



# Perforins Expression by Cutaneous Gamma Delta T Cells

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Gamma delta (GD) T cells are an unconventional T cell type present in both the epidermis and the dermis of human skin. They are critical to regulating skin inflammation, wound healing, and anti-microbial defense. Similar to CD8+ cytotoxic T cells expressing an alpha beta (AB) TCR, GD T cells have cytolytic capabilities. They play an important role in elimination of cutaneous tumors and virally infected cells and have also been implicated in pathogenicity of several autoimmune diseases. T cell cytotoxicity is associated with the expression of the pore forming protein Perforin. Perforin is an innate immune protein containing a membrane attack complex perforin-like (MACPF) domain and functions by forming pores in the membranes of target cells, which allow granzymes and reactive oxygen species to enter the cells and destroy them. Perforin-2, encoded by the gene *MPEG1*, is a newly discovered member of this protein family that is critical for clearance of intracellular bacteria. Cutaneous GD T cells express both Perforin and Perforin-2, but many questions remain regarding the role that these proteins play in GD T cell mediated cytotoxicity against tumors and bacterial pathogens. Here, we review what is known about Perforin expression by skin GD T cells and the mechanisms that contribute to Perforin activation.

**Keywords:** pore forming proteins, perforin, perforin-2, mpeg-1, gamma delta T cells, skin, cytotoxicity

## INTRODUCTION

Gamma delta (GD) T cells are an unconventional T cell type that constitutes about 1–5% of circulating lymphocytes (1, 2). Despite low numbers in circulation, GD T cells are enriched in barrier tissues including the skin, gut, and reproductive tract (3–8). The skin is an epithelial tissue that serves as a barrier to protect against physical and chemical insults as well as potentially pathogenic microorganisms. It is composed of two main layers, the epidermis and dermis, and GD T cells are present in both layers in both mice and humans (2, 9). Epidermal GD T cells in mice are referred to as dendritic epidermal T cells (DETCs) because of the dendritic processes they use to survey surrounding keratinocytes for signs of stress or damage (10, 11). These cells all express an identical invariant TCR and are potent producers of IFN- $\gamma$  (12–14). Dermal GD T cells on the other hand are not dendritic and they produce IL-17 (5, 6). Human GD T cells, however, exist primarily in the dermis (15, 16). Small numbers are observed in the epidermis in steady state, but unlike mouse DETCs, they do not exhibit dendritic processes (17). Human GD T cells also express an invariant TCR. Skin resident cells express a delta 1 TCR, while circulating GD T cells express a delta 2 TCR (18–20).

In recent years, researchers have begun to elucidate the vast roles that GD T cells play in skin inflammation and antimicrobial defense. GD T cells serve as bridges between the innate and adaptive immune system. Although they express a recombinant TCR as alpha beta (AB) T cells do, they can bind to and recognize antigens directly without processing and presentation by MHC molecules (21). They can also respond to antigens without the need for pre-stimulation (6, 22). These innate-like characteristics position GD T cells to respond quickly to signs of stress caused by damage or infection. GD T cells play an important role in the response to pathogens in the skin. They produce IL-17 and IL-22 upon stimulation with IL-23, which triggers antimicrobial peptide production by keratinocytes as well as neutrophil recruitment to the site of infection (5, 9, 23). GD T cells also contribute to wound healing, particularly through their production of fibroblast growth factor-7 (FGF-7) and insulin-like growth factor-1 (IGF-1), which promote keratinocyte proliferation and reepithelialization (24, 25), as well as through their production of IL-17 (26). They also secrete fibroblast growth factor-9 (FGF-9), which triggers Wnt activation in wound fibroblasts and modulates hair follicle regeneration after wounding (27). In addition, GD T cells play a role in tumor surveillance. They have the ability to kill a variety of cutaneous tumors including melanoma and carcinomas, and they express cytotoxic molecules including Perforins and granzymes (18, 28, 29). Although researchers have documented Perforin production by GD T cells, the mechanisms regulating Perforin production and the pathways through which it stimulates GD T cell cytotoxicity are not fully understood. This review will cover what is known about the role of Perforin as well as the novel pore forming protein Perforin-2 in GD T cells in the skin.

