T-bet optimizes CD4 T-cell responses against influenza through CXCR3-dependent lung trafficking but not functional programming

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Although clearance of many intracellular pathogens requires T-bet-dependent CD4 T cell programming, the extent to which T-bet is needed to direct protective CD4 responses against influenza is not known. Here, we characterize wild-type and T-bet-deficient CD4 cells during murine influenza infection. Surprisingly, although T-bet expression has broad impacts on cytokine production by virus-specific CD4 cells, the protective efficacy of T-bet-deficient effector cells is only marginally reduced. This reduction is due to lower CXCR3 expression, leading to suboptimal accumulation of activated T-bet-deficient cells in the infected lung. However, T-bet-deficient cells outcompete wild-type cells to form lung-resident and circulating memory populations following viral clearance, and primed T-bet-deficient mice efficiently clear supralethal heterosubtypic influenza challenges even when depleted of CD8 T cells. These results are relevant to the identification of more incisive correlates of protective T cells and for vaccines that aim to induce durable cellular immunity against influenza.

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INTRODUCTION

CD4 T cells combat pathogens through direct effector functions and by helping to maximize the protective activities of other leukocytes.1 There is increasing interest in improving the ability of vaccines to prime CD4 immunity against threats like Influenza A virus (IAV) that can escape antibody-mediated protection. Prerequisite for such approaches is establishing the kinds of CD4 responses needed to clear a given microbe. This question has been framed for the last 30 years by the expanded paradigm that categorizes CD4 cells largely based on their cytokine production. In general, protection against intracellular pathogens is believed to require Th1-polarized cells characterized by strong IFNγ production and a broader differentiation program guided by the ‘master’ transcription factor T-bet.2

A number of functionally distinct subsets of CD4 T cells combat IAV using multiple mechanisms that provide synergizing and redundant layers of protection.3,4 A complete description of the distinct mechanisms brought to bear as part of this integrated response is still evolving, but an implicit assumption is that T-bet-dependent programming is crucial to successful CD4 T cell-mediated IAV clearance. Seminal work found that Th1-polarized clones recognizing IAV could transfer immunity to unprimed hosts while Th2 clones could not.5 Subsequent studies showed that IAV-specific Th1 effector or memory cells also protect naïve mice while Th2 and unpolarized (Th0) cells do not.6,7 Furthermore, IFNγ production is the hallmark of CD4 cells responding to IAV and in some models CD4 T cell protection is IFNγ-dependent.8,9 Indeed, IFNγ remains by far the most measured CD4 attribute across human and animal IAV studies, supporting the consensus that Th1 responses underlie effective CD4 T cell immunity.

Some evidence, however, indicates that prototypical Th1 cells may not be needed for robust immunity against IAV. For example, IFNγ-deficient mice have been shown to be no more susceptible to IAV than WT mice,10 and we found IFNγ neutralization not to compromise the ability of Th1-polarized memory cells to protect naïve WT mice.9 In fact, ablating IFNγ signaling can reduce morbidity during IAV infection, correlating with improved innate lymphoid cell function11 and reduced viral spread.12 Additionally, IAV-specific Th17 cells can protect naïve mice against IAV13 and may contribute to vaccine-primed immunity.14

To determine how T-bet expression affects the overall development of protective CD4 effector and memory responses we analyzed WT and T-bet-deficient (Tbx21−/−) T cell receptor transgenic as well as polyclonal CD4 cells responding against IAV. Whereas T-bet does not impact activation, it has a broad impact on effector cytokine production, highlighted by decreased IFNγ and increased IL-17 production by Tbx21−/− T cells. CD4 T cell-intrinsic T-bet is also required for maximal effector accumulation in the lung. Higher expression of the known T-bet-regulated chemokine receptor CXCR315 is alone responsible for increased WT vs. Tbx21−/− CD4 responses in the lung. We transferred effector cells primed in vitro under “Th1” conditions to naïve mice and challenged with IAV to determine the extent that T-bet impacts their anti-viral capacity. While WT and Tbx21−/− effectors both protect against lethal IAV, ultimately the input of fewer WT donors is required, reflecting the marginally compromised lung trafficking of Tbx21−/− effectors.

Tbx21−/− effectors at the peak of the anti-viral response display hallmarks of a phenotype more associated with memory precursor cells than do WT effectors and they outcompete WT CD4 cells to...
form lung-resident and circulating memory cells after viral clearance. Finally, we find IAV-primed WT and Tbx21−/− mice are similarly protected against supralethal heterosubtypic IAV challenge, even when the primed mice are depleted of CD8 cells prior to secondary challenge. Our results indicate that the establishment of protective CD4 cell memory and robust secondary CD4 T cell responses against IAV do not require CD4 cell-intrinsic or extrinsic T-bet expression.

In summary, we find that classic Th1 programming is not needed for CD4 effectors to promote efficient IAV clearance. However, two important findings emerge with relevance to vaccination. First, T-bet-dependent CXCR3 levels maximize CD4 effector accumulation in the lung. In fact, we suggest CXCR3 expression is the most insightful T-bet-dependent correlate of effector accumulation in the lung. In fact, we suggest CXCR3 expression is the most insightful T-bet-dependent correlate of effector accumulation in the lung.

RESULTS

T-bet-deficiency prevents development of Th1 but not Th2 or Th17 cells in vitro
To determine the role of T-bet in CD4 responses against IAV, we bred Tbx21−/− OT-II TCR transgenic mice recognizing an epitope of ovalbumin (OVA) expressed by the A/PR8-OVAi virus. Prior to experiments with IAV, we confirmed the phenotype of Tbx21−/− effector cells generated in defined priming environments in vitro using antigen presenting cells and OVAi peptide. As expected, Tbx21−/− cells primed in “Th1” conditions did not show the strong IFNγ production seen from WT cells (Supplementary Fig. 1). Th2 cytokines were not produced by Tbx21−/− “Th1” effectors (not shown), nor was IL-17 (Supplementary Fig. 1). T-bet-deficiency did not alter expression of key transcription factors or cytokines in Th2 or Th17 cells, or in un polarized Th0 cells (Supplementary Fig. 1). Finally, T-bet did not impact expansion in any condition (Supplementary Fig. 1). These results confirm a need for T-bet to program prototypical ‘Th1’ responses marked by strong IFNγ production.

