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# CD96 Blockade Ameliorates Imiquimod-Induced Psoriasis-like Dermatitis via Suppression of IL-17A Production by Dermal γδ T Cells

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**Psoriasis is a chronic inflammatory skin disease. IL-23 plays a critical role in its pathogenesis by inducing production of IL-17A from pathological Th17 cells and IL-17A–producing γδ T cells. However, the mechanisms regulating the IL-23/IL-17 axis in psoriasis are incompletely understood. In this study, we show that, in comparison with wild-type mice, those deficient in the CD96 immunoreceptor had lower production of IL-17A in their dermal γδ T cells and milder psoriasis-like dermatitis after topical application of imiquimod (IMQ). Moreover, transfer of CD96-deficient dermal γδ T cells into the skin of Rag1-deficient mice resulted in them developing milder IMQ-induced dermatitis compared with Rag1-deficient mice transferred with wild-type dermal γδ T cells. In γδ T cells in vitro, CD96 provides a costimulatory signal for the production of IL-23–induced IL-17A. In mice given an anti-CD96 neutralizing Ab, IL-17A production from dermal γδ T cells decreased and IMQ-induced dermatitis was milder compared with mice given a control Ab. These results suggest that CD96 is a potential molecular target for the treatment of psoriasis.** ***The Journal of Immunology*****, 2022, 209: 2313–2321.**

Psoriasis is a chronic inflammatory skin disease that affects 2–4% of the population (1). The lesions are usually manifested as raised erythematous plaques with adherent silvery scales due to epidermal hyperplasia and aberrant differentiation. Accumulating evidence indicates that IL-23 plays a critical role in the pathogenesis of psoriasis by inducing production of IL-17A from pathological Th17 cells and IL-17A–producing γδ T cells (2, 3). Blockade of the IL-23/IL-17 axis by either an anti–IL-23 mAb, an anti-p40 subunit of IL-12/IL-23 mAb, an anti–IL-17A mAb, or an anti–IL-17 receptor mAb is used for the treatment of psoriasis (4). Although useful in this context, these Abs also cause side effects such as severe infections due to systemic immunosuppression.

Imiquimod (IMQ), a TLR7/8 agonist used topically for treatment of actinic keratosis and superficial basal cell carcinoma, can exacerbate symptoms in patients with otherwise well-controlled psoriasis (5, 6). Daily application of IMQ to the shaved dorsal skin of mice induces inflamed scaly skin lesions resembling plaques seen in psoriasis (7). Activation of TLR7/8 by IMQ triggers IL-23 production from macrophages and dendritic cells, inducing IL-17A production from dermal γδ T cells (3, 8). Dermal γδ T cells have been demonstrated to be the major producers of IL-17A in mouse skin (3, 9). However, the mechanisms regulating γδ T cells in the skin remain incompletely understood.

CD96 is a member of the Ig superfamily and is expressed on most lymphocytes, including NK cells, $CD8^+$ T cells, and γδ T cells (10). The ligand for CD96 is poliovirus receptor CD155 and its family member nectin-1 (CD111, also called poliovirus receptor-related family 1) (11–13). CD155 and CD111 are broadly distributed on myeloid cells, epithelial cells, and endothelial cells in many tissues (14, 15). CD96 was first demonstrated to function as an activating receptor using freshly established NK cell lines, and accumulating evidence shows that interactions between CD96 on NK and $CD8^+$ T cells and its ligands CD155/CD111 on target cells enhance cell-mediated cytotoxicity, cytokine induction, and cell proliferation (10, 11, 16–20). CD96 deficiency or anti-CD96 blockade has been shown to suppress the activation of $CD8^+$ T cells infiltrated into CT26 colorectal carcinoma (19), indicating that CD96 functions as an activating receptor. However, in contrast to studies showing an activating role, mouse studies using CD96-deficient ($Cd96^{-/-}$) mice or an anti-mouse CD96 neutralizing mAb have found evidence of an inhibitory role of CD96 on NK cells in several disease models, such as LPS-induced endotoxicosis (10), and lung tumor metastasis models (10, 17). In addition, it has been demonstrated using an MCA1956-induced fibrosarcoma model that mouse CD96 on $CD8^+$ T cells has an inhibitory function (20). These results suggest that the function of CD96 may differ according to cell type or disease condition. Although previous reports have shown that CD96 is also expressed on γδ T cells in humans and mice, little is known about the function of CD96 on γδ T cells (10).

In this study, we demonstrated that CD96 delivered a costimulatory signal and enhanced the production of IL-17A in mice. Consistent

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Abbreviations used in this article: BM, bone marrow; cIg, control Ig; dLN, draining lymph node; DNAM-1, DNAX accessory molecule 1; IMQ, imiquimod; pAb, polyclonal Ab; WT, wild-type.

with this result, CD96 was found to exacerbate IMQ-induced psoriasis by induction of IL-17A from dermal γδ T cells. We also demonstrated that blockade of CD96 by an anti-CD96 neutralizing mAb suppressed IMQ-induced psoriasis-like dermatitis.

## Materials and Methods

### *Mice*

C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). CD45.1$^+$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CD96-deficient (*Cd96*$^{-/-}$) C57BL/6J mice were generated in our laboratory, as described previously (21). All mice were housed and bred under specific pathogen-free conditions at the Laboratory Animal Resource Center at the University of Tsukuba.

### *Abs and reagents*

Anti-mouse neutralizing CD96 mAb (TX111 clone, IgG2b, Igκ) was generated by immunization of *Cd96*$^{-/-}$ mice with a BW5147 transfectant expressing mouse CD96 (CD96/BW5147) and a soluble protein consisting of the extracellular portion of mouse CD96 fused with the human IgG Fc portion.

mAbs to mouse CD96 (3.3), CD16/32 (2.4G2), CD3 (145-2C11), CD45.1 (A20), CD45.2 (104), CD11b (M1/70), Ly-6G (1A8), CD69 (H1.2F3), CD25 (PC61), CD4 (RM-4-5), CD8 (53-6.7), NK1.1 (DX5), CD11c (N418), Ly6c (HK1.4), CD127 (A7R34), and CD207 (4C7) were purchased from BioLegend (San Diego, CA). mAbs to mouse TCRβ (H57 – 597), TCRγδ (GL3), Vγ5 (536), CD90.2 (53-2.1), IL-17A (TC11-18H10), and RORγt (Q31-378) were purchased from BD Biosciences (San Jose, CA). Polyclonal Ab (pAb) to phospho-Erk (catalog no. 9101) was purchased from Cell Signaling Technology (Danvers, MA). mAb to mouse Vγ4 (UC3-10A6) was purchased Thermo Fisher Scientific (Waltham, MA). mAb to mouse IL-17A (17F3) and TNF-α (XT3.11) for in vivo use was purchased Bio X Cell (West Lebanon, NH, USA). mAbs to human CD96 (NK92.39), CD14 (M5E2), and CD25 (BC96) were purchased from BioLegend. mAbs to human CD3 (HIT-3a), TCRγδ (B1.1), Vδ2 (B6), CD19 (HIB19), and CD69 (FN50) were purchased from BD Biosciences. Secondary Abs PE-conjugated pAb to rabbit IgG (catalog no. 406421), PE-conjugated streptavidin (catalog no. 405203), allophycocyanin-conjugated pAb to mouse IgG (catalog no. 405308), and PE-conjugated pAb to human IgG (catalog no. 410708) were purchased from BioLegend.

