Programmed cell death ligand 1 alleviates psoriatic inflammation by suppressing IL-17A production from programmed cell death 1–high T cells

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Background: Psoriasis is one of the most common chronic inflammatory diseases of the skin. Recently, IL-17–producing T cells have been shown to play a critical role in psoriatic inflammation. Programmed cell death 1 (PD-1) is a coinhibitory receptor expressed on T cells in various chronic inflammatory diseases; however, the expression and function of PD-1 during psoriatic inflammation have not previously been characterized.
Objective: We examined PD-1 expression on IL-17A–producing T cells from imiquimod-treated mice and patients with psoriasis. Additionally, we investigated the therapeutic effect of recombinant programmed cell death ligand 1 (PD-L1) protein on imiquimod-induced psoriatic inflammation.
Methods: PD-1 expression on IL-17A–producing γδ T cells from imiquimod-treated mice was examined by means of multicolor flow cytometric analysis. In the psoriatic skin of patients, PD-1 and IL-17A expression was analyzed by using immunofluorescence. The therapeutic effect of PD-L1–Fc fusion protein (PD-L1-Fc) was assessed in imiquimod-treated mice *ex vivo* and *in vivo*.
Results: During imiquimod-induced psoriatic inflammation, PD-1 is overexpressed on $CD27^{-}V\gamma1^{-}$ γδ T cells. Furthermore, PD-1 expression on $IL\text{-}17A^{+}$ T cells was confirmed in psoriatic skin tissues from patients and imiquimod-treated mice. In the $CD27^{-}V\gamma1^{-}$ γδ T-cell population, $V\gamma4^{-}$ γδ T cells with Vγ6 mRNA expression showed a high level of PD-1 expression. Furthermore, these $PD\text{-}1^{hi}V\gamma4^{-}$ ($V\gamma6^{+}$) γδ T cells were specialized for anti-CD3–induced IL-17A production, which was inhibited by PD-L1-Fc treatment. In imiquimod-treated mice PD-L1-Fc reduced psoriatic inflammation when given alone and enhanced the therapeutic effect of anti-p40 when given in combination.
Conclusion: PD-1 is overexpressed in IL-17A–producing T cells in both imiquimod-treated mice and patients with psoriasis. Moreover, recombinant PD-L1-Fc alleviates psoriatic inflammation in imiquimod-treated mice. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Psoriasis, programmed cell death 1, programmed cell death ligand 1, IL-17A, T cell*

Psoriasis is one of the most common chronic inflammatory skin diseases and manifests as relapsing scaling plaques and erythematous patches on the skin. Histologic findings show proliferation of keratinocytes, massive lymphocytic infiltration, and neutrophil condensation called Munro microabscess.[1] Although IL-17–mediated inflammation plays a major role in the pathogenesis of psoriasis, it is not fully understood how T cells are stimulated to produce IL-17.[2] During the initiation of psoriasis, dendritic cells are known to be activated by Toll-like receptor (TLR) 7 and TLR8, which can be stimulated by the antimicrobial peptide LL37 and RNA complexes.[3] Similarly, when the TLR7/8 ligand imiquimod is repeatedly applied to murine skin, psoriasis-like inflammation presents both clinically and histologically.[4] In this imiquimod-induced model of psoriasis, it is IL-17–secreting γδ T (γδT17) cells that predominantly contribute to psoriatic inflammation.[5,6]

IL-23 and IL-1β are well known to serve critical roles in the induction and activation of IL-17–producing T cells in patients with psoriasis[7] and imiquimod-induced psoriatic inflammation.[5,6] Indeed, γδT17 cells respond to produce IL-17 rapidly on IL-23 and IL-1β stimulation in mice.[8] Therefore IL-23 has been targeted for treatment of psoriasis by using an antibody against p40, a common subunit of IL-23 and IL-12.[9] In addition to stimulation with IL-23 and IL-1β, T-cell receptor (TCR) engagement also contributes to activation of pathogenic T cells in patients with psoriasis. In fact, oligoclonality of T cells is observed in skin lesions of patients with psoriasis.[10] Moreover, T cells recognizing the autoantigens keratin 17 and LL37 have been detected in patients with psoriasis and are believed to contribute to disease pathogenesis.[11-13]

Programmed cell death 1 (PD-1) is a coinhibitory receptor, which, on interaction with its ligands (ie, programmed cell death ligand 1 [PD-L1; B7-H1] or programmed cell death ligand 2 [PD-L2; B7-DC]), inhibits TCR-mediated signaling by recruiting the tyrosine phosphatase SHP2.[14,15] PD-1 expression is increased

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Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication May 21, 2015; revised November 5, 2015; accepted for publication November 20, 2015.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.11.021

Abbreviations used
APC: Allophycocyanin
FITC: Fluorescein isothiocyanate
gMFI: Geometric mean fluorescence intensity
LN: Lymph node
PD-1: Programmed cell death 1
PD-L1: Programmed cell death ligand 1
PD-L1-Fc: PD-L1-Fc fusion protein
PE: Phycoerythrin
γδT17: IL-17–secreting γδ T
TCR: T-cell receptor
TLR: Toll-like receptor

by continuous or repetitive TCR stimulation of T cells,[14] and thus PD-1 expression on T cells is observed in various chronic inflammatory conditions.[15] Furthermore, inflammation is exacerbated by the absence or blockade of PD-1, and in contrast, administration of recombinant PD-L1 protein attenuates disease severity in murine models of colitis[16] and arthritis.[17] Nevertheless, it remains to be elucidated whether PD-1 is overexpressed on T cells in patients with psoriasis and whether recombinant PD-L1 protein alleviates psoriatic inflammation.

In the present study we examined PD-1 expression on IL-17–producing T cells in imiquimod-induced psoriatic inflammation of mice and in psoriatic lesions from patients. In particular, we investigated which subset of γδT17 cells overexpresses PD-1. We also studied the therapeutic effect of recombinant PD-L1 protein in the imiquimod-induced model of psoriatic inflammation.

METHODS

Mice

C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Me) and bred in-house in a specific pathogen-free animal facility at the Korea Advanced Institute of Science and Technology (KAIST). All housing, breeding, and experimental procedures involving mice were approved by the Animal Care Committee of KAIST.

Imiquimod-induced model of psoriatic inflammation and *in vivo* treatment

Mice were used at 8 to 12 weeks of age. Forty milligrams of imiquimod cream 5% (Aldara; 3M Pharmaceuticals, Leicestershire, United Kingdom) or a vehicle cream (Zeroid; Neopharm, Daejeon, Korea) was applied daily on the skin of either ears or the back for 6 consecutive days. Ear thickness was measured with a dial thickness gauge caliper (Peacock G; Ozaki MFG, Tokyo, Japan) daily.

For measuring ear thickness *in vivo* PD-L1 treatment study, imiquimod or vehicle cream (20 mg/mouse/d) was applied on both ears daily. PD-L1-Fc fusion protein (PD-L1-Fc; 200 μg; Genexine, Seongnam, South Korea)[16] or anti-p40 (100 μg; eBioscience, San Diego, Calif) was administrated intraperitoneally on days 1, 2, 4, and 6. For immunofluorescent staining and mRNA analysis, imiquimod or vehicle cream (30 mg/mouse/d) was applied on the back daily. PD-L1-Fc (20 μg) or anti-p40 (10 μg) was injected intradermally into the back every 12 hours. On day 7, mice were killed and analyzed.

Cell preparation from skin tissue

Whole skin cells were prepared from mouse ear skin. The cartilage of the ear was removed, and dorsal and ventral pieces were separated. For separation of the epidermis and dermis, ear halves were incubated in 2 mg/mL Dispase II (Roche, Indianapolis, Ind) for 30 minutes at 37°C. The dermis was cut into small pieces and digested with RPMI containing 10% FBS, 1 mg/mL Collagenase IV (Roche), and 0.1 mg/mL DNase I (Roche) for 1 hour at 37°C. Single-cell suspensions were prepared with a syringe.