T-bet promotes maximal lung CD4 responses and broadly impacts cytokine production
We transferred 1 × 10^6 naive CFSE-labeled WT or Tbx21−/− OT-II cells to CD45.1^+ B6 hosts and challenged with a sublethal (0.2 LD_{50}) dose of A/PR8-OVAi. We first analyzed donor cells in the dLN at 5 dpi, before their appreciable migration to the lung. Similar numbers of WT and Tbx21−/− donor cells were present, and all were CFSE_{low} (not shown). WT and Tbx21−/− cells displayed similar IL-2 production, but more IL-2^{high} cells were seen in Tbx21−/− cells, resulting in increased MFI at the population level (Fig. 1a). Consistent with results in vitro, Tbx21−/− cells made less IFNγ (Fig. 1b) but a small percentage also produced IL-17 while almost no IL-17^{+} WT cells were seen (Fig. 1c). Both IFNγ^– and IFNγ^+ Tbx21−/− cells were IL-17^{–} (Fig. 1d), indicating diverse cytokine production patterns within the Tbx21−/− effectors. Thus, T-bet does not impact the kinetics of CD4 T cell activation but does alter the functional potential of early effector cells primed by IAV infection.

The frequency and number of WT and Tbx21−/− cells at 7 dpi (the peak of donor CD4 responses) were similar in the spleen and dLN, but Tbx21−/− cells in the lung reached only about one half of the WT response (Figs. 2a, b). Lower Tbx21−/− counts were not due to less proliferation as Ki67 staining in WT and Tbx21−/− cells was comparable (Fig. 2c). Decreased Tbx21−/− cell accumulation correlated with their slightly lower expression of the integrin CD11a (Fig. 2d) and the chemokine receptor CXCR3 (Fig. 2e). CD11a supports CD4 lung trafficking during mycobacterial challenge, and CXCR3, a known T-bet target, optimizes lung CD4 trafficking in a parainfluenza model. In contrast, Tbx21−/− cells expressed slightly more CCR4 (Fig. 2f), which is also linked to CD4 lung trafficking during IAV infection, but both cell types expressed many other chemokine receptors similarly including CXCR5, CCR5, CCR6, and CCR7 (not shown). Thus, T-bet expression by CD4 cells is required for maximal effector responses in the lung, possibly through regulating cell trafficking.

Analysis of cytokine production at 7 dpi by effectors responding in the lung revealed reduced IFNγ and increased IL-2 by Tbx21−/− cells (Fig. 3a), a pattern also seen in the spleen and dLN (Supplementary Fig. 2). A sizable cohort of Tbx21−/− but not WT effectors produced IL-17 (Fig. 3a), while little IL-17 was seen.

Fig. 1  Increased IL-17 and diminished IFNγ production from early Tbx21−/− effectors primed by IAV. 1 × 10^6 naive WT or Tbx21−/− OT-II cells were adoptively transferred to congenic (CD45.1^+) WT hosts and challenged with a sublethal dose of PR8-OVAi. At 5 dpi, dLNs were harvested and cytokine production assessed from donor cells by intracellular staining. a Representative staining for IL-2 from WT (black) and Tbx21−/− (grey) cells and control staining of WT cells without re-stimulation (solid) along with a summary of the percent of IL-2^{+} positive cells (left) and MFI (right) for each population (n = 4 mice/group). b IFNγ production with representative staining (left) and the percent of positive cells (right). c Representative staining for IL-17 from WT (left) and Tbx21−/− (right) cells with summary from 4 mice/group, and (d) representative staining of IL-17 vs. IFNγ from Tbx21−/− cells. Results presented from 1 of 2 similar experiments.
outside of the lung (Supplementary Fig. 2). There was no difference in IL-10 production, which was limited to the lungs as established previously\(^6\) (Fig. 3a). TNF levels were not impacted by T-bet (Fig. 3a and Supplementary Fig. 2), but increased IL-4 and reduced GM-CSF were seen from \(\text{Tbx21}^{-/-}\) cells in all organs tested (Fig. 3a and Supplementary Fig. 2). T-bet expression thus impacts diverse aspects of cytokine production by IAV-primed CD4 T cells, especially in the lung (Fig. 3b). WT and \(\text{Tbx21}^{-/-}\) cells expressed similar levels of Granzyme B, with higher levels in lung tissues (Fig. 3c), a pattern consistent with previous studies.\(^6\)

Virtually all WT effector cells in the lung expressed T-bet at 7 dpi (Fig. 3c) that, together with high IFN\(\gamma\) and low IL-4 and IL-17 production, clearly mark the WT response against IAV as strongly Th1-polarized. Increased IL-17 and IL-4 production by \(\text{Tbx21}^{-/-}\) cells correlated with \(<30\%\) staining for Ror\(\gamma\) and \(<4\%\) for GATA-3 (Fig. 3d). These results indicate CD4 T cell-intrinsic T-bet represses Th17 and Th2 elements that can otherwise be directed by infection-induced signals. Very few FoxP3\(^+\) donor cells were detected, indicating that \(\text{Tbx21}^{-/-}\) cells are not more prone to Treg differentiation than are WT cells (Fig. 3d). Finally, we observed similar frequencies of follicular helper cells (T\(\text{FH}\)) within WT and \(\text{Tbx21}^{-/-}\) effectors in the spleen and dLN (Fig. 3e).

