ATP Drives Lamina Propria TH17 Cell Differentiation
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LETTERS

ATP drives lamina propria T_H17 cell differentiation

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Interleukin (IL)-17-producing CD4$^+$ T lymphocytes (T_H17 cells) constitute a subset of T-helper cells involved in host defence and several immune disorders[1,2]. An intriguing feature of T_H17 cells is their selective and constitutive presence in the intestinal lamina propria[3]. Here we show that adenosine 5′-triphosphate (ATP) that can be derived from commensal bacteria activates a unique subset of lamina propria cells, CD70^{high}CD11c^{low} cells, leading to the differentiation of T_H17 cells. Germ-free mice exhibit much lower concentrations of luminal ATP, accompanied by fewer lamina propria T_H17 cells, compared to specific-pathogen-free mice. Systemic or rectal administration of ATP into these germ-free mice results in a marked increase in the number of lamina propria T_H17 cells. A CD70^{high}CD11c^{low} subset of the lamina propria cells expresses T_H17-prone molecules, such as IL-6, IL-23p19 and transforming-growth-factor-β-activating integrin-αV and -β8, in response to ATP stimulation, and preferentially induces T_H17 differentiation of co-cultured naive CD4$^+$ T cells. The critical role of ATP is further underscored by the observation that administration of ATP exacerbates a T-cell-mediated colitis model with enhanced T_H17 differentiation. These observations highlight the importance of commensal bacteria and ATP for T_H17 differentiation in health and disease, and offer an explanation of why T_H17 cells specifically present in the intestinal lamina propria.

The intestinal mucosa has a unique and complicated immune system composed of a variety of cell populations. Among these, T_H17 cells, a subset of CD4$^+$ T cells characterized by their STAT3-dependent expression of RORγt (encoded by *Rorc*) and production of IL-17, IL-22 and IL-21, control a variety of bacterial and fungal infections at mucosal surfaces[1–7]. Importantly, aberrant T_H17 responses have been implicated in the pathogenesis of inflammatory bowel diseases[8]. The development of T_H17 cells has been shown to be controlled by the local cytokine milieu, including IL-6, transforming growth factor-β (TGF-β) and IL-23 (refs 1, 2, 7, 9–12). However, the mechanism of T_H17 development in the intestine is as yet not fully understood.

IL-17-expressing cells constitute a considerable proportion of CD4$^+$ cells in the intestinal lamina propria, even in healthy mice kept under specific-pathogen-free (SPF) conditions (Supplementary Fig. 1a and ref. 3). The colonic lamina propria CD4$^+$ cells also express messenger RNAs for IL-17, IL-17F and RORγt, representing the hallmarks of T_H17 cells (Supplementary Fig. 1b). The number of these 'naturally occurring' T_H17 cells in the colonic lamina propria increases with age (Supplementary Fig. 1c). Although interferon (IFN)-γ-positive CD4$^+$ cells are similarly observed in the lamina propria and spleen, IL-17-producing cells are rarely observed in the spleen, mesenteric lymph node (MLN) or Peyer's patches (Supplementary Fig. 1a and ref. 3). Furthermore, the lamina propria IL-17-producing CD4$^+$ cells were normally observed in Peyer's-patch- and colonic-patch-null mice[13] (Supplementary Fig. 2). These observations suggest that a specific environment in the lamina propria supports the generation of T_H17 cells *in situ*.

To investigate whether intestinal commensal bacteria are responsible for the generation of lamina propria T_H17 cells, we evaluated the numbers of T_H17 cells in germ-free mice. Although the numbers of colonic lamina propria CD4$^+$ cells were not significantly changed (Supplementary Fig. 3a), the numbers of IL-17-positive CD4$^+$ cells were greatly reduced in the large intestines of germ-free mice compared to those in SPF mice (Fig. 1a, b and Supplementary Fig. 3b). Consistent with previous reports[14], the germ-free mice also exhibited severe reductions in their faecal IgA levels (Supplementary Fig. 3c), demonstrating that commensal bacteria contribute to the provision of a particular environment for lamina propria T_H17 cells as well as IgA-producing cells. To examine the role of commensal bacteria further, we treated SPF mice with a combination of vancomycin and metronidazole by oral administration, and analysed lamina propria T_H17 cells. The vancomycin- and metronidazole-treated mice showed marked reductions in both their faecal IgA levels and their numbers of IL-17-producing CD4$^+$ cells (Supplementary Fig. 4a–c).

