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IL-27 Induces Th17 Differentiation in the Absence of STAT1 Signaling

Anneli Peters,*†‡, Kevin D. Fowler,*†‡, Fanny Chalmin,‡ Doron Merkler,‡§, Vijay K. Kuchroo,*‡ and Caroline Pot‡,¶

It is known that differentiation of Th17 cells is promoted by activation of STAT3 and inhibited by activation of STAT1. Although both transcription factors are activated by several cytokines, including IL-6, IL-21, and IL-27, each of these cytokines has a very different effect on Th17 differentiation, ranging from strong induction (IL-6) to strong inhibition (IL-27). To determine the molecular basis for these differences, we measured STAT3 and STAT1 activation profiles for IL-6, IL-21, and IL-27, as well as for cytokine pairs over time. We found that the ratio of activated STAT3/activated STAT1 is crucial in determining whether cytokines promote or inhibit Th17 differentiation. IL-6 and IL-21 induced p-STAT3/p-STAT1 ratios > 1, leading to the promotion of Th17 differentiation, whereas IL-27 or IL-6+IL-27 induced p-STAT3/p-STAT1 ratios < 1, resulting in inhibition of Th17 differentiation. Consistent with these findings, we show that IL-27 induces sufficient p-STAT3 to promote Th17 differentiation in the absence of STAT1. Furthermore, IL-27–induced STAT1-deficient T cells were indistinguishable from bona fide highly proinflammatory Th17 cells because they induced severe experimental autoimmune encephalomyelitis upon adoptive transfer. Our results suggest that the ratio of p-STAT3/p-STAT1 induced by a cytokine or cytokine pairs can be used to predict whether they induce a competent Th17-differentiation program. The Journal of Immunology, 2015, 195: 4144–4153.

D4+ Th cells are key orchestrators of the adaptive immune system. Depending on activation conditions and the cytokine milieu, these cells can differentiate into a variety of effector T cell subsets that are characterized by the cytokines they secrete. It was originally believed that there were two types of effector Th cells: Th1 and Th2 cells (1, 2). However, more recently, another lineage of effector T cells, called Th17 cells, was discovered in the context of autoimmune inflammation (3–5) and was shown to induce CNS inflammation during experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (6). These proinflammatory effector cells can be induced in vitro by culturing naïve CD4+ T cells under activating conditions in the presence of IL-6 and TGF-β (7–9).

IL-6 signals primarily through the JAK/STAT pathway (10), whereas TGF-β signals primarily through the SMAD pathway (11). IL-6 binds to IL-6R, causing JAKs to activate members of the STAT family by phosphorylation. Activated STATs (p-STATs) form dimers, revealing a nuclear-targeting sequence that allows them to translocate to the nucleus and initiate transcriptional programs. IL-6 is thought to primarily activate STAT3 (12), and STAT3 is the primary input to the genetic network governing Th17 differentiation (13, 14). It was demonstrated that STAT3 is crucial for the induction of Th17 cells, as evidenced by a nearly complete loss of IL-17 secretion in its absence (15). However, IL-6 is just one of several cytokines that activates STAT3, but it remains the most potent inducer of IL-17–producing cells. Other cytokines that activate STAT3 include IL-21 (16), which induces weaker IL-17 production in the absence of TGF-β than does IL-6 (17), and the anti-inflammatory cytokines IL-10 (18, 19), which does not induce Th17 differentiation, and IL-27 (20–23), a potent inhibitor of Th17 differentiation (24).

How these different STAT3-inducing cytokines cause such a large range of effects on Th17 differentiation is an unresolved question in the field (25). Part of the answer may be found in another STAT protein induced by these cytokines: p-STAT1 (25). Three forms of p-STAT1 and p-STAT3 can exist in the nucleus as a result of JAK/STAT signaling: these two particular p-STATs can form homodimers (p-STAT1–p-STAT1, p-STAT3–p-STAT3) or a heterodimer (p-STAT1–p-STAT3) (26–28). Importantly, each dimer binds to different DNA sequences and, thus, has different effects on transcription. It was shown that IL-27 completely loses its ability to inhibit Th17 differentiation in STAT1–knockout (S1KO) mice (25, 29), suggesting that p-STAT1 is an inhibitor of the Th17-differentiation pathway. A simple theory to account for the different effects of these cytokines on Th17 differentiation is that each cytokine induces different amounts of activated STAT3 and STAT1, with IL-6 inducing primarily p-STAT3, IL-27 inducing primarily p-STAT1, and IL-21 inducing a combination of
the two. One way to test this theory is to comparatively measure STAT1 and STAT3 activation among the cytokines. Previous studies used Western blots or flow cytometry to measure STAT activation by IL-21 (30), IL-6 and IL-10 (18, 31), and IL-6 and IL-27 (22) in a variety of cell types, but not in the same study or same cell type, so it is impossible to compare the kinetics and amplitude of STAT activation for all cytokines.

