Leukotriene B$_4$ driven neutrophil recruitment to the skin is essential for allergic skin inflammation

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Summary

Scratching triggers skin flares in atopic dermatitis (AD). We demonstrate that scratching of human skin, and tape stripping of mouse skin, causes neutrophil influx. This influx in mice was largely dependent on the generation of leukotriene B$_4$ (LTB$_4$) by neutrophils and their expression of the LTB$_4$ receptor BLT1. Allergic skin inflammation in response to epicutaneous (EC) application of ovalbumin to tape-stripped skin was severely impaired in *Ltb4r1$^{-/-}$* mice, and required expression of BLT1 on both T cells and non-T cells. Co-transfer of WT neutrophils, but not neutrophils deficient in BLT1 or the LTB$_4$ synthesizing enzyme LTA$_4$H, restored the ability of WT CD4$^+$ effector T cells to transfer allergic skin inflammation to *Ltb4r1$^{-/-}$* recipients. Pharmacologic

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blockade of LTB_4 synthesis inhibited allergic skin inflammation elicited by cutaneous antigen challenge in previously EC-sensitized mice. Our results demonstrate that a neutrophil-T cell axis reliant on LTB_4-BLT1 interaction is required for allergic skin inflammation.

Introduction

Eicosanoids are biologically active lipid mediators that are rapidly generated at sites of injury or inflammation. Leukotrienes (LTs) and prostaglandins are two classes of eicosanoids generated by the metabolism of arachidonic acid through the 5-lipoxygenase (5-LO) pathway and the cyclooxygenase pathway, respectively (Funk, 2001). 5-LO expression and LT generation are generally restricted to myeloid leukocytes, particularly neutrophils, eosinophils, monocytes, macrophages, and mast cells (Werz, 2002). LTs consist of LTB_4 and of cysteinyl leukotrienes (cys-LTs), which include LTC_4, LTD_4, and LTE_4. The relative amounts of LTB_4 and cys-LTs depend on the relative expression of the 5-LO distal enzymes LTA_4 hydrolase (LTA_4H) and LTC_4 synthase (LTC_4S), respectively. LTB_4 is generated from innate immune cells such as neutrophils, macrophages, and mast cells in response to a variety of stimuli (Peters-Golden and Henderson, 2007). It primarily acts on myeloid leukocytes causing activation of integrins, adhesion to endothelium walls, and chemotaxis (Goodarzi et al., 2003; Huang et al., 1998; Patcha et al., 2004).

Two receptors for LTB_4 have been identified: BLT1 and BLT2. BLT1 is a high-affinity receptor for LTB_4 predominantly expressed on neutrophils, macrophages, eosinophils, and activated T cells, with little expression, if any, on resting T cells. BLT2 exhibits low-affinity binding for LTB_4 and is ubiquitously expressed (Friedrich et al., 2003). All LTB_4 effects on both neutrophils and T cells are lost in $Ltb4r1^{-/-}$ mice (Tager et al., 2003; Tager et al., 2000). LTB_4 has been implicated in the pathogenesis of allergic diseases (Ohnishi et al., 2008). Nocturnal concentrations of LTB_4 and cys-LTs are elevated in the bronchoalveolar lavage (BAL) fluid of patients with nocturnal asthma (Wenzel et al., 1995). Zileuton, a specific inhibitor of 5-LO, has been shown to decrease LTB_4 concentrations in BAL fluid, raising the possibility that its beneficial effect in asthma may involve decreased production of both cys-LTs and LTB_4 (Wenzel et al., 1995). Studies on $Ltb4r1^{-/-}$ mice have suggested an important role of the LTB_4-BLT1 pathway in the recruitment of effector CD4^{+} and CD8^{+} T cells to antigen-challenged airways in a classical mouse model of asthma (Miyahara et al., 2005; Tager et al., 2003; Terawaki et al., 2005).

Atopic dermatitis (AD) is an inflammatory skin disease characterized by itching and scratching, dermal infiltration with Th2 cells and a systemic Th2 cell-mediated response with elevated serum IgE and eosinophilia. Neutrophils from AD patients have elevated LTA_4H activity (Okano-Mitani et al., 1996), and release more LTB_4 in response to several stimuli (Hilger et al., 1991). LTB_4 concentrations are elevated in lesional skin of patients with AD (Fogh et al., 1989; Koro et al., 1999; Ruzicka et al., 1986; Thorsen et al., 1990). However, little is known about the role of LTB_4 in AD. To this end, we have developed a mouse model of allergic skin inflammation with many similarities to AD. In this model, repeated epicutaneous (EC) sensitization of tape-stripped skin with ovalbumin (OVA) results in a Th2 cell dominated systemic immune response characterized by dermal infiltration of CD4^{+} T cells and eosinophils and increased local expression of Th2 cell-derived cytokines (Spergel et al., 1998; Spergel et al., 1999). We have used this model to examine the potential role of LTB_4 in allergic skin inflammation. Our findings indicate that LTB_4-mediated attraction of neutrophils to skin subjected to tape stripping, a surrogate for scratching, is essential for the development of allergic inflammation at cutaneous sites of antigen introduction.

Results

Scratching and tape stripping increase neutrophils and neutrophil-derived LTB$_4$ and BLT1 in the skin

Mechanical injury to the skin by scratching triggers flares in patients with AD (Fleischer and Boguniewicz, 2010). We examined whether scratching triggers a cellular infiltrate in the skin. There were virtually no detectable neutrophils in the dermis of unscratched skin. In contrast, neutrophils, which were easily differentiated from other inflammatory cells by their characteristic multilobed nucleus, accumulated in the dermis of scratched skin (Fig. 1A,B). There was also an increase of one cell layer in epidermal thickness in scratched skin sites (Fig. 1A). LTB$_4$ is a potent chemoattractant for neutrophils, which express the LTB$_4$ receptor BLT1 (Peters-Golden and Henderson, 2007). Levels of LTB$_4$ and BLT1 mRNA were significantly elevated in scratched skin compared to control unscratched skin (Fig. 1C,D).

We examined whether tape stripping, a surrogate for scratching, causes neutrophil accumulation in mouse skin. Shaved dorsal skin of C57BL/6 mice was tape-stripped six times with Tegaderm and examined 24 hrs later. Histologic examination revealed that there were virtually no detectable neutrophils in the dermis of shaved non tape-stripped skin. In contrast, neutrophils were abundant in the dermis of tape-stripped skin (Fig. 1E,F). There was an increase in epidermal and dermal thickness in tape-stripped skin sites compared to non tape-stripped skin (27.24 ± 1.4 mm vs. 8.04 ± 0.4 mm, and 252.9 ± 11.4 mm vs. 154.9 ± 11.9 mm, n=4, p<0.001 each). The amount of LTB$_4$ and BLT1 mRNA were significantly elevated in tape-stripped skin compared to non tape-stripped skin (Fig. 1G,H). An influx of neutrophils was also observed in ear skin 24 hrs after tape stripping six times with Tegaderm, as determined by flow cytometry analysis of cell suspensions from trypsin digested skin for the presence of CD11b$^+$Gr-1$^+$ cells (Fig. 1I).

Neutrophils, but not mast cells, are the source of elevated LTB$_4$ in tape-stripped mouse skin

Neutrophil influx, LTB$_4$ accumulation, and expression of BLT1 mRNA in tape-stripped skin were comparable in mast cell-deficient WBB6F1/*J-Kit^W/Kit^{W-v}* (*Kit^W/Kit^{W-v}*) mice and WT WBB6F1 controls (Fig. 2A-C). Neutrophil depletion in C57BL/6 mice by Gr-1 mAb resulted in the loss of >99% of the CD11b$^+$Gr-1$^+$ cells from the blood (Fig. S1A), and strongly impaired the accumulation of neutrophils, LTB$_4$ and BLT1 mRNA in tape-stripped ear skin, compared to treatment with isotype control antibody (Fig. 2D-F). The role of neutrophils in the accumulation of LTB$_4$ and BLT1 mRNA in tape-stripped skin was confirmed in mice depleted of neutrophils by treatment with cyclophosphamide (CTX) (Fig. 2G-I), which resulted in >98% depletion of circulating blood neutrophils (Fig. S1B). These results suggest that the accumulation of LTB$_4$ and BLT1 mRNA in tape-stripped mouse skin is largely dependent on neutrophils.

