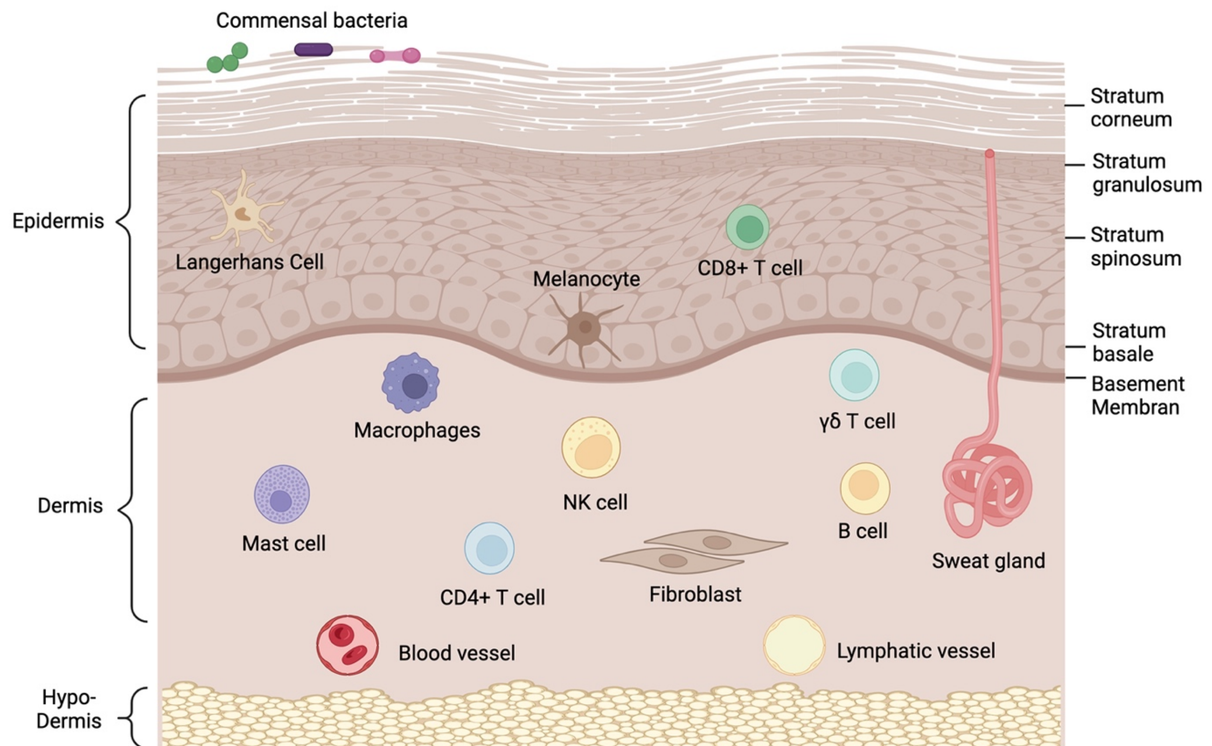

Figure 1: The skin immune system. The skin comprises the epidermis, dermis, and hypodermis. The epidermis contains four layers of keratinocytes in their different differentiation stages. Additionally, the epidermis is home to Langerhans cells, melanocytes, and CD8+ T cells. Fibroblasts serve as structural cells in the dermis, where several immune cells, such as mast cells, macrophages, NK cells, B cells, CD4+ T cells, and yd T cells, are present. These cells may either by tissue resident or infiltrate the skin through lymphatic or blood vessels. This illustration was created with BioRender.

Keratinocytes

Beyond their structural function within the skin, keratinocytes are actively involved in the innate immune defense of the skin under both homeostatic and inflammatory conditions. As the primary cells of the epidermis, they are the first cells to sense invading pathogens, such as *S. aureus*. Therefore, they play a critical role in initiating and maintaining inflammation to ensure effective host defense and subsequent restoration of homeostasis [5]. They express multiple pattern recognition receptors (PRRs) with which they sense pathogen-associated-molecular patterns (PAMPs) [15]. A detailed description of PRRs and their downstream signaling is provided in section 3.1.2. In brief, the binding of PAMPs to PRRs initiates the activation of an intracellular signaling cascade. The most common pathways activated by PRRs signaling are the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway as well as the mitogen-activated protein kinase (MAPK) signaling pathway [16]. Activation of these pathways results in the expression and secretion of immune mediators including cytokines, chemokines and antimicrobial peptides (AMPs) [17]. This enables keratinocytes to both directly attack pathogens and to recruit immune cells to the

infection site and activate them to further promote immune responses [5]. Besides sensing exogenous factors such as PAMPs from invading bacteria, PRRs expressed by keratinocytes can also sense endogenous factors, e.g. damage associated molecular patterns (DAMPs) secreted by cells under stress or cell death and indicate tissue damage [18]. In addition to PRRs, keratinocytes express a variety of cytokine and chemokine receptors allowing them to sense and react to immunomodulatory factors secreted by surrounding immune cells. In the context of *S. aureus* skin infection, keratinocyte-derived IL-1 is a critical factor in orchestrating skin immune responses. IL-1 mediates signaling via the IL-1R/MyD88 pathway and induces the production of chemoattractants in keratinocytes, resulting in the recruitment of neutrophils which is essential for bacterial clearance [19] [20,21] . On the other hand, keratinocyte-derived IL-1 activates T cells that further drive the skin immune responses in epicutaneous *S. aureus* infections [22]. This highlights the role of keratinocyte-derived IL-1 in orchestrating the immune defense against *S. aureus*.

Keratinocytes are also significantly involved in the pathogenesis of several inflammatory skin diseases, including AD [23]. For instance, keratinocyte-derived alarmins such as thymic stromal lymphopoietin (TSLP) activate T cells to produce Th2 cytokines which can induce skin barrier dysfunction [24-26].

In summary, keratinocytes not only provide barrier functions to the skin, but they are also an important part in the initial sensing of invading pathogens such as *S. aureus* and the initiation of subsequent immune responses. The interplay between keratinocytes and other immune cells in the skin and the skin microbiome will be further discussed in the following sections.

Neutrophils

Neutrophils are the initial responders to invading pathogens like *S. aureus* and are rapidly recruited to the site through chemokines released by skin-resident cells like keratinocytes [19,27]. Upon reaching the site of infection, neutrophils mediate the clearance of bacteria by employing diverse antimicrobial mechanisms encompassing the production of reactive oxygen species (ROS), degranulation, phagocytosis, and the release of neutrophil extracellular traps (NETs) [28,29]. Furthermore, neutrophils actively shape immune responses by the secretion and sensing of several immunomodulatory mediators [30,31]. A comprehensive description of neutrophils,

elucidating their antimicrobial strategies, immunomodulatory functions and their significance in combating *S. aureus* skin infection is provided in sections 3.2 and 3.3.

Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) present in the epidermis and dermis of the skin. They are activated by inflammatory mediators released by keratinocytes, for example by thymic stromal lymphopoietin (TSLP) [32,33]. A specific type of DC that populates the epidermis are the **Langerhans Cells (LCs)** [34]. LCs are leukocytes derived from yolk-sac derived myeloid progenitor cells that populate the skin before birth [35]. They express cytoplasmic Birbeck granules and langerin, which they use for antigen-sensing and processing [36]. In addition, they express a diverse set of surface markers such as adhesion molecules allowing the anchoring to neighboring keratinocytes or T cells [37].

Being APCs, LCs express major histocompatibility complex (MHC) class II molecules and Fc- and complement receptors on their surface [38-40]. LCs can use their dendrites to take up antigens that are too large to cross the tight junction barrier, thereby initiating immune responses against external pathogens [41]. This process requires a close interaction with keratinocytes and ensures tight junction integrity during antigen uptake [42]. LCs then either present the antigen to skin-resident T cells or migrate through the dermal lymphatic vessel to the skin-draining lymph node and there present the antigen to naïve cluster of differentiation (CD) 4+ T cells in a CD1a-dependent manner, ultimately leading to differentiation of the naïve T cells to a T helper (Th) 1, Th2, or Th17 effector phenotype [43]. As LCs migrate to lymph nodes, the expression of the surface molecules on LCs is modified, allowing them to detach from keratinocytes and optimize antigen presentation to T cells [37]. LC migration takes place under steady-state conditions; however, it is significantly increased under inflammatory conditions [44]. In contrast to other DCs that are resident in lymphoid organs or the airway mucosa, LCs in the epidermis are long-lived and repopulate themselves from dividing skin-resident LC stem cells and are therefore not dependent on circulating precursor cells [45,46]. However, under inflammatory conditions, LCs can also be repopulated by circulating monocytes in a CC chemokine receptor (CCR) 2/CCR6 dependent manner [46-48]

In terms of functional properties, LCs can either stimulate or regulate the immune system, depending on the situation. During steady-state conditions, LCs activate the

proliferation of CD4+ memory regulatory T cells (Treg) located in the skin, which leads to the suppression the proliferation of skin-resident effector memory T cells (Tem) [49,50]. Conversely, under inflammatory conditions, LCs induce the proliferation of pathogen-specific skin-resident Tems, thereby stimulating the immune response [50]. Due to their location in the epidermis, LCs contribute significantly to the immune response against *S. aureus* infections of the skin. LCs detect *S. aureus* surface proteins and wall teichoic acids (WTA) β-N-Acetylglucosamine (GlcNAc) via their PRR langerin and initiate subsequent immune responses in the skin including the proliferation of CD4+ T cells [51,52]. Langerin specifically senses *S. aureus* and does not recognize coagulase-negative staphylococci [51]. Notably, stimulation of LCs by AD-associated *S. aureus* strains induced greater LC-mediated T cell proliferation and a shift towards a Th2 response compared to standard *S. aureus* strains or *S. epidermidis* [52]. Furthermore, epicutaneous application of *S. aureus* to filaggrin (Flg) - deficient mice, which exhibit an impaired skin barrier similar to that seen in AD, resulted in LCs-mediated IL-17 responses in the skin [53]. This suggests a possible involvement of LCs in promoting skin inflammation in AD. Indeed, LCs have been connected with the pathogenesis of AD. Studies using mouse models for AD revealed that keratinocyte derived TSLP, abundantly expressed in the lesional skin of AD patients, enhanced LC proliferation. This, in turn, induced T cell proliferation, ultimately triggering a Th2 immune response and immunoglobulin (Ig) production. Furthermore, inflammation decreased when LCs were depleted [54]. In summary, LCs show a dynamic and context-dependent role in the skin. During steady-state conditions, they primarily exhibit immunoregulatory functions, maintaining a balance in the immune system. However, when faced with inflammatory conditions, they contribute to aggravating of the inflammation of the skin.

In addition to LCs, different subtypes of DCs populate the dermis, playing a pivotal role in orchestrating skin immunity through the regulation of humoral responses and the promotion of antibody production. Unlike LCs, dermal DCs have a short lifespan and rely on a continuous supply of bone marrow-derived precursors [55]. Similar to LCs, they possess antigen-presenting capabilities and are proficient in inducing T cell responses in the skin-draining lymph nodes [56].

Macrophages

Macrophages are a subset of leukocytes that can be found in various tissues including the skin, where they are located in the dermis and hypodermis [1,57]. Dermal-

resident macrophages originate from both fetal-derived and monocyte-derived macrophages. [58-60]. They are long-lived cells, ranging from weeks to months in mice and up to several years in humans and they proliferate under steady-state conditions in the tissue in which they reside [61-63]. In principle, macrophages are divided into two subtypes: the "classically activated" macrophages (M1) with a proinflammatory phenotype, induced by IFN-y and LPS, and the "alternatively activated" macrophages (M2) with an anti-inflammatory phenotype, induced by IL-4, IL-10 or IL-13 [64,65]. However, this classification is somewhat rigid and additional subtypes of macrophages have been identified [66].

Macrophages are professional phagocytes that aid in the host defense against *S. aureus* skin infections through phagocytosis and the production of ROS [67]. In addition, they play a critical role in preserving tissue homeostasis through the removal of apoptotic or necrotic cells and cell debris in both steady state and inflammatory conditions, in a process called efferocytosis [68,69]. Efferocytosis typically induces an anti-inflammatory M2 phenotype in macrophages accompanied by decreased proinflammatory cytokine and chemokine expression. Ultimately, efferocytosis induces macrophage apoptosis, further supporting inflammation resolution [70,71].

In addition to their role in tissue repair and anti-inflammatory functions, macrophages express a variety of TLRs allowing them to sense invading bacteria and produce pro-inflammatory cytokines and chemokines to actively participate in the immune response [67]. In case of an intradermal *S. aureus* challenge, resident macrophages promote rapid, but controlled, neutrophil recruitment in a MyD88-dependent way to allow efficient bacterial killing and abscess resolution [72]. Infiltrated neutrophils release NETs upon contact with bacteria, thereby immobilize the bacteria for macrophages to kill them by phagocytosis. Macrophages can then phagocytize neutrophils and NETs from the inflammation site, promoting inflammation resolution and restoration of homeostasis [73]. The significance of macrophages in the host defense against intradermal *S. aureus* infection is underscored by a recent study demonstrating that CD169+ macrophages contribute to the defense against intradermal *S. aureus* infections by activating IL-17-producing $\gamma\delta$ T cells. Interestingly, CD196 expression on macrophages was regulated by commensal bacteria, highlighting the interplay between macrophages, commensal bacteria, and adaptive immune cells in shaping an effective defense strategy [74]. Additionally, Feuerstein et al. found that dermal resident macrophages mediate innate memory against intradermal

S. aureus infections, leading to increased bacterial killing and reduced neutrophil infiltration after a second challenge with *S. aureus*. This effect was dependent upon STAT1 and CXCL9 signaling and was independent of MyD88 signaling. Depletion of skin-resident macrophages prior to the second challenge prevented the enhanced bacterial killing [75]. This memory effect, however, was only transient for 6-12 weeks post infection. In contrast to their protective role against invading pathogens, macrophages can exert harmful effects and be involved in the pathogenesis of AD. The abundance of macrophages in AD skin is significantly increased compared to healthy skin [76]. Furthermore, in a 2,4-Dinitrochlorbenzol (DNCB)-induced AD mouse model, an increased macrophage infiltration was observed, correlating with scratching behavior and skin inflammation [77]. This suggests macrophages might be involved in the pathogenesis of AD by driving skin damage and inflammation.

In summary, dermal macrophages play a crucial role in skin immunity to invading pathogens by actively contributing to immune response and subsequent restoration of homeostasis. Nevertheless, under pathological conditions, they can also contribute to skin inflammation.

Mast cells

Mast cells (MCs) are granulocytes that are present in all vascularized tissues where they contribute to host protection [78]. They are derived from circulating hematopoietic progenitors that infiltrate tissues and mature through cytokine-mediated mechanisms [59,79]. MCs harbor a wide range of chemical mediators, including histamine, cytokines, chemokines, and proteases among others, stored within cytoplasmic granules [78]. Activation of MCs can be triggered by several endogenous peptides and proteins, as well as microbial products, leading to the secretion of cytokines or chemokines and degranulation. Notably, the primary activator is immunoglobulin (Ig) E binding to Fc receptors on MCs [80-82]. MCs are classified into subtypes, based on their protease content. In the skin, the predominant subtype is represented by the tryptase-positive, chymase-positive MCs (MC_{TC}s), primarily located in the dermis, either directly beneath or within the epidermis. The number of MCs in the skin significantly increases during inflamed conditions, such as those seen in AD or psoriasis [83,84]. MCs play a role in regulating both the epidermal barrier function and antigen exposure. In an AD mouse model deficient in MCs, the expression of genes related to the epidermal differentiation

complex was decreased, coupled with an increase in antigen uptake [85]. This highlights the involvement of MCs in maintaining skin homeostasis.

Due to their surface expression of TLRs, FC- and cytokine receptors, MCs are capable of sensing bacterial products and mediators released by nearby cells, enable them to actively contribute to the immune responses of the skin during inflammatory or infectious conditions [86]. In *S. aureus* skin infections, studies have shown that γ-toxin from *S. aureus* triggers mast cell degranulation in a PI3K-dependent manner resulting in allergic skin disease [87]. Additionally, in a murine epicutaneous colonization model, lipoteichoic acid (LTA) derived from *S. aureus* stimulates keratinocytes to produce stem cell factor, thereby promoting the proliferation and maturation of mast cells [88]. Further experiments revealed that AD-associated *S. aureus* strains trigger mast cell degranulation, with IgE further exacerbates degranulation [87]. Mast cells produce IL-4 and IL-13, which both contribute to the pathogenesis of AD [82] Additionally, mast cells produce TSLP, which drives skin inflammation in a MC903-AD mouse model, suggesting a potential role of mast cells in AD development [89]. Conversely, MCs may also be protective in AD, as MC depletion led to worse inflammation in several mouse models of AD [90,91]. Furthermore, MCs stimulate regulatory T cells through IL-2 production, indicating an immunosuppressive role for MCs [92]. Collectively, these findings highlight the multifaceted and contextual influence of MCs in the pathogenesis of AD, especially in relation to *S. aureus*-triggered skin inflammation.

Innate lymphoid cells

In addition to the myeloid-derived effector cells, lymphocytes participate in skin immunity. The innate lymphoid cells (ILCs) represent one cell type involved in this process. Based on their cytokine stimulus, ILCs differentiate into ILC1s, ILC2s and ILC3s, promoting type 1, 2, and 3 immune responses, respectively [93]. In addition, natural killer (NK) cells belong to the type 1 ILCs. While NK cells and ILC1s are circulating, ILC2s and ILC3s are tissue-resident, with the most abundant ILC found in the skin being the ILC2s [94-96]. NK cells and ILC1s respond to intracellular microbial infections by generating IFNγ, granzyme, and perforin. In addition, NK cells possess potent cytotoxic capabilities and can eliminating infected cells [97]. ILC2s are associated with type 2 immunity against extracellular parasites and produce a variety of cytokines including IL-4, IL-5, IL-9, and IL-13 in response to keratinocyte-derived alarmins TSLP, IL-33 or IL-25 [98-100]. ILC3s promote type 3 immunity through the production of TNF, IL-

22, and IL-17 for protection against extracellular bacteria and fungi. [93]. Skin-resident ILCs can drive skin inflammation in mouse models of type 3 and type 2 immunity [95,101]. In addition, skin-resident ILCs regulate the expansion of commensal microbiota by controlling AMP production through sebaceous glands, indicating their role in maintaining skin homeostasis [15]. Furthermore, ILC-derived cytokines have been implicated in the pathogenesis of inflammatory skin disorders, including AD [102]. Therefore, ILCs have a diverse impact on the complex balance between immune system and skin health.

T cells

T cells are lymphocytes derived from bone marrow precursors and are key players in the adaptive immune system by coordinating immune responses to pathogens [103]. The development and maturation of T cells takes place in the thymus, and they are then distributed to a variety of different tissues, performing essential functions such as initiating and sustaining immune responses, ensuring homeostasis, and preserving immunological memory [103].

Naïve T cells are predominantly located in lymph nodes, where they encounter antigens, presented to them on MHCs by migrating APC, such as LCs or DCs. This induces the differentiation and proliferation of the naïve T cells into effector T cell subtypes with specific phenotypes that defines their functionality in the tissue they migrate into [104]. Naive CD4+ T cells differentiate into Th1, Th2, or Th17 phenotypes depending on the antigen stimulus and the cytokines expressed by the APC. Each phenotype expresses a distinct profile of T cell receptor (TCR) and produces specific cytokines and chemokines [105]. Th1 cells participate in immune responses against intracellular pathogens, whereas Th2 cells are implicated in allergic inflammation and responses to extracellular parasites. Th17 cells are crucial for fighting extracellular bacteria and fungi [106]. The effector T cells then migrate to the respective tissue, such as the skin where they combat invading pathogens as part of the immune response. Once the immune response is restrained, the majority of T cells in the skin die, while some remain as tissue resident memory T cells (TRMs). These T cells proliferate in situ, ensuring long-lasting memory and a prompt immune response upon subsequent antigen exposure [107]. Additionally, TRMs perform immunosuppressive functions and ensure tissue homeostasis. In the human epidermis, CD8+ skin TRMs are predominant with primed cytotoxic functions, while CD4+ skin TRMs are present in the dermis [108].

In addition to skin TRMs, immunosuppressive, CD4+ Foxp3+ regulatory T cells (Tregs) can be found in the skin. In early life, numerous antigen encounters drive the differentiation of naïve CD4+ T cells into Tregs, ensuring immune tolerance against harmless and self-antigens [109]. Fibroblast-derived cytokines and keratinocyte-derived transforming growth factor-β1 (TGFβ-1) can induce Tregs proliferation under inflammatory conditions and promote inflammation resolution [110,111]. Tregs secrete IL-10 and TGFβ, which decrease neutrophil recruitment and induce apoptosis. Additionally, they inhibit the proliferation and subsequent production of cytokines by other skin-resident T cells [110,112,113]. Furthermore, Tregs interact with keratinocytes and regulate epidermal barrier repair and innate immune responses during *S. aureus* infections of the skin in a TGFβ-mediated manner [111]. In addition, Tregs infiltrating the neonatal mouse skin are critical in mediating tolerance against skin commensals, further underscoring their integral role in orchestrating immune homeostasis within the skin microenvironment [114].

Besides the classical CD4+ αβ T cells, another subtype of T cells is located in the skin, the so-called γδ T cells which inhabit the skin directly from the thymus [115,116]. These cells differ from conventional αβ T cells in that they are not dependent on MHC-antigen presentation [117]. In mice, the resident epidermal T cells are exclusively γδ T cells, also referred to as dendritic epidermal T cells (DETCs) [118]. In humans, this cell type does not exist and both the αβ- and γδ T cell populations reside in the dermis [119,120]. The γδ T cells are critical for maintaining tissue homeostasis, participating in wound healing, and ensuring epidermal barrier function. Similar to αβ T cells, γδ T cells can produce proinflammatory cytokines, such as IL-17A or IFNγ and actively shape immune responses [84]. For instance, γδ T cells are important for the immune response against intradermal and epicutaneous *S. aureus* infections by releasing IL-17 in response to stimulation with IL-1β and IL-36α, respectively [121,122].

T cells are central players in multiple inflammatory skin disorders including atopic dermatitis or psoriasis. In AD, T cells promote skin inflammation and can mediate skin barrier dysfunctions by the secretion of Th2 cytokines [123,124]. Targeting Th2 cytokine signaling with dupilumab, an antagonist for IL-4Rα, the receptor used by IL-4 and IL-13, results in the improvement of AD symptoms [125,126]. Interestingly, it also led to reduced *S. aureus* colonization on AD skin, thus further emphasizing the link between Th2 skin inflammation and *S. aureus* skin colonization in the pathogenesis of AD [127].

In conclusion, T cells play a central role in orchestrating immune responses and ensuring immune tolerance in the skin. However, uncontrolled T cell responses may disrupt the skin's immune system and trigger inflammatory skin conditions like those seen in atopic dermatitis and psoriasis [128].

B cells

B cells are antibody-producing cells that resemble the humoral arm of the adaptive immunity and play a critical role in the host's systemic inflammatory response. In the bone marrow, naïve B cells mature from hematopoietic stem cells and then enter secondary lymphoid organs (SLO) in an integrin- and chemokine-dependent manner [129]. Following binding of antigens to their B cell receptor (BCR), naïve B cells differentiate into plasma cells (PCs). These PCs produce antigen-specific antibodies to ensure efficient adaptive immune responses [130]. In addition, naïve B cells can differentiate into memory B cells that persist for a long time ensuring a rapid response to re-encounters with the antigen. B cell activation is typically T-cell dependent, requiring several days to produce high-affinity antibodies [130]. However, B cells can also function independently of T cells. A specific B cells subset, called innate-like B-1 cells, can be activated by PAMPs through TLRs. This alternative pathway allows for a rapid response to pathogens but leads to the production of low affinity antibodies [131]. In addition to their ability to produce antibodies, B cells also participate in the immune response through cytokine production and by acting as APCs, enabling them to activate T cells [132-134].

Although B cells are mainly located in SLOs, they are also present in human skin even under healthy conditions and their frequency increases significantly under inflammatory conditions [135]. B cells are thought to migrate into the skin in a CCR6/CCL20 dependent way [136]. Both, conventional B cells and B-1 cells, are present in the skin and they may play either immunoregulatory or immunostimulatory roles [135]. Regulatory B cells (Bregs) are essential for regulating skin inflammation by releasing the anti-inflammatory cytokine IL-10. For example, in psoriasis, a reduced number of Bregs in the bloodstream leads to the decreased expression of IL-10, which is associated with an upregulation of proinflammatory cytokines [137]. Additionally, B cell depletion by rituximab treatment was associated with the induction or exacerbation of psoriatic lesions [138]. Furthermore, Jiang et al. reported a correlation between reduced level of IL-10-producing Bregs and an increased T follicular helper (Tfh)-induced

immune response and disease severity in children with extrinsic AD [139]. These findings underscore the important role that Bregs play in regulating skin inflammation.

B cells cannot only regulate but also contribute to skin inflammation. For example, B cells can produce harmful antibodies against the host's self, leading to the generation of antinuclear and poly-reactive antibodies [140]. Under steady-state conditions, checkpoints exist to eliminate autoreactive B cells during their maturation [141]. Failure to remove them can result in an increase in auto-reactive B cells which have been involved in the development of various autoimmune diseases with cutaneous manifestations, including systemic lupus erythematosus (SLE) [142]. B cells also contribute the pathogenesis of inflammatory skin diseases by activating T cell responses. In a mouse model of AD induced by ovalbumin sensitization, the quantity of CD4+ T cells and epidermal and dermal thickening were decreased in mice, deficient in CD19, suggesting that the expression of CD19 on B cells is important for T cell activation in AD-like skin inflammation [143]. In addition, Aranda et al. reported an increased presence of IL-4R+IgG+ memory B cells, which was associated with increased circulating immunoglobulin (Ig) E, which can then trigger mast cell degranulation, another hallmark of AD [144]. Interestingly, B cells also play a role in the skin's immune response to *S. aureus* infections. In a murine model of epicutaneous *S. aureus* infection, keratinocyte-mediated IL-36 signaling activates PC differentiation which was associated with elevated levels of serum IgE. Further experiments provided clinical relevance for this finding by showing elevated levels of IL-36α in the epidermis of AD patients and patients with an IL-36RA deficiency show increased serum IgE levels [145]. Taken together, the dual role of B cells, acting as both regulators and contributors to skin inflammation, highlights the complexity of their interactions within the skin microenvironment.

3.1.2 Pattern recognition receptors in the skin

The induction of a rapid and effective immune response to pathogens requires tools for efficient pathogen sensing. As discussed in the previous sections, various cells within the skin immune system express PRRs that can recognize PAMPs. Keratinocytes, as the first cells to sense *S. aureus*, express several PRRs, such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs), which are expressed on the surface of the cell or in the endosomal compartment [146]. In addition to sensing PAMPs, PRRs also serve to

detect tissue damage by recognizing DAMPs [5]. In the following section, the largest family of PRRs, the TLRs, and their associated signaling will be discussed in detail and is illustrated in Figure 2.

TLRs represent the most extensively studied family of PRRs within the innate immune system. They bind PAMPs and therefore play a critical role in the skin immune defense against invading pathogens [147]. Besides PAMPs, TLRs also bind DAMPs, like High-Mobility-Group-Box 1 (HMGB1), released by stressed or dying cells during tissue damage [148]. They are type I transmembrane proteins with an extracellular glycoprotein domain, responsible for the recognition of microbial patterns, a transmembrane domain and a cytoplasmic tail, known as the Toll/IL1R (TIR) domain, responsible for initiating intracellular signaling cascades [149]. So far, ten human and twelve mouse TLRs have been discovered [150]. While TLRs 1, 2, 4, 6 and 10 are located on the surface of the cell and sense extracellular PAMPs, TLRs 3, 7, 8 and 9 are expressed intracellularly in endosomes and lysosomes and can detect nucleic acids released from viruses or bacteria that are degraded within endosomes and lysosomes within the cell [147]. For optimal ligand binding and initiation of signaling cascades, TLRs form homo- or heterodimers. In addition, TLRs interact with cofactors that participate in TLR activation by facilitating TLR ligand binding [151]. TLRs 1, 2, 3, 4, 5, 6 and 9 are constitutively expressed by keratinocytes while the expression of TLR7 can be caused by TLR3 activation [152,153].

Each TLR recognizes distinct PAMPs or DAMPs. TLR2 recognizes peptidoglycan of most bacterial species and LTA of gram-positive bacteria along with CD36 as its co-receptor. Additionally, TLR2 senses bacterial tri- or diacyl lipopeptides by forming heterodimers with TLR1 and TLR6, respectively [154]. TLR3 detects double-stranded ribonucleic acid (RNA) and plays a vital role in host antiviral defense [155]. TLR4 in coordination with CD14 is responsible for detecting the presence of lipopolysaccharide (LPS) from gram-negative bacteria [156]. TLR2 and TLR4 can also detect various DAMPs, including HMGB1, S100 proteins, and heat shock proteins (HSPs) [148]. The ligands for TLR5 are bacterial flagellin protein components [16]. TLR7&8 identify viral and bacterial single-stranded RNA [157,158]. Lastly, TLR9 perceives hypomethylated CpG motifs of bacterial and viral double-stranded deoxyribonucleic acid (DNA) [159]. The PAMP or DAMP that is recognized by TLR10 remains unidentified; however, literature suggests that TLR10 has anti-inflammatory properties [160].

The sensing of PAMPs and DAMPs by TLRs activates a downstream signaling cascade and is illustrated in Figure 2. This cascade leads to the activation of the NFκB and MAPK signaling pathways which subsequently result in the expression of immunomodulatory mediators, including cytokines, chemokines or AMPs [147]. This signaling cascade is divided into two pathways based on the adaptor proteins involved. All TLRs use MyD88 activation during this signaling cascade, except TLR3 which utilizes a MyD88-independent pathway via TIR domain-containing adaptor molecule 1 (TICAM1). This pathway can also be used by TLR4. The activation of TICAM1 not only activates NFκB and MAPK signaling, but also induces type I interferon production, which plays a critical role in antiviral defense [161].

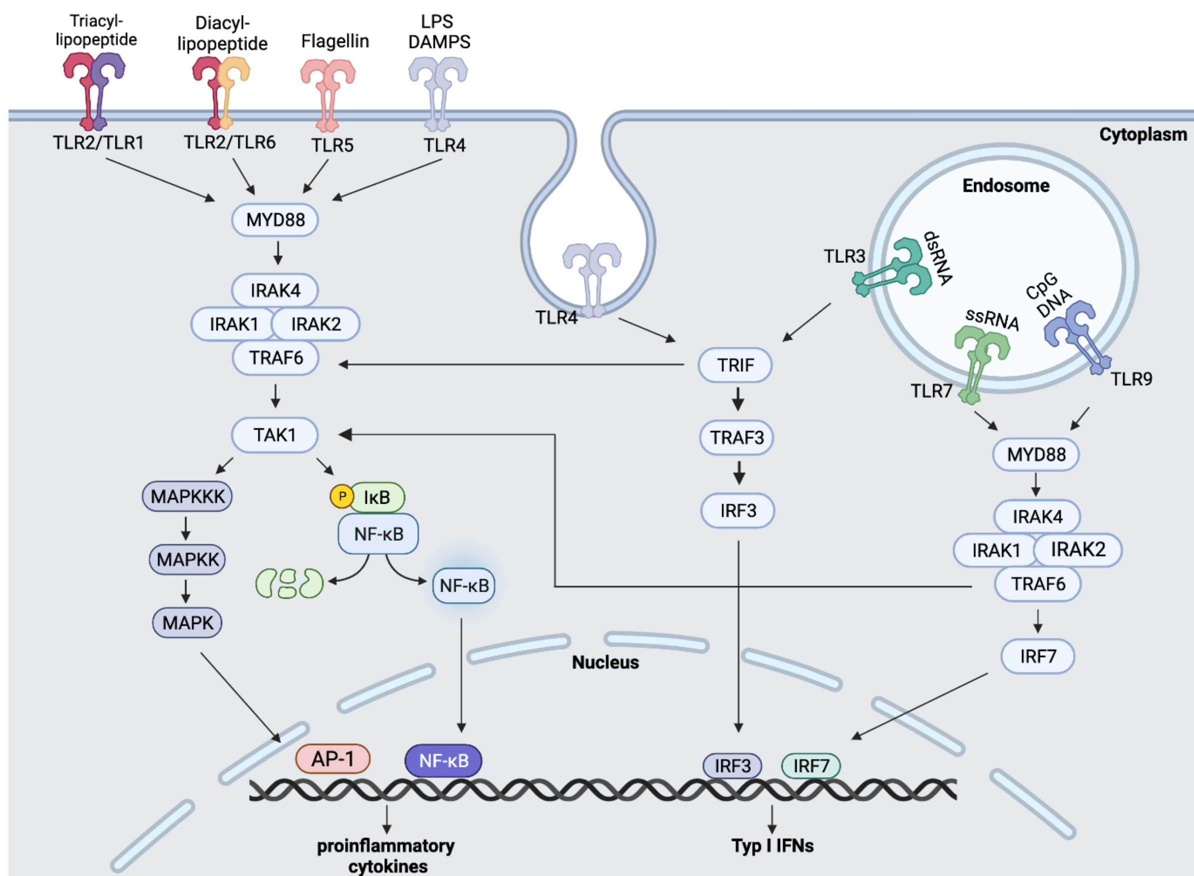

Figure 2: TLR signaling in keratinocytes. Keratinocytes express TLR1, TLR2, TLR4 and TLR5 on their surface and TLR3, TLR9 and TLR7 in the endosome. Binding of the indicated ligands to the TLRs activates a downstream signaling cascade. All TLRs use MyD88-dependent signaling except TLR3. The adaptor protein MyD88 activates the IRAK kinase members IRAK4, IRAK1 and IRAK3 which subsequently leads to the activation of the adaptor protein TRAF6. This results in the activation of MAPK and NFκB signaling. In addition, when TLR7 or TLR9 signaling is induced, TRAF6 activates the transcription factor IRF7 which activates Type I IFN gene expression. Instead of MyD88, TLR3 uses TRIF as adaptor protein which ultimately activates of Type I IFN gene expression mediated by the transcription factor IRF3. This signaling pathway can also be used by TLR4. This illustration was created with BioRender

In the context of sensing *S. aureus* in the skin, TLR2 plays a significant role. TLR2-deficiency in mice correlates with enhanced susceptibility to systemic *S. aureus*

infection [162,163]. In addition, TLR2 signaling triggers innate immune responses such as the induction of AMPs (e.g., HBD3) and cytokines in keratinocytes in response to *S. aureus* [164,165]. Furthermore, TLR2 signaling plays an important role in maintaining an intact epidermal barrier during infection, as *S. aureus* activation of TLR2 signaling induces the upregulation of tight junction proteins in keratinocytes and TLR2 knock-out mice exhibited impaired barrier recovery after tape-stripping [166]. Besides *S. aureus*, other members of the skin microbiome induce innate immune responses in keratinocytes through TLR2. For instance, a newly discovered AMP called Lugdunin produced by the commensal *S. lugdunensis* induces IL-8 secretion and neutrophil recruitment to the skin in a TLR2/MyD88 dependent way [167]. In addition, the skin commensal *S. epidermidis* induces the expression of AMPs in keratinocytes via TLR2 [168,169]. This highlights the role of TLR2 in the commensal-mediated protective immune responses against *S. aureus*.

While TLRs are critically involved in ensuring an efficient immune response to both exogeneous and endogenous insults, their activation must be tightly controlled in order to prevent excessive inflammation. Several mechanisms for the negative regulation of TLR signaling have been identified, including the release of soluble receptors or cofactors that can bind to ligands and inhibit membrane-bound receptor binding [170-172]. In addition, TLR signaling can be downregulated by feedback inhibition or transcriptional regulation [173,174]. Moreover, the influence of microRNAs on TLR signaling regulation has also been described [175-177].

Dysregulated activation of TLR signaling can be detrimental to the host. Overactivated or impaired TLR signaling can cause chronic inflammation or increased susceptibility to invading pathogens, resulting in inflammatory skin diseases. For example, keratinocytes in psoriatic skin exhibit increased expression of TLR2 and TLR4. Furthermore, a correlation between disease severity in AD and a deficiency in TLR2 signaling has been observed [178,179]. This is supported by findings that macrophages and monocytes in the peripheral blood of patients with AD exhibit downregulated TLR2 expression and impaired TLR2 activation in response to *S. aureus* by LCs in AD skin [180-182]. As TLR2 signaling in keratinocytes regulates the expression of tight junction proteins and AMPs, it is believed that TLR2 deficiency in AD allows pathogens to penetrate the skin [166].

3.1.3 Signaling pathways activated by PRRs in the skin

NFκB signaling

PAMPs or DAMPs that bind to PRRs trigger a downstream signaling cascade that activates the NFκB pathway. NFκB is a transcription factor that regulates the gene expression of a wide range of immunomodulatory mediators including cytokines, chemokines, AMPs, and adhesion molecules. It therefore plays a crucial role in shaping the immune response. In addition, NFκB signaling controls the expression of regulators of the cell cycle and pro-survival factors, thereby exerting a protective effect on cells [88].

The NFκB transcription factor family comprises five members, p105/p50 (NFκB1), p100/p52 (NFκB2), p65/RelA, RelB and c-Rel [183]. In principle, two main mechanisms of NFkB signaling have been reported: the canonical or classical pathway and the non-canonical or alternative pathway which differ in terms of their activation and signaling [184]. Here, only the canonical pathway will be discussed. Under steady-state conditions, the members of the NFκB family exist as homo- or heterodimers, with the p50/p65 heterodimer being the dominant form. These dimers are present in the cytosol and bind to inhibitory proteins of the IκB family. In the presence of an activating stimuli, the IκB kinase (IKK) complex which consists of IKKα, IKKβ, and the regulatory component NFκB essential modulator (NEMO), selectively phosphorylates the inhibitory IκB protein, leading to its ubiquitination and subsequent proteasomal degradation. As a result, the NFκB family proteins are released and able to enter the nucleus, bind to the DNA and exert their role as transcription factors [185].

NFκB signaling plays a crucial role in coordinating the immune response by regulating neutrophil recruitment, M1 polarization in macrophages, and T cell activation and differentiation, among other functions [88]. Precise regulation of NFκB signaling is essential and achieved in part by a negative feedback mechanism whereby activation of NFκB signaling induces inhibitory proteins such as IκBα, p105, or A20 [186]. Dysregulation of NFκB signaling is involved in the pathogenesis of inflammatory skin disorders, including AD. Numerous AD mouse models have shown overactive of NFκB signaling, and nuclear p65 expression is increased in the lesional skin of patients with AD compared to healthy skin [88,187,188]. This suggests that inhibition of NFκB signaling may be a promising treatment approach. However, inhibition of NFκB signaling can paradoxically lead to exacerbated inflammation. Previous studies have demonstrated

that mouse models with a specific deletion of I IKKβ in keratinocytes exhibited a lack of NFκB signaling, resulting in heightened skin inflammation shortly after birth and death within 10 days [189]. These findings were further validated by additional studies using different mouse models with distinct defects in NFκB signaling, thereby underscoring the indispensable role of NFκB signaling in the maintenance of skin homeostasis [190,191]. Moreover, a recent study indicates that restraining NFκB signaling leads to the necroptosis of keratinocytes and subsequent skin inflammation [192]. Possible explanations suggest that this could result from the pro-survival characteristics of NFκB and potentially elevated keratinocyte apoptosis in its absence [190,193]. Additionally, NFκB signaling provides an important communication way between keratinocytes and other immune cells which is disrupted upon inhibition [193].

MAPK signaling

In addition to the NFκB pathway, the downstream signaling of TLRs includes the activation of MAPKs which contribute to the innate immune response by promoting the activation of both pro-inflammatory and anti-inflammatory mediators [194]. The MAPK family can be divided in three pathways, the (classical) extracellular signal-regulated kinase (ERK) the stress-activated p38a and the Jun N-terminal kinase (JNK) pathway, with the ERK pathway being the most researched. These pathways vary in terms of their activating stimuli and downstream signaling response [195].

There is evidence that dysregulated MAPK signaling is involved in inflammatory skin diseases. A study by Zeze et al. showed that p-ERK levels are elevated in the skin of patients with AD and AD mouse models. Targeting p-ERK activation in the AD mouse model improved skin barrier function, immune cell infiltration and dermatitis [196]. Moreover, ERK-signaling is involved in the induction of epidermal barrier defects in AD. Ryu et al. demonstrated that in IL-33 transgenic mice, which display an AD-like phenotype, exhibit a notable reduction in CLDN1 expression in the epidermis. Subsequent experiments indicated that IL-33 downregulates CLDN1 in keratinocytes through ERK signaling [197].

3.1.4 Cytokines and chemokines induced after PRR activation in the skin

The sensing of DAMPs and PAMPs through PRRs and the subsequent downstream signaling, including NFκB and MAPK pathways, results in the enhanced production of

immune mediators such as cytokines and chemokines [198]. This activation ensures an effective immune response to both exogenous and endogenous insults. Secreted cytokines and chemokines induce the infiltration of immune cells into the skin and facilitate communication between different cell types, further initiating the immune response and bridging the innate and adaptive immune systems [2]. The following section and Figure 3 highlight the diverse array of cytokines and chemokines that play crucial roles in *S. aureus* skin infections. Their dynamics in both an intact skin barrier as well as in the context of an impaired skin barrier, as commonly observed in AD will be discussed.

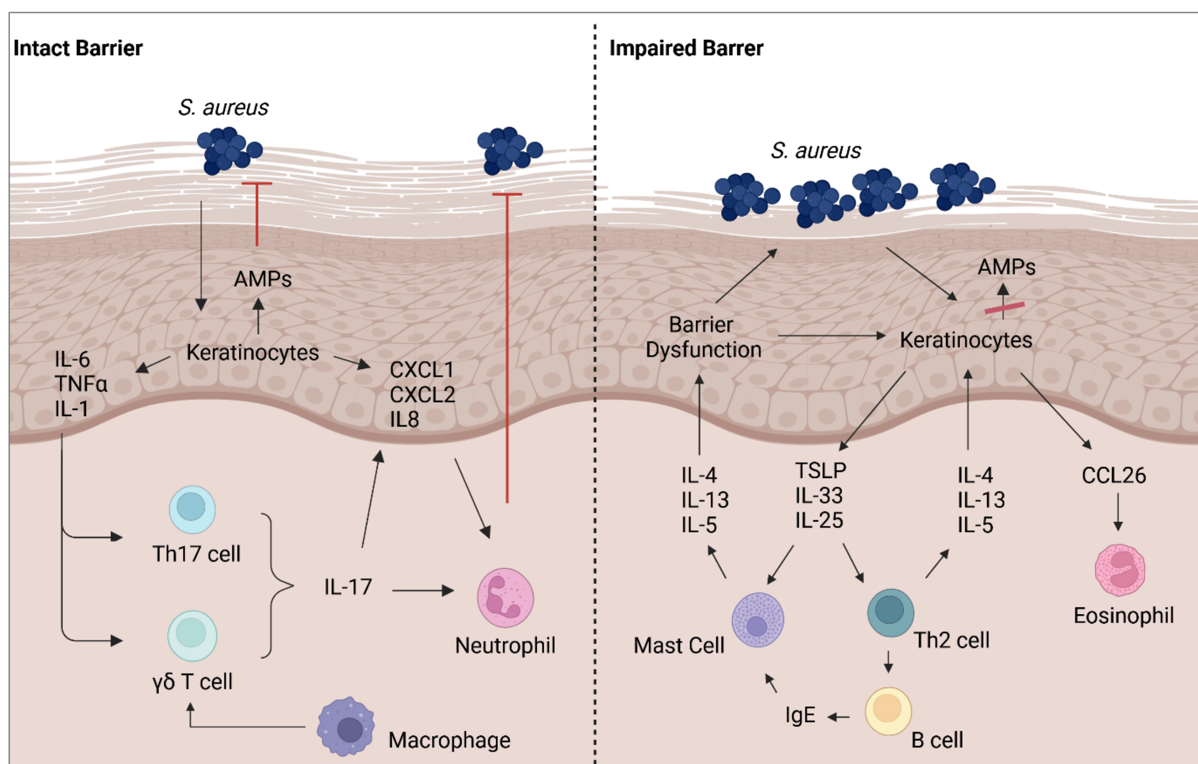

Figure 3: Cytokines involved in skin immunity. Epicutaneous *S. aureus* infection induces the secretion of IL-6, TNFα and IL-1 by keratinocytes, which induces the production of IL-17 by T cells. Additionally, macrophages induce IL-17 production by T cells. IL-17 leads to neutrophil recruitment to the skin and induces chemokines in keratinocytes which lead to further neutrophil recruitment that mediate bacterial clearance. Furthermore, AMPs induced by keratinocytes are bactericidal against *S. aureus*. In atopic dermatitis, the impaired skin barrier induces the secretion of the alarmins TSLP, IL-33 or IL-25 by keratinocytes. These alarmins induce the production of Th2 cytokines which further promote skin barrier dysfunction and IgE production by B cells which induces mast cell degranulation. Skin barrier dysfunction promotes *S. aureus* skin colonization, which further induces alarmin secretion by keratinocytes. In addition, Th2 cytokines induce CCL26 secretion by keratinocytes, which induces eosinophil recruitment to the skin. This illustration was created with BioRender

IL-1 family

One large cytokine family involved in skin immunity is the IL-1 cytokine family, which includes 11 members - all expressed in the epidermis. The proinflammatory members,

IL-1α, IL-1β, IL-33, IL-36α, and IL-18, contribute to inflammation by inducing the expression of proinflammatory cytokines and chemokines. On the other hand, the anti-inflammatory cytokines IL-37, IL-38, IL-1RA, and IL-36RA regulate IL-1-mediated inflammation by blocking signaling cascades through feedback inhibition or receptor blockade for instance [199].

Each IL-1 family cytokine binds to a specific IL-1 receptor, all of which comprise three extracellular Ig domains, a transmembrane domain as well as an intracellular TIR domain that shares homology with TLRs [199]. Like the TLRs, each IL-1 receptor forms complexes with specific accessory proteins for signal transduction [200]. The downstream signaling cascade of IL-1 receptors involves MyD88 and leads to the activation of MAPKs, NFκB, and activator protein 1 (AP-1). This leads to the subsequent activated gene expression of proinflammatory cytokines, including IL-12, IL-6, TNFα, and IL-23, which drive the immune response [201-203].

IL-1α is constitutively expressed in keratinocytes. Although proteolytic processing enhances the activity of IL-1α, it is not required, as full-length IL-1α already exhibits full biological activity [204]. IL-1α is characterized as an alarmin and exhibits dual functionality. In addition to its function as an extracellular cytokine, it also contains DNA binding sites and can translocate into the nucleus and function as a transcription factor and thus is involved in the regulation of the immune response on a transcriptional level. For example, nuclear IL-1α can upregulate the gene expression of *IL-8*, a chemokine highly chemotactic for neutrophils [205].

Unlike IL-1α, IL-1β is not constitutively expressed in keratinocytes but can be induced by microbial products, for instance. Moreover, IL-1β can induce its own gene expression [206]. It is stored in the cytosol in its biologically inactive, full-length form and requires proteolytic cleavage for activation. Inflammasome-activated caspase-1 is responsible for this cleavage process, both intracellularly and extracellularly [206]. Furthermore, other proteases such as neutrophil-derived proteases like neutrophil elastase (NE) or proteinase 3 can process IL-1β. However, this form of cleaved IL-1β is not as active as the one cleaved by capase-1. This suggests that neutrophils infiltrating inflamed skin can further contribute to skin inflammation by processing IL-1β, IL-1α, and IL-36 cytokines [207].

IL-36 can induce the expression of various genes in keratinocytes including pro-inflammatory cytokines like further IL-36, IL-1β or IL-8, but also anti-inflammatory

mediators such as A20 or NFKBIA suggesting that IL-36 plays a regulatory role in the immune response. [208]. Furthermore, IL-36 also affects the adaptive immune response by stimulating the differentiation of naïve T cells to Th1 cells [209,210].

IL-1 cytokines promote wound repair by mediating keratinocyte and fibroblast proliferation as well as ECM protein production [211]. The skin microbiome-mediated promotion of skin regeneration relies on IL-1β signaling in keratinocytes [212]. In addition, a recent study in mice revealed that IL-1β mediates immune tolerance to skin commensal bacteria, while inducing inflammation in response to pathogens like *S. aureus* by negatively regulating *S. aureus*-specific Treg cells [114]. This underscores the significance of IL-1 signaling in skin homeostasis and tolerance to commensal bacteria. Furthermore, IL-1 cytokines play a pivotal function in regulating skin infections induced by *S. aureus*. Interestingly, the IL-1 cytokine employed in response to *S. aureus* infections of the skin depends on the depth of the infection. During intradermal *S. aureus* infection, neutrophil-derived IL-1β, and IL-1R signaling is critical for the clearance of bacteria [21]. In contrast to that, in epicutaneous *S. aureus* skin infection models, keratinocytes induce skin inflammation by releasing the alarmins IL-1α and IL-36α resulting in a T cell-driven immune response [88]. Blocking IL-36 signaling using IL-36R-ko mice led to a reduction in skin inflammation and serum IgE levels, indicating a role for IL-36 in the onset of atopic inflammation in response to *S. aureus* [213]. Additionally, skin lesions of AD patients demonstrated an increase in mRNA expression of IL-36α and IL-36γ in epidermal keratinocytes, further supporting the role of IL-1 family cytokines in AD pathogenesis [213]. Furthermore, a recent study showed that *S. aureus* infection activates the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing (NLRP) 1 inflammasome in keratinocytes, resulting in IL-18 and IL-1β secretion [214]. Additionally, this study found a correlation between NLRP1 and the expression of IL-1β and IL-18 mRNA expression in AD patients [214]. Moreover, IL-33, also an IL-1 family cytokine, is secreted by stressed or damaged cells, is associated with a type 2 immune response and causes the downregulation of filaggrin and HBD2 in the skin, potentially increasing the susceptibility to bacteria in AD [215-217]. However, IL-33 also contributes to the improved clearance of *S. aureus* in an intradermal infection model by inducing NET formation in neutrophils, thus enhancing their antimicrobial properties [218].

