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Crosstalk: Skin cells and immune cells in inflammatory skin diseases

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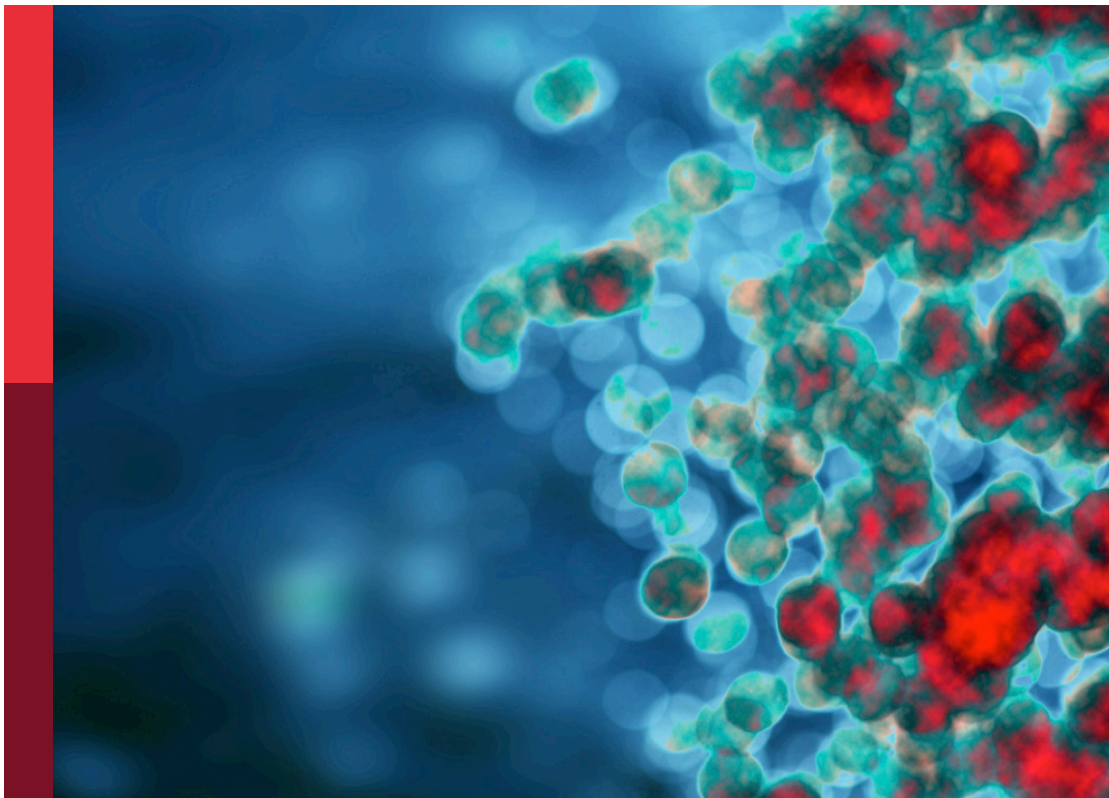

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Crosstalk: Skin cells and immune cells in inflammatory skin diseases

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Editorial: Crosstalk: skin cells and immune cells in inflammatory skin diseases

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KEYWORDS

skin, immune cells, crosstalk, inflammation, atopic dermatitis, psoriasis

Editorial on the Research Topic
Crosstalk: skin cells and immune cells in inflammatory skin diseases

The skin is the human body's largest organ consisting of two layers: epidermis and dermis, and appendages: hair and sweat glands. The skin not only wraps the body but also protects it from external stimuli and infection, perceives sensations such as pain and itch, and coordinates with various circulating immune cells for immune response/regulation. Recent studies have shown that inflammatory skin diseases, including psoriasis and atopic dermatitis, harbor systemic inflammation/immune abnormalities such as strong Th activation and expansion of specific immune cell subsets. Novel biologics and small molecule inhibitors targeting specific biomarkers and immune signals are much more effective and safer than conventional systemic therapies for these skin diseases. This Research Topic focuses on the interaction between the skin and immune cells and explores how skin cells and immune cells interact with each other and contribute to the pathogenesis of those skin diseases.

The review from Dainichi and Iwata can be considered the grand opening of the Research Topic. The epithelial–immune microenvironment (EIME) of epithelial tissues has five common elements (1): microbial flora (2), barrier (3), epithelial cells (4), immune cells, and (5) peripheral nerve endings. EIME provides both constant defense and situation-specific protective responses through three-layered mechanisms comprising barriers, innate immunity, and acquired immunity. The interactions between the five EIME elements of the skin protect against external dangers from the environment. They show five EIME models: atopic dermatitis, psoriasis, SLE, alopecia areata, and acne to simplify the disease pathologies.

We should know more about epidermal cells to understand the interactions between the skin and other cells. Epidermal keratinocytes can recognize various cytokines and pathogen-associated molecular patterns and produce a wide variety of inflammatory

cytokines, chemokines, and antimicrobial peptides. Morizane et al. summarize the foundation of knowledge on the cytokines recognized or produced by epidermal keratinocytes.

The common inflammatory skin diseases atopic dermatitis (AD) and psoriasis are now known as systemic immune skin diseases with Th skewing. Although these two inflammatory diseases often overlap clinically, it has been reported that each can be distinguished by its own genetic biomarkers (1). AD is a Th2-dominant chronic inflammatory skin disease, and acquired immunity plays an important role in the pathogenesis of AD. On the other hand, there is increasing evidence that toll-like receptors (TLRs) are involved in the pathomechanisms of not only infectious diseases, but also non-infectious inflammatory diseases. It has been demonstrated that TLRs recognize both exogenous threats, e.g. bacteria and viruses, and endogenous danger signals related to inflammation, cell necrosis, or tissue damage. Tamagawa-Mineoka review the current understanding of the roles of TLR signaling in the pathogenesis of AD, with particular emphasis on skin barrier function and inflammation.

Recently, Th balances in AD have reported to vary with the age of onset and ethnicity (2, 3). Li et al. investigate that the H2 antigen associated with blood type O is expressed in the granular and horny layers of the skin and plays a protective role in AD-like inflammation.

The factors that can potentially trigger or contribute to AD include genetic factors, family history, dietary choices, immune triggers, and environmental factors (Afshari et al.). Narrow targeting agents blocking IL-4, IL-13, and IL-31 signaling have exhibited significant clinical benefits in patients with AD (4). Stimulation of IL-31 cognate receptors on C-fiber nerve endings believe to activate neurons in the dorsal root ganglion, causing the itch. The IL-31 receptor is a heterodimer of oncostatin M receptor (OSMR) β and IL31RA subunits, and OSMRβ can also bind OSM, a pro-inflammatory cytokine released by monocytes/macrophages, dendritic cells, and T lymphocytes. Suehiro et al. investigate OSM, released from monocytes in the skin, modulates the sensitivity of dorsal root ganglion neurons to Th2 inflammatory cytokines and thereby the severity of AD-associated skin itch. *Staphylococcus aureus* (*S. aureus*) can be frequently found on the skin of AD patients where it actively contributes to skin inflammation. Focken and Schittek describe that *in vitro* co-culture, polymorphonuclear neutrophils (PMNs), and primary human keratinocytes (PHKs) induce inflammatory responses in PHKs which are further exacerbated in the presence of *S. aureus* and induces further PMN recruitment thus fueling skin inflammation. Interestingly, infection of PHKs with the skin commensal *S. epidermidis* reduces the inflammatory effects of PMNs in the skin and has an anti-inflammatory effect. Yu et al. have established a novel mitochondrial-based molecular signature that considers IDH3A, BAX, MRPS6, and GPT2. Their study combines bioinformatics analysis and machine learning to increase their understanding of the crosstalk relationship among these key genes, AD immune infiltration, and mitochondrial metabolic function. In addition, they describe that plasma circulating cell-free mitochondrial DNA may be a key indicator of AD progression, providing evidence of mitochondrial oxidative stress damage during the advancement of AD in adult patients with moderate-to-severe AD.

Psoriasis is associated with various systemic diseases, including cardiovascular disease, diabetes, metabolic syndrome, and several autoimmune diseases. Several biomarkers and signaling pathways are candidates as predictors of cardiovascular disease in patients with psoriasis (5). Clinical studies have suggested a bidirectional association between non-alcoholic steatohepatitis (NASH) and psoriasis, affecting each other's development and severity. Takezaki et al. investigate the co-occurrence of NASH exacerbate psoriatic skin changes associated with increased serum inflammatory cytokine levels and decreased serum adiponectin levels. They suggest that therapeutic intervention for co-occurring NASH is essential to achieve a favorable prognosis of psoriasis in clinical practice. Kamata and Tada give a general overview of the pathogenesis of psoriasis. Various immune cells are involved in the pathogenesis of psoriasis, including dendritic cells, Th17 cells, and resident memory T cells. Furthermore, keratinocytes play a role in the development of psoriasis as immune cells by secreting antibacterial peptides, chemokines, tumor necrosis factor-α, IL-36, and IL-23. These immune cells and skin cells interact and drive the aberrant differentiation and proliferation of keratinocytes. Among those peptides and chemokines, Liang et al. focus on S100 proteins as potential therapeutic targets and diagnostic biomarkers in psoriasis. Regulatory T cells (Tregs) maintain immune tolerance and prevent autoimmune diseases. They suppress the activation and proliferation of other immune cells, thereby controlling immune responses and reducing inflammation. Tregs diminish during psoriatic inflammation. Lee et al. investigate that treatment with cytotoxic T lymphocyte antigen-4 signaling peptide diminishes psoriatic skin inflammation with increased Treg cell proportion and reduces IL-17 production by T cells.

The crosstalk between the skin and immune cells is important not only in inflammatory skin diseases but also in other skin conditions. Zhang et al. show CD64 play a crucial role in wound healing, especially in diabetes mellitus conditions, where it is associated with $CD163^{+}$ M2 macrophage infiltration. Cuproptosis is a copper-induced cell death reported by Tsvetkov et al. in 2022 (6). Song et al. describe identification of several cuproptosis-related genes as novel therapeutic targets for hypertrophic scar using single-cell analysis and machine learning techniques. Feng et al. discuss the role of macrophages in acne vulgaris. Macrophages embody a paradoxical role in acne development, serving as both sentinels and provocateurs. Their vital functions include the regulation of lipid concentrations and facilitating the elimination of *Cutibacterium acnes*. However, an excessive immune reaction can provoke inflammation and subsequent acne scarring. It is imperative to comprehend their intricate roles to maintain physiological equilibrium and circumvent adverse pathological outcomes. Lu et al. report the case of Sweet syndrome during the lymphoma treatment, with rare clinical presentations of local crater-like suppurative skin lesions. The incidence of Sweet syndrome is high in hematological malignancy but rare in lymphomas.

This Research Topic describes how the skin interacts with immune and other cells, microbes, and nerves, leading to specific inflammation and immune imbalances. The skin and its cells interact with other cells and organs through a "conversation" with cytokines and chemokines, regulating the clinical condition of various skin diseases. A deeper understanding of these interactions in the skin may facilitate the development of targeted therapeutic approaches for skin diseases.

Author contributions

KY: Writing – original draft, Writing – review & editing. HK: Writing – review & editing. JK: Writing – review & editing.

Conflict of interest

The 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

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Atypical Sweet syndrome: skin sinus tracts in an acutely febrile patient after lymphoma treatment: a case report

Shi-Ying Lu[1], Hui-Fang Yang[2], Qing-Li Zeng[2], Peng Chen[2], Li Chen[2], Jing Gao[2], Xue-Kui Gu[2], Hai Lan[3] and Man Luo[2]*

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Sweet syndrome (SS) is an uncommon inflammatory disease that involves painful skin, edematous, red papules, plaques, or nodules often accompanied by fever and leukocytosis. SS has three subtypes, including classical, malignant-tumor associated, and drug-induced SS (DISS). Patients with DISS have clear histories of recent drug exposure. The incidence of SS is high in hematological malignancy but rare in lymphomas. Glucocorticoid treatment is the recommended treatment for all subtypes of SS. This case study describes a male patient who had a history of sALCL(Systemic anaplastic large cell lymphoma) and was treated with multiple cycles of monoclonal-antibody (mAb) therapy. They also received the G-CSF injection at the site where skin lesions later developed. They met the diagnosis criteria for DISS, which was considered to be caused by the G-CSF injection. In addition, BV(Brentuximab vedotin) administration might predispose them to DISS. This case illustrates the first reported SS during the lymphoma treatment, with rare clinical presentations of local crater-like suppurative skin lesions. This case expands the available literature on SS and hematologic neoplasms and reminds clinicians to promptly recognize and diagnose SS to minimize patient morbidity and long-term sequelae.

KEYWORDS

drug-induced Sweet syndrome, local sinus ulcer, systemic anaplastic large-cell lymphoma, granulocyte colony-stimulating factor, brentuximab vedotin

1 Introduction

Sweet syndrome (SS), or acute febrile neutrophilic dermatosis, is an uncommon inflammatory disorder characterized by the abrupt appearance of painful, edematous, and erythematous papules, plaques, nodules, or honeycomb-like or bullous changes on the skin. In addition, affected patients can have fever and leukocytosis, as well as eye,

musculoskeletal, and internal organ involvement. SS was initially described by Dr. Sweet in 1964 (1). In 1971, the first cancer-related SS was reported (2). Later, drug-induced SS (DISS) was presented and its diagnostic criteria were proposed in 1986 (3). Researchers have since classified SS into three subtypes based on the etiologies, including classical, malignant-tumor associated, and DISS. Most SS subtypes can be directly distinguished by etiology.

Herein, we report on a case of SS that was considered to be related to two factors, including a history of lymphoma and anti-tumor drugs. The atypical clinical presentations of the skin sinus tracts in the patient made diagnosing SS challenging.

2 Case description

A 30-year-old male patient was admitted to our hospital for 8 days with redness and swelling of the abdominal wall and left arm. The patient had systemic anaplastic large cell lymphoma (sALCL). He had received immune-chemotherapy with a BV-CHP regimen, including brentuximab vedotin (BV, 1.8 mg/kg on day 1), cyclophosphamide (750 mg/m^2 on day 1), doxorubicin (50 mg/m^2 on day 1), and prednisone (100 mg on days 1–5) (4) for four cycles and achieved complete remission (CR) during the second cycle. Ten days after his last treatment, the patient received granulocyte colony-stimulating factor (G-CSF 300 μg for 2 days) injections in the abdomen wall and left arm due to agranulocytosis. Seventy-two hours after G-CSF injections, the patient developed rapidly expanding erythema and induration in the areas of administration. In addition, he also reported low-grade fever. At hospital admission, the size of the abdominal wall induration had reached 10×10 cm^2 (Figure 1A). The patient had no family history of neoplastic disease or severe dermatosis.

After admission, laboratory tests reported the following results: white blood cell count (WBC) 16.27×10^9/L, neutrophil count 12.69×10^9/L, lymphocyte count 0.89×10^9/L, hemoglobin (Hgb) 113 g/L, platelet count (PLT) 257×10^9/L, C-reactive protein (CRP) 139 mg/L, procalcitonin (PCT) 0.8 ng/mL, and serum amyloid A (SAA): >200 mg/L. Doppler ultrasound of the abdominal induration revealed swelling with limited fluid collection. The patient received antibiotic treatment with cefoperazone–sulbactam empirically. However, the skin erythema and induration gradually progressed to become ulcers (Figure 1B). His pain intensity increased and their body temperature also rose to >39°C, with chills. Antibiotic treatment was switched to linezolid, moxifloxacin, and topic mupirocin ointment. One week later, the repeat laboratory tests showed that CRP and PCT levels decreased to 77.2 mg/L and 0.57 ng/mL, respectively. However, the patient's skin lesions continued to expand (Figure 1C). The ulcerated areas on his skin gradually developed abscesses. Antibiotics were then switched to imipenem, vancomycin, and ornidazole. However, the patient's symptoms did not improve. He required frequent analgesics for pain and antipyretics for fever. We then further switched his antibiotics to piperacillin–tazobactam and sulfamethoxazole (Figure 2). During this process, wound secretions and blood were cultured several times. The tissue samples were also sent for metagenomic next-generation sequencing to detect potential pathogens. However, all these tests reported negative results. His skin lesions had a poor response to antibiotic treatment (Figure 1D).

The patient had a history of lymphoma. Although the previous treatment had achieved CR, we considered possible tumor recurrence and skin invasion due to the poor response of skin lesions to the antibiotic treatments. We performed a skin lesion tissue biopsy. The pathological report showed diffuse neutrophil

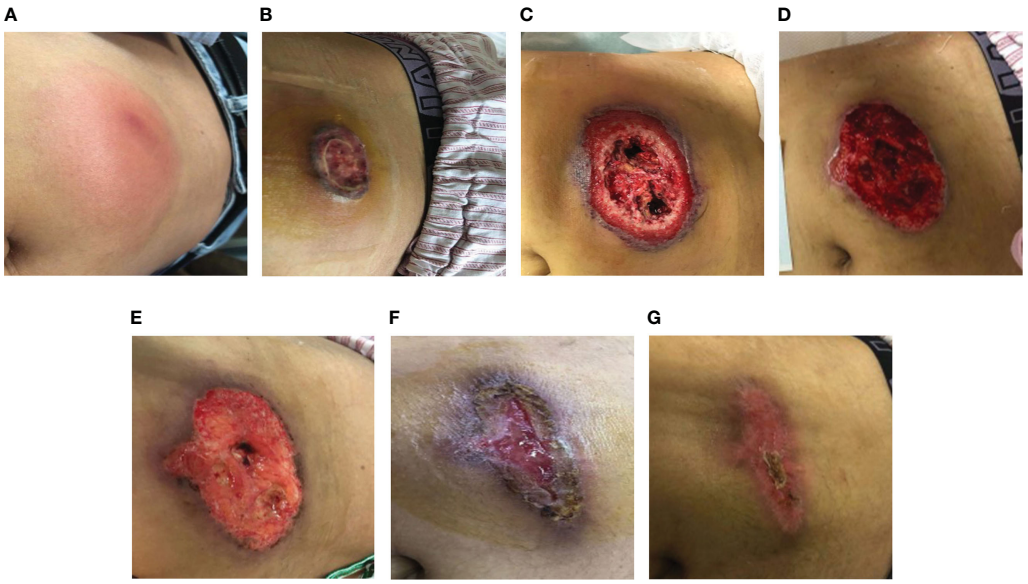

FIGURE 1
(A) The patient developed a subcutaneous induration in the abdominal wall. Redness, swelling, and pain worsened within 4 days. **(B)** Abdominal-wound tissue necrosis; ulcerated area: 2×2 cm. **(C)** Abdominal-ulcer worsened; ulcerated area: 10×10 cm. **(D)** The skin lesions had a poor response to the antibiotic treatment. **(E–G)**. After treatment with prednisone, the patient's crater-like ulcer gradually healed.

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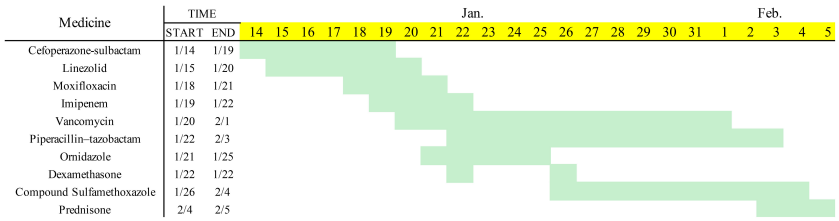

FIGURE 2
Record of drug use.

infiltrations in the muscle and adipose tissue without lymphoma cells (Figure 3). In addition, a positron emission tomography/computed tomography examination showed a negative result for lymphoma (Figure 4). Therefore, we ruled out tumor recurrence and skin invasion based on these two evaluations.

We then noticed that the patient had used two drugs (BV and G-CSF) before the development of the skin lesions. Therefore, we considered whether the skin lesions might be related to these two drugs.

After MDT(Multi-Disciplinary Treatment), combined with the patient's current clinical signs and the wound's pathological findings of massive neutrophil infiltrations, we considered the diagnosis of SS. According to his recent history of G-CSF injection and BV treatment, we speculated the subtype of DISS (5, 6) (diagnostic criteria shown in Table 1), since his presentation was consistent with a diagnosis of DISS based on the available clinical information. Therefore, we stopped all antibiotics and administrated glucocorticoid treatment 20 days after admission (prednisone, 40 mg q.d. initially and then gradually tapered and maintained for 1 month). After the initiation of prednisone, his temperature dropped rapidly, and the skin lesion steadily improved. On February 3, the patient's body temperature returned to normal. The ulcer then gradually closed and the wound healed one month later (Figures 1E–G).

Treatment results indicated that the patient was responsive to steroid therapy. This patient met all five diagnostic criteria for DISS (5, 6) (Table 1). Severe cutaneous-to-muscular sinus ulcers caused by SS had not been previously reported. These ulcers might be related to the history of sALCL and the G-CSF and BV use in this patient.

3 Discussion

SS induced by G-CSF has been reported in scientific literature (7, 8). The most commonly involved common skin wounds are papular lesions. Bullous ulcers are occasionally seen and can be complicated by pyoderma gangrenosum. SS is common in hematological malignancies. The inflammation is confined to the dermis. Our case's unique features included rare sinus ulcers, a history of malignant tumors, and immunocompromised status from recent immuno-chemotherapy. It should also be noted that SS is a generalized cutaneous inflammatory lesion (9). Affected patients commonly have skin lesions all over the body. However, our patient only had skin wounds at the sites of G-CSF injection.

In this case, skin inflammation progressed rapidly, became necrotic, and quickly penetrated the subcutaneous and fatty tissue to form sinus tracts to the muscles. These SS skin lesions were atypical and difficult to distinguish from infection, which made clinical diagnosis and management difficult.

Meanwhile, ulcerative SS needs to be distinguished from Pyoderma Gangrenosum(PG). Both SS and PG belong to

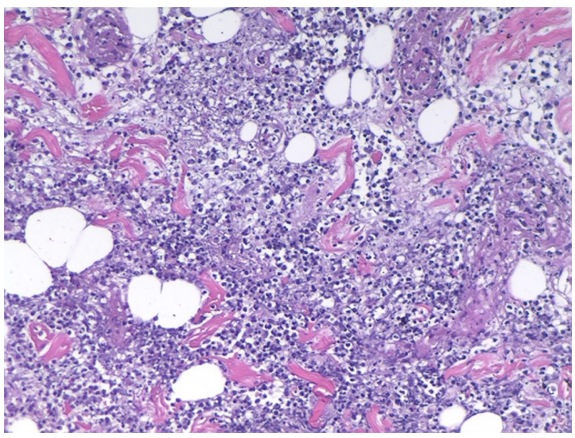

FIGURE 3
Muscle and adipose-tissue pathology results: diffuse and extensive neutrophil infiltrations.

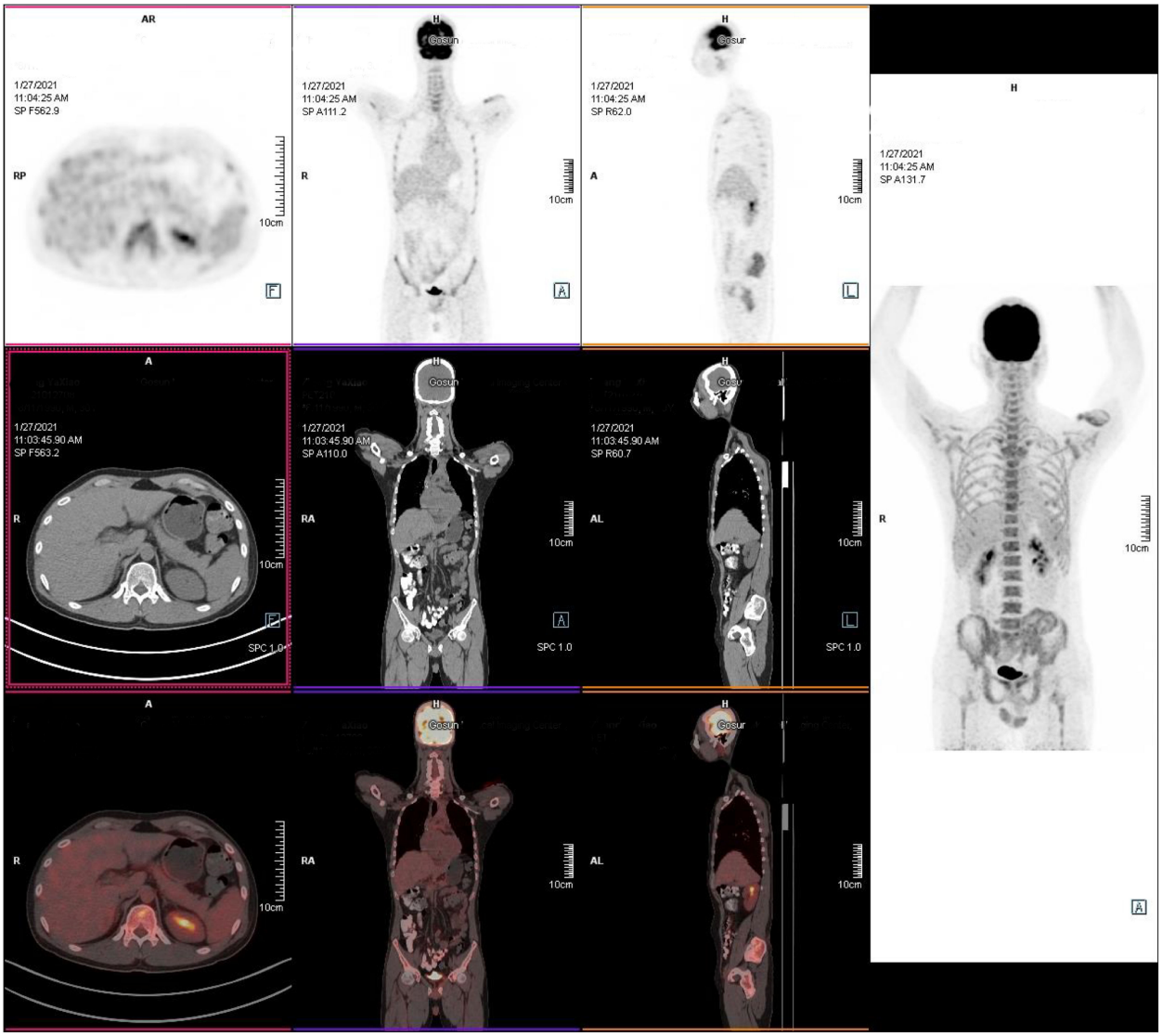

FIGURE 4
Positron emission tomography/computed tomography examination. The tumor was in remission without radiographic recurrence. (1) There was no increase in lesion metabolism in the original mediastinal, neck, axillar, supraclavicular, and internal mammary areas; the Deauville score was 1, which was considered complete remission after treatment. (2) Left upper-arm and lower-left abdominal-wall skin and subcutaneous inflammatory changes.

neutrophilic dermatosis. In the diagnosis of SS, the patient's peripheral blood white blood cells and granulocytes can be significantly increased. At the time of admission, the patient had only honeycomb-like tissue changes of the skin, without ulcers, and significantly increased white blood cells and granulocytes in peripheral blood. The time from skin change to ulcer progression was fast in PG, while most SS only showed skin-like changes. The patient's skin in our case took 11 days from local redness, redness, and swelling to rupture and form ulcers. The ulcer surface gradually expanded and deepened after formation, and the development of the ulcer surface slowed down after debridement. The skin did not form "wrinkled paper" changes after ulcers (10, 11) healing. Due to the specificity of the ulcer, in the later stage of this patient's treatment, the clinical diagnosis was a special manifestation subtype of SS.

TABLE 1 Diagnostic criteria for drug-induced Sweet syndrome (DISS) (4, 5).

1. Abrupt onset of painful erythematous plaques or nodules
2. Histopathological evidence of dense neutrophilic infiltration without evidence of vasculitis
3. Pyrexia, >38°C
4. Temporal relationship between drug ingestion and clinical presentation, or temporally related recurrence after oral challenge
5. Temporally related resolution of lesions after drug withdrawal or treatment with systemic corticosteroids

SS is a rare inflammatory disorder characterized by painful, edematous red papules, plaques, or nodules of the skin, often accompanied by fever and leukocytosis. It can also involve the eyes, muscles, bones, and internal organs (12, 13) (the diagnostic criteria are outlined in Table 2). SS is classified into three subtypes, classical SS, malignant-tumor–associated SS, and DISS (9).

TABLE 2 Major and minor diagnostic criteria for Sweet syndrome.

Criteria	Findings
Major	1. Abrupt onset of painful erythematous plaques or nodules
	2. Histopathological evidence of a dense neutrophilic infiltrate without evidence of leukocytoclastic vasculitis
Minor	1. Pyrexia, >38°C
	2. Association with an underlying hematological or visceral malignancy, inflammatory disease, or pregnancy; or preceded by an upper-respiratory or gastrointestinal inflection or vaccination
	3. Excellent response to treatment with systemic corticosteroids or potassium iodide
	4. Abnormal laboratory values at presentation (three or four): *Erythrocyte sedimentation rate (ESR) > 20 mm/h *White blood cell count (WBC) > 8×109/L *Neutrophil count > 70% *High C-reactive protein (CRP) levels

Classical SS is the main form of the syndrome and is associated with infection, inflammatory bowel disease, and pregnancy. It can be diagnosed only by ruling out malignancy and drug exposure (9, 12–14). SS associated with malignant tumors is mainly reported in patients with hematological malignancies (6, 15). The most common malignancy involved is acute myeloid leukemia, followed by myelodysplastic syndrome, Hodgkin lymphoma, and non-Hodgkin lymphoma (15, 16). The current literature suggests that SS can be a harbinger of impending malignancy and in some cases be the first sign of cancer recurrence (14).

The main characteristic of DISS is concurrence with drug use. It usually develops within 2 weeks after drug use (17). Repeated use of the same drug at the same site can cause DISS recurrence. G-CSF is currently thought to be the most common drug that induces DISS (18). Antitumor drugs can also lead to neutrophilic dermatosis (19, 20). To date, drugs that have been reported in DISS include azacitidine (21, 22), bortezomib (23, 24), imatinib (25–27), lenalidomide (19), and all-trans retinoic acid (28). With the increasing use of immune checkpoint inhibitors, it has been reported that SS could be induced by mAbs(Monoclonal antibodies) (29).

In our patient, it was unknown whether BV or CHP induced or predisposed him to DISS. BV has been reported to cause toxic necrosis of the skin and subcutaneous tissue. However, according to pharmacological studies of BV, the drug's half-life is no more than 7 days. BV can cause epidermolytic poisoning, which often happens within 7 days (30). In our patient, the period from the last BV treatment and the initial onset of skin lesions was >10 days. The patient's clinical manifestation was local skin ulceration, which was not consistent with the BV-induced skin lesions of generalized dermatitis. Therefore, we consider that his SS was less likely due to BV administration. The same consideration to the chemotherapy of CHP regimen, the most common adverse events are acute cystitis and myocardial cell injury. However, we could not exclude the possibility of BV as a predisposing factor. BV is an antibody drug conjugate, a combination of Cluster of Differentiation 30 (CD30)–directed antibody, and anti-tubulin monomethyl obestatin E (31) that is used as targeted immunotherapy against CD30 (32). Adverse skin reactions and subcutaneous-tissue diseases from BV have been reported, such as toxic epidermal necrolysis and Stevens–Johnson syndrome (33). In this case, multiple courses of BV treatments might cause increased immune activity that leads to hypersensitivity to the subsequent G-CSF injection.

Pathological studies indicate that CD30 can also be expressed in subcutaneous myoepithelial cells. This patient had a history of multiple BV injections, which might cause small amounts of BV immune complex accumulations in his subcutaneous tissue. After subcutaneous injection of G-CSF, the patient's local tissues were stimulated to accumulate many neutrophils, which, together with the immune complex, triggered a local hypersensitivity reaction. Therefore, we could not rule out his severe ulcers as an Arthus reaction (34, 35).

Since the mechanism of SS is not fully understood, the dual effects of G-CSF and BV might have led to the co-existence of cytokine storm and hypersensitivity response, possibly causing severe sinus ulcerative SS in this patient.

4 Conclusion

This patient had a rare presentation of SS, characterized by hyperpyrexia and local sinus ulcers. These symptoms are not consistent with common systemic epidermal reaction in SS and are difficult to differentiate from local severe skin infections or tumor cell infiltrations. With the glucocorticoid therapy, his symptoms were relieved and the skin ulcers were fully healed. In this case, the onset of SS was associated with G-CSF injection. In addition, the skin lesions might have been related to his recent history of BV medication, which requires further investigation and attention during clinical practice.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

S-YL performed data analysis and drafted the manuscript. H-FY performed data analysis and prepared the manuscript. Q-LZ performed data analysis and prepared the manuscript. LC performed data analysis and prepared the manuscript. PC reviewed the article critically for important intellectual content. JG reviewed the article critically for important intellectual content. X-KG reviewed the article critically for important intellectual content. HL reviewed the article critically for important intellectual content. ML reviewed the

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Conflict of interest

The 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

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Pathogenic role of S100 proteins in psoriasis

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Psoriasis is a chronic inflammatory skin disease. The histopathological features of psoriasis include excessive proliferation of keratinocytes and infiltration of immune cells. The S100 proteins are a group of EF-hand Ca^{2+}-binding proteins, including S100A2, -A7, -A8/A9, -A12, -A15, which expression levels are markedly upregulated in psoriatic skin. These proteins exert numerous functions such as serving as intracellular Ca^{2+} sensors, transduction of Ca^{2+} signaling, response to extracellular stimuli, energy metabolism, and regulating cell proliferation and apoptosis. Evidence shows a crucial role of S100 proteins in the development and progress of inflammatory diseases, including psoriasis. S100 proteins can possibly be used as potential therapeutic target and diagnostic biomarkers. This review focuses on the pathogenic role of S100 proteins in psoriasis.

KEYWORDS

psoriasis, S100 proteins, interaction, signaling pathways, inflammation

Introduction

Psoriasis is a chronic, adaptive immune-mediated inflammatory skin disease with a strong genetic predisposition and autoimmune pathogenic features (1–4). Immune-mediated inflammatory dermatosis is characterized by complex multifactorial etiologies and is clinically very different from each other despite sharing a chronic inflammatory background (Table 1). Psoriasis presents a notable burden, with about 0.2 billion people worldwide suffering from psoriasis. The prevalence of psoriasis varies from country to country (5, 6). The pathogenesis of psoriasis is attributable to many factors, including ethnicity, genetics, gene variants, and environment (7, 8).

Psoriasis primarily comprises plaque psoriasis (the common form, approximately 90% of psoriatic cases are chronic plaque-type psoriasis), guttate psoriasis (after the streptococcal infection), inverse psoriasis (flexural psoriasis), pustular psoriasis (the rare and unstable), and erythrodermic psoriasis (systemic inflammation). Histopathologically, psoriasis is characterized by infiltration of immune cells, epidermal hyperproliferation, and abnormal keratinocyte (KC) differentiation (9). The recruitment of circulating leukocytes to the epidermis and the production of pro-inflammatory factors, such as TNF-α, IFN-γ, IL-6, IL-8, IL-23, IL-1β, and IL-17A play a crucial role in the development of psoriasis (10).

In addition, psoriasis can be accompanied by comorbidities such as cardiovascular diseases, hyperlipidemia, hypertension, coronary artery disease, and diabetes (11–16).

Originally, psoriasis was thought to be due to dysregulation of keratinocyte proliferation (17), increasing evidence indicates a crucial role of the immune system in the pathogenesis of psoriasis (18). Psoriasis is caused by chronic interaction between KCs and activated immune cells (19). One of the most important features of psoriatic lesions is upregulation of expression levels of KC-originated antimicrobial peptides and proteins such as S100 protein subfamily, an important multifunctional player in inflammatory dermatoses (20, 21). The expression and secretion of S100 protein in keratinocytes, leads to the production of an array of pro-inflammatory cytokines, promoting dendritic cell maturation and CD4+ T cell proliferation (22), contributing to autoimmune system activation and psoriasis pathogenesis.

S100 protein family

The S100 protein family is a type of Ca^{2+}-binding protein composed of a multigene family of low molecular mass proteins. The S100 proteins are expressed in a cell- and tissue-specific manner and engaged in multiple functions in various cell types and tissues (23, 24). The expression levels of S100 proteins are altered in various diseases such as cardiomyopathies, neurodegenerative, inflammatory disorders, and cancers (25–29). S100 proteins act as calcium sensors that regulate the function and subcellular distribution of specific target proteins participating in a variety of signaling pathways and playing a key role in diverse cellular processes such as cell proliferation, migration, differentiation, energy metabolism, apoptosis, etc. (30). In addition, S100 proteins have an extracellular activity in response to inflammatory stimuli (30–32).

The S100 protein is the largest subgroup within the superfamily of calcium-binding EF-hand motif. Genes of the S100 protein family are encoded in epidermal differentiation complex (EDC) and located within the cluster on human chromosome 1q21 (33). The members of the S100 protein family have a similar low molecular weight of 10-12 kDa and share 10% - 98% similarity in the amino acid sequences. S100 proteins are typically symmetric dimers, and each subunit contains two helix-loop-helix EF-hand motifs that are separated by a flexible hinge region and flanked by conserved hydrophobic residues at the amino- and carboxy-terminal ends (34) (Figure 1). The calcium-binding affinity of S100 proteins is low in the absence of target proteins, but the affinity is significantly increased by several orders of magnitude in the presence of specific target proteins (35, 36). The canonical C-terminal EF-hand motif binds to calcium with 100-fold higher than the N-terminal non-canonical EF-hand (37).

The 3D structures of S100 protein family have been resolved in three different states: Ca^{2+}-free apo state, bound to Ca^{2+} and bound to its target protein (38). Upon calcium binding, the S100 protein undergoes a large conformational change that creates a special target protein recognition site and allows interaction with different target proteins, including cell surface receptors and other S100

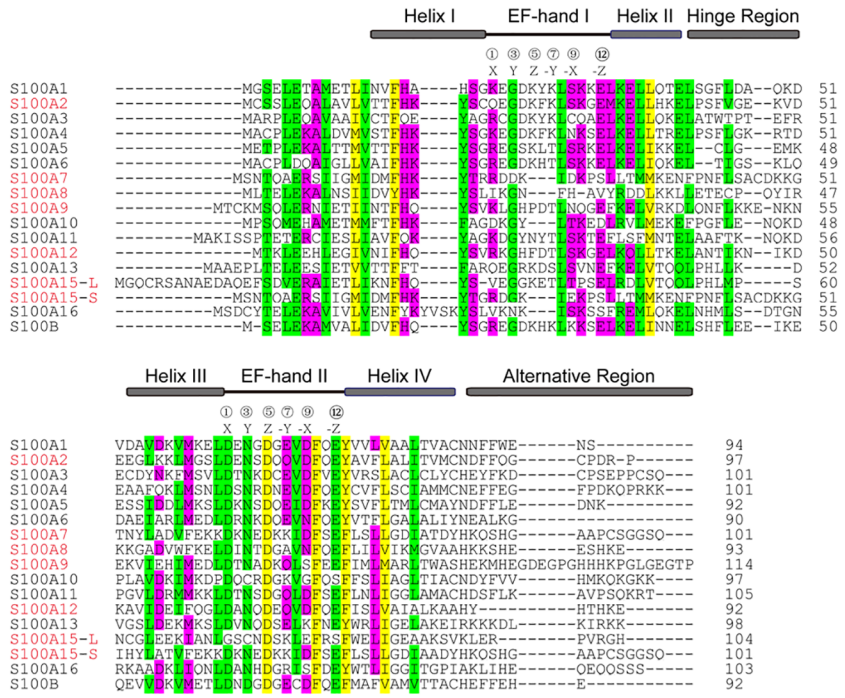

FIGURE 1
Alignment of the amino acid sequences of S100 from homo sapiens. The position of two EF-hands is indicated by black lines and the residues at positions 1, 3, 5, 7, 9, and 12 for Ca^{2+}-coordination are labeled X, Y, Z, -Y, -X, -Z, respectively. Four α-helical segments, a hinge region, and an alternative region are indicated by grey boxes. The sequences highlighted in red are discussed in the text.

proteins (39). Moreover, S100 proteins present as a non-covalent anti-parallel homo- or heterodimers form, and the target protein can bind to opposite ends of S100 protein dimer. Thus, S100 protein dimer is a cross-bridge between two target proteins.

Because of multiple functions of the S100 proteins, many diseases, including psoriasis, are associated with altered expression levels of S100 proteins. Recent studies suggest the potential roles of S100 in keratinocyte proliferation, differentiation, and stress response. This review will focus on the expression and function of S100 protein-target protein interaction, and the signaling pathways of each S100 protein in psoriasis.

S100A2

S100A2 (S100 calcium-binding protein A2) is encoded by a gene located in human chromosome 1q21 (31), which was first identified as a tumor-suppressor gene by subtractive hybridization in human mammary epithelial cells (40). The three-dimensional crystal structure of a cysteine-deficient S100A2 in the calcium-free form is similar to other S100 proteins. S100A2 contains an N-terminal specific EF-hand loop by helix I and helix II, and a C-terminal classical EF-hand motif between helix III and helix IV, which are linked by a hinge region (41) (Figures 1, 2A, B). In addition to Ca^{2+}, S100A2 also binds to Zn^{2+} with higher affinity, and the binding affinity of S100A2 to calcium is significantly reduced upon binding to zinc (42). Ca^{2+}- and Zn^{2+}-ions have opposite effects on the stability of S100A2, with Ca^{2+} acting as a protein stabilizer and Zn^{2+} as a protein destabilizer (43).

S100A2 is a member of the S100 EF-hand calcium-binding family found in mammary epithelial cells and other organs or tissues, including the lungs, kidneys, liver, and prostate gland (44). S100A2 has distinct functional roles in epithelial tissue of different origins (45). S100A2 is primarily expressed in the nucleus and moderately expressed in the cytoplasm of normal human keratinocytes (46). In the skin, S100A2 is co-localized with cytokeratin K14, an intermediate filament protein expressed primarily in basal proliferative KCs. Expression levels of S100A2 are correlated positively with the levels of cytokeratin K14. Expression levels of S100A2 gene is markedly upregulated in the involved skin of inflammatory dermatoses such as psoriasis and atopic dermatitis, and correlate positively with the severity of inflammatory skin disorders (47).

Many factors can regulate the distribution and expression levels of S100A2. For example, either oxidative stress or changes in intracellular Ca^{2+} levels enhance the translocation of S100A2 from the nucleus to the cytoplasm. Expression levels of the S100A2 gene can also be upregulated by multiple factors such as epidermal growth factor (EGF) (48), transforming growth factor β (TGF-β) (49), and interferon α (50). In keratinocyte cultures, EGF treatment significantly upregulated the expression levels of S100A2, while S100A2, but not EGF, is an effector of the regenerative hyperplasia pathway of epidermal differentiation (51). The binding of p53 to the binding site at the promoter of S100A2 can upregulate S100A2 expression (52–54). S100A2 protein is a direct transcriptional target of p73β and ΔNp63α, which both are required for the developmental and differentiation processes of keratinocytes (55). Moreover, ΔNp63α can interact with BRCA1, the breast/

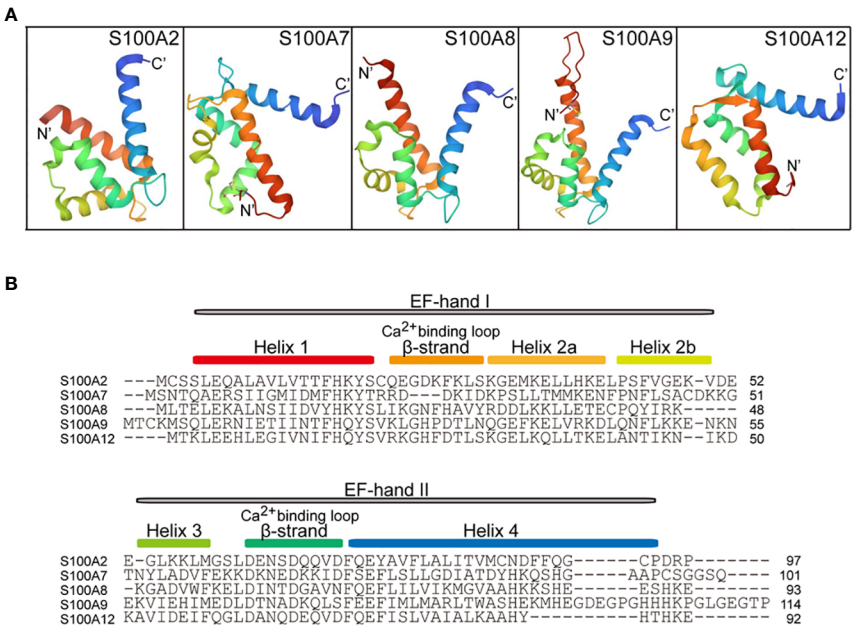

FIGURE 2
Cartoon representation of the tertiary structure in S100A proteins (PDB ID: 4DUQ, 2WOS, 1XK4, 6ZDY and 2WCF). **(A)**, comparison of the crystal structure between S100A2, S100A7, S100A8, S100A9, and S100A12. The N-terminal and the C-terminal of S100 proteins indicate N′ and C′. **(B)**, the primary secondary structure of S100A proteins. The red box indicates the helix 1 at the N-terminal, the Ca^{2+}-binding loop 1, Helix 2a, Helix 2b, Helix 3, Ca^{2+}-binding loop 2, and Helix 4 are indicated by the yellow box, orange box, yellow-orange box, green box, cyan box, and blue box, respectively. EF-hand regions are colored in grey boxes.

ovarian cancer susceptibility gene, upregulating S100A2 expression and enhancing tumor growth (56). Thus, multiple factors can regulate S100A2-mediated physiological and pathological reactions (47).

The S100A2 is an important regulator of keratinocyte differentiation, proliferation and wound healing. Epithelial-specific S100A2 transgenic mice exhibit increased proliferation and delayed skin wound repair (57). S100A2 and tumor suppressor factor p53, an important regulator in the wound healing process, can form a positive feedback loop to regulate the wound repair process. Moreover, S100A2 interacts with p53 to increase its transcriptional activity, and posttranslational modification of p53 increases its interaction with S100A2 (58). Electrophoresis and mass spectrometry assays showed a cross-link formation of S100A2 and S100A4 *via* copper-mediated oxidation of cysteine residues, resulting in increases in activation of NF-κB and secretion of TNF-α (59).

S100A2 interacts with KPNA2, a nucleocytoplasmic transport protein karyopherin α, to form a cotransport complex, transporting the tumor-associated transcription factor and regulating nucleocytoplasmic transport (60). Pulldown and coimmunoprecipitation assays revealed that the interaction between S100A2 and Hsp70/Hsp90-organizing protein or kinesin-light chain through tetratricopeptide domain modulates protein complex folding and KLC-cargo interaction (61). S100A2 can be recognized by and interact with erythropoietin, being involved in the development of tumors and other diseases (62).

RAGE (receptor for advanced glycation end products) is a member of an immunoglobulin protein family, including the extracellular part (composed of one variable like V-domain and two constants like C-type domains), transmembrane spanning helix, and a cytosolic domain (63). RAGE interacts with structurally diverse ligands, and plays an important role in human diseases. Although earlier studies did not show the interaction between S100A2 and RAGE, recent studies showed a micromolar affinity and strict calcium-dependent interaction between S100A2 and RAGE *via* the V-domain of RAGE (64) (Figure 3). However, further studies are needed to determine the functional significance of the interaction between S100A2 and RAGE.

S100A7

S100A7, also called psoriasin, was first found as a protein overexpressed in psoriasis-involved skin and later identified as a biomarker of psoriasis-involved skin (65–67). S100A7 is an EF-hand type calcium-binding protein with a molecular weight of 11.4 kDa localized in the cytoplasm of keratinocytes and distributed at the cell periphery in terminally differentiated keratinocytes (68). The three-dimensional crystal structure of S100A7 is typically a symmetric dimer containing four α helices (69). S100A7 contains two Ca^{2+}-binding domains: an N-terminal non-canonical EF-hand domain and a C-terminal canonical EF-hand domain (Figures 1, 2). The amino acid and carboxyl ends are connected by a hinge region, which consists of 10-12 amino acid residues, and is crucial for target interactions. S100A7 lacks the ability of Ca^{2+}-binding at the amino acid EF-hand motif, due to the absence of three amino acid residues and non-conserved substitution at position 12 of the EF-hand loop region (69). But S100A7 has a zinc-binding site composed of three histidines and an aspartate residue. The affinity of S100A7 for calcium is higher than that for zinc (70). Moreover, absence of zinc induces reorganization of the adjacent empty and distorted EF-hand loop in S100A7 structure, similar to a Ca^{2+}-binding EF-hand (71). Contrary to most S100 proteins, S100A7 binding to calcium does not cause significant conformational changes.

Although S100A7 is constitutively expressed at relatively low levels in normal keratinocytes, its expression levels are dramatically increased in psoriatic lesions (72), suggesting its key role in response to inflammatory stimuli (73, 74) and the pathogenesis of psoriasis (68, 75). The altered expression of S100A7 is associated with keratinocyte differentiation and poor prognosis of psoriasis. Moreover, psoriasis-related cytokines and chemokines can upregulate S100A7 expression in normal and pathological conditions (76–81). S100A7 can also be produced by circulating cells, possibly contributing to systemic inflammation and psoriasis-associated comorbidities (10).

S100A7 exerts many functions, depending on its interaction with specific target proteins. Early studies suggested that epidermal fatty acid binding protein (E-FABP) is a candidate interaction target protein of S100A7 (82, 83). S100A7 and E-FABP form a complex participating in focal adhesion-related functions. Moreover, the S100A7/E-FABP complex binds to oleic acid to regulate oleic acid metabolism and transport. The S100A7/E-FABP complex is also involved in lipid transport and metabolism during epidermal barrier formation, and modulation of cell differentiation and migration in some dermatoses such as psoriasis (31, 68, 84).

Ran-binding protein M (RanBPM), a nucleoporin component of the nuclear pore complex, is another potential binding partner for S100A7. Both yeast two-hybrid and co-immunoprecipitation assays show an interaction of S100A7 with RanBPM, suggesting the involvement of S100A7/RanBPM complex in nucleocytoplasmic transport (85). Because expression levels of both nucleoporins Ran-binding protein 2 (RanBP2) and Ran-GTPase-activating protein 1 (RanGAP1) on the nuclear envelope are upregulated in the epidermis of psoriatic lesion, adequate expression of these nuclear envelope proteins is likely a prerequisite for nucleocytoplasmic transport in keratinocytes of the psoriatic epidermis (86).

Interaction of S100A7 with c-Jun activation domain-binding protein 1 (Jab1), a multifunctional signaling molecule, increases the activity of nuclear factor-κB (NF-κB) and phosphor-Akt (87, 88). The effects of S100A7 on NF-κB and phosphor-Akt pathway are dependent on the Jab1-binding domain and the interaction with Jab1 because mutation at the Jab1 binding domain of S100A7 does not stimulate phosphor-Akt (88). Treatment of keratinocytes with S100A7 also increases expression levels of both mRNA and protein of transglutaminase I and III *via* activation of MAPK signaling pathway. Moreover, psoriasis-involved skin displays higher expression levels of transglutaminase and alteration in the interaction between S100A7 and transglutaminase (89).

S100A7 can activate a variety of intracellular signaling pathways, and many S100A7 functions depend on the receptor

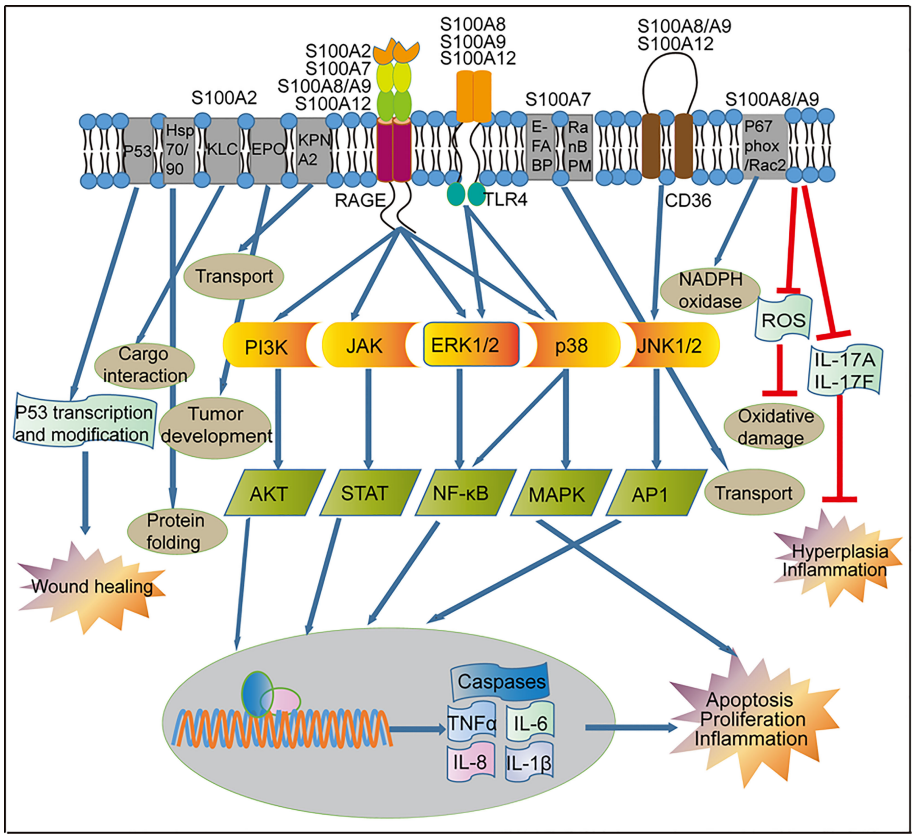

FIGURE 3
Regulation of signaling pathways by S100 proteins in psoriasis. Activation of RAGE by S100 proteins released from inflammatory cells can lead to the activation of the PI3K/AKT, JAK/STAT, and NF-κB pathways, which ultimately activate transcription of pro-inflammatory factors and other pathways that leads to proliferation and apoptosis. RAGE-mediated signaling can also activate the p38/MAPK signaling pathway in the immune cells, resulting in the up-regulated of genes involved in cell proliferation and inflammation. S100A8, S100A9 and S100A12 through the TLR4 receptor to activate NF-κB and MAPK. S100A8/A9 and S100A12 through the CD36 receptor activate the JNK1/2/AP1 signaling pathway and up-regulation of genes involved in cell inflammatory reactions. S100A2 and p53 can form a positive feedback loop to regulate the wound repair process in keratinocytes. S100A2 interacts with KPNA2 to form a cotransport complex, regulating nucleocytoplasmic transport in cancer cells, S100A2 interacts with Hsp70/ Hsp90-organizing protein or kinesin-light chain, modulates protein complex folding and KLC-cargo interaction in fibroblasts. In keratinocytes, S100A7 promotes transport *via* interaction with E-FABP or RanBPM. In addition, S100A8/A9 reduces skin hyperplasia by inhibiting the production of IL-17A and IL-17F in the mouse keratinocytes. KLC, Kinesin-light chain; EPO, Erythropoietin; KPNA2, nucleocytoplasmic transport protein karyopherin α; E-FABP, epidermal fatty acid binding protein; RanBPM, Ran-binding protein M; RAGE, receptor for advanced glycation end products; TLR4, toll-like receptor 4; CD36, cluster of differentiation 36; PI3K, phosphatidylinositol 3-kinases; JAK, Janus kinase; ERK1/2, extracellular signaling-related kinase 1/2; JNK1/2, c-Jun N-terminal kinase; AKT, AGC kinase family; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor kappa B, MAPK, mitogen-activated protein kinase; AP1, activator protein 1; TNF-α, tumor necrosis factor alpha; IL, interleukin.

for advanced glycation end products (RAGE) (90). Interaction of S100A7 with RAGE activates p38 MAPK (mitogen-activated protein kinase) and ERK (extracellular signal-regulated kinase) signaling pathways, leading to production of multiple inflammatory mediators involved in psoriasis, including IL-1α, IL-1β, IL-6, IL-8, TNF-α (91, 92) (Figure 3). S100A7 promotes cell proliferation and suppresses cell differentiation by inhibition of GATA3/Caspase14 (signal transducer and activator of transcription 3) signaling pathway (93, 94). Moreover, S100A7 can stimulate cell proliferation and angiogenesis through a RAGE-dependent up-regulation of endothelial growth factor (95). Recent study showed that lysine crotonylation at position 49 of S100A7 was suppressed in psoriatic lesions. Lysine crotonylation affects gene expression and epigenome, thereby affecting cell function and regulating immune responses (96, 97). Because of the important role of S100A7 in amplifying inflammatory process in psoriatic lesions, S100A7 becomes a potential diagnostic and therapeutic target for psoriasis.

S100A8/S100A9

S100A8 (MRP8) and S100A9 (MRP14) are Ca^{2+}- and Zn^{2+}-binding proteins, with damage-related molecular patterns (98). Human S100A8 and S100A9 are composed of 93 and 114 amino acid residues (Figures 1, 2B), respectively, and S100A9 has an isoform of 110 amino acid residues. The three-dimensional structure shows that both S100A8 and S100A9 have two helix-loop-helix motifs with charged amino acid residues (Figures 2A, B). The affinity of Ca^{2+}-binding in the C-terminal EF-hand loop is higher than that in the N-terminal. S100A8 lacks the ability of Ca^{2+}-

TABLE 1 Immune-mediated inflammatory skin diseases.

Disease	Type	Prevalence	Inducing factors	Distribution	Clinical Features
Psoriasis	Plaque, guttate, inverse, pustular, erythrodermic psoriasis	At any age, affecting 2-4% of the worldwide population	Ethnicity, genetics, environment	Scalp, elbows, knees, lumbosacral area, body folds	Scaling papules and plaques,well-circumscribed, circular, red papules or plaques with a grey or silvery-white, dry scale, relapsing
Atopic dermatitis	IgE-high, extrinsic, and IgE-normal, intrinsic subtypes	Affects approximately 5-20% of children and 1-3% of adults	Genetics, barrier dysfunction Abnormal immunity, Environment	Scalp, elbows, knees, body folds, head, face	Persistent itching, allergy, eczematous lesions, relapsing
Hidradenitis suppurativa	Stage I, stage II, stage III	Mainly in the young and middle-aged women, 1% of the general population	Tobacco smoking, obesity, psoriasis,	Axillary, inguinal, perineal, pubic anogenital regions	Deep-seated nodules, abscesses, fistulae, sinus tracts, lesion recurrence, fibrotic
Bullous diseases	Pemphigus and pemphigoid	Primarily the elderly, dramatically increasing incidence rates in recent decades	Genetics, drugs, viruses, environment	Limbs, buttocks, sacral region, neck, face, scalp	Flaccid blisters and erosions, erosions heal with crusting and scaling, pruritic eruption
vitiligo	Nonsegmental and segmental vitiligo	0.5-2% of the population worldwide	Genetic, metabolic, oxidative stress, Immune responses environment	Distal extremities, abdomen, trunk, scalp, face	Selective loss of melanocytes, nonscaly, chalky-white macules.

binding in the N-terminal EF-hand motif, due to the absence of Ca^{2+}-coordination necessary amino acid residues. S100A9 is different from other S100 proteins due to its long, very flexible C-terminal α-helix extension region (71).

S100A8 and S100A9 are predominantly expressed in myeloid cells (99, 100), epithelial cells (101), keratinocytes (102), and endothelial cells (103). S100A8 and S100A9 can form a stable homodimer or heterodimer by non-covalent bonding in the absence of calcium. S100A8 and S100A9 tend to form heterotetramers in the presence of calcium (104). The tetramer form is exposed to two high-affinity zinc ion binding sites (the crucial sites for functions) at the interface of the S100A8/S100A9 (calprotectin) complex (105, 106). S100A8 and S100A9 are the most abundant damage-associated molecular patterns in many inflammatory diseases. Expression levels of S100A8 and S100A9 are significantly elevated in psoriatic keratinocytes and leukocytes, and their expression levels correlate positively with the severity of psoriasis (107). The S100A8/S100A9 is a reliable biomarker for monitoring inflammatory disease activity (108–110).

S100A8/S100A9 complex plays a prominent role in regulation of inflammatory processes and immune response, including modulation of cell signaling transduction, differentiation, migration, apoptosis, regulation cytoskeleton, and so on (108, 111). S100A8/S100A9 complex promotes the polymerization of microtubules through direct interaction with tubulin, and modulates the cytoskeleton during the process of transendothelial migration (112). S100A8/S100A9 also stimulates neutrophil microbicidal activity by induction of reactive oxygen accumulation, resulting in activation of NADPH-oxidase (113). S100A8/S100A9-induced increase in NADPH-oxidase activity is *via* S100A9 transport of the cofactor arachidonic acid to the NADPH oxidase complex, and interaction of S100A8 with cytosolic *phox* proteins facilitating the enzyme assembly (114). Overexpression of S100A8/S100A9 induces cellular apoptosis (115), increases the production of pro-inflammatory cytokines and adhesion molecules, and enhances leukocyte adhesion and migration (116, 117). In addition, S100A8/S100A9 complex exerts bactericidal effects (118).

The role of S100A8 and S100A9 in the development of inflammation has been well demonstrated in murine models. For example, S100A8 deficiency induces a severe phenotype of psoriasis-like cutaneous inflammation, and rapid synchronous early resorption of the mouse embryo (119). Thus, S100A8 is an indispensable gene for mouse survival. Surprisingly, S100A9-deficient mice show slight decreases in the response of neutrophils to stimulation with chemoattractant (120) and slight aggravation of psoriasis symptoms compared with control mice (121), indicating functional differences between S100A8 and S100A9. In general, knock-out of the S100A8 or S100A9 leads to skin hyperplasia and aggravation of psoriatic symptoms in mouse models (121–123). S100A8 and S100A9 negatively regulate psoriatic skin inflammatory responses in mouse models. Combined with previous studies, it is suggested that S100A8 and S100A9 have both pro- and anti-inflammatory dualistic biological functions.

Several targets for S100A8/S100A9 have been identified, including Toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD 2). S100A8 interacts with TLR4-MD2 complex,

TABLE 2 S100 proteins in psoriasis: interacting partners and associated functions.

Proteins	Target proteins	Functions
S100A2	P53	Promotes p53 transcription and post-translation modification, regulates wound repair
	KPNA2	Regulating nucleocytoplasmic transport
	Hsp70/Hsp90-organizing protein	Modulating protein folding
	Kinesin-light chain	Promoting cargo transportation
	Erythropoietin	Involved in the development of tumors
	RAGE	Not reported
S100A7	E-FABP	Participating in focal adhesion-related functions, regulating material metabolism and transport
	RanBPM	Involved in nucleocytoplasmic transport
	Jab1	Activates NF-κB and AKT, modulating progression and survival
	RAGE	Activates p38 MAPK and ERK, stimulating cell proliferation and angiogenesis
S100A8/S100A9	Tubulin	Promotes polymerization of microtubules
	P67phox/Rac2	Activates NADPH-oxidase, stimulating neutrophil microbicidal activity
	TLR4	Enhancing inflammatory cascade response in cells
	RAGE	Activates NF-κB and MAPK, promoting cell growth
S100A12	RAGE	Activates NF-κB and ERK, regulating cell proliferation and inflammatory responses
	Phospholipid bilayers	Involved in monocytes migration
	TLR4	Modulating inflammatory response and migration of monocytes
	CD36	Promotes the interaction of S100A12 with CacyBP/SIP
S100A15	Gi protein-coupled receptor	Not reported

enhancing inflammatory response in target cells (122) (Figure 3). Activation of TLR4 signaling pathway *via* S100A8/S100A9 appears to be crucial for inflammatory cascade response and systemic autoimmunity (123). In addition, S100A8/S100A9 complex interacts with p67phox and Rac2, increasing NADPH oxidase activity (114). S100A8/S100A9 complex is also involved in transcellular eicosanoid metabolism *via* interaction with scavenger receptor CD36 (124). Moreover, S100A8/S100A9 complex binds to endothelial cells *via* the S100A9 subunit interacting chiefly with heparin and heparan sulfate proteoglycans (125), contributing to the immobilization of the myeloid cell-derived S100A8/S100A9 complex on endothelium in human inflammatory diseases.

A line of evidence suggests the involvement of NF-κB signaling pathway in the action of S100A8 and S100A9. First, S100A8/S100A9 promotes tumor cell growth through the activation of the NF-κB signaling pathway in a RAGE-dependent manner (126), while S100A8/S100A9 can interact with RAGE (127). Second, amplification of pro-inflammatory cytokine response in macrophages by S100A8/S100A9 complex is *via* activation of MAPK and NF-κB signaling (128). Third, oxidative and carbonyl stresses can induce production of CML-motified (N^{ϵ}-carboxymethyllysine) S100A8/S100A9. The latter binds to RAGE, leading to an increase in NF-κB-dependent pro-inflammatory gene expression, suggesting that CML-S100A8/S100A9 generated in inflammatory lesions can elicit a RAGE-dependent inflammatory response (129). Additionally, carboxylated glycans promotes the binding of S100A8/S100A9 to RAGE, resulting in activation of NF-κB signaling and cell proliferation (130) (Figure 3).

S100A12

S100A12, also named calgranulin C or EN-RAGE, is a pro-inflammatory protein, mainly expressed in keratinocytes of various inflammatory dermatoses (131, 132). In the psoriatic epidermis, S100A12 is expressed in the suprabasal epidermal layers (133), and its expression levels correlate positively with psoriasis severity (134). S100A12 has also been identified in neutrophils, macrophages, and lymphocytes, and functions as an innate immune defense against microorganisms. S100A12 can translocate from the cytosol to the membrane upon calcium activation.

S100A12 belongs to the S100 proteins family of EF-hand Ca^{2+}-binding proteins, and its nucleotide sequence is localized within the epidermal differentiation complex on human chromosome 1q21. The predicted size of S100A12 is 92 amino acid residues with a molecular weight of 10.6 kDa (135) (Figures 1, 2B). The crystal structure of S100A12 reveals that monomeric subunits have four α-helices and two EF-hand motifs linked by a hinge domain (136, 137) (Figures 2A, B). The N-terminal domain of the target-binding site has two residues, Glu 5 and Glu 9, that are the most highly conserved in S100 proteins (136).

The crystal structure of Zn^{2+} or Ca^{2+}/Cu^{2+}-bound S100A12 shows that Zn^{2+} and Cu^{2+} share the same binding site. The type of

ion binding to S100A12 determines its biological function (138). Binding of S100A12 to Cu^{2+} probably is essential in early immune reactions. Intracellular S100A12 is an anti-parallel homodimer form in the presence of zinc- and calcium- ions (138–142). Extracellular S100A12 is either a homodimer or hexamer form, with cytokine-like features.

Several mechanisms are involved in the action of S100A12. Interaction of S100A2 with target proteins regulates a variety of cellular processes and is linked to certain autoimmune responses (143). S100A12 recruits both mast cells and monocytes to inflammatory sites by its flexible hinge region during early inflammatory stages (144–146). Interaction of calcium-activated S100A12 with RAGE promotes cell proliferation and pro-inflammatory responses (147–149). The binding of S100A12 to the RAGE V domain increases cellular inflammatory response to oxidative stress (150) and participates in the pathogenesis of psoriasis (Figure 3). Moreover, S100A12 interacts with phospholipid bilayers by specific lipid and divalent ions in physiological responses (149), suggesting that it is involved in signaling events, such as migration of monocytes, located in the cell membrane (151). S100A12 binds more closely to negatively charged lipids in the presence of Ca^{2+} or Zn^{2+}, both of which can change the conformation and enhance the thermal stability of S100A12 protein, further enhancing protein-membrane interaction (152). Furthermore, interaction of S100A12 with TLR4 activates TLR4 signaling pathway (Figure 3), resulting in enhanced inflammatory response and migration of monocytes (153). Additionally, interaction of S100A12 with CacyBP/SIP (Calcyclin binding protein/Siah-1 interacting protein) is *via* the CD36 (a class B scavenger) receptor (154, 155). Notably, RAGE upregulates while TLR4 downregulates the expression levels of CD36 (156, 157).

S100A12 exhibits pro-inflammatory cytokine-like activities and antimicrobial activity (139, 158, 159). The antimicrobial activity of S100A12 is mainly attributed to its ability to chelate metal ions during the nutritional immunity process (160–163). The binding of S100A12 to TLR4 activates TLR4 signaling pathway, and increases the expression levels of pro-inflammatory cytokines (164, 165). Carboxylated glycans on the V-domain of RAGE enhances its binding to S100A12, subsequently activating NF-κB/ERK signaling pathway (164, 166) and upregulating pro-inflammatory molecules (165). Thus, interaction of S100A12 with its specific target proteins increases pro-inflammatory immune response and induces inflammation (143).

S100A15

S100A15, also called koebnerisin or S100A7A, is recently identified as a member of the S100 protein family, and its amino acid sequences are 93% identical to S100A7 (167), suggesting the similarity of functions between S100A15 and S100A7. S100A15 protein is predictively composed of 101/104 amino acid residues, in which 13-48 residues contain the N-terminal non-canonical EF-hand domain, and 50-85 residues contain the canonical C-terminal EF-hand motif (168) (Figure 1). Interestingly, S100A15 is different from other S100 proteins because of its lack of acidic amino acids and the increased basic amino acids in the C-terminal (167).

The S100A15 gene has an alternative splicing site and two mRNA isoforms, i.e., the long isoform S100A15-L (104 amino acids) and the short isoform S100A15-S (101 amino acids) with exon 1 being spliced out (10, 90, 169). S100A15-L and S100A15-S variants are differentially expressed in normal, non-lesional and lesional skin of psoriasis. More S100A15-L than S100A15-S isoform is expressed in the skin. In comparison to normal individuals, expression levels of S100A15 are upregulated in both the uninvolved and involved skin of psoriatic subjects, with a more prominent upregulation in the involved skin.

S100A15 is an antimicrobial peptide and can increase the production and secretion of immunotropic cytokines such as TNF-α, IL-6, and IL-8 in keratinocytes, leading to the development of cutaneous inflammation (21, 170). S100A15 directly acts as a chemoattractant, promoting the infiltration of inflammatory cells and amplifying the pro-inflammatory cascade in the skin. Moreover, S100A15 act as a danger-associated molecular pattern or an alarmin factor by priming immune cells to produce pro-inflammatory mediators (168).

In contrast to S100A7, S100A15 is not able to either bind to Jab1 or interact with RAGE to activate respective signaling pathways. But it can interact with an as-yet-unknown Gi protein-coupled receptor. So far, little is known about the roles of S100A15 in multiple pathways involved in cell proliferation, migration and inflammation.

Conclusions

The S100 protein family consists of a series of homologous proteins that are involved in a wide range of intracellular processes, including inflammatory responses. The expression levels of several S100 proteins, such as S100A2, S100A7, S100A8/S100A9, S100A12, and S100A15, are significantly up-regulated in psoriasis-involved skin. The S100 protein family interacts with specific target proteins to regulate cell proliferation, differentiation, migration, signal transduction, apoptosis, energy metastasis, and more (Table 2). In addition, S100 protein family plays an important pathogenic role in inflammatory diseases such as psoriasis, and can be used as a potential biomarker to estimate the prognosis and severity of diseases. However, whether the S100 protein family can be used as drug target in the management of psoriasis and possibly other inflammatory dermatoses remains to be explored.

Author contributions

HL: data curation, visualization, writing-original draft preparation and funding acquisition; JL: reviewing and editing, funding acquisition; KZ: supervision, funding acquisition, reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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

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Deciphering the contributions of cuproptosis in the development of hypertrophic scar using single-cell analysis and machine learning techniques

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Hypertrophic scar (HS) is a chronic inflammatory skin disease characterized by excessive deposition of extracellular matrix, but the exact mechanisms related to its formation remain unclear, making it difficult to treat. This study aimed to investigate the potential role of cuproptosis in the information of HS. To this end, we used single-cell sequencing and bulk transcriptome data, and screened for cuproptosis-related genes (CRGs) using differential gene analysis and machine learning algorithms (random forest and support vector machine). Through this process, we identified a group of genes, including ATP7A, ULK1, and MTF1, as novel therapeutic targets for HS. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to confirm the mRNA expression of ATP7A, ULK1, and MTF1 in both HS and normal skin (NS) tissues. We also constructed a diagnostic model for HS and analyzed the immune infiltration characteristics. Additionally, we used the expression profiles of CRGs to perform subgroup analysis of HS. We focused mainly on fibroblasts in the transcriptional profile at single-cell resolution. By calculating the cuproptosis activity of each fibroblast, we found that cuproptosis activity of normal skin fibroblasts increased, providing further insights into the pathogenesis of HS. We also analyzed the cell communication network and transcription factor regulatory network activity, and found the existence of a fibroblast-centered communication regulation network in HS, where cuproptosis activity in fibroblasts affects intercellular communication. Using transcription factor regulatory activity network analysis, we obtained highly active transcription factors, and correlation analysis with CRGs suggested that CRGs may serve as potential target genes for transcription factors. Overall, our study provides new insights into the pathophysiological mechanisms of HS, which may inspire new ideas for the diagnosis and treatment.

KEYWORDS

hypertrophic scar, cuproptosis, single-cell, machine learning, GEO

Introduction

Hypertrophic scar (HS) is a result from chronic inflammation in the reticular dermis and is an abnormal scar that forms as a result of improper wound healing (1). This condition occurs when there is an excessive production of collagen proteins in response to skin injuries such as burns or surgical incisions (2). HS is a skin lesion that results from excessive proliferation of newly formed connective tissue in the dermis or deeper tissues, where fibroblasts play a key role (3, 4). HS is usually limited to the boundaries of the original wound and may cause physical and psychological discomfort, including itching, pain, and functional loss (5). Overall, the formation of HS is a complex process involving multiple cell types and molecular mechanisms. Although various treatment options for HS exist, such as surgery, steroid injections, laser and skin grafting, there is currently no guaranteed cure. The current focus of research is to understand the molecular mechanisms of HS formation and to identify new therapeutic approaches for preventing or treating HS.

Excessive proliferation and activation of fibroblasts are characteristics of fibrosis, and promoting cell apoptosis appears to be an effective solution for preventing tissue scarring and reversing fibrosis that has already formed (6, 7). Ferroptosis, as another form of programmed cell death, has been shown to be involved in alleviating fibrotic diseases (8, 9). Cuproptosis is a newly discovered form of programmed cell death that is directly linked to the acylation of components of the tricarboxylic acid (TCA) cycle by copper, resulting in toxic protein stress and ultimately cell death (10). Cuproptosis has been shown to be potentially involved in alleviating pulmonary fibrosis, but its role in dermal fibroblasts has not yet been studied (11). Currently, little is known about the role of cuproptosis in HS.

The emergence of single-cell RNA sequencing (scRNA-seq) technology provides unprecedented molecular information and is the most important methodological advance and breakthrough, playing a critical role in exploring various disease mechanisms (12). In this study, through the integration of single-cell sequencing with bulk transcriptome sequencing, we investigated for the first time the relationship between the occurrence and development of HS and cuproptosis, deepening our understanding of the underlying mechanisms of HS and providing new emerging potential therapeutic strategies and research foundations for inhibiting scar formation.

Materials and methods

Data acquisition

The Gene Expression Omnibus (GEO) is a database created by the NCBI that stores a large amount of gene expression data. We obtained the dataset GSE181540 from GEO (https://www.ncbi.nlm.nih.gov/geo/), which includes 3 HS and 3 NS tissues, as well as 6 NS tissues from GSE158395, 3 NS tissues from GSE190626, and 3 HS tissues from GSE210434. In addition, we obtained dataset OEP002674 from The National Omics Data Encyclopedia (NODE, https://www.biosino.org/), which includes 5 HS and 5 NS samples. Based on previous studies on cuproptosis-related genes (CRGs), we identified a set of 56 genes related to cuproptosis (10, 13–15).

Data processing and differential gene expression analysis

We annotated, normalized, and performed log2 transformation on different datasets using R. Then, we used the "sva" R package to merge the aforementioned datasets, correct batch effects, and extract CRGs from the merged dataset. We performed differential gene analysis on the processed dataset using the "limma" package and screened for differentially expressed genes. The threshold was set to $|\log2 \text{ Fold change (FC)}| > 1$ and p-value <0.05.

Functional and pathway enrichment analysis

We performed Gene Ontology (GO) enrichment analysis on the genes from three aspects: biological process, cellular component, and molecular function, in order to identify the biological functions of the genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a primary public database related to pathways. We used the hypergeometric test to identify significantly enriched pathways in the genes. We performed functional and pathway enrichment analysis on the differentially expressed genes using these two methods, and set the P-value threshold to 0.05.

Machine learning

We used the "randomForest" R package to perform Random Forest (RF) feature selection on the dataset. The RF algorithm is an ensemble learning method that uses multiple decision trees to form a regressor. It is used to screen for the importance of genes in diseases. Its main advantages are its good performance in processing high-dimensional data and insensitivity to outliers. Then, we used the "e1071" R package to perform support vector machine-recursive feature elimination (SVM-RFE) feature selection on the gene set. SVM-RFE scores and ranks gene features using binary classification and selects the top few genes with the lowest error rate. We took the intersection of the results from these two machine learning methods and the differentially expressed genes that we previously screened for, and used the 11 intersected genes as hub genes for further analysis.

Differential validation of hub genes in external dataset

We selected 5 HS samples and 3 NS samples from the dataset GSE188952 in GEO to perform external differential expression validation of the hub genes. We calculate the differences and significance of the genes between the disease group and the

control group, and plotted the gene differential boxplot. We selected the genes that were validated and further analyzed them.

Construction and evaluation of diagnostic model

The three selected genes were subjected to multivariable logistic regression modeling. Using the generalized linear model with binary classification, the entire dataset was used as the training set to construct the model. ROC curve was plotted and the area under the curve (AUC) was calculated. Finally, bootstrap validation was performed with 1000 resampling iterations to calculate the sensitivity and specificity of the model. Further evaluation of the stability and reliability of the model was conducted through construction of nomogram and decision curves.

Immune infiltration and inflammation factor analysis

Previous studies have demonstrated a significant association between cuproptosis and cellular immunity (16–18). In this study, we performed a Pearson correlation analysis using the corrplot R package to investigate the relationship between the mRNA expression of the 3 CRGs. Additionally, we used the "IOBR" R package to apply the CIBERSORT and MCPcounter algorithms for quantifying the degree of immune cell infiltration in the samples (19). We also analyzed the correlation between the 3 CRGs and immune cell infiltration as well as the expression of inflammatory factors.

Consensus clustering analysis

We utilized the "ConsensusClusterPlus" R package to perform a k-means consensus clustering analysis, aiming to identify distinct subtypes that are associated with the expression of CRGs.

Functional enrichment analysis between two subtypes

The differentially expressed genes(|log2 FC|>1, p-value<0.05) identified between two subtypes was used for GO biological function enrichment analysis and KEGG pathway enrichment analysis by using R package "clusterprofiler" and "enrichplot"

Single-cell RNA statistical processing

Single-cell sequencing dataset analysis has been used to explore a variety of skin diseases with promising results (20–22). In our study, we used the "Seurat" R package to create a Seurat object and normalize and scale cells. By excluding cells with less than 200 expressed genes, more than 2500 expressed genes, or mitochondrial gene content higher than 10%, we retained 45095 cells. We selected the top 3000 highly variable genes for principal component analysis with a setting of 15 principal components for subsequent dimensionality reduction and clustering. We integrated samples and removed batch effects using the "harmony" function, obtained unsupervised cell clusters using a graph-based clustering method (with a resolution of 0.5), and visualized them using t-SNE plots. We used the "FindAllMarkers" function and Wilcoxon rank-sum test algorithm to identify marker genes for each cell cluster, with conditions including |log2 FC|>0.25, P<0.05, and a minimum percentage>0.1. To better identify fibroblast clusters, we selected clusters of fibroblast cell types for further t-SNE analysis, graph-based clustering, and marker gene analysis. We calculated the score of the cuproptosis in each cell using the "AddModuleScore" function and divided cells into high and low groups based on the median score.

Cell-cell communication analysis

We used the recently developed tool "CellChat" R package to perform comprehensive analysis of cell-cell communication molecules. This tool can generate and plot the probability and interaction strength of cell-cell communication from single-cell transcriptome data, allowing for in-depth analysis of cell-cell communication. The normalized count and cell types by Seurat were used for this analysis.

Scenic transcription factor regulatory network analysis

The SCENIC transcription factor inference was performed using the R SCENIC package following the recommended pipeline steps (23).

Patient and ethical declaration

We collected 3 NS tissues and 3 HS tissues. Prior to surgery, we provided patients with a detailed explanation of the research purpose and process, and obtained their consent to participate in the study. We also obtained written informed consent from all patients or their legal guardians and received approval from the Ethics Committee of the Xijing Hospital, Fourth Military Medical University.

Real-time quantitative polymerase chain reaction

The tissues were subjected to RNA extraction using the TRIzol reagent (Invitrogen, Camarillo, CA, United States). The extracted RNA was then reverse transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Remover (Takara, Shiga, Japan) at a concentration of 1,000 ng. For qRT-PCR, the TB Green Premix Ex Taq II (Takara, Shiga, Japan) was utilized, and the BIO-RAD CFX

Connect Real-Time System (Bio-Rad, Munich, Germany) was used for the analysis. The expression levels of target genes were normalized to GAPDH. Forward 5′-GCACCGTCAA GCTGAGAAC-3′ and reverse 5′-TGGTGAAGACGC CAGTGGA-3′ for human GAPDH, forward 5′-GGCAAG TTCGAGTTCTCCCG-3′ and reverse 5′-CGACCTCCAA ATCGTGCTTCT-3′ for human ULK1, forward 5′-CAGTGC GGAGAACACTTGC-3′ and reverse 5′-TGCACATAA CCCTGGGACATT-3′ for human MTF1, forward 5′-TGGG ACCATATCCAAAGCACA-3′ and reverse 5′-CAAGCTCC TGACTTTTTCTCCAG-3′ for human ATP7A.

Results

Differential gene expression analysis

The research process of this study can be seen from the graphic flow chart (Figure 1). The "Limma" R package was used to identify differentially expressed CRGs in the dataset. The filtering criteria were set as |log2 FC| > 1 and p-value <0.05, and boxplots and volcano plots of the differentially expressed genes were generated (Figures 2A, B). Figure 2C shows the correlation between differentially expressed genes. Among the 56 CRGs, 15 genes were found to be significantly differentially expressed, including 14 downregulated genes and 1 upregulated gene.

Enrichment analysis of differentially expressed genes

The differentially expressed genes were subjected to enrichment analysis using the GO and KEGG methods, and enrichment circle plots were generated for both (Figures 2D, E). In GO analysis, the genes were mainly enriched in cellular copper ion homeostasis, copper ion transport, and copper ion homeostasis, while in KEGG analysis, the genes were mainly enriched in the HIF-1 signaling pathway, VEGF signaling pathway, and Mineral absorption pathways.

Machine learning-based selection of hub genes

Machine learning methods are widely used in dermatological diseases (24–26). The SVM-RFE and RF machine learning methods were used to further screen candidate hub genes. First, the RF algorithm was used to rank the importance of each gene (Figures 3A, B), and the top 20 genes with the highest importance ranking were selected: ULK1, LOXL2, VEGFA, MAP2K1, ATP7A, PDE3B, LIAS, DBT, CDKN2A, PDHA1, COA6, COX11, SLC25A3, DLAT, SOD1, AOC3, MTF1, TYR, DBH, ATOX1. Then, the top 20 genes with the highest accuracy in the SVM-RFE results were selected: LOXL2, ULK1, DBT, PDE3B, LIAS, COX11, TYR, CCS, ATP7A, ATOX1, VEGFA, DLAT, SCO1, PDK1, MAP2K1,

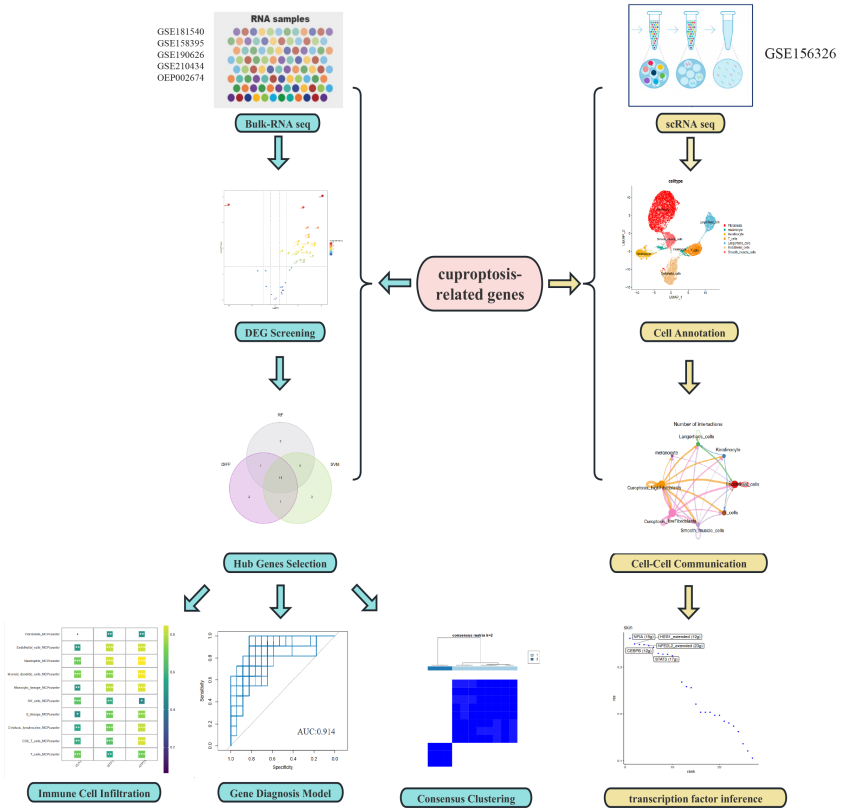

FIGURE 1
Flowchart of the study.

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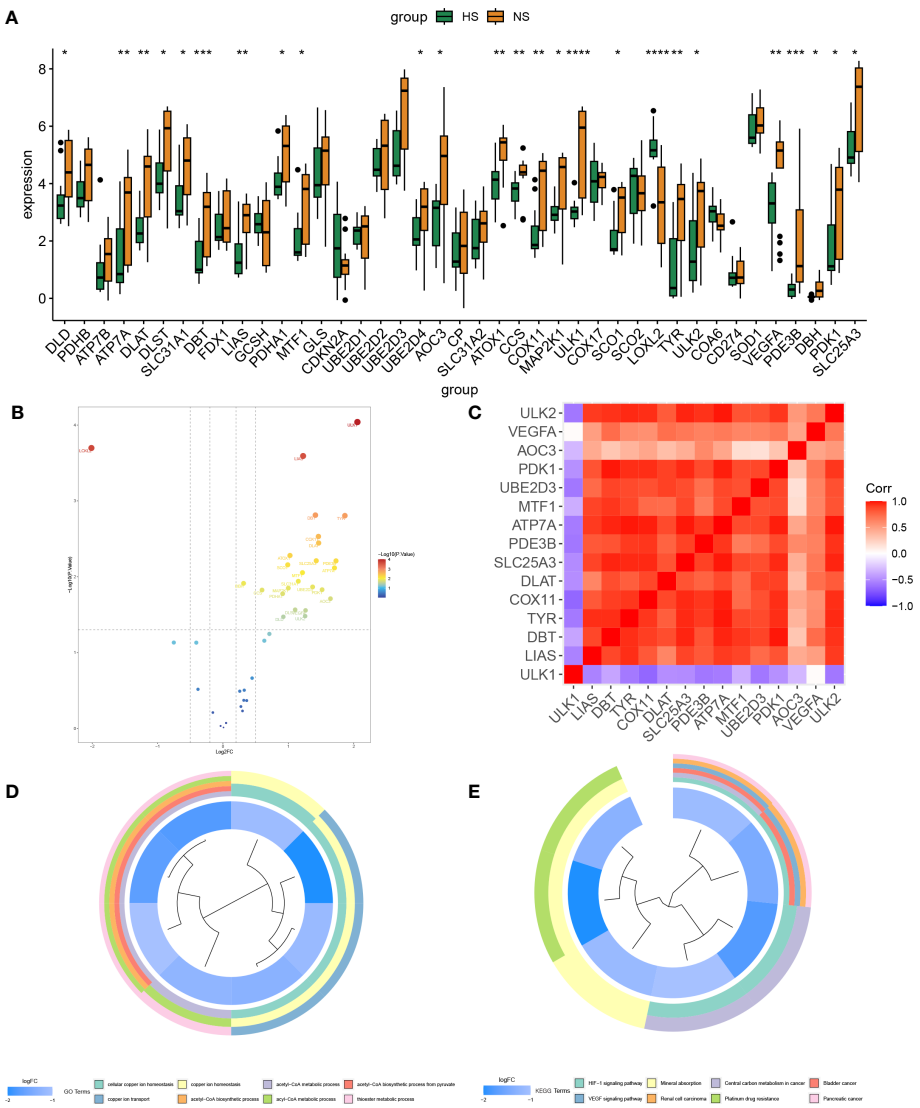

FIGURE 2
Screening and correlation analysis of differentially expressed genes and their functional enrichment analysis. **(A, B)** Differential box plot and volcano plot of CRGs in HS. **(C)** Heat map of correlation between differentially expressed genes. **(D, E)** GO, KEGG enrichment analysis of differentially expressed genes, different colors represent various significant pathways and related enriched genes. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

SLC25A3, PDHA1, DLST, DBH, MTF1 (Figure 3C). The intersection of the two machine learning methods' results was taken as the candidate hub genes for further analysis.

