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Interplay between CXCR2 and BLT1 Facilitates Neutrophil Infiltration and Resultant Keratinocyte Activation in a Murine Model of Imiquimod-Induced Psoriasis

Hayakazu Sumida,*† Keisuke Yanagida,*‡ Yoshihiro Kita,*§ Jun Abe,‖ Kouji Matsushima,‖ Motonao Nakamura,* Satoshi Ishii,* Shinichi Sato,‡ and Takao Shimizu*†§

Psoriasis is an inflammatory skin disease with accelerated epidermal cell turnover. Neutrophil accumulation in the skin is one of the histological characteristics of psoriasis. However, the precise mechanism and role of neutrophil infiltration remain largely unknown. In this article, we show that orchestrated action of CXCR2 and leukotriene B4 receptor BLT1 plays a key role in neutrophil recruitment during the development of imiquimod (IMQ)-induced psoriatic skin lesions in mice. Depletion of neutrophils with anti-Ly-6G Ab ameliorated the disease severity, along with reduced expression of proinflammatory cytokine IL-1β in the skin. Furthermore, CXCR2 and BLT1 coordinately promote neutrophil infiltration into the skin during the early phase of IMQ-induced inflammation. In vitro, CXCR2 ligands augment leukotriene B4 production by murine neutrophils, which, in turn, amplifies chemokine-mediated neutrophil chemotaxis via BLT1 in autocrine and/or paracrine manners. In agreement with the increased IL-19 expression in IMQ-treated mouse skin, IL-1β markedly upregulated expression of anacanthosis-inducing cytokine IL-19 in human keratinocytes. We propose that coordination of chemokines, lipids, and cytokines with multiple positive feedback loops might drive the pathogenesis of psoriasis and, possibly, other inflammatory diseases as well. Interference to this positive feedback or its downstream effectors could be targets of novel anti-inflammatory treatment. The Journal of Immunology, 2014, 192: 4361–4369.

Psoriasis is a persistent inflammatory skin disease thought to arise as a result of infiltration of inflammatory cells and activation of keratinocytes. Psoriasis has been considered as a classical type 1 autoimmune disease; recently, however, Th17 cells are attracting much interest. Biological drugs targeting the IL-23/IL-17 pathway achieved successful outcomes in psoriasis patients (1). Moreover, a massive amount of neutrophils and activated T cells infiltrates into psoriatic plaques (1). A role for IL-17 in neutrophil-mediated inflammation (2) suggests that neutrophils may also participate in the pathogenesis of psoriasis.

Neutrophils have been traditionally considered as an effector arm of innate immunity. However, recent studies suggest an intimate association of neutrophils with acquired immunity (3), suggesting a crucial role of neutrophils in the pathogenesis of a wide variety of diseases, including infections, autoimmunity, chronic inflammation, and cancer (4). In fact, various neutrophil chemoattractants are known to have unique functions in a number of human diseases (5). In psoriasis, neutrophils first infiltrate into the dermis at the early phase and later into the epidermis at the chronic phase (6). Moreover, neutrophil chemoattractants, including CXCL1, CXCL8 (also known as IL-8), and leukotriene B4 (LTB4), are upregulated in psoriatic skin (7, 8). A case report documented psoriasis remission during drug-induced agranulocytosis and its reappearance after the recovery of neutrophil numbers in the blood (9), suggesting a critical role of neutrophils in psoriatic skin inflammation. However, the detailed kinetics of neutrophil infiltration and its pathological role in psoriasis are still unknown.

Knowledge on the pathology of psoriasis has been obtained mostly through the study of human samples. However, the lack of appropriate animal models hindered addressing the mechanisms for the cellular and molecular events associated with the development of psoriatic lesion. Recently, topical application of imiquimod (IMQ) on the mouse skin has been reported to induce psoriasis-like inflammation (10). In this model, IMQ is suggested to exert its effects through adenosine receptor, but not TLR7 or TLR8, on keratinocytes, leading to the induction of proinflammatory cytokines (11). Importantly, IMQ model recapitulates the hallmarks of human psoriasis, including hyperkeratosis, erythema, scaling, neutrophil microabscesses in epidermis, and infiltration of γδ T cells and Th17 cells (10).

