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ARTICLE

BLT1 in dendritic cells promotes Th1/Th17 differentiation and its deficiency ameliorates TNBS-induced colitis

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Leukotriene B4 (LTB4) synthesis is enhanced in the colonic mucosa in patients with inflammatory bowel disease (IBD). BLT1, a high-affinity receptor for LTB4, exhibits no effect on the progression of dextran sodium sulfate (DSS)-induced colitis, which mostly relies on innate immunity. Here, we reported that BLT1 regulates trinitrobenzene sulfonic acid (TNBS)-induced colitis, which reflects $CD4^+$ T-cell-dependent adaptive immune mechanisms of IBD. We found that BLT1 signaling enhanced the progression of colitis through controlling the production of proinflammatory cytokines by dendritic cells (DCs) and modulating the differentiation of Th1 and Th17. $BLT1^{-/-}$ mice displayed an alleviated severity of TNBS-induced colitis with reduced body weight loss and infiltrating cells in the lamina propria. BLT1 deficiency in DCs led to reduced production of proinflammatory cytokines, including IL-6, TNF-α, and IL-12, and these results were further confirmed via treatment with a BLT1 antagonist. The impaired cytokine production by $BLT1^{-/-}$ DCs subsequently led to reduced Th1 and Th17 differentiation both in vitro and in vivo. We further performed a conditional DC reconstitution experiment to assess whether BLT1 in DCs plays a major role in regulating the pathogenesis of TNBS-induced colitis, and the results indicate that BLT1 deficiency in DCs also significantly reduces disease severity. The mechanistic study demonstrated that BLT1-regulated proinflammatory cytokine production through the Gαi βγ subunit-phospholipase Cβ (PLCβ)-PKC pathway. Notably, we found that treatment with the BLT1 antagonist also reduced the production of proinflammatory cytokines by human peripheral blood DCs. Our findings reveal the critical role of BLT1 in regulating adaptive immunity and TNBS-induced colitis, which further supports BLT1 as a potential drug target for adaptive immunity-mediated IBD.

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INTRODUCTION

Leukotrienes are lipid mediators produced by leukocytes upon activation. Leukotrienes can be grouped into two classes. One class includes cysteinyl leukotrienes containing a thioether linkage, including LTC4, LTD4, and LTE4. These leukotrienes activate cysteinyl leukotriene receptors CysLT1 and CysLT2. The other class includes Leukotriene B4 (LTB4), which is devoid of a thioether-linked peptide.[1] B leukotriene receptor 1 and 2 (BLT1, BLT2) are two-specific receptors for LTB4. 5-Lipoxygenase (5-LO) initiates the synthesis of leukotrienes from arachidonic acid when cells are activated. Leukotriene A4 (LTA4) is further converted into LTB4 by LTA4 hydrolase (LTA4H)[2] or is conjugated with glutathione by LTC4 synthase to form cysteinyl leukotriene.[3]

Inflammatory bowel disease (IBD) is characterized by chronic and spontaneously relapsing inflammation of the intestine. Ulcerative colitis and Crohn's disease are the two primary forms of IBD.[4] 2,4,6-Trinitrobenzene sulfonic acid (TNBS)-induced colitis is a well-established model of intestinal inflammation that expresses important histological and biochemical features of human IBD.[5,6] Highly activated T cells, especially Th1 and Th17, exhibit key pathogenic roles in TNBS-induced colitis.[7-9]

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in peripheral lymphoid tissue that innate and adaptive immunity. DC-derived costimulatory molecules and cytokines drive T-cell activation and differentiation. DC-derived IL-6 and TNF-α induce the differentiation of naïve $CD4^+$ T cells into Th17 cells, and IL-12 promotes differentiation of naïve $CD4^+$ T cells into Th1 cells.[10-13] Previous studies showed that DC-derived IL-23 enhanced Th17 expansion contributes to the process of enteritis in murine models of colitis.[14-16] Therefore, DC-derived cytokine production and subsequently induction of $CD4^+$ T-cell differentiation are important process during the pathogenesis of colitis.

Previous studies demonstrated that leukotrienes are increased in the colonic mucosa in IBD patients.[17] Animal studies further demonstrated that reduced leukotrienes production alleviates the degree of infiltrating inflammatory cells and colonic injury in 5-LO knockout mice,[18] indicating that leukotriene signaling plays a major role in IBD. To understand the mechanism of leukotriene signaling in colitis, two types of receptors for leukotrienes, cysteinyl leukotriene receptor, and BLT receptor, need to be studied in the pathogenesis of colitis. Cysteinyl leukotriene

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receptor antagonist montelukast exhibits limited potential to ameliorate acute TNBS-induced colitis.[19] BLT2 is expressed in colon cryptic cells and protects against dextran sodium sulfate (DSS)-induced colitis. However, BLT1$^{-/-}$ mice exhibited a similar severity of DSS-induced colitis compared with wild-type (WT) mice.[20] DSS-induced colitis is a model to study the innate immune mechanisms involved in the development of IBD. Innate cells, such as neutrophils and macrophages, play critical roles in DSS-induced colitis, and mucosal inflammation develops in the absence of T cells mediating adaptive immunity, such as that noted in SCID and Rag2$^{-/-}$ mice.[21-23] These reports indicated that BLT1 has no obvious effect on innate immunity, so we utilized a TNBS-induced colitis model to further explore the role of BLT1 in adaptive immunity in colitis.

Using BLT1 knockout mice, we revealed that BLT1 plays a critical role in controlling Th1 and Th17 differentiation by regulating DC-derived proinflammatory cytokines production. BLT1$^{-/-}$ mice exhibited alleviated symptoms of TNBS-induced colitis with less body weight loss and infiltrating cells in lamina propria. We also demonstrated that BLT1 regulates proinflammatory cytokine production by DCs through the Gαi βγ subunit-phospholipase Cβ (PLCβ)-protein kinase C (PKC) pathway.

MATERIALS AND METHODS

Mice

BLT1$^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratory. CD11c-DTR mice expressing the diphtheria toxin (DTx) receptor under the *Cd11c* promoter were obtained from Xuetao Cao, The Second Military Medical University. Green Fluorescent Protein (GFP) mice were provided by the animal center of Tongji University. GFP OT-II mice were generated by breeding GFP mice with OT-II mice. All mice were housed in pathogen-free conditions in Tongji University and provided standard laboratory chow and water ad libitum. All experiments were performed with approval of the Animal Care Committee of Tongji University.

Reagents

The BLT1 agonist LTB4 and antagonist U75302 were purchased from Cayman Chemical Company. The PLCβ inhibitor U73122 and PKC inhibitor Ro31-8220 were purchased from ApexBio Technology, USA. Lipopolysaccharides from *Escherichia coli* O55:B5 (LPS 055:B5) were purchased from Sigma. MOG_{35-55} was purchased from GL Biochem (Shanghai, China) with >95% purity. Complete Freund's adjuvant was purchased from Sigma-Aldrich. DTx and the $OVA_{323-339}$ peptide were purchased from Sigma. Lymphoprep™ was purchased from Stemcell. 2,4,6-TNBS was obtained from Sigma.