Differential gene validation using external dataset

The candidate hub genes selected using machine learning and the 15 significantly differentially expressed genes obtained from differential analysis were intersected to obtain 11 genes (Figure 3D): MTF1, ULK1, ATP7A, TYR, DBT, SLC25A3, LIAS, COX11, DLAT, VEGFA, PDE3B. The external dataset GSE188952 was used for validation of differential gene expression (Figure 3E), and 3 CRGs with significant differential expression in the external dataset were identified: MTF1, ULK1, ATP7A.

Validation of the expression of three cuproptosis-related mRNA in HS and NS

To further verify the expression levels of the three CRGs, we collected specimens from 3 patients with NS and 3 HS with patients. The mRNA expression level of MTF1, ULK1 and ATP7A in NS was significantly higher than that in HS, which also consistent with the expression of bulk RNA-seq datasets (Figure 3F).

Establishment and evaluation of the diagnostic model

Firstly, a correlation graph between 3 CRGs was drawn (Figure 4A), and ROC curve was plotted to evaluate the diagnostic specificity and sensitivity of each gene, and the area

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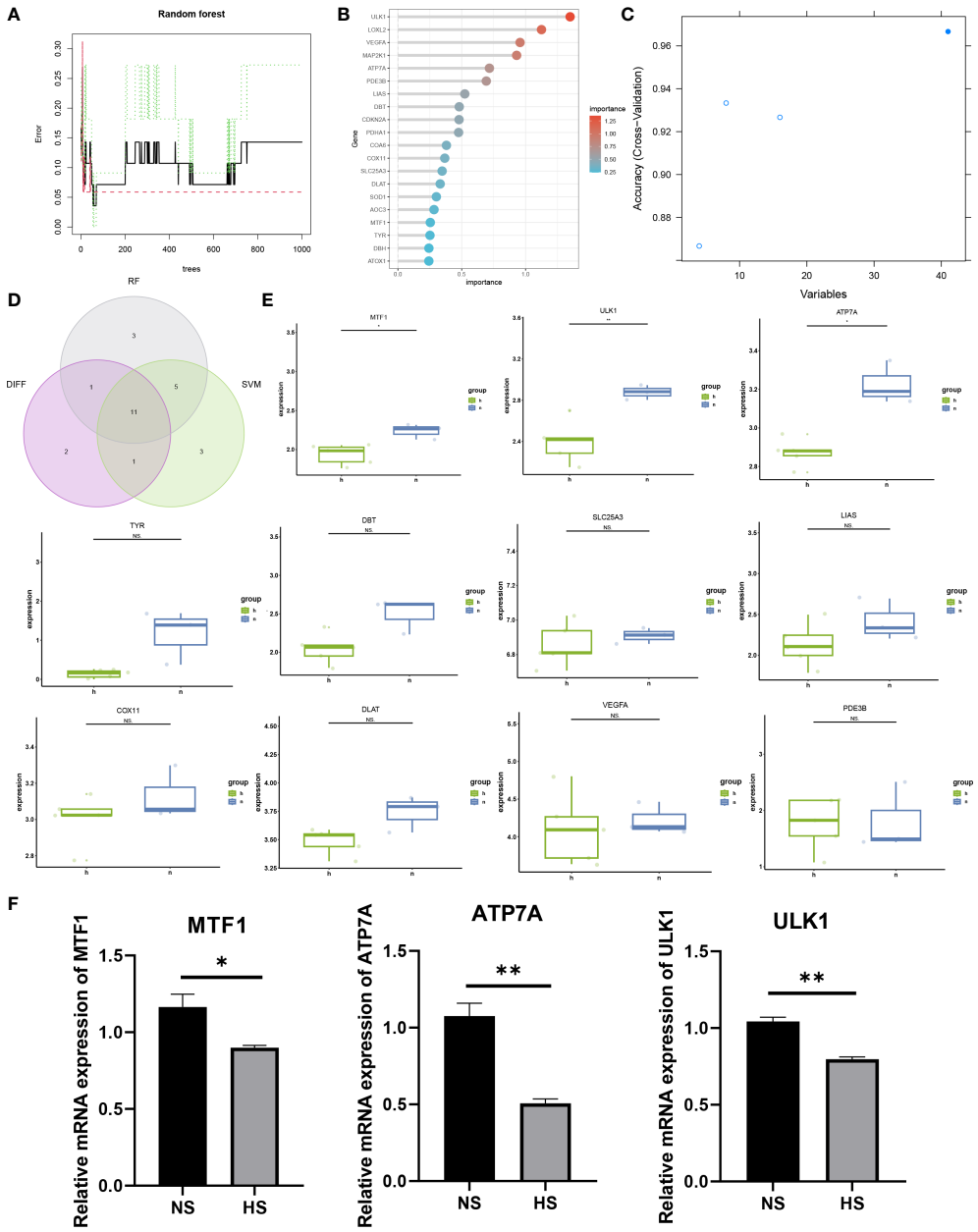

FIGURE 3
Screening of candidate hub genes and validation of differential expression. **(A, B)** Ranking the importance of genes using the random forest algorithm. **(C)** SVM-REF algorithm selects genes with the highest accuracy. **(D)** The intersection genes of the top 20 genes of importance in the RF algorithm, the top 20 genes with the highest accuracy in the SVM-REF algorithm, and differentially expressed genes. **(E)** Validation of differential expression of candidate hub genes in external dataset. **(F)** Differential expression validation using qRT-PCR for candidate hub genes. (*p < 0.05, **p < 0.01 and ns = not significant).

under the curve (AUC) was calculated. ATPA (AUC=0.79), ULK1 (AUC=0.92), and MTF1 (AUC=0.76) were obtained (Figure 4B). We performed multivariable logistic regression modeling on three hub genes, and the model was evaluated using ROC curve (Figure 4C). The AUC was calculated to be 0.914. Further verification was performed using the bootstrap algorithm with R=1000 resampling times, and the average AUC value was0.879893, with a 95% confidence interval (CI) of 0.7303-0.9189 (Figure 4D). These results indicate that the diagnostic model has high diagnostic value. A nomogram of the model was drawn, and the total score of the sample was calculated (Figure 4E). Finally, the Decision Curve Analysis (DCA) curve was plotted (Figure 4F), and it showed that the diagnostic model composed of MTF1, ULK1, and ATP7A had the highest diagnostic performance.

Immune infiltration and inflammatory factor analysis

The "IOBR" package in R was used to conduct MCPcounter and CIBERSORT immune infiltration analysis on the 3 CRGs

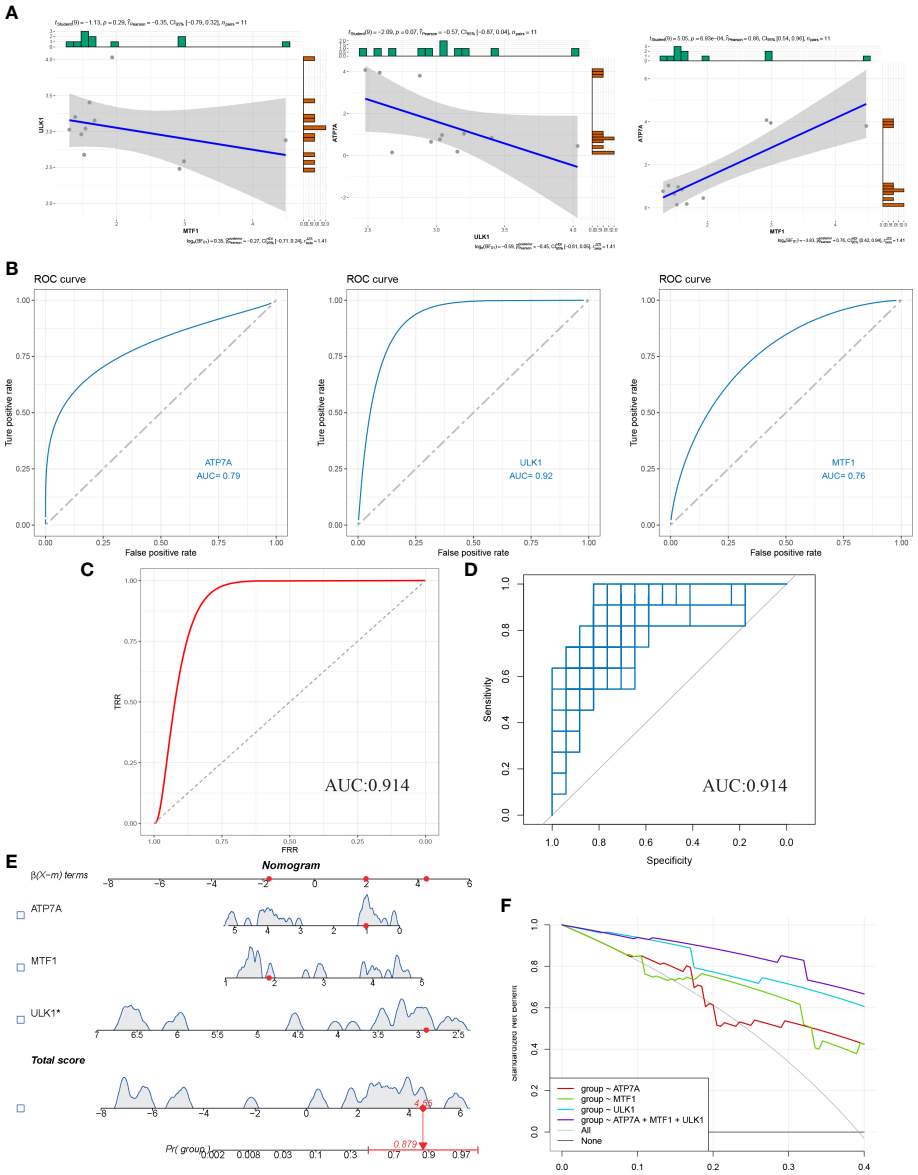

FIGURE 4
Diagnostic model of HS was constructed and evaluated. **(A)** Correlation analysis of three candidate hub genes. **(B)** Nomogram of candidate hub genes in the hyperplastic scar dataset and the area under the curve (AUC). **(C)** ROC curve and AUC using SVM-REF and DEGs diagnostic models. **(D)** Bootstrap resampling algorithm to verify the model. **(E)** Prediction of hypertrophic scarring using nomogram. **(F)** The decision curve evaluates the predictive performance of the model.

(Figures 5A, B). In MCPcounter, almost all immune cells showed a significant positive correlation with 3 CRGs, which were upregulated in the NS group. In CIBERSORT, dendritic cells resting showed a significant positive correlation with CRGs, T regulatory cells showed a positive correlation with ULK1 and ATP7A, and macrophages M0 showed a negative correlation with MTF1 and ATP7A. In addition, we generated correlation plots of the top 5 associations between CRGs and the corresponding immune cells using the CIBERSORT and MCPcounter algorithms (Figure 5C). In the analysis of inflammatory factors (Figure 5D), IL5, TNF, IL7, PDGFA, CD4, CSF1, HLA-DRB5, HLA-DRB1, HLA-DRA, and other factors showed significant positive correlations with 3 CRGs, while CSF3, IL6, and other factors showed significant negative correlations. Through the analysis of immune infiltration and inflammatory factors, it can be concluded that the CRGs have an extremely important influence on the immune and inflammatory reactions in the development of the disease.

Consensus clustering analysis of HS

We performed consensus clustering analysis to group the dataset of HS, setting the maximum number of clusters to 5, and scoring the 2-5 clusters (Figures 6A, B). The results showed that when the number of clusters was k=2, the inter-cluster correlation

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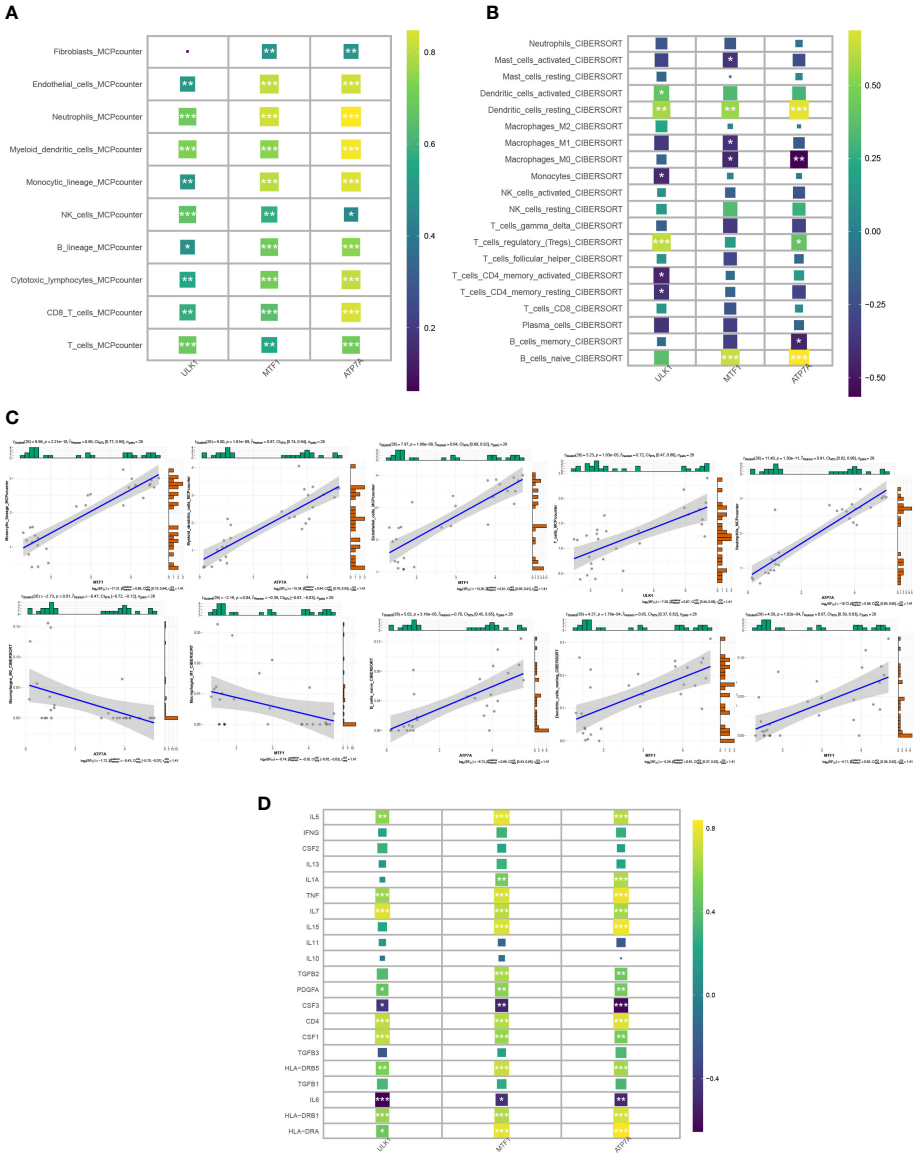

FIGURE 5
Correlation of candidate genes with immune cell infiltration. **(A, B)** Heat map of the correlation between 3 CRGs and immune cell infiltration in the MCPcounter and Cibersort algorithms. **(C)** Top 5 correlation plots in Cibersort and MCPcounter. **(D)** Heat map of correlation between candidate genes and inflammatory factors.

was the lowest and the intra-cluster correlation was the highest, and the score was the highest. Therefore, we chose k=2 to group the dataset of HS and analyzed the gene expression of the two groups, and generated heatmaps and differential boxplots (Figures 6C, D). Finally, we further analyzed the expression differences of inflammatory factors and immune cells in the two groups (Figures 6E, F).

Functional and pathway enrichment analysis

To investigate functional and pathway disparities between subtypes and unravel potential mechanisms of disease progression, we conducted GO and KEGG enrichment analysis using the differentially expressed genes identified between the two subtypes. In the BP category, the genes were mainly enriched in functions such as epidermis development, skin development, axon development, and regulation of neuron projection development. In the CC category, the genes were mainly enriched in functions such as cell-substrate junction, cell leading edge, focal adhesion, and chromosomal region. In the MF category, the genes were mainly enriched in functions such as GTPase regulator activity, nucleoside-triphosphatase regulator activity, and cadherin binding (Figures 7A, B). In the KEGG analysis, the genes were mainly enriched in metabolic pathways such as Human papillomavirus infection, Rap1 signaling pathway, Ras signaling pathway, and Focal adhesion (Figures 7C, D).

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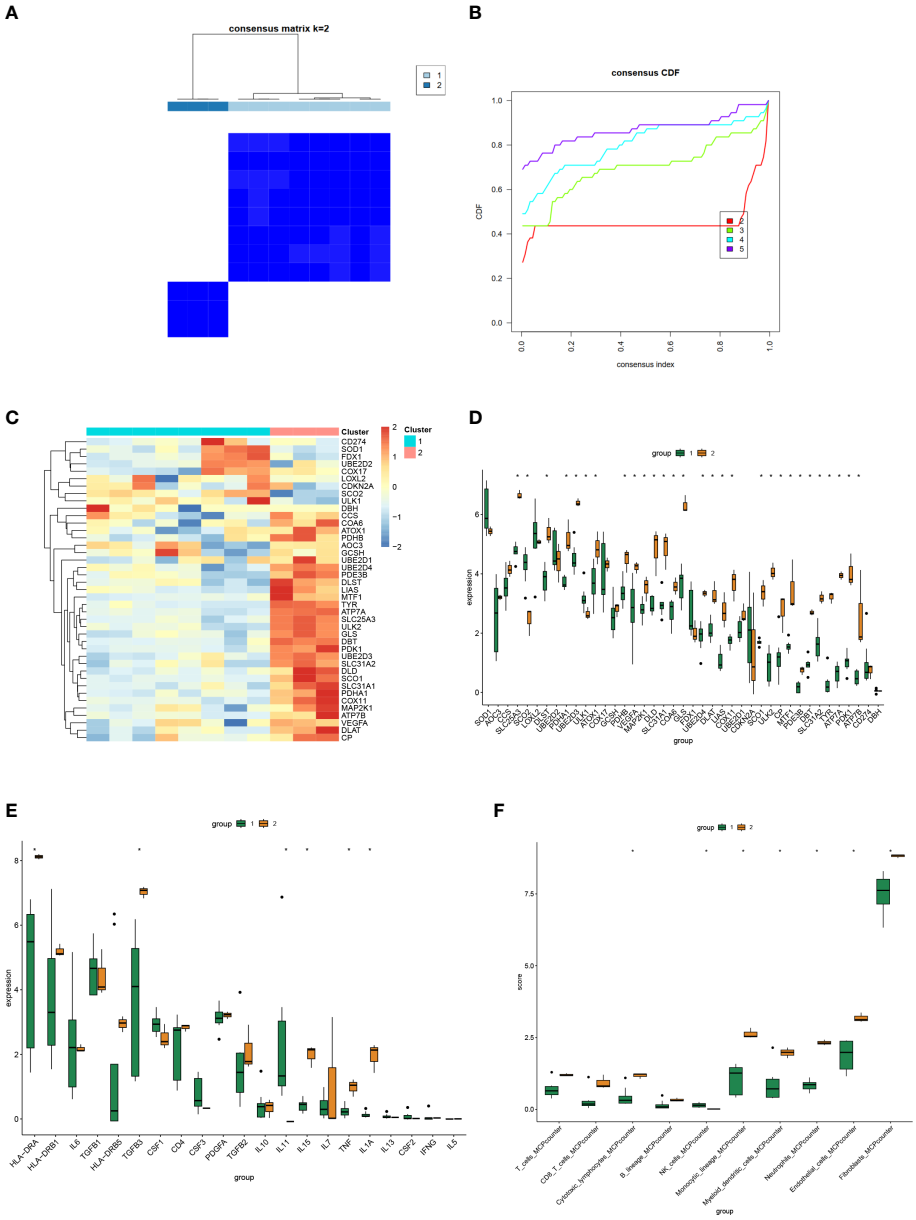

FIGURE 6
Consensus Clustering analysis of HS. **(A)** The circular manhattan (CM) plot exhibited the cluster at k = 2. **(B)** The area change under the empirical cumulative distribution function (CDF) curve when k=2-5. **(C, D)** Heat map and box plot of the expression distribution of Cuproptosis-related genes among different clusters. **(E, F)** Box plots of expression distribution of inflammatory factors and immune cell infiltration among different clusters. (*P < 0.05).

Analysis of high cellular heterogeneity in human HS tissue through single-cell RNA-seq profiling

We utilized scRNA-seq datasets containing 3 NS and 3 HS samples from the GEO database to unveil the intrinsic cellular heterogeneity within the skin tissue. After strict quality control, low-quality cells were excluded, and a total of 45095 cells were selected for subsequent analysis. Using dimensionality reduction clustering, we identified 16 cell clusters, which displayed high heterogeneity between different cell populations (Figure 8A). Based on marker genes identified in previous studies, we annotated 8 cell types, including Melanocytes (cluster 14, marked by MITF), Langerhans cells (clusters 8, 9, marked by Langerhans cells), T cells (clusters 7, 12, marked by PTPRC), Sweat gland cells (cluster 18, marked by SCGB1B2P, SCGB1D2), Fibroblasts (clusters 0, 2, 4, 5, 10, 15, marked by FBLN1, COL1A1, APOE, and APCDD1), Keratinocytes (clusters 11, 13, marked by KRT1 and KRT14), Smooth muscle cells (cluster 3, marked by ACTA2 and RGS5), and Endothelial cells (clusters 1, 6, marked by THBD and SELE) (Figure 8B). We then explored the TOP5 essential genes in seven previously annotated cell clusters and visualized the results (Figure 8C). Figure 8D also shows the proportion of fibroblasts in NS and HS. Further analysis of fibroblasts was performed by extracting them and reanalyzing them using tSNE and UMAP, which resulted in their re-clustering into 8 clusters. We scored the

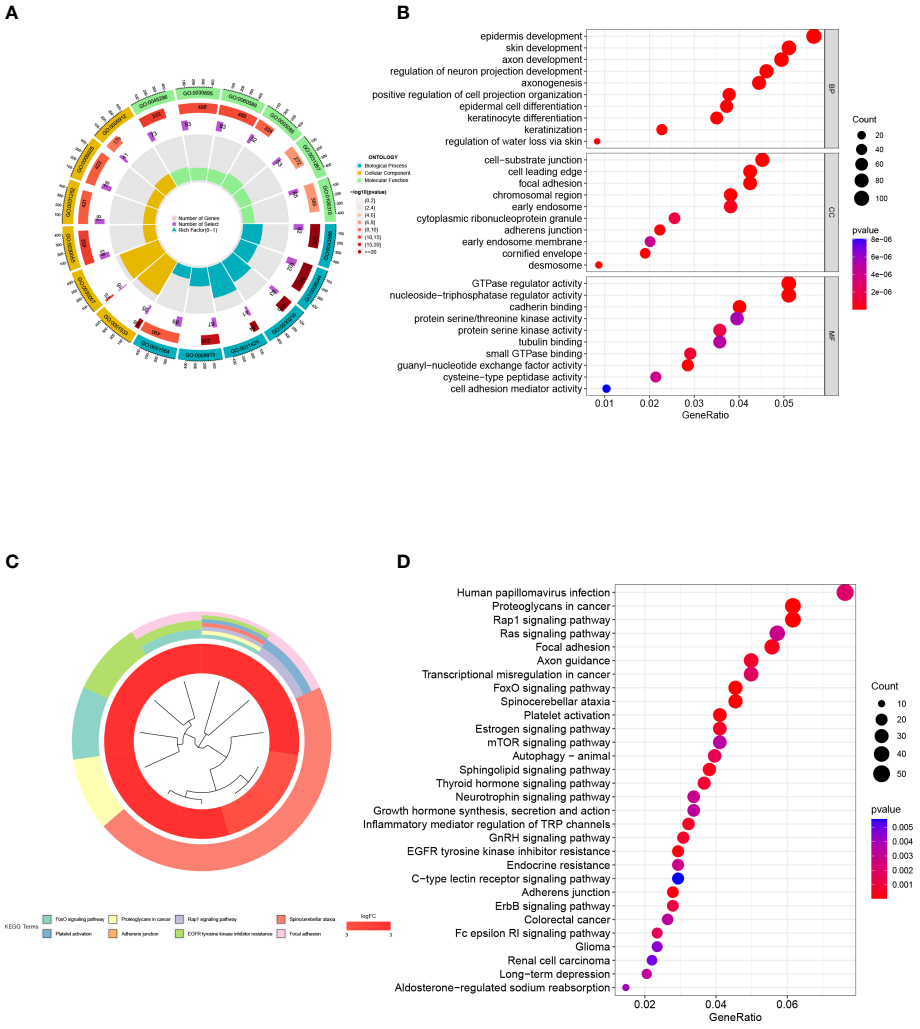

FIGURE 7
Functional enrichment analysis. **(A, B)** GO enrichment analysis of inter-subtype DEGs. **(C, D)** KEGG enrichment analysis of inter-subtype DEGs.

activity of cuproptosis in each cell using the AddModuleScore function, and found that the cuproptosis score of fibroblasts in NS was higher than that in HS (Figure 8E). Specifically, clusters 1 and 6 had the highest activity of cuproptosis (Figure 8F).

Cell-cell communication

To decipher cell-cell signaling, we performed cell-cell communication analysis between different cell types using the "cellchat" R package. We classified fibroblasts into two types based on their previous cuproptosis activity scores, dividing them into high and low cuproptosis activity groups using the median value. Aggregated cell-cell communication networks were constructed based on interaction numbers (Figure 9A) and interaction weights (Figure 9B). The interaction strengths of cells transmitting and receiving signals were plotted in Figure 9C, which showed that fibroblasts played a crucial role as information senders in cell-cell communication. Low cuproptosis activity fibroblasts had weaker signal transmission strengths than high cuproptosis activity fibroblasts. We compared the interactions between the two types of fibroblasts and other cells. Compared to fibroblasts with low pathway activity, those with high pathway activity can engage in additional cell communication through the MIF pathway and with T cells and Langerhans cells. Additionally, they can communicate with smooth muscle cells via PROS1-AXL interactions.

SCENIC transcription factor activity prediction and correlation analysis with CRGs

Figures 10A, B show the top 5 transcription factors with the highest activity, including NFIA, HES1, CEBPB, NFE2L2, and STAT3 in NS and CREB3L1, FOSB, JUND, KLF6, and KLF4 in HS. Figure 10C displays the differential expression of transcription factors between the two groups. We further analyzed the correlation between these transcription factors and CRGs at the bulk transcriptome level, and found that REL, TGIF1, NFIA, IRF1, BCLAF1, NFE2L2, STAT3, KLF9, MAFF, NFIC, MAFB, KLF10,

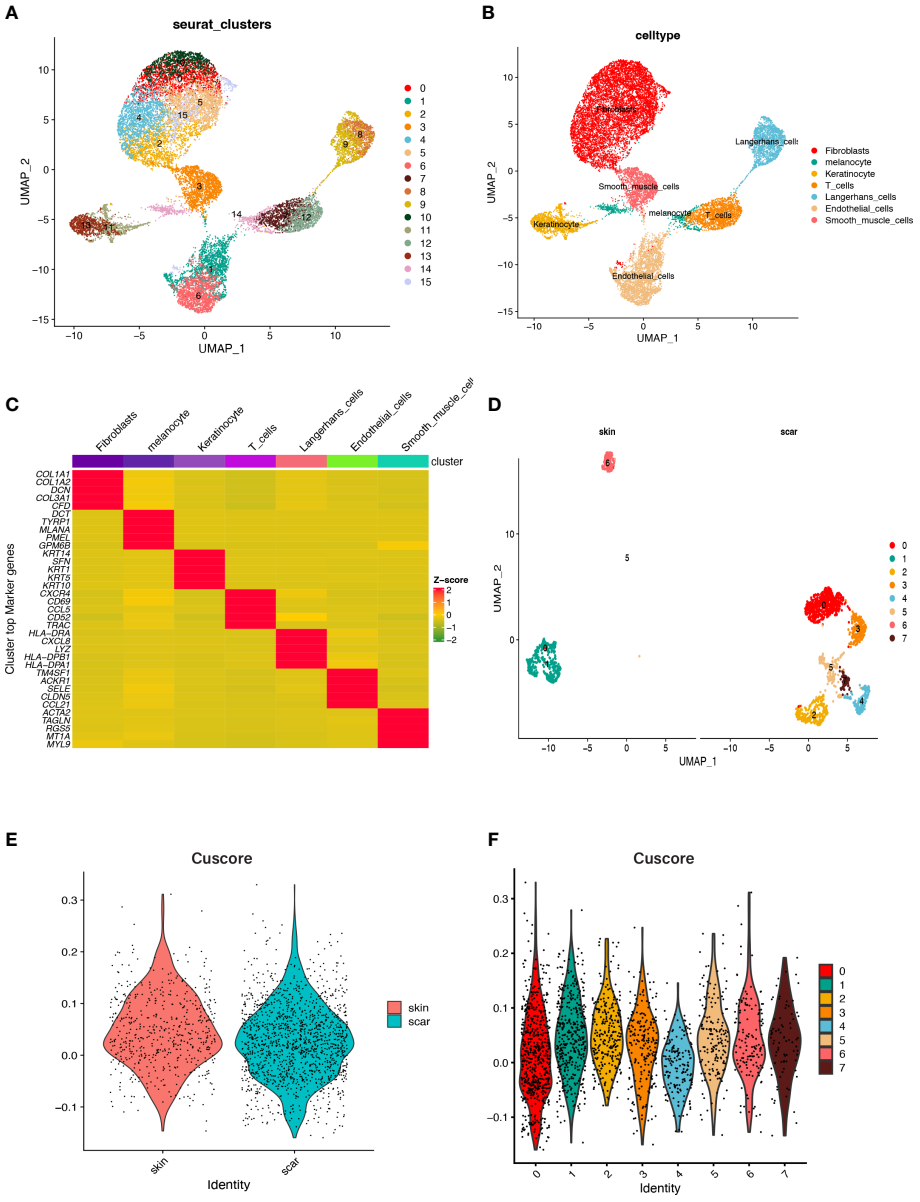

FIGURE 8
Cell populations and marker genes in HS and NS. **(A)** The cell clusters visualized by the dimensional reduction of t-SNE. **(B)** Cell subgroup annotation results. **(C)** A heatmap to display the TOP5 significant genes associated with each cell cluster, following differential analysis aimed at identifying marker genes. **(D)** Fibroblasts subpopulation clustering and dimensionality reduction. **(E)** The cuproptosis score of fibroblasts in HS and NS. **(F)** The cuproptosis score of the fibroblast subpopulations that were downscaled again.

KLF6, CEBPB, KLF4, and other transcription factors are positively correlated with most CRGs, suggesting a potential network of positive regulation. The remaining transcription factors are negatively correlated with most CRGs.

Discussion

HS is a fibrous proliferative skin disease characterized by excessive deposition of extracellular matrix (ECM) and is also a chronic inflammatory disease. Its treatment, including laser therapy, surgery, and cryotherapy, is often challenging and there is no universally effective treatment (27). Early diagnosis and timely and effective targeted interventions are important, so there is a need to identify novel biomarkers that inhibit proliferation and reduce proliferative scar formation. Copper can cause aggregation of lipid acylated proteins and loss of iron-sulphur (Fe-S) cluster proteins by directly binding to the lipid acylated components of the tricarboxylic acid (TCA) cycle and increasing protein hydrotoxic stress, ultimately leading to cell death. This novel form of cell death is quite distinct from known forms of cell death (e.g. scorch death, apoptosis, ferroptosis and necroptosis) and is defined as Cuproptosis (10). The involvement of cuproptosis in the progression of fibrotic diseases (e.g. pulmonary fibrosis, myocardial fibrosis, oral mucosal fibrosis, etc.) has been demonstrated and is considered as a potential therapeutic strategy (11, 28, 29). However, the role of cuproptosis in skin fibrotic diseases has not been studied. Our

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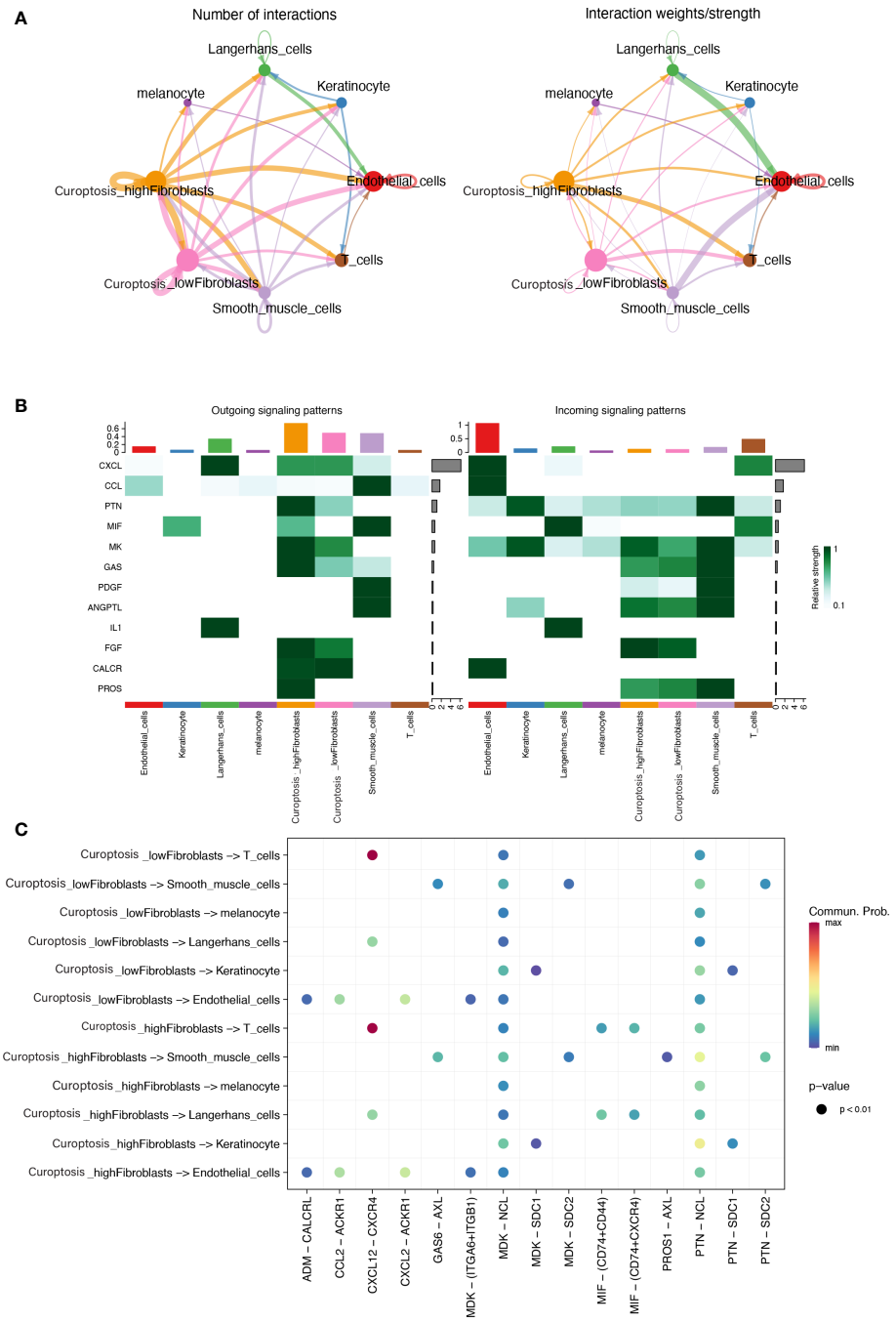

FIGURE 9
(A) An integrated cell-cell communication network mapped by the number and weight of interactions. **(B)** A visualization showing the relative strength of interactions, in terms of incoming and outgoing signals, across 8 different types of cells **(C)** A dot plot illustrating the signal pathways of incoming and outgoing interactions in two distinct subtypes of fibroblasts.

study comprehensively analyzed the role of cuproptosis in the occurrence and development of HS for the first time through joint bulk and single-cell transcriptomics. We constructed a CRG diagnostic model for HS using machine learning and classified HS according to the cuproptosis pathway. We investigated the role of cuproptosis in cell differentiation, communication, and transcription factor regulation in fibroblasts.

In this study, 3 CRGs (ATP7A, ULK1, MTF1) were screened by differential gene analysis and machine learning algorithms (RF and SVM). ATP7A acts as a copper transporter protein and provides energy for copper transport. When intracellular copper levels are low, ATP7A recycles TGN and transports copper from the cytoplasm to the Golgi apparatus. Induction of ATP7A has been shown to cause renal fibrosis (30).MiRNA-1297 inhibits myocardial fibrosis by downregulating ULK1 (31),and short-chain fatty acids attenuate renal fibrosis via the HDAC2/ULK1 axis in metallotranscription factor-1 (MTF-1) has been shown to promote fibrosis (32). Saffronin ameliorates liver fibrosis by

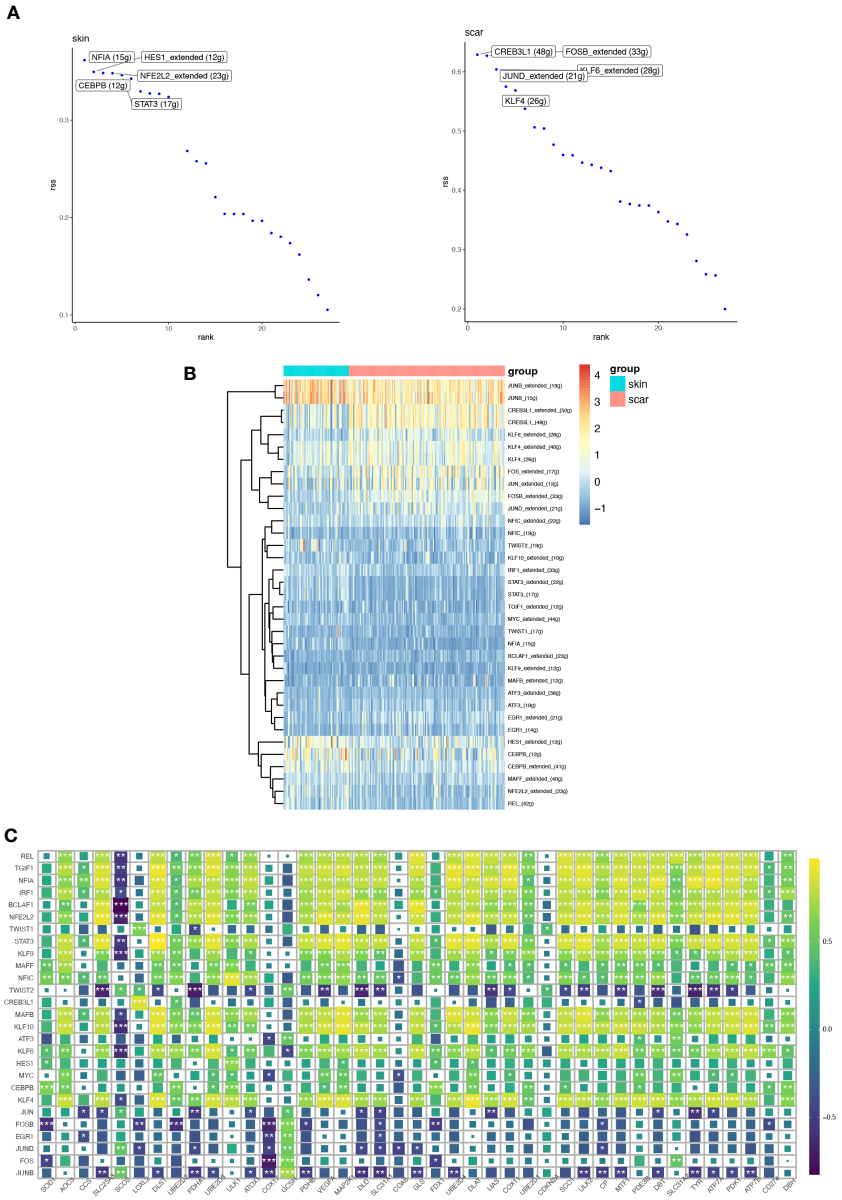

FIGURE 10
The transcription factors (TFs) and regulatory network involved in HS and NS. **(A)** The transcription factors regulating TOP5 activity in normal skin and hypertrophic scar fibroblasts. **(B)** Heatmaps showing the expression distribution of key transcription factors involved in differential transcription activity in HS and NS. **(C)** Correlation heatmap between key transcription factors involved in differential transcription activity and CRGs. (*P < 0.05, **P < 0.01, ***P < 0.001).

inhibiting HSC activation via the lnc-LFAR1/MTF-1/GDNF axis (33). These genes, which have not yet been found in HS, have significant potential for research. We validated the expression of the three genes in HS and NS by qRT-PCR and our results were consistent with expression differences in the RNA-seq dataset. We performed a multifactorial logistic regression approach to construct a diagnostic model using these three genes and validated the model as having high diagnostic value by bootstrap resampling.

Since immune cells and inflammatory factors play an important role in the development of HS (1), we applied the CIBERSORT, MCP algorithm to calculate the expression profile of immune cells in HS and to calculate the correlation of key genes associated with cuproptosis. Based on the results, we speculate that immune cells are extensively involved in the cuproptosis process in HS. CD4+ T cells and CD8+ T cells have been shown to potentially participate in hypertrophic scar formation. Tregs are beneficial to the heart by inhibiting excessive inflammatory responses and promoting early stable scar formation in cardiac injury (34). Macrophages undergo distinct phenotypic and functional changes at different stages of the pathogenesis of HS and are considered potential therapeutic targets (35). We hypothesize that cuproptosis may influence scar formation and development through the regulation of immune T cells and macrophages. MTF1, ULK1, ATP7A are associated with multiple inflammatory factors such as IL5, TNF, IL7, IL15, TGFB2, PDGFA, CSF3, CSF1, CD4, IL6, HLA-DRB1, HLA-DRA, etc., thus we found the excellent potential value of cuproptosis in the immunotherapy

of HS. Since the clinical treatment of HS is often unsatisfactory, we divided HS into two clusters by cuproptosis pathway, in which immune cells such as cytotoxic lymphocytes, NK cells, myeloid dendritic cells, neutrophils were significantly different between the two subtypes (P value<0.05), demonstrating that they can be studied in depth as important targets for therapy.

As fibroblasts play an important role in HS (36), the changes in cuproptosis in skin fibroblasts were further explored in this study using the single cell dataset GSE156326. We divided fibroblasts into 8 cell clusters and calculated cuproptosis activity for each fibroblast. Based on our results, we found that cuproptosis activity was higher in fibroblasts from NS than HS, and we speculate that cuproptosis may alleviate skin fibrosis.

We compared the interaction of both types of fibroblasts with other cells, and high pathway activity fibroblasts could additionally communicate cellularly with Langerhans cells via the MIF pathway and T cells. MIF has been shown to have anti-fibrotic effects (37).The overexpression of protein S in lung cells reduces bleomycin-induced pulmonary fibrosis through the interaction between PROS1-AXL and communication with smooth muscle cells (38). Changes in cuproptosis activity in fibroblasts may affect other cell types and thus function through these specific receptor ligands. With these findings, we have further demonstrated that high cuproptosis activity can act as an inhibitor of fibrosis. We further analyzed the regulatory activity of transcription factors in skin fibroblasts and found that NFIA regulatory activity was highest in normal skin fibroblasts, and it has been shown that most DM rats developed retinopathy and lens fibrosis after inhibition of NFIA gene expression using RNA silencing (39). In HS fibroblasts, CREB3L1 regulatory activity was highest and CREB3L1 has been identified as a key transcription factor involved in HS myofibroblasts (21). The correlation between transcription factors with high activity in skin fibroblasts and CRGs was explored and from our results we know that CRGs are strongly positively and negatively correlated with a large number of transcription factors. Moreover, the transcription factors highly expressed in HS fibroblasts showed a negative correlation with CRGs, further confirming the potential of cuproptosis in alleviating skin fibrosis. This suggests that CRGs may act as potential target genes of transcription factors to influence phenotypic changes in skin fibroblasts.

While our study has yielded valuable insights into the relationship between certain CRGs and the development of HS, it is important to acknowledge certain limitations. One such limitation is the relatively small number of samples available in existing public databases, which may introduce statistical errors. Additionally, the clinical information available for these samples was limited, making it difficult to account for various analytical factors. It is clear that further research on CRGs is necessary to improve the overall predictive accuracy of our findings. Furthermore, there is a need for additional studies to investigate the molecular mechanisms underlying the influence of these CRGs on the formation and progression of HS.

Conclusion

In conclusion, we explored the potential role of the cuproptosis in HS and provided a set of gene markers and constructed a diagnostic model, including ATP7A, ULK1, MTF1, which were validated by qRT-PCR in HS and NS and played a critical role in the development of HS. Subsequently, we further revealed the changes in the activity of cuproptosis in skin fibroblasts and its role in cell communication and transcription factor regulation activity through single-cell analysis. Therefore, our data provided insight into the development of more effective therapeutic interventions to improve the healing of HS.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Fourth Military Medical University's Xijing Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: BYS, WL, and YZ; data curation and methodology: YZ, LC, and YP; analysis and interpretation of data: BG and ZC; writing of the manuscript: BYS and WL; review of the manuscript: LC, ZY, and BQS; study supervision: ZY and BQS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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

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Co-occurrence of non-alcoholic steatohepatitis exacerbates psoriasis associated with decreased adiponectin expression in a murine model

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Introduction: Clinical studies have suggested a bidirectional association between non-alcoholic steatohepatitis (NASH) and psoriasis, affecting each other's development and severity. Here, we explored bidirectional causal linkages between NASH and psoriasis using a murine model.

Methods: NASH was induced in mice by streptozotocin injection at 2 days of age and by high-fat diet feeding (STAM™ model). Psoriasis was induced by topical application of imiquimod (IMQ) on the ear. The severities of liver damage and psoriatic skin changes were determined using histological analysis. Gene expression in the skin tissues was evaluated using quantitative PCR analysis. Serum cytokine levels were determined using enzyme-linked immunosorbent assay. To examine the innate immune responses of normal human epidermal keratinocytes (NHEKs), the cells were treated with interleukin (IL)-17A, tumor necrosis factor (TNF)-α, and AdipoRon, an adiponectin receptor agonist.

Results and Discussion: There were no differences in the degree of liver tissue damage (fat deposition, inflammation, and fibrosis) between NASH mice with and those without psoriasis. Conversely, the co-occurrence of NASH significantly augmented psoriatic skin changes, represented by epidermal hyperplasia, in psoriatic mice. Pro-inflammatory cytokines were expressed in the inflamed skin of psoriatic mice, and the expression of genes, especially *Il23a*, *Il1b*, *Il36g*, and *Mip2*, was significantly upregulated by the co-occurrence of NASH. The expression of keratinocyte activation marker genes *Defb4b* and *Krt16* was also upregulated by the co-occurrence of NASH. The serum TNF-α and IL-17 levels were increased by the co-occurrence of NASH and psoriasis. The serum adiponectin levels decreased in NASH mice compared with that in non-NASH mice. In NHEK culture, TNF-α and IL-17A synergistically upregulated *CXCL1*, *CXCL8*, and *IL1B* expression. The upregulated pro-inflammatory gene expression

was suppressed by AdipoRon treatment, reflecting the anti-inflammatory capacity of adiponectin.

Conclusion: The co-occurrence of NASH exacerbated psoriatic skin changes associated with increased serum inflammatory cytokine levels and decreased serum adiponectin levels. Combined with *in vitro* findings, increased inflammatory cytokine levels and decreased adiponectin levels likely promote innate immune responses in epidermal keratinocytes in psoriatic skin lesions. Overall, therapeutic intervention for co-occurring NASH is essential to achieve a favorable prognosis of psoriasis in clinical practice.

KEYWORDS

psoriasis, non-alcoholic steatohepatitis, adiponectin, tumor necrosis factor-α, interleukin-17, epidermal keratinocyte

1 Introduction

Psoriasis is a chronic inflammatory skin disease characterized by thick, scaly, erythematous plaques; its global adult prevalence is approximately 2%–3% (1). Psoriasis is associated with obesity and metabolic diseases such as metabolic syndrome, dyslipidemia, and diabetes (2). Clinical studies have shown strong associations between obesity and psoriasis (3, 4). Both conditions increase the prevalence and severity of diseases bidirectionally (5). Metabolic disease-associated chronic inflammation is considered to activate a vicious cycle of inflammation. For instance, in our previous study, we found that obesity exacerbates psoriatic skin changes via increased leptin secretion and the subsequent innate immune responses of epidermal keratinocytes (6).

Non-alcoholic fatty liver disease (NAFLD) is a pathological liver condition affecting individuals who consume little to no alcohol. It ranges from intracellular fat deposition without inflammation (simple steatosis) to non-alcoholic steatohepatitis (NASH), which is accompanied by inflammation, cell damage, and fibrosis (7). The global prevalence of NAFLD and NASH is approximately 30% and 5%, respectively (8). NAFLD and NASH have become global health issues, as their prevalence has increased by 50% in the last 20 years (8).

NAFLD is a multisystem disease affecting various extrahepatic organs and increases the risk of cardiovascular disease, type 2 diabetes, and chronic kidney disease (9). Psoriasis is also recognized as an extrahepatic complication of NAFLD, with its prevalence increasing in patients with NAFLD (10, 11). Furthermore, the severity of psoriasis is higher in patients with NAFLD than in those without NAFLD (12). An inverse relationship has also been noted; patients with chronic psoriasis have nearly twice the odds of developing NAFLD compared to healthy controls (12). Bidirectional relationships between psoriasis and NAFLD have been suggested; however, the underlying causative relationships have not yet been clarified.

Adiponectin is an adipokine primarily secreted by adipose tissue (13). It plays a crucial role in regulating various physiological processes such as metabolism and inflammation (13). Adiponectin enhances the body's sensitivity to insulin (13). Adiponectin also has anti-inflammatory properties (14, 15). It can suppress the production of pro-inflammatory cytokines and promote the secretion of anti-inflammatory cytokines (14, 15). Studies have shown that adiponectin levels are often decreased in patients with psoriasis (16, 17). This reduction in adiponectin level is suggested to contribute to persistent inflammation; however, the mechanisms are not fully elucidated. Therefore, in this study, we aimed to clarify the pathogenic links between NASH and psoriasis in a murine model focusing on adiponectin.

2 Materials and methods

2.1 Reagents

Imiquimod (IMQ) cream (Beselna Cream) was provided by Mochida Pharmaceutical (Tokyo, Japan). Streptozotocin (STZ) was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Recombinant human TNF-α, IL-17A, and AdipoRon were purchased from Chemicon (Temecula, CA, USA), R&D Systems (Minneapolis, MN, USA), and Focus Biomolecules (Plymouth Meeting, PA, USA), respectively.

2.2 Mice and diets

Wild-type pregnant mice (C57BL/6J background) were purchased from Japan SLC, Inc. (Shizuoka, Japan) at 14 days of gestation. Neonatal male pups obtained after spontaneous parturition were used in this study. All mice were maintained at 23°C under a 12-h light/12-h dark cycle with free access to water and chow in SMC Laboratories, Inc. (Tokyo, Japan). All mice were fed a normal diet (ND; CE-2; CLEA Japan, Tokyo, Japan) for 4 weeks of weaning. After weaning, the healthy and IMQ group mice

were fed ND, and the NASH (STAM) group mice were fed a high-fat diet (HFD; HFD32; CLEA Japan, Tokyo, Japan) (see Figure 1A). The mice were administered the diets until the end of the experiment.

2.3 NASH mouse model (STAM™ model)

To recapitulate the progression of NASH, we used a STAM™ murine model (18). Briefly, a single dose of STZ (200 μg/animal) was subcutaneously administered to the back of the mice at the age of 2 days to reduce insulin secretory capacity. The STAM mice were fed HFD from 4 weeks of age until the end of the experiments. The mice developed steatohepatitis at 8 weeks of age and progressive fibrosis at approximately 11 weeks of age, consistent with the development of NASH in mice (18, 19).

2.4 Imiquimod-induced psoriasis model

Psoriatic dermatitis was induced in mice by the topical application of IMQ, as described previously (6, 20). Mice were treated with 25 mg of 5% IMQ cream on the right ear for 5 consecutive days at the age of 10 weeks. Vaseline was used as a control. Ear thickness was measured daily using a digital thickness gauge. The mice were dissected 1 day after the last treatment. After euthanizing the mice, blood and tissue samples were collected.

STAM mice treated with IMQ were divided into four experimental groups, as shown in Figure 1A. For statistical power, we used eight mice per group based on our experience.

2.5 Histological analyses of the liver

After weighing the sampled liver, the left lobe was divided into three equal parts. The two pieces were fixed in Bouin's fixative for 24 h, embedded in paraffin, and sectioned using a microtome. The sections were then subjected to hematoxylin–eosin (HE) and Sirius Red staining to evaluate intrahepatic fat deposition and fibrosis, respectively. The histological severity of steatohepatitis was evaluated using the NAFLD activity score, which was calculated according to Kleiner's criteria based on images of HE-stained specimens (7). This score includes a numerical score for steatosis (0–3), hepatocyte ballooning (1–2), and lobular inflammation (0–3) (7).

Another piece of the liver was fixed in 10% formalin fixative for 24 h, immersed in sucrose solution, embedded in O.C.T. compound, and immediately frozen with liquid nitrogen. The

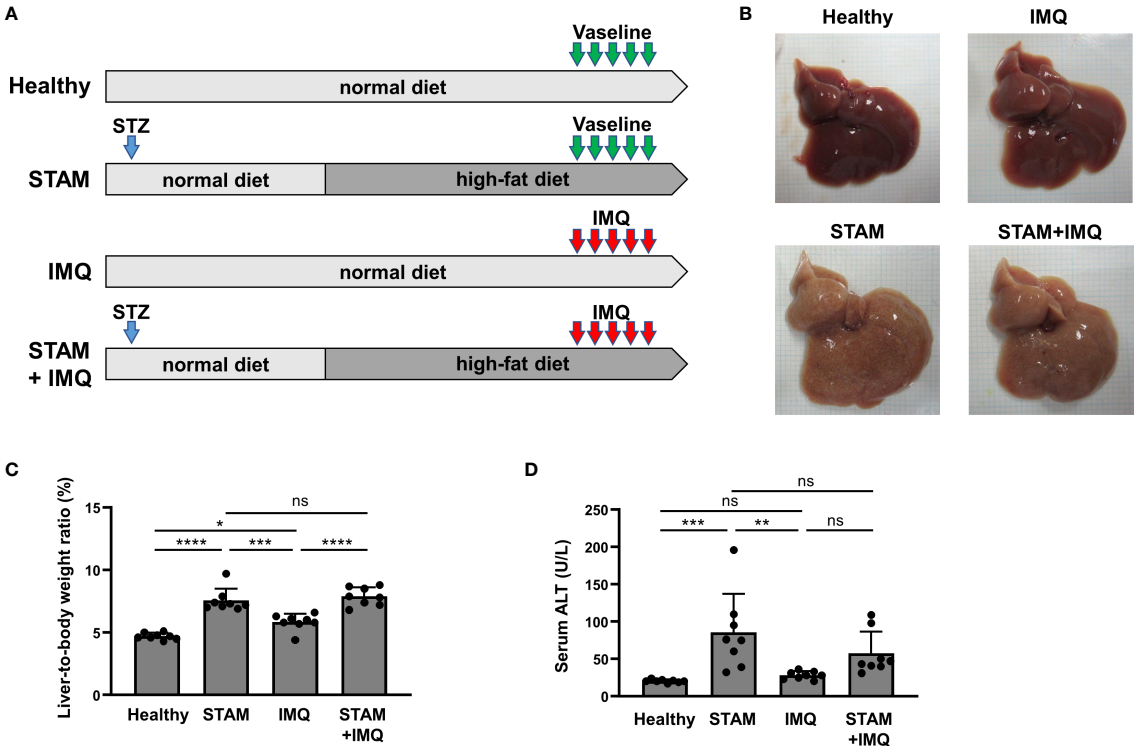

FIGURE 1
Generation of a murine model with both NASH and psoriasis. **(A)** Study protocol. Healthy mice were fed a normal diet (ND) for 11 weeks. Two-day-old male pups assigned to the STAM groups were subcutaneously injected with streptozotocin (STZ; 200 μg/mouse) on the back and fed the ND for 4 weeks until weaning, after which they were fed a high-fat diet (HFD) for 7 weeks. Imiquimod cream (IMQ; 25 mg/mouse) or Vaseline (as control) was topically applied to the ear of the mice at the age of 10 weeks for 5 consecutive days (n = 8 per group). Blood, liver, and skin samples were collected 1 day after the last treatment. **(B)** Photographs of the liver. **(C)** Liver-to-body weight ratio. **(D)** Serum alanine aminotransferase (ALT) level. Each dot denotes an individual mouse. Values are presented as mean with standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. indicated group. NASH, non-alcoholic steatohepatitis. ns, not significant.

frozen blocks were sectioned using a cryostat and subjected to Oil Red O staining. Five images per section were captured at a 200× field of view centered on the central vein. Oil Red O or Sirius Red staining-positive areas were measured using ImageJ (version 1.54d; NIH, Bethesda, MD, USA) (18).

2.6 Histological analyses of the ears

Histological analysis of the ear tissue was performed as described previously (21). The ear tissues were fixed in 10% formalin fixative for 24 h, embedded in paraffin, and sectioned using a microtome. The sections were stained with HE. Epidermal hyperplasia was quantified by measuring the thickness of the epidermis, excluding the stratum corneum, on the stained sections using ImageJ. Ten representative areas of the epidermis per mouse were measured, and the average values were calculated.

2.7 Real-time quantitative polymerase chain reaction analysis

Real-time quantitative polymerase chain reaction (qPCR) analysis was performed as described previously (22, 23). The total RNA was extracted from the right ear and liver using RNAiso Plus (Takara Bio, Shiga, Japan) and solubilized in ribonuclease-free water. Complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit (Takara Bio). Real-time quantitative polymerase chain reaction was performed using the TB Green PCR Master Mix (Takara Bio) on the QuantStudio1 System (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression levels were calculated using the ΔΔCt method and normalized to their levels in control samples indicated in each experiment. *36b4* was used as a housekeeping gene for murine samples and *GAPDH* was used for human epidermal keratinocyte samples. Real-time qPCR was performed using the primers listed in Table S1. All qPCRs yielded products with single-peak dissociation curves.

2.8 Measurement of biochemical parameters

Murine blood samples were centrifuged to separate the serum, and serum alanine aminotransferase (ALT) level was measured using a colorimetric method (GPT/ALT-PIII; FUJIFILM Co., Tokyo, Japan), following the manufacturer's instructions.

2.9 Enzyme-linked immunosorbent assay

The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-17, and adiponectin were measured in murine serum samples using the respective Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following the manufacturer's protocol.

2.10 Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were obtained from Thermo Fisher Scientific (C-001-5C) and cultured in serum-free EpiLife Medium (Thermo Fisher Scientific) supplemented with 0.06 mM Ca^{2+} and EpiLife Defined Growth Supplement (Thermo Fisher Scientific) in 12-well flat-bottom plates at 37°C with 5% CO_2. The cells were maintained for a maximum of eight passages in this medium supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). When the cells reached 70% confluence, they were stimulated with IL-17A (30 ng/mL) and TNF-α (30 ng/mL) for 24 h. Thereafter, AdipoRon (10, 30, 100, and 300 μM) was added 30 min before or 60 min after IL-17A and/or TNF-α treatment. RNA samples were then collected using RNAiso Plus and used for qPCR analysis.

2.11 Ethical approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of SMC Laboratories, Inc. (SLMN#043-2209-4). All experimental procedures were conducted in accordance with the institutional and NIH guidelines for the humane use of animals.

2.12 Statistical analysis

All values are presented as mean with standard deviation, except the values of the time course of the ear thickness, which are presented as mean ± standard error of the mean. A one-way analysis of variance, followed by Tukey's *post-hoc* test, was used to compare three or more groups. All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3 Results

3.1 Generation of the murine model with both NASH and psoriasis

To investigate the bidirectional pathogenic links between NASH and psoriasis, we generated a murine model with both NASH and psoriasis. NASH was induced by STZ injection and HFD feeding (STAM model), and psoriasis was induced by the topical application of IMQ to the ear. The mice were divided into four groups, as shown in Figure 1A.

The livers isolated from STAM group mice were yellow (Figure 1B). The liver-to-body weight ratio of STAM group mice was higher than that of healthy and IMQ mice (Figure 1C). The serum ALT level of STAM group mice was higher than that of healthy and IMQ mice (Figure 1D).

3.2 Effects of psoriasis on the severity of NASH

To examine whether psoriasis affects the severity of NASH, we performed liver histological analyses. The liver specimens of STAM mice showed balloon-like degeneration of hepatocytes, deposition of large and small fat droplets, and infiltration of inflammatory cells, including lymphocytes and neutrophils (Figure 2A), which were comparable to the pathology of human NASH. IMQ-induced psoriasis did not augment the severity of NASH, as indicated by the NAFLD activity scores (Figure 2B). Moreover, psoriasis did not enhance the severity of liver fibrosis and fat deposition (Figures 2C, D).

3.3 Effects of NASH on the severity of psoriasis

Next, we investigated whether the presence of NASH affects the severity of psoriasis; for this purpose, the development of psoriatic lesions was examined after topical IMQ treatment. The IMQ treatment aggravated skin thickening over time, and the co-occurrence of NASH tended to worsen skin thickening (Figure 3A). In the Vaseline-treated group, the co-occurrence of NASH did not significantly affect ear thickness (Figure 3A). In the histological analysis, we found epidermal thickening and inflammatory cell infiltrates in the skin of IMQ-treated mice (Figure 3B). Additionally, the co-occurrence of NASH

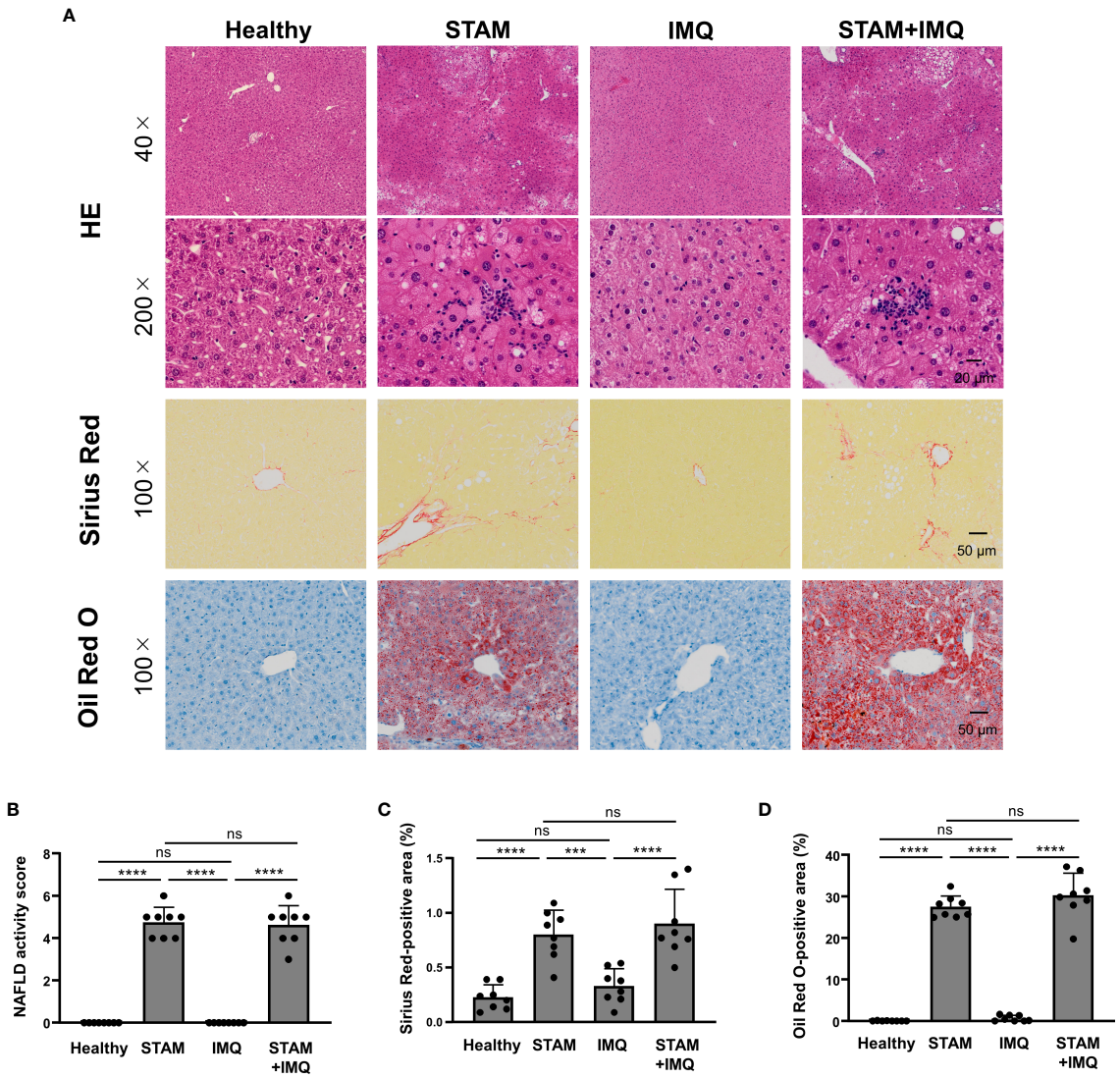

FIGURE 2
Histological analyses of the liver. Non-alcoholic steatohepatitis (NASH) and psoriatic skin changes were induced as described in Methods and Figure 1. Liver tissues were collected 1 day after the last treatment. **(A)** Representative images of the liver stained by hematoxylin–eosin, Sirius Red, and Oil Red O. **(B)** Non-alcoholic fatty liver disease (NAFLD) activity score according to Kleiner's criteria. **(C, D)** Percentage of Sirius Red- and Oil Red O-stained areas. Each dot denotes an individual mouse. Values are presented as mean with standard deviation. *** $p < 0.001$, **** $p < 0.0001$ vs. indicated group. ns, not significant.

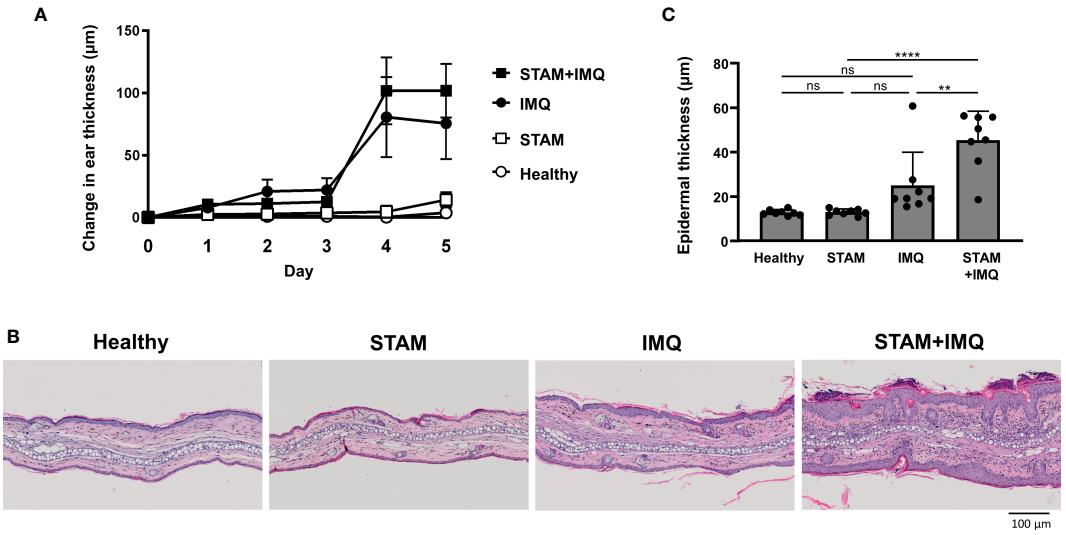

FIGURE 3
Co-occurrence of NASH exacerbated psoriatic skin changes. **(A)** Changes in ear thickness. Mice were topically treated with imiquimod (IMQ) cream (25 mg/mouse) or Vaseline (control; n = 8 per group). The ear thickness of each mouse was measured using a digital thickness gauge daily. Values are presented as mean ± standard error of the mean. **(B)** Representative images of hematoxylin–eosin-stained ears of indicated mice. Bar = 100 μm. **(C)** Histological analysis of epidermal thickness. Ten representative areas of the epidermis were measured for individual mice, and their average value was calculated. Each dot denotes an individual mouse. Values are presented as mean with standard deviation. ** $p < 0.01$, **** $p < 0.0001$ vs. indicated group. NASH, non-alcoholic steatohepatitis. ns, not significant.

significantly enhanced epidermal thickness, parakeratosis, and inflammatory cell infiltrates in psoriatic mice (Figure 3B). The exacerbated epidermal thickness in IMQ-treated STAM mice was confirmed by the quantitative analysis of the skin (Figure 3C).

3.4 Enhanced pro-inflammatory cytokine expression in the inflamed skin by NASH

To elucidate the mechanisms by which accompanying NASH exacerbates psoriatic skin phenotypes, we assessed the expression of pro-inflammatory cytokine genes in inflamed skin lesions. The expression of pro-inflammatory cytokine was moderately upregulated in IMQ-treated mice compared with that in healthy mice (Figure 4). Interestingly, the co-occurrence of NASH significantly upregulated the gene expression of *Il23a*, *Il1b*, *Il36g*, and *Mip2*, compared with that in IMQ-treated mice without NASH (Figure 4). In addition, the expression of the antimicrobial peptide gene *Defb4* and the keratinocyte proliferation marker *Krt16* was upregulated by the co-occurrence of NASH (Figure 4).

Notably, in the groups not treated with IMQ, the co-occurrence of NASH moderately increased the expression of some genes, such as *Il17a* and *Il36g* (Figure 4), although the changes were not significant owing to the large variation in the data.

3.5 Altered serum cytokine levels by NASH

Psoriasis and NAFLD have common feature of chronic inflammation, and both exert systemic effects. We next evaluated the systemic inflammatory factors that account for the exacerbation of psoriasis in NASH mice. The serum levels of TNF-α and IL-17 were synergistically elevated by the co-occurrence of NASH and psoriasis (Figures 5A, B).

Notably, the serum adiponectin level significantly decreased in NASH mice compared with that in non-NASH mice (Figure 5C).

3.6 Suppressive effect of adiponectin on the pro-inflammatory response in epidermal keratinocytes

The reduced serum adiponectin level in NASH mice led us to speculate about the involvement of adiponectin in the pathogenesis of exacerbated skin inflammation in IMQ-treated STAM mice. To investigate the potential involvement of adiponectin, we evaluated the effect of adiponectin on epidermal keratinocytes. Epidermal keratinocytes act as initiator and amplifier cells in the pathogenesis of psoriasis through the production of chemokines and inflammatory cytokines (24). In terms of the importance of epidermal keratinocytes in the pathology of psoriasis, we have previously clarified the essential roles of epidermal keratinocytes in psoriasis (6, 25–27).

Cultured NHEKs were treated with AdipoRon, an adiponectin receptor agonist, in combination with IL-17A and/or TNF-α. We found that treatment with IL-17A, TNF-α, and their combination significantly upregulated the expression of *CXCL1*, *CXCL8*, and *IL1B* (Figure 6A). AdipoRon pretreatment significantly suppressed the pro-inflammatory gene expression (Figure 6A). In addition, 60 min after treatment of AdipoRon significantly suppressed the gene expression to levels similar to those of the pretreatment experiment (Figure 6B). These findings reflect the anti-inflammatory nature of adiponectin (13).

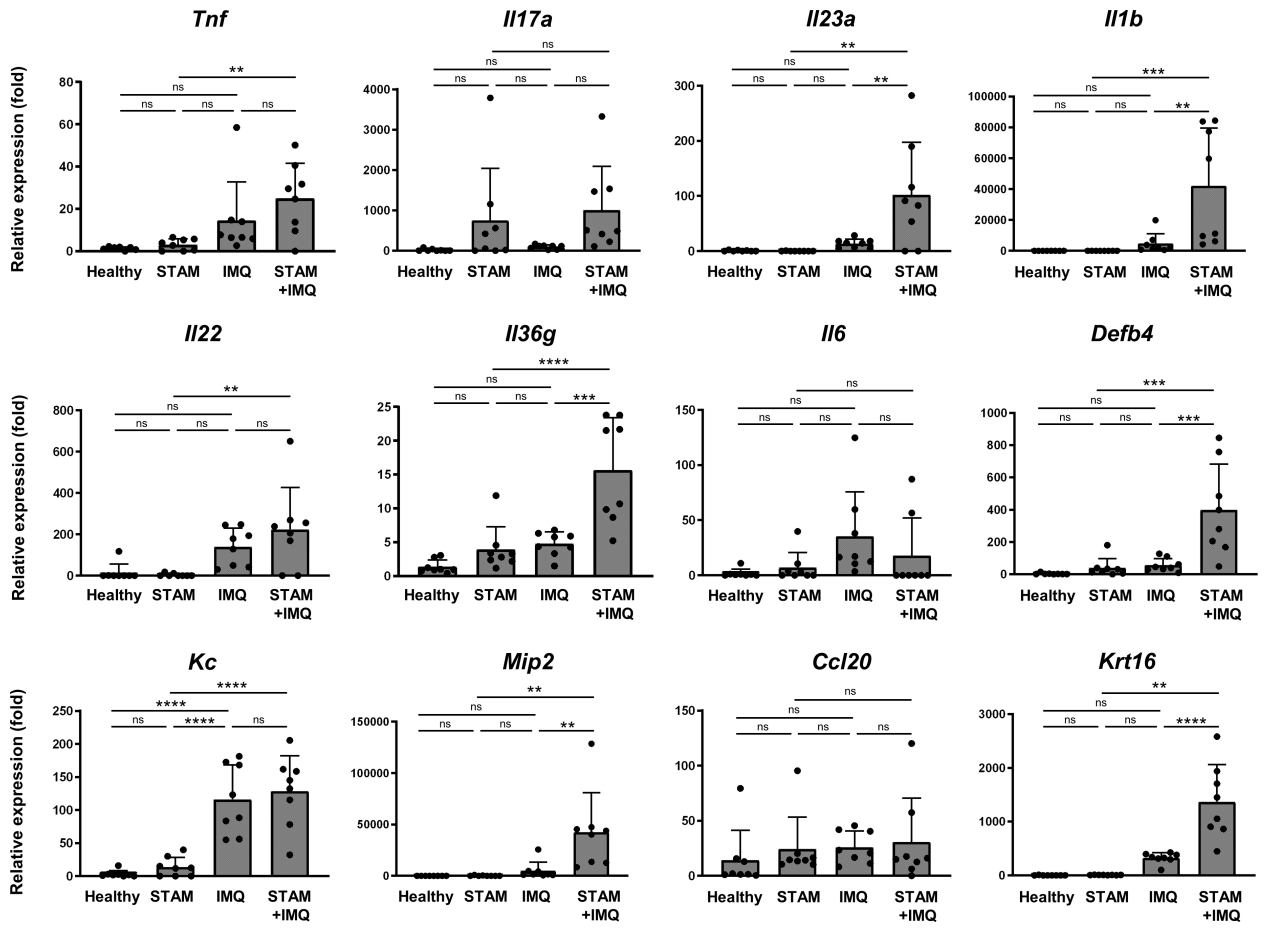

FIGURE 4
Quantitative polymerase chain reaction analysis of the ear. Alterations in the mRNA expression of pro-inflammatory cytokines associated with psoriasis. RNA samples were extracted from the ear tissues of the indicated mice. mRNA expression levels were determined using quantitative PCR analysis. The levels were calculated relative to the level of *36b4* and normalized to the expression level in the healthy group. Each dot denotes an individual mouse. Values are presented as mean with standard deviation. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. indicated group. IMQ, imiquimod. ns, not significant.

4 Discussion

In this study, the co-occurrence of NASH significantly augmented epidermal hyperplasia in psoriatic mice, which was associated with decreased serum adiponectin levels. Adiponectin is an adipokine produced by adipose tissue, and its secretion is reduced by obesity (13). It serves as an anti-inflammatory cytokine (13). In the present study, the serum adiponectin level significantly decreased in NASH mice. In *in vitro* experiments using NHEKs, stimulation of adiponectin receptors significantly suppressed TNF-α- and IL-17A-induced pro-

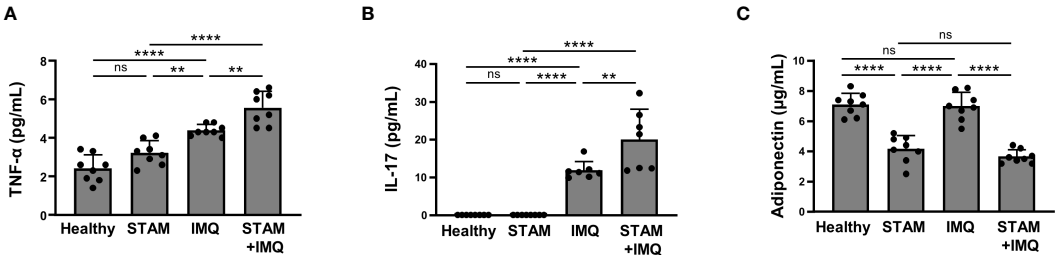

FIGURE 5
Serum cytokine levels. Blood samples were collected from the indicated mice treated with imiquimod (IMQ) cream (25 mg/mouse) or Vaseline (control; $n = 8$ per group). **(A–C)** Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-17, and adiponectin were measured using enzyme-linked immunosorbent assay (ELISA). Each dot denotes an individual mouse. Values are presented as mean with standard deviation. *** $p < 0.001$, **** $p < 0.0001$ vs. indicated group. ns, not significant.

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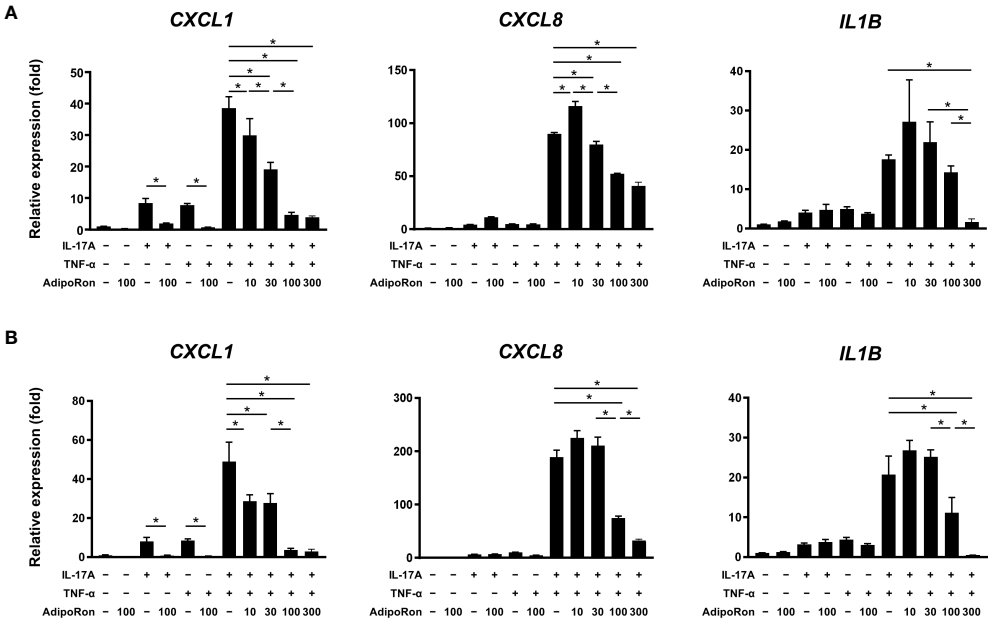

FIGURE 6
AdipoRon suppressed innate immune responses of epidermal keratinocytes. Normal human epidermal keratinocytes were stimulated with interleukin (IL)-17A (30 ng/mL) and tumor necrosis factor (TNF)-α (30 ng/mL) for 24 h. AdipoRon (10, 30, 100, and 300 μM) was added 30-min before **(A)** or 60-min after **(B)** the IL-17A and/or TNF-α treatment. Gene expression levels relative to *GAPDH* level were calculated and normalized to the levels in the non-stimulated control. Values are presented as mean with standard deviation. * $p < 0.05$ vs. indicated group.

inflammatory cytokine gene expression. These findings suggest that the presence of NASH reduces adiponectin production, which in turn decreases the anti-inflammatory effect of adiponectin, resulting in exacerbated psoriatic skin changes, at least partially, by directly acting on epidermal keratinocytes. Supporting this notion, a previous clinical study reported associations between psoriasis and decreased serum adiponectin levels (28–30). Additionally, an earlier study in a murine model has shown the involvement of adiponectin in the pathogenesis of psoriasis (31). In a murine model, adiponectin deficiency exacerbated psoriasiform dermatitis by promoting the infiltration of IL-17-producing dermal γδT cells (31). Therefore, adiponectin is suggested to contribute to the exacerbation of dermatitis in patients with psoriasis as a NASH-associated systemic factor.

In addition to adiponectin, pro-inflammatory cytokines are likely to contribute to the worsening of psoriatic skin changes as NASH-associated systemic factors. NASH is known to exert systemic effects, leading to the development or exacerbation of cardiovascular diseases, type 2 diabetes, chronic kidney disease, and hypothyroidism (32). In terms of mechanism, the involvement of intrahepatic Th17 cells and serum IL-6, IL-17, and IL-23 has been demonstrated in a murine NASH model and human patients with NAFLD (33, 34). The serum levels of TNF-α and IL-6 are elevated in patients with NASH and are associated with disease severity (35, 36). As inflammatory cytokines are also critically involved in the pathogenesis of psoriasis, NASH-associated inflammatory cytokines are likely to be exacerbating factors of psoriatic skin changes. This notion is supported by our *in vitro* findings, showing that IL-17A and TNF-α augment innate immune responses of epidermal keratinocytes, represented by increased chemokine and inflammatory cytokine production.

The effect of psoriasis on the development or severity of NAFLD remains controversial in clinical practice. Some studies have reported a higher prevalence and more severe phenotypes of NAFLD in patients with psoriasis (37, 38), while others have found no significant association between psoriasis and liver fibrosis (39, 40). For instance, the risk of patients with new-onset psoriasis developing NAFLD in the future is 1.28-fold higher than that of patients without psoriasis (11). Besides, a meta-analysis showed that 9.66% of patients with psoriasis were at an increased risk of developing advanced liver fibrosis (39). Although the co-occurrence of psoriasis did not significantly augment the severity of liver damage in our experimental model, the findings need to be re-evaluated by using other murine models with prolonged psoriasis. As the psoriatic skin phenotypes are induced in a short period (5 days) in the IMQ-induced psoriatic model, the disease duration may have been too short to worsen NAFLD pathology. Therefore, other murine models that present prolonged psoriasis, such as genetically modified psoriasis models (41, 42), would be required to evaluate the effects of chronic psoriasis on the development or progression of NAFLD.

Interestingly, we found that the co-occurrence of NASH moderately increased *Il17a* and *Il36g* expression in the skin tissues without IMQ treatment, although the changes did not reach significance. This observation suggests that NASH can induce subclinical skin inflammation without direct skin stimulation. In our previous study, we found that aberrant metabolic conditions, obesity and dyslipidemia, induce characteristic expression patterns of pro-inflammatory cytokines in the skin (6). HFD feeding tended to elevate *Ccl20* expression in the skin, whereas *Apoe* deficiency-mediated dyslipidemia upregulated *Il19* expression in the skin (6).

Another group reported similar findings, indicating that short-term exposure to a Western diet composed of high fat content and simple sugars induced psoriasiform dermatitis in mice (43). Thus, our current and previous study findings suggest that aberrant metabolic conditions predispose the skin to psoriasis, a so-called pre-psoriatic state.

In addition to adiponectin, other factors have been suggested to be involved in the liver–skin axis. Leucine-rich α-2 glycoprotein (LRG) modulates the liver–skin axis and is involved in the pathogenesis of psoriasiform inflammation (44). LRG deficiency resulted in mild psoriatic skin changes associated with decreased inflammatory cytokine expression in a murine model of psoriasis. Thus, clarifying the detailed mechanisms of the liver–skin axis in psoriasis is necessary to develop novel treatments.

There may be some possible limitations to this study. A more comprehensive analysis would be required to comprehensively clarify the pathological link between psoriasis and NASH. Although we have shown the possible involvement of adiponectin, the effects of factors related to metabolic abnormalities on skin inflammation are diverse and complex. Various metabolic disease-associated factors other than adiponectin have been reported to affect psoriatic skin phenotypes, such as leptin (6, 45), chemerin (46), resistin (16), and free fatty acids (6, 47). Additionally, metabolic disease-associated factors could directly act on immune cells, including macrophages (48), lymphocytes (15, 49), and innate lymphoid cells (50, 51), not just on epidermal keratinocytes.

Another concern is that STZ-induced hyperglycemia and HFD may have acted as confounding factors to the exacerbation of psoriasis. Two groups have reported that STZ and HFD-induced diabetic mice exhibit more severe psoriatic skin changes after IMQ treatment than non-diabetic control mice (52, 53). Although the timing of STZ injection and duration of HFD are different between their study and our study, their findings suggest that STZ-induced hyperglycemia and/or HFD could affect the severity of psoriatic skin changes.

In conclusion, our findings suggest that the co-occurrence of NASH exacerbates psoriasis associated with decreased serum adiponectin level. Further research is required to clarify possible causal relationships between decreased adiponectin level and psoriasis exacerbation. Such studies could help develop effective treatment strategies for psoriasis accompanying NAFLD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by the Institutional Animal Care and Use Committee of SMC Laboratories, Inc. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DT, KI, TH, and YSh performed the experiments and analyzed the data. SM, MI, YSa, YK, SN, and TM helped in the interpretation of the data. DT and TM wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

TM received scholarship donations from Eli Lilly Japan, Inc., Sun Pharma, and AbbVie. SM received research grants from AbbVie, Sun Pharma Japan, and Maruho and honoraria for lectures from Pfizer, Sanofi, Eli Lilly, Boehringer Ingelheim, Novartis, Kyowa Kirin, AbbVie, Sun Pharma Japan, and Maruho. YK received a research grant from Maruho.

Authors TH and YSh are employed by SMC Laboratories, Inc.

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/fimmu.2023.1214623/full#supplementary-material

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Toll-like receptors: their roles in pathomechanisms of atopic dermatitis

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The skin functions as a physical barrier and represents the first line of the innate immune system. There is increasing evidence that toll-like receptors (TLRs) are involved in the pathomechanisms of not only infectious diseases, but also non-infectious inflammatory diseases. Interestingly, it has been demonstrated that TLRs recognize both exogenous threats, e.g. bacteria and viruses, and endogenous danger signals related to inflammation, cell necrosis, or tissue damage. Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease, which is associated with impaired skin barrier function, increased skin irritability to non-specific stimuli, and percutaneous sensitization. The impairment of skin barrier function in AD allows various stimuli, such as potential allergens and pathogens, to penetrate the skin and activate the innate immune system, including TLR signaling, which can lead to the development of adaptive immune reactions. In this review, I summarize the current understanding of the roles of TLR signaling in the pathogenesis of AD, with special emphasis on skin barrier function and inflammation.

KEYWORDS

atopic dermatitis, barrier, inflammation, innate immunity, stratum corneum, skin, toll-like receptors

1 Introduction

With respect to pathogen recognition by the innate immune system, characteristic molecular patterns that are exhibited by bacteria or viruses are recognized by pathogen sensors, which are termed pattern recognition receptors (PRRs) (1–3). The primary PRRs include toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, and retinoic acid-inducible gene-I-like receptors. Regarding TLRs, ten kinds of TLRs have been found, and they are expressed on a variety of cells in humans (Figure 1) (1–3). TLR signaling can induce the activation of nuclear factor-kB (NF-kB), interferon-regulatory factor, and their target genes, resulting in the release of numerous antimicrobial and proinflammatory mediators (1–3).