### *IMQ-induced psoriasis-like dermatitis*

For 4 or 5 consecutive days, 62.5 mg of IMQ cream (5% Beselna cream; Mochida Pharmaceutical, Tokyo, Japan) was applied once a day to the shaved dorsal skin of mice. Petroleum jelly was applied as a control. A scoring system based on the Psoriasis Area and Severity Index was used to assess the IMQ-induced psoriasis-like dermatitis (5). Skin thickness was measured using a dial thickness gauge (catalog no. G-2.4N, Peacock, Ozaki Manufacturing, Tokyo, Japan).

### *Histology*

Excised dorsal skin tissue samples were fixed in 10% formalin and embedded in paraffin, and 4-μm sections were cut and stained with H&E. The thickness of the skin epidermal layer was measured and analyzed by light microscopy. Epidermal thickness was measured at five random locations in each field and in five fields per mouse.

### *Generation of bone marrow chimeric mice*

Mice were irradiated (10 Gy) and then i.v. injected with $1 \times 10^7$ bone marrow (BM) cells.

### *Skin cell preparation*

To isolate skin cells from IMQ-naive and IMQ-treated mice, dorsal skin samples were minced with scissors and incubated for 1 h in collagenase IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium containing DNase I (50 U/ml; Worthington Biochemical, Lakewood, NJ) and 10% FBS. Additional dissociation and homogenization were performed by using a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell preparations were filtered through a 55-μm nylon mesh to obtain single-cell suspensions.

### *Isolation of mouse γδ T cells*

Splenic and dermal γδ T cells were isolated by negative selection using biotinylated mouse mAbs against lineage markers (CD4, CD8, and B220) and streptavidin microbeads (Miltenyi Biotec), followed by sorting into CD45$^+$ TCRβ$^-$TCRδ$^+$Vγ4$^+$γδ T cells (splenic γδ T cells) and CD45$^+$TCRβ$^-$ TCRδ$^+$Vγ5$^-$γδ T cells (dermal γδ T cells) with FACSAria flow cytometers (BD Biosciences, San Jose, CA).

### *In vitro stimulation of mouse γδ T cells*

Dermal γδ T cells were cultured in 96-well U-bottom culture plates for 4 d under 5% $CO_2$ at 37°C in complete RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, 100 U of penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 100 μM MEM nonessential amino acids in the presence of IL-2 (2 ng/ml), IL-6 (30 ng/ml), IL-7 (20 ng/ml), and IL-15 (20 ng/ml), which were purchased from R&D Systems (Minneapolis, MN).

Sorted splenic γδ T cells and cultured dermal γδ T cells were stimulated with 0.1 μg/ml plate-coated anti-CD3 mAb (145-2C11; BioLegend) and 20 μg/ml plate-coated anti-CD96 mAb (6A6; BioLegend) or rat IgG2a (catalog no. 553927; BD Biosciences) for 18 h under 5% $CO_2$ at 37°C in the complete medium in the presence of mouse IL-2 (2 ng/ml). For IL-17A detection,

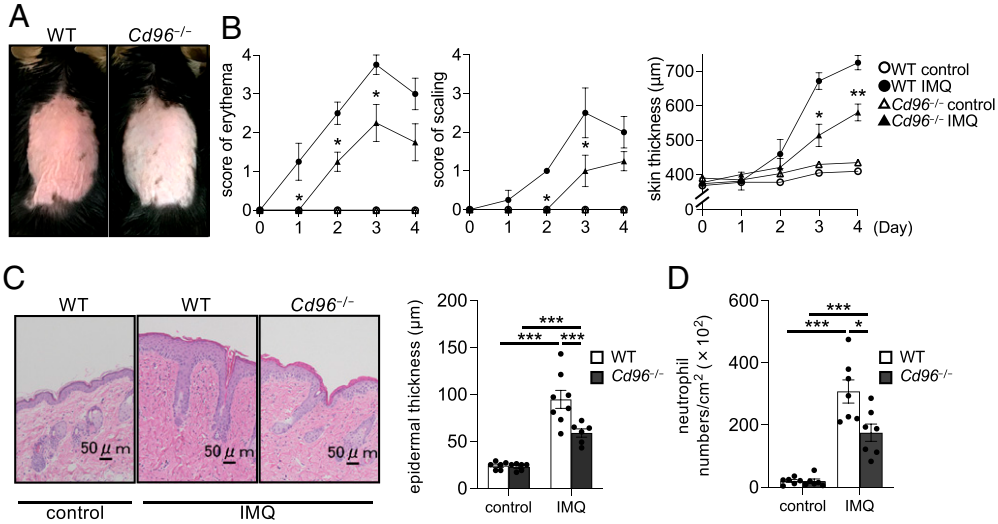


**FIGURE 1.** CD96-deficient mice were more resistant to IMQ-induced psoriasis-like dermatitis than were WT mice. Wild-type (WT) mice ($n = 4$) or *Cd96*$^{-/-}$ mice ($n = 4$) were treated once a day with IMQ or petroleum jelly on the shaved dorsal skin for 4 d. (**A**) Phenotypical presentation of IMQ-treated mouse dorsal skin on day 2. (**B**) Changes in erythema, scaling, and thickening of dorsal skin. (**C**) Representative histology (H&E staining) and epidermal thickness of dorsal skin on day 3. (**D**) Numbers of neutrophils (CD45$^+$, CD11b$^+$, Ly6G$^+$) in the skin (1 cm$^2$) on day 3. Error bars indicate SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are representative from two independent experiments (B) or two pooled experiments (C and D).

sorted splenic γδ T cells and cultured dermal γδ T cells were stimulated with 0.1 μg/ml plate-coated anti-CD3 mAb and 20 μg/ml plate-coated anti-CD96 mAb or rat IgG2a for 18 h under 5% $CO_2$ at 37°C in the complete medium in the presence of mouse IL-2 (2 ng/ml) and 0.1 ng/ml IL-23 (R&D Systems). To examine the phosphorylation of Erk, splenic γδ T cells were incubated with 10 μg/ml anti-CD3 mAb and 30 μg/ml anti-CD96 mAb or mouse IgG2b (catalog no. 400348) for 30 min on ice, followed by crosslinking by 10 μg/ml anti-hamster IgG (catalog no. 107-005-142; Jackson ImmunoResearch Laboratories) and anti-mouse IgG (catalog no. A90-136A; Bethyl Laboratories, Montgomery, TX) at 37°C.

### *Preparation of human γδ T cells and CD4$^+$ T cells*

PBMCs were collected from the peripheral blood of healthy donors after obtaining their written informed consent by using Lymphoprep (catalog no. 07851, STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. γδ T cells or CD4$^+$ T cells were isolated by