Splenic DC isolation and stimulation

Splenic DCs were isolated from WT and BLT1$^{-/-}$ spleens using a MagniSort® Mouse CD11c Positive Selection Kit (8802-6861-74, eBioscience). Then, DCs were stimulated with lipopolysaccharide (LPS) (100 ng/ml) and BLT1-related regents for 24 h. The protein levels of cytokines in supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Cells were collected, and surface markers were stained with the following relevant Abs: CD11c, CD80, and CD86 (all were purchased from Biolegend).

Bone marrow-derived dendritic cell (BMDC) isolation, culture, and stimulation

BMDCs were isolated from the femur and tibia of WT mice and BLT1$^{-/-}$ mice and subsequently cultured in RPMI 1640 containing 10% FBS, 2-ME (50 μM), glutamine (2 mM), murine GM-CSF (25 ng/ml), and murine IL-4 (1 ng/ml) for 7 days and stimulated by LPS (100 ng/ml) and BLT1-related regents for 24 h. The cytokines in supernatants were measured by ELISA.

T-cell separation and differentiation

Naïve CD4^{+} T cell were separated by magnetic cell separation (Invitrogen) from the spleen of 6–8-week-old WT and BLT1$^{-/-}$ mice and then stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml). For Th1 differentiation, IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) were added in the culture media. For Th17 differentiation, anti-IL-4 (10 μg/ml), anti-IFN-γ (10 μg/ml), IL-6 (30 ng/ml), TNF-α (10 ng/ml), TGF-β1 (3 ng/ml), and IL-1β (10 ng/ml) were added to the culture media.

Reverse transcription and real-time PCR

Total RNA was extracted from colon samples of WT and BLT1$^{-/-}$ mice 4 days after TNBS was administered intrarectally using TriPure Isolation Reagent (Roche) and then reverse transcribed into cDNA with M-MLV Reverse Transcriptase (Promega) and random hexamer primers. Gene expression was analyzed by real-time PCR performed with Biotool™ 2× SYBR Green qPCR Master Mix. Gene expression was normalized to β-actin.

Flow cytometry staining

Cells were stimulated for 5 h at 37 °C with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (750 ng/ml; Sigma-Aldrich), and brefeldin A (1.0 mg/ml; Sigma-Aldrich). Then, cells were stained for surface markers. After completing surface staining, cells were fixed in Fixation/Permeabilization solution (Cytofix/Cytoperm kit; BD Pharmingen), and intracellular cytokine staining was performed according to the manufacturer's protocol.

Induction of IBD

The acute colitis model was induced by TNBS as described by a previous protocol.[7] Eight- to ten-week-old mice were immunized with (150 μl) TNBS pre-sensitization solution dissolved in acetone and olive oil in a 4:1 volume ratio by vortexing rigorously. Briefly, 4 volumes of acetone/olive oil was mixed with 1 volume of 5% (wt/vol) TNBS (p2297, Sigma) solution to obtain 1% (wt/vol) TNBS. After 7 days, 100 μl of 2.5% (wt/vol) TNBS (diluted in 50% ethanol) was administered intrarectally through a catheter inserted into the colon 4 cm proximal to the anus. The weight was monitored daily. Cytokine expression and colon histology were examined until day 4 after TNBS treatment. Whole blood was collected from the orbit, and serum was collected for ELISA. BMDC transfer experiments were performed as described in a previous report.[24] CD11c-DTR mice first undergo DC depletion when 100 ng DTx is administered to recipient mice. Six hours later, BLT1$^{-/-}$ BMDCs and WT BMDCs were transferred to recipient mice, respectively. Three days later, colitis was induced by TNBS in these recipient mice.

Colon organ culture

A segment of the colon was excised and washed twice in clean phosphate-buffered saline containing penicillin and streptomycin. Then, the colon was further cut into 1-cm^2 pieces and placed in 24-well flat bottom-well culture plates with 1 ml RPMI 1640 supplemented with penicillin and streptomycin at 37 °C for 24 h. The supernatant was collected, and cellular debris was removed by centrifugation. Cytokine levels in the supernatant were quantified by ELISA.

Cell adoptive transfer

Naïve GFP OT-II T cells (2×10^6) were injected into the lateral tail vein of C57BL/6 mice. One day later, 2×10^5 splenic DCs isolated from BLT1$^{-/-}$ and WT mice were pulsed with 50 μg/ml OVA and 500 ng/ml LPS for 8 h, extensively washed, and injected into recipient mice. Seven days later, these mice were killed to analyze the Th1 and Th17 cell proportion in the spleen.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (GraphPad Software). Data are presented as the means ± SEM. Student's *t*-test

