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A role for BATF3 in T_H9 differentiation and T cell driven mucosal pathologies

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Abstract

T helper 9 (T_H9) cells are important for the development of inflammatory and allergic diseases. The T_H9 transcriptional network converge signals from cytokines and antigen presentation but is incompletely understood. Here, we identified TL1A, a member of the TNF superfamily, as strong inducer of mouse and human T_H9 differentiation. Mechanistically, TL1A induced the expression of the transcription factors BATF and BATF3 and facilitated their binding to the *Il9* promoter leading to enhanced secretion of IL-9. BATF- and BATF3-deficiencies impaired IL-9 secretion under T_H9 and T_H9-TL1A polarizing conditions. *In vivo*, using a T cell transfer model we demonstrated that TL1A promoted IL-9-dependent, T_H9 cell-induced intestinal and lung inflammation. Neutralizing IL-9 antibodies attenuated TL1A-driven mucosal inflammation. *Batf3*$^{-/-}$ T_H9-TL1A cells induced reduced inflammation and cytokine expression *in vivo* compared to WT cells. Our results demonstrate that TL1A promotes T_H9 cell differentiation and function and define a role of BATF3 in T cell driven mucosal inflammation.

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Author Contributions

M.T., H.H., L.T., M.W., B.S., M.W., J.N., J.S., A.N.-W., R.B., Y.W., J.T., V.F., and K.M. performed experiments, analyzed data, and critically reviewed the manuscript. A.P., J.T., and Y.W. performed RNA-Seq data analysis. M.T., S.T., and K.M. designed the experiments. M.T. and K.M. wrote the manuscript.

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Disclosure

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Introduction

Specialized subsets of T helper (T_H) cells play an integral role in maintaining tissue homeostasis and during the development of inflammatory diseases at mucosal surfaces. T_H9 cells have been recently identified as an independent T_H cell subset that produces mainly IL-9 but also IL-10 and IL-21 [1, 2]. T_H9 cells have been implicated in several diseases, including allergic lung inflammation, experimental autoimmune encephalomyelitis (EAE), colitis, parasitic worm infections, and cancer [1, 3, 4, 5]. Recently, T_H9 cells have been reported to play a role in inflammatory bowel diseases (IBD) [6, 7, 8, 9]. Ulcerative colitis (UC) patients have elevated numbers of mucosal IL-9^+ T cells and the IL-9 receptor (IL-9R) is up-regulated on the intestinal epithelium. IL-9 deficiency suppresses the development of acute and chronic oxazolone-induced colitis, a model that mimics UC [6]. In Crohn's disease (CD) patients, high serum IL-9 levels correlate with severe disease [8, 9, 10].

T_H9 cells differentiate from naïve $CD4^+$ T cells in the presence of TGF-β1 and IL-4. Several transcription factors down-stream of T cell receptor (TCR), TGF-β1 and IL-4 receptors are required for the differentiation of T_H9 cells including IRF4, STAT6, GATA3, PU.1, NF-κB, and Basic leucine zipper transcription factor ATF-like (BATF) [1, 11, 12, 13, 14, 15]. However, the transcriptional program and inflammatory triggers that drive the differentiation of T_H9 cells are still not well understood and a lineage-defining factor associated with IL-9 expression has not been identified [16].

The family of BATF transcription factors is comprised of BATF, BATF2, and BATF3 and belong to the AP-1 family of transcription factors that include JUN and FOS. Transcription factors in the BATF family are composed of a DNA-binding domain and leucine zipper motif but lack a transactivation domain and were initially described as negative regulators of AP-1 activity [17]. However, recent studies have demonstrated that BATF family members interact with IRF transcription factors including IRF4 and IRF8, and bind to AP-1-IRF composite element sequences to regulate their target genes in T cells and dendritic cells [18]. BATF has been shown to be required for the development of T_H9 cells as well as T_H2, T_H17 and T_{FH} cells [15, 19]. BATF3 has been described to control the development of $CD8\alpha^+$ and $CD103^+$ dendritic cells [20, 21]. In contrast to BATF, a non-redundant function of BATF3 in the development of T_H cells has not been demonstrated. In addition, the extracellular stimuli that can activate the BATF3 pathway in $CD4^+$ T_H cells remains to be elucidated.

We have recently shown that TL1A, a TNF superfamily member that plays an important role in immune mediated diseases including IBD [22, 23, 24, 25], induces the secretion of IL-9 in T_H17 cells [26]. Furthermore, a recent publication demonstrated that TL1A, via its receptor DR3, promotes T_H9 differentiation through IL-2 and STAT5-dependent but PU.1 and STAT6-independent mechanisms in allergic lung inflammation [27]. However, the transcriptional programs induced by TL1A and the pathogenicity of TL1A-induced T_H9 cells in intestinal inflammation and IBD remains to be elucidated.

Here, we demonstrate that TL1A promotes the differentiation of human and murine T_H9 cells via a novel signaling pathway. We define BATF3 as a novel transcriptional regulator for the differentiation of T_H9 cells. TL1A leads to chromatin remodeling at the *Il9* locus and

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recruitment of the pioneer transcription factors IRF4, and BATF, which are important components for IL-9 transcriptional activation, and BATF3 to conserved regions within the *Il9* promoter. Furthermore, TL1A upregulates the expression of BATF and BATF3 in a STAT6-dependent manner. *Batf*- and *Batf3*-deficient T_H9 and TL1A-induced T_H9 (T_H9-TL1A) cells are impaired in their IL-9 production. T_H9-TL1A cells are highly pro-inflammatory *in vivo* when adoptively transferred into *Rag1*$^{-/-}$ mice as evident by severe mucosal inflammation in intestines and lungs, which was attenuated by treatment with neutralizing IL-9 antibodies. Adoptive transfer of *Batf3*$^{-/-}$ T_H9-TL1A cells into *Rag1*$^{-/-}$ mice leads to significantly reduced mucosal inflammation and cytokine production in comparison with WT T_H9-TL1A cells. Our data demonstrate a novel role for TL1A and BATF3 in developing pathogenic effector T_H9 cells and identify this signaling pathway as a potential therapeutic target in T_H9-driven pathologies, including IBD and allergic lung disease.

Results

TL1A enhances murine and human T_H9 cell differentiation in vitro

To determine the effect of TL1A on T_H9 differentiation, we stimulated naïve CD4$^+$ T cells under T_H9 conditions with or without TL1A. TL1A significantly enhanced IL-9 secretion and *Il9* mRNA expression (Figure 1a, Supplementary Figure 1a). TL1A alone did not induce IL-9 secretion, suggesting that TL1A acts synergistically with TGF-β1 and IL-4 to promote IL-9 production. Secretion and mRNA expression of T_H9-associated cytokines IL-10 and IL-13 was also enhanced by TL1A (Figure 1a, Supplementary Figure 1a, b). Consistent with previous reports, time-course experiments of intracellular IL-9 staining revealed transient IL-9 expression with a maximal induction under T_H9 conditions at day 3 and a decline back to baseline at day 5 (Supplementary Figure 1b)[28, 29]. TL1A induced a significantly higher percentage of IL-9 producing cells throughout the time-course of the experiments with a shift in kinetics towards an earlier and stronger IL-9 induction that even at an early time-point exceeded the maximum of IL9 production induced under T_H9 conditions (Supplementary Figure 1b). We also observed an increase of IL-9$^+$IL-10$^+$ cells in the presence of TL1A (Supplementary Figure 1b). We did not observe any shift towards other T_H subsets (Supplementary Figure 1c). The enhancement of T_H9 differentiation by TL1A was completely dependent on its receptor DR3 (Supplementary Figure 1d). Taken together, these data suggest that TL1A synergizes with TGF-β1 and IL-4 in T_H9 differentiation and IL-9 production.

TL1A also significantly enhanced T_H9 differentiation in an antigen-specific setting using OVA-specific OT-II cells (Supplementary Figure 1e, f). TL1A did not affects cell proliferation during T_H9 differentiation (Supplementary Figure 1g), as has been demonstrated for memory CD4$^+$ T cells [30]. These data suggest that TL1A enhanced T_H9 differentiation without affecting proliferation. Furthermore, we examined IL-9 production in cells that were differentiated under T_H9 and T_H9-TL1A conditions for 3 days and re-stimulated with anti-CD3ε/CD28. We observed increased IL-9 production in T_H9-TL1A cells compared to T_H9 cells after secondary stimulation that was further enhanced in the

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presence of TL1A (Supplementary Figure 1h, i). These data suggest that TL1A also enhanced IL-9 production from differentiated T_H9 cells.

Next, we determined the effect of TL1A on the differentiation of human T_H9 cells. Naïve $CD4^+$ T cells were isolated from PBMC from healthy donors and stimulated under T_H9 condition in the presence of human TL1A. Consistent with our data from mouse T_H9 cells, addition of TL1A significantly enhanced IL-9 secretion from human T_H9 cell cultures (Figure 1b).

