Cutting Edge: STAT1 Is Required for IL-6–Mediated Bcl6 Induction for Early Follicular Helper Cell Differentiation

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Cutting Edge: STAT1 Is Required for IL-6–Mediated Bcl6 Induction for Early Follicular Helper Cell Differentiation

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Bcl6 is required for CD4 T cell differentiation into T follicular helper cells (Tfh). In this study, we examined the role of IL-6 in early processes of in vivo Tfh differentiation, because the timing and mechanism of action of IL-6 in Tfh differentiation have been controversial in vivo. We found that early Bcl6 CXCR5 Tfh differentiation was severely impaired in the absence of IL-6; however, STAT3 deficiency failed to recapitulate that defect. IL-6R signaling activates the transcription factor STAT1 specifically in CD4 T cells. Strikingly, we found that STAT1 activity was required for Bcl6 induction and early Tfh differentiation in vivo. IL-6 mediated STAT3 activation is important for downregulation of IL-2Rα to limit Th1 cell differentiation in an acute viral infection. Thus, IL-6 signaling is a major early inducer of the Tfh differentiation program unexpectedly mediated by both STAT3 and STAT1 transcription factors. The Journal of Immunology, 2013, 190: 000–000.

Follicular helper (Tfh) CD4 T cells are a subset of differentiated CD4 T cells with specialized B cell help functions (1). Tfh differentiation of murine and human CD4 T cells is programmed by the transcription factor Bcl6 (1, 2). Effector CD4 T cell differentiation is controlled by induction of key transcription factors by AgR signals, costimulatory receptors, and cytokine receptor–mediated activation signals (3). IL-6 was proposed to induce Tfh differentiation. CXCR5 CD4 T cell frequencies were decreased in vivo in the absence of IL-6 in one study (4). Perplexingly, following protein immunization and acute viral infections, Tfh differentiation was found to be near normal in the absence of IL-6 at the peak of the CD4 T cell response (5, 6). CD4 T cells were shown to acquire the expression of some Tfh genes in vitro in the presence of IL-6 (7–9). However, other studies found that Tfh differentiation is not readily recapitulated with in vitro culture of pure CD4 T cells plus IL-6 (10). STAT3 is the best-recognized transcription factor downstream of IL-6R (11). Similar to IL-6, there have been variable outcomes regarding whether STAT3 is required for Tfh differentiation in vivo (4, 12). Tfh differentiation is certainly a multifactorial process (10); nevertheless, recent studies highlighted that IL-6 signaling is important to Tfh cell biology, because there is a dramatic IL-6 requirement for sustaining Tfh cells in a chronic viral infection of mice (13), and IL-6 strongly correlated with Tfh cell frequency and function in SIV-infected macaques (14), the best available animal model of HIV infection.

Recently, we showed that early signaling events are sufficient for fate-committed differentiation of CD4 T cells into Tfh versus Th1 cells during the primary immune response to an acute viral infection (15). In this study, we examined the roles of IL-6 in this process. Through cell transfers, as well as genetic and molecular approaches, we demonstrate that IL-6 provides critical signals for CD4 T cells to induce Bcl6 and CXCR5 in vivo. We identify that both STAT1 and STAT3 activation is required for IL-6–mediated Bcl6 induction and early Tfh differentiation.

Materials and Methods
Mice and viral infections
C57BL/6j (B6) and IL-6–deficient mice (The Jackson Laboratory) and CD4-Cre mice (Taconic Farms) were purchased. CD45.1 SMARTA (SM; lymphocytic choriomeningitis virus [LCMV] gp66-77-IAα specific) (16) and STAT3high SM (17) mice were obtained from in-house breeders at La Jolla Institute for Allergy and Immunology, IL.21−/− mice were obtained from the Zajac laboratory (18). LCMV Armstrong strain and recombinant acute vaccinia virus (VACV) that expresses LCMV gp protein (VACV-gpc) (19) were used. A total of 1 or 0.5 × 106 PFU LCMV Armstrong strain or 50 or 20 × 106 PFU of VACV-gpc was injected i.p. for analysis at day 2 or 3 postinfection, respectively. All animal experiments were performed in compliance with approved animal protocols at La Jolla Institute for Allergy and Immunology. Naïve or RV+ SM cells were transferred into recipient mice via the retro-orbital sinus. For the analysis of SM cells after LCMV infection, 1 × 105 and 4–5 × 105 SM CD4+ T cells were used for day 2 and 3 experiments; in VACV-gpc infection system, 3 × 105 and 1.5 × 105 SM cells were used for day 2 and 3 experiments. Statistical analyses for all experiments were conducted using Prism 5.0 (GraphPad) software, and p values were obtained using two-tailed unpaired t tests with a 95% confidence interval. Data are depicted as mean ± SEM.