Neutrophil influx in tape-stripped mouse skin is largely dependent on BLT1 and neutrophil production of LTB$_4$

Kinetic analysis revealed that neutrophil accumulation in tape-stripped ear skin of WT mice, peaked at 24 hrs and waned by 72 hrs (Fig. 3A), as recently reported (Gregorio et al., 2010; Guiducci et al., 2010). There was markedly less accumulation of neutrophils in tape-stripped ear skin of *Ltb4r1$^{-/-}$* mice compared to WT controls at all time points (Fig. 3A). The residual accumulation of neutrophils in *Ltb4r1$^{-/-}$* mice may be due to robust induction by tape stripping of mRNA for neutrophil chemoattracting chemokines, such as CXCL2, interleukin-1β (IL-1β and IL-6; both the rapid induction (within 6 hrs) of these cytokines and neutrophil influx into tape-stripped skin, were blunted in mice lacking myeloid

differentiation primary response gene 88 (*Myd88*) (Fig. 3B,C). These results indicate that neutrophil influx in tape-stripped mouse skin is largely dependent on BLT1.

To examine whether BLT1 expression by neutrophils is important for their accumulation in tape-stripped skin, we compared the ability of adoptively transferred neutrophils purified from bone marrow of CD45.2$^+$ WT and *Ltb4r1*$^{-/-}$ mice to accumulate in tape-stripped ears of CD45.1$^+$ WT recipients depleted of neutrophils by CTX. Because of the short half-life of neutrophils, the recipients' ears were tape-stripped 20 hrs before neutrophil transfer to allow for expression of inflammatory cytokines in injured skin. Adoptively transferred WT neutrophils, but not *Ltb4r1*$^{-/-}$ neutrophils, accumulated in recipient ears 4 hrs, and, to a lesser extent, 24 hrs, post transfer (Fig. 3D). The rise in blood neutrophil count and the percentages of CD45.2$^+$ donor neutrophils in the spleens 4 hrs post transfer were comparable in recipients of WT and *Ltb4r1*$^{-/-}$ neutrophils (data not shown), indicating that the general circulation of *Ltb4r1*$^{-/-}$neutrophils was not impaired.

Neutrophil accumulation in ear skin 24 hrs after tape stripping was severely diminished in *Lta4h*$^{-/-}$ mice compared to WT controls (Fig. 3E), and in WT mice treated with the LTA$_4$H inhibitor bestatin (Fig. 3F). Since most of the LTB$_4$ that accumulated in tape-stripped skin was derived from neutrophils, we examined whether neutrophil-derived LTB$_4$ was important for the optimal accumulation of neutrophils in tape-stripped skin. There was minimal influx of adoptively transferred CD45.2$^+$ *Lta4h*$^{-/-}$ neutrophils, which cannot synthesize LTB$_4$, into tape-stripped ears of CTX-treated CD45.1$^+$ WT recipients, compared to CD45.2$^+$ WT neutrophils (Fig. 3G). The percentages of CD45.2$^+$ donor neutrophils in the spleens were comparable in recipients of *Lta4h*$^{-/-}$ and WT neutrophils (data not shown).

Neutrophil-derived LTB$_4$ may act in an autocrine and/or paracrine manner to promote neutrophil recruitment to tape-stripped skin. To address this issue, we performed co-transfer experiments in which *Lta4h*$^{-/-}$, *Ltb4r1*$^{-/-}$ or WT neutrophils loaded with CMTMR dye (red) were mixed with equal numbers of WT neutrophils loaded with CMFDA dye (green) and adoptively transferred into CTX-treated WT recipients. Four hours later, ear cells were analyzed and the homing index of *Lta4h*$^{-/-}$ and *Ltb4r1*$^{-/-}$ neutrophils was calculated relative to that of WT neutrophils, set at 1. Co-administration of WT neutrophils partially, but not completely, restored the influx of *Lta4h*$^{-/-}$ neutrophils to tape-stripped skin (Fig. 3H), indicating both paracrine and autocrine roles of neutrophil-derived LTB$_4$ in the recruitment of neutrophils to mechanically injured skin. *Ltb4r1*$^{-/-}$ neutrophils co-transferred with WT neutrophils accumulated significantly less than *Lta4h*$^{-/-}$ neutrophils co-transferred with WT neutrophils (Fig. 3H), supporting a paracrine component to the action of neutrophil-derived LTB$_4$.

The role of LTB$_4$ in neutrophil recruitment to the skin was not limited to mechanical injury induced by tape stripping. Treatment of mice with bestatin resulted in a significant reduction in neutrophil recruitment to sites of cutaneous infection with *S. aureus* (Fig. S2A) and in increased bacterial counts and development of larger lesions at these sites (Fig. S2B,C). Bestatin treatment also significantly inhibited the recruitment of neutrophils in response to sterile wounding of the skin (Fig. S2D), and resulted in significantly larger skin lesions on day 1 post-wounding (Fig. S2E).

BLT1 expression by both T cells and non-T cells is essential for allergic skin inflammation

BLT1 expression on T cells has been shown to be important in a mouse model of antigen-driven allergic airway inflammation (Tager et al., 2003). Consistent with this finding, *Ltb4r1*$^{-/-}$ mice failed to develop allergic skin inflammation following EC sensitization with OVA, as evidenced by their failure to increase dermal infiltration by CD4$^+$ T cells and eosinophils, and upregulate IL-4 and IL-13 mRNA expression in OVA-sensitized skin (Fig.

S3A,B). Impaired skin inflammation in *Ltb4r1*^{−/−} mice was not due to failure to mount a systemic immune response to EC sensitization as their OVA specific antibody concentrations and cytokine secretion by OVA-stimulated splenocytes were comparable to WT controls (Fig. S3C,D).

To investigate the role of BLT1 expression on T and non-T cells in allergic skin inflammation, CD4$^+$ donor T cells from EC-sensitized WT and *Ltb4r1*$^{−/−}$ mice were examined for their capacity to transfer allergic skin inflammation to WT and *Ltb4r1*$^{−/−}$ recipients. OVA stimulated CD4$^+$ splenocytes from EC-sensitized mice were adoptively transferred by intravenous (*i.v.*) injection to naïve recipients, which were challenged with OVA on shaved and tape-stripped back skin on days 0 and 3 after transfer, and examined on day 7. As previously reported in BALB/c mice (He et al., 2008), CD4$^+$ T cells from C57BL/6 WT donors EC-sensitized with OVA transferred allergic skin inflammation to WT recipients, as evidenced by significant increases in epidermal and dermal thickness, number of CD4$^+$ T cells, and IL-4 and IL-13 mRNA expression at sites of OVA challenge compared to sites of saline challenge (Fig. 4 and data not shown). CD4$^+$ T cells from *Ltb4r1*$^{−/−}$ donors EC-sensitized with OVA failed to transfer allergic skin inflammation to WT recipients. Unexpectedly, CD4$^+$ T cells from WT donors EC-sensitized with OVA failed to transfer allergic skin inflammation to *Ltb4r1*$^{−/−}$ recipients (Fig. 4A-D), indicating a critical role for BLT1 expression on non-T cells in our model. As expected, CD4$^+$ T cells from *Ltb4r1*$^{−/−}$ donors EC-sensitized with OVA failed to transfer allergic skin inflammation to *Ltb4r1*$^{−/−}$ recipients. Recipients of CD4$^+$ T cells from OVA-sensitized donors failed to develop allergic skin inflammation in response to skin challenge with saline (data not shown). Transfer of CD4$^+$ T cells from saline-sensitized mice failed to elicit detectable skin inflammation in OVA-challenged skin of recipients (data not shown).

Expression of BLT1 by neutrophils is important for the development of allergic skin inflammation

To determine whether BLT1 expression on neutrophils is important for the development of allergic skin inflammation, we compared WT and *Ltb4r1*$^{−/−}$ neutrophils for their capacity to rescue the transfer allergic skin inflammation by OVA-activated CD4$^+$ effector T cells from EC-sensitized WT mice to *Ltb4r1*$^{−/−}$ recipients. Neutrophils were administered *i.v.* (10^7 per mouse) at days 0 and 3 of the one week-long epicutaneous challenge with OVA (Fig. 5A). Administration of WT neutrophils rescued the ability of WT CD4$^+$ T cells to transfer allergic skin inflammation to *Ltb4r1*$^{−/−}$ recipients. This was evidenced by significant increases in epidermal and dermal thickness, numbers of CD4$^+$ T cells in the dermis and expression of IL-4 and IL-13 mRNA, compared to skin of mice that received only WT CD4$^+$ T cells (Fig. 5A-D). In contrast, administration of *Ltb4r1*$^{−/−}$ neutrophils failed to rescue the ability of WT CD4$^+$ T cells to transfer allergic skin inflammation to *Ltb4r1*$^{−/−}$ recipients. These results demonstrate that BLT1 expression by neutrophils is critical for the development of allergic skin inflammation.

