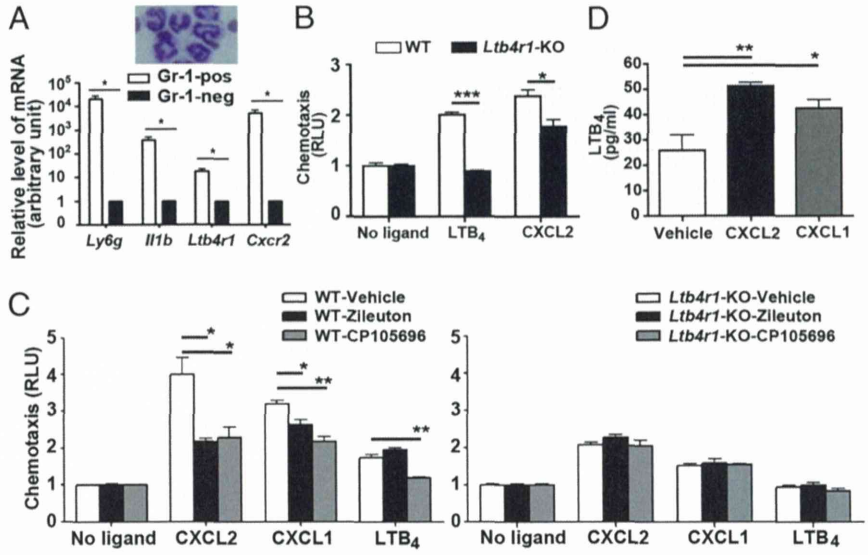

FIGURE 5. LTB_4 production induced by CXCR2 ligands positively amplifies CXCR2-mediated neutrophil chemotaxis. (**A**) Characterization of Gr-1^+ cells isolated from thioglycolate-induced peritoneal exudates cells by Giemsa staining (*upper panel*) and qPCR. Relative mRNA levels of Gr-1^- cells were normalized to 1 ($n = 6$ pairs of samples, each sample was pooled from 2–3 mice). (**B**) Neutrophil chemotaxis toward LTB_4 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 LTB_4 ($n = 4$ wells for each group). (**D**) Release of LTB_4 from neutrophils after stimulation with CXCL2 or CXCL1 ($n = 4$ 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). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

assay of thioglycolate-elicited Gr-1^+ peritoneal neutrophils that express high levels of IL-1β, BLT1, and CXCR2 mRNA (Fig. 5A). Migration toward LTB_4 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 LTB_4 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 LTB_4 secretion. In addition, IL-1β production by neutrophils was not amplified by chemoattractants such as CXCL1, CXCL2, and LTB_4 (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-1β production (Supplemental Fig. 2B).

IL-19 as a downstream effector of neutrophil-derived IL-1β 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

