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Novel aspects of the immunological and structural barrier of the epidermis

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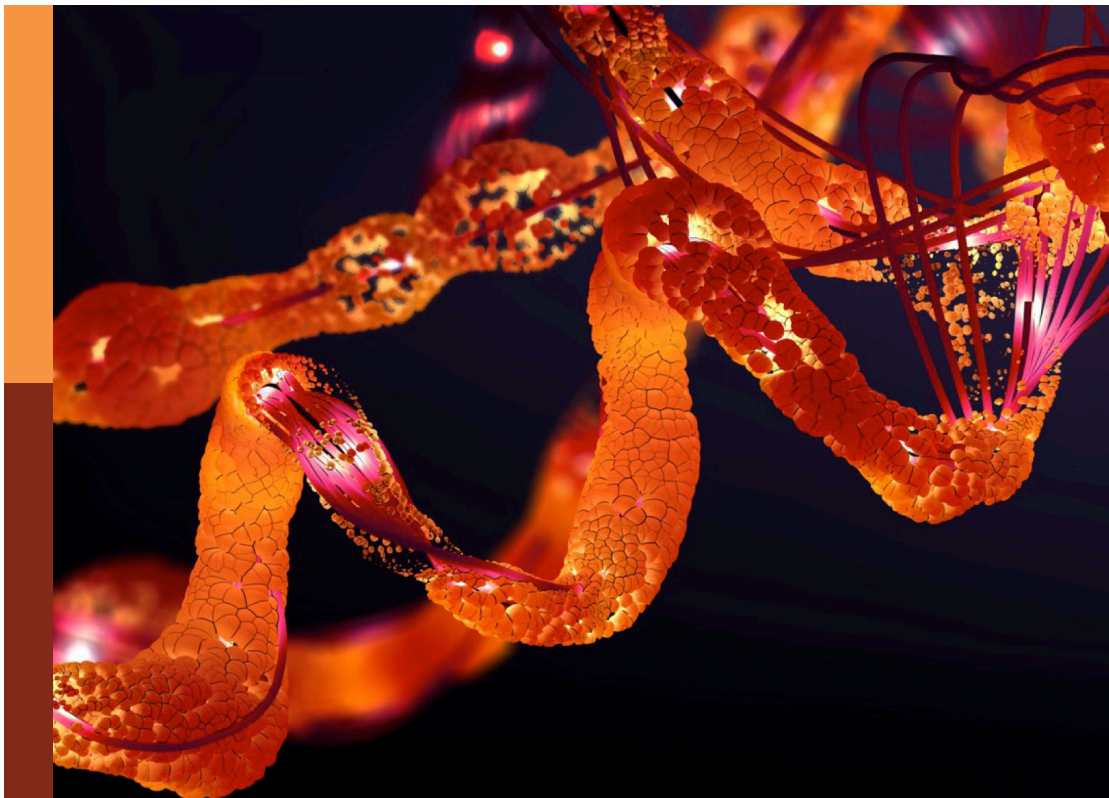

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Novel aspects of the immunological and structural barrier of the epidermis

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Table of contents

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Editorial: Novel aspects of the immunological and structural barrier of the epidermis

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KEYWORDS

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Editorial on the Research Topic
Novel aspects of the immunological and structural barrier of the epidermis

The importance of the epidermal barrier as a multifaceted mechanistic unit supporting whole-body homeostasis continues to grow in prominence. The spotlight again points towards this vital barrier tissue with the recent realization of significant importance for novel aspects in preventing skin diseases and skin infections, that have been tightly associated with atopic dermatitis (AD) and psoriasis. Unchecked, percutaneous defects progress to atopic diathesis and linked systemic manifestations. To this end, the skin provides interlinked mechanical, immunological, and antimicrobial protection, formed through keratinocyte differentiation, generating a multi-layered tissue with a structural and protective function. In addition, multiple cell types including keratinocytes, melanocytes, and immune cells crosstalk within the skin to create the first line of protection against pathogens and environmental threats, through the production of immunological mediators, recruitment of humoral and cellular immunity as well as the hosting of a commensal microbiome. The goal of this Research Topic was to bring together the latest advances and insights of epidermal biology, emphasizing both its immunological and physical aspects, providing a platform to present novel findings and summarise current knowledge, from both basic science and the clinical perspective. To this end, ten publications were accepted covering a broad range of research areas for epidermal barrier function in the form of 4 original articles and 6 reviews.

Multiple functions related to effective epidermal homeostasis has been implicated in various skin diseases. Protein and lipid-related barrier functions are associated with common, complex disorders such as atopic dermatitis (AD) but also many rarer and monogenic disorders with gene defects that result in ichthyosis or scaling phenotypes of the epidermis. One such condition is recessive X-linked ichthyosis. This disorder is caused by genetic defects in steroid sulfatase (*STS*) gene resulting in a cutaneous phenotype but also additional syndromic consequences. The mechanisms related to X-linked ichthyosis are still unclear and therefore McGeoghan et al. investigated the transcriptomic and lipidomic profiles related to *STS* gene deficiency. Findings included major alterations in epidermal differentiation and lipid metabolism as well as differentially expressed genes (DEGs) related to corneal transparency and

behavioural disorders that are syndromic with the cutaneous phenotype. This work provides novel pathomechanistic data that will be important for future studies on X-linked ichthyosis.

Pemphigus represents a group of rare disorders with severe autoimmune blistering of the skin and mucosa. Autoantibodies are produced in pemphigus that target desmosomal adhesion proteins such as desmoglein 3 and desmoglein 1. The desmosome is a major junctional component of the skin responsible for maintaining tissue integrity and acting as an intracellular signalling platform within the epidermis. Lim et al., review the current literature around pemphigus discussing predisposing factors, underlying triggers for autoantibody production, pathogenic mechanisms, and the current emerging therapeutics. The review also provides an update on clinical phenotypes as well as detailed description of the cellular contribution related to T cells and myeloid cells pathogenesis.

Atopic dermatitis (AD) is a chronic inflammatory skin disease with an itchy erythematous rash. The pathomechanism of AD is complex with major contributions from the skin barrier, inflammation and the exposome. The complex genetics of AD has revealed over 50 potential loci that are associated with the phenotype of which the strongest genetic risk factor is *filaggrin* (*FLG*) gene, a skin barrier protein expressed in the upper layers of the epidermis. There is still much work to be done to further investigate DEGs in inflammatory skin diseases such as AD and Li et al., have taken a bioinformatics approach to this challenge. To identify potential effective diagnostic biomarkers in AD the team investigated gene expression from the Gene Expression Omnibus (GEO) database using comprehensive bioinformatics analysis. They identified biomarkers that were subsequently validated with immunohistochemistry showing CCR7, CXCL10, IRF7, MMP1, and RRM2 as potential biomarkers with diagnostic value. Finally, CIBERSORT algorithm was used to evaluate immune cell infiltration with CCR7 correlating with high levels of $CD4^+$ naïve T cells.

Continuing the theme on skin inflammation, Dainichi et al., and co-workers have identified an orphan receptor, G protein-coupled receptor 15 ligand (GPR15L), encoded by *C10Orf99* gene as an important protein increased in frequently occurring skin diseases atopic dermatitis (eczema) and psoriasis. Over-expression of GPR15L reduces the expression of cornified envelope and keratinocyte differentiation markers keratin 10, filaggrin and loricrin, and increased the inflammatory response. Gene expression analyses revealed that GPR15L upregulated pathways and cognate transcription factors involved in signal transduction and stress inducible transcription, while reducing lipid metabolism, all responses that have previous been associated with skin barrier dysfunction.

The study by Kobiela et al., has reported alteration of cellular communication originating from epidermal keratinocytes, as the effect of allergic milieu and AD-relevant skin pathogens. Specifically, the study determined that the combined presence of AD cytokines and a common AD pathogen, *C. albicans*, resulted in an increased propensity of small extracellular vesicles (sEVs), a vehicle of long-distance communication, secreted by keratinocytes to interact with immunosuppressive Siglec receptors. The study also implicated the upregulation of specific enzymes involved in cellular glycosylation, i.e., β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) in remodelling of sEV surface glycans to allow for this keratinocyte related signalling effect to take place.

The theme of the microbiome is further addressed in the review by Baquero et al., who discussed the dynamic interaction between the epidermis and microbes and involving mechanisms of innate cell control. The authors explained how the threshold control is executed over overgrown pathogenic populations while allowing commensals to re-establish the original population density. The review further discussed the role of the epidermis in pathogen transmission and the factors behind interindividual variability in transmission efficiency.

The manuscript by Visscher et al., discusses the formation of the epidermal barrier from the foetal stage through birth and into adolescence, encompassing detailed information on the process of barrier maturation. The authors highlight the remodelling of the barrier proteome and lipidome as well as the importance of the vernix at the various life stages, and also touch on the role of the skin microbiome evolution past birth. The review by Çetinarslan et al. and co-workers provide an update on the contribution of microbiome imbalance, or "dysbiosis", in atopic dermatitis (AD). This covers the complex interplay between innate immunity, lipid metabolism and dysbiosis, discussing the microbiome beyond the role of *Staphylococcus aureus*.

Arginase 1 is a key modulator of the skin microbiome but also regulates various mechanisms related to wound healing. In the review by Szondi et al. and co-workers, the multiple roles of Arginase 1 are discussed, including the well-characterised role in macrophage polarity, and also pro- and anti-inflammatory responses via innate immunity modulation. The review is concluded by describing how defects in Arginase 1 or Arginase 1 function can alter the microbiome of wounds contributing to chronic and non-healing wounds.

Another key component of innate immunity is the IL-1 family of cytokines. The review by MacLeod et al., provides a comprehensive description of the multiple functions of the IL-1 family in detecting external threats, controlling physical barrier function and inflammation. The authors also describe the deleterious effects on skin immunity when the IL-1 signalling pathways are defective. Finally, the potential role the IL-1 family has for translational therapeutics is reviewed with perspectives for future developments.

In conclusion, the articles in this Research Topic present original findings and aggregate prior knowledge on the novel aspects of the epidermal barrier. Appreciation of the importance of the epidermal barrier in physiology and pathology provides an important basis for development of new therapeutic strategies to address the diverse patient groups suffering from epidermal-associated skin diseases and their linked co-morbidities.

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REVIEW
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Arginase Signalling as a Key Player in Chronic Wound Pathophysiology and Healing

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Arginase (ARG) represents an important evolutionarily conserved enzyme that is expressed by multiple cell types in the skin. Arg acts as the mediator of the last step of the urea cycle, thus providing protection against excessive ammonia under homeostatic conditions through the production of L-ornithine and urea. L-ornithine represents the intersection point between the ARG-dependent pathways and the urea cycle, therefore contributing to cell detoxification, proliferation and collagen production. The ARG pathways help balance pro- and anti-inflammatory responses in the context of wound healing. However, local and systemic dysfunctionalities of the ARG pathways have been shown to contribute to the hindrance of the healing process and the occurrence of chronic wounds. This review discusses the functions of ARG in macrophages and fibroblasts while detailing the deleterious implications of a malfunctioning ARG enzyme in chronic skin conditions such as leg ulcers. The review also highlights how ARG links with the microbiota and how this impacts on infected chronic wounds. Lastly, the review depicts chronic wound treatments targeting the ARG pathway, alongside future diagnosis and treatment perspectives.

Keywords: wound healing, arginase (ARG), chronic wounds, polyamines, diabetic foot ulcers (DFU), venous leg ulcers (VLU)

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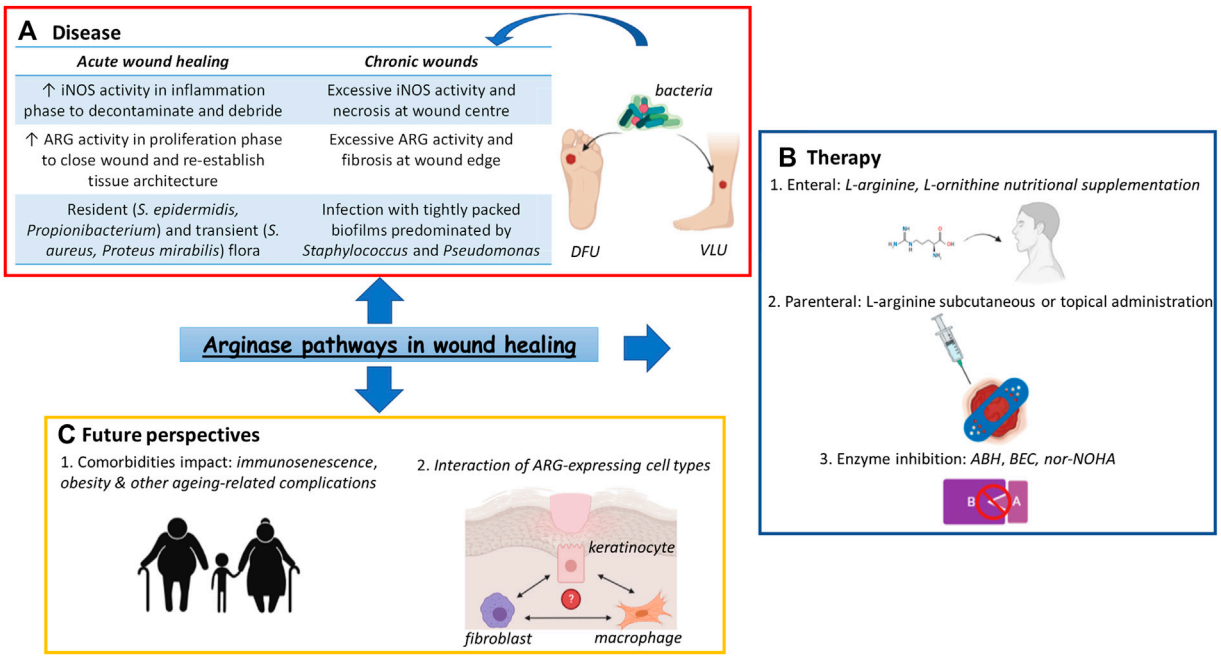
INTRODUCTION

The skin represents a multifaceted organ with a complex architecture and biology. Due to the vast array of skin components and functions, it follows that cutaneous wound healing has to be a well-coordinated cascade of intricate events to ensure a re-establishment of protection against environmental hazards and infections.

Over the last decades, there has been an increasing interest in the Arginase (ARG) pathways and their link to the proper progression through the wound healing cascade. ARG has been shown to have ample physiological implications, due to being the nexus of upstream signalling events as well as downstream metabolism of polyamines and proline (Caldwell et al., 2018). ARG dysfunction has been extensively linked to cardiovascular and neuropathic conditions (Gao et al., 2007; Pernow and Jung 2013; Caldwell et al., 2015). More and more studies are also highlighting a link between this enzyme and impaired wound healing (Jude et al., 1999; Abd-El-Aleem et al., 2000; Campbell et al., 2013; Abd El-Aleem et al., 2019). However, the implications of a malfunctioning ARG enzyme in wound chronicity are not well-understood.



GRAPHICAL ABSTRACT | Arginase (ARG) pathways in wound healing. **(A)** Dysfunctionalities of ARG pathways have been regarded as contributing factors to the occurrence of chronic wounds such as DFUs or VLUs, the presence of bacterial populations adding a further layer of complexity to the pathophysiology of these conditions **(B)** Various treatment options based on the activity of the ARG pathways have been devised, popular choices being amino acid supplementation by both enteral and parenteral routes as well as enzyme inhibitors, such as the potent drug nor-NOHA. **(C)** However, ARG pathways-based monotherapies have showed limited success, highlighting the need to focus on patient comorbidities as well as ARG-expressing cell interactions when devising future chronic wound treatments. Abbreviations: ARG, arginase; ABH, 2(S)-amino-6-boronohexanoic acid; BEC, S-(2-boronoethyl)-L-cysteine; DFU, Diabetic Foot Ulcer; iNOS, inducible Nitric Oxide Synthase; nor-NOHA, Nω-hydroxy-nor-arginine; VLU, Venous Leg Ulcer.

This review aims to give an overview of ARG activity and the current understanding of how it relates to wound healing.

Arginase Pathways Overview

ARG represents an evolutionarily conserved ureohydrolase enzyme involved in the final step of the urea cycle (Dzik 2014). This manganese (Mn)-containing metalloenzyme catalyses the conversion of L-arginine into L-ornithine and urea, thus being crucial for the disposal of toxic nitrogen resulting from amino acid (AA) and nucleotide metabolism (Meijer et al., 1990; Wu and Morris 1998).

The expression and activity of the enzyme ARG has been associated with several intracellular signalling events such as the activation of Rho kinase, Mitogen-Activated Protein Kinase (MAPK) and Protein Kinase A (PKA) (Shatanawi et al., 2011; Chandra et al., 2012). Moreover, multiple wound healing-related cytokines like Transforming Growth Factorβ (TGFβ), Interleukin-4 (IL-4), IL-6, IL-8, IL-13 and Tumour Necrosis Factor α (TNFα) have also been linked to the modulation of ARG activity, TGFβ being one of the most potent activators of ARG1 (Gordon 2003; Gao et al., 2007; Chandra et al., 2012). The Reactive Oxygen Species (ROS) produced early in the wound microenvironment might also modulate ARG activity, the ARG1 promoter region containing potential redox-sensitive elements (Kawamoto et al., 1987). ARG1 is also a CCAAT/Enhancer-Binding Protein-β (C/EBP-β) target gene (Ruffell et al., 2009). The activity of C/EBP-β is antagonised by Protein phosphatase 6 (Pp6), an epidermis-specific Ser/Thr phosphatase is essential for skin homeostasis (Lou et al., 2020).

Enzyme Isoforms and their Distribution

ARG has two isoforms ARG1 and ARG2, each one being encoded by different genes located on separate chromosomes. ARG1 is localised in the cytosol and, although it is mainly expressed in the liver and immune cell populations such as alternatively activated macrophages (AAMs) (Pauleau et al., 2004), it is also found in multiple cell types involved in the wound healing process including fibroblasts, keratinocytes and endothelial cells (Albina et al., 1990; Witte et al., 2002; Kampfer et al., 2003). Endothelial cells express both ARG isoforms (Buga et al., 1996; Bachetti et al., 2004), while smooth muscle cells possess ARG1 activity only (Durante et al., 1997). In fibroblasts ARG1 activity is upregulated by certain growth factors like TGFβ and haemodynamic forces including cyclic stretch (Durante et al., 2000).

ARG2 is present in the mitochondria and is highly expressed in tissues such as the kidneys, brain and retinas (Caldwell et al., 2018). ARG1 consists of 322 AAs (Dizikes et al., 1986), while ARG2 comprises 354 AAs (Gotoh et al., 1996). The two isoforms having a 60% homology in AA residues, whilst the areas critical to their catalytic function have 100% homology (Vockley et al., 1996).

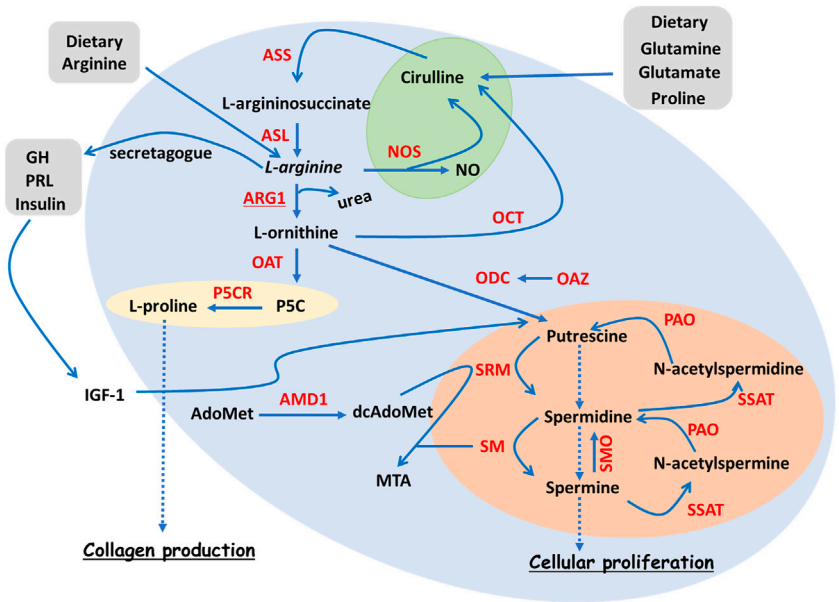


FIGURE 1 | Overview of L-arginine metabolism. Simplified diagram summarising the ARG pathways, together with polyamine and collagen synthesis. The NOS branch of the ARG pathways is highlighted in green, polyamine production in orange and collagen production in yellow. L-arginine is an AA that can be derived either from diet or from recycled L-citrulline deriving from glutamate, glutamine or proline. L-arginine can be metabolised by ARG1 during the urea cycle, L-ornithine and urea being its breakdown products. L-ornithine, in turn, can be the precursor of several molecular events including the synthesis of L-proline via OAT, the synthesis of the polyamine putrescine *via* ODC, and the production of L-citrulline *via* OCT. L-proline represents a key collagen building block. Putrescine, together with its downstream products spermidine and spermine, make up the reversible polyamine pathway that contributes to cell proliferation. An important rate-limiting enzyme of the polyamine pathway is AMD1. L-arginine is also a secretagogue for growth hormone (GH), which contributes to cell proliferation *via* IGF-1-dependent polyamine pathway enhancement. Finally, L-arginine can be metabolised by NOS, the reaction products being NO and L-citrulline. Enzymes are highlighted in red. Abbreviations: AA, amino acid; AdoMet, S-Adenosyl methionine; AMD1, AdoMet decarboxylase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; dcAdoMet, decarboxylated AdoMet; GH, growth hormone; IGF-1, insulin-like growth factor 1; MTA, 5′-methylthioadenosine; NO, nitric oxide; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; OAZ, ornithine decarboxylase antizyme; OCT, ornithine carbamoyl transferase; ODC, ornithine decarboxylase; P5C, L-Δ1-pyrroline-5-carboxylate; P5CR, P5C reductase; PAO, polyamine oxidase; PRL, prolactin; SM, spermine synthase; SMO, spermine oxidase; SRM, spermidine synthase; SSAT, spermidine / spermine N1 -acetyltransferase.

As illustrated in **Figure 1**, Nitric Oxide Synthase (NOS), the antagonist of ARG, is an enzyme catalysing the conversion of L-arginine into L-citrulline and Nitric Oxide (NO) (Schwentker and Billiar 2003). There are three different isoforms of NOS with slightly different functions and cellular localisation, namely neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) (Witte and Barbul 2002; Schwentker and Billiar 2003).

Whilst nNOS and eNOS are discretely expressed in the brain and blood vessels respectively, iNOS is located in a variety of tissues, including the skin (Frank et al., 2002; Nieves and Langkamp-Henken 2002; Witte and Barbul 2003). Both eNOS and nNOS represent Ca^{2+}-dependent isoforms that are constitutively expressed in their specific tissues (Nathan and Xie 1994), whilst iNOS is Ca^{2+}-independent and mainly produced in pathological conditions (Ischiropoulos et al., 1992). This review will mainly focus on the functions of the iNOS and eNOS isoforms.

L-arginine in ARG and NOS Homeostatic Push-And-Pull

L-arginine is a dibasic, conditionally non-essential AA derived from either protein turnover or diet (**Figure 1**). This AA is tightly linked to several metabolic pathways involved in the synthesis of creatine, agmatine, urea, nitric oxide (NO) and polyamines (Witte and Barbul 2003). Decreased L-arginine plasma levels are observed in patients with trauma, burn wounds or those undergoing surgery, this reduction in its availability being associated with the increased metabolic demand triggered by the systemic inflammatory responses (Nijveldt et al., 2000; Ochoa et al., 2001; Yu et al., 2001). Randomised clinical trials in patients with severe skin burns highlight the beneficial effects of L-arginine enteral administration on burn healing, further suggesting a necessary increase in L-arginine catabolism (Yan et al., 2007). This increased L-arginine metabolism in the context of skin wounds is a first clue to the implications of ARG pathway in wound healing.

L-arginine represents the common substrate that ARG and NOS compete for (**Figure 1**). Therefore, under homeostatic conditions, the activities of these two enzyme systems are mutually exclusive. Certain regulatory mechanisms account for further activity control; Nitrite (NO_2), the stable metabolite of NO, suppresses ARG (Hrabak et al., 1996), while the polyamine spermidine, an ARG downstream metabolite, can suppress NOS activity (Szabo et al., 1994).

ARG Pathway and Polyamine Production

Due to being a secretagogue for Growth Hormone (GH) (Nieves and Langkamp-Henken 2002), L-arginine indirectly induces the secretion of Insulin-like Growth Factor-1 (IGF-1) (Merimee et al., 1965; Merimee et al., 1967). IGF-1, which is also important for cell growth, acts, in turn, on Ornithine Decarboxylase (ODC; **Figure 1**) which is the first rate-limiting enzyme involved in polyamine synthesis. ODC decarboxylates L-ornithine into putrescine (Regunathan and Reis 2000; Witte and Barbul 2003). Putrescine is then sequentially converted into spermidine and spermine through the addition of an aminopropyl group from dcAdoMet. dcAdoMet results from the decarboxylation of AdoMet *via* Adenosylmethionine Decarboxylase 1 (AMD1), the second rate-limiting enzyme of the polyamine pathway (**Figure 1**) (Casero et al., 2018).

The polyamines are important for cell growth and proliferation (Langkamp-Henken et al., 1998; Li et al., 2001), being able to modulate a wide range of cellular functions including transcription, translation, cytoskeleton assembly and ion transport (Iarashi and Kashiwagi 2010; Lightfoot and Hall 2014).

ARG, Collagen Production and Fibrosis

Besides polyamines, L-ornithine is also the precursor for proline, the AA produced through the activity of Ornithine Aminotransferase (OAT; **Figure 1**). Proline is one of the main constituents of collagen and its increased production contributes to the post-traumatic increase in wound tensile strength, parenteral administration of L-arginine enhancing wound hydroxyproline levels in the dorsal skin incisions of both control and diabetic Lewis rats (Witte and Barbul 2003; Caldwell et al., 2018). Enhanced collagen production can lead to connective tissue thickening and subsequent fibrosis, as shown by studies based on both the activity of proline hydroxylase and the collagen incorporation rate of radiolabelled proline in skin tissue explants (Craig 1975; Rockwell et al., 1989). The enzymatic activity of proline hydroxylase correlates well with the rate of collagen synthesis, and the activity of this enzyme is markedly increased in the fibrotic areas of hypertrophic and keloid scars, when compared to normal scars (Craig 1975), thus putatively linking an enhanced collagen synthesis to the aetiology of fibrosis. The collagen synthesis rate was determined to be 3 times higher in hypertrophic scars and 20 times higher in keloids, when compared to normal scars (Rockwell et al., 1989). Fibrotic areas can also be seen in the dermal tissue surrounding chronic wounds (Abd-El-Aleem et al., 2000; Eming et al., 2014).

Wound healing represents an intricate multifactorial process whose slight dysregulations can impair its overall success. Recent studies have implicated the ARG pathway in several wound healing-related cell types and stages with macrophages being the most extensively studied (Campbell et al., 2013). However, given its wide expression in the wound site it is likely that arginase in cell types such as fibroblasts and keratinocytes will play an important role in healing and their contribution is less well-defined but should not be underestimated. Irrespective of the sources of ARG in the wound it is important to consider the impact of ARG on would healing. The following sections will review the involvement of ARG in key stages of wound healing, while emphasising the current understanding of the link between the ARG pathways and skin healing impairments.

ARG and iNOS in acute Wound Healing

In acute wounds, iNOS activity predominates in the early inflammatory stage where the wound microenvironment is cytotoxic. Infiltrating inflammatory cells such as macrophages and neutrophils synthesize large amounts of NO (Lyons et al., 1992). The effects of NO release, including anti-microbial activity (Granger et al., 1988), vasodilation (Stuehr et al., 1989), and anti-platelet aggregation activity (Salvemini et al., 1989), are important for the initial wound decontamination and debridement.

In the late healing stages, iNOS levels return to normal and ARG activity predominates (Albina et al., 1990), leading to the production of proline for collagen, and polyamines which are essential for cell growth, differentiation and matrix modelling (Sarhan et al., 1992; Shearer et al., 1997). Intracellular polyamine levels increase in response to cellular damage, this increase highlighting their putative role in the proliferative stage of wound healing (Casero and Pegg 2009; Gao et al., 2013). Moreover, a change in the putrescine/spermine ratio is important for epithelial tongue migration and wound re-epithelialisation *via* Urokinase-type Plasminogen Activator (uPA)/uPA receptor (uPAR)-driven actin cytoskeleton reorganisation (Lim et al., 2018).

Due to the complexity of the wound healing phases, the variable skin architecture of the outbred human population and differing microbial residency, impairments of skin-related molecular/cellular mechanisms can arise, leading to the occurrence of chronic wounds. The next sections are focused on the implication of the ARG pathways in the pathophysiology of chronic skin conditions, including ulcers and infected chronic wounds. Attention is given to the role that ARG plays in key cells of the wound healing process such as macrophages and fibroblasts. Current ARG pathway-based chronic wound treatments are also described, and future perspectives are emphasised.

MACROPHAGES, ARG PATHWAY AND WOUND HEALING

Macrophages represent a crucial immune cell population with extensive physiological implications, among which is the regulation of wound healing progression. The importance of macrophages in the wound microenvironment is going to be detailed in the next section, emphasis being given to the activity of the ARG pathway in this cell population.

Macrophages as Master Regulators of Wound Healing

Whilst macrophages seem to be dispensable in early foetal wounds (Martin et al., 2003), several murine knockout models have highlighted the need for macrophages in normal adult

wound healing (Nagaoka et al., 2000; Peters et al., 2005; Ishida et al., 2008).

Macrophages persist throughout the entire healing response, their numbers increasing during the inflammatory stage, peaking during the tissue formation phase and finally declining during the maturation step (Martin and Leibovich 2005). The use of Diphtheria Toxin (DT)-mediated macrophage depletion in Lysozyme M (LysM) Cre/inducible Diphtheria Toxin receptor (iDTR) mice at early, mid and late wound repair stages showed the importance of macrophage involvement throughout the entire healing process. Inflammatory phase-restricted macrophage depletion led to impaired granulation tissue formation and re-epithelialisation, while the mid proliferative stage-restricted macrophage ablation resulted in severe wound site haemorrhage (Lucas et al., 2010).

In light of their ubiquity and extensive functions, Lawrence et al. have described macrophages as the "orchestra leaders" of adult wound healing (Lawrence and Diegelmann 1994). Critical to the function of macrophages in the healing process is their plasticity.

Macrophage Plasticity and ARG Pathway

As far as the implications of macrophage-related L-arginine metabolism are concerned, the initially high levels of wound site pro-inflammatory cytokines, such as Interferon γ (IFNγ), TNFα and IL-1β, stimulate the iNOS-dependent production of large amounts of NO by pro-inflammatory classically activated (CAM) or M1 macrophages (Witte and Barbul 2002). NO may then spontaneously react with O_2^-, thus yielding toxic peroxynitrous acids, peroxynitrite and hydroxyl (OH^-) radicals involved in pathogen killing (Beckman et al., 1990; Radi et al., 1991; Jude et al., 1999; Goldman 2004). In contrast, macrophage stimulation via cytokines such as IL-4, IL-10, IL-13 and IL-33 leads to a tissue reparative alternatively activated (AAM) M2 macrophage phenotype (Locati et al., 2013; Wang et al., 2014) associated with an enhanced ARG1 expression and activity (Munder et al., 1998; Pauleau et al., 2004). This cytokine-based phenotype switch highlights the plasticity of these heterogeneous leukocytes and its dependency on their surrounding milieu (Modolell et al., 1995; Shapouri-Moghaddam et al., 2018).

Studies focusing on the dichotomous far ends of the macrophage phenotypic spectrum, namely CAMs/M1 and AAMs/M2, have suggested that local macrophage ARG1 activity is vital for proper cutaneous wound healing (Gordon and Taylor 2005; Mosser and Edwards 2008). Differentiated macrophages appear to have distinct roles in the healing process-the activity of iNOS predominating in the early stages of wound healing in CAMs followed by high ARG activity later in the healing process in AAMs (Campbell et al., 2013; Krzyszczyk et al., 2018). Moreover, both the local pharmacological inhibition of ARG using nor- N^{ω}-hydroxy-L-arginine (nor-NOHA) (Tenu et al., 1999; Takahashi et al., 2010) and the Tie2-mediated conditional ablation of ARG1 (El Kasmi et al., 2008) triggered pronounced delays in wound healing, suggesting a possible link between chronic wounds, ARG dysfunction and macrophage phenotypic transition.

This phenotypic transition from a M1 pro-inflammatory to a M2 anti-inflammatory macrophage was manipulated by Kim et al. *via* an exosome-guided reprogramming of cell polarity, with the purpose of accelerating wound healing. The subcutaneous administration of high purity M2-exosomes into the excisional wound edge of 5-week-old male Balb/c mice led to better wound healing outcomes by means of a successful M1 to M2 phenotypic switch although only CD86, iNOS and CD206, ARG1 were used to distinguish M1 and M2 macrophages respectively and fuller characterisation was not done (Kim et al., 2019). Even if promising, the therapeutic viability of this *in situ* exosome-based phenotypical transition remains questionable as the study did not test their effectiveness in animal models with underlying pathological conditions. Moreover, a challenge in studying macrophage function includes the usage of iNOS and ARG1 as markers of M1 vs. M2 phenotypes, as certain macrophages (such as the alveolar macrophages) express both markers (Thomas and Mattila 2014). Mouse age may pose a further difficulty, especially from a translational perspective, the incidence of wound healing problems and chronicity increasing with age (Jackson et al., 2017).

Due to their pervasive role in the wound healing process, it becomes obvious that macrophage malfunctions are a likely contributor to the pathophysiology of chronic wound impairments. The next section will focus on the underlying causes of skin ulcers while highlighting the implication of ARG1 and macrophage dysfunction in impaired healing/chronicity.

ARG1 in Chronic Wounds

Owing to its expression in several cell types involved in wound healing as well as its ability to influence cell proliferation and collagen deposition by means of its downstream metabolites (Caldwell et al., 2018), it can be assumed that ARG might play a role in the non-healing status of chronic wounds. Ulcers represent prevalent chronic lesions that arise as a result of halted progression through the wound healing cascade (Agren et al., 2000). Given that ARG is involved in wound healing and skin ulcers represent a sequela of impaired cutaneous healing, the possible relationship between a malfunctioning ARG pathway and the underlying aetiology of ulcers is important to consider.

Ulcers represent a major concern from both a clinical and economic point of view, as the number of patients suffering from chronic wounds is reaching epidemic proportions (Brem and Tomic-Canic 2007; Sen et al., 2009; Guest et al., 2018). Moreover, the aging, multi-morbid population poses a further burden to the clinical world, the management of ulcers having to cater for both the systemic conditions such as diabetes as well as the local wound disorders (Eming et al., 2014). Depending on the underlying causes, there are several types of ulcers (Eming et al., 2014). For the purpose of this review, Venous Leg Ulcers (VLUs) and Diabetic Foot Ulcers (DFU) will be the main focus. However, it is worth noting that Arterial Ulcers (AU) and Pressure Ulcers (PU) represent important clinical challenges as well (Eming et al., 2014).

TABLE 1 | Summary of key ARG pathway-related pathological commonalities between DFUs and VLUs. Although NOS and ARG are known to have competitive enzymatic activities when expressed within the same cell, they both contribute to DFUs and VLUs, as they show discrete wound expression sites. iNOS is mainly present in endothelial cells and macrophages present at the ulcer base, contributing to defective matrix deposition and necrosis *via* excessive NO. ARG is mainly expressed in the dermal fibroblasts of the skin surrounding the ulcer itself. The increased ARG activity of both ulcer types is associated with fibrosis surrounding the ulcerations, resulting in either calluses or lipodermatosclerosis in DFUs and VLUs respectively. The enhanced levels of the cytokine TGF-β noted in the sclerotic skin adjacent to the venous ulcer further contributes to this excessive ARG activity.

Category	Feature	References
ARG activity and distribution	Hyperactive ARG in the fibroblasts, macrophages and endothelial cells of surrounding dermis leading to pathological fibrosis surrounding ARG-hindered ulceration	Herrick et al. (1992) Jude et al. (1999) Abd-El-Aleem et al. (2000)
NOS activity and distribution	Upregulated e/iNOS activity in wound bed M1 pro-inflammatory macrophages leading to large amounts of NO and subsequent necrosis/ulceration	Albina et al. (1993) Messmer et al. (1994) Lin et al. (1995) Jude et al. (1999)
Inflammatory status	Perpetuated M1 pro-inflammatory macrophages contributing to protracted inflammation *via* TNFα, IL1 and IL6 within ulceration, and hindering transition to ARG-related proliferative stage	Sindrilaru et al. (2011)
Expression and distribution of proteolytic enzymes	Excessive wound bed ECM breakdown due to abnormal levels of macrophage/neutrophil MMP2 and MMP9 overriding ARG-dependent ECM deposition	Herrick et al. (1997)
ROS and tissue oxidative stress	Excessive ROS-dependent DNA damage causing fibroblast senescence within wound bed and impairing ECM deposition, despite enhanced ARG activity	di Fagagna et al. (2003) Garinis et al. (2009)
Growth factors	High levels of ARG-inducing TGF-β in sclerotic skin adjacent to ulcer, but drastically reduced within ulcer per se	Higley et al. (1995) Boutard et al. (1995) Stevens et al. (1997)
Angiopathies	Perivascular cuffs and reduced compliance associated to proliferative and matrix deposition effects of excessive ARG activity	Hagenfeldt et al. (1989) Pieper and Dondlinger (1997) Kovamees et al. (2016)

VLUs represent a common form of leg ulcers with increased incidence among the elderly. VLUs are one of the most severe symptoms of low extremity chronic venous insufficiency (Bergan et al., 2006; Raju and Neglén 2009). DFUs are regarded as one of the most common reasons for hospital admission among diabetic patients (Williams 1985), leading to 50–70% of all non-traumatic amputations (Boulton 1997; Margolis et al., 2005). Even more worrying is the anticipation that the prevalence of Type 2 Diabetes Mellitus (T2DM) will increase from 6.4 to 8% in the world population by the year 2030 making the incidence of DFUs potentially even more common (Nolan et al., 2011).

VLU and DFU Pathophysiology Overview

VLUs are characterised by venous hypertension, persistent inflammation, hemosiderin deposition and lipodermatosclerosis (Eming et al., 2014). This chronic inflammatory state suggests a failure of VLUs to progress through the physiological pattern of wound healing stages (Agren et al., 2000; Smith 2001; Wlaschek and Scharffetter-Kochanek 2005). The hallmarks of DFUs are hyperglycaemia, micro-/macroangiopathy, neuropathy and infection, the condition usually culminating in foot deformities (Eming et al., 2014). Similar to VLUs, diabetic wounds show an unresolved inflammatory state, suggesting a hindrance of the transition from the pro-inflammatory to the proliferative stage of wound healing (Sibbald and Woo 2008).

Increased NOS Activity in Both VLUs and DFUs

In chronic venous ulcer histological sections, the expression of both eNOS and iNOS was increased when compared to normal skin samples (Shimizu et al., 1997; Abd-El-Aleem et al., 2000). This enhanced NOS expression correlated with an increase in NOS activity, suggesting a higher NO production than that of normal skin tissue (Abd-El-Aleem et al., 2000). In addition, a venous stasis-dependent accumulation of NOS-expressing M1 macrophages is one of the prevalent features of VLUs (Rosner et al., 1995; Abd-El-Aleem et al., 2000), with hypoxia and the low shear stress contributing to the transmigration of such "trapped" leukocytes into the tissue. Once there, they release high amounts of ROS such as Fenton reaction-derived OH^- (Yeoh-Ellerton and Stacey 2003), proteolytic enzymes like MMP2 or MMP9 (Wysocki et al., 1993), and proinflammatory cytokines such as TNFα, IL-1 and IL-6 (Tarnuzzer and Schultz 1996). These contribute to extensive connective tissue breakdown and subsequent ulcerations (Wlaschek and Scharffetter-Kochanek 2005). The large amounts of NO produced *via* iNOS activity interact with the ROS resulting from the respiratory burst of the macrophages/neutrophils present at the wound site, leading to the formation of peroxynitrite (Beckman et al., 1990). Both NO and peroxynitrite cause apoptotic cell death, thus contributing to the damage seen in chronic venous ulcers (Albina et al., 1993; Messmer et al., 1994; Lin et al., 1995). Moreover, Sindrilaru et al. have directly linked the persistent inflammation and ECM breakdown of VLUs with a perpetuated M1 proinflammatory state of iron-overloaded macrophages, as iron chelation, etanercept-dependent TNFα inhibition and clodronate-induced macrophage depletion improve the phenotype of these ulcers (Sindrilaru et al., 2011).

As far as diabetic ulcers are concerned, eNOS is increased at the base and edge of the ulcer in DFUs when compared to normal and diabetic human skin, thus showing similarities to the eNOS

expression seen in VLUs (**Table 1**). In addition, an increased iNOS expression was noted at the ulcer margins, predominantly localised to the smooth muscle cells of the blood vessels as well as the infiltrated macrophages (Jude et al., 1999). The similarity between this iNOS localisation and that seen in VLUs provides a further commonality between these two ulcer types (**Table 1**). As well as expression of eNOS and iNOS, there is an increased total NOS activity in DFUs, when compared to normal and diabetic skin and the NOS is thought to be largely macrophage derived (Jude et al., 1999). Similar findings have been reported in rodent models (Stevens et al., 1997). Upregulated catalytic activity in DFU patients was also mirrored by increased plasma NO_2 levels, and treatment with insulin lowered NO_2 concentration in these sufferers (Stevens et al., 1997; Jude et al., 1999). The excessive levels of nitrite may provide a likely explanation for the DFU-associated neuropathies. The increased NOS activity of ulcers was further corroborated in a recent study by Dixit et al. conducted in 61 patients with chronic wound cases, DFUs and VLUs being the most predominant lesions. By comparing the tissue and serum enzyme activities at initial patient presentation and 12-weeks follow-ups, an increased expression and activity of NOS was noted at the second time point (Dixit et al., 2021). Thus, this further highlights the putative metabolic implications of the NOS isoforms in chronic wound healing.

Increased ARG Activity in Both VLUs and DFUs

Perhaps surprisingly given the high levels of NOS, VLUs show increased ARG activity concentrated in fibroblast- and macrophage-like cells present in both the epidermis and the dermis surrounding the wound bed (Abd-El-Aleem et al., 2000; Sindrilaru et al. 2011). However, these macrophages fail to fully switch from a pro-inflammatory M1 state to a tissue repair M2 one and transiently co-express high levels of M1 associated markers such as TNFα and C-C chemokine receptor type 2 (CCR2) as well as intermediate levels of M2 markers like ARG, CD36 and CD206 (Sindrilaru et al., 2011).

Despite the upregulation of ARG in chronic venous ulcers, the matrix deposition was defective. The upregulation of certain proteases like Ser proteases and neutrophil elastases might explain this discrepancy, as their excessive proteolytic activity leads to the breakdown of fibronectin within the wound bed (Herrick et al., 1997). The lack of a fibronectin scaffold for fibroblast integrin-dependent migration hinders their matrix deposition capabilities. In addition, the enhanced release of ROS, especially OH^- and peroxynitrite, by the macrophage population described by Sindrilaru et al. is a likely contributor to this defective matrix deposition. The extensive ROS-related DNA damage (Szabo et al., 2007) triggers a senescent program in skin resident fibroblasts (di Fagagna et al., 2003; Garinis et al., 2009), thus impairing their tissue repair capabilities (Sindrilaru et al., 2011). Higher ARG levels may, therefore, be explained, in part, as a consequence of a failed compensatory mechanism for the extensive wound bed tissue breakdown.

Akin to VLUs, ARG expression is high in dermal fibroblasts and endothelial cells (Jude et al., 1999, **Table 1**). This ARG upregulation was shown to be involved in the pathological fibrosis seen in the callus that surrounds the DFU (Jude et al., 1999) and has also been described in rodent studies (Kampfer et al., 2003; Miao et al., 2012). Similarly, significantly increased ARG has been reported in chronic skin wounds 12 weeks after initial presentation (Dixit et al., 2021). It is not fully known why these defective transitions occur in ulcers but one factor may be due to a lack of critical cytokines such as TGFβ1 in the wound environment.

Lack of TGFβ as an Ulceration-Related Factor

When compared to both normal and diabetic skin, macrophage TGFβ1 expression in DFUs is drastically reduced or even absent (Stevens et al., 1997; Jude et al., 1999). Given that TGFβ increases the ARG activity at the expense of NOS activity (Boutard et al., 1995), this lack of TGFβ1 in the ulcer macrophages translates into an impaired transition from the tissue debridement to the tissue repair stage. This provides a possible reason for the NO-rich necrotic area characteristic of DFUs (Ferguson et al., 1996), a raised and sustained NOS activity being deleterious. Moreover, the diabetes-specific hyperglycaemic environment impairs the normal phenotypic transition of wound macrophages as well as their iNOS/ARG activity with compromised responses to their instructive cytokines being noted (Gordon 2003; Gordon and Taylor 2005; Miao et al., 2012). However, much remains to be learnt about factors involved in driving macrophage polarisation with the chronic wound bed.

Systemic Complications of Excessive Ulcer ARG1 Activity

The high levels of ARG1 noted in several cell types present within and around the granulation tissue of both VLU and DFUs, including fibroblasts, macrophages, endothelial and vascular smooth muscle cells, might contribute to systemic complications as well. A polyamine-driven hyperplasia or hypertrophy of blood vessels may lead to the underlying venous hypertension characteristic of VLU patients. What is more, the ARG1-triggered excessive collagen deposition further contributes to this hypertensive phenotype, due to the thickening and stiffening of blood vessels (**Table 1**). These blood vessel architecture-related assumptions are corroborated by studies showing that coronary arteries isolated from diabetes patients had increased ARG1 expression and exhibited an impaired endothelium-dependent vasorelaxation (Bagi et al., 2013). Furthermore, other studies highlighted a correlation between increased ARG activity, decreased plasma L-arginine levels and endothelial dysfunction in type 2 diabetic patients and diabetic rats, reinforcing the idea of ARG-dependent pathological vascular remodelling, fibrosis and decreased compliance in DFUs (**Table 1**) (Hagenfeldt et al., 1989; Pieper and Dondlinger 1997; Kovamees et al., 2016). However, this hyperactive ARG does not help with tissue deposition and repair within the wound per se.

This section emphasises the possible implications of ARG in ulcers at both local and systemic levels while highlighting the

similarities between DFUs and VLUs. This suggests that there might be a certain molecular common ground between these two ulcer types which would be worth exploiting when it comes to therapeutical options. Indeed studies have highlighted the etiological commonalities between different ulcerations (Chen and Rogers 2007).

In addition to the putative implications of ARG in conditions like ulcers, its activity is also associated with the balance micro-organismal populations residing on the skin. Skin infection in chronic wounds is a major risk factor in poor healing. Therefore, the next section studies the implications of ARG in the context of chronic infected wounds.

ARG AND THE SKIN MICROBIOTA

The microbial composition of wounds also play an important role in the healing process and alterations in skin bacterial communities have been associated with VLUs and DFUs, among other skin conditions (Hannigan and Grice 2013; Weyrich et al., 2015). ARG activity has been linked to the presence of certain pathogens within the wound microenvironment, further suggesting the far-reaching implications of this enzyme in wound healing (Debats et al., 2006). This section will explore the changes seen in wound-related microbiota, as opposed to normal skin microorganisms, and how these impact on the activity of the ARG pathway and vice versa.

Homeostatic and Wound-Related Skin Microbiota

The skin is heavily populated by bacteria, viruses, archaea and fungi, all contributing to the holobiont. There is a constant crosstalk between the skin microorganism and the host as changes in the microbial communities populating the skin can trigger the expression of antimicrobial peptides and inflammatory molecules (Zeeuwen et al., 2012). Even in the absence of overt signs of infection, the impairment seen in certain chronic wounds has been associated with the presence of a specific bioburden consisting of bacteria belonging to the *Staphylococcus, Pseudomonas, Peptoniphilus, Enterobacter, Stenotrophomonas, Finegoldia, and Serratia* genera (Gardner et al., 2013; Eming et al., 2014; Misic et al., 2014; Rahim et al., 2017). These polymicrobial communities exist mainly in the form of tightly packed aggregates with synergistic virulence known as biofilms (James et al., 2008). The biofilm composition differs from that of normal skin, the latter consisting mainly of resident flora such as *S. epidermidis* or *Propionibacterium*, and transient flora such as *S. aureus* or *Proteus mirabilis*, different body parts having varying micro-organismal proportions or abundances (Rahim et al., 2017).

Hallmarks of Infected Chronic Wounds and Impact on L-arginine Metabolism

According to a comparative study between patients displaying acute, chronic non-infected and chronic infected wounds, acute wounds have considerably lower indicators of inflammation and a lack of infection signs, with no bacteria cultured from their wound fluid (Debats et al., 2006) whereas infected chronic wounds are often associated with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus mirabillis* (Debats et al., 2006). Considerably decreased levels of plasma L-arginine were noted in patients with infected chronic wounds, when compared with both acute and non-infected chronic wound patients (Debats et al., 2006). Despite decreased plasma L-arginine, no differences in systemic inflammatory markers such as Erythrocyte Sedimentation Rate (ESR) and albumin were noted when infected chronic wounds were compared with their non-infected counterparts (Debats et al., 2006) indicative of a local rather than a systemic response. Therefore, this suggests an increased uptake and metabolism of L-arginine at the site of chronic infected lesions, the infection being the likely driver of this increased metabolic activity. However, it should be pointed out that both ESR and albumin are crude, non-specific markers of systemic inflammation, certain endogenous pyrogens, such as IL-6 and TNFα, representing better options.

Wound fluid L-citrulline, L-ornithine and ARG1 levels are significantly increased in infected chronic wounds, when compared to non-infected wounds and normal skin (Debats et al., 2006). These increased levels of certain AA at the wound site may be explained by an extensive proteolysis. However, L-ornithine is a non-proteinogenic AA and, hence, it is not incorporated into polypeptide chains during protein biosynthesis. Therefore, it is unlikely that L-ornithine is released by proteolysis. This seems to suggests that the increase in wound fluid L-ornithine is the likely consequence of an increased ARG1 activity (Debats et al., 2006). Although the synthesis and breakdown of L-ornithine are important for producing wound healing-related metabolites (**Figure 1**) (Bachrach et al., 2001; Urdiales et al., 2001), these excessive L-ornithine levels paradoxically contribute to wound chronicity rather than accelerated wound closure.

Significantly decreased levels of the NO metabolites nitrite and nitrate were noted in infected chronic wounds, while their plasma levels were similar to that of acute and non-infected chronic lesions (Debats et al., 2006). This, together with enhanced ARG levels, strongly suggests that the NOS activity within the chronic infected wound is reduced. However, the increase in wound citrulline production then seems paradoxical. An explanation for the unexpected increase in citrulline is provided by the presence of Arginine Deiminase (ADI)-expressing bacteria like *P. aeruginosa* and *E. coli*. This enzyme can catalyse the direct conversion of L-arginine into citrulline (Galkin et al., 2004; Debats et al., 2006). Indeed, when cultured in the fluid derived from infected wounds, these bacteria use L-arginine in order to generate energy in the form of ATP (Theil et al., 1969; Lu et al., 1999), therefore the inherent bacterial catalytic ability may account for overall increased wound citrulline. Moreover, the decreased production of bactericidal NO may further contribute to defective barrier function in infected wounds where microorganism growth is not being hindered by a NO-rich microenvironment. This seems to suggest that the excessive ARG activity may contribute to bacterial growth and thus

impaired wound healing of infected lesions. However, macrophage ARG1 activity has been shown to play a central role in restricting bacterial growth and restraining tissue damage in hypoxic tuberculosis granulomas (Duque-Correa et al., 2014), suggesting that the relationship between ARG activity and bacterial burden might be tissue- and pathology-specific.

In light of what has been said, the presence of an infection is a likely contributor to the hindered progression of the wound healing process and it is notable that successful wound closure is associated with a low microbial count (Edwards and Harding 2004). Nevertheless, it is worth saying that certain studies emphasise the importance of bacterial colonisation, setting a threshold of 10^4 Colony Forming Units (CFUs)/ml as a delineation between colonisation and wound healing-impeding infection (Trengove et al., 1996). Moreover, isolates from different parts of the same wound house different organisms (Schneider et al., 1983). Furthermore, the stability of the chronic wound microbiota is important as successful healing is associated with a flexible dynamic microbiota (Loesche et al., 2017).

Owing to the intricate interplay between molecular, cellular and microbial components associated with impaired wound healing, the difficulty of devising highly effective chronic wound treatments becomes obvious. The following section highlights some of the most common chronic wound therapeutical approaches based on the modulation of the ARG pathway.

ARG PATHWAY-BASED CHRONIC WOUND TREATMENT OPTIONS

Given the far-reaching implications of the ARG pathway in wound healing and pathology as well as the extensive web of molecular components contributing to this pathway, the search for its druggable targets seems to be a sensible step in the attempt to design a multimodal treatment for chronic skin conditions. While some treatments focus on the AAs of this pathway by means of nutritional or parenteral supplementation, other current monotherapies aim to target the enzymatic nodes of the ARG pathway via pharmacological modulation. Both of these approaches will be discussed in this section.

Nutritional Therapy

Nutritional factors have a crucial role in the development of chronic wounds (Schaffer et al., 1997). Nutritional therapy involving the intake of vitamins A and C (MacKay and Miller 2003) or proteins (Ruberg 1984; Breslow et al., 1993) in malnourished patients with chronic wounds has led to an improvement in wound healing, although complete wound closure was not observed (Thomas 2001; Houwing et al., 2003). A more targeted strategy proposes using the supplementation of specific AAs known to be of great importance in the wound healing cascade of events (Angele et al., 2002; Witte et al., 2002; Shi et al., 2003) such as L-arginine and L-Ornithine (Barbul et al., 1990; Albina et al., 1993; Efron and Barbul 2000). Unfortunately, little is known about the effects of the addition of exogenous L-arginine to chronic wounds, as to date enteral L-arginine supplementation was mainly studied in acute artificially induced wounds, in both humans and rodents. Parenteral means of administration have also been studied, intravenous, intraperitoneal, subcutaneous and dressing-based topical applications of L-arginine being the most common approaches. Both enteral and parenteral supplementation contributed to increased collagen synthesis and enhanced wound breaking strength (Stechmiller et al., 2005).

L-arginine supplementation has been associated with enhanced production of NO at the chronic wound site (Norris et al., 1995; Schaffer et al., 1996; Pollock et al., 2001) which may then contribute to granulation tissue formation, immune responsiveness and angiogenesis (Yamasaki et al., 1998; Schaffer et al., 1999; Witte and Barbul, 2002). Furthermore, L-arginine supplementation has been regarded as an adjunct treatment for either normalising or enhancing wound healing, since the body does not produce sufficient amounts of this AA during metabolically-demanding times of stress (Albina et al., 1988; Witte and Barbul 2003). Proper levels of L-ornithine are crucial for the production of proline (Albina et al., 1993), an essential AA for collagen synthesis (Morris 1992), and polyamines, key players in cell growth and proliferation (Dolynchuk et al., 1994; Wu and Morris 1998). L-ornithine may also regulate the catalytic activity of ARG via a feedback loop mechanism as high AA levels could be an inhibitor of ARG activity (Hunter and Downs 1945). Dietary polyamines should not be excluded as a treatment option either, as systemic administration of spermidine led to accelerated skin wound healing in a mouse excisional back wound model (Ito et al., 2021).

ARG-promoting treatments might also be worth considering. A biomaterial-based controlled release of IL-4 prompted a switch from M1 to ARG1-expressing M2 phenotype in RAW 264.7 macrophages, while the treatment of the same macrophage cell line with Echinacea-derived alcohol extracts enhanced their ARG activity (Zhai et al., 2009; Yang et al., 2018). These treatments, even though restricted to macrophage alternative activation and the enhancement of their ARG enzymatic activity, might prove to have overarching anti-inflammatory implications in the overall chronic wound healing outcome. However much remains to be elucidated about arginase modulation or its downstream metabolites supplementation in the healing response.

Pharmacological Antagonism of ARG Activity

Pharmacological inhibitors of ARG or NOS represent other therapeutic means intended for targeting the activity of ARG itself. DFMO, a potent inhibitor of OCD, acts as a weak, nonspecific inhibitor of ARG (**Table 2**) (Morris 2009). The ARG inhibitory effect of the DFMO treatment can be mediated by the accumulation of L-ornithine feedbacking on ARG and limiting its activity (Hunter and Downs 1945; Caldwell et al., 2018). The potent inhibition of OCD by DFMO would also limit the polyamine production, this limitation preventing the backward metabolism of spermidine/spermine by PAO (**Figure 1**), therefore reducing the oxidative stress of the wound site (Caldwell et al., 2018). Two potent

TABLE 2 | Summary of key ARG-pathway related drug pharmacodynamics. The inhibitory mechanisms of ARG pathway-targeting drugs are given together with their inhibition constants (Ki). * iNOS-specific range.

Drug	Pharmacology	K_i	References
ABH	commercially available for preclinical use; highly specific competitive ARG inhibitor; its boronic head binds to the catalytic Mn(II) cluster of ARG	0.25 μM	Baggio et al. (1999) Colleluori and Ash (2001) Berkowitz et al. (2003) Caldwell et al. (2018)
AG	selective irreversible inhibitor of cytokine-induced iNOS; L-arginine analogue competitive at substrate and gene level	NA	Salinas-Carmona et al. (2020)
BEC	commercially available for preclinical use; highly selective competitive ARG inhibitor; its boronic head binds to the catalytic Mn(II) cluster of ARG	0.31 μM	Baggio et al. (1999) Colleluori and Ash (2001) Berkowitz et al. (2003) Caldwell et al. (2018)
DFMO	weak indirect inhibitor of ARG via potent ODC inhibition and L-ornithine accumulation feedback	3.9 mM	Selamnia et al. (1998) Morris (2009)
L-NAME	non-selective competitive reversible NOS inhibitor	4–65 μM *	Rees et al. (1990) Furfine et al. (1993)
NOHA	potent competitive endogenous ARG inhibitor; L-arginine analogue binding the ARG Mn(II) cluster; also acts as an intermediate in NOS-dependent L-citrulline production, thus being a bisubstrate	10 μM	Daghigh et al. (1994) Custot et al. (1997) Caldwell et al. (2018)
nor-NOHA	commercially available for preclinical use; reversible selective ARG inhibitor; more potent than its longer NOHA analogue; binds ARG Mn(II) cluster	0.5 μM	Custot et al. (1997) Caldwell et al. (2018)
SC-842	selective iNOS inhibitor; no further details found in literature	NA	Bell et al. (2007)

pharmacological inhibitors of ARG are NOHA and its analogue nor-NOHA which has a longer half-life (**Table 2**). However, the shortcoming of NOHA is that it is an intermediate metabolite in the NOS-mediated pathway of L-arginine breakdown (Caldwell et al., 2018).

Competitive ARG inhibitors with high specificity have been developed, their design being based on the determination of the crystal structure of human ARG1/2 (Kanyo et al., 1996; Di Costanzo et al., 2005). These highly selective ARG inhibitors are S-(2-boronoethyl)-L-cysteine (BEC) and 2(S)-amino-6-boronohexanoic acid (ABH) (Baggio et al., 1999) and their strong competitive inhibition results from the binding of their boronic heads to the Mn cluster found at the catalytic site of ARG (**Table 2**) (Berkowitz et al., 2003). Despite their specificity for ARG, the drawback of using these pharmacological inhibitors is that they are not isoform-selective (Caldwell et al., 2018). Furthermore, there may be unintended consequences of blocking arginase indiscriminately as it expressed by so many cell types in the skin. The proper design of isotype-selective, cell-specific inhibitors may facilitate the future development of strategies aimed at modulating the enzymatic activity of ARG in order to restore its homeostatic activity in pathological conditions.

Of the ARG inhibitors described in this section, nor-NOHA, BEC and ABH are commercially available and have been used in preclinical animal models as well as in humans with promising results reported for the small-scale treatment of patients with hypertension (Holowatz and Kenney 2007) and T2DM (Shemyakin et al., 2012), however, there is no information currently available on any clinical trials in patients with chronic wounds.

Chronic wounds were also associated with a sustained upregulation of iNOS (Abd El-Aleem et al., 2000). Therefore, targeting this enzyme of the ARG pathways might also represent a therapeutic option. Given the known homeostatic push-and-pull mechanism between iNOS and ARG in the context of acute wound healing, it would be expected that iNOS inhibition might trigger the pro-healing activity of ARG. However, an *in vivo* study of incisional skin wound healing in hairless but immunocompetent SKH-1 female mice showed no difference in wound tensile strength or histopathological features (such as epidermal hyperplasia, fibrin deposition and oedema) upon iNOS inhibition *via* SC-842 (Bell et al., 2007). Moreover, iNOS inhibition by means of both selective (aminoguanidine; AG; **Table 2**) and non-selective (N-Nitro-L-Arginine Methyl Ester; L-NAME; **Table 2**) inhibitors delayed wound closure and increased scarring in an *in vivo* acute incisional back wound model involving adult male, Sprague Dawley rats (Abd El-Aleem et al., 2020).

Natural Products with Wound Healing Properties

In addition to conventional drug-based treatments to specifically target ARG pathways, there is increasing interest in the use of traditional medicines as adjunctive therapies for chronic skin conditions (Kulprachakarn et al., 2017), and it is possible that these will modulate ARG pathways. Further study and validation are needed to understand the underlying mechanisms involved in their restorative effects. Notably, enhanced wound healing has been linked to the bioactive constituents of organic products such as *Achillea asiatica* (Dorjsembe et al., 2017), *Aloe vera* (Daburkar et al., 2014), catechins (Kapoor et al., 2004), grapefruit (Kandhare et al., 2014), honey (Vijaya and Nishteswar 2012) and turmeric (Ibrahim et al., 2018). The wound-healing related attributes of these constitutes include antibacterial, anti-inflammatory, antioxidant, and pro-collagen synthesis properties (Ibrahim et al., 2018). Given these attributes are closely linked to the

effector functions of ARG, the possibility of their interaction with members of the ARG pathways cannot be excluded. Indeed, *in vivo* model studies of *Aloe vera*, grapefruit extracts (Kulprachakarn et al., 2017) and the catechin epicatechin galatte (ECG) (Kapoor et al., 2004) showed that there was modulation of the ARG pathways. A significant upregulation of iNOS activity was noted in the early stages of the wound healing process (days 1–3), whilst a significant decrease in ARG1 activity was observed during the same time interval (Kapoor et al., 2004). The applicability of natural compounds in clinical practice has seen an improvement in recent years, due to being incorporated into different formulations based on hydrogels, micelles or nanoparticles (Ibrahim et al., 2018). These formulations have helped overcome pharmacodynamic problems such as poor aqueous solubility and fast degradation rates, therefore enhancing medicine delivery and bioavailability (Ibrahim et al., 2018; Kant et al., 2021). As more of these products become more pharmacokinetically available, further work may assess and target the molecular components of the ARG pathways.

Even though several current options for the ARG-dependent treatment of chronic wounds and their pitfalls have been presented in this section, the fast-paced development of new technologies as well as our ever-improving understanding of the chronic wound aetiology should help us develop better preclinical and patient-related procedures in the near future. In clinical practice, chronic wound monotherapies have led to unsatisfactory results. Thus, the need for the design of a multimodal treatment for chronic skin conditions is imperative.

DISCUSSION, FUTURE OPTIONS AND PERSPECTIVES

The activity of ARG may be modulated by intrinsic differences in human skin. ARG activity in the context of wound healing may vary depending on the sex, age and demographics of the patients (Wong et al., 2016). Sex-related differences are important, as skin is thinner in women than men (Sandby-Møller et al., 2003) which is further exacerbated during the menopause due to depletion of oestrogen (Chen et al., 2001). Age differences between patients should also be taken into consideration, as the diminished elasticity, xerosis and immunosenescence noted in aging skin can have a considerable impact on the outcome of studies involving the ARG pathway (Leyden 1990).

In patients with chronic wounds, underlying systemic factors such as advanced age, compromised immunological status and constant mechanical stress can contribute to poor healing, as is the case of the ulcers described in this review. Due to the need to target both systemic comorbidities, like diabetes or hypoperfusion, and local/regional skin impairments, Eming et al. raise the question of possible drug-drug interactions in patients with chronic wounds. Therefore, the best treatment approach would be to normalise the underlying systemic condition while administering local treatment, paying close attention to the pharmacodynamics of the multiple drugs used (Eming et al., 2014). In addition, given that the chronic wound aetiology is multifactorial, a synergising multimodal treatment targeting several growth factors, ECM components and cell types, rather than the ARG pathway alone, may improve the quality of future treatments (Eming et al., 2014). As far as prospective clinical trials are concerned, their large-scale successfulness may depend on the improvement of inclusion criteria, proper patient stratification and standardisation. These factors may also facilitate the implementation and interpretation of future meta-analyses.

While ulcers highlight the likely implications of a malfunctioning ARG pathway in macrophages and fibroblasts, the pathological activity of ARG in other wound healing-related cells known to express this enzyme should not be neglected either. Due to inappropriate re-epithelialisation, the aetiology of chronic skin conditions has been strongly linked to keratinocytes. However, little attention has been given to the implications of ARG-expressing epidermal cells in the context of chronic wounds. Since the downstream metabolites of the ARG pathway (especially the polyamines) have been extensively associated with cell proliferation and differentiation, the links between the ARG pathway, the polyamines and inappropriate re-epithelialisation might be worth highlighting in the context of chronic wounds. Therefore, this represents a future research avenue that may improve our understanding of ARG in the context of non-healing wounds.

CONCLUSION

To sum up, the ARG pathway is important in wound healing with multiple roles as discrete points of the healing cascade. Defects in arginase signalling, expression and/or function are common in delayed healing wounds. However, more must be known about its cell specific roles and how different morbidities and factors such as age and sex impact it. Indeed, the translational potential of emerging therapies aiming to fill the bench-to-bedside gap in delivering arginase linked therapies will likely depend on a better understanding of patient heterogeneity and stratification criteria. Nevertheless, manipulation of the arginase pathway is an exciting and important area to consider targeting to enhance healing and improve patient outcomes.

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Epidermis as a Platform for Bacterial Transmission

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The epidermis constitutes a continuous external layer covering the body, offering protection against bacteria, the most abundant living organisms that come into contact with this barrier. The epidermis is heavily colonized by commensal bacterial organisms that help protect against pathogenic bacteria. The highly regulated and dynamic interaction between the epidermis and commensals involves the host's production of nutritional factors promoting bacterial growth together to chemical and immunological bacterial inhibitors. Signal trafficking ensures the system's homeostasis; conditions that favor colonization by pathogens frequently foster commensal growth, thereby increasing the bacterial population size and inducing the skin's antibacterial response, eliminating the pathogens and re-establishing the normal density of commensals. The microecological conditions of the epidermis favors Gram-positive organisms and are unsuitable for long-term Gram-negative colonization. However, the epidermis acts as the most important host-to-host transmission platform for bacteria, including those that colonize human mucous membranes. Bacteria are frequently shared by relatives, partners, and coworkers. The epidermal bacterial transmission platform of healthcare workers and visitors can contaminate hospitalized patients, eventually contributing to cross-infections. Epidermal transmission occurs mostly *via* the hands and particularly through fingers. The three-dimensional physical structure of the epidermis, particularly the fingertips, which have frictional ridges, multiplies the possibilities for bacterial adhesion and release. Research into the biology of bacterial transmission *via* the hands is still in its infancy; however, tribology, the science of interacting surfaces in relative motion, including friction, wear and lubrication, will certainly be an important part of it. Experiments on finger-to-finger transmission of microorganisms have shown significant interindividual differences in the ability to transmit microorganisms, presumably due to genetics, age, sex, and the gland density, which determines the physical, chemical, adhesive, nutritional, and immunological status of the epidermal surface. These studies are needed to optimize interventions and strategies for preventing the hand transmission of microorganisms.

Keywords: epidermis microbiota, bacterial transmission, protection pathogens, heterogeneity transmitters, skin tribology

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INTRODUCTION

The transmission of infectious microorganisms between individuals through skin contact has long been known, driving the development of hygiene because of the impracticality of perpetual skin sterilization. Our knowledge of the skin microbiota has increased considerably since the introduction of massive sequencing techniques, particularly for bacteria and fungi, demonstrating the existence of specific ecosystems in differentiated areas. As with other colonized body surfaces, the detailed composition of a healthy microbiota has not yet been fully defined. Interest in bacterial transmission *via* the skin has been limited to the spread of pathogens; however, most skin microorganisms can be classified as both commensal and pathogenic, as is the case for *Cutibacterium acnes*, which is commensal in almost all patients but also causes acne (1).

Skin microbiota can be transmitted by direct contact but is also released into the atmosphere through the desquamation process. The environment is an almost infinite source of microorganisms suspended in the air and water systems or deposited on surfaces (2), where individuals exchange microorganisms as donors and recipients. The current SARS-CoV-2 pandemic has highlighted the importance of determining certain microorganisms' skin transmission ability (3), particularly the high transmission between individuals in enclosed and crowded spaces such as public transport and swimming pools (2, 4, 5). After being transmitted, the foreign microorganism can colonize the skin, sometimes causing an infection. The epidermis also acts as a platform for transmission to other individuals/environments. While differences in bacterial transmission capacity have been extensively studied, there are still unknown human factors favoring or limiting the transmission of exogenous bacteria by the skin.

Continuous external microbial exposure ensures the frequently transient diversity of the skin microbiome. In the hospital setting, the skin microbiome's (both for patients and healthcare workers) should be carefully controlled, as it is a key source for transmission events involving pathogenic bacteria. In fact, nosocomial infection often follows skin colonization by antibiotic multiresistant pathogenic bacteria. Hand washing by healthcare workers is still the best strategy for preventing the transmission of nosocomial pathogens, as has been demonstrated for *Clostridioides difficile* whose spores are resistant to the action of alcohol gels and standard disinfectants (6) and should be eliminated by shedding through standardized structured washing techniques (7).

EPIDERMIS, THE BORDER WITH THE MICROBIAL ENVIRONMENT

Our epidermis, the outermost side of the skin that covers 2 m^2 on average, is not only exposed to a multiplicity of environmental organisms but also repeatedly makes contact and rubs against contaminated natural and artificial surfaces. The skin is a compartmentalized habitat, with specific microecological spaces such as keratinized space, sebaceous glands, and apocrine and eccrine glands where the bacterial density can differ considerably. There is also a critical epidermis-mucosal border, where mucosal microbial populations can coalesce with those established on the epidermal surface.

Despite the considerable increase in recent years in our knowledge of human microbiota, more investigation is needed into the interactions between the two microbial worlds of muco-cutaneous junctions, from the point of view of biochemistry, microecology, and immunology (8). It seems clear that there is no sharp border but rather a gradient of conditions, likely dependent on physical variables such as humidity and temperature. These transitional areas are obvious in the lips, where we observe the non-keratinized epithelium of the labial mucosa transitioning to the buccal mucosa, with surfaces changing with age (9–11). Similar "borders" occur at the eyes, rectum, and vaginal mucosa and the neighboring keratinized epidermis. The microbiota at these borders has been poorly characterized.

The common use of fingers for exploring and washing mucosal orifices and during sexual activities is another important source of interactions between epidermal and mucosal microbes. Artificial mucosal-epidermis interfaces are frequently created in surgery (e.g., in ileostomies), sometimes with pathogenic consequences (12). In contrast, certain less exposed skin regions, such as interdigital and other skin folds, could constitute a potential healthy microbiome reservoir for re-colonization of altered epidermal communities.

The microbial dialogue with the skin's immune system, both with the innate and adaptive cells, determines the tolerance or inflammation response. Although the response to microbial infections is well known, the determinants of a tolerance status are not. Dermatological research is currently focused on the interplay between the immune system and skin microbiota for diseases such as acne and seborrheic dermatitis (13). In these skin conditions, the pathogenic process is probably based on an excessive response of the local innate immunity against members of commensal microbiota, including bacteria such as *Cutibacterium acnes*, fungi, as *Malassezia furfur*, and viruses, as Merkel cell polyomavirus or herpesvirus.

A proinflammatory gut microbiota has been reported in patients with alopecia areata universalis (14), and there have been supporting epidemiological hypotheses such as the relationship between rosacea and Parkinson's disease on one hand and the skin-gut-brain axis on the other (15). Detailed studies of the local microbiome, such as the follicular microbiome, provide valuable information on the etiopathogenesis of chronic inflammatory diseases such as primary cicatricial alopecia and facilitates their understanding and classification (16). There has recently been major interest in microbiota composition as a predictor of drug response, particularly for the gut ecosystem and immunotherapy in melanoma, and it has been proposed the transference of fecal microbiota from immunotherapy responders to non-responders (17, 18).

THE EPIDERMAL MICROBIOME AND MODULATION STRATEGIES

Is the epidermal microbiome representative of the dermal microbiome? **Table 1** shows the bacterial genera which are shared in the dermal and epidermal compartments. In general, bacterial composition of the stratum corneum does not significantly differ from that of the full skin (20, 21). Sampling by swabbing therefore yields analogous results to sampling by tapping (22). The "constant" epidermal microbiome is a minority subset of the dermal microbiome, which constitutes a "real microbiota", extremely stable and universal in human hosts (19), where bacteria are adapted to the nutritional conditions of the dermal compartment. However, the shared bacteria are much more abundant in number in the epidermis, which also contains a high density of diverse and transient bacterial organisms, as expected by the frequency of environmental contacts, and consequently variable among individuals. Given that our focus is on transmission, we will not go into detail on the detailed skin microbiome composition, a topic that has been covered by other authors (21, 23, 24).

At birth, single clones of a variety of microorganism colonize human skin. The environmental pressures and particular conditions experienced by the individual lead to microevolutions that result in lineage diversification, even in commensal *S. epidermidis* (25). During diversification, commensal microbiota can experience genetic acquisition or loss after punctual interactions with the transient transmitted bacteria. In addition, virulent and antibiotic-resistant clones, such as the Panton-Valentine leukocidin-producing methicillin-resistant *S. aureus* clone USA300, are habitually transmitted by skin contact and cause major outbreaks (26, 27).

The pathogenicity of particular *C. acnes* lineages has been reported not only in acne but also in systemic diseases such as prostatitis/prostate cancer, synovitis-acne-pustulosis-hyperostosis-osteitis syndrome, sarcoidosis, sciatica, and implant-associated infections. Skin microbiota transplantation from a healthy donor has been proposed to treat dermatologic conditions (28, 29), mainly to eradicate virulent *C. acnes* and *S. aureus* clones, or at least to replace the bacteria with non-virulent ones. The utility of bacteriophages for skin infections, particularly those caused by *Pseudomonas* in extensive burns, has recently been recently reviewed (30).

BACTERIAL NUTRITION AND GROWTH ON THE EPIDERMIS

Lipids excreted by sebaceous glands (frequently in the facial epidermis) contain anti-bacterial substances and protective compounds and are a nutrition source (31). Gram-positive bacteria in the skin, mainly *Staphylococcus* and *Cutibacterium*, release exoenzymes to enhance the recovery of nutrients from the environment, particularly proteases for amino acid liberation from skin proteins such as keratins, collagen and elastin (32). For instance, lipase production for triglyceride lipid degradations is significantly higher around comedones, causing inflammation in acne (21, 33). Other exoenzymes include bacterial hyaluronidase, which enable the obtention of glucuronic acid and N-acetyl-D-glucosamine from long-chained hyaluronic acid, and DNase, which likely degrades the extracellular DNA from apoptotic keratinocytes or corneocytes. *C. acnes* can induce keratinocyte autophagy by stimulating the CD36-CD14-TLR2/4-TLR6 signaling module, triggering ROS generation through nicotinamide adenine dinucleotide phosphate oxidase and the TRAF6-ECSIT-NLRX1 pathway and evoking mitochondrial dysfunction (34, 35). However, we still lack the full picture of microbial nutrition in the skin, particularly regarding the role of protocooperative actions among bacterial species in nutrient exploitation.

The critical factors of bacterial nutrition and growth in the epidermis are pH and water availability, which also determine the concentration of free amino acids and lactate. For example, the water content of the stratum corneum of Japanese individuals (as measured by Raman spectroscopy) ranges from 30% at the

TABLE 1 | Bacterial microbiota in the epidermal and dermal compartments.

Epidermal-Dermal Genera Not Phylum Proteobacteria	Epidermal-Dermal Genera Phylum Proteobacteria	Epidermal-Dermal Genera Anaerobes
Corynebacterium	***Pelomonas***	*Finegoldia*
Staphylococcus	***Acinetobacter***	*Peptoniphilus*
Micrococcus	***Moraxella***	***Anaerococcus***
Streptococcus	*Pseudomonas*	*Blautia*
Paracoccus		*Porphyromonas*
Brachyobacterium		*Fenollaria*
Kocuria		***Veillonella***
Dietzia		*Cylindrospermum*
Actinomyces		*Prevotella*
Brevibacterium		*Dialister*
Tepidimonas		*Bifidobacterium*

This Table was inspired by the publication referenced as Bay et al. (19).

Epidermal microbiota is much more abundant and variable (strongly affected by environmental contacts) than dermal microbiota. A subset of most frequent members of the epidermic microbiota constitute a very stable and universal (preserved in different individuals) dermal bacterial community, adapted to the nutrients of the dermal compartment. In the boxes below, listed by frequency, the epidermal genera with high representation in the dermal compartment are highlighted in bold characters.

surface to 70% in the deeper layers (36). Prolonged water exposure significantly increases the epidermal water content; however, the external part of the stratum corneum gradually dries out after the forced hydration is discontinued (36). The water content of the skin surface is then mainly based on excretion by eccrine sweat glands. However, free amino acids, glycerol, sphingolipids and particularly metabolites of filaggrin, a large protein bound to lipids and keratin, such as urocanic acid and pyrrolidone carboxylic acid, also function in the outer skin as natural moisturizing factors, able to absorb large amounts of water and maintain a low pH (37). The epidermal water content depends on age, anatomical site and season. Certain molecules (such as teichoic acids) on the external surface of Gram-positive bacteria can produce local proinflammatory effects (38), which results in greater water availability resulting from increased vascularity. Osmolarity increases with water paucity. Water tends to be retained in the epidermal keratinocytes outer layer, particularly in the valleys where it is protected from evaporation; in addition, under osmotic stress more water-channels are produced, as aquaporin-3 (39). The skin's retention of transmitted bacteria depends strongly on water (40). Sweat increases water availability, transports molecules with bacterial impact, and facilitates bacterial transmission through signals contained in their extracellular vesicles.

