



Tumor necrosis factor-like cytokine 1A plays a role in inflammatory bowel disease pathogenesis

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The binding of tumor necrosis factor-like cytokine 1A (TL1A) to death receptor 3 (DR3) plays an important role in the interaction between dendritic cells (DCs) and T cells and contributes to intestinal inflammation development. However, the mechanism by which DCs expressing TL1A mediate helper T (Th) cell differentiation in the intestinal lamina propria (LP) during the pathogenesis of inflammatory bowel disease remains unclear. In this study, we found that TL1A/DR3 promoted Th1 and Th17 cell differentiation in T-T and DC-T cell interaction-dependent manners. TL1A-deficient CD4⁺ T cells failed to polarize into Th1/Th17 cells and did not cause colonic inflammation in a T cell transfer colitis model. Notably, TL1A was located in the cytoplasm and nuclei of DCs, positively regulated the DC-specific ICAM-grabbing nonintegrin/RAF1/nuclear factor κB signaling pathway, enhanced the antigen uptake ability of DCs, and promoted TLR4-mediated DC activation, inducing naive CD4⁺ T cell differentiation into Th1 and Th17 cells. Our work reveals that TL1A plays a regulatory role in inflammatory bowel disease pathogenesis.

TL1A | inflammatory bowel disease | dendritic cell

TL1A (tumor necrosis factor-like cytokine 1A, TNFSF15) is a member of the TNF protein superfamily (TNFSF) that binds to death domain receptor 3 (DR3) and decoy receptor DcR3. TL1A exerts pleiotropic effects on cell proliferation, activation, differentiation, and cytokine production by subsets of effector T cells and is expressed on various cell types, including myeloid lineage-, activated T-, and endothelial cells (1). TL1A's expression and its two receptors are up-regulated in inflammatory diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and psoriasis, and TL1A/DR3 signaling facilitates disease progression (1).

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic and spontaneously relapsing intestinal inflammation. The amount of TL1A protein in biopsied LP from patients with UC and CD correlated with the severity IBD inflammation (2). Animal studies demonstrated that increased TL1A production exacerbates the degree of disruption of villi architecture, inflammatory cell infiltration of the LP, goblet cell hyperplasia, and thickening of the muscularis propria in TL1A-tg mice, indicating that TL1A/DR3 signaling plays a major role in IBD (1). Furthermore, transgenic mice with TL1A-overexpressing T lymphocytes display more severe intestinal inflammation and that TL1A may promote Th9 cell differentiation (3). However, the TL1A/DR3 axis alleviates inflammation in an acute colitis model, reflecting its complex involvement in IBD pathogenesis (4, 5).

IBD pathogenesis involves several cell types, including dendritic cells (DCs), which contribute to multiple aspects of intestinal inflammation (6). DCs are the most potent antigen-presenting cells (APCs). They can prime naive T cells and induce their differentiation into inflammatory (e.g., Th1 and Th17) (7). Mice with ileitis showed that TL1A was primarily expressed on CD11c^{high} DCs within the LP. The interaction between TL1A expressed on DCs and DR3 expressed on lymphocytes may play a significant role in the pathogenesis of IBD by promoting excess secretion of IFN-γ (8). Thus, cytokines produced by DCs and the subsequent induction of CD4⁺ T cell differentiation are critical to IBD pathogenesis. Furthermore, TL1A up-regulated chemokine receptors lead to enhanced function of bone marrow-derived dendritic cells (BMDCs), including T cell-dependent stimulation in TL1A tg BMDCs, exacerbating dextran sodium sulfate (DSS)-induced colitis (9). Co-IP and immunofluorescence assays unequivocally demonstrated that endogenous TL1A interacted with RAF1 directly in BMDCs. However, the mechanism by which TL1A regulates the DCs function to impact IBD remains unknown.

In this study, we examined the possible contribution of TL1A in Th1 and Th17 differentiation in T-T and DC-T cell interactions *in vivo* and *ex vivo* using multiple colitis mouse models.

Significance

We utilized various mouse models of IBD to demonstrate that TL1A expression on the surface of DCs is increased in inflamed intestines. Moreover, *Tl1a*^{-/-} naive CD4⁺ T cells exhibited impaired ability for Th1 or Th17 differentiation *ex vivo*, and we used *Tl1a*^{-/-}/*Rag1*^{-/-} and *Rag1*^{-/-} mice to explore how TL1A influences T cell differentiation *in vivo*. Our findings indicate that TL1A plays a crucial role in the interaction between T-T cells and APC-T cells and contributes to the development of intestinal inflammation by binding to DR3. Additionally, we observed that TL1A was located in the cytoplasm and nucleus of DCs, where it bound to RAF1 and positively regulated the RAF1/p65 signaling pathway, enhancing the antigen uptake ability of BMDCs *in vitro*.

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Results

TL1A is Highly Expressed in Inflamed Intestinal Tissues. First, we retrieved microarray data from the Gene Expression Omnibus database to analyze the differences in *TL1A* expression in patients with IBD. *TL1A* mRNA expression was up-regulated in inflamed CD and UC colon tissues compared with noninflamed and normal tissues (*SI Appendix*, Fig. S1A). We found similar results in a DSS colitis model in wild-type (WT) mice, in which the expression of *Tl1a* mRNA and TL1A protein was significantly enhanced on day 6 after DSS treatment compared with those in normal mice (*SI Appendix*, Fig. S1B and C). Then, we detected the dynamic changes in TL1A or DR3 expression in different cell subsets in the colon LP during intestinal inflammation development. Although TL1A was detected in activated CD4⁺ T cells, it was primarily expressed on myeloid dendritic cells (mDCs, CD11c⁺CD11b⁺B220⁺), macrophages (CD11b⁺F4/80⁺), neutrophils (CD11b⁺Ly6G⁺), and monocytes (CD11b⁺Ly6C⁺) (*SI Appendix*, Fig. S2A). Unlike TL1A, DR3 was primarily expressed on CD4⁺ effector T cells (CD44⁺CD4⁺) (*SI Appendix*, Fig. S2B). Moreover, TL1A and DR3 levels significantly increased with colitis progression. Therefore, high TL1A and DR3 expression in colon-infiltrating immune cells were involved in DSS-induced colitis pathogenesis.

TL1A Deficiency Alleviates DSS-Induced Colonic Inflammation. To directly evaluate the role of TL1A in colitis, we treated age-matched *Tl1a*^{-/-} or WT mice with 3% DSS to compare their acute colitis phenotype. Notably, intestinal inflammation in *Tl1a*^{-/-} mice was significantly alleviated after day 6 of DSS administration compared with WT mice, as characterized by lower body weight loss, lower disease activity index score, longer survival time, and longer colons length (Fig. 1 A–D). Consistently, histopathological analysis using hematoxylin and eosin staining

showed that TL1A deficiency decreased inflammatory cell infiltration with less mucosal epithelium disruption in response to DSS treatment (Fig. 1E). Moreover, endoscopy showed bleeding and ulcers in the colonic wall of WT mice; these symptoms were significantly alleviated in *Tl1a*^{-/-} mice (Fig. 1F). Similarly, *Tl1a* gene deletion also significantly improved the symptoms of DSS-induced chronic colitis in mice (*SI Appendix*, Fig. S3 A–E). Together, these results indicated that TL1A plays a proinflammatory role in IBD pathogenesis in mice.

To further characterize the role of TL1A in colitis as a positive regulator of intestinal inflammation, we analyzed cytokine expression in colon tissues of WT and *Tl1a*^{-/-} colitis mice using real-time quantitative PCR (RT-qPCR). Compared with WT mice, the relative mRNA expression levels of Th1-related (*Il12*, *Ifng*, and *Tnfa*) and Th17-related (*Il23*, *Il1β*, *Il6*, and *Il17a*) cytokines significantly decreased, whereas those of the Th2-related cytokine *Il4* increased in *Tl1a*^{-/-} colitis mice (*SI Appendix*, Fig. S4A). We also detected a significant decrease in the frequencies and absolute numbers of colon-infiltrating macrophages (CD45⁺CD11b⁺F4/80⁺Ly6G⁻) and monocytes (CD45⁺CD11b⁺Ly6C⁺Ly6G⁻) and a marked increase in neutrophils colonic infiltration (CD45⁺CD11b⁺F4/80⁺Ly6C^{low}Ly6G⁺) in *Tl1a*^{-/-} mice relative to those in WT mice on day 6 (*SI Appendix*, Fig. S4B). Notably, the number of mDCs (CD45⁺CD11c⁺CD11b⁺B220⁺) was markedly reduced, while plasmacytoid DCs (pDCs, CD45⁺CD11c⁺CD11b⁻B220⁺) increased in the inflamed colon of *Tl1a*^{-/-} mice compared with that in WT (Fig. 1G). Similar results were found in the chronic colitis model (*SI Appendix*, Fig. S3 F–H). Thus, TL1A deficiency suppresses colonic inflammatory responses and myeloid-derived monocyte and macrophage infiltration, especially mDCs, during colitis.

