Tfh Cell Differentiation: Missing Stat3 Uncovers Interferons' Interference

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Viral infection induces a number of cytokines that shape T cell responses. In this issue of *Immunity*, Ray et al. (2014) describe how CD4$^+$ T cells decide on T follicular helper (Tfh) or T helper 1 (Th1) cell skewed gene expression during acute viral infection.

Follicular helper T (Tfh) cells are uniquely specialized to provide B cell help. Understanding their differentiation program will be the key for future vaccination strategies and new therapeutic options in autoimmune diseases like systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). However, we might not be able to make use of or manipulate the underlying program without studying how Tfh cell differentiation and plasticity connect to alternative programs and how genetic and environmental impacts select between these differentiation programs in specific immunological contexts.

CD4$^+$ T cell differentiation starts with antigen recognition by a naive T cell that causes dramatic cellular changes in the activation status, metabolism, and cell cycle. CD4$^+$ T cells can differentiate into the T helper 1 (Th1), Th2, Th17, regulatory T (Treg) or Tfh cell subsets characterized by distinct gene-expression profiles. These relate to the expression of a subset-specifying transcription factor and to specific effector functions due to the ability to express hallmark cytokines. The activity of the signaling molecules within the differentiating T cell are placed in a complex regulatory network in order to create a differential gene-expression profile that enables appropriate effector T cell phenotypes. It integrates diverse signals from the T cell receptor (TCR) and costimulatory receptors as well as the humoral context. Not surprisingly, the strength of TCR stimulation and the input from specific cytokines have been determined as major factors. Consistently, Tfh differentiation depends on increased TCR signal strength and is stimulated by interleukin-6 (IL-6) and IL-21 cytokines (Yamane and Paul, 2012). The Tfh cell phenotype is characterized by expression of the subset-determining transcription factor Bcl6 and is associated with downregulated expression of its antagonistic transcription factor Blimp-1. These cells have the ability to produce IL-21 and are localized in the B cell follicle as a result of expression of the chemokine receptor CXCR5. In addition, they are characterized by high expression of the costimulatory molecules PD-1, BTLA, and ICOS. Signal transduction by IL-21 and IL-6 converge on the activation of STAT3 and STAT1 (Yamane and Paul, 2012). However, the relative roles of STAT1 and STAT3 in Tfh cell induction during acute viral infection have not been fully understood.

Ray et al. now shed light on this issue by analyzing the immune response against lymphocytic choriomeningitis virus (LCMV) in mice with STAT3-deficient T cells. Compared to wild-type (WT) mice, they find that Tfh cell differentiation was impaired 8 days after infection and that both polyclonal and GP_{66}-specific Tfh cell numbers were decreased. This reduction in Tfh cells led to defective germinal center generation accompanied by reduced production of LCMV-specific antibodies. These findings greatly expand the importance of STAT3 compared to earlier results by Choi et al. (2013), who suggested that STAT1 was required, whereas STAT3 function only contributed to Tfh cell differentiation in the early phase of LCMV infection. However, those results were obtained only with TCR-transgenic T cells and did not include analyses of germinal center B cell differentiation and antibody formation. Performing a comprehensive gene-expression analysis, Ray et al. demonstrated that STAT3-deficient Tfh cells expressed less of the subset-specifying transcription factor Bcl6, as well as hallmark cytokine IL-21, and instead upregulated markers of Th1 cell differentiation including CD25 and T-bet. The detailed comparison of gene expression in Tfh cells from WT or STAT3-deficient mice also revealed that *Cxcr5*, *Icos* and *IL6st* were downregulated, whereas *Prdm1*, the gene that encodes Blimp-1, the antagonist of Bcl6, was highly increased. Most strikingly, cluster analysis of different mRNA samples demonstrated that STAT3-deficient Tfh cells appeared more closely related to Th1 cells located in the T cell zone (Ly6C^{lo}, PSGL-1^{hi}) than to Tfh cells from WT mice. The lack of STAT3 was at the same time associated with a higher expression of interferon (IFN)-inducible genes.

An earlier publication has generated strong evidence for a connection of IFN-γ with the Tfh cell subset by investigating the *sanroque* mice, which express the posttranscriptional regulator Roquin-1 only in its point-mutated (M199R) form. *Sanroque* mice develop lupus-like disease due to accumulation of Tfh cells. In fact, experimentally introduced deficiency for the IFN-γ receptor rescues Tfh cell accumulation and Tfh cell-mediated pathology in the *sanroque* mouse strain (Lee et al., 2012). However, in the present study by Ray et al. there was no significant effect of IFN-γ blockade on Tfh cell differentiation in response to acute viral infection. Together, these findings rather suggest a role for IFN-γ in Tfh

