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IL-36α from Skin-Resident Cells Plays an Important Role in the Pathogenesis of Imiquimod-Induced Psoriasiform Dermatitis by Forming a Local Autoamplification Loop

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IL-36α (gene symbol *Il1f6*), a member of the IL-36 family, is closely associated with inflammatory diseases, including colitis and psoriasis. In this study, we found that *Il1f6*$^{-/-}$ mice developed milder psoriasiform dermatitis upon treatment with imiquimod, a ligand for TLR ligand 7 (TLR7) and TLR8, whereas *Il1f6*$^{-/-}$ mice showed similar susceptibility to dextran sodium sulfate–induced colitis to wild-type mice. These effects were observed in both cohoused and separately housed conditions, and antibiotic treatment did not cancel the resistance of *Il1f6*$^{-/-}$ mice to imiquimod-induced dermatitis. Bone marrow (BM) cell transfer revealed that IL-36α expression in skin-resident cells is important for the pathogenesis of dermatitis in these mice. Following stimulation with IL-36α, the expression of *Il1f6* and *Il1f9* (IL-36γ), but not *Il1f8* (IL-36β), was enhanced in murine BM-derived Langerhans cells (BMLCs) and murine primary keratinocytes but not in fibroblasts from mice. Upon stimulation with agonistic ligands of TLRs and C-type lectin receptors (CLRs), *Il1f6* expression was induced in BMLCs and BM-derived dendritic cells. Furthermore, IL-36α stimulation resulted in significantly increased gene expression of psoriasis-associated Th17-related cytokines and chemokines such as IL-1α, IL-1β, IL-23, CXCL1, and CXCL2 in BMLCs and fibroblasts, and IL-1α, IL-1β, IL-17C, and CXCL2 in keratinocytes. Collectively, these results suggest that TLR/CLR signaling–induced IL-36α plays an important role for the development of psoriasiform dermatitis by enhancing Th17-related cytokine/chemokine production in skin-resident cells via a local autoamplification loop. *The Journal of Immunology*, 2018, 201: 167–182.

The IL-36 family members have been recently reclassified on the basis of homology with the IL-1 family members (1). The IL-36 family is composed of five members including three agonists (IL-36α, IL-36β, and IL-36γ [gene symbols: *Il1f6*, *Il1f8*, and *Il1f9*, respectively]) and two antagonists (IL-36 receptor [IL-36R] antagonist [*Il36rn*] and IL-38 [*Il1f10*]) (2–4). The three agonists bind to IL-36R (*Il1rl2*) in a form of heterocomplex with IL-1 receptor accessory protein (IL-1RAcp), leading to activation of GM-CSF–induced dendritic cells (GM-DCs) (5–7); expansion of Th cell subsets such as Th1, Th2, and Th17 cells (5, 8, 9); and abnormal differentiation and hyperproliferation of keratinocytes (10–12). IL-36 binding to IL-36R recruits myeloid differentiation primary response gene 88 (MyD88) to Toll/IL (TIR) homology domain of IL-36R and activates NF-κB and MAPK in the downstream, inducing the expression of inflammatory mediators such as cytokines, chemokines, and antimicrobial peptides (13–15). In another report, however, association of IL-36R with MyD88 is not observed, although IL-36–induced IL-6 production in ovarian cancer cells depends on MyD88 (16). IL-36R antagonist competes for IL-36R binding with three IL-36 agonists (2), whereas IL-38 inhibits IL-36R signaling (17). IL-36 cytokines induce their own expression in keratinocytes in an autocrine/paracrine manner (18).

Recent studies indicate the importance of the IL-36–IL-36R axis in inflammatory diseases, particularly in inflammatory bowel disease (IBD) and psoriasis (19–21). IBD consists of ulcerative colitis (UC) and Crohn's disease (CD), and dysregulation of gut immune responses and/or imbalance of intestinal commensal microbiota are suggested to be involved in the pathogenesis (22). Several reports indicate that expression of IL-36 genes is upregulated in patients with CD and UC (23–25), suggesting possible involvement of IL-36 cytokines in intestinal inflammation. Recently, Medina-Contreras et al. (26) showed that IL-36R signaling is required for the wound healing of the colon in mice. However, the differential roles of IL-36 family members in colitis remain to be elucidated.

Psoriasis is a chronic inflammatory skin disease characterized by thickening and redness of the skin with keratinocyte

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Abbreviations used in this article: Abx, antibiotic; BM, bone marrow; BMC, BM cell; BMLC, BM-derived Langerhans cell; CD, Crohn's disease; DC, dendritic cell; DSS, dextran sodium sulfate; ES, embryonic stem cell; Flt3L-DC, Fms-like tyrosine kinase 3 ligand–induced DC; GM-DC, GM-CSF–induced dendritic cell; IBD, inflammatory bowel disease; ILC3, group 3 innate immune cell; IL-36R, IL-36 receptor; IMQ, imiquimod; LC, Langerhans cell; MEF, mouse embryonic fibroblast; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid DC; qPCR, quantitative PCR; rDNA, rRNA-encoding DNA; UC, ulcerative colitis; WT, wild-type.

hyperproliferation, skin inflammation associated with inflammatory cell infiltration in the epidermis and dermis, and aseptic abscess formation in severe cases (27). The involvement of IL-36 in the pathogenesis is suggested because mutations in the *IL36RN* gene are associated with general pustular psoriasis, the most severe psoriasis (28–31). Expression levels of IL-36 genes in skin biopsy specimens from patients with psoriatic dermatitis are dramatically elevated in comparison with nonlesional skin from the same individuals or healthy controls (15). Furthermore, Krt14 promoter–driven IL-36α–overexpressing transgenic mice display transient psoriasis-like dermatitis and exacerbated psoriasis-like dermatitis after 12-*O*-tetradecanoylpholbol-13-acetate (TPA) treatment (10, 32). Imiquimod (IMQ) is a ligand for TLR7/8 and causes dermatitis resembling psoriasis vulgaris with erythema, skin thickening, scaling, acanthosis, and parakeratosis in mice (33) and plaque-type dermatitis in patients with psoriasis (34–36). Although *Il1rl2*$^{-/-}$ mice are refractory to IMQ-induced psoriasiform dermatitis, *Il36rn*$^{-/-}$ mice develop more severe symptoms in this psoriasis model (11). Recently, Milora et al. (12) reported that IL-36α, but not IL-36β and IL-36γ, promotes psoriasis-like skin inflammation in mice. Furthermore, increasing evidence suggests that skin-commensal microbiota are closely associated with skin health and diseases (37–40). A recent study showed that antibiotic (Abx) treatment of mice affects the susceptibility to experimental psoriasis (41). However, it is not known how commensal microbiota influence the development of IL-36α–mediated psoriasiform dermatitis.

It is well established that IL-17A plays important roles in host defense against infection and development of inflammatory diseases in animal models, including rheumatoid arthritis, multiple sclerosis, IBD, and psoriasis (42–44). Actually, it has been shown that anti–IL-17A or anti–IL-17RA is effective in treating inflammatory diseases such as psoriasis, psoriatic arthritis, and ankylosing sclerosis in humans (45, 46). IL-17A is produced by several types of cells, such as Th17 cells, and specific innate immune cells such as γδ T cells, group 3 innate immune cells (ILC3s), NKT cells, neutrophils, and myeloid cells and activates cells to produce cytokines and chemokines through a receptor complex consisting of IL-17RA and IL-17RC, which are expressed in many cells, including lymphoid cells, epithelial cells, endothelial cells, keratinocyte, and fibroblasts (47, 48). This signaling induces expression of inflammatory cytokines and chemokines such as TNF, IL-6, IL-8, CXCL1, CXCL2, and CCL2, leading to activation and recruitment of immune cells, such as neutrophils, ILC3s, and γδ T cells to the site of inflammation. Recently, Carrier et al. (18) demonstrated that the expression of IL-36 genes in keratinocytes is regulated by IL-17A.

Other Th17 signature cytokines such as IL-17F and IL-22 also participate in the pathogenesis of IBD and psoriasis. Genes encoding IL-17A, IL-17F, and IL-22 are highly expressed in biopsy specimens from patients with IBD and psoriasis (49–51). Studies using genetically modified mice demonstrate that IL-17A, IL-17F, and IL-22 play distinct roles in the pathogenesis of colitis and psoriasiform dermatitis (52, 53). Additionally, it has been reported that IL-17C is required for the development of IMQ-induced psoriasiform dermatitis in mice (54). IL-17C is produced in epithelial cells and keratinocytes upon stimulation with proinflammatory cytokines and activates epithelial cells and keratinocytes to produce cytokines and chemokines through the receptor consisting of IL-17RA and IL-17RE. Therefore, IL-17 family members are suggested to play important roles in the development of inflammatory diseases in humans. However, little is known about the effect of IL-36α on IL-17–mediated regulation of IBD and psoriasis.

In this study, we investigated the roles of IL-36α in the development of dextran sodium sulfate (DSS)–induced colitis, a model for UC, and IMQ-induced dermatitis, a model for psoriasis, using *Il1f6*$^{-/-}$ mice. Because commensal microbiota are suggested to be involved in the pathogenesis of these diseases, we carried out these experiments under two different housing conditions (separately housed and cohoused) to exclude possible effects of IL-36α deficiency on the commensal microbiota. We found that the development of IMQ-induced dermatitis is attenuated in *Il1f6*$^{-/-}$ mice compared with wild-type (WT) mice, whereas the development of DSS-induced colitis is similar between two strains. These results were similarly observed both in cohoused and separated groups and in Abx-treated mice, suggesting that the observed effect of IL-36α efficiency is not mediated by commensal microbiota. We also found that IL-36α expression in skin-resident cells, rather than bone marrow (BM)–derived cells, plays an important role in the pathogenesis of IMQ-induced dermatitis. Furthermore, the expression of Th17 cytokines and chemokines was reduced in *Il1f6*$^{-/-}$ mice after IMQ treatment. These results suggest that IL-36α is involved in the development of IMQ-induced psoriasiform dermatitis by inducing Th17-related cytokines and chemokines, whereas IL-36α is dispensable for the development of colitis.

