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Effector γδ T Cell Differentiation Relies on Master but Not Auxiliary Th Cell Transcription Factors

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γδ T lymphocytes are programmed into distinct IFN-γ–producing CD27$^+$ (γδ27$^+$) and IL-17–producing CD27$^-$ (γδ27$^-$) subsets that play key roles in protective or pathogenic immune responses. Although the signature cytokines are shared with their αβ Th1 (for γδ27$^+$) and Th17 (for γδ27$^-$) cell counterparts, we dissect in this study similarities and differences in the transcriptional requirements of murine effector γδ27$^+$, γδ27$^-$CCR6$^-$, and γδ27$^-$CCR6$^+$ γδ T cell subsets and αβ T cells. We found they share dependence on the master transcription factors T-bet and RORγt for IFN-γ and IL-17 production, respectively. However, Eomes is fully dispensable for IFN-γ production by γδ T cells. Furthermore, the Th17 cell auxiliary transcription factors RORα and BATF are not required for IL-17 production by γδ27$^-$ cell subsets. We also show that γδ27$^-$ (but not γδ27$^+$) cells become polyfunctional upon IL-1β plus IL-23 stimulation, cosecreting IL-17A, IL-17F, IL-22, GM-CSF, and IFN-γ. Collectively, our in vitro and in vivo data firmly establish the molecular segregation between γδ27$^+$ and γδ27$^-$ T cell subsets and provide novel insight on the nonoverlapping transcriptional networks that control the differentiation of effector γδ versus αβ T cell subsets. ***The Journal of Immunology*****, 2015, 196: 000–000.**

Interferon-γ and IL-17 are potent proinflammatory cytokines involved in protection against infections, but when deregulated can become highly pathogenic in several chronic inflammatory and autoimmune diseases (1–4). Although these cytokines can be produced by various leukocyte subsets, γδ and αβ T cells have been shown to be major providers at early and late stages of immune responses, respectively (5, 6). The earlier contributions of γδ T cells, which impact on experimental models of arthritis, colitis, psoriasis, and multiple sclerosis (3, 4, 7, 8), are likely to stem from their developmental programming in the thymus and innate-like responsiveness at peripheral sites (3, 5, 9–11). This contrasts with the dynamics of peripheral αβ CD4 T cells, which undergo a prolonged differentiation program associated with intense proliferation upon Ag recognition in the presence of instructive cytokines (6).

Our understanding of the molecular events that govern the secretion of IFN-γ and IL-17 has largely emerged from studies on CD4 T cells. Naive CD4 T cell activation in the presence of IL-12 leads to the expression of the master transcription factor T-bet that induces IFN-γ production and thus polarization toward the Th1 cell fate (6). CD8 T cells use a different T-box family member, Eomes, to regulate IFN-γ production (12). In contrast, Th17 cell differentiation relies on IL-6, in cooperation with TGF-β and IL-1β, to trigger the expression of the master transcription factor RORγt (encoded by *Rorc*) that governs IL-17-expression, whereas maintenance is controlled by IL-23 (13). Furthermore, Th17 cells are endowed with functional plasticity, as IL-12 or IL-23 induces IFN-γ production (14–18). Strikingly, these IFN-γ$^+$ (Th1-like) Th17 cells are associated with pathogenicity in various autoimmune models (14, 16, 19). The pathogenic signatures of IFN-γ$^+$ Th17 cells are under extensive scrutiny, and although the inflammatory cytokines IL-17F, IL-22, TNF-α, and GM-CSF seem consistent (20), the role of T-bet remains controversial (14, 15, 17, 18, 20–22).

The master transcription factors T-bet and RORγt are strictly necessary and sufficient for Th1 versus Th17 cell differentiation, as they act as lineage-specifying factors that bind to multiple target loci, including those of the signature cytokines (23). In addition, various other auxiliary factors act downstream of the signaling pathways initiating Th lineage development. In Th1 cells, STAT4 and STAT1 induce T-bet, which then works in conjunction with Eomes, Hlx, and Runx3. In Th17 cells, STAT3, BATF, IFN regulatory factor (IRF)4, and RORα are known to play critical roles (24). The specific role(s) of these auxiliary factors is still being uncovered, but they have been shown to cooperate with master regulators to initiate and propagate the chromatin modifications, to allow preservation of the distinctive transcriptional profiles and maintenance of selective effector functionality. For

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; IRF, IFN regulatory factor; MOG, myelin oligodendrocyte glycoprotein; WT, wild-type.

example, BATF and IRF4 open the chromatin at Th17 cell–specifying loci to allow access to RORγt, and by doing so are termed pioneering factors (24). Importantly, both IRF4 and BATF-deficient mice have impaired Th17 cell differentiation and are resistant to EAE (25–27). The role of RORα is more subtle, and *Rora* deficiency alone had little effect on Th17 cells or EAE development, whereas the combined deletion of *Rorc* and *Rora* completely abolished Th17 differentiation (28).

Critically, whereas these concepts have been established for CD4 Th cells, they remain unexplored in γδ T cells. In fact, given their rather distinct differentiation dynamics, it is questionable whether the same principles apply to both T cell lineages. Consistent with this, IRF4 has been recently shown to be dispensable for the differentiation of IL-17–producing γδ T cells (29, 30). Although IRF4 and BATF cooperate in conventional CD4 T cells, it has been shown that BATF has a direct impact on IL-17 expression by innate-like iNKT cells (31), whereas IRF4 was not required (29). Therefore, building on these foundations, and on our previous identification of effector γδ T cell subsets segregated on the basis of CD27 expression (10, 32–34), we have in this work assessed the specific roles of the transcription factors T-bet, Eomes, RORγt, BATF, and RORα in the production of IFN-γ and IL-17 by γδ T cells in vitro and in vivo.

Materials and Methods

Mice

All mice used were adults 6–18 wk of age. C57BL/6, *Rorcγt*$^{GFP/GFP}$ (hereafter referred as *Rorc*$^{-/-}$), *Batf*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were from The Jackson Laboratory (Bar Harbor, ME). *Rorα*$^{sg/sg}$ were provided by A. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, U.K.). *Rorc*$^{-/-}$ and *Rorα*$^{sg/sg}$ were maintained by crossing heterozygous mice, and wild-type (WT) littermates were always used as controls for all type of experiments. *Eomes* floxed mice (provided by S. Reiner, Columbia University, New York, NY) were crossed with Rag1-cre on the C57BL/6 background to generate *Rag1*-cre-*Eomes* fl/+. These mice were intercrossed to generate *Rag1*-cre-*Eomes* fl/fl mice (hereafter referred to as *Eomes*$^{-/-}$). For *Rag1*-cre-*Eomes* fl/fl, *Batf*$^{-/-}$, and *Tbx21*$^{-/-}$ mice, purchased age- and gender-matched C57BL/6 mice (The Jackson Laboratory) were used as WT controls. Mice were bred and maintained in the specific pathogen-free animal facilities of Instituto de Medicina Molecular (Lisbon, Portugal) or the Babraham Institute (Cambridge, U.K.). All experiments involving animals were done in compliance with the relevant laws and institutional guidelines and were approved by local and European ethic committees.

Cell preparation, flow cytometry, cell sorting, and analysis

Cell suspensions were obtained from spleens, lymph nodes, or spinal cord. Erythrocytes were osmotically lysed in RBC lysis buffer (BioLegend). Cells were filtered through 70-μm cell strainers (BD Biosciences). For cell surface staining, single-cell suspensions were incubated, in presence of 2.4G2 (anti-FcγR; BD Biosciences) and 2% normal mouse serum, for 30 min with saturating concentrations of combination of the following mAbs that were purchased from BD Biosciences, eBiosciences, or BioLegend: eFluor450 anti-CD4 (RM4-5), brilliant violet 510 anti-CD8 (53-6.7), brilliant violet 421 anti–TCR-γδ (GL3), PE-Cy7 anti-CD27 (LG.7F9), Alexa-Fluor 647 anti-CCR6 (29-2L17), and allophycocyanin-Cy7 anti-CD3ε (17A2).

For the preparation of spinal cord mononuclear cells, mice were perfused through the left cardiac ventricle with cold PBS. The spinal cord was dissected and tissue was cut into pieces, dilacerated between two opaque slides, and digested with collagenase type IV (0.2 mg/ml; Lorne Laboratories) and DNase I (0.15 mg/ml) (Sigma-Aldrich) in RPMI 1640 at 37°C for 30 min, followed by 5-min treatment with 10 mM EDTA (Sigma-Aldrich). Mononuclear cells were isolated by passing the tissue through a 70-μm cell strainer, followed by a 33% Percoll (Sigma-Aldrich) gradient and 30-min centrifugation at 2000 rpm. Mononuclear cells were recovered from the pellet, resuspended, and used for further analysis.

For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) (Sigma-Aldrich) for 4 h at 37°C; 10 μg/ml brefeldin A (Sigma-Aldrich) was added during the last 2 h. Cells were fixed and permeabilized according to the manufacturer's instructions with