In this study, we investigated the significance of neutrophils in psoriasis using this IMQ-induced psoriasis model. Our data demonstrate that CXCR2 and BLT1 (also known as LTB4 receptor 1 [LTb4r1]) cooperatively act to promote neutrophil recruitment into psoriatic inflamed skin. Furthermore, we present evidence that the recruited neutrophils participate in the induction of psoriatic inflammation through IL-1β production.
Materials and Methods

Mice

Ltb4r1-knockout (KO) mice were described previously (12). Ccr2-KO mice were purchased from The Jackson Laboratory. All mice used in this study were on a C57BL/6 background and kept under specific pathogen-free conditions with food and water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of The University of Tokyo.

IMQ-induced psoriasis model

Mice at 8–12 wk of age received a daily topical dose of 30 mg 5% IMQ cream (Beselna Cream; Mochida Pharmaceutical, Tokyo, Japan) on shaved backs for 6 consecutive days. Based on a previously described objective scoring system called Psoriasis Area and Severity Index (10), erythema, scaling, and thickness were scored independently on a score from 0 to 4: none; 1, slight; 2, moderate; 3, marked; 4, very marked. The cumulative score (erythema plus scaling plus thickness) served as the measure of the severity (score 0–12).

Quantitative real-time PCR

After sacrificing the mice, 6-mm punch biopsies were obtained from the back skin, and total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (QIAGEN). Using 1 µg total RNA template, we prepared cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen Life Technologies). mRNA levels were measured by quantitative real-time PCR (qPCR) analysis using the LightCycler System (Roche; for primers, see Supplemental Table I). The PCRs were set up in microcapillary tubes in a volume of 20 µl, consisting of 2 µl cDNA solution, 1 × FastStart DNA Master SYBR Green I, and 0.5 µM each sense and antisense primers. The PCR program was as follows: denaturation at 95˚C for 3 min and 45 cycles of amplification consisting of denaturation at 95˚C for 15 s, annealing at 65˚C for 5 s, and extension at 72˚C for 7 s. Data were normalized to Rplp0 (also known as 36B4) expression levels.

Measurement of cytokine and chemokine levels in IMQ-treated skin

Cytokine and chemokine concentrations in the total skin were measured by ELISA as described previously with minor modifications (13). In brief, three samples of 3-mm full-thickness punch biopsies were obtained from IMQ-treated mouse back skin and were incubated in 450 µl PBS with Complete protease inhibitors (Roche). The biopsy fragments were shaken in the solution at 4˚C for 3 h. The supernatant was collected after centrifugation at 12,000 × g for 5 min at 4˚C, and protein concentrations were measured with commercially available ELISA kits (R&D Systems).

Immunohistochemical staining of skin sections for Gr-1

Dorsal skin samples were obtained from 6-mm punch biopsies, embedded in OCT compound (Tissue-Tek, FL; Sakura Finetek, Tokyo), and snap-frozen in liquid nitrogen. Immunohistochemical staining (IHS) was performed using the Vectastain ABC peroxidase kit (Vector Laboratories). In brief, sections were blocked with diluted normal blocking serum and were incubated with primary rat anti-mouse Gr-1 (clone RB6-8C5) mAb (eBioScience) diluted at 1:200. Sections were further incubated with biotinylated donkey anti-rat IgG secondary Ab (Jackson ImmunoResearch Laboratories). Immunoreactivity was detected by incubating with the 3,3′-diaminobenzidine peroxidase substrate kit (Vector Laboratories). Sections were then counterstained with hematoxylin.

In vivo administration of anti-Ly-6G mAb to deplete neutrophils

As reported previously (14), wild-type (WT) mice were i.p. injected every other day from days 2–2 to 4 with 500 µg rat anti-mouse Ly-6G Ab (clone 1A8; BioXcell) or rat IgG2a (clone 2A3; BioXcell) dissolved in 200 µl PBS. Skin samples on day 2 and 4 were fixed in 10% formalin for H&E staining or embedded in OCT compound and frozen for immunohistochemical analysis. IHS of day 2 skin samples revealed successful depletion of Gr-1+ cells by anti-Ly-6G mAb treatment (Fig. 1D).

In vivo administration of SB225002

Mice received daily i.p. injection of 1 mg/kg selective CXCR2 antagonist SB225002 (Cayman Chemical) or vehicle from days −1 to 5. SB225002 was dissolved in saline containing 0.33% Tween 80 just before use.

Cell culture

HaCaT cells were routinely cultured in DMEM (Nacalai Tesque) supplemented with 10% FBS. In some experiments, cells were serum starved for indicated times. For the stimulation, cells were treated with IMQ (5 µg/ml; Invivogen), IL-1β (10 ng/ml; Wako), or vehicle for the indicated times.