Materials and Methods

Mice

Rag2$^{-/-}$ mice on the C57BL/6J background were obtained from Central Institute for Experimental Animals (Kanagawa, Japan). *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ mice were generated by crossing *Il1f6*$^{-/-}$ mice with *Rag2*$^{-/-}$ mice. Commensal microflora was controlled as shown in Fig. 1A: WT and *Il1f6*$^{-/-}$ pregnant mice were cohoused a few days before the birth of their offspring and continued cohousing until the offspring were weaned. After genotyping, WT and *Il1f6*$^{-/-}$ mice were separately grown in separate cages for more than 4 wk (separate group). In the case of the cohoused group, WT and *Il1f6*$^{-/-}$ mice were further housed in the same cage for more than 4 wk after weaning. Age-matched mice (8–12-wk-old) were used for all experiments. All the mice were bred under specific pathogen-free conditions in the clean rooms at the Institute of Medical Science, The University of Tokyo, and in the Research Institute for Biomedical Science, Tokyo University of Science, and provided with gamma ray–sterilized normal F1 diet (Funabashi Farm, Chiba) and acidified tap water (0.002 N HCl). All experiments were approved by the committees of Life Science Research Ethics and Safety of The University of Tokyo and the Animal Care and Use Committee of Tokyo University of Science and were conducted according to the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments.

Generation of Il1f6$^{-/-}$ *mice*

Genomic DNA containing *Il1f6* gene was isolated from a 129/SvJ genomic phage library (Stratagene, La Jolla, CA). The *Pst*I-*Bam*HI fragment (0.8 kbp), which contains the initiation codon ATG, was replaced by neomycin resistance and enhanced GFP (*NeoR* and *EGFP*) genes to disrupt the *Il1f6* gene (Supplemental Fig. 1A). A diphtheria toxin A fragment, under the control of MC1 promoter, was ligated at the 5′ end of the targeting vector for negative selection. Homologous arms of 5′ and 3′ ends were 4.1 and 3 kbp, respectively. The targeting vector was linearized by *Sal*I digestion and electroporated into embryonic stem cells (ESs) (E14.1). The ES colonies were selected in the presence of G418 (250 μg/ml; Life Technologies, Grand Island, NY). Homologous recombinants of ES genome were confirmed by Southern blot hybridization analysis with 5′ probe (Supplemental Fig. 1B). An obtained ES clone (2B3) was used for generation of chimera mice by an aggregation method. Chimeric mice were mated with C57BL/6J female mice. Germline-transmitted mice were backcrossed to C57BL/6J mice for nine generations. Genotypes were determined by PCR with a primer set (Supplemental Fig. 1C; Common primer, 5′-GGGACCTTGTGACGCTTGGTTTAG-3′; WT primer, 5′-GGCTACTCACCTGGAACTGTTTGC-3′; Mutant primer, 5′-CGATGCCCTTCAGCTCGATG-3′). The expression of *Il1f6* transcript was confirmed by quantitative PCR (qPCR) with a PCR primer pair (Table I, Supplemental Fig. 1D). They were fertile and showed no apparent phenotypic abnormalities under specific pathogen-free conditions.

Table I. Primers used for qPCR analysis

	Forward	Reverse
Cxcl1	5′-AGCCACACTCAAGAATGGTC-3′	5′-GCCATCAGAGCAGTCTGTC-3′
Cxcl2	5′-CACTGCGCCCAGACAGAAGTC-3′	5′-TCCTCCTTTCCAGGTCAGTTATCC-3′
Cxcl9	5′-TGCACGATGCTCCTGCA-3′	5′-AGGTCTTTGAGGGATTTGTAGTGG-3′
Ccl20	5′-CTTGCTTTGGCATGGGTACT-3′	5′-TGTACGAGAGGCAACAGTCG-3′
Gapdh	5′-TTCACCACCATGGAGAAGGC-3′	5′-GGCATGGACTGTGGTCATGA-3′
Il1a	5′-CGAAGACTACAGTTCTGCCATT-3′	5′-GACGTTTCAGAGGTTCTCAGAG-3′
Il1b	5′-GCAACTGTTCCTGAACTCAACT-3′	5′-ATCTTTTGGGGTCCGTCAACT-3′
Il1f6	5′-CTACAGCTTGGGGAAGGGAACATA-3′	5′-CCCTTTAGAGCAGACAGCGATGAA-3′
Il1f8	5′-ACAAAAAGCCTTTCTGTTCTATCAT-3′	5′-CCATGTTGGATTTACTTCTCAGACT-3′
Il1f9	5′-ATGGACACCCTACTTTGCTG-3′	5′-TGTCCGGGTGTGGTAAAACA-3′
Il1rl2	5′-AAACACCTAGCAAAAGCCCAG-3′	5′-AGACTGCCCGATTTTCCTATG-3′
Il6	5′-GAGGATACCACTCCCAACAGACC-3′	5′-AAGTGCATCATCGTTGTTCATACA-3′
Il10	5′-GTGGAGCAGGTGAAGAGTGATTT-3′	5′-TCCCTGGATCAGATTTAGAGAGC-3′
Tgfb	5′-GGTTCATGTCATGGATGGTGC-3′	5′-TGACGTCACTGGAGTTGTACGG-3′
Il17a	5′-TTTAACTCCCTTGGCGCAAAA-3′	5′-CTTTCCCTCCGCATTGACAC-3′
Il17c	5′-CTGGAAGCTGACACTCACG-3′	5′-GGTAGCGGTTCTCATCTGTG-3′
Il17f	5′-CAAAACCAGGGCATTTCTGT-3′	5′-ATGGTGCTGTCTTCCTGACC-3′
Il23a	5′-AATAATGTGCCCCGTATCCA-3′	5′-AGGCTCCCCTTTGAAGATGT-3′
Il22	5′-TGACGACCAGAACATCCAGA-3′	5′-AGCTTCTTCTCGCTCAGACG-3′
Rorc	5′-AGCAGTGTAATGTGGCCTAC-3′	5′-GCACTTCTGCATGTAGACTG-3′
S100a8	5′-TCAAGACATCGTTTGAAAGGAAATC-3′	5′-GGTAGACATCAATGAGGTTGCTC-3′
S100a9	5′-AAAGGCTGTGGGAAGTAATTAAGGAG-3′	5′-GCCATTGAGTAAGCCATTCCC-3′
Tnf	5′-GCCTCCCTCTCATCAGTTCT-3′	5′-CACTTGGTGGTTTGCTACGA-3′
Iab	5′-CTGTCTGGATGCTTCCTGAGTTT-3′	5′-CAGCTATGTTTTGCAGTCCACC-3′
Cd40	5′-TTGTTGACAGCGGTCCATCTA-3′	5′-GCCATCGTGGAGGTACTGTTT-3′
Krt6	5′-TGAAGGAGTACCAGGAACTC-3′	5′-CACCACAGAGATGTTGACTG-3′
16SrDNA	5′-AGAGTTTGATCMTGGCTCAG-3′	5′-CTGCTGCCTYCCGTA-3′

DSS-induced colitis

DSS-containing water was prepared by dissolving DSS (molecular weight 35–50; MP Biomedicals, Irvine, CA) in tap water at 2%, followed by filtration using a Sterile Disposable Bottle Top Filter (Thermo Scientific, Waltham, MA). Mice were administrated with the DSS-containing water (2%) for 7 d, followed by acidified tap water for 14 d. Survival and body weight were monitored daily. The extent of colitis was evaluated daily. Blood in stool was scored as follows: 0 = normal, 1 = visible blood in stool, 2 = reddish stool and slight bleeding around anus, and 3 = gross bleeding around anus. Diarrhea was scored as follows: 0 = solid, 1 = loose stools, 2 = very soft, and 3 = diarrhea. Disease activity index was calculated with the cumulative scores.

IMQ-induced psoriasiform dermatitis

Mice were applied daily with ∼14 mg of Beselna Cream (5% IMQ; Mochida Pharmaceutical, Tokyo, Japan) on the ventral surface of both ears. Ear thickness was measured daily in three fields per ear lobe using a micrometer caliper. Ear swelling was shown as percentage of thickness compared with the ears at day 0. Redness was assessed in two fields per ear as follows: 0 = no clinical signs, 1 = slight, 2 = marked, and 3 = very severe. The global score was the sum of local redness scores. Scaling was evaluated in three areas per ear as follows: 0 = no clinical sign, 1 = <50% of area, 2 = >50%. The total score was the sum of local scale scores.

Abx treatment

An Abx mixture was prepared by mixing drinking water with ampicillin sodium salt (1 g/l; Nacalai Tesque, Kyoto, Japan), vancomycin hydrochloride (1 g/l; Wako Pure Chemical, Osaka, Japan), neomycin sulfate (1 g/l; Nacalai Tesque), and metronidazole (1 g/l; Nacalai Tesque). Mice were given drinking water containing the Abx mixture for 4 wk after weaning, and the Abx treatment was continued until the end of the IMQ-induced dermatitis experiment (Fig. 1D).

DNA isolation from skin

Sheets from shaved dorsal skin were cut into 235.5-mm^2 pieces and stored at −80°C. The frozen sheets were disaggregated and incubated with 100 μg/ml Proteinase K (Sigma-Aldrich, St. Louis, MO) in 100 mM NaCl, 10 mM Tris-Cl (pH 8), and 1 mM EDTA for 4 h at 55°C. DNA was extracted from the homogenate by phenol-chloroform extraction.

BM cell transplantation

Recipient mice on the *Rag2*$^{-/-}$ background were used because conventional T and B cells are completely depleted in these mice. These mice were irradiated lethally with gamma rays using a Gamma Cell 40 (5.5 Gy, twice at 6 h intervals; Nordion, Ottawa, Canada). BM cells (BMCs) were obtained from femurs and tibias of donor mice by flushing. Single-cell suspensions (1×10^7) were intravenously injected into the recipient mice. Four weeks later, chimeric mice were used for the induction of IMQ-induced dermatitis. The ratio of cell chimerism in the skin was analyzed by flow cytometry (Supplemental Fig. 3A).

Isolation of primary ear skin cells

Ear lobes were cut out, minced into small pieces (<2 mm^2), and digested with 0.03% hyaluronidase (Sigma-Aldrich), 0.27% collagenase XI (Sigma-Aldrich), 10 U/ml DNase I (Sigma-Aldrich), and 10 mM HEPES in RPMI 1640 medium for 2 h at 37°C. The homogenate was disaggregated by vigorous pipetting, and single-cell suspension was prepared by filtrating through a cell strainer (70 μm).

Immunohistochemistry

Mouse ear biopsy specimens were fixed with 10% neutral formalin, dehydrated, and paraffin-embedded. Five-micrometer sections were deparaffinized and stained with H&E, and they were mounted using Mount-Quick (Daido Sangyo, Saitama, Japan). Images were acquired using a fluorescent microscope, BZ-9000 (Keyence, Osaka, Japan). Thickness of the epidermis was analyzed using ImageJ (W. Rasband, National Institutes of Health), an image-processing program. Psoriasiform disease indices (redness and scaling) were assessed by two persons independently under strict criteria. Histology score was determined by the sum of the following pathological scores, in support of the dermatologists (Y.N. and H.M.). Hyperkeratosis (thickness of keratin layers in stratum corneum) was scored as follows: normal, 0; mild, 1; and severe, 2. Parakeratosis (presence of nuclei in a stratum corneum) was scored as follows: absence, 0; mild, 1; and severe, 2. Microabscess (abundance of aggregates of lymphocytes on stratum corneum) were scored as follows: absence, 0; present, 1; and abundant, 2. Spongiosis (edema in dermis) was scored as follows: mild symptoms, 1; severe symptoms all over the epidermis, 2; and severe symptoms with infiltrates, 3. Acanthosis (thickening of epidermal layers) was scored as follows: less than 30 μm, 0; 30 to ∼50 μm, 1; and >50 μm, 2.