Taken together, these findings demonstrate that IL-1 cytokine signaling is critically involved in the skin immune defense against *S. aureus,* but it also contributes to the pathogenesis of AD by driving skin inflammation.

TNFα

TNFα is a cytokine found in low concentrations in the upper epidermis and exhibits a significant increase in expression in keratinocytes during inflammation [219]. Both *S. aureus* infection and tape-stripping are stimuli that induce TNFα production in the skin [220,221]. TNFα signaling occurs through two receptors, TNFR1 and TNFR2, with keratinocytes predominantly utilizing TNFR1 [222]. Binding of TNFα to its receptor activates intracellular signaling resulting in the activation of NFκB and AP-1, among others, and subsequently triggers the expression of inflammatory cytokines and chemokines involved in innate immunity and inflammation [223]. TNFα plays a significant role in wound healing and considerably impacts the motility of keratinocytes [223]. Interestingly, TNFR2 agonists have demonstrated therapeutic efficacy against intradermal *S. aureus* skin infections, involving increased formation of neutrophil extracellular traps which mediate bacterial clearance [224].

IL-6

S. aureus infection stimulates IL-6 expression in both keratinocytes and tape-stripped skin. In mice, tape-stripping of the skin results in a rapid expression of IL-6 in keratinocytes throughout the epidermis and facilitates swift barrier repair [34]. Binding of IL-6 to its receptor, IL-6R activates an intracellular signaling cascade that involves the transcription factors STAT1 and STAT3 [225]. The production of IL-6 is regulated by TLR and IL-1 signaling, as well as TNFα. IL-6 can transmit signals through both a conventional membrane-bound mechanism and a trans-signaling pathway. In the latter, soluble IL-6 binds to soluble IL-6R, allowing IL-6 signaling in cells deficient in IL-6R expression, while augmenting IL-6 signaling in cells expressing IL-6R [226].

IL-6 is actively involved throughout the entire inflammation process from initial infiltration of neutrophils to T cell differentiation, thus building a a bridge between the innate and adaptive immune system [227]. Importantly, IL-6 contributes significantly to the control of bacterial infection, and impaired IL-6 signaling can enhance susceptibility to *S. aureus* infections [228]. However, IL-6 signaling is also involved in the development

of AD. The concentration of soluble IL-6R is increased in the plasma of patients with AD, and a functional variant of IL-6R correlates with heightened AD persistence [229].

IL-17 family

The IL-17 family cytokines, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25), and IL-17F, play a crucial role in the immune response to *S. aureus* skin infections. Binding to their specific receptors initiates a signaling cascade leading to the production of chemokines and consequent neutrophil migration, as well as the production of AMPs and cytokines, thereby amplifying the inflammatory response [230]. The main producers of IL-17 are CD4+ T cells that have been differentiated into a Th17 phenotype mediated by cytokines like IL-1β, IL-21, IL-6, IL-23, and TGFβ [231]. Apart from Th17 cells, IL-17 cytokines can also be expressed by innate immune cells, CD8+ T cells, B cells, or keratinocytes, for instance [232-234].

IL-17 cytokines have an important function in the fight against *S. aureus* skin infections. Upon *S. aureus* infection, keratinocyte-derived IL-1α and IL-36α induce the production of IL-17 by γδ T cells, resulting in neutrophil recruitment and subsequent bacterial clearance [121,235]. Deficiencies in IL-17 or IL-17RA are associated with an enhanced susceptibility to staphylococcal infections and mice with a specific deletion of IL-17RA in keratinocytes exhibited impaired clearance of *S. aureus* from the skin [236-238]. IL-17 has been implicated in the pathogenesis of several skin disorders and has been used effectively used as a target to treat psoriasis [239]. Some studies have proposed the involvement of IL-17 in AD, considering the increased levels of IL-17 expression levels in AD skin compared to healthy skin and the correlation between increased levels of IL-17-producing T cells in the blood of patients with AD and AD severity [240,241]. Deletion of IL-17 reduced Th2 induction in two mouse models of AD, further implying a potential contribution of IL-17 to AD pathogenesis [242]. However, a recent study using secukinumab, a monoclonal antibody targeting IL-17A, reported that targeting IL-17A is not suitable for the treatment of AD [243].

Th2 cytokines

Th2 cytokines are elevated in AD skin and have been implicated in the pathogenesis of the disease [244-247]. These cytokines include IL-4, IL-13, or IL-5 and are primarily derived from Th2 T cells, ILC2s, or MCs [248]. The Th2 response can be induced by the

epidermal-derived alarmins IL-33, IL-25 or TSLP, which are induced in keratinocytes upon injury [249,250]. For example, tape stripping leads to the secretion of TSLP from keratinocytes, which triggers the DC-mediated activation of IL-13- and IL-4-producing T cells [251]. In addition, stimulation of keratinocytes with pro-inflammatory cytokines including TNFα or Th2 cytokines induces TSLP secretion [252]. Interestingly, *S. aureus* infection of the skin, triggers TSLP and IL-33 secretion by keratinocytes and subsequent induction of a Th2 response [253]. This suggests that the increased *S. aureus* colonization of the skin, as observed in AD skin, may further drive Th2 responses in the skin and thus exacerbate the disease.

Th2 cytokines act in a number of ways. They can induce the expression of CCL26 in keratinocytes, resulting in the recruitment of eosinophils to the skin, a hallmark of AD [254]. However, IL-4 and IL-13 also promote skin barrier dysfunction by reducing the expression of filaggrin, involucrin and loricrin, and also affect the antimicrobial system of the skin by impairing the expression of human beta-defensins (HBDs) [255]. Therefore, the elevated expression of Th2 cytokines in AD skin might explain why these patients are more susceptible for to bacterial infections, which then further promote Th2 responses thus creating a vicious cycle of inflammation. Targeting Th2 cytokines with dupilumab, an antibody against IL-4Rα, impairs both IL-4 and IL-13 signaling and has been successfully used to treat AD [256]. Interestingly, recent evidence shows that dupilumab treatment leads to a rapid reduction in *S. aureus* skin colonization on AD skin [127,257]. In addition, another recent study showed that IL-4Ra blockade significantly reduced *S. aureus*-mediated skin inflammation in an ovalbumin-sensitized mouse model. This correlated with an increased expression of IL-17A and associated antimicrobial peptide genes which they propose as the underlying mechanism for the reduced *S. aureus* colonization [258].

Chemokines

The establishment and maintenance of an effective immune system relies on the dynamic migration of immune cells to, from and within tissues, both during homeostasis and in response to inflammatory stimuli. Chemoattractant cytokines, known as chemokines, orchestrate the rapid influx of immune cells into the skin, while also directing the migration of APCs from the skin to the skin-draining lymph nodes by selectively binding to their corresponding G-protein-coupled receptors (GPCRs) located on the surface of immune cells [259]. As a result, these chemokines guide

immune cells in a chemotactic gradient-dependent manner, ensuring their precise localization within the designated target tissue. Based on their cysteine configurations, chemokines are divided into subfamilies: CC, CXC, CX_3C, and XC [260].

Immune cells change their chemokine receptor expression pattern depending on their maturation status, location, and function. For example, upon activation, the expression pattern of naïve αβ T cells changes from CCR7+ CXCR4+ to a more skin-specific phenotype, including the upregulation of CCR4, CCR6, CCR8 and CCR10 on the surface. The ligands for these receptors are produced by skin-resident keratinocytes and fibroblasts as well as APCs [261,262]. In mice, keratinocyte-derived CCL27 is important in facilitating the migration of γδ T cell progenitors into the skin via CCR10 receptor binding [263]. Dermal γδ T cells can migrate to the epidermis via CCL20/CCR6 signaling, where they produce IL-22 and IL-17 and thus contribute to the pathogenesis of psoriasis [264]. The migration of skin-resident DCs and LCs to the skin-draining lymph node depends on the interaction of their chemokine receptor CCR7 and the chemokine CCL27, which is located on the lymphatic vessel and in complex with collagen IV in the basement membrane [265]. This is the main pathway by which immune cells navigate into the lymphatic vessels. Inflamed skin contains high levels of CCL2, CCL7 and CCL13 which bind to CCR2, highly expressed on monocytes, and initiate the entry of monocytes into the circulation and the epidermis [266]. In addition, IL-8, CXCL1, CXCL2, CCL2, CCL3, and CCL4 induce neutrophil infiltration into inflamed skin and are expressed by keratinocytes, neutrophils, and macrophages, for instance [267,268]. Changes in chemokine receptors on the surface of neutrophils lead to their eventual remigration back to the bone marrow for subsequent clearance [269]. A detailed description of the migration of neutrophil from the circulation to inflammation sites is given in section 3.2.

3.1.5 Antimicrobial peptides and the skin microbiome

Antimicrobial peptides

Besides the immunological and physical barriers, the skin possesses a chemical barrier involved in the fight against exogenous pathogenic bacteria. This consists mainly of AMPs, which are expressed by a variety of skin-resident or skin infiltrating cells. AMPs are either constitutively expressed or their expression can be induced or enhanced by pro-inflammatory cytokines or PAMPs [270]. They are small, amphipathic, mostly cationic peptides that, due to their positive charge, have a high affinity for the

negatively charged bacterial membranes and induce cell lysis [271]. AMPs have potent antimicrobial and immunomodulatory properties and thus contribute to the effective skin defense against invading pathogens [272].

One group of AMPs in the skin are the **human β-defensins** (hBDs) consisting of hBD1-4. HBD1 is constitutively expressed in the skin. In addition, proinflammatory cytokines or PAMPs induce the expression of hBD2, hBD3 and hBD4 [273,274]. The different hBDs vary in their antimicrobial properties. While hBD1 and hBD2 are mainly active against gram-negative bacteria [275], hBD3 is the most potent antibiotic against a broad spectrum of bacteria including *S. aureus* [276]. The significance of hBD3 in controlling *S. aureus* skin infections is highlighted by studies showing that a positive outcome of *S. aureus* skin infections is associated with higher inducibility of hBD3 in the skin [277]. Due to their antimicrobial functions, hBDs have been proposed as therapeutic target for AD [278]. AD skin is characterized by a Th2-dominated inflammation with an impaired barrier function and high *S. aureus* skin colonization. Th2-cytokines reduce the expression of hBD2 and hBD3 in keratinocytes [279,280]. HBDs not only act antibacterial, but they contribute to an effective skin barrier by regulating gene expression for epidermal barrier and tight junction proteins [281,282]. A recent study shows that hBD3 regulates the epidermal barrier function. They demonstrate that in an AD mouse model, the administration of the mouse homologue of hBD3, mBD-14, results in improved tight junction barrier function and reduced Th2 cytokines which was dependent on autophagy induction in keratinocytes [283]. In addition to their antimicrobial properties, hBDs harbor important immunomodulatory functions by inducing the chemotaxis of several immune cells [273,284,285].

In addition to hBDs, the AMP **RNase 7** is constitutively expressed by human keratinocytes and contributes to the host defense against invading bacteria [286,287]. Its expression can further be induced by proinflammatory cytokines or PAMPs [288,289]. RNase 7 is highly bactericidal against *S. aureus* and the decreased expression of RNase 7 is linked to higher *S. aureus* skin infections [290]. Similar to hBDs, RNase 7 is immunomodulatory and has been described to reduce Th2 cytokine production by T cells [291]. Moreover, a recent study has revealed a potential role for RNase 7 as an alarmin, by binding self-DNA and subsequently inducing danger signals in keratinocytes [292].

Another AMP in the skin is the cathelicidin **LL-37**. It is abundant in the specific granules of neutrophils [293]. Furthermore, its expression can be induced in keratinocytes

upon inflammatory conditions [167]. LL-37 is a small, highly cationic peptide that shows a wide range of antimicrobial activity against both gram-negative and gram-positive bacteria, including *S. aureus* [294,295]. In addition, LL-37 has immunomodulatory functions and induces FPR1-mediated recruitment of immune cells including neutrophils, monocytes, and T cells [293]. Furthermore, LL-37 can synergistically enhance the proinflammatory functions of immune mediators like IL-1β [296]. As hBDs, LL-37 contributes to a functional skin barrier by regulating expression of epidermal barrier genes and tight junction proteins and by promoting autophagy in keratinocytes [297,298].

Dermcidin is generated exclusively by human sweat gland cells and can be found abundantly in sweat [299]. Upon proteolytic processing, it harbors antimicrobial activity against skin pathogens including *S. aureus*. In contrast to the other AMPs described above, dermcidin is not cationic but carries a negative charge and thus exerts a different antimicrobial mechanism. Interestingly, AD sweat contains less dermcidin which is associated with enhanced susceptibility to skin infections, underscoring its role in the protection against invaders [300].

In summary, AMPs are valuable mediators in the interplay between the physical, chemical, and immunological barriers in the skin to provide a functional skin barrier and an effective host defense against invading pathogens.

The skin microbiome

Our skin is a habitat for a multitude of microorganism communities, defined as skin commensals. They are in a symbiotic relationship with our skin; on the one hand the skin provides them with a habitat and nutrients, while on the other hand skin commensals protect us from pathogenic bacteria. They do this in various ways, by producing antibiotics that directly interfere with invading pathogens or by interacting with skin host cells and shaping our cutaneous immune system [301,302].

The skin is a harsh environment to live in and is scarce of nutrients. Commensal bacteria express enzymes including proteases and lipases to make nutrients from the skin available from the skin [303]. Lipases also help to release free fatty acids from lipids present in the stratum corneum, which in turn promote bacterial adherence [304,305]. The composition of the skin microbiome undergoes changes with puberty and after that it stays stable [306]. Furthermore, the skin microbiome composition also depends on the body site; dry, moist, or sebaceous. Moreover, changes have been observed under

inflammatory conditions [301,307]. Interestingly, keratinocytes do not induce skin inflammation in response to skin commensals. Exposure to skin commensals early in life drives the development of *S. epidermidis*-specific Tregs in the skin that provide immune tolerance [308,309].

Skin commensals are important for the establishment of the skin barrier [310]. Uberoi et al. reported that germ-free mice had impaired barrier functions associated with aberrant keratinocyte differentiation and increased transepithelial electrical resistance (TEER). This could be retained by topical application of skin commensals highlighting the importance of the skin microbiome in the development of an effective skin barrier mediated by the induction of aryl hydrocarbon receptor (AhR) signaling in keratinocytes [162 311]. Moreover, *S. epidermidis* contributes to the maintenance of skin barrier homeostasis by providing ceramides to the skin via its sphingomyelinase [312].

Many components of our skin microbiome act beneficial against *S. aureus* skin infections [313]. For instance, *S. epidermidis* produces a serine protease that degrades proteins used by *S. aureus* for adhesion and biofilm formation [229,314]. Moreover, lantibiotics, produced by skin commensals have potent antibacterial activity against *S. aureus* and can synergize with each other and with AMPs derived from skin cells. For example, lantibiotics produced by the coagulase-negative *staphylococci S. epidermidis* and *S. hominis* and Lugdunin, produced by *S. lugdunensis* acts synergistically with LL-37 and dermcidin, derived from the host, in killing *S. aureus* [167,315]. Skin commensals can also be immunomodulatory. For example, *S. epidermidis*-specific T cells in the skin can induce AMP activation in keratinocytes which in turn act synergistically with *S. epidermidis*-derived bacteriocins to provide an effective defense against invading pathogens [316,317].

A dysbiosis in the skin microbiome is associated with inflammatory skin diseases. In AD, a shift in the skin microbiome has been observed which is associated with an overabundance of *S. aureus* on the skin, influencing the local immune response and contributing to inflammation [10]. This underscores the crucial role of the microbiome and its interplay with the skin in maintaining skin homeostasis

3.2 Neutrophils

Neutrophils, which make up the majority of leukocytes in the human blood, are a key component of the innate immune system and play a vital role in fighting bacterial infections [31]. They are the primary cells that enter a site of infection where they can

deploy a diverse set of antimicrobial mechanisms to ensure a robust initial defense against invading pathogens such as *S. aureus* to ensure host protection [318]. Beyond their antimicrobial functions, neutrophils actively modulate the immune response by detecting and releasing inflammatory mediators. This triggers the recruitment and activation of additional immune cells and facilitates the resolution of inflammation [319]. In addition, neutrophils actively contribute to tissue repair by degrading cell debris and promoting revascularization of tissues [320].

Studies showing that dysfunction or low counts of neutrophils are associated with recurrent severe infections underscore the importance of neutrophils in fighting *S. aureus* [321]. However, excessive neutrophil activity can result in chronic inflammation and autoimmune disorders [67,322]. The following sections will delve into the role of neutrophils in the innate immune system, covering their life cycle, antimicrobial mechanisms, and their implications in the pathogenesis of skin diseases.

3.2.1 Neutrophil life cycle

In the bone marrow, neutrophils differentiate from a common myeloid progenitor into terminally differentiated, mature neutrophils and subsequently enter the circulation in a granulocyte colony-stimulating factor (G-CSF) mediated manner [323]. Large numbers of neutrophils circulate in the bloodstream from where they can rapidly infiltrate tissues to respond to sterile or infectious inflammation [324]. In addition, neutrophils can also infiltrate and reside in naïve tissues including the spleen or the liver [325]. Neutrophils are considered short-lived cells and are constantly replenished by the bone marrow with an estimated turnover rate of 5.07 +/- 0.87 x 10^9 cells/kg per day [326]. Given the high production rate with about 10^{11} neutrophils per day, circulating neutrophils must be removed in a programmed manner to ensure homeostasis. Upon release of neutrophils into the circulation, their phenotype undergoes gradual changes with a decrease of CD62L surface expression and increase of CXCR4 surface expression over time in accordance with circadian rhythms [325,327]. These changes affect their migratory capability towards inflamed tissues and induce the clearance of neutrophils from the circulation [325,327]. Neutrophils re-enter the bone marrow, become apoptotic and are removed by macrophages by efferocytosis. Efferocytosis also takes place in the liver and the spleen [328,329]. In addition to macrophages, neutrophils can also be eliminated by DCs [330]. Neutrophil apoptosis and their clearance are key steps for inflammation resolution and important for maintaining immune homeostasis and

preventing the release of toxic substances from dying neutrophils which otherwise might induce tissue damage [331,332]. Ren et al. reported that septic mice showed enhanced survival mediated by apoptotic neutrophils which had a reduced LPS binding and subsequent macrophage clearance [333]. In addition, apoptotic neutrophils induce a pro-resolving phenotype in macrophages associated with the IL-23-mediated downregulation of G-CSF and subsequent inhibition of neutrophil infiltration [334].

There is still a debate regarding the lifespan of neutrophils. Studies using mice models have proposed that circulating neutrophils have a half-life of about 12h-18h [335]. Additionally, an investigation of the lifespan of murine neutrophils across various tissues – bone marrow, blood, liver, lung, spleen, intestine, and skin –revealed that the longevity of neutrophils depends upon the specific tissue they infiltrate [336]. Intriguingly, this study demonstrates that neutrophils rapidly adopt a tissue-specific phenotype and transcriptional profile [336]. This adaptation likely contributes to effective immune responses tailored to the demands of each particular tissue. The findings from these mouse studies underscore that the lifespan of neutrophils is linked to their location and function.

However, the lifespan of human circulating and tissue neutrophils is poorly understood. Early studies using an *ex vivo* labeling and reinfusing model, revealed an estimated half-life of circulating neutrophils of about 7-9 hours in humans [326,337]. A more recent study by Pillay et al. using *in vivo* labeling of human neutrophils with ^2H_2O showed that under homeostatic conditions, circulating human neutrophils have an estimated lifespan of 5.4 days [338]. However, doubts have been risen concerning this finding as the method they used also labels neutrophils in the bone marrow [339].

Inflammation is a key factor influencing the lifespan of neutrophils. The presence of inflammation leads to a notable increase, up to 10-fold, in the number of mature neutrophils in circulation [340]. This is orchestrated by to emergency granulopoiesis, a process triggered by the TLR-dependent cytokine production such as G-CSF and IL-6 [341,342]. Notably, several studies have shown that the extension of neutrophil lifespan under inflammatory conditions and multiple proinflammatory cytokines and PAMPs can delay neutrophil apoptosis [336,340,343-345]. In addition, inflammatory stimuli induce a primed state in neutrophils, associated with several phenotypic and transcriptional alterations that affect the adhesion, transmigration, and chemotaxis of neutrophils. Primed neutrophils exhibit elevated ROS production and enhanced antimicrobial responses against invading pathogens [346]. This suggests that the prolonged lifespan

observed under inflammatory conditions ensures the efficient use of neutrophil defense mechanisms to effectively eradicate infectious agents.

3.2.2 Neutrophil migration into tissues

Circulating neutrophils can rapidly migrate from the circulation into tissues during sterile or infectious inflammation to provide an effective first line of defense. This requires a multi-step process. In response to inflammatory signals, the expression of adhesion receptors like selectins on endothelial cells is increased. This enables circulating neutrophils to interact with these selectins, initiating the leukocyte adhesion cascade [347]. The transient binding of neutrophils to selectins leads to their rolling on endothelial cells [348]. This slowing of movement allows neutrophils to detect proinflammatory cytokines and chemokines at inflamed sites on the endothelial surface, inducing the upregulation of integrins on the surface of neutrophils [349,350]. These integrins can firmly bind to receptors on endothelial cells, slowing neutrophil rolling and eventually causing their arrest [351]. The subsequent transmigration of neutrophils through the endothelium to reach the inflammation site is known as diapedesis. This process involves alterations in neutrophil morphology, characterized by cell flattening and polarization, where neutrophils develop a leading edge, rich in filamentous actin and a trailing edge rich in myosin filaments. These structural changes facilitate their directed movement, aiding in the search for a suitable site to transmigrate through the endothelium into the extravascular space [352-354]. For the subsequent diapedesis, neutrophils can employ either the paracellular pathway, passing between adjacent endothelial cells, or the transcellular pathway, passing directly through the endothelial cell. The paracellular pathway is the predominant method, involving the formation of a gap between endothelial cells through the disruption of vascular endothelial cadherin contacts [355-357]. Once in the extravascular space, neutrophils migrate directionally to the site of inflammation in an integrin-dependent manner and guided by chemoattractant gradients [348].

Various chemoattractants, including CXCL8 family chemokines, lipid mediators like Leukotriene B4 (LTB4), PAMPs derived from bacteria, or tissue-derived DAMPs, can facilitate the migration of neutrophils [358,359]. Neutrophils display a range of surface receptors such as GPCRs, FPRs, and TLRs with which they can sense chemoattractants. Interestingly, neutrophils priorize chemoattractants as they show much higher chemotaxis towards bacterial stimuli than to endogenous chemokines [360].

Neutrophil migration can be divided into two phases. Early phase neutrophil recruitment is mediated by short-term signals such as bacterial products or DAMPs sensed by tissue-resident cells which subsequently release chemokines [361]. For example, skin inflammation caused by tape-stripping of mouse skin leads to rapid upregulation of the neutrophil chemoattractants CXCL2 and IL-1β in the skin and is associated with an initial influx of neutrophils to the skin. Interestingly, these neutrophils then produce LTB4, which leads to the recruitment of additional neutrophils and initiates neutrophil swarming [361-363]. Additionally, during intradermal *S. aureus* infection, early infiltrating neutrophils phagocytose the bacteria resulting in the inflammasome-mediated secretion of IL-1β. In turn, this IL-1β triggers surrounding cells to generate additional neutrophil chemokines, consequently amplifying the recruitment of neutrophils to the skin [21,152].

Interestingly, neutrophils can also migrate away from the inflammation site in a process called reverse transmigration. It is known that neutrophils can leave the inflammation site and relocalize to the lymph nodes or bone marrow, where they can be removed via efferocytosis by macrophages and activate T lymphocytes [364]. Furthermore, several studies using intravital microscopy have shown that neutrophils can reenter the bloodstream and disseminate to secondary organs where they can cause tissue damage. This is associated with severe systemic inflammation [357,365,366]. Neutrophils that underwent reverse transmigration are characterized by a prolonged lifespan and a proinflammatory phenotype [357,367]. However, further investigations concerning the reverse transmigration of neutrophils are necessary.

3.2.3 Antimicrobial mechanisms of neutrophils

Under homeostatic conditions, circulating neutrophils are in a quiescent state [340]. However, when exposed to proinflammatory factors, neutrophils are primed, enabling them to rapidly respond to activating stimuli. Primed neutrophils exhibit various phenotypic changes, including enhanced NADPH oxidase activation, granule release, cytokine and lipid synthesis, improved adhesion and transmigration, enhanced chemotaxis, and delayed apoptosis [346]. The subsequent sections provide a detailed discussion of the diverse antimicrobial mechanisms employed by neutrophils to combat pathogens. Conversely, *S. aureus* has evolved numerous evasion strategies to escape neutrophil-mediated killing, which are elucidated in section 3.3.

One mechanism that neutrophils use against *S. aureus* is **phagocytosis**. In this process, neutrophils engulf *S. aureus* in phagosomes and subsequently kill the bacteria by releasing an arsenal of antimicrobial substances including ROS and cytosolic granules into the phagosome. This process requires recognition and binding of the bacterium by neutrophils. Neutrophils bind microbial structures via PRRs, such as TLRs, expressed on their surface and subsequently activate signaling cascades that initiate cellular antimicrobial responses that contribute to bacterial killing after ingestion [368]. In addition, phagocytosis is enhanced by opsonization, a process in which the bacterium is coated with serum Igs and complement system factors. These factors are able to interact with Fc- and complement receptors, such as lectin, present on the surface of neutrophils, thereby facilitating bacterial binding and uptake [369]. Human serum contains specific Igs against *S. aureus* cell wall proteins including wall teichoic acids (WTA) or peptidoglycans. Upon receptor binding, the plasma membrane engulfs *S. aureus*, leading to the formation of a vacuole called a phagosome [369]. Once inside the phagosome, neutrophils employ several mechanisms to kill *S. aureus*. The fusion of granule membranes with the phagosome membrane results in the release of granule proteins into the phagosome. Myeloperoxidase (MPO), a granule present in azurophilic granules, generates highly cytotoxic ROS [370]. The generation of ROS by MPO requires the assembly and the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the phagosomal membrane. By electron transfer, NADPH oxidase generates superoxide anions in the phagosome, which are subsequently dismutated to H_2O_2. MPO then catalyzes the reaction of chloride and H_2O_2 resulting in the production of the ROS hypochlorous acid (HOCl), which exerts potent microbicidal capacities [370,371]. Interestingly, patients with defective NADPH oxidase activity suffer from recurrent, life-threatening infections [372], underscoring the importance of ROS production by neutrophils.

In addition to ROS production in the cytosol, *S. aureus* killing is mediated by the release of cytosolic granule contents into the phagosome in a process called **degranulation**. Neutrophils contain over 300 pre-formed proteins stored in cytoplasmic granules. These granules include azurophilic granules, specific granules, gelatinase granules, and secretory vesicles [373]. They are produced at different stages of granulopoiesis starting from the promyelocyte stage, with the azurophilic granules being produced first, followed by the specific granules, gelatinase granules and finally the secretory vesicles in that order in a targeting by timing model [374]. They differ greatly

in their content. Azurophilic, or primary granules contain the most toxic compounds with potent bactericidal properties including the above-mentioned MPO as well as hydrolytic serine proteases including neutrophil elastase (NE), proteinase 3, and cathepsin G, and antimicrobial pore-forming α-defensins [375]. The specific, or secondary granules contain lactoferrin, which sequesters iron to limit bacterial growth [376], as well as defensins and MMPs. The gelatinase, or tertiary granules, contain MMPs, lysozyme and lipocalin-2 (LCN2), which also acts bactericidal by sequestering iron, among others [377]. Finally, the secretory vesicles contain membrane receptors including CR1, CD14, CD16, and FPRs, as well as cytokines and albumin, indicative of endocytosis, and regulate the inflammatory response [378]. Thus, neutrophils can adapt to their environment by changing the expression of surface receptors through degranulation. Granule release is precisely regulated to reduce host damage and is dependent on the intensity of the stimulus [340,379,380]. Initial release occurs from the secretory vesicles, stimulated by chemokines or selectin signaling. Gelatinase granules are exocytosed at a higher threshold of stimulus concentration, and specific granules at even higher thresholds. Azurophilic granules, which contain the most toxic proteins, are released last [340].

In addition to fusion with the phagosome membrane, cytosolic granules can also fuse with the plasma membrane leading to the expression of the granules on the cell surface as well as their secretion into the extracellular space where they can exert their function [381]. Degranulation is tightly regulated to prevent excessive tissue damage [375]. An illustration of neutrophil phagocytosis and degranulation is provided in Figure 4.

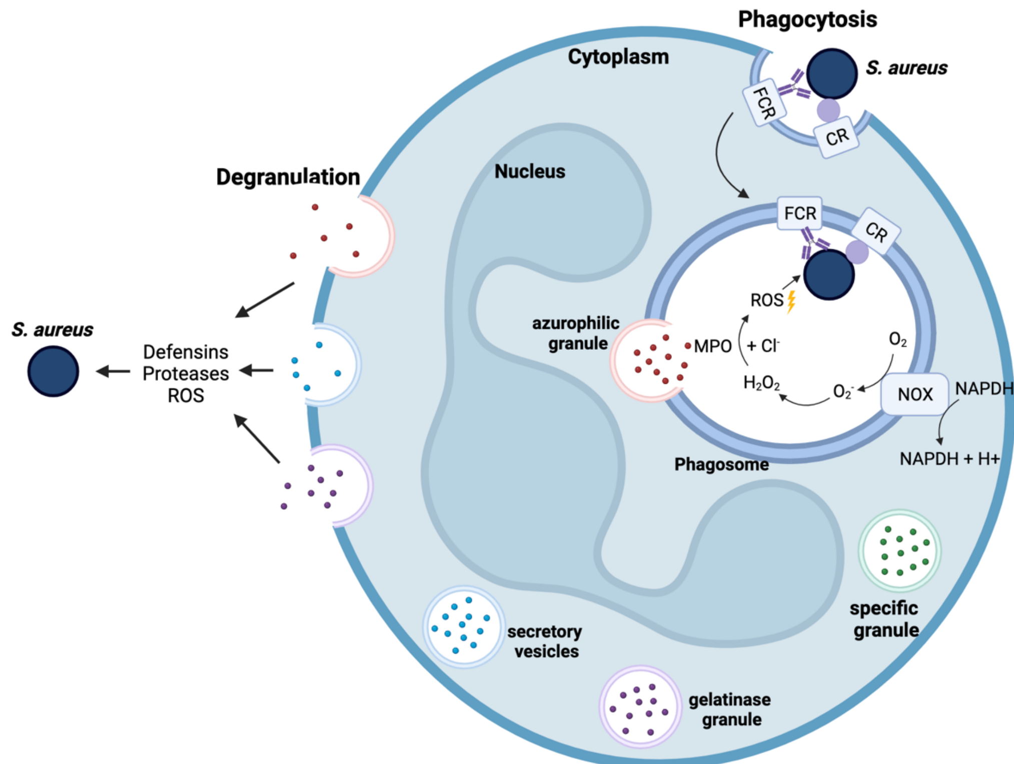

Figure 4: Neutrophil phagocytosis and degranulation. Phagocytosis of *S. aureus* is facilitated by opsonization of *S. aureus* with Igs and complement factors, which bind to FCRs and CRs on the neutrophil surface. Within the phagosome, the killing of *S. aureus* is orchestrated through the coordinated production of ROS by NADPH oxidase and MPO. In addition, cytosolic granules fuse with the phagosome membrane and cell membrane and expose *S. aureus* to defensins, serine protease and ROS. This illustration was created with BioRender.

Another potent antimicrobial mechanism used by neutrophils involves the generation and release of **neutrophil extracellular traps (NETs)** through a mechanism called NETosis [382]. NETs are chromatin structures enriched in antimicrobial peptides and histones [383]. Prominent substances present in NETs include histones, serine proteases, MPO, calprotectin, cathelicidins, and defensins [384]. Interestingly, NET formation does not occur in resting neutrophils and requires a priming stimulus [385]. NET formation is initiated by a wide variety of stimuli, including proinflammatory cytokines, activated platelets, bacteria and bacteria-derived products [386]. In the case of bacteria-induced NETosis, the size of the microorganism appears to determine whether phagocytosis or NETosis is triggered [387]. NETs act as antimicrobial agents by trapping pathogens, preventing them from spreading and killing them by exposing them to a high local concentration of antimicrobial peptides [387,388]. Several receptors appear to work synergistically to induce effective NET formation as more NET formation is induced by microorganisms compared to stimulation with one single TLR ligand [386,389].

Two mechanisms of NET formation have been reported: the lytic or suicidal NETosis and non-lytic or vital NETosis formation, as illustrated in Figure 5. Lytic NETosis results in cell death, while neutrophils undergoing non-lytic NETosis remain viable [383,390]. **Lytic NETosis** occurs within 45-240 minutes after the activation of neutrophils and involves several steps including chromatin decondensation, loss of nuclear membrane integrity, merging of chromatin and cytoplasmic components, disruption of the cell membrane, and eventual expulsion of NET structures into the surrounding space [386,389]. Over the years, a more detailed mechanistic understanding of the lytic NETosis have been described. In lytic NETosis, a stimulus triggers Raf-MEK-ERK-mediated activation of the NADPH oxidase, resulting in the production of ROS by neutrophils. This causes MPO and NE to exit the azurosome into the cytosol. NE then binds and breaks down F-actin filaments, facilitating its translocation into the nucleus. NE in the nucleus mediates chromatin decondensation by proteolytic processing of histones [391]. Additionally, H_2O_2 and increased intracellular calcium levels enhance the activation of peptidyl-arginine deiminase type 4 (PAD4), which triggers histone citrullination through the conversion of arginine residues, which have a positive charge, to citrulline, which is neutral. This modification affects histone-DNA interactions, causing chromatin decondensation [392]. In order for chromatin to exit the nucleus, protein kinase C and PAD4 catalyze lamin B1 phosphorylation and gasdermin D forms pores in the nuclear membrane upon activation by caspases or NE, leading to nuclear disintegration [393] [394,395]. To release NET structures into the extracellular space, cytoskeleton disassembly and plasma membrane rupture are necessary [393]. PAD4 plays a critical role in this intricate process. For example, PAD4 facilitates NRLP3 inflammasome activation, which in turn contributes to envelope or plasma rupture and the release of NETs [393,395-397].

In contrast to lytic NETosis, **non-lytic NETosis** is a much more rapid process, only taking about 5-60 minutes [398]. A prominent stimulus of non-lytic NETosis is *S. aureus*. Pilsczek et al. demonstrated that neutrophils release NETs within minutes following exposure to *S. aureus* and interestingly, this form of NETosis was independent of ROS production and did not require cell lysis to release the NETs [398]. The nucleic DNA is packaged into vesicles and secreted into the extracellular space, where the vesicles lyse and mix with the secreted granule content. These NETs exhibit strong bactericidal activity against *S. aureus* [398]. In non-lytic NETosis, a viable anuclear cytoplast with intact membranes is left behind that retains phagocytic capabilities. This viable form of

NET formation enables the neutrophils to trap bacteria and later phagocytize bacteria, keeping them local in the initial stages of infection and preventing subsequent systemic spread and infection [399].

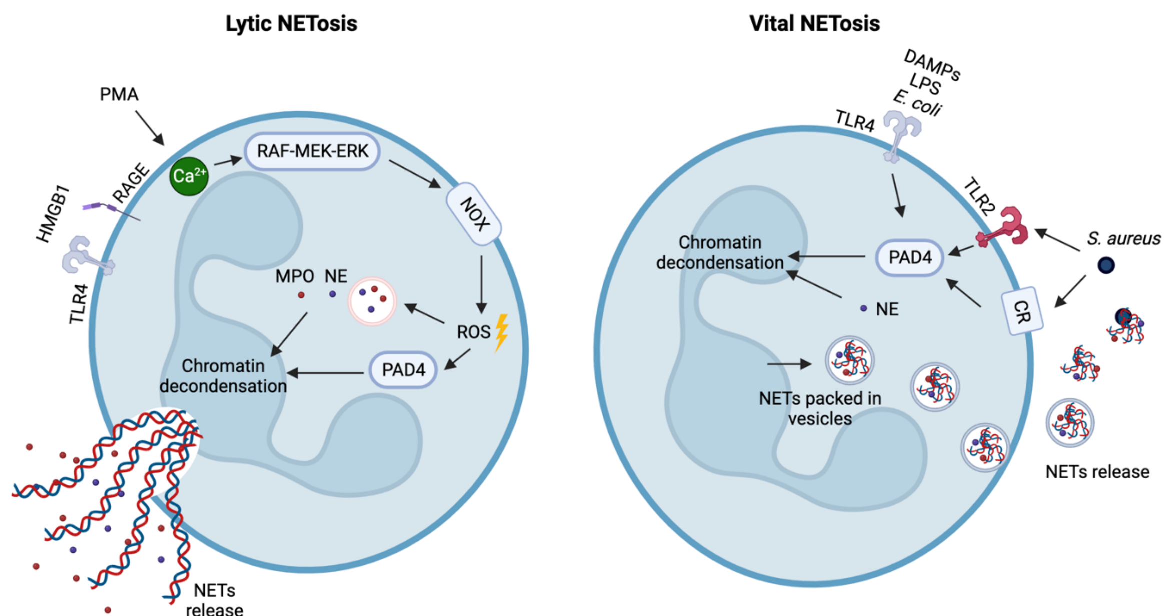

Figure 5: Mechanism of NET Formation. During lytic NETosis, stimuli activate the RAF-MEK-ERK-dependent activation of NADPH oxidase and subsequent ROS production. ROS induces the translocation or NE, MPO and PAD4 into the nucleus where they promote chromatin decondensation. Lytic NETosis is accompanied by cell lysis. In vital NETosis, stimuli activate PAD4 and NE which translocate into the nucleus and promote chromatin decondensation. This process is independent of ROS. NETs are packed in vesicles and are secreted to the extracellular space. An anuclear cytoplast remains. This illustration was created with BioRender.

As NETs are enriched in components that possess potent cytotoxic and inflammatory properties, the timely clearance of NETs from sites of inflammation is critical. Macrophages are capable of removing NETs through endocytosis and successive lysosomal degradation. The prior degradation of NETs by endogenous DNase I facilitates the ingestion of NETs [400].

3.2.4 The role of neutrophils and NETs in the skin immune system

Besides their antimicrobial functions, neutrophils actively shape the innate immune response by expressing various PRRs, including TLRs and cytoplasmic sensors of ribonucleic acids like NOD1 and FPRs [401-404]. In addition, neutrophils express several cytokine and chemokine receptors and produce various pro- and anti-inflammatory cytokines and chemokines. This allows neutrophils to detect and respond to immune reactions, as well as to recruit and activate immune responses in other immune cells. Neutrophils therefore both exert antimicrobial mechanisms and actively shape the immune response [343,405].

Although neutrophils primarily exist in the bone marrow and blood, some have also been observed in the skin even under steady-state conditions [406]. A recent study revealed a role of a keratinocyte-neutrophil crosstalk in maintaining skin microbiome diversity. Neutrophils express specific GPCRs, Mrgpra2, which bind to keratinocyte-derived defensins. Interestingly, the disruption of this defensin/Mgrpra2a signaling by creation of knock-out mice resulted in a loss of the skin microbiome diversity with a shift toward a higher abundance of *Staphylococcus* species on the skin [407]. This demonstrates the involvement of neutrophils in preventing dysbiosis of the skin microbiome. Additionally, neutrophils have an active role in skin wound healing and repair. In a murine model where skin injury was caused by tape-stripping, skin-infiltrated neutrophils were stimulated by the skin microbiota to generate CXCL10, which forms complexes with skin commensal derived DNA. These complexes induce plasmacytoid DCs (pDCs) to produce type I IFNs, triggering the production of growth factors by macrophages and fibroblasts which promote wound healing [408].

Neutrophils interact with several immune cells in the skin, including keratinocytes, DCs, NK cells, B cells, and T cells, as evidenced by several studies [409]. Neutrophils-produced chemokines like CCL2, CXCL9, CXCL10, CCL2, and CCL20 facilitate the recruitment of Th1 and TH17 cells to inflammation sites [410]. On the other hand, activated Th17 cells are able to induce additional recruitment and activation of neutrophils and stimulate granulopoiesis through upregulation of G-CSF mediated by IL-17 [411]. Additionally, cytokines produced by macrophages lead to neutrophil recruitment and prolong their survival, which provides optimal conditions for effective clearance of exogenous agents [412]. This dynamic interplay highlights the complexity and mutual influence of the immune system.

Effective neutrophil function requires a delicate balance between neutrophil infiltration, activation, and subsequent clearance from sites of inflammation. There is increasing evidence suggesting that dysregulated activation of neutrophils and increased formation of NETs are associated with the pathogenesis of several skin disorders [413]. In a murine model of allergic skin inflammation induced by tape-stripping and ovalbumin sensitization, neutrophils infiltrating the skin produced LTB4, which led to the recruitment and accumulation of more neutrophils as well as CD4+ T cells, triggering allergic skin inflammation [362]. Interestingly, individuals with AD have heightened LTB4 production in their neutrophils, and elevated levels of LTB4 have been observed in AD skin lesions [414-417]. Additionally, tissue-resident CD4+ T cells

induce neutrophil recruitment, which is associated with chronic skin inflammation in AD [418]. Moreover, skin-infiltrating neutrophils were found to induce itch in a MC903 murine AD model by inducing CXCL10 [419]. These findings strongly suggest that neutrophils contribute to the initiation of allergic skin inflammation in the context of AD. Furthermore, Shibuya et al. showed that neutrophils are involved in skin inflammation development in a murine model of irritant contact dermatitis [420]. The study found that the application of surfactants induced the accumulation of neutrophils in the skin, which was dependent on CCR2-mediated IL-1β production in the skin. Depletion of neutrophils eliminated skin inflammation signs, including epidermal thickening and TEWL, underscoring the key role of neutrophils in this model [420].

The role of neutrophils and NETs in skin pathogenesis has been well described in psoriasis [421]. Patients with psoriasis exhibit elevated levels of NETotic neutrophils in their bloodstream, which correlate with disease activity [422,423]. Furthermore, there is an increase in neutrophil granule proteins in the bloodstream, which may amplify the inflammatory response [424]. Psoriasis patients often display increased keratinocyte proliferation and neutrophil microabscesses in the skin, which are hallmarks of the disease [425,426]. The interaction between neutrophils and keratinocytes induces proinflammatory responses in both cell types. For instance, neutrophil proteases can cleave and thereby activate IL-1 and IL-36 cytokines, promoting keratinocyte proliferation [425,426]. In addition to neutrophils, NETs can induce inflammatory responses in keratinocytes by activating TLR4/IL36R-induced MyD88/NFκB signaling pathways. This leads to the production of LCN2, which further enhances neutrophil recruitment both *in vitro* and *in vivo* [422]. Moreover, NETs are capable of activating inflammation and NET formation in neutrophils, thus further fueling skin inflammation [427]. The ability of NETs to promote skin inflammation is underscored by a recent study which found that NETs trigger activation of the AIM2-inflammasome in keratinocytes through a MAPK-p38-dependent mechanism resulting in the production and secretion of IL-1β by keratinocytes [428]. Neutrophils and NETs are also involved in inducing the IL-23/IL-17 response in the skin, which is dominant in psoriasis. They function as a source of IL-17 and trigger IL-17R activation in keratinocytes, ultimately leading to psoriatic immune responses in an imiquimod murine model of psoriasis [429-431]. Aside from keratinocytes, neutrophils and NETs can also stimulate plasmacytoid DCs to initiate an immune response and enhance T cell responses [432-434]. NETs can induce Th17 cell expansion from PBMC-derived memory T cells [429]. Taken together, these findings highlight the

role of neutrophils and NETs in the inflammatory process mediated by IL-23/IL-17, demonstrating their significant involvement in the development of psoriasis.

Due to their high concentration of endogenous proteins, NETs are a source of autoantigens and are involved in the pathogenesis of various autoimmune diseases including SLE [435,436]. Patients with SLE demonstrate increased levels of circulating NETotic neutrophils and pathogenic autoantibodies such as anti-DNA antibodies. Additionally, NETotic neutrophils are observed in the skin of patients with lupus [437,438]. NET-derived LL37-DNA complexes activate B cells in a TLR9-dependent manner, including LL-37 specific memory B cells, thereby promoting the development of anti-LL37 autoantibodies [439]. Furthermore, NET-derived LL-37-DNA complexes stimulate the production of IFNα by pDCs [440]. Therefore, these findings reveal a further role of NETs in promoting inflammation by activating self-reactive B cells or pDCs. Additionally, NETs contribute to inflammatory responses in lupus macrophages by instigating NLRP3-mediated release of IL-1β and IL-18 [441]. Figure 6 illustrates the proinflammatory properties of NETs.

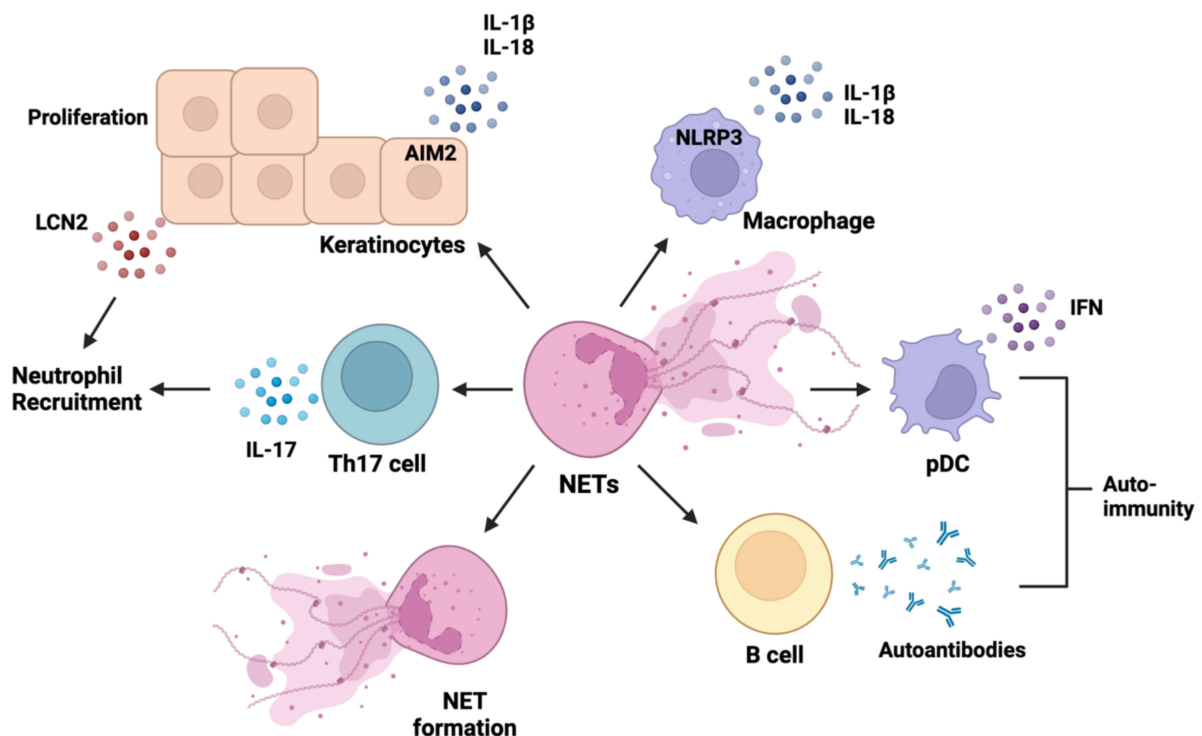

Figure 6: Inflammatory function of NETs: NETs active immune responses in various cells. They mediate the secretion of IL-1β and IL-18 in an AIM2- and NLRP3-inflammasome mediated manner in keratinocytes and macrophages, respectively. Additionally, NETs cause hyperproliferation of keratinocytes, a hallmark of psoriasis, and can induce neutrophil recruitment to the skin by triggering the secretion of LCN2 by keratinocytes and IL-17 by Th17 cells. Furthermore, NETs stimulate NET formation in neutrophils. NETs also mediate autoimmune responses by eliciting the production of autoantibodies in B cells and IFN responses in pDCs. This illustration was created with BioRender.

In addition to their immunomodulatory functions, agents derived from activated neutrophils and NETs such as proteolytic enzymes, or the overproduction of ROS can induce tissue damage and impair the skin barrier [442,443]. For example, NETs can delay the healing of cutaneous wounds [444].

In summary, excessive neutrophil activation and NET formation are associated with the pathogenesis of several inflammatory skin diseases. Targeting neutrophils and NETs may be a promising approach, as it has been demonstrated to improve symptoms in a murine model of psoriasis [445]. Numerous strategies have been reported to target neutrophils or NETs [413,446]. These include modulators that can influence the function of neutrophils either by blocking their recruitment, such as anti-IL-17 antibodies, CXCR2 antagonists, or anti-G-CSF receptors, or by directly interfering with neutrophil activation and NET formation, such as PAD4 inhibitors, antioxidants, or MPO- or NE inhibitors [446].

