



# Transcriptional factor ATF3 protects against colitis by regulating follicular helper T cells in Peyer's patches

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**Disruption of mucosal immunity plays a critical role in the pathogenesis of inflammatory bowel disease, yet its mechanism remains not fully elucidated. Here, we found that activating transcription factor 3 (ATF3) protects against colitis by regulating follicular helper T (T<sub>FH</sub>) cells in the gut. The expression of ATF3 in CD4<sup>+</sup> T cells was negatively correlated with the severity of ulcerative colitis in clinical patients. Mice with ATF3 deficiency in CD4<sup>+</sup> T cells (CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>) were much more susceptible to dextran sulfate sodium-induced colitis. The frequencies of T<sub>FH</sub> cells, not other T cell subsets, were dramatically decreased in Peyer's patches from CD4<sup>cre</sup>Atf3<sup>fl/fl</sup> mice compared with Atf3<sup>fl/fl</sup> littermate controls. The defective T<sub>FH</sub> cells significantly diminished germinal center formation and IgA production in the gut. Importantly, adoptive transfer of T<sub>FH</sub> or IgA<sup>+</sup> B cells caused significant remission of colitis in CD4<sup>cre</sup>Atf3<sup>fl/fl</sup> mice, indicating the T<sub>FH</sub>-IgA axis mediated the effect of ATF3 on gut homeostasis. Mechanistically, B cell lymphoma 6 was identified as a direct transcriptional target of ATF3 in CD4<sup>+</sup> T cells. In summary, we demonstrated ATF3 as a regulator of T<sub>FH</sub> cells in the gut, which may represent a potential immunotherapeutic target in colitis.**

activating transcription factor 3 | follicular helper T cell | Peyer's patches | intestinal mucosal immunity | colitis

**D**efects in the gut mucosal immune system play an important role in the pathogenesis of inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis (UC). Gut microbiota promote the development of gut-associated lymphoid tissues (GALTs), which are responsible for the production of secretory IgA (sIgA) in the gut (1, 2). sIgA in lumen functions to maintain the indigenous members of microbiota and prevent the colonization of harmful microbes (3, 4). Once this delicate balance is disrupted, the hosts usually suffer from pathogenic conditions, especially IBD. sIgA, therefore, plays a protective role in IBD.

The production of IgA could be T cell-independent or T cell-dependent, with the latter as the dominant manner (2, 3). The major site of T cell-dependent IgA production occurs in Peyer's patches (PPs), which are the organized follicular structures present along intestinal walls. Indeed, follicular helper T (T<sub>FH</sub>) cells play a critical role in the facilitation of T cell-dependent production of IgA in PPs, through promoting germinal center (GC) formation and differentiation of B cells into IgA-producing plasmablasts. The plasmablasts then relocate to lamina propria and secrete high-affinity IgA into the intestinal lumen (5).

The major biological function of T<sub>FH</sub> cells is to facilitate GC formation, affinity maturation, and antibody production in activated B cells (6). The importance of T<sub>FH</sub> cells has been well recognized in host defense against viral infections (7), deliberate vaccination (8), and autoimmune diseases (9). In contrast to

intensive studies on systemic T<sub>FH</sub> cells, the mechanism regulating gut T<sub>FH</sub> cells remains poorly understood (6, 10).

Activating transcription factor 3 (ATF3) is a member of the ATF/cAMP response element-binding (ATF/CREB) family (11). ATF3 is rapidly induced by a multitude of stimuli which directly or indirectly alter the expression of a variety of genes in immune cells to limit excessive inflammation (12, 13). The participation of ATF3 in host immune responses against pathogens and certain inflammatory diseases, such as sepsis (12, 13), asthma (14), and hepatic steatosis (15), has been reported. However, its role in gut homeostasis remains to be fully understood. Expression of ATF3 was significantly induced in patients with Crohn's disease (16). Several studies have indicated the protective role of ATF3 in the maintenance of intestinal barrier function and the pathogenesis of IBD, although distinct mechanisms may contribute (17, 18). Here, we identified ATF3 as a regulator of T<sub>FH</sub> cells in the gut. Expression of ATF3 in CD4<sup>+</sup> T cells was negatively correlated with the severity of UC disease in clinical patients. Deficiency of ATF3 in CD4<sup>+</sup> T cells significantly aggravated colitis in mice, which could be rescued by transfer of T<sub>FH</sub> or IgA<sup>+</sup> B cells. We further demonstrated that the regulation of T<sub>FH</sub> cells by ATF3 was intrinsic to T cells and dependent on B cell lymphoma 6 (Bcl6). Collectively, these observations shed light on the contribution of ATF3 to gut mucosal homeostasis, which indicates its potential therapeutic value in IBD.