It is critical to confirm key results made with transgenic OT-II cells with endogenous polyclonal CD4 T cell responses. Because T-bet is expressed by adaptive and innate immune cells,\(^20\) and could thus indirectly impact CD4 T cell responses in \(\text{Tbx21}^{-/-}\) mice, we created chimeras using 50/50% WT (Thy1.1/Thy1.2) and \(\text{Tbx21}^{-/-}\) (Thy1.2) bone marrow to reconstitute lethally irradiated Thy1.1 B6 hosts. This approach allows WT and \(\text{Tbx21}^{-/-}\) cells responding in the same environment to be clearly resolved (Fig. 4a). After validating chimera establishment, we challenged with A/PR8, and IAV-specific CD4 cells were visualized using a tetramer for the major I-A\(^d\) restricted epitope NP\(_{311-325}\) (Fig. 4b). Similar frequencies of WT and \(\text{Tbx21}^{-/-}\) NP\(_{311}\) cells were seen in the spleen and dLN at 9 dpi, but >70% of tetramer\(^+\) cells in the lungs were WT (Fig. 4c). Combined with lower CXCR3 expression by polyclonal \(\text{Tbx21}^{-/-}\) cells (Fig. 4d), these findings are consistent with results from OT-II cells above. The lung CD4 \(^{+}\)CD44\(^{+}\) \(\text{Tbx21}^{-/-}\) cells were also marked by reduced IFN\(\gamma\) and increased IL-17 and IL-2 (Figs. 4e, f), again confirming hallmarks of the WT vs. \(\text{Tbx21}^{-/-}\) OT-II response.

CXCR3-mediated signals alone lead to improved lung responses from WT CD4 cells

We next addressed the extent to which CXCR3-mediated signals account for greater WT vs. \(\text{Tbx21}^{-/-}\) CD4 T cell accumulation in infected lungs. We co-transferred equal numbers of WT (Thy1.1/Thy1.2) and \(\text{Tbx21}^{-/-}\) (Thy1.2) OT-II cells to CD45.1\(^+\) B6 mice, challenged with PR8-OVA\(_{489}\), and treated one group of recipients with blocking antibody against CXCR3 from 3–6 dpi and the other with PBS (Fig. 5a). We did not block CXCR3 directly after infection as CXCR3-mediated interactions can impact CD4 T cell activation.\(^21\) At 7 dpi we confirmed CXCR3 blockade (Figs. 5b, c) and assessed donor cell accumulation in the lungs. While \(\text{Tbx21}^{-/-}\) responses were largely unaffected by CXCR3 blockade, WT cells were reduced by about half (Fig. 5d), leading to similar numbers of WT and \(\text{Tbx21}^{-/-}\) cells. We asked if a particular
subset of WT effectors was impacted by CXCR3 blockade, but extensive phenotyping (not shown), and analysis of cytokine production (Fig. 5e) revealed similar cohorts of WT cells in the lung with or without CXCR3 blockade. T-bet-dependent CXCR3-mediated signaling is thus entirely responsible for improved accumulation of WT vs. T-bet-deficient CD4 effectors in IAV-infected lungs.

Tbx21$$^{-/-}$$ CD4 effectors are protective against lethal IAV infection. For decades, effective CD4 T cell responses against IAV have been characterized as strongly Th1-polarized. This conclusion rests largely on using differentially-polarized effector cells to transfer immunity to unprimed mice. We used this approach to compare the protective efficacy of an equal number of WT or Tbx21$$^{-/-}$$ OT-Ii effectors generated in 'Th1' conditions (as in Supplementary Fig. 1). We first transferred 3 x 10^6 cells and found that both effectors protected against lethal PR8-OVA, but that recipients of Tbx21$$^{-/-}$$ cells lost around 10% more weight (Fig. 6a). Nevertheless, recipients of both effector types similarly gained weight back and showed no difference in viral clearance vs. control mice not receiving donor cells that succumbed to infection (Fig. 6b). T-bet programming is thus largely dispensable for CD4 effector-mediated protection even against lethal IAV challenge.

The responding Tbx21$$^{-/-}$$ and WT effectors displayed similar differences in cytokine production as effectors arising from naive CD4 cells shown in Fig. 3, highlighted by reduced IFN$$\gamma$$ and GM-CSF, and enhanced IL-2, IL-4, and IL-17 (not shown). Given that increased IL-17 production is the most remarkable functional distinction between WT and Tbx21$$^{-/-}$$ cells, and that IL-17 may either be protective during IAV infection or drive immunopathology and weight loss, we treated mice receiving Tbx21$$^{-/-}$$ effectors with antibody to neutralize IL-17 or an isotype control. Blocking IL-17 caused only a minor reduction in weight loss (Fig. 6c) and had no impact on viral control (Fig. 6d). IL-17 is thus neither a critical component of the protective Tbx21$$^{-/-}$$ CD4 response, nor a major driver of weight loss during IAV challenge.

CXCR3 is required to maximize the efficacy of CD4 T cell effector-mediated protection. We next assessed the number of WT or Tbx21$$^{-/-}$$ donor effectors at 5 dpi, the peak of their response in this model. No differences were seen in the spleen or dLN, but fewer Tbx21$$^{-/-}$$ effectors were seen in lungs (Fig. 6e), similar to findings using naive cell transfers. We hypothesized that titration of the effectors transferred should thus reveal a defect in Tbx21$$^{-/-}$$ effector-mediated protection, as their number in the lung could drop below a required threshold earlier than for WT effectors. Indeed, when 1 x 10^6 effector cells were transferred, WT recipients began to recover by 9 dpi while mice receiving Tbx21$$^{-/-}$$ cells did not (Fig. 6f) and had higher viral titers (Fig. 6g).

