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STAT1-Activating Cytokines Limit Th17 Responses through Both T-bet–Dependent and –Independent Mechanisms

Alejandro V. Villarino, Eugenio Gallo, and Abul K. Abbas

Given the association with autoimmune disease, there is great interest in defining cellular factors that limit overactive or misdirected Th17-type inflammation. Using in vivo and in vitro models, we investigated the molecular mechanisms for cytokine-mediated inhibition of Th17 responses, focusing on the role of STAT1 and T-bet in this process. These studies demonstrate that, during systemic inflammation, STAT1- and T-bet–deficient T cells each exhibit a hyper-Th17 phenotype relative to wild-type controls. However, IL-17 production was greater in the absence of T-bet, and when both STAT1 and T-bet were deleted, there was no further increase, with the double-deficient cells instead behaving more like STAT1-deficient counterparts. Similar trends were observed during in vitro priming, with production of Th17-type cytokines greater in T-bet<sup>−/−</sup> T cells than in either STAT1<sup>−/−</sup> or STAT1<sup>−/−</sup> T-bet<sup>−/−</sup> counterparts. The ability of IFN-γ and IL-27 to suppress Th17 responses was reduced in T-bet–deficient cells, and most importantly, ectopic T-bet could suppress signature Th17 gene products, including IL-17A, IL-17F, IL-22, and retinoic acid-related orphan receptor γT, even in STAT1-deficient T cells. Taken together, these studies formally establish that, downstream of IFN-γ, IL-27, and likely all STAT1-activating cytokines, there are both STAT1 and T-bet–dependent pathways capable of suppressing Th17 responses. The Journal of Immunology, 2010, 185: 6461–6471.

During an adaptive immune response, CD4<sup>+</sup> Th cells differentiate into multiple effector subsets, each characterized by their transcriptional profile and cytokine output. Among these, the recently identified Th17 subset has been increasingly linked to autoimmunity with its signature products, IL-17A and IL-17F, implicated in the pathogenesis of numerous inflammatory disorders including arthritis, multiple sclerosis, graft-versus-host disease, and psoriasis (1). Although multiple factors are known to influence Th17 responses, cytokines have emerged as the key negative regulators, with IFN-γ, the signature product of Th1 effector cells, considered one of the most potent inhibitors. Deletion of IFN-γ or its receptor leads to increased IL-17 production and severe clinical manifestations in many of the experimental models where Th17 cells are considered pathogenic, including arthritis, experimental autoimmune encephalitis (EAE), and experimental autoimmune uveitis (2–6). IFN-γ deficiency also results in a hyper-Th17 phenotype during in vitro T cell differentiation, and consistent with a Th1-intrinsic mode of action, the addition of exogenous IFN-γ yields a converse hypo-Th17 phenotype (7–9). These findings imply an inverse relation between the Th1 and Th17 subsets, but it should also be noted that cells producing both IL-17A and IFN-γ have been reported in numerous inflammatory settings, and that conversion of IL-17–producing Th17 cells into IFN-γ–producing Th1 cells has been shown to occur both in vivo and in vitro, which points toward developmental plasticity and, perhaps, a linear relation (2, 10–14).

IFN-γ exerts its functions through a high-affinity cell surface receptor that is composed of two chains (IFN-γR1/IFN-γR2) and is expressed on range of immune and nonimmune cells. In CD4<sup>+</sup> T cells, IFN-γ propagates a Jak/STAT signaling cascade leading to robust activation of STAT1 and, to a lesser extent, of STAT3 (15). Among its principal STAT1-dependent activities in CD4<sup>+</sup> T cells is the induction of T-bet, a transcription factor (TF) that is both necessary and sufficient for Th1 differentiation. T-bet drives expression of several hallmark Th1 genes, including IL-12Rβ2, and through its ability to drive IFN-γ production, it establishes a positive feedback loop where IFN-γ induces T-bet, which, in turn, triggers more IFN-γ (16–20). T-bet deficiency confers resistance in several models of T cell-dependent autoimmunity, like diabetes, EAE, and colitis (21–24); but in some inflammatory settings, like allograft rejection and myocarditis, it leads to increased pathology, typically characterized by a profound a Th1 defect and a corresponding increase in Th17-type cytokines (13, 25–27). Thus, although generally considered proinflammatory, T-bet–driven Th1 responses can also have anti-inflammatory consequences, best exemplified by their ability to limit Th17-mediated disease.