## Significance

**The mechanisms underlying gut follicular helper T (T<sub>FH</sub>) cells and their role in mucosal homeostasis is not fully understood. In this study, we demonstrate that activating transcription factor 3 (ATF3) represents a regulator of gut TFH cells, which dictates the susceptibility of colitis. Bcl6 was identified as a transcriptional target of ATF3 in gut T<sub>FH</sub> cells.**

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## Results

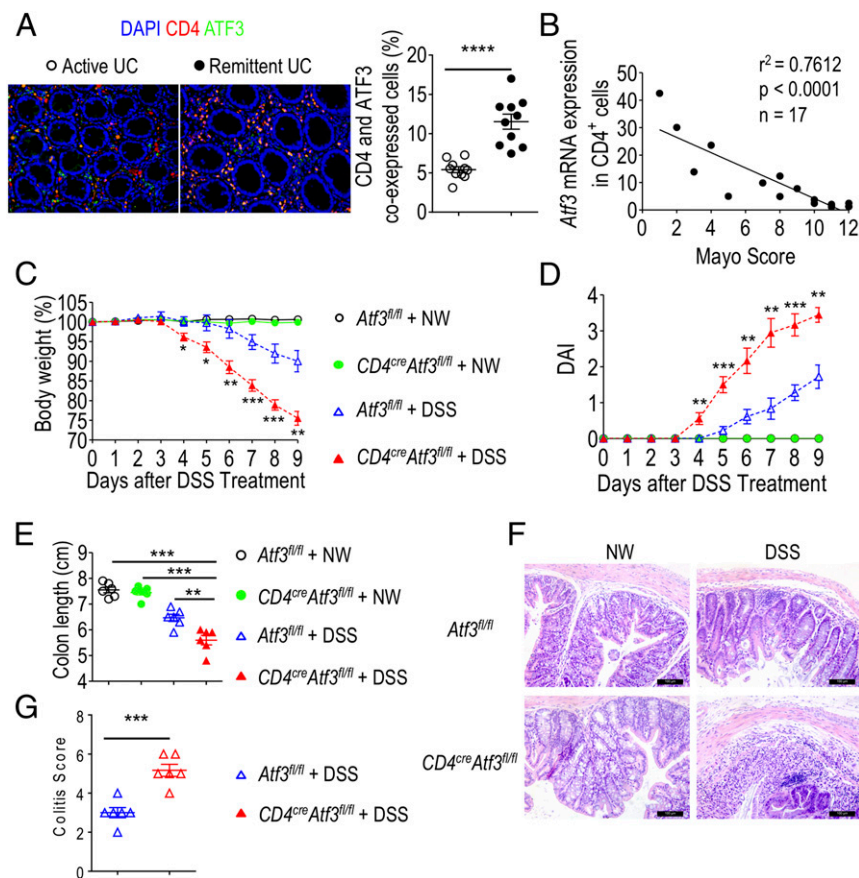
**ATF3 Deficiency in CD4<sup>+</sup> T Cells Aggravates Murine Colitis.** Expression profiling of distinct tissues revealed that ATF3 was highly expressed in GALTs including colon, PPs, and mesenteric lymph nodes, both in mRNA and protein levels (*SI Appendix, Fig. S1 A and B*). Its levels in GALTs were comparable to that in lung and even higher than that in liver (*SI Appendix, Fig. S1 A and B*), where ATF3 is known to play important roles (14, 15). To explore the role of ATF3 in the gut, we evaluated its level in CD4<sup>+</sup> T cells and B cells, the major cell types in PPs, during the induction of dextran sulfate sodium (DSS)-induced murine colitis (19). Results showed that the expression of ATF3 in CD4<sup>+</sup> T cells, not B cells, in PPs was highly sensitive to colitis, with highest levels within 24 h after DSS administration, and decreased thereafter (*SI Appendix, Fig. S1C*). CD4<sup>+</sup> T cells from the peripheral blood of colitic mice displayed a pattern of ATF3 expression similar to those from the gut; however, their magnitude changes were much smaller (*SI Appendix, Fig. S1D*). UC is the corresponding disease of DSS-induced murine colitis (19). Immunofluorescence staining showed that ATF3 was dominantly expressed in CD4<sup>+</sup> T cells in colonic mucosa, and patients with remittent UC displayed an approximately twofold higher level of colocalization between ATF3 and CD4 than those from active colitis (Fig. 1A). ATF3 level in CD4<sup>+</sup> T cells from peripheral blood was negatively correlated with disease severity as indicated by Mayo Score (20)

(Fig. 1B). These observations indicate a potential link between ATF3 in CD4<sup>+</sup> T cells and mucosal homeostasis of the gut.

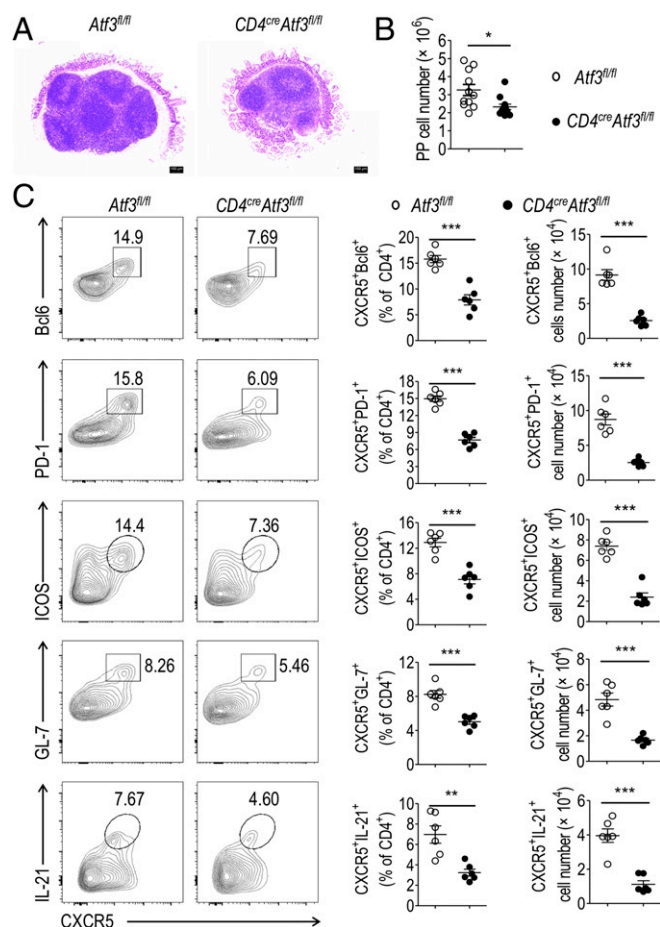
Next, ATF3 was deleted in CD4<sup>+</sup> T cells by cross-breeding *Atf3*-floxed mice with CD4-Cre mice, named *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* hereafter (*SI Appendix, Fig. S1 E–G*). DSS-induced colitis was next performed. Results showed that *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* mice encountered significantly more severe clinical symptoms than *Atf3<sup>fl/fl</sup>* control littermates, as revealed by loss of body weight (Fig. 1C), disease activity index (DAI) (Fig. 1D), and shortened colon length (Fig. 1E). The disease severity was further confirmed by H&E staining (Fig. 1F) and quantitated as colitis score (Fig. 1G). These results suggest that deletion of ATF3 in CD4<sup>+</sup> T cells renders mice more highly susceptibility to colitis.

