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T Follicular Helper Cells restricted by IRF8 contribute to T Cell-Mediated Inflammation

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Abstract

The follicular helper T cell (T_{FH}) are established regulators of germinal center (GC) B cells, whether T_{FH} have pathogenic potential independent of B cells is unknown. Based on *in vitro* T_{FH} cell differentiation, *in vivo* T cell transfer animal colitis model, and intestinal tissues of inflammatory bowel disease (IBD) patients, T_{FH} and its functions in colitis development were analyzed by FACS, ChIP, ChIP-sequencing, WB, ELISA and PCR. Herein we demonstrate that intestinal tissues of patients and colon tissues obtained from *Rag1*$^{-/-}$ recipients of naïve CD4^{+} T cells with colitis, each over-express T_{FH}associated gene products. Adoptive transfer of naïve

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Competing Interest Statement

The authors declare that they have no competing interests.

Bcl6$^{-/-}$ CD4^{+} T cells into *Rag1*$^{-/-}$ recipient mice abrogated development of colitis and limited T_{FH} differentiation *in vivo*, demonstrating a mechanistic link. In contrast, T cell deficiency of interferon regulatory factor 8 (IRF8) resulted in augmentation of T_{FH} induction *in vitro* and *in vivo*. Functional studies showed that adoptive transfer of IRF8 deficient CD4^{+} T cells into *Rag1*$^{-/-}$ recipients exacerbated colitis development associated with increased gut T_{FH}-related gene expression, while *Irf8*$^{-/-}$/*Bcl6*$^{-/-}$ CD4^{+} T cells abrogated colitis, together indicating that IRF8-regulated T_{FH} can directly cause colon inflammation. Molecular analyses revealed that IRF8 suppresses T_{FH} differentiation by inhibiting transcription and transactivation of the TF IRF4, which is also known to be essential for T_{FH} induction. Our documentation showed that IRF8-regulated T_{FH} can function as B-cell-independent, pathogenic, mediators of colitis suggests that targeting T_{FH} could be effective for treatment of IBD.

Keywords

IRF8; TFH; Colitis; IRF4

1. Introduction

T cell help to B cells is a fundamental mechanism for the generation of protective humoral immunity, but over-activation of B cells by T cells can result in excessive humoral immune responses, pathologic inflammation, and autoimmunity[1–3]. A subset CD4^{+} T cells, termed follicular helper T cells (T_{FH}), are specialized regulators of T cell help to B cells and are required for induction of germinal center (GC) B cell responses[4–5].

T_{FH} stably express C-X-C chemokine receptor 5 (CXCR5), which mediates chemotaxis toward GCs upon ligation by C-X-C chemokine ligand 13 (CXCL13) expressed by follicular dendritic cells (fDCs)[6–8]. Expression of transcription factor (TF) B-cell lymphoma 6 (Bcl-6), among other TFs (e.g. IRF4, c-MAF, Ascl2), requisitely orchestrates T_{FH} differentiation[9–16]. T_{FH} also express an elaborate network of cell surface molecules that promote T-B cell collaboration in GCs [7–8], including the co-stimulatory receptor inducible co-stimulatory molecule (ICOS) which engages ICOS ligand (ICOSL) on GC B cells[17–18]. ICOS expression is essential for the generation and maintenance of T_{FH} cells including production of the cytokine interleukin-21 (IL-21)[19–20], which promotes GC B-cell proliferation, class switch recombination (CSR), memory B cell formation and plasma cell differentiation[21–22]. Antigen-experienced T_{FH} also rapidly up-regulate the expression of CD154 (CD40L)[23–24], which ligates the B cell surface receptor CD40 to induce B cell activation, proliferation, somatic hypermutation (SHM) and class switch recombination (CSR). Engagement of GC B cell-expressed programmed cell death 1 ligand 1 (PD-L1) and/or PD-L2[25–26] to PD-1 on T_{FH} cells negatively regulates the size and function of the induced T_{FH} response. Activated T_{FH} express the highest levels of CD40L, ICOS, PD-1 and IL-21 among T cell subsets[27–29].

Through decades of research by many investigators, T helper cells including T_H1 and T_H2 cells were initially implicated in the pathogenesis of inflammatory bowel diseases (IBD). Following the discovery of T_H17 cells, which specifically produce T_H17 family cytokines

(e.g. IL-17A, IL-17F, and IL-22), investigators reconsidered the T_H1/T_H2 cytokine balance hypothesis, and posited that T_H17 cells are potentially instrumental in IBD pathogenesis. Despite the strong associative evidence between T_H17-associated genes/mutations and IBD from murine and human studies, clinical trials targeting IL-17A proved ineffective in Crohn's disease patients and in fact paradoxically worsened disease in a set of patients. In addition, naïve $CD4^+$ T cells from IL-17 deficient mice induced more severe colitis in recipient mice. Thus, the mechanism through which T helper cells mediate inflammatory bowel diseases remains elusive. Interestingly, T_{FH} signature genes have been expressed in several inflammatory diseases including IBD, suggesting that T_{FH} may contribute to the development of inflammatory diseases.

Multiple transcription factors, including C-Maf, Batf, Irf4, STAT1, STAT3, and Ascl2, are actively involved in the development and function of T_{FH}[9–16], but maintenance and full differentiation of T_{FH} critically requires expression of Bcl-6[9–10]. In addition, the T_{FH} differentiation pathway is also opposed by other factors including Blimp-1, Foxo1 and Foxp1[30–31]. However, the molecular mechanism for the regulation of T_{FH} is incompletely understood, especially how T_{FH} are negatively regulated.

IRF8 is a member of the evolutionarily conserved IRF family of transcription factors with diverse and important regulatory roles in the growth, differentiation, and function of innate and adaptive immune cells. IRF8 is expressed by a wide spectrum of immature and mature hematopoietic cells including B cells, dendritic cells (DCs), macrophages, and activated T cells [32–33]. It has an N-terminal DNA-binding domain (DBD) and a C-terminal IRF association domain (IAD), the latter of which is responsible for heterodimerization with other transcription factors[33–34]. IRF8 can function as either a transcriptional repressor or an activator, depending on the specific heterodimeric DNA-binding complexes produced with its varied partners[35–41]. Previously published work showed that germline *Irf8*$^{-/-}$ mice develop a chronic myeloid leukemia-like syndrome with impaired T_H1 immunity, but how IRF8 controls T cell function was seldom discussed[33–35,40]. We previously reported that IRF8 negatively regulates T_H17 cell differentiation[42] raising the possibility that this TF could serve as a negative regulator of other T_H subsets including T_{FH}.

Herein, we provide new evidence that T_{FH} can mediate intestinal pathology independent of B cells and show that this pathogenic function is regulated by IRF8 inhibition of IRF4. In addition to providing paradigm shifting mechanistic insight into the functions of T_{FH} our new findings raise the possibility that $CD4^+$ T cell-intrinsic IRF8 expression critically regulates other pathogenic autoimmune responses driven by T_{FH}.

2. Materials and Methods

2.1. Mice

C57BL/6J (B6, stock#000664), *Rag1*$^{-/-}$ (on B6 background, B6.129S7-*Rag1*^{tm1Mom}/J, stock#002216), *Bcl-6*$^{-/-}$ mice (B6.129S(FVB)-Bcl6<tm1.1Dent>/J, stock# 023727) were obtained from the Jackson laboratory. *Irf8*$^{-/-}$ and Lck-Cre$^+$*Irf8*$^{fl/fl}$ were maintained in the barrier facility at the Icahn School of Medicine at Mount Sinai. The animal study protocols

were approved by the Institutional Animal Care and Use Committees of Icahn School of Medicine at Mount Sinai.

2.2. Human colon tissue

Human colon tissues from Crohn's disease patients and control patients undergoing resection for cancer screening were obtained from the Mount Sinai Hospital with a protocol approved by the Institutional Review Board of Icahn School of Medicine at Mount Sinai.

2.3. Antibodies

The following antibodies against mouse antigens and conjugated to FITC, PE, PE-Cy5, PerCP-Cy5.5 or APC were purchased from BD Biosciences (CD4 (L3T4), eBioscience: biotin-rat anti-mouse CXCR5 (2G8), plus streptavidin-APC-eFluor 780 or eBioscience: PD-1 (J43), ICOS (7E17G9), Bcl-6 (mG1191E), IL-21 (mhalx21), CD8 (53–6.7), CD3ε (145–2C11), CD62L (MEL-14) and isotype controls.