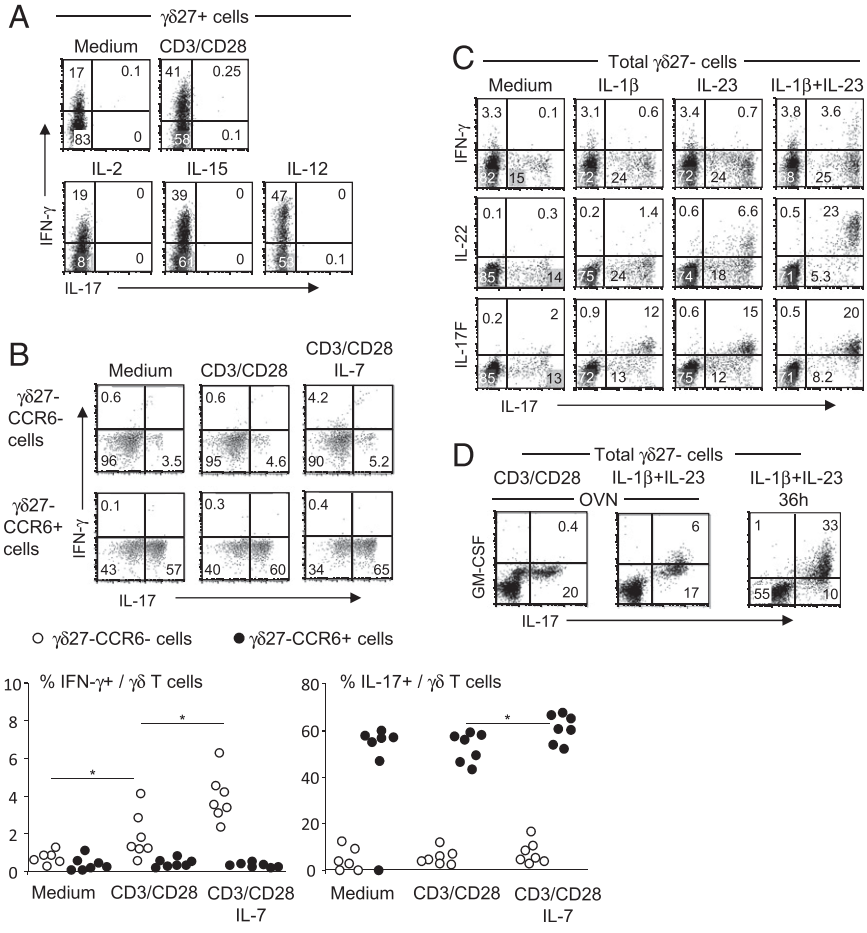

FIGURE 1. Distinct and diversified profiles of cytokine production by γδ27$^+$ and γδ27$^-$ cells. γδ T cell subsets were FACS sorted from peripheral spleen and lymph nodes, of normal C57BL6 mice, as follows: CD27$^+$ (γδ27$^+$), total CD27$^-$ (total γδ27$^-$), or CD27$^-$CCR6$^-$ (γδ27$^-$CCR6$^-$) and CD27$^-$CCR6$^+$ (γδ27$^-$CCR6$^+$) γδ T cells. (**A**) γδ27$^+$ cells were stimulated overnight in the presence of coated anti-CD3 plus anti-CD28 mAb, or the cytokines IL-2, IL-12, IL-15, and then assessed for IFN-γ and IL-17 production. (**B**) Specific production of IL-17 and IFN-γ by γδ27$^-$ and γδ27$^-$CCR6$^+$ γδ T cells was assessed after stimulation with coated anti-CD3 plus anti-CD28 mAb in the presence or not of IL-7 overnight. The graphs show percentage of γδ T cells producing IFN-γ (*left*) or IL-17 (*right*). (**C**) γδ27$^-$ cells were stimulated overnight in the presence of IL-1β, IL-23, or both and then were assessed for IFN-γ, IL-17, IL-17F, and IL-22 production. (**D**) Shows GM-CSF and IL-17 production by γδ27$^-$ cells stimulated in the presence of coated anti-CD3 plus anti-CD28 mAb overnight, or in the presence of IL-1β or IL-23 overnight or 36 h. Medium refers to cells left resting overnight without stimulation. *$p < 0.05$ (Mann–Whitney two-tailed test).

the Cytofix/Cytoperm kit from BD Biosciences. The following mAbs purchased from BD Pharmingen, eBiosciences, or BioLegend were used to stain cytokines: FITC anti–IL-17A (TC11-18H10), PE anti–IL-22 (1H8PWSR), PE anti–GM-CSF (MP1-22E9), PerCP-eFluor710 anti–IL-17F (18F10), and allophycocyanin anti–IFN-γ (XMG1.2).

For intracellular transcription factor staining, cells were fixed and permeabilized according to the manufacturer's instructions with the transcription factor fixation/permeabilization kit from eBiosciences. The following mAbs purchased from eBiosciences were used: PE anti–T-bet (4B10) and PE anti-RORγt (AFKJS-9).

Cells were sorted on FACSAria I (BD Biosciences) or analyzed on FACSFortessa or FACSCalibur (BD Biosciences). All graphical output was performed using FlowJo (Tree Star, Costa Mesa, CA).

In vitro γδ T cell stimulation and CD4 Th cell polarization

$CD27^+$ and $CD27^-$ γδ T cells were FACS sorted and subjected to various stimulation conditions overnight or for 36 h. Cells were incubated on plate-bound anti-CD3ε (145.2C11) plus anti-CD28 mAb (37.51) (both at 2 μg/ml) or, alternatively, in the presence of murine IL-1β (50 ng/ml), IL-2 (10 ng/ml), or IL-12 (5 ng/ml), or the cross-reactive human IL-15 (20 ng/ml) or IL-23 (50 ng/ml).

For Th1 cell culture conditions, cells were incubated with plate-bound anti-CD3ε (145.2C11) and soluble anti-CD28 mAb (37.51) (both at 2 μg/ml) in the presence of IL-12 (5 ng/ml) and neutralizing anti–IL-4 mAb (11B11) (5 μg/ml). For Th17 cell culture conditions, cells were incubated with plate-bound anti-CD3ε and soluble anti-CD28 (both at 2 μg/ml), and TGF-β (2 ng/ml), IL-1β (50 ng/ml), IL-6 (20 ng/ml), IL-21 (100 ng/ml), IL-23 (50 ng/ml), and neutralizing anti–IFN-γ (10 μg/ml) were added to the medium. CD4 T cell polarization cultures were kept for 5 d.

All cytokines were from PeproTech, except TGF-β and IL-23, which were from R&D Systems. The Abs were from eBiosciences or BD Biosciences.

Real-time PCR

mRNA was prepared from FACS-sorted cell populations using High Pure RNA Isolation kit (Roche). Reverse transcription was performed with random oligonucleotides (Invitrogen) using Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h at 42°C. Relative quantification of specific cDNA species to endogenous references *β-2 microglobulin* or *Actinb* was carried out using SYBR or TaqMan probe chemistry on ABI ViiA7 cycler (Applied Biosystems). The C_T for the target gene was subtracted from the C_T for endogenous references, and the relative amount was calculated as $2^{-\Delta CT}$. Primers were designed using Primer Express software (Applied Biosystems), and their sequences are listed in Supplemental Table 1.

EAE induction

For active EAE, C57BL/6 mice were immunized s.c. in both flanks with 100 μg myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) (Biopolymers Laboratory, Harvard Medical School) emulsified in CFA solution (4 mg/ml mycobacteria in IFA) (Difco Laboratories). On the day of immunization and 2 d after, mice received 200 ng pertussis toxin (List Biological Laboratories) in 100 μl PBS i.v.

Listeria monocytogenes *infection*

We used the *L. monocytogenes* strain EGDe, carrying a recombinant InlA with a mutation in S192N and Y369S, as previously described (35). Briefly, all mice were deprived of food and water overnight prior to infection, housed individually with minimal bedding, and given a ~0.5-cm^3 piece of mashed food inoculated with 2×10^9 CFUs *L. monocytogenes* in PBS. Naive mice were sham infected with mashed food inoculated with PBS.

Murid herpesvirus-4 infection

Mice were infected i.p. with 10^6 PFU murid herpesvirus-4, and cells from the spleen were collected after 5 or 8 d.

Statistical analysis

Statistical analysis was performed using a two-tailed nonparametric Mann–Whitney *U* test. The *p* values <0.05 were considered significant and are indicated on the figures.

Results

Segregation of proinflammatory cytokine production within γδ T cell subsets

This study initiated with an assessment of the diversity of proinflammatory cytokines produced by distinct lymphoid γδ T cell subsets. We have previously demonstrated that IFN-γ–producing

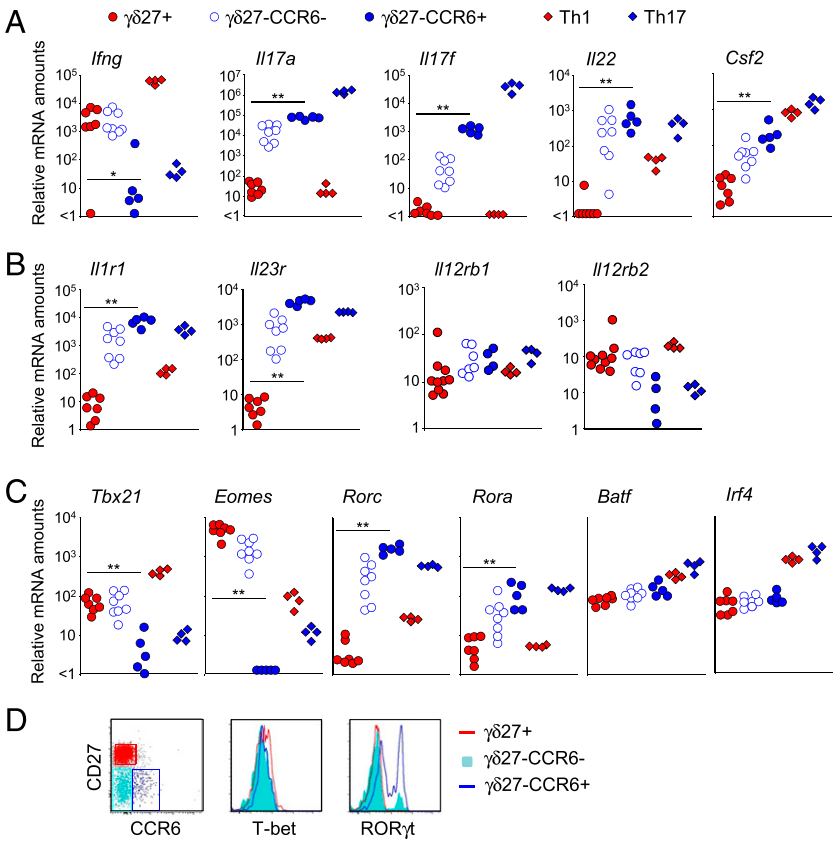

FIGURE 2. Cosegregation of cytokine and transcription factor expression in $γδ27^+$, $γδ27^-CCR6^-$, and $γδ27^-CCR6^+$ cells. (**A**) γδ T cell subsets were FACS sorted from peripheral spleen and lymph nodes, as follows: $CD27^+$ ($γδ27^+$), $CD27^-CCR6^-$ ($γδ27^-CCR6^-$), and $CD27^-CCR6^+$ ($γδ27^-CCR6^+$) γδ T cells. In addition, in vitro generated Th1 and Th17 CD4 T cells were used. Graphs represent the results of RT-PCR data for expression of (A) *Ifng*, *Il17*, *Il17f*, *Il22*, and *Csf2*; (**B**) *Il1r1*, *Il12rb1*, *Il12rb2*, and *Il23r*; and (**C**) the transcription factors *Tbx21*, *Eomes* (type 1 factors), *Rorc*, *Rora*, *Batf*, and *Irf4* (type 17 factors). Each dot represents T cells isolated from one individual mouse. All gene expressions are calculated relative to endogenous gene reference *b2m* or *Actb* or *Hprt*. $*p < 0.05$, $**p < 0.01$ (Mann–Whitney two-tailed test). (**D**) $CD3ε^+TCRδ^+$ cells were gated from spleen, and dot plots show γδ T cell subsets according to CD27 and CCR6 expression. Histograms display protein expression of T-bet and RORγt in $γδ27^+$, $γδ27^-CCR6^-$, and $γδ27^-CCR6^+$ γδ T cells. Representative transcription factor expression from six different mice is shown.