BACTERIAL DEATH ON THE EPIDERMIS

Keratinocytes contribute to the innate immune response system by sensing microbial cell density by detecting pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) (21). Such detection results in the induction of release of cytokines, chemokines, and antimicrobial peptides (AMPs) such as human beta-defensins HBD-1, HBD-2, and HBD-3, cathelicidin LL-37, and the antimicrobial proteins RNase 7 (from the RNase A superfamily) and Perforin-2, the last being able to kill intracellular organisms (41–43).

The response is modulated by molecules on the surface or those released by commensal organisms (44). The conditions favoring the overgrowth and immunological tolerance of skin commensals are frequently those that are also beneficial for the growth of certain skin pathogens (**Figure 1**). The "bacterial overgrowth" signal is therefore likely triggered by commensals, resulting in decreased commensal density; however, this decrease is deleterious for less numerous pathogens. The overgrowth of skin commensals likely releases immunity-stimulating signals, including structural bacterial molecules such as teichoic acids or proteins released from the bacterial cell wall, microbial-secreted substances such as porphyrins, and molecules, as oleic acid, resulting from the bacterial metabolism of local lipid substrates (45). Overgrowth is eventually followed by intracellular engulfment and the release of more signaling molecules such as complement and interleukin-1. Perforin-2 upregulation following *S. epidermidis* overgrowth increases the intracellular killing of *S. aureus* (41). The innate lymphoid cells regulate the production of antimicrobial lipids (such as palmitoleic fatty acids), reducing the population density of *Staphylococcus* and, in general, all Gram-positive bacteria (46).

In normal conditions, a "normal"-sized population of commensals (such as *S. epidermidis*) stimulates only the innate defense system (47). Pathogens such as *S. aureus* have evolved mechanisms for subverting immune stimulation, such as modifying their PAMPS (48); however, these bacterial protection mechanisms are expected to be effective only after reaching a cell-density threshold.

The strategy of eliminating pathogens by regulating commensal overgrowth (**Figure 1**) is based on the differences between highly adapted organisms (commensals, those with large populations) and less adapted organisms (occasional pathogens, those with small populations) that compete for the same resources (47). Commensals are better endowed to reconstruct the original population density after a challenge. In fact, the population density of commensals is by itself a limiting factor for pathogenic colonization, either indirectly (e.g., by nutritional competition) or directly (e.g., antagonistic substances). Direct antagonism between commensal and pathogenic staphylococci is frequently mediated by secondary metabolites such as low molecular weight bacteriocins, frequently of the lantibiotics type, the equivalent to microcins in intestinal Enterobacterales (49, 50). Lantibiotics released from commensal *Staphylococcus* are synergistic with the human cathelicidin antimicrobial peptide LL-37 in reducing *S. aureus* populations (51). *S. epidermidis* might also interfere with *S. aureus* biofilm-type colonization by expressing the serine-type protease Esp. In fact, protection against *S. aureus* involvement in atopic dermatitis by increasing the density of commensal microbes is being explored. The immunological response against pathogens might in fact depends on resident commensals. The shedding of the stratum corneum acts as a sink for microorganisms adhered to the skin surface; however, these organisms persist by colonizing the deeper layers of the epidermis thereby keeping the population density stable.

GRAM-POSITIVE *VERSUS* GRAM-NEGATIVE

The conditions of low moisture, high osmolarity, low pH, and AMPs presence clearly favors organisms with a thick peptidoglycan envelope and lacking an outer membrane, as well as Gram-positive organisms, with the interesting exception of *Enterococcus* species, which are not usually found in the epidermis. Gram-negative organisms are not permanent colonizers of the outer skin, with the exception of *Acinetobacter* in moist intertriginous areas. In experimental transmission studies, Gram-positive organisms, such as *E. faecium* and *S. aureus*, exhibited the highest transmission efficiency, whereas Gram-negative organisms were less efficient, particularly *E. coli*, which had the lowest transmission rate (52). The recovery of phages from Gram-negative *Proteobacteria* might simply reflect the occasional, transient colonization of these microorganisms, with the possible exception of individuals with primary

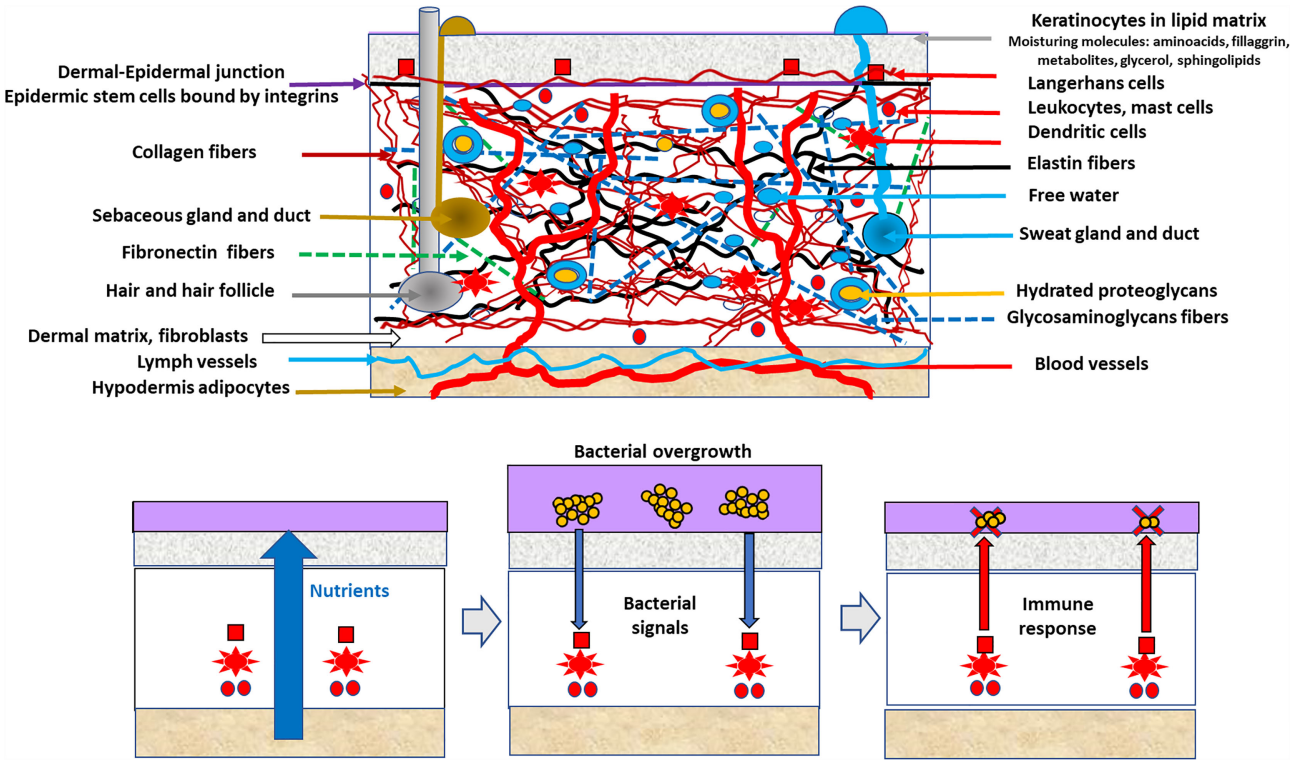

FIGURE 1 | The hypothesis of indirect epidermal bacterial clearance of a pathogen. Up in the figure, a schematic view of the skin is presented, particularly of the components of the dermal compartment, determining the commensal microbiota of the epidermis. Below, the indirect epidermal bacterial clearance of a pathogen (clusters of yellow circles) in the violet layer (surface commensal microbiota). Conditions facilitating the growth of commensals (vertical blue thick arrow) also favors pathogens, but the commensals overgrowth stimulate (narrow blue arrows) the defense system based on cellular local immune innate response involving cellular (red squares, circles, stars) and glands secretion. Antimicrobial defense (red arrows) reduces the overall bacterial density, but the lower-numbered pathogens are eliminated (red X symbol), whereas the dominant commensal population is reduced to its normal density (sequence from the second to the third box at the bottom).

immunodeficiencies (53, 54). However, some commensal species from the Phylum Proteobacteria, as *Pelomonas*, are consistently present in the dermal compartment, and might migrate to the epidermis (**Table 1**). Important potential human pathogens such as *Enterobacterales* and *Enterococcus* are rare in normal epidermis, despite expected frequent external contamination from the intestinal microbiota. It is certainly plausible that Gram-positive commensals might elicit the production of human antimicrobial peptides able to eradicate small populations of Gram-negative bacteria, using the strategy illustrated in **Figure 1**.

Skin osmolarity also favors Gram-positive bacteria. Certain *Proteobacteria* can be detected by 16S rDNA sequencing from the skin surface, including *Acinetobacter, Enterobacter, Klebsiella, Pseudomonas, Serratia* and *Sphingomonas*, all of them environmental organisms and likely transient "skin-landing" bacteria. In short, the epidermis should not be considered a niche for *Enterobacterales* or *Enterococcus*, given that it does not support the significant growth of these critical human pathogens (55). If these organisms are just transiently bound to the epidermis by weak unspecific adhesion, the possibility of being transmitted between hosts could be enhanced.

A rarely considered aspect of the epidermal bacterial microecology (and transmission) is the spatial structure of bacterial cells, ranging from homophilic clumps to larger biofilms. In particular, *Staphylococcus* tends to clump into multicellular aggregates (staphyle is the Greek name for a bunch of grapes), facilitating adhesion to the skin and likely colonization. The intercellular homophilic bonds of *Staphylococcus* depend on a polycationic polysaccharide intercellular adhesin; however, the role of cell wall-anchored proteins (self-aggregating molecules) appears to be critical in the process. These proteins (e.g., the bacterial surface serine-aspartate repeat protein SdrC) might also be considered a multifunctional adhesin involved in hydrophobic interactions with surfaces. In fact, clumping-negative *S. aureus* mutants are less adhesive to surfaces, including keratinocytes (56).

The interaction between bacteria and the colonizable surfaces is mediated by cell appendages and specific molecules located at the cell surface. In particular, a large variety of adhesins assures the close contact required to exploit structured environments. Many of these adhesins are involved in the attachment to the host tissues including skin, and also contribute to in the formation of interbacterial interactions resulting in the formation of local biofilms (bacterial multicellular aggregates), increasing the

resilience (permanence) of adhered bacterial populations. The molecular determinants mediating specific bacterial adhesion to the outer layers of the epidermis remain scarcely investigated for most bacterial taxa. On the contrary, excellent information is accessible for some pathogenic Gram-positive bacteria, including a thematic Research Topic in Frontiers in Microbiology (57). Seminal reviews are available on *Staphylococcus* adhesion mediated by surface proteins, mostly comprising the hydrophobic LPXTG transmembrane motif assuring cell-wall anchoring. Many of these surface components recognize adhesive matrix molecules as fibronectin, vitronectin, laminin, fibrinogen or elastin, the MSCRAMM family. Fibronectin-binding adhesins (FnBPA and FnBPB in *S. aureus*) regulated by *agr* and *sar* operons, are critical in the adhesion to the squamous epithelium (58–61).

Interestingly, *Staphylococcus* LPXTG proteins and some extracellular adherence proteins also promote bacterial internalization by keratinocytes, assuring the permanence of the bacterial population in the epidermal compartment (62). Populational resilience is enhanced by biofilm formation, involving, among others, Aap and SdrF proteins (63). Interbacterial adhesion on the epidermis is also favored by the small basic protein (Sbp). This protein favors the formation of amyloid fibrils structuring the biofilm matrix derived from the effect of adhesins, comprehending microbial polysaccharide (mostly β-1-6-linked N-acetylglucosamine), intercellular adhesin surface proteins, DNA, and proteins from dead cells (60). Other surface components of Gram positives, as teichoic acids, also contribute to skin adhesion (64).

Consider that in *S. aureus* there are 24 different LPXTG adhesins with different specificities and roles. *S. epidermidis* expresses more than 10 adhesins (60, 61). *Staphylococcus lugdunensis* is also a frequent commensal in the outer layers of the skin and contains the LPXTG protein SrtA (65, 66).

In the case of *Cutibacterium* bacteria, mostly probably originated in the sebaceous glands-ducts where they have their niche (67, 68), they migrate to the epidermis and remain attached by their fibronectin-binding surface proteins, also inducing keratinocytes internalization, which assures the local permanence (35, 62).

The skin adhesion of bacterial spores, including those of pathogenic organisms such as *C. difficile*, is poorly known, but approximately 5% of hospital health workers are carriers of these organisms (69, 70). Future advances in the "ecology of adhesion" might result from applying confocal laser scanning microscopy, given that it has been used for human mucosal surfaces (71).

THE ROLE OF THE EPIDERMIS IN TRANSMITTING HUMAN PATHOGENS

Although certain key human pathogens are not permanently established on the surface of the normal epidermis, they use the surface as a platform for host-to-host transmission. In the case of *Enterobacterales*, the hands of intensive care unit (ICU) staff are frequently (nearly 20%) contaminated by the same *Klebsiella* clones that infect patients; however, the absolute count by culture does not exceed 10^3 per hand, and the survival time was estimated at approximately 2–3 h (72). In fact, in abiotic dry surfaces, such as computer keyboards in the ICU used by nurses and doctors, only Gram-positive skin bacteria are recovered, mostly *S. epidermidis* (73). As in the case of ICU health workers, environmentally linked individuals tend to share bacterial organisms. In fact, easy transmission of antibiotic-resistant *Enterobacterales*, presumptively by hand contamination, occurs among household members and individuals who visit friends and relatives (74–76). In closed communities, a common community epidermal microbiota is expected.

Most of the published works on the epidermal transmission of human pathogens are derived from hand-washing studies, one of the key "rituals" in preventive medicine. Although the efficacy of hand washing is extremely difficult to estimate, hand washing is undeniably highly effective in decreasing the risk of contaminating sterile tissues, mechanical devices and food by low-numbered pathogenic bacteria, as shown in the seminal work by Semmelweis (77). The effectiveness of hand washing in eliminating microbial organisms is inversely proportional to the skin's bacterial load and frequently has only a marginal, transient effect on heavily inoculated fingertips (78). It is noteworthy that Semmelweis discarded as irrelevant the use of microscopes to explain his results (77). That resulted in an epistemologically utilitarian "Semmelweis Complex" recommending that the focus be on efficacy and not the scientific reasons for explaining the effect. Such scientifically deleterious Complex remains very much alive in public health. To quote a modern reference, "studies on practical and efficient means to increase compliance with hand hygiene guidelines and to influence behavior surely are needed more than are elaborate and sophisticated studies on the effects of hand washing" (79). Such generalized "practical" view has delayed experimental studies, and consequently there are numerous issues in hand-washing biology that are unknown or poorly understood. For instance, if the hand's commensal bacteria can prevent the growth of potential pathogens, the controlled reduction of these commensals might be more advisable than full eradication (80). Increasing our knowledge of the biology of epidermal bacterial transmission appears to be the only option for improving our interventions.

EXPERIMENTAL BACTERIAL TRANSMISSION

The biology of hand-to-hand transmission is a recent field of research. In a seminal study published in 2014 (81), broth suspensions of potentially pathogenic well-defined clones of *E. faecium*, a non-skin commensal Gram-positive organism, were gently deposited and spread on both thumb tip surfaces (approx. 10^7 cells over 1.32 cm^2) of 30 healthy volunteers (4 experiments per individual, spread over 6 months). After complete drying, the organisms from a sample surface of 0.78 cm^2 on one of the thumbs were suspended by shaking them in a saline solution. The second contaminated thumb was put in close

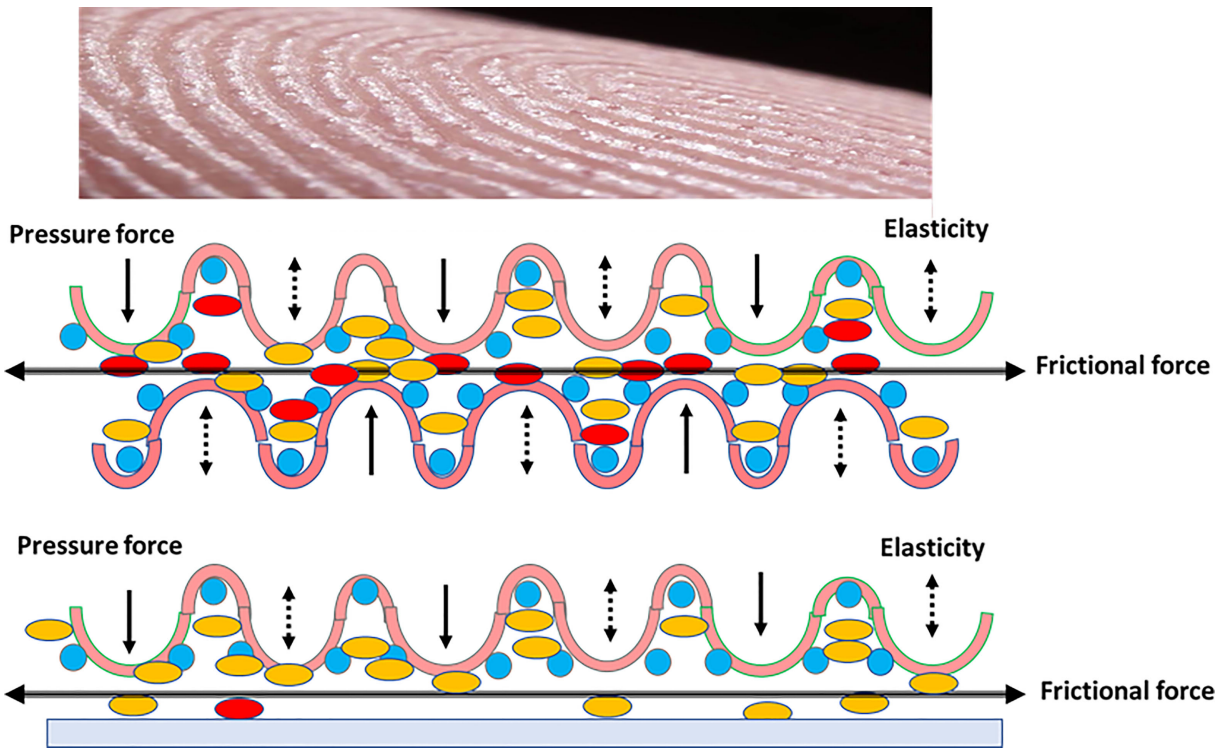

FIGURE 2 | Epidermis tribology. Up in the figure, the surface of a finger pad, showing the epithelial pad crests. Below, two rubbing series of friction (beheaded arrow) finger pad crests. Because of the pressure and frictional forces (black arrows), and elasticity of the dermal compartment, the (yellow) bacteria of the upper epithelium frequently migrate to the lower epithelium, and only some of the (red) bacteria are transmitted to the green epithelium. The asymmetry of transmission might be due to microecological differences, for instance water content (blue circles). Down in the slide, if the epidermis is rubbed on a smooth surface, the transmission is much less effective.

static contact (with minimal pressure and preventing twists or wipes) with the index fingertip of the other hand of the same individual for 10 s, and the transmitted organisms were also suspended. Over several steps involving the fingers of both hands, a quantitation curve of the proportion of finger-to-finger transmission was obtained. The bacterial count showed an exponential decay in sequential finger-to-finger transfers in most of the volunteers, typically a decay of 1.5 log in successive counts. This result is consistent with that obtained in similar experiments on skin bacterial survival and hand transmission (82–84). Interestingly, in the *E. faecium* experiment, the frequency distribution of the exponential decay parameter estimated for all individuals clearly showed an asymmetrical right tail containing an overrepresentation of high transmitter individuals (13% of the volunteers) with their epidermis exponential decay parameters close to zero. A variable transmission rate of the various *Enterococcus* clones was also observed, supporting the suggestion that a number of bacterial variants (including Gram-negative bacteria, such as *Proteobacteria*) could be better adapted than others to the environmental skin circulation (81, 85). A further analysis of the data proposed 3 transmission efficiency categories: poor, medium, and high finger-to-finger bacterial transmitters. All 10 male volunteers were classified as poor or high intrahost transmitters, whereas almost all 20 of the female participants were grouped in the medium category (53).

Experimental fingertip transmission studies extended to interhost transmission have shown that the success of the transmission chain depends on the position of a poor transmitter in the series. Poor-transmitter volunteers had the ability to cut off the transmission chain independently of their position (53). Certainly, these preliminary observations might foster further research to determine the individual risks of foodborne transmission and healthcare workers.

THE BASIS FOR INDIVIDUAL VARIABILITY IN BACTERIAL TRANSMISSION

The density and diversity of skin commensal bacteria might affect the fate of pathogenic bacteria contacting the surface. In contrast to the low variability of dermal microbiota across humans, the epidermis shows a higher polymorphic set of bacterial species, probably because they reflect the host's lifestyle and changing environment (e.g., altitude or temperature) (19, 86). Interindividual genetic differences in epidermal microbes cannot be totally ruled out, given that

variations occur with organisms in fish skin and that of sea mammals (87, 88). In humans, these genetic differences might occur (45, 89, 90), but the effects of race have not been sufficiently evaluated, although peculiarities of the skin microbiota of Chinese populations have been suggested (90). Genetics and sex can affect the density of eccrine, apocrine, and sebaceous glands determining the physical, chemical, adhesive, nutritional, and immunological status of the epidermal surface and thereby the density and type of organisms. The influence of age, stress, and hormonal status also cannot be overlooked. In the above-mentioned experiments regarding *E. faecium* finger-to-finger transmission, no significant association for transmission decay was found between fingertip temperature measurements and finger pressure (53, 81). There is a need for studying the intraindividual and interindividual transmission of organisms from various epidermic sites, such as sebaceous areas (e.g., face and back), moist areas (e.g., the armpits and the webbed parts of the fingers/toes), dry areas (e.g., forearms and buttocks), and sites containing varying densities of hair follicles, skin folds, and skin thicknesses. The role of epidermal friction ridges is a possible line for further research into the biology of the epidermal transmission of bacteria.

TRIBOLOGY OF SKIN: FRICTION RIDGES ON THE FINGERTIPS

Tribology is the science of interacting surfaces in relative motion, including, as said before, friction, wear and lubrication. Tribological phenomena occur on a large scale, and include microbiotribology, as in the case of homophilic bacterial cell interactions, giving rise to frictional phenomena, possibly creating electromagnetic fields that influence collective and individual cell behavior (91, 92). For human surfaces, a greater understanding is needed of the nonlinear effects of plasticity, adhesion, friction, wear, lubrication and surface chemistry, all of which play a part in skin tribology (**Figure 2**) (93).

Fingertips, which are critical surfaces in human-to-human bacterial transmission, are covered with friction skin (94, 95). Papillary ridges in friction skin likely evolved to assist in grasping and holding onto objects and are important in the biology of primates and koalas (96). The equilibrium between friction ridges and optimal hydration (eccrine glands) of the keratin layer maximizes friction (97). In humans, skin friction and microbial transmission depend on the tribological configuration; the surface roughness and the finger pad sweat rates. Age modifies friction forces by reducing skin thickness, dermal elasticity, and by ridge flattering, possibly in relation with collagen reduction (98). Dynamic optical coherence measurements by tomography will likely be essential for determining the frictional behavior of human finger pads (99, 100). We expect these studies to be applied soon to the human-to-human transmission of microorganisms (101, 102). Nothing is known about the triboelectric effects of ridge friction (which creates magnetic fields) on bacterial adhesion and repulsion. Multivariate models for predicting the frictional behavior of human skin, thereby identifying "high-transmitter" populations (81), could have obvious applications in public health, from food microbiology to hospital-based cross-infections.

AUTHOR CONTRIBUTIONS

FB and RdC wrote the manuscript; CS, DMM and LB provided information on microbiology, SGV and OMMA supervised and provided dermatological information. The final manuscript is approved by all authors. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Immunological Impact of IL-1 Family Cytokines on the Epidermal Barrier

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The skin barrier would not function without IL-1 family members, but their physiological role in the immunological aspects of skin barrier function are often overlooked. This review summarises the role of IL-1 family cytokines (IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-38) in the skin. We focus on novel aspects of their interaction with commensals and pathogens, the important impact of proteases on cytokine activity, on healing responses and inflammation limiting mechanisms. We discuss IL-1 family cytokines in the context of *IL-4/IL-13* and *IL-23/IL-17* axis-driven diseases and highlight consequences of human loss/gain of function mutations in activating or inhibitory pathway molecules. This review highlights recent findings that emphasize the importance of IL-1 family cytokines in both physiological and pathological cutaneous inflammation and emergent translational therapeutics that are helping further elucidate these cytokines.

Keywords: IL-36 cytokine family, IL-1 family cytokines, IL-33, proteolytic regulation, IL-1alpha, IL-1beta, pathogen/commensal discrimination, IL-18

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INTRODUCTION

The IL-1 family is a large group of cytokines, all of which are expressed in human epidermis, and have well established roles in skin immunopathology. Collectively these cardinal immunological functions encompass anti-microbial host protection and barrier function maintenance or restoration *via* inflammation and inflammation resolution mechanisms. In addition to established pathological roles this family of cytokines are vital for maintaining the skin's immunological barrier and wound repair, yet this physiological role often gets overlooked. This review aims to give an overview of the role IL-1 family proteins play in skin, focusing on novel findings in their physiological role, and provides context for a deeper understanding of pathway derangement with resultant cutaneous disease.

THE IL-1 FAMILY CYTOKINES

The IL-1 superfamily (**Figure 1**) can be split into three smaller subfamilies according to structural similarities amongst the cytokine IL-1 consensus sequence and their primary ligand binding

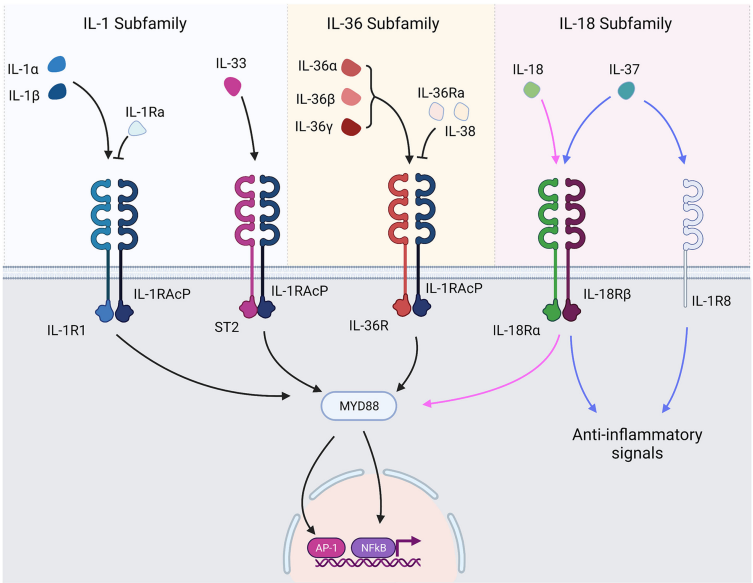

FIGURE 1 | IL-1 superfamily cytokines and receptors. IL-1 family cytokines bind their respective receptors and transduce signals through TIR/MyD88 signalling pathways. The major signalling pathways result in activation of NF-κB and AP-1 and subsequent transcription of genes under their control. Receptor antagonists bind their respective receptors but prevent the recruitment of the accessory protein, thereby preventing signal transduction. IL-37 exerts anti-inflammatory signals following interaction with IL-1R8 and IL-18Rα.

receptors: the IL-1, IL-18 and IL-36 subfamilies (1). The IL-1 subfamily has three agonists (IL-1β, IL-1α and IL-33) and a receptor antagonist (IL-1Ra). The IL-18 subfamily contains IL-18 as well as the anti-inflammatory cytokine IL-37 and IL-18 binding protein (IL-18BP). The IL-36 subfamily consists of three agonists – IL-36α, IL-36β and IL-36γ – and the antagonist IL-36Ra. It also contains the cytokine IL-38 whose function is not wholly understood yet is considered predominantly anti-inflammatory.

With the exception of IL-1Ra, all IL-1 family cytokines lack an N-terminal signal peptide and are therefore not secreted in a classical manner (2). Each cytokine is produced as an immature precursor that undergoes maturation by proteolytic truncation, the specificity of which differs from cytokine to cytokine. The activity of IL-1 family cytokines is hugely affected by this proteolytic maturation. Some cytokines such as IL-1α and IL-33 have biological activity in their immature form, yet others such as IL-1β and the IL-36 cytokines must undergo maturation to become active (1–3). Within the proteolytic maturation itself there exists more levels of regulation as variation in the specific truncations generated can affect the biological activity of the resulting mature cytokine. Therefore, changes to the proteolytic environment to which the IL-1 family cytokines are exposed can significantly affect the resulting inflammatory outcome and their subsequent impact on skin.

Signalling within the IL-1 family is a highly conserved process, with agonists binding their cognate receptors causing signal transduction *via* Toll/Interleukin-1 Receptor (TIR) and MyD88-dependent signalling pathways culminating in the activation of NF-κB and MAPK and subsequent NF-κB and AP-1 dependent gene transcription (4). As a result, many IL-1 family members generate similar pro-inflammatory responses. Nuances in cellular and tissue compartment expression, activation of IL-1 family cytokines and the responsive cell types, however, means IL-1 family cytokines can generate a huge variation of cellular responses and impart a broad impact on the immune system. The IL-1 family cytokines signal through IL-1 family receptors, some of which are shared amongst cytokines (**Figure 1**). IL-1 receptor biology is a complex topic in itself that is beyond the remit of this review and has been extensively reviewed elsewhere (5). However, to provide a contextual overview, all IL-1 family cytokines transduce signals in a similar manner. Agonists bind their cognate receptor, which subsequently recruits a membrane-bound accessory protein. Interaction between the ligand-bound receptor and the recruited accessory protein allows a signal to be transduced to the cell (6). As outlined in greater detail by **Figure 1**, some of the receptors are shared amongst IL-1 family cytokines. IL-1α, and IL-1β bind IL-1R1, IL-33 binds ST2, IL-18 and IL-37 bind IL-18Ra, IL-37 also binds a decoy receptor IL-1R8 through which it mediates some anti-inflammatory activity, and the IL-36 cytokines all bind IL-36R.

All IL-1 family cytokines are expressed, with some variation, within the skin. IL-1α is constitutively expressed by keratinocytes, yet is retained as intracellular stores (7, 8). IL-1β has a more specialised expression profile across the body, however in the skin most cell types present can express the cytokine, notably keratinocytes, fibroblasts and both resident and infiltrating myeloid cells (9, 10). Unlike IL-1α, IL-1β is only expressed following cell activation. IL-33 is only weakly

expressed by keratinocytes in healthy human skin, but is strongly induced in the epidermis of inflamed skin (11). IL-18 is also expressed throughout the epidermis. Similarly to IL-1α, IL-18 is constitutively expressed by keratinocytes and retained within cells as intracellular stores, yet is only cleaved into its mature form following cell activation in the same manner as IL-1β (12). IL-37 exists in 5 isoforms termed IL-37a-e as a result of alternative splicing. The largest and most common isoform, IL-37b, is expressed by keratinocytes, monocytes and other immune cells within skin (13). Of the IL-36 cytokines, IL-36γ is the most strongly expressed in skin. Tape strip analysis of human skin show basal levels of IL-36γ are present in the outer layers of the epidermis, even in healthy skin (14). IL-36γ is constitutively expressed at low levels by keratinocytes, and strongly upregulated following activation (15, 16). The IL-36 cytokines are not constitutively expressed by other cell types present in skin but are inducible in myeloid cells following activation (17). In addition to functioning as cytokines, IL-1α, IL-33 and some isoforms of IL-37 are known to translocate to the nucleus where they can regulate gene expression through their DNA-binding capabilities (8, 13, 18).

SENSING ENVIRONMENTAL THREATS

A number of IL-1 family proteins have a significant role in detecting environmental threats encountered in skin. Many have been added to the group of damage-associated molecular patterns (DAMPs) or "alarmins".

Protease Sensors

Several factors in IL-1 family member biology and expression characteristics make these cytokines excellent sensors of protease activity, alerting the immune system to potential threats. Although IL-1α, IL-33 and IL-37 exhibit a reduced level of activity in their immature form, IL-1β, IL-18, and the IL-36 cytokines require N-terminal truncation in order to engage their respective receptors (19). Proteolytic activation of IL-1 family cytokines is a tightly controlled process in order to license inflammation only when necessary. IL-1β and IL-18 activity is controlled by regulation of caspase-1, which itself remains inactive until inflammasome assembly occurs (20). The IL-36 agonists require precise cleavage to achieve maturity; truncation a single amino acid upstream or downstream of the correct site will generate an inactive cytokine (2). Indeed, as will be discussed later, a breakdown in the regulation of IL-1 proteolysis can lead to significant inflammatory disease.

Nevertheless, despite strict endogenous controls on the activity of IL-1 family cytokines, several have been shown to be activated by exogenous pathogen-derived and environmental proteases. IL-1β and IL-36γ are both processed to their active forms by the protease SpeB, secreted by *Streptococcus pyogenes* during infection (15). IL-36γ is also processed to its active form by Alp1, secreted by *Aspergillus fumigatus*, and both IL-36γ and IL-36α have been shown to be activated by *Staphylococcus aureus* and *Trichophyton rubrum* culture filtrates (15). Active IL-1β and IL-36 cytokines promote the development of Type I and Type III immune responses, which will facilitate clearance of bacterial and fungal pathogens. As such, the requirement of IL-1 family cytokines to undergo proteolytic cleavage to reach biological maturity also enables the cytokines to function as sensors of exogenous proteases and signal the presence of a threat.

Alarmin Function

IL-1α has long been associated with an alarmin role owing to its constitutive expression maintaining an intracellular store of the cytokine, and its ability to bind its receptor and transduce signals in its immature form. In the event of cellular damage or programmed cell death IL-1α is released into the extracellular space and able to warn surrounding cells of damage or infection (21). Furthermore, the skin barrier is subjected to mechanical stressing as a physiological phenomenon and this too regulates the release of IL-1α (22). Whilst IL-1α has been long established in this role, recent work supports the case that other IL-1 family members may also function in an alarmin capacity despite not wholly fitting the classical definition.

IL-36γ, in particular, is well suited as an epidermal alarmin as it is constitutively expressed in healthy human skin and is found even in the upper layers of the stratum corneum, where it is most likely to come into contact with invading pathogens following injury (14, 16). Its sensitivity to exogenous proteolytic activation and presence in the epidermal layers as a store of inactive inflammatory cytokine means that a small amount of pathogen-derived protease capable of activating IL-36γ can rapidly generate a strong inflammatory signal without relying on protein synthesis. **Figure 2** outlines the mechanism by which IL-1 family cytokines may sense environmental threats and warn the surrounding tissue to potential damage or infection.

Although not all IL-1 family cytokines act with an alarmin function in skin, several still have a crucial role in communicating the presence of environmental threats through their regulation by inflammasomes. Upon recognition of PAMPs and DAMPs by inflammasome sensors such as NLRP3, NLRC4, NAIP and AIM2, inflammasome complexes form and act as platforms for the activation of IL-1β, IL-18 and IL-37 (20). Inflammasome-mediated activation of the pore-forming protein gasdermin D then facilitates release of these cytokines into the surrounding tissue, thereby signalling the presence of an environmental threat (23). Whilst IL-1β must be induced and expressed prior to activation and secretion, IL-18 is constitutively expressed in the epidermis and even secreted in its immature form and is therefore available for activation immediately upon inflammasome formation (24). Furthermore, the inflammasome complexes themselves can be released into the surrounding extracellular tissue following activation which allows for maturation of extracellular IL-18 and IL-1β, amplifying the inflammatory response (25).

Microbial Interaction

In addition to alerting the skin to potential threats, many aspects of IL-1 family biology also help the immune system discriminate harmful pathogenic microbes from commensals. IL-1β has been

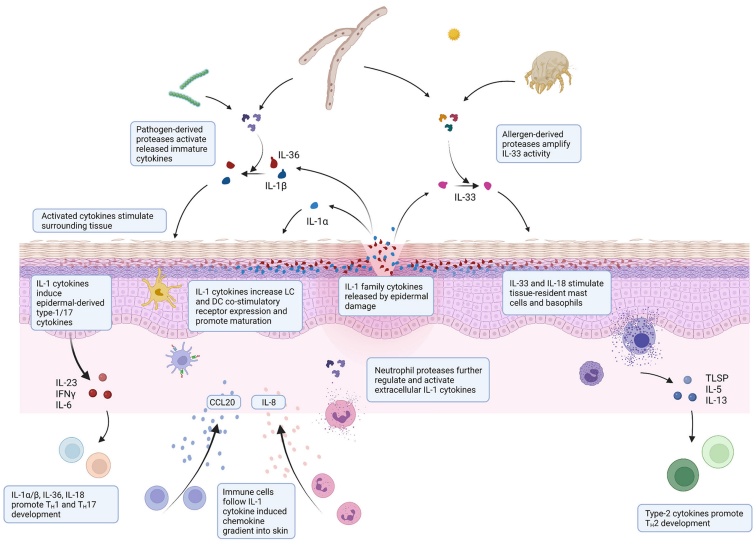

FIGURE 2 | Environmental interaction of IL-1 family cytokines in skin injury. Schematic illustrating the mechanisms by which IL-1 family cytokines can sense environmental threats and orchestrate a subsequent immunological response. Epidermal damage through infection or injury releases IL-1 cytokines. Immature cytokines are activated by environmental proteases expressed by pathogens during infection or allergens present in the environment. Active released cytokines stimulate surrounding epidermis and resident immune cells to facilitate immune cell recruitment to mount an appropriate immune response.

shown to have a role facilitating the discrimination of pathogens from commensals in skin through its influence on peripheral tolerance. Colonisation of skin by commensals during early life gives rise to commensal-specific regulatory T cell populations, which provide the host with commensal-specific tolerance and prevent unwanted inflammation (26). Recent evidence in mouse models shows IL-1β can influence which colonising microbes the immune system tolerates by negatively regulating T_{reg} cells (27). During early life colonisation of skin by *S. aureus*, virulence factors and cytotoxic proteins expressed by *S. aureus* that commensals lack induce expression of IL-1β. The induced IL-1β prevents the expansion of *S. aureus*-specific T_{reg} cells, meaning subsequent exposure to *S. aureus* does not result in the expansion of a strong tolerogenic population of T_{reg} cells, thereby allowing an inflammatory response to the skin pathogen (27).

Unlike IL-1β and IL-18, IL-36 cytokines are not activated by caspase-1 following inflammasome activation (3, 28). Whilst the mechanisms of IL-36 cytokine release have not been definitively outlined, cell death induced by pathogen-mediated damage has been demonstrated as one method. Indeed, the literature highlights a trend of IL-1 family cytokines to be released and activated by proteins associated with pathogen virulence. Phenol-soluble modulin (PSM)α virulence peptides secreted by *S. aureus* release IL-1α and IL-36 cytokines from keratinocytes through cellular damage (29, 30). As previously highlighted, the virulence factors SpeB and Alp 1 secreted during infection by *S. pyogenes* and *A. fumigatus* respectively activate IL-36γ, and SpeB has been shown to activate extracellular pro-IL-1β (15, 31). Recently the *Yersinia pestis* virulence factor YopJ, which inhibits NF-kB signalling, has been shown to trigger release of IL-1β and IL-18 *via* caspase-8-dependent gasdermin D activation (32, 33). By requiring either active or pathogen-dependent release from a damaged or activated cell, or activation by a pathogen-derived protease, or a combination of the two, these cytokines help the skin's immune system discriminate the harmful from the harmless and allow appropriate anti-microbial or wound-healing responses.

IL-33 is an immunological outlier of the IL-1 family of cytokines given it is associated with the paradigmatic Type II diseases rather than with the innate immunopathology driven diseases associated with the other members. It has been shown to contribute to the downregulation of both filaggrin and human beta defensin 2 expression (34, 35). However, IL-33 shares many characteristics with its fellow IL-1 family cytokines in its alarmin function and as a sensor of environmental threats. IL-33 also appears to have a role in detecting cellular stress. Keratinocytes increase expression of IL-33 when experiencing hypo-osmotic stress. The induced IL-33 was observed to localise at the nucleus rather than being secreted, leading the authors to hypothesise that the increased expression acts as a failsafe in circumstances where stress progresses to damage, thus releasing IL-33 and inducing inflammation (36). In addition to the above mentioned IL-33 functions, this may be of relevance in atopic dermatitis (AD) and eczema where a loss of barrier integrity leaves the skin more exposed to external osmotic stress. Indeed, exposure to fresh water is known to exacerbate AD and eczema (37). A similar concept of increasing alarmin expression as a failsafe has also been suggested to occur with IL-1α, whereby the presence of the commensal *Staphylococcus epidermidis* allows the epidermis to maintain a level of intracellular IL-1α, keeping it primed for an efficient response in the event of damage (38). Similar to IL-1 and IL-36 cytokines, IL-33 has been shown to be truncated to potently

active forms by numerous exogenous proteases, including pollen- and house dust mite-derived proteases (39). IL-33 is retained in the cell nucleus and cytosol as an intracellular store, is active in its immature form, and is released upon cell damage or programmed cell death (40). Yet, proteolytic truncation can dramatically increase its biological activity and will therefore generate a more potent alarmin once exposed to the external environment. In contrast to the other IL-1 family cytokines, IL-33 drives Type II immune responses, so susceptibility to proteolytic activation by an alternative set of exogenous proteases may function as a method of fine-tuning the inflammatory alarm depending upon the proteolytic environment experienced by the alarmin once released from the cell.

Pathological Consequences

Whilst IL-1 family cytokines are well suited as sensors of potential threats, their sensitivity to proteolytic activation and interaction with environmental microbes may also lead to the development of pathological inflammation. IL-36γ and *Cutibacterium acnes* have also been implicated in the pathomechanism of drug-induced acneiform skin toxicity (41). Satoh et al. report that EGFR/MEK inhibition in patients colonised with the commensal causes synergistic expression of IL-36γ. *C. acnes* mediated activation of NF-kB combined with increased expression of Krüppel-like factor 4 as a result of EGFRi/MEKi treatment strongly upregulated IL-36γ expression, which in turn lead to inflammatory skin-based toxicity (41). *S. pyogenes* tonsillar infection has a well-established strong aetiological association with guttate psoriasis – an acute eruptive form of psoriasis seen largely in children – and is the strongest environmental factor in triggering and exacerbating psoriatic skin lesions (42, 43). It has therefore been hypothesised that *S. pyogenes*-mediated proteolysis of IL-1β and IL-36γ during infection may trigger inflammation and lead to the development of inflammatory disease. The importance of balanced proteolytic regulation between host and commensal in epidermal homeostasis is underlined in patients with Netherton Syndrome (NS), in which the endogenous protease inhibitor LEKTI-1 is non-functional. Patients with NS suffer predominantly IL-17- and IL-36-driven cutaneous inflammation in conjunction with a *S. aureus* and *S. epidermidis* dominated microbiome (44, 45). A recent study conducted in mice has demonstrated the lack of adequate endogenous protease inhibition imparted by LEKTI-1 promotes dysbiosis, which in turn alters the proteolytic balance of the epidermis through the secretion of proteolytic virulence factors, inducing skin damage and promoting inflammation (45).

The pathological consequence of protease sensitivity is not limited to exogenous proteases as many endogenous proteases are also capable of truncating IL-1 family precursors to biologically active forms. IL-1α can be truncated to a more potent form by numerous proteases including calpain, granzyme B and neutrophil proteases to a more biologically potent form (46). Yet 'unplanned' truncation by endogenous proteases often generates less biologically active forms than their intended activators. IL-1β, for example, can be truncated by neutrophil elastase, proteinase-3 and cathepsin G, however the resultant forms are less active than caspase-1-truncated IL-1β (47–49). Nevertheless these mechanisms of activation are implicated in a number of inflammatory conditions. Proteases secreted by neutrophils, which are prominent in psoriasis plaques, have been shown to truncate immature IL-1 family cytokines including IL-1α, IL-1β, and the IL-36 cytokines, thereby potentially amplifying the inflammatory environment once recruited to the skin (50). Cathepsin G, neutrophil elastase and proteinase-3 have been demonstrated to activate IL-36α and IL-36γ (16, 51). There is some debate over the inflammatory impact of neutrophil-derived proteases as they are also involved in activating immature IL-36Ra and may also be involved in proteolytically de-activating IL-1 family cytokines following prolonged exposure (3, 52). Given the abundance of neutrophils and IL-36γ in psoriasis plaques, it seems likely neutrophils will exacerbate IL-36-mediated inflammation in psoriasis, however the contradictions present in the literature demonstrate there is still a lack of understanding on the overall neutrophil-mediated impact on IL-1 and IL-36 mediated inflammation *in vivo*.

In addition to promoting inflammatory disease, proteolysis and microbe-induced expression of IL-1 family members can promote atopic disease. Whilst IL-18 is primarily associated with promoting Type I immune responses, IL-18 secretion is known to be induced by *S. aureus* colonisation in cases of AD (53). This is particularly interesting when considered in conjunction with mast cell digestion allowing activation of extracellular IL-18 in the absence of caspase-1 activity (54). In a mouse model of atopic dermatitis, epicutaneous *S. aureus* has been shown to induce IL-36-dependent signalling and a subsequent increase in IgE and IL-4 production (55). However, as yet there are no data implicating this mechanism is present in humans and there is no comprehensive link between IL-36 signalling and factors driving immunoglobulin class switching.

IL-1α has been demonstrated to be instrumental in the development of chronic inflammation in filaggrin-deficient mice. In these mice, skin injury and dysbiosis drive IL-1α expression and secretion from keratinocytes, which in turn causes chronic inflammation (56). IL-33 has also been implicated in the environmental allergen-mediated triggering of allergic inflammation. Recent evidence shows environmental allergens including house dust mites, *Alternaria alternata*, *Aspergillus fumigatus* and cat dander can initiate intracellular cleavage and subsequent secretion of active IL-33 as a result of RIPK-caspase-8 ripoptosome activation in epithelial cells (57). The released active IL-33 is then free to induce pro-atopic responses from surrounding tissue. Considering the evidence that IL-33 is also susceptible to proteolytic activation by many proteases produced by these environmental allergens when in the extracellular space, it seems likely pathological IL-33-mediated signalling would result from exposure to these allergens.

ORCHESTRATING IMMUNE RESPONSES

Once released and activated, IL-1 family cytokines help to orchestrate an appropriate immune response through interaction with their local cellular environment (**Figure 2**). IL-1 family cytokines have an impact to some extent on all cells

within the skin and elicit a response from neighbouring keratinocytes and infiltrating immune cells. In doing so they influence both the innate and adaptive response.

Effects on the Keratinocyte Barrier

Within the epidermis, keratinocytes are strong responders to IL-1 family cytokines. Given their abundance in comparison to infiltrating immune cells, they are arguably the most important initial responders to IL-1 family cytokines and act to both fight infection through release of anti-microbial peptides and amplify inflammatory signals through release of a range of chemokines and cytokines. As many IL-1 family cytokines transduce signal through MyD88, keratinocytes respond to pro-inflammatory IL-1 family cytokines in a very similar fashion. Indeed, a recent study examining transcriptional differences in keratinocytes following IL-1β and IL-36 cytokine stimulation found nearly all IL-1β responses were replicated by IL-36 stimulation (58). In response to IL-1α, IL-1β, and IL-36 stimulation, keratinocytes express and release a host of antimicrobial peptides including human beta defensins, LL-37, and S100 proteins which directly combat infection (59, 60). They increase protein levels of many key pro-inflammatory cytokines including IL-23, IL-17C, TNFα, as well as other IL-1 family cytokines that will further amplify an inflammatory response (1, 61). Keratinocytes also secrete chemokines following stimulation with IL-1 family cytokines. IL-1α, IL-1β, and IL-36 cytokines all induce secretion of IL-8 (CXCL8) and CCL2 (aka MCP-1) – strong inducers of neutrophil and myeloid cell chemotaxis respectively – and CCL20 – responsible for the influx of cells expressing CCR6, which include T_H17, gamma delta (γδ) T cells and Type III innate lymphoid cells (ILC3s) (58, 62). Keratinocytes also secrete CXCL10 in response to IL-18 stimulation; a chemoattractant of CXCR3-expressing cells including CD8 and CD4 T cells (63).

In addition to inducing expression and secretion of cytokines, chemokines and peptides from keratinocytes, IL-1 family cytokines can also induce cellular changes in keratinocytes that influence their barrier function. Recently IL-1β has been shown to promote cell-intrinsic immunity in epithelial cells by inducing activation of IRF3 and therefore driving an innate anti-viral response. IL-1β was found to induce the release of mitochondrial DNA into the cytosol of stimulated cells, subsequently activating the cGAS-STING pathway and activating IRF3 (64). There is evidence to suggest IL-1α stimulation of keratinocytes reduces the adherence of *S. pyogenes*, thereby reducing the likelihood of invading bacteria establishing an infection. This was hypothesised to be a result of changes in their expression of receptors utilised by *S. pyogenes* in adhering to keratinocytes (65). IL-18 stimulation of keratinocytes is known to increase expression of molecules including MHC-II, increasing their capacity to present antigens to infiltrating T cells (63).

Effects on the Skin's Immune Compartment

In addition to their effects on the epidermis, the IL-1 family cytokines have a significant effect on the immune compartment. Langherans cells (LCs) and dendritic cells (DCs) reside in the epidermis and dermis, respectively, sampling their environment for threats to respond to. LCs express high levels of IL-36R and IL-1R in comparison to blood-derived myeloid cells and respond to stimulation by secreting pro-inflammatory mediators and expressing co-stimulatory receptors. Whilst traditionally IL-1β has been thought of as a crucial cytokine along with TNFα for the maturation and migration of LCs (66), IL-36β has been shown to be equally as potent as IL-1β in stimulating LCs (67). Dendritic cells, on the other hand, are slightly more varied in their expression and response to IL-1β and IL-36 cytokines. $CD3a^+$ DCs express high levels of IL-36R yet have limited expression of IL-1RAcP, and conversely $CD14^+$ DCs express high levels of IL-1RAcP but do not express IL-36R. As a result, dermal DCs are not very responsive to IL-36 stimulation but respond well to IL-1β by similarly expressing pro-inflammatory cytokines and co-stimulatory receptors (67).

Neutrophils and macrophages recruited to the skin during inflammation are also influenced by IL-1 family cytokines. IL-1α, IL-1β and IL-18 have been shown to activate neutrophils, increasing oxidative burst and inducing calcium-dependent degranulation (68, 69). Macrophages react variably to IL-1 family cytokines depending on their differentiation. M0 macrophages are responsive to both IL-1β and IL-36 cytokines, but more responsive to IL-1β than IL-36. M1 macrophages respond to IL-1β but not to IL-36, whilst M2 macrophages are equally responsive to both IL-36 cytokines and IL-1β (67). IL-18, originally termed IFN-gamma (IFN-γ) inducing factor, plays a significant role in the activation of mononuclear cells facilitating improved clearance of both intracellular and extracellular bacterial infections as a result of its induction of IFN-y from multiple cellular sources (70).

Innate lymphoid cells (ILCs) both reside within the dermis and epidermis as tissue-resident cells and are actively recruited to the skin during inflammation. IL-1β has recently been shown to facilitate plasticity in ILCs. ILC2s stimulated with IL-1β begin to express low levels of T-bet and IL-12Rβ2, allowing a phenotypic switch to ILC1s following IL-12 stimulation (71). Similarly, ILC2s treated with IL-1β, TGF-b and IL-23 can adopt an ILC3 phenotype by expressing RORγt and producing IL-17 (72). IL-18 in combination with IL-15 has been shown to be important in the proliferation of ILC3s and can contribute to their role in epithelial barrier function by inducing expression of IL-22 (73).

In addition to their effects on cells of the innate immune system, IL-1 family cytokines significantly influence adaptive immunity, both directly and indirectly. IL-1β is well established to potentiate production of IFN-γ and act as a driver of T_H17 differentiation through direct stimulation (74). Recently, IL-1β has also been shown to promote T_H17 differentiation indirectly *via* activation of DCs. IL-1β-primed DCs increased expression of CD14 and induced RORγt expression and IL-17 production in CD4 memory T cells in a CD14 dependent manner (75). Whilst murine T cells are well established as expressing IL-36R and are directly responsive to IL-36 stimulation, the sensitivity of human T cells to IL-36 cytokines is less clear. Early research could not demonstrate a direct effect of IL-36 stimulation on human T cells, however a later report by Penha et al. show expression of IL-36R in blood- and intestinal-derived T cells and dose-

dependent CD4 T cell expansion following stimulation with IL-36β (62, 67, 76). IL-36 cytokines have also similarly been shown to promote CD4 T cell proliferation through their activation of DCs, other antigen presenting cells, and the induction of polarising cytokines (62, 77, 78). Interestingly, in mice IL-36γ has been shown to prevent the development of T_{reg} cells, instead promoting development of T_H9 effector cells (79).

Unlike the other IL-1 family members, IL-33's influence on the immune compartment drives Type II immunity and is strongly linked with promoting T_H2-driven diseases such as AD. IL-33 has been shown to induce the expression of CCL17 from AD-derived keratinocytes; a chemokine responsible for attracting CCR4 positive cells associated with Type II immune responses (80). Mast cells undergo activation and maturation following IL-33 stimulation and become more responsive to IgE and IgG. These cells also respond to both IL-18 and IL-33 by producing Type II polarising cytokines such as TSLP, IL-4, and IL-13 (81–83) and therefore promote T_H2 development. T_H2 cells themselves are also known to express ST2 and respond directly to IL-33 stimulation by producing IL-5 and IL-13 (84, 85).

In addition to driving T_H2 development, IL-33 has been well described as a driver of ILC2 proliferation (86). ILC2s are thought to play a prominent role in the pathogenesis of AD. Their numbers are found at increased levels in the skin of AD patients when compared to that of psoriasis patients (87). Indeed mouse models of AD induced by transgenic IL-33 expression can be effectively treated by the specific depletion of ILC2s (88). Despite their role in promoting pathological Type II inflammation, IL-33 and ILC2s are also associated with protection. Numerous publications demonstrate their importance for protection against gut tissue damage and gastro-intestinal infections (89–91), and similarly has been shown as promoting ILC2-mediated wound healing in skin (92).

RESOLVING AND REGULATING IL-1 FAMILY INFLAMMATION

All IL-1 family members are tightly controlled (93) on multiple levels including RNA stability, translation, secretion and activation by proteases as well as soluble receptors, receptor antagonists and binding proteins (**Table 1**). This significant level of control may also illustrate the powerful pro-inflammatory properties of IL-1 family members and disturbance in any of these mechanisms can lead to auto-inflammatory (sometimes clinically dramatic) disease manifestations.

There is limited knowledge about how counter-regulatory IL-1 family mechanisms are activated and regulated. While counter-regulatory activity happens on the level of receptor expression, soluble receptor release, signalling molecule stability/ubiquitination, translocation, activation and mRNA stability, here we highlight protein antagonists for each IL-1 family member. The cutaneous dynamics of all counter-regulatory mechanisms have not been well investigated and as such no complete picture is available for the IL-1 family relevant resolution phase of inflammatory epithelial responses *in vivo*. For blood derived monocytes, persistent versus resolving inflammation experimental setups have been investigated (96) and showed that IL-1β, IL-1Ra and soluble receptors are all released between 4 and 14h during the course of both resolving and persistent inflammation *in vitro*.

IL-1Ra is the first described endogenous receptor antagonist. Different variants, including an intracellular form lacking a leader sequence (97), have been described which add to its complex biology. In the skin, IL-1Ra is produced abundantly by keratinocytes, infiltrating neutrophils and infiltrating or resident antigen presenting cells. IL-1Ra binds to the IL-1R1 but fails to induce recruitment of the co-receptor IL-1R3 (aka IL-1RAcP), thus preventing signal transduction *via* the IL-1R. This is how it works as a competitive antagonist for both IL-1α and IL-1β. IL-1R type 2 (CD121b) is a decoy receptor which binds to IL-1α and IL-1β with high affinity and IL-1RA with lower affinity. While IL-1R2 interacts with IL-1R3 this interaction does not result in signal transduction. In addition to its membrane bound form IL-1R2 has also been found as a soluble protein either as a result of shedding or alternative splicing and has been shown to be active in the cytosol binding to IL-1α and preventing its cleavage and activation. Similarly, soluble receptors have been described for IL-33, sequestering

TABLE 1 | Table showing the regulatory mechanisms controlling IL-1 family mediated signalling.

IL-1 family member	Antagonist	Other inflammation limiting mechanisms
IL-1α (signal transduction by receptor complex IL-1R1+IL-1R3)	IL-1Ra, IL-1R2, soluble receptors (sIL-1R1 & sIL-1R2), IL-37/SIGIRR	RNA stability, protein secretion, sheddase activity; miRNA (94)
IL-1β	IL-1Ra, IL-1R2, soluble receptors, IL-37/SIGIRR *Note: the affinity of sIL-1R2 for IL-1β is increased by the formation of trimeric soluble complexes with sIL-1R3 (95)*	RNA stability, caspase activation, protein secretion, sheddase activity; reduced inflammasome activity/caspase 1
IL-36 agonists	IL-36Ra IL-37 IL-38	Protease availability; activation of IL-36Ra by elastase
IL-18	IL-18BP, IL-37	
IL-33	sST2	Caspase-3/-7 mediated inactivation, oxidation, nuclear sequestration
IL-37		Enhanced IL-18BP activity Binds to IL-18R1 and SIGIRR
IL-38		Uncharacterised mechanism

IL-1Ra, IL-1 receptor antagonist; BP, binding protein.

extracellular IL-33 and limiting subsequent signalling (98). All IL-1α/β relevant molecules mentioned, including the IL-1RA and type 2 IL-1R are expressed by keratinocytes (99). The IL-36 cytokines are also regulated through a receptor antagonist, IL-36Ra. IL-36Ra functions in an analogous manner to IL-1Ra, binding its cognate receptor IL-36R without recruiting the accessory protein IL-1RAcP, thus preventing signal transduction.

IL-38 is another anti-inflammatory cytokine belonging to the IL-36 sub-family. Whilst this cytokine is thought to be an important regulator of IL-36 dependent inflammation and has been shown to be a general regulator of IL-1-family mediated inflammation, its precise mechanism of action is not wholly understood. There is evidence to suggest it may directly antagonise IL-36R like IL-36Ra, however this has not yet been conclusively demonstrated. Nevertheless, it has been shown to inhibit IL-36γ-induced NF-kB activity in keratinocytes and seems to be important in regulating inflammation in psoriasis. Its expression is lower in psoriatic skin than in healthy skin and increases following anti-IL-17A treatment in a manner that positively correlates with therapeutic efficacy (100).

While those cells producing IL-1α/β also harbour the full repertoire of antagonistic molecules the same is not necessarily true for the IL-18 system. Here keratinocytes are recognised producers of IL-18 as of relevance in psoriasis, cutaneous lupus and chronic eczema, however a main source of IL-18BP in the skin organ are fibroblasts (101) which are not known to secrete IL-18. Yet it has recently been shown that the cleaved pro-peptide from IL-18 can interact with mature IL-18, potentially abrogating its ability to engage with its receptor, highlighting an innate method of limiting IL-18 signalling without requiring expression of IL-18BP (102).

It is of interest that another anti-inflammatory molecule impacting on IL-1 family members, IL-37 (103), uses the IL-18R in addition to SIGIRR (IL-1R8) for its downstream effects (104). IL-1R8 interacts with IRAK and TRAF6 thus competing with IL-1 or TLR activated signalling. As previously mentioned, Gudjonsson et al. have clearly shown that the signalling events induced by IL-36 are mostly identical to IL-1 induced signalling (58). The difference in their pathophysiology relies on the compartment where they are expressed and events leading to cleavage of pro-forms into bioactive molecules. Regarding IL-1R8, it can attenuate inflammatory responses induced by IL-1, IL-18, IL-33, IL-36, and TLR. A recent study in *SIGIRR*$^{-/-}$ mice demonstrates its importance in regulating IL-36-mediated inflammation (105). The anti-inflammatory molecule IL-37 can also bind to IL-18BP and seems to enhance its effects. IL-37 along with all IL-36 members and the IL-36 receptor antagonist IL-36Ra are all expressed by human keratinocytes.

Studies examining the regulation of IL-33 have revealed some interesting mechanisms of limiting IL-1 cytokine activity. Whilst proteolytic regulation of the IL-1 family cytokines is often associated with their activation, it has been demonstrated that IL-33, already active in its native form, is inactivated following truncation by caspase-3 and caspase-7 during apoptosis (106, 107). Interestingly, this inhibition is mediated by truncation of a specific amino acid sequence that is absent in all other IL-1 family cytokines, suggesting specific regulation of IL-33 during apoptosis (106). Intriguingly IL-33 has recently been demonstrated to exhibit an intrinsic method of limiting prolonged activity. IL-33 quickly loses its biological function following release into the extracellular environment as oxidation of critical cysteine residues in IL-33 causes a conformational switch to an inactive form (108). As other IL-1 family cytokines also contain free cysteine residues it is possible other IL-1 family members are regulated in a similar manner. Indeed Cohen et al. demonstrate IL-1α, IL-18, IL-36α and IL-36γ all exhibit similar oxidative changes when exposed to an oxidative environment (108).

Within the skin compartment much inflammatory activity related to the IL-1 family is based in the epidermis. As mentioned, dermal resident cells such as fibroblasts are less able to produce IL-1 family agonists but contribute to antagonist secretion including IL-18BP and IL-1Ra. Thus, infiltrating macrophages aside, the overall property of the dermal versus epidermal compartment is more inflammation limiting than pro-inflammatory with regard to IL-1 family molecules.

The importance of both adequate regulation and resolution of inflammation by the IL-1 family cytokines is most clearly demonstrated when examining monogenic mutations that directly affect the regulation of IL-1 family cytokines (summarized in **Figure 3**). This is most striking in conditions that result from the loss of the IL-1 family antagonists. Deficiency of the IL-1 Receptor Antagonist **(DIRA)** is an autosomal recessive disease caused by mutations of *IL1RN* that results in non-expression of the IL-1 receptor antagonist and subsequent unopposed IL-1 receptor activation by IL-1α and IL-1β (109). DIRA is most commonly associated with a pustular rash, joint swelling and periostitis. DIRA has been successfully treated with the anti-IL-1 biologics Anakinra (recombinant IL-1Ra) (110, 111), Canakinumab (112) (IL-1β blocking antibody) and Rilonacept (fusion protein of IL-1 receptor and IgG Fc) (**Figure 4**) (113).

Similarly, loss of function mutations in *IL36RN*, which encodes IL-36Ra, leads to the development of DITRA (Deficiency of interleukin-36 receptor antagonist) in which unchecked IL-36 signalling causes significant systemic inflammation (114). In addition to the immediate inflammatory consequence of enhanced IL-36 signalling, the lack of IL-36-specific inhibition has been shown to promote extensive antigen-dependent expansion of T_H17 cells and subsequent production of IL-17A, promoting T cell-mediated inflammation (115). Affected patients develop GPP psoriasis with a systemic inflammation and fever. GPP has successfully been treated with anti-IL-36R therapy (**Figure 4**) (116). If left untreated, both DIRA and DITRA can prove fatal, demonstrating the importance of proper regulation of IL-1 and IL-36 signalling (117, 118).

Numerous genetic defects that occur in the inflammasome-activation pathway demonstrate the importance of properly regulated inflammasome activation. Examples of both gain of function mutations in inflammasome activation and loss of function mutations in inflammasome regulation have been well reported. Cryopyrin-Associated Periodic Syndrome (CAPS) represents autosomal dominant gain of function mutations that

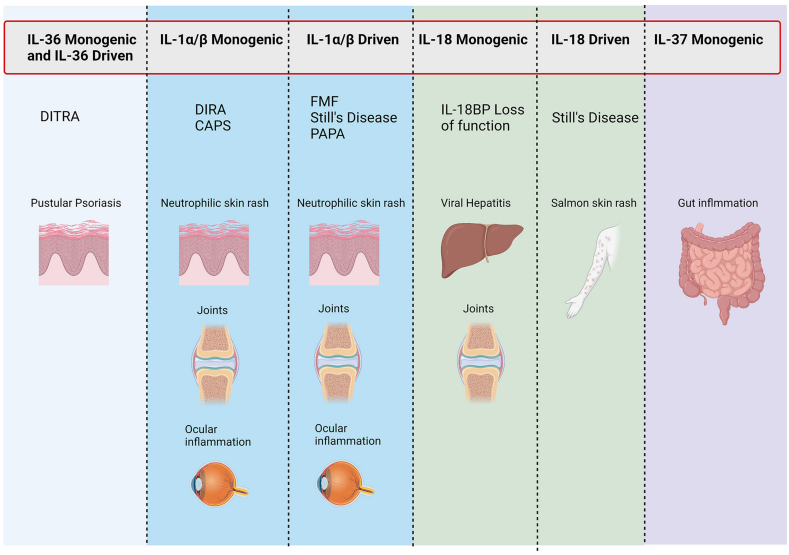

FIGURE 3 | Conditions associated with IL-1 family genetic mutations. Figure outlining symptoms associated with disease-causing mutations in monogenic and IL-1 family driven conditions.

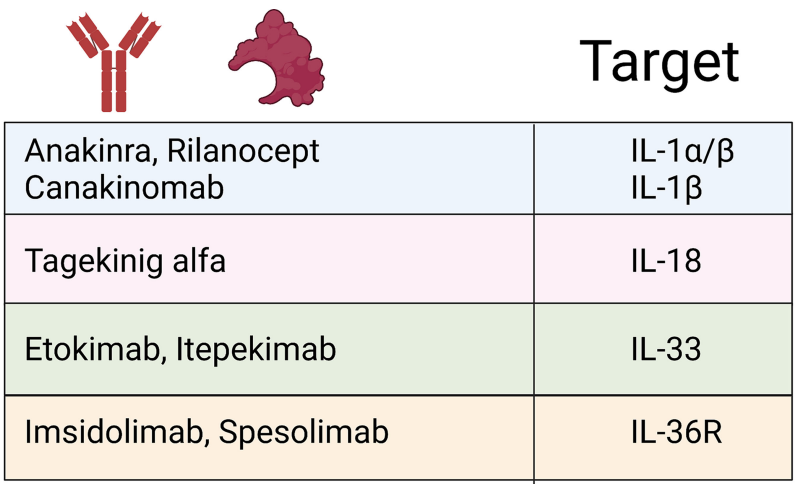

FIGURE 4 | IL-1 family biologics. Schematic outlining current FDA-approved biologics targeting IL-1 family cytokines and signalling pathways that have demonstrated efficacy treating IL-1 cytokine-driven conditions.

results in over activation of the inflammasome and increased conversion of pro-caspase-1 to activate caspase-1 and this results in increased IL-1β and IL-18 protein levels (119, 120). These conditions include Familial Cold Autoinflammatory Syndrome (FCAS) and Muckle-Wells Syndrome (MWS) caused by mutations in the CIAS1 gene (NLRP3) which encodes cryopyrin (121–123). CINCA (chronic infantile neurologic, cutaneous and arthritis syndrome) was later revealed to be caused by a spontaneous mutation in the same gene and called, Neonatal Onset Multisystem Inflammatory Disease (NOMID) (124, 125). Recent publications have also identified gain of function mutations in *NLRC4* resulting in similar aberrant activation of the NLRC4 inflammasome (126). Amongst severe systemic symptoms such as macrophage activation syndrome and periodic fever, patients also exhibit severe urticarial rashes that are marked by significant neutrophil infiltrates. Interestingly, patients with NLRC4 mutations exhibit higher levels of IL-1β and IL-18 than those with NLRP3 mutations, and have been shown to respond well to IL-18BP treatment (127–129). CAPS patients respond well to IL-1

blockade, with Anakinra, Rilonacept and Canakinumab all showing efficacy (130–135).

FMF (Familial Mediterranean Fever) results from a breakdown in regulation of inflammasome activity caused by mutations in the MEFV gene which encodes pyrin (136, 137). The precise role of pyrin in the pathogenesis of FMF is heavily debated. Pyrin interacts with ASC, a component of the inflammasome and sequesters ASC, making unable to form an inflammasome (138, 139). Wildtype pyrin has also been demonstrated to bind to caspase-1 and inhibit IL-1β secretion (138, 140). FMF is commonly treated with colchicine, however, there are reports of IL-1 blockade being successful in the form of Anakinra and Canakinumab (141, 142).

Whilst not common, there are also examples of IL-37 and IL-18BP loss of function giving rise to inflammatory disease. An infant with non-functional IL-37 has been identified to suffer inflammatory bowel disease, however, as yet shows no sign of skin manifestations. The loss of functional IL-18BP resulted in a case of fulminant viral hepatitis and the death of the patient (143, 144).

The IL-1 family cytokines are also well known to have central roles in complex multifactorial inflammatory conditions such as psoriasis, ichthyosis conditions, hidradenitis suppurativa (HS) and AD. As these topics have been extensively reviewed elsewhere (93, 145, 146) this review shall highlight recent examples of inflammatory conditions involving IL-1 family cytokines.

Systemic-onset juvenile idiopathic arthritis (sJIA) and adult-onset Still's disease (AoSD) are rare systemic auto-inflammatory polygenic disorders, which are accompanied by a broad spectrum of manifestations. The diseases, considered part of a spectrum continuum, are associated with fever, arthritis and skin rashes that are salmon pink in appearance. Skin biopsies show neutrophilic inflammation (147). Both diseases considered can significantly overlap with macrophage activation syndrome (MAS) and feature a cytokine storm. The causes of these entities and immunogenetics are complex and are discussed elsewhere (148). However, a central mechanism appears to be the dysregulated production of IFN-y, IL-1, IL-6 and IL-18, the latter driving IFN-y (148). Targeting of IL-1α/β (Anakinra (149) or Canakinumab (150), as well as recombinant IL-18BP (151) (tadekinig alfa) has shown some success. A hair follicle disease hidradenitis suppurativa (HS), which is dominated by pathological neutrophils, has recently been shown to significantly involve the IL-1 and IL-36 pathways in driving its proinflammatory pathways (152, 153) IL-1β-driven transcription present in HS lesions was found to induce immune cell infiltration to lesions and extracellular matrix degradation (152). IL-36 cytokines were also found to be significantly upregulated, and their regulatory counterparts downregulated, in lesional HS (153).

IL-1 FAMILY CYTOKINES IN THE PROMOTION OF WOUND HEALING

Wound healing is essential in maintaining the skin integrity and barrier function. It is a complex process, defined by four consecutive but overlapping stages, starting with haemostasis, followed by inflammation, proliferation, and finally tissue remodelling (154, 155). Imbalance in any of these stages can lead to delayed or impaired wound healing. IL-1 family members are known to play a key role in the wound healing process. IL-1α and IL-1β levels are immediately increased following tissue injury, reaching their peak in the first 12 – 24h, underscoring their alarmin function, and return to normal once the proliferation stage of wound healing is complete (156). Interestingly, reinforcing the importance of proteolytic regulation of IL-1 family cytokines, thrombin has recently been demonstrated to truncate IL-1α to a more potent form following its activation in the coagulation cascade (157). Indeed, a mouse model expressing IL-1α that cannot be truncated by thrombin exhibits delayed wound closure (157).

As already alluded to, IL-1 cytokines promote the initial neutrophil recruitment to the site of injury, facilitating the removal of debris and the prevention of a potential bacterial infection (158). Similarly to TNFα, both IL-1α and IL-1β facilitate wound healing by inducing fibroblast and keratinocyte proliferation, the production and degradation of extracellular matrix (ECM) proteins (collagens, elastins, fibronectins and laminins), fibroblast chemotaxis, and by exerting immune modulatory functions (156). The majority of these effects are obtained *via* the production of various growth factors by IL-1 stimulated cells. *In vitro* experiments showed that IL-1 produced by human keratinocytes stimulate the production of transforming growth factor alpha (TGF-α) in an autocrine fashion (159). TGF-α increases keratinocyte migration (160) and proliferation (161–163) as well as their expression of K6 and K16, markers of a hyperproliferative state (164). Furthermore, keratinocyte derived IL-1 stimulates fibroblasts to produce other growth factors with important roles in keratinocyte proliferation, including keratinocyte growth factor (KGF) (165–167) and granulocyte–macrophage colony-stimulating factor (GM-CSF), creating a double paracrine loop (168, 169). In addition, IL-1 indirectly induces dermal fibroblast and arterial smooth muscle cell proliferation by stimulating their production of platelet-derived growth factor (PDGF) AA (170). The newly forming tissue during wound repair has a high oxygen demand, therefore, neovascularisation is an essential part of normal wound healing. IL-1β has been shown to induce vascular endothelial growth factor (VEGF) production by keratinocytes, a key factor in neovascularisation, although to a lesser extent compared to TNFα (171, 172). Interestingly, the bacterial microbiome has been shown to be important in promoting wound healing by inducing IL-1α and IL-1β expression during wounding. A recent study has shown a low bacterial quantity and diversity in mice skin correlates with poor wound-induced hair follicle neogenesis (WIHN), and that increasing bacterial counts, even pathological *S. aureus*, improves WIHN (173). This microbiome-induced promotion of skin regeneration was shown to be dependent on keratinocyte-specific IL-1β signalling. Furthermore, in a small human trial the use of topical antibiotics prior to wounding was demonstrated to prolong the healing process (173).

The various growth factors resulting from the keratinocyte-fibroblast crosstalk are essential in the proliferation of the epithelial stem cell pool (174). Epithelial stem cell mobilisation is crucial in the proliferative phase of wound healing. Progenitor cells derived from interfollicular epidermal and hair follicle stem cells are mobilised to rapidly restore skin integrity (175, 176). However, the signals that trigger the activation of these stem cells proximal to the wound are not fully understood. Recent work conducted in mice indicates the synergistic involvement of IL-1α and IL-7, secreted by keratinocytes, in the expansion of epidermal γδ T cells (177). γδ T cells are resident epidermal cells, which upon activation secrete KGF and induce the proliferation of epidermal stem cells (178, 179). Moreover, it has been shown that in mice IL-1β is indispensable in the recall of inflammatory memory of epidermal stem cells, facilitating wound healing following repeated insults (180).

Interestingly, IL-1β and IL-18 have recently been implicated in the activation of cutaneous mucosal associated invariant T (MAIT) cells following their interaction with local microbiota, which results in the promotion of wound healing. Following interaction with *S. epidermidis* in human epidermis, cutaneous MAIT cells have been shown to expand, produce IL-17A and exhibit a transcriptional profile consistent with leukocyte activation and tissue repair in a manner dependent on IL-1 and IL-18 signalling (181). Local IL-1α signalling was shown to be crucial for MAIT cell IL-17A production, whilst IL-18 was required for their expansion. As previously mentioned, the presence of *S. epidermidis* allows the epidermis to maintain a level of IL-1α expression, thus is primed for allowing IL-1 signalling following tissue damage, promoting MAIT cell activation and subsequent tissue repair. IL-1 family cytokines have also been implicated in promoting wound healing through their impact on commensal-specific tissue resident T cells (T_{RM}). A recent study identified *S. epidermidis*-specific $CD8^+$ T_C17 cells to co-express GATA3 and constitutively express a Type II transcriptome, including *IL5* and *IL13*. Despite their expression of the Type II transcriptome, protein synthesis was only licenced following stimulation by tissue damage-associated alarmins (182). Stimulation of *S. epidermidis*-specific T_C17 cells with IL-1α, IL-33 and IL-18 in conjunction with T cell receptor activation induced production of IL-13, which has been shown to promote wound healing (183). Indeed, the authors demonstrated these Tc17 cells were able to recover *S. epidermidis*-accelerated wound healing in IL-13 deficient mice (182).

Less is known on the role of other IL-1 family members in wound healing apart for an important role for IL-33 that is upregulated following cutaneous wounding in mice, peaking 5 days post-injury (92). Unlike IL-1β, IL-33 promotes reepithelization by facilitating the shift from a pro-inflammatory to an anti-inflammatory macrophage profile and stimulates ECM production in both normal (184) and diabetic mice (185). Furthermore, it enhances neoangiogenesis *via* the production of VEGF and von Willebrand factor (185). IL-33-sensitive ILC2 cells have also been shown to play a role in the wound healing process. Rak et al. identified an increase in $ST2^+$ ILC2 numbers at sites of healing wounds in both human and mouse cutaneous tissue. The authors demonstrated that IL-33 knockout mice exhibited significantly smaller numbers of activated ILC2s at wound sites and were significantly slower in closing wounds (92). Oshio et al. also demonstrate IL-33 knockout mice exhibit slower wound closure, and identify the importance of nuclear IL-33 in limiting excessive NF-kB-mediated inflammation (186). IL-36 cytokines have also been implicated in wound healing as a result of injury. Jiang et al. demonstrate that release of TLR3 agonists following tissue damage induces expression of IL-36γ, which in turn induces expression of REG3A in keratinocytes to promote re-epithelialization and wound healing (187).

A wound healing response does not always have a beneficial outcome. Tissue trauma and the subsequent wound healing response can contribute to resurgence of dormant tumours under certain circumstances. The production of growth factors, increase in inflammatory mediators and promotion of re-epithelialisation and proliferation have all been implicated in promoting metastasis and tumour dormancy escape (188). Furthermore, inflammation induced in wound healing does not always resolve completely, and can in some cases lead to the development of chronic wounds (189). Indeed, whilst IL-1 family cytokines are important in the wound healing process, their dysregulation can contribute to pathological outcomes. IL-1 family members have been found to play roles in such inflammatory override, with an essential role being attributed to IL-1β (190). It has been shown that non-microbial danger signals resulting from hyperglycaemia and obesity can lead to inflammasome activation and release of IL-1β and IL-18 from a number of different cell types, including monocytes/macrophages, known to be involved in all stages of wound healing (191–196). IL-1β then sustains the inflammasome activity of macrophages, creating a positive feedback loop (197). Moreover, a recent study in diabetic mice showed that beside maintaining an inflammatory macrophage profile, increased levels of IL-1β also result in the persistence of inflammatory cells at the wound site, senescence of fibroblasts and high levels of matrix metalloproteinases (190), which in turn degrade growth factors and extracellular matrix proteins with an important role in wound healing.

Murine models showed that topical inhibition of caspase 1, the inflammasome, IL-1β or its signalling through IL-1R1, results in the switch from a sustained pro-inflammatory macrophage stage to an anti-inflammatory macrophage profile, reduced levels of proinflammatory cytokines (IL-1β, TNFα, IL-6, CXCL1), chemokines (the murine homologue of IL-8, MIP-1α), and matrix metalloproteases, but increased the levels of anti-inflammatory cytokines (TGF-β1, IL-4, IL-10), tissue inhibitor of metallopeptidase-1 (TIMP-1) and growth factors (FGF-2, PDGF-BB, VEGF-A), facilitating effective wound healing (190, 197–200). Treatment with IL-1RA has been also shown to reduce postoperative wound pain due to the reduced inflammatory response (200). These studies highlight IL-1β as the biggest culprit in the maintenance of chronic, non-healing wounds in diabetic patients, suggesting the inflammasome as a potential therapeutic target in the treatment of non-healing diabetic wounds.