TL1A induces the expression of the transcription factors BATF and BATF3s

To elucidate signaling pathways induced by TL1A, we used RNA sequencing to assess transcriptomic profile in T_H9 and T_H9-TL1A cells. TL1A did not substantially alter the T_H9 transcriptional profile indicating that TL1A did not globally skew T_H9 differentiation (data not shown). However, 219 genes were differentially expressed in T_H9-TL1A cells (Figure 2a, Supplementary Table 1, 2). Pathway enrichment analysis identified significant changes in genes involved in cytokine-cytokine receptor and JAK-STAT signaling pathways in T_H9-TL1A conditions (Supplementary Table 1, 2, data not shown). BATF3 and several BATF-regulated genes were significantly up-regulated under T_H9-TL1A conditions (Supplementary Table 3) suggesting that the transcription factors BATF3 and BATF might be involved in the differentiation of T_H9-TL1A cells. BATF has been described to be required for the development of T_H17 and T_H9 cells [15, 31, 32, 33, 34]. To determine whether TL1A activates BATF family members, we first assessed the expression level of *Batf* in different T_H subsets and detected strong *Batf* mRNA expression in T_H9 and T_H2 cells (Figure 2b). TL1A further enhanced the expression of *Batf* under T_H9 conditions, particularly on day 1 when T_H9 conditions alone are not sufficient to induce *Batf* mRNA (Figure 2c). TL1A alone induced *Batf* mRNA to a similar degree as T_H9 conditions (Figure 2c). Intracellular staining for BATF confirmed that T_H9-TL1A stimulation significantly increased the percentage of $BATF^+$ cells (Figure 2d). Furthermore, TL1A alone increased the percentage of $BATF^+$ cells and more importantly the percentage of $BATF^+IRF4^+$ cells compared to T_H9 conditions suggesting that TL1A might enhance the formation of IRF4-BATF cooperative complexes (Figure 2d). T_H9-TL1A stimulation also enhanced the percentage of $BATF^+IL\text{-}9^+$ cells (Figure 2e).

In addition, human T_H9 cells had significantly higher percentage of IL-9-producing cells when stimulated with TL1A (Figure 2f, g). Notably, the majority of IL-9-producing cells co-expressed BATF. Co-staining of BATF and IRF4 showed that addition of TL1A increased the percentage of $BATF^+IRF4^+$ cells during differentiation of human T_H9 (data not shown), suggesting that the enhancement of IL-9 production via the BATF signaling pathway by TL1A is conserved between mouse and human T_H9 cells.

In contrast to BATF, the role of BATF3 in the differentiation of T_H cells is less defined. To confirm our RNA-seq data, we determined the expression level of *Batf3* in T_H9 cells and other T_H subsets and found that *Batf3* mRNA expression was highest in T_H9, T_H1, and T_H2 cells (Figure 2h). TL1A enhanced the expression of *Batf3* under T_H9 conditions (Figure 2i). Intracellular staining for BATF3 confirmed that T_H9-TL1A stimulation significantly increased the percentage of $BATF3^+$ $IL\text{-}9^+$ cells (Figure 2j, k).

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In human T_H9 cells TL1A significantly enhanced the expression of $BATF3^+IL\text{-}9^+$ cells (Figure 2l, m). Similar to BATF, the majority of IL-9-producing cells co-expressed BATF3.

BATF and BATF3 are critical transcription factors in TL1A-induced T_H9 differentiation

To further define the role of BATF and BATF3, we performed ChIP assays to determine binding at two *Il9* conserved noncoding sequences (*Il9* CNS1, CNS0) [35]. TL1A alone induced significantly greater binding of BATF to *Il9* CNS1 compared to T_H9 conditions and T_H9-TL1A further enhanced BATF binding (Figure 3a). At the more upstream *Il9* CNS0, TL1A also enhanced BATF binding under T_H9 conditions while TL1A alone had no effect (Figure 3b). TL1A enhanced IRF4 binding under T_H9 conditions at CNS1 and CNS0 (Figure 3a, b). Interestingly, binding of acetylated histone 3 (AcH3) at CNS1 and CNS0 that was previously described to facilitate chromatin remodeling at the *Il9* locus during T_H9 differentiation [14] was also enhanced under T_H9-TL1A conditions (Figure 3a, b). BATF3 binding to CNS1 was upregulated in T_H9 cells and the binding was further enhanced under T_H9-TL1A conditions while BATF3 did not bind to CNS0 (Figure 3a, data not shown). These data suggest that TL1A enhances BATF, BATF3, and IFR4 binding to the *Il9* promoter under T_H9 conditions to induce *Il9* expression.

To confirm roles for BATF and BATF3 in TL1A-dependent T_H9 differentiation, we used *Batf*$^{-/-}$ and *Batf3*$^{-/-}$T cells. IL-9, IL-10, and IL-13 production was severely impaired in *Batf*$^{-/-}$ cells under T_H9 conditions (Figure 4a-d). However, TL1A stimulation at least partially overcame the requirement for BATF for IL-9 and IL-13 production during T_H9 differentiation (Figure 4a, b, d). In the absence of BATF3 the induction of IL-9 was significantly reduced, particularly under T_H9-TL1A conditions. (Figure 4e, f, Supplementary Figure 2a). In contrast to BATF, BATF3 was not required for IL-10 or IL-13 expression (Figure 4g, h, Supplementary Figure 2b, c). To define the signaling pathways that lead to BATF and BATF3 upregulation in the presence of TL1A we used *Stat6*$^{-/-}$ and *p50*$^{-/-}$ cells. *p50*$^{-/-}$ T_H9 cells had reduced percentages of $BATF^+IRF4^+$ cells and the increase in the percentages of $BATF^+IRF4^+$ cells under TL1A stimulation was almost completely abolished (Supplementary Figure 3a, b). Upregulation of *Batf* and *Batf3* mRNA under T_H9 conditions was p50-independent while the enhancement of *Batf* and *Batf3* expression under T_H9-TL1A conditions was completely p50-dependent (Supplementary Figure 3c, d). *Stat6*$^{-/-}$ T_H9 cells had reduced percentages of $BATF^+IRF4^+$ cells and their increase under T_H9-TL1A stimulation was completely abolished (Supplementary Figure 4a, b). Similarly, upregulation of *Batf* and *Batf3* mRNA was completely dependent on STAT6 under T_H9 and T_H9-TL1A conditions (Supplementary Figure 4c, d). We observed no significant effects of BATF or BATF3-deficiency on the expression of *Batf3* and *Batf*, respectively, suggesting that there is no compensation between the two transcription factors (Supplementary Figure 5). Together these data suggest that BATF and BATF3 play important and distinct roles during T_H9 differentiation in the presence of TL1A and that increased BATF and BATF3 expression under TL1A stimulation is dependent on STAT6 and the canonical NF-κB pathway.

Next, we performed RNA sequencing on WT and *Batf3*$^{-/-}$ T_H9-TL1A cells. 139 genes were differentially expressed in WT versus *Batf3*$^{-/-}$ T_H9-TL1A cells (Figure 4i, Supplementary Table 4). Gene ontology pathway enrichment analysis identified significant changes in genes

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involved in regulation of transcription, cell proliferation, and intracellular signal transduction (Supplementary Table 5, 6). These results reveal a role for BATF3 in the differentiation of T_H9-TL1A cells.

T_H9-TL1A cells are highly pathogenic in vivo

To determine the pathogenic potential of T_H9-TL1A cells *in vivo*, we adoptively transferred *ex vivo* differentiated CD45.1$^+$ T_H9 or T_H9-TL1A cells into *Rag1*$^{-/-}$ mice. Mice that received T_H9 cells did not develop significant weight loss (Figure 5a). However, mice that received T_H9-TL1A cells lost weight and developed more severe intestinal inflammation, particularly in the small intestine (Figure 5a-c). We also observed severe pulmonary inflammation, characterized by peribronchial and perivascular cellular infiltration of inflammatory cells in T_H9-TL1A recipients. Staining with Alcian Blue periodic acid-Schiff (AB-PAS) showed hyperproliferation of mucin-producing cells in the airway epithelium of T_H9-TL1A recipients (Supplementary Figure 6a, b). We observed higher cellularity in spleens and lamina propria mononuclear cells (LPMC), and higher percentages and absolute numbers of splenic, MLN, and LPMC CD4$^+$ cells in T_H9-TL1A recipients (Figure 5d, e) suggesting that T_H9-TL1A cells have a higher proliferative potential *in vivo*. Ki67 staining confirmed that cells from MLN, spleen, and LPMC from T_H9-TL1A recipients are more proliferative even 6 weeks after T cell transfers (Figure 5f). Significantly higher secretion of IL-13, and IL-17 in anti-CD3/anti-CD28 restimulated MLN cells from T_H9-TL1A recipients was observed (Figure 5g), and significantly higher secretion of IL-9 and IL-13 in anti-CD3/anti-CD28 restimulated LPMC from T_H9-TL1A recipients (Figure 5h). We observed a higher absolute number of splenic and MLN IL-9$^+$, IL-13$^+$, and IL-17$^+$ cells and a higher absolute number of MLN, splenic, and LPMC CCR6$^+$ and CD103$^+$ T cells in T_H9-TL1A recipients consistent with a role of these cells at mucosal surfaces (Figure 5i, j).

IL-9 is required for in vivo inflammation driven by T_H9-TL1A cells

To examine whether increased IL-9 production by T_H9-TL1A cells contributed to intestinal and lung inflammation, we treated mice receiving T_H9-TL1A cells with neutralizing IL-9 or control antibodies for the duration of the T cell transfer experiment. Histopathological analysis demonstrated significantly reduced intestinal inflammation in anti-IL-9 treated mice compared to isotype controls (Figure 6a, b). We also observed attenuated lung inflammation with decreased peribronchial infiltrates and reduction in mucus-producing cells in anti-IL-9 treated mice (Supplementary Figure 6c). Total cell numbers and total CD4$^+$CD45.1$^+$ T cells in MLN were reduced in anti-IL-9 treated mice compared to isotype controls (Figure 6c). Anti-IL-9 treatment also reduced production of IL-13 and IL-17 in MLN and spleens while IFN-γ production was unchanged (Figure 6d, data not shown). Together these data demonstrate that T_H9-TL1A cells were potent effector cells and resulted in intestinal and lung inflammation *in vivo*, and that the pathogenic effect was IL-9-dependent.