2.4. Intracellular staining and flow cytometry

Naive $CD4^+$ T cells ($CD62L^+CD44^{lo}$) were prepared by fluorescence-activated cell sorting (FACS) from spleens and lymph nodes of *Irf8*$^{-/-}$ mice or WT littermates. Anti-mouse CD4 microbeads (L3T4, Miltenyi Biotec) from spleen and lymph nodes of mice using AutoMACS separator (Miltenyi Biotec). The sorted cells were stimulated for 72 hours with plate-bound anti-CD3 (1 μg/ml; 145–2C11; BD Biosciences) and soluble anti-CD28 (1 μg/ml; 37.51; BD Biosciences) with or without IL-21. The cells were then re-stimulated for 5 hours with PMA and ionomycin in the presence of Brefeldin A, cells were fixed with IC Fixation Buffer (BD), incubated with permeabilization buffer, and stained with PE–, APC– or PE–Cy 5.5 anti–mouse antibodies and intracellular cytokines were measured by flow cytometry. Flow cytometry was performed on a FACSCalibur (BD). Cells stimulated under neutral conditions were defined as T_H0 cells. Cells were stimulated to differentiate into T_{FH} cells by the supplementation with 10 ng/ml IL-21 (R&D Systems). Flow cytometry was performed on a FACSCalibur or LSR Fortessa analyzer (BD Biosciences).

2.5. T cell transfer model and histology

T cell transfer experiment was performed as previously described[42]. In brief, purified $CD4^+CD45RB^{hi}$ T cells from WT, *Irf8*$^{-/-}$ and *Bcl6*$^{-/-}$ mice with or without $CD19^+B220^+$ B cells (1×10^6 cells per mouse in 200 μl sterile PBS) from WT mice were transferred intraperitoneally into *Rag1*$^{-/-}$ recipients (6×10^5 cells per mouse in 200 μl sterile PBS). Mice were weighed every week throughout the course of experiments. After 5 weeks, mice were sacrificed, their spleens and mesenteric lymph nodes excised then and analyzed by flow cytometry. Spleens and colon tissues from WT, *Irf8*$^{-/-}$ or *bcl6*$^{-/-}$ mice were fixed in 10% neutral buffered formalin and embedded in paraffin or in frozen section. 5μm sections of tissue were stained with fluorochrome-conjugated antibodies. The degree of inflammation in the epithelium, submucosa and muscularis propria of colon tissue was scored separately as described by Totsuka *et al*[43].

2.6. Mice immunization

Mixing anti-mouse CD3 antibody (BD Biosciences, San Jose, CA) 5μg/mouse and anti-mouse CD28 antibody (BD Biosciences, San Jose, CA) 2μg/mouse in 100μL PBS, injection intraperitoneally two times interval three days. NP(40)-OVA/alum was prepared by mixing NP(40)-OVA (Biosearch Technologies, Petaluma, CA) in PBS with alum (Pierce, Rockford, IL) at a 1:1 ratio. NP-OVA/alum immunizations consisted of 100 μg given intradermal injection (i.d) four times interval one week.

2.7. RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted using an RNeasy plus kit (QIAGEN) and cDNA was transcribed using the Superscript II system (Invitrogen) with an oligo-dT primer followed by analysis using iCycler PCR with SYBR Green PCR master Mix (Applied Biosystems) using the primers in Table S1 and S2. Results were normalized based on the expression of ubiquitin.

2.8. Immunoblot

Cells were washed with cold phosphate-buffered saline and lysed for 15 min on ice in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 280 mM NaCl, 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol and 1 mM dithiothreitol) containing protease inhibitors. Cell lysates were performed for immunoblotting. Anti-IRF4 and anti-IRF8 (SantaCruz Biotechnology) and anti-β-actin (Sigma) antibodies were used according to the manufactures' instructions. Secondary antibodies were purchased from SantaCruz Biotechnology.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP was performed using a kit following the manufacturers' instruction (Upstate Biotechnology). Briefly, cells were fixed by 1% formaldehyde for 10 min at 37 °C. Nuclei were purified and sonicated to obtain DNA fragments. Chromatin fractions were pre-cleared with protein A-conjugated agarose beads followed by immunoprecipitation overnight at 4°C with 3 μg of anti-IRF8 or control antibody. Crosslinking was reversed at 65 °C for 4 h, followed by proteinase K digestion. DNA was purified and subjected to qPCR. The input DNA was diluted 200 times before PCR amplification. The input and immunoprecipitated DNAs were amplified by qPCR using the primers targeting.

2.10. Chromatin immunoprecipitation sequence (ChIP-Seq)

Purified $CD4^+$ T cells from WT mice were stimulated with or without anti-CD3 and anti-CD28 for 48h in the presence of IL-21. The cell pellet was cross-linked and sonicated, the chromatin was ready for MOWChIP-seq. The mixed beads of protein A beads and protein G beads were incubated with IRF8 antibody. After washing, the IRF8-beads were loaded to chamber and incubate with sonicated chromatin samples. After ChIP, the washed immune complexes were collected and resuspended in the reverse crosslinking buffer to incubate. The DNA was extracted and precipitation. Sequencing libraries were prepared by Accel-NGS 2S Plus DNA Library Kit (Swift). The libraries were sequenced on an Illumina HiSeq 4000 with single-end 50-nt reads.

2.11. Statistical analysis

Statistical analysis was performed using Student's *t*-test for most of the experiments. $P<0.05$ was considered statistically significant.

3. Results

3.1. T_{FH} are critically involved in intestinal inflammation

While the primary known function of T_{FH} is to provide helper signals that drive GB B cell differentiation[5–8], it is possible that T_{FH} can directly function as pathogenic mediators of autoimmune injury independent of their helper functions. To investigate the possibility that T_{FH} cells contribute to intestinal inflammation, we first examined the expression of T_{FH} signature genes in the intestinal tissues of Crohn's disease patients. These analyses revealed that T_{FH}-associated gene products including IL-21, CXCR5, ICOS, PD1 and Bcl-6 were significantly up-regulated in intestinal tissues of Crohn's disease patients compared to normal controls (Fig. S1). Similarly, when we analyzed colon tissue of a murine model in which adoptive transfer of $CD4^+$ T cells together with or without B cells into syngeneic *Rag1*$^{-/-}$ hosts reproducibly results in colitis[42–43] (Fig. S2A and data not shown), we observed significantly higher expression of the same T_{FH}-related gene products (Fig. S2B). Histological staining showed that Bcl-6 was highly expressed in the intestinal tissues with colitis (Fig. S2C) and flow cytometry analysis of spleen cells showed detectable $CXCR5^+ICOS^+PD1^+BCL\text{-}6^+$ T_{FH} only in *Rag1*$^{-/-}$ recipients of WT naïve $CD4^+$ T cells (Fig. S2D).

As Bcl-6 is one of several TFs required for T_{FH} differentiation[9–10] we directly tested the pathogenicity of T_{FH} by adoptively transferring naïve *Bcl-6*$^{-/-}$$CD4^+$ T cells into *Rag1*$^{-/-}$ recipients (Fig. 1A-B). Distinct from recipients of WT $CD4^+$ T cells, recipients of *Bcl-6*$^{-/-}$ $CD4^+$ $CD45Rb^{hi}$ cells appeared phenotypically normal and maintained rather than lost weight. Histological analyses of the intestinal tissue 6 weeks post-transfer revealed less inflammatory cell infiltration and lower pathology scores in the *Rag1*$^{-/-}$ recipients of *Bcl-6*$^{-/-}$ $CD4^+$ cells (Fig. 1C). When we analyzed T cell phenotypes of mesenteric lymph nodes at 6 weeks we observed significantly fewer T_{FH} in adoptive recipients of *Bcl-6*$^{-/-}$ $CD4^+$ cells (Fig. 1D). Taken together, the results support the conclusion that that T_{FH} rather than T_H1 or T_H17 cells (data not shown) are pathogenic mediators of intestinal inflammation in this model.