Cell-surface staining, intracellular cytokine staining, and flow cytometric analysis

Cells were obtained from skin tissues or draining lymph nodes (LNs) and Fc receptors blocked with anti-CD16/32 (eBioscience). Fluorochrome-labeled mAbs for surface staining included anti-CD3e–V500 (500A2), PerCP-Cy5.5, or allophycocyanin (APC)–Cy7 (145-2C11), anti-TCRβ-V450 or Alexa Fluor 700 (H57-597), anti-TCRγδ–phycoerythrin (PE) or fluorescein isothiocyanate (FITC; GL3), anti-Vγ4–FITC (UC3-10A6), anti-Vγ5–FITC (536), anti-CD27–V450 or PE (LG.3A10), anti-CD19–Alexa Fluor 700 (1D3), anti–PD-1–PE (J43), anti–inducible T-cell costimulator–PE (17G9), and anti-CD44–PE (all from BD Biosciences, Franklin Lakes, NJ); anti-CD127–PE (A7R34; eBioscience); and anti-TCRγδ–PerCP-Cy5.5 or PE-Cy7 (GL3), anti-Vγ1–FITC or APC (2.11), anti-Vγ4–PE or APC (UC3-10A6), anti-Vγ5–APC (536), anti-CD127–PE-Cy7 (A7R34), anti-CD43–APC-Cy7 and anti–PD-1–PE-Cy7 (29F. 1A12; all from BioLegend, San Diego, Calif). For intracellular staining, anti–IL-17A–APC-Cy7 (TC11-18H10, BD Biosciences) was used. Dead cell staining was performed with either ethidium monoazide (Invitrogen, Carlsbad, Calif), Live/Dead aqua, or red fluorescent reactive dye (Invitrogen). Cells were fixed and permeabilized according to the manufacturer's recommendation (BD Biosciences and eBioscience). Cells from LNs were stimulated with recombinant IL-1β (10 ng/mL; PeproTech, Rocky Hill, NJ) and recombinant IL-23 (50 ng/mL, eBioscience) or anti-mouse CD3e (2 μg/mL, BD Biosciences) precoated in 96-well plates for total of 6 hours and in the presence of brefeldin A (GolgiPlug, BD Biosciences) for 4 of those hours at 37°C. To evaluate the effect of PD-L1 treatment, 96-well plates were precoated with PD-L1-Fc (2 or 8 μg/mL; Genexine) or Fc control protein (8 μg/ml; Genexine) overnight at 4°C. Stained cells were acquired with an LSR II (BD Biosciences), and fluorescence-activated cell sorting analysis was performed with FlowJo software (Tree Star, Ashland, Ore).

Cell sorting

For molecular analysis of Vγ subtypes, pooled LN cells were labeled with anti-CD4, anti-CD8, anti-CD11c, and anti-B220 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and labeled cells were depleted with an LD column (Miltenyi Biotec), according to the manufacturer's instructions. The negative cell population was stained with anti-TCRγδ–PE, anti-TCRβ–V450, anti-CD27–V450, anti-CD3ε–APC-Cy7, anti-Vγ1–FITC, anti-Vγ4–APC, and ethidium monoazide, and $CD27^{-}V\gamma1^{-}V\gamma4^{+}$ and $CD27^{-}V\gamma1^{-}V\gamma4^{-}$ T cells were sorted with a FACSAria II (BD Biosciences). The purity of isolated populations was 99.0% and 98.6%, respectively. For *in vitro* PD-L1 treatment assays, γδ T cells were sorted from ear draining LNs by using a TCRγδ isolation kit (Miltenyi Biotec), according to the manufacturer's recommendations. The purity of isolated γδ T cells was greater than 97%.

RNA extraction and real-time quantitative PCR

RNA was extracted with TRIzol (Invitrogen), according to the manufacturer's protocol. The quantity of RNA was confirmed with a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, Mass). cDNA was synthesized with a cDNA Synthesis Master Mix (Legene, San Diego, Calif). For molecular subtyping of Vγ, SYBR Green PCR was performed in triplicate to determine mRNA levels in Vγ chains. The amount of specific mRNA was calculated by using Sequence Detector software (Applied Biosystems, Foster City, Calif) by using standard curves and normalized to mean levels of β-actin mRNA. A combination of the following primers was used. Forward primers were as follows: Vγ1/2, 5′-ACA CAG CTA TAC ATT GGT AC-3′; Vγ4, 5′-TGT CCT TGC AAC CCC TAC CC-3′; Vγ5, 5′-TGT GCA CTG GTA CCA ACT GA-3′; Vγ6, 5′-GGA ATT CAA AAG AAA ACA TTG TCT-3′; Vγ7, 5′-AAG CTA GAG GGG TCC TCT GC-3′; and β-actin, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. Reverse primers were as follows: Cγ, 5′-CTT ATG GAG ATT TGT TTC AGC-3′; and

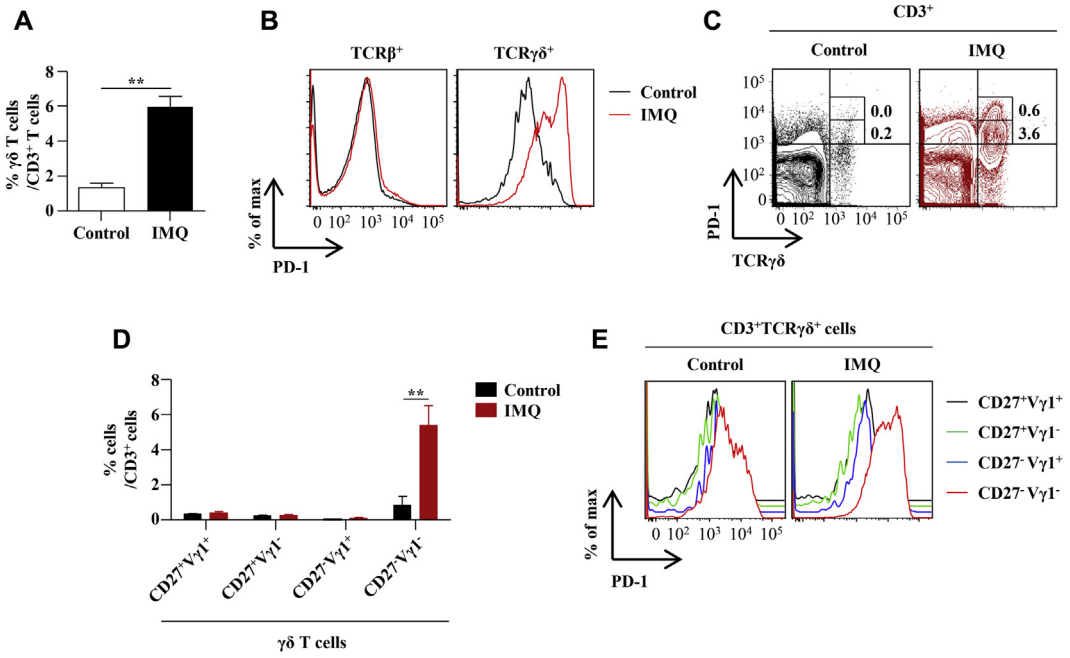

FIG 1. PD-1 expression on CD27$^-$Vγ1$^-$ γδ T cells in imiquimod *(IMQ)*–treated mice. Cells were isolated from ear draining LNs on day 7 (n = 24). **A,** The relative frequency of γδ T cells in the CD3$^+$ T-cell population was analyzed. **B,** PD-1 expression of TCRγδ$^+$ and TCRβ$^+$ CD3$^+$ T cells was examined. **C,** PD-1 expression in the CD3$^+$ T-cell population was analyzed. PD-1^{hi} and PD-1^{lo} cells were distinctly detected in the TCRγδ$^+$ T-cell population in imiquimod-treated mice. **D,** The relative frequencies of CD27$^+$Vγ1$^+$, CD27$^+$Vγ1$^-$, CD27$^-$Vγ1$^+$, and CD27$^-$Vγ1$^-$ γδ T cells in the CD3$^+$ T-cell population were analyzed. **E,** PD-1 expression of CD27$^+$Vγ1$^+$, CD27$^+$Vγ1$^-$, CD27$^-$Vγ1$^+$, and CD27$^-$Vγ1$^-$ γδ T cells was compared by overlaying histograms. $^{**}P < .005$.

β-actin, 5′-TGG AAT CCT GTG GAC TCC ATG AAA C-3′. For evaluation of IL-17A mRNA levels, mouse back tissues were homogenized with Precellys 24 homogenizer (Bertin technologies, Montigny-le-Bretonneux, France), RNA was extracted, and cDNA was synthesized, as described above. TaqMan real-time PCR was performed to determine mRNA levels of IL-17A and CD45 by using the TaqMan gene expression assay (Applied Biosystems). IL-17A mRNA levels were normalized to CD45 mRNA levels.

Tissue preparation for histologic analysis

Mouse skin samples were fixed overnight in 10% formalin and embedded in paraffin. After deparaffinization, sections were stained with hematoxylin and eosin. Epidermal thickness was measured with MetaMorph software (Molecular Devices, Sunnyvale, Calif).

