A STAT3-dependent transcriptional circuitry inhibits cytotoxic gene expression in T cells

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CD8$^+$ T cells are preprogrammed for cytotoxic differentiation in the thymus as they acquire expression of the transcription factor Runx3. However, a subset of effector CD8$^+$ T cells (Tc17) produce IL-17 and fail to express cytotoxic genes. Here, we show that the transcription factors directing IL-17 production, STAT3 and RORγt, inhibit cytotoxicity despite persistent Runx3 expression. Cytotoxic gene repression did not require the transcription factor Thpok, which in CD4$^+$ T cells restrains Runx3 functions and cytotoxicity; and STAT3 restrained cytotoxic gene expression in CD8$^+$ T cells responding to viral infection in vivo. STAT3-induced RORγt represses cytotoxic genes by inhibiting the functions but not the expression of the "cytotoxic" transcription factors T-bet and Eomesodermin. Thus, the transcriptional circuitry directing IL-17 expression inhibits cytotoxic functions. However, by allowing expression of activators of the cytotoxic program, this inhibitory mechanism contributes to the instability of IL-17–producing T cells.

CD8 T cells | cytotoxicity | IL-17 | STAT3 signaling

T cells are essential to fight intracellular pathogens, including viruses, bacteria, and protozoans. MHC I-restricted CD8$^+$ T cells differentiate into cytotoxic (Tc1) effectors that produce the cytokine IFNγ and cytolytic molecules, including perforin and granzymes (1). Whereas acquisition of cytotoxic functions is not typical of MHC II-restricted CD4$^+$ T cells, IFNγ secretion by Th1 CD4$^+$ effector T cells is essential to combat intracellular pathogens (2). The differentiation of both Tc1 (CD8$^+$) and Th1 (CD4$^+$) T cells involves the transcription factor Runx3 and the T-box factors T-bet or Eomesodermin (Eomes). Runx3 is up-regulated during the differentiation of MHC I-restricted T cells in the thymus (3, 4) and remains expressed in postthymic resting and activated CD8$^+$ T cells (5). Although not expressed in naïve CD4$^+$ T cells, Runx3 is induced in differentiating CD4$^+$ Th1 effectors (6, 7). While neither T-bet nor Eomes are expressed in resting T cells, they are up-regulated in differentiating Th1 and Tc1 effectors, in which they sustain production of IFNγ and cytotoxic molecules (2, 8, 9).

CD4$^+$ T cells are also involved in the control of extracellular microbes, including bacteria, yeast, and fungi, through their production of IL-17 and related cytokines (10, 11). The differentiation of IL-17–producing CD4$^+$ T cells (Th17) requires the transcription factors STAT3 and RORγt (12–15). There is evidence that the transcriptional circuitry directing IFNγ and cytotoxic gene expression in Th1 or Tc1 cells inhibits Th17-related gene expression (16). Mechanistically, T-bet and Eomes directly antagonize the expression of RORγt (17–19) and thereby restrain IL-17 production.

Because MHC I molecules typically present peptide antigens synthesized intracellularly, it had been considered that CD8$^+$ T cells were not involved in IL-17–mediated control of extracellular pathogens. Nonetheless, CD8$^+$ T cells producing IL-17 (Tc17) are found at effector sites both in humans and in experimental models, and there is evidence that such cells have potential pathogenic properties (20–23). Moreover, the differentiation of Tc17 cells involves STAT3 and RORγt, as does that of Th17 CD4$^+$ effectors (20, 24). This indicates that a common transcriptional circuitry, called "$T_{eff}17$" hereafter, directs IL-17 production in Th17 and Tc17 cells.

It was noted that Tc17 cells show reduced cytotoxic activity and cytotoxic gene expression relative to Tc1 cells (20, 24, 25). However, how this is achieved has not been investigated. Here, we demonstrate that repression of cytotoxic genes is an intrinsic property of the $T_{eff}17$ circuitry, which we show acts in Tc17 CD8$^+$ T cells by inhibiting the function but not the expression of Runx3. Such inhibition depends on the transcription factor STAT3, in part through its ability to promote RORγt expression. Accordingly, the $T_{eff}17$ circuitry represses cytotoxic genes independently of Thpok in CD4$^+$ T cells. Last, we show that RORγt itself restrains the activation of cytotoxic genes but fails to inhibit the expression of T-bet or Eomes. We propose that such persistent expression of key activators of cytotoxic differentiation contributes to the instability of IL-17–producing T cells.

Results

$T_{eff}17$ Transcriptional Circuitry Represses Cytotoxic Functions Despite Persistent Runx3 Expression. Upon antigen stimulation, naïve CD8$^+$ T cells typically differentiate into Tc1 killer cells that express molecules essential for cytotoxicity, including perforin, granzymes A, B, and K, and the cytokine IFNγ. In contrast, CD8$^+$ T cells signaled with TGF-β and IL-6 (Tc17 culture conditions) produce IL-17 and show little if any cytotoxic activity (Fig. 1*A* and Fig. S1*A*). Of note, CD8$^+$ T cells activated in the presence of either TGF-β or IL-6 alone maintained cytotoxic activity (Fig. S1*B*), suggesting that repression of cytotoxic differentiation is characteristic of the $T_{eff}17$ transcriptional circuitry, rather than resulting from signaling by either cytokine.