Retroviral vector production and CD4 T cell transduction
STAT1 (antisense sequence: 5′-TGACAAAGCTCATCCTTTGTT-3′; Thermo Scientific) or a nonfunctional microRNA-adopted short hairpin RNA expressing

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Abbreviations used in this article: B6, C57BL/6j; LCMV, lymphocytic choriomeningitis virus; SM, SMARTA; Tfh, T follicular helper cell; VACV, vaccinia virus; VACV-gpc, vaccinia virus that expresses lymphocytic choriomeningitis virus gp protein; WT, wild type.

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retroviral vectors (pLMA-mAmetrine) (20) was used to produce virions from the Plat-E cell line. SM cells were stimulated in vitro with 8 μg/ml anti-CD3 and anti-CD28 (Bio X Cell) and then transduced with retroviral virions 24 and 36 h poststimulation.

**Flow cytometry**

Splenocytes were prepared for staining as previously described (20). Naive or R7 SM cells were cultured in a 96-well plate with IL-6 or IL-12 (20 ng/ml) at 37°C for 15–30 min for staining with anti–p-STAT1 (14/P-STAT1), anti–p-STAT4 (38/P-STAT4; all from BD Biosciences) or RV+ SM cells were cultured in a 96-well plate with IL-6 or IL-12 (20 ng/ml) at 37°C for 15–30 min for staining with anti–p-STAT1 (14/P-STAT1), anti–p-STAT4 Abs (38/P-STAT4; all from BD Biosciences).

**Results**

**IL-6 is required for early Tfh differentiation**

Bcl6⁺CXCR5⁺ Tfh cells are present at day 3 after LCMV infection (20, 21) and are fate committed (15). We carefully examined the role of IL-6 during early Tfh differentiation by analyzing SM cells in B6 or IL-6–deficient recipient mice at early time points after LCMV infection. At day 2 after LCMV infection, Tfh cells could be phenotypically identified as IL-2Rα⁺CXCR5⁺Bcl6⁺ (Supplemental Fig. 1A, 1B). Bcl6 induction was severely impaired in IL-6⁻/⁻ mice (Fig. 1A). IL-2Rα⁺CXCR5⁺Bcl6⁺ Tfh cells were very rare at 48 h after LCMV infection in the absence of IL-6 (Fig. 1B, p = 0.0001). Bcl6⁺CXCR5⁺ Tfh cells were not observed in IL-6⁻/⁻ mice at day 3 after LCMV infection (Fig. 1C, p = 5.2 × 10⁻⁴).

Defective early Tfh differentiation was not due to a CD4 T cell–activation defect (Supplemental Fig. 1C).

IL-2 signaling inhibits Tfh differentiation (21, 22). Bcl6⁺ CXCR5⁺ Tfh cells strongly downregulate IL-2Rα, the high-affinity subunit of IL-2R, by day 3 after LCMV infection (20, 21). Interestingly, IL-6 was required for downregulation of IL-2Rα on CD4 T cells. At days 2 and 3 after LCMV infection, SM cells retained a much higher level of IL-2Rα in IL-6⁻/⁻ mice (Fig. 1D). Excessive IL-2Rα expression was also observed on CXCR5⁻ Tfh SM cells in IL-6⁻/⁻ mice (Fig. 1E). These findings suggest a direct antagonism between the IL-6– and IL-2–signaling pathways in differentiating CD4 T cells during an antiviral immune response. This is consistent with binding competition between STAT3 and STAT5 to the IL-2 signaling inhibitory site of STAT5 to the IL-2 signaling inhibitory site of STAT5 (23). Bcl6 through the STAT3 gene of CD4 T cells during Tfh cell differentiation (23). IL-21, in addition to IL-6, is associated with Tfh differentiation (4, 24) and can signal via partially overlapping pathways. However, IL-21 is not required for development of Tfh cells at the peak of the CD4 T cell response in multiple acute viral infections and protein immunizations (5, 6, 24), nor is it required for Tfh cell priming (Supplemental Fig. 1D, 1E). IL-21 plays an important role in the absence of IL-6 later in acute viral infections and protein immunizations (5, 24). Collectively, our data show that IL-6, but not IL-21, is required for early Tfh differentiation in vivo.