TL1A Deficiency Attenuates Th1/Th17 Cell Immune Responses in Colitis Mouse Intestines. To determine whether *Tl1a* gene deletion improves intestinal inflammation symptoms by

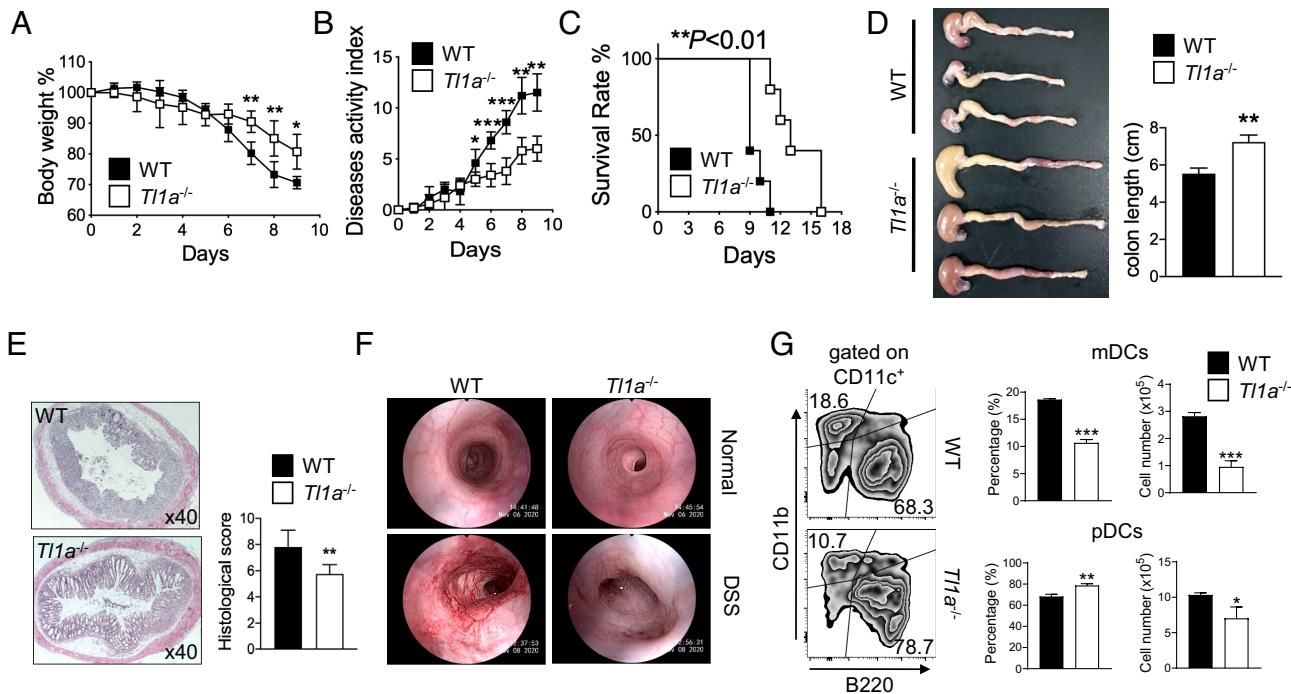


Fig. 1. Susceptibility of *Tl1a*^{-/-} mice to DSS-induced acute colitis. (A) Weight loss, (B) disease activity index, (C) Survival rates were daily observed, (D) Colon length, (E) histopathology severity scores, (F) Macroscopic changes of colon measured on day 6 after 3% DSS administration. (G) Flow cytometry analysis of mDCs and pDCs in LP of the large intestine from DSS-induced WT and *Tl1a*^{-/-} mice. The absolute number of each subset was calculated by multiplying the total number of LP cells by the percentage of each subset. Values of each column and vertical bar indicate means \pm SD for three to five mice within each group. Representative data are shown from three independent experiments. Statistically significant differences are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

inhibiting the differentiation of Th cells, we examined intracellular cytokines production by CD4⁺ Th cells in colonic LP after DSS treatment. The percentage and number of Th1 (IFN- γ ⁺CD4⁺), Th17 (IL-17A⁺CD4⁺), and Th1/Th17 (IL-17A⁺IFN- γ ⁺CD4⁺) subsets were significantly reduced in the acute inflamed colonic LP of *Tl1a*^{-/-} mice as compared with those in WT mice (Fig. 2A). In contrast, *Tl1a* deficiency resulted in a relative increase in Tregs (CD4⁺CD25⁺IL-10⁺) in colonic LP of mice after DSS-induced acute colitis (Fig. 2A). Furthermore, the frequencies and absolute numbers of CD4⁺TNF- α ⁺ and Th17-related cell subsets (IL-17F⁺CD4⁺ and IL-17F⁺IL-22⁺CD4⁺) were significantly decreased, while Th2 (IL-4⁺CD4⁺) and Th22 (IL-22⁺CD4⁺) cell subsets were significantly increased in colonic LP from *Tl1a*^{-/-} mice compared with those in

WT mice on day 6 (Fig. 2A). Similar results were found in the chronic colitis model (*SI Appendix*, Fig. S5A).

Next, we detected the expression of Th cell differentiation-related nuclear transcription factors in colonic LP during colitis. The percentage of T-bet and RORyt in CD4⁺ T-LPL were significantly reduced in *Tl1a*^{-/-} mice compared with those in WT mice with both DSS-induced acute and chronic colitis. Conversely, GATA3 or Foxp3 levels were markedly higher in CD4⁺ T-LPL of *Tl1a*^{-/-} mice than in WT in both DSS colitis models (Fig. 2B and *SI Appendix*, Fig. S5B). We compared cytokine levels produced by activated T-LPL in *Tl1a*^{-/-} and WT mice with DSS-induced colitis. Under TCR-stimulation, the secretion of Th1- and Th17-related cytokines (IFN- γ , TNF- α , IL-17A, IL-17F, and IL-21) by