To determine the impact of the $T_{eff}17$ transcriptional circuitry on the cytotoxic program, we compared gene expression in Tc1 vs. Tc17 CD8$^+$ T cells by microarray analyses. We identified

Significance

The acquisition of cytotoxic function by CD8$^+$ T cells is critical for antiviral and antitumor responses. While cytotoxic differentiation is preprogrammed during CD8$^+$ T-cell development in the thymus, the regulation of T-cell cytotoxic capacities by inflammatory cues is poorly understood, notably in cases of immune dysfunction observed in tumor-infiltrating lymphocytes or during chronic infections. Here, we demonstrate that the program underlying IL-17 production dampens cytotoxic function in both CD4$^+$ and CD8$^+$ T cells. Specifically, we show that two transcription factors involved in IL-17 production, STAT3 and RORγt, repress cytotoxic differentiation. These results highlight the role of the inflammatory environment on T-cell responses and have implications for the development of T cell-based immunotherapies.

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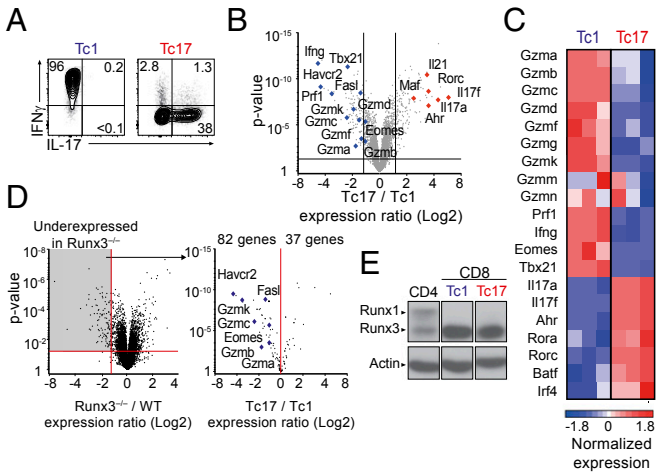

Fig. 1. The Tc17 transcriptional program represses cytotoxic functions. (*A*) Contour plots of IL-17 vs. IFNγ expression on CD8$^+$ T cells cultured under Tc1 and Tc17 conditions. (*B*) Volcano plot displays Tc17/Tc1 expression ratios ($\log_2$ values, full gene set) vs. *P* values; each symbol represents a distinct gene. Relevant genes are indicated. Data are from three replicates. Lines represent 1.5-fold change, *P* value 0.05. (*C*) Heatmap displays normalized expression on selected genes in Tc1 and Tc17 cells (Z score, color scale at *Bottom*). Data are from three replicates. (*D*) Volcano plot (*Left*) displays expression ratios ($\log_2$ values, full gene set) vs. *P* values of differential expression in *Runx3*$^{-/-}$ over wild-type CD8$^+$ T cells; original data are from ref. 26. The *Right* volcano plot displays Tc17/Tc1 expression ratio vs. *P* values of differential expression for genes significantly underexpressed in *Runx3*$^{-/-}$ cells (1.5-fold change, $P < 0.05$, gray shading on *Left* plot). Each symbol represents a gene; relevant genes are indicated. (*E*) Immunoblot analyses of Runx protein expression in effector CD4$^+$ (ThN) or CD8$^+$ T cells cultured under Tc1 or Tc17 conditions. Data are representative of five (*A*) or two (*B*–*D*) mice analyzed in four (*A*) or two (*B*–*D*) independent experiments.

269 genes differentially expressed (1.5-fold change, $P < 0.05$) between these two subsets (Fig. 1*B*). Consistent with previous reports (21, 24), expression of genes associated with IL-17 production, such as *Il17a*, *Il17f*, *Rorc* (encoding RORγt), and *Ahr*, was higher in Tc17 than Tc1 cells (Fig. 1*C*). Strikingly, we found that Tc17 differentiation was associated with a broad repression of the cytotoxic program, including genes encoding T-bet (*Tbx21*, called *T-bet* here), Eomes (*Eomes*), and cytotoxic molecules (Fig. 1*C*). Quantitative RT-PCR (qPCR) experiments confirmed lower expression in Tc17 than in Tc1 cells of genes encoding granzymes A and B, and perforin (*Gzma*, *Gzmb*, and *Prf1*, respectively) (Fig. S1*C*). These observations suggest that the transcriptional circuitry involved in Tc17 differentiation broadly inhibits cytotoxic gene expression.

The transcription factor Runx3 promotes cytotoxic gene expression and IFNγ production in CD8$^+$ effector T cells (5); in addition, both Runx3 and the related protein Runx1 promote the production of IFNγ by "pathogenic" Th17 CD4$^+$ T cells (17, 18). Given that Tc17 effectors expressed neither IFNγ nor cytotoxic genes, we predicted that they would express little or no Runx3. Consistent with this idea, many previously identified Runx3-dependent genes (Fig. 1*D*, *Left*) (26) were underexpressed in Tc17 compared with Tc1 cells (Fig. 1*D*, *Right*), including canonical cytotoxic genes *Gzma*, *Gzmb*, *Gzmc*, *Fasl*, or *Havcr2* (encoding Tim-3). However, and contrary to the prediction, immunoblot analyses showed equivalent amounts of Runx3 protein in Tc1 and Tc17 cells (Fig. 1*E*); importantly, Runx1 was not detected in either subset. These findings indicate that the $T_{eff}17$ transcriptional circuitry inhibits Runx3-dependent expression of cytotoxic genes without affecting the expression of Runx3 itself.