BATF3 contributes to in vivo inflammation driven by T_H9-TL1A cells

To determine whether BATF3 plays a role in T_H9-driven pathologies *in vivo* we adoptively transferred *ex vivo* differentiated WT or *Batf3*$^{-/-}$ T_H9-TL1A into *Rag1*$^{-/-}$ mice. Mice that received *Batf3*$^{-/-}$ T_H9-TL1A cells develop significantly less pulmonary (Figure 7a, b, Supplementary Figure 6e) and a trend towards reduced intestinal inflammation (Histoscore:

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3.6 vs. 1.2) (Figure 7a). We observed reduced cellularity in lungs and LPMC, and lower percentages of MLN and lung $CD4^+$ T cells in *Batf3*$^{-/-}$ T_H9-TL1A recipients compared to WT T_H9-TL1A recipients (Figure 7c, Supplementary Figure 6d). Significantly lower percentages of IL-13^+, and IL-17^+ cells in spleens, MLN, and lungs from *Batf3*$^{-/-}$ T_H9-TL1A recipients were observed (Figure 7d). Collectively, our data indicate that BATF3 plays a role in IL-9 production and T_H9 development *in vitro* and in T_H9-TL1A-driven mucosal inflammation *in vivo*.

Discussion

T_H9 cells are important for the development of inflammatory and allergic diseases. However, the transcriptional program and inflammatory triggers that drive the differentiation of T_H9 cells remain incompletely understood. In this study, we identified the pro-inflammatory cytokine TL1A as a potent inducer of murine and human T_H9 differentiation and identified BATF3 as an important transcription factor involved in the differentiation of T_H9 cells. The transcriptional network that was induced by TL1A included the transcription factors BATF and BATF3 and several BATF-regulated genes (Supplementary Figure 7). *In vivo*, in adoptive transfer experiments T_H9-TL1A cells were highly pro-inflammatory leading to intestinal and lung inflammation. The pro-inflammatory propensity of T_H9-TL1A cells was dependent on IL-9 production. Transfers of *Batf3*$^{-/-}$ T_H9-TL1A cells resulted in reduced mucosal inflammation and cytokine expression *in vivo*.

Although T_H9 cells have been identified as a distinct T helper subset, a transcription factor that acts as a master regulator or solely identifies the T_H9 phenotype has not been identified. Instead, several transcription factors act in concert to regulate the differentiation of T_H9 cells. BATF has been shown to functionally cooperate with IRF4 and to bind to composite elements within the promoter regions of responsive genes. BATF along with IRF4 have been recently proposed as “pioneer factors” in the differentiation of T_H17 cells by contributing to the initial chromatin accessibility and facilitating the access of other transcription factors [36]. We observed that TL1A has a direct effect on BATF expression and it synergizes with TGF-β1 and IL-4 to maximally induce BATF expression particularly early in T_H9 differentiation. In support of this notion, stimulation with TL1A alone leads to the binding of BATF to the *Il9* promoter while it has no direct effect on the binding of other transcription factors (IRF4, BATF3). However, T_H9-TL1A conditions synergistically enhances the binding of BATF3 and IRF4 as well as acetylation of histone H3, a permissive chromatin modification that correlates with *Il9* promoter activity [11, 14]. In line with previously published data BATF is required for the expression of IL-9 and IL-10 under T_H9 conditions [15]. TL1A is at least partially able to overcome the requirement for BATF in the induction of IL-9 and IL-13 most likely through enhancement of other transcription factors that might be able to compensate the loss of BATF. One candidate for functional compensation is BATF3 which is transcriptionally induced by TL1A. It has been proposed that BATF and BATF3 are functionally interchangeable for T_H17 and T_H2 development and overexpression of BATF3 in *Batf*$^{-/-}$ T cells can restore IL-17 production in T_H17 cells and IL-4 and IL-10 production in T_H2 cells [18, 32, 37, 38]. However, the role of BATF3 in the development of T_H cells under physiological conditions and potential interaction between BATF and BATF3 during T_H9 differentiation has not been defined. We demonstrate here that TL1A facilitates the binding

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of BATF3 to the *Il9* promoter. As a functional consequence, BATF3 contributes to IL-9 production under T_H9 conditions particularly in the context of TL1A stimulation. Surprisingly, BATF3 is dispensable for the early differentiation of T_H9 cells but might be required to stabilize or maintain the T_H9 phenotype and IL-9 secretion at later time-points. Our findings of a role of BATF3 in T_H9 differentiation are in contrast with previous reports of BATF3 being dispensable for T_H1, T_H2, and T_H17 differentiation [21, 37]. In contrast to BATF, BATF3 only contributes to IL9 secretion and not to other T_H9 cytokines such as IL-10 and IL-13, suggesting that BATF3 specifically binds to the *Il9* promoter and induces IL-9 secretion. Further studies are required to determine additional BATF3 target genes during T_H9 differentiation and its role in other T_H subsets.

Although other pro-inflammatory cytokines or mediators such as IL-1β, OX40, TSLP have been described to enhance T_H9 differentiation, TL1A seems to be unique in up-regulating BATF, thereby opening chromatin for the recruitment of other transcription factors including BATF3 [35, 39, 40]. Our RNA sequencing data support this conclusion identifying a subset of BATF-dependent genes significantly up-regulated by TL1A. Furthermore, our RNA-sequencing data reveal that BATF3 regulates the activity of transcription factors, cell proliferation, and intracellular signal transduction suggesting an important role of BATF3 during T_H9 differentiation. *In vivo, Batf3*$^{-/-}$ T_H9-TL1A cells resulted in reduced mucosal inflammation and cytokine expression. We did not observe impaired migration of *Batf3*$^{-/-}$ T_H9-TL1A cells to the intestine, which is in contrast to *Batf*$^{-/-}$ $CD4^+$ T cells that fail to upregulate intestinal migration markers and do not populate the intestines [41]. Our data support a role of BATF3 in the pathophysiological function of T_H9-TL1A cells.

IL-9 and T_H9 cells have been recently associated with the pathogenesis of IBD [6, 7, 8, 9, 10]. IL-9^+ $CD4^+$ T cells were enriched in patients with UC and high serum levels of IL-9 correlated with severe disease in patients with CD[6, 7, 8, 9, 10]. In an experimental model of hapten-induced UC-like disease, IL-9- and PU.1-deficient mice are protected from intestinal inflammation and anti-IL-9 antibodies are protective in a chronic prevention model [6]. However, we did not observe any effects of TL1A on PU.1 expression under T_H9 conditions. We have shown that TL1A stimulation results in upregulation of BATF, and BATF3 but not of PU.1. Although, PU.1 has been described as a master regulator of T_H9 differentiation, it is only required for the expression of a subset of T_H9 genes and binding of PU.1 is not changed in *Batf*$^{-/-}$ mice suggesting a complete independency of PU.1 and BATF [15].

We demonstrate that T_H9-TL1A cells are highly pathogenic *in vivo* and induce intestinal and lung inflammation. Intestinal inflammation in T_H9-TL1A recipients was mainly confined to the small intestine. Furthermore, we observed an increased number of $CCR6^+$ and $CD103^+$ cells in T_H9-TL1A recipients *in vivo.* These data are consistent with findings demonstrating that the chemokine receptor CCR6 and the integrin CD103 are expressed on T_H9 cells and facilitate migration to inflammatory sites and mucosal surfaces [15, 42]. The increased number of $CCR6^+$ cells in T_H9-TL1A recipients *in vivo* is consistent with our observation of mainly small intestinal inflammation since the CCR6/CCL20 axis has been shown to specifically facilitate the migration of T_H17 cells to the small intestine and it is plausible that similar mechanisms apply to T_H9 cells [43]. Our findings are also consistent with recent findings of elevated IL-9 serum levels in CD patients, particularly with severe disease [8].

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TL1A enhances T_H1, T_H2, and T_H17 differentiation and although we did not see any indication of a global shift from T_H9 cells into other T_H subsets *in vitro*, it could suggest that T_H9 cells differentiated with TL1A might be unstable *in vivo* and "trans-differentiate" into other subsets. The potential of trans-differentiation of T_H9 cells has been controversial and might be dependent on the context, disease model, and immune status of the host. In EAE, trans-differentiation of T_H9 to IFN-γ producing cells occurs, however, the T_H9 phenotype seems to be stable in cancer models [3, 39]. Surprisingly, *in vivo* T_H9-TL1A cells continued to produce IL-9 and down-stream cytokines such as IL-17 and IL-13 and did not trans-differentiate. This notion is also supported by our data showing that anti-IL-9 treatment alone is sufficient to block the pathogenic effects of T_H9-TL1A cells. Anti-IL-9 antibody treatment alone reduced IL-13 production and attenuated intestinal and lung inflammation back to T_H9 levels. Development of allergic lung inflammation requires the corporation of T_H2 and T_H9 cells. However, our model of chronic, non-allergic lung inflammation seems to be mainly driven by IL-9-producing T_H9 cells, although we can't exclude a contribution of IL-9-producing non-T cells in pathogenesis in our model.

In summary, TL1A is a strong inducer of murine and human T_H9 differentiation and we elucidated the signaling pathways involved. Given the pro-inflammatory properties of T_H9-TL1A cells *in vivo* targeting the TL1A-BATF3-IL-9 axis may be a promising therapeutic approach in T_H9-driven pathologies such as in IBD or allergic lung disease.

Methods

Mice

C57BL/6J, C57BL/6 *Rag1*$^{-/-}$, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1), *Stat6*$^{-/-}$ (B6.129S2(C)*Stat6*^{tm1Gru}/J), *p50*$^{-/-}$ (B6.Cg-*Nfkb1*^{tm1Bal}/J), B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II), *Batf*$^{-/-}$ (B6.129S-Batf$^{tm1.1kmm}$/J), and *Batf3*$^{-/-}$ (B6.129S(C)-Batf3^{tm1kmm}/J) mice were purchased from the Jackson Laboratory. *Dr3*$^{-/-}$ mice have been described elsewhere [44]. Mice were maintained under SPF conditions. All animal studies were approved by the Cedars-Sinai Medical Center Animal Care and Use Committee.