3.3 *Staphylococcus aureus*

S. aureus is a gram-positive coagulase-positive, facultative pathogen that either transiently or permanently colonizes about 30% of humans primarily in the nose [447,448]. However, *S. aureus* causes the majority of skin infections worldwide and can lead to serious systemic infections which can be life-threatening [449]. Nasal carriers of *S. aureus* are at great risk of *S. aureus* infections and targeting *S. aureus* in the nose temporarily protects against infections [450] [451].The rise of community-associated methicillin-resistant *S. aureus* (CA-MRSA) has been described. As these strains quickly develop resistance to a wide range of antibiotics, they pose a major problem for our health [452]. Therefore, new therapeutic strategies to effectively control *S. aureus* infections are required. For this, the further understanding of the mechanism regulating *S. aureus* infection is of high importance.

3.3.1 *S. aureus* colonization as a driver of inflammatory skin diseases

AD is a chronic inflammatory skin condition with a compromised skin barrier [6-8]. The pathogenesis of AD is complex and various factors contribute to the development and progression of the disease. Additionally, there is a dysbiosis and a reduced diversity of the skin microbiome, with a shift towards *S. aureus* in AD skin. Approximately 90 % of AD patients exhibit colonization of *S. aureus* of their skin [453]. This impedes the skin

microbiome-mediated protection against *S. aureus*, including skin microbiome-derived AMPs, thus facilitating further growth of *S. aureus* [315]. Subsequently, epidermal *S. aureus* colonization exacerbates skin barrier defects and inflammation in AD skin [454,455]. Studies have demonstrated a positive correlation between AD severity and *S. aureus* abundance on the skin and targeting *S. aureus* colonization has been shown to alleviate AD severity [453,456,457].

S. aureus possesses multiple tools that facilitate colonization, inflammation, and immune evasion, as illustrated in Figure 7. These consist of virulence factors, including phenol-soluble modulins (PSMs), pore-forming toxins, and superantigens, as well as extracellular enzymes, cell wall associated proteins, and PAMPs that trigger inflammation [458].

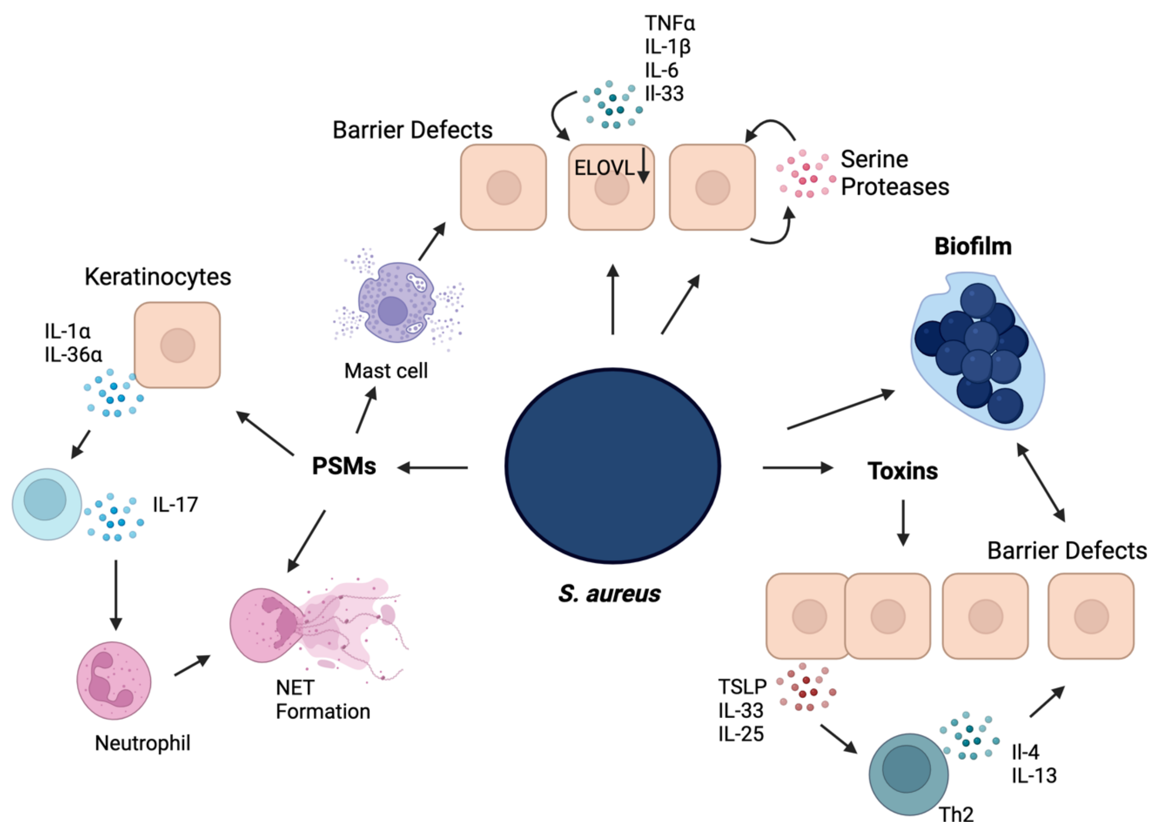

Figure 7*: S. aureus* as driver of skin inflammation. *S. aureus* produces a variety of factors contributing to skin inflammation. Skin barrier defects as induced by PSMs, toxins, proteases, or biofilm formation, either directly or by inducing Th2 responses in the skin. Furthermore, *S. aureus* induces the production of serine proteases in keratinocytes and inhibits the fatty acid elongase enzymes causing the accumulation of shorter chain fatty acids in the skin. *S. aureus* PSMs drive skin inflammation by inducing IL-1 cytokine secretion by keratinocytes which leads to the activation of IL-17 producing Th17 cells and neutrophil recruitment. *S. aureus* PSM then can induce NET formation in the recruitment of neutrophils. This illustration was created with BioRender.

The vast majority of the virulence factors is regulated by the quorum-sensing accessory gene regulatory system (agr), which is activated in response to bacterial density [459]. One noteworthy group of virulence factors comprises the PSMs, which

include PSM α, β and γ [460]. PSMα induces skin inflammation by stimulating keratinocytes to release IL-1α and IL-36α which, in turn, induces IL-17 production by $\gamma\delta$ T cells and neutrophil infiltration [235]. *S. aureus* can subsequently induce NETs in the infiltrating neutrophils [461,462]. The ability of PSMα to trigger skin inflammation has been verified by a recent study describing the induction of various proinflammatory cytokines and chemokines by keratinocytes in response to synthetic PSMα, as well as the lack of inflammation induction upon stimulation with *S. aureus* PSM mutants [463]. Additionally, *S. aureus*-derived PSMδ induces mast cell degranulation in lesional skin of AD by inducing IL-4 and IgE production [87,456]. Moreover, PSMs derived from *S. aureus* demonstrate high cytotoxicity and the ability to lyse cells via pore formation. Lysis of neutrophils post-phagocytosis enables *S. aureus* to evade immune responses [464]. Furthermore, PSMs play a crucial role in the biofilm formation of *S. aureus* [465]. A recent study reported that *S. aureus* colonization on AD skin can activate NLRP1 in keratinocytes, leading to the activation of IL-1β and IL-18 [214]. Moreover, Di Domenico et al. showed that proinflammatory cytokines IL-1β and IFNγ, which are elevated in AD skin lesions and can be induced in keratinocytes by *S. aureus* itself, enhance the growth of both planktonic and biofilm cultures of *S. aureus* in a concentration-dependent manner [466]. This suggests that *S. aureus* creates favorable growth conditions for itself by provoking inflammation in the skin.

The formation of biofilms by *S. aureus* could account for the heightened antibiotic resistance observed. AD-associated *S. aureus* strains are proficient to generate biofilms, which displays a significant correlation between the severity of AD, the dysfunction of skin barrier, and the probability of biofilm generation by *S. aureus* [467]. A study by Gonzales et al. sheds light on this relationship by revealing that an increased propensity of *S. aureus* to form biofilms is linked to a reduction in FLG expression in the skin [468]. These results demonstrate the clinical significance of biofilm formation in AD-associated *S. aureus* strains and highlight the complex interrelation between biofilm dynamics, AD severity, and skin barrier integrity.

Skin barrier defects facilitate colonization of *S. aureus*, and interestingly, *S. aureus* colonization further promotes skin barrier dysfunction. In lesional AD skin, *S. aureus* triggers the release of inflammatory cytokines including TNFα, IL-1β, IL-6. and IL-33 in keratinocytes, inhibiting the expression of fatty acid elongase enzymes, the so-called elongation of very long chain fatty acids proteins (ELOVL) in keratinocytes. This, in turn, leads to an accumulation of shorter chain fatty acids in the skin, which are

associated with skin barrier dysfunction [469]. Additionally, *S. aureus* triggers Th2 inflammation in the skin by secreting enterotoxin, which interacts with MHCII and TCR of Th2 cells leading to the production of Th2 cytokines which are associated with skin barrier dysfunctions. Furthermore, toxins secreted by *S. aureus* may cause cell lysis and subsequent death of keratinocytes, resulting in the secretion of DAMPs that activate the production of Th2 cytokines [470]. As discussed in section 1.1.4, skin barrier dysfunction is closely linked to Th2 inflammation, highlighting the direct contribution of *S. aureus* to this inflammatory process. Furthermore, *S. aureus* can induce the expression of serine proteases in keratinocytes, which results in the degradation of epidermal barrier proteins and thus further driving skin barrier defects [471].

In summary, *S. aureus* colonization on AD skin plays an active role in the progression of the disease through various mechanisms. As such, targeting *S. aureus* colonization appears to be a promising avenue for treatment. Targeting *S. aureus* using antibiotics is inefficient and leads to the rapid recolonization of the skin by *S. aureus* within weeks [472]. This approach is impractical in light of the current antibiotic resistance crisis. A promising alternative is the use of skin commensals, such as *S. epidermidis* or *S. hominis* or *S. lugdunensis* which produce AMPs that provide both direct bactericidal and immunomodulatory protection against *S. aureus* [315,167]. Additionally, a recent study demonstrated that *S. aureus* skin colonization in AD skin quickly decreased by treatment of dupilumab, an IL-4R inhibitor that blocks IL-4 and IL-13 signaling, as a therapy for AD. This was also linked to decreased AD severity [127]. Further comprehension of the regulation of *S. aureus* skin colonization is vital for the development of new approaches.

3.3.2 Role of neutrophils in *S. aureus* skin infection

As outlined in section 3.2, neutrophils are the initial cells to infiltrate the skin during *S. aureus* infection, and therefore serve as the first layer of defense in clearing bacteria and preventing systemic spread [473-475]. Defects in the function of neutrophils correlate with severe relapsing infections, highlighting the significance of neutrophils in *S. aureus* skin infections [321].

Interestingly, there is a different role for neutrophils in mouse models of epicutaneous or intradermal *S. aureus* infection. In intradermal *S. aureus* infection, neutrophil recruitment to the skin and subsequent clearing of bacteria from the skin is

dependent on IL-1R/Myd88 signaling initiated by inflammasome-dependent production of IL-1β in the skin, which subsequently induces IL-17 production in skin-resident γδ T cells [21,121,476,477]. A recent study further showed that during an intradermal *S. aureus* infection, keratinocyte-derived defensins activate neutrophils to release IL-1β and CXCL2, which increases further neutrophil recruitment and subsequent bacterial clearing [407]. Neutrophil depletion in the intradermal *S. aureus* infection resulted in increased pathogen loads and skin lesions in the skin, highlighting the importance of neutrophils in bacterial clearance in this model which is proposed to be mediated by NADPH oxidase-dependent phagocytosis [478]. In addition to relocating into the skin to fight *S. aureus* directly at the infection site, studies have revealed that intradermal *S. aureus* infection triggers neutrophil migration through high endothelial venules to the skin-draining lymph nodes, where they prevent systemic *S. aureus* dissemination and activate lymphocyte proliferation [474,479,480]. Interestingly, the early infiltration of neutrophils into the skin-draining lymph node was only observed following *S. aureus* infection but not in sterile lesions [480]. Neutrophil clearance in the skin-draining lymph nodes is regulated by conventional DCs, which helps to control skin inflammation in response to *S. aureus* skin infection [481].

Interestingly, the immune response of neutrophils differs during epicutaneous *S. aureus* infection where keratinocytes are the first sensors of the pathogen. Although neutrophil depletion reduced the disease score in an epicutaneous infection model, it had no effect on the total bacterial loads on the skin. However, it led to the invasion of *S. aureus* into the dermis, suggesting a role of neutrophils in preventing *S. aureus* dissemination in the skin [478]. Additionally, a recent study demonstrated that after epicutaneous *S. aureus* infection, defensins produced by keratinocytes activate Mrgpra2, a neutrophil-specific GPCR essential for the clearance of *S. aureus* from the skin. Mice with neutrophils lacking these receptors exhibited prolonged persistence of *S. aureus* on the skin, highlighting the critical role of Mrgpra2 in the immune response to *S. aureus* infection [407]. Furthermore, Schulze et al. used a humanized model to demonstrate that the epicutaneous colonization of a non-invasive MRSA strain on the stratum corneum triggered the recruitment of neutrophils via IL-8 signaling, which subsequently eliminated *S. aureus* from the skin. This finding reveals a vital role for neutrophils in regulating transient *S. aureus* colonization of the skin [482].

Although neutrophils are considered indispensable for *S. aureus* skin infection, there is increasing evidence of a correlation between neutrophils and *S. aureus* skin

colonization. In AD skin, elevated levels of IL-8 and GM-CSF, known chemoattractants for neutrophils, correlate with increased neutrophilic infiltrates in the dermis [483,484]. The increased colonization of *S. aureus* in lesional AD skin is associated with elevated levels of IL-8 within the stratum corneum, indicating a connection with the severity of AD and skin barrier dysfunction [485]. Moreover, the upregulation of IL-8 in an epidermal skin model with FLG knockdown is linked to enhanced *S. aureus* colonization of the skin [486]. Skin barrier disruption by tape-stripping enhances *S. aureus* persistence on the skin *in vivo* [220]. This enhanced *S. aureus* colonization of the skin is mediated by the interaction of NETs, formed by infiltrating neutrophils, and skin-resident keratinocytes [221]. Interestingly, a recent study showed that epicutaneous *S. aureus* colonization can induce NET formation in infiltrating neutrophils which subsequently triggers the development of autoantibodies as they occur in SLE [487]. There is evidence that *S. aureus* colonization might be increased on the lesional skin of SLE subjects who also exhibit skin barrier dysfunctions [488].

Taken together these findings suggest a potential role for neutrophils in increasing *S. aureus* colonization of the skin through the release of NETs. The resulting increase in *S. aureus* skin colonization may further contribute to skin inflammation and potentially play a role in the development of autoimmune inflammation.

3.3.3 Evasion strategies of *S. aureus*

S. aureus has developed a number of mechanisms to evade neutrophil-mediated killing, as illustrated in Figure 8. Especially highly virulent strains, which are increased in AD skin lesions, are capable of avoiding the antimicrobial response while at the same time exhibiting a potent colonization ability [164,489].

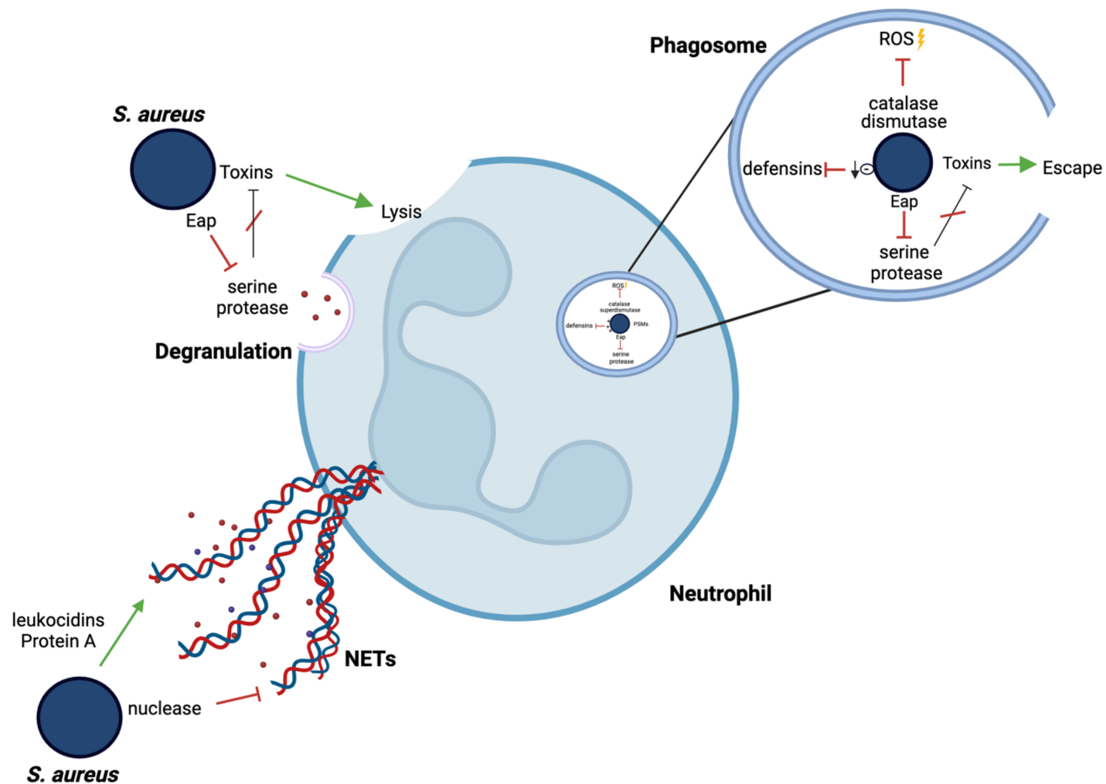

Figure 8: Strategies of *S. aureus* for evading neutrophil-mediated killing. Neutrophils employ various antimicrobial mechanisms including phagocytosis, degranulation, and NET formation to eliminate *S. aureus*. *S. aureus* can escape phagosomal killing by expressing catalase and superoxide dismutase, which neutralize ROS, lowering their negative charge to evade killing by cationic defensins and by expressing protease inhibitors Eap that inhibit serine proteases responsible for toxin degradation. Toxins produced by *S. aureus* can lyse the phagosome membrane, leading to the escape of the bacterium, as well as lysing the cell membrane, resulting in the death of neutrophils. Moreover, *S. aureus* induces NET formation via toxins or protein A and subsequently evades NET-mediated killing by expressing nucleases that degrade NETs. This illustration was created with BioRender.

One notable evasion strategy employed by *S. aureus* involves inhibiting neutrophil recruitment mediated by chemotaxis inhibiting proteins (CHIPs) in a C5a- and FPR-mediated manner [490]. Furthermore, several factors of *S. aureus*, including Spa and clumping factor A (ClfA) have been identified to inhibit neutrophil phagocytosis-mediated killing [491,492]. *S. aureus* can evade ROS-mediated killing phagosome by expressing enzymes like catalase and superoxide dismutase which neutralize ROS within the phagosome [493]. In addition, *S. aureus* can activate itaconate production in neutrophils, which blocks the production of ROS [494]. By reducing its negative charge on the cell wall via dlt-mediated incorporation of D-alanine into teichoic acids or multiple peptide resistance factor (mprf)-mediated L-lysine into phosphatidylglycerol, *S. aureus* becomes less susceptible to killing by cationic AMPs such as α-defensins from neutrophils [495,496]. The expression of protease inhibitors, such as the extracellular adherence protein (Eap), adds another layer of evasion strategy by inhibiting the activation of neutrophil-derived serine proteases, thereby preventing the degradation of its virulence factors, particularly PSMs [497,498]. Moreover, *S. aureus* can evade

phagosomal killing and escape the phagosome via cell lysis. Toxins like leukocidins and PSMs actively participate in this process by lysing neutrophils through pore formation, ultimately facilitating the escape of *S. aureus* from the cell [464].

In addition to phagocytosis, neutrophils can employ NET formation to eliminate *S. aureus*. Interestingly, *S. aureus* has the ability to both induce NET formation and subsequently evade NET-mediated killing. *S. aureus*-induced NET formation can be mediated by several virulence factors including leukocidin LukAB or Spa [499,500]. To counteract NET-mediated killing, *S. aureus* has developed several strategies. *S. aureus* expresses nucleases that effectively degrade NETs, preventing their entrapment and subsequent killing [501]. Thammavongsa et al. showed that intravenous *S. aureus* infection triggers a two-step process. *Staphylococcal* nuclease initially degrades the DNA of NETs to deoxyadenosine monophosphate, and subsequently, staphylococcal adenosine synthase A (AdsA) converts this into deoxyadenosine (dAdo), inducing apoptosis in macrophages [502]. NETs can enhance bacterial killing of *S. aureus* by macrophages [503]. Therefore, by degrading NETs and potentially inhibiting their microbicidal enhancement of macrophages, *S. aureus* manipulates the immune response to its advantage.

A study by Kwiecinski et al. reported the involvement of the ArlRS two-component system, comprising ArlRs and MrgA, in regulating the expression of factors involved in immune evasion. Mutant strains have a lower nuclease expression and exhibit a decreased capacity to degrade NETs., highlighting the critical role of this regulatory system. Additionally, the expression of various *S. aureus* toxins, including leukocidins, which are linked to NET development and immune evasion, was also impaired in mutants [504].

In conclusion, the extensive range of evasion mechanisms demonstrates *S. aureus*'s resilience against neutrophil-mediated immune responses, emphasizing the complexity of host-pathogen interaction. The further understanding of these sophisticated mechanisms is crucial for developing effective strategies to counteract *S. aureus* infections.

3.4 Aim of the Thesis

S. aureus is the cause of the majority of skin infections, and it is highly prevalent on the skin of patients with AD where it actively contributes to the pathogenesis of the disease by exacerbating skin inflammation [456]. Neutrophils are usually critical for controlling *S. aureus* skin infections. However, there is growing evidence that neutrophils may have an opposing effect in inflammatory skin conditions by potentially creating a favorable environment for *S. aureus* skin colonization. In particular, an interaction between NETs and keratinocytes has been demonstrated to increase *S. aureus* skin colonization. However, the underlying mechanisms of this phenomenon are as yet unknown. Thus, the purpose of this thesis was to examine in detail the interplay between neutrophils, NETs and keratinocytes that underlies the increased *S. aureus* colonization in inflamed skin. In addition, this thesis aimed to comprehend how the crosstalk between keratinocytes and neutrophils influences both cell types and contributes to skin inflammation in both a sterile environment and during *S. aureus* infection.

The specific objectives for the manuscripts included were as follows:

Manuscript I: The aim of this manuscript was to analyze in detail the effect of neutrophils and NETs on keratinocytes in promoting *S. aureus* colonization and persistence in inflamed skin. The specific objectives of this study were (i) to examine the role of neutrophils in the increased *S. aureus* colonization and persistence on tape-stripped skin *in vivo* using an epicutaneous murine infection model, (ii) to analyze the innate immune response triggered in keratinocytes by neutrophils and NETs responsible for the increased colonization of *S. aureus in vitro* using a human co-culture system, and (iii) to demonstrate the clinical significance of the results using CODEX analysis and immunofluorescence staining of the skin of AD patients.
This manuscript is published in Cell Reports [505].

Manuscript II: The aim of this manuscript was to examine the effect of neutrophil and keratinocyte crosstalk on immune response modulation in inflamed skin under sterile conditions and during *S. aureus* infection. The specific aims of this study were (i) to analyze how the communication between keratinocytes and neutrophils affects the lifespan and activation of neutrophils, as well as the proinflammatory responses in

keratinocytes, (ii) to analyze how the communication between neutrophils and keratinocytes affects the proinflammatory responses of both cell types during *S. aureus* skin infection and lastly, (iii) to investigate if and how the skin microbiome regulates neutrophil-mediated skin inflammation.

This manuscript is published in Frontiers in Immunology [506]

4 Manuscript I

This work is published in Cell Reports [505]:

Focken J, Scheurer J, Jäger A, Schürch CM, Kämereit S, Riel S, Schaller M, Weigelin B, Schittek B. Neutrophil extracellular traps enhance S. aureus skin colonization by oxidative stress induction and downregulation of epidermal barrier genes. Cell Rep. 2023 Oct 31;42(10):113148. doi: 10.1016/j.celrep.2023.113148.

Personal Contribution

- Together with B. Schittek, I was responsible for conceptualizing, designing, and supervising this study and developing all required methods
- I performed all the experiments with the help of J. Scheurer (mouse experiments, flow cytometry), S. Kämereit (immunofluorescence microscopy), S. Riel (confocal microscopy) and B. Weigelin (live-cell imaging)
- A. Jäger performed and analysed CODEX experiments in guidance of C.M. Schürch
- I did the data analysis and writing of the manuscript under the supervision of B. Schittek
- All other co-authors contributed by proof-reading the manuscript

Neutrophil extracellular traps enhance *S. aureus* skin colonization by oxidative stress induction and downregulation of epidermal barrier genes

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18
Summary

19 *Staphylococcus aureus* is the most common cause of bacterial skin infections in
20 humans including patients with atopic dermatitis (AD). Polymorphonuclear neutrophils
21 (PMNs) are the first cells to infiltrate an infection site where they usually provide an
22 effective first-line of defense including neutrophil extracellular trap (NETs) formation.
23 Here we show that infiltrating PMNs in inflamed human and mouse skin enhance *S.*
24 *aureus* skin colonization and persistence. Mechanistically we demonstrate that a
25 crosstalk between keratinocytes and PMNs results in enhanced NET formation upon
26 *S. aureus* infection which in turn induces oxidative stress and expression of danger-
27 associated molecular patterns such as high-mobility-group-protein B1 (HMGB1) in
28 keratinocytes. In turn, HMGB1 enhances *S. aureus* skin colonization and persistence
29 by promoting skin barrier dysfunctions by the downregulation of epidermal barrier
30 genes. Using patient material, we show that patients with AD exhibit enhanced
31 presence of PMNs, NETs and HMGB1 in the skin demonstrating the clinical relevance
32 of our finding.

33

34
Keywords

35 Neutrophil extracellular traps, neutrophils, Staphylococcus aureus, atopic dermatitis,
36 skin barrier, oxidative stress, DAMPs

Introduction

Staphylococcus aureus is a facultative pathogen and leading cause of skin infections in humans including patients with atopic dermatitis (AD) [1]. AD is a chronic and relapsing inflammatory skin disease and is often associated with skin barrier defects [2]. *S. aureus* frequently colonizes the skin of patients with AD. *S. aureus* burden correlates with AD severity and targeting *S. aureus* colonization improves AD indicating that *S. aureus* skin colonization contributes to skin inflammation and promotes the disease [2-5].

Polymorphonuclear neutrophils (PMNs) are the first cells that infiltrate an infection site where they provide an effective first-line of defense [6]. The importance of PMNs in host defense against *S. aureus* is emphasized by studies showing that defects in PMN function correlate with immunodeficiency and recurrent severe infections [7]. PMNs are endowed with various antimicrobial mechanisms, including phagocytosis, reactive oxygen species [507] production, and degranulation of cytosolic granules [8]. In addition, PMNs can release neutrophil extracellular traps (NETs) in response to *S. aureus* [9]. NETs are web-like chromatin structures decorated with histones and antimicrobial peptides that trap *S. aureus,* prevent it from spreading and expose it to high local concentrations of antimicrobial peptides [10]. Besides their antimicrobial role, NETs also exert immunomodulatory functions. For example, they can induce immune responses in keratinocytes via a Toll-like receptor 4(TLR4)interleukin-36 receptor (IL-36R) crosstalk and activate ROS production and IL-8 secretion in PMNs [11,12].

Our recent results and those of other groups indicated that PMNs and NETs play a functional role in promoting *S. aureus* skin colonization [13,14]. These data suggest that skin infiltrating PMNs and NETs are important to keep infections local in the initial stages of colonization. However, the underlying mechanism of the crosstalk of skin-derived cells such as primary human keratinocytes (PHKs) with PMNs/NETs to enhance *S. aureus* skin colonization is not yet understood. In this work, we elucidate in depth the mechanisms of PMN- and NET- mediated enhanced *S. aureus* skin colonization using an *in vitro* human co-culture model and an *in vivo* epicutaneous mouse skin infection model. Furthermore, we analyze the clinical relevance of our findings by immunofluorescence analysis of skin samples from AD patients.

70 # Results

71 ## Neutrophils enhance the persistence of *S. aureus* in inflamed skin

72 We showed previously that tape-stripping of mouse skin results in the induction of
73 proinflammatory cytokines and recruitment of PMNs to the skin, which enhances *S.*
74 *aureus* skin colonization in an epicutaneous mouse infection model [15,16]. To elucidate
75 how long-lasting this effect is and how this correlates with the number of PMNs in the
76 skin we determined the number of bacteria on the skin of C57BL/6 mice 1, 3 and 7
77 days after epicutaneous *S. aureus* application with or without previous tape-stripping.
78 As shown in Figure 1 A, tape-stripping of mouse skin significantly increased *S. aureus*
79 colonization and persistence compared with non-tape-stripped skin. Tape-stripping of
80 mouse skin increased the number of skin infiltrating neutrophils (live CD45+ CD11b+
81 Ly6G+ Ly6C+) until 7 days after tape stripping compared with non-tape-stripped skin
82 (Figure 1 B). *S. aureus* application on the skin further increased the number of PMNs
83 under both conditions. However, in tape-stripped skin, the number of infiltrated PMNs
84 was clearly higher compared with non-tape-stripped skin as observed by flow
85 cytometry and by Myeloperoxidase (MPO) staining of skin sections (Figure 1 C and
86 D). Furthermore, NET formation was detected in tape-stripped, *S. aureus* infected skin
87 as detected by immunofluorescence staining for citH3 and MPO and western blot
88 analysis for citH3 (Figure S1 A and B). To elucidate whether PMNs actively contribute
89 to the enhanced persistence of *S. aureus* on tape-stripped skin, we depleted PMNs by
90 intraperitoneal (i.p.) injection of anti-Ly6G antibody 24 h before epicutaneous *S. aureus*
91 application and analyzed the number of colonizing *S. aureus* at days 1, 3 and 7 after
92 infection. Antibody injection efficiently reduced the number of skin infiltrating PMNs in
93 tape-stripped skin up to day 7 after epicutaneous *S. aureus* application as observed
94 by flow cytometry and immunofluorescence staining for MPO as a PMN marker similar
95 to the experiment shown in Figure 1D (Figure S1 B and D). Furthermore, PMN
96 depletion significantly reduced the number of *S. aureus* 1 to 7 days after infection in
97 tape-stripped skin compared to the control isotype treated mice (Figure 1 E). These
98 data indicate that skin infiltrating PMNs are responsible for enhanced *S. aureus* skin
99 colonization and persistence in inflamed skin.

100

101

Keratinocytes prime PMNs for NET formation which enhances *S. aureus* skin colonization

We showed previously that co-incubation of PHKs with PMNs overnight in a transwell chamber system *in vitro* enhances *S. aureus* adhesion to PHKs by the induction of NET formation [15]. To decipher the mechanism behind this, we first performed kinetic experiments where PMNs and PHKs are co-incubated for different time periods prior to *S. aureus* infection and subsequently analyzed S. *aureus* colonization and NET formation (Figure 2 A). We observed that *S. aureus* adhesion steadily increases with extended co-incubation time of PHKs with PMNs (Figure 2 B). This correlated with increased NET formation by PMNs in response to *S. aureus* infection as determined by the measurement of extracellular DNA using SYTOX Green staining (Figure 2 C). We confirmed the induction of NETs in the co-culture upon *S. aureus* infection by live cell imaging (Figure 2 D; Video S1). Treatment of PMNs with phorbol 12-myristate 13-acetate (PMA) served as a positive control. Whereas no NET formation was visible in the co-culture before the infection or after *S. epidermidis* infection, we could observe NET formation in the co-culture after *S. aureus* infection and PMA treatment. This suggests the involvement of *S. aureus* secreted factors in the induction of NET formation. NETs produced by PMNs in the co-culture are responsible for the colonizing-enhancing effect as shown by the co-culture of PHKs with NETs and by the loss of the colonizing-enhancing effect after DNase I-treatment of the co-culture with PMNs or with NETs (Figure 2 E).

To validate on a functional level that NETs induced in the co-culture after infection are responsible for enhanced *S. aureus* skin colonization, we treated PHKs overnight with supernatants of PHKs without PMN co-culture or of PHKs after an 18h co-culture with PMNs and either before and after *S. aureus* infection and determined the efficiency of *S. aureus* adhesion. Interestingly, only the supernatant of PHKs in the co-culture after infection increased *S. aureus* adhesion whereas the PHK supernatant without PMN co-culture and all the PHK supernatants before infection did not have an effect (Figure 2 F). Moreover, only the PMN supernatant of the co-culture after and not before infection could induce NET formation in freshly isolated PMNs as observed by Sytox Green staining (Figure 2 G). These data indicate that a crosstalk between PHKs and PMNs during the co-incubation time primes PMNs for enhanced NET formation upon *S. aureus* infection which in turn modulates the PHKs in a way favorable for *S. aureus* adhesion.

136 **PMN- and NET-mediated oxidative stress induction in PHKs is responsible for**
137 **enhanced *S. aureus* skin colonization**

138 Next, we wanted to elucidate how PMNs and NETs influence PHKs to enhance *S.*
139 *aureus* adherence. We hypothesized that infiltrating PMNs in tape-stripped skin induce
140 oxidative stress in PHKs and that it is not induced solely by tape-stripping of the skin.
141 To test this, we tape-stripped human skin explants and quantified levels of
142 Malondialdehyde (MDA), a marker for lipid peroxidation which is a result of oxidative
143 stress widely used as indirect ROS marker [17,18] , 24 h after tape-stripping. Indeed, tape-
144 stripping alone did not significantly induce oxidative stress in the skin explants (Figure
145 S2 A). Interestingly, when skin explants were co-cultured with PMNs, tape-stripping
146 does significantly induce oxidative stress (Figure S2 B). Next, we used our *in vitro* co-
147 culture model with PHKs and PMNs/NETs to test whether PMNs/NETs can induce ROS
148 in PHKs. ROS induction in PHKs was measured by flow cytometry using H2DCFDA,
149 a non-fluorescent dye that is taken up by the cells and converted to fluorescent DCF
150 in the presence of intracellular ROS. Interestingly, ROS induction in PHKs steadily
151 increases with PMN co-incubation time (Figure 3 A). Furthermore, co-incubation with
152 NETs also induced ROS formation in PHKs (Figure 3 B). To analyze whether oxidative
153 stress induction in PHKs is responsible for enhanced *S. aureus* skin colonization we
154 treated PHKs in the co-culture with PMNs or NETs with N-Acetylcysteine (N-Ac), which
155 efficiently prevented induction of ROS (Figure 3 C) in PHKs and is not toxic to the cells
156 (Figure S2 C). N-Ac treatment of the co-culture does also not significantly affect NET-
157 production by the PMNs (Figure S2 D). Most importantly, inhibition of ROS induction
158 in PHKs by N-Ac treatment prevented the PMN/NET-mediated *S. aureus* colonizing-
159 enhancing effect (Figure 3 D).

160 To validate the results *in vivo* we analyzed oxidative stress induction 1, 3 and 7 days
161 after tape-stripping of mouse skin by quantifying levels of MDA. Indeed, MDA levels
162 were significantly elevated in tape-stripped skin compared to non-tape-stripped skin at
163 all analyzed time points, indicating the induction of oxidative stress (Figure 3 E).
164 Immunohistology staining for MDA of the mouse skin samples validated the results and
165 revealed that oxidative changes are induced in keratinocytes in the tape-stripped skin
166 24h after epicutaneous *S. aureus* application (Figure S2 E). As we showed above this
167 correlated with enhanced *S. aureus* skin colonization (Figure 1 A). To investigate
168 whether infiltrating PMNs are responsible for oxidative stress induction in the skin after
169 *S. aureus* infection, we depleted PMNs *in vivo* by injecting an anti-Ly6G antibody or an

170 isotype control antibody i.p. and investigated MDA levels in tape-stripped or not tape-
171 stripped skin. Interestingly, depleting PMNs prevented the oxidative stress induction
172 upon tape-stripping and *S. aureus* infection as indicated by reduced MDA levels
173 (Figure 3 F). Therefore, our data show that the induction of oxidative stress in skin is
174 mediated by skin infiltrating PMNs and by NET induction which correlates with
175 enhanced *S. aureus* skin colonization and persistence.

176

177 **ROS induction by PMNs and NETs activate NFκB signaling in PHKs which**
178 **enhances *S. aureus* skin colonization**

179 ROS can exert immunomodulatory functions and are able to activate proinflammatory
180 signaling pathways in target cells. We analyzed signaling pathways activated in PHKs
181 by PMNs or NETs in our *in vitro* co-culture model by western blot. We found that
182 especially the NFκB and MAPK-signaling pathways are activated in PHKs in the co-
183 culture with PMNs or NETs in a time-dependent fashion as indicated by increased
184 phosphorylation of ERK and p65, respectively (Figures 4 A and B). Interestingly,
185 activation of MAPK- and NFκB -signaling in PHKs in the co-culture was prevented by
186 the addition of N-Ac (Figures 4 A and B). This further indicates that activation of these
187 signaling pathways is mediated by oxidative stress induction by the PMNs/NETs in
188 PHKs. Next, we analyzed the effect of inhibiting these signaling pathways on *S. aureus*
189 skin colonization. Inhibition of NFκB- or MAPK- signaling using celastrol or trametinib,
190 respectively, significantly reduced the observed *S. aureus* colonizing-enhancing effect
191 of PMNs or NETs in the co-culture (Figures 4 C and D) and was not toxic to the cells
192 (Figures S3 A and B). These data indicate that PMNs and NETs induce ROS in PHKs,
193 which activates NFκB- and MAPK-signaling pathways enabling enhanced *S. aureus*
194 skin colonization.

195 To validate the importance of the NFκB signaling pathway in the PMN/NET-mediated
196 enhanced *S. aureus* skin colonization *in vivo,* we used NFκB1-ko mice, lacking the p50
197 subunit of NFκB. Tape-stripping of mouse skin significantly enhanced *S. aureus* skin
198 colonization in WT mice but not in NFκB1-ko mice (Figure 4E). This correlated with low
199 numbers of infiltrating PMNs in the skin of NFκB1-ko mice, which did not increase after
200 tape-stripping as observed by MPO staining of skin sections and quantification by flow
201 cytometry (Figures 4 E and F). Interestingly, *S. aureus* skin colonization in NFκB1-ko
202 mice was already lower compared to WT mice and correlated with lower infiltrated
203 PMNs also under non-tape-stripped conditions indicating a pivotal role of the NFκB

204 signaling pathway already in *S. aureus* skin colonization in addition to the colonizing-
205 enhancing effect mediated by PMNs/NETs (Figures 4 E and F). These results indicate
206 that NFκB signaling promotes *S. aureus* skin colonization under non-inflamed
207 conditions. Under inflammatory conditions after tape-stripping activation of NFκB-
208 signaling in PHKs increases PMN recruitment to the skin, which induces ROS and
209 enhances *S. aureus* skin colonization.
210

211 **PMNs and NETs mediate the induction and release of HMGB1 by PHKs**

212 Oxidative stress can result in the induction and secretion of damage-associated
213 molecular patterns (DAMPs). Co-incubation of PHKs with PMNs or NETs induces the
214 expression of several DAMPs in PHKs including HMGB1, S100A8, HSP70 and HSP27
215 (Figures 5 A and B). We found especially a time-dependent increase in intracellular
216 protein levels of HMGB1 in PHKs in the co-culture (Figure 5 C). Furthermore, secreted
217 levels of HMGB1 are increased in the co-culture and further increased after *S. aureus*
218 infection (Figure 5 D). In a similar way, co-culture of PHKs with NETs instead of PMNs
219 increased intracellular and secreted HMGB1 levels (Figures 5 E and F). Induction of
220 HMGB1 in PHKs in the co-culture is prevented after inhibition of ROS induction by
221 addition of N-Ac to the co-culture (Figure S4 A) indicating that ROS induces these
222 DAMPs in PHKs. Interestingly, NETs also contain HMGB1 as observed by ELISA
223 analysis (Figure S4 B). To validate the role of PMNs in HMGB1 induction in PHKs and
224 analyze the role of the NFkB signaling pathway in this respect, we analyzed protein
225 expression of HMGB1 in tape-stripped and non-tape-stripped skin of PMN-depleted
226 WT mice (Ly6G) or in NFKB1-ko mice, who have reduced PMN infiltration. We found
227 that tape-stripping strongly increases HMGB1 protein expression in the skin of WT but
228 not NFkB ko mice (Figure 5 G). Furthermore, PMN-depletion in WT mice (Ly6G) does
229 not induce HMGB1 protein expression in skin compared to isotype-treated control mice
230 (Figure 5 H). This further strengthens our conclusion that PMNs induce HMGB1
231 expression in PHKs.

232 Since HMGB1 is abundantly secreted in the co-culture, we studied the effect of HMGB1
233 on ROS induction and *S. aureus* skin colonization. Stimulation of PHKs with
234 recombinant HMGB1 highly induced intracellular ROS levels (Figure 5 I) and
235 significantly enhanced *S. aureus* skin colonization on PHKs and human skin explants
236 (Figures 5 J and K). This effect is specific for *S. aureus* since HMGB1 cannot increase
237 skin colonization of the skin commensals *S. epidermidis* and *S. lugdunensis* (Figure

238 S4 C). In addition, neither treatment with neither S100A8/A9 nor HSP70 affected *S.*
239 *aureus* skin colonization indicating that HMGB1 has a prominent role in the colonizing-
240 enhancing effect (Figures S4 D). Next, we analyzed whether HMGB1 can induce NETs
241 in PMNs. Stimulation of freshly isolated PMNs with different concentrations of
242 recombinant HMGB1 induced NET formation in a concentration-dependent manner as
243 observed by live cell imaging and SYTOX Green staining (Figure 5 L). These results
244 indicate that skin infiltrating PMNs induce oxidative stress in PHKs leading to the
245 upregulation and secretion of HMGB1 which additionally induces ROS in PHKs
246 followed by subsequent NET formation by PMNs and enhanced *S. aureus* skin
247 colonization.
248
249 **The *S. aureus* skin colonizing enhancing effect depends on TLR4 and RAGE**
250 **signaling**
251 We further aimed to investigate how PMNs/NETs/DAMPs can promote *S. aureus* skin
252 colonization focusing on the role of TLRs, which are known to bind to DAMPs [19]. We
253 used mice deficient for TLR2, TLR3, TLR4, TLR7 and TLR9 (5xTLR-ko) and applied
254 *S. aureus* epicutaneously on tape-stripped or non-tape-stripped skin. 24 h after *S.*
255 *aureus* application the number of PMNs and the number of colonizing *S. aureus* were
256 analyzed. Interestingly, whereas tape-stripping increased *S. aureus* skin colonization
257 in the WT mice, this colonizing-enhancing effect was not detected in the 5xTLR-ko
258 mice (Figure 6 A). By analyzing the number of skin-infiltrating PMNs via flow cytometry
259 and immunofluorescence staining of skin sections for MPO, we found that 5xTLR-ko
260 mice show an impaired PMN recruitment upon *S. aureus* infection in both tape-stripped
261 and not tape-stripped skin (Figures 6 A and B). These data indicate that PMN
262 recruitment upon tape-stripping and the resulting enhanced *S. aureus* skin colonization
263 depend on TLR signaling.
264 Since HMGB1 plays a pivotal role in PMN recruitment, NET formation, and ROS
265 induction [20,21], we focused in the following on the two main receptors for HMGB1: TLR4
266 and RAGE. Interestingly, we found increased protein expression of TLR4 and RAGE
267 in PHKs co-incubated with PMNs or NETs and also in skin explants co-incubated with
268 PMNs (Figures S5 A-C). Inhibition of TLR4 signaling by TAK-242 or RAGE signaling
269 by RAGE-AP in PHKs prevented enhanced *S. aureus* skin colonization by recombinant
270 HMGB1 (Figure 6 C) and was not toxic to the cells (Figures S5 D and E). In addition,
271 inhibition of each receptor alone prevented the enhanced *S. aureus* skin colonization

272 in the PMN- or NET-co-culture (Figure 6 D) highlighting the importance of HMGB1 and
273 these receptors. Furthermore, NET-mediated induction of ROS in PHKs is dependent
274 on TLR4 and RAGE signaling as measured by reduced DCF fluorescence after TLR4
275 or RAGE inhibition (Figure 6 E). Moreover, secretion of HMGB1 in the PMN co-culture
276 is significantly decreased in the presence of TAK-242 or RAGE-AP (Figure 6 F). These
277 results indicate that NETs induce oxidative stress in PHKs via TLR4/RAGE signaling
278 leading to the secretion of HMGB1 from the PHKs which in turn signals via TLR4/RAGE
279 to enhance *S. aureus* skin colonization.
280

281 **PMNs, NETs and increased HMGB1 expression are found in the skin of patients**
282 **with AD**

283 To elucidate the clinical relevance of PMNs, NETs and HMGB1 expression in inflamed
284 *S. aureus* infected skin we analyzed the skin of five patients with AD, an inflammatory
285 skin disease associated with abundant *S. aureus* colonization, for the presence of
286 PMNs, NETs, and for expression of HMGB1 using CODEX analysis.

287 In the skin of these patients, we found an enhanced presence of PMNs, showing high
288 expression of neutrophil elastase (NE) and significantly increased HMGB1 expression.
289 Interestingly, in patients with AD, HMGB1 was more expressed in the cytoplasm than
290 in the nucleus compared with healthy control subjects (Figure 7 A; Figure S6). This
291 correlated with enhanced presence of *S. aureus* in the skin (Figure 7 A). Furthermore,
292 immunofluorescence staining for MPO and 8-Oxoguanin-glycosylase enzyme (OGG1)
293 revealed the presence of NETs in the stratum corneum of AD patients (Figure 7 B;
294 Figure S7). OGG1 is an enzyme responsible for the excision of 8-Hydroxyguanosine
295 (8-OHdG), which is elevated in NET DNA, previously described as an ideal marker for
296 NETs [17,22,23]. These data indicate that PMNs and NETs in the skin of patients with AD
297 might drive the disease and increase *S. aureus* skin colonization by the induction of
298 ROS and increasing levels of HMGB1.
299

300 **NETs and HMGB1 regulate the gene expression of epidermal barrier genes in**
301 **PHKs**

302 Finally, as we found that PMNs, NETs and HMGB1 are elevated in the skin of patients
303 with AD, we hypothesized that they could promote skin barrier dysfunctions. To test
304 this hypothesis, we analyzed the expression of genes encoding for the epidermal

305 barrier molecules filaggrin, involucrin and tight-junction protein claudin-1 in PHKs co-
306 cultured with NETs or stimulated with recombinant HMGB1 for 18h. Co-incubation with
307 NETs and stimulation with HMGB1 both significantly decreased the expression of *FLG*,
308 *IVL* and *CLDN-1* (Figure 7 C). We confirmed these data on protein level in skin explants
309 treated with NETs or recombinant HMGB1 (Figure S8 A). Interestingly, the NET-
310 mediated downregulation of the epidermal barrier genes *FLG* and *IVL* in PHKs is
311 completely prevented by N-Ac treatment indicating the involvement of a ROS-
312 dependent mechanism in epidermal barrier gene regulation (Figure S8 B).

313 These results indicate that the enhanced presence of NETs and HMGB1 in the skin
314 promotes skin barrier dysfunctions by the downregulation of epidermal barrier genes,
315 which promotes *S. aureus* skin colonization.

316

317 **Discussion**

318 In our study we show that PMNs, NETs and HMGB1 are elevated in the skin of patients
319 with AD, which downregulates epidermal barrier gene expression promoting an
320 epidermal barrier defect. It is well known that patients with AD exhibit skin barrier
321 defects with downregulation of epidermal barrier genes including *FLG*, *IVL*, and tight
322 junction protein *CLDN-1*. These skin barrier defects have been considered an
323 important step in developing AD [24-26] and also promote *S. aureus* skin colonization, a
324 hallmark of AD. For instance, the lesional and non-lesional skin of patients with AD,
325 where *S. aureus* is abundantly present, show reduced expression of filaggrin and
326 filaggrin-like stratum corneum proteins [27.] Moreover, filaggrin knockdown promotes
327 enhanced *S. aureus* colonization in an epidermal skin model [28]. Interestingly, AD-
328 associated proinflammatory cytokines IL-4 and IL-13 can also induce the
329 downregulation of filaggrin in the skin thus further promoting *S. aureus* colonization
330 and the development of AD [29]. In addition, as we show here, the crosstalk of skin
331 infiltrating PMNs and colonizing *S. aureus* promotes NET-induction, which induces
332 expression of DAMPs such as HMGB1 in keratinocytes, which downregulates
333 epidermal barrier genes further promoting disease progression.

334 We found an increased number of PMNs and NETs in the skin of patients with AD,
335 which correlated with enhanced presence of *S. aureus* in the skin. There is increasing
336 evidence that the enhanced presence of PMNs in the skin of patients with AD
337 contributes to increased *S. aureus* colonization and persistence on AD skin. For

338 example, gene expression of the neutrophil chemoattractants IL-8 and GM-CSF and
339 neutrophilic infiltrates in the dermis are significantly increased in AD skin compared
340 with in healthy control subjects and elevated PMN levels in AD skin are associated with
341 *S. aureus* infection [30,31]. Furthermore, *S. aureus* colonization is higher on the lesional
342 skin of patients with AD compared to the non-lesional site and this correlates with
343 higher IL-8 levels in the stratum corneum which in turn correlates with AD severity and
344 skin barrier dysfunction [32-34]. Moreover, *S. aureus* colonization is enhanced in an *FLG*
345 knockdown epidermal skin model which correlated with enhanced IL-8 expression [28].
346 In addition, here we found the enhanced presence of PMNs and NETs especially in
347 the stratum corneum of patients with AD. As NETs play an important role to keep *S.*
348 *aureus* local and prevent dissemination of the pathogen, their presence in the stratum
349 corneum might prevent *S. aureus* from invading deeper tissues [14]. Since Nakatsuji et
350 al. showed that *S. aureus* is more present in the dermis of lesional skin from patients
351 with AD [35] the invasion of *S. aureus* past the NETs into the dermis of AD skin might be
352 due to its several escape mechanisms to evade NET-mediated killing [9,36]. However, all
353 these findings indicate that PMNs play a role in the pathogenesis of AD.