Like IFN-γ, other STAT1-activating cytokines can affect Th17 responses. One pertinent example is IL-27, which mirrors IFN-γ in three key ways: 1) it is a potent STAT1 activator; 2) it induces expression of T-bet; and 3) it inhibits key Th17 gene products, like IL-17A, IL-17F, and retinoic acid-related orphan receptor γT (RORγT) (28). Consistent with this analogy, IL-27R– and IL-27R–deficient mice, similar to IFN-γ<sup>−/−</sup> and IFN-γR<sup>−/−</sup> mice, exhibit severe pathology and increased Th17 responses in models of T cell-dependent autoimmunity, including toxoplastic encephalitis and EAE (9, 29, 30). In vitro studies have shown that IFN-γ and IL-27 cannot suppress IL-17 production in the absence of STAT1, which suggests a common mechanism, but it remains unclear whether this is due to direct effects (i.e., STAT1 binding to Th17-associated loci) and or indirect effects (i.e., STAT1 regulating other inductive/inhibitory factors) (9, 29–33). The role of T-bet in this process is also poorly understood. It is known that
IFN-γ and IL-27 can each induce expression of T-bet, and that ectopic T-bet expression can suppress IL-17 production, but whether this is due to a cell-intrinsic mechanism or its ability to drive IFN-γ–mediated, STAT1-dependent inhibition has not been resolved (34, 35). Furthermore, although numerous studies have shown that IL-27 can limit Th17 responses in the absence of T-bet, thus demonstrating that it is not required for STAT1-mediated inhibition, the possibility remains that T-bet–dependent mechanisms are still working in parallel to or in concert with STAT1 (9, 30, 31). The data presented in this study address these latter issues, formally establishing that, downstream of STAT1-activating cytokines, there are two distinct anti-Th17 pathways: first, the previously described STAT1-dependent, T-bet–independent pathway; and second, a T-bet–dependent, STAT1-independent pathway.

Materials and Methods

Animals

Gene-deficient donor mice were generated by crossing DO11.10 TCR transgenics (Jackson Laboratories, Bar Harbor, ME) with the following BALB/c strains: IFN-γ–/– (Jackson Laboratories), T-bet–/– (from L. Glimcher, Harvard University, Cambridge, MA) (36), IL-17A–/– (from Y. Iwakura, University of Tokyo, Tokyo, Japan) (37), and IL-2–/– (Taconic, Germantown, NY) (38). STAT1–/– mice from Taconic were used in compliance with their Research Cross-breeding Agreement. These were backcrossed (more than eight generations) onto the BALB/c background and then bred with either wild-type (WT) or Rag2–/– mice purified by positive selection using magnetic beads. These LNs and spleens were dissected from WT or gene-deficient mice, pooled, and cultured for 48 h and then collected (no restimulation). For retroviral experiments, T cells expressing more than one transgene (GFP–Thy1.1+) were purified by high-speed cell sorting (1–2 × 10^7 per group). For in vitro studies, T cells were cultured for 48 h and then collected (no restimulation). For retroviral experiments, T cells expressing more than one transgene (GFP–Thy1.1+) were purified by high-speed cell sorting (1–2 × 10^7 per group; Supplemental Fig. 4). For all PCR studies, mRNA was extracted from purified T cells and converted to cDNA using oligo-dT priming and SuperScript III reverse transcriptase (100–250 ng RNA per reaction; Invitrogen). PCR amplification was performed with SYBR green master mix (10–50 ng cDNA per reaction; Applied Biosystems, Foster City, CA) using an iQ5 real-time PCR thermal cycler (Bio-Rad, Hercules, CA). Primer sequences and relevant information are provided in Supplemental Table I. Reactions were performed in duplicate, Ct values normalized to β-actin levels, and fold induction (n > 1) or reduction (n < 1) of each gene calculated (ΔΔCt) with respect to the indicated controls (n = 1).

Retroviral Gene Transduction

RORγT cDNA was PCR-amplified from Th17-polarized BALB/c CD4+ T cells using a high-fidelity polymerase (Easy A, Stratagene, Cedar Creek, TX). PCR products were then digested, ligated into a modified MIG-R1 vector (directly upstream of the internal ribosome entry site and Thy1.1 marker), and recombined clones with ~100% sequence homology to the National Center for Biotechnology Information GenBank mRNA entry (accession No. AJ132394) were selected for further amplification (restricting enzymes and T4 ligase from NEB, Ipswich, MA; PCR sequencing by Sequtech, Mountain View, CA; Mini- and Maxi-prep kits from Qiagen, Germantown, MD). MIG-R1 expressing T-bet followed by an IRES and GFP was generated as described and kindly provided by S. Reiner (University of Pennsylvania, Philadelphia, PA) (19). RORγT and T-bet vectors, together with corresponding “empty” controls, were transfected into Phoenix packaging cells (together with pCL-Eco helper plasmid), and the resulting culture supernatants were used to infect T-bet–/– or STAT1–/– T cells. These were cultured under nonpolymerizing conditions (± anti–IFN-γ) for 36 h, exposed to viral supernatant for 1 h (at 2200 rpm, 19°C), and then cultured for an additional 36 h.

Statistics

Paired Student t test (one-tailed) was used to measure statistical deviation between experimental groups. In all figures, an asterisk represents significant differences (p < 0.05–0.08); when this symbol is not used, p values are given.