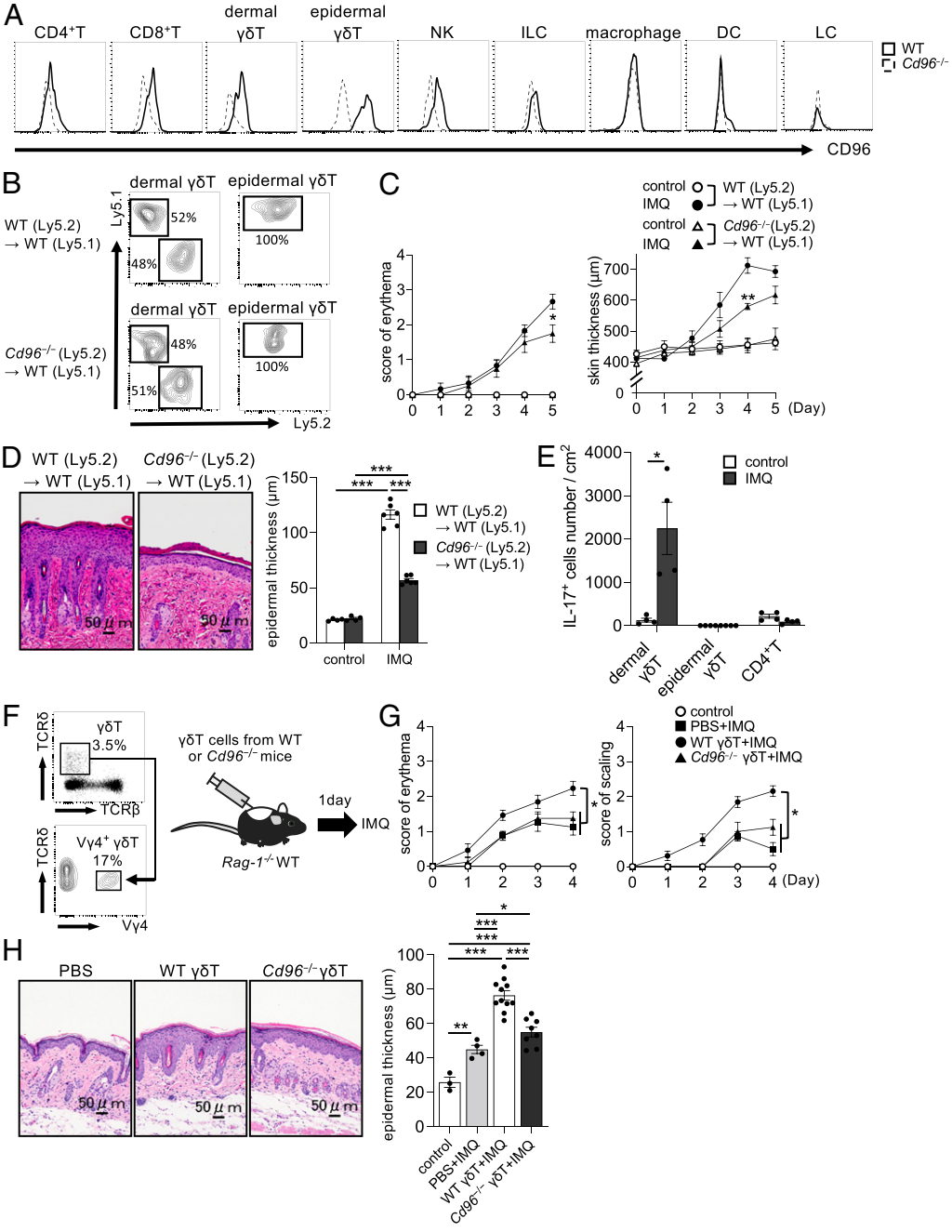


**FIGURE 2.** CD96 on dermal γδ T cells is involved in IMQ-induced psoriasis-like dermatitis. (**A**) CD96 expression on lymphocytes and myeloid cells in the skin from naive WT or *Cd96*$^{-/-}$ mice. DC, dendritic cell; ILC, innate lymphoid cell; LC, Langerhans cell. (**B**–**D**) Bone marrow (BM) chimeric mice were generated by reconstituting irradiated Ly5.1$^+$ WT mice with BM cells from Ly5.2$^+$ WT ($n = 6$) or *Cd96*$^{-/-}$ ($n = 6$) mice. (B) Representative plot showing the chimerism of dermal (TCRδ$^+$Vγ5$^-$) and epidermal (TCRδ$^+$Vγ5$^+$) γδ T cells 12 wk after BM transplantation. (C) Changes in erythema and skin thickening in BM chimeric mice treated once a day with IMQ or petroleum jelly on the shaved dorsal skin for 5 d. (D) Representative histology (H&E staining) and epidermal thickness of dorsal skin. (**E**) WT mice were treated once a day with IMQ or petroleum jelly (control) on the shaved dorsal skin for 3 d. The IL-17A–producing cell numbers/cm$^2$ of dermal γδ T cells, epidermal γδ T cells, and CD4$^+$ T cells ($n = 4$) are shown. (**F**–**H**) Vγ4$^+$ γδ T cells isolated from the spleen of WT or *CD96*$^{-/-}$ mice were injected into the shaved dorsal skin of *Rag1*$^{-/-}$ mice. (F) From the next day, IMQ was applied once a day to the shaved dorsal skin for 4 d. (G) Changes in erythema and scaling of dorsal skin. (H) Representative histology (H&E staining) and the epidermal thickness of dorsal skin after IMQ treatment for 4 d (control, $n = 3$; PBS+IMQ, $n = 4$; WT γδ T+IMQ, $n = 11$; CD96$^{-/-}$ γδ T+IMQ, $n = 8$). Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are pooled from four experiments (G and H).

negative selection using biotinylated mouse mAbs (CD8, CD19, and CD14) and streptavidin microbeads (Miltenyi Biotec), followed by sorting into $CD45^+CD4^-TCR\delta^+V\delta2^+$ cells and $CD45^+CD4^+$ cells, respectively, by FACSAria flow cytometers (BD Biosciences). The study was approved by the Ethics Committee of the University of Tsukuba.

### *In vitro stimulation of human γδ T cells and CD4$^+$ T cells*

Sorted $V\delta2^+$ γδ T cells ($2 \times 10^5$/well) and $CD4^+$ T cells ($2 \times 10^5$/well) were stimulated with 0.1 μg/ml plate-coated anti-CD3 mAb (HIT-3a; BD Biosciences) and 20 μg/ml plate-coated anti-CD96 mAb (NK92.39; BioLegend) or mouse IgG1 (catalog no. 400102; BioLegend) for 48 or 96 h under 5% $CO_2$ at 37°C in RPMI 1640 complete medium in the presence of mouse IL-2 (2 ng/ml). For IL-17A detection, sorted $V\delta2^+$ γδ T cells and $CD4^+$ T cells were stimulated with 0.1 μg/ml plate-coated anti-CD3 mAb and 20 μg/ml plate-coated anti-CD96 mAb or mouse IgG1 for 48 or 96 h under 5% $CO_2$ at 37°C in RPMI 1640 complete medium in the presence of 2 ng/ml mouse IL-2, 50 ng/ml human TGF-β, 10 ng/ml human IL-1β, 20 ng/ml human IL-23, 50 ng/ml human IL-6, and 10 ng/ml human IL-12 (all cytokines were purchased from R&D Systems).

### *Flow cytometry*

Flow cytometric analysis was performed by using FACS LSRFortessa (BD Biosciences). FlowJo software (Tree Star, Ashland, OR) was used for data analyses. Dead cells were stained and excluded by using Zombie NIR or Zombie Violet fixable viability kits (BioLegend).

### *Intracellular IL-17A staining*

Cells were treated for 2 h with BD GolgiStop (1:1500 dilution; BD Biosciences), fixed, permeabilized, and washed with an eBioscience Foxp3/transcription factor staining buffer set (Thermo Fisher Scientific), and then stained with PE-conjugated anti-mouse IL-17A mAb.