### *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* Mice Display Reduced Levels of T<sub>FH</sub> Cells in PPs Under Colitis.

Further histological analysis reveals that PPs from *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* mice were markedly shrunk in size under colitis (Fig. 2A), with ~1.5-fold reduction in cellularity compared with *Atf3<sup>fl/fl</sup>* controls (Fig. 2B). Gene expression showed that ATF3 was highly expressed in T<sub>FH</sub> cells, compared with other cell types from PPs, including B cells, GC B cells, and CD8<sup>+</sup> and CD4<sup>+</sup> T cells (*SI Appendix, Fig. S2A*). The level of ATF3 in T<sub>FH</sub> cells was also clearly higher than other T cell subsets, as well as naïve CD4<sup>+</sup> T cells, non-T<sub>FH</sub> cells, and pre-T<sub>FH</sub> cells (*SI Appendix, Fig. S2 B and C*). These observations indicate a potential role of ATF3 in T<sub>FH</sub> cells from PPs.



**Fig. 1.** ATF3 deficiency in CD4<sup>+</sup> T cells aggravates murine colitis. (A) Representative immunofluorescence staining of colonic tissues from active and remittent UC patients (Left). Red, CD4; green, ATF3; blue, DAPI ( $n = 10$  per group, 400 $\times$  magnification). The colocalization of CD4 and ATF3 was quantitated using ImageJ software (Right). (B) Correlation between ATF3 expression in CD4<sup>+</sup> T cells from peripheral blood with Mayo Score in UC patients. (C–G) *Atf3<sup>fl/fl</sup>* and *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* mice were challenged with 2.5% (weight per volume) DSS to induce colitis; normal water (NW) was used as control. The severity of colitis was monitored, including loss of body weight (C), DAI (D), shortened colon length (E), and representative H&E staining of proximal colon tissues (F) (magnification, 200 $\times$ ). (G) Histological scores of colitis from F. In all plots, mean  $\pm$  SEM are shown. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , using two-tailed Student's test. Data are representative of three independent experiments.

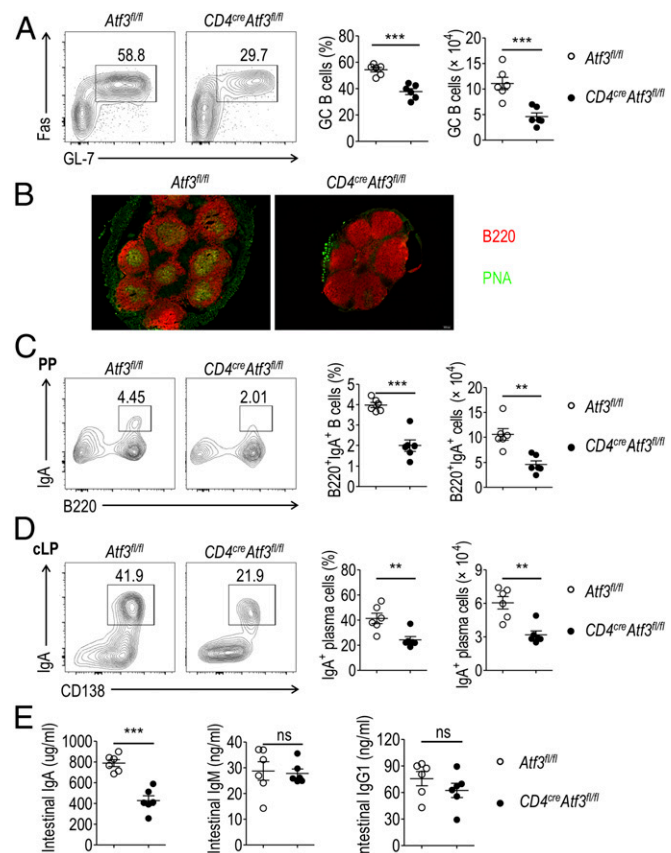


**Fig. 2.**  $CD4^{cre}Atf3^{fl/fl}$  mice displayed reduced level of  $T_{FH}$  cells in PPs under colitis. (A) Representative H&E staining of PPs from mice under colitis. (B) Absolute cell numbers of PPs pooled from the entire small intestine of mice in A. (C)  $Atf3^{fl/fl}$  and  $CD4^{cre}Atf3^{fl/fl}$  mice were challenged with DSS to induce colitis ( $n = 6$  mice per group). At day 9, flow cytometric analysis was performed. The frequencies of  $T_{FH}$  cells in PPs were evaluated by flow cytometry. Both representative results (Left, pregated on  $CD4^{+}$ ) and mean  $\pm$  SEMs from all mice (Right) are shown. In all plots, mean  $\pm$  SEM from three independent experiments are shown. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , using two-tailed Student's test.