Finally, we asked if we could recapitulate the slightly impaired protection provided by Tbx21$$^{-/-}$$ effectors by blocking CXCR3 in recipients of 3 x 10^6 WT effectors. Indeed, anti-CXCR3 treatment from 1–7 dpi significantly increased weight loss and delayed recovery of adoptive hosts that were nevertheless still protected (Fig. 6h). These results indicate that T-bet-dependent CXCR3 expression by CD4 effectors is needed for their optimal protective potential, but that T-bet-dependent cytokine production is not needed for them to mediate efficient anti-viral responses.
Improved IAV-primed memory generation from T-bet-deficient CD4 T cells

The ability of primed CD4 cells to contribute to immunity depends entirely on their ability to form memory. We thus tested how T-bet impacts memory generation following IAV priming. We first assessed expression of markers associated with differential survival potential of effector cells at the peak of the OT-II (7 dpi) and endogenous (8 dpi) CD4 T cell response. Tbx21−/− cells expressed reduced Ly6C (Fig. 7a), a marker of terminal differentiation in some models,24,25 and increased IL-7 receptor alpha chain (CD127) (Fig. 7b), increased levels of which correlate with improved fitness of effector CD4 T cells to form memory in this model.26 Tbx21−/− effector cells also expressed more CD25 (Fig. 7c), consistent with their enhanced IL-2 production, and fitting with the need for IL-2 signaling to effector cells at this timepoint to induce sustained CD127.27

The phenotypic analysis above indicates that Tbx21−/− cells may outcompete WT cells for memory niches. To test this in the most direct way, we transferred equal numbers of naïve WT (Thy1.1/Thy1.2) and Tbx21−/− (Thy1.2) OT-II cells to CD45.1+ B6 hosts and primed with PR8-OVAII as in Fig. 5. We assessed the

Fig. 4 Polyclonal Tbx21−/− CD4 responses display hallmarks of the Tbx21−/− OT-II response. a Bone marrow chimeric mice were made as depicted. b Following sublethal PR8 infection, IAV-specific cells were detected at 9 dpi using the NP311 tetramer compared to staining with CLIP control tetramer as shown in representative panels. c The average frequency of WT vs. Tbx21−/− within the polyclonal NP311+ population is shown for 3 mice/group for spleen (S), dLN (D), and lungs (L). d Representative staining of CXCR3 from WT and Tbx21−/− NP311+ cells in the lung (left) and MFI analysis from 3 mice/group (right), and (e) representative intracellular staining for IFNγ vs. IL-17 from WT (left panel) vs. Tbx21−/− (right panel) CD44high CD4 cells in the lung at 9 dpi (e). f Summary from 3 mice/group is shown for the stated cytokines detected from CD44high CD4 cells in the lung. Results from one of two similar experiments.

Fig. 5 CXCR3-mediated signals are required for maximal CD4 responses in the lung. a 1×10^6 of both naïve WT (Thy1.1/Thy1.2) and Tbx21−/− (Thy1.2) OT-II cells were transferred to WT CD45.1+ hosts, challenged with sublethal PR8-OVAII, and either treated with CXCR3 blocking antibody or PBS alone from 3–6 dpi. On 7 dpi, CXCR3 blockade was confirmed by FACS analysis assessing CXCR3 on WT OT-II cells using the same antibody clone, with (b) representative staining (c) and summary MFI analysis from WT donor cells 3 mice/group. d the absolute number of WT (left) and Tbx21−/− (right) donor cells responding in the lung is shown. e Representative cytokine production from WT donor cells in the lung from mice either treated (right panel) or not (left panel) with CXCR3-blocking antibody. Results from one of three similar experiments.
with different genotypes to populate memory niches. Only express higher CD127 than WT cells at 28 dpi (Fig. 7e), which we previous studies (Fig. 7g). Indeed, we observed similar high the lung at 28 dpi, and low frequencies of CD69 high in the spleen (Fig. 7h). Virtually no FoxP3

Tbx21 −/− effectors were challenged with 2 LD50 PR8-OVA and were treated throughout the first week of infection with either IL-17 neutralizing antibody or with an isotype control antibody. The frequency of donor WT and Tbx21 −/− effectors in stated organs is shown at 5 dpi. Adoptive hosts were given 1 × 10^6 effector cells and shown is the (f) weight loss and (g) pulmonary viral titer analysis from 4 mice/group at 9 dpi. Recipients of 3 × 10^6 WT effectors were treated with CXCR3-blocking antibody from 0–7 dpi or with PBS alone. H. Weight loss and recovery is shown from four mice/group. Results representative of at least two independent experiments.

frequency of WT vs Tbx21 −/− memory cells within the donor gate at 28 dpi, a well-accepted memory timepoint and an approach we have used previously to directly compare the fitness of CD4 cells with different genotypes to populate memory niches.26,28 Only about 20% of donor cells recovered at 28 dpi were WT, indicating a decisive advantage for Tbx21 −/− cells to form memory and to survive long-term (Fig. 7d). Indeed, Tbx21 −/− cells continued to express higher CD127 than WT cells at 28 dpi (Fig. 7e), which we find is the most robust marker of CD4 T-cell memory fitness in this model.30

We next re-stimulated the memory cells and assayed for IFNγ and IL-17 and IL-2 production at 28 dpi to determine the functional relationship of the Tbx21 −/− memory cells to the effector cells analyzed at 7 dpi. Tbx21 −/− cells produced reduced but still substantial IFNγ vs. WT memory cells, retained a strong IL-17 response component while virtually no IL-17 was detected from WT cells, and more Tbx21 −/− memory cells were IL-2+ (Fig. 7f). This analysis indicates that cytokine production potential of Tbx21 −/− cells as they transition from the effector to the memory state does not significantly shift in terms of prototypical Th1 vs Th17 functions.