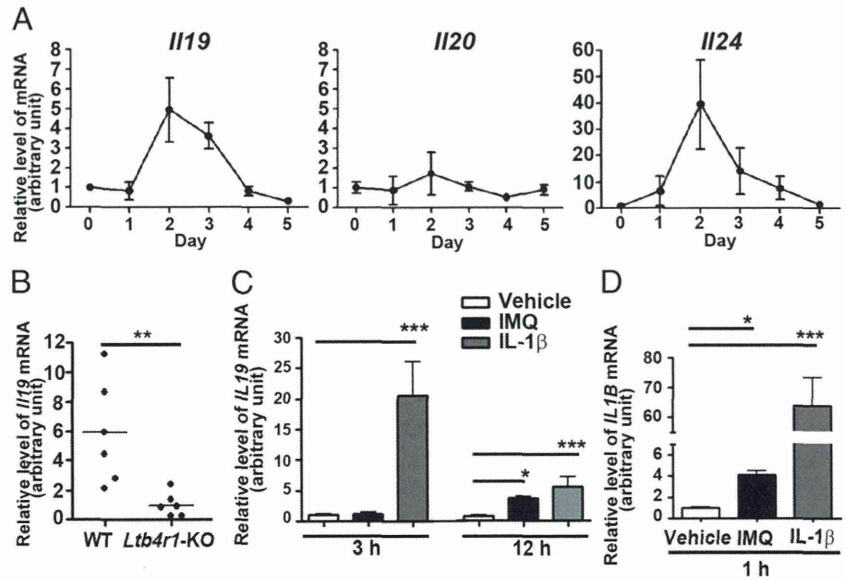

FIGURE 6. IL-1β–induced IL-19 mRNA elevation in keratinocytes as the potential link between neutrophil infiltration and psoriatic phenotypes. (**A**) qPCR analysis for IL-19 family cytokines in IMQ-treated skin from WT mice ($n = 5$ mice for each time point). (**B**) IL-19 mRNA levels in WT and *Ltb4r1*-KO skin on day 2. Each dot represents 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-1β after serum starvation for 12 h (pooled data, $n = 13$ wells for each group). (**D**) Expression of IL-1β mRNA in HaCaT cells stimulated for 1 h with vehicle, IMQ, or IL-1β 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

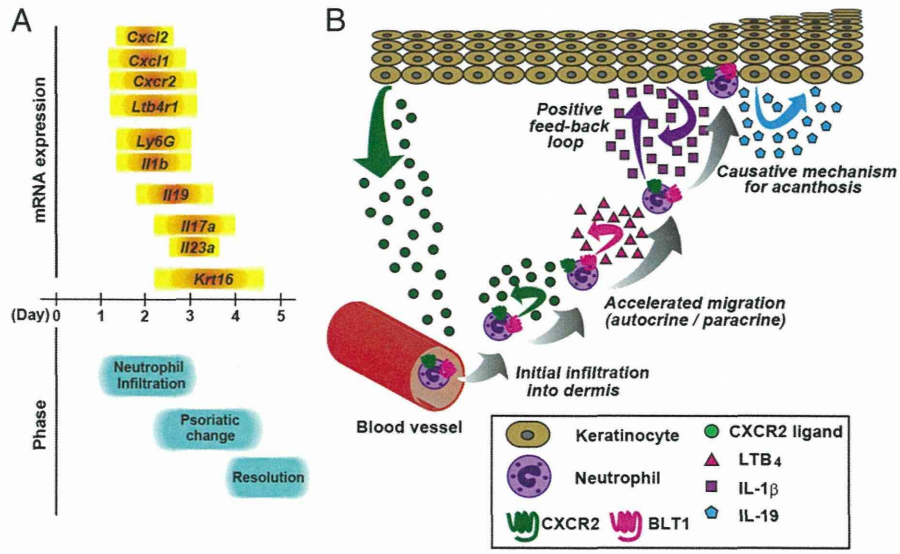

FIGURE 7. Characterization of the IMQ model and proposed model for human psoriasis. (**A**) mRNA expression profile during the development of IMQ-induced skin inflammation. (**B**) A putative model for neutrophil infiltration in psoriasiform skin inflammation. Keratinocyte-derived CXCR2 ligands serve as the initial trigger for neutrophil extravasation from blood vessels into the dermis. Infiltration of neutrophils is amplified by CXCR2 ligands and LTB_4 in an autocrine and/or paracrine fashion. IL-1β produced by neutrophils stimulates keratinocytes, followed by the production of IL-1β and acanthosis-inducing IL-19 by keratinocytes.

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 LTB_4 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 LTB_4-BLT1 signaling as a novel component that contributes to psoriasiform dermatitis. This finding is consistent with previous studies implicating LTB_4-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 LTB_4 as the initial neutrophil chemoattractant in rheumatoid arthritis, cascade was ordered as chemokine-lipid-cytokine in our psoriasis model. Specifically, CXCR2 ligands act in the earliest stage of psoriasis, which are then amplified by LTB_4 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.

LTB_4 production from infiltrated neutrophils could be enhanced by previously unappreciated positive feedback between LTB_4 and CXCR2 ligands, although LTB_4 levels in the skin remain relatively stable in the context of psoriatic inflammation. However, local LTB_4 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 LTB_4 production by neutrophils has never been documented. This mechanism is reminiscent of the case of formyl peptides in vitro (43) and C5aR in autoantibody-induced arthritis model (44). In addition, a recent study revealed the central role of neutrophil-derived LTB_4 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 LTB_4 in the pathogenesis of psoriasis was strongly suspected by the abundance of LTB_4 in human psoriatic skin and the fact that topical application of LTB_4 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 LTB_4–BLT1 axis in neutrophils. CXCR2 ligands enhance the local LTB_4 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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Disclosures

The authors have no financial conflicts of interest.