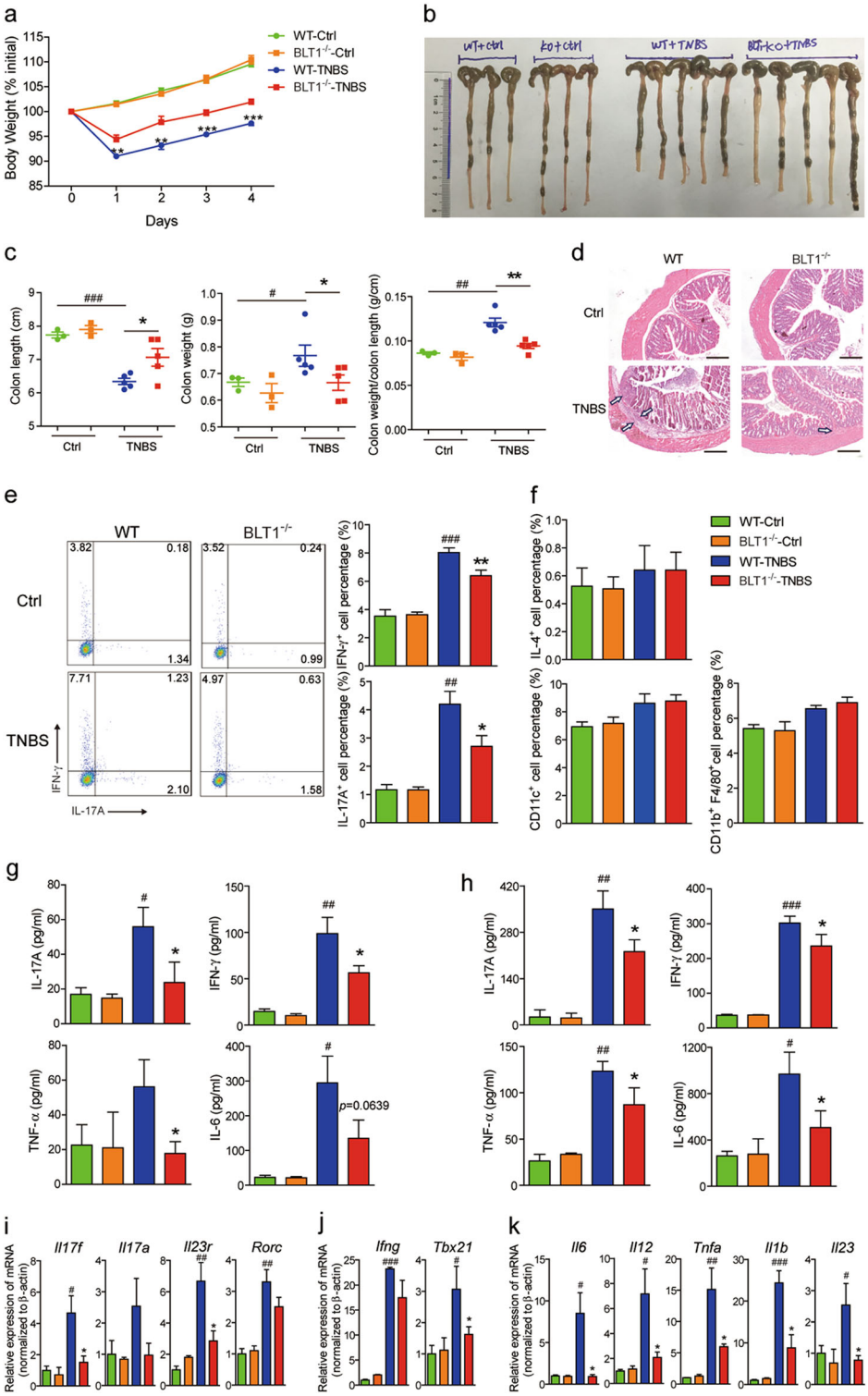

Fig. 1 BLT1 deficiency alleviates clinical symptoms of colitis. **a**–**k** WT and BLT1$^{-/-}$ mice were induced with TNBS ($n = 5$ mice per group) to develop colitis. Control mice ($n = 3$ per group) received ethanol only. Mouse body weight was measured daily (**a**). At day 4, all the mice were killed, and colon images were obtained (**b**). In addition, colon length, colon weight, and colon weight/colon length (**c**) were measured. Representative images from H&E staining of colon samples. Scale bars, 200 μm (**d**). Splenocytes from the WT-Ctrl, BLT1$^{-/-}$-Ctrl, WT-TNBS, and BLT1$^{-/-}$-TNBS group were separated and analyzed by FACS. The percentages of Th1 (IFN-γ$^+$ cells in CD4$^+$ T cells gate) and Th17 (IL-17A$^+$ cells in CD4$^+$ T cells gate) cells were analyzed (**e**), and Th2 (IL-4$^+$ cells in CD4$^+$ T cells gate), DCs (CD11c$^+$ cells), and macrophages (CD11b$^+$ F4/80$^+$ cells) were also detected (**f**). Serum was collected, and IL-17A, IFN-γ, IL-6 and TNF-α production were detected by ELISA (**g**). Colons from control and TNBS group of WT and BLT1$^{-/-}$ mice were dissected on day 4. The colon was thoroughly washed, cut into 1-cm pieces and cultured overnight for cytokine production assay. IL-17A, IFN-γ, IL-6, and TNF-α were detected in supernatants of organ culture (**h**). QPCR analysis of the expression of major genes related to Th17, Th1 and DCs in colons. The results were normalized to β-actin expression in the same sample and then normalized to the WT-Ctrl group (**i**–**k**). Data are presented as the mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT-TNBS group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus WT-Ctrl group (one-tailed Student's *t*-test). Data from WT-Ctrl and BLT1$^{-/-}$-Ctrl are derived from one experiment. Data from WT-TNBS and BLT1$^{-/-}$-TNBS are representative of three independent experiments with similar results