3.2. IRF8-deficient mice display enhanced T_{FH} cell differentiation

Building upon our previous observation that IRF8 inhibits T_H17 differentiation[42] we tested the impact of IRF8 on differentiation of naïve $CD4^+$ T cells toward the T_{FH} phenotype using an *in vitro* culture system (Fig. 2). Three days after stimulating purified naïve *Irf8*$^{-/-}$ or WT $CD4^+$ T cells with anti-CD3/CD28 under T_{FH}-inducing conditions, we analyzed cytokine, surface marker and TF profiles in the responding T cells. These assays showed higher proportions of $CXCR5^+PD\text{-}1^+$, $CXCR5^+ICOS^+$ and $CXCR5^+Bcl\text{-}6^+$ cells (Fig. 2A−C) and more IL-21, within the *Irf8*$^{-/-}$ $CD4^+$ T cells vs. WT controls (Fig. 2D and 2E). Quantitative PCR assays showed higher *Icos*, *Pdcd1*, *Cxcr5* and *Il21* gene expression in the *Irf8*$^{-/-}$ $CD4^+$ T cells (Fig. 2F). Analysis of $CD4^+$ T cells obtained from mice with IRF8 deficiency

restricted to T cells (Lck-Cre^{+}*Irf8*$^{fl/fl}$) confirmed enhanced T_{FH} cell differentiation (Fig. 3A, 3B and 3C). Proliferative responses of WT and *IRF8*$^{-/-}$ CD4^{+} T cells cultured under T_{FH}-inducing conditions did not differ (Fig. S3), and IRF8 deficiency did not alter *in vitro* induction of IFNγ^{+} T_H1, IL-4^{+} T_H2 or Foxp3^{+} T_{reg} cells (Fig. S4)[42], together demonstrating that IRF8 specifically inhibits the T_{FH} differentiation program.

To begin to assess how the above observed effects of T cell IRF8 deficiency apply *in vivo*, we injected groups of Lck-Cre^{+}*Irf8*$^{fl/fl}$ and Lck-Cre^{+}*Irf8*$^{wt/wt}$ mice with anti-CD3 mAb (Fig. 4) and independently we immunized groups of animals with 4-hydroxy-3nitrophenylacetyl-conjugated ovalbumin (NP-OVA, Fig. S5). Under both conditions, flow cytometry analyses revealed ~2-fold higher frequencies of T_{FH} cells in the spleens of treated Lck-Cre^{+}*Irf8*$^{fl/fl}$ mice (Fig. 4A, and S5). Quantification of T_H1, T_H2 and T_{reg} did not differ between groups in either set of experiments (Fig. S4), validating that the *in vitro* findings (Fig. 2) apply *in vivo* and supporting the concept that IRF8 specifically regulates T_{FH} differentiation[42].

3.3. IRF8-deficient T_{FH} cells are pathogenic mediators of colitis

To directly test the hypothesis that IRF8 negatively regulates T_{FH} capable of mediating colonic pathology, we adoptively transferred WT or *Irf8*$^{-/-}$ CD4^{+} T cells into groups of *Rag1*$^{-/-}$ mice and followed the animals for up to 6 weeks, analyzing and comparing clinical disease expression, colon histology and splenic T cell responses between groups. These analyses showed *Rag1*$^{-/-}$ recipients of *Irf8*$^{-/-}$ CD4^{+} T cells exhibited greater and more rapid weight loss with more severe histological changes in the colons (Fig. 5A, 5B) and higher frequencies of splenic T_{FH} (Fig. 5C).

To confirm that absence of T cell IRF8 exacerbates colitis through a T_{FH}–dependent mechanism, we adoptively transferred naïve *Irf8*$^{-/-}$*Bcl-6*$^{-/-}$ CD4^{+} T cells into *Rag1*$^{-/-}$ recipients. These experiments showed diminished colitis severity (Fig 6A-C), similar to that observed in the recipients of *Bcl-6*$^{-/-}$ CD4^{+} T cells (Fig. 1B). Frequencies of splenic T_{FH} were also reduced in *Irf8*$^{-/-}$*Bcl-6*$^{-/-}$ recipient mice compared to WT controls (Fig. 6D). Taken together, the data support the conclusion that IRF8 suppresses inflammation via the inhibiting differentiation of pathogenic T_{FH}.

3.4. Mice with T cell-specific IRF8 deficiency exhibit exaggerated B cell differentiation

The data above suggests that IRF8-regulated T_{FH} cells play an important role in T cell-mediated inflammation. To determine whether the enhanced T_{FH} signature functionally affects B cell development, we analyzed mice with germline (*Irf8*$^{-/-}$) or T cell-specific IRF8 deficiency (Lck-Cre^{+}*Irf8*$^{fl/fl}$). These mice had enlarged spleens and lymph nodes (Fig. S6A and B) that harbored increased numbers of CD19^{+}CD138^{+} plasma cells (Fig. S6C) and germinal center B cells (Fig. S6D). Consistently, Lck-Cre^{+}*Irf8*$^{fl/fl}$ mice had increased levels of serum IgG and IgM (Fig. S6E). The expansion of plasma cells in secondary lymphoid organs of *Irf8*$^{-/-}$ mice was not due to infection or autoimmunity, as CD11c^{+} DCs were not increased in any immune organ or tissue analyzed (Fig. S7A), and there was no autoimmune kidney damage, such as cellular infiltration and glomerular crescents, in *Irf8*$^{-/-}$ mice (Fig. S7B). There is no significant expansion in population of T cells but as reported data that myeloid cells were obviously expanded (Fig. S7A). To determine whether *Irf8*$^{-/-}$ CD4^{+} T

cells also induce more B cell proliferation *in vivo*, we co-transferred WT B cells with either WT or *Irf8*$^{-/-}$ CD4^{+} T cells into *Rag1*$^{-/-}$ mice. Splenomegaly after adoptive transfer was markedly increased in recipients of *Irf8*$^{-/-}$ CD4^{+} T cells (Fig. S6F), and elevated percentage of B cells was found in the spleen and lymph nodes (Fig. S6G). These results suggest that IRF8 controls the magnitude of humoral immunity by regulating the differentiation and function of T_{FH} cells. In addition to regulating the function of CD4^{+} T cells in GC B cell response, IRF8 expression in CD4^{+} T cells may influence B cell differentiation prior to the GC stage. To test this possibility, we co-transferred C57BL/6 (H-2^{b}) bone marrow cells depleted of T cells with either WT or *Irf8*$^{-/-}$ C57BL/6 (H-2^{b}) CD4^{+} T cells into irradiated allogeneic BALB/c (H-2^{d}) recipients. Co-transferred *Irf8*$^{-/-}$ CD4^{+} T cells induced more B cell development from H-2^{b} bone marrow cells than co-transferred WT CD4^{+} T cells (Fig. S8). Therefore, IRF8 in CD4^{+} T cells also restrains the ability of CD4^{+} T cells to support B cell development *in vivo*.

3.5. IRF8 suppresses IRF4 expression in CD4^{+} T cells

IRF4 is important for the differentiation of various T helper cell subsets, including T_{FH}, T_H2, T_H9 and T_H17 cells[11,14,34]. IRF4 and IRF8 represent immune-specific members of the interferon regulatory family and co-operate to play major roles in controlling the development and functioning of T cell subsets and other immune cells. To better understand the molecule mechanism of IRF8 in controlling of T_{FH} cell function, we purified CD4^{+} T cells from WT or *Irf8*$^{-/-}$ mice and stimulated with or without antiCD3 and anti-CD28 under T_{FH} cells culture condition. The results clearly indicated that *Irf4* and *Il21* expression levels were significantly increased in *Irf8*$^{-/-}$ mice compared with WT mice by microarray analysis (Fig. 7A). Furthermore, we evaluated the *Irf4* gene by Quantitative real-time-PCR analysis (Fig. 7B) and protein expression levels of CXCR5, Bcl-6, IRF4 and IRF8 by western blotting (Fig. 7E) in WT and *Irf8*$^{-/-}$ CD4^{+} T cells under T_{FH} condition for time course. The results showed that both the *Irf4* gene and IRF4 protein were significantly up-regulated in *Irf8*$^{-/-}$ mice compared with WT mice (Fig. 7A, B and E). We performed ChIP-seq assay to understand whether IRF8 binds to the promoter region of IRF4 gene in T_{FH} cell condition. The result showed that IRF8 indeed bound to the promoter region of IRF4 gene (Fig. 7C). Furthermore, the ChIP assays also showed more IRF4 protein bound to the *Il21* promoter in CD4^{+} T cells of *Irf8*$^{-/-}$ mice under T_{FH} cell culture condition when compared with WT mice (Fig. 7D). The results suggested that IRF8 suppresses T_{FH} differentiation by inhibiting the DNA biding activity of IRF4 to the promoter region of *Il21* gene.