T cell isolation and differentiation

Naïve T cells (CD62L^{high}CD44^{low}CD25^{-}; clones MEL-14, IM7, PC61.5; eBioscience) were isolated from spleens and MLN using the EasySep™ Mouse CD4^{+} Isolation Kit (Stem Cell Technologies) followed by cell sorting (MoFlo™, Beckman Coulter). Cells were cultured in RPMI-1640 medium (10 % FBS) with anti-CD3ε (145–2C11; BD Biosciences), anti-CD28 (37.51; eBioscience) (T_H0 conditions), murine TL1A (100 ng/ml; R&D Systems) under T_H9 conditions (human TGF-β1 [3 ng/ml], BioLegend, IL-4 [20 ng/ml], PeproTech). After 2 days, IL-2 (20 U/ml) was added. To evaluate cell proliferation, sorted cells were labeled with carboxy fluorescein succinimidyl ester (CFSE; Invitrogen), differentiated for 5 days, and CFSE dilution was assessed by flow cytometry. To evaluate antigen-specific T_H9 differentiation, naïve CD4^{+} T cells from OT-II mice were stimulated for 3 days with 1 μg/ml $OVA_{323–339}$ peptide in the presence of syngeneic APCs (ratio 1:3). APCs were prepared by depletion of CD90.2^{+} T cells from splenocytes (Stem Cell Technologies) followed by Mitomycin C treatment.

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Isolation of human CD4+ T cells and differentiation

Blood was obtained from healthy volunteers after informed consent in accordance with procedures established by the Cedars-Sinai Institutional Review Board (IRB # 3358, 2673). Naïve $CD4^+$ T cells ($CD4^+CD25^-CD45RA^+CD45RO^-$) were isolated from PBMC using the EasySep™ Human $CD4^+$ T cell Enrichment Kit (Stem Cell Technology) followed by cell sorting. Cells were cultured with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) with human TL1A (100 ng/ml; Fitzgerald) under T_H9 conditions (human TGF-β1 [5 ng/ml], human IL-4 [10 ng/ml]).

ELISA

Cytokine concentration in culture supernatants was assayed by ELISA for murine or human IL-9, IL-10, IL-13 (eBioscience).

Intracellular staining

Cells were re-stimulated with 50 ng/ml PMA (phorbol 12-myristate 13-acetate), 500 ng/ml ionomycin, and Monensin (eBioscience) for 4 h, stained with anti-CD4 (RM4–5, eBioscience), fixed and permeabilized using the FoxP3 staining buffer set (eBioscience) and stained with antibodies against murine IL-9 (RM9A4, BioLegend), IL-10 (JES5–16E3), IL-13 (13A), IL-17A (eBio17B7), IL-17F (eBio18F10), IFN-γ (XMG1.2), IL-4 (BVD6–24G2), IL-22 (1H8PWSR), Ki67 (SolA15), BATF (MBM7C7), IRF4 (3E4), human IL-9 (MH9A4, all eBioscience), and BATF3 (841792, R&D Systems). Samples were analyzed using a CyAn™ ADP flow cytometer (Dako Cytomation) and FlowJo software (TreeStar Inc.).

Quantitative RT-PCR

Total RNA was isolated using RNeasy kits and reverse transcribed into cDNA with Omniscript RT kit (both Qiagen). QPCR was performed using the Mastercycler® ep realplex^2 System (Eppendorf). Platimum® Quantitative PCR SuperMix-UDG (Invitrogen) and TaqMan probes and primers were used for *Actb, Il9, Il10, Il13, Batf* (IDT) (Supplementary Table 7). RT^2 SYBR® Green qPCR Mastermix (Qiagen) and primer sets were used for *Actb, Batf3* (IDT). mRNA expression of target genes was normalized to the expression of *Actb*. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using EZ ChIP chromatin immuno-precipitation kit (Millipore) followed by qPCR analysis. 1×10^6 cells were stimulated, fixed, sonicated, and immunoprecipitated using 2 μg of ChIP grade antibodies: anti-BATF (sc-100974), anti-BATF3 (sc-162246), normal mouse IgG (sc-2025), anti-IRF4 (sc-6059), normal goat IgG (sc-2028) (all Santa Cruz Biotechnology), anti-Acetyl-Histone H3 (17–615), and normal rabbit IgG (Millipore). Immunoprecipitated DNA was reverse-cross-linked, purified using spin columns, and analyzed by qPCR (Primer Sequences: Supplementary Table 7). To quantify immunoprecipitated DNA, we generated a standard curve from serial dilutions of input DNA. Data are presented as percentage of input DNA based on normalization against the amount of input DNA.

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RNA-Sequencing (GEO accession number: GSE60362, and GSE106926)

Low-input RNA-Seq (T_H9 vs. T_H9-TL1A): Clontech's SMARTer® Ultra™ Low Input RNA Kit for Sequencing v3 was employed to generate double-stranded cDNA libraries from 1.5 to 2.5 ng total RNA from each sample per manufacturer's recommendations. Double-stranded cDNA libraries were individually fragmented and ligated with Ion Torrent Ion Xpress™ Barcode Adapters using Ion Xpress™ Plus Fragment Library Kit with limited modifications including size-selected final libraries with a double bead clean-up and ½ volume of Ion Adapters. RNA-Seq libraries were assessed for concentration and length using Invitrogen's Qubit® dsDNA HS Assay Kit and Agilent High Sensitivity DNA Kit, respectively. Samples were multiplexed to obtain >10 million reads each for sequencing. The pooled libraries were amplified onto Ion Sphere™ and sequenced using the Ion PI™ Sequencing 200 Kit v2, Ion Torrent™.

mRNA-Seq (WT vs. Baft3$^{-/-}$ T_H9-TL1A): Illumina TruSeq Stranded mRNA library preparation kit was employed for mRNASeq. 1 μg of total RNA per sample was used for poly-A mRNA selection using streptavidin-coated magnetic beads. cDNA was synthesized from enriched and fragmented RNA using reverse transcriptase (Super-Script II, Invitrogen) and random primers. The cDNA was converted into double-stranded DNA, and enriched with PCR for library preparation. The PCR-amplified library was purified using Agencourt AMPure XP beads (Beckman Coulter). The concentration of the amplified library was measured with a NanoDrop spectrophotometer and on an Agilent 2100 Bioanalyzer. Samples were multiplexed to obtain > 20 million reads/sample and sequenced on a NextSeq 500 platform (Illumina) using 75bp singleend sequencing.

Data analysis: Raw reads were filtered and trimmed by FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and aligned using Tophat version 2.0.8 with UCSC GRCm38/mm10 mouse reference genome annotation (http://genome.ucsc.edu). Gene read counts were generated using HTSeq (v0.5.4) then normalized by the trimmed mean of the Mvalues normalization method with edgeR (v3.0.8) in Bioconductor in R (v2.15), which uses a weighted trimmed mean of the log expression ratios. For all analysis, only quality signals (threshold: 10 counts per million in at least two out of four samples) were used. Unsupervised analysis was performed using the top 100 ranked genes by IQR (Interquantile Range) then hierarchical clustering was generated using gplots (v2.11) using two-way Pearson correlations to visualize unbiased pervasive gene expression patterns. Supervised analysis using a modified fisher's exact test in edgeR and a false discovery rate cutoff of 10% using Benjamini and Hochberg procedure was used to determine differential expressions between T_H9 and T_H9-TL1A or WT and *Batf3*$^{-/-}$ T_H9-TL1A. Pathway enrichment analysis was performed using DAVID 6.7 (http://david.abcc.ncifcrf.gov/) with count threshold = 5 and ease score threshold = 0.05.

T cell transfer model

$CD4^+CD62L^{high}CD44^{low}CD25^-$ T cells from CD45.1 mice were differentiated into T_H9 or T_H9TL1A cells for 3 days. Male *Rag1*$^{-/-}$ recipient mice were injected i.p. with 0.5×10^6 T_H9, or T_H9TL1A cells. Mice were weighed for 6 to 8-weeks. For neutralization experiments, mice were injected i.p. with anti-IL-9 or IgG_{2b} control antibody (both 50 μg/

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dose; R&D Systems) 3 times per week for 4 weeks and twice per week for the remaining time starting at the day of T cell transfer. Tissues were formalin-fixed, paraffin-embedded, and stained with H & E or AB-PAS. Inflammation was scored by a semi-quantitative scoring system by a trained pathologist blinded to the experimental conditions [45, 46]. Lamina propria mononuclear cells (LPMC) were isolated from the large intestine as previously described [47]. Lung tissue was digested for 45 min. at 37ºC with 10 ml HBSS containing 15 μg/ml Liberase™ (Roche) and 25 μg/ml DNase I (Sigma) and filtering through a 40 μm cell strainer followed by red blood cell lysis. Single-cell suspensions were stained with anti-CD4, anti-CD45.1 (A20), anti-CCR6 (29–2L17), anti-CD103 (2E7, all eBioscience), and anti-Ki-67 (SolA15, BD PharMingen) antibodies for flow cytometry or restimulated with anti-CD3ε/anti-CD28 antibodies for 3 days. Cytokine levels in supernatants were measured by ELISA.