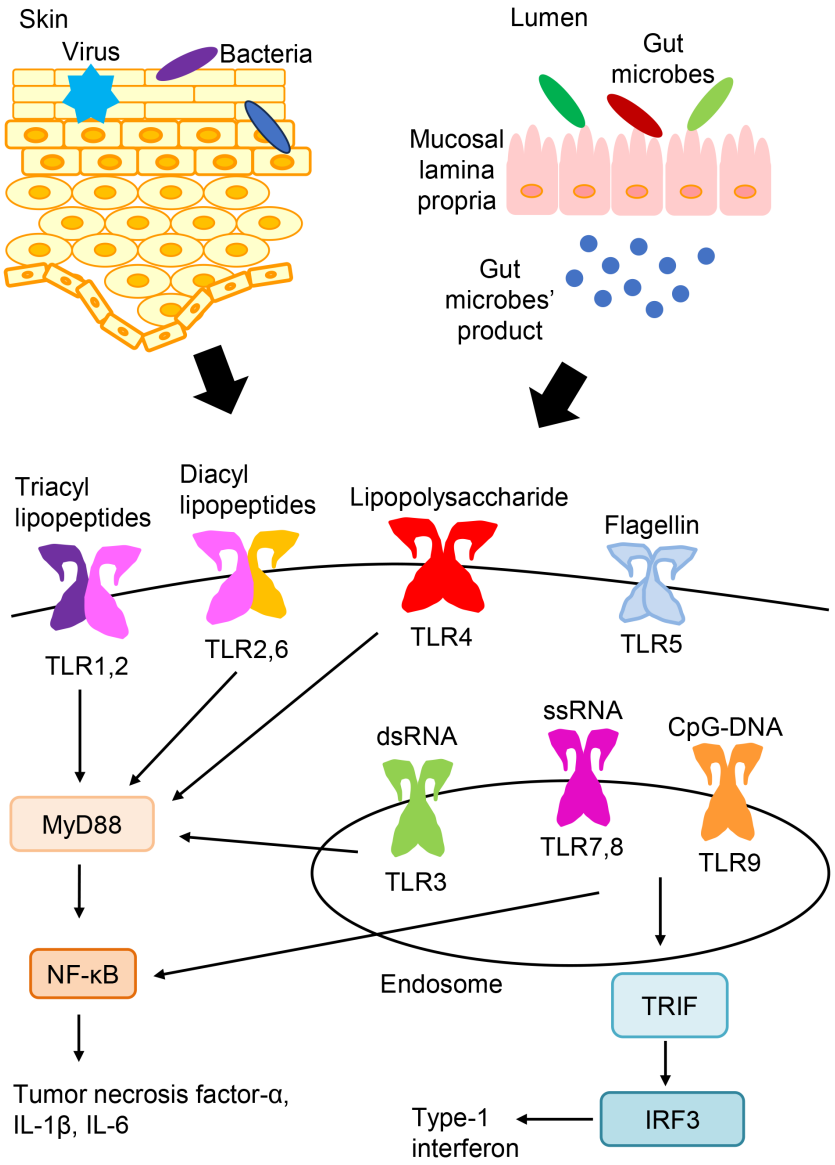

FIGURE 1
TLR expression and signaling. TLRs express on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or in endosomes (TLR3, TLR7, TLR8, and TLR9). TLR ligands from bacteria, viruses, and so on are recognized and stimulate TLR signaling pathways. And, TLR signaling leads to the activation of NF-kB, interferon-regulatory factor, and their target genes, which induces the production of various antimicrobial and proinflammatory cytokines.

Atopic dermatitis (AD) is a common chronic inflammatory skin disorder, affecting 2–20% of the general population, although its frequency varies with age and ethnicity (4, 5). The major manifestations of AD include dry skin, intense pruritus, and recurrent eczematous lesions. The pathomechanisms of AD are associated with impaired skin barrier function, enhanced skin irritability in response to non-specific stimuli, and percutaneous sensitization (4). In AD, a variety of complicating factors, e.g., irritants, house dust mites, pollen, food, microbial organisms, sweat, contact allergens, and scratching, can induce the development of skin inflammation (6). Recently, the innate immune system, including TLR signaling, has been shown to be involved in the pathomechanisms of AD. In this review, I summarize the roles of TLRs in cutaneous barrier function and inflammation in AD.

2 Stimulation of TLR signaling

2.1 TLR expressions in the skin

The skin consists of the epidermis, dermis, and subcutaneous layers. Various cell types express TLRs in the skin. In the epidermis, keratinocytes express TLRs 1-6, 9, and 10, and Langerhans cells express TLRs 1-6, 8, and 10 (7). Melanocytes express TLRs 2-4, 7,

and 9 (7). In the dermis, resident cells, including mast cells (TLRs 1-7 and 9) and fibroblasts (TLRs 1-10), also express TLRs (7).

2.2 Exogenous ligands

TLRs recognize the common pathogen-derived molecular patterns shown by bacteria or viruses, which are termed pathogen-associated molecular patterns (3). The recognition of triacyl and diacyl lipopeptides requires the dimerization of TLR2 with TLR1 and TLR6, respectively. TLR3 detects double-stranded RNAs (dsRNAs). TLR4 recognizes bacterial lipopolysaccharides. TLR5 recognizes flagellin that makes up the flagella of bacteria. Both TLR7 and TLR8 detect single-stranded RNAs. TLR9 detects non-methylated cytosine-phosphate-guanine (CpG) DNA. TLRs express on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or in endosomes (TLR3, TLR7, TLR8, and TLR9) (3).

Microbes, including bacteria, fungi, and viruses, are found on the skin surface and constitute a population that is peculiar to the skin, which is called the indigenous skin microbial flora (8). Such microbes can stimulate TLR signaling and are associated with cutaneous immune and inflammatory responses (Figure 1). Common skin commensal bacteria include *Propionibacterium acnes*, *Staphylococcus epidermitis* (*S. epidermitis*), and *S. aureus* (8). Bacterial colonization by *S. aureus* is the most common skin infection in AD patients (8). *S. aureus* is sensed by several PRRs, including TLR2 and TLR4 (1, 2). By acting on TLR2, *Propionibacterium acnes* may induce the cytokine release from follicular keratinocytes and macrophages, leading to inflammation (9). It has been reported that *Adam17*$^{fl/fl}$*Sox9*-^{Cre} mice, an animal model ADAM17-deficiency in human, developed AD-like skin lesions with naturally occurring dysbiosis (10). *Corynebacterium mastitidis*, *S. aureus*, and *Corynebacterium bovis* sequentially appeared in the skin lesions. Furthermore, antibiotic specific for these bacterial species reversed dysbiosis and improved the skin lesions. These findings suggest that skin dysbiosis in the microbiome is related to the pathomechanisms of AD.

Demodex mites live in hair follicles or sebaceous glands (11). Demodex mites occur in all human ethnic groups, and they infect about 100% of the population up to old age (11). It was reported that the density of Demodex mites was increased in rosacea skin (11, 12). Sebum with an abnormal composition may induce the proliferation of Demodex mites in rosacea skin, leading to skin inflammation (11, 12). Furthermore, chitin, a polysaccharide present in the exoskeletons of insects, is known to stimulate TLR2 on keratinocytes (13).

Malassezia also exists in the human skin and is thought to be etiological factors for various skin disorders, including AD (6). Park et al. identified viral dsRNA segments in several clinical isolates of Malassezia species and revealed that the viral nucleic acid induced a TLR3-dependent immune response of dendritic cells (13). These findings suggest that a viral element included in Malassezia may be associated with the development of skin inflammation via TLR3 signaling.

In addition to the skin microbe, the gut microbiota is closely involved in the pathomechanisms of AD (14, 15). The gut microbiota and their products can be recognized by TLRs (Figure 1) (14). In the patients with AD, there are changes in the gut microbiota. While the good microbes including *Bifidobacterium* and *Lactobacillus* are reduced in AD patients (16, 17), there are higher proportions of *Clostridium*, *Staphylococcus* and so on in AD patients than those in non-allergic individuals (16, 18). Zachariassen et al. showed that a high and a low responding phenotype of AD, e.g. dermatitis and increased cytokine expressions, can be transferred with the gut microbiota to germ-free mice (19).

2.3 Endogenous ligands

Endogenous ligands stimulate TLRs and induce inflammation in non-infectious diseases (1). Many of the endogenous ligands are known to be released as a result of inflammation, cell necrosis, or tissue damage (1). Chaperones, such as heat shock protein and high mobility group box 1 (HMGB1) nuclear protein, are released from dead cells, and hyaluronic acid and biglycan are released from the extracellular matrix during inflammation (20). Moreover, TLR stimulation by low-density lipoprotein, fatty acids, or ω-2-carboxyethyl pyrrole was reported to be associated with dyslipidemia or oxidative stress (21). Such endogenous ligands act on TLR2 and TLR4, which function on the cell surface (1). In addition, TLR3, TLR7, TLR8, and TLR9 respond to self-derived nucleic acids (1).

3 Impaired skin barrier function in AD

3.1 Skin barrier function

The epidermis, which is the outermost layer of the skin, has important functions in the maintenance of skin barrier function (22–24). It consists of the stratum corneum, and granular, prickle, and basal cells. The stratum corneum contains three factors that are important for skin barrier function, sebum, intercellular lipids, and the natural moisturizing factor (22). Sebum derives from fat secreted by the sebaceous glands. A mixture of sebum and sweat covers the skin surface, preventing water transpiration by forming a sebum barrier. Intercellular lipids are lipids, e.g., ceramide and fatty acids, that fill the gaps between corneocytes (23). They play a role in the adhesive bonding of corneocytes and help to retain water in the skin. The natural moisturizing factor, which is responsible for water retention, is the name given to amino acids and their metabolites in the horny layer (23). These three factors function to maintain the skin's barrier function and retain skin moisture.

3.2 Decreased filaggrin levels in AD

The granular-layer cells contain keratohyalin granules, the main component of which is profilaggrin, a phosphorylated histidine-rich high-molecular-weight protein (22, 24). In the keratinization phase, profilaggrin is decomposed to filaggrin monomers in the horny

layer (22, 24). These filaggrin monomers aggregate to form keratin fibers, contributing to horny-layer cytoskeleton formation and the maintenance of physical strength. In addition, they are decomposed into amino acids in the most superficial layer of the horny layer. These amino acids and their metabolites are collectively termed the natural moisturizing factor. These factors are involved in the moisturization of the horny layer. Several studies have reported that 15-50% of the AD patients in Europe have filaggrin gene mutations, although there are regional differences (25–27). Filaggrin gene mutations are considered to prevent the production of normal profilaggrin and impair the barrier function of the skin.

3.3 Increased protease levels in AD

Other molecules that are important for the skin barrier formation include tissue kallikrein, a serine protease (28). In humans, 15 types of tissue kallikrein have been reported. In keratinocytes, the expression levels of kallikrein 5 (trypsin-type serine protease) and kallikrein 7 (chymotrypsin-type serine protease) are particularly high (29). In normal skin, these kallikreins may promote dekeratinization of the horny layer, with cell adhesion molecules used as a substrate (30). Interestingly, the overexpression of several types of kallikrein has been detected in the lesion sites of AD patients (31). In human kallikrein 7 transgenic mice, the spontaneous onset of chronic dermatitis was observed (32). On the other hand, lympho-epithelial Kazal-type inhibitor (LEKTI) tightly controls the activity of serine proteases, such as kallikrein 5 and kallikrein 7, in the epidermis (33). In LEKTI-deficient mice, horny-layer barrier hypofunction and skin inflammation are induced after the promotion of filaggrin decomposition (34). LEKTI is known to be an important molecule for the skin barrier function, as demonstrated by the fact that mutations in the gene of SPINK5, which encodes LEKTI protein, resulting in Netherton syndrome (35). In patients with this condition, AD-like symptoms and high serum IgE levels are seen (36).

3.4 Impaired tight junctions in AD

Tight junctions are structures that have an essential role in maintaining the skin barrier function (22, 24). The granular layer on the inside of the horny layer consists of 3 layers of cells, and the cells in the 2nd layer adhere tightly to each other through tight junctions, preventing the migration of water, ions, or soluble proteins (24). Tight junctions are consisted of transmembrane proteins, e.g. occludin, claudins, junction adhesion molecule proteins, and cytosolic scaffold proteins, including zonula occludens (22). Claudin-1 expression is greatly decreased in the skin of AD patients compared with that in non-atopic subjects (37). In addition, an association between claudin-1 polymorphisms and susceptibility to AD has been shown (37). These findings suggest that tight junction impairment is associated with the skin barrier dysfunction seen in AD patients.

3.5 Role of TLR signaling in skin barrier function of AD

In the skin of patients with AD, a significant imbalance in the microbiome, involving marked *S. aureus* colonization, is seen (8). *S. aureus* is sensed by several PRRs, including TLR2 (38). *S. aureus*-derived peptidoglycan, a TLR2 agonist, upregulates the expression of the tight junction proteins claudin-1, claudin-23, occludin, and zonulae occludens-1 in human keratinocytes (38). In addition, a TLR2 agonist increases the recovery of barrier function in the skin wounded by tape-stripping (38). Furthermore, TLR2 deficiency delays the skin barrier recovery after tape-stripping (38). In patients with AD, epidermal TLR2 expression is lower than that in non-atopic individuals, and is inversely correlated with transepidermal water loss (38). Yuki et al. also reported that tight junction-related barrier function was increased by a TLR2 agonist and the mechanism was inhibited by the knockdown of TLR signaling adaptor protein MyD88 (39). With regard to filaggrin-related barrier function, removal of profilaggrin/filaggrin products through extracellular vesicles in keratinocytes was done to regulate intracellular free filaggrin monomer content and prevent premature cell death during stratification (40). Such phenomenon was enhanced via TLR2-mediated sensing of *S. aureus* (40). These findings suggest that TLR2 signaling is involved in upregulation of the skin barrier function.

TLR3 signaling is also associated with skin barrier formation (41). The stimulation of TLR3 enhances the expression of several important genes that are related to skin barrier formation, e.g. the genes of ATP-binding cassette subfamily A, member 12; glucocerebrosidase; acid sphingomyelinase; serine palmitoyltransferase; glucosylceramide synthase; and transglutaminase 1 (41). In addition, TLR3 activation increases lamellar bodies and keratohyalin granules of keratinocytes, including profilaggrin (41). Borkowski et al. showed that the deficiency of TLR3 delayed the recovery of skin barrier function following ultraviolet B exposure (42). These findings indicate that TLR3 signaling contributes to the recovery of skin barrier function after skin injuries.

With regard to kallikrein and LEKTI, TLR1/2, 3, 5, and 2/6 ligands was reported to induce the expression of LEKTI in cultured human keratinocytes (43). On the other hand, trypsin and chymotrypsin-like serine protease activity are upregulated in human keratinocytes after the stimulation with a TLR3 agonist (43). The mRNA expression levels of kallikrein 6, kallikrein 10, kallikrein 11, and kallikrein 13 are also increased by a TLR3 agonist (43). TLR signaling may regulate kallikrein and LEKTI expression in keratinocytes.

4 Skin inflammation in AD

4.1 Immune and inflammatory responses in AD

In AD, an impaired skin barrier allows non-specific stimuli and potential allergens and pathogens to penetrate the skin, which can then activate immune and inflammatory responses (4–6). Recent

studies have demonstrated that epithelial cell-derived cytokines, such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-33, and IL-25 act on immune cells, including type-2 innate lymphoid cells (ILC2); induce type 2 immunity; and are deeply involved in the pathogenesis of AD (4, 5). Furthermore, not only type 2 helper T cells (Th2 cells), but also Th17 cells, Th1 cells, and Th22 cells have been reported to be involved in tissue remodeling, which is observed in the chronic phase of AD. Cytokines, such as IL-31 and TSLP, are also involved in itching, which can initiate a vicious cycle of inflammation (5).

4.2 Roles of TLR1/TLR2 in inflammation in AD

Single nucleotide polymorphisms in TLR1 have been suggested to contribute to susceptibility to AD (44). House dust mite allergens can be a complicating factor in AD. Jang et al. reported that upregulated mRNA and protein expression of TLR1, TLR6, IL-25, and IL-33 were detected in human AD skin lesions that had been subjected to high house dust mite sensitization (45). In addition, house dust mite extract upregulates the expression of TLR1, TLR6, IL-25, and IL-33 in cultured human keratinocytes (45). Furthermore, the knockdown of TLR1 inhibits the release of IL-25 and IL-33 (45). These findings suggest that house dust mite allergen-induced activation of TLR1 may induce polarization toward a type 2 immune response via the release of IL-25 and IL-33.

4.3 Role of TLR2/TLR6 in inflammation in AD

It is considered that microbial organisms, including *S. aureus*, may contribute to the pathomechanisms of AD (8). Iwamoto et al. reported that the expression level of TLR2, which senses *S. aureus*, on Langerhans cells in AD skin lesions was lower than that seen in healthy skin (46). Stimulation of Langerhans cells from normal skin with a TLR2 ligand leads to the maturation and increase of migratory activity (46). On the other hand, Langerhans cells from AD skin are less responsive to the TLR2 ligand (46). These findings suggest that TLR2-mediated signals are impaired in Langerhans cells from AD skin.

In addition, the associations between TLRs and high-affinity IgE receptor (FcεRI) polymorphisms have been reported (47). More severe skin conditions are observed in AD patients having TLR2 gene (*TLR2*) rs4696480 major homozygotes and the FcεRI α-chain gene (*FCER1A*) rs2252226 minor allele than in those characterized by the remaining combined rs2252226 and rs4696480 genotypes (47). These findings imply the involvement of TLRs-FcεRI interactions in the pathomechanisms of AD.

Yu et al. showed that the basal mRNA expression level of thymus and activation-regulated chemokine (TARC)/C-C motif chemokine ligand 17 (CCL17) was elevated in peripheral blood mononuclear cells (PBMCs) from AD patients compared with that in PBMCs from healthy controls, while the basal mRNA expression levels of CCL8 and monocyte chemotactic protein-4 (MCP-4)/CCL13 were decreased in patients with AD (48). After stimulation with TLR2 ligands, the mRNA expression levels of regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, MCP-2/CCL8, MCP-4/CCL13, pulmonary and activation-regulated chemokine (PARC)/CCL18, and macrophage-derived chemokine (MDC)/CCL22 were higher in PBMCs from AD patients than in those from healthy controls (48)). In addition, Jang et al. reported that TLR6 deficiency suppressed the house dust mite allergen-induced upregulation of IL-25 or IL-33 expression (48)). These findings imply that TLR2 and TLR6 may induce polarization toward the type-2 immune response via the release of IL-25 and IL-33.

4.4 Role of TLR3 in inflammation in AD

TLR3 is expressed in the keratinocytes of both AD patients s and healthy individual (7, 49). The TLR3 expression levels of stratum corneum were elevated in the affected skin of AD patients compared with those in the unaffected skin of AD patients or healthy controls (49). Interestingly, the TLR3 expression levels of stratum corneum were correlated with the total intensity score, erythema score, oozing/crusting score, edema/papule score, excoriation score, lichenification score, and xerosis score (49). In addition, the water content was inversely correlated with the TLR3 expression levels in the stratum corneum of AD patients (49). Moreover, TLR3 (50–52) and its transcription factor interferon regulatory factor (53), especially in epithelial cells, is deeply involved in the mechanisms of allergic inflammation via cytokine and chemokine release in several murine models, including AD models.

Carbonic anhydrases are the enzymes that reversibly hydrate carbon dioxide and produce bicarbonate and protons, leading to regulation of pH and osmotic balance (54). The carbonic anhydrase II mRNA and protein expressions were increased in human primary keratinocytes upon treatment with a TLR3 agonist or the type 2 cytokines IL-4 and IL-13, seen elevated in AD (54). These findings suggest that carbonic anhydrase II may be involved in TLR3-related pathways in AD.

TLR3 signaling is also associated with the mechanisms of itching (7, 55). In the periphery, skin-resident cells, including mast cells and keratinocytes, can release various mediators, including nerve growth factor (NGF) and cytokines, that cause itching sensations. Deficiency of TLR3 suppresses the expression of NGF and scratching behavior in dry skin (55). Furthermore, the TSLP derived from keratinocytes acts upon primary sensory neurons, leading to occurrence of itching (56). Because the stimulation of TLR3 causes the production of TSLP by epithelial cells (57), the TSLP released from keratinocytes may stimulate primary sensory neurons through TLR3 signaling in the lesions of AD. In the spinal cord, TLRs, including TLR3, TLR4, and TLR7, are expressed in sensory neurons in dorsal root and trigeminal ganglions (55, 58, 59). TLR3 is expressed in small primary sensory neurons of dorsal root ganglions. Deficiency of TLR3 inhibits the histamine-dependent and -independent itching (55). These findings suggest that TLR3 signaling is associated with occurrence of itching.

4.5 Role of TLR4 in inflammation in AD

Many AD patients produce IgE against house dust mites or ticks (6). Trompette et al. reported that a tick component, Derp2, enhanced lipopolysaccharide-related TLR4 signals as a TLR4 adaptor-resembling molecule (60). In addition, they reported that the transnasal administration of Derp2 and lipopolysaccharide to mice increased the total IgE level, inducing tracheal inflammation, and that no such IgE induction was seen in TLR4-deficient mice (60). In addition to a Th2 immune responses, Th22 cells infiltrate the skin lesions of AD and release IL-22 that mediates epidermal thickening (5). Yoon et al. reported that endogenous TLR4 ligands were induced by tape-stripping, and caused the release of IL-23 from keratinocytes. This cytokine polarized dendritic cells to drive an IL-22 response in $CD4^+$ T cells (61). These findings indicate that TLR4-mediated innate immune signals induce or enhance allergic responses.

On the other hand, some reports have suggested that TLR4 can regulate the development of AD (62, 63). In neonates, the occurrence of AD was related to decreased IL-10 production via TLR4, implying that TLR4-mediated immunomodulation during early life might affect the onset of AD (62). Lin et al. reported the immune regulatory function of TLR4 signaling in AD murine model induced by repeated epicutaneous application of a hapten, TLR4-deficient mice showed more severe AD symptoms and higher expression levels of inflammatory cytokines than wild-type mice after hapten challenge (63). In addition, the skin expression of TSLP was increased in TLR4-deficient mice compared with that in wild-type mice (63). Furthermore, the migration of dendritic cells into draining lymph nodes was increased in TLR4-deficient mice following hapten challenge (63). Therefore, the role of TLR4 signaling on the immune reactions in adult AD patients might differ depending on the experimental conditions.

With regard to the gut microbe, a previous cohort study showed a significant multiplicative interaction between *E. coli* and TLR4 SNP rs10759932 in children (64). In addition, *E coli* colonization was related to a decreased risk of sensitization only in children with the rs10759932 TT genotype (64). Furthermore, West et al. reported that *Ruminococcaceae* in stool samples was decreased in the infants developing AD compared with that in healthy individuals and was inversely related to TLR2-induced IL-6 and tumor necrosis factor (TNF)-α. *Enterobacteriaceae* (a genus of Proteobacteria phylum) was negatively associated with TLR4-induced TNF-α (65). Taken together, these findings suggest that the gut microbe can affect the development of AD via TLR signaling in early life.

5 Conclusions

In conclusion, TLRs are expressed on various types of cells, including leukocytes and skin-resident cells. They detect not only exogenous threats, including bacteria and viruses, but also endogenous danger signals related to inflammation, cell necrosis, or tissue damage. In the skin of AD patients, several cell types can release numerous mediators via TLR signaling and are deeply involved in skin barrier formation and inflammation. These findings could lead to the establishment of novel TLR signaling-based treatments for AD based on the concept of innate immunity.

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Alleviating psoriatic skin inflammation through augmentation of Treg cells via CTLA-4 signaling peptide

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Psoriasis is a chronic inflammatory skin disease characterized by hyperplasia of keratinocytes and immune cell infiltration. The IL-17-producing T cells play a key role in psoriasis pathogenesis, while regulatory T (Treg) cells are diminished during psoriatic inflammation. Current psoriasis treatments largely focus on IL-17 and IL-23, however, few studies have explored therapeutic drugs targeting an increase of Treg cells to control immune homeostasis. In this study, we investigated the effects of a cytotoxic T lymphocyte antigen-4 (CTLA-4) signaling peptide (dNP2-ctCTLA-4) in Th17, Tc17, γδ T cells, Treg cells *in vitro* and a mouse model of psoriasis. Treatment with dNP2-ctCTLA-4 peptide showed a significant reduction of psoriatic skin inflammation with increased Treg cell proportion and reduced IL-17 production by T cells, indicating a potential role in modulating psoriatic skin disease. We compared dNP2-ctCTLA-4 with CTLA-4-Ig and found that only dNP2-ctCTLA-4 ameliorated the psoriasis progression, with increased Treg cells and inhibited IL-17 production from γδ T cells. *In vitro* experiments using a T cell-antigen presenting cell co-culture system demonstrated the distinct mechanisms of dNP2-ctCTLA-4 compared to CTLA-4-Ig in the induction of Treg cells. These findings highlight the therapeutic potential of dNP2-ctCTLA-4 peptide in psoriasis by augmenting Treg/Teff ratio, offering a new approach to modulating the disease.

KEYWORDS

psoriasis, dNP2-ctCTLA-4, CTLA-4-Ig, Treg cells, IL-17A

Introduction

Psoriasis is a chronic inflammatory skin disease that affects approximately 2-3% of adults worldwide (1). Clinical features of psoriasis present as erythematous plaques with silvery-scale (2). It is characterized by systemic inflammation, which is associated with comorbid diseases such as arthritis, inflammatory bowel diseases, and cardiovascular diseases (3). The pathogenesis of psoriasis is complex and involves the infiltration of various immune cells, such as T cells and dendritic cells, into the skin (4). The activation of IL-17-secreting T cells through the IL-23-IL-17A axis plays a crucial role in the exacerbation of inflammation. Pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β secreted from keratinocytes, as well as effector cytokines such as IL-12 and IL-23 secreted from dendritic cells, contribute to the activation of Th17 and Tc17 cells (5, 6). In addition, innate lymphoid cell 3 (ILC3) and γδ T cells are also involved in skin inflammation by secreting IL-17A cytokine (7–12).

Psoriasis treatment strategies are determined based on the severity of the patient (13–15). Topical steroids and/or calcipotriol are commonly used to treat mild cases. For moderate-to-severe patients, systemic therapy including cyclosporin A, methotrexate, and narrow-band ultraviolet B is administered, and more recently, biologics and small molecule drugs have been used to target specific molecules involved in inflammation (16). These drugs include secukinumab and ixekizumab, which target the IL-17A cytokine, and guselkumab and risankizumab, which simultaneously inhibit IL-23 by targeting the p19 molecule (2, 3, 17–19).

Regulatory T (Treg) cells play a crucial role in maintaining immune homeostasis and regulating inflammatory responses (20, 21). These cells can be divided into two subsets: Natural Treg cells that develop in the thymus and induced Treg cells that are induced in the periphery and express the transcription factor Forkhead box P3 (Foxp3), which confers immunosuppressive function (20). Treg cells suppress effector T cell responses by depleting IL-2 signaling through high CD25 (IL-2Rα) expression, regulating co-stimulatory signaling through constitutive expression of CTLA-4, and secreting suppressive cytokines such as IL-10 (22–24). Defects in the number or suppressive function of Treg cells have been implicated in autoimmune diseases, including type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, and psoriasis (25–29). The conversion of Treg cells to IL-17A-secreting cells that have lost their suppressive function has been shown in several autoimmune diseases including psoriasis (30–32). In animal models of psoriasis, depletion of Treg cells results in increased disease severity (33–35), highlighting their crucial role in disease regulation. Despite this knowledge, there are currently no treatments available specifically targeting Treg cell augmentation in psoriasis. In this study, we investigated a synthesized peptide that combines the signaling domain of CTLA-4 with a cell-penetrating peptide (CPP) to promote the generation of $Foxp3^+$ Treg cells during psoriatic skin inflammation. Our findings indicate that the dNP2-ctCTLA-4 peptide holds promise as a therapeutic agent for psoriasis by inducing endogenous Treg cells *in vivo*.

Results

Induction of $Foxp3^+$ Treg cells by dNP2-ctCTLA-4 peptide

Previously we generated the recombinant protein and a synthetic peptide of the cytoplasmic domain of the cytotoxic T lymphocyte antigen-4 (CTLA-4) in conjugation with a CPP dNP2 (dNP2-ctCTLA-4) (36–38). This protein and peptide showed successful immune modulatory effects on T cell activation during autoimmune or allergic disease with an increase of Foxp3 expressing Treg cells (36, 38–40). Building on this knowledge, we aimed to investigate whether the dNP2-ctCTLA-4 could be effective in treating psoriasis with increased Treg cells. As both Th17 (CD4) and Tc17 (CD8) cells are important pathogenic cells in psoriasis, we examined the functional effects of the dNP2-ctCTLA-4 peptide on mouse CD4 and CD8 T cells *in vitro*. In our experiments, we cultured $TCR\alpha\beta^+CD4^+CD25^-CD62L^{high}CD44^{low}$ naïve CD4 T cells under Treg condition (TGF-β, IL-2) with or without the peptide. The results demonstrated a significant increase in $CD25^+Foxp3^+$ Treg cells when the peptide was present (Figures 1A, B). Furthermore, when we cultured these naïve CD4 T cells under Th17+IL-2 conditions (TGF-β, IL-6, IL-1β, IL-23, IL-2), dNP2-ctCTLA-4 exhibited inhibitory effects on IL-17 production while simultaneously increasing Foxp3 expression (Figures 1C, D). Similar observations were made with $TCR\alpha\beta^+CD8^+CD25^-CD62L^{high}CD44^{low}$ naïve CD8 T cells, which were differentiated under Treg condition (TGF-β, IL-2) in the presence of dNP2-ctCTLA-4. The peptide treatment led to a significant increase in $CD25^+Foxp3^+$ CD8 T cells (Figures 1E, F). Moreover, when naïve CD8 T cells were cultured under Tc17+IL-2 conditions (TGF-β, IL-6, IL-1β, IL-23, IL-2), the peptide demonstrated a reduction in IL-17 production while promoting Foxp3 expression (Figures 1G, H). To investigate whether dNP2-ctCTLA-4 can intrinsically increase TGF-β mediated Foxp3 expression, naïve CD8 T cells were stimulated with anti-CD3 and TGF-β in the presence or absence of dNP2-ctCTLA-4 peptide. The dNP2-ctCTLA-4 peptide reduced SMAD2 linker region phosphorylation and the amount of p-ERK. This result is consistent with our previous research confirmed in the splenocytes (38), suggesting that dNP2-ctCTLA-4 peptide intrinsically enhances TGF-β signaling in CD8 T cells, leading to an increase in $Foxp3^+$ T cells (Figure 1I). In summary, our findings demonstrate that the dNP2-ctCTLA-4 peptide has the ability to induce Foxp3 expression in both CD4 and CD8 T cells under various stimulatory conditions. These results suggest the therapeutic potential of the peptide in modulating Th17/Tc17 responses associated with psoriasis.

Amelioration of psoriasis-like skin inflammation by dNP2-ctCTLA-4

To assess the potential of the dNP2-ctCTLA-4 peptide in inducing Treg cells and alleviating psoriatic skin inflammation, we conducted *in vivo* experiments using the well-established imiquimod (IMQ)-induced psoriasis mouse model (41), which closely resembles human psoriasis (42). The IMQ-induced psoriasis mouse model was established by daily

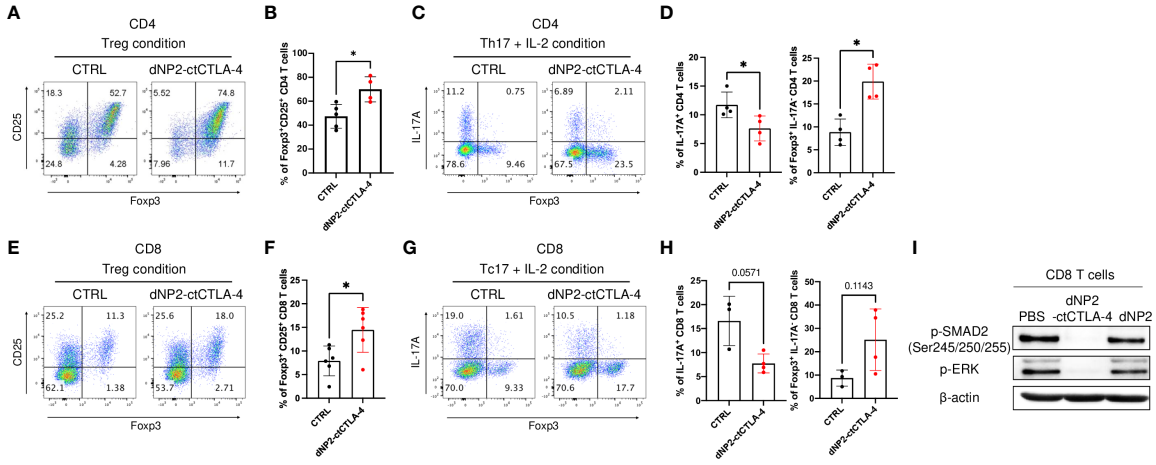

FIGURE 1
dNP2-ctCTLA-4 effectively induces Foxp3$^+$ Treg cells in both CD4 and CD8 T cells. **(A-D)** Mouse naïve CD4 T cells were stimulated with anti-CD3/CD28 mAb in the presence or absence of dNP2-ctCTLA-4 for 3 or 4 days. Representative flow cytometric dot plot **(A)** and bar graphs **(B)** of expression of CD25 and Foxp3 under Treg differentiation condition (n=4~5). Representative flow cytometric dot plot **(C)** and bar graphs **(D)** of expression of IL-17A and Foxp3 under Th17 with IL-2 differentiation condition (n=4). **(E-H)** Mouse naïve CD8 T were stimulated with anti-CD3/CD28 mAb in the presence or absence of dNP2-ctCTLA-4 for 3 days. Representative flow cytometric dot plot **(E)** and bar graphs **(F)** of expression of CD25 and Foxp3 under Treg differentiation condition (n=6). Representative flow cytometric dot plot **(G)** and bar graphs **(H)** of expression of IL-17A and Foxp3 under Tc17 with IL-2 differentiation condition (n=3-4). **(I)** Mouse naïve CD8 T cells were stimulated with anti-CD3/CD28 mAb and TGF-β in the presence of dNP2-ctCTLA-4 or dNP2 peptide. The lysate was analyzed by immunoblotting of the p-SMAD2 linker region and p-ERK. Data are presented as mean ± S.D. Statistical significance was determined by the Mann-Whitney test. n.s. = nonsignificant, *$p < 0.05$.

application of IMQ to the back skin of mice until day-5. Concurrently, the mice received intraperitoneal administration of 100 μg of the dNP2-ctCTLA-4 peptide three times daily from day-1 to 5 (Figure 2A). Our findings demonstrated that the dNP2-ctCTLA-4 peptide significantly inhibited the progression of psoriasis (Figure 2B) and effectively improved the psoriasis area and severity index (PASI) score (Figure 2C). Histological analysis of dorsal tissue samples revealed a reduction in epidermal hyperplasia and immune cell infiltration (Figure 2D). Flow cytometry analysis of skin-draining lymph nodes (SDLN) showed a notable increased in the proportion of Foxp3$^+$ CD4 T cells, accompanied by a reduction in IL-17A-producing γδ T cells (Figures 2E, F). Taken together, these results highlight the potential of the dNP2-ctCTLA-4 peptide to induce Foxp3$^+$ Treg cells *in vivo*, consequently ameliorating psoriasis-like skin inflammation. This induction of Treg cells leads to a significantly increased Treg/Teff ratio (Figure 2G), underscoring the therapeutic efficacy of the dNP2-ctCTLA-4 peptide in the treatment of psoriatic skin inflammation.

Suppression of psoriatic skin inflammation by dNP2-ctCTLA-4, but not CTLA-4-Ig

CTLA-4-Ig (Abatacept) is a US Food and Drug Administration (FDA)-approved drug primarily used for rheumatoid arthritis treatment. It consists of the extracellular domain of CTLA-4 fused with the Fc region of an immunoglobulin (43, 44). By binding to B7 molecules, CTLA-4-Ig effectively inhibits the co-stimulatory signal of T cells, thereby preventing their activation (45). In contrast, the dNP2-ctCTLA-4 peptide utilized in our study employs the cytoplasmic domain of CTLA-4 to modulate intracellular signaling and result in Foxp3-expressing Treg cells. Considering the distinct domains of CTLA-4 targeted by these two agents (Figure 3A), we hypothesized that they would exhibit different mechanisms of action in treating psoriasis. To evaluate this hypothesis, equal doses of CTLA-4-Ig and dNP2-ctCTLA-4 peptide were administered to mice with IMQ-induced psoriatic skin inflammation, following the same protocol as in the previous experiment (Figure 3B). As shown in the figure, CTLA-4-Ig did not effectively inhibit psoriatic skin inflammation, while the dNP2-ctCTLA-4 peptide significantly reduced psoriasis symptoms and PASI score (Figures 3C, D). Additionally, only the group treated with the dNP2-ctCTLA-4 peptide demonstrated a decrease in epidermal layer thickness and infiltration of inflammatory cells (Figure 3E). These compelling findings suggest that the dNP2-ctCTLA-4 peptide, which specifically targets the intracellular signaling events related to the cytoplasmic domain of CTLA-4, would be more effective in regulating psoriatic skin inflammation compared to CTLA-4-Ig. The differential mechanisms of action of these two agents provide further insights into their distinct therapeutic potential for managing psoriasis.

Induction of CD25$^+$ Foxp3$^+$ Treg cells and suppression of IL-17A-producing effector T cells by dNP2-ctCTLA-4, but not CTLA-4-Ig

To investigate the impact of dNP2-ctCTLA-4 peptide and CTLA-4-Ig on lymphoid cells in psoriasis-like skin inflammation, we conducted a flow cytometry analysis. Intriguingly, the dNP2-ctCTLA-4 peptide significantly induced CD25$^+$ Foxp3$^+$ Treg cells, whereas CTLA-4-Ig could not in SDLN (Figures 4A, B). Neither

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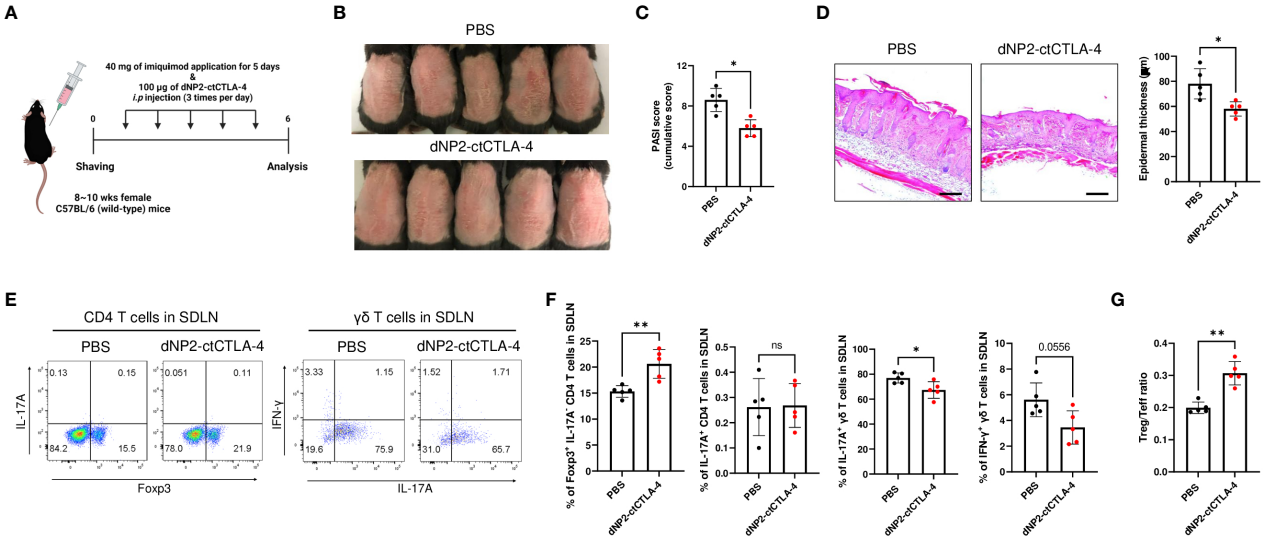

FIGURE 2
dNP2-ctCTLA-4 ameliorates psoriatic skin inflammation, accompanied by an increase in Foxp3$^+$ CD4 T cells. **(A)** Experimental scheme of drug treatment in the imiquimod-induced psoriasis murine model. **(B)** Comparison of representative gross back skin phenotypes after the last IMQ application between the control and dNP2-ctCTLA-4 treated groups (n=5 per group). **(C)** Cumulative PASI scores (erythema, thickness, scaling). **(D)** Histological analysis of H&E-stained back skin samples (left, x100 magnification, scale bar = 200 μm) and epidermal thickness measured by ImageJ software (right). **(E)** Representative dot plots and **(F)** bar graphs of Foxp3, IL-17A, IFN-γ expression of isolated lymphocytes (CD4 T cells, γδ T cells) in SDLN. **(G)** The Treg/Teff ratio in SDLN. Data are presented as mean ± S.D. Statistical significance was determined by Mann–Whitney test. n.s., nonsignificant; *p < 0.05, **p < 0.01.

treatment showed a statistically significant tendency to inhibit the production of IL-17A and IFN-γ in CD4 T cells (Figure 4B). Although the dNP2-ctCTLA-4 peptide resulted in a slightly reduced tendency in the proportion of IL-17A-producing γδ T cells (Figures 4C, D), it exhibited a significantly increased Treg/Teff ratio compared to CTLA-4-Ig, confirming its potential as an effective regulator of psoriatic skin inflammation (Figure 4E). Consistent with these findings, the dNP2-ctCTLA-4 peptide also demonstrated a significant reduction in the absolute cell number of infiltrated CD45$^+$ lymphocytes into the skin tissue, as compared to control group (Figures 4F, G). However, it is worth noting that neither CTLA-4-Ig nor the dNP2-ctCTLA-4 peptide appeared to reduce the proportion of IL-17$^+$ γδ T cells in the skin. Overall, this result suggests that CTLA-4-Ig might not be an effective molecule for controlling psoriatic skin inflammation, whereas the dNP2-ctCTLA-4 peptide could serve as a promising novel therapeutic option, as it exhibits a mechanism of increasing the Treg/Teff ratio.

Distinct mechanisms of action by dNP2-ctCTLA-4 and CTLA-4-Ig during Th17 and Treg differentiation in T cell -antigen presenting cells (APC) coculture system

To gain further insights into the distinct mechanisms underlying T cell regulation by dNP2-ctCTLA-4 peptide and CTLA-4-Ig, we performed experiments utilizing FACS-sorted naïve CD4 T cells derived from 2D2 mice. These cells were stimulated with MOG_{35-55} peptide in the presence of APCs to induce Th17 and Treg cells, while being exposed to either the dNP2-ctCTLA-4 peptide or CTLA-4-Ig. Our findings revealed noteworthy disparities between the two agents. Specifically, the dNP2-ctCTLA-4 peptide demonstrated induction of Foxp3$^+$ Treg cells under Th17 differentiation conditions (IL-6, TGF-β), which was not observed with CTLA-4-Ig (Figures 5A, B). Intriguingly, CTLA-4-Ig exhibited a tendency to inhibit IL-17$^+$ CD4 T cells, a response not observed with the dNP2-ctCTLA-4 peptide. We further examined key functional molecules in Treg functions including CD25 and CD39. Notably, the dNP2-ctCTLA-4 peptide significantly upregulated both CD25 and CD39 expression in Foxp3$^+$ Treg cells compared to CTLA-4-Ig (Figures 5C, D). Moreover, the dNP2-ctCTLA-4 peptide induced CD25$^+$ Foxp3$^+$ Treg cells under Treg conditions, while CTLA-4-Ig failed to elicit a similar response (Figures 5E, F). We observed an increase in the CD25^{high} CTLA-4^{high} population within Foxp3$^+$ Treg cells under Treg conditions when treated with the dNP2-ctCTLA-4 peptide, but not with CTLA-4-Ig (Figures 5G, H). Notably, the expression level of CTLA-4 tended to be increased in Foxp3$^+$ CD4 T cells by the dNP2-ctCTLA-4 peptide, whereas CTLA-4-Ig did not elicit a similar effect (Figures 5I, J). Collectively, these findings highlight that the dNP2-ctCTLA-4 peptide induces Foxp3$^+$ Treg cells with enhanced Treg characteristics, while CTLA-4-Ig suppresses Th17 responses. Thus, our results suggest that the mechanisms underlying the actions of CTLA-4-Ig and the dNP2-ctCTLA-4 peptide are fundamentally different which provides a plausible explanation for the divergent outcomes observed in the psoriasis experiments.

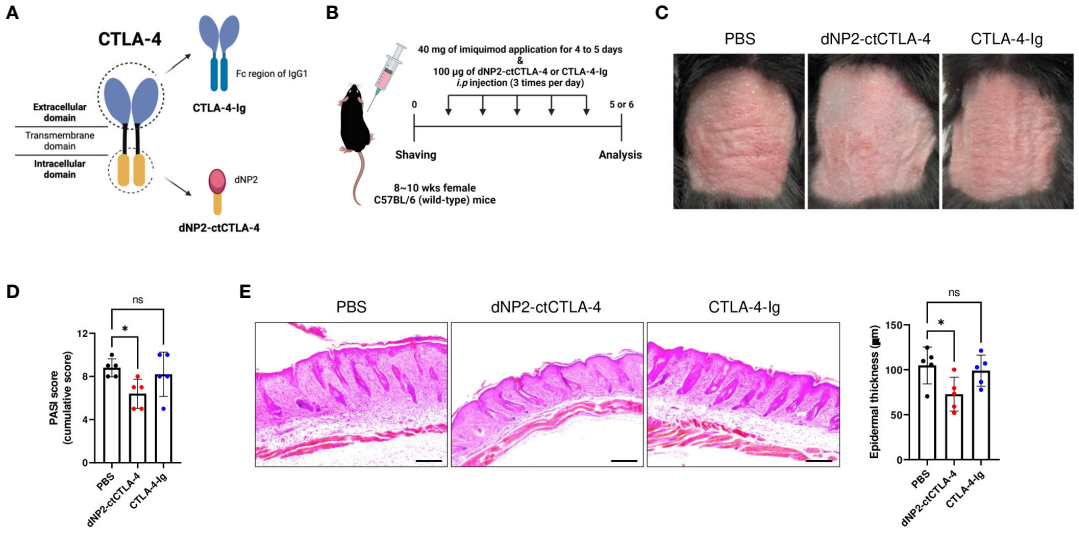

FIGURE 3
dNP2-ctCTLA-4 effectively improves psoriatic skin inflammation, while CTLA-4-Ig does not. **(A)** Schematic images of dNP2-ctCTLA-4 and CTLA-4-Ig utilizing each domain of CTLA-4. **(B)** Experimental scheme of drug treatment in the IMQ-induced psoriasis murine model. **(C)** Comparison of representative back skin phenotypes after last IMQ application between dNP2-ctCTLA4 and CTLA4-Ig treated group (n=5 per group). **(D)** Cumulative PASI scores (erythema, thickness, scaling). **(E)** Histological analysis of H&E-stained back skin samples (left, x100 magnification, scale bar = 200 μm) and epidermal thickness measured by ImageJ software (right). Data are presented as mean ± S.D. Statistical significance was determined by the Kruskal-Wallis test. n.s., nonsignificant; *p < 0.05.

Discussion

In this study, we conducted a comprehensive investigation into the therapeutic potential of a dNP2-ctCTLA-4 peptide utilizing an IMQ-induced psoriasis-like skin inflammation mouse model (Figure 6). Our findings substantiated the effectiveness of dNP2-ctCTLA-4, which employs a CPP fused with the cytoplasmic domain of CTLA-4. The peptide demonstrated its remarkable efficacy in inducing Foxp3-expressing T cells in both CD4 and CD8 T cells by inhibiting the p-ERK and the p-SMAD2/3 linker region *in vitro*. *In vivo* studies further demonstrated its ability to effectively ameliorate the progression of psoriatic skin inflammation and augment the population of $Foxp3^+$ CD4 T cells. Notably, the Treg cells induced by the dNP2-ctCTLA-4 peptide exhibited heightened expression of functional markers, such as CD25, CD39, and CTLA-4 suggesting the induction of potent suppressive Treg cells. In contrast, CTLA-4-Ig, which employs the extracellular domain of CTLA-4, displayed a propensity to reduce Treg cell populations *in vitro* and provided ineffective in inhibiting psoriasis progression. Our findings shed light on Treg induction as a novel strategy for mitigating psoriasis-like skin inflammation and underscore the potential of dNP2-ctCTLA-4 as a promising therapeutic agent for autoimmune diseases, including psoriasis.

Psoriasis, a chronic inflammatory skin disease, is characterized by an immunological imbalance, resulting in the excessive secretion of IL-17A cytokine. This dysregulation occurs through the IL-23/IL-17A axis and involves multiple immune cell types. While IL-17-secreting αβ T-cells (Th17, Tc17) are the major contributor to pathogenesis in human psoriasis patients, γδ T cells are primarily responsible for inflammatory responses in mouse models (46–48). The γδ T cells expressing the Vγ4 and Vγ6 chain are found in the dermal layer of skin tissue (49–51) and known to secrete IL-17A in response to stimuli such as IL-1β and IL-23 (52, 53). They also migrate to inflamed areas by expressing C-C motif chemokine receptor 6 (CCR6) on their surface (54–56). Although there is evidence of Treg cells suppressing the activation of γδ T cells (57–59), the interplay between these cell types remains relatively unexplored. In this study, we demonstrated that dNP2-ctCTLA-4 peptide induces Treg cells in an inflammatory environment. It effectively suppresses IL-17A production by γδ T cells in SDLN, consequently reducing immune cell infiltration into the skin tissue. Furthermore, the dNP2-ctCTLA-4 peptide promotes the generation of $Foxp3^+$ Treg cells while inhibiting IL-17A production during Th17 and Tc17 differentiation *in vitro*. In our previous report, we confirmed that the dNP2-ctCTLA-4 peptide can be delivered to T cells (36, 60) and inhibit T cell activation. We also demonstrated the peptide interferes with PKC-η to increase Treg cells, inhibiting progression of experimental autoimmune encephalomyelitis (EAE) (38). In the induced psoriasis model involving the ear, our findings once again demonstrated the efficacy of the dNP2-ctCTLA-4 peptide in effectively suppressing psoriatic skin inflammation (Supplementary Figures S1A, B in Supplementary Material). Notably, we observed a significant reduction in *Il17a* mRNA expression within the skin (Supplementary Figure S1C in Supplementary Material). Furthermore, we observed that the activation of γδ T cells, which are known to be activated by IL-1β and IL-23, could be effectively inhibited by the dNP2-ctCTLA-4 peptide (Supplementary Figures S1D, E in Supplementary Material). Therefore, we have expected that dNP2-ctCTLA-4 peptide could regulate both IL-17-secreting αβ T cells and IL-$17A^+$ γδ T cells. These results provide compelling evidence that the dNP2-ctCTLA-4 peptide acts as a potent regulator of immune

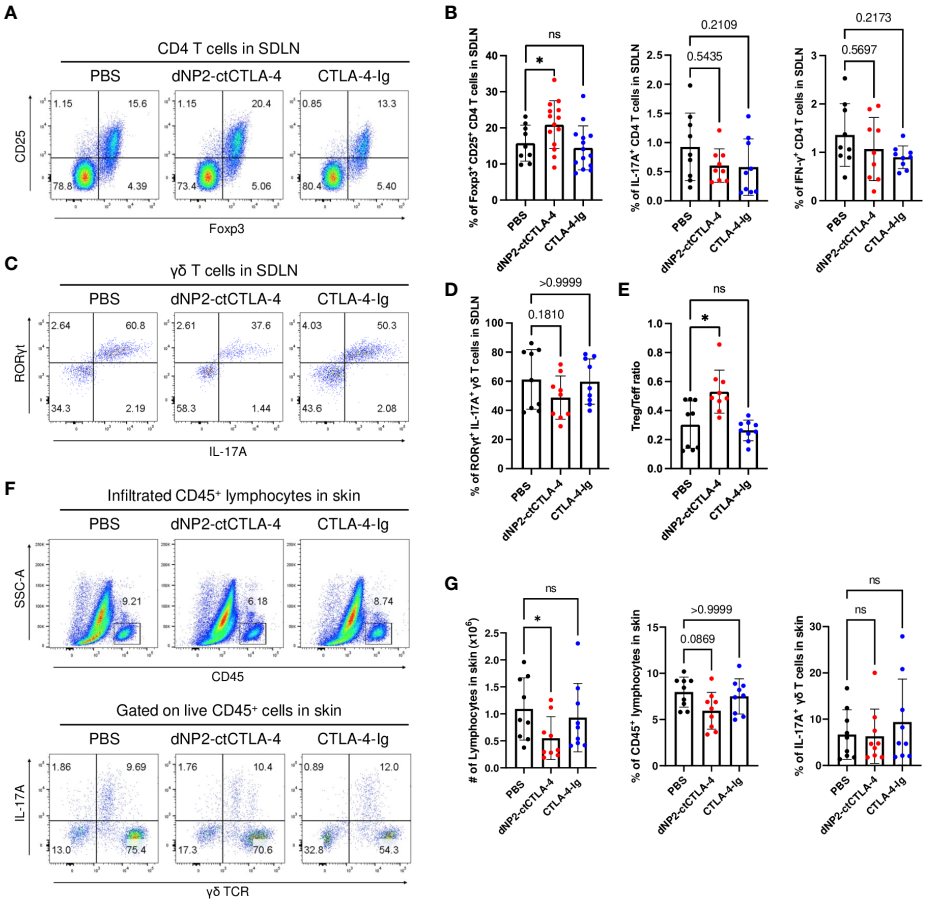

FIGURE 4
dNP2-ctCTLA-4 increases CD25$^+$ Foxp3$^+$ Treg cells in the SDLN of the mice, whereas CTLA-4-Ig does not. **(A)** Representative dot plots and **(B)** bar graphs of flow cytometric analysis in SDLN to identify CD25$^+$ Foxp3$^+$ CD4 T cells (n=9-14 per group), IL-17A$^+$ CD4 T cells (n=9 per group) and IFN-γ$^+$ CD4 T cells (n=9 per group). **(C)** Representative dot plots and **(D)** bar graphs of flow cytometric analysis in SDLN to identify IL-17A$^+$ RORγt$^+$ γδ T cells (n=9 per group). **(E)** The Treg-Teff ratio in SDLN (n=9 per group). **(F)** Representative dot plots and **(G)** bar graphs of flow cytometric analysis in the skin tissue to identify infiltrated lymphocytes, and IL-17A$^+$ γδ T cells (n=9 per group). Data are presented as mean ± S.D. Statistical significance was determined by the Kruskal-Wallis test or Brown-Forsythe and Welch ANOVA tests. n.s., nonsignificant; *p < 0.05.

responses in the context of psoriatic skin inflammation. Its ability to attenuate the expression of IL-17A by Th17 and Tc17, a critical driver of psoriasis pathology, and inhibit the activation of γδ T cells further support its therapeutic potential in this disease.

CTLA-4 (CD152) is an immune checkpoint molecule that plays a crucial role in regulating T cell activity by competing with CD28 signaling to bind to the B7 molecules expressed on the surface of APCs (61–64). CTLA-4-Ig (Abatacept), a drug that combines the extracellular domain of CTLA-4 with the Fc region of an immunoglobulin, has been FDA-approved as a treatment for rheumatoid arthritis, active psoriatic arthritis. Our findings showed that only the dNP2-ctCTLA-4 peptide was effective in increasing Treg cells and Treg/Teff ratio, while CTLA-4-Ig did not show any therapeutic effect. In our T cell-APC coculture experiment, we observed that CTLA-4-Ig successfully suppressed antigen-specific T cell activation. However, when it came to alleviating psoriatic skin inflammation, CTLA-4-Ig did not yield the same results as the dNP2-ctCTLA-4 peptide. This discrepancy may be attributed to the fact that the IMQ-induced psoriasis model predominantly relies on the involvement of γδ T cells. Additionally, several studies have shown that CTLA-4-Ig reduces Treg cells (65, 66) and combination therapies involving rapamycin, which induces Treg cells, have been attempted to overcome this limitation (67). Clinically, CTLA-4-Ig has not been successful in treating psoriasis. Although CTLA-4-Ig was studied in a phase 1 clinical trial for patients with psoriasis vulgaris (NCT00306878, NCT0027722); the study was discontinued in 2011, and no results have been reported. While CTLA-4-Ig is used in patients with psoriatic arthritis, it only had a moderate impact on psoriasis symptoms (68). Moreover, it has been reported that CTLA-4-Ig failed to prevent psoriasis relapse after discontinuation of Ustekinumab treatment (69). Obviously, the cytoplasmic and extracellular domains of CTLA-4 play distinct roles in modulating inflammation, each operating through different mechanisms. The cytoplasmic domain of CTLA-4 encompasses various functional residues, including a crucial lysine residue that interacts with PKC-η protein (70). Our previous study demonstrated that the mutant form of the lysine residue of dNP2-ctCTLA-4 peptide failed to induce Foxp3$^+$ Treg cells and ameliorate the progression of EAE disease, whereas the wild-type form of dNP2-ctCTLA-4 peptide effectively regulated EAE by

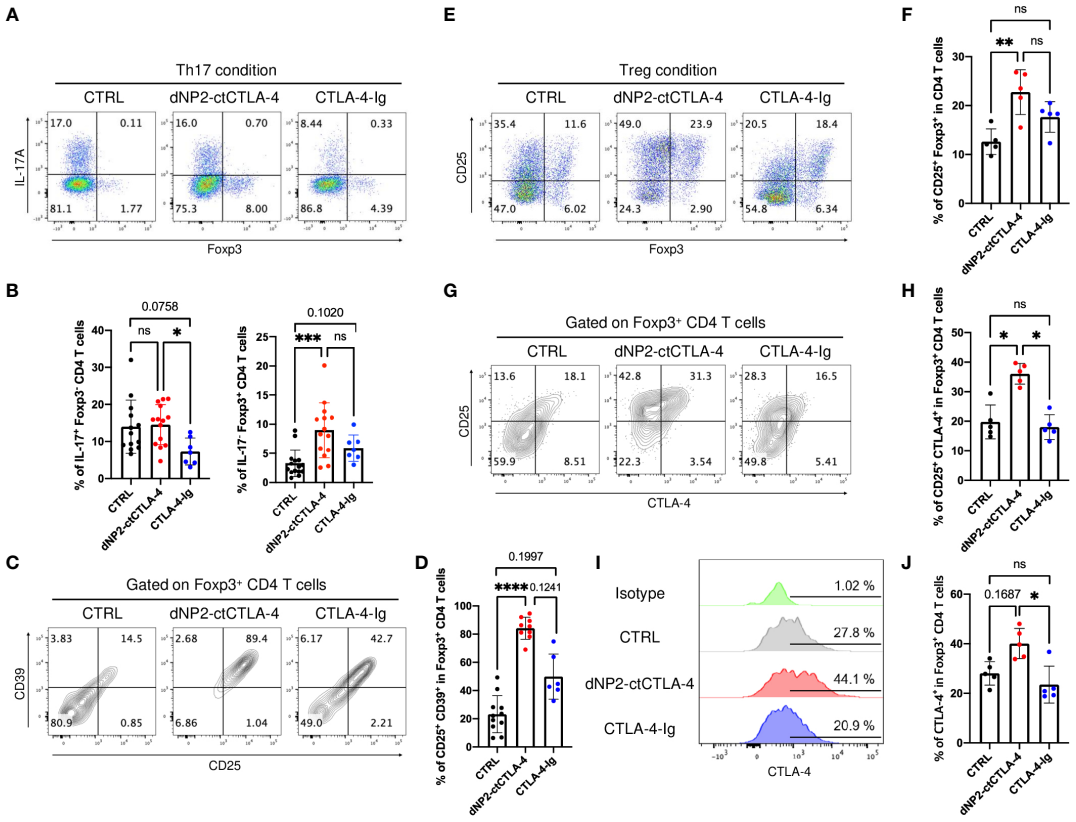

FIGURE 5
dNP2-ctCTLA-4 and CTLA-4-Ig exhibit distinct functional effects on T cell activation and differentiation. **(A-D)** Naïve CD4 T cells from 2D2 mice were stimulated with MOG_{35-55} peptide loaded by APCs in the presence of 3~5 μg/ml of dNP2-ctCTLA-4 or CTLA-4-Ig under Th17 condition. Representative flow cytometric dot plot **(A)** and bar graphs **(B)** of expression of IL-17 and Foxp3 under Th17 differentiation condition (n=6-14). Representative flow cytometric dot plot **(C)** and bar graphs **(D)** of Treg markers including CD25 and CD39 in $Foxp3^+$ CD4 T cells (n=6-11). **(E-J)** Naïve CD4 T cells from 2D2 mice were stimulated with MOG_{35-55} peptide loaded by antigen-presenting cells in the presence of 3~5 μg/ml of dNP2-ctCTLA-4 or CTLA-4-Ig under Treg condition (n=5). Representative flow cytometric dot plot **(E)** and a bar graph **(F)** of Treg induction. Representative flow cytometric dot plot **(G)** and a bar graph **(H)** of Treg marker including CD25 and CTLA-4 in $Foxp3^+$ CD4 T cells. Representative flow cytometric histogram **(I)** and bar graphs **(J)** of CTLA-4 expression in $Foxp3^+$ CD4 T cells. Data are presented as mean ± S.D. Statistical significance was determined by the Kruskal-Wallis test. n.s., nonsignificant; *p < 0.05, **p<0.01, ***<0.001, ****<0.0001.

inducing Treg cells (38). The dNP2-ctCTLA-4 peptide enhances TGF-β-Smad2/3 signaling, leading to increased Foxp3 expression in CD4 T cells through its binding with PKC-η protein. These findings highlight the importance of considering the differential mechanisms of action when designing therapies targeting CTLA-4 for the management of psoriasis.

Biologics have emerged as effective treatments for psoriasis, targeting specific cytokines, such as IL-17A and IL-23 (17, 71). However, despite their success, there are still some non-responders (72, 73) and currently available biologics do not offer a complete cure for psoriasis, often leading to relapse upon discontinuation. Treg cells play a critical role in maintaining immune tolerance and prevent autoimmune diseases. In a notable study, it was demonstrated that inducing Treg cells by topical application of short-chain fatty acids was shown to inhibit psoriatic skin inflammation reducing *Il17a* mRNA levels while increasing *Foxp3* and *Il10* mRNA levels in the skin (74). Moreover, the vitamin D analog, Maxacalcitol, significantly inhibits IMQ-induced psoriasiform dermatitis and reduces IL-17A mRNA expression in the skin, while also increasing $Foxp3^+$ Treg cells (75). These findings support the notion that inducing Treg cells could serve as an effective treatment strategy for psoriasis, offering sustained therapeutic efficacy without recurrence after the cessation of therapy. Therefore, given the potential of Treg cells in regulating immune responses and the observed benefits of Treg cell induction in psoriasis, there is a promising opportunity to develop therapies that specifically target and modulate Treg cells, providing a novel approach for the treatment of psoriasis and other autoimmune conditions. Furthermore, the combination therapy of IL-17 or IL-23 blockade with Treg-inducing molecules such as dNP2-ctCTLA-4 peptide may hold therapeutic potential for more complete regulation of the disease.

Taken together, our findings suggest that the dNP2-ctCTLA-4 peptide holds promise as a novel therapeutic approach for psoriasis, offering the potential to modulate immune responses, suppress inflammation, and mitigate the pathogenic effects associated with controlling IL-17A producing Th17, Tc17, and γδ T cells via increasing Treg cells. Further research is warranted to fully elucidate the underlying mechanisms and explore the translational potential of the dNP2-ctCTLA-4 peptide as a targeted treatment for psoriasis.

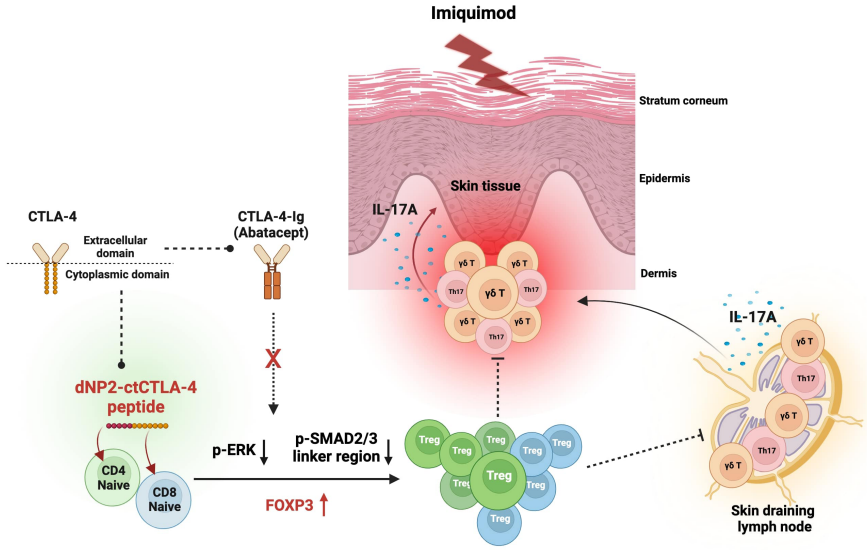

FIGURE 6
Graphical summary of the effects of dNP2-ctCTLA-4 on psoriatic skin inflammation. Diagram created with BioRender (BioRender.com). The dNP2-ctCTLA-4 peptide is composed of dNP2, a cell-penetrating peptide, and the cytoplasmic domain of CTLA-4. In contrast, CTLA-4-Ig (Abatacept) is a fusion protein that combines the Fc region of immunoglobulin IgG1 and the extracellular domain of CTLA-4. The dNP2-ctCTLA-4 peptide induces Foxp3 expression in both CD4 and CD8 T cells by inhibiting the p-ERK and p-SMAD2/3 linker regions. However, CTLA-4-Ig is incapable of inducing Foxp3^{+} Treg cells. The dNP2-ctCTLA-4 peptide can suppress IL-17A production in γδ T cells, Th17 and Tc17 cells while inducing Treg cells, thereby presumably alleviating psoriatic skin inflammation.

Materials and methods

Mice

Female C57BL/6 mice aged 8 to 10 weeks were purchased from Daehan Biolink (DBL, Korea), while 2D2 TCR-transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed and bred in a specific pathogen-free animal facility at Hanyang University under controlled conditions of constant temperature (21 ± 1°C), humidity (50 ± 5%), and a 12-hour light/dark cycle, and provided with regular chow and autoclaved water. All mouse experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committees of Hanyang University (Animal Experiment Approval Number: 2022-0002A) and all mice studies were randomized in a blinded manner in psoriasis mouse models.

Imiquimod-induced psoriasis-like mouse model

To induce psoriasis, 8- to 10-week-old female C57BL/6 mice purchased from Daehan Biolink (DBL, Korea) were used. On Day 0, hair was removed from the dorsal skin using a clipper and hair removal cream. Starting from Day 1, 40 mg of 5% Aldara Cream (3M Pharmaceuticals, Leicestershire, United Kingdom) was topically applied to the dorsal skin for 4 to 5 consecutive days. Psoriasis was induced under deep anesthesia with zoletil (30 mg/kg; Virvac, Carros, France) and rompun (10 mg/kg; Bayer Korea, Ansan, Korea). During disease induction, 100 μg of either dNP2-ctCTLA-4 or CTLA-4-Ig were administered intraperitoneally three times a day. On day 5 or 6, mice were sacrificed and the severity of inflammation in each tissue was assessed using flow cytometry (FACS) or histological analysis with hematoxylin and eosin (H&E) staining. Skin lesion severity was evaluated using the PASI scoring criteria, which considers three signs of erythema, scaling, and thickening, with a scale ranging from 0 (no symptoms) to 4 (very marked). To investigate the effect on ears, we applied 20 mg of 5% Aldara cream daily for six days, and administered 100 μg of protein via intraperitoneal injection. We measured the thickness of the ears using a micrometer (Mitutoyo, Kawasaki, Japan) every day and analyzed the data on day 7.

Skin cell preparation

To acquire single cells, the fat was initially scraped off, and the dorsal skin tissue of the mouse was then separated into the epidermal and dermal layers at 37°C for 90 minutes using 2.4 U/ml of dispase II (04 942 078 001; Roche). After separation, the tissue was cut into small pieces and digested in DMEM media containing 1 mg/ml of collagenase D (11 088 866 001; Sigma-Aldrich), 0.2 mg/ml of DNase I (10 104 159 001; Sigma-Aldrich), and 2% FBS (S 001 01; Welgene) at 225 rpm for 1 hour at 37°C. The enzymatic reaction was stopped by adding 0.5 M ethylenediaminetetraacetic acid (EDTA), followed by filtering through a 40 μm cell strainer and washing with PBS. Single cells were finally obtained, and flow cytometry analysis was performed after antibody staining.

Flow cytometry

Cells isolated from each tissue were analyzed using flow cytometry. Cells were restimulated with Cell Stimulation Cocktail (00-4975-03; eBioscience) to determine intracellular cytokine levels for 4 h at 37°C. To exclude dead cells, cells were stained with the Zombie Aqua Fixable Viability Kit (423101; BioLegend) at room temperature for 10 min. After washing with PBS, the surface proteins were stained with specific monoclonal antibodies for 20 min at 4°C. After washing with PBS, cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (00-5523-00; eBioscience) for 30 min at room temperature. Finally, intracellular cytokine proteins were stained with monoclonal antibodies for 30 min at room temperature. FACS Canto II or FACS Symphony A3 was used to acquire data and FlowJo software version 10.8.0 was used to analyze the data.

Histology

For histological analysis of mouse back skin samples, the tissues were fixed in 4% paraformaldehyde overnight at 4°C, followed by embedding in paraffin. Sections of the skin tissue blocks were prepared and stained with hematoxylin and eosin to reveal the cellular architecture. The resulting H&E-stained sections were then mounted with a cover glass using a mounting solution. Subsequently, the sections were observed under an inverted microscope (Leica DMi8, Leica Microsystems), and images were captured and analyzed using Image J software version 2.0.0

In vitro T cell differentiation

Naïve CD4 T cells ($TCR\alpha\beta^+CD4^+CD25^-CD62L^{high}CD44^{low}$) or naïve CD8 T cells ($TCR\alpha\beta^+CD8^+CD25^-CD62L^{high}CD44^{low}$) were isolated from splenocytes of 8- to 10-week-old C57BL/6 mice using a FACSAria Fusion (BD Biosciences). Purified naïve CD4 T cells were activated with plate-bound anti-CD3 (2 μg/ml; 553057; BD Pharmingen) and anti-CD28 (2 μg/ml; 553294; BD Pharmingen) antibodies and differentiated with the following cytokine cocktails in a 96-well plate for 3 or 4 days: 20 U/ml of IL-2 (212-12; Peprotech), 2 ng/ml of TGF-β (240-B-002; R&D Systems) for Treg differentiation; or 5 μg/ml of anti-IL-4 (11B11; 16-7041-85; eBioscience), 5 μg/ml of anti-IFN-γ (XMG1.2; 16-7311-85; eBioscience), 30 ng/ml of IL-6 (406-ML; R&D Systems), 2 ng/ml of TGF-β, 20 ng/ml of IL-1β (401-ML-010; R&D Systems), 20 ng/ml of IL-23 (1887-ML-101; R&D Systems), 20 U/ml of IL-2 for Th17 differentiation. Additionally, cells were co-treated with 2 μM of dNP2-ctCTLA-4. Purified naïve CD8 T cells were activated with plate-bound anti-CD3 (3 μg/ml) and anti-CD28 (3 μg/ml) antibodies and differentiated with the following cytokine cocktails in a 96-well plate for 3 days: 25 U/ml of IL-2, 0.5 ng/ml of TGF-β for Treg differentiation; or 5 μg/ml of anti-IL-4, 5 μg/ml of anti-IFN-γ, 30 ng/ml of IL-6, 2 ng/ml of TGF-β, 20 ng/ml of IL-1β, 20 ng/ml of IL-23, 20 U/ml of IL-2 for Tc17 differentiation. Additionally, cells were co-treated with 2 μM of dNP2-ctCTLA-4.

In vitro co-culture assay

Naïve CD4 T cells ($CD4^+TCR\text{-}V\beta11^+CD25^-CD62L^{high}CD44^{low}$) were isolated from splenocytes of 8- to 10-week-old 2D2 mice using the CD4 T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions, followed by FACS sorting. The antigen-presenting cells were irradiated with a 3500 rad gamma ray and seeded with the sorted naïve CD4 T cells at a 5:1 ratio. T cells were activated using 40 μg/ml of MOG_{35-55} (AnyGen, Korea), and differentiated using the following cytokine cocktails in a 96-well flat bottom plate for 3 days: 5 μg/ml of anti-IL-4 (11B11; 16-7041-85; eBioscience), 5 μg/ml of anti-IFN-γ (XMG1.2; 16-7311-85; eBioscience), 30 ng/ml of IL-6 (406-ML; R&D Systems), 2 ng/ml of TGF-β (240-B-002; R&D Systems) for Th17 differentiation; or 5 ng/ml of TGF-β for Treg differentiation. Additionally, cells were co-treated with dNP2-ctCTLA-4 or CTLA-4-Ig.

Immunoblotting

Naïve CD8 T cells were isolated and stimulated with 5μg/ml of anti-CD3/CD28 mAb and 5 ng/ml of TGF-β at 37°C, 30 minutes. Afterward, the cells were washed with PBS and lysed with RIPA buffer (Cell Signaling, Beverly, MA) containing 1 μM of NaF and 1 μM of PMSF for 30 minutes on ice. Immunoblotting was performed on PVDF membranes (Bio-Rad) using the following primary antibodies: phospho-Smad2, phospho-ERK, and β-actin mouse mAb.

Recombinant protein purification and peptide synthesis

dNP2-conjugated recombinant proteins were purified as previously described (36). Briefly, dNP2-ctCTLA-4 was purified by Ni-NTA affinity chromatography (Qiagen, Chatsworth, CA, USA) and desalted using a PD-10 Sephadex G-25 column (GE Healthcare, Uppsala, Sweden). To obtain highly purified proteins, an additional ion-exchange protein purification step was performed using SP Sepharose High Performance (GE Healthcare, Uppsala, Sweden). For the peptide form of dNP2-ctCTLA-4 (from AnyGen, Korea), the peptide was synthesized by solid-phase peptide synthesis as previously described (38). The purity of the final peptide products was more than 95%.

RNA extraction and quantitative PCR

To extract RNA from ear tissue, we used TRI Reagent RT (Molecular Research Center, Inc.) following the manufacturer's protocol. The extracted RNA was then quantified using a UV spectrophotometer (CellTAGen, Inc., Seoul, Korea). Subsequently, cDNA synthesis was performed using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). For quantitative RT-PCR

analysis, we used iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with a Bio-Rad CFX Connect real-time PCR detection system. To ensure accuracy, we normalized expression levels to CD45.

Antibodies

The following monoclonal antibodies were used for cell surface staining: anti-mouse CD45 (30-F11; BioLegend), anti-mouse TCRβ (H57-597; eBioscience), anti-mouse TCRγδ (eBioGL3 (GL-3, GL3); eBioscience), anti-mouse Vγ1 (2.11; BioLegend), anti-mouse CD4 (RM4-5; BioLegend), anti-mouse CD25 (PC61; BioLegend), anti-mouse CD27 (LG.3A10; BD Biosciences), anti-mouse CD39 (Duha59; BioLegend), anti-mouse CD44 (IM7; BioLegend), anti-mouse CD62L (MEL-14; eBioscience). The following antibodies were used for intracellular staining: anti-mouse Foxp3 (FJK-16s; eBioscience), anti-RORγt (Q31-415 378; BD Biosciences), anti-mouse IL-17A (eBio17B7; eBioscience), anti-mouse IFN-γ (XMG1.2; eBioscience), anti-mouse CTLA-4 (UC10-4B9; eBioscience).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Mann–Whitney test, Kruskal–Wallis test and Brown-Forsythe and Welch ANOVA tests were employed for data analysis. The data are presented as mean ± S.D. or ± S.E.M., and $p \leq 0.05$ was considered statistically significant. Additional information regarding sample size and specific statistical analyses can be found in the corresponding figure legend.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committees of Hanyang University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

J-MC designed and supervised the project. W-SL conducted and analyzed an experiment using a psoriasis mouse back skin model. K-HN designed comparative analysis study using dNP2-ctCTLA-4 and CTLA-4-Ig by an *in vitro* T cell-APC co-culture assay and also performed the Tc17 experiment. JHK and W-JK initiated this study by analyzing psoriasis mouse ear model experiment and *in vitro* γδ T cell experiments. G-RK provided support recombinant proteins and synthetic peptides and performed *in vitro* Th17, Treg differentiation, and immunoblotting experiment. JEK and E-CS provided valuable technical advice on the psoriasis mouse model and contributed insightful comments on the results. W-SL, K-HN, and G-RK prepared the original draft and J-MC revised the draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1233514/full#supplementary-material

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Inflammatory loops in the epithelial–immune microenvironment of the skin and skin appendages in chronic inflammatory diseases

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The epithelial–immune microenvironment (EIME) of epithelial tissues has five common elements: (1) microbial flora, (2) barrier, (3) epithelial cells, (4) immune cells, and (5) peripheral nerve endings. EIME provides both constant defense and situation-specific protective responses through three-layered mechanisms comprising barriers, innate immunity, and acquired immunity. The skin is one of the largest organs in the host defense system. The interactions between the five EIME elements of the skin protect against external dangers from the environment. This dysregulation can result in the generation of inflammatory loops in chronic inflammatory skin diseases. Here, we propose an understanding of EIME in chronic skin diseases, such as atopic dermatitis, psoriasis, systemic lupus erythematosus, alopecia areata, and acne vulgaris. We discuss the current treatment strategies targeting their inflammatory loops and propose possible therapeutic targets in the future.

KEYWORDS

EIME, atopic dermatitis, psoriasis, systemic lupus erythematosus, alopecia areata, and acne

1 Introduction

The epithelial–immune microenvironment (EIME) provides both constant defense and situation-specific protective responses in several organs, such as the skin, gut, and lungs, which are located at the interface between the environment and an organism (1). The host defense system can be classified into three layers: (constant and nonspecific) barriers, innate immunity, and acquired immunity (2). There are five common elements in the microenvironments of these organs: microbial flora, barriers, epithelial cells, immune cells, and peripheral nerve endings (Figure 1). The interaction between these five elements provides protection against dangers from the environment. This dysregulation can result in the generation of inflammatory loops in chronic inflammatory diseases (1).

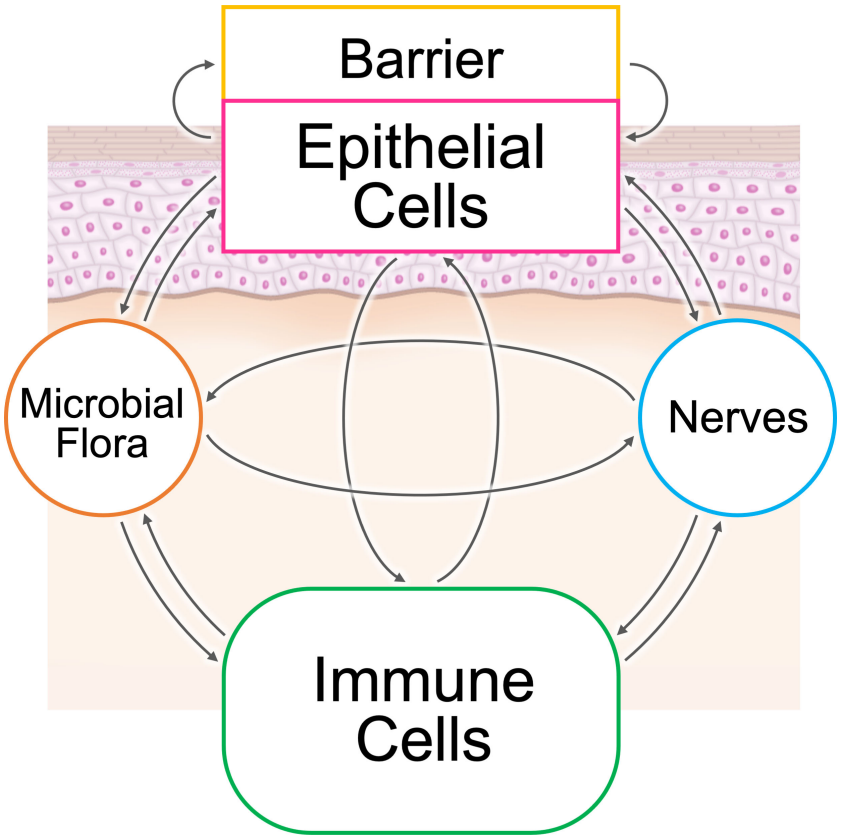

FIGURE 1
The epithelial–immune microenvironment (EIME) of the skin and skin appendages. There are five common elements in the microenvironment of epithelial tissues: microbial flora, barrier, epithelial cells, immune cells, and peripheral nerve endings. The interaction between these five elements provides protection against dangers from the environment.

The skin is one of the largest organs in the host defense system (3). Here, we propose an understanding of the EIME in five chronic skin diseases: atopic dermatitis (AD), psoriasis, systemic lupus erythematosus (SLE), alopecia areata (AA), and acne vulgaris. We discuss current treatments targeting inflammatory loops and propose possible therapeutic strategies for the future.

2 Loops in atopic dermatitis

Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by chronic pruritic eczematous skin lesions (1). AD is an atopic disorder characterized by elevated serum concentrations of immunoglobulin E (IgE) (4). AD has two age peaks (infancy and the third decade of life) in its prevalence and is spontaneously ameliorated (1). The onset of AD is often followed by serial occurrence of allergic diseases that represent the atopic march (5). AD lesions affect predilection sites including the cubital and popliteal fossae that are predominantly colonized by *Staphylococcus aureus* (1). Topical therapies with moisturizers and corticosteroids are first-line treatment (6). The blockade of interleukin (IL)-4 or IL-13 is highly effective, indicating that T_H2-type inflammation is essential for its pathogenesis (1).

A relationship chart of the elements in type 2 EIME of AD depicts double loops (Figure 2A) (1). This redundancy results in the partial efficacy of IL-4/13 blockade therapies in AD, in contrast to the almost perfect efficacy of the IL-17-blockades in psoriasis. The first is a positive feedback loop between keratinocytes and immune cells. Keratinocytes produce epithelial type 2 mediators including thymic stromal lymphopoietin (TSLP), IL-33, granulocyte–macrophage colony-stimulating factor (GM-CSF), and IL-25. In contrast, type 2 cytokines, such as IL-4 and IL-13, produced by immune cells, activate keratinocytes via IL-4/13 receptors. The other is a positive feedback involving dysbiosis of the microbial flora and peripheral nerve sensing of pruritus. Impaired barrier formation in the skin results in *S. aureus*-predominant dysbiosis in AD. *S. aureus* activates type 2 immune responses. In contrast, IL-4 and IL-13 directly dampen barrier formation via IL-4/13 receptors in keratinocytes, and several type 2 cytokines indirectly damage the skin barrier by activating sensory nerve endings via receptors for IL-4, IL-13, IL-31, IL-33, and TSLP that cause pruritus and subsequent scratching behavior (7). Additional activation of G-protein-coupled receptors (GPCRs) and ion channels in sensory nerve endings may be involved in the itch–scratch cycle in AD (7).

IL-31 from immune cells enhances the release of brain-derived natriuretic peptide (BNP) from dorsal root ganglionic neurons

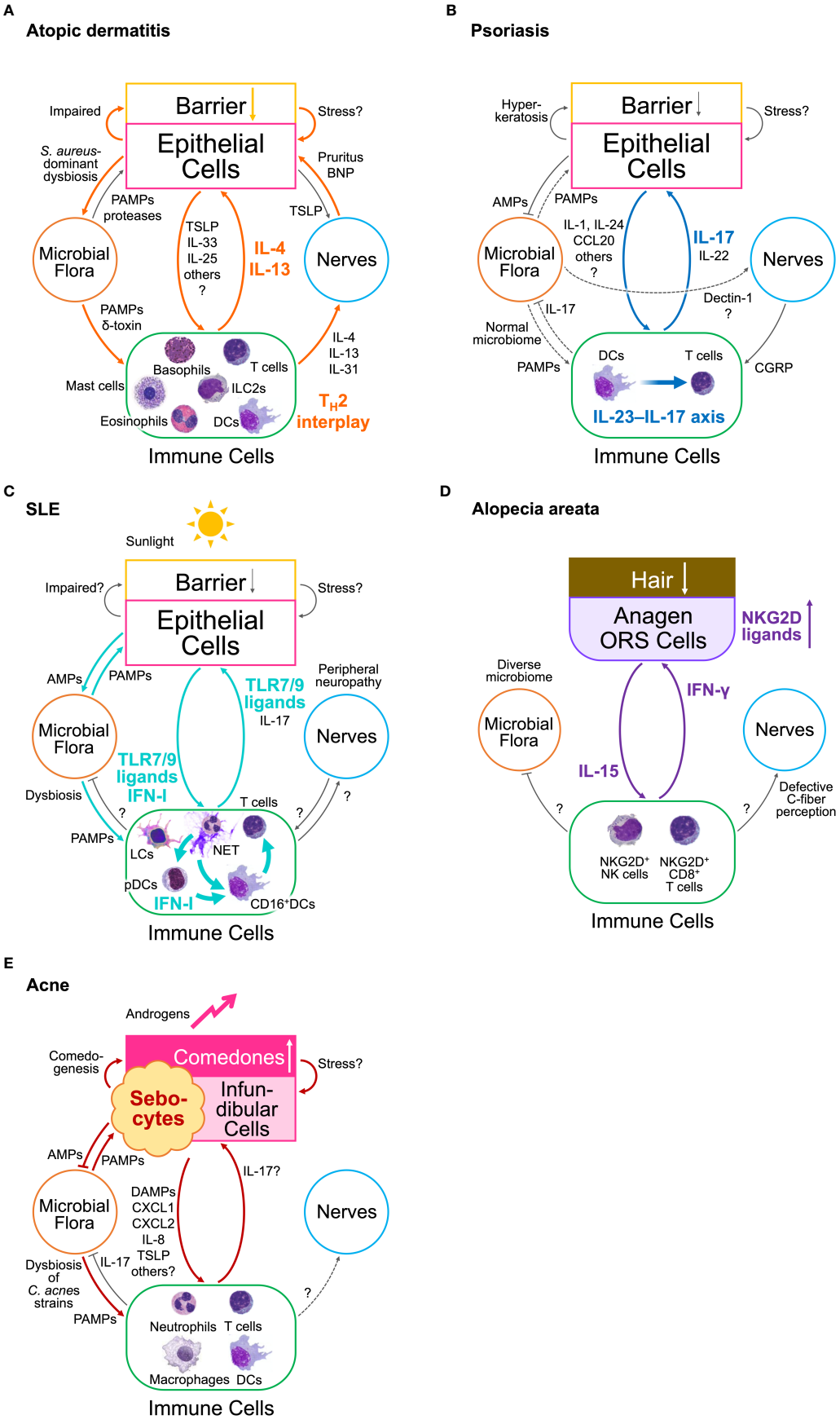

FIGURE 2 (Continued)

FIGURE 2 (Continued)
Inflammatory loops in the epithelial–immune microenvironment (EIME) of chronic inflammatory diseases **(A)** Loops in atopic dermatitis. Two inflammatory loops drive type 2 EIME in skin lesions in AD. One is the loop between epithelial and immune cells, which constructs T_H2 interplay. The other is a loop involving *S. aureus*-dominant dysbiosis and abnormal sensory nerve endings that cause pruritus. **(B)** A loop in psoriasis. A single inflammatory loop between epithelial and immune cells in the interleukin (IL)-23–IL-17 axis drives type 17 EIME in lesional skin in psoriasis. The skin microbiome remains unchanged, suggesting less involvement of microbial flora, whereas *C albicans* colonization elicits a type 17 response by directly stimulating the sensory nerve endings. **(C)** Loops in systemic erythematosus (SLE). An inflammatory loop between epithelial cells, microbial flora, and immune cells is drawn in the EIME of lesional skin in SLE. Another loop may be organized without microbial flora. Keratinocyte damage caused by sunlight or microbial flora can trigger these loops. The plasmacytoid dendritic cells (pDCs) promote these loops only during the initiation phase by releasing type I interferons (IFN-I). The constitutive activation of Toll-like receptor (TLR)7/9 drives type-I IFN loops. Neutrophil extracellular traps (NET) activate pDCs at an early stage and promote disease propagation. **(D)** A loop in alopecia areata (AA). A single inflammatory loop of interferon (IFN)-γ and IL-15 is driven by the EIME of hair follicles (HFs) in the anagen phase. The outer root sheath (ORS) cells of HFs express abnormal or ectopic major histocompatibility complex (MHC) molecules and NKG2D ligands. IFN-γ produced from $NKG2D^+$ T cells and NK cells induce hair loss and promote the expression of these molecules, and IL-15, from the HF ORS cells. IL-15 activates IFN-γ-producing cells. **(E)** Loops in acne vulgaris. The inflammatory loops in acne vulgaris involve sebocytes, infundibular cells, *Cutibacterium acnes*, and immune cells. An increase in androgen levels triggers these loops. Comedogenesis is a bottleneck in acne pathophysiology. AMPs, antimicrobial peptides; BNP, brain-derived natriuretic peptide; CCL, C-C motif ligand; CGRP, calcitonin gene-related peptide; CXCL, C-X-C motif ligand; DCs, dendritic cells; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; LCs, Langerhans cells; LTC_4, leukotriene C4; NET, Neutrophil extracellular trap; NK, natural killer; ORS, outer root sheath; PAMPs, pathogen-associated molecular patterns; pDCs, plasmacytoid dendritic cells; T_H2, T helper type 2; TLR, Toll-like receptor.

(DRGs). BNP induces the activation of glycogen synthase kinase 3 (GSK3) and production of matrix metalloproteinase (MMP)9 in cultured human keratinocytes (8). These results suggest that the activation of sensory nerves directly affects keratinocyte activation and may impair the skin barrier, regardless of the induction of scratching behavior in the EIME of AD.

Basophils are involved in both chronic itch and itch flares in AD (9). In chronic AD skin lesions, keratinocytes produce TSLP that primes basophils to release IL-4, and activation of IL-4 receptors in sensory neurons drives chronic itch. In contrast, during allergen-stimulated AD itch flares, the epithelial barrier disruption allows increased allergen infiltration. $IgE\text{-}R^+$ basophils recruited to the skin release leukotriene C4 (LTC_4) and drive itch sensations via LTC_4 receptors in sensory nerve endings (10).