The transcription factor Thpok antagonizes Runx-mediated expression of cytotoxic genes in CD4$^+$ T cells and is expressed, although at modest levels, in activated CD8$^+$ T cells (27–30). Thus, we considered the possibility that Thpok may contribute to cytotoxic gene repression in Tc17 cells. To address this, we assessed wild-type (WT) and Thpok-deficient Tc17 effector cells for the expression of granzyme B, a sensitive marker of Thpok repression in both CD4$^+$ and CD8$^+$ T cells (28, 31). To ensure that Tc17 effectors were MHC I restricted, they were derived from naïve CD8$^+$ T cells obtained from *Cd4*-cre$^+$ *Thpok*$^{fl/fl}$ mice expressing the MHC I-restricted P14 transgenic TCR. Thpok disruption did not increase granzyme B expression (Fig. S1*D*), supporting the conclusion that the transcriptional circuitry of Tc17 cells overcomes Runx3-mediated activation of the cytotoxic program independently of Thpok.

Stat3 Represses Cytotoxic Gene Expression. The preceding findings suggested that repression of the cytotoxic program was integral to the $T_{eff}17$ transcriptional circuitry. Because the transcription factor STAT3, activated by IL-6, is required for the differentiation of both Th17 and Tc17 cells (20, 32), we examined whether it represses cytotoxic gene expression. We differentiated CD8$^+$ T cells from *Cd4*-cre$^+$ *Stat3*$^{fl/fl}$ mice (called here *Stat3*$^{-/-}$) under Tc17 conditions. To avoid noncell-intrinsic effects, we compared *Stat3*$^{-/-}$ and wild-type CD8$^+$ T cells cocultured in the same environment (Fig. S2*A*). Unlike control cells in the same coculture, *Stat3*-deficient CD8$^+$ T cells failed to produce IL-17, and they displayed increased granzyme B expression (Fig. 2*A*), suggesting that STAT3 represses cytotoxic genes. To further evaluate this possibility, we performed microarrays on RNAs prepared from *Stat3*$^{-/-}$ and wild-type CD8$^+$ T cells purified after coculture in Tc17 conditions. In parallel, we analyzed RNAs from wild-type and *Stat3*$^{-/-}$ CD8$^+$ T cells cocultured in Tc1 conditions. Gene expression in *Stat3*$^{-/-}$ Tc17 cells was highly similar to that in wild-type Tc1 cells (Fig. 2*B*). Specifically, *Stat3*$^{-/-}$ Tc17 cells were skewed

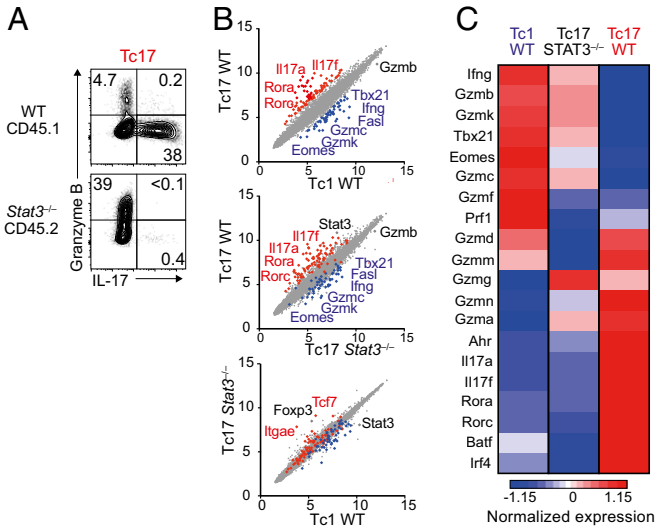

Fig. 2. STAT3 represses cytotoxic gene expression in CD8$^+$ T cells. (*A*) Contour plots of IL-17 vs. granzyme B intracellular expression in CD8$^+$ T cells cocultured under Tc17 conditions as shown in Fig. S2*A*. Data are gated on WT CD45.1$^+$ or *Stat3*$^{-/-}$ CD45.2$^+$ cells and are representative of three mice per genotype analyzed in three independent experiments. (*B*) Scatterplots show microarray gene expression ($\log_2$ values, full gene set) in indicated cell populations after sorting from mixed cultures set as in Fig. S2*A*. Genes with 1.5-fold or greater expression change in wild-type Tc17 vs. Tc1 cells ($P < 0.05$) are defined in the *Top* plot and shown in red and blue in all three plots. Relevant genes are indicated. Data are from three replicates. (*C*) Heatmap displays normalized expression on selected genes in Tc1 WT, Tc17 WT, and Tc17 *Stat3*$^{-/-}$ cells (Z score, color scale at *Bottom*). Data are from three replicates.

toward expression of cytotoxic genes, including those encoding granzymes B, C, and K, T-bet, and Eomes, in addition to their impaired expression of canonical Tc17 genes, including *Rorc*, *Il17a*, or *Il17f* (Fig. 2*C*). In contrast STAT3 disruption had no detectable effect on the transcriptome of in vitro Tc1 effectors, which display high-level expression of cytotoxic genes (Fig. S2*B*).