Statistics

Statistical significance was calculated using SPSS software (IBM SPSS Statistics 20, IBM Machines Corp., Armonk, NY, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc unpaired two-tailed Student *t*-test, or Mann-Whitney *U*-test as indicated. Differences were considered significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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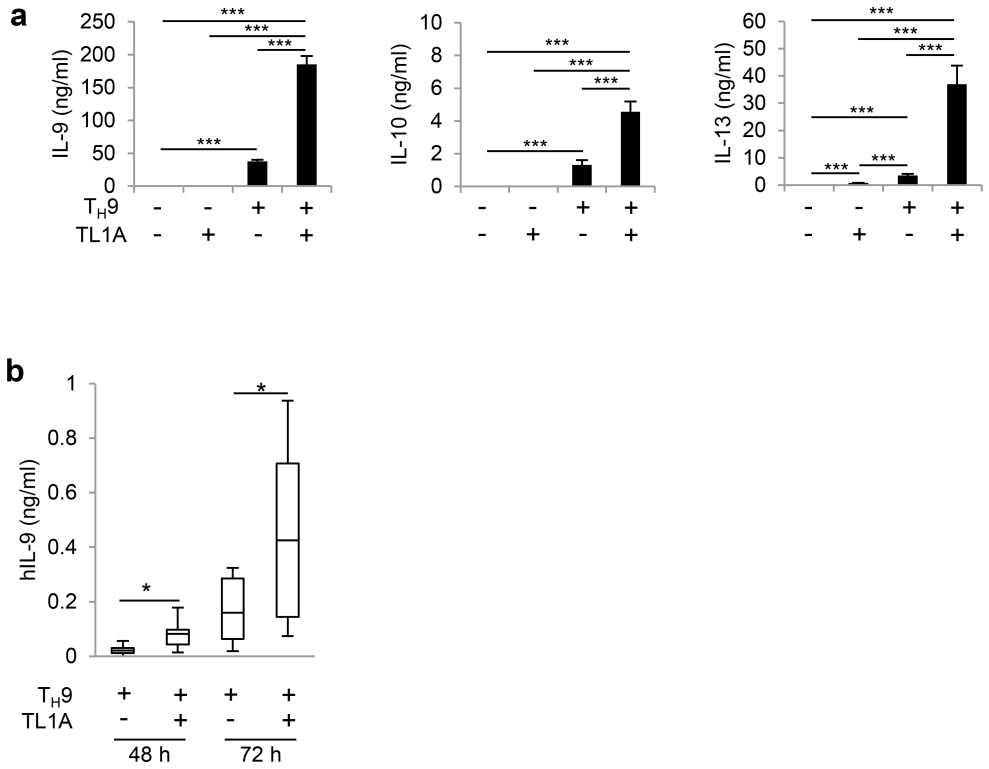

Figure 1. TL1A enhances the differentiation of murine and human T_H9 cells.

(**a**) Naïve murine $CD4^+$ T cells were differentiated under T_H0- or T_H9-polarizing conditions with or without TL1A for 3 days. (**a**) ELISA analysis of IL-9, IL-10, and IL-13 secretion. (**b**) Naïve human $CD4^+$ T cells ($CD4^+CD45RA^+CD45RO^-CD25^-$) were isolated from PBMCs of healthy donors and differentiated under T_H9-polarizing conditions with or without TL1A for 3 days. ELISA analysis of human IL-9 secretion at 48 and 72 h. Box-whisker plot showing the IL-9 concentrations in culture supernatants for T_H9 and T_H9-TL1A treated cells. The boxes represent the median and 25th and 75th percentile, and the whiskers represent the minimum and maximum values of the distribution. N= 7–9. Data represent means ± SD of one independent experiment out of four (**a**) independent experiments. *, $p<0.05$, ***, $p<0.005$ determined by Student's *t*-test.

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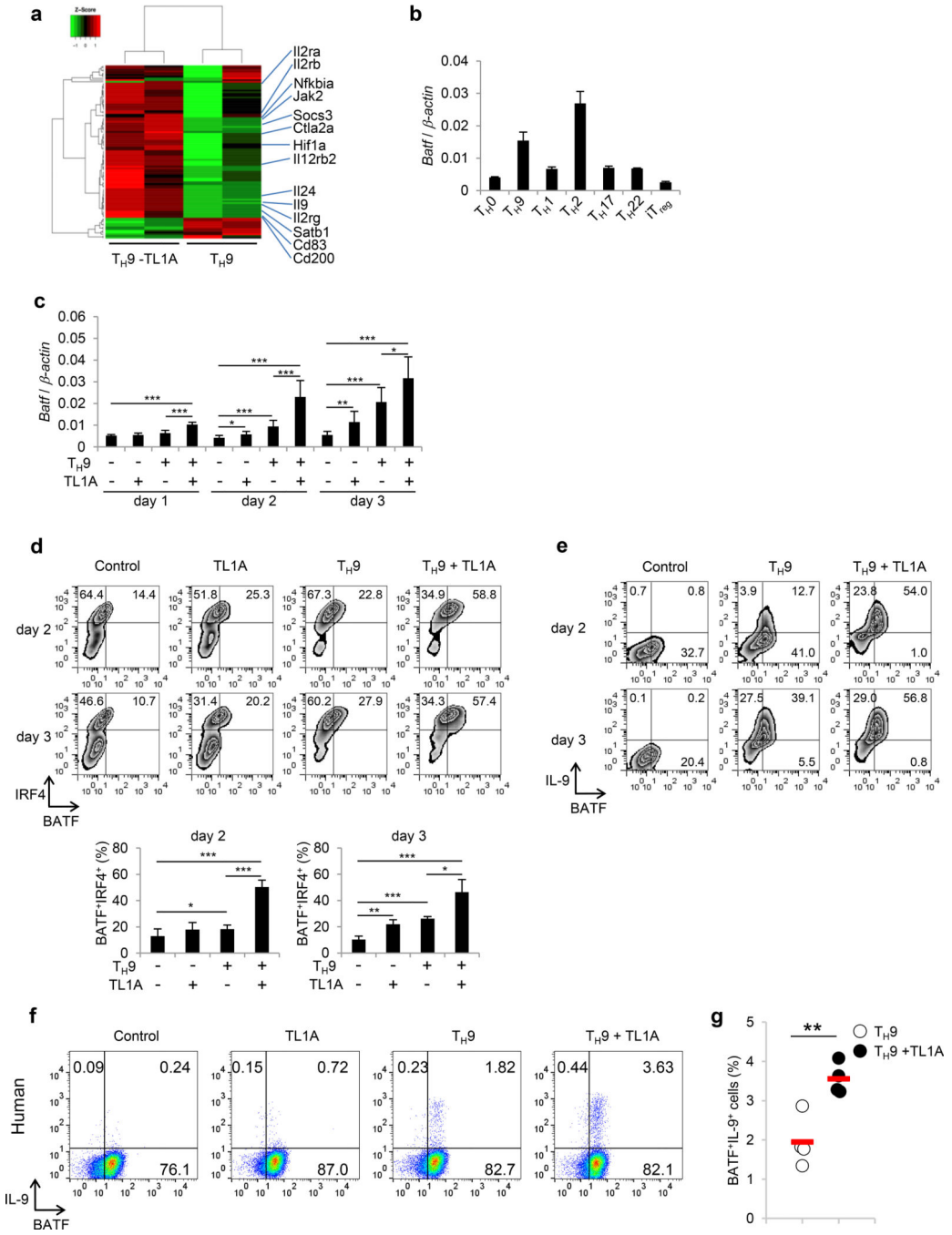

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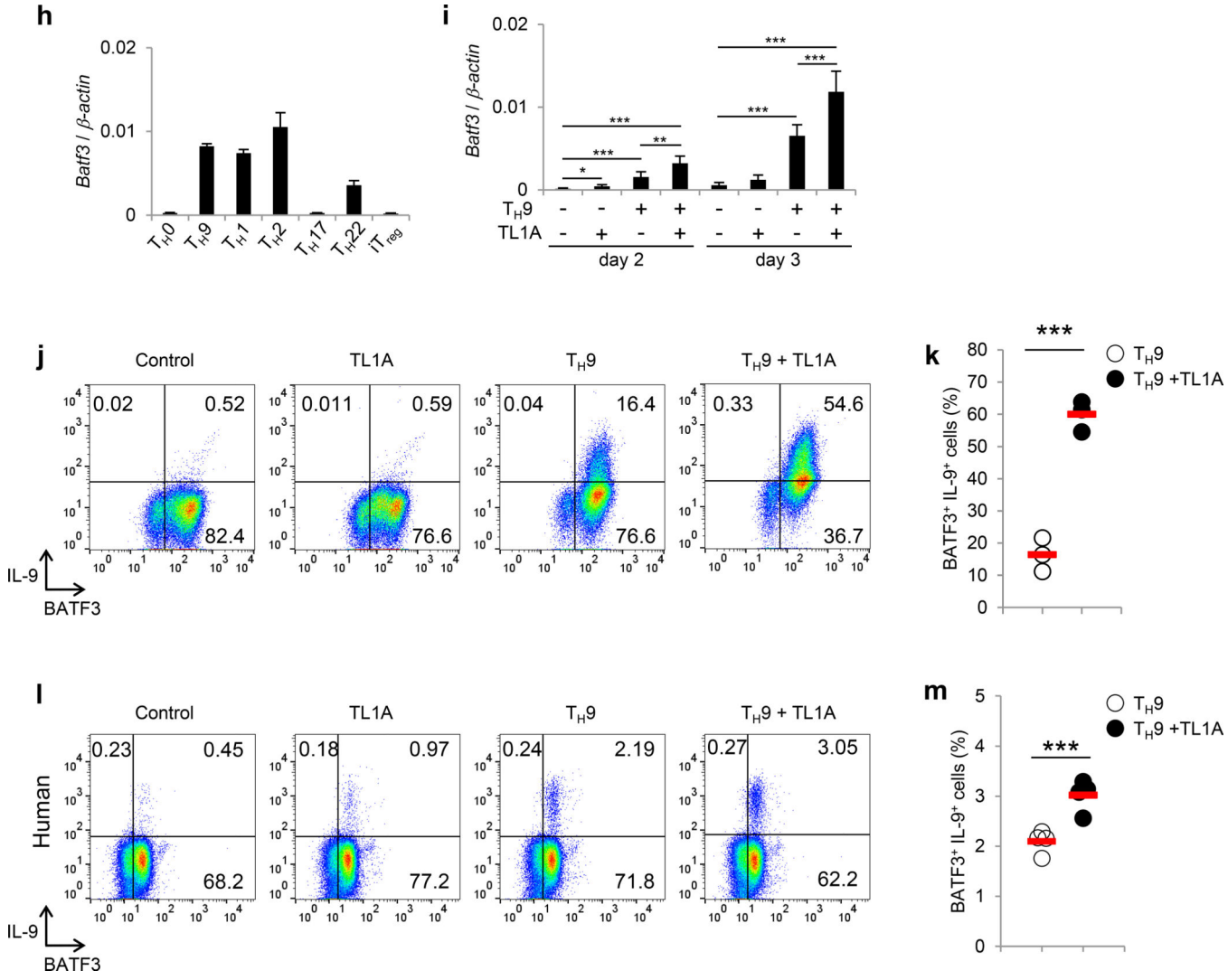

Figure 2. TL1A upregulates BATF and BATF3 expression during T_H9 differentiation.