**FIGURE 1.** IL-6 signals are required for development of early Bcl6⁺CXCR5⁺ Tfh cells. Naive SM (CD45.1⁺) CD4 T cells were transferred into B6 and IL-6⁻/⁻ mice and analyzed at days 2 and 3 after LCMV infection. (A) Bcl6 graphs of SM cells in infected mice (colors). Naive CD4 T cells of uninfected mice (gray). (B) IL-2Rα⁺CXCR5⁺Bcl6⁺ cells were gated and calculated as the percentage of total SM cells. (C) Bcl6⁺CXCR5⁺ Tfh cells at day 3 postinfection (left panel). The percentage of Tfh cells was calculated (right panel). (D) IL-2Rα graphs of SM cells in infected mice (colors). Naive CD4 T cells of an uninfected mouse (shaded graph) (left panels). IL-2Rα geometric MFIs were calculated (right panels). (E) IL-2Rα geometric MFIs of day-3 CXCR5⁻ SM cells. Data are representative of three independent experiments (n = 4–5 mice/group). **p < 0.01, ***p < 0.001.

**FIGURE 2.** STAT3 contributes to early Bcl6 induction and Tfh differentiation. Naive WT or STAT3⁻/⁻ SM (CD45.1⁺) cells were transferred into B6 mice that were infected with LCMV. SM cells were analyzed for Tfh differentiation at day 2 (A, B) and day 3 (C, D) postinfection. (A) Bcl6 graphs of WT (red) and STAT3⁻/⁻ (blue) SM cells. Naive CD4 T cells of an uninfected mouse (shaded graph). (B) Bcl6⁺CXCR5⁺ SM cells were gated and calculated as the percentage of total SM cells. (C) Gates indicate Bcl6⁺CXCR5⁺ SM cells at day 3 after LCMV infection (left panel). Percentage of Tfh cells among total SM cells (right panel). (D) IL-2Rα graph of WT (red) and STAT3⁻/⁻ (blue) SM Tfh cells, gated in (C) (left panel). IL-2Rα geometric MFIs (right panel). Data are representative of three independent experiments (n = 4–5 mice/group). ***p < 0.001.
STAT3 contributes to IL-6-mediated Bcl6 induction and Tfh differentiation

STAT3 binds to IL-6R and is activated by JAK-mediated phosphorylation (p-STAT3) upon IL-6 stimulation (17). Therefore, we investigated whether a IL-6R-mediated Tfh differentiation signal is delivered by STAT3. Wild type (WT) and STAT3<sup>−/−</sup> CD4<sup>+</sup>Cxcr5<sup>−/−</sup> (referred to hereafter as STAT3<sup>−/−</sup>) SM cells were transferred into B6 mice and analyzed for Tfh differentiation at days 2 and 3 after LCMV infection. STAT3 deficiency resulted in severely impaired Tfh differentiation during the first 48 h postinfection. Bcl6 induction was defective (Fig. 2A), and Bcl6<sup>+</sup>Cxcr5<sup>+</sup> CD4<sup>+</sup> T cells were severely reduced in STAT3<sup>−/−</sup> SM cells (Fig. 2B, p = 0.0007). Quite surprisingly, however, STAT3<sup>−/−</sup> SM cells differentiated into Bcl6<sup>+</sup>Cxcr5<sup>+</sup> Tfh cells in a comparable fashion as did WT SM cells within an additional 24 h (Fig. 2C). Interestingly, STAT3 activation controlled IL-2R<sup>α</sup> downregulation downstream of IL-6R. In comparison with WT SM cells, STAT3<sup>−/−</sup> SM cells were severely impaired in downregulation of IL-2R<sup>α</sup> at both day 2 (Supplemental Fig. 2A, p = 0.01) and day 3 (Fig. 2D, p = 2.6 × 10<sup>−5</sup>) after LCMV infection. Nonetheless, the relatively normal Tfh differentiation of STAT3<sup>−/−</sup> SM cells at day 3 postinfection was a sharp contrast to the severely defective Tfh differentiation in IL-6R<sup>−/−</sup> mice at the same time point (Fig. 1C), strongly implicating the presence of a second pathway downstream of IL-6R signaling for Bcl6 induction. Collectively, our data show that STAT3 participates in Bcl6 induction and IL-2R<sup>α</sup> downregulation downstream of IL-6R signaling.