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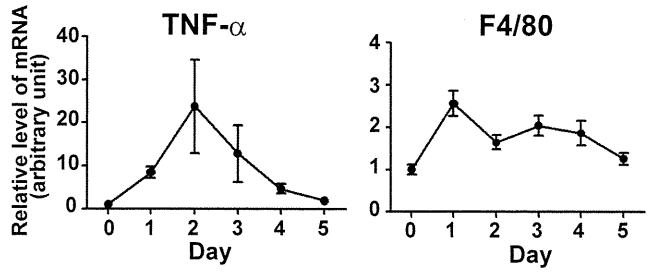
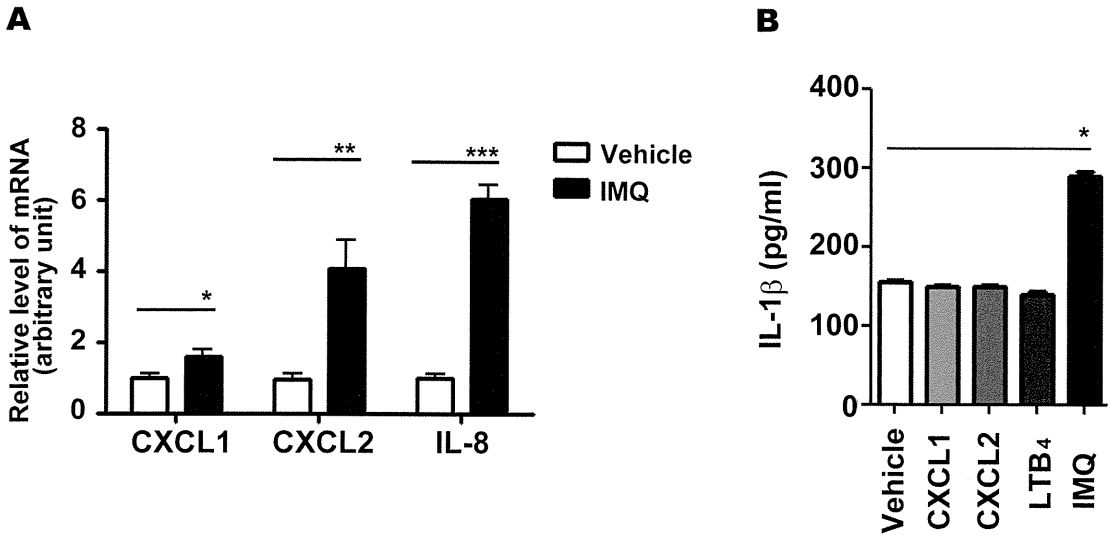
Supplemental Fig. 1