In this study, we investigated cytokine specificity in the context of Th17 differentiation by measuring the STAT1 and STAT3 activation profiles for three cytokines (IL-6, IL-21, and IL-27) in a systematic way. We show that, among the three cytokines, IL-6 activates the most STAT3, as well as the most STAT1, raising the question of how it can be such a potent inducer of Th17 differentiation when it activates the highest amount of the Th17-inhibitory transcription factor, STAT1. Our experiments suggest that it is actually the ratio of p-STAT3/p-STAT1, rather than the total amount of either p-STAT3 or p-STAT1, which determines the ability of a cytokine to induce Th17 differentiation. This led to the finding that, in the absence of STAT1, IL-27 becomes a potent inducer of Th17 differentiation and that IL-27–induced S1KO CD4+ T cells are indistinguishable from bona fide Th17 cells with regard to their expression of key Th17-associated factors and their encephalitogenic properties.

Materials and Methods

Mice

Six- to eight-week-old C57BL/6J mice were obtained from The Jackson Laboratory, and 6- to 8-wk-old 129S6/SvEvTac mice (wild-type [WT]) and 129S6/SvEv-Stat1(tm1Rds) (S1KO) mice (32) were obtained from Taconic. S1KO mice on the C57BL/6 background were a kind gift from Prof. Dr. M. Mueller (Institute of Animal Breeding and Genetics, University of Veterinary Medicine, Vienna, Austria) (33). 2D2 mice were described previously (34) and were crossed with C57BL/6 S1KO mice. Mice were housed in a specific pathogen–free, viral Ab–free animal facility at the Harvard Institutes of Medicine or at the University of Geneva. All breeding and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Harvard Medical School or by the local veterinary office (Geneva, Switzerland), according to Swiss ethical regulations.

Intracellular staining of p-STAT1, p-STAT3, and p-STAT5

T cells were isolated from spleen and lymph nodes of WT or S1KO mice. CD4+ T cells were purified using magnetic beads coated with anti-CD4 Ab, according to the manufacturer’s instructions (Miltenyi Biotec). CD4+ T cells were cultured for various times at a concentration of 1.0–2.0 × 10^6/ml with IL-6 (10 ng/ml), IL-21 (20 ng/ml), IL-27 (25 ng/ml), or IL-7 (10 ng/ml) in the presence of plate-bound anti-CD3 Ab (2 μg/ml) (clone 145-2C11) and anti-CD28 Ab (2 μg/ml) (clone PV-1; both from Bio X Cell). All cytokines were purchased from R&D Systems. After the desired incubation times, the cells were collected and fixed with 4% paraformaldehyde (EM Grade) for 12 min at 37°C. The cells were then rinsed and permeabilized in 90% methanol for 30 min on ice. Subsequently, the cells were rinsed with Permeabilization Buffer (eBioscience) and stained with anti-p-STAT1 and anti-p-STAT3 Abs. Anti-STAT1 (pY701; clone 4a), anti-STAT3 (pY705; clone 4/P-STAT3), and anti-STAT5 (pY694; clone 47/STAT5) Abs were purchased from BD Biosciences. All flow cytometry data were acquired on a BD FACS Calibur and analyzed with FlowJo software (TreeStar).

Th17 differentiation in vitro

T cells were isolated from spleen and lymph nodes of WT or S1KO mice. CD4+ T cells were purified using magnetic beads coated with anti-CD4 Ab, according to the manufacturer’s instructions (Miltenyi Biotec). Subsequently, CD4+ T cells were cultured with anti-CD4, anti-CD25, and anti-CD62L Abs and sorted into naive CD4+CD25-CD62Llo T cells with a BD FACS Aria. Naive cells were cultured at a concentration of 1.0–1.5 × 10^6/ml. Cells were stimulated in the presence of plate-bound anti-CD3 Ab (1 μg/ml) and anti-CD28 Ab (1 μg/ml) in the presence of plate-bound anti-CD3 Ab (1–3 μg/ml) and anti-CD28 Ab (1–2 μg/ml) (clone PV-1; all from Bio X Cell). For the generation of Th17 cells, naive T cells were cultured with IL-6 (30 ng/ml), TGF-β (3 ng/ml), and IL-27 (50 ng/ml). In some experiments, Th17 cells were supplemented with 10 ng/ml IL-23 after 48 h. Cytokines were purchased from R&D Systems or eBioscience. After 4 d, production of IFN-γ was measured by ELISA.