Keratinocytes play a pivotal role in driving the inflammatory loop of type 2 inflammation in AD (1). Single-cell RNA sequencing of skin lesions from patients with AD who underwent long-term treatment with the IL-4Rα blocker dupilumab demonstrated that transcriptomic dysregulation in keratinocytes was completely normalized, whereas the AD signature in dendritic cells (DCs) and T lymphocytes persisted for up to a year after clinical remission (11). These results suggest that keratinocytes are the major target of dupilumab in AD, and that IL-4/13 signaling in keratinocytes is essential for the inflammatory loop of type 2 EIME in AD, regardless of the persistent activation of DCs and T lymphocytes.

3 Loops in psoriasis

Psoriasis is a common chronic inflammatory disease characterized by both cutaneous and systemic manifestations (1, 12). It is clinically characterized by red scaly papules and plaques, and can be associated with psoriatic arthritis. Its prevalence is estimated to be 1–3% worldwide. Psoriasis typically develops in genetically predisposed middle-aged individuals and is commonly associated with metabolic syndrome. Genetic predisposition is related to keratinocyte pro-inflammatory signaling and type 17 responses. The efficacy of selective biologics targeting tumor necrosis factor (TNF), IL-23, and IL-17 has demonstrated their pivotal roles in the pathogenesis of psoriasis (1).

Psoriasis simulates the protective machinery of the body opposing dermatophytes. The skin removes them together with the stratum corneum by accelerating its turnover and neutrophil attacks, mediated by the T_H17 response, which is called 'psoriasiform dermatitis,' characterized by epithelial hyperplasia and neutrophil infiltration (1).

p38 mitogen-activated protein kinase (MAPK)-dominant activation of the TNF receptor–associated factor 6 (TRAF6) pathway in keratinocytes may be involved in triggering psoriasis (13). Many psoriasis-susceptibility genes, such as *IL36RN* and *CARD14*, are related to skin-specific p38 activation. In addition, psoriasis develops during middle age, and the p38 pathway activation in the skin of aged individuals is more inducible than that of young subjects (14). Furthermore, skin scrubbing elicits psoriatic lesions (Koebner's phenomenon), and physiologically scrubbed stresses immediately induce p38 activation in keratinocytes (15). Moreover, keratinocyte TRAF6 signaling is necessary for releasing proinflammatory cytokines and chemokines, such as IL-1, IL-6, C-X-C motif ligand (CXCL)1, and C-C motif ligand (CCL)20, and for the activation and propagation of the IL-23–IL-17 axis in psoriatic inflammation (16), while the cutaneous p38 activation is sufficient to induce psoriatic inflammation (15).

In contrast to the type 2 EIME in AD, the type 17 EIME in psoriasis depicts a single-loop circuit (Figure 2B) (1). This is consistent with the efficacy of biologics targeting IL-17, IL-23, and TNF in this loop in type 17 EIME in psoriasis. transient receptor potential vanilloid 1 $(TRPV1)^+$ sensory nerves sense *Candida albicans* and drives type 17 protective cutaneous immunity (17). By contrast, microbiota-induced *S. aureus*-specific T_H17 cells accelerate sensory neuronal regeneration (18). However, the fungal and bacterial skin microbiota in lesional skin of patients with psoriasis are similar to those in non-lesional or healthy skin (1). Therefore, despite the bidirectional interaction between skin microbiota and sensory nerves in an acute protective response (17, 18), these two elements do not appear to contribute to the

formation of a closed circuit between other elements in the EIME during chronic inflammation. Collectively, the contribution of skin microbiota and sensory nerves to the inflammatory loop in type 17 EIME remains obscure in psoriasis.

4 Loops in systemic lupus erythematosus

SLE is a systemic syndrome that affects multiple organs including the skin, kidneys, brain, and vasculature, with a profound clinical heterogeneity (19–21). SLE has long been considered a systemic autoimmune disease. However, recent progress suggests that the initial trigger probably involves recognition of self or foreign molecules, especially nucleic acids, by innate sensors (22).

SLE can be spontaneously triggered by exposure to environmental stimuli such as ultraviolet light or infection (21). Dysregulation of apoptosis and nuclear debris clearance is a characteristic of SLE and contributes to multi-organ autoimmunity (23). Studies in mice and humans have shown definitive roles of neutrophils, plasmacytoid DCs (pDCs), Toll-like receptor (TLR) activation, and type I interferon (IFN) production in SLE, and increased IL-17 production may contribute to this process (20).

The skin of patients with SLE shows LC defects and reduced epidermal epidermal growth factor receptor (EGFR) phosphorylation, and topical EGFR ligands reduce photosensitivity (24). These results suggest that a defective Langerhans cell–keratinocyte axis protects against photosensitivity and triggers keratinocyte apoptosis and subsequent events in SLE.

SLE patients display an increased capacity to form neutrophil extracellular traps (NETosis). NETs harboring self- and foreign RNA and DNA antigens are poorly cleared and stimulate pDCs to produce type I IFN via TLR7 and TLR9 stimulation. It induces an innate immune response and the propagation of proinflammatory T_H17 cells that are involved in disease expression and promote NETosis (20). The blockade of type I IFN receptor by treatment with anifrolumab is effective for reducing the disease activity in patients with SLE (25).

In patients with cutaneous lupus erythematosus (CLE), interfollicular keratinocytes exhibit a type I IFN–rich signature in pre-lesional skin (26). pDCs dominated the perifollicular region in non-lesional skin but not in lesional skin. In contrast, $CD16^+$ DCs arise from non-classical monocytes, migrate into the non-lesional skin, and undergo IFN education for inflammation in the CLE.

TLR7 gain-of-function gene mutations and single nucleotide polymorphisms (SNPs) in the TLR trafficking chaperone UNC93B1 are found in patients with SLE (27, 28). Epicutaneous application of TLR7 agonists for four weeks led to a significant increase in *Ifna* expression in the spleen and the development of SLE-like systemic autoimmunity (29). These results indicate that dysregulation of EIME in the skin results in systemic autoimmunity.

Gut barrier defects associated with microbial dysbiosis have been observed in SLE patients and mouse models (30, 31). Additionally, the skin microbiota of patients with SLE is distinct from that of healthy individuals (32, 33). Notably, *S. aureus* skin colonization in epithelial cell–specific IκBζ-deficient ($Nfkbiz^{\Delta K5}$) mice promotes SLE-like autoimmune inflammation via caspase-mediated keratinocyte apoptosis and the subsequent activation of neutrophils and the IL-23–IL-17 axis (34).

Among patients with SLE, 7.6% experience peripheral nervous system events, including peripheral neuropathy (35). SLE may have an early effect on peripheral nerve function in patients without clinical or electrophysiological neuropathy (36). However, little is known about the interactions among peripheral nerves in the EIME of the skin. Peripheral blood mononuclear cells (PBMCs) from SLE patients are highly susceptible to apoptosis induced by calcitonin gene-related peptide (CGRP), a neuropeptide produced by the central and peripheral nerves (37). CGRP from the peripheral nerves drives dermal DCs to produce IL-23 in a type 17 response to cutaneous *C. albicans* infection (17). Notably, increased serum levels of procalcitonin, an alternative transcription product of CGRP, are diagnostic markers of bacterial infection in patients with SLE (38).

Thus, an inflammatory loop of type I IFN between keratinocytes and immune cells emerges in the EIME of the skin in patients with SLE (Figure 2C). Increased susceptibility to keratinocyte cell death induces the release of danger-associated molecular patterns (DAMPs), neutrophil recruitment, and NETosis, which trigger the activation of the TLR7 and TLR9 pathways in pDCs and their production of type I IFN. The type I IFN-rich signature of the skin primes $CD16^+$ DCs and propagates a type 17 immune response involving keratinocyte activation and NETosis. However, the contribution of dysbiosis and peripheral neuropathy and the difference in the role of the type 17 immune response in psoriasis and SLE remain obscure.

5 Loops in alopecia areata

Alopecia areata (AA) is a common, acquired, non-scarring hair loss that affects 2% of the global population and is intractable in severe and relapsing cases (39).

AA is an autoimmune disease resulting from a disruption in hair follicle immune privilege, a structure or a system that protects vital organs, including the central nervous system, testes, placenta, eyes, and hair follicles (HFs), from the potential harm of immune recognition (40). Immune-privileged sites in HFs prevent natural killer (NK) cells from activating them. Specifically, suppressed expression of major histocompatibility complex (MHC) class I and NKG2D ligands in healthy HFs protected them from NK cell attack and subsequent hair loss. In contrast, HFs in patients with AA show abnormal expression of major histocompatibility complex (MHC) class I and II molecules and NKG2D ligands. Indeed, a genome-wide association study (GWAS) in 1,024 patients with AA and 3,278 controls identified *ULBP3*, which encodes an NKG2D ligand, as the responsible gene (41). Histologically, late anagen HFs in patients with AA show perifollicular infiltration of mononuclear cells, including $CD4^+$ or $CD8^+$ $NKG2D^+$ T cells and $CD56^+$ $NKG2D^+$ NK cells (42).

In AA, an inflammatory loop of IFN-γ and IL-15 emerges between immune cells and HF epithelial cells in the EIME of the lesional scalp, and is thought to be the driving force of the disease state (Figure 2D) (43, 44). IFN-γ induces abnormal expression of MHC molecules and NKG2D ligands in the anagen hair bulb,

leading to the collapse of the HF immune privilege. IFN-γ also acts on the HF epithelial cells to enhance the expression of IL-15. The expression levels of IL-15 and IL-15 receptor α in the outer root sheath of HF were higher in patients with AA and in animal models than in healthy controls (45). Furthermore, IL-15 signaling enhances $CD8^+$ memory T cell survival, expansion, and maintenance of T and NK cells, and $CD8^+$ T cell production of IFN-γ (46, 47). Consistently, serum levels of IFN-γ and IL-15 are higher in patients than in controls and correlate with disease activity (46, 47). In contrast, IL-15 prolongs anagen phase, stimulates proliferation, and suppresses apoptosis in the hair matrix of human scalp hair follicles (48). Of note, the IFN-γ pathway depends on Janus kinase (JAK)1/2 and the IL-15 pathway depends on JAK1/3, respectively (43, 44). Therefore, peroral JAK inhibitors selective for either JAK1/3 provide a clear example of the treatment development process via the blockade of the inflammatory loop in EIME (44, 49, 50).

The microbial flora of the lesional scalp may be less involved in AA pathogenesis because the scalp microbiome is more diverse in patients with AA than in healthy controls, but is not significantly different according to the severity of AA (51).

The AA scalp shows defective C-fiber sensory perception (52). However, the involvement of sensory nerves in the EIME of patients with AA remains largely unknown.

6 Loops in acne vulgaris

Acne vulgaris is a chronic inflammatory condition involving the pilosebaceous units of skin on the face, neck, chest, or back (53). Acne vulgaris affects approximately 85% of people aged 12–24 years, 18% of women, and 8% of men aged ≥ 25 years. Acne accounts for approximately 16% of the dermatological disease burden (54), and the global market size is estimated at USD 10.48 billion in 2022 (55). GWAS identified the possible link to genes related to androgen metabolism, inflammation processes, the tumor growth factor-β (TGF-β) pathway, and hair follicle development (56–58). A possible relationship between acne and diet, such as a high-glycemic-load diet or chocolate, has been suggested (59–61).

The development of acne involves the interplay of multiple factors, including (i) hormonal influences on sebum production and composition, (ii) follicular hyperkeratinization, and (iii) inflammation involving colonization with *Cutibacterium acnes* (62, 63). The specific relationship between these key factors remains to be defined, although an older study suggested that inflammation precedes hyperkeratinization (64).

Sebum production by the sebaceous glands is regulated by many factors and is primarily controlled by androgens that are produced both outside (gonads and adrenal glands) and inside the pilosebaceous unit (62, 63). Androgen levels are elevated during the neonatal period and puberty and have a significant impact on triggering the development of acne. Acne is associated with alterations in sebum composition. The sebum of patients with acne contains fewer essential free fatty acids (65) and increased levels of monounsaturated fatty acids (MUFAs) and lipoperoxides that influence keratinocyte proliferation and differentiation compared to healthy people (66–68). Notably, the topical application of fatty acids is sufficient to facilitate follicular hyperkeratosis in animal model (69).

Comedo, a hyperkeratotic plug in the infundibulum, is a diagnostic clue to acne vulgaris and can differentiate it from other acneiform eruptions. Microcomedo is the precursor of all acne lesions and a bottleneck in acne formation (62). However, the inciting event for microcomedo formation remains obscure whereas IL-1α may be involved (64).

C. acnes is a Gram-positive anaerobic/microaerophilic rod and is a commensal organism in the pilosebaceous unit. The amount of *C. acnes* is similar between patients with acne and healthy controls and was not correlated with disease severity; however, the strain populations differed between patients with acne and healthy individuals (70, 71). *C. acnes* and associated lipopolysaccharide (LPS) activate the TLR2/4 pathway and nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) inflammasome and induces the release of proinflammatory mediators, such as IL-8, TNF, IL-1α, IL-1β, and GM-CSF in human sebocytes and keratinocytes. In addition, *C. acnes* promote T_H17 and T_H1 response pathways, which are activated in acne lesions, by inducing the secretion of IL-17A and IFN-γ from $CD4^+$ T cells (63). The type 17 immune response can affect keratinocyte proliferation and differentiation at the infundibulum and sebaceous duct in the pilosebaceous unit, and promote the infiltration of neutrophils via the release of their chemoattractants.

The facial skin of patients with acne is highly innervated and the sebaceous glands express receptors for several neuropeptides. Their activation in human sebocytes modulates cytokine production, cell proliferation, cell differentiation, lipogenesis, and androgen metabolism. The expression levels of substance P in the peripheral nerves and neutral endopeptidase, which degrades substance P, in the sebaceous glands of the facial skin are higher in patients with acne than in healthy individuals (72). However, the interaction between peripheral nerves in the EIME of the skin in acne remains poorly investigated.

In acne, factors other than the five major components of EIME, such as pre-adipocytes and triggering receptors expressed on myeloid cells 2 $(TREM2)^+$ macrophages, have also been suggested (73, 74). Their interplay in the EIME is also expected to be critical for the pathogenesis and treatment of acne.

Thus, more than one inflammatory loops involving the pilosebaceous unit including sebocytes, *C. acnes*, and immune cells, emerge in the EIME of the skin in patients with acne vulgaris (Figure 2E), and may be primarily triggered by hormonal changes that influence sebum composition during puberty.

7 Discussion and concluding remarks

The epithelium senses external factors on the body surface in the earliest stages, determines the type of immune response, and constructs an optimal EIME that is best suited for defense. Epithelial stem cells memorize tissue invasion of the skin and respond rapidly to a second attack (75). This mechanism also induces allergic inflammation in the respiratory tract (76).

If chronic inflammation is a pathological mimic of host defense, the epithelium could also determine the type of inflammation in chronic inflammatory diseases by constructing each EIME, such as type 2 EIME in atopic dermatitis and type 17 EIME in psoriasis. This perspective raises several questions: What determines whether immune responses terminating in healthy skin are also terminated in chronic inflammatory diseases? What mechanisms of the epithelium determine the type of immune response? Do EIME in other epithelial organs, such as the gut and lungs, share common or unique mechanisms that govern biological defense and chronic inflammation?

The EIME concept will facilitate the development of new therapeutic targets for chronic inflammatory diseases because it simplifies the model of each disease. In addition, drawing the EIMEs for multiple diseases will clarify the contradictions involved in each existing model. Targeting disease-specific interrelationships between immune cells and non-immune factors will lead to the development of new therapies in the future.

Author contributions

TD: Writing – original draft, Writing – review & editing. MI: Writing – review & editing.

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Conflict of interest

The 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

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"Input/output cytokines" in epidermal keratinocytes and the involvement in inflammatory skin diseases

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Considering the role of epidermal keratinocytes, they occupy more than 90% of the epidermis, form a physical barrier, and also function as innate immune barrier. For example, epidermal keratinocytes are capable of recognizing various cytokines and pathogen-associated molecular pattern, and producing a wide variety of inflammatory cytokines, chemokines, and antimicrobial peptides. Previous basic studies have shown that the immune response of epidermal keratinocytes has a significant impact on inflammatory skin diseases. The purpose of this review is to provide foundation of knowledge on the cytokines which are recognized or produced by epidermal keratinocytes. Since a number of biologics for skin diseases have appeared, it is necessary to fully understand the relationship between epidermal keratinocytes and the cytokines. In this review, the cytokines recognized by epidermal keratinocytes are specifically introduced as "input cytokines", and the produced cytokines as "output cytokines". Furthermore, we also refer to the existence of biologics against those input and output cytokines, and the target skin diseases. These use results demonstrate how important targeted cytokines are in real skin diseases, and enhance our understanding of the cytokines.

KEYWORDS

epidermal keratinocytes, input cytokines, output cytokines, biologics, inflammatory skin diseases

1 Introduction

In recent years, many biologics targeting cytokines have been clinically used for inflammatory skin diseases. Therefore, we must understand the importance of cytokines in the pathogenesis of the diseases. It is widely known that cytokines mainly function among immunocytes such as lymphocytes, but in fact, epidermal keratinocytes, which are resident cells, also recognize and produce various cytokines.

Epidermal keratinocytes occupy 90% or more of the epidermis, form a physical barrier (1). On the other hand, epidermal keratinocytes also form an innate immunological barrier with the potential to mount an innate immune response. For example, epidermal keratinocytes also express a variety of cytokine receptors, and microbial sensors such as Toll-like receptor (TLR) 1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, MDA5 (melanoma differentiation-associated gene 5) and RIG-I (retinoic acid-inducible gene-I) (2–6). Also, epidermal keratinocytes are capable of producing inflammatory cytokines and chemokines (5, 6). In addition, the cells show antibacterial activity by expressing antibacterial peptides such as defensins, cathelicidin, and S100 proteins (7). Through these immunological functions, epidermal keratinocytes play an important role in the pathogenesis of inflammatory skin diseases including atopic dermatitis (AD), psoriasis, several pustular dermatoses and so on (8–10).

The purpose of this review is to provide foundation of knowledge on the cytokines which are recognized or produced by epidermal keratinocytes. Since a number of biologics for skin diseases have appeared, it is necessary to fully understand the relationship between epidermal keratinocytes and the cytokines. We here focus on pro- or anti-inflammatory cytokines except growth factors in epidermal keratinocytes. The cytokines directly recognized by epidermal keratinocytes are specifically introduced as "input cytokines", and the produced cytokines as "output cytokines". Furthermore, we also refer to the existence of biologics against those input and output cytokines and the target skin diseases. Some of these biologics have already been approved and are in use, while others have not been shown to be effective. Recognizing these findings will enhance our understanding of the cytokines.

2 "Input cytokines" in epidermal keratinocytes

"Input cytokines" in epidermal keratinocytes include IL-1α/β/Ra, IL-4, IL-13, IL-17A/AF/C/F, IL-18, IL-19, IL-20, IL-21, IL-22, IL-24, IL-26, IL-27, IL-31, IL-36α/β/γ/Ra, IL-37, IL-38, IFN-α/β/ε/γ/κ/λ1/λ2/λ3/λ4/ω, oncostatin M (OSM) and TNF-α (Figures 1-3, Table 1).

2.1 IL-1 family cytokines

The IL-1 family consists of 11 cytokines which are further divided into inflammatory cytokines with agonistic activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and anti-inflammatory cytokines with antagonistic activity (IL-1Ra, IL-36Ra, IL-37, IL-38) (11). They are also classified into three subfamilies (IL-1, IL-18, IL-36 subfamily) according to their structures and receptors (11). Most of the human IL-1 family cytokine genes are located on chromosome 2, and the IL-18 and IL-33 genes are located on chromosomes 11 and 9, respectively (12). Among them, epidermal keratinocytes recognize IL-1α, IL-1β, IL-18, IL-36α, IL-36β, IL-36γ, IL-1Ra, IL-36Ra, IL-37, and IL-38 (Figure 1, Table 1).

IL-1α and IL-1β bind to IL-1R1, and this binding signals via TIR-MyD88, leading to NF-κB and MAPK activation (Figure 1, Table 1) (13). IL-1RAcP is the co-receptor for IL-1R1 (Figure 1, Table 1). IL-1-bound IL-1RI associates with IL-1RAcP to form a heterodimer. Signal transduction requires the presence of IL-1RI and IL-1RAcP molecules. IL-1α is produced as precursors and activated by calpain (10). IL-1β is also produced as precursors and

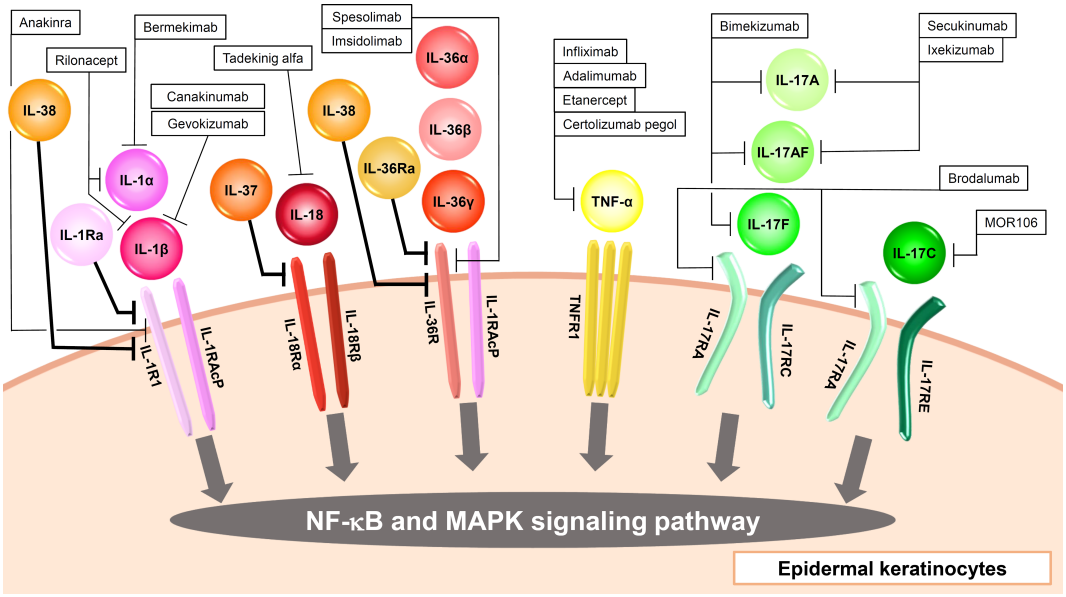

FIGURE 1
Input cytokines which activate NF-κB and MAPK signaling pathway in epidermal keratinocytes.

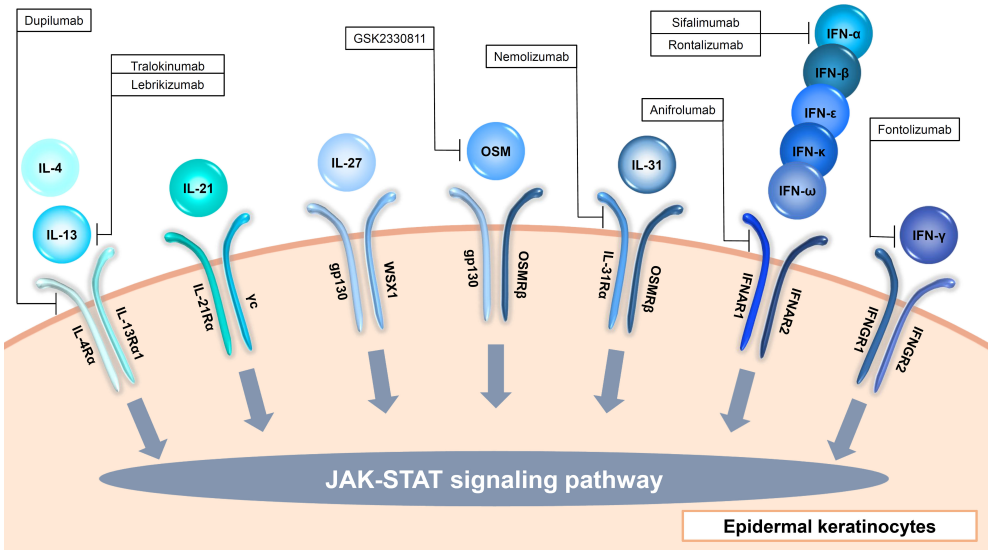

FIGURE 2
Input cytokines which activate JAK-STAT signaling pathway in epidermal keratinocytes (γc cytokines, IL-6 family cytokines, type I IFNs and type II IFN).

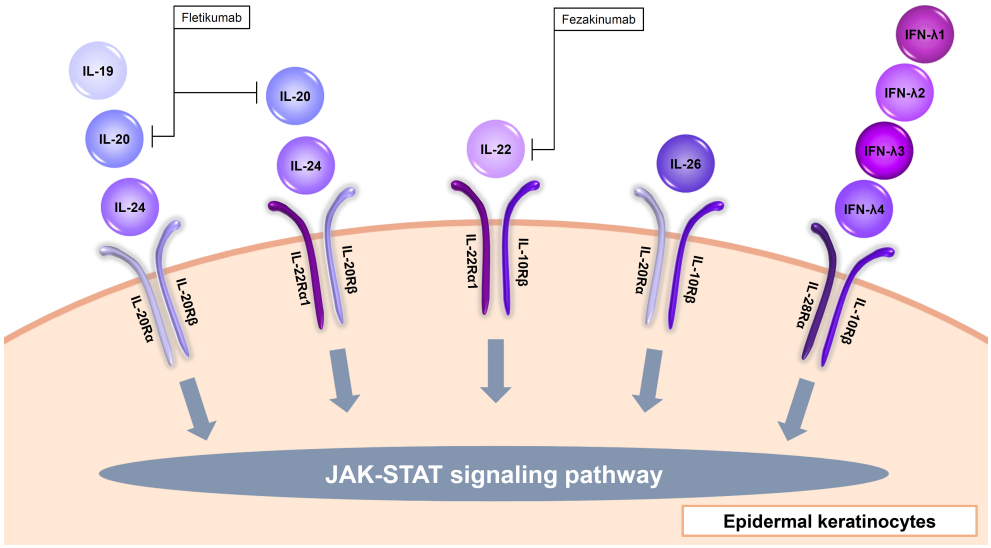

FIGURE 3
Input cytokines which activate JAK-STAT signaling pathway in epidermal keratinocytes (IL-20 family cytokines and type III IFNs).

activated by casepase-1. IL-1α and IL-1β induce inflammatory cytokines including TNF-α and IL-6, and chemokines including IL-8 in epidermal keratinocytes (14). IL-1Ra binds to IL-1R1, and this cytokine work as the inhibitor of IL-1α and IL-1β (Figure 1, Table 1) (13).

IL-1 signaling is thought to play an important role in not only autoinflammatory diseases but also various inflammatory skin diseases. Therefore, it has attracted attention as a therapeutic target. Anakinra, a recombinant IL-1Ra which blocks the activities of the proinflammatory cytokines IL-1α and IL-1β, is clinically used for rheumatoid arthritis (RA), neonatal-onset multisystem inflammatory disease (NOMID), cryopyrin-associated periodic syndromes (CAPS), systemic juvenile idiopathic arthritis (sJIA), adult-onset Still disease (AOSD), Schnitzler's Syndrome (SS), and deficiency of IL-1RA (DIRA) (15). However, it was not significantly effective in a phase II randomized, double-blind clinical trial for palmoplantar pustulosis (PPP) (16). On the other hand, another group reported that anakinra up to 300mg daily showed positive responses with localized and generalized pustular psoriasis (GPP) in a phase II

TABLE 1 Input cytokines in epidermal keratinocytes.

Cytokine	Classification	Receptor	Signaling
IL-1α/β	IL-1 family	IL-1R1/IL-1RAcP	NF-κB and MAPK
IL-1Ra	IL-1 family	IL-1R1	Act as IL-1R antagonist
IL-4	γc family	IL-4Rα/IL-13Rα1	JAK-STAT
IL-13	IL-4 like cytokine	IL-4Rα/IL-13Rα1	JAK-STAT
IL-17A/AF/F	IL-17 family	IL-17RA/IL-17RC	NF-κB and MAPK
IL-17C	IL-17 family	IL-17RA/IL-17RE	NF-κB and MAPK
IL-18	IL-1 family	IL-18Rα/IL-18Rβ	NF-κB and MAPK
IL-19	IL-20 family	IL-20Rα/IL-20Rβ	JAK-STAT
IL-20/24	IL-20 family	IL-20Rα/IL-20Rβ, IL-22Rα1/IL-20Rβ	JAK-STAT
IL-21	γc family	IL-21Rα/γc	JAK-STAT
IL-22	IL-20 family	IL-22Rα1/IL-10Rβ	JAK-STAT
IL-26	IL-20 family	IL-20Rα/IL-10Rβ	JAK-STAT
IL-27	IL-6 family	gp130/WSX1	JAK-STAT
IL-31	IL-6 family	IL-31Rα/OSMRβ	JAK-STAT
IL-36α/β/γ	IL-1 family	IL-36R/IL-1RAcP	NF-κB and MAPK
IL-36Ra	IL-1 family	IL-36R	Act as IL-36R antagonist
IL-37	IL-1 family	IL-18Ra	Act as IL-18R antagonist
IL-38	IL-1 family	IL-1R1 or IL-36R	Act as IL-1R or IL-36R antagonist
IFN-α/β/ε/κ/ω	Type I IFN	IFNAR1/IFNAR2	JAK-STAT
IFN-γ	Type II IFN	IFNGR1/IFNGR2	JAK-STAT
IFN-λ1/λ2/λ3/λ4	Type III IFN	IL-28Rα/IL-10Rβ	JAK-STAT
OSM	IL-6 family	gp130/OSMRβ	JAK-STAT
TNF-α	TNF family	TNFR1	NF-κB and MAPK

open-label trial (17). Anakinra has also been used in hidradenitis suppurativa (HS) by several groups with controversial results (10). Rilonacept is an IL-1 receptor fusion protein consisting of the Fc portion of human IgG1 and the human IL-1 receptor which traps both IL-1α and IL-1β, and clinically used for familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and recurrent pericarditis (15). A clinical trial for cold contact urticaria (CCU) is currently ongoing with this agent (18). Canakinumab is a human anti-IL-1β monoclonal antibody, and clinically used for FCAS, MWS, CAPS, familial Mediterranean fever (FMF), mevalonate kinase deficiency (MKD), tumor necrosis factor receptor-associated periodic syndrome (TRAPS), and AOSD (15). Canakinumab has also shown contradictory efficacy results in HS (10). In an open-label prospective study, this agent was effective for pyoderma gangrenosum (PG) (10). Bermekimab, a human anti-IL-1α monoclonal antibody, showed efficacy in phase II open-label studies in HS patients (10). Gevokizumab is a humanized anti-IL-1β monoclonal antibody, and clinical trials for PG are currently ongoing with this agent (18).

IL-18 binds to IL-18Rα, and this binding signals via TIR-MyD88, leading to NF-κB and MAPK activation (Figure 1, Table 1) (13). IL-18Rβ is the co-receptor for IL-18Rα (Figure 1, Table 1). Like IL-1 signaling, the signal transduction requires the heterodimerization of IL-18Rα and IL-18Rβ (Figure 1, Table 1) . IL-18 is produced as precursors and activated by casepase-1 (10). Epidermal keratinocytes express IL-18Rα and IL-18Rβ. When IL-18 binds to these receptors on the surface of keratinocytes, it triggers a signaling cascade within the cells, leading to various cellular responses such as the induction of CXCL9, CXCL10, CXCL11, major histocompatibility complex (MHC) class I, and MHC class II expression (19, 20). IL-18 is considered to be involved in the pathogenesis of psoriasis, AD, and AOSD, and tadekinig alfa, a human recombinant IL-18-binding protein, is currently investigated in a phase II open-label clinical trial on patients with AOSD (18).

IL-36α, IL-36β, and IL-36γ bind to IL-36R, and these binding signal via TIR-MyD88, leading to NF-κB and MAPK activation (Figure 1, Table 1) (13). IL-1RAcP is the co-receptor for IL-36R

(Figure 1, Table 1). Like IL-1 and IL-18 signaling, the signal transduction requires the heterodimerization of IL-36R and IL-1RAcP (Figure 1, Table 1). IL-36 cytokines are produced as precursors and activated by neutrophil-derived proteases (10). Similar to IL-1α and IL-1β, IL-36α, IL-36β, and IL-36γ induce TNF-α, IL-6, IL-8, G-CSF, GM-CSF, CXCL1, CXCL10, CCL20, and RANTES in epidermal keratinocytes (21, 22).

IL-36Ra binds to IL-36R, and this cytokine works as the inhibitor of IL-36α, IL-36β, and IL-36γ (Figure 1, Table 1) (13). Deficiency of IL-36Ra develop GPP, which suggests the importance of IL-36 signaling in the disease (23). In fact, spesolimab, a humanized anti-interleukin-36 receptor monoclonal antibody which blocks human IL-36α-, IL-36β-, and IL-36γ-induced IL-36R activation, show significant clinical improvement in GPP (24). Additional studies of spesolimab are currently being performed in patients with PPP and HS (10). Imsidolimab is also a humanized anti-interleukin-36 receptor monoclonal antibody which blocks human IL-36α-, IL-36β-, and IL-36γ-induced IL-36R activation, and clinical trials for HS and GPP are currently ongoing with this agent (10).

IL-37 is an anti-inflammatory cytokine, and reported to suppress the production of CXCL8, IL-6, and S100A7 which are induced by the mixture of five proinflammatory cytokines in human keratinocyte cell line HaCaT cells (25). Extracellularly, IL-37 binds to IL-18Ra and recruits IL-1R8 to form the IL-37/IL-1R8/IL-18Ra complex, inhibiting IL-18R-dependent inflammation (10).

IL-38 is also anti-inflammatory cytokine, and reported to inhibit IL-36γ-induced inflammatory molecules in epidermal keratinocytes (26). IL-38 binds to IL-1RAcP or IL-36R, and works as the inhibitor of IL-1α/β or IL-36α/β/γ, respectively (10).

2.2 Gamma chain cytokines

The γc cytokines family consists of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and among them, IL-4 and IL-21 are input cytokines in epidermal keratinocytes (Figure 2, Table 1) (27). IL-13, another type 2 cytokine which shares IL-4Rα and IL-13Rα1 with IL-4, is also an input cytokine in the cells (Figure 2, Table 1) (28). Th2 cells release IL-4 and IL-13, and type 2 innate lymphoid cells produce IL-13 (29). The IL-4 and IL-13 signaling in the cells decrease the expression of filaggrin, loricrin, an involucrin via JAK-STAT pathway (30, 31). These cytokines also suppress ceramide synthesis and inhibit the expression of elongases which lengthen fatty acid chain in the cells (32–34). In addition, antimicrobial peptides expression is also suppressed by IL-4 and IL-13 in the cells, which enhances the susceptibility to infection (35). Furthermore, IL-4 and IL-13 increase serine protease KLK7 expression and function in the cells (36). Recently, these cytokines were also reported to impair TLRs-mediated barrier functions in the early phases of AD (37). These findings suggest that IL-4 and IL-13 contribute to not only allergic inflammation but also barrier dysfunction. The importance of IL-4 and IL-13 in skin diseases is found in recent biologics. Anti-IL-4Rα antibody dupilumab which blocks both IL-4 and IL-13 signaling and anti-IL-13 antibody including tralokinumab and lebrikizumab show clinical efficacy in AD (38–41). In addition, dupilumab represents significant improvement in prurigo nodularis (PN) (42).

IL-21 is produced by NKT and CD4(+) T cells, and signals via JAK-STAT pathway (Figure 2, Table 1) (43). IL-21R is up-regulated in patients with systemic sclerosis (SSc) and might be involved in the pathogenesis of SSc via induction of VEGF (44). IL-21 is also highly expressed in the skin of individuals with psoriasis, and stimulates epidermal keratinocytes to proliferate and causes epidermal hyperplasia (45).

2.3 IL-6 family cytokines

The IL-6 family consists of 11 cytokines and shares 130-kDa signal-transducing β-receptor subunit gp130, except IL-31 (46–48). All the cytokines activate JAK-STAT signaling pathway. Among them, IL-27 induces CXCL9, CXCL10, CCL2, CCL5, and enhance anti-viral activity in epidermal keratinocytes (49–51). Another IL-6 family member, OSM, is also recognized via gp130 and OSM receptor beta (OSMRβ) by epidermal keratinocytes (Figure 2, Table 1) (52). OSM is produced by T cells, monocytes, macrophages, hepatocytes and endothelial cells (52). OSM is involved with innate immunity, angiogenesis, adhesion, motility, tissue remodeling, cell cycle and transcription in epidermal keratinocytes (52, 53). Since this cytokine show synergy with TNF-α, IL-1α, IL-17A, and IL-22 in production of antimicrobial peptides, it is considered to be involved in pathogenesis of psoriasis (53). OSM is also implicated in the pathogenesis of SSc, and a randomized phase 2 study ofan anti-OSM monoclonal antibody GSK2330811 in SSc was conducted. However, its effects were not different from placebo (54). IL-31 is also an input cytokine which signals through heterodimeric receptors composed of the OSMRβ and the interleukin 31 receptor alpha (IL-31Rα) (Figure 2, Table 1) (48). IL-31 is mainly produced by Th2 cells, and suppresses the skin barrier protein expression such as filaggrin and involucrin and induces the expression of several chemokines in epidermal keratinocytes (55, 56). IL-31Rα is also expressed in sensory nerves and IL-31 promotes nerve fiber extension, suggesting that IL-31 is involved in pruritus in AD (57). Actually, nemolizumab, a humanized monoclonal antibody against IL-31Rα which blocks signaling from IL-31, provides improvement of pruritis in patients with AD in a 16-week, double-blind, phase 3 trial (58).

2.4 IL-17 family cytokines

The IL-17 family consists of 6 homodimers IL-17A to IL-17F and 1 heterodimer IL-17AF (59). On the other hand, the IL-17 receptor family consists of 5 molecules, IL-17RA-RE (59). IL-17RA is a common receptor and forms heterodimeric complexes with IL-17RB, IL-17RC and IL-17RE. Epidermal keratinocytes recognize IL-17A, IL-17C, IL-17F, and IL-17AF and then strongly produce inflammatory cytokines, chemokines, and antimicrobial peptides (Figure 1, Table 1) (60). IL-17A, IL-17AF, and IL-17F are mainly produced by Th17 cells, and share the heterodimeric receptor of IL-17RA and IL-17RC (Figure 1, Table 1) (59). Binding of these

cytokines to their receptors recruits Act1 to which TRAF6 binds. TAK1-NF-κB and MAPK-AP1 axes are activated downstream of TRAF6. IL-17C are produced by epithelial cells rather than immune cells, and binds to the heterodimeric receptor of IL-17RA and IL-17RE, and shows similar activation to IL-17A (Figure 1, Table 1). However, the ability to induce inflammation in epidermal keratinocytes is reported to be stronger in the order of IL-17A, IL-17AF, IL-17F, and IL-17C (60). These cytokines, especially IL-17A, are considered to play a critical role in the pathogenesis of psoriasis, and anti-IL-17A antibody including secukinumab and ixekizumab, anti-IL-17A/IL-17F antibody bimekizumab, and anti-IL-17RA antibody brodalumab which blocks the signaling of IL-17A, IL-17A/F, IL-17F, IL-17C, and IL-17E, show high clinical efficacy in psoriasis (61–64). Secukinumab and bimekizumab are also reported to be clinically effective in HS (65, 66).

2.5 IL-20 family cytokines

The IL-20 family consists of IL-19, IL-20, IL-22, IL-24, IL-26, and type III IFNs. IL-19, IL-20, and IL-24 signal through the IL-20Rα/IL-20Rβ heterodimer. Furthermore, IL-20 and IL-24 also signal through the IL-22Rα1/IL-20Rβ heterodimer (Figure 3, Table 1) (67). IL-19, IL-20, and IL-24 is mainly produced by myeloid cells but can also be produced by epidermal keratinocytes (68). TNF-α and IFN-γ enhance IL-20Rα expression in the cells (69). These cytokines all induce epidermal keratinocytes to proliferate and to express inflammatory and immunomodulatory mediators through activation of STAT3 (67). IL-20 was considered to be involved in the pathogenesis of psoriasis, and a phase I study with an anti-IL-20 monoclonal antibody fletikumab for psoriasis was conducted, however the study was terminated due to lack of efficacy (70).

IL-22 is known to exert protective functions in barrier defense, tissue repair, and homeostasis depending on the context, in various organs including the skin (71). Epidermal keratinocytes recognize IL-22 through the IL-22Rα1 and IL-10Rβ heterodimer (Figure 3, Table 1) (72). IL-22 is mainly produced by Th1, Th17, and Th22 cells and also type 3 innate lymphoid cells (72–74). IL-22 up-regulates, in a dose-dependent manner, the expression of S100A7, S100A8, S100A9, a group of proinflammatory molecules belonging to the S100 family of calcium-binding proteins, as well as the matrix metalloproteinase 3, the platelet-derived growth factor A, and the CXCL5 chemokine (75). IL-22 also down-regulates the expression of genes associated with keratinocyte differentiation such as filaggrin (75). In addition, IL-22 strongly induces hyperplasia of reconstituted human epidermis (75). Therefore, IL-22 is considered to contribute to the acanthosis in psoriasis and lichenification in AD. However, the inhibitors of and IL-22 (fezakinumab) did not show sufficient improvement in psoriasis (70). On the other hand, fezakinumab, anti-IL-22 antibody, showed clinical efficacy in moderate-to-severe AD (76).

IL-26 is an input cytokine in epidermal keratinocytes. IL-26 is produced mainly by Th1, Th17, or natural killer cells (77, 78). IL-26R is a heterodimer composed of two receptor proteins: IL-20Rα and IL-10Rβ (Figure 3, Table 1) (79). IL-26 enhances the production of FGF1, FGF2, and FGF7 from epidermal keratinocytes and vascular endothelial cells (80). These may promote angiogenesis in patients with T cell-mediated skin inflammation, including psoriasis (80). IL-26 enhanced IL-8, IL-1β, CCL20, IL-33, and β-defensin 2 expression via JAK1, JAK2, and TYK2 in normal human epidermal keratinocytes (81). These may be involved in the pathogenesis of AD (81).

2.6 type I interferons

Type I interferon (IFN) members consist of IFN-α, IFN-β, IFN-ϵ, IFN-κ and IFN-ω, and bind to the heterodimeric receptor of IFN-α/β receptor 1(IFNAR1) and 2(IFNAR2), resulting in the activation of JAK1 and non-receptor tyrosine kinase 2 (TYK2) and the formation of STAT1-STAT2-IRF9 complex which is called ISGF3 (82). Almost all cell types produce type I IFNs (82). Since epidermal keratinocytes express both IFNAR1 and IFNAR2, the cells recognize type I IFNs (Figure 2, Table 1) (83). For example, IFN-κ induces IFN-κ expression itself and enhances the anti-viral activity against HSV-1 in epidermal keratinocytes (84). In addition, IFN-α and IFN-κ increase IL-6 production in the cells, which is considered to be associated with the pathogenesis of cutaneous lupus erythematosus (85).

2.7 type II interferon

Epidermal keratinocytes also recognize Type II IFN, IFN-γ which is produced by T cells, B cells, NK cells, NKT cells, and dendritic cells (86). IFN-γ binds to the heterodimeric IFN-γ receptor (IFNGR) complex comprising IFNGR1 and IFNGR2 (Figure 2, Table 1) (86). The signal phosphorylates and activates JAK1, JAK2, and STAT1, which leads to the homodimerization of STAT1 (86). Stimulation with IFN-γ in epidermal keratinocytes increases terminal differentiation of cells, inhibits proliferation, and enhance anti-viral activities (87, 88). Furthermore, IFN-γ cooperates with TNF-α and IL-17A to induce the production of cytokines, chemokines, and antimicrobial peptides (89–91). IFN-γ is increased in the skin lesions of psoriasis, and the disease was previously considered to be a Th1 disease. Therefore, a clinical trial with humanized anti–IFN-γ antibody (Fontolizumab) for moderate-severe plaque psoriasis was performed, however, no significant clinical changes were observed (92).

2.8 type III interferons

Type III IFNs, including IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4, are involved in inhibiting viral infection similar to type I IFNs (93, 94). Type III IFNs act via the heterodimer of IL-28Rα and IL-10Rβ (Figure 3, Table 1) (93). These cytokines are input cytokines in epidermal keratinocytes, for example, IFN-λ1 is shown to enhance anti-viral activity through an increase in TLR3 in the cells (95).

2.9 tumor necrosis factor

Epidermal keratinocytes recognize TNF-α. TNF-α was discovered as a necrotic cytokine in solid tumors and later turned out to be a major cytokine involved in inflammation (96). TNF-α is produced from almost all cells, and are thought to exist both upstream and downstream of the pathological cascade of various inflammatory diseases. Since epidermal keratinocytes express TNFR1 receptors and produce TNF-α, autocrine phenomena are observed and an inflammatory loop is formed (Figure 1, Table 1) (97). The importance of TNF-α in various skin diseases is easily found in the clinical use of anti-TNF-α antibodies against the diseases. TNF-α inhibitors including infliximab, adalimumab, etanercept, and certolizumab pegol are clinically effective in psoriasis (8). Infliximab and adalimumab also show clinical efficacy in HS, and PG (98–101).

3 "Output cytokines" in epidermal keratinocytes

"Output cytokines" in epidermal keratinocytes include IL-1α/β/Ra, IL-6, IL-7, IL-15, IL-17C, IL-17E (IL-25), IL-18, IL-19, IL-20, IL-24, IL-33, IL-34, IL-36α/β/γ/Ra, IL-37, IL-38, IFN-α/β/ε/κ/λ1, thymic stromal lymphopoietin (TSLP), and TNF-α (Figure 4, Table 2).

3.1 IL-1 family cytokines

Epidermal keratinocytes produce inflammatory IL-1 family cytokines with agonistic activity including IL-1α, IL-β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ and anti-inflammatory IL-1 family cytokines with antagonistic activity including IL-1Ra, IL-36Ra, IL-37, and IL-38 (Figure 4, Table 2). Since IL-1α, IL-β, IL-18, IL-36α, IL-36β, and IL-36γ are also input cytokines and are capable of inducing themselves, inflammation loops are formed in the cells (Figures 1, 4, Tables 1, 2) (14, 102).

IL-33 is an IL-1-family cytokine that is over-expressed in the keratinocytes of patients with AD (103, 104). IL-33 is also in an activated state in the precursor and is rather inactivated when cleaved by caspase-1 or caspase-3 (105). IL-33 activates type 2 innate lymphoid cells which induce type 2 inflammation by producing IL-5 and IL-13 (103, 106). Therefore, IL-33 is thought to be involved in the pathogenesis of AD. However, anti-IL-33 antibody LY3375880 and etokimab or anti-IL-33 receptor ST2 antibody astegolimab did not show significant clinical improvement in AD (107–109).

3.2 Gamma chain cytokines

Among γc cytokines, epidermal keratinocytes produce IL-7 and IL-15 (Figure 4, Table 2) (27). IL-7 is produced under the stimuli with IFN-γ (110). IL-15 expression is increased in vitiligo epidermis, and is induced by oxidative stress via NF-κB (111). IL-7 and IL-15 derived from hair follicle keratinocytes regulate skin-resident memory T cell homeostasis (112). In a mouse model of alopecia areata, blockade of IL-7 signaling with anti-mouse IL-7Rα antibody suppressed inflammatory responses and reversed alopecia areata (113). Also, in a mouse model of vitiligo, blocking IL-15 signaling with an antibody reversed the disease symptoms (114).

Epidermal keratinocytes express TSLP which is an epithelial-derived IL7-like cytokine and initiate or perpetuate the Th2-type allergic inflammation via dendritic cells or group 2 innate lymphoid cells (Figure 4, Table 2) (115, 116). TSLP mediates STAT5 phosphorylation via kinases JAK1 and JAK2 (Table 2) (117). The

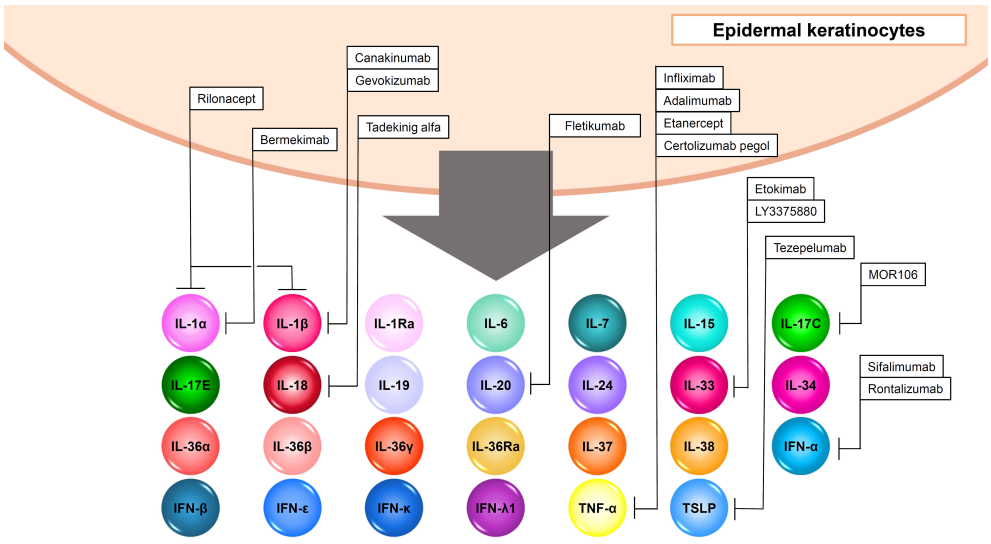

FIGURE 4
Output cytokines in epidermal keratinocytes.

TABLE 2 Output cytokines in epidermal keratinocytes.

Cytokine	Classification	Receptor	Signaling
IL-1α/β	IL-1 family	IL-1R1/IL-1RAcP	NF-κB and MAPK
IL-1Ra	IL-1 family	IL-1R1	Act as IL-1R antagonist
IL-6	IL-6 family	IL-6R/gp130	JAK-STAT
IL-7	γc family	IL-7Rα/γc	JAK-STAT
IL-15	γc family	IL-2/15Rβ/γc	JAK-STAT
IL-17C	IL-17 family	IL-17RA/IL-17RE	NF-κB and MAPK
IL-17E	IL-17 family	IL-17RA/IL-17RB	NF-κB and MAPK
IL-18	IL-1 family	IL-18Rα/IL-18Rβ	NF-κB and MAPK
IL-19	IL-20 family	IL-20Rα/IL-20Rβ	JAK-STAT
IL-20/24	IL-20 family	IL-20Rα/IL-20Rβ, IL-22Rα1/IL-20Rβ	JAK-STAT
IL-33	IL-1 family	ST2/IL-1RAcP	NF-κB and MAPK
IL-34	CSF-1-like	CSF-1R Syndecan-1 PTP-ζ	JAK-STAT NF-κB and MAPK, Caspase, AMPK/ULK1, PI3K/AKT
IL-36α/β/γ	IL-1 family	IL-36R/IL-1RAcP	NF-κB and MAPK
IL-36Ra	IL-1 family	IL-36R	Act as IL-36R antagonist
IL-37	IL-1 family	IL-18Ra	Act as IL-18R antagonist
IL-38	IL-1 family	IL-1R1 or IL-36R	Act as IL-1R or IL-36R antagonist
IFN-α/β/ε/κ	Type I IFN	IFNAR1/IFNAR2	JAK-STAT
IFN-Λ1	Type III IFN	IL-28Rα/IL-10Rβ	JAK-STAT
TNF-α	TNF family	TNFR1	NF-κB and MAPK
TSLP	IL-7-like cytokine	TSLPR/IL-7Rα	JAK-STAT

levels of TSLP is significantly increased in the lesional skin of AD, indicating that TSLP is important for initiating the systemic Th2 immunity favorable for the development of allergic inflammation (115). Against this background, a randomized phase 2a clinical trial of the anti-TSLP monoclonal antibody tezepelumab in the treatment of moderate-to-severe AD patients was conducted but did not reach the targeted level of efficacy (118).

3.3 IL-6 family cytokines

IL-6, the first cytokine discovered in the IL-6 family, activates the JAK-STAT pathway and induces inflammation (46, 47). Epidermal keratinocytes also produce IL-6 under the stimuli with some TLR ligands, UVB, TNF-α, IL-17, IFN-γ, and so on (Figure 4, Table 2) (119–122). Anti-IL-6 receptor antibody such as tocilizumab shows clinical efficacy in rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), giant cell arteritis (GCA), and Castleman's disease (CD) (123). The efficacy of tocilizumab in morphea, SSc, psoriasis, AD, vitiligo or PG has been also reported in case series, however, higher-level evidences have not been shown in these skin diseases (123).

3.4 IL-17 family cytokines

Epidermal keratinocytes produce IL-17C and IL-17E (IL-25) (Figure 4, Table 2). IL-17C controls the innate immune activity of epithelial cells in an autocrine manner (124). IL-17C is induced by TNF-α, IL-17A, and IFN-γ in epidermal keratinocytes (91). Anti-IL-17C antibody MOR106 showed no significant clinical improvement in AD although it was reported to be effective in mouse experiments (125, 126). IL-17E is produced by various cell types and induces Th2 responses (59). In AD, IL-17E derived from epidermal keratinocytes activates type 2 innate lymphoid cells, which drive IL-13 production (127). Therefore, IL-17E is considered to play an important role in the pathogenesis in AD. IL-17E is shown to be induced by IL-17A and IL-22 in epidermal keratinocytes (Figure 4, Table 2) (128).

3.5 IL-20 family cytokines

As described above, IL-19, IL-20, and IL-24 are input and output cytokines in epidermal keratinocytes (Figures 3, 4, Tables 1, 2). TNF-α, IL-17A, and IL-22 induces IL-19, IL-20, and

IL-24 production in the cells (68, 129–131). These cytokines are considered to enhance psoriatic inflammation (130, 131).

3.6 type I interferons

Type I IFNs including IFN-α, IFN-β, IFN-ϵ, and IFN-κ are also output cytokines in epidermal keratinocytes (Figure 4, Table 2) (84). These cytokines are induced by TLR3 and TLR9 signaling, or Type I IFNs themselves in the cells (2, 84, 132). Since type I IFNs are considered to stimulate myeloid dendritic cells which produce IL-23 and contribute to the pathogenesis of psoriasis, randomized, double-blind, placebo-controlled, phase I study of MEDI-545 (Sifalimumab), an anti-IFN-α monoclonal antibody for plaque psoriasis was performed, however, it showed no significant clinical improvement (133). Sifalimumab was also expected to be a treatment for systemic lupus erythematosus (SLE), but the clinical trial was discontinued in favor of anifrolumab (134). Anifrolumab, a monoclonal antibody that binds to IFNAR1, therefore blocking the activity of all type I IFNs, are demonstrated to improve skin and joint disease activity in patients with SLE (134). Rontalizumab is also a monoclonal antibody, and did not show clinical efficacy including a phase 2 trial in SLE patients (134).

3.7 type III interferons

Among type III IFNs, IFN-λ1 is shown produced by epidermal keratinocytes stimulated with TLR3 ligand poly (I:C) or vesicular stomatitis virus (Figure 4, Table 2) (135). IFNλ and the IFNλ receptor are strongly expressed in the epidermis of cutaneous lupus erythematosus (CLE), SLE, lichen planus (LP) and dermatomyositis (135).

3.8 tumor necrosis factor

TNF-α is also an input and output cytokine as described above (Figures 1, 4, Tables 1, 2). TNF-α is induced by TNF-α itself, IL-1β, IL-17A, TLR ligands including poly (I:C), LPS, flagellin, CpG, ultraviolet light, anisomysin, palmitic acid and so on (4, 131, 136, 137).

3.9 Others

IL-32 is a proinflammatory cytokine which is produced by a variety of cells, including NK cells, T cells, monocytes, and epithelial cells (138, 139). IL-32 expression is increased in the epidermis of AD lesions, and the expression is induced by TNF–α and/or IFN-γ in cultured epidermal keratinocytes (139). However, IL-32 is not secreted by the cells and remains in the cells; therefore, this cytokine cannot be called an output cytokine in epidermal keratinocytes (139). This cytokine is considered to modulate keratinocyte apoptosis and contribute to the pathogenesis of AD (139).

IL-34 is an output cytokine in epidermal keratinocytes (Figure 4, Table 2). It exists as a homodimer consisting of 39 kDa monomers (140). IL-34 has no evident sequence homology with other cytokines (141). Likewise, IL-34 has only a 26% sequence homology with colony-stimulating factor 1 (CSF-1), yet they share a common receptor known as CSF-1R (Table 2) (141, 142). Furthermore, IL-34 has exhibits interactions with two distinct receptors: protein-tyrosine phosphatase (PTP)-ζ, and syndecan-1 (Table 2) (141). Through the investigation of IL-34-deficient (Il34LacZ/LacZ) reporter mice, it was found that keratinocytes and neurons were the main sources of IL-34 (143). Especially, IL-34 is highly expressed in the epidermis during murine embryogenesis (144). CSF-1R is expressed by dendritic cells (DCs) and macrophages, excluding CD11c+ precursors of DCs, whereas PTP-ζ is expressed by neural progenitors, glia, glioblastoma, B cells, and kidney tubular cells (141). Syndecan-1 is expressed by many cancers, such as myeloma, melanoma (141). IL-34 is considered to regulate major cellular functions, including cell adhesion, motility, proliferation, differentiation, survival, metabolism, and cytokine/chemokine expression (141).

IL-39 is a cytokine composed of IL-23Ap19 and Epstein–Barr virus-induced (EBI) 3 heterodimer which was firstly reported in

TABLE 3 Disease names and the abbreviations.

Disease name	Abbreviation
Adult-onset Still disease	AOSD
Atopic dermatitis	AD
Castleman's disease	CD
Cryopyrin-associated periodic syndrome	CAPS
Cold contact urticaria	CCU
Cutaneous lupus erythematosus	CLE
Deficiency of IL-1RA	DIRA
Familial cold autoinflammatory syndrome	FCAS
Familial Mediterranean fever	FMF
Giant cell arteritis	GCA
Generalized pustular psoriasis	GPP
Hidradenitis suppurativa	HS
Juvenile idiopathic arthritis	JIA
Lichen planus	LP
Mevalonate kinase deficiency	MKD
Muckle–Wells syndrome	MWS
Palmoplantar pustulosis	PPP
Prurigo nodularis	PN
Pyoderma Gangrenosum	PG
Rheumatoid arthritis	RA
Schnitzler's syndrome	SS
Systemic lupus erythematosus	SLE
Systemic sclerosis	SSc
Tumor necrosis factor receptor-associated periodic syndrome	TRAPS

2015 (145). This cytokine is shown to be produced by B cell lymphocytes and activate neutrophils (146, 147). Our group researched about the expression of IL-39 in human epidermal keratinocytes, however our ELISA experiment and LC-Ms/Ms analyses did not detect the heterodimeric cytokine IL-39 in epidermal keratinocytes (148). So far, this cytokine cannot be called an output cytokine in epidermal keratinocytes Table 3.

4 Conclusion

In this review, we introduced that epidermal keratinocytes recognize and produce a large number of cytokines and are deeply involved in the pathogenesis of these diseases. The number of output cytokines appears to be lower compared to that of input cytokines in the cells. This might suggest that epidermal keratinocytes are cells that are responsible for innate immunity rather than adaptive immunity, and that they are excellent at functioning as sensor cells rather than the control tower. The immunological functions of epidermal keratinocytes in innate immunity requires further investigation.

We also referred to the existence of biologics against those input and output cytokines and the target skin diseases. Current biologics have a significant impact on immune cells throughout the body, which can lead to side effects such as serious infections. If we could target only cytokines derived from epidermal keratinocytes through the development of drug delivery that specifically acts on cells, it will be possible to suppress only excessive immune reactions in the skin caused by pathological activation of epidermal keratinocytes, which should be a safer treatment.

Author contributions

SM, YK, and KS wrote the manuscript. TM, KT, and MO contributed to writing and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Crosstalk: keratinocytes and immune cells in psoriasis

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In the past, psoriasis was considered a skin disease caused only by keratinocyte disorders. However, the efficacy of immunosuppressive drugs and biologics used to treat psoriasis proves that psoriasis is an immune-mediated disease. Indeed, a variety of immune cells are involved in the pathogenesis of psoriasis, including dendritic cells, Th17 cells, and resident memory T cells. Furthermore, keratinocytes play a role in the development of psoriasis as immune cells by secreting antibacterial peptides, chemokines, tumor necrosis factor-α, interleukin (IL)-36, and IL-23. These immune cells and skin cells interact and drive the aberrant differentiation and proliferation of keratinocytes. This crosstalk between keratinocytes and immune cells critical in the pathogenesis of psoriasis forms an inflammatory loop, resulting in the persistence or exacerbation of psoriasis plaques.

KEYWORDS

psoriasis, crosstalk, immune cell, skin cell, keratinocyte

1 Introduction

Psoriasis is a chronic inflammatory skin disease clinically characterized by indurated scaly erythema and pathologically by abnormal differentiation and proliferation of keratinocytes. Therefore, in the past, psoriasis was considered a skin disease caused only by keratinocyte disorders. However, reports of psoriasis successfully treated with cyclosporine have altered our understanding of the pathogenesis of psoriasis. In addition, the efficacy of immunosuppressive drugs and biologics used to treat psoriasis proves that psoriasis is an immune-mediated disease (1, 2).

To date, many studies have revealed how a variety of immune cells are involved in the pathogenesis of psoriasis. Furthermore, keratinocytes are not only the consequences of immune reactions (namely, phenotype), but also themselves play a role in the development of psoriasis as immune cells. These immune cells and keratinocytes interact, consequently driving the aberrant differentiation and proliferation of keratinocytes.

In this review article, we focus on this crosstalk mechanism and discuss its importance in the pathogenesis of psoriasis.

2 Crosstalk: immune cells to keratinocytes

In the pathogenesis of psoriasis, interleukin (IL)-17 plays a key role. Moreover, IL-17 induces the proliferation and abnormal differentiation of keratinocytes (3). Keratinocytes simulated with

IL-17 and tumor necrosis factor (TNF)-α produce various inflammatory cytokines, chemokines, and antibacterial peptides (AMPs) (4–6), as discussed later. IL-22 also activates keratinocytes, resulting in the proliferation and production of these inflammatory substances (7–10). In this section, we focus on immune cells that affect keratinocytes in psoriasis (Figure 1).

2.1 Pathogenic Th17 cells induced by dendritic cells

Th17 cells play a pivotal role in the pathogenesis of psoriasis. Murine studies have revealed that transforming growth factor (TFG)-β and IL-6 are required to activate a unique transcription factor known as retinoid-related orphan receptor-γt (RORγt). RORγt in association with other transcription factors, increases both IL-23R and IL-17A in Th17 cells. Subsequent exposure of IL-23 to developing Th17 cells enhances Th17 cytokines, including IL-17 (11). Human Th17 cells produce mainly IL-17A, IL-17F, and IL-22 in addition to TNF-α (12, 13). Their cytokines drive keratinocytes to their aberrant differentiation and proliferation, as well as producing pro-inflammatory substances. IL-23 promotes Th17 cells to become highly pathogenic. It also regulates the development and maintenance of the Th17 population (14–16). The main source of IL-23 is thought to be inflammatory dendritic cells (DC), as described in our previous review article (16), including TNF-α and inducible nitric oxide synthase (iNOS)-producing DC (Tip-DC) and slanDC.

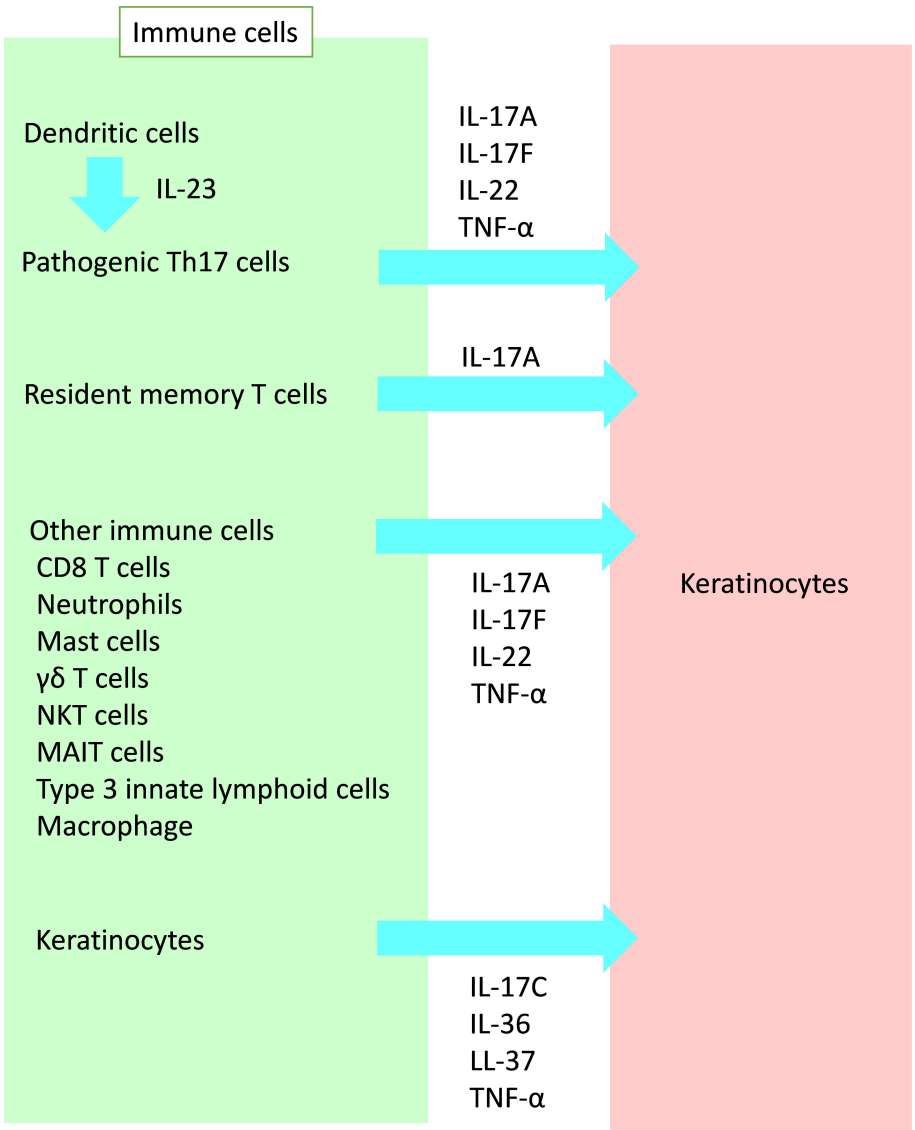

FIGURE 1
Crosstalk from immune cells to keratinocytes in psoriasis. A variety of immune cells affect keratinocytes in psoriasis. IL, interleukin; TNF, tumor necrosis factor; NKT, natural killer T; MAIT, mucosal-associated invariant T.

2.2 Resident memory T cells

Recently, skin resident memory T (Trm) cells have recieved attention, especially as the cells contributing to relapse or Köbner phenomenon (3, 17–20). In resolved psoriatic skin lesions, a population of Trm cells are observed, which are responsible for local relapse of psoriasis (17–19, 21–23). Epidermal $CD8^+CD103^+$ Trm cells are considered to be one of the major immune cells in resolved skin and are capable of IL-17A (22, 24–26). Gallais Sérézal et al. confirmed through NanoString analysis that $CD49a^-CD103^+CD8^+$ Trm cells were capable of triggering psoriasiform tissue response (27). These results suggest that IL-17 –producing $CD49a^-CD103^+CD8^+$ Trm cells are responsible for psoriasis relapse (20, 28, 29).

In addition to skin Trm cells, memory-like γδT cells (30), and skin structural cells with inflammatory memory (31, 32) could be involved in psoriasis relapse (20).

2.3 Other immune cells producing IL-17A

In addition to Th17 cells, IL-17A is produced by various cells of the innate and adaptive immune systems (11). $CD8^+$ IL-17-producing T cells are observed in psoriatic lesions, and they produce both Th1- and Th17-related cytokines, including interferon (IFN)-γ, TNF-α, IL-17A, IL-21, and IL-22 (33–35).

Since neutrophils and mast cells staining positive for IL-17 were identified at higher densities than $IL\text{-}17^+$ T cells in psoriatic lesions, neutrophils and mast cells are considered other significant potential sources of IL-17A in psoriasis (36–38). However, whether these cells synthesize and secrete IL-17A or whether positive staining represents cytokine uptake has yet to be determined (11). Mashiko et al. reported that human mast cells are major IL-22 producers in patients with psoriasis (39). Further investigation is needed to elucidate the role of mast cells and neutrophils in the pathogenesis of psoriasis.

IL-17A and IL-17F are also secreted by innate immune cells, such as group 3 innate lymphoid cells (ILC3s), and innate-like lymphocytes (ILLs), such as γδT cells, mucosal-associated invariant T (MAIT) cells, and natural killer T (NKT) cells (40–45).

Under the condition of abundant IL-23 in psoriasis lesional skin, some macrophages may produce IL-17A, IL-22 and IFN-γ in addition to TNF-α as described in our previous review article (16).

2.4 Keratinocytes

Keratinocytes also act as immune cells. Some cytokines secreted by keratinocytes, including IL-17C and IL-36, act on keratinocytes in an autocrine way (46). IL-36 cytokines, such as IL-36α/β/γ, are produced by keratinocytes following stimulation by TNF-α, IL-17A, IL-22, and IL-1β. IL-36 stimulates keratinocytes to produce TNF-α and IL-17C (47, 48). IL-17C is expressed by (and acts on) epithelial cells (49). Keratinocytes, the main producers of (and responders to) IL-17C in the skin, contribute to psoriatic inflammation (50–52). IL-17C has been identified as a functional regulator of the initial psoriatic cytokine network, suggesting its role during the early stages of psoriatic inflammation, or the "priming" for plaque formation (53).

Cathelicidins are a class of AMPs. LL-37, one of cathelicidins, produced by skin injury and bacterial infection, activates toll-like receptor (TLR)8 in keratinocytes and induces IL-17C through the induction of IL-36γ (47). Inhibition of IL-17 results in normalization of IL-36γ and IL-17C to levels associated with non-lesional skin (54).

3 Crosstalk: keratinocytes to immune cells

Reciprocally, keratinocytes also produce various substances that affect immune cells. In this section, we focus on these substances and their effects on immune cells (Figure 2).

3.1 Antibacterial peptides, including β-defensins, S100 proteins and cathelicidin

In non-lesional skin in psoriasis patients, trauma, injury, infection, or medication causes the production of various autoantigens from stressed or damaged keratinocytes (20, 55, 56). Among them, cationic AMPs [including LL-37, human beta-defensin (hBD)-2, and hBD-3], develop with DNA or RNA to form multimeric AMP–nucleic acid complexes, which induce the production of interferon (IFN)-α and IFN-β through TLR7 or 8 in plasmacytoid dendritic cells (pDCs) or increase the amounts of IL-6 and TNF-α by myeloid dendritic cells (mDCs) (20, 57, 58). IL-6, together with TGF-β, drives naïve T cell differentiation into Th17 cells, as described above. IFN-α and TNF-α further activate mDCs to produce IL-12 and IL-23 (16, 20, 59, 60). This process could be involved in the mechanism underlying Köbner phenomenon in psoriasis (3, 61).

In psoriatic lesions, various AMPs such as hBD-2, hBD-3, S100 proteins, and cathelicidin, are also highly expressed (15, 62, 63). hBD-2 and hBD-3 are induced by TNF-α and IFN-γ in keratinocytes (64, 65). hBD-2 is also induced by IL-17A and IL-22 (66). S100 proteins, such as S100A7 (psoriasin), S100A8 (calgranulin A), S100A9 (calgranulin B), S100A12 (calgranulin C), and S100A15, are abundantly expressed in psoriatic lesions, and some are elevated in the serum of psoriatic patients (67).

In a study by Liang et al., IL-22 in conjunction with IL-17A or IL-17F synergistically induced the expression of hBD-2 and S100A9 and additively enhanced the expression of S100A7 and S100A8 in keratinocytes (68). S100A7 may also havechemotactic potential in psoriasis (15, 69). The LL-37 high expression in the psoriatic epidermis may also accelerate inflammation through its capacity

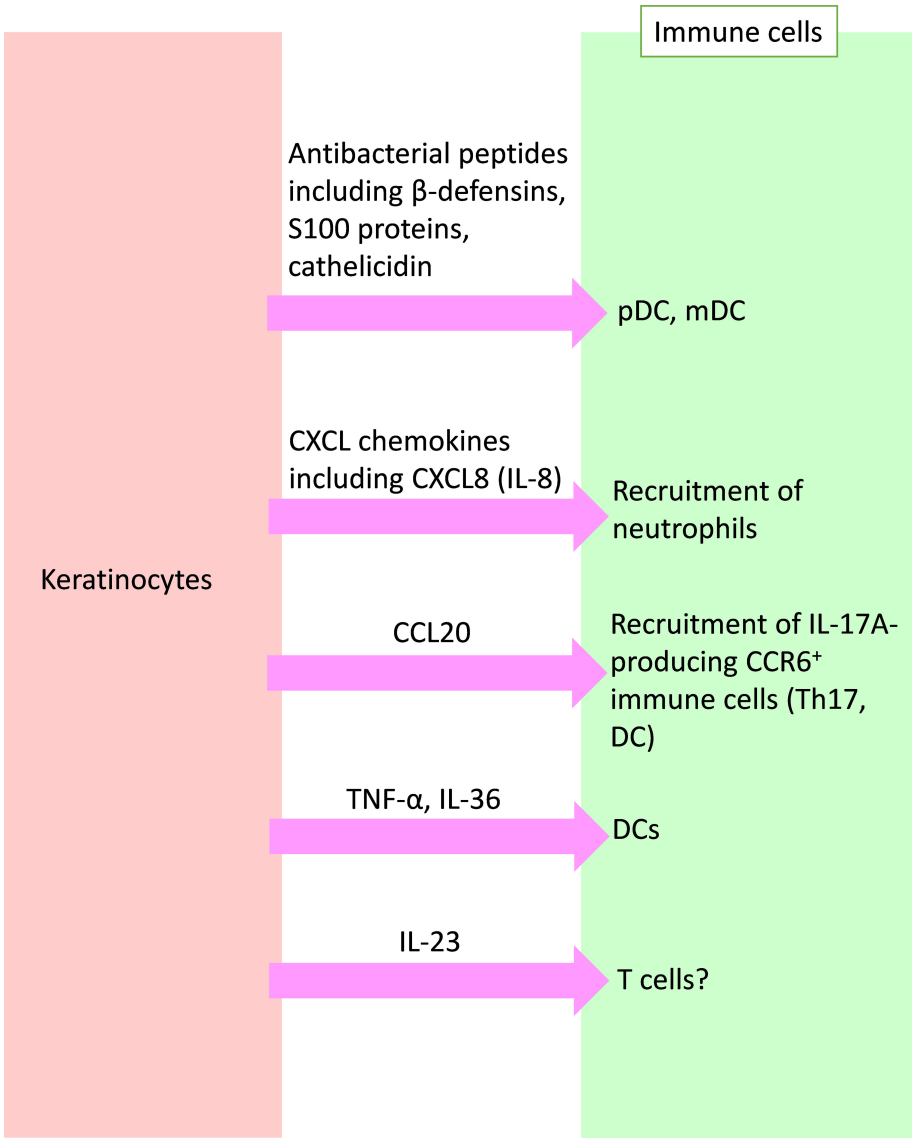

FIGURE 2
Crosstalk from keratinocytes to immune cells in psoriasis. Activated keratinocytes produce antibacterial peptides, chemokines, and inflammatory cytokines, and affect immune cells in psoriasis. pDC, plasmacytoid dendritic cell; mDC, myeloid DC; CXCL, C-X-C motif chemokine ligand; CCL, C-C motif chemokine ligand; IL, interleukin; TNF, tumor necrosis factor.

to enable pDC to recognize self-DNA via TLR9 (58). These AMPs affect various immune cells resulting in triggering, sustaining, and/or exacerbating psoriatic inflammation.

3.2 Chemokines, including CXCL1, CXCL2, CXCL8 (IL-8), and CCL20

Keratinocytes stimulated with IL-17 showed increased expression of multiple chemokines, including C-X-C motif chemokine ligand (CXCL)1, CXCL2, CXCL3, CXCL5, CXCL8 (IL-8), and C-C motif chemokine ligand (CCL)20 (4, 70–72). The CXCL chemokines likely attract neutrophils to the psoriatic epidermis (3, 11). CCL20 may recruit $CCR6^+$ cells, including Th17 and dendritic cells, to the skin (70). Inhibition of IL-17 normalizes expression of CXCL1, CXCL8 (IL-8), and CCL20 to the levels associated with non-lesional skin (54).

3.3 TNF-α, and IL-36

Keratinocytes stimulated with TNF-α, IL-17A, IL-22, and IL-1β produce IL-36 (46). IL-36 stimulates keratinocytes themselves to produce TNF-α and IL-17C (47, 48). TNF-α activates mDCs, leading to production of IL-23. IL-36 drives IFN-α production in pDCs, as well as IL-1β, IL-6, and IL-23 production in mDCs (46). These cytokines secreted by keratinocytes form an aggravating inflammatory loop in the pathogenesis of psoriasis.

3.4 IL-23

Several reports indicate that keratinocytes produce IL-23. Moreover, immunostaining of psoriatic lesions revealed enhanced expression of IL-23 in keratinocytes (73, 74). Park et al., using publicly available single-cell RNA sequencing data from human samples, revealed that IL-23 expression was detectable in psoriatic keratinocytes as well as DCs (75). Kelemen et al. reported that psoriasis-associated inflammatory conditions induced IL-23 mRNA expression in normal huma epidermal keratinocytes (76). Li et al., using a genetic mouse model, showed that keratinocyte-produced IL-23 was sufficient to cause chronic skin inflammation with an IL-17 profile and that epigenetic control of IL-23 expression in keratinocytes was important for chronic skin inflammation (77). However, whether the expression of IL-23 in keratinocytes in psoriasis contributes to the development of psoriasis remains to be elucidated.

4 Effect of IL-17 or IL-23 inhibition on immune cells and keratinocytes in psoriasis

As mentioned above, IL-23 and IL-17 play important roles in the pathogenesis of psoriasis. Indeed, biologics, including IL-23 inhibitors and IL-17 inhibitors, greatly impact keratinocytes and immune cells in psoriasis.

Secukinumab (an anti-IL-17 antibody) and guselkumab (an anti-IL-23 antibody) decrease the frequencies of inflammatory monocyte-like cells, inflammatory DC-like cells, and $CD4^+CD49a^-CD103^-$ T cells (78). Furthermore, bimekizumab (an anti-IL17A/F antibody) induces normalization of keratinocyte-related gene products, including CXCL1, CXCL8, CCL20, IL-36γ, and IL-17C, to levels associated with non-lesional skin (54). Krueger et al. reported that secukinumab caused reductions in critical downstream targets of IL-17A in the skin, including the AMPs (DEFB4A/β-defensin 2 and the S100 family) in addition to reductions in IL-23 and IL-17 in transcriptomic analyses (79). Inhibition of IL-23 or TNF-α also caused reductions in the gene expression of Th17-induced mediators by keratinocytes, including antimicrobial peptides (80, 81). Mehta et al. reported that inhibition of IL-23 reduced memory T cells while maintaining regulatory T cells, and vice versa for secukinumab (78). Furthermore, Whiley also revealed that clinical anti-IL-23 therapy depleted IL-17-producing Trm cells from the skin of patients with psoriasis (82).

5 Conclusion

In psoriasis, a variety of immune cells activate keratinocytes (mainly through Th17 cytokines), resulting in their abnormal differentiation and proliferation. Activated keratinocytes produce AMPs, chemokines, and various cytokines, which cause further inflammation and the recruitment of inflammatory cells. In addition, keratinocytes activate themselves by producing IL-36, IL-17C, and TNF-α. The crosstalk between immune cells and keratinocytes contributes to the development and maintenance of psoriasis.

Author contributions

MK: Conceptualization, Writing – original draft. YT: Supervision, Validation, Writing – review & editing, Conceptualization.

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Conflict of interest

The 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.

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Oncostatin M suppresses *IL31RA* expression in dorsal root ganglia and interleukin-31-induced itching

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by intermittent itchy rash. Type 2 inflammatory cytokines such as interleukin (IL)-4, IL-13, and IL-31 are strongly implicated in AD pathogenesis. Stimulation of IL-31 cognate receptors on C-fiber nerve endings is believed to activate neurons in the dorsal root ganglion (DRG), causing itch. The IL-31 receptor is a heterodimer of OSMRβ and IL31RA subunits, and OSMRβ can also bind oncostatin M (OSM), a pro-inflammatory cytokine released by monocytes/macrophages, dendritic cells, and T lymphocytes. Further, OSM expression is enhanced in the skin lesions of AD and psoriasis vulgaris patients.

Objective: The current study aimed to examine the contributions of OSM to AD pathogenesis and symptom expression.

Methods: The expression levels of the OSM gene (*OSM*) and various cytokine receptor genes were measured in human patient skin samples, isolated human monocytes, mouse skin samples, and mouse DRG by RT-qPCR. Itching responses to various pruritogens were measured in mice by counting scratching episodes.

Results: We confirmed overexpression of *OSM* in skin lesions of patients with AD and psoriasis vulgaris. Monocytes isolated from the blood of healthy subjects overexpressed *OSM* upon stimulation with IL-4 or GM-CSF. Systemic administration of OSM suppressed *IL31RA* expression in the mouse DRG and IL-31-stimulated scratching behavior. In contrast, systemic administration of OSM increased the expression of IL-4- and IL-13-related receptors in the DRG.

Conclusion: These results suggest that OSM is an important cytokine in the regulation of skin monocytes, promoting the actions of IL-4 and IL-13 in the DRG and suppressing the action of IL-31. It is speculated that OSM released from monocytes in skin modulates the sensitivity of DRG neurons to type 2 inflammatory cytokines and thereby the severity of AD-associated skin itch.

KEYWORDS

oncostatin M, atopic dermatitis, IL-31, itch, pruritus, IL31RA

1 Introduction

Eczema or atopic dermatitis (AD) is a chronic inflammatory skin condition manifesting as intermittent flares of itchy skin. The lifetime prevalence is roughly 15%–30% in children and adolescents and 2%–10% in adults (1). The normal barrier function provided by the stratum corneum is deficient in AD, resulting in invasion of allergens or pathogens and leading to activation of lymphocytes, monocytes/macrophages, eosinophils, and dendritic cells. In turn, these immune cells release pro-inflammatory cytokines that stimulate receptors on skin keratinocytes, fibroblasts, and sensory neurons, inducing inflammation and itch (1, 2). In particular, type 2 inflammatory cytokines such as interleukin (IL)-4, IL-13, and IL-31 produced by Th2 cells and type 2 innate lymphoid cells are primary drivers of the inflammatory process (3). As well as causing and prolonging inflammation, these cytokines suppress the production of filaggrin and loricrin by keratinocytes and thereby exacerbate the loss of epidermal barrier function (4). Itching is caused in part by histamine from mast cells, as well as serotonin, interleukins, and thymic stromal lymphopoietin (TSLP) produced by epidermal keratinocytes, which stimulate the C-fiber nerve endings of dorsal root ganglion (DRG) neurons. Interleukin-31 produced by activated CD4+ Th2 cells also activates C-fiber nerve endings of DRG neurons, causing itch (5). In addition to AD, IL-31 is considered the initiating pruritogen involved in itchy skin diseases such as prurigo nodularis, psoriasis vulgaris, and chronic urticaria (6).

The IL-31 receptor is a heterodimer of OSMRβ and IL31RA proteins, and mutations in the OSMR gene encoding OAMRβ cause familial localized cutaneous amyloidosis, a disease associated with intense itch (7, 8), suggesting that OSMRβ is an important signaling component in itch generation. Oncostatin M (OSM), another ligand for OSMRβ, is an inflammatory cytokine released by monocytes/macrophages, dendritic cells, and T lymphocytes under pro-inflammatory conditions (9, 10). The receptor for OSM is a heterodimer of OSMRβ and gp130, and its downstream signals have been found to function in hematopoiesis, mesenchymal stem cell differentiation, liver regeneration, cardiac remodeling, nociception, inflammation, and metabolism (11). However, OSM functions in skin remain unclear. Both OSMRβ and gp130 are expressed in keratinocytes, fibroblasts, and DRG neurons, suggesting that OSM may modulate skin inflammation and sensation by acting on skin component cells and sensory nerves. In fact, expression of OSM is enhanced in AD and psoriasis vulgaris lesions, although the mechanisms underlying this upregulation are unknown. Recently, it was reported that OSM does not directly cause itch but rather induces hypersensitivity to itch by enhancing the neuronal response to the pruritogens histamine and leukotriene (12). In this study, the conditions leading to OSM release by skin monocytes and the effects of OSM on IL-4, IL-13, and IL-31 receptors in skin cells and DRG neurons were examined to elucidate potential contributions to AD pathogenesis and skin pruritus symptoms.

2 Methods

2.1 Harvesting of skin samples from AD and psoriasis vulgaris patients

Fixed paraffin-embedded biopsy samples of skin from AD and psoriasis vulgaris patients were obtained from Hiroshima University Hospital. All samples (10 from AD patients and 10 from psoriasis vulgaris patients) were extracted within the past three years. Fifteen normal skin tissues from mapping skin biopsies were used as controls. Briefly, samples were extracted using a 4-mm punch biopsy, promptly fixed in formalin, embedded in paraffin, and sectioned at 10 μm thickness. Total RNA was isolated using the RNeasy FFPE kit (Qiagen, Hilden, Germany) for subsequent gene expression analysis by RT-qPCR (Section 2.9).

2.2 Purification and culture of human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers in Leucosep™ tubes (Greiner Bio-One Co, Tokyo. Japan) by density gradient centrifugation with Ficoll (GE Healthcare Japan, Tokyo. Japan). Monocytes were then purified from PBMCs using the Human Monocyte Enrichment Kit (STEMCELL Technologies, Tokyo, Japan). Monocytes were cultured in RPMI medium supplemented with 5% human serum at 37°C under a 5% CO_2 atmosphere for 24 h, then seeded on 24-well plates and treated for 1 h with RPMI medium containing 50 ng/mL of various test substances, including IL-4 (R&D Systems, Minneapolis, MN, USA), IL-13 (R&D Systems), IL-17A (PeproTech, Cranbury, NJ, USA), IL-33 (R&D Systems), IL-10 (PeproTech), GM-CSF (R&D Systems), and TNF-α (R&D Systems).

2.3 Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of Hiroshima University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Hiroshima University. Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories Japan.

2.4 Maintenance of a human epidermal keratinocyte cell line

The keratinocyte cell line Human Epidermal Keratinocytes, pooled (HEK) was obtained from Gibco (Gibco ThermoFisher, NY, USA) and cultured in Keratinocyte SFM (Gibco ThermoFisher)

supplemented with 5 µg/mL human recombinant epidermal growth factor (Gibco ThermoFisher), 50 µg/mL bovine pituitary extract (Gibco ThermoFisher), and 100 IU/mL penicillin/streptomycin (Gibco ThermoFisher). Cells were stimulated with recombinant human OSM (100 ng/mL, R&D Systems) for 1, 3, 6, and 24 h, and gene expression changes analyzed by RT-qPCR (Section 2.9). In addition, cells were stimulated with various concentrations of recombinant human OSM (0.1–100 ng/mL, R&D Systems) for 3 h. and gene expression changes were analyzed by RT-qPCR (Section 2.9).

2.5 Quantitation of *Osmr* and *Il31ra* expression in isolated (ex vivo) OMS-stimulated mouse DRG

Male C57BL/6 mice aged 8 to 12 weeks were euthanized with carbon dioxide and DRGs promptly dissected out and cultured in Dulbecco's modified Eagle's medium (Gibco ThermoFisher) supplemented with 10% fetal bovine serum (Gibco ThermoFisher) and penicillin/streptomycin at 37°C under a humidified atmosphere of 5% CO_2. Cultured DRGs were stimulated with recombinant mouse OSM protein (100 ng/mL, R&D Systems) for 3, 6, 12, and 24 h, and total RNA was extracted using a RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) for subsequent measurement of gene expression changes.

2.6 Quantitation of *Osmr* and *Il31ra* expression in dispersed culture mouse DRGs

DRGs were extracted using the same procedure as in Section 2.5 and sequentially dissociated in collagenase type III (Worthington, California, USA) and trypsin (Gibco ThermoFisher), which digest all intercellular connections. Neurons were sorted using Percoll (GE Healthcare) and cultured for 3 days to adjust cell numbers. Cultured DRGs were stimulated with recombinant mouse OSM protein (100 ng/mL, R&D Systems) for 3, 6, and 24 h, and total RNA was extracted using a RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany).

2.7 Quantitation of cytokine receptor expression in OMS-stimulated mouse skin and DRGs

Mice were administered four doses of recombinant mouse OSM protein (R&D Systems) at a dose of 200 ng in 50 µL saline every 8 h or equal-volume saline (control) by tail vein injection. Four h after the final injection, all mice were euthanized with carbon dioxide. Skin tissues were extracted from four treated and four control mice using a 4-mm Derma punch® (Maruho, Tokyo, Japan), while DRGs were collected following a previous described method of "Dispersed Cultures of Mature Rat DRG Neurons and Their Applications.".