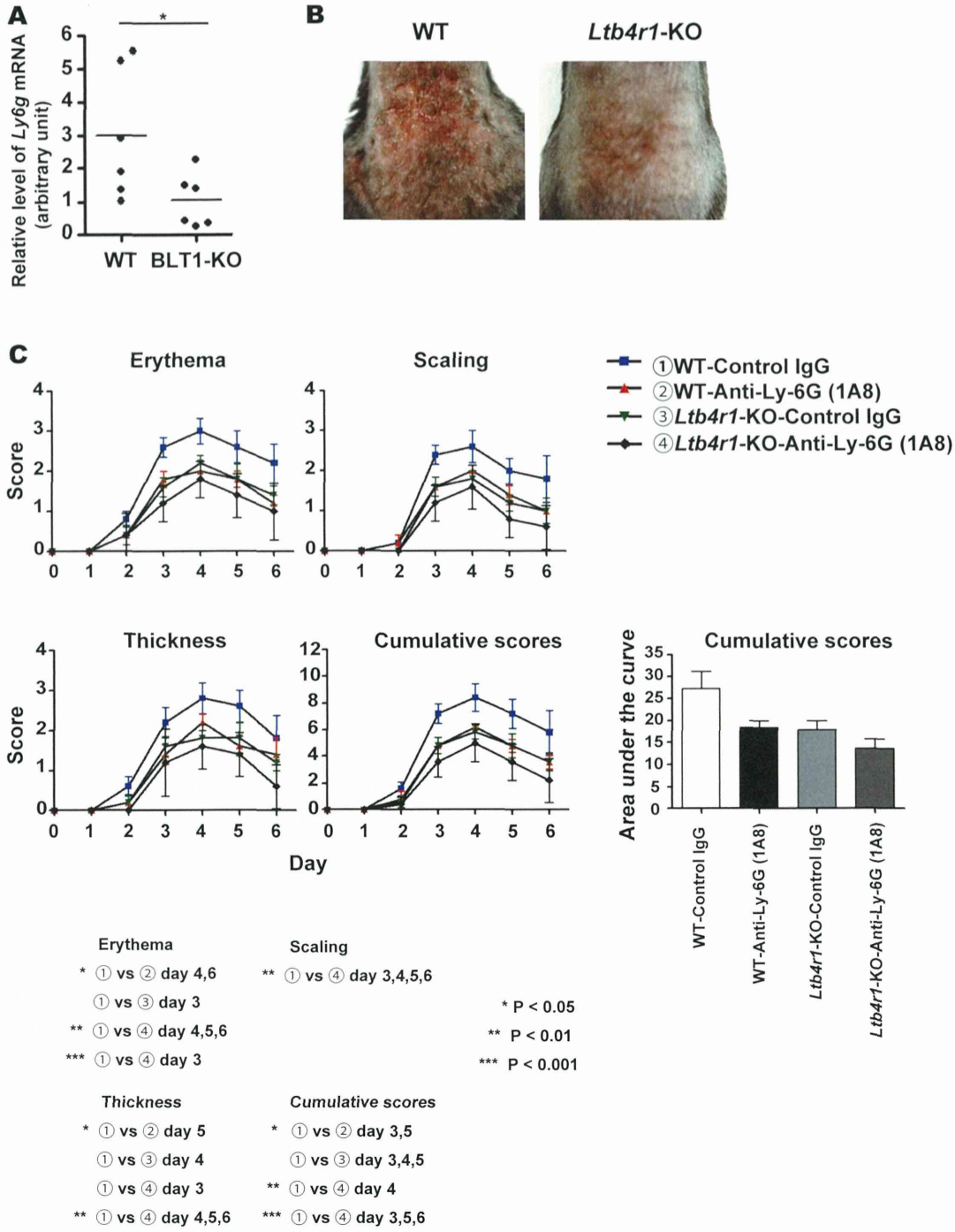
Supplemental Fig. 1. Characterization of the IMQ-induced psoriatic inflammation. Kinetics of the mRNA levels of TNF-α and Emr1 in IMQ-treated skin tissue (n = 5 in each time point). Representative data from one of two independent experiments are shown.

Supplemental Fig. 2



Supplemental Fig. 2. IMQ-induced keratinocyte and neutrophil activations. (**A**) Chemokine production by IMQ-stimulated human keratinocyte cell line HaCaT. Expression of CXCL1, CXCL2, and IL-8 mRNA in HaCaT cells stimulated for 1 h with vehicle or IMQ (5 μg/ml) after serum-starvation for 12 h. Pooled data from three independent experiments are shown. Two-tailed Student's *t*-test; *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. (**B**) Released IL-1β from murine peritoneal neutrophils. Isolated peritoneal neutrophils (2×10^6 cells suspended in 200 μl of Hanks' balanced salt solution) were stimulated for 6 h with CXCL1 (100 ng/ml), CXCL2 (100 ng/ml), LTB_4 (10 nM), and IMQ (5 μg/ml) after serum-starvation for 12 h. Released IL-1β was analyzed in cell-cultured supernatants using ELISA kit (R&D Systems). Representative data from one of three independent experiments are shown. One–way ANOVA followed by Dunnett's post hoc test; *, $P < 0.0001$.

Supplemental Fig. 3



Supplemental Fig. 3. Role of BLT1 in IMQ-induced psoriasis-like skin inflammation. (**A**) Expression of Ly-6G mRNA in WT and *Ltb4r1*-KO skin on day 2. (**B**) IMQ-treated back skin of WT or *Ltb4r1*-KO mice on day 4. (**C**) Time-course of the scores (erythema, scaling, thickness, and cumulative scores) and area under the curve (only cumulative scores) in WT or *Ltb4r1*-KO mice administered rat IgG2a or anti-Ly-6G mAb ($n = 5$ in each group). Representative data from one of two independent experiments are shown (**A** and **C**). Two-tailed Student's *t*-test (**A**). Two-way ANOVA followed by Bonferroni's post hoc test (**C**). *, $P < 0.05$.