Results

Kinetics of Th1 and Th17 responses during lymphopenia-induced autoimmune disease

To investigate mechanisms of T cell-mediated autoimmunity, we have developed a mouse model where naive, TCR-transgenic T cells (DO11.10) are adoptively transferred into lymphopenic hosts that express their cognate Ag, chicken OVA, as a soluble protein in the bloodstream (sOVA Rag2–/–). This highly immunogenic environment results in an aggressive T cell response characterized by distinct waves of effector subsets, with a rapid expansion of Th17 cells followed by a more prolonged Th1-type response. Previous work has shown that, in this setting, IFN-γ and T-bet–deficient T cells exhibit a hyper-Th17 phenotype, thereby illustrating the anti-Th17 capacity of the IFN-γ/T-bet axis and validating this as a robust in vivo model for studying the regulation of Th17 responses (13).

For our basic experimental setup, naive CD4+ T cells were purified from DO11.10 Rag2–/– mice and adoptively transferred...
into sOVA Rag2−/− hosts or, as controls, into lymphopenic Rag2−/− hosts that lack the sOVA transgene. From 3 to 10 d later, LNs were dissected from recipient animals, restimulated overnight, and cytokine production measured by intracellular flow cytometry (IFC). We found that, soon after transfer (days 3–4), there was a massive accumulation of Th17 cells in sOVA Rag2−/− hosts, with about half of the donor cells producing IL-17A and a quarter producing IL-17F (Fig. 1A). At later time points (days 7–9), this Th17 response was abolished, as evidenced by the >2-fold reductions in IL-17A and IL-17F, whereas a Th1-type response dominated, as evidenced by sustained production of IFN-γ. In control Rag2−/− hosts, there were few IL-17A− or IL-17F−positive donor T cells at the early time point, but, surprisingly, these were readily detected at the later time point, which suggests that, even in the absence of a high-affinity Ag, lymphopenia itself is permissive for Th17 differentiation. However, it should also be noted, that in contrast with sOVA Rag2−/− hosts, this “homeostatic” Th17 response was slower, lesser in magnitude (in terms of total cytokine-producing cells), and ultimately, did not elicit autoimmune disease, which suggest that Ag is still key for determining the kinetics and pathogenicity of donor T cell responses. In addition, few IFN-γ+ donor cells could be detected in Rag2−/− hosts (Fig. 1A). Given that T cells are known to produce IFN-γ under such lymphopenic conditions (39, 40), this finding reflects both the importance of Ag and the fact that, although day 7 is a late time point for moribund sOVA Rag2−/− hosts, it is a relatively early time point compared with previous studies on “Ag-independent” homeostatic Th1 responses.

To further investigate the inflammatory response in sOVA Rag2−/− mice, we purified donor T cells and used real-time time PCR to measure Th17-associated cytokines, receptors, and TFs. Consistent with our IFC measurements, we found the early time point was associated with prominent induction of IL-17A, IL-17F, IL-22, and RORγT, whereas the later time point was associated with a >2-fold decline in all of these transcripts (Fig. 1B). Two Th17-associated receptors, IL-1R1 and IL-23R, were also strongly induced, but in those cases, mRNA levels continued to escalate throughout the course of study. Th1-type transcripts IFN-γ, IFN-γR2, T-bet, and IL-12Rβ2 were highly expressed at all time points, as was IL-21, which is associated with both Th1- and Th17-type inflammation (Fig. 1B). Together with our IFC studies, these data suggest a complex relation between Th17 and Th1 responses; there appears to be some chronology, with the former preceding the latter, but there is also evidence for concurrent expression of both Th1- and Th17-type factors within the donor population, if not within individual donor cells.

To explore the relation between Th17 and Th1 cells in sOVA Rag2−/− hosts, we used IFC to measure coexpression of IL-17A and IFN-γ. Consistent with previous work (13), we found that a large fraction of donor T cells produced both cytokines, and that,

FIGURE 1. Kinetics of Th1 and Th17 responses during systemic autoimmune disease. A. Naive DO11.10 CD4+ T cells were transferred into Rag2−/− or sOVA Rag2−/− hosts. Three to 9 d later, lymphocytes from recipient mice were restimulated ex vivo and cytokine production measured by IFC. Shown is the percentage of cytokine-positive donor cells (CD4+ DO11.10+) at the indicated time points. Day 0 represents cytokine production in naive controls. Data are compiled from 5 experiments (5–10 mice/group) and shown is the fold change for sOVA Rag2−/− hosts comparing the early and late time points. B. Adoptive transfers were performed as in A. CD4+ DO11.10 CD44high donor cells were purified by high-speed cell sorting and mRNA levels quantified by real-time PCR. Data are representative of four experiments and are presented as the fold increase (x > 1) or decrease (x < 1) relative to naive controls (x = 1). Shown is the fold change for the indicated mRNAs comparing the early and late time points. C. Adoptive transfers and restimulations are performed as in A. Dot plots indicate the percentage of donor T cells producing IL-17A and or IFN-γ/IL-17F in sOVA Rag2−/− hosts. Bottom panels, Histograms denote the percentage of donor T cells expressing detectable levels of IL-17F (see Supplemental Fig. 1 for additional IL-17F analysis). D. IL-17A/IFN-γ and IL-17A/IL-17F coexpression data are compiled from three to four independent experiments.
over time, these double-positive cells declined with similar kinetics to IL-17A single-positive counterparts (Fig. 1C,1D). We also found that, in both sOVA Rag2−/− and Rag2−/− hosts, most IL-17A+ donor cells coexpressed IL-17F. However, there was a notable difference in the flow cytometry for IL-17A and IL-17F, with the former exhibiting a bimodal distribution and the latter exhibiting a more unimodal distribution and a whole-sale population shift rather than a clear demarcation between positive and negative events (Fig. 1C, bottom panels). Whether this reflects the actual protein output or a technical issue (i.e., Ab affinity) remains unknown, but given this caveat, we performed a detailed analysis to better gauge IL-17F production. This examination revealed that, whereas cells expressing low levels IL-17F+ cells were better visualized using histograms than dot plots, all of the observed trends, including time-dependent inhibition and coexpression with IL-17A, were similar between these two types of analysis (Supplemental Fig. 1). In addition, both show that, although most IL-17A+ cells (>60%) expressed detectable levels of IL-17F, there were some that did not, which is consistent with published reports on the heterogeneity of Th17 cells (10, 41, 42).