STAT1 is required for early Tfh differentiation

The disparate requirements for IL-6 and STAT3 led us to investigate whether another transcription factor is required for IL-6–mediated early Tfh differentiation. Interestingly, although IL-6 stimulation of IL-6R activates STAT3 in multiple hematopoietic cell types, STAT1 is strongly activated by IL-6 selectively in CD4<sup>+</sup> T cells (Fig. 3A) (25). Therefore, we investigated the role of the transcription factor STAT1 in early Tfh differentiation. STAT1 expression was inhibited by a STAT1-specific microRNA-adapted short hairpin RNA (STAT1<sup>KD</sup> hereafter) (Fig. 3A); p-STAT1 MFI was reduced by ~80%. STAT1<sup>KD</sup> activity was specific for STAT1, because activation of both STAT3 (Fig. 3A) and STAT4 (Supplemental Fig. 2B) in vitro was normal upon IL-6 and IL-12 stimulation, respectively. We then examined the role of STAT1 in Tfh differentiation in vivo. Although IL-6 stimulation led to p-STAT1 in day-3 WT SM cells, STAT1 activity was further elevated in STAT3<sup>−/−</sup> SM cells (Supplemental Fig. 2C). Forty-eight hours after LCMV infection, STAT1<sup>KD</sup> SM cells failed to differentiate into Bcl6<sup>+</sup>Cxcr5<sup>+</sup> and IL-2R<sup>α</sup><sup>+</sup>Cxcr5<sup>+</sup> cells (Fig. 3B, p = 0.0004; Supplemental Fig. 2D, p = 0.001).

Although a major defect in Bcl6 and Cxcr5 expression was seen in the absence of STAT1 expression at day 2 after LCMV infection, like STAT3 the STAT1 defect could be compensated for; STAT1<sup>KD</sup> SM cells regained Bcl6 and Cxcr5 expression that was comparable to control SM cells by day 3 in vivo (Supplemental Fig. 2E). This again was in contrast to the requirement for IL-6 signaling through day 3 of CD4 T cell priming in vivo (Fig. 1C). Therefore, we investigated whether STAT1 and STAT3 cooperate to induce Tfh differentiation. To test this, STAT1 activity was re-

FIGURE 3. Both STAT1 and STAT3 are required for early Tfh differentiation of CD4 T cells. (A) Ctrl SM or STAT1<sup>KD</sup> SM (CD45.1<sup>+</sup>) cells were stimulated with IL-6 for p-STAT stainings. Graphs of p-STAT1 and p-STAT3. (B) Ctrl SM or STAT1<sup>KD</sup> SM cells were transferred into B6 mice that were infected with LCMV. Gates indicate Bcl6<sup>+</sup>Cxcr5<sup>+</sup> at day 2 postinfection (left panel). Percentage of Bcl6<sup>+</sup>Cxcr5<sup>+</sup> Tfh cells among total SM cells (right panel). (C) Ctrl SM, STAT1<sup>KD</sup> SM, STAT3<sup>−/−</sup> SM, or STAT3<sup>−/−</sup>STAT1<sup>KD</sup> SM cells were transferred into B6 mice. SM cells were analyzed at day 3 postinfection. (C) Gates indicate Bcl6<sup>+</sup>Cxcr5<sup>+</sup> Tfh cells. (D) The percentage of Tfh cells was calculated. (E) Cxcr5 mean fluorescent intensities (MFIs) were normalized to cell size of donor cells (Cxcr5<sub>WT</sub>SM/FSCa<sub>WT</sub>SM). Data are representative of two (C–E) or three (A, B) independent experiments (n = 4–5 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 4. STAT1 is necessary for early Tfh differentiation after VACV infection. Ctrl SM or STAT1<sup>KD</sup> SM (CD45.1<sup>+</sup>) cells were transferred into B6 mice that were infected with VACV-gpc. Bcl6<sup>+</sup>Cxcr5<sup>+</sup> Tfh cells were gated (left panel) and calculated as the percentage of total SM cells (right panel) at day 2 (A) and day 3 (B) postinfection. Data are representative of two independent experiments (n = 4–5 mice/group). *p < 0.05, ***p < 0.001.
pressed in STAT3−/− SM cells (STAT1KDSTAT3−/−). In vitro stimulation with IL-6 of STAT3−/−, STAT1KD, or STAT1KDSTAT3−/− SM cells confirmed the expected STAT1 and STAT3 phosphorylation defect(s) (data not shown). Quite strikingly, we found that STAT1KDSTAT3−/− SM cells failed to develop into Bcl6+CXCR5+ Tfh cells at 72 h post-LCMV infection (Fig. 3C–E, *p = 0.005*). T-bet was upregulated by CXCR5+ Th1 STAT1KDSTAT3−/− SM cells (Supplemental Fig. 2F). These findings indicate strong cooperation between STAT1 and STAT3 downstream of IL-6 in Tfh cell priming.

