Navigating double negatives: new pathways for regulating T_{FH} differentiation

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The transcription factors Foxp1 and Foxo1 inhibit the differentiation of follicular helper T cells. On the other hand, the E3 ligase Itch targets Foxo1 for degradation to promote such differentiation.

Help provided by T cells is necessary for efficient B cell responses. Investigation of helper T cell differentiation has so far focused largely on identifying positive regulators of each subset and how they antagonize each other to stabilize lineage commitment. However, studies by Wang *et al.*[1] and Xiao *et al.*[2] in this issue of *Nature Immunology* demonstrate that negative regulators also have an important role in the differentiation of helper T cells.

Cognate help from T cells is required for B cells to undergo affinity maturation and generate B cell memory, which happens mainly in a specialized lymphoid structure: the germinal center. Follicular helper T cells (T_{FH} cells) are a subset of helper T cells specialized to support the proliferation and selection of B cells and their differentiation in germinal centers[3,4]. Sufficient T_{FH} cell function is required for robust antibody responses to control infections, whereas excessive T_{FH} cell activity promotes the production of autoantibodies and can lead to autoimmune diseases[5–7].

The differentiation of T_{FH} cells is induced by antigen stimulation in synergy with costimulation from CD28, CD40L, the inducible costimulator ICOS, receptors of the SLAM ('signaling lymphocyte activation molecule') family and a cytokine milieu that includes interleukin 21 (IL-21), IL-6 and IL-12. This differentiation program is directed by the key transcription factor Bcl-6 and is facilitated by several other transcription factors, including STAT3, c-Maf and Ascl2 (refs. 4,7).

In contrast to the considerable body of knowledge about the positive regulators of T_{FH} cell differentiation, understanding of the negative regulation of this process is limited. IL-2–STAT5 signaling has been reported to suppress T_{FH} cell differentiation, probably through a mechanism that induces expression of the transcriptional repressor Blimp-1, which subsequently antagonizes Bcl-6 function[7]. Now Wang *et al.* and Xiao *et al.* identify previously unknown pathways that suppress T_{FH} cell differentiation[1,2]. These new reports substantially extend the knowledge of the sophisticated molecular program that regulates T_{FH} cell differentiation. Indeed, while Wang *et al.* study the involvement of the transcription factor Foxp1 in negatively regulating T_{FH} cell differentiation[1], Xiao *et al.* investigate the mechanism by which a second negative regulator, the transcription factor Foxo1, is itself inhibited by via its degradation by the E3 ubiquitin ligase Itch[2] (**Fig. 1a**).

Foxp1, which belongs to the forkhead box ('Fox') family of transcription factors, maintains the quiescence of naive T cells[8]. Foxp1-deficient $CD4^+$ or $CD8^+$ T cells in the periphery spontaneously acquire an activated phenotype, increase their proliferation and rapidly produce cytokines upon stimulation via the T cell antigen receptor (TCR). However, as this prevents investigation of the role of Foxp1 in peripheral $CD4^+$ T cell responses, Wang *et al.* use a strategy in which deletion of Foxp1 is induced by tamoxifen[1]. After mice are immunized with protein antigens, naive $CD4^+$ T cells in which Foxp1 has been deleted 'preferentially' differentiate into T_{FH} cells. The increased T_{FH} differentiation of Foxp1-deficient cells supports considerably enhanced formation of germinal centers and leads to more production of antigen-specific isotype-switched antibodies.

Notably, among the four alternative splice isoforms of Foxp1 (Foxp1A–Foxp1D), Foxp1A is constitutively expressed in $CD4^+$ T cells and Foxp1D is the only isoform induced upon stimulation with antibody to the invariant signaling protein CD3 (anti-CD3) and anti-CD28. In mice with transgenic expression specifically of Foxp1A or Foxp1D, overexpression of either isoform suppresses T_{FH} cell differentiation. Furthermore, deletion of one copy of *Foxp1* (which results in reduced expression of both Foxp1A and Foxp1D) or selective deletion of Foxp1D enhances T_{FH} cell differentiation, although it is less pronounced than that achieved by complete deletion of *Foxp1*. These observations allow Wang *et al.* to propose that Foxp1 serves as a rate-limiting 'double-check' negative regulator for T_{FH} cell differentiation: sustained Foxp1A is a homeostatic limiting factor for T_{FH} cell differentiation, and induced Foxp1D is a 'gatekeeper' for such differentiation[1].