Taken together, these studies demonstrate that, when primed under lymphopenic conditions, helper T cells can differentiate into “classic” Th1 and Th17 effectors, as well as a hybrid subset that shares the defining characteristics of both. They also suggest a hierarchy, with IFN-γ-producing Th1 cells eventually dominating over IL-17–producing “Th17-like” subsets.

T-bet and STAT1 limit Th17 responses during systemic autoimmune disease

To investigate the role of IFN-γ in limiting Th17 responses, we crossed both our donor and recipient mouse strains onto an IFN-γ–deficient background and performed adoptive transfer experiments with each of the four possible donor/host combinations. We found that host-derived IFN-γ had the greatest impact, with production of IL-17A and IL-17F greater in IFN-γ–deficient hosts than in WT sOVA Rag2−/− counterparts (Fig. 2A, 2B, and Supplemental Fig. 1). A similar trend was observed when donor T cells were transferred into IFN-γ–deficient Rag2−/− mice only; in this study, a combination host and donor-derived IFN-γ was required to limit “homeostatic” Th17 responses (Fig. 2C and Sup-

FIGURE 2. T cell and non-T cell-derived IFN-γ can limit Th17 responses during systemic autoimmune disease. A, Naive CD4+ T cells from WT or IFN-γ−/− DO11.10 donors were adoptively transferred into either WT or IFN-γ−/− sOVA Rag2−/− hosts. Shown is the percentage of cytokine-positive donor cells after 7 d. B, Data are compiled from three experiments (see Supplemental Fig. 1 for additional IL-17F analysis). C, Naive CD4+ T cells from WT or IFN-γ−/− Rag2−/− DO11.10 donors were adoptively transferred into either WT or IFN-γ−/− Rag2−/− hosts (no Ag). Shown is the percentage of cytokine-positive donor cells compiled from 3 experiments (n = 3–6 mice/group). B and C, Single asterisk denotes significant differences between the indicated group and the WT into WT group. Double asterisk denotes significant differences between the indicated group and the WT into IFN-γ−/− group. p < 0.05.
Aside from IL-17 production, there were other notable differences in the behavior of STAT1- and T-bet–deficient donors in sOVA Rag2\(^{-/-}\) hosts. Whether T-bet\(^{-/-}\) or T-bet\(^{+/+}\), STAT1-deficient T cells did not proliferate to the same extent as either WT or T-bet\(^{-/-}\) counterparts (Fig. 4B). Unlike the hyper-Th17 phenotype, this hypoproliferative phenotype cannot be explained by the loss of IFN-γ since, paradoxically, donor T cells were hyper-proliferative in the absence of IFN-γ (Fig. 4A). Compared to T-bet\(^{-/-}\) counterparts, STAT1\(^{-/-}\) donors also produced more IL-4 and IL-13, two known Th17 inhibitors (Fig. 4C) (7, 8, 43). To determine the impact of this increased Th2-type response, we compared IL-17 production from donors lacking STAT1 with that of donors lacking STAT1 and IL-4Rα, a shared receptor component for IL-4 and IL-13. Relative to STAT1-deficient counterparts, the compound loss STAT1 and IL-4Rα had little effect on IL-17 but did lead to significant reductions in IL-4 and IL-13 (data not shown).