For immunofluorescent staining of skin samples, 10% formalin-fixed tissues were embedded in OCT compound before sectioning. Antigen retrieval was performed in citrate buffer (pH 6.0; DAKO, Glostrup, Denmark) before incubation with primary antibodies. Sectioned human normal and psoriatic tissues were stained overnight with mouse anti-human TCRγδ (5A6.E91, Thermo Fisher Scientific); mouse anti-human CD8 (HIT8a, BD Biosciences); mouse anti-human CD3 (PS1), mouse anti-human CD4 antibody, and polyclonal rabbit anti-human IL-17 (all from Abcam, Cambridge, United Kingdom); polyclonal goat anti-human PD-1 and polyclonal goat anti-human IL-17 (both from R&D Systems, Minneapolis, Minn); rabbit anti-human CD3 antibody, rabbit anti–c-kit antibody (both from DAKO); mouse anti-human neutrophil elastase (NP57; Santa Cruz, Dallas, Tex); and mouse anti-human CD56 antibody (Cell Signaling, Danvers, Mass) as primary antibodies. Sectioned mouse skin tissues were stained with hamster anti-mouse TCRγδ (UC7-13D5, eBioscience), rat anti-mouse IL-17 (50101) and polyclonal goat anti-mouse PD-1 (both from R&D Systems) as primary antibodies. Rabbit and goat IgG isotypes (both from R&D Systems) and mouse IgG isotype (Sigma, St Louis, Mo) were used as controls of primary antibodies. Alexa Fluor 488–conjugated donkey anti-rabbit IgG and Alexa Fluor 594–conjugated donkey anti-goat IgG (both from Jackson Immunoresearch, West Grove, Pa), Alexa Fluor 647–conjugated donkey anti-mouse IgG (Abcam), and Alexa Fluor 488–conjugated goat anti-rat IgG and Alexa Fluor 647–conjugated goat anti-mouse IgG and goat anti-hamster IgG (both from Molecular Probes, Carlsbad, Calif) were used as secondary antibodies. Sections were finally stained for 5 minutes with 4′-6-diamidino-2-phenylindole (Invitrogen). Images were captured with an LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany). The number of fluorescent cells from human skin was counted in whole dermis along the tile scans with a ×400 field. In mouse skin we calculated the average number of cells from 3 independent z-stacked images with a ×200 field.

Statistical analysis

We present all data as means with SDs. We used a paired *t* test to compare the geometric mean fluorescence intensity (gMFI) between PD-1^{lo} and PD-1^{hi} T cells. In other analyses we used the Student *t* test. *P* values of less than .05 were considered statistically significant.

RESULTS

PD-1 is expressed on expanded CD27$^-$Vγ1$^-$ γδ T cells in imiquimod-induced psoriatic inflammation

First, we studied expansion of γδ T cells and their PD-1 expression in the mouse model of imiquimod-induced psoriatic inflammation. As shown in Fig 1, the relative frequency of γδ T cells was increased in the CD3$^+$ T-cell population of the ear draining LNs (Fig 1, *A*), and PD-1 was overexpressed in γδ T cells but not in αβ T cells after imiquimod application (Fig 1, *B*). In particular, PD-1^{hi} and PD-1^{lo} cells were distinctly detected in γδ T cells from imiquimod-treated mice (Fig 1, *C*). Among γδ T cells, IL-17 is known to be produced by CD27$^-$ γδ

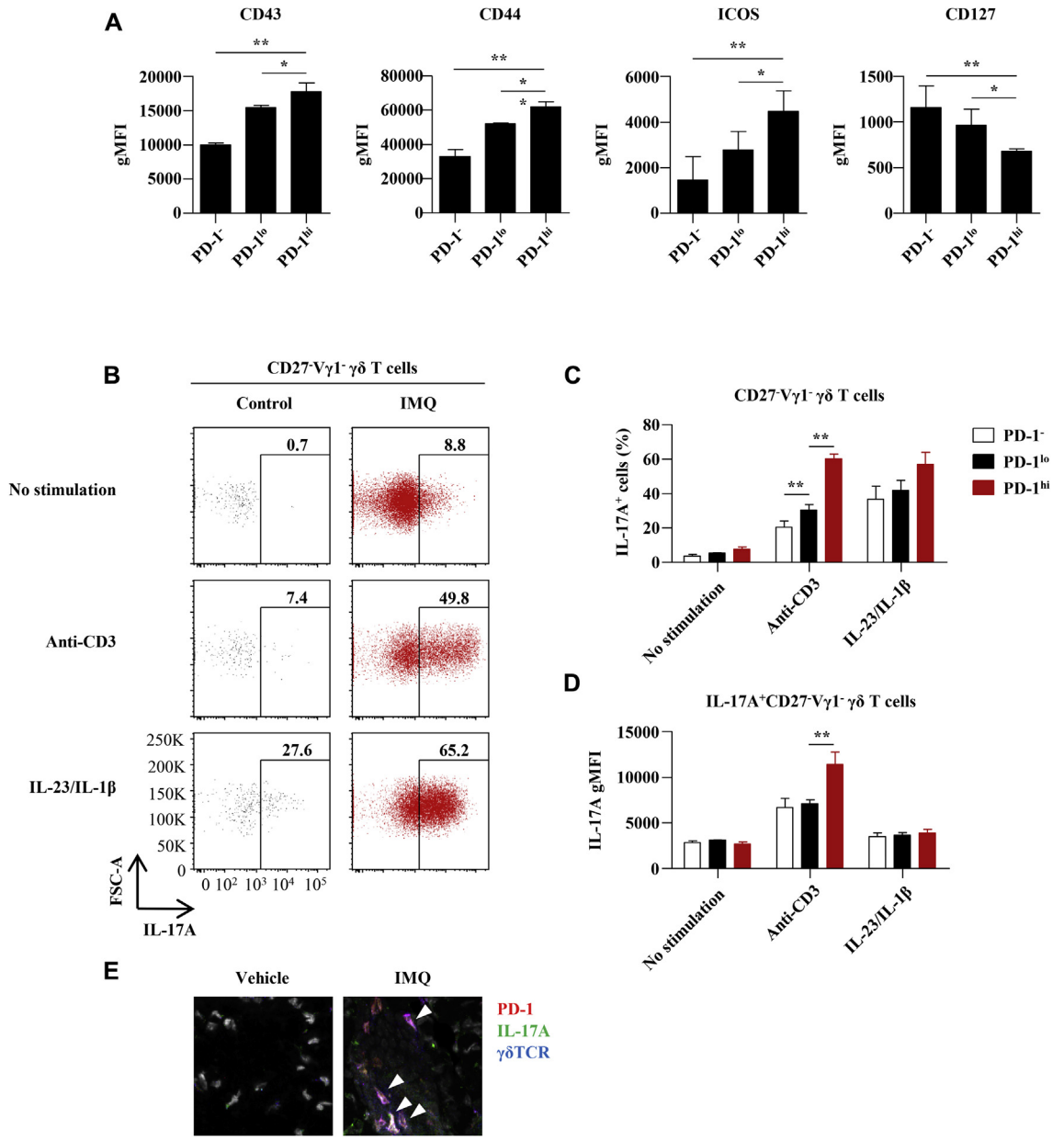

FIG 2. Surface phenotypes and IL-17A production of PD-1^{-}, PD-1^{lo}, and PD-1^{hi} subpopulations of CD27^{-}Vγ1^{-} γδ T cells from imiquimod *(IMQ)*–treated mice. Cells were isolated from ear draining LNs on day 7. **A,** gMFIs of CD43, CD44, inducible T-cell costimulator *(ICOS)*, and CD127 were analyzed in PD-1^{-}, PD-1^{lo}, and PD-1^{hi} subpopulations among CD27^{-}Vγ1^{-} γδ T cells (n = 3). **B-D,** IL-17A production was analyzed in CD27^{-}Vγ1^{-} γδ T cells after stimulation with anti-CD3 or IL-23/IL-1β and intracellular cytokine staining (n = 6). Representative flow cytometric plots are presented (Fig 2, *B*). The relative frequency of IL-17A^{+} cells (Fig 2, *C*) and gMFIs of IL-17A in IL-17A^{+} cells (Fig 2, *D*) were analyzed. **E,** Immunofluorescent staining of PD-1, IL-17A, and TCRγδ is presented. *White arrowheads* indicate triple-stained cells (IL-17A^{+}PD-1^{+} γδ T cells). **P* < .05 and ***P* < .005.

T cells, which express the TCRγ chains Vγ1, Vγ4, or Vγ6.[18,19] Thus we further analyzed T-cell expansion and PD-1 expression in γδ T-cell subsets according to CD27 and Vγ1 expression. The gating strategy for the flow cytometric analysis is presented in Fig E1 in this article's Online Repository at www.jacionline.org. The frequency of CD27^{-}Vγ1^{-} γδ T cells in the CD3^{+} T-cell population was significantly increased after imiquimod application (Fig 1, *D*), and PD-1 overexpression was predominantly detected in CD27^{-}Vγ1^{-} γδ T cells, particularly in imiquimod-treated mice (Fig 1, *E*). Collectively, these data show that PD-1 is overexpressed by expanded CD27^{-}Vγ1^{-} γδ T cells in imiquimod-treated mice.