To delineate the mechanism of Foxp1-mediated negative regulation of T_{FH} cells, Wang *et al.* compare the gene-expression profiles of wild-type and Foxp1-deficient $CD4^+$ T cells and find that IL-21 expression is increased in Foxp1-deficient $CD4^+$ T cells[1]. Foxp1-deficient $CD4^+$ T cells produce more IL-21, and chromatin immunoprecipitation reveals that Foxp1 directly binds to the *Il21* promoter region, which suggests that Foxp1 directly targets *Il21* to inhibit its expression. In addition, Foxp1-deficient $CD4^+$ T cells have higher expression of ICOS than do wild-type cells upon activation, although the expression of *Icos* mRNA is unchanged. Activation of the kinases MEK and Erk has been shown to amplify ICOS expression[4], and MEK-Erk signaling is enhanced in Foxp1-deficient T cells[8]. Therefore, Foxp1 acts to limit the expression of IL-21 (directly) and ICOS (indirectly) to suppress T_{FH} cell differentiation.

Foxp1 is not the only member of the Fox family of transcription factors that has been shown to suppress T_{FH} cell differentiation. Conditional deletion of Foxo1 in $CD4^+$ T cells leads to enhanced T_{FH} cell differentiation and spontaneous germinal center formation in mice[9,10]. Although how Foxo1 regulates T_{FH} cell differentiation remains elusive, Xiao *et al.* demonstrate that Itch targets Foxo1 for ubiquitination and degradation[2]. By downregulating this negative regulator, Itch promotes T_{FH} cell differentiation.

After infection with vaccinia virus, Itch-deficient mice have fewer T_{FH} cells, germinal centers and plasma cells and lower titers of virus-specific antibodies than do wild-type mice. The phenotype is entirely recapitulated in mice with conditional deletion of Itch in T cells, which suggests that Itch regulates the antibody response in a T cell–intrinsic manner. Itch is required in $CD4^+$ T cells for T_{FH} cell differentiation from its early stage. In further studies, Xiao *et al.* infect mice with lymphocytic choriomeningitis virus (LCMV) and track $CD4^+$ T cells expressing a TCR specific for LCMV in the infected mice; they find that T_{FH} cell differentiation is severely impaired from day 3 after infection[2]. The ICOS–Bcl-6

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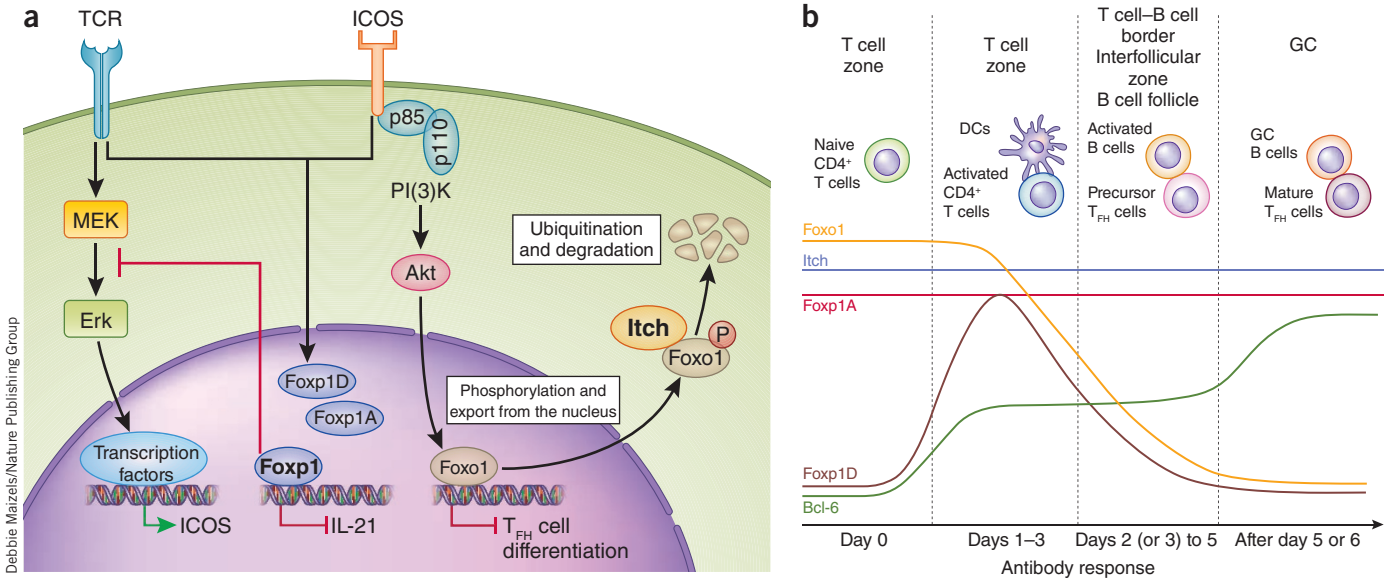