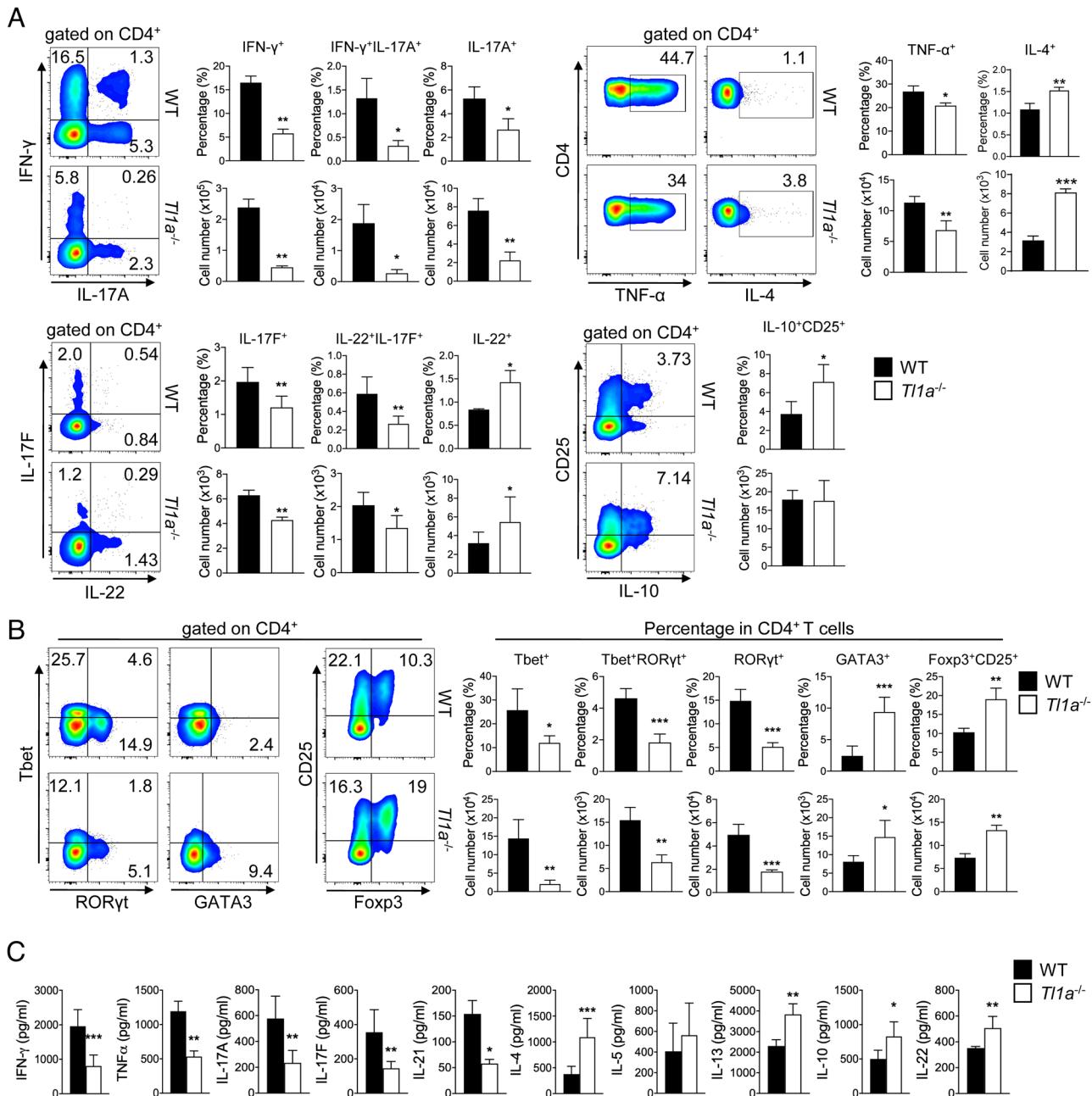


Fig. 2. TL1A deficiency attenuates Th1/Th17 responses in the intestine of acute colitis mice. Cytokine production and intracellular cytokine expression by LP cells of *Tl1a*^{-/-} Mice with DSS-induced acute colitis. (A) The proportions and absolute numbers of cytokine producing-CD4⁺ T cell subsets in LPL of colon; (B) The proportions and absolute numbers of T-bet⁺CD4⁺, GATA3⁺CD4⁺, RORyt⁺CD4⁺, Foxp3⁺CD4⁺CD25⁺ T cells in LPL of colon; (C) Cytokine production of LPL in large intestines after 48 h cultured with anti-CD3/CD28 mAbs. Each column and vertical bar indicate means \pm SD for three to five mice of each group. Data of a representative experiment are shown from three independent experiments. Statistically significant differences are shown (*P < 0.05, **P < 0.01, ***P < 0.001).

T-LPL from *Tl1a*^{-/-} mice decreased, while the secretion of Th2-and Treg-related cytokines (IL-4, IL-13, and IL-10) and IL-22 increased (Fig. 2C and *SI Appendix*, Fig. S5C). These data suggested that TL1A positively regulates colonic Th1 and Th17 differentiation during DSS-induced intestinal inflammation.

Naive CD4⁺ T Cells Require TL1A for Differentiation and Colitis Promotion. Given the requirement for TL1A in Th cell development, we examined whether naive CD4⁺ T cell deficient in TL1A can develop into Th1 or Th17 ex vivo. Naive CD4⁺ T cells sorted from normal *Tl1a*^{-/-} and WT mice were cultured under TCR-stimulation for 48 h, which showed that TL1A and DR3 expression was significantly enhanced in a time-dependent manner (Fig. 3A). Moreover, under Th1-, Th17-, or Treg-polarizing conditions ex vivo, *Tl1a*^{-/-} naive CD4⁺ T cells exhibited a greater Th1 and Th17 differentiation impairment and greater promotion of T cell differentiation into Treg cells than those exhibited by WT naive CD4⁺ T cells (Fig. 3B). These changes were characterized by decreased expression and secretion of IFN- γ and IL-17A (Fig. 3C) and reduced expression of the transcription factors T-bet and ROR γ t, which are specific to Th1 and Th17 cells, respectively (*SI Appendix*, Fig. S6 A and B). We also showed that the TL1A/DR3 signal is necessary for Th1 and Th17 differentiation ex vivo by using anti-DR3 agonistic and anti-TL1A blocking antibodies (*SI Appendix*, Fig. S6 C–F), while another non-TNF family ligand for DR3, PGRN inhibited the interaction between DR3 and TL1A (10). Although TL1A was not involved in inducing PGRN expression (*SI Appendix*, Fig. S6I), PGRN significantly inhibited Th1 and Th17 differentiation, especially in the absence of TL1A (*SI Appendix*, Fig. S6 G and H). These results suggested that PGRN inhibits Th1 and Th17 differentiation by binding DR3 competitively with TL1A. All these results indicated that DR3 signal transduction is vitally important for Th1 or Th17 polarization through T-T cell interaction.

We then used a T cell transfer colitis model to investigate whether the interaction between TL1A and DR3 is necessary in the Th1 and Th17 differentiation in vivo. WT and *Tl1a*^{-/-} naive CD4⁺ T cells were used as donors in the CD4⁺CD62L⁺ T cell transfer colitis model. Four weeks after *Rag1*^{-/-} mice received *Tl1a*^{-/-} CD4⁺ T cell transplants, the inflammatory symptoms in colonic tissues of mice receiving *Tl1a*^{-/-} CD4⁺ T cells transplantation were significantly reduced, when compared with the control group (Fig. 3 D–F). Compared to WT CD4⁺ T cells, there was a significant reduction in the percentage and absolute number of total CD4⁺, IFN- γ ⁺, IFN- γ ⁺IL-17A⁺, and IL-17A⁺ CD4⁺ cells in the colonic LP (*SI Appendix*, Fig. S7 A and B and Fig. 3G). Moreover, the levels of IFN- γ , IL-17A, TNF- α , and IL-6 produced by *Tl1a*^{-/-} colonic LPL in the presence of TCR-stimulation were significantly reduced and a decrease in chemokine expression, including *Ccl17*, *Ccl21*, *Ccr7*, *Ccl2*, *Ccl5*, and *Ccl7* in colon tissues compared to those in *Rag1*^{-/-} mice transferred with WT T cells (Fig. 3H and *SI Appendix*, Fig. S7C). However, we found no significant change in the proportion and cell number of DCs between two groups (*SI Appendix*, Fig. S7D), suggesting that TL1A expression on T cells is necessary for Th1 or Th17 cell development and mediation of chronic intestinal inflammation.

TL1A Deficiency Suppressed mDCs-Mediated Th Cell Polarization

Ex Vivo. mDCs mediate Th1 or Th17 differentiation by secreting IL-12 and IL-23, respectively, and play a role in the development of chronic intestinal inflammation (11). To investigate the role of TL1A in these mDC functions, BMDCs of WT (WT-BMDCs) and *Tl1a*^{-/-} mice (*Tl1a*^{-/-}-BMDCs) were prepared and cultured with lipopolysaccharide (LPS), a TLR4 ligand. Consistent with

previous results (*SI Appendix*, Fig. S2A), the expression of TL1A on the surface of WT-BMDCs gradually increased under LPS stimulation (Fig. 4A). Furthermore, upon LPS-induced BMDCs activation, the expression of activation markers, including MHC II, CD86, and CD80, in *Tl1a*^{-/-}-BMDCs was significantly lower than in WT-BMDCs (Fig. 4B). The ability of activated *Tl1a*^{-/-}-BMDCs to secrete IL-12 and IL-23 was significantly weaker than that of WT-BMDCs (Fig. 4C).

Next, we investigated whether TL1A is required for the interaction between DC and T cell during T cell polarization. Activated BMDCs and naive CD4⁺ T cells from WT or *Tl1a*^{-/-} mice were prepared and cocultured for 4 d. IFN- γ - or IL-17A-producing CD4⁺ T cells were detected. When naive CD4⁺ T cells lacked TL1A, normal BMDCs-induced Th1 and Th17 differentiation was slightly inhibited, but the difference was not statistically significant (Fig. 4D). However, *Tl1a*^{-/-}-BMDCs failed to induce normal Th1 and Th17 cell differentiation, regardless of whether naive CD4⁺ T cells expressed TL1A. In addition, *Tl1a*^{-/-}-BMDCs specifically enhanced Treg differentiation compared to WT BMDCs. These findings established that the expression of TL1A on mDCs is not only essential for inducing naive CD4⁺ T cells into Th1 and Th17 cells but also inhibits Treg differentiation ex vivo.

TL1A is Necessary for Colonic mDCs to Drive T Cell-Mediated Colitis.