Total RNA was prepared from the treated cells by using the RNeasy Mini Kit (Qiagen).

Neutrophil isolation from the IMQ-treated skin

For neutrophil isolation from the skin tissue, harvested skin samples were cut in pieces with scissors and digested with 0.14 U/ml Liberase DH (Roche) and 100 µg/ml DNase I (Roche) in RPMI 1640 medium (Sigma) for 1.5 h. Single-cell suspension was prepared by passing the digested tissues through a 70-µm cell strainer (BD) and shredding with gentleMACS Dissociator (Miltenyi Biotec). Gr-1+ cells were enriched by positive selection using autoMACS (Miltenyi Biotec). Successful separation was confirmed by flow cytometry.

RT-PCR analysis

Total RNA was purified from MACS-sorted Gr-1+ and Gr-1− cells obtained from IMQ-treated skin tissue as described earlier. cDNA was synthesized using SuperScript III reverse transcriptase and random primers. The resultant cDNA was amplified by PCR. The protocol and sequences of primers used to measure Ltb4r1, arachidonate 5-lipoxigenase (Alox5), and Actb (also known as β-actin) levels were described previously (15).

Quantification of LTB4 levels in skin tissue

LTB4 levels were measured as described previously (16). In brief, dorsal skin samples were obtained from 6-mm punch biopsies, frozen immediately in liquid nitrogen, and stored at −80˚C until use. The frozen tissues (50–100 mg) were powdered with Auto-Mill (Tokken, Chiba City, Japan), and lipids were extracted for 1 h at 4˚C with methanol. LTB4 levels were quantified by reversed phase HPLC electrospray ionization-tandem mass spectrometry methods.

In vivo administration of zileuton and CP105696

Mice were given daily oral administration of 50 mg/kg 5-lipoxigenase (5-LOX) inhibitor zileuton (Sigma), 10 mg/kg BLT1 antagonist CP105696 (a kind gift from Pfizer), or vehicle (0.5% methylcellulose), starting 1 d before the first IMQ treatment.

Isolation of peritoneal neutrophils

Neutrophils were obtained from the peritoneal exudates 3–4 h after i.p. injection of 3% thiglycollate at a dose of 2 ml/25 g (BD). For cell cultures, Gr-1+ cells were isolated from peritoneal exudates by using an autoMACS cell separator. Purity of the isolated Gr-1+ cells was >95%. Purified cells were subjected to chemotaxis assay or RNA extraction using the RNeasy Mini Kit (Qiagen).

Chemotaxis assays

Chemotaxis assays were performed by using HTS Transwell 96-well plates with 3-µm pores (Corning). Mouse peritoneal neutrophils were resuspended in RPMI 1640 medium supplemented with 0.25% BSA (fatty acid free; Sigma). The cells were added into the upper wells (5 × 105 cells/well), and 100 ng/ml CXCL2 (Biologend), 100 ng/ml CXCL1 (Biologend), or 10 nM LTB4 (Cayman) was added to the lower wells. In some experiments, resuspended neutrophils were pretreated with vehicle, zileuton (50 µM), or CP105696 (1 µM) for 30 min. The plates were incubated at 37˚C for 3 h. After removing the upper wells, we determined the number of migrated cells by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Data were normalized with the results from vehicle-treated samples and expressed as relative light unit.

Quantification of released LTB4 from peritoneal neutrophils stimulated by CXCR2 ligands

The effect of CXCL2 or CXCL1 on LTB4 production was studied using peritoneal neutrophils (1 × 106 cells suspended in 200 µl HBSS), challenged either with CXCL2 (100 ng/ml, 10 min, 37˚C) or CXCL1 (100 ng/ml, 10 min, 37˚C) after preactivation with GM-CSF (50 ng/ml, 30 min, 37˚C). Incubations were terminated by rapid pelleting of cells at 4˚C. Supernatants were collected and frozen immediately at −80˚C. LTB4 levels in supernatant samples were determined by ELISA kits (Cayman Chemical) following the manufacturer’s instructions.
**Results**

Infiltration of Gr-1⁺ cells and elevated IL-1β level in IMQ-treated skin during the development of psoriatic lesions