354 Here, using a mouse model resembling the epidermal barrier defect in patients with
355 AD, we show that PMNs actively contribute not only to the initial colonization but also
356 to the enhanced persistence of *S. aureus* on tape-stripped and thus inflamed skin. We
357 found that, although the overall numbers decreased over time, *S. aureus* skin
358 colonization was significantly elevated up to day 7 on tape-stripped skin compared with
359 non-tape-stripped skin. This enhanced *S. aureus* persistence was mediated by the
360 enhanced presence of PMNs in the tape-stripped skin as observed by PMN depletion
361 *in vivo*. Although the difference in the bacterial abundance on the skin of tape-stripped
362 and non-tape-stripped mouse skin or with or without PMN-depletion seems to be low
363 (between 36.000 and 57.000 CFU/skin punch), this small difference is clinically
364 relevant, as demonstrated in a recent study that describes the treatment of AD patients
365 with dupilumab. Already similar low changes in the abundance of *S. aureus* on the skin
366 of these patients after therapy resulted in a significant decrease in AD severity [37].

367 In healthy skin, PMNs mediate the transient colonization of *S. aureus* on the stratum
368 corneum. Schulz et al reported that an epicutaneous challenge of *S. aureus* induces
369 IL-8 secretion in the epidermis leading to PMN infiltration which ultimately reduces
370 bacterial numbers on the stratum corneum [38]. In non-tape-stripped skin, we detect
371 something similar with initial PMN infiltration to the skin and a decrease of *S. aureus*

372 and PMNs over time. The clearing of PMNs from the infection site is considered a
373 crucial factor for inflammation resolution and restoring homeostasis [8]. In tape-stripped
374 skin, we also detected overall decreasing numbers of *S. aureus* colonization and PMNs
375 over time. However, *S. aureus* colonization was still higher in tape-stripped skin
376 compared with in non-tape-stripped skin indicating that the infiltration of PMNs upon
377 tape-stripping prior to *S. aureus* infection creates an environment favorable for *S.*
378 *aureus* colonization. Indeed, we could show *in vitro* that a prolonged co-incubation of
379 PHKs and PMNs correlates with enhanced *S. aureus* skin colonization further
380 indicating that a crosstalk between skin infiltrating PMNs and PHKs underlies this
381 colonizing-enhancing effect. Interestingly, Matsumoto et al. showed that in non-
382 inflamed skin, PMN-depletion does not affect bacterial numbers on the skin on day 7
383 after epicutaneous *S. aureus* application [39]. This suggests that the *S. aureus*
384 colonizing-enhancing effect of PMNs depends on an inflammatory skin environment
385 and on an already established crosstalk of infiltrating PMNs with PHKs. Indeed, we
386 could show that a prolonged co-incubation of PHKs and PMNs *in vitro* correlates with
387 enhanced *S. aureus* skin colonization.

388 We found that the enhanced presence of PMNs in tape-stripped mice skin induces
389 oxidative stress in the skin which correlates with enhanced *S. aureus* colonization.
390 Further *in vitro* studies revealed that PMNs and NETs are able to induce ROS in PHKs.
391 This induction is responsible for the enhanced *S. aureus* skin colonization as targeting
392 ROS induction by N-Ac prevented the enhanced *S. aureus* colonization. Interestingly,
393 oxidative stress has been involved in the pathogenesis of AD. Targeting ROS in a 2D
394 AD model by the inhibition of NADPH oxidase promotes survival of keratinocytes [40,41].
395 With our findings, we could link oxidative stress induction with *S. aureus* skin
396 colonization thus proposing inhibition of oxidative stress induction as a potential
397 treatment strategy for AD.

398 Along with oxidative stress induction, we observed an upregulation of DAMPs in PHKs
399 after co-culture with PMNs or NETs. Especially elevated levels of secreted HMGB1
400 were observed in the supernatant of the co-culture. Oxidative stress is a central
401 regulator of the function of HMGB1 [42] and the upregulation of HMGB1 could be
402 prevented by N-Ac addition suggesting that ROS induction promotes HMGB1
403 secretion. This finding is supported by Cui et al, who described the release of HMGB1
404 under the induction of ROS via hydrogen peroxide treatment by human keratinocytes
405 and melanocytes [43]. Furthermore, blocking the two prominent HMGB1 receptors TLR4

406 and RAGE prevented NET-mediated enhanced *S. aureus* skin colonization. Inhibition
407 of TLR4 and RAGE signaling, respectively, also prevented the induction of ROS in
408 PHKs, and the secretion of HMGB1 in the co-culture and treatment of PHKs with
409 recombinant HMGB1 induced oxidative stress in PHKs. The fact that already individual
410 blockade TLR4 or RAGE reduces ROS, and HMGB1 levels and *S. aureus* skin
411 colonization suggests that TLR and RAGE signaling can influence each other. Indeed,
412 there is evidence that RAGE and TLR4 are both required for HMGB1-induced
413 inflammation in bone marrow derived macrophages and that RAGE and TLR4 can
414 interact with each other to regulate the surface expression of both - TLR4 and RAGE
415 on macrophages [44].

416 HMGB1 has been shown to be involved in the pathogenesis of AD and targeting
417 HMGB1 ameliorated inflammation and AD symptoms in AD mouse models [45,46]. Here,
418 we show that HMGB1 expression is significantly enhanced in the skin of patients with
419 AD. Interestingly, we observed increased cytoplasmic HMGB1 expression and
420 decreased nuclear expression of HMGB1 in AD skin compared with healthy controls
421 indicating to a translocation of HMGB1. HMGB1 translocation from the nucleus to the
422 cytoplasm is induced in response to stress and often results in the subsequent release
423 of HMGB1 [47,48]. Indeed, Wang et al. reported enhanced HMGB1 expression in the
424 epithelial intercellular space indicating HMGB1 secretion in AD [49]. These results
425 therefore indicate the possible activation of HMGB1 in the pathogenesis of AD.

426 Interestingly, we found that stimulation of PHKs with recombinant HMGB1 was able to
427 enhance *S. aureus* skin colonization *in vitro*, thereby revealing a functional role for
428 HMGB1, suggesting it as a potential target in the treatment of AD. However, the exact
429 mechanism by which HMGB1 enhances *S. aureus* skin colonization is unclear. A
430 possible explanation is suggested by Nygaard et al. who showed that HMGB1 can
431 downregulate skin barrier proteins [50]. Indeed, we found that treatment of PHKs with
432 recombinant HMGB1 significantly downregulates the expression of genes encoding for
433 epidermal barrier proteins including filaggrin, involucrin and tight-junction proteins
434 claudin-1 providing one mechanism underlying enhanced *S. aureus* skin colonization.
435 Furthermore, our data indicate that epidermal barrier gene expression is regulated in
436 a ROS-dependent manner. This ROS-mediated downregulation of epidermal barrier
437 genes has already been reported for Th2 cytokines IL-13 and IL-4 [51].

438 In summary, our work revealed a mechanistic understanding of the functional role of
439 PMNs and NETs in the colonization and persistence of *S. aureus* on inflamed skin. We

440 propose, as an underlying mechanism, that the enhanced persistence of PMNs and
441 NETs in inflamed skin induces oxidative stress in keratinocytes leading to the secretion
442 of DAMPs such as HMGB1, which induces skin barrier dysfunction thus driving *S.*
443 *aureus* skin colonization. The enhanced *S. aureus* skin colonization further
444 exacerbates skin inflammation thus promoting a vicious cycle with further PMN
445 infiltration and epithelial barrier disruption as seen in AD. In healthy skin, on the other
446 hand, infiltration of neutrophils in response to epicutaneous *S. aureus* infection
447 mediates transient colonization and ensures host protection.
448

449 ## Limitations of the study

450 We do not yet know how specific the PMN/NET/HMGB1/ROS-mediated effect on
451 epidermal barrier gene expression and *S. aureus skin* colonization is for AD. Does it
452 also play a role in other inflammatory skin diseases, such as psoriasis, and affect the
453 skin colonization of other bacterial species? Further studies are needed to solve this
454 question. Furthermore, since several biologically distinct populations of neutrophils
455 exist [508] future studies should include additional markers other than Ly6G for their
456 identification to better represent the different PMN subpopulations and differentiation
457 stages.
458

459 ## Acknowledgements

460 This work was supported by the Deutsche Forschungsgemeinschaft with funding grant
461 number TR156, SCHI510/12-1 and the Cluster of Excellence EXC2124 (B.S.)
462 ‘Controlling Microbes to Fight Infections’. A.J. was supported by a graduate stipend of
463 the Medical Faculty of Tübingen (Promotionskolleg Medizin 2022-1-22). The graphical
464 abstract was created with Biorender.com
465

466 ## Author contributions

467 J.F. and B.S. designed the experiments and wrote the manuscript with critical input
468 from J.S., A.J., C.M.S., S.K., S.R. and B.W. Mouse experiments, immune cell isolation
469 and flow cytometry were performed by J.F. and J.S. Immunofluorescence microscopy
470 was performed by J.F. and S.K. Adhesion and invasion assays, viability assays, NET
471 isolation and quantification, western blot studies, ROS quantification, ELISA and
472 qPCRs were performed by J.F. Confocal laser scanning microscopy was performed by

473 S.R. and J.F. Live cell imaging was performed by J.F. and B.W. CODEX experiments
474 were performed by A.J. and C.M.S.

475

476 Declaration of interest

477 The authors state no competing interests.

478

479 Inclusion and diversity

480 We support inclusive, diverse, and equitable conduct of research

481

482 Figure legends

483 **Figure 1: Neutrophils enhance the persistence of *S. aureus* on inflamed skin**
484 **A-E:** The dorsal skin of C57BL/6 mice was tape-stripped or left untreated. After 24h,
485 1x10^8 *S. aureus* (**A&C-E)** or PBS (**B**) was epicutaneously applied onto the skin. **A:**
486 On day 1, 3 and 7 after *S. aureus* application, 4 mm skin punches were used for CFU
487 determination. Horizontal lines represent the mean of each group ± SEM. One dot
488 represents one mouse. Significant differences were analyzed by multiple unpaired t-
489 tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B&C:** On day 1, 3 and 7 after
490 epicutaneous PBS (**B**) or *S. aureus* (**C**) application, immune cells present in the skin
491 were analyzed in 4 mm skin punches by flow cytometry. Shown is the mean percentage
492 of PMNs (Ly6G+ Ly6C+) pre-gated on CD11b+ CD45+ live cells in the dorsal skin of
493 mice (gating strategy shown in Figure S1 A). One dot represents one mouse.
494 Horizontal lines represent the mean of each group ± SEM. Significant differences were
495 analyzed by multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P <
496 0.0001. **D:** Representative MPO staining of tape-stripped and non-tape-stripped skin
497 sections on day 1, 3 and 7 after epicutaneous *S. aureus* application. Scale bar = 100
498 μm. MPO+ cells were quantified using Fiji. Significant differences were analyzed by
499 multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **E:** PMNs
500 were depleted in C57BL/6 mice by a single intraperitoneal injection of 100 μg anti-
501 Ly6G or isotype control. After 24 h, the dorsal skin was tape-stripped and 1x10^8 *S.*
502 *aureus* was epicutaneously applied. On days 1, 3 and 7 after application, *S. aureus*
503 skin colonization was analyzed in 4 mm skin punches. Horizontal lines represent the
504 mean of each group ± SEM. One dot represents one mouse. Significant differences

were analyzed by multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. TS = tape-stripping; MPO = myeloperoxidase; CFU = colony forming units

Figure 2: Co-Culture with PHKs primes PMNs for NET formation which positively correlates with *S. aureus* skin colonization

A: Graphical illustration of the *in vitro* co-culture system. **B:** PHKs were co-incubated with PMNs for 2, 4, 6 or 18 h before infection with *S. aureus* (MOI = 30) for 1.5 h. Subsequently, PHKs were lysed, serial dilutions were plated onto TSB agar plates, and CFUs were quantified. One representative experiment of three independent experiments is shown. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **C:** NET formation was analyzed by quantifying extracellular DNA in the PMN well of the co-culture at different co-incubation times after *S. aureus* infection by SYTOX Green staining. The amount of extracellular DNA was normalized to the data of Triton-X treated cells representing the total DNA amount. One representative experiment of three independent experiments is shown. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **D:** NET formation in the co-culture upon *S. aureus* infection was visualized by live cell imaging. Scale bar = 100 μm. FIJI/ImageJ was used to quantify the SytoxGreen-+ area fraction of the image at each time-point. **E:** PHKs were co-cultured with PMNs or NETs in the presence or absence of DNAse I. After 18 h, PHKs were infected with *S. aureus* for 1.5 h and subsequently lysed and serial dilutions were plated onto TSB agar plates and CFUs were quantified. One experiment of three independent experiments is shown. Significant differences were analyzed by one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **F:** The supernatant of PHKs without PMN co-culture or of PHKs after an 18 h co-culture with PMNs and either before and after *S. aureus* infection was filter sterilized and added to fresh PHKs. After 18 h, PHKs were infected with *S. aureus* (MOI = 30) for 1.5 h. PHKs were lysed, serial dilutions were plated onto TSB agar plates and CFUs were quantified. One representative experiment of three independent experiments is shown. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **G:** NET formation in PMNs stimulated with the supernatant of the PMN well of the co-culture before and after infection was visualized by live cell imaging. Representative images and quantifications are shown. Scale bar = 200 μm NET formation is indicated by SYTOX Green+ cells. FIJI/ImageJ was used

to quantify the SytoxGreen+ area fraction of the image at each time-point. PHKs, primary human keratinocytes; PMNs, polymorphonuclear neutrophils; NETs, neutrophil extracellular traps; CFUs, colony forming units; MOI, multiplicity of infection

Figure 3: PMN- and NET-mediated oxidative stress induction in PHKs is responsible for enhanced *S. aureus* skin colonization

A: PHKs were co-incubated with PMNs. After 3, 6 and 18h, intracellular ROS induction in PHKs was analyzed by quantifying DCF fluorescence. Non-co-cultured PHKs were used as control. Shown is one experiment of three independent experiments. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B:** PHKs were co-incubated with PMNs or NETs. After 18 h, intracellular ROS levels in PHKs were analyzed by quantifying DCF fluorescence. 1 hour treatment with VitC (8 mM) served as positive control. Shown is one experiment of three independent experiments. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **C:** PHKs were co-incubated with PMNs or NETs for 18 h in the presence or absence of N-Ac (5 mM) and intracellular ROS was measured by quantifying DCF fluorescence. One representative experiment of three independent experiments is shown. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001,

****P < 0.0001. **D:** PHKs were co-incubated with PMNs or NETs in the presence or absence of N-Ac (5 mM). After 18 h, PHKs were infected with *S. aureus* (MOI = 30) for 1.5 h. PHKs were subsequently lysed, serial dilutions were plated onto TSB agar plates and CFUs were quantified. Shown is one experiment of three independent experiments. Significant differences were analyzed by multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **E:** The dorsal skin of C57BL/6 WT mice was either tape-stripped or left untreated. On days 1, 3 and 7, lipid peroxidation in the skin was analyzed by quantification of MDA levels in tissue lysates. Horizontal lines represent the mean of each group ± SD. One dot represents one mouse. Significant differences were analyzed by multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **F:** C57BL/6 WT mice were injected intraperitoneally with 100 µg anti-Ly6G antibody, for neutrophil depletion, or isotype control. After 24 h, 1x10^8 *S. aureus* were applied epicutaneously on tape-stripped or non-tape-stripped dorsal skin. After 1 day, lipid peroxidation in the skin was analyzed by quantification of MDA levels in tissue lysates. Horizontal lines represent the mean of each group ± SD. One dot

represents one mouse. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PHKs, primary human keratinocytes; DCF, dichlordihydrofluorescein; PMNs, polymorphonuclear neutrophils; NETs, neutrophil extracellular traps; N-Ac, N-Acetylcysteine, VitC, Vitamin C, MDA, Malondialdehyde; TS, tape-stripping; MOI, multiplicity of infection

Figure 4: ROS induction by PMNs or NETs activates NFkB signaling in PHKs which enhances *S. aureus* skin colonization

A&B: PHKs were co-cultured with PMNs (**A**) or NETs (**B**) in presence or absence of N-Ac (5 mM). At different time points, activated signaling pathways in PHKs were analyzed by western blot studies. Protein expression of β-actin was used as loading control. **C&D:** PHKs were co-cultured with PMNs (**C**) or NETs (**D**) in the presence or absence of Trametinib (15 nM) or Celastrol (1μM). PHKs were subsequently infected with *S. aureus* (MOI = 30) for 1.5 h. *S. aureus* skin colonization was analyzed by quantifiying CFUs. Non-co-cultured PHKs served as control. Shown is one representative experiment of three independent experiments. Significant differences were analyzed by One-Way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **E:** The dorsal skin of WT of NFkB1-KO mice was tape-stripped or left untreated and 1x10^8 *S. aureus* was epicutaneously applied. After 24 h, *S. aureus* skin colonization was analyzed by quantifying CFUs. Significant differences were analyzed by One-Way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Horizontal lines represent the mean of each group ± SD. One dot represents one mouse. **F:** Presence of neutrophils in the skin 24 h after S. aureus application was analyzed by flow cytometry. Shown is the mean percentage of neutrophils (Ly6G+ Ly6C+) pre-gated on CD11b+ CD45+ live cells. One dot represents one mouse. Significant differences were analyzed by Two-Way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **G:** Representative MPO staining of tape-stripped and non-tape-stripped skin of WT and NFkB1-KO mice. Scale bar = 100 μm. MPO+ cells were quantified using ImageJ. One dot represents one mouse. Significant differences were analyzed by Two-Way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PHKs, primary human keratinocytes; PMNs, polymorphonuclear neutrophils; NETs, neutrophil extracellular traps; WT, wildtype; NFkB1, nuclear factors kappa-light-chain-enhancer of activated B cells; TS, tape-stripping; MPO, myeloperoxidase; MOI, multiplicity of infection

606 **Figure 5: PMNs and NETs mediate induction and release of HMGB1 by PHKs**
607 **A&B:** PHKs were co-cultured with PMNs (**A**) or NETs (**B**). After 18 h, gene expression
608 of DAMPs was analyzed by qPCR. Shown is one representative experiment of three
609 independent experiments. Significant differences were analyzed by multiple t-tests *P
610 < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **C:** Intracellular levels of HMGB1 in
611 PHKs co-incubated with PMNs were detected at different time points via western blot.
612 Protein expression of β-actin was used as loading control. **D:** Secreted levels of
613 HMGB1 by PHKs co-incubated with PMNs before and after *S. aureus* infection were
614 analyzed by ELISA. Shown is one representative experiment of three independent
615 experiments. Significant differences were analyzed by One-Way-ANOVA *P < 0.05, **P
616 < 0.01, ***P < 0.001, ****P < 0.0001. **E:** Intracellular levels of HMGB1 in PHKs co-
617 incubated with NETs were analyzed at different time points via western blot. Protein
618 expression of β-actin was used as loading control. **F:** Secreted levels of HMGB1 by
619 PHKs after 18 h co-culture with NETs were analyzed by ELISA. Shown is one
620 representative experiment of three independent experiments. Significant differences
621 were analyzed by an unpaired t-test *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
622 **G & H:** HMGB1 expression in skin tissue lysates of tape-stripped and non-tape-
623 stripped WT and NFkB1-KO mice **(G)** and in anti IgG2a control and anti-Ly6G treated
624 mice **(H)** 24 h after *S. aureus* infection was analyzed via western blot. Expression of
625 β-actin was used as loading control. **I:** PHKs were treated either with medium (Ctrl.) or
626 with recombinant HMGB1 for 18 h and intracellular ROS levels were analyzed by
627 quantifying DCF fluorescence. Shown is one representative experiment of three
628 independent experiments. Significant differences were analyzed by an unpaired t-test
629 *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **J:** PHKs were treated with
630 recombinant HMGB1 for 18h and subsequently infected with *S. aureus* (MOI = 30).
631 After 1.5 h, PHKs were
632 lysed, serial dilutions were plated onto TSB plates and CFUs were quantified. Shown
633 is one representative experiment of three independent experiments. Significant
634 differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001,
635 ****P < 0.0001. **K:** Skin explants of two donors were treated with HMGB1 or PBS for
636 18h before *S. aureus* (1x10^8) was applied to the skin explants. 4 mm skin punches
637 were used for CFU determination. Significant differences were analyzed by unpaired
638 two-tailed t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **L:** Freshly isolated
639 PMNs were stimulated with different concentrations of HMGB1, and NET formation

640 was analyzed by live-cell imaging via analyzing extracellular DNA by SYTOX Green
641 staining. Scale bar = 200 μm. FIJI/ImageJ was used to quantify the SytoxGreen+ area
642 fraction of the image at each time-point. PHKs, primary human keratinocytes; PMNs,
643 polymorphonuclear neutrophils; NETs, neutrophil extracellular traps; HMGB1, high-
644 mobility-group-protein B1; HSP, heat-shock-protein; DCF, dichlordihydrofluorescein;
645 DNA, deoxyribonucleic acid; MOI, multiplicity of infection; TS, tape-stripped; WT, wild-
646 type

647

648 **Figure 6: The *S. aureus* skin colonization enhancing effect depends on TLR4 and**
649 **RAGE signaling**

650 **A:** The dorsal skin of C57BL/6 WT mice and 5xTLR-ko mice was either tape-stripped
651 or left untreated. After 24 h, 1x10^8 *S. aureus* was epicutaneously applied to the skin.
652 *S. aureus* skin colonization was assessed 24 h after infection in 4 mm skin punches.
653 Horizontal lines represent the mean of each group ± SEM. One dot represents one
654 mouse Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01,
655 ***P < 0.001, ****P < 0.0001. Presence of neutrophils in the skin 24 h after S. aureus
656 application was analyzed by flow cytometry. Shown is the mean percentage of
657 neutrophils (Ly6G+ Ly6C+) pre-gated on CD11b+ CD45+ live cells. One dot represents
658 one mouse. Significant differences were analyzed by Two-Way ANOVA *P < 0.05, **P
659 < 0.01, ***P < 0.001, ****P < 0.0001. **B:** Representative MPO stainings of tape-stripped
660 and non-tape-stripped WT and 5xTLR-ko mice (Scale bar = 100 μm). MPO+ cells were
661 quantified using Fiji. One dot represents one mouse. Significant differences were
662 analyzed by Two-Way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
663 **C&D:** PHKs were stimulated with HMGB1 (**C**) or co-incubated with PMNs/NETs (**D**) for
664 18 h in the presence or absence of TLR4 inhibitor (TAK-242) or RAGE-inhibitor (RAGE-
665 AP) before *S. aureus* infection (MOI = 30). After 1.5 h, PHKs were lysed, serial dilutions
666 were plated onto TSB agar plates. Shown is one representative experiment of three
667 independent experiments. Significant differences were analyzed by one-way ANOVA
668 *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **E:** PHKs were co-incubated with
669 NETs in the presence or absence of TAK-242 or RAGE-AP for 18 h. Intracellular ROS
670 levels were analyzed by quantifying DCF fluorescence. Shown is one representative
671 experiment of three independent experiments. Significant differences were analyzed
672 by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **F:** Extracellular
673 levels of HMGB1 in the PMN well of the co-culture in the presence or absence of TAK-

674 242 or RAGE-AP was analyzed by ELISA. Shown is one representative experiment of
675 three independent experiments. Significant differences were analyzed by one-way
676 ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. WT, wild type; TLR, toll-
677 like receptor; TS, tape-stripping; CFU, colony forming units; MPO, myeloperoxidase,
678 PHKs, primary human keratinocytes; PMNs, polymorphonuclear neutrophils; NETs,
679 neutrophil extracellular traps; RAGE, receptor for advanced glycation end products;
680 HMGB1, high-mobility-group-protein B1; MOI, multiplicity of infection

681

682 **Figure 7: PMNs, NETs and HMGB1 are present in the skin of patients with AD**

683 **A:** Codex Analysis of skin sections of patients with AD or healthy controls for *S. aureus,*
684 NE and HMGB1. Marker intensities were quantified using ImageJ. Significant
685 differences between healthy skin and AD skin were analyzed by an unpaired t-test *P
686 < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. In the analysis, 5 patients with AD and
687 5 healthy control subjects were included. **B:** Representative confocal images of healthy
688 skin (a) and AD skin (b) stained for MPO (scale bar: 200 μm). Presence of NETs in AD
689 skin was analyzed by immunofluorescence staining for MPO and OGG1 (c&d). Scale
690 bars = 10 μm (c) and 5 μm (d). **C:** Expression of *FLG*, *IVL* and *CLDN-1* in PHKs co-
691 incubated with NETs or stimulated with HMGB1 for 18 h. Non-co-cultured PHKs were
692 used as control. Shown is one representative experiment of three independent
693 experiments. Significant differences were analyzed by multiple t-tests. *P < 0.05, **P <
694 0.01, ***P < 0.001, ****P < 0.0001. AD, atopic dermatitis; NE, neutrophil elastase;
695 HMGB1, high-mobility-group-protein B1; MPO, myeloperoxidase; NETs, neutrophil
696 extracellular traps, OGG1, 8-Oxoguanine DNA glycosylase**;** PHKs, primary human
697 keratinocytes

698

699
STAR★Methods

700
Resource availability

701
Lead contact

702 Further information and requests for resources and reagents should be directed to and
703 will be fulfilled by the Lead Contact, Birgit Schittek (birgit.schittek@uni-tuebingen.de).

704

705
Materials availability

706 This study did not generate new unique materials.

707

708 **Data and code availability**

709 - All data reported in this paper will be shared by the lead contact upon request.
710 - This paper does not report original code.
711 - Any additional information required to reanalyze the data reported in this paper
712 is available from the lead contact upon request.

713 Experimental model and study participant details

714 **Humans**

715 Primary human keratinocytes were isolated from human foreskin after routine
716 circumcision from the Loretto Clinic in Tübingen as previously described [13, 52].
717 Keratinocyte isolation from human foreskin was approved by the ethics committee of
718 the medical faculty of the University Tübingen (654/2014BO2) and performed
719 according to the principles of the Declaration of Helsinki. Immune cell isolation from
720 human blood was approved by the ethics committee of the medical faculty of the
721 University Tübingen (054/2017BO2). Sample analysis of atopic dermatitis patients and
722 healthy controls (4 males, 6 females; 41-63 years old) were performed in full
723 agreement with institutional guidelines with the approval of the Ethic committee of the
724 medical faculty of the University Tübingen (654/2014B02; 093/2019B02).

725

726 **Mice**

727 All mouse experiments were conducted in accordance with the German regulations of
728 the Gesellschaft für Versuchstierkunde/Society for Laboratory Animal Science (GV-
729 SOLAS) and the European Health Law of the Federation of Laboratory Animal Science
730 Associations (FELASA). All mouse experiments were approved (HT1/17) by the local
731 authorities (Regierungspräsidium Tübingen). Animal studies were performed with 6-8-
732 week-old female C57BL6 WT/5xTLR-ko/NFκB-1ko mice.

733

734 Method Details

735 ***Bacterial strains and culture conditions***

736 In this study, *Staphylococcus aureus* USA300 LAC was used. The bacteria were
737 aerobically grown in tryptic soy broth (TSB) at 37 °C and orbital shaking. All
738 experiments were performed with logarithmically grown (OD = 0.5) bacteria.
739
740 ***In-vivo skin colonization model***
741 The *in vivo* skin colonization model was performed as previously described [13, 52.]
742 Mouse skin was shaved 3 days before starting the experiments to allow healing of
743 potential microwounds and recovery of the skin from shaving. Skin inflammation was
744 induced by repeated tape-stripping (7 times) of the shaved dorsal skin. After 24h, finn
745 chambers with filter discs containing 1x10^8 *S. aureus* were epicutaneously applied
746 on the shaved, dorsal skin. Finn chambers were fixated with fixomull. On day 1, 3 and
747 7 after *S. aureus* infection, mice were euthanized, and samples were taken. *S. aureus*
748 skin colonization was analyzed by scraping 4 mm skin punches as previously
749 described [13, 52].
750
751 ***Neutrophil Depletion in vivo***
752 Neutrophils were depleted using an anti-Ly6G antibody as previously described [13].
753 Briefly, 100 ug of anti-Ly6G or IgG control antibody (BioXCell, West Lebanon, NH,
754 clone 1A8 and clone 2A3) were injected intraperitoneally 24h before epicutaneous *S.*
755 *aureus* application on the skin. Successful depletion was ensured by flow cytometry.
756
757 ***Mouse immune cell isolation and staining procedure***
758 Isolation of immune cells from mouse skin was performed as previously described [221].
759 Briefly, relevant dorsal skin was removed and placed in PBS+2% FCS. Subcutaneous
760 fat and blood vessels were removed using a razor blade and skin samples were
761 subsequently placed into a reaction tube containing RPMI with
762 0.05 mg/ml DNAse I (Roche) and 0.25 mg/ml Liberase (Roche). For digestion, tissue
763 samples were disintegrated by scissors and incubated at 37 °C for 70 min. The
764 digestion reaction was stopped by addition of 250 µl FCS. Single cell suspensions
765 were generated using 70 µm cell strainers. After washing with PBS + 2% FCS, single
766 cell suspensions were treated with TruStain fcXanti-CD16/32 (1:50, BioLegend) and
767 subsequently surface stained with the following monoclonal antibodies: CD45.2
768 (1:200, BioLegend, clone 104, Cat#109824), CD11b (1:200, BioLegend, clone M1/70,

769 Cat#101227), CD11c (1:200, BioLegend, clone N418, Cat#117337), Ly6G (1:200,
770 BioLegend, clone 1A8, Cat#127614), Ly6C (1:200, BioLegend, clone HK1.4,
771 Cat#128014). To exclude dead cells, a fixable viability dye eFluor520 (1:1000,
772 eBioscience) was used. All samples were measured using a BD LSRII flow cytometer
773 (CD Bioscience) and analyzed with FlowJo (TreeStar).
774

775 ***Immunofluorescence staining for MPO***

776 For immunofluorescence staining for MPO, 2 μm skin sections were deparaffinized.
777 Antigen retrieval was performed for MPO in EDTA buffer (pH = 9) for 7 min using a
778 pressure cooker. After slow cooling down, tissue sections were washed with 1x PBS
779 and blocked for 90 min with 1xPBS + 0.05% Triton-X containing 5% donkey serum
780 before incubation in primary MPO antibody (Abcam, 1:75) diluted in blocking buffer
781 overnight at 4 °C. The next day, tissue sections were washed 3 times with 1xPBS and
782 incubated in Cy3-labeled goat anti-rabbit IgG antibody (Abcam, 1:250) for 1.5 h at RT.
783 Nuclear DNA was detected by incubating tissue slides with Hoechst 33342 (Thermo
784 Fisher) for 20 min at RT. Immunofluorescence images were acquired using the Zeiss
785 Axiovert 200 microscope (Zeiss) with the VisiView software (Visitron systems,
786 Puchheim, Germany). MPO+ cells were quantified using Fiji.
787

788 ***Confocal laser scanning microscopy for NETs in mouse skin***

789 4 μm skin sections were deparaffinized and antigen retrieval was performed for MPO
790 and citH3 in EDTA buffer (pH = 9) for 15 min using a pressure cooker. After cooling
791 down, tissue sections were blocked for 90 min at RT with 1xPBS + 0.05% Triton-X
792 containing 5% donkey serum and subsequently incubated in primary MPO (1:50, R&D)
793 and citH3 (1:50, abcam) diluted in blocking buffer overnight at 4°C. After three washing
794 steps in 1xPBS, tissue sections were incubated in Alexa-400-labeled donkey anti-goat
795 IgG antibody and Cy3-labeled goat anti-rabbit IgG antibody (both Abcam, 1:250) for
796 1.5 h at RT. Nuclear DNA was detected by DAPI (Sigma) staining for 20 min at RT.
797 Confocal laser scanning microscopy was performed using an LSM 800 (Zeiss,
798 Oberkochen, Germany).
799
800

801 ***Immunohistochemical staining for Malondialdehyde***

802 For immunohistochemical staining for MDA, 4 μm skin sections were deparaffinized.
803 Antigen retrieval was performed for MDA in citrate buffer (pH = 6) for 15 min using a
804 pressure cooker. After slow cooling down, tissue sections were washed with 1x PBS
805 and blocked for 90 min with 1xPBS + 0.05% Triton-X containing 5% donkey serum
806 before incubation in primary MDA antibody (Novusbio, 1:100) diluted in blocking buffer
807 overnight at 4 °C. The next day, tissue sections were washed 3 times with 1xPBS and
808 incubated with primary enhancer (Lab Vision UltraVision LP Detection System, Thermo
809 Fisher Scientific) for 20 min at room temperature and subsequently incubated with AP
810 polymer (Lab Vision UltraVision LP Detection System, Thermo Fisher Scientific) for 30
811 min at room temperature in a humid chamber. After three washing steps, skin sections
812 were stained with the liquid fast red substrate system (Lab Vision UltraVision, Thermo
813 Fisher Scientific) and subsequently hematoxylin-eosin (H&E, Agilent/Dako) was
814 performed for 2 min.

815

816 ***Confocal laser scanning microscopy for NETs in patients***

817 2 μm skin sections of atopic dermatitis patients and healthy control were
818 deparaffinized. Antigen retrieval was performed in Citrate buffer (pH = 6) for 10 min
819 and subsequent EDTA buffer (pH = 9) for 7 min using a pressure cooker. After slow
820 cooling down, tissue sections were washed with 1x PBS and blocked for 90 min with
821 1xPBS + 0.05% Triton-X containing 5% donkey serum before incubation in primary
822 antibodies for MPO (R&D, 1:50) and OGG1 (Novus bio, 1:50) in blocking buffer
823 overnight at 4 °C. The next day, tissue sections were washed three times with 1xPBS
824 and incubated in Cy3-labeled goat anti-rabbit IgG (Abcam, 1:250) and Alexa488-
825 labeled donkey anti-goat IgG (Abcam, 1:250) for 1.5h at RT. Nuclear DNA was detected
826 by incubating tissue slide with DAPI (Sigma) for 20 min at RT. Confocal laser scanning
827 microscopy was performed using an LSM 800 (Zeiss, Oberkochen, Germany).

828

829 ***CODEX Analysis***

830 CODEX was performed as previously described [53,54,55,56]. Briefly, FFPE blocks of skin
831 punches were evaluated on corresponding H&E staining and epidermal regions were
832 annotated for preparation of a Tissue Micro Array of 1 mm diameter including 5
833 samples of healthy skin, 5 sample from AD. 4 µm thick sections of the TMA were cut

834 and mounted on a Vectabond-treated coverslip for CODEX analysis. For tissue
835 staining the samples were deparaffinized, rehydrated and antigen retrieval was
836 performed. The tissue was then bleached for 90 min, followed by washing and blocking
837 steps. Afterward an antibody cocktail containing all markers in the panel design was
838 added and incubated overnight in a humidity chamber at 4°C. All antibodies were
839 conjugated to a unique oligonucleotide and tested and validated beforehand.
840 Oligonucleotide conjugation was performed as previously described [53]. For all details
841 concerning antibodies and oligonucleotides see Table S2.

842 Staining was followed by washing and fixation of the tissue for imaging. Automated
843 image acquisition was then performed using the Akoya CODEX phenocycler. For
844 multicycle imaging of the TMA spots, the multipoint function of the BZ-X viewer
845 software was manually programmed to the center of each TMA spot. All data was
846 processed using the CODEX processor, creating hyperstacked images of all
847 fluorescent channels following previous background subtraction. Processed images
848 were analyzed using ImageJ. Single marker pictures were created using the CODEX-
849 Yury tool for combination of selected channels from focused CODEX stack.
850 Quantification of marker intensities was performed by manual selection of epidermal
851 regions using freehand selection and the Measure tool for Mean Marker Expression
852 provided in ImageJ. Statistical data was then analyzed and visualized in Prism.

853

854 ***Cell Culture and in-vitro co-culture model***

855 Keratinocytes were cultured in collagen-coated tissue flasks (Corning, BioCoat) in
856 epidermal keratinocyte medium (CELLnTEC) at 37°C, 5% CO_2. Keratinocytes were
857 differentiated 24h before the experiments with keratinocyte bare medium (CELLTECH)
858 containing 1.7 mM $CaCl_2$. The *in vitro* co-culture model was performed as previously
859 described [13221221]. Keratinocytes were seeded in collagen-coated transwell inserts (0.4
860 µm pores). PMNs were isolated as described above and seeded in a concentration of
861 2x10^6/ml in a 24 well plate. Transwell inserts containing the differentiated
862 keratinocytes were placed above the PMNs and co-incubated for indicated times.
863 Where indicated, inhibitors were added to the upper and lower well of the co-culture
864 and remained there during the whole experiment. For inhibition of TLR4, RAGE-AP,
865 NFkB, MEK, ROS formation final concentrations of 1 µM TAK-242, 5 µM RAGE-AP, 1
866 µM Celastrol, 15 nM Trametinib or 5 mM N-Actelycystein were used, respectively.

867

868 ***Skin Explants***

869 After removal of fact and blood vessels, human foreskins were incubated overnight at
870 37 °C in epidermal keratinocyte medium (CellnTEC,) containing 0.2 % gentamycin
871 (Sigma-Aldrich). After two washing steps with Hank's Balanced Salt Solution (Sigma-
872 Aldrich), skin explants were cut into pieces and transferred in a 12 well plate and
873 supplied with 700 µl epidermal keratinocyte medium. A filter disc containing 100 ng/ml
874 recombinant HMGB1, or PBS was topically applied on the skin explants for 18h.
875 Subsequently, the filter disc was replaced with a filter disc containing 1x10^8 *S. aureus*.
876 After 18h, the filter disc was removed, and *S. aureus* adherence was determined in 4
877 mm skin punches.

878

879 ***PMN isolation from whole human blood***

880 Whole blood from healthy donors was mixed with dextran solution (2% Dextran, 0.9%
881 NaCl) in a 1:1 ratio and incubated for 30 min until two phases have formed. The upper
882 phase was layered on BioColl (1.077 g/ml, Bio&Sell) in a 3:2 ratio following density
883 gradient centrifugation for 30 min @ 1600 rpm in a swinging bucket rotor without brake.
884 After removing the plasma, PBMC ring and the BioColl layer, the pellet containing
885 erythrocytes and granulocytes was resuspended in hypotonic erythrocyte lysis buffer
886 (C-C-Pro), incubated for 10 min, and subsequently centrifuged for 10 min @ 1600 rpm
887 in a swinging bucket rotor without brake. The remaining pellet containing PMNs was
888 washed once in PBS and was then resuspended in keratinocyte base medium
889 (CELLTECH) containing 1.7 mM $CaCl_2$.

890

891 ***Isolation of Neutrophil extracellular traps***

892 NETs isolation was performed as previously described [57]. PMNs were isolated as
893 described above and seeded in a concentration of 2x10^6/ml in a 6 well plate. NET
894 formation was induced by addition of 500 nM PMA (Sigma). After 4h,

895 supernatant was carefully removed, and NETs were collected by washing the plate
896 with cold PBS followed by a centrifugation step for 10 min @ 450g and 4°C. The
897 supernatant was removed and centrifuged @ 18,000g @ 4°C. After removal of the
898 supernatant, the pellet was resuspended in cold water and DNA concentrations were
899 measured. Indicated concentrations of isolated NETs were used for the co-incubation
900 with keratinocytes instead of PMNs.

901 ***Viability Assay***

902 Toxicity of reagents used in this study on keratinocytes were tested using 4-
903 methylumbelliferyl heptanoate (MUH). Briefly, cells were treated with the respective
904 reagents for 24h and subsequently incubated with 100 µg/ml MUH (Sigma-Aldrich) in
905 PBS for 1h @ 37°C. The fluorescence intensity was quantified using a Fluoroskan II
906 (Labsystems).

907 ***Adhesion and invasion assay***

908 For analyzing *S. aureus* skin colonization *in vitro,* adhesion invasion assays were
909 performed as previously described [13]. Briefly, keratinocytes were either co-incubated
910 with PMNs as described above or incubated alone in the presence or absence of
911 inhibitors. After different co-incubation times, keratinocytes were infected with *S.*
912 *aureus* (MOI = 30; OD = 0.5) for 1.5h. After the infection, keratinocytes were washed
913 twice with Phosphate Buffered Saline (Sigma-Aldrich) and subsequently lysed with a
914 lysis buffer containing PBS, 2.5% Trypsin and 1% Triton-X in a 7:2:1 ratio. Serial
915 dilutions of the lysates were plated onto TSB agar plates and incubated at 37 °C
916 overnight. The next day, *S. aureus* skin colonization was analyzed by quantifying CFU.

917

918 ***Neutrophil extracellular traps quantification assay (SYTOX)***

919 Quantification of Neutrophil extracellular traps was performed as previously described
920 [13]. Briefly, keratinocytes and PMNs were co-incubated for 2h, 4h, 6h or 18h following
921 infection of keratinocytes with *S. aureus* (MOI = 30, 1.5h). After 3h of infection, 100 µl
922 of the supernatant of the neutrophil well was transferred into a 96 well plate and
923 incubated for 10 min in the dark with 5 µM SYTOX Green (Thermo Fisher). SYTOX
924 Green fluorescence (λex: 488 nm; λem: 523 nm) was quantified using a Fluoroskan II
925 (Labsystems). Treatment of PMNs with 500 nM PMA served as a positive control and
926 treatment with 10% Triton X-100 served as a total DNA content control.

927

928 ***Live Cell Imaging of NETosis***

929 NET formation of PMNs was analyzed by live-cell imaging using the Incucyte SX1
930 (Sartorius) or a Thunder Imaging System (Leica Microsystems, 20x NA0.8 objective in
931 24h recordings with a frame rate of 5 min). Briefly, freshly isolated PMNs were
932 resuspended in keratinocyte base medium (CELLTECH) with 1.7 mM $CaCl_2$ containing
933 1 µM DRAQ5 and 0.2 µM SYTOX Green for staining of intracellular and extracellular

934 DNA, respectively. PMNs were further co-incubated for 18h with keratinocytes
935 following the infection of keratinocytes with *S. aureus* (MOI = 30, 1.5h). PMNs
936 undergoing NET formation upon the *S. aureus* infection were indicated by positive
937 SYTOX Green staining. Treatment of PMNs with 500 nM PMA (Sigma) was used as
938 control for NET formation. Furthermore, NET formation by freshly isolated PMNs
939 stimulated with the supernatants of keratinocyte and neutrophil well of the co-culture
940 before and after *S. aureus* infection was analyzed.
941

942 ***RNA isolation and cDNA synthesis***

943 Isolation of total RNA of keratinocytes was performed using the Nucleospin RNA kit
944 (Macherey-Nagel) according to the manufacturer's protocol. Following RNA isolation,
945 complementary DNA was synthesized using the Reverse-Transcriptase Kit (Thermo
946 Scientific) with 1 µg of RNA, 4 µl of 5x RT buffer, 0.5 µl Maxima reverse transcriptase
947 (200 U/ml), 1 µl of random hexamer primer (100 µM), dNTP (1mM) and RNAse-free
948 water to a total volume of 20 µl. RNA was first pre-incubated with RNAse-free water at
949 70°C for 10 min before the master mix was added and cDNA was synthesized for 10
950 min @ 25°C and 45 min @ 50 °C followed by a heat-inactivation step for 5 min @
951 85°C.
952

953 ***Quantitative reverse transcription-polymerase chain reaction***

954 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was
955 performed in a 10 µl reaction volume with SYBR Green PCR Master Mix (Thermo
956 Fisher) according to the manufacturer's instructions using a LightCycler 96 (Roche Life
957 Science). The PCR program consisted of a denaturation step for 5 min @ 95°C
958 followed by 40 cycles with 10s each for denaturation @ 95°C, annealing at individual
959 temperature and elongation @ 72°C. Primer Sequences are listed in. (actin, Nrf2,
960 DAMPs). To screen genes upregulated in keratinocytes co-incubated with PMNs
961 before and after *S. aureus* infection, the antibacterial RT Profiler Array (Quiagen) was
962 used according to the manufacturer's instructions. Briefly, keratinocytes were co-
963 incubated with PMNs or NETs. To analyze the effect of the co-culture, not co-culture
964 keratinocytes were used as control.
965
966

967 ***Western Blot***

968 Analysis of activated signaling pathways in keratinocytes was analyzed by western blot
969 using whole cell lysates. Briefly, keratinocytes were co-incubated with PMNs for 3h, 6h
970 and 18h in the presence or absence of inhibitors and keratinocytes were subsequently
971 lysed in a lysis buffer containing protease and phosphatase inhibitors. Lysates were
972 separated via SDS-polyacrylamide gel electrophoresis and plotted onto PVDF
973 membranes. After 60 min blocking in PBS + 0.1% Tween + 5% dry milk, membranes
974 were incubated overnight @ 4°C in the following antibodies: p-p65 and p-65 (1:1000
975 Cell Signaling), p-ERK and ERK (1:1000 Cell Signaling), TLR4 (1:500 Santa Cruz), β-
976 actin (1:1000 Cell Signaling). As secondary antibodies, horseradish peroxidase-
977 conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:2000) were used. ECL
978 (Thermo Fisher Scientific) or ECL prime (GE Healthcare Lifesciences) was used as
979 chemiluminescence reagents, and an Amersham Imager 600 (General Electric) was
980 used for detection.

981

982 **Enzyme-linked immunosorbent assay**

983 For HMGB1 ELISA, ELISA plates (Nunc) were coated with 100 µl of cell culture
984 supernatant or 2-fold dilutions of recombinant HMGB1 starting from 8 µg/ml overnight
985 at 4 °C. After three washing steps with PBS+0.05%Tween, 100 µl primary antibody
986 against HMGB1 (Biolegend) at 37 °C for 1h. After three washing steps, 100 µl
987 secondary antibody (Cell Signaling) were added and the plate was incubated @ 37°C
988 for 1h. After three washing steps, 100 µl TMB substrate solution (Cell Signaling) was
989 added to each well. Reaction was stopped with 50 µl 2 N H2SO4 and absorbance at
990 450 nm was measured using a Fluoroskan II (Labsystems).

991

992 ***Measurement of oxidative stress***

993 Oxidative stress in tape-stripped and not tape-stripped C57BL/6 WT mice was
994 detected by measuring lipid peroxidation by quantifying malondialdehyde (MDA) in skin
995 tissue lysates using a commercial kit (Sigma-Aldrich, St. Louis, USA) according to the
996 manufacturer's instructions.

997 For measuring oxidative stress in-vitro, PHKs and PMNs were incubated with
998 H2DCFDA (ThermoFisher) for 30 min and intracellular ROS levels were measured as
999 DCF fluorescence via flow cytometry as described elsewhere.

Quantification and statistical analysis

Significant differences between the means of the different treatments were evaluated using GraphPad Prism 9.0 (GraphPad Software, Inc.). Either unpaired, two-tailed Student's t test or one-way analysis of variance followed by Dunnett's multiple comparisons test was used for statistical analysis and indicated in the respective figure legends. Differences were considered statistically significant with a p value of <0.05. Data were visualized using GraphPad 9.0 (GraphPad Software Inc.), MS Excel (Microsoft Corporation), or FlowJo (TreeStar).