FIGURE 1. p-STAT1 and p-STAT3 expression over time in response to different cytokines. Flow cytometry measurement of p-STAT3 and p-STAT1 at different time points in CD4+ T cells (CD4+CD25-CD62Llo) cultured with anti-CD3/anti-CD28 for 1 h and then IL-6, IL-21, or IL-27 for up to 4 h. p-STAT3 (A) and p-STAT1 (B) levels for all cytokines over time. Results are the averages of 4–13 MFI measurements at a given time point and a given condition, normalized using Eq. 1. Error bars represent SEM. Curves were significantly different (p < 0.0001), as determined by two-way ANOVA with a Bonferroni post test. (C–E) The cytokine fingerprints illustrate how the relative amounts of p-STAT1 and p-STAT3 develop over time.
measured by intracellular cytokine staining and subsequent flow cytometry. After 1 or 2 d, cells were collected for RNA extraction, and mRNA levels were analyzed by quantitative PCR, as described below.

**Quantitative real-time PCR**

RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized with random hexamers and Superscript II reverse transcriptase (Invitrogen) and was used as the template for quantitative PCR. PCR was performed with the Real-Time PCR Detection System (Applied Biosystems StepOne plus) and SYBR Green Supermix (KAPA SYBR FAST Universal; Labgene). Expression of Tbet (FW: 5'-AGGGAGCGCTTAGTGTT-3' and Rev: 5'-TTCTCTATACGGCCCTGGTT-3') and Rorc (FW: 5'-CAGCTGCGAGGCTGAAGAC-3' and Rev: 5'-GGAAACTCGGGAAAGCTAC-3') and actin (FW: 5'-CCTGATGCTCTGAGCTGTA-3' and Rev: 5'-CATCTCCCTGCGAAGTCT-3') was assessed with specific primers and probes. Gene expression was normalized to expression of the housekeeping gene actin.

**Measurement of cytokines**

Secreted cytokines were measured after 48 h by ELISA (purchased at eBioscience) if not otherwise specified.

**In vitro T cell proliferation**

Proliferation was measured by incorporation of [3H]thymidine (Perkin-Elmer) for the last 16 h of a 3 d culture. After 48 h, cultures were supplemented with IL-23; after 4 d, cultures were stimulated for 4 h with PMA and ionomycin, followed by intracellular cytokine staining and subsequent flow cytometry measurement of p-STAT3 and p-STAT1 at different time points in CD4+ T cells cultured with IL-6, IL-21, or IL-27.

**Histology**

For analysis of CNS inflammation, mice were sacrificed 20 days after immunization and perfused with cold PBS, followed by 4% paraformaldehyde fixation. Brain and spinal cord tissue were embedded in paraffin. Sections were stained with H&E for light microscopy. Images of tissue sections were scanned using a Mirax slide scanner (Zeiss). Sections were stained with H&E, and consecutive sections were examined by immunohistochemistry. Immunostaining was performed to assess the numbers of activated macrophages/microglia (Mac3, clone M3/84; BP Pharmingen) and T cells (CD3, clone CD3-12; AbD Serotec). The avidin-biotin technique with 3,3-diaminobenzidine was used for the visualization of bound primary Abs.

**Results**

Normalization method allows for cytokine comparison

We sought to find a method that would allow us to reproducibly and reliably characterize the signaling pathways of the three cytokine cocktails. Five million cells were adoptively transferred i.p. Animals were monitored daily for the development of classical and atypical signs of EAE, as previously described (6, 35), according to the following criteria: 0, no disease; 1, decreased tail tone or mild balance defects; 2, hind limb weakness, partial paralysis, or severe balance defects that cause spontaneous falling over; 3, complete hind limb paralysis or very severe balance defects that prevent walking; 4, front and hind limb paralysis or inability to move body weight into a different position; and 5, moribund state.

**Statistics**

Statistical evaluations of gene expression data were performed with the unpaired Student t test. p-STAT profiles of different cytokines were evaluated using two-way ANOVA with the Bonferroni post test. The p values < 0.05 were considered significant.
cytokines of interest (IL-6, IL-21, and IL-27) early during T cell differentiation to determine why they behave differently despite activating the same two transcription factors: STAT1 and STAT3. To measure the expression of p-STAT1 and p-STAT3, CD4+ T cells were activated with anti-CD3/anti-CD28 Abs in the presence of cytokines of interest. Measurements of p-STAT1 and p-STAT3 levels were made only during the first 3–4 h after T cell activation to ensure that the STAT activation levels were entirely attributable to the cytokine of interest and not due to secondary effects of other cytokines secreted by the T cells over time.