PD-1^{hi} CD27^{-}Vγ1^{-} γδ T cells produce IL-17A after anti-CD3 stimulation

Because 3 distinct populations of γδ T cells (PD-1^{-}, PD-1^{lo}, and PD-1^{hi} cells) were detected in draining LNs from

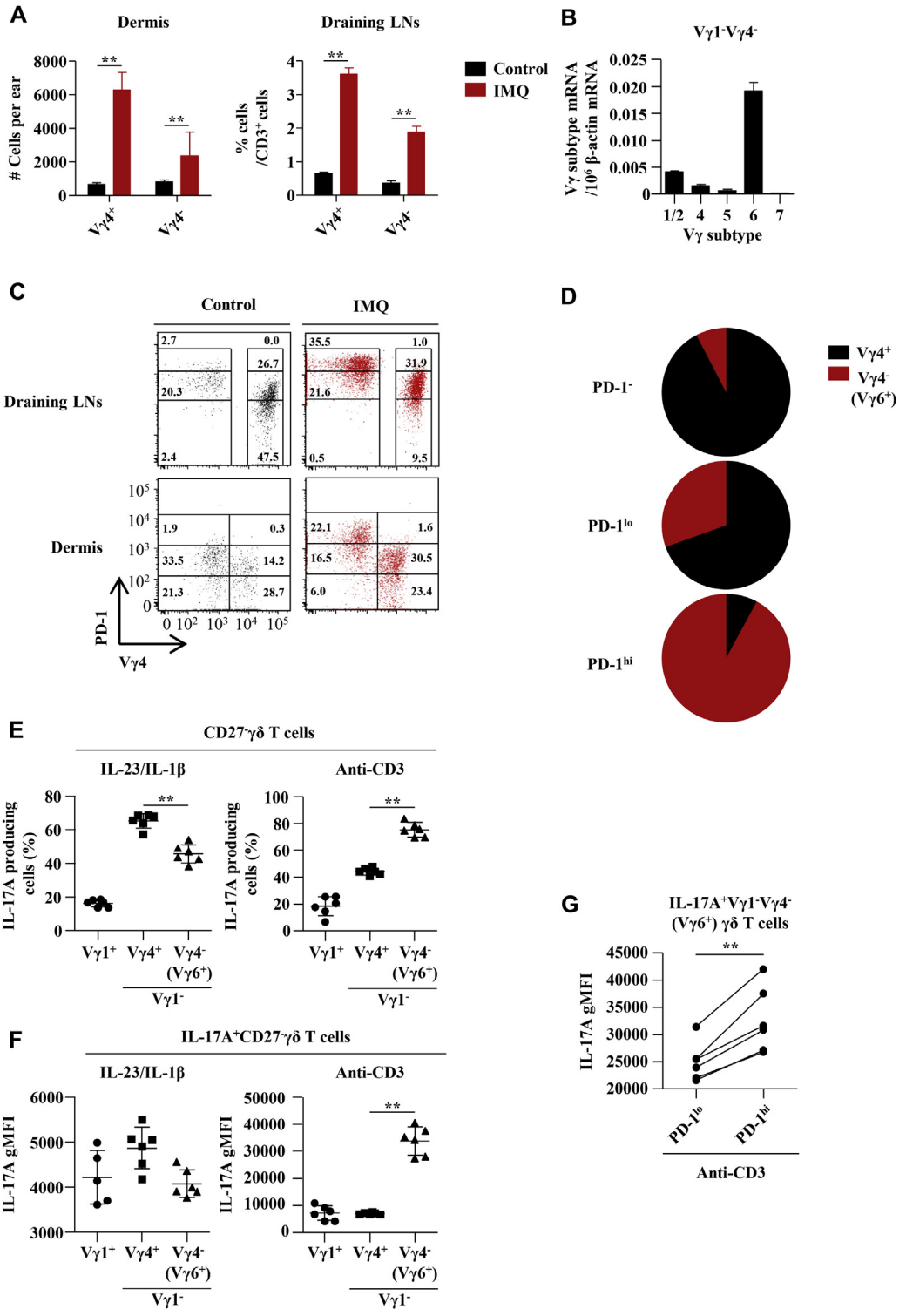

FIG 3. PD-1 expression and IL-17A production in CD27$^-$Vγ1$^-$ γδ T-cell population subsets from imiquimod *(IMQ)*–treated mice. Cells were isolated from the dermis or ear draining LNs on day 7. **A,** CD27$^-$Vγ4$^+$ and CD27$^-$Vγ4$^-$ γδ T cells in the dermis were quantified. The relative frequency of CD27$^-$Vγ1$^-$Vγ4$^+$ and CD27$^-$Vγ1$^-$Vγ4$^-$ γδ T cells were calculated in the CD3$^+$ cell population (n = 3). **B,** Expression of Vγ chain mRNA in CD27$^-$Vγ1$^-$Vγ4$^-$ γδ T cells was analyzed. **C,** A representative flow cytometric plot for PD-1 and Vγ4 expression in CD27$^-$Vγ1$^-$ γδ T cells is presented. PD-1^{hi} cells are detected in the Vγ4$^-$ cell population. **D,** Among CD27$^-$Vγ1$^-$ γδ T cells, frequencies of Vγ4$^+$ and Vγ4$^-$ (Vγ6$^+$) cells were analyzed in the PD-1$^-$, PD-1^{lo}, and PD-1^{hi} cell populations (n = 6). **E-G,** Intracellular staining for IL-17A production by CD27$^-$ γδ T cells was performed after stimulation with anti-CD3 or IL-23/IL-1β (n = 6). The relative frequency of IL-17A$^+$ cells (Fig 3, *E*) and gMFIs of IL-17A in IL-17A$^+$ cells (Fig 3, *F*) were analyzed in the Vγ1$^+$, Vγ1$^-$Vγ4$^+$, and Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) cell populations. After anti-CD3 stimulation, gMFIs of IL-17A in IL-17A$^+$ cells were compared between PD-1^{lo} and PD-1^{hi} cells in the Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) cell populations (Fig 3, *G*). $^{**}P < .005$.

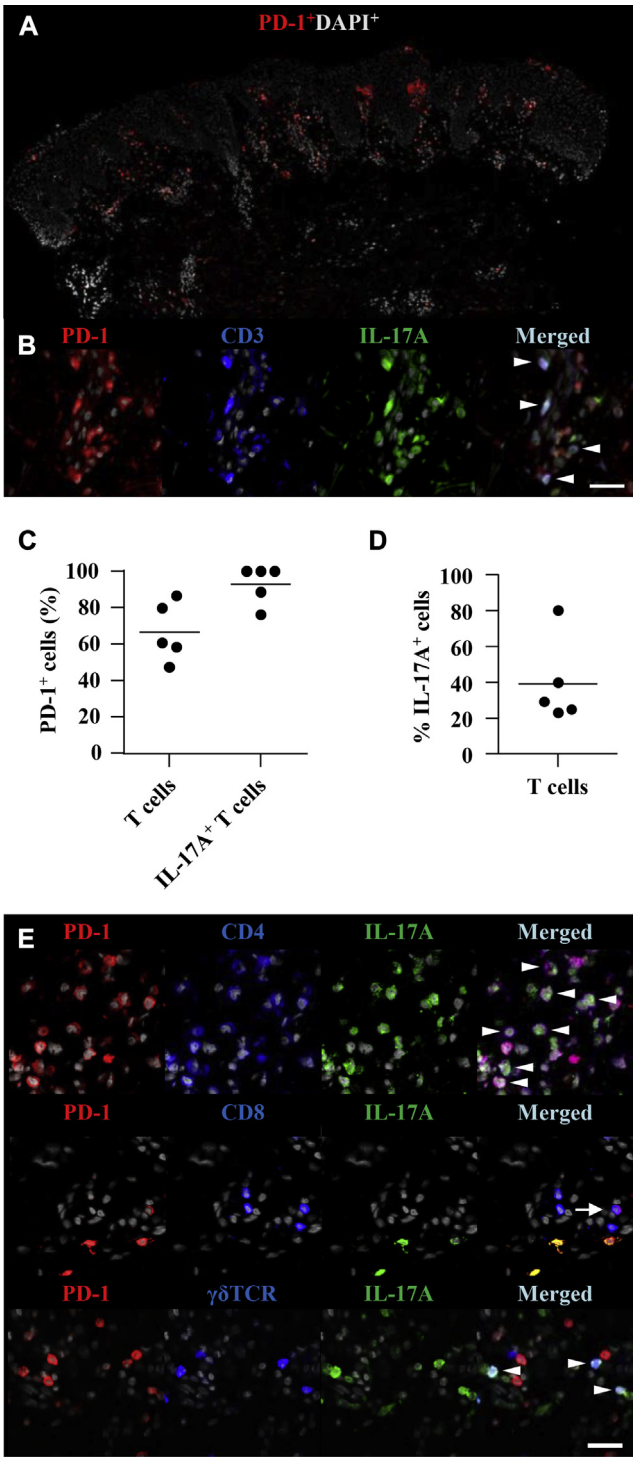

FIG 4. PD-1 expression on IL-17A$^+$ T cells in psoriatic skin of patients. **A-D,** Expression of PD-1, CD3, and IL-17A in skin biopsy specimens from patients with psoriasis was examined by means of immunofluorescence and confocal microscopy. Representative images from a single patient are presented (Fig 4, *A* and *B*). PD-1 expression was observed in the upper dermis (Fig 4, *A*). PD-1, IL-17A, and CD3 were costained (*scale bar* = 20 μm; Fig 4, *B*). Colocalization of PD-1, IL-17A, and CD3 is marked by *white arrowheads*. Colocalized PD-1$^+$ cells were quantified among CD3$^+$ T cells or IL-17A$^+$CD3$^+$ T cells (n = 5; Fig 4, *C*). Colocalized IL-17A$^+$ cells were quantified among CD3$^+$ T cells (n = 5; Fig 4, *D*). **E,** PD-1 and IL-17A were costained with CD4, CD8, or TCRγδ. *White arrowheads* indicate triple-stained cells and a *tailed white arrow* indicates IL-17A$^-$PD-1$^+$ CD8 T cells.