γδ T cells express the costimulatory receptor CD27 (hereafter γδ27$^+$ cells), whereas IL-17–producing γδ T cells lack CD27 (in this work noted as γδ27$^-$ cells) (10, 34). We also used the chemokine receptor CCR6 that is an additional marker that delineates further the IL-17–producing γδ27$^-$ cells (32, 33). An improved understanding of γδ T cell differentiation requires the elucidation of the upstream regulators of cytokine production, and emerging evidence supports that γδ27$^+$ cells preferentially respond to TCR signaling, whereas γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ cells mostly respond to cytokine-derived stimuli (11, 32–34, 36–38). In this study, we aimed at a more detailed dissection of the extracellular stimuli that trigger cytokine production by each γδ T cell subset (FACS sorted from spleen and lymph nodes on the basis of CD27 and when indicated CCR6 expression levels).

When stimulated with PMA plus ionomycin for 4 h, ∼20% of γδ27$^+$ cells expressed IFN-γ intracellularly (Fig. 1A). This proportion increased upon addition of anti-CD3/28 mAb and/or several cytokines overnight (Fig. 1A, Supplemental Fig. 1A). The most dramatic response was observed to IL-12, which per se induced ∼50% of γδ27$^+$ cells to produce IFN-γ. Moreover, IL-12 synergized with CD3/CD28 signaling to induce IFN-γ production by 70% of γδ27$^+$ cells (Supplemental Fig. 1A). Albeit to a lesser extent, IL-2 and IL-15 also synergized with CD3/CD28 signals, and induced a 2-fold increase in the frequency of IFN-γ–secreting γδ27$^+$ T cells (Supplemental Fig. 1A). These data show that the cytokines IL-2, IL-12, and IL-15 are important cofactors in TCR-mediated stimulation of γδ27$^+$ cells. In stark contrast, γδ27$^-$ T cells did not respond with increased IL-17 production upon stimulation with CD3/CD28 or IL-15, whereas IL-2 and IL-12 induced a small proportion of IFN-γ single producers (Supplemental Fig. 1B). Noteworthily, only a minute proportion (<5%) within the γδ27$^-$CCR6$^-$ subset, but essentially none of the γδ27$^-$CCR6$^+$ cells, produced IFN-γ in response to TCR engagement (Fig. 1B). Importantly, neither γδ27$^-$CCR6$^-$ nor γδ27$^-$CCR6$^+$ cells increased IL-17 production upon CD3/CD28 stimulation overnight, even in the presence of IL-7, which has been reported to promote specific survival of the IL-17–biased γδ subsets (37, 39).

We (34, 36) and others (3) previously showed that γδ27$^-$ cells respond to the combination of IL-1β and IL-23. Besides their impact on the proportion of IL-17A producers and on the expression levels of IL-17A per γδ27$^-$ cell (Fig. 1C, Supplemental Fig. 1B), IL-1β plus IL-23 strikingly induced the simultaneous coproduction of IL-17F, IL-22, IFN-γ, and GM-CSF (Fig. 1C, 1D). Importantly, IL-1β and IL-23 showed strong synergistic effects in the induction of these inflammatory cytokines (Fig. 1C), consistent with results obtained upon intranasal administration in vivo (40). These data demonstrate that γδ27$^-$ (but not γδ27$^+$) cells are endowed with striking polyfunctionality that is induced by IL-1β and IL-23.

Then, we performed gene expression analysis to gain further insights into the potential functional and transcriptional partition between γδ27$^+$, γδ27$^-$CCR6$^-$, and γδ27$^-$CCR6$^+$ cell subsets. Consistent with our previous report (34), γδ27$^+$ cells expressed high levels of *Ifng* but negligible amounts of *Il17a*, *Il17f*, *Il22*, and *Csf2* (encoding GM-SCF) transcripts (Fig. 2A). Conversely, γδ27$^-$CCR6$^-$ cells expressed high levels of *Il17a*, *Il17f*, *Il22*, and *Csf2* mRNA. Importantly, these differences were further amplified in γδ27$^-$CCR6$^+$ cells, suggesting a more terminal differentiation stage. Of note, the most extreme profile of cytokine gene transcription as seen between γδ27$^+$ and γδ27$^-$CCR6$^+$ cells mirrored that of in vitro differentiated CD4 Th1 and Th17

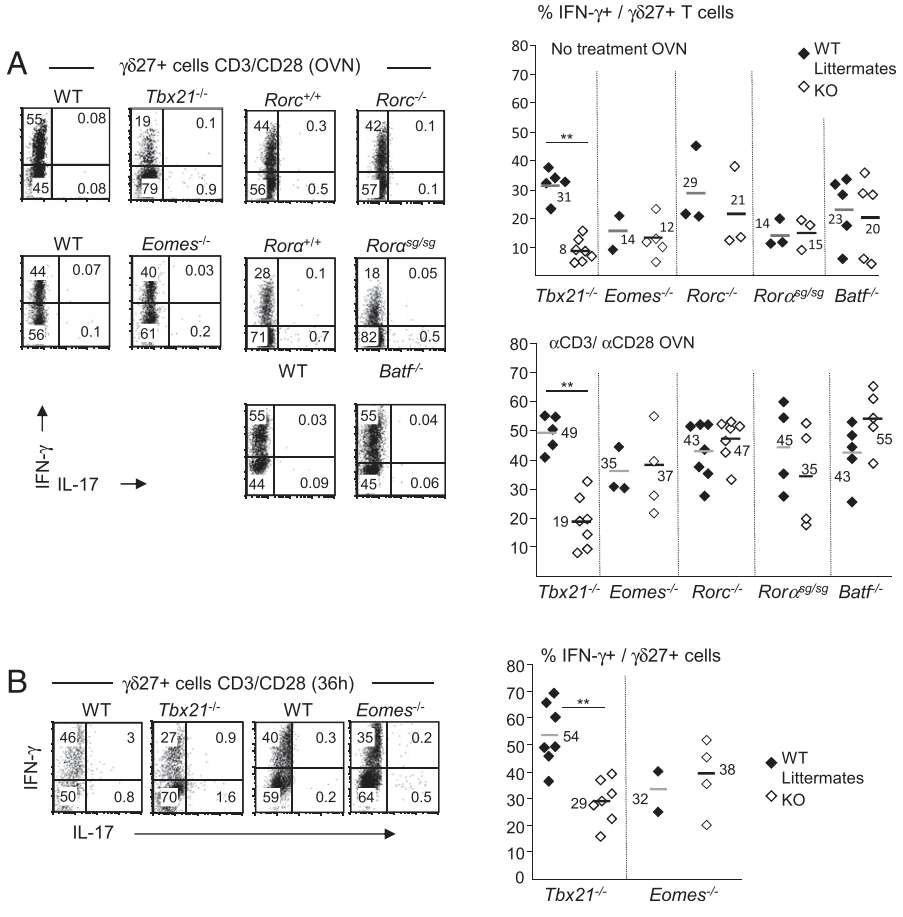

FIGURE 3. T-bet regulates IFN-γ production in γδ27$^+$ T cells in vitro. γδ27$^+$ cells were FACS sorted from each following *Tbx21*$^{-/-}$, *Eomes*$^{-/-}$, *Rorc*$^{-/-}$, *Rora*$^{sg/sg}$, and *Batf*$^{-/-}$ mouse strain, and (**A**) left untreated or restimulated with coated anti-CD3 plus anti-CD28 mAb overnight or for (**B**) 36 h. IFN-γ and IL-17A production was assessed by intracellular flow cytometry. The graphs show percentage of γδ27$^+$ T cells producing IFN-γ after (A) resting overnight (*top*) and CD3/CD28 stimulation overnight (*bottom*) and (B) 36 h. The graphs result from two or more experiments. $^{**}p < 0.01$ (Mann–Whitney two-tailed test).

cells, respectively (Fig. 2A). Importantly, these data suggest that CCR6$^+$γδ27$^-$ cells (but not γδ27$^+$ cells) are programmed to produce all five proinflammatory cytokines. However, surprisingly, despite the presence of transcripts for these cytokines in γδ27$^-$ cells ex vivo (Fig. 2A), the corresponding proteins were not expressed upon mere PMA plus iono stimulation but required additional (14 h) activation with IL-1β and IL-23 (Fig. 1C, 1D). The responsiveness of γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ cell subsets to IL-1β and IL-23 is consistent with their high (100-fold) expression of *Il1r1* and *Il23r* (Fig. 2B). The *Il12rb1* chain shared between the receptor for IL-12 and IL-23 is expressed at constant levels between the three subsets. The specific response of γδ27$^+$ cells to IL-12 is most likely attributable to their higher expression of the *Il12rb2* chain, which is required for the recognition of IL-12.

Altogether, these data establish the distinct TCR and cytokine responsiveness of lymphoid γδ T cell subsets and their markedly different patterns of effector cytokine (gene and protein) expression.

Differential expression of candidate transcription factors in γδ T cell subsets

To gain additional insight into the differentiation programs that regulate the production of selective cytokines by γδ27$^+$ versus γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ cells, we assessed the expression of transcription factors known to control IFN-γ or IL-17 expression in their αβ counterparts (23). The type 1 cell transcription factors, *Tbx21* and *Eomes*, were expressed at similar levels between γδ27$^+$ and γδ27$^-$CCR6$^-$ cells, but were clearly downregulated in the γδ27$^-$CCR6$^+$ subpopulation (Fig. 2C). Namely, *Tbx21* transcripts were 100-fold less abundant in γδ27$^-$CCR6$^+$ cells than in γδ27$^-$CCR6$^-$ or γδ27$^+$ cells. Even more strikingly, *Eomes* mRNA was absent in γδ27$^-$CCR6$^+$ cells, whereas it was highly expressed in γδ27$^-$CCR6$^-$ or γδ27$^+$ cells. These data show that γδ27$^-$CCR6$^-$ cells contain the type 1 transcriptional machinery that supports their polyfunctionality.