### *cDNA synthesis and real-time PCR*

Total RNA was extracted by using Isogen reagent (Nippon Gene, Tokyo, Japan). cDNA was synthesized by using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA). Expression of genes was measured through quantitative real-time PCR analysis by using SYBR Green master mix (Applied Biosystems) and appropriate primers. Primer sequences of the target genes are as follows: mouse *Il23R*, forward, 5′-GCA ACA TGA CAT GCA CCT GG-3′, reverse, 5′-GAC AGC TTG GAC CCA TAC CA-3′; mouse *Il12Rβ1*, forward, 5′-ATG GCT GCT GCG TTG AGA A-3′, reverse,



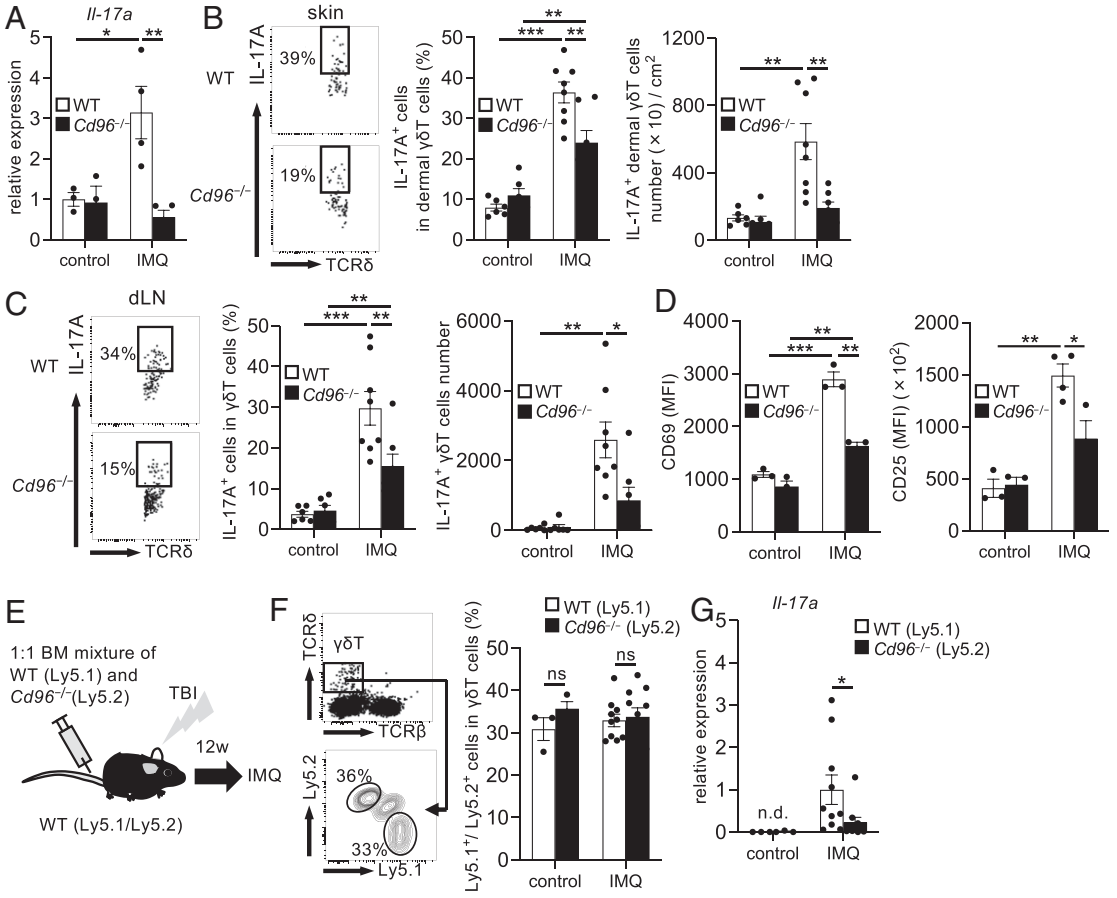


**FIGURE 3.** CD96 on dermal γδ T cells augments IL-17A expression after IMQ treatment. (**A**–**D**) WT or *Cd96*$^{-/-}$ mice were treated once a day with IMQ or petroleum jelly (control) on the shaved dorsal skin for 3 d. (A) *Il17a* mRNA levels of dermal γδ T cells in the skin of WT mice (IMQ, $n = 4$; control, $n = 3$) and *Cd96*$^{-/-}$ mice (IMQ, $n = 4$; control, $n = 3$). (B and C) The proportions and the numbers of IL-17A–producing dermal γδ T cells in (B) the skin (1 $cm^2$) and (C) the draining lymph nodes (dLNs) of WT mice (IMQ, $n = 8$; control, $n = 6$) and *Cd96*$^{-/-}$ mice (IMQ, $n = 8$; control, $n = 6$). (D) The mean fluorescence intensity (MFI) of CD69 expression on γδ T cells in dLNs from WT mice (IMQ, $n = 3$; control, $n = 3$) and *Cd96*$^{-/-}$ mice (IMQ, $n = 3$; control, $n = 3$) 3 h after IMQ treatment. The MFI of CD25 expression on γδ T cells in dLNs from WT mice (IMQ, $n = 4$; control, $n = 3$) and *Cd96*$^{-/-}$ mice (IMQ, $n = 3$; control, $n = 3$) after IMQ treatment for 3 d is shown. (**E**) BM chimeric mice were generated by reconstituting irradiated $Ly5.1^+Ly5.2^+$ mice with a 1:1 mixture of BM cells from $Ly5.1^+$ WT and $Ly5.2^+$ *Cd96*$^{-/-}$mice. (**F**) BM chimeric mice 12 wk after BM transplant were treated once a day with IMQ or petroleum jelly (control) on the shaved dorsal skin for 3 d. Representative plot and ratio of donor-derived $Ly5.1^+$ WT and $Ly5.2^+$ *Cd96*$^{-/-}$ γδ T cells in the dLNs (IMQ, $n = 10$; control, $n = 3$). (**G**) *Il17a* mRNA levels of $Ly5.1^+$ WT and $Ly5.2^+$ *Cd96*$^{-/-}$ γδ T cells of dLNs (IMQ, $n = 10$; control, $n = 3$). Error bars indicate SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. nd, not detected; ns, not significant. Data are representative from two independent experiments (A–C) or pooled from two experiments (F and G).

5′-AGC ACT CAT AGT CTG TCT TGG A-3′; mouse *Il17a*, forward, 5′-TTT AAC TCC CTT GGC GCA AAA-3′, reverse, 5′-CTT TCC CTC CGC ATT GAC AC-3′; mouse *Il23a*, forward, 5′-CAG CTC TCT CGG AAT CTC TGC-3′, reverse, 5′-GAC CTT GGC GGA TCC TTT GC-3′; mouse *Cd155*, forward, 5′-CAA CTG GTA TGT TGG CCT CA-3′, reverse, 5′-ATT GGT GAC TTC GCA CAC AA-3′; mouse *Cd96*, forward, 5′-GAG ACT CGA GGT GTG GGA AG-3′, reverse, 5′-TCT CCT GGA GCA TTG CAT CA-3′; mouse *Actβ*, forward, 5′-ACT GTC GAG TCG CGT CCA-3′, reverse, 5′-GCA GCG ATA TCG TCA TCC AT-3′. The *β-actin* level was measured as an internal control to normalize data. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

### *Generation of a mouse CD96-expressing transfectant as well as chimeric proteins of mouse CD96 or CD155 fused with human IgG Fc portion*

A BW5147 transfectant expressing CD96 was established as described elsewhere (22). Chimeric fusion proteins consisting of the extracellular portion of mouse CD96 or CD155 and the Fc portion of human IgG were generated with chimeric cDNA of the entire extracellular domain of mouse CD96 or CD155 with human IgG Fc portion as described elsewhere (23).

### *Statistical analysis*

Statistical analyses were performed using the unpaired or paired Student *t* test. A $p$ value of $<0.05$ was considered to be significant.