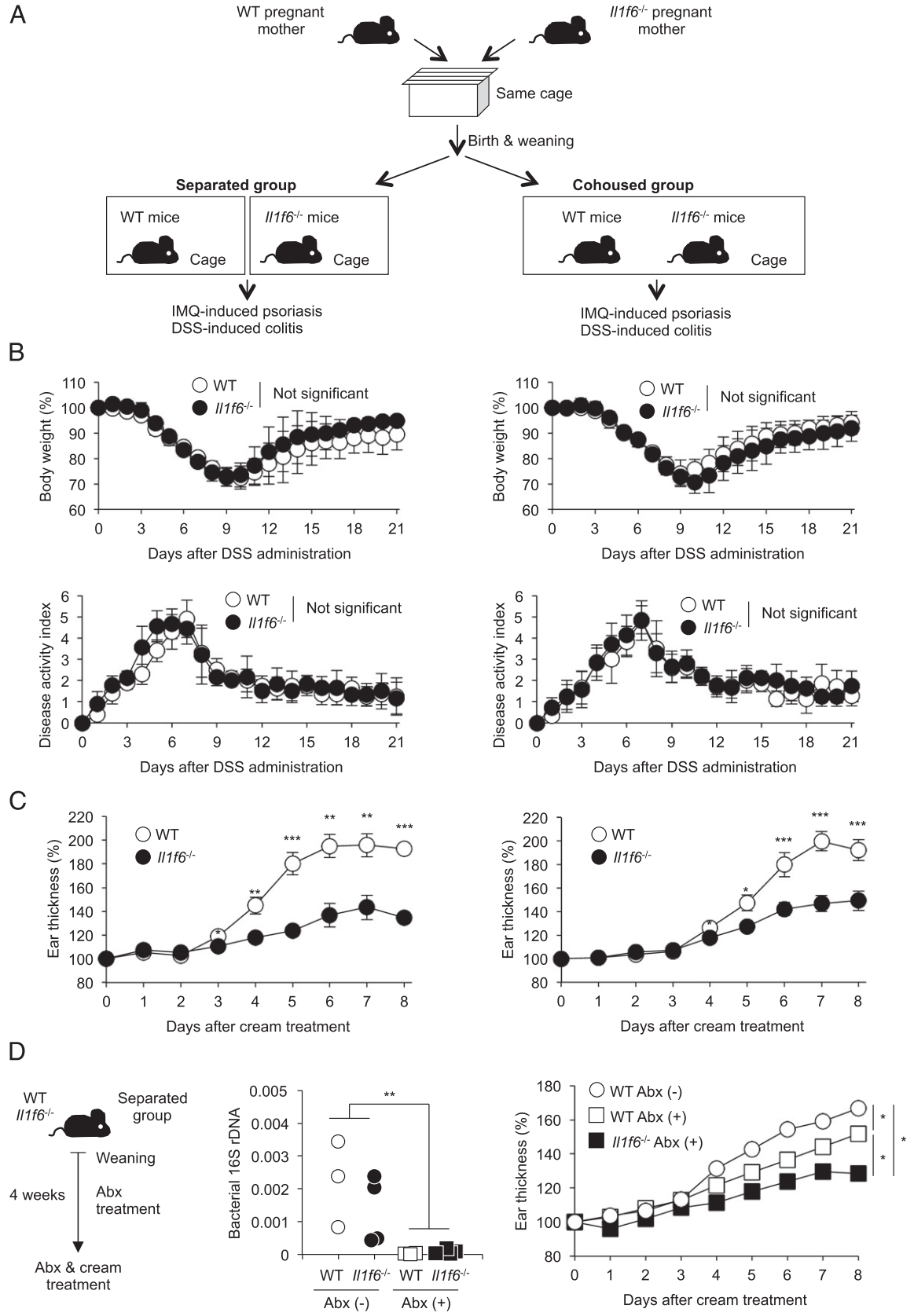

FIGURE 1. The development of IMQ-induced psoriasiform dermatitis, but not DSS-induced colitis, is attenuated in *Il1f6*$^{-/-}$ mice. (**A**) Experimental settings for DSS-induced colitis and IMQ-induced psoriasiform dermatitis under separated or cohoused conditions. (**B**) Mice were administrated with 2% DSS in drinking water for 7 d followed by 2 wk of normal drinking water. Body weight (upper panels) and disease activity index (lower panels) were daily evaluated (separated group, $n = 9$ each; cohoused group, $n = 12$ each). Data are mean ± SD. (**C**) Ear lobes were treated daily with IMQ cream for 8 d. Ear lobe thickness was measured daily using a caliper. Ear swelling is shown as percentage of thickness compared with the thickness at day 0. Data are mean ± SEM. Separated group: WT = 11, *Il1f6*$^{-/-}$ = 8. Cohoused group: WT = 10, *Il1f6*$^{-/-}$ = 12. (**D**) After weaning, mice were fed with drinking water containing Abx mixture for 4 wk followed by 8 d of daily topical application of IMQ cream. The contents of bacterial 16S rDNA in the *(Figure legend continues)*

Table II. Abs and streptavidin

Name	Clone	Manufacturer
Biotin-conjugated anti-TER119	TER119	BD Pharmingen, San Diego, CA
Biotin-conjugated anti-CD8α	53-6.7	BioLegend, San Diego, CA
Biotin-conjugated anti-CD11b	M1/70	Biolegend
Biotin-conjugated anti-DC49b	DX5	BioLegend
Biotin-conjugated anti-B220	RA3-6B2	BioLegend
Biotin-conjugated anti-γδTCR	UC7-13D5	eBioscience, San Diego, CA
Biotin-conjugated anti-CD207	929F3.01	Dendritics, Lyon, France
APC/Cy7-conjugated anti-CD3e	17A2	BioLegend
PE-conjugated anti-γδTCR	UC7-13D5	BioLegend
Pacific blue–conjugated anti-βTCR	H57-597	BioLegend
PE/Cy7-conjugated anti-CD4	GK1.5	BD Pharmingen
APC-conjugated anti-CD25	PC61.5	eBioscience
Pacific blue–conjugated anti-CD62L	MEL-14	BD Pharmingen
PE/Cy7-conjugated anti-CD11c	N418	BioLegend
APC-conjugated anti-B220	RA3-6B2	BioLegend
APC/Cy7-conjugated I-E/I-A	M5/114.15.2	BioLegend
Pacific blue–conjugated anti-CD45.1	A20	BioLegend
FITC-conjugated anti-CD45.2	104	BioLegend
PE/Cy7-conjugated anti-CD11b	M1/70	BioLegend
APC-conjugated streptavidin	—	BioLegend

—, not applicable.

Reverse transcription and qPCR

RNAs from tissues and cells were extracted using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), respectively. The resulting RNA was reverse-transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). qPCR analysis was performed using an iCycler system (Bio-Rad, Hercules, CA) with either SYBR Premix Ex Taq or SYBR Premix Ex Taq II qPCR kits (TaKaRa, Kyoto, Japan) and primer sets (Table I). The ΔΔCt method was used for analysis, and the expression of target genes was normalized with *Gapdh*. Bacterial rRNA-encoding DNA (rDNA) in the skin was also determined by qPCR with the primer set in Table I. Relative contents of bacterial rDNA were calculated by the ΔCt method.

Generation of BM-derived myeloid cells

BM-derived myeloid cells were prepared as described previously (55). Briefly, BMCs were obtained by flushing femurs and tibiae. For preparation of Fms-like tyrosine kinase 3 ligand–induced DCs (Flt3L-DCs), BMCs were cultured in the presence of 100 ng/ml recombinant mouse Flt3L for 10 d (PeproTech, Rocky Hill, NJ). Plasmacytoid DCs (pDCs) were isolated by flow cytometry after Ab staining (described below). For generation of GM-DCs, BMCs were cultured in the presence of 20 ng/ml recombinant mouse GM-CSF (PeproTech) for 10 d. Nonadherent cells were collected as GM-DCs. For generation of BM-derived Langerhans cells (BMLCs), BMCs were cultured with 25 ng/ml recombinant mouse GM-CSF, 25 ng/ml recombinant mouse IL-4 (PeproTech), and 8 ng/ml recombinant human TGF-β (PeproTech) for 7 d. For generation of M-CSF–induced macrophages, BMCs were cultured with 20 ng/ml recombinant mouse M-CSF (R&D Systems, Minneapolis, MN) for 7 d.

Preparation of T and B cells

T and B cells were isolated from the spleen using an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD90.2– and anti-B220–conjugated microbeads (Miltenyi Biotec), respectively, in accordance with the manufacturer's instructions. For preparation of naive T cells ($CD25^{lo}$ $CD62L^{hi}CD4^{+}$), cells from the spleens and lymph nodes were labeled with biotin-conjugated Abs (Table II) followed by anti-biotin–conjugated microbeads (Miltenyi Biotec). The labeled cells were negatively selected using an autoMACS. The cells were further purified by flow cytometry as describe below.

Preparation of primary keratinocytes

Primary keratinocytes were generated according to CELLnTEC's instruction. Neonates were anesthetized with isoflurane and sterilized with 70% ethanol. The dorsal skin was separated from the body and floated on the CnT-Prime medium (CELLnTEC, Bern, Switzerland) containing 5 mg/ml dispase II (Wako), 500 U/ml penicillin (Life Technologies), and 500 μg/ml streptomycin (Life Technologies) at 4°C overnight. Epidermal sheets were peeled off, incubated with TrypLE Select (Life Technologies) for 30 min at room temperature, and disaggregated by vigorous pipetting. Single-cell suspension was seeded on 96-well plates and incubated at 35°C in 5% CO_2 incubator. Three days later, the cells (1×10^5) were stimulated with indicated cytokines for 6 and 24 h at 35°C.

Preparation of mouse embryonic fibroblasts

Mice on day 13.5–15.5 of pregnancy were anesthetized with isoflurane, and embryos were separated. After removal of the head and internal organs, the embryos were minced and treated with 0.1% trypsin/EDTA (Sigma-Aldrich) with gentle shaking for 15 min. Single-cell suspensions were seeded on 0.1% gelatin-coated dishes.