Whilst IL-36 signalling has been shown to have some beneficial roles in the wound healing process, excessive signalling also seems detrimental. Studies performed in mice lacking expression of IL-36Ra demonstrated these mice experienced a 3-day delay in wound closure as a result. Similar to IL-1β, the delay in wound closure was associated with an increased influx of inflammatory cells such as neutrophils and macrophages and increased expression of inflammatory cytokines. Interestingly, normal wound healing was restored in this mouse model by inhibition of TLR4, which the authors attributed to the inhibition of hyaluronic acid-mediated activation of TLR4 and subsequent induction of IL-36 signalling (201).

CONCLUDING REMARKS

This review has provided an overview of the role IL-1 family cytokines play in maintaining the skin's immunological barrier emphasising recent findings that contribute to our understanding. As has been outlined above, IL-1 family cytokines significantly impact on all stages of an immune response in skin, from sensing threats and alerting surrounding tissue to danger, to facilitating the closure of a wound and restoring the skin's barrier function. However, IL-1 family cytokines are potent cytokines that require tight regulation and balanced signalling. As has been highlighted, a breakdown in adequate regulation often contributes to, if not causes, disease. The dynamic nature of their regulation, particularly at the post-translational level *via* proteolysis, makes understanding their inflammatory outcome in a given biological system difficult. Yet, given the significant impact they have on almost every aspect of immune function in skin, it is important to consider.

AUTHOR CONTRIBUTIONS

TM prepared and wrote the manuscript. AB, CB, and MW contributed to the content of the review. MS, DM and MW directed focus. All authors listed contributed intellectually and approved the manuscript for publication.

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REVIEW
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Autoimmune Pemphigus: Latest Advances and Emerging Therapies

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Pemphigus represents a group of rare and severe autoimmune intra-epidermal blistering diseases affecting the skin and mucous membranes. These painful and debilitating diseases are driven by the production of autoantibodies that are mainly directed against the desmosomal adhesion proteins, desmoglein 3 (Dsg3) and desmoglein 1 (Dsg1). The search to define underlying triggers for anti-Dsg-antibody production has revealed genetic, environmental, and possible vaccine-driven factors, but our knowledge of the processes underlying disease initiation and pathology remains incomplete. Recent studies point to an important role of T cells in supporting auto-antibody production; yet the involvement of the myeloid compartment remains unexplored. Clinical management of pemphigus is beginning to move away from broad-spectrum immunosuppression and towards B-cell-targeted therapies, which reduce many patients' symptoms but can have significant side effects. Here, we review the latest developments in our understanding of the predisposing factors/conditions of pemphigus, the underlying pathogenic mechanisms, and new and emerging therapies to treat these devastating diseases.

Keywords: pemphigus, pemphigus treatment, autoimmunity, autoimmune bullous diseases, advance in pemphigus

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INTRODUCTION

Autoimmune bullous diseases (AIBDs) occur when self-reactive immune responses target the basement membrane or desmosomes of the skin and mucous membrane, leading to loss of epithelium integrity and varying severities of painful blistering (Schmidt et al., 2019). As a primary role of the skin is to protect from invading microorganisms, patients with bullous diseases can suffer recurrent, and potentially life-threatening infections, as well as chronic pain and lowered quality of life (Hsu et al., 2016). AIBDs are separated in two main subtypes, pemphigus and pemphigoid diseases. Pemphigus, from the Greek word "pemphix" (blister), is a heterogenous group of AIBDs affecting the stratified squamous epithelia, responsible for intraepithelial blistering, which is caused by inter-keratinocytic deposits of autoantibodies. Pemphigoid group in contrast affects the subepithelial layers of the skin and/or mucous membranes, responsible for subepithelial blistering, and thus exhibit distinct clinical features. In comparison with subepithelial AIBDs, most types of pemphigus do not involve the basement membrane. Among the pemphigus family are those more common variants, pemphigus vulgaris (PV) and pemphigus foliaceus (PF), as well as the rarer paraneoplastic pemphigus (PNP), pemphigus herpetiformis (PH), and IgA pemphigus (Joly and Litrowski, 2011; Kasperkiewicz et al., 2017).

TABLE 1 | Types of pemphigus.

Clinical variant	General frequency	Ig subtype	Autoantigen	Clinical hallmarks
Pemphigus vulgaris	70 to 90%	IgG	Dsg3, Dsg1	Mainly oral mucosal lesions affecting the gingiva, palate, floor of the mouth, buccal mucosa, lips, tongue, nasal, pharyngeal, laryngo-oesophageal, urethral, genital (glans penis, vulva), perianal and conjunctival mucosae
Pemphigus foliaceus	10 to 30%	IgG	Dsg1	Flaccid and fragile blisters involving skin; no mucous lesions; erythroderma, puff pastry-like scales and crusts affecting seborrheic areas (scalp, face, presternal, interscapular skin)
Paraneoplastic pemphigus	5%	IgG	Dsg3, Dsg1, desmoplakin I and II, envoplakin, periplakin, plectin, BP230, BP180, A2ML1, Dsc1, epiplakin	Severe mucocutaneous lesions with erosions and ulcers involving tongue, lips and vermillion border, nasal, conjunctival and genital mucosa; skin involvement are polymorphic, with pustular, erythema multiform-like or lichenoid lesions; association with lymphoproliferative disorder, lymphoma, leukaemia, carcinomas, sarcomas
Pemphigus vegetans	1–2%	IgG	Dsg3, Dsg1, periplakin	Hypertrophic papillomatous vegetative skin lesions affecting intertriginous sites (axillae, inframammary folds, umbilicus, inguinal creases, anogenital skin, scalp, neck), flaccid blisters and erosions with fetid-odoured
IgA pemphigus	<2%	IgA	Dsg3, Dsg1, Dsc1	Annular erosive plaques, pustules affecting the skin of the trunk, proximal extremities, and intertriginous regions; uncommon mucosal involvement
Pemphigus herpetiformis	<2%	IgG	Dsg3, Dsg1, Dsc	Pruritic herpetiform blisters, annular urticarial plaques with or without erosions, scaly erosive patches mimicking eczema

Dsg, desmoglein; Dsc, desmocollin; BP230 or 180, bullous pemphigoid 230 or 180; A2ML1, α-2 microglobulin-like 1.

Although clinically and aetiologically distinct, all pemphigus variants induce the formation of flaccid blisters and erosions on the skin and/or mucous membranes, and are characterised histologically by acantholysis, in which keratinocytes separate from each other (Amagai et al., 1991). In this case, acantholysis is caused by autoimmune disruption of the desmosomes - specialized adhesive protein complexes that connect neighbouring keratinocytes to each other - as a result of the generation and deposition of IgG auto antibodies against the desmosomal structural proteins desmoglein (Dsg) 1 and/or Dsg3 (Amagai et al., 1991). While the main antigenic targets of these autoimmune skin conditions were discovered several decades ago, our understanding of the factors underpinning the initiation and progression of pemphigus remains incomplete. Moreover, despite recent advances in the treatment of pemphigus, many patients continue to suffer repeated relapses of their condition and/or treatment side-effects, and a significant proportion of cases remain refractory to currently available therapies. Pemphigus mortality rates is elevated compared to the general population (Kridin et al., 2017; Jelti et al., 2019).

In this review we will bring together recent progress in our understanding of the aetiology and pathogenic mechanisms of pemphigus and note those areas in which our knowledge remains incomplete. We will also discuss the latest advances and clinical trials aiming to offer better treatment of this challenging set of diseases and highlight key areas for future research and development.

THE PEMPHIGUS FAMILY OF AUTOIMMUNE SKIN DISEASES: CLINICAL FEATURES AND DIAGNOSIS

All patients with pemphigus variants exhibit autoantibodies against desmosomal structural proteins, but the discrimination of each variant is based on specificities in clinical presentation and/or histological features or in most commonly auto-antibodies encountered (Hertl et al., 2015) (**Table 1**). Understanding the features that distinguish between variants and sub-variants is necessary to allow accurate and rapid diagnosis; moreover, it is hoped that, as we increased our understanding, variant-specific therapies will begin to emerge as part of personalised treatment strategies that take into account any comorbidities in this fragile patient population.

Pemphigus Vulgaris

Pemphigus vulgaris (PV) ("*vulgus*" *which* means "common" in Latin) accounts for up to 70% of pemphigus cases (Joly and Litrowski, 2011; Jelti et al., 2019) with an estimated incidence of between one and five cases per million inhabitants per year (Bastuji-Garin et al., 1995, 1996; Jelti et al., 2019). However, incidence varies greatly between different regions and ethnicities: while PV occurs at a rate of less than 0.76 per million population in Finland or less than 0.5 per million population in Germany (Hübner et al., 2016), its incidence is more than twenty times higher in the United States and Israel (Hammers and Stanley,

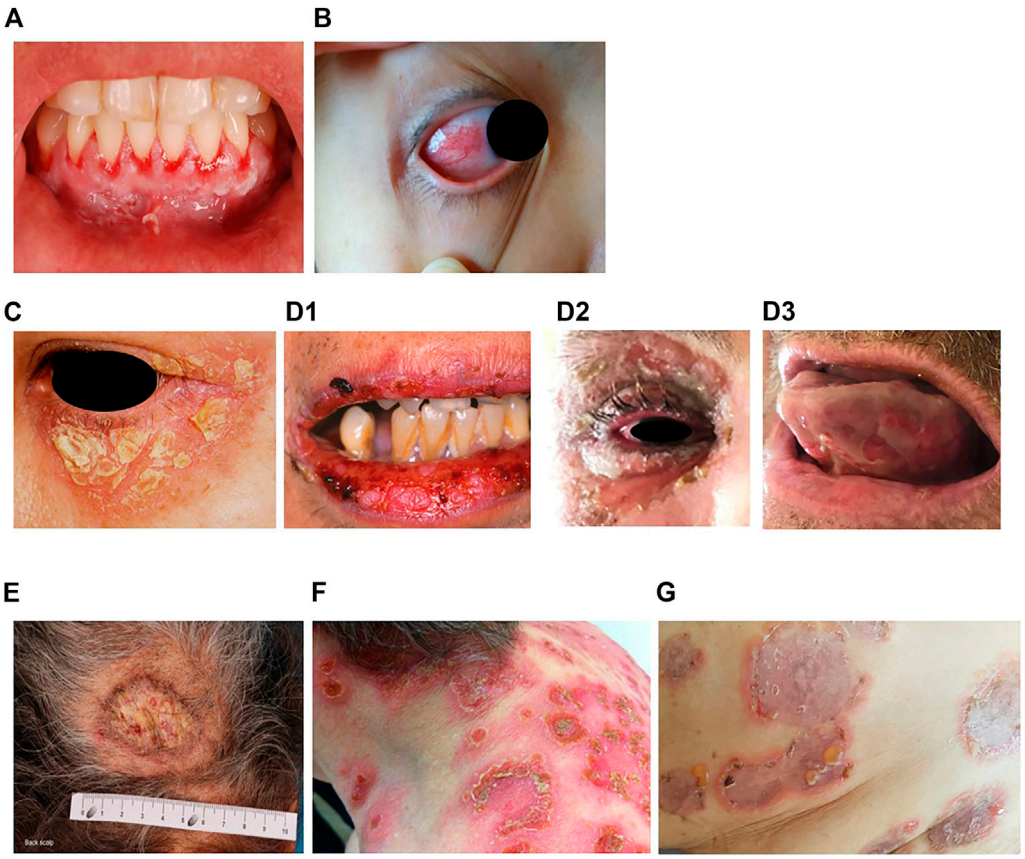

FIGURE 1 | Erosions of the alvealor gingiva and attached mucosa **(A)** and conjuctival involvement with episcleritis **(B)** in patients with pemphigus vulgaris. Puff pastry-like scales and crusts on the periorbital skin of a patient with pemphigus foliaceus **(C)**. Severe stomatitis crusting and bleeding erosions on the lips that extended beyond the vermilion **(D1)**, severe pseudomembranous conjuctivitis with mucus discharge and eyelid erosions **(D2)**, diffuse lingual erotions **(D3)** in patient with paraneoplastics pemphigus. Vegetative plaque of pemphigus vegetans on the patient's occipital scalp **(E)**. Post-pustular crusts with rounded or annular disposition in the trunk and neck in a patient with profuse IgA pemphigus **(F)**. Trunk lesions with annular lesions in a pemphigus herpetiformis patient **(G)**.

2016; Kridin, 2018). Regardless of the population, PV typically affects adults between the age of 45 and 65 years, with a slight female predominance (Joly and Litrowski, 2011; Didona et al., 2019). PV cases are extremely rare in children and its incidence increases with age (Kneisel and Hertl, 2011b). Almost all PV cases will develop oral mucosal lesions during the course of the disease; oral mucosal lesions being the first site involved in more 50–70% (Shamim et al., 2008; Pollmann et al., 2018) with lesions affecting the gingiva, palate, floor of the mouth, buccal mucosa, lips and tongue (Kneisel and Hertl, 2011a). Blistering in these sites can cause symptoms ranging from mild discomfort to such severe pain during eating that rapid weight loss ensues. Other mucous membranes may also be involved, including the nasal, pharyngeal, laryngo-oesophageal, urethral, genital (glans penis, vulva), perianal and conjunctival mucosae (Bystryn and Rudolph, 2005; Kneisel and Hertl, 2011a) (**Figures 1A,B**).

In muco-cutaneous variants, non-scarring skin lesions (flaccid blisters, crusts, erosions) arise during the course of the disease. Although any parts of the body can be affected, the predilection sites of PV lesions encompass face, neck, axillary folds, scalp and trunk, notably in seborrheic areas or exposed to mechanical stress. Recent studies have also revealed other manifestations, such as nail involvement in patients with extensive disease (Pietkiewicz et al., 2018; De et al., 2019), and post-inflammatory hyperpigmentation that can last for months in patients with darker skin (Tamazian and Simpson, 2020). In case of cutaneous involvement, erosions can be induced in perilesional skin by applying tangential pressure, also known as Nikolsky's sign. The diagnosis of pemphigus is evoked upon clinical examination; histological analysis of a skin/mucous membrane biopsy taken in a lesional area and showing acantholysis is required to support the diagnosis. Direct immunofluorescence examination of biopsy samples taken in perilesional areas is considered the "gold standard" to confirm PV diagnosis, revealing intercellular deposits of IgG and/or C3 on the surface of keratinocytes in the epidermis or mucosa (Harman et al., 2017; Joly et al., 2020), but with the disadvantage of requiring invasive sampling. Additional immunological tests are carried out in parallel, including detection of autoantibodies in serum targeting the epithelial cell by indirect immunofluorescence (IIF) on monkey oesophagus or human skin, and by enzyme-linked immunosorbent assays (ELISA) to

detect circulating anti-desmoglein autoantibodies surface (anti-Dsg3 alone where there is mucosal involvement only, or anti-Dsg3 and anti-Dsg 1 in patients with mucocutaneous disease) (Joly et al., 2020). Of note, anti-Dsg ELISA is positive in 95% of cases and anti-Dsg values correlate with disease activity (Joly et al., 2020).

Pemphigus Foliaceus

Pemphigus foliaceus (PF) ("*folium*" meaning "leaf" in Latin) is the second most common type of pemphigus and accounts for 27% of cases in France (Gonçalves et al., 2011; Jelti et al., 2019). Unlike PV, PF overall affects a broader age range of individuals due to the varied age of onset of several PF sub-variants (Chapman et al., 2018). PF-associated acantholysis occurs within the subcorneal layer of the epidermis, leading to more flaccid and fragile blisters that involve the skin and spare the mucous membranes (Joly and Litrowski, 2011). PF blistering and erosions typically exhibit "puff pastry-like" scales and crusts which tend to affect the seborrheic areas on the body: the scalp, face, presternal and interscapular skin (**Figure 1C**). In severe forms, PF might present as an erythroderma (chronic erythema involving more than 90% of the body's surface area), in contrast with the large erosive areas found in PV. Subcorneal pustules or acantholytic clefts in the granular layer of the epidermis are distinguishing histological features of PF, which are important because both direct and indirect immunofluorescence findings are similar to those seen in PV. However, only anti-Dsg1 antibodies are present, accounting for the absence of mucosal lesions in PF (see Dsg 1/Dsg 3 compensation theory, below, and Mahoney et al. (1999)).

Several clinical sub-variants of PF which have been characterised have interesting and distinctive features. Notably, pemphigus erythematosus (PE) (also known as Senear-Usher syndrome) has a milder presentation with lesions often confined to or first seen in the malar area of the face in a butterfly shape, as typically found in lupus erythematosus (Amerian and Ahmed, 1985; Khachemoune et al., 2006; Hobbs et al., 2021). More extensive involvement might be seen in photo-distributed areas. Patients with PE also present with laboratory findings suggestive of systemic lupus erythematosus, including the presence of anti-nuclear antibodies in 30–80% of cases (Malik and Ahmed, 2007; Oktarina et al., 2012; Pérez-Pérez et al., 2012). It is not yet known how or whether these antibodies are related to the pathology seen in PE.

In addition to PE, three PF sub-variants restricted to particular locations or populations have been described. The first such "endemic" form to be described, in 1903, was fogo selvagem, which is found in subtropical areas of Brazil (Diaz et al., 1989; Aoki et al., 2015), where its prevalence can be as high as 3–5% of inhabitants (Gonçalves et al., 2011). While PF in children is generally rare (Kanwar and Kaur, 1991; Mintz and Morel, 2011), up to 30% of those affected by fogo selvagem are children or young adults (Diaz et al., 1989; Aoki et al., 2015). Colombian pemphigus foliaceus is a PF sub-variant that occurs within the El Bagre area of Northern Colombia (Abrèu-Velez et al., 2003a; Abrèu-Velez et al., 2003b), and closely resembles fogo selvagem, except for its male preponderance (Abrèu-Velez et al., 2003b). The third endemic form is found in north African Arab countries, mostly in the southern area of Tunisia but also in Algeria and Morocco (Morini et al., 1993; Abida et al., 2009), and some sub-Saharan countries, such as Mali (Mahé et al., 1996). A predominance of cases among young women aged 25 to 34 years had been described in Tunisia (Bastuji-Garin et al., 1995, 1996) and familial cases have also been reported (Morini et al., 1993). The geographical restriction, the age and the more extensive lesions of these sub-variants pose intriguing questions on the aetiology of the disease, which in some cases has been partially resolved (see below).

Paraneoplastic Pemphigus

Paraneoplastic pemphigus (PNP) was first recognised in 1990 (Anhalt et al., 1990), and represents about 5% of pemphigus cases (Paolino et al., 2017). PNP is seen almost exclusively in patients with co-occurring neoplasms including lymphoproliferative disorders such as Castleman's disease and non-Hodgkin lymphoma, as well as thymoma, chronic lymphocytic leukaemia, and various carcinomas and sarcomas (Anhalt et al., 1990; Joly et al., 2000; Leger et al., 2012; Ohzono et al., 2015; Yatim et al., 2019). PNP predominantly affects patients between 45 and 70 years of age, but may also occur in children, particularly when associated with Castleman's disease (Wieczorek and Czernik, 2016). The condition typically presents as PV with severe mucocutaneous lesions, often with erosions and ulcers involving the tongue, and lesions on the lips that frequently extend to the vermillion border (Yatim et al., 2019). Other mucous membrane, such nasal, conjunctival and genital mucosa, may also be involved (Yatim et al., 2019): alongside, PNP-suggestive features of skin involvement are polymorphic, with pustular, erythema multiform-like or lichenoid lesions in addition to the classical flaccid bullous form and typical erosions (Anhalt, 2004; Leger et al., 2012; Ohzono et al., 2015) (**Figures 1D1–D3**). PNP may also affect internal organs, particularly the lung, and, more rarely, the gastrointestinal mucosa (Odani et al., 2020). The severity of PNP, possibility of multi-organ involvement, and co-occurrence with underlying malignancy together combine to give a high mortality rate (Leger et al., 2012; Ohzono et al., 2015; Wieczorek and Czernik, 2016; Jelti et al., 2019).

Histologically, PNP is characterized by a "tombstone" appearance of acantholytic basal keratinocytes, associated with an interface or lichenoid dermatitis caused by self-reactive $CD4^+$ and $CD8^+$ T cells, and with epidermal exocytosis and dyskeratotic or necrotic keratinocytes (Joly et al., 2020). Linear IgG/C3 deposits at the dermal-epidermal junction might also be present, in addition to positive intercellular labelling of the epithelium by DIF (Joly et al., 2020). Patients with PNP may develop IgG autoantibodies against a diverse array of antigens, including: Dsg3 (Amagai et al., 1998; Brandt et al., 2012), and several members of the plakin family (Mahoney et al., 1998) including the desmosomal plaque proteins desmoplakin I and II (Hashimoto et al., 1995), envoplakin (Kim et al., 1997), periplakin (Mahoney et al., 1998; Kazerounian et al., 2000), as well as against hemi-desmosomal adhesion molecules such as plectin and bullous pemphigoid antigen (BP) 230 (also known as

dystonin-e), BP180 (also known as collagen α-1[XVII] chain) (Maier et al., 2017) and the proteinase inhibitor α2-macroglobulin-like protein 1 (Schepens et al., 2010). In addition, approximately 75% of patients exhibit antibodies against desmocollins (Dsc) 1, 2 or 3 (Ishii et al., 2015; Ohzono et al., 2015). Anti-epiplakin antibodies are especially significant, both clinically and diagnostically as they are thought to be responsible for the occurrence of often-fatal bronchiolitis obliterans, which results from loss of adhesion of the lung epithelium (Tsuchisaka et al., 2016; Tsuchisaka et al., 2016). Considering these immunological features of PNP, IIF examination of the serum is usually performed using rat bladder to investigate reactivity against plakin proteins (Joly et al., 2000). ELISA for anti-Dsg and anti-envoplakin antibodies are usually made with immunoblots with epidermal extracts or extracts from cultured keratinocytes to investigate IgG reactivity against other antigens, especially anti-plakins (Probst et al., 2009; Joly et al., 2020). In the case of PNP, co-morbid malignancy is clear, and for some time the mechanism of association has been known. The emergence of PNP appears to be related to the generation of antibodies against tumour antigens which cross-react with epithelial antigens, including both desmosomal and hemi-desmosomal proteins. This process is similarly seen in the case of some environmental antigens (see below) in endemic PV, but similar "trigger antigens" have yet to be investigated or defined in other pemphigus variants.

Pemphigus Vegetans

Pemphigus vegetans, a rare clinical variant of PV which represents 1–2% of all pemphigus cases (von Köckritz et al., 2017). Due to it's rarity, only few papers in the literature are specifically devoted to it with most of them are case reports (Mergler et al., 2017; Cuellar et al., 2020; Liu et al., 2020; Verma et al., 2020). Pemphigus vegetans is characterized by hypertrophic papillomatous vegetative skin lesions that preferentially affect intertriginous sites, such as the axillae, inframammary folds, umbilicus, inguinal creases, anogenital skin, scalp, and neck (Jain et al., 1989; Huang et al., 2005; Cozzani et al., 2007) (**Figure 1E**). Depending on the morphology of the initial lesions and the clinical course, pemphigus vegetans is further subdivided into two types: Neumann and Hallopeau. The Neumann type presents with flaccid blisters and erosions, evolving into fetid-odoured, whitish, macerated, hypertrophic, papillomatous plaques in the intertriginous body folds and lips, and more rarely on the trunk and extremities; the oral mucosa is usually involved (Leroy et al., 1982; Ahmed and Blose, 1984). By contrast, the Hallopeau type is associated with localised, pustular lesions that rupture and coalesce into vegetative erosions, and does not exhibit oral mucosa involvement (Zaraa et al., 2011; Imchen et al., 2014). Autoantibodies in pemphigus vegetans are against Dsg1 and Dsg3, as well as other proteins constituting the desmosome (Hashimoto et al., 1994; Cozzani et al., 2007).

IgA Pemphigus

IgA pemphigus, even rarer among the pemphigus family, is characterized clinically by annular erosive plaques rimmed by pustules and affects the skin of the trunk, proximal extremities, and intertriginous regions (**Figure 1F**) (Geller et al., 2014). Mucosal involvement is uncommon (Kridin et al., 2020) and acantholysis is usually absent (Tsuruta et al., 2011). Here, IgA autoantibodies recognising various dermosomal proteins bind the epidermal cell surface and are detectable by direct immunofluorescence; while immune-serological assays are used to detect circulating IgA autoantibodies in these patients (Nishikawa et al., 1991; Geller et al., 2014). Depending on the level of pustule formation, IgA pemphigus can be divided into two major clinical and histological subtypes: intraepidermal neutrophilic dermatosis (IEN), which is characterized by suprabasilar pustules located at the lower or entire epidermis and associated with the presence of IgA antibodies against Dsg1 or Dsg3, and other yet-unidentified antigens; and subcorneal pustular dermatosis (SPD), in which patients exhibit subcorneal pustules in the upper epidermis and auto-reactive IgA binds desmocollin 1 (Dsc1) (Hashimoto et al., 1997; Karpati et al., 2000; Yasuda et al., 2000). IgA pemphigus may also be associated with IgA gammopathies, haematological disorders and ulcerative colitis (Hashimoto et al., 1997; Kridin et al., 2020).

Neonatal Pemphigus Vulgaris

Neonatal pemphigus vulgaris (NPV) is a transient immunobullous disease induced by transplacental transfer of maternal autoantibodies from a mother with PV to her newborn (Fenniche et al., 2006; Gushi et al., 2008; Turrentine et al., 2014; Carvalho et al., 2019; Fenner et al., 2020; Foster et al., 2021). First reported in 1975 (Salihbegović-Opalić et al., 1975; Zhao et al., 2016), few cases exist in the literature, as female patients with pemphigus can experience infertility, premature delivery and stillbirth (Ouahes et al., 1997). NPV has various clinical manifestations, including skin and mucosa defects, blisters and erosions at birth, and extensive skin exfoliation which can arise early after birth. As maternal-derived antibody levels drop, NPV resolves spontaneously or with mild topical corticosteroid treatment within 4 weeks of birth (Kardos et al., 2009). Importantly, before the development of the neonatal mouse model of PV, clinical observation of NPV gave the first evidence of the direct pathogenicity of pemphigus autoantibodies.

Pemphigus Herpetiformis

Pemphigus herpetiformis (PH), first named in 1975 (Jablonska et al., 1975), is a rare subtype of pemphigus. It clinically resembles to dermatitis herpetiformis, with patients exhibiting pruritic herpetiform blisters, and/or annular urticarial plaques with or without erosions, or scaly erosive patches mimicking eczema (Tay et al., 2018) (**Figure 1G**). However, immunological findings are consistent with pemphigus (Kasperkiewicz et al., 2014; Costa et al., 2019). Direct and indirect immunofluorescence demonstrate intercellular deposits of IgG and/or C3, with Dsg as the main target of autoantibodies, with additional recognition of Dsc (1,2 3) and other hemidesmosomal antigens, seen in some patients (Kozlowska et al., 2003; Ohata et al., 2013; Ueda et al., 2013; On et al., 2015). The histology of PH is less specific than that of PV and ranges from intraepidermal eosinophils or neutrophilic spongiosis (Hashimoto et al., 1983) to intraepidermal vesicles

filled with neutrophils or eosinophils, and may also encompass dermal papillary micro-abscesses (Costa et al., 2019).

THE PATHWAY TO PEMPHIGUS: RISK FACTORS FOR DEVELOPING THE DISEASE

Many autoimmune diseases have a complex aetiology, and pemphigus is no exception. Factors as diverse as genetics, environment, pre-existing health conditions, medication use and even post-vaccine reactions have been linked with the emergence of pemphigus. In this section we will review some of the most recent observations that are building up the picture of circumstances and pathways leading to the clinical manifestation of pemphigus.

Genetic Risk Factors

The familial clustering of certain variants of pemphigus, combined with its predilection for some ethnicities and co-occurrence of other autoimmune diseases in patients, all point towards a significant genetic component to susceptibility. To date, the strongest link has been reported for the association between PV and HLA class II genes (Gazit and Loewenthal, 2005; Tron et al., 2006; Vodo et al., 2018). Studies have shown that HLA alleles DRB1*04:02 and DQB1*05:03 represent the most common PV associated alleles (Ahmed et al., 1991; Carcassi et al., 1996; Delgado et al., 1996; Lee et al., 1998; Lombardi et al., 1999; Loiseau et al., 2000; Miyagawa et al., 2002; Geng et al., 2005; Liu et al., 2008; Shams et al., 2009; Tunca et al., 2010; Párnická et al., 2013; Brochado et al., 2016), with the majority of the patients with PV expressing one of these two alleles. While some of the HLA types are more population-specific, there are others that are associated with PV across numerous ethnic groups: a link between HLA-DRB1*04:02 and DQB1*03:02 with PV in the Jewish population has been highlighted (Gazit and Loewenthal, 2005), while HLA-DQB1*05:03 was found in association with PV in non-Jewish populations (Ahmed et al., 1991). Studies in Han Chinese patients with PV have highlighted DRB1*04, DRB1*14 and DQB1*05:03 as relevant risk alleles (Zhou et al., 2003; Geng et al., 2005; Liu et al., 2008; Gao et al., 2018), as well as HLA-DRB1*03 and HLA-CW*14 in the same Chinese population with PNP (Martel et al., 2003; Liu et al., 2008). Meta-analyses of the correlation between PV occurrence and HLA-DRB1 and HLA-DQB1 have shown that DRB1*04, DRB1*08, DRB1*14, DQB1*03:02 and DQB1*05:03 are significant susceptibility factors for PV while DRB1*03, DRB1*07, DRB1*15, DQB1*02, DQB1* 03:03, DQB1*05:01 and DQB1*06:01 were less common in patients with PV compared to healthy individuals (Yan et al., 2012; Li et al., 2018). In addition to HLA class II alleles, there may be some association between PV and certain HLA class I alleles within specific ethnic groups, for example, HLA-A10 and -B15 in the Japanese population (Hashimoto et al., 1977; Miyagawa et al., 2002), and HLA-A3, -A26, and -B60 in the Han Chinese population (Gao et al., 2018). However, although numerous studies have suggested that HLA status could be a key driver of pemphigus disease activity, the mechanistic link between the HLA genetic profile and the clinical picture within patients remains unclear.

Alongside the HLA susceptibility studies, other research has uncovered associations between PV and autoantigen or immune gene sequences: specific *Dsg3* haplotypes were significantly linked with PV in both British and Indian cohorts (Capon et al., 2006); while single nucleotide polymorphisms within the variable region of the immunoglobulin heavy chain *VH3* gene were associated with PF in two patients (Yamagami et al., 2009). Genetic variants of the cytokine genes *TNF-α*, *IL-6* and *IL-10* gene have also been linked with PV (Torzecka et al., 2003; Pereira et al., 2004; Eberhard et al., 2005; Mosaad et al., 2012; Toumi et al., 2013; Dar et al., 2016; Vodo et al., 2016); as have those affecting the *TAP2* gene, which encodes a protein involved in peptide assembly and transport to HLA class I (Niizeki et al., 2004; Slomov et al., 2005). Immune-associated SNPs that have been linked with pemphigus include those within the *ST18* gene, which encodes a transcription factor involved in inflammation and apoptosis (Sarig et al., 2012; Vodo et al., 2016; Etesami et al., 2018; De Bonis et al., 2019; Radeva et al., 2019; Assaf et al., 2021); and those within the *CTLA4* and *CD86* genes, whose protein products are expressed on antigen-presenting cells (APC) and are involved in activating T-cells and stimulating IgG production by B cells (Dalla-Costa et al., 2010; Tanasilovic et al., 2017).

While strong linkages have been found to exist between pemphigus and some of the above HLAs, the vast majority of individuals who carry the PV-associated HLA susceptibility alleles do not develop the disease. This prompted a group of researchers to discover a subset of PV-related differentially expressed genes which suggests a "protection" signature in genetically susceptible individuals from developing PV (Dey-Rao et al., 2013).

Knowing the genetic susceptibility in pemphigus may spur further research to better understand the interactions between various genetic susceptibility factors, the role of epigenetics and the functional effects of the identified genetic variants in disease-relevant cells, and hopefully, ultimately, their influence on response to treatment and severity of disease (Petzl-Erler, 2020).

Environmental Risk Factors

The lived-in environment is a complex mixture of factors that act across time and space, interacting with genetic susceptibility to affect health outcomes. Studying this intricate web of influences has led to the identification of significant factors linked with the development of pemphigus in specific populations.

In the case of endemic pemphigus foliaceus in Brazil, the observation that many of the adults affected by fogo selvagem were outdoor workers living in conditions of poor hygiene and low-quality housing, built in forest areas adjacent to rivers and streams (Diaz et al., 1989; Aoki et al., 2015), led researchers to seek environmental factors causing the disease. These investigations resulted in the identification of sand flies as a triggering factor the salivary protein LJM11 of *Lutzomyia longipalpis* (also known as black fly) (Culton et al., 2008) cross reacts with Dsg1 (Warren et al., 2000; Qian et al., 2012).

Ultraviolet radiation has been reported to exacerbate or cause new-onset of pemphigus (Fryer and Lebwohl, 1994; Muramatsu

et al., 1996; Aghassi, 1998; Kano et al., 2000; Makino et al., 2014). A study using medical records review showed that the majority of pemphigus patients had the first manifestation of their disease in spring and summer of Sofia, Bulgaria (Tsankov et al., 2000). Hospitalisation primarily for pemphigus was also noted to be higher in days with higher UV index for Hispanic pemphigus patients in United States (Ren et al., 2019). In a more recent study which looked at seasonal patterns and triggering factors in non-endemic pemphigus foliaceous in Turkey (Sayar and Küçükoğlu, 2021), it was also observed that new onset and relapses of non-endemic pemphigus foliaceous occurred more often during the spring-summer season where UV radiation is at its peak, further supporting the possible role of UV radiation as a trigger factor in this disease. While it has been demonstrated that UVB induces acantholysis and epidermal intercellular deposition of immunoreactants in Fogo selvagem patients (Reis et al., 2000), the molecular pathway still needs to be fully elucidated.

Other environmental risk factors for the development of pemphigus include trauma (Daneshpazhooh et al., 2016), stress (Cremniter et al., 1998; Morell-Dubois et al., 2008), diet (Ruocco et al., 2001). Interestingly, smoking seems to confer a protective effect on pemphigus (Valikhani et al., 2007). Cigarette smoking has also been found to improve pemphigus disease and those who smoked had earlier disease remission (Valikhani et al., 2008). It is postulated that interaction of nicotine and other nicotinic agonists with nicotinic acetylcholine receptors on keratinocytes may promote cell–cell adherence (Grando and Dahl, 2000).

Therapeutic Risk Factors

Drugs have been reported to trigger pemphigus. The three main groups of drugs which has been reported to be associated with new onset or exacerbation of pemphigus are the thiol drugs, phenol drugs and non-thiol/non-phenol drugs. Thiol drugs have sulfhydryl (-SH) group in their chemical structure and are probably the better studied group of drugs in the pathogenesis of pemphigus. In a systematic review of 170 drug-induced pemphigus (Ghaedi et al., 2021), penicillamine, captopril and bucillamine, which belong to the thiol drugs, were the most commonly reported drugs to induce pemphigus.

Biological thiol substances such as cysteine and glutathione has been shown to induce acantholysis in human skin fragments under certain experimental conditions (Ruocco et al., 1982). *In vitro* experiments subsequently demonstrated that thiol drugs (D-penicillamine, captopril, thiopronine and piroxicam) induce acantholytic splitting in human skin fragments or skin cultures in the absence of pemphigus antibodies (i.e biochemical acantholysis) (Ruocco et al., 1993). It is postulated that thiol drugs form drug-cysteine disulfides which directly interfere with desmoglein adhesions between keratinocytes, or indirectly through antigen modification and autoantibodies production (Wolf and Ruocco, 1997). Plasminogen activator inhibitor, a proteolytic enzyme, has been shown to be important in preventing immunoglobulin-induced acantholysis in pemphigus (Hashimoto et al., 1989). Thiol drugs, on the other hand, stimulate plasminogen activator through inhibition of their natural inhibitors, leading to promotion of acantholysis (Hashimoto et al., 1984; Lombardi et al., 1993).

Other than drugs, vaccines have also been reported to trigger or exacerbate pemphigus. Amongst them are influenza (Mignogna et al., 2000; De Simone et al., 2008), hepatitis B (Berkun et al., 2005), rabies (Yalçin and Alli, 2007), tetanus (Cozzani et al., 2002) and more recently, SARS-COV2 vaccines (Damiani et al., 2021; Koutlas et al., 2021; Lua et al., 2021; Solimani et al., 2021; Thongprasom et al., 2021). While the mechanisms behind vaccine-induced pemphigus has not been worked out, there are several hypotheses other than those proposed for drug-induced pemphigus. These include autoimmunity arising from molecular mimicry of vaccines or their adjuvants with self-antigens (Shoenfeld et al., 2000), and non-specific activation of innate immunity and expansion of autoreactive T cells (Horwitz and Sarvetnick, 1999). It is however interesting to note that infections which these vaccines are used against, have not been reported as triggers for pemphigus (Brenner et al., 2002).

AUTOIMMUNE PATHWAYS AND PROCESSES IN PEMPHIGUS

Autoantibodies

Following the identification of Dsg 1 and 3 as critical pemphigus autoantigens (Eyre and Stanley, 1987, 1988; Hashimoto et al., 1990; Amagai et al., 1991; Mahoney et al., 1999; Amagai and Stanley, 2012), many studies have investigated the autoantibodies and autoreactive B cells that drive pemphigus pathogenesis. The extracellular domains, EC1 and EC2, of Dsg have been defined as the main target region recognised by autoantibodies from pemphigus patients (Amagai et al., 1992, 1995; Futei et al., 2000; Sekiguchi et al., 2001; Payne et al., 2005; Ishii et al., 2008; Chan et al., 2010; Di Zenzo et al., 2012), with some evidence of minor EC4 recognition, and IgG4 is now known to be the major pathogenic antibody isotype (Dmochowski et al., 1992; Allen et al., 1993; Wilson et al., 1993). However, other isotypes may also be pathogenic in some variants or circumstances (Payne et al., 2005; Saleh et al., 2015). Interesting new data have revealed that DSG3-specific memory B cells have an activated phenotype and show signs of ongoing affinity maturation and clonal selection (Cho et al., 2019).

Although Dsg-specific autoantibody titre was long-thought to correlate with disease activity in pemphigus (Amagai et al., 1999; Daneshpazhooh et al., 2007; Abasq et al., 2009; Schmidt et al., 2010), more recent studies have detected anti-Dsg3 IgG antibodies in patients who were in clinical remission, suggesting the existence of non-pathogenic Dsg-specific IgG (Belloni-Fortina et al., 2009; Yoshida et al., 2017; Chernyavsky et al., 2019). This concept of pathogenicity has been confirmed by *in vivo* and *in vitro* studies demonstrating that pathogenic IgG autoantibodies preferentially target the NH2-terminal portion of Dsg3. Non-pathogenic autoantibodies recognize epitopes of the membrane proximal COOH-terminus of the Dsg ectodomains (Amagai et al., 1992; Futei et al., 2000; Bhol and Razzaque Ahmed, 2002; Tsunoda et al., 2003; Chan et al., 2010; Lo et al., 2016). Comparing conventional and EDTA-pretreated anti-Dsg3 commercial ELISA, Kamiya et al. demonstrated the presence

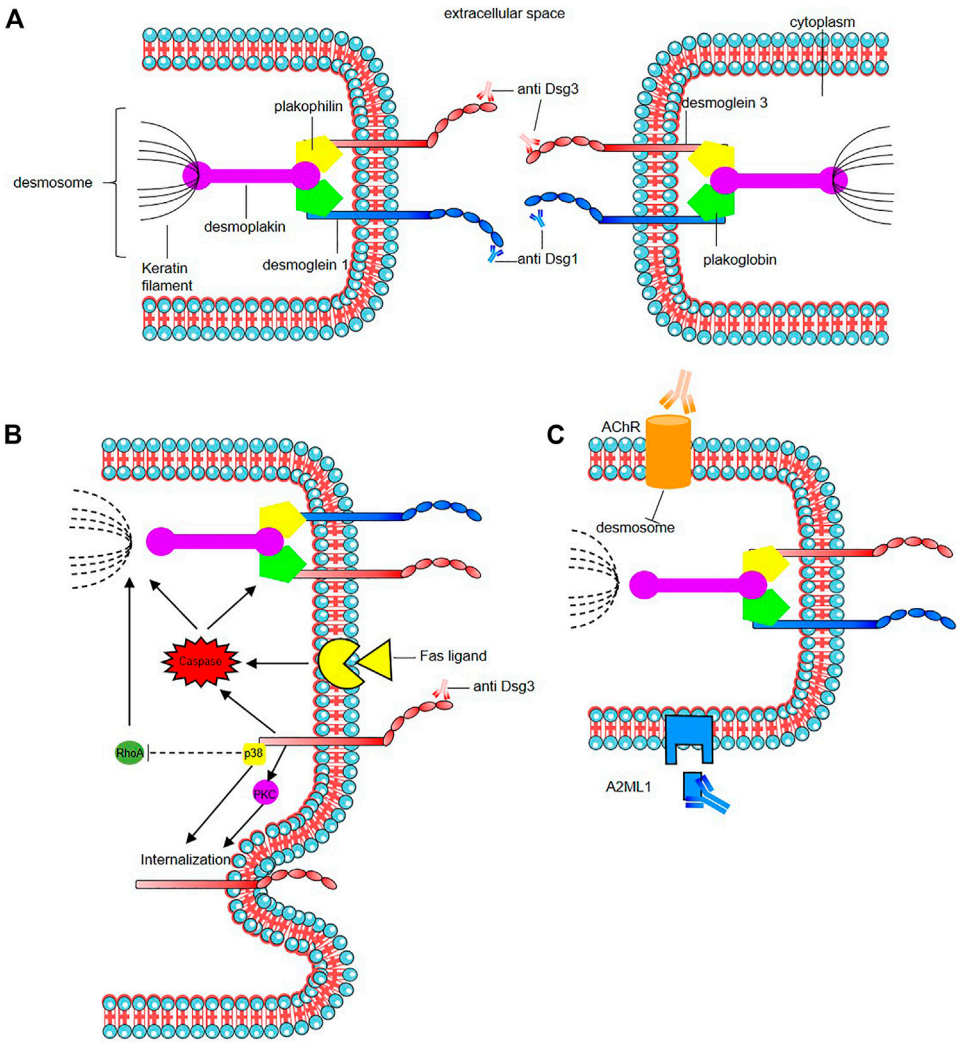

FIGURE 2 | Mode of action of the pathogenic autoantibodies in pemphigus. **(A)** Interaction of pathogenic autoantibodies the NH2-terminal of desmosomal protien leading to steric hindrance of trans-Dsg binding. **(B)** Binding of autoantibodies to desmoglein induce an alteration of cellular signaling affecting components of multiple pathways including p38 MAPK (p38), protien kinase C (PKC) including an internalization of Dsg3. The autoantibodies binding reduce the RhoA activity in the p38MPAK-dependent manner which impact the reorganization of the actin cytoskeleton and drive further loss of desmosomal integrity. Caspace signaling may be activated by signal induced by the binding of autoantibodies to desmoglein by the Fas ligand/receptor pathway including pre-apoptotic caspase signaling. **(C)** Anti-keratinocyte α-acetycholine receptor (AChR) IgG autoantibodies induce signal causing disassembly of desmosomes, leading to acantholysis and blistering. In PNP, autoantibody binding also seems to inhibit A2ML1 (α-2 microglobulin-like 1) impacting the activation of a protease inhibitor. For a recent in-depth review see Schmitt and Waschke (2021).

of anti-Dsg antibodies directed against calcium-dependent epitopes targeting the extracellular domains of Dsg3 (Kamiya et al., 2012) and found a higher correlation between antibodies against calcium-dependent epitopes of Dsg3 and clinical activity than with total anti-Dsg3 antibodies (Kamiya et al., 2013). The pathogenic activity of these IgG directed against Ca-dependent epitope is sustained by their ability to induce the keratinocyte dissociation *in vivo* (Kamiya et al., 2012).

In PF and PV subtypes, Dsg1 and Dsg3 specific antibodies might directly affect the clinical presentation due to their differential expression in skin and mucous membranes. While patients with PV often experience mucosal and cutaneous blistering, and possess both anti-Dsg1 and anti-Dsg3 antibodies, those with PF have antibodies only to Dsg1 and present with cutaneous but not mucosal involvement. Dsg1 is mainly expressed in the upper epidermis and upper layers of the mucosa, while Dsg3 is predominantly expressed in the suprabasal layers of the epidermis and throughout the mucosal epithelium. This differential expression of Dsg1 and Dsg3 in the epidermis and mucosa together with the postulation that the Dsg can compensate for each other's adhesive function when expressed on the same keratinocyte: this is the Dsg1/Dsg3 compensation theory (Amagai, 2009; Amagai and Stanley, 2012). Unfortunately, this concept cannot explain all clinical forms of pemphigus,

especially those with atypical features, and the factors affecting the ability or extent of functional compensation have yet to be revealed. With better immunological profiling of pemphigus, a modification of the compensation theory and new alternative pathogenic mechanisms need to be evaluated (Koga et al., 2012; Sardana et al., 2013; Carew and Wagner, 2014; Ahmed et al., 2016; Öktem et al., 2018).

The mode of action of the pathogenic autoantibodies found in pemphigus patients is now also well-defined (**Figure 2**). The desmosomal depletion that leads to acantholysis can be divided into two major mechanisms: firstly, the interaction of IgG autoantibodies with the NH2-terminal EC1 subdomains of Dsg1 and Dsg3 in PF and PV, respectively, leads to steric hindrance of trans-Dsg binding (Heupel et al., 2008; Jennings et al., 2011; Saito et al., 2012), and also disrupts the interaction between Dsg and flotillin (Völlner et al., 2016), together leading to the loss of desmosomal integrity (**Figure 2A**). The second mechanism is an alteration to cellular signalling that is induced by autoantibody binding affecting components of multiple pathways including p38 MAPK, protein kinase C (PKC), c-Jun N-terminal kinases (JNK), RhoA and caspases 3, 6, 8 and 9, which leads to Dsg endocytosis and depletion driving further loss of desmosomal integrity and adhesion (Puviani et al., 2003; Berkowitz et al., 2005, 2006, 2008; Frušić-Zlotkin et al., 2005; Kawasaki et al., 2006; Lee et al., 2009; Cirillo et al., 2010; Mao et al., 2011) (**Figure 2B**). In PNP, autoantibody binding also seems to inhibit A2ML1 (α-2 microglobulin-like 1) impacting the activation of a protease inhibitor (Inaoki et al., 2001; Schepens et al., 2010; Numata et al., 2013) (**Figure 2C**). For a recent in-depth review of this topic see (Schmitt and Waschke, 2021).

In addition to anti-Dsg IgG autoantibodies, various antibodies targeting other autoantigens have been identified, notably with proteomics studies (Kalantari-Dehaghi et al., 2013), in the sera of a minority of patients with various forms of pemphigus with an unclear pathogenic role (Nguyen et al., 2000b; Ahmed et al., 2016; Amber et al., 2018). IgG and IgA autoantibodies against desmocollins (Dsc1, Dsc2 and Dsc3 being Ca^2+-dependent cadherins involved in desmosome assembly) have been detected in variable percentage of pemphigus patients (5–60%), according cohorts, Dsc subfamily and the type of pemphigus variant (Dmochowski et al., 1993; Müller et al., 2009; Ishii et al., 2015; Mindorf et al., 2017). Their rates are higher in rare variants such as IgA pemphigus, PNP, and pemphigus herpetiformis (Hashimoto et al., 1997; Müller et al., 2009; Ishii et al., 2015; Mindorf et al., 2017). It has been demonstrated that anti-Dsc3 react against extra cellular domains of Dsc3 in human skin model resulting in intraepidermal blister formation, and in loss of intercellular cohesion in keratinocyte culture induced by targeting of the Dsc3/Dsg1 binding on keratinocyte cell surface as Dsc and Dsg are interacting by homophilic and heterophilic trans-interaction (Spindler et al., 2009). Anti-keratinocyte α-acetylcholine receptor (AChR) IgG autoantibodies have been also reported in 85% of PV and PF patients (Nguyen et al., 1998; Lakshmi et al., 2017). Inhibition of these receptors which control keratinocyte adhesion and motility may induce signals causing disassembly of desmosomes, leading to acantholysis and blistering (**Figure 2C**). Autoantibodies against muscarinic AchRs but also against mitochondrial nAChR were found (Chernyavsky et al., 2015). A correlation of anti-muscarinic AchRs antibody titers and anti-Dsg antibody titers with the severity of disease in patients with pemphigus has been reported (Lakshmi et al., 2017). The role of these autoantibodies found in sera from pemphigus is unclear, as the fact that they could result from epitope spreading following the loss of tolerance or have a synergistic action with anti-Dsg in pemphigus pathogenesis (Nguyen et al., 2000a; Grando, 2000). Some react mainly against structural components of adhesion molecules such as desmosomes like autoantibodies against desmoplakin 1, desmoplakin 2 (Kim et al., 2001), Dsg4 (Kljuic et al., 2003; Nagasaka et al., 2004), E-cadherin (Evangelista et al., 2008; Oliveira et al., 2013), plakoglobin (Korman et al., 1989; Ishii et al., 2001), plakophilin 3 (Lambert et al., 2010), FCεR1 (Fiebiger et al., 1998), and pemphaxin (Nguyen et al., 2000a) while other impact protease inhibitors such as anti- A2ML1 or anti-hSPCA1 (Ca21/Mg21-ATPase encoded by ATP2C1 gene) which is involved in Golgi apparatus trafficking and of which impairment could lead to the impairment of proteic assembly and adress to the membrane as in hailey-hailey disease (Kalantari-Dehaghi et al., 2013). Although mouse models have been designed to incorporate some of these autoantibodies (Chen et al., 2008; Rafei et al., 2011), their role in pemphigus pathogenesis is still unclear (Spindler et al., 2018) and more complex mouse model need to be designed.

T Cells

While autoantibodies produced by B cells are the key and direct pathogenic mediators of pemphigus, recent studies are beginning to illuminate the broader autoimmune picture, with an emphasis on the role of T cell subsets. The disruption of peripheral T cell-mediated tolerance to self-antigens can be a critical event leading to autoantibody generation, inflammation, and tissue infiltration by immune cells: thus, a role for autoreactive helper T cells in driving pemphigus has long been postulated. Patients with pemphigus possess Dsg3-reactive Th1 and Th2 cells (Lin et al., 1997; Rizzo et al., 2005), with some studies finding comparable frequencies of the two subsets (Eming et al., 2000), and others detecting greater numbers of autoreactive Th1 compared to Th2 cells (Veldman et al., 2003). Studies in a humanized HLA-DRB1*04:02 transgenic mouse model showed that T-lymphocytes recognize human desmoglein 3 epitopes in the context of HLA-DRB1*04:02 (Eming et al., 2014). This recognition was associated with a CD40-CD40L-dependent T-cell—B-cell interaction that lead to the induction of pathogenic IgG autoantibodies, which in turn triggered intraepidermal blister formation. Although it seems likely that both Dsg3-reactive Th1 and Th2 cell are important for pemphigus development, most studies have focused on the role of Th2 cells and shown them be critical for the development of the disease. Frequencies of Dsg3-reactive Th2 cells are significantly higher in patients than controls, and their levels are positively correlated with disease activity (Rizzo et al., 2005). In addition to Th2 cells, Th2 cytokine levels are significantly higher in the serum of patients with PV than

controls, suggesting an imbalance between Dsg3-reactive Th2 and Th1 cells in the pathogenesis of PV (Satyam et al., 2009; Lee et al., 2017). However, the role of other T cell subsets in pemphigus pathogenesis remained unclear.

Th17 cells are identified by their specific expression of the transcription factor RORγt and have been implicated in the autoimmune pathology of a range of conditions including psoriasis (Fitch et al., 2007), ankylosing spondylitis (Taams et al., 2018) and, more recently, pemphigus (Xu et al., 2013; Timoteo et al., 2017; Jmaa et al., 2018). In patients with PV, the frequency of Th17 cells in the peripheral blood is significantly higher than in healthy controls, especially during the acute onset and active chronic stages (Holstein et al., 2021). Moreover, the levels of Th17-associated molecules (IL-17A and CCL20) in the serum, and the relative expression levels of RORγt, CCR6 and CCL20 is also relatively higher in patients with PV than in controls (Asothai et al., 2015). Th17 cells are also present in lesional skin (Arakawa et al., 2009): accordingly, a comparison of the expression profiles of lesional versus perilesional and healthy skin from a patient with pemphigus identified an IL-17A-dominated immune signature, with high expression of genes involved in the IL-17A signalling pathway (Holstein et al., 2021).

Alongside Th1, Th2 and Th17 cells are T follicular helper cells (Tfh): these are a subset of $CD4^+$ T lymphocytes expressing the transcription factor BCL6, and CXCR5, which enable them to migrate into germinal centres where they are required for the selection of high-affinity antibody-producing B cells and the development of memory B cells (Morita et al., 2011; Craft, 2012). Tfh cells also express the surface receptors programmed cell death protein-1 (PD-1), inducible T cell costimulator, and CD40L, and secrete IL-21 to promote B cell growth, activation, immunoglobulin class-switching and differentiation into memory B cells and plasmablasts (Zotos et al., 2010). Early indications of a role for Tfh cells in pemphigus came from studies showing that the frequency of circulating Tfh cells (defined as $CD4^+CXCR5^+$ T cells) and plasma concentrations of IL-21 were both significantly higher in patients with PV compared to controls (Holstein et al., 2021); and identified autoreactive IL-21-secreting cells in 50% of patients with PV (Hennerici et al., 2016). In addition, frequencies of Th17 and Tfh17 cells in the blood of patients with pemphigus correlate with levels of Dsg-specific $CD19^+CD27^+$ memory B cells (Maho-Vaillant et al., 2021), while patients with acute pemphigus exhibit higher levels of Dsg3-autoreactive Tfh17 cells (Hennerici et al., 2016). Moreover, *in vitro* coculture experiments revealed that Tfh17 cells are primarily responsible for inducing Dsg-specific autoantibody production by B cells (Holstein et al., 2021). Leading on from this, targeting the inducible co-stimulator expressed on $CXCR5^+PD\text{-}1^+$ Tfh cells suppressed the progression of PV in a murine model (Kim et al., 2020). All these findings show that Tfh17 cells are critically involved in the pathogenesis of pemphigus and offer novel targets for therapeutic intervention.

A major cell type tasked with preventing autoimmunity in the body is regulatory T cells (Treg), which exhibit a $TCR\alpha\beta^+Foxp3^+CD4^+$ phenotype and develop in the thymus (Sakaguchi et al., 1995; Peterson, 2012). Treg cells possess potent immune-suppressive activity and their main role is to control inflammation and immune responses to self-antigens. Various studies have demonstrated that the proportion of Treg cells in the blood of patients with PV is markedly lower than in healthy donors (Veldman et al., 2004). Similar trends are observed in patients with PV in the acute onset and remittent stages: frequencies of Th17 and Treg cell populations are inversely correlated, indicating that the balance of Th17/Treg cells is disrupted in PV (Xu et al., 2013; Asothai et al., 2015, 4). Similarly, a lower ratio of Dsg3-specific type 1 regulatory T cells (Tr1) to Th2 cells in patients with PV has been described (Veldman et al., 2006). *In vivo*, mouse models of PV have demonstrated that Tregs control anti-Dsg3 antibody production, and that adoptive transfer of Tregs or the depletion of endogenous Tregs suppresses and increases anti-Dsg3 antibody production, respectively (Yokoyama et al., 2011). Alongside, *in vitro* studies have shown that Dsg3-specific Tr1 cells secrete IL-10, TGF-β, and IL-5 upon autoantigen stimulation and inhibit the proliferation of Dsg3-responsive, auto-reactive helper T clones in an autoantigen- and cell-number- dependent manner (Veldman et al., 2004). This study suggests that Treg cells are involved in the maintenance and restoration of tolerance against autoantigens in PV. Recent work also suggests that Treg cells from patients with PV may have defective CCR4-CCL22 ligand interactions (Asothai et al., 2015), which could lead to reduced homing to the sites of skin lesions.

The skin also provides a niche for long term tissue-resident memory T (TRM) cells to accumulate, where they can mediate durable protective immunity but may also be involved in driving inflammatory diseases (Ho and Kupper, 2019). Recently, a study reported the over-representation of $CD4^+$ TRM in the lesional skin of patients with pemphigus (Zou et al., 2021). These T follicular helper-like $CD4^+$ TRM cells were thought to promote local autoantibody production, which could result in the formation and recurrence of lesions. Another study suggested that the long-lasting response to rituximab in pemphigus could rely on the decrease of Dsg-specific circulating T follicular helper cells, which correlates with a sustained depletion of IgG-switched memory autoreactive B cells, leading to the disappearance of anti-Dsg antibody-secreting cells (Maho-Vaillant et al., 2021). These results support the therapeutic targeting of Tfh-like TRM cells in pemphigus treatment, perhaps via IRF4 which might serve as potential therapeutic target (Zou et al., 2021). Understanding the changes in phenotype, functions, and interactions of these, and other, T cell subsets is key to understanding the pathogenesis of pemphigus and for the development of new targeted therapeutics.

Myeloid Cells

The myeloid cell population represents a major compartment of the immune system and includes dendritic cells, macrophages, monocytes and neutrophils (Bassler et al., 2019). Dermal dendritic cells (cDC1, cDC2) and epidermal Langerhans cells (LCs) represent the specialized antigen presenting cells of the skin and the functional "bridge" between innate and adaptive immunity. They are considered as sentinels of the skin that defend against pathogens by controlling the innate immune cell wave infiltrating the area during infection (Del Fresno

et al., 2018; Janela et al., 2019) as well as by shaping the B and T cell mediated immune response: accordingly, they are key players in the maintenance of tolerance. Despite this, little is known of the role of the myeloid compartment in PV pathogenesis.

The frequency of myeloid DCs in the blood of patients with pemphigus is increased, and these cells exhibit altered expression of DC-associated stimulatory (CD40 and CD80) and inhibitory (PSGL1 and ILT3) markers compared to healthy donors (Das et al., 2020). These observations suggest a potential role of DC dysregulation in the immunopathogenesis of pemphigus. A recent study demonstrated that high numbers of LCs are present in perilesional skin from patients with pemphigus (Das et al., 2020); while *in vitro* work showed that LCs were able to capture the epidermal antigen Dsg3 via langerin and to present this antigen to T cells (Kitashima et al., 2018). Interestingly, LCs express the IL-2 receptor complex, and disruption of IL-2 signalling in LCs inhibits LC-mediated regulatory T cell expansion *in vitro* (Kitashima et al., 2018). Therefore, LCs may be important mediators of peripheral tolerance against epidermal autoantigens via IL-2 signalling, though this has yet to be directly demonstrated in pemphigus.

Another population of cells that exhibits potentially significant changes in pemphigus is myeloid-derived suppressor cells (MDSCs). This heterogeneous group of immature myeloid cells incorporates granulocytic or polymorphonuclear-like MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) (Gabrilovich and Nagaraj, 2009), which inhibit T cell activity via several mechanisms (Nagaraj et al., 2010). A population of CD66b + CD11b + PMN-MDSCs is expanded in the peripheral blood of patients with active pemphigus (Oktem et al., 2021), but not those in remission (Neri et al., 2021). *In vitro*, these PMN-MDSCs can suppress allogeneic T-cell proliferation and exhibit high levels of expression of characteristic effector molecules such as arginase I and interleukin-10 (Neri et al., 2021). In addition, a correlation between MDSC frequency and Th2/Th1 cell ratio has been highlighted, which suggests a possible role of these cells as regulators of Th cell responses in pemphigus (Neri et al., 2021).

Plasmacytoid DCs (pDCs), which were long-thought to be part of myeloid lineage but in fact come from a lymphoid progenitor (Dress et al., 2019), could also have a possible role in pemphigus pathogenesis. pDCs seems to be recruited at very high level into the lesional skin of pemphigus patients (Ramadan et al., 2019). It is thus possible that pDCs might be involved in the initial mechanisms leading to autoantibody production, but this has yet to be formally investigated.

It has been demonstrated in autoimmune diseases such as lupus, rheumatoid arthritis and small vessel vasculitis that neutrophil extracellular traps (NETs) are linked with immune complexes and responsible for tissue inflammation and polyclonal activation of B-cell as well as memory B-cell activation (Yu and Su, 2013; Delgado-Rizo et al., 2017; Apel et al., 2018; O'Neil et al., 2019). As neutrophil can be found in the skin of pemphigus, a clinical trial aims to assess the effects on B-cell activation and the phenotypic changes in B-cell population from pemphigus patients after stimulation by NETs (NCT04117529). The understanding of the link between innate and adaptive immunity is essential for a direct targeting of the different actors of the diseases.

COMPREHENSION OF THE PEMPHIGUS BY USING *IN VIVO* MODEL OF THE DISEASE

As discussed above, there are many aspects of the aetiology, pathogenesis and treatment of pemphigus that we have yet to fully understand: these diseases are rare–very rare in the case of some variants and sub-variants–which limits opportunities for clinical studies, and robust insights can be hard to glean from the type of mechanistic experiments that are possible using human cells *in vitro*. In the past three decades, experiments using different mouse models of pemphigus have led directly or indirectly to the identification of the dominant autoantigens, the discovery of autoreactive T cells and B cells and their roles in the production of pathogenic IgG autoantibodies. These findings represent the sum of multiple studies employing different *in vivo* models, each focused on specific aspects of the autoimmune cascade, from loss of immunological tolerance on the level of T and B cells to the pathogenic effects of autoantibodies upon binding to their target autoantigen.

The first mouse model of pemphigus was the passive transfer model, in which IgG from the sera of patients with PV is injected intraperitoneally into neonatal mice, resulting in the formation of cutaneous blisters and erosions and reproducing the clinical, histologic, ultrastructural and immunologic features of the disease (Anhalt et al., 1982). Studies in these mice provided the first evidence that PV IgG alone are pathogenic *in vivo*, and facilitated the dissection of the mechanism of blister formation (Schulze et al., 2012). Later, the need to study the mechanisms leading to the generation of pathogenic autoantibodies in PV drove the development of an active disease model: $Dsg3^{-/-}$ mice, lacking self-tolerance against naturally expressed Dsg3 (Koch et al., 1997), were immunized against Dsg-3 to generate a source of Dsg3-reactive T and B cells, which were then isolated and injected into immune-deficient ($Rag2^{-/-}$) but $Dsg3^{+/+}$ recipients to induce a Dsg3-specific autoimmune response *in vivo* (Amagai et al., 2000; Tsunoda et al., 2003; Aoki-Ota et al., 2004). The recipient mice developed oral erosions with suprabasal acantholysis that was induced by the stable production of a panel of Dsg3-specific autoantibodies (Ohyama et al., 2002). A deeper analysis of the active pemphigus mouse model revealed that, in these mice, a single Dsg3-specific $CD4^+$ T-cell clone was able to induce a clinical phenotype in recipient mice by activating Dsg3-reactive B cells (Takahashi et al., 2008).

A major criticism of the passive-transfer and active pemphigus mouse models is that they cannot take account of the genetic components of pemphigus predisposition, which are known from human studies to be significant determinants of disease. In response, a humanized HLA-class II transgenic mouse model of PV was developed (Eming et al., 2014). In this mouse, antigen presentation to $CD4^+$ T cells is restricted to human HLA alleles, which allows the characterization of the loss of self-tolerance

against human Dsg3 in an HLA-restricted *in vivo* model system (Eming et al., 2014). The immunization of HLA-DRB1*04:02-transgenic mice with immunodominant Dsg3 peptides generates a $CD4^+$ T cell-dependent immune response against human Dsg3 along with the production of pathogenic Dsg3 reactive IgG antibodies (Eming et al., 2014). This model therefore allows investigation of the communication between B cells and T cells that underpins the production of anti-Dsg3 IgG, leading to the discovery that the use of anti-CD40L or the depletion of $CD4^+$ T cells abolishes the induction of pathogenic anti-Dsg3 IgG. Furthermore, when Treg were induced in these mice by injection with anti-CD28 antibodies, researchers observed a reduced humoral Dsg3-specific immune response, which supports the hypothesis that the Dsg3-specific $CD4^+$ T-cell dependent immune pathogenesis of PV is modulated by Treg (Schmidt et al., 2016).

Whilst these studies, in conjunction with clinical data, have supported major steps forward in our understanding and treatment of pemphigus in recent years, significant challenges remain. For example, our knowledge of the initiation of pemphigus is severely limited as we lack a spontaneous model of the disease. Similarly, our understanding of immune-pathogenesis is almost completely restricted to Dsg3 as the target autoantigen, despite knowledge from human patients that this is only one part of the pemphigus picture. A pressing need in the field is the development of a model that reproduces the full complexity of the disease, including multi-pathogenic mechanisms that target different autoantigens. The impact of the lack of such a model is nowhere clearer than in the development of novel therapeutics to treat pemphigus disease.

PEMPHIGUS TREATMENT

Prior to the era of systemic corticosteroids, 75% of patients with pemphigus were dying within the first year of the disease (Bystryn, 1996). Complications such as infections or nutritional deficiency contributed significantly towards this high mortality. The introduction of systemic corticosteroids and subsequent use of other immunomodulatory or immunosuppressive agents has brought a marked improvement in survival (Bystryn, 1996), however the level of immunosuppression required to ameliorate symptoms has created its own problems. More recently, advances in our understanding of the pathogenesis of pemphigus and the biological mechanisms of therapeutics have led to a paradigm shift in the treatment of this disease, from blanket immunosuppression towards a more targeted restriction of autoimmunity.

Conventional Systemic Immunosuppressants/Immunomodulators

Immunosuppression with systemic corticosteroids in the form of oral prednisone or prednisolone frequently remain either the initial or mainstay of treatment for pemphigus. Different dosing schedules are employed depending on the severity of the disease: they are typically initiated at a dose of 0.5 to 1.5 mg/kg/day to achieve initial control of the disease (Hertl et al., 2015; Harman et al., 2017); while more extreme cases may be treated with either intravenous pulse methylprednisolone (500–1,000 mg daily for three to five consecutive days) or dexamethasone 100 mg daily for three consecutive days, with or without concomitant cyclophosphamide (Pasricha et al., 1995; Werth, 1996; Saha et al., 2009). Corticosteroids are effective in autoimmune diseases as they exert strong anti-inflammatory effects and induce apoptosis of lymphocytes (Shimba and Ikuta, 2020). However, as morbidity and mortality from cumulative long-term corticosteroid use is significant (Razzaque Ahmed and Moy, 1982), various non-steroid immunosuppressive agents have been trialled in a bid to improve outcomes in these patients.

Initial attempts to treat pemphigus with non-steroid immunosuppressive agents such as azathioprine, mycophenolate mofetil and cyclophosphamide achieved mixed results: when analysed together, they seemed to reduce the risk of relapse in pemphigus (Atzmony et al., 2015). However, these medications individually were not shown to be better in achieving remission, reducing deaths or reducing relapse compared to corticosteroids alone (Martin et al., 2009). Nonetheless, both azathioprine and cyclophosphamide showed steroid-sparing effects and mycophenolate demonstrated a significant effect on disease control (Martin et al., 2009). Azathioprine is often used at a dose of 2–3 mg/kg/day, mycophenolate mofetil at a dose of 2–3 g/day and cyclophosphamide at 75–150 mg/day orally or 500–1,000 mg monthly intravenously. Due to its toxicity, cyclophosphamide is not widely used and is usually only considered in severe recalcitrant cases where other therapeutic options are not available or are contraindicated (Atzmony et al., 2015).

Methotrexate, a dihydrofolate reductase inhibitor, is the earliest steroid-sparing agent used in the treatment of pemphigus. It fell out of favour due to its associated toxicities when used at higher dose. It was not until later, when low to moderate doses (ranging 15–20 mg/week) were employed that it re-emerged as a safe and effective adjunct therapy in pemphigus (Gürcan and Razzaque Ahmed, 2009). Dapsone, with its ability to interfere with neutrophil chemo-attractants, is often used in the treatment of pemphigus with a predominantly neutrophilic infiltrate, such as pemphigus foliaceus, IgA pemphigus and pemphigus herpetiformis (Kasperkiewicz et al., 2014; Porro et al., 2019; Kridin et al., 2020). Dapsone is also an appropriate adjunctive steroid-sparing agent for the treatment of patients with PV whose disease was initially controlled with corticosteroids (Werth et al., 2008).

Anti-CD20 Antibody Therapy

The latest game changer in the treatment of pemphigus is rituximab, a murine-human chimeric anti-CD20 monoclonal antibody (human Fc portion associated with a murine variable region) which targets B cells expressing CD20, and was initially developed and used to treat B cell malignancies (Grillo-López, 2000). First trialled in PNP in 2001 (Heizmann et al., 2001) and in PV in 2002 (Salopek et al., 2002), rituximab is now considered by many to be the primary therapeutic option in pemphigus.

Injection of rituximab induces rapid depletion of B cells, including autoreactive B cells, from the peripheral blood of patients, with the B cell pool being reconstituted over the next 6–12 months from non-depleted progenitors (Mouquet et al., 2008). CD20 is expressed on the surface of B cells from the late pre-B-cell stage in the bone marrow, through naïve follicular B cells and into the memory B cell population; but the long-lived plasma cells do not express this molecule and survive in specific niches including the bone marrow, gut-associated lymphoid tissue (GALT) and skin-associated lymphoid tissue (iSALT) (Lightman et al., 2019; Zhou et al., 2020). Thus, depletion of mature B cells and short-lived plasma cells in patients with PV leads to lowering of autoreactive anti-Dsg antibodies in the serum, while antibodies produced by long-lived plasma cells, such as those directed against tetanus and pneumococcus, remain unchanged (Mouquet et al., 2008). Although rituximab is more expensive than broad spectrum immunosuppressants, a recent study showed that the initially higher cost of rituximab was almost completely off-set by costs related to management of flares and relapses in patients treated with the standard corticosteroid regimen (Hébert et al., 2020).

The therapeutic efficacy of rituximab treatment in patients with pemphigus seems to operate on multiple levels. Prolonged and continuous repopulation of naïve B cells bearing a new repertoire and a markedly delayed reappearance of memory B cells are seen after rituximab treatment: numbers of $CD19^+$ B-lymphocytes even 6 years after treatment are much lower than at baseline (Colliou et al., 2013). This prolonged blockage of B cell maturation also inhibits the IgM to IgG class switching process, thereby reducing levels of autoimmune IgG^+ circulating B lymphocytes and autoantibodies (Colliou et al., 2013): such long-lasting modification of the naive/memory-B-cell ratio accounts for the prolonged therapeutic effect of rituximab in patients with pemphigus. In addition, transitional B-cell and IL-10-secreting regulatory B cell (B reg) populations seem to expand during the B cell repopulation (Colliou et al., 2013); this could be significant because B reg cells can down-regulate inflammation and may be involved in the maintenance of long term immune tolerance (Lund and Randall, 2010; Mauri and Menon, 2017; Cao et al., 2019).

However, post-rituximab relapses do occur, and are thought to be linked to the re-emergence of anti-Dsg B cell clones that have lost self-tolerance (Hammers et al., 2015). Relapse occurs in more than 80% of PV patients over a median period of 79 months (Colliou et al., 2013), and is more likely when patients have severe disease at treatment outset and/or persistently high anti-Dsg1/3 antibody levels 3 months after treatment (Mignard et al., 2020): conversely, older patients and those given higher doses of the drug are less likely to experience early relapse (Kushner et al., 2019). It is plausible that higher doses of rituximab achieve a deeper B cell depletion and that the weaker immune systems of the elderly make the re-emergence of anti-Dsg B cell clones less likely. Alongside, long-term follow-up of auto-reactive B cells and antibodies in rituximab-treated patients with pemphigus demonstrated a complex regulatory process: in patients in remission, there were fewer autoreactive B cells than in patients with active pemphigus, and within that B cell population there was a higher proportion of IgM $Dsg3^+$ cells than IgG $Dsg3^+$ cells, in association with a rearrangement in Ig repertoire which had switched from an oligoclonal to polyclonal profile (Colliou et al., 2013; Hébert et al., 2019). In addition, the remaining autoreactive anti-Dsg antibodies detected in patients in remission seem to target non-pathogenic epitopes of Dsg (Müller et al., 2010).

It may be possible to reduce relapse rates, or lengthen the time to relapse, by modifying the approach to the use of rituximab in pemphigus patients. In a recent randomised control trial, 89% of patients treated with two infusions of 1 g of rituximab given a fortnight apart at baseline, and with 0.5 g at 12 and 18 months, combined with short-term prednisolone (0.5–1.0 mg/kg/day for 3–6 months) were in complete remission off therapy at 2 years, compared with only 34% of those given prednisone alone (1.0–1.5 mg/kg/day) (Joly et al., 2017). Importantly, the cumulative prednisolone dose used, and the number of severe adverse events in the rituximab-treated group, was three times and two times lower respectively, when compared to the prednisone-only group.

Alongside the relapse-rate, unfortunately, 10–20% of patients with pemphigus seem to be resistant to rituximab therapy (Joly et al., 2017). Trying to understand this phenomenon, a recent study showed that memory and germinal autoreactive B cells may persist in lymphoid tissues or ectopic lymphoid-like structures in PV lesions in cases of rituximab resistance (Zhou et al., 2020). Furthermore, the authors showed similar persistence of autoreactive $CD4^+$ Th cells, which provide a crucial help to B cells for the secretion of autoantibodies. Post rituximab treatment, the disruption of immune tolerance could lead to the appearance of new autoreactive B cells, and the non-depletion of the long-lived autoreactive plasma cells with persistent production of anti-Dsg 3 antibodies could similarly explain the resistance to treatment in some patients (Hammers et al., 2015). There may also be a link between the production of human anti-chimeric antibodies (HACA) to the murine fragments of rituximab which may hamper the effectiveness of subsequent doses and contribute to a lack of therapeutic response (Lunardon and Payne, 2012). In addition, a downregulation of CD20 in some $CD27^+$ memory B cells and the presence of an alternative transcript of the CD20 (D393–CD20), which was described in lymphoma and pemphigus could explain an impairment of the rituximab binding and the resultant lack of B cell depletion (Gamonet et al., 2014). Taken together, multiple pathways of incomplete/non-responsiveness to rituximab treatment have been suggested, and ways of overcoming these limitations and/or new therapies remain urgently needed.

Rituximab's targeting of B-cells also has the side-effect of leaving patients highly susceptible to bacterial infections, particularly those of the respiratory tract and skin (Goh et al., 2007; Kamran et al., 2013). Similarly, viral infections or reactivation of latent viruses such as herpes simplex (HSV), cytomegalovirus (CMV), hepatitis B (HBV) and hepatitis C (HCV) viruses have been described in lymphoma or pemphigus patients who received rituximab (Suzan et al., 2001; Goh et al., 2007; Yeo et al., 2009; Nooka et al., 2011). Screening or prophylaxis for HSV and CMV infections is

generally not regarded as necessary, but pre-treatment screening for HBV and HCV is (Sagnelli et al., 2012; Pattullo, 2015; Reddy et al., 2015). However, establishing a direct causal link between infections and rituximab therapy is often confounded by the concomitant use of other immunosuppressive agents and generalised immune dysfunction induced by the underlying diseases for which rituximab is used as therapy. As rituximab does not affect $CD4^+/CD8^+$ T-cell circulating numbers, susceptibility to bacterial infection and opportunistic infections after rituximab therapy may then be partly explained by the disruption of B cell's role in T-cell activation and optimal $CD4^+$ memory response. Hypogammaglobulinemia, which has been associated with multiple infusions of rituximab for rheumatic autoimmune diseases and lymphoma, is a risk factor for serious infections (Casulo et al., 2013; Yusof et al., 2019). However, hypogammaglobulinaemia has not been reported in pemphigus patients and it remains to be seen with longer term follow up of pemphigus patients undergoing multiple rituximab infusions. Concerns of progressive multifocal leukoencephalopathy has largely been reassured by the findings of post-marketing surveillance that this rare opportunistic infection is mainly seen in oncology (lymphoma) patients (Focosi et al., 2019).

The use of rituximab has undeniably revolutionised the treatment of pemphigus for many patients but its high initial cost, the high rate of relapse (Colliou et al., 2013) lowered resistance to infection and further lower immunogenicity to several vaccines remain significant drawbacks. These factors have driven more research aiming to discover and develop more targeted therapeutics with improved safety and efficacy profiles.

Adjunct Therapies

In the absence of a single therapy that works across all patients with pemphigus, different treatments have been trialled in combination with more conventional therapies, aiming to enhance the overall effect. One such adjunct therapy for severe recalcitrant pemphigus is high dose intravenous immunoglobulins (IVIG), given at 2 g/kg across five daily doses per month (Amagai et al., 2009) in addition to conventional immunosuppressive agents or rituximab (see below) (Jolles, 2001; Grando, 2019). Initial IVIG therapy rapidly elevates total circulating Ig levels, which is thought to stimulate homeostatic antibody-catabolic mechanisms, leading to lowering of serum levels of IgG1 and IgG4 anti-Dsg 1 and Dsg 3 antibodies (Green and Bystryn, 2008) while normal antibodies are replaced by those in subsequent IVIG doses: this is a particularly useful treatment when fast onset of therapy and lower risk of infection is needed. Combining rituximab and IVIG can be effective for the treatment of refractory pemphigus cases and may even induce long-term complete remission with lower risk of infection (Hamadah et al., 2019): accordingly, a clinical trial was started in June 2020 to evaluate the efficacy and safety of early use of rituximab with or without IVIGs in patients with moderate to severe pemphigus (NCT04400994). Immunoadsorption, which also rapidly removes anti-Dsg IgG from the circulation, has also been trialled in conjunction with conventional immunosuppressive treatment and intravenous rituximab (Eming and Hertl, 2006; Behzad et al., 2012). In its current form, immunoglobulins are non-specifically adsorbed and removed, but studies are underway to develop anti-Dsg immunoglobulin-specific adsorption (Langenhan et al., 2014); it is, however, not easily available in many parts of the world, limiting its potential for widespread use.

Complementary to systemic therapeutic options, local treatment of mucosal - especially oral - lesions of pemphigus should not be overlooked. Poor oral hygiene can be a contributory factor for persistent oral erosions in pemphigus (Gambino et al., 2014), and topical analgesics or anaesthetics (e.g., lidocaine 2% gel) can be used prior to eating or brushing of teeth. Topical medium or high potency corticosteroids (e.g., clobetasol propionate 0.05% in adhesive paste) can also be applied directly to the lesions (Lozada-Nur et al., 1994) or used as mouth gargle. Alongside, the use of topical calcineurin inhibitors and cyclosporine mouthwash may be also beneficial (Gooptu and Staughton, 1998; Hodgson et al., 2003). Candida infection is a common occurrence in patients with mucosal pemphigus who are treated with topical or systemic glucocorticoids (Lozada-Nur et al., 1994): oral nystatin swish-and-swallow or mouthwash can be used as prophylaxis. Dietary advice in favour of a soft diet and the avoidance of spicy or very hot foods can also be helpful for patients.