**Direct evidence for both T-bet– and STAT1-dependent regulation of Th17 responses**

To complement our in vivo studies, we used an in vitro model where Th17 differentiation could be monitored in the presence or absence of STAT1-activating cytokines. As expected, we found that WT T cells produced IFN-γ and almost no IL-17 in nonpolarizing cultures. The inverse was true in Th17-polarizing cultures, where they produced little IFN-γ and significantly more IL-17 (Fig. 5A). Recombinant IFN-γ had little impact on WT T cells, whereas IL-27 had dramatic effects, inducing IFN-γ production and suppressing Th17-type cytokines (Fig. 5A, 5B). IFN-γ–deficient T cells produced almost twice as much IL-17 as WT counterparts under Th17 conditions, and in this case, both IFN-γ and IL-27 could suppress the Th17 response (Fig. 5A, 5B). Given that IFN-γ could suppress IL-17 production only in an IFN-γ–deficient

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**FIGURE 3.** T-bet– and STAT1-deficient T cells exhibit a hyper-Th17 phenotype during systemic autoimmune disease. A, Naive T cells were purified from WT, T-bet\(^{-/-}\), STAT1\(^{-/-}\), or T-bet\(^{-/-}\)× STAT1\(^{-/-}\) DO11.10 mice and adoptively transferred into WT sOVA Rag2\(^{-/-}\) hosts. Shown is the percentage of cytokine-positive donor cells (CD4\(^{+}\) DO11.10^+) on day 7 posttransfer. B, Data are compiled from three to five experiments. Single asterisk denotes statistically significant differences between the indicated group and WT donors. Double asterisk denotes significant differences between the indicated group and T-bet\(^{-/-}\) donors. \(p < 0.05\). See Supplemental Fig. 1 for additional IL-17F analysis.
setting, these data imply that IFN-γ responsiveness is rapidly saturated by autocrine IFN-γ production in WT cells. In contrast, because IL-27 could suppress in either WT or IFN-γ−/− cultures, these data also suggest that its anti-Th17 capacity is independent of its pro-Th1 capacity and, perhaps, hint at a level of cooperation, with IL-27 retaining the ability to suppress IL-17 production when cells become insensitive to IFN-γ.

As with the in vivo model, T-bet− and STAT1-deficient T cells exhibited a hypo-Th1, hyper-Th17 phenotype during in vitro differentiation. Under Th17 polarizing conditions, they each produced

FIGURE 4. Phenotypic differences between T-bet− and STAT1-deficient donor T cells in sOVA Rag2−/− mice. A, Naive T cells were purified from either WT or IFN-γ−/− DO11.10 mice and then adoptively transferred into either WT or IFN-γ−/− sOVA Rag2−/− hosts. At 7 d posttransfer, lymphocytes were stained directly ex vivo for flow cytometry. Shown are the percentages of total and activated donor T cells (CD4+ DO11.10+, CD25+ or CD44hi). B, Naive T cells were purified from WT, T-bet−/−, STAT1−/−, or T-bet−/− × STAT1−/− DO11 mice and transferred into sOVA Rag2−/− hosts. Shown are the percentages of total and activated donor T cells after 7 d. A and B, Data are compiled from three individual experiments. An asterisk denotes statistically significant differences between the indicated group and WT controls, p < 0.05. C, Adoptive transfers were performed as in B. At 7 d posttransfer, lymphocytes were restimulated and stained for IFC. Shown are the percentages of IL-4+ and IL-13+ donor T cells (gated on CD4+ DO11.10 TCR+). Data are representative of three individual experiments.

FIGURE 5. STAT1-activating cytokines fail to suppress Th17 responses in the absence of STAT1. A, CD4+ T cells were purified from WT, IFN-γ−/−, T-bet−/−, and STAT1−/− mice. These were cultured under nonpolarizing or Th17 conditions for 72 h and shown is the percentage of cytokine-positive cells (CD4+). B, Data are compiled from three to five experiments (Th17 conditions) and are presented as the log2 fold change in cytokine-positive cells on exposure to IFN-γ (black bars) or IL-27 (white bars). Untreated groups have a relative value of 0, whereas cytokine-treated groups have values >0 or <0, depending on whether they have an enhancing (x >0) or inhibitory effect (x <0). Gray area denotes log2 values that are >−1 but <1. Error bars represent the SD between all fold changes for each group. Asterisk denotes >2-fold, statistically significant differences between the indicated group and corresponding Th17 controls, p < 0.05.
more IL-17 than WT counterparts, but again, T-bet deficiency led to a greater increase, and when both TFs were deleted, the double-deficient cells behaved like STAT1−/− counterparts (Fig. 5A, 5B). IFN-γ had a muted effect in T-bet-deficient cultures, prompting only small reductions in IL-17A and IL-17F. IL-27 was more potent, leading to >2-fold reductions in both, though it should be noted that the level of inhibition was less than what was observed in WT or IFN-γ−/− cultures. Neither IFN-γ nor IL-27 impacted IL-17 production in STAT1-deficient cultures, whether T-bet deficient or sufficient, thereby illustrating the central role of STAT1 in this process (Fig. 5A, 5B).