We compared different techniques to measure differences in STAT activation. Given the number of conditions that we sought to measure, it became clear that flow cytometry was the best technique because of the ease of measurement and the availability of a quantitative comparable result. Because it was not feasible to do all of the flow cytometry measurements in 1 d, we devised a way to display all of our data collected over several months of separate experiments in one graph. Using the mean fluorescence intensity (MFI), we were able to extract just one number from each FACS measurement that could be plotted and compared with other samples. Daily variations in FACS measurements and mouse-to-mouse variations could be corrected for by determining the conditions resulting in the highest and the lowest signal in every experiment. By testing numerous conditions and time points, we determined that the maximum signal obtained for both p-STAT1 and p-STAT3 was always measured in cells that received 15 min of IL-6 stimulation, whereas the minimum signal for both p-STATs was always measured in unstimulated cells (Supplemental Fig. 1). Therefore, these two conditions were included in every experiment and used to normalize that day’s measurements to all other measurements using Eq. 1:

\[
\text{Normalized } p-\text{STAT} = \frac{MFI_{\text{Sample}} - MFI_{\text{Untreated}}}{MFI_{15\text{min IL-6}} - MFI_{\text{Untreated}}}
\]

In each experiment, IL-6 at 15 min was assigned a value of 100 for p-STAT1 and for p-STAT3, whereas a value of 0 indicated a basal level of STAT activation measured in cells cultured without cytokines. It is important to note that p-STAT1 and p-STAT3 were normalized using different MFI values; thus, a value of 100 for p-STAT1 and a value of 100 for p-STAT3 do not indicate that there are equivalent amounts of the two transcription factors at the molecular level. The values observed for MFI_{IL6,15min}/MFI_{Untreated} were typically 1.5–2.0 for p-STAT3 and 8.0–15.0 for p-STAT1. As a result, because of the greater separation between the maximum signal and the background, less variation was observed in the normalized p-STAT1 measurements compared with the p-STAT3 measurements.

Cytokines induce different STAT activation profiles

We measured STAT3 and STAT1 activation profiles in CD4+ T cells treated with the three cytokines for different time periods and normalized the profiles using the method described above. This enabled us to compare the activation profiles of STAT1 and STAT3 among the different cytokines (Fig. 1).

IL-6 clearly induced the most p-STAT3 of the three cytokines at all time points measured (Fig. 1A). Comparatively, IL-21 activated roughly half the amount of STAT3 during that time period. IL-27 had peak STAT3 activation at 15–30 min, reaching ~33% of the p-STAT3 levels induced by IL-6 at those time points. Activation of STAT3 by IL-27 then steadily decreased to basal levels during the remainder of the first 2 h. Given that STAT3 is vital to Th17 differentiation (15), it is logical that IL-6 is the best inducer of Th17 cells (in combination with TGF-β), because it induced the most p-STAT3. IL-21 is a weak inducer of Th17 cells, and it induces roughly half the amount of p-STAT3 as IL-6. IL-27 does not induce Th17 cells and showed the lowest induction of p-STAT3. These data prompted the question whether there is a threshold of p-STAT3 induction below that of IL-21 and above that of IL-27 that needs to be reached for a cytokine to induce Th17 cells.

It was generally believed that IL-27 inhibits Th17 differentiation by inducing high amounts of p-STAT1, because IL-27 fails to inhibit Th17 differentiation in S1KO mice (25, 29). Therefore, we expected IL-27 to induce higher levels of p-STAT1 than either of the other cytokines. Indeed, IL-21 induced relatively small amounts of p-STAT1 compared with IL-27. However, we observed that IL-6 induced more p-STAT1 than IL-27 at almost all time points in the first 4 h (Fig. 1B). At its peak induction of p-STAT1 at 15 min, IL-27 only induced ~60% of the amount induced by IL-6. These data raised the question of how IL-6 can induce the most p-STAT1, which is known to inhibit Th17 differentiation, and still be the strongest inducer of Th17 cells.

Visualizing the data in the form of cytokine “fingerprints” might assist in addressing this question. To create a “fingerprint” of a given cytokine’s signaling pattern, the time courses of p-STAT1 and p-STAT3 levels are combined in the same graph. This allows for a comparison of the relative amounts of p-STAT3 and p-STAT1 induced at all of the time points for a given cytokine.
(Fig. 1C–E). As mentioned previously, the values for p-STAT1 and p-STAT3 cannot be compared directly; however, regardless of the absolute number of STAT1 or STAT3 molecules, it is still valid to compare the ratio of p-STAT3/p-STAT1 over time among the different cytokines, because all of the data were normalized the same way. The fingerprint for IL-6 showed that T cells treated with IL-6 for 15 min had a p-STAT3/p-STAT1 ratio of 1. At 3 h, the ratio of p-STAT3/p-STAT1 increased to 5 (Fig. 1C). Similarly, IL-21 induced a p-STAT3/p-STAT1 ratio of 2–3 in T cells during the first 3 h (Fig. 1D). In contrast, IL-27 was the only cytokine to induce a higher relative amount of p-STAT1 than p-STAT3. In fact, at all time points, T cells treated with IL-27 had more p-STAT1 than p-STAT3 (Fig. 1E). These observations suggest that the ratio of p-STAT3/p-STAT1 is an important parameter in determining whether a cytokine will induce Th17 differentiation: cytokines, such as IL-6 and IL-21, which induce a p-STAT3/p-STAT1 ratio $\leq$ 1 at most or all time points promote Th17 differentiation, whereas cytokines, such as IL-27, which induce a p-STAT3/p-STAT1 ratio $\geq$ 1 at all time points inhibit Th17 differentiation.