Conversely, the type 17 cell transcription factors, *Rorc* and *Rora*, were strongly enriched in γδ27$^-$CCR6$^-$ cells, and once again the more extreme phenotype was observed in the γδ27$^-$CCR6$^+$ cell subset (Fig. 2C). By contrast, *Batf* and *Irf4* were expressed at similar levels across all γδ T cell subpopulations (with small difference between Th1 and Th17 cells).

In addition, we confirmed that at the protein level T-bet was expressed in γδ27$^+$ cells, whereas RORγt was present in a small proportion of γδ27$^-$CCR6$^-$, but in most γδ27$^-$CCR6$^+$ cells (Fig. 2D). Thus, this interesting profile of transcription factor expression provided the grounds to assess their specific roles in the effector functions of γδ27$^-$ and γδ27$^+$ T cell subsets.

In vitro production of IFN-γ by γδ T cell subsets depends on T-bet but not Eomes

We employed murine models for single gene deficiency for *Tbx21*, *Eomes*, *Rorc*, *Rora*, and *Batf* to characterize cytokine production by γδ T cell subsets. We assessed whether the mouse strains display normal γδ27$^+$, γδ27$^-$CCR6$^+$, and γδ27$^-$CCR6$^+$ cell subsets (Supplemental Fig. 2). Only mice that are deficient in *Rorc* specifically lack the γδ27$^-$CCR6$^+$ cell subset, as previously observed (34), whereas the *Tbx21*$^{-/-}$, *Rora*$^{sg/sg}$, and *Batf*$^{-/-}$ mice have normal frequency of all γδ T cell subsets. For γδ27$^+$ cells, given their restricted functional potential, we focused on IFN-γ production induced by stimulation with anti-CD3/CD28 mAb overnight (~14 h) or for 36 h. We chose anti-CD3/CD28 mAb treatment over all other conditions assessed (Fig. 1, Supplemental Fig. 1B) because it induced ~40% of γδ27$^+$ cells to produce IFN-γ, over a short-term in vitro activation, and T-bet expression is known to be increased by TCR signaling in γδ T cells (41). In line with some published data on total γδ T cells (41, 42), we found that T-bet (*Tbx21*) deficiency significantly reduced IFN-γ production by γδ27$^+$ cells after 14 and 36 h of TCR stimulation (Fig. 3). This dependence on T-bet was readily observed in the absence of any exogenous stimulation (Fig. 3A, see no treatment

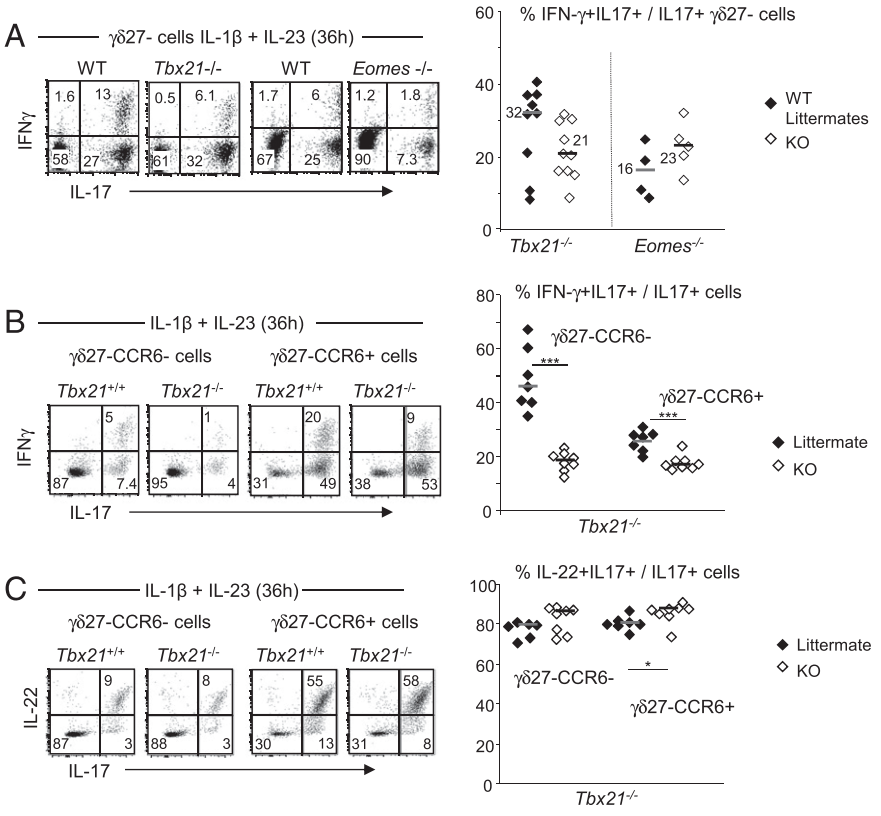

FIGURE 4. T-bet regulates IFN-γ production in γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ T cells in vitro. (**A**) Total γδ27$^-$ cells were FACS sorted from *Tbx21*$^{-/-}$ and *Eomes*$^{-/-}$ mice, restimulated 36 h with IL-1β plus IL-23, and assessed for IFN-γ and IL-17 production. The graph depicts the percentage of γδ27$^-$ T cells that coproduce IFN-γ and IL-17 of the total IL-17$^+$γδ27$^-$ T cells. (**B** and **C**) γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ cells were FACS sorted from *Tbx21*$^{-/-}$ mice, restimulated 36 h with IL-1β plus IL-23, and assessed for (B) IFN-γ and IL-17 production and (C) IL-22 and IL-17 production. The graphs on the *right* depict the percentage of γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ cells that coproduce IFN-γ/IL-17 and IL-22/IL-17 of the total IL-17$^+$ γδ27$^-$ T cells. The graphs result from two or more experiments. *$p < 0.05$, ***$p < 0.005$ (Mann–Whitney two-tailed test).

overnight), suggesting a major role for T-bet in the developmental programming of IFN-γ–producing γδ27$^+$ cells. By contrast, Eomes is fully dispensable for IFN-γ expression in γδ27$^+$ T cells (Fig. 3).

For γδ27$^-$ cells, we sought to dissect the role of T-bet and Eomes in the production of IFN-γ that is induced by IL-1β plus IL-23 signaling (Fig. 1B). We found that Eomes was completely dispensable (Fig. 4A), whereas T-bet was required in both γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ subtypes to produce IFN-γ in response to proinflammatory cytokine stimulation (Fig. 4B). Finally, T-bet has been proposed to control IL-22 production in ILC22 (43, 44) and Th22 (45) cells. This contrasts with the ability of IL-1β/IL-23–stimulated γδ27$^-$CCR6$^-$ and γδ27$^-$CCR6$^+$ subsets to produce IL-22 in the absence of T-bet (Fig. 4C). Based on these in vitro observations, we next questioned the role of T-bet during physiological responses to infectious microorganisms in vivo.

T-bet is indispensable for IFN-γ production by both γδ27$^+$ and γδ27$^-$ cells in vivo

Given that during infection with murine herpesviruses γδ T cells play important protective roles (46), with γδ27$^+$ cells providing IFN-γ (36), we infected *Tbx21*$^{-/-}$ mice with murid herpes virus-4. Splenocytes were harvested 5 and 8 d postinfection and restimulated with PMA/ionomycin for 4 h. This showed that, in the absence of *Tbx21*, IFN-γ production by γδ27$^+$ cells was significantly reduced both in frequencies and absolute numbers (Fig. 5A). Of note, IFN-γ production also depended on *Tbx21* in CD4 Th1 cells, but not in CD8 T cells (Supplemental Fig. 3).

Next, to assess the in vivo IFN-γ response of γδ27$^-$ cells, we infected mice orally with *L. monocytogenes* that has been shown to induce the generation of polyfunctional γδ27$^-$ cells capable of coproducing IFN-γ and IL-17 (35). Splenocytes were harvested 8 d postinfection and restimulated for 4 h either with PMA/ionomycin or in the presence of IL-1β/IL-23. Both stimulations allowed detection of similar proportion of IL-17$^+$ γδ27$^-$ cells, but, in our hands, the latter conditions revealed higher amounts of *L. monocytogenes*-induced IL-17$^+$ IFN-γ$^+$ γδ27$^-$ cells (Fig. 5B). This short ex vivo restimulation also induced single-producer IFN-γ$^+$ γδ27$^-$ cells. This was observed by Sheridan et al. (35), although they used restimulation of splenocytes with αCD3$^-$ and αCD28 mAb-coated beads for 5 h. Importantly, lack of T-bet impaired both IL-17$^+$ IFN-γ$^+$ γδ27$^-$ cells and IFN-γ$^+$ γδ27$^-$ cells (Fig. 5B). These data revealed, to our knowledge, for the first time, a strict dependence on T-bet for IFN-γ production by γδ T cell subsets in vivo.