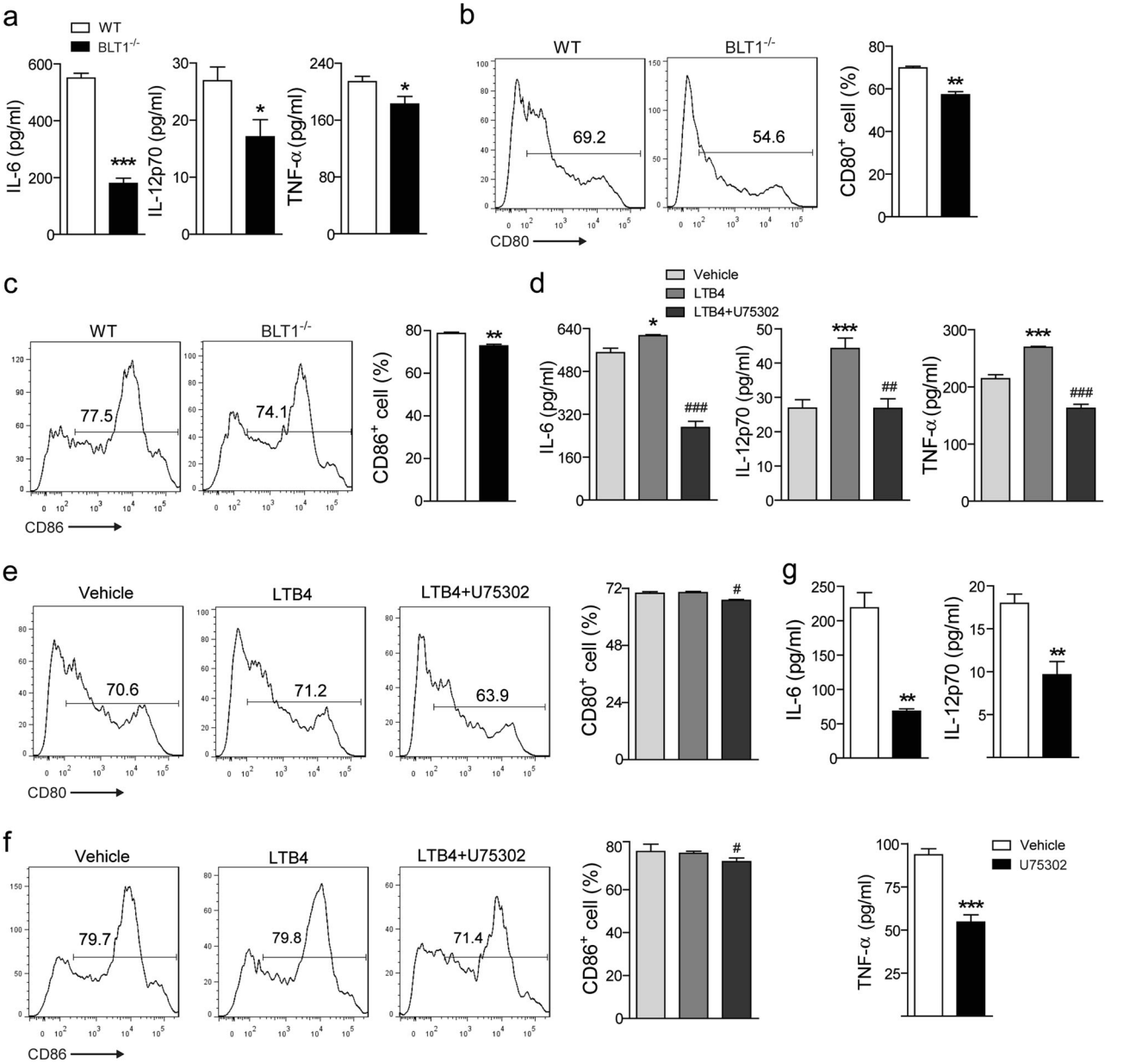

Fig. 2 BLT1 regulates proinflammatory cytokine production by DCs in vitro and in vivo. **a**–**c** WT and BLT1$^{-/-}$ splenic DCs were isolated and stimulated by LPS (100 ng/ml). IL-6, IL-12p70, and TNF-α production in supernatants was detected by ELISA (**a**). Cells were collected for surface staining of CD80 and CD86 with antibodies (**b**, **c**). **d**–**f** WT splenic DCs were isolated and stimulated by LPS alone or in the presence of the BLT1 agonist LTB4 (300 nM) or the combination of LTB4 (300 nM) and the antagonist U75302 (5 μM) for 24 h. IL-6, IL-12p70, and TNF-α levels in supernatants were detected by ELISA (**d**). Cells were collected for surface staining of CD80 and CD86 with antibodies (**e**, **f**). **g** Splenic DCs separated from TNBS-induced WT mice were incubated with or without U75302 (5 μM) ($n = 3$), and the production of IL-6, TNF-α, and IL-12p70 in supernatants was analyzed by ELISA. Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus WT + LTB4 group (Student's *t*-test). Data are representative of three independent experiments with similar results

was applied for comparison of means to identify differences between groups. *P*-values < 0.05 were considered statistically significant.

RESULTS

Deficiency of BLT1 alleviates TNBS-induced colitis

We first investigated the effect of BLT1 in the pathogenesis of colitis. We employed an inflammatory disease of colitis induced by 2,4,6-TNBS, the pathogenic progression of which is largely determined by Th1 and Th17 in vivo.[25–27] Mice in the control group exhibited continuous body weight gain compared with the TNBS-induced group. BLT1$^{-/-}$ mice exhibited alleviated symptoms of colitis induced by TNBS compared with WT mice characterized by reductions in body weight loss (Fig. 1a), colon shortening and enlargement, and weight per unit length of intestine (Fig. 1b, c). H&E staining of colon sections revealed reduced infiltrating cells in the lamina propria in BLT1$^{-/-}$ mice (Fig. 1d). To investigate the immune response involved in the pathogenesis of TNBS-induced colitis in WT and BLT1$^{-/-}$ mice, the percentages of immune cells in spleen, including Th1, Th2, Th17, macrophage, and DCs, were analyzed by flow cytometry. We found that TNBS-induced colitis induces an inflammatory