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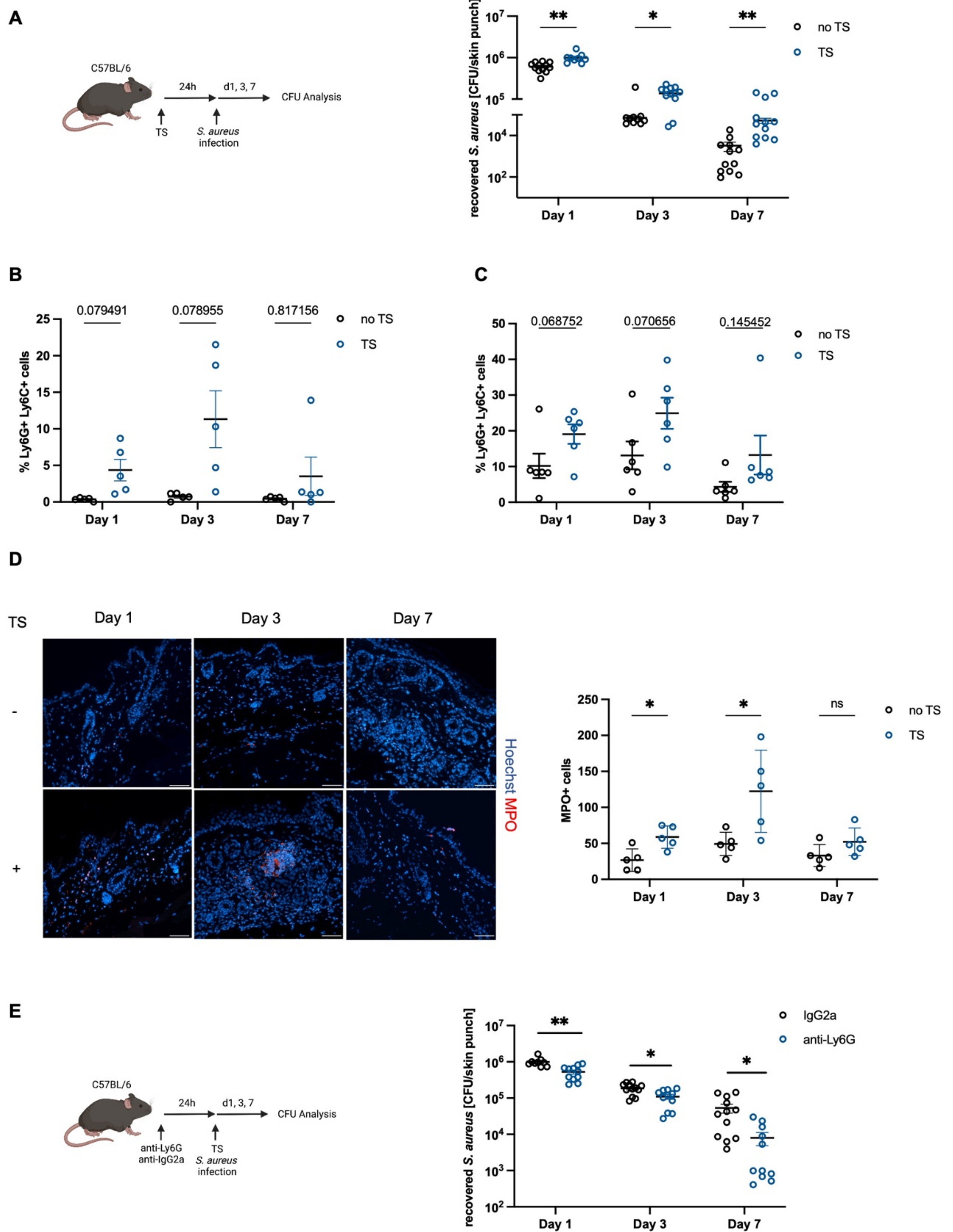
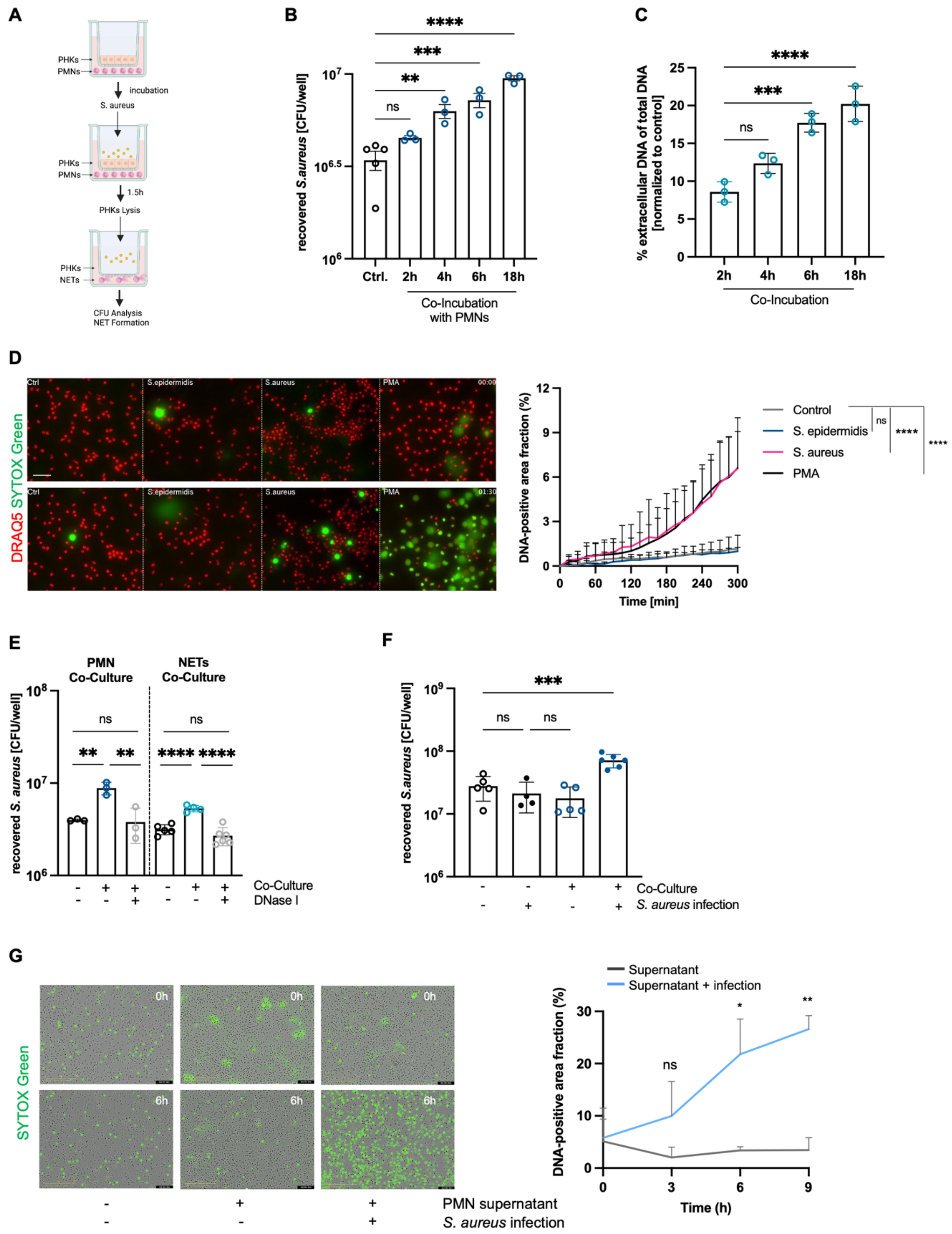


Figure 1



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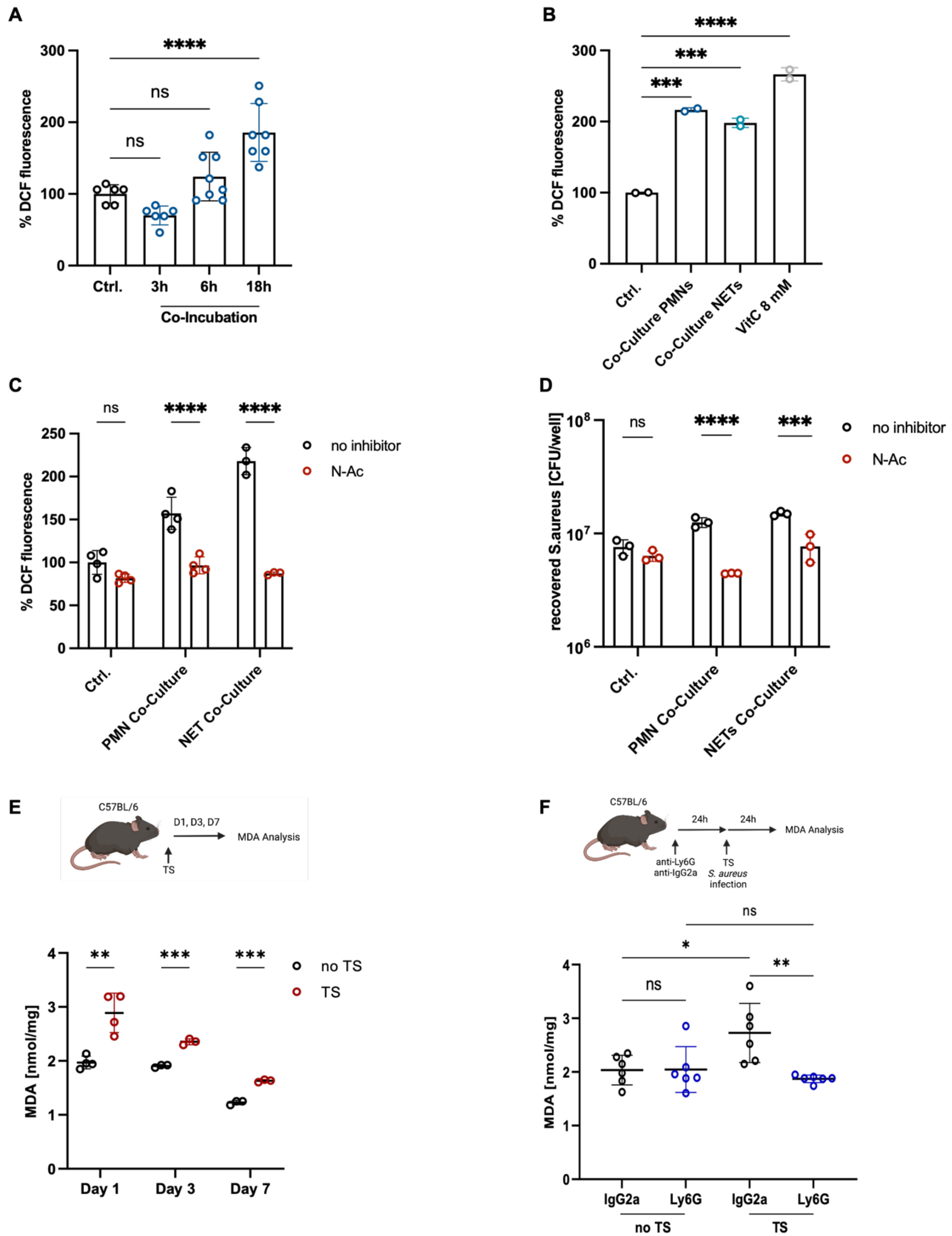

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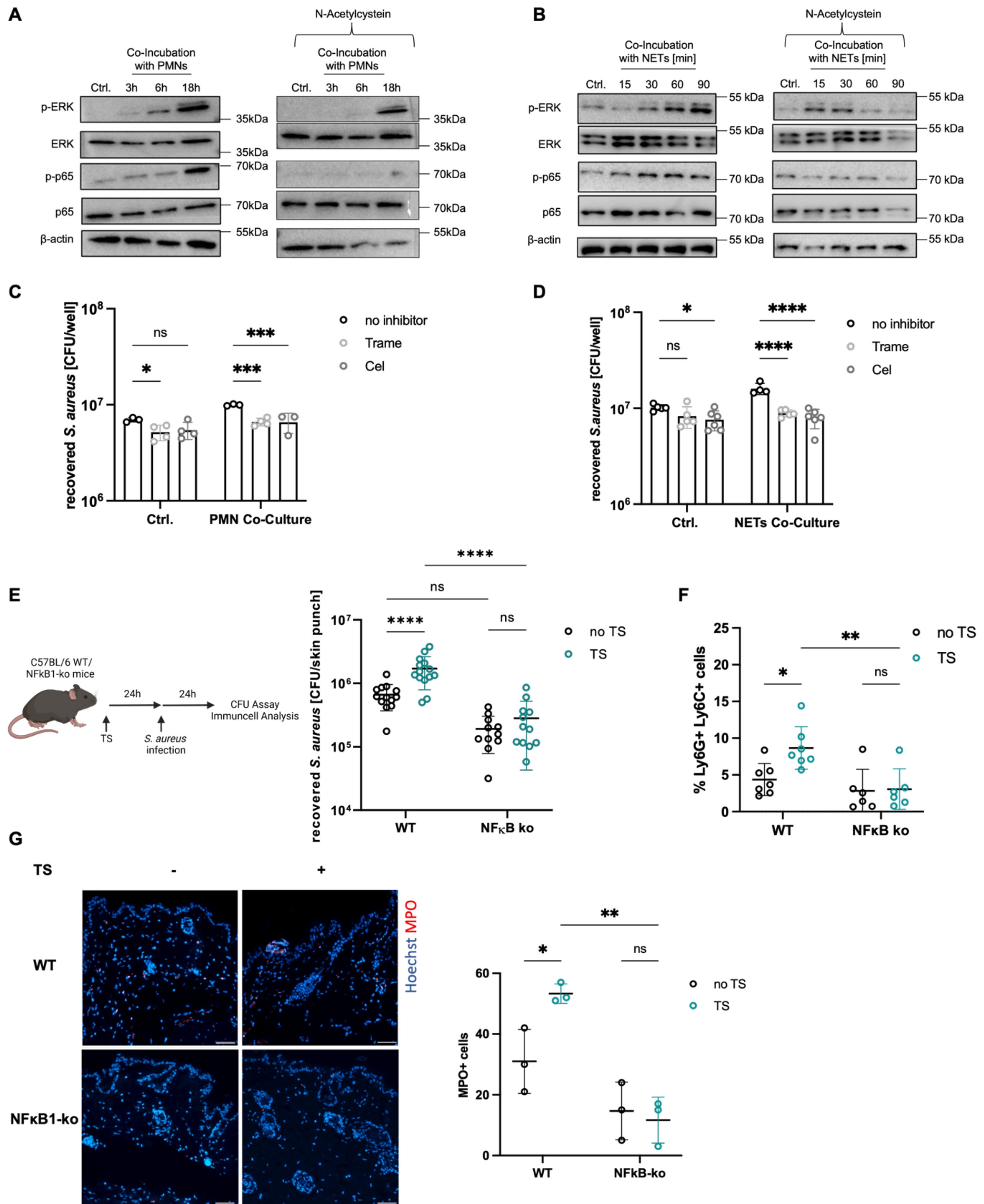

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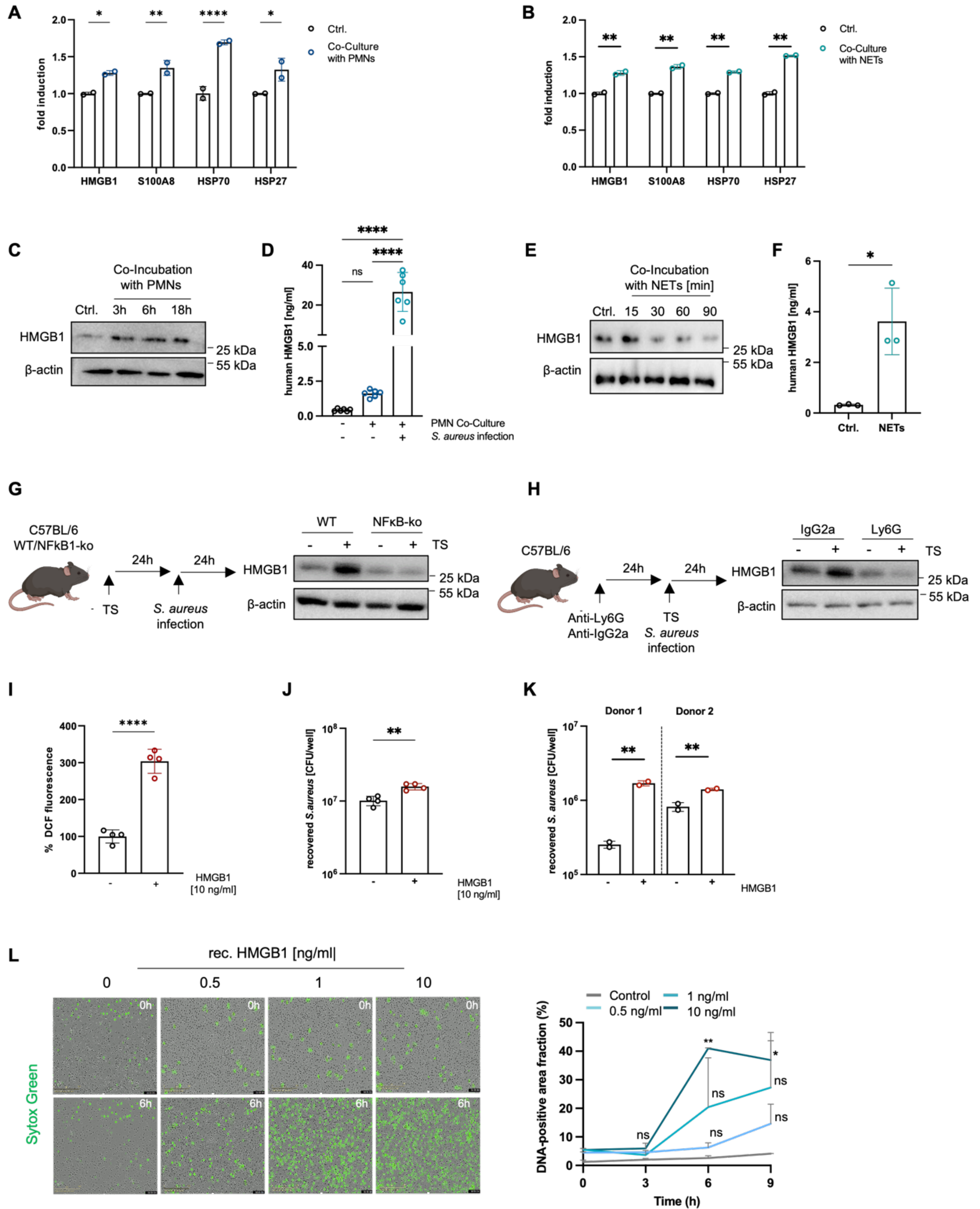

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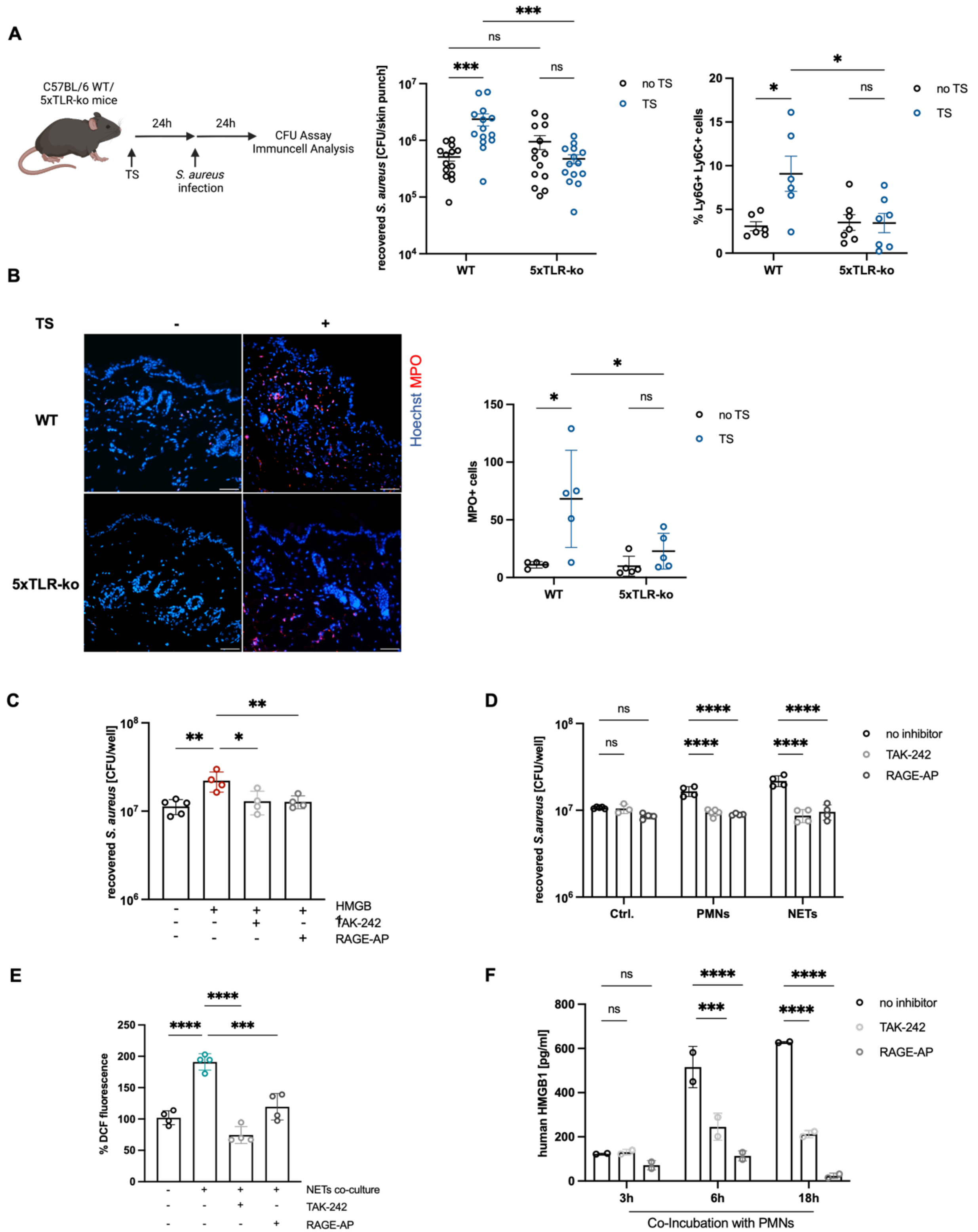

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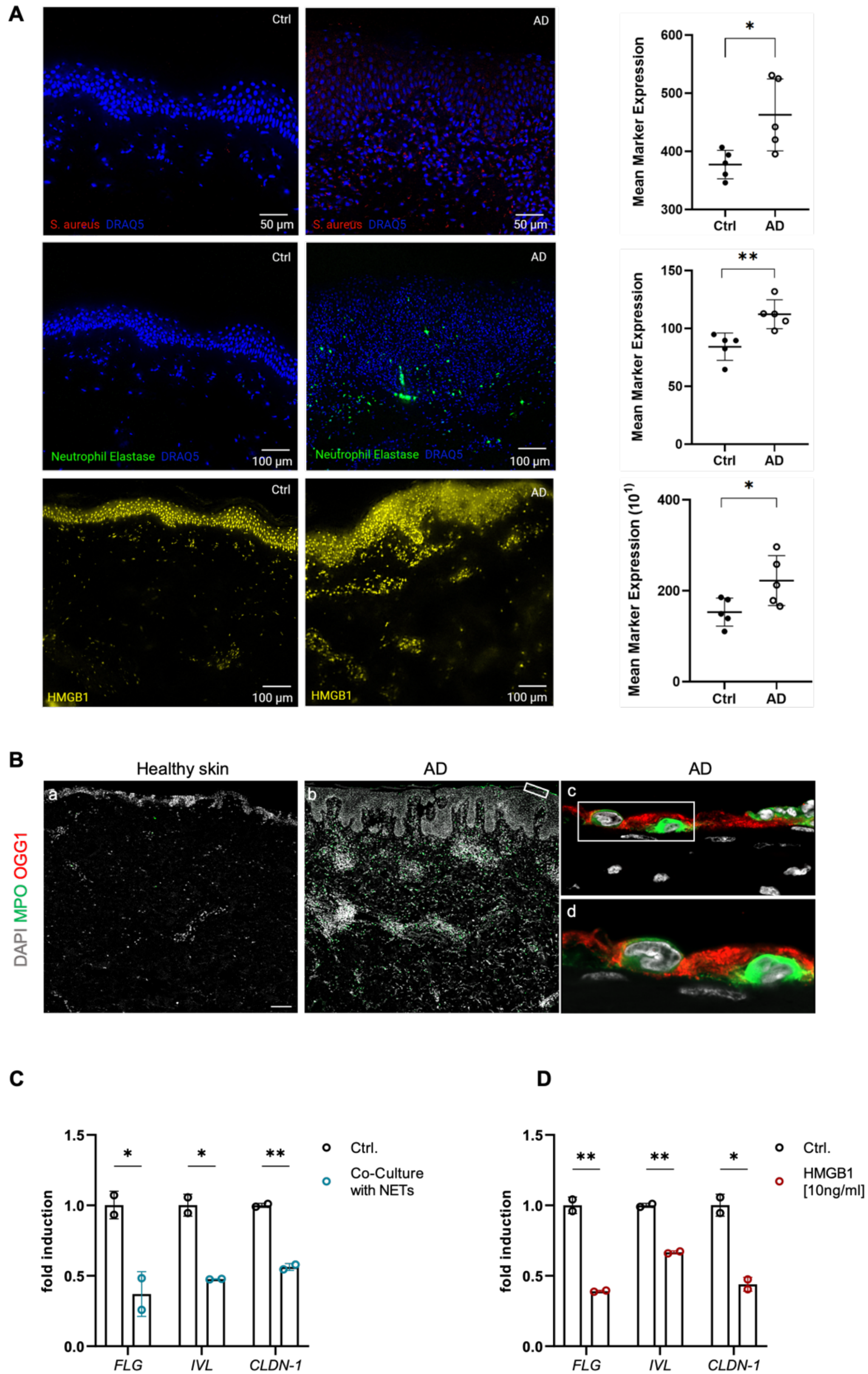

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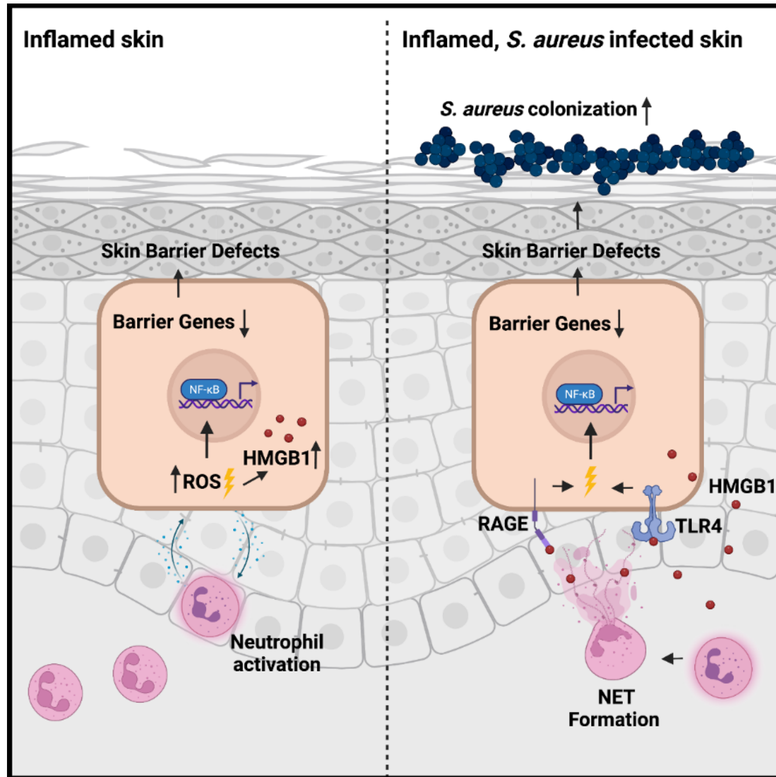

Graphical Abstract

Supplementary Figure legends

Figure S1: NETs Detection and quantification of PMNs after PMN depletion

A: Presence of citH3 in skin lysates of mice +/- tape-stripping and +/- S. aureus infection was analyzed after 24h by western blot. Expression of β-actin was used as loading control. **B:** Representative image of immunofluorescence staining for citH3 and MPO, indicating NETs, in tape-stripped mouse skin after 24h epicutaneous *S. aureus* application. Scale bar = 50 µm **C**: Gating Strategy for immune cell analysis in mouse skin. Isolated cells were first gated for viability and CD45+ positivity (live CD45+). Then the live CD45+ cells were gated for CD11b. Neutrophils were then gated from the CD11b+ cells and defined as live CD45+ CD11b+ Ly6G+ Ly6C+ cells. **D**: Efficiency of PMN depletion was analyzed by quantifying numbers of PMNs (live CD45+ CD11b+ Ly6G+ Ly6C+) in the tape-stripped skin of mice injected intraperitoneal with an anti-Ly6G antibody or isotype control (IgG2a) on days 1, 3 and 7 after epicutaneous *S. aureus* application via flow cytometry. Shown is the mean of each group ± SD. One point represents one mouse. **E:** MPO staining of tape-stripped skin of PMN-depleted (Ly6G) or isotype-treated (IgG2a) mice on day 3 and 7 after epicutaneous *S. aureus* application to proof efficient PMN depletion. Scale bar = 50 µm. MPO+ cells were quantified using Fiji. Significant differences were analyzed by multiple unpaired t-tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S2: Lipid Peroxidation assay of skin explants and viability assay of PHKs after N-Acetylcystein treatment

A: Human skin explants of three donors either tape-tripped or left untreated. After 24h, signs of oxidative stress were analyzed in the skin by quantifiying MDA levels in tissue lysates. Significant differences were analyzed by multiple unpaired t-tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B:** Human skin explants were either tape-

stripped or left untreated and subsequently incubated alone or in co-culture with PMNs. After 24h, MDA levels in the tissue lysates was analyzed. Significant differences were analyzed by One-Way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **C:** PHKs were stimulated with 5 mM N-Acetylcysteine (N-Ac) for 18h or left unstimulated and subsequently infected with *S. aureus* (MOI = 30) for 1.5h or left uninfected. Viability of the PHKs was analyzed using 4-methylumbelliferyl heptanoate (MUH). As positive control, cells were treated with 1% Triton-X. Shown is one representative experiment of three independent experiments. Significant differences were analyzed using one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **D**: NET formation in the co-culture upon S. aureus infection was analyzed in the presence or absence of N-Acetylcysteine by SYTOX Green staining. The amount of extracellular DNA was normalized to the data of Triton-X treated cells representing the total DNA amount. Significant differences were analyzed by One-Way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **E:** Representative immunohistochemistry staining for MDA of the skin of tape-stripped and not-tape-stripped mouse skin 24h after epicutaneous *S. aureus* application. Scale bar = 50 µm

Figure S3: Viability assay of PHKs after Trametinib or Celastrol treatment

A&B: PHKs were treated with MEK inhibitor Trametinib (**A**) or NFκB inhibitor Celastrol (**B**) for 18h or left untreated and subsequently infected with *S. aureus* (MOI = 30). After 1.5h, viability of the PHKs was analyzed using 4-methylumbelliferyl heptanoate (MUH). As positive control, cells were treated with 1% Triton-X. Shown is one significant experiment of three independent experiments. Significant differences were analyzed using one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

Figure S4: Effect of DAMPs on *S. aureus* skin colonization and effect of HMGB1 treatment on skin colonization of skin commensals

A: Intracellular protein expression of HMGB1 in PHKs co-incubated with PMNs in the presence of 5 mM N-Acetylcystein was investigated via western blot analysis. Protein expression of β-actin was used as loading control. **B:** Presence of HMGB1 in isolated NETs was analyzed by ELISA. Supernatant of PHKs was used as negative control. **C:** PHKs were treated with recombinant HMGB1 (10 ng/ml) for 18h or left untreated and

subsequently infected with *S. aureus*, *S. epidermidis* or *S. lugdunensis* (all MOI = 30). After 1.5h infection, PHKs were lysed, and CFU were quantified the next day. Significant differences were analyzed using multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **D:** PHKs were treated with recombinant HSP70 (1 µM) or S100A8/A9 (10 ng/ml) for 18h or left untreated and subsequently infected with S. aureus (MOI = 30). After 1.5h PHKs were lysed and CFU were quantified. Shown is one representative experiment of three independent experiments. Significant differences to the untreated control were analyzed by an unpaired t-test *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S5: Protein Expression of TLR4 in PHKs and skin explants and viability assay of PHKs after TAK-242 and RAGE-AP treatment

A&B: PHKs were co-incubated with PMNs (**A**) or NETs (**B**). After different time-points, protein expression of TLR4 and RAGE in PHKs was analyzed by western blot. Protein expression of β-actin was used as loading control. **C:** Human skin explants were either tape-stripped (TS) or left untreated and then placed in a transwell insert and co-incubated with PMNs or alone. After 18h, protein expression of TLR4 was analyzed by western blot analysis. Protein expression of β-actin was used as loading control. **D&E:** PHKs were treated with TLR4 inhibitor TAK-242 or RAGE inhibitor RAGE-AP for 18h or left untreated and subsequently infected with *S. aureus* (MOI = 30). After 1.5h, viability of the PHKs was analyzed using 4-methylumbelliferyl heptanoate (MUH). As positive control, cells were treated with 1% Triton-X. Shown is one representative experiment of three independent experiments. Significant differences were analyzed using one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S6: HMGB1 expression in the skin of AD patients compared to healthy controls

A-C: Total, nuclear and cytoplasmic HMGB1 expression was quantified in the skin of AD patients compared to healthy control using QuPath. Significant differences were analyzed by unpaired two-tailed t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

93 **Figure S7: NETs are present in the skin of atopic dermatitis patients**

94 Representative immunofluorescence images of skin sections of three atopic dermatitis
95 patients for NETs (a, c, e). Scale bar = 200 µm. NET formation, defined by the co-
96 localization of OGG1 and MPO, is indicated by white arrows (b, d, f). Scale bar = 20
97 µm.
98
99 **Figure S8: NETs downregulate epidermal barrier genes in PHKs via ROS**

100 **A:** Protein expression of involucrin and filaggrin was investigated in skin explants 24h
101 after topical application of NETs (25 ug/ml) or HMGB1 (100 ng/ml). Protein expression
102 of ®-actin was used as loading control. Semi-quantification was performed using
103 ImageJ and significant differences to the control were analyzed using One-Way
104 ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B:** Differentiated PHKs
105 were co-cultured with NETs for 24h in the presence or absence of ROS scavenger N-
106 Acetylcysteine. After 24h co-culture, gene expression of epidermal barrier genes
107 filaggrin (FLG) and involucrin (IVL) was analyzed by qRT-PCR. Shown is one
108 representative experiment of three independent experiments. Significant differences
109 were analyzed using One-Way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P <
110 0.0001.
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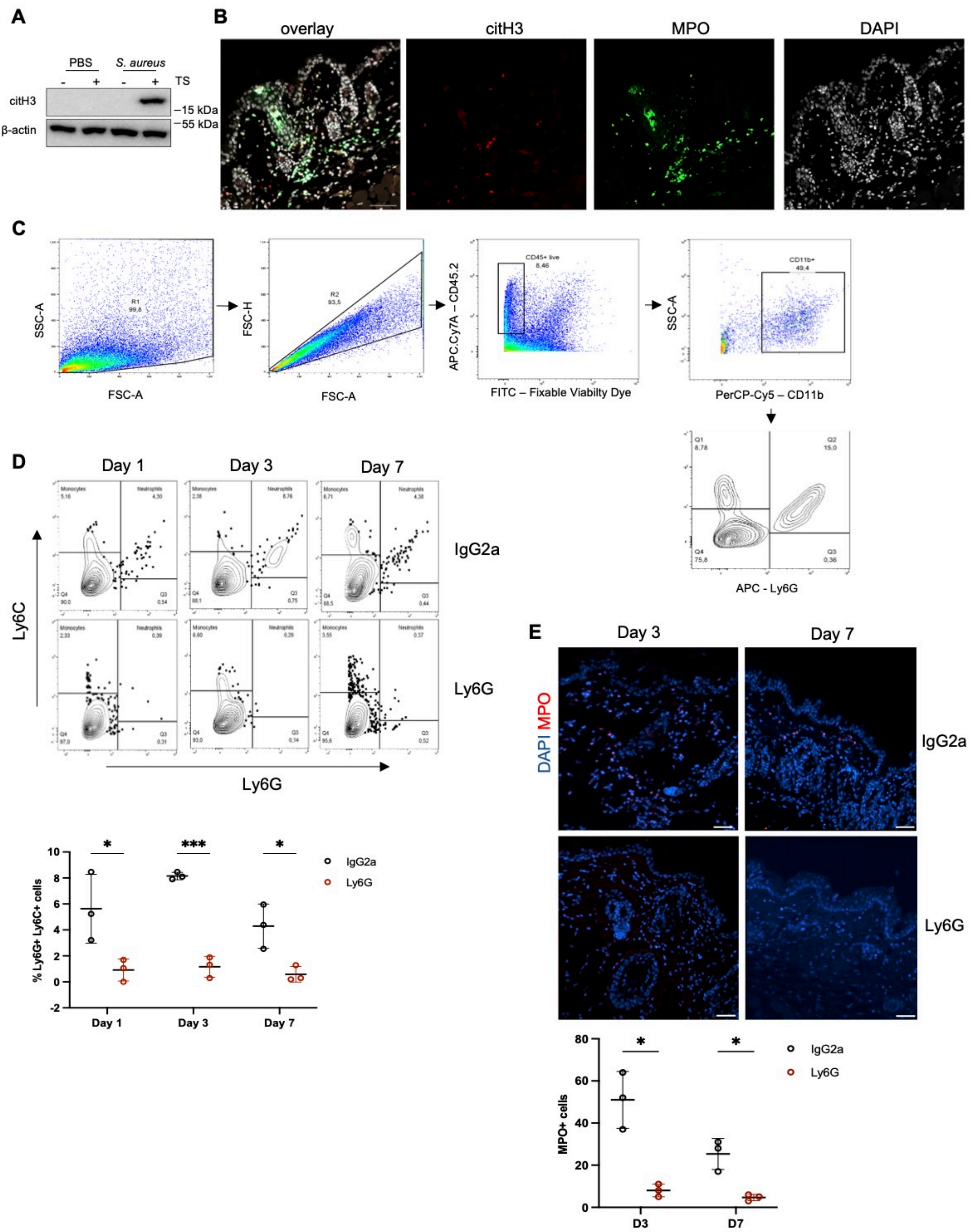

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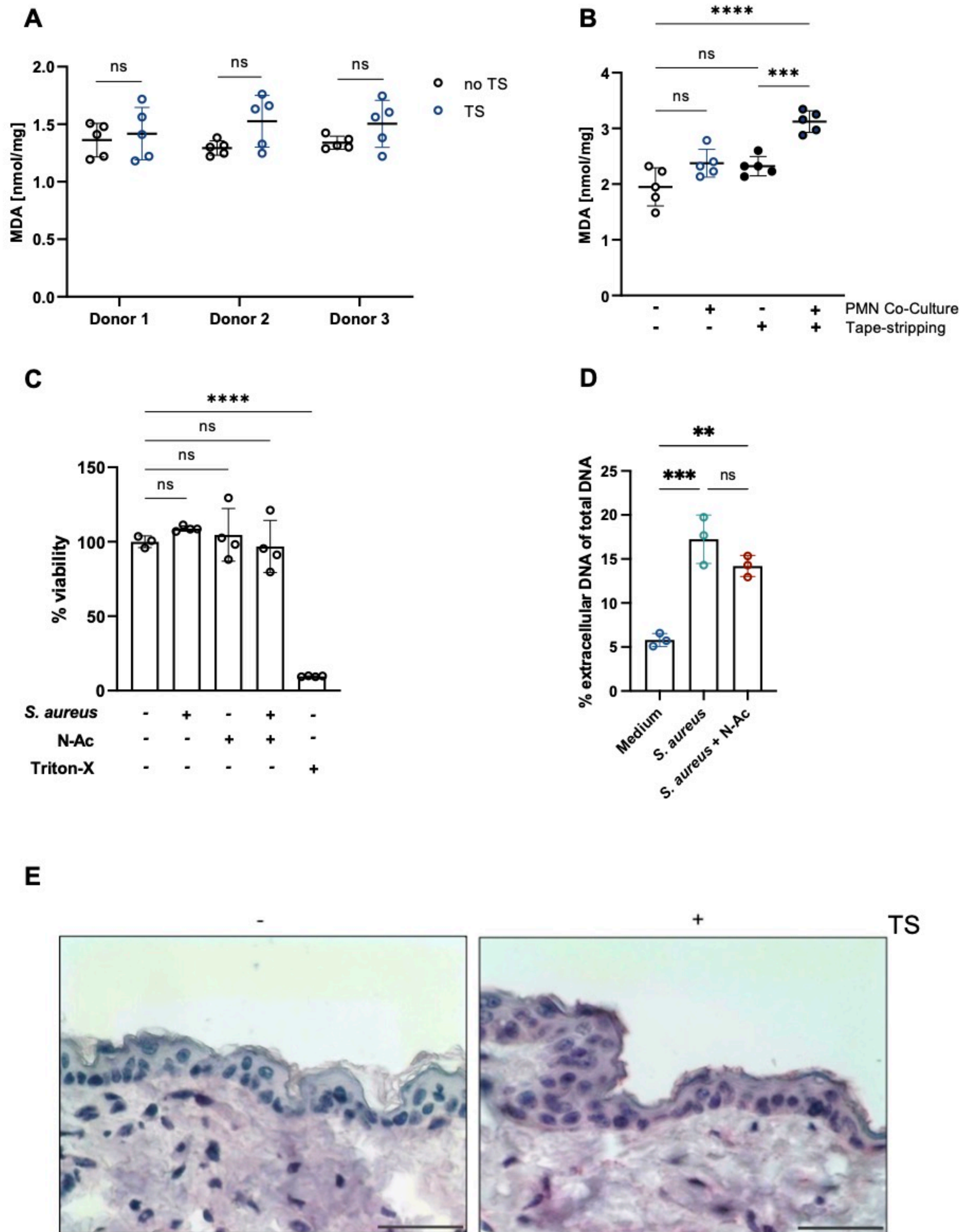

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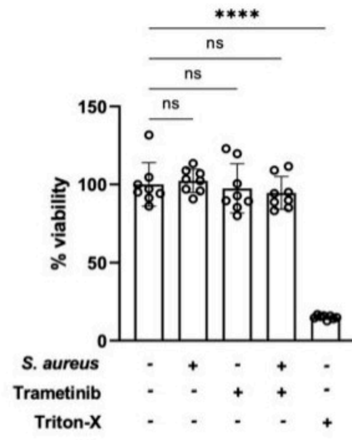

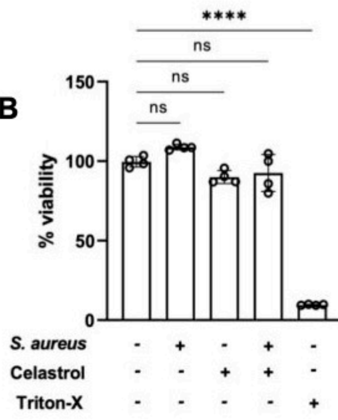

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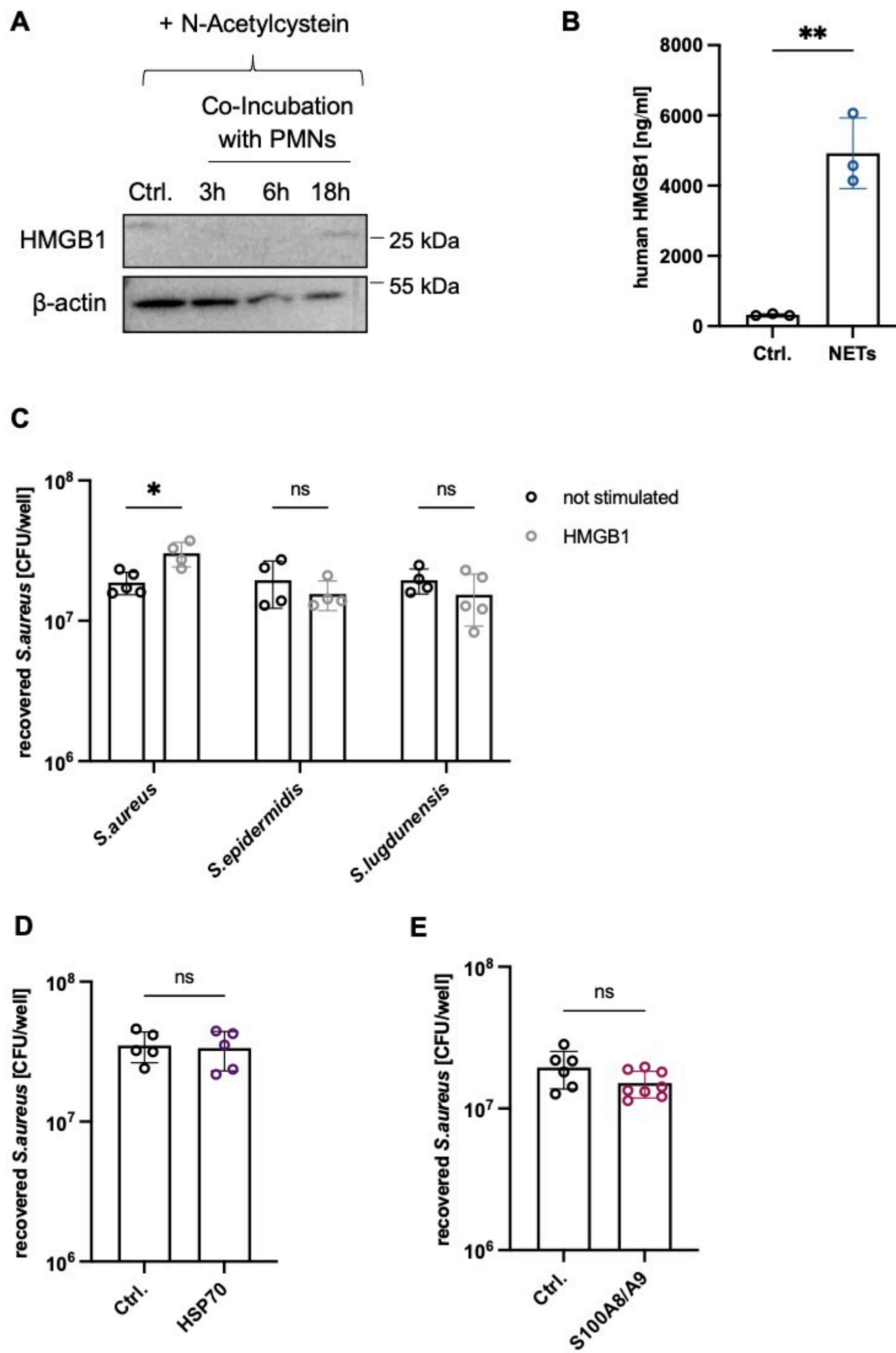

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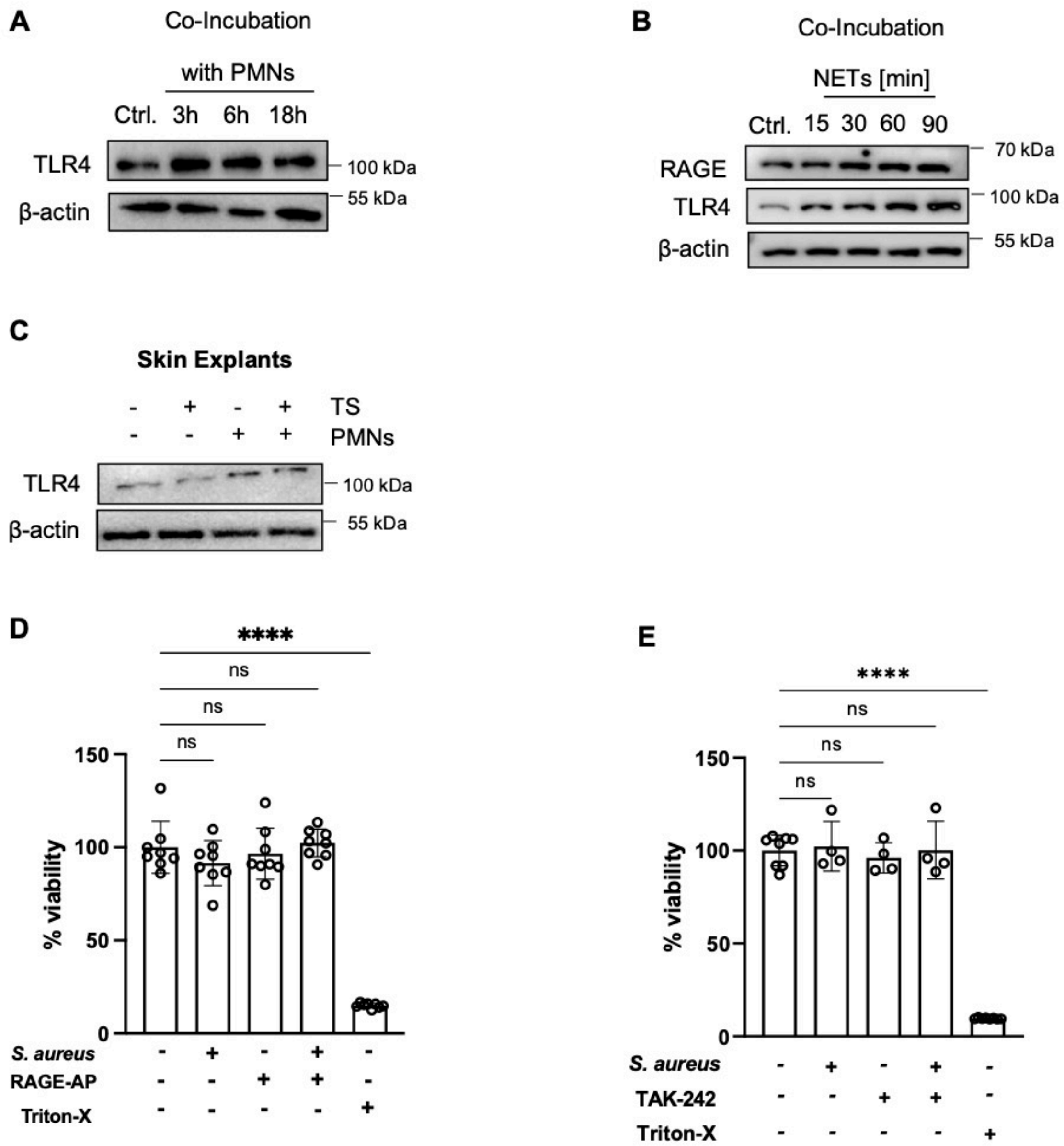