To assess the molecular basis for the commensal-bacteria-driven T_H17 differentiation, we examined the contribution of Toll-like receptor (TLR) signalling using *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice, which lack all TLR signalling. There was no detectable difference in the numbers of lamina propria IL-17-producing CD4$^+$ cells between control and mutant animals (Fig. 1c, d and Supplementary Fig. 3d), indicating that the development of lamina propria T_H17 cells is independent of TLR signalling. It is worth noting that *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice showed impaired secretion of IgA in their faecal pellets (Supplementary Fig. 3e), indicating that the development of intestinal T_H17 cells and IgA-producing cells are both dependent on microflora, but are regulated by different mechanisms.

ATP has recently been shown to modulate immune cell functions by means of activation of the ATP sensors, P2X and P2Y receptors[15–18]. In addition, bacteria have been shown to generate and secrete large amounts of ATP[19]. Indeed, ATP concentrations in faecal samples without bacterial lysis were much higher in SPF mice than in germ-free mice (Fig. 1e). Consistent with this result, ATP concentrations were reduced in faecal samples from SPF mice treated with vancomycin and metronidazole (Supplementary Fig. 4d). Furthermore, high ATP concentration was detected in the supernatant of *in vitro* cultured intestinal commensal bacteria derived from faeces of SPF mice (Supplementary Fig. 5). Therefore, although there might be other cellular sources of ATP such as dead epithelial cells, commensal bacteria may be a major source of intestinal luminal ATP. Interestingly, the faecal ATP concentrations were not reduced in

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Introduction

The intestinal mucosa has a unique and extremely complicated immune system composed of a variety of cell populations, such as immunoglobulin A (IgA)-producing B cells, regulatory T cells, γδT cells, and T cells that are dominated by a TH1 or TH2 phenotype. Among these, the best-characterized is the IgA-producing B cells, which are regulated by the presence of microflora for their own development and, in turn, regulate gut microbial ecology. Another cell population gaining much attention in the intestinal immune system is TH17 cells. TH17 cells are a subset of activated CD4+ T cells characterized by their Stat3-dependent expression of the transcription factor RORγt and production of IL-17 and IL-22 and thought to be required for the control of a variety of bacterial and fungal infections at mucosal surfaces (*1*) (*2*). More importantly, it has been shown that aberrant TH17 responses play leading roles in inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease. The development of TH17 cells has been shown to be controlled by the local cytokine milieu including IL-6, TGF-β and IL-23; however, the mechanism of TH17 development in the intestine is at present not fully understood..

TH17 induction by commensal bacteria

We first confirmed the presence of high frequencies of TH17 cells in the small and large intestinal lamina propria (LP) but not the spleen, mesenteric lymph nodes or Peyer's patches of healthy mice housed in a specific-pathogen-free (SPF) environment. It was noted that the frequency of TH17 cells appeared to increase with age. Since the temporal accumulation of TH17 in the LP correlated with the development of commensal bacteria, we analyzed TH17 in germ-free mice as well as mice treated with the antibiotics. In both situations elimination of commensal bacteria resulted in a marked reduction in the frequency of TH17 cells as well as fecal IgA. These observations suggested that the microflora somehow contributes to the development of TH17 cells in the intestine.

ATP derived from commensal bacteria promotes TH17 differentiation

Extracellular ATP has recently been shown to modulate immune cell functions via the activation of ATP sensors--P2X and P2Y receptors (*3*). In addition, bacteria have been shown to generate and secrete high amounts of extracullular ATP. Indeed, fecal samples from SPF mice exhibited dramatically higher levels of ATP than did samples derived from gnotobiotic or antibiotic-treated mice. Additionally, freshly isolated commensal bacteria produced high levels of ATP in supernatants during in vitro culture. To directly examine the role of ATP in TH17 differentiation, we used several complimentary approaches including treating mice with 1) a non-hydrolysable ATP homologue, ATPγS; 2) an ATP-hydrolyzing enzyme, apyrase; and 3) an antagonist of P2X receptors for ATP, TNP-ATP. Remarkably, administration of ATPγS to gnotobiotic mice resulted in a significant increase in the frequency of TH17 cells. This effect was specific for CD4+ T cells producing IL-17 since IFN-γ-producing T cells were not affected by this treatment. Alternatively, TH17 cells were significantly diminished in SPF mice treated with apyrase or TNP-ATP. Collectively these results suggested that ATP plays a vital role in LP TH17 differentiation.