We also sought to determine if IRF8 controls the expression of CD40 ligand (CD40L), a molecule crucial to the function of T_{FH} cells. Compared to WT CD4^{+} T cells, *Irf8*$^{-/-}$ CD4^{+} T cells expressed higher protein and mRNA levels of CD40L following *ex vivo* stimulation in T_{FH} conditions (Fig. 7F, 7G, and 7H). The same results for protein expression were also seen in *ex vivo* stimulated CD4^{+} T cells from Lck-Cre^{+}*Irf8*$^{fl/fl}$ mice (Fig. 7I). Upon TCR stimulation by anti-CD3 Ab *in vivo*, CD40L expression was higher on CD4^{+} T cells of Lck-Cre^{+}*Irf8*$^{fl/fl}$ mice than those of WT control mice in mesenteric lymph nodes (Fig. S9A). Considering that IRF8 can function as a transcriptional repressor, we performed a chromatin immunoprecipitation (ChIP) assay to determine if IRF8 can repress the *Cd40lg* gene in CD4^{+} T cells. IRF8 bound to three of four putative IRF8-binding sites in the *Cd40lg*

promoter and one experiment of ChIP data shown as Fig. 7J. Consistently, IRF8 suppressed CD40L expression in $CD4^+$ T cells in a cell-intrinsic fashion, as evidenced by reduced cell surface CD40L levels in $CD4^+$ T cells transduced by an *Irf8*-encoding retrovirus (Fig. S9B), as compared to marked increases in CD40L staining in cells transfected with empty virus. Taken together, these results suggest that IRF8 represses CD40L expression at both the transcriptional and protein levels in T_{FH} cells.

4. Discussion

Our data newly and uniquely demonstrate a proinflammatory role for T_{FH} as mediators of colitis independent of their ability to provide helper signals to B cells. We show that intestinal tissues of inflammatory bowel disease (IBD) patients and colon tissues obtained from *Rag1*$^{-/-}$ recipients of naïve $CD4^+$ T cells with colitis, each over-express T_{FH}-associated gene products. Adoptive transfer of naïve *Bcl6*$^{-/-}$ $CD4^+$ T cells into *Rag1*$^{-/-}$ recipient mice abrogated development of colitis and limited T_{FH} differentiation *in vivo*, demonstrating a mechanistic link. In contrast, T cell deficiency of interferon regulatory factor 8 (IRF8) resulted in augmentation of T_{FH} induction *in vitro* and *in vivo*. Functional studies showed that adoptive transfer of *Irf8*$^{-/-}$ $CD4^+$ T cells into *Rag1*$^{-/-}$ recipients exacerbated colitis development associated with increased gut T_{FH}-related gene expression, while *Irf8*$^{-/-}$/*Bcl6*$^{-/-}$ $CD4^+$ T cells abrogated colitis, together indicating that IRF8-regulated T_{FH} can directly cause colon inflammation. Molecular analyses revealed that IRF8 suppresses T_{FH} differentiation by inhibiting transcription and transactivation of the TF IRF4, which is also known to be essential for T_{FH} induction.

T_{FH} cells are a distinct T helper cell subset shown to coordinate generation of the germinal center (GC) responses by initiating help for antigen specific B cells. Through this B cell help mechanism, T_{FH} have been shown to be pathogenic in a number of autoimmune diseases. Increased frequencies of T_{FH}-like cells in peripheral blood are observed in subsets of patients with Sjogren's syndrome, juvenile dermatomyositis, and systemic lupus erythematosus[44–45], each of which is associated extensive autoantibody production. However, several observations in the literature hint that T_{FH} have other functions beyond providing helper signals for B cell differentiation and antibody switching. T_{FH} are found within injured organs and tissues in subjects with lupus nephritis[46], multiple sclerosis[47], inflammatory arthritis, type 1 diabetes[48], and intestinal tissues of patients with IBD[49]. Our data using adoptive transfer of Bcl-6 deficient naïve $CD4^+$ T cells into *Rag1*$^{-/-}$ recipients provide direct evidence in support of this hypothesis linking T_{FH} to intestinal inflammation.

T_{FH}-produced IL-21, which is the key cytokine shown to drive B cell differentiation in GCs[19–22]. Early studies revealed that IL-21 is markedly overproduced in inflamed guts of patients with IBD compared to non-inflamed controls[49]. Mice lacking IL-21 are protected against chemically induced colitis; moreover, wild type mice given a neutralizing IL-21R/Fc fusion protein exhibit less experimental colitis as compared to control mice. IL-21 has been shown to activate macrophages, participate in granuloma formation, and inhibit induction of regulatory T cells (Treg) in murine graft vs host diseases. These findings together raise the possibility that T_{FH}/IL-21 have pathogenic potential. In the present study, we demonstrate that IL-21 is highly expressed in intestinal tissues of IBD patients and also in the colon

tissues of recipient mice transferred with naïve $CD4^+$ T cells with colitis. Based these published findings and our new data, including the strong association of T_{FH}-related genes to human IBD (Fig S1), we speculate that T_{FH}-produced IL-21 is one pathological mediator of intestinal inflammation.

IRF8 plays critical roles in the differentiation of myeloid cells, B cells, dendritic cells and T cells, and hence the regulation of both innate and adoptive immune responses[11,32–42]. A recent genome-wide association (GWA) study has strongly implicated a variant near the IRF8 gene in SLE susceptibility in Europeans[50]. In addition, a different genetic variation in the IRF8 gene has been implicated in multiple sclerosis[51]. A GWA study identified the IRF8 gene as strongly associated with development of Crohn's disease[52], but provided no mechanistic explanation. Our findings in which we demonstrate that IRF8 deficiency a) favors T cell differentiation toward the T_{FH} lineage and b) exacerbates $CD4^+$ T cell-mediated colitis provide a potential explanation to account for this GWAS association. The observations support the testable hypothesis that IRF8 mutations that diminish production or function of IRF8 lead to augmented T_{FH} differentiation which in turn contributes to the development of IBD.

Published evidence demonstrates that IRF8 cooperates with other TFs including Bcl-6, IRF4, and PU.1 to regulate immune cell function[11,33–42]. IRF8 and IRF4 are immune-specific members of the IRF TF family and have evolved not only to interact with specific members of the Ets superfamily, e.g. PU.1 and Spi-B, but also with particular members of the AP-1 superfamily, e.g. BATF-containing heterodimers[40–41]. IRF4 is important for the differentiation of various T helper cell subsets, including T_{FH}, T_H2, T_H9 and T_H17 cells[11,14,34]. Adding to this literature, in the present study, we provide new insight into mechanistic links among IRF8, IRF4 and IL-21 producing T_{FH}. We demonstrate that IRF8 binds to the promoter region of IRF4 gene and that in the absence of IRF8, T cells express more IRF4. In addition, IRF8 deficiency significantly enhanced the IRF4 binding to the promoter region of IL-21 gene, together supporting the conclusion that IRF8 suppresses T_{FH} differentiation by inhibiting transcription and transactivation of the TF IRF4.

Taken together, our murine and human data has uncovered potentially important pathogenic role for T_{FH} cells in inflammatory diseases, and suggests that the suppressive function of IRF8 in T_{FH} differentiation and the function may be potentially targeted to treat these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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our study reveals that T_{FH} cells are critically involved in the development of T cell-mediated inflammatory diseases and defines IRF8 as an important intrinsic suppressor of T_{FH} differentiation, and our studies are really novel and will lead to more exciting investigations concerning the contributions of T_{FH} cells in the development of various T cell-mediated inflammatory diseases in future.