To elucidate the important role of TL1A in the interaction between mDCs and CD4⁺ T cells in vivo, we generated DKO (*Tl1a*^{-/-}*Rag1*^{-/-}) mice. We did not observe a difference in mDC, macrophages, neutrophils, and monocytes of LPL and IL-12 or IL-23 expression in colon tissues between *Rag1*^{-/-} and DKO mice in original state (*SI Appendix*, Fig. S8 A–E). We then transferred WT-naive CD4⁺ T cells into *Rag1*^{-/-} and DKO mice to establish a T cell-mediated colitis model. Compared to *Rag1*^{-/-} recipients, DKO recipients showed significantly reduced disease activity (Fig. 5 A–C). The DC populations of colonic LP in *Rag1*^{-/-} recipients were composed of mDCs (CD11c⁺MHC II⁺CD11b⁺B220⁻), whereas pDCs (CD11c⁺MHCII⁺CD11b⁻B220⁺) were hardly detected (Fig. 5D). However, the mDCs in colonic LP of DKO recipients were significantly lower than those in *Rag1*^{-/-} recipients after T cell transfer (Fig. 5D). The expression of CD80 and CD86 on colonic mDCs in DKO recipients was significantly lower than that in *Rag1*^{-/-} recipients (Fig. 5E). Flow cytometric analysis of mDCs recovered from the inflamed colonic LP identified four populations of CD11b- and CD103-expressing cells in *Rag1*^{-/-} recipients (12). The CD103⁺CD11b⁺ phenotype was the most prominent (90%) (Fig. 5F). However, CD103⁺CD11b⁺ mDCs were significantly reduced in DKO recipients compared with those in *Rag1*^{-/-} recipients (Fig. 5F). Furthermore, significantly reduced IL-12 and IL-23 levels were observed in chronic inflammatory colonic LP of DKO recipients transferred with naive CD4⁺ T cells, compared with those in *Rag1*^{-/-} recipients (Fig. 5G). Significant Th1 and Th17 differentiation in colonic LP was observed in *Rag1*^{-/-} recipients transferred with WT-naive CD4⁺ T cells (Fig. 5H). In contrast, a significant reduction in IL-17A⁺ and IFN- γ ⁺ CD4⁺ T cells were observed in the colonic LP of DKO recipients. Specifically, double-positive cells (IFN- γ ⁺IL-17A⁺), considered the major effector T cells during IL-23-mediated intestinal inflammation (13), were hardly detected in DKO recipients (Fig. 5H). Finally, Th1-related cytokines (IFN- γ and TNF- α) and Th17-related cytokines (IL-17A and IL-6) produced by colonic LP-T cells decreased significantly in DKO recipients compared to those in *Rag1*^{-/-} recipients (Fig. 5I). Since TL1A is expressed on several nonhematopoietic cells (14), we further constructed mixed bone-marrow (BM) chimera using *Rag1*^{-/-} and DKO mice

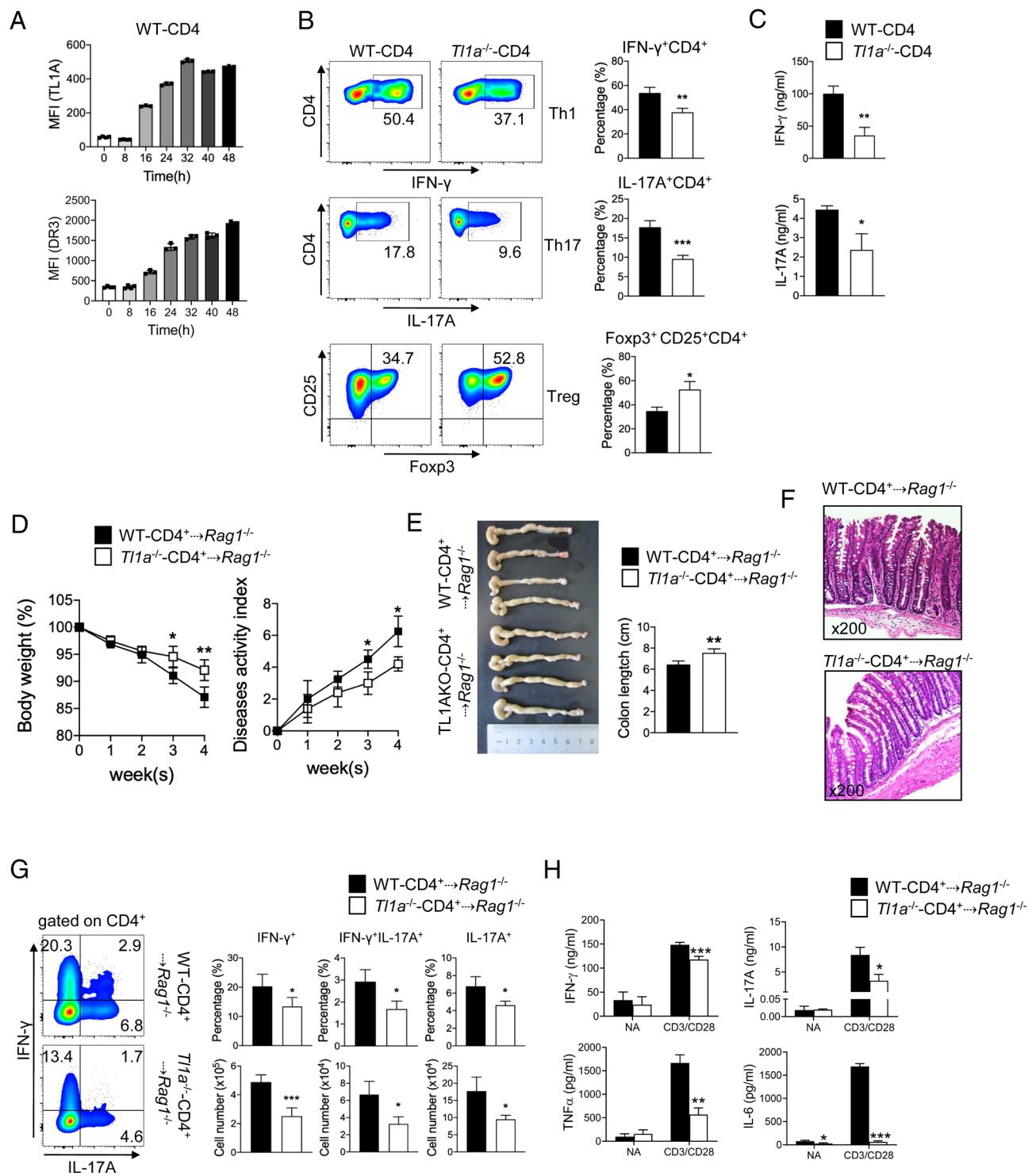


Fig. 3. Naive CD4⁺ T cells require TL1A for differentiation and driving colitis. (A) Naive CD4⁺ T cells were sorted from spleens of WT and *Tl1a*^{-/-} mice and were cultured with anti-CD3/anti-CD28 mAbs, TL1A and DR3 expression were analyzed at the indicated time points by FACS; (B) Induction of Th1, Th17 or Treg differentiation in vitro, IFN- γ , IL-17A- or Foxp3 expression was analyzed by FACS, (C) the levels of IFN- γ and IL-17A were analyzed by ELISA. WT and *Tl1a*^{-/-} naive CD4⁺ T cells were used as donors in the CD4⁺CD62L⁺ T cell transfer colitis model. Four weeks after transfer into *Rag1*^{-/-} recipients, (D–F) body weight, DAI, colon length and histology scores were observed; (G) IFN- γ and IL-17A expression in CD4⁺ T cells of LPL were analyzed by FACS; (H) ELISA analysis of IFN- γ , IL-17A, IL-6 and TNF α production from LPL after ex vivo restimulation with anti-CD3/anti-CD28 after 48 h. Values of each column and vertical bar indicate means \pm SD for three to five mice within each group. Representative data are shown from three independent experiments. Statistically significant differences are shown (*P < 0.05, **P < 0.01, ***P < 0.001).

(*SI Appendix*, Fig. S8F). Four weeks after the T cell-mediated colitis model, we found that nonhematopoietic cell-derived TL1A was not involved in colitis (*SI Appendix*, Fig. S8 G–J). Therefore, TL1A production from hematopoietic cells is necessary for mDC-induced T cell differentiation and colitis development.