To examine whether STAT3 restrains cytotoxic gene expression in vivo, we evaluated the response of STAT3-deficient $CD8^+$ T cells during infection by the Armstrong strain of lymphocytic choriomeningitis virus (LCMV). While LCMV Armstrong is cleared by a strong cytotoxic $CD8^+$ T-cell response (16, 33), it causes acute IL-6 production (34), allowing us to assess the potential impact of STAT3 activation on cytotoxic genes. Consistent with our hypothesis, disruption of *Stat3* increased Eomes expression and IFNγ production in effector $CD8^+$ T cells at the peak of the LCMV response (Fig. 3 *A* and *B*); analyses in mixed bone-marrow chimeras (*Stat3* deficient: wild type; 1:1) showed that this effect is cell intrinsic (Fig. S2 *C* and *D*).

This suggested that STAT3 represses cytotoxic genes in vivo. Accordingly, we speculated that ectopic activation of STAT3 in Tc1 cells should counteract their cytotoxic differentiation. To test this, we used a Cre-inducible allele (*Rosa26*$^{Stat3C\text{-}GFP}$) in which the *Rosa26* locus contains a floxed transcription termination site followed by a bicistronic insert encoding both a constitutively active version of STAT3 (STAT3C) and GFP as a reporter for Cre expression (35). To avoid constitutive STAT3 activity in developing thymocytes and resting T cells, we generated *Rosa26*$^{Stat3C\text{-}GFP/+}$ mice carrying *Ox40*-cre, which is expressed in 10–15% of effector $CD8^+$ T cells after LCMV infection (Fig. S2*E*) but not in naïve $CD8^+$ T cells. As controls, we used *Rosa26*$^{YFP/+}$*Ox40*-cre mice, in which YFP identifies cells with a history of Cre expression (Fig. S2*E*). In LCMV-infected *Rosa26*$^{Stat3C\text{-}GFP/+}$ *Ox40*-cre$^+$ mice, expression of STAT3C resulted in a significant inhibition of canonical Tc1 markers, as shown by the reduced frequency of cells expressing granzyme B, IFNγ, T-bet, and Eomes (Fig. 3 *C* and *D*). Thus, both loss- and gain-of-function experiments support the conclusion that STAT3 inhibits cytotoxic gene expression in $CD8^+$ T in vivo.

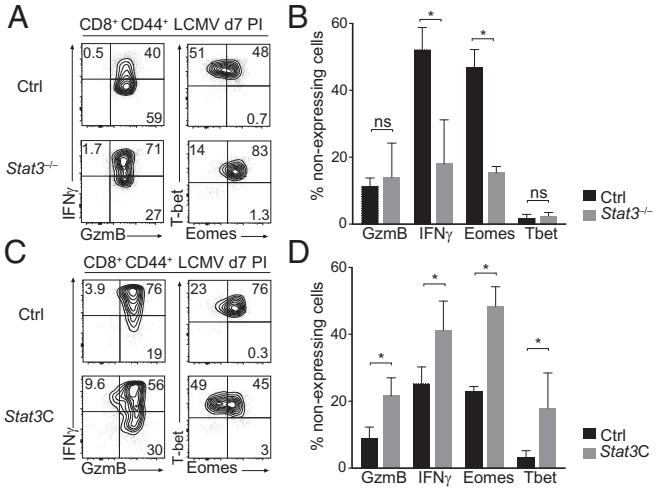

Fig. 3. STAT3 opposes $CD8^+$ T cell cytotoxic differentiation in vivo. (*A* and *B*) $CD8^+$ $CD44^+$ T cells were sorted from the spleen of *Stat3*$^{-/-}$ or control mice 7 d after LCMV infection. (*A*) Contour plots show intracellular expression of granzyme B vs. IFNγ (*Left*) and Eomes vs. T-bet (*Right*) in $CD44^{hi}$ $CD8^+$ T cells. (*B*) Percentage of cells with no detectable expression of the indicated protein among $CD8^+$ T cells. (*C* and *D*) GFP^+ or YFP^+ $CD8^+$ $CD44^{hi}$ T cells were sorted from the spleen of *Ox40*-cre$^+$ *Rosa26*$^{Stat3C\text{-}GFP}$ (*Stat3C*) or *Ox40*-cre$^+$ *Rosa26*^{YFP} (Ctrl) animals 7 d after LCMV infection. (*C*) Contour plots show intracellular expression of granzyme B vs. IFNγ (*Left*) or Eomes vs. T-bet (*Right*). (*D*) Percentage of cells with no detectable expression of the indicated protein among $CD8^+$ T cells. Note that, in control animals, a greater fraction of YFP^+ $CD8^+$ cells express granzyme B and IFNγ, relative to YFP^- $CD8^+$ cells in the same mouse (which represent the vast majority of $CD8^+$ responders) (Fig. S2*E*). This is consistent with the preferential expression of Ox40 on highly activated $CD8^+$ T cells (73). (*A*–*D*) Data are representative of two independent experiments, each with two mice of each genotype. $*P < 0.05$; ns, not significant.