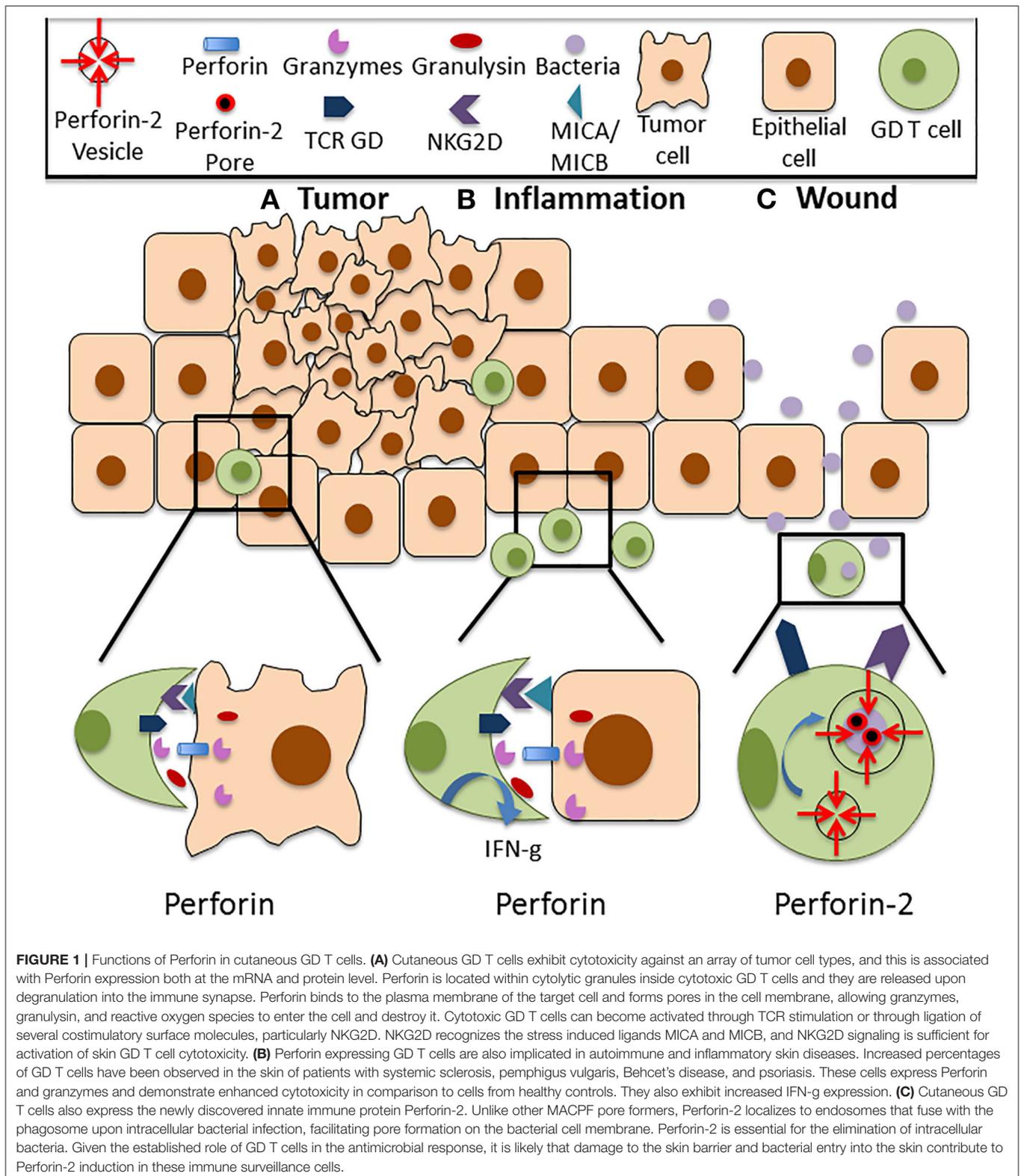
## OVERVIEW OF THE PERFORINS

Some of the most well-characterized pore forming proteins include the pore forming toxins expressed by pathogenic bacteria (30). One example is the cholesterol dependent cytolysins (CDC) which are produced by several gram-positive bacterial species. These virulence factors promote bacterial pathogenesis by lysing or permeabilizing host cell membranes or intracellular organelles (30–32). CDC proteins share a complex core fold with proteins from the membrane attack complex/perforin (MACPF) superfamily (33–35). This structural homology underlies the similar mechanism of pore formation and membrane disruption shared by both protein families. Pore forming proteins in the MACPF family are named as such because they all contain a domain that is shared by the proteins that form the membrane attack complex and the Perforins (36). Hundreds of MACPF domain-containing proteins have been identified, but some of the most well-characterized are the mammalian MACPF immune proteins, which include complement proteins C6–C9, Perforin, and Perforin-2 (34, 37, 38). Perforin, encoded by the gene *PRF1*, is located within cytolytic granules inside cytotoxic T cells and natural killer (NK) cells (39). When the cytotoxic cell recognizes a transformed or infected target cell, the granules containing Perforin, granzymes, and granulysin migrate to the

cell membrane and release their contents into the immune synapse (40). Perforin binds to the plasma membrane of the target cell and forms pores in the cell membrane, allowing delivery of cytolytic effector proteins and subsequent destruction of the cell (39–41). Perforin-2, encoded by the gene *MPEG1*, is a recently discovered innate immune protein that is highly conserved throughout the animal kingdom (42–44). Perforin-2 is the more ancient of the two Perforins and it is thought that Perforin originated as a gene duplication of *MPEG1* (45). Perforin-2 differs from other MACPF pore formers in that it has a transmembrane domain and localizes to endosomal membranes. The Perforin-2 cytosolic tail directs the endosomes to bacteria-encapsulating phagosomes (46). Acidification of the phagosome stimulates reconfiguration of the MACPF domain, resulting in pore formation on the bacterial cell membrane (46, 47). Our group was the first to demonstrate the essential role of Perforin-2 in eliminating intracellular bacterial infections (48, 49), confirming the importance of this protein as an antimicrobial effector protein expressed by both phagocytic and tissue forming cells.