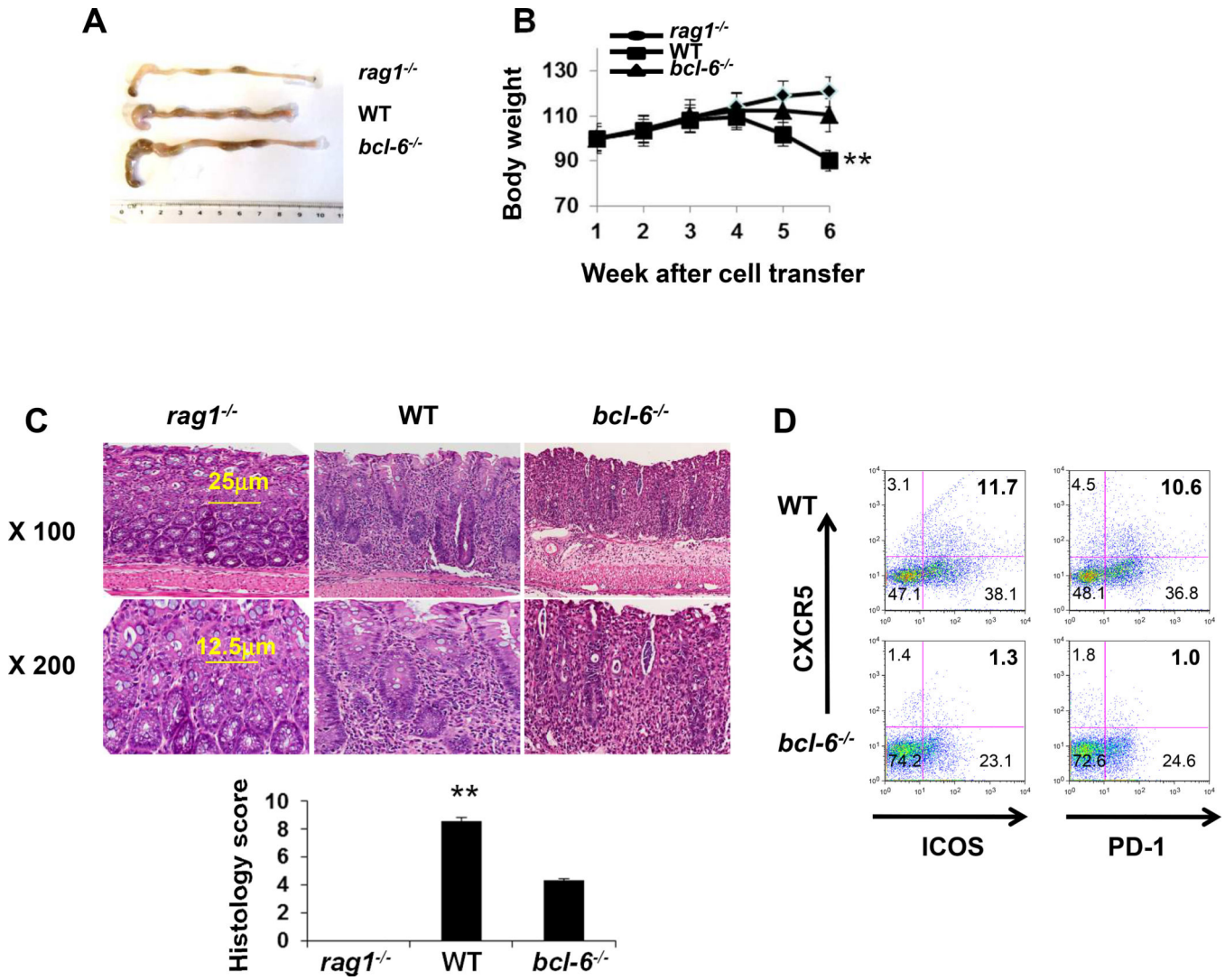

Figure 1. T_{FH} signature is up-regulated in autoimmune and inflammatory diseases.

(A and B) Colon morphology and body weight of *Rag1*$^{-/-}$ recipient mice after receiving 6×10^5 purified WT (n = 5) or *Bcl-6*$^{-/-}$ (n = 5) CD4$^+$ T cells. (C) H&E staining and histology score of colon tissues of *Rag1*$^{-/-}$ recipient mice after receiving 6×10^5 purified WT (n = 5) or *Bcl-6*$^{-/-}$ (n = 5) CD4$^+$ T cells. (D) The percentages of CXCR5$^+$ICOS$^+$ and CXCR5$^+$PD-1$^+$ CD4$^+$ T cells were compared between *Rag1*$^{-/-}$ recipients of WT CD4$^+$ T cells (n = 5) and those of *Bcl-6*$^{-/-}$ CD4$^+$ T cells (n = 5).

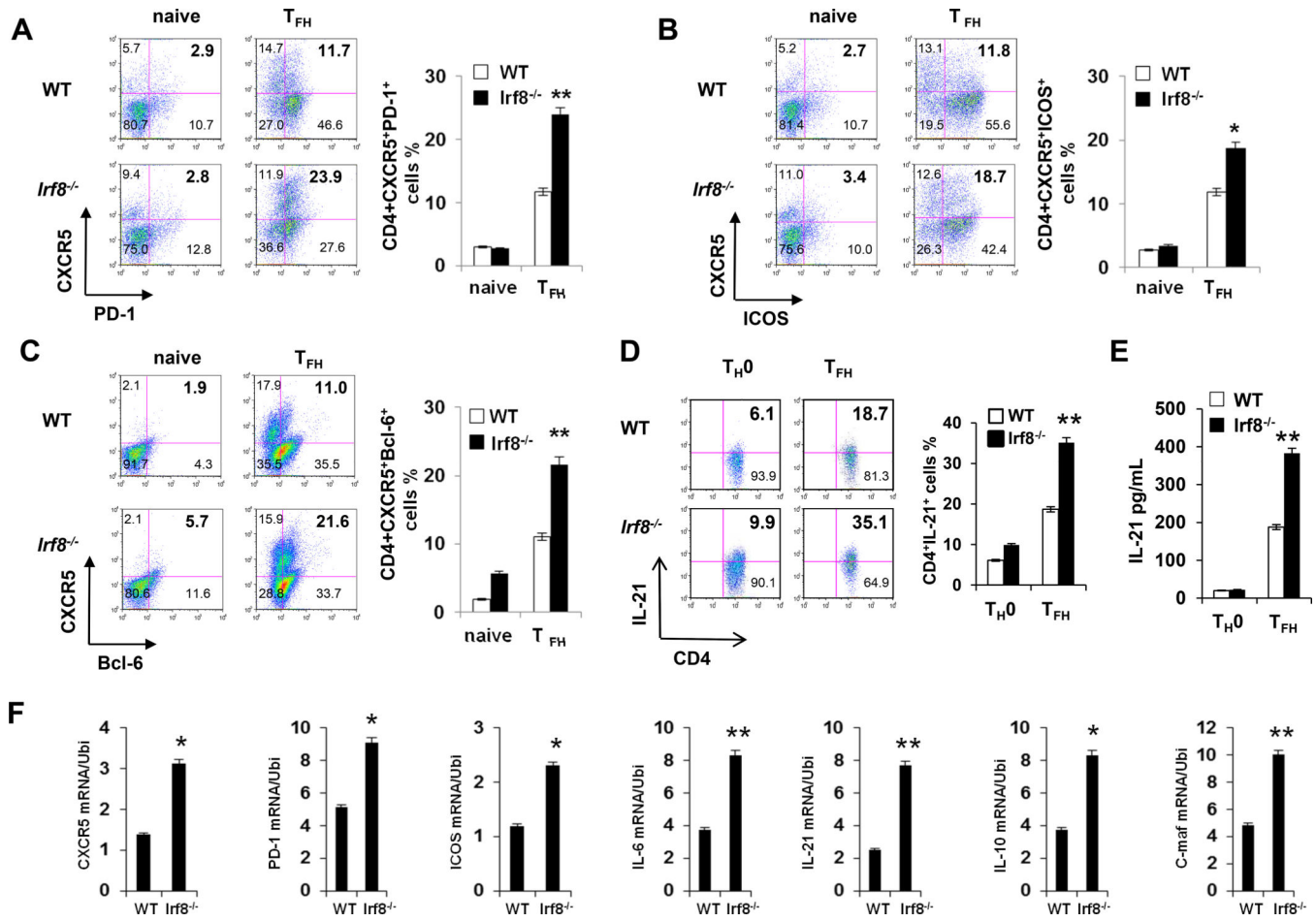

Figure 2. IRF8-deficient CD4$^+$ T cells show enhanced T_{FH} polarization after *ex vivo* stimulation.