Recent studies indicate that CD4 cells with reduced T-bet expression establish lung-resident memory after IAV priming with greater efficiency.35 In agreement with these findings, the majority of Tbx21 −/− memory cells in the lung fit criteria of lung tissue-resident memory by expression of a CD69<sup>high</sup> CD127<sup>low</sup> phenotype, combined with shielding from labeling by fluorescent anti-CD4 antibody given to the mice just prior to organ harvest (Fig. 7g). In contrast, Tbx21 −/− memory cells in the spleen displayed a CD69<sup>low</sup> CD127<sup>high</sup> phenotype, consistent with our previous studies.38 (Fig. 7g). Indeed, we observed similar high frequencies of CD69<sup>high</sup> WT and Tbx21 −/− OT-II memory cells in the lung at 28 dpi, and low frequencies of CD69<sup>high</sup> cells in the spleen (Fig. 7h). Virtually no FoxP3<sup>+</sup> CD25<sup>−</sup> memory cells were seen within either donor population (not shown), supporting results from Fig. 3 indicating that loss of T-bet does not promote Treg development. These results indicate that Tbx21 −/− cells outcompete WT CD4 T cells to populate both tissue-resident and conventional memory niches.

T-bet is not required for CD4 T cell-dependent heterosubtypic immunity against IAV.

We next determined if T-bet expression is needed for IAV-primed mice to mount robust heterosubtypic immunity, which relies on IAV-specific memory T cells. We thus primed WT and Tbx21 −/− mice with sublethal PR8 (H1N1) and challenged at 28 dpi with A/Philippines (H3N2) to test if T-bet expression, even by non-T cells, contributes to recovery against primary or secondary infection. When primed with 0.5 LD<sub>50</sub> PR8, WT and Tbx21 −/− mice lost equivalent weight and began to recover on the same dpi (Fig. 8a). Histological analysis revealed no clear distinctions in lung pathology at 7 dpi (Fig. 8b), and viral control was equivalent (Fig. 8c). Total PR8-specific IgG levels were similar in WT and Tbx21 −/− mice. Given that high titers of long-lived IgG in IAV-primed mice require T<sub>FH</sub> help,30 this indicates similar T<sub>FH</sub> activity in WT and Tbx21 −/− mice. In agreement with analysis in Fig. 3, IgG2a production was impaired in Tbx21 −/− mice while IgG1 was enhanced (Fig. 8d). To test the protective capacity of the anti-PR8 serum generated in WT or Tbx21 −/− mice, we passively transferred 50 μL of immune serum to naive WT mice that were then challenged with 10 LD<sub>50</sub> PR8 as in a previous study. Recipients of WT or Tbx21 −/− serum were protected from lethal challenge while controls succumbed by 10 dpi (Fig. 8e). As protection in this model requires T<sub>FH</sub>-dependent titers of PR8-specific IgG,30 this indicates similar protective quality of CD4 T cell-dependent humoral immunity in the WT and Tbx21 −/− mice.

We next re-challenged the PR8-primed mice at 28 dpi with a supralethal (150 LD<sub>50</sub>) dose of A/Philippines. As compared to
unprimed WT controls that lost ~30% of their weight, primed WT and Tbx21−/− mice lost minimal weight and recovered by 7 dpi (Fig. 8f). Analysis of viral titer revealed equivalent control in WT and Tbx21−/− OT-II cells were co-transferred to CD45.1+ hosts then challenged with sublethal PR8-OVA<sub>a</sub>. The frequency of WT and Tbx21−/− cells within the donor gate at 28 dpi (4 mice/group). e The MFI of CD127 expression by lung donor cells is shown at 28 dpi from 4 mice/group. The frequency of WT and Tbx21−/− donors in the spleen (open) and lung (shaded) for CD69 (left histogram) and CD127 (right histogram), as well as lung for donor cell protection from the i.v. administered anti-CD4 antibody. h The frequency of CD69<sup>hi</sup> WT (black) and Tbx21−/− (open) OT-II cells in the lung and spleen is shown at 28 dpi from 3 mice/group. Results representative of two independent experiments.

**DISCUSSION**

Defining the attributes of CD4 T cells needed to combat specific pathogens is critical to improve the ability of vaccines to induce protective cellular immunity. The clearance of intracellular pathogens is strongly linked with responses orchestrated by Th1 cells that require T-bet for their programming. Indeed, outcomes of *Leishmania donovani*, *Toxoplasma gondii*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Francisella tularensis*, and *Trypanosoma cruzi* infection are all more severe in Tbx21−/−/− mice than in WT mice. Similarly, T-bet is required for optimal protection against Herpes Simplex virus 2 and *Vaccinia*. Rabies, and while not required to clear Rhinovirus, infected Tbx21−/− mice develop severe asthma-like inflammation. It is thus surprising that even
though CD4 T cells primed by IAV express high levels of T-bet, T-bet is not required for efficient clearance of primary or heterosubtypic IAV infection.

Although many changes in cytokine production distinguish WT and Tbx21−/− CD4 cells responding to IAV, IFNγ is still the predominant cytokine made by Tbx21−/− cells, albeit at reduced levels vs. WT effectors. STAT4-dependent signals may be sufficient to direct this residual IFNγ production, a hypothesis supported by some IL-12-dependent IFNγ seen from Tbx21−/− effectors in “Th1” conditions in vitro. A non-mutually exclusive possibility is that Eomesodermin (Eomes) can substitute for T-bet. Eomes has been shown to direct robust IFNγ production in CD4 T cells responding to IAV in terms of cytokine production. However, we found IL-17 neutralization not to markedly impact protection mediated by Tbx21−/− effectors. Whether other aspects of Th17 programming underlie the ability of Tbx21−/− CD4 T cells to combat IAV remains to be determined, but this possibility is suggested by a significant population of Rorγt+ Tbx21−/− effector cells detected in the lungs of IAV-infected mice.