To characterize the IMQ-induced skin inflammation, we first investigated the kinetics of expression of Krt16 as an epithelial differentiation marker, Il23a (also known as IL-23 p19) as a cytokine driving the development of Th17 cells, and Il17a as a Th17 cytokine. In agreement with published studies (10), these markers were induced with a peak on day 3 (Fig. 1A). The mRNA level of TNF-α, another key proinflammatory cytokine in psoriasis, was also found to be increased transiently on day 2 (Supplemental Fig. 1). Associated with these findings, mRNA encoding neutrophil marker Ly-6G was found to show a transient increase that peaked on day 2 (Fig. 1A). Accordingly, we also observed the infiltration of Gr-1 (also known as Ly-6G/C)-positive cells in IMQ-treated skin on day 2 (Fig. 1B). Furthermore, polymorphonuclear leukocytes infiltrated into IMQ-treated skin were observed on day 2 by H&E staining (Fig. 1C). In support of these findings, the kinetics of mRNA expression and protein level of a neutrophil-derived differentiation marker, Ly-6G (clone 1A8) Ab (18) in the IMQ model. IHS confirmed the successful depletion of neutrophils by anti–Ly-6G Ab treatment (Fig. 2A). Assessment of disease severity by using Psoriasis Area and Severity Index score (10) revealed that neutrophil depletion alleviated the psoriatic symptoms compared with control IgG-treated mice (Fig. 2B, 2C). In addition, histological examination showed that inflammatory cellular infiltration, epidermal thickening, and dermal thickening in neutrophil-depleted mice were all attenuated (Fig. 2D). In stark contrast, mice deficient in the gene encoding CCR2 with defective monocyte egress from bone marrow (19) exhibited comparable scores with those of WT mice (Fig. 2E). These results indicate that neutrophils, but not macrophages, contribute to psoriatic skin inflammation in this model.

Involvement of CXCR2 in neutrophil recruitment in IMQ-induced psoriatic skin

Next, we sought to identify the chemokine responsible for the initial neutrophil recruitment in the IMQ model. The mRNA of CXCR2, one of the representative molecules responsible for neutrophil recruitment, is upregulated in epidermis and detected in infiltrated dermal polymorphonuclear cells in human psoriatic skin (20), we first focused on the role of CXCR2 in this model. mRNA level of CXCR2 in IMQ-treated skin was increased with progression of psoriatic skin (Fig. 3A). Both mRNA and protein levels of CXCR2 ligands CXCL2 (also known as MIP-2) and CXCL1 (also known as keratinocyte-derived chemokine) were transiently elevated (Fig. 3A, 3B) in parallel with Ly6g and Cxcr2 expression levels and neutrophil infiltration. Consistent with a previous study, IMQ induced the expression of CXCR2 ligands in human keratinocyte cell line HaCaT cells in vitro (Supplemental Fig. 2A), suggesting that keratinocytes stimulated with IMQ may initiate neutrophil recruitment in the IMQ model. These results prompted us to hypothesize that CXCR2 and its ligands might contribute to initial neutrophil recruitment in early phase of psoriasis. We tested...
this by using a selective CXCR2 antagonist, SB225002. As expected, Gr-1+ neutrophil infiltration was reduced by SB225002 administration (Fig. 3C), demonstrating an important role of CXCR2 in neutrophil recruitment in IMQ-induced dermatitis. Of note, SB225002 treatment also attenuated symptoms (Fig. 3D), which is consistent with the effect of neutrophil depletion by anti–Ly-6G Ab (Fig. 2B).

FIGURE 2. Attenuated psoriatic skin inflammation by neutrophil depletion. (A) IHS analysis of neutrophil infiltration on day 2 in IMQ-treated skin from mice administered rat IgG2a (left) or anti–Ly-6G mAb (right). Images are representative of four mice from two independent experiments in each group. (B) Time course of the scores in mice administered rat IgG2a or anti–Ly-6G mAb (n = 6 mice for each group in each time point). Two-way ANOVA followed by Bonferroni’s post hoc test. (C) IMQ-treated back skin of mice administered rat IgG2a (left panel) or anti–Ly-6G mAb (right panel) on day 4. Images are representative of six mice from three independent experiments in each group. (D) H&E staining of IMQ-treated skin from mice administered rat IgG2a (left panel) or anti–Ly-6G mAb (right panel) on day 4. Images are representative of five mice from two independent experiments in each group. (E) Time course of scores in WT or Ccr2-KO mice (n = 6 mice for each group in each time point). Representative data from one of two (E) or three (B) independent experiments are shown. Scale bars, 100 μm (A and D). *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 3. Role of CXCR2 in IMQ-induced psoriasis-like skin inflammation. (A) mRNA of neutrophil-related chemokines and their receptors in IMQ-treated skin tissue from individual WT mice (n = 5 mice for each time point). (B) CXCL2 and CXCL1 protein levels in IMQ-treated skin from individual WT mice (n = 4–7 mice for each time point). (C) Number of Gr-1+ cells in IMQ-treated back skin of WT mice administered vehicle or SB225002 (n = 11 mice for each group). (D) Time course of scores in WT mice administered vehicle or SB225002 (n = 7 mice for each group in each time point). Representative data from one of two independent experiments are shown. Two-tailed Student t test (C). Two-way ANOVA followed by Bonferroni’s post hoc test (D). *p < 0.05, **p < 0.01, ***p < 0.001.
Involvement of BLT1 in neutrophil recruitment in IMQ-induced psoriatic skin

Although SB225002 alleviated psoriatic skin inflammation, it was not as efficient as neutrophil depletion. We surmised that this was due to the presence of additional chemoattractants involved in this process. LTB₄, the ligand for the G protein–coupled receptor BLT1 (21), is a potent chemoattractant for neutrophils (22) and could serve as such a candidate. Expression of BLT1 mRNA transiently increased on day 2 (Fig. 4A), as observed for Ly-6G mRNA expression and neutrophil infiltration. The source of this transient upregulation might be due to the presence of additional chemoattractants involved in this process (23, 24). In contrast with the increase in BLT1 mRNA levels, LTB₄ levels remained constant in IMQ-treated mouse skin from days 0 to 6 (Fig. 4I). Despite the absence of upregulation of LTB₄, mice treated with a 5-LOX inhibitor zileuton showed disease symptoms as mild as those treated with a BLT1 antagonist CP105696 (Fig. 4J). These results and the expression of Alox5 in Gr-1⁺ neutrophils (Fig. 4B) suggest that LTB₄ production in the proximity of neutrophils may be involved in the exacerbation of psoriatic inflammation, while the role of BLT1 in psoriasis pathogenesis remains to be elucidated.

**IMQ-induced psoriatic skin requires 5-LOX pathway**

LTB₄ is a major product of arachidonic acid metabolism and is synthesized via the 5-LOX, also known as Alox5, pathway (23, 24). By activating LTB₄ receptors, LTB₄ exerts its biological effects in host immune response and in pathogenesis of various inflammatory diseases (25). In contrast with the increase in BLT1 mRNA levels, LTB₄ levels remained constant in IMQ-treated mouse skin from days 0 to 6 (Fig. 4I). Despite the absence of upregulation of LTB₄, mice treated with a 5-LOX inhibitor zileuton showed disease symptoms as mild as those treated with a BLT1 antagonist CP105696 (Fig. 4J). These results and the expression of Alox5 in Gr-1⁺ neutrophils (Fig. 4B) suggest that LTB₄ production in the proximity of neutrophils may be involved in the exacerbation of psoriatic inflammation. Another possibility is that basal LTB₄ level in mouse skin is sufficient for the induction of psoriatic dermatitis.

**Amplification of CXCR2 ligand–induced neutrophil chemotaxis by LTB₄–BLT1 axis in vitro**

The concurrent expression of Cxcr2 and Ltb4r1 prompted us to hypothesize that these factors cooperatively regulate neutrophil infiltration. To test this hypothesis, we performed in vitro chemotaxis.
assay of thioglycolate-elicited Gr-1+ peritoneal neutrophils that express high levels of IL-1\(\beta\), BLT1, and CXCR2 mRNA (Fig. 5A). Migration toward LTB4 was completely blocked by BLT1 deficiency (Fig. 5B). Moreover, the migration of Ltb4r1-KO neutrophils induced by CXCL2 was significantly reduced compared with WT (Fig. 5B). This prompted us to examine whether pharmacological blockade of BLT1 signaling using CP105696 or zileuton would also inhibit CXCR2 ligand–induced migration. Similar to the Ltb4r1-KO neutrophils, neutrophils treated with these compounds did not show the enhanced migration toward CXCL2 and CXCL1 (Fig. 5C, left panel). Meanwhile, the antagonists exerted no additional inhibitory effect on the migration of Ltb4r1-KO neutrophils toward CXCR2 ligands (Fig. 5C, right panel). Unexpectedly, CXCR2 ligands induced LTB4 secretion from neutrophils (Fig. 5D), suggesting a previously unprecedented positive feedback mechanism whereby chemokine-induced neutrophil chemotaxis is positively amplified by the autocrine and paracrine actions of chemokine-induced LTB4 secretion. In addition, IL-1\(\beta\) production by neutrophils was not amplified by chemoattractants such as CXCL1, CXCL2, and LTB4 (Supplemental Fig. 2B). These results suggest that increased chemoattractants affect neutrophil recruitment but do not directly regulate cytokine production. In contrast, IMQ could directly activate neutrophils and promote IL-1\(\beta\) production (Supplemental Fig. 2B).

**IL-19 as a downstream effector of neutrophil-derived IL-1\(\beta\) in psoriatic skin**

We next explored the link between CXCR2-BLT1 signaling and effector cytokine production in IMQ-induced psoriatic lesions. IL-19 family members (IL-19, IL-20, and IL-24) are upregulated in human psoriatic skin (26) and induce acanthosis in the reconstituted keratinocyte model (Fig. 6). Immunofluorescence staining revealed that LTB4 treatment significantly increased IL-19 expression in human keratinocytes (Fig. 6B). Moreover, IL-19 mRNA levels were higher in Ltb4r1-KO skin compared to WT skin (Fig. 6B). These results indicate that increased IL-19 expression in keratinocytes is related to the blockade of BLT1 signaling in neutrophils.

**FIGURE 5.** LTB4 production induced by CXCR2 ligands positively amplifies CXCR2-mediated neutrophil chemotaxis. (A) Characterization of Gr-1+ cells isolated from thioglycolate-induced peritoneal exudates by Giemsa staining (upper panel) and qPCR. Relative mRNA levels of Gr-1+ cells were normalized to 1 (n = 6 pairs of samples, each sample was pooled from 2–3 mice). (B) Neutrophil chemotaxis toward LTB4 or CXCL2 (n = 6 wells for each group). (C) Chemotaxis assay of neutrophils from WT (left panel) or Ltb4r1-KO (right panel) mice pretreated with vehicle, zileuton, or CP105696 before stimulation with CXCL2, CXCL1, or LTB4 (n = 4 wells for each group). Representative data from one of three independent experiments are shown. Wilcoxon matched-pairs signed rank test (A). Two-tailed Student t test (B). One-way ANOVA followed by Dunnett’s post hoc test (C and D). *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 6.** IL-1\(\beta\)-induced IL-19 mRNA elevation in keratinocytes as the potential link between neutrophil infiltration and psoriatic phenotypes. (A) qPCR analysis for IL-19 family cytokines in IMQ-treated skin from WT mice (n = 5 mice for each time point). (B) IL-19 mRNA levels in WT and Ltb4r1-KO skin on day 2. Each dot represents an individual mouse (n = 6 mice for each group). (C) Expression of IL-19 mRNA in HaCaT cells stimulated for 3 or 12 h with vehicle, IMQ, or IL-1\(\beta\) after serum starvation for 12 h (pooled data, n = 13 wells for each group). (D) Expression of IL-1\(\beta\) mRNA in HaCaT cells stimulated for 1 h with vehicle, IMQ, or IL-1\(\beta\) after serum starvation for 12 h (pooled data, n = 11 wells for each group). Representative data from one of two independent experiments are shown (A and B). Pooled data from three independent experiments are shown (C and D). Two-tailed Student t test (B). One-way ANOVA followed by Dunnett’s post hoc test (C and D). *p < 0.05, **p < 0.01, ***p < 0.001.
epidermis (27), thus serving as candidate effector cytokines. The mRNA level of IL-19 in skin tissue was increased by IMQ application, peaking on days 2–3 (Fig. 6A), which was slightly later than the peak in Ly6g and Il1b (Figs. 1A, 7A). Similarly, IL-24 mRNA also showed a transient increase on day 2, although the IL-20 mRNA level was not affected (Fig. 6A). In particular, we focused on IL-19 because of its eminent involvement in the proliferation of keratinocytes in psoriasis (28). IL-19 mRNA level in IMQ-treated Ltb4r1-KO skin on day 2 was significantly lower than in similarly treated WT skin (Fig. 6B), supporting a possible role for IL-19 as a causative factor of the differential pathology observed between WT and Ltb4r1-KO.