## PERFORIN EXPRESSION BY SKIN GD T CELLS

The tumor-lysing capabilities of GD T cells have been well-documented in human skin (**Figure 1A**). Human skin derived GD T cells were purified using single cell sorting and tested in cytotoxicity assays against a variety of melanoma cell lines. They demonstrated cytotoxicity against SK-Mel2 and HS-294 melanoma cells, resulting in up to 90% cell death. This was comparable to the cytotoxic activity of the CD8+ AB T cells and NK cells that were also tested (18). GD T cells, CD8+ AB T cells, and NK cells only expressed Perforin after being cultured in the presence of IL-2, which is a previously established mechanism of Perforin induction in cytotoxic CD8+ T cells (18, 50, 51). Murine cutaneous Vdelta1+ GD T cells also express Perforin both at the mRNA and protein levels (51). They exhibited cytotoxicity against several tumor cell lines and also expressed granzyme B in amounts comparable to cytotoxic CD8+ AB T cells. Cytotoxic GD and AB T cells both produced IFN- $\gamma$  and TNF- $\alpha$  (18, 52, 53). Additionally, increased numbers of circulating CD3+TCR GD+ cells were observed in melanoma patients in comparison to healthy controls. These cells highly expressed Perforin in both normal individuals and melanoma patients, which may be important to anticancer surveillance (54). However, a study using a mouse model of skin carcinoma reported that circulating IL-17 producing GD T cells supported cutaneous tumor progression by promoting angiogenesis (55). In contrast to cytotoxic skin resident GD T cells, these non-skin resident IL-17 producing GD T cells that infiltrated the skin after tumor formation expressed low levels of Perforin and increased levels of the tumor-promoting factor COX-2. Although this paper did not establish a causative link between reduced Perforin expression and IL-17 production by circulating GD T cells, it implies that low levels of Perforin in these cells may contribute to their lack of cytotoxic activity and allow them to acquire a



pro-tumor GD T cell phenotype. These results underscore the importance of Perforin as an effector molecule in GD T cell mediated cytotoxicity in the skin.