Accumulation of effector T cells to antigen-challenged skin requires BLT1 expression by neutrophils

Failure of *Ltb4r1*$^{−/−}$ neutrophils to rescue the ability of WT CD4$^+$ T cells to transfer allergic skin inflammation to *Ltb4r1*$^{−/−}$ recipients prompted us to directly investigate the role of BLT1 expression by neutrophils in the accumulation of antigen specific effector CD4$^+$ T cells into cutaneous sites of antigen challenge. We examined the ability of CD4$^+$ OT-II T cells, activated with OVA peptide and antigen presenting cells (APCs) for 4 days *in vitro*, to accumulate into the tape-stripped ear skin of recipients following challenge with OVA +cholera toxin (CT) or CT alone, as control. Vα2$^+$Vβ5$^+$ WT OT-II effector T cells accumulated significantly more in antigen-challenged skin than in control challenged ear

skin of WT recipients (Fig. 5E). In contrast, WT OT-II effector T cells failed to accumulate in antigen-challenged skin of $Ltb4r1^{-/-}$ recipients (Fig. 5E), indicating that BLT1 expression by non-T cells is important for the homing of $CD4^+$ effector T cells to the skin. $Ltb4r1^{-/-}$ OT-II effector T cells failed to accumulate in antigen-challenged skin of WT recipients (Fig. S4A), despite normal proliferation to OVA (data not shown) and normal expression of the skin homing receptor CCR4 and memory marker CD44 (Fig. S4B,C), confirming the role of BLT1 expression on T cells in homing to the skin.

Co-administration of WT neutrophils *i.v.* significantly increased the accumulation of adoptively transferred WT OT-II effector T cells into antigen-challenged skin of WT recipients (Fig. 5F). More importantly, it completely rescued the ability of WT OT-II effector T cells to accumulate in antigen-challenged skin of $Ltb4r1^{-/-}$ recipients (Fig. 5F). These results demonstrate that BLT1 expression by neutrophils is critical for the accumulation of $CD4^+$ effector T cells into cutaneous sites of antigen challenge and for allergic skin inflammation.

Neutrophil-derived LTB$_4$ is important for the development of allergic skin inflammation

We next investigated whether LTB$_4$ production by skin-infiltrating neutrophils important for the development of allergic skin inflammation. To this purpose, we examined whether $Lta4h^{-/-}$ neutrophils rescued the ability of OVA-activated $CD4^+$ effector T cells from EC-sensitized WT mice to transfer allergic skin inflammation to $Ltb4r1^{-/-}$ recipients. Because $Lta4h^{-/-}$ neutrophils failed to migrate to tape-stripped skin, neutrophils from $Lta4h^{-/-}$ mice and WT controls were injected intradermally (*i.d.*) on days 0 and 3 at the site of EC challenge. Intradermal administration of WT neutrophils rescued the ability of WT $CD4^+$ T cells to transfer allergic skin inflammation to $Ltb4r1^{-/-}$ recipients, evidenced by significant increases in epidermal and dermal thickness, $CD4^+$ T cell infiltration in the dermis, and expression of IL-4 and IL-13 mRNA, compared to antigen-challenged skin of mice that received only WT $CD4^+$ T cells (Fig. 6A-D). In contrast, *i.d.* injection $Lta4h^{-/-}$ neutrophils in $Ltb4r1^{-/-}$ recipients failed to rescue the ability of WT $CD4^+$ T cells to transfer allergic skin inflammation to $Ltb4r1^{-/-}$ recipients. In addition, *i.d.* administration of $Ltb4r1^{-/-}$ neutrophils, which have an intact ability to produce LTB$_4$, caused an increase in the accumulation of OT-II cells in EC challenged ear skin of $Ltb4r1^{-/-}$ recipients, and in ear scratching, comparable to those caused by *i.d.* administration of WT neutrophils (Fig. 6E,F).

LTB$_4$ induces itch-associated scratching response in mice (Kim et al., 2007). Tape stripping induced significantly more scratching in WT mice than in $Ltb4r1^{-/-}$ and $Lta4h^{-/-}$ mice, suggesting that LTB$_4$ is important in the scratching response to mechanical injury (Fig. S5A). EC-sensitized with OVA exhibited increased scratching (Fig. S5B), and LTB$_4$ concentrations were elevated in OVA sensitized mouse skin sites (Fig. S5C), as in AD skin lesions. These results suggest that generation of LTB$_4$ by infiltrating neutrophils is critical for their ability to promote T cell mediated allergic skin inflammation.

Pharmacologic blockade of LTB$_4$ synthesis inhibits acute skin flares in previously EC-sensitized mice

Cutaneous exposure to antigens provokes skin flares in AD patients (Nosbaum et al., 2010; Sicherer and Leung, 2007). Therefore, we next investigated whether administration of bestatin to EC-sensitized mice prevented the development of allergic skin inflammation following cutaneous antigen challenge. C57BL/6 mice were EC-sensitized with OVA for 7 weeks then immediately challenged by application of OVA to a previously unsensitized shaved and tape-stripped skin site on the back. Mice were treated by intraperitoneal (*i.p.*) injection with bestatin (0.4 mg/day) or vehicle control, daily for 7 days beginning 2 days before the seven-day challenge (Fig. 7A). Mice treated with vehicle alone developed allergic

skin inflammation in response to cutaneous OVA challenge, as evidenced by significantly increased epidermal and dermal thickness, dermal infiltration with $CD4^+$ T cells, and expression of Th2 cell-derived cytokines, compared to challenge with saline (Fig. 7B-E). In contrast, mice treated with bestatin failed to develop allergic skin inflammation in response to cutaneous OVA challenge. These results suggest that inhibition of LTB_4 production prevents allergic skin inflammation driven by cutaneous antigen exposure in previously sensitized mice.

Discussion

In this study, we have identified a critical role for neutrophils in allergic skin inflammation in a mouse model with features of AD. We demonstrated that LTB_4 production by neutrophils was essential for their accumulation in mechanically injured skin and for the recruitment of effector $CD4^+$ T cells to cutaneous sites of antigen exposure and development of allergic skin inflammation.

Mechanical skin injury by scratching in humans, and its surrogate, tape stripping in mice, caused robust infiltration of neutrophils and accumulation of LTB_4 and mRNA for its receptor, BLT1, in the dermis. The slight increase in epidermal thickness in injured human and mouse skin is possibly due to the release of cytokines that promote keratinocyte proliferation, which include IL-6 (Werner et al., 2007). Accumulation of neutrophils and BLT1 mRNA was substantially greater in tape-stripped mouse skin than in scratched human skin, possibly because mouse skin is much thinner than human skin and might be more susceptible to mechanical skin injury. The relatively comparable elevation of LTB_4 concentrations in tape-stripped mouse skin and scratched human skin suggests a more rapid washout of LTB_4 from injured mouse skin. The baseline concentration of LTB_4 may reflect a background signal intrinsic to the assay used and/or be derived from resident cells in the skin and may have not been biologically active, since neutrophils were not detected in unmanipulated skin. Studies in $Lta4h^{-/-}$ and $Ltb4r1^{-/-}$ mice demonstrated that LTB_4 and BLT1 are both important for neutrophil accumulation into tape-stripped mouse skin. The role of LTB_4 in neutrophil recruitment to the skin is supported by the observation that epicutaneous application of LTB_4 in normal healthy volunteers results in local neutrophil infiltration (de Jong et al., 1992). LTB_4 induces itch-associated scratching response in mice (Kim et al., 2007). Tape stripping induced significantly more scratching in WT mice than in $Ltb4r1^{-/-}$ and $Lta4h^{-/-}$ mice, suggesting that LTB_4 is important in the scratching response to mechanical injury.

Neutrophils, but not mast cells, were the major source of the increased LTB_4 in mouse skin 24 hrs following mechanical injury. LTB_4 failed to accumulate in mice depleted of neutrophils using Gr-1 mAb or CTX, but accumulated normally in the mechanically injured skin of mast cell-deficient Kit^W/Kit^{W-v} mice. Although mast cells could release LTB_4 early after mechanical injury, mast cell-derived LTB_4 was clearly not essential for neutrophil influx into mechanically injured skin. This is consistent with our previous observation that allergic skin inflammation elicited by EC sensitization of tape-stripped skin with OVA is intact in mast cell-deficient Kit^W/Kit^{W-v} mice (Alenius et al., 2002).