Purified CD4$^+$ T cells from WT or *Irf8*$^{-/-}$ mice (A, B, and C) were stimulated with or without anti-CD3 and anti-CD28 for 48h in the presence of IL-21. Cell surface expression of PD-1, ICOS, CXCR5 and intracellular expression of Bcl-6 were analyzed by flow cytometry. The percentages of CXCR5$^+$PD1$^+$, CXCR5$^+$ICOS$^+$ and CXCR5$^+$Bcl6$^+$ cells were compared between WT (n = 5) and *Irf8*$^{-/-}$ (n = 5) CD4$^+$ T cell cultures. (D) Flow cytometry evaluation of IL-21$^+$CD4$^+$ T cells in WT and *Irf8*$^{-/-}$ CD4$^+$ T cells under T_{FH} condition (n = 5). (E) ELISA of IL-21 levels in culture supernatant of WT and *Irf8*$^{-/-}$ CD4$^+$ T cells cultured under T_{FH} condition (n = 4). (F) Quantitative real-time RT-PCR analysis of T_{FH}-associated genes in WT and *Irf8*$^{-/-}$ CD4$^+$ T cells under T_{FH} condition (n = 5). The ubiquitin gene (*Ubi*) was used as an internal control. Results shown are representative of three independent experiments. Data are given as means ± SEM.

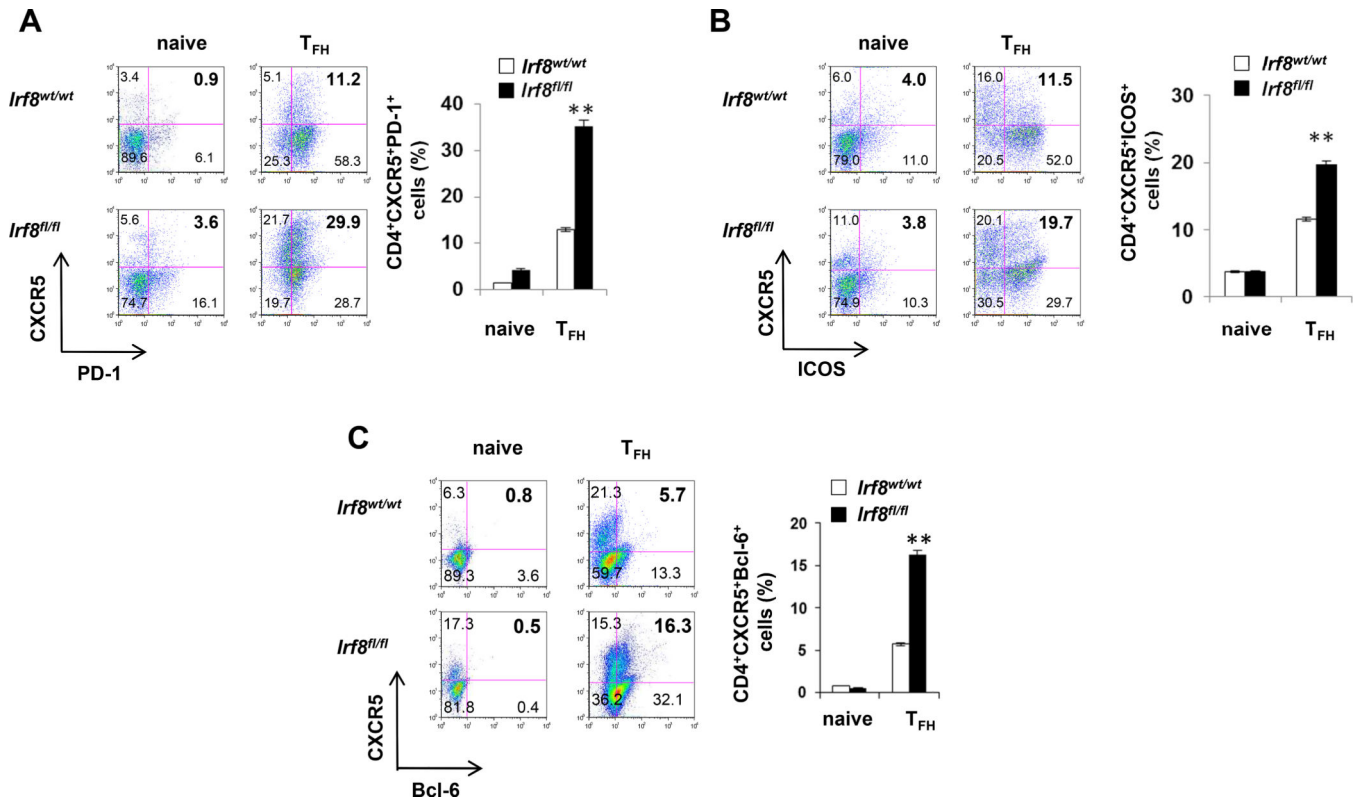

Figure 3. IRF8-deficient in T cells show enhanced T_{FH} polarization after *ex vivo* stimulation.

Purified $CD4^+$ T cells from *Irf8*$^{wt/wt}$ or *Irf8*$^{dck/lck}$ mice (A, B, and C) were stimulated with or without anti-CD3 and anti-CD28 for 48h in the presence of IL-21. Cell surface expression of PD1, ICOS, CXCR5 and intracellular expression of Bcl-6 were analyzed by flow cytometry. The percentages of $CXCR5^+PD1^+$, $CXCR5^+ICOS^+$ and $CXCR5^+Bcl6^+$ cells were compared between *Irf8*$^{wt/wt}$ (n = 5) and *Irf8*$^{dck/lck}$ (n = 5) $CD4^+$ T cell cultures. Results shown are representative of three independent experiments. Data are given as means ± SEM.

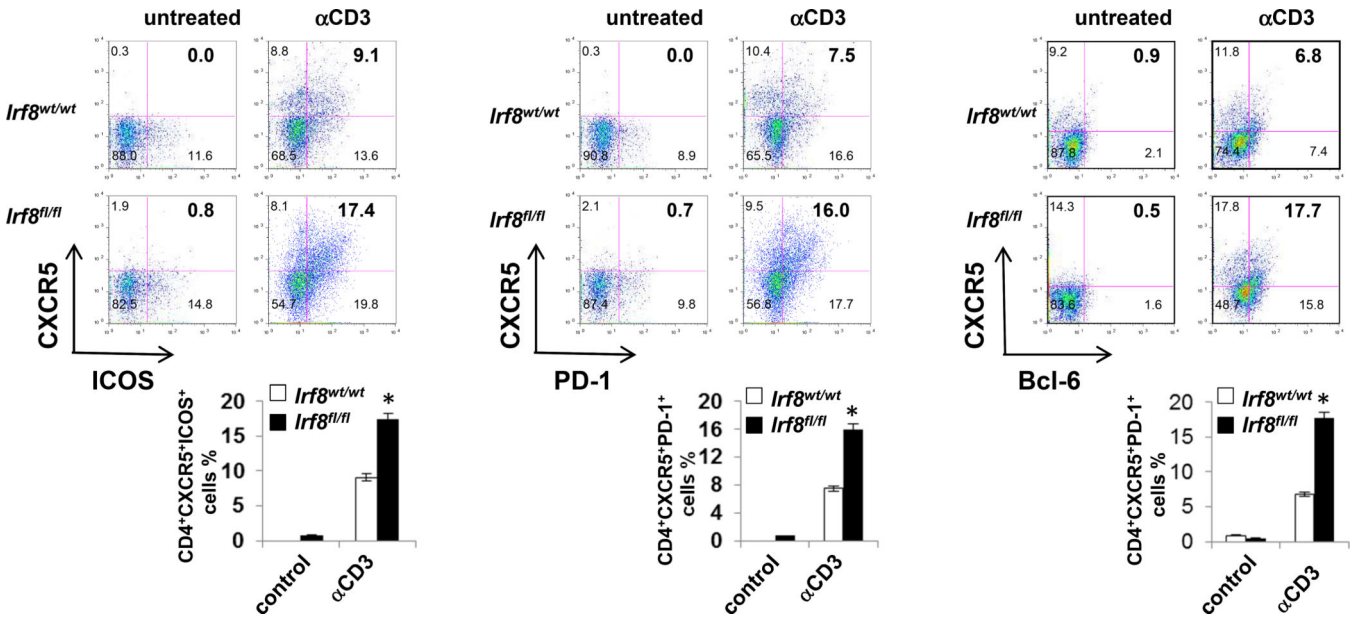

Figure 4. T_{FH}-associated signatures were significantly increased in IRF8-deficient T cells *in vivo*.

Cell surface expression of ICOS, PD-1, CXCR5 and intracellular expression of Bcl-6 in $CD4^+$ T cells of Lck-Cre^+ *$Irf8^{wt/wt}$* or Lck-Cre^+ *$Irf8^{fl/fl}$* mice after anti-CD3 administration two times interval three days intraperitoneally, analyzed by flow cytometry. The percentages of $CXCR5^+ICOS^+$, $CXCR5^+PD1^+$ and $CXCR5^+Bcl6^+$ cells in $CD4^+$ T cells of spleen were compared between Lck-Cre^+ *$Irf8^{wt/wt}$* (n = 6) and Lck-Cre^+ *$Irf8^{fl/fl}$* (n = 6) mice. Results shown are representative of three independent experiments. Data are given as means ± SEM.