imiquimod-treated mice, we next compared PD-1$^-$, PD-1^{lo}, and PD-1^{hi} CD27$^-$Vγ1$^-$ γδ T cells in regard to their surface phenotypes and ability to produce IL-17A. PD-1^{hi} cells expressed significantly higher levels of CD43 and CD44 than did PD-1$^-$ or PD-1^{lo} cells (Fig 2, *A*), indicating that PD-1^{hi} cells are antigen-experienced effector T cells. Moreover, PD-1^{hi} cells expressed a higher level of inducible T-cell costimulator, a costimulatory receptor, and a lower level of CD127, the IL-7 receptor α (Fig 2, *A*). This low level of CD127 indicates that PD-1^{hi} cells are recently activated T cells.[20]

Next, we examined IL-17A production from CD27$^-$Vγ1$^-$ γδ T cells after stimulation with IL-23/IL-1β or anti-CD3 antibody. Although both types of stimulation increased IL-17A production in CD27$^-$Vγ1$^-$ γδ T cells from control and imiquimod-treated mice, IL-17A production was more dramatically increased in CD27$^-$Vγ1$^-$ γδ T cells from imiquimod-treated mice (Fig 2, *B*). We also compared IL-17A production between PD-1$^-$, PD-1^{lo}, and PD-1^{hi} CD27$^-$Vγ1$^-$ γδ T cells from imiquimod-treated mice. Although there was no significant difference in the relative frequency of IL-23/IL-1β–induced IL-17A$^+$ cells among the 3 populations, the relative frequency of anti-CD3–induced IL-17A$^+$ cells was increased in concert with PD-1 expression (Fig 2, *C*). A similar result was observed when the gMFI of IL-17A was analyzed (Fig 2, *D*). Coexpression of IL-17A and PD-1 in γδ T cells was confirmed by means of immunofluorescent staining of imiquimod-treated psoriatic skin tissue (Fig 2, *E*). Taken together, we conclude that PD-1^{hi}CD27$^-$Vγ1$^-$ γδ T cells are recently activated effector T cells with a high capacity to produce IL-17A after TCR ligation.

CD27$^-$Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) γδ T cells are PD-1^{hi} cells and produce IL-17A robustly on anti-CD3 stimulation

It was previously shown that Vγ4$^+$ and Vγ6$^+$ γδ T cells are the major effector T-cell populations in psoriatic inflammation.[21] Therefore we studied which of the CD27$^-$Vγ1$^-$ γδ T-cell populations obtained from imiquimod-treated mice, Vγ4$^+$ or Vγ6$^+$ γδ T cells, were expanded and expressed high levels of PD-1. In the dermis and draining LNs of imiquimod-treated mice, the relative frequency of both Vγ4$^+$ and Vγ4$^-$ γδ T cells was increased significantly (Fig 3, *A*). Molecular analysis of Vγ expression revealed that Vγ4$^-$ cells expressed a high level of Vγ6 mRNA, indicating that Vγ6$^+$ cells are the main constituents of the Vγ4$^-$ cell population (Fig 3, *B*). Flow cytometric analysis of Vγ4 and PD-1 expression in the CD27$^-$Vγ1$^-$ γδ T-cell population revealed that PD-1^{hi} expression was detected only in Vγ4$^-$ (Vγ6$^+$) cells from both the draining LNs and dermis of imiquimod-treated mice (Fig 3, *C*, and see Fig E2 in this article's Online Repository at www.jacionline.org). The major population of PD-1^{hi} cells (>94%) was composed of Vγ4$^-$ (Vγ6$^+$) cells, whereas a majority of PD-1$^-$ cells (>88%) were Vγ4$^+$ cells. Around 30% of the PD-1^{lo} cell population were Vγ4$^-$ (Vγ6$^+$) cells (Fig 3, *D*).

Next, we examined IL-17A production by CD27$^-$ γδ T cells expressing various Vγ chains after IL-23/IL-1β or anti-CD3 stimulation. The frequency of IL-23/IL-1β–induced IL-17A$^+$ cells was highest in the Vγ1$^-$Vγ4$^+$ population (Fig 3, *E*). As expected because of the finding that the major population of PD-1^{hi} cells is composed of Vγ4$^-$ (Vγ6$^+$) cells, the frequency of anti-CD3–induced IL-17A$^+$ cells was highest in the

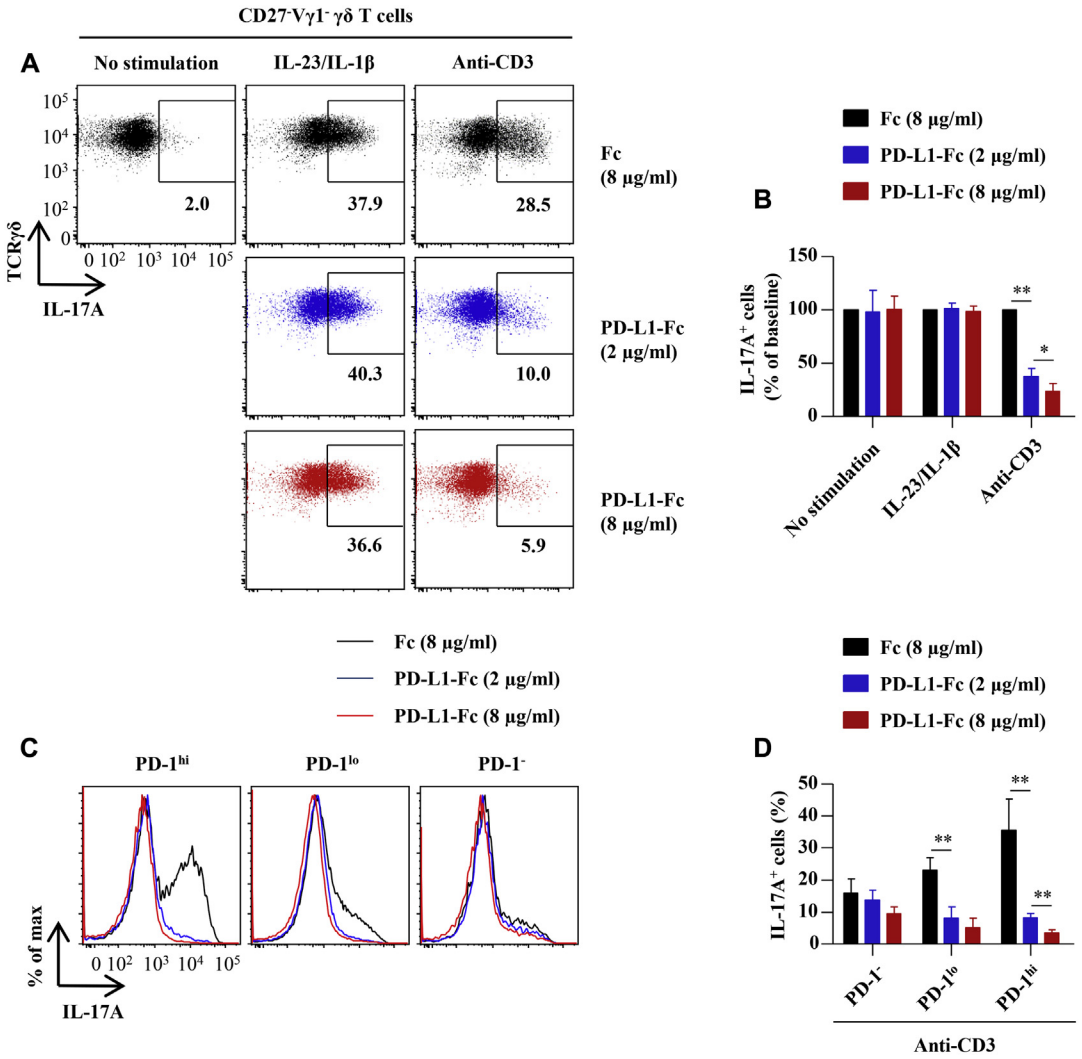

FIG 5. *In vitro* effect of PD-L1-Fc on inhibition of IL-17A–producing CD27$^-$Vγ1$^-$ γδ T cells. γδ T cells were purified from ear draining LNs of imiquimod *(IMQ)*–treated mice and stimulated with anti-CD3 or IL-23/IL-1β in the presence of PD-L1-Fc or Fc control protein, followed by intracellular cytokine staining (n = 4). **A** and **B,** IL-17A production was analyzed in CD27$^-$Vγ1$^-$ γδ T cells. Representative flow cytometric plots are presented (Fig 5, *A*). IL-17A$^+$ cells with Fc control treatment were used to set the baseline (100%), and then the relative change in IL-17A$^+$ cells from baseline was calculated (Fig 5, *B*). **C** and **D,** IL-17A production was analyzed in PD-1$^-$, PD-1^{lo}, and PD-1^{hi} cells among CD27$^-$Vγ1$^-$ γδ T cells. Representative histograms are presented (Fig 5, *C*). The relative frequency of IL-17A production was analyzed in each gate (Fig 5, *D*). *P < .05 and **P < .005.

Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) population (Fig 3, *E*). A similar result was observed when the gMFI of IL-17A was analyzed (Fig 3, *F*). Intriguingly, on CD3 ligation, PD-1^{hi} cells showed a significantly higher gMFI of IL-17A staining than did PD-1^{lo} cells when IL-17A$^+$Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) γδ T cells from draining LNs of imiquimod-treated mice were analyzed (Fig 3, *G*). Taken together, these data demonstrate that PD-1^{hi} cells are comprised mainly of CD27$^-$Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) γδ T cells, which robustly produce IL-17A on TCR ligation.

IL-17A$^+$ T cells express PD-1 in psoriatic skin of patients

Because PD-1^{hi} cells from imiquimod-treated mice efficiently produce IL-17A on anti-CD3 stimulation, we further studied PD-1 expression on IL-17A$^+$ T cells in the skin of patients with psoriasis before the initiation of antipsoriasis treatment. To this end, we performed immunofluorescent staining for CD3, PD-1, and IL-17A. The specific staining was confirmed by means of isotype control antibody staining (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). In contrast to normal human skin, PD-1 was widely expressed in the upper dermis of psoriatic skin (Fig 4, *A*, and see Fig E3, *B*), and around 70% of T cells expressed PD-1 (Fig 4, *B* and *C*). A minor proportion of PD-1$^+$ cells were CD3$^-$CD56$^+$ natural killer cells, which were PD-1^{dim} (see Fig E3, *C*). IL-17A was detected in 40% of T cells (Fig 4, *D*) and 30% of CD45$^+$ cells (see Fig E3, *D*). Approximately 60% of IL-17A$^+$ cells were T cells (see Fig E3, *E*), and a few IL-17A$^+$ cells were c-kit$^+$ mast cells or neutrophil elastase–positive neutrophils (see Fig E3, *F*). Importantly, more

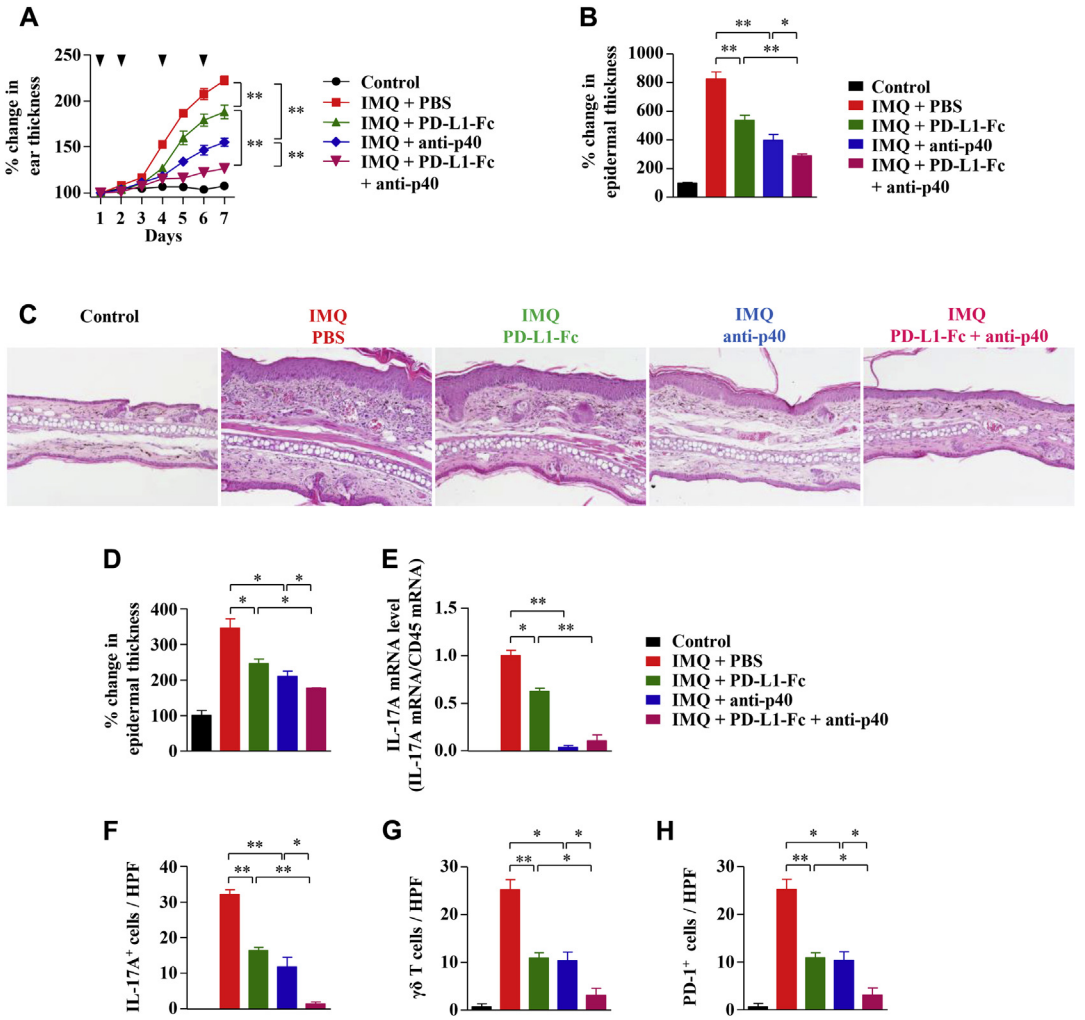

FIG 6. *In vivo* effects of PD-L1-Fc on imiquimod *(IMQ)*–treated mice. **A-C,** PD-L1-Fc, anti-p40, or both were administered intraperitoneally on days 1, 2, 4, and 6 in imiquimod-treated mice, and mice were killed on day 7 (n = 5). Ear skin or epidermal thickness is expressed as the relative percentage over the measurement in untreated mice (Fig 6, *A* and *B*). Mouse ear skin thickness was measured during the course of imiquimod treatment (Fig 6, *A*). *Black downward arrowheads* indicate days on which mice received PD-L1-Fc, anti-p40, or both. Epidermal thickness of the dorsal ear was analyzed from tissue histology (Fig 6, *B*). Representative images of hematoxylin and eosin staining of ear skin are displayed (Fig 6, *C*). **D-H,** PD-L1-Fc, anti-p40, or both were injected intradermally every 12 hours on the back skin of imiquimod-treated mice, and mice were killed on day 7 (n = 3). Epidermal thickness (Fig 6, *D*) and IL-17A mRNA levels (Fig 6, *E*) in skin tissues were determined. Immunofluorescent staining for IL-17A (Fig 6, *F*), TCRγδ (Fig 6, *G*), and PD-1 (Fig 6, *H*) and confocal microscopy was performed on frozen sections from the skin. The stained cells were counted from the images per ×200 high-power field. **P* < .05 and ***P* < .005.

than 95% of IL-17A$^+$ T cells expressed PD-1 (Fig 4, *C*), and these IL-17A$^+$PD-1$^+$ T cells were TCRγδ$^+$ or CD4$^+$ cells (Fig 4, *E*). These findings demonstrate that PD-1 is expressed by IL-17A$^+$ T cells in the lesional skin of patients with psoriasis, suggesting that PD-1 can be targeted to suppress IL-17A$^+$ T cells for the treatment of psoriatic inflammation.