Adoptive transfer experiments demonstrated that neutrophil influx in tape-stripped mouse skin was largely dependent on BLT1 and on both paracrine and autocrine actions of neutrophil-derived LTB_4. LTB_4 played a role in neutrophil recruitment to the skin in response to bacterial infection and sterile wounding. In addition, neutrophil-derived LTB_4 is required for the recruitment of neutrophils to the joints and for the development of inflammatory arthritis (Chen et al., 2006; Chou et al., 2010; Kim et al., 2006). These observations suggest that LTB_4 generated by neutrophils is important for their recruitment to

the skin in response to a variety of inflammatory stimuli, as well as for their recruitment to other tissues.

Additional factors, which include inflammatory chemokines and cytokines, produced by resident skin cells may contribute to the initial neutrophil influx in the tape-stripped skin, as suggested by the residual neutrophil infiltrate observed of $Ltb4r1^{-/-}$ mice. Furthermore, production of IL-1b and IL-6 by skin infiltrating WT neutrophils may explain the finding that $Ltb4r1^{-/-}$ neutrophils could be detected in tape-stripped skin when co-transferred with WT neutrophils, but not when transferred alone. Both the rapid induction (within 6 hrs) of CXCL2, IL-1b and IL-6 mRNA and neutrophil influx into tape-stripped skin, were blunted in $Myd88^{-/-}$ mice. This observation suggests that signaling via TLR receptors, possibly by endogenous alarmins (Guiducci et al., 2010) and/or signaling via IL-1 receptor (IL-1R) family members released from damaged keratinocytes, plays an important role in the initial neutrophil influx into injured skin.

A central finding of the present study is that CD4^{+} T effector cells from OVA-sensitized WT mice fail to transfer allergic inflammation to $Ltb4r1^{-/-}$ recipients. Taken together with the observation that $Ltb4r1^{-/-}$ CD4^{+} T cells fail to transfer allergic inflammation, this result indicates that the expression of BLT1 on both non-T cells and T cells is important for allergic skin inflammation. Co-transfer of WT neutrophils, but not $Ltb4r1^{-/-}$ neutrophils, rescued the ability of CD4^{+} effector T cells to accumulate in antigen-challenged skin of $Ltb4r1^{-/-}$ recipients and cause allergic skin inflammation. Intradermal administration of WT, and $Ltb4r1^{-/-}$, but not $Lta4h^{-/-}$, neutrophils rescued the ability of WT CD4^{+} effector T cells to transfer allergic skin inflammation to $Ltb4r1^{-/-}$ recipients. This finding demonstrates that neutrophil-derived LTB$_4$ is critical for the ability of CD4^{+} effector T cells to cause allergic skin inflammation. These observations demonstrate that LTB$_4$ production by neutrophils is critical both for the BLT1 dependent accumulation of neutrophils and the BLT1 dependent recruitment of antigen specific effector CD4^{+} T cells to cutaneous sits of antigen introduction.

The LTB$_4$ synthesis inhibitor bestatin blocked neutrophil influx into tape-stripped skin and inhibited the development of allergic inflammation at sites of cutaneous antigen challenge in mice. This result suggests that blockade of LTB$_4$-BLT1 interaction may be a useful strategy in preventing acute flares that are precipitated in quiescent skin in patients with AD by scratching and introduction of previously encountered antigen.

LTB$_4$ concentrations were elevated in OVA sensitized mouse skin sites, as in AD skin lesions, and mice EC-sensitized with OVA exhibited increased scratching. However, unlike AD skin lesions, which contain few, if any, neutrophils (Leiferman, 1994), EC-sensitized mouse skin sites exhibit neutrophil infiltration (Spergel et al., 1998). Neutrophils, like eosinophils, may not persist intact in chronic AD lesions. It thus remains possible that scratching established skin lesions induces a transient acute neutrophil influx, which triggers LTB$_4$-dependent recruitment of BLT1^{+} effector T cells, resulting in the exacerbation of allergic skin inflammation.

Scratching is a hallmark of AD and causes further disruption of an already abnormal skin barrier, allowing cutaneous introduction of antigen. Our finding that the LTB$_4$-BLT1 axis is implicated in two sequential steps in allergic inflammation at sites of cutaneous antigen exposure, namely recruitment of neutrophils and neutrophil-dependent recruitment of effector T cells, make the LTB$_4$-BLT1 axis a potential therapeutic target in AD.

Experimental Procedures

Human skin biopsy

Two healthy non-allergic adult subjects were asked, after obtaining informed consent, to scratch the inner side of their forearm, for a total of 12 times over a period of 24 hrs (every hour 8 times on day 0, and 4 times on day 1) using each time twenty strokes of their index fingernail. A 4 mm punch biopsy was obtained an hour after the last scratching from the scratched site and from the corresponding unscratched site of the contralateral forearm. Histologic examination was performed on H&E stained sections by an experienced dermatopathologist who was blinded to the source of the material.

Mice and Sensitization

$Ltb4r1^{-/-}$ and $Ltb4r1^{-/-}$ OT-II mice on C57BL/6 background, were previously described (Tager et al., 2003). $Lta4h^{-/-}$ mice, a kind gift of Dr. Koller (Byrum et al., 1999) were bred on C57BL/6 background for 10 generations. WT C57BL/6 mice were obtained from Charles River Laboratory. $J\text{-}Kit^{W}/Kit^{W\text{-}v}$ (WBB6F1) mice and congenic WBB6F1 WT mice, OT-II, and CD45.1$^+$ mice on C57BL/6 background were obtained from the Jackson Laboratory. LysEGFP reporter mice on a C57BL/6 genetic background were previously described (Faust et al., 2000).

The animals were kept in a specific pathogen-free environment. All procedures performed on the animals were in accordance with the Animal Care and Use Committee of the Boston Children's Hospital or UCLA Chancellor's Animal Research Committee.

To cause mechanical injury, the shaved back skin or the ear skin were tape-stripped 6 times with Tegaderm (Westnet Inc, Canton, MA). For neutrophil depletion, WT mice were treated with CTX or PBS control as described previously (Zuluaga et al., 2006). In selected experiments, WT mice were treated by *i.p.* injection with 100 μg Gr-1 mAb (RB6-8C5, ebioscience) or isotype control at day -3 and -1 of tape stripping. Neutrophil depletion was confirmed by flow cytometry at day 0 before tape stripping. Mice were treated by *i.p.* injection with 0.4 mg/day bestatin (Cayman Chemical) or vehicle control, daily for 2 days before tape stripping.

EC sensitization of six- to eight-week-old female mice was performed as described previously (Spergel et al., 1998). Briefly, the skin of anesthetized mice was shaved and tape-stripped 6 times by transparent IV dressing (Tegaderm®; Owens & Minor Inc., Franklin, Massachusetts, USA). One hundred micrograms of chicken egg ovalbumin (OVA Grade V; Sigma-Aldrich, St. Louis, Missouri, USA) in 100 μL of normal saline, or placebo (100 μL of normal saline), were placed on a patch of sterile gauze (1 × 1 cm), which was secured to the skin with a transparent bio-occlusive dressing. Each mouse had a total of three one-week exposures to the patch separated from each other by two-week intervals. In the acute skin challenge model, mice were EC-sensitized with OVA for 7 weeks then challenged on days 0 and 3 after sensitization (days 50 and 53 from the start) by means of application of OVA or saline to shaved and tape-stripped, previously unsensitized skin and were studied on day 7 of challenge (day 57 from the start). Mice were treated by *i.p.* injection with 0.4 mg/day bestatin or vehicle control, daily for 7 days beginning 2 days before the seven-day challenge as shown in Fig. 7A.

Antibodies and flow cytometry analysis of ear skin

Ear skin was used for flow cytometry analysis due to a technical difficulty of preparing cell suspension from the dorsal skin. Cells from ears were isolated as described previously (He et al., 2008). Briefly, ears were separated in two halves and floated on trypsin-EDTA (Cellgro)

for 30 min at 37°C to allow dissociation of the epidermis. Both epidermal and dermal sheets were incubated for a further 1-2 hr at 37°C. The resulting cell suspensions were stained with the appropriate antibodies and analyzed by flow cytometry.