Figure 1 Dynamic regulation of T_{FH} cell differentiation by negative regulators. (**a**) Foxp1 suppresses T_{FH} cell differentiation by directly inhibiting IL-21 expression and indirectly limiting ICOS expression, presumably through the MEK-Erk pathway. Foxp1A is constitutively expressed, while Foxp1D expression is transiently induced by signaling via the TCR and costimulation. Foxo1 also suppresses T_{FH} cell differentiation by a mechanism that remains to be elucidated. ICOS signaling activates the phosphatidylinositol-3-OH kinase (PI(3)K)-Akt pathway, which induces phosphorylation (red 'P') of Foxo1 and its export from the nucleus. Itch in the cytosol then targets Foxo1 for ubiquitination and degradation and promotes T_{FH} cell differentiation. (**b**) Bcl-6 is a positive regulator that directs T_{FH} cell differentiation. Bcl-6 expression is upregulated during the priming of T cells by dendritic cells (DCs). Cognate interaction with B cells induces a second wave of Bcl-6 upregulation for the terminal differentiation of T_{FH} cells. Foxp1A and Foxp1D are negative regulators of T_{FH} cell differentiation. Foxp1A is constitutively expressed during T_{FH} cell differentiation, while Foxp1D expression is transiently induced during T cell priming. Foxo1 is a negative regulator of T_{FH} cell differentiation and its expression decreases during this differentiation. Itch targets Foxo1 for degradation after Foxo1 is exported to the cytosol and is therefore a positive regulator of T_{FH} cell differentiation. The expression of Itch persists during T_{FH} cell differentiation. GC, germinal center.

axis 'instructs' early T_{FH} differentiation[3,4,7]. ICOS upregulates Bcl-6 expression to promote T_{FH} differentiation through the phosphatidylinositol-3-OH kinase (PI(3)K)–kinase Akt pathway[4]. How activation of PI(3)K-Akt induces Bcl-6 remains unknown. Interestingly, Itch deficiency in $CD4^+$ T cells substantially reduces Bcl-6 expression without impairing ICOS expression, which suggests that Itch acts downstream of ICOS. Identifying the function of Itch might help to reveal the regulatory circuit of ICOS–PI(3)K–Akt–Bcl-6.

Itch has been shown to target the transcription factors JunB and c-Jun for degradation to inhibit differentiation into the T helper type 2 subset of helper T cells[11]. Xiao *et al.* establish that Itch targets Foxo1 for ubiquitination and degradation[2]. Coimmunoprecipitation analyses show that Itch directly interacts with Foxo1. In addition, overexpression of Itch enhances the ubiquitination of Foxo1. Notably, stimulation with anti-CD3 and anti-ICOS enhances the Foxo1-Itch interaction and subsequently induces degradation of Foxo1. The induced degradation of Foxo1 is forfeited in Itch-deficient cells. This elegant study reveals how ICOS regulates Foxo1 expression. Although Itch is constitutively expressed in the cytosol of $CD4^+$ T cells, it mediates the degradation of Foxo1 only upon signaling via the TCR and ICOS, presumably through a mechanism of nuclear-to-cytosolic translocation of Foxo1, which then 'opens Foxo1 up' to Itch-mediated degradation. Signaling via the TCR and ICOS activates Akt, which has been reported to phosphorylate Foxo1 to trigger its export from the nucleus[10]. Finally, a functional study proves that genetic ablation of Foxo1 restores the ability of Itch-deficient $CD4^+$ T cells to differentiate into T_{FH} cells and to support the formation of germinal centers.