PD-L1-Fc inhibits anti-CD3–induced IL-17A production of CD27$^-$Vγ1$^-$ γδ T cells

Because PD-1 was overexpressed on CD27$^-$Vγ1$^-$Vγ4$^-$ (Vγ6$^+$) γδ T cells of imiquimod-treated mice, we investigated whether exogenous PD-L1 protein could suppress IL-17A production by γδ T cells. Thus γδ T cells were isolated from draining LNs of imiquimod-treated mice and stimulated with IL-23/IL-1β or anti-CD3 antibody in the presence of recombinant PD-L1-Fc or Fc control protein. Interestingly, PD-L1-Fc suppressed anti-CD3–induced IL-17A production from CD27$^-$Vγ1$^-$ γδ T cells, whereas it did not suppress IL-23/IL-1β–induced IL-17A production (Fig 5, *A* and *B*). Moreover, a higher concentration of PD-L1-Fc resulted in increased suppression of anti-CD3–induced IL-17A production by CD27$^-$Vγ1$^-$ γδ T cells (Fig 5, *B*). We next analyzed the effect of PD-L1-Fc with regard to the expression level of PD-1 on CD27$^-$Vγ1$^-$ γδ T cells. As described above (Fig 2, *C* and *D*), IL-17A was produced mainly by PD-1^{hi} cells, rather than PD-1^{lo} or PD-1$^-$ cells, after anti-CD3 stimulation. As expected, PD-L1-Fc strongly suppressed, in a dose-dependent manner, the

ability of PD-1^{hi} cells to produce IL-17A in response to anti-CD3 (Fig 5, *C* and *D*). Activation of PD-1^{lo} cells was also suppressed by PD-L1-Fc, whereas PD-1^{-} cells were not significantly inhibited (Fig 5, *D*). Collectively, this suggests that PD-L1-Fc can be used for treatment of psoriatic inflammation because of its ability to inhibit anti-CD3–induced IL-17A production by PD-1^{+} γδ T cells.

PD-L1-Fc alleviates psoriatic inflammation in imiquimod-treated mice

Finally, we investigated the therapeutic effect of PD-L1-Fc *in vivo*. Over the course of imiquimod-induced psoriatic inflammation in the ears, PD-L1-Fc was administrated intraperitoneally on days 1, 2, 4, and 6. Compared with control treatment with PBS, PD-L1-Fc treatment significantly reduced disease activity, as represented by ear swelling and epidermal thickness (Fig 6, *A-C*). Ear and epidermal thickness were also decreased after treatment with anti-p40, which blocks a common subunit of IL-23 and IL-12 (Fig 6, *A-C*). Combination therapy with PD-L1-Fc and anti-p40 resulted in an additive effect, suggesting that these 2 therapeutic agents target distinct mechanisms of psoriatic inflammation. A similar result was observed in imiquimod-induced psoriatic inflammation in the back (Fig 6, *D-H*). In this model we injected PD-L1-Fc intradermally every 12 hours and evaluated disease activity not only based on epidermal thickness but also based on IL-17A mRNA levels and immunofluorescent staining for IL-17A, TCRγδ, and PD-1. As a result, PD-L1-Fc or anti-p40 reduced disease activity evaluated by using these various parameters, and the combined administration of PD-L1-Fc and anti-p40 overcomes the limited efficacy of anti-p40 for the treatment of psoriatic inflammation.

DISCUSSION

In the present study we demonstrate PD-1 expression on IL-17–producing T cells in a murine model of psoriasis and in human patients with psoriasis. In addition, we also show that recombinant PD-L1 protein suppresses disease activity in a murine model of psoriasis.

In imiquimod-induced psoriatic inflammation PD-1 is highly expressed by CD27^{-}Vγ1^{-}Vγ4^{-} γδ T cells, which express Vγ6 mRNA (Fig 3, *B-D*). When stimulating these cells to produce IL-17A, anti-CD3 antibody was more effective than IL-23/IL-1β, indicating that CD27^{-}Vγ1^{-}Vγ4^{-} (Vγ6^{+}) γδ T cells are specialized for TCR-stimulated IL-17A production (Fig 3, *E* and *F*). Another main source of IL-17A in imiquimod-induced psoriatic inflammation was CD27^{-}Vγ1^{-}Vγ4^{+} γδ T cells (Fig 3, *E* and *F*). In contrast to CD27^{-}Vγ1^{-}Vγ4^{-} (Vγ6^{+}) γδ T cells, CD27^{-}Vγ1^{-}Vγ4^{+} γδ T cells were efficiently stimulated by IL-23/IL-1β to produce IL-17A but not by anti-CD3 (Fig 3, *E* and *F*). In agreement with previous findings, these data indicate that CD27^{-}Vγ1^{-}Vγ4^{+} γδ T cells are specialized for IL-23/IL-1β–induced IL-17A production.[5] The γδ T-cell subset responsible for anti-CD3–induced IL-17A production has not previously been defined in imiquimod-induced psoriatic inflammation. Herein, we demonstrate that CD27^{-}Vγ1^{-}Vγ4^{-} (Vγ6^{+}) γδ T cells are PD-1^{hi} and contribute to anti-CD3–induced IL-17A production.

In mice γδT17 cells are known to be hyporesponsive to TCR stimulation.[22,23] However, the TCR responsiveness of γδT17 cells can be enhanced by cytokines or inflammatory stimuli. For example, IL-7 helps γδT17 cells, making them more responsive to TCR stimulation.[24] Moreover, imiquimod treatment also leads to increased TCR responsiveness of γδT17 cells.[25] In the present study we confirmed that anti-CD3–stimulated IL-17A production is greatly enhanced in CD27^{-}Vγ1^{-} γδ T cells after imiquimod treatment (Fig 2, *B*). In further analyses we found that major cellular sources of anti-CD3–stimulated IL-17A production were PD-1^{hi}CD27^{-}Vγ1^{-}Vγ4^{-} (Vγ6^{+}) γδ T cells (Fig 3, *E* and *F*). However, it remains unclear which antigens activate γδ T cells through their TCRs to produce IL-17A in imiquimod-induced psoriatic inflammation. In the setting of human psoriasis, autoantigens, including LL37, are known to stimulate IL-17A–producing T cells.[12] Future studies will be required to identify antigens for stimulation of γδT17 cells, particularly of CD27^{-}Vγ1^{-}Vγ4^{-} (Vγ6^{+}) γδ T cells, in imiquimod-induced psoriatic inflammation.

PD-1, a coinhibitory receptor, is expressed after continuous or repetitive TCR stimulation of T cells. As a result, PD-1 is overexpressed on T cells under chronic inflammatory conditions, and these cells are considered to be crucial players in inflammatory diseases.[15] Furthermore, blockade of the PD-1 axis exacerbates disease activity in murine models of systemic lupus erythematosus,[26] autoimmune dilated cardiomyopathy,[27] insulitis,[28] experimental autoimmune encephalomyelitis,[29] and autoimmune enteritis.[30] Thus *in vivo* application of the PD-L1 protein has been investigated as a therapeutic approach for treatment of chronic inflammatory diseases. In fact, recombinant PD-L1 protein has been found to reduce disease severity in murine models of rheumatoid arthritis and inflammatory bowel diseases.[16,17] Nevertheless, the therapeutic effects of PD-L1 protein for psoriatic inflammation have not previously been tested.

In the present study we evaluated imiquimod-induced psoriatic inflammation after *in vivo* application of PD-L1-Fc, anti-p40, or both. Anti-p40 binds to the p40 subunit of IL-23 and IL-12 and inhibits IL-23–induced IL-17A production. Hence anti-p40 has been developed for the treatment of psoriasis and approved for clinical use.[9] Based on the clinical data, anti-p40 considerably reduces psoriatic inflammation. However, residual lesions in some patients still exist after treatment of anti-p40,[31] suggesting additional therapeutic agents are needed to control non–IL-23–related psoriatic inflammation. In the current study both recombinant PD-L1-Fc and anti-p40 were found to alleviate psoriatic inflammation when administered individually. Importantly, disease activity was further alleviated after combination therapy with PD-L1-Fc and anti-p40 (Fig 6). This enhanced therapeutic effect when combining treatments is likely attributable to the fact that PD-L1-Fc and anti-p40 target distinct γδT17 cell populations. PD-L1-Fc inhibits PD-1^{hi} γδT17 cells, which are CD27^{-}Vγ1^{-}Vγ4^{-} (Vγ6^{+}) γδ T cells specialized for anti-CD3–induced IL-17A production (Fig 5, *C* and *D*). In contrast, anti-p40 targets IL-23 and thus inhibits IL-23–induced IL-17A production from CD27^{-}Vγ1^{-}Vγ4^{+} γδ T cells (Fig 5, *C* and *D*). Considering the current data, use of recombinant PD-L1 protein in combination with anti-p40 for the treatment of psoriasis is warranted.

In the therapeutic use of recombinant PD-L1 protein, possible side effects should be considered. PD-L1 attenuates the activation and proliferation of effector T cells, and it is known that anti-p40 treatment increases the possibility of various infections.[32] Therefore PD-L1 protein might aggravate anti-p40–related infections

when used in combination. A chance of potential side effects of recombinant PD-L1 protein might be reduced by nonsystemic administration. In the current study we demonstrated that PD-L1-Fc alleviated psoriatic inflammation by means of not only intraperitoneal (Fig 6, *A-C*) but also intradermal (Fig 6, *D-H*) administration.