For surface staining, the following antibodies were used: fluorochrome-labeled anti-CD11b, Gr-1, CD4, CD44, Vα2, Vβ5, CD45.1 and CD45.2 (all from eBioscience). Cells were analyzed on FACSCanto and the data was analyzed using Flowjo software.

Measurement of skin LTB$_4$ concentrations

LTB$_4$ concentrations in human and mouse skin were determined using solid phase extraction followed by EIA kit measurement according to the manufacturer's instructions (Cayman Chemical).

Neutrophil preparation from bone marrow

Mouse neutrophils were prepared from the femur and tibia of WT and *Ltb4r1*$^{-/-}$ mice using discontinuous Percoll density centrifugation as previously described (Jia et al., 2007). Preparations were >90% pure as determined by flow cytometry analysis with double staining using Gr-1 and CD11b mAbs.

Adoptive transfer of neutrophils

CD45.1$^+$ WT recipient mice were treated with CTX as described above and ear skin were tape-stripped 20 hrs before neutrophil transfer. 1×10^7 bone marrow neutrophils were isolated from CD45.2$^+$ WT, *Ltb4r1*$^{-/-}$, and *Lta4h*$^{-/-}$ mice and *i.v.* injected into CTX-treated WT mice. The percentage of adoptively transferred neutrophils recruited to the ear skin was analyzed 4 hrs and 24 hrs after transfer using a FACSCanto (BD Biosciences).

In co-transfer experiment, 5×10^6 neutrophils from WT and *Ltb4r1*$^{-/-}$ mice were labeled with 1 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) and 15 μM CellTracker Orange CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (Invitrogen) at 37°C for 10 minutes. *Ltb4r1*$^{-/-}$ or WT neutrophils loaded with CMTMR dye (red) were mixed with 5×10^6 WT neutrophils loaded with CMFDA dye (green) at an input ratio of ~1:1 and adoptively transferred by *i.v.* into CTX-treated WT recipients whose ears were tape-stripped 20 hrs earlier. Four hours later, the ratio of green:red fluorescent cells in the ears was determined, adjusted to the input ratio and the relative skin homing index was calculated.

Adoptive transfer of allergic skin inflammation

Splenocytes from EC-sensitized WT or *Ltb4r1*$^{-/-}$ mice were cultured with OVA for 5 days, then CD4$^+$ T cells were purified by AutoMACS and injected *i.v.* into naïve recipients (5×10^6 CD4$^+$ T cells per mouse) which were challenged the same day by application of 100 mg OVA to shaved and tape-stripped dorsal skin. Skin challenge was repeated on day 3 and the skin site was examined on day 7. In selected experiments, 5×10^6 splenic CD4$^+$ T cells from WT mice EC-sensitized with OVA were injected *i.v.* into naïve *Ltb4r1*$^{-/-}$ recipients on day 0 and WT, *Ltb4r1*$^{-/-}$, or *Lta4h*$^{-/-}$ neutrophils were administered *i.v.* (1×10^7) or *i.d.* (1×10^6) on day 0 and day 3 of one-week EC challenge.

Skin homing of CD4$^+$ T cells

CD4$^+$ T cells were prepared from the spleen of OT-II mice on WT or *Ltb4r1*$^{-/-}$ background and activated with irradiated syngeneic splenocytes (3000 rads) (1:10 ratio) as antigen-presenting cells (APCs) in the presence of OVA (100 mg/ml) and IL-2 (10 ng/ml) for 5 days. 5×10^6 activated transgenic CD4$^+$ T cells from WT or *Ltb4r1*$^{-/-}$ background were

injected *i.v.* into naïve recipients followed by application of OVA+cholera toxin (CT) or CT alone to tape-stripped ear skin as previously described (He et al., 2008). In selected experiments, recipients received WT or *Ltb4r1*$^{-/-}$ neutrophils *i.v.* (1×10^7) or *i.d.* (1×10^6) on day 0 and again on day 3 of one-week EC challenge. At the end of one-week EC challenge, single cell suspension was prepared from ear skin as described above, stained with the appropriate antibodies, and analyzed by flow cytometry.

Quantitative PCR analysis of cytokines and histological analysis

Total RNA was extracted from homogenized skin tissue or from cultured cells with RNAqueous extraction kit (Ambion Inc) following the manufacturer's instructions. cDNA was generated with iScript cDNA synthesis kit (Bio-rad Laboratory). Quantitative real-time PCR was done using Taqman Gene Expression Assay, universal PCR master mix and the ABI Prism 7300 sequence detection system with commercial primers and probes, all from Applied Biosystems. Fold induction of target gene expression was calculated using the comparative method for relative quantitation by normalization to the internal control β2-microglobulin, as described previously (He et al., 2007). For histological analysis, skin specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4 mm sections of skin were stained with hematoxylin and eosin (H&E). Neutrophils were counted in ten randomly chosen areas 25×25 μm in size. CD4 staining of skin sections was performed as previously described (Spergel et al., 1998). Epidermal and dermal thickening, eosinophils, and CD4$^+$ T cells were counted blinded in 10-15 high-power fields (HPFs) at a magnification of 400×.

Immunoglobulin determinations

Mice were bled, and serum concentrations of OVA-specific IgE, IgG1, and IgG2a Abs were measured using modified OVA-specific ELISAs as previously described (He et al., 2008).

Response of splenocytes to OVA

Single-cell suspensions from spleen of EC-sensitized mice were cultured in complete RPMI 1640 (Invitrogen) supplemented by 10% FCS, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin at 3×10^6/ml in 24-well following the stimulation with OVA (100 μg/ml) for 4 days. Supernatants were collected for cytokine measurements by ELISA.

Statistical analysis

Two-tailed Student's t-test or one-way ANOVA was used to determine statistical differences between groups. A *p* value smaller than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Scratching of human and mouse skin causes LTB_4-BLT1 dependent neutrophil influx