STAT3 Target RORγt Represses Cytotoxic Effector Genes. In addition to STAT3, expression of $T_{eff}17$ genes involves the transcription factors Irf4, Batf, and RORγt (13, 36–40). Both Irf4 and Batf are expressed in Tc1 cells and promote IFNγ and cytotoxic gene expression in vivo during viral infection (41–44). In contrast, RORγt is specific to the $T_{eff}17$ program. Because STAT3 promotes RORγt expression, we considered the possibility that RORγt would repress cytotoxic genes. To evaluate this, we expressed RORγt in *Stat3*$^{-/-}$ $CD8^+$ T cells cocultured with WT $CD8^+$ T cells under Tc17 conditions. Enforced RORγt expression failed to restore IL-17 production to wild-type levels, but strongly repressed *Gzmk* and to a lesser extent *Gzmb* (Fig. 4 *A* and *B*). However, even though it inhibited expression of IFNγ, a prototypical T-bet target (Fig. 4*C*), RORγt failed to affect expression of *T-bet* or *Eomes*, the "master regulators" of cytotoxic genes (Fig. 4*D*). Consistent with these results, reanalysis of previously published ChIP-seq data from Th17 $CD4^+$ T cells detected STAT3 binding at *T-bet*, but little or no binding at *Ifng*, *Gzmb*, and *Gzmk*, which were bound by RORγt (Fig. S3). Of note, RORγt binding sites also recruited T-bet in Th1 cells (45) (Fig. S3).

This suggested that RORγt inhibits the function of T-bet or Eomes rather than their expression and prompted us to examine whether ectopic expression of RORγt in WT Tc1 cells, which express T-bet and Eomes, would dampen the cytotoxic program. Indeed, retroviral RORγt transduction impaired both granzyme B and IFNγ expression in wild-type Tc1 $CD8^+$ effectors (Fig. 4 *E* and *F*). We conclude from these experiments that RORγt inhibits cytotoxic differentiation at least in part independently of STAT3, and that it acts by restraining the function but not the expression of T-bet and Eomes.

$T_{eff}17$ Effector Program Represses Cytotoxic Differentiation in $CD4^+$ T Cells. While $CD8^+$ T cells are preprogrammed for cytotoxic differentiation, $CD4^+$ T cells are preprogrammed to repress cytotoxic genes by their expression of Thpok, which inhibits Runx3 expression and functions (28, 29, 46–48). Accordingly, we previously showed that postthymic Thpok deletion diverts Th1 and Th2 $CD4^+$ effectors toward cytotoxic differentiation (29). However, the preceding findings raised the possibility that Th17 $CD4^+$ T cells, which also express STAT3 and RORγt (12–14, 49), would restrain cytotoxic gene expression independently of Thpok. We addressed this question by activating naïve $CD4^+$ T cells from *Ox40*-cre$^+$ *Thpok*$^{fl/fl}$ mice (called *Thpok*^{AD} for "activation deleted") in which *Thpok* disruption occurs during $CD4^+$ T cell activation (50, 51). In line with previous results (29, 52), Thpok disruption did not impair IL-17 production (Fig. S4*A*). Importantly, Thpok was dispensable for the repression of *Prf1*, *Gzma*, and *Gzmb* in Th17- but not in Th1-activated cells (Fig. 5*A*). We previously reported that Thpok prevents CD8α reexpression in naïve and Th1 effector $CD4^+$ T cells (29). In contrast, Th17 effector $CD4^+$ T cells restrained CD8α expression despite *Thpok* disruption (Fig. 5*B*). To determine whether repression of cytotoxic genes requires Thpok in Th17 effectors in vivo, we examined the small intestine lamina propria (siLP), a site highly enriched in effector T cells in unmanipulated mice. Using cytokine capture assays, we isolated T cells producing IFNγ (Th1) or IL-17 (Th17) (Fig. S4*B*). Similar to in vitro analyses, repression of *Gzma* and *Prf1* in Th17 cells was independent of Thpok, unlike in Th1 cells in which both genes were up-regulated after