Flow cytometric analysis showed that the proportions of T helper type 1 (Th1), Th2, Th17, or regulatory T (Treg) cells in PPs displayed no obvious differences between  $CD4^{cre}Atf3^{fl/fl}$  and  $Atf3^{fl/fl}$  mice after induction of colitis (SI Appendix, Fig. S2D). However, a dramatic reduction was observed in  $T_{FH}$  cells in PPs from  $CD4^{cre}Atf3^{fl/fl}$  mice under colitis (Fig. 2C).  $T_{FH}$  cells were identified by coexpression of C-X-C chemokine receptor type 5 (CXCR5), Bcl6, programmed cell death-1 (PD-1), inducible T cell costimulator (ICOS), and GL-7 markers as well as secretion of IL-21 (Fig. 2C) (6, 21). Further analysis of Foxp3 showed that this reduction was mainly derived from immunostimulatory  $T_{FH}$  cells (Foxp3<sup>-</sup>), rather than follicular Treg cells ( $T_{FR}$ , Foxp3<sup>+</sup>) (22) (SI Appendix, Fig. S2E). The mean fluorescence intensity (MFI) of multiple  $T_{FH}$  cell markers was clearly reduced in ATF3-deficient  $T_{FH}$  cells under colitis (SI Appendix, Fig. S2F). In addition, neither the proliferation nor apoptosis of  $T_{FH}$  cells displayed noticeable changes under colitis (SI Appendix, Fig. S2G). In contrast with the clear reduction of  $T_{FH}$  in PPs, no differences were observed in the frequency of circulating  $T_{FH}$  in the peripheral blood in colitic mice (SI Appendix, Fig. S2H). Importantly, the reduction of  $T_{FH}$  frequencies in PPs from

$CD4^{cre}Atf3^{fl/fl}$  mice was also observed under homeostasis (SI Appendix, Fig. S3A). These observations indicate that deletion of ATF3 in  $CD4^{+}$  T cells impaired the level of  $T_{FH}$  cells in the gut under both colitis and steady-state conditions.

**Impaired  $T_{FH}$  Diminishes GC Reaction and IgA Production in Gut from  $CD4^{cre}Atf3^{fl/fl}$  Mice.** In support of the reduced levels of  $T_{FH}$  cells, the frequency of GC B cells ( $B220^{+}IgD^{lo}GL7^{+}Fas^{+}$ ) was profoundly decreased in PPs from  $CD4^{cre}Atf3^{fl/fl}$  mice under colitis, as represented by both flow cytometry (Fig. 3A) and immunofluorescence staining of peanut agglutinin (PNA)-positive GCs within B cell follicles (Fig. 3B).  $T_{FH}$ -dependent GC reactions in PPs instruct  $IgA^{+}$  plasmablasts to migrate to the lamina propria of both the small and large intestine, which leads to secretion of IgA into the intestinal lumen (4). The reduced GC reaction led to significantly lower levels of  $IgA^{+}$  B cells ( $B220^{+}IgA^{+}$ ) in PPs (Fig. 3C) and  $IgA^{+}$  plasma cells ( $B220^{-}CD138^{+}IgA^{+}$ ) in colonic lamina propria (cLP) (Fig. 3D) in  $CD4^{cre}Atf3^{fl/fl}$  mice compared with  $Atf3^{fl/fl}$  controls under colitis. The concentration of free IgA, but not IgG1 or IgM, in the intestinal lumen was consistently decreased in  $CD4^{cre}Atf3^{fl/fl}$  colitic mice (Fig. 3E). Consistent results