In the present study we also examined PD-1 expression on IL-17–producing T cells in the skin of patients with psoriasis and found that IL-17A–producing T cells were almost all PD-1^+ (Fig 4, *B* and *C*). Furthermore, another recent study also reported increased PD-1 expression on PBMCs in patients with psoriatic arthritis.[33] Taken together, these data indicate that PD-1 is overexpressed on T cells not only in the mouse model of psoriasis but also in patients with psoriasis, underscoring the promise of the PD-L1 protein as a therapy for patients with psoriasis. However, an important difference between IL-17A–producing T cells in the mouse model and human patients with psoriasis needs to be noted. In imiquimod-induced psoriatic inflammation IL-17A is produced mainly by γδ T cells, whereas in patients with psoriasis, IL-17A is produced by T_H17 cells with an TCRαβ. Future studies will be required to determine whether this difference influences the effectiveness of PD-L1 protein for treatment of psoriatic inflammation.

In summary, PD-1 is overexpressed by IL-17A–producing T cells in both imiquimod-treated mice and in human psoriatic skin. Furthermore, PD-1^{hi} γδT17 cells in imiquimod-treated mice are $CD27^-V\gamma1^-V\gamma4^-$ ($V\gamma6^+$) γδ T cells and respond robustly to anti-CD3 stimulation. Recombinant PD-L1 protein inhibits PD-1^+ γδT17 cells and thus alleviates psoriatic inflammation in imiquimod-treated mice. Importantly, combination therapy with PD-L1 protein and anti-p40 results in an additive effect, suggesting that PD-L1 protein overcomes the limited efficacy of anti-p40 for the treatment of psoriatic inflammation.

We thank Sung Yong Choi, Si-Hyung Lee, Wonhyo Seo, and Eunee Lee for technical support with histologic assays.

Key messages

- **PD-1 is overexpressed on IL-17A–producing T cells in imiquimod-treated mice and patients with psoriasis.**
- **$CD27^-V\gamma1^-V\gamma4^-$ ($V\gamma6^+$) γδ T cells express high levels of PD-1 and are specialized for anti-CD3–induced IL-17A production in imiquimod-treated mice.**
- **In imiquimod-treated mice, PD-L1-Fc alleviates psoriatic inflammation alone and shows an additive effect when combined with anti-p40.**

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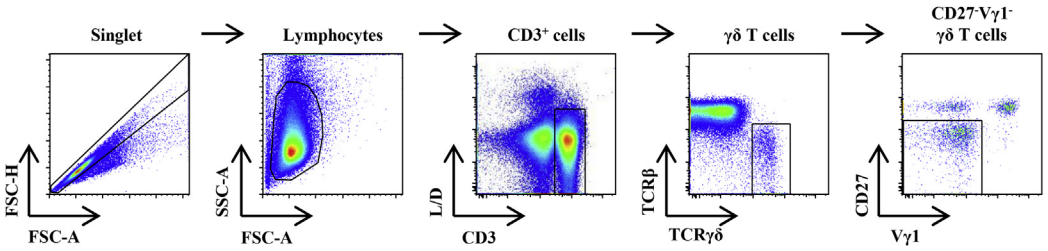

FIG E1. Gating strategy for $CD27^{-}V\gamma1^{-}$ γδ T cells is presented in flow cytometric analysis. *FSC*, Forward scatter; *L/D*, live/dead; *SSC*, side scatter.

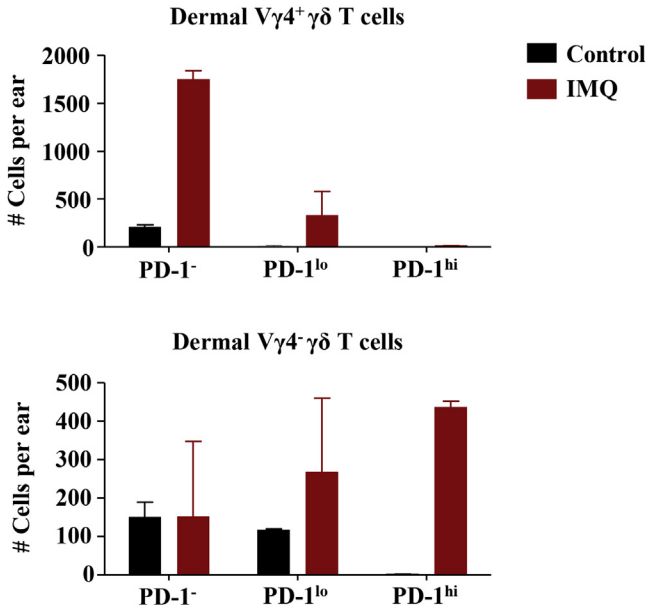

FIG E2. Cell numbers per ear of PD-1^{-}, PD-1^{lo}, and PD-1^{hi} cells are shown from the dermal CD27^{-}Vγ4^{+} and CD27^{-}Vγ4^{-} (Vγ6^{+}) $\gamma\delta$ T cells. Cells were isolated from the dermis of vehicle- or imiquimod *(IMQ)*–treated mice at day 7. By using flow cytometric analysis, percentages of PD-1^{-}, PD-1^{lo}, and PD-1^{hi} cells were determined in CD27^{-}Vγ4^{+} or CD27^{-}Vγ4^{-} (Vγ6^{+}) $\gamma\delta$ T cells, and absolute PD-1^{-}, PD-1^{lo}, and PD-1^{hi} cell numbers per ear were calculated.

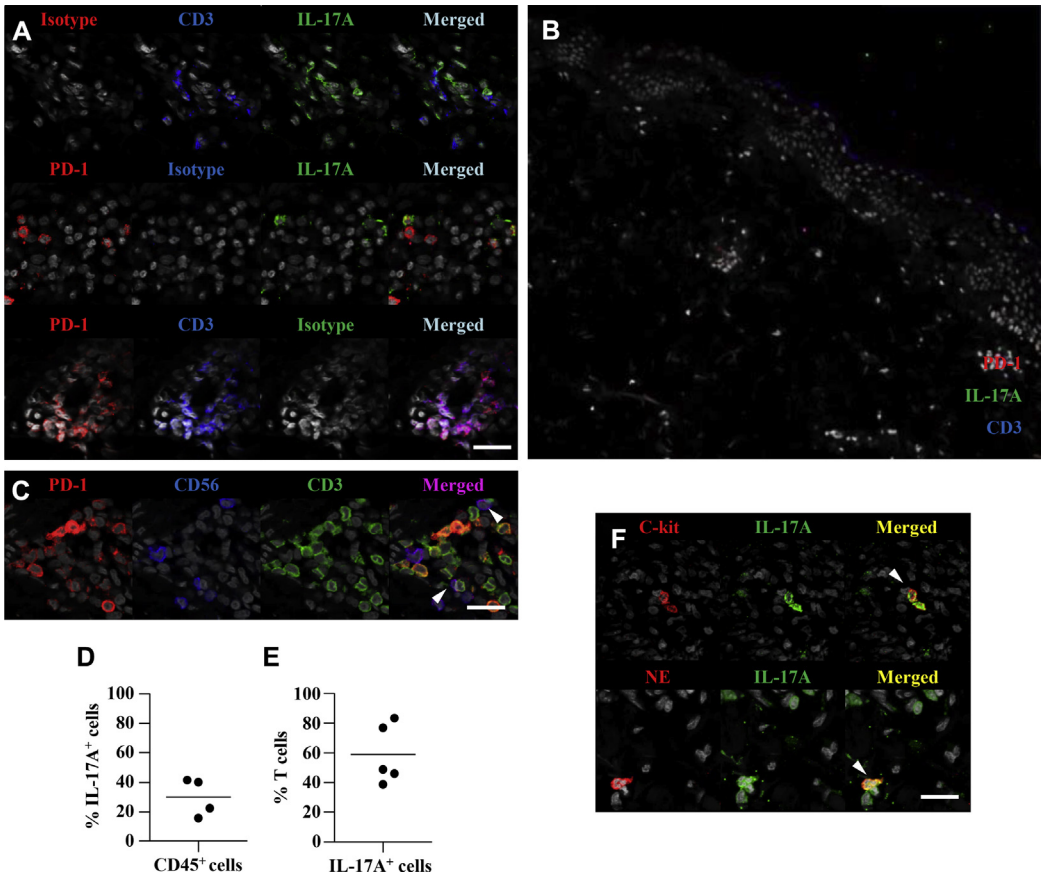

FIG E3. Immunofluorescent staining and confocal microscopy were performed in skin biopsy specimens from patients with psoriasis or a healthy control subject (*scale bar* = 20 μm). **A,** Immunofluorescent staining was performed with isotype control antibodies for anti-CD3, anti–PD-1, or anti–IL-17A. **B,** Immunofluorescent staining of CD3, PD-1, and IL-17A was performed in a healthy control subject. **C,** PD-1, CD56, and CD3 were costained. *White arrowheads* indicate PD-$1^{dim}CD56^{+}CD3^{-}$ cells. **D** and **E,** Frequencies of $IL\text{-}17A^{+}$ cells among $CD45^{+}$ cells (n = 4; Fig E3, *D*) and T cells among $IL\text{-}17A^{+}$ cells (n = 5; Fig E3, *E*) were calculated in whole dermal sections. **F,** IL-17A was costained with c-kit or neutrophil elastase *(NE)*. *White arrowheads* indicate double-stained cells.