$p$-STAT1/p-STAT3 ratio determines whether a cytokine pair inhibits or induces Th17 cells

To further investigate the importance of the p-STAT3/p-STAT1 ratio for the ability of cytokines to induce Th17 differentiation, we began looking at STAT activation profiles for pairs of cytokines. We were interested in the STAT-activation profiles for pairs of cytokines for three reasons. First, in experiments showing that IL-27 inhibits Th17 differentiation, IL-27 was added in addition to IL-6 and TGF-β at time 0 (25, 29). Therefore, the cell is exposed to both IL-6 and IL-27 at the same time, so their individual profiles are not directly applicable. Second, we may gain new mechanistic insight by determining how individual profiles combine. If the sum of the individual profiles does not equal that of the cytokine pair’s profile, then there are saturating or inhibitory effects. Finally, the profiles of cytokine pairs will help us to define which profiles lead to Th17 cell differentiation.

We measured the fingerprints of IL-6 (Fig. 2A), IL-6 with IL-21 (Fig. 2B), and IL-6 with IL-27 (Fig. 2C). All measurements were normalized as before using the MFI from untreated T cells as the baseline and the MFI from T cells treated with IL-6 for 15 min as the maximum. Assuming that the profiles are additive, it was now possible for the cytokine pairs to induce p-STAT values $>100$, because IL-6 was present in all pairs, and all measurements were normalized to IL-6 at 15 min. Thus, the addition of either IL-21 or IL-27 to IL-6 increased the value of p-STAT1 to $>100$ at 15 min and shifted the p-STAT1 curve upward in value at all time points while maintaining the same shape as the p-STAT1 curve in the IL-6 fingerprint (Fig. 2A–C). Interestingly, the p-STAT1 profiles in the IL-6+IL-21 fingerprint and the IL-6+IL-27 fingerprint were nearly identical. This similarity, together with the fact that IL-27 inhibits Th17 differentiation via STAT1, suggests that the addition
IL-27 induces Th17-like cells in S1KO T cells

Given the similarity between the p-STAT3 profiles induced by IL-6 and IL-27 in S1KO T cells, we predicted that, in the absence of IL-21 at time 0 should also inhibit Th17 differentiation. To test this hypothesis, we measured Th17 differentiation in the presence of TGF-β+IL-6 (Fig. 2D), TGF-β+IL-6+IL-27 (Fig. 2E), and TGF-β+IL-6+IL-27 (Fig. 2F) by flow cytometry. We measured a similar frequency of IL-17+ T cells in the presence of TGF-β+IL-6 and TGF-β+IL-6+IL-21, whereas the presence of IL-27 almost completely inhibited Th17 differentiation. These data raise the question of why IL-21 does not inhibit Th17 differentiation although it induces an almost identical p-STAT1 profile as IL-27. Again, the answer can be obtained by considering the relative p-STAT1 and p-STAT3 profiles. Addition of IL-21 to IL-6 caused an upward shift in both the p-STAT1 and p-STAT3 curves in the IL-21+IL-6 fingerprint, resulting in a similar p-STAT3/p-STAT1 ratio as observed in the IL-6 fingerprint (Fig. 2A–C). However, when IL-27 was added to IL-6, the p-STAT1 curve was shifted upward, and the p-STAT3 curve was shifted downward, resulting in a significant change in the p-STAT3/p-STAT1 ratio compared with the IL-6 fingerprint (Fig. 2A–C). Therefore, considering just the p-STAT1 profiles alone is not sufficient for making conclusions about what effect a cytokine has on Th17 differentiation, because one would infer that IL-21 should be inhibitory to Th17 differentiation. Instead, these data suggest that determining the ratio of p-STAT3/p-STAT1 induced by a cytokine is superior to individual profiles for predicting the effect that the cytokine may have on Th17 differentiation.