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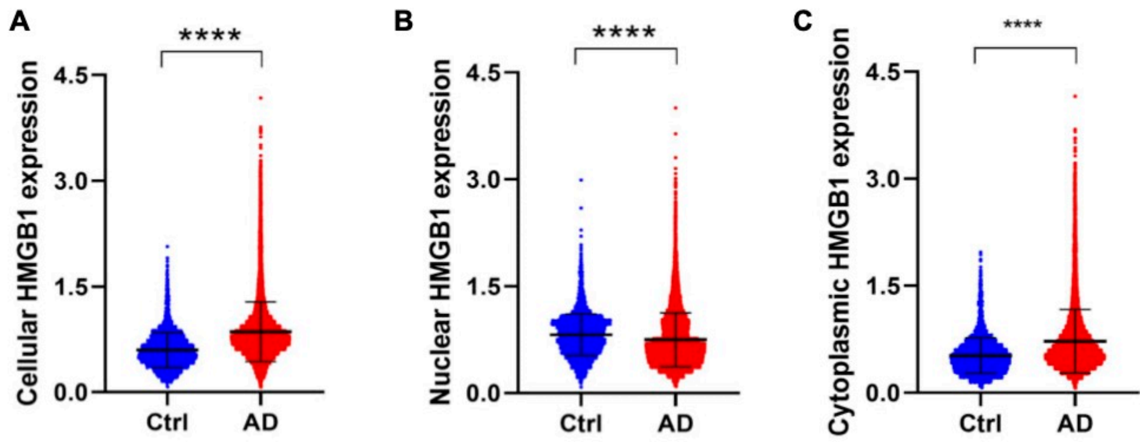

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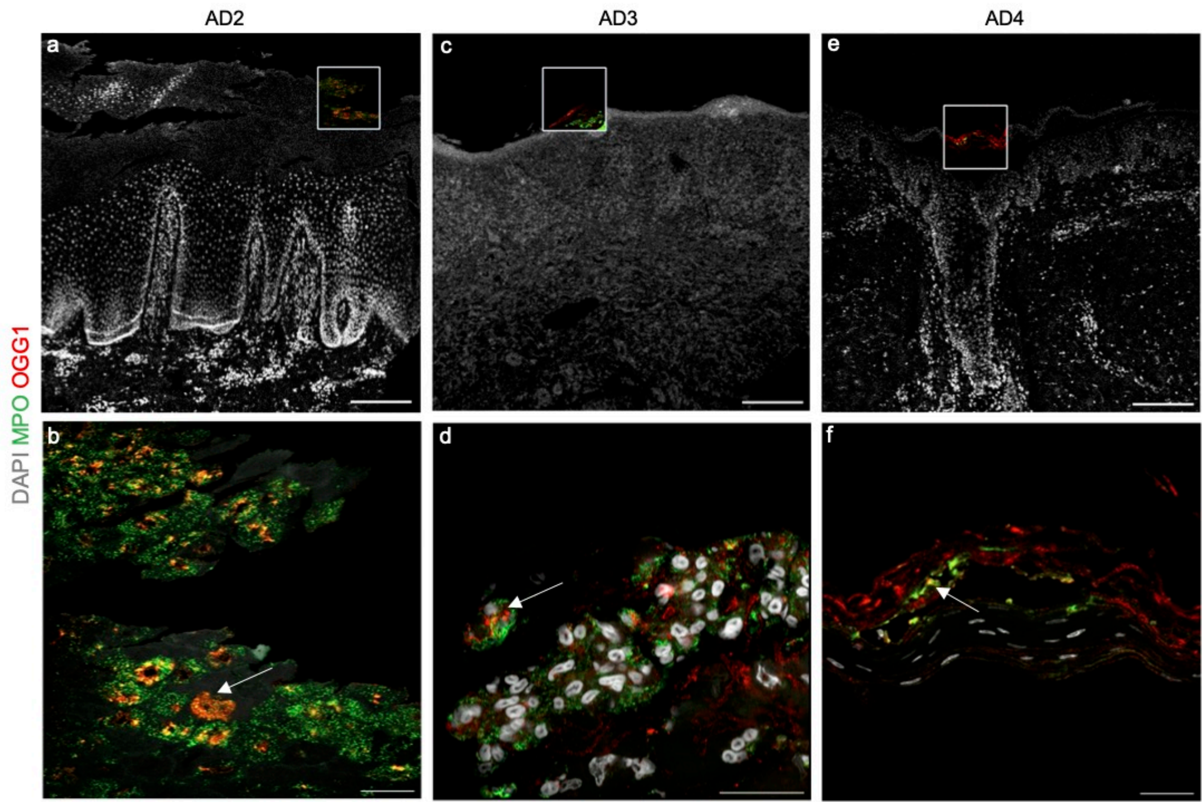

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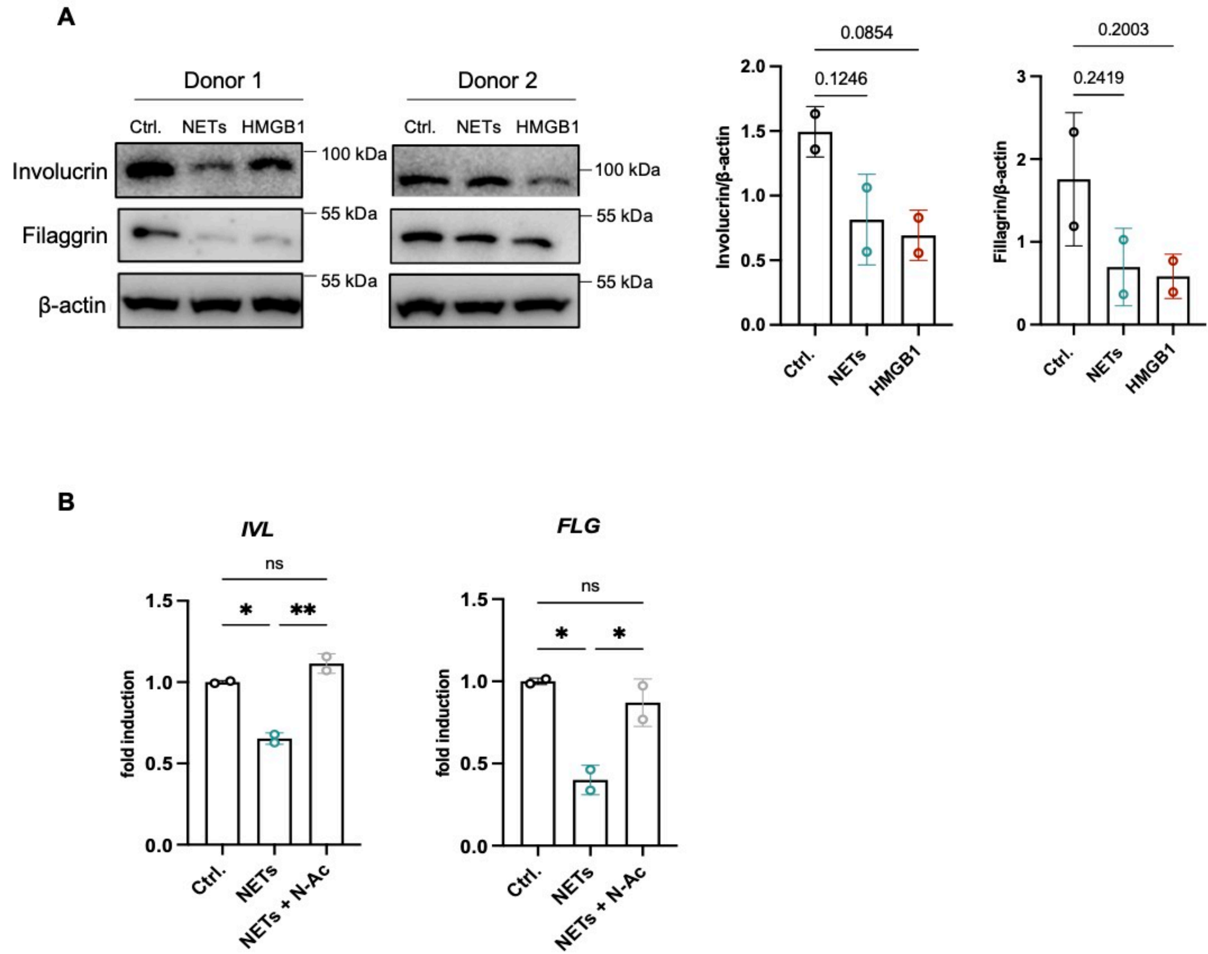

Figure S8

Table S1 Primer Sequences. Related to Figure 7

Primer	Sequence	Annealing Temp
ACTB fw	TTGTTACAGGAAGTCCCTTGCC	60 °C
ACTB rv	ATGCTATCACCTCCCCTGTGTG	
CLDN1 fw	GCTTCTCTCTGCCTTCTGGG	60 °C
CLDN1 rw	TCACACGTAGTCTTTCCCGC	
FLG fw	ACTTCACTGAGTTTCTTCTGATGGTATT	60 °C
FLG rv	TCCAGACTTGAGGGTCTTTTTCTG	
IVL fw	ACTTATTTCGGGTCCGCTAGGT	60 °C
IVL rv	GAGACATGTAGAGGGACAGAGTCAAG	

Table S2 Antibodies used in CODEX analysis. Related to Figure 7

Antibody	Clone(s)	Manufacturer	Cat#	Oligo	Oligosequence
S. aureus	Poly	Novus	NB100-64499	A11	/5Alex647N/ACGAGTGTATAACCC
HMGB1	Polyclonal	R&D Systems	MAB1690	A60	/5ATTO550N/CGACAGCAGTTTTGT
Neutrophilen Elastase	ELA2	R&D Systems	MAB91671-100	A75	/5Alex647N/TAAACACCCAAGCG

5 Manuscript II

This work is published in Frontiers in Immunology [506].

Focken J, Schittek B. Crosstalk between keratinocytes and neutrophils shapes skin immunity against *S. aureus* infection. Front. Immunol. 2024 Feb 16;15:1275153. doi: 10.3389/fimmu.2024.1275153

Personal Contribution

- I designed, performed, and analyzed all experiments under the supervision of B. Schittek
- I wrote the manuscript together with B. Schittek

Crosstalk between Keratinocytes and Neutrophils shapes skin immunity against *S. aureus* infection

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Keywords: Neutrophils, Keratinocytes, *Staphylococcus aureus*, Skin Inflammation, Skin Immune System

14 **Abstract**

15 Introduction: *Staphylococcus aureus* (*S. aureus*) infection of the skin leads to a rapid
16 initial innate immune response with keratinocytes in the epidermis as the initial
17 sensors. Polymorphonuclear neutrophils (PMNs) are the first innate immune cells to
18 infiltrate infection sites where they provide an effective first-line of defense. Previous
19 work of our group showed that in inflamed skin a crosstalk between PMNs and
20 keratinocytes results in enhanced *S. aureus* skin colonization.
21
22 Methods: In this work, we used an *in vitro* co-culture model to studied the crosstalk
23 between primary human keratinocytes (PHKs) and PMNs in a sterile environment and
24 upon *S. aureus* infection. We investigated the influence of PHKs on PMN activation by
25 analyzing PMN lifespan, expression of degranulation markers and induction of
26 proinflammatory cytokines. Furthermore, we analyzed the influence of PMNs on the
27 inflammatory response of PHKs. Finally, we investigated the influence of the skin
28 microbiome on PMN-mediated skin inflammation.
29
30 Results: We show that co-culture of PMNs with PHKs induces activation and
31 degranulation of PMNs and significantly enhances their lifespan compared to PMN
32 cultivation alone by an IL-8 mediated mechanism and, furthermore, primes PMNs for
33 enhanced activity after *S. aureus* infection. The prolonged incubation with PMNs also
34 induces inflammatory responses in PHKs which are further exacerbated in the
35 presence of *S. aureus* and induces further PMN recruitment thus fueling skin
36 inflammation. Interestingly, infection of PHKs with the skin commensal *S. epidermidis*
37 reduces the inflammatory effects of PMNs in the skin and exhibits an anti-inflammatory
38 effect.
39
40 Discussion: Our data indicate that skin infiltrating PMNs and PHKs influence each
41 other in such a way to enhance skin inflammation and that commensal bacteria are
42 able to reduce the inflammatory effect.
43
44
45
46

47 **Introduction**
48
49 The skin immune barrier depends on the interplay of different cell types to ensure
50 homeostasis under physiological conditions and protection against invading pathogens
51 (Nguyen and Soulika, 2019). Keratinocytes are the predominant constituents of the
52 epidermis and thus the first cells to sense an invading pathogen such as
53 *Staphylococcus aureus* (*S. aureus*) and therefore are critical in initiating and
54 maintaining skin inflammation (Bitschar et al, 2017). They contain pattern-recognition
55 receptors that help to sense pathogen-associated-molecular-patterns (PAMPs) on the
56 microbes which initiates secretion of cytokines, chemokines, and antimicrobial
57 peptides (AMPs) and the recruitment of immune cells to infection site (Bitschar et al,
58 2017).

59 Polymorphonuclear neutrophils (PMNs) are the most abundant leucocytes in the
60 human blood (Rosales, 2018). Upon skin infection, they are the first cells to arrive at
61 the site of infection and ensure an effective first-line of protection (Burn et al., 2021,
62 Kolaczkowska and Kubes, 2013) To ensure a fast response at infection sites, PMNs
63 contain preformed molecules stored in cytoplasmic granules that can be rapidly
64 mobilized via degranulation (Ley et al., 2018, Lacy, 2006). However, excessive
65 degranulation can cause enormous collateral damage to surrounding tissue and lead
66 to systemic inflammation. Therefore, PMN activation and degranulation needs to be
67 tightly controlled and requires receptor-coupled mechanisms (Lacy, 2006). After
68 completing their tasks, PMNs undergo apoptosis and are cleared by macrophages.
69 This prevents excessive inflammation and helps restoring homeostasis (Hidalgo et al.,
70 2019, Summers et al., 2010, Gordy et al., 2011).

71 *S. aureus* is a gram-positive facultative pathogen responsible for the majority of skin
72 infections in humans. It asymptomatically colonizes about 30 % of the human
73 population, mainly in the nose (Wertheim et al., 2005, Hanselman et al., 2009). *S.*
74 *aureus* can be frequently found on the skin of patients with atopic dermatitis where it
75 actively participates in skin inflammation (David and Daum, 2017, Eyerich et al., 2015).
76 The recruitment of PMNs has been shown to be crucial for the clearance of S. aureus
77 infections (Millet et al., 2006, Molne et al., 2000). However, we and others provide *in*
78 *vitro* and *in vivo* evidence that PMNs promote *S. aureus* skin colonization and
79 persistence through the interaction of PHKs with neutrophil extracellular traps (NETs)
80 (Bitschar et al., 2020, Yipp et al., 2012). These data indicate that an interaction of PHKs

81 with PMNs via NETs are important for maintaining local infection during the early
82 stages of colonization. Further studies of us revealed that PMNs and NETs present in
83 the inflamed skin induce oxidative stress in PHKs which results in the secretion of
84 HMGB1. NETs and HMGB1 can downregulate the expression of epidermal barrier
85 genes thus promoting skin barrier defects which favors *S. aureus* skin colonization
86 (Focken et al., 2023).

87 Here, we studied the interaction between keratinocytes and PMNs independent of
88 NETs in a sterile and an infectious environment. We investigated the influence of PHKs
89 on activation of PMNs by analyzing PMN lifespan, expression of degranulation markers
90 and induction of proinflammatory cytokines. Vice versa, we analyzed the influence of
91 PMNs on the inflammatory response of PHKs. Finally, we investigated the influence of
92 the skin microbiome on PMN-mediated skin inflammation. Our data highlight a
93 sophisticated crosstalk of PMNs with PHKs in the skin which shapes immune
94 responses to invading bacteria.

95

96 **Material & Methods**

97 **Isolation of Primary Human PMNs**

98 Peripheral blood from healthy donors was mixed with dextran solution (2% Dextran,
99 0.9% NaCl) and incubated for 30 min at RT. The upper phase was applied on BioColl
100 (1.077 g/ml, Bio&Sell) in a 3:2 ratio and density gradient centrifugation was performed
101 for 30 min at 1600 rpm in a swing-out bucket rotor with no brakes. Remaining
102 erythrocytes in the cell pellet were lysed in hypotonic lysis buffer (C-C-Pro). After 10
103 min incubation time, a second centrifugation step at 1600 rpm for 10 min without brake
104 was performed. After one washing step with PBS, the remaining cell pellet containing
105 the PMNs was resuspended in keratinocyte basal medium (CELLnTECH)
106 supplemented with $CaCl_2$ (1.7 mM). PMN isolation from human blood was permitted
107 by the ethics committee of the medical faculty of the University of Tübingen
108 (054/2017BO2).

109

110 **Cell Culture and *in vitro* co-culture system**

111 Primary human keratinocytes (PHKs) were isolated from human foreskin as previously
112 described (Burian et al., 2017, Bitschar et al., 2020, Bitschar et al., 2019). PHK isolation
113 was permitted by the ethics committee of the medical faculty of the University of

114 Tübingen (654/2014BO2) and conducted in accordance with the principles of the
115 Declaration of Helsinki. PHKs were cultured at 37°C and 5% CO_2 in tissue flasks
116 (Corning, BioCoat™) coated with collagen in epidermal keratinocyte medium
117 (CELLnTEC). 24h prior the experiments, 1.7 mM $CaCl_2$ was applied to keratinocyte
118 base medium (CELLnTECH) to induce PHK differentiation. The *in vitro* co-culture was
119 conducted as described previously (Bitschar et al., 2020). Briefly, PHKs were grown in
120 collagen-coated transwell inserts (pore size: 0.4 µm) and differentiated after reaching
121 confluency. Isolated PMNs were then and seeded into 24 well plates in a concentration
122 of 2x10^6/ml. The inserts containing differentiated PHKs were placed on top of the
123 PMNs, and the cells were co-incubated for the indicated times.
124
125 **Flow cytometry**
126 1x10^6 PMNs were incubated with the following surface antibodies for 20 min on ice
127 in the dark. Surface antibodies included PerCP-Cy5.5-anti-CD11b, APC-anti-CD63,
128 APC-anti-CD66b (all Biolegend). Exclusion of dead cells was ensured using a fixable
129 viability dye. Flow cytometry was performed using an LSR II (BD Bioscience) and
130 FlowJo (TreeStar) was used for analysis.
131
132 **Annexin-V staining**
133 Annexin-V staining was performed as described previously. Briefly, 1x10^6 PMNs were
134 resuspended in Annexin binding buffer containing Annexin and incubated for 15 min at
135 RT in the dark. After one washing step with annexin binding buffer, PMNs were
136 measured at the LSR II (BD Bioscience) and FlowJo (TreeStar) was used for analysis.
137
138 **Live Cell Imaging**
139 The Incucyte SX1(Sartorius) was used to investigate PMN viability over time. PMNs
140 were seeded in a 24 well plate in a concentration of 1x10^6/ml and were either co-
141 incubated with PHKs or stimulated with the PMN supernatant of the 18h co-culture.
142 Non-co-cultured PMNs or PMNs incubated with medium served as control. DRAQ5 (1
143 uM; Thermofisher) was used for staining the intracellular DNA and Sytox Green (0.2
144 uM; Thermofisher) was used for staining the extracellular DNA, thus indicating cell
145 death. Pictures were taken every hour when PMNs were stimulated with the

146 supernatant or after 6h, 18h and 30h when co-cultured with PHKs. Percentage of Sytox
147 Green positive cells were quantified using Fiji/ImageJ.
148

149 **Western blot**

150 Capase-3 cleavage in whole cell lysates of PMNs was analyzed by western blot. Cell
151 lysis was performed with a lysis buffer supplemented with protease- and phosphatase
152 inhibitors. Lysates were subjected to SDS-polyacrylamide gel electrophoresis for
153 separation followed by transfer to PVDF membranes. To block nonspecific binding
154 sites, membranes were incubated in PBS+0.1%Tween+5% dry milk for 30 min followed
155 by incubation in caspase-3 antibody (1:1000, Cell Signaling) at 4°C overnight.
156 After a 60 min blocking step in PBS + 0.1% Tween + 5 % dry milk, the membranes
157 were incubated in anti-caspase-3 antibody (1:1000, Cell Signaling) at 4°C overnight.
158 The next day, the membrane was washed three times with PBS + 0.1% Tween before
159 incubation in a secondary antibody, a horseradish peroxidase-conjugated anti-rabbit
160 IgG (1:2000, Cell Signaling). The chemiluminescent reagent used was ECL
161 (ThermoScientific). Detection was performed with an Amersham Imager 600 (General
162 Electric)
163

164 **LEGENDplex^{TM} multiplex cytokine analysis**

165 Cytokine analysis was performed with 25 µl of cell culture supernatants of either the
166 PHK well or the PMN well of the co-culture using the LEGENDplex^{TM} human cytokine
167 panel 2 and essential immune response panel (BioLegend) according to the
168 manufacturer’s instructions. Samples were acquired using a LSRII flow cytometry (BD
169 Biosciences) and analyzed using the LEGENDplex^{TM} Software (BioLegend). We
170 assured that the cytokines we detect in the respective wells are derived from PMNs or
171 PHKs, respectively.
172

173 **Enzyme-linked immunosorbent assay**

174 Secreted MPO and LCN2 in cell culture supernatants were analyzed using DuoSet
175 ELISA Kits from R&D in accordance with the manufacturer’s instructions. Briefly, ELISA
176 plates (Nunc) were coated with 50 µl capture antibody overnight at 4°C. The next day,
177 the plate was washed three times with PBS + 0.05% Tween before being incubated in
178 PBS + 1% BSA for 1h at RT. After three washing steps with PBS + 0.05% Tween, 50

179 µl cell culture supernatant or standards were added, and the plate was incubated for
180 2h at RT. The plate was washed three times and incubated with a biotinylated detection
181 antibody for 2h at RT. After three washing steps, the plate was incubated in HRP-
182 conjugated streptavidin for 20 min at RT in the dark. The plate was subsequently
183 washed and TMB substrate solution (Cell Signaling) was given to the samples. 2N
184 H_2SO_4 was used to stop the reaction and a Fluoroskan II (Labsystems) was used to
185 measure absorbance at 450 nm.

186

187 **RNA Isolation & cDNA generation**

188 Total RNA from PHKs was isolated with the RNA kit (Macherey-Nagel) in accordance
189 with the manufacturer's instructions. After isolating the RNS, complementary DNA
190 synthesis was performed with a reverse transcriptase kit (ThermoScientific) using 1 µg
191 of RNA, 4 µl of 5x RT buffer, 0.5 µl Maxima reverse transcriptase (200 U/ml), 1 µl of
192 random hexamer primer (100 µM), dNTP (1mM) and RNAse-free water in 20 µl volume
193 total. Following an incubation step of RNA in RNase-free water at 70°C for 10 min the
194 master mix was given to the samples and cDNA synthesis was performed for 10 min
195 at 25°C and 45 min at 50°C and a final step for 5 min at 85°C for heat-inactivation.

196

197 **RT^2 Profiler^{TM} PCR Array**

198 RT^2 Profiler^{TM} PCR Array Antibacterial Response (PAHS-148Z) was used for the
199 analysis of genes involved in inflammation and immune responses in PHKs after 18h
200 co-incubation with PMNs. Non-co-cultured PHKs served as control. The assay and
201 subsequent data analysis was performed in accordance with the manufacturer's
202 instructions. Samples were analyzed in triplicates.

203 **Bacterial strains**

204 *Staphylococcus aureus* USA300 LAC and *Staphylococcus epidermidis* 1457 was used
205 in this study. Bacteria were grown aerobically in tryptic soy broth (TSB) at 37 °C with
206 orbital shaking. Logarithmically grown bacteria (OD = 0.5) were used for the
207 experiments.

208

209 **Neutrophil Recruitment Assay**

210 For the PMN recruitment assay, 1 µM Calcein (eBioscience) was used for labeling
211 PMNs for 30 min, washed and seeded into a transwell insert with 3 µm pores

(Sarstedt). The transwell insert was then placed above a well containing different stimuli and incubated for 1 hour. The migrated PMNs in the lower well were then lysed in 1% Triton X-100. The fluorescence of the lysates was quantified in triplicates using a Fluoroskan II (Labsystems). As stimuli, the supernatants of the PHK well (upper well) or the PMN well (lower well) of the 18h co-culture with or without *S. aureus* infection was used. As controls, the supernatants of non-co-cultured PHKs with and without *S. aureus* infection and non-co-cultured PMNs was included. As positive control, N-formyl-met-leu-phe was used (fMLF). Prior to the migration assay, the supernatants were centrifuged, and filter sterilized. A standard curve was included to calculate the absolute number of migrated PMNs.

Statistical Analysis

GraphPad Prism 9.0 (GraphPad Software, Inc.) was used for analysis of significant differences between the means of the different treatments. Statistical analyses were performed using either unpaired two-tailed Student’s t-test, one-way ANOVA followed by Dunnett’s multiple comparison test, or two-way ANOVA followed by Šídák's multiple comparison tests, as indicated in figure legends. P-value <0.05 was used to determine statistical significance. Data visualization was performed with GraphPad 9.0 (GraphPad Software Inc.), FlowJo (TreeStar), MS Excel (Microsoft Corporation), or Fiji/ImageJ.

234 **Results**

235 **Co-Incubation with PHKs prolongs the lifespan of PMNs**

236 First, we were interested whether the viability of PMNs changes when we co-cultured
237 with PHKs. Therefore, we analyzed the viability of PMNs by SYTOX Green staining, a
238 non-permeable dye indicating dead cells, at different time points after co-culture with
239 PHKs. We used an established *in vitro* co-culture transwell chamber model (Bitschar
240 et al., 2020) and compared it to the results of the non-co-cultured PMNs (Fig.1A). We
241 found that the percentage of SYTOX Green positive cells steadily increases overtime
242 in the non-co-cultured PMNs. In contrast to this, co-cultured PMNs exhibit a
243 significantly extended lifespan, with only little increase in Sytox Green positive cells in
244 the first 18h. However, the induction of cell death was not completely prevented in co-
245 cultured PMNs as we see a clear increase in SYTOX Green positive cells at 30h co-
246 incubation time. Therefore, we conclude that the co-culture with PHKs significantly
247 delays the induction of cell death in PMNs. We calculated the delay of cell death
248 induction between the co-cultured and non-co-cultured PMNs using interpolation and
249 found a delay of 9.3h until 50% of the cells are dead (S.Fig.1A). To unravel the type of
250 cell death, we analyzed the induction of apoptosis in co-cultured and non-co-cultured
251 PMNs by caspase-3 cleavage and Annexin-V staining (Fig.1B&C). After 18h, apoptosis
252 induction of non-co-cultured PMNs was indicated by cleaved caspase-3 which was not
253 observed in co-cultured PMNs (Fig.1B). Furthermore, we used Annexin-V staining,
254 which detects cells in the early or late apoptosis phase before the cells lose their
255 membrane integrity and get Sytox positive. Interestingly, Annexin-V staining after
256 different incubation times revealed that apoptosis induction was significantly delayed
257 in co-cultured PMNs compared to non-co-cultured PMNs (Fig.1C) confirming our Sytox
258 experiments. Together, these data indicate that in the co-culture a beneficial interaction
259 between PHKs and PMNs significantly extend the viability of PMNs by delaying
260 apoptosis induction.

261

262 **The prolonged lifespan of PMNs in the co-culture with PHKs is mediated by**
263 **secreted IL-8**

264 We hypothesized that the delayed apoptosis induction in PMNs is mediated by soluble
265 factors released by the PMNs itself during the co-incubation with PHKs. To test this
266 hypothesis, we co-cultured PMNs with PHKs for 18h and subsequently collected the

267 supernatant of the PMN well, filter-sterilized it and used it for stimulation of freshly
268 isolated PMNs. Non-co-cultured PMNs incubated in medium were used as control. We
269 investigated cell death of PMNs over time by Sytox Green staining and live cell imaging
270 (Fig.2A). While the percentage of Sytox-Green-positive cells increased significantly
271 after 5 hours in control PMNs cultured in medium, the induction of cell death was
272 delayed in PMNs incubated with PMN supernatant. Here, a significant increase in
273 Sytox-Green-positive cells was observed after 11 hours, which then steadily increased
274 over time. We calculated the delay in cell death induction between PMNs incubated in
275 medium or PMN supernatant by interpolation and found a delay of 8.3 hours until 50%
276 of the cells were dead (S.Fig.1B). This delay (8.3 hours) is comparable to the delay
277 found in co-cultured vs non-co-cultured PMNs (9.3 hours), observed in Figure 1A which
278 indicates that the soluble factors released by PMNs during the co-culture are mainly
279 accountable for the extended lifespan. To identify these factors, we performed
280 LEGENDplex analysis of the PMN well after 3h, 6h and 18h co-culture with PHKs and
281 compared these to secreted factors from non-co-cultured PMNs. We detected
282 significantly increased amounts of secreted Interleukin (IL)-8 and IL-1α in PMNs co-
283 cultured with PHKs compared to non-co-cultured PMNs after 18h (Fig.2B). Other
284 examined cytokines were not significantly induced (S.Fig.2A). To test whether the IL-
285 1α and IL-8 are responsible for the enhanced viability of PMNs co-cultured with PHKs,
286 we co-cultured PMNs with PHKs for 18h in the presence of absence of either an anti-
287 IL-8, anti-Il-1α or both antibodies together and analyzed cell viability by Sytox Green
288 staining. We compared the results to the non-cocultured PMNs. Interestingly, addition
289 of the anti-IL-8 antibody led to a significant reversal of the life-prolonging effect of the
290 co-culture, whereas IL-α had no significant life-prolonging effect (Fig.2C-D&S.Fig.2B-
291 C). Interestingly, the combined treatment with an anti-IL8 and anti-IL-1α antibody still
292 reverted the life-prolonging effect of the co-culture indicating that the effect of IL-8
293 dominates compared to IL-1α in the life prolongation of PMNs in the co-culture
294 (Fig.2E&S.Fig.2D). Interestingly, recombinant IL-8 delayed apoptosis in freshly
295 isolated PMNs in a concentration-dependent manner, further highlighting the anti-
296 apoptotic properties of IL-8 (S.Fig.2E).

297

298

299

300 **Co-Culture with PHKs activates PMNs**

301 We next analyzed whether along with extending their lifespan, the co-culture with
302 PHKs activates PMNs. Activated PMNs are characterized by degranulation [509]. Indeed,
303 we observed a decrease in the side scatter (SSC) of co-cultured
304 PMNs after 18h compared to non-co-cultured PMNs or freshly isolated PMNs (0h),
305 indicative for reduced granularity and thus degranulation (Fig.3A). We further analyzed
306 degranulation by analyzing surface expression of CD11b, CD66b and CD63 and
307 extracellular levels of MPO and LCN2, all markers for degranulation. We observed that
308 PMNs co-cultured with PHKs for 18h released significantly more MPO and LCN2
309 compared to non-co-cultured PMNs or freshly isolated PMNs (Fig.3B). Furthermore,
310 while we did not detect a significant difference in the surface expression of CD11b or
311 CD66b, surface expression of CD63 was significantly increased in co-cultured PMNs
312 compared to non-co-cultured or freshly isolated PMNs (S.Fig.3). Together, these data
313 indicate that PMNs are in an activated state after co-culture with PHKs.

314 We next hypothesized that already activated state leads to an enhanced activation of
315 PMNs in response to an infectious stimulus. To investigate this, we infected freshly
316 isolated PMNs or PMNs non-co-cultured or co-cultured with PHKs for 18h with *S.*
317 *aureus* (MOI = 10) and analyzed PMN activation by the analysis of extracellular levels
318 of MPO and LCN2 as markers for degranulation. Interestingly, while *S. aureus* infection
319 resulted in increased levels of secreted MPO and LCN2 in all conditions, the increase
320 in MPO and LCN2 levels was especially prominent in co-cultured PMNs (Fig. 3C).
321 Together, these data indicate that the co-culture with PHKs primes PMNs for enhanced
322 activation in response to an infectious stimulus.

323

324 **Co-incubation with PMNs activates an inflammatory response in PHKs**

325 Our results show that the interaction between PHKs and PMNs boosts the activity of
326 PMNs. Next, we analyzed whether the interaction with PMNs affects, on the other
327 hand, also the PHKs and induces a proinflammatory response in PHKs. For this, we
328 co-cultured PHKs with PMNs for 18h and subsequently analyzed in PHKs the
329 expression of 84 different genes involved in inflammation using an RT-Profiler PCR
330 Array and compared gene expression to 18h non-co-cultured PHKs. Interestingly, we
331 detected significant upregulation of genes associated with immune cell recruitment
332 (CXCL1, CXCL2, IL-8, CCL5), TLR signaling (CD14, LY96), regulation of apoptosis

333 (FADD, CARD6, BIRC3), NFκB signaling (CHUK, NFKB1a, IL-1β) as well as stress
334 response (MAP2K3) in co-cultured PHKs compared to the non-co-cultured PHKs
335 (Fig.4A). We further analyzed the induction of a proinflammatory response in PHKs by
336 comparing the secretion of cytokines and chemokines by PHKs co-cultured with PMNs
337 to the non-co-cultured PHKs at different time points using Legendplex analysis. With
338 increasing incubation time, we detected in co-cultured PHKs elevated levels of
339 secreted IL-1α, IL-1β, IL-8, IL-6, GM-CSF, and CXCL-10 compared to the non-co-
340 cultured PHKs (Fig.4B). Other analyzed cytokines and chemokines were not
341 significantly induced (S.Fig.4). Our results indicate that co-culture with PMNs induce
342 induces a proinflammatory state in PHKs.
343

344 **Crosstalk between PHKs and PMNs exacerbates immune responses to *S. aureus***
345 **infection**

346 Next, we analyzed whether the proinflammatory state in PHKs after co-culture with
347 PMNs under sterile conditions results in an exacerbated response of PHKs after *S.*
348 *aureus* infection. To explore this, we co-cultured PHKs with PMNs for 18h followed by
349 infection of the PHKs with *S. aureus*. Subsequently, we examined the release of
350 proinflammatory cytokines by PHKs using Legendplex analysis. Interestingly, our
351 results revealed that *S. aureus* infection of the PHKs further enhanced the secretion of
352 CXCL10, IL-1β and IL-8 by PHKs in the co-culture. Additionally, *S. aureus* infection
353 induced the secretion of MCP-1, and IL-33 by PHKs, and notably, this induction was
354 significantly higher in co-cultured PHKs compared to non-co-cultured PHKs (Fig.5A).
355 The induction of other analyzed cytokine and chemokines were not significantly
356 different between co-cultured and non-co-cultured PHKs (S.Fig.5A). Furthermore, *S.*
357 *aureus* infection of co-cultured PHKs significantly enhances the secreted levels of IL-
358 1β, IL-8 and IL-1α in the co-cultured PMNs (Fig. 5B). Interestingly, the supernatant of
359 co-cultured PHKs and to a lesser extent of non-co-cultured PHKs after *S. aureus*
360 infection significantly enhanced migration of PMNs (Figure 5C). These findings
361 demonstrate that the co-culture with PMNs significantly enhances the *S. aureus*-
362 induced inflammatory response in PHKs and PMNs.
363

364 **The skin microbiome reduces PMN-mediated inflammation and induces**
365 **apoptosis in PMNs**

366 Previous results of our group showed that the skin microbiome has a beneficial role in
367 preventing *S. aureus* colonization of the skin in a non-inflammatory milieu (Burian et
368 al., 2017). Interestingly, the decreased *S. aureus* colonization was accompanied by
369 reduced skin inflammation and PMN recruitment (Bitschar et al., 2020). Our results
370 also showed that in an inflammatory environment induced by tape-stripping, skin
371 infiltrating PMNs enhance *S. aureus* skin colonization (Bitschar et al., 2020). Based on
372 these results, we hypothesized that the skin microbiome affects PMN-mediated skin
373 inflammation. To test this, we analyzed inflammatory responses in PHKs co-cultured
374 with PMNs without or upon infection with the skin commensal *S. epidermidis*.
375 Interestingly, we observed a significant decrease in the secreted levels of IL-1β and
376 CXCL-10 by co-cultured PHKs upon *S. epidermidis* infection compared to the
377 cocultured non-infected PHKs (Fig.6A). No significant difference between infected and
378 not infected co-cultured PHKs were observed on other analyzed cytokines and
379 chemokines (S.Fig.6A). This indicates that the skin microbiome reduces the
380 inflammatory responses in PHKs induced by PMNs. Furthermore, we found that *S.*
381 *epidermidis* infection of PHKs significantly reduces the secreted levels of IL-8 and IL-
382 1α by PMNs in the co-culture (Fig.6B) and significantly enhanced apoptosis induction
383 in PMNs compared to the non-infected and co-cultured PMNs (Fig. 6C). These results
384 indicate that the skin microbiome reduces PMN-mediated skin inflammation by
385 downregulation of PMN-induced proinflammatory cytokines in PHKs and induction of
386 apoptosis in PMNs.
387

388 **Discussion**

389 PMNs are the most common type of leucocytes in the human blood and are important
390 for the innate immune system (Kobayashi and DeLeo, 2009). Upon inflammation,
391 PMNs are rapidly recruited from the circulation to infection sites where they ensure
392 effective initial protection. Several groups have shown that PMNs play a crucial role in
393 clearing *S. aureus* skin infections (Miller et al., 2006, Molne et al., 2000). Here we
394 demonstrate that the interaction between infiltrating PMNs and skin-resident PHKs
395 enhances the proinflammatory responses of both cells upon *S. aureus* infection.
396 PMNs have a relatively short lifespan. They are continually replenished from the bone
397 marrow and released into the bloodstream. In circulation, they actively patrol for
398 infection of tissue and in the absence of an inflammatory stimulus, PMNs undergo

spontaneous apoptosis and are cleared by macrophages, maintaining immune balance (Hidalgo et al., 2019). The lifespan of circulating murine PMNs is estimated to be about 12h (Pillay et al., 2010), however, this can be significantly extended upon inflammation (Kolaczkowska and Kubes, 2013). During inflammation, PMNs are rapidly recruited to the affected site. Inflammatory signals promote their survival and activation, enabling them to combat invading pathogens by releasing antimicrobial agents and engaging in phagocytosis (Fox et al., 2010). However, excessive PMN activation can cause tissue damage, so mechanisms exist to regulate inflammation resolution including the induction of PMN apoptosis and their subsequent clearance by macrophages for immune homeostasis (Fox et al., 2010).

In their investigation of murine PMN lifespan in various tissues (bone marrow, blood, liver, lung, spleen, intestine, and skin) Ballesteros et al. discovered that the half-life of neutrophils varies depending on the specific tissue they infiltrate into (Ballesteros et al., 2020). Notably, this study reveals that PMNs quickly adopt a tissue-specific phenotype and transcriptional profile, likely contributing to efficient immune responses against invading pathogens. Interestingly, they showed that the half-life of PMNs was highest in the skin with about 18h.

While several studies have examined the lifespan of PMNs in various tissues of mice, there is limited knowledge regarding the lifespan of PMNs in the human system, especially in the skin. Here, we demonstrate for the first time that the presence of PHKs during co-incubation significantly prolongs the lifespan of PMNs in a human *in vitro* co-culture model. Compared to PMNs cultured alone, co-cultured PMNs exhibit a noticeable delay in apoptosis induction. This extended lifespan is facilitated by the secretion of IL-8, which increases progressively as the incubation time is prolonged. We observed a reversal of this effect when an anti-IL-8 antibody was added to the co-culture. The ability of IL-8 to delay spontaneous apoptosis induction of PMNs is also described by previous studies (Kettritz et al., 1998). Interestingly, we did find that depletion of IL-1α, which is also induced in the co-culture, further prevents cell death induction thus indicating that IL-1α is capable of inducing cell death in PMNs. The proapoptotic functions of IL-1α have been described for other cells (Gou et al., 2016), however, to the best of our knowledge not in PMNs.

Nevertheless, the co-culture with PHKs and IL-8 stimulation did not entirely inhibit PMN apoptosis; instead, it caused a delay in the process, eventually leading to its induction within the co-culture system. Our findings revealed that co-cultured PMNs are

433 activated and released their granules after 18h incubation time. Excessive activation
434 of PMNs can cause tissue damage, so mechanisms exist to regulate inflammation
435 resolution including PMN apoptosis and their subsequent clearance by macrophages
436 for homeostasis (Fox et al., 2010). Therefore, we hypothesize that the eventual
437 induction of apoptosis in PMNs is required to minimize tissue damage and restore
438 homeostasis.

439 Interestingly, we found that co-cultured PMNs displayed an elevated activation and
440 responsiveness towards an infectious stimulus in comparison to non-co-cultured or
441 freshly isolated PMNs. This was demonstrated by increased degranulation upon *S.*
442 *aureus* infection by co-cultured PMNs compared to non-co-cultured PMNs or freshly
443 isolated PMNs. PMNs play a pivotal role in the immune response against *S. aureus*
444 skin infections (Cho et al., 2012, Miller et al., 2006). The reactivity of circulating PMNs
445 towards inflammatory stimuli is intentionally constrained to avoid tissue damage and
446 uphold homeostasis (Miralda et al., 2017). However, this reactivity can be significantly
447 enhanced by the exposure to inflammatory stimuli such as cytokines, chemokines,
448 pathogen- or damage-associated molecular patterns (PAMPs or DAMPs,
449 respectively). The exposure to such stimuli primes the PMNs for enhanced
450 responsiveness towards invading pathogens (Elbim et al., 1994, El-Benna et al., 2008,
451 Malachowa et al., 2013, Miralda et al., 2017). Some studies showed that PMNs primed
452 by proinflammatory cytokines derived from PBMCs exert enhanced killing capacities
453 against *S. aureus* (Ferrante et al., 1993, Bates et al, 1991). Therefore, we hypothesize
454 that the crosstalk with PHKs contributes to shaping the immune response against *S.*
455 *aureus* skin infections by priming PMNs for enhanced activation. The co-culture
456 environment seems to provide a beneficial influence on the PMNs, enhancing their
457 ability to combat *S. aureus* skin infections. This finding highlights the significance of
458 cellular interactions and their impact on the immune system’s effectiveness in mounting
459 a robust response against infectious agents.

460 Moreover, we found that the extended co-incubation not only has a priming effect on
461 PMNs but also induces a proinflammatory state in PHKs. This was characterized by
462 increased secretion of IL-1α, IL-1β, IL-6, IL-8, GM-CSF and CXCL10 by PHKs.
463 CXCL10 and GM-CSF are chemoattractants for other immune cells besides PMNs,
464 such as monocytes, macrophages, T cells, NK cells or dendritic cells (Booth et al.,
465 2002, Kajah et al., 2011). This suggests that the extended crosstalk between PHKs
466 and PMNs initiates the recruitment of other immune cells to the skin to facilitate the

467 advance of the immune response. The induction of these proinflammatory mediators
468 is independent on direct cell-to-cell contact but is rather mediated by secreted factors.
469 This finding is supported by previous studies describing the induction of inflammation
470 in PHKs by PMNs (Liu et al., 2022, Shao et al., 2019). For example, Lieu et al showed
471 that indirect co-culture with PMNs leads to the induction of several pro-inflammatory
472 genes and secretion of IL-8 in HaCaT cells (Liu et al., 2022). Moreover, Shao et al
473 showed that exosomes derived from PMNs are able to induce pro-inflammatory gene
474 expression in PHKs (Shao et al., 2019). Interestingly, although the expression of
475 neutrophilic chemoattractants such as CXCL-1/2 and IL-8 was highly elevated in PHKs
476 after 18h co-culture with PMNs, the supernatant containing these chemoattractants
477 was not able to induce PMN migration. This might be that the concentrations of the
478 respective cytokines were too low for PMN migration. Another reason might be that
479 some factor stops PMN migration as excessive PMN migration and activation can lead
480 to tissue damage.

481 As PHKs are the main constituents of the epidermis, the uppermost layer of our skin,
482 they are constantly exposed to exogenous bacteria such as beneficial skin
483 commensals like *S. epidermidis* or pathogenic bacteria like *S. aureus* and are therefore
484 crucial in the initiation of skin inflammation (Bitschar et al., 2017). Here, we found that
485 a previous co-culture with PMNs significantly elevated the level of proinflammatory
486 cytokines and chemokines by PHKs induced by *S. aureus* infection. *S. aureus* is a
487 facultative pathogen which is abundant on AD skin, exacerbating skin inflammation.
488 There is evidence that PMNs are elevated in AD skin and contribute to its pathogenesis
489 (Choy et al., 2012, Dhingra et al., 2013, Amarbayasgalan et al., 2013). Furthermore,
490 previous results of our group showed that the PMNs can enhance *S. aureus* skin
491 colonization (Bitschar et al., 2020, Focken et al, 2023). As a mechanism for this we
492 show that PMNs infiltrating inflamed skin are primed by PHKs for NET formation in
493 response to *S. aureus* infection. The increased presence of PMNs and NETs in the
494 inflamed skin induces oxidative stress in PHKs which results in the secretion of
495 HMGB1, which induces further oxidative stress in PHKs and NET formation in PMNs.
496 Moreover, both NETs and HMGB1 induce the downregulation of epidermal barrier
497 genes in PHKs, thus inducing a skin barrier defect which favors *S. aureus* skin
498 colonization (Focken et al., 2023). Here, we observed that in addition to promoting skin
499 barrier defects which could enhance *S. aureus* skin colonization, the co-culture with

500 PMNs exacerbates *S. aureus* induced skin inflammation. We believe that the PMNs
501 secrete

502 IL-8 during the co-culture, thereby prolonging their lifespan and becoming activated.
503 The activated PMNs then induce an inflammatory state in PHKs and boost their
504 inflammatory response against *S. aureus*.

505 The eventual induction of PMN apoptosis in the co-culture might be necessary to not
506 further prolong inflammation. Interestingly, we found that *S. epidermidis* infection of
507 PHKs in the co-culture reduced the proinflammatory responses in PHKs mediated by
508 the co-culture with PMNs. Furthermore, we found that *S. epidermidis* infection of PHKs
509 induced apoptosis in PMNs in the co-culture. Previous work of our group showed that
510 the skin microbiome has a protective role against *S. aureus* skin infections for example
511 by reducing *S. aureus*-mediated skin inflammation and PMN recruitment (Bitschar et
512 al., 2020, Burian et al., 2017). Our results here further show that the skin commensal
513 bacteria could play a role in preventing PMN-mediated excessive skin inflammation.

514 In conclusion, here we show for the first time that the interaction between PHKs and
515 PMNs in the skin shaped the immune responses against *S. aureus* infections (see
516 graphical abstract). On the one hand, the crosstalk delays the induction of apoptosis
517 in PMNs, prolonging their lifespan and enhancing their activation and responsiveness
518 against *S. aureus*. This effect is achieved through the release of IL-8 rather than direct
519 cell-to-cell contact. However, it is important to note that prolonged incubation with
520 PMNs also lead to inflammatory responses in keratinocytes. This inflammatory

521 response is further exacerbated in the presence of *S. aureus*. Interestingly, the skin
522 commensal *S. epidermidis* reduces the PMN-mediated skin inflammation in PHKs and
523 induces apoptosis in activated PMNs which indicates a beneficial role of the skin
524 microbiome in preventing excessive inflammation.