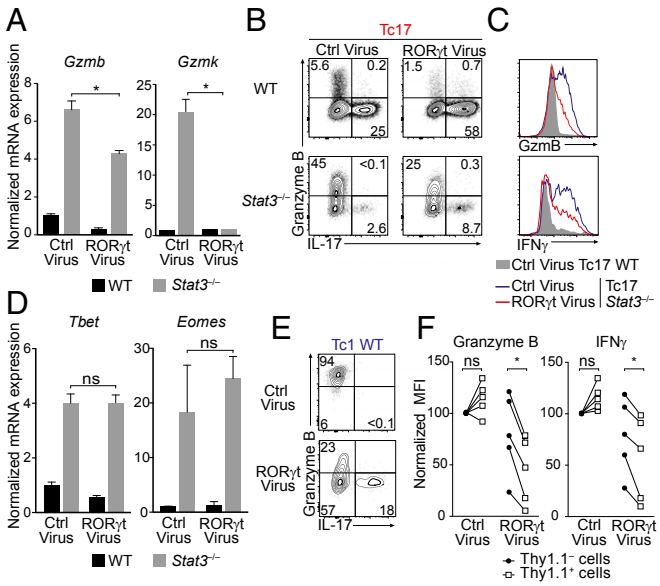

Fig. 4. RORγt antagonizes cytotoxic functions in CD8$^+$ T cells. (*A* and *D*) RT-qPCR experiments assess expression of *Gzmb* and *Gzmk* (*A*) or *T-bet* and *Eomes* (*D*) from WT (black bars) or *Stat3*$^{-/-}$ (gray bars) cells cocultured in Tc17 conditions as described in Fig. S2*A*, transduced with RORγt or control (empty) retroviruses, and sorted for CD45 allele expression before RNA preparation. Data are expressed relative to expression in WT Tc17 cells transduced with control virus (set to 1) and is representative of two mice per genotype analyzed in two independent experiments. (*B*) Contour plots show intracellular expression of IL-17 vs. granzyme B on WT or *Stat3*$^{-/-}$ cells cocultured in Tc17 conditions as described in Fig. S2*A* and retrovirally transduced as indicated. Data are gated on transduced cells and representative of three mice per genotype in three independent experiments. (*C*) Overlaid histograms show intracellular granzyme B and IFNγ expression in WT (gray shaded) or *Stat3*$^{-/-}$ (transduced as indicated) CD8$^+$ T cells assessed as in *B*. Data are representative of three mice per genotype in three independent experiments. (*E* and *F*) WT CD8$^+$ T cells were transduced with RORγt or control Thy1.1-expressing retrovirus and cultured in Tc1 conditions. (*E*) Contour plots show intracellular expression of IL-17 vs. granzyme B, gated on retrovirus-expressing (Thy1.1$^+$) cells. (*F*) Before–after plots compare intracellular granzyme B and IFNγ expression in Thy1.1$^+$ (empty squares) and Thy1.1$^-$ (filled circles) cells within the same culture. Data [mean fluorescent intensity (MFI)] is expressed relative to that in Thy1.1$^-$ cells in control virus-transduced cultures, set to 100 within each mouse. Each pair of symbols represents a separate culture; data are from five mice analyzed in three independent experiments *$P < 0.05$; ns, not significant.

Thpok disruption (Fig. 5*C*); the same was true of repression of CD8α (Fig. 5*D*).

The preceding findings demonstrate that Th17 effectors repress cytotoxic genes independently of Thpok, both in vitro and in vivo. To examine the potential role of STAT3 in such repression, we compared expression of IFNγ and granzyme B in *Stat3*$^{-/-}$ and control CD4$^+$ T cells cultured under Th17 conditions. STAT3 disruption increased expression of both molecules (Fig. S4*C*), a result consistent with previous transcriptome analyses (36, 53). However, the up-regulation of granzyme B and IFNγ expression in *Stat3*$^{-/-}$ CD4$^+$ T cells was lower than in *Stat3*$^{-/-}$ CD8$^+$ T cells cultured in the same conditions (Fig. S4*C*), consistent with a STAT3-independent inhibition by Thpok.

In addition to repressing cytotoxic genes, Thpok promotes expression of genes characteristic of the helper program, including *Cd40lg*, encoding a surface protein essential for helper activity. In Th1 cells, Thpok activation of *Cd40lg* is mediated in part through antagonism of Runx functions (29). In contrast to Th1 cells, Thpok was dispensable for CD40L expression in Th17 effectors (Fig. 5*E*), supporting the conclusion that the Th17 effector program of CD4$^+$ T cells antagonizes Runx functions independently of Thpok.

In CD4$^+$ T cells, Thpok serves in part redundantly with the related transcription factor LRF (encoded by *Zbtb7a*, called *Lrf* here) (29, 54). Thus, we considered that LRF could repress cytotoxic genes in Thpok-deficient Th17 effectors. To address this question, we cultured CD4$^+$ T cells that postthymically delete both Thpok and LRF [from *CD2*-cre *Thpok*$^{fl/fl}$ *Lrf*$^{fl/fl}$ mice (29)] under Th1 and Th17 conditions. Double-deficient Th17 cells fully repressed granzyme B expression (Fig. 5*F*) and, as previously reported (29), produced IL-17. In contrast, double-deficient Th1 cells failed to repress the expression of cytotoxic molecules compared with controls. Thus, repression of cytotoxic gene expression requires neither Thpok nor LRF in Th17 cells, unlike in other helper effector subtypes (29).

Discussion

The present report demonstrates that the transcriptional circuitry involved in IL-17 production in T cells broadly represses