TL1A Promotes Antigen Uptake and DC Activation In Vitro. Immature DCs have strong antigen uptake ability. Following antigen stimulation, they develop into mature DCs and present peptides to naive T cells to induce T cell differentiation (15). Membrane-bound TL1A plays an important role during T-T and

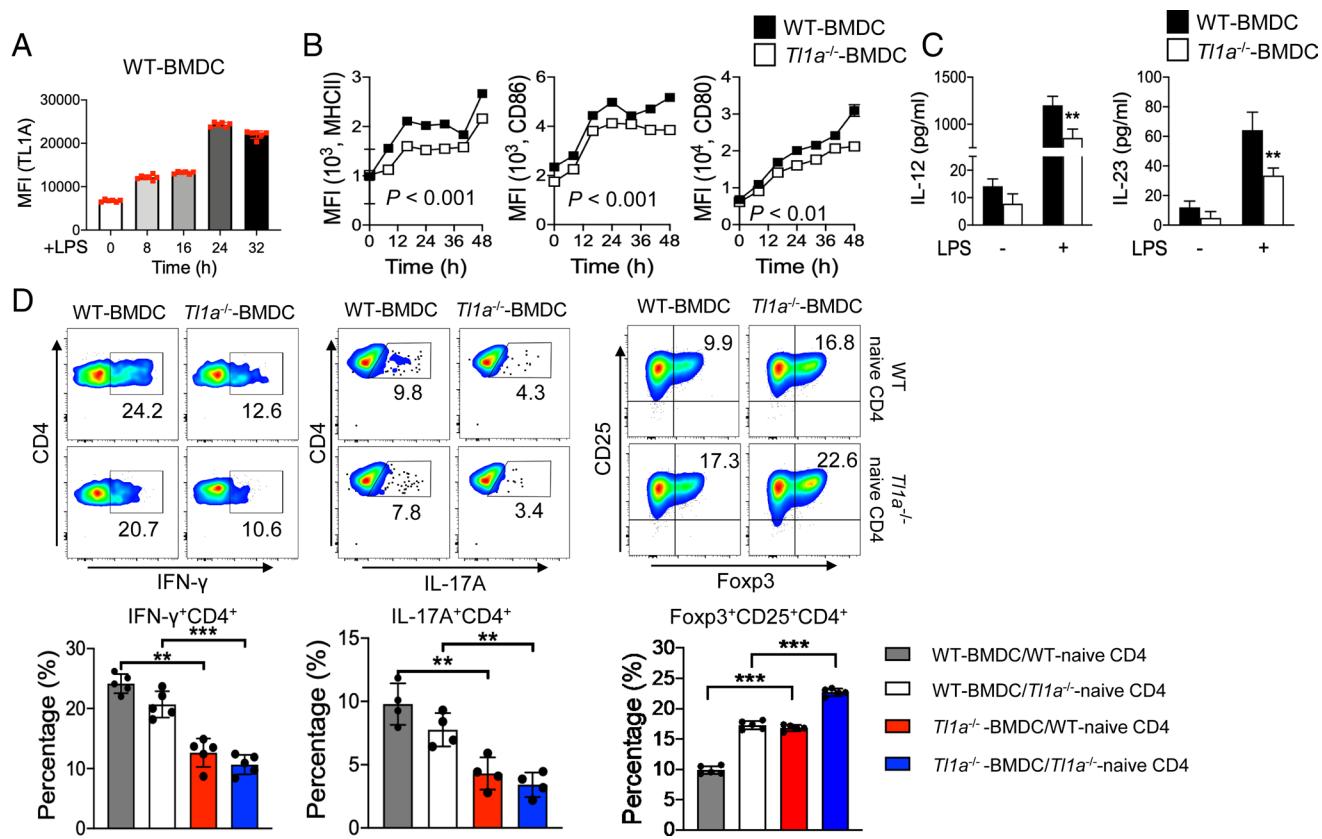


Fig. 4. TL1A deficiency suppressed the function of mDCs to mediate Th cell polarization ex vivo. (A) FACS analysis of TL1A expression on WT BMDCs stimulated with LPS (1 μ g/mL) at the indicated time points; (B) FACS analysis MFI of CD80, CD86 and MHCII expression on WT and *Tl1a*^{-/-} BMDCs stimulated with LPS at the indicated time points; (C) BMDCs from WT or *Tl1a*^{-/-} mice were stimulated with LPS for 24 h, ELISA analysis of IL-12 and IL-23 secretion. (D) Th1, Th17 and Tregs differentiation in the in vitro DC-T cell coculture system was monitored by FACS analysis. Values of each column and vertical bar indicate means \pm SD for three to five mice within each group. Representative data are shown from three independent experiments. Statistically significant differences are shown (** P < 0.01, *** P < 0.001).

DC-T cell interactions in vivo and ex vivo. Unexpectedly, TL1A was also detected in the cytoplasm and nuclei of BMDCs using fluorescence confocal microscopy, and its expression was significantly enhanced upon LPS stimulation (Fig. 6A). Interestingly, TL1A deficiency alone resulted in the inability of BMDCs to be fully activated, even in the presence of LPS, characterized by the reduction of IL-12 and IL-23 protein expression (Fig. 6B). As BMDCs do not express DR3 (*SI Appendix*, Fig. S2C), we hypothesized that TL1A has a regulatory effect on antigen phagocytosis and DC activation. Hence, we examined whether TL1A contributes to BMDCs antigen uptake using a dextran phagocytosis test and found that TL1A deficiency significantly reduced the dextran phagocytic capacity of BMDCs in vitro (Fig. 6C and D). Dextran is recognized by the DC-SIGN (intercellular adhesion molecule-grabbing nonintegrin) family receptors SIGN-R1 and SIGN-R3, and activates the DC-SIGN/serine/threonine kinase RAF-1(RAF1)/p65/nuclear factor (NF) κ B signaling pathway, enhancing TLR4 signaling-mediated DC activation and maturation (16). *Tl1a* gene deletion significantly reduced the total protein and phosphorylation levels of RAF1 and p65, suggesting that TL1A may contribute to DC antigen uptake and activation by enhancing RAF1/p65 signaling in vitro (Fig. 6E and *SI Appendix*, Fig. S9).

To verify these findings, we prepared TL1A-overexpressing BMDCs (TL1A-BMDCs) (Fig. 6F and G). BMDC's phagocytic ability was significantly enhanced by TL1A overexpression in vitro, compared with the control (NC-BMDCs) (Fig. 6H). TL1A-BMDCs expressed higher CD80, CD86, and MHC II levels and secreted

more IL-12 and IL-23 after LPS stimulation (Fig. 6I and J). Moreover, the total protein and phosphorylation levels of RAF1 and p65 were significantly up-regulated in TL1A-BMDCs (Fig. 6K and *SI Appendix*, Fig. S10). Furthermore, the co-immunoprecipitation (Co-IP) assay verified the interaction between TL1A and RAF1 (Fig. 6L), and immunofluorescence analysis confirmed the colocalization of intracellular TL1A and RAF1 (*SI Appendix*, Fig. S11), further suggesting that TL1A promotes TLR4 signaling-mediated DC activation through the DC-SIGN/RAF1/NF- κ B pathway, releasing IL-12 and IL-23, and thereby enhancing Th1 and Th17 differentiation in vitro.

Discussion

Although TL1A plays an important proinflammatory role in IBD pathogenesis (17), the underlying mechanism is unclear. Using colitis models, we confirmed that TL1A/DR3 regulates colonic inflammatory responses by inducing naive T cell differentiation and participating in IBD occurrence and development. Moreover, we revealed that TL1A is highly expressed in the cytoplasm and nuclei of activated DCs. The expression of TL1A enhances DCs' antigen uptake capacity by activating the DC-SIGN/RAF1/p65/NF- κ B signaling pathway and synergistically promotes TLR4 signaling-mediated DC activation.