2.8 ELISA for OSM production

The ELISA assay for human OSM was performed using the Human Oncostatin M (OSM) ELISA kit (Ray Biotech, Peachtree Corners, GA, USA) according to the manufacturer's protocol.

2.9 Isolation of RNA

Total RNA was isolated from paraffin-embedded tissue sections using the RNeasy FFPE kit, from monocytes using the RNeasy Mini Kit, and from DRGs and skin samples was using the RNeasy Fibrous Tissue Mini Kit (all from Qiagen) according to the manufacturer's instructions.

2.10 Real-time quantitative PCR (RT-qPCR)

First-strand cDNA was synthesized from isolated RNA using a QuantiTect® Reverse Transcription Kit (Qiagen), and RT-qPCR was performed using a QuantStudio 3 real-time PCR system (ThermoFisher) with the following thermocycle: denaturation at 95°C for 15 s, annealing at 60°C for 60 s. Expression of the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was measured as the internal control. Primer pairs for detecting *OSM, IL31, IL4, IL31RA, OSMR, CCL2, Il31ra, Osmr, Il4ra, Il13ra1, Il2rg*, and *Il13ra2* were obtained from ThermoFisher Scientific.

2.11 Measurement of scratching behavior

Itch-related scratching behavior in treated mice was analyzed using a SCLABA®-Real system (Noveltec, Kobe, Japan). Briefly, mice were preinjected with OSM (100 ng) in 50 µL saline via the tail vein. After 11.5 h, four mice were placed in an acrylic cage (19.5 × 24 × 35 cm) for at least 30 min to acclimate then injected with histamine (55.6 µg), serotonin (2.5 µg), or IL-31 (500 ng) in 50 µL saline. Scratching behavior was monitored remotely for 30 or 120 min at an ambient room temperature of 22°C.

2.12 Statistical analysis

Data are expressed as the mean ± SD or mean ± SEM as indicated. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). Two sample groups were compared by independent samples t-test and three or more groups by analysis of variance with Tukey's multiple comparisons tests. A $p < 0.05$ was considered statistically significant for all tests. In figures, significance level is represented by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

2.13 Study approval

Mouse experiments were approved by the animal experimentation ethics committee of Hiroshima University and

informed-consent was obtained from all human subjects following approval by the ethics committee of Hiroshima University.

3 Results

3.1 Expression of *OSM* is upregulated in human AD and psoriasis vulgaris lesions

To elucidate the contributions of OSM signaling to AD and psoriasis vulgaris, we first examined if *OSM* expression is upregulated in lesions compared to healthy skin tissue. Indeed, expression was undetectable in 14 of 15 healthy control skin biopsy samples but detected in 8 of 10 AD lesion samples and 5 of 10 psoriasis vulgaris lesion samples (Figure 1A). The expression of *OSMR* in lesional skin showed a tendency to be lower compared to healthy skins, but no significant difference was observed (Figure S1). The expression of *IL31RA* could not be detected in human skin tissues (Data not shown).

3.2 OSM protein levels in the serum of healthy humans and AD patients

No significant difference in OSM levels was observed among serum samples obtained from 20 patients with AD currently undergoing treatment at our hospital and 20 healthy humans (Figure 1B).

3.3 Expression of *OSM* is upregulated in healthy human monocytes by IL-4 and CM-CSF stimulation

To investigate if the upregulation of OSM in skin lesions of patients with AD and psoriasis vulgaris results from a local pro-inflammatory cytokine environment, *OSM* expression was measured in human monocytes isolated from healthy human subjects following stimulation by cytokines known to be upregulated in AD and psoriasis lesions. Stimulation with IL-4 or GM-CSF (both at 50 ng/mL for 1 h) significantly increased *OSM* expression compared to vehicle-treated control samples (Figure 1C), and co-stimulation with IL-4 and GM-CSF enhanced *OSM* expression (Figure 1D).

3.4 OSM stimulation modulates IL-31 receptor gene expression in isolated mouse DRG

Stimulation of IL-31 receptors expressed on sensory neurons is believed to promote itch in AD. To investigate the potential contribution of *OSM* upregulation to this process, we examined changes in IL-31 receptor gene expression in isolated mouse skin samples and DRGs treated with OSM (100 ng/mL for up to 24 h). In DRGs, expression of *Osmr* increased immediately after OSM stimulation (Figure 2A), while the expression of *Il31ra* decreased starting after 12 h of treatment (Figure 2B). In contrast, no expression changes were observed in mouse skin samples (Figures 3D, E). Conversely, in dispersed cultured DRGs, there was an observed trend of increased expression for both *Osmr* and *Il31ra* after OSM stimulation (Figures S2A, B).

3.5 OSM stimulation modulates IL-31 receptor gene expression in cultured HEK

Stimulation of HEKs with OSM upregulated *OSMR* expression and downregulated *IL31RA* expression (Figures 2C, D).

3.6 OSM stimulation increases *CCL2* gene expression in HEKs

CCL2 gene expression increased in a concentration-dependent manner upon OSM stimulation (Figure 2E). In fact, a notable trend of increased *CCL2* expression was observed in both AD and psoriasis lesions (Figure 2F).

3.7 OSM stimulation modulates IL-31, IL-4 and IL-13 receptor expression in mouse skin and DRG

To examine if OSM stimulation also modulates the expression of IL-31 receptors and other cytokine receptors *in vivo*, we measured *Osmr* and *Il31ra* expression in mouse skin and DRG samples freshly excised 28 h following the last of 4 OSM systemic injections (200 ng at 8-hour intervals in the tail vein) (Figure 3A). Consistent with observations in culture, the expression of *Osmr* was upregulated (Figure 3B) while that of *Il31ra* was downregulated in freshly excised DRG tissue (Figure 3C). In contrast, there were no significant changes in *Osmr* and *Il31ra* expression in skin tissue (Figures 3D, E), suggest that longer-term changes in IL-13 signaling occur at the level of the neuronal cell body. Furthermore, OSM also increased the expression of genes encoding IL-4 and IL-13 receptor subunits IL-4Rα, IL-4Rγc, IL-13Rα1, and IL-13Rα2 (*Il4ra*, *Il2rg*, *Il13ra1*, *Il13ra2*) in freshly excised mouse DRG (Figures 3F–I).

3.8 OSM suppresses scratching behavior in mice

Based on our findings that OSM reduced IL-31RA expression in mouse DRG, we predicted that the behavioral response to IL-31 would be reduced by OSM stimulation. For these tests, 100 ng of OSM was injected into the tail vein 8 h prior to subcutaneous treatment with various pruritogens including IL-31. To first examine if OSM alone initiates itch, scratching behavior was compared between mice receiving OSM or saline starting 2-h post treatment. The number of scratch events was slightly but not significantly higher following OSM administration compared to

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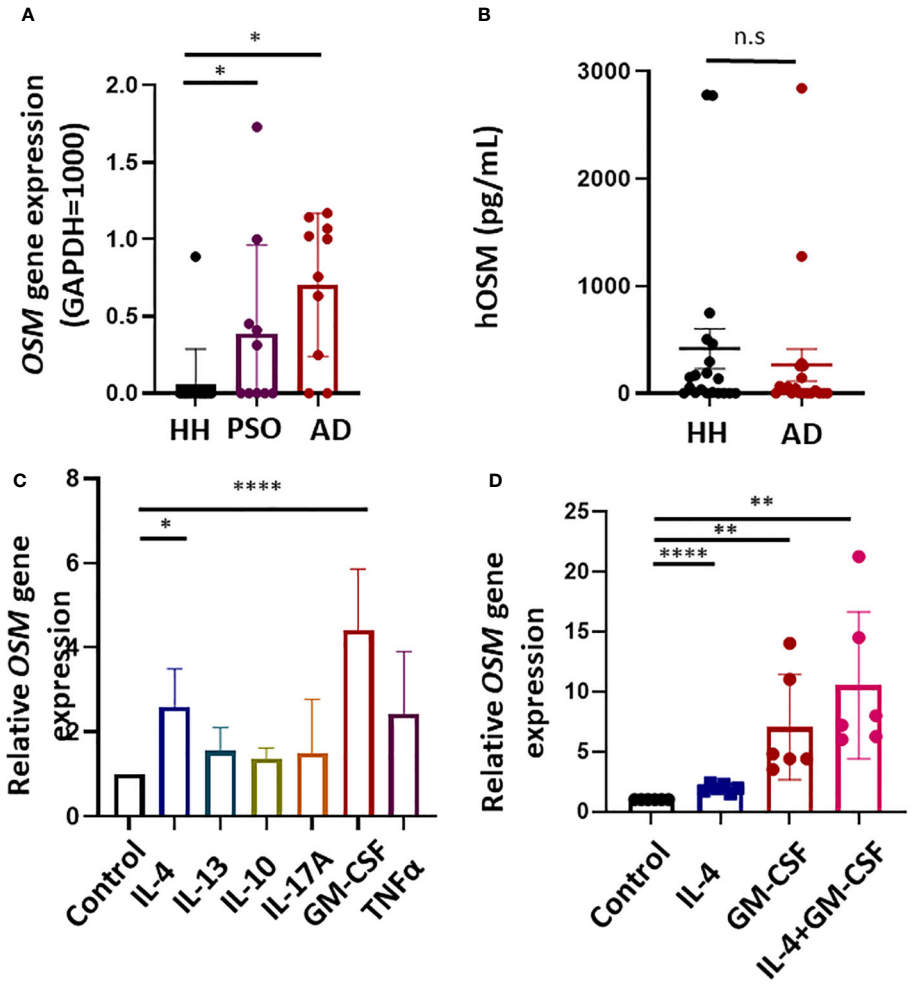

FIGURE 1
Expression of the gene encoding oncostatin M (*OSM*) is upregulated in atopic dermatitis (AD) lesions and cytokine-stimulated human monocytes. **(A)** Expression of *OSM* was undetectable in 14 of 15 healthy human (HH) skin samples but was detected in 8 of 10 AD lesion samples and 5 of 10 psoriasis (PSO) lesion samples by RT-qPCR. **(B)** OSM protein levels in serum did not differ significantly between healthy humans and patients with AD (n = 20). **(C)** Stimulation of monocytes isolated from healthy humans with IL-4 (50 ng/mL) or GM-CSF (50 ng/mL) increased *OSM* expression (n = 6 samples from 6 human volunteers). **(D)** Stimulation of monocytes from healthy humans with both IL-4 and GM-CSF (50 ng/mL) increased *OSM* (n = 6 monocyte samples from 6 human volunteers). All results presented as mean ± SD. * $p < 0.05$, **< 0.01 and **** $p < 0.0001$ by Tukey's multiple comparisons test.

saline administration (61.9 ± 8.8 vs. 52.1 ± 10.5) (Figure 4A), suggesting little direct pruritogenic action. However, systemic pretreatment with OSM did suppress subsequent IL-31-induced itch (Figure 4B) as the number of scratching events observed over a 2-h period following IL-31 administration was significantly lower among mice receiving OSM (100 ng) 12 h earlier compared to mice receiving saline (213.8 ± 21.0 times vs. 321.7 ± 34.0 times) (Figure 4C). Alternatively, systemic OSM had no effect on scratching behavior induced by histamine or 5-HT (Figures 4D, E).

4 Discussion

This study provides evidence that oncostatin M can reduce cytokine-induced itch associated with AD by suppressing pro-inflammatory cytokine signaling in keratinocytes and sensory neurons. This is the first report demonstrating the negative regulatory role of OSM in itching. OSM may be an important endogenous negative regulator of AD pathogenesis and symptom expression.

The expression of *OSM* was substantially elevated in skin lesions from AD and psoriasis vulgaris patients (Figure 1), consistent with a previous report (13). Multiple inflammatory cell types infiltrate AD and psoriasis vulgaris lesions, including OSM-producing monocytes/macrophages. Monocytes are known to release OSM in response to GM-CSF stimulation (14). In the present study as well, GM-CSF and IL-4 enhanced *OSM* expression in isolated human monocytes, and there is a trend toward higher CCL2 expression in the skin lesions of AD and psoriasis vulgaris (Figure 2). In the acute phase of AD, there is a substantial increase in the number of cells in skin expressing IL-4, IL-5, and IL-13 mRNAs, while in the chronic phase, skin lesions exhibit greater numbers of cells expressing IL-5, GM-CSF, IL-12, and IFN-α mRNAs (15). Monocytes differentiate into dendritic

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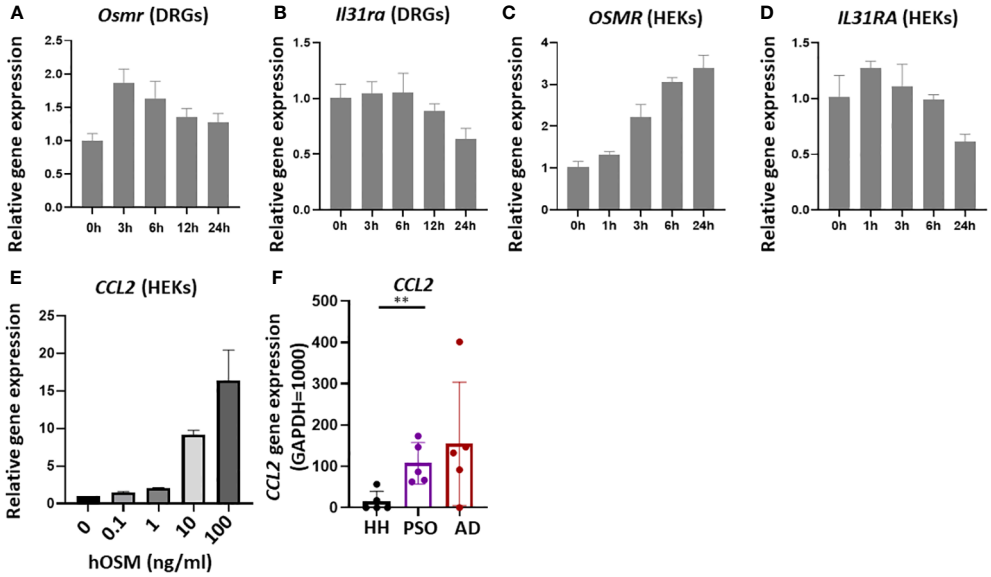

FIGURE 2
Stimulation of isolated mouse dorsal root ganglia (DRG) and cultured human keratinocytes (HEKs) with OSM modulated IL-31 receptor subunit gene expression. **(A, B)** In isolated mouse DRG, OSM treatment (100 ng/mL) rapidly upregulated *Osmr* expression, followed by a slow return to baseline, and more slowly downregulated *Il31ra* expression. **(C, D)** In HEKs as well, OSM progressively increased *OSMR* expression **(C)** and more slowly reduced *IL31RA* expression **(D)**. **(E)** In HEKs, *CCL2* gene expression increases in an OSM concentration-dependent manner. **(F)** In lesions of AD and psoriasis vulgaris patients, there was a tendency for higher *CCL2* gene expression compared with healthy humans (HH). All results presented as mean ± SD. ** $p < 0.01$ by unpaired t-test. All experiments except F were performed ≥three times.

cells in the presence of GM-CSF and IL-4 (16–18), suggesting that the chronic phase of AD is associated with the differentiation of OSM-expressing monocytes into dendritic cells. Furthermore, keratinocytes produce CCL2 upon OSM stimulation, which attracts additional monocytes into the dermis (19). We suggest that monocytes and keratinocytes are mutually activated via OSM and CCL2 release in the presence of IL-4 and GM-CSF within AD skin lesions.

It has been reported that IL-31 transmits itch stimuli to the central nervous system by binding receptors expressed on DRG

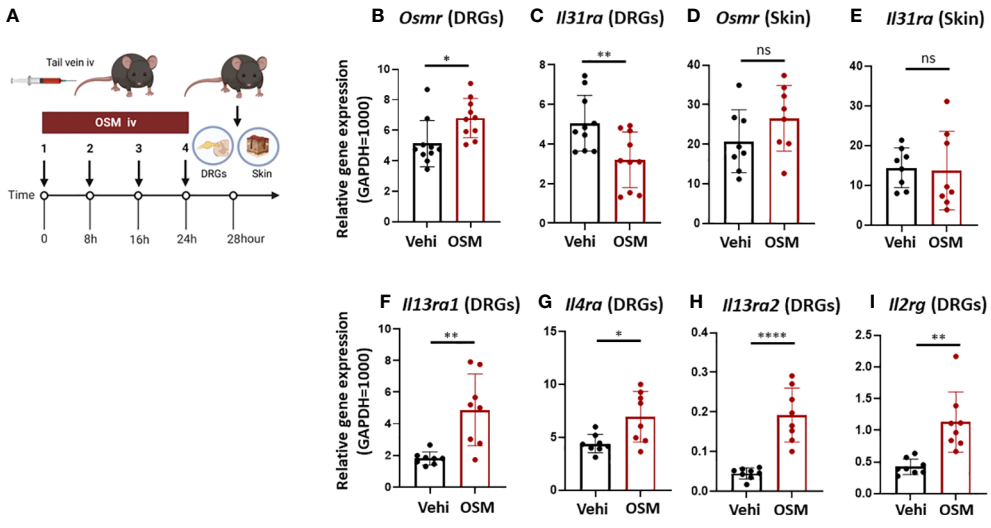

FIGURE 3
High systemic OSM modulates IL-31 receptor subunit gene expression in mouse DRG. **(A)** Experimental schema. Mice were injected with OSM (200 ng) into the tail vein every 8 h for a total of four doses. At 28 h after the final dose, samples of the dorsal root ganglion (DRG) and dorsal skin were collected. **(B, C)** Injection of OSM increased DRG expression of *Osmr* **(B)** and decreased DRG expression of *Il31ra* **(C)** compared to vehicle (Vehi) injection (n = 10 DRG samples from 10 injected mice). **(D, E)** Alternatively, systemic injection of OSM did not alter IL-31 receptor subunit expression in mouse skin (n = 8 skin samples from 8 mice). **(F–I)** Systemic injection of OSM also increased DRG expression levels of IL-4 and IL-13 receptor subunits (n = 8 DRG samples from 8 mice). All results are presented as mean ± SD. *$p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ by unpaired t-test. ns, non-significant.

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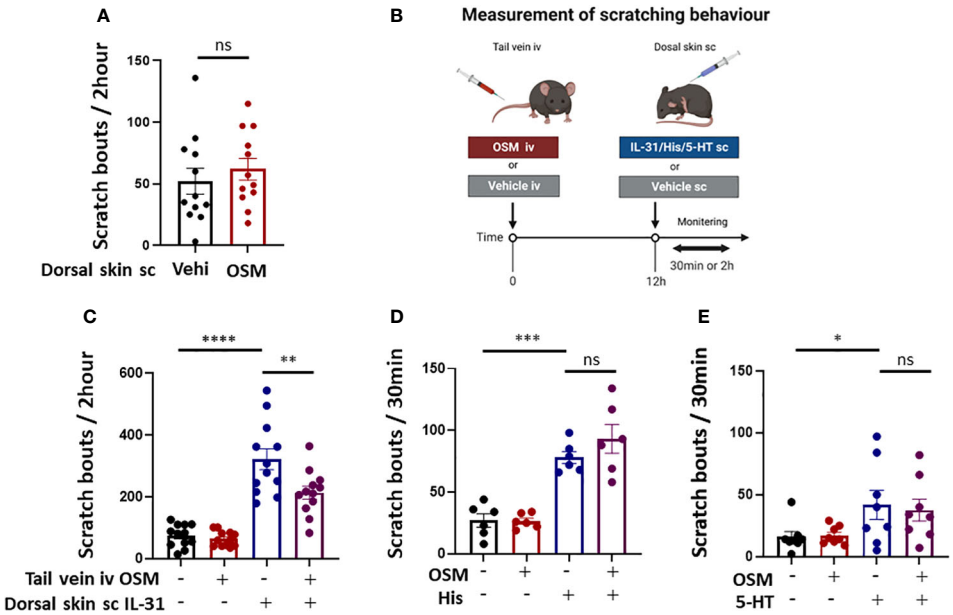

FIGURE 4
Systemic OSM injection suppresses IL-31-induced scratching behavior in mice. **(A)** Intradermal administration of OSM alone did not induce itching. (n = 12 mice per treatment group). **(B)** The schedule of OSM and pruritogen administration followed by monitoring of scratching behavior. **(C)** Intravenous (iv) injection of OSM into the tail vein 12 h prior to subcutaneous (sc) injection of IL-31 reduced scratching behavior compared to iv vehicle (Vehi) injection (n = 12 mice per treatment group). **(D, E)** Tail vein injection of OSM did not alter scratching behavior induced by sc injection of **(D)** histamine (His) or **(E)** serotonin (5-HT) (n = 6 mice per treatment group). All results expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by Tukey's multiple comparisons test. n.s, non-significant.

neurons and activating the ERK signaling pathway (20). Consistent with this mechanism, systemic OSM treatment reduced IL-31-induced scratching behavior in mice. Interleukin-31 is believed to act as a pruritogenic agent in multiple skin diseases. In fact, the humanized anti-IL31RA nemolizumab was recently approved in Japan for the relief of AD-associated pruritus. In contrast to IL31-induced itch, OSM had no effect on itch induced by histamine or serotonin. Endogenous itch caused by histamine, leukotrienes, and serotonin is also dependent on stimulation of cognate receptors expressed by keratinocytes and DRGs (21, 22). Thus, the effects of OMS appear specific to IL-31 receptor expression and itch transmission.

It is speculated that keratinocytes and DRG neurons expressing IL-31 receptors directly contribute to itch and that the associated transduction pathways are modulated by various other factors, including other cytokines, through effects on IL-13 receptor activity. Miake and colleagues reported that IL-4 stimulation enhanced *IL31RA* gene expression and IL31-induced production of CCL17 and CCL22 in BMDCs (23), suggesting that *IL31RA* expression level influences the responsiveness of cells to IL-31. Tail vein administration of OSM enhanced the expression of *Osmr* but suppressed *Il31ra* expression in the mouse DRG (Figures 3B, C). In mouse skin tissue, however, the expression levels of these receptor-related genes were unchanged by OSM, whereas OSM stimulation enhanced the expression of *OSMR* and suppressed *IL31RA* expression in primary human cultured keratinocytes (HEKs) (Figures 2C, D, 3D, E). Furthermore, systemic OSM suppressed phosphorylation of ERK by IL-31 in HEKs. We speculate that OSM may suppress IL-31-induced pruritus by reducing IL31RA expression and downstream ERK activity in both DRGs and keratinocytes. In contrast, OSM stimulation enhanced the expression of receptor subunits for IL-4 and IL-13 in the mouse DRG (Figures 3F–I). Both IL-4 and IL-13 receptors are also upregulated in the lesional skin of AD patients (24), suggesting that OSM suppress itch by promoting IL-4 and -13 responses and by concomitantly suppressing IL-31 responses in the DRG.

Previous reports have concluded that OSM acts as a modulator rather than an initiator of itch. Tseng and colleagues simultaneously administered pruritogenic substances and OSM subcutaneously to mice and found that OSM enhanced histamine- and leukotriene C4 (LTC4)-induced itch (12). In mouse DRG, *Il31ra* gene expression was not immediately affected by OSM stimulation but decreased substantially after 12 h (Figure 2B). Alternatively, expression levels of histamine H1 and H4 receptor genes were not influenced by OSM (data not shown). Systemic administration of OSM 12 h before subcutaneous administration of pruritogenic substances (i.e., a time sufficient to downregulate *Il31ra*) suppressed IL-31-induced scratching behavior, but did not alter histamine- or serotonin-induced scratching behavior. The discrepancies between the present study and previous reports may be explained by the different intervals between systemic OSM and subcutaneous pruritogen administration, as OSM-induced modulation of histamine receptor subunit expression is rapid but transient and so may have subsided after 12 h. The effects of OSM on pruritic stimulation pathways are likely to be diverse and require further validation.

Primary sensory neurons in the DRG have been classified into 11 subsets based on comprehensive transcriptome analysis, with

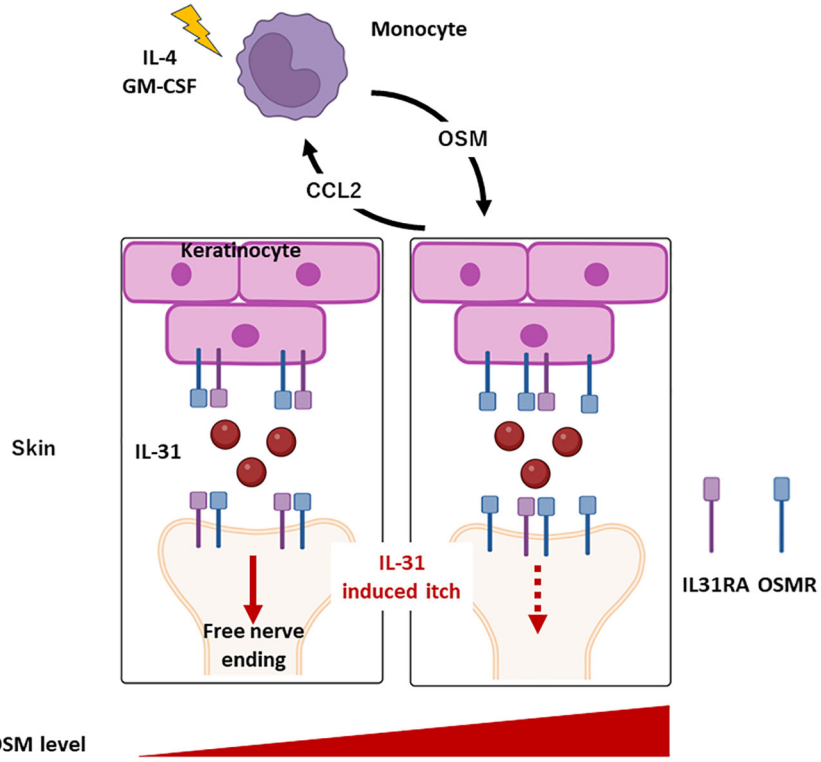

FIGURE 5
Roles of monocyte and OSM in AD. Monocytes produce OSM in response to stimulation by IL-4 and GM-CSF. OSM triggers the release of CCL2 from keratinocytes, inducing monocytes infiltration into the skin. Under conditions of elevated OSM in skin, *IL31RA* expression is downregulated while *OSMR* expression is upregulated in keratinocytes and DRG. Consequently, suppression of IL-31-induced itch is triggered.

only one type expressing *IL31RA* and two mechanoreceptive subtypes expressing OSM receptors (25). In DRGs, 50.6% of small-diameter neurons (<30 μm) expressed IL31RA, whereas expression was not detected in the more numerous large-diameter neurons (>50 μm). As a result, only a small proportion of DRG neurons (< 4%) responded to IL-31 (20). In the current study, OSM stimulation of isolated mouse DRGs reduced *IL31RA* expression (Figures 2C, D), whereas in primary cultured DRG neurons, *IL31RA* expression was increased by OSM (Figure S2). During culture, the number of differentiated DRG neurons decreased and undifferentiated DRG neurons with proliferative potential increased and eventually were the majority. Therefore, cultures may not be a reliable model to assess cytokine responses in DRG neurons. Future work is needed to examine whether there are differences in cellular responses to OSM stimulation among DRG neuron subtypes.

This study has several limitations. It is not known whether OSM can suppress IL-31-induced itch in humans for AD treatment. The correlation between the degree of itching in atopic dermatitis and psoriasis vulgaris and the level of OSM in the skin or serum remains unclear and is a subject for further investigation. We also need to confirm that IL31RA surface expression (like mRNA expression) is reduced by OSM and elucidate the specific signaling pathways involved. Finally, the safety of OSM treatment and potential effects on other sensory processes requires further investigation.

In the present study, we confirmed that *OSM* expression is upregulated in AD lesions and further demonstrated that upregulation can be induced in skin cells by IL-4 and GM-CSF. Systemic OSM elevation was found to reduce IL-31 receptor subunit expression in the mouse DRG as well as IL-31-induced scratching behavior (Figure 5). At the same time, OSM enhanced the expression of IL-4 and IL-13 receptor subunits in the DRG. Collectively, these findings suggest that OSM contributes to AD pathogenesis and symptom expression by modulating the expression of IL-31, IL-4, and IL-13 receptor subunits on DRG neurons, thereby altering intracellular signaling pathways responsible for regulating the transmission of itch signals to the central nervous system.

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 in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Hiroshima University Clinical Research Review Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed

consent to participate in this study. The animal study was approved by Hiroshima University Animal Experimentation Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MS and AT made contributions to the conception of the work and were major contributors in writing the manuscript. MS, TN, RS, KU, TK, and AT collected the data. YY, YI, and JM supervised the project and confirmed the authenticity of all raw data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AT has received honoraria from Eli Lilly, Kaken Seiyaku, Sanofi, Taiho Pharma, Abbie, Pfizer, Kyorin Pharmaceutical, Mitsubishi-Tanabe, Torii Pharmaceutical, and Maruho as a speaker as well as research grants from Eli Lilly, Sanofi, Teijin Pharma, Taiho Pharma, Mitsubishi-Tanabe, Torii Pharmaceutical, and Maruho.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed 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/fimmu.2023.1251031/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Gene expression in lesions of AD and psoriasis vulgaris patients. *OSMR* gene expression tends to be high in healthy humans (n = 5). Although *IL31*, *IL4*, *GM-CSF* and *IL31RA* gene expression was investigated, expression could not be detected.

SUPPLEMENTARY FIGURE 2
Oncostatin M modulated the expression levels of *Osmr* and *Il31ra* in dispersed cultured mouse DRGs. **(A)** The expression of *Osmr* increased with time after OSM stimulation. **(B)** The expression of *Il31ra* increased up to 3 h and then gradually decreased below baseline. All experiments were performed ≥three times.

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

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Introduction: *Staphylococcus aureus* (*S. aureus*) infection of the skin leads to a rapid initial innate immune response with keratinocytes in the epidermis as the initial sensors. Polymorphonuclear neutrophils (PMNs) are the first innate immune cells to infiltrate infection sites where they provide an effective first-line of defense. Previous work of our group showed that in inflamed skin a crosstalk between PMNs and keratinocytes results in enhanced *S. aureus* skin colonization.

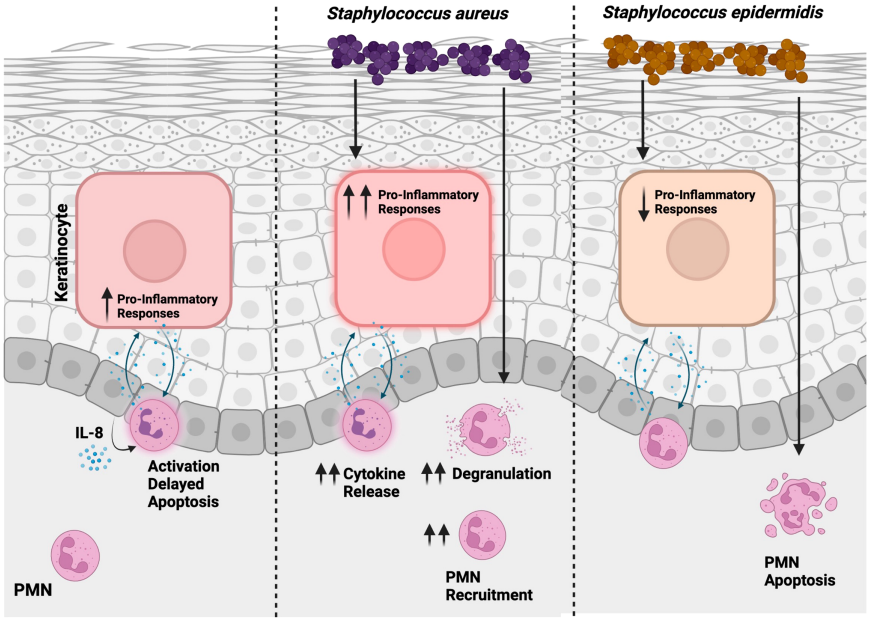
Methods: In this work, we used an *in vitro* co-culture model to studied the crosstalk between primary human keratinocytes (PHKs) and PMNs in a sterile environment and upon S. aureus infection. We investigated the influence of PHKs on PMN activation by analyzing PMN lifespan, expression of degranulation markers and induction of proinflammatory cytokines. Furthermore, we analyzed the influence of PMNs on the inflammatory response of PHKs. Finally, we investigated the influence of the skin microbiome on PMN-mediated skin inflammation.

Results: We show that co-culture of PMNs with PHKs induces activation and degranulation of PMNs and significantly enhances their lifespan compared to PMN cultivation alone by an IL-8 mediated mechanism and, furthermore, primes PMNs for enhanced activity after *S. aureus* infection. The prolonged incubation with PMNs also induces inflammatory responses in PHKs which are further exacerbated in the presence of *S. aureus* and induces further PMN recruitment thus fueling skin inflammation. Interestingly, infection of PHKs with the skin commensal *S. epidermidis* reduces the inflammatory effects of PMNs in the skin and exhibits an anti-inflammatory effect.

Discussion: Our data indicate that skin infiltrating PMNs and PHKs influence each other in such a way to enhance skin inflammation and that commensal bacteria are able to reduce the inflammatory effect.

KEYWORDS

neutrophils, keratinocytes, *Staphylococcus aureus*, skin inflammation, skin immune system



GRAPHICAL ABSTRACT
Proposed model of the interplay between PMNs and PHKs during skin inflammation. In a sterile environment, PMNs undergo activation, with their lifespan extended through an IL-8 dependent way. Concurrently, proinflammatory responses are initiated in PHKs. Skin infection with *S. aureus* intensifies inflammation, leading to enhanced recruitment of PMNs. Conversely, *S. epidermidis* exerts an anti-inflammatory influence by mitigating PMN-mediated skin inflammation and facilitating apoptosis in PMNs. This illustration was created with BioRender.

Introduction

The skin immune barrier depends on the interplay of different cell types to ensure homeostasis under physiological conditions and protection against invading pathogens (1). Keratinocytes are the main constituents of the epidermis and thus the first cells to sense an invading pathogen such as *Staphylococcus aureus* (*S. aureus*) and therefore play a crucial role in initiating and maintaining skin inflammation (2). They contain pattern-recognition receptors that help to sense pathogen-associated-molecular-patterns (PAMPs) on the microbes which initiates secretion of cytokines, chemokines, and antimicrobial peptides (AMPs) and the recruitment of immune cells to the site of infection (2).

Polymorphonuclear neutrophils (PMNs) are the most abundant leucocytes in the human blood (3). Upon skin infection, they are the first cells to infiltrate the infected site where they provide an effective first-line of defense (4, 5). To ensure a fast response at infection sites, PMNs contain preformed molecules stored in cytoplasmic granules that can be rapidly mobilized via degranulation (6, 7). However, excessive degranulation can cause enormous collateral damage to surrounding tissue and lead to systemic inflammation. Therefore, PMN activation and degranulation needs to be tightly controlled and requires receptor-coupled mechanisms (6). After completing their tasks, PMNs undergo apoptosis and are cleared by macrophages. This prevents excessive inflammation and helps restoring homeostasis (8–10).

S. aureus is a gram-positive facultative pathogen responsible for the majority of skin infections in humans. It asymptomatically colonizes about 30% of the anterior nares of the human population (11, 12). *S. aureus* can be frequently found on the skin of atopic dermatitis patients where it actively contributes to skin inflammation (13, 14). It has been shown that upon *S. aureus* infection of the skin, the recruitment of PMNs is critical in clearing the infection (15, 16). However, we and others show that the presence of PMNs in the skin of mice or in a human *in vitro* coculture system of PHKs and PMNs enhances *S. aureus* skin colonization and persistence by the interaction of PHKs with neutrophil extracellular traps (NETs) (17, 18). These data indicate that an interaction of PHKs with PMNs via NETs are important to keep infections local in the initial stages of colonization. Further studies of us revealed that PMNs and NETs present in the inflamed skin induce oxidative stress in PHKs which results in the secretion of HMGB1. NETs and HMGB1 can downregulate the expression of epidermal barrier genes thus promoting skin barrier defects which favors *S. aureus* skin colonization (19).

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

Materials and methods

Isolation of primary human PMNs

Peripheral blood was drawn from healthy donors, mixed with dextran solution (2% Dextran, 0.9% NaCl) and incubated for 30 min at RT. The upper phase was layered onto BioColl (1.077 g/ml, Bio&Sell) in a 3:2 ratio and density gradient centrifugation was performed for 30 min at 1600 rpm in a swinging bucket rotor without brake. The cell pellet was resuspended in hypotonic erythrocyte lysis buffer (C-C-Pro). After 10 min incubation time, a second centrifugation step at 1600 rpm for 10 min without brake was performed. After one washing step with PBS, the remaining cell pellet containing the PMNs was resuspended in keratinocyte base medium (CELLnTECH) containing 1.7 mM $CaCl_2$. PMN isolation from human blood was approved by the ethics committee of the medical faculty of the University of Tübingen (054/2017BO2).

Cell culture and *in vitro* co-culture system

Primary human keratinocytes (PHKs) isolated from human foreskin after routine circumcision from the Loretto Clinic in Tübingen as previously described (18, 20, 21). PHK isolation was approved by the ethics committee of the medical faculty of the University of Tübingen (654/2014BO2) and performed according to the principles of the Declaration of Helsinki. PHKs were cultured in collagen-coated tissue flasks (Corning, BioCoat™) in epidermal keratinocyte medium (CELLnTEC) at 37°C, 5% CO_2. 24h before the experiments, PHKs were differentiated with keratinocyte base medium (CELLnTECH) containing 1.7 mM $CaCl_2$. The *in vitro* co-culture model was performed as previously described (18). Briefly, PHKs were seeded into collagen-coated transwell inserts (pore size: 0.4 µm) and differentiated after reaching confluency. PMNs were isolated as described above and seeded into 24 well plates in a concentration of 2x10^6/ml. The inserts containing differentiated PHKs were placed on top of the PMNs, and the cells were co-incubated for the indicated times.

Flow cytometry

1x10^6 PMNs were incubated with the following surface antibodies for 20 min on ice in the dark. Surface antibodies included PerCP-Cy5.5-anti-CD11b, APC-anti-CD63, APC-anti-CD66b (all Biolegend). To exclude dead cells, a fixable viability dye was included. Flow cytometry was performed using an LSR II (BD Bioscience) and analyzed with FlowJo (TreeStar).

Annexin-V staining

Annexin-V staining was performed as previously described. Briefly, 1x10^6 PMNs were resuspended in Annexin binding buffer containing Annexin and incubated for 15 min at RT in the dark. After one washing step with annexin binding buffer, PMNs were measured at the LSR II (BD Bioscience) and analyzed with FlowJo (TreeStar).

Live cell imaging

The Incucyte SX1(Sartorius) was used to investigate PMN viability over time. PMNs were seeded in a 24 well plate in a concentration of 1x10^6/ml and were either co-incubated with PHKs or stimulated with the PMN supernatant of the 18h co-culture. Non-co-cultured PMNs or PMNs incubated with medium served as control. DRAQ5 (1 uM; Thermofisher) was used for staining the intracellular DNA and Sytox Green (0.2 uM; Thermofisher) was used for staining the extracellular DNA, thus indicating cell death. Pictures were taken every hour when PMNs were stimulated with the supernatant or after 6h, 18h and 30h when co-cultured with PHKs. Percentage of Sytox Green positive cells were quantified using Fiji/ImageJ.

Western blot

Analysis of capase-3 cleavage in PMNs was performed by western blot using whole cell lysates. Briefly, PMNs were lysed in a lysis buffer containing protease and phosphatase inhibitors. Lysates were separated via SDS-polyacrylamide gel electrophoresis and plotted onto PVDF membranes. After 60 min blocking in PBS + 01% Tween + 5% dry milk, membranes were incubated in anti-caspase-3 antibody (1:1000, Cell Signaling) at 4°C overnight. The next day, the membrane was washed three times with PBS + 0.1% Tween before incubation in a secondary antibody, a horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Cell Signaling). ECL (Thermo Fisher Scientific) was used as chemiluminescence reagents, and an Amersham Imager 600 (General Electric) was used for detection.

LEGENDplex™ multiplex cytokine analysis

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

Enzyme-linked immunosorbent assay

Secreted MPO and LCN2 in cell culture supernatants were analyzed using DuoSet ELISA Kits from R&D according to the manufacturer's instructions. Briefly, ELISA plates (Nunc) were coated with 50 µl capture antibody overnight at 4°C. The next

day, the plate was washed three times with PBS + 0.05% Tween before being incubated in PBS + 1% BSA for 1h at RT. After three washing steps with PBS + 0.05% Tween, 50 µl cell culture supernatant or standards were added, and the plate was incubated for 2h at RT. The plate was washed three times and incubated with a biotinylated detection antibody for 2h at RT. After three washing steps, the plate was incubated in HRP-conjugated streptavidin for 20 min at RT in the dark. The plate was subsequently washed and a TMB substrate solution (Cell Signaling) was added to each well. The reaction was stopped with 2N H_2SO_4 and absorbance at 450 nm was measured using a Fluoroskan II (Labsystems).

RNA isolation & cDNA generation

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

RT^2 Profiler™ PCR Array

RT^2 Profiler™ PCR Array Antibacterial Response (PAHS-148Z) was used for the analysis of genes involved in inflammation and immune responses in PHKs after 18h co-incubation with PMNs. As control, non-co-cultured PHKs were used. The assay and subsequent data analysis was performed according to the manufacturer's instructions. Samples were acquired in triplicates.

Bacterial strains

Staphylococcus aureus USA300 LAC and *Staphylococcus epidermidis* 1457 was used in this study. Bacteria were aerobically grown in tryptic soy broth (TSB) at 37°C and orbital shaking. All experiments were performed with logarithmically grown bacteria (OD = 0.5).

Neutrophil recruitment assay

For the PMN recruitment assay, freshly isolated PMNs were labeled with 1 µM Calcein (eBioscience) for 30 min, washed and seeded into a 3 µm transwell insert (Sarstedt). The transwell insert was then placed above a well containing different stimuli. After 1h, the inserts were removed and PMNs migrated into the lower well were lysed using 1% Triton X-100. The fluorescence of the lysates was quantified in triplicates using a Fluoroskan II (Labsystems). As stimuli, the supernatants of the PHK well (upper well) or the PMN well (lower well) of the 18h co-culture with or without *S. aureus* infection was used. As controls, the supernatants of non-co-cultured PHKs with and without *S. aureus* infection and non-co-cultured PMNs was included. As positive control, N-formyl-met-leu-phe was used (fMLF). Prior to the migration assay, the supernatants were centrifuged, and filter sterilized. To calculate the absolute numbers of migrated PMNs, a standard curve was included.

Statistical analysis

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

Results

Co-incubation with PHKs prolongs the lifespan of PMNs

First, we were interested whether the viability of PMNs changes when we co-cultured with PHKs. Therefore, we analyzed the viability of PMNs by SYTOX Green staining, a non-permeable dye indicating dead cells, at different time points after co-culture with PHKs. We used an established *in vitro* co-culture transwell chamber model (18) and compared it to the results of the non-co-cultured PMNs (Figure 1A). We found that the percentage of SYTOX Green positive cells steadily increases overtime in the non-co-cultured PMNs. In contrast to this, co-cultured PMNs exhibit a significantly extended lifespan, with only little increase in Sytox Green positive cells in the first 18h. However, the induction of cell death was not completely prevented in co-cultured PMNs as we see a clear increase in SYTOX Green positive cells at 30h co-incubation time. Therefore, we conclude that the co-culture with PHKs significantly delays the induction of cell death in PMNs. We calculated the delay of cell death induction between the co-cultured and non-co-cultured PMNs using interpolation and found a delay of 9.3h until 50% of the cells are dead (Supplementary Figure 1A). To unravel the type of cell death, we analyzed the induction of apoptosis in co-cultured and non-co-cultured PMNs by caspase-3 cleavage and Annexin-V staining (Figures 1B, C). After 18h, apoptosis induction of non-co-cultured PMNs was indicated by cleaved caspase-3 which was not observed in co-cultured PMNs (Figure 1B). Furthermore, we used Annexin-V staining, which detects cells in the early or late apoptosis phase before

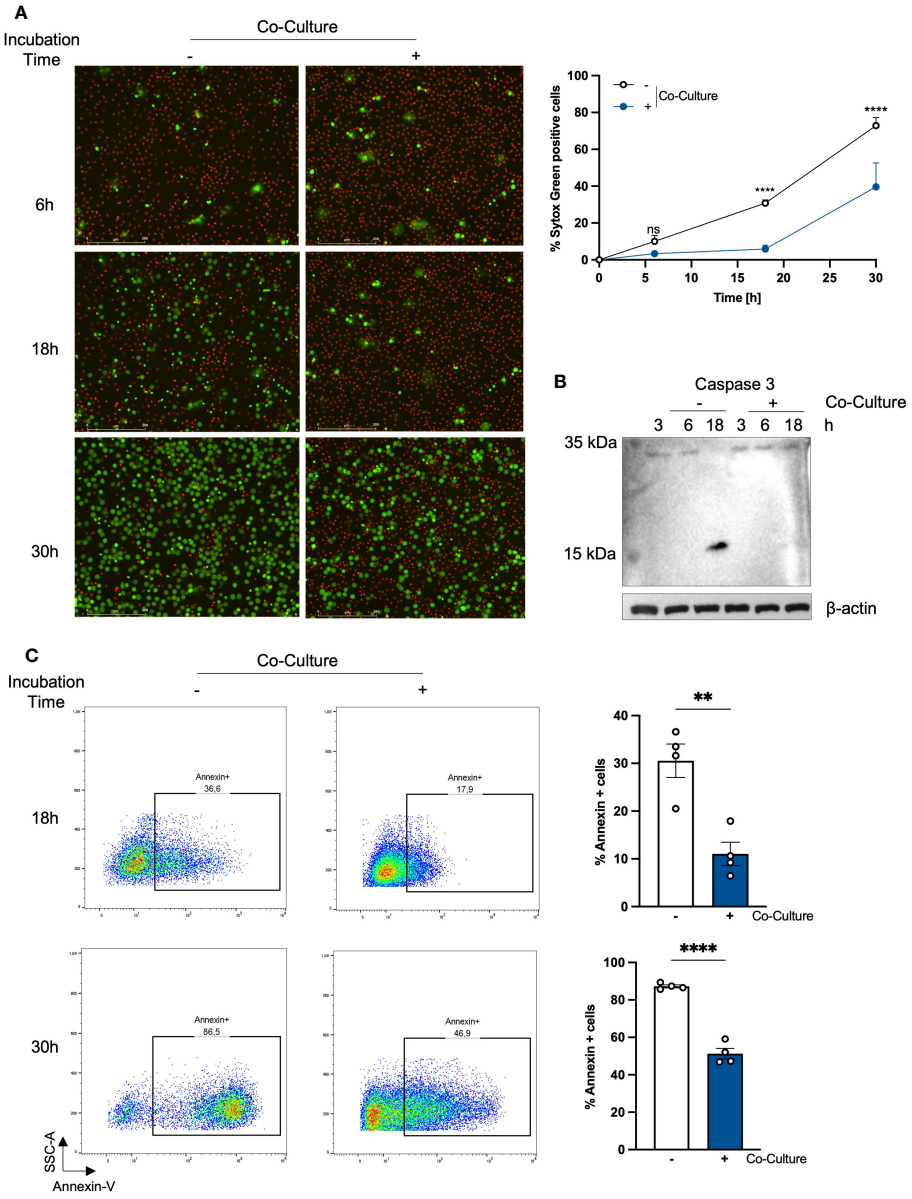


FIGURE 1
Co-incubation with PHKs prolongs the lifespan of PMNs. **(A)** PMNs (2x10^6/ml) were either incubated alone or in co-culture with differentiated PHKs seeded in a transwell insert (pore size 0.4 µm) and placed above the PMNs. Cell viability was analyzed at different times by quantifying the percentage of Sytox Green positive cells (n = 4). Representative pictures of one experiment after 6h, 18h and 30 are shown. Scale bars = 200 µm. Significant differences between non-co-cultured and co-cultured PMNs at the different time point were analyzed by two-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Shown are the mean of four different experiments + SEM. **(B)** Induction of apoptosis in non-co-cultured and co-cultured PMNs was analyzed at 3h, 6h and 18h incubation time by investigating caspase-3 cleavage via western blot. Protein expression of β-actin was used as loading control. **(C)** Apoptosis induction in non-co-cultured and co-cultured PMNs was analyzed at 18h and 30h by Annexin-V staining. Annexin positive cells were quantified and represent apoptotic cells. Shown are the mean of four different experiments + SEM. Significant differences were analyzed by unpaired two-tailed t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. On representative experiment of three independent experiments is shown. PHKs, primary human keratinocytes; PMNs, polymorphonuclear neutrophils; SEM, standard error of the mean.

the cells lose their membrane integrity and get Sytox positive. Interestingly, Annexin-V staining after different incubation times revealed that apoptosis induction was significantly delayed in co-cultured PMNs compared to non-co-cultured PMNs (Figure 1C) confirming our Sytox experiments. Together, these data indicate that in the co-culture a beneficial interaction between PHKs and PMNs significantly extend the viability of PMNs by delaying apoptosis induction.

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

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

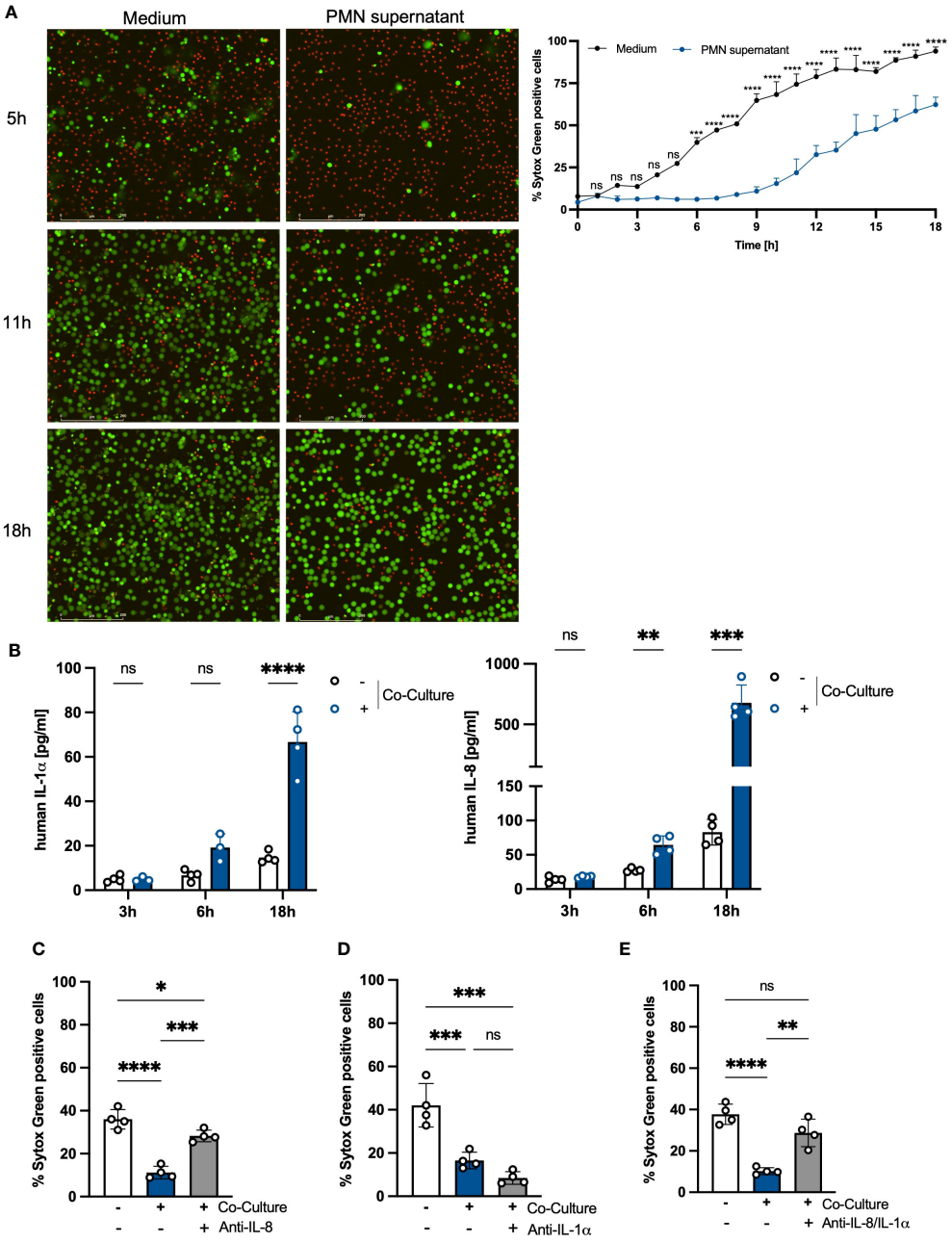


FIGURE 2
The prolonged lifespan of PMNs in the co-culture with PHKs is partly mediated by secreted IL-8. **(A)** Freshly isolated PMNs (2x10^6/ml) were incubated either in medium or in the PMN supernatant of the 18h co-culture. Cell viability was analyzed over time by Sytox Green staining and live cell imaging. The percentage of Sytox Green positive cells, indicating dead cells, was quantified every hour for 18h using Fiji/ImageJ (n = 3). Representative images of the same cell area after 5h, 11h and 18h incubation are shown. Scale bars = 200 µm. **(B)** Freshly isolated PMNs were co-cultured with differentiated PHKs or alone. After 3h, 6h and 18h, secreted factors in the PMN well were analyzed using Legendplex analysis. Significant differences between the non-co-cultured and co-cultured PMNs were analyzed for each time point by multiple unpaired t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Shown is one representative experiment of four independent experiments + SD. **(C–E)** PMNs were either incubated alone or in co-culture with differentiated PHKs in the presence or absence of an anti-IL-8 antibody **(C)** or anti-IL-1α antibody **(D)** or both **(E)**. Induction of cell death was analyzed after 18h by quantifying Sytox Green positive cells using Fiji/Image J (n = 4). Shown is one representative experiment of four independent experiments + SD. Significant differences were analyzed between the percentage of SYTOX Green positive cells by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. PMNs, polymorphonuclear neutrophils; PHKs, primary human keratinocytes; IL-1α, interleukin 1α; IL-8, interleukin 8; SD, standard deviation. ns, non significant.

of the PMN well, filter-sterilized it and used it for stimulation of freshly isolated PMNs. Non-co-cultured PMNs incubated in medium were used as control. We investigated cell death of PMNs over time by Sytox Green staining and live cell imaging (Figure 2A). While the percentage of Sytox-Green-positive cells increased significantly after 5 hours in control PMNs cultured in medium, the induction of cell death was delayed in PMNs incubated with PMN supernatant. Here, a significant increase in Sytox-Green-positive cells was observed after

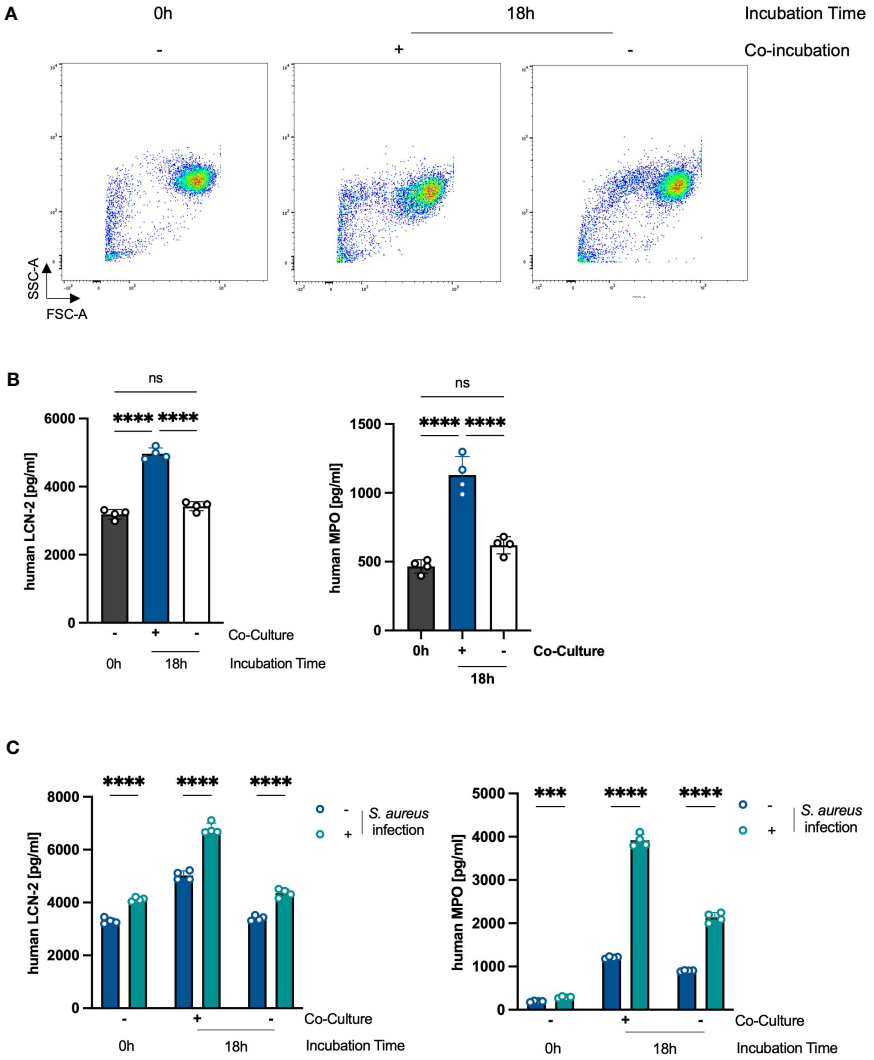


FIGURE 3
Co-Culture with PHKs activates PMNs. **(A)** Forward Scatter (FSC) and Side Scatter (SSC) of freshly isolated PMNs and PMNs co-cultured with differentiated PHKs or alone for 18h were analyzed by flow cytometry. **(B)** Secreted levels of MPO and LCN-2 by co-cultured and non-co-cultured PMNs after 18h incubation was analyzed by ELISA and compared to freshly isolated PMNs (0h). Shown is one representative experiment of three independent experiments + SD. Significant differences were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One representative experiment of three independent experiments is shown. **(C)** Freshly isolated (0h), 18h co-cultured and 18h non-co-cultured PMNs were infected directly with *S. aureus* (MOI = 10) or left uninfected. After 2h, secreted MPO and LCN2 levels were analyzed by ELISA. Significant differences between uninfected and infected PMNs were analyzed by unpaired two-tailed t-tests *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Shown is one representative experiment of three independent experiments + SD. PMNs, polymorphonuclear neutrophils; PHKs, primary human keratinocytes; MOI, multiplicity of infection; MPO, myeloperoxidase; LNC2, lipocalin-2. ns, non significant.

11 hours, which then steadily increased over time. We calculated the delay in cell death induction between PMNs incubated in medium or PMN supernatant by interpolation and found a delay of 8.3 hours until 50% of the cells were dead (Supplementary Figure 1B). This delay (8.3 hours) is comparable to the delay found in co-cultured *vs* non-co-cultured PMNs (9.3 hours), observed in Figure 1A which indicates that the soluble factors released by PMNs during the co-culture are mainly responsible for the extended lifespan. To identify these factors, we performed LEGENDplex analysis of the PMN well after 3h, 6h and 18h co-culture with PHKs and compared these to secreted factors from non-co-cultured PMNs. We detected significantly increased amounts of secreted Interleukin (IL)-8 and IL-1α in PMNs co-cultured with PHKs compared to non-co-cultured PMNs after 18h (Figure 2B). Other examined cytokines were not significantly induced (Supplementary Figure 2A). To test whether the IL-1α and IL-8 are responsible for the enhanced viability of PMNs co-cultured with PHKs, we co-cultured PMNs with PHKs for 18h in the presence of absence of either an anti-IL-8, anti-Il-1α or both antibodies together and analyzed cell viability by Sytox Green staining. We compared the results to the non-cocultured PMNs. Interestingly, addition of the anti-IL-8 antibody led to a significant reversal of the life-prolonging effect of the co-culture, whereas IL-α had no significant life-prolonging effect (Figures 2C, D; Supplementary Figures 2B, C). Interestingly, the combined

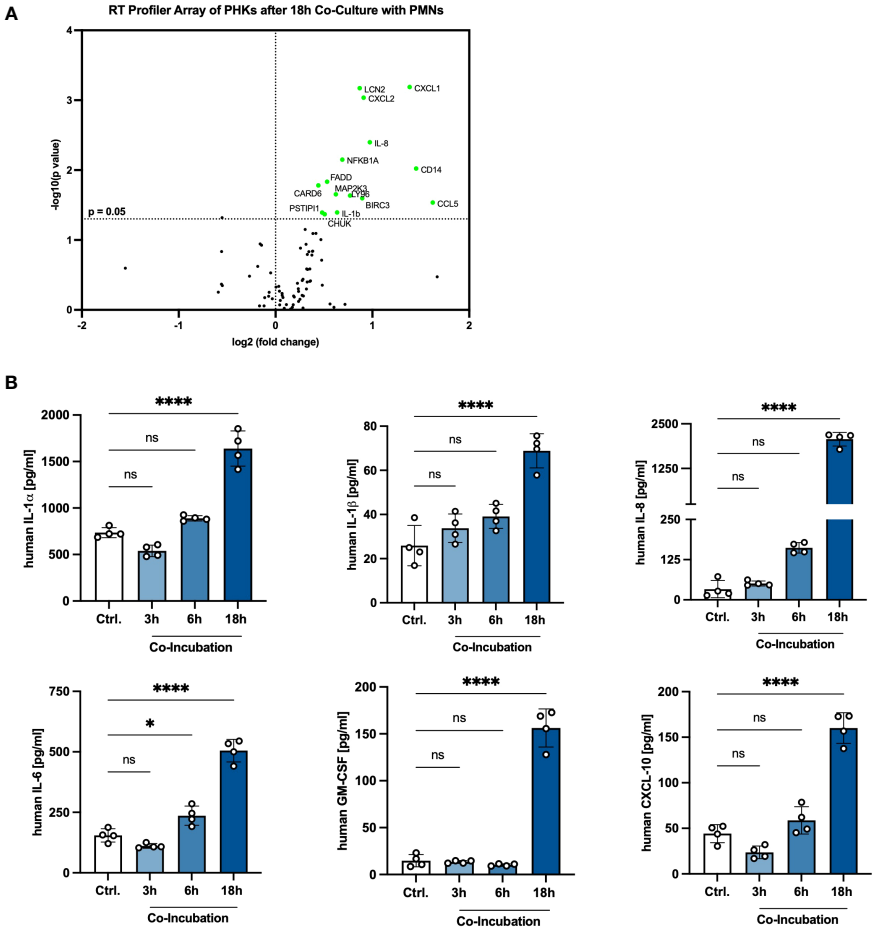


FIGURE 4
Extended co-incubation activates a proinflammatory state in PHKs. **(A)** Differentiated PHKs were co-incubated with freshly isolated PMNs. After 18h, the expression of 84 different genes was analyzed in PHKs by a RT-Profiler Array (n = 3). Significantly upregulated genes in co-cultured PHKs compared to non-co-cultured PHKs are marked green. **(B)** Secreted factors by PHKs were analyzed after 3h, 6h and 18h of co-incubation with PMNs. Ctrl., non-co-cultured PHKs. Shown is one representative experiment of four independent experiments + SD. Significant differences to the control were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One representative experiment of three independent experiments is shown. PHKs, primary human keratinocytes; PMNs, polymorphonuclear neutrophils; RT, real-time. ns, non significant.

treatment with an anti-IL8 and anti-IL-1α antibody still reverted the life-prolonging effect of the co-culture indicating that the effect of IL-8 dominates compared to IL-1α in the life prolongation of PMNs in the co-culture (Figure 2E; Supplementary Figure 2D). Interestingly, recombinant IL-8 delayed apoptosis in freshly isolated PMNs in a concentration-dependent manner, further highlighting the anti-apoptotic properties of IL-8 (Supplementary Figure 2E).

Co-culture with PHKs activates PMNs

We next analyzed whether along with extending their lifespan, the co-culture with PHKs activates PMNs. Activated PMNs are characterized by degranulation (22). Indeed, we observed a decrease in the side scatter (SSC) of co-cultured PMNs after 18h compared to non-co-cultured PMNs or freshly isolated PMNs (0h), indicative for reduced granularity and thus degranulation (Figure 3A). We further analyzed degranulation by analyzing surface expression of CD11b, CD66b and CD63 as well as extracellular levels of MPO and LCN2, all markers for degranulation. We observed that PMNs co-cultured with PHKs for 18h released significantly more MPO and LCN2 compared to non-co-cultured PMNs or freshly isolated PMNs (Figure 3B). Furthermore, while we did not detect a significant difference in the surface expression of CD11b or CD66b, surface expression of CD63 was significantly increased in co-cultured PMNs compared to non-co-cultured or freshly isolated PMNs (Supplementary Figure 3). Together, these data indicate that PMNs are in an activated state after co-culture with PHKs.

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

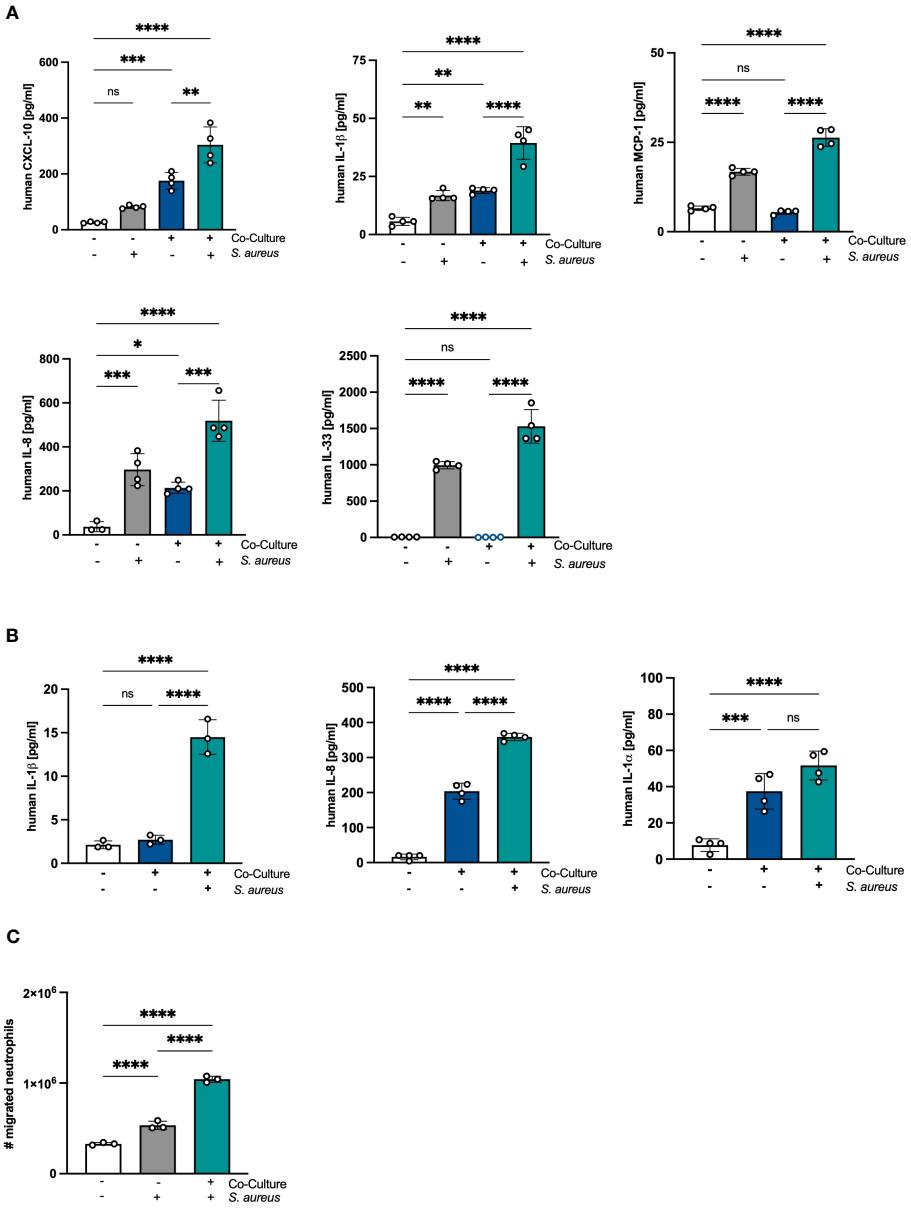


FIGURE 5
Crosstalk between PHKs and PMNs exacerbates immune responses to *S. aureus* infection. **(A, B)** Differentiated PHKs were co-incubated with freshly isolated PMNs or alone. After 18h, PHKs were infected with *S. aureus* (MOI = 30) for 1.5h or left uninfected. Secreted factors in the PHK well **(A)** and PMN well **(B)** were analyzed by Legendplex analysis. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One representative experiment of three independent experiments is shown. **(C)** Freshly isolated PMNs were seeded into transwell inserts with 3 µm pores and placed above a well containing supernatants of either non-co-cultured or co-cultured PHKs after *S. aureus* infection. After 1h, the number of migrated PMNs were quantified. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One representative experiment of three independent experiments is shown. PHKs = primary human keratinocytes; PMNs, polymorphonuclear neutrophils; MOI, multiplicity of infection; ns, non significant; SD, standard deviation.

Co-incubation with PMNs activates an inflammatory response in PHKs

Our results show that a crosstalk between PHKs and PMNs boosts the activity of PMNs. Next, we analyzed whether the interaction with PMNs affects, on the other hand, also the PHKs and induces a proinflammatory response in PHKs. For this, we co-cultured PHKs with PMNs for 18h and subsequently analyzed in PHKs the expression of 84 different genes involved in inflammation using an RT-Profiler PCR Array and compared gene expression to 18h non-co-cultured PHKs. Interestingly, we detected significant upregulation of genes associated with immune cell recruitment (CXCL1, CXCL2, IL-8, CCL5), TLR signaling (CD14, LY96), regulation of apoptosis (FADD, CARD6, BIRC3), NFκB signaling (CHUK, NFKB1a, IL-1β)

as well as stress response (MAP2K3) in co-cultured PHKs compared to the non-co-cultured PHKs (Figure 4A). We further analyzed the induction of a proinflammatory response in PHKs by comparing the secretion of cytokines and chemokines by PHKs co-cultured with PMNs to the non-co-cultured PHKs at different time points using Legendplex analysis. With increasing incubation time, we detected in co-cultured PHKs elevated levels of secreted IL-1α, IL-1β, IL-8, IL-6, GM-CSF, and CXCL-10 compared to the non-co-cultured PHKs (Figure 4B). Other analyzed cytokines and chemokines were not significantly induced (Supplementary Figure 4). Our results indicate that co-culture with PMNs induce induces a proinflammatory state in PHKs.

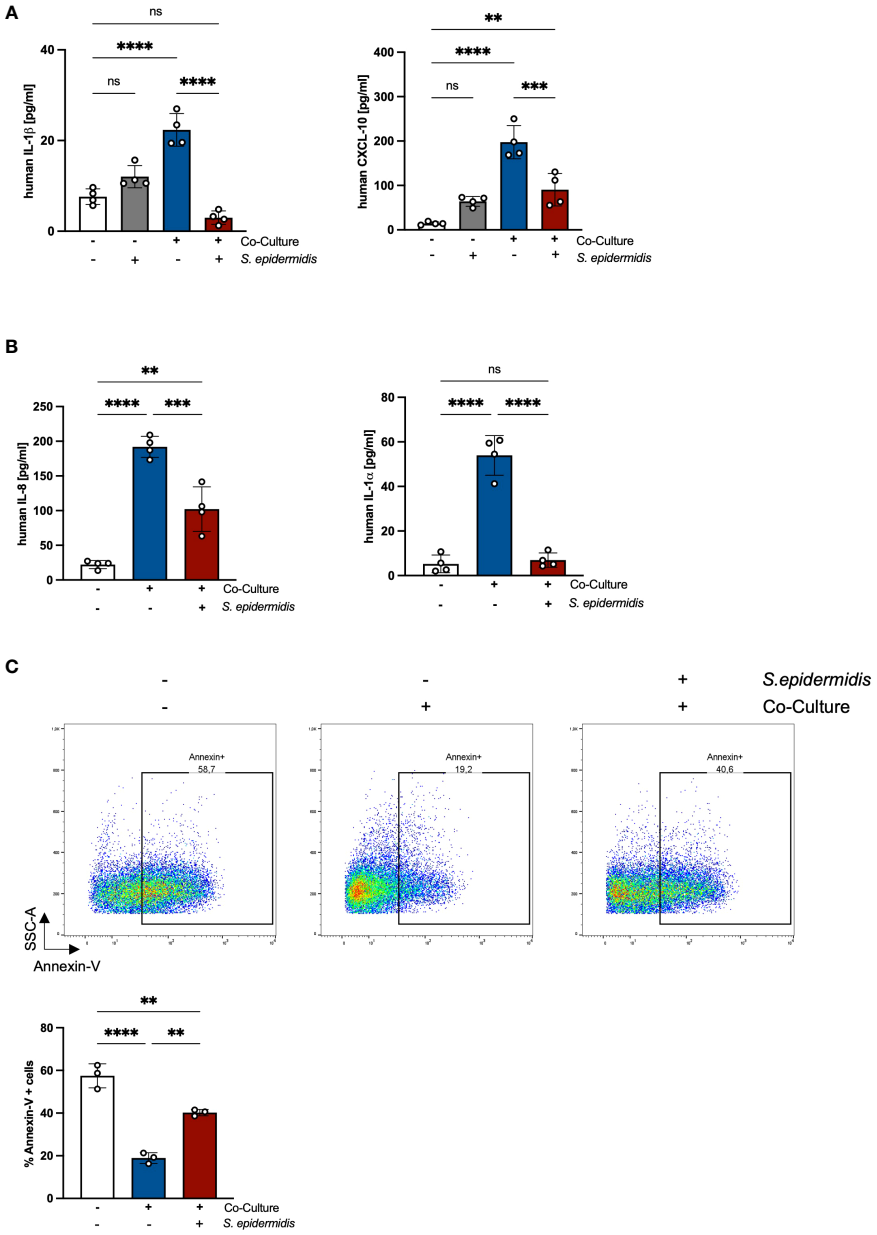


FIGURE 6
The skin microbiome reduces PMN-mediated inflammation and induces apoptosis in PMNs. **(A, B)** Differentiated PHKs were co-incubated with freshly isolated PMNs or alone. After 18h, PHKs were infected with *S. epidermidis* (MOI = 30) for 1.5h or left uninfected. Secreted factors in the PHK well **(A)** and the PMN well **(B)** were analyzed by Legendplex analysis. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. One representative experiment of three independent experiments is shown. **(C)** Freshly isolated PMNs were incubated alone or in co-culture with PHKs. After 18h, PHKs were infected with *S. epidermidis* or left uninfected (MOI = 30) for 1.5h. Apoptosis induction in PMNs was analyzed by Annexin-V staining. Shown is one representative experiment of four independent experiments + SD. Significant differences between the samples were analyzed by one-way ANOVA *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, non significant.

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

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

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

Previous results of our group showed that the skin microbiome has a beneficial role in preventing *S. aureus* skin colonization in a non-inflammatory environment (20). Interestingly, the decreased *S. aureus* colonization was accompanied by reduced skin inflammation and PMN recruitment (18). Our results also showed that in an inflammatory environment induced by tape-stripping, skin infiltrating PMNs enhance *S. aureus* skin colonization (18). Based on these results, we hypothesized that the skin microbiome affects PMN-mediated skin inflammation. To test this, we analyzed inflammatory responses in PHKs in the co-culture with PMNs without or upon infection with the skin commensal *S. epidermidis*. Interestingly, we observed a significant decrease in the secreted levels of IL-1β and CXCL-10 by co-cultured PHKs upon *S. epidermidis* infection compared to the cocultured non-infected PHKs (Figure 6A). No significant difference between infected and not infected co-cultured PHKs were observed on other analyzed cytokines and chemokines (Supplementary Figure 6A). This indicates that the skin microbiome reduces the inflammatory responses in PHKs induced by PMNs. Furthermore, we found that *S. epidermidis* infection of PHKs significantly reduces the secreted levels of IL-8 and IL-1α by PMNs in the co-culture (Figure 6B) and significantly enhanced apoptosis induction in PMNs compared to the non-infected and co-cultured PMNs (Figure 6C). These results indicate that the skin microbiome reduces PMN-mediated skin inflammation by downregulation of PMN-induced proinflammatory cytokines in PHKs and induction of apoptosis in PMNs.

Discussion

PMNs are the most abundant type of leucocytes in the human blood and play an important role in the innate immune system (23). Upon inflammation, PMNs are rapidly recruited from the circulation to infection sites where thy provide an effective first-line defense. Several groups have shown that PMNs play a crucial role in clearing *S. aureus* skin infections (15, 16). Here we demonstrate that a crosstalk between skin-infiltrating PMNs and skin-resident PHKs enhances the proinflammatory responses of both cells upon *S. aureus* infection.

PMNs have a relatively short lifespan. They are continually replenished from the bone marrow and released into the bloodstream. In circulation, they actively patrol for infection of tissue and in the absence of an inflammatory stimulus, PMNs undergo spontaneous apoptosis and are cleared by macrophages, maintaining immune balance (10). The lifespan of circulating murine PMNs is estimated to be about 12h (24), however, this can be significantly extended upon inflammation (4). During inflammation, PMNs are rapidly recruited to the affected site. Inflammatory signals promote their survival and activation, enabling them to combat invading pathogens by releasing antimicrobial agents and engaging in phagocytosis (25). However, excessive PMN activation can cause tissue damage, so mechanisms exist to regulate inflammation resolution including the induction of PMN apoptosis and their subsequent clearance by macrophages for immune homeostasis (25).

In their investigation of murine PMN lifespan in various tissues (bone marrow, blood, liver, lung, spleen, intestine, and skin) Ballesteros et al. discovered that the half-life of neutrophils varies depending on the specific tissue they infiltrate into (26). Notably, this study reveals that PMNs quickly adopt a tissue-specific phenotype and transcriptional profile, likely contributing to efficient immune responses against invading pathogens. Interestingly, they showed that the half-life of PMNs was highest in the skin with about 18h.

While several studies have examined the lifespan of PMNs in various tissues of mice, there is limited knowledge regarding the lifespan of PMNs in the human system, especially in the skin. Here, we demonstrate for the first time that the presence of PHKs during co-incubation significantly prolongs the lifespan of PMNs in a human *in vitro* co-culture model. Compared to PMNs cultured alone, co-cultured PMNs exhibit a noticeable delay in apoptosis induction. This extended lifespan is facilitated by the secretion of IL-8, which increases progressively as the incubation time is prolonged. We observed a reversal of this effect when an anti-IL-8 antibody was added to the co-culture. The ability of IL-8 to delay spontaneous apoptosis induction of PMNs is also described by previous studies (27). Interestingly, we did find that depletion of IL-1α, which is also induced in the co-culture, further prevents cell death induction thus

indicating that IL-1α is capable of inducing cell death in PMNs. The proapoptotic functions of IL-1α have been described for other cells (28), however, to the best of our knowledge not in PMNs.

Nevertheless, the co-culture with PHKs and IL-8 stimulation did not entirely inhibit PMN apoptosis; instead, it caused a delay in the process, eventually leading to its induction within the co-culture system. Our findings revealed that co-cultured PMNs are activated and released their granules after 18h incubation time. Excessive activation of PMNs can cause tissue damage, so mechanisms exist to regulate inflammation resolution including PMN apoptosis and their subsequent clearance by macrophages for homeostasis (25). Therefore, we hypothesize that the eventual induction of apoptosis in PMNs is required to minimize tissue damage and restore homeostasis.

Interestingly, we found that co-cultured PMNs displayed an elevated activation and responsiveness towards an infectious stimulus in comparison to non-co-cultured or freshly isolated PMNs. This was demonstrated by increased degranulation upon *S. aureus* infection by co-cultured PMNs compared to non-co-cultured PMNs or freshly isolated PMNs. PMNs play a pivotal role in the immune response against *S. aureus* skin infections (16, 29). The reactivity of circulating PMNs towards inflammatory stimuli is intentionally constrained to avoid tissue damage and uphold homeostasis (22). However, this reactivity can be significantly enhanced by the exposure to inflammatory stimuli such as cytokines, chemokines, pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively). The exposure to such stimuli primes the PMNs for enhanced responsiveness towards invading pathogens (22, 30–32). Some studies showed that PMNs primed by proinflammatory cytokines derived from PBMCs exert enhanced killing capacities against *S. aureus* (33, 34). Therefore, we hypothesize that the crosstalk with PHKs contributes to shaping the immune response against *S. aureus* skin infections by priming PMNs for enhanced activation. The co-culture environment seems to provide a beneficial influence on the PMNs, enhancing their ability to combat *S. aureus* skin infections. This finding highlights the significance of cellular interactions and their impact on the immune system's effectiveness in mounting a robust response against infectious agents.

Moreover, we found that the extended co-incubation not only has a priming effect on PMNs but also induces a proinflammatory state in PHKs. This was characterized by increased secretion of IL-1α, IL-1β, IL-6, IL-8, GM-CSF and CXCL10 by PHKs. CXCL10 and GM-CSF are chemoattractants for other immune cells besides PMNs, such as monocytes, macrophages, T cells, NK cells or dendritic cells (35, 36). This suggests that the extended crosstalk between PHKs and PMNs initiates the recruitment of other immune cells to the skin to facilitate the advance of the immune response. The induction of these proinflammatory mediators is independent on direct cell-to-cell contact but is rather mediated by secreted factors. This finding is supported by previous studies describing the induction of inflammation in PHKs by PMNs (37, 38). For example, Lieu et al. showed that indirect co-culture with PMNs leads to the induction of several pro-inflammatory genes and secretion of IL-8 in HaCaT cells (38). Moreover, Shao et al. showed that exosomes derived from PMNs are able to induce pro-inflammatory gene expression in PHKs (37). Interestingly, although the expression of neutrophilic chemoattractants such as CXCL-1/2 and IL-8 was highly elevated in PHKs after 18h co-culture with PMNs, the supernatant containing these chemoattractants was not able to induce PMN migration. This might be that the concentrations of the respective cytokines were too low for PMN migration. Another reason might be that some factor stops PMN migration as excessive PMN migration and activation can lead to tissue damage.

As PHKs are the main constituents of the epidermis, the outermost layer of our skin, they are constantly exposed to exogenous bacteria such as beneficial skin commensals like *S. epidermidis* or pathogenic bacteria like *S. aureus* and are therefore crucial in the initiation of skin inflammation (2). Here, we found that a previous co-culture with PMNs significantly elevated the level of proinflammatory cytokines and chemokines by PHKs induced by *S. aureus* infection. *S. aureus* is a facultative pathogen which frequently colonizes the skin of atopic dermatitis patients where it actively contributes to inflammation. There is evidence that PMNs are elevated in AD skin and contribute to its pathogenesis (39–41). Furthermore, previous results of our group showed that the PMNs can enhance *S. aureus* skin colonization (18, 19). As a mechanism for this we show that PMNs infiltrating inflamed skin are primed by PHKs for NET formation in response to *S. aureus* infection. The increased presence of PMNs and NETs in the inflamed skin induces oxidative stress in PHKs which results in the secretion of HMGB1, which induces further oxidative stress in PHKs and NET formation in PMNs. Moreover, both NETs and HMGB1 induce the downregulation of epidermal barrier genes in PHKs, thus inducing a skin barrier defect which favors *S. aureus* skin colonization (19). Here, we observed that in addition to promoting skin barrier defects which could enhance *S. aureus* skin colonization, the co-culture with PMNs exacerbates *S. aureus* induced skin inflammation. We believe that the PMNs secrete IL-8 during the co-culture, thereby prolonging their lifespan and becoming activated. The activated PMNs then induce an inflammatory state in PHKs and boost their inflammatory response against *S. aureus*.

The eventual induction of PMN apoptosis in the co-culture might be necessary to not further prolong inflammation. Interestingly, we found that *S. epidermidis* infection of PHKs in the co-culture reduced the proinflammatory responses in PHKs mediated by the co-culture with PMNs. Furthermore, we found that *S. epidermidis* infection of PHKs induced apoptosis in PMNs in the co-culture. Previous work of our group showed that the skin microbiome has a protective role against *S. aureus* skin infections for example by reducing *S. aureus*-mediated skin inflammation and PMN recruitment (18, 20). Our results here further show that the skin commensal bacteria could play a role in preventing PMN-mediated excessive skin inflammation.

In conclusion, here we show for the first time that a crosstalk between PHKs and PMNs in the skin shaped the immune responses against *S. aureus* infections (see graphical abstract). On the one hand, the crosstalk delays the induction of apoptosis in PMNs, prolonging their lifespan and enhancing their activation and responsiveness against *S. aureus*. This effect is achieved through the release of IL-8 rather than direct cell-to-cell contact. However, it

is important to note that prolonged incubation with PMNs also lead to inflammatory responses in keratinocytes. This inflammatory response is further exacerbated in the presence of *S. aureus*. Interestingly, the skin commensal *S. epidermidis* reduces the PMN-mediated skin inflammation in PHKs and induces apoptosis in activated PMNs which indicates a beneficial role of the skin microbiome in preventing excessive inflammation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by University of Tübingen Ethics committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BS: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft. JF: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft.

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Conflict of interest

The 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.

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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.2024.1275153/full#supplementary-material

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Unraveling the skin; a comprehensive review of atopic dermatitis, current understanding, and approaches

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Atopic dermatitis, also known as atopic eczema, is a chronic inflammatory skin disease characterized by red pruritic skin lesions, xerosis, ichthyosis, and skin pain. Among the social impacts of atopic dermatitis are difficulties and detachment in relationships and social stigmatization. Additionally, atopic dermatitis is known to cause sleep disturbance, anxiety, hyperactivity, and depression. Although the pathological process behind atopic dermatitis is not fully known, it appears to be a combination of epidermal barrier dysfunction and immune dysregulation. Skin is the largest organ of the human body which acts as a mechanical barrier to toxins and UV light and a natural barrier against water loss. Both functions face significant challenges due to atopic dermatitis. The list of factors that can potentially trigger or contribute to atopic dermatitis is extensive, ranging from genetic factors, family history, dietary choices, immune triggers, and environmental factors. Consequently, prevention, early clinical diagnosis, and effective treatment may be the only resolutions to combat this burdensome disease. Ensuring safe and targeted drug delivery to the skin layers, without reaching the systemic circulation is a promising option raised by nano-delivery systems in dermatology. In this review, we explored the current understanding and approaches of atopic dermatitis and outlined a range of the most recent therapeutics and dosage forms brought by nanotechnology. This review was conducted using PubMed, Google Scholar, and ScienceDirect databases.

KEYWORDS

atopic dermatitis, nano-dermatology, skin immunology, epidermal barrier dysfunction, immune dysregulation, nano-delivery systems, filaggrin gene mutations, Th Lymphocytes

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Introduction

Atopic dermatitis (AD), also referred to as atopic eczema, is a phenotypically heterogeneous chronic inflammatory skin disease. It typically arises due to environmental triggers in individuals who are genetically predisposed to the disease. AD is characterized by pruritus, particularly worsening at night, dry and leathery indurated skin, covered by intensively itchy papules which may release clear fluid when scratched. Additionally, xerosis, the term used for roughness of skin, and ichthyosis, a category of skin keratinization disorder, are the two terms often used with AD (1). The prevalence of AD is about 20% in childhood and 1-3% in adults, with female predominance, following a bimodal curve that peaks at early childhood and middle-aged adult population (2). AD was ranked 15th in the global burden of disease study in 1990-2017 among all nonfatal diseases, and ranked 1st among skin diseases measured in disability-adjusted life-years (DALYs) (3).The treatment of AD requires frequent physician visits and treatment compliance. The Out of pocket cost related to AD in the USA is $600 on average per person per year (4). AD is a member of a triad known as the "atopic triad", along with allergic rhinitis and bronchial asthma. Recent studies indicate a sequential development of atopic disorders, referred to as atopic march, including asthma, allergic rhinitis, and food allergies in later childhood closely associated with infantile AD (5).

AD can be classified based on the age of onset. Pediatric patients are categorized into three main groups. The "very early onset" group includes patients who manifest their first signs of the disease as early as three months old. A significant portion of these patients experience complete remission by the age of two. This group accounts for more than two-thirds of all cases of AD. Patients aged between two and six years fall into the "early onset" category, while those patients between six and fourteen years are considered to have a "childhood onset" of AD. The "adolescent onset" group includes patients aged between fourteen and eighteen with limited available data. In contrast, adults are categorized into two main groups. The "adult-onset" group comprises patients between the ages of twenty and sixty, while the "very late onset" group includes patients over the age of sixty. The majority of adult patients are women with limited spectrum of sensitization (6).

In elder patients, it is recommended to carefully differentiate the potential diagnosis as other medical conditions, such as scabies, psoriasis, erythroderma, and cutaneous T-cell lymphoma, may manifest similarly to those of AD during their early stages. The diagnosis of AD relies on clinical observations as there are no validated, specific laboratory assays. In case of questionable or borderline diagnoses, skin biopsies may be recommended (7).