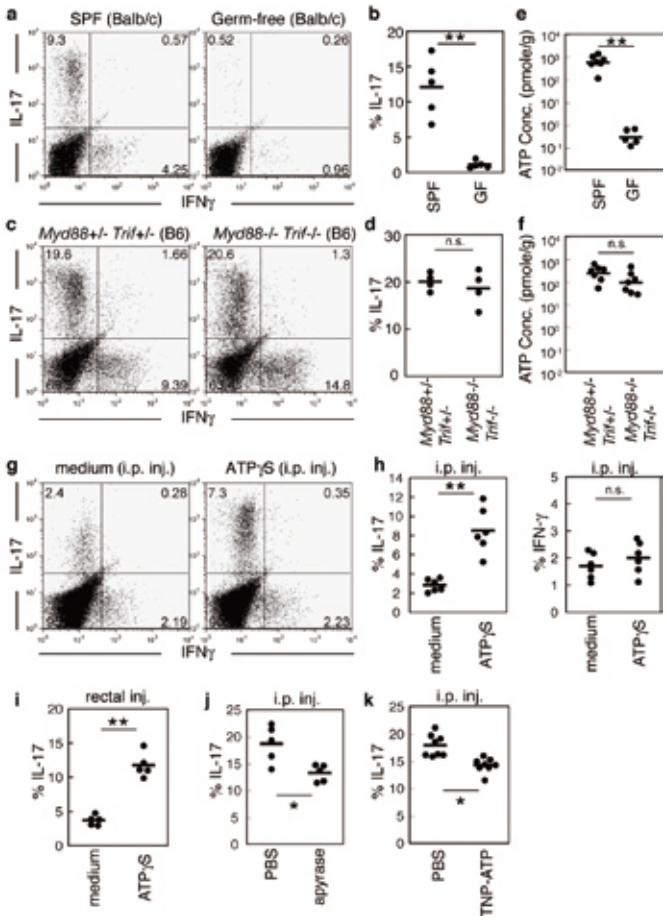

Fig. 1 The administration of ATP leads to a marked increase in the lamina propria TH17 cells in otherwise TH17-lacking germ-free mice. **a–d**, Representative FACS dot plots gated on colonic lamina propria CD4+ cells in the indicated mice are shown in **a** and **c**, and the percentages of IL-17-producing CD4+ cells of individual mice are shown in **b** and **d**. GF, germ free. **e**, **f**, Fecal ATP levels (pmol per g faeces) in the indicated individual mice. **g–k**, Germ-free mice (ICR) were daily injected intraperitoneally (i.p.) or rectally with medium or ATPS (**g–i**). SPF mice were i.p. injected with PBS or apyrase, or with TNP-ATP (**j**, **k**). All mice were processed for FACS as in **a–d**. Horizontal bars indicate the means. **P < 0.01, *P < 0.05; NS, not significant.

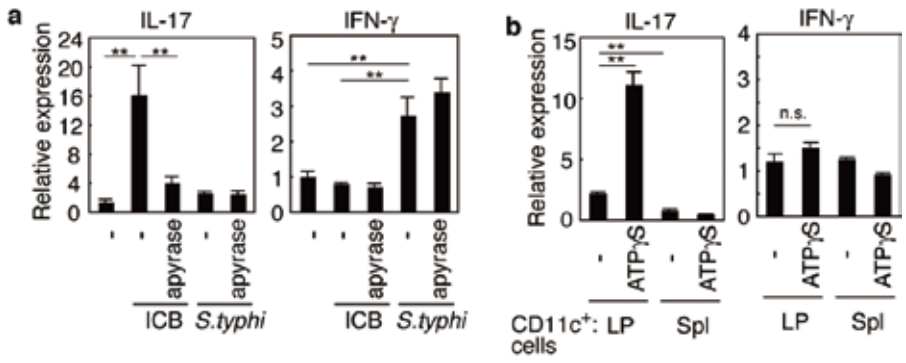

Fig. 2 ATP induces differentiation of naïve CD4+ T cells into TH17 cells. **a**, Splenic naive CD4+ T cells were co-cultured with colonic lamina propria CD11c+ with 20% conditioned medium from cultures of intestinal commensal bacteria (ICB) or S. typhimurium (S. typhi) in the presence or absence of apyrase. After 4 days, T cells were collected, restimulated and assayed for expression of *Il17* and *Ifng* by rRT–PCR. **b**, rRT–PCR analyses for the indicated genes of splenic naive CD4+ T cells cultured with colonic lamina propria (LP) or splenic CD11c+ cells in the presence of ATPγS. Error bars, s.d. *P < 0.05, **P < 0.01.