## Results

### *CD96-deficient mice were more resistant to IMQ-induced psoriasis-like dermatitis than were wild-type mice*

To assess the role of CD96 in the pathogenesis of psoriasis, we applied IMQ to the dorsal skin of wild-type (WT) or *Cd96*$^{-/-}$ mice once a day for 4 or 5 consecutive days. WT mice developed erythema, scaling, and skin thickening 1 d after IMQ application. The dermatitis scores peaked on day 3 or 4. In contrast, *Cd96*$^{-/-}$ mice showed lower dermatitis scores and milder skin thickening compared with WT mice (Fig. 1A, 1B). Consistent with these observations, histological analysis demonstrated that epidermal acanthosis was milder in *Cd96*$^{-/-}$ mice than in WT mice (Fig. 1C). Furthermore, there were fewer infiltrating neutrophils in the skin on day 3 in *Cd96*$^{-/-}$ mice than in WT mice (Fig. 1D). These results demonstrate that CD96 exacerbates IMQ-induced psoriasis-like dermatitis.

### *CD96 on dermal γδ T cells is involved in IMQ-induced psoriasis-like dermatitis*

To clarify which cells expressing CD96 are involved in IMQ-induced dermatitis, we analyzed the expression of CD96 on immune cells in the skin. Flow cytometry analyses demonstrated that CD96 was expressed on lymphocytes such as CD4$^+$ and CD8$^+$ T cells, epidermal and dermal γδ T cells, NK cells, and innate lymphoid cells, but not on myeloid cells, including macrophages, dendritic cells, or Langerhans cells, in the skin (Fig. 2A). The frequencies and numbers of CD96-expressing lymphocytes, including CD4$^+$ T cells, CD8$^+$ T cells, dermal γδ cells, and epidermal γδ cells, in the skin of naive WT and *Cd96*$^{-/-}$ mice were comparable (Supplemental Fig. 1). We found that the level of CD96 expression on epidermal γδ T cells was highest (Fig. 2A). Therefore, we first investigated whether CD96 on epidermal γδ T cells is involved in IMQ-induced dermatitis.

Previous reports have shown that epidermal γδ T cells are resistant to radiation (24). Indeed, when Ly5.1$^+$ WT mice that had been subjected to a lethal dose of radiation received transplants of BM cells from Ly5.2$^+$ WT or *Cd96*$^{-/-}$ mice, most epidermal γδ T cells continued to be derived only from the recipient lineage (Fig. 2B). In contrast, half of the dermal γδ T cells in recipient mice were replaced with Ly5.2$^+$ donor-derived γδ T cells (Fig. 2B). These chimeric mice were treated with IMQ or petroleum jelly (control) for 5 d. Mice transplanted with *Cd96*$^{-/-}$ BM cells showed milder phenotypes of erythema and skin thickening compared with mice transplanted with WT BM cells (Fig. 2C). Consistent with these observations,

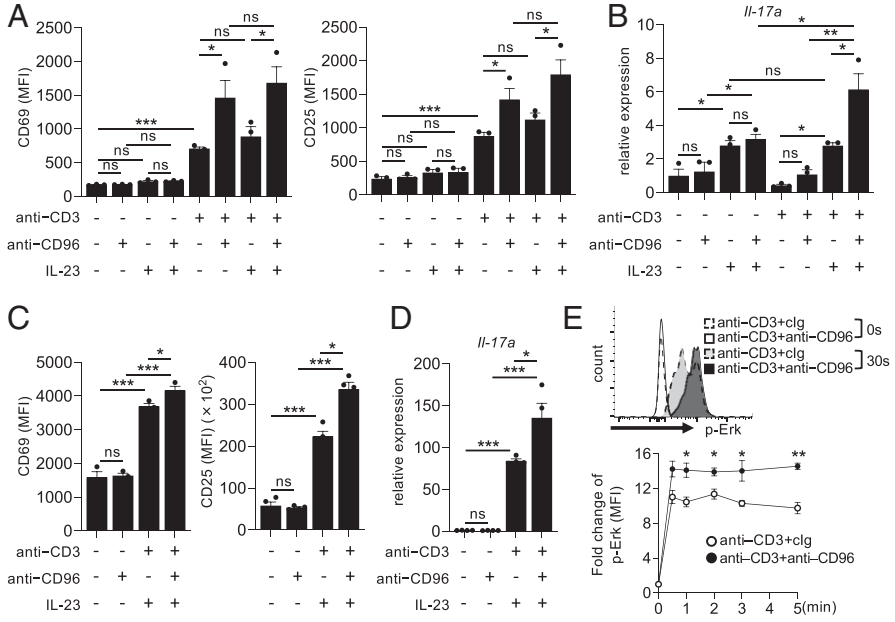


**FIGURE 4.** CD96 mediates a costimulatory signal in γδ T cells. (**A** and **B**) Splenic γδ T cells of WT mice were stimulated with or without anti-CD3 mAb in combination with or without anti-CD96 mAb and/or mouse IL-23 for 18 h. (A) MFI of CD69 and CD25 expressions ($n = 3$ in each column). (B) Quantitative real-time PCR analysis of *Il17a* mRNA levels ($n = 3$ in each column). (**C** and **D**) Dermal γδ T cells of WT mice were isolated and cultured in the presence of IL-2, IL-6, IL-7, and IL-15 for 4 d and stimulated with or without anti-CD3 mAb, anti-CD96 mAb, and/or mouse IL-23 for 18 h. (C) MFI of CD69 expression ($n = 3$ in each column) and CD25 expression ($n = 4$ in each column). (D) *Il17a* mRNA levels ($n = 4$ in each column). (**E**) Splenic γδ T cells of WT mice were stimulated with anti-CD3 mAb and either anti-CD96 mAb or mouse IgG2b (cIg) and analyzed for the expression of p-ERK. Representative flow cytometry analysis data and the relative expression of p-ERK ($n = 3$ in each group) are shown. Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant. Data are representative from two independent experiments.

histological analysis demonstrated that epidermal acanthosis of the skin after IMQ treatment was significantly milder in mice transplanted with *Cd96*$^{-/-}$ BM cells than in those transplanted with WT BM cells (Fig. 2D). Moreover, although the development of IMQ-induced psoriasis-like dermatitis depends on IL-17 (7), dermal, but not epidermal, γδ T cells produced IL-17 after IMQ treatment (25) (Fig. 2E). These results suggest that CD96 on dermal γδ T cells, rather than epidermal γδ T cells, is involved in the pathogenesis of IMQ-induced dermatitis.

To directly demonstrate that CD96 on dermal γδ T cells, which are characterized by expression of the Vγ4$^{+}$ γδ TCR (26), is involved in the pathogenesis of IMQ-induced dermatitis, Vγ4$^{+}$ γδ T cells isolated from the spleen of WT or *Cd96*$^{-/-}$ mice were injected into the shaved dorsal skin of *Rag1*$^{-/-}$ mice. IMQ was then applied to the skin once a day for the next 4 consecutive days (Fig. 2F). Although all *Rag1*$^{-/-}$ mice developed dermatitis after IMQ treatment, the dermatitis scores and epidermal thickening were greater in *Rag1*$^{-/-}$ mice injected with the WT γδ T cells than in those that did not receive the γδ T cells (Fig. 2G, 2H). Importantly, *Rag1*$^{-/-}$ mice injected with WT γδ T cells showed higher dermatitis scores and greater epidermal thickening than did *Rag1*$^{-/-}$ mice injected with *Cd96*$^{-/-}$ γδ T cells (Fig. 2G, 2H). Furthermore, *Rag1*$^{-/-}$ mice injected with *Cd96*$^{-/-}$ γδ T cells showed dermatitis scores and epidermal thickening after IMQ treatment that were comparable to *Rag1*$^{-/-}$ mice that did not receive the γδ T cells (Fig. 2G, 2H). These results suggest that CD96 plays a pivotal role in the function of dermal γδ T cells in the development of IMQ-induced psoriasis-like dermatitis.