According to the diagnostic criteria established by the United Kingdom working party, diagnosis of AD requires the presence of itchy skin with three or more minor criteria. These minor criteria include flexural dermatitis or skin lesions, a history of generalized dry skin, asthma and the presence of a rash before the age of two (8). Noteworthy, these criteria are not applicable in very young children particularly infants. In infants and younger pediatric patients, the condition often affects extensor surfaces of extremities excluding diaper area, face, neck, and scalp. Papules on cheeks and cradle cap are commonly seen in infants in the first few months after birth (9). With further maturation into adolescence and adulthood, lesions tend to get fixated to more classical areas, namely the head, hands, neck, and flexural areas. Eyelid's AD is also commonly seen in adult female patients. Clinicians acknowledge that, regardless of the specific phenotype of AD, pruritus remains the number-one complaint and first sign of AD in 87% of patients. The insomnia and anxiety stemming from excessive itch are the initial steps towards subsequent social and psychological burdens. The unpleasant sensation forces patients to scratch their skin, also raising the risk of skin barrier disruption, the likelihood of superimposed infections, and the sensation of pain (10). In cases of AD, hyperactivity of the nerves surrounding the atopic loci has been documented which may account for tingling and burning sensation. The density and diameter of nerves distribution was much higher in these patients compared to healthy controls (11).

Besides clinical features which aid us in accurate diagnosis of AD, validated scoring systems such as EASI (Eczema Area and Severity Index) and SCORAD (SCORing Atopic Dermatitis) subjectively and objectively stratify the severity of the disease into mild, moderate, or the severe forms (12). For many years, it was believed that clinical manifestations of AD were consistent across all ethnicities. However, recent studies suggest that the variation in transcriptomic profile of Asian patients, particularly in Th17 lymphocytes polarization, accounts for the difference in the clinical picture of chronic inflammation in Asians compared to Caucasians (13). Another notable difference is the mutation of filaggrin gene in Africans and Caucasians, as filaggrin deficiency is not observed in most Africans but is prevalent among Caucasian patients with AD (14).

The concept of precision medicine coupled with society's growing demand to prevent or treat diseases in the most cost-effective manner, has led researchers to further investigate individual's sensitization profile or detect biomarkers which predict the severity and identify patients before any clinical signs appear. One practical method to assess individual's sensitization profile in AD is to assess total IgE and particularly allergen-specific IgE against common allergens such as food allergens or pollen allergens, and calculate the ratio of specific to total IgE (15). Measuring serum or plasma biomarkers is also promising approach proposed for diagnosis of AD.

Skin; immunological perspective

The skin, the largest organ in the human body, originates embryologically from the surface ectoderm, neural crest, and mesoderm. Skin serves as an insulator of the internal surface from the external world by shielding against invasion of pathogens and toxins. The thickness of skin varies across different body sites and protects against physical trauma and chemical injuries. Additionally, skin preserves water and electrolytes and contributes to the body's temperature regulation. In conjugation with the endocrine system, skin plays a role in hormone production (16). Various types of cells, including some originating from bone marrow, are distributed throughout the skin, and serve as immune

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or non-immune cells. The skin compromises three main layers, in order from top to base, the epidermis, the dermis, and the hypodermis. The epidermis itself is further divided into stratum corneum, lucidum, granulosum, spinosum and basale.

Keratinocytes, which represent the most abundant cell type in the skin and the fundamental building blocks of the epidermis, continually undergo proliferation and migration to maintain a balance between the ongoing terminal differentiation and desquamation occurring at the uppermost layer of epidermis. The transformation of keratinocytes into corneocytes are known as cornification. Corneocytes are a-nucleated keratinocytes with high keratin content and no inner organelles, covered in the cornified envelope, ceramides, free fatty acids, and cholesterol to form the first line of defense. Changes in the quantity and composition of ceramides are linked to pathogenesis of AD (17). Keratinocytes together with neutrophils and natural killer cells(NKs), are major producers of antimicrobial peptides (AMPs). AMPs protect skin against infections and inflammation, by forming a chemically stable cover on the skin surface. Patients with AD have been documented to exhibit reduced levels of AMPs, specifically cathelicidin and β-defensins, exposing them to greater risk of Staphylococcus aureus infections (18). Keratinocytes express Toll-like receptors (TLRs), which activate Th1 lymphocytes to produce interferon-gamma (INF-γ) and pro-inflammatory interleukins (ILs) such as IL-1 β and IL-18, which further facilitate leukocyte migration to the skin (19).

Langerhans cells (LCs) are resident immune cells of the epidermis. They are resident macrophages which function like dendritic cells. They operate as a binary system in response to foreign invaders. LCs are primarily known for their negative regulatory immune actions and tolerization (20). Research has shown that LCs induce CD4+ regulatory T lymphocytes, and promote tolerance in CD8+ T lymphocytes, and cause production of IL-10 by Langerhans cells which suppress contact dermatitis (21). These processes represent rapid and nonspecific primary responses known as the innate immune response, thus LCs are classified as innate immune cells. Langerhans cells possess long processes called dendrites; these processes are elongated to reach the epidermal tight junctions allowing contact to pathogens via pattern recognition receptors (PRRs). After internalization of pathogens associated molecular patterns (PAMPs), they are processed into antigenic fragments subsequently bound to human leukocyte antigen II (HLA-II) molecules, this happens during migration of LC into skin draining lymph nodes through dermal lymphatic vessels. Finally, they will present the processed linear antigenic peptide bounds by major histocompatibility complex (MHC)class II molecules to the T lymphocytes, thereby initiating an adaptive immune response (22). Within the epidermis, several other cell types can be found, including pigment-producing melanocytes and sensory Merkel cells for skin sensation. Memory T lymphocytes may also be found in healthy epidermis, although much more extensive infiltration of T lymphocytes and neutrophils are seen in AD (23).

Dermis, the second and thickest layer of the skin, hosts various cells that contribute to the immune system. There are couple of non-immune cells in the dermis which play a dual role in maintaining dermal integrity and providing a pathway for transportation of immune cells. Fibroblasts in the dermis produce structural proteins that act as a supporting framework. Endothelial cells, lining the skin's blood vessels, express adhesion molecules and produce cytokines facilitating oriented passage of immune cells to the upper epidermis.

Dermal dendritic cells (dDCs) are positioned just beneath the epidermal-dermal junction and possess fewer dendrites compared to Langerhans cells. This reduction result in greater mobility for immune interactions. Dermal dendritic cells express epithelial-cell adhesion molecules as well as IL-10. Their distinctive function involves stimulating B cells to produce various classes of immunoglobulin, such as IgM, for immunosurveillance against pathogens through cytokines and chemokines productions. This activity highlights their significance in both innate and adaptive immunity of the dermis (24, 25).

Monocytes are found circulating in the bloodstream serving as a precursor for macrophages. Monocytes remain in a state of readiness waiting for a cue to be recruited into the dermis. Upon differentiation to macrophages, they transform to highly mobile phagocytic cells capable of initiating immune response by PRRs and release of cytokines (26). Recent studies have provided evidence that in the normal state, monocytes function as tissue antigen presenters to T lymphocytes. Additionally, skin-resident macrophages are established in the dermis during embryonic development and are renewed only by *in situ* proliferation (27).

Another large group of innate immune cells occupying the dermis are granulocytes. Neutrophils, basophils, eosinophils, and mast cells contain granules filled with proteases and antimicrobial peptides. The degranulation of basophils and mast cells is IgE-dependent. The crosslinking of FcεRI receptors occupied by IgE immunoglobulins, which are recognizing specific allergens, causes the release of content of cytoplasmic granules. Overall, when macrophages and dendritic cells get activated, they recruit granulocytes to the site of encounter with invaders; neutrophils are the initial cells to get recruited (28).

Mast cells represent another cell type present in the dermis. These cells contain histamine and act as effector cells in IgE-mediated hypersensitivity reactions (29). Mast cells are tryptase and chymase positive. These enzymes are important in degrading a pathway that allows invasion of activated immune cells to the epidermis (30). In addition to enzymes production, mast cells activate other immune cells by direct contact or cytokine production. In AD, mast cells, along with other immune cells such as NKs, eosinophils, and basophils, produce IL-4, which results in overt Th2 polarization of the antigen stimulated naïve CD4 lymphocytes (31). In a study performed on normal human keratinocytes and skin equivalent models, the inhibitory effect of INF-γ produced by mast cells on tight junctions, specifically claudin-1 was revealed, resulting in compromised barrier integrity (32). Mast cells engage with other cells through expression of TLRs and HLA I and II. Their production of co-stimulatory molecules such as CD80 or CD86 further facilitates antigen presentation. The interaction of mast cells with Langerhans and dermal dendritic cells is pivotal for dendritic cells maturation, migration, and antigen presentation, accomplished through production of TNF-α and histamine. Mast cells are capable of inducing immune tolerance by production of IL-10, transforming

growth factor-β (TGF-β) and an increase in the number of T-regulatory lymphocytes (33). TGF-β can modulate B lymphocytes activity by limiting the germinal center formation and promoting IgA class switching. Furthermore, TGF-β was found to be driving the fibrotic process following the damage caused by inflammation in AD (34).

The final category of innate immune cells in the dermis, known as NKs, share some characteristics that bridge both innate and adaptive immunity. They function as innate cells by detecting surface HLA-I molecules and operate on basis of PRRs. When the cellular expression of surface HLA-I is reduced, as seen in virus-infected or cancerous cells, the NKs become activated. Additionally, They can perform an antibody-dependent cytotoxic attack, by binding to the IgG-labelled pathogens or cancerous cells, releasing their intracellular granules or inducing apoptosis of these cells (35).

Dermis is the home to most adaptive immune cells in the skin. While adaptive immune cells travel through skin for surveillance, some such as memory T lymphocytes can also be found in healthy epidermis. T lymphocytes can be grouped to several subpopulations but the most extensively studied groups are cytotoxic T lymphocytes and T helper lymphocytes. Cytotoxic T lymphocytes, also known as CD8+ T lymphocytes, are the effector cells recognizing surface antigens represented by antigen presenting cells. Upon recognizing HLA I class molecules occupied by foreign antigenic linear peptide on the cell surfaces, a programmed cell death process is initiated (36). After effective antigen recognition, CD4+ T lymphocytes are clonally expanded and functionally polarized into different subsets, such as Th1, Th2, Th17 and Treg lymphocytes. Polarized T helper lymphocytes, support effector cells by producing cytokines and aiding in maturation of dendritic cells. The cytokines produced by T helper lymphocytes (CD4+) couple with another group of adaptive immune cells, known as the B lymphocytes. Recently there are new findings proving B lymphocytes are passively and actively participating in maintaining immunity in the skin through specific-antibodies production. Mature B lymphocytes, after antigenic stimulation, are clonally expanded and terminally differentiated into plasma cells. This process is tightly regulated by Th2 polarized T lymphocytes. Plasma cells which can produce specific antibodies, reside in lymph nodes, and bone marrow. Their secreted antibodies reach the target site by circulation. Recent data indicates that B lymphocytes are actively involved in both inflammation and immunosuppression within the skin. They produce proinflammatory cytokines and anti-inflammatory IL-10, highlighting the hemostatic role of B lymphocytes in host defense (37). B lymphocytes can also serve as very effective antigen presenting cells to T lymphocytes and important source of the spectrum of immunoregulatory cytokines. There are as many as 20 billion T and B lymphocytes in the skin, making the skin a potential candidate as a peripheral lymphoid organ (38).

Immune dysregulation in atopic dermatitis

While the epidermal barrier impairment is commonly recognized as the initial trigger of AD, it is crucial to acknowledge the dysregulation of innate and adaptive immune system in the pathogenesis of AD. According to the "Inside-out" hypothesis, the barrier impairment in AD is the consequence of immune responses to irritants and allergens (39).

Innate immunity is expected to be the primary defense against the invaders of the skin layers, although it's impairment is documented in the AD (40). It is also documented that patients suffering from AD are predisposed to infections as the process of pathogen recognition and invasion control is impaired (41).

Keratinocytes, Langerhans cells, stromal and endothelial cells, macrophages, and various other immune cells residing in the skin are equipped with PRRs which serve as initiator of epidermal immune reaction through recognition of pathogenic patterns known as PAMPs. PRRs are responsible for pathogen recognition by activating the innate immune system and antigen-specific adaptive immunity (42). PRRs encompass several families of receptors, such as TLRs. This support is unreplaceable for isotypic switching and somatic mutation of antigen stimulated B lymphocytes. Activation of downstream signaling pathways result in activation of various transcription factors, which are regulating several hundreds of genes with proinflammatory activities, promoting production of pro-inflammatory proteins. The inflammatory cytokines along with the antimicrobial peptides are the rapid primary responses against pathogens, however, in some instances these factors are unable to combat the pathogens sufficiently, alternatively, TLRs also shape the adaptive immune response by activating the maturation of dendritic cells as antigen presenting cells, and influencing T and B lymphocytes function to act as a second line of defense (43).

Inflammatory cytokines, such as IL-6 and TNF-α, can stimulate neighboring cells to produce chemokines and adhesion molecules to further recruit additional immune cells to the site of invasion. TLRs can also recognize both damage-associated molecular patterns (DAMPs), expressed by damaged skin cells, and pathogen-associated molecular patterns (PAMPs) expressed by invading pathogens. Such recognition enables innate and adaptive immune system activation as well as enhancing host defense molecules production such as AMPs and proinflammatory cytokines. Excessive TLRs activation can inadvertently cause T lymphocyte mediated autoimmunity, further predisposing individuals to undesired inflammation and skin conditions, such as AD. Keratinocytes, dermal fibroblasts, and Langerhans cells are equipped with PRRs and are also capable of production of AMPs and cytokines. When activated by DAMPs or PAMPs, these cells will recruit other immune cells including neutrophils, macrophages, and T lymphocytes to establish an adaptive immune response, and a memory for possible re-invasion (44).

Keratinocytes, the abundant cell type on the skin surface, are equipped with TLRs. TLR 2 and TLR 3 are the main receptors of pathogens in keratinocytes. As bacterial lipopeptides are recognized by TLR 2, proinflammatory cytokines such as IL-6 and tumor necrosis factor-alpha TNF-α are produced. In a study performed on Leiden epidermal models, it was proven that TNF-α alone or in combination with other cytokines, such as IL-4 or IL-13, decrease the length of long chain free fatty acid and ceramides leading to altered barrier function (45). TLR 3 on the other hand, ensures a

normal barrier function by inducing expression and function of tight junction as well as ensuring re-epithelization, granulation, and vascularization for wound healing (19).

Dermal fibroblasts are equipped with TLR 2 and 4, allowing them to recognize bacterial lipopeptides and fungal pathogens, promoting proinflammatory cytokines production. They are also able to differentiate to preadipocytes upon dermal infection with Staphylococcus aureus. The role of adipocytes in prevention of further invasion of Staphylococcus aureus is still an ongoing area of research (46).

Dendritic cells of the skin and the LCs are dependent on TLRs for their functioning. TLRs enable these cells to present antigens to T lymphocytes and induce production of cytokines and chemokines. Upon detection of the PAMPs and DAMPs, they migrate to lymph nodes to prime T cells for further immune responses. Langerhans cells possess several TLRs, such as TLR 2,3,4,7 and 8. TLRs in LCs can produce both cytokines and chemokines such as proinflammatory cytokines, IL-12, IL-8, and CCL3. IL-12and IL-8 were further studied for correlation between skin samples levels or serum levels and the severity or progression of AD. IL-8 was elevated in stratum corneum samples of AD subjects with highest correlation to the severity scores (47). In another study, the measured serum sample level of IL-12 correlated with the severity score, SCORAD, in a concentration dependent manner (48). Chemokines such as CXCL9, CXCL10, and CXCL11, used as biomarkers of AD, are expressed when TLR3 is activated in the LCs (49).

AD has been associated with impaired TLR2 function, as evident by genetic polymorphism of TLR2 and the down-regulation of its expression in macrophages and peripheral blood mononuclear cells of the patients (50). In a research performed by Yu et al. on the mentioned cells, samples treated with TRL2 ligands produced less of Th1 and Th17 cytokines compared to Th2 cytokines, supporting the Th2 polarization in AD (51). Additionally, confocal microscopy of skin sections of AD patients revealed less TLR2 expressed in basal keratinocyte compared to the whole width expression in the normal skin (52). In normal skin, TLR2 activation in keratinocytes leads to an increase in the expression of tight junction proteins, such as claudin, and antimicrobial peptides such as cathelicidine. However decreased levels of tight junctions and AMPs are commonly observed in all individuals with dermatitis (53).

Thymic stromal lymphopoietin (TSLP), is a highly expressed cytokine in atopic dermatitis. Upon TSLP expression, the innate and adaptive immune system develops a connection to demonstrate an atopic reaction. TSLP is not confined to skin barrier surfaces, but it is also found in lungs and gastrointestinal tract (GIT). The TSLP receptors are also found on variety of cells, including dendritic cells and monocytes. TSLP exists in two isoforms, the one known as lfTSLP, has a long chain, while the latter is referred to as sfTSLP (54). TSLP-1 was found to be upregulated in lesioned skin of AD patients. Elevated TSLP-1 levels may be detected before development of any AD phenotypes, making TSLP-1 a candidate biomarker for early detection of AD. Studies involving both humans and mice have revealed the elevation of TSLP levels upon activation by TLRs ligands. TSLP is a key cytokine which triggers the Th2 cytokine associated inflammation in atopic diseases including asthma and allergic rhinitis (55). Keratinocytes and mast cells are among the main producers of TSLP in AD. Further, TSLP promotes the activation and migration of dendritic cells in the dermis. These dendritic cells produce Th2 attracting chemokines in order to notify the differentiation of naïve CD4 T lymphocytes to Th 2 lymphocytes (56). Furthermore, a study conducted on skin explants showed that TSLP induce allergic inflammation by inducing production of proinflammatory cytokines such as IL-4 and IL-13. In return, TSLP is further upregulated by TNF-α, IL- 1α, IL-4 and IL-13. The collaboration of these cytokines synergize and result in the initiating role of TSLP in allergic responses (57).

The severity of AD goes hand in hand with the higher cytokine expression. In a study performed on mice models, it was revealed that the absence of IL-4 enhances the epidermal barrier function and innate defense mechanisms (58). More recently, the association of IL-4 and IL-13 has been studied, owning to similarities in their course of action in AD. While both cytokines act on the IL-4Rα receptors, it was evident that IL-13 rather participate more peripherally compared to IL-4, which is expressed in lymph nodes by T lymphocytes. IL-13 is locally-expressed in the skin, down-regulating OVOL1-FLG pathway, which results in epidermal barrier dysfunction and increased trans-epidermal water loss (TEWL) (59). IL-13 and IL-4 reduce the production of AMP by keratinocytes, predisposing the skin to dysbiosis and penetration of pathogenic entities (60). Further, experiments on mice models have shown that IL-4 and IL-13 reduce the sensitivity threshold of sensory nerves to pruritogenic stimuli, such as alarmins, and can also directly induce itching behavior in the experimental models (61).

IL-5 modulates the clinical activity and severity of AD, through regulation of IgE synthesis and eosinophilic activation (62). IL-5, which is produced by Th2 lymphocytes, co-expressed with IL-4 and IL-13, can increase vascular permeability and muscular contractibility. IL-5 together with other chemo-attractants, such as CCL-11, draws eosinophils to vascular compartments, resulting in inflammation. IL-5 also prolongs eosinophils activities and survival by reducing their peripheral apoptosis and increasing their bone marrow genesis (63).

The studies on human skin and mice models have shown the over-expression of IL-31 and its receptor, IL-31RA, in dermatitis models. In mice models, IL-31 was identified as a pruritogen, independent of mast cells (64). Protease activated receptor 2 (PAR-2) activation cause IL-31 and TSLP release, which stimulate the immune cells, and the pruriceptive sensory nerve fibers to induce itch. In a study by Takaoka et.al, the overexpression of IL-4 and IL-31 were detected in the epidermis and hair follicles of the mice (65). Further, IL-31 stimulate sprouting and branching of sensory nerves, playing a major role in the chronic itch in AD, and the hyperkinesis and alloknesis phenomenon. Hyperkinesis and alloknesis both contribute to the itch-scratch cycle in AD, complicating the nature of pruritus (61). In another study on human skin affected by dermatitis, the addition of staphylococci toxin to the serum of the subject group produced higher IL-31 expression compared to the control group, further proving the role of staphylococci in overexpression of cytokines leading to dermatitis features (50).

IL-17 is a Th 17 lymphocyte cytokine, which was found to be increased in the peripheral blood of AD patients with high disease severity. IL-17 amplifies the production of IL-4 by Th2 lymphocytes, causing epidermal barrier dysfunction (66). It is a cytokine that is enhanced by superantigen staphylococcal enterotoxin B and negatively correlates with IgE levels and eosinophils count (67). The IL-23/IL-17 axis holds significance in chronic atopic dermatitis. Th17 cells, activated under the influence of IL-23 produced by immune cells and keratinocytes, produce IL-17A and F, IL-6 and TNF-α. IL-17 suppresses the synthesis of filaggrin in keratinocytes, impairing the skin integrity and barrier (68). Interestingly, in a study by Mizutani et.al, co-stimulation of keratinocytes and skin equivalent models with IL-17A resulted in restoration of tight junctions dysfunction induced by INF-γ (32).

Pathogenesis of atopic dermatitis

Etiopathogenesis of AD is not yet very clear. Although genetics, environmental, and immunologic factors play a significant role in its development. In a recent meta-analysis, it was documented that the probability of children developing AD is increased by approximately 40% if they have at least one parent with atopic history. This probability rises even further to 60-80% when both parents suffer from atopic diseases (69). Furthermore, Genome-wide linkages studies have identified potential associations with AD on various chromosomes including chromosome 1,3,5,11,15, and17, despite no specific loci on genes were pinpointed (70).

Another aspect of genetics in AD are the filaggrin gene loss of function mutations. In short, filaggrin (FLG) is one of the key proteins in the terminal differentiation of keratinocytes and of the protective skin barrier (71). The skin uses by-products of FLG metabolism to produce natural moisturizing factors (NMFs), which are crucial for skin hydration and prevention of Staphylococcus aureus over-growth (72). FLG metabolism ends in the production of amino acids which may have a role in the acidification of skin pH and a factor facilitating the action of antimicrobial peptides. For many years, it was believed that deficiency of FLG is a single agent causing AD. Later it was proved that not all patients suffering from AD have FLG gene loss of function mutations, but most with barrier dysfunction are the carriers of the mutation. The deficiency of FLG was pronounced in Europeans and Asians who are carriers of two null FLG mutations. Approximately forty loss of function mutations in genes coding for FLG were found in affected populations, except in Africans, therefore the mutations in FLG gene were rather considered as predisposing factor for AD (73).

The word "filaggrin" is a short form for filament aggregation protein. FLG acts as an aggregator that compacts the keratin intermediate filaments, which are necessary in keratinization of skin cells. The differentiation process of keratinocytes to corneocytes is dependent on a calcium gradient. The calcium gradient is higher in the stratum granulosum compared to stratum corneum. Thus, the gradient difference drives the differentiation while the keratohyalin granules in stratum granulosum release their content, profilaggrin (74).

Profilaggrins, under the influence of serine proteases, become FLG monomers. Being the most important component of the cornified envelope, the FLG monomers cross link the intermediate keratin filaments to form bundles and promote dehydration of keratinocytes and subsequently cause the cellular flattening and collapse. The uppermost layer of epidermis is supported by a bilayer of lipid-rich extracellular matrix, resembling the analogy of mortar and bricks, where bricks are the corneocytes and the extracellular matrix is the mortar. The mentioned layer of cells are very efficient in protecting deeper layer of skin when they are intact (75).

Studies have shown that patients with FLG gene mutations, have marked falls in NMF concentration and keratohyalin granules. A generalized decrease in density of corneo-desmosomes along with disordered architecture of extracellular lipid matrix are also observed. The organization of keratin filaments, loading of lamellar body contents for secretion of lipids and enzymes, and an increase in pH, which favors inflammatory reactions, are also the consequence of FLG deficiency (76). FLG gene mutations are capable of recruiting immune cells to the site of invasion, as induced keratinocytes will produce more IL-1 (77). FLG deficiency was also linked to IL-33 up regulation in mice models (78). In another study, higher serum level of IL-33 was showed in AD patients compared to healthy controls (79). Further, in a study by Howell et.al, the effect of IL-4 and IL-13, which are Th2 inflammatory cytokines, on FLG expression was studied. The aim of the study was to determine if those cytokines modulate FLG expression during keratinocytes differentiation. The IFN-γ was used as Th1 cytokine control. Five days differentiation of keratinocyte in presence or absence of IL-4 and IL-13 resulted in significant reduction of FLG expression. In contrary, differentiation of keratinocytes, in the presence of IFN-γ, augmented the expression of FLG (80).

The increased barrier permeability, by FLG mutations, facilitates the passage of allergens to deeper layers of skin and subsequently increases the chance for the encounter of allergens with LCs, initiating a complex immune response. In a study performed by H. Kondo, the percutaneous sensitization through disturbed skin barrier was proved in the mice models, accompanied by predomination of Th2 mediated immune responses proving polarization in adaptive immunity (81). The local increase in the number of eosinophils and mast cells, as well as increased allergen specific IgE and total IgE, added to the increased TSLP-1 and IL-1α production by disturbed keratinocytes, further favors the immune polarization (82).

In a study carried out in Copenhagen on 411 children born to mothers with a history of asthma, a specific endotype of AD was documented. The FLG gene mutations predispose to an endotype with an earlier onset of the dermatitis with a more severe course (83). In another study carried out in Pennsylvania on African-American children, it was noted that mutation in FLG-2 was accompanied with a more persistent course of disease continuing to adulthood (84).

Familial or ethnic background affects the outcome of the disease, but the increased global prevalence of atopic diseases

cannot be solely dependent on genetics. It is likely that the interplay between genetic predisposition and environmental exacerbation are the possible explanation for the increased prevalence, thus recognition of the environmental factors triggering the onset or recurrence of AD could be a window to prevention.

It was previously believed that prenatal exposure to cigarette smoke and maternal alcohol consumption may increase the likelihood of childhood atopies (85). Although more recent studies could not reproduce the same findings, passive exposure to cigarette smoke is proved to be associated with increased AD prevalence (86). Multiple studies on the effect of breastfeeding or hydrolyzed formula feeds and their duration with or without halting of solid foods has not shown a clear change in prevalence or outcome of AD (87). In contrary, several studies have proved the positive effect of maternal fish ingestion prenatally on lowering the chances of developing AD (88). Further, the postnatal ingestion of fish at one year of age has shown to decrease the prevalence of AD, hence increased dietary fish intake is advised (89).

The "hygiene hypothesis" is another theory regarding the effect of environment on the development of atopic diseases in children. In this theory it is proposed that children living in countries with lower socioeconomic status and rural living standards, who may be exposed to farm animals and unpasteurized milk, are less prone to development of AD. Exposure to dirt and pathogens such as helminths and Herpesviridae has shown to modulate the immune system, favoring less occurrence of allergic inflammations (90).

Although it was documented that exposure to bacterial endotoxins and early exposure to infections in daycare may be a protective factor against AD, not all contracted pathogens have positive protective roles. The respiratory syncytial virus (RSV) and herpes simplex virus (HSV) were documented to increase the risk of developing dermatitis (91). The hygiene theory is further supported by the study showing that prenatal exposure to antibiotics, increases the chances of developing AD, and exposure to probiotics and omega 3 fatty acids, decreases the probability. The early exposure to antibiotics harm the microbiota of skins and guts of fetuses and newborns. In contrast, the use of probiotics strengthen the microbiota (92).

Skin microbiota has always been a question to researchers pertaining to AD. The skin microbiota contribute to the fight against pathogens by inducing production of AMPs and maturating the innate immune system from day one of human life (93). The skin microbiota of newborns delivered vaginally is similar to the vaginal flora, further strengthening the microbial diversity of skin compared to newborns delivered by c-section. Therefore, infants may be predisposed to AD already at birth only according to the mode of delivery (94). Commensal species, such as Staphylococcus epidermidis, suppress Staphylococcus aureus over-growth and toxin production by inducing AMPs production by keratinocytes, through activation of Toll-like receptor 2 (TLR-2) and nuclear factor kappa B (NF-κB) signaling pathway. They are also able to abrogate NF-kB suppression induced by pathogenic species such as Staphylococcus aureus (95). The global influence of AD on lesioned and non-lesioned skin microbiota was studied by Clausen et al. proving that FLG mutation also contributes to the disturbance of the symbiotic balance (96).

Upon epidermal barrier dysfunction, the balance between commensal and pathogenic species is disturbed. The organic diversity is challenged as species such as Staphylococcus aureus colonize the skin, outnumbering the other commensal species. The disease progression in AD is tightly bound to pathogenic colonization. The severity and chronicity are among the consequences of pathogenic colonization (97). The pathogenic proteins of Staphylococcus aureus induce production of proinflammatory cytokines such as IL-4, IL-5, IL-6, IL-17 and TSLP in human and animal models (98). Staphylococcal protein A (SpA) can induce production of alarmin IL-33 by keratinocytes (99), which in turn reduces the expression of filaggrin and claudin-1 responsible for the skin barrier integrity and chronic itch (100). Th2 polarization and allergen sensitization predispose AD patients to a viral complication. AD patients display low INF-γ and its receptor, resulting in defective response to viral multiplication, as the T cell polarization is shifted from Th1 to Th2 (101). The impact of AD-related cytokines and Staphylococcus aureus on adult human keratinocytes was investigated. The study showed that the heat-killed Staphylococcus aureus and Staphylococcus epidermidis, in presence or absence of T Lymphocytes-derived cytokines, induce a distinctly different gene expression in keratinocytes. The cooperation of cytokines and Staphylococcus aureus contributed to the exacerbation of AD-associated transcriptomes, which provide us a new understanding about the role of Staphylococcus aureus in the AD flares (102).

Fungal sensitization is often observed among AD patients. Among fungal species are Candida albicans and Malassezia furfur. The sensitization may elicit IgE response along with cytokine production and self-reactive T lymphocytes stimulation (103). The molecular mimicry and cross-reactivity are thought to be the mechanism behind fungal aggregation resulting in atopic dermatitis (104).

Skin pH is another pivotal physical factor in the integrity of the skin. The skin pH is 4.7 on average (105). The acidic pH is the result of different intrinsic processes maintaining not only the acidity but also moisture and barrier integrity. It is proved that acidic milieu, is a protective factor for atopic dermatitis as it supports antimicrobial activity and barrier function (106). There are number of non-preventable factors which fluctuate the skin pH. Skin pH is age dependent; newborns are born with skin of neutral pH although by the age of one, the pH is decreased to more acidic pH (107). The elder's skin pH is more basic, as there is ceramide deficiency and subsequent stratum corneum dehydration. This phenomenon may be the explanation to sudden increase in prevalence of AD in older age. Among the preventable causes of pH disturbances are the use of personal hygiene care products. All these agents act as an emulsifier of the skin surface lipid. Sodium lauryl sulfate and surfactants, constituents of many hygiene products, are known for their negative effect on skin surface as they increase TEWL (108).

Stratum corneum can be externally damaged by meteorological variables, such as low ambient humidity and extreme ambient temperatures. In AD, decreased ambient humidity exponentially increases the gradient of TEWL, leading to dryer skin (109). Although higher humidity hinders excessive TEWL, the increased perspiration provoked by humidity may worsen the severity in the

established disease (110). There is a need for vaster epidemiological studies to explore the exact role of humidity in provoking or preventing AD. In a study in the Swiss high-mountain area of Davos, it was noted that increased temperature from very low to moderate temperature of +18°C, decreased the pruritus and severity of eczema (111). On the contrary, in an American study, it was noted that higher temperatures are associated with poorly controlled AD (112). Although individuals living in warmer countries are better exposed to eczema-protective factor, UV-light, the overall harm caused by excessive perspiration and subsequent Th2 activation may outweigh the benefits. The prevalence of AD could also be increased with the rise of absolute latitude. The observation in Australia showed that AD prevalence is higher among children living in the central and southern regions of Australia as these areas are further from the equator (113).

Human life is industrializing at an undoubtedly faster pace than expected. Air pollution is one of the many detrimental effects of industrialization and urbanization on human lives. Air pollution may also exacerbate skin barrier defects and heighten immune responses (114). In a study carried out on AD patients and healthy controls, the effect of nitrogen dioxide, which is shed from diesel engines in high concentration in urban areas due to heavy traffic, and formaldehyde, present in most indoor environments, was investigated to differentiate whether air pollution can further challenge skin barrier function. The study showed that upon exposure to nitrogen dioxide, the TEWL is increased in the eczematous skin at the state of barrier dysfunction compared to TEWL in eczema patients prior to the test and healthy controls (115). Other studies showed an increase in NF-κB production of mice models (116) and an increased production of IL-6 and IL-1β by cultured human keratinocytes, resulting in further induction of proinflammatory cytokines production (117, 118).

Recently researchers have focused on the interplay between food allergies or sensitization and AD. Although it is believed that AD preceded food allergies but not vice versa, the mechanism is not very clear. Number of studies have proved that patients with more severe phenotypes of AD, show more frequent diagnosis of food allergies or sensitization (119). It is believed that food sensitization could occur percutaneously. The impaired skin barrier in AD patients is a great entry point for food allergens via skin. As food sensitivity and allergy are two different entities with notable differences in severity; It may be recommended that children diagnosed with AD undergo some skin prick test or oral challenge test for common food allergens, to eliminated foods which may be triggering co-expression of food allergy and AD. These tests may prevent excessive food avoidance, which may cause nutritional issues in children (120).

Understanding the role of epidermal barrier in the pathogenesis of AD is crucial. The "Inside Out or Outside In theories" are still at the center of current research. It is not completely proven if barrier disruption causes the AD or the vice versa, nonetheless, the barrier dysfunction causes excessive TEWL and increased permeability to different irritants and pollutants to the deeper layers of the skin (121).

Diverse functions of epidermal barrier, from preventing excessive TEWL and microbial invasion to its photoprotective role is mostly mastered in the uppermost layer of the epidermis. There are exogenous and endogenous factors which alter the integrity, inevitably causing a disturbance in barrier integrity.

Skin desquamation is delicately controlled by different types of proteolytic enzymes, named as epidermal proteases, and their inhibitors. These enzymes hydrolyze peptide bonds of corneo-desmosomal adhesions and subsequently result in detachment of upper cellular layers of epidermis. Four families of proteases have been identified of which serine proteases are the most researched proteases found in the epidermis. Kallikrein-related peptidases (KLKs), a member of serine proteases family, account for most of the proteolytic function in the desquamation process. The up regulation of proteases can stimulate overexpression of proinflammatory cytokines through protease activated receptor 2 (PAR2), resulting in production of chemokines and cytokines involved in pathogenesis of AD (122). PAR2 receptors are also found on keratinocytes and sensory nerves. They play a major role in the production of neuropeptides, such as substance P and calcitonin gene-related peptides, which stimulate mast cells to release tryptase and induce non-histaminergic pruritus in AD (61).

The most important members of KLKs in the pathogenesis of AD are KLK-5,7 and 14. They are synthesized among their inhibitors, Lympho-epithelial Kazal-type-related inhibitor (LEKTI), in the stratum granulosum, where pH is neutral. The neutral pH changes to more acidic pH of 4.5-5.5 in the stratum corneum where the mentioned proteases are activated to perform their proteolytic function in a cascade manner. The KLK 5 is able to autoactivate itself and activate other KLKs such as KLK-7 and 14. Although KLK-5 is capable of enzymatic activity at neutral pH of the stratum granulosum, the LEKTI in their vicinity inhibit their function (123).

In a recent study, Zhu et al. described that persistently upregulated KLK-5, independent of PAR2, stimulate secretions of IL-8, IL-10, and TSLP-1 (124). In another study, the mass level of different KLKs were measured in healthy and AD patients. Although mass volume of KLK-7 and KLK-11 were increased in AD skin, the KLK-5 mass volume remained unchanged (125). Lastly, reduced function of LEKTI, due to 420K variant, has been observed in the AD, further explaining the decreased inhibitory action against KLK-5 (126).

Variations in genes encoding for proteases such as KLK-7 and their inhibitors such as serine peptidase inhibitor Kazal type 5 (SPINK- 5), increase the proteolytic activity in the stratum corneum and subsequently cause barrier disruption and dysfunction (127, 128). Furthermore, FLG gene variants with their resulting reduction of NMF production may increase the pH, which facilitates the activation of protease cascade (129). Finally, exogenous proteases, such as staphylococcal proteases, or other barrier aggravating agents such as soaps and detergents may alter the balance between exogenous and endogenous proteases and their inhibitors, ending in excessive desquamation and degradation (130).

Stratum corneum is made of a combination of lipids such as cholesterol, ceramides, and free fatty acid. Altered stratum corneum lipids were documented in patients with AD. The ceramides in stratum corneum are critical for skin barrier function and prevention of TEWL. There are number of mechanisms

attributed to ceramides reduction in AD. Current research shows that there is a disturbance in the length of the fatty acid chains of ceramides in patient with AD, where the amount of long chained fatty acid ceramides is decreased and the short chained fatty acid ceramides are increased. The length of fatty acid chain of ceramides is inversely proportional to the TEWL and barrier function of the skin. In addition, there is a significant correlation between disease severity and changes in lipid composition and organization in AD (131). It is documented that loricrin and involucrin, which are protein component of skin barrier, are significantly decreased in both acute and non-lesioned skin of AD subjects, further proving the flaw in the skin barrier of AD patients (132).

Tight junctions (TJs) are groups of adhesive proteins which are placed on opposing membranes of keratinocytes in stratum granulosum. They are crucial in maintaining skin barrier integrity as they control the passage of fluids and solutes paracellularly acting as a selective barrier. They may also be recognized as a second physical barrier beneath the stratum corneum. Claudin-1 and claudin-23 are among the most affected TJs proteins in AD. In a study conducted by De Benedetto et al. on expression or function of TJ proteins such as claudin-1 in epithelium of AD and non-atopic subjects, a striking reduction in expression of TJ proteins in AD subjects were observed. The expression of claudin-1 inversely correlated with Th 2 lymphocyte polarization biomarkers such as serum total IgE and total eosinophil count (133). In another study on animal models of AD, it was revealed that upregulation of claudin-1 attenuated the severity and natural course of AD (134). Further, it was observed that cytokines produced by Th-2 and Th-17 lymphocytes, such as IL-17, reduce the expression of tight junction proteins claudin-4 and 8 in AD patients (135). Reduced expression of tight junction proteins such as claudin-1 and 4 have been observed during infection with pathogenic strains of Staphylococcus. During infection, the TJs are primarily promoted to maintain barrier function although later their expression is reduced (136).

Atopic dermatitis treatments; conventional to novel delivery systems

The chronic course of AD is characterized by periods fluctuating between remission and exacerbation. The exacerbation periods are also known as flares. The flares are crucial in dosing of any treatment, as in these periods' patients may require intensified doses. In many cases patients and caregivers judge the success of the treatment according to the frequency and severity of the flare-ups, hence it is crucial to educate patients regarding signs and symptoms of flares to improve the adherence and compliance with treatment and subsequently the treatment outcome. There are two approaches in long term management of AD with chronic tendency. The approaches are denoted as reactive or proactive. The reactive approach involves daily moisturizing and skin hydration plus an anti-inflammatory of choice. Conversely, proactive management involves intermittent application of anti-inflammatories and emollients to affected, newly affected or unaffected areas of skin. The effectivity of proactive management in controlling flare-ups has been shown in multiple studies, hence it is recommended to patients who are experiencing symptoms of moderate to severe AD with compromised quality of life (137).

The first understanding and treatment of eczema dates to 400 BC, when Hippocrates described AD as a cutaneous manifestation of internal diseases or a disequilibrium in humor. In that era, it was believed that symptoms of eczema, such as oozing or itching, should not be treated promptly as it may mask or worsen the internal disease (138). Nearly a thousand years ago Avicenna, a Persian polymath, accumulated medical knowledge known to human about eczema into a comprehensive text called Canon. The proportional relation between long bathing and dry skin was described in an anecdote, recommending shorter baths to prevent eczema (139).

The ancient therapeutic approaches focused on the cure of the internal disease as recommended by Hippocrates. Patients applied mercury ointments near lesions to form a blister, or ingested sulfur or arsenic to treat the internal disease. Leeches, body wrapping in rubber or laxatives were also used as detoxifying methods to clear eczema. Later an Austrian dermatologist, Hebra, described eczema as a solo entity with chronic course which could not be cured fast and recommended topical application of soaps and oils and other natural lotions to treat it (140).

In 1933, AD was the new term introduced for description of eczema. Along with the change in the terminology, new therapies emerged in late 19th century. Physicians understood the role of food in manifestation of AD, and recommended food avoidance in patients with positive skin tests. Further, they understood that first symptoms could manifest very early even in infants who are exclusively breastfed. In 1952, the use of compounds containing hydrocortisone was proposed. The introduction of corticosteroids revolutionized management of AD (141). Nowadays, with a rise in so-called steroid phobias, many are going back to primitive remedies. The modern practice has added multiple effective therapies to topical corticosteroids.

Non-medical treatments for AD are essential in managing this chronic skin condition by addressing triggers and contributing factors that can exacerbate symptoms. These non-medical approaches primarily focus on skincare practices and lifestyle modifications. Gentle skincare routines, which include the use of fragrance-free, hypoallergenic moisturizers, and mild non-soap cleansers, are essential to maintain skin hydration and prevent flare-ups. Avoiding harsh soaps, hot water, and excessive scrubbing can help protect the skin's natural barrier. Lifestyle modifications often involve identifying and minimizing environmental triggers such as allergens, irritants, and stress. Implementing dietary changes and stress-reduction techniques like meditation or yoga may also complement medical treatments, offering individuals with AD a holistic approach to managing their condition and improving their quality of life (142).

Corticosteroids are commonly used as the first-line treatment of AD, offering relief from itching, inflammation, and redness through inhibiting antigen processing by dendritic cells and Langerhans cells

and production of inflammatory cytokines (143). Among the most frequently prescribed corticosteroids for AD are hydrocortisone, triamcinolone, and betamethasone. These medications are available in various forms, including creams, ointments, and lotions, providing flexibility in application. Treatment regimens should be individualized with lower-potency corticosteroids, such as hydrocortisone 1%, preferred for the face and neck and more potent options, such as clobetasol, for other areas of body. A thin layer is applied to the affected skin once or twice daily followed by a moisturizer, gradually tapering as symptoms improve to minimize potential side effects like striae, atrophy or skin thinning (144). Hydrocortisone-loaded poly ϵ-caprolactone nanoparticles (PCLNPs) in ointment form, have showed better permeation and controlled release with no change in the toxicity compared to the conventional dosage form (145). Betamethasone-17-valerate prepared under hot high-pressure homogenization in the form of solid lipid nanoparticles (SLNs) have also shown greater depot in both intact and impaired skin barrier compared to the conventional ointments (146). Additionally, clobetasol propionate has been nanotechnologically formulated *in vivo*, to a nano-emulsion composed of eucalyptus oil and surfactant with improved solubility which reduces edema notably (147).

Topical calcineurin inhibitors (TCIs), such as tacrolimus and pimecrolimus, are important non-steroidal options as the second line of treatment for AD. They function by reducing calcineurin-dependent T lymphocytes activation and inhibiting genes coding for transcription of inflammatory cytokines. These medications are especially useful in sensitive areas such as face and skin folds, where corticosteroids may pose risks of atrophy or thinning of the skin. Tacrolimus is available in ointment form, while pimecrolimus comes in form of cream. The typical regimen involves applying a thin layer of the medication to affected areas twice daily with special care regarding the age of the patient, as tacrolimus 0.1% should only be prescribed to patients older than 15 years. Among usual side effects of the use of TCIs are skin burning and itching (148). Tacrolimus ointments prepared nanotechnologically with ionic gelation method has shown improved permeation at reduced doses. The hydrophilic nature of chitosan and nicotinamide increase the entrapment efficiency of hydrophobic tacrolimus, resulting in greater permeation and retention (149). Further, polymeric nanoparticles of tacrolimus decorated by hyaluronic acid formulated under high pressure homogenization-evaporation method has shown targeted sustained release pattern compared to previous formulations (150). Other nanotechnologically designed tacrolimus, such as thermosensitive SLNs or microemulsions have also shown better penetration and retention in the skin. The thermosensitive SLNs showed penetration to up to 500 µm compared to ointment penetration of 150 µm *in vivo*, which suggest high drug loading efficiency (151).

Although the itch associated with AD is mostly of non-histaminic origins, antihistamines of the first and the second generations may also be prescribed to patients who experience problems with sleeping or scratching during sleep. As first-generation antihistamines, such as diphenhydramine or hydroxyzine, have sedative characteristics and may not be a reasonable option for patients at work or school, the use of oral second-generation antihistamines, such as cetirizine or levocetirizine, is advised. Recently, levocetirizine and cetirizine hydrochloride have been nanotechnologically formulated in gel form, available for topical use to prevent itch and erythema. The niosome and chitosan nanoparticles ensure optimized skin retention (152).

Systemic immunosuppressive agents such as cyclosporine, azathioprine, and methotrexate are added to the management when conventional treatments prove insufficient. Barbosa et al., developed a fucoidan/chitosan nanoparticle for topical treatment by methotrexate. The formulation ensures a strong inhibition of pro-inflammatory cytokines production along with safe drug delivery to deeper layers of skin (153). Additionally, Verma and Fahr utilized a lipid mixture to develop a vesicle for enhanced topical delivery of cyclosporin A. The study proved effective delivery dependent on concentration of ethanol and size of the vesicles (154). These medications work by dampening the overactive immune response responsible for the skin inflammation characteristic of AD. These drugs are usually prescribed in case of treatment- resistant AD or very severe AD. Patients who are candidate to receive systemic therapies should undergo pre-treatment renal and hepatic screen, as these medications predispose to kidney or liver dysfunction (155).

Many patients with AD do not achieve adequate symptom control with conventional treatments, leading to persistent discomfort and reduced quality of life. Patients may have varying responses to conventional treatments, emphasizing the need for more tailored and effective therapies. A better understanding of the immunological and genetic background of AD has highlighted the potential targets for novel therapies that can address the disease's root causes more effectively. Dupilumab is an FDA-approved breakthrough treatment for AD. This monoclonal antibody targets IL-4 and IL-13 signaling pathway, inhibiting Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway of signaling. Patients typically receive dupilumab as a subcutaneous injection every two weeks, and it is often used in conjunction with other therapies (156). One of the side-effects associated with the use of dupilumab is conjunctivitis. The eye redness accompanied by burning and foreign body sensation was reported by 28% of patients (157). Janus kinase (JAK) inhibitors and phosphodiesterase-4 (PDE-4) inhibitors represent promising novel therapeutic approaches in the treatment of AD, addressing the underlying inflammatory processes. PDE4 is a key player in generations of cytokines such as IL-4 and IL-13, subsequently its inhibition in animal models have shown immunosuppression by increasing AMP and reducing IL-4 and TNFα (158). JAK inhibitors target the Janus kinases which are key signaling pathways in cytokine productions bridging various cytokine receptors with STAT transcription factors. IL-2, IL-4 and many other cytokines are expressed on basis of such signaling, thus JAK inhibitors could be a promising therapy for AD. Tofacitinib is an example of a JAK-1 and 3 inhibitors found to be effective in oral and topical form against AD (159).

In a recent clinical trial, the safety and efficacy of etrasimod, an oral selective sphingosine-1-phophate receptor $_{1,4,5}$ modulator, was

assessed. Etrasimod regulates the migration of lymphocytes and modulates migration of dendritic cells to lymph nodes. Further, etrasimod prevent the release of certain subsets of lymphocytes from lymphatic tissues, reducing the final number of available lymphocytes for recruitment to the site of inflammation. Although percent changes in EASI, and improvements in validated Investigator's Global Assessment score were observed in patients receiving etrasimod 2-mg compared to the placebo group, the primary outcome of EASI-75 was not met (160).

Further, Rocatinlimab, an anti-OX40 antibody for subcutaneous injections, was evaluated in a phase 2b, randomized, placebo-controlled clinical trial. The trial showed a significant reduction in the EASI of patients receiving the treatment compared to the placebo group. The clinical response was maintained twenty weeks after the treatment cessation. The OX40 is crucial in the expansion and survival of T lymphocytes and subsequent memory formation, making anti-OX40 a great candidate for the treatment of moderate to severe AD (161).

While extensive research has been conducted in the field of dermatology and the immunological aspects of AD, numerous questions are left without answers regarding pathogenesis and treatment of this condition. The advances in the field of nanotechnology and the introduction of novel topical and biological therapies have brought about significant advancements, substantially enhancing patient care, and elevated the quality of life for those affected. Although progress has been made, there remains an untouched area of more individualized treatments and identification of specific types of biomarkers, such as serum or plasma biomarkers. This unexplored area promises a fresh avenue for monitoring the disease's progression, comprehending its trajectory, and assessing the effectiveness of appointed therapies of different characteristic. The potential discovery of such biomarkers could revolutionize the way we understand pathogenesis of AD, leading to better outcomes for patients and improved therapeutic approaches as well as early diagnosis and prevention.

Author contributions

MA: Writing – original draft, Writing – review & editing, Conceptualization, Investigation. MK: Writing – review & editing. MR: Writing – review & editing. JC: Supervision, Writing – review & editing. JK: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The 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.

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Glossary

AD	atopic dermatitis
DALYs	disability-adjusted life-years
EASI	eczema area and severity index
SCORAD	SCORing Atopic Dermatitis
AMP	antimicrobial peptide
TLR	toll-like receptor
INF-γ	interferon-gamma
IL	interleukin
LC	Langerhans cells
PRR	pathogen recognition receptor
PAMP	pathogen associated molecular pattern
HLA	human leukocyte antigen
MHC	major histocompatibility complex
DAMP	damage associated molecular pattern
TNF-α	tumor necrosis factor-alpha
dDCs	dermal dendritic cells
NK	natural killers
TGFβ	transforming growth factor β
TSLP	thymic stromal lymphopoietin
GIT	gastrointestinal tract
TEWL	trans-epidermal water loss
NMF	natural moisturizing factor
FLG	filaggrin
NF-κB	nuclear factor kappa B
RSV	respiratory syncytial virus
HSV	herpes simplex virus
SpA	Staphylococcal protein A
KLK	kallikrein-related peptidase
PAR2	protease activated receptor 2
LEKTI	lympho-epithelial Kazal type related inhibitor
SPINK5	serine peptidase inhibitor Kazal type 5
TJ	tight junction
PCL	poly ϵ-caprolactone
NPs	nanoparticles
SLNs	solid lipid nanoparticles
TCI	topical calcineurin inhibitor
JAK-STAT	Janus kinase/signal transducers and activators of transcription
PDE	phosphodiesterase

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CD64 plays a key role in diabetic wound healing

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Introduction: Wound healing poses a clinical challenge in diabetes mellitus (DM) due to compromised host immunity. CD64, an IgG-binding Fcgr1 receptor, acts as a pro-inflammatory mediator. While its presence has been identified in various inflammatory diseases, its specific role in wound healing, especially in DM, remains unclear.

Objectives: We aimed to investigate the involvement of CD64 in diabetic wound healing using a DM animal model with CD64 KO mice.

Methods: First, we compared CD64 expression in chronic skin ulcers from human DM and non-DM skin. Then, we monitored wound healing in a DM mouse model over 10 days, with or without CD64 KO, using macroscopic and microscopic observations, as well as immunohistochemistry.

Results: CD64 expression was significantly upregulated (1.25-fold) in chronic ulcerative skin from DM patients compared to non-DM individuals. Clinical observations were consistent with animal model findings, showing a significant delay in wound healing, particularly by day 7, in CD64 KO mice compared to WT mice. Additionally, infiltrating $CD163^+$ M2 macrophages in the wounds of DM mice decreased significantly compared to non-DM mice over time. Delayed wound healing in DM CD64 KO mice correlated with the presence of inflammatory mediators.

Conclusion: CD64 seems to play a crucial role in wound healing, especially in DM conditions, where it is associated with $CD163^+$ M2 macrophage infiltration. These data suggest that CD64 relies on host immunity during the wound healing process. Such data may provide useful information for both basic scientists and clinicians to deal with diabetic chronic wound healing.

KEYWORDS

diabetes mellitus, CD64, $CD163^+$ M2 macrophage, wound healing, CD68/CD80 M1 macrophages

Introduction

Compromised wound healing presents a considerable challenge, especially for immunocompromised individuals, causing substantial physical and psychological burdens worldwide (1). Among the various factors contributing to delayed wound healing, diabetes mellitus (DM) is recognized for its detrimental effects on the process. This is primarily attributed to compromised host immunity, often leading to prolonged wound healing and, in severe cases, potential amputation in susceptible patients (2).

The wound healing process is intricate, encompassing four overlapping phases: coagulation, inflammation, proliferation, and remodeling (3). Various immune cells and both pro- and anti-inflammatory mediators play pivotal roles in this process (3). Studies have documented elevated levels of pro-inflammatory mediators such as IL-1β (4), TNF (5, 6), IL-23 (7), IL-27 (8), alongside anti-inflammatory mediators including TGFβ1 (9), IL-10 (10), IL-35 (11). In previous research, we established that wound healing is significantly impaired in the context of diabetes mellitus (DM), with GM-CSF-regulated macrophages playing a mediating role (12). Specifically, during the inflammatory phase of diabetic chronic wound healing, immune cell dysfunction, notably involving a subset of macrophages known as M2 macrophages, has been shown to be crucial (13). Therefore, comprehending these immune-related mechanisms is vital for the development of effective strategies to enhance wound healing outcomes not only in diabetic patients but also in other immunocompromised individuals.

CD64, also known as IgG Fc segment receptor 1 (Fcgr1), belongs to the immunoglobulin superfamily and holds a pivotal role in the immune response, by recognizing immunoglobulins and binding to IgG (14). CD64 is predominantly expressed on myeloid cells, including monocytes, macrophages, neutrophils, and dendritic cells (15). Previous studies have indicated that CD64 plays a significant role in neutrophil recruitment during acute infectious diseases (16). Furthermore, there is evidence suggesting its involvement in certain chronic autoimmune conditions such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and atopic dermatitis (17–19).

However, the precise role of CD64 in wound healing among DM individuals remains uncertain. In the present study, our objective was to explore the connection between CD64 and wound healing in both DM patients and an animal model. These findings may offer valuable insights into the mechanisms underlying chronic ulceration in DM patients and contribute to a better understanding of this intricate process.

Materials and methods

Clinical cases

Demographic information was obtained from the electronic database of Tongren Hospital, including 56 patients with type II DM who underwent amputation due to diabetic gangrene and 10 patients who underwent surgical procedures for non-DM (Table 1).

TABLE 1 Clinical information of skin cases in diabetic and non-diabetic groups.

Variable	Diabetes Mellitus n=56	Control n=10	P-value
Sex, male:	42 (75.0)[a]	8 (80.0)[a]	1.000
Age, years: **<45** **≥45-<65** **≥65**	63.5 (58.0, 71.0)[b] 2 28 26	56.5 (44.5, 69.0)[b] 2 5 3	0.124
BMI, kg/m²: **<25** **≥25-<30** **≥30**	23.3 (20.8, 25.9)[b] 40 13 3	23.8 (20.3, 24.9)[b] 8 2 0	0.892
Systolic pressure, mmHg: **<140** **≥140**	142.790 ± 2.720[c] 34 22	127.000 ± 5.461[c] 8 2	0.017
Duration of diabetes, years: **<5** **≥5-<10** **≥10**	10.870 ± 1.150 17 7 32	/	/
FBG, mmol/L: **<7** **≥7-<11.1** **≥11.1**	9.720 ± 0.439 [c] 12 27 17	5.140 ± 0.502 [c] 10 0 0	<0.001
HbA1c, %:	8.835 ± 2.223 [c]	/	/
GA, %:	25.250 ± 7.771 [c]	/	/

BMI, body mass index; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; GA, glycated serum albumin. [a]Data are the numbers (percentage). [b]Data are the medians (interquartile range). [c]Data are the means ± standard deviations.

Skin were collected from these patients and sent to the Department of Pathology for routine histopathological confirmation.

Tissue array, histology and immunohistology of patients

Paraffin skin samples were collected from the Department of Pathology at Shanghai Tongren Hospital, China, between 2017 and 2022. The study received approval from the Ethics Committee of Tongren Hospital, Shanghai Jiaotong University School of Medicine. Informed consent was obtained from each patient prior to surgery. From each specimen, relatively normal skin tissue was carefully selected from the wax blocks to create tissue chips containing 4 x 5 cores of 5 mm each. The blocks were sectioned at 5 μm for HE staining, as described (20).

Immunohistochemistry was performed as described in the relevant reference. The primary antibody against CD64 with ab203349 at 1/800 dilution (Abcam, Cambridge, UK) was used to detect CD64 expression. The secondary antibody was provided by BOND Polymer Refine Detection (Leica Biosystems Newcastle Ltd),

and the color development was performed according to the manufacturer's instructions. CD64 expression levels were measured using the HALO image analysis platform in a double-blind fashion for tissues from both DM and non-DM patients (21, 22).

Construction of CD64 knockout mice

CD64 knockout (KO) mice were provided by Shanghai Model Organism (Shanghai, China) (https://www.modelorg.us/?lang=en-us). For easy understanding the CD64 knockout approach, a schematic figure was presented (Supplementary Figure S1). DNA fragments that contain a 567 bp sequence in Wt mice and an ≈1000 bp sequence in CD64 KO mice were amplified by PCR using the primers listed in Supplementary Table S1. PCR confirmation of CD64 KO mice was depicted in Supplementary Figure S2.

Induction of diabetic mice

Male, 14 week old CD64 KO and Wt mice on a C57 background were procured from Shanghai Model Organism, Shanghai, China (https://www.modelorg.us/?lang=en-us). The CD64 KO mice were validated by PCR (Supplementary Figure S2). The mice were housed in groups of four per cage under SPF conditions, with a 12-hour light/dark cycle *ad libitum*. All experimental procedures were conducted in accordance with the guidelines approved by the Institutional Laboratory Animal Care and Use Committee.

To induce diabetes, both CD64 KO and WT mice were administered STZ (Sigma, U.S.A.). Multiple low doses of STZ (40 mg/kg/d, i.p.) in 50 mM sodium citrate buffer (pH 4.5) on five consecutive days, as described while non-diabetic mice received vehicle only (23). Each set of experiments involved a group of 16 mice. Daily recordings of body weights were collected throughout the study. Diabetic status was confirmed after 9 days through tail vein blood glucose testing using an automated Accu-Chek glucometer. Mice with non-fasting blood glucose levels exceeding 11.1 mmol/L on two consecutive non-concurrent days were considered diabetic (12, 23), whereas those with lower values were excluded from the study. The maintenance of a diabetic state was confirmed by weekly tail vein blood glucose measurements.

Wound protocol

The backs of the animals were carefully shaved and then swabbed with 70% alcohol after anesthetic. Full-thickness skin wounds (1.0 x 1.0 cm) were created in a sterile manner and left unsutured without any dressing, following the method described in (12). To prevent traumatic injury caused by other mice, all animals were housed individually after the surgery. Skin samples were collected at specific time points: days 1, 3, 7, and 14 post-wounding, which included a 4-mm margin of unwounded skin around each wound. Each specimen was fixed in Histochoice to enable subsequent histopathological analyses.

Wound healing analysis

Digital photographs of the skin wound sites in each animal, along with a scale bar, were taken at various time points: days 0, 1, 3, 5, 7, 10, and 14 post-wounding. The assessment of wound closure was performed in a double-blind manner using Image J software. The wound areas were standardized by comparing them with the original wound size and expressed as a percentage of wound closure using the formula: [(day 0 area) - (day n area)]/(day 0 area) x 100. The data were presented as mean ± standard deviation (SD) and subjected to multiple comparisons using one-way analysis of variance (ANOVA) to analyze differences among different groups.

Histology, immunohistology and immunofluorescence of mice

Tissues were sectioned at a thickness of 5 μm and subjected to both HE staining and immunohistochemical staining following specified procedures. The antibodies utilized for immunohistochemistry were sourced from AbCam, based in Cambridge, UK. Detailed information, including catalog numbers and dilutions for each antibody, is explicitly provided, such as CD64 (ab203349, 1/800), CD163 (ab182422, 1/800), CD31 (ab281583, 1/8000), and TGFβ1 (ab215715, 1/250).

The procedures for immunohistological staining and the specific antibodies used were previously described in detail (12, 24, 25). TGFβ1 labelling is performed as described previously (26). Representative images are provided in accordance with the details outlined.

The numbers of $CD163^+$ macrophages within the wound beds were quantified using the HALO image analysis platform (22). Specifically, 12 randomly chosen visual fields (at 400x magnification) of each mouse's section, stained with the anti-CD163 antibody, were analyzed, and the mean count was calculated. Similarly, CD31 staining was assessed using Image J in the same manner, with 12 randomly selected visual fields (at 400x magnification) of each mouse's section, and the mean count was determined. For both $CD163^+$ and CD31 measurements, all analyses were performed under double-blind conditions to minimize bias and ensure unbiased results.

Immunohistochemistry was conducted to identify $CD64^+$ cells, and the quantification of these positive cells per field was performed using HALO image analysis platform (22). The kinetics of $CD64^+$ cells in wounds from Wt mice are depicted in Figure 1. The corresponding images are presented accordingly.

Immunofluorescence double staining was utilized to assess the expression of macrophages. Tissue sections were incubated with specific antibodies, including anti-F4/80 (ab111101, 1/250, Abcam, Cambridge, UK), anti-CD80 (ab254579, 1/500, Abcam, Cambridge, UK), and anti-CD163 (ab182422, 1/500, Abcam, Cambridge, UK), overnight at 4°C. Following this, the sections were treated with the appropriate fluorescent secondary antibodies: anti-F4/80 antibody paired with its counterpart from Beijing Panovue Biological Technology, China, and CD80 or CD163 with their respective

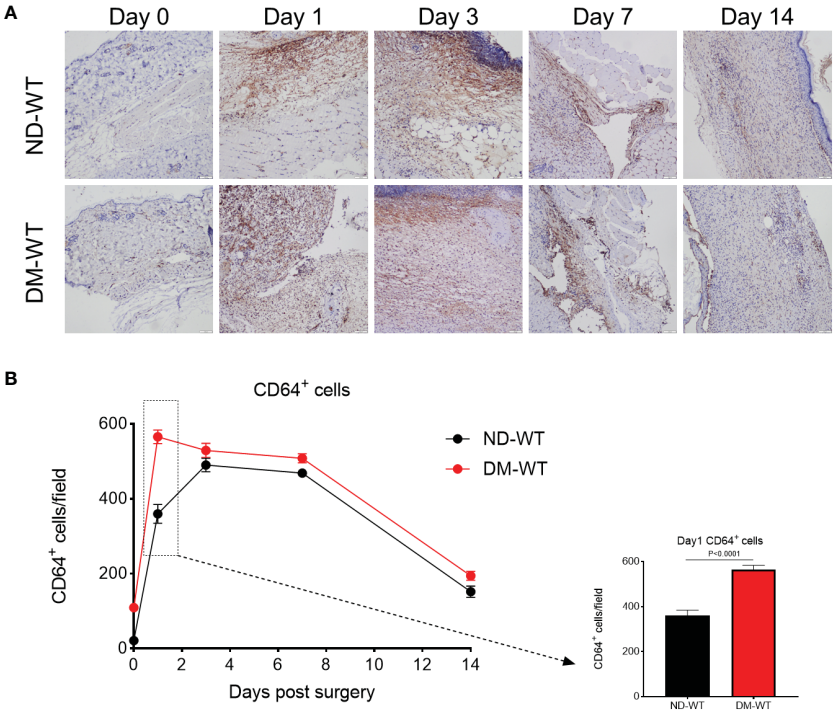

FIGURE 1
CD64$^+$ cells in the wounds across different groups from Wt. The representing photos are presented in **(A)**, while the quantification is presented in **(B)**. The X-axis denotes the days post-surgery, while the Y-axis represents the number of CD64$^+$ cells per high power field.

fluorescent secondary antibodies from Beijing Panovue Biological Technology, China. Nuclei were identified using DAPI (Beijing Panovue Biological Technology, China). Co-localization was visualized using a BX60 Olympus fluorescence microscope, and quantitative analysis was conducted using Halo digital imaging analysis software 2.0 (Indica Labs, USA).

Trichrome staining was used for collagen identification. The ratio of collagen-positive blue area to that of the total tissue area was calculated by collagen volume fraction (27), using the computer-imaging software Image J. For each section ten horizontal photographs were randomly (× 400) evaluated, whereby collagen volume fraction = (collagen area)/(full tissue area), and taking the average.

The experiment has been approved by Human and Animal Ethic Committee, Shanghai Tongren Hospital.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, United States). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was employed to compare group means for CD64$^+$ cells of patients. Two-way ANOVA was used to compare group means followed by Holm-Sidak *post-hoc* test for CD64$^+$ cells of mice. Three-way ANOVA followed by Holm-Sidak *post-hoc* test was conducted for group mean comparisons related to wound closure, CD163$^+$ macrophages, TGF-β$^+$ cells, CD31$^+$ cells of mice. Clinical information, such as age, sex, and BMI, was tested using the Mann–Whitney U-test and Pearson's chi-squared test. A significance level of $P < 0.05$ was considered statistically significant.

Results

CD64 was closely associated with diabetic wound skin

CD64 expression in mice skin from DM-WT group was higher than in ND-WT tissues (Figure 1). Following surgery, CD64 expression in the diabetic group increased and peaked at day 1, whereas the expression of CD64 in the non-diabetic group reached its peak on the 3rd day. The difference between DM-WT and ND-WT was most pronounced on day 1: CD64 expression in skin from DM mice was approximately 1.5-fold higher than in non-DM tissues ($P<0.001$). There was no detectable CD64 from ko mice, as expected and confirmed by the commercial supplier.

The patients with diabetic foot were predominantly male, with a majority being elderly (almost half of them aged >65). Their fasting blood glucose (FBG) levels were mostly elevated (> 7 mmol/L), along with high levels of HbA1c (mean = 8.835%) and GA (mean = 25.25%). In both DM and non-DM patients, CD64 expression was mainly observed in the dermal layer of the skin (Figure 2A). Remarkably, CD64 expression in skin from DM patients was approximately 3-fold higher than in non-DM tissues.

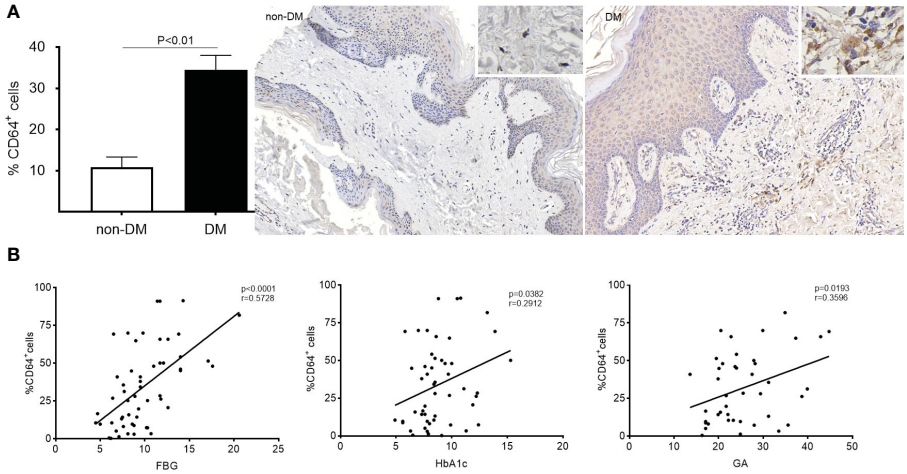

FIGURE 2
Expression of CD64 in diabetic and non-diabetic skin detected by immunohistochemistry. Data are presented as mean ± standard deviation in the left graph **(A)**. The correlation between %CD64$^+$ cells and FBG, HbA1c and GA was analysed **(B)**.

Furthermore, a significant correlation was observed between CD64 expression and FBG (P<0.001), CD64 expression and GA (P<0.05), as well as CD64 expression and HbA1c (P<0.05) in diabetic patients (Figure 2B). These findings indicate the potential relevance of CD64 in diabetic wound healing and its association with glycemic control markers in diabetic patients.

CD64 gene knockout delayed wound healing in diabetic and non-diabetic skin

All wounds from the four groups (non-diabetic with WT mice - ND-WT, non-diabetic with CD64 KO mice - ND-KO, diabetic with WT mice - DM-WT, and diabetic with CD64 KO mice - DM-KO) were carefully monitored and captured daily for quantification following a full-thickness skin incision that removed a 1.0×1.0 cm area of skin at macroscopic level (Figure 3A), which is consistent with microscopic level in HE staining (Supplementary Figure S3).

The three-way ANOVA showed that diabetes mellitus (P<0.0001) and CD64 KO (P<0.001) significantly influenced the wound closure. Specifically, wound closure progressed rapidly in the ND-WT mice, with almost complete closure observed on day 10 post-surgery. In contrast, wound closure in the DM-WT mice was significantly delayed, showing almost a 3-fold difference between ND-WT and DM-WT on days 1, 3, 5, and 7. However, the difference gradually reduced on days 10 and 14, although complete wound closure had not been achieved by day 14 following surgery. Consequently, wound closure in the DM-WT mice was delayed by nearly 1 week compared to the ND-WT mice. Quantitatively, wound closure was substantially impaired at all-time points in the DM-WT mice. Specifically, at days 1, 3, 5, 7, 10, and 14, wound closure in the DM-WT mice was 3.2%, 14.9%, 26.5%, 38.8%, 20.5%, and 8.6% lower than their ND-WT counterparts, respectively (Figure 3B). These findings highlight the considerable delay in wound healing observed in the DM-WT mice compared to the non-diabetic group with WT mice.

The wound closure in CD64 KO mice was significantly delayed compared to WT mice, particularly on days 5, 7, and 10 post-surgery. Quantitatively, at days 5, 7, and 10, wound closure in the ND-KO mice was 14.6%, 13.6%, and 9.9% lower than in their ND-WT counterparts, respectively. Furthermore, the closure in DM-KO mice was 7.9%, 13.0%, and 10.7% lower than in their DM-WT counterparts on the same respective days. These data clearly demonstrate that the loss of CD64 due to CD64 KO leads to delayed wound healing. Importantly, the extent of delayed wound healing did not differ significantly between the diabetic and non-diabetic mice groups.

In summary, the images of wound closure revealed a considerable delay in wound healing in diabetic mice compared to non-diabetic mice. Additionally, it showed a significant delay in wound healing in CD64 KO mice compared to WT mice. Moreover, there was no synergistic effect of CD64 depletion and diabetes on wound healing. These findings underscore the importance of CD64 in the wound healing process and suggest its potential therapeutic relevance in both diabetic and non-diabetic conditions.

CD64 KO had a detrimental effect on diabetic wound healing, primarily through the reduction of CD163$^+$ macrophages and TGFβ1 production

The three-way ANOVA showed that diabetes mellitus (p<0.0001) and CD64 KO (p<0.0001) significantly influenced the CD163$^+$ macrophages. Specifically, after day 1 post-surgery, only a small number of CD163$^+$ macrophages were observed in the skin in all four groups (Figure 4A). The infiltration of CD163$^+$ macrophages gradually increased, reaching a plateau on the 7th day, followed by a gradual reduction until day 14. On the 3rd day after surgery, the number of CD163$^+$ macrophages was higher in the non-diabetic group compared to the diabetic group. The number of CD163$^+$ macrophages in ND-KO mice was similar to that in ND-WT mice, but in DM-KO

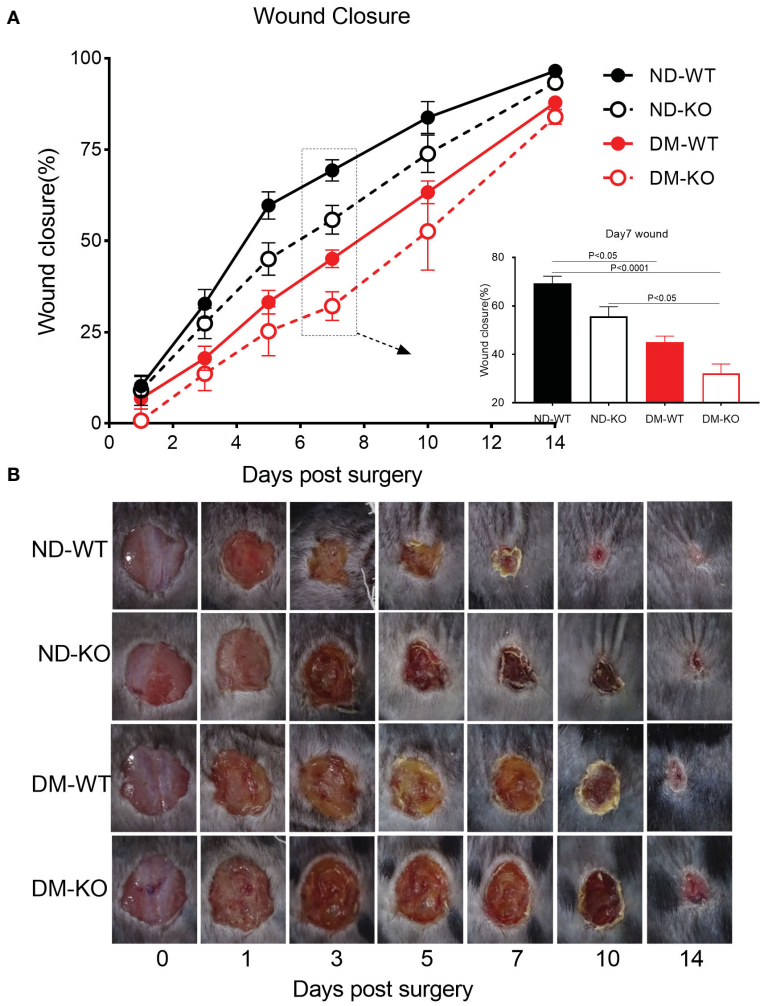

FIGURE 3
Wound closure of mice in each group. The mean ± standard deviation of wound closure rate was demonstrated **(A)**. The inset shows data for day 7. The macroscopic wound closure at the days post-surgery was showed in the photographs **(B)**.

mice, it was lower than in DM-WT mice, nearly twice as low on day 3. The difference between the four groups was most pronounced on day 7: ND-KO had lower CD163$^+$ macrophage levels than ND-WT, and the levels in DM-KO were significantly lower than in DM-WT, being nearly four times lower than DM-KO. In summary, both the diabetic condition and CD64 gene knockout compromised wound healing, possibly through compromised inflammatory mediators and neovascularization, leading to a reduction of M2 macrophages at the wound site. However, the effect on M2 macrophages was greater in the diabetic state (Figure 4B).

To verify the assumption of CD163$^+$ cells are M2 macrophages, we further explore the subsets of M1 and M2 macrophages, using immunofluorescent double staining, i.e. F480/CD80 (Figure 5A) and F480/CD163 (Figure 5B) labelling.

A very similar pattern of F4/80$^+$ macrophages infiltration to the wounds with CD163$^+$ cells was observed (Figure 5C). Upon stratifying F4/80$^+$ macrophages into M1 (F4/80$^+$/CD80$^+$) and M2 (F4/80$^+$/CD163$^+$) cells, we demonstrated that infiltrating M1 cells were elevated on day 3, plateaued on day 7, and gradually decreased in non-diabetic ND-WT and ND-KO wounds (Figure 5D). Notably, infiltrating M1 macrophages exhibited a persistent increase throughout the entire wound healing period in both DM-WT and DM-KO mice.

Conversely, the infiltrating M2 macrophages in the wound were consistent with wound healing, especially under diabetic conditions (Figure 5E), showing that there was compromised M2 macrophage infiltration DM-WT and DM-KO mice (Figure 5E). It is noteworthy that there were some differences observed in infiltrating CD163$^+$ cells alone and F4/80$^+$/CD163$^+$ M2 macrophages between CD163 single staining and CD163/F4/80 double staining in our current study (Figures 4, 5E).

Similarly, the three-way ANOVA showed that diabetes mellitus ($p<0.05$) and CD64 KO ($p<0.01$) significantly influenced the TGFβ1 production. TGFβ1 expression in the unwounded skin was almost undetectable in all four groups at day 0 (Figure 6). Following surgery, TGFβ1 expression increased and peaked at day 7, gradually down-regulating in all groups. However, there was a significant difference in TGFβ1 production among the groups. Notably, TGFβ1 production was highest in ND-WT, followed by ND-KO, and lowest in DM-WT, with ND-WT showing 1.5 times

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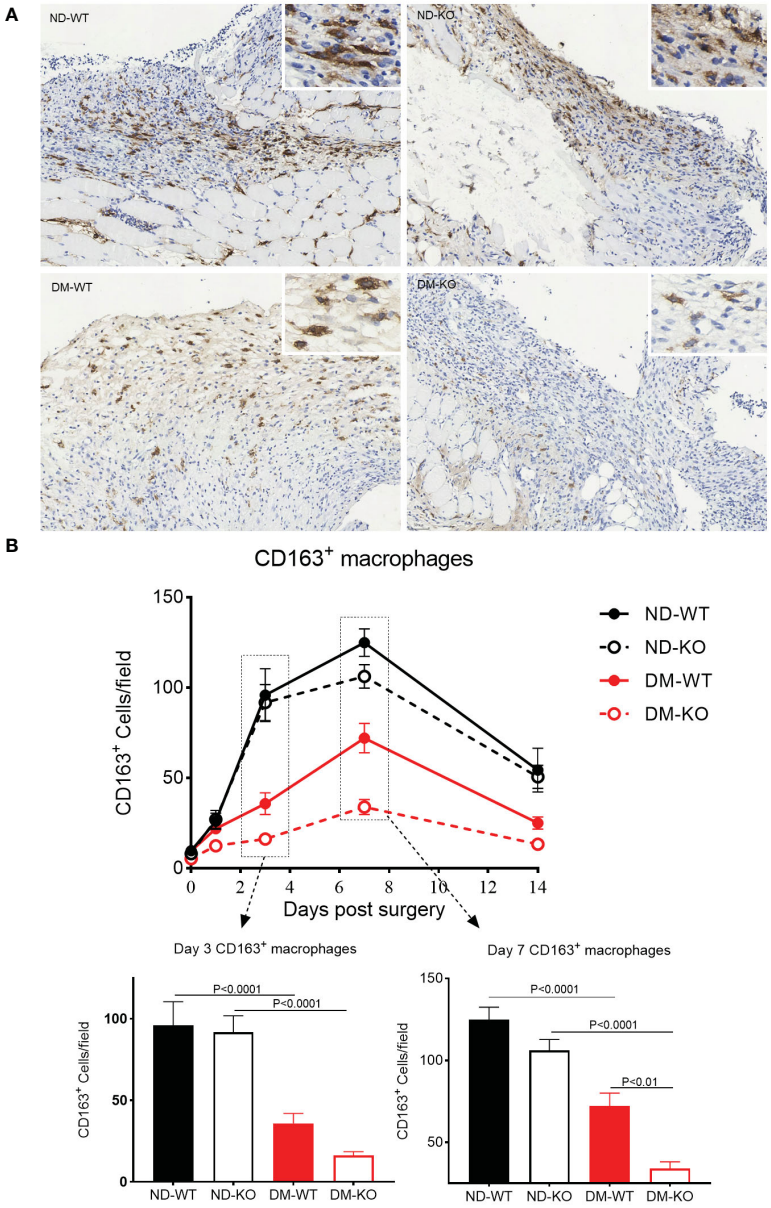

FIGURE 4
Expression of CD163$^+$ macrophages in wounds across different groups detected by immunohistochemistry. CD163$^+$ macrophages infiltration at the day 7 post-surgery was showed in the photomicrographs **(A)**. The mean ± standard deviation of CD163$^+$ cells/filed was showed **(B)**. The inset shows data for days 3 and 7. The X-axis denotes the days post-surgery, while the Y-axis represents the expression of CD163$^+$ macrophages in image units.

higher production than DM-WT ($p<0.001$). There were no significant differences among the other groups.

The effect of CD64 gene knockout on angiogenesis and collagen deposition in wound healing was not significant

Angiogenesis in the wounds was identified through CD31 immunostaining for microvessels (Figure 7). High-resolution images depicting CD31 labelling at higher magnification are provided (Figure 7A), as detailed in our previous publications (12, 25). Three-factor analysis of variance showed that diabetes had a statistical significance for angiogenesis ($p<0.05$), while CD64 KO had no statistical significance for angiogenesis ($p=0.8787$). No significant difference in microvessel number was observed among all groups in uninjured skin. Following surgery, angiogenesis in the wounds gradually increased in ND-WT, ND-KO, and DM-WT mice, peaking at day 7 (Figure 7B). In the DM-KO group, the area of CD31$^+$ microvessels in the wounds increased steadily from day 1 to 14. A significant difference in angiogenesis was observed on day 7 between DM and non-DM groups. The area of CD31$^+$ microvessels in DM-WT was lower than that in ND-WT, being ~ 1.3 times higher in ND-WT (at day 7). The area of CD31$^+$ microvessels in ND-KO was nearly 1.4 times higher than in DM-KO on day 7. However, the difference between KO and WT was not significant.

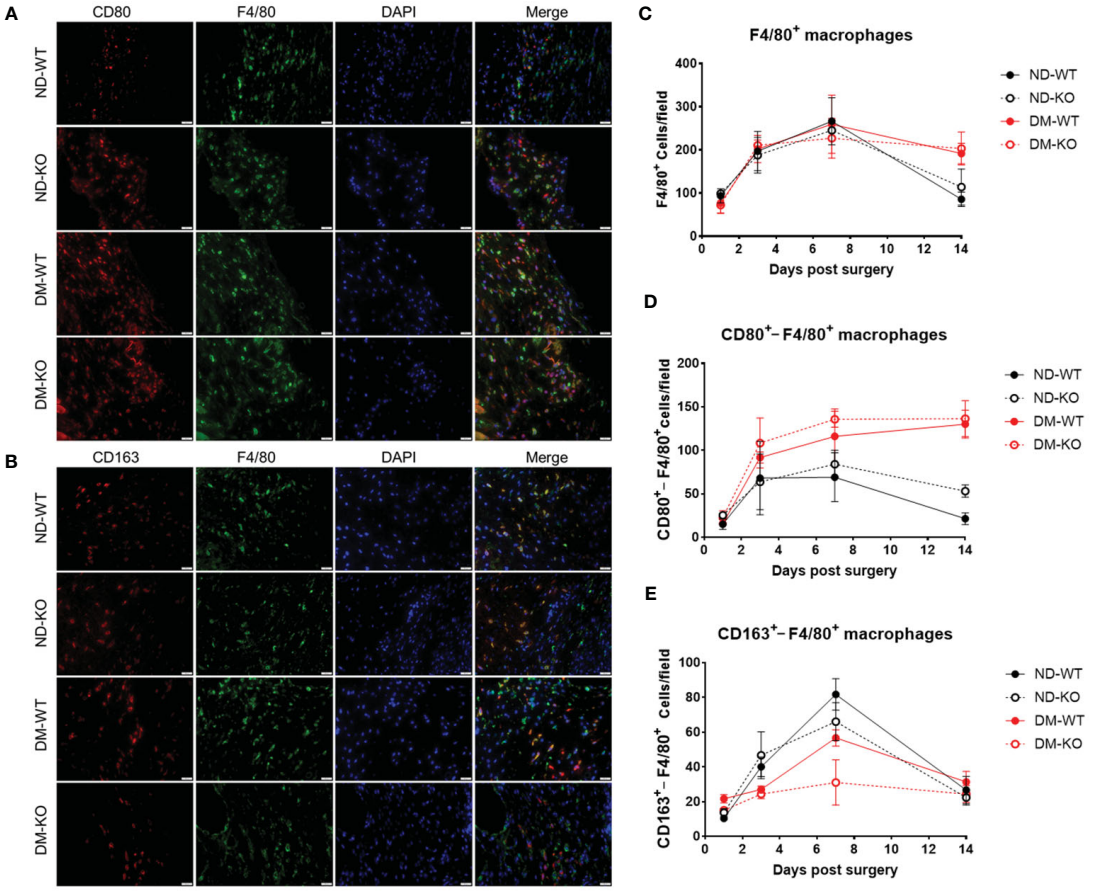

FIGURE 5
It illustrates the presence of macrophages in wounds among different groups, as detected by immunofluorescence double staining. Photomicrographs **(A)** showcase immunofluorescence staining of CD80 (in red) and F4/80 (in green) on day 7 post-surgery. Similarly, photomicrographs **(B)** display immunofluorescence staining of CD163 (in red) and F4/80 (in green) on the same day. Quantitative analysis reveals the mean ± standard deviation of $F4/80^+$ cells per field **(C)**, $CD80^+$ cells per field **(D)**, and $CD163^+$ cells per field **(E)**. The X-axis denotes the days post-surgery, while the Y-axis represents the positive cells per field in image units.

In uninjured skin, all groups showed a collagen volume fraction (CVF) of about 55% (Figure 8A). Collagen deposition increased gradually in all groups following injury. There was no significant difference in collagen deposition among all groups at days 1 and 3. However, a significant difference was observed at days 7 and 14 (Figure 8B). Notably, collagen deposition was highest in ND-WT, followed by ND-KO, DM-WT, and DM-KO, in descending order. The difference in collagen deposition between DM and non-DM groups was statistically significant, whereas the difference between WT and KO groups was not statistically significant.

In summary, the delayed wound healing observed in diabetes correlated well with microvessel formation and collagen deposition. However, the connection between CD64 KO and microvessel formation or collagen deposition was minimal.

Discussion

In the current study, CD64 was significantly up-regulated in the chronic ulcerative skin of DM patients compared to non-DM skin. CD64 expression correlated with FBG, GA, and HbA1c levels. Clinical observations were supported by findings in CD64 KO mice, which exhibited significant delays in wound healing, particularly in the context of diabetes. This delay was associated with compromised inflammatory mediators, emphasizing the critical role of inflammation in wound healing. However, the precise underlying mechanism of CD64 in diabetic wound healing remains to be clarified and will be further verified in clinical samples through *ex vivo* manipulation of CD64 and/or experiments *in vivo*, using animal models.

Significant delays in wound healing, particularly in diabetes, was associated with compromised inflammatory mediators, providing compelling evidence for the critical role of inflammation in the wound healing process (28). These results underscore the potential importance of CD64 in diabetic wound healing and highlight the relevance of inflammatory pathways in this complex biological process.

Wound healing involves the interplay of host immunity and inflammatory responses (5). DM patients experience considerable impairment in wound healing (29), primarily due to compromised host immunity (30). CD64, known to be up-regulated on neutrophils in various conditions such as septicemia (31), neonatal severe respiratory infection (32) and septic shock (33), plays a role in host defense against microbial invasion (34).

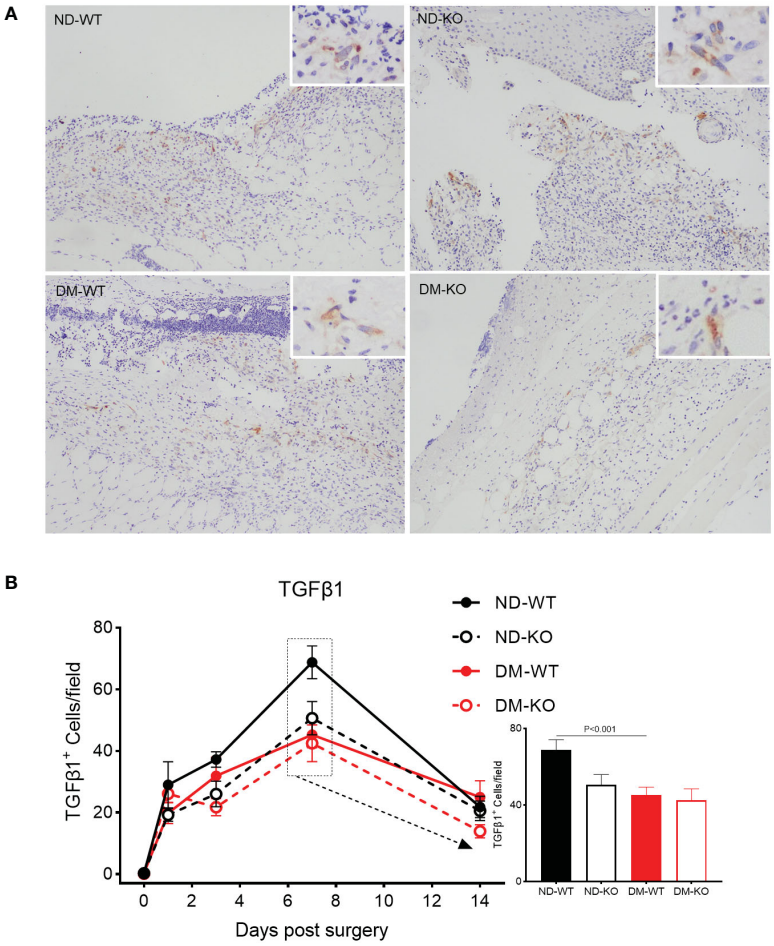

FIGURE 6
Expression of TGFβ1 in wounds across different groups detected by immunohistochemistry. TGFβ1 expression at the day 7 post-surgery was showed in the photomicrographs **(A)**. The mean ± standard deviation of TGFβ1$^+$ cells/filed was showed **(B)**. The inset shows data for day 7. The X-axis denotes the days post-surgery, while the Y-axis represents the expression of TGFβ1 in image units.

While compromised wound healing was evident in CD64 KO mice, the potential influence of wound contraction on the observed delayed closure cannot be conclusively ruled out. Future experiments employing splinting techniques will further investigate its impact on the wound healing process, correlating with TGFβ1 expression. To address the possibility of delayed wound closure being attributed to wound contraction, upcoming experiments using splinting techniques will confirm its impact on the healing process (35).