Signaling via the TCR and ICOS is the main driving force for T_{FH} cell differentiation, but the mechanism of this action remains a puzzle[4]. With the key pieces identified by these two studies, the whole picture should be revealed soon. Coincidentally, both studies demonstrate that signaling via the TCR and costimulation induces T_{FH} cell differentiation by removing negative regulation. Naive $CD4^+$ T cells have high expression of the negative regulators Foxo1 and Foxp1A. Foxp1A is a homeostatic controller that prevents T cell activation and remains at steady levels during T_{FH} cell differentiation[1]. In contrast, Foxo1 expression is reduced soon after activation, and this is required for the first wave of T_{FH} cell differentiation[2]. Foxo1 decreases gradually throughout T_{FH} cell differentiation, and mature T_{FH} cells have a low abundance of Foxo1, an effect that now seems to occur mainly via Itch-mediated degradation. Although there is high expression of Itch even in naive T cells, Itch mediates Foxo1 degradation only upon stimulation via the TCR and ICOS. The expression of Foxp1D is low in naive T cells and is transiently induced by priming[1]. Foxp1D might work in synergy with Foxp1A to limit cell activation and ICOS expression. Intriguingly, after reaching its peak at the priming stage, Foxp1D expression decreases in a manner similar to that of Foxo1 expression. Such a reduction in Foxp1D is considered to be induced by signaling via the TCR and costimulation through cognate interactions with B cells. Removal of the negative regulator Foxp1D probably promotes the production of IL-21 and terminal differentiation of T_{FH} cells. Together these new reports represent a substantial advance in the understanding of the mechanism that regulates two waves of T_{FH} cell differentiation (**Fig. 1b**).

Several interesting questions are raised by these studies. Are these regulatory mechanisms unique to T_{FH} cells and not present in other helper T cell subsets? How does Foxo1 suppress T_{FH} cell differentiation? Why does signaling via the TCR and costimulation regulate Foxp1D expression but not Foxp1A expression? Discoveries of previously unknown regulatory circuits have highlighted negative regulation as an important checkpoint in T_{FH} cell differentiation. Targeting pathways of negative regulation, such as CTLA-4 or PD-1, has achieved great success in cancer immunotherapy. Researchers can also now start to consider how

to apply the new knowledge acquired from these papers to potentially modulate T_{FH} cell function to treat autoimmune disease, enhance vaccination and control chronic infection[7].

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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A gut reaction to IL-9

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The pathogenesis of inflammatory bowel disease is orchestrated by specific subsets of cytokine-secreting T cells. The interleukin 9–producing subset of helper T cells contributes to the pathogenesis of inflammatory bowel disease in part by disrupting intestinal barrier function and impairing tissue-repair mechanisms.

Inflammatory bowel disease (IBD), which encompasses Crohn's disease (CD) and ulcerative colitis (UC), involves sustained inflammation, mucosal barrier defects and frequent intestinal infections, probably as a result of a dysfunctional immune response. $CD4^+$ T cells promote the chronic inflammatory response and probably determine the nature of the disease. Although enhanced responses of the T_H17 subset of helper T cells and diminished responses of regulatory T cells correlate with disease in both CD and UC, there is a distinction between the inflammatory milieu of these diseases in that patients with CD have increased amounts of T helper type 1 (T_H1)-related cytokines, while patients with UC exhibit a more pronounced T helper type 2 (T_H2)-related immune response[1,2]. In a study in this issue of *Nature Immunology*, Gerlach *et al.* define the important role that anothersubset of helper T cells, interleukin 9 (IL-9)-producing T_H9 cells, serves in the pathogenesis of IBD, particularly UC, by regulating intestinal barrier integrity and immunological function[3].