### *CD96 on dermal γδ T cells augments IL-17A expression after IMQ treatment*

We next investigated how CD96 on dermal γδ T cells exacerbates IMQ-induced dermatitis. We found no significant difference in expression of *Il23R* and *Il12Rβ1* in dermal γδ T cells between WT



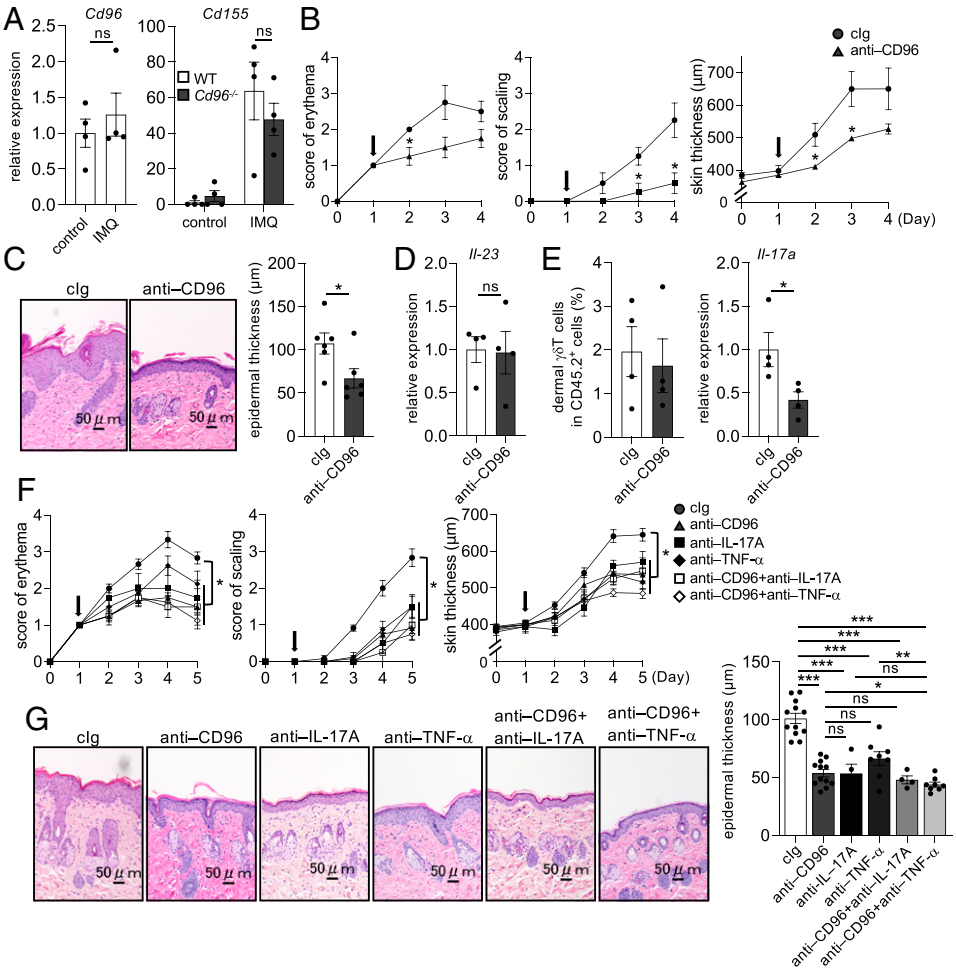


**FIGURE 5.** Blockade of CD96 ameliorates IMQ-induced psoriasis-like dermatitis. WT or *Cd96*$^{-/-}$ mice were treated once a day with IMQ or petroleum jelly on the shaved dorsal skin for 3 d. (**A**) Quantitative real-time PCR analysis of *Cd96* mRNA levels of dermal γδ T cells in WT mice ($n = 4$ in each column) and *Cd155* mRNA levels in nonhematopoietic cells in WT and *Cd96*$^{-/-}$ mice ($n = 4$ in each column). (**B**–**E**) Mice were i.p. injected with 100 μg of cIg or anti-CD96 mAb on day 1 after treatment with IMQ or petroleum jelly. (B) Changes in erythema, scaling, and thickening of dorsal skin ($n = 4$ in each group). (C) Representative histology (H&E staining) and epidermal thickening ($n = 6$ in each group) of mouse dorsal skin on day 4. (D) *Il23* mRNA levels of dermal myeloid cells on day 4 ($n = 4$ in each group). (E) The proportion of dermal γδ T cells in CD45.2$^{+}$ cells of dorsal skin ($n = 4$ in each group). *Il17a* mRNA levels of dermal γδ T cells on day 4 ($n = 4$ in each group) are shown. (**F** and **G**) WT mice were treated once a day with IMQ cream on the shaved dorsal skin from day 0 to day 4. On day 1, mice were i.p. injected with 100 μg of cIg, anti-CD96 mAb, anti–IL-17A mAb, anti–TNF-α mAb, anti-CD96 mAb plus anti–IL-17A mAb, or anti-CD96 mAb plus anti–TNF-α mAb. (F) Changes in erythema, scaling, and thickening of dorsal skin ($n = 4$–12 in each group). (G) Representative histology of dorsal skin (H&E staining) and epidermal thickness of dorsal skin on day 5 ($n = 4$–12 in each group). Error bars indicate SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns, not significant. Data are representative from two independent experiments (B) or pooled from three experiments (F and G).

mice and *Cd96*$^{-/-}$ mice that had not been treated with IMQ (Supplemental Fig. 2). However, in both skin and draining lymph nodes (dLNs), *Il17a* expression in dermal γδ T cells and the numbers of IL-17A–producing dermal γδ T cells were lower in *Cd96*$^{-/-}$ mice than in WT mice on day 3 after IMQ treatment (Fig. 3A–C). Moreover, γδ T cells in the dLNs of WT mice showed upregulated expression of T cell activation markers CD69 and CD25 compared with those of *Cd96*$^{-/-}$ mice after IMQ treatment (Fig. 3D). Because γδ T cells in dLNs were derived from the dermis after IMQ treatment (27), these results suggest that CD96 augments activation of, and IL-17A production by, dermal γδ T cells.

To analyze whether CD96 has an intrinsic or extrinsic effect on the activation of dermal γδ T cells, we generated mixed BM chimeric mice by transplantation of BM from Ly5.1$^+$ WT mice and Ly5.2$^+$ *Cd96*$^{-/-}$ mice at a 1:1 ratio into lethally irradiated Ly5.1$^+$Ly5.2$^+$ mice (Fig. 3E). The mixed BM chimeric mice had a comparable number of WT and *Cd96*$^{-/-}$ γδ T cells in the dLNs 12 wk after BM transplantation (Fig. 3F). After treatment with IMQ for 3 d, *Il17a* expression was lower in *Cd96*$^{-/-}$ γδ T cells than in WT cells of the BM chimeric mice (Fig. 3G). These results suggest that CD96 on dermal γδ T cells augmented IL-17A production in a cell-intrinsic manner after IMQ treatment.