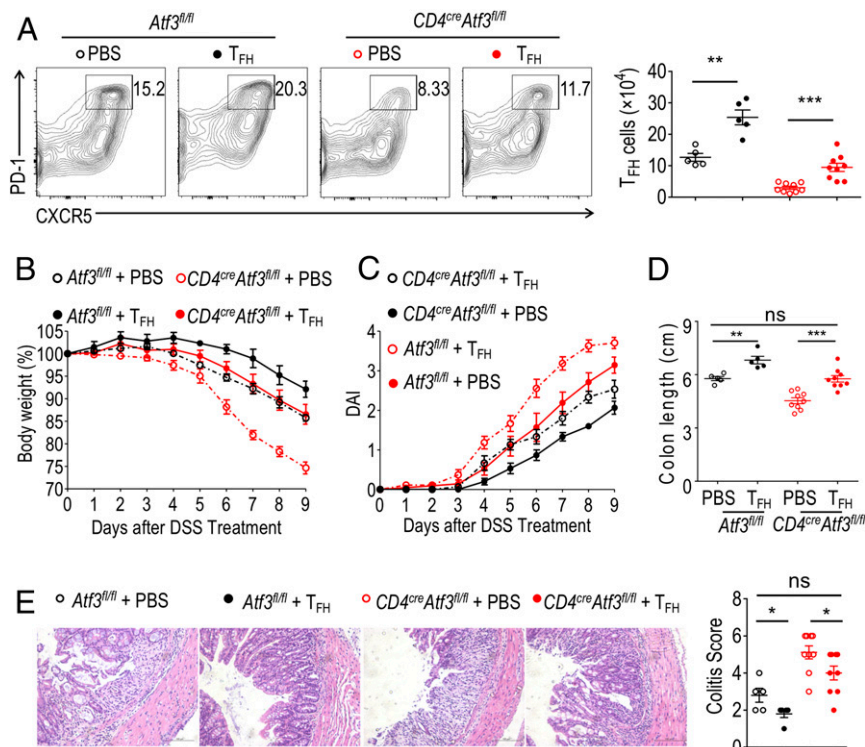
**Fig. 3.** Impaired  $T_{FH}$  diminishes GC reaction and IgA production in gut from  $CD4^{cre}Atf3^{fl/fl}$  mice. (A–E)  $Atf3^{fl/fl}$  and  $CD4^{cre}Atf3^{fl/fl}$  mice were challenged with DSS to induce colitis ( $n = 6$  mice per group). Mice were killed at day 9 for analysis. (A) Flow cytometric analysis of GC B cells in PPs, pregated on  $B220^{+}IgD^{lo}$ . (B) Representative immunofluorescence staining of B220 and PNA in PPs ( $n = 5$  mice per group). (C) Flow cytometric analysis of  $IgA^{+}$  B cells in PPs. (D) Flow cytometric analysis of  $IgA$ -secreting plasma cells in cLP, pregated on the  $B220^{-}$  population. (E) The concentration of intestinal immunoglobulins was measured by ELISA. Mean  $\pm$  SEM are shown ( $n = 6$  mice per group). (A, C, and D) Both representative results (Left) and mean  $\pm$  SEMs from all mice (Right) are shown. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, no significance, using two-tailed Student's test. Data are representative of three independent experiments.

were observed under steady-state conditions (*SI Appendix, Fig. S3 B–E*). Collectively, these observations suggest that defective  $T_{FH}$  impaired GC reaction and reduced IgA production in the gut from  $CD4^{cre}Atf3^{fl/fl}$  mice.

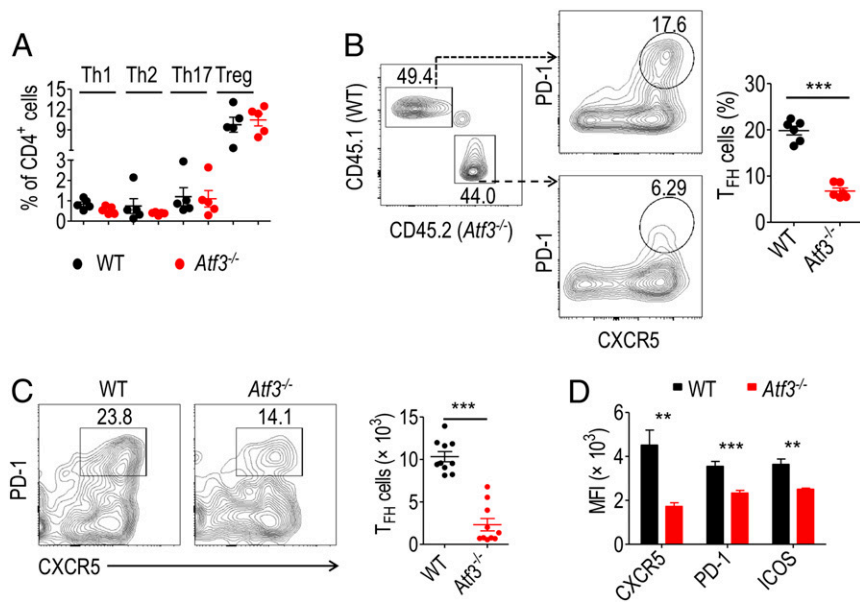
**Transfer of  $T_{FH}$  or IgA<sup>+</sup> B Cells Alleviates Colitis in  $CD4^{cre}Atf3^{fl/fl}$  Mice.** To establish the causal relationship between the defects in the gut  $T_{FH}$ -IgA axis and the aggravated colitis observed in  $CD4^{cre}Atf3^{fl/fl}$  mice, adoptive transfer experiments were performed. First,  $T_{FH}$  cells from PPs of WT  $CD45.1^{+}$  congenic mice were adoptively transferred into  $CD4^{cre}Atf3^{fl/fl}$  or  $Atf3^{fl/fl}$  recipients ( $CD45.2^{+}$ ), followed by DSS challenge. Flow cytometric analysis showed donor  $T_{FH}$  cells ( $CD45.1^{+}$ ) contributed roughly 35% of total  $T_{FH}$  cells in PPs of recipients (*SI Appendix, Fig. S4A*). As expected, a significant elevation of  $T_{FH}$  cells was observed in PPs of both  $CD4^{cre}Atf3^{fl/fl}$  and  $Atf3^{fl/fl}$  recipients upon  $T_{FH}$  cell transfer (Fig. 4A), which substantially enhanced GC reaction in PPs (*SI Appendix, Fig. S4B*) and caused a higher level of IgA-producing B cells in PPs and IgA concentration in the gut lumen (*SI Appendix, Fig. S4 C and D*). Consequently, the severity of colitis in  $CD4^{cre}Atf3^{fl/fl}$  recipients was significantly ameliorated upon receiving donor  $T_{FH}$  cells, which were even comparable to those of  $Atf3^{fl/fl}$  controls receiving PBS, including body weight loss, disease signs, shortened colon length, and remission of colon histology (Fig. 4 B–E). Remission of colitis by  $T_{FH}$  cells was also observed in  $Atf3^{fl/fl}$  recipients, as expected (Fig. 4 B–E). Consistently, adoptive transfer of PP IgA<sup>+</sup> B cells from WT  $CD45.1^{+}$  congenic mice also significantly relieved colitis symptoms in  $CD4^{cre}Atf3^{fl/fl}$  recipients compared with IgG1<sup>+</sup> B cells control (*SI Appendix, Fig. S5 A–D*). The elevation of total IgA<sup>+</sup> B cells in PPs as well as the luminal IgA levels in recipients indicated the success of B cell transfer (*SI Appendix, Fig. S5 E–G*). Together,