**IL-19 production in human keratinocytes stimulated by positive feedback loop of IL-1β**

To correlate neutrophil recruitment with effector cytokine production by keratinocytes, we investigated the influence of IL-1β on IL19 expression using a human keratinocyte cell line HaCaT, which exhibits morphological and functional properties similar to normal human keratinocytes. We found that IL-1β stimulation caused a marked increase in IL-19 transcript levels in HaCaT cells (Fig. 6C), potentially linking neutrophil infiltration to the pathogenesis of psoriatic lesions. Moreover, IL-1β stimulation also increased IL-1β mRNA levels in HaCaT cells (Fig. 6D). Hence our results raise the possibility that decreased neutrophil infiltration causes reduced IL-1β levels, which, in turn, result in lower production of IL-1β and IL-19 by keratinocytes, leading to the attenuated psoriatic phenotypes (Fig. 7B). Incidentally, IMQ stimulation also caused an increase in IL-19 transcript levels, but later than IL-1β stimulation did (Fig. 6C). A possible reason for this delay might be that IL-19 production by keratinocytes after IMQ stimulation follows rapid IL-1β production induced by IMQ (Fig. 6D).

**Discussion**

In this study, we showed the significance of neutrophils in the psoriasis model and the potential molecular mechanism that initiates and promotes neutrophil infiltration during early phase of the disease. We demonstrated that CXCR2 and BLT1 coordinately facilitate the neutrophil chemotaxis, leading to neutrophil accumulation during development of IMQ-induced psoriatic lesions. Furthermore, we provided the evidence that infiltrated neutrophils producing IL-1β potentially contribute to the development of psoriatic hyperkeratosis in early phase by promoting IL-19 production by keratinocytes. In addition, IL-1β production from keratinocytes was enhanced by IL-1β.

Gene expressions associated with neutrophil infiltration were transiently elevated before those associated with psoriatic features. Eventually, these expressions of characteristic markers were decreased in parallel with the resolution of IMQ-induced psoriatic skin inflammation (Fig. 7A). Our findings reinforce the similarity of IMQ model to human disease in which IL-1β and CXCR2 ligands are upregulated (17, 29). Furthermore, IL-19 family cytokines were upregulated, as well as human psoriatic lesion (26) and IL-23–induced murine psoriasis-like dermatitis (26). Moreover, this IL-23–induced murine model also showed transient erythema and epidermal thickening (26), just as the IMQ-induced murine model did. In addition, it is well-known that neutrophils predominantly infiltrate into dermis rather than epidermis in early phase of psoriatic lesion (6), as observed in IMQ-treated skin on day 2. Considering these symptomatic, histological, and molecular similarities, the IMQ model seemingly recapitulates the early phase of psoriasis and is therefore suited for analyzing the induction mechanism of the disease. Incidentally, with our results of neutrophil and keratinocyte activations by IMQ in mind, the IMQ model might reveal the significance of these activations in the early phase of psoriasis. This clinically means that any stimulus could activate neutrophils/keratinocytes and induce psoriasis. Therefore, our results will provide clues for novel treatment of human psoriasis.

In human psoriasis, neutrophil infiltration in the skin is observed not only in the early phase but also in the chronic phase (6). Therefore, for the maintenance of chronic psoriatic lesion, neutrophils need to be constantly recruited into lesions via circulation because of their short half-lives. Our findings suggest that neutrophils recruited by the action of CXCR2 and BLT1 increase IL-1β levels in the skin, which might be because of increased numbers of infiltrated neutrophils rather than enhanced capacity of neutrophils to release IL-1β. IL-1β upregulation likely results in the amplification of IL-17 production from γδ T cells (30), which contributes to the early phase of psoriasis-like dermatitis (31). IL-17, in turn, would promote further neutrophil recruitment (2), resulting in the persistent infiltration of neutrophils into the skin. Furthermore, IL-1β also promotes acanthosis-inducing IL-19 production by keratinocytes. Thus, increased IL-1β could act as a bridge between neutrophils and IL-23/IL-17 axis, and as an inducer of terminal effector cytokine, promoting sustained psoriatic inflammation. Of note, another IL-1 family cytokine, IL-1α, and its receptor...
were shown to play a role in keratinocyte activation and up-regulation of CXCR2 ligands (32). Taken together, IL-1 family cytokines potentially serve as a key for feedback loop between keratinocyte activation and neutrophil recruitment in the context of skin inflammation. Given that TNF-α also participates in psoriatic inflammation (33, 34) and IL-19 expression in keratinocytes (26), inhibitors of IL-1 and TNF-α signaling could be exploited for the treatment of psoriasis.