Decreased levels of the chemokine receptor CXCR3 characterized the most striking phenotypic distinction between Tbx21−/− and WT CD4 T cells responding to IAV. Furthermore, high CXCR3 expression is the most relevant T-bet-dependent correlate of protective CD4 cells identified herein. This conclusion is similar to recent findings of impaired Toxoplasma gondii control in peripheral tissues of Tbx21−/− vs. WT mice that was associated with reduced T cell accumulation with less CXCR3. We note, however, that CXCR3 blockade only decreased WT effectors in the lungs by about one half vs. the 5–10-fold decrease seen in CXCR3-deficient CD4 cell lung trafficking during Sendia virus infection. This reflects strong CXCR3-independent recruitment operating during IAV infection. IAV drives not only CXCL9 and CXCL10 (ligands for CXCR3) but also CCL3, a ligand for CCR4, which was increased on Tbx21−/− cells, as well as several other major chemokines. Multiple layers of redundancy may thus provide for CXCR3-independent CD4 trafficking sufficient to control even higher doses of IAV. A similar explanation may at least in part

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underlie intact immunity against LCMV and Listeria monocytogenes reported in Tbx21−/− mice.47,48

T-bet-deficient CD4 effector cells outcompete WT cells for memory after IAV priming. Our results are consistent with studies linking higher T-bet and Ly6C expression by CD4 effector cells responding to LCMV49 and to murine γ-herpesvirus 6850 with a terminal fate. The Tbx21−/− effectors primed by IAV also express higher CD127. CD127 is positively regulated by IL-2 during IAV responses,51 suggesting that enhanced IL-7 receptor expression by Tbx21−/− cells may be linked to their increased production of IL-2 vs. WT cells. While our findings support work indicating that T-bet restricts lung-resident CD4 memory potential,52 we also saw improved Tbx21+/− memory in the spleen and dLN. In contrast to the IL-7-dependence of memory homeostasis in secondary lymphoid organs, survival of at least some lung-resident CD4 memory is IL-7-independent.53 T-bet expression may thus restrict both canonical and non-canonical pathways impacting diverse memory subsets primed by IAV. It is interesting to speculate that T-bet-dependent control of the glycolysis pathway in CD4 T cells may contribute differential memory fitness of WT and Tbx21−/− effector cells, given the prime role for cellular metabolism that is emerging in the governance of T cell memory states.50 A full investigation of how T-bet impacts metabolic programming of IAV-primed effector and memory CD4 T cell subsets may provide novel targets for vaccines to improve the establishment and durability of cellular immunity.

T-bet-deficient mice retain the ability to clear even high doses of primary IAV infection as well as WT mice. These results are in agreement with a recent study51 finding low-dose IAV-primed Tbx21−/− mice are better protected against secondary bacterial infection than are WT animals. We extend these observations to show that IAV-primed Tbx21−/− mice develop robust hetero-subtypic immunity with no discernable defects compared to WT mice following supralethal IAV challenges. While the focus of this study is how CD4 T cell-intrinsic T-bet expression impacts this subset’s response potential, our limited experiments analyzing primary and heterosubtypic IAV challenge in WT and Tbx21−/− provide important insights into T-bet’s roles in regulating other aspects of the IAV-immune state. First, as the experiments assessing heterosubtypic immunity were done in full T-bet-deficient animals, our findings indicate neither adaptive nor innate immune cells require T-bet expression to clear primary or secondary IAV infection. Our results cannot determine if the role of individual innate immune subtypes is gained or lost importance in contributing to viral control in the absence of T-bet. However, they do indicate that T-bet induction is not prerequisite to program protective “trained innate immunity” that can synergize with memory T cell responses to clear even supralethal heterosubtypic IAV challenge in primed mice. Second, our data indicates that virus-specific IgG2a is not a required element of heterosubtypic immunity, which is of interest in gaining further understanding of the roles of non-neutralizing antibodies in contributing to vaccine-induced protection against IAV. That similar titers of PR8-specific IgG were detected in WT and Tbx21−/− mice is indicative of intact Th1 activity, as virus-specific IgG in IAV-primed C57BL/6 mice lacking Tbx2 is reduced by 1 to 2 logs vs. WT mice.52 Indeed, Th1 were detected at equal frequencies within WT and Tbx21−/− cells responding to IAV in WT hosts. Recent observations indicate that Tbx2 may contain subsets that mirror conventional effector states (i.e., Th1, Th2, Th17).54 Given this framework, reduced IgG2a but increased IgG1 in Tbx21−/− mice may reflect decreased IFNγ+ “Th1” Tbx2 and increased IL-4+ “Th2” Tbx2. However, B cells can also express T-bet with important functional consequences.53 Further studies are thus required for a full understanding of how T-bet regulates humoral immunity against IAV.

In summary, our studies indicate that T-bet-dependent programming is not required to generate effector or memory CD4 cells with strong anti-viral functions. However, upregulation of CXCR3, which is T-bet-dependent, is needed to maximize the efficacy of CD4 responses against IAV by promoting accumulation of effector cells in the infected lung. Finally, our results indicate that the goal of promoting strong Th1 responses against IAV may limit the ability of such effector cells to form protective memory. Our results indicate the need to better characterize and differentiate CD4 cells that are defined as “Th1” based on IFNγ production alone. Such efforts will help to uncover more decisive correlates of protective cells in specific disease settings and also allow for the development of novel vaccine strategies that induce specific attributes in effector cells best suited for responses against specific pathogens à la carte rather than through sweeping polarization programs.