NOVEL TREATMENT IN THE PIPELINE

As we learn more about the pathogenesis of pemphigus, the development of more targeted therapeutic approaches with improved safety and efficacy profiles is gradually becoming possible.

Recent studies characterising the immune features of pemphigus lesions themselves have paved the way for innovative local treatment strategies. For example, intralesional delivery of rituximab, which has already been used in the treatment of lymphoma (Davies et al., 2017), has been proposed as a way of targeting the diffuse ectopic lymphoid-like structures that are commonly seen in lesions of both PV and PF and to treat refractory oral pemphigus vulgaris (Vinay et al., 2015; Zhou et al., 2020). Disruption of these lymphoid-like structures, which are composed of T cells, dendritic cells, centroblasts, plasmablasts and plasma cells might therefore disrupt the niche that supports the *in situ* B cell differentiation, clonal expansion and production of autoreactive antibody in the skin of patients with pemphigus. Identification of the cell composition and exploration of the impact of the injection of steroids into lesions of patients with pemphigus that harbour ectopic lymph node-like structures is ongoing (NCT04509570).

Some novel approaches target specific obstacles within current therapeutic settings. Aiming to restore the efficacy of anti-CD20 therapy in rituximab patients with HACA, subcutaneous injection of Ofatumumab, a fully human anti-CD20 IgG_1 antibody with increased binding affinity for CD20, has been successfully trialled in a single patient (Rapp et al., 2018), but

has yet to be assessed in a larger cohort. Similarly, veltuzumab, a second generation humanized anti-CD20 antibody, has been successfully used in a case of rituximab-refractory PV (Ellebrecht et al., 2014). The development of these second and third generation anti-CD20 antibodies, which possess superior B cell-depleting qualities and higher binding affinities compared to rituximab, might represent an important step forward in the treatment of pemphigus and other autoimmune B cell diseases.

A different type of treatment obstacle can be simply a practical limitation, as in the case of IVIG: these products are generally considered safe and effective, but their production requires an abundant supply of human plasma in order to generate the large doses of product (up to 2 g/kg body weight) needed for therapy.

To overcome this supply limitation, antibodies with high affinity for the neonatal Fc receptor (FcRn) have been developed. These antibodies represent a key determinant actor for IgG levels and functions (Pyzik et al., 2019). FcRn-targeting therapeutics aim to block the binding of IgG and IgG immune complexes to the FcRn, thereby accelerating their breakdown and inducing a reduction in overall plasma IgG levels, including the levels of pathogenic autoantibodies (Blumberg et al., 2019).

Data from a phase 1b/2a study using ALXN1830 (NCT03075904), a humanized affinity-matured IgG4-kappa monoclonal antibody with high affinity for the neonatal Fc receptor, has shown major improvements in both cutaneous and mucosal disease, and an overall acceptable safety and tolerability profile (Werth et al., 2021). In this trial, ALXN1830-associated clinical improvement was accompanied by a similarly rapid and significant decrease in levels of total IgG, all individual IgG subclasses, and IgG immune complexes. In addition, this study thereby provides evidence that IgG circulating immune complexes may be involved in the pathogenesis of pemphigus. Another promising anti-FcRn therapeutic is also being trialled in pemphigus. Efgartigimod is a human IgG1 antibody Fc-fragment, a natural ligand of FcRn, that has been engineered for increased affinity to FcRn compared with endogenous IgG (Zuercher et al., 2019). Proven safe and effective in patients with myasthenia gravis (Howard et al., 2021), results from a completed phase two trial of efgartigimod in patients with PV (NCT03334058) have shown that it is well-tolerated and exhibited an early effect on disease activity and outcome parameters, providing support for further evaluation as a therapy for pemphigus (Goebeler et al., 2021). Two phase 3 clinical trials assessing the early and long-term efficacy and safety of a subcutaneous formulation of efgartigimod in adults with pemphigus is on-going (NCT04598451 and NCT04598477).

Focussing on the immune-associated SNPs that have been linked with pemphigus, Assaf S et al. recently demonstrated through a series of experiments on how ST18 contributed to destabilization of cell-cell adhesion in a tumour necrosis factor (TNF)-α-dependent manner, potentially opening up new therapeutic option of using TNF-α inhibitors in the treatment of pemphigus (Assaf et al., 2021).

Targeting another aspect of the biology of B cells, such as their survival or their differentiation into plasma cells, is a useful approach that has been tested in SLE and could be applied to the treatment of pemphigus. The use of belimumab, a monoclonal human IgG1 antibody that binds to soluble B lymphocyte stimulator (BLyS) or B cell activating factor belonging to the TNF family (BAFF) (Möckel et al., 2021), or atacicept, a fully human recombinant fusion protein that blocks BLyS and the proliferation-inducing ligand (APRIL) (Kaegi et al., 2020), might be a promising treatment of pemphigus. This is further supported by the recent finding that a modification of the BAFF/BAFF receptor axis in patients with pemphigus could explain the high number of relapses following standard corticosteroid treatment alone versus with rituximab (Hébert et al., 2021). New therapies such as Bruton's tyrosine kinase (BTK) inhibitors have also emerged as a potential treatment option: BTK inhibitors seem to be able to neutralize pathogenic autoantibodies, to inhibit new autoantibody production and to possess anti-inflammatory effects (Weber et al., 2017). A trial involving the use of a BTK inhibitor, PRN 1008, in patients with PV (NCT02704429) has been completed and recently published (Murrell et al., 2021). The study suggests that BTK inhibition may be a promising treatment strategy and supports further investigation of such inhibitor for the treatment of pemphigus.

Recently, chimeric antigen receptor (CAR) T cell technology has revolutionized cancer immunotherapy (reviewed in Mohanty et al. (2019)). This approach uses T cells from the patient's own blood that are genetically manipulated in research laboratories to express a CAR capable of recognizing a specific cell type uniquely expressing the target antigen. In the case of pemphigus, researchers have engineered a chimeric autoantibody receptor (CAAR), with Dsg3 as the extracellular domain in order to generate CAAR-T cells that recognize the Dsg3-specific BCR on autoreactive B cells and induce their elimination (Ellebrecht et al., 2016). Preclinical study has demonstrated that DSG3-CAART could be a precise therapy for PV (Lee et al., 2020). A phase one clinical trial to determine the maximum tolerated dose of Dsg3-CAART in mucosal dominant PV patients is ongoing (NCT04422912). Whilst this is potentially a promising step forwards, the efficacy of Dsg3-CAAR-T cell therapies in pemphigus may be limited by the single antigen focus, and so the repertoire of recognition might need to be expanded in future trials if this approach is found to be safe and well-tolerated.

VACCINATION AND PEMPHIGUS

Vaccination strategy and Pemphigus treatment are in need of more studies especially in this time of COVID-19 pandemic. The effect of rituximab and other anti-CD20 monoclonal antibodies on vaccine response has been studied for inactivated vaccines (Baker et al., 2020). These studies have suggested that rituximab recipients mount attenuated yet meaningful vaccine responses. Concerning live attenuated vaccines, no study addressing their immunogenicity has been started due to the safety concerns regarding the use of these vaccines in rituximab recipients. In addition, no studies have evaluated yet the safety and immunogenicity of messenger RNA vaccines or viral vector vaccines, which are among the leading COVID-19 vaccine

candidates. To gain knowledge on COVID-19 vaccine and pemphigus, a clinical trial has been started recently in august 2021 to compare the immune response to different COVID-19 vaccine booster doses (Moderna COVID-19 vaccine, Pfizer-BioNTech COVID-19 vaccine, or Janssen COVID-19 vaccine) in participants with autoimmune disease requiring immunosuppressive medications, including pemphigus patients (NCT05000216).

CONCLUSION

Alongside advances in our understanding of pemphigus pathogenicity, the number of therapeutic options to treat pemphigus has increased over the last decade, with more targeted therapies and refined diagnostic techniques beginning to emerge. However, with better definition of the clinical subtypes of pemphigus, the aetiology and immune pathogenesis of these diseases is revealed to be more and more complex, and still requires further investigation. Better comprehension of the early stages of pemphigus, the role of innate and adaptive immune cells - most notably dendritic cells and full analysis of the involved B cells' biology will be required. To support this, the field should develop new mouse models that incorporate all the immune players necessary for the emergence of pemphigus variants, and that can be used to test new and innovative therapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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C10orf99/GPR15L Regulates Proinflammatory Response of Keratinocytes and Barrier Formation of the Skin

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The epidermis, outermost layer of the skin, forms a barrier and is involved in innate and adaptive immunity in an organism. Keratinocytes participate in all these three protective processes. However, a regulator of keratinocyte protective responses against external dangers and stresses remains elusive. We found that upregulation of the orphan gene *2610528A11Rik* was a common factor in the skin of mice with several types of inflammation. In the human epidermis, peptide expression of G protein-coupled receptor 15 ligand (GPR15L), encoded by the human ortholog *C10orf99*, was highly induced in the lesional skin of patients with atopic dermatitis or psoriasis. *C10orf99* gene transfection into normal human epidermal keratinocytes (NHEKs) induced the expression of inflammatory mediators and reduced the expression of barrier-related genes. Gene ontology analyses showed its association with translation, mitogen-activated protein kinase (MAPK), mitochondria, and lipid metabolism. Treatment with GPR15L reduced the expression levels of filaggrin and loricrin in human keratinocyte 3D cultures. Instead, their expression levels in mouse primary cultured keratinocytes did not show significant differences between the wild-type and *2610528A11Rik* deficient keratinocytes. Lipopolysaccharide-induced expression of *Il1b* and *Il6* was less in *2610528A11Rik* deficient mouse keratinocytes than in wild-type, and imiquimod-induced psoriatic dermatitis was blunted in *2610528A11Rik* deficient mice. Furthermore, repetitive subcutaneous injection of GPR15L in mouse ears induced skin inflammation in a dose-dependent manner. These results suggest that C10orf99/GPR15L is a primary inducible regulator that reduces the barrier formation and induces the inflammatory response of keratinocytes.

Keywords: EIME, keratinocyte, GPR15L, C10orf99, 2610528A11Rik, atopic dermatitis, psoriasis

INTRODUCTION

The defense system of the skin has three layers: barrier, innate immunity, and acquired immunity, and is organized to elicit effective responses to protect an organism from external dangers and stresses (1). The barrier is a constitutive machinery at the outermost sites of the organism physically, chemically, and biologically (2). At the same time, the other two layers depend on inducible responses. Defects in the barrier can give rise to abnormal responses in subsequent layers, resulting in inflammatory skin diseases (1). We have previously proposed a concept that interactions in "a loop" among epithelial–immune microenvironment (EIME)-specific factors—microbiota, barrier, epidermis, immune cells, and peripheral nerve endings—are observed in chronic inflammatory skin diseases such as atopic dermatitis and psoriasis (3) and epidermal keratinocytes play central roles in the initiation and propagation of the inflammatory responses (3, 4). However, a shared molecule in keratinocytes that responds to diverse dangers and stresses and governs the protective machinery of the skin, and induces various types of inflammatory responses remains obscure.

G protein-coupled receptor 15 ligand (GPR15L) is a 9 kDa polypeptide encoded by orphan genes *C10orf99* in humans and *2610528A11Rik* in mice (5, 6). GPR15L is a ubiquitous membrane/secretion protein expressed in epithelial tissues such as gastrointestinal mucosa, cervix, and skin. Its orthologs display a signal peptide, four conserved cysteines forming intramolecular disulfide bridges, and a highly conserved C-terminal domain, which is involved in receptor activation and signaling (5, 6). *2610528A11Rik* deficient mice are fertile and develop normally but exhibit an increased ovalbumin-specific IgG2a response (7). A *C10orf99* peptide has broad antimicrobial activity against different organisms, including *Staphylococcus aureus*, *Aspergillus*, *Mycoplasma*, and lentiviruses (8). In addition, several functions of GPR15L have been suggested in the context of molecular interaction: the binding of *C10orf99* peptides with sushi containing domain-2 (SUSD2) induces the growth inhibition of intestinal epithelial cancer cell lines (9), involvement of *C10orf99* in the insulin growth factor-like (IGFL)–Akt pathway (10), and a chemoattractant activity as the ligand of GPR15, an orphan G protein-coupled receptor (5, 6). Of note, gene expression levels of *C10orf99/2610528A11Rik* are highly increased in the lesional skin of human psoriasis (10–14) and murine psoriasis models (15), as well as in atopic dermatitis (16–19) and its murine models (19). However, details of the mechanical involvement of GPR15L in the barrier and inflammatory responses of the skin remain elusive.

GPR15 was originally identified as a G protein-coupled receptor (GPCR) family member (20). It was identified as a receptor for the infection of simian and human immunodeficiency viruses (21). GPR15 is expressed by memory B cells, plasmablasts, memory/effector and regulatory T cell subsets, and directs their homing to the colon to regulate colitis (22–25). In particular, GPR15 mediates dendritic epidermal T-cell recruitment (5, 6, 26). Specifically, dendritic epidermal T-cell precursors from GPR15-deficient mice failed to migrate in response to GPR15L (6). However, the role of GPR15L–GPR15 interaction in skin inflammation remains controversial. Transgenic overexpression of GPR15L seems to confer significant protection in the murine imiquimod-induced psoriatic dermatitis model (5), which is blunted by local depletion of GPR15L (27). Nevertheless, GPR15 deficiency does not alter the course of disease in an imiquimod-induced or IL-23-induced psoriatic dermatitis model (28). These results suggested more intrinsic functions of GPR15L beyond its antimicrobial activity or GPR15-mediated chemotactic activity in the skin.

Here we report that GPR15L plays a primary role in the innate response of keratinocytes and barrier formation in the skin. GPR15L expression is promptly induced during keratinocyte differentiation while it was impaired by GPR15L treatment *in vitro*. In addition, GPR15L depletion leads to defective lipopolysaccharide (LPS)-induced transcriptional response of keratinocytes *in vitro* and blunted skin inflammation by imiquimod treatment *in vivo*. Furthermore, a drastic increase in GPR15L expression levels is found in a wide variety of skin inflammation regardless of the type of immune response, suggesting a fundamental role for GPR15L in the immunological and structural barrier of the epidermis.

MATERIALS AND METHODS

Animals

Eight- to 12-week-old female mice were used in this study. All animals used were of the C57BL/6 genetic background. *2610528A11Rik-/-* mice have been previously generated in a mouse knockout library for secreted and transmembrane proteins (7). Heterozygous littermates were used as wild-type controls unless otherwise indicated.

Skin Inflammation Models

For DNFB-induced contact hypersensitivity, mice were sensitized to shaved abdominal skin with 25 μL 0.5% (w/v) DNFB in acetone/olive oil (4:1). Five days after sensitization, the ears were challenged with an application of 20 μL 0.3% DNFB. For the papain occlusive dressing technique, 100 μg of papain (Calbiochem, San Diego, CA, USA) diluted in 10 μL PBS was applied to the shaved and tape-stripped dorsal skin and fixed. Each mouse had a total of three 4-day exposures to the patch, separated by 3-day intervals. Mice were euthanized at the end of the third cycle of sensitization. For the imiquimod-induced psoriasis model, a daily dose of 10 mg of imiquimod-containing cream, Aldara (Beselna Cream 5%, Mochida Pharmaceuticals, Tokyo, Japan), was applied to the mouse ear for 2–6 consecutive days. For irritant contact dermatitis, mice were anesthetized with diethyl ether, and 20 μL of 1% (v/v) croton oil in acetone was applied to the ear skin. All animals were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals at Kyoto University Graduate School of Medicine. The sample sizes were determined based on previous studies (4, 29). The sample size reflects the number of independent biological replicates for each experiment and is provided in the figure legend.

Human Specimens

We obtained biopsy specimens of skin lesions from patients with atopic dermatitis or psoriasis at Kyoto University Hospital.

Histological Analyses

Skin tissues were fixed in 10% formalin in PBS and then embedded in paraffin. Sections of 5 μm thickness were prepared and stained with hematoxylin and eosin (H&E). For immunohistochemistry, formalin-fixed, paraffin-embedded tissue samples were processed for immunohistochemistry using the following primary antibodies: anti-C10orf99 antibody (1:500, ab151109, Abcam, Cambridge, UK), anti-filaggrin antibody (1:250, sc-66192, Santa Cruz Biotechnology Inc., Dallas, TX, USA), and anti-loricrin antibody (1:1,000, RPB-145P, Covance, Princeton, NJ, USA). Sections were deparaffinized and processed for antigen retrieval by incubating in 10 mM sodium citrate buffer (pH 6.0) at 95–100°C for 10 min. Tissue samples were incubated overnight with primary antibodies at 4°C. The sections were incubated with the biotinylated secondary antibody and with streptavidin-HRP (VECTASTAIN® Elite® ABC Kit Peroxidase (HRP) PK-6100, Vector Laboratories, Burlingame, CA), and developed with DAB reagents (Vector® DAB, Vector Laboratories), following the manufacturer's instructions.

Quantitative PCR

Total RNA was isolated using TRIzol (Invitrogen, Waltham, MA, USA) or RNeasy kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA was reverse-transcribed using the Prime Script RT reagent kit (Takara Bio, Kusatsu, Japan). Quantitative RT-PCR was performed as previously described (4). All the primers were obtained from Greiner Bio-One Co., Tokyo, Japan. Primer sequences are listed in **Table S1** in the **Supplementary Material**.

Cell Culture

Mouse primary keratinocytes were isolated from newborn mice, cultured in a low-calcium medium (0.05 mM Ca^{2+}), and induced to differentiate by raising calcium to 1.3 mM, as described previously (30). For cell-stimulation experiments, cells were serum-starved for 24 h before stimulation. The culture was maintained at 32°C in a humidified chamber containing 5% CO_2. NHEKs (Kurabo, Osaka, Japan) were inoculated at a concentration of 2,500 cells/cm^2 in culture medium, HuMedia-KG2 (Kurabo) supplemented with insulin (10 μg/mL), human epidermal growth factor (0.1 ng/mL), hydrocortisone (0.5 μg/mL), bovine pituitary extract (0.4% v/v), gentamicin (50 μg/mL), and amphotericin B (50 ng/mL). The culture was maintained at 37°C in a humidified chamber containing 5% CO_2. For the 3D-cultured human epidermis, we used the LabCyte EPI-MODEL 24, 6-day culture kit, as a model for the developing human epidermis (Japan Tissue Engineering Co., Ltd., Gamagori, Japan).

Peptides

GPR15L synthetic peptides (mouse, 54 AA; human full-length, 57 AA; and human ΔC, 47 AA) were obtained from Zhejiang Ontores Biotechnologies Co., Ltd., Hangzhou, Zhejiang Province, China. The peptides were dissolved in PBS at the indicated concentrations. For animal treatment, peptide solution or PBS were subcutaneously injected into both ears of each mouse using a 30-gauge needle every other day for ten days.

Transfection Analysis

The human *C10orf99* expression vector was generated by inserting a *C10orf99* open reading frame (NM_207373) into the multiple cloning sites of pIRES-EGFP (Clontech Laboratories, Mountain View, CA, USA). According to the manufacturer's instructions, the human *C10orf99* expression vector (or the mock vector) was transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). The cells were harvested for quantitative RT-PCR analyses before and after 2-day culture.

RNA-Seq Library Preparation

Total RNA was extracted from primary cultured normal human epidermal keratinocytes (NHEK) following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific, Waltham, MA, USA) followed by a Qiagen RNeasy Micro clean-up procedure (Qiagen, Hilden, Germany). RNA was analyzed on Agilent Bioanalyser for quality assessment with RNA Integrity Number (RIN) range from 9.6 to 9.8 and median of RIN 9.8. cDNA libraries were prepared using 2 ng of total RNA using the SMARTSeq v2 protocol (31) with the following modifications: 1. Addition of 20 μM TSO; 2. Use of 200 pg cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, San Diego, CA, USA). The length distribution of the cDNA libraries was monitored using a DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip (Perkin Elmer, Waltham, MA, USA). All samples were subjected to an indexed paired-end sequencing run of 2x151 cycles on an Illumina HiSeq 4000 system (Illumina) (31 samples/lane).

Transcriptome Analyses

The 2 × 151 bp paired-end reads from Illumina sequencing were quality checked using fastqc (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and after quality checks mapped to the GRCh38 human genome assembly using the STAR alignment tool (32). Reads mapped to genes were counted in each sample using featureCounts (33), where the reference gene annotations were obtained from GENCODE v26 (34). Gene counts were loaded into the R/Bioconductor package edgeR (35) for differential gene expression analysis. Gene counts were normalized across samples using the trimmed mean of M-values (TMM) method. Genes with expression lower than one count per million in all samples were excluded from the analysis. A negative binomial generalized linear model was fitted to the data. The estimateDisp function was used to estimate the common, trended, and tagwise dispersion terms. The glmFit and glmLRT functions were used to fit a negative binomial generalized log-linear model to the read counts for each gene and conduct a genewise statistical test of the difference in mean expression between sample groups. Differentially expressed genes were selected based on a false discovery rate (Benjamini-Hochberg multiple testing corrected *p*-value) of < 0.05. RNA-seq

data were deposited in the Gene Expression Omnibus (GEO) database; accession number GSE189751.

Gene ontology analysis was performed based on the database for annotation, visualization, and integrated discovery (DAVID) (36) and Enrichr (37).

Parametric enrichment analysis based on the KEGG functional hierarchy (38) was performed using the GAGE algorithm (39). Briefly, we compared the average gene expression of the target genes of a TF against that of the whole gene. A weighted t-test procedure was used in the parametric gene set enrichment analysis to weigh the target genes with high-frequency binding. The binding frequency of TFs to their target genes was assessed based on a number of high-throughput chromatin immunoprecipitation (ChIP) experiments obtained from the Gene Expression Omnibus database (GEO, www.ncbi.nlm.nih.gov/geo/). A weighted t-statistic was used as the enrichment score. The p values calculated by GAGE were then corrected for multiple testing using the Benjamini-Hochberg procedure. FuncTree was used to visualize enrichment results (40). Transcription factor enrichment analysis based on numerous ChIP-seq data was performed as previously described (41).

Statistical Analysis

Unless otherwise indicated, data are presented as the mean ± standard deviation. A two-tailed Student's t-test or an analysis of covariance (ANCOVA) was performed to assess statistical significance. Statistical significance was set at $P < 0.05$ and is indicated in the figures.

RESULTS

Lesional Skin From Several Immune Types of Dermatitis Commonly Expresses GPR15L in Mice and Humans

The results of our previous transcriptomic profiling by microarrays on biopsy specimens from four animal models of atopic dermatitis (NC/Nga, flaky tail, *Flg*-mutated, and ovalbumin-challenged mice) revealed that *2610528A11Rik* encoding GPR15L is one of four genes, whose expression levels were increased in all the models (19).

We next wonder whether the induction of GPR15L expression in the lesional skin is widely shared in mice with several inflammatory skin conditions with different immune types. We analyzed the gene expression levels of *2610528A11Rik* in the lesional skin of mice with croton oil-induced irritant dermatitis (a model for T cell-independent innate response) (42), DNFB-induced contact hypersensitivity (a model for allergic contact dermatitis; type 1 immunity) (43), papain-induced dermatitis (a model for atopic dermatitis; type 2 immunity) (44), and imiquimod-induced dermatitis (a model for psoriasis; type 17 immunity) (45). We found that gene expression levels of *2610528A11Rik* were more than 100 times higher in lesional skin with croton oil-induced irritant contact dermatitis than in healthy skin (**Figure 1A**). The higher expression levels of GPR15L in the lesional skin were common to all the other dermatitis models (**Figure 1A**).

Next, we evaluated the protein expression levels of GPR15L in the lesional skin of humans with representative inflammatory skin diseases, including atopic dermatitis and psoriasis, since the increase in gene expression levels of GPR15L in the lesional skin has been demonstrated in multiple transcriptomic studies in both diseases (10–14, 16–19). As expected, immunohistochemistry revealed much higher expression levels of GPR15L protein in the lesional skin of patients with atopic dermatitis or psoriasis than in healthy skin (**Figure 1B**). Specifically, the induction of GPR15L is mainly localized in the epidermis. In addition, its expression levels were higher in the suprabasal cells (especially in the upper layer) than in the basal cells of the epidermis and higher on the upper side than on the lower side of each epidermal keratinocyte (**Figure 1B**). These patterns were similar to the expression of human β-defensin 2, a microbial peptide remarkably induced in the epidermis of patients with psoriasis (46) though the intracellular expression patterns of GPR15L in the epidermis were unclear in a previous study (27). Regardless of immune cell infiltration, there was incontinence of GPR15L into the extracellular spaces of the papillary dermis of the lesional skin from some patients with psoriasis (**Figure S1B**).

These results suggest that GPR15L is a highly inducible molecule in epidermal keratinocytes in response to skin inflammation, regardless of the immune type of inflammation.

High Calcium-Induced Differentiation Triggers GPR15L Expression in Mouse Primary Keratinocytes

The mechanism of GPR15L gene expression induction has not yet been fully elucidated. In newborn mice, GPR15L was mainly expressed in the epidermis (**Figure S1A**). Therefore, we first evaluated the nature of GPR15L gene expression in mouse keratinocytes during calcium-induced differentiation *in vitro*. Switching experimentally from low to high calcium induces phosphoinositide-dependent kinase 1 (PDK1)-dependent cell differentiation in keratinocytes (30). Quantitative RT-PCR showed that GPR15L gene expression as well as gene expression of *Krt10* (encoding keratin 10), a suprabasal cell marker, and *Ivl* (involucrin), *Flg* (filaggrin), and *Lor* (loricrin), late differentiation markers, was induced by high calcium levels in primary cultured mouse keratinocytes (**Figure 2A**). These results suggest that GPR15L expression is triggered by keratinocyte differentiation during the basal-to-suprabasal switch or a late differentiation phase mediating barrier formation in the epidermis.

GPR15L Treatment *In Vitro* Attenuates Late Keratinocyte Differentiation

Gene expression levels of keratinocyte differentiation markers in newborn epidermis were comparable between wild-type and *2610528A11Rik* deficient mice (**Figure S2A**). To investigate the relationship between GPR15L expression and keratinocyte differentiation, we evaluated the calcium-induced differentiation of GPR15L-deficient keratinocytes. On day three of the high-calcium culture conditions, the gene expression levels of *Krt10*, *Ivl*,

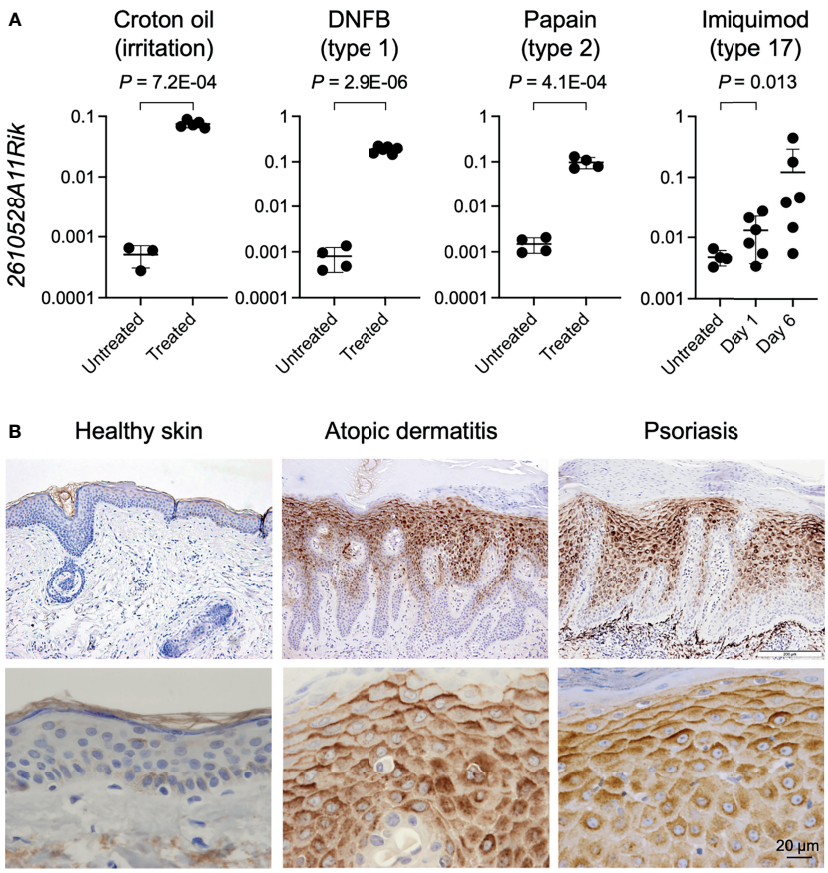

FIGURE 1 | GPR15L expression in the skin with several types of inflammation in mice and humans. **(A)** Quantitative RT-PCR analysis of GPR15L mRNA levels in the lesional skin of mice with helper T lymphocyte-independent irritant dermatitis and with three immune types of skin inflammation. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression (error bars, SD; n ≥ 5 per group). **(B)** GPR15L protein expression in healthy human skin and lesional skin from patients with atopic dermatitis or psoriasis. Representative results from the three or more cases are shown. Upper panels, low power (a scale bar, 200 μm); Lower panels, high power (a scale bar, 20 μm).

Flg, and *Lor* were comparable between GPR15L-deficient keratinocytes and wild-type keratinocytes (**Figure 2B**), although only a few specimens revealed higher levels of *Flg*, and *Lor*, late differentiation markers for keratinocytes, in GPR15L-deficient keratinocytes.

To further analyze the relationship between GPR15L and barrier formation of the epidermis, we treated 3D-cultured normal human epidermal keratinocytes with GPR15L synthetic peptides during their stratification *in vitro*. GPR15L treatment downregulated the gene expression levels of filaggrin and loricrin in a dose-dependent manner (**Figure 2C**). Consistently, immunohistochemistry of the 3D-cultured epidermis showed that protein expression levels of filaggrin and loricrin were lower in the GPR15L-treated group than in the non-treated group (**Figure 2D**). In addition, thickness of the filaggrin-positive layers in the 3D-culturted epidermis were less in the GPR15L-treated group in a dose-dependent manner (**Figure 2E**). Thickness of the loricrin-positive layers were also less in the GPR15L-treated group in both 1 μM and 10 μM concentrations than in the non-treated group (**Figure 2E**).

These results suggest that GPR15L negatively regulates the late differentiation of keratinocytes and barrier formation of the epidermis. In contrast, defective GPR15L does not affect normal keratinocyte differentiation.

Next, we tested several stimulations or cellular stresses to induce GPR15L gene expression in keratinocytes. We stimulated mouse primary cultured keratinocytes with NF-κB activators, phorbol 12-myristate 13-acetate (PMA), interleukin (IL)-1β, interferon (IFN)-γ, or lipopolysaccharide (LPS) because the *2610528A11Rik* gene has several κB sites in the promoter region and the first intron. All these stimuli induced IL-1β transcription in mouse primary cultured keratinocytes by 4 hours (**Figure S2B**). We found that the up-regulation of GPR15L gene expression in mouse keratinocytes was detectable in 1 hour and enhanced by 4 hours after stimulation with IFN-γ *in vitro* (**Figure 2F**). In contrast, the other stimuli or stress conditions failed to induce GPR15L expression (**Figure 2F**). These results suggest that GPR15L expression in keratinocytes is induced during cell-intrinsic differentiation and is augmented by a certain external inflammatory mediator, such as IFN-γ.

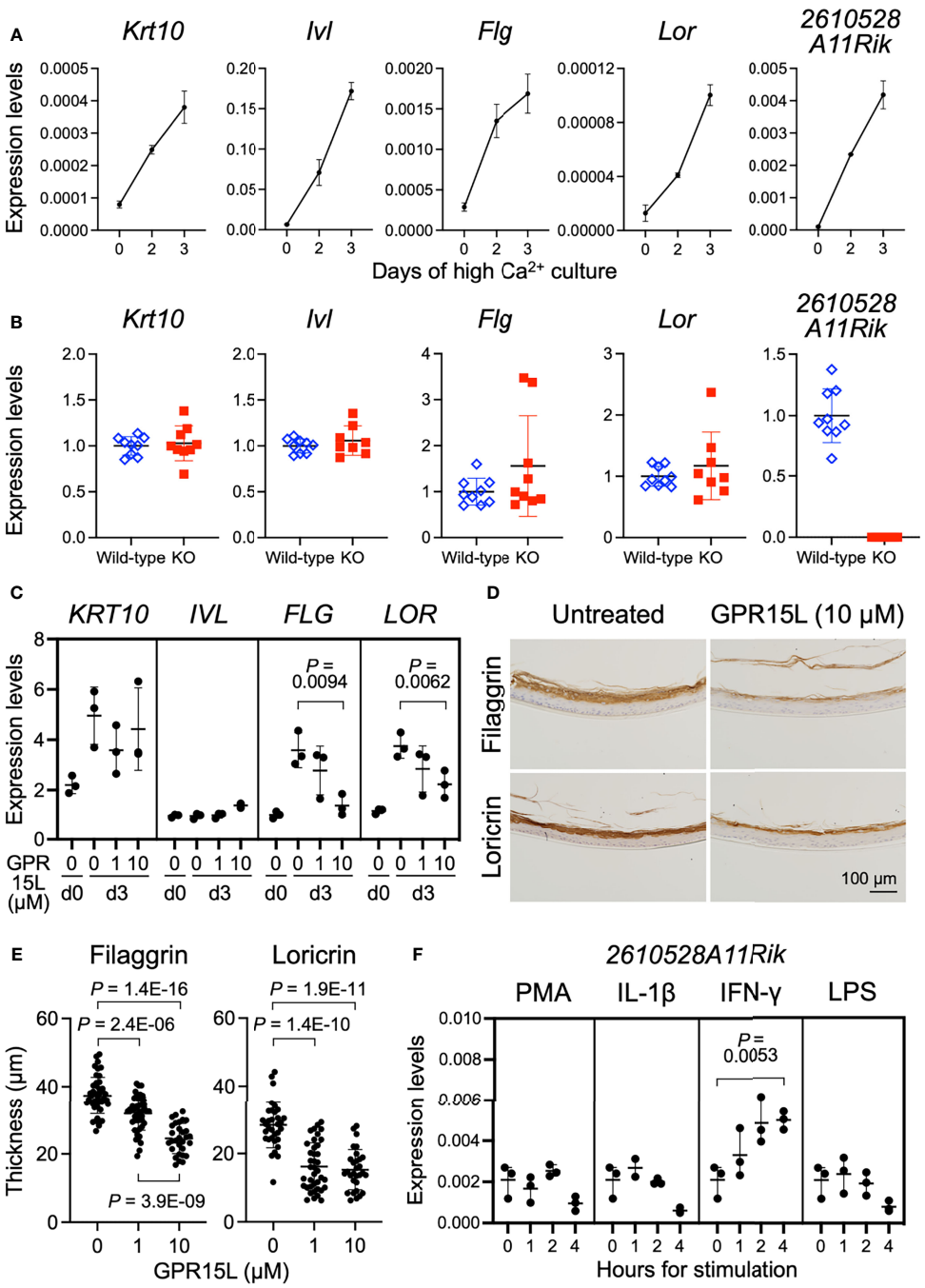

FIGURE 2 | GPR15L expression in keratinocytes and GPR15L-mediated control for epidermal differentiation. **(A)** Quantitative RT-PCR analysis of mRNA levels of keratinocyte differentiation markers and GPR15L in mouse primary cultured keratinocytes during Ca^{2+}-induced differentiation. Results were normalized to *Gapdh* expression (error bars, SD; n = 3 per group). **(B)** Quantitative RT-PCR analysis of mRNA levels of keratinocyte differentiation markers in primary cultured keratinocytes from wild-type or GPR15L-null mice on day three of Ca^{2+}-induced differentiation. Results were normalized to *Gapdh* expression and indicated as expression levels relative to those of the wild-type (error bars, SD; n ≥8 per group). **(C)** Quantitative RT-PCR analysis of mRNA levels of keratinocyte differentiation markers in 3D-cultured human epidermis treated with GPR15L during development *in vitro*. Results were normalized to *GAPDH* expression (error bars, SD; n = 3 per group). **(D)** Protein expression levels of keratinocyte differentiation markers in 3D-cultured human epidermis treated with or without 10 μM GPR15L during development *in vitro*. Representative results from triplicates are shown (a scale bar, 100 μm). **(E)** Thickness of the filaggrin-positive and loricrin-positive layers in the 3D-cultured human epidermis treated with or without GPR15L during development *in vitro*. Thickness of filaggrin or loricrin-positive layers of three sections from each of the triplicates were measured at three or more sites (error bars, SD; n ≥ 30 per group). **(F)** Quantitative RT-PCR analysis of GPR15L mRNA levels in mouse primary cultured keratinocytes with indicated stimulations. Results were normalized to *Gapdh* expression (error bars, SD; n = 3 per group).

GPR15L Gene Transfection Induces Inflammatory Gene Expression and Reduces Barrier Gene Expression in Normal Human Epidermal Keratinocytes

The inducible responses of keratinocytes against external dangers and stresses have been demonstrated to play a critical role in triggering and propagating different types of skin inflammation (4, 47). To evaluate the role of GPR15L in the proinflammatory response of keratinocytes, we transfected NHEKs with GPR15L-expressing plasmids or empty plasmids. We found that the gene expression levels of representative proinflammatory mediators, such as IL-1β, IL-6, chemokine C-X-C motif ligand 1 (CXCL1), thymic stromal lymphopoietin (TSLP), and β-defensin 4, from keratinocytes after the overnight transfection were higher in GPR15L-expressing NHEKs than in empty plasmid-transfected NHEKs (**Figure 3A**).

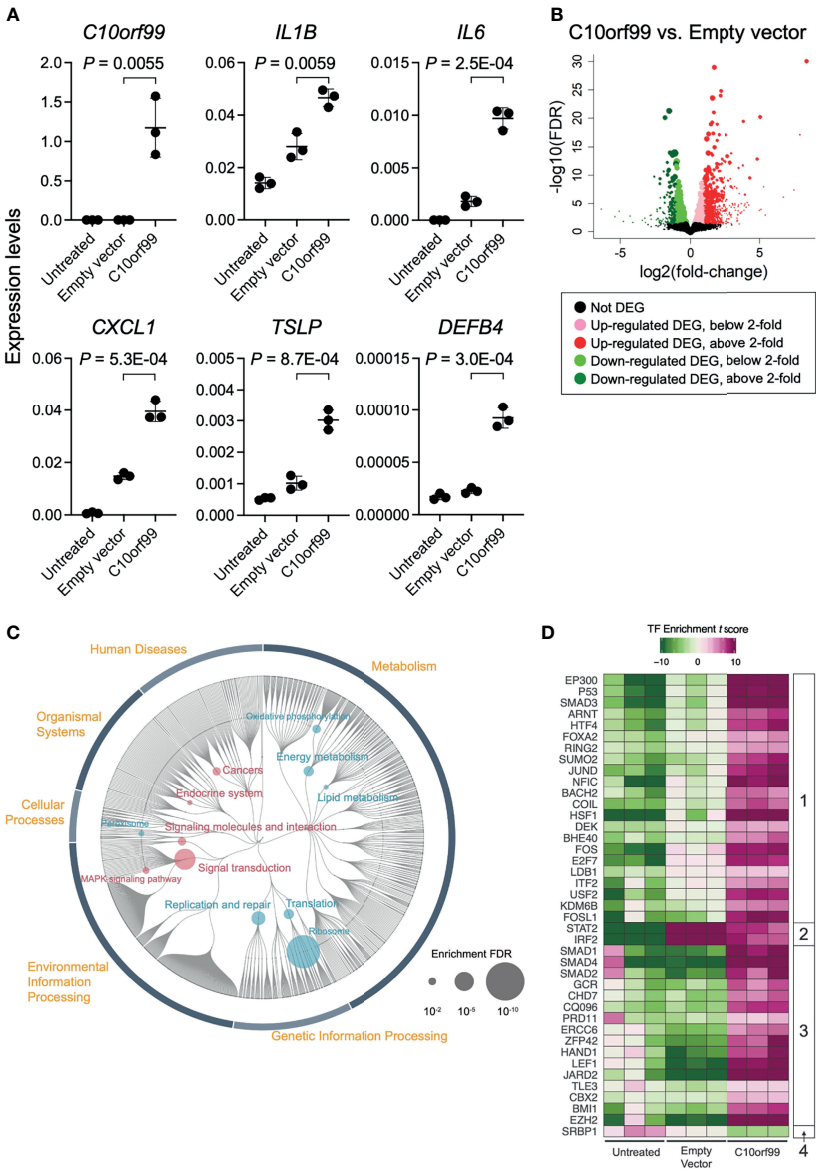

FIGURE 3 | Gene expression profiles in normal human epidermal keratinocytes (NHEKs) transfected with *C10orf99*. **(A)** Quantitative RT-PCR analysis of mRNA levels in untreated NHEKs transfected with empty vector or with *C10orf99*. The results were normalized to *GAPDH* expression levels (error bars, SD; n = 3). **(B)** Volcano plots of the relative differences in gene expression levels using the differentially expressed genes (DEGs) in NHEKs transfected with empty vector or *C10orf99* and analyzed by RNA-seq analysis. Each point represents a unique gene, and the size of a point is proportional to the average absolute expression level of the gene (log2 CPM) over all samples. **(C)** Parametric enrichment analysis of the DEGs. The analysis was based on the KEGG functional hierarchy. **(D)** Transcription factor (TF) enrichment analysis of DEGs. The analysis was based on ChIP-seq data. The TFs were divided into four groups based on their changes in the enrichment *t* scores.

To further characterize the GPR15L-induced transcriptional response of keratinocytes, we performed RNA-Seq analyses on keratinocytes. The quality of transcriptomes from untreated NHEKs, empty vector-transfected NHEKs, or *C10orf99*-transfected NHEKs (n = 3) was validated by sample clustering analyses (**Figure S3**). We detected 2,186-differentially expressed genes (DEGs) between the *C10orf99*-transfected NHEKs and empty plasmid-transfected NHEKs (**Figure 3B** and **Table S2**). The results of database for annotation, visualization, and integrated discovery (DAVID) and Enrichr gene ontology analyses suggested the highest relation of C10orf99 to "lipid/cholesterol biosynthesis, followed by "cadherin/cell adhesion," and "mitochondrion" (**Table S3**).

The up-regulated DEGs in the GPR15L-expressing NHEKs included a set of genes related to skin inflammation: *CSF1* (encoding M-CSF, macrophage colony-stimulating factor), *IL24* (IL-24), *SOCS3*, *IL6* (IL-6), *MAP3K11, CSF2* (GM-CSF, granulocyte-macrophage colony-stimulating factor), *IL20* (IL-20), *RELB, IL7R* (a receptor for IL-7) and *TSLP* (thymic stromal lymphopoietin), *PTGER4* (prostaglandin E2 receptor EP4), *CIITA*, and *SEMA3E* (**Table 1**). In addition, genes encoding mitochondrial proteins, such as *MARC1*, *PREP*, and *COQ2*, were highly up-regulated (**Table 1**). Of note, some genes whose known functions have not been investigated in keratinocytes are listed in **Table 1**; for example, *MAFF*, a transcriptional cofactor for keratinocyte differentiation (48), was recently reported as a central regulator of atherosclerosis connecting inflammation and cholesterol metabolism (49). *LINC00702*, a long-noncoding RNA, regulates *PTEN via* miR-510, suppresses proliferation and invasion in non-small cell lung cancer (50), and activates the Wnt/β-catenin pathway during meningioma progression (51).

The down-regulated DEGs in the GPR15L-expressing NHEKs included a set of genes related to keratinocyte differentiation and cell adhesion: *CASP14* (coding caspase 14), *LY6D*, *DSG1* (desmoglein 1), *IVL* (involucrin), and *DSC1* (desmocollin 1) (**Table 2**). A series of psoriasis-related genes, such as *S100A8*, *S100A9*, *KRT6A* (keratin 6A), *KRT6B*, *KRT6C*, *KRT16*, and *MMP9* (metalloproteinase 9), were also down-regulated (**Table 2**). In particular, several genes related to barrier formation of the stratum corneum, such as *TGM1* (transglutaminase 1), *SERPINB3* (serpin family B member 3), and *SPINK5* (LEKTI, lympho-epithelial Kazal-type-related inhibitor), were down-regulated (**Table 2**). In addition, a set of genes related to cholesterol and lipid metabolism, such as *HSD3B7*, *PLA2G4E*, *SULT2B1*, and *ALOX15B*, were down-regulated (**Table 2**). Of note, a set of genes related to antiviral response, such as *RAB7B* (Ras-related protein Rab-7b), *IFIT3* (interferon-induced protein with tetratricopeptide repeats 3), *IFIT2*, and *IFIT1*, were included in the down-regulated DEGs (**Table 2**).

These results suggest that GPR15L induces inflammatory gene expression and reduces barrier gene expression in epidermal keratinocytes.

Pathway Analysis: GPR15L Induces Signal Transduction and Reduces Energy and Lipid Metabolism in Keratinocytes

To characterize the impact of GPR15L in keratinocytes more comprehensively, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and transcription factor enrichment analysis of the RNA-Seq data. We compared the results of *C10orf99*-transfected NHEKs to those of the empty vector-transfected NHEKs unless otherwise indicated because lipofection induced an inflammatory response, which was not found in untreated NHEKs, in both groups.

The results of KEGG pathway analysis revealed that genes related to "Signal transduction" and "Signaling molecules and interaction," especially "Mitogen-activated protein kinase (MAPK) signaling pathway," were up-regulated by the expression of GPR15L in NHEKs (**Figure 3C**, and **Table S4**). On the other hand, down-regulation of genes related to "Energy metabolism," "Lipid metabolism," and "Translation" was considered to be a unique change in GPR15L-transfected NHEKs (**Figure 3C**, and **Table S4**). At the same time, the relation to ribosome might not correspond to the gene function in this analysis.

TF Enrichment Analysis: GPR15L Enhances Stress-Inducible Transcription

The results of transcription factor (TF) enrichment analysis indicated average changes in the expression levels of their target genes. We detected 40 TFs that showed significant changes in the TF enrichment *t* scores in untreated and *C10orf99*-transfected NHEKs, and empty vector vs. *C10orf99*-transfected NHEKs (**Figure 3D**).

They were divided into four groups based on their changes in the TF enrichment *t* scores among each condition: *Group 1*, down in untreated, up in empty vector, and more up in C10orf99; *Group 2*, down in untreated, up in empty vector, and less up in C10orf99; *Group 3*, comparable or less in untreated and empty vector, and up in C10orf99; and *Group 4*, up in untreated and down in C10orf99 (**Figure 3D**).

In *Group 1*, there were a set of ubiquitous stress-inducible TFs, such as *EP300* (coding p300), *SMAD3*, *ARNT* (aryl hydrocarbon receptor nuclear translocator), *JUND*, *FOS*, and *HSF1* (heat shock transcription factor 1) (**Figure 3D**). TF p300 is an acetyl-transferase with chromatin binding and subsequent histone acetylation as key functions for the transcriptional activation of stress-inducible genes (52). In addition, *P53*, a tumor suppressor that induces growth arrest or apoptosis *via* chromatin remodeling, was also included in this group. These results indicate that genes that respond to these TFs are strictly regulated in untreated keratinocytes but are released from this regulation under stress conditions by lipofection and their expression is further enhanced by GPR15L expression.

In *Group 2*, *STAT2* and *IRF2* showed a strong increase in the empty vector group compared to the untreated group, despite a weak increase in the *C10orf99*-transfected group (**Figure 3D**). These results indicate that transcription of STAT2- and IRF2-target genes is from a group of lipofection-induced stress responses, which GPR15L negatively regulates. Both STAT2 and IRF2 are negative regulators of type I IFN signaling (53, 54). These results suggest that GPR15L inhibits the transcription of the stress-induced group 2-target genes by direct inhibition of type I IFN signaling or indirect inhibition by enhancing STAT2- or IRF2-mediated transcriptional regulation.

TABLE 1 | Thirty representative DEGs up-regulated in the GPR15L-transfected NHEKs.

Symbol	Fold*	logCPM	LR	P-Value	FDR	Protein	Category**
C10orf99	**326.97**	6.66	588.23	6.09E-130	9.83E-126	chromosome 10 open reading frame 99	–
CTGF	**32.95**	6.05	103.23	2.99E-24	6.03E-21	connective tissue growth factor	KC differentiation
DUSP1	**28.95**	4.95	66.37	3.74E-16	1.89E-13	dual specificity phosphatase 1	Inflammation
SDF4	**20.12**	4.77	49.91	1.61E-12	3.42E-10	stromal cell derived factor 4	KC differentiation
INA	**15.79**	2.03	34.66	3.92E-09	3.42E-07	internexin neuronal intermediate filament protein alpha	Others
MAFF	**14.77**	4.32	99.02	2.50E-23	4.03E-20	MAF bZIP transcription factor F	Others
MARC1	**11.77**	2.00	58.63	1.91E-14	6.28E-12	mitochondrial amidoxime reducing component 1	Mitochondria
LINC00702	**8.74**	2.30	25.52	4.38E-07	2.18E-05	(long intergenic non-protein coding RNA 702)	Others
CSF1	**7.39**	2.51	62.13	3.21E-15	1.20E-12	colony stimulating factor 1	Inflammation
PREP	**6.56**	6.18	69.31	8.40E-17	4.52E-14	prolyl endopeptidase	Mitochondria
COQ2	**6.10**	4.35	20.57	5.74E-06	1.83E-04	coenzyme Q2, polyprenyltransferase	Mitochondria
BTG1	**5.95**	5.18	64.32	1.06E-15	4.87E-13	BTG anti-proliferation factor 1	Others
IL24	**4.61**	5.59	126.04	3.02E-29	1.62E-25	interleukin 24	Inflammation
MMP3	**4.50**	2.67	56.37	6.02E-14	1.77E-11	matrix metallopeptidase 3	KC differentiation
CAPN8	**4.34**	2.48	49.60	1.89E-12	3.96E-10	calpain 8	Inflammation
TRAF4	**4.24**	4.90	87.18	9.90E-21	8.87E-18	TNF receptor associated factor 4	Inflammation
SOCS3	**3.18**	2.18	18.18	2.01E-05	5.26E-04	suppressor of cytokine signaling 3	Inflammation
IL6	**3.05**	2.08	27.16	1.87E-07	1.02E-05	interleukin 6	Inflammation
NGF	**3.04**	2.02	12.49	4.10E-04	6.04E-03	nerve growth factor	Others
MAP3K11	**3.00**	3.59	12.42	4.24E-04	6.17E-03	mitogen-activated protein kinase kinase kinase 11	Inflammation
CSF2	**2.84**	2.22	21.38	3.77E-06	1.30E-04	colony stimulating factor 2	Inflammation
IL20	**2.81**	2.47	26.15	3.16E-07	1.63E-05	interleukin 20	Inflammation
TXNRD3	**2.67**	3.24	32.90	9.70E-09	7.83E-07	thioredoxin reductase 3	Inflammation
RELB	**2.60**	2.45	18.18	2.01E-05	5.26E-04	RELB proto-oncogene, NF-kB subunit	Inflammation
IL7R	**2.50**	3.54	20.23	6.87E-06	2.12E-04	interleukin 7 receptor	Inflammation
MAP2K5	**2.26**	3.81	18.06	2.14E-05	5.49E-04	mitogen-activated protein kinase kinase 5	Inflammation
PTGER4	**2.24**	3.84	12.79	3.49E-04	5.32E-03	prostaglandin E receptor 4	Inflammation
CIITA	**2.22**	2.13	11.18	8.26E-04	1.05E-02	class II major histocompatibility complex transactivator	Inflammation
SEMA3E	**2.15**	3.03	13.26	2.70E-04	4.40E-03	semaphorin 3E	Inflammation
JUN	**2.14**	7.61	28.73	8.34E-08	4.98E-06	proto-oncogene c-Jun	Inflammation

*Shading with orange ≥ 10, light orange ≥ 3. **Colored according to the category.*

In *Group 3*, *SMAD1*, *SMAD2*, *SMAD4*, *JARD2*, and *EZH2* indicate the GPR15L standalone role for their response genes regardless of lipofection-mediated changes (**Figure 3D**). In particular, *JARD2* (JARID2, jumonji and AT-rich interaction domain containing 2) and *EZH2* increased in the enrichment score in the *C10orf99*-transfected group despite decreasing the enrichment score in the empty vector group (**Figure 3D**). JARID2 and EZH2 are components of the polycomb complex 2 (PRC2), a negative regulator of gene transcription *via* chromatin remodeling and histone modification (55). These results suggest that GPR15L may be involved in SMAD-mediated transcription and PRC2-mediated transcriptional regulation, regardless of the stress conditions.

In *Group 4*, only one gene, *SRBP1* (SREBP1, sterol regulatory element-binding protein 1), was included. SREBP1 is a key transcription factor that regulates the expression of genes involved in cholesterol biosynthesis and lipid homeostasis (56). Therefore, SREBP1 is expected to be a major downstream effector of GPR15L in the regulation of lipid metabolism.

Inflammatory Skin Response is Impaired by GPR15L Deficiency and Induced by GPR15L Treatment

We addressed whether GPR15L has a keratinocyte-intrinsic role in the induction of skin inflammation. We stimulated primary-cultured, wild-type mouse keratinocytes with LPS, which *per se* did not induce the GPR15L expression (**Figure 2F**). Stimulation of mouse primary keratinocytes with LPS triggered the transcription of pro-inflammatory mediators, such as IL-1β and IL-6 (**Figure 4A**). However, the expression levels of *Il1b* encoding IL-1β were lower in the GPR15L-deficient keratinocytes than in the wild-type keratinocytes after LPS stimulation (**Figure 4A**). In addition, the induction of *Il6* transcription was almost completely defective in GPR15L-deficient keratinocytes (**Figure 4A**). These results suggest that GPR15L plays an essential role in the LPS-induced response of keratinocytes.

Next, we tested whether GPR15L has a positive role in the induction of skin inflammation in an animal model of psoriasis because the role of GPR15L in psoriatic skin inflammation remains controversial (27, 28). The increased ear thickness in the psoriatic lesion was milder, and the expression levels of inflammatory genes, *Ccl20* and *Defb4*, in the lesional skin were lower, in the GPR15L-deficient mice than in the wild-type mice (**Figure 4B**). These findings suggest that GPR15L contributes to the development of skin inflammation under a certain pathophysiological condition.

Finally, we tested whether the cutaneous administration of synthetic peptides of GPR15L can induce dermatitis *in vivo*. Subcutaneous injection of GPR15L in mouse ears every other day for ten consecutive days induced a significant increase in ear thickness (**Figure 4C**). However, administration of GPR15L lacking a C-terminal region, which is conserved across species, failed to induce skin inflammation (**Figure 4C**), although their dose-dependent increases in ear thickness were not statistically

TABLE 2 | Thirty representative DEGs down-regulated in the GPR15L-transfected NHEKs.

Symbol	Fold*	logCPM	LR	PValue	FDR	Protein	Category**
CASP14	0.22	4.20	45.11	1.87E-11	3.11E-09	caspase 14	KC differentiation
RAB7B	0.22	4.60	49.23	2.28E-12	4.62E-10	RAB7B, member RAS oncogene family	Inflammation
CDHR1	0.23	2.25	23.19	1.47E-06	6.04E-05	cadherin related family member 1	KC differentiation
S100A8	0.27	3.11	55.50	9.34E-14	2.47E-11	S100 calcium binding protein A8	Inflammation
LY6D	0.27	3.80	32.86	9.91E-09	7.96E-07	lymphocyte antigen 6 family member D	KC differentiation
IFIT3	0.28	8.40	102.78	3.75E-24	6.72E-21	interferon induced protein with tetratricopeptide repeats 3	Inflammation
S100A9	0.32	4.64	63.63	1.50E-15	6.55E-13	S100 calcium binding protein A9	Inflammation
KRT6B	0.35	10.66	108.92	1.69E-25	4.54E-22	keratin 6B	KC differentiation
HSD3B7	0.35	2.61	19.35	1.09E-05	3.08E-04	3 beta-hydroxysteroid dehydrogenase type 7	Lipid
TGM1	0.35	7.07	57.17	3.99E-14	1.24E-11	transglutaminase 1	KC differentiation
PLA2G4E	0.35	2.31	16.76	4.25E-05	9.90E-04	phospholipase A2 group IVE	Lipid
TAF9B	0.36	4.62	46.18	1.08E-11	1.87E-09	TATA-box binding protein associated factor 9b	Others
SPRR1B	0.36	5.99	42.34	7.67E-11	1.10E-08	small proline rich protein 1B	KC differentiation
SERPINB3	0.36	3.97	33.52	7.07E-09	5.91E-07	serpin family B member 3	KC differentiation
GJB6	0.37	3.86	36.88	1.25E-09	1.37E-07	gap junction protein beta 6	KC differentiation
GJB2	0.37	8.41	72.54	1.64E-17	1.20E-14	gap junction protein beta 2	KC differentiation
MMP9	0.38	3.22	23.99	9.68E-07	4.28E-05	matrix metallopeptidase 9	KC differentiation
IFIT2	0.39	7.64	27.60	1.49E-07	8.21E-06	interferon induced protein with tetratricopeptide repeats 2	Inflammation
SULT2B1	0.39	4.22	20.39	6.31E-06	1.97E-04	sulfotransferase family 2B member 1	Lipid
ALOX15B	0.41	5.78	47.39	5.81E-12	1.07E-09	arachidonate 15-lipoxygenase, type B	Lipid
DSG1	0.41	3.11	18.15	2.04E-05	5.30E-04	desmoglein 1	KC differentiation
A2ML1	0.42	6.29	48.60	3.14E-12	6.02E-10	alpha-2-macroglobulin like 1	KC differentiation
IVL	0.42	4.26	25.99	3.42E-07	1.75E-05	involucrin	KC differentiation
KRT6C	0.42	9.59	69.90	6.25E-17	3.47E-14	keratin 6C	KC differentiation
IFIT1	0.43	8.59	31.23	2.29E-08	1.64E-06	interferon induced protein with tetratricopeptide repeats 1	Inflammation
KRT16	0.43	9.75	50.04	1.51E-12	3.25E-10	keratin 16	KC differentiation
KRT6A	0.46	13.58	72.19	1.96E-17	1.35E-14	keratin 6A	KC differentiation
SPINK5	0.49	2.76	12.17	4.87E-04	6.86E-03	serine peptidase inhibitor, Kazal type 5	KC differentiation
KRT15	0.50	6.94	29.06	7.00E-08	4.26E-06	keratin 15	KC differentiation
DSC1	0.50	2.28	8.30	3.97E-03	3.39E-02	desmocollin 1	KC differentiation

*Shading with blue ≤ 0.3, light blue ≤ 0.4. ** Colored according to the category.*

different. The dose-dependent increase in IL-1β gene expression levels in the GPR15L-treated skin was statistically significant (**Figure 4C**). These results suggest that GPR15L is involved in the proinflammatory response of keratinocytes and the development of inflammatory skin conditions.

DISCUSSION

We propose a new role for GPR15L in the proinflammatory response and late differentiation of keratinocytes, beyond its previously reported role, such as a chemoattractant (5, 6, 26), and an antimicrobial peptide (8). Our results highlight a keratinocyte-intrinsic role for GPR15L. In NHEK or primary cultured mouse keratinocytes, GPR15L increased the gene expression levels of proinflammatory molecules and reduced the expression levels of barrier molecules. In contrast, GPR15L-deficient keratinocytes showed a defective response to LPS. Previous findings and our results indicate that GPR15L is a highly inducible peptide on a variety of skin inflammatory disorders. These results suggest that GPR15L has new roles in the protective response and barrier formation of the epidermis.

The findings of the new role of GPR15L prompted us to consider whether GPR15L drives these roles in keratinocytes in a GPR15-dependent or a GPR15-independent manner. It has been shown that expression of GPR15 is selective in T lymphocytes (22, 23) and detectable in macrophages, monocytes, and neutrophils (57). In contrast, we could not detect GPR15 gene expression by RT-qRCR and RNA-Seq analyses in NHEK and mouse primary cultured keratinocytes but detected it in skin tissues. We cannot rule out the possible involvement of the GPR15 pathway in keratinocytes in an autocrine manner. However, these findings suggest the GPR15-independent roles for GPR15L in keratinocytes.

Previous studies and our present study proposed possible GPR15-independent roles for GPR15L. It has been demonstrated that antibody-mediated skin inflammation is alleviated in GPR15-deficient mice (58) while imiquimod-induced psoriatic dermatitis is not blunted (28). However, treatment of GPR15L-siRNA reduced the skin inflammation in the imiquimod-induced psoriatic dermatitis model at the treated sites, and the treatment with lentiviral GPR15L overexpression increased the disease severity in this animal model (27). Consistently, we demonstrated that the development of imiquimod-induced psoriatic dermatitis was milder in GPR15L-deficient mice. In addition, the results of TF enrichment analysis in the present study suggested a broader role for GPR15L in keratinocyte transcriptional responses. It is unclear whether all these responses are driven by the known GPR15 pathway reducing cyclic AMP (cAMP) production *via* Gi/o-coupled (59). These findings suggest that GPR15L may contribute to skin inflammation and barrier regulation beyond the GPR15L–GPR15 axis.

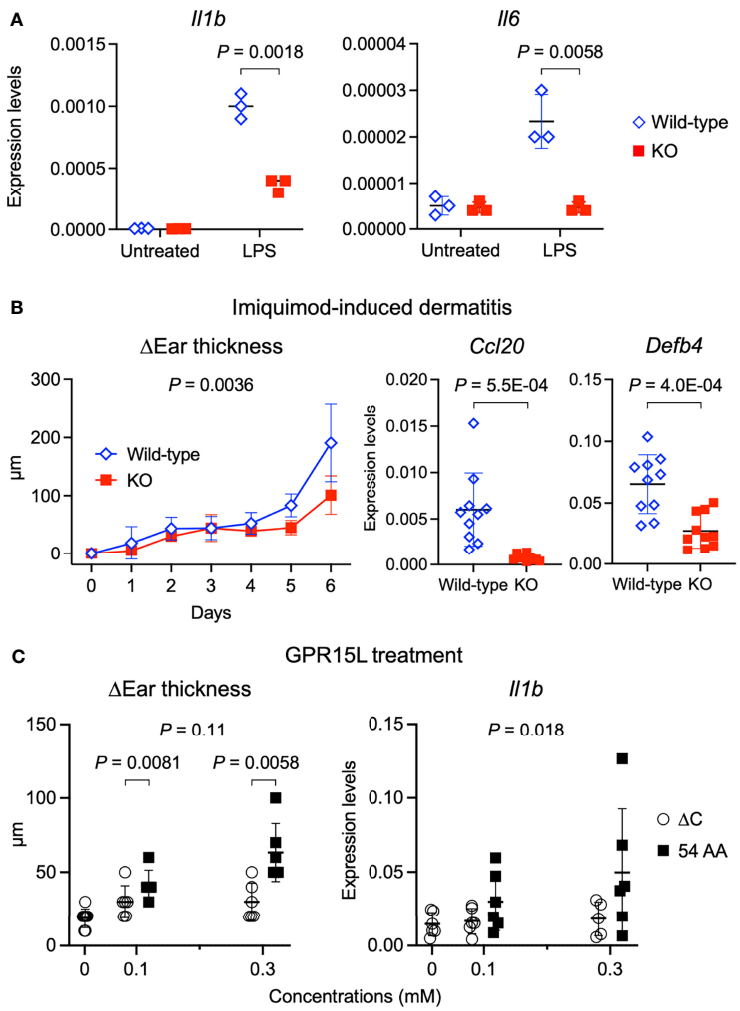

FIGURE 4 | GPR15L deficiency impairs inflammatory responses of keratinocytes and in the skin. **(A)** Quantitative RT-PCR analysis of mRNA levels in primary cultured keratinocytes from GPR15L-deficient (KO) mice or their +/+ littermates (wild-type) with or without LPS stimulation. Results were normalized to *Gapdh* expression (error bars, SD; n = 3). **(B)** Time course of changes in the ear thickness (n ≥ 5 per group) from wild-type or GPR15L-deficient (KO) mice treated daily with or without topical imiquimod for six consecutive days. The thickness was measured daily before treatment. Values are shown as means ± SD. The difference in two curves were evaluated by ANCOVA. *Ccl20* and *Defb4* mRNA levels in the lesional skin from wild-type or GPR15L-deficient (KO) mice treated daily with topical imiquimod for six consecutive days. RT-PCR results were normalized to *Gapdh* expression (error bars, SD; n ≥ 6 per group). We performed RT-PCR experiments with 6-month-old GPR15L deficient mice and wild-type C57BL/6 mice that were co-housed for 1 week before the experiments. The results were reproducible in the two experiments. **(C)** Changes in the ear thickness and *Il1b* mRNA levels analyzed by quantitative RT-PCR (n ≥ 5 per group) from mice intracutaneously treated with GPR15L lacking the C-terminus (ΔC) or the full-length (54 AA) every other day for ten days. RT-PCR results were normalized to *Gapdh* expression (error bars, SD; n ≥ 5 per group). The differences in dose-dependent curves between two groups was evaluated by ANCOVA and the differences in each dose between two groups were evaluated by unpaired t-test.

Our results suggest that the induction of GPR15L is involved in the impaired barrier during skin inflammation. In contrast, it is not essential for the development and maintenance of steady states. Our *in vitro* study demonstrated the impaired expression of the barrier proteins by GPR15L. Still, we failed to reveal an increase in GPR15L-null keratinocytes. These findings are consistent with the normal skin development in GPR15L-deficient mice (5). However, the extremely lower expression levels of GPR15L in healthy skin compared to inflamed skin suggest that GPR15L expresses and governs its major functions under stressful conditions. Its activity is strictly controlled in healthy states.

The precise mechanism for the induction of GPR15L expression in keratinocytes during skin inflammation remains to be elucidated. We demonstrated that GPR15L is highly inducible during skin inflammation in both mice and humans, regardless of the immune type. Our *in vitro* results suggest that the IFN-γ pathway is a possible upstream inducer of GPR15L. Activation of the IFN-γ pathway triggers the transcription of a series of target genes through activation of the canonical NF-κB pathway (60). Consistently,

there are several κB sites in the promoter region and the first intron of *2610528A11Rik*. However, other stimuli that activate the canonical NF-κB pathway, such as LPS, failed to trigger GPR15L transcription in keratinocytes (**Figure 2F**). Other mechanisms or common triggers under stressful conditions may be involved in inducible GPR15L expression at the outermost surface of the body. Our findings also stimulate us to investigate the dynamics of GPR15L expression levels during pathological and protective conditions, such as superficial skin infections and wound healing.

The GPR15L–SREBP1 axis may be critical in the crosstalk between lipid metabolism and inflammation, the regulation of which has been proposed for a long time (61–63). We previously demonstrated that topical activation of cholesterol metabolism *via* the liver X receptor (LXR) alleviates imiquimod-induced psoriatic dermatitis. In contrast, LXR expression levels are decreased in the lesional skin and human psoriasis (64). LXR binds to the SREBP1c promoter and initiates SREBP1c transcription (65). In activated $CD4^+$ T cells, the T cell receptor/CD28–mammalian target of rapamycin complex 1 (mTORC1) signaling axis controls fatty acid uptake and biosynthesis through the induction of PPARγ and the activation of SREBP1 (66). In the kidney, SREBP1 regulates transforming growth factor-β (TGF-β) activity *via* a positive feedback loop with the TGF-β–Smad3 pathway in mediating fibrosis (67). Therefore, it would be of great interest to know whether GPR15L addresses the LXR pathway and TGFβ–Smad3 *via* GPR15, unidentified receptors, or intracellularly.

There are a few limitations in the present study. First, our findings *in vivo* are not only explained by the new roles of GPR15L in keratinocytes found in the present study *in vitro*. Specifically, blunted imiquimod-induced dermatitis in GPR15L-deficient mice, or the GPR15L peptide injection-induced dermatitis, could in part be explained by the known role of GPR15L in the GPR15L–GPR15 axis and subsequent recruitment of dendritic epidermal T-cells (5, 6, 26), or in keratinocyte proliferation (27). Second, it remains unclear whether inflammation, or barrier disruption, triggers GPR15L expression in the epidermis in stressed situations *in vivo*, because it is very difficult to distinguish the effect of barrier disruption from subsequent inflammation (or vice versa). This issue is a shared research-associated conundrum in the field of skin barrier and protective response; and hence should be addressed with elegant and elaborate strategies in the future.

In conclusion, we found a new role for GPR15L, the product of a former orphan gene, in regulating proinflammatory response and late differentiation of keratinocytes. We propose that cellular stress, during the breakdown of skin homeostasis, triggers the expression of GPR15L, which contributes to the organization of the protective response and subsequent pathological conditions of the skin. Therefore, our findings suggest that GPR15L production is a key event in various types of skin inflammation and a potential therapeutic target.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. RNA-seq data were deposited in the Gene Expression Omnibus (GEO) database; accession number GSE189751.

ETHICS STATEMENT

All procedures for animal experiments were reviewed and approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (approval Med Kyo 20532) and performed according to the institutional guidelines. The study using human specimens was approved by the Graduate School of Medicine, Kyoto University (R0743). Written informed consent was obtained from all individuals.

AUTHOR CONTRIBUTIONS

TD initiated and supervised the research, obtained funding, performed experiments, and wrote the manuscript. YN, HD, and RM performed the experiments. SatN, VN, RMT, and EK performed the transcriptome analyses. SaeN, TF, EG-Y, and TL predicted GPR15L gene properties. OD, PW, and BR provided methodological support. KK obtained funding and supervised the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.825032/full#supplementary-material

Supplementary Table 1 | Primer sequences used for the quantitative RT-PCR analyses.

Supplementary Table 2 | DEGs between the *C10orf99*-transfected NHEKs and empty plasmid-transfected NHEKs (false discovery rate: FDR < 0.05).

Supplementary Table 3 | The results of database for annotation, visualization, and integrated discovery (DAVID) and Enrichr gene ontology analyses.

Supplementary Table 4 | The results of parametric enrichment analysis based on the KEGG functional hierarchy using the GAGE algorithm.

Supplementary Figure 1 | (A) Quantitative RT-PCR analysis of GPR15L mRNA levels in the skin from newborn mice. Results were normalized to *Gapdh* expression (error bars, SD; n = 3 per group). **(B)** GPR15L protein expression in the upper dermis of lesional skin from a patient with psoriasis. Representative results from the five cases are shown (a scale bar, 20 μm).

Supplementary Figure 2 | (A) Quantitative RT-PCR analysis of mRNA levels in the epidermis of newborn mice. Results were normalized to *Gapdh* expression

(error bars, SD; n = 5 per group). **(B)** Quantitative RT-PCR analysis of IL-1β mRNA levels in mouse primary cultured keratinocytes with indicated stimulations. Results were normalized to *Gapdh* expression (error bars, SD; n = 3 per group).

Supplementary Figure 3 | Sample clustering analyses of the RNA-Seq data. **(A)** Hierarchical clustering. **(B)** Multidimensional scaling analysis. **(C)** Principal component analysis (PCA).

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REVIEW
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Epidermal Immunity and Function: Origin in Neonatal Skin

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The fascinating story of epidermal immunity begins *in utero* where the epidermal barrier derives from the ectoderm and evolves through carefully orchestrated biological processes, including periderm formation, keratinocyte differentiation, proliferation, cornification, and maturation, to generate a functional epidermis. Vernix caseosa derives from epidermal cells that mix with sebaceous lipids and coat the fetus during late gestation, likely to provide conditions for cornification. At birth, infants dramatically transition from aqueous conditions to a dry gaseous environment. The epidermal barrier begins to change within hours, exhibiting decreased hydration and low stratum corneum (SC) cohesion. The SC varied by gestational age (GA), transformed over the next 2–3 months, and differed considerably versus stable adult skin, as indicated by analysis of specific protein biomarkers. Regardless of gestational age, the increased infant SC proteins at 2–3 months after birth were involved in late differentiation, cornification, and filaggrin processing compared to adult skin. Additionally, the natural moisturizing factor (NMF), the product of filaggrin processing, was higher for infants than adults. This suggests that neonatal skin provides innate immunity and protection from environmental effects and promotes rapid, continued barrier development after birth. Functional genomic analysis showed abundant differences across biological processes for infant skin compared to adult skin. Gene expression for extracellular matrix, development, and fatty acid metabolism was higher for infant skin, while adult skin had increased expression of genes for the maintenance of epidermal homeostasis, antigen processing/presentation of immune function, and others. These findings provide descriptive information about infant epidermal immunity and its ability to support the newborn's survival and growth, despite an environment laden with microbes, high oxygen tension, and irritants.

Keywords: epidermal barrier, immunity, stratum corneum, neonatal, skin, proteomics, genomics, vernix caseosa

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1 INTRODUCTION

Epidermal immunity is prominent in the major global health issue of high neonatal mortality due to sepsis. Among nearly three million live births in 14 countries from 1979–2019, there were 29,608 sepsis cases, corresponding to 2,842 in 100,000 live births and 17.9 percent mortality (Fleischmann et al., 2021). Unfortunately, the rate was 1.4 times higher for the most recent decade (2009–2018). The cost of neonatal sepsis was 469 billion US dollars, as of 2014 (Ranjeva et al., 2018). Skin-based infant care practices, including kangaroo mother care where there is uninterrupted contact with the

infant and mother (chest to chest) and only breastfeeding for nutrition (Conde-Agudelo and Diaz-Rossello, 2016), newborn umbilical cord treatment with chlorhexidine (Imdad et al., 2013), and repetitive application of topical emollients, such as sunflower oil, in hospitalized premature infants (Darmstadt et al., 2004; Darmstadt et al., 2005; Darmstadt et al., 2008; Darmstadt et al., 2014), have reduced infection rates compared to no intervention.

Despite advances in medical care, premature birth remains high, at about 11% of births worldwide (WHO, 2012). Late-onset sepsis is the cause of mortality and morbidity in this population (Stoll et al., 2002). Clinical practice changes, specifically the implementation of evidence-based catheter insertion practices in 22–29 weeks gestation infants, have reduced catheter-related bloodstream infections significantly, but to a lesser degree than expected (Kaplan et al., 2011). Poor skin integrity may be a major predisposing factor for neonatal sepsis. The development of interventions to enhance stratum corneum (SC) formation is a global priority (Lawn et al., 2006) and essential for reducing the consequences of epidermal barrier immaturity (Rutter, 1996; 2000).

In this review, we discuss epidermal immunity from its origins, namely during fetal development, late gestation, at birth, and over the first few months of life. The inherent benchmark for epidermal immunity is adult epidermis, viewed as a stable, steady-state condition. The intrinsic self-renewal feature distinguishes the skin and, thereby, the dependence on the provision of continual immune function. The newborn infant provides a truly unique opportunity to observe its rapid evolution, namely, during adaptation from a water-based vessel to the gaseous, somewhat hostile terrestrial environment. We consider the role of vernix caseosa in this process and investigate epidermal immunity from the origin, using proteomic and genomic techniques and discuss the implications for improving clinical outcomes.

2 FETAL SKIN DEVELOPMENT

A single epithelial layer forms from ectoderm during embryogenesis under the influence of fibroblast growth factors, bone morphogenic proteins, and Notch signaling (Fuchs, 2007). A basal epidermal layer and one periderm layer have been created by gestational week 4 (Lane, 1986). The periderm covers the basal layer and forms tight junctions during fetal development. Melanocytes appear in the basal layer during weeks 5–8. Three epidermal layers appear by weeks 8–11. Proliferation and maturation of basal keratinocytes produce the spinous layer beneath the periderm and begin to stratify (Koster and Roop, 2007) (Blanpain and Fuchs, 2009). Four to five epidermal layers appear throughout gestational weeks 16–23. When the periderm regresses around week 23, fetal suprabasal cells adhere to other cells to create a barrier structure (Sumigray and Lechler, 2015). By 26 gestational weeks, the epidermis consists of one basal layer, 2-3 spinous layers, one granular layer, and 5-6 stratum corneum layers (Holbrook and Odland, 1975). Eight distinct phases of differentiation occur over gestational weeks 5 and 26 (Holbrook and Odland, 1975).

Hair follicles begin to form throughout weeks 9–14. In the second of eight phases, the epidermis begins to thicken in certain regions, giving rise to hair pegs (stage 3) (Hu et al., 2018). Fibroblasts from the dermis collect at the lower end of the peg to generate a sphere-shaped dermal papilla. The root sheath moves up the hair follicle and the follicle grows in a downward direction in stage 6. The hair extends out through the skin surface in stage 6. The sebaceous glands develop near the upper hair follicle at gestational weeks 13–14. Eccrine glands develop at about the same time and continue to develop through gestational week 24 (Fu et al., 2005).

Leukocytes that are positive for the human leukocyte antigen dendritic major histocompatibility complex cell surface receptor (HLA-DR) develop in the fetus about gestational week 5 and in the skin at week 7 (Schuster et al., 2009). Predecessors of Langerhans cells (LC) emerge and produce antigens at gestational weeks 7–9 (Foster et al., 1986; Fujita et al., 1991). Adult LC is characterized by the presence of Birbeck granules, CD207/langerin, and CD1a but these features are not seen in the fetus until gestational week 11 (Foster et al., 1986; Schuster et al., 2009). Mast cells appear only after gestational week 11 and then increase in the second trimester (Schuster et al., 2012).

3 BIRTH

Few events are as dramatic and "life changing" as birth, when the human infant abruptly transitions from warm, wet, nurturing, serene *in utero* conditions to a cooler, dry, gaseous, microbe laden environment. The infant immediately relies on a robust innate immune system, provided by the epidermal barrier, and begins self-sufficiency with air-breathing, nutrient intake, and growth. Epidermal differentiation generates the stratum corneum (SC), the essential innate immune interface between the living infant and the external environment. For the full-term infant, the SC provides 1) a barrier to water loss from within and irritants from outside, 2) thermal regulation, 3) sensation and tactile discrimination, 4) an acid mantle, 5) immunosurveillance and infection control and 6) tractability to mechanical trauma.

This extraordinary process begins from ~5 to 26 weeks gestation when the periderm shields the epidermis from amniotic fluid. At ~ 23.5 weeks, the periderm is no longer present and keratin-containing cells are noted in the interfollicular spaces and along the hair follicle (Holbrook and Odland, 1975). Starting at weeks 18–19, the stratum corneum barrier forms, that is, cornification of epidermal corneocytes takes place. Initially, SC formation occurs around/along the hair follicle, then on the head (week 23), and later on the abdomen (week 25) (Hardman et al., 1999).