2. BLT1 expression on T and non-T cells is required for allergic skin inflammation

3. Transfer of allergic skin inflammation requires neutrophil-derived LTB_4 and BLT1

4. Pharmacologic blockade of LTB_4 synthesis inhibits allergic skin inflammation
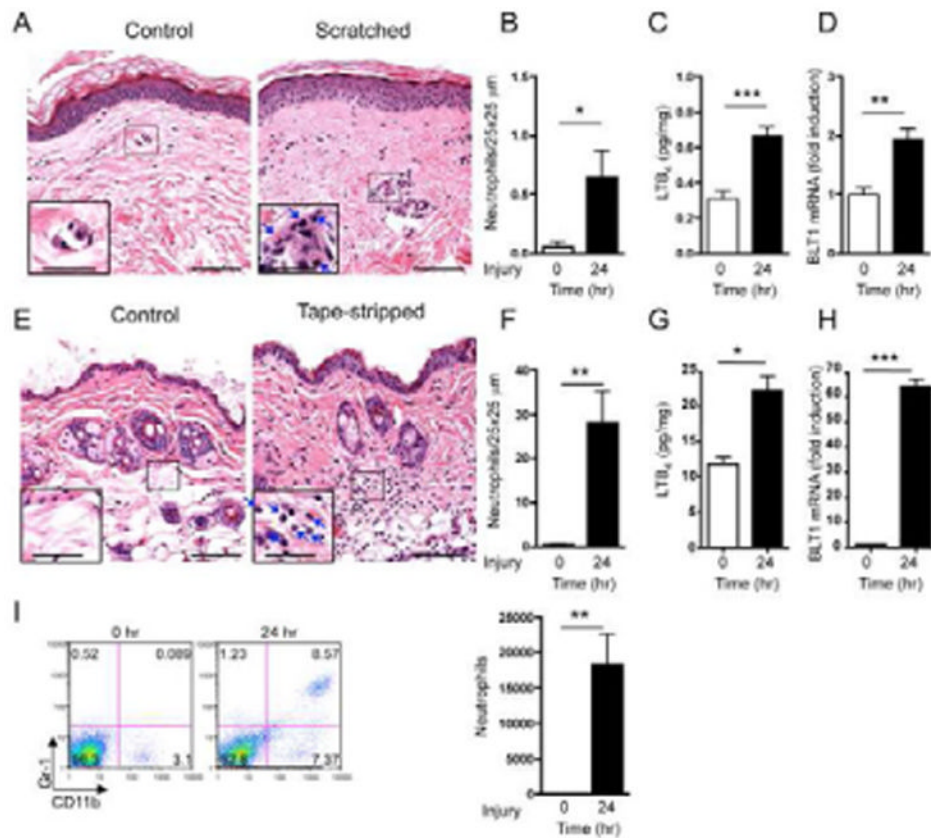
Figure 1. Mechanical skin injury results in the accumulation of neutrophils, LTB$_4$ and BLT1 mRNA in human and mouse skin
A-D. Representative photomicrographs (A), quantification of neutrophils (B), LTB$_4$ concentrations (pg/mg wet skin tissue) (C), and BLT1 mRNA expression (D) in scratched and unscratched human skin (n=2). Arrows indicate neutrophils. Magnification 200×, inset 400×. Scale bars: 70 μm, inset 35 μm. BLT1 mRNA levels are expressed as fold induction relative to unmanipulated skin. **E-H.** Representative photomicrographs (E), quantification of neutrophils (F), LTB$_4$ concentrations (pg/mg wet skin tissue) (G), and BLT1 mRNA expression (H) in tape-stripped and unmanipulated back skin of C57BL/6 mice (n=4). **I.** Representative flow cytometry analysis and quantification of CD11b$^+$Gr-1$^+$ cells in tape-stripped and unmanipulated ear skin of C57BL/6 mice (representative of 4). Columns and error bars represent mean and SEM. *p<0.05, **p<0.01, ***p<0.001.
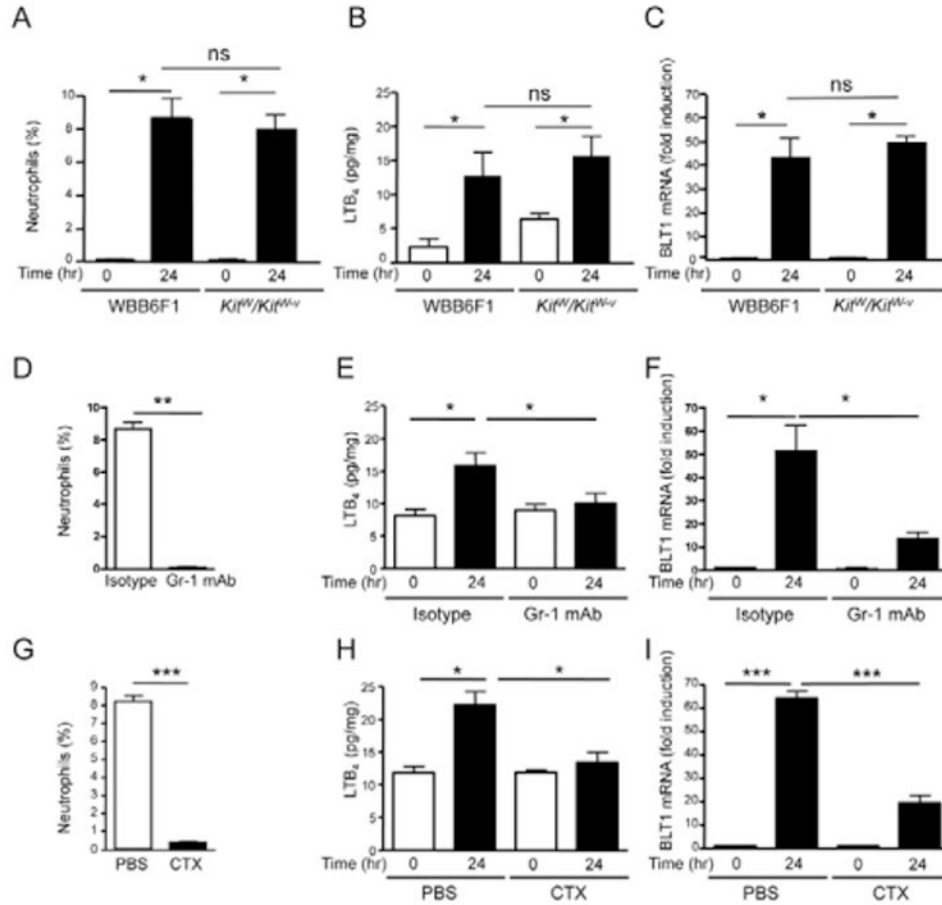
Figure 2. Neutrophils but not mast cells are the source of increased LTB$_4$ in tape-stripped skin
A-I. CD11b$^+$Gr-1$^+$ neutrophils (% of total ear cells), LTB$_4$ concentrations (pg/mg wet skin tissue), and BLT1 mRNA expression in tape-stripped and unmanipulated ear or back skin of Kit^W/Kit^{W-v} mice and WBB6F1 controls (A-C), of tape-stripped C57BL/6 mice treated with Gr-1 mAb or isotype control (D-F), and of tape-stripped C57BL/6 mice treated with CTX or vehicle (G-I). Ear skin was used for A, D, and G. n=3 per group for A-I. BLT1 mRNA levels are expressed as fold induction relative to unmanipulated skin. Columns and error bars represent mean and SEM. *p<0.05, **p<0.01, ***p<0.001. ns= not significant. See also Figure S1.
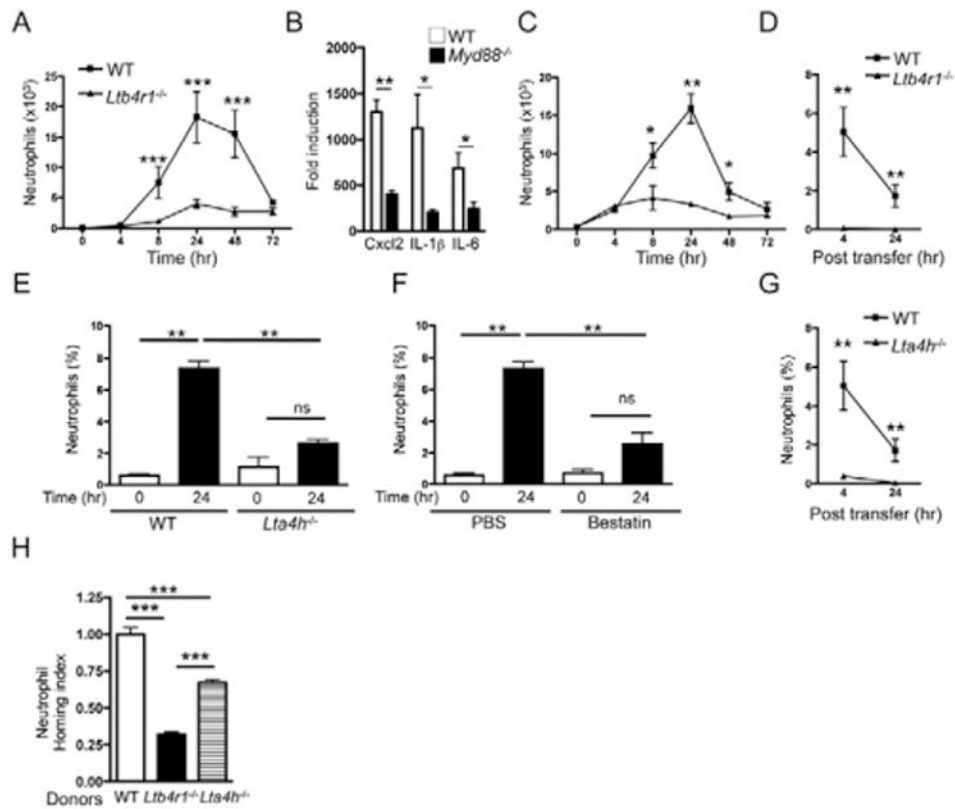
Figure 3. Neutrophil accumulation in tape-stripped skin is dependent on their expression of BLT1 and LTA$_4$H