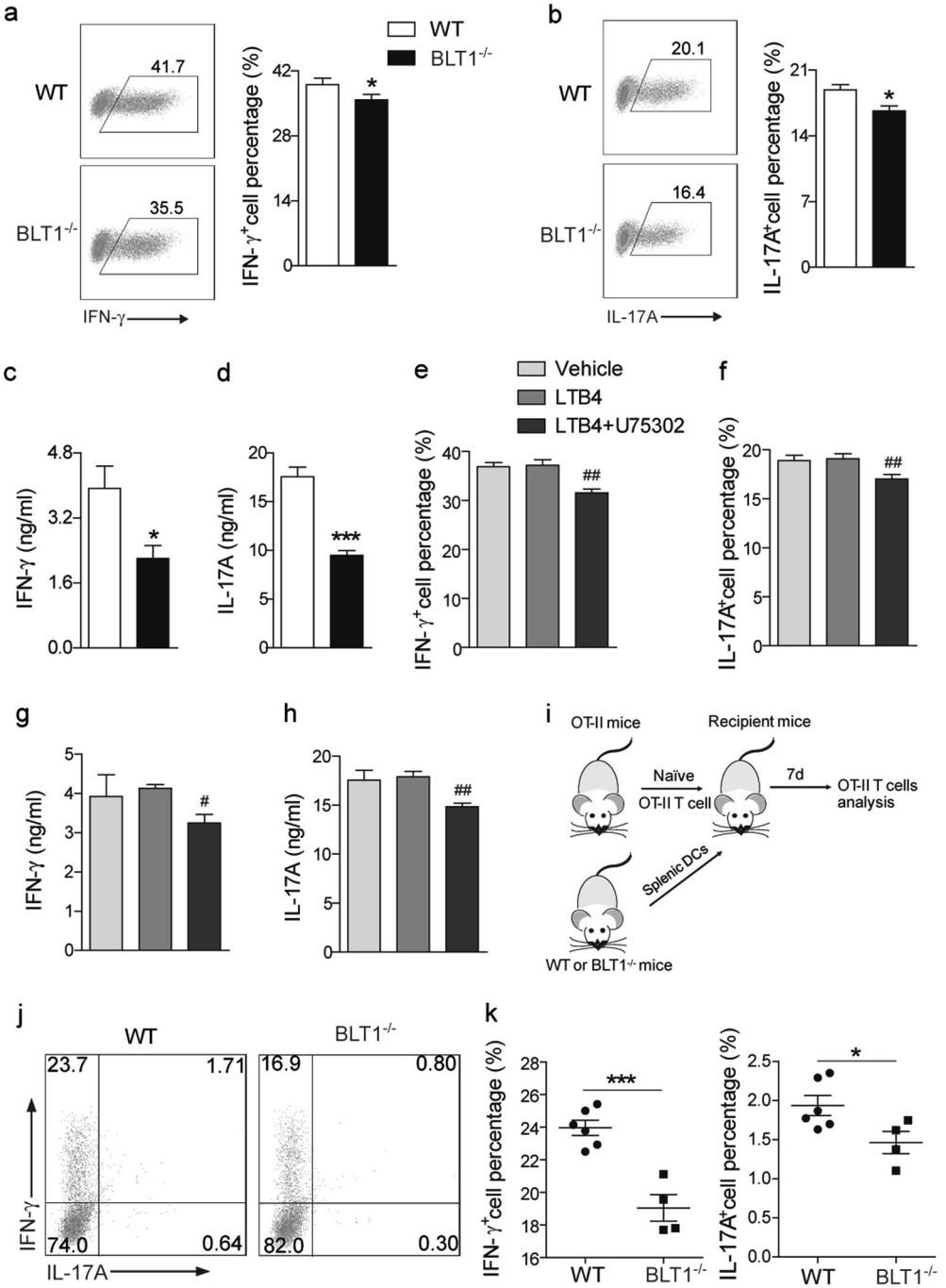

Fig. 3 BLT1 in DCs is required for inducing Th1 and Th17 differentiation in vitro and in vivo. **a**–**d** Th1 and Th17 differentiation in the in vitro DC–T-cell coculture system was monitored by FACS analysis. WT and BLT1$^{-/-}$ BMDCs were stimulated by LPS (100 ng/ml) for 24 h, washed, and then cocultured with naïve CD4^{+} T cells in presence of anti-IL4 (Th1 differentiation) or anti-IL4 and anti-IFN-γ (Th17 differentiation). The representative FACS and statistical data are presented (**a**, **b**). IFN-γ and IL-17A levels in supernatants were detected by ELISA (**c**, **d**). **e**–**h** Th1 and Th17 differentiation was analyzed by FACS in the in vitro DC–T-cell coculture system. WT BMDCs were stimulated by LPS alone or in the presence of BLT1 agonist LTB4 (300 nM) or the combination of LTB4 (300 nM) and antagonist U75302 (5 μM) for 24 h, washed, and then cocultured with naïve CD4^{+} T cells in the presence of anti-IL4 (Th1 differentiation) or anti-IL4 and anti-IFN-γ (Th17 differentiation). Statistical data are presented (**e**, **f**). IFN-γ and IL-17A levels in supernatants were detected by ELISA (**g**, **h**). **i**–**k** WT and BLT1$^{-/-}$ splenic DCs (2×10^5) were stimulated by OVA (50 μg/ml) and LPS (500 ng/ml) for 8 h, extensively washed, and then transfer into C57BL/6 recipient mice that received naïve GFP OT-II CD4^{+} T cells (2×10^6) from GFP OT-II mice. After 7 days, GFP OT-II CD4^{+} T-cell differentiation was analyzed in vivo (**i**). OT-II Th1 and Th17 cells in spleen of recipient mice in the GFP gate were analyzed by intracellular staining of IFN-γ and IL-17A, respectively. The representative FACS (**j**) and the statistical data (**k**) are presented. Data are representative of three independent experiments (mean ± SEM; $n =$ 4–6). *$p < 0.05$, ***$p < 0.001$ versus WT group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus WT + LTB4 group. *P*-values were determined using Student's *t*-test

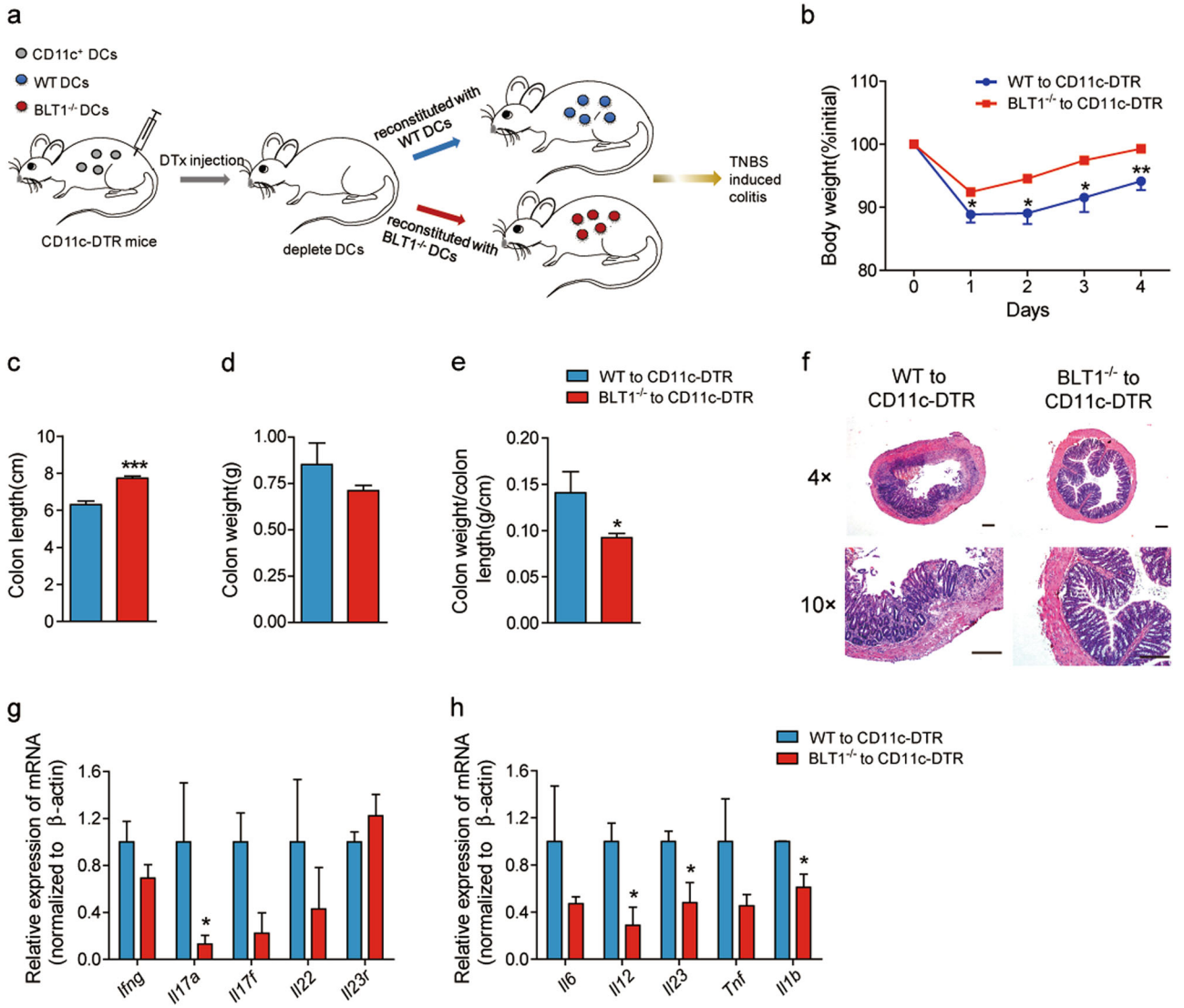

Fig. 4 BLT1 acts on DCs to mediate the pathogenesis of colitis. **a**–**h** CD11c-DTR mice are subject to DC depletion upon the administration of 100 ng diphtheria toxin (DTx) to serve as recipient mice. Six hours later, WT DCs and BLT1$^{-/-}$ DCs were transferred to recipient mice, separately. Three days later, colitis was induced by TNBS in these recipient mice ($n = 5$ mice per group) (**a**). Body weight loss was measured daily in the mice (**b**). The mice were killed, and the colon length (**c**), colon weight (**d**), and colon weight/colon length (**e**) were measured after 4 days of TNBS-induced colitis. Representative images from H&E staining of colon samples. Scale bars, 200 μm (**f**). QPCR analysis of the expression of Th1-, Th17- and DC-related gene expression in colons (**g**, **h**). Data are mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus WT to CD11c-DTR group (one-tailed Student's *t*-test). Data are representative of two independent experiments with similar results