Our previous research demonstrated substantial impairment in wound healing in GM-CSF depleted animals with DM (24), accompanied by reduced recruitment of neutrophils and macrophages (12). However, the deficiency in wound healing in DM could be restored with exogenous GM-CSF, along with increased pro-inflammatory cytokines (12). These findings underscore the crucial role of inflammation in acute wound healing, and any impairment in inflammatory responses may lead to delayed wound healing (36). Understanding the intricate interplay between host immunity, inflammation, and wound healing in the context of diabetes could offer valuable insights for potential therapeutic interventions in improving wound healing outcomes for DM patients.

Considering CD64 is involved in host inflammatory responses during acute inflammation (14) and its association with certain autoimmunities (37), it is reasonable to speculate that CD64 may negatively impact wound healing via dysregulating local host immunity in DM skin. This speculation aligns with our current finding of substantially increased CD64 expression in the dermal layer of DM chronic ulcerative skin compared to non-DM skin. The upregulated expression of CD64 might be compensating for compromised host immunity in diabetic wounds, resulting in delayed wound healing. This observation is consistent with the up-regulation of inflammatory mediators, such as IL-1β, TNF, and IL-8, in DM skin, further supporting the potential role of CD64 in the wound healing process within chronic ulcerative skin.

To validate the role of CD64 in diabetic wound healing, we found a significant reduction in wound healing in CD64 KO mice compared to WT mice, especially under diabetic conditions. Such findings highlight the essential role of CD64 in the diabetic wound healing process, consistent with the previously observed

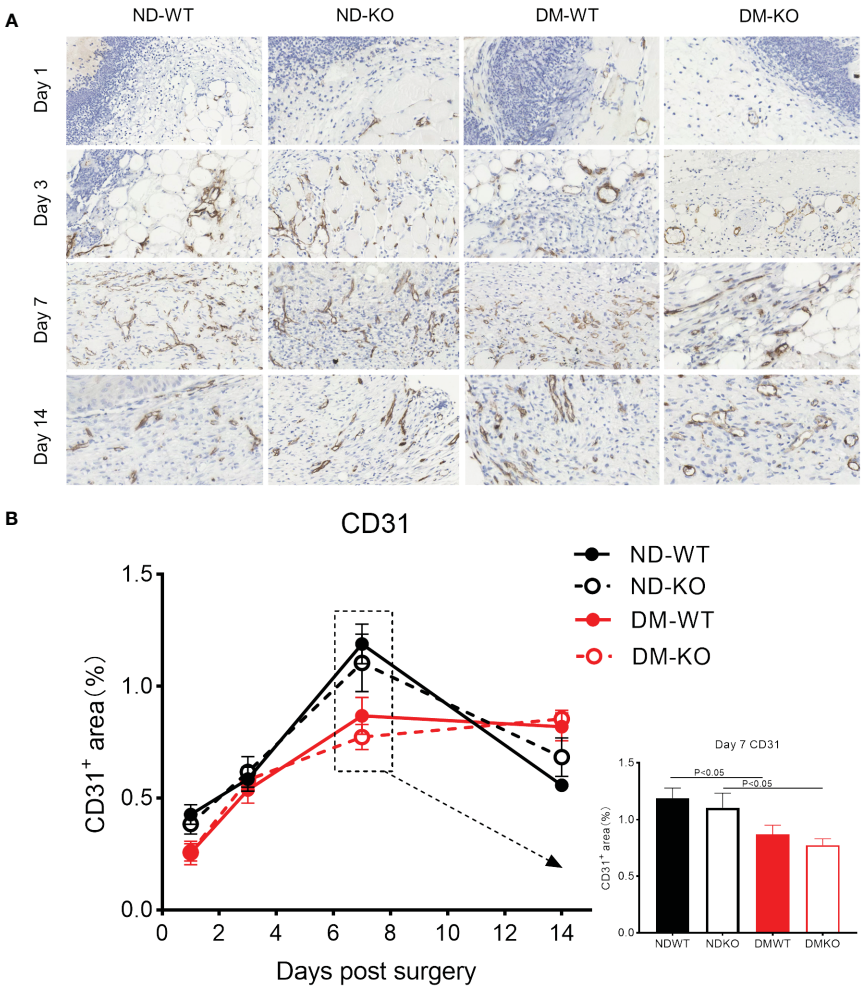

FIGURE 7
Expression of CD31 in wounds across different groups detected by immunohistochemistry. $CD31^+$ microvessels at the days post-surgery was showed in the photomicrographs **(A)**. The mean ± standard deviation of $CD31^+$ microvessels area rate was showed **(B)**. The inset shows data for day 7. The X-axis denotes the days post-surgery, while the Y-axis represents the area rate of $CD31^+$ microvessels in image units.

compromised host immunity (38). Notably, there wasn't a synergistic effect between CD64 expression and the DM condition in this acute wound healing model. This could be attributed to the short duration of the current study, which may limit the impact of long-term host immunity disturbances. Therefore, further investigation is needed to explore the correlation between CD64 and long-term DM in wound healing and its underlying mechanisms.

Nevertheless, our data may offer valuable insights for managing chronic ulceration in DM patients, using CD64 as a potential therapeutic target. Furthermore, it encourages further research to better understand the complexities of wound healing in diabetes and its association with CD64 mediated inflammatory responses. However, we recognize that the current wound healing in the diabetic animal model can't fully reflect the clinical chronic wounds and/or ulcerations in patients with diabetes mellitus due to differences in species and disease progression (weeks in animal models versus years in humans).

Previous studies identified characteristic markers for M1 and M2 cells, including CD80, CD86, CD38, HLA-DR for M1, and CD163, CD206, CD204, MerTK for M2 (39). Our earlier publications emphasized the role of mature macrophages in diabetic wound healing (12, 24), without distinguishing between M1 and M2. $CD163^+$ M2 macrophages contribute significantly to wound healing by promoting anti-inflammatory responses and releasing IL-10 and IL-35 (40). Thus, we used the CD163 biomarker to explore mechanisms of diabetic wound healing.

$CD163^+$ macrophages, representing the main subset of polarized M2 macrophages, play a pivotal role in wound healing by producing elevated levels of anti-inflammatory cytokines and growth factors, including IL-10, IL-1β, and TGFβ. These macrophages are associated with critical wound healing processes such as angiogenesis, matrix maturation, phagocytosis, and anti-inflammatory effects, collectively accelerating wound healing (41–44). Results revealed a reduction in $CD163^+$ macrophages, particularly in DM mice with CD64 depletion, suggesting impaired diabetic wound healing by decreasing $CD163^+$ M2 macrophages, possibly *via* active cytokine secretion, including TGFβ1 (45). This aligns with the gradual upregulation of TGFβ1 in all groups, more significantly in the ND-WT group. Further

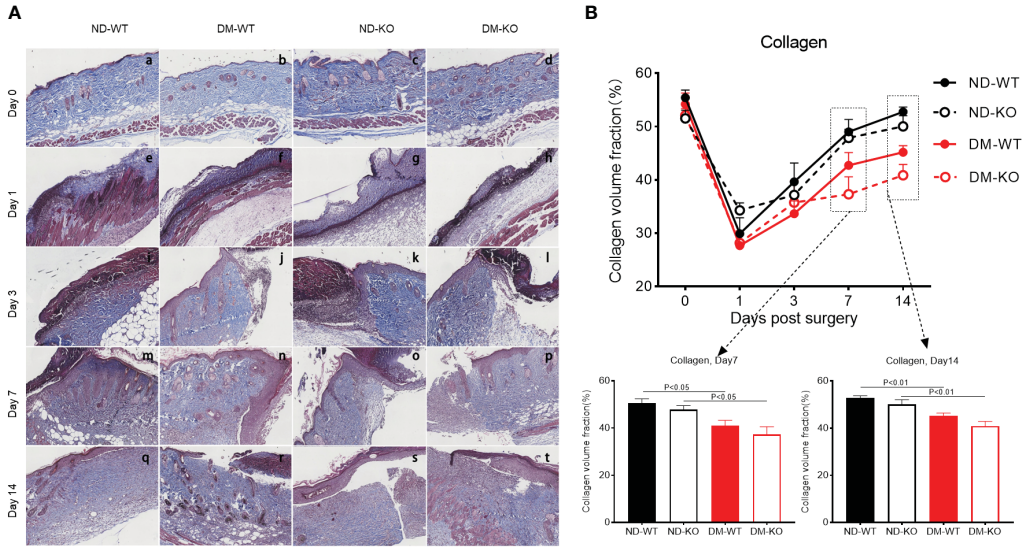

FIGURE 8
Collagen deposition in the wounds across different groups detected by Trichrome staining. The collagen deposition at the days post-surgery was showed in the photographs **(A)**. The mean ± standard deviation of CVF was showed **(B)**. The inset shows data for day 7 and 14. The X-axis denotes the days post-surgery, while the Y-axis represents the collagen volume fraction in image units.

investigations are warranted to elucidate these interactions and their impact on wound healing in diabetics.

Verification of M1 and M2 macrophage infiltration suggest that M1 may play a crucial role in chronic wound clearance, particularly under diabetic conditions, this is supported by the finding that high glucose impairs keratinocyte migration via inducing M1 macrophage polarization (46). Such finding is also aligned with our macroscopic findings, showing substantial compromised wound healing *in vivo*. It is noteworthy that there were some differences observed in infiltrating $CD163^+$ cells alone and $CD163^+/F4/80^+$ M2 macrophages between CD163 single staining and CD163/F4/80 double staining in our current study. This discrepancy could be attributed to variations in sensitivity between immunohistochemical staining and immunofluorescent staining. Additionally, it is important

to acknowledge that not all $CD163^+$ cells necessarily represent macrophages, e.g. dendritic cells may also express CD163 (47), and we plan to verify this point in future studies.

Neo-vascularization increased gradually, peaking at day 7, aligning with our previous publications (12, 24), supporting the importance of neo-vascularization in wound healing. The area of neo-vascularization was highest in ND-WT, followed by ND-KO, DM WT, and DM-KO, consistent with macroscopic observations, suggesting that neo-vascularization enhances wound healing.

In DM, wound healing is suppressed, inhibiting wound closure by suppressing neutrophil and macrophage recruitment, delaying fibroblast proliferation, and collagen secretion (30). Our findings demonstrate delayed wound healing in CD64 KO mice, particularly in the presence of DM, suggesting a role for CD64 expressed neutrophils in wound healing (14). Additionally, we observed reduced fibrosis in DM-KO mice but increased inflammation and vascular congestion. The delayed wound healing in diabetes correlates with collagen deposition, but the connection between CD64 depletion and collagen deposition appears minimal, warranting further investigation.

Initially, we planned to utilize 8-12 week-old mice to synchronize and minimize the effects of epithelial hair follicle stem cells, and to investigate the subsequent contribution of anagen hair follicles to wound healing. However, our plan experienced significant delays due to a mandatory lockdown for two months in Shanghai, triggered by the Omicron variant of the SARS-CoV-2 viral outbreak (48). As a result, we had to use 14-week-old mice, facing financial, staffing, and ethical challenges in discarding the original batch of animals. Using 14-week-old mice may introduce issues related to epithelial hair follicle stem cells, yet this study serves as a proof of concept, highlighting the significant role of CD64 in diabetic wound healing. Future research will explore other contributing factors. To mitigate potential genetic effects on wound healing, we employed CD64 knockout (ko) mice, which underwent over six generations of backcrossing with C57B/6 mice. Moving forward, we plan to conduct the study using het and het crosses to further minimize such potential influences.

Conclusions

In conclusion, our study highlights the significant role of CD64 in wound healing, particularly in the context of diabetes. The correlation between $CD163^+$ macrophages and wound healing indicates that CD64 is closely linked to host immunity during the wound healing process. These findings hold promise for providing valuable insights to clinicians in the management of diabetic chronic ulceration. Understanding the intricate interplay between CD64, host immunity, and wound healing pathways may pave the way for potential therapeutic strategies to improve wound healing outcomes in diabetic patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee, Tongren Hospital, Shanghai Jiaotong University School of Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XZ: Investigation, Data curation, Project administration, Writing – original draft. ZT: Writing – review & editing, Investigation, Methodology. HW: Writing – review & editing. FC: Writing – review & editing. LY: Writing – review & editing. JH: Writing – original draft. PW: Writing – review & editing. HB: Writing – review & editing. SB: Conceptualization, Writing – review & editing. TK: Conceptualization, Formal analysis, Funding acquisition, Writing – review & editing.

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Conflict of interest

The 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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1322256/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Schematic diagram illustrating PCR amplification for the identification of Wt or CD64 KO mice.

SUPPLEMENTARY FIGURE S2
PCR confirmation of CD64 KO mice, with the top band exclusively representing CD64 KO mice, the bottom band exclusively indicating Wt mice, and double bands indicative of Het mice. The red circles represent CD64 KO male mice.

SUPPLEMENTARY FIGURE S3
Hematoxylin and eosin staining of wound tissues at the days post-surgery.

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Macrophages in acne vulgaris: mediating phagocytosis, inflammation, scar formation, and therapeutic implications

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Macrophages serve as a pivotal nexus in the pathogenesis of acne vulgaris, orchestrating both the elimination of *Cutibacterium acnes* (*C. acnes*) and lipid metabolic regulation while also possessing the capacity to exacerbate inflammation and induce cutaneous scarring. Additionally, recent investigations underscore the therapeutic potential inherent in macrophage modulation and challenge current anti-inflammatory strategies for acne vulgaris. This review distills contemporary advances, specifically examining the dual roles of macrophages, underlying regulatory frameworks, and emergent therapeutic avenues. Such nuanced insights hold the promise of guiding future explorations into the molecular etiology of acne and the development of more efficacious treatment modalities.

KEYWORDS

acne vulgaris, macrophages, *Cutibacterium acnes*, inflammation, therapeutics

1 Introduction

Acne vulgaris (AV) denotes a dermatological disorder exhibiting a high prevalence especially in adolescents, primarily impacting follicular sebaceous gland units (1, 2). In regions notably the face, chest, and back, enriched with sebaceous glands, the condition primarily presents as lesions characterized by open (blackheads) and closed (whiteheads) comedones, accompanied by additional dermatological features such as papules, pustules, and subsequent scarring (3). Although not life-threatening, the profound psychological ramifications stemming from acne due to its detriment to appearance and self-esteem are significant, encompassing anxiety, depression, and potential suicidal tendencies (4). The intricate pathogenesis of acne involves four key processes, including hyperkeratosis of the hair follicle, androgen-induced alterations in sebaceous products, *C. acnes* colonization, and associated inflammatory responses (5). While inflammatory events pervade AV, their precise immunological underpinnings warrant further investigation (6).

Macrophages, pivotal elements of the immune system, pervade skin tissues, orchestrating immune responses, inflammation regulation, and tissue regeneration (7). Macrophage

polarization, coupled with the ensuing metabolic reprogramming, commands a pivotal position in contemporary immune regulation research. A confluence of studies elucidates the characterization of macrophages in disparate immune microenvironments, spotlighting seminal regulators of macrophage polarization and underscoring the instrumental role of metabolic reprogramming in orchestrating the activation toward pro-inflammatory and anti-tumor polarization (8, 9). While within dermatology, dysregulation of macrophage polarization significantly influences the pathogenesis of inflammatory skin diseases, such as psoriasis, Behçet's disease, and rosacea (10–12).

Recent investigations delineate the multifaceted roles macrophages assume in acne formation: they contribute beneficially by clearing pathogens and regulating lipid metabolism, yet exert adverse impacts on inflammation and scarring. Despite converging evidence, strategies targeting macrophage regulation in acne vulgaris treatments remain varied. Delving deeper into the roles of macrophages in acne vulgaris is imperative for pioneering therapeutic interventions. This review elucidates recent insights into macrophage involvement in the pathogenesis of acne vulgaris, aiming to foster further research and therapeutic advancements.

2 The role of macrophages in cutaneous tissues

Skin macrophages are classified into tissue-resident and infiltrating types. Tissue-resident macrophages, originating from precursor cells during embryogenesis, are maintained through adulthood via self-renewal (13). Conversely, infiltrating macrophages differentiate from monocytes that, originating from bone marrow, infiltrate tissues in response to stimuli such as Macrophage Colony-Stimulating Factor (M-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Interleukin (IL)-4, IL-10, and IL-13.

In skin, macrophages play crucial roles in maintaining homeostasis and mediating immune responses. Under normal conditions, they sustain tissue homeostasis by clearing cellular debris and secreting cytokines (14). In sebum-rich areas, they maintain lipid equilibrium by phagocytosing sebum (15). Upon injury, they modulate inflammatory responses and mediate adaptive immunity by presenting antigens to T cells (16). In later stages, macrophages secrete growth factors and cytokines to promote cell proliferation and matrix remodeling, aiding wound healing.

Macrophage polarization is pivotal for their role in skin. Lipopolysaccharide (LPS), GM-CSF, Tumor Necrosis Factor (TNF)-α, and Interferon (IFN)-γ produced by Th1 cells mediate the classical activation pathway, polarizing macrophages to M1 type, which produce high levels of reactive oxygen species (ROS) and pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and IL-12, enhancing inflammation and eliminating pathogens (17). Conversely, chemokine (C-C motif) ligand 6 (CCL6), M-CSF, linoleic acid, oleic acid, IL-4, and IL-13 produced by Th2 cells mediate the alternative activation pathway, polarizing to M2 type (18). M2 macrophages suppress the inflammatory response by producing IL-10. They further promote fibroblast proliferation, collagen synthesis, and angiogenesis through the release of Transforming Growth Factor (TGF)-β, Platelet-Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF), facilitating tissue repair (19). In contrast, Matrix Metalloproteinases (MMPs) expressed by M1 macrophages degrade collagen, inhibiting fiber formation. IL-12 and IL-10, in turn, stimulate the differentiation of T cells to Th1 and Th2 cells, respectively (20) (Figure 1). Comprehensively, M1 macrophages within the dermal ecosystem play a pivotal role in instigating inflammatory responses that not only target pathogens but also contribute to the resultant tissue damage. Simultaneously, M2 macrophages, in their pivotal role, regulate and suppress excessive inflammatory reactions to forestall damage, also facilitating tissue repair and wound healing. The interplay between shifts in the cytokine milieu and macrophage plasticity enables a flexible modulation of M1 and M2 functionalities, a cornerstone for preserving systemic equilibrium. Disruption of this equilibrium heralds pathological sequelae and the inception of disease states. For instance, single-cell RNA sequencing insights from hidradenitis suppurativa illustrate that the STAT1/IFN axis propels macrophages towards an M1-skewed pro-inflammatory phenotype, exacerbating inflammation and promoting immune cell ingress (21). Conversely, the predilection for M2 macrophage polarization not only underpins tumorigenesis but also catalyzes fibrosis and granuloma evolution, potentially correlating with the systemic manifestations observed in sarcoidosis (22, 23).

3 The central role of inflammation in the pathogenesis of acne vulgaris

The onset of acne lesions is characterized by the emergence of microcomedones, consequent to hyperkeratinization occurring within the funnel section of the follicular canal, causing pore obstruction and the subsequent development of comedones (24). Upon exposure to air, blackheads undergoes oxidation and darkening, contrasting with whiteheads, which remains subcutaneous, manifesting a white or normal-skinned appearance. *C. acnes* multiply within these obstructed follicles, inciting an inflammatory response manifested by redness, swelling, and pus (25). Without adequate intervention, such lesions may intensify into painful pustules, cysts, or nodules, risking irreversible damage and scarring in the absence of proper treatment or when exacerbated.

The manifestation of acne lesions is precipitated by the synergistic interaction of four predominant causative factors (26). The upsurge in sebum production and augmented epithelial keratinization within hair follicles, instigated by abnormalities in androgen metabolism, cultivate an environment optimal for the prolific expansion of *C. acnes*. Metabolites of *C. acnes* incite inflammation within follicular and perifollicular regions, coupled with an ensuing immune cell infiltration of the follicular wall, which may culminate in follicular rupture. Subsequent to this, the

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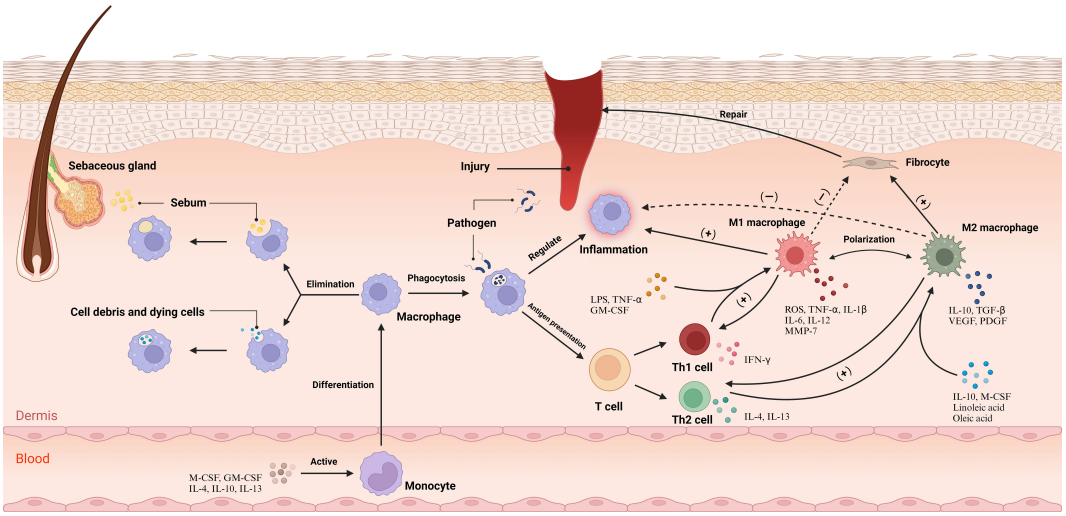

FIGURE 1
Under physiological conditions, macrophages play pivotal roles, namely the clearance of cellular remnants and apoptotic cells, phagocytosis of lipids, and the sustenance of dermal tissue equilibrium. Upon the manifestation of a wound, Cytokines incite monocytes within the bloodstream, catalyzing their differentiation into macrophages, which subsequently permeate the dermal tissue. Macrophages undertake the phagocytosis of pathogens and orchestrate the inflammatory responses while mediating antigen presentation to T cells. This interaction induces the differentiation of T cells into Th1 and Th2 subsets. Subsequently, Th1 cells augment M1 polarization, and Th2 cells enhance M2 polarization. M1 macrophages primarily emit pro-inflammatory cytokines and attenuate fibroblast function, while M2 macrophages release cytokines that mitigate inflammation and elevate fibroblast activity, promoting tissue repair. The cytokines secreted by M1 and M2 macrophage subtypes stimulate Th1 and Th2 cells, respectively, maintaining a balanced immune response within the dermal ecosystem. CCL6, chemokine (C-C motif) ligand 6; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; IFN-γ, Interferon-gamma; IL-1, Interleukin-1; LPS, Lipopolysaccharide; M-CSF, Macrophage Colony-Stimulating Factor; MMP-7, Matrix Metallopeptidase 7; PDGF, Platelet-Derived Growth Factor; ROS, reactive oxygen species; TGF-β, Transforming Growth Factor-beta; Th1, T helper 1 cell; TNF-α, Tumor Necrosis Factor-alpha; VEGF, Vascular Endothelial Growth Factor. Adapted from "Skin (Layout)" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

materials within pimples permeate the dermal tissue, escalating the inflammation and thereby engendering pustules, granulomas, and symptoms indicative of systemic inflammation.

Inflammatory reactions are omnipresent throughout acne's progression. Research conducted by Jeremy et al. has revealed prominent escalations in CD3+ and CD4+ T cells, macrophages, and IL-1α in the dermis surrounding unaltered hair follicles in individuals afflicted with acne, suggesting inflammation as a precursor to the manifestation of acne (6). Importantly, IL-1α is capable of activating basal keratinocytes through autocrine production, prompting the augmented proliferation of suprabasal cells and subsequently inducing hyperkeratosis in the follicular funnel (27). Subsequently, *C. acnes* facilitates the release of inflammatory cytokines, including TNF-α, IL-6, and IL-8 from dendritic cells and keratinocytes, initiating an extensive adaptive immune response (28). This series of events leads to the mobilization of macrophages and lymphocytes to the site, enhancing perifollicular cell infiltration due to elevated cytokine release, resulting in the formation of inflamed papules. Post follicular destruction, components such as keratin and hair elements incite severe inflammatory reactions, culminating in the development of pustules or granulomas. A significant correlation is observed between the severity and duration of inflammation and the inception of scars, as demonstrated by Carlavan et al., attributed to delayed inflammatory reactions enhancing the susceptibility to scarring in acne patients due to the dysregulation of the innate immune response (29). This investigation further identified elevated expression of various immunoglobulin genes and the pervasive infiltration of mature B cells within the long-lasting lesions of scarring patients. Concurrently, Holland and colleagues documented that lesions in scarring individuals exhibit diminished HLA-DR expression alongside sparse CD4+ T cell infiltration (30). Collectively, these observations underscore the potential role of adaptive immunity dysregulation in the etiology of acne scar formation.

4 Macrophages in acne: a dual facet in pathogenesis

Macrophages embody a paradoxical role in acne development, serving as both sentinels and provocateurs. Their vital functions include the regulation of lipid concentrations and facilitating the elimination of *C. acnes*. However, an immoderate immune reaction can provoke inflammation and subsequent acne scarring. It is imperative to comprehend their intricate roles to maintain physiological equilibrium and circumvent adverse pathological outcomes.

4.1 Macrophages and lipid metabolism regulation

Individuals with AV often exhibit abnormal plasma lipid profiles, characterized by elevated plasma lipoproteins and a

decrease in High-Density Lipoprotein (HDL) cholesterol levels, particularly in severe acne conditions (31). These abnormalities in serum total cholesterol levels act as a substrate for androgen synthesis by the adrenal glands and gonads, intensifying the progression of AV through the increase of androgens (32).

In the context of the aforementioned lipid metabolism disruption, macrophages are crucial regulators, orchestrating the assimilation of oxidized low-density lipoprotein (LDL) and cholesterol. Macrophage scavenger receptor 1 (MSR1) facilitates the incorporation and breakdown of modified LDL, leading to intracellular cholesterol accumulation and influencing macrophage functionality (33). AbdElneam et al. found that of the four common haplotypes of the MSR1 gene, the most common haplotypes in patients with AV are TCAC and CAGG, whereas the most common haplotypes in healthy populations are TAAC and CCAC (34). Additionally, they observed a linkage disequilibrium in the MSR1 gene, indicating that genetic elements might significantly influence the development of AV by altering macrophage-mediated lipid regulation.

4.2 Engulfment and clearance of *C. acnes* by macrophages

Macrophage phagocytosis in acne lesions is prominently orchestrated by *C. acnes*, a Gram-positive, parthenogenetic anaerobic bacillus. This bacterium is pivotal, predominantly inhabiting the sebaceous glands of hair follicles in acne patients and playing a vital role in acne pathogenesis (35). It triggers the expression of IL-15 and GM-CSF via Toll-like receptor 2 (TLR2), fostering the differentiation of monocytes into CD209+ macrophages and CD1b+ dendritic cells (36). Comparatively, CD209+ macrophages exhibit superior efficacy in phagocytosing and curtailing the growth of *C. acnes*, becoming a major line of host defense against this bacterium.

Detailed analysis of skin biopsies from inflammatory acne lesions reveals macrophages' ability to recognize and phagocytose *C. acnes* strains, with sebum playing a role in enhancing this ability (15). These macrophages utilize a variety of enzymes, such as lipoxygenase and myeloperoxidase, to produce antimicrobial agents ROS and reactive nitrogen intermediates (RNI) in lysosomes. These agents then fuse with phagosomes to eliminate the pathogens using ROS and RNI (37).

Notably, Do et al. found that excess squalene production by keratin-forming cells in acne lesions scavenges ROS (38). This also leads to a reduction in the expression of genes encoding oxidative enzymes produced by ROS and RNI, including the NADPH oxidase genes NOX1, NOS2, and NFKB. Furthermore, the expression of genes encoding ROS-induced oxidative pathway enzymes, such as the mitogen-activated protein kinase genes MAPK8, MAPK14, and NFKB, is also reduced. This renders TREM2 macrophages virtually devoid of antimicrobial activity, blocking the antimicrobial response and increasing the bacterial load. Consequently, this leads to the secretion of IL-18 and upregulation of inflammatory chemokine expression, instigating a disease site-specific inflammatory response. Remarkably, this phenomenon elucidates the efficacy of benzoyl peroxide in the clinical management of acne through the peroxidative destruction of *C. acnes*. Remarkably, this study illuminates an additional mechanism by which topical benzoyl peroxide combats acne, beyond its direct antibacterial action against *C. acnes*, anti-inflammatory effects, and comedolytic properties (4). It achieves this by generating oxygen radicals that negate squalene's capacity to scavenge oxygen radicals, thereby countering the suppression of squalene's antimicrobial potency on TREM2 macrophages.

Nakatsuji et al. elucidated that the synergistic interaction of the Christie, Atkins, Munch-Peterson (CAMP) factor from *C. acnes* and acidic sphingomyelinase (ASMase) from the host cells triggers an inflammatory response and inflicts cytotoxicity to macrophages (39). Based on these findings, Nakatsuji formulated two plausible hypotheses: *C. acnes* may evade the immune response by utilizing host ASMase, or the CAMP factors might manipulate host ASMase, thereby amplifying bacterial virulence, undermining macrophages, and obstructing the efficient clearance of *C. acnes* (Figure 2).

4.3 Macrophage-mediated inflammatory regulation in acne lesions

Macrophages exhibit a central role in mediating inflammatory responses within acne, governed by diversified pathways.

Sebaceous gland-originating lipids are crucial modulators of macrophage activities. A study led by Lovászi et al. elucidated that sebum is imperative for IL-1β secretion in *C. acnes*-activated macrophages, where components like oleic acid and squalene intensify IL-1β secretion (15). Intriguingly, oleic acid selectively moderates the expression of IL-6 and TNF-α, introducing a paradox considering its role in promoting M2 polarization and IL-1β secretion amplification. In contrast, linoleic acid exerts anti-inflammatory properties, reducing the secretion of prominent inflammatory mediators. On a different note, Tang et al.'s study provides insights into the synergistic secretion of IL-1β and TNF-α by sebaceous cell glands and macrophages in acne conditions (40). Sebaceous cell glands induce macrophage polarization to the M1 phenotype, exacerbating inflammatory reactions. Cannabidiol (CBD) is recognized to mitigate this interplay effectively.

Furthermore, keratinocytes residing in acne lesions orchestrate macrophage inflammatory responses. Research by Graham highlighted that *C. acnes* and its GroEL protein trigger keratinocytes to release IL-1α, Chemokine (C-X-C motif) Ligand 8 (CXCL8), GM-CSF, TNF-α, and human β-defensin-2 (hBD-2) via the TLR, enhancing macrophage activation and recruitment and intensifying inflammation (41, 42). Additionally, squalene, produced by keratinocytes, prompts macrophages to increase TREM2 receptor expression, amplifying inflammatory gene expression (38).

C. acnes plays a cardinal role in inciting the production of pro-inflammatory cytokines such as IL-1β, largely by the direct activation of Pattern Recognition Receptors (PRR) in macrophages, occurring primarily through Pathogen-Associated Molecular Patterns (PAMP) (43). This encompasses entities like TLR and Nod-like receptors (NLR). Tsai et al. delineated that *C. acnes* engages the MAPK and

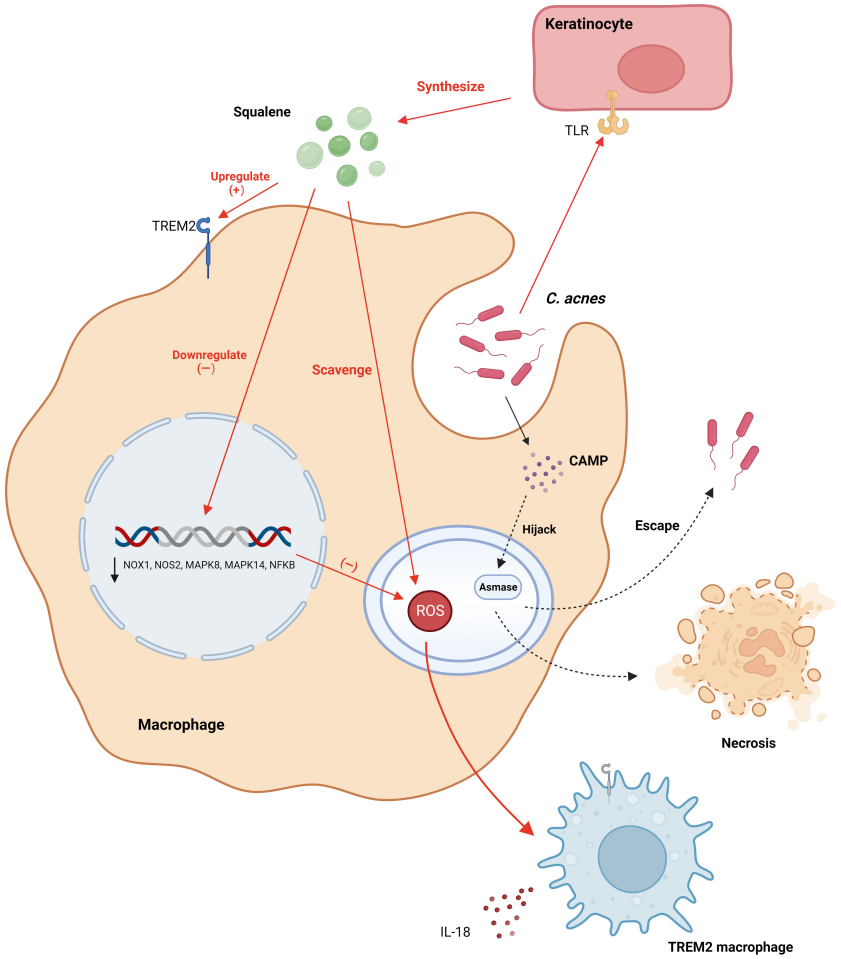

FIGURE 2
C. acnes modulates the secretion of squalene from keratinocyte cells via TLR receptors, elevating the expression of TREM2 receptors. This secretion mitigates reactive oxygen species within lysosomes, leading to the downregulation of genes encoding oxidative and pathway enzymes responsible for the production of ROS and RNI. Consequently, this leads to the transformation of macrophages into TREM2 macrophages, which primarily release pro-inflammatory factors and exhibit diminished proficiency in eliminating *C. acnes*. Additionally, *C. acnes* synthesizes the CAMP factor, which, facilitated by Asmase, either evacuates from macrophages or provokes macrophage necrosis. CAMP, Christie, Atkins, Munch-Peterson; TLR, Toll-Like Receptor; TREM2, Triggering Receptor Expressed on Myeloid cells 2. Created with BioRender.com.

NF-κB signaling cascades via the TLR2 receptor on macrophages, thereby modulating the expression of inducible Nitric Oxide Synthase (iNOS)/NO and Cyclooxygenase-2 (COX-2)/Prostaglandin E2 (PGE2) (44). This interaction is reliant upon the nuanced phosphorylation of protein kinases JNK and EPK along with ROS-enhanced IKK protein phosphorylation, cumulatively activating AP-1 and NF-κB and consequently augmenting the production of NO and PGE2. Fischer and colleagues delineated that the activation of *C. acnes* is dependent on the junctional protein TIR-domain-containing adapter-inducing interferon-β (TRIF), initializing the cGAS-STING pathway and inducing the type I interferon axis in macrophages, catalyzing additional signaling pathways, including the JAK-STAT pathway, and modulating the inflammatory response (45). TRIF, predominantly a downstream of TLR3/4, does not ordinarily recognize Gram-positive bacteria. Fischer posited that TRIF signaling is coupled with TLR2, aiding macrophages in the activation of the IFN-I axis after the recognition of *C. acnes*. It's worth noting that TLR4 also discerns Gram-positive bacteria, and, as previously highlighted, *C. acnes* can stimulate keratinocytes via both TLR2 and TLR4 (41, 42, 46). Consequently, the intricate mechanisms by which *C. acnes* activates the cGAS-STING pathway via TRIF remain to be explicated comprehensively.

C. acnes intricately modulates IL-1β secretion through NOD-like receptor family, pyrin domain-containing 3 (NLRP3), amplifying the transcription of both IL-1β and NLRP3 genes predominantly via the NF-κB pathway (47). Once transcribed within macrophages, NLRP3 emerges as a pivotal sensor, converging with the effector cysteine protease (caspase)-1 to orchestrate the inflammasome, thereby catalyzing the metamorphosis of pro-IL-1β to matured IL-1β for ensuing release (48). This mechanism, elucidated by Qin et al., is contingent upon K+ efflux (Figure 3). Regrettably, the absence of subsequent comprehensive studies renders this field ripe for further exploration. Indeed, inflammasomes are also capable of processing Gasdermin D (GSDMD), integrating it into the plasma membrane and creating pore structures to release pro-inflammatory cytokines, ultimately inducing cellular pyroptosis——a distinct, inflammation-

associated form of programmed cell death instrumental in host defense and implicated in anti-tumor activities in certain cancers (47). However, in dermatological conditions, it predominantly manifests as a pathogenetic mechanism. Deng et al. delineated how SpeB, an exotoxin of Streptococcus pyogenes, triggers cutaneous keratinocyte pyroptosis through the cleavage of GSDMA (49). While Lian et al. highlighted the role of GSDMD-mediated keratinocyte pyroptosis in fostering hyperproliferation and aberrant differentiation, contributing to the development of psoriasis (50). Hence, probing the potential induction of macrophage cell pyroptosis by *C. acnes* is pivotal and promises profound insights into the pathogenesis of acne vulgaris.

Finally, genetic elements play a significant role in shaping the manner in which macrophages regulate inflammation. Resistin, a protein primarily secreted by macrophages, acts to elevate the expression of inflammatory mediators (51). A significant revelation made by Akcılar et al. was the identification of a protective association linked to the CG genotype of the resistin gene, correlating prominently with a decreased risk of acne vulgaris.

4.4 Influence of macrophages on the progression of acne scarring

Primary lesions in acne vulgaris frequently culminate in scarring, which, by altering physical appearance, can engender significant social and psychological ramifications (52). The genesis of scarring is multifaceted, implicating not only the intensity and duration of inflammation but also the intricate interplay of cytokines secreted by diverse macrophage subtypes, orchestrating dermal fibroblasts' activity (53). Disruption in the equilibrium of pro- and anti-fibrinogen cytokines and growth factors precipitates aberrant extracellular matrix protein synthesis

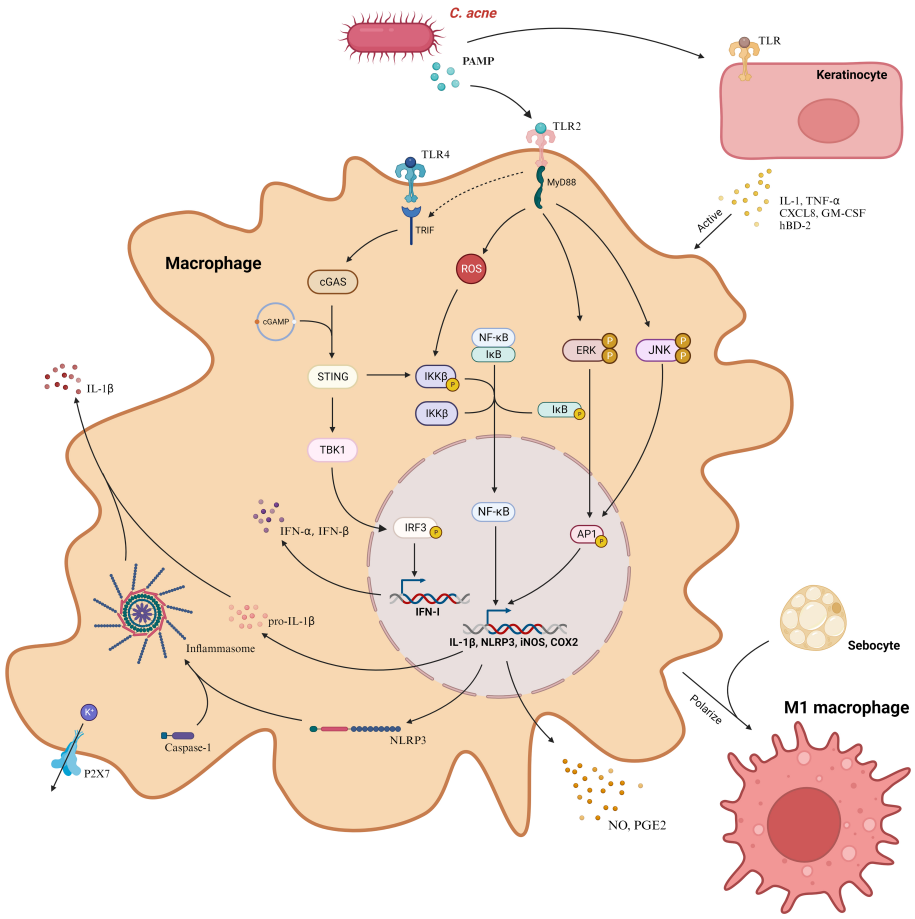

FIGURE 3
C. acnes predominantly instigates three distinctive signaling cascades in macrophages through PAMP, mediated via TLR2. These cascades encompass the ERK, JNK, and NF-κB pathways, which amplify the transcription of iNOS and COX2 genes, facilitating the liberation of NO and PGE2. Concurrently, *C. acnes* incites the cGAS-STING signaling cascade via TRIF, initiating the type I interferon axis. This bacterium also enhances the expression of NLRP3, which collaborates with caspase-1 to form the inflammasome. This assembly, reliant on K+ efflux, processes pro-IL-1β into IL-1β, enabling its release from the cell. Additionally, *C. acnes* induces keratinocytes to discharge cytokines, in turn, activating macrophages. Furthermore, in the presence of *C. acnes*, sebocytes facilitate the polarization of macrophages to the M1 phenotype. COX2, Cyclooxygenase-2; CXCL8, Chemokine (C-X-C motif) ligand 8; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; hBD-2, Human Beta-Defensin 2; IFN-I, Interferon Type I; IL-1β, Interleukin-1 beta; iNOS, Inducible Nitric Oxide Synthase; MyD88, Myeloid Differentiation Primary Response Protein 88; NLRP3, NOD-like Receptor 3; P2X7, Purinergic Receptor P2X7; PAMP, Pathogen-Associated Molecular Pattern; PGE2, Prostaglandin E2; TLR, Toll-Like Receptor; TNF-α, Tumor Necrosis Factor-alpha; TRIF, TIR-Domain-Containing Adapter-Inducing Interferon-β Protein. Created with BioRender.com.

and compromises fibroblast functionality, culminating in wound remodeling, contraction, and subsequent scar manifestation (54).

Holland et al. elucidated disparate macrophage trajectories between not-scar-prone (NSP) and scar-prone (SP) patients (30). In NSP patients, pervasive macrophage infiltration coupled with augmented HLA-DR expression marked efficient antigen clearance and cellular activation, reverting to basal levels as lesions resolved. In contrast, SP patients evidenced sustained macrophage elevations and initial subdued, but progressively accentuated, HLA-DR expressions, indicating an escalating, specialized immune response, fostering angiogenesis and exacerbated inflammation, and culminating in scarring. Complementing this, Carlavan et al. detected enduring macrophage recruitment and irreversible glandular disruptions in SP patients, implying an elongated inflammatory state (29).

Macrophage polarization may further play a pivotal role in the pathogenesis of acne scarring. Under normal conditions, M1 macrophages predominantly clear pathogens and debris during wound healing's initial phase. As healing advances to the proliferative phase, a critical shift to M2 macrophages occurs, promoting tissue regeneration and reducing inflammation. In the remodeling phase, M2 macrophages regulate collagen, essential for tissue restoration (55). Saint-Jean et al. discerned elevated IL-2 and IL-10 and attenuated MMP-9 proteins in SP patients, implicating a predominance of M2 macrophages (56). Besides, relationship between macrophage polarization and acne scarring still lacks more empirical elucidation, but there have been many solid proofs for other scarring phenomena. It is imperative to acknowledge that a heightened prevalence of pre-injury M2 cells, a suppression of M1 expression in preliminary phases, and a subsequent deferred M2 expression in terminal phases, are associated with the evolution of pathological scarring (57). The accentuated presence of M2 cells along scar peripheries and within the superficial dermis underscores the potential ramifications of macrophage hyperactivity in disrupting the equilibrium of collagen synthesis and catabolism, thereby precipitating hyperfibrosis (58, 59). This scenario is analogous to the complexities observed in numerous chronic wounds, wherein the activation of M1 macrophages is inhibited, preventing a full transition to the M2 phenotype and thus arresting the repair mechanism within the inflammatory stage (60). Recent investigations have illuminated that SPP1+ macrophages exhibiting an M2 macrophage phenotype, in conjunction with POSTN+ fibroblasts, precipitate the development of fibrotic scars in acne keloidalis through interactions mediated by the SPP1 axis (61).

5 Therapeutic strategies and potential approaches addressing macrophages in acne vulgaris

The comprehensive understanding of the pathogenesis of acne vulgaris is yet to be fully attained. Nonetheless, numerous empirical studies have highlighted the ability of existing therapeutic strategies to alleviate acne by modulating macrophage functionality. Furthermore, multiple approaches, which focus specifically on macrophages, display considerable potential for therapeutic advancement in acne vulgaris, signaling new directions in research and application. Intriguingly, our scrutiny of acne treatment strategies targeting macrophages uncovered a general trend towards dampening inflammatory signaling pathways and cytokine production, with strategies encompassing M2 macrophage polarization among others. Remarkably, photodynamic therapy (PDT), used to treat severe acne, delineates an antithetical mechanism by advocating for M1 macrophage polarization, thereby escalating inflammation (62). This tiered therapeutic paradigm for acne articulates a groundbreaking perspective: advocating pro-inflammatory interventions for individuals grappling with severe acne, in stark divergence from the anti-inflammatory modalities prescribed for mild conditions. The discrepancy between fostering and mitigating inflammation unmistakably signals the presence of an alternative to current therapeutic modalities, signaling a departure from established treatments and underscoring the pressing need for further investigation to validate these novel approaches.

5.1 Retinoids

Retinoids, esteemed in dermatological domains, are quintessential in acne vulgaris therapy, administered both topically and orally. They exemplify diverse pharmacological competence, optimizing follicular keratinization, suppressing the proliferation of *C. acnes*, exhibiting anti-inflammatory traits, and forestalling scarring (63). Notably, All-trans retinoic acid (ATRA) and adapalene (AD) are paramount in this pharmacological class.

Retinoids enhance macrophage phagocytosis and clearance via the inhibition of squalene sebum (38). Liu et al. elucidated that ATRA not only moderates the expression of TLR2, attenuating C. acnes-induced inflammatory cytokine production, but also orchestrates the differentiation of monocytes into macrophages over dendritic cells, intensifying the phagocytosis of C. acnes and curtailing its proliferation (64). Further, Ji et al. validated the therapeutic efficacy of the electrostatically optimized adapalene-loaded emulsion, illustrating its significant inhibitory impact on the macrophage expression of pro-inflammatory substances (65).

5.2 Natural product drugs

Natural entities, including specific plant and animal extracts, have long been integral to acne treatment methodologies, predominantly as ethnomedicines, across diverse cultures. Several of these derivatives are renowned for their anti-inflammatory capacities, with a focus on modulating signaling pathways in macrophages (Table 1).

Kang et al. elucidated that ethyl acetate extract from *Angelica Dahuricae* Radix (EAAD) effectively inhibits iNOS and COX-2 expression and concurrently reduces the production of NO, PGE2, and TNF-α in macrophages by blocking the NF-κB and MAPK

pathways (66). Analogously, substances such as quercetin, apigenin, and cembrene diterpenoids from the cultured soft coral *Sinularia flexibilis* inhibit the MAPK pathway in macrophages, thereby alleviating inflammation (67–69). On the other hand, *Kaempferia parviflora* extract and licochalcone A demonstrate anti-inflammatory effects by obstructing the NF-κB pathway and NLRP3 inflammasomes respectively (70, 71). In a distinctive study, Sekiguchi et al. revealed that JHT, a conventional Japanese medicine for acne, promotes the differentiation of monocytes to M1-type macrophages and intensifies macrophage infiltration, suggesting a potential improvement in acne symptoms through the enhancement of M1-type macrophage functions (72).

5.3 Photodynamic therapy

5-Aminolevulinic Acid Photodynamic Therapy (ALA-PDT) is emerging as a groundbreaking therapeutic approach for acne vulgaris, distinguished by its unequivocal efficacy, minimal invasiveness, and lack of systemic side effects (73). Post-ALA-PDT, a significant initial symptom is a severe inflammatory response, evidenced by erythema and pustules, with the intensity of the response being directly proportional to the effectiveness of the treatment (74).

Zhang et al. delineated that ALA-PDT activates the p38 MAPK pathway, instigating CXCL8 expression in sebaceous gland cells and subsequently enlisting macrophages to augment the inflammatory response (75). Subsequently, Liu et al. elucidated that keratinocytes subjected to ALA-PDT exhibit escalated COX2 expression, releasing PGE2 to elevate TREM1 receptor expression via TLR4 in macrophages, fostering macrophage M1 polarization and triggering the p38 and NF-κB signaling pathways, thereby intensifying inflammation (62) (Figure 4). It's hypothesized that by directing macrophage M1 polarization to enhance inflammation, ALA-PDT may offer a revolutionary solution for acne treatment.

5.4 Traditional Chinese medicine interventions

Auriculotherapy, an integral component of Traditional Chinese Medicine (TCM), has been revered as a therapeutic intervention for conditions like inflammation, pain, and drug addiction. Zuo et al. executed auriculotherapy interventions, which encompass auricular bloodletting therapy (ABT), auricular point sticking (APS), and their combination (ABPS), on a rat model representing acne (76). The results unveiled that ABT, APS, and ABPS were proficient in prompting the initiation of M2-type macrophages via meticulous modulation of macrophage polarization. This resulted in the alleviation of acne symptoms and a reduction in serum levels of TNF-α and IL-1β in rats. Notably, APS also exhibited a significant reduction in the expression of the TLR2/NF-κB signaling pathway. The discoveries from this study reinforce the mechanistic underpinning of TCM auricular acupoint therapy in addressing inflammatory conditions like acne, adding substantial validation to the application of auriculotherapy in unconventional acne treatments.

5.5 Exploration of alternative therapeutic strategies

5.5.1 Antimicrobial peptides

Antimicrobial peptides, inherent immune molecules in organisms, exhibit versatile antimicrobial properties. Research by Wu et al. elucidated that Cath-MH, originating from frog skin, exhibits potent antimicrobial efficacy against *C. acnes* by inhibiting the proliferation of *C. acnes* and curtailing inflammatory cytokine production (77). This is facilitated through the suppression of macrophage TLR expression via PAMP binding, highlighting antimicrobial peptides as potent candidates for acne interventions.

TABLE 1 Natural product drugs employed for Acne Vulgaris intervention by addressing action on macrophages.

Chemical	Description	Mechanism of action	Source	References
EAAD	Ethyl acetate extract from Angelica Dahuricae Radix	Inhibition of inflammatory responses through suppression of the NF-κB and MAPK pathways	Angelica dahurica	(66)
Quercetin	Plant polyphenolic of flavonoid	Inhibition of inflammatory responses through suppression of the MAPK pathway	Fruits, leaves, and vegetables	(67)
Cembrene Diterpenoids	Obtained from Soft Coral Sinularia flexibilis	Inhibition of inflammatory responses through suppression of the MAPK pathway	*S. flexibilis*	(68)
Apigenin	Phenolic compound	Inhibition of inflammatory responses through suppression of the MAPK pathway	Fruits, leaves, and vegetables	(69)
Kaempferia parviflora Extract	Plant Extract	Inhibition of inflammatory responses through suppression of the NF-κB pathway	*Kaempferia parviflora*	(70)
licochalcone A	Chalconoid isolated from the root of Glycyrrhiza inflate	Inhibition of inflammatory responses through suppression of NLRP3 inflammasome activation	*Glycyrrhiza inflate*	(71)
Jumihaidokuto (JHT)	Pharmaceutical-grade traditional medicine	Promotion of inflammatory responses through induction of M1 polarization	Japanese medicine	(72)

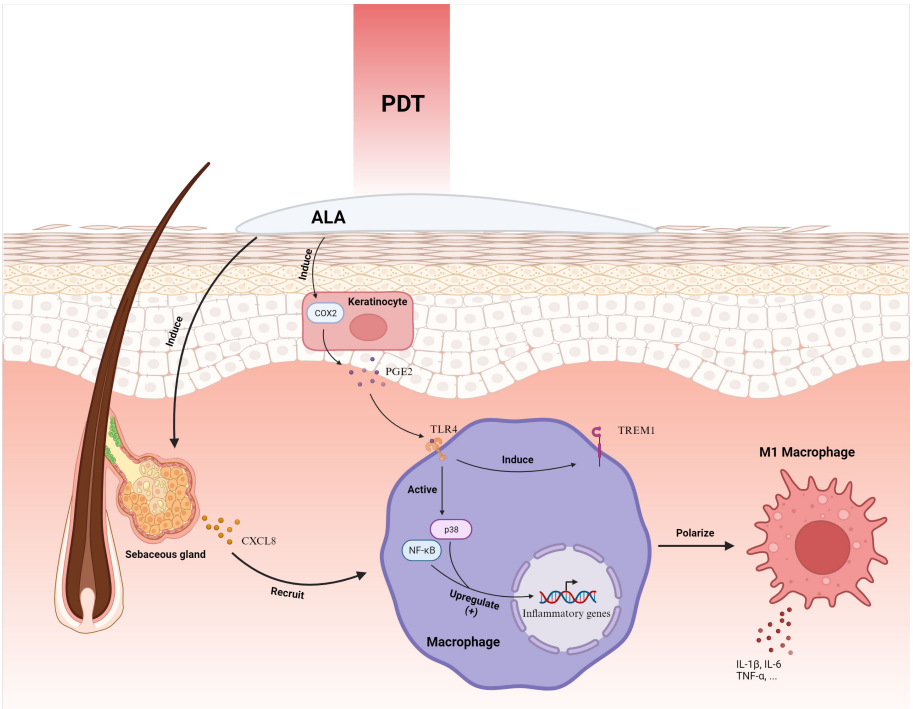

FIGURE 4
ALA-PDT orchestrates the mobilization of macrophages by inciting the secretion of CXCL8 from sebaceous gland cells. Furthermore, it intensifies the expression of COX2 in keratinocytes, culminating in the augmentation of PGE2. This molecule interacts with the TLR4 receptor on macrophages, instigating the activation of p38 and NF-κB pathways and ensuing in the elevation of inflammatory gene expression. Concurrently, it enhances the manifestation of TREM1 receptors, steering the polarization of macrophages toward the M1 phenotype, thereby amplifying the inflammatory response. ALA-PDT, Aminolevulinic Acid Photodynamic Therapy; COX2, Cyclooxygenase-2; CXCL8, Chemokine (C-X-C motif) ligand 8; IL-1β, Interleukin-1 beta; PGE2, Prostaglandin E2; TLR4, Toll-Like Receptor 4; TNF-α, Tumor Necrosis Factor-alpha; TREM1, Triggering Receptor Expressed on Myeloid cells 1. Adapted from "Skin (Layout)" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

5.5.2 Organic compounds

Several organic compounds have unveiled promising therapeutic efficacies for acne through macrophage modulation. A study by Shin et al. depicted the suppressive role of pyrrolidine dithiocarbamate (PDTC) on multiple inflammatory cytokines, achieved by inhibiting NF-κB signaling and NLRP3 activation in macrophages under the influence of *C. acnes* (78). Additionally, investigations by Kim et al. concluded that the benzoxathiolone BOT-64 could deter the synthesis of inflammatory mediators by blocking the NF-κB signaling pathway through the inhibition of IKKβ in macrophages (79). Concurrently, research by Chung et al. demonstrated the capacity of LYR-71, another benzoxathiolone, to inhibit IFN-γ-induced inflammatory responses, accomplished by uncoupling the tyrosine phosphorylation of STAT-1 in macrophages (80).

5.5.3 Additional therapeutic strategies

Non-steroidal anti-inflammatory drugs (NSAIDs) offer significant promise in acne treatment through novel mechanisms. Specifically, a study by Yang et al. substantiated that kinofen has the potential to inhibit NLRP3 inflammasome activation and reduce IL-1β expression, suggesting its applicability in treating acne vulgaris (81). Furthermore, innovative approaches such as microcurrent stimulation (MC) have been proposed by Lee et al., capable of mitigating inflammatory responses by modulating protein expression levels involved in TLR2/NF-κB signaling within macrophages (82). This highlights the potential of MC as a novel acne treatment method. Additionally, the role of probiotics in skin homeostasis and inflammation regulation cannot be overlooked. Wang et al. demonstrated that Bifidobacterium fermentum lysate (BFL) effectively mitigates LPS-induced secretion of IL-8, TNF-α, and COX-2 expression in macrophages (83). Collectively, these studies underscore the diverse and promising therapeutic strategies for acne treatment, ranging from NSAIDs and microcurrent stimulation to probiotics, each contributing uniquely to the understanding and management of acne (Table 2).

6 Concluding remarks and perspectives

Macrophages indeed exemplify a double-edged sword in the pathogenesis of acne vulgaris. They are fundamental for sustaining homeostasis, modulating lipid, and neutralizing pathogens. Nevertheless, the overactivation of macrophages, underscored by the proinflammatory responses to *C. acnes*, can augment the severity of acne symptoms (Figure 5). The intricate mechanisms are being meticulously unraveled, albeit the methodology through which ALA-PDT modulates macrophage polarization and escalates the inflammatory response remains obscure. This ambiguity may be

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TABLE 2 Supplementary approaches for acne vulgaris focused on macrophage involvement.

Category	Chemical	Description	Mechanism of action	Source	References
Antimicrobial peptide	Cath-MH	Antimicrobial peptide from the skin of the frog Microhyla heymonsivogt	Killing bacteria and downregulating TLR expression	Microhyla heymonsivogt	(77)
Organic Compounds	PDTC	Hiol compound	Inhibition of the inflammatory response through suppression of the NF-κB pathway and activation of the NLRP3 inflammasome	Pyrrolidine dithiocarbamate	(78)
Organic Compounds	BOT-64	The novel small-molecule benzoxathiole 6,6-dimethyl-2-(phenylimino)-6,7-dihydro-5H-benzo-[1,3]oxathiol-4-one	Inhibition of inflammatory responses through suppression of the NF-κB pathway	Benzoxathiole derivatives	(79)
Organic Compounds	LYR-71	6-methyl-2-propylimino-6,7-dihydro-5H-benzo[1,3]oxathiol-4-one	Restraining IFN-gamma-induced inflammatory responses through uncoupling the tyrosine phosphorylation of STAT-1	Benzoxathiolone derivatives	(80)
NSAIDs	Auranofin	Anti-Rheumatic Gold Compound	Inhibition of inflammatory responses through suppression of NLRP3 inflammasome activation	/	(81)
Micro-current stimulation	MC	/	Inhibition of inflammatory responses through suppression of the TLR2/NF-κB pathway	/	(82)
Probiotic	BFL	Bifida Ferment Lysate	Reduction in the secretion of IL-8 and TNF-α, as well as downregulation of COX-2 expression	Bifidobacterium	(83)

attributed to the advanced extermination of bacteria and the attenuation of scarring, necessitating more rigorous studies for verification. Given the pivotal role of macrophage polarization in the pathogenesis of myriad skin diseases, the imperative for intensified exploration of its implications in acne vulgaris is underscored (84).

In addressing acne, therapeutic interventions necessitate a comprehensive consideration of immune responses, anti-

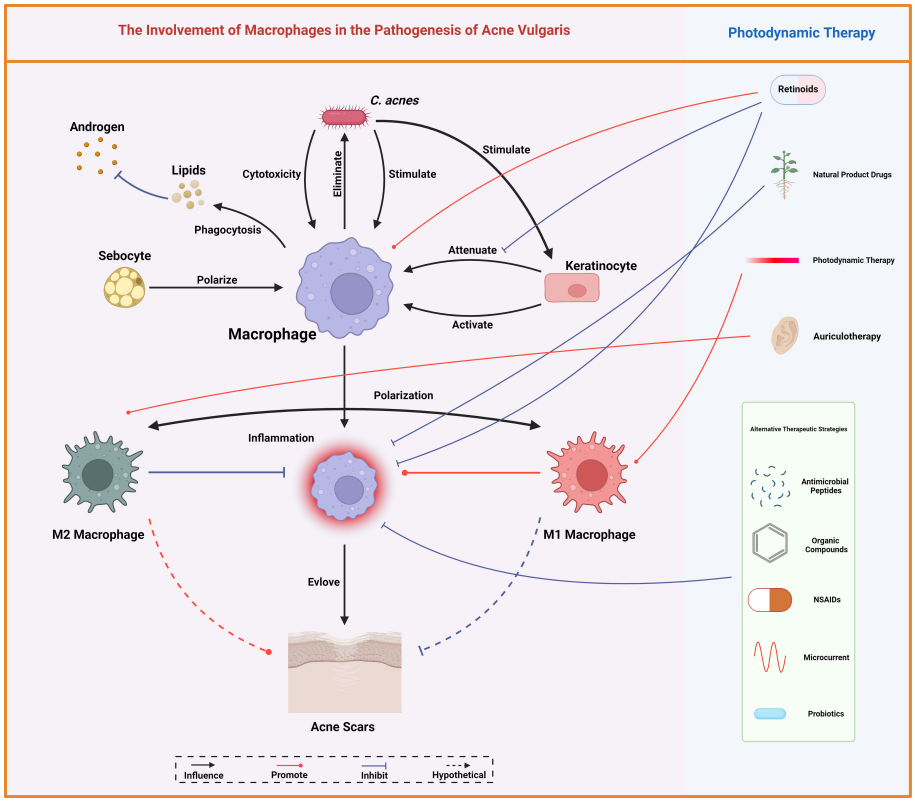

FIGURE 5
Macrophages mediate lipid homeostasis, bacterial eradication, immune modulation, and scar tissue formation, central to acne vulgaris pathobiology. Conventional therapeutic measures for acne vulgaris like retinoids and photodynamic therapy, alongside various other interventions, modulate these macrophage functions, offering therapeutic leverage against acne. Created with BioRender.com.

inflammatory, and antimicrobial approaches. Probing the alterations in macrophage activity in acne vulgaris promises enhanced comprehension of this prevalent dermatological ailment's pathogenesis. Current interventions primarily employ anti-inflammatory strategies, with emerging evidence supporting the feasibility of macrophage modulation as a potential therapeutic avenue. The unveiling of novel therapeutic pathways, such as the employment of statins to inhibit mevalonate, is underway (85). Nevertheless, the pro-inflammatory properties of ALA-PDT pose significant challenges to existing anti-inflammatory paradigms. This brings forth pivotal queries: Should we consider the implementation of M1 macrophage polarization in patients manifesting suitable conditions (86)? Additionally, can ALA-PDT therapy be administered in patients with mild conditions to halt disease progression, serving as an expansion of the existing indications for ALA-PDT (87)? Such inquiries indubitably mandate more extensive and focused research endeavors into the pathogenesis and treatment methodologies of acne vulgaris.

Author contributions

YF: Writing – original draft. JL: Writing – original draft. XM: Writing – review & editing. QJ: Writing – review & editing.

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Conflict of interest

The 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

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Screening mitochondria-related biomarkers in skin and plasma of atopic dermatitis patients by bioinformatics analysis and machine learning

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Background: There is a significant imbalance of mitochondrial activity and oxidative stress (OS) status in patients with atopic dermatitis (AD). This study aims to screen skin and peripheral mitochondria-related biomarkers, providing insights into the underlying mechanisms of mitochondrial dysfunction in AD.

Methods: Public data were obtained from MitoCarta 3.0 and GEO database. We screened mitochondria-related differentially expressed genes (MitoDEGs) using R language and then performed GO and KEGG pathway analysis on MitoDEGs. PPI and machine learning algorithms were also used to select hub MitoDEGs. Meanwhile, the expression of hub MitoDEGs in clinical samples were verified. Using ROC curve analysis, the diagnostic performance of risk model constructed from these hub MitoDEGs was evaluated in the training and validation sets. Further computer-aided algorithm analyses included gene set enrichment analysis (GSEA), immune infiltration and mitochondrial metabolism, centered on these hub MitoDEGs. We also used real-time PCR and Spearman method to evaluate the relationship between plasma circulating cell-free mitochondrial DNA (ccf-mtDNA) levels and disease severity in AD patients.

Results: MitoDEGs in AD were significantly enriched in pathways involved in mitochondrial respiration, mitochondrial metabolism, and mitochondrial membrane transport. Four hub genes (BAX, IDH3A, MRPS6, and GPT2) were selected to take part in the creation of a novel mitochondrial-based risk model for AD prediction. The risk score demonstrated excellent diagnostic performance in both the training cohort (AUC = 1.000) and the validation cohort (AUC = 0.810). Four hub MitoDEGs were also clearly associated with the innate immune cells' infiltration and the molecular modifications of mitochondrial hypermetabolism in AD. We further discovered that AD patients had considerably greater plasma ccf-mtDNA levels than controls (U = 92.0, $p < 0.001$). Besides, there was a significant relationship between the up-regulation of plasma mtDNA and the severity of AD symptoms.

Conclusions: The study highlights BAX, IDH3A, MRPS6 and GPT2 as crucial MitoDEGs and demonstrates their efficiency in identifying AD. Moderate to severe AD is associated with increased markers of mitochondrial damage and cellular stress (ccf=mtDNA). Our study provides data support for the variation in mitochondria-related functional characteristics of AD patients.

KEYWORDS

atopic dermatitis, mitochondria, diagnostic biomarker, mitochondrial metabolism, immune infiltration, circulating cell-free mitochondrial DNA

1 Introduction

Atopic dermatitis (AD) is one of the most prevalent chronic inflammatory skin diseases with a high burden of disease ranking among non-fatal diseases worldwide and is strongly associated with increased risks for asthma, rhinitis, and food allergy (1, 2). Regretfully, the majority of therapies concentrate on relieving symptoms because of our limited understanding of AD pathogenesis, which makes it difficult for early prevention and control of the disease. Along with the progression of AD, the deficiency of antioxidant capacity and the accumulation of large amounts of oxidants together build the oxidative stress (OS) milieu in AD (3–5). Existing studies have shown that the components of the OS milieu are distinct contributors to the epithelial immune microenvironment (EIME) in AD, which may be attributed to the dynamic cross-talk between reactive oxygen species (ROS) and type 2 immune inflammation (3, 6, 7). However, the unequivocal OS pathophysiological mechanism describing the development of AD remains poorly understood.

Mitochondria are particularly rich in the most metabolically active organs such as skin and serve a key role in providing energy and maintaining somatic homeostasis via oxidative phosphorylation (OXPHOS) and the generation of natural by-products of OXPHOS (mitochondria ROS) (8). Mitochondrion is a main source of intracellular ROS (9). Although epidermal differentiation and pigmentation benefit from mitochondrial ROS, perturbations of mitochondrial homeostasis are frequently reported in skin aging and skin cancer (8, 10, 11). In terms of mechanisms, impaired mitochondrial energetics in skin cells would accelerate a surge in mitochondria ROS production and mitochondrial DNA (mtDNA) mutations, thereby triggering a vicious cycle of OS and mtDNA damage (8, 12). It could explain abnormally elevated levels of OS markers (such as 8-OHdG) and an mtDNA variant localized to the MT-ND6 gene in the skin tissue and blood samples of AD patients (3, 13).

It is proposed that mitochondria play an important role in the pathogenesis of AD. Abnormal levels of mitochondrial metabolism and mitochondrial respiration in pro-oxidative situations have been gradually identified as a potential distinguishing characteristic of inflammatory illnesses like AD (5, 14, 15). Topical application of MitoQ, a mitochondrial targeting antioxidant, has been shown to effectively ameliorate AD-like eczema in mice through anti-inflammatory and antioxidant effects (14). It is necessary to uncover novel critical mitochondria-related genes, in light of aberrant mitochondria activity in the course of AD, to better understand the potential mechanism of AD and provide new ideas for molecular diagnosis and therapy for these patients. Through the setup of a large sample database and the development of Biochip, bioinformatic techniques have allowed us to gain an understanding of the components that contribute to disease at the multi-omics level. However, to the best of our knowledge, disease-specific biomarkers that correlate with AD have not been found using bioinformatics analysis of mitochondria-related genes.

In this work, we applied a combination of protein-protein interaction (PPI) network analysis and machine learning techniques to locate hub mitochondria-related differentially expressed genes (MitoDEGs) in the AD skin transcriptome based on the relevant microarray data from the Gene Expression Omnibus (GEO) database. Preliminary investigations were also conducted into potential correlations between hub MitoDEGs and the EIME of AD as well as mitochondrial metabolic function. In addition, it is essential to look for mitochondria-related indicators at the transcriptome level of AD blood to evaluate mitochondrial dysfunction. Circulating cell-free mitochondrial DNA(ccf-mtDNA) refers to “free-floating” non-encapsulated double-stranded DNA fragments that emerge from any cell type in the body (16). Damaged mitochondria often release ccf-mtDNA into the bloodstream, where it functions as a damage-related molecular pattern (DAMP) in intercellular communication and the cellular innate immune inflammatory response (17). Due to its high detectability in human bodily fluids (blood, urine, saliva), ccf-mtDNA has been identified as a promising biomarker for the estimation of related inflammatory diseases. Psoriasis and lupus are two examples of inflammatory skin diseases for which ccf-mtDNA are being actively investigated (16, 18, 19). We therefore chose plasma ccf-mtDNA as a biomarker of systemic mitochondrial damage and investigated the connection between ccf-mtDNA levels and the risk of AD subpopulations to further substantiate the involvement of mitochondrial dysfunction in AD etiology.

2 Materials and methods

2.1 Recruitment of participants and sample collection

57 participants (AD, n=38; healthy control, HC, n=19)were recruited from the Dermatology Department of the Second Affiliated Hospital of Harbin Medical University, Harbin, China. The Chinese criteria (20, 21) was used to diagnose AD participants, and the Eczema Area and Severity Index (EASI) criteria was used to assess the degree of clinical symptoms. Healthy subjects were defined by a lack of history of visible signs of skin damage that is indicative of AD. All participants who had EASI scores less than 8 points or who currently had a medical condition—such as an autoimmune or metabolic disease, malignant tumor, or hematological disease—were excluded. AD patients had not been treated with oral glucocorticoids or other immunosuppressive agents at least 1 month before study evaluation and blood draw. The demographic and clinical information of participants were summarized in Table 1. The study was authorized by the local ethics council (Medical Ethics Committee of the Second Affiliated Hospital of Harbin Medical University) and conformed with the Declaration of Helsinki principles. Following the signing of a written informed consent form by each participant, clinical data and samples were gathered for the study.

Before being sampled, the recruited individuals were told not to use body wash or lotion for 24 hours, nor to use topical treatments for 7 days. In addition, all participants had non-fasting peripheral blood drawn via venipuncture into EDTA tubes, and the blood was processed in four hours. Following extraction using the FICOLL separation method (Haoyang Biological Manufacture, Tianjin, China), supernatants were separated, aliquoted, and kept at -80°C until laboratory examination. The skin lesions, of 6 AD patients and 6 HCs were fixed with formaldehyde for subsequent laboratory examination. As previously mentioned, their peripheral blood mononuclear cells and supernatants were also acquired.

2.2 Dataset collection and preparation

Three AD and one psoriasis gene expression profiles, including RNA sequencing dataset GSE121212, microarray dataset GSE120721, GSE16161, and GSE109248, were taken from the NCBI GEO online public database (http://www.ncbi.nlm.nih.gov/geo/) (22). Supplementary Table S1 provides a summary of the comprehensive baseline data. GSE121212, consisting of 27 AD lesional samples, 27 AD non-lesional samples, and 38 healthy samples, served as the training cohort, while GSE120721, which included 15 AD lesional samples and 22 healthy samples, GSE16161, including 9 AD lesional samples and 9 healthy samples, GSE109248, including 17 psoriasis samples and 14 healthy samples, were employed for external validation. To standardize these datasets, the R v4.3.0 "DESeq2" and "Limma" packages were utilized. All single data that lacked transcript IDs were eliminated. We selected the transcript ID with the average expression when a gene has several transcript IDs. Using the "Batch correction" method, GSE120721 and GSE16161 were combined into an expression matrix after the elimination of batch effects and normalization. For all ensuing downstream analyses, the raw gene expression data were quantile normalized and log2 transformed.

2.3 Identification of MitoDEGs and functional enrichment analysis

Using the R program "DESeq2," DEGs between AD and HC in GSE121212 were assessed. Statistical significance was determined by $|log2FoldChange| > 0.5$ and an adjusted p-value< 0.05. To retrieve human genes with high certainty of mitochondrial localization, the mitochondrial protein database MitoCarta3.0 (http://www.broadinstitute.org/mitocarta) (23) was used. By overlapping DEGs and the genes localized in the mitochondria, MitoDEGs in AD were discovered. After that, up- and down-regulated MitoDEGs underwent pathway enrichment analyses using the R packages "clusterProfiler" and "org.Hs.eg.db" for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), respectively, with a p-value< 0.05 being statistically significant.

2.4 PPI network analysis

The MitoDEGs were prepared for PPI analysis using the STRING database (https://string-db.org/) (24), and Cytoscape

TABLE 1 Baseline demographics of AD and HC individuals.

	HC(n=19)	AD(n=38)	Statistical test	p value
Female sex, n (%)	11 (57.9%)	16 (42.1%)	X = 1.267	0.26
Age (mean (SD))	28.68 (2.76)	26.97 (3.198)	U = 270.5	0.125
Ccf-mtDNA (log, (mean (SEM))	3.42 (0.19)	4.61 (0.10)	t = 6.264	< 0.001
EASI score (mean (SEM))	–	21.96 (1.39)	t = 10.049	< 0.001
IgE (mean (SEM))	–	526.14 (71.45)	t = 5.964	< 0.001
EO% (mean (SEM))	–	5.62% (0.86%)	t = 0.723	0.474

AD, atopic dermatitis; HC, healthy controls; EO, eosinophils.

3.9.1 was used to show the resulting interactions as a network. With Cytoscape 3.9.1, the "MCODE" plug-in was used to further identify the PPI network's essential subnetwork. The primary screening parameters were max. depth = 100, K-core = 2, and node score cutoff = 0.2. Furthermore, using the "cytoHubba" plug-in of Cytoscape 3.9.1, each node gene was scored by 12 algorithms: EcCentricity, Closeness, Radiality, Betweenness, Stress, Clustering Coefficient, Edge Percolated Component (EPC), BottleNeck, Maximal Clique Centrality (MCC), Density of Maximum Neighborhood Component (DMNC), Maximum Neighborhood Component (MNC), and Degree. The key clusters identified from the MCODE plugin and the junction genes from the 12 algorithms of the cytoHubba plugin were merged for the following analysis.

2.5 Identification and validation of hub MitoDEGs

The training set GSE121212's machine learning-based creation process for mitochondria-relate diagnostic markers was taken from an earlier article (25) and was described as follows: (1) To screen for critical variables that could differentiate AD from control situations, the random forest (RF) algorithm (26) was used, with a filter requirement of Mean Decrease Gini (MDG) greater than 0.25. (2) The merging genes found in the PPI network and these RF-screened significant genes intersected, and the resulting genes were chosen as candidate modeling genes. (3) Using the R "glmnet" packages, the least absolute shrinkage and selection operator (LASSO) logistic regression (27) was used to further reduce the range of potential modeling genes. Ultimately, the hub MitoDEGs in AD were chosen using ten-fold cross-validation to determine the optimum λ and the risk score for each sample was computed using the method that follows:

$$\text{risk score} = \sum_{i=1}^{N} \beta i \times Ei$$

where N, β, and E represent the total number of selected marker genes, the coefficient index of each gene calculated by LASSO regression, and the gene expression value of each gene, respectively.

Using the R package "rms," a nomogram model (28) based on the differentially expressed hub MitoDEGs was built to calculate the diagnosis probability of AD patients. The receiver operating characteristic (ROC) curve analysis was used to show the prediction performance of hub MitoDEGs and risk score. The GSE109248 psoriasis dataset and the combined AD dataset (GSE120721 and GSE16161) were utilized as external validation cohorts to assess the model's resilience and diagnostic capacity.

2.6 Gene set enrichment analysis

The important biological pathway changes of hub MitoDEGs in AD were found using GSEA (http://www.broadinstitute.org/gsea) (29). The predefined gene sets were chosen from the KEGG gene set (c2.cp.kegg.v2023.1.Hs.symbols.gmt) by the Java application from the Molecular Signatures Database (MSigDB) (http://software.broadinstitute.org/gsea/msigdb/). Maximum and minimum gene set sizes of 500 and 15 genes, respectively, were used to filter gene sets. Gene sets with p-value< 0.05 were deemed significantly enriched following 100 permutations.

2.7 Correlations between hub MitoDEGs and mitochondrial metabolism in AD

The genes involved in the mitochondrial metabolism were extracted and classified from MitoCarta3.0 database, and the correlations between mitochondrial metabolism and hub MitoDEGs were computed with the Mantel test and the Pearson correlation coefficient in AD non-lesional and AD lesional groups via the R package "ggcor".

2.8 Immune infiltration analysis

Using RNA-seq or microarray data, the CIBERSORT algorithm, a deconvolution approach founded on the linear support vector regression principle, can determine the infiltration abundance of 22 immune cell types in a sample (30). Using the R package's "CIBERSORT" algorithm, the abundance of 22 immune cell types in AD and healthy samples (GSE121212) was determined. Using Spearman's rank correlation, the relationship between each hub MitoDEG and the 22 immune cells was examined and the results were displayed as lollipop charts and heatmaps.

The "GSVA" R package, which is widely used in immune infiltration-related bioinformatics research, employs the ssGSEA algorithm to evaluate the relative infiltration abundance of 28 immune cells in AD and normal skin tissues (31). The "ComplexHeatmap" package in R was used to create heatmaps and clustering analyses that illustrate the correlation.

2.9 Immunohistochemical verification

Extracted fresh skin tissues were fixed with 4% formaldehyde buffer overnight and 5-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 2 min and quenched in 0.3% H_2O_2 for 15 min for endogenous peroxidase activity. Then, sections were treated with immunol staining blocking buffer (Beyotime, Shanghai, China) for 30 min, and incubated overnight at 4°C with primary antibodies against IDH3A(Proteintech, Wuhan, China, dilution 1:200), BAX (Proteintech, Wuhan, China, dilution 1:2000), MRPS6(Bioss, Beijing, China, dilution 1:300), and GPT2(Proteintech, Wuhan, China, dilution 1:300). These sections were treated with HRP-conjugated secondary antibodies (ZSGB-BIO, Beijing, China) for 30 minutes at 37°C and the DAB substrate. Micrographs of the stained sections were captured by light microscopy (Zeiss Imager A2, Germany), and three fields were randomly selected for each skin tissue section. We then used the Image J v1.54f software (NIH,

Wayne Rasband, USA) to measure the integrated optical density (IOD) value and the positive area of each visual field image. The average OD (AOD) of the positive sites (IOD/area) was used to evaluate the relative expression of the target genes.

2.10 DNA isolation and measurement of ccf-mtDNA content in plasma

Thawed plasma at room temperature was followed by centrifugation at 10000×g for 10 min to remove cells and cellular debris. According to the protocol of Serum/Plasma Free DNA Extraction Kit (Tiangen, Beijing, China), we isolated total DNA in plasma with 30 μl of elution buffer and quantified using spectrophotometric analysis at 260/280 nm in NanoPhotometer ® P-Class (Implen, Westlake Village, CA, US). It is essential to have all the different samples under study adjusted to the same concentration (8-12ng/μL).

Quantitative analysis of the ccf-mtDNA content in human plasma by real-time PCR has been reported (32). Briefly, we first amplified MT-ND1 (GGCTATATACAACTACGCAAAGGC, GGTAGATGTGGCGGGTTTTAGG) to obtain purified PCR products from a control individual and calculated the copy number per 1μL of the purified DNA by the following equation (32):

$$\frac{\text{Avogadro's constant}}{330\text{Da} \times 2 \times \text{the size of the PCR fragment (117bp)}} \times \text{Concentration of DNA(ng/μL)}$$

The ccf-mtDNA copy number was reported as copies per microliter (copies/μL). We performed serial dilution of the purified PCR product by calculating copy numbers to create the standard curve. The reaction efficiencies of the standard curves ranged from 90% to 110%, with an ideal $R^2 \approx 1$. The crossing-point values from the testing samples were compared with the standard curve to quantify the DNA concentration.

The PCR reaction was performed using SYBR (SEVEN, Beijing, China) in a 7900HT Fast Real-Time PCR apparatus (Applied Biosystems, USA). Each reaction contained 10ng of template, 0.4μL of each primer(10μM), 5μL SYBR MIX, and 3.2μL of nuclease-free water. PCR program: initial denaturation at 95°C for 30 s, followed by 40 cycles consisting of 95°C in 15 s (melting) and 60°C for 15s (annealing and extension). The program ended with a melting curve analysis measuring fluorescence continuously from 60 to 95°C.

2.11 Statistical analysis

R (version 4.3.0) and IBM® SPSS® Statistics (version 19.0, Chicago, IL, USA) were applied for statistical analysis. The Shapiro-Wilk test ($n \leq 50$) or the Kolmogorov-Smirnov test ($n>50$) was used to check the normality of the data. Categorical variables were compared using the chi-square test. The Mann-Whitney U test or Student's t-test was used to test for differences between AD and HC for continuous variables with non-normal or normal distribution, respectively. Correlations between two variables were evaluated using Spearman's rho or Pearson test. Statistical significance was set at $p<0.05$.

3 Results

3.1 Identification of MitoDEGs and functional enrichment analysis associated with AD

The workflow of this study was shown in Figure 1. We performed DEGs analysis on the GEO dataset GSE121212 by DESeq2, and the results showed that a total of 4773 DEGs were differentially expressed between AD and HC samples on the criteria of $|\log2FoldChange| > 0.5$ and $p < 0.05$. The volcano plot of DEGs between the two groups was shown in Figure 2A, in which, 2459 genes were up-regulated and 2314 genes were down-regulated in the AD group. The heatmap representing the most significant DEGs was shown in Figure 2B. Taking the intersection of these DEGs with 1136 mitochondria-related genes retrieved from the MitoCarta3.0 database, 203 overlapped MitoDEGs (127 up-regulated and 76 down-regulated) were detected in AD skin tissue (Figures 2C, D).

GO and KEGG pathway analyses were performed to explore these MitoDEGs' biological characteristics in more detail. Figures 2E, F displayed the MitoDEGs' most enriched GO keywords, which include biological process, molecular function, and cellular component. The redox reaction, energy metabolism, mitochondrial respiration, and mitochondrial membrane transport were all linked to the up-regulated MitoDEGs for AD. The pathways related to metabolism, thermogenesis, apoptosis and necroptosis, OXPHOS, ribosome, peroxisome, and other processes dominated the most enriched KEGG pathways of the MitoDEGs (Figures 2G, H).

3.2 Identification of hub MitoDEGs from PPI analysis and machine learning

The PPI network of MitoDEGs was analyzed using the STRING database and visualized as a network with the Cytoscape (Figure 3A). The MCODE plug-in of Cytoscape software was utilized to extract significant modules (gene clusters) from the PPI network, resulting in 16 candidate genes (Figure 3B). Meanwhile, 12 candidate genes were segregated from the PPI network using 12 algorithms of plug-in CytoHubba (Figure 3C). A total of 24 genes were obtained after the combination. In addition, based on the above 203 MitoDEGs, 26 genes were further selected as key variables capable of distinguishing AD and HC samples through RF algorithm analysis (MDG ≥ 0.25, $p<0.05$, Figures 3D, E). We eventually acquired 8 candidate hub genes for the final LASSO regression modeling to further narrow the gene number by intersecting the significant genes acquired via RF with the candidate genes discovered in the PPI network (Figure 3F). The

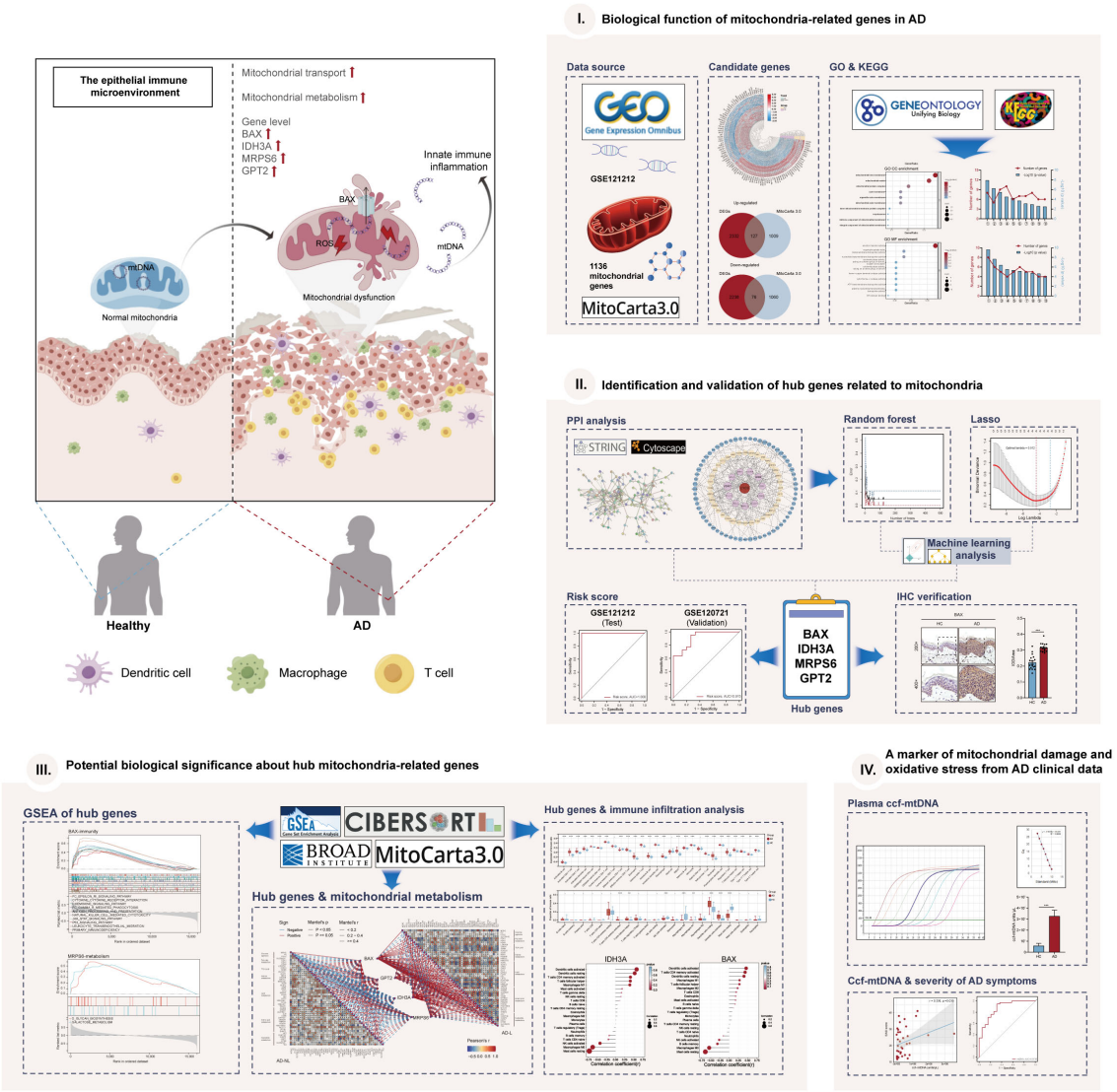

FIGURE 1
The graphical abstract and workflow of this study. By Figdraw.

results showed the lambda values ranged from 0.01027782 to 0.05484964. Plots for LASSO regression coefficients over different values and tenfold cross-validation for the penalty term were shown in Figures 3G, H. The lambda.min was confirmed as 0.01027782 where the optimal lambda resulted in 4 non-zero coefficients. In the end, 4 hub MitoDEGs identified by LASSO regression included BAX, IDH3A, GPT2, and MRPS6 (Table 2).