METHODS

Mice

C57BL6 (B6) mice knocked out for T-bet (Tbx21−/−) or wild-type B6.CD45.1 mice were used at least 8 weeks old for IAV infection. Donor CD4 cells for adoptive transfer experiments were obtained from 4–8 week old OT-II TcR transgenic mice on a WT (Thy1.1/Thy1.2) or Tbx21−/− (Thy1.2) background. The OT-II TcR recognizes aa 323–339 of chicken ovalbumin (OVA). For bone marrow chimeras, B6.Thy1.1 mice were lethally irradiated and reconstituted intravenously with 2 × 10^7 T and B cell-depleted bone marrow cells from B6.CD45.1 or T-bet−/− mice. Reconstitution was verified by FACS analysis of peripheral blood prior to use in experiments. All mice were originally obtained from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Central Florida. All experimental animal procedures were approved by and conducted in accordance with the University of Central Florida’s Animal Care and Use Committee’s guidelines.

CD4 T cell isolation, effector and memory generation, and cell transfer

Naïve CD4+ cells from OT-II donor mice were obtained from pooled spleen and lymph nodes of unmanipulated mice. Single cell suspensions were incubated on nylon wool for one hour followed by Percoll gradient separation and then positive MACS selection using CD4 microbeads (Miltenyi Biotec, Auburn, CA). The resulting effector cells were analyzed at 4 days. Effector cultures were fed with fresh media and IL-2 at 2 days, and thoroughly washed prior to adoptive transfer experiments.

Effector cells were generated as previously described13,54 by thoroughly washing effector cells, resting the cells 15 ug/mL, IL-6 at 20 ng/mL, IL-23 at 25 ng/mL, IL-21 at 50 ng/mL, TGFβ at 0.5 ng/mL, IL-1 at 10 ng/mL, TNF at 10 ng/mL. All blocking antibodies were purchased from BioXcell (West Lebanon, NH). All other reagents were purchased from Peprotech (Rocky Hill, NJ). Effector cultures were fed with fresh media and IL-2 at 2 days, and the resulting effector cells were analyzed at 4 days. Effectors were thoroughly washed prior to adoptive transfer experiments. Memory populations were generated and assessed as previously described54 by thoroughly washing effector cells, resting the cells for at least 3 days in fresh media without added cytokines or peptide, followed by isolation of live cells using lympholyte M (Cedarlane, Burlington, NC).

Naïve, effector, and memory CD4 cells were adoptively transferred to host mice under light anesthesia in 200μL of RPMI media alone via retro-orbital injection.
Viral stocks and infections and in vivo antibody treatments
A/PR8 and A/PR8-OVA 

infection. In other experiments, mice were treated with 250 μg of neutralizing antibody against IL-17 (clone 17F3) administered by intraperitoneal injection on days 1, 3, 5, and 7 post IAV infection. In other experiments, mice were treated with 250 μg blocking antibody against CXCR3 (clone BE2Q49) by intraperitoneal injection daily throughout the first week of IAV infection. All antibodies used to treat mice were purchased from BioXcell (West Lebanon, NH).

In some experiments, 50 μL of serum obtained from PR8-primed mice at 28 dpi was transferred to naive WT mice by intraperitoneal injection. Two hours later, the mice were challenged with 10 LD50 PR8.

Flow cytometry
Single-cell suspensions were washed, resuspended in FACS buffer (PBS plus 0.5% BSA and 0.02% sodium azide) and incubated on ice with 1 μg of anti-FcR (2.4G2) and optimized concentrations of the following fluorochrome-labeled antibodies for surface staining: anti-Thy1.1 (OX-7), anti-Thy1.2 (53–2.1), anti-CD4 (RM4.5), anti-CD69 (H12F3), anti-CD25 (PC6.15), anti-CD127 (A7R34), anti-CD44 (IM7), anti-CD45.2, anti-CXCR3 (CXCR3-173), anti-CCR4 (2G12), anti-Ly-6C (HK1.4), anti-CCR7 (4B12), anti-CD44 (IM7), anti-CCR6 (29-2L17), anti-CCR5 (HM-CCR5), anti-CXCR5 (SPRCL5), anti-CD11a (Ly-6C) (HK1.4), and anti-CD11c (ED1). To detect IAV-specific (NP311−325), anti-Granzyme B (NGZB) fluorescently labeled antibodies for 20 min.

Real time-PCR
Viral titers were determined by quantification of viral RNA prepared from whole lung homogenates using TRizol (Sigma-Aldrich). 2.5 μg of RNA was reverse transcribed into cDNA using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen). Quantitative PCR was performed to amplify the polymerase (PA) gene of PR8 and A/Phil using an ABI Prism 7700 Sequence Detector (Applied Biosystems) with 50 ng of cDNA per reaction and the following primers and probe: forward primer, 5'-CGGCTCATTTCCGTGCA-3'; reverse primer, 5'-CATTGGTCTTCCATCCCA-3'; probe, 5'-6-FAM-CCAAGTGATGAGAGGAGGAATACCCGC-3'. Data were analyzed with Sequence Detector v1.7a (Applied Biosystems). The copy number of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid of known concentration as a standard.

Statistical analysis
Unpaired, two-tailed, Students t-tests, \( \alpha = 0.05 \), were used to assess whether the means of two normally distributed groups differed significantly. The Welch-correction was applied when variances were found to differ. One-way ANOVA analysis with Bonferroni's multiple comparison post-test was employed to compare multiple means. Significance is indicated as *\( P < 0.05 \); **\( P < 0.005 \); ***\( P < 0.001 \); and ****\( P < 0.0001 \). The Log Rank test was used to test for significant differences in Kaplan–Meier survival curves. All error bars represent standard deviation.

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AUTHOR CONTRIBUTIONS
K.D. and C.F. performed all experiments. K.D. and K.K.M. analyzed data and wrote the manuscript. S.S. performed blinded analysis of histopathology. T.M.S. provided key reagents, reviewed and critiqued the manuscript and contributed to interpretation of the findings.