4 VERNIX CASEOSA

4.1 Vernix Origins and Formation

Histological and microscopic examination of vernix caseosa found ovid or polygonal cells without nuclei or organelles while some had nuclear ghosts (Agorastos et al., 1988; Pickens

et al., 2000). The cellular acid phosphatase activity was variable, from none to very high. The cytoplasm and cell membranes showed no alkaline phosphatase activity. The cells were thin (1–2 μm) and differed from regular to irregular with 5-6 sides with microvilli projections on the surface. The degree of keratinization varied, suggesting that they were from the outermost fetal stratum corneum and deeper levels, that is, less mature keratinocytes. Alternatively, the cells may originate during the transition from periderm to keratinized epidermis. There were few keratin filaments, lacking in orientation, and no evidence of desmosomes. In micrographs, the lipids between cells were generally amorphous, with occasional lamellae (Pickens et al., 2000). The cell diameter was ~40 μm, larger than stratum corneum cells, perhaps due to absorption of water from amniotic fluid, and individual cellular hydration varied (Pickens et al., 2000).

Vernix caseosa is an amorphous, white waxy mixture of water-containing cells covered by a mixture of lipids (Pickens et al., 2000; Rissmann et al., 2006). It may appear on the fetal eyebrows at gestational week seventeen. Over time, it covers the fetal skin surface, advancing from head to toe and back to front (Visscher et al., 2005). Placental or hypothalamic corticotropic-releasing factors (CRF) may signal the pituitary gland to release adrenocorticotropic hormone (ACTH), causing the adrenal gland to release androgenic steroids (Zouboulis et al., 2003). They become active androgens and function within the sebaceous gland. Hair follicles have a local hypothalamic-pituitary-adrenal-like axis (Ito et al., 2005) that may be involved in vernix formation. Several vernix lipid types are also produced by the sebaceous glands, namely triglycerides, wax esters, and squalene (Nicolaides et al., 1972; Rissmann et al., 2006). Fetal cells possibly originate from the hair follicles (Kurokawa et al., 2009), mix with sebaceous lipids, extrude through the hair shaft, and continue to form and spread over the interfollicular epidermis during latter gestation (Hardman et al., 1998). Vernix cells may also come from the infundibular portion of sebaceous glands (Rissmann et al., 2006). Vernix films (i.e., spread on a porous substrate) *in vitro* are hydrophobic, due to the lipid cover on the hydrated cells (Youssef et al., 2001).

Vernix lipids cover the hydrated vernix cells to create a hydrophobic coating during latter gestation, thereby protecting the underlying fetal epidermis from exposure to amniotic fluid (Youssef et al., 2001). Vernix films are non-occlusive and permit water vapor transport through them (Tansirikongkol et al., 2007a). *In utero*, cornification of the fetal epidermis is incomplete thereby permitting a high water flux potential driven by osmotic gradients. Water gradients occur in skin homeostasis. Specifically, the SC exhibits a water gradient with higher hydration in the lower layers and decreased hydration at the skin surface (Warner et al., 1988; Caspers et al., 2003; Verdier-Sevrain and Bonte, 2007). The ability of the SC barrier to recover after the damage is due in part to the transepidermal water gradient and subsequently increased synthesis of DNA and lipids (Proksch et al., 1991; Denda et al., 1998a; Denda et al., 1998b; Fluhr et al., 1999). Vernix may serve as a semi-regulated barrier and/or physiological gradient for transepidermal water and nutrients *in utero*. This process, in turn, prompts epidermal cornification by increasing the synthesis of DNA and lipids.

As full-term gestation approaches, the mature fetal lungs secrete phospholipid surfactants that cause some of the vernix to detach from the skin surface (Narendran et al., 2000). This process causes the amniotic fluid to become cloudy. The infant swallows the amniotic fluid and, thereby, vernix provides nutrients to prepare the intestine for extra-utero feeding.

4.2 Vernix Composition and Function

Vernix is composed of ~80% water, associated with the cells, 10.3% protein, and 9.7% lipids (Pickens et al., 2000; Hoath et al., 2006). The non-lamellar lipid mixture that covers the flattened vernix cells comprises both non-polar, as the predominant fraction, and polar lipids, including fatty acids, ceramides, squalene, cholesterol, wax esters, and triglycerides (Rissmann et al., 2006). Epidermal barrier lipids, that is, cholesterol, fatty acids, and ceramides compose 10–30% of the vernix lipid fraction (Hoeger et al., 2002; Rissmann et al., 2006), with ceramide lipids comprising 4.9% of the total vernix lipid fraction (Rissmann et al., 2006). Vernix ceramide profiles (weight percent of total ceramides) compared to adult stratum corneum (SC) ceramides are in **Figure 1A** (Rissmann et al., 2006). Ceramide AH (AH contains α-hydroxy acids and sphingosines) was the most abundant in vernix, followed by ceramide NS (NS contains non-hydroxy fatty acids and sphingosines), ceramide AS/NH (AS contains α-hydroxy fatty acids and sphingosines and NH contains non-hydroxy fatty acids and 6-hydroxysphingosines) and ceramide EOS (EOS contains ester-linked fatty acids, ω-hydroxy fatty acids, and sphingosine). The distributions were similar while relative ceramide levels were higher in vernix compared to adult SC, except for ceramides AP (AP contains α-hydroxy fatty acids and phytosphingosine) and NP (NP contains non-hydroxy fatty acids and phytosphingosine) that were lower in vernix. The ceramide profiles in vernix, fetal SC (16–20 weeks GA), mid-gestational SC (23–25 weeks GA), infant SC (1–11 months), and child 1-6 years were compared, shown in **Figure 1B** (Hoeger et al., 2002) and the relative abundance of ceramides was similar to that of Rissmann (Rissmann et al., 2006). The vernix ceramide AH level was higher and vernix NP and NS levels were lower than those of infants aged 1–11 months ($p < 0.05$). Ceramide levels in vernix and premature infant stratum corneum at 23–25 gestational weeks were comparable, except for ceramide AP that was higher in vernix ($p < 0.05$). Collectively, these results reveal the uniqueness but also the similarities between vernix and SC, as well as the dynamic, variable nature of SC ceramide composition over time.

The fatty acid profile of vernix lipids includes branched-chain fatty acids (BCFA), a species that is not present in epidermal barrier lipids, as well as saturated, mono-unsaturated, and poly-unsaturated fatty acids (**Table 1**). Additionally, the fatty acid distribution in vernix lipids differed significantly by GA. Premature infants (29–36 wks GA) had significantly higher saturated and poly-unsaturated FAs and lower BCFA and mono-unsaturated FAs than full-term infants (≥37 wks GA) (Li et al., 2021).

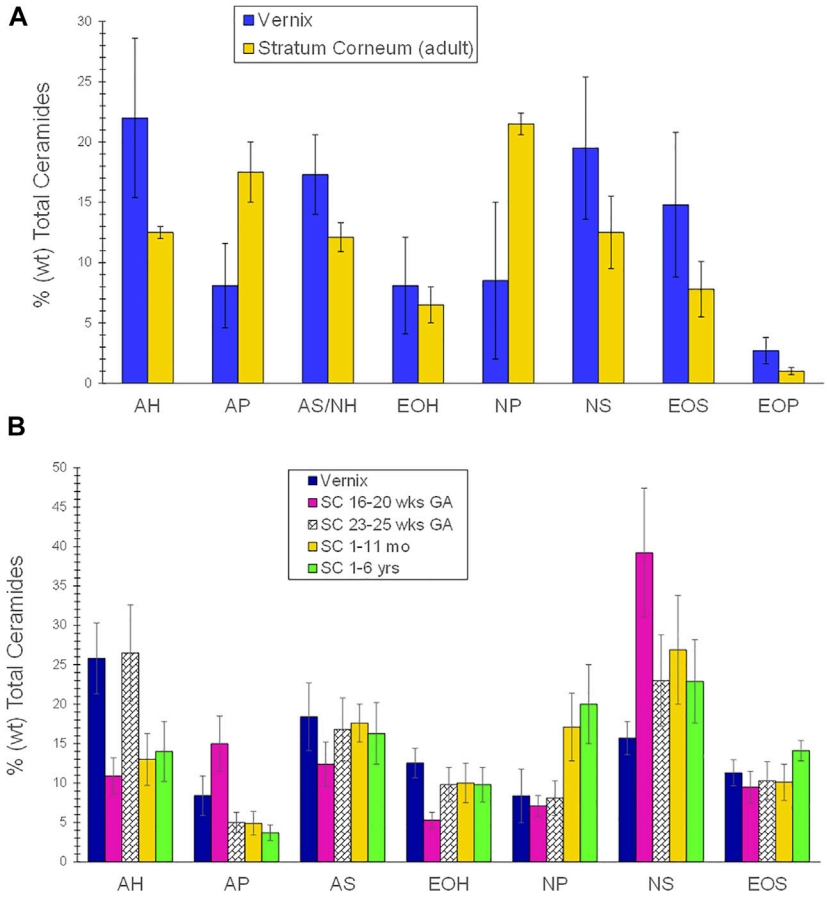

FIGURE 1 | (A) A comparison of the ceramide profile of vernix caseosa and adult stratum corneum. The ceramide profile of vernix was compared to that of adult stratum corneum. Samples of vernix caseosa were collected at birth from full-term infants and adult tissues were obtained during cosmetic surgery (Rissmann et al., 2006). The lipids were extracted, separated by high-performance thin layer chromatography, and quantified. Values are given as percent weight as mean and ± standard deviations. Ceramide AH (AH contains α-hydroxy acids and sphingosines) was the most abundant, followed by NS (NS contains non-hydroxy fatty acids and sphingosines), AS/NH (AS contains α-hydroxy fatty acids and sphingosines and NH contains non-hydroxy fatty acids and 6-hydroxy sphingosine and EOS (EOS contains ester-linked fatty acids, ω-hydroxy fatty acids, and sphingosine). The relative ceramide levels were higher in vernix compared to adult SC except for ceramides AP (AP contains α-hydroxy fatty acids and phytosphingosine) and NP (NP contains non-hydroxy fatty acids and phytosphingosine) that were lower in vernix. The ceramide distributions were similar in vernix and adult SC. Statistical comparisons were not reported. **(B)** A comparison of the ceramide profiles in vernix, fetal stratum corneum (16–20 weeks GA), mid-gestational SC (23–25 weeks GA), infant SC (1–11 months), and child SC (1–6 years). Vernix caseosa from healthy full-term infants, tissue samples that required surgery, and fetal tissue from spontaneous abortions were quantified by high-performance thin layer chromatography (Hoeger et al., 2002). Ceramide (AH) was the highest fraction, followed by AS, NS, EOS, and EOH, with AP and NP being the lowest species. Vernix ceramide AH was significantly higher and vernix NP and NS were significantly lower than for infants of 1–11 months ($p < 0.05$). Ceramide levels in vernix and premature infant stratum corneum at 23–25 GA were comparable, except for ceramide AP that was higher in vernix ($p < 0.05$).

TABLE 1 | Profile of the fatty acid classes in vernix caseosa from premature and full-term infants (Li et al., 2021).

Species	Premature infants (% weight)	Full-term infants (% weight)
Saturated fatty acids	61.2 ± 1.22	35.8 ± 3.23
Monounsaturated fatty acids	3.0 ± 0.31	16.6 ± 5.09
Polyunsaturated fatty acids	10.5 ± 0.42	5.5 ± 0.02
Branched-chain fatty acids	25.3 ± 0.51	43.0 ± 1.87

Understanding the composition of vernix, which the infant ingests before birth, could have important pathophysiological consequences. For example, 20–30% of premature infants are affected by necrotizing enterocolitis, a potentially fatal intestinal inflammatory condition. In an animal model, dietary supplementation with 20% of vernix-type BCFAs reduced necrotizing enterocolitis by 50%, increased the intestinal microbiota diversity, and increased IL-10 three-fold versus the

control whose diet was lacking in BCFA, indicating a protective role for this fatty acid in newborn intestines (Ran-Ressler et al., 2011). Additionally, data from an *in vitro* model demonstrated that induction of inflammation in intestinal cells with lipopolysaccharide (LPS) was associated with, a 20% reduction in cell viability was observed (Yan et al., 2018). However, when cells were treated with either vernix monoacylglycerides or vernix free fatty acids, the cell viability was restored. This study showed that the intestinal cells assimilated the BCFAs after treatment with vernix lipids. A putative role in prevention was suggested by experiments in which cells that were pretreated with vernix monoacylglycerides or vernix free fatty acids, followed by LPS exposure, expressed lower levels of IL-8 and NF-kB, suggesting that pretreatment with BCFA attenuated LPS-induced inflammation.

How epidermal lipids might mediate skin inflammation and immune function is unknown, but the mechanisms could include keratinocyte production of antimicrobial compounds, fibroblast migration, regulation of the rate of wound healing, and/or regulation of dendritic cells, for example, antigen uptake and activation of T cells (Kendall and Nicolaou, 2013). The impact of gestational age and gender has been studied with vernix samples from 156 infants in 3 GA categories, that is, 36–38 weeks, 39–40 weeks, and 41–42 weeks, revealing 54 lipid mediators (coefficient of variation <30% and in >70% of samples (Checa et al., 2015). Three classes of lipids were identified, namely, sphingolipids (n = 23), oxylipins (n = 43) and endocannabinoids (n = 14), and gender differences were noted (Checa et al., 2015). Sphingolipids are of interest for their potential role in skin barrier integrity and function, particularly to facilitate skin maturation and immunity in very premature infants (i.e., <28 weeks GA) who lack exposure to vernix. Within the sphingolipids, sphingomyelins increased with gestational age. The ceramide/sphingomyelin ratio (corrected for gender and maternal lifestyle) was significantly higher with increasing gestational age for chain lengths 12:0, 16:0, 18:0, 18:1, 24:0, and 24:1.

It is noteworthy that vernix from healthy full-term infants contained cytokines TNFα, IL8, IL1α, IL1β, IL6, MCP1, and IP10 (Narendran et al., 2010). The levels were substantially lower than in skin surface (stratum corneum) samples from premature infants, full-term infants, and adults (Narendran et al., 2010). This finding is consistent with the reduction of IL-8 and NF-kB in LPS-mediated intestinal cells (Yan et al., 2018). IL1α from the vernix covering may accelerate SC barrier maturation after birth (Jiang et al., 2009).

4.3 Vernix Lipids, Inflammation, and Filaggrin

Qiao et al. investigated the effects of vernix lipids (n = 10 infants) on the expression of a critically important skin protein, filaggrin (FLG), and markers of inflammation in normal human epidermal keratinocytes *in vitro* (Qiao et al., 2019). Inflammation, evidenced by increased amounts of cytokines tumor necrosis factor alpha (TNFα) and thymic stromal lymphopoietin (TSLP) was provoked by exposure of keratinocytes to polyinosinic:polycytidylic acid (poly I:C), a synthetic double-stranded RNA. This resulted in a dose-dependent reduction in cell viability (Qiao et al., 2019). Exposure to vernix lipids attenuated TNFα and TSLP levels, further supporting the antiinflammatory potential of vernix. In this work, keratinocytes treated with Poly I:C decreased FLG expression while the addition of 25, 50, and 100 μg/ml of vernix lipids increased filaggrin (FLG) expression relative to cells that were not treated with Poly I:C. (Qiao et al., 2019). In contrast, FLG expression decreased in keratinocytes that were treated with poly I:C. This work may have broader relevance to newborn infants as FLG is a precursor of the Natural Moisturizing Factor. FLG mutations are implicated in atopic dermatitis. Further investigation of the effect of vernix on atopic dermatitis is warranted.

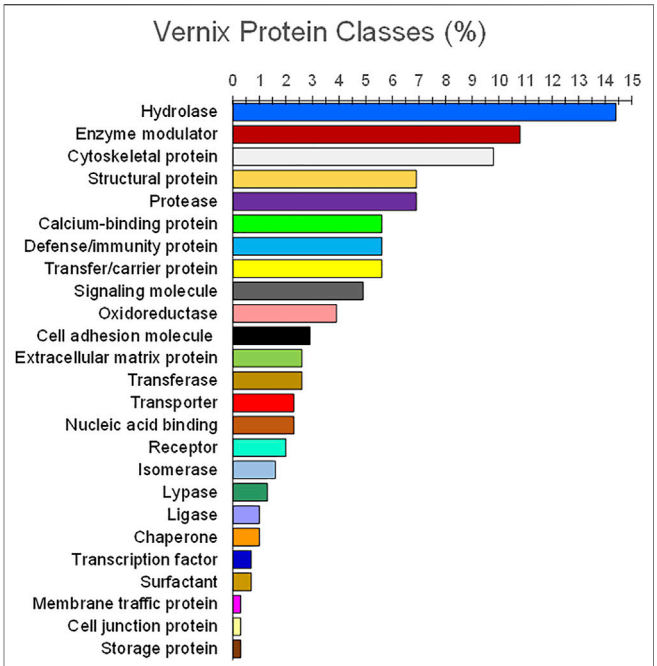

FIGURE 2 | Functional classes of vernix proteins. Vernix was extracted and digested with trypsin, quantified by liquid chromatography–tandem mass spectrometry, and analyzed against the Swiss-Prot protein database (Holm et al., 2014). Proteins (n = 203) for $p < 0.05$ and belonging to 25 functional classes were identified (percent by weight). Hydrolases, proteases, and enzyme modulators encompassed 29, 22, and 22 proteins, respectively, with 11 proteins classified as immunity/defense.

4.4 Vernix Proteins

Analysis of vernix proteins with 2D gel electrophoresis identified 41 proteins, including 16 associated with innate immunity (Tollin et al., 2006). They were: UBB (ubiquitin), S100A8, S100A9, S100A7, LYZ, NGAL, H2AC11, H2BC1, RNASE7, SLPI, CAMP (LL-37), MUC7, BPIFA1, PSMB2, ARG1, and SOD1. Additionally, the first 12 genes (UBB to MUC7) demonstrated antimicrobial properties (Tollin et al., 2006). Vernix contains antimicrobial proteins lysozyme and lactoferrin, localized in "granules" that may facilitate "quick release" in the presence of infectious agents (Akinbi et al., 2004). Vernix decreased specific

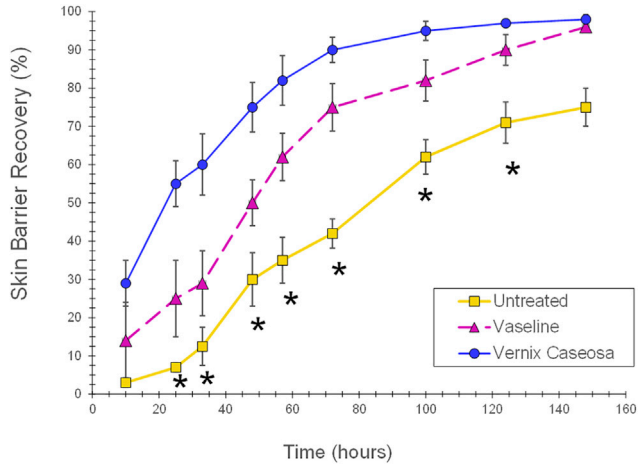

FIGURE 3 | Skin barrier repair following application of vernix or petrolatum versus untreated skin. Skin barrier damage was created by repeatedly tape stripping the SC in the hairless mouse model. The damaged skin sites were treated with 5 mg/cm^2 of vernix, 5 mg/cm^2 petrolatum, or left untreated as controls and barrier recovery monitored over time (one-way ANOVA, posthoc Bonferroni correction, and $p < 0.05$) (Oudshoorn et al., 2009b). Vernix treated skin demonstrated a significantly increased rate of SC barrier repair compared to untreated, damaged control skin. In the same study, treatment of damaged skin with petrolatum also significantly increased the SC barrier repair rate versus the untreated control, but the skin was more erythematous and thickened compared to the vernix treated skin ($p < 0.05$). *Indicates significant difference for untreated skin versus vernix and petrolatum treated sites ($p < 0.05$).

perinatal pathogens, namely group B *Streptococcus*, K *pneumoniae,* and L *monocytogenes* (Akinbi et al., 2004). Holm, et al., analyzed 34 individual vernix samples using liquid chromatography tandem mass spectrometry and identified 203 proteins (Holm et al., 2014). Their analysis with multivariate and classification methods revealed 25 functional classes. Hydrolases, proteases, and enzyme modulators encompassed 29, 22, and 22 proteins, respectively, with 11 proteins classified as immunity/defense and generally consistent with Tollin et al., (2006); Holm et al., (2014). The 34 vernix samples were from 16 infants who had developed atopic dermatitis by 2 years of age and 18 non-atopic healthy controls. A comparison of the proteomic data found significantly reduced levels of both UBC (polyubiquitin-C) and CALM5 (calmodulin-like protein 5) in vernix of infants who later developed atopic eczema versus vernix from infants who did not develop eczema, shown in **Figure 2** (Holm et al., 2014). Furthermore, investigation to determine whether these biomarkers are early indicators of atopic disease is clearly warranted, given the increased incidence and morbidity associated with this condition.

4.5 Vernix as a Skin Protectant

Vernix has demonstrated multiple "protective" functions. Evidence of these actions includes the following. 1) Vernix was spread on a highly permeable fiber substrate to create films of known thicknesses *in vitro*. The vernix films impeded exogenous chymotrypsin transport and maintained the native enzyme activity that is necessary for epidermal development (Tansirikongkol et al., 2007b). 2) Normal adult skin was treated with native vernix and common skin creams petrolatum, Aquaphor and Eucerin, and an untreated control. Vernix-treated skin had a significantly higher peak water sorption value than all the cream treatments and the control, indicating that it binds exogenous water to the skin (Bautista et al., 2000). 3) In parallel cohorts of full-term infants, vernix was retained on the skin of one group and removed from the other group. The skin covered with vernix was significantly more hydrated, less erythematous, and had a lower surface pH than skin where the vernix was removed (Visscher et al., 2005). These differences were observed immediately after birth and 24 h later. 4) The SC from the vernix retained and vernix removed groups was sampled 24 h after birth and analyzed for the free amino acid (FAA) component of the natural moisturizing factor. Free amino acid levels were significantly higher for infants with vernix retained versus those with vernix removed where FFAs were extremely low or undetectable (Visscher et al., 2011b). The FFA appeared to originate from the vernix that was retained on the skin after birth. That is, native vernix contained FFAs. 5) Skin barrier damage was created by repeatedly tape stripping the SC in the hairless mouse model. The damaged skin, treated with vernix, demonstrated a significantly increased rate of SC barrier repair compared to untreated, damaged control skin (Oudshoorn et al., 2009a). In the same study, treatment of damaged skin with petrolatum also significantly increased the SC barrier repair rate versus the untreated control, but the skin was more erythematous and thickened compared to the vernix treated skin (**Figure 3**). 6) Wounds that were produced with 25 microns of laser energy (animal model) showed an increased rate of barrier recovery after 2 days of treatment with either vernix or a petrolatum-based cream compared to a wounded, untreated control (Visscher et al., 2011a).

In summation, the literature suggests that vernix protects the infant throughout fetal development and at birth, supporting its role in the innate immune function of the epidermis. Vernix appears during the last 10 weeks of gestation. Consequently, premature infants, particularly those <29 weeks GA at birth, lack exposure to significant amounts of vernix, raising these questions. What is the effect of exposure to vernix caseosa during gestation on the development of the innate immune system? How might the presumed positive effects of vernix be implemented to facilitate innate immune system development in very premature infants?

5 EPIDERMAL BARRIER AFTER BIRTH

5.1 Full-Term Infants

The dramatic transition from aqueous *in utero* conditions to a dry, gaseous environment at birth initiates changes in the skin that are required for the full-term infant to survive and thrive. Remarkably, the epidermal barrier is intact and functional, despite submersion in amniotic fluid. This is in marked contrast to skin maceration and SC lipid disruption with prolonged water exposure in older children and adults

(Warner et al., 1999; Ogawa-Fuse et al., 2019). Within minutes after birth, full-term skin hydration changes and varies due to the presence of vernix, infant care practices, for example, exposure to radiant warming, and body site (Visscher et al., 1999; Visscher et al., 2005). Despite prolonged exposure to water during gestation, a rapid decrease in hydration occurs consistently within the first day, followed by an increase over the first 2 weeks and suggesting SC adaptation to the drier environment (Visscher et al., 1999; Visscher et al., 2000; Visscher et al., 2005; Fluhr et al., 2012). The low transepidermal water loss (TEWL) observed in full-term newborn skin indicated a well-functioning epidermal barrier (Hammarlund et al., 1979; Fluhr et al., 2012; Ludriksone et al., 2014). A rapid humidity decrease (hairless mice) lead to increased DNA synthesis, lower free amino acid levels, dry skin, and lower filaggrin immunoreactivity, due to decreased epidermal keratohyalin granules (Scott and Harding, 1986; Denda et al., 1998a; Visscher et al., 1999; Katagiri et al., 2003).

Full-term skin pH is nearly neutral at birth, decreases significantly by day 4 (Visscher et al., 2000) and then gradually continues to decrease over the next few months. An acidic skin pH is important in establishing the skin barrier as it promotes the effective functioning of enzymes required for SC development and integrity, that is, lipid metabolism, bilayer structure formation, ceramide synthesis, lipid bilayer formation, and desquamation (Rippke et al., 2002; Schmid-Wendtner and Korting, 2006). The skin pH reduction after birth is due to multiple mechanisms, including 1) filaggrin proteolysis to amino acids, pyrrolidone carboxylic acid, and urocanic acid; 2) secretory phospholipase hydrolysis to FFA; 3) acidification in the lower SC by a Na^+H^+ antiporter mechanism (NHE1); 4) melanin granule dispersion to release H+; and 5) cholesterol sulfate production of H^+(Elias, 2017).

Full-term skin microbiota colonization begins at birth (Capone et al., 2011; Cuenca et al., 2013) and is populated by *Lactobacillus*, *Propionibacterium*, *Streptococcus,* and *Staphylococcus*, differing by body site at 6 weeks of life (Chu et al., 2017). Skin microbiota contributes to innate immunity by regulating antimicrobial peptides, including cathelicidins and β-defensins, and responding to inflammation via IL1α (Naik et al., 2012). *S. epidermidis* and *hominis* produce antimicrobial peptides that are noxious to *S. aureus* (Chen et al., 2018a; b). Skin bacteria and yeasts hydrolyze sebaceous gland triglycerides to glycerin and free fatty acids (Yosipovitch et al., 2000) that, in turn, have antimicrobial properties and contribute to skin surface acidity (Eyerich et al., 2018).

5.2 Premature Infants

The epidermal barrier is under-developed in premature infants at birth, particularly those <29 weeks GA, putting them at risk for infection and increased permeability to both internal water loss and external deleterious agents (Evans and Rutter, 1986; Cartlidge, 2000; Rutter, 2000). The skin is easily injured or torn due to deficiencies in dermal structural proteins (Eichenfield and Hardaway, 1999). Although epidermal barrier development is rapid upon exposure to a dry environment at birth (Harpin and Rutter, 1983; Okah et al., 1995; Agren et al., 2006), very premature stratum corneum is not fully competent at 1 month of life, as indicated by a considerably higher transepidermal water loss (TEWL) compared to full-term infants (Agren et al., 1998).

The preterm skin surface pH decreased following birth but the rate was slower for infants weighing less than 1,000 g than for infants weighing more than 1,000 g. The decrease was faster during postnatal week 1 versus weeks 2–4 (Fox et al., 1998). The interaction of GA and postnatal age significantly influenced the rate of pH reduction (Green et al., 1968).

Infection is one cause of premature birth. In utero exposure to infectious agents and/or to antibiotics before birth is likely to impact the microbiome soon after birth. Infants <32 weeks GA demonstrated a decrease in bacterial richness during postnatal weeks 1 and 2, followed by an increase. However, the bacterial diversity was lower in premature infants than it was in full-term infants (Pammi et al., 2017). Firmicutes and Proteobacteria were the most abundant phyla in premature infants. The implications of the skin microbiome and maternal antibiotic use are areas that warrant further investigation.

6 EPIDERMAL BARRIER MATURATION-PROTEOMICS

The risks of infection and skin damage in premature infants are considerable. Consequently, the facilitation of epidermal barrier maturation and immune function is a critically important aspect of clinical practice. A fundamental understanding of the biological processes governing skin maturation will enable the implementation of effective skincare practices, for example, humidification, topical treatments, and implementation of appropriate antisepsis measures.

The relatively recent emergence of quantitative, noninvasive analytical methods has enabled simultaneous measurements of protein and non-protein biomarkers of epidermal barrier status and immune function. A highly specific, quantitative analysis of the outer stratum corneum via noninvasive collection techniques revealed important differences in innate immune biomarkers in premature infants ≤32 weeks GA compared to full-term infant and adult samples. Proinflammatory cytokines IL1β, IL6, MCP1, and IL8 and structural proteins involucrin and albumin were significantly higher in premature infants ($p < 0.05$), and involucrin and albumin levels were inversely related to GA (Narendran et al., 2010). These initial findings prompted a more detailed investigation. Stratum corneum biomarkers of antimicrobial function and late cornification were hypothesized to be lower in premature infants than in full-term infants and later normalize over 3–4 months after birth.

Subsequently, targeted proteomic analysis of skin surface (stratum corneum) biomarkers and established biophysical measures of barrier function were used to determine changes over time. The cohorts included 61 newborn infants grouped by GA, specifically: premature <34 wks GA (PT), late premature ≥34-< 37 wks GA (LPT), and full-term ≥ 37 wks GA (FT) (Visscher et al., 2020). Infant biomarkers were compared to adult values (i.e., a widely studied, established steady-state

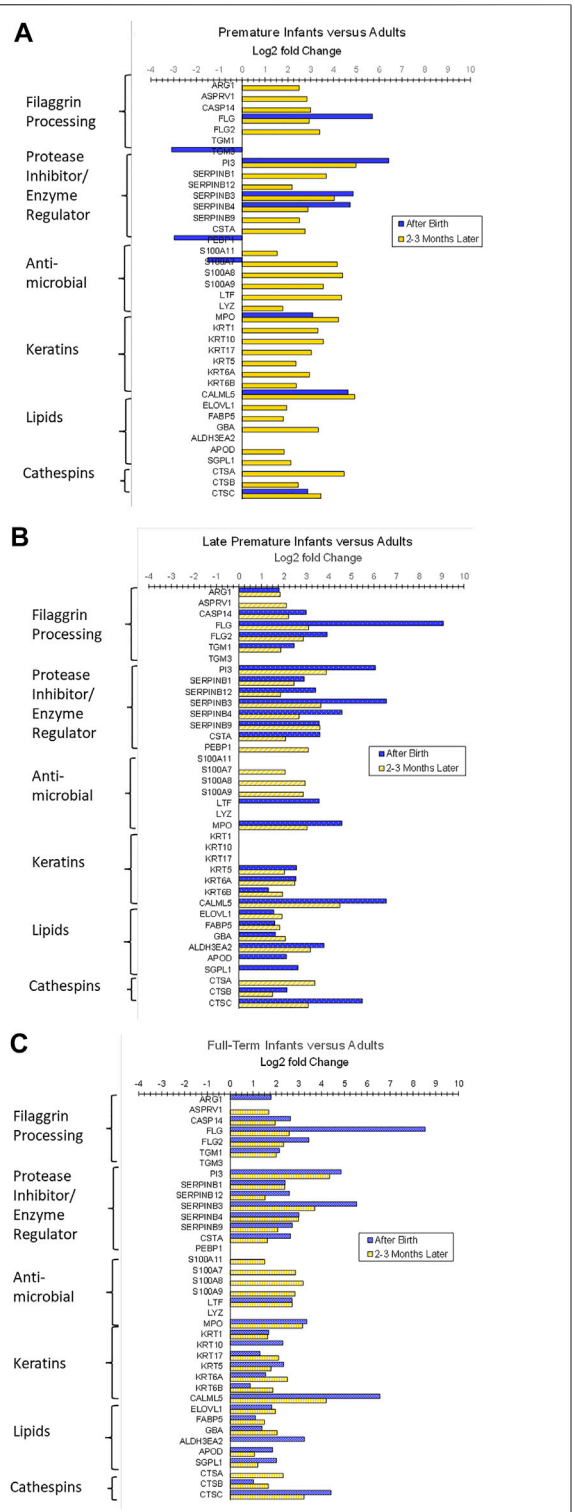

FIGURE 4 | Differentially expressed biomarkers for infants 4–8 days after birth and 2–3 months later at corrected GAs of 46–48 weeks versus adult skin. Two sequential skin surface samples (stratum corneum) were collected from the lower legs of 61 infants at each time and from the volar forearms of 34 adults (parent) at one time. Samples were extracted, quantified with liquid chromatography tandem mass spectrometry, and analyzed with

(*Continued*)

FIGURE 4 | targeted proteomics ($p < 0.05$) (Visscher et al., 2020). The proteins classified by function were: filaggrin processing, protease inhibitor/ enzyme regulators, antimicrobials, keratins/structural proteins, lipid processing, and cathepsins ($p < 0.05$). **(A–C)** show the log2 fold changes for the specific proteins in each class versus adults for PT, LPT, and FT infants at both times. The differentially expressed biomarkers were decidedly different for infant skin compared to stable adult skin. For PT infants, the differentially expressed proteins increased from 12 to 54 versus adults over 2–3 months, suggesting substantial adaptive changes over time.

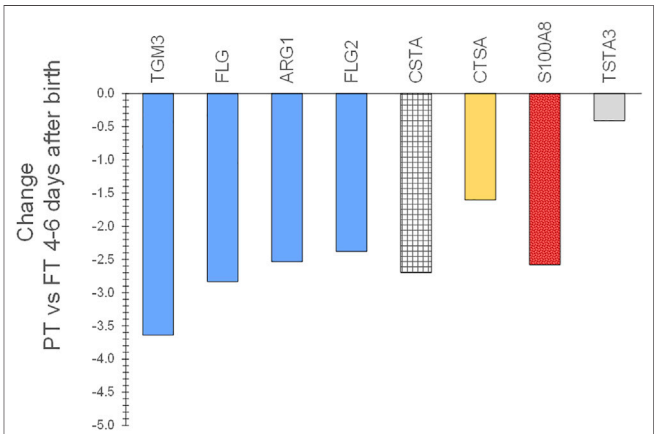

FIGURE 5 | Changes in SC proteins for PT infants compared to FT infants 4–8 days after birth. Two sequential skin surface samples (stratum corneum) were collected from the lower legs of 61 infants at each time and from the volar forearms of 34 adults (parent) at one time. Samples were extracted, quantified with liquid chromatography tandem mass spectrometry, and analyzed using targeted proteomics ($p < 0.05$) (Visscher et al., 2020). PT infant SC had decreased expression of filaggrin processing biomarkers FLG, FLG2, AGR1, and TGM3, antimicrobial S100A8, protease inhibitor CSTA, and protective protein CTSA (cathepsin A) soon after birth compared to FT infant SC.

condition subject parents, $n = 34$) at two-time points, 4–8 days after birth, and 2–3 months later when the three infant groups were at comparable gestational ages of 46–48 weeks.

The sets of differentially expressed biomarkers at both time points were decidedly different than those in stable adult skin. After birth, the expression of 40 biomarkers in FT, 38 in LPT, and 12 in PT was higher compared to adults ($p < 0.05$). Two-three months later, the expression of 40 biomarkers in FT, 38 in LPT, and 54 in PT was higher versus adults ($p < 0.05$). The differentially expressed proteins classified by function were: filaggrin processing, protease inhibitor/enzyme regulators, antimicrobials, keratins/structural proteins, lipid processing, and cathepsins ($p < 0.05$). **Figures 4A–C** show the log2 fold changes for specific proteins by class for PT, LPT, and FT infants versus adults at both times. The number of differentially expressed proteins increased from 12 to 54 for PT infants versus adults over the 2–3 month time period, suggesting substantial adaptive changes over time.

Expression patterns of SC biomarkers between the infant groups were examined to gain insight into the effects of gestational age at birth and after 2–3 months of life. PT infant

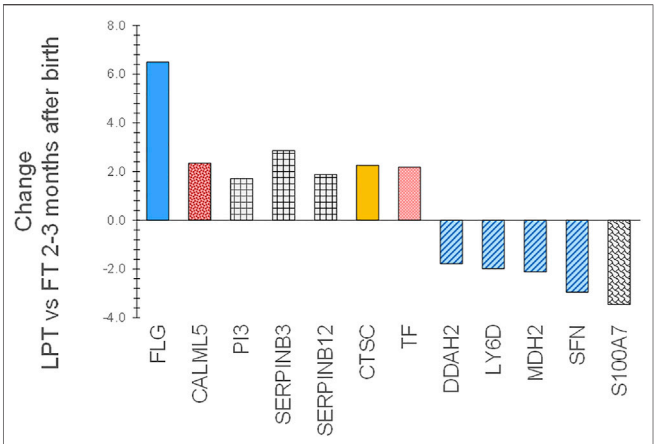

FIGURE 6 | Changes in SC proteins for LPT infants compared to FT infants 2–3 months after birth at comparable corrected GA. Two sequential skin surface samples (stratum corneum) were collected from the lower legs of 61 infants at each time and from the volar forearms of 34 adults (parent) at one time. Samples were extracted, quantified with liquid chromatography tandem mass spectrometry, and analyzed using targeted proteomics ($p < 0.05$) (Visscher et al., 2020; Visscher et al., 2021). LPT infants had increased expression of protease inhibitors PI3, SERPINB3, and SERPINB12, as well as FLG, CALML5, CTSC, and TF versus FT infants. Expression of S100A7, LY6D, SFN, MDH2, and DDAH2 was lower in LPT compared to FT 2–3 months later. These findings suggest that the rate of change of specific aspects of epidermal barrier development may vary with GA and/or time from birth.

SC had decreased expression of filaggrin processing biomarkers FLG, FLG2, AGR1, and TGM3, antimicrobial S100A8, protease inhibitor CSTA, and protective protein CTSA (cathepsin A) soon after birth compared to FT infant SC (**Figure 5**). The protein expression did not differ for PT versus FT 2–3 months later. LPT and FT infants had comparable protein expression soon after birth but LPT had increased expression of protease inhibitors PI3, SERPINB3, and SERPINB12, as well as FLG, CALML5, CTSC, and TF (**Figure 6**). Expression of S100A7 (antimicrobial), LY6D, SFN, MDH2, and DDAH2 was lower in LPT compared to FT at similar corrected GA 2–3 months later. These findings suggest that the rate of change of specific aspects of epidermal barrier development may vary with GA and/or time from birth.

6.1 Epidermal Immunity

Expression patterns for biomarkers of innate immunity by GA and over time warrant further comment. Soon after birth, increased expression of the antimicrobial proteins MPO (all infants) and LTF (LPT, FT) were noted in adults. Two to three months later, the expression of biomarkers S100A8, S100A9, S100A7, and S100A11, as well as MPO, LTF, and LYZ (PTs), had increased significantly versus adults (**Figures 4A–C**). S100A7, S100A8, and S100A9 prompt keratinocytes to produce cytokines and chemokines. In turn, cytokines can promote the production of S100A7, S100A8, and S100A9, and, thereby, respond to threats (stressors) to facilitate immunity against pathogens (Lee et al., 2012; Lesniak and Graczyk-Jarzynka, 2015). Increased expression of S100A7, S100A8, S100A9, and S100A12 occur in inflammatory skin conditions with epidermal barrier defects, that is, atopic dermatitis and psoriasis (Oestreicher et al., 2001; Sugiura et al., 2005; Benoit et al., 2006; Wolk et al., 2006). Two to three months after birth, several clinical measures of barrier status demonstrated higher TEWL (FT) versus adults, higher visual dryness (PT, FT) and lower SC cohesion (PT, FT) (Visscher et al., 2020). Consequently, the increased S100 protein expression levels may occur in response to multiple factors, including pathogen exposure and minor barrier injury.

The increased expression of filaggrin processing biomarkers FLG, FLG2, ASPRV1, CASP14, and TGM1 for all infants compared to adults 2–3 months after birth was associated with changes in the products of filaggrin proteolysis, that is, natural moisturizing factor (NMF), histidine, proline, urocanic acid, and pyrrolidone carboxylic acid (PCA) that were quantified with reverse phase high-performance liquid chromatography and tandem mass spectrometry (Wei et al., 2016). NMF, PCA, histidine, and proline amounts were significantly higher for every infant group versus adults 2–3 months later ($p < 0.05$). In contrast, after birth, NMF, PCA, histidine, and proline levels were lower for all three infant groups versus adult samples ($p < 0.05$). The NMF increase was associated with a skin surface pH reduction for all infant groups (data not shown), and this acidification of the epidermal barrier processes is necessary to provide immunity via the promotion of colonization with effective microbiota.

All three infant groups (PT, LPT, FT) had higher levels of 9 biomarkers versus adults shortly after birth (FLG, SERPINB3, SERPINB4, PI3, MPO, CALML5, CTSC, ALB, TF, **Figures 4A–C**), likely indicating their importance in newborn skin development and maturation. The functions and possible implications of these proteins are discussed below. Increased FLG was associated with reduced NMF in infants, potentially due to inhibition of FLG proteolysis at high humidity described *in utero* (Scott and Harding, 1986). SERPINB3 and SERPINB4 are protease (e.g., serine, cysteine) inhibitors, including proteases generated by infectious pathogens (Sun et al., 2017). PI3 inhibited keratinocyte desquamation prior to terminal differentiation (Nakane et al., 2002) and kallikrein proteolysis (McGovern et al., 2017) and is a component of the corneocyte envelope (Steinert and Marekov, 1995), functions that are important to the provision of the physical epidermal barrier. While identified in the SC, MPO was produced in immune cells, for example, neutrophils, and lymphocytes (Khan A. A. et al., 2014; Liu et al., 2015), was higher in infected wounds (Gabr and Alghadir, 2019), and was higher under conditions of oxidative stress (Khan et al., 2018) and inflammation (Voss et al., 2018). Higher CALML5 levels were implicated in terminal differentiation (Sun et al., 2015) and barrier repair (atopic dermatitis) (Donovan et al., 2013). CTSC prompted serine protease generation in immune cells (Meyer-Hoffert, 2009). High ALB was associated with reduced skin hydration, consistent with our observation of lower hydration/skin dryness, particularly in LPT and FT shortly after birth. TF was associated with inflammation (Mehul et al., 2017).

Over the time from birth until 2–3 months later, a greater number of biomarkers were differentially expressed for infants versus adults,

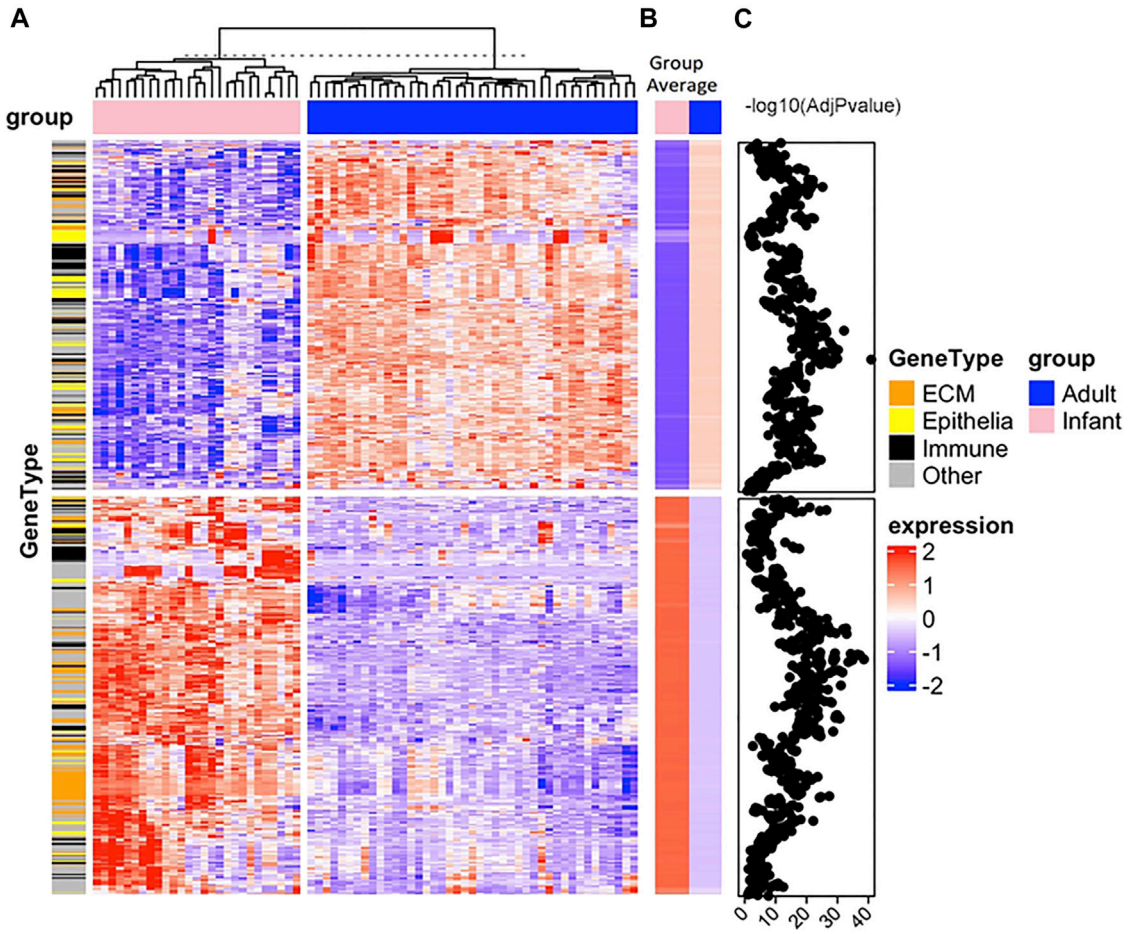

FIGURE 7 | Hierarchical clustering analysis of differentially expressed genes in newborn infant and adult skin samples. Full-thickness tissue samples (body site, non-foreskin) from 27 infants were collected at non-elective surgery and buttocks tissue (protected from ultraviolet radiation exposure) from 43 adults was processed to collect total RNA (Visscher et al., 2021). Gene expression was determined from mRNA using Affymetrix GeneTitan U219 array plates. The lowest 30% of the 49,386 gene transcripts were removed, assayed for quality, data was normalized and Log2 transformed, analyzed using linear models and differential expression analyses and analyzed and analysis of variance with a term for the combination was conducted, as previously described (Merico et al., 2010; Supek et al., 2011; Yu et al., 2012; Gu et al., 2016; Szklarczyk et al., 2019; Visscher et al., 2021). Rigorous quality control was applied and all data were MIAME compliant. The Empirical Bayes method (limma R-package) was used to test comparisons. Test statistics were moderated with the Empirical Bayes method (limma R-package). The Benjamini–Hochberg correction was used to control for false discovery rates. The complete linkage method using the R hclust function was used to perform hierarchical clustering. Genes that were significantly expressed were analyzed for enrichment of biologic themes (Gene Ontology) using the clusterProfiler package (Yu et al., 2012), EnrichmentMap (Merico et al., 2010), g:profiler (Raudvere et al., 2019), Revigo (Supek et al., 2011) and String database (Szklarczyk et al., 2019). The transcriptomics is in the NCBI Gene Expression Omnibus (GEO) repository with the dataset accession number GSE181022. Panel **(A)** is a heatmap of the normalized expression values (based on z-score) of the 1,086 differentially regulated genes with adjusted *p* value <0.05 and absolute fold change ≥1.5 for the two groups, infants (pink) and adults (blue). Euclidean distances between each sample were determined and cluster analysis was performed with an unsupervised hclust algorithm. Samples formed two clusters. From a hierarchical cluster, analysis genes were grouped for similarity where each column is an individual sample and each row is a single gene. Extracellular matrix (ECM, orange), immune-related (black), and epithelial (yellow) genes are indicated on the annotation bar under the gene type. Panel **(B)** is a heatmap of group average values for infants (pink) and adults (blue). Panel **(C)** are the values of the negative $\log_{10}$ of the adjusted *p* values from the Limma testing for adults versus infants for each gene. The z-scores are shown in the blue-white-red gradient where 2 is the darkest red color, 0 is white and -2 is the darkest blue color shown on the right and labeled as "expression". Many negative $\log_{10}$ adjusted *p* values were greater than 10, indicating large differences.

in addition to those involving filaggrin processing. S100A7, S100A8, S100A9, and MPO were significantly higher for all infants versus adults (**Figures 4A–C**). Protease inhibitors/enzyme regulators, PI3, SERPINB3, and SERPINB4 remained higher for all infant groups versus adults, and SERPINB1, SERPINB9, SERPINB12 and CSTA became significantly higher over time (**Figures 4A–C**). SERPINB1, located in macrophages and the cytoplasm and granules of neutrophils (Majewski et al., 2016), functions as an antimicrobial in infections and can guard against apoptosis. SERBINB9 has been described to react with enzymes in bacteria, yeasts, and fungi (Meyer-Hoffert, 2009) and serves in host-defense against bacteria and viruses in the lung, another epithelial tissue (Askew and Silverman, 2008). SERPINB12 is ubiquitous in human tissue, including the epidermis and eccrine duct, and is thought to guard macrophages from their internal protease inhibitors as well as from exogenous sources (Niehaus et al., 2015).

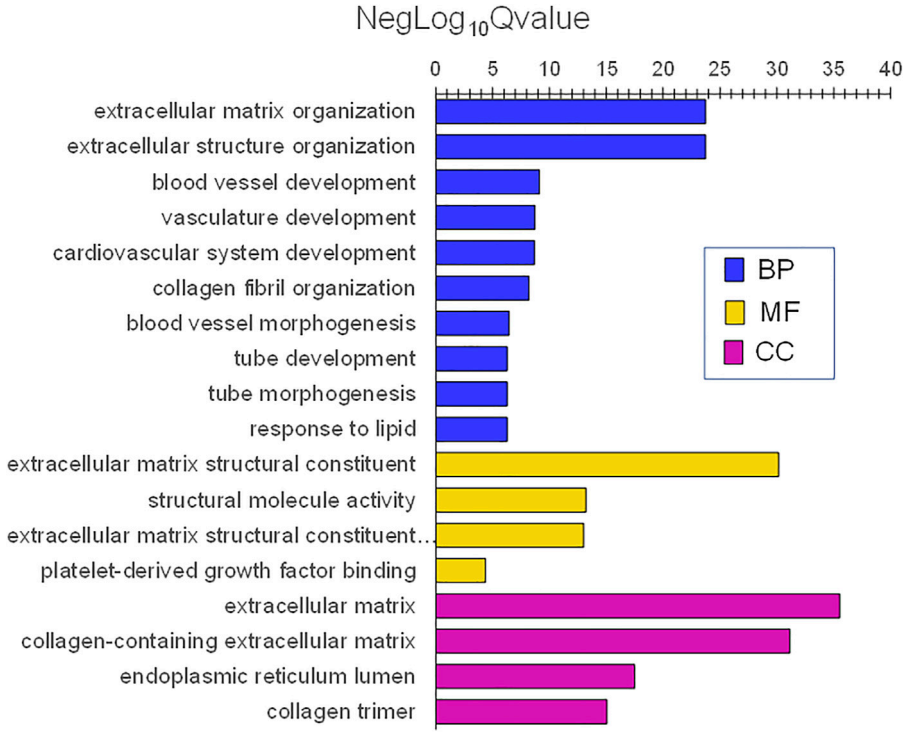

FIGURE 8 | Gene ontology themes with enriched gene expression in infant skin. Significantly expressed genes were analyzed for enrichment of biologic themes (Gene Ontology) using the clusterProfiler package (Yu et al., 2012), EnrichmentMap (Merico et al., 2010), and g:profiler (Raudvere et al., 2019). Significant pathways were selected using the false discovery rate (FDR)adjusted *p* values. $NegLog_{10}Qvalue$ indicates $-Log_{10}FDR$ adjusted *p* value. The lowest adjusted *p* values (highest $NegLog_{10}Qvalue$) were biological processes (BP) extracellular matrix (ECM) organization and ECM structure organization. Others included system development (e.g., blood vessel, cardiovascular, tube), and response (e.g., to lipid). The most significant molecular functions (MF) were ECM matrix structural constituent and structural molecule activity. ECM and collagen-containing ECM were the most significant cell components (CC).

Collectively, these unique protein expression profiles suggest that the processes and pathways regulated by these proteins continue to be important for the provision of epidermal immunity well after birth. Neonates respond to multiple system transitions at birth by 1) producing NMF and lowering skin pH, 2) mitigating desquamation by inhibiting specific protease activity, and 3) increasing the antimicrobial features of the epidermal barrier. Neonatal skin must adapt over time to provide a sufficient level of epidermal immunity and maturation.

7 INFANT SKIN GENOMICS

Neonatal epidermal development and immunity were further examined via genomic analysis of full-thickness skin samples from newborns who required surgery. The hypothesis was that infant skin would exhibit increased expression of innate immunity genes and adult skin would have increased expression of epidermal barrier genes. Genomic analysis of newborn infant skin (n = 27) was compared to ultraviolet radiation protected adult skin (age 20–60 years, n = 43) to differentiate the physiological and structural features at the biological, molecular, and cellular levels as previously described (Visscher et al., 2021).

There were numerous differences across biological processes, with 1,086 probes differentially expressed in infant skin versus adult skin with 508 probes increasing while 578 probes decreased. Hierarchical clustering analysis of the probe normalized expression values ($|FC| \geq 1.5$, adjusted *p* value <0.05) was performed. Limma testing (negative $\log_{10}$ (AdjPvalue)) showed many values over 10 for infants versus adults, representing large differences (**Figure 7**).

Infant skin was enriched in genes implicated in many gene ontology (GO) themes for biological processes, molecular functions, and cell components. The lowest adjusted *p* values (highest $NegLog_{10}Qvalue$) as categorized by GO biological processes (BP) were extracellular matrix (ECM) organization and ECM structure organization. Others included system development (e.g., blood vessel, cardiovascular, tube) and response (e.g., to lipid) (**Figure 8**). The most significant GO molecular functions (MF) were ECM matrix structural constituent and structural molecule activity. GO cell components (CC) with the lowest adjusted *p* values were ECM and collagen-containing ECM.

In contrast, adult skin was enriched in genes involved with skin and epidermis development. The lowest adjP values for BPs were skin development, epidermis development, keratinocyte differentiation, keratinization, and cornification. Additionally, immune BPs including antigen processing and presentation of exogenous antigen, major histocompatibility protein complex, and antigen-

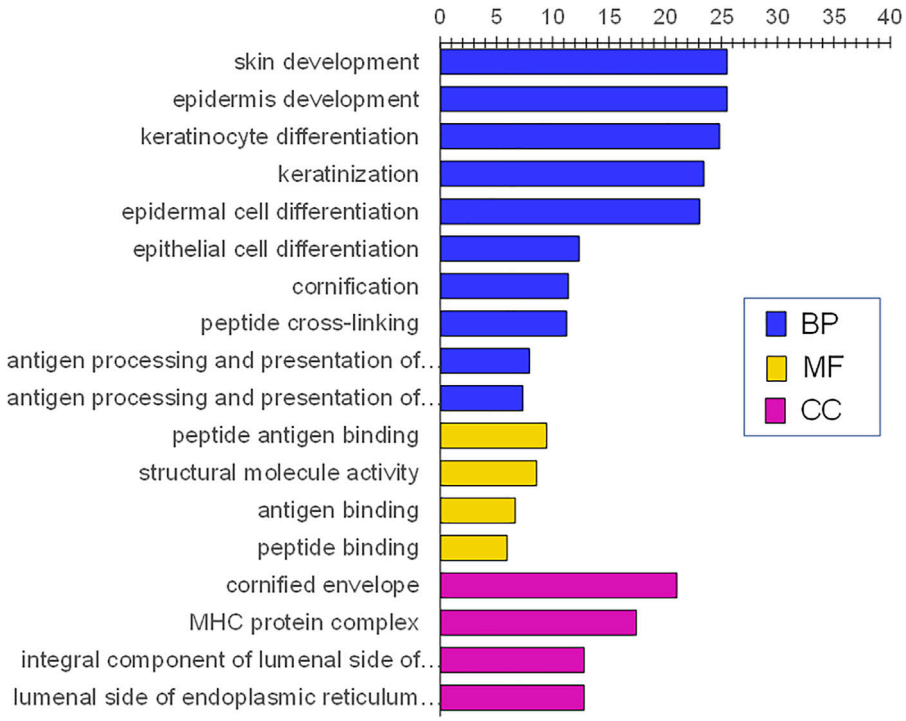

FIGURE 9 | Gene ontology themes with enriched gene expression in adult skin. Significantly expressed genes were analyzed for enrichment of biologic themes (Gene Ontology) using the clusterProfiler package (Yu et al., 2012), EnrichmentMap (Merico et al., 2010), and g:profiler (Raudvere et al., 2019). Significant pathways were selected using the false discovery rate (FDR)adjusted *p* values. $NegLog_{10}$Qvalue indicates $-Log_{10}$FDR adjusted *p* value. The lowest adjusted *p* values for BP were skin development, epidermis development, keratinocyte differentiation, keratinization, and cornification. Immune BPs included antigen processing and presentation of exogenous antigen, major histocompatibility protein complex, and antigen-binding were also prominent. Highly significant MF was peptide antigen binding and structural molecule activity and CC was a cornified envelope and major histocompatibility complex (MHC) protein complex.

binding were also prominent (**Figure 9**). Highly significant MF were peptide antigen binding and structural molecule activity and those for CC were cornified envelope and major histocompatibility complex (MHC) protein complex (**Figure 9**). Complete lists of the infant and adult processes for adjusted *p* values <0.0001 are listed in **Supplementary Tables S1 and S2**.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed 15 enriched pathways in infant tissues wherein protein digestion and absorption, PI3K-Akt signaling pathway, human papillomavirus infection, ECM receptor interaction, and focal adhesion had the lowest adjusted *p* values. Adult tissues had 38 enriched KEGG pathways, including those associated with *Staphylococcus aureus* infection, allograft rejection, immune disease, infectious disease, cancer, transport and catabolism, endocrine or endocrine disease and antigen processing and presentation had the lowest adjusted *p* values.

Analysis of data using REACTOME revealed 20 pathways with the genes differentially expressed in infant tissues, with adjusted *p* values <0.001 compared with adults (**Table 2**). Extracellular matrix organization, a top-level pathway, had the lowest adjusted *p* value. Also enriched in infants were sub-pathways degradation of the extracellular matrix, ECM proteoglycans, collagen formation, integrin cell surface, non-integrin membrane-ECM interactions, and laminin interaction. REACTOME analysis showed 5 pathways with genes differentially expressed in adult tissues, with adjusted *p* values <0.001 compared with infants (**Table 2**). The pathways included keratinization and formulation of the cornified envelope, that is, epidermal barrier, as well as interferon gamma signaling and endosomal/vacuolar pathway, that is, immune function.

Gene expression analyses were conducted on infants, adults, children, and adolescents to understand the progression of atopic dermatitis (AD) over time. Lesional and nonlesional tissue samples from individuals with AD were compared, with samples from non-AD infants, adults, children, and adolescents serving as normal controls (Renert-Yuval et al., 2021). The infant-adult control comparison revealed more differentially expressed genes than any other. Several immune genes were increased in infants, namely, S100A7, CTLA4, S100P, CXCR4, CCL4L1, CCL25, CCL4, CSCL2, IL6, IL10, CCL3, IL32, TNFRSP4, TNFB3 and CCL16 (criteria of the fold change of 2 and false discovery rate of <0.05) (Renert-Yuval et al., 2021). The following epidermal barrier genes were significantly increased in non-atopic adults versus infants: LCE1F, LCE2B, LCE2C, LC#2D, KRT2, SCEL, CLDN11, EREG, FLG, ELOVL5, FADS1, FADs2, CLDN1, FABP7, SCPP1, and CLDN8. In

TABLE 2 | REACTOME pathways with the genes differentially expressed in infant tissues with adjusted *p* values <0.001 compared with adults and for adult tissues with adjusted *p* values <0.001 compared with infants.

Increased in infants	*p* value	Increased in adults	*p* value
Extracellular matrix organization	1.00E-28	Keratinization	1.01E-23
Degradation of the extracellular matrix	5.62E-19	Formation of the cornified envelope	2.36E-23
ECM proteoglycans	1.51E-17	Developmental Biology	1.79E-08
Collagen formation	9.23E-16	Interferon gamma signaling	4.00E-06
Collagen biosynthesis and modifying enzymes	2.54E-13	Endosomal/Vacuolar pathway	8.29E-06
Assembly of collagen fibrils and other multimeric structures	6.27E-13		
Collagen chain trimerization	1.35E-12		
Collagen degradation	3.86E-11		
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	2.12E-10		
Integrin cell surface interactions	3.53E-09		
Post-translational protein phosphorylation	1.59E-07		
Crosslinking of collagen fibrils	3.76E-07		
Non-integrin membrane-ECM interactions	5.64E-07		
MET activates PTK2 signaling	1.9735E-06		
Laminin interactions	1.9735E-06		
NCAM1 interactions	3.2334E-06		
Metal sequestration by antimicrobial proteins	7.9462E-06		
MET promotes cell motility	3.1644E-05		
Signaling by PDGF	4.2549E-05		
Signaling by receptor tyrosine kinases	6.1897E-05		

comparison, the immune genes S100A7, S100P, and CXL2 were significantly increased in newborn infants versus adults, and the epidermal barrier genes LCE1F, LCE2B, LCE2C, LCE2D, KRT2, and CLDN1 were significantly increased in adults compared to newborn infants in the previous study (Visscher et al., 2021). The infants in Renert-Yuval, et al., were somewhat older 14 ± 10 months (range 3–36 months) at tissue compared to 1.5 ± 2.6 months (range 0.1–11.3) in Visscher, et al. The age difference may account for the differences in gene expression. The TH17/TH22 genes IL20, IL22, S100A7, S100A9, S100A12, S100A8, CCL20, and PI3 were significantly increased in infants compared to the older groups in Renert-Yuval, et al. Likewise, the expression of S100A7, S100A8, S100A9 and PI3 were significantly increased in infants versus adults in Visscher, et al.

8 DISCUSSION

Compared to adult skin, infant skin exhibited increased gene expression for extracellular matrix and development, among multiple processes. For adult skin, compared to infant skin, gene expression was higher for epidermal homeostasis and antigen processing/presentation, that is, adaptive immune function, and others. The adult epidermal barrier is constantly renewing while the infant barrier development is "in progress". The newborn infant depends upon the innate immune system, including the extracellular matrix, to protect against microbiota and the relatively hostile environment after birth, while stimulating adaptive immunity. **Table 3** provides an overview of the most important features of premature and full-term infants at birth and over the first few postnatal months relative to the benchmark of normal, healthy adult skin.

Overexpression of ECM genes in infant skin versus adult skin suggests their importance in newborn skin adaptation. ECM components influence cell proliferation, adhesion, apoptosis (Daley et al., 2008), barrier repair (Sumigray and Lechler, 2015) and connect the epidermis and dermis for tissue integrity. The rate of ECM modification and renewal is high in wound healing and response to infection (Sumigray and Lechler, 2015). ECM organization and structure organization were increased in infant skin. The ADAM9 gene, for example, produces MMP9 and facilitates wound healing by regulating keratinocyte migration collagen VII shedding (Mauch et al., 2010). Fetal skin demonstrates rapid and scarless wound healing and differs in inflammation, cytokine response, and ECM composition versus adult skin (Hu et al., 2018). We observed increased expression of fibronectin genes FNDC3B and FNDC3A. GNDC1, FLRT2, and FLRT3 in infants. With injury or infection, immune cells produce enzymes (e.g., MMPs, ADAMs, ADAMTSs) that promote inflammation (Tomlin and Piccinini, 2018). Collagen, laminin, and fibronectin, bind to microorganisms that can degrade the ECM (Arora et al., 2021). Immune cells control ECM synthesis, assembly, remodeling, and degradation, and respond to infection (Bhattacharjee et al., 2019).

For infants, the reduced expression for keratin genes, including KRT2, KRT25, KRT27, and KRT31, that provide structural integrity, for late cornified envelope genes, including LCE1C, LCE1D, LCE1 E, LCE1F, LCE2B, and for hair adaptation, that is, KRTAP genes (Khan I. et al., 2014), indicate the aspects of the epidermal barrier that develop over time after birth.

At birth, the newborn infant is equipped to survive and flourish, despite entering a vastly different environment replete with microbes, potential irritants, and high oxygen tension. While newborn skin is considered adaptive, the environmental exposures immediately after birth may alter the "intended" programmed trajectory resulting in

TABLE 3 | Overview of the most important features of premature and full-term infants at birth and over the first few postnatal months relative to the benchmark of normal, healthy adult skin.

Skin feature	Premature infant	Full-term infant
Barrier integrity (TEWL, g/m^2/hr)	Initially higher vs. FT and adult then decreases over time	Comparable to adults at birth
Hydration	Higher initially vs. FT and adult, decreases then increases	Initially lower vs. PT and adult, then increases
pH	Comparable to FT, higher vs. adult then decreases more slowly vs. FT	Comparable to PT, higher vs. adult, then decreases
Visual dryness/scaling	Lower vs. FT, comparable to adult then increases before decreasing	Higher vs. PT and adult initially, then decreases
Visual erythema	Higher vs. adult at birth and 2–3 months later	Higher vs. adult at birth and 2–3 months later
SC cohesion	Initially comparable to adults then decreasing before increasing again	Lower vs. PT and adult initially then increasing
SC thickness	Thinner vs. FT, adult	Thinner vs. adult
Microbiome	Less bacterial diversity than FT; decrease in richness followed by an increase	More diverse than PT
NMF level	Lower vs. adult, comparable to FT initially then increasing to higher than adult	Lower vs. adult, comparable to PT initially then increasing to higher than adult
Filaggrin (FLG) (stratum corneum)	Lower vs. FT at birth, higher vs. adults at birth and 2–3 months later	Higher vs. adults at birth and 2–3 months later
Filaggrin processing proteins (SC)[a]	Higher vs. adult 2–3 months after birth	Higher vs. adult 2–3 months after birth
Protease inhibitors/enzyme regulators (SC)[b]	Higher vs. adult at birth and 2–3 months later	Higher vs. adult at birth and 2–3 months later
Antimicrobial proteins (SC)[c]	Higher vs. adult 2–3 months after birth	Higher vs. adult 2–3 months after birth

[a]*Filaggrin processing biomarkers: FLG, FLG2, CASP14, ASPRV1, TGM1.*
[b]*Protease inhibitors/enzyme regulators: PI3, SERPINB3, SERPINB4.*
[c]*Antimicrobial proteins: S100A7, S100A8, S100A9, LFT, MPO.*

aberrant skin or diseased states. Immune functions provided by the epidermis are, arguably, never more essential that at birth, particularly for infants born prematurely with underdeveloped skin. Gene expression in infant skin increased for processes including extracellular matrix and development while adults had increased gene expression for epidermal homeostasis and antigen processing/presentation of immune function. Newborn infant stratum corneum contained protease inhibitors/enzyme regulators to interact with microorganisms and moderate desquamation to ensure a barrier. Antibacterial proteins were higher in infants compared to adults well after birth, suggesting a role in immune function.

In many cases, the literature on early epidermal immunity is limited to descriptive information. Clearly, additional research is needed to delineate the gaps in our current knowledge of skin development, particularly regarding the rates of change in the epidermal barrier adaptation process as a function of gestational age. It does not yet include information among the most premature infants, names those of 22–28 gestational age. As the limit of viability decreases, the need to implement clinical practices to facilitate epidermal maturation and effective function become essential. Further research is important to understand the factors, including environmental conditions, microbiome development, and skin-device interactions, that trigger specific phenotypes, for example, atopic dermatitis, during gestation, and following birth. Reduction in neonatal mortality and morbidity is a global priority and a challenge that requires multiple research and clinical specialties. The continuous advances in research tools of genomics, proteomics, metabolomics, and bioinformatics and the availability of precious data from repositories will prime the research system for further advances. The present, available results collectively serve to guide clinical practice and the implementation of strategies to facilitate robust infant barrier integrity and function.

AUTHOR CONTRIBUTIONS

MV contributed to the manuscript conception, critical review, synthesis of the content, and preparation of the first draft. AC and VN contributed to revising the manuscript critically for important content and final approval of the version being submitted.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.894496/full#supplementary-material

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Exposure of Keratinocytes to *Candida Albicans* in the Context of Atopic *Milieu* Induces Changes in the Surface Glycosylation Pattern of Small Extracellular Vesicles to Enhance Their Propensity to Interact With Inhibitory Siglec Receptors

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Candida albicans (C. albicans) infection is a potential complication in the individuals with atopic dermatitis (AD) and can affect clinical course of the disease. Here, using primary keratinocytes we determined that atopic *milieu* promotes changes in the interaction of small extracellular vesicles (sEVs) with dendritic cells and that this is further enhanced by the presence of *C. albicans*. sEV uptake is largely dependent on the expression of glycans on their surface; modelling of the protein interactions indicated that recognition of this pathogen through *C. albicans*-relevant pattern recognition receptors (PRRs) is linked to several glycosylation enzymes which may in turn affect the expression of sEV glycans. Here, significant changes in the surface glycosylation pattern, as determined by lectin array, could be observed in sEVs upon a combined exposure of keratinocytes to AD cytokines and *C. albicans*. This included enhanced expression of multiple types of glycans, for which several dendritic cell receptors could be proposed as binding partners. Blocking experiments showed predominant involvement of the inhibitory

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Siglec-7 and -9 receptors in the sEV-cell interaction and the engagement of sialic acid-containing carbohydrate moieties on the surface of sEVs. This pointed on ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) as potential enzymes involved in the process of remodelling of the sEV surface glycans upon *C. albicans* exposure. Our results suggest that, in combination with atopic dermatitis *milieu*, *C. albicans* promotes alterations in the glycosylation pattern of keratinocyte-derived sEVs to interact with inhibitory Siglecs on antigen presenting cells. Hence, a strategy aiming at this pathway to enhance antifungal responses and restrict pathogen spread could offer novel therapeutic options for skin candidiasis in AD.

Keywords: Candida albicans, glycosylation, extracellular vesicle, keratinocyte, siglec, atopic dermatitis (AD), immune evasion, exosomes

INTRODUCTION

Candida albicans (*C. albicans*) is a pathogen which can colonise the skin of atopic dermatitis (AD) patients, contributing to exacerbation of clinical symptoms (1, 2). Suspected mechanisms beyond the spread of the pathogen suggest that the exposure to *C. albicans* in the context of atopic inflammation promotes complex cytokine responses, with a pronounced involvement of Th17 cells (3–5), as confirmed by increased candidiasis risk in patients undergoing anti-IL-17 (6) therapy, in whom these cells are lacking. In effect, IgE-mediated hypersensitivity may follow (5), as a consequence of class-switching events (7) involving the antibodies directed against the yeast (1, 8). In addition, reduced lymphocyte proliferation upon *C. albicans* stimulation was observed in early AD studies (9); all this may compound the pathology.

C. albicans can be recognised by numerous innate receptors (10); epidermal keratinocytes, which form the uppermost layer of the skin and naturally come in contact with *C. albicans* are involved in the innate response directed against the fungus. Specifically, keratinocytes sense the invasion through pattern recognition receptors (PRRs) (11), i.e., Toll-like receptors (TLR) -2, -4 and -9, C-type lectins (dectin-1, DC-SIGN, mannose receptor), galectin-3 as well as NOD-like receptor NLRP3; some of those receptors are only expressed in activated keratinocytes and not in the steady state (11–14). Interestingly however, *C. albicans* has not been reported to be directly recognised by Siglec-type receptors which are abundantly expressed by the Langerhans cells. It has been documented that keratinocytes respond to the fungal threat by secretion of immune cell-attracting cytokines and chemokines (11).

In addition to the soluble factors, keratinocytes also secrete a very different kind of mediators, i.e. membranous organelles known as extracellular vesicles (EVs); of those, the fraction of exosome-enriched small EVs (sEVs) seems to be involved in long-distance communication. sEV membrane can either non-specifically fuse with the membrane of the recipient cell or participate in receptor-ligand interaction; both may result in the sEV uptake (15, 16). In addition, binding itself can impact processes occurring in the target cell (17, 18). Keratinocytes secrete sEVs (19–21) containing antigens that the cells are exposed to (19) and mediators which promote response against pathogens (22). Only one study so far investigated keratinocyte response to infection in the EV context; the authors showed that sEVs may be carriers for the staphylococcal enterotoxin and stimulate polyclonal T cell responses (22). Little is known on the role of keratinocyte-derived sEVs in the defense against other skin pathogens, including in atopic skin disease which predisposes to difficult to treat infections.

Here, we focused on the primary events at the initiation of the immune response, i.e. the process of sEV interaction with immune cells. We investigated the adhesiveness of sEVs secreted by keratinocytes during their differentiation process as well as the modifying effect of the AD *milieu* and exposure of the cells to common skin pathogens, i.e. *Candida albicans* and *Staphylococcus aureus*. Using lectin array we next profiled carbohydrate moieties present on the surface of the adhesive sEVs and identified glycosylation patterns which could be correlated with the increase in the propensity of sEVs to interact with dendritic cells (DCs). Modelling of those carbohydrate patterns onto DC receptors identified potential binding partners; these were validated experimentally. We found that Siglec-7 and Siglec-9 blockade reduced interaction of keratinocyte-derived sEVs, suggesting the role for those receptors in the process of information transfer between keratinocytes and antigen presenting cells, with relevance to the setting of allergic skin inflammation and *C. albicans* infection. Further analysis of the carbohydrate moieties suggested ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) as enzymes likely contributing to the changes on the sEV surface. Hence, targeting either this sialyltransferase or inhibitory Siglecs during sEV-cell interaction could be explored as a novel therapeutic strategy to enhance antifungal response in the patients.

RESULTS

Atopic Dermatitis Inflammatory Milieu Promotes Changes *in sEVs Secreted by Proliferatory and* Differentiated Keratinocytes Leading *to* Their Differential Interaction With Denritic Cells

Keratinocyte gene expression is heavily remodelled during the process of differentiation and this affects all organelles (23).

Keratinocyte-derived extracellular vesicles, and especially exosome-enriched sEVs may provide both an efficient source of antigenic information and contain innate mediators (24–26); their uptake may alert residing skin immune cells. Previous studies documented secretion of sEVs by epidermal murine and human keratinocytes (19, 20, 27, 28); however, little is known about functional effects of keratinocyte differentiation state on the fate of those vesicles and their downstream effect on immune responses to pathogens. Hence, we set out to investigate if the differentiation advancement of keratinocytes may have any effects on the process of sEV interaction with antigen presenting cells. To begin, we isolated primary normal human epidermal keratinocytes (NHEK) from skin samples. Cultures were brought to near-confluence; at this point we performed "calcium switch" on the cells, by either keeping them in the proliferation (low calcium; 0.06 mM) or differentiation-promoting (high calcium; 1.5 mM) medium. We harvested conditioned media after 72h, isolated sEV fractions (100K pellet) using ultracentrifugation method (**Figure 1A**) and assessed their size and morphology (**Figures 1B, C**). 100K pellets contained cup-shaped vesicles enriched in exosomal markers (CD63, CD9 and syntenin), while being negative for calnexin (**Figure 1D**), as expected (29).

Next, we set out to test for differentiation-dependent changes in the capacity of NHEK-derived sEVs to transfer the pathogen-dependent signals to antigen presenting cells. To this end, we generated dendritic cell models (iMDDC, immature dendritic cells and mMDDC, mature dendritic cells) and subjected them to the PKH67-labelled sEVs, to allow fluorescent tracking of their interaction with the cells by flow cytometry. To this end, since the technique itself does not allow to discriminate between the sEV binding and uptake, for the purpose of this study we interpret positive signal as the 'interaction' between sEVs and recipient cells. However, it is highly likely that the uptake may occur given the nature of these interactions. Here we found that both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ interacted with mature and immature dendritic cells; the signal observed was higher for the latter, as expected given the efficient phagocytic ability of those cells. However, although we observed a trend towards more effective interaction of $sEV_{0.06NHEK}$, this difference was not significant, suggesting that in the healthy skin this interaction is not dependent on the differentiation status of the secreting steady-state keratinocytes (**Figures S1A–F**). Nevertheless, we anticipated that keratinocytes might communicate *via* sEVs differently when activated through specific conditions, such as

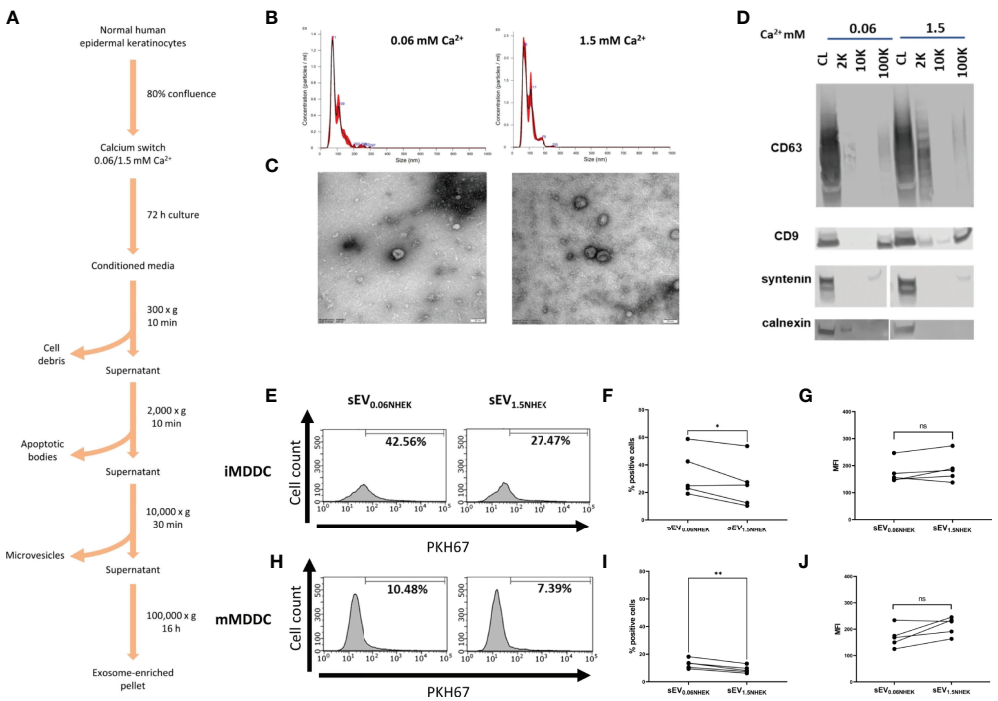


FIGURE 1 | Atopic dermatitis *milieu* alters properties of keratinocyte-derived small extracellular vesicles (sEVs) which leads to their differential interaction with dendritic cells. **(A)** Protocol for sEV isolation from NHEK-conditioned medium; **(B)** size distribution of NHEK-derived sEVs (100K pellet) by Nanoparticle Tracking Analysis (NTA); **(C)** electron microscopy pictures confirming enrichment of cup-shaped exosomes within 100K pellet sEV fraction; scale bar: 50nm; **(D)** marker expression pattern in 2K, 10K and 100K fractions during isolation, confirming enrichment of exosomal makers in the sEV fraction (100K pellet); **(E)** iMDDC interaction with sEV_{NHEK} secreted under AD cytokine stimulation; example FACS data; **(F)** iMDDC interaction with sEV_{NHEK} under AD cytokine stimulation; % positive cells data pooled from n = 5 donors; **(G)** iMDDC interaction with sEV_{NHEK} under AD cytokine stimulation; MFI data pooled from n = 5 donors; **(H)** mMDDC interaction with sEV_{NHEK}; example FACS data; **(I)** mMDDC % positive cells data pooled from n = 5 donors; **(J)** mMDDC interaction with sEV_{NHEK} under AD cytokine stimulation; MFI data pooled from n = 5 donors; *p < 0.05; **p < 0.01 (paired t-test). ns, not significant.

inflammation in AD. To test this we subjected the cells to the "AD cytokine cocktail" (containing IL-4, IL-13, IL-22 and TSLP) at the time of the calcium switch. Here, we observed that $sEV_{0.06NHEK}$ were interacting more than $sEV_{1.5NHEK}$ when produced by keratinocytes in the "AD inflammatory context"; the difference was not very pronounced, i.e. only 23% and 30% reduction for the imMDDC and mMDDC models, respectively; yet observed as significant and consistent for different donors (**Figures 1E–J**).

Keratinocyte-Derived sEVs Are Enriched in Glycoproteins Involved in Adhesion

Next, to better understand how keratinocyte differentiation state in combination with AD *milieu* may affect the sEVs in the context of cell adhesion, we analyzed $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ proteomes by LC-MS/MS and further profiling using Gene Ontology (GO), STRING and Reactome Pathway Database. We first started with the re-analysis of the proteomic dataset published by Chavez-Muñoz et al. (28), which contained results of proteomic profiling of exosome-enriched sEVs (sucrose cushion purified) derived from NHEKs in a very similar model to ours (0.07 and 1.8 mM calcium was used in that study, similar to our work). These results suggested that during calcium-induced differentiation steady state cells increase content of sEV_{NHEK} proteins which could be assigned the 'cell adhesion' term (GO:0007155) by GO analysis, albeit this is not very pronounced (**Figure 2A**).

When we exposed sEV-secreting NHEKs to AD cytokines, we detected a greater variety of proteins in $sEV_{0.06NHEK}$ in comparison to $sEV_{1.5NHEK}$, suggesting that the differentiation process in keratinocytes leads to a shift to a more profiled sEV proteome (**Table S1**). Interestingly, all of the proteins identified in $sEV_{1.5NHEK}$ were also found in $sEV_{0.06NHEK}$. Similarly to the steady state conditions, we also observed a higher proportion of proteins assigned with the 'cell adhesion' term present in $sEV_{1.5NHEK}$ compared to $sEV_{0.06NHEK}$ (**Figure 2B**). Further STRING analysis of the 'cell adhesion'-related proteins revealed strong predicted interactions between the vast majority of those (**Figure 2C**). A similar proportion of proteins among both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ proteomes was predicted to interact with 10 or more partners within their corresponding datasets (pink circle in **Figure 2C**) and we did not observe any substantial differences between the conditions, suggesting that the presence of adhesion-relevant proteins alone in not sufficient to define the sEV_{NHEK} propensity for differential interaction with a cell. However, the adhesive properties of EVs have been shown to also depend on their surface glycosylation pattern. Hence, we next assessed the content of glycoproteins which may undergo such a modification. Nevertheless, the number of glycoproteins implicated in cell or extracellular matrix (ECM) adhesion detected in both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ was similar (**Figure 2D**). Further analysis of the sEV_{NHEK} proteome against Reactome Pathway Database also revealed similar extent of overrepresentation in glycosylation-related pathways in both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ (**Figure 2E** and **Figure S2A**). Interestingly, we also noted that a number of enzymes involved in protein glycosylation or N-linked carbohydrate processing during glycoprotein turnover were also present in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ (**Figure S2B**).

Exposure to *C. Albicans* but Not *S. Aureus* in the Context of AD Milieu Promotes sEV Cell Interaction

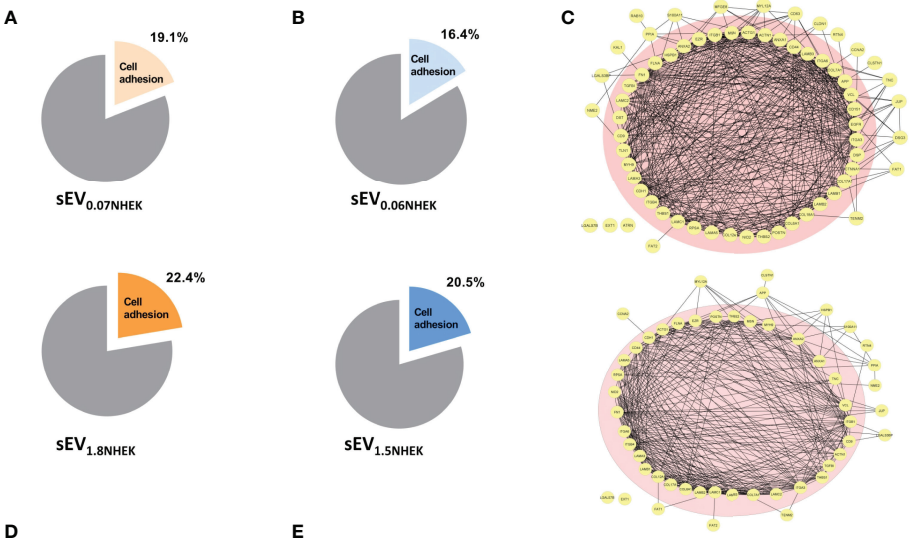
Since the previous results did not provide any strong indications on the functional differences we observed, i.e. sEV_{NHEK} secreted by both steady-state and "AD *milieu*-exposed" NHEKs seemed to have similar content of adhesion-relevant proteins, including glycoproteins, we deepened our analysis by the addition of another AD-relevant factor. Specifically, given that the allergic-type AD inflammation *milieu* is often clinically overlayed with an infection by AD-related pathogens, we aimed to investigate the effect of a combined stimulation of keratinocytes by AD cytokines and either *S. aureus* or *C. albicans* on sEV_{NHEK}-cell interaction. Here, in the case of iMDDCs we noted increased interaction of the $sEV_{0.06NHEK}$ secreted by AD/*C. albicans*-stimulated cells in comparison to the $sEV_{0.06NHEK}$ obtained from keratinocytes treated only with the cytokines; no similar difference was noted for the $sEV_{1.5NHEK}$ interaction (**Figure 3A**). In contrast, while differential interaction was also observed between AD/*C. albicans* vs AD-control in the mMDDC model, this was noted for $sEV_{1.5NHEK}$ rather than $sEV_{0.06NHEK}$ (**Figure 3B**). Intrestingly, we did not see any of those effects with sEVs produced upon the exposure of cells to AD/*S. aureus*, suggesting that the pathways which lead to the difference in the sEV interaction may be more specifically activated by the fungus and not broadly relevant to all skin pathogens or general keratinocyte activation.

Pathways of Innate Recognition Of *C. Albicans and AD* Cytokine Signalling Are Linked *to the* Glycosylation Enzyme Network in Keratinocytes

Identification of additional conditions promoting sEV_{NHEK}-cell interaction allowed us to further hypothesize on the mechanisms involved. Specifically, we asked whether *C. albicans* recognition by keratinocytes may affect pathways involved in glycosylation. To this end we next modelled protein networks between the pathogen recognition receptors (PRR) and glycosylation enzymes, based on the work of Schjoldager et al. (30) (**Table S2**). Given differential results between the two AD pathogens, for this analysis we only included PRRs known to be involved in the recognition of *C. albicans*, but excluded those implicated in the detection of *S. aureus*. The networks identified 11 enzymes with recognised links to PRR-mediated signalling (**Figures 3C, D**; full list, including references in **Table S3**), implying that exposure of keratinocytes to this pathogen may impact protein glycosylation.

Increased Expression of Glycosylation Enzymes in the *AD* Skin Is Disease-Specific and Not Observed in Psoriasis

Next, we investigated the levels of the identified enzymes in the AD skin, by analysing publically available transcriptome



D

Glycoproteins

0.06	1.5
Syndecan-1	Syndecan-1
Syndecan-4	Syndecan-4
Heparan sulfate proteoglycan 2	Heparan sulfate proteoglycan 2
Fibronectin-1	Fibronectin-1
Tenascin-C	Tenascin-C
Galectin-3-binding protein	Galectin-3-binding protein
Glypican-1	Glypican-1
Integrin ITGA3	Integrin ITGA3
Integrin ITGA6	Integrin ITGA6
Integrin ITGB1	Integrin ITGB1
Integrin ITGB4	Integrin ITGB4
Thrombospondin-1	Thrombospondin-1
Thrombospondin-2	Thrombospondin-2
E-cadherin	E-cadherin
Agrin	Agrin
CD44	CD44
Desmoglein-3	

E

Glycosylation-related Reactome pathway analysis

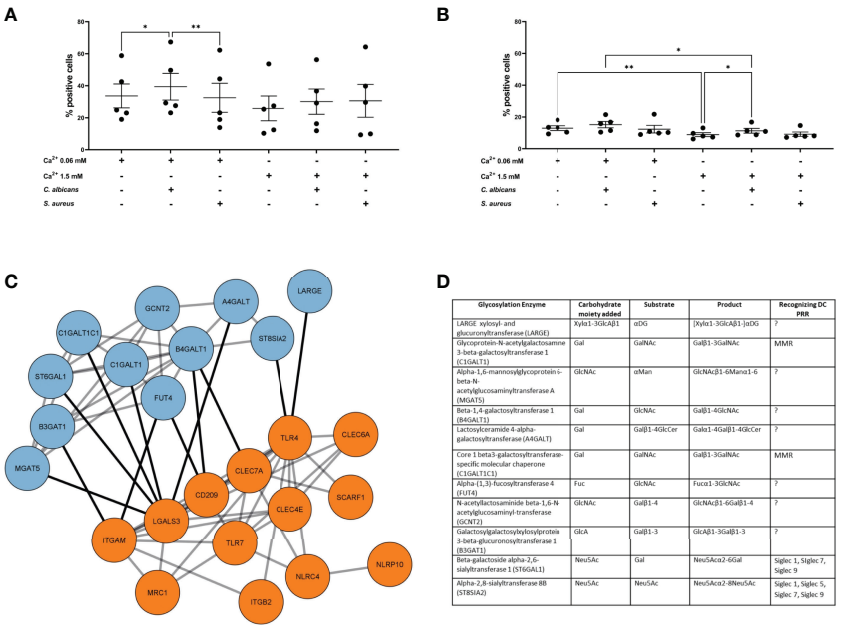
Ca2+ concentration (mM)	Pathway identifier	Pathway name	FDR
0.06	R-HSA-446203	Asparagine N-linked glycosylation	1.334E-4
	R-HSA-3000178	ECM proteoglycans	1.477E-4
	R-HSA-532668	N-glycan trimming in the ER and Calnexin/Calreticulin cycle	1.364E-3
	R-HSA-3560782	Diseases associated with glycosaminoglycan metabolism	3.435E-3
	R-HSA-879415	Advanced glycosylation endproduct receptor signaling	0.037
1.5	R-HSA-3000178	ECM proteoglycans	1.407E-6
	R-HSA-3560782	Diseases associated with glycosaminoglycan metabolism	2.445E-4
	R-HSA-532668	N-glycan trimming in the ER and Calnexin/Calreticulin cycle	5.231E-3
	R-HSA-3781865	Diseases of glycosylation	6.072E-3
	R-HSA-1630316	Glycosaminoglycan metabolism	0.023

FIGURE 2 | Keratinocyte-derived sEVs are enriched in glycoproteins involved in adhesion in steady state and in AD *milieu*. **(A)** Enrichment of adhesion proteins involved in cell adhesion in sEV_{NHEK} secreted in steady-state keratinocytes; Reactome - Gene Ontology terms re-analysis of the data available from Chavez-Muñoz et al. (28); **(B)** enrichment of adhesion proteins involved in cell adhesion in sEV_{NHEK} secreted by NHEKs exposed to AD *milieu* as identified by Reactome - Gene Ontology terms analysis; **(C)** interaction network for sEV adhesion-relevant proteins identified in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$; **(D)** cell adhesion-relevant glycoproteins identified by mass spec in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$; **(E)** Reactome-Gene Ontology identified term enrichment for the proteins in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$; FDR, False Discovery Rate; N.B. classical exosomal glycoprotein markers are included in the supplementary data (**Figure S2D**); mass spectrometry data based on n=3 biological replicates.

profiling datasets published by He et al., Leung et al. and Esaki et al. (31–33). This analysis revealed differential expression for 7 out of 11 glycosylation enzymes listed in **Figure 3D**. Specifically, while the identified proteins differed between the studies, we consistently noticed a positive change in expression (upregulation) for all the differentially regulated genes in AD; this was observed both in the epidermal samples obtained by tape stripping (He et al. and Leung et al.) and those harvested by laser-capture microdissection (Esaki et al.), increasing our confidence in the physiological relevance of the obtained data. Here we noted several enzymes differentially expressed, however, only FUT4 and ST6GAL1 were detected as upregulated in at least two of those datasets (**Figure 4A**). Furthermore, a comparison of the levels of the enzymes in AD vs psoriatic epidermis suggested a degree of disease-specificity, with B4GALT1, FUT4 and ST6GAL1 found expressed at levels significantly higher than in psoriasis. Interestingly, of all the enzymes, the highly upregulated expression of ST6GAL1 in lesional AD epidermis compared to the healthy epidermis was the most consistent and significant across all the three datasets analyzed. However, the single cell dataset that we sourced from the Skin Cell Atlas (34) was somehow different, showing increased expression in both those inflammatory skin diseases (**Figures S5A, B**), including in proliferating, undifferentiated and differentiated keratinocytes (**Figures 4B**, **S5B**).

Upregulation of Glycosylation Enzymes in the *AD* Skin Is Driven by Atopic Milieu and Not by Filaggrin Insufficiency

Next, having gathered substantial AD-relevant evidence, we aimed to provide additional insights into the causes leading to the expression changes we next attempted to identify links



D

Glycosylation Enzyme	Carbohydrate moiety added	Substrate	Product	Recognizing DC PRR
LARGE xylosyl- and glucuronyltransferase (LARGE)	Xylα1-3GlcAβ1	αDG	[Xylα1-3GlcAβ1-]αDG	?
Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GALT1)	Gal	GalNAc	Galβ1-3GalNAc	MMR
Alpha-1,6-mannosylglycoprotein i-beta-N-acetylglucosaminyltransferase A (MGAT5)	GlcNAc	αMan	GlcNAcβ1-6Manα1-6	?
Beta-1,4-galactosyltransferase 1 (B4GALT1)	Gal	GlcNAc	Galβ1-4GlcNAc	?
Lactosylceramide 4-alpha-galactosyltransferase (A4GALT)	Gal	Galβ1-4GlcCer	Galα1-4Galβ1-4GlcCer	?
Core 1 beta3-galactosyltransferase-specific molecular chaperone (C1GALT1C1)	Gal	GalNAc	Galβ1-3GalNAc	MMR
Alpha-(1,3)-fucosyltransferase 4 (FUT4)	Fuc	GlcNAc	Fucα1-3GlcNAc	?
N-acetyllactosaminide beta-1,6-N acetylglucosaminyl-transferase (GCNT2)	GlcNAc	Galβ1-4	GlcNAcβ1-6Galβ1-4	?
Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1 (B3GAT1)	GlcA	Galβ1-3	GlcAβ1-3Galβ1-3	?
Beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1)	Neu5Ac	Gal	Neu5Acα2-6Gal	Siglec 1, Siglec 7, Siglec 9
Alpha-2,8-sialyltransferase 8B (ST8SIA2)	Neu5Ac	Neu5Ac	Neu5Acα2-8Neu5Ac	Siglec 1, Siglec 5, Siglec 7, Siglec 9

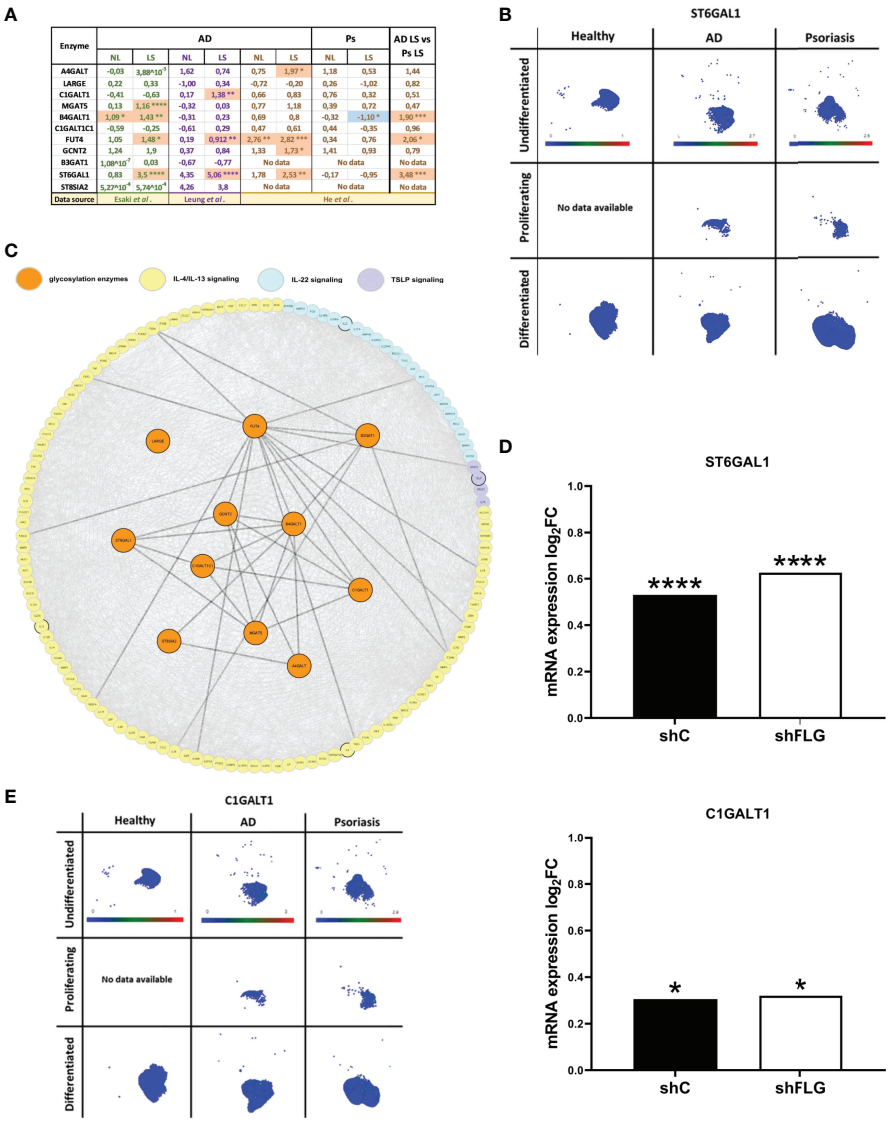
FIGURE 3 | AD *milieu* and *C albicans* exposure affects sEV_{NHEK}-cell interaction by interfering with glycosylation enzyme network in keratinocytes. **(A)** iMDDC interaction with sEV_{NHEK} under combined AD cytokine/pathogen stimulation; data pooled from n=5 donors; **(B)** mMDDCs interaction with sEV_{NHEK} under combined AD cytokine/pathogen stimulation; data pooled from n = 5 donors; *p < 0.05; **p < 0.01 (A-B: one-way ANOVA), means and SEM are shown; N.B. some comparisons with significant p-values are not labelled for the clarity of the graph; **(C)** STRING analysis of the protein networks between *C. albicans*-stimulated signalling from pattern recognition receptors in human keratinocytes (PRRs; in orange) and linked glycosylation-relevant enzymes (in blue); **(D)** Carbohydrate moiety/substrate/product-specificity of glycosylation enzymes identified in the network linked to *C. albicans*-specific PRRs (full list, including references in **Table S3**); DC, Dendritic cell; PRR, Pattern recognition receptor; PAMP, Pathogen associated molecular pattern; LacNAc, N-acetyllactosamine; GalNAc, N-acetylglucosamine; GlcNAc, N-acetylglucosamine; Fuc, Fucose; Gal, Galactose; Man, Mannose; Neu5Ac, N-acetylneuraminic acid; MMR, Macrophage mannose receptor; MGL, Macrophage galactose type lectin; DCIR, Dendritic cell immunoreceptor; BDCA2, Blood dendritic call antigen 2; CLEC, C-type lectin domain family; Siglec, sialic acid-binding immunoglobulin-type of lectin.

between the enzymes and signalling networks of AD cytokines (**Figure 4C**). This highlighted several connections to their downstream pathways which may suggest potential modulatory effect on the glycosylation network. To this end, we noticed that those connections were present mainly downstream from the IL-4/IL-13 pathway. To this end, we identified a transcriptomic dataset which reported an increase in expression of ST6GAL1 in normal keratinocytes (reconstructed into an organotypic model) upon IL-4/IL-13 treatment (35), further suggesting that atopic cytokines may affect this pathway.

Downregulation of filaggrin (FLG) in AD skin is one of the hallmarks of the disease; the extensive impact of this downregulation is a reflection of multifaceted role FLG carries out in the skin (36, 37), also at the keratinocyte biology level. Hence, it is plausible that FLG insufficiency itself could also affect the expression of the glycosylation enzymes; in which case the effect in the skin would not be exclusive to the *milieu* but could potentially require the underlying FLG insufficiency background providing synergestic effect. To test this, we used filaggrin-insufficient keratinocytes (shFLG), in which FLG expression was reduced by shRNA interference (38–40) and the control (shC) line; we assessed mRNA expression in both lines upon exposure to IL-4/IL-13. We determined that two of the enzymes, i.e. ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) were upregulated by this treatment (**Figure 4D**); expression of both of those enzymes was increased in AD epidermis as detected before in AD skin. Importantly, however, there was no difference between shC and shFLG cells, ruling out that FLG status is prerequisite to the observed effect. Given that C1GALT1 is a core extension enzyme and its activity in generating O-linked glycans is prerequisite to the action of the ST6GAL1 (as a capping enzyme), we also investigated the distribution of its expression reported by Skin Atlas. Here, we noted that C1GALT1 levels are pronouncedly upregulated in both AD and psoriatic keratinocytes, with no visibly significant difference between the two conditions (**Figure 4E**), which is in line with the analysis of the skin transcriptomic data (**Figure 4A**). This may suggest that the expression of the two enzymes may be regulated differently with the involvement of either both C1GALT1 and ST6GAL1 or exclusively ST6GAL1 for AD and psoriasis, respectively.

sEVs Secreted Under Exposure to *C. Albicans* and *AD* Cytokines Express Altered Surface Glycosylation Pattern

Next, to investigate the glycosylation pattern on the surface of sEVs which were characterised by increased cell interaction, we proceeded with the identification of the sEV membrane-exposed



Enzyme	AD				Ps		AD LS vs Ps LS
	NL	LS	NL	LS	NL	LS	
A4GALT	-0,03	3,88^10^7	1,62	0,74	0,75	1,97 *	1,18	0,53	1,44
LARGE	0,22	0,33	-1,00	0,34	-0,72	-0,20	0,26	-1,02	0,82
C1GALT1	-0,41	-0,63	0,17	1,38 **	0,66	0,83	0,76	0,32	0,51
MGAT5	0,13	1,16 ****	-0,32	0,03	0,77	1,18	0,39	0,72	0,47
B4GALT1	1,09 *	1,43 **	-0,31	0,23	0,69	0,8	-0,32	-1,10 *	1,90 ***
C1GALT1C1	-0,59	-0,25	-0,61	0,29	0,47	0,61	0,44	-0,35	0,96
FUT4	1,05	1,48 *	0,19	0,912 **	2,76 **	2,82 ***	0,34	0,76	2,06 *
GCNT2	1,24	1,9	0,37	0,84	1,33	1,73 *	1,41	0,93	0,79
B3GAT1	1,08^10^7	0,03	-0,67	-0,77	No data		No data		No data
ST6GAL1	0,83	3,5 ****	4,35	5,06 ****	1,78	2,53 **	-0,17	-0,95	3,48 ***
ST8SIA2	5,27^10^4	5,74^10^4	4,26	3,8	No data		No data		No data
Data source	Esaki et al.		Leung et al.		He et al.				

FIGURE 4 | Glycosylation enzymes are upregulated in the epidermis of AD patients and keratinocytes exposed to AD *milieu*. **(A)** The expression of glycosylation enzymes in the epidermis of AD and Ps patients; analysis of the transcriptome profiling data available as datasets in Esaki et al., Leung et al. and He et al. (values in the table show expression log_2FC compared to healthy epidermis); *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; (moderated t-test for Esaki et al. and He et al., multiple unpaired t-tests for Leung et al.); NL, non-lesional epidermis; LS, lesional epidermis; **(B)** UMAP plots of single cell expression of ST6GAL1 enzyme in keratinocyte subpopulations in the skin; data available through Human Developmental Cell Atlas; **(C)** protein network links between AD-relevant cytokine pathways and the previously identified 11 glycosylation-relevant enzymes; **(D)** mRNA expression of glycosylation enzymes differentially regulated upon exposure to IL-4/IL-13 stimulation in filaggrin-insufficient (shFLG; knockdown) keratinocytes and control (shC) keratinocytes (log2 fold change shown); combined data for n=3 biological replicates; *p < 0.05; ****p < 0.0001 (moderated t-test); **(E)** UMAP plots of single cell expression of C1GALT1 enzyme in keratinocyte subpopulations in the skin; data available through Human Developmental Cell Atlas; ST6GAL1, β-Galactoside α-2,6-Sialyltransferase 1; C1GALT1, Core 1 β,3-Galactosyltransferase 1.

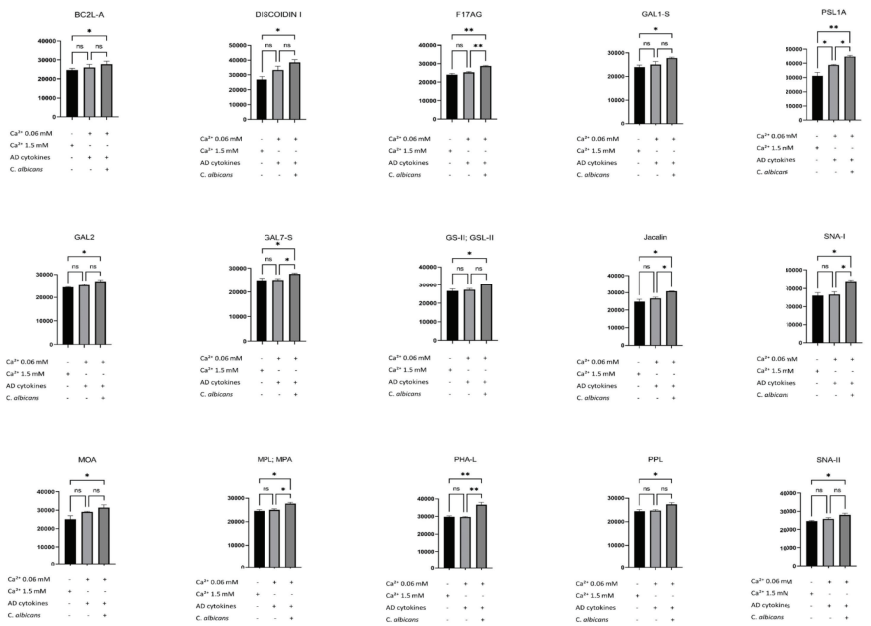
carbohydrate moieties by lectin array. Lectin array is a useful tool for glycosylation pattern identification; lectins on the slide have binding specificity towards defined carbohydrate moieties which allows identification of glycosylation of the bound molecules (either soluble or displayed on sEV (41)). To dissect the differences in the functional outcomes, we selected conditions on a spectrum of the interaction characteristics, i.e. AD/*C. albicans* $sEV_{0.06NHEK}$ vs AD cytokines $sEV_{0.06NHEK}$; we also included the condition which resulted in the lowest level of this interaction observed in our experiments, i.e. $sEV_{1.5NHEK}$ (at steady state). We observed substantial binding of sEVs to the array for 17 out of 70 lectins reporting significant changes between the conditions (**Figure 5A**, **Figure S2C**); we identified enrichment rather than *de novo* appearance of any additional carbohydrate patterns on sEVs secreted upon the combined AD/*C. albicans* stimulation. The results were filtered to identify lectins for which sEV binding to the array followed the functional results of the cell interaction (the highest for the AD/*C. albicans* $sEV_{0.06NHEK}$, the lowest for

$sEV_{1.5NHEK}$ and intermediate for AD cytokines $sEV_{0.06NHEK}$). This yielded 15 lectins binding glycans enriched in sEVs characterised by increased interaction; the binding from the majority (almost 90%) of the lectins with differential outcomes represented the trend, indicating unidirectional alterations in the sEV glycan profile. The resulting panel of the identified carbohydrate moieties was next matched to the innate carbohydrate recognition receptors on the antigen presenting cells, yielding several potential binding partners suitable for experimental validation (**Figure 5B**; full list, including references in **Table S4**).

Siglec-7 and Siglec-9 Receptors Are Involved in the Interaction Between Keratinocyte-Derived sEVs and Antigen Presenting Cells

Since the binding to the array revealed a considerable level of detected glycans on sEV_{NHEK}, we reasoned that these

A



B

Lectin	Carbohydrate Specificity (PAMP)	DC PRR
BC2L-A	High mannose	MMR, Dectin 2, DC-SIGN, Langerin, CLEC2, Mincle, DCIR, BDCA2
Discoidin 1	GalNAc	Langerin, MGL
	Galβ1-3GalNAc	MMR
	GalNAcβ1-3Gal	MGL
	αGalNAc (Tn antigen)	MGL
	Gal	MGL, MMR
	GlcNAcβ1-3Gal	Langerin
	LacNAc	BDCA, DCIR, Galectin 1
F17AG	GlcNAc	Langerin, BDCA2
	pNP-GlcNAc	?
	GlcNAcβ1-3Gal	Langerin
GAL1S	β-galactoside	Galectin
	Gal	MGL, MMR
	LacNAc	Galectin 1
	branched-LacNAc	Galectin
Gal2	GalNAcα1-3Gal (Blood group A)	MGL, DC-SIGN
	Lac	BDCA2, CLEC2B
	branched-LacNAc	Galectin
Gal7s	Gal	MGL, MMR
	Galβ1-3GlcNAc	DC-SIGN
GS1, GSL2, BSL1	αGal	MMR, MGL
	α3GalNAc	MGL
Jacalin	Galβ1-3GalNAc	MMR
MOA	Galα1-3Gal	MMR
	Galα1-3Galβ1-4GlcNAc	DC-SIGN
	Galα1-3Fucα1-2Galβ1-4GlcNAc (Blood group B)	DC-SIGN, Langerin
MPL, MPA	Gal	MMR, MGL
	GalNAc	MGL
	Galβ1-3GalNAc	MMR
PHA-L	Man	MMR, Langerin, DC-SIGN, BDCA2
	Gal	MGL, MMR
	GlcNAc	Langerin, DCIR2, BDCA2
	Galβ1-4GlcNAcβ2-6GlcNAcβ1-2Manα3Manα3	?
PPL	GalNAc	MGL
	Gal	MGL, MMR
	α/βGalNAc	MGL,
PSL1a	Neu5Acα2-6 linkages [α2-6 Sialic acid]	Siglec 1, Siglec 2, Siglec 3, Siglec 5, Siglec 7, Siglec 8, Siglec 9, Siglec 10
	Neu5Acα2-6Galβ1-4GlcNAc	Siglec 1, Siglec 9
Sna1	Neu5Acα2-6Gal	Siglec 1, Siglec 7, Siglec 9
	NeuAcα2-6Galβ1-4Glc	Siglec 9
	Neu5Acα2-6GalNAc	Siglec 2, Siglec 3, Siglec 5, Siglec 7, Siglec 9
	GalNAc	MGL
	Lac	BDCA2, CLEC2B
	GalNeu5Acα2-6Gal	?
Sna2	Gal	MMR, MGL
	GalNAc	MGL
	Neu5Acα2-6Lac	Siglec 1, Siglec 2, Siglec 3, Siglec 5, Siglec 7, Siglec 8, Siglec 9, Siglec 10
	GalNAcα1-3Gal	MGL, DC-SIGN

FIGURE 5 | Lectin array-based identification of carbohydrate moieties on the adhesive sEV_{NHEK} map to potential receptors on antigen presenting cells. **(A)** Lectin binding to carbohydrate moieties on the surface of sEV_{NHEK}; data summarizes biological duplicates with n=3 keratinocyte donors; *$p < 0.05$; **$p < 0.01$; **(B)** carbohydrate moieties enriched in the more adhesive sEVs as identified by lectin array, together with potential binding receptors on antigen presenting cells as identified by the literature search (full list, including references in **Table S4**). ns, not significant.

glycosylation patterns may also promote interaction between sEVs and antigen presenting cells in the steady state. Hence, to identify the receptor route involved, we next performed receptor blocking experiments, using IL-4/GM-CSF-differentiated THP-1 cells, serving as antigen presenting cells and N/TERT-1 immortalised keratinocytes (42, 43) as an efficent sEV source (**Figure S3**). Given the previously identified carbohydrate moieties involved, we focused on the C-type Lectin Receptors (CLRs), i.e. Langerin (CD207), Macrophage Mannose Receptor (MMR; CD206), Dendritic Cell-Specific Intercellular adhesion molecule (DC-SIGN; CD209). In addition, we also investigated Sialic acid-binding Immunoglobulin-like Lectins known to recognize sialic acid. Specifically, we included Siglec-2, Siglec-7 and Siglec-9, as these receptors could be matched to the recognized carbohydrate moieties detected by the array but they differ in specificity and affinity to the same glycans (44). In this model differences could be observed in the expression level for the selected receptors during the differentiation process; a positive population was present for each of the markers and further increased during THP-1 differentiation (**Figure S4A**). Of those, DC-SIGN expression was the highest and seen for nearly all of the cells; on the other hand, Langerin was the least abundant marker, as expected, but also showed an increase in expression. However, the differences in the outcome of the blocking experiments with specific antibodies could not be simply explained by the variation in the receptor expression levels. Specifically, while we observed no clear effect of the anti-MMR, -Langerin, -DC-SIGN (**Figure S4B**) and Siglec-2 antibodies, blocking with Siglec-7- and Siglec-9-specific antibodies significantly decreased cell interaction of either $sEV_{0.06NHEK}$ or both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$, respectively (**Figure 6A**); the most profound effect was observed upon Siglec-9 blockade. We also observed the p-value approaching significance (p=0.063) for the anti-Siglec-7 blocking of the $sEV_{1.5NHEK}$ interaction; as well as (a non-significant) trend for anti-Siglec-2 blokade. Siglec-7 and Siglec-9 are known inhibitory receptors which decrease PRR-dependent activation of the cell upon sialic acid binding (45, 46). Their expression within the epidermis is almost exclusively confined to the population of Langerhans cells (**Figure 6B**). In the whole skin samples those receptors have broader and elevated expression in inflammatory skin diseases, i.e. AD and psoriasis (Ps). However, the expression is, still mainly confined to the myeloid cell populations, as identified by single cell analysis available through Skin Cell Atlas (34). This includes macrophages, monocyte-derived DCs, LCs, etc., which can serve as antigen presenting cells in the skin (**Figure 6C**; **Figure S5A**). Combined, these results suggest that sialic acid moieties could provide a specific targeting motif directing keratinocyte-derived sEVs to antigen presenting cell populations in the tissue.

Changes in Glycosylation Pathways Are Implicated *in* Sialylation Pattern *on* Keratinocyte-Derived *sEVs and* Define Their Interaction With *Siglec-7 and Siglec-9* Receptors

Finally, we set out to determine the specific enzyme(s) implicated in the observed process of sEV surface glycan remodelling. Indeed, the ST6GAL1 enzyme identified by us earlier within the glycosylation enzyme network linked to the *C. albicans*-stimulated PRR signalling (**Figure 3C**) and most consistently upregulated in AD skin and keratinocyte cultures by IL-4/IL-13 (**Figure 4**) was an immediate match to the glycosylation pattern observed, as it catalyses the reaction of the addition of sialic acid *via* an α2-6 linkage (**Figure S6A**). The associated GO terms for this enzyme reflected as enrichment of the term GO: 0005515 "Enables protein binding" (**Figure S6B**). We were not able to match other enzymes which we found upregulated by AD cytokines or in patient epidermal samples, including FUT4 enzyme, highly differentially expressed and consistent between the three studies. Likewise, C1GALT1 also does not seem to be of the primary importance in our model given that it despite that it produces Galβ1-3GalNAc linkage detected in the array; however, this linkage is recognised by the MMR receptor (47), blockade of which did not affect the strength of sEV-cell interactions (**Figure S4B**).

Finally, with the aim of identifying sEV_{NHEK} proteins that could be targeted by glycosylation changes we carried out further analysis, focusing on the direct interactomes of ST6GAL1 and C1GALT1 (**Figure 6D**). This revealed links to the other glycosylation-relevant proteins. In addition, both enzymes link to several mucins as expected given their inclusion in the mucin formation pathways. In addition, a node connection between ST6GAL1 and galectin-3 (LGALS3) can be also seen. This suggests potential substrates for the enzymes, displayed on the sEV_{NHEK} surface; unfortunately, none of those were found in our mass spectometry dataset, thus not providing us with any indications regarding the identity of the potential targets.

DISCUSSION

Small extracellular vesicles (sEVs) produced by virtually all living cells, including those of non-immune origin have been shown to participate in immune responses, both innate and adaptive (48, 49). sEV-specific role depends on their capacity to directly interact with cells, where receptor-ligand interactions could lead to a downstream effect. This cellular interaction is also critical initial event enabling sEV uptake and intracellular transfer of sEV cargo, enriched in antigens and innate signalling molecules or other immune-relevant compounds. Experiments with various cell lines indicated that sEV glycan composition, which is important during cellular adhesion of sEVs, is dependent on the cellular source. Importance of glycans as potential targeting motifs for the recipient cell was previously indicated (50).

To this end, our results suggest that the exposure of keratinocytes to stimulation relevant to atopic dermatitis (AD), i.e. allergic inflammatory *milieu* and *C. albicans* may induce relevant changes in the sEV surface glycosylation patterns and are translated into differential functional outcomes; here we showed certain increase in the propensity for sEV interaction with dendritic cells. By carbohydrate moiety identification with lectin array we subsequently determined that these alterations include enhanced expression of forms of sialic acid. Modelling

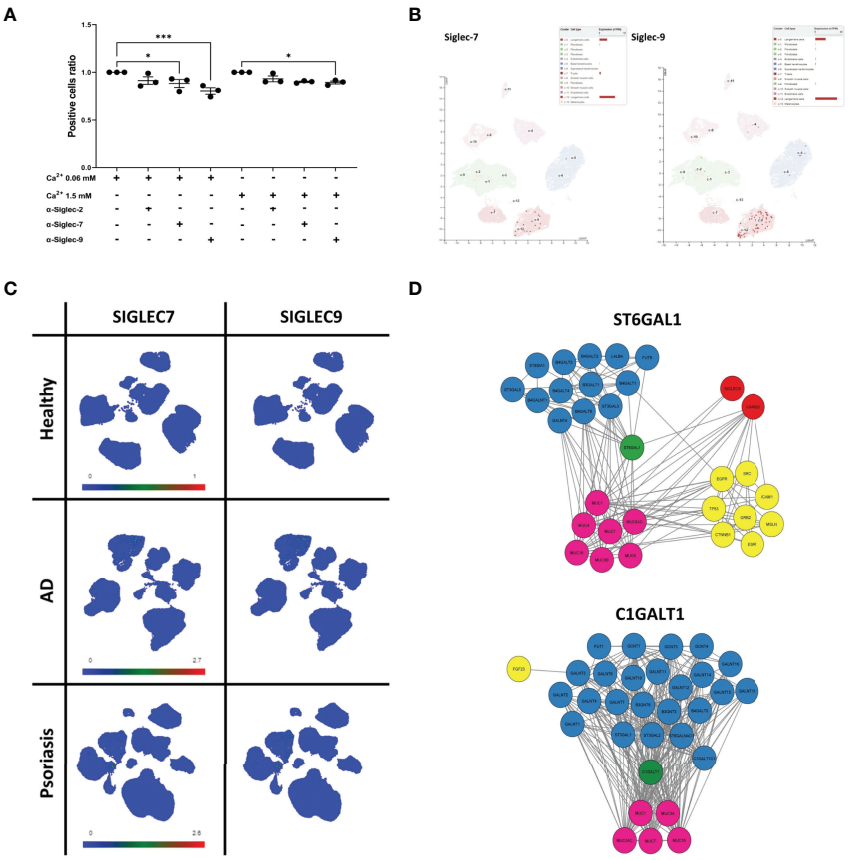


FIGURE 6 | Sialyltransferase ST6GAL1 is implicated in sialylation pattern on keratinocyte-derived sEVs and defines their cell interaction propensity *via* Siglec-7 and Siglec-9 receptors. **(A)** Identification of binding receptors for $sEV_{N/TERT-1}$ cell interaction by blocking experiments with anti Siglec-type receptors on differentiated THP-1 cells; combined data for n=3 biological replicates; **(B)** single-cell RNA expression levels of Siglec-7 and Siglec-9 in the epidermis of the skin; data available through ProteinAtlas; **(C)** UMAP plots of single cell expression of SIGLEC7 and SIGLEC9 receptors in the skin; data avalabale through Human Developmental Cell Atlas; **(D)** protein interaction network of ST6GAL1 and C1GALT1 enzymes obtained *via* STRING analysis. * - p<0.05, *** - p<0.001.

indicated that the effect downstream of the PRR signalling induced by *C. albicans* on the activity of sialyltransferase ST6GAL1 could provide a plausible explanation for this effect. Sialic acid-containing motifs were abundant on keratinocyte-derived sEVs and enriched further in vesicles characterised by enhanced propensity for cell interaction (41).

Innate recognition of sialic acid is mediated by a group of Sialic acid-binding Immunoglobulin-like Lectins (Siglecs) and we determined that the cell interaction of keratinocyte-derived sEVs in our model is Siglec-7 and Siglec-9-mediated. Furthermore, the dependence on either both Siglecs or exclusively Siglec-9 for the undifferentiated vs. differentiated keratinocytes as sEV sources, respectively, mirrors the difference observed by us earlier at the functional level. Specifically, higher interaction efficiency could be observed for $sEV_{0.06NHEK}$, for which the interaction seems to be aided by both of these receptors. Being two members of the CD33-related Siglec family both Siglec-7 and Siglec-9 are considered "endocytic" receptors (51, 52); therefore the sEV binding may promote their efficient uptake by the cells expressing those receptors. From the first glance this could appear as benefiting the host, since provision of *C. albicans* antigens and other stimulatory signals within the cargo should promote T cell responses. However, it has been shown that signalling through Siglec-7 and Siglec-9, which contain tyrosine-based inhibition motif (ITIM) within their intracellular domains dampens proinflammatory responses by inhibiting NF-κB-dependent TLR4 signalling pathway (53); as a result, stimulation of these "inhibitory Siglecs" provides strong negative signal. Interestingly, data published by Varchetta et al. (54) stands in contrast to the studies implicating the inhibitory role of Siglec-7 in immune response, suggesting a potential role of sialic acid-*independent* stimulation of this receptor in triggering the release of proinflammatory cytokines by monocytes. In this particular study Siglec-7 was activated by either antibody-mediated crosslinking or zymosan, a yeast cell wall-derived particle devoid of any forms of sialic acid. Here, since we propose a mechanism critically dependent on the sialic acid engagement of Siglec-7, the study might be less relevant in the context of our findings. However, it brings up a potentially important question regarding the possibility of different

functional outcomes upon the activation of the Siglec-7 pathway depending on the chemical composition of the ligand. Furthermore, while we did not establish the content of the sEV_{NHEK} cargo, these may contain RNA species, incl. regulatory, proteins and other effector molecules which could potentially also contribute to the effect.

While *C. albicans* expresses several PRR agonists, it does not seem to express enough sialic acid to stimulate Siglec receptors (10) and switch off the NF-κB signalling by Siglec-dependent inhibition. Besides, as a predominantly intracellular pathogen, *C. albicans* may not have any effective means to directly interact with the membrane-expressed Siglecs on the antigen presenting cells. Hence, the proposed mechanism could potentially increase the chances of the pathogen to achieve the induction of tolerogenicity, if *C. albicans* exploit the host's enzymatic machinery to induce sialic acid coating on sEVs. It has been also demonstrated that antigen sialylation results in the inhibition of Th1 and Th17 cells and induction of Treg subsets (55); given the Th1/Th17-dominated effective antifungal response, such an effect would similarly benefit *C. albicans*. Intrestingly, the inhibitory Siglec pathways seem to be hijacked by numerous PRRs-stimulating pathogens escaping immunosurveillance (46). To our best knowledge we are the first to report a possibility of remodelling of the host sEV surface glycosylation by a pathogen which could also constitute a potentially attractive and resource-saving immune evasion strategy. However, the relevance of this mechanism would have to be confimed in the dedicated immune evasion *in vivo* studies.

In the context of the skin disease, this mechanism may potentially have an important effect on the Siglec-7 and Siglec-9 expressing Langerhans cells (LCs). LCs switch between immunomodulation and immunactivation by integration of incoming stimuli (56); in the healthy skin this would mean tolerogenic phenotype upon encounter of non-threatening signals and proinflammatory one during skin infection. By preventing LC activation with inhibitory signalling, *C. albicans*-induced sEV sialylation could target those cells predominantly, given the LC-exclusive expression of these Siglecs in the epidermis. Deeper in the tissue, these receptors are also expressed by additional myeloid cells, all of which could both present antigens and react to innate stimuli, e.g. macrophages or dendritic cells. Hence, sialic acid-dependent exosome/sEV-mediated activation inhibition could have a potential to affect clearance of *C. albicans* from the AD skin and lead to its enhanced spreading.

Recently, the inhibitory Siglec-type receptors were proposed as a novel class of immune checkpoints targeting myeloid cells with inhibitors suggested for clinical application (45). In addition, in the cancer setting T cells may also express inhibitory Siglec-7 and Siglec-9, meaning that they could be directly targeted; in agreement with this, sialic acid-dependent exosome/sEV-mediated direct T cell inhibition was also previously shown (57). Interestingly, candidiasis itself increases risk of many malignancies (58); hence, the question that needs further addressing is if *C. albicans*-remodelled sEVs could suppress anticancer response and promote tumour growth.

Mammalian proteins involved in the glycosylation processes show great diversity (30). In our study, we identified 11 enzymes which could be linked to the innate response to *C. albicans* in keratinocytes. Of those, ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) seemed to be involved in the changes in the glycosylation pattern induced by the fungus on keratinocyte-derived sEVs, i.e. both enzymes are upregulated in AD and Ps and expressed in the majority of skin cell populations, including all subopulations of (proliferating, undifferentiated and differentiated) keratinocytes. It is important to note that Siglec-7 may also have additional specificities i.e also recognise 2-8 and 2-3-linked moieties (59, 60) (these were not identified by our lectin array screen) as well as detecting more complex epitopes, beyond isolated glycans (61). Interestingly, recent genome-wide CRISPR-aided screen highlighted the importance of additional enzymes (62) for generaton of Siglec-7 ligands, including C1GALT1. This makes sense as C1GALT1 is a core extension enzyme acting at the beginning of the synthesis pathway which generates core of the O-glycans exposed on the sEV surface, hence required as an anchor for the exposed ST6GAL1-generated moieties added subsequently. Recent study by Büll et al (61) indicated that the core extension feature prevails over the capping glycan features and the binding is completely abolished in the C1GALT1C1 (C1GLAT1-specific chaperone; COSMC) knockout. Hence, given the role of O-glycosylation for Siglec-7 recognition, it seems that the changes in the expression of this enzyme that we noted upon analysis of data from both the epidermal samples and cytokine-stimulated keratinocytes (normal (35) and FLG insufficient) may also critically contribute to the observed effect. It is unclear, however, if the direct product(s) of the enzyme (T-antigen) may be detected by Siglec-7; further glycan modifications by sequential enzymatic action of additional capping enzymes are likely required for the recognition; this is where ST6GAL1 may execute its role in our system as moiety exposed as a part of complex Siglec-7-recognized epitopes; additional role of sulfation has to be also considered given that a group of carbohydrate sulfotransferases (CHSTs) has been implicated in the binding affinity for both Siglecs we found important for sEV-cell interactions (61, 63). Some moieties may be of course less involved, e.g. our negative blocking data for MMR receptor which has high affinity to Gal1β-3GalNAc glycans (47) suggest considerably lower importance of this linkage in our model. In our study we were not able to match our data to enzymes implicated in the binding of Siglec-9 ligands, ST3GAL4/6 (61). Overall, our results support the notion on the complexity in the Siglec system; e.g. in our experiments we only observed slight disruption of the cellular interaction with Siglec-2 blokage (not significant, despite shared receptor specificity to the 2-6 linkage). This may be dependent on the breadth of the accepted ligand pool which is constricted for this Siglec and the differences in affinities of specific glycans containing the linkage in comparison to that of Siglec-7 and -9 as shown in detail by Blixt et al. (44).

Interestingly, ST6GAL1 seems to be an important enzyme during influenza infection, since the virus uses sialic acid-containing glycans as cellular entry points (64). It has been

shown that ST6GAL1 expression also correlates with poor tumour prognosis (65) and affects multiple mechanisms related to cancer (66), suggesting that the immune effect is not limited to infection. ST6GAL1 was also recently shown as enzymatically active cargo of both exosome-like sEVs and exomeres, capable of transferring sialyltransferase activity to receipient cells and inducing expression of sialylated proteins on the cell membrane (67). As for the C1GALT1 enzyme, it has also been implicated in cancer; however, the role is less clear-cut, with contradicting data on the pro-/antitumorogenic effect (68). The enzyme has also been linked to IgA nephropathy by deposition of galactose-deficient IgA1 (Gd-IgA1) circulating in the patients with *C1GALT1* mutation (69).

The exact identity of the sEV-expressed proteins which may be modified by the ST6GAL1 enzyme is not known, although we identified several proteins present within keratinocyte-derived sEVs which are likely to undergo such modification. Literature indicates some potential examples in sEVs, e.g. in a study in ovarian cancer-derived vesicles galectin-3-binding protein (LGAL3BP), was previously identified as a sialoprotein (70). LGAL3BP is a known sialic acid-dependent ligand for CD33-related Siglec family (71) (including Siglec-7 and Siglec-9), so could be potentially important in the sEV-mediated communication in the skin. Similarly, β1 integrins (72), also present in our samples, could be similarly modified; lipid molecules which may also be sialylated, as shown for ganglioside GD3 delivering a direct T cell inhibitory signal *via* sialic acid (57). With respect to successful adhesion and delivery of the inhibitory signal, also the spatial distribution of sialylated proteins on the surface of keratinocyte-derived sEVs could potentially affect the outcome, especially if the proteins segregate into rafts or microdomains on the surface of sEVs to mediate specific interaction by formation of so called "glycosynapses" (73); however, we did not determine this in this study.

In summary, our study showed that in the context of AD *C. albicans* promotes sialic acid-enriched glycosylation pattern on the host sEVs to increase their interaction with inhibitory Siglec receptors. We may predict potential future applicability of targeting this glycosylation-sEV-Siglec-dependent pathway as a novel adjuvant therapy in skin candidiasis in AD patients; however, we cannot exclude potential applicability also beyond the skin.

MATERIALS AND METHODS

Samples

Ethical approvals for the study were obtained from the Independent Bioethics Committee for Scientific Research at Medical University of Gdansk, ethical approval numbers: NKBBN/559/2017-2018 and NKBBN/621-574/2020. Perioperative skin samples (2-3 cm^2) were obtained from the individuals undergoing surgery at the Department of Surgical Oncology, Medical University of Gdansk, Poland. Until isolation, the material was stored in PBS (Sigma-Aldrich, St. Louis, MO, USA), with 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), in 4°C. Buffy coats were obtained as a byproduct from blood donations coming from healthy donors at the Regional Blood Centre in Gdansk.

Keratinocyte Isolation and Culture

Skin samples were washed in PBS with 100 U/ml penicillin + 100 μg/ml streptomycin, subcutaneous adipose tissue was removed and the samples were cut into small pieces. Epidermis was removed from the dermis after 2-3 hour incubation in dispase (12U/mL, Corning, NY, USA) at 37°C and digested in a 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) with trypsin inhibition with EpiLife Medium supplemented with EpiLife™ Defined Growth Supplement (EDGS) (Thermo Fisher Scientific, Waltham, MA, USA), antibiotics and 10% FBS (Sigma-Aldrich, St. Louis, MO, USA). Keratinocytes were seeded in a collagen IV-coated dishes (Corning, NY, USA) in EpiLife medium supplemented with EDGS, 100 U/ml penicillin + 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS. The next day, the medium was changed to a serum-free EpiLife with EDGS and antibiotics with regular changes every 2 days of culture at 37°C, 5% CO_2. For experiments, pooled NHEK cultures from n=3-4 donors were used. N/TERT-1 keratinocytes obtained as a kind gift from Prof Jim Rhinwald were cultured in Keratinocyte SFM medium (Thermo Fisher Scientific, Waltham, MA, USA) + 25 μg/mL bovine pituitary extract (Thermo Fisher Scientific, Waltham, MA, USA) + 0.2 ng/mL Epidermal Growth Factor (Thermo Fisher Scientific, Waltham, MA, USA) + 0.4 mM Ca^{2+}. ShC and shFLG cells were grown in Dulbecco's Modified Eagle's Medium (DMEM-high glucose, Sigma-Aldrich, St. Louis MO, USA). Media used for EV isolation contained no animal products or were supplemented with EV-depleted FBS.

Calcium Switch and AD-Relevant Treatments

Upon reaching 80% confluence in a serum-free EpiLife with EDGS supplemented with antibiotics (free from animal products), cells were washed and cultured for 3 days as undifferentiated (in 0.06 mM Ca^{2+}) or differentiated (in 1.5 mM Ca^{2+}) cells in the following conditions: untreated or treated with atopic dermatitis (AD) cytokine cocktail (20 ng/mL IL-4, and 10 ng/mL IL-13, IL-22 and TSLP each; Peprotech, London, UK). For pathogen treatment addition of 75 ng/mL of a selected AD-relevant inactivated pathogens; *Candida albicans* (prick test; Inmunotek, Madrid, Spain) and *Staphylococcus aureus* (heat-killed) was applied.

Heat-Killed Bacteria

1 ml of overnight culture of *S. aureus* "Newman" (2.4 x 10^9 CFU/mL) was centrifuged at 1700 x g. The cell pellet was washed with PBS and centrifuged 1700 x g, 5 min (2x) and resuspended in 1 ml PBS, followed by heat treatment with shaking (80°C, 30 min, 1000 rpm). The resulting suspension of heat-killed bacteria was cooled on ice and protease inhibitors (final concentration: 1 μM of E-64, 0.5 μg/mL of pepstatin A and 5 μM of leupeptin) were added after heat treatment in order to retain their stability and stored in -20°C.

EV Isolation

The exosome-enriched fraction of sEVs (100K pellet) secreted by keratinocytes was isolated by the differential ultracentrifugation protocol as in **Figure 1A**. Briefly, conditioned medium was first centrifuged at 300 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min to remove the cellular debris, followed by 2,000 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min to remove soluble proteins and apoptotic bodies (AP; 2K pellet). The supernatant was ultracentrifuged at 10,000 x g (maximum rotation speed) for 30 min (OptimaTM L-90K or OptimaTM LE-80K ultracentrifuge, Beckman Coulter, Brea, CA, USA) to isolate microvesicles (MVs; 10K pellet). The supernatant was then ultracentrifuged at 100,000 x g (maximum rotation speed) for 16 h to pellet exosome-enriched fraction (exosome-enriched sEVs; 100K pellet). The 100K pellet was washed in PBS by additional spin and stored at -20°C for further use.

sEVs were labelled using the PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. In brief, sEVs corresponding to 6 x 10^6 keratinocytes for a given culture condition were resuspended in 100 µl of Diluent C and incubated with 5 µM PKH67 for 5 minutes. The labelling reaction was quenched by the addition of 2x volume of EV-depleted complete RPMI medium (Sigma-aldrich, St. Louis, MO, USA) (supplemented with 10% EV-depleted FBS and 100 U/ml penicillin + 100 µg/ml streptomycin), and samples were then washed in PBS (100,000 x g (maximum rotation speed), 16 h, 4°C). Labelled sEVs were resuspended in EV-depleted complete RPMI medium and used directly for MDDC interaction assessment.

Western Blot

Cell lysates were prepared in RIPA buffer (Cell Signalling Technology, Danvers, MA, USA) supplemented with the cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA), vortexed well and centrifuged for 15 min at 4°C in 13,000 rpm. The supernatant was collected, and 4X Bolt™ LDS Sample Buffer (Thermo Fisher Scientific, Waltham, MA, USA) (10x diluted) was added. The same amount of the loading buffer was added to the EV samples in PBS (10x diluted). The samples were heated for 10 min at 80°C. EV samples (an equivalent of EVs isolated from 1.71 mln cells per well) were then loaded onto the Bolt™ 4 to 12% Bis-Tris precast gel (Thermo Fisher Scientific, Waltham, MA, USA) and ran for 30-60 min at 150V and then transferred onto nitrocellulose membranes (iBlot™ 2 Transfer Stacks, Thermo Fisher Scientific, Waltham, MA, USA) in the iBlot transfer system (iBlot™ 2 Gel Transfer Device, Thermo Fisher Scientific, Waltham, MA, USA). Next, the membranes were blocked in 5% fat-removed powdered milk (Carl Roth, Karlsruhe, Germany) in PBS for an hour on the shaker, and next incubated with primary antibodies overnight at 4°C on the shaker (all primary Abs were diluted 1:250, only CD63 was 1:500 diluted). The next day membranes were washed 3x for 5 min in PBS-T (PBS + 0.5ml/l Tween 20) and secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) in PBS-T (1:25,000) were added. After 30 min of incubation with the secondary antibodies the membranes were washed 3x for 5 min in PBS-T and once in PBS, paper-dried and scanned using the Odyssey CLx Near Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

EV Characterisation

Electron microscopy imaging was carried out as a paid service by the University of Gdansk Electron Microscopy Facility. Briefly, samples were adsorbed onto formvar/carbon-coated copper grids size 300 mesh (EM Resolutions, Sheffield, UK), stained with 1.5% uranyl acetate (BD Chemicals Ltd.), and imaged by Tecnai electron microscope (Tecnai Spirit BioTWIN, FEI, Hillsboro, OR, USA). Nanoparticle Tracking Analysis was carried out using NS300 NanoSight NTA (Malvern Panalytical, Malvern, UK), the EV samples were diluted 1000x in PBS.

Dendritic Cell Models

PBMCs were separated from buffy coat samples with Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada). $CD14^+$ cells were isolated by the MojoSort™ Human CD14 Selection Kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. Cells were seeded in 24-well plates in complete RPMI medium (supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin + 100 µg/ml streptomycin) at a density of 1 x 10^6 cells in 1 ml of medium per well. Cells were cultured for 7 days at 37°C and 5% CO_2 in the presence of 50 ng/mL (500 U/mL) GM-CSF and 200 ng/mL (1000 U/mL) IL-4 for the generation of immature monocyte-derived dendritic cells (iMDDCs). Cytokine-supplemented medium was replaced on day 2 and 4 of the culture. To generate mature monocyte-derived dendritic cells (mMDDCs) 1 µg/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) was added on day 6 of the culture.

EV Cell Interaction Assessment and Blocking

On day 7, iMDDCs and mMDDCs were washed, resuspended in EV-depleted complete RPMI medium and seeded on 96-well round-bottom plates at a density of 0.066 x 10^6 cells/well. Cells were then incubated for 4 h in a total volume of 100 µl/well with PKH67-labelled sEVs obtained from 1 x 10^6 of keratinocytes cultured as previously described. Cells were then washed with PBS (10 min, 300 x g), fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and the cell interaction of sEVs by MDDCs was analyzed by flow cytometry using the Millipore Guava EasyCyte Flow Cytometer (Merck Millipore, Burlington, MA, USA).

For the blocking experiment with a THP-1-based model, N/TERT-1-derived sEVs were used. sEVs were isolated by differential centrifugation as described before and quantified using NanoSight NS300 NTA (Malvern Panalytical, Malvern, UK). Before use sEVs were resuspended in Diluent C and labelled with PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich, St. Louis, MO, USA), for a mock control Diluent C alone was used for labelling. THP-1 cells were differentiated by culturing them in the presence of 1000 U/mL IL-4 (PeproTech, London, UK) and 50 ng/mL GM-CSF (PeproTech, London, UK) for 7 days. On days 2 and 4 of the

culture the whole medium was replaced; fresh medium was supplemented with cytokines as before. On day 7 cells were collected, washed, and treated with CD206, CD207, CD209, Siglec-2, Siglec-7 or Siglec-9 antibodies (Biolegend, San Diego, CA, USA) at 10 µg/mL for 1 hour at 37°C. Next, cells were washed twice with PBS and exposed to either PKH67-labelled N/TERT-1-derived sEVs or mock control, and incubated for 4 hours at 37°C. Cells were then washed, fixed Bioligand in 4% Formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and analyzed using the Millipore Guava EasyCyte Flow Cytometer (Merck Millipore, Burlington, MA, USA).

Lectin Array

Lectin array 70 product (GA-Lectin-70-14) was purchased from RayBiotech (Peachtree Corners, GA, USA) and the assay was run according to the manufacturer's protocol, with an anjustment of using PHK67-labelled sEVs directly as the sample source with the omission of flouorophore-conjugated detection antibody. The array slide was imaged with the Amersham Typhoon RGB scanner (Cy2 525BP20 filter) (Marlborough, MA, USA) at adjusted PMT voltages (intensities).

Mass Spectrometry

After lysis of sEVs with 1% SDS and cysteine residues' reduction with dithiothreitol, samples were processed in a standard Multi-Enzyme Digestion Filter Aided Sample Preparation (MED-FASP) procedure with cysteine alkylation by iodoacetamide and consecutive proteolytic digestion by LysC, trypsin, and chymotrypsin. Peptides obtained after each digestion were separately desalted on a C18 resin in a STAGE Tips procedure, and subsequently measured in the data-dependent acquisition mode on a Triple TOF 5600+ mass spectrometer (SCIEX, Farmingham, MA, USA) coupled with an Ekspert MicroLC 200 Plus System (Eksigent Technologies, Redwood City, CA, USA). All measurement files were subjected to joint database search in the MaxQuant 1.6.2.6a against the Homo sapiens SwissProt database (version from 09.11.2020). Resulting intensities were normalized using Total Protein Approach and protein concentrations in pmol/mg were calculated. Concentrations were imported into Perseus software and log2-transformed, data was restricted to 50% valid values, missing values were imputed from normal distribution and all values were normalized by z-score. T-tests between the test groups were conducted, and the results with p-value lower than 0.05 were considered to be statistically significant. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier: PXD031729.

Microarray

For the microarray study, shC and shFLG cells were grown to 80% confluence and then exposed to IL-4 and IL-13 (Peprotech, London, UK) at 50 ng/mL for the incubation time of 24 h. RNA was extracted with RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The microarray was performed by ServiceXS (Leiden, Netherlands) on an Illumina HT12v4 BeadArray platform (Illumina, San Diego, CA, USA) and the data were normalized using lumi (74) and analysed with LIMMA (75). The microarray dataset has been deposited to the Gene Expression Omnibus (GEO) repository and assigned the accession number: GSE203409.

Analysis

Data was analysed by Graph Pad Prism version 7 with one-way ANOVA (Holm-Sidak correction); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Cell adhesion-related proteins in sEVs were identified using the Gene Ontology tool, available at http://geneontology.org/ (76, 77). Interactions between the proteins of interest were identified using the STRING (78) database available in the Cytoscape 3.8.2 software (https://cytoscape.org/) (79) *via* the stringApp. Glycoproteins and glycosylation-relevant enzymes within NHEKs sEVs MS dataset were identified by literature search. Glycosylation-related pathways were identified using the Reactome Pathway Database (https://reactome.org/). For the STRING analysis protein lists were subjected to STRING database analysis (78). Generated networks were obtained with confidence mode of display of network edges. As a source of interactions between proteins we used "textmining", "experiments" and "databases" only with medium confidence interaction score (0.4) applied. Networks were not further expanded. Graphical adjustment was done using Cytoscape software platform. Single cell data on protein expression in skin population was obtained from the Human Developmental Cell Atlas available at https://developmentcellatlas.ncl.ac.uk/.

Transcriptomic data from Esaki et al. (GSE120721) was analyzed using the GEO2R tool available through the Gene Expression Omnibus (GEO) database (80, 81).

In datasets analyzed for the expression of glycosylation enzymes, i.e. Esaki et al., Leung et al. and He et al. p-values were adjusted using the Benjamini & Hochberg method.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below:

https://www.ebi.ac.uk/pride/archive/, PXD031729.

https://www.ncbi.nlm.nih.gov/geo/, GSE203409.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Independent Bioethics Committee for Scientific Research at Medical University of Gdansk. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AKo, JF, ABi, LH, ABog performed experiments, analysed data and contributed to the writing and figure preparation. AKr and MD performed experiments. JL performed data analysis. JZ provided surgical samples. SG, GSO, MP interpreted the data and participated in manuscript writing. DG-O provided funding, planned experiments and analysed the data, wrote the first and

subsequent paper drafts. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.884530/full#supplementary-material

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Identification of Effective Diagnostic Biomarkers and Immune Cell Infiltration in Atopic Dermatitis by Comprehensive Bioinformatics Analysis

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Background: Atopic dermatitis (AD) is a dermatological disorder characterized by symptoms such as chronically inflamed skin and frequently intolerable itching. The mechanism underlying AD development is still unclear. Our study aims to identify the diagnostic and therapeutic biomarkers for AD and provide insight into immune mechanisms at the molecular level through bioinformatics analysis.

Methods: The GSE6012, GSE32924, and GSE36842 gene expression profiles were obtained for analysis from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were segregated using the "Batch correction" and "RobustRankAggreg" methods. Weighted gene co-expression network analysis (WGCNA) was performed to screen for module genes with AD traits. Then, common DEGs (co-DEGs) were screened out via combined differential expression analysis and WGCNA. Functional enrichment analysis was performed for these co-DEGs using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG), followed by protein-protein interaction network analysis. Candidate hub genes were identified using the "cytoHubba" plugin in Cytoscape, and their value for AD diagnosis was validated using receiver operating characteristic curve analysis in the external database GSE120721. Immunohistochemical staining was performed for further validation. The CIBERSORT algorithm was used to evaluate skin samples obtained from healthy controls (HCs) and lesions of AD patients, to determine the extent of immune cell infiltration. The association between the identified hub genes and significant differential immune cells was analyzed using Pearson correlation analysis.

Results: A total of 259 DEGs were acquired from the intersection of DEGs obtained by the two independent procedures, and 331 AD-trait module genes were separated out from the blue module via WGCNA analysis. Then, 169 co-DEGs arising from the intersection of the 259 DEGs and the 331 AD-trait module genes were obtained. We found that co-DEGs were significantly enhanced in the type I interferon and IL-17 signal transduction pathways. Thirteen potential hub genes were identified using Cytoscape. Five hub genes (CCR7,

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CXCL10, IRF7, MMP1, and RRM2) were identified after screening via external dataset validation and immunohistochemical analysis. We also identified four significant differential immune cells, i.e., activated dendritic cells, plasma cells, resting mast cells, and $CD4^+$ naïve T cells, between AD patients and HCs. Moreover, the relationship between the identified hub genes and significant differential immune cells was analyzed. The results showed that the CCR7 expression level was positively correlated with the number of $CD4^+$ naïve T cells ($R = 0.42$, $p = 0.011$).

Conclusion: CCR7, CXCL10, IRF7, MMP1, and RRM2 could be potential diagnostic and therapeutic biomarkers for AD. CCR7 expression level was positively correlated with the number of $CD4^+$ naïve T cells in AD. These findings need to be corroborated in future studies.

Keywords: atopic dermatitis, immune cells infiltration, diagnostic biomarkers, bioinformatics analysis, immunohistochemical verification

INTRODUCTION

Atopic dermatitis (AD) is a dermatological disorder that presents as chronically inflamed skin and often intolerable itching (Langan et al., 2020). Globally, it is one of the most prevalent skin conditions, affecting approximately 2.1–4.9% of adults and 20% of children (Nutten, 2015; Barbarot et al., 2018). AD causes severe psychological and social hardships and is associated with a high risk of depression, anxiety, work absenteeism, and suicidal tendencies (Eckert et al., 2017; Patel et al., 2019; Sandhu et al., 2019; Schonmann et al., 2020).

The occurrence of AD could be attributed to various factors, including environmental factors, hereditary tendencies, epidermal dysfunction, skin microbiome abnormalities, and immune dysregulation. Immune dysregulation plays a substantial role in the progression of AD (Li et al., 2021). The main immunological mechanisms underlying AD have been attributed to an imbalance of T helper (Th)1/Th2 differentiation. A Th2 cell-mediated response triggers the acute phase, while the chronic phase is triggered by a shift to a Th1 cell-mediated response (Salvati et al., 2021). Damage to the epidermal barrier results in penetration of the skin by antigens, causing the release of alarmins such as interleukin (IL) -25, IL-33, and thymic stromal lymphopoietin (Salimi et al., 2013). These events result in the stimulation of innate type 2 lymphoid cells and inflammatory epidermal dendritic cells. Naïve $CD4^+$ T cells also gravitate towards Th2 cells, following the generation of IL-4, IL-5, and IL-13 (Cosmi et al., 2019). In addition to triggering type 2 inflammation, immune cells including Th1, Th17, and mast cells are involved in mixed inflammatory responses (Ho and Kupper, 2019; Abreu and Kim, 2021). However, the immune mechanisms underlying AD have not been described adequately. Investigation of the role played by immune cells and the crucial genes related to associated immune responses needs to be conducted in a methodical manner, and was the focus of the current study.

Several novel genes and biomarkers have been identified for various diseases following the widespread use of bioinformatics analysis techniques and the development of various algorithms. Weighted gene co-expression network analysis (WGCNA) is a sophisticated data processing tool that is widely used to detect clusters or modules of highly correlated genes. It can identify a cluster of genes with similar biological functions, and it can be used to investigate connections between clinical characteristics and gene expression (Langfelder and Horvath, 2008). Besides, an algorithm known as cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) has been developed for estimating numbers of immune cells (Chen et al., 2018). This tool has been utilized to measure the extent of immune cell infiltration in various immune-mediated skin disorders, including psoriasis and acne vulgaris (Yang et al., 2021; Zhang et al., 2021).

Here, we aimed to identify diagnostic and therapeutic biomarkers for AD via the analysis of gene expression omnibus (GEO) database using R packages and online bioinformatics tools. We also aimed to determine the pathways related to AD pathogenesis and mechanisms of immune cell infiltration. We believe this is the first study to apply both the WGCNA and CIBERSORT methods for integration of datasets from various platforms to understand the molecular mechanisms involved in AD, and to verify the results via immunohistochemical method. We are confident that this study would provide a new direction to research on AD management.

MATERIALS AND METHODS

Data Acquisition and Workflow

The GEO database (http://www.ncbi.nlm.nih.gov/geo) was used to segregate gene expression profiles. Gene expression profiles were included if 1) high-throughput sequencing or array-based mRNA expression profiling had been performed; 2) it was possible to obtain lesion skin tissues and healthy skin tissues from AD patients and healthy controls (HCs), respectively; and 3) a minimum of six specimens were included in the dataset. Datasets with drug, placebo, vehicle administration, or other treatments were excluded. Finally, the datasets GSE6012, GSE32924, GSE36842, and GSE120721 were selected for use in

TABLE 1 | Baseline information regarding the selected datasets.

GEO datasets	Platform	Samples (Number)			Sample characteristics				Experiment type	Attribute	Author (Reference)
		Total	Patients	Controls	Racial group	Age, y	Lesion phase	Severity			
GSE6012	GPL96	20	10	10	Caucasian	Patients: 40.3 (21–50); Controls: 43 (25–50)	NR	NR	Array	Test	Olsson et al. (2006) Mobini et al. (2009)
GSE32924	GPL570	21	13	8	NR	Patients: 38.9 (16–81); Control: NR	Acute exacerbation of chronic disease	Moderate-to-severe	Array	Test	Suárez-Fariñas et al. (2011)
GSE36842	GPL570	31	16	15	NR	Patients: 44 (20–67); Control: NR	Acute and chronic	Moderate-to-severe	Array	Test	Gittler et al. (2012)
GSE120721	GPL570	37	15	22	NR	Patients: 39.4 (27–59); Control: NR	Chronic	Moderate-to-severe	Array	Validation	Esaki et al. (2015)

NR, not reported.

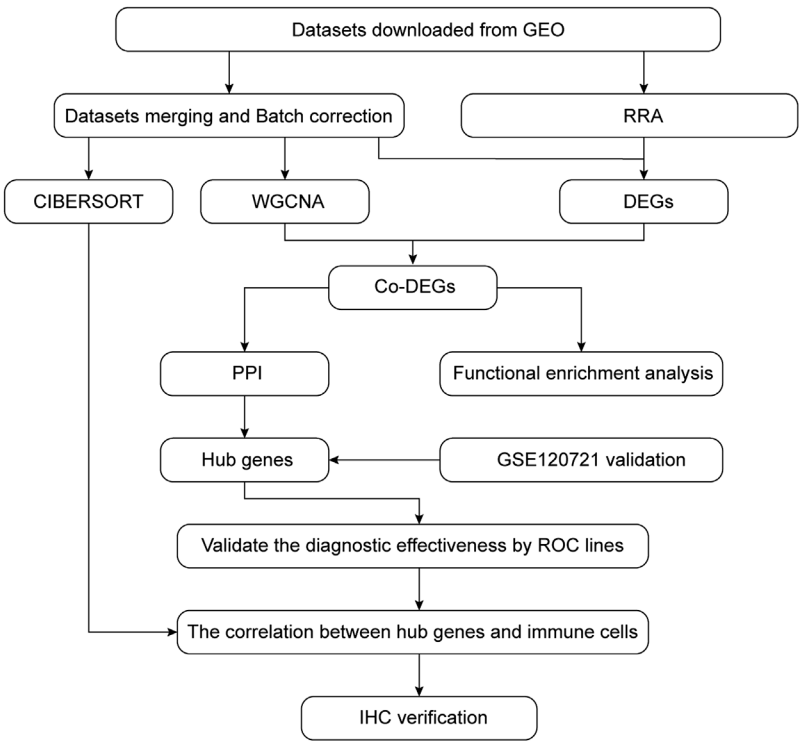

FIGURE 1 | Flow chart summarizing the procedures used in this study.

our study. The data are summarized in **Table 1** and the workflow is depicted in **Figure 1**.

Data Pre-processing

Series matrix files and platform annotation information were downloaded for three microarray datasets, namely, GSE6012, GSE32924, and GSE36842. The three-probe expression matrixes were converted into gene expression matrixes using Perl script. The average value was derived if there was more than one probe corresponding to a gene. Batch effects were then eliminated using the R package "sva." Thus, a merged, normalized gene expression matrix was obtained for further

analysis. The gene expression matrix for the external validation dataset GSE120721 was obtained in a similar manner.

Construction of WGCNA

The "WGCNA" R package was used to derive modules identified with clinical characteristics, and to identify hub genes. The merged gene matrix was checked to eliminate anomalous specimens which might have escaped from clustering of samples. The topological overlap matrix (TOM) was derived from the adjacency matrix. Genes were divided into modules based on their dissimilarity in TOM. The cut height, minimal module size, and soft-thresholding power were set at 0.25, 30, and 4 (scale-free $R^2 = 0.85$), respectively. The absolute value of the correlation coefficient between traits and genes was used to determine the relationship between clinical traits and module eigengenes. A gene was considered to be significant based on the absolute correlation coefficient between traits and genes. A gene was considered to be a member of a module based on its association with certain expression profiles. Genes with a gene significance (GS) > 0.2 and module membership (MM) > 0.8 in the module most relevant to clinical traits were considered as module genes with AD traits (Hu et al., 2020).

Screening for Differentially Expressed Genes

Two independent methods were used to obtain the most robust differentially expressed genes (DEGs) from several microarray cohorts. In the first approach ("Batch correction"), the three downloaded raw datasets were combined into an expression matrix after the elimination of batch effects and normalization. Then, DEGs were analyzed using the R package "limma". In the second approach ("RobustRankAggreg, RRA"), the R package "limma" was used to analyze the DEGs of the downloaded gene matrixes. The DEGs from each such derived dataset were then combined using the R package "RRA" (Zhou et al., 2021). The final DEGs identified by the two methods were determined from their intersection using a Venn diagram. A |log fold change| > 1 was set along with an adjusted p value <0.05, as the threshold points for DEGs.

Functional Annotation and Pathway Enrichment Analysis

The gene from the module of interest and the final DEGs obtained by the two methods were intersected to identify the common DEGs (co-DEGs) using a Venn diagram. Then, the co-DEGs were subjected to functional enrichment analysis. Gene names to gene ID conversion of the co-DEGs was achieved using the R package "org.Hs.eg.db." Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for co-DEGs was conducted using the R package "clusterProfiler". Metascape (http://metascape.org/gp/index.html) was then used for comprehensive data analysis. Significance was set at a p value <0.05 and q value <0.05.

Identification and Validation of Hub Genes

The protein-protein interaction (PPI) network for the co-DEGs was constructed using the STRING tool (https://string-db.org/). The interaction file was downloaded and each node gene was scored by 12 algorithms (Maximal Clique Centrality (MCC), Density of Maximum Neighborhood Component (DMNC), Maximum Neighborhood Component (MNC), Degree, Edge Percolated Component (EPC), BottleNeck, EcCentricity, Closeness, Radiality, Betweenness, Stress, and Clustering Coefficient), using the "cytoHubba" plugin of Cytoscape (v 3.7.2). Candidate hub genes were identified from the genes at the junction of these 12 algorithms, then visualized using the R package "UpSet". The preciseness of these genes was evaluated in the gene expression profile GSE120721 by ROC curve analysis using the R package "pROC". Hub genes were validated with an area under the curve (AUC) criterion of >0.8 and a differential expression level significance of $p < 0.05$ using the Wilcoxon test (Sun et al., 2021).