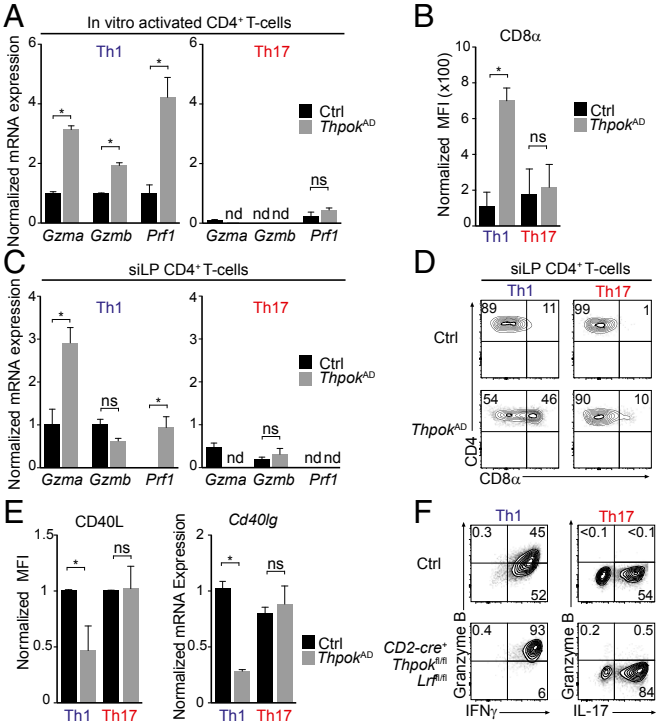

Fig. 5. The T$_{eff}$17 effector program represses cytotoxic gene expression in CD4$^+$ T cells. (*A* and *C*) RT-qPCR expression of *Gzma*, *Gzmb*, and *Prf1* on Th1 or Th17 cells from *Thpok*^{AD} (*Ox40*-cre$^+$ *Thpok*$^{fl/fl}$, gray bars) or control (*Ox40*-cre$^+$ *Thpok*$^{+/+}$, black bars) mice. Data are shown for effectors derived in vitro from naïve CD4$^+$ T cells (*A*) or for CD4$^+$ T cells isolated from the siLP (*C*), and is shown relative to gene expression values in control Th1 cells, set to 1 (except in *C* for *Prf1*, set to 1 on *Thpok*^{AD} Th1 cells). (*B*) Bar graphs show the MFI of surface CD8α expression on effectors CD4$^+$ T cells derived as in *A*. (*D*) Contour plot show CD8α vs. CD4 expression on TCRβ$^+$ CD4$^+$ CD44$^+$ IFNγ$^+$ (Th1) and IL-17$^+$ (Th17) siLP cells from control or *Thpok*^{AD} animals; data are gated on YFP$^+$ cells (as an indicator of Cre activity; mice carried a Rosa26YFP allele). (*E*) Bar graphs show the MFI of surface CD40L expression or RT-qPCR expression of *Cd40lg* (encoding CD40L) on effector CD4$^+$ T cells derived in vitro as in *A*. Data are expressed relative to values in control Th1 cells, set to 1. (*F*) Contour plots show intracellular expression of IFNγ (*Left*) or IL-17 (*Right*) vs. granzyme B in CD4$^+$ effectors derived in Th1 or Th17 culture conditions from *CD2*-cre$^+$ *Thpok*$^{fl/fl}$ *Lrf*$^{fl/fl}$ or control (*CD2*-cre$^-$ *Thpok*$^{fl/fl}$ *Lrf*$^{fl/fl}$). Data are representative of two (*A*, *E*, and *F*) or three (*B*–*D*) mice per genotype analyzed in two (*A*, *B*, *E*, and *F*) or three (*C* and *D*) independent experiments. *$P < 0.05$; nd, not detected; ns, not significant.

cytotoxic functions. Such repression is dependent on the transcription factor STAT3, in part via the induction of RORγt. Importantly, RORγt represses expression of cytotoxic effector genes despite persistent expression of canonical transcription factors Runx3, T-bet, and Eomes, implying that persistent inhibition of cytotoxic functions in Tc17 cells is highly dependent on cytokine-activated STAT3.

While T-bet and Eomes had been shown to restrain RORγt expression and thereby Th17 or Tc17 differentiation (16–18), whether STAT3 or RORγt reciprocally inhibit cytotoxic gene expression had not been elucidated. Although Th1-related and cytotoxic genes are not expressed in Th17 $CD4^+$ T cells (36, 55), this observation did not imply repression by the $T_{eff}17$ circuitry because Th17 $CD4^+$ T cells express Thpok, which itself inhibits expression of cytotoxic genes (28, 29). In fact, Batf and Irf4, key components of the $T_{eff}17$ circuitry, are also needed for proper Tc1 responses to viral infection (41–44). Here, we demonstrate that a STAT3–RORγt-based $T_{eff}17$ transcriptional circuitry represses cytotoxic gene expression and the development of cytotoxic functions in Tc17 $CD8^+$ T cells.

While RORγt represses effector genes (including those encoding granzymes), it does not inhibit *T-bet* or *Eomes* expression, in contrast to T-bet inhibition of RORγt gene expression. Such an asymmetric control has important functional implications. Whereas T-bet repression of RORγt stabilizes Tc1 differentiation, the inability of RORγt to repress *T-bet*, *Eomes*, and *Runx3* compromises the stability of IL-17–producing T cells. In circumstances where STAT3 activation is not sustained (e.g., by IL-6 signaling), or is counteracted through signaling by other cytokines (e.g., IL-12), the persistent expression of T-bet, Eomes, and Runx3 would favor the reemergence of cytotoxic gene expression.