(**a**) Transcriptional profiling of T_H9 and T_H9-TL1A cells by RNA sequencing. Heat map displaying RNA sequencing data using the top 100 genes with the largest IQR (Interquantile Range). The dendrograms to the left and above the heat map represent clustering of genes (rows) and samples (columns). (**b**) Naïve $CD4^+$ T cells were differentiated into different T_H subsets and Tregs for 3 days. Relative *Batf* mRNA expression was analyzed by qPCR. (**c**) Naïve $CD4^+$ T cells were differentiated under T_H0- or T_H9-polarizing conditions with or without TL1A for the indicated time periods. QPCR analysis of relative *Batf* mRNA expression. (**d**) Co-staining of BATF and IRF4 (top). Quantitative analysis of percentages of $BATF^+IRF4^+$ cells (bottom). (**e**) Co-staining of BATF and IL-9. (**f-g**) Naïve human $CD4^+$ T cells were isolated from PBMCs of healthy volunteers and differentiated under T_H9-polarizing conditions with or without TL1A. (**f**) Representative intracellular staining of IL-9 and BATF at 48 h. (**g**) Frequency of $BATF^+IL\text{-}9^+$ T cells differentiated under T_H9 and T_H9-TL1A conditions. Means are shown. Each symbol represents an individual donor. N=4. (**h**) Relative *Batf3* mRNA expression in different murine T_H subsets and Tregs was analyzed by

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qPCR. (**i**) Relative *Batf3* mRNA expression at indicated time points. (**j**) Representative co-staining of BATF3 and IL-9. (**k**) Frequency of $BATF3^{+}IL\text{-}9^{+}$ T cells differentiated under T_H9 and T_H9-TL1A conditions. Means are shown. Each symbol represents an individual experiment. N=3. (**l-m**) Naïve human $CD4^{+}$ T cells were differentiated under T_H9-polarizing conditions with or without TL1A. (**l**) Representative intracellular staining of IL-9 and BATF3 at 48 h. (**m**) Frequency of $BATF3^{+}IL\text{-}9^{+}$ T cells differentiated under T_H9 and T_H9-TL1A conditions. Means are shown. Each symbol represents an individual donor. N=4. Data represent means ± SD of one independent experiment out of two (a) or at least three (b-e, h, i) independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.005$ as determined by Student's *t*-test.

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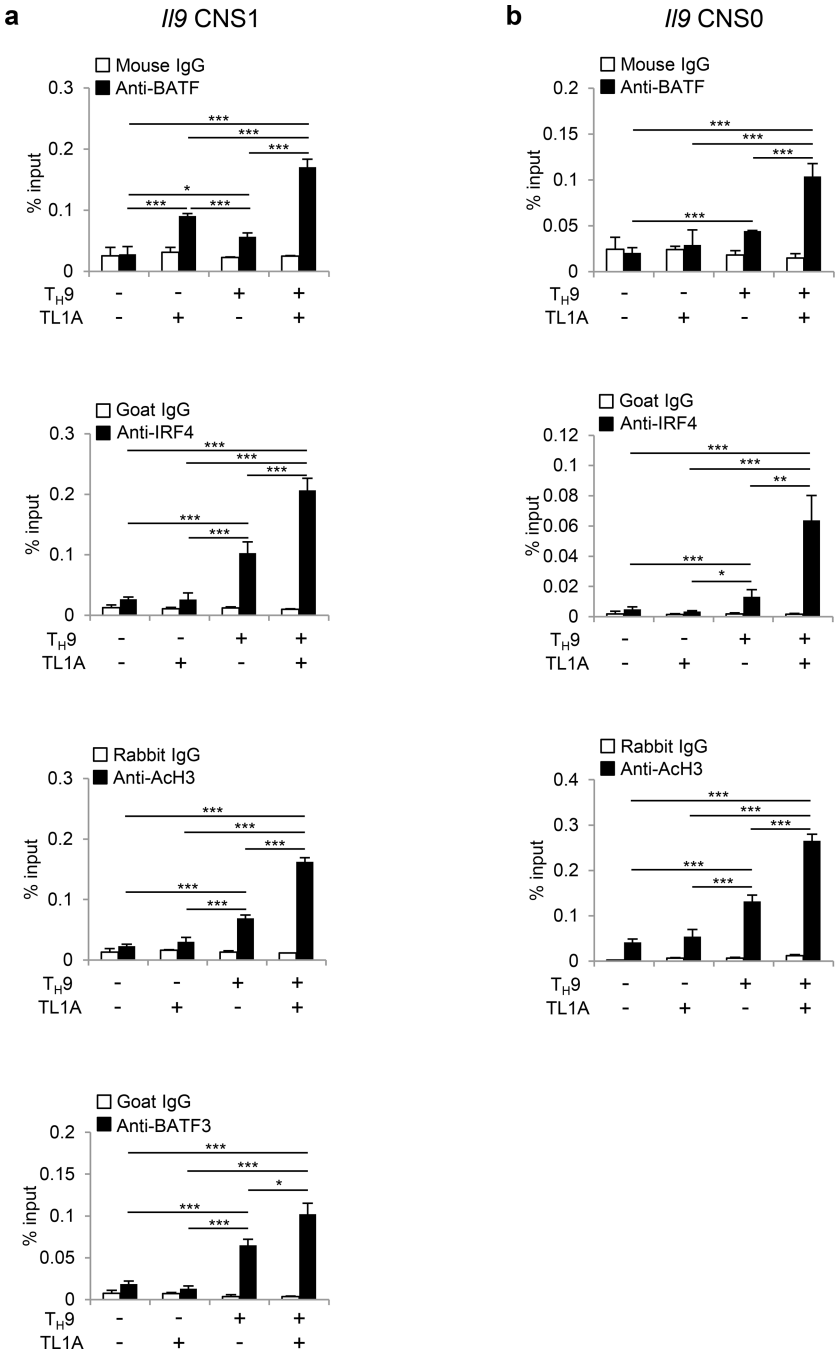

Figure 3. TL1A enhances binding of BATF, IRF4, and BATF3 to the *Il9* promoter during T_H9 differentiation.

(**a-b**) Naïve $CD4^+$ T cells were differentiated under T_H0- or T_H9-polarizing conditions with or without TL1A for 2 (BATF, IRF4, AcH3), or 3 days (BATF3). ChIP assays were performed for BATF, IRF4, H3AC, or BATF3 binding to conserved noncoding sequences (CNS) 1 (**a**) and CNS0 (**b**) of *Il9*. Data represent means ± SD of one independent experiment out of two independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.005$ determined by Student's *t*-test.

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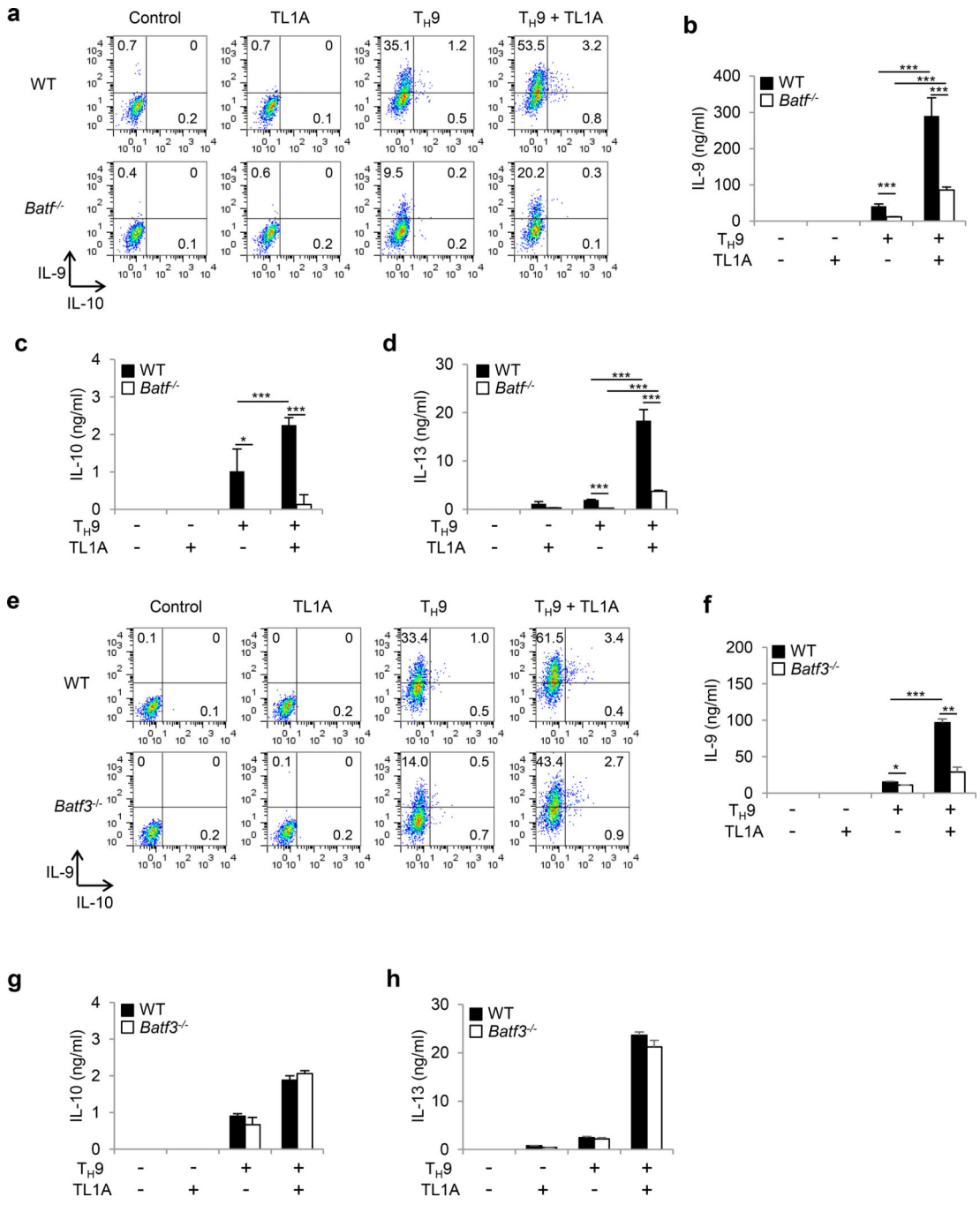