ATP acts on denderitic cells to promote TH17 differentiation

Dendritic cells (DCs) play a pivotal role in dictating CD4+ T cells to differentiate into TH1, TH2 or TH17 cells (*4*). Thus, we next tested whether extracellular ATP promotes TH17 differentiation via stimulation of the LP DCs. LP DCs significantly higher levels of mRNA for numerous P2X and P2Y receptors for ATP compared to DCs in other organs. In response to ATP, LP DCs expressed mRNA for several genes believed to be involved in TH17 differentiation. We also observed TH17 differentiation in co-cultured naïve CD4+ T cells with the lamina propria DCs in the presence of ATPγS in vitro. These results suggest that ATP acting on LP DCs induces IL-6 and TGF-β and subsequent TH17 differentiation.

Finally, we demonstrated a role for ATP in regulating intestinal inflammation in vivo using the well-established naïve CD4+ transfer model of colitis. Repeated administration of ATP exacerbated the symptoms of colitis including weight loss and diarrhea, and led to enhanced intestinal edema, inflammatory cell infiltrate, epithelial hyperplasia and goblet cell depletion. Importantly, increased disease severity correlated with the enhanced frequency of TH17 cells. Overall, these results suggest that ATP may play a role in promoting the differentiation of TH17 cells involved in the pathogenesis of intestinal inflammation.

Conclusion

In the present study, we identified extracellular ATP as a commensal bacteria-derived factor responsible for the TH17 differentiation in the lamina propria. The physiologic nature of these TH17 cells in the intestine will be an interesting future issue to be addressed in the context of the maintenance of homeostasis of intestinal mucosa against commensal and pathogenic bacteria. The elucidation of the entire picture of the regulation of development of intestinal regulatory T cells, TH17 cells, and other types of cells by ATP and its metabolites (ADP and adenosine) will provide valuable information on our understanding of the complicated intestinal mucosal immunity as well as establishment of an innovative ATP-targeted approach to treat patients with inflammatory bowel diseases.

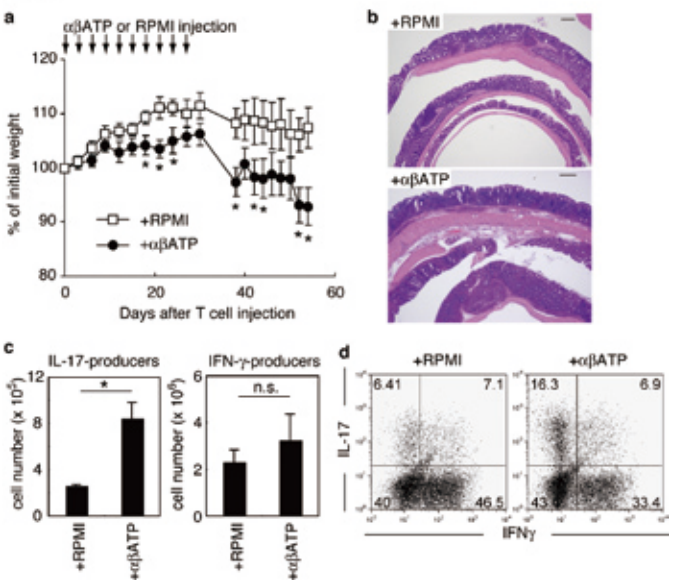

Fig. 3 Treatment with ATP exacerbated experimental colitis induced by adoptive transfer of naïve CD4+ T cells. SCID mice were transferred with naive CD4+ T cells on day 0, and were intraperitoneally injected with ATP (1.5 mg per mouse) or RPMI 1640 medium every 3 days for 1 month. **a**, The mean body weights of SCID mice injected with ATP or RPMI (n = 6 per group) were monitored. Error bars, s.e.m. *P < 0.05 versus RPMI-injected mice. **b**, Haematoxylin- and eosin-stained colon sections of SCID mice treated with ATP or RPMI. The numbers of IL-17- and IFN-γ-producing colonic lamina propria CD4+ cells (n = 3 per group) are shown in c, and representative flow cytometry plots of CD4+ T cells are shown in d. Error bars, s.d. *P < 0.02. NS, not significant. Two independent experiments were performed with similar results.

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