Consistent with this asymmetric antagonism, IFNγ and IL-17 double-producing $CD8^+$ T cells are found in experimental colitis (22). Similar dual producers contribute to graft versus host disease (GVHD) after allogeneic stem cell transplantation (21), and therefore are presumably equivalent to pathogenic Th17 cells described in experimental models of colitis and multiple sclerosis (18, 56, 57). While these IFNγ- and IL-17–producing $CD8^+$ T cells expressed T-bet, they showed reduced expression of *Eomes* and cytotoxic genes, including *Gzmb*. Consistent with the idea that Tc17 cells are unstable, they were shown by fate-mapping analyses to revert to a cytotoxic fate (21).

In contrast to Tc17 $CD8^+$ T cells, in which inhibition of cytotoxic gene expression relies on the STAT3-driven $T_{eff}17$ circuitry, both that circuitry and the $CD4^+$ lineage-specific transcription factor Thpok contribute to restrain cytotoxic genes in Th17 $CD4^+$ T cells. Of note, Thpok-mediated repression of IFNγ can be overcome by Th1-inducing environmental cues, despite persistent Thpok expression (28, 29, 58). Accordingly, Th17 effectors, which harbor epigenetic marks of activity at Th1 loci, can acquire IFNγ production and contribute to immunopathology during inflammation (59, 60).

STAT3 and RORγt may inhibit cytotoxic genes hierarchically, as suggested by ChIP binding results: in this scenario, STAT3 acts on transcriptional regulators T-bet, Eomes, and RORγt, which themselves control cytotoxic effector genes. Mechanistically, STAT3 may serve by opposing the positive effect of STAT5 on cytotoxic genes, including *T-bet* and *Eomes* (61). As STAT3 competes with STAT5 for DNA binding genome-wide (62), sustained STAT3 activation may displace STAT5 and thereby inhibit expression of cytotoxic genes. Additionally, because STAT5 and Runx3 molecules directly interact (63), the competition between STAT3 and STAT5 may affect Runx3-dependent genes, including Eomes (5).

In cells that coexpress RORγt and T-bet or Eomes, the present study indicates that RORγt can counteract T-bet and Eomes and restrain cytotoxic gene expression. The binding of RORγt to *Ifng*, *Gzmb*, and *Gzmk cis*-regulatory regions suggests that such an effect could be direct, through RORγt recruitment to these genes. Because RORγt binds *cis*-regulatory elements that can also recruit T-bet, it is possible that competition between these factors for DNA binding controls cytotoxic gene expression. Challenging this idea, RORγt and T-bet recognize distinct DNA sequences (36, 64). Alternatively, RORγt could inhibit T-bet or Eomes without affecting their DNA binding, e.g., by affecting their recruitment of transcriptional coactivators.

Cytotoxic gene repression by STAT3 and RORγt is expected to reduce the antitumor potential of $CD8^+$ T cells in inflammatory tumor microenvironments. Indeed, *Stat3* disruption promotes responses against experimental tumors (65). Even though the exact mechanisms by which STAT3 inhibits antitumoral activity remain to be elucidated, a growing number of reports suggest a critical role of STAT3 and IL-6 signaling in T cells and natural killer cells, consistent with an effect on cytotoxic gene expression (66–69). Thus, the ability to manipulate and target this pathway might be a valuable approach to enhance antitumor responses in cancer immunotherapy strategies.

Materials and Methods

Mice. Mice carrying floxed alleles for *Thpok* (28), *Stat3* (70), *Rosa26*$^{Stat3C\text{-}GFP}$ (35), or *Lrf* (71) were from our own colony or obtained from J. O'Shea (National Institutes of Health, Bethesda), S. Koralov (New York University, New York), and P. P. Pandolfi (Harvard University, Boston), respectively. Additional strains are described in *SI Materials and Methods*. Animal procedures were approved by the National Cancer Institute Animal Care and Use Committee.

In Vitro Cell Procedures. Sorted naïve ($CD44^{lo}$) T cells were activated with anti-CD3 and anti-CD28, in the presence of T cell-depleted irradiated WT splenocytes and cytokines and anti-cytokines antibodies as described in *SI Materials and Methods*. Retroviral transductions were performed as previously described (31), using either MIGR-RORγt-Thy1.1 or PMRX-Thy1.1 retroviruses (72). In vitro cytotoxicity was determined using pan-T-depleted WT splenocytes coated with relevant GP33 (KAVYNFATM) or irrelevant (SIIFNEKL) peptides, labeled with distinct CFSE concentrations, and cocultured with in vitro derived $CD8^+$ effector T cells for 24 h.

Microarrays and ChIP-Seq Data. Affymetrix Mouse Exon 2.0 ST arrays were processed as described in *SI Materials and Methods* and analyzed with Partek Genomic Suite; data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under accession nos. GSE104143 and GSE104144. The Runx3 dataset (26) was obtained from the GEO (accession no. GSE50131). The STAT3 and RORγt (36) and T-bet ChIP-seq datasets (45) were obtained from the GEO (GSE40918 and GSE40623, respectively), aligned to the mouse genome (mm10 release) using the Bowtie package and analyzed with Partek Flow on the National Institutes of Health high-performance computing Biowulf cluster.

Statistical Analyses. All statistical analyses were performed using Prism software. Bars in graphs indicate average ± SEM. Comparisons were performed by two-tailed unpaired *t* test. **P* values <0.05.

Additional information is available in *SI Materials and Methods*.

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