response with increased percentages of Th1, Th17, macrophage, and DCs in spleen. Compared with WT-TNBS group, the BLT1$^{-/-}$-TNBS group exhibited a significantly reduced percentage of Th1 and Th17 cells in spleen, whereas no significant difference was noted in the percentage of macrophage and DC cells, indicating an impaired Th1 and Th17 response in BLT1$^{-/-}$ mice with TNBS-induced colitis (Fig. 1e, f). IL-17A, IFN-γ, TNF-α, and IL-6 levels decreased significantly in serum from BLT1$^{-/-}$ mice compared with WT mice in the TNBS-induced group (Fig. 1g). We further detected these cytokines in colon organ culture supernatants, and the production of IL-17A, IFN-γ, TNF-α, and IL-6 was impaired in BLT1$^{-/-}$ mice (Fig. 1h). Moreover, we also observed reduced upregulation of Th17- and Th1-related gene expression, including *Il17a*, *Il17f*, *Il23r*, *Rorc*, *Ifng*, and *Tbx21*, in colon from BLT1$^{-/-}$ mice with TNBS-induced colitis (Fig. 1i, j). The expression of proinflammatory cytokines, including *Il6, Il12a, Il23a, Tnfa*, and *Il1b* that are associated with DCs, was reduced in the colon of BLT1$^{-/-}$ mice[28] (Fig. 1k). To analyze whether BLT1 has a direct effect on T-cell differentiation, in vitro Th1 and Th17 differentiation was induced under different polarizing conditions as previously described.[29] Flow cytometric analysis revealed that BLT1 receptor agonist LTB4 treatment did not affect Th1 and Th17 differentiation either alone or combination with the antagonist U75302. BLT1-deficient T cells also exhibited similar results, suggesting that BLT1 had no obvious in vitro effect on Th1 and Th17 differentiation (Supplementary Figures 1 a, b, d and e). IFN-γ and IL-17A protein levels in supernatants also exhibited no significant changes when treated with LTB4 alone or in combination with U75302 both in WT and BLT1$^{-/-}$ T cells (Supplementary Figures 1c and f). Taken together, our data suggested that BLT1 regulates the development of colitis but not through affecting CD4^{+} T cells differentiation directly.

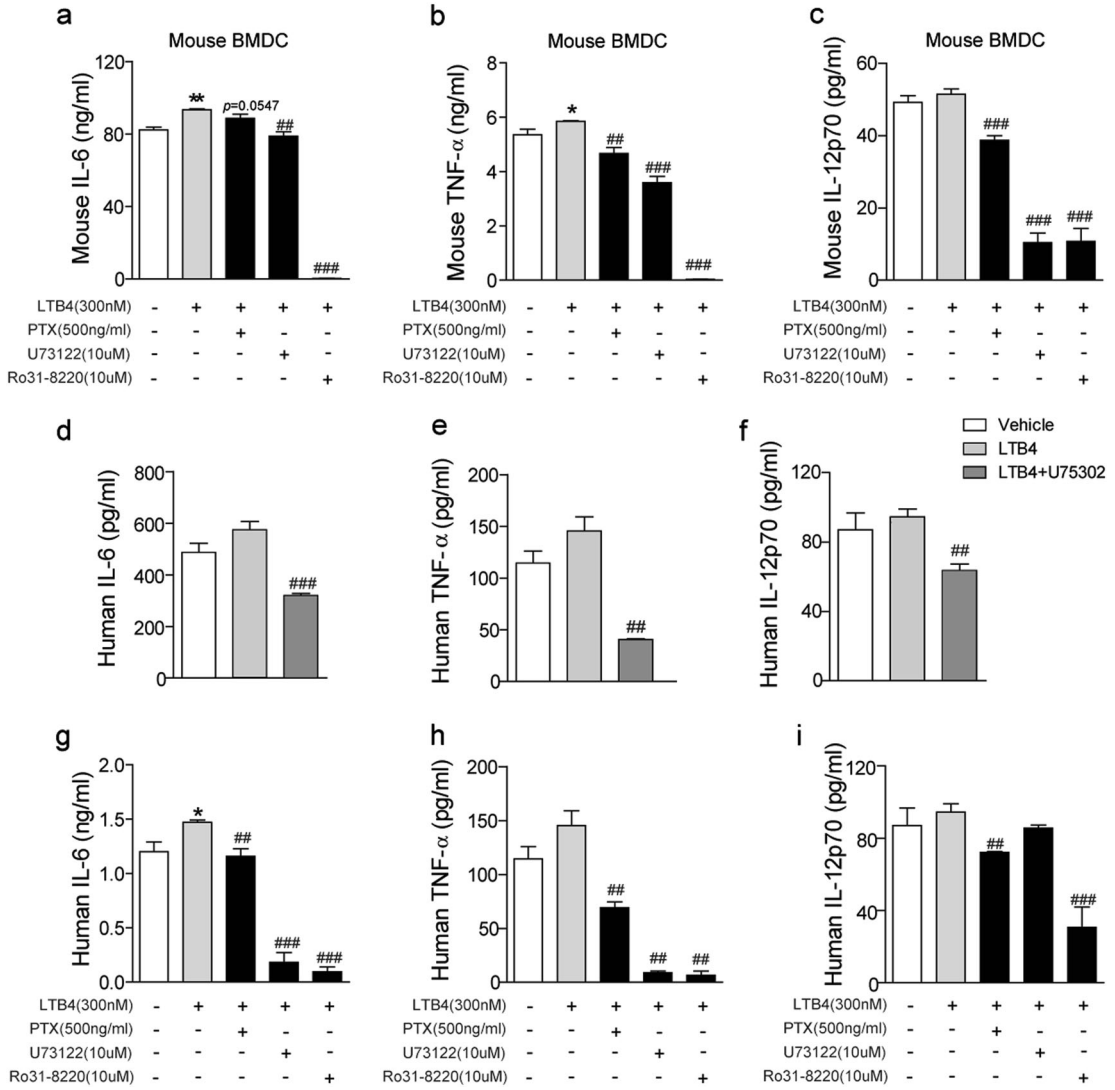

Fig. 5 The downstream pathways involved in BLT1 regulate mouse and human proinflammatory cytokine production by DCs. **a**–**c** WT and BLT1$^{-/-}$ BMDCs were prepared by culturing bone marrow cells in RPMI 1640 with GM-CSF and IL-4 for 7 days followed by stimulation with LPS (100 ng/ml) alone or in the presence of Gαi-sensitized PTX, the PLC inhibitor U73122, and the PKC inhibitor Ro31-8220 combined with the BLT1 agonist LTB4 for 24 h. IL-6 (**a**), TNF-α (**b**), and IL-12 (**c**) levels in supernatants were detected by ELISA. **d**–**f** Human CD11c^{+} DCs were isolated from peripheral blood mononuclear cells and then stimulated by LPS (100 ng/ml) alone or in the presence of the BLT1 agonist LTB4 or the antagonist U75302 for 24 h. Proinflammatory cytokine IL-6 (**d**), TNF-α (**e**), and IL-12 (**f**) levels in supernatants were detected by ELISA. **g**–**i** Human CD11c^{+} DCs stimulated by LPS alone or in the presence of various pathway inhibitors combined with the BLT1 agonist LTB4 for 24 h. The effects of Gαi-sensitized PTX, the PLC inhibitor U73122, and the PKC inhibitor Ro31-8220 on IL-6 (**g**), TNF-α (**h**), and IL-12 (**i**) by human DCs. Data are representative of three independent experiments (mean ± SEM; $n = 4$–6). $^{*}p < 0.05$, $^{**}p < 0.01$ versus DCs treated with LPS alone; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus DCs treated LPS combined with LTB4 (one-tailed Student's *t*-test)

BLT1 regulates DC-derived proinflammatory cytokine production in vitro and in vivo