IL-6 and IL-27 induce similar p-STAT3 profiles in S1KO T cells

It was shown that inhibition of Th17 differentiation via IL-27 is dependent on STAT1, because S1KO T cells stimulated with TGF-β, IL-6, and IL-27 generate a similar percentage of IL-17–producing cells as S1KO T cells stimulated with TGF-β and IL-6 alone (25, 29). Based on the relatively low amount of p-STAT3 induced by IL-27 compared with IL-6 (Fig. 1), and given the similarity in Th17 induction, we expected IL-6 with and without IL-27 to induce similar p-STAT3 profiles in S1KO T cells. To test this, we measured the p-STAT3 profiles over time in WT (Fig. 3A) and S1KO (Fig. 3B) T cells after stimulation with IL-6, IL-27, or IL-6+IL-27. In WT T cells, IL-6 induced the most p-STAT3 at all times, and IL-27 induced the least amount of p-STAT3. As early as 15 min, a decrease in p-STAT3 was observed in WT T cells upon addition of IL-27 to IL-6, so that the p-STAT3 profile of WT T cells stimulated with IL-6+IL-27 fell in between the p-STAT3 profiles of IL-6– and IL-27–treated WT cells (Fig. 3A). In contrast, in S1KO cells, the STAT3-activation profiles for IL-6 and IL-6+IL-27 were almost identical, implying that the presence of STAT1 is somehow able to reduce the level of activation of STAT3. In addition, we observed that IL-27 induced only slightly of STAT1 is somehow able to reduce the level of activation of STAT3. In addition, we observed that IL-27 induced only slightly

IL-27 induces Th17-like cells in S1KO T cells

Given the similarity between the p-STAT3 profiles induced by IL-6 and IL-27 in S1KO T cells, we predicted that, in the absence of STAT1, IL-27 induces enough p-STAT3 to drive Th17 differentiation when combined with TGF-β. To test our prediction, we cultured naive CD4+ T cells (CD4+CD45+CD62L(+) from either WT or S1KO mice with anti-CD3 and anti-CD28 Abs and with no cytokines (Th0), TGF-β, TGF-β+IL-6, TGF-β+IL-6+IL-27, or TGF-β+IL-27. After 4 d, we measured IL-17 production (Fig. 4A). Consistent with previous studies, IL-27 potently inhibited Th17 differentiation in WT cells treated with IL-6+IL-27+TGF-β, but it did not inhibit Th17 differentiation in S1KO cells treated with IL-6+IL-27+TGF-β (25, 29). As expected, IL-27 and TGF-β did not induce any Th17 differentiation in WT cells. In contrast, treatment of S1KO T cells with IL-27 and TGF-β generated a significant percentage of IL-17+ cells (Fig. 4A), as we predicted based on the p-STAT3 profiles. Accordingly, S1KO T cells treated with IL-27+TGF-β displayed a Th17-like phenotype: they produced and secreted similar amounts of IL-17 and expressed similar RORγt (Rorc) mRNA levels as did WT Th17 cells generated via IL-6+TGF-β. In addition, IL-27+TGF-β–treated S1KO T cells did not express Th1-associated markers [i.e., they did not produce IFN-γ nor did they express the Th1 transcription factor T-bet (Tbx21)] (Fig. 4B, 4C).

**FIGURE 5.** IL-27–induced S1KO Th17 cells respond to IL-23. CD4+ T cells from WT or S1KO mice were cultured with plate-bound anti-CD3 and anti-CD28 in the presence of the indicated cytokines. RNA was isolated after 24 h of culture, and mRNA levels of IL-23R (A), IL-12Rβ2 (C), and IL-12Rβ1 (D) relative to β-actin were measured by real-time PCR. (B) Sorted naïve 2D2 CD4+ T cells from WT or S1KO mice were stimulated with irradiated APCs and anti-CD3 in the presence of the indicated polarizing cytokines and supplemented with IL-23 after 48 h. Proliferation was determined after 24 h of culture, and mRNA levels of IL-23R (A), IL-12Rβ2 (C), and IL-12Rβ1 (D) relative to β-actin were measured by real-time PCR. (B) Sorted naïve 2D2 CD4+ T cells from WT or S1KO mice were stimulated with irradiated APCs and anti-CD3 in the presence of the indicated polarizing cytokines and supplemented with IL-23 after 48 h. Proliferation was measured by [3H]thymidine incorporation (cpm ± SD). Data are representative of at least three separate experiments. ***p ≤ 0.001, ****p ≤ 0.0001.
levels of IL-23R mRNA, whereas IL-27 did not induce IL-23R expression in WT T cells. In contrast, IL-27 and IL-6 both induced high levels of IL-23R mRNA in S1KO CD4 T cells (Fig. 5A). These data suggest that IL-27–induced S1KO Th17 cells can form a functional IL-23R and, thus, respond to IL-23. To test this, we measured proliferation of IL-27–induced S1KO Th17 cells and WT Th17 cells in response to IL-23 using a radiolabeled thymidine assay. Importantly, S1KO CD4+ T cells stimulated with IL-23 proliferated just as well as S1KO and WT T cells stimulated with IL-6, whereas WT T cells cultured with IL-27 did not proliferate in the presence of IL-23 (Fig. 5B). In addition, expression of IL-12Rβ2 was increased by IL-27 in WT cells but not in S1KO cells (Fig. 5C), indicating that IL-27 acts via STAT1 to induce IL-12Rβ2 expression. The IL-12Rβ1 subunit was constitutively expressed in all CD4+ T cells subsets (Fig. 5D).