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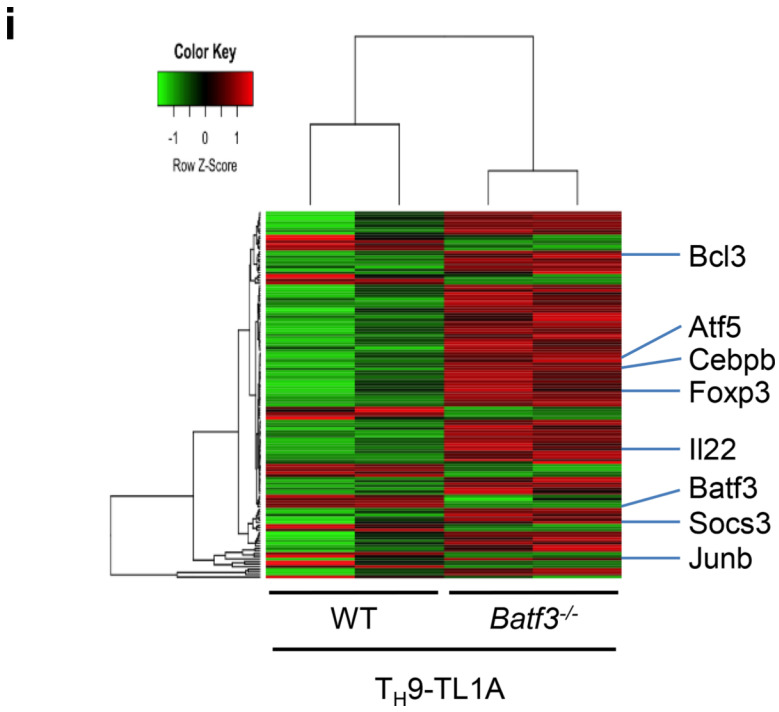

Figure 4. Effects of BATF and BATF3 deficiencies on T_H9 differentiation.

(**a-d**) Naïve $CD4^+$ T cells from *Batf*$^{-/-}$ or WT mice were differentiated under T_H0- or T_H9-polarizing conditions with or without TL1A for 3 days. (**a**) Intracellular staining of IL-9 and IL-10. (**b**) ELISA analysis of IL-9 production. (**c**) ELISA analysis of IL-10 production. (**d**) ELISA analysis of IL-13 production. (**e-h**) Naïve $CD4^+$ T cells from *Batf3*$^{-/-}$ or WT mice were differentiated under T_H9-polarizing conditions with or without TL1A for 3 days. (**e**) Intracellular staining of IL-9 and IL-10. (**f**) ELISA analysis of IL-9 production. (g) ELISA analysis of IL-10 production. (h) ELISA analysis of IL-13 production. (**i**) Transcriptional profiling of WT and *Batf3*$^{-/-}$ T_H9-TL1A cells by RNA sequencing. Heat map displaying RNA sequencing data of genes differentially expressed with $p<0.01$. The dendrograms to the left and above the heat map represent clustering of genes (rows) and samples (columns). Data represent means ± SD of one independent experiment out of two (i) or three (a-h) independent experiments. *, $p<0.05$, ***, $p<0.005$ determined by Student's *t*-test.

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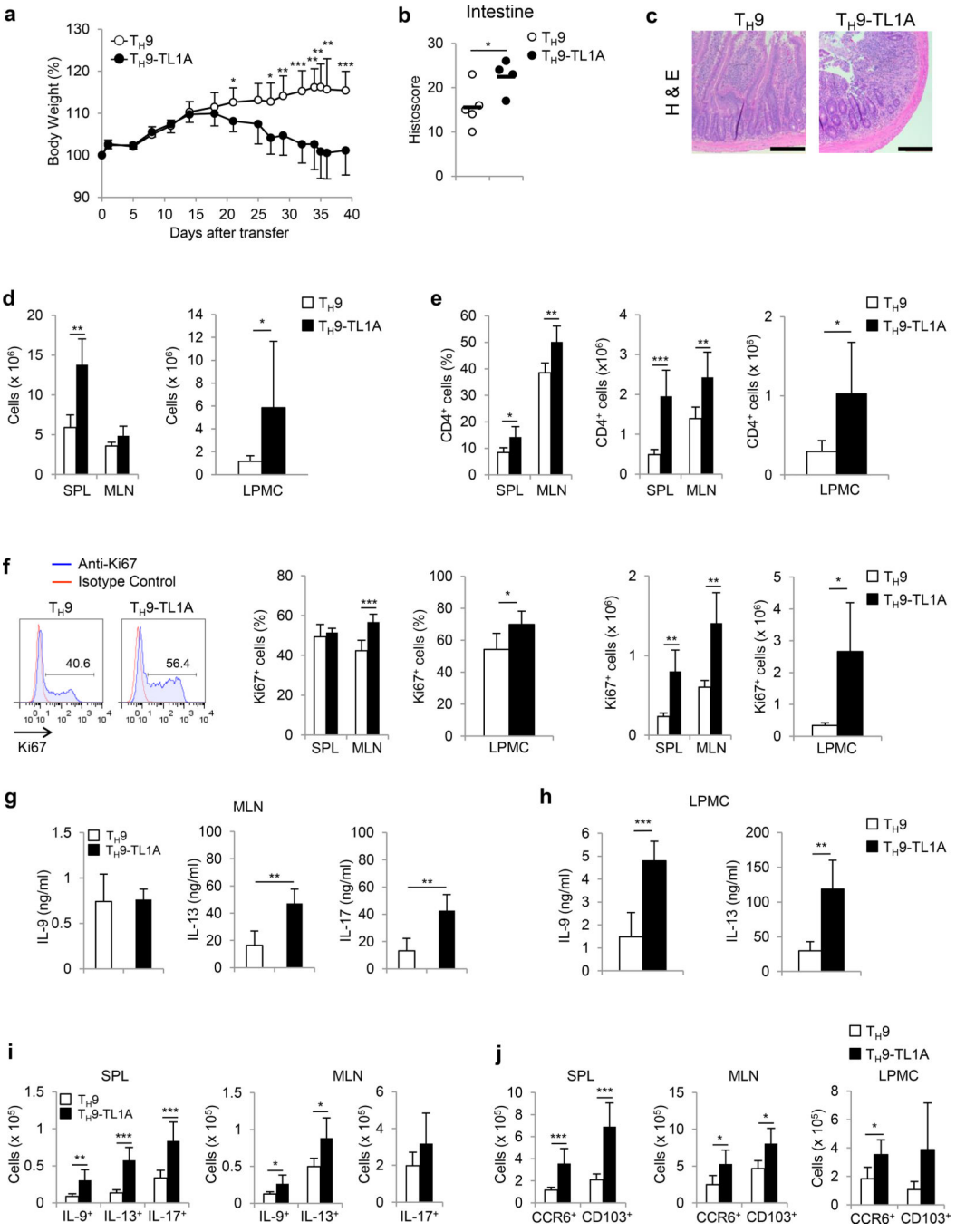

Figure 5. T_H9 cells differentiated in the presence of TL1A induce more severe mucosal inflammation.

Naïve $CD4^+$ T cells from CD45.1 mice were differentiated under T_H9-polarizing conditions (T_H9) or T_H9 with TL1A (T_H9-TL1A) for 3 days. T_H9 or T_H9-TL1A cells were injected into *Rag1*$^{-/-}$ mice.(**a**) Body weight changes after T cell transfer. (**b**) Histology scores for intestine. (**c**) Representative H & E stains of duodenum. Scale bar: 200 mm. (**d**) Total cell counts in spleens, MLN, and LPMC. (**e**) Frequency of $CD4^+$ cells (left). Total cell counts of $CD4^+$ cells (right). (**f**) Representative histograms of Ki67 staining of MLN cells (left).