Evaluation of Immune Cell Infiltration and Correlation Analysis Between Hub Genes and Immune Cells

The immune cell proportions were determined using the CIBERSORT R package (http://cibersort.stanford.edu/download.php), with data from the normalized gene expression matrix of AD patients and HCs and the data from expressed reference signature genes (LM22, http://cibersort.stanford.edu/download.php) (Zhang et al., 2021). A violin plot was used to visualize the distribution of these immune cells using the "vioplot" package in R software. A significance criterion of $p < 0.05$ was used for this calculation. The correlation between the identified hub genes and the infiltrating immune cells was conducted by using Pearson correlation analysis. The resulting relationships were studied using the "ggplot2" and "ggpubr" packages.

Patient Recruitment

The study proposal was reviewed and approved by the Ethics Committee of Shengjing Hospital of China Medical University. AD patients and HCs were included in the study after obtaining written informed consent. AD was diagnosed as per the Hanifin-Rajka criteria (Hanifin and Rajka, 1980). Full thickness skin samples were collected from 12 individuals. These included six AD patients and six HCs matched for age, sex, and race. Details of the study subjects are shown in **Supplementary Table S1**.

Immunohistochemical Verification

Extracted skin tissues were fixed with 4% formaldehyde buffer, and 4-μm-thick sections were obtained from paraffinized specimens. Tissue sections were incubated at 60°C for 2 h before the dewaxing process. For antigen retrieval, the sections were autoclaved in a citric acid buffer (pH 6.0) at 115°C for 3 min and quenched in 0.3% H2O2 for 15 min for endogenous peroxidase activity. Then, sections were treated with goat serum, which was used as a blocking solution, for 45 min, and incubated overnight at 4°C with primary antibodies against CCR7

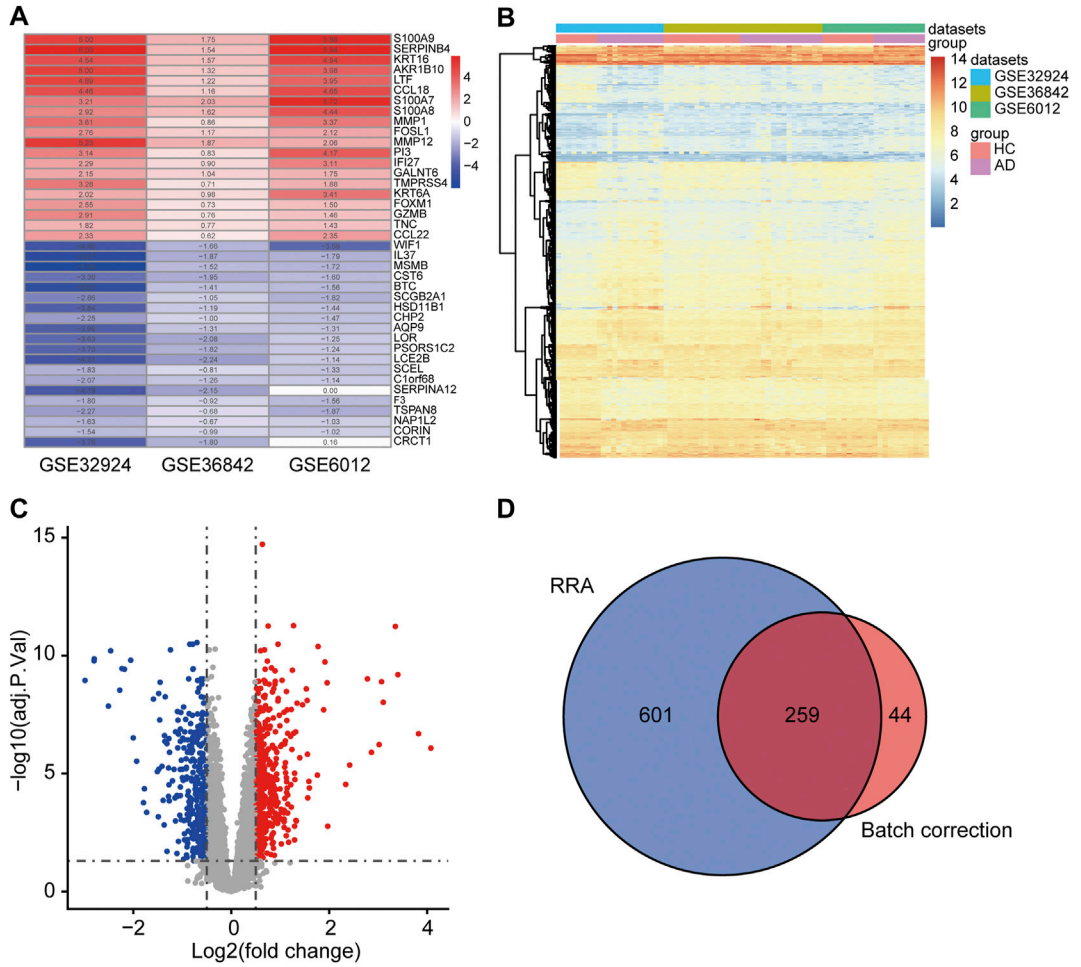

FIGURE 2 | Determination of DEGs. **(A)** The first 20 upregulated and downregulated DEGs of the three datasets merged using the "RRA" approach. **(B)** Heat map of the DEGs obtained via the "Batch correction" approach. **(C)** Volcano map of the DEGs obtained via the "Batch correction" approach. **(D)** Venn diagram of the intersection of the DEGs screened using the two methods. DEGs, differentially expressed genes; RRA, RobustRankAggreg.

(Abmart, dilution 1:100), CCNA2 (Abmart, dilution 1:200), CXCL10 (Abmart, dilution 1:200), IRF7 (Abmart, dilution 1:100), ISG15 (Abmart, dilution 1:100), MKI67 (Abmart, dilution 1:100), MMP1 (Abmart, dilution 1:200), NCAPG (Abmart, dilution 1:200), and RRM2 (Abmart, dilution 1:200). These sections were treated with the goat anti-rabbit secondary antibody for 30 min at room temperature. Then, 3,3′-diaminobenzidine was used to visualize protein expression and a Nikon Eclipse 80i microscope (Nikon Corporation) was used to capture images. The public domain program "Image-Pro Plus 6.0" was used to measure the immunohistochemical integral optical density (IOD). Sum IODs and areas of each photo were measured, then average optical densities in different groups were compared.

Statistical Analysis

R studio (version 4.0.3) was used for the analysis of data and GraphPad Prism 7 software (version 7.0, San Diego, CA, United States) was used for the generation of images. Immunohistochemical data were assessed using the Student's *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Identification of DEGs

DEGs were identified by two methods using three microarray datasets, including 39 AD and 33 HC samples. A total of 303 DEGs were acquired by "RRA." These consisted of 139 upregulated and 164 downregulated genes, some of which are shown in **Figure 2A**. A total of 860 DEGs were obtained by "Batch correction." These consisted of 478 upregulated genes and 382 downregulated genes, which were visualized via a heatmap (**Figure 2B**) and a volcano map (**Figure 2C**). A total of 259 DEGs, including 130 upregulated genes and 129 downregulated genes, were acquired from the intersection of the DEGs obtained by the two procedures (**Figure 2D** and **Supplementary Table S2**).

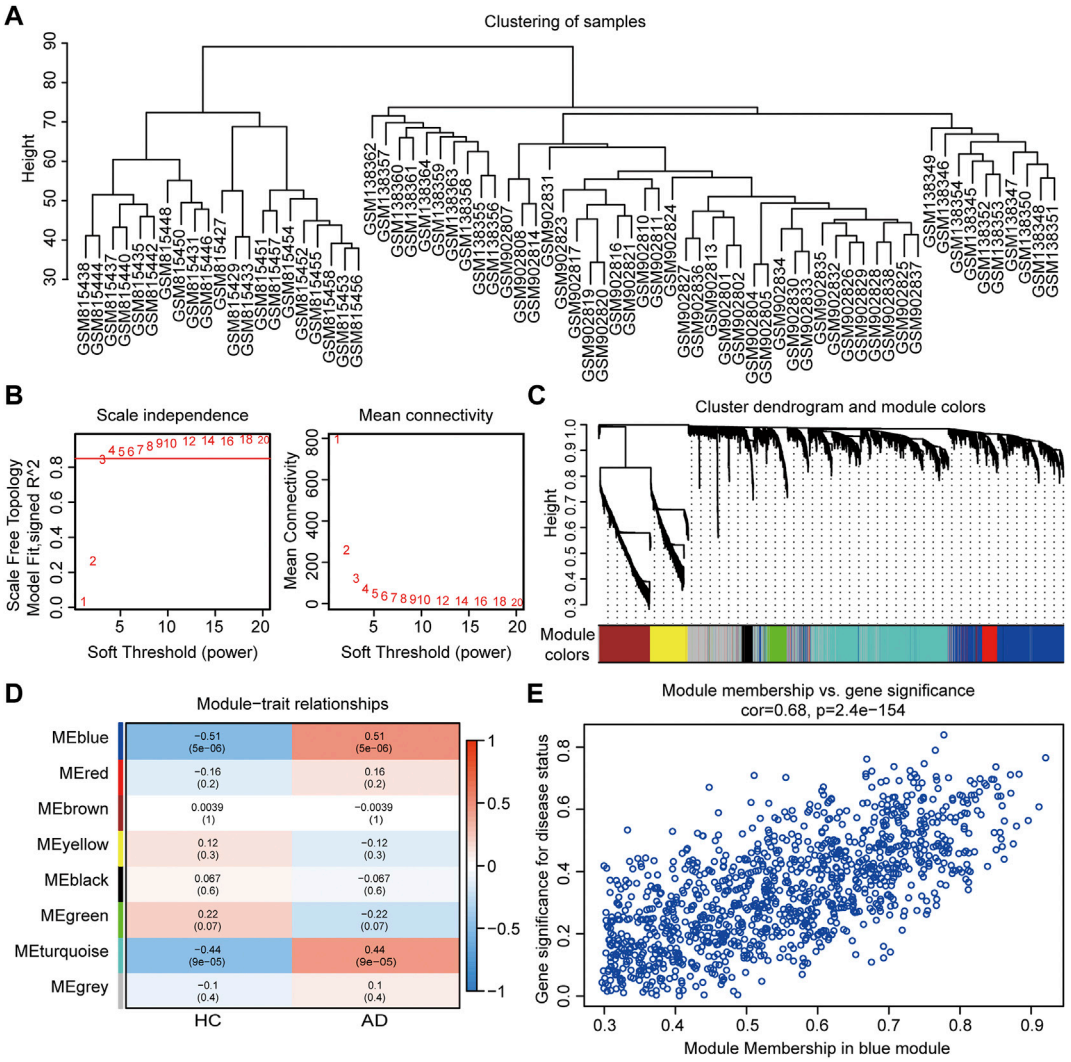

FIGURE 3 | Co-expression network assessment using WGCNA. **(A)** Clustering dendrograms of DEGs from "Batch correction." **(B)** Assessment of the scale-free fitness index and mean connectivity for various soft-thresholding powers. **(C)** Dendrogram of all the genes clustered based on dissimilarity in the topological overlap. **(D)** Heatmap exhibiting the relationship between module eigengenes and clinical traits. Each row represents a color module, and every column represents a clinical trait. The correlation coefficient and corresponding *p* value are shown in each cell. **(E)** Scatter plot exhibiting genes in the blue module. WGCNA, weighted gene co-expression network analysis; DEGs, differentially expressed genes.

WGCNA and Module Analysis

In WGCNA analysis, the merged gene matrix from "Batch correction" was clustered to exclude outliers and abnormal samples which might have escaped during sample clustering (**Figure 3A**). The soft-thresholding power β was set to 4 to attain a scale-free network evaluation coefficient R^2 of 0.85 (**Figure 3B**). Similar modules from the co-expression network were combined using a cut height of 0.25 to obtain a total of eight modules (**Figure 3C**). Correlations between clinical traits and module eigengenes are shown in **Figure 3D**. The genes of blue module were determined to be the most relevant to AD, based on the module-trait relationship heatmap (correlation coefficient 0.51, $p < 0.001$). The MM vs. GS for AD in blue module were calculated and presented with a scatter diagram (**Figure 3E**). Finally, 331 module genes with AD traits from the blue module were identified for further studies (**Supplementary Table S3**).

Identification of Co-DEGs and Functional Enrichment Analysis

A total of 169 co-DEGs were identified from the intersection of the 331 AD traits module genes and 259 DEGs (**Figure 4A**). GO and KEGG pathway analyses were used to further investigate the biological properties of these co-DEGs. We identified several biological properties, including cellular components (CCs), biological processes (BPs), and molecular functions (MFs), using GO annotation enrichment terms. With regard to BPs, the co-DEGs were substantially enhanced in the cellular response to type I

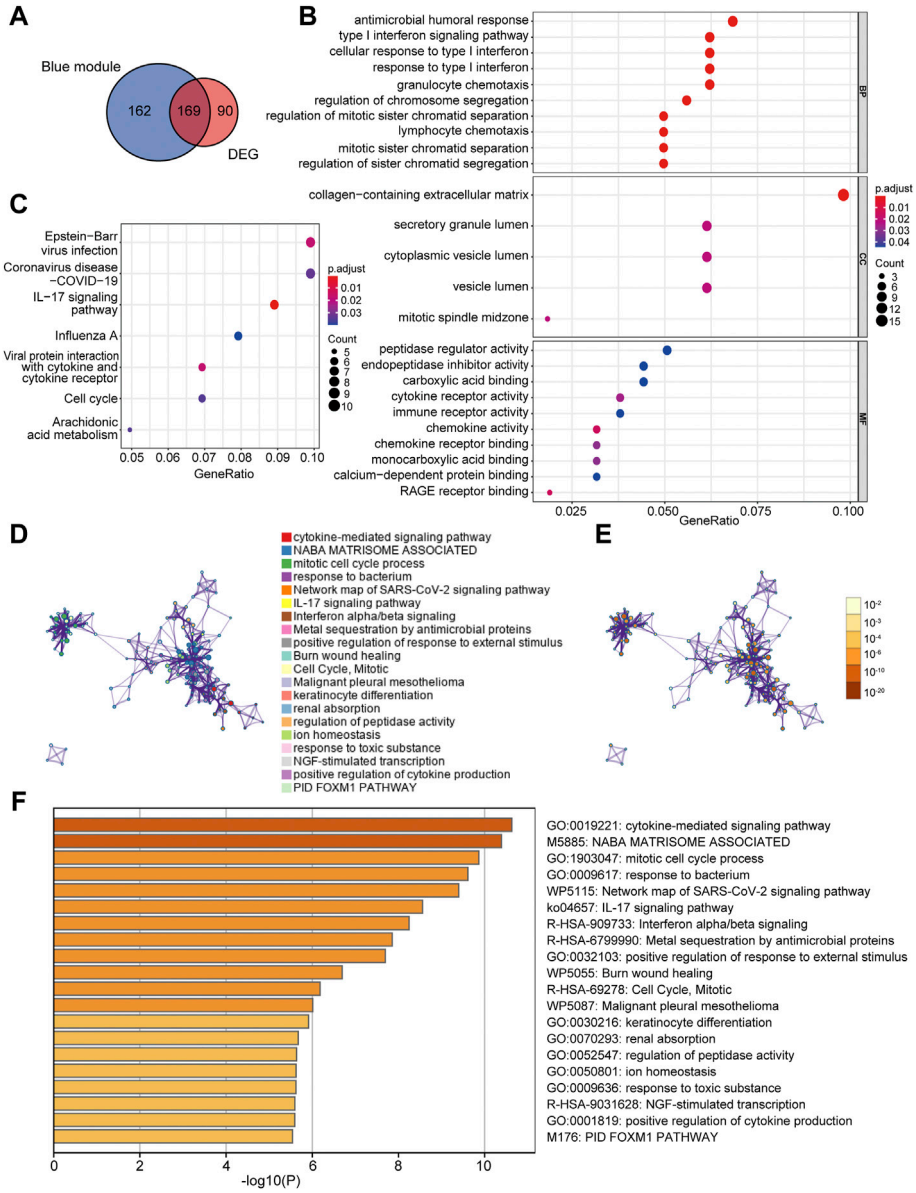

FIGURE 4 | Determination of co-DEGs and functional enrichment analysis. **(A)** Venn diagram illustrating the co-DEGs screened from the intersection of the DEGs and the module genes with AD traits. **(B)** Remarkably enriched GO terms of co-DEGs. **(C)** Remarkably enriched KEGG cascades of co-DEGs. **(D)** Network illustrating terms occurring abundantly in co-DEGs. Every node designates an enriched term colored based on its cluster identity. **(E)** The same enriched network with nodes colored via *p*-value. **(F)** The top 20 pathways linked to co-DEGs based on a comprehensive enrichment assessment. co-DEGs, common differentially expressed genes; AD, atopic dermatitis; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

interferon (IFN) and the type I IFN signaling pathway. As for CC and MF, co-DEGs were substantially enhanced in the collagen-containing extracellular matrix and peptidase regulator activation, respectively (**Figure 4B**). We identified critical signaling pathways using KEGG enrichment analysis. The co-DEGs were substantially enhanced in the IL-17 signaling pathway (**Figure 4C**). Metascape analysis indicated that co-DEGs were substantially enhanced in the cytokine-mediated signaling pathway (**Figures 4D–F**).

Identification and Validation of Hub Genes

The PPI network of co-DEGs was constructed by STRING as shown in **Figure 5A**. 12 algorithms were used to calculate the score of each node gene. Then, 13 candidate hub genes were segregated, including CCR7, CCNA2, CXCL10, CXCL11, IRF7, ISG15, KIAA0101, MKI67, MMP1, NCAPG, RRM2, SELE, and SERPINB3 (**Figure 5B**). To make the result more reliable, GSE120721 was employed to validate these candidate hub genes. The expression levels of 11 candidate hub genes, including CCR7, CCNA2, CXCL10, IRF7, ISG15, KIAA0101,

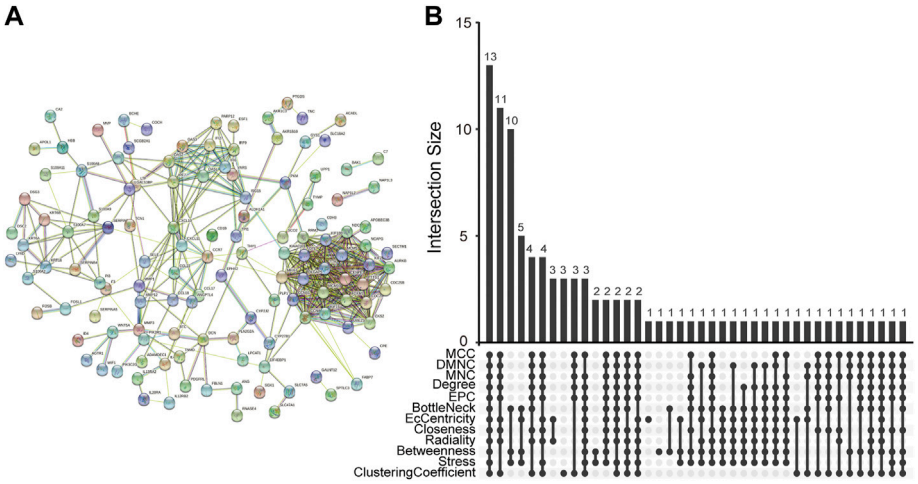

FIGURE 5 | Determination of candidate hub genes. **(A)** The PPI network of co-DEGs. **(B)** Candidate hub genes were screened out using 12 algorithms. PPI, protein-protein interaction; co-DEGs, common differentially expressed genes.

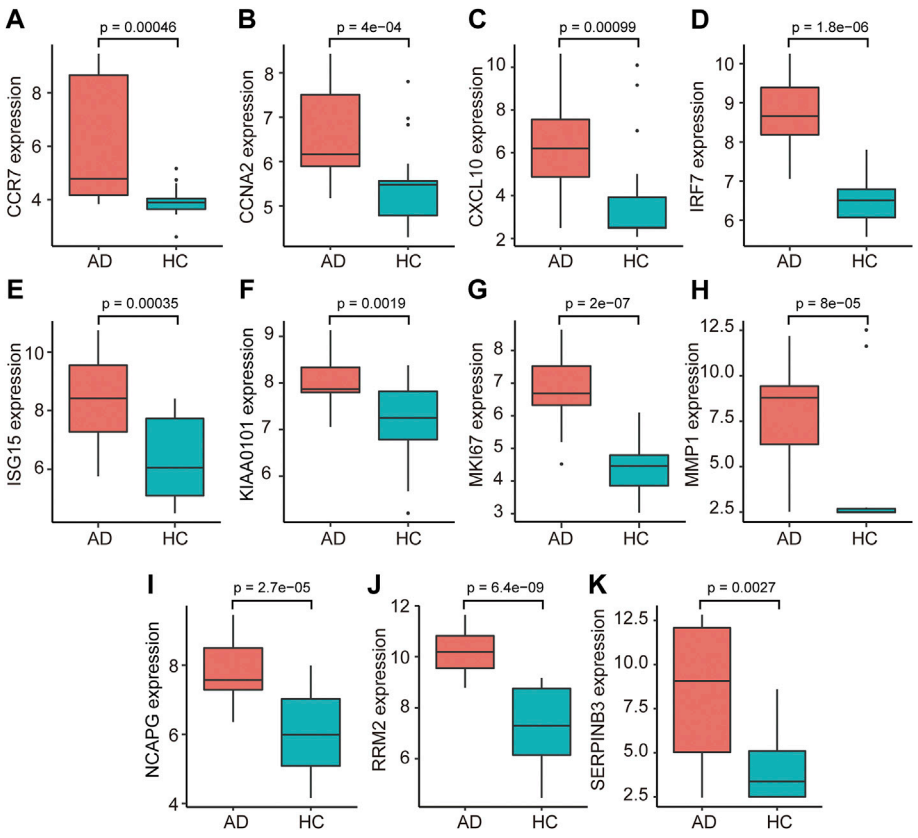

FIGURE 6 | The relative expression levels of 11 candidate hub genes, i.e., (**(A)** CCR7, **(B)** CCNA2, **(C)** CXCL10, **(D)** IRF7, **(E)** ISG15, **(F)** KIAA0101, **(G)** MKI67, **(H)** MMP1, **(I)** NCAPG, **(J)** RRM2, and **(K)** SERPINB3, validated using GSE120721. AD, atopic dermatitis; HC, healthy control.

MKI67, MMP1, NCAPG, RRM2, and SERPINB3, were significantly higher in AD patients (**Figure 6**). These 11 candidate hub genes were subjected to ROC analysis. An AUC >0.8 was considered indicative of AD, with excellent specificity and sensitivity. Nine genes (CCR7, CCNA2, CXCL10, IRF7, ISG15, MKI67, MMP1, NCAPG, and RRM2) with an AUC

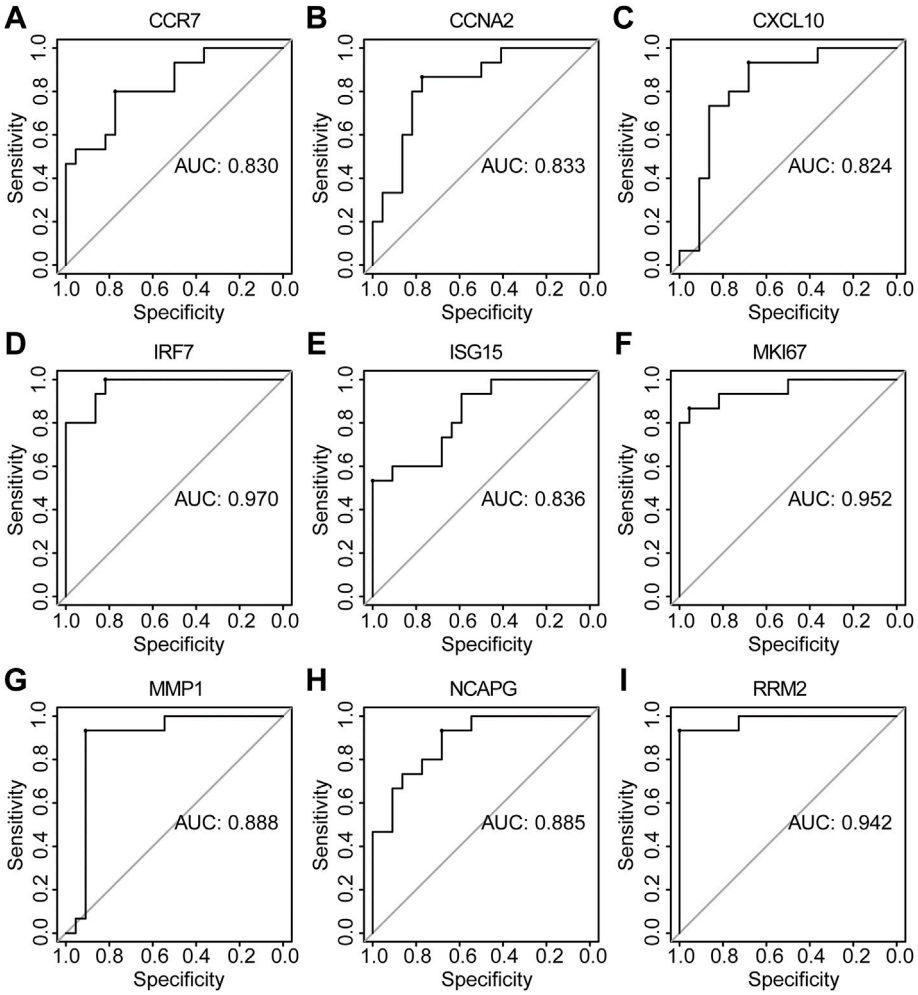

FIGURE 7 | The diagnostic effectiveness of nine hub genes (**(A)** CCR7, **(B)** CCNA2, **(C)** CXCL10, **(D)** IRF7, **(E)** ISG15, **(F)** MKI67, **(G)** MMP1, **(H)** NCAPG, and **(I)** RRM2 was validated using GSE120721.

>0.8 and significantly different expression levels ($p < 0.05$ as determined by the Wilcoxon test) were identified as hub genes. Among these, IRF7 exhibited the best specificity and sensitivity for the diagnosis of AD (AUC = 0.97) (**Figure 7**).

Immunohistochemical Analysis

We found that the IOD/area values of CCR7, CXCL10, IRF7, MMP1, and RRM2 in AD tissue were significantly higher than those in HC tissue ($p < 0.05$) (**Figure 8**). However, the IOD/area values of CCNA2, ISG15, MKI67, and NCAPG were comparable between the AD and HC tissues (**Supplementary Figure S1**).

Ananalysis of Immune Cell Infiltration and the Relationship Between Identified Hub Genes and Differential Immune Cells in AD

A total of 23 AD and seven HC tissues met the criteria for analysis with CIBERSORT ($p < 0.05$). The constitutions of 22 types of immune cells in each specimen are shown in **Figure 9A**. Compared with HC tissues, AD tissues exhibited a higher number of activated dendritic cells (DCs), $CD4^+$ naïve T cells, and plasma cells, whereas the number of resting mast cells was relatively lower (**Figure 9B**). Relationships between five identified hub genes (CCR7, CXCL10, IRF7, MMP1, and RRM2) and four significant differential immune cells (naive $CD4^+$ T cells, plasma cells, activated DCs, and resting mast cells) in AD tissue were analyzed (**Figure 9C**). Significantly related hub genes and immune cells were segregated using thresholds of $R > 0.4$ and $p < 0.05$. We found that the CCR7 expression level was positively correlated with the number of $CD4^+$ naïve T cells ($R = 0.42$, $p = 0.011$) (**Figure 9D**).

DISCUSSION

Though research into the molecular processes involved in AD has made rapid advances, the mechanisms of pathogenesis and progression of this disease are still poorly understood, which

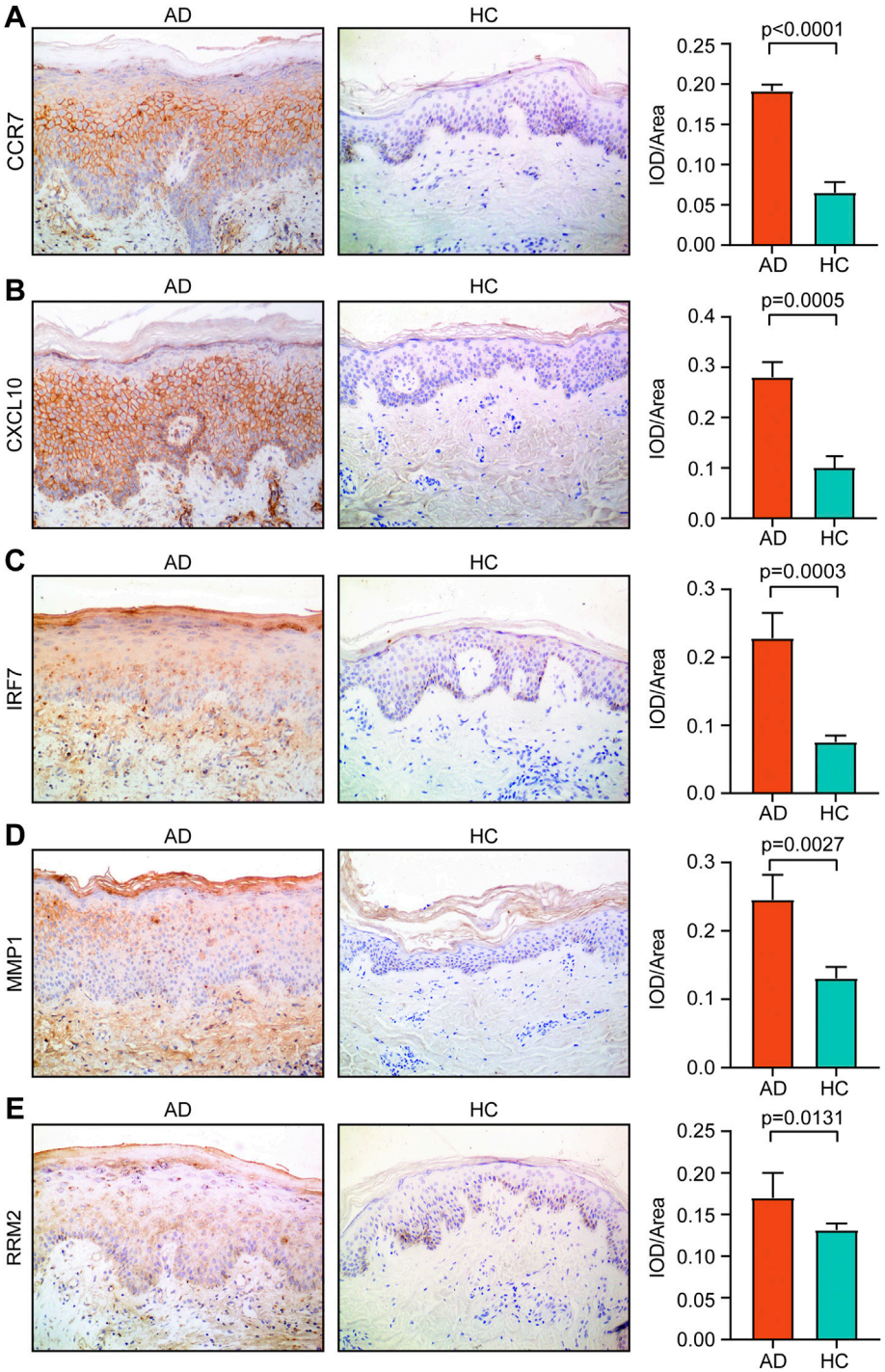

FIGURE 8 | Expression of **(A)** CCR7, **(B)** CXCL10, **(C)** IRF7, **(D)** MMP1, and **(E)** RRM2 in AD tissue and healthy tissue. Magnification, ×200. AD, atopic dermatitis; HC, healthy control.

makes the diagnosis and treatment of this disease a challenge. In this study, we methodically segregated potential new biomarkers for AD and evaluated the extent of infiltration of the skin tissue by immune cells in AD patients. We screened DEGs using two independent methods to improve the precision of the results. We also screened 169 final co-DEGs in combination with WGCNA and differential expression analysis. The results of GO and KEGG analyses showed that co-DEGs were substantially enhanced in the type I IFN and IL-17 signaling pathways.

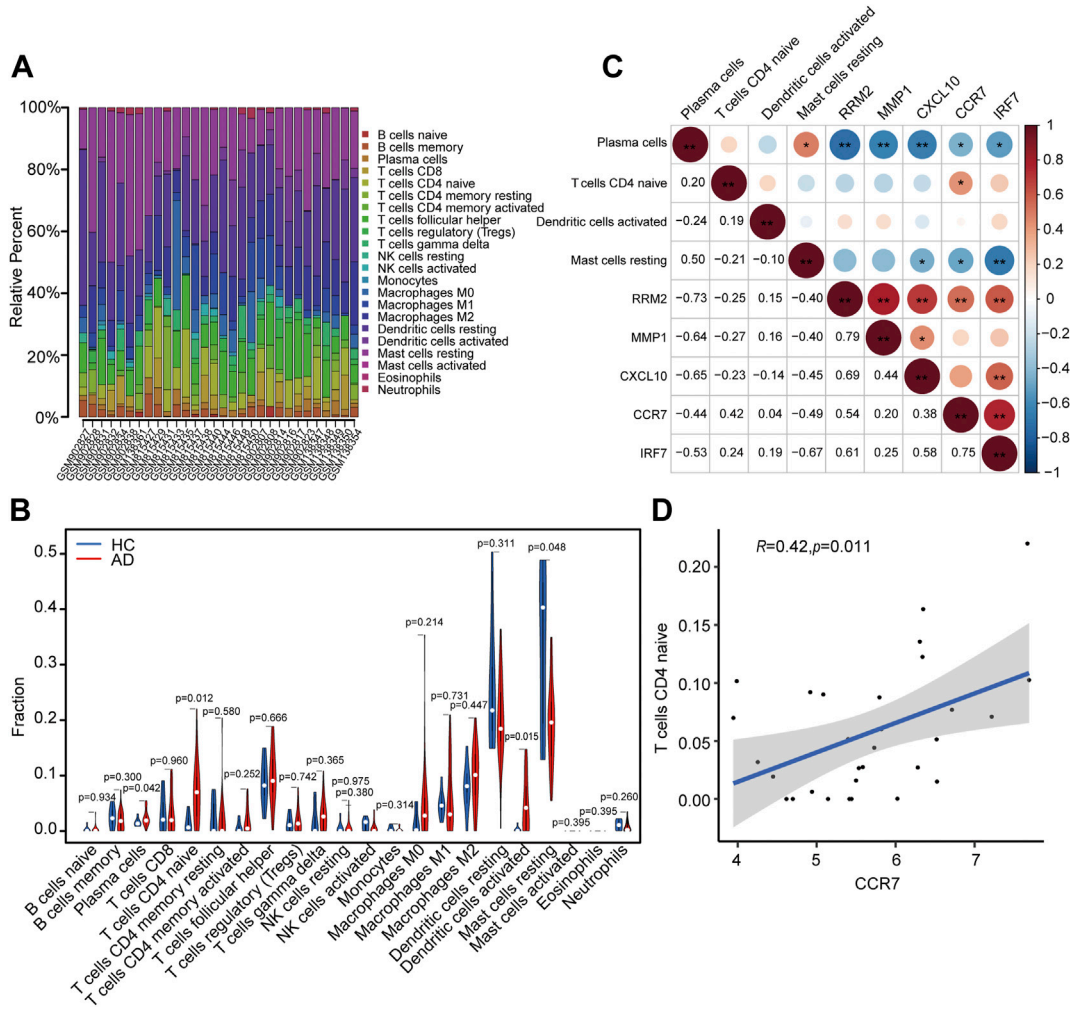

FIGURE 9 | Analysis of immune cell infiltration and the relationship of hub genes with differential immune cells in individuals with AD. **(A)** Relative fraction of 22 sub-populations of immune cells in AD samples. **(B)** The differences of 22 sub-populations of immune cells among the AD and HC tissues. **(C)** Correlation among four remarkable differential immune cells and five identified hub genes. **(D)** Remarkably related hub genes and immune cells screened by the criteria R > 0.4 and $p < 0.05$.

Multiple IFNα isotypes and IFNβ of the type I family have been extensively studied. Type I IFNs can activate crucial processes of innate and adaptive immune systems, including the presentation of antigens and the production of cytokines responsible for triggering the activation of B cells, T cells, and natural killer cells. These contribute substantially to the pathogenesis of AD (Kader et al., 2021; Li et al., 2021). IL-17 is consistently upregulated in AD in both the acute and chronic phases (Gittler et al., 2012). It may promote abnormalities in immune regulation in AD by upregulating S100A7/8/9 in combination with IL-22 (Nograles et al., 2008). The S100A proteins are highly upregulated in AD, and can operate as both promoters of inflammation and as antimicrobials (Mansouri and Guttman-Yassky, 2015). It has been reported that IL-17 downregulated filaggrin and promotes abnormalities in the skin barrier. It has also been shown to affect the expression of genes in keratinocytes related to cell adherence, thereby increasing the risk of AD (Gutowska-Owsiak et al., 2012).

In the present study, 13 prospective hub genes were screened using STRING and the cytoHubba plugin in Cytoscape. We found nine hub genes (CCR7, CCNA2, CXCL10, IRF7, ISG15, MKI67, MMP1, NCAPG, and RRM2) with high diagnostic sensitivity and specificity by external dataset GSE120721 validation and ROC curve analysis. For experimental validation, we performed immunohistochemistry analysis for 12 individuals, including 6 AD patients and 6 age-matched, race-matched and sex-matched healthy individuals, to detect expression levels of these nine identified hub genes. The expression levels of CCR7, CXCL10, IRF7, MMP1, and RRM2 were found to be upregulated in AD patients, as compared to the levels observed in HCs. This was in accordance with the results obtained using bioinformatics analysis. Expression levels of CCNA2, ISG15, MKI67, and NCAPG did not differ significantly in AD patients and HCs, though AD patients had higher expression levels of all four genes. We hypothesize that the non-significance of these differences could be attributed to the

small sample size. This was because a greater number of skin samples could not be obtained from AD patients due to ethical reasons. Moreover, we only performed immunohistochemistry analysis. We could not simultaneously investigate protein expression levels via western blotting because of the small amount of skin tissue collected.

In this study, we identified CCR7, CXCL10, IRF7, MMP1, and RRM2 to be potential diagnostic biomarkers for AD. The chemokine receptor CCR7 was reportedly present on DCs as well as naïve, regulatory, and memory T cells (Förster et al., 2008). The movement of skin DCs to lymphoid tissue has been shown to be conditionally dependent upon the activation of the chemokine receptor CCR7 under both stable and inflammatory conditions (Ohl et al., 2004). After $CCR7^{-/-}$ mouse bone marrow-derived DCs were subcutaneously injected into wild-type mice, these cells were unable to travel to the lymph nodes and access lymphatic drainage, which indicates that DCs also need to express CCR7 to facilitate their homing to lymph nodes (Hintzen et al., 2006). Moreover, it has been reported that the movement of neutrophils from skin to skin-draining lymph nodes via lymphatic vessels was also mediated by CCR7 (Özcan et al., 2022). In the present study, we found that the CCR7 expression level was upregulated in AD patients. As AD is a complex disease involving various immune cells such as DCs and neutrophils (Novak, 2012; Dhingra et al., 2013), it could be speculated that CCR7 might mediate the movement of DCs and neutrophils to skin-draining lymph nodes in AD and induce subsequent immune responses.

CXCL10, a 10 kDa protein classified as a Th1-chemokine, binds to CXCR3 receptors. CXCL10 exhibits chemotactic activity toward activated T lymphocytes and monocytes in the peripheral blood and is produced by activated T cells, monocytes, endothelial cells, and keratinocytes (Qi et al., 2009). In AD patients, the serum levels of CXCL10 and the expression levels of CXCL10 in skin lesions were significantly increased in comparison to the levels observed in HCs (Esaki et al., 2016; Brunner et al., 2017; Lang et al., 2021). This is consistent with our findings. CXCL10 can recruit activated T cells expressing CXCR3 (especially Th1 cells). These recruited Th1 cells secrete IFN- γ, stimulate local CXCL10 production, and further facilitate Th1 cell recruitment (Colvin et al., 2004). As T lymphocytes play vital roles in AD pathogenesis (Sroka-Tomaszewska and Trzeciak, 2021), lymphocyte chemotaxis regulated by CXCL10 may be involved in the AD immune response.

IFN regulatory factor 7 (IRF7) belongs to the IFN regulatory transcription factor family, which plays a vital role in the control of several biological processes, including inflammation, apoptosis, and immune response generation. He *et al.* found that the expression of IRF7 is increased in type 2 lymphoid cells during allergic inflammation, whereas an IRF7 deficiency leads to its remission (He et al., 2019). Cohen *et al.* reported that the phenotypic transformation from pro-inflammatory macrophages to anti-inflammatory macrophages (M1-to-M2) is modulated by IRF7, which is down-regulated by the transforming growth factor-beta 1 pathway (Cohen et al., 2014).

MMP-1, an interstitial collagenase, cleaves type I and II collagen, which are major constituents of the dermis (Parks et al., 2004). It has been shown that the serum levels of MMP-1 were significantly higher in the AD group than in HCs, and may correlate with the degree of damage to the epidermal barrier, which is represented by the extent of trans-epidermal water loss (Basałygo et al., 2021). Harper *et al.* reported that an increase in MMP-1 levels was associated with a corresponding increase in the severity of AD (Harper et al., 2010). Another report demonstrated that the severity of AD was proportional to the severity of damage to the epidermal barrier (Weidinger and Novak, 2016). MMP-1 also triggers other MMPs, such as MMP-9, which is highly specific for substrates such as dermal elastin and fibrillin (Tsoureli-Nikita et al., 2006). The cleavage of the components in the basement membrane allows T cells to cross the basement membrane and enter the epidermal compartment during skin inflammation (Wölfle et al., 2015).

RRM2, a small subunit of ribonucleotide reductase, is overexpressed by tumors, and plays a role in resistance to chemotherapy (Zhan et al., 2021). Tang *et al.* reported that there was a positive correlation between the expression level of RRM2 and the extent of infiltration by neutrophils and macrophages. They also reported that RRM2 inhibition effectively suppressed macrophage infiltration, and affected the balance of macrophage polarization, which promoted M1 phenotype polarization and suppressed the M2 phenotype *in vitro* and *in vivo* (Tang et al., 2021). However, there have been no reports about the relationship between RRM2 and AD until now. We found that RRM2 was upregulated in AD skin tissue. This may contribute to the chronic inflammation involved in AD, since RRM2 is known to promote macrophage infiltration and polarization. However, this hypothesis needs to be validated by further studies.

Immune cell infiltration is a hallmark of AD. Therefore, we studied infiltration of immune cells, and relationships between the identified hub genes (CCR7, CXCL10, IRF7, MMP1, and RRM2) and significant differential immune cells (naive $CD4^+$ T cells, plasma cells, activated DCs, and resting mast cells) in AD. We found that CCR7 expression level was positively correlated with the number of $CD4^+$ naïve T cells ($R = 0.42$, $p = 0.011$) in AD patients. It has been shown that CCR7 mediates the homing of naive T cells. Studies in CCR7-deficient ($CCR7^{-/-}$) mice have demonstrated that on examination of lymph nodes and Peyer's patches, naive T cell numbers are reduced. When the T cells of $CCR7^{-/-}$ mice were adoptively transferred to wild-type mice, they were unable to home to Peyer's patches and draining lymph nodes (Förster et al., 1999). We hypothesized that CCR7 might also mediate the homing of naive T cells in AD. However, more *in vitro* and *in vivo* studies need to be conducted to validate this hypothesis.

Several limitations are associated with our study. First, the human sample size was limited. Additional samples are required to validate the present findings. Second, we used only immunohistochemistry techniques to validate hub genes *in vitro*. Additional experiments with more quantitative methodologies will be needed for further confirmation of our results. Finally, the precise role of identified hub genes in AD requires additional elucidation both *in vitro* and *in vivo*. Further research is essential to determine whether CCR7, CXCL10, IRF7, MMP1, and RRM2 could be used as predictive biomarkers for the diagnosis and treatment of AD.

CONCLUSION

To summarize, our study uses both WGCNA and CIBERSORT methods to understand the molecular mechanism of AD, and to verify the result by immunohistochemical technology. Our study has not only identified effective biomarkers for AD but has increased our understanding of how immune cells related to AD. CCR7, CXCL10, IRF7, MMP1, and RRM2 could be used as biomarkers in AD for both diagnostic and therapeutic purposes. Additionally, we have shown that CCR7 expression level was positively correlated with the number of $CD4^+$ naïve T cells. We therefore have confidence that our study supports a new trajectory for investigation into the management of AD and provides new knowledge on the immune, cellular, and molecular mechanisms for its pathogenesis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/, accession numbers: GSE6012, GSE32924, GSE36842, and GSE120721.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Shengjing Hospital of the China Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CL downloaded and analyzed the data, prepared charts, and wrote the draft of the paper. YL initiated and planned the experiments. XH initiated and planned the experiments and reviewed and revised the article. We declare that all authors have read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.917077/full#supplementary-material

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RNA sequencing and lipidomics uncovers novel pathomechanisms in recessive X-linked ichthyosis

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Recessive X-linked ichthyosis (RXLI), a genetic disorder caused by deletion or point mutations of the steroid sulfatase (*STS*) gene, is the second most common form of ichthyosis. It is a disorder of keratinocyte cholesterol sulfate retention and the mechanism of extracutaneous phenotypes such as corneal opacities and attention deficit hyperactivity disorder are poorly understood. To understand the pathomechanisms of RXLI, the transcriptome of differentiated primary keratinocytes with STS knockdown was sequenced. The results were validated in a stable knockdown model of STS, to confirm STS specificity, and in RXLI skin. The results show that there was significantly reduced expression of genes related to epidermal differentiation and lipid metabolism, including ceramide and sphingolipid synthesis. In addition, there was significant downregulation of aldehyde dehydrogenase family members and the oxytocin receptor which have been linked to corneal transparency and behavioural disorders respectively, both of which are extracutaneous phenotypes of RXLI. These data provide a greater understanding of the causative mechanisms of RXLI's cutaneous phenotype, and show that the keratinocyte transcriptome and lipidomics can give novel insights into the phenotype of patients with RXLI.

KEYWORDS

ichthyosis, skin barrier, steroid sulfatase, ceramides, lipidomics

Introduction

Recessive X-linked ichthyosis (RXLI) is a genetic disorder occurring in approximately 1: 1500 males (Craig et al., 2010). The disease presents in the first year of life with scaling and erythema developing with time into brown polygonal scales most marked on the shins, neck and abdomen but generally sparing the palmoplantar skin and flexures. There are often extracutaneous features, which include asymptomatic corneal opacities, neurological features (autism-related traits and attention deficit hyperactivity disorder (ADHD)) and hypogonadism (Crane and Paller, 2022). In an Italian study, ADHD was reported in 9 out of 33 RXLI patients (Diociaiuti et al., 2019). A study of female carriers showed increased rates of autism-related traits and post-partum depression compared to controls (Cavenagh, Chatterjee, and Davies, 2019).

A recent study using UK Biobank identified an association with atrial arrhythmias, haemorrhage after surgery and palmar fascial fibromatosis (Brcic et al., 2020), (Brcic

et al., 2022). An online survey showed self-reported abnormal heart rhythms in 28%–35% of male and female carriers of Xp22.31 deletions and suggested that cardiac screening might be important (Wren et al., 2022).

The causative gene steroid sulfatase (STS) encodes a protein which hydrolyses cholesterol sulfate (CSO_4) to cholesterol (Koppe et al., 1978) (Tiepolo et al., 1980; Yen et al., 1987). In the epidermis this occurs in the upper granular layer and stratum corneum, where STS activity increases up to 20-fold compared to the basal layer (Williams et al., 1983). In RXLI there is a total loss of STS activity in all layers of the epidermis, leading to adverse effects on the lipid content, delayed desquamation and acanthosis which is thought to result from CSO_4 levels increasing up to 20-fold. In addition, mothers giving birth to babies with RXLI often experience prolonged labour (Paige et al., 1994).

Previous research *in vitro* has indicated CSO_4 can affect the activity and expression of transglutaminase (TGM) 1, a cross-linking enzyme important for the formation of the cornified envelope (CE) and the cornified lipid envelope (CLE) (Nemes et al., 2000). Analysis of RNA sequencing of human keratinocyte HaCaT cells treated with cholesterol and cholesterol sulfate identified increased Yippee-like 3 (*YPEL3*), a gene expected to affect keratinocyte differentiation. Increased expression of YPEL3 in STS-deficient cell lines promoted cellular senescence and increased involucrin and loricrin (Baek et al., 2021). In addition, CSO_4 has been shown *in vitro* to be a serine protease inhibitor, so it is theorised there is inhibition of kallikreins (KLK) 5 and 7 in RXLI, which would prevent desquamation (Sato et al., 1998). Thus far, these data have not been confirmed in RXLI samples and there is no data on the pathogenesis of extracutaneous signs, other than in those patients with contiguous deletions.

Here, we confirm reduced TGM1 expression and activity *in vivo* using RXLI skin samples and show upregulated expression of desmoglein-1 and reduced expression of KLK5. Furthermore, we report the results of transcriptome sequencing from primary keratinocytes with STS knockdown and validation in an STS knockdown model and RXLI skin. Immunostaining showed reduced epidermal expression of UDP-glucose ceramide glycosyltransferase (UGCG) and alkaline ceramidase 1 (ACER1), two important components of the ceramide synthesis pathway. Corneal opacities were linked to aldehyde dehydrogenase 1 family member A1 (ALDH1A1) and aldehyde dehydrogenase 3 family member A1 (ALDH3A1) downregulation, both of which are involved in corneal transparency (Lassen et al., 2007). A reduction was seen in oxytocin receptor (OXTR) expression which has been linked to behavioural disorders and prolonged labour (Fuchs et al., 1982) (Jacob et al., 2007). These data provide novel explanations for the development of extracutaneous features in RXLI, and further the understanding of how CSO_4 and STS interact and regulate epidermal differentiation and lipid synthesis.

Results

RNA sequencing in primary keratinocytes with siRNA knockdown of STS

We analysed a data set of triple biological replicates from primary keratinocytes with siRNA knockdown of STS. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE232622 (Barrett et al., 2013). After applying a cut-off point of $p < 0.05$ there were 3,454 upregulated and 3,608 downregulated genes in the transcriptome data set. Genes with log-fold change >2 were inputted into the Gene Ontology GO Enrichment Analysis using the PANTHER Classification System to identify enriched biological processes. Table 1 shows the top 20 downregulated biological processes. Of interest, these included enrichment of genes involved in cholesterol efflux (4.9-fold), astrocyte development (4.55-fold), keratinization (4.22-fold), keratinocyte differentiation (3.7-fold) and cardiac septum morphogenesis (3.19-fold). Table 2 shows the top 20 upregulated biological processes. This included enrichment of genes involved in negative regulation of epithelial cell differentiation (6.2-fold), cell substrate junction assembly and organisation (5.53 and 5.39-fold, respectively) and response to oestrogen (4.22-fold).

Development of RXLI model and characterization of transglutaminase expression and desquamation

To validate genes of interest, skin biopsies were obtained from three RXLI patients. To confirm any changes observed were due to loss of STS, we also created using lentiviral particles STS shRNA knockdown (STS KD1 and STS KD2) and non-targeting control (NTC) cell lines in immortalised N/TERT keratinocytes. Knockdown was confirmed using qPCR showing greater KD in STS KD 2 (Supplementary Figure S1A). These cell lines were used to make a 3D model of RXLI (Supplementary Figure S1B). The 3D model of RXLI had decreased expression of TGM1 and decreased activity of TGM1 and TGM3 (pH 8.4) with a greater decrease in STS KD2 skin equivalents (Figure 1A). Decreased activity was also seen in the RXLI 3D model at pH 7.4 (Supplementary Figure S2), confirming that TGM1 activity alone was also reduced. In RXLI skin samples, there was a significant decrease of TGM1 expression and activity (Figures 1B, C). Desquamation requires a KLK cascade and CSO_4 has been previously shown to inhibit KLK5 and KLK7 (Sato et al., 1998). We confirmed reduction of KLK5 in RXLI skin. KLK5 self-activates and then begins to activate other kallikreins, potentially making it the most important kallikrein for desquamation (Caubet et al., 2004). A reduction of KLK5 expression as observed here would likely affect the rest of the cascade, resulting in a reduction of several kallikreins required for desquamation resulting in the delay of desquamation seen in RXLI. Interestingly, loss of STS increased desmoglein 1 (*DSG1*) expression (Log Fold Change 1.049) in the RNA sequencing data set. Cell adhesion proteins are targets of KLKs including DSG1, desmocollin 1 (DSC1) and corneodesmosin (CDSN). The increase in DSG1 was confirmed by immunostaining in both the RXLI model (more obvious in STS KD2) in Figure 1A and in RXLI skin (Figures 1B, C), consistent with the reduced desquamation observed.

Reduced expression of lipid metabolism enzymes in the RXLI model and skin

Differentially expressed genes in primary keratinocytes with STS knockdown included *ACER1* and *UGCG*, which encode enzymes important for lipid metabolism in the epidermis (Amen et al., 2013) (Sun et al., 2008). Alkaline ceramidase 1 hydrolyses long-chain

TABLE 1 Gene Ontology annotation of biological processes downregulated in keratinocytes with siRNA knockdown of STS. FDR = False discovery rate. Expected = expected number of genes downregulated. Fold-enrichment- Actual number of genes downregulated/expected.

GO biological processes	Reference list	Downregulated (1292)	Expected	Fold-enrichment	Raw *p*-value	FDR
antigen processing and presentation of endogenous peptide antigen (GO:0002483)	19	8	1.19	6.71	1.17E-04	1.06E-02
response to interferon-alpha (GO:0035455)	23	8	1.44	5.54	3.36E-04	2.39E-02
neural tube patterning (GO:0021532)	33	11	2.07	5.31	3.67E-05	4.39E-03
positive regulation of cholesterol efflux (GO:0010875)	26	8	1.63	4.9	6.61E-04	3.95E-02
antigen processing and presentation of endogenous antigen (GO:0019883)	26	8	1.63	4.9	6.61E-04	3.94E-02
astrocyte development (GO:0014002)	35	10	2.2	4.55	2.38E-04	1.90E-02
keratinization (GO:0031424)	83	22	5.21	4.22	1.68E-07	6.44E-05
ventricular septum morphogenesis (GO:0060412)	41	10	2.57	3.89	6.90E-04	4.05E-02
keratinocyte differentiation (GO:0030216)	138	32	8.66	3.7	4.75E-09	3.39E-06
intermediate filament organization (GO:0045109)	70	16	4.39	3.64	3.81E-05	4.49E-03
negative regulation of viral genome replication (GO:0045071)	57	13	3.58	3.63	2.03E-04	1.66E-02
SMAD protein signal transduction (GO:0060395)	63	14	3.95	3.54	1.48E-04	1.31E-02
embryonic digit morphogenesis (GO:0042733)	59	13	3.7	3.51	2.71E-04	2.03E-02
roof of mouth development (GO:0060021)	92	20	5.77	3.46	8.17E-06	1.41E-03
aorta development (GO:0035904)	56	12	3.51	3.41	5.73E-04	3.54E-02
epidermal cell differentiation (GO:0009913)	201	42	12.61	3.33	3.18E-10	2.94E-07
BMP signaling pathway (GO:0030509)	91	19	5.71	3.33	2.23E-05	3.09E-03
cardiac septum morphogenesis (GO:0060411)	70	14	4.39	3.19	3.80E-04	2.61E-02
intermediate filament cytoskeleton organization (GO:0045104)	92	18	5.77	3.12	7.58E-05	7.62E-03
intermediate filament-based process (GO:0045103)	93	18	5.84	3.08	8.56E-05	8.28E-03

ceramides to sphingosine, which is then phosphorylated to form sphingosine-1-phosphate, regulated by sphingosine 1 kinase (SPHK1). The UGCG enzyme catalyses the first step of glycosphingolipid production, synthesising glucosylceramides (GluCers) from ceramides and UDP-glucose. Loss of these enzymes could affect bioactive lipid content within the epidermis, so RXLI skin samples and the 3D model were immunostained for ACER1 and UGCG (Figures 2A, B). In the 3D model, expression of all 3 proteins was reduced in both STS KD models (Figure 2A). As previously shown, ACER1 was expressed in differentiated epidermis with a clear reduction in RXLI skin (see Figures 2B, C). The UGCG expression was strong in normal basal epidermis with weaker expression in the upper epidermis and was markedly decreased in RXLI skin (see Figures 2B, C). Expression of SPHK1 was seen throughout the epidermis in both the NTC 3D model and in normal control skin. A reduction was seen in both STS KD models and in RXLI epidermis (see Figures 2A–C).

Reduced expression of aldehyde dehydrogenases and the oxytocin receptor in the RXLI model and skin

The transcriptome of primary keratinocytes with STS knockdown showed altered expression of *ALDH1A1*, *ALDH3A1* and *OXTR*. Investigation of the literature indicated

TABLE 2 Gene Ontology annotation of biological processes upregulated in keratinocytes with siRNA knockdown of STS. FDR = False discovery rate.

GO biological processes	Reference list	Upregulated (745)	Expected	Fold-enrichment	Raw *p*-value	FDR
endoderm formation (GO:0001706)	54	13	1.95	6.65	4.61E-07	3.28E-04
endodermal cell differentiation (GO:0035987)	44	10	1.59	6.28	1.51E-05	3.70E-03
negative regulation of epithelial cell differentiation (GO:0030857)	49	11	1.77	6.2	6.29E-06	1.97E-03
regulation of cell migration involved in sprouting angiogenesis (GO:0090049)	38	8	1.38	5.82	1.71E-04	2.34E-02
regulation of transforming growth factor beta production (GO:0071634)	39	8	1.41	5.67	2.00E-04	2.64E-02
cell-substrate junction assembly (GO:0007044)	40	8	1.45	5.53	2.33E-04	2.97E-02
regulation of blood coagulation (GO:0030193)	70	14	2.53	5.53	1.17E-06	5.56E-04
negative regulation of blood coagulation (GO:0030195)	46	9	1.66	5.41	1.11E-04	1.70E-02
cell-substrate junction organization (GO:0150115)	41	8	1.48	5.39	2.70E-04	3.36E-02
regulation of hemostasis (GO:1900046)	72	14	2.61	5.37	1.57E-06	6.84E-04
negative regulation of hemostasis (GO:1900047)	47	9	1.7	5.29	1.28E-04	1.86E-02
regulation of coagulation (GO:0050818)	75	14	2.71	5.16	2.40E-06	9.40E-04
negative regulation of coagulation (GO:0050819)	50	9	1.81	4.97	1.93E-04	2.62E-02
positive regulation of blood vessel endothelial cell migration (GO:0043536)	54	9	1.95	4.61	3.22E-04	3.88E-02
regulation of wound healing (GO:0061041)	130	21	4.7	4.46	7.01E-08	8.45E-05
endoderm development (GO:0007492)	81	13	2.93	4.44	2.30E-05	5.08E-03
response to estrogen (GO:0043627)	72	11	2.61	4.22	1.43E-04	1.99E-02
negative regulation of wound healing (GO:0061045)	69	10	2.5	4.01	4.15E-04	4.72E-02
positive regulation of smooth muscle cell proliferation (GO:0048661)	85	12	3.08	3.9	1.42E-04	1.98E-02
regulation of ERBB signaling pathway (GO:1901184)	78	11	2.82	3.9	2.68E-04	3.36E-02

they could be linked to extracutaneous features of RXLI. There is evidence that ALDH1A1 and ALDH3A1 are important for transparency of the cornea (Lassen et al., 2007; Arrowsmith and Wray, 2014), whereas OXTR has been linked to behavioural disorders and is known to regulate parturition (Rijlaarsdam et al., 2017) (Arrowsmith and Wray, 2014). Thus, immunostaining for ALDH1A1, ALDH3A1 and OXTR was performed in the RXLI model (Figure 3A), while the expression of ALDH1A1, ALDH3A1 and OXTR was also investigated in normal control skin and RXLI skin (Figure 3B). All three had decreased expression in the RXLI model compared to NTC (Figure 3A). ALDH1A1 was expressed in the stratum corneum and basal epidermis close to the basement membrane in control skin and markedly reduced expression was observed in RXLI skin (Figures 3B, C). ALDH3A1 was expressed in suprabasal epidermis in normal skin and was markedly reduced in RXLI skin. OXTR expression was present in the basal epidermis in normal skin and was markedly decreased in RXLI skin (Figure 3B).

Lipidomic analysis of the RXLI model

In order to understand the effect of STS knockdown further, changes in lipid profiles induced in the RXLI model were analysed using lipidomics as described in the Methods. We initially applied an untargeted approach to the whole lipid extracts from 3D epidermis. Only entities whose abundance appeared to be affected by the STS

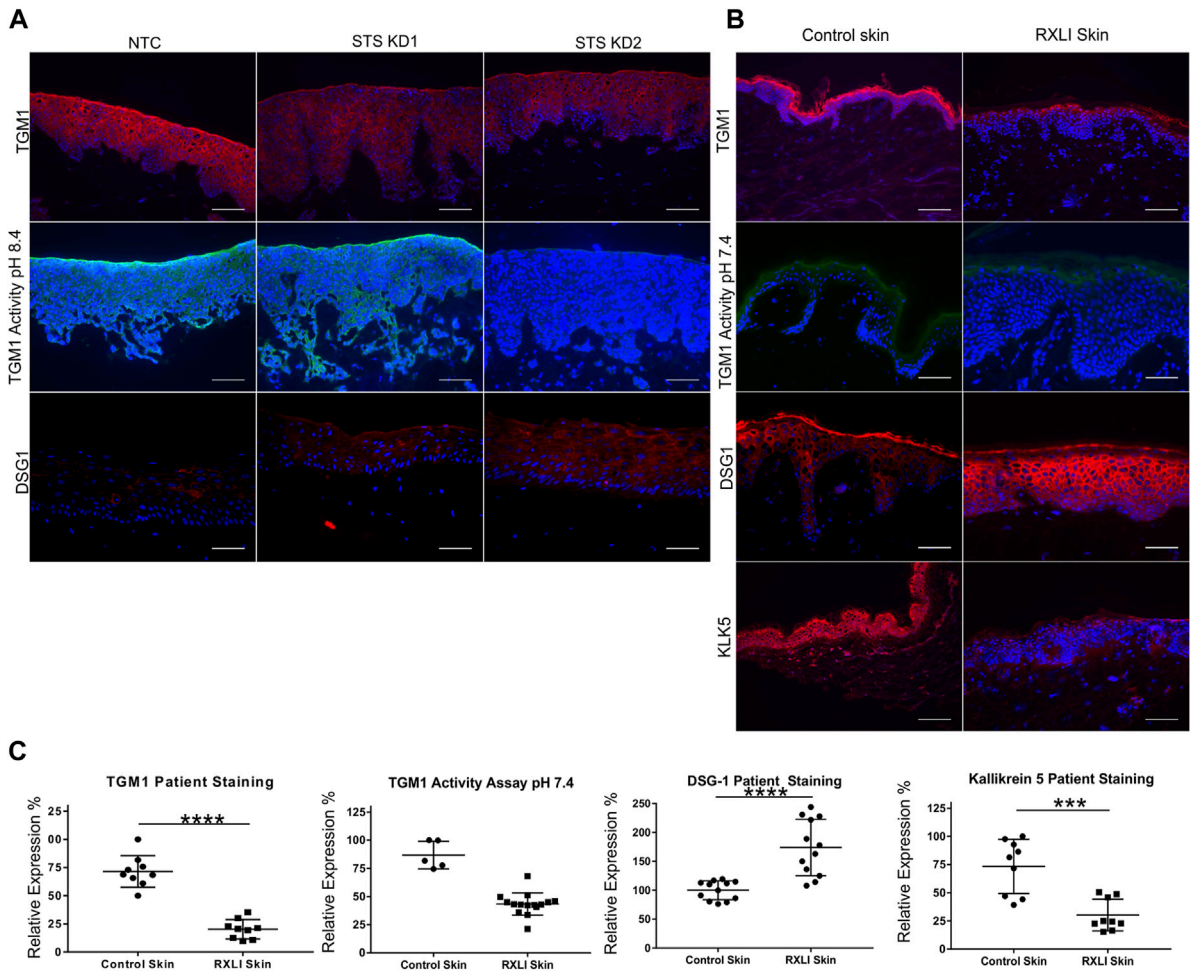

FIGURE 1
Loss of steroid sulfatase alters differentiation of the epidermis. **(A)** Immunohistochemistry of TGM1 expression in red (upper panel), TGM1 activity at pH 8.4 in green (middle panel) and DSG1 in red (lower panel) in 3D organotypics. **(B)** Immunohistochemistry of TGM1 expression (red) and activity at pH 7.4 (green), DSG1 (red) and KLK5 (green) expression in control skin and RXLI skin samples. DAPI (4′,6-diamidino-2-phenylindole) nuclear staining is blue. **(C)** Quantification of immunostaining shown in **(B)**. Three 40 X images were taken per sample (n = 3 for control and RXLI skin) for quantification of TGM1 and KLK5 expression. Five 40 X images were taken per sample (n = 1 for control skin and n = 3 for RXLI skin) for quantification of the TGM activity assay. Scale bar: 100 μm ***p < 0.001 and ****p < 0.0001 by unpaired 2-tailed t-test.

knockdown at a statistically significant extent were further processed for their identification. As shown in Figure 4A, CSO_4 expression was significantly increased in STS KD 2 epidermis, validating the model as CSO_4 retention is seen in RXLI. Principal component analysis (see Figure 4B) showed that there was a high degree of overlap between NTC and STS KD1, while there was an appreciable difference between NTC and STS KD2. The volcano plot in Figure 4C represents the changes of lipid levels in STS KD2 compared to NTC. Table 3 shows detailed changes that satisfied the cut-offs of fold change >1.4 and significance ≤0.05 in regulation of lipid species in STS KD2 versus NTC, as depicted in the volcano plot in Figure 4C. Supplementary Figure S3 shows hierarchical clustering of lipids modulated in STS KD1 and KD2 compared to NTC again confirming major changes in STS KD2 (lipid species are detailed in Supplementary Table S4). Several ceramide species belonging to the non-hydroxy fatty acyl dihydrosphingosine (NDS) subclass class consisting of non-hydroxy fatty acids (N) and dihydrosphingosine base (DS), which is also known as sphinganine, were upregulated. Ceramide nomenclature is as described in these references (Masukawa et al., 2008; Liebisch et al., 2020). The increase of these ceramides may reflect a promoted *de novo* synthesis of ceramides or defective desaturation to the sphingosine base by sphingolipid desaturases, such as DEGS1. One member of the non-hydroxy fatty acyl sphingosine (NS) ceramides was upregulated. Ceramides containing deoxysphinganine as the sphingoid base were also upregulated. The increase of these anomalous ceramides may be due to condensation of alanine (instead of serine) with palmitoyl-CoA (Muthusamy et al., 2020). Several members of the tri hexosyl ceramides (Hex3Cer) were downregulated in KD2. Decreased levels of these sphingolipid metabolites in KD2 indicates deregulated sphingolipid synthesis/metabolism and trafficking (Iwabuchi et al., 2015). Moreover, the significantly decreased levels of N-acylphosphatidylethanolamine (NAPE) suggests deranged metabolism of endocannabinoids which may be related to the decrease of phosphatidylethanolamine (PEs) species (Coulon et al., 2012). The significance of a moderate, but

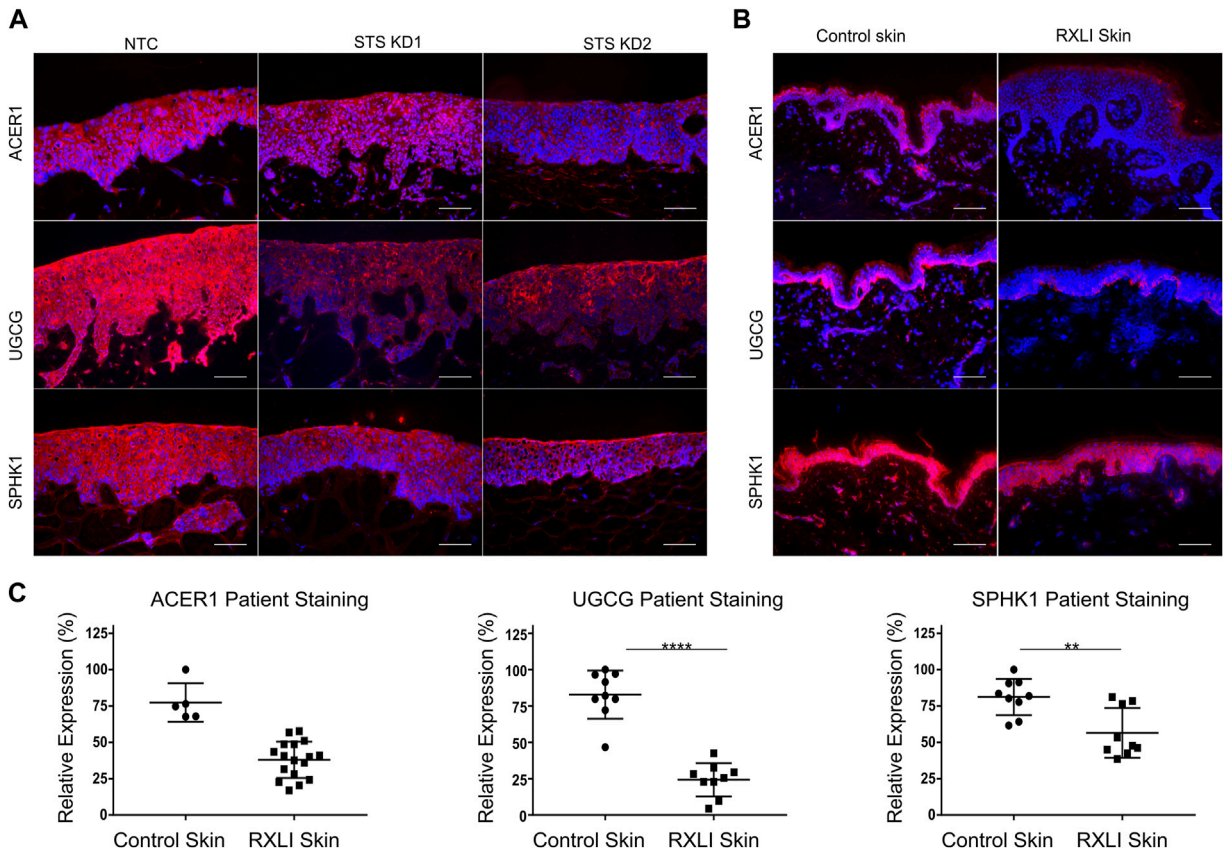

FIGURE 2
Loss of steroid sulfatase alters lipid metabolism in the epidermis. **(A)** Immunohistochemistry of ACER1, UGCG and SPHK1 expression in 3D organotypics. Scale bar: 100 μm **(B)** Immunohistochemistry of ACER1, UGCG and SPHK1 expression in control skin and RXLI skin samples. All antigens of interest are in red. DAPI nuclear staining is blue. **(C)** Quantification of immunostaining shown in **(B)**. For UGCG and SPHK1 quantification three 40 X images were taken per sample (n = 3 each for control and RXLI skin). For ACER1 five 40 X images were taken per sample (n = 1 for control skin and n = 3 for RXLI skin). Scale bar: 100 μm $^{**}p < 0.01$ and $^{****}p < 0.0001$ by unpaired 2-tailed t-test.

statistically significant, decrease of etherPEs requires further investigation (Fontaine et al., 2020).

Discussion

Apart from deletion of *STS* and retention of CSO_4, little is known about the underlying pathomechanisms in RXLI, or how extracutaneous phenotypes develop. These data further the understanding of RXLI pathomechanisms in skin, by showing loss of STS results in decreased expression of TGM1, KLK5, ACER1, UGCG, SPHK1 and decreased TGM activity. Taken together, these data suggest STS is important for regulating epidermal homeostasis, lipid metabolism, proliferation, differentiation, and desquamation. The data presented also provide insights into pathomechanisms involved in corneal opacities, behavioural changes and prolonged labour in female carriers of RXLI. Although, we have not explored further, the presence of a gene cluster involved in cardiac septum morphogenesis may be relevant to the development of arrhythmias in some RXLI patients.

Mice with *Acer1* knockout have increased levels of ceramides and sphingomyelin, and decreased levels of sphingosine and sphingosine-1-phosphate (S1P). Furthermore, the mice have dry, hyperplastic skin and a thickened stratum corneum (Liakath-Ali et al., 2016). The phenotype of these mice is similar to that of RXLI, particularly the dry skin and thickened stratum corneum, which suggests loss of ACER1 contributes to RXLI skin pathology. The activity of SPHK1 is crucial for generating S1P, which has anti-proliferative effects on keratinocytes (Di Nardo et al., 2000). Less SPHK1 will generate less S1P, indicating the loss of SPHK1 observed will result in a proliferative phenotype and altered differentiation.

A knockout mouse model of *Ugcg* presented with dry skin, thickening of the epidermis and hyperkeratosis, all phenotypes of RXLI. The knockout mice had extended expression of K14 up through the suprabasal layers, and overexpression of K6, which would both indicate a proliferative phenotype (Amen et al., 2013) (Jennemann et al., 2007). Importantly, *Ugcg* knockout mice have reduced levels of free-extractable glucosylceramides and increased free-extractable ceramides. However, protein-bound ceramides are severely reduced, indicating UGCG is important for producing protein-bound ceramide precursors (protein-bound glucosylceramides). It is probable that the reduction of UGCG in RXLI would alter ceramide metabolism by lowering the glucosylceramide content of the epidermis, reflected by the decreased Hex3Cer shown in Table 3. As shown in mouse

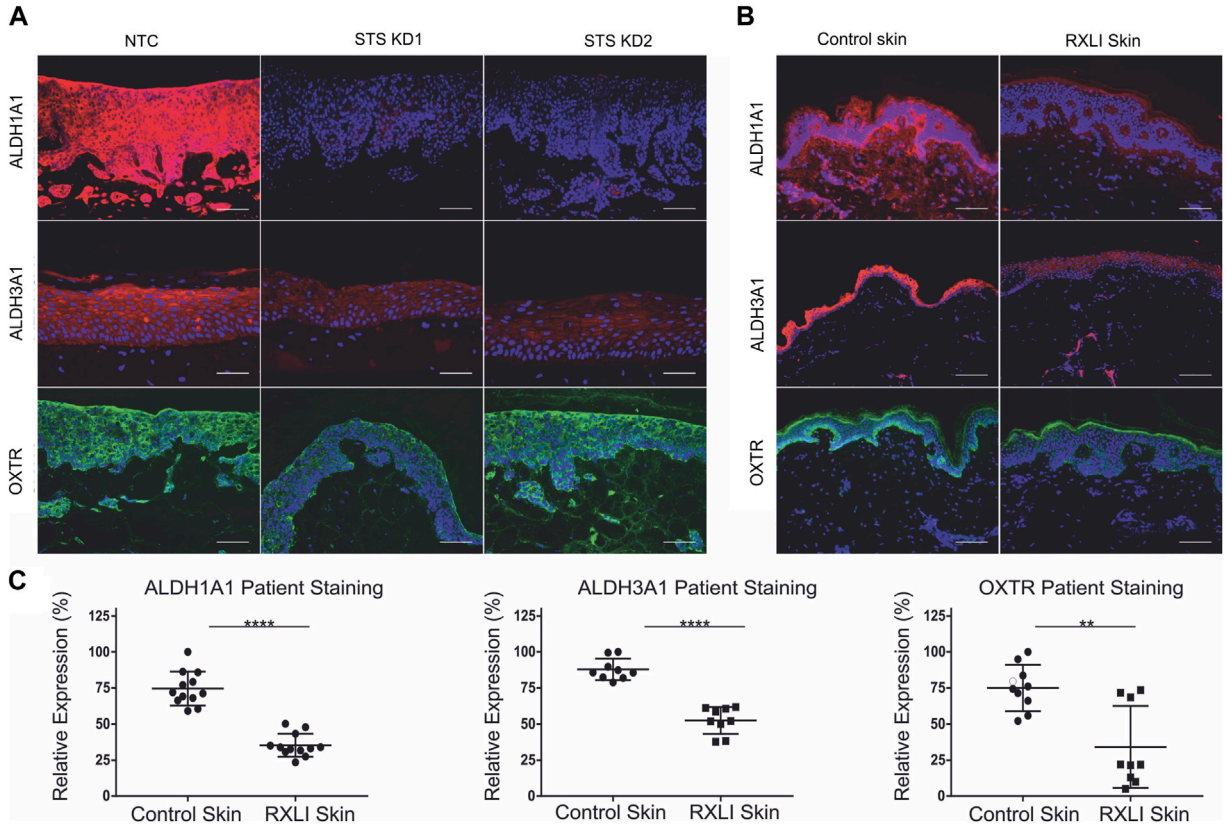

FIGURE 3
Loss of steroid sulfatase results in altered expression of genes linked to extracutaneous features of RXLI. **(A)** Immunohistochemistry of ALDH1A1 (red), ALDH3A1 (red) and OXTR (green) expression in 3D organotypics. Scale bar: 100 μm. **(B)** Immunohistochemistry of ALDH1A1 (red), ALDH3A1 (red) and OXTR (green) expression in control skin and RXLI skin samples. DAPI nuclear staining is blue. **(C)** Quantification of immunostaining shown in **(B)**. Three (ALDH3A1 and OXTR) or four (ALDH1A1) 40 X images were taken per sample (n = 3 each for control and RXLI skin) for quantification. Scale bar: 100 μm $^{**}p < 0.01$ and $^{****}p < 0.0001$ by unpaired 2-tailed t-test.

models, this would prevent the formation of protein bound ceramides, and impact on the cornified lipid envelope.

Both ALDH1A1 and ALDH3A1 are important in reducing UVR damage in the cornea and lens as their loss results in higher levels of ROS and thus damage to the eye. It is believed this is how the corneal opacities develop in *Aldh1a1/Aldh3a1* knockout mice (Lassen et al., 2007). It was also noted previously that knockdown of OXTR in keratinocytes and fibroblasts caused increased levels of ROS and decreased levels of glutathione, an important antioxidant (Deing et al., 2013). Therefore, loss of OXTR may also contribute to corneal opacities by further reducing the cornea's protection against stressors such as ultraviolet radiation. With our confirmation of reduced ALDH1A1, ALDH3A1 and OXTR expression in RXLI, we propose this is a contributing factor to the corneal opacities. Interestingly, the majority of corneal opacities in RXLI patients do not develop until the second or third decade of life. Damage caused by UVR may accumulate over time and act in combination with loss of these proteins in the development of corneal opacities, explaining why these develop later in life. As only half of patients develop corneal opacities, there may be other genetic or environmental factors involved. The aldehyde dehydrogenase family members are important components of the retinoic acid signalling pathway which is important in regulating epithelial proliferation (Kumar et al., 2017). A reduction in ALDH1A1 and ALDH3A1 may also affect keratinocyte proliferation.