Consistent with our protein measurements, exogenous IFN-γ had little effect on IL-17A and IL-17F mRNA levels in WT cells but suppressed both transcripts in IFN-γ−/− T cells (Fig. 6). A similar trend was observed for IL-22, another Th17-associated cytokine, and for RORγT and RORα, two key Th17-associated TFs (Fig. 6). As before, IL-27 displayed potent activity in either WT or IFN-γ−/− cells, suppressing IL-17A, IL-17F, and other Th17-associated transcripts, whereas at the same time promoting Th1-associated transcripts. IFN-γ had little effect on Th17-associated mRNAs in T-bet-deficient cells, and although IL-27 could still prompt >2-fold reductions, its inhibitory capacity was less in T-bet−/− cells than in WT or IFN-γ−/− counterparts (Fig. 6). As noted earlier, neither IFN-γ nor IL-27 affected Th17-associated transcripts in STAT1-deficient cells (Fig. 4). Also noteworthy, and consistent with a recent report (44), IL-27 was a powerful STAT1 and T-bet-independent inducer of IL-21 mRNA (Fig. 6). Together with our flow cytometry studies, these data confirm that STAT1-dependent pathways are critical for limiting Th17 responses. In addition, given the reduced capacity of STAT1-activating cytokines to suppress in T-bet-deficient cells, they also suggest a T-bet-dependent, STAT1-independent pathway.

To directly test whether T-bet can suppress Th17 responses independently of STAT1, we used retroviral gene transduction to overexpress T-bet and/or RORγT in a STAT1-deficient setting (Supplemental Fig. 2). As expected, we found that ectopic RORγT was a potent Th17 stimulus, prompting a dramatic increase in the percentage of IL-17+ cells whether in T-bet−, STAT1−, or double-deficient T cells (Fig. 7A–C). However, when T-bet was also introduced, RORγT-driven IL-17 production was significantly decreased, and when these “double-infected” cells were cultured without neutralizing anti–IFN-γ mAb, there was a further reduction in T-bet-deficient but not STAT1-deficient cells. These latter findings establish that T-bet can suppress Th17 responses independently of STAT1, and that, in WT cells, STAT1- and T-bet-mediated pathways may cooperate in achieving this shared function.

Consistent with our protein measurements, we also found that ectopic T-bet had significant impact on RORγT-driven Th17 responses at the mRNA level, prompting reduced expression of IL-
17A, IL-17F, and IL-22 in both T-bet−/− and STAT1−/− cells. Removing the anti–IFN-γ led to further reductions only in T-bet−/− cells, which, again, hints at a degree of cooperation between T-bet and STAT1 in this process (Fig. 8A, 8B). We also noted that the ability of T-bet to suppress RORγT and RORα was modest by comparison, and that it had little influence, positive or negative, on expression of IL-23R. Thus, although T-bet clearly influences the output of Th17-type cytokines, it does not appear to do so by “locking down” transcription of ROR family TFs.

Discussion

In the preceding studies, we used in vivo and in vitro models of T cell differentiation to establish that, downstream of IFN-γ, IL-27 and likely all STAT1-activating cytokines, there are both STAT1- and T-bet–dependent mechanisms capable of suppressing Th17 responses. As evidence for STAT1-mediated inhibition, and consistent with published reports (8, 9, 29–31), we demonstrate that STAT1-deficient T cells exhibit a hyper-Th17 phenotype and are refractory to the anti-Th17 effects of IFN-γ and IL-27. As evidence for T-bet–mediated inhibition, we demonstrate that T-bet–deficient T cells also exhibit a hyper-Th17 phenotype, that T-bet deficiency hinders the ability of STAT1-activating cytokines to suppress Th17 responses, and most importantly, that ectopic T-bet expression can suppress Th17 responses in the complete absence of STAT1. Previous studies have also suggested that STAT1 and T-bet might play independent roles in this process, but because T-bet is both upstream (an inducer) and downstream (induced by) of STAT1, they could not definitively exclude the possibility that

![FIGURE 7. Direct evidence for T-bet–dependent regulation of Th17 responses.](image)

![FIGURE 8. T-bet limits transcription of key Th17-associated genes.](image)
T-bet may be arbitrating STAT1-dependent inhibition through its well-known ability to drive IFN-γ production (26, 34). We overcame the “chicken-and-egg” problem by using retroviral vectors to restore T-bet expression in either T-bet or STAT1-deficient T cells, finding that, indeed, there are two pathways at work, with STAT1 not required for T-bet-mediated inhibition and T-bet not required for STAT1-mediated inhibition.

Aside from those operating through STAT1, other cytokines have been implicated in the regulation of Th17 responses, including IL-2 and IL-4, which are known to act primarily through STAT5 and STAT6, respectively. Similar to STAT1 deficiency, genetic ablation of these STATs is associated with increased Th17 responses, but whether this shared outcome is achieved through a common mechanism is yet to be resolved (8, 45). The most direct way that STATs could limit Th17 responses is by binding to promoter/enhancer regions of Th17-associated genes and thereby obstructing the transcriptional machinery. There is some evidence for this, with STAT5 having been shown to bind the promoter of IL-17A, but whether this interaction is what determines the ability of STATs to suppress IL-17 production was not determined (45).