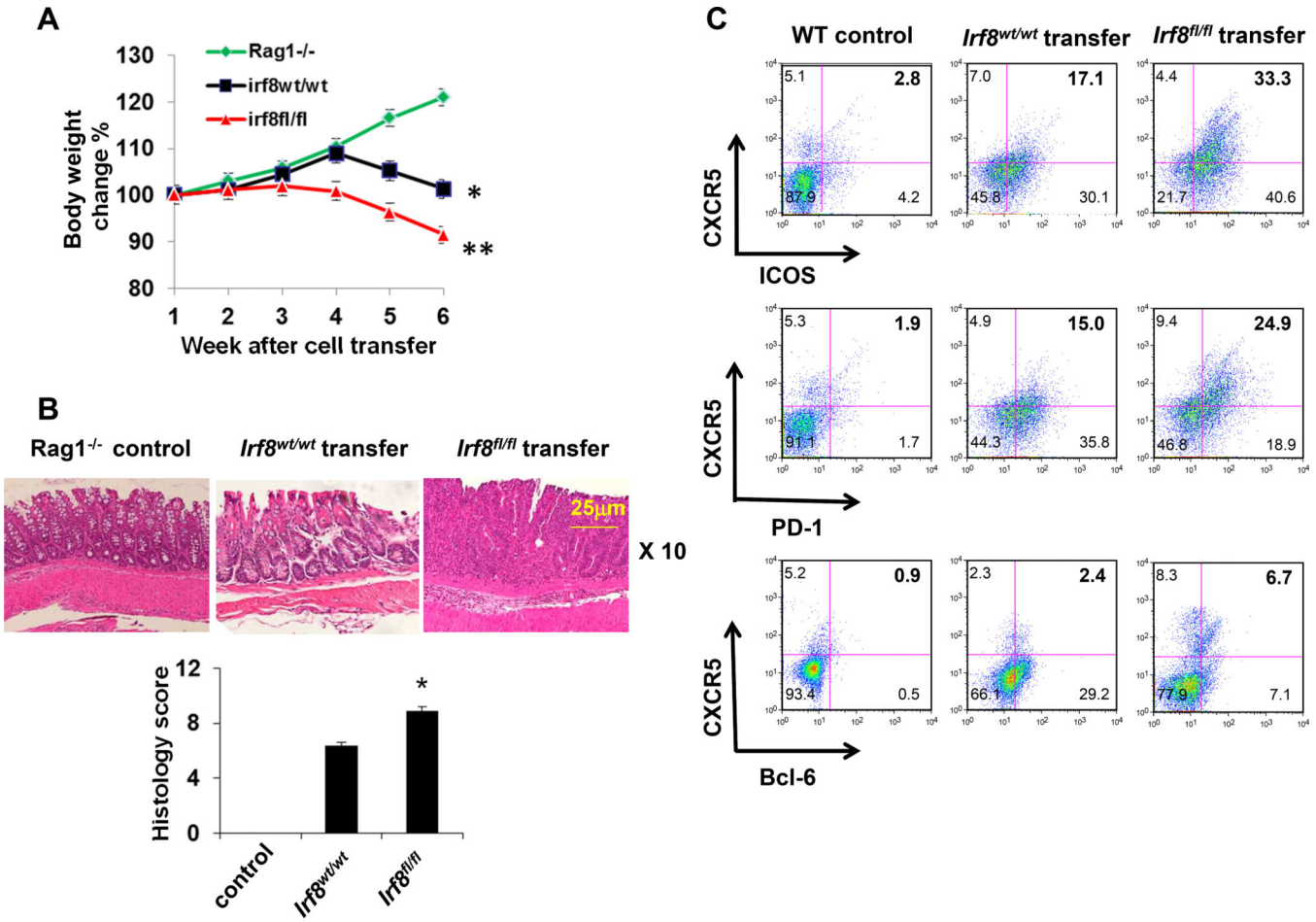

Figure 5. T_{FH} signature is up-regulated in autoimmune and inflammatory diseases.

(A) Body weight of *Rag1*$^{-/-}$ recipient mice after receiving 6×10^5 purified CD4$^+$ T cells from LckCre$^+$*Irf8*$^{wt/wt}$ (n = 5) or Lck-Cre$^+$*Irf8*$^{fl/fl}$ (n = 5) mice with CD19$^+$B220$^+$ B cells from WT mice. (B) Histological staining of colon tissues and histology score of *Rag1*$^{-/-}$ recipients of LckCre$^+$*Irf8*$^{wt/wt}$ CD4$^+$ T cells (n = 5) and those of Lck-Cre$^+$*Irf8*$^{fl/fl}$ CD4$^+$ T cells (n = 5). (C) The percentages of CXCR5$^+$ICOS$^+$, CXCR5$^+$PD-1$^+$ and CXCR5$^+$Bcl6$^+$ CD4$^+$ T cells were compared between *Rag1*$^{-/-}$ recipients of Lck-Cre$^+$*Irf8*$^{wt/wt}$ CD4$^+$ T cells (n = 5) and those of Lck-Cre$^+$*Irf8*$^{fl/fl}$ CD4$^+$ T cells (n = 5) and WT as control (n=5). Results shown are representative of three independent experiments. Data are given as means ± SD.

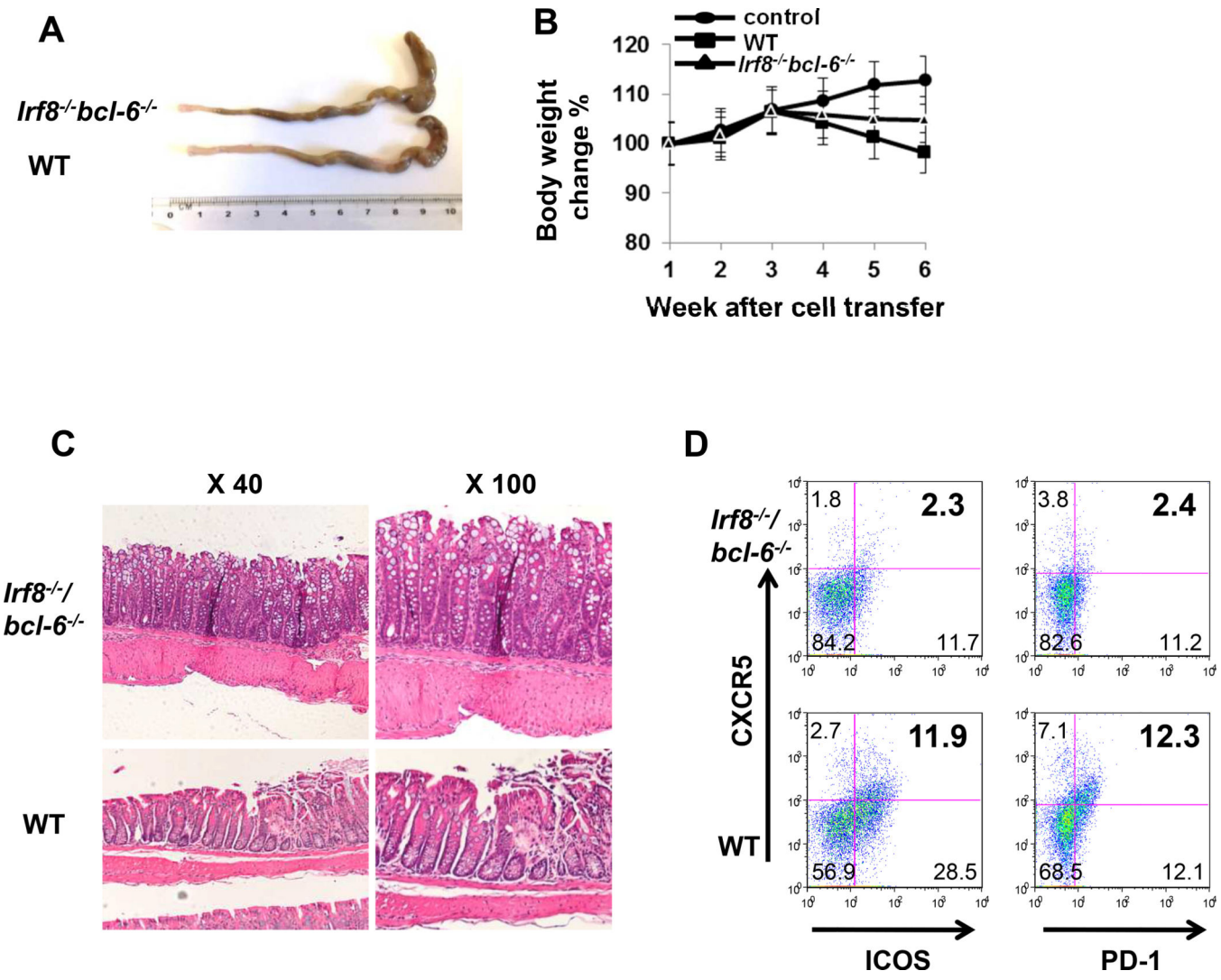

Figure 6. T_{FH} signature is up-regulated in autoimmune and inflammatory diseases.