**IL-27–induced S1KO Th17 cells are encephalitogenic upon adoptive transfer**

In the absence of STAT1, IL-27 induces CD4+ T cells that resemble Th17 cells because they express the lineage-specific transcription factor RORγt, the cytokine IL-17A, and IL-23R. However, we and other investigators showed that Th17 cells come in different flavors that do not necessarily harbor identical pathogenicity (39). To test the pathogenic potential of IL-27–induced S1KO Th17 cells, we performed adoptive-transfer experiments using CD4+ T cells from either 2D2 mice (which genetically express a TCR specific for myelin oligodendrocyte glycoprotein) or S1KO 2D2 mice. Naïve CD4+ T cells were differentiated in vitro into Th17 cells, as previously described (6) and as represented in Fig. 6A. Confirming our previous result, IL-27 induced IL-17 production in S1KO CD4+ T cells to the same extent as did IL-6 in WT and S1KO T cells (Fig. 6B). Upon adoptive transfer, we observed that S1KO CD4+ T cells differentiated with IL-27-induced EAE disease similar to WT Th17 cells or S1KO T cells differentiated with IL-6 (Fig. 6C). Although Th1 cell transfer is known to induce classical EAE, which is characterized by an ascending paralysis, Th17 cell recipients were described to also develop atypical EAE signs characterized by an unbalanced gait and severe axial and barrel rotary defects (40). When the mice were scored over 20 d, recipients of WT Th17 cells, IL-27–induced S1KO Th17 cells, and IL-6–induced S1KO Th17 cells displayed a similar disease course, with similar disease severity and onset (Fig. 6D, Supplemental Table I). As a result of the limited proliferative capacity shown in Fig. 5B, we were not able to sufficiently...
expand WT CD4 T cells differentiated with IL-27 for adoptive transfer. In summary, IL-27–induced S1KO Th17 cells are highly pathogenic and capable of inducing EAE with similar severity as IL-6–induced Th17 cells.

**IL-27–induced S1KO Th17 cells lead to cerebellum and brain stem lesions**

Atypical EAE is associated with lesions in the brain, in particular in the cerebellum and the brainstem. Because Th17 cell recipients exhibited an atypical neurologic disease with severe ataxia, we compared the localization of CNS lesions in sick mice from the different groups (Fig. 7A). Histological and immunohistochemistry analysis revealed that recipients of IL-27–induced S1KO Th17 cells exhibited immune cell infiltration that was as severe as in recipients of classically differentiated Th17 cells (Fig. 7B). Cellular infiltrates were particularly abundant in the cerebellum and brainstem. Anti-CD3 and anti–Mac3 staining further showed marked infiltration of T cells and myeloid cells. Our histologic analysis showed that WT Th17 cells and S1KO Th17 cells induced by IL-27 or IL-6 caused a very similar pattern and degree of inflammation, cellular infiltration, and tissue destruction.

**Discussion**

IL-6 (18, 22, 31), IL-21 (30), and IL-27 (22) all activate STAT3 but have very different effects on Th17 differentiation. In this study, we systematically measured and compared the p-STAT3 and p-STAT1 profiles in T cells in response to all three cytokines separately and in response to cytokine pairs. In these analyses, we made the assumption that STAT activation in the first 4 h is relevant in determining a cytokine’s ability to induce Th17 differentiation, a process spanning several days. However, we observed that STAT3 reaches its peak expression within 2 h during Th17 differentiation and quickly drops off to low levels for the remainder of the differentiation process (K.D. Fowler and V.K. Kuchroo, unpublished observations), confirming that it is relevant to measure STAT activation at early times.

IL-6 activated the most STAT3 of the three cytokines, followed by IL-21 and IL-27, suggesting that the amount of p-STAT3 induced by a cytokine may directly correlate with its relative ability to induce Th17 differentiation. Surprisingly, we observed that, of the three cytokines, IL-6 also induced the most p-STAT1, which is known to inhibit Th17 differentiation. Because IL-6 is the best inducer of Th17 cells, and STAT1 is entirely responsible for IL-27’s ability to potently inhibit Th17 differentiation (25, 29), we expected IL-27 to induce the most p-STAT1 among these cytokines. These data indicate that considering the total amounts of p-STAT1 or p-STAT3 induced by a cytokine separately is not sufficient to predict its effect on Th17 differentiation. Measuring both p-STAT3 and p-STAT1 profiles in response to stimulation with cytokine pairs revealed that the p-STAT3/p-STAT1 ratio is important in determining whether cytokines will induce or inhibit Th17 differentiation. Thus, T cells treated with IL-21+IL-6 had a high p-STAT3/p-STAT1 ratio, similar to T cells treated with IL-6 alone, and they showed a similar degree of Th17 differentiation. In contrast, the p-STAT3/p-STAT1 ratio was significantly lowered in T cells treated with IL-27+IL-6 compared with IL-6 alone.