T-bet suppresses IL-17 expression by γδ27$^-$ cells in vivo

Given that an additional role ascribed to T-bet, in conventional T cells, is to inhibit IL-17 production (47, 48), we next questioned whether it operated similarly in γδ T cells. We observed a 3-fold increase in the frequency of IL-17–producing γδ T cells upon *L. monocytogenes* infection in the absence of T-bet (Figs. 5B, 6A). In addition, we used EAE, the most commonly used animal model of multiple sclerosis, which is accompanied by an accumulation of IL-17$^+$ γδ T cells in the CNS (3, 4). We immunized WT or knockout mice with MOG peptide emulsified in CFA and treated them with pertussis toxin to induce disease. In this response, we

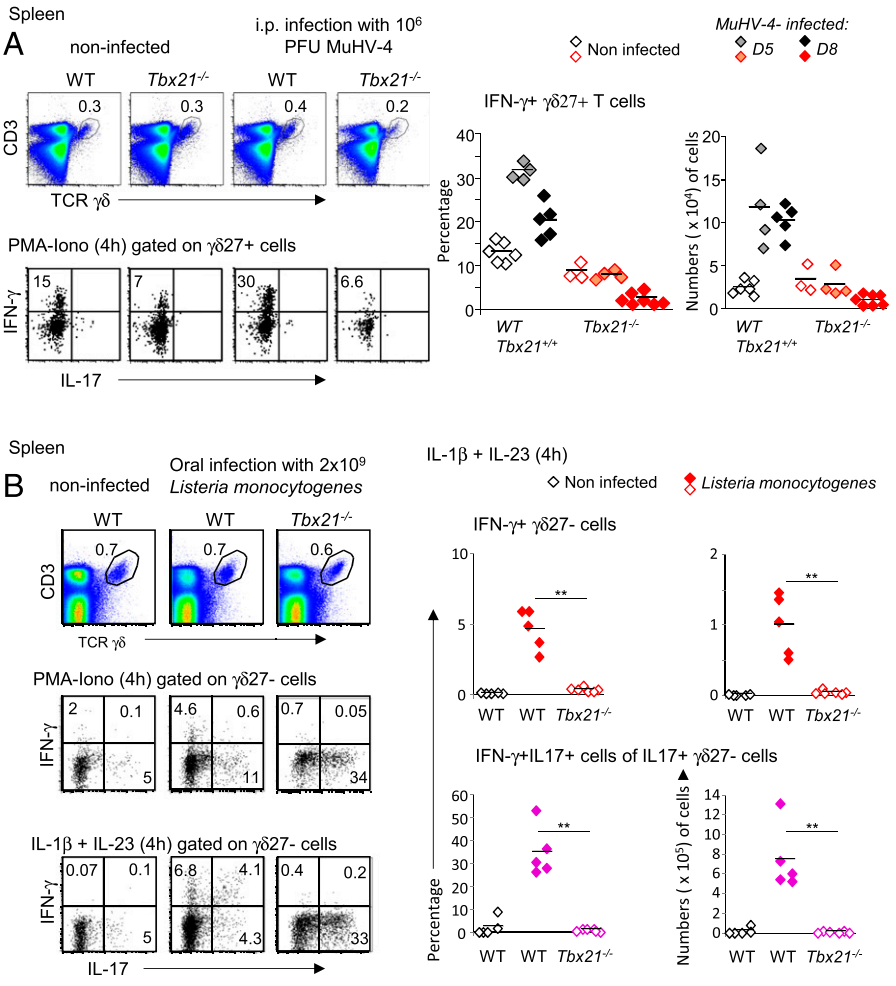

FIGURE 5. T-bet is required for in vivo IFN-γ responses to infection by both γδ27$^+$ and γδ27$^-$ cells. (**A**) WT and *Tbx21*$^{-/-}$ mice were infected with murid herpesvirus-4 i.p., and splenocytes were harvested 5 or 8 d later. Total splenocytes were restimulated with PMA/ionomycin for 4 h and assessed for production of IFN-γ and IL-17 by intracellular cytokine staining gated on γδ27$^+$ cells. (**B**) WT and *Tbx21*$^{-/-}$ mice were infected orally with *L. monocytogenes*, and splenocytes were harvested 1 wk later. Total splenocytes were restimulated with PMA/ionomycin or IL-1β/IL-23 for 4 h and assessed for production of IFN-γ and IL-17 by intracellular cytokine staining gated on total γδ27$^-$ T cells. The graphs on the *right* show percentages and numbers of (A) IFN-γ$^+$γδ27$^+$ cells after murid herpesvirus-4 infection and (B) IFN-γ$^+$γδ27$^-$ cells (*top*) and IFN-γ$^+$IL-17$^+$γδ27$^-$ cells (*bottom*) after *L. monocytogenes* infection, from two independent experiments, with each dot representing value for one individual mouse. $*p < 0.05$, $**p < 0.01$ (Mann–Whitney two-tailed test).

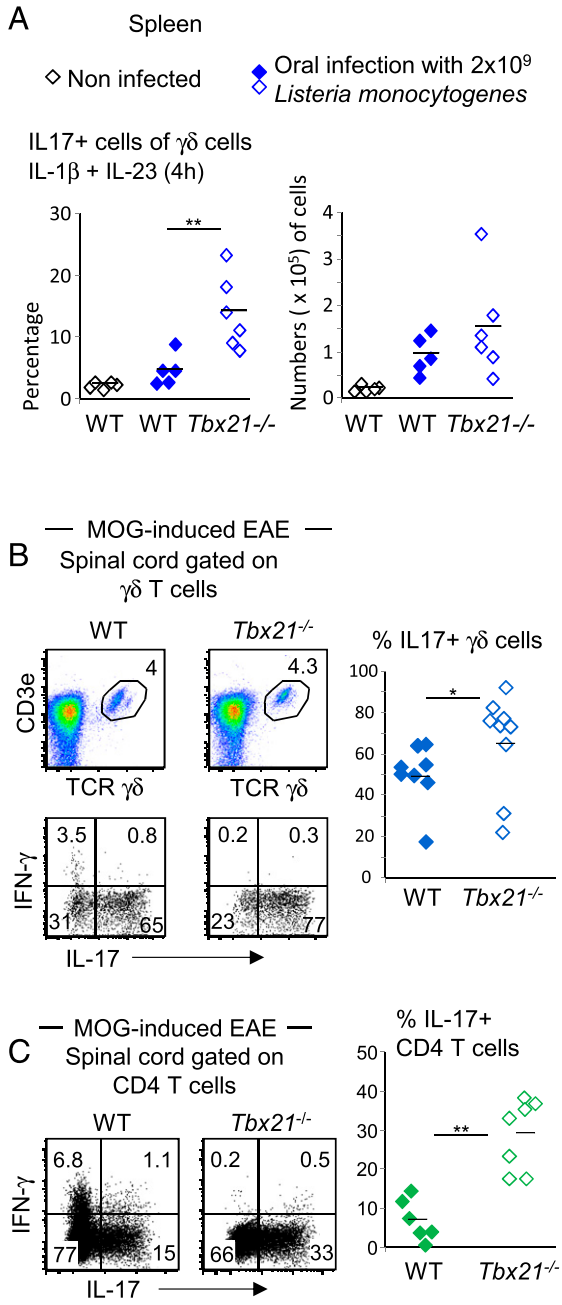

FIGURE 6. T-bet represses IL-17 production by γδ T cells and CD4 T cells in vivo. (**A**) WT and *Tbx21*$^{-/-}$ mice were infected orally with *L. monocytogenes*, and splenocytes were harvested 1 wk later. Total splenocytes were restimulated with IL-1β/IL-23 for 4 h and assessed for production of IFN-γ and IL-17 by intracellular cytokine staining gated on γδ T cells, as in Fig. 4B. The graphs show percentages and numbers of IL-17–producing cells from total γδ T cells after *L. monocytogenes* infection, from two independent experiments, with each dot representing value for one individual mouse. (**B** and **C**) WT and *Tbx21*$^{-/-}$ mice were induced to develop EAE with MOG injection, and cells were harvested from the spinal cord 2 wk later. Total cells from spinal cords were restimulated with PMA/ionomycin for 4 h and assessed for production of IFN-γ and IL-17 by intracellular cytokine staining gated on γδ T cells (B) and CD4 T cells (C). The graphs on the *right* show percentages of (B) IL-17$^+$ cells of total γδ T cells and (C) IL-17$^+$ CD4 T cells of total CD4 T cells during EAE, from two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ (Mann–Whitney two-tailed test).

also observed higher proportions of IL-17 producers among both γδ27$^-$ cells (Fig. 6B) and CD4 T cells (Fig. 6C) accumulating within the spinal cord of mice lacking T-bet.

Collectively, these results show that the T-bet–dependent mechanisms that induce IFN-γ and repress IL-17 in CD4 T cells are shared by γδ T cells in vivo.

IL-17 production by γδ27$^-$ cells does not depend on auxiliary Th17 transcription factors

A similar approach was used to determine whether γδ27$^-$ cells share the main transcriptional regulators of IL-17 production with conventional CD4 Th17 cells.

The production of IL-17 by γδ27$^-$ cells was absolutely dependent on RORγt (Fig. 7). Conversely, and unexpectedly, we found that RORα and BATF were not required for production of IL-17, nor IL-17F or IL-22, by γδ27$^-$ cells (Fig. 7). In fact, contrary to our expectation, the absence of RORα in γδ27$^-$ cells led almost to a 2-fold increase in IL-17–producing γδ27$^-$ cells. Although viable, the *Rora* mutant mice are very small and die at ~3–4 wk of age, which prevented us from assessing the role of RORα in infections or autoimmune responses in vivo. Ongoing work aims at establishing a mouse model that allows conditional deletion of RORα in T cells to decipher its role in the regulation of IL-17 in γδ T cells.

The independence on BATF was validated in vivo in two responses that, as shown above, induced strong IL-17 production by γδ T cells—*L. monocytogenes* infection and EAE. Thus, IL-17$^+$ γδ27$^-$ cells were induced to similar frequencies and numbers in *Batf*$^{-/-}$ mice infected orally with *L. monocytogenes* as compared with WT controls (Fig. 8A). *Batf*$^{-/-}$ mice are resistant to EAE induction most likely because lymphocytes do not infiltrate the spinal cord or brain (27). Consequently, we assessed IL-17$^+$ γδ27$^-$ cells in the inguinal lymph nodes that drain the site of MOG/CFA injection, as this induced a marked increase in IL-17$^+$ γδ27$^-$ cells. Once again, no difference was observed for frequencies and numbers of IL-17$^+$ γδ27$^-$ cells in WT and *Batf*$^{-/-}$ mice (Fig. 8B). Together with published findings on IRF4, also dispensable for IL-17 expression in γδ27$^-$ cells (29, 30), our data demonstrate that IL-17 production by γδ27$^-$ cells, although dependent on the master transcription factor RORγt, does not rely on auxiliary transcriptional partners (BATF, RORα, IRF4) that promote CD4 Th17 cell differentiation.

Discussion

We have previously shown that γδ27$^+$ cells are epigenetically committed to express IFN-γ but not IL-17, whereas γδ27$^-$CCR6$^+$ cells make IL-17, but can be induced to produce IFN-γ under inflammatory conditions (34). In this study, by revealing clearly distinct transcriptional requirements for their functional differentiation, our data strengthen the divergence between γδ27$^+$, γδ27$^-$CCR6$^-$, and γδ27$^-$CCR6$^+$ T cell subsets.