Despite the clear importance of Perforin expressing cytotoxic GD T cells in the cutaneous anti-tumor response, these cells have the potential to develop into aggressive T cell lymphomas

(56, 57). Primary cutaneous GD T cell lymphomas constitute a subgroup of aggressive T cell lymphomas that express a mature cytotoxic phenotype. These tumors are characterized by their expression of T-cell-restricted intracellular antigen-1 (TIA-1), granzyme B, and Perforin (56–58) and there is also a strong correlation between CD30 expression and cytotoxic protein production (59). Cutaneous GD T cell lymphomas, while rare, have a poor prognosis; therefore, appropriate tumor phenotyping could be a useful tool for diagnosing this disease.

Perforin expressing GD T cells are also implicated in autoimmune and inflammatory skin diseases (Table 1). Patients with the autoimmune disease systemic sclerosis exhibit an increased percentage of Vdelta 1+ GD T cells that demonstrate cytotoxic activity in the skin (69, 70). Increased percentages of CD27+ GD T cells expressing Perforin and granzyme B are also present (71). Patients with Pemphigus vulgaris, an autoimmune disease that causes skin blistering, also have an increased percentage of circulating GD T cells. These cells demonstrate increased activation and cytotoxic activity in comparison to healthy controls (72). Behcet's disease, a disorder that causes blood vessel inflammation, is also associated with the expansion of GD T cells exhibiting increased Perforin and granzyme A expression (73, 74). Increased Perforin and granzyme B expression by CD3+ T cells is also evident in the epidermis of psoriasis patients (75–77). Only the CD8+ and CD4+ T cell subsets were analyzed, so Perforin expression by GD T cells in psoriasis has not yet been confirmed. It has been confirmed, however, that dermal GD T cells are recruited to the epidermis in psoriasis by activated keratinocytes that produce the chemokines CCL2 and CCL20 (78). Recruited dermal GD T cells induce an innate-like immune response by producing IL-17 and IL-22 upon IL-1 and IL-23 stimulation. IL-1 is expressed by keratinocytes while IL-23 is expressed by dermal dendritic cells and macrophages. IL-17 and IL-22 production further drives keratinocyte hyperplasia, neutrophil recruitment and disease progression (5). Since pro-inflammatory GD T cells are recruited to psoriatic skin, they are likely to be among the Perforin expressing CD3+ T cells observed in psoriasis patients (Figure 1B). Overall, studies have shown that GD T cells in the skin express Perforin, and this is associated with an activated cytotoxic phenotype. This can contribute positively to skin immune function via induction of the anti-tumor immune response, or it may have deleterious effects in the case of autoimmune and inflammatory skin disorders or cutaneous GD T cell lymphomas.

## MECHANISMS REGULATING PERFORIN EXPRESSION IN SKIN GD T CELLS

Although it is clear that cutaneous cytotoxic GD T cells express Perforin, the mechanisms that regulate its expression are still unclear. Perforin is copiously expressed once these cells become activated (18). In contrast to CD8+ AB T cells which require TCR binding to MHC for activation (79), cytotoxic GD T cells can become activated through a non-MHC-restricted mechanism (61). In fact, recognition and killing of target cells can proceed