Flow cytometry

Abs used for flow cytometric analysis are listed in Table II. Flow cytometry was performed as described previously (55, 56). Briefly, cells were stained with fluorescence-conjugated Abs for 30 min at 4°C after blocking with 2.4G2. Dead cells were stained with 7-AAD (Sigma-Aldrich). Cells were analyzed by a flow cytometer, FACSCanto II (Becton Dickinson, Sparks, MD) with FlowJo (Tree Star, Ashland, OR). For intracellular CD207 staining, cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% saponin, followed by staining with biotin-conjugated anti-Langerin Ab and APC-conjugated streptavidin. For preparation of highly purified cells, the following fluorescence-labeled cells were sorted by a MoFlo XDP (Beckman Coulter, Miami, FL): naive T cells, $CD4^{+}CD25^{lo}$ $CD62L^{hi}$ cells; γδ T cells, $CD3\varepsilon^{+}\beta TCR^{-}\gamma\delta$ TCR^{+} cells; pDCs, $B220^{+}$ $CD11c^{+}$ cells.

Induction of IL-36α in cell culture

Cells were cultured in the presence of indicated cytokines (IL-36α [R&D Systems], 10 and 100 ng/ml; TGF-β, 20 ng/ml; IL-6 [PeproTech], 20 ng/ml; IL-23 [R&D Systems], 20 ng/ml; and IL-1β [PeproTech], 10 ng/ml) or indicated pathogen-associated molecular patterns (PAMPs) (IMQ [InvivoGen],

skin of untreated and Abx-treated mice were analyzed by qPCR. WT Abx (–) = 3, *Il1f6*$^{-/-}$ Abx (–) = 4, WT Abx (+) = 3, and *Il1f6*$^{-/-}$ Abx (+) = 5. Ear lobe thickness was measured daily. Ear swelling is expressed as percentage of thickness compared with the thickness at day 0. Data are mean ± SEM. WT Abx (–) = 4, WT Abx (+) = 8, and *Il1f6*$^{-/-}$ Abx (+) = 8. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student *t* test.

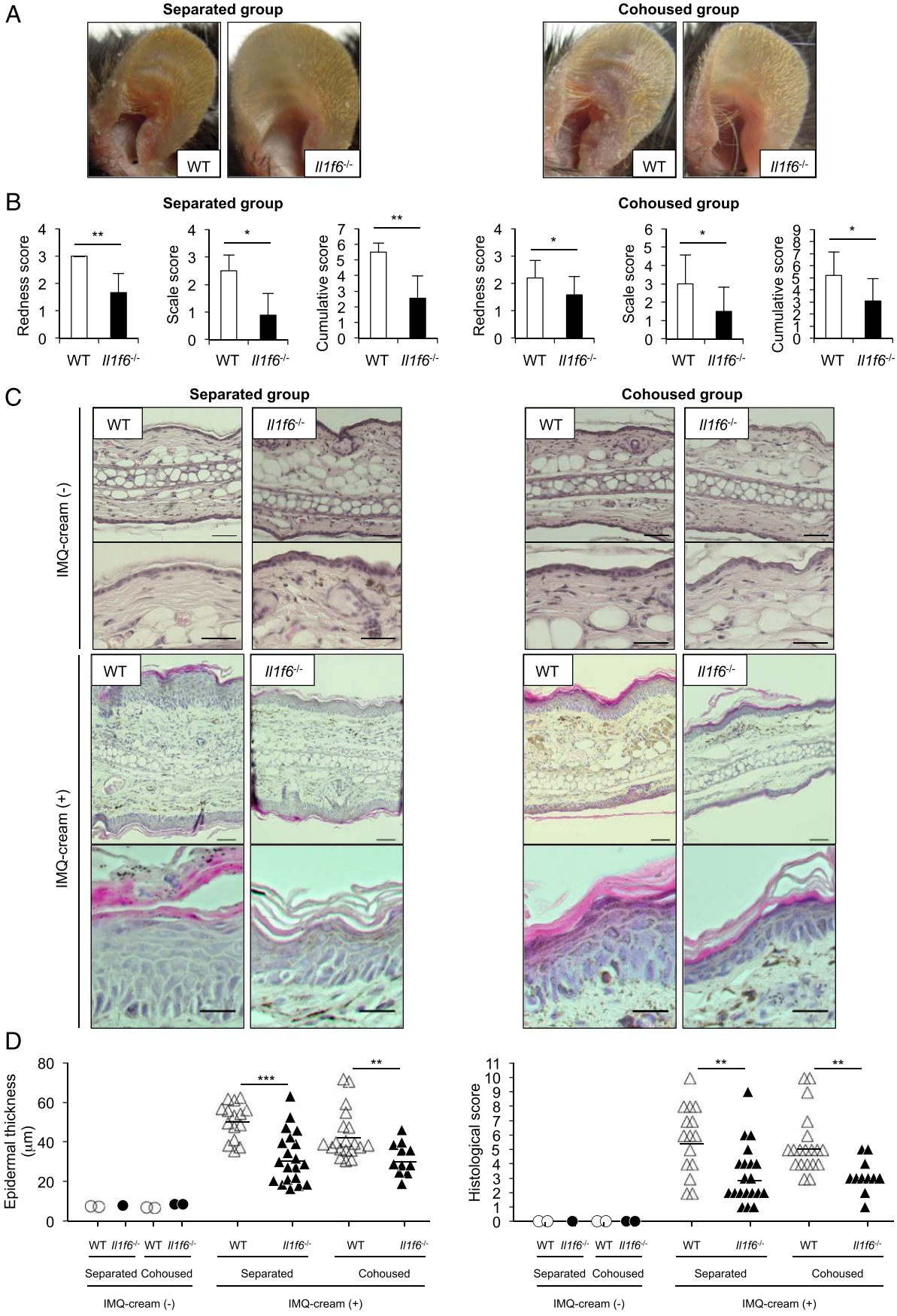

FIGURE 2. The severity of psoriasis-like dermatitis in *Il1f6*$^{-/-}$ mice is low compared with WT mice, irrespective of the housing conditions. (**A**) Photographs of the psoriatic ear lesions of WT and *Il1f6*$^{-/-}$ mice at day 8. (**B**) Clinical assessment (scale, redness, and cumulative score) of the ears of WT and *Il1f6*$^{-/-}$ mice at day 8 after IMQ cream treatment. Data are mean ± SD. Separated group: WT = 4, *Il1f6*$^{-/-}$ = 9. Cohoused group: WT = 10, *Il1f6*$^{-/-}$ = 12. *$p < 0.05$, **$p < 0.01$, Mann–Whitney U test. (**C**) Earlobe sections of WT and *Il1f6*$^{-/-}$ mice at day 0 and day 8 after *(Figure legend continues)*

5 and 20 μg/ml; LPS [Sigma-Aldrich], 1 and 10 ng/ml; ODN D19 [Operon], 0.1 and 1 μg/ml; Poly(I:C) [InvivoGen], 1 and 10 μg/ml; and zymosan [Sigma-Aldrich], 10 and 100 μg/ml) for indicated time.

Statistics

The Mann–Whitney *U* test was used for clinical and histological scores of IMQ-induced dermatitis and DSS-induced colitis. Log rank test was applied for the differences in survival rate. The two-tailed Student *t* test was used for other experiments. The *p* values <0.05 were considered significant: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

IL-36α deficiency ameliorates the development of IMQ-induced psoriasiform dermatitis, but not DSS-induced colitis, in mice in cohoused and separately housed experimental conditions

We generated *Il1f6*$^{-/-}$ mice by homologous recombination techniques, as described in Supplemental Fig. 1A. Homologous recombination was confirmed by Southern blot hybridization analysis, and *Il1f6* deficiency was verified by qPCR with mRNAs from stomach and IMQ cream–treated ears (Supplemental Fig. 1B–D). Because commensal microbiota affect the development of colitis and psoriasis (22, 39, 41), we designed the experiments so as to evaluate the contribution of commensal microbiota, as shown in Fig. 1A. WT and *Il1f6*$^{-/-}$ pregnant mothers were cohoused in the same cages, and their offspring were further nursed together until weaning. After genotyping by PCR analysis at ∼4 wk of age, WT and *Il1f6*$^{-/-}$ mice were separately housed in one group (separated group), whereas in another group, both WT and *Il1f6*$^{-/-}$ mice were further cohoused (cohoused group). More than 1 mo after weaning, these mice were administered with 2% DSS–containing tap water to induce colitis or with IMQ cream spread over ear lobes to induce dermatitis. The development of colitis, in terms of the severity and the onset time, was similarly observed in both *Il1f6*$^{-/-}$ and WT mice (Fig. 1B, Supplemental Fig. 2). In contrast, we found that the severity of dermatitis was much milder in *Il1f6*$^{-/-}$ mice compared with WT mice, in both separately housed and cohoused groups. No significant difference of ear thickness was observed between separately housed and cohoused groups (Fig. 1C). These results suggest that IL-36α deficiency causes suppression of IMQ-induced dermatitis, but not through an effect on commensal microbiota. To confirm this, we induced IMQ-induced dermatitis in mice orally administered with an Abx mixture, which reduced the contents of commensal bacteria in the skin of mice (Fig. 1D). Although the extent of ear thickness in Abx-treated WT mice was significantly decreased compared with Abx-untreated WT mice (Fig. 1D), consistent with a previous report (41), Abx-treated *Il1f6*$^{-/-}$ mice still showed milder ear swelling than Abx-treated WT mice. These results suggest that the effect of *Il1f6* deficiency on the development of IMQ-induced dermatitis is not mediated by the change of commensal microbiota.

Psoriasiform dermatitis is suppressed in Il1f6$^{-/-}$ *mice irrespective of their housing conditions*

The psoriasiform skin pathology was attenuated in *Il1f6*$^{-/-}$ mice in both separate and cohousing conditions (Fig. 2A). Severity scores of redness and scale were significantly reduced in *Il1f6*$^{-/-}$ mice compared with WT mice (Fig. 2B). Histological analysis by H&E staining revealed that thickness of epidermis was significantly reduced in *Il1f6*$^{-/-}$ mice compared with WT mice (Fig. 2C, 2D), and histological scores representing the extent of hyperkeratosis (Supplemental Fig. 3B; green line), parakeratosis (blue asterisk), spongiosis, acanthosis (yellow line), and formation of microabscess (pink arrow) were much milder in earlobes of *Il1f6*$^{-/-}$ mice, in both separated and cohoused groups. Notably, no significant difference of the dermatitis severity was observed in *Il1f6*$^{-/-}$ mice between separate and cohoused groups (Fig. 2B, 2D).

The gene expression of Th17-associated cytokines and chemokines is downregulated in Il1f6$^{-/-}$ *mouse skin after treatment with IMQ*

To know the role of IL-36α in IMQ-induced psoriasiform dermatitis, we examined gene expression of cytokines, chemokines, and antimicrobial peptides relevant to psoriasiform skin inflammation. The expression of *Il6*, *Il1a*, *Il1b*, *Il17c*, *Il17f*, *Cxcl2*, and *S100a9* in IMQ cream–treated ears of *Il1f6*$^{-/-}$ mice was significantly reduced compared with those of WT mice (Fig. 3). *Il17a* expression also tended to decrease in IMQ cream–treated *Il1f6*$^{-/-}$ mouse skin.

IL-36α expression in skin-resident cells is important for the pathogenesis of IMQ-induced psoriasiform dermatitis

Next, we examined the producer cells of IL-36α upon treatment with IMQ. First, we examined IL-36α, from which tissue-resident or BM-derived cells are responsible for the pathogenesis of IMQ-induced dermatitis. For this, *Rag2*$^{-/-}$ and *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ mice were lethally irradiated with gamma rays, and then BMCs from WT and *Il1f6*$^{-/-}$ mice were transplanted, followed by the induction of IMQ-induced dermatitis. *Rag2*$^{-/-}$ recipient mice transplanted with WT or *Il1f6*$^{-/-}$ BMCs developed severe dermatitis with increased ear thickness and notable epidermal pathology (Fig. 4A, 4B). In contrast, *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ recipient mice transplanted with WT or *Il1f6*$^{-/-}$ BMCs developed much milder dermatitis. Severity scores of scaling were significantly reduced in *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ recipient mice transferred with WT and *Il1f6*$^{-/-}$ BMCs, although redness was not changed significantly (Fig. 4C). Decreased epidermal thickness in WT or *Il1f6*$^{-/-}$ BMC-transplanted *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ recipient mice was observed compared with WT or *Il1f6*$^{-/-}$ BMC-transplanted *Rag2*$^{-/-}$ recipient mice (Fig. 4D, 4E, Supplemental Fig. 3C). Under our experimental conditions, ∼80% dermal DCs were substituted with donor BM-derived cells, whereas almost all the LCs remained as the original recipient type after 1 mo of BM transfer (Supplemental Fig. 3A). These results suggest that IL-36α producer cells upon treatment with IMQ reside in the skin.

Il1f6 expression is induced in DCs and LCs following stimulation with PAMPs

Because DCs and LCs in the skin are key initiators/amplifiers of cutaneous immune responses, we analyzed the expression of *Il1f6* in GM-DCs and BMLCs following stimulation with PAMPs.

IMQ cream treatment were stained with H&E, and histology was examined by microscope. One of representative sections is shown. Scale bars in upper panels, 50 μm; scale bars in lower panels, 20 μm. (**D**) Epidermal thickness was analyzed by ImageJ with H&E sections, and histology score was assessed as described in the *Materials and Methods*. Separated group (IMQ, –): WT = 2, *Il1f6*$^{-/-}$ = 1. Cohoused group (IMQ, –): WT = 2, *Il1f6*$^{-/-}$ = 2. Separated group (IMQ, +): WT = 15, *Il1f6*$^{-/-}$ = 20. Cohoused group (IMQ, +): WT = 19, *Il1f6*$^{-/-}$ = 11. Data from two to three independent experiments are combined. Left panel, $**p < 0.01$, $***p < 0.001$, Student *t* test. Right panel, $**p < 0.01$, Mann–Whitney *U* test.

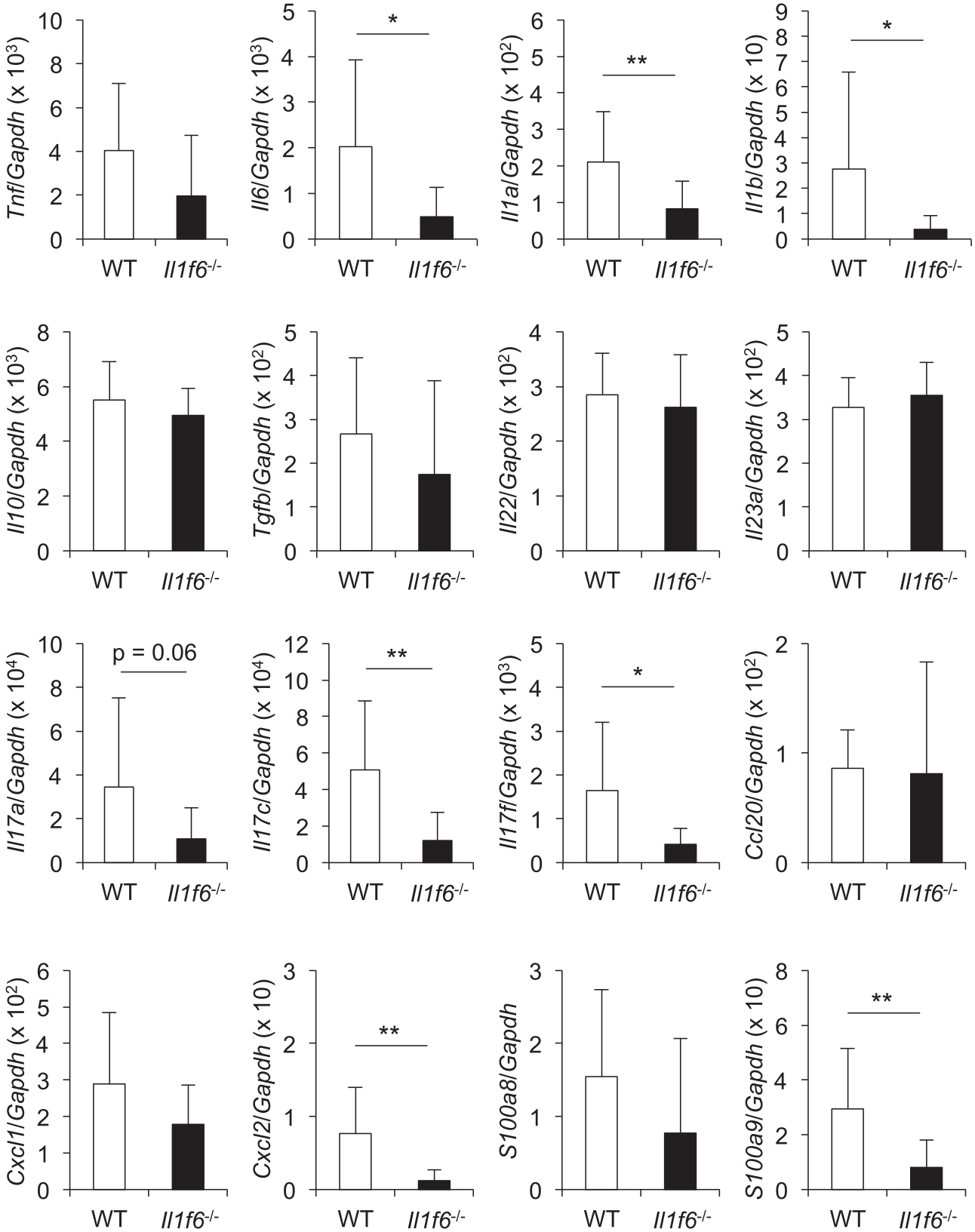

FIGURE 3. Expression of proinflammatory cytokine and chemokine genes is impaired in the ears of *Il1f6*$^{-/-}$ mice after IMQ cream treatment. Expression of psoriasis-related inflammatory genes in the ears of WT and *Il1f6*$^{-/-}$ mice at day 5 after IMQ cream treatment was examined by qPCR. Data from three independent experiments are combined (WT = 11, *Il1f6*$^{-/-}$ = 13). Data are mean ± SD. *$p < 0.05$, **$p < 0.01$, Student *t* test.

qPCR analysis revealed that *Il1f6* expression was strongly induced in GM-DCs following stimulation with zymosan (TLR2 and Dectin-1/-2 ligands, 6.6- and 11.0-fold), LPS (TLR4 ligand, 1.6 and 4.1) and IMQ (TLR7/8 ligand, 4.7 and 12.9), but not in Poly(I:C) (TLR3 ligand) and ODN D19 (TLR9 ligand) (Fig. 5A). In BMLCs, zymosan (76.8 and 157.3) and IMQ (21.1 and 32.2),

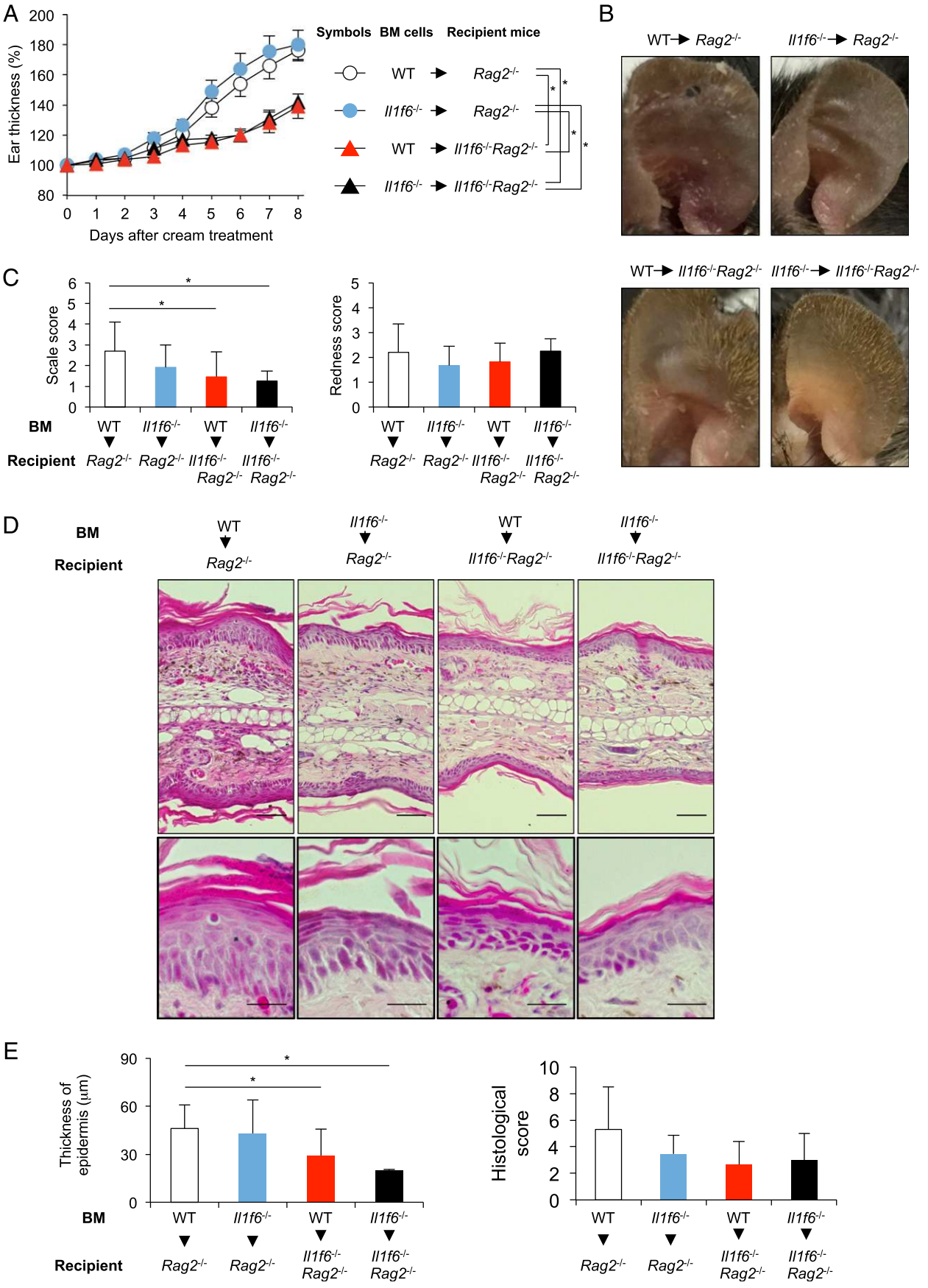

FIGURE 4. IL-36α expression in skin-resident cells is important for the pathogenesis of IMQ-induced dermatitis. (**A**) BMCs from WT or *Il1f6*$^{-/-}$ mice were transferred into gamma ray–irradiated *Rag2*$^{-/-}$ and *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ recipient mice, and after 4 wk, the recipient mouse ears were treated daily with IMQ cream. Ear thickness was measured daily using a caliper. Ear swelling is shown as a percentage of thickness compared with the ears at day 0. Data are mean ± SEM. WT BMCs → *Rag2*$^{-/-}$ = 10, *Il1f6*$^{-/-}$ BMCs → *Rag2*$^{-/-}$ = 12, WT BMCs → *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ = 11, and *(Figure legend continues)*

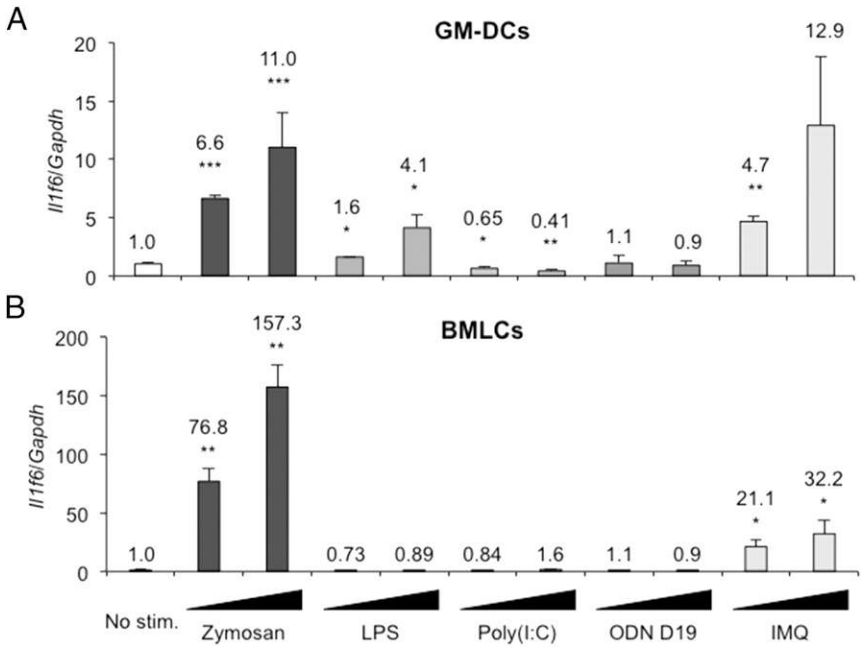

FIGURE 5. *Il1f6* is induced in GM-DCs and BMLCs upon stimulation with PAMPs. GM-DCs (**A**) and BM-LCs (**B**) were stimulated with zymosan (10 and 100 μg/ml), LPS (1 and 10ng/ml), Poly(I:C) (1 and 10 μg/ml), ODN D19 (0.1 and 1 μg/ml), and IMQ (5 and 20 μg/ml) for 24 h. Expression levels of *Il1f6* transcripts in these cells were analyzed by qPCR. Data are mean ± SD. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student *t* test.

but not with LPS; Poly(I:C); and ODN D19 enhanced the expression of *Il1f6* (Fig. 5B). These results suggest that DCs and LCs in the skin can respond to various innate stimulations to produce IL-36α.

Il1f6 expression is induced in mouse embryonic fibroblasts, BMLCs, and keratinocytes upon stimulation with IL-36α

Next, we examined expression of *Il1f6* and *Il1rl2* in cells of the skin under physiological conditions. *Il1f6* gene was strongly expressed in keratinocytes, but not in mouse embryonic fibroblasts (MEFs) and BMLCs (Fig. 6A), whereas *Il1rl2* transcript was detected in keratinocytes, MEFs, and BMLCs (Fig. 6B). Because it was reported that IL-36 signaling augments IL-36 expression by forming a self-amplification loop in human keratinocytes (18), we investigated the induction of *Il1f6* and *Il1rl2* in keratinocytes, MEFs, and BMLCs after stimulation with IL-36α to examine if such a self-amplifying loop is also observed in IMQ-stimulated mouse skin. Expression of *Il1f6* was markedly induced in BMLCs (12.0- and 27.8-fold; Fig. 6C) and less markedly induced in keratinocytes (1.1 and 2.2; Fig. 6D), but not in MEFs (Fig. 6E), after stimulation with IL-36α. Furthermore, *Il1f9* expression was also induced in MEFs (5.1 and 11.6; Fig. 6F), BMLCs (6.4 and 6.8; Fig. 6G), and keratinocytes (1.4 and 2.1; Fig. 6H), whereas *Il1f8* expression was not induced in these cells (Fig. 6F–H). These results suggest that an IL-36α self-amplification loop also exists in mouse skin after stimulation with IMQ.

Proinflammatory cytokines and chemokines are induced in MEFs, BMLCs, and keratinocytes by IL-36α stimulation

Many cytokines, chemokines, and anti-micropeptides such as TNF, IL-1α, IL-1β, IL-17A, IL-17F, IL-17C, IL-22, IL-23, CCL20, CXCL1, CXCL2, CXCL9, S100a8, and S100a9 are suggested to be involved in the development of psoriasis (57–60). Thus, we examined the induction of these psoriasis-related gene expressions in MEFs, BMLCs, and keratinocytes after 24-h stimulation with IL-36α in vitro. qPCR analysis revealed that the expression of *Tnf*, *Il1a*, and *Il1b* was increased in MEFs, BMLCs, and keratinocytes (Fig. 7), whereas *Il23a* was induced in BMLCs. *Cxcl1* and/or *Cxcl2*, but not *Cxcl9*, were induced in MEFs, BMLCs, and keratinocytes, whereas *Ccl20* was induced in MEFs. *S100a8* and *S100a9* were selectively induced in keratinocytes. However, IL-17 family cytokine genes, such as *Il17a*, *Il17c*, *Il17f*, and *Il22* were not induced in MEFs, BMLCs, and keratinocytes. We also examined differentiation-related markers (57, 61). *Cd40* was induced in MEFs, BMLCs, and keratinocytes, whereas *Iab* was not induced in BMLCs and keratinocytes. *Krt6* was not induced in keratinocytes.

IL-36α selectively induces Il17c expression from keratinocytes

Finally, we examined IL-17 family cytokine induction in keratinocytes by IL-36α in detail. We examined the effect of IL-36α in combination with TGF-β, IL-6, IL-23, and IL-1β. qPCR analysis showed that keratinocytes selectively induced *Il17c*, but not *Il17a*, *Il17f*, or *Il22* after 6-h stimulation, but we could not observe any promoting effects of TGF-β + IL-6 + IL-23 + IL-1β (Fig. 8). Consistent with this, the expression of RAR-related orphan receptor γ (RORγt), a transcription factor that is required for the production of IL-17A, IL-17F, and IL-22 (62), was not changed (Fig. 8). These results suggest that IL-36α selectively induces *Il17c* from keratinocytes.

Discussion

In this article, we presented evidence that the pathogenic roles of IL-36α are different between DSS-induced colitis and IMQ-induced

Il1f6$^{-/-}$ BMCs → *Il1f6*$^{-/-}$*Rag2*$^{-/-}$ = 4. Data from three independent experiments are combined. $*p < 0.05$, Student *t* test. (**B**) Photographs of psoriatic ear lesions at day 8. (**C**) Severity score of scale and redness of the psoriatic ear lesions as shown in (B). Data are mean ± SD. $*p < 0.05$, Mann–Whitney *U* test. (**D**) Earlobe sections were stained with H&E, and histology was examined by microscope. One of representative sections is shown. Scale bars in upper panels, 50 μm; scale bars in lower panels, 20 μm. (**E**) Epidermal thickness was analyzed by ImageJ with H&E sections, and histological score was assessed as described in the *Materials and Methods*. Epidermal thickness: Histological score: (Mann–Whitney *U* test). Data are mean ± SD. $*p < 0.05$, Student *t* test.

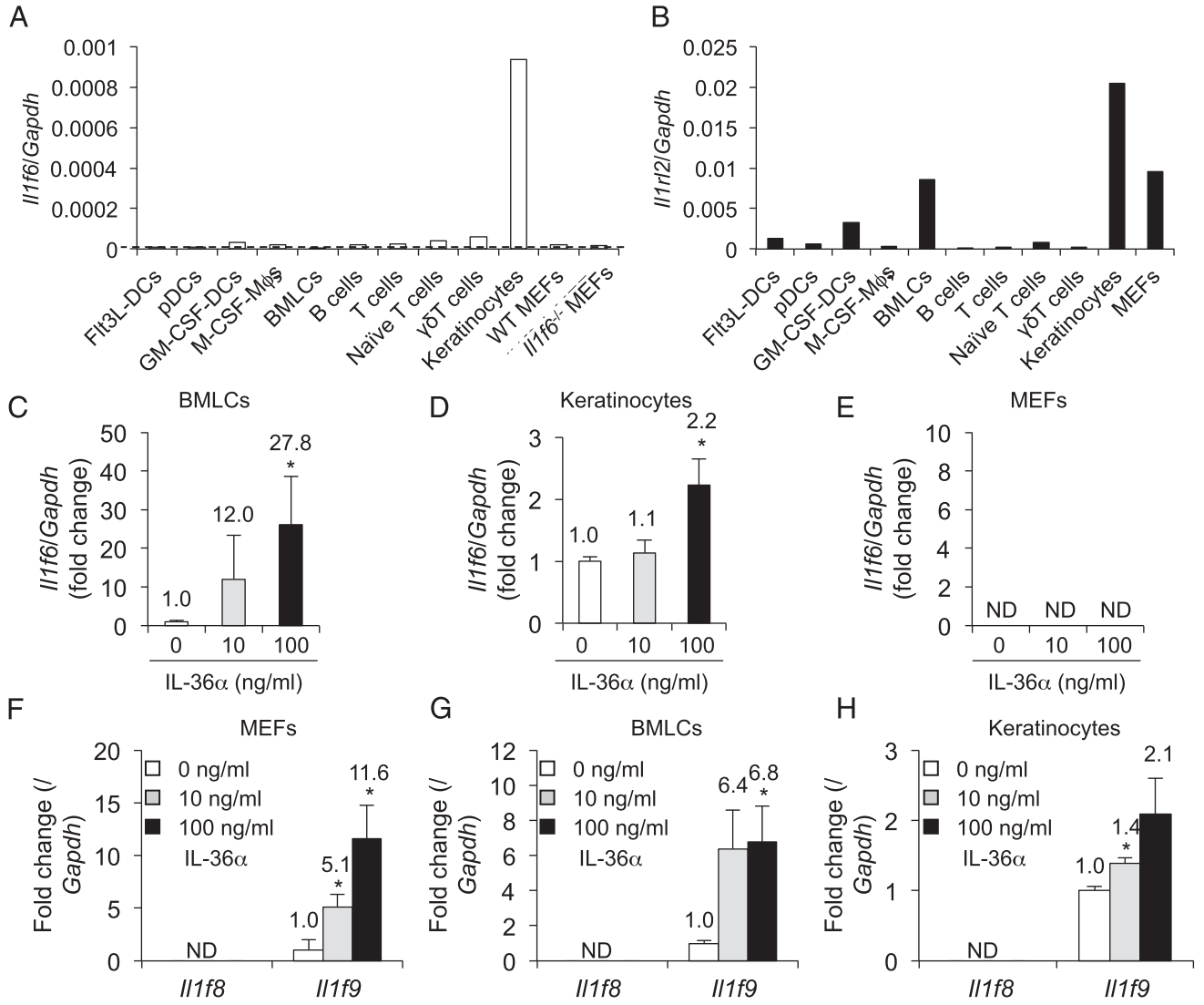

FIGURE 6. IL-36α activates *Il1f6* and *Il1f9* genes in MEFs, BMLCs, and keratinocytes. (**A** and **B**) Flt3L-DCs, pDCs, GM-DCs, M-CSF macrophages (Mϕ), and BMLCs were induced from WT BMCs in vitro, and other cells were prepared from WT or *Il1f6*$^{-/-}$ mice. The spontaneous expression of *Il1f6* (A) and *Il1rl2* (B) in indicated cells without any treatment was analyzed by qPCR. (**C**–**E**) Cells were stimulated with IL-36α (10 and 100 ng/ml) for 24 h. Expression of *Il1f6* transcripts in BMLCs (C), keratinocytes (D), and MEFs (E) was analyzed by qPCR. Data are mean ± SD. Data are representative of two independent experiments. (**F**–**H**) Cells were stimulated with IL-36α (10 and 100 ng/ml) for 24 h. Expression of *Il1f8* and *Il1f9* genes in MEFs (F), BMLCs (G), and keratinocytes (H) was analyzed by qPCR. Data are mean ± SD. Data are representative of two independent experiments. *$p < 0.05$, Student *t* test. ND, not detected.

psoriasiform dermatitis; IL-36α is important for the development of IMQ-induced dermatitis, whereas IL-36α is dispensable for the pathogenesis of DSS-induced colitis. Because DSS-induced colitis is impaired in *Il1rl2*$^{-/-}$ mice (26), and IL-36α, IL-36β, and IL-36γ expression levels are increased in patients with IBD (23–25), it is likely that IL-36 family members other than IL-36α are involved in the development of colitis. Consistent with this idea, it was reported that IL-36γ is the major product in the intestinal mucosal samples of colitis-induced mice and patients with IBD (26). Thus, it seems likely that IL-36γ, instead of IL36α, plays a major role in the development of colitis in mice. However, in humans, the importance of IL-36α is suggested in IBD (21, 63, 64). The expression of human *IL1F6* is increased in patients with UC and CD (24), and *IL1F6* expression is also largely positive in colonic sections from patients with UC (24). Nishida et al. (25) also demonstrated enhanced expression of *IL1F6* in patients with IBD. Thus, the roles of IL-36α in colitis in humans could be more important than those in mice.

It was reported that *Il1rl2*$^{-/-}$ mice are protected from IMQ-induced dermatitis, and *Il36rn*$^{-/-}$ mice showed exacerbated pathology (11), suggesting that IL-36 signaling plays an important role for the development of IMQ-induced dermatitis. Recently, Milora et al. (12) showed that *Il1f6*$^{-/-}$ mice exhibit diminished psoriasiform dermatitis, whereas both *Il1f8*$^{-/-}$ and *Il1f9*$^{-/-}$ mice similarly develop dermatitis as WT mice, suggesting that IL-36α plays a pivotal role for the development of IMQ-induced psoriasiform dermatitis in mice. Consistent with these reports, we showed that *Il1f6*$^{-/-}$ mice are refractory to IMQ-induced dermatitis. Thus, it is suggested that each IL-36 family member has its own roles in the development of inflammatory diseases in a tissue-specific manner. Although we examined the role of IL-36α in the development of psoriasis using an IMQ-induced model, IMQ-induced dermatitis only reproduces limited features of human psoriasis vulgaris, such as erythema, skin thickening, scaling, and epidermal alteration (33). In addition, this model shows some features of chronic atopic dermatitis (65). Therefore, further

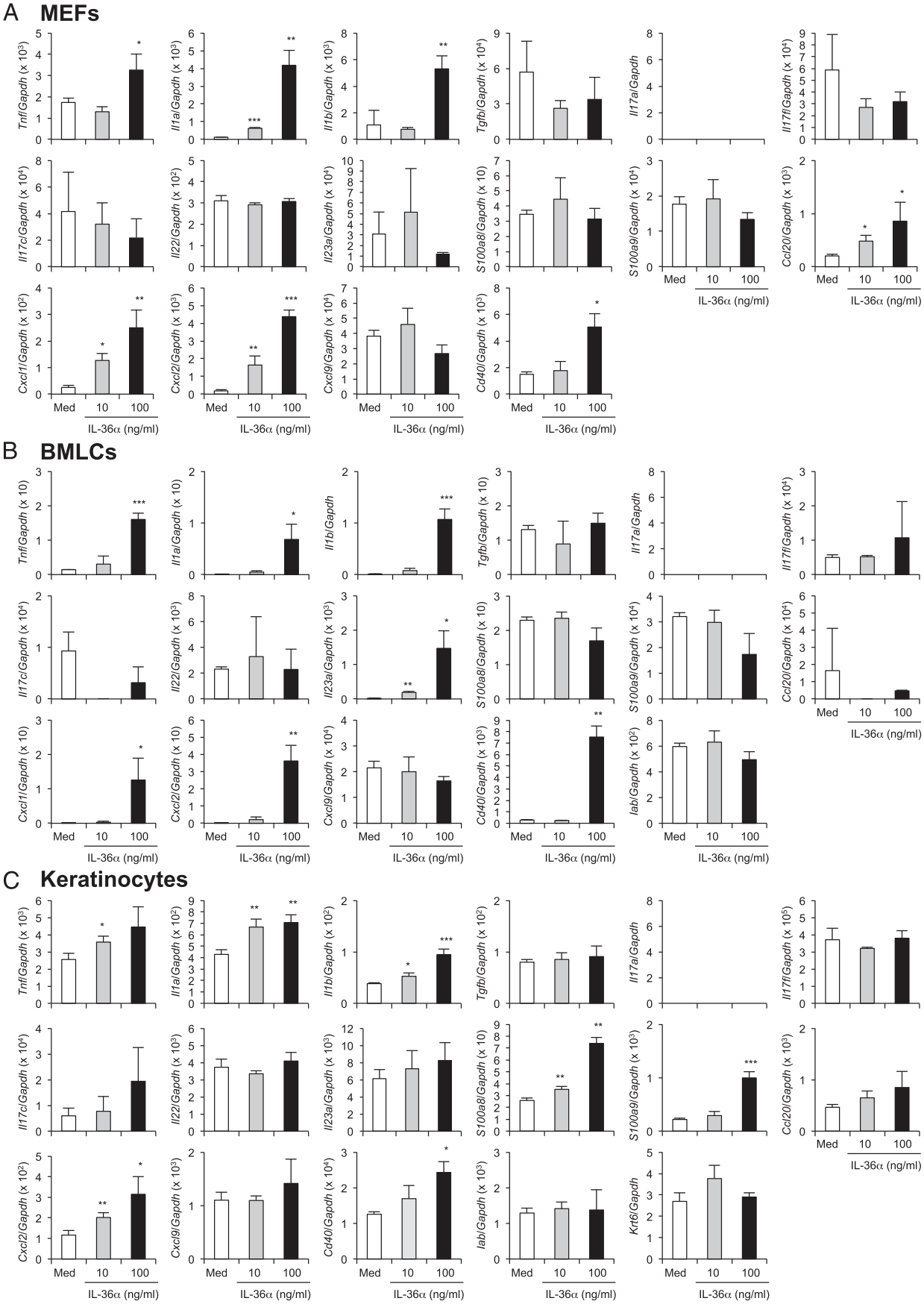

FIGURE 7. Induction of inflammatory cytokine, chemokine, and antimicropeptide gene expression in MEFs, BMLCs, and/or keratinocytes after treatment with IL-36α. Cells were stimulated with IL-36α (10 and 100 ng/ml) for 24 h. Expression of psoriasis-related genes in MEFs (**A**), BMLCs (**B**), and keratinocytes (**C**) was determined by qPCR. Data are mean ± SD. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student t test. ND, not detected.