525

526 **Figure Legends**

527 **Figure 1: Co-incubation with PHKs prolongs the lifespan of PMNs**

528 **A:** PMNs (2x10^6/ml) were either incubated alone or in co-culture with differentiated
529 PHKs seeded in a transwell insert (pore size 0.4 μm) and placed above the PMNs. Cell
530 viability was analyzed at different times by quantifiying the percentage of Sytox Green
531 positive cells (n = 4). Representative pictures of one experiment after 6h, 18h and 30
532 are shown. Scale bars = 200 μm. Significant differences between non-co-cultured and
533 co-cultured PMNs at the different time point were analyzed by two-way ANOVA *P <
534 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Shown are the mean of four different
535 experiments + SEM. **B:** Induction of apoptosis in non-co-cultured and co-cultured
536 PMNs was analyzed at 3h, 6h and 18h incubation time by investigating caspase-3
537 cleavage via western blot. Protein expression of β-actin was used as loading control.
538 **C:** Apoptosis induction in non-co-cultured and co-cultured PMNs was analyzed at 18h
539 and 30h by Annexin-V staining. Annexin positive cells were quantified and represent
540 apoptotic cells. Shown are the mean of four different experiments + SEM. Significant
541 differences were analyzed by unpaired two-tailed t-tests *P < 0.05, **P < 0.01, ***P <
542 0.001, ****P < 0.0001. On representative experiment of three independent experiments
543 is shown. PHKs = primary human keratinocytes; PMNs = polymorphonuclear
544 neutrophils; SEM = standard error of the mean

545

546 **Figure 2: The prolonged lifespan of PMNs in the co-culture with PHKs is partly**
547 **mediated by secreted IL-8**

548 **A:** Freshly isolated PMNs (2x10^6/ml) were incubated either in medium or in the PMN
549 supernatant of the 18h co-culture. Cell viability was analyzed over time by Sytox Green
550 staining. The percentage of Sytox Green positive cells, indicating dead cells, was
551 quantified every hour for 18h using Fiji/ImageJ (n = 3). Representative pictures after
552 5h, 11h and 18h incubation are shown. Scale bars = 200 μm. **B:** Freshly isolated PMNs
553 were co-cultured with differentiated PHKs or alone. After 3h, 6h and 18h, secreted
554 factors in the PMN well were analyzed using Legendplex analysis. Significant
555 differences between the non-co-cultured and co-cultured PMNs were analyzed for
556 each time point by multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P
557 < 0.0001. Shown is one representative experiment of four independent experiments +
558 SD. **C-E:** PMNs were either incubated alone or in co-culture with differentiated PHKs

559 in the presence or absence of an anti-IL-8 antibody (**C**) or anti-IL-1α antibody (**D**) or
560 both (**E**). Induction of cell death was analyzed after 18h by quantifiying Sytox Green
561 positive cells using Fiji/Image J (n = 4). Shown is one representative experiment of four
562 independent experiments + SD. Significant differences were analyzed between the
563 percentage of SYTOX Green positive cells by one-way ANOVA *P < 0.05, **P < 0.01,
564 ***P < 0.001, ****P < 0.0001. PMNs = polymorphonuclear neutrophils; PHKs = primary
565 human keratinocytes; IL-1α = interleukin 1α; IL-8 = interleukin 8; SD = standard
566 deviation
567

568 **Figure 3: Co-Culture with PHKs activates PMNs**

569 **A:** Forward Scatter (FSC) and Side Scatter (SSC) of freshly isolated PMNs and PMNs
570 co-cultured with differentiated PHKs or alone for 18h were analyzed by flow cytometry.
571 **B:** Secreted levels of MPO and LCN-2 by co-cultured and non-co-cultured PMNs after
572 18h incubation was analyzed by ELISA and compared to freshly isolated PMNs (0h).
573 Shown is one representative experiment of three independent experiments + SD.
574 Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P
575 < 0.001, ****P < 0.0001. One representative experiment of three independent
576 experiments is shown. **C:** Freshly isolated (0h), 18h co-cultured and 18h non-co-
577 cultured PMNs were infected directly with *S. aureus* (MOI = 10) or left uninfected. After
578 2h, secreted MPO and LCN2 levels were analyzed by ELISA. Significant differences
579 between uninfected and infected PMNs were analyzed by unpaired two-tailed t-tests
580 *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Shown is one representative
581 experiment of three independent experiments + SD. PMNs = polymorphonuclear
582 neutrophils; PHKs = primary human keratinocytes; MOI = multiplicity of infection; MPO
583 = myeloperoxidase; LNC2 = lipocalin-2
584

585 **Figure 4: Extended co-incubation activates a proinflammatory state in PHKs**

586 **A:** Differentiated PHKs were co-incubated with freshly isolated PMNs. After 18h, the
587 expression of 84 different genes was analyzed in PHKs by a RT-Profiler Array (n = 3).
588 Significantly upregulated genes in co-cultured PHKs compared to non-co-cultured
589 PHKs are marked green. **B:** Secreted factors by PHKs were analyzed after 3h, 6h and
590 18h of co-incubation with PMNs. Ctrl. = non-co-cultured PHKs. Shown is one
591 representative experiment of four independent experiments + SD. Significant

592 differences to the control were analyzed by one-way ANOVA *P < 0.05, **P < 0.01,
593 ***P < 0.001, ****P < 0.0001. One representative experiment of three independent
594 experiments is shown. PHKs = primary human keratinocytes; PMNs =
595 polymorphonuclear neutrophils; RT = real-time

596

597 **Figure 5: Crosstalk between PHKs and PMNs exacerbates immune responses**
598 **to *S. aureus* infection**

599 **A&B:** Differentiated PHKs were co-incubated with freshly isolated PMNs or alone. After
600 18h, PHKs were infected with *S. aureus* (MOI = 30) for 1.5h or left uninfected. Secreted
601 factors in the PHK well (**A**) and PMN well (**B**) were analyzed by Legendplex analysis.
602 Shown is one representative experiment of four independent experiments + SD.
603 Significant differences between the samples were analyzed by one-way ANOVA *P <
604 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One representative experiment of three
605 independent experiments is shown. **C:** Freshly isolated PMNs were seeded into
606 transwell inserts with 3 µm pores and placed above a well containing supernatants of
607 either non-co-cultured or co-cultured PHKs after *S. aureus* infection. After 1h, the
608 number of migrated PMNs were quantified. Shown is one representative experiment
609 of four independent experiments + SD. Significant differences between the samples
610 were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
611 One representative experiment of three independent experiments is shown. PHKs =
612 primary human keratinocytes; PMNs = polymorphonuclear neutrophils; MOI =
613 multiplicity of infection; SD = standard deviation

614

615 **Figure 6: The skin microbiome reduces PMN-mediated inflammation and**
616 **induces apoptosis in PMNs**

617 **A&B:** Differentiated PHKs were co-incubated with freshly isolated PMNs or alone. After
618 18h, PHKs were infected with *S. epidermidis* (MOI = 30) for 1.5h or left uninfected.
619 Secreted factors in the PHK well (**A**) and the PMN well (**B**) were analyzed by
620 Legendplex analysis. Shown is one representative experiment of four independent
621 experiments + SD. Significant differences between the samples were analyzed by
622 one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One
623 representative experiment of three independent experiments is shown. **C:** Freshly
624 isolated PMNs were incubated alone or in co-culture with PHKs. After 18h, PHKs were

625 infected with *S. epidermidis* or left uninfected (MOI = 30) for 1.5h. Apoptosis induction
626 in PMNs was analyzed by Annexin-V staining. Shown is one representative experiment
627 of four independent experiments + SD. Significant differences between the samples
628 were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

629
630 **References**
631

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Experiments were designed and the manuscript was written by J.F. and B.S. The experiments were performed and analyzed by J.F.

Funding & Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft with the funding grant number TR156 and the Cluster of Excellence EXC2124 ‘Controlling Microbes to Fight Infections’.

Data Availability Statement

The corresponding author will share all data reported in this paper upon request.

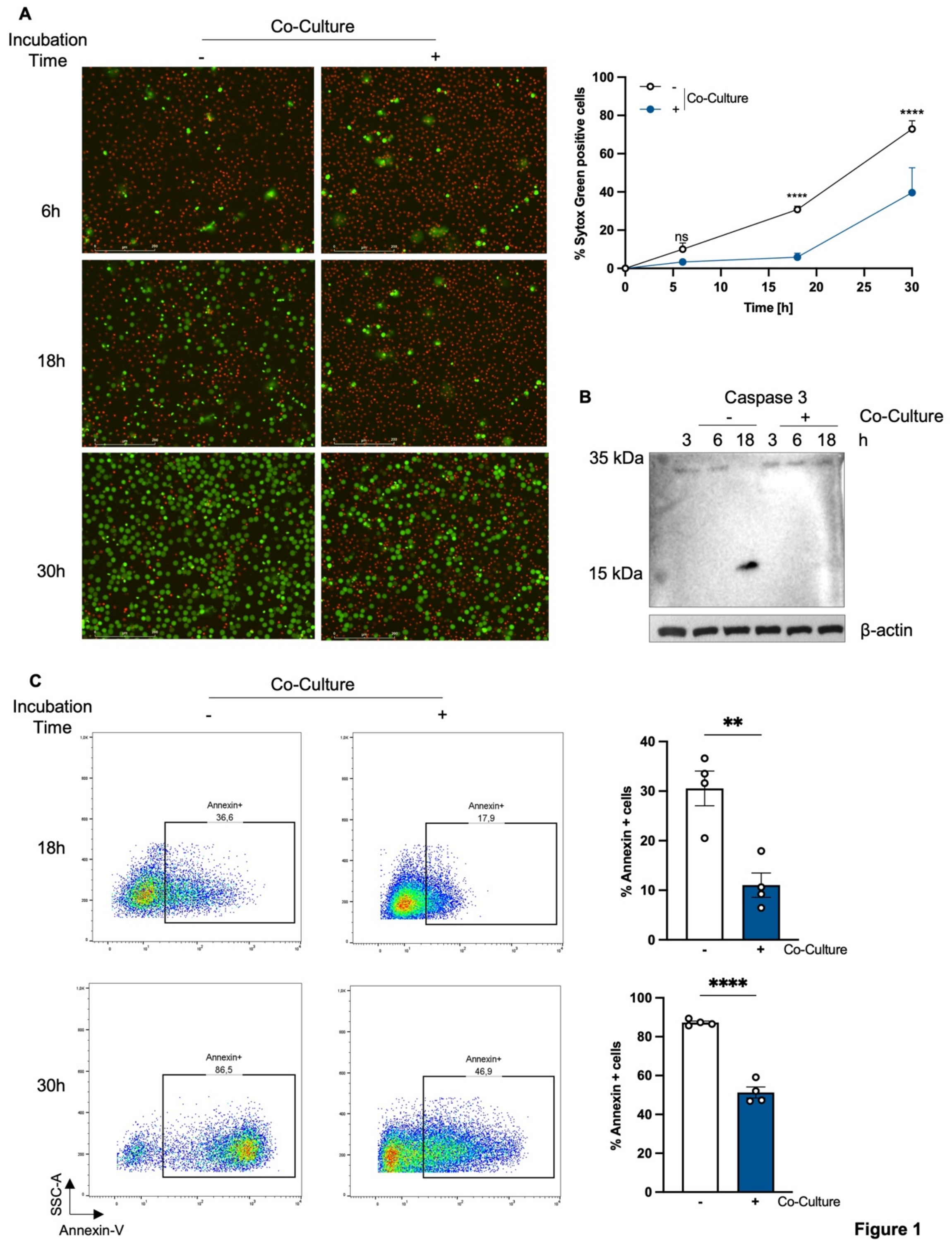

Figure 1

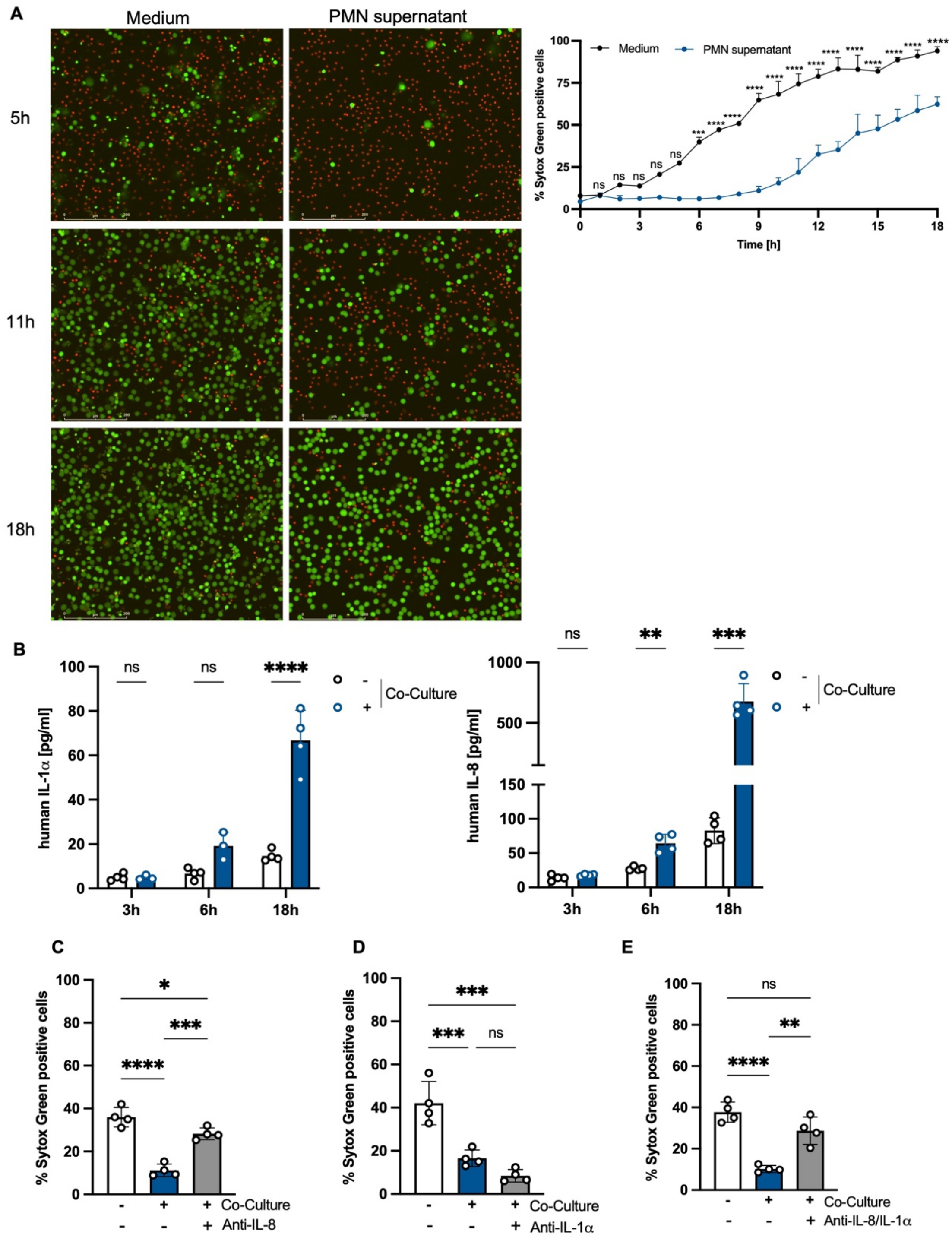

Figure 2

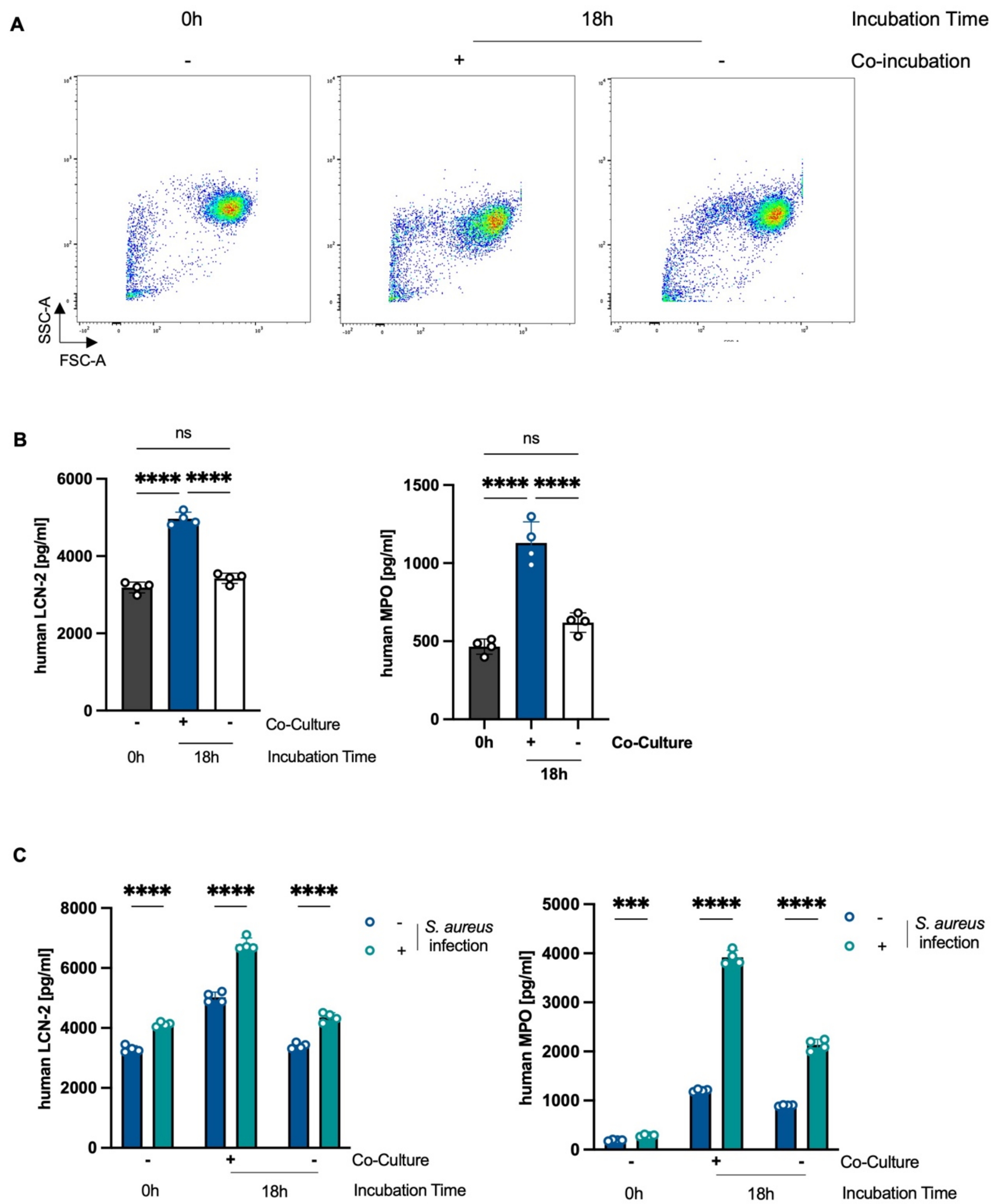

Figure 3

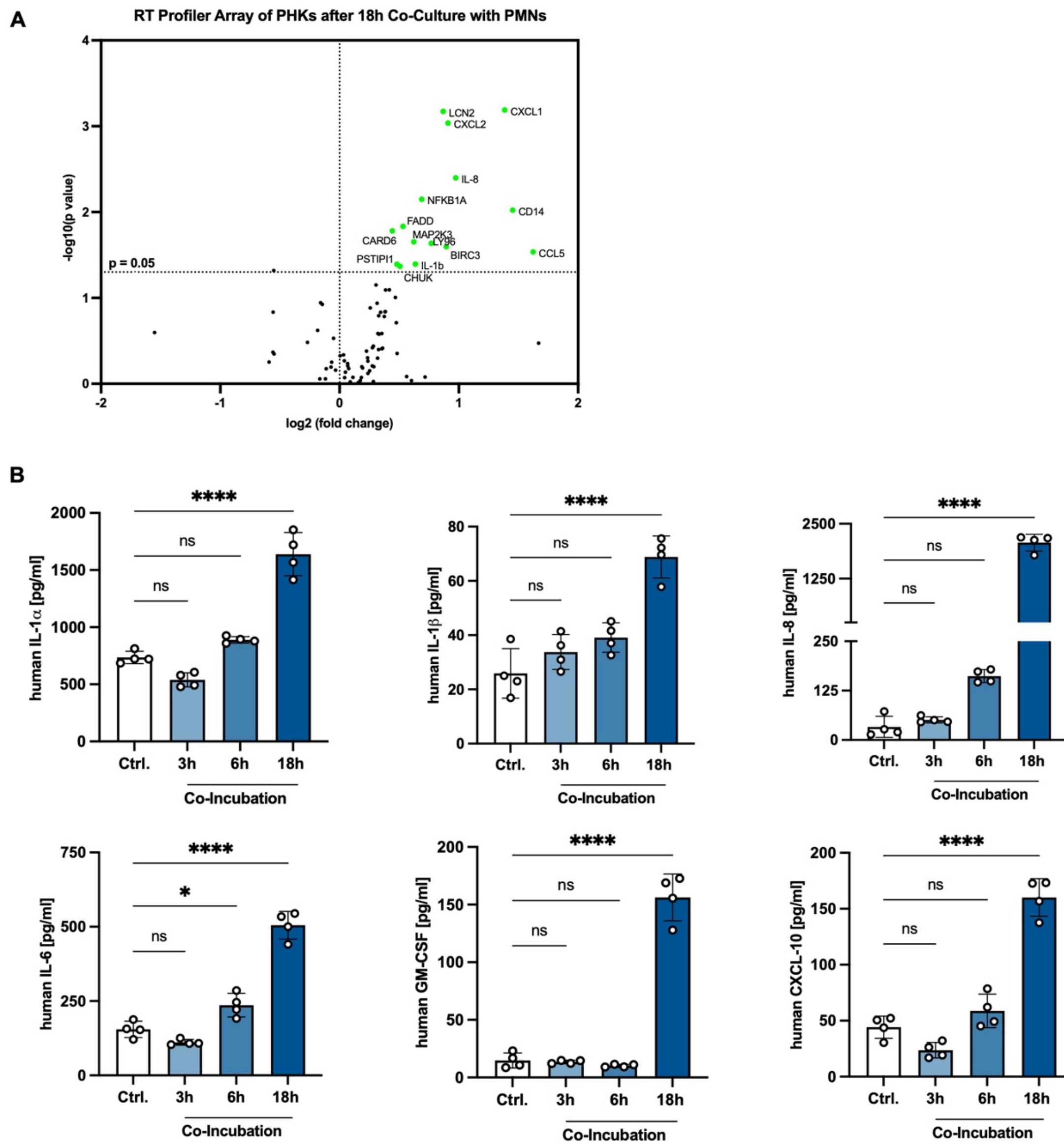

Figure 4

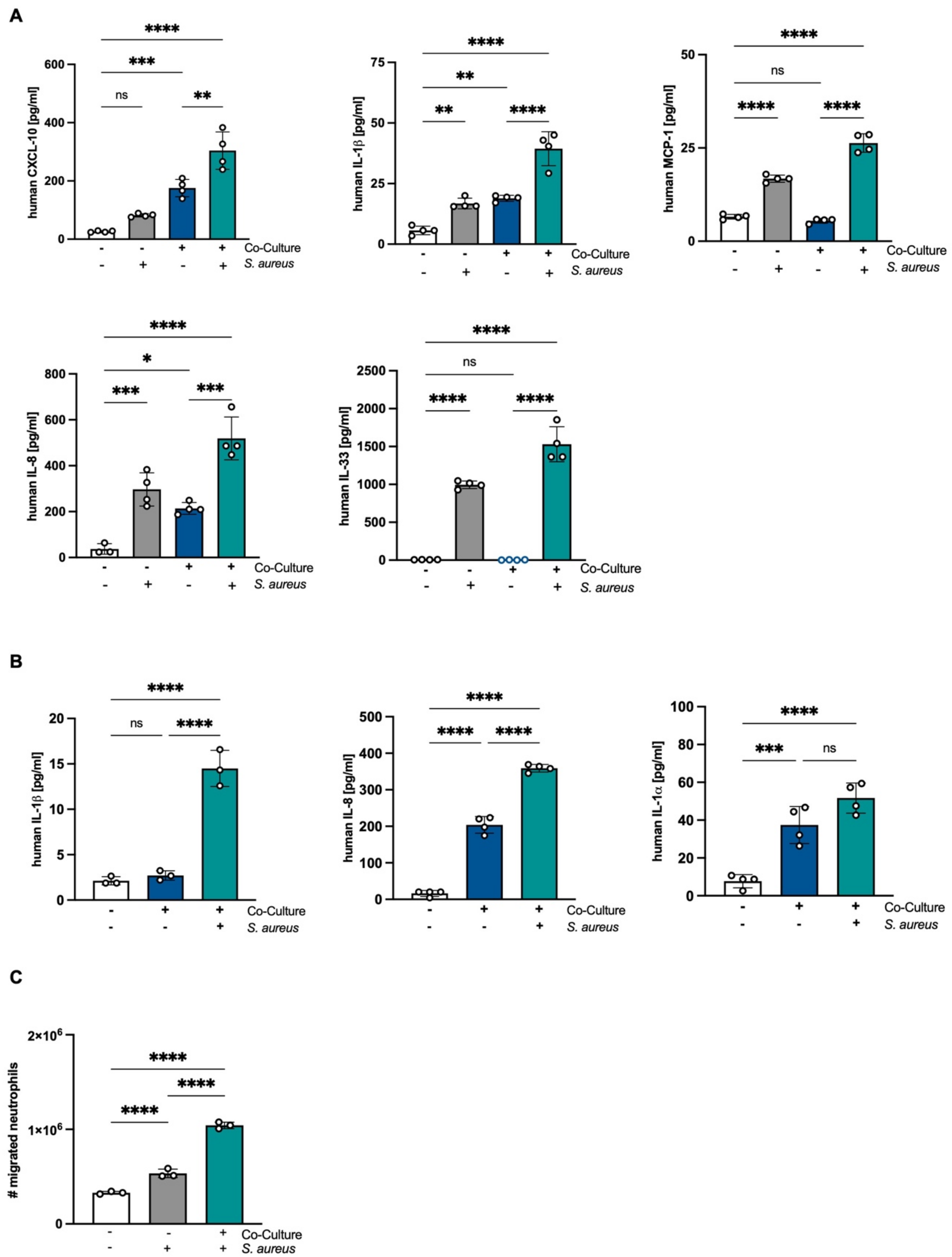

Figure 5

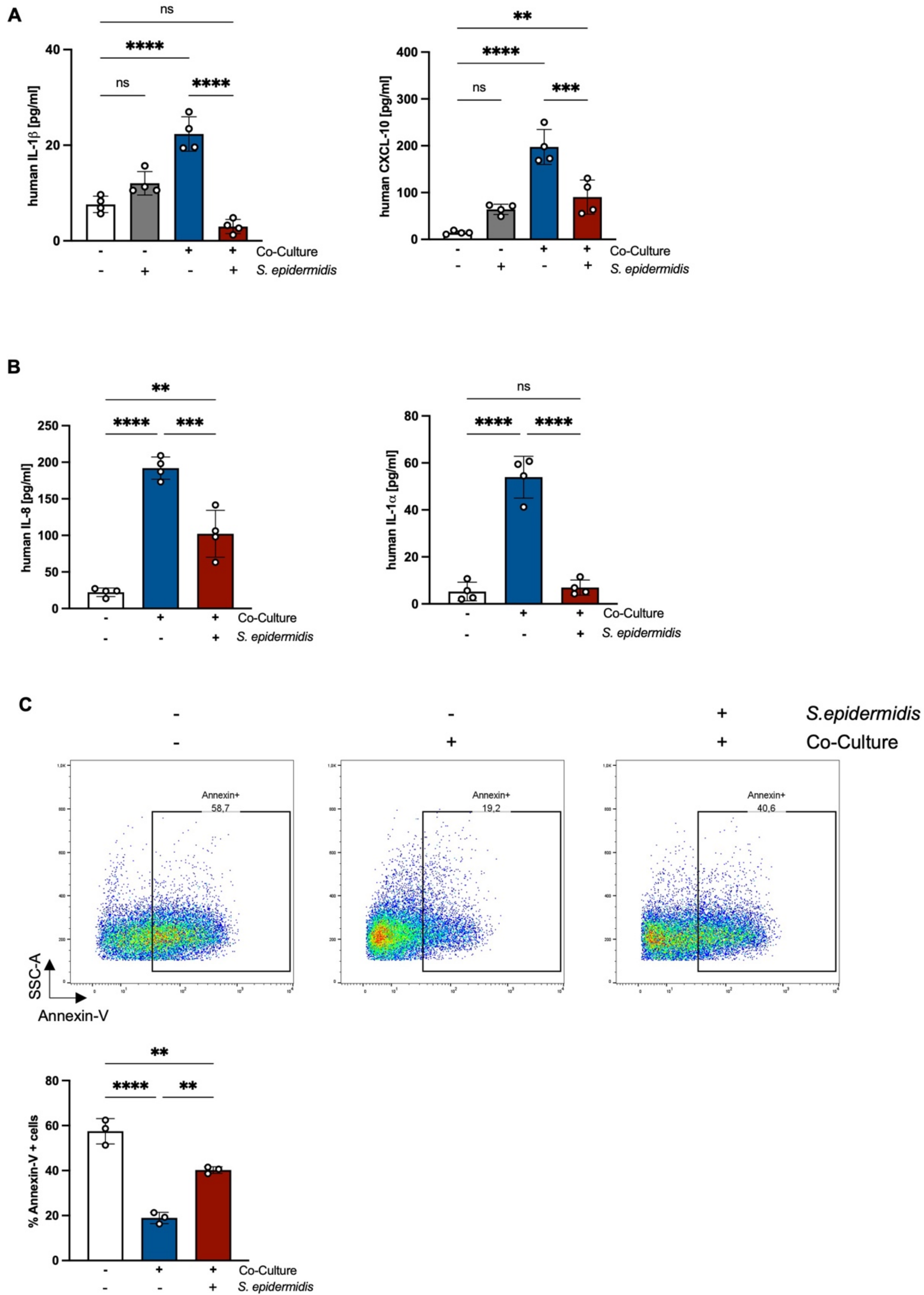

Figure 6

Supplementary Figure legends

Supplementary Figure 1: Calculation of the delay of cell death induction using interpolation A&B: Calculation of cell death delay between co-cultured and non-co-cultured PMNs (**A**) and PMNs incubated in medium or PMN supernatant (**B**) using interpolation. Shown are the mean of four different experiments + SEM. PMNs = polymorphonuclear neutrophil; SEM = standard error of the mean

Supplementary Figure 2: Legendplex analysis, viability analysis of PMNs after IL-8 and IL-1α blockade. A: Non-significantly induced cytokines and chemokines in PMNs co-cultured with PHKs or alone for different time points. Non-co-cultured PMNs served as control. Shown is one representative experiment of four different experiments + SD Significant differences to the control were analyzed by two-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B-D:** PMNs were co-cultured with PHKs for 18h in the presence or absence of anti-IL-8 (**B**), anti-IL-1a (**C**), or both (**D**). After 18h, cell viability was analyzed by SYTOX Green staining. Representative pictures are shown. **E:** freshly isolated PMNs were stimulated with different concentrations of recombinant human IL-8. After 18h, apoptosis induction was analyzed by Annexin-V staining and flow cytometry. Shown is one representative experiment of three independent experiments + SD. Percentage of apoptotic cells was compared to unstimulated PMNs by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PMN = polymorphonuclear neutrophils; PHK = primary human keratinocyte; SD = standard deviation

Supplementary Figure 3: Surface expression of activation markers on co-cultured and non-co-cultured PMNs after 18h. Histograms and quantification of surface expression of CD63, CD66b, CD11b on freshly isolated, 18h co-cultured and non-co-cultured PMNs. Shown is one representative experiment of three independent experiments + SD. Significant differences between the samples were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PMNs = polymorphonuclear neutrophils; SD = standard deviation

Supplementary Figure 4: Non-significantly induced cytokines in PHKs co-incubated with PMNs. To see, if proinflammatory responses upon *S. aureus* infection are affected by the co-culture, differentiated PHKs were co-incubated with PMNs for

3h, 6h and 18h, Secreted cytokines were analyzed by Legendplex. Uninfected PHKs were used as control. Shown is one representative experiment of four independent experiments + SD. Significant differences to the control were analyzed by one-way ANOVAs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PHKs = primary human keratinocytes; PMNs = polymorphonuclear neutrophil; *S. = staphylococcus aureus;* SD = standard deviation

Supplementary Figure 5: Non-significantly induced cytokines in PHKs and PMNs after *S. aureus* infection in the co-culture. **A:** To see, if proinflammatory responses upon *S. aureus* infection are affected by the co-culture, differentiated PHKs were co-incubated with PMNs for 18h and then infected with *S. aureus* or left uninfected for 1.5h. Secreted cytokines were analyzed by Legendplex. Uninfected PHKs were used as control. Shown is one representative experiment of four independent experiments + SD. Significant differences to the control were analyzed by two-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B:** PMNs were either co-cultured with PHKs or alone for 18h and subsequently, PHKs were infected with *S. aureus* (MOI = 30). Secreted cytokines were analyzed by Legendplex analysis. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by two-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PHKs = primary human keratinocytes; PMNs = polymorphonuclear neutrophils; *S. = Staphylococcus;* SD = standard deviation

Supplementary Figure 6: Non-significantly induced cytokines in PHKs and PMNs after *S. epidermidis* infection in the co-culture. A: To see, if proinflammatory responses upon *S. epidermidis* infection are affected by the co-culture, differentiated PHKs were co-incubated with PMNs for 18h and then infected with *S. epidermidis* (MOI = 30) or left uninfected for 1.5h. Secreted cytokines were analyzed by Legendplex. Uninfected PHKs were used as control. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by two-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **B:** PMNs were either co-cultured with PHKs or alone for 18h and subsequently, PHKs were infected with *S. epidermidis* (MOI = 30). Secreted cytokines were analyzed by Legendplex analysis. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by two-

70 way ANOVA $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. PHKs = primary human
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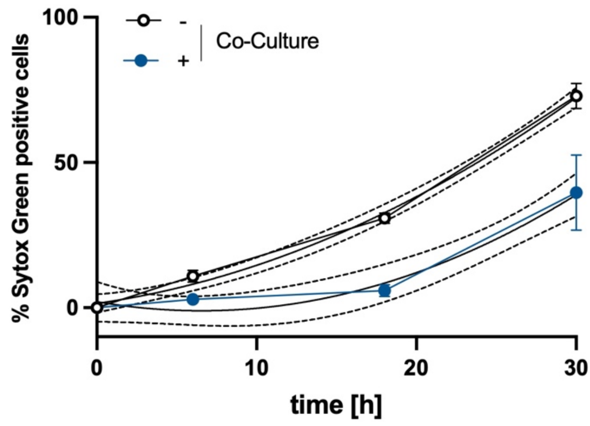
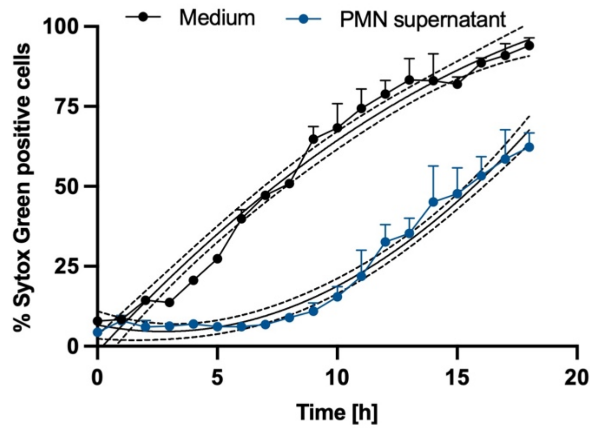
72 multiplicity of infection; SD = standard deviation

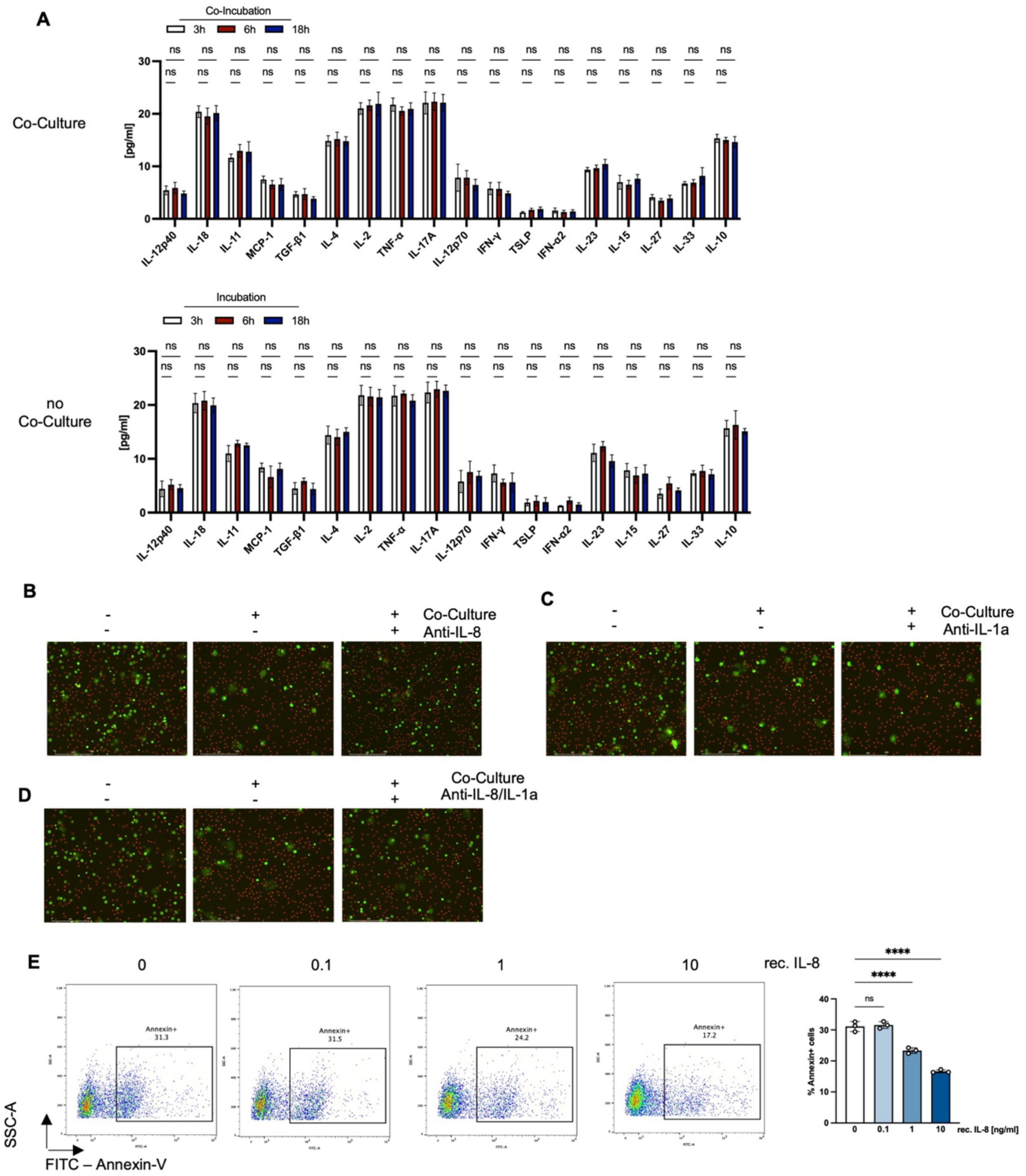


Sytox Green positive cells	Time [h] Interpolated		Delay [h]
	No Co-Culture	Co-Culture	
50%	23.8 h	33.1 h	9.3 h



Sytox Green positive cells	Time [h] Interpolated		Delay [h]
	Medium	PMN Supernatant	
50%	7,39 h	15,7 h	8.31 h

S. Figure S1



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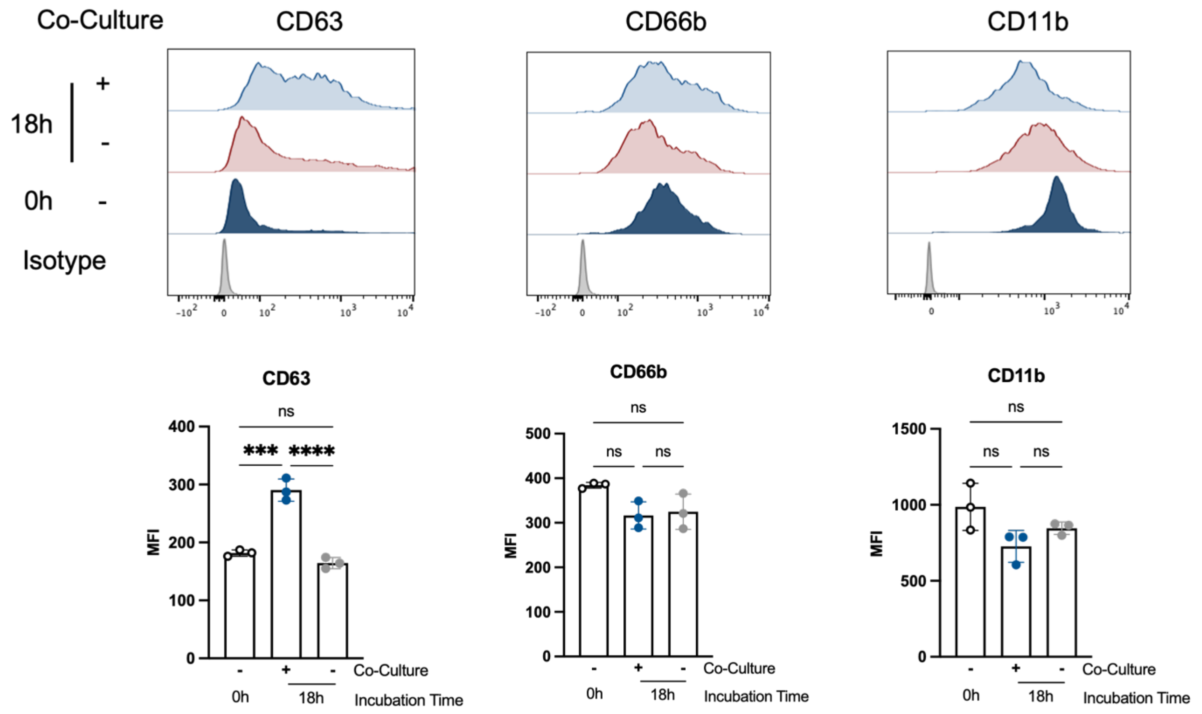

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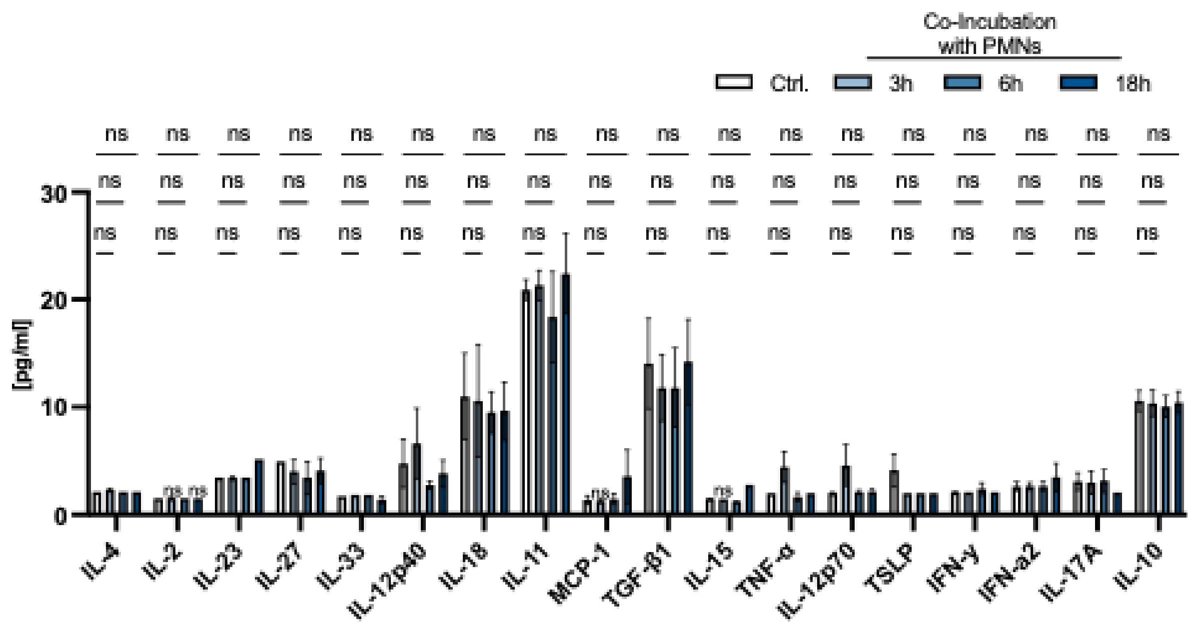

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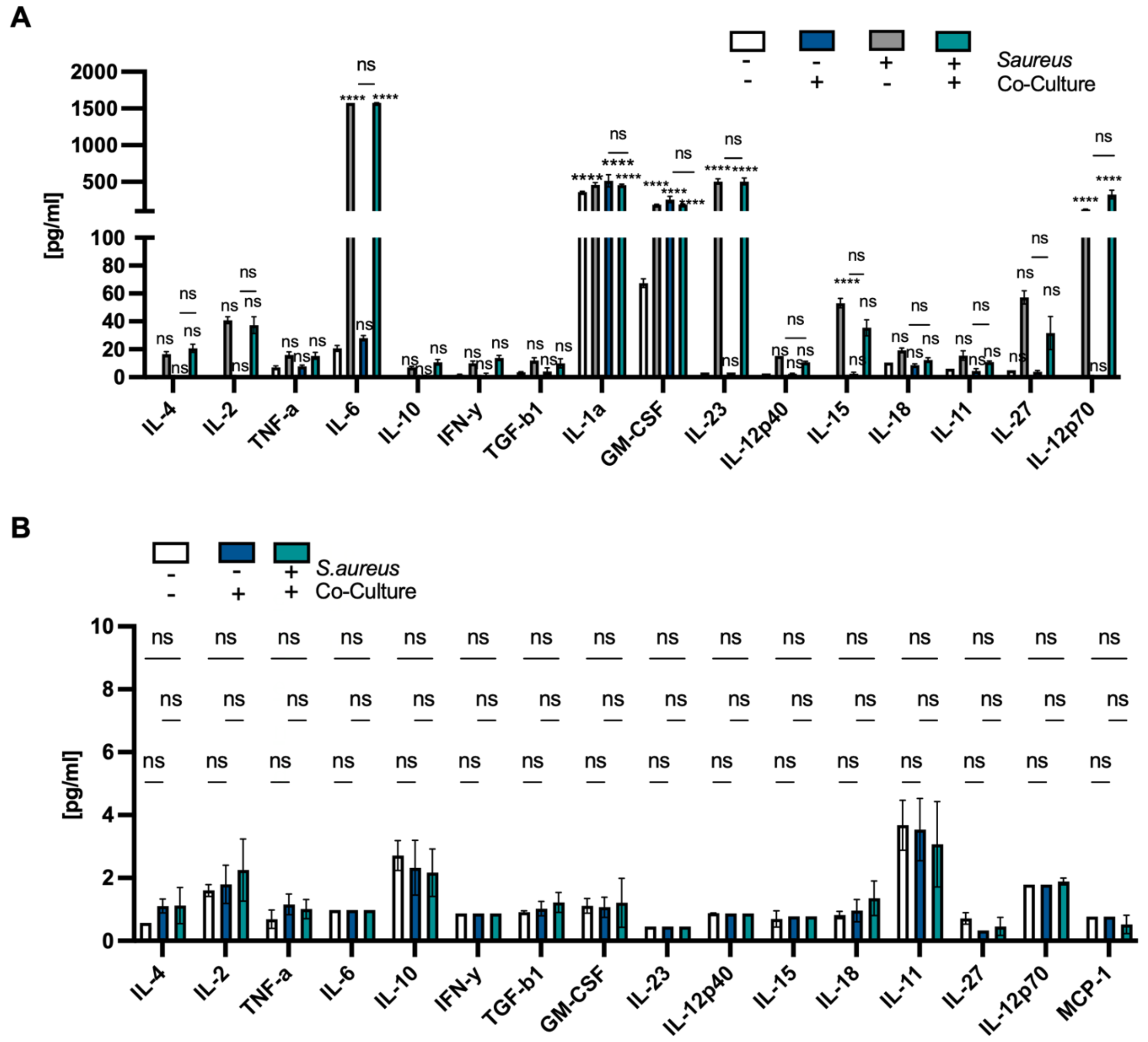

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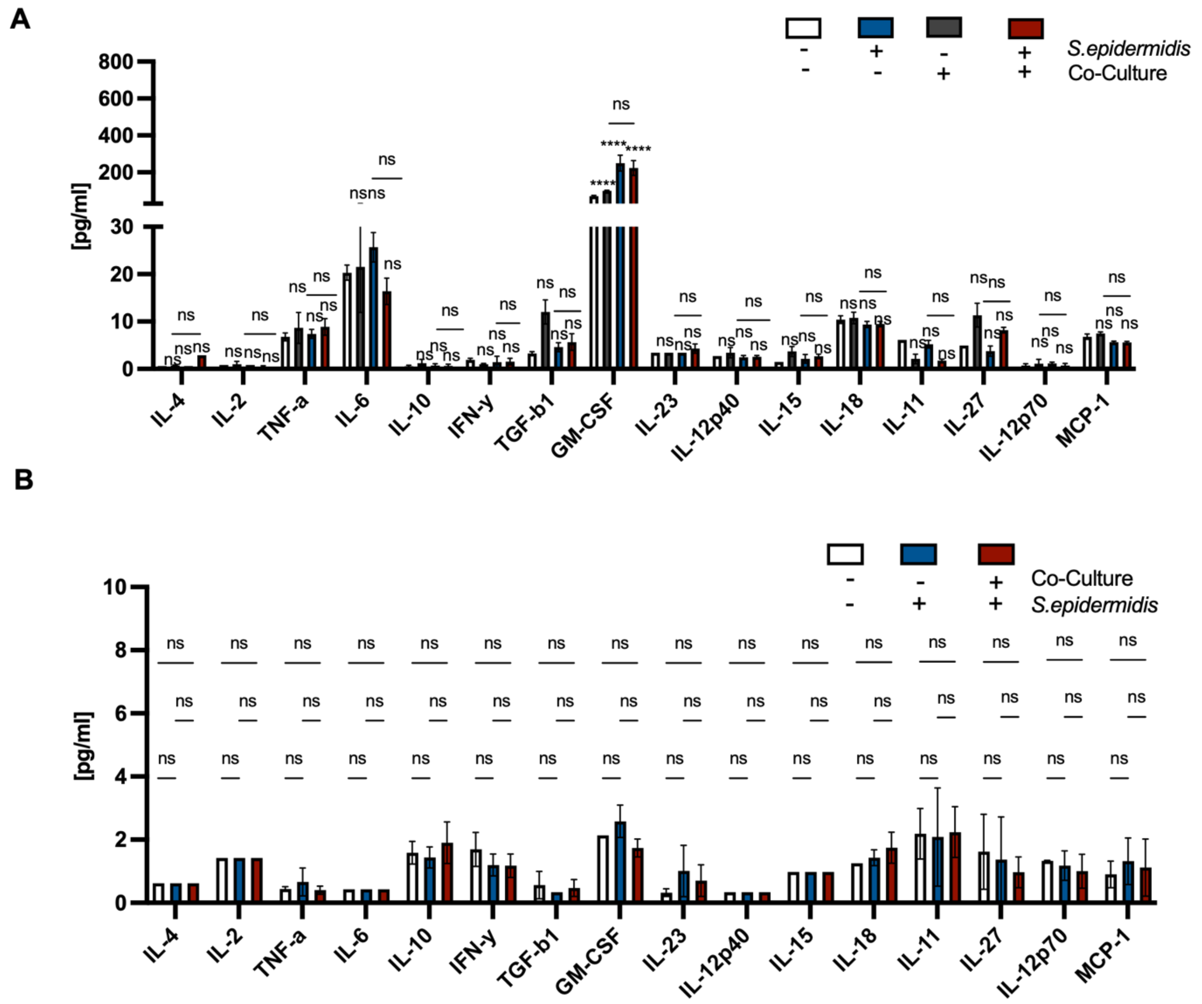

S. Figure 6

6 Results and Discussion

In the skin, a highly regulated interplay between keratinocytes, immune cells and the skin microbiome provides a protective barrier against skin infections caused by *S. aureus*. Dysregulation of this interplay can result in increased *S. aureus* colonization on the skin. In the context of AD, skin barrier defects, exacerbated skin inflammation and a loss of the skin microbiome diversity are associated with increased *S. aureus* skin colonization [510,511]. The abundant presence of *S. aureus* actively contributes to the pathogenesis of AD by further exacerbating skin inflammation and skin barrier defects [512,513]. Targeting *S. aureus* skin colonization has been associated with decreased AD severity, underscoring the active role of *S. aureus* in the disease [514]. Therefore, it is essential to gain a comprehensive understanding of how interactions within the skin immune system impact *S. aureus* skin colonization in order to develop novel treatment approaches. Neutrophils play a vital role in *S. aureus* skin inflammation and can target *S. aureus* using various antimicrobial mechanisms including NET formation. Additionally, they exhibit immunomodulatory functions that promote their interaction with different cells and shape immune responses in both sterile and infectious conditions. However, dysregulated neutrophil activation has been linked to exacerbated skin inflammation in several diseases. Recent studies suggest that neutrophils infiltrating inflamed skin can enhance the early colonization of *S. aureus* skin by releasing of NETs [221]. However, the exact mechanism behind this phenomenon is still unknown. Therefore, the purpose of this study was to investigate the role of neutrophils in *S. aureus* colonization and persistence, particularly on inflamed skin. Manuscript I and Manuscript II conducted an in-depth analysis of the interaction between skin-infiltrating neutrophils, NETs, and skin-resident keratinocytes as well as their impact on skin inflammation and on the colonization of the skin by *S. aureus*.