A. Number of neutrophils in WT and *Ltb4r1*$^{-/-}$ ear skin at different time points (hr) after tape stripping. **B.** Induction of CXCL2, IL-1β, and IL-6 mRNA expression 6 hrs after tape stripping in the skin of WT and *Myd88*$^{-/-}$ mice on BALB/c background. mRNA levels are expressed as fold induction relative to unstripped WT skin (0 hrs) (n=4 per group). **C.** Percentages of neutrophils (% total ear cells) in ear skin of *Myd88*$^{-/-}$ mice and WT controls at different time points after tape stripping (n=5 per group). **D.** Percentage of neutrophils (% total ear cells) from WT and *Ltb4r1*$^{-/-}$ donors in tape-stripped ears of CTX-treated WT recipients (n=4 per group). **E, F.** Percentage of neutrophils (% total ear cells) in WT and *Lta4h*$^{-/-}$ ear skin 24 hrs after tape stripping (E), and in WT mice treated with bestatin or vehicle control prior to tape stripping (F). **G.** Accumulation of neutrophils (% total ear cells) from WT and *Lta4h*$^{-/-}$ donors in tape-stripped ears of CTX-treated WT recipients (n=4 per group). **H.** Homing index of *Ltb4r1*$^{-/-}$ and *Lta4h*$^{-/-}$ neutrophils in tape-stripped ear skin of CTX-treated WT recipients (n=4 per group). Experiments D and G were performed together and thus share the same WT controls. Columns and error bars represent mean and SEM (n=4 mice per group). *p<0.05, ** p<0.01, *** p<0.001. ns = not significant. See also Figure S2.
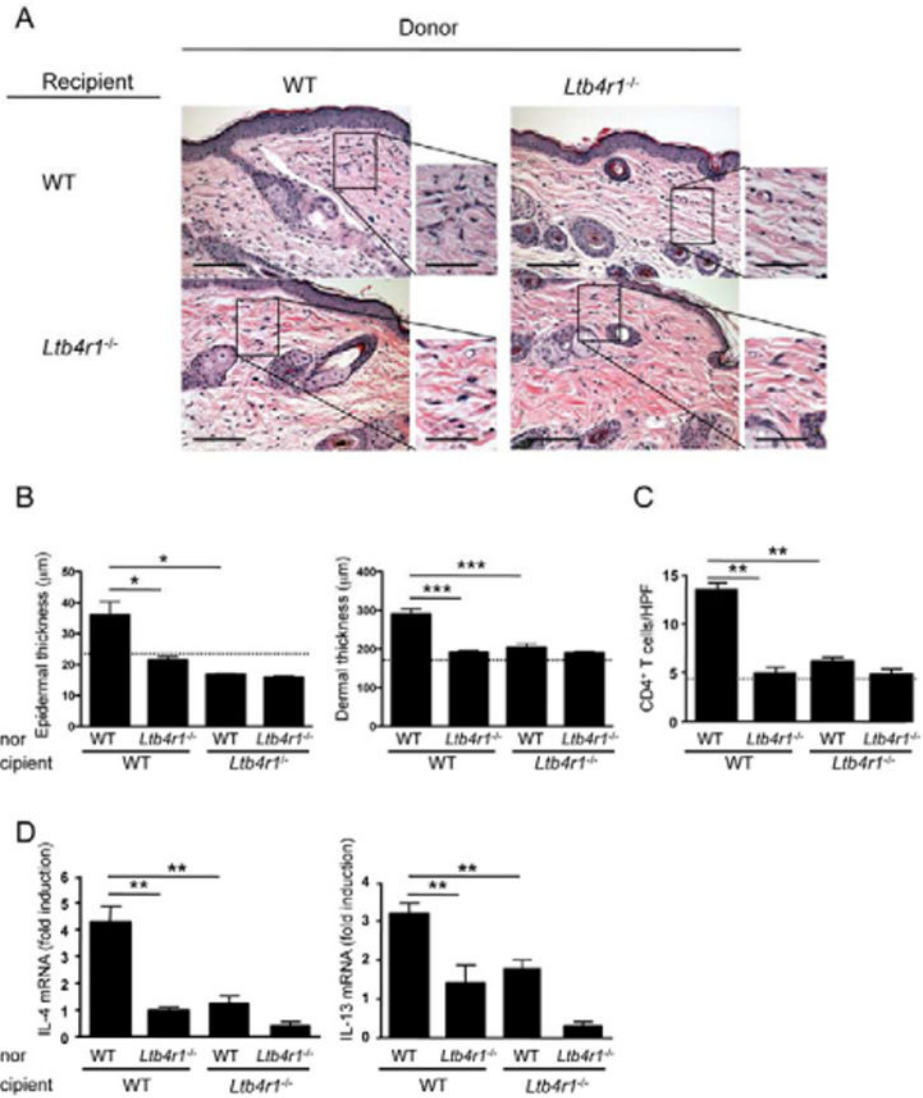
Figure 4. BLT1 expression by both T cells and non-T cells is essential for allergic skin inflammation

A. Representative photomicrographs of H&E sections from OVA-challenged skin of WT and $Ltb4r1^{-/-}$ recipients of CD4^{+} T cells from WT or $Ltb4r1^{-/-}$ mice EC-sensitized with OVA. n=5 for each of the 4 groups. Magnification 200×, inset 400×. Scale bars: 100 μm, inset 50 μm. **B, C.** Epidermal and dermal thickness (B), and numbers of infiltrating CD4^{+} T cells (C). The dotted line in B and C represents the mean value for OVA-challenged skin of recipients of CD4^{+} T cells from saline-sensitized WT donors. **D.** mRNA levels for IL-4 and IL-13 expressed as fold induction relative to OVA-challenged skin of recipients of CD4^{+} T cells from saline-sensitized WT donors (n=5). Columns and error bars represent mean and SEM. *p<0.05, **p<0.01, *** p<0.001. See also Figure S3.
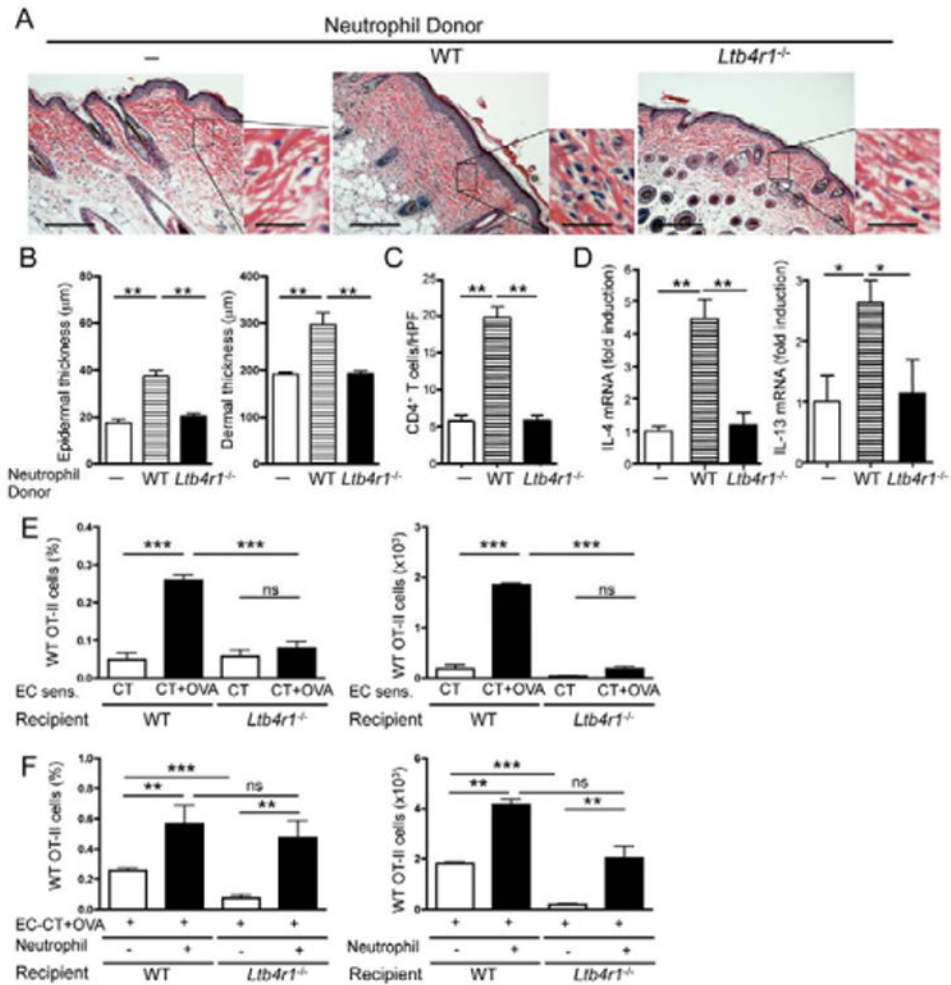
Figure 5. WT, but not *Ltb4r1*−/− neutrophils restores allergic skin inflammation in *Ltb4r1*−/− recipients