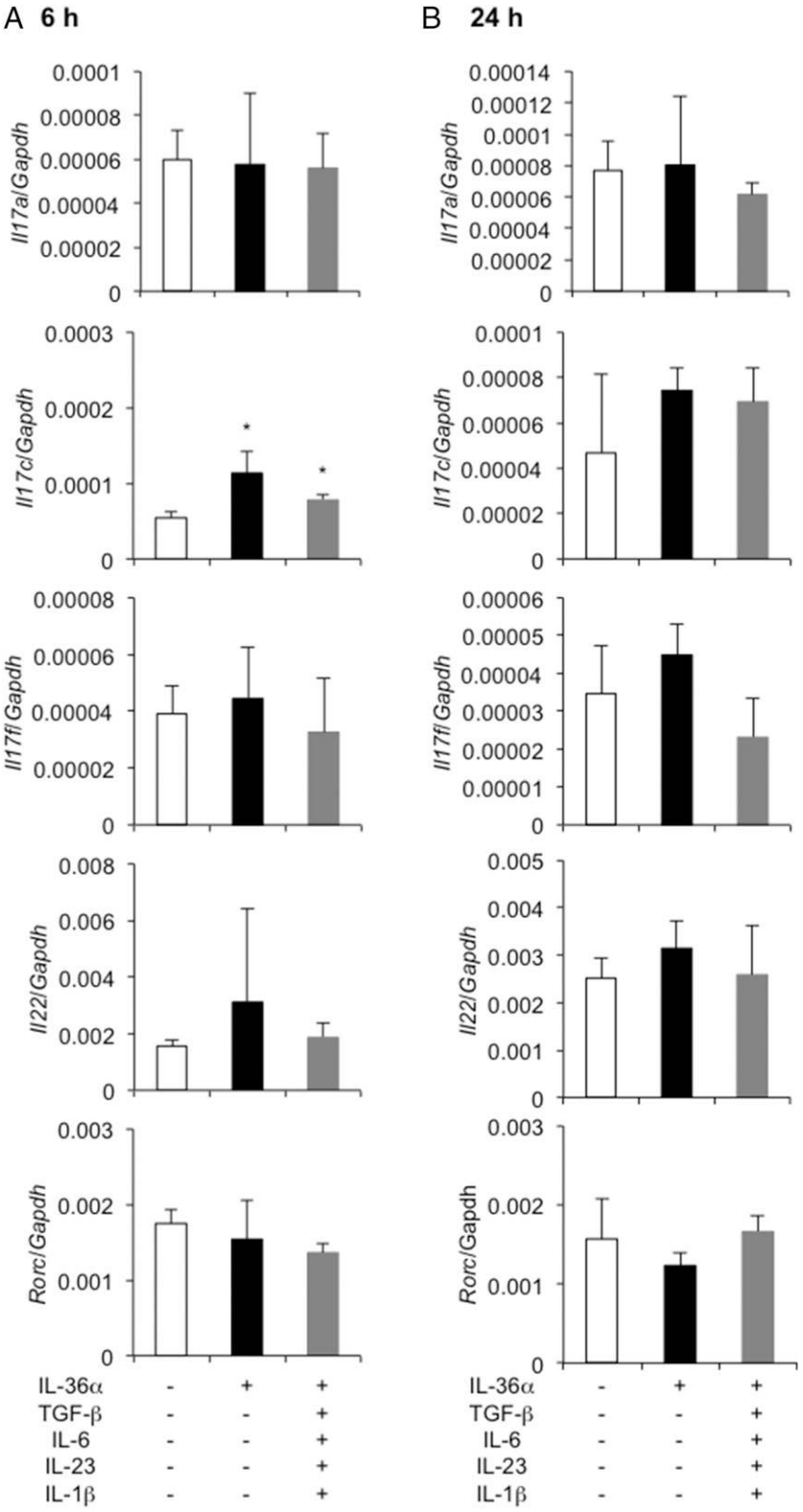

FIGURE 8. IL-17C gene is selectively induced in keratinocytes following stimulation with IL-36α. (**A** and **B**) Keratinocyte culture was stimulated with 100 ng/ml of IL-36α, 20 ng/ml of TGF-β, 20 ng/ml of IL-6, 20 ng/ml of IL-23, and 10 ng/ml of IL-1β for 6 (A) and 24 h (B). Expression of IL-17–related cytokine genes was determined by qPCR. Data are mean ± SD. Data are representative of two independent experiments. $*p < 0.05$.

analysis in humans is needed for understanding the precise roles of IL-36α in psoriasis.

IL-36 induces various inflammatory cytokines, chemokines, and antimicrobial peptides (15, 20) and can control Th1 and Th17 cell differentiation in the downstream (5, 6). Thus, it seemed likely that IL-36 regulates the microbiota in the skin and/or intestine. Because both commensal bacteria/fungi of the skin and intestine are involved in the homeostasis of the skin/intestine and the development of dermatitis and colitis (38, 66, 67), we carefully examined the possibility that the effect of IL-36α is mediated by a change of commensal microbiota of the skin and/or intestine. Interestingly, in both DSS-induced colitis and IMQ-induced psoriasis models, the development of diseases was not affected whether WT and *Il1f6*$^{-/-}$ mice were cohoused or separately housed. Furthermore, the development of IMQ-induced dermatitis was also suppressed in *Il1f6*$^{-/-}$ mice, even

after Abx treatment, although the severity was reduced in Abx-treated mice. These observations suggest that the effect of IL-36α deficiency on the development of psoriasiform dermatitis is not mediated by the regulation of microbiota in the skin or intestine.

We showed that IL-36α is produced in radiation-resistant skin-resident cells in the skin. Furthermore, we showed that IL-36α is induced directly from myeloid-lineage tissue-resident cells, DCs, and LCs, by the activation of TLRs and CLRs. However, because DCs are mostly replaced after BMC transfer, and LCs are the only resident cells that remain after irradiation, LCs are suggested to be the main producer of IL-36α in the skin. These results suggest that commensal microbiota or some pathogens induce production of IL-36α from LCs through activation of TLRs and CLRs, leading to the induction of proinflammatory cytokines, including IL-1 and IL-23, which can activate γδ T cells and ILC3s to produce IL-17A, IL-17F, and IL-22, as well as chemokines to recruit these IL-17–producing cells (48, 68). It should be noted that not only TLR7/8, but also TLR2/4, and even Dectin-1/-2 can induce IL-36α production in GM-DCs, whereas Dectin-1/2 and TLR2/7/8 are mainly involved in the induction of IL-36α in BMLCs. Thus, Dectin-1/2 and TLR2/4/7/8 ligands on commensal and/or pathogenic bacteria and fungi may trigger development of IL-36α–mediated dermatitis through activation of DC and LCs. Keratinocytes, another skin-resident cell, can produce IL-36α when these cells are infected with *Staphylococcus aureus* (69). In this case, however, IL-36α is not produced through activation of innate immune receptors but is released as an alarmin upon exposure to a cytolytic virulence peptide, *S. aureus*–expressed phenol-soluble modulin α. Furthermore, keratinocytes can produce IL-36α upon stimulation with IL-36α in an autocrine manner. Thus, keratinocytes are considered to be the main amplifier of IL-36α action and produce various cytokines that eventually induce production of IL-17A and IL-17F to cause psoriasiform dermatitis. Whether keratinocytes produce IL-36α by directly recognizing PAMPs and/or alarmins remains to be elucidated (61).

We found that the expression of *Il17a*, *Il17c*, *Il17f*, and *Cxcl2* was decreased in the skin of *Il1f6*$^{-/-}$ mice after IMQ treatment, although the change of *Il17a* was rather marginal, suggesting that IL-36α controls IL-17–type immune responses in the skin. Furthermore, we found that IL-36α can induce cytokines such as *Tnf*, *Il1a*, and *Il1b*, as well as chemokines such as *Cxcl1* and *Cxcl2* in MEFs and keratinocytes, in which *Il1rl2* expression was strongly induced. The induction of *Ccl20* and *Il23a* was observed in MEFs and BMLCs, respectively. Thus, although the expression of *Il17a*, *Il17f*, and *Il22* was not directly induced in these cells upon stimulation with IL-36α, chemokines such as *Cxcl1*, *Cxcl2*, and *Ccl20* may recruit IL-17–producing cells, including Th17, γδ T cells, and ILC3s to the skin. Because IL-36R was reported to be expressed on γδ T cells from the salivary glands of patients with Sjogren's syndrome (70), we examined whether IL-36α directly induce IL-17 in γδ T cells. However, we could not detect induction of *Il17a* or *Il17f* expression in γδ T cells (Supplemental Fig. 4A). Thus, these observations suggest that enhanced production of IL-17A, IL-17F, and IL-22 in skin lesions is induced indirectly by IL-36α–induced cytokines such as IL-1 and IL-23 (48, 68). Notably, however, IL-36α can directly induce *Il17c* from keratinocytes.

Transcriptional regulation of IL-17C induction is barely understood. RORγt, *Il17a*, and *Il17f* were not induced in keratinocytes after stimulation with IL-36α, suggesting that the induction mechanisms are different between IL-17C and IL-17A/IL-17F. Because IL-17C is also involved in the development of psoriasis (54, 71), IL-36α may also facilitate development of psoriasis by directly inducing IL-17C from keratinocytes. We also found that the expression of *Il1f6* was enhanced in BMLCs and keratinocytes after IL-36α stimulation. *Il1f9* expression was also induced by IL-36α in BMLCs, MEFs, and keratinocytes, indicating the formation of a self-amplification loop in the skin. These findings suggest that IL-36α expression in LCs, DCs, fibroblasts, and keratinocytes in the skin contributes to the pathogenesis of IMQ-induced dermatitis through induction of Th17 cytokines.

It is known that LCs are originated from BMCs and primitive yolk sac and can self-renew in the epidermis (72, 73). It is difficult to prepare live LCs from the skin by using flow cytometry–based cell sorting because we have to stain Langerin, a signature protein of LCs, intracellularly, which is localized at the cytoplasmic organelles Birbeck granules (74, 75). We therefore generated LCs from BMCs in vitro and used these cells for in vitro experiments, although we cannot exclude completely the possibility that immune responses of BMLCs are different from those of skin LCs.

Taken together, we demonstrate that IL-36α is required for the development of IMQ-induced dermatitis, but not for DSS-induced colitis. IL-36α expression in skin-resident cells promotes the development of psoriasiform dermatitis through direct induction of proinflammatory cytokines, such as IL-1, TNF, IL-23, and IL-17C, and chemokines, resulting in the over-production of Th17 cytokines that cause the development of psoriasis (Supplemental Fig. 4B). This promoting effect of IL-36α on psoriasiform dermatitis is not mediated by the regulation of commensal microbiota. Instead, microbiota of the skin may be important for the induction of IL-36α. Given that human *IL1F6* is highly associated with the development of psoriasis (10, 15, 32) and that mutations in *IL36RN* are reported to associate with general pustular psoriasis and psoriasis vulgaris (31, 76), it seems likely that IL-36α is involved in the development of psoriasis in humans and is an attractive target for the development of medicine.

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Disclosures

The authors have no financial conflicts of interest.

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