through ligation of several costimulatory surface molecules in the absence of TCR stimulation (53). One of these surface molecules is the activating NK receptor NKG2D. It recognizes the MHC class I polypeptide-related sequence A and B (MICA and MICB), which are induced on stressed cells (53). NKG2D is expressed by most NK and CD8+ AB T cells, but it was found that both mouse and human GD T cells constitutively express NKG2D as well (53, 80, 81). Its expression is maintained during cell culture of freshly isolated GD T cells (13, 18, 53). NKG2D ligation stimulates epidermal GD T cell degranulation using a phosphatidylinositol 3-kinase (PI3K)-dependent pathway, and killing of NKG2DL expressing target cells occurs in the absence of CD3 and TCR signaling (60). Treatment of skin GD T cells with anti-NKG2D antibodies impaired lysis of A20 B cell lymphoma cells, but treatment with anti-TCR antibodies had no effect, indicating that NKG2D signaling is sufficient to activate cytotoxicity in skin GD T cells (53). Skin GD T cells also express the surface glycoprotein 2B4. Removal of IL-2 from GD T cell culture media reduced surface expression of 2B4 and also reduced their capacity to lyse target cells (61). Their cytotoxicity was enhanced by treatment with a stimulatory anti-2B4 antibody, indicating the importance of this receptor in cutaneous GD T cell mediated cytotoxicity. B7-1 (CD80) also provides costimulatory signals to both AB and GD T cells by binding to CD28 on the T cell surface, increasing adhesion to the target cell. B7-1 surface expression on Pam212, murine squamous cell carcinoma cells, stimulated cutaneous GD T cell proliferation and increased carcinoma cell sensitivity to GD T cell mediated lysis (62). Cutaneous GD T cell cytotoxicity is regulated through expression of several inhibitory receptors that block cytotoxicity upon binding with their appropriate ligands. Murine skin GD T cells express the inhibitory receptors Ly49 and CD94/NKG2. Presentation with Qdm, the ligand for CD94/NKG2, prevented the epidermal GD T cells from killing target cells (67).

GD T cell cytotoxicity is also activated by their recognition of stress-induced molecules on target cell surfaces. Macrophages and neutrophils externalize the heat shock proteins Hsp60 and Hsp70 under inflammatory conditions to target them for destruction, thus resolving the inflammation and preventing excess tissue damage. Cytotoxic GD T cells recognize these molecules and kill the target cells via direct cell-cell interactions (64). Activation of Perforin expressing cytotoxic GD T cells is also mediated through cytokine expression. IL-2, IL-15, and IL-12 enhance cytolytic activity of cutaneous GD T cells, and the transcription factor IFN regulatory factor-1 (IRF-1) is essential for induction of cytotoxicity (61, 63). The Perforin dependent anti-tumor properties of GD T cells can be enhanced pharmacologically. For example, rapamycin enhances the perforin-dependent cytotoxicity of human GD T cells against squamous cell carcinomas *in vitro* and in a mouse xenograft model of human squamous cell carcinoma (65). Additionally, Resveratrol enhances Perforin expression in NK cells through an NKG2D dependent pathway, implicating its potential to increase Perforin expression by NKG2D expressing GD T cells as well (82). Given the deleterious effects of Perforin expressing cells in the context of autoimmune and inflammatory disorders, blocking therapeutics targeting Perforin may benefit patients

**TABLE 1** | Perforins expressed by cutaneous GD T cells.

	Perforin	Perforin-2
Location in the cell	Intracellular cytolytic granules (39)	Membranes of intracellular endosomes (46, 47)
Function in GD T cells	Lysis of transformed or infected cells (39–41)	Destruction of intracellular bacteria (46, 47)
Stimulatory factors	Surface receptors: - GD TCR (53) - NKG2D (13, 18, 53, 60) - 2B4 (61) - B7-1 (62) Cytokines: - IL-2 (61) - IL-12 (63) - IL-15 (63) Stress induced molecules: - Hsp60 (64) - Hsp70 (64) Transcription Factors: - IRF-1 (63) Therapeutics: - Rapamycin (65)	Commensal bacteria: - <i>S. epidermidis</i> (66)
Inhibitory factors	Surface receptors: - Ly49 (67) - CD94 (67)	Pathogenic bacteria: - <i>S. aureus</i> (68)
Role in disease	Expressed by cutaneous GD T cell lymphomas (56–59) Upregulated in autoimmune and inflammatory diseases: - Systemic sclerosis (69–71) - Pemphigus vulgaris (72) - Behcet's disease (73, 74) - Psoriasis (75–77)	Upregulated in response to wounding (68) Downregulation may contribute to persistent wound infection (68)