Type I IFN signal is critical for CD4 T cell survival in LCMV-infected mice (26), and STAT1 activation is critical for type I IFN signaling. As a consequence, SM cells were 5–10-fold less abundant when STAT1 activity was curtailed in STAT1KD cells (data not shown). However, CD4 T cell type I IFN signaling is not required in other infections (26). Therefore, VACV was used as an independent model (Supplemental Fig. 2G, 2H). STAT1KD SM cells failed to differentiate into Tfh cells 2 d after VACV-gpc infection (Fig. 4A, *p = 0.0001*). Notably, STAT1KD CD4 T cells had continued Tfh differentiation defects at day 3 after VACV-gpc infection, even in the presence of STAT3 (Fig. 4B, *p = 0.047*). Residual STAT1 in STAT1KD cells may have low-level activity. Taken together, our data demonstrate that STAT1 is an important transcription factor directing IL-6–dependent Bcl6 induction and Tfh differentiation during the dendritic cell–priming stage of acute viral infections.

**Discussion**

Cytokine-dependent STAT activation has roles in many T cell–differentiation and –survival processes (3). Defective IL-6 signaling resulted in a significant loss of Tfh cell maintenance during a chronic LCMV clone 13 infection (13). The ability of SIV-infected macaques to make germinal centers and high-affinity anti-Env IgG responses correlated with Tfh cell abundance, which correlated with IL-6 availability (14). Those observations highlighted the likely importance of Tfh cells in HIV infection and other viral infections that are major public health burdens (27). In this study, we examined the roles of IL-6 during the early stage of in vivo Tfh differentiation. Our study demonstrates that Bcl6 induction and CXCR5 expression are specifically impaired in the absence of IL-6 during the dendritic cell–priming phase of the CD4 T cell response; surprisingly, this depends on both STAT1 and STAT3. Through STAT3 activation, IL-6 also can compete with IL-2 signaling by restricting surface expression of IL-2Rx. Hence, these findings may explain mechanisms by which IL-6 affects Tfh cells in numerous contexts. The early Tfh-differentiation defect is not permanent in the response to an acute LCMV infection in IL-6−/− mice (5, 6, 13). We infer that compensatory signals become available at later time points of LCMV infection, which can largely compensate for the lack of IL-6. Having multiple redundant pathways to signal Tfh differentiation is almost certainly a highly evolutionarily evolved system to prevent pathogen evasion, because Ab responses are valuable for control and clearance of most pathogens, whether viral, bacterial, fungal, or parasitic (10). Acute LCMV infection is a very robust immunogen, and it is likely that some other immunogens do not engage compensatory pathways effectively. Recent studies indicate that there are nonredundant roles for IL-6 in Tfh cell biology in important physiological conditions (13, 14). In summary, the data presented in this article show quite strikingly that IL-6 is a dominant factor during the Tfh-priming stage.

The findings reported in this article have intriguing implications for understanding human Tfh cells and multiple human genetic diseases. Humans with Job’s syndrome have heterozygous dominant-negative STAT3 gene mutations (28). In those patients, Th17 cell differentiation is completely lost, demonstrating the central importance of STAT3 for Th17 cells (28). In contrast, in those STAT3mut patients Tfh cell frequencies are only moderately reduced (CD45RA−CXCR5+) (29). Our data confirm that STAT3 contributes to IL-6–dependent Bcl6 induction, but that contribution overlaps with critical roles of STAT1 delivering IL-6 signals to instruct Tfh differentiation in vivo. Notably, a trend of reduced Tfh frequencies was observed in patients with dominant-negative STAT1 mutations (29). Interestingly, three patients with gain-of-function mutations in STAT1 exhibited unexpected increases in blood Tfh cell frequencies (29). We infer that excessive activation of STAT1 downstream of IL-6R may explain the phenotype of increased Tfh frequencies in those patients. Hence, phenotypes of both STAT1mut and STAT3mut patients may be explained by the demonstration in this study that both STAT1 and STAT3 contribute to IL-6–mediated Bcl6 induction and Tfh differentiation. Further understanding of the sources of IL-6 production and the pathways downstream of the STAT1 and STAT3 transcription factors are likely to provide mechanistic insights that can contribute to the design of more efficacious vaccines against infectious diseases.

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**Disclosures**

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**References**