Colon morphology and body weight of *Rag1*$^{-/-}$ recipient mice after receiving 6×10^5 purified WT (n = 5) or *Irf8*$^{-/-}$*Bcl6*$^{-/-}$ (n = 6) CD4$^+$ T cells with CD19$^+$B220$^+$ B cells from WT mice (A and B). (C) H&E staining of colon tissues of *Rag1*$^{-/-}$ recipient mice after receiving 6×10^5 purified WT (n = 5) or *Irf8*$^{-/-}$*Bcl6*$^{-/-}$ (n = 6) CD4$^+$ T cells. (D) The percentages of CXCR5$^+$ICOS$^+$ and CXCR5$^+$PD-1$^+$ CD4$^+$ T cells were compared between *Rag1*$^{-/-}$ recipients of WT CD4$^+$ T cells (n = 5) and those of *Irf8*$^{-/-}$*Bcl6*$^{-/-}$ CD4$^+$ T cells (n = 6). Results shown are representative of five mice. Data are given as means ± SD.

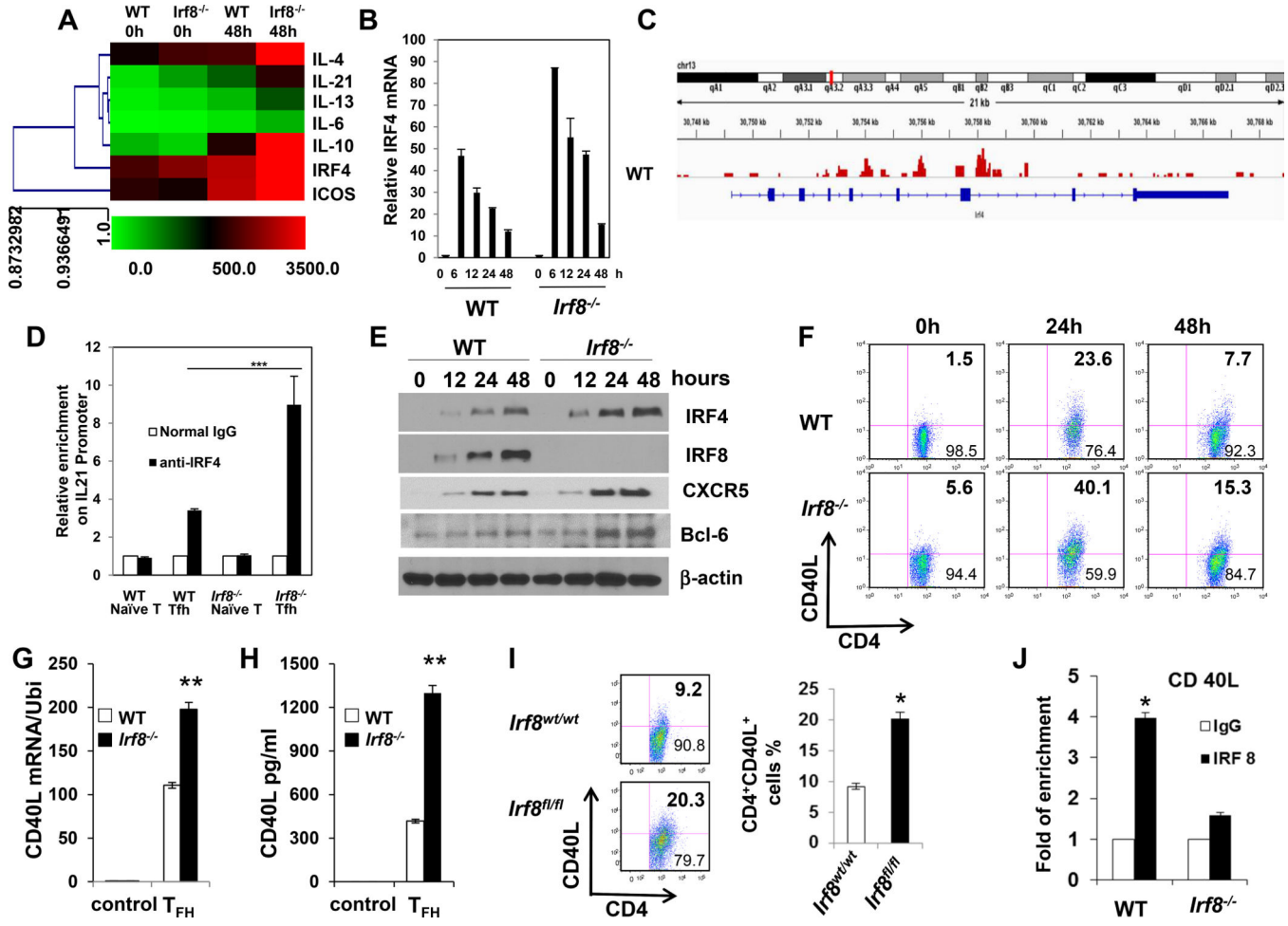

Figure 7. Regulation of IRF4 expression by IRF8.

(A) Purified CD4$^+$ T cells from WT or *Irf8*$^{-/-}$ mice were stimulated with or without anti-CD3 and anti-CD28 for 48h in the presence of IL-21. Microarray analysis of the gene expression by WT and *Irf8*$^{-/-}$ CD4$^+$ T cells under T_{FH} condition. (B) Quantitative real-time RT-PCR analysis of irf4 gene level in WT and *Irf8*$^{-/-}$ CD4$^+$ T cells under T_{FH} condition for time course (n = 5). The ubiquitin gene (*Ubi*) was used as an internal control. (C) IRF8 ChIP-seq signals at the IRF4 gene locus. Blue line denotes the range of the IRF4 gene locus, and the peak patterns in red represent the ChIP-seq signals of IRF8 binding. Signals represent DNA fragments that were captured by the IRF8 antibody through ChIP. (D) Purified CD4+ T cells from WT or *Irf8*$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 for 48h in the presence of IL-21, followed by ChIP assay. Three micrograms of an anti-IRF4 antibody or isotype-matched IgG as control antibody were used in the immunoprecipitation step. PCR was used to quantify the amount of precipitated DNA with primers flanking the irf4-binding site of the IL-21 promoter region. Each bar represents mean ± S.D. from three independent experiments, unpaired Student's t-test, *P<0.05, versus WT cells. (E) Western blot analysis of IRF4, IRF8, Bcl-6 and CXCR5 protein levels in WT and *Irf8*$^{-/-}$ CD4$^+$ T cells under T_{FH} condition for time course. The β-actin was used as an internal control. (F) Purified CD4$^+$ T cells from WT or *Irf8*$^{-/-}$ mice were stimulated with anti-CD3 and anti-

CD28 antibody for 48h. CD40L expression was analyzed by flow cytometry gated on $CD4^+$ T cells and used isotype control. (G) *Cd40lg* RNA level was analyzed by real-time RT-PCR (n = 5). (H) Soluble CD40L protein level in culture media was measured by ELISA. (I) The experiment was repeated by using purified $CD4^+$ T cells of Lck-Cre^+ $Irf8^{wt/wt}$ (n = 6) or Lck-Cre^+ $Irf8^{fl/fl}$ (n = 6) mice. (J) ChIP assay of $CD4^+$ T cells from WT and $Irf8^{-/-}$ mice was analyzed for binding of IRF8 to the *Cd40lg* promoter (n = 4). Results shown are representative of three independent experiments. Data are given as means ± SEM.