Supplemental Table I

Primer sequences for the quantification of mouse and human gene expression using the LightCycler system.

Mouse

Gene	Forward primer	Reverse primer
Ly6g (Gr-1)	5'-CTTCTCTGATGGATTTTGCGTTG-3'	5'-AGTAGTGGGGCAGATGGGAAG-3'
Krt16	5'-CCACTCCTCCTCACAGCACTC-3'	5'-CCTGGAACTCTGACTTTGGCTCT-3'
Il23a	5'-CGTATCCAGTGTGAAGATGGTTGT-3'	5'-CATGGGGCTATCGGGAGTAGAG-3'
Il17a	5'-CACCTCACACGAGGCACAAG-3'	5'-GCAGCAACAGCATCAGAGACA-3'
Il1b	5'-ATAACCTGCTGGTGTGTGACGTT-3'	5'-AAGGCCACAGGTATTTTGTCGTT-3'
Cxcr2	5'-GCTCACAAACAGCGTCGTAGAA-3'	5'-GAGTGGCATGGGACAGCATC-3'
Ccr1	5'-GGTTGGGACCTTGAACCTTG-3'	5'-GTAGTTGTGGGGTAGGCTTCTGTG-3'
Cxcl2	5'-ACTCTCAAGGGCGGTCAAAAA-3'	5'-AGGCACATCAGGTACGATCCA-3'
Cxcl1	5'-CAAACCGAAGTCATAGCCACAC-3'	5'-TGGGGACACCTTTTAGCATCTTT-3'
Ltb4r1 (BLT1)	5'-GGACCCTGGCACTAAGACAGA-3'	5'-CAGTAGAACAATGGGCAACAGAGA-3'
Il19	5'-TGACGTTGATTCTCTGCTCAGTTC-3'	5'-AGGGACAGGATGGTGACATTTTT-3'
Il20	5'-GACCCCTGACCACCATACCC-3'	5'-CCATTGCTTCTTCCCCACAA-3'
Il24	5'-AGAACCAGCCACCTTCACACA-3'	5'-CCCAAATCGGAACTCTTGACC-3'
Tnf	5'-GCCACCACGCTCTTCTGTCT-3'	5'-ACTCCAGCTGCTCCTCCACTT-3'
Emr1 (F4/80)	5'-GGCTTCAGTGGGATGTACAGATG-3'	5'-TGAAAGTTGGTTTGTCCATTGCT-3'
Rplp0 (36B4)	5'-CTGAGATTCGGGATATGCTGTTG-3'	5'-AAAGCCTGGAAGAAGGAGGTCTT-3'

Human

Gene	Forward primer	Reverse primer
IL1B	5'-ATGACTTGTTCTTTGAAGCTGATGG-3'	5'-GCCCTTGCTCTAGTGGTGGT-3'
IL19	5'-GTGTGTTTCCCTTTGGCTCCT-3'	5'-CTTGGATGGCTCTTTTGATTTCTT-3'
CXCL1	5'-AATCCTGCATCCCCCATA-3'	5'-TGTCTCTCTTTCCTCTTCTGTTCCT -3'
CXCL2	5'-CAAACCGAAGTCATAGCCACAC -3'	5'-TTGCCATTTTTCAGCATCTTTTC -3'
IL8	5'-TCTGTCTGGACCCCAAGGAA-3'	5'-GCATCTGGCAACCCTACAACA -3'
RPLP0(36B4)	5'-AAATGTTTCATTGTGGGAGCAGA-3'	5'-GTTTCTCCAGAGCTGGGTTGTTT-3'

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Visualization of the pH-dependent dynamic distribution of G2A in living cells