these results suggested that the impaired  $T_{FH}$ /IgA axis plays a major role in the aggravated colitis in  $CD4^{cre}Atf3^{fl/fl}$  mice.

**Regulation of  $T_{FH}$  by ATF3 in PPs Is Intrinsic to T Cells.** We next investigated whether the defect of  $T_{FH}$  cells is intrinsic to T cells. Mixed bone marrow chimeras were generated by transplantation of bone marrow cells from  $Atf3^{-/-}$  ( $CD45.2^{+}$ ) and WT ( $CD45.1^{+}$ ) mice at a 1:1 ratio into  $Tcra^{-/-}$  recipients (*SI Appendix, Fig. S6A*). The reconstitution of total B cells and distinct T cell subsets was comparable between  $Atf3^{-/-}$  and WT donors 8 wk after transplantation (Fig. 5A and *SI Appendix, Fig. S6B*). However, a dramatic decrease was observed in PPs  $T_{FH}$  cells derived from  $Atf3^{-/-}$  donors, compared with that from WT controls (Fig. 5B). In these chimeric mice there were no noticeable differences in the proportion of GC B cells and IgA-producing B cells in PPs between  $Atf3^{-/-}$  and WT donor cells (*SI Appendix, Fig. S6 C and D*); one explanation for this is that B cells in  $Tcra^{-/-}$  recipients could have simultaneously interacted with  $T_{FH}$  cells from both  $Atf3^{-/-}$  and WT donors. These results suggest that the regulation of  $T_{FH}$  cells by ATF3 is cell-intrinsic. For further confirmation, naïve  $CD4^{+}$  T cells from  $Atf3^{-/-}$  or WT mice were transferred into  $Tcra^{-/-}$  mice (*SI Appendix, Fig. S6E*). No obvious defects were observed in other  $CD4^{+}$  T cell subsets in PPs 2 wk after transfer (*SI Appendix, Fig. S6F*). As expected, the frequency of  $T_{FH}$  cells in PPs was markedly reduced in recipients receiving  $Atf3^{-/-}$  donor cells (Fig. 5C), which led to a reduction in both GC B and IgA<sup>+</sup> B cells (*SI Appendix, Fig. S6 G and H*). The important markers for  $T_{FH}$  cells, including CXCR5, PD-1, and ICOS, were consistently down-regulated in  $Atf3^{-/-}$  donor-derived  $T_{FH}$  cells (Fig. 5D). Taken together, these results indicate that ATF3 regulates the development of PP  $T_{FH}$  cells in T cell-intrinsic manner.



**Fig. 4.** Transfer of  $T_{FH}$  cells from PPs alleviates colitis in  $CD4^{cre}Atf3^{fl/fl}$  mice.  $T_{FH}$  cells from PPs of WT congenic mice ( $CD45.1^{+}$ ) were transferred into  $Atf3^{fl/fl}$  ( $n = 5$ ) or  $CD4^{cre}Atf3^{fl/fl}$  ( $n = 9$ ) recipients, followed by DSS treatment to induce colitis. PBS was used as vehicle control. (A) Flow cytometric analysis of  $T_{FH}$  cells in PPs of recipients. Both representative (pregated on  $CD4^{+}$  cells) and statistical results are shown. (B–E) The severity of colitis was monitored, including body weight loss (B), DAI (C), and colon length (D). (E) H&E staining of proximal colon (magnification, 200 $\times$ ) and quantitated colitis scores. (Scale bars, 100  $\mu$ m.) In all plots, mean  $\pm$  SEM are shown. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, no significance, using two-tailed Student's test. Data are representative of three independent experiments.