**FIGURE 7.** IL-27–induced Th17 cells drive infiltration of the brainstem and cerebellum. Histological analysis of brainstem and cerebellum of the three recipient mice groups showing severe cellular infiltration, as visualized at low magnification by H&E staining (A) and high magnification with H&E staining and immunohistochemistry using anti-CD3 and anti-Mac3 Abs (B) of the representative regions in (A) indicated by the rectangles in the cerebellum.
resulting in inhibition of Th17 differentiation. Consistent with this concept, IL-27 was the only cytokine that induced a higher amount of p-STAT1 compared with p-STAT3 on the normalized scale, leading to a p-STAT3/p-STAT1 ratio < 1 and inhibition of Th17 differentiation.

Because IL-6 and IL-27 both require the receptor subunit gp130, paired with the specific subunits IL-6R and WSX-1, respectively, to signal through their receptors, competition for gp130 could be an issue. Thus, it is possible that the IL-6+IL-27 fingerprint reflects the action of the dominant cytokine rather than equal action of both cytokines. Several arguments point against this hypothesis. First, we could not drive Th17 cell differentiation with IL-27 in the absence of WSX-1 (data not shown). Furthermore, although IL-6R is highly expressed on naïve T cells, it is downregulated upon T cell activation (41), whereas WSX-1 is upregulated upon activation of naïve T cells (23). Thus, by activating our T cells before adding cytokines, we reduced the competition for gp130. Considering also that IL-6+IL-27 generates a different fingerprint than IL-6 or IL-27 alone, it is reasonable to assume that the IL-6+IL-27 fingerprint reflects the action of both cytokines. Thus, we believe that our observations directly reflect the differences in p-STAT profiles and that these observations cannot be explained by competition for gp130.

The p-STAT3/p-STAT1 ratio affects the distribution of the three dimeric forms: conceivably the highest relative amount of heterodimer is formed when p-STAT1 and p-STAT3 are present in similar quantities (1:1 ratio). The relative amount of heterodimer decreases as one of the STATs starts dominating in numbers over the other, and the homodimeric form of the dominant STAT will be present in the higher relative amount. Thus, a high p-STAT3/p-STAT1 ratio leading to Th17 differentiation is characterized by the dominance of p-STAT3–p-STAT3 homodimers, whereas a low p-STAT3/p-STAT1 ratio is characterized by the abundance of p-STAT1–p-STAT1 homodimers, leading to inhibition of Th17 differentiation. We attempted to determine the relative amounts of homo- and heterodimers in response to different cytokines more precisely using an EMSA. However, we found that in our hands, EMSA lacked both precision and reproducibility to account for the differences. Thus, one could argue that IL-27 only induces Th17 differentiation in S1KO cells because they are more prone to Th17 differentiation as a result of the significant reduction in IFN-γ in these cells, which is known to inhibit Th17 cells. However, treatment of IFN-γ−/− T cells with IL-27 and TGF-β did not induce any Th17 differentiation (data not shown), confirming that the Th17 differentiation seen in S1KO cells in response to IL-27 is not due to the lack of IFN-γ. Thus, we believe that our results and verified predictions can be largely explained by considering p-STAT1 and p-STAT3 profiles and the resulting ratio.

Interestingly and consistent with our results, STAT1 and STAT3 imbalance also affects Th17 responses in humans. Thus, patients with STAT1 gain-of-function mutations, who presumably have a low p-STAT3/p-STAT1 ratio, suffer from chronic mucocutaneous candidiasis due to an impaired Th17 response (45, 46). Similarly, patients with STAT3 loss-of-function mutations also suffer from recurrent Candida and Staphylococcus infections (47, 48). In contrast, patients with STAT3 gain-of-function mutations, who presumably have a very high p-STAT3/p-STAT1 ratio, develop multorgan autoimmune disease (49). Although the underlying mechanisms are still unclear and likely involve defects in several cell types, including regulatory T cells, increased Th17 responses may contribute to this immune dysregulation, because one patient showed elevated IL-17 levels and responded well to anti–IL-6R therapy (50). These results imply that the p-STAT3/p-STAT1 ratio could play an important role for both beneficial and pathogenic Th17 differentiation in mice, as well as in humans. In conclusion, the current study demonstrates the relevance and predictive value of the p-STAT3/p-STAT1 ratio for the induction of pathogenic Th17 cells. Modulation of the STAT activation balance could be foreseen as a novel therapeutic approach to fine-tune the immune response during infections or autoimmune disorders.

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References