Although IL-9 is a pleiotropic cytokine, serving as a growth factor for various cell types, activating mast cells and triggering mucous production by epithelial cells[4], it remains an understudied cytokine in many disease models. T_H9 cells are major producers of IL-9 and develop *in vitro* after naive T cells are activated in the presence of IL-4 and transforming growth factor-β (TGF-β)[5,6] (**Fig. 1**). T_H9 differentiation requires transcription factors that include PU.1, IRF4 and BATF[7–9]. IL-9 and T_H9 cells have been shown to provide protection against helminth infection, to mediate allergic inflammation and to promote antitumor immunity[10]. The function of T_H9 cells in autoimmune disease is less clear. The initial description of T_H9 cells noted that the transfer of T_H9 cells into lymphopenic recipient mice deficient in the RAG recombinase worsens colitis[5], although the mechanism of this exacerbation and how IL-9 might contribute to the pathogenesis of the colitis were not detailed. Gerlach *et al.* address these issues by examining the role of IL-9 in mouse models of colitis and by demonstrating that IL-9 expression is associated with the severity of human gastrointestinal disease[3].

Initially, Gerlach *et al.* examine the expression of genes encoding proinflammatory cytokines in colonic biopsies obtained from patients with active or inactive IBD[3]. *IL9* expression is significantly elevated in patients with active UC and is highest in patients with the greater disease severity. Interestingly, increased expression of *IL9* mRNA does not simply 'track with' intestinal inflammation, as patients with active CD do not exhibit higher *IL9* expression. Because IL-9 can be derived from multiple cell types, the authors demonstrate, through the use of surgical resections from patients with IBD and control patients, that IL-9-producing cells in patients with active UC are $CD4^+$. Furthermore, patients with active UC have more $CD4^+PU.1^+$ T cells in the intestine than do control patients or patients with CD. Expression of the receptor for IL-9 (IL-9R) is higher on the intestinal epithelial cells of patients with UC than on those of control patients, which suggests a role for signaling by IL-9 in IBD pathogenesis.

To address the function of IL-9 and T_H9 cells in IBD experimentally, Gerlach *et al.* study mouse models of acute and chronic colitis[3]. Similar to the observations obtained with human patients with active UC, IL-9-producing cells and IL-9R expression on intestinal epithelial cells are increased during colitis. Gerlach *et al.* use a novel IL-9 reporter mouse to identify $CD4^+$ cells, rather than alternative cell types such as innate lymphoid cells, as the principal source of this cytokine *in vivo*[3]. To address the function of IL-9, they induce colitis in mice given a neutralizing antibody to IL-9 or in mice with genetic deficiency in IL-9. In both models, colitis is reduced considerably in the absence of IL-9, as measured by weight loss, the generation of reactive oxygen species and clinical scores; this suggests that IL-9 is a pivotal cytokine in the promotion of colitis disease. Furthermore, PU.1 deficiency in $CD4^+$ T cells, which blocks the differentiation of T_H9 cells[7], protects mice from colitis, which indicates T_H9 cells are also vital to the pathogenesis of colitis.

Gerlach *et al.* proceed to explore potential mechanisms by which IL-9 might promote colonic disease[3]. The authors note alterations in tight junctions in the intestinal wall in both patients with active UC and colitis models. In oxazolone-induced colitis (a form of colitis with T_H2 characteristics), expression of the pore-forming factors claudin-1 and claudin-2 is increased, in contrast to the diminished expression of claudin-3 and occludin, which are known to increase barrier function. Because IL-9R expression is enhanced on intestinal epithelial cells, the authors hypothesize that stimulation of the intestinal epithelial cells with IL-9 might impair intestinal barrier function. Indeed, in the colitis models, intestinal membrane permeability (as measured by the translocation of bacteria or with the permeability tracer fluorescein isothiocyanate–dextran) is markedly diminished in the absence of IL-9. The authors then investigate whether IL-9 directly affects barrier function

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