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ABSTRACT **G2A (from G2 accumulation) receptor is a member of the proton-sensing G-protein coupled receptor (GPCR) family and induces signal transduction events that regulate the cell cycle, proliferation, oncogenesis, and immunity. The mechanism by which G2A-mediated signal transduction is regulated by the extracellular pH remains unresolved. Here, we first visualize the pH-dependent G2A distribution change in living cells by a sortase A-mediated pulse labeling technology: the short-peptide tag-fused human G2A on human embryo kidney HEK293T cell surfaces was labeled with a small fluorescent dye in the presence of lysophosphatidylcholine, and the labeled G2A was chased at acidic and neutral pHs in real time by microscope time course observations. G2A internalization from cell surfaces into intracellular compartments was observed to be inhibited under acidic pH conditions, and this inhibition was relieved at neutral pH. Additionally, the internalized G2A was redistributed onto cell surfaces by jumping from a neutral to an acidic pH. From quantitative image analysis data, we conclude the amount of G2A on the cell surface was controlled by suppressing the G2A internalization rate by one-tenth in response to the extracellular acidic pH, and this acidic pH-induced G2A accumulation on cell surfaces may be explained by proton-induced dissociation of G2A from endocytic machinery.—Lan, W., Yamaguchi, S., Yamamoto, T., Yamahira, S., Tan, M., Murakami, N., Zhang, J., Nakamura, M., Nagamune, T. Visualization of the pH-dependent dynamic distribution of G2A in living cells. *FASEB J.* 28, 3965–3974 (2014). www.fasebj.org**

Key Words: GPCR · internalization · pH dependence · pulse labeling

G-PROTEIN-COUPLED RECEPTORS (GPCRs) are important targets in medical and pharmaceutical research fields because a wide variety of disease-related processes, including cell proliferation, activation, tumor cell invasiveness, and inflammation, are critically controlled by the signal transduction mediated by GPCRs (1–3). In particular, proton-sensing GPCRs that are activated under acidic pH conditions have been actively studied recently (4–8). Although the extracellular pH is maintained in a quite narrow range of 7.4 under physiological conditions (9), a decrease in pH is well known to occur locally in certain pathological regions, cancer tissues and inflammatory sites (10–15). In addition, the proton-sensing properties of GPCRs have been often found in cancer- or inflammation-related GPCRs such as ovarian cancer GPCR 1(OGR1/GPR68) and T-cell death-associated gene 8 (TDAG8/GPR65) (16–19). Accordingly, elucidation of the mechanism by which extracellular pH regulates the activities of proton-sensing GPCRs is directly linked to both an understanding of various pathogenic mechanisms and to the discovery of new therapeutics for these diseases.

G2A, named for its ability to cause G_2/M arrest in the cell cycle, is a member of the proton-sensing GPCR family and has structural similarities to OGR1/GPR68 and TDAG8/GPR65 (20–23). Human G2A-mediated

Abbreviations: AF488-NHS, Alexa Fluor 488 carboxylic acid, succinimidyl ester, mixed isomers dye; AF647, Alexa Fluor 647; cAMP, cyclic adenosine, monophosphate; DMEM, Dulbecco's modified Eagle's medium; EPPS, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HEK293T, human embryonic kidney 293T; HEM, HEPES/EPPS/MES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LPC, lysophosphatidylcholine; MES, 2-(N-morpholino)ethanesulfonic acid; OGR1, ovarian cancer G-protein-coupled receptor 1; PAFR, platelet-activating factor receptor; RMS, root-mean-square; RP-HPLC, reversed-phase high-performance liquid chromatography; SrtA, sortase A; TDAG8, T-cell death-associated gene 8

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signal transduction was observed to be enhanced only by lowering the extracellular pH without the addition of any ligands, and this low-pH-dependent enhancement was antagonized by treatment with a bioactive lysophospholipid, *i.e.*, lysophosphatidylcholine (LPC) (24). However, similar to other lysophospholipids, such as sphingosylphosphorylcholine, lysophosphatidic acid, and sphingosine-1-phosphate (25–27), LPC has been identified mainly as an agonist of G2A and is reported to stimulate a broad array of cellular events, such as Ca^{2+} accumulation (25), cAMP accumulation (28), and chemotaxis (29) in a G2A-dependent manner. In the chemotaxis of T cells to LPC, the low-pH-induced reduction of migration was briefly mentioned as a negative effect of the extracellular pH on G2A-mediated signaling (29). These findings raise the possibility that, if the diverse responses of G2A are due to its conformational changes, depending on whether it is bound to ligands/proton, then at neutral pHs G2A might associate with inactivation machinery, *e.g.*, receptor internalization, rather than signaling events.