The fact that CXCR2 ligands potentially provided by keratinocytes trigger dermal neutrophil infiltration implies that persistent production of CXCR2 ligands by keratinocytes might be the key to the chronicity of psoriatic lesions. In agreement with this, CXCR2 density is elevated on neutrophils from psoriatic individuals compared with those from healthy volunteers (35), along with increased IL-8 and CXCL1 levels in psoriatic epidermis (29). Furthermore, cathelicidin antimicrobial peptide (also known as LL-37), another potential ligand for CXCR2 (36), is overexpressed in human psoriatic keratinocytes and has been suggested to play a role in the pathogenesis of inflammatory skin diseases including psoriasis (37, 38). Much to our interest, cathelicidin triggers LTB4 synthesis from human neutrophils (39), as well as CXCL1 and CXCL2, as shown in Fig. 5D. Therefore, besides CXCL1 and CXCL2, other CXCR2 ligands such as cathelicidin, CXCL5, and CXCL7 might be deeply committed to IMQ-induced skin inflammation in the same manner.

Our study added LTB4-BLT1 signaling as a novel component that contributes to psoriasisform dermatitis. This finding is consistent with previous studies implicating LTB4-BLT1 signaling in the pathogenesis of rheumatoid arthritis (40) and atopic dermatitis (41). Although our findings and a previous report (42) share common molecular components involved in neutrophil recruitment, they act in a different order. Whereas a previous report (42) proposed lipid-cytokine-chemokine cascade with LTB4 as the initial neutrophil chemoattractant in rheumatoid arthritis, cascade was ordered as chemokine-lipid-chemokine in our psoriasis model. Specifically, CXCR2 ligands act in the earliest stage of psoriasis, which are then amplified by LTB4 and result in IL-19 production by keratinocytes through the elevated IL-1β. It is likely that interplay of chemokines, lipids, and cytokines plays an important role in a wide variety of inflammatory disorders but may differ in regard to the initiator cues and terminal effectors under different conditions.

LTB4 production from infiltrated neutrophils could be enhanced by previously unappreciated positive feedback between LTB4 and CXCR2 ligands, although LTB4 levels in the skin remain relatively stable in the context of psoriatic inflammation. However, local LTB4 level around neutrophils may be increased by the BLT1-CXCR2 feedback loop. This idea is consistent with limited dissemination of lipid mediators due to their short half-lives (24). To our knowledge, chemokine-driven LTB4 production by neutrophils has never been documented. This mechanism is reminiscent of the case of formyl peptides in vitro (43) and C5aR in autoimmune-induced arthritis model (44). In addition, a recent study revealed the central role of neutrophil-derived LTB4 in swarming behavior of neutrophils in wound or infected area (45). Detailed pathway for the signaling cross talk between CXCR2 and BLT1 might be interesting to further elucidate molecular mechanism for neutrophil swarming in inflamed tissues.

As reported previously (46, 47), we observed the beneficial effect of CP105696 on the intradermal neutrophil accumulation. However, a randomized, controlled trial of another BLT1 antagonist, VML295, in patients with psoriasis did not alleviate the psoriasis severity (48). Although the clinical utility of BLT1 antagonists for psoriasis still remains controversial, the involvement of LTB4 in the pathogenesis of psoriasis was strongly suspected by the abundance of LTB4 in human psoriatic skin and the fact that topical application of LTB4 on the skin induces flare and epidermal hyperproliferation in humans (8). Therefore, the unsuccessful therapeutic treatment of psoriasis by BLT1 antagonists may reflect suboptimal pharmacokinetics of currently available compounds or the necessity of combination therapy. Blockade of CXCR2, BLT1, and/or IL-1β in conjunction with current anti-inflammatory drug regimens may achieve more effective therapies for neutrophil-mediated skin diseases like psoriasis.

In conclusion, we propose a novel mechanism underlying the pathogenesis of psoriasis, in which coordinated chemokine, lipid, and cytokine signaling and the resulting positive feedback loops drive the early phase of psoriatic inflammation. CXCR2 ligands produced by stimulated keratinocytes initiate neutrophil infiltration into the skin, which is further driven by the LTB4→BLT1 axis in neutrophils. CXCR2 ligands enhance the local LTB4 production and facilitate neutrophil recruitment. Subsequent secretion of IL-1β from neutrophils results in keratinocyte activation, leading to increased IL-19 levels and disease progression (Fig. 7B). Given the diverse functions of neutrophils in immune systems, these insights may be extrapolated to a broad spectrum of inflammatory and immune disorders, and may aid in the rational design of novel anti-inflammatory treatments.

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References