Likewise, STAT1 has been shown to bind upstream of the RORα and RORγ loci in human HELA cells, but the nature of this interaction, be it stimulatory or inhibitory, and whether it happens in primary T cells, are questions that remain unanswered (46). Another, more indirect way that STATs could impact Th17 responses is by inducing or promoting the function of auxiliary anti-Th17 factors. There is strong evidence for this because cytokines with anti-Th17 activity are already known to induce “Th17 inhibitors,” like T-bet, Ets1, and Gfi-1, and this is not likely to be an exhaustive list of indirect targets (47–49). STATs could also influence Th17 responses by interfering with pro-Th17 TFs or signaling pathways, as is the case with the ability of IL-27 to induce expression of SOCS3, which is known to curb STAT3-dependent pathways, as is the case with the ability of IL-27 to induce expression of SOCS3, which is known to curb STAT3-dependent pathways. These pathways was confounded by the wide-ranging effects of STAT1 deficiency.

As with the STATs, T-bet could suppress Th17 responses in a variety of ways. A recent genome-wide mapping of T-bet binding sites did not reveal significant enrichment near the IL-17A, IL-17F, IL-22, or ROR loci, making a direct interaction between T-bet and relevant Th17-associated promoters seem unlikely (52). However, a direct protein–protein interaction between T-bet and pro-Th17 TFs remains a possibility, especially because T-bet is known to interact with and thereby limit the function of other TFs, including GATA-3 and RelA (53, 54). Consistent with this latter point, our studies demonstrate that T-bet can suppress IL-17 production even in the face of ectopic RORγT, which is driven by a retroviral promoter and, thus, is impervious to transcriptional effects. These data suggest a physical interaction between T-bet and elements of the Th17 differentiation machinery, if not RORγT itself, though it should also be noted that T-bet might influence Th17 responses through more indirect means. Adding further complexity, T-bet has a functional homolog, Eomes, which is expressed in T cells and is known to exhibit anti-Th17 activity. Recent studies have shown that ectopic expression of Eomes can suppress IL-17 production, but whether this is due to a cell-intrinsic effect or its ability to drive IFN-γ-mediated suppression was not resolved (35). It is also known that, unlike T-bet and STAT1, genetic ablation of both T-bet and Eomes results in a compound hyper-Th17 phenotype, but again, the increase in IL-17 production was mirrored by a corresponding reduction in Th1 responses, making it unclear whether the phenotype was due to direct effects or a lack of IFN-γ/STAT1-dependent inhibition (26). Based on the data presented in this paper, we propose that both are true: that T-bet and Eomes can each limit Th17 responses through at least two shared mechanisms, one involving STAT1, with IFN-γ as an intermediary, and the other completely STAT1 independent.

Although our findings establish that STAT1 and T-bet influence Th17-type cytokines through genetically distinct pathways, we noted that T cells lacking both TFs did not exhibit an additive, or compound, phenotype. Instead, the hyper-Th17 phenotype of T-bet–deficient cells was always more severe than that of STAT1- or double-deficient counterparts, which, despite the well-known ability of STAT1 to drive T-bet expression, is also inconsistent with the notion that they operate within the same pathway. Taken together, these contradictory observations suggest epistasis, meaning that the loss of STAT1 affects cellular processes that, although not directly related to Th17 differentiation, impact the overall quality of T cell responses, thereby hindering production of Th17-type cytokines. We found that, beyond Th17 responses, T-bet and STAT1-deficient T cells behaved differently in vivo, with the latter exhibiting reduced proliferation and increased Th2-type cytokine production. We also found evidence for epistasis during in vitro differentiation. Those studies confirmed that STAT1 is required for IFN-γ and IL-27 to suppress IL-17 production, but also showed that, compared with WT counterparts, expression of many Th17-type mRNAs was not grossly increased in STAT1-deficient cells, which, perhaps, indicates posttranscriptional effects (Supplemental Fig. 3). Thus, although we can still conclude that STAT1 and T-bet influence Th17 responses through both divergent and convergent mechanisms, it must be noted that genetic dissection of these pathways was confounded by the wide-ranging effects of STAT1 deficiency.

The ability of signature Th1-type factors, like IFN-γ, STAT1, and T-bet, to inhibit signature Th17-type factors, like IL-17, IL-22, and RORγT, has led to the idea that there is an inverse relation between the Th1 and Th17 subsets. However, despite this antagonism, T cells producing IFN-γ and IL-17 are known to occur in multiple inflammatory settings, which suggests a more nuanced relation (2, 10–14). This work illustrates both sides of this paradox. On one hand, we have shown that T cells produce either IFN-γ or IL-17 when primed in vitro; on the other hand, they can produce both when primed in vivo (in highly immunogenic sOVA Rag2−/− mice). We also report that, when T-bet and RORγT were both highly expressed in the same cells, the result is dichotomous, with some cells expressing one cytokine or the other, but rarely both (Fig. 1D, 7D). Based on this last finding, and the fact that T-bet is known to promote long-term Th1 lineage commitment, we propose that “double-positive” T cells represent a transitional phase in a linear progression from IL-17–producing Th17 cell to IFN-γ–producing Th1 cell. Inherent to this hypothesis is the idea that Th17 cells can convert to other subsets, which has strong experimental support (10), and that such conversion is a part of normal immune responses, which is now supported by recent studies demonstrating that Th17-type cytokines are required for the development of Th1 responses during infection (55).