TL1A-DR3 interactions affect gut mucosal immunity during homeostatic conditions and various inflammatory states (18, 19). We found that TL1A was mainly expressed on the surface of DCs in the intestinal LP. Compared with mice in the control group,

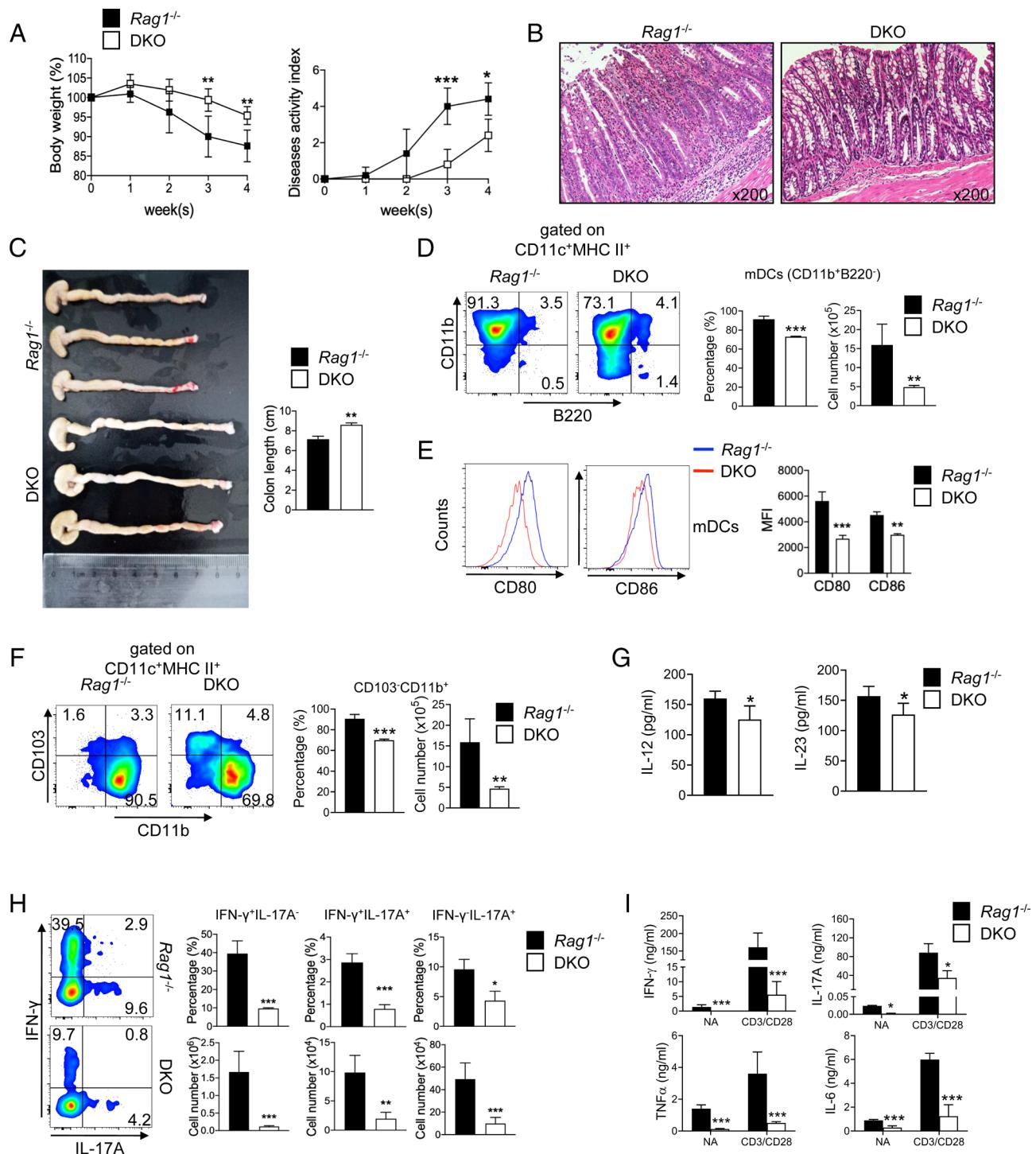


Fig. 5. TL1A is necessary for colonic mDCs to drive T cell-mediated colitis. Naive CD4⁺ T cells from WT mice were sorted and transferred into *Rag1*^{-/-} or DKO mice to induce T cell-mediated colitis. (A–C) Body weight changes, DAL, colon length and histology scores for the intestine after transfer. (D and E) Representative flow plots for expression of mDCs, and MFI of CD80 and CD86 in mDCs. (F) The frequency and numbers of CD103⁺CD11b⁺ DCs from LP of colon were analyzed by FACS after transfer. (G) ELISA analysis of IL-12 and IL-23 production from LPL without any stimulation. (H) IFN- γ - or IL-17A-producing CD4⁺ T-LPL were analyzed by FACS. (I) ELISA analysis of IFN- γ , IL-17A, TNF- α , IL-6 production from LPL after ex vivo restimulation with/without anti-CD3 and anti-CD28 after 48 h. Values of each column and vertical bar indicate means \pm SD for three to five mice within each group. Representative data are shown from three independent experiments. Statistically significant differences are shown (* P < 0.05, ** P < 0.01, *** P < 0.001).

the expression of TL1A on DCs in the intestinal LPL was significantly increased after DSS induction and further increased with continuous activation. Furthermore, DR3 was expressed on the surface of active CD4⁺ T cells in the LP after DSS treatment. The expression of TL1A on DCs and DR3 on CD4⁺ T cells increased after DSS induction, suggesting that the TL1A/DR3 signaling

may be involved in IBD development. Consistent with a previous study (17), we found that mice with TL1A deficiency were resistant to DSS-induced acute and chronic colitis, which mainly manifested in the decrease of Th1- and Th17-mediated inflammatory responses in the colonic LP, while the Treg response was enhanced (17).