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Frequency of Ki67$^+$ cells in MLN, Spleens, LPMC (middle). Total cell counts of Ki67$^+$ cells in MLN, Spleens, LPMC (right). (**g**) ELISA analysis of IL-9, IL-17, and IL-13 production from MLN after *ex vivo* re-stimulation with anti-CD3 and anti-CD28. (**h**) ELISA analysis of IL-9, and IL-13 production from LPMC after *ex vivo* re-stimulation with anti-CD3 and anti-CD28. (**i**) Total cell numbers of IL-9$^+$, IL-13$^+$, and IL-17$^+$ CD4$^+$CD45.1$^+$ T cells in spleens (left) and MLN (right). (**j**) Total cell numbers of CD4$^+$CCR6$^+$, and CD4$^+$CD103$^+$ T cells in spleens (left), MLN (middle), and LPMC (right). Data represent means ± SD of one independent experiment out of three independent experiments. n=4–5/group. *, $p<0.05$, **, $p<0.01$, ***, $p<0.005$ as determined by Student's *t*-test.

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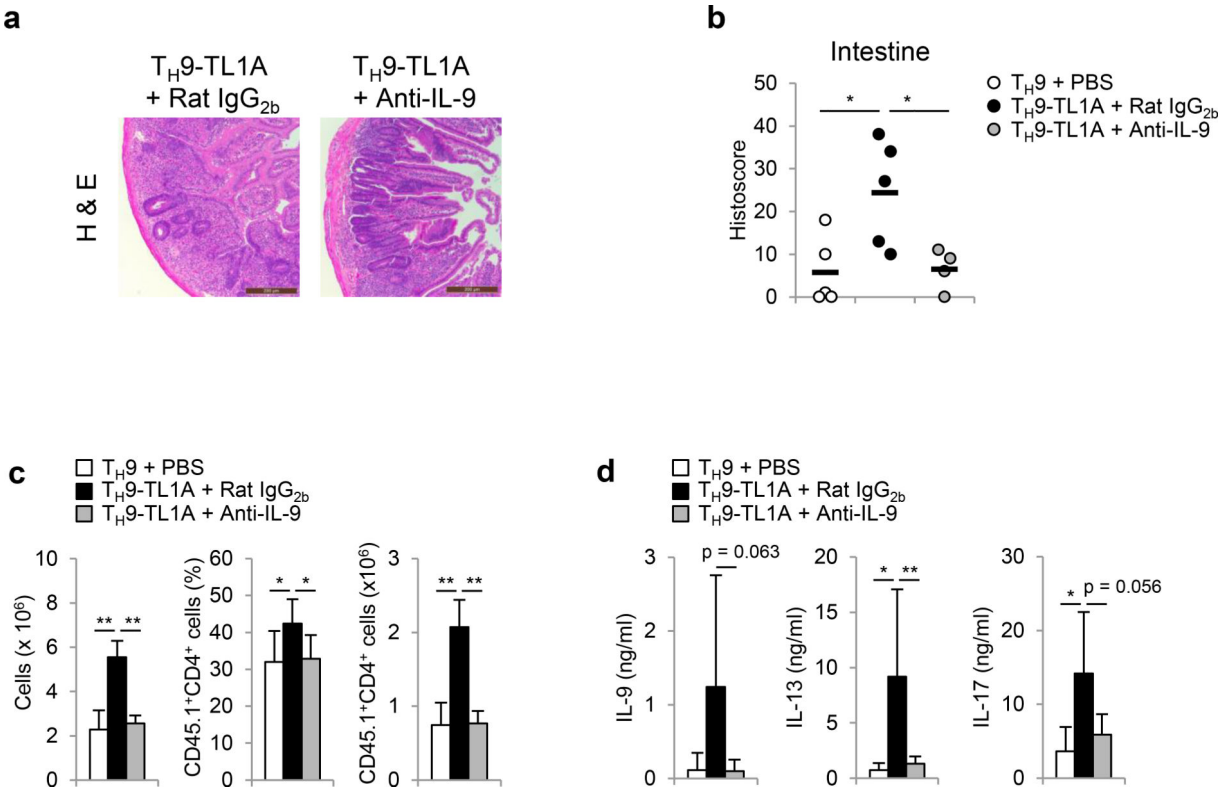

Figure 6. IL-9 is required for *in vivo* mucosal inflammation driven by T_H9-TL1A cells.

T_H9 or T_H9TL1A cells were injected into *Rag1*$^{-/-}$ mice and mice were treated three times per week with anti-IL-9 antibody or isotype control (Rat IgG_{2b}). (**a**) Representative H & E stainings of duodenum. Scale bar: 200 μm. (**b**) Histology scores for intestine. (**c**) Total cell counts (left), frequency of CD45.1$^+$ cells (middle), total cell counts of CD45.1$^+$ cells (right). **(d)** ELISA analysis of IL-9, IL-17, and IL-13 production from MLN after *ex vivo* re-stimulation with anti-CD3 and anti-CD28. Data represent means ± SD of one independent experiment out of two independent experiments. n=4–5/group. *, $p<0.05$, **, $p<0.01$, ***, $p<0.005$ as determined by Student's *t*-test (**b**) or Mann-Whitney *U*-test (**c, d**).

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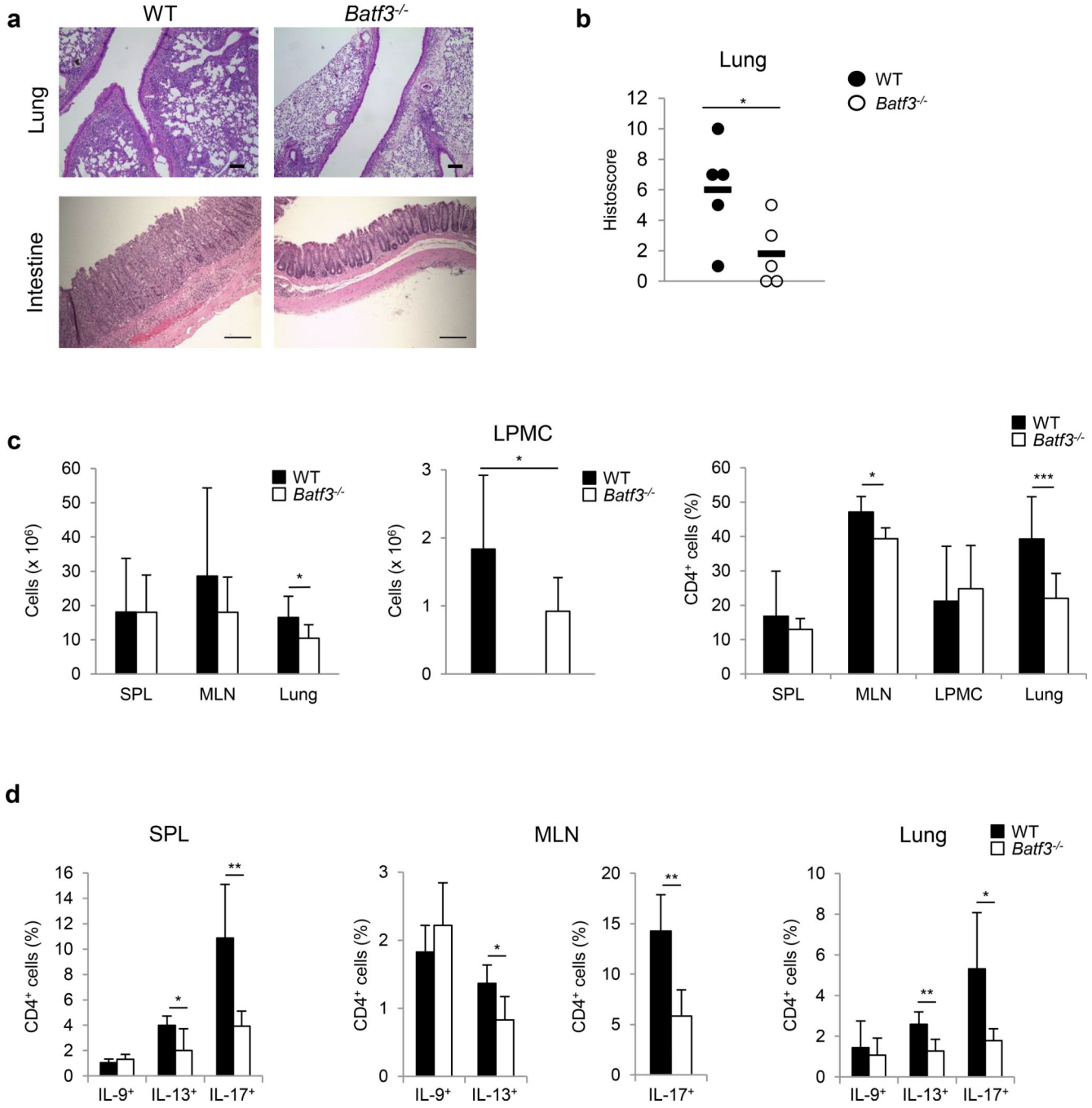

Figure 7. Batf3-deficiency leads to reduced mucosal inflammation driven by T_H9-TL1A cells.

Naïve WT or *Batf3*$^{-/-}$ cells were differentiated into T_H9-TL1A cells and injected into *Rag1*$^{-/-}$ mice. (**a**) Representative H & E stainings of lungs (top panels) and large intestines (bottom panels). Scale bar: 200 mm. (**b**) Histology scores for lungs. (**c**) Total cell counts (left, middle), frequency of CD4$^+$ T cells (right). Data represent means ± SD. n=10/group. **(d)** Intracellular staining of IL-9, IL-13, and IL-17 from Spleen, MLN, and lungs after *ex vivo* restimulation with PMA + Ionomycin. Data represent means ± SD. n=5/group. One

representative experiment out of three independent experiments is shown. *, $p<0.05$, **, $p<0.01$ as determined by Student's *t*-test.

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