### *CD96 mediates a costimulatory signal in γδ T cells*

To investigate how CD96 augments production of IL-17A by γδ T cells, we stimulated γδ T cells isolated from the spleen with plate-coated anti-CD3 mAb in combination with or without anti-CD96 mAb. We found that stimulation with anti-CD3 mAb upregulated CD69 and CD25 expressions, which was further augmented by additional stimulation with anti-CD96 mAb (Fig. 4A). However, addition of IL-23 to the culture had no effect on the expression of CD69 and CD25 (Fig. 4A). In contrast, *Il17* expression required IL-23 stimulation in γδ T cells (Fig. 4B). Moreover, IL-23–induced *Il17* expression was higher in γδ T cells stimulated with anti-CD3 mAb together with anti-CD96 mAb than in those stimulated with anti-CD3 mAb alone (Fig. 4B). Taken together, these results demonstrated that CD96 mediates a costimulatory signal for activation and IL-17A expression in splenic γδ T cells.

To examine whether this is also the case for dermal γδ T cells, we isolated these cells from the skin and induced them to proliferate by stimulating with IL-2, IL-7, and IL-15, and them stimulated them with anti-CD3 mAb together with or without anti-CD96 mAb in the presence of IL-23. We observed that the expressions of CD69, CD25, and *Il17* were higher in dermal γδ T cells stimulated with anti-CD96 mAb and anti-CD3 mAb than in those stimulated with anti-CD3 mAb alone in the presence of IL-23 (Fig. 4C, 4D). Moreover, Erk phosphorylation was higher in splenic γδ T cells stimulated with anti-CD96 mAb and anti-CD3 mAb than in those stimulated with anti-CD3 mAb alone (Fig. 4E). Taken together, these results suggest that CD96 mediates a costimulatory signal for activation of, and IL-17A expression by, γδ T cells in the skin as well as in the spleen.

### *Blockade of CD96 ameliorates IMQ-induced psoriasis-like dermatitis*

To examine whether CD96 can be a potential target for immunotherapy against psoriasis, we generated an anti-mouse CD96 neutralizing mAb (Supplemental Fig. 3A), which was able to inhibit the binding of CD96 to the soluble form of CD155 (CD155-Fc, the



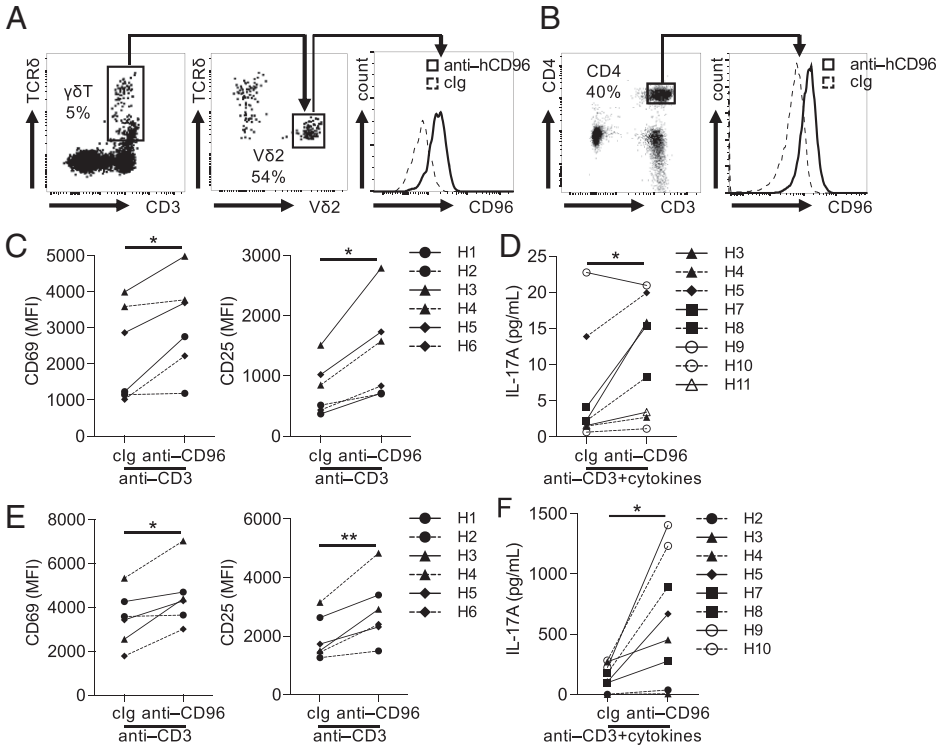


**FIGURE 6.** CD96 delivers a costimulatory signal in human γδ T cells and CD4$^+$ T cells. (**A** and **B**) Representative CD96 expression profile of human Vδ2$^+$ γδ T cells (A) and CD4$^+$ T cells (B). (**C**) Vδ2$^+$ γδ T cells derived from the peripheral blood of six healthy donors (H1–H6) were stimulated with anti-CD3 mAb and either anti-CD96 mAb or cIg for 18 h and analyzed for the expression of CD69 and CD25. (**D**) Vδ2$^+$ γδ T cells derived from eight healthy donors (H3–H5 and H7–H11) were stimulated with anti-CD3 mAb and either anti-CD96 mAb or mouse IgG1 (cIg) in the presence of TGF-β, IL-1β, IL-23, IL-6, and IL-12 for 48 h and analyzed for IL-17A production in the culture. (**E**) CD4$^+$ T cells derived from six healthy donors (H1–H6) were stimulated with anti-CD3 mAb and either anti-CD96 mAb or cIg for 18 h and analyzed for CD69 and CD25 expressions. (**F**) CD4$^+$ T cells derived from eight healthy donors (H2–H5 and H7–H10) were stimulated with anti-CD3 mAb and either anti-CD96 mAb or cIg in the presence of TGF-β, IL-1β, IL-23, IL-6, and IL-12 for 96 h and analyzed for IL-17A production in the culture. Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$.

fusion protein of the extracellular portion of CD155 with the Fc portion of human IgG) (Supplemental Fig. 3B). We found that although *Cd96* expression was not changed on dermal γδ T cells in mice 3 d after being treated with IMQ, *Cd155* expression was more than 50-fold upregulated in the skin tissue after IMQ application (Fig. 5A), suggesting that the contribution of CD96 to the pathogenesis of IMQ-induced psoriasis-like dermatitis is augmented as a result of increased CD96-mediated signaling in dermal γδ T cells. Indeed, treatment with the anti-CD96 mAb by i.p. injection on day 1 after IMQ application decreased dermatitis scores such as erythema and scaling as well as skin thickening compared with treatment with a control Ab (control Ig [cIg]) (Fig. 5B). Histological analyses also showed that IMQ-induced epidermal thickening was milder in mice treated with the anti-CD96 mAb than in those treated with cIg (Fig. 5C). Treatment with the anti-CD96 mAb did not alter *Il23* mRNA levels in dermal myeloid cells (Fig. 5D). Although the proportion of dermal γδ T cells in CD45.2$^+$ cells was comparable between cIg or anti-CD96 mAb treatment, the *Il17a* mRNA expression level in dermal γδ T cells was significantly reduced by treatment with the anti-CD96 mAb (Fig. 5E).