Summary of the information currently known about Perforin and Perforin-2 expression by cutaneous GD T cells. Listed are the location of the Perforins within the cell, functions of the Perforins in GD T cells, Perforin stimulatory and inhibitory factors, and the role of Perforins in cutaneous diseases.

suffering from these illnesses. Compounds identified as Perforin inhibitors in NK and AB T cells include diarylthiophenes and benzenesulfonamide based therapeutics (83–85). In short, cutaneous GD T cells express Perforin upon activation and this activation can be achieved through a variety of mechanisms including stimulation of the TCR, ligation of costimulatory molecules, and cytokine stimulation (**Table 1**). The critical role of Perforin in executing GD T cell mediated cytotoxicity in the skin warrants further studies on the mechanisms that induce Perforin expression by these cells.

## PERFORIN-2 EXPRESSION BY GD T CELLS

Perforin-2 is a recently identified member of the MACPF domain containing pore forming family of innate immune proteins that plays a critical role in clearance of intracellular bacterial infections. However, the mechanisms behind Perforin-2 activation and the extent of its contributions to host immunity have not been fully characterized. Our group was the first to

report Perforin-2 expression in a variety of cell types in human skin including keratinocytes, fibroblasts and GD T cells (68). Although it is known that both murine and human GD T cells express Perforin-2, the role that it plays in GD T cell mediated cytotoxicity toward bacterial pathogens is still unknown. We demonstrated that Perforin-2 expression is upregulated in CD45+ cells upon wounding in an *ex vivo* human skin model. However, infection of the wounds with *Staphylococcus aureus* inhibited Perforin-2 expression in these cells (68). This indicates a potential role for Perforin-2 in promoting skin homeostasis and barrier integrity by aiding in the clearance of bacteria from the wound site (**Figure 1C**). This was confirmed by our finding that human keratinocyte cells constitutively expressing a Perforin-2-GFP fusion protein demonstrate improved clearance of intracellular *S. aureus* infection in comparison to control cells (68). Our group has also demonstrated that Perforin-2 deficient mice infected epicutaneously are unable to clear *S. aureus* and eventually succumb to the infection (48). Interestingly, in this issue we provide evidence that *S. epidermidis*, a skin commensal microorganism, induces Perforin-2 in GD T cells in human skin *ex vivo* (66). Given the established role of GD T cells in the antimicrobial response and in keratinocyte proliferation and migration upon wounding, it is likely that disruption of the epidermal barrier and signals from microorganisms can result in Perforin-2 induction. This warrants further studies on Perforin-2 regulation and its function as an effector protein in GD T cell mediated skin immune responses.

## CONCLUSIONS

Maintaining skin homeostasis and barrier function is essential for protection against physical and chemical stress, infections, and malignancies. Recent research has highlighted the significant contributions of GD T cells to skin health through their role in bacterial clearance, wound healing, and tumor killing. Expression of both Perforin and Perforin-2 by GD T cells has been implicated in the activation of these effector functions (**Figure 1, Table 1**). GD T cells lyse a variety of tumor cell lines and exhibit many characteristics of conventional cytotoxic T cells. Although GD T cells have been shown to produce Perforin, little is known about the signals that stimulate its expression. Additionally, Perforin-2 was recently identified as another member of the MACPF domain containing pore forming protein family. It is critical for clearance of intracellular bacterial infections, and it is expressed constitutively by GD T cells. Given the established role of GD T cells as first responders to bacterial infections in the skin, it is important to further investigate the regulation of Perforin-2 in this GD T cell function. Understanding the signals that activate Perforin expression by GD T cells can help to elucidate the mechanisms governing their diverse roles in skin immunity.

## AUTHOR CONTRIBUTIONS

KO'N wrote the manuscript and prepared the figures. IP, MT-C, and NS revised the manuscript and provided critical comments. 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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