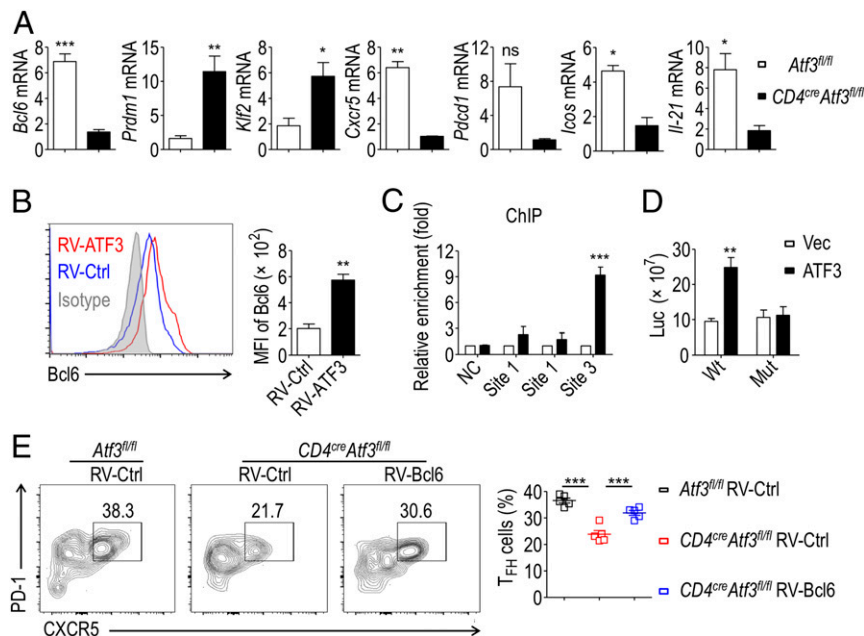


**Fig. 5.** Regulation of  $T_{FH}$  by ATF3 in PP is intrinsic to T cells. (A and B) Bone marrow cells from WT (CD45.1<sup>+</sup>) and *Atf3*<sup>-/-</sup> mice (CD45.2<sup>+</sup>) were mixed at 1:1, followed by transfer into irradiated *Tcrα*<sup>-/-</sup> mice. The reconstitution of distinct T cells in recipients' PPs were analyzed after 8 wk ( $n = 5-6$  mice per group), including Th1, Th2, Th17, and Treg (A) and  $T_{FH}$  (B). (C) Naïve CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD62<sup>hi</sup>CD44<sup>lo</sup>) from WT or *Atf3*<sup>-/-</sup> mice were transferred into *Tcrα*<sup>-/-</sup> mice. The proportions of  $T_{FH}$  cells in PPs from recipients' PPs were evaluated 2 wk after transfer ( $n = 10$ ). (D) The MFI of  $T_{FH}$  cell markers from mice in C was evaluated by flow cytometry ( $n = 6$ ). In all plots, mean  $\pm$  SEM are shown. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , using two-tailed Student's test. Data are representative of three independent experiments.

We next investigated whether ATF3 affects systemic  $T_{FH}$  responses. First, it was found that PP  $T_{FH}$  expressed significantly higher levels of ATF3 than  $T_{FH}$  from draining lymph nodes (dLN) and spleen (SI Appendix, Fig. S7 A and B). Further immunization of keyhole limpet hemocyanin (KLH) mouse model failed to reveal any noticeable differences between *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* and *Atf3<sup>fl/fl</sup>* littermate controls in either  $T_{FH}$  or GC B cells from dLN and spleen (SI Appendix, Fig. S7 C and D). The in vitro  $T_{FH}$ -like culture system showed that deficiency of ATF3 did not cause a clear defect in the generation of  $T_{FH}$  from naïve CD4<sup>+</sup> T cells of peripheral lymph nodes (SI Appendix, Fig. S7E). No obvious differences were observed in the differentiation of other CD4<sup>+</sup> T cells under specific polarizing culture conditions, as expected (SI Appendix, Fig. S7E). These results indicated that  $T_{FH}$  cells in the gut are more sensitive to ATF3 expression.

**Bcl6 Mediates the Effect of ATF3 on  $T_{FH}$  Cells in PPs.** To explore the mechanism by which ATF3 regulates  $T_{FH}$  cells in PPs, the transcription of  $T_{FH}$  signature genes was profiled by qRT-PCR. The expression of *Bcl6* was dramatically down-regulated and its antagonist *Prdm1* (encodes Blimp1) and negative transcriptional target *Klf2* were up-regulated in PP  $T_{FH}$  cells from *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* mice, compared with *Atf3<sup>fl/fl</sup>* controls (Fig. 6A). The mRNA levels of  $T_{FH}$  effector molecules, including CXCR5, PD-1, ICOS, and IL-21, were simultaneously down-regulated (Fig. 6A). Retroviral overexpression of ATF3 up-regulated *Bcl6* expression in CD4<sup>+</sup> T cells in vitro (Fig. 6B) with an infection efficiency of roughly 25% (SI Appendix, Fig. S8A). Bioinformatics analysis of the *Bcl6* regulatory region revealed three potential ATF3 binding sites (at  $-50 \sim -1,783$  bp upstream of the transcriptional

**Fig. 6.** *Bcl6* mediates the effect of ATF3 on  $T_{FH}$  cells in the gut. (A) The mRNA expression of indicated genes in  $T_{FH}$  cells from PPs under steady state was evaluated by qRT-PCR. (B) CD4<sup>+</sup> T cells were infected with retrovirus (RV) overexpressing ATF3 or vector control (with GFP tag), and *Bcl6* expression was measured by flow cytometry within the infected cells (GFP<sup>+</sup>). (C) ChIP assay was performed on PPs CD4<sup>+</sup> T cells from WT mice under steady state using anti-ATF3 or anti-IgG antibodies; the presence of ATF3 binding site on *Bcl6* promoter was measured by qPCR. Data were normalized against input DNA and presented as fold increase over IgG control. (D) HEK-293T cells were cotransfected with plasmid overexpressing ATF3 and *Bcl6* reporter containing ATF3 binding sites, either WT or site-directed mutation (Mut). Luciferase activity was measured 48 h posttransfection. (E) Naïve CD4<sup>+</sup> T cells from *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* mice were infected with retrovirus expression *Bcl6* (RV-*Bcl6*) or vector control (RV-Ctrl); CD4<sup>+</sup> T cells from *Atf3<sup>fl/fl</sup>* mice infected with vector control were used as control. Three days later, GFP<sup>+</sup> cells were sorted and adoptively transferred into *Tcrα*<sup>-/-</sup> mice ( $n = 5$  mice per group). The levels of  $T_{FH}$  cells in PPs of *Tcrα*<sup>-/-</sup> recipient mice were evaluated by flow cytometry 7 d after adoptive transfer. Both representative results (Left, pregated on CD4<sup>+</sup> cells) and mean  $\pm$  SEMs from all mice (Right) are shown. In all plots, mean  $\pm$  SEM are shown. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , using two-tailed Student's test. Data are representative of three independent experiments.