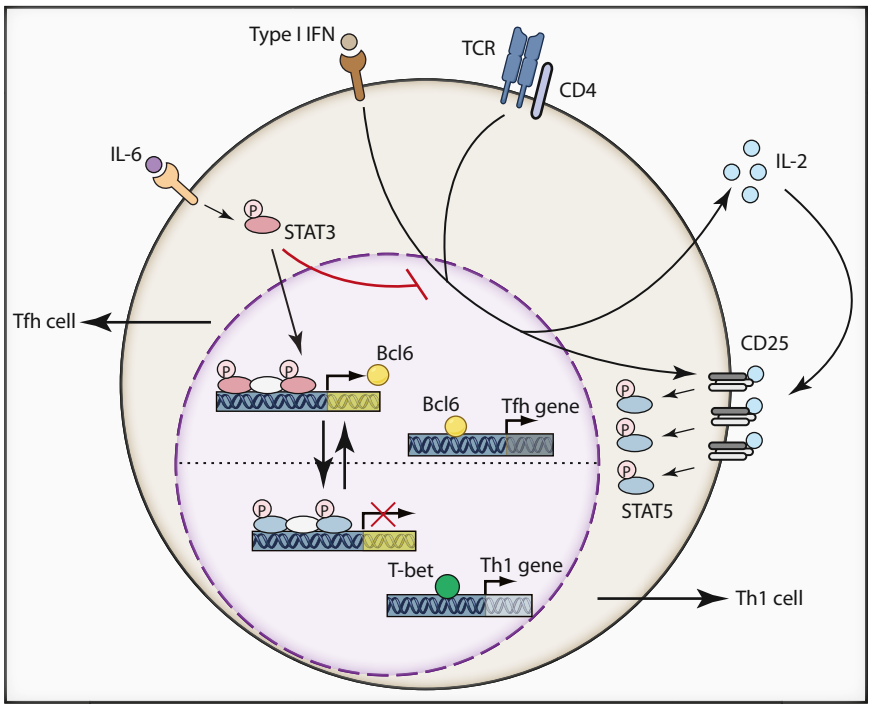

Figure 1. Naive CD4$^+$ T Cells Integrate TCR, Type I Interferon, IL-2, and IL-6 Signaling to Decide on Tfh or Th1 Cell Differentiation

IL-6 signaling activates STAT3, which then binds and activates the Bcl6 promoter. Type I interferon signaling induces IL-2 and CD25 expression, which in turn activate STAT5. The scheme also depicts the implicated competitive replacement of STAT3 by STAT5 on the Bcl6 locus. In addition, STAT3 inhibits the type I IFN–IL-2–CD25–STAT5 signaling pathway by a not-yet-defined molecular mechanism. Please note that for simplicity reasons, this representation omits previously demonstrated regulatory mechanisms, including the upregulation of Blimp1 by STAT5, the antagonism of Blimp1 and Bcl6, the induction of T-bet by STAT1, and the regulation of Bcl6 binding to DNA by T-bet, which might act in concert.

cell differentiation or homeostasis in the specific context of the *sanroque* mutation.

In the next step, Ray et al. tested whether type I IFN would have an impact on Tfh differentiation during viral infection. Indeed, blocking antibodies against IFN-αβR brought back the frequencies of STAT3-deficient Tfh cells to WT and could also rescue the germinal center B cell and antibody responses. At the same time, the lack of IFN-αβ signaling led to a strong reduction of Th1 cell differentiation in WT, as well as in STAT3-deficient T cells. Thus, one role of STAT3 is to counteract the inhibitory effects of type I IFN signaling during Tfh cell differentiation in vivo. These findings are in contrast to very recently published data showing that addition of type I interferon in cell culture experiments was able to induce a Tfh-like phenotype. However, these in vitro differentiation conditions only induced an incomplete phenotype, because they did not bring about IL-21 production and so far lack in vivo confirmation (Nakayamada et al., 2014).

To describe the mechanism, Ray and colleagues analyzed IFN-αβR-deficient T cells that are virus-specific due to expression of a TCR transgene. After adoptive transfer into WT mice, LCMV infection yielded a higher percentage of Tfh cells for IFN-αβR-deficient compared to WT TCR transgenic T cells. In vitro studies with peptide stimulation of these TCR-transgenic T cells provided additional important mechanistic insight. In fact, the presence of IFN-β together with IL-2 induced the expression of CD25 and led to a drastic activation of STAT5 (Figure 1). Importantly, chromatin immunoprecipitation experiments showed that in this way STAT3, which was bound to the *Bcl6* locus after IL-6 stimulation, was replaced by STAT5 upon IFN-β stimulation. These findings nicely connect to the observation that deficiency of STAT3 correlated with decreased protein amounts of Bcl6 and increased protein amounts of CD25 and T-bet protein in Tfh cells. Therefore, type I interferon signaling via the IL-2–CD25–STAT5 axis inhibits Tfh cell differentiation and modulates the immune response toward a Th1 cell phenotype. STAT3 is counteracting this pathway upstream by inhibiting type I IFN-induced CD25 expression, as well as downstream by competing with STAT5 for binding to the Bcl6 locus (Figure 1). One key question for future studies will be to find out how the competitive binding to the same *cis*-regulatory elements by STAT5 or STAT3 actually translate into Bcl6 gene repression or activation, respectively. Another issue for future consideration is which molecular targets of STAT3 can explain its inhibitory effect on IL-2–CD25 signaling.