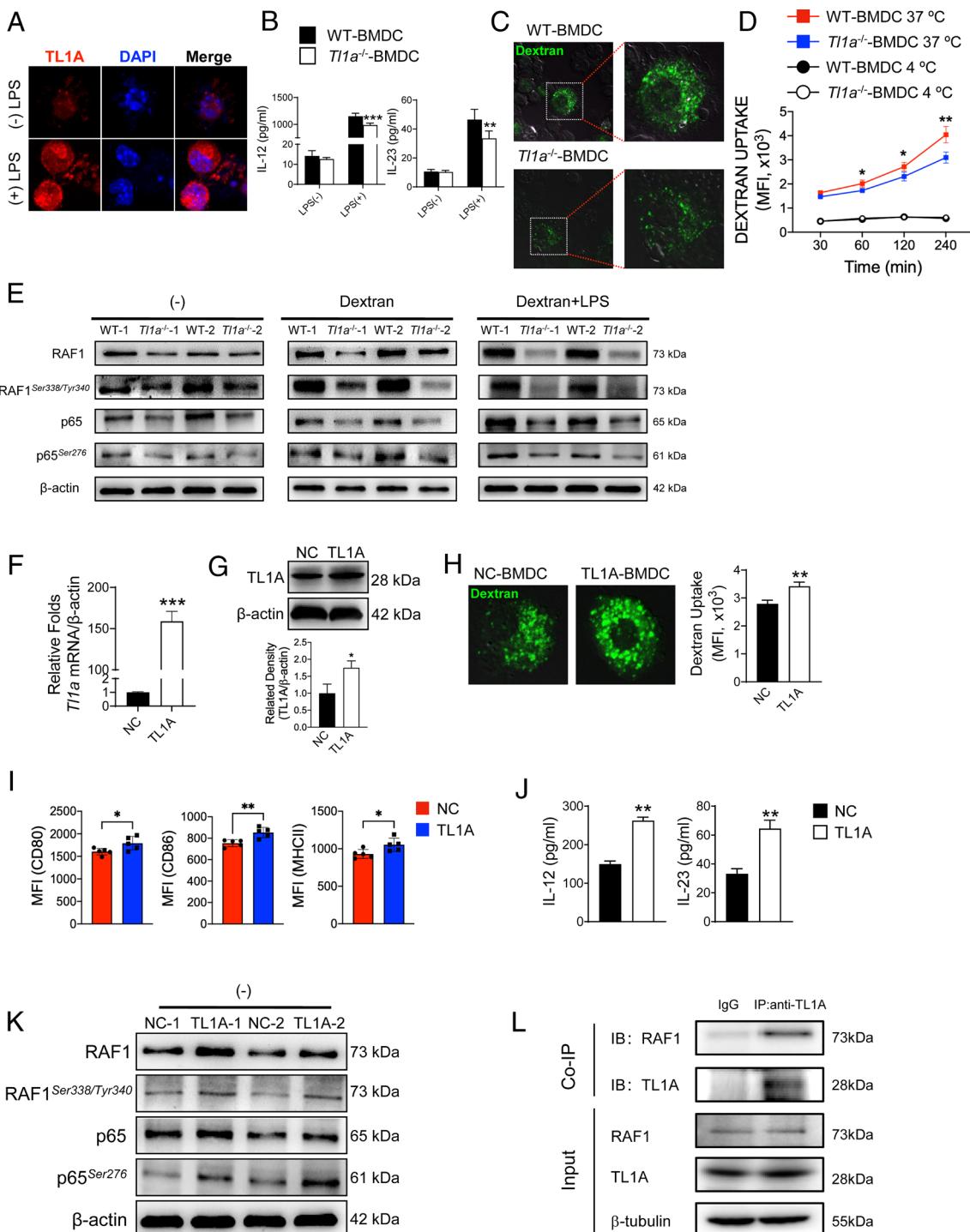


Fig. 6. TL1A promotes antigen uptake and activation of DCs. (A) Immunofluorescent staining for TL1A (red) or DAPI (blue) expression from WT BMDCs stimulated with LPS for 24 h using confocal microscopy; (B) 1×10^6 of BMDCs from WT or $Tl1a^{-/-}$ mice were cultured with or without LPS for 24 h and the protein levels of IL-12 and IL-23 in the culture supernatant were measured by ELISA analysis; (C) Immunofluorescent analysis of phagocytosis of WT- or $Tl1a^{-/-}$ -BMDCs; (D) A time course of phagocytosis from WT and $Tl1a^{-/-}$ BMDCs was assessed using FACS; (E) Immunoblot analysis of RAF1, pRAF1, p65, pp65 in the lysate (cells) from WT or $Tl1a^{-/-}$ BMDCs; (F and G) WT BMDCs were transfected with negative control (NC) plasmid or $Tl1a$ plasmid for 48 h, harvested and analyzed for $Tl1a$ mRNA by qPCR and TL1A protein by western blotting; (H) Immunofluorescent analysis of phagocytosis from NC or $Tl1a$ overexpression BMDCs; (I) FACS analysis MFI of CD80, CD86 and MHC II expression, and (J) ELISA analysis of IL-12 and IL-23 production from 2×10^5 of NC or $Tl1a$ overexpression BMDCs stimulated with LPS for 24 h; (K) Immunoblot analysis of RAF1, pRAF1, p65, pp65 in the lysate (cells) from NC or $Tl1a$ overexpression BMDCs; (L) Interaction between endogenous TL1A and RAF1 in BMDCs. Cell lysates of endogenous TL1A were prepared and used for Co-IP. The coimmunoprecipitates were analyzed by western blotting. Values of each column and vertical bar indicate means \pm SD for three to five mice within each group. Representative data are shown from three independent experiments. Statistically significant differences are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Immature DCs have a strong antigen uptake ability and present antigen peptides to naive T cells to induce T cell activation. Antigen-activated DCs differentiate into mature DCs and secrete

a variety of cytokines to induce activated T cells to differentiate into Th1 or Th17 (20, 21). These superfluous Th1 and Th17 responses in the IBD were due, at least partly, to an increase in

the LP-DC population, which has been identified as a potent inducer of Th1 and Th17 differentiation (12). Moreover, *Tl1a*^{-/-} LP-DCs exhibited reduced maturity and MHC II expression, as well as costimulatory molecules CD80 and CD86. In fact, IL-12 and IL-23 produced by DCs are required for intestinal Th1 and Th17 differentiation, respectively (12, 22).

In contrast to earlier studies, *Tl1a*^{-/-} mice were less susceptible to DSS-induced colitis. Jia et al. (23) showed a protective role of the TL1A/DR3 axis in acute DSS colitis in *Tl1a*^{-/-} mice by contracting the Treg pool. In contrast, we found an enhanced presence of Tregs in LPLs in acute and chronic experimental colitis models, demonstrating that deletion of TL1A leads to Treg upregulation. There are several explanations could explain the discrepancy between these findings. First, the genetic background determines the susceptibility of experimental mice to DSS-induced colitis. In contrast to published reports (18, 23), we used *Tl1a*^{-/-} mice with a C57BL/6 genetic background instead of a mixed 129/sv-C57BL/6 background. A previous study demonstrated that the number of CD4⁺CD25⁺ Treg cells was significantly reduced in C57BL/6 mice compared with those in 129SV mice (24). Second, in the T cell transfer colitis model, TL1A-tg Tregs were unable to protect against colitis; in contrast, Tregs expressing low levels of TL1A were able to suppress T cell transfer colitis (25). Finally, TL1A release can be regulated by bacteria and TL1A-mediated intestinal inflammation depending on specific microbial populations (26, 27). These differences in the results across the different studies could potentially be explained by the differences in intestinal microbiota. Based on our results that showed an increased proportion and number of Tregs in *Tl1a*^{-/-} mice, we speculate that the depletion of TL1A on Tregs, which enhanced immunosuppression, acts on Th cells due to a lack of interaction between TL1A and DR3.

TL1A triggers an interaction between DR3 and its adaptor protein, TNFR-associated death domain, which activates mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB signaling to induce immune cells activation and cytokine secretion (28). T-bet is critical for IFN-γ production in Th1 cells, while NF-κB deficiency can impair T-bet production (29). Similarly, the important role played by NF-κB in the regulation of RORyt, and by extension in the differentiation and functioning of Th17 cells, has been demonstrated (30). In this study, T cell differentiation experiments indicated that the TL1A/DR3 signal could up-regulate the expression of T-bet and RORyt within the T-T cell interaction pattern, and promote the differentiation of Th1 and Th17. This was consistent with our results in the intestinal LP from mice with DSS-induced colitis. Our results and other reports showed that TL1A was expressed not only on the surface of activated T cells, but on the surface of DCs (27, 31). Moreover, as another ligand for DR3, PGRN acts as a competitive inhibitor for TL1A signaling by binding to DR3 (10). PGRN significantly inhibits the differentiation of Th1 and Th17 cells in vitro, especially for naive *Tl1a*^{-/-} CD4⁺ T cells, although TL1A was not involved in inducing PGRN expression. PGRN inhibits T cell differentiation in both naive WT and *Tl1a*^{-/-} CD4⁺ T cells means that the *Tl1a*^{-/-} phenotype could be partly due to the increased relative inhibitory signaling from PGRN to DR3 in the absence of TL1A. This indicates that simultaneously targeting TL1A and PGRN may produce greater benefits in IBD than separately targeting these factors might. Collectively, we believe that the TL1A/DR3 signal promotes Th1 and Th17 differentiation by enabling T-T cell interactions in vitro, while mediating Th cell differentiation by regulating DC function in the LP of the intestinal mucosa in vivo.

In the adoptive-transfer model, *Tl1a*^{-/-} CD4⁺ T cells attenuated the Th1/Th17-dominant immune response, inconsistent with previous studies that showed that transferring TL1A-tg naive T cells into *Rag1*^{-/-} mice resulted in an upregulation of Th1 and almost complete absence of IL-17A production (32). These results differ from those of our in vivo and ex vivo experiments, where we found that TL1A deficiency negatively affected Th17 differentiation. However, Robert et al. (32) tested Th1/Th17 only in the spleen and MLN, not in colitis-associated LPLs. Therefore, different cytokines may mediate T cell polarization in LP than those found ex vivo. Furthermore, *Tl1a*^{-/-} CD4⁺ T cell transferred mice exhibited significantly reduced transcript-level expression of *Ccl17*, *Ccl21*, *Ccr7*, *Ccl2*, *Ccl5*, and *Ccl7*, whereas no change was observed in the transcript-level expression of *Cx3cl1*, *Cxcl12*, or *Ccl19*. After WT-CD4⁺ or *Tl1a*^{-/-}-CD4⁺ T cell transplantation, there was no change in the recruitment of total DCs (MHCII⁺ CD11c⁺) in inflammatory intestinal tissues. Chemokines can be secreted by a range of cell types (including monocytes and macrophages) and can also act on a variety of cells. We hypothesize that mDCs-mediated intestinal Th1 and Th17 differentiation is necessary to establish a T cell-mediated chronic colitis model. Therefore, we think that the absence of TL1A in mDC results in the inhibition of effector Th cell differentiation and reduction in pro-inflammatory cytokine secretions (e.g., IFN-γ and IL-17A), and suppression of innate immune cells (e.g., macrophages and monocytes) activation and subsequent chemokine production, which can further affect the accumulation of inflammatory cells in the intestinal mucosa and attenuate the symptoms of intestinal inflammation. The presence of intestinal microbial flora may also induce specific immune responses in the gut. To further examine the intrinsic role of T cells in the absence of TL1A on APCs in regulating Th cell differentiation, we used DKO mice to construct an adoptive-transfer model in vivo. The reduction in mDCs levels in the transplantation model was not observed in the absence of transplanted T cells, and the mDCs produced cytokines normally. These findings suggest that the reduction in mDC levels in the DKO mice is due to impaired chemokine secretion. Furthermore, the robust Th1 responses were attributed to an increase in the CD103⁺CD11b⁺ LP-DC population, which is a potent inducer of Th1/Th17 differentiation and activation (12). Moreover, LP-DCs in DKO hosts exhibited reduced maturity and expression of the costimulatory molecules CD80 and CD86. IL-12 produced by CD103⁺CD11b⁺ DCs is required for intestinal Th1 differentiation (12). In addition to the expression in hematopoietic cells, TL1A is also expressed on nonhematopoietic cells, such as synovial fibroblasts and endothelial cells (14). To investigate whether non-hematopoietic cell-derived TL1A affects disease progression, a T cell-mediated colitis model was established by mixed bone-marrow chimera using *Rag1*^{-/-} mice and DKO mice, which showed that TL1A production from stroma cells did not play an important role in this model.