3.3 Efficiency of hub MitoDEGs screened by LASSO model

The expression levels of these four hub MitoDEGs were considerably higher in both AD lesion and non-lesion samples compared to HC samples (Figure 4A; Supplementary Figure S1A). The algorithm below was used to calculate each patient's risk score based on the expression of the four model genes:

$$\text{Risk score} = 1.246 * \text{IDH3A} + 0.793 * \text{BAX} + 0.746 * \text{MRPS6} + 0.363 * \text{GPT2}$$

We noticed that the group with a high MitoDEGs score was more likely to develop AD than the group with a low score (Figure 4E; Supplementary Figure S1C). ROC curves with AUC values were constructed to evaluate the predictive power of diagnostic risk models for AD. As shown in Figures 4B, E, the AUCs of IDH3A, BAX, MRPS6, GPT2, and risk score for AD diagnosis were, respectively, 0.991, 0.964, 0.986, 0.951, and 1.000, indicating that the efficiency of these 4 hub MitoDEGs and the model were good. Additionally, a nomogram model was created to assess the diagnostic probability of AD using four hub MitoDEGs (Figure 4C). The best prediction performance was nearly matched by the nomogram-predicted AD, as demonstrated by the calibration curve (C-index=1) (Figure 4D). Interestingly, the diagnostic efficacy of these marker genes and the composition

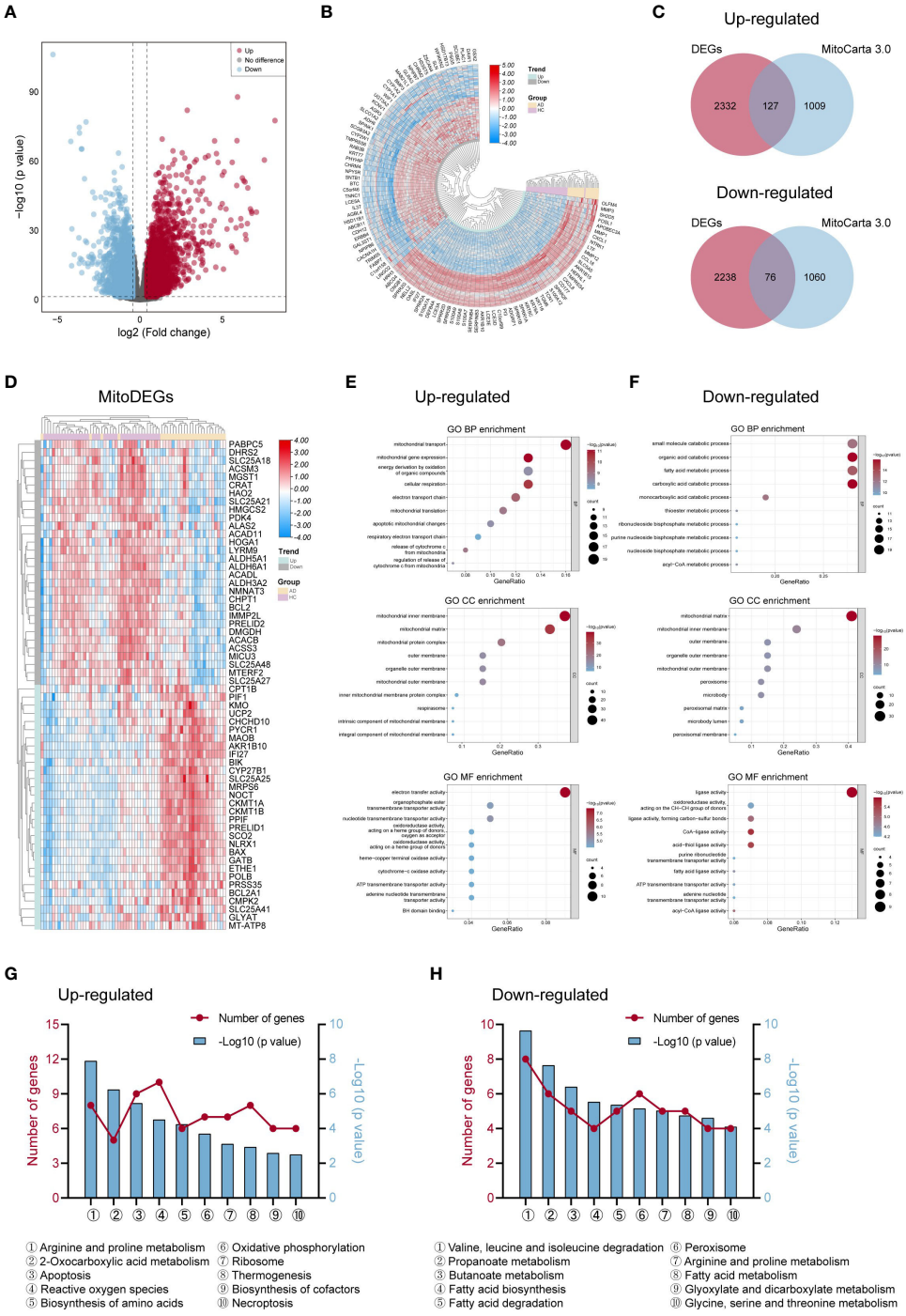

FIGURE 2
Differentially expressed genes linked to mitochondria and their roles in AD. **(A, B)** The volcano map **(A)** and heat map **(B)** of DEGs in AD and control groups. **(C)** Venn diagrams displayed the number of MitoDEGs chosen from MitoCarta 3.0's mitochondria-related genes and up- and down-regulated DEGs. **(D)** The heat map of the expression of the top 60 MitoDEGs. **(E, F)** GO enrichment analysis of up-regulated **(E)** and down-regulated **(F)** MitoDEGs. **(G, H)** KEGG pathway analysis of up-regulated **(G)** and down-regulated **(H)** MitoDEGs. AD, atopic dermatitis; DEG, differentially expressed gene; MitoDEGs, mitochondria-related differentially expressed genes; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes.

model chosen by LASSO regression remained good when the AD group was substituted with non-lesional skin samples (Supplementary Figures S1B-D). To increase the reliability of the result, the marker genes were further validated using the combined AD dataset. The expression and AUC values of the 4 hub MitoDEGs were shown in Supplementary Figures S2A, B. Similar to the findings in GSE121212, the risk score was still higher in the AD group, and the AUC of the probability value was

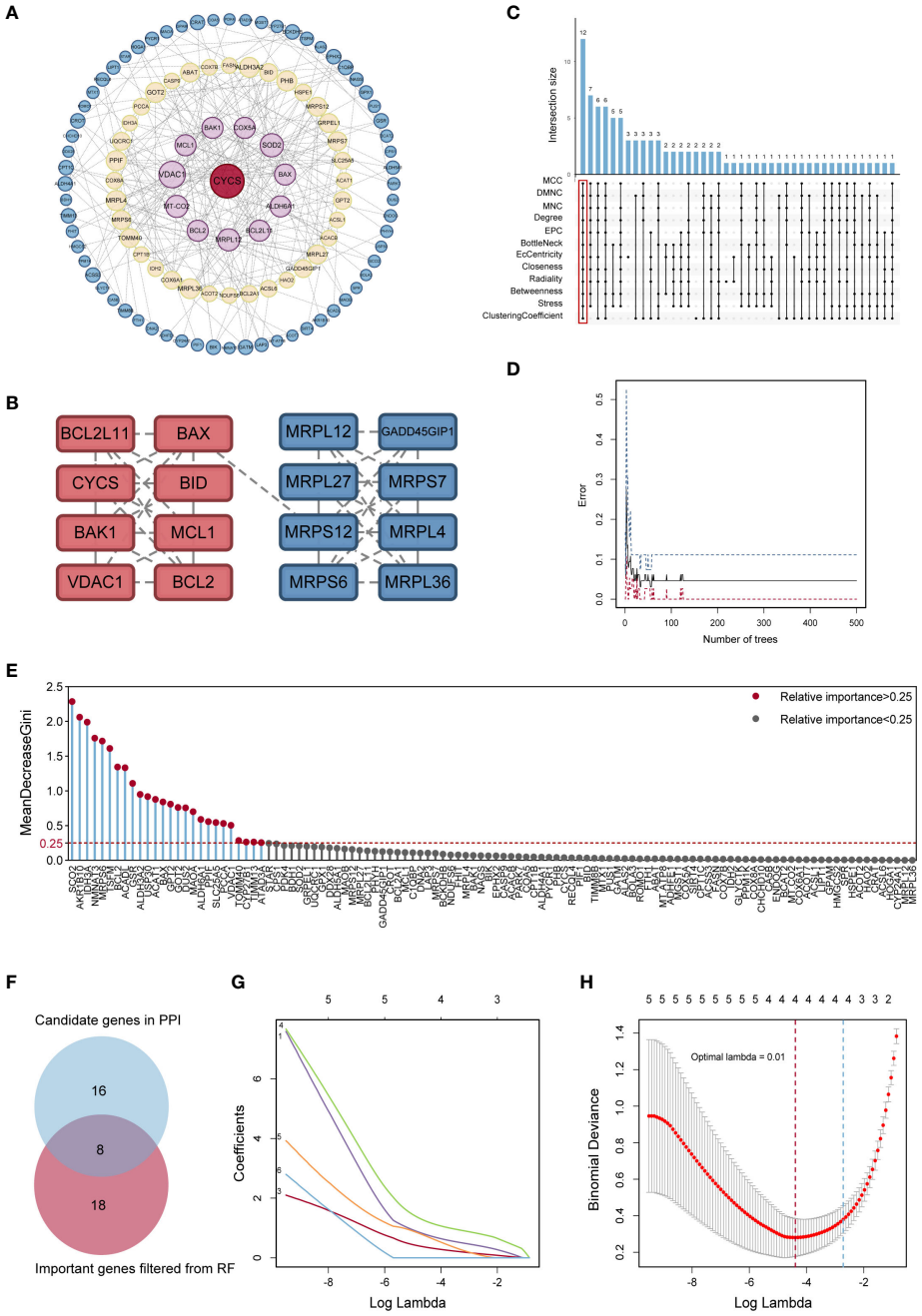

FIGURE 3
Identification of potential hub genes. **(A)** The PPI network of MitoDEGs. **(B)** MCODE highlighted a significant cluster of 16 genes. **(C)** The 12 node genes were intersected by the 12 algorithms of the cytoHubba plugin. **(D)** The model error and the number of RF trees in a correlation plot. **(E)** Relative significance of 26 MitoDEGs determined using RF. **(F)** Venn diagram representing the points where key gene variables from RF pre-screening and PPI analysis overlap. **(G)** Candidate hub MitoDEGs were screened out using LASSO regression. **(H)** Ten-fold cross-validation for tuning parameter selection in the LASSO regression. PPI, protein-protein interaction; RF, random forest; LASSO, least absolute shrinkage and selection operator.

0.810 (Figure 4F). The nomogram calibration curves (C-index=0.909) also displayed obvious concordance between the predicted results and observations in GSE121212 (Supplementary Figure S2C). Additionally, these critical genes were tested using LASSO regression in the validation set for psoriasis to investigate the hub gene expression and the specificity of its model in AD. The distinction was that, in terms of both gene expression and ROC curve, the only element in the psoriasis validation set that matched the trend of the AD dataset was BAX (Supplementary Figures S3A, B). It didn't seem that the risk score produced using the aforementioned formula was appropriate for psoriasis diagnosis (Supplementary Figure S3C).

TABLE 2 The information of 4 hub MitoDEGs.

Gene symbol	Gene ID	Full name	Location	Function of the encoded protein
IDH3A	3419	NAD (+) 3 catalytic subunit alpha	Mitochondria	NAD(+)-dependent isocitrate dehydrogenases are thought to play a major role in the allosterically regulated rate-limiting step of the tricarboxylic acid cycle.
BAX	581	BCL2 associated X	Cytoplasm	BAX is reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome c and mtDNA.
MRPS6	64968	Mitochondrial ribosomal protein S6	Mitochondria	MRPS6 encodes a 28S subunit protein that belongs to the ribosomal protein S6P family. As a protein coding gene, MRPS6 has been implicated in numerous biological functions including mitochondrial translation and metabolism of proteins.
GPT2	84706	Glutamic-pyruvic transaminase 2	Mitochondria	This gene encodes a mitochondrial alanine transaminase, a pyridoxal enzyme that catalyzes the reversible transamination between alanine and 2-oxoglutarate to generate pyruvate and glutamate.

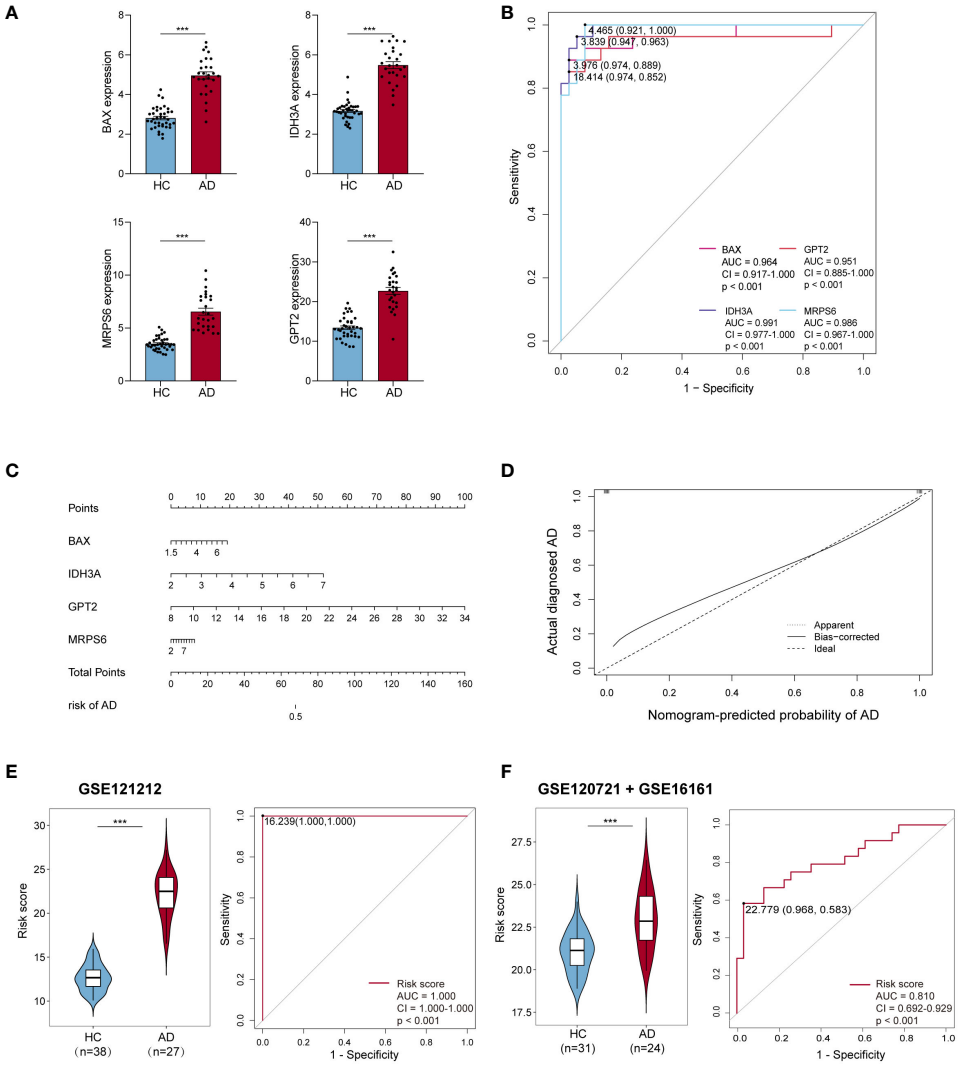

FIGURE 4
Efficiency of hub MitoDEGs. **(A)** Expression of the 4 hub MitoDEGs between AD and HC groups. **(B)** The ROC curve of four hub MitoDEGs for AD diagnosis. **(C)** The nomogram of LASSO regression. **(D)** The calibration curve of the hub genes model. **(E, F)** Distribution of risk scores constructed by four hub MitoDEGs between ADs and controls and ROC analysis of risk scores in GSE121212 **(E)** and GSE120721+GSE16161 **(F)**. Mean ± SEM, ***p<0.001. HC, healthy control; ROC, receiver operating characteristic.

3.4 Biological significance underlying hub MitoDEGs

We applied the GSEA method to obtain a deeper insight into the function of hub MitoDEGs. GSEA showed that overexpression of BAX and MRPS6 were mainly involved in pathways related to glycometabolism and immunity, including N/O glycan biosynthesis, amino sugar and nucleotide sugar metabolism, arachidonic acid metabolism, galactose metabolism, NK cell-mediated cytotoxicity, cytokine-cytokine receptor interaction, chemokine, primary immunodeficiency, antigen processing and presentation, and the JAK-STAT signaling pathway (Figures 5A, B, D, E). The metabolic pathways linked to galactose metabolism, amino acid and amino sugar metabolism, and N glycan biosynthesis were similarly significantly enriched in the pathways that IDH3A changed (Figure 5G). Furthermore, they displayed numerous cellular biology processes related to cell adhesion, apoptosis, and the cytosolic DNA sensing pathway (Figures 5C, F, H). It's noteworthy to note that we discovered a substantial correlation between the signaling pathways related to metabolism and

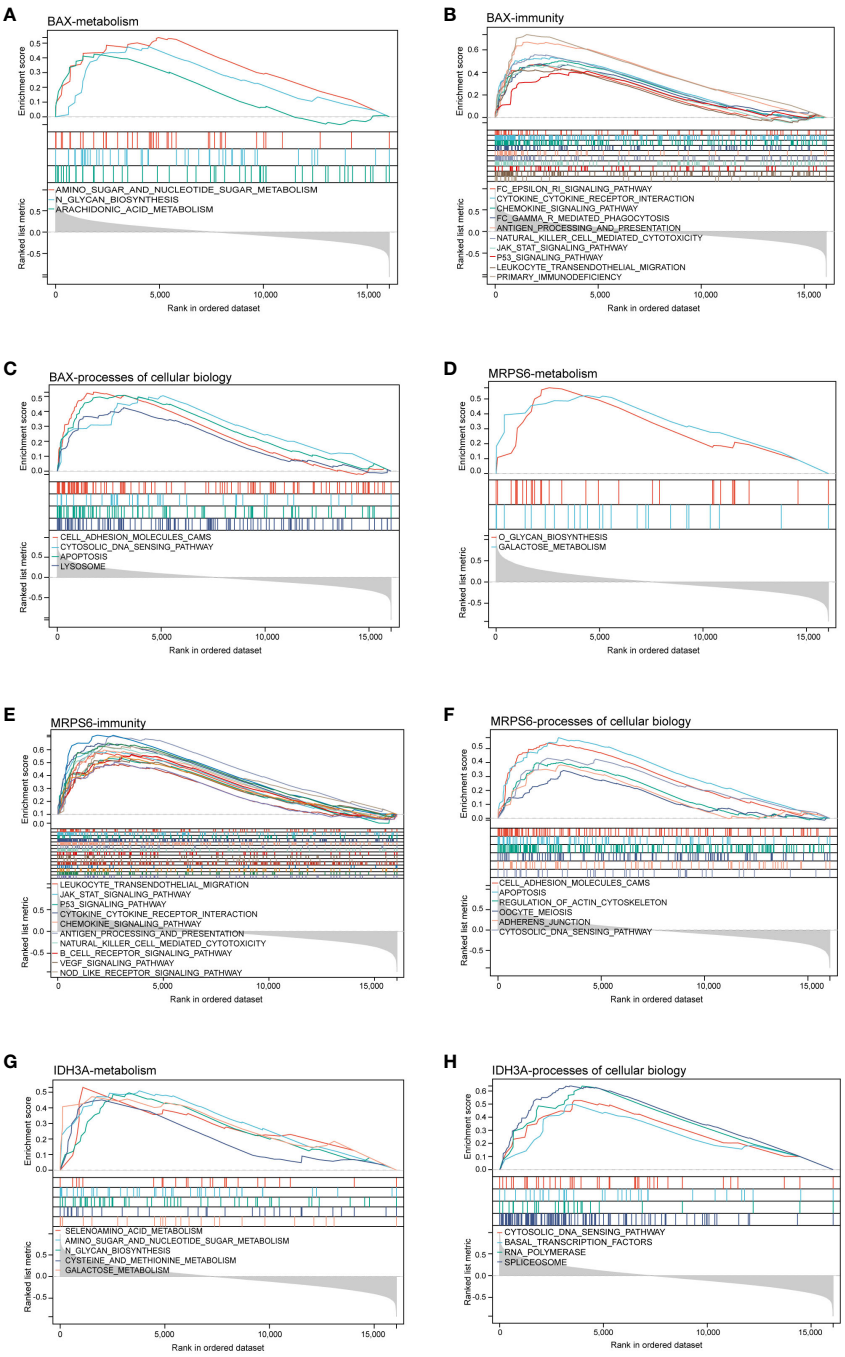

FIGURE 5
GSEA analysis of hub MitoDEGs. **(A–C)** BAX. **(D–F)** MRPS6. **(G, H)** IDH3A. GSEA, gene set enrichment analysis.

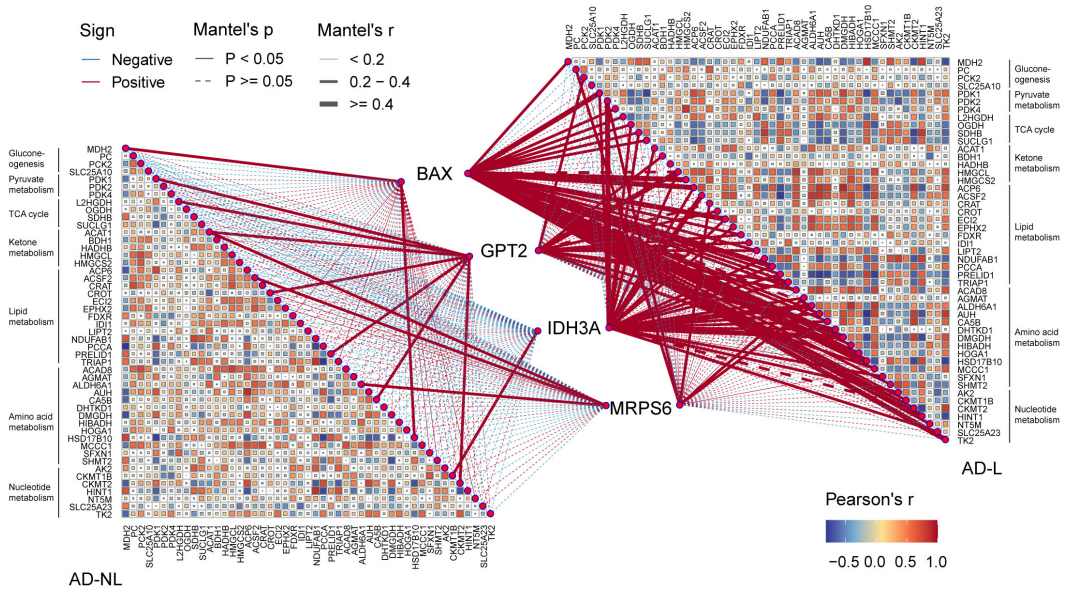

FIGURE 6
Correlation between hub MitoDEGs and mitochondrial metabolism. Color represents Pearson's correlation coefficient r of each hub gene versus mitochondrial metabolism-related genes, with red color indicating a positive correlation (Pearson's r< 0), and blue color indicating a negative correlation (Pearson's r > 0). Statistical analysis was done with the Mantel test, with a full line indicating p value<0.05 and, a dotted line indicating p ≥0.05.

immunity and the three mitochondria-related genes mentioned above.

Thus, we investigated the possible relationship between mitochondrial metabolism and the four hub MitoDEGs. In the AD-lesional group, the hub genes developed a strong positive correlation with the majority of mitochondrial metabolic pathways, including pyruvate/ketone/lipid/amino acid/nucleotide metabolism, TCA cycle, and gluconeogenesis, as Figure 6 illustrates. These findings suggested that the lesional skin tissue of AD may be undergoing biological alterations related to mitochondrial hypermetabolism, which these hub MitoDEGs may partially reflect.

3.5 Immune cell infiltration and the association between hub MitoDEGs and differential immune cells in AD

SsGSEA and CIBERSORT were performed to understand the differences in immunological function better. The majority of immune cell subtypes were shown to be significantly expressed in AD skin tissue by ssGSEA analysis, indicating an environment of excessive immunological activation (Figures 7A, C). Moreover, CIBERSORT analysis revealed significant differences (p< 0.05) in the infiltration of eight immune cell types into skin tissue between the AD and HC groups. In particular, the AD group had significantly more activated memory $CD4^+$ T cells, T follicular helper cells (Tfh), M1 macrophages, and resting/active dendritic cells (DC); in contrast, the HC group had significantly more activated NK cells, M0 macrophages, and resting mast cells (Figures 7B, D). We also further explored the correlation between these four hub MitoDEGs and immune cells (Figure 7E). Figures 7F–I demonstrates that hub MitoDEGs had a negative correlation with resting mast cells, activated NK cells, and M0 macrophages, but a positive correlation with innate and adaptive immune cells like DC, M1 macrophages, activated memory $CD4^+$ T cells, and Tfh cells. These results suggested that the hub MitoDEGs could reflect immune cell infiltration in the skin tissue of AD patients.

3.6 Expression level of the hub MitoDEGs in skin tissue and peripheral blood of AD patients

The AOD values of BAX, GPT2, IDH3A, and MRPS6 in AD skin tissue were considerably higher than those in HC skin tissue (p< 0.001), according to the results of skin tissue IHC between six AD patients and six healthy controls (Figures 8A–D). These outcomes agreed with the GSE121212 dataset's findings in human skin tissues. These four hub genes were mainly located in the cytoplasm and nucleus of epidermal basal cells in HC skin tissues, where they had a light yellow or brown-yellow color. Nevertheless, the positive degree of gene expression progressively diminished or even became negative as one moved from the deep spinous layer to the stratum corneum. These four hub genes were unevenly expressed, with brownish-yellow or brown coloring across the cytoplasm of the entire epidermal layer in the lesions of AD patients. The four hub MitoDEGs with distinct differential AOD values were also analyzed for their associations with EASI scores in the AD group(Supplementary Figure S4). We did not find a significant correlation between EASI scores and BAX (rho = 0.321, p = 0.536), GPT2 (rho = -0.072, p = 0.892), IDH3A(rho =

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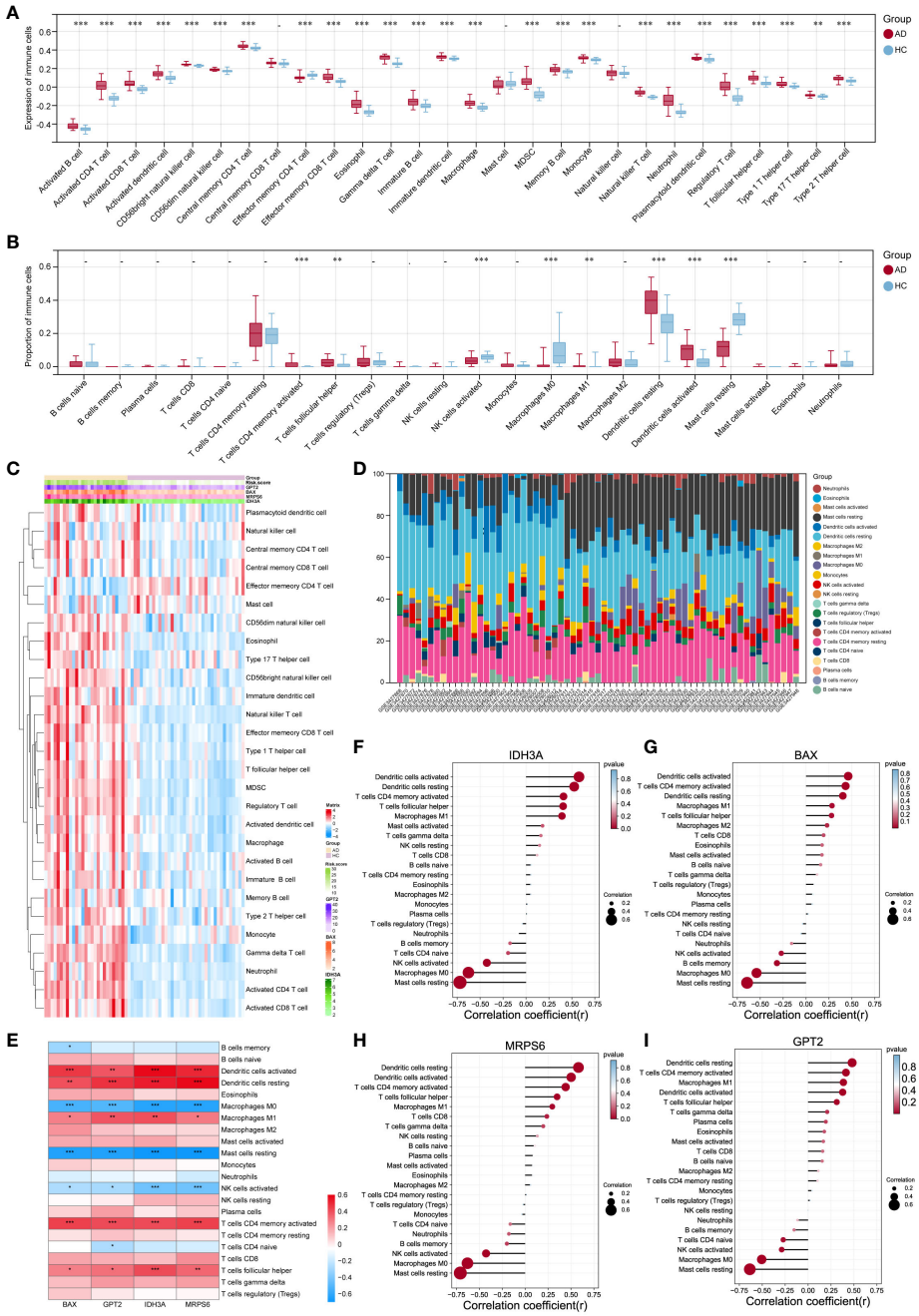

FIGURE 7
Examination of the infiltration of immune cells and the connection between hub genes and distinct immune cells in AD patients. **(A, B)** The violin plots showed the expression **(A)** of immune cells and their percentage **(B)** in the AD and HC tissues. **(C)** Heatmap of the expression of 28 immune cell types. **(D)** Stacked bar chart of the immune cell. **(E-I)** The link between immune cells and hub MitoDEGs in AD was depicted using the heat map **(E)** and lollipop plots, including IDH3A **(F)**, BAX **(G)**, MRPS6 **(H)** and GPT2 **(I)**. Mean ± SEM, *p< 0.05, **p<0.01, ***p<0.001.

0.147, p = 0.780) and MRPS6 (rho = -0.073, p = 0.891). Peripheral expression of 4 hubgenes was also validated in 12 recruiters mentioned above. Their expressions in the AD group tended to rise in comparison to the HC group (Supplementary Figure S5A). For the diagnosis of AD, the risk score obtained from the skin transcriptome and the blood transcriptome demonstrated similar accuracy and stability. The AUC of the probability value and the c-index were both 1.000 (Supplementary Figures S5B-D).

3.7 Correlation between plasma ccf-mtDNA and the severity of AD individuals

We evaluated the plasma ccf-mtDNA concentration in individuals who had moderate-to-severe AD. The findings showed that the peripheral plasma of people with moderate-to-severe AD had higher levels of ccf-mtDNA (455,533 ± 108,599 copies/μL) than did healthy people (57,705 ± 22,844 copies/μL) (U

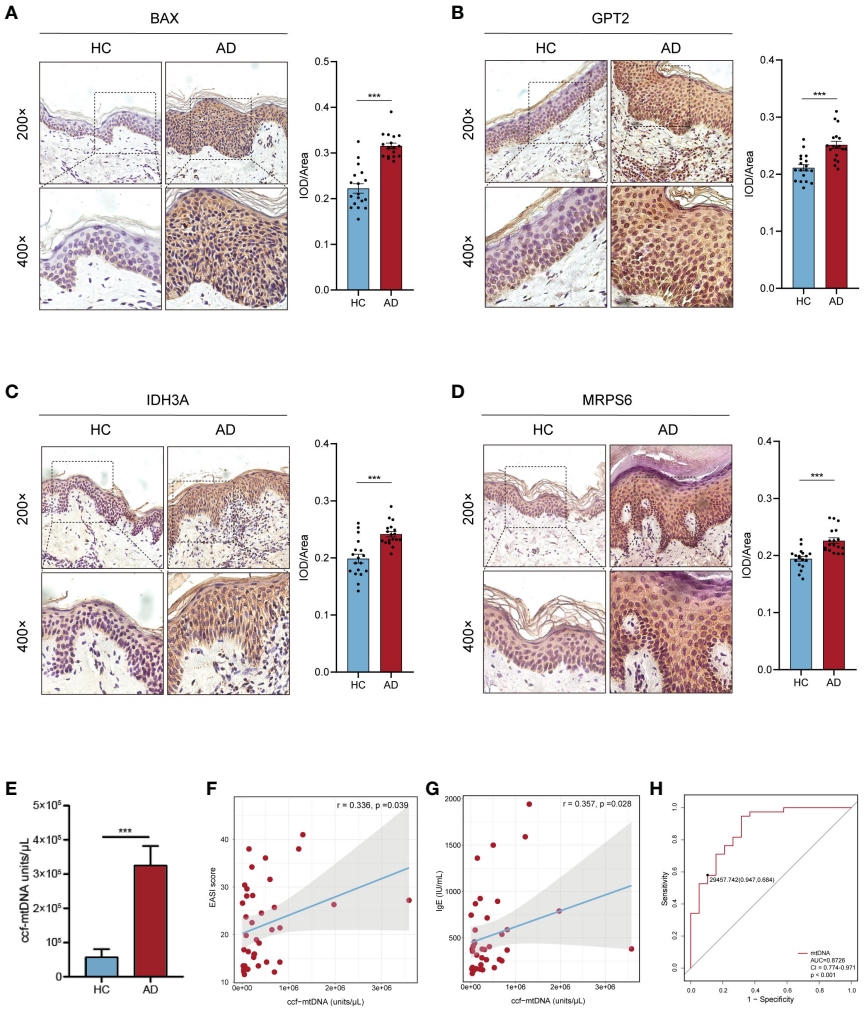

FIGURE 8
Confirmation of hub MitoDEGs expression and the diagnostic value of ccf-mtDNA in moderate to severe AD. **(A-D)** Expression of BAX **(A)**, GPT2 **(B)**, IDH3A **(C)**, and MRPS6 **(D)** in AD tissue and HC tissue. **(E)** Measurement of plasma ccf-mtDNA copy number levels between AD and HC groups. **(F, G)** Correlations between EASI score **(F)**, IgE **(G)**, and plasma ccf-mtDNA in AD patients. **(H)** The ROC curve of plasma ccf-mtDNA for AD diagnosis. Mean ± SEM, ***p<0.001. ccf-mtDNA, circulating cell-free mitochondrial DNA.

TABLE 3 Correlation between ccf-mtDNA and clinical diagnostic indicators in AD individuals.

Parameters	rho	p value
Ccf-mtDNA		
Age	-0.232	0.082
Sex	-0.161	0.231
EASI score	0.336	0.039
IgE	0.357	0.028
EO%	0.185	0.267

AD, atopic dermatitis; Ccf-mtDNA, circulating cell-free mtDNA; EO, eosinophils.

= 92.0, p<0.001) (Figure 8E). We next examined the relationship between the participants' baseline characteristics and ccf-mtDNA in plasma samples (Table 3, Figures 8F, G). The concentration of ccf-mtDNA was found to be positively correlated with EASI scores (rho = 0.336, p = 0.039) and the levels of IgE (rho = 0.357, p = 0.028) when we included only participants with AD. We did not find a significant correlation between ccf-mtDNA concentration and age (rho = -0.232, p = 0.082), sex (rho = -0.161, p = 0.231), EO% (rho = 0.185, p = 0.267) (Table 3). We also did a ROC analysis to evaluate the diagnostic properties of ccf-mtDNA to identify AD cases (Figure 8H). The optimum cut-off point was greater than 29458, with a sensitivity of 94.7% and specificity of 68.4%. The area under the curve was 0.873 ± 0.05, p<0.001. An analysis was conducted on the relationship between the AOD values of the four hubgenes in the corresponding IHC results of six AD patients and the plasma

ccf-mtDNA expression level. In AD patients, there was no link with other genes, but a significant positive association was found between the AOD value of BAX and the ccf-mtDNA copy number (rho = 0.934, p = 0.006) (Supplementary Figure S6).

4 Discussion

Although mitochondria can regulate many other cellular processes in skin cells such as energy metabolism, redox balance, growth/differentiation, and apoptosis, few studies have evaluated the role of mitochondria-related genes in the pathogenesis of AD (8). For the first time, we have comprehensively shown in this work the various pathobiological modifications of mitochondria in the AD environment, including gene expression levels, cellular infiltration, and biological pathways. We also screened out four mitochondria-related biomarkers in AD and checked their validity with machine-learning classifiers. Besides, the increased plasma ccf-mtDNA levels in AD patients indicated its role in the progress of AD. These findings may provide new insights into the AD pathogenesis.

Previous targeted microarray-based studies have demonstrated de-coordinated anti-oxidative response in AD epidermis (14). The fact that some down-regulated antioxidant genes support mitochondrial quality control in multiple ways is noteworthy. They regulate mitochondrial metabolism (mitophagy and oxygen homeostasis) through a series of cascade reactions (33–35), which in turn modulate mitochondrial stress response (reducing mitochondrial ROS clusters) (36), mitochondrial respiratory function (oxygen transport and cytochrome C oxidase maturation) (33, 37), and programmed cell death (ferroptosis) (38). Meanwhile, our results reveal that mitoDEGs participated in OHPHOS and redox reactions in addition to processes linked to mitochondrial membrane transport and small molecule catabolism, including amino acid metabolism, according to GO and KEGG analysis. These results extend our knowledge of mitochondrial activity in AD beyond its well-known role in bioenergetics.

Different from prior screening methods, the combined application of biological analysis and machine learning revealed IDH3A, BAX, MRPS6, and GPT2 as key MitoDEGs in AD. This was achieved in response to the growing scale and complexity of biological databases (39). The diagnostic utility of the potential biomarker genes and the risk model they comprised were also examined. The nomogram model and ROC curve results indicate that these four hub genes have favorable effects on AD diagnosis. In particular, the identification of these four mitochondria-related biomarkers from the blood transcriptome offers a promising avenue for the future development of non-invasive diagnostic methods. Remarkably, the risk model outperformed the individual four-hub MitoDEGs in terms of diagnosis accuracy. A diagnostic model constructed by multi-genes is more comprehensive and effective than a single gene.

We further deduced that there might be anomalies in mitochondrial biological processes in the epidermis of AD lesions, given that the altered protein expression patterns of these four hub genes are primarily seen in the superficial layer of the epidermis. BAX, a key apoptosis regulator that mediates the decisive step of mitochondrial outer membrane permeabilization, is recruited and co-assembled with BAK which is a pro-apoptotic member of the BCL2 family to induce apoptotic pore expansion, mtDNA release, and activation of cGAS/STING signaling pathway (40). According to reports, pro-inflammatory factor release via the STING signaling pathway is induced by tissue-released mtDNA, aggravating atopic processes from the skin to the gut (41). Interestingly, our results highlighted that increased BAX expression in AD promoted ccf-mtDNA accumulation in extracellular fluid (plasma), which may provide strong support for the mitochondria-related hypothesis that BAX mediates pathophysiological changes in AD EIME through a mtDNA-induced proinflammatory mechanism. Taking part in the oxidative decarboxylation of isocitrate into α-ketoglutarate, IDH3A is an essential enzyme that produces ATP in the mitochondrial tricarboxylic acid (TCA) cycle (42). According to past research, AD patients' no-lesion skin has a higher energy metabolism than healthy skin (43). This finding may be related to glutamine's attempt to speed up the TCA cycle turnover rate by reversing the keratinocytes' significantly lower levels of citrate/isocitrate expression in non-lesional areas of AD (15, 44). Without a doubt, OS and mitochondrial ROS overproduction are progressively triggered by the activation of mitochondrial energy metabolism (45). Our findings could support the idea that IDH3A is involved in the pathophysiology of AD by dysregulating mitochondrial activity and isocitrate metabolism. Research on MRPS6 and GPT2 has mostly examined their role in increasing tumor cell proliferation and metastasis (46–49), with little attention paid to their role in AD. Unexpectedly, we found that the up-regulation of hub MitoDEGs was not statistically significantly correlated with the severity of AD individuals. Perhaps these initially explored mitochondrial-related biomarkers do not seem to help assess the severity of AD. Additional research is required, given the insufficient validation sample size.

Mitochondria not only integrate cellular metabolism and physiology, but they are also a major source of immunity (50). Moreover, immune cell metabolism and the activation of associated signaling pathways depend on mitochondria. For example, amino acid metabolism is a key modulator of redox balance in immune cells and supports essential metabolic reprogramming for immune cell activation (51). A crucial TCA cycle enzyme called fumarate causes the cytosol to release mtDNA, which in turn changes the mitochondrial network to activate innate immunity (52). GSEA analysis showed BAX, IDH3A, and MRPS6 were importantly involved in pathways related to metabolism, immunity, and cellular biology. After more research, we discovered that four hub MitoDEGs are strongly associated with the mitochondrial metabolic pathways in AD, which include pyruvate/ketone/lipid/amino acid metabolism, TCA cycle, and gluconeogenesis. All of the findings showed that the pathophysiology of AD and mitochondrial immunity and metabolism interact and overlap. Recently, Thomas et al. reported the single-cell transcriptomics and proteomics results obtained through skin aspiration blisters and highlighted the prominent role of DC and macrophages in maintaining the typical immune microenvironment of AD (53). This is following our findings when examining AD immune cell infiltration using the

CIBERSORT or ssGSEA methods. Due to a study on lesional DC in AD patients, DC polarity expands or activates memory T cells, which in turn maintains the state of inflammation, rather than directly driving differential T-cell subset responses (54). Uncertainty surrounds the involvement of MI/M2 macrophages in AD; the majority of studies have shown an increase in both macrophage subtypes (55, 56). Furthermore, macrophage-produced CCL13 is regarded as a new inflammatory cytokine in the AD EIME (54). In our study, Tfh cells, a subset of $CD4^+$ T cells, were enriched in AD patients over controls. A negative relationship between IL-10^+ Breg cells and Tfh cell differentiation in children with extrinsic AD has been observed (57), which may promote researchers focusing on the function of Tfh cells. Interestingly, there was a close relationship between the above-mentioned immune cells and the hub MitoDEGs, which promoted our understanding of the interaction among mitochondria and innate immune cells in the EIME of AD patients.

That is why we monitor if mtDNA, the bridge connecting mitochondrial OS damage and innate immune inflammatory processes, makes a difference in AD patients. Wang et al. recently reported that children with AD have higher levels of ccf-mtDNA in their peripheral plasma when compared to HCs (41), yet there is a shortage of information on adult AD patients. Our results refine this section and re-emphasize the important role of ccf-mtDNA in AD pathogenesis. When OS-damaged mitochondria are released into the cytoplasm, interstitial space, and circulation, mtDNA can function as a damage-associated molecular pattern that disrupts mitochondrial autophagy (58) and programmed cell death (59), in addition to inciting a cascade of uncontrollable inflammatory reactions (17). These studies provided more context for the rising amount of mt-DNA in adult AD patients' peripheral plasma. Our findings, however, did not support the hypothesis that the patient's age or the level of mt-DNA were related. This finding may have been caused by the limited sample size and the absence of a vertical design.

A few other limitations need to be noted as well. Our study first built the risk model using a tiny sample of public databases, and the verification part was comparatively weak. An independent prospective cohort study with a large sample of clinical data is necessary. Furthermore, while we have detected differences in the expression of hub MitoDEGs and ccf-mtDNA in AD patients, the possible mechanisms of their interaction with the EIME remain unclear. Our next research will concentrate on the recruitment and activation of these four genes, particularly BAX, in the innate immune cells of the AD EIME, as well as their function in controlling mtDNA cytoplasmic escape.

5 Conclusion

To summarize, we established a novel mitochondrial-based molecular signature that takes into account IDH3A, BAX, MRPS6, and GPT2. Our study combined bioinformatics analysis and machine learning to increase our understanding of the crosstalk relationship among these key genes, AD immune infiltration and mitochondrial metabolic function. In addition, we found that plasma ccf-mtDNA may be a key indicator of AD progression, providing evidence of mitochondrial OS damage during the advancement of AD in adult patients with moderate-to-severe AD. Our results may provide a new research trajectory for AD pathogenesis.

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 in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

HY: Writing – original draft, Conceptualization, Data curation, Formal analysis, Writing – review & editing. JL: Conceptualization, Data curation, Writing – original draft. JY: Project administration, Writing – review & editing. XS: Project administration, Writing – review & editing. CW: Formal analysis, Writing – original draft. BB: Funding acquisition, Project administration, Writing – review & editing.

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Conflict of interest

The 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.

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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.2024.1367602/full#supplementary-material

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Impact of fucosyltransferase 1-mediated epidermal blood group antigen H on anti-inflammatory response in atopic dermatitis

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The presence of the blood group H2 antigen on the membrane of red blood cells determines blood type O in individuals and this H2 antigen serves as a precursor to the A and B antigens expressed in blood types A and B, respectively. However, the specific involvement of ABH antigens in skin diseases is unknown. Therefore, we aim to investigate the expression of ABH antigens in skin tissue of patients with atopic dermatitis (AD) and MC903-induced AD-like mice. We demonstrated that the expression of ABH antigen is primarily located in the granular and horny layers of the skin in healthy control individuals. However, in patients with AD, the expression of the ABH antigen was absent or diminished in these layers, while the H2 antigen expression increased in the spinous layers of the affected skin lesions. Then, we investigated the biological function of blood group H antigen mediated by fucosyltransferase 1 (Fut1) in the skin, utilizing an AD mouse model induced by MC903 in wild-type (WT) and *Fut1*-knockout mice. After the application of MC903, Fut1-deficient mice, with no H2 antigen expression on their skin, exhibited more severe clinical signs, increased ear swelling, and elevated serum IgE levels compared with those of WT mice. Additionally, the MC903-induced thickening of both the epidermis and dermis was more pronounced in Fut1-deficient mice than that in WT mice. Furthermore, Fut1-deficient mice showed a significantly higher production of interleukin-4 (IL-4) and IL-6 in skin lesions compared with that of their WT counterparts. The expression of chemokines, particularly *Ccl2* and *Ccl8*, was notably higher in Fut1-deficient mice compared with those of WT mice. The infiltration of $CD4^+$ T cells, eosinophils, and mast cells into the lesional skin was significantly elevated in Fut1-deficient mice compared with that in WT mice. These findings demonstrate the protective role of H2 antigen expression against AD-like inflammation and highlight its potential therapeutic impact on AD through the regulation of blood group antigens.

KEYWORDS

ABH antigens, fucosyltransferase 1, atopic dermatitis, cytokines, chemokines

Introduction

Atopic dermatitis (AD) is a multifaceted and chronic inflammatory skin disease, representing one of the most prevalent skin disorders (1). Current estimates indicate that 10–20% of children and 3% of adults in developed countries are affected by this condition (1–4). AD represents a highly heterogenous clinical and molecular phenotypes (4–6). While the pathogenesis of AD is not fully understood, the disorder is attributed to a combination of genetic and environmental factors, microbial imbalance, immune dysregulation, and abnormalities in the skin barrier (6–9). The amplification of skin inflammation in AD is primarily associated with a T helper-2 (Th2) immune response, characterized by high expression levels of interleukin (IL)-4 and IL-5 (10, 11). The lesional skin of patients with AD exhibited T-cell infiltration, primarily characterized by CD4 expression (1, 12). IL-4 from Th2 cells promotes class switching to immunoglobulin E (IgE) in B cells, leading to the production of antigen-specific IgE (1, 13, 14). Eosinophils and mast cells infiltrate the skin in cases of AD, and their presence was correlated with the severity of the disease (15, 16). Consequently, these immune cells are regarded as potential effector cells due to their production of inflammatory molecules associated with AD (17–19). Moreover, epidermal keratinocytes highly express C-C motif chemokine ligand (CCL) 2 and CCL8 (20, 21) in the skin of patients with AD. CCL2 signaling recruits immune cell infiltration and elicits itch behavior in a mouse model of allergic contact dermatitis (22, 23). CCL8 exhibits chemotactic properties, attracting activated and highly differentiated Th2 cells to the skin during chronic allergic inflammation (24).

The weakening of the skin barrier promotes inflammation and T cell infiltration, subsequently compromising barrier function, intensifying itching, and facilitating microbial dysbiosis, such as colonization with *Staphylococcus aureus* (1, 25, 26). Under inflammatory conditions, keratinocytes produce the epidermal alarmins, such as thymic stromal lymphopoietin (TSLP), leading to tissue damage and driving the recruitment of type 2 inflammatory cells (1, 27–29).

Fucosyltransferase-1 (FUT1) is pivotal in the synthesizing the blood group H2 antigen, a critical determinant for blood type O. This H2 antigen is indispensable for the subsequent formation of A and B antigens, thereby determining blood types A and B, respectively (30, 31). While it is established that ABH antigens determine individual blood types through their expression on the erythrocyte membrane (32), our prior research, along with other studies, have reported that these antigens are predominantly distributed in various tissues. Notably, they are concentrated in regions in direct contact with the external environment, such as the skin, intestines, oral cavity, and liver (33–36). Additional studies have revealed that the expression of FUT1 is restricted to a limited extent within epithelial cells (37–39). The association between the expression of ABH antigens and inflammatory responses, including those induced by UV irradiation, breast and ovarian cancer, respiratory diseases, and pathogen infections, has been explored (33, 39–43). Despite this, the specific involvement of ABH antigens in skin diseases has remained largely elusive. This study sheds light on the anti-inflammatory role of ABH antigens, especially the H2 antigen, within the context of MC903-induced AD-like inflammation in mouse model.

Materials and methods

Antibodies and reagents

Antibodies against A (Z2B-1), B (Z5H-2), H2 (BRIC231), FUT1 (G-13), Filaggrin (N-20), and β-Actin (C4) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). FUT1 antibody (17956-1) was purchased from Proteintech (Chicago, IL, USA). Antibodies against CD4 (4SM95) and F2RL1 were purchased from Invitrogen (Carlsbad, CA, USA). Eosinophil antibody (BMK-13) was purchased from Novus Biologicals (Littleton, CO, USA). Antibodies against Loricrin (Poly19051) and IL-4 (11B11) were purchased from BioLegend (San Diego, CA, USA). Keratin 10 antibody (EP1607IHCY) was purchased from Abcam (Waltham, Massachusetts, USA). Mcpt6 antibody (MAB3736) was purchased from R&D systems (Minneapolis, MN, USA). MC903 was purchased from Tocris (Bristol, UK). Toluidine blue solution was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Patients and specimens

Skin tissue samples included 39 patients diagnosed with AD (27 males and 12 females, age range = 19 to 46 years) and 66 healthy controls (10 males and 56 females, age range = 20 to 81 years). Informed consent was obtained from all participants before their involvement, and the study received ethical approval from the Institutional Review Board (IRB No. C-1312-084-543) of Seoul National University Hospital. The study adhered to the principles outlined in the Declaration of Helsinki. Patients with AD who had concurrent acute illnesses were excluded from the study.

Mice

Fut1-deficient mice (B6.129-Fut1tm1Sdo/J) and wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). They were housed in a pathogen-free barrier facility. Male mice aged six weeks were used in this study, which was approved by the Animal Care and Use Committee (IACUC No. 20-0112-S1A0) of Seoul National University Hospital. The study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemical staining

Briefly, 4 μm sections from the formalin-fixed paraffin-embedded skin specimens of lesional and nonlesional skin from

patients with AD and healthy skin were dewaxed in xylene, rehydrated in graded alcohol, and washed with distilled water. For frozen tissue specimens, the sections were fixed in acetone for 5 min at -20°C. Endogenous peroxidase activity was quenched by a 6-min incubation with a 3% hydrogen peroxide solution. Following a 30-min block with the blocking solution from the SPlink HRP Broad Bulk Kit (Golden Bridge International, Mukilteo, WA, USA), the specimens were incubated with monoclonal primary antibodies against A, B, or H2 antigen in a humidified chamber for 18 h at 4°C. Subsequently, they were incubated with biotinylated secondary antibodies for 15 min and then with streptavidin-horseradish peroxidase conjugate for 15 min at 25°C (Golden Bridge International). Immunostaining signals were developed using 3-amino-9-ethylcarbazole (Golden Bridge International) for 3 to 5 min, depending on the types of antibodies, and the slides were mounted with Paramount medium (DAKO, Carpinteria, CA, USA). Images were captured using a Leica DFC280 camera (Leica, Heerbrugg, Switzerland) coupled with an Olympus BX51 microscope (Olympus, Tokyo, Japan). Semiquantitative evaluation of the images was independently performed by three dermatologists on a scale of 0–4. Standard images, ranging from of grade 0 (no staining) to grade 4 (strongest staining), were selected from all staining images. The grade values of each group were statistically analyzed using the Mann–Whitney U Test for comparison between healthy skin and lesional or nonlesional skin from patients with AD. The Wilcoxon signed-rank test was applied for paired samples of lesional and nonlesional skin from the same patients with AD.

MC903-induced AD-like skin inflammation in mice

In the MC903-induced AD-like model, 1.0 nmol of MC903 in 25 μL ethanol was topically applied on both ears for 12 d (44). The ear thickness was measured daily using a digital caliper (Mitutoyo Corp., Tokyo, Japan). At the end of experiment on day 13, the ear skin was snap-frozen in liquid nitrogen for RNA and protein isolation, and the ear was embedded in Optimal Cutting Temperature compounds (Invitrogen) to prepare frozen sections.

Histology, immunofluorescence analysis, and toluidine blue staining

Skin was fixed in 4% paraformaldehyde at 4°C overnight, paraffin-embedded, sectioned into 4 μm, and stained with hematoxylin and eosin. Images were acquired using a Nikon ECLIPSE Ci-L microscope (Nikon Instruments Inc., USA). Epidermal and dermal thicknesses were measured using ImageJ 1.53e software (NIH). For immunofluorescence, skin sections were blocked in pre-blocking solution (GBI Labs, Bothell, WA, USA) and stained with the indicated primary antibodies at 4°C overnight. After washing, the sections were incubated with an Alexa Fluor 488-conjugated secondary antibody at 25°C for 1 h and stained with 4′-6-diamidino-2-phenylindole dihydrochloride (R37606; Thermo Fisher Scientific, Waltham, MA, USA) at 25°C for 5 min. Images were acquired using a confocal microscope (Leica STED CW; Leica Microsystems, Mannheim, Germany). For the detection of mast cells, ear sections were stained with 0.5% toluidine blue at 25°C for 15 min and then washed three times in PBS. Images were acquired using a Nikon ECLIPSE Ci-L microscope. The number of immune cells was counted every five fields (40× objective), and the average was calculated.

ELISA

The level of *in vivo* cytokine and IgE production in mice was measured using ELISA according to the manufacturer's protocol (BioLegend).

Quantitative PCR

Total RNA was isolated from skin and cells using RNAiso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Subsequently, 1.0 μg of the total RNA was converted to cDNA using a First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Quantitative RT-PCR was performed using AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer, Daejeon, Korea), and an ABI 7500 Real-Time PCR instrument (ABI, Indianapolis, IN, USA) was used to measure relative mRNA expression levels. Each sample was analyzed in duplicate, and the expression levels were normalized to those of the housekeeping gene, *36B4*.

Flow cytometry

Single-cell suspensions from the ear-draining lymph nodes (LNs) were pre-treated with an anti-CD16/32 antibody (BD Biosciences, San Jose, CA, USA) prior to staining with the specified antibodies. Intracellular staining was conducted using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) in accordance with the manufacturer's protocol. Samples were assessed with the BD FACSCanto™ II (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences).

Western blot analysis

Tissue lysates were prepared in 1×RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% NP-40), supplemented with a complete mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA), and a phosphatase inhibitor cocktail (Sigma-Aldrich). Subsequently, total protein extracts were loaded and resolved on a 12% SDS-polyacrylamide gel, then transferred onto a polyvinyl difluoride membrane. After blocking with 5% skim milk in PBS/Tween 20 (0.1%) overnight at 4°C with specified primary antibodies, the membrane underwent PBS/Tween washes. Afterwards, the membrane was incubated with HRP-

conjugated secondary antibodies for 1 h at 25°C and visualized using WestGlow™ PICO PLUS or FEMTO (Biomax Co., Ltd., Seoul, Korea).

Statistical analysis

Statistical analyses were performed using Prism software (version 9.0; GraphPad Software, La Jolla, CA, USA). Dunnett's multiple range test or Student's t-tests were used to compare differences between two groups, whereas comparisons between multiple groups utilized the two-way ANOVA. All graphs are presented as mean ± standard error. A significance threshold of $p < 0.05$ was set for all analyses.

Results

Aberrant expression of ABH antigen was observed in the skin of patients with AD

We have determined that ABH antigen expression was primarily localized in the granular and horny layers for each blood type (A, B, or O) of healthy skin (Figure 1A,

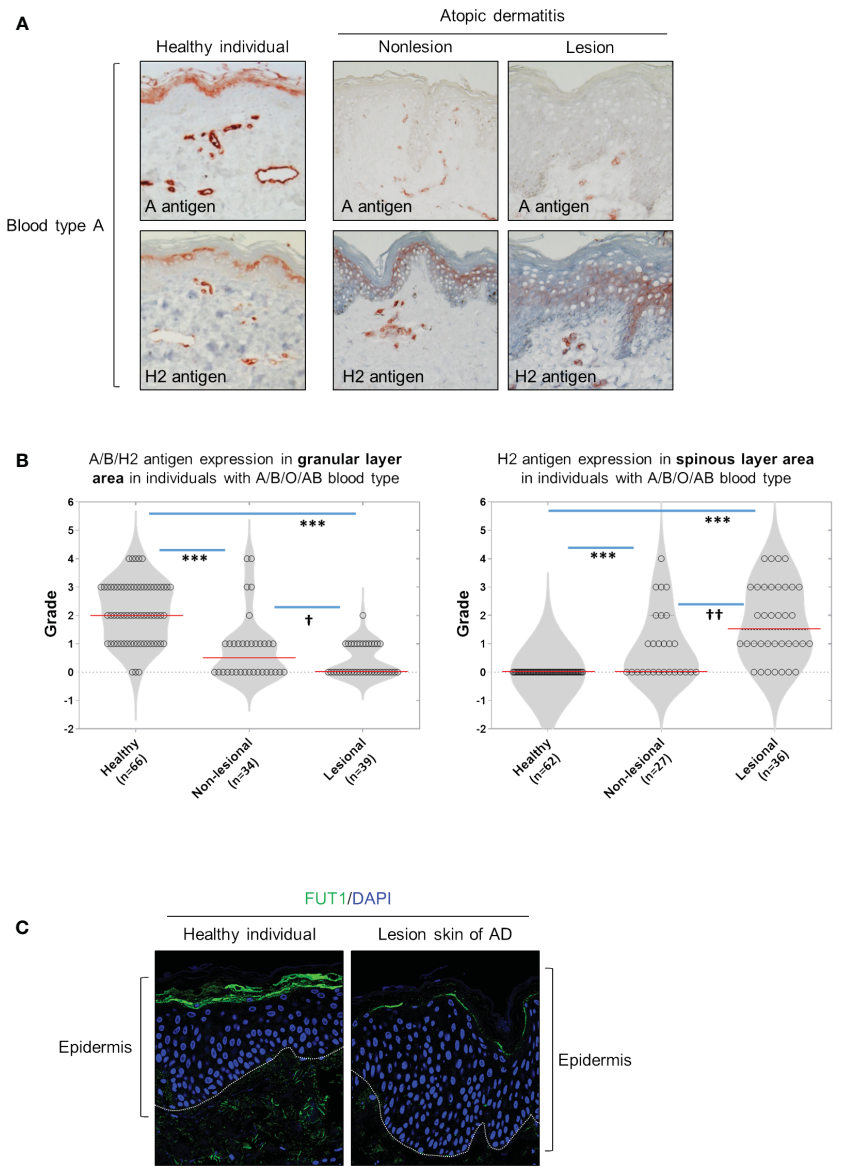

FIGURE 1
The granular layers of healthy skin exhibit ABH antigen and FUT1 expression which is diminished in nonlesional and lesional AD skin. **(A)** Immunohistochemical staining to detect A and H2 antigens in healthy skin, as well as on nonlesion and lesioned AD skin from individuals with blood type A. **(B)** Evaluating the expression of A, B, and H2 antigens in the granular (the left graph) and spinous layers (the right graph) of healthy skin, and comparing these expressions in nonlesion and lesional AD skin among individuals with blood types A, B, O, or AB. **(C)** Immunofluorescent labeling of FUT1 (green) and DAPI (blue) in healthy skin and lesioned AD skin. The data are representative of the mean ± SEM from 39 patients and 66 healthy controls. † < 0.05, †† p < 0.01, *** p < 0.001. AD, atopic dermatitis.

Supplementary Figure S1). However, the expression of ABH antigens was absent or diminished in both the granular and horny layers of nonlesioned and lesioned skin from patients with AD (Figure 1A; Supplementary Figure S1). Moreover, H2 antigen expression abnormally manifested in the spinous layers, exhibiting a randomly dispersed pattern in many cases of patients with AD (Figure 1A; Supplementary Figure S1). The grading of immunostaining intensity revealed a significant decrease in the staining of A, B, and H2 antigens in granular layers in both lesional and nonlesional skin of patients with AD compared with those in healthy skin (Figure 1B). Additionally, the staining of H2 antigen in the spinous layers significantly increased in both lesional and nonlesional skin of patients with AD compared with that in healthy skin (Figure 1B). Notably, these changes were more pronounced in lesional skin than in nonlesional skin (Figure 1B). Furthermore, FUT1 expression was identified in the granular layers of healthy skin, while in the lesional skin of AD, it was either absent or reduced in these layers. Conversely, FUT1 expression exhibited no increase in the spinous layers of the lesional skin of AD (Figure 1C). The data showed a correlation between the patterns of FUT1 expression and H2 antigen expression in the granular layers, while no such correlation was observed in the spinous layers. These findings indicate the potential relationship between ABH antigen expression and inflammation in AD.

FUT1 deficiency enhances AD-like inflammation in a mouse model

Subsequently, we investigated the biological implications of ABH antigen expression in context of AD-like inflammation. To do this, we conducted a comparative analysis of MC903-induced AD-like inflammation in both WT and Fut1-deficient mice. H2 antigen and FUT1 expression were exhibited in the granular layers of control skin of WT mice, whereas they were notably absent in vehicle-treated Fut1-deficient mice (Figures 2A, B). The expression of the H2 antigen decreased in the granular layer, but increased in the spinous layers of WT mice treated with MC903 on day 12, whereas no such expression was observed in *Fut1*-knockout (KO) mice (Figures 2A, B). Additionally, the expression of Fut1 mRNA and protein in the skin of WT mice treated with MC903 decreased notably compared to the vehicle-treated skin of WT mice (Supplementary Figures S2A, B). Conversely, no expression of Fut1 mRNA and protein was detected in the skin of *Fut1*-KO mice treated with either vehicle or MC903 (Supplementary Figures S2A, B).

Upon daily application of 1 nmol MC903 or EtOH (vehicle) on the ears for 12 d, the ear skin of Fut1-deficient mice exhibited greater redness and attained higher clinical scores compared with that of WT mice (Figures 2C, D). From day 6 of MC903 application onward, the caliper measurements indicated a significantly higher ear thickness in Fut1-deficient mice compared with that of WT mice (Figure 2E). Fut1-deficient mice exhibited elevated serum IgE levels compared with that in WT mice following MC903 induction (Figure 2F). The expression levels of keratinocyte differentiation markers, such as Loricrin, Filaggrin, and Keratin 10, showed no significant differences between the epidermal layers of vehicle-treated WT and *Fut1*-KO mice, as well as between MC903-treated WT and *Fut1*-KO mice (Figures 2G–I). This indicates that the diminished expression of the H2 antigen was not caused by abnormal epidermal differentiation. Our findings suggest that FUT1 plays a crucial role in providing protection against AD-like inflammation.

FUT1 deficiency exacerbates both epidermal and dermal thickening and inflammatory cell infiltration following MC903 application

Histological analysis revealed increased inflammatory cell infiltration as well as greater epidermal and dermal thickening in Fut1-deficient mice compared with that in WT mice following MC903 application (Figures 3A–C). Nevertheless, in the control skin, there were no discernible differences in epidermal and dermal thickness between WT and Fut1-deficient mice (Figures 3A–C). To identify the specific immune cell types infiltrating the skin, we conducted staining on ear skin using CD4, Eosinophil marker, and toluidine blue for the detection of $CD4^+$ T cells, eosinophils, and mast cells, respectively. The results revealed elevated numbers of infiltrating $CD4^+$ T cells in the epidermis and dermis, along with increased counts of eosinophils and mast cells in the dermis of Fut1-deficient mice compared with those in WT mice following MC903 application (Figures 3D–I). However, no apparent differences in the numbers of $CD4^+$ T cells, eosinophils, and mast cells were observed in the control skin between WT and Fut1-deficient mice (Figures 3D–I). Nevertheless, the expression levels of mast cell activation markers, F2RL1 and Mcpt6, exhibited variability depending on mast cell morphology in the MC903-treated skin of *Fut1*-KO mice (Supplementary Figures S3A, B). Our findings disclosed that Fut1 deficiency leads to heightened infiltration of inflammatory immune cells, concurrently resulting in increased dermal and epidermal thickening in AD-like inflammation.

FUT1 deficiency amplifies the expression of AD-related cytokines and chemokines following MC903 application

We have discerned differences in the expression of AD-related cytokines and chemokines between WT and Fut1-deficient mice. ELISA results revealed elevated expression of AD-related cytokines, such as IL-4 and IL-6, in the skin of Fut1-deficient mice compared with that in WT mice following MC903 application (Figures 4A, C). Nevertheless, no noticeable differences were observed in the production of IL-5 and TSLP (Figures 4B, D). Quantitative PCR analysis showed a significant increase in the expression of *Ccl2* and *Ccl8* mRNA in the skin of Fut1-deficient mice compared with those in WT mice (Figures 4E, F). Our results conclusively demonstrated that the enhanced expression of chemoattractive factors, specifically CCL2 and CCL8, contributes to immune cell infiltration, subsequently leading to increased expression of IL-4 and IL-6 from immune cells in the skin of Fut1-deficient mice.

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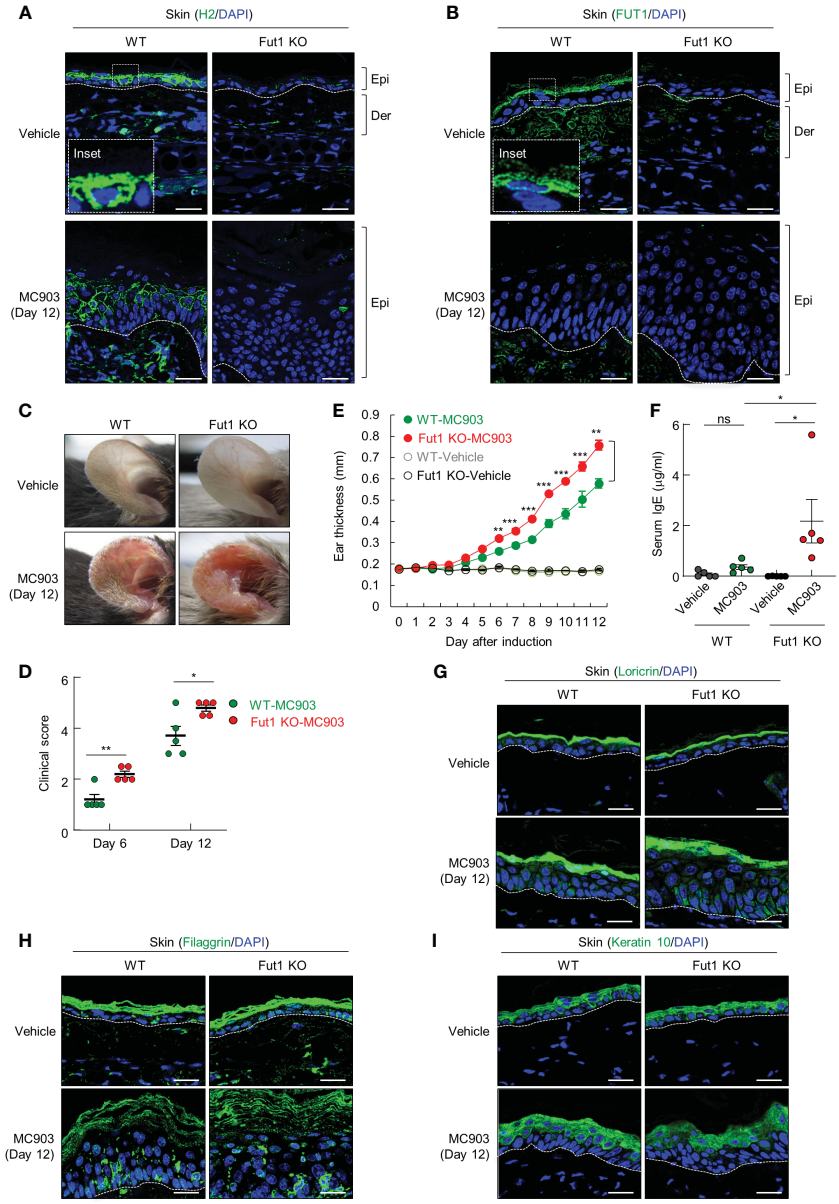

FIGURE 2
The deficiency of FUT1 exacerbates AD-like inflammation induced by MC903 in a murine model. **(A)** Immunofluorescent labeling of H2 antigen (green) and DAPI (blue) in the ears of both WT and *Fut1*-KO mice treated with either vehicle or MC903 on day 12. Scale bars, 20 μm. **(B)** Immunofluorescent labeling of FUT1 (green) and DAPI (blue) in ear sections from both WT and *Fut1*-KO mice. Scale bars, 20 μm. **(C)** Ear skin inflammation induced by MC903 in both WT and *Fut1*-KO mice on day 12. **(D)** Clinical scores of MC903-induced ear skin inflammation in both WT and *Fut1*-KO mice on days 6 and 12. **(E)** Measurement of ear swelling in both WT and *Fut1*-KO mice treated with MC903 using a digital caliper. **(F)** ELISA analysis of serum IgE levels in both WT and *Fut1*-KO mice treated with either vehicle or MC903 on day 12. **(G–I)** Immunofluorescent labeling of Loricrin, Filaggrin, or Keratin 10 (green), and DAPI (blue) in ear sections on day 12. The data are representative of the mean ± SEM of three independent experiments, with five mice per group. ns., not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. AD, atopic dermatitis; WT, wild-type.

FUT1 deficiency enhances the population of CD4$^+$ T cells producing IL-4 in the ear-draining LNs following MC903 application

Finally, we performed FACS analysis to evaluate the population of IL-4 producing CD4$^+$ T cells in the ear-draining LNs of both WT and *Fut1*-KO mice after a 12-d treatment with MC903. The results revealed an elevated ratio and greater numbers of IL-4 producing CD4$^+$ T cells in the ear-draining LNs of *Fut1*-KO mice compared with those in WT mice (Figures 5A, B). This observation correlated with elevated levels of IL-4 production and a greater number of CD4$^+$ T cells in the skin of *Fut1*-KO mice compared with that in WT mice. This suggests an increased proliferation of IL-4 producing CD4$^+$ T cells in the ear-draining LNs.

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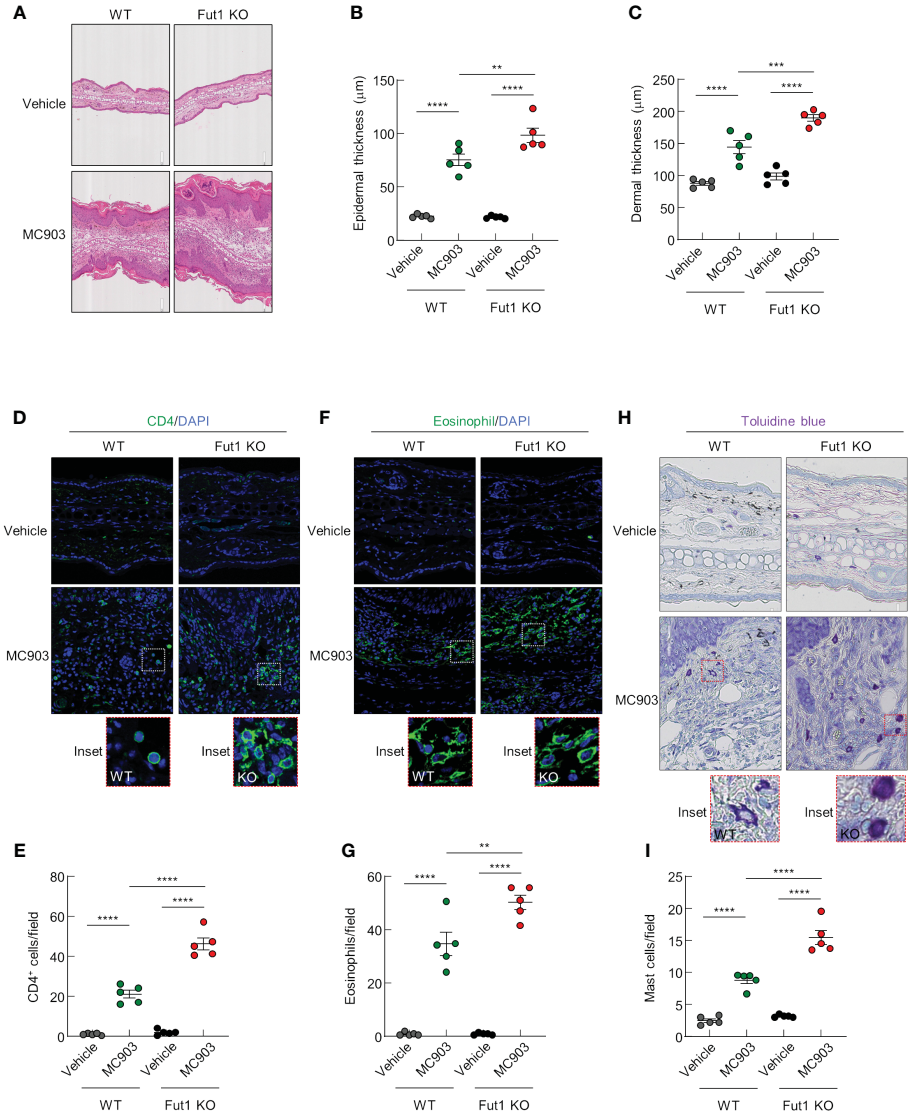

FIGURE 3
FUT1 deficiency amplifies MC903-induced ear thickening and immune cell infiltration. **(A)** H&E staining of the ear in both WT and *Fut1*-KO mice treated with either vehicle or MC903 on day 12. **(B)** Epidermal thicknesses measurements derived from H&E-stained ear sections. **(C)** Dermal thicknesses measurements obtained from H&E-stained ear sections. **(D)** Immunofluorescent labeling of CD4 (green) and DAPI (blue) in ear sections on day 12. **(E)** Quantitation of $CD4^+$ T cells in immunofluorescent-labeled ear sections. **(F)** Immunofluorescent labeling of Eosinophil (green) and DAPI (blue) in ear sections on day 12. **(G)** Quantitation of eosinophils in immunofluorescent-labeled ear sections. **(H)** Toluidine blue staining of mast cells in ear sections. **(I)** Quantitation of mast cells in toluidine blue-stained ear sections. The data are representative of the mean ± SEM of three independent experiments, with five mice per group. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. AD, atopic dermatitis; H&E, hematoxylin and eosin; WT, wild-type.

Discussion

In this study, we elucidated the protective role of H2 antigen expression, induced by FUT1, in the epidermal layers against AD-like inflammation triggered by MC903 treatment in a murine model. Additionally, we demonstrated the diminished expression of ABH antigens in the granular and horny layers of lesional skin in AD compared with that in healthy skin. However, there is an observed increase in H2 antigen expression in spinous layers of lesional skin in AD. This result aligns with our earlier findings, indicating a significant decrease in the expression of ABH antigens in the granular layers of photoaged skin and acutely UV-irradiated skin (33). Notably, there is an elevation in H2 antigen expression in the spinous layers of these skin conditions (33). Our findings suggest a robust link between skin inflammation and the diminished expression of ABH antigens in the epidermal granular layers, accompanied by an elevation of H2 antigens in the spinous layer. This heightened expression of the H2 antigen, a precursor to A and B antigens, in the spinous layers of inflamed skin may represent compensatory mechanisms in response to the reduced expression of ABH antigens in the granular and horny layers. Alternatively, it could be attributed to a decrease in ABO glycosylatransferase expression or activation. Consequently, this may result in the accumulation of H2 antigen without its

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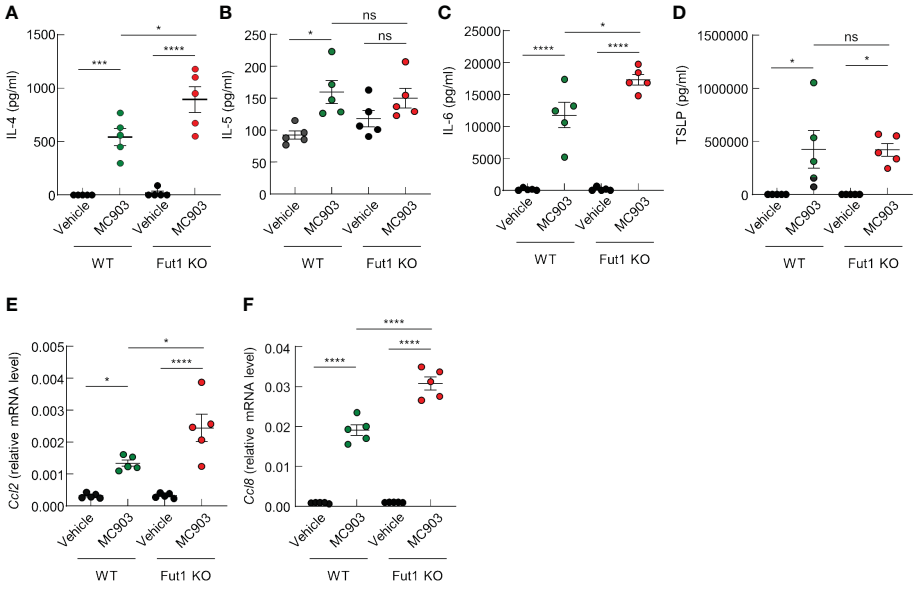

FIGURE 4
FUT1 deficiency enhances the production of AD-associated cytokines in ear skin induced by MC903. **(A–D)** ELISA analysis of IL-4 **(A)**, IL-5 **(B)**, IL-6 **(C)**, and TSLP **(D)** levels in the ear skin of both WT and *Fut1*-KO mice treated with either vehicle or MC903 on day 12. **(E, F)** Relative mRNA expression of *Ccl2* **(E)** and *Ccl8* **(F)** in the ear skin of both WT and *Fut1*-KO mice treated with vehicle or MC903 on day 12. The data are representative of the mean ± SEM of three independent experiments, with five mice per group. ns., not significant; * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$. AD, atopic dermatitis; TSLP, thymic stromal lymphopoietin.

conversion into A or B antigens. Previous investigations showed a decrease in *ABO* gene expression in ovarian tumor tissues and human bladder tumors (41, 45). The ABO polymorphism has been linked to specific infectious diseases, such as *Helicobacter pylori* infection, *Plasmodium falciparum* malaria, and SARS-CoV-2 (46–49). These findings underscore the broader clinical relevance of the ABH antigens, reaching beyond their role in blood transfusion and transplantation. Moreover, these antigens play a crucial role in various clinically significant aspects, including inflammatory responses, cancer, and other diseases (50–53).

Furthermore, our results suggest that the regulation of H2 antigen expression in the skin epidermal layers is predominantly governed by FUT1, as demonstrated using *Fut1*-KO mice. Fut1-deficient mice do not exhibit H2 antigen expression in the granular layers of control skin. FUT1 expression was also observed in the granular layers of healthy human and mouse skin. However, FUT1 expression was notably absent in the spinous layers of AD and MC903-treated mice skin, despite an elevated expression of the H2 antigen in these layers. This observation could be a potential short half-life for FUT1 expression in the spinous layers following H2

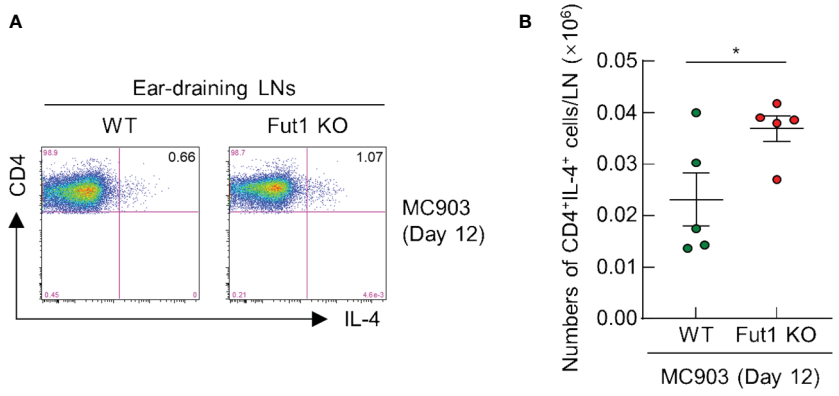

FIGURE 5
FUT1 deficiency heightens the number of IL-4-producing $CD4^+$ T cells in the ear-draining LN following MC903 treatment. **(A)** FACS analysis of IL-4-producing $CD4^+$ T cells in in the ear-draining LN after 12 d of MC903 treatment. **(B)** Quantification of IL-4-producing $CD4^+$ T cells in the ear-draining LN on day 12. The data are representative of the mean ± SEM of two independent experiments, with five mice per group. * $p < 0.05$. LN, lymph node.

antigen induction. Further elucidation is needed to clarify this aspect. In addition, our confirmation that MC903 treatment results in decreased levels of Fut1 mRNA and protein in the skin suggests direct involvement of MC903 in downregulating the H2 antigen expression. This cumulative evidence suggests that the control of H2 expression in the skin is predominantly controlled by FUT1. Importantly, this downregulation does not result from abnormal epidermal differentiation, as evidenced by the expression of epidermal differentiation markers, including loricrin, filaggrin, and keratin 10, within the granular and horny layers of both vehicle and MC903-treated WT and *Fut1*-KO mice. This implies that the absence of H2 antigen expression in the granular and horny layers of MC903-treated skin does not stem from impaired terminal differentiation after MC903 treatment.

Under physiological conditions, the H1 antigen in the intestine serves a protective role by maintaining a balance of commensal bacteria (54, 55). Loss-of-function mutations in FUT2 result in an altered gut microbiome, contributing to the development of inflammatory bowel diseases (55, 56). Nevertheless, amid the inflammatory response, the function of the H1 antigen in the intestine and lung remains controversial, suggesting its potential involvement in either mitigating or exacerbating inflammation (57–62). Our findings indicate that H2 antigens contribute to a protective role against AD-like inflammation triggered by MC903 in the skin. In the absence of H2 antigens in *Fut1*-KO mice, MC903-induced ear swelling, as well as epidermal and dermal thickening, exceeded that observed in WT mice. Corresponding with the heightened dermal thickening, the infiltration of immune cells, including $CD4^+$ T cells, eosinophils, and mast cells into the dermis, was more pronounced in *Fut1*-KO mice compared with those in WT mice.

The topical application of MC903 on the skin swiftly initiates TSLP production, considered as an initial trigger for Th2 inflammation, in keratinocytes, beginning on day 2 after the initial application (44). Nevertheless, our study revealed no apparent distinction in TSLP production between the skin of WT and *Fut1*-KO mice on day 12, a relatively late time point. This observation could be attributed to the substantial accumulation of TSLP production over the 12-day period with daily application of MC903. IL-4 is primarily produced by Th2 cells and, as non-T cells, by eosinophils (63, 64). The production of IL-4, occurring in the late response following MC903 application, was more pronounced in *Fut1*-KO mice compared with that in WT mice. This correlated with a higher number of infiltrating $CD4^+$ T cells and eosinophils in the skin of *Fut1*-KO mice compared with those in WT mice. IL-4 stimulates the expansion and activation of mast cells, as well as induces B cells to produce IgE (65–70). This aligns with our findings, which demonstrate higher IgE production and a greater number of mast cells in *Fut1*-KO mice compared with that in WT mice. A study has elucidated that mast cells in MC903-induced AD-like skin are sustained through both the local proliferation of tissue-resident mast cells and the infiltration/differentiation of bone marrow-derived mast cell progenitors. This finding provides a novel perspective on mast cell heterogeneity in allergic conditions (71). Such heterogeneity may contribute to the variability observed in mast cell morphology and the different expression levels of mast cell activation markers (F2RL1 and Mcpt6) in MC903-treated skin in our study, despite the ongoing ambiguity surrounding the functional distinctions between these cell types.

A prior study demonstrated elevated IL-6 production by T cells derived from patients with AD (72). Additionally, IL-6 is generated by various cell types, including dendritic cells, mast cells, eosinophils, and keratinocytes (73–76). After the application of MC903 in *Fut1*-KO mice, there was a significantly elevated production of IL-6 in the skin, along with increased infiltration of mast cells and eosinophils compared to WT mice. IL-5 is recognized as the most crucial cytokine in eosinophil maturation within the bone marrow and their subsequent release into the blood (77–79). In humans, IL-5 affects eosinophils and basophils, influencing their growth, activation, and survival (80, 81). However, our findings indicate that IL-5 production did not exhibit a significant difference between the skin of WT and *Fut1*-KO mice. This implies that in mice, alternative chemokines may exert a substantial influence on eosinophil recruitment into the skin, or it could be essential to explore different time points to discern any divergence in IL-5 production. Furthermore, our findings indicate that the expression of chemokines, CCL2 and CCL8, was notably higher in the skin of *Fut1*-KO mice compared with those in WT mice on day 12 after MC903 application. CCL8 has the potential to regulate the recruitment of immune cells in various tissues during inflammatory diseases (82). Mouse CCL8 is constitutively expressed in the skin and may contribute to chronic eosinophilic inflammation by inducing the accumulation of $CD4^+$ Th2 cells in a mouse model of chronic AD (24). CCL2 secretion in the skin plays a role in recruiting inflammatory monocytes, dendritic cells, and memory T cells (22, 83–85). Mice deficient in CCL2 exhibited diminished recruitment of monocytes and eosinophils in an irritant contact dermatitis model (86). CCL2 induces DC maturation and migration from the skin to draining lymph nodes, facilitating antigen presentation to T cells (87). The data strongly support our findings, indicating that heightened production of CCL2 and CCL8 is likely to contribute to the augmented recruitment of immune cells into the skin of *Fut1*-KO mice. Additionally, our results reveal an increased presence of IL-4-producing $CD4^+$ T cells in the ear-draining LNs of *Fut1*-KO mice compared with that in WT mice.

We investigated the synthesis of the H2 antigen by Fut1 and its expression patterns within healthy human and murine skin, particularly within the granular cells and stratum corneum. Notably, these patterns differed in skin lesions in patients with AD and in MC903-treated mice. In these pathological contexts, both H2 antigen levels and FUT1 expression within the granular layers and stratum corneum were markedly decreased but were significantly increased in the spinous layers of lesional skin. Based on these findings, complete loss of H2 antigen expression in patients with AD may exacerbate inflammation associated with this condition. Although inflammation typically correlates with reduced H2 antigen expression in the granular cells and stratum corneum of healthy skin, compensatory upregulation of H2 antigen expression throughout the spinous layers in lesional skin suggests a mechanism for counteracting this loss. Further studies are required to fully understand the underlying mechanisms.

The importance of Fut1 or the H2 antigen in biological processes may vary depending on the context. Both Fut1-mediated terminal fucosylation and the resultant H2 antigen may contribute distinct but interconnected roles in cellular interactions and signaling pathways. The presence or absence of FUT1 can influence interactions between cells and molecules in the immune system, as well as cell adhesion and signaling (88–91). The absence of functional FUT1 enzyme activity leads to the Bombay phenotype, in which individuals lack H2 antigen expression; this absence can have important biological consequences (92, 93). Similarly, the presence of the H2 antigen and its modifications may contribute to various physiological processes (94, 95).

In summary, our study reveals that ABH antigens are expressed in the granular and honey layers of healthy human and mice skin. However, in the context of AD and MC903-treated mice skin, there is a significant reduction in the expression of these antigens within these layers. Notably, our findings indicate that the H2 antigen plays a role in a protective capacity against AD-like inflammation, leading to reductions in clinical scores, skin thickening, and the production of AD-related cytokines. While our results underscore the potential therapeutic significance of the H2 antigen in AD by regulating blood group antigens, further investigation is essential to elucidate the specific mechanisms through which the H2 antigen contributes to its protective role in AD inflammation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by The Institutional Review Board (IRB No. C-1312-084-543) of Seoul National University Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by The Animal Care and Use Committee (IACUC No. 20-0112-S1A0) of Seoul National University Hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. NL: Data curation, Investigation, Writing – review & editing. J-HO: Writing – review & editing, Funding acquisition, Investigation. JS: Resources, Writing – review & editing. S-PJ: Data curation, Writing – review & editing. DL: Conceptualization, Funding acquisition, Writing – review & editing. JC: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The 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/fimmu.2024.1365430/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The granular layers of healthy skin exhibit ABH antigen expression which is diminished in nonlesional and lesional AD skin. Immunohistochemical staining to detect B and H antigens in healthy skin, as well as on nonlesion and lesioned AD skin from individuals with blood types B or O. AD, atopic dermatitis.

SUPPLEMENTARY FIGURE 2
Reduced expression levels of Fut1 mRNA and protein in the skin of WT mice treated with MC903. **(A)** Relative mRNA expression of *Fut1* in the ear skin of both WT and *Fut1*-KO mice treated with vehicle or MC903 on day 12. **(B)** The protein expression of Fut1 and β-Actin in the ear skin of both WT and *Fut1*-KO mice treated with vehicle or MC903 on day 12. The data represent the mean ± SEM of three independent experiments, with five mice per group. ns., not significant; **** $p < 0.0001$.

SUPPLEMENTARY FIGURE 3
Expression of mast cell activation markers in the skin. **(A)** Immunofluorescent labeling of F2RL1 (green) and DAPI (blue) in ear sections on day 12. **(B)** Immunofluorescent labeling of Mcpt6 (green) and DAPI (blue) in ear sections on day 12.

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