Our results show that T-bet is critical for IFN-γ production by γδ27$^+$ cells responding to TCR engagement in vitro or to Herpes virus infection in vivo. Additionally, T-bet also operates in γδ27$^-$ cells, particularly during *L. monocytogenes* in vivo. In contrast, although γδ27$^+$ T cells express high levels of *Eomes* mRNA (compared with γδ27$^+$ CCR6$^+$ T cells and Th1 cells), this transcription factor is not required for their production of IFN-γ. Of note, Eomes has been reported to play a critical role in the differentiation of tumoricidal Vγ4 γδ T cells by controlling perforin expression and cytolytic activity (49). The role of these transcriptional regulators, their functional partition and cooperation in controlling cytotoxic or IFN-γ effector functions, as well as potential memory formation, remains to be fully elucidated in γδ T cells. In any case, our data set firmly establishes that T-bet is the main regulator of IFN-γ production by γδ T cells. Moreover, in the absence of T-bet, we also found higher proportions of IL-17$^+$

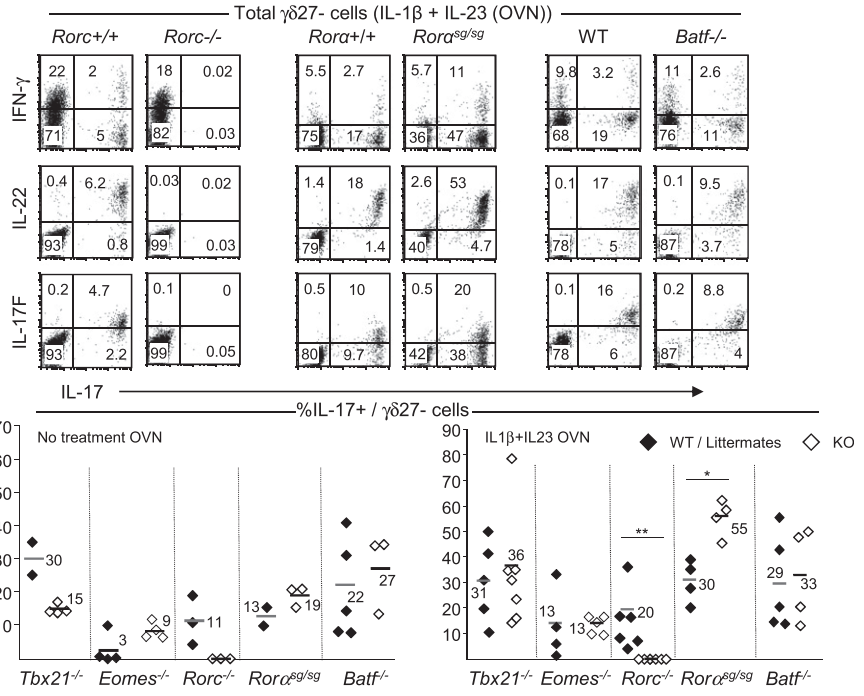

FIGURE 7. Production of IL-17, IL-17F, and IL-22 by total γδ27$^-$ cells depends on RORγt but not RORα or BATF. Analysis of effector γδ27$^-$ cells derived from *Tbx21*$^{-/-}$, *Eomes*$^{-/-}$, *Rorc*$^{-/-}$, *Rora*$^{sg/sg}$, and *Batf*$^{-/-}$ mice. γδ27$^-$ cells were FACS sorted from each strain and restimulated overnight with IL-1β/IL-23. Intracellular flow cytometry that assesses IFN-γ, IL-17, IL-17F, and IL-22 production is shown. The graphs show percentages of γδ27$^-$ T cells producing IL-17 after resting overnight (*left*) and IL-1β plus IL-23 stimulation (*right*) from two or more experiments. *$p < 0.05$, **$p < 0.01$ (Mann–Whitney two-tailed test).

γδ T cells and Th17 cells during *L. monocytogenes* infection and in response to EAE. Therefore, induction of IFN-γ and inhibition of IL-17 are T-bet–dependent functions conserved between γδ and CD4 T cells.

Conversely, RORγt deficiency completely abolished the production of IL-17 by γδ27$^-$ cells. This is consistent with previous studies (50–52), and our own data supporting a key role for RORγt in the development of the γδ27$^-$CCR6$^+$ cell subset (34). This fits well with the established role of RORγt in the emergence of innate cell types, including ILC3 and LTi [reviewed in (53)]. In contrast, it is interesting to note that several other transcription factors have been specifically implicated in the intrathymic development of IL-17–producing γδ T cells. These include Hes-1, a component of the Notch-signaling pathway (50); RelB, a member the NF-κB family (30); and Sox13, Sox4, and the Ets family member ETV5, which have been described as key regulators of the development of the

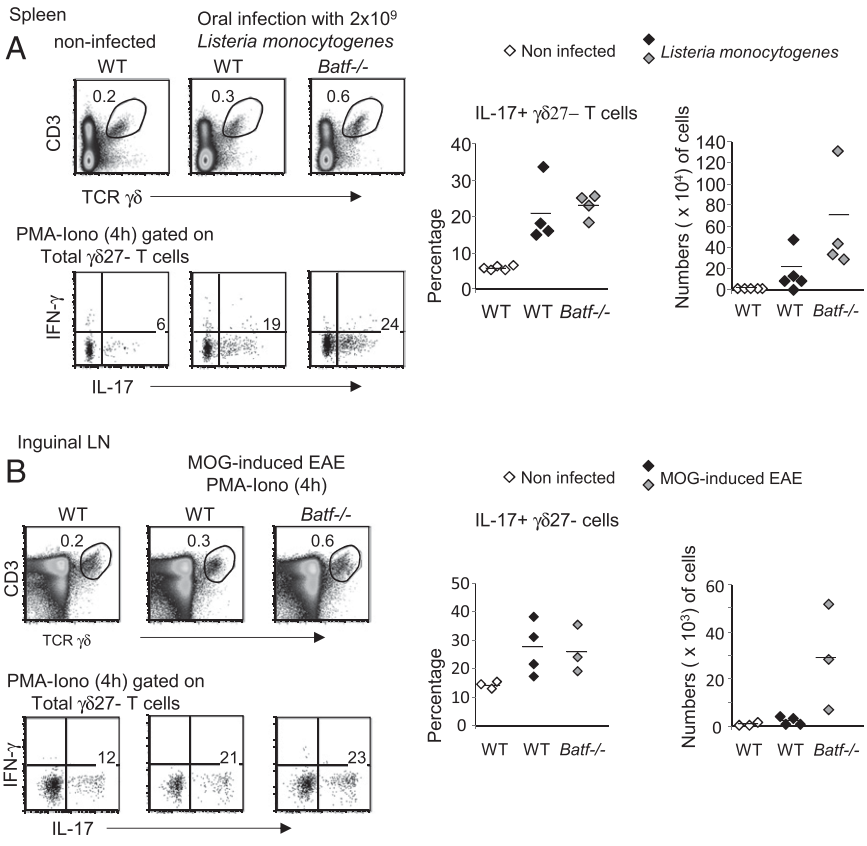

FIGURE 8. BATF is dispensable for IL-17 production by total γδ27$^-$ cells in vivo. (**A**) WT and *Batf*$^{-/-}$ mice were infected orally with *L. monocytogenes*, and splenocytes were harvested 1 wk later. Total splenocytes were restimulated with PMA/ionomycin for 4 h and assessed for production of IFN-γ and IL-17 by intracellular cytokine staining gated on γδ27$^-$ cells. (**B**) WT and *Batf*$^{-/-}$ mice were induced to develop EAE with MOG injection, and cells were harvested from the inguinal lymph nodes (draining the site of injection) 2 wk later. Total lymph node cells were restimulated with PMA/ionomycin for 4 h and assessed for production of IFN-γ and IL-17 by intracellular cytokine staining gated on γδ27$^-$ cells. The graphs on the *right* show the percentage of γδ27$^-$ T cells producing IL-17 from mice infected with *L. monocytogenes* (*top*) or induced to develop EAE (*bottom*). Each dot represents value for one individual mouse.

IL-17–producing γδ27$^-$CCR6$^+$ cell subset (54–56). However, it remains to be established whether (and how) these factors form a cooperative network with RORγt, and whether they operate in the periphery to control IL-17 responses by γδ27$^-$ cells.

Although RelB promotes the differentiation of thymic precursors into IL-17$^+$ γδ T cells possibly by controlling the expression of both RORγt and RORα, we found that IL-17 production by γδ T cells was independent of RORα. Additionally, although in Th17 cells RORγt binding requires chromatin remodelling via the cooperative action of IRF4 and BATF, we found that γδ T cells lacking BATF produce normal amount of IL-17. In the studies by Powolny-Budnicka et al. and Raifer et al. (29, 30), it was shown that γδ T cells from mice deficient in IRF4 can readily produce IL-17. In our study, we extended this type of analysis to mice deficient in RORα or BATF and found their γδ T cells differentiate into IL-17 producers similarly to WT controls. Thus, whereas our understanding of the functional differentiation of γδ T cells is still lagging behind that of αβ T cells, the present work highlights the existence of lineage-specific transcriptional mechanisms, namely an unconventional control of IL-17 production by innate-like γδ27$^-$ T cells.

This study was focused on peripheral γδ T cell subsets, whose functional properties can be either preprogrammed in the thymus (9, 10, 57) or induced upon peripheral activation (38). Whether these two differentiation processes rely on the same transcriptional regulators remains unknown. Differences in thymic development and peripheral differentiation may justify lineage-specific auxiliary transcription factor segregation between γδ and CD4 T cells for IL-17 induction. This underlies that CD4 T cells differentiating in the periphery rely on prolonged activation and expansion to implement the molecular machinery controlling IL-17 production. γδ27$^-$CCR6$^+$ T cells, however, are precommitted for IL-17 production with expression of RORγt at the protein level, which keeps the *Il17a* locus transcriptionally active with high amount of *Il17a* mRNA present and ready to receive environmental signaling to trigger its conversion into protein. This suggests that posttranscriptional mechanisms, such as small noncoding RNAs, are most likely playing a role in rapidly regulating cytokine translation, and we are currently investigating these possibilities. As indicated above, thymic preprogramming of IL-17$^+$ γδ T cells in vivo relies on a transcriptional network that strongly differs from that of peripheral differentiation of naive CD4 T cells into Th17 cells. This is consistent with exclusive signaling pathways operating in conventional CD4 T cells for production of IL-17 and in thymic precommitment of IL-17$^+$ γδ T cells. Thus, whereas the production and maintenance of Th17 cells require multiple cytokines, including IL-1β, IL-6, IL-21, IL-23, and TGF-β, only TGF-β has been reported to be critical for thymic development of IL-17$^+$ γδ T cells (58).