Type I interferon signaling has also been involved in the reprogramming of established Th2 cells to generate a stable subset of "Th2+1" cells that mount protective responses against viral infection. This has been demonstrated in experiments involving adoptive transfer of in vitro differentiated virus-specific Th2 cells into mice that are subsequently infected with LCMV (Hegazy et al., 2010). In this model, type I and type II interferons are proposed to act as "door openers" to enable STAT1–STAT4-mediated induction of T-bet in addition to a maintained GATA3 expression. Interestingly, the infection of mice with influenza virus reveals similar plasticity from Tfh to Th1 cells, because Tfh cells established during a primary infection lose Tfh cell marker and effector molecule expression and are converted toward a Th1 cell phenotype following adoptive transfer and secondary influenza infection of the host mice (Lüthje et al., 2012). However, the type I IFN-mediated skewing toward the Th1 cell subset is likely to impact early in the differentiation of CD4$^+$ T cells. This type of negative regulation by IL-2 and STAT5 signaling has been well established in Tfh cell differentiation (Johnston et al., 2012; Nurieva et al., 2012; Oestreich et al., 2012). Moreover, the CD25–STAT5 signaling is important in Th1 and Th2 differentiation and favors Th1 over Th17 cell differentiation (Yamane and Paul, 2012). Yet, the study by Ray et al. connects the molecular aspects by showing that not only increased stimulation by IL-2, but also IFN-β signaling, indirectly

impinge on the *Bcl6* locus where STAT5 can outcompete STAT3 on shared binding sites to repress *Bcl6* transcription (Figure 1). Rather than opening closed doors in $CD4^+$ T cell differentiation, type I interferons' interference in Tfh deviates differentiation toward Th1 cells, if STAT3-activating signals are missing. Besides the known adjuvant effect of type I interferon on non-T cells, the present study demonstrates a crucial importance for balanced cytokine signals to enable efficient Tfh cell differentiation in vaccination strategies. At the same time, it highlights the potential of STAT3 as a target to treat autoimmune diseases that involve Tfh cells.

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Clec12a: Quieting the Dead

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Immune activation as a result of the recognition of damage-associated molecular patterns needs to be controlled. In this issue of *Immunity*, Neumann et al. (2014) demonstrates that Clec12a is a receptor for dead cells through the recognition of uric acid crystals and contributes to the dampening of the responses.

C-type lectin receptors (CLRs) are pattern-recognition receptors (PRRs) that recognize microbial pathogen-associated molecular patterns (PAMPs), which leads to the induction of host immune responses against many pathogens (Robinson et al., 2006). In addition to acting as PRRs for PAMPs, some CLRs also function as receptors for damage-associated molecular patterns (DAMPs), which are exposed or released upon cell death by noninfectious insults such as tissue injury, ischemia, and infarction. For example, Lox-1 and MGL-1 are CLRs known to recognize dead cells and are likely to act as phagocytic receptors for dead cells (Robinson et al., 2006). Some CLRs coupled with immunoreceptor tyrosine-based activation motif (ITAM) or hemITAM (hemi-immunoreceptor tyrosine-based activation motif), such as Mincle (Clec4e) and DNGR-1 (Clec9a), have also been shown to recognize dead cells (Sancho et al., 2009; Yamasaki et al., 2008). These CLRs sense nonhomeostatic cell death and thereby induce inflammation or promotion of antigen presentation. These immune responses against "damaged self" are thought to be beneficial to maintain homeostasis of the organisms. In contrast, the "anti-self" responses should be immediately terminated to prevent tissue damage or autoimmunity caused by prolonged halmful immune reaction against self. To date, however, negative regulatory CLRs for dead cells have never been identified. Several CLRs possesses immunoreceptor tyrosine-based inhibitory motif (ITIM) within their own cytoplasmic tails. Upon receptor engagement, tyrosine residues within ITIM are phosphorylated and thus provide docking sites for cytosolic negative regulatory proteins such as SHP-1, SHP-2, or SHIP. In T cells, ITIM-containing costimulatory inhibitory receptors CTLA-4 and PD-1 play critical roles in terminating activatory signals delivered through ITAM-containing T cell receptor (TCR) complexes in order to prevent autoimmunity. Likewise, it is possible that unknown inhibitory CLR(s) contribute to the negative regulation of immune responses against damaged self.

Clec12a (also called myeloid inhibitory C-type lectin-like receptor, MICL) was originally described as an ITIM-containing inhibitory CLR expressed by human granulocytes and monocytes (Marshall et al., 2006). It was suggested that Clec12a recognizes some endogenous ligands as soluble Clec12a could bind to single-cell suspensions isolated from various murine tissues (Pyz et al., 2008). In this issue of *Immunity*, Neumann et al. (2014) identified Clec12a as an inhibitory CLR for dead