Behaviours on the autistic spectrum and ADHD are more common in RXLI patients (25% and 40%, respectively) than the general population (Kent et al., 2008; Deing et al., 2013). It has been shown that loss of STS due to point mutations alone can result in RXLI patients developing ADHD. Interestingly, dysregulation of ceramides, alkaline ceramidases and S1P have been implicated in neurodegenerative disorders, indicating the potentially altered sphingolipids in RXLI may contribute to the behavioural disorders associated with RXLI (Cutler et al., 2004; Ben-David and Futerman, 2010; Wang et al., 2015). There are now numerous studies which conclude that polymorphisms in the *OXTR* gene confer a risk of behavioural disorders (Jacob et al., 2007; Lerer et al., 2008; Liu et al., 2010). It is theorised this is a result of oxytocin being unable to properly elicit its neurological effects due to loss of its receptor.

In RXLI, reduced placental STS results in prolonged labour. Oxytocin is used to induce uterine contractility (Fuchs et al., 1982). A reduction of placental OXTR expression may contribute to prolonged labour. Transport of OXT has been shown to occur from mother to

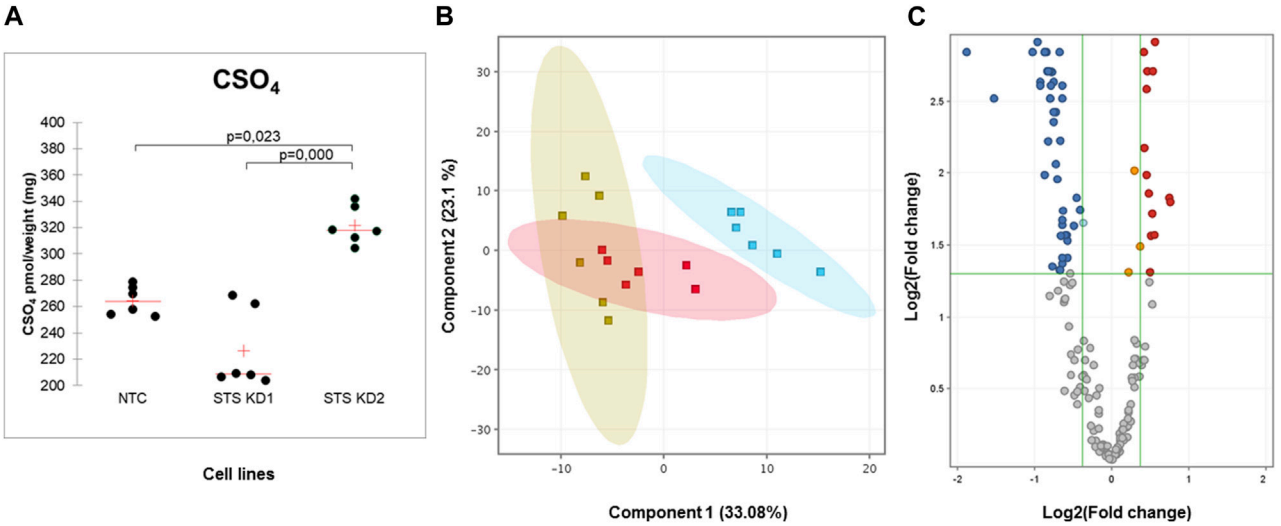

FIGURE 4
Retention of cholesterol sulfate and altered lipidome of RXLI model. **(A)** Quantitative assessment (LCMS) of cholesterol sulfate (CSO_4) in NTC, STS KD1, and STS KD2. Quantitative amounts are reported as pmol/weight (mg) of the 3D organotypics. **(B)** Principal components analysis (PCA) of the lipids detected in NTC (red), STS KD1 (yellow) and STS KD2 (blue) organotypics. **(C)** Volcano plot indicating the changes in the lipid abundance in STS KD2 compared to control (NTC). Each dot represents one lipid species. Differential abundance is reported as log2 transformed fold change (FC) vs. NTC. Lipid species diminished or increased at a statistically different level were indicated with blue and red dots, respectively. Identities, molecular mass, and regulation of species that passed significance = 0.05 and FC = 1.3 cut-offs are reported in Table 3. Upregulated and downregulated species that did not pass the significance cut-off of 0.05 are displayed in orange and light blue, respectively.

foetus and *vice versa*, which helps explain how loss of OXTR due to RXLI could potentially affect the mother and parturition regulation (Malek, Blann, and Mattison, 1996). This reduction of OXTR could be a result of decreased oestrogen, or a concurrent mechanism that also contributes to prolonged parturition.

In summary, in this paper, we show that loss of STS in human epidermis causes major changes to ceramide and sphingolipid synthesis. This expands our understanding of RXLI pathogenesis and has implications for the skin barrier. The changes in sphingolipids may also be associated with the neuropsychiatric symptoms experienced by these patients. The connection between ALDH1A1 and ALDH3A1 loss and corneal opacities, and OXTR loss and prolonged labour and behavioural disorders provides a new explanation for the extracutaneous features of RXLI. In addition, the RNA-Seq data showed altered RNA expression of several genes linked to behavioural disorders and brain and cardiac development which could be explored further. Further work could be performed in a mouse model or using patient-derived induced pluripotent stem cells which could be differentiated into cells of interest including cardiomyocytes or neurons. A better understanding of the biological mechanisms underlying RXLI may lead to new treatments in the future.

Materials and methods

Study approval

This study was conducted according to the Declaration of Helsinki Principles and was approved by the East London and City Health Authority Research Ethics Committee. Written informed consent was obtained from patients before samples were taken.

Control skin samples and RXLI skin samples

Dermal fibroblasts were isolated from neonatal foreskins. Redundant normal skin was used as a control for RXLI skin samples. Samples of RXLI skin were obtained from 3 individuals diagnosed with RXLI, confirmed by either steroid sulfatase activity assays or genetic testing.

Cell culture and Knockdown cell lines

The human keratinocyte telomerase reverse-transcriptase–immortalized (h/TERT-immortalized) N/TERT-1 cell line derived from clinically normal foreskin tissue and supplied by James Rheinwald (Department of Dermatology, Harvard University Medical School, Boston, Massachusetts, United States) (Dickson et al., 2000) was grown in RM + growth media (DMEM/F-12, 10% FBS, 1× penicillin streptomycin [P/S], 0.4 μg/mL hydrocortisone, 0.5 μg/mL insulin, 10 ng/mL epidermal growth factor, 0.1 nM cholera toxin, 5 μg/mL transferrin, 20 p.m. liothyronine) and incubated at 37°C, 5% CO2. Human primary fibroblasts isolated from fresh redundant skin were grown in fibroblast growth media (DMEM, 10% FBS, 1× P/S) and incubated at 37°C, 5% CO2. Primary keratinocytes were isolated from neonatal foreskin and grown in Epilife media. SMARTvector

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TABLE 3 Lipid species upregulated and downregulated in STS KD2 cells compared to control.

Compound	Mass	FC (STS KD2 Vs. NTC)	Log FC (STS KD2 Vs. NTC)	Regulation (STS KD2 Vs. NTC)
Cholesterol sulfate	466,3115	1,49	0,57	up
Cer(d18:0/22:0)	623,6191	1,45	0,53	up
Cer(d18:0/24:0)	651,6520	1,40	0,49	up
Cer(d18:0/26:0)	679,6835	1,38	0,46	up
Cer(d18:0/28:0)	707,7141	1,34	0,42	up
Cer(d18:1/22:0)	621,6017	1,42	0,51	up
Cer(m18:0/20:0)	579,5908	1,42	0,50	up
Cer(m18:0/22:0)	607,6240	1,48	0,56	up
Cer(m18:0/24:0)	635,6570	1,46	0,54	up
Cer(m18:0/24:1)	633,6417	1,37	0,46	up
Cer(m18:0/26:0)	663,6881	1,34	0,43	up
Cer(m18:0/26:1)	661,6730	1,37	0,46	up
EtherPC 40:2	827,6639	1,69	0,75	up
SM 40:2;O2	784,6430	1,70	0,76	up
NAPE(48:2)	925,7069	−1,56	−0,64	down
Hex3Cer(d18:0/14:1)	995,6363	−1,32	−0,41	down
Hex3Cer(d18:0/16:1)	1023,670	−1,71	−0,78	down
Hex3Cer(d18:0/18:1)	1051,699	−1,48	−0,57	down
Hex3Cer(d18:0/20:1)	1079,731	−1,48	−0,57	down
Hex3Cer(d18:0/24:1)	1135,794	−1,60	−0,68	down
Hex3Cer(d18:1/14:0)	995,6363	−1,36	−0,45	down
Hex3Cer(d18:1/16:0)	1023,670	−1,71	−0,77	down
Hex3Cer(d18:1/18:0)	1051,699	−1,56	−0,64	down
Hex3Cer(d18:1/18:1)	1023,670	−1,71	−0,78	down
Hex3Cer(d18:1/20:0)	1079,730	−1,50	−0,58	down
Hex3Cer(d18:1/20:1)	1051,699	−1,54	−0,63	down
Hex3Cer(d18:1/24:0)	1135,794	−1,59	−0,66	down
Hex3Cer(d18:1/24:1)	1133,778	−1,82	−0,87	down
Hex3Cer(d18:1/26:1)	1161,809	−1,70	−0,76	down
PE 31:2	673,4664	−1,58	−0,66	down
PE 32:1	689,5011	−1,60	−0,67	down
PE 32:0	691,5161	−1,55	−0,63	down
PE 34:4	711,4799	−1,56	−0,64	down
PE 34:3	713,5015	−1,62	−0,70	down
PE 34:2	715,5223	−1,89	−0,92	down
PE 34:1	717,5291	−1,94	−0,96	down
PE 35:5	727,5234	−1,51	−0,59	down
PE 36:4	739,5121	−1,73	−0,79	down

(Continued on following page)

TABLE 3 (*Continued*) Lipid species upregulated and downregulated in STS KD2 cells compared to control.

Compound	Mass	FC (STS KD2 Vs. NTC)	Log FC (STS KD2 Vs. NTC)	Regulation (STS KD2 Vs. NTC)
PE 36:3	741,5337	−1,68	−0,75	down
PE 36:2	743,5466	−2,03	−1,02	down
PE 36:1	745,5616	−1,89	−0,92	down
PE 38:4	767,5443	−1,71	−0,78	down
PE 38:3	769,5597	−1,77	−0,82	down
PE 38:2	771,5786	−1,78	−0,83	down
PE 40:8	787,5107	−1,55	−0,63	down
PE 40:7	789,5289	−1,68	−0,75	down
PE 40:6	791,5450	−1,79	−0,84	down
EtherPE 32:2	673,5048	−1,55	−0,63	down
EtherPE 34:3	699,5168	−1,65	−0,72	down
EtherPE 34:2	701,5359	−1,57	−0,65	down
EtherPE 36:6	721,5056	−1,82	−0,87	down
EtherPE 36:5	723,5194	−1,74	−0,80	down
EtherPE 36:4	725,5350	−1,64	−0,72	down
EtherPE 36:2	729,5682	−1,77	−0,82	down
EtherPE 38:6	749,5345	−1,77	−0,82	down
EtherPE 38:5	751,5484	−1,73	−0,79	down
EtherPE 40:7	775,5501	−1,68	−0,75	down
EtherPE 40:6	777,5673	−1,82	−0,86	down
SM 32:1;O2	674,5364	−1,40	−0,49	down

2.0 lentiviral shRNA particles (GE Healthcare, UK) targeting exons five and ten were used to transduce N/TERT keratinocytes with an shRNA construct in order to generate a stable knockdown of STS (see Supplementary Table S1). A non-targeting shRNA construct was transduced into keratinocytes and used as a control. The construct included a GFP reporter to allow confirmation of successful transduction.

3D model of RXLI

Collagen and Matrigel gels were prepared by mixing 3.5 volumes of collagen I with 3.5 volumes of Matrigel, one volume of MEM, one volume of FBS and 1 volume of primary fibroblasts. 1 ml of gel mix was added per well required of a 24 well plate and incubated at 37°C for 1 h. Subsequently, 1 ml of media were added to the top of the gel and incubated overnight at 37°C. The media were aspirated from the gel, and N/TERT keratinocytes infected with shRNA lentiviral particles were seeded on top of the gel and incubated at 37°C overnight. The gels were then raised to the air/liquid interface on a steel grid placed in a 6 well plate. The gels were incubated for a further 14 days.

Preparation of tissues

Tissues were fixed in 4% PFA and embedded in paraffin using an automated tissue processor. Sections (5 µm-thick) of 4% PFA-fixed and paraffin embedded samples were cut using a Reichert-Jung 2035 microtome and placed on SuperFrost Plus slides. Sections were then deparaffinized in xylene and then rehydrated through a graduated ethanol series and water before immunostaining. Cryosections (10 µm-thick) of frozen samples mounted in OCT (Tissue-Tek, NL) were cut in a cryostat, placed on SuperFrost Plus slides and stored at −80°C. When used, sections were left to air dry for 10 min then washed once in PBS before immunostaining.

Immunostaining

Sections were washed in PBS for 5 min, then blocked by incubating in IFF (1% BSA w/v, 2% FBS v/v in PBS) containing 5%–10% goat serum for 1 hour. Sections were then probed with primary antibody diluted in PBS containing 1% BSA and incubated on the section at RT for 2 hours or overnight at 4°C. Sections were washed three times with PBS before

addition of the secondary antibody, Alexa Fluor 568-red or 488-green, goat anti-rabbit or goat anti-mouse (Invitrogen, CA, United States) at a 1:500 dilution for 1 hour at room temperature. Sections were washed three times in PBS before DAPI at a 1:1000 dilution was used as a nuclear stain. Following a further three washes in PBS the sections were mounted using Immu-mount (Thermo Fisher Scientific MA, United States). Negative controls consisted of mouse or rabbit IgG diluted to the same concentration as the primary antibody. Images were obtained using a Leica MM epi-fluorescence microscope. Antibodies used are detailed in Supplementary Table S2.

Transglutaminase assay

Cryosections were washed with PBS for 5 min. Four sections were used per sample. Sections were blocked by incubation in 0.1 M Tris with 3% BSA at either pH 7.4 or pH 8.4. Sections were then incubated with 0.1 M Tris with 3% BSA containing either 20 µl 10 mM biotinylated monodansylcadaverine +20 µl 0.5 M $CaCl_2$ per ml (positive sample) or 20 µl 10 mM biotinylated monodansylcadaverine +20 µl 0.5 M EDTA per ml (negative sample) at either pH 7.4 or pH 8.4 for 1 hour. The reaction was stopped by incubation of sections for 10 min with PBS +20 µl 0.5 M EDTA per ml. The sections were then washed three times with PBS. Streptavidin-Alexa488 diluted 1:2000 and DAPI diluted 1:1000 in PBS was applied to the sections for 30 min. The sections were then washed a further three times and mounted using Immu-mount. Images were obtained using a Leica MM epi-fluorescence microscope.

Quantification

The staining of RXLI patient and normal control skin was quantified using Cell Profiler (3.0.0) which measured the intensity of staining across each image taken. This allowed us to calculate an average intensity per cell for control skin samples and RXLI patient samples, which were then averaged across multiple images from the same section to produce an overall value.

RNA work

Total RNA was extracted from cells in a six well plate and purified using the RNAeasy kit (Qiagen, NL) according to the manufacturer's instructions. Concentration of RNA samples was determined by measuring the absorbance at 260 nm with a spectrophotometer after RNA extraction (Nanodrop, ND-1000 Spectrophotometer) and stored at −80°C. cDNA was synthesised from total RNA using SuperScript VILO cDNA synthesis kit (Invitrogen, CA, United States) according to the manufacturer's instructions.

qPCR

The qPCR was performed with 5 ng of cDNA using the KAPA SYBR FAST qPCR kit (Biosystems, ES). cDNA was mixed with 1x KAPA SYBR FAST qPCR Master Mix, forward primer (200 nM), reverse primer (200 nM), and carboxy-X-rhodamine (ROX) reference dye low. The mix was adjusted to a reaction volume of 10 µl with water. The qPCR reaction was carried out using the 7,500 Real Time PCR system (Life Technologies, CA, United States) with the following cycle sequence: initiation at 95°C for 5 min, then 40 cycles of melting at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s. Each sample was analysed in triplicate and data analysis was performed using the 7,500 System Detection Software v1.4 (Life Technologies, CA, United States) and Microsoft Excel 2011. HPRT was used as an internal control. Primers used are detailed in Supplementary Table S3.

RNA Sequencing

HISAT2 was used to align sequencing reads to a reference genome. This tool uses the Burrows-Wheeler transform and the FM index to align reads with Bowtie2 as the algorithmic core. Once the reads are aligned, HTSeq is used to produce counts for each gene of how many aligned reads overlap its exons. This preprocesses the RNA-Seq data for differential expression analysis. The counts are fed into DESeq2, a tool used for differential expression analysis based on a model using negative binomial distribution.

Statistics

Statistical analysis was determined by a Student's unpaired two-tailed *t*-test using GraphPad Prism 7.03 (GraphPad Software). A *p*-value of 0.05 or less was considered statistically significant.

Sample processing for the analysis of lipids

Organotypics (NTC, n = 3, STS KD1 n = 3, and STS KD2 n = 3) were treated as a whole. Organotypics were weighed and lipids extracted with a chloroform/methanol mixture 2:1 after addition of the internal standard mixture containing SPLASH Lipidomix®, LM6002, and d31CerNS (Avanti Polar Lipids, United States), and in-house mixed deuterated standards supplied by C/D/N isotopes and Toronto Research Chemicals, both from Canada. Aliquots of dissolved lipid extracts were analysed in duplicate by GCMS for the quantification of cholesterol, desmosterol, and free fatty acids. The dissolved lipid extracts were further analysed in duplicate by untargeted LCMS in positive and negative ion mode. The results of the untargeted approach were normalised by the internal standard d31CerNS and the weight of each sample. Quantitative results from both GCMS and LCMS were normalised by the mg of tissue weight and reported as pmol/weight (mg of tissue).

Gas chromatography-mass spectrometry

Gas chromatography coupled to electron ionisation mass spectrometry (GCMS) dual scan-selected ion monitoring was employed to determine target compounds in the lipid extracts.

Samples were analysed with a GC 7890 A coupled to the MS 5975 VL analyzer (Agilent Technologies, CA, United States). Analysis of free cholesterol was performed simultaneously to free fatty acids (FFAs) analysis by a GCMS method as previously reported (Singh et al., 2018). 20 µL of the lipid extracts dissolved in 200 µL of $CHCl_3$/MeOH 2:1 mixture were dried under nitrogen and derivatized with 50 µL BSTFA added with 1% trimethylchlorosilane (TCMS) in pyridine to generate the trimethylsilyl (TMS) derivatives. The reaction was carried out at 60°C for 60 min. GC separation was performed with the 30 m–0.250 (i.d.) GC DB-5MS UI fused silica column (Agilent Technologies, CA, United States), chemically bonded with a 5% diphenyl 95% dimethylpolysiloxane cross-linked stationary phase (0.25 mm film thickness). Helium was used as the carrier gas. Samples were acquired in scan mode by means of electron impact (EI) MS. Cholesterol and FFAs were determined against d7Cholesterol and d31C16:0, respectively, with the MassHunter quantitative software (Agilent Technologies, CA, United States). Analyses were run in duplicate.

Liquid chromatography-mass spectrometry

The chromatographic apparatus consisted of the 1260 Infinity II series LC system (Agilent Technologies, CA, United States). High resolution reversed phase liquid chromatography (RPLC) was perforfed with a Zorbax SB-C8 HT (2.1 × 100 mm, 1.8 µm p. s.) with a maximal operational backpressure at 600 Bar (Agilent Technologies, CA, United States). Lipid mixtures were eluted a gradient of (A) 5 mM ammonium formate in water, (B) methanol, (C) acetonitrile, (D) isopropanol. The elution program was as follows: A/B/C/D 60/28/8/40 at time 0 and held for 1 min, brought to A/B/C/D 1/70/20/9 in 10 min and held up to 20 min. The flow rate was maintained at 400 µL/min during the entire LC run. The column was thermostated at 60°C. The injection volume was 0.20 µL. The separation of polar lipids, e.g., phosphatidylcholines (PCs), phosphatidylethanolamins (PEs), and sphingomyelins (SMs), was performed on a HILIC stationary phase. The HaloHILIC column (2.1 × 50 mm, 2.7 µm p. s.) was purchased from Advanced Materials Technologies (PN 9281-1204, Wilmington, DE, US). The separation of lipids in HILIC mode was performed with a gradient of (A) 5 mM ammonium formate in water, (B) methanol, and (C) acetonitrile. The elution program was as follows: A/B/C 1/2/97 at time 0 and held for 1 min, brought to A/B/C 18/2/80 in 20 min and held up to 10 min. The flow rate was maintained at 400 µL/min during the entire LC run. The column was thermostated at 40°C. The injection volume was 1 µL. The mobile phases were filtered through 0.45 µm glass filters and continuously degassed under vacuum. The injector needle was washed with the mobile phase in the wash port during the LC runs.

Accurate mass measurements in full MS and auto MS/MS were conducted with a G6545B series LC-QTOF (Agilent Technologies, United States) equipped with a JetStream Technology electrospray interface (ESI) interface operating in both positive and negative ion mode. Analytes eluted from the LC system were introduced into the Q-TOF apparatus at the operating chromatographic flow rate (see chromatographic conditions). Nitrogen was used as the nebulizing and desolvation gas. The temperature and the flow of the drying gas temperature were 200°C, and 12 L/min, respectively. The temperature and the flow of the sheath gas were 350°C and 12 L/min, respectively. The nebulizer pressure was 40 psi. The capillary and the fragmentor voltage were 4,000 and 180 V, respectively. Full scan mass spectra were acquired in the positive and negative ion modes in the range from m/z 100 to m/z 1600. To enhance accurate mass measurement for the ion species a reference solution of two compounds with m/z 121.050873 and 922.009798, respectively, was vaporized in continuum in the spray chamber by means of a separate nebulizer. Analyses were performed in duplicate in each mode.

Extraction of MS features

Molecular features, defined by an m/z, RT and signal intensity value, were extracted from the raw LCMS data files using the untargeted or the targeted batch recursive feature extraction in the MassHunter Profinder software (Agilent Technologies, United States). The features extracted were exported into a compound exchange format (CEF) reporting RT, the accurate mass and the absolute abundance for each entity to be processed in the subsequent chemometric analysis as previously reported (Ludovici et al., 2018; Singh et al., 2018).

Data analysis (lipidomics)

Agilent Mass Profiler Professional (MPP version 15.1) was used to process the LCMS untargeted and targeted data. Retention times (RT) were aligned by setting a RT window of 0.6 min, whereas m/z binning was performed by setting windows at 10 ppm. Absolute abundance of each entity was normalised by the absolute abundance of the d31CerNS internal standard. Data were filtered by frequency of detection, which reflects the number of samples that presented particular features. A frequency filter was applied to data extracted from MPP and only entities present in 100% of samples belonging to at least one of the investigated groups were retained for the statistical analysis. Fold changes of filtered entities were compared between groups volcano plots in the MPP tools. Fold changes with p values <0.05 after Bonferroni's correction were considered as significant. Identification of entities within the MPP workflow was performed based on the METLIN Metabolomics Database (http://metlin.scripps.edu/) and the Lipid Annotator software (Agilent Technologies, CA, United States). Quantitative assessment of cholesterol sulfate (CSO_4) was performed with the deuterium labelled internal standard d7CSO_4.

Data availability statement

The data presented in this study are deposited in the NIH GEO repository under GSE 232622.

Ethics statement

The studies involving human participants were reviewed and approved by East London and City Health Authority. The patients/participants provided their written informed consent to participate in this study.

Author contributions

This project was conceived by EO who obtained funding. EO, EC, and AE supervised the work. MC helped with day to day supervision. All of the experimental work was performed by FM, PD, MMe, EC, and MMa. Patients were recruited and biopsied by SZ and EO. The manuscript was written by FM, EC and EO with comments by all the authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

EO has had grant funding from Palvella Therapeutics and Kamari Pharma. She has consulted for Palvella Therapeutics, Kamari Pharma and Azitra Inc. She has had PhD studentships co-funded with GSK and Unilever. She has been a speaker for Almirall. All money goes to the university. MD was employed by Senzo Health Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2023.1176802/full#supplementary-material

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The immunological and structural epidermal barrier dysfunction and skin microbiome in atopic dermatitis-an update

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Atopic dermatitis (AD) is a common, chronic and relapsing inflammatory skin disease with various clinical presentations and combinations of symptoms. The pathophysiology of AD is complex and multifactorial. There are several factors involved in the etiopathogenesis of AD including structural and immunological epidermal barrier defect, imbalance of the skin microbiome, genetic background and environmental factors. Alterations in structural proteins, lipids, proteases, and their inhibitors, lead to the impairment of the stratum corneum which is associated with the increased skin penetration and transepidermal water loss. The elevated serum immunoglobulin E levels and blood eosinophilia have been shown in the majority of AD patients. Type 2 T-helper cell immune pathway with increased expression of interleukin (IL)-4, IL-5, and IL-13, has an important role in the etiopathogenesis of AD. Both T cells and keratinocytes contribute to epidermal barrier impairment in AD via a dynamic interaction of cytokines and chemokines. The skin microbiome is another factor of relevance in the etiopathogenesis of AD. It has been shown that during AD flares, *Staphylococcus aureus* (*S. aureus*) colonization increased, while *Staphylococcus epidermidis* (*S. epidermidis*) decreased. On the contrary, *S. epidermidis* and species of *Streptococcus*, Corynebacterium and Propionibacterium increased during the remision phases. However, it is not clear whether skin dysbiosis is one of the symptoms or one of the causes of AD. There are several therapeutic options, targeting these pathways which play a critical role in the etiopathogenesis of AD. Although topical steroids are the mainstay of the treatment of AD, new biological therapies including IL-4, IL-13, and IL-31 inhibitors, as well as Janus kinase inhibitors (JAKi), increasingly gain more importance with new advances in the therapy of AD. In this review, we summarize the role of immunological and structural epidermal barrier dysfunction, immune abnormalities, impairment of lipids, filaggrin mutation and skin microbiome in the etiopathogenesis of AD, as well as the therapeutic options for AD and their effects on these abnormalities in AD skin.

KEYWORDS

skin microbiome, atopic dermatitis, keratinocytes, epidermal barrier, filaggrin

1 Introduction

Atopic dermatitis (AD) is a common chronic and relapsing skin disease which usually occurs in the first years of life and affects ~20% of children worldwide (Bylund et al., 2020). The prevalence of AD is increasing. in both children and adults (Fuxench et al., 2019; Lee et al., 2019). AD is characterized by chronic inflammation which is associated with animpaired immunological response and epidermal barrier dysfunction. This chronic inflammation leads to itching in AD patients due to dry skin, mechanical injury and allergic sensitization to environmental antigens (Oyoshi et al., 2009).

In general, AD is associated with other atopic comorbidities such as asthma, rhinitis, conjunctivitis, and food allergy (Paller et al., 2019a; Kim et al., 2019). The acute phase of AD is characterized by erythematous papules and vesicles, accompanied by itching. With the evolution to the chronic phase, lichenified lesions occur as a result of dermal fibrosis. The location of AD lesions varies with the age of the patient. In adults, it predominantly occurs in the skin flexures, face and extremities, while patients under 1 year of age generally present widely distributed lesions. The cheeks are usually the first affected area in infantil period (Bieber, 2022).

The most important factors in the etiopathogenesis of AD are genetic background, imbalance of the skin microbiome, environmental factors, as well as structural and immunological epidermal barrier defect which leads to transepidermal water loss (TEWL) (Figure 1). The increased TEWL which is associated with increased permeability of the stratum corneum (SC), is a characteristic finding of both lesional and non lesional skin of AD patients (Dizon et al., 2018). The magnitude of increase in TEWL is also correlated with the disease severity (Dizon et al., 2018). Dry skin which is one of the hallmarks of AD, occurs due to increased water loss and it leads to pruritus that impairs AD patients' quality of life (Goerdt et al., 1999).

This review will focus on the immunological and structural epidermal barrier dysfunction in AD and the role of keratinocytes, filaggrin (FLG) mutation, lipid alterations in SC, as well as skin microbiome in AD and the therapeutic options in the treatment of AD and their effects on AD skin.

The immune abnormalities in AD and their contribution to epidermal barrier dysfunction

AD is a complex, multifactorial disease in which both innate and adaptive immune system contribute to its etiopathogenesis (Figure 1). Previously, it has been thought that AD is simply a Th2-mediated inflammatory disease since the majority of patients have increased serum immunoglobulin E (IgE) levels and high numbers of circulating eosinophils (Tokura and Hayano, 2022). However, it has been demonstrated that there is a biphasic switch from Th2 to Th1 responses in both acute and chronic skin lesions of AD patients (Leung, 2000). Moreover, we know that the immunological pathway of AD is not simple and also characterized by a dysfunction in the immune system, with a dominant Th2/Th22 skewing, and variable activation of Th17/Th1 subtypes (Tokura and Hayano, 2022). In chronic AD lesions, it has been reported that there is a complex inflammation pathway between T helper cells (Th1/Th2/Th17/Th22) and hyperproliferative

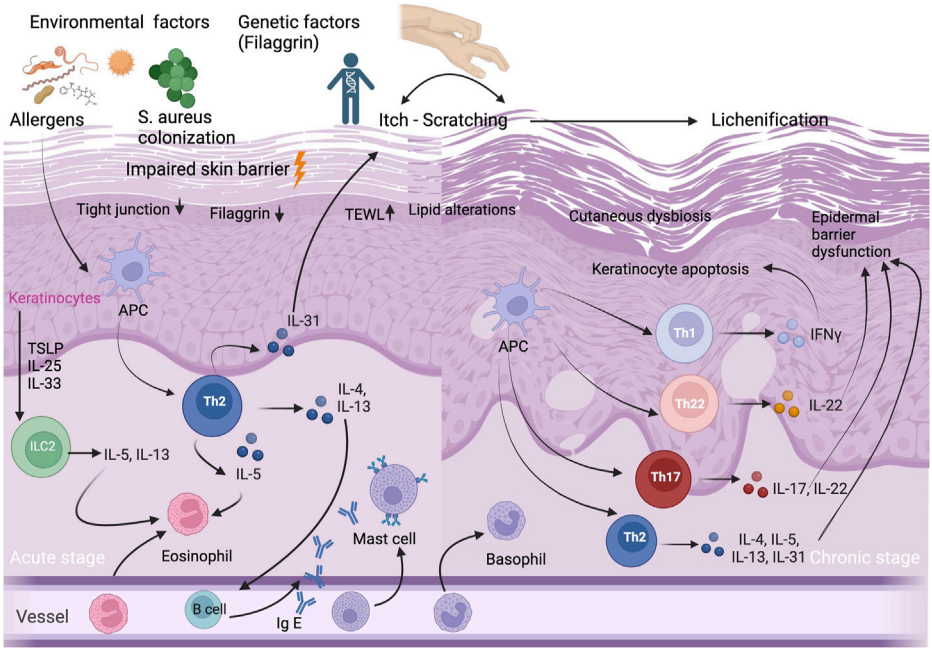

FIGURE 1
Immunopathogenesis of AD. Acute and chronic stages of AD and selected T-cell subpopulations and interleukins, other inflammatory cytokines and IgE antibodies, and selected cell populations ILC2, APCs, and eosinophils, mast cells, basophils, eosinophils and keratinocytes that play an important role in AD etiopathogenesis. Impaired skin barrier is associated with decrease in filaggrin protein, increase in TEWL, lipid alterations and cutaneous dysbiosis. Abbreviations: APC, antigen presenting cell; IFN, interferon; Ig E, immunoglobulin E; IL, interleukin; ILC2, innate lymphoid cell 2; S.aureus, *Staphylococcus aureus*; TEWL, transepidermal water loss; Th, T helper; TSLP, thymic stromal lymphopoietin cell.

keratinocytes which are characterized by altered terminal differentiation (Renert-Yuval et al., 2021).

IL-4 and IL-13 are two major cytokines in the etiopathogenesis of AD and play a critical role in the differentiation of Th2 cells and the production of IgE. The increased skin expression of Th2 cytokines including IL-4, IL-5, and IL-13, has been shown in acute AD skin lesions (Oyoshi et al., 2009). These cytokines stimulate IgE antibodies and eosinophils in both skin and peripheral blood (Matsunaga and Yamauchi, 2016). IL-4 and IL-13 disrupt the epidermal barrier integrity by decrease of main terminal differentiation proteins including filaggrin, loricrin, and involucrin (Cork et al., 2006).

Additionally, IL-4 decreases the expression of genes in the epidermal differentiation complex in keratinocytes and it leads to epidermal barrier dysfunction and impaired innate skin immune system, and consequently to an increased risk of infections (Sehra et al., 2010). IL-13 overexpression has been found in both lesional and non-lesional skin of AD patients (Tsoi et al., 2019) and its levels correlate with AD disease severity (Szegedi et al., 2015; Guttman-Yassky et al., 2019; Tsoi et al., 2019). Moreover, it has been reported that IL-13 messenger RNA and protein levels are higher compared to IL-4, although both cytokines play a major role in the pathogenesis of AD (Tsoi et al., 2019). This highlights the importance of IL-13 inhibition in AD therapy.

The impairment of the epidermis due to various factors such as infectious agents, allergens or mechanical trauma may stimulate inflammation processes and leads to production of proinflammatory mediators such as TSLP (Thymic stromal lymphopoietin), which is an IL-7–like cytokine, IL-4, IL-13, IL-25, and IL-33 (Camelo et al., 2017; Klonowska et al., 2018). Thus, these increased inflammatory cytokines induce immune cell accumulation, which leads to formation of nitrogen oxide and reactive oxygen species (Mittal et al., 2014).

Various antimicrobial peptides play an important role in the innate immunity (Lehrer et al., 1993). The expression of human β-defensin 2 (hBD-2) and LL-37, is triggered by the inflammation of the skin (Frohm et al., 1997). In some AD patients, it has been reported that the decreased antimicrobial and immunomodulatory peptides such as LL-37, hBD-2, and hBD-3, are associated with skin infections (Niyonsaba et al., 2017; Nguyen et al., 2020). Host defense peptides (HDPs) induce cytokine and chemokine production and promote cell proliferation and migration (Reinholz et al., 2012; Pahar et al., 2020), and also contribute to normal epidermal barrier function against TEWL via organizing the distrubiton of tight junction protiens (Akiyama et al., 2014). The predominance of Th2-related cytokines, which is associated with an inhibitory effect against LL-37, hBD-2, and hBD-3 production, might be one of the reasons for reduced HDP levels in AD patients (Reinholz et al., 2012; Akiyama et al., 2014; Nguyen et al., 2020).

Keratinocytes play a major role in immunological and structural epidermal barrier impairment in AD

The human epidermis is a unique protective barrier against transcutaneous water loss, penetration of microbial pathogens and ingress of toxins and allergens (Marks, 2004; Elias, 2005). The epidermis consists of four layers: the stratum basale, the stratum spinosum, the stratum granulosum and the SC (Matsui and Amagai, 2015). The SC, which is the outermost layer of skin and the most important part of the epidermal barrier, comprises 20 layers of corneocytes, which are embedded in intercellular lipids (Oyoshi et al., 2009). The impairment of the SC, which protects against environmental factors, allergens and water loss, leads to epidermal barrier dysfunction in AD (Schleimer and Berdnikovs, 2017).

Keratinocytes have a critical role both in the pathophysiology of epidermal barrier defect and in the activation of the innate immune response. They receive both mechanical and inflammatory stimuli, and produce antimicrobial peptides (AMPs) and proinflammatory cytokines. In patients with AD, the epidermis is characterized by a block in terminal keratinocyte differentiation (Guttman-Yassky et al., 2009) which leads to allergen penetration through the epidermis and systemic IgE sensitization (Jensen et al., 2004), and reduced expression of skin barrier proteins including FLG, involucrin, loricrin, as well as AMPs (Ong et al., 2002; Howell et al., 2007). AMPs including LL-37, hBD-2, and hBD-3, play a major role in repairing the impaired epidermal barrier. They also show an autocrine function on keratinocytes, which produce pro-inflammatory cytokines including IL-25, IL-33, and TSLP. This inflammatory cytokines activate the innate lymphoid cell 2 (ILC2), dendritic cells and langerhans cells, and initiate the differentiation of type 2 immune response (Nakatsuji et al., 2017; Radi et al., 2022). ILC2s have an important role in homeostasis and produce a variety of cytokines, primarily IL-5 and IL13 (Mielke et al., 2013). ILC2s which present CD1a, have been found to be increased and activated in AD lesional skin (Hardman et al., 2017) (Figure 1).

TSLP which is secreted by epidermal keratinocytes, also induces other cells such as dendritic cells, T cells, as well as mast cells. Mast cells play an important role in IgE-mediated hypersensitivity and allergic diseases, as well as in AD. It has been shown that mast cells are significantly increased in AD skin (Kawakami et al., 2009). Not only keratinocytes and mast cells, but also T cells have a major role in skin barrier dysfunction via cytokine secretion (Humeau et al., 2022). Kallikreins (KLK) are proteases which have various functions including the induction of pro-inflammatory cytokine production by keratinocytes, the degradation of intercellular adhesion molecules, as well as the regulation of barrier integrity (Briot et al., 2009). In the SC of AD patients, the levels of KLK5 and KLK7 increase by the stimulation of IL-4 and IL-13 (Komatsu et al., 2007). IL-4, IL-13, IL-31, and IL-22 expressed by Th2 and Th22 cells, may disturb epidermal barrier function via scratching due to itch in AD patients (Furue et al., 2018). Although the predominance of type 2 response in AD is well-known, there is a complex pathway, including Th2, Th22, Th17 and Th1 subtypes, associated with regulatory T cell (Treg) dysfunction (Humeau et al., 2022).

In patients with AD, various abnormalities manifest not only in the SC, but also in other epidermal layers. Degradation in the cells of the stratum spinosum and stratum granulosum with a concomitant expansion of cells in the stratum basale has been described (Jensen et al., 2004). Totsuka et al. investigated the changes of structural proteins and adhesion molecules in the stratum spinosum of AD lesional skin, and the effect of Th2 cytokines including IL-4 and IL-13 on expression of these proteins (Totsuka et al., 2017). In AD lesional skin, they found decreased expression of keratin 1, keratin 10, desmoglein 1 and desmocollin 1, which is supressed by Th2 cytokines (Totsuka et al., 2017).

However, it is not certain that the epidermal skin barrier defect in lesional skin of AD is a primary factor or a process due to disease activity. The epidermal barrier abnormality has been reported not only in lesional skin, but also in non-lesional skin of AD patients (Proksch et al., 2006). Moreover, the epithelium of AD, which is not affected by skin lesions, is also characterized by bioelectric abnormalities in tight junctions (Pelc et al., 2018). Tight junctions are intercellular junction units and have an important role in the formation of the epidermal barrier against the transition of water, ions, and macromolecules (Basler et al., 2016). Dysfunction of tight junctions as a result of cutaneous inflammation in AD skin impairs epidermal barrier permeability by altering the pH of the SC which leads disruption of the mature lamellar structures, polar lipid formation and keratohyalin granules (Yuki et al., 2013). As a result, epidermal barrier permeability increases and it leads to increased ease of transition of the various bacterial agents and allergens, leading to a vicious circle of epidermal barrier dysfunction and cutaneous inflammation (Yokouchi et al., 2015; Katsarou et al., 2023).

Lipid alterations in AD

The epidermis contains basal keratinocytes, which are highly proliferative and have differentiation capability (van Smeden et al., 2014). They are characterized by the organized expression of specific proteins, intercellular junctions, enzymes such as proteases/antiproteases, as well as lipid components (van Smeden et al., 2014). The main SC lipids including ceramides (CERs), free fatty acids (FFAs) and cholesterol and its esters, are essential for a healthy skin barrier (Pappas, 2009; Jungersted and Agner, 2013; Loiseau et al., 2013; Li et al., 2016). SC lipids are secreted by keratinocytes into the extracellular space and move to the stratum corneum via lamellar bodies, which consist mostly of phospholipids, sphingolipids, and cholesterol (Wertz, 2018). In lamellar bodies, these lipids are metabolized by various enzymes such as sphingomyelinase, glucocerebrosidase and phospholipase (Wertz, 2018).

The intercellular lipid membrane shows a barrier role against infectious agents and maintains the integrity of the SC (Elias, 2005). An ideal ratio of the different SC lipids is one of the most important factors for healthy epidermal barrier function. AD is a common dermatological disease, characterized by impaired lipid barrier function. Mutations in lipid metabolizing enzymes or mutations that lead to increased protease activity or decreased protease inhibitor activity, could be associated with epidermal barrier dysfunction in AD. The lipid group most commonly reported to be deficient in AD is CERs (Levin et al., 2013). AD is characterized by abnormal skin lipids that are stimulated by hyperactivated type 2 immune response (Berdyshev et al., 2018). Inflammatory cytokines (IL-4, IL-13 and IL-31) reduce the expression of main CER synthesizing enzymes which are essential for lipid formation (Danso et al., 2017). This abnormality in SC lipids seems to occur independently of FLG mutations (Janssens et al., 2012; Joo et al., 2015). The metabolism of CERs in AD is affected by the immune/inflammatory response. Decreased total CER levels and variety in chain length such as increased short chain CERs, diminished long-chain CERs, increase in short chain FFAs, reduction in long chain FFAs and decrease in hydroxy-FFAs, have been shown in the etiopathogenesis of AD (van Smeden et al., 2014b; van Smeden and Bouwstra, 2016).

The enrichment of the skin microbiota with ceramidase-secreting bacteria, has been reported as a possible cause of reduced levels of CERs in AD skin (Ohnishi et al., 1999). Although *S. aureus* colonization is an important factor in AD patients, *S. aureus* does not show ceramidase activity (Ohnishi et al., 1999). Furthermore, so far no ceramidase-producing bacteria have been found in the skin of patients with AD (Pavel et al., 2022). One alternative hypothesis which seems more reasonable to explain the reduced levels of CERs, is the stimulation by Th2 cytokines such as IL-4 which leads to reduced CER synthesis in keratinocytes (Hatano et al., 2005).

These alterations in lipids lead to increased TEWL in AD skin (Janssens et al., 2012; Meckfessel and Brandt, 2014; Danso et al., 2017). Moreover, the decreased lipid levels are not only limited to lesional skin, but also have been demonstrated in non-lesional skin of patients with AD (van Smeden et al., 2014b; Joo et al., 2015; Toncic et al., 2020). This indicates that AD is not limited to visible skin lesions only.

The key protein filaggrin in AD

FLG is major epidermal structural protein which is provided by upper-layer epidermal keratinocytes. FLG is produced as a polymer profilaggrin and it is located in the outer nucleated layers of the epidermis. FLG degradation products maintain the pH balance, skin hydration and antimicrobial function of the epidermal barrier (Brown and McLean, 2012; Harding et al., 2013; Drislane and Irvine, 2020; Kim and Lim, 2021). FLG mutations are the most well-known genetic risk factors in AD (Morar et al., 2007; Esparza-Gordillo et al., 2009). The Th2 pathway plays an important role in AD and other atopic comorbidities, such as allergic rhinitis, asthma, and food allergy. Th2-related (IL-4, IL-13, IL-25), and Th22-related (IL-22) cytokines, which lead to reduced FLG levels in keratinocytes, are involved in the etiopathogenesis of AD (Fenner and Silverberg, 2018).

FLG mutations which are the most common, affecting 30%–50% of white AD patients, are associated with an increased skin permeability (Patrick et al., 2021). FLG deficiency is a complex combination of dysregulation of molecules involved in inflammatory, proteolytic and cytoskeletal functions (Elias et al., 2017). FLG deficiency is associated with dry skin, in consequence of the impaired skin barrier function and increased TEWL. This altered epidermal barrier function also makes the skin vulnerable against irritants, haptens and allergens, which penetrate the skin and induce allergic sensitization (Ständer, 2021).

In murine models of AD, it has been shown that FLG deficiency changes the construction of keratinocytes and secretion of lipids (Man et al., 2008; Kawasaki et al., 2012; Thyssen and Kezic, 2014; Elias et al., 2017). The decreased skin lipids lead to the diminished production of epidermal AMPs and consequently an altered skin microbiome (Langan et al., 2020; Patrick et al., 2021). In support of this, in AD patients with FLG mutations, an elevated *S. aureus* colonization has been shown (Clausen et al., 2017). In addition to the imbalance of the skin microbiome, FLG mutation is also

associated with higher risk of early onset of the disease, high serum levels of IgE and other manifestations of atopy, as well as the persistence of AD into adulthood (Zaniboni et al., 2016).

Haftek et al., examined biomechanical characteristics of corneocytes in children with AD with FLG mutations (Haftek et al., 2020). In these corneocytes, they showed a decreased elastic modulus which strongly correlated with FLG degradation products and TEWL, but not with SCORAD (SCORing Atopic Dermatitis) (Haftek et al., 2020). They suggested that AD patients have decreased corneocyte stiffness, which correlates with reduced levels of FLG degradation products and skin barrier function (Haftek et al., 2020). Furthermore, FLG metabolites, such as urocanic acid and pyrrolidone carboxylic acid, were shown to contribute to moisturization and maintenance of acidic pH of the SC. Both of these molecules may be crucial to epidermal barrier homoeostasis by regulating the activity of multiple enzymes which control desquamation, lipid synthesis and inflammation in AD skin (Meckfessel and Brandt, 2014; Moosbrugger-Martinz et al., 2022).

FLG deficiency may lead to impaired skin barrier function in AD through multiple pathways. It has been also shown in murine models, that the genetic modifications may affect microbial colonization (Kobayashi et al., 2015; Nakatsuji et al., 2016). However, FLG mutations have not been demonstrated in all of AD patients and also some patients with FLG mutations do not present dysbiosis of the microbiome (Kobayashi et al., 2015; Nakatsuji et al., 2016; Williams et al., 2020). Further studies seem to be necessary to clarify the relationship between the skin microbiome and FLG mutations. It is also clear that the FLG mutation is not the only and/or absolute factor in the etiopathogenesis of AD.

The environmental factors associated with AD

Two hypotheses have been suggested for the development of AD lesions. The first one is the "inside-outside hypothesis". This hypothesis is explained with epidermal barrier impairment in AD as a secondary result of the inflammatory response to irritants and allergens (Leung, 2000). On the other hand, the second one is the "outside-inside hypothesis" which favors that the xerosis (Denda et al., 1998), and the abnormal permeability of the barrier (Elias et al., 1999) or both may lead to AD lesions (Elias et al., 1999; Chamlin et al., 2002).

Microbial pathogens including bacteria, viruses and fungi may trigger AD and initiate allergic sensitization. These factors are modifiable and recognized by cell receptors. Thus, the skin immune cells are activated and secrete inflammatory cytokines causing the development of AD (Cork et al., 2009). Aeroallergens are also one of the AD triggers and include indoor aeroallergens such as house dust mite (HDM), pet dander, fur, cockroach, and mold, and outdoor aeroallergens such as tree, grass, and weed pollen (Werfel et al., 2015; Chong et al., 2022). HDM is a common sensitizing factor in pediatric AD patients and it has been also reported that children with a strong skin prick test reaction to HDM have more severe disease (Kutlu et al., 2013).

Various chemicals and irritants have been shown to influence AD, including soaps and detergents, as well as washing with hard water (Danby et al., 2018). Epidermal barrier dysfunction makes the skin vulnerable against these environmental factors, resulting in epidermal barrier damage. Detergents which contain irritating ingredients such as surfactants, may cause skin dryness, tightness and roughness, resulting in erythema and swelling (Ananthapadmanabhan et al., 2004). In AD patients, Callahan et al. showed lower thresholds to irritancy by sodium lauryl sulphate, which is a chemical agent in hand cleansers or shampoos (Callahan et al., 2013). Mechanical epidermal trauma could also exacerbate the symptoms of AD (Lee, 2020).

Climate is one of the associated factors with AD. It has been suggested that warm temperatures, high sun exposure or UV index, and higher humidity are associated with decreased AD prevalence, while low UV exposure, low temperatures and indoor heating may increase the risk of disease onset (Silverberg et al., 2013). However, higher temperatures and increased sun exposure may exacerbate the disease in some AD patients (Sargen et al., 2014; Kantor and Silverberg, 2017). These factors could be associated with the alterations in keratinocyte metabolism and immune dysregulation, as well as the degradation of FLG, which is affected by climate changes (Kantor and Silverberg et al., 2017). Vocks et al. showed that the temperature change from very cold (−17°C) to moderate (+18°C) was associated with decreased pruritus severity in AD patients (Vocks et al., 2001). Moreover, it has been suggested that people living in warmer climates may spend more time outdoors, thus they have more UV exposure, which may protect against AD. Contraversely, people living in warmer climates use less indoor heating, which may aggravate AD (Silverberg et al., 2013).

The relationship between birth season and AD has been investigated. Calov et al. (2020) found a significant association between AD and fall birth and winter birth when compared to spring birth. They suggested that the higher prevalance of AD in specific seasons could be explained by reduced ultraviolet radiation exposure, as well as increased air pollution (Calov et al., 2020) Air pollution is one of several factors that people are exposed to in daily life, and a contributor to AD. Air pollutants may arise from indoor and/or outdoor environments and they could enter the systemic circulation through penetration of the skin (Ahn, 2014). Rutter et al. found that the exposure to heavy traffic during the 12 months before measurement was significantly associated with eczema symptoms in children (Rutter et al., 2019) In contrast, Huls et al. showed no association between the traffic-related air pollution and AD in the general population (Huls et al., 2019). However, they suggested that the prevalence of childhood AD is correlated with oxidative stress and inflammation (Huls et al., 2018). They also reported that the risk scores from glutathione S-transferase P1, tumor necrosis factor, Toll-like receptor (TLR)-2, and TLR-4 single-nucleotide polymorphisms are associated with AD up to the age of 2 years (Huls et al., 2019; Ahn et al., 2020).

Exposure to certain foods may induce an immunological response in the skin and exacerbate the symptoms of some AD patients through allergic and non-allergic hypersensitivity reactions. It has been shown that more severe disease activity is correlated with increased frequency of food allergy (Burks et al., 1998; Eigenmann and Calza, 2000). Children with food allergies have positive skin tests and/or presence of serum IgE antibodies against particularly eggs, milk, wheat, soy and peanuts (Wassmann-Otto et al., 2018;

Wollenberg et al., 2020). In a large population-based study, it has been reported that infants with AD are 6 times more likely to have egg allergy and 11 times more likely to have peanut allergy by 12 months than infants without AD at 12 months of age (Martin et al., 2014). However, restricted diet is not recommended for most AD patients (Rustad et al., 2022) since the possible tolerance of food allergies is being developed until the age of three (Akdis et al., 2006; Pelc et al., 2018). Elimination diet should be recommended in case a food is clearly identified as an exacerbarating factor (Papapostolou et al., 2022).

The relationship between immune abnormalities, keratinocytes and cutaneous microbiome in AD

As the skin microbiome gains more and more interest as a key determinant of skin health, much effort is made to unravel the complex interplay of host cells and skin residents. In the last decade, it was shown in multiple studies that skin immunology and therefore also inflammatory processes are tightly and inevitably linked to the skin's microbiota. Especially in the last few years a lot of evidence beginning to unravel the complex symbiosis between host and microorganisms was collected. The epidermis is colonized by bacteria, fungi, viruses and other microorganisms, and there is a complex interplay between host cells and the so-called commensals. From birth on, the skin of the new-born is colonized by microorganisms, with differences according to the mode of delivery (natural birth versus caesarean section). Based on environmental and individual factors, the microbiota stabilizes during the first years of life (Luna, 2020) Due to the importance of early microbial colonization, it can unsurprisingly be linked to the development of allergic diseases like food allergy or AD (Peroni et al., 2020). Therefore, human health is not only dependant on the actual microbial environment but also on the microbes that were encountered in the past, especially the first few years of life.

The cutaneous microbiome is important for both immune maturation and epidermal barrier function. There are many factors, including skin immune system, pH and water balance, the epidermal lipid composition and the expression of antimicrobial peptides, that contribute to the microbial balance in the epidermis (Zhang and Gallo, 2016; Wohlrab et al., 2018; Fölster-Holst, 2022). Because the skin's microbiota (as well as microorganisms in other habitats) affects immunologic maturation, characteristic changes in skin colonization like the overrepresentation of pathogenic bacteria or the lack of contact to "good" ones in early life may have consequences for inflammatory skin diseases and might be even involved in their pathogenesis. For instance, this has been demonstrated for AD. Meylan and others found that *S. aureus* colonization in infancy was positively associated with the development of AD, and this colonization preceded disease onset (Meylan et al., 2017). In contrast, the presence of *Staphylococcus* hominis at age 3 months tended to be negatively associated with AD development, underscoring that different bacteria exert different effects to the skin and its immune system (Meylan et al., 2017). Moreover, during AD flares, the variety of skin bacteria colonization alters and *S. aureus* colonization increased and S. epidermidis decreased (Bjerre et al., 2017). On the contrary, S. epidermidis and species of *Streptococcus*, Corynebacterium and Propionibacterium increased during the remission phases (Kong et al., 2012). However, it has been reported that antibacterial agents against *S. aureus* are not superior to other non-antimicrobial treatments in AD (Bath-Hextall FJ et al., 2010). *S. aureus* may not be a major pathogenic factor in the etiopatogenesis of AD, nevertheless the presence of S. aureus could be associated with disease severity (Moosbrugger-Martinz et al., 2021). In adult patients with severe AD during disease flare, increased levels of *S. aureus* in lesional skin have been shown (Clausen et al., 2018; Smits et al., 2020). Moreover, *S. aureus* colonization in non-lesional skin and even twice more in lesional skin have been shown in patients with AD (Baker, 2006; Totté et al., 2016; Nowicka et al., 2022). However, it has not been found in all AD patients (Paller et al., 2019b) and it is not certain whether *S. aureus* promotes the inflammation in AD or whether the inflammation is the reason for the presence of *S. aureus* due to an impaired skin barrier. *S. aureus* secretes toxins and superantigens and stimulates the activation of T cells, thus contributes to skin inflammation in patients with AD (Schlievert et al., 2010). In support of this, Skop et al. suggested that the application of Staphylococcal enterotoxin B to non-lesional skin may induce eczematous lesions (Skov et al., 2000). On the other hand, the colonization with some "good" bacteria on the skin may have protective effects in AD (Cho et al., 2001). In patients with AD and Netherton syndrome, increased S. epidermidis and S. hominis in the post flare phase have been shown (Byrd et al., 2017; Moosbrugger-Martinz et al., 2021). Some strains of S. epidermidis may improve innate immunity and protect the skin against infection with pathogens by activating IL-17- expressing $CD8^+$ T cells (Prescott et al., 2017).

The present microbes and their metabolites also prime the maturation of the host immune system. Innate as well as adaptive immune responses are different in neonates compared to adults. In order to ensure tolerance to self- and foreign antigens, neonates are less prone to inflammation, which is crucial for the rapidly developing tissues (Paller et al., 2019b). During this educational period for the host immune system, the foundation for health or disease in later life could be established, as demonstrated in animal models, e.g., for inflammatory bowel disease and asthma (Gensollen et al., 2016). For the skin, this was shown with the commensal *Staphylococcus* epidermidis, which is considered a beneficial bacterium in this tissue. Adult immunologic responses to this commensal were different in mice according to the time of introduction of *S. epidermidis*. Exposure in early life circumvented inflammation induced by contact in adult animals, and this effect was attributed to the development of regulatory T-cells in answer to early exposure (Scharschmidt et al., 2015).

Keratinocytes are directly involved in the regulation of commensal-specific T cells. Epidermal accumulation of lymphocytes is a phenomenon induced by the microbiota, with type 1 and type 17 cells present in these clusters. Commensal induced Th1 cells thereby are regulated by MHC class II expressing keratinocytes in an IL-22 dependent fashion (Tamoutounour et al., 2019). The epidermal microbiota seems to be more individual than the dermal one, which shows less inter-individual variability (Bay et al., 2020). Host cells like keratinocytes

establish an environment in favour of beneficial microbes by secreting specific antimicrobial components including innate cytokines, pathogen associated molecular pattern (PAMP) molecules, the inflammasome, and AMPs, targeting unwanted microorganisms, while providing nutrition for those who can metabolize special lipids (Nakatsuji et al., 2010; Dahlhoff et al., 2016).

Next to specific antimicrobial substances, bacteria are influenced by other host signals whose primary function is the communication between host cells. For example, it was found that neuropeptides like substance P exert effects on the virulence of bacteria, thereby contributing to microbiota homeostasis (N'Diaye et al., 2017).

The high diversity of the gut microbiome affects the immunity of the whole body, including skin. It improves the levels of regulatory T cells and short-chain fatty acids (Belkaid and Hand, 2014). On the other hand, an imbalance of the gut microbiome and increased levels of noxious microorganisms lead to secondary skin infections and immune-related diseases including AD (Belkaid and Hand, 2014). Infants with a diverse gut microbiome have a lower risk of development of AD (Ihekweazu and Versalovic, 2018; Park et al., 2021). In addition to this, infants with AD are more often colonized by *Clostridium difficile* and *Escherichia coli* than infants without AD (Penders et al., 2007). The priming of the cutaneous immune system enables the mature tissue to select for beneficial microorganisms, which at the same time prevent the outgrowth of pathogenic species. By competing for space and nutrition and by specific antagonistic mechanisms, commensal bacteria protect their respective niche. This delicately balanced homeostasis is disrupted in different disease settings. Dysbiosis, which means an adversely altered microbiota, can trigger inflammatory processes, e.g., by inducing IL-1α release by keratinocytes (Archer et al., 2019). Archer and others demonstrated in a filaggrin deficient mouse model, that skin injury and dysbiosis resulted in chronic inflammation in the animals, and that keratinocyte-derived IL-1α was the driver of this development. This suggests that skin microbiome modulation could be beneficial for AD patients.

Several regulators and signalling pathways were found to be involved in the skin-commensal crosstalk and the cutaneous microbiota homeostasis. In mice, dendritic epidermal T cells are regulated by the G protein coupled receptor 15 (GPR15) (Sezin et al., 2021). Gpr15−/− mice exhibit a severe deficiency in this cell type, with an overall reduced number of T cells in the epidermis, but not the dermis. The expression of GPR15 on T cells in turn is influenced by the skin as well as the gut microbiome (Kim et al., 2013; Jacob et al., 2020), and knock-out animals show alterations of their cutaneous microbiomes compared to wildtype littermates. Interestingly, GPR15 expression in human $CD4^+$ cells is regulated by aryl hydrocarbon receptor (Ahr) (McAleer et al., 2018), and since dendritic epidermal T cells depend on Ahr signalling (Kadow et al., 2011), Sezin et al. speculated about GPR15 as a downstream target of Ahr (Sezin et al., 2021). Furthermore, keratinocyte Ahr is essential for barrier integrity in murine skin (Haas et al., 2016), demonstrating the importance of the Ahr pathway in different cell types in the epidermis. Just like Gpr15−/− mice, Ahr-deficient mice demonstrate an altered, more variable and probably more unstable microbiome (Haas et al., 2016). Ahr is activated by indole-3-aldehyde, a microbial tryptophan metabolite, and Ahr activation leads to production of anti-inflammatory IL-10 by Langerhans cells and subsequently inhibited $CD4^+$ T cell proliferation (Liu et al., 2020). In addition, Ahr signalling in keratinocytes is influenced by the microbiota, with consequences for skin barrier function and repair (Uberoi et al., 2021).

A further player in the dialogue between skin immune cells and microbiota is the transcription factor JunB, which is expressed by keratinocytes. Mice deficient in epidermal JunB exhibit an AD-like phenotype, including proneness to spontaneous skin colonization by *S. aureus*. JunB negatively regulates MyD88 in keratinocytes, upon loss an inflammatory response cascade was reported. As inflammation was exacerbated in Rag1−/− mice, it was concluded that the adaptive immune system, presumably due to the production of IL-17A by T cells, is necessary to prevent *S. aureus* infection (Uluçkan et al., 2019).

Another example of the complexity of the regulation of epithelial cells, immune cells and commensals is the homeostatic control of sebaceous glands by innate lymphoid cells residing in hair follicles. Innate lymphoid cells express mediators that restrict sebocyte growth, thereby regulating the expression of sebaceous gland derived antimicrobial lipids. In this way, the commensal bacteria equilibrium is maintained (Kobayashi and Nagao, 2019). Hair follicles themselves rely on the endopeptidase ADAM10-Notch signalling axis, with a disruption in this pathway resulting in skin dysbiosis and destruction of hair follicles (Sakamoto et al., 2021).

While the interactions between host skin cells and commensals are already a complex and delicately balanced system, another layer of complexity is added when considering the skin as part of the whole human body. Not only keratinocytes and epidermal immune cells receive signals from within the body, so do the microorganisms which stand in contact with other microbial habitats from their host organism. Especially the gut as the most heavily colonized region is thought to act as a central signalling node for all peripheral microbial communities (Martínez et al., 2021). The cutaneous microbiome therefore is not only important for immune maturation and regulation but is directly involved in epidermal function. In conclusion, future work should be directed at further unravelling the network of interactions and molecular signalling mechanisms that include keratinocytes, skin immune cells and microorganisms.

Cutaneous inflammation, AD and cancer risk

The relationship between inflammatory skin disorders including AD, and cancer has been investigated (Zhu et al., 2022; Wan et al., 2023). AD has been found to be significantly correlated with an increased risk of non melanoma skin cancer (NMSC) (Zhu et al., 2022). In line with this, in another study, a greater risk of NMSC in children with mild AD was reported, however, a lower risk of melanoma in children with moderate AD was found (Vittrup, 2023). Further, an increased risk of lymphoma among children with severe AD has been found (Vittrup, 2023). In adults, a slightly increased risk of haematological malignancy, aslightly higher skin cancer risk and lower risk of solid organ malignancy has been reported (Vittrup, 2023). The authors suggested that although there is no overall association between AD and malignancy, AD may have heterogeneous effects by cancer subtype (Vittrup, 2023).

Although the mechanism of skin cancer in AD patients is not certain, skin cancer progression has been associated with the

dysregulation of microbiome (Woo et al., 2022). Furthermore, it can be suggested that the increased risk of skin cancer in patients with AD may be a long-term side effect of phototherapy treatment. However, the reduced risk of melanoma contradicts this hypothesis (Vittrup, 2023). Moreover, in recents studies, no strong overall malignancy risk in AD but a possible increased lymphoma risk in patients with severe AD (Wan et al., 2023), particularly NHL, that increased with eczema severity (Mansfield et al., 2020), has been reported.

On the other hand, decreased risk of malignancy in the esophagus, stomach, colorectum, and liver, has been found in patients with allergic diseases including allergic rhinitis, asthma, and AD (Choi et al., 2023). This decreased risk in GI cancers in AD patients could be explained by the immunosurveillance hypothesis. According to this theory, excessive stimulation of T-helper cell type II immune response and other immune cells such as mast cells, natural killer cells and eosinophils can prevent the onset of cancer by detecting and destroying the damaged cells before the onset of carcinogenesis (Ji et al., 2009; Choi et al., 2023). On the contrary, it is known that a Th2-dominant environment downregulates tumor immunity (Morimura et al., 2021). However, Morimura et al., suggested that a high level of CCL17, known as thymus and activation-regulated chemokine, may work as a "safety-net" to reduce the risk of malignant tumors and positively contributes to tumor immunity via decreasing myeloid-derived suppressor cells in the Th2-dominant environment in AD patients (Morimura et al., 2021). This may be one of the explanations for the normal incidence of cancer among patients with AD, regardless of the Th2-dominant environment. However, it is clear that the incidence of cancer in patients with AD needs further investigation.

Therapeutic options for AD and their effects on the immunological and structural epidermal barrier dysfunction and skin microbiome

AD presents quite heterogeneously, both clinically and immunologically (Czarnowicki et al., 2019). There are various treatment options for AD according to age, the severity of pruritus, involved body-surface area and the clinical stage of the disease (mild, moderate, or severe) (Sidbury et al., 2014; Wollenberg et al., 2018). A multi-therapeutic approach is essential in the management of AD. Short-term management aims to control symptoms in the periods of exacerbation of AD, while long-term treatment aims to prevent from new lesions and prolong the time between flares (Boulos and Yan, 2018). The most important aim of AD treatment is disease prevention due to potential toxicity of immunosuppressive therapies (Diaz and Guttman-Yassky, 2019).

Although it is not possible to mention all of the drugs marketed or being tested, there are approved topical and systemic treatment options available in the treatment of AD. Basic therapy contributes to barrier function through improving skin hydration and regeneration of intercellular lipid lamellae and is always required in addition to other topical and systemic therapeutics (Staubach and Lunter, 2014). Moisturizers are used both for the therapy and prevention of AD, providing repair to the epidermal lipid matrix (Elias et al., 2019). Moisturizers also reduce the use of inflammatory products and improve epidermal barrier function (Loden, 2003; Szczepanowska et al., 2008; Czarnowicki et al., 2016; Giam et al., 2016). In premature newborns, moisturizers also decrease bacterial colonization, TEWL and disease severity (Nopper et al., 1996; Darmstadt et al., 2007). Additionally, petrolatum increases the expression of FLG and loricrin and also induces upregulation of major AMPs such as LL-37, lipocalin 2 and peptidase inhibitor 3 (Czarnowicki et al., 2016). Moreover, although there are opposing views, it has been reported that early moisturizing in high-risk newborns alters the skin microbiome and pH levels (Glatz et al., 2015).

In 1991, wet-wrap dressing was applied to pediatric AD patients for the first time (Goodyear et al., 1991). For such dressings, the patient takes a bath in warm water and dries off the water, after this, the lesions are covered with wet gauze. This wet material is then covered with a second layer of dry material for a certain time. Wet-wrap dressing may also be applied with moisturizer or weak topical corticosteroids according to the clinical findings of the disease (Twitchen and Lowe, 1998). Wet-wrap therapy is associated with improvement of epidermal barrier function in patients with AD. Lee et al. (2007) showed that wet-wrap dressing is associated with decreased SCORAD, increased epidermal water content, and decreased TEWL. However, they observed no change in keratinocyte differentiation and calcium ion gradient with wet-wrap dressing (Lee et al., 2007).

Application of topical corticosteroids (TCSs) remains to be the mainstay in the treatment of AD. TCSs have anti-inflammatory, anti-proliferative, and immunosuppressive effects which contribute to the treatment of AD (Del Rosso and Friedlander, 2005). Glucocorticoids show suppressive effects against neutrophils, monocytes, lymphocytes, Langerhans cells and cytokines such as IL-1α, IL-1β, IL-2, tumor necrosis factor, and granulocyte-monocyte colony stimulating factor (Del Rosso and Friedlander, 2005). In contrast to the beneficial effects of steroids, they also inhibit the synthesis of cholesterol, ceramides, and free fatty acids and disrupt the anti-bacterial function of the epidermis (Pelc et al., 2018). Long-term use of TCSs leads to various side effects including skin atrophy, striae distensae, teleangiectasia, and impaired skin barrier (Schoepe et al., 2006; Shlivko et al., 2014). Therefore, topical corticosteroids should be used only for a certain period of time and in appropriate amounts.

There are also other topical therapeutic options such as topical calcineurin inhibitors (TCIs) and topical phosphodiesterase 4 (PDE4) inhibitors with advantages of minimal side effects and possible long-term use (Kim et al., 2020; Freitas et al., 2022). TCIs inhibit the transcription of proinflammatory cytokine genes, including IL-2 (Bornhovd et al., 2001). TCIs are favorable particularly in skin folds and at the face, which are highly sensitive areas (Simpson, 2010). Jensen et al. reported improvement in all epidermal barrier parameters including TEWL in AD patients which were treated with both a topical steroid (betamethazone valerate) and a topical calcineurin inhibitor (pimecrolimus). Although both treatments normalized epidermal differentiation and reduced epidermal hyperproliferation, betamethazone valerate has been found to be more effective in reducing clinical symptoms and epidermal proliferation, however it induced epidermal thinning unlike

pimecrolimus (Jensen et al., 2009). Their alternate use may be an option in AD lesions.

Crisaborole ointment 2% is a nonsteroidal PDE4 inhibitor for the treatment of mild-to-moderate AD. The inhibition of PDE4 may decrease the inflammatory processes associated with AD without significant serious adverse event incidences. PDE4 inhibitors reduce the occurrence of AD exacerbation, however they have a statistically significant risk of producing pain (Martín-Santiago et al., 2022). Moreover, roflumilast (Calverley et al., 2007) and apremilast (Papp et al., 2013) have several adverse effects after systemic administration, but topical application has been found to decrease exposure and minimize adverse effects including burning of skin, pruritus and skin infections (Ashcroft et al., 2005; Broeders et al., 2016; Abed and Pawliczak, 2019).

The gut-skin-axis is involved in several dermatological diseases including AD (De Pessemier et al., 2021). In inflammatory skin diseases like AD or psoriasis, increasing interest is directed at the microbial skin component as therapeutic target. Influencing skin commensals and/or pathogenic bacteria like *S. aureus* with probiotics could happen via the gut-skin axis with selected bacteria (Szántó et al., 2019). Topical or oral probiotics may have some beneficial effects on AD symptoms associated with gut microbiome dysbiosis, including the alteration of the abundance of skin commensals and/or pathogenic bacteria like *S. aureus* (Szántó et al., 2019; Fang et al., 2020). It has been reported that *Lactobacillus* johnsonii caused significant improvement in skin symptoms of AD patients (Szántó et al., 2019) and also *Lactobacillus* plantarum significantly decreased SCORAD index and influencds the gut microbiota composition (Fang et al., 2020). Other approachs are even more direct and are supposed to modulate the skin microbiome by immediate application of specific bacteraial strains to the skin. Isolation of Roseomonas mucosa strains from healthy volunteers and transplantation to adult and peadiatric AD patients was successfully performed and led to reduced *S. aureus* colonization (Myles et al., 2018; Myles et al., 2020). Furthermore, SCORAD and Eczema Area and Severity Index (EASI) decreased and patients needed less glucocorticoid treatment. The authors found that Roseomonas mucosa produced beneficial sphingolipids and induced TNFR2-mediated epithelial repair mechanisms (Myles et al., 2020).

In another study aiming at the decrease of *S. aureus* colonization, autologous transplantation of specific bacterial strains like *S. epidermidis* and *S. hominis* after screening for antimicrobial activity achieved good results in AD patients and demonstrated the importance of skin commensals for AD disease pathology (Nakatsuji et al., 2017). In a corresponding phase 1 trial, a *S. hominis* strain isolated from healthy human skin was used as bacteriotherapy for AD and decreased *S. aureus* (Nakatsuji et al., 2021). Although eczema severity was not significantly changed over all patients, a specific subgroup seemed to especially benefit from the *S. hominis* treatment. This finding highlights that when it comes to the microbiome and its role in diseases, there will be huge differences between patients and individualized therapy approaches are or will be warranted.

In recent years, new therapeutic options such as biologic drugs have gained more importance with better understanding of AD pathogenesis (Kim et al., 2020; Freitas et al., 2022). Dupilumab is a human monoclonal antibody against IL-4 receptor α. Berdyshev et al. investigated the role of dupilumab in the regulation of skin barrier structure and function. They reported that blocking IL-4/IL-13 signalling with dupilumab decreased the TEWL in AD lesions, normalized the lipid composition and increase the ceramide chain length in lesional as well as non-lesional SC of AD patients (Berdyshev et al., 2022). Recently, the effect of dupilumab on *S. aureus* colonization and microbial diversity of the skin has been investigated (Callewaert et al., 2020). During dupilumab therapy, Callewaert et al. showed in both, lesional and non-lesional skin, increased microbial diversity and decreased S. aureus colonization, which was correlated with clinical improvement of AD (Callewaert et al., 2020). Dupilumab seems to affect many factors such as immunological markers, lipid composition and microbial colonization in the etiopathogenesis of the AD.

With immunological discoveries in the etiopathogenesis of AD, new biological therapies are gaining importance in the treatment of the disease. IL-13 is a potential therapeutic target for patients with AD (Zhang et al., 2022). In AD patients, increased expression of IL-13 in lesional tissues and elevated serum IL-13 levels have been found when compared to healthy controls (Tazawa et al., 2004). Moreover, it has been reported that an elevated IL-13 level is positively correlated with AD disease severity (Tazawa et al., 2004; Ungar et al., 2017). Tralokinumab is a human IgG4 monoclonal antibody, preventing IL-13 from binding to both IL-13Rα1 and IL-13Rα2 (Popovic et al., 2017). In a recent report from 2,285 patients, tralokinumab has been found to be a well-tolerated agent both in combination with TCS and as monotherapy, with long-term use up to 52 weeks for moderate-to-severe AD (Simpson et al., 2022). Lebrikizumab, which is also one of the new biological therapies, is a selective monoclonal antibody that targets IL-13. IL-13 plays an important role in multiple itch pathways and may contribute to the persistence of chronic itch in AD. The improvement of chronic itch in AD patients by lebrikizumab seems to be related to neuronal effects via IL-13 inhibition (Miron et al., 2022). A potential advantage of IL13 inhibitors is that the conjunctivitis, which is a common side effect of dupilumab (up to 22% in clinical trials), is less frequent under lebrikizumab (6.3%) and tralokinumab (6.2%) (Akinlade et al., 2019; Zhang et al., 2022).

Nemolizumab is a subcutaneously administered humanized monoclonal antibody against IL-31 receptor A. IL-31 blockage shows a direct effect against pruritus in AD patients (Nemoto et al., 2016; Oyama et al., 2018). Nemolizumab may also improve skin barrier function (Feld et al., 2016; Singh et al., 2016) and reduce the overall severity of AD (Liang et al., 2022). In a recent study, it has been reported that nemolizumab achieves TIMEACLIR-Itch (MEAningful CLInical Response for itch reduction) more quickly than anti-IL-4 or -IL-4/13 agents (Lin et al., 2022). In 2022, nemolizumab has been approved in Japan for adults and children above the age of 13 years in the treatment of itch associated with AD, refractory to current treatments (Maruho, 2022). Additionally, numerous clinical trials are ongoing which are investigating the efficacy of nemolizumab in AD (Keam, 2022).

Topical and oral JAKi are potential treatment options which significantly improve the clinical symptoms of AD patients with unsatisfactory response to conventional therapeutics (Honstein and Werfel, 2020). IL-4, IL-13, and IL-31 are the major cytokines which influence AD pathogenesis via the JAK-STAT signalling pathway. Systemic JAKi including baricitinib (JAK1/2i), upadacitinib (JAK1i)

and abrocitinib (JAK1i) are current treatment options in moderate to severe AD (Klein et al., 2022).

Ruxolitinib is also a selective JAK1/2 inhibitor available in both topical and oral administration options. Ruxolitinib 1.5% topical cream is the first topical JAKi, approved by US Food and Drug Administration (FDA) in patients (≥12 years) with mild-to-moderate AD (Owji et al., 2022). It has been reported that ruxolitinib and delgocitinib (pan JAKi) significantly improve pruritus and EASI in patients with moderate to severe AD (Kim et al., 2020; Nakagawa et al., 2020). In patients with mild to moderate AD with 2%–20% body surface area involvement, the efficacy of tofacitinib, which is a topical JAK 1/3 inhibitor, has also been reported (Bissonnette et al., 2016). In a recent meta-analysis, it has been found that particularly tofacitinib 2% has superior Investigator's Global Assessment response over other included JAKi and PDE4 inhibitors, followed by ruxolitinib 1.5% and delgocitinib 3% (Zhang et al., 2021). However, it is clear that more studies comparing the effect of topical and/or biological agents in AD patients, are needed.

2 Conclusion

AD is a chronic inflammatory skin disease in which many factors such as immunological and structural epidermal barrier dysfunction, immune abnormalities, lipid alterations, FLG mutations and skin microbiome alterations are involved in its etiopathogenesis. Although much progress has been made regarding the pathophysiology of AD and its clinical manifestations in adults and children, there are still many points that need to be clarified including unclear, complex molecular and cellular mechanism and metabolics, and also the specific mechanism of the alterations of lipid compositions.

Although topical corticosteroids are the cornerstone of AD treatment, new biological therapies also achieve promising results. However, it is also necessary to monitor the long-term effects of biological treatments. With new discoveries in the etiopathogenesis of AD, the symptoms of the disease could be controlled more effectively with new targeted therapies.

Author contributions

TÇ: literature search, data acquisition, data analysis, manuscript preparation, manuscript editing, and manuscript review. LK: literature search, data acquisition, data analysis, manuscript preparation, manuscript editing, and manuscript review. RF-H: literature search, data acquisition, data analysis, manuscript preparation, manuscript editing, and manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

LK is working as a Medical Advisor Dermatology for the company of MEDICE. However she worked on the text while on parental leave without any financial support by MEDICE and without using MEDICE resources. General conflicts of interest of Regina Fölster-Holst: RFH has been an advisor or speaker for Biogen, Johnson & Johnson, La Roche Posay, LEO Pharma, Neubourg, Novartis, Pfizer, Pierre Fabre, Regeneron and Sanofi.

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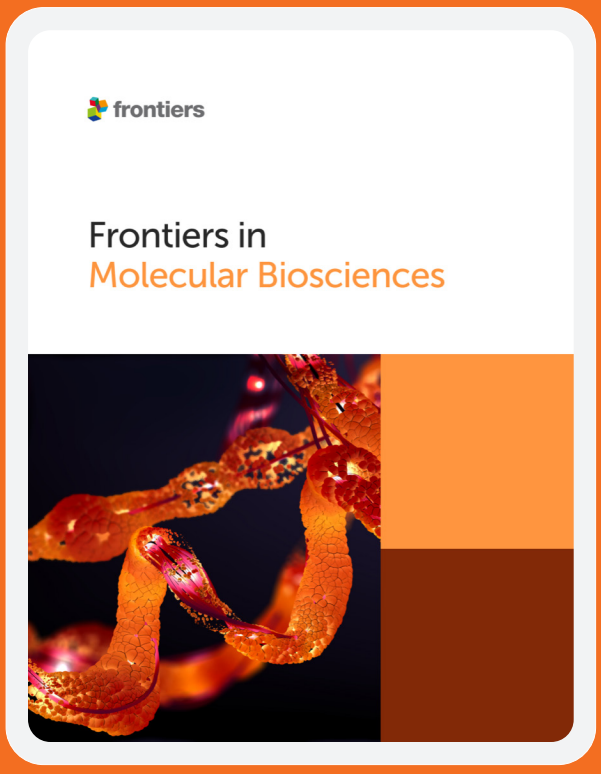

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