Although Th17 responses can have important, host-protective functions, it is also widely accepted that, when dysregulated, they can promote autoimmune disease. Given the current and prospective use of STAT1-activating cytokines as therapeutics for Th17-associated pathologies, best exemplified by the use of IFN-β to treat multiple sclerosis, it is critical to understand exactly how they
suppress Th17-type inflammation. The studies presented in this paper provide an important piece of mechanistic information, demonstrating that the ability of STAT1 to limit Th17-type responses is intimately linked to T-bet, a TF that is at once a potent anti-Th17 effector and, through its ability to drive IFN-γ production, an essential STAT1 stimulus. Although we have focused on CD4+ T cells, which we interrogated in a select few model systems, this relation between STAT1 and T-bet is likely to impact other IL-17–producing lineages, such as CD8+ T cells or NKT cells, and is likely to influence Th17 responses in a variety of immune and autoimmune settings, making these pathways acutely relevant in the context of inflammatory disease cause and cytokine-based drug design.

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Disclosures

A.V.V. has a patent pending on the anti-inflammatory properties of IL-27. The other authors have no financial conflicts of interest.

References


STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and independent mechanisms

Alejandro V. Villarino, Eugenio Gallo and Abul K. Abbas

Supplemental Figure Legends

Supplemental Figure 1. Analysis of donor T cell IL-17F production. (A) Naïve DO11.10 CD4⁺ T cells were transferred into Rag2/-/- or sOva Rag2/-/- hosts. 3-9 days later, lymphocytes from recipient mice were re-stimulated and IL-17F production measured by intracellular flow cytometry. Open histograms denote IL-17F levels in donor cells (CD4⁺ DO11.10⁺) while grey histogram denote naïve controls. Right panel: Data are compiled from 4 experiments (4-8 mice/group). Star indicates statistically significant differences between early (days 3-4) and late (days 7-9) time points (p<0.05). (B) Adoptive transfers and re-stimulations were performed as in (A). Shown is IL-17F production in either IL-17A⁻ (grey histograms) or IL-17A⁺ (open histograms) donor T cells. (C) Adoptive transfers were performed as in Figure 2A. Open histograms denote IL-17F levels in donor cells (CD4⁺ DO11.10⁺) while grey histogram denote naïve controls. (D) IL-17F was measured in total and IL-17A⁺/- donor T cells from WT and IFN-γ/- sOva Rag2/-/- (left) or Rag2/-/- hosts (right). Data are compiled from 3 experiments. (E) Adoptive transfers were performed as in Figure 3. Open histograms denote IL-17F levels in donor cells (CD4⁺ DO11.10⁺) while grey histogram denote naïve controls. Lower panel: Data are compiled from 3 experiments. (D-E) One star denotes significant differences between the indicated group and WT controls.

Supplemental Figure 2. Host-derived IFN-γ production in sOva Rag2/-/- mice. Naïve T cells were purified from either WT or IFN-γ/- DO11.10 mice and then adoptively transferred into
either WT or IFN-\(\gamma\)-/- sOva Rag2-/ hosts. At 7 days post-transfer, lymphocytes were re-stimulated and cytokine production measured as in Figure 1. Shown are the percentages of TNF-\(\alpha^+\) and IFN-\(\gamma^+\) cells in the non-T cell fraction (CD4- DO11.10 TCR`). Data are representative of >5 individual experiments.

**Supplemental Figure 3. PCR analysis of Th17-associated genes in T-bet and STAT1-deficient T cells.** Naïve T cells were cultured under non-polarizing or Th17 conditions (+/- IFN-\(\gamma\) or IL-17) and PCR used to measure the indicated transcripts. Data are color (by genotype) and are presented as the fold increase (X>1) or decrease (X<1) relative to WT controls (X=1). Graphs are representative of 3 experiments and error bars represent the standard deviation within replicate measurements.

**Supplemental Figure 4. Purifying retrovirally transduced T cells.** T-bet-deficient T cells were cultured under non-polarizing conditions and transduced with retroviral vectors expressing T-bet (marked by GFP), ROR\(\gamma T\) (marked by Thy1.1), and or ‘empty’ GFP or Thy1.1 controls. 72 hours later, cells were stained for high-speed cell sorting and those infected with two viral vectors were purified (CD4+ GFP+ Thy1.1+; upper right quadrant). Shown are representative data from one of 3 independent experiments.
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Villarino et al. Supplemental Figure 4
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<th>Gene</th>
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<th>3' Primer (Anti-Sense)</th>
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* Primer set does not distinguish between the γ and γt isoforms produced by RORc locus

** Primer set amplifies only endogenous RORγt transcripts (not retro-viral transcripts)

*** Primer set amplifies only RORα transcript variant 4 (Yang et al., Immunity 2008)