ADDITIONAL INFORMATION
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REFERENCES

1. Swain, S. L., McKinstry, K. K. & Strutt, T. M. Expanding roles for CD4(+) T cells in immunity to viruses. Nat. Rev. Immunol. 12, 136–148 (2012).

2. Szabo, S. J. et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100, 655–669 (2000).

3. McKinstry, K. K. et al. Memory CD4(+) T cells protect against influenza through multiple synergizing mechanisms. J. Clin. Invest 122, 2847–2856 (2012).

4. Strutt, T. M. et al. Mice engineered with an inducible embryonic lethal mutation for T-bet demonstrate T-bet-expressing CD4(+) T-cell effector and memory responses cooperate to provide potent immunity against respiratory virus. Immunol. Rev. 255, 149–164 (2013).

5. Graham, M. B., Braciale, V. L. & Braciale, T. J. Influenza virus-specific CD4(+) T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. J. Exp. Med. 180, 1273–1282 (1994).

6. Brown, D. M., Dilzer, A. M., Meents, D. L. & Swain, S. L. CD4(+) T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. J. Immunol. 177, 2888–2898 (2006).

7. Teijaro, J. R., Verhoeven, D., Page, C. A., Turner, D. & Farber, D. L. Memory CD4(+) T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. J. Virol. 84, 9217–9226 (2010).

8. Brown, D. M., Lee, S., Garcia-Hernandez Mde, L. & Swain, S. L. Multifunctional CD4 cells expressing gamma interferon and perforin mediate protective action against lethal influenza virus infection. J. Virol. 86, 6792–6803 (2012).

9. Bot, A., Bot, S. & Bona, C. A. Protective role of gamma interferon during the recall response to influenza virus. J. Virol. 72, 6637–6645 (1998).

10. Graham, M. B. et al. Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. J. Exp. Med. 178, 1725–1732 (1993).

11. Califano, D. et al. IFN-gamma increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. Mucosal Immunol. 11, 209–219 (2018).

12. Nicol, M. Q. et al. Lack of IFNgamma signaling attenuates spread of influenza A virus in vivo and leads to reduced pathogenesis. Virology 526, 155–164 (2018).

13. McKinstry, K. K. et al. IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge. J. Immunol. 182, 7353–7363 (2009).

14. Eliasson, D. G. et al. M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection. Mucosal Immunol. 11, 273–289 (2018).

15. Lord, G. M. et al. T-bet is required for optimal proinflammatory CD4(+) T cell function. Blood 129, 6266–6273 (2014).

16. Strutt, T. M., McKinstry, K. K., Kuang, Y., Bradley, L. M. & Swain, S. L. Memory CD4(+) T cells can cooperate to provide potent immunity against respiratory virus infection. J. Virol. 86, 6792–6803 (2012).

17. Ghosh, S., Chackerian, A. A., Parker, C. M., Ballantyne, C. M. & Behar, S. M. The LFA-1 adhesion molecule is required for protective immunity during pulmonary Mycobacterium tuberculosis infection. J. Immunol. 176, 4914–4922 (2006).

18. Kohlmeier, J. E. et al. CXCX3 directs antigen-specific effector CD4(+) T cell migration to the lung during parainfluenza virus infection. J. Immunol. 183, 4378–4384 (2009).

19. Mikhail, Z., Strassner, J. P. & Luster, A. D. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. J. Exp. Med. 210, 1855–1869 (2013).

20. Lazarevic, V., Glimcher, L. H. & Lord, G. M. T-bet: a bridge between innate and adaptive immunity. Nat. Rev. Immunol. 13, 777–789 (2013).

21. Groom, J. R. et al. CXCX3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. Immunity 37, 1091–1103 (2012).

22. Wang, X. et al. IL-17A Promotes Pulmonary B-1a Cell Differentiation via Induction of Blimp-1 Expression during Influenza Virus Infection. PLoS Pathog. 12, e1005367 (2016).

23. Crowe, C. R. et al. Critical role of IL-17RA in immunopathology of influenza infection. J. Immunol. 183, 5301–5310 (2009).

24. Marshall, H. D. et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. Immunol. 35, 633–646 (2011).

25. Hsu, Z., Blackman, M. A., Kaye, K. M. & Usherwood, E. J. Functional heterogeneity in the CD4(+) T cell response to murine gamma-herpesvirus 68. J. Immunol. 194, 2746–2756 (2015).

26. McKinstry, K. K. et al. Effector CD4 T-cell transition to memory requires late cognate interactions that induce autocrine IL-2. Nat. Commun. 5, 5377 (2014).

27. Oestreich, K. J. et al. Blc-6 directly represses the gene program of the glycolysis pathway. Nat. Immunol. 15, 957–964 (2014).

28. van der Windt, G. J. & Pearce, E. L. Metabolic switching and fuel choice during T-cell differentiation and memory development. Immunol. Rev. 249, 27–42 (2012).

29. Er J. Z., Koean R. A. G., Ding J. L. Loss of T-bet confers survival advantage to Listeria monocytogenes infection in the absence of T-bet. J. Immunol. 173, 5918–5922 (2004).

30. McKinstry, K. K. et al. Rapid default transition of CD4 T cell effectors to functional memory cells. J. Exp. Med. 204, 2199–2211 (2007).

31. Ferrer, J., Retana, I., Hernández-Balboa, D. & Del Carpio-Mora, M. Expression of IFN-gamma and perforin mediate protection against lethal influenza infection through multiple synergizing mechanisms. J. Clin. Invest 122, 2847–2856 (2012).

32. Koean R. A. G., Ding J. L. Loss of T-bet confers survival advantage to Listeria monocytogenes infection in the absence of T-bet. J. Immunol. 173, 5918–5922 (2004).

33. McKinstry, K. K. et al. T-bet optimizes CD4 T-cell responses against influenza through...