We showed the role of *Tl1a*^{-/-} DC in DSS-induced colitis in vivo, however, the exact mechanism by which the absence of TL1A on DCs attenuated T cell differentiation is not completely clear. To explore this further, we conducted several experiments and identified a function of TL1A-deficient BMDCs in inhibiting phagocytosis in vitro. DC-SIGN is a receptor expressed by DCs ex vivo and in vivo (33, 34). DC-SIGN receptors in mice have eight orthologs, including SIGN-R1 to SIGN-R8 (35), and dextran uptake via the DC-SIGN family receptors SIGN-R1 and SIGN-R3 has been reported in mice (36, 37). The DC-SIGN signalosome is a complex composed of the scaffold proteins LSP1, KSR1, and CNK and is required for the constitutive association

of DC-SIGN with RAF1 and the association or isolation of the compounds with more proinflammatory effects (16). RAF1 is a serine/threonine MAP kinase involved in the acetylation of the NF- κ B subunit p65 and TLR4–DC-SIGN cross-talk up-regulates IL-12 and IL-6 expression (16). We validated total RAF1 and found that RAF1 at Ser338 or Tyr340 proteins decreased in *Tl1a*^{-/-} BMDCs, resulting in p65 phosphorylation at Ser276 and innate immune response modulation. TL1A is considered a type II transmembrane protein (38), predominantly expressed on the cell surface. In a recent study, nuclear localization of TL1A did not occur under steady-state conditions but could represent a unique inflammation-driven phenomenon (39). RAF1 and p65 total proteins decreased in *Tl1a*^{-/-}-BMDCs but increased in TL1A-overexpressing BMDCs. Thus, TL1A may up-regulate RAF1 and p65 expression, leading to enhanced BMDCs activation. We speculate that decreased phagocytosis is related to TL1A depletion, which is consistent with our Co-IP and immunofluorescence experiments that identified RAF1 as a binding partner of endogenous TL1A and that the loss of TL1A attenuates RAF1 expression in BMDCs. However, further investigation is required to explore the exact mechanism underlying TL1A regulation of RAF1 and p65 expression. In our study, *Tl1a*^{-/-}-BMDCs exhibited decreased maturity with reduced expression of MHC II and costimulatory molecules CD80 and CD86 when stimulated with LPS compared to WT-BMDCs. We describe a function of TL1A in regulating DC activity, including phagocytosis and antigen presentation in vitro. Immature DCs come into contact with pathogens and mature by forming peptide-MHC II complexes, that are then redistributed from the cytosolic compartment to the cell surface for efficient antigen presentation to naive CD4⁺ T cells to induce antigen-specific T cell responses. We examined the role of TL1A in the regulation of DC function, which is responsible for Th1 and Th17 differentiation ex vivo. Several microbes can induce TL1A expression in primary human monocytes and monocyte-derived DCs (27). Our results showed that TL1A expression increased after LPS stimulation. Furthermore, in the monocytic cell line U937, TL1A transcription was induced by LPS via the NF- κ B pathway. By binding to TLR4, LPS activates the MAPK and IKK pathways, which activate NF- κ B that is then translocated into the nuclei, suggesting that LPS-induced TL1A expression may occur via the TLR4/NF- κ B signaling pathway (40). We found that TL1A regulated proinflammatory cytokine secretion produced by BMDCs. NF- κ B drives the production of IL-12/IL-23 in DCs to amplify the inflammatory response (41, 42). Finally, ex vivo coculture experiments with DCs and T cells further verified that TL1A deficiency in BMDCs decreased Th1 and Th17 differentiation.

In summary, we report that TL1A expression on DCs regulates Th1/Th17 differentiation and DSS-induced colitis pathogenesis by regulating the TL1A/DR3 axis and the production of proinflammatory cytokines, including IL-12 and IL-23. More importantly, this study provides a theoretical framework for further investigations of the TL1A/DR3 axis. Our results suggest that targeting this pathway could provide beneficial therapeutic options for IBD patients.

Materials and Methods

Mice. *Tl1a*^{-/-} and *Rag1*^{-/-} mice were created by Nanjing Biomedical Research Institute of Nanjing University, Jiangsu, China, and were crossed with WT C57BL/6 mice to generate age- and sex-matched littermate controls. The progeny of a cross of *Tl1a*^{-/-} mice to *Rag1*^{-/-} mice were intercrossed to generate *Tl1a*^{-/-}*Rag1*^{-/-} mice (DKO) and WT C57BL/6 littermate controls. All mice were bred and maintained in individually ventilated cages under specific pathogen-free conditions

and were offered food and water ad libitum. All mice were used at 6 to 8 wk of age. This study was approved by the Animal Welfare and Ethics Committee of China Medical University (IACUC No. 2018154). Experiments were carried out under the control of the Guidelines for Animal Experiments.

Induction of acute or chronic colitis by DSS. Induction of DSS-induced acute and chronic colitis was performed as previously described (43). Details are provided in *SI Appendix*.

T cell transfer colitis. For naive T cell transfer colitis, 1×10^6 of CD4⁺CD62L⁺ T cells from WT or *Tl1a*^{-/-} mice were intraperitoneally injected (i.p.) into *Rag1*^{-/-} recipients. A total of 1×10^6 CD4⁺CD62L⁺ T cells from WT were injected i.p. into *Rag1*^{-/-} recipients or *Tl1a*^{-/-}*Rag1*^{-/-} recipients. Mice were killed 4 wk later.

Bone Marrow-Derived DCs. Tibial and femoral bone marrow from 6 to 8-wk-old mice as described was isolated. After lysing red blood cells, whole BM cells (2×10^6 cells/mL) were used to prepare BMDCs according to the methods described in the literature (44). The purity of prepared CD11c⁺ DCs was more than 90%.

Immunofluorescent Staining. BMDCs were cultured as described above. After 7 d, BMDCs were washed with PBS and blocked with BSA blocking for 2 h. For the TL1A expression experiment, the BMDCs were incubated with primary antibodies for anti-TL1A (1:200, abcam, 85,566) for 16 h at 4 °C, and followed by PE-conjugated secondary antibodies (Servicebio, GB21303). For colocalization experiment, the BMDCs were incubated with primary antibodies for anti-TL1A (1:200, bioss, bs-5092R) and anti-RAF1 (1:400, proteintech, 66592-1-Ig) for 16 h at 4 °C, and followed by FITC and PE-conjugated secondary antibodies (Servicebio, GB21303), respectively. After BMDCs were cytopsin onto glass slides and stained by DAPI (Solarbio, S2110), sections were examined with a confocal microscope (Thermo).

Phagocytosis Assay. On day 7, immature BMDCs (1×10^6 /mL) were incubated in 24-well plates with a final concentration of 1 mg/mL FITC-dextran (42,000 Da; Sigma, FD40s). One plate was incubated at 37 °C and the second was incubated on ice and was used as a background control for every time point. Endocytosis was halted at the indicated time points and cells were rapidly cooled on ice, then washed three times with cold PBS and FITC-dextran stained DCs were analyzed by flow cytometry or confocal microscopy. The geometric mean fluorescent intensity difference between 37 °C and 4 °C was considered as the result of antigen uptake.

Co-immunoprecipitation. BMDCs were lysed for 30 min in protein lysis buffer (Sigma-Aldrich) on ice. After centrifugation, the protein concentration was detected by a BCA Protein Assay kit (Beyotime, P0012). Then, cell lysates were incubated with antibodies against TL1A (ALX-804-859-C100; Enzo) at 4 °C on a shaker overnight. The immunoprecipitation of TL1A-tagged proteins was performed by using Protein A/G PLUS-Agarose beads (cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) incubated for 2 h. After precipitation, the beads were washed three times with protein lysis buffer and then combined with 30 μ L of 2× loading buffer (1:1). The samples were boiled for 5 min at 100 °C and loaded onto the SDS-PAGE gels.

Statistical Analysis. The data were analyzed with GraphPad Prism 8 and are presented as the mean \pm SD. A Student's *t*-test was used when two conditions were compared, while ANOVA with Bonferroni or Newman-Keuls correction was used for multiple comparisons. Probability values < 0.05 were considered significant; two-sided Student's *t*-tests or ANOVA were performed. (**P* < 0.05 ; ***P* < 0.01 ; ****P* < 0.001 or *****P* < 0.0001).

Data, Materials, and Software Availability. The microarray data are available from the Gene Expression Omnibus under accession number GSE73094 (45). All study data are included in the article and/or *SI Appendix*.

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