The amount of GPCRs on the cell surface is well known to be controlled by internalization in response to extracellular conditions for protecting cells against overstimulation (30, 31). Against agonist-induced overstimulation, the receptors are desensitized through internalization *via* arrestin/clathrin-mediated endocytosis through ligand-induced conformational changes of the receptor. Internalized receptors are either degraded in lysosomes or maintained in intracellular recycling compartments (31, 32). Alternatively, most of the constitutively active GPCRs are spontaneously internalized in the absence of any ligands, and inverse to agonist-induced internalization. Such a constitutive internalization can be prevented by binding to a ligand (33). Similar to such constitutively active GPCRs, G2A was observed previously to be spontaneously internalized into endosomal compartments using a murine G2A-green fluorescence protein (GFP) fusion construct (34). Furthermore, the elaborate pulse-chase analysis of G2A-GFP successfully clarified that the surface localization of G2A was enhanced and stabilized by LPC treatment. Moreover, this LPC-induced G2A distribution onto cell surfaces led to the activation of the G2A-mediated signaling pathway. However, in this pioneer study, it was also reported that any effects of pH on G2A localization in the absence or presence of LPC were not observed (34). Accordingly, the mechanism by which G2A is activated by extracellular protons had been assumed to be different from the receptor redistribution observed in LPC-induced activation.

In this study, we reexamined the effect of pH on G2A intracellular trafficking by using a site-specific protein pulse-labeling method. Post-translational labeling of tag-fused proteins with small chemical probes represents powerful tools for visualizing the innate localization and dynamic trafficking of a target protein in living cells (35–37) when compared with large fluorescent proteins being fused to a target protein. Moreover, small molecular probes potentially have less influence on the intracellular localization and functions of target proteins, and the timing of labeling can be more easily and strictly set up for chasing target proteins.

Our group and the others recently reported the methods for sortase-mediated C- and N-terminal labeling of cell surface proteins on living cells (37–41). Sortase A (SrtA) from *Staphylococcus aureus* is a member of transpeptidases, which catalyze the ligation of two substrate peptides; SrtA specifically cleaves one substrate peptide (LPETGG) between threonine and glycine and then links the exposed carboxyl group of the threonine to the amino group of the N-terminal oligoglycine on another peptide (37, 42). In the present SrtA-mediated labeling method, highly site-specific, stable, and rapid labeling of target membrane proteins could be achieved only by appending a fluorescent short peptide to the terminals of target proteins. Furthermore, by using this site-specific N-terminal labeling method (39), the agonist-induced trafficking of a GPCR, platelet-activating factor receptor (PAFR) was successfully evaluated (40). Therefore, in this study, the short peptide tag-fused G2A was fluorescently labeled on cell surfaces by the SrtA-mediated N-terminal labeling method, and the labeled G2A from cell surfaces was continuously observed under either low- or high-pH conditions by a confocal laser microscope (**Fig. 1**). Our data clearly showed that the internalization of G2A into intracellular compartments is suppressed under acidic condi-

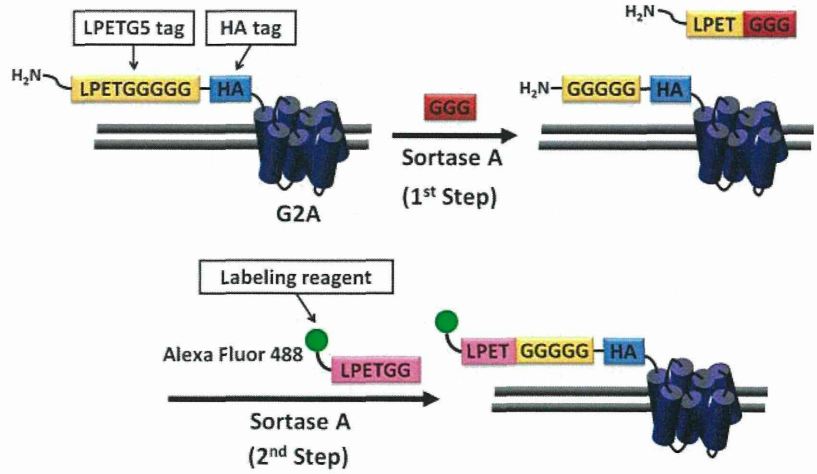

Figure 1. Schematic illustration of the sortase-mediated fluorescent labeling of G2A. Human G2A containing the LPETGGGGG tag at the N-terminus is expressed on the cell surface. Initially, the LPETGGGGG sequence is site-specifically degraded through sortase-mediated transpeptidation with a triglycine peptide. Next, at the second step, the Alexa Fluor 488-conjugated LPET peptide was linked to the exposed N-terminal pentaglycine on G2A by the sortase.