start site) (*SI Appendix, Fig. S8B*). ChIP confirmed the direct binding of ATF3 protein with site 3 (−1,775 ~ −1,783) (Fig. 6C). Overexpression of ATF3 enhanced the activity of Bcl6 reporter, whereas mutation of the ATF3 binding site abrogated this effect (Fig. 6D). Moreover, overexpression of Bcl6 in CD4<sup>+</sup> T cells from *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* mice rescued their defect of differentiation into T<sub>FH</sub> cells in PPs upon transfer into *Tcrα<sup>-/-</sup>* recipient mice (Fig. 6E and *SI Appendix, Fig. S8C*). These results identified Bcl6 as a direct target of ATF3 in CD4<sup>+</sup> T cells.

## Discussion

In this study, we have demonstrated that stress-inducible transcription factor ATF3 plays a critical role in the prevention of colitis by dictating the development of T<sub>FH</sub> cells in the gut. Bcl6, the master regulator of T<sub>FH</sub> cells (23, 24), was identified as the direct transcriptional target of ATF3. The significance of ATF3 in gut T<sub>FH</sub> cells was evidenced by its impact on the susceptibility of murine colitis as well as its negative correlation with the severity of clinical UC disease.

The role of T<sub>FH</sub> cells in the systemic immune responses has been well recognized in the context of viral infections (7, 25), vaccination (8), and autoimmunity (9); its regulatory mechanism in the gut (4, 6), however, remains largely unknown. Although deficiency of ATF3 in CD4<sup>+</sup> T cells caused aggravation of colitis, no obvious defects were observed in systemic T<sub>FH</sub> responses upon KLH immunization. Furthermore, ATF3 failed to cause noticeable defects in the circulating T<sub>FH</sub> cells under colitis. The generation of T<sub>FH</sub> cells was not apparently affected by ATF3 deletion when naïve CD4<sup>+</sup> T cells from peripheral lymphoid organs were cultured in vitro. Importantly, the expression level of ATF3 in PP-derived T<sub>FH</sub> cells was much higher than those from the peripheral lymphoid tissues. These observations indicate that T<sub>FH</sub> cells in the gut may be more sensitive to ATF3 expression. Exploration of the specific signaling underlying the development of gut T<sub>FH</sub> cells will benefit the better understanding of mucosal immunity and the pathogenesis of colitis.

There is increasing evidence about the importance of ATF3 in inflammatory diseases. It was reported that ATF3 was induced by lipopolysaccharide and functions as a negative regulator of Toll-like receptor (12). ATF3 was also a negative regulator of allergic pulmonary inflammation (14). It was recently reported that ATF3 sustains gut mucosa homeostasis via STAT3 signaling in epithelium (18), whereas deficiency of ATF3 protected mice against bacterial and fungal infections under conditions of reactive oxygen species

stress (13). Here we demonstrated that ATF3 represents a positive regulator of gut T<sub>FH</sub> cells, which contributes to the maintenance of gut homeostasis. Therefore, the exact role of ATF3 in inflammatory disorders may be context- or tissue-dependent.

Although ATF3 levels in UC patients were negatively correlated with disease severity of UC, the clinical significance of ATF3-mediated T<sub>FH</sub> cells in colitis needs further investigation before firm conclusions can be drawn. Due to the limitation of colonic mucosa from patients, peripheral blood samples were collected for the clinical correlation analysis in this study. Although CD4<sup>+</sup> T cells from the peripheral blood displayed trends of ATF3 expression similar to those from the gut in the murine colitis model, their magnitude changes were much smaller. Furthermore, there were no obvious changes in T<sub>FH</sub> cells in the peripheral blood between *CD4<sup>cre</sup>Atf3<sup>fl/fl</sup>* and *Atf3<sup>fl/fl</sup>* controls under colitis. These observations indicate that T<sub>FH</sub> cells in the gut may be more sensitive to ATF3 expression than T<sub>FH</sub> cells from the peripheral blood. Therefore, the blood samples from UC patients may not fully reflect the local mucosal tissues. In summary, we identified ATF3 as a regulator of gut T<sub>FH</sub> cells, which dictates the susceptibility of colitis.

## Materials and Methods

Detailed materials and methods can be found in *SI Appendix*, including information on mouse strains, human samples, DSS-induced colitis, flow cytometric analysis and sorting, adoptive transfer, in vitro differentiation of T cells, mixed bone marrow chimera, qRT-PCR, ELISA, ChIP, luciferase assay, statistics, and other methods.

All experimental procedures with mice were performed in accordance with the Animal Care and Use Committee of Sun Yat-sen University. The study of UC patients was approved by the Clinical Ethics Review Board of the Sixth Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from all the participants or their legal guardians at the time of admission.

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