Promotion of an IL-17 response in peripheral γδ T cells relies on cytokines (3, 34). Although STAT3 signaling is not required for the development of IL-17$^+$ γδ T cells (50), in the periphery they rapidly respond to IL-1β as well as STAT3-dependent IL-7 and IL-23 signaling (3, 37, 59). By contrast, peripheral γδ27$^-$ cells have been reported to be hyporesponsive to TCR stimulation, particularly when compared with γδ27$^+$ cells (11). Given that BATF and IRF4 in conventional αβ T cells are triggered in part by TCR engagement, it is possible that the lack of responsiveness to TCR activation of γδ27$^-$ cells underlies their independence on BATF and IRF4. It was also shown that IRF4 was dispensable for IL-17 production by LTi/ILC3 and NKT cells (29), and, although the roles of BATF and RORα in these cell types remain to be elucidated, this might suggest an alternative IL-17 pathway in innate-like lymphocytes. The resolution of this putative alternative pathway is highly relevant given that IL-17$^+$ γδ T cells have been shown to contribute to diverse chronic inflammatory or autoimmune diseases such as colitis (7, 8, 60), psoriasis (61, 62), experimental autoimmune encephalomyelitis (3, 4), uveitis (63), as well as cancer progression in mice (64–66) and humans (67).

Even though IFN-γ and IL-17 are produced by a large diversity of cells, coproduction is a rare event that only occurs under certain specific inflammatory conditions. The acquisition of IFN-γ expression by Th17 cells has been well documented (14, 68, 69), but the transcriptional regulators of these Th1-like Th17 cells are still controversial. Essentially, it is unclear as to whether T-bet is dispensable (18, 22) or required (15, 17) for the emergence of Th1-like Th17 cells in vitro and in vivo. These discrepancies could be due to signaling differences, as both IL-12 and IL-23 have been shown to induce IFN-γ production in Th17 cells. However, we found that only IL-23, in synergy with IL-1β, but not IL-12, could induce the production of IFN-γ, IL-17F, IL-22, and GM-CSF in γδ27$^-$ cells. Surprisingly, despite the presence of transcripts for *Il17f*, *Il22*, and *Csf1* in γδ27$^-$ cells ex vivo, the corresponding proteins were not expressed upon PMA plus iono stimulation, but only after short-term (14-h) stimulation with IL-1β plus IL-23. This calls the attention to the stimulatory conditions used to trigger specific effector functions in γδ T cells in vitro. Importantly, upon IL-1β plus IL-23 treatment, the generation of IL-17$^+$ IFN-γ$^+$ γδ27$^-$ cells was impaired in the absence of T-bet; and those cells were completely absent in *Listeria*-infected T-bet–deficient mice. Thus, T-bet is the key regulator of IFN-γ production in both γδ27$^+$ and γδ27$^-$ T cell subsets.

Whereas both Th17 cells and γδ27$^-$ cells display a high degree of functional plasticity, their Th1 cells and γδ27$^+$ counterparts seem stably committed to IFN-γ production. However, a recent study challenged the stability of the γδ27$^+$ cell subset. One fourth of the Ly6C$^+$CD44^{int} γδ27$^+$ cells stimulated for 4 d with coated anti-CD3 and anti-CD28 mAb in the presence of IL-6, IL-23, and TGF-β1 were capable of differentiating into IL-17–producing γδ T cells (70). We never observed IL-17 production from γδ27$^+$ cells upon stimulation with IL-1β plus IL-23, which was performed for 36 h and in absence of TCR engagement. Moreover, the plasticity of γδ27$^+$ cells proposed by Lombes et al. (70) remains to be observed during in vivo responses.

In conclusion, we provide evidence for a common core transcriptional program dedicated to IFN-γ production and governed by T-bet in both CD4 and γδ T cells. By contrast, despite depending on RORγt, γδ27$^-$ cells are clearly distinct from Th17 cells as the auxiliary transcription factors BATF and RORα (and IRF4) are completely dispensable. These findings are likely to have important implications for the manipulation of proinflammatory γδ T cell responses in infection, cancer, and autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Damsker, J. M., A. M. Hansen, and R. R. Caspi. 2010. Th1 and Th17 cells: adversaries and collaborators. *Ann. N. Y. Acad. Sci.* 1183: 211–221.
2. Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 cells. *Annu. Rev. Immunol.* 27: 485–517.

3. Sutton, C. E., S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31: 331–341.
4. Petermann, F., V. Rothhammer, M. C. Claussen, J. D. Haas, L. R. Blanco, S. Heink, I. Prinz, B. Hemmer, V. K. Kuchroo, M. Oukka, and T. Korn. 2010. γδ T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism. *Immunity* 33: 351–363.
5. Hayday, A. C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 31: 184–196.
6. Zhu, J., H. Yamane, and W. E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annu. Rev. Immunol.* 28: 445–489.
7. Do, J. S., A. Visperas, C. Dong, W. M. Baldwin, III, and B. Min. 2011. Cutting edge: generation of colitogenic Th17 CD4 T cells is enhanced by IL-17+ γδ T cells. *J. Immunol.* 186: 4546–4550.
8. Park, S. G., R. Mathur, M. Long, N. Hosh, L. Hao, M. S. Hayden, and S. Ghosh. 2010. T regulatory cells maintain intestinal homeostasis by suppressing γδ T cells. *Immunity* 33: 791–803.
9. Jensen, K. D., X. Su, S. Shin, L. Li, S. Youssef, S. Yamasaki, L. Steinman, T. Saito, R. M. Locksley, M. M. Davis, et al. 2008. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity* 29: 90–100.
10. Ribot, J. C., A. deBarros, D. J. Pang, J. F. Neves, V. Peperzak, S. J. Roberts, M. Girardi, J. Borst, A. C. Hayday, D. J. Pennington, and B. Silva-Santos. 2009. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat. Immunol.* 10: 427–436.
11. Wencker, M., G. Turchinovich, R. Di Marco Barros, L. Deban, A. Jandke, A. Cope, and A. C. Hayday. 2014. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat. Immunol.* 15: 80–87.
12. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, et al. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302: 1041–1043.
13. Kuchroo, V. K., and A. Awasthi. 2012. Emerging new roles of Th17 cells. *Eur. J. Immunol.* 42: 2211–2214.
14. Hirota, K., J. H. Duarte, M. Veldhoen, E. Hornsby, Y. Li, D. J. Cua, H. Ahlfors, C. Wilhelm, M. Tolaini, U. Menzel, et al. 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat. Immunol.* 12: 255–263.
15. Lee, Y. K., H. Turner, C. L. Maynard, J. R. Oliver, D. Chen, C. O. Elson, and C. T. Weaver. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30: 92–107.
16. Muranski, P., Z. A. Borman, S. P. Kerkar, C. A. Klebanoff, Y. Ji, L. Sanchez-Perez, M. Sukumar, R. N. Reger, Z. Yu, S. J. Kern, et al. 2011. Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity* 35: 972–985.
17. Wang, Y., J. Godec, K. Ben-Aissa, K. Cui, K. Zhao, A. B. Pucsek, Y. K. Lee, C. T. Weaver, R. Yagi, and V. Lazarevic. 2014. The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon-γ-producing T helper 17 cells. *Immunity* 40: 355–366.
18. Duhen, R., S. Glatigny, C. A. Arbelaez, T. C. Blair, M. Oukka, and E. Bettelli. 2013. Cutting edge: the pathogenicity of IFN-γ-producing Th17 cells is independent of T-bet. *J. Immunol.* 190: 4478–4482.
19. Bending, D., H. De la Peña, M. Veldhoen, J. M. Phillips, C. Uyttenhove, B. Stockinger, and A. Cooke. 2009. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J. Clin. Invest.* 119: 565–572.
20. Lee, Y., A. Awasthi, N. Yosef, F. J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D. A. Hafler, et al. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nat. Immunol.* 13: 991–999.
21. Yang, Y., J. Weiner, Y. Liu, A. J. Smith, D. J. Huss, R. Winger, H. Peng, P. D. Cravens, M. K. Racke, and A. E. Lovett-Racke. 2009. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J. Exp. Med.* 206: 1549–1564.
22. Grifka-Walk, H. M., S. J. Lalor, and B. M. Segal. 2013. Highly polarized Th17 cells induce EAE via a T-bet independent mechanism. *Eur. J. Immunol.* 43: 2824–2831.
23. Kanno, Y., G. Vahedi, K. Hirahara, K. Singleton, and J. J. O'Shea. 2012. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annu. Rev. Immunol.* 30: 707–731.
24. Ciofani, M., A. Madar, C. Galan, M. Sellars, K. Mace, F. Pauli, A. Agarwal, W. Huang, C. N. Parkurst, M. Muratet, et al. 2012. A validated regulatory network for Th17 cell specification. *Cell* 151: 289–303.
25. Huber, M., A. Brüstle, K. Reinhard, A. Guralnik, G. Walter, A. Mahiny, E. von Löw, and M. Lohoff. 2008. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc. Natl. Acad. Sci. USA* 105: 20846–20851.
26. Brüstle, A., S. Heink, M. Huber, C. Rosenplänter, C. Stadelmann, P. Yu, E. Arpaia, T. W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 8: 958–966.
27. Schraml, B. U., K. Hildner, W. Ise, W. L. Lee, W. A. Smith, B. Solomon, G. Sahota, J. Sim, R. Mukasa, S. Cemerski, et al. 2009. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* 460: 405–409.
28. Yang, X. O., B. P. Pappu, R. Nurieva, A. Akimzhanov, H. S. Kang, Y. Chung, L. Ma, B. Shah, A. D. Panopoulos, K. S. Schluns, et al. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28: 29–39.
29. Raifer, H., A. J. Mahiny, N. Bollig, F. Petermann, A. Hellhund, K. Kellner, A. Guralnik, K. Reinhard, E. Bothur, M. Huber, et al. 2012. Unlike αβ T cells, γδ T cells, LTi cells and NKT cells do not require IRF4 for the production of IL-17A and IL-22. *Eur. J. Immunol.* 42: 3189–3201.
30. Powolny-Budnicka, I., M. Riemann, S. Tänzer, R. M. Schmid, T. Hehlgans, and F. Weih. 2011. RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in γδ T cells. *Immunity* 34: 364–374.
31. Jordan-Williams, K. L., S. Poston, and E. J. Taparowsky. 2013. BATF regulates the development and function of IL-17 producing iNKT cells. *BMC Immunol.* 14: 16.
32. Haas, J. D., F. H. González, S. Schmitz, V. Chennupati, L. Föhse, E. Kremmer, R. Förster, and I. Prinz. 2009. CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. *Eur. J. Immunol.* 39: 3488–3497.
33. Martin, B., K. Hirota, D. J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 31: 321–330.
34. Schmolka, N., K. Serre, A. R. Grosso, M. Rei, D. J. Pennington, A. Q. Gomes, and B. Silva-Santos. 2013. Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory γδ T cell subsets. *Nat. Immunol.* 14: 1093–1100.
35. Sheridan, B. S., P. A. Romagnoli, Q. M. Pham, H. H. Fu, F. Alonzo, III, W. D. Schubert, N. E. Freitag, and L. Lefrançois. 2013. γδ T cells exhibit multifunctional and protective memory in intestinal tissues. *Immunity* 39: 184–195.
36. Ribot, J. C., M. Chaves-Ferreira, F. d'Orey, M. Wencker, N. Gonçalves-Sousa, J. Decalf, J. P. Simas, A. C. Hayday, and B. Silva-Santos. 2010. Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-γ- or IL-17-producing γδ T cells upon infection. *J. Immunol.* 185: 6421–6425.
37. Michel, M. L., D. J. Pang, S. F. Haque, A. J. Potocnik, D. J. Pennington, and A. C. Hayday. 2012. Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing γδ cells. *Proc. Natl. Acad. Sci. USA* 109: 17549–17554.
38. Zeng, X., Y.-L. Wei, J. Huang, E. W. Newell, H. Yu, B. A. Kidd, M. S. Kuhns, R. W. Waters, M. M. Davis, C. T. Weaver, and Y. H. Chien. 2012. γδ T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. *Immunity* 37: 524–534.
39. Corpuz, T. M., J. Stolp, H.-O. Kim, G. V. Pinget, D. H. D. Gray, J.-H. Cho, J. Sprent, and K. E. Webster. 2016. Differential responsiveness of innate-like IL-17- and IFN-γ-producing γδ T cells to homeostatic cytokines. *J. Immunol.* 196: 645–654.
40. Price, A. E., R. L. Reinhardt, H. E. Liang, and R. M. Locksley. 2012. Marking and quantifying IL-17A-producing cells in vivo. *PLoS One* 7: e39750.
41. Yin, Z., C. Chen, S. J. Szabo, L. H. Glimcher, A. Ray, and J. Craft. 2002. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-gamma by gammadelta T cells. *J. Immunol.* 168: 1566–1571.
42. Chen, L., W. He, S. T. Kim, J. Tao, Y. Gao, H. Chi, A. M. Intlekofer, B. Harvey, S. L. Reiner, Z. Yin, et al. 2007. Epigenetic and transcriptional programs lead to default IFN-γ production by gammadelta T cells. *J. Immunol.* 178: 2730–2736.
43. Sciumé, G., K. Hirahara, H. Takahashi, A. Laurence, A. V. Villarino, K. L. Singleton, S. P. Spencer, C. Wilhelm, A. C. Poholek, G. Vahedi, et al. 2012. Distinct requirements for T-bet in gut innate lymphoid cells. *J. Exp. Med.* 209: 2331–2338.
44. Klose, C. S. N., E. A. Kiss, V. Schwierzeck, K. Ebert, T. Hoyler, Y. d'Hargues, N. Göppert, A. L. Croxford, A. Waisman, Y. Tanriver, and A. Diefenbach. 2013. A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. *Nature* 494: 261–265.
45. Basu, R., D. B. O'Quinn, D. J. Silberger, T. R. Schoeb, L. Fouser, W. Ouyang, R. D. Hatton, and C. T. Weaver. 2012. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity* 37: 1061–1075.
46. Sciammas, R., P. Kodukula, Q. Tang, R. L. Hendricks, and J. A. Bluestone. 1997. T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis. *J. Exp. Med.* 185: 1969–1975.
47. Lazarevic, V., X. Chen, J.-H. Shim, E.-S. Hwang, E. Jang, A. N. Bolm, M. Oukka, V. K. Kuchroo, and L. H. Glimcher. 2011. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt. *Nat. Immunol.* 12: 96–104.
48. Guo, S., D. Cobb, and R. B. Smeltz. 2009. T-bet inhibits the in vivo differentiation of parasite-specific CD4+ Th17 cells in a T cell-intrinsic manner. *J. Immunol.* 182: 6179–6186.
49. He, W., J. Hao, S. Dong, Y. Gao, J. Tao, H. Chi, R. Flavell, R. L. O'Brien, W. K. Born, J. Craft, et al. 2010. Naturally activated V gamma 4 gamma delta T cells play a protective role in tumor immunity through expression of eomesodermin. *J. Immunol.* 185: 126–133.
50. Shibata, K., H. Yamada, T. Sato, T. Dejima, M. Nakamura, T. Ikawa, H. Hara, S. Yamasaki, R. Kageyama, Y. Iwakura, et al. 2011. Notch-Hes1 pathway is required for the development of IL-17-producing γδ T cells. *Blood* 118: 586–593.
51. Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121–1133.
52. Lochner, M., L. Peduto, M. Cherrier, S. Sawa, F. Langa, R. Varona, D. Riethmacher, M. Si-Tahar, J. P. Di Santo, and G. Eberl. 2008. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J. Exp. Med.* 205: 1381–1393.