6.1 Neutrophil extracellular traps promote *S. aureus* skin colonization by the induction of oxidative stress and the downregulation of epidermal barrier genes

Skin barrier defects induced by tape-stripping have been shown to induce skin inflammation and enhance *S. aureus* skin colonization [510]. A recent study from our group demonstrated that tape-stripping induced the infiltration of neutrophils into the skin. These neutrophils have been shown to actively contribute to increased skin colonization by *S. aureus* by releasing NETs, which then interact with keratinocytes to create a milieu that favors *S. aureus* skin colonization [221]. However, the underlying mechanism of this phenomenon remains to be elucidated. Therefore, the aim of this study is to gain a comprehensive understanding of the mechanism of PMN- and NET-mediated increase in *S. aureus* colonization of the skin.

6.1.1 *S. aureus* persistence on inflamed skin is enhanced by the presence of Neutrophils

Previous studies have examined the role of neutrophils in the increased *S. aureus* skin colonization only at 24h after epicutaneous *S. aureus* application. Therefore, we first investigated how long the increase in *S. aureus* colonization of the skin mediated by PMNs persisted. For this, we applied *S. aureus* epicutaneously to the tape-stripped and non-tape-stripped skin of C57BL/6 WT mice and subsequently analyzed the number of colony-forming units (CFU) and PMNs in the skin on day 1, 3 and 7 following epicutaneous *S. aureus* infection. We observed that *S. aureus* colonization was significantly increased in tape-stripped skin compared to non-tape-stripped skin on all days (Manuscript I, Figure 1A). The observed changes in *S. aureus* CFUs may appear small, however, a recent study suggests that even small changes in *S. aureus* CFUs following dupilumab treatment can have a beneficial impact on skin inflammation and AD severity [127]. Therefore, we conclude that the relatively small changes in bacterial CFUs between tape-stripped and non-tape-stripped skin have biological significance.

Notably, the amount of PMNs in tape-stripped skin was notably increased in comparison to non-tape-stripped skin as detected using flow cytometry (CD45+ Ly6G+ Ly6C+) and immunofluorescence staining for MPO on day 1 and day 3. Furthermore, *S. aureus* infection further elevated PMN numbers in the skin (Manuscript I, Figure

1B-D). Based on the correlation between the presence of PMNs and increased *S. aureus* colonization in tape-stripped skin, we hypothesized that PMNs play an active role in this effect. To address this question, we depleted PMNs in mice with onetime injection of either anti-Ly6G antibody or isotype control. Effective depletion was confirmed by the absence of PMNs, as demonstrated by flow cytometry analysis and immunofluorescence staining for MPO of corresponding skin samples (Manuscript I, S. Figure 1D&E). Notably, PMN depletion significantly reduced *S. aureus* skin colonization in the tape-stripped skin (Manuscript I, Figure 1E). These results suggest that PMNs are critical for shaping the initial and prolonged colonization of *S. aureus* on inflamed skin. Increasing evidence links PMNs present in the skin to *S. aureus* colonization. *S. aureus* skin colonization is increased in AD skin [307]. Studies have reported that AD skin contains high levels of cutaneous PMN chemoattractants, including IL-8 and GM-CSF. Accordingly, there is an increased neutrophilic infiltrate in the dermis [515,516]. The stratum corneum of AD's lesional skin has higher levels of IL-8 compared to non-lesional skin which is associated with increased *S. aureus* colonization of the skin and skin barrier dysfunction and has been proposed as a marker of severity of skin inflammation [516-518]. In addition, a recent study by Moos et al. showed that spontaneous *S. aureus* skin infection correlates with an increased level of activated PMNs in the skin [519]. In addition to the mentioned literature, our data further suggest that PMNs present in inflamed skin actively promote the persistence of *S. aureus* on the skin. This PMN-mediated increase in *S. aureus* skin colonization appears to be specific to inflamed skin. A recent study using an epicutaneous *S. aureus* colonization model showed that PMN depletion had no impact on total bacterial CFU load in non-inflamed, non-tape-stripped skin 7 days after the application of *S. aureus*. However, PMNs prevented *S. aureus* invasion into the dermis [520]. Furthermore, Schulz et al. showed that *S. aureus* applied epicutaneously to uninflamed skin induced the production of IL-8 and subsequent infiltration of PMNs into the skin which reduced the bacterial load on the stratum corneum [521]. Additionally, in our work, we found a reduction in CFUs and PMNs over time in non-tape-stripped skin, aligning with the findings of the above-mentioned studies.

Taken together, this indicates that in healthy, non-inflamed skin, PMN prevents the dissemination of *S. aureus* in the skin and mediates transient colonization. This suggests that the increased *S. aureus* colonization of the skin mediated by PMNs

requires an inflammatory environment and an interaction between PMNs and PHKs prior to *S. aureus* infection.

6.1.2 Enhanced *S. aureus* colonization increases with extended co-incubation

Our next aim was to further analyze the interaction between primary human keratinocytes (PHKs) and PMNs that contributes to increased *S. aureus* colonization of the skin. We performed time-kinetic experiments using our established *in vitro* co-culture system [221] to co-culture PMNs and PHKs for varying time periods before infecting the PHKs with *S. aureus.* Notably, we observed an increase in *S. aureus* adhesion to PHKs with extended co-culture time (Manuscript I, Figure 2B). This correlated with an increase in NET formation by the co-cultured PMNs following *S. aureus* infection as detected with SYTOX Green staining (Manuscript I, Figure 2B&C). This suggests that PMNs are primed for NET formation by a crosstalk with PHKs.

We have further confirmed the formation of NETs in the co-culture using live cell imaging and staining with Sytox Green. Infection of the PHKs with *S. aureus* or stimulation with PMA clearly induced NET formation in the co-cultured PMNs, whereas no NETs were detected in the uninfected co-culture or upon *S. epidermidis* infection of the PHKs (Manuscript I, Figure 2D). In order to confirm these results *in vivo*, we examined the presence of NETs in the skin of C57BL/6 WT mice with and without tape-stripping and with or without epicutaneous *S. aureus* application. Interestingly, citH3, a NET marker, was only observed by western blot analysis in the tape-stripped, *S. aureus*-infected skin. Furthermore, immunofluorescence staining for citH3 and MPO further confirmed the presence of NETs in the tape-stripped skin 24h after epicutaneous *S. aureus* infection (Manuscript I, S. Figure 1A&B). This confirmed our *in vitro* findings that PMNs co-cultured with PHKs, are primed for NET formation after *S. aureus* infection.

We investigated the influence of NET formation on the increased adherence of *S. aureus* observed in the co-culture further by specifically targeting NETs by the addition of DNase I to the PMN well of the co-culture. Interestingly, DNase I treatment prevented the colonizing-enhancing effect in the co-culture (Manuscript I, Figure 2E). Furthermore, treatment of PHKs with the filter-sterilized supernatant from co-cultured, *S. aureus*-infected PHKs increased *S. aureus* adherence, while no effect was observed for supernatants from uninfected, co-cultured PHKs or non-co-cultured, infected PHKs

(Manuscript I, Figure 2F). This suggests that NETs induced in the co-culture have a functional impact on shaping PHKs to promote *S. aureus* adherence. Furthermore, treatment of freshly isolated PMNs with the filter-sterilized PMN supernatant from the *S. aureus*-infected co-culture induces NET formation (Manuscript I, Figure 2G). A recent study showed that NETs themselves can induce NET formation in PMNs [522].

Since the supernatant of the PMNs from the *S. aureus*-infected co-culture contains NETs, we hypothesize that these NETs stimulate freshly isolated PMNs to form NETs. Further experiments using PAD4-ko mice, which exhibit impaired NET formation, would further elucidate the role of NETs in the *in vivo* setting further. In conclusion, these data suggest that the interaction between PMNs and PHKs prior to *S. aureus* infection activates PMNs to form more NETs, which promotes the *S. aureus* colonization of the skin.

6.1.3 Oxidative stress induced in PHKs by PMNs, or NETs correlates with increased *S. aureus* colonization of the skin

Activated PMNs are characterized by increased production of ROS, which has been linked to oxidative stress induction in neighboring tissues [523,524]. Oxidative stress results in an imbalance between oxidant production and antioxidant mechanisms and can lead to tissue damage, including lipid peroxidation [525]. Oxidative stress is involved in several skin disorders, including AD [526-529]. We hypothesized that the increased amount of activated PMNs and NETs leads to the induction of oxidative stress in the skin. Therefore, we analyzed oxidative stress induction in PHKs in our *in vitro* co-culture model after co-culture with NETs or PMNs using 2',7'-Dichlorodihydrofluorescein-diacetat (H_2DCFDA), which detects intracellular ROS. Interestingly, prolonged co-culture time with PMNs and 18h co-culture with NETs increased the intracellular levels of ROS in PHKs (Manuscript I, Figure 3A&B). Since the induction of ROS in PHKs correlates with increased *S. aureus* adherence in the co-culture, we hypothesized that this ROS induction might affect *S. aureus* adherence in the co-culture model. To test this hypothesis, we targeted ROS induction in PHKs co-cultured with PMNs or NETs by the addition of the ROS scavenger N-Acetylcysteine (N-Ac). This effectively inhibited the induction of ROS in the PHKs (Manuscript I, Figure 3C). Subsequent analysis of the *S. aureus* adherence in this setting revealed a functional role for ROS induction in PHKs, as the previously observed enhanced *S. aureus* adherence in the co-culture was prevented (Manuscript I, Figure 3D). We

further analyzed the induction of ROS in our *in vivo* epicutaneous infection model by measuring malondialdehyde (MDA) levels in skin tissue lysates. MDA is an indicator of lipid peroxidation, a result of excessive ROS and is used as an indirect marker of oxidative stress [530]. Interestingly, tape-stripping of mouse skin resulted in elevated MDA levels compared to non-tape-stripped skin (Manuscript I, Figure 3D). Based on our *in vitro* findings, we hypothesized that PMNs, infiltrating the skin during tape-stripping were responsible for the elevated MDA levels. To test this, we depleted PMNs in mice and analyzed levels of MDA in the skin of tape-stripped and non-tape-stripped mice 24h after epicutaneous *S. aureus* application. Notably, tape-stripping led to an increase of MDA levels in the skin of PMN-sufficient but not PMN-depleted mice (Manuscript I, Figure 3E).

We further investigated the impact of PMNs in the induction of oxidative stress in the skin using skin explants. We co-cultured tape-stripped and non-tape-stripped human skin explants with PMNs or alone and observed elevated MDA levels when PMNs were present, but not when PMNs were absent, suggesting that PMNs mediate ROS induction in the skin rather than tape-stripping alone (Manuscript I, S. Figure 2A&B). Since PMNs themselves are a significant source of ROS, we performed immunohistochemical staining for MDA of tape-stripped and non-tape-stripped skin to see if ROS induction can be detected in the keratinocytes. We detected positive signals particularly in the epidermis of tape-stripped skin, indicating elevated MDA levels in keratinocytes, which were not observed in non-tape-stripped skin (Manuscript I, S. Figure 2E). This confirms that PMNs and NETs, present in tape-stripped, *S. aureus*-infected skin, induce ROS in PHKs in the skin. PHK-derived ROS have been suggested to contribute to the progression of Th2-mediated inflammation, which is dominant in AD and causes skin barrier dysfunctions [531]. Additionally, inhibiting ROS induction has been suggested as a possible therapeutic approach for AD [532]. Our findings suggest that a correlation exists between the induction of oxidative stress in PHKs and the increased *S. aureus* colonization of the skin. This further indicated that targeting ROS induction in PHKs could serve as a possible target for the treatment for AD.

6.1.4 NFκB signaling is activated in PHKs by PMNs or NETs in a ROS-dependent manner which correlates with increased *S. aureus* colonization of the skin

Both PMNs and NETs can activate pro-inflammatory responses in PHKs [533,534]. Additionally, it is well known that ROS are immunomodulatory and can contribute to skin inflammation [535]. We next analyzed the activation of proinflammatory signaling pathways in PHKs co-cultured with PMNs or NETs. Western blot analysis showed that co-culture with PMNs or NETs induced the activation of the NFκB- and MAPK-ERK signaling pathways in PHKs (Manuscript I, Figure 4A&B). Interestingly, supplementation of the co-culture with N-Ac prevented the activation of both signaling pathways in PHKs, suggesting that this is mediated by ROS induction in PHKs. (Manuscript I, Figure 4A&B). As previously described in section 3.1.3 inhibiting ROS in the co-culture prevented the enhanced adherence of *S. aureus*. Therefore, we hypothesized that targeting the ROS-activated signaling pathways in PHKs would impact *S. aureus* adherence. To test this hypothesis, we targeted the activation of MAPK-ERK and NFκB in PHKs co-cultured with PMNs or NETs by addition of trametinib or celastrol, respectively. Interestingly, the inhibition of each signaling pathway reduced *S. aureus* adherence in the PMN and NETs co-culture (Manuscript I, Figure 4C&D) highlighting the importance of both signaling pathways in this effect.

NFκB signaling is fundamental to the immune response by regulating the transcription of several immune mediators and providing a communication pathway between cells involved in the immune response [536]. Interestingly, overactivation of both NFκB- and MAPK-ERK signaling is involved in the pathogenesis of inflammatory skin diseases such as AD. For example, p-ERK levels and nuclear p65 expression are elevated in AD lesional skin compared to healthy skin [537,538]. In addition, p-ERK and NFκB activation has been reported in AD mouse models [537,539,540]. We further investigated the involvement of the NFκB pathway in *S. aureus* colonization of the skin *in vivo* using NFκB1-ko mice, which are deficient in the p50 subunit [541]. We analyzed *S. aureus* skin colonization and the numbers of PMNs in the tape-stripped and non-tape-stripped skin of WT and NFκB1-ko mice 24h after epicutaneous *S. aureus* application. We already observed a decreased *S. aureus* colonization of the non-tape-stripped skin of NFκB1-ko mice compared to WT mice (Manuscript I, Figure 4E). Furthermore, additional tape-stripping did not result in an increase in *S. aureus* colonization of the skin of NFκB1-ko mice in comparison to WT mice (Manuscript I, Figure 4E). Moreover, PMN recruitment to the skin after tape-stripping was impaired in

NFκB1-ko mice as observed by immunofluorescence staining for MPO and flow cytometry (Manuscript I, Figure 4F&G). These findings imply that NFκB signaling promotes PMN infiltration into the skin during inflammatory conditions, ultimately leading to increased *S. aureus* skin colonization. However, as these mice already exhibit impaired PMN infiltration into the skin, no conclusion can be drawn regarding the involvement of NFκB signaling in the crosstalk between PMNs and NETs *in vivo*.

In conclusion, our findings indicate that NFκB signaling in the skin triggers the recruitment of PMNs, which cause inflammation in PHKs via ROS-mediated activation of NFκB and MAPK-ERK pathways. Furthermore, in the context of an epicutaneous *S. aureus* infection, the subsequent release of NETs amplifies NFκB and MAPK-ERK signaling in PHKs. This amplification of signaling pathways exacerbates the inflammatory response provoked by *S. aureus* skin infection and promotes increased *S. aureus* colonization of the skin.

6.1.5 PMNs and NETs trigger HMGB1 induction and secretion from PHKs

So far, our results suggest that PMNs and NETs trigger ROS-mediated inflammatory induction in PHKs, which is associated with increased *S. aureus* skin colonization. Oxidative stress can induce the secretion of DAMPs, which are endogenous molecules secreted by stressed or damaged cells that can have immunomodulatory effects on neighboring cells [542,543]. The gene expression of several DAMPs in PHKs was upregulated after 18h co-incubation with PMNs or NETs, as observed by RT-qPCR (Manuscript I, Figure 5A&B). In particular, we observed an increase in intracellular HMGB1 in PHKs when co-cultured with PMNs, which was dependent on ROS induction (Manuscript I Figure 5C & S. Figure 4A). Additionally, co-cultured PHKs secreted elevated levels of HMGB1, which were further increased after infection with *S. aureus* (Manuscript I, Figure 5D). NETs also promote HMGB1 induction and secretion from PHKs, as observed by western blot and enzyme-linked immunosorbent assay (ELISA) studies (Manuscript I, Figure 5E&F). Notably, HMGB1 is also a component of NETs, as detected by ELISA (Manuscript I, S. Figure 4B).

Based on our *in vitro* findings, we further investigated whether PMNs and NETs also induce HMGB1 *in vivo*. We examined HMGB1 protein levels in skin lysates of WT, PMN-depleted (Ly6G), or NFkB1-ko mice with impaired PMN recruitment. Interestingly,

we found increased levels of HMGB1 in the skin of tape-stripped, *S. aureus*-infected WT mice, compared to the skin PMN-depleted of NFkB1-ko mice (Manuscript I, Figure 5G&H). This confirms our *in vitro* findings and further suggests that PMNs and NETs, present in tape-stripped, *S. aureus*-infected skin, trigger the production of HMGB1 in the skin.

HMGB1 is a conserved, non-histone DNA-binding protein that typically resides in the nucleus where it facilitates DNA bending [544]. However, inflammation may cause the relocation of HMGB1 to the cytoplasm and its release from stressed or dying cells [545]. Oxidative stress can affect HMGB1 functionality, and a recent study by Cui et al. reported that H_2O_2 treatment of human keratinocytes and melanocytes induced HMGB1 secretion, consistent with our findings [546,547]. Extracellular HMGB1 functions as an alarmin and can exert proinflammatory effects [548]. It is linked to the development of inflammatory skin disorders, such as AD. For example, HMGB1 expression is increased in the intercellular spaces of the epithelium in lesional AD skin, suggesting HMGB1 secretion [88]. Additionally, several mouse models have reported the translocation of HMGB1 from the nucleus to the cytoplasm. Inhibition of HMGB1 signaling ameliorated AD symptoms in these models [549 187].

Based on the increased amount of HMGB1 secreted in the co-culture, we hypothesized that this could be related to the enhanced *S. aureus* colonization of the skin. To analyze this, we conducted experiments to investigate if HMGB1 could induce ROS in PHKs. Our findings demonstrated that stimulating PHKs with recombinant HMGB1 resulted in increased intracellular ROS levels in PHKs after 18h, as observed by DCF fluorescence measurement (Manuscript I, Figure 5I). Furthermore, treatment of PHKs or topical application of recombinant HMGB1 to human skin explants significantly increased *S. aureus* adherence (Manuscript I, Figure 5J&K). Moreover, HMGB1 stimulation triggered isolated PMNs to form NETs in a concentration-dependent fashion, as detected by staining with Sytox Green (Manuscript I, Figure 5L). This result confirms previous studies of HMGB1-induced NET formation [550,551].

We further aimed to investigate the mechanisms underlying the increase in *S. aureus* colonization of the skin mediated by HMGB1, PMNs and NETs and we next examined the receptors involved in this process. Various DAMPs, including HMGB1, have been shown to bind to TLRs [552]. To evaluate the impact of TLRs on *S. aureus* colonization of the skin, we used 5xTLR-ko mice that lack TLR2, TLR3, TLR4, TLR7, and TLR9 [553]. Tape-stripping of the skin of WT mice resulted in increased PMN infiltration and

subsequent increased *S. aureus* skin colonization. Notably, this effect was impaired in 5xTLR-ko mice, indicating a functional role for TLR signaling in PMN recruitment during inflammation (Manuscript I, Figure 6A&B). No differences were observed in *S. aureus* skin colonization or PMN recruitment between WT and 5xTLRo-ko mice in non-tape-stripped skin (Manuscript I, Figure 6A&B), confirming results from previous studies [21,235]. Based on our findings, it appears that HMGB1 plays a functional role in the increase of *S. aureus* colonization of the skin. In further experiments, we concentrated on the two primary HMGB1 receptors: TLR4 and the receptor for advanced glycation endproducts (RAGE). Although HMGB1 can also bind to TLR2, previous work of our groups has demonstrated that the colonizing-enhancing effect is unrelated to TLR2. This is evident from the increased *S. aureus* skin colonization observed in tape-stripped skin of TLR2-ko mice [221]. Therefore, we did not investigate TLR2 signaling in the subsequent experiments. Interestingly, co-incubation with PMNs or NETs increased the protein levels of TLR4 and RAGE in PHKs and human skin explants (Manuscript I, S. Figure A-C). Since we found that treating PHKs with HMGB1 resulted in increased adherence of *S. aureus*, we investigated *S. aureus* adherence in PHKs treated with HMGB1 with and without TAK-242 or an antagonist peptide (AP) to RAGE to directly target TLR4 and RAGE signaling, respectively (Manuscript I Figure 6C). This suggests that HMGB1-mediated enhanced *S. aureus* skin colonization requires both receptors. Previous studies have noted an interaction between TLR4 and RAGE, which promotes the surface expression of each other and that TLR4/RAGE signaling is required for HMGB1-mediated inflammation in macrophages [554]. Notably, targeting TLR4 or RAGE reduces *S. aureus* adherence in PMN or NETs co-culture (Manuscript I, Figure 6D). Furthermore, inhibition of TLR4 or RAGE prevented both NET-mediated ROS induction in PHKs and HMGB1 secretion in PMN co-culture (Manuscript I, Figure 6E&F).

In conclusion, our results demonstrate that the presence of PMNs initiates the induction of HMGB1 in PHKs through ROS-mediated mechanisms in inflamed skin. Consequently, HMGB1 performs a dual function by inducing NET formation in PMNs and stimulating ROS production in PHKs, ultimately leading to increased *S. aureus* skin colonization. This process is likely coordinated through activation of TLR4 and RAGE signaling pathways.

6.1.6 Atopic dermatitis skin contains elevated levels of HMGB1, PMNs and NETs

Next, we aimed to demonstrate the clinical significance of our findings. For this, we used CODEX analysis to assess the presence of *S. aureus*, PMNs and HMGB1 in healthy skin and the skin of AD patients. As expected, *S. aureus* was abundant in the skin of AD patients compared to healthy skin. We observed that the epidermis and dermis of AD patients contain elevated levels of PMNs, as observed by NE staining, in contrast to healthy skin. This indicates a potential crosstalk between keratinocytes and PMNs in AD skin (Manuscript I, Figure 7A). Furthermore, HMGB1 expression was significantly increased in AD skin. Interestingly, we detected the expression of HMGB1 predominantly in the cytoplasm rather than in the nucleus where it is located at steady state conditions (Manuscript I, Figure 7A, S. Figure 6A-C). This indicates that HMGB1 translocates from the nucleus to the cytoplasm, resulting in potential HMGB1 secretion [475,88,545,555]. These findings support the results of a previous study that showed increased expression of HMGB1 in intercellular spaces of lesional AD skin [68]. The study additionally demonstrated elevated expression of TLR4 in AD lesional skin and proposed the involvement of HMGB1-TLR4 signaling in the pathogenesis of AD [88].

We further investigated whether NET formation could be detected in the skin of AD patients. Using immunofluorescence staining for MPO and OGG1, a suitable NET marker [535], we confirmed that NETs are present in the skin of AD patients and are located in the stratum corneum (Manuscript I, Figure 7B & S. Figure 7). This localization may prevent the spread of *S. aureus* to deeper skin layers, as NETs are known to be critical in keeping *S. aureus* infections local [399]. However, according to a study by Nakatsuji et al. *S. aureus* can be found in the dermis of the lesional skin from AD patients [556], which we confirmed in our analysis (Manuscript I, Figure 7A). One possible explanation for this could be the various escape strategies developed by *S. aureus* to evade NET-mediated killing. Interestingly, AD-associated *S. aureus* strains are distinct and produce higher levels of toxins. For example, *S. aureus* strains isolated from lesional AD skin had increased IL-8 induction in PHKs and increased ability to recruit neutrophils in comparison to non-lesional strains which also correlated with their ability to adhere to PHKs [164]. Further research should investigate the potential of AD-associated *S. aureus* strains to evade NET-mediated killing. This explanation would suggest that in AD, S. *aureus* triggers NET formation in infiltrating PMNs, thereby preventing PMNs from using other killing mechanisms. *S. aureus* subsequently evades

NET-mediated killing, while NETs cause skin inflammation that further promotes *S. aureus* colonization.

The results reported in this section demonstrate the clinical significance of our findings and further suggest that PMNs and NETs present in AD skin might induce ROS-mediated HMGB1 secretion, subsequently promoting inflammation and *S. aureus* skin colonization.

6.1.7 NETs and HMGB1 reduce epidermal barrier gene expression in PHKs

We further sought to investigate the role of NETs and HMGB1 in promoting enhanced skin colonization by *S. aureus*. AD patients have an epidermal barrier defect that is thought to contribute to the development of the disease and facilitate *S. aureus* skin colonization and subsequent inflammation [557]. For example, the decreased filaggrin expression in lesional and non-lesional AD skin is associated with increased *S. aureus* skin colonization [417]. In addition, *S. aureus* skin colonization is increased in a filaggrin knockdown epidermal skin model [486,558]. Furthermore, by reducing the expression of epidermal barrier proteins, HMGB1 can impair the function of the skin barrier in human skin equivalents [559]. We aimed to determine whether the NETs and HMGB1 could lead to skin barrier dysfunction. To investigate this, we co-incubated PHKs with NETs or stimulated PHKs with HMGB1 for 18h and subsequently assessed the expression of epidermal barrier genes encoding for filaggrin (*FLG*), involucrin (*IVL*), or the tight junction protein claudin-1 (*CLN-1*). Interestingly, both HMGB1 and NETs reduced the gene expression of *FLG*, *IVL,* and *CLN-1* in PHKs (Manuscript I, Figure 7C&D). In addition, topical application of recombinant HMGB1 or NETs to skin explants reduced the protein expression of filaggrin and involucrin in the skin (Manuscript I, S. Figure 8A). These results suggest that NETs and HMGB1 can cause epidermal barrier defects. Previous studies have shown that the Th2 cytokines IL-13 and IL-4 can reduce the expression of epidermal barrier genes in PHKs through a ROS-dependent mechanism [560]. Since NETs induce ROS in PHKs, we analyzed the impact of ROS inhibition on the expression of epidermal barrier genes. To test this, we co-cultured PHKs with NETs with and without N-Ac for 18h and examined the expression of the genes *IVL* and *FLG* in PHKs. Interestingly, inhibition of ROS induction prevented the decrease in *IVL* and *FLG* gene expression in PHKs (Manuscript I, S. Figure 8B).

Taken together, these findings suggest that NETs and HMGB1 in inflamed skin cause skin barrier dysfunction, potentially leading to increased *S. aureus* skin colonization. Our observations show that the skin of AD patients contains elevated levels of NETs and HMGB1, which may further elucidate the skin barrier defects in AD and highlight potential therapeutic targets.

6.1.8 Conclusion

In this work, we provide a detailed mechanistic analysis of how PMNs and NETs promote *S. aureus* skin colonization in inflamed skin. We propose that PMNs and NETs induce oxidative stress in the skin, which then stimulates the secretion of HMGB1. Extracellular HMGB1 further induces oxidative stress in PHKs and NET formation in infiltrating PMNs. Interestingly, both HMGB1 and NETs induce a decrease in the expression of in epidermal barrier protein, which facilitates *S. aureus* colonization of the skin. We provide clinical relevance by showing that HMGB1, PMN, and NETs can be found in AD skin. NETs normally trap bacteria and prevent them from spreading. However, we observed the dissemination of *S. aureus* in the skin of patients with AD despite the presence of NETs in the stratum corneum. This suggests that NETs were ineffective in preventing *S. aureus* invasion. It is plausible that *S. aureus* triggers NETs and subsequently employs mechanisms to escape NET-mediated killing, while exacerbating skin inflammation, which favors its colonization.

In conclusion, the role of PMNs in *S. aureus* skin infections appears to be context dependent. In non-inflamed skin, PMNs infiltrate the skin to prevent *S. aureus* invasion into the dermis and mediate transient *S. aureus* colonization, whereas in inflamed skin, PMNs promote *S. aureus* skin colonization. Our findings are illustrated in Figure 9.

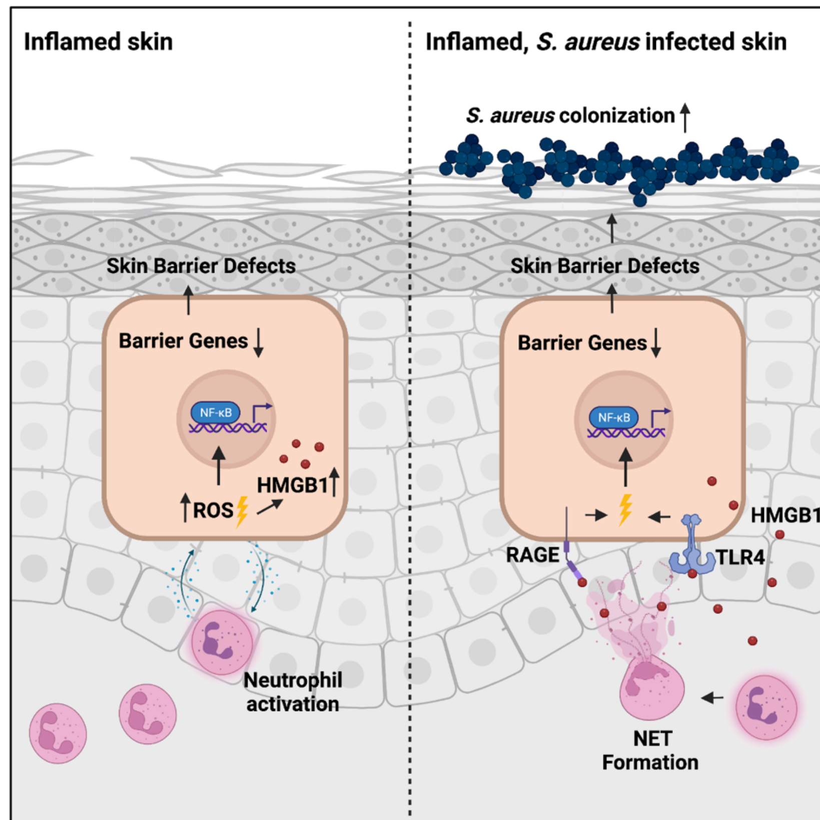

Figure 9: Proposed model of how NETs enhance *S. aureus* skin colonization (Manuscript I, [505]). PMNs infiltrate inflamed skin and induce the ROS-mediated induction of HMGB1 and activation of NFκB activation in PHKs. *S. aureus* infection induces NET formation, triggering HMGB1 secretion by PHKs, which in turn induces skin barrier gene downregulation in a ROS-dependent manner and promotes *S. aureus* skin colonization. This Illustration was created with BioRender.

6.2 Crosstalk between neutrophils and keratinocytes shapes skin immunity against *S. aureus* infections

Skin inflammation results in the rapid infiltration of neutrophils into the skin in both a sterile and infectious setting [350,561]. Inflammatory conditions shape the phenotype of neutrophils, leading to a prolonged lifespan and increased activation. This adaptation ensures optimal neutrophil functionality in effectively combating invading pathogens [340]. However, the enhanced presence of activated neutrophils can also result in exacerbated skin inflammation. The purpose of this work was to examine how neutrophils and keratinocytes influence each other in both a sterile environment and during *S. aureus* infection.

6.2.1 PMN lifespan is prolonged by co-incubation with PHKs in an IL-8 dependent manner

PMNs are typically short-lived cells that rapidly undergo spontaneous apoptosis upon completion of their task [340]. A recent study proposed that the lifespan of PMNs depends on the tissue into which they infiltrate and has found that the lifespan of PMNs is highest in the skin [336]. Based on the literature, we hypothesized that the close contact with keratinocytes in the skin influences the lifespan of PMNs. To test this, we co-cultured PMNs with PHKs in our *in vitro* co-culture system. We then analyzed PMN viability over time using Sytox Green staining. Indeed, PMNs co-cultured with PHKs exhibited a significantly prolonged lifespan compared to non-co-cultured PMNs with a delay of 9.3h until 50% of the cells were dead (Manuscript II, Figure 1A, S. Figure 1A). Further investigation into the mechanism of cell death revealed a spontaneous induction of apoptosis in non-co-cultured PMNs. This induction was not observed in co-cultured PMNs after 18h of incubation (Manuscript II, Figure 1B&C). Nevertheless, the induction of apoptosis was not completely prevented in co-cultured PMNs but rather was delayed and eventually induced, and after 30h of co-culture approximately 50% of the PMNs were apoptotic (Manuscript II, Figure 1C). Therefore, our findings indicate that co-culturing with PHKs creates a beneficial, lifespan-extending environment for PMNs.

Induction of spontaneous apoptosis by PMNs is considered a non-inflammatory form of programmed cell death required for inflammation resolution and homeostasis restoration [323]. However, various stimuli such as proinflammatory cytokines or

chemokines can significantly extend the lifespan of PMNs, likely to ensure their optimal antimicrobial function in the presence of an infectious stimuli [340]. We hypothesized that soluble factors, released during the co-culture mediate the extended lifespan of PMNs co-cultured with PHKs. To test this, we incubated freshly isolated PMNs in the sterile-filtered PMN supernatant from the 18h co-culture and analyzed PMN viability over time using Sytox Green staining. Interestingly, freshly isolated PMNs incubated with co-culture PMN supernatant exhibited a significantly prolonged lifespan compared to PMNs incubated in medium. The delay in cell death induction was comparable to that observed in the co-culture (Manuscript II, Figure 2A & S. Figure 1B). By analyzing the secreted factors present in the PMN supernatant that may promote the extended lifespan, we observed increasing levels of IL-8 and IL-1α with prolonged co-culture (Manuscript II, Figure 2B). Notably, targeting these cytokines with neutralizing-antibodies revealed that IL-8, but not IL-1α, was involved in enhancing PMN viability in the co-culture (Manuscript II, Figure 2C).

Our findings are supported by previous studies, which reported that IL-8 can delay spontaneous and inflammation-induced apoptosis in PMNs [562]. As mentioned in section 2.1, elevated IL-8 levels in the skin are associated with the disease severity of AD [518]. Our findings here suggest that the elevated levels of IL-8 in AD skin could potentially prolong the lifespan of infiltrating PMNs.

6.2.2 PMNs are activated by co-culture with PHKs

The extension of PMN lifespan can potentially increase PMN activity [563]. Therefore, we hypothesized that co-culture with PHKs would both extend the lifespan and activate PMNs. Strikingly, we observed higher levels of activation markers in co-cultured PMNs, including increased surface expression of CD63 and elevated secretion of MPO and LCN-2, compared to non-co-cultured PMNs (Manuscript II, Figure 3B, S. Figure 3). This indicates that the co-culture of PMNs with PHKs leads to an activated phenotype. Activated PMNs display an enhanced antimicrobial response to invading pathogens [509]. For instance, pro-inflammatory cytokines from PBMCs prime PMNs, leading to stronger killing of *S. aureus* [564,565]. Given the critical role of PMNs in *S. aureus* skin inflammation, we hypothesized that co-culture with PHKs would prime PMNs for more potent reactivity against infectious stimuli. Exposure of PMNs co-cultured with PHKs to *S. aureus* for 18h resulted in a significant increase in reactivity and secretion of

higher levels of MPO and LCN-2 (Manuscript II, Figure 3C). This effect was not observed in freshly isolated or non-co-cultured PMNs incubated in medium for 18h, suggesting a possible role for PHK crosstalk in shaping the PMN immune response to infectious stimuli such as *S. aureus*. PMNs are critical for immune defense against *S. aureus* in mouse models of intradermal infection, where PMNs come into direct contact with *S. aureus* [21]. In these models, PMN depletion is associated with increased bacterial burden and excessive inflammation [520]. Given the findings here, it is possible that crosstalk with PHKs in the skin enhances PMN activity, resulting in increased host protection and killing of *S. aureus* infection.

6.2.3 PHKs co-cultured with PMNs exhibit a proinflammatory state

We hypothesized that extended co-culture with activated PMNs leads to inflammation in PHKs. We assessed the gene expression of 84 distinct genes in PHKs co-cultured with PMNs for 18 hours. We discovered an increased expression of genes associated with immune cell migration, TLR- and NFκB signaling, pro-survival genes, and stress response (Manuscript II, Figure 4A). Moreover, extended co-culture led to the increased secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF, and CXCL-10 by co-cultured PHKs compared to non-co-cultured PHKs (Manuscript II, Figure 4B). These results imply that PMN co-culture stimulates an inflammatory state in PHKs, thus confirming the results of previous studies regarding PMN-mediated inflammation in PHKs via soluble factors [27,534]. Interestingly, some of the induced factors we discovered act as chemoattractants for other immune cells, such as monocytes, macrophages, T cells, NK cells, and DCs [566,567]. This supports the potential role of PHKs and PMNs crosstalk in recruiting additional immune cells to the skin to enhance the skin's immune response.

6.2.4 Immune responses to *S. aureus* infection is exacerbated by the crosstalk between PHKs and PMNs

We further investigated whether the co-culture of PHK with PMNs affects the inflammatory responses of PHKs in response to *S. aureus* infection. To analyze this, we co-cultured PHKs and PMNs for 18h before infecting the PHKs with *S. aureus.* We then analyzed the induction of inflammatory mediators. Interestingly, co-culturing PHKs with PMNs and infecting them with *S. aureus* resulted in an increase of

proinflammatory responses within the PHKs. This is evident in the enhanced levels of CXCL-10, IL-1β and IL-8. Additionally, the co-culture environment results in increased secreted levels of IL-33 and MCP-1 induced in PHKs during *S. aureus* infection (Manuscript II, Figure 5A). Notably, IL-33 is elevated in the lesional skin of patients with AD and can induce the production of type 2 cytokines, which are associated with AD pathogenesis, by skin ILC2s [215,568]. These findings collectively demonstrate that previous crosstalk with PMNs exacerbates the immune response of PHKs to *S. aureus*. We further observed that the supernatant of co-cultured PHKs and, to a lesser extent, non-co-cultured PHKs after *S. aureus* infection had the ability to induce PMN migration (Manuscript II, Figure 5C), which could potentially further fuel skin inflammation.

Previous research has reported a correlation between IL-8 levels (a potent PMN chemoattractant) and *S. aureus* skin colonization [516-518,569,570]. *S. aureus* in turn actively contributes to the pathogenesis of AD by aggravating skin inflammation [571]. Our results, combined with the literature, suggest that an interaction between PHKs and PMNs in the skin may exacerbate *S. aureus*-induced skin inflammation in AD.

6.2.5 The skin microbiome reduces inflammation mediated by PMNs and induces PMN apoptosis

Previous results from our group have shown that the skin microbiome has a protective effect on the skin in response to *S. aureus* by reducing *S. aureus* skin colonization and associated skin inflammation [221,572,573]. Interestingly, *S. epidermidis* also reduced *S. aureus*-induced PMN recruitment to the skin [221]. Based on these findings and the results mentioned above, we hypothesized that the skin microbiome might have a preventive role in PMN-mediated inflammation in the skin. In order to test this, we analyzed the induction of proinflammatory mediators in the co-culture both before and after infecting the PHKs with *S. epidermidis*. We found that *S. epidermidis* infection significantly decreased the secreted levels of IL-1β and CXCL-10 as well as IL-8 and IL-1α in the co-culture by PHKs and PMNs, respectively (Manuscript II, Figure 6A&B). This suggests that the skin microbiome can reduce PMN-mediated skin inflammation. Additionally, *S. epidermidis* infection of the co-cultured PHKs induced apoptosis in PMNs, which was a particularly intriguing finding (Manuscript II, Figure 6C). As mentioned previously, apoptosis of PMNs is crucial for both resolving inflammation and re-establishing homeostasis [340]. In healthy skin, the skin microbiome comprises a diverse set of beneficial commensals that actively contribute to skin

homeostasis [574]. However, in individuals with AD, this diversity in the skin microbiome is lost and is associated with a higher abundance of *S. aureus* on the skin [307]. Based on our previous research, our data suggests that the skin microbiome in healthy skin might prevent PMN-mediated skin inflammation by exerting an anti-inflammatory effect, and that this effect is lost in AD skin. However, further investigations are necessary to confirm this hypothesis.

6.2.6 Conclusion

The results of this work demonstrated that the interaction between PHKs and PMNs in the skin has a significant impact on the immune response to *S. aureus* skin infections. A proposed model of our results is illustrated in Figure 10. Co-culture of PMNs with PHKs resulted in a significant increased lifespan, mediated by secreted IL-8. Moreover, the co-culture primes PMNs for enhanced activity against *S. aureus*. On the other hand, we discovered that prolonged co-culture with PMNs caused inflammation in PHKs, which was worsened by *S. aureus* infection. Notably, the skin commensal *S. epidermidis* decreased PMN-mediated skin inflammation in PHKs and induced apoptosis in activated PMNs. This implies that the skin microbiome has a constructive role in preventing excessive inflammation. Since several studies suggest that PMNs may be involved in AD development, the interaction between PHKs and PMNs may serve as a target to combat *S. aureus*-induced inflammation and offer a promising therapeutic approach for AD.

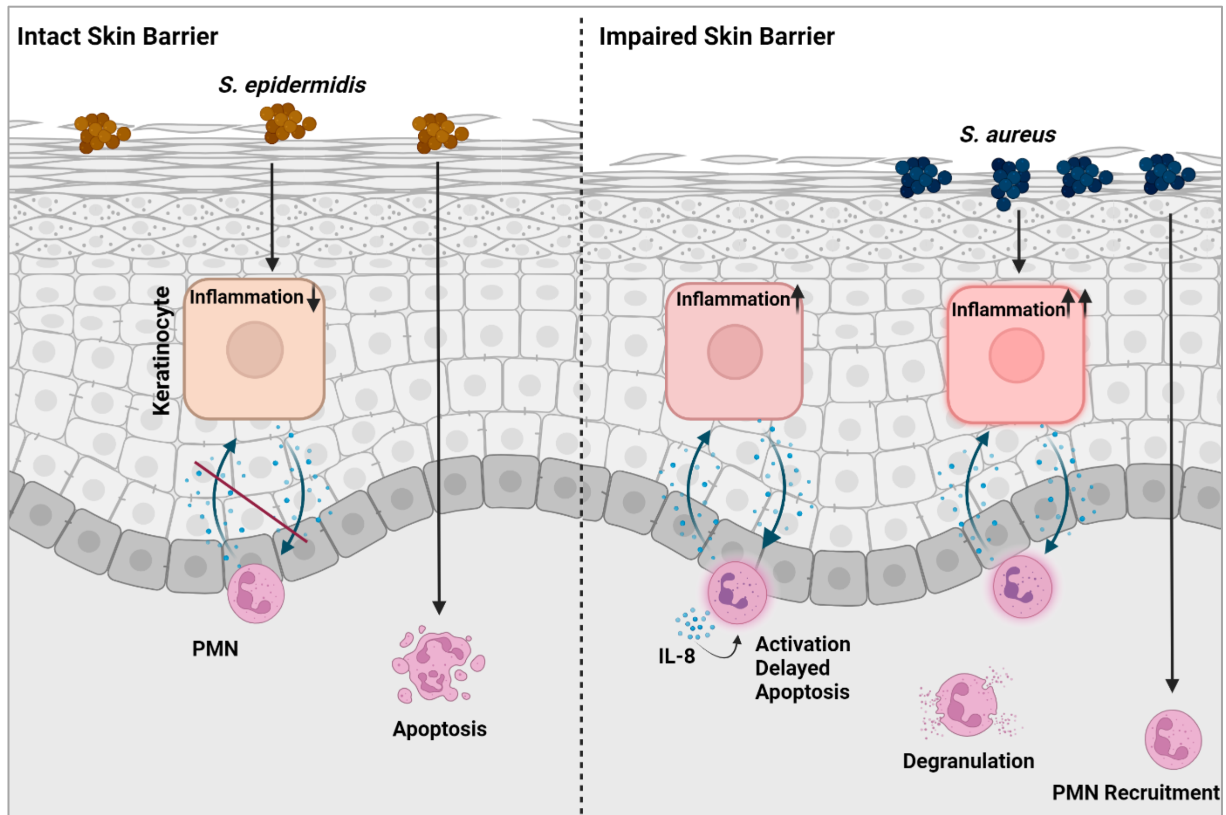

Figure 10: Proposed model of the PMNs/PHK crosstalk during skin inflammation. In a sterile environment, PMNs are activated, and their lifespan is prolonged in an IL-8-dependent manner. Additionally, proinflammatory responses are activated in PHKs. *S. aureus* infection exacerbates skin inflammation and triggers further PMN recruitment. In contrast, *S. epidermidis* infection exerts an anti-inflammatory effect by reducing PMN-mediated skin inflammation and promoting apoptosis in PMNs. This Illustration was created with BioRender.

6.3 Conclusive remarks and future perspective

This work provides the intriguing finding that the interaction between PMNs and PHKs plays a vital role in *S. aureus* skin infections in inflamed skin. First, PMNs are primed for NET formation by interacting with PHKs, which increases *S. aureus* colonization of the skin by causing skin barrier defects in a ROS-dependent manner. Second, the interplay between PMNs and PHKs exacerbates skin inflammation induced by *S. aureus* infection. Therefore, these results propose that blocking the interaction between PMNs, NETs and PHKs could serve as a potential treatment approach for skin disorders associated with *S. aureus* colonization, such as AD. However, our findings have several limitations that need to be addressed in future studies.

The role of NETs in enhanced skin colonization by *S. aureus* can be further investigated using PAD4-ko mice, which have impaired NET formation. This would not only provide further evidence that NETs are involved in the enhanced *S. aureus* colonization *in vivo*, but it would also provide more information on how NETs influence the immune response in inflamed, *S. aureus*-infected skin, as NETs have immunomodulatory properties and can recruit and activate various immune cells. This can be achieved using approaches like CODEX analysis, spatial transcriptomics and single cell RNA sequencing of non-tape stripped- and tape stripped skin under both sterile conditions and after epicutaneous *S. aureus* infection to reveal the presence of different immune cells as well as their phenotype and localization within the skin.

In addition to analyzing the immune response within the skin, the bacterial component should also be considered. *S. aureus* has developed several escape strategies to evade neutrophil/NET-mediated killing, for example by degrading NETs using their nucleases. It would be interesting to use mutant strains of *S. aureus* that lack nuclease to see if their colonization is also enhanced by NETs or if they are more susceptible to NET-mediated killing. In addition, different *S. aureus* strains, preferably those associated with AD, should be analyzed for their ability to escape neutrophil-mediated killing. This would provide information on whether NETs in inflamed skin fail to kill *S. aureus* but promote skin barrier defects that are beneficial for *S. aureus* skin colonization.

Furthermore, it remains unclear how distinct the effects of PMNs/NETs and HMGB1 observed here are for AD. NETs and HMGB1 are also elevated in skin diseases that are not typically associated with *S. aureus* skin colonization, including psoriasis or SLE.

Approaches such as CODEX analysis could be used to better understand the differences in immune responses between the diseases.

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8 Acknowledgments

This thesis is the result of a long journey full of hard work, success, frustration, joy, and self-development. Along the way, I have been fortunate enough to be accompanied by many people who have supported me tremendously.

I want to start by thanking my supervisor, Prof. Dr. Birgit Schittek, for giving me the opportunity to pursue my PhD in her lab, for always supporting me and for her trust and the opportunity to also work independently and follow my own ideas.

Next, I would like to thank Prof. Dr. Andreas Peschel, for agreeing to be my second supervisor.

A big thank you to all the members of the lab who accompanied me during my PhD journey for their support and the daily lunch breaks. I would like to mention three people in particular: Thank you, Lena, for being with me from the beginning. You have become a valuable friend outside of the lab, and I am grateful to have you in my life. Thank you, Jasmin, for your support with all the mouse experiments and flow cytometry experiments. Thank you, Lisa, for supporting me all these years with your passion and all your ideas and for being a friend in and out of the lab and for helping me to improve not only my lab skills but also my MTB skills. I will always remember our fun biking trips in the forest.

Finally, I would like to thank my friends and family for always supporting me and believing in me. Max, thank you for being by my side for so many years and for giving me your unconditional love and support. Thank you for being there after long working days and through all the mental breakdowns and for giving me the confidence to believe in myself. There are no words that can describe how grateful I am to have you in my live.