A. Representative photomicrographs of H&E sections from OVA-challenged skin of *Ltb4r1*−/− recipients of CD4+ T cells from OVA-sensitized WT mice transferred alone (n=3) or with neutrophils from WT (n=5) or *Ltb4r1*−/− donors (n=4). Magnification 100×, inset 400×. Scale bars: 200 mm, inset 50 mm. **B-D.** Epidermal and dermal thickness (B), number of infiltrating CD4+ T cells (C), and mRNA levels of IL-4 and IL-13 expressed as fold induction relative to recipients of CD4+ T cells from OVA-sensitized WT mice transferred without neutrophils (D). **E.** Percentage (% total ear cells) and number of Vα2+Vβ5+ transgenic OT-II CD4+ T cells from WT donors accumulating in OVA-challenged ear skin of WT and *Ltb4r1*−/− recipients. **F.** Effect of co-administration of WT neutrophils on the percentage (% total ear cells) and number of Vα2+Vβ5+ transgenic OT-II CD4+ T cells from WT donors which accumulate in OVA-challenged ear skin of WT and *Ltb4r1*−/− recipients. Columns and error bars represent mean and SEM (n=3-5 mice per group). *p<0.05, **p<0.01, *** p<0.001. ns = not significant. See also Figure S4.
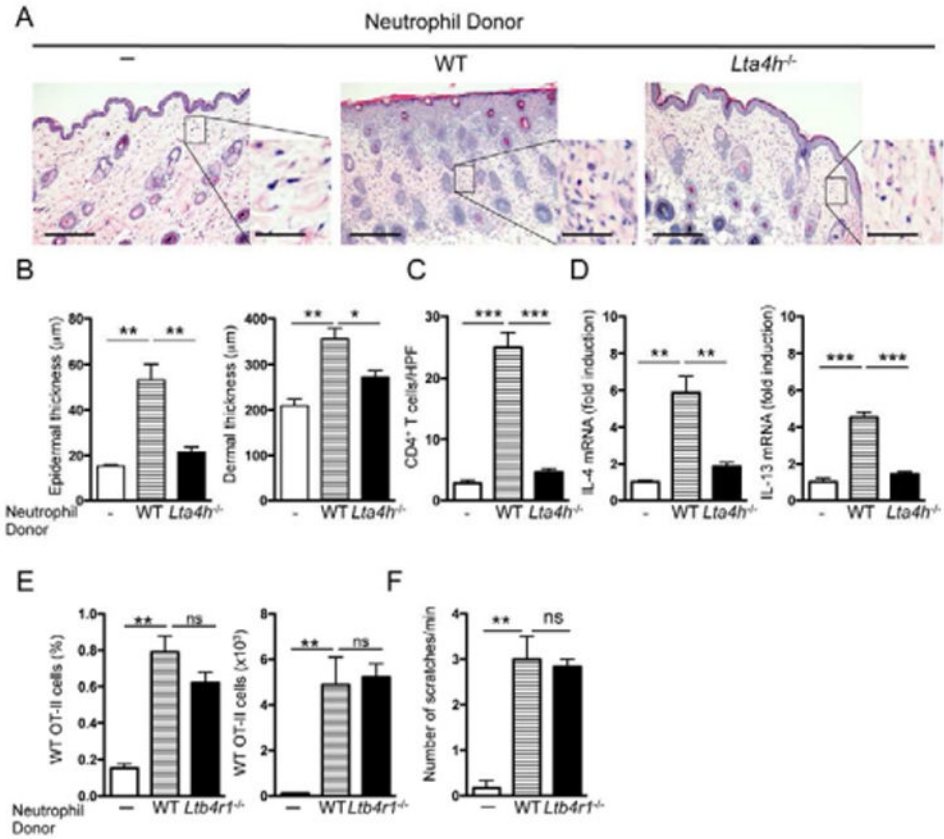
Figure 6. Intradermal injection of *Lta4h*$^{-/-}$ neutrophils fails to restore allergic skin inflammation in *Ltb4r1*$^{-/-}$ recipients

A. Representative photomicrographs of H&E sections from OVA-challenged skin of *Ltb4r1*$^{-/-}$ recipients of CD4$^+$ T cells from OVA-sensitized WT mice transferred alone or with neutrophils from WT or *Lta4h*$^{-/-}$ donors (n=3 per group). Magnification 100×, inset 400×. Scale bars: 200 μm, inset 50 μm. **B-D.** Epidermal and dermal thickness (B), number of infiltrating CD4$^+$ T cells (C), and mRNA levels of IL-4 and IL-13 expressed as fold induction relative to recipients of CD4$^+$ T cells from OVA-sensitized WT mice transferred without neutrophils (D). **E, F.** Effect of *i.d.* injection of WT and *Ltb4r1*$^{-/-}$ neutrophils on the homing of WT OT-II cells to OVA challenged skin of *Ltb4r1*$^{-/-}$ recipients (E) and on ear scratching by the recipients (F). Columns and error bars represent mean and SEM. n=3-4 mice per group. *p<0.05, **p<0.01, ***p<0.001. See also Figure S5.
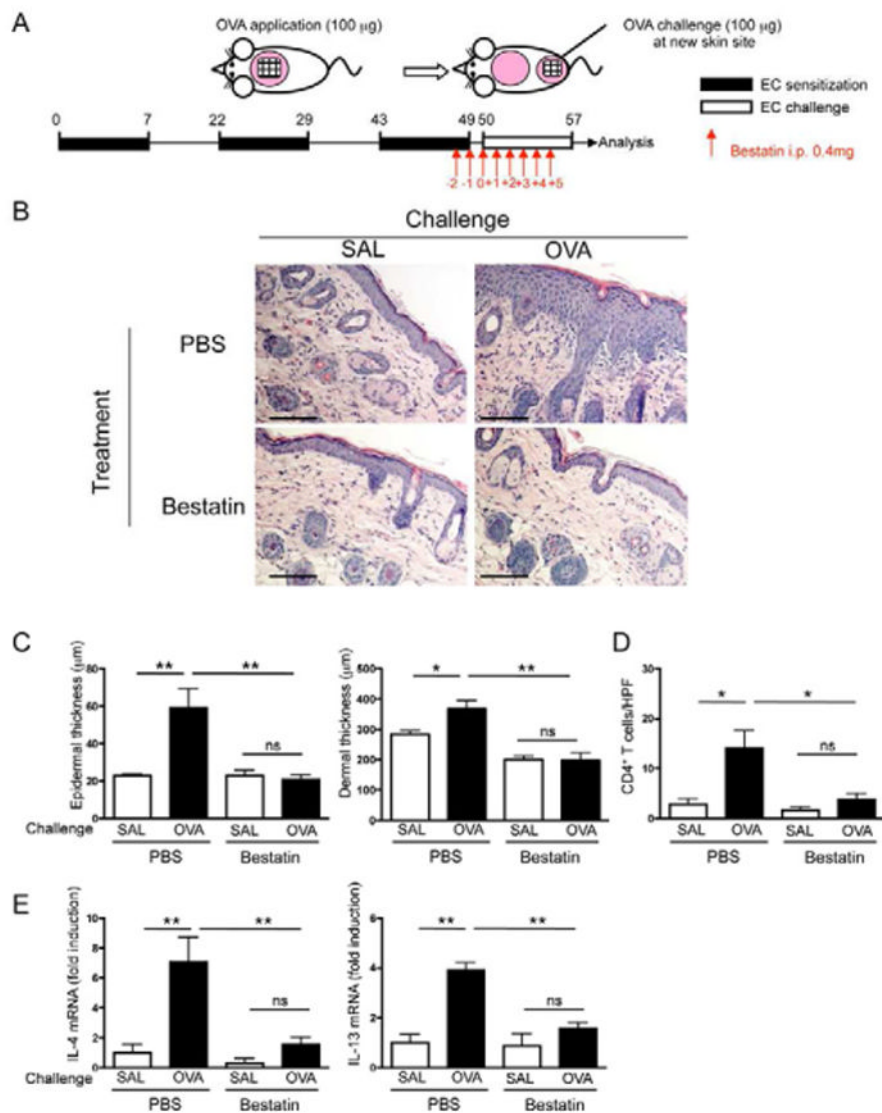
Figure 7. Pharmacologic blockade of LTB$_4$ synthesis inhibits acute skin flares in previously EC-sensitized mice

A. Experimental protocol. **B.** Representative photomicrographs of H&E stained sections from challenged skin of OVA-sensitized mice treated with bestatin or vehicle (PBS) control. Magnification 200×. Scale bars: 100 μm. **C-E.** Epidermal and dermal thickness (C), number of infiltrating CD4$^+$ T cells (D), and mRNA levels of IL-4 and IL-13 expressed as fold induction relative to saline challenged untreated mice (E). Columns and error bars represent mean and SEM. n=3 mice per group. *p<0.05, **p<0.01. ns = not significant.