53. Shih, H. Y., G. Sciumè, A. C. Poholek, G. Vahedi, K. Hirahara, A. V. Villarino, M. Bonelli, R. Bosselut, Y. Kanno, S. A. Muljo, and J. J. O'Shea. 2014. Transcriptional and epigenetic networks of helper T and innate lymphoid cells. *Immunol. Rev.* 261: 23–49.
54. Jojic, V., T. Shay, K. Sylvia, O. Zuk, X. Sun, J. Kang, A. Regev, D. Koller, A. J. Best, and J. Knell, ARegev, , and DKoller, , Immunological Genome Project Consortium. 2013. Identification of transcriptional regulators in the mouse immune system. *Nat. Immunol.* 14: 633–643.
55. Malhotra, N., K. Narayan, O. H. Cho, K. E. Sylvia, C. Yin, H. Melichar, M. Rashighi, V. Lefebvre, J. E. Harris, L. J. Berg, and J. Kang, Immunological Genome Project Consortium. 2013. A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity* 38: 681–693.
56. Gray, E. E., F. Ramírez-valle, Y. Xu, S. Wu, Z. Wu, K. E. Karjalainen, and J. G. Cyster. 2013 Deficiency in IL-17-committed Vγ4(+) γδ T cells in a spontaneous Sox13-mutant CD45.1(+) congenic mouse substrain provides protection from dermatitis. *Nat. Immunol.* 14: 584–592.
57. Zarin, P., E. L. Y. Chen, T. S. H. In, M. K. Anderson, and J. C. Zúñiga-Pflücker. 2015. Gamma delta T-cell differentiation and effector function programming, TCR signal strength, when and how much? *Cell. Immunol.* 296: 70–75.
58. Do, J. S., P. J. Fink, L. Li, R. Spolski, J. Robinson, W. J. Leonard, J. J. Letterio, and B. Min. 2010. Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism. *J. Immunol.* 184: 1675–1679.
59. Duan, J., H. Chung, E. Troy, and D. L. Kasper. 2010. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe* 7: 140–150.
60. Do, J.-S., A. Visperas, M. L. Freeman, Y. Iwakura, M. Oukka, and B. Min. 2014. Colitogenic effector T cells: roles of gut-homing integrin, gut antigen specificity and γδ T cells. *Immunol. Cell Biol.* 92: 90–98.
61. Cai, Y., X. Shen, C. Ding, C. Qi, K. Li, X. Li, V. R. Jala, H. G. Zhang, T. Wang, J. Zheng, and J. Yan. 2011. Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35: 596–610.
62. Pantelyushin, S., S. Haak, B. Ingold, P. Kulig, F. L. Heppner, A. A. Navarini, and B. Becher. 2012. Rorγt+ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J. Clin. Invest.* 122: 2252–2256.
63. Liang, D., A. Zuo, H. Shao, W. K. Born, R. L. O'Brien, H. J. Kaplan, and D. Sun. 2013. IL-23 receptor expression on γδ T cells correlates with their enhancing or suppressive effects on autoreactive T cells in experimental autoimmune uveitis. *J. Immunol.* 191: 1118–1125.
64. Ma, S., Q. Cheng, Y. Cai, H. Gong, Y. Wu, X. Yu, L. Shi, D. Wu, C. Dong, and H. Liu. 2014. IL-17A produced by γδ T cells promotes tumor growth in hepatocellular carcinoma. *Cancer Res.* 74: 1969–1982.
65. Rei, M., N. Gonçalves-Sousa, T. Lança, R. G. Thompson, S. Mensurado, F. R. Balkwill, H. Kulbe, D. J. Pennington, and B. Silva-Santos. 2014. Murine CD27(-) Vγ6(+) γδ T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. *Proc. Natl. Acad. Sci. USA* 111: E3562–E3570.
66. Coffelt, S. B., K. Kersten, C. W. Doornebal, J. Weiden, K. Vrijland, C.-S. Hau, N. J. M. Verstegen, M. Ciampricotti, L. J. C. Hawinkels, J. Jonkers, and K. E. de Visser. 2015. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 522: 345–348.
67. Wu, P., D. Wu, C. Ni, J. Ye, W. Chen, G. Hu, Z. Wang, C. Wang, Z. Zhang, W. Xia, et al. 2014. γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity* 40: 785–800.
68. Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Filì, S. Ferri, F. Frosali, et al. 2007. Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204: 1849–1861.
69. Boniface, K., W. M. Blumenschein, K. Brovont-Porth, M. J. McGeachy, B. Basham, B. Desai, R. Pierce, T. K. McClanahan, S. Sadekova, and R. de Waal Malefyt. 2010. Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage. *J. Immunol.* 185: 679–687.
70. Lombes, A., A. Durand, C. Charvet, M. Rivière, N. Bonilla, C. Auffray, B. Lucas, and B. Martin. 2015. Adaptive immune-like γ/δ T lymphocytes share many common features with their α/β T cell counterparts. *J. Immunol.* 195: 1449–1458.