To explore whether BLT1 acts on DCs in the pathogenesis of colitis, we tested the role of BLT1 in regulating DCs activation and proinflammatory cytokine production. Splenic DCs were isolated from WT and BLT1$^{-/-}$ mice and activated with LPS. BLT1$^{-/-}$ DCs exhibited decreased IL-6, IL-12p70, and TNF-α production compared with WT DCs (Fig. 2a). Further, we analyzed the effect of BLT1 on DCs maturation. The results indicated that BLT1$^{-/-}$ DCs exhibited reduced CD80 and CD86 expression compared with WT DCs (Fig. 2b, c). The BLT1 agonist LTB4 and antagonist U75302 were also used to validate the effect of BLT1 on DCs. WT splenic DCs were treated with LPS alone or LTB4 alone or in combination with U75302. Pre-treatment with the BLT1 agonist LTB4 significantly enhanced the production of IL-6, IL-12p70, and TNF-α by DCs, and these enhancements were abolished when treated with the BLT1 antagonist U75302 (Fig. 2d). Pre-treatment with the BLT1 antagonist U75302 reduced CD80 and CD86 expression (Fig. 2e, f). To explore whether BLT1 is responsible for proinflammatory cytokine production in vivo, we evaluated proinflammatory cytokine production in the serum of WT and BLT1$^{-/-}$ mice after i.p. injection of LPS (10 mg/kg). Compared with WT mice, IL-6, TNF-α, and IL-12p70 serum levels were remarkably reduced in BLT1$^{-/-}$ mice at 3 h (Supplementary Figure 2a, b and c). To determine whether BLT1 signaling has an effect on DC cytokine production directly or acts on DC maturation, we separated WT DCs from the spleen of TNBS mice and detected cytokine production after treatment with the BLT1 antagonist U75302. BLT1 signaling blockade reduced IL-6, TNF-α, and IL-12p70 protein levels in supernatants of DCs, indicating that BLT1 signaling affects cytokine production directly (Fig. 2g). Collectively, our results demonstrated that BLT1 plays an

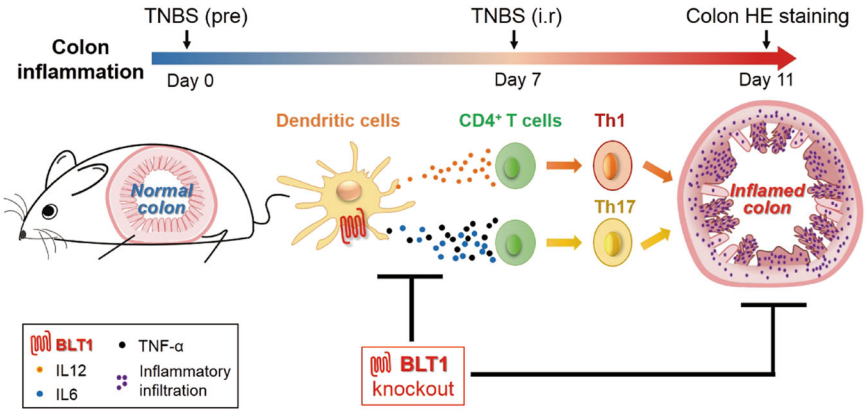

Fig. 6 The working model in which BLT1 in DCs promotes Th1 and Th17 differentiation and its deficiency ameliorates TNBS-induced colitis. BLT1$^{-/-}$ DCs exhibit impaired proinflammatory cytokine production and decreased ability to induce Th1 and Th17 differentiation. The impaired inflammatory response in adaptive immunity ameliorates clinical symptoms of TNBS-induced colitis in BLT1$^{-/-}$ mice

important role in DC-derived proinflammatory cytokine production in vitro and in vivo.

BLT1 in DCs is required for initiating Th1 and Th17 differentiation in vitro and in vivo

DCs control T-cell differentiation via secreting polarizing cytokines.[30] IL-6 and TNF-α production by DCs polarizes naïve T cells to Th17 differentiation. IL-12 secretion by DCs induces naïve CD4$^+$ T cells to undergo Th1 differentiation.[31-33] To further investigate the role of BLT1 in DCs in initiating effector CD4$^+$ T-cell differentiation, we employed an in vitro DC–T coculture system to examine whether BLT1 affects proinflammatory cytokine production by DCs to regulate the differentiation of CD4$^+$ T cells. WT and BLT1$^{-/-}$ BMDCs were stimulated by LPS, washed and then cocultured with naïve CD4$^+$ T cells for 4 days. We found that BLT1 deficiency in DCs led to a reductioninTh1 and Th17 differentiation (Fig. 3a, b). IFN-γ and IL-17A protein levels decreased significantly in supernatants of the BLT1$^{-/-}$ group (Fig. 3c, d). Then, we applied the agonist LTB4 and BLT1 antagonist U75302 to further validate that BLT1 in DCs could affect Th1 and Th17 differentiation. DCs treated with U75302 reduced Th1 and Th17 differentiation in cocultured naïve CD4$^+$ T cells (Fig. 3e, f). IFN-γ and IL-17A were also reduced in supernatants in the U75302 group (Fig. 3g, h).

To further determine whether BLT1 in DCs is required to induce effector CD4$^+$ T-cell differentiation in vivo, splenic DCs obtained from WT and BLT1$^{-/-}$ mice were primed with LPS and OVA, washed, and then injected into two groups of recipient mice that previously received naïve GFP OT-II T cells. Then, Th1 and Th17 differentiation was analyzed (Fig. 3i). Mice that received BLT1$^{-/-}$ DCs exhibited decreased Th1 and Th17 differentiation of GFP OT-II T cells in spleen compared with the group transferred WT DCs. The Th1 percentage decreased by approximately 25%, and the Th17 percentage decreased by approximately 21% in mice that received BLT1$^{-/-}$ splenic DCs compared with WT control (Fig. 3j, k). Therefore, these data demonstrated that BLT1 in DCs is required for initiating Th1 and Th17 differentiation both in vitro and in vivo via regulating proinflammatory cytokine production by DCs.

BLT1 in DCs is responsible for the pathogenesis of colitis

DCs play an important role in colitis by inducing a proinflammatory cytokine response.[34] To confirm that BLT1 acts on DCs to mediate the development of colitis, we utilized a BMDC transfer model in colitis that was previously established.[24] A conditional DC reconstitution experiment was implemented by transferring WT or BLT1$^{-/-}$ DCs to CD11c-DTR mice that were depleted of DCs after the administration of DTx, and colitis was induced by TNBS (Fig. 4a). In mice reconstituted with BLT1$^{-/-}$ DCs, the severity of colitis was reduced compared with those reconstituted with WT DCs. Specifically, reduced body weight loss (Fig. 4b), relieved colon shortening and enlargement, and decreased weight per unit length of intestine were observed (Fig. 4c–e). H&E staining of colon sections further revealed that reconstitution of BLT1$^{-/-}$ DCs reduced mononuclear cell infiltration and goblet cell destruction in colon (Fig. 4f). The expression of the Th1-related gene *Ifng* and Th17-related genes *Il17a* and *Il17f* was downregulated in the BLT1$^{-/-}$ DCs group (Fig. 4g). The expression of proinflammatory related to DCs, including *Il6*, *Il12a*, *Il23a*, *Tnfa*, and *Il1b*, was also decreased in the BLT1$^{-/-}$ DCs group (Fig. 4h). Thus, our results suggested BLT1 regulates the development of colitis by acting on DCs.

BLT1 regulates mouse and human DC-derived inflammatory cytokine production through the Gαi βγ subunit-PLCβ-PKC pathway

As a typical G-protein coupled receptor, BLT1 is involved in various signaling pathways, including Gαi-cAMP-kinase A, the Gαi βγ subunit-PLCβ-PKC and Gαq-PLCβ-PKC. To investigate the pathways involved in BLT1-mediated proinflammatory cytokine production by DCs, we applied Gαi-sensitized pertussis toxin (PTX), the PLC inhibitor U73122, and the PKC inhibitor Ro31-8220 combined with the BLT1 agonist LTB4 during the activation of DCs induced by LPS. The results demonstrated that IL-6, TNF-α, and IL-12p70 were decreased upon treatment with PTX, suggesting that Gαi is involved in BLT1-regulated proinflammatory cytokine production by DCs. Treatment with the PLC inhibitor U73122 and PKC inhibitor Ro31-8220 significantly reduced IL-6 and TNF-α production by DCs, indicating that PLCβ and PKC were also associated with BLT1 by regulating DC-derived proinflammatory cytokine production (Fig. 5a–c). To further investigate the role of BLT1 in human DCs, we isolated DCs from human peripheral blood. Remarkably, we observed that treatment with the BLT1 agonist LTB4 enhances the production of IL-6, TNF-α, and IL-12p70, whereas BLT1 antagonist U75302 treatment significantly reduced the production of IL-6, TNF-α, and IL-12p70 (Fig. 5d–f). We next examined the downstream pathways that were associated with BLT1 in human DCs. Consistent with the results from mouse DCs, treatment with PTX, U73122, and Ro31-8220 significantly reduced the production of proinflammatory cytokines by human DCs (Fig. 5g–i). Together, these data indicated that BLT1 regulates mouse and human DC-derived inflammatory cytokine production through the Gαi βγ subunit-PLCβ-PKC pathway.

In summary, we describe herein that BLT1 in DCs promotes Th1 and Th17 differentiation by regulating proinflammatory cytokines production, which contributes to TNBS-induced colitis. BLT1$^{-/-}$ mice exhibited a reduction in colitis severity with less body weight loss and infiltrating cells compared with WT mice (Fig. 6). These data suggested that BLT1 is a potential therapeutic drug target for intervention in DC-mediated diseases.

DISCUSSION

BLT1 is involved in regulating the immune response in various animal experimental models.[35] BLT1 promotes monocyte infiltration into adipose tissue and chronic inflammation in obesity. BLT1$^{-/-}$ mice exhibited reduced inflammation in liver and adipose tissue and reduced hepatic triglyceride accumulation.[36] Selective inhibition of BLT1 with LSN2792613 reduced inflammation and infarct size in a mouse model of myocardial ischemia-reperfusion injury.[37] BLT1$^{-/-}$ mice exhibited delayed onset and less severe symptoms of experimental autoimmune encephalomyelitis compared with WT mice.[38] Consistent with these reports, we found that BLT1 deficiency alleviated the development of TNBS, the pathogenic progression of which is largely determined by Th1 and Th17 cells. These data support a proinflammatory role of BLT1 in mediating the pathogenesis of disease.

BLT1 is expressed on a variety of immune cells, including DCs and effector CD4$^+$ T-cell lineages Th1 and Th17, which participate in mediating the migration of various types of leucocytes.[39,40] BLT1 in DCs can regulate the Th2 response and allergen-induced airway hyperresponsiveness. BLT1 deficiency decreased IL-13 production in bronchoalveolar lavage fluid during allergen-induced airway hyper-responsiveness.[41] LTB4-BLT1 signaling participates in DCs motility in a contact hypersensitivity model. Blocking BLT1 signaling attenuated cutaneous acquired immune responses by inhibiting cutaneous DCs motility.[42] Deficiency of BLT1 in BMDCs led to the reduced production of IL-12p70 and IFN-γ in an allogeneic mixed lymphocyte reaction.[43] DCs are professional APCs. The cytokines produced by DCs control Th1 and Th17 differentiation. However, the role of BLT1 in regulating DCs maturation and proinflammatory cytokine production is unclear. In addition, studies assessing the role of BLT1 in DCs controlling Th1 polarization are quite limited, and the role of BLT1 in DCs involved in Th17 differentiation remains unknown. In this work, BLT1$^{-/-}$ DCs exhibited reduced maturity with reduced expression of costimulatory molecules CD80 and CD86 compared with WT DCs. Our results are consistent with previous studies. Upon treatment with BLT1 antagonist, TLR2, and NF-κB activation were reduced.[44,45] These signaling pathways are essential for DCs maturation.[46] In addition, we comprehensively elucidated the role of BLT1 in regulating splenic DC function, which is responsible for Th1 and Th17 differentiation in vitro and in vivo. We found that BLT1-regulated proinflammatory cytokines, including IL-6, TNF-α, and IL-12, produced by DCs. In vitro DC–T-coculture experiments further verified that BLT1 deficiency in DCs led to decreased Th1 and Th17 differentiation due to the reduction in proinflammatory cytokine production by BLT1$^{-/-}$ DCs. To further assess whether BLT1 acts on DCs to mediate the pathogenesis of colitis, we applied a conditional DC reconstitution experiment to confirm that BLT1 in DCs contributes to inflammatory autoimmune disease of colitis induced by TNBS, the pathogenesis of which is thought to be related to Th1 and Th17.[47,48] We demonstrated that transfer of BLT1$^{-/-}$ DCs led to reduced IL-6, TNF-α, and IL-12 production; subsequently reduced the expression of Th1- and Th17-related genes, including *Ifng*, *Il17a*, and *Il17f*; and alleviated the severity of TNBS.

Recent studies have determined several signaling pathways coupled with BLT1 receptors. LTB4 binds BLT1 to activate phosphatidylinositol 3-kinase (PI3K) through Gαi. In addition, inhibition of PI3K by wortmannin or LY290042-reduced degranulation of granulocytes.[49] BLT1 receptor activation resulted in IP3-mediated calcium release, which is sensitive to the activity of PKC.[50] According to these reports, we employed Gαi-sensitized PTX, the PLC inhibitor U73122 and the PKC inhibitor Ro31-8220 to determine which pathway is involved in BLT1 in DCs. Similarly, we found that the Gαi βγ subunit-PLCβ-PKC pathway is essential for BLT1-mediated DC activation.

In summary, we report that BLT1 signaling in DCs controls Th1 and Th17 differentiation and the pathogenesis of TNBS-induced colitis via regulating the production of proinflammatory cytokines, including IL-6, TNF-α and IL-12. Mechanistically, the Gαi βγ subunit-PLCβ-PKC pathway is crucial for BLT1-mediated DC-derived proinflammatory cytokine production by both human and mouse DCs. Our work might provide a new potential therapeutic targets for IBD.

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ADDITIONAL INFORMATION

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