Next, the effect of the anti-CD96 neutralizing mAb on dermatitis was compared with the effect of anti–IL-17A mAb or anti–TNF-α mAb, because anti-human IL-17A mAb and anti-human TNF-α mAb are currently used for treatment of patients with psoriasis. The effect of anti-CD96 mAb on the dermatitis was comparable to that of anti–IL-17A mAb and anti–TNF-α mAb (Fig. 5F, 5G). Of note, although the combination of anti-CD96 mAb plus anti–IL-17A mAb had a comparable effect on dermatitis to that of each mAb alone, the combination of anti-CD96 mAb plus anti–TNF-α mAb resulted in milder epidermal thickening than did either of the mAbs alone (Fig. 5F, 5G). These results suggest that the blockade of CD96 ameliorated IMQ-induced dermatitis via suppression of IL-17A production by dermal γδ T cells, and thus the anti-CD96 mAb has an additive effect in combination with the anti–TNF-α mAb, but not in combination with the anti–IL-17A mAb.

### *CD96 delivers a costimulatory signal in human γδ T cells and CD4$^+$ T cells*

γδ T cells and Th17 cells play crucial roles in the pathogenesis of psoriasis in humans (2, 3, 28, 29). Because circulating Vγ9Vδ2 cells are rapidly recruited to inflamed skin to produce IL-17A in psoriasis patients (30), we first examined the expression of CD96 on Vδ2$^+$ γδ T cells in PBMCs. Flow cytometry analysis demonstrated that CD96 was expressed on Vδ2$^+$ γδ T cells derived from PBMCs (Fig. 6A). We also found that CD4$^+$ T cells in the PBMCs expressed CD96 (Fig. 6B). Stimulation of isolated Vδ2$^+$ γδ T cells and CD4$^+$ T cells with plate-coated anti-CD3 mAb together with plate-coated anti-CD96 mAbs enhanced the expression of T cell activation markers CD69 and CD25 compared with stimulation with plate-coated anti-CD3 mAb alone (Fig. 6C, 6E). Stimulation of these isolated cells with plate-coated anti-CD3 mAb together with plate-coated anti-CD96 mAb in the presence of IL-23 and several cytokines enhanced the expression of IL-17A compared with stimulation with plate-coated anti-CD3 mAb alone in the presence of IL-23 (Fig. 6D, 6F). These results demonstrated that CD96 mediates a costimulatory signal for IL-17A production also in human Vδ2$^+$ γδ T cells and CD4$^+$ T cells and is likely to be involved in the pathogenesis of psoriasis in humans (Fig. 7).

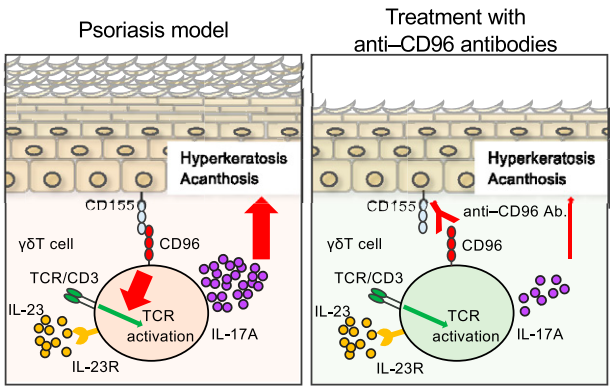


**FIGURE 7.** Proposed model. The CD96-mediated signal costimulates the TCR signal and upregulates IL-17A production from γδ T cells, which in turn exacerbates the IMQ-induced psoriasis-like dermatitis. Consistent with this model, the pathology of IMQ-induced psoriasis-like dermatitis is suppressed when CD96-mediated signaling is blockaded by using an anti-CD96 mAb.

## Discussion

We demonstrated that CD96 mediated a costimulatory signal in both human and mouse γδ T cells and enhanced IL-17A production, which in mice resulted in the exacerbation of IMQ-induced psoriasis-like dermatitis (Fig. 7).

The intracellular domain of human and mouse CD96 contains an ITIM, which interacts with SHP-1, SHP-2, and/or SHIP that suppress cell activation (19, 31). Although human CD96 also has a PI3K-binding motif (YxxM), mouse CD96 does not have this motif but instead has an immunoreceptor tyrosine tail–like motif (YxN) (19, 31), which recruits growth factor receptor–bound protein 2 (Grb2), leading to activation of Vav-1, the PI3K–AKT pathway, and phospholipase C-γ1 (32). Accordingly, CD96 likely mediates its costimulatory signal via the PI3K-binding motif in human γδ T cells and via the immunoreceptor tyrosine tail–like motif in mouse γδ T cells. However, it remains undetermined how CD96 activated these motifs, rather than the ITIM, in human and mouse dermal γδ T cells in vitro and in IMQ-induced dermatitis in mice. Previous studies have demonstrated that antitumor immunity mediated by NK cells is augmented in CD96-deficient mice (10, 17), suggesting that CD96 delivers an inhibitory signal in NK cells. Whether CD96 induces an activating or an inhibitory signal may be dependent on cell types and/or microenvironmental factors such as ligand expression and inflammatory state.

γδ T cells are considered to be innate immune cells that rapidly produce IL-17A following stimulation with IL-23 or IL-1β (33, 34). In mice with IMQ-induced psoriasis-like dermatitis, the major IL-17A producers are dermal γδ T cells (3). In patients with psoriatic disease, in contrast, the main sources of IL-17A are Vδ2$^+$ γδ T cells and Th17 cells (2, 30). We showed that CD96 delivered a costimulatory signal and enhanced IL-17A production from both Vδ2$^+$ γδ T cells and CD4$^+$ T cells in human PBMCs. We showed that blockade of the CD96-mediated signal with an anti-CD96 neutralizing mAb ameliorated psoriasis-like dermatitis by suppressing IL-17A production from dermal γδ T cells in mice (Fig. 7). This may also be the case in patients with psoriasis, in which blockade of CD96 may suppress IL-17A production by γδ T cells and CD4$^+$ T cells. These results suggest that CD96 may be a good candidate as a molecular target for the treatment of IL-17A–related inflammatory diseases, including psoriasis, autoimmune diseases (e.g., multiple sclerosis, rheumatoid arthritis), and fibrosis in various organs.

Importantly, although the CD96 ligand CD155 is ubiquitously expressed on both hematopoietic and nonhematopoietic cells at a weak level under normal physiological conditions (14, 15), it was strongly upregulated in the inflamed skin regions after IMQ treatment, suggesting that CD96-mediated signaling is enhanced in dermal γδ T cells, which further exacerbates dermatitis, in a vicious

circle. Therefore, an anti-CD96 neutralizing mAb may break this vicious circle, particularly in the inflamed regions, in which CD155 expression is upregulated. Thus, anti-CD96 neutralizing mAb is considered to have an effect mainly in the inflamed tissue. In contrast, mAbs against IL-23, IL-17A, TNF-α, and their receptors have broad systemic effects in addition to their action on affected skin regions and can cause side effects such as severe infections.

In addition, CD155 is a ligand for the activating receptor DNAM-1 (DNAX accessory molecule 1, CD226) (23, 35, 36) and the inhibitory receptor TIGIT (T cell Ig and ITIM domain) (37). TIGIT is not expressed on γδ T cells, but DNAM-1 is highly expressed on γδ T cells (38). Therefore, CD155 may enhance the DNAM-1–mediated activation signal in γδ T cells when CD96-mediated signaling is deficient in *CD96*$^{-/-}$ mice or in mice treated with an anti-CD96 neutralizing mAb. However, γδ T cell activation and psoriasis were suppressed in *CD96*$^{-/-}$ mice and by using CD96 neutralizing Abs. This means that CD96-mediated signal transduction promotes γδ T cell activation regardless of the contribution of DNAM-1 in γδ T cells.

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## Disclosures

The authors have no financial conflicts of interest.

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