ARTICLE

T-helper 22 cells develop as a distinct lineage from Th17 cells during bacterial infection and phenotypic stability is regulated by T-bet

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CD4^{+} T-helper 22 (Th22) cells are a phenotypically distinct lymphocyte subset that produces high levels of interleukin (IL)-22 without co-production of IL-17A. However, the developmental origin and lineage classification of Th22 cells, their interrelationship to Th17 cells, and potential for plasticity at sites of infection and inflammation remain largely undefined. An improved understanding of the mechanisms underpinning the outgrowth of Th22 cells will provide insights into their regulation during homeostasis, infection, and disease. To address this knowledge gap we generated 'IL-17A-fate-mapping IL-17A/IL-22 reporter transgenic mice' and show that Th22 cells develop in the gastrointestinal tract and lung during bacterial infection without transitioning via an Il17a-expressing intermediate, although in some compartments alternative transition pathways exist. Th22-cell development was not dependent on T-bet; however, this transcription factor functioned as a promiscuous T-cell-intrinsic regulator of IL-17A and IL-22 production, in addition to regulating the outgrowth, phenotypic stability, and plasticity of Th22 cells. Thus, we demonstrate that at sites of mucosal bacterial infection Th22 cells develop as a distinct lineage independently of Th17 cells; though both lineages exhibit bidirectional phenotypic flexibility within infected tissues and their draining lymph nodes, and that T-bet plays a critical regulatory role in Th22-cell function and identity.

INTRODUCTION

CD4^{+} T-helper lymphocytes (Th cells), under strict regulation, enable immunological specificity and diversity to orchestrate appropriate immune responses to external challenge. Divergent differentiation programs, directed by cell-extrinsic and -intrinsic factors, give rise to distinct and highly specialized Th subsets known as Th1, Th2, Th17, Th9 and follicular T-helper lineages, that are well characterized.[1] A Th22-cell subset, originally identified in patients with inflammatory skin disease,[2–5] has also been proposed based on their predominant interleukin (IL)-22 production. Although Th22 cells are associated with a range of bacterial infections[6,7] and immune disorders including psoriasis,[5] atopic dermatitis (AD)[4] and asthma,[8,9] our understanding of their differentiation, regulation, and immunological role remains limited. This is attributable to our inability to directly identify and isolate Th22 cells from complex inflammatory milieus and discern their relative contributions in relation to all other IL-22 producing cells.

IL-22 is considered a critical conduit between the immune system and barrier tissues, due to the limited expression of its cognate receptor, IL-22RA1, on non-immune cells such as epithelial cells of the respiratory and gastrointestinal (GI) tracts.[10]

IL-22 may have protective or deleterious functions contingent on the disease context, inflammatory milieu and cellular source.[11–15] The contribution of Th22-cell-derived IL-22 to effector functions remains unknown as Th17 cells (produce IL-17A, IL-17F and IL-21[16]), γδ T cells, and innate lymphoid cells (ILCs) also produce this cytokine.[13] The presence of Th22 cells (which do not produce IL-17A, but may have variable IFNγ production)[2,3,8,17] at mucosal sites and their ability to upregulate antimicrobial factors from epithelial cells suggest important roles in adaptive host defense. Definitive evidence of a direct role during infectious disease is limited to a single elegant study whereby transfer of in vitro generated Th22 cells provided protection to IL-22-deficient hosts against *Citrobacter rodentium* (*Cro*) infection of the GI tract, which was not afforded by Th17 cell-derived IL-22.[15] However, the use of mixed populations of Th cells, owing to insufficient tools to isolate pure populations of in vitro generated Th22 cells, limited the specificity of the aforementioned study, and the plasticity of the transferred cells was not assessed.

As the discovery of the Th17-lineage[18,19] predates that of Th22 cells, functions ascribed to Th-derived IL-22 have historically been linked to Th17 cells.[16,20] Furthermore, available evidence

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T-helper 22 cells develop as a distinct lineage from Th17 cells during…
JL Barnes et al.

1078

based on *Il22* fate-mapping studies has suggested that Th-derived IL-22 during infection may be transiently associated with Th17 cells rather than a dedicated Th22 lineage.[21] Notably, Th22 cells accumulate at sites of inflammation, both alongside Th17 cells and independently of IL-17A co-expression in multiple contexts.[2–5,8,15,21–23] In common with Th17 cells, Th22-cell differentiation is contingent on IL-6 signaling and supported by IL-1β and IL-23.[15,24–26] However, TGFβ signaling acts as a critical molecular switch, potently suppressing Th22 differentiation in vitro, while promoting Th17 cell outgrowth,[15,23,24,27–31] and similarly induces Th17 cell differentiation and their production of IL-22 in vivo, but not that of Th22 cells.[23] As developing Th17 cells retain considerable plasticity,[30] it may be that Th17-precursors (Th17p) acquire a Th22 phenotype, contingent on TGFβ signaling or other environmental cues. However, we have previously demonstrated that Th22 development occurs without transitioning *via* an *Il17a*-expressing stage in vitro, suggesting that Th22 cells may represent a distinct lineage in vivo.[24] Furthermore, analysis of putative Th22 cells isolated from inflamed human skin[5] and cell cultures[15,24] identified unique molecular profiles by contrast to Th17 cells. Whether Th22 cells represent a distinct lineage or an intermediary of the Th17-lineage in vivo remains unclear.[15,17,21,32]

In contrast to other Th lineages,[1] a unique transcriptional signature sufficient to specify the Th22-cell program has not been identified, though multiple transcription factors positively regulate their IL-22 production in vitro, including Runx1,[33] Rorγt,[24,33] HIF-1α,[34] and AhR.[3,15] While we have shown that T-bet potently suppresses Th22-cell differentiation in vitro,[24] others have shown Th22 development in vivo is dependent on this transcription factor.[15] Delineating the contribution of T-bet in Th22 cells during their developmental program in vivo in response to infection will provide important insights into the regulation of transcription and plasticity of this cell.

Currently, there is no unified understanding of the development of Th22 cells as a distinct lineage in vivo, their interrelationship with Th17 cells or phenotypic stability, during microbial infections. Here we performed a detailed characterization of the relationship between Th17- and Th22-cell development in the GI tract and lung following bacterial infection. Collectively our data demonstrate, for the first time, that Th22 cells (defined as IL-22+IL-17A−CD4+ T cells) develop as a stable lineage distinct from Th17 cells during bacterial infection, and identify a critical role for T-bet as a negative regulator of Th22-cell development and plasticity.

RESULTS

To definitively and directly map the spatial distribution and origins of Th22 cells in vivo, we generated a transgenic mouse strain by crossing an *Il17a*^eGFP*Il22*^tdTom dual-reporter[24] with an IL-17A fate-mapping strain (Th17-lineage^eYFP; *Il17a*^Cre *R26R*^eYFP[35]), referred to as triple-reporter mice. This unique system enables direct visualization of active IL-17A and IL-22 expression *via* eGFP and tdTomato fluorescence, and identification of cells with previously activated *Il17a* gene-expression *via* eYFP.

We first characterized triple-reporter Th cells in our culture system.[24] Under Th22 differentiation conditions, we identified a distinct population of putative Th22 cells that were IL-22 positive without current or previous IL-17A production (eYFP-eGFP-tdTom+) (Supplementary Fig. 1). Thus, the Th triple-reporter system enables quantification of active IL-17A and IL-22 expression and previous IL-17A expression, and confirms our previous findings that Th22 cells develop independently of the Th17 lineage in vitro.[24]

Distinct Th22 cells develop in vivo following GI *Citrobacter rodentium* infection that do not transition *via* an IL-17A-expressing stage

Although Th22 cells have been reported to emerge in the GI tract and gut-associated lymphoid tissues (GALT) during the mid-to-late phase of *Cro* infection (8–21 days post infection (dpi)) and may play a direct role in host protection *via* IL-22,[15,21,27,36] their developmental origin and interrelationship with Th17 cells remains unresolved. We initially assessed the emergence of Th17 and Th22 cells during *Cro* infection of dual-reporter (*Il17a*^eGFP*Il22*^tdTom) mice. Robust populations of both subsets emerged in the colon lamina propria (CLP), mesenteric lymph nodes (MLN) and Peyer's patches (PP) at 14 and 19 days after *Cro* infection (Supplementary Fig. 2).

Next, we performed Th17-lineage tracing in Th-cell (CD3+CD4+TCRβ+) subsets from triple-reporter mice 14 days after *Cro* infection and revealed that populations of Th17 cells producing IL-17A alone (9.5% ±0.9), or together with IL-22 (18.7% ±1.5), accumulated in the CLP, the majority of which (90.1% ±2.6 and 84.0% ±5.1, respectively) were eYFP+ (Fig. 1a, b). There was a 90% concordance between the IL-17A reporters, confirming reports that the fidelity of the fate-mapping mouse is much higher in the in vivo setting compared to in vitro cultures.[35] Within the CLP, Th cells actively expressing IL-22 and not IL-17 were equally split between eYFP+ (7.9% ±0.6; Th22 cells derived from Th17p cells, that have ceased active IL-17A production) and eYFP− (7.4% ±0.4; putative Th22 cells developing independently of *Il17a* expression) (Fig. 1a, b).

By contrast to the CLP, few Th cells in the MLN expressed the IL-17A fate-mapping locus (1.4% ±0.7) and cells expressing active IL-17A were negligible (Fig. 1c, d). IL-17-IL-22+ cells represented the highest frequency (2.3% ±0.3) of cytokine-reporting MLN Th cells, the majority having developed independently of *Il17a* expression (eYFP-; 84.5% ±1.0; Fig. 1c, d). We also observed a lack of IL-17A fate-mapped Th22 cells in MLN at 0, 4, and 8 days after *Cro* infection (Supplementary Fig 2) indicating this population does not develop in MLN during infection before egress to effector tissues. Among PP Th cells, 6.1% ±0.9 of cells expressed the IL-17A fate-mapping locus and 29.9% ±3.0 of these cells co-expressed IL-17A and IL-22, whereas 14.9% ±1.7 expressed IL-17A alone (Fig. 1e, f). Like the MLN, the highest frequency of cytokine-reporting Th cells in the PP exhibited a Th22 phenotype (eGFP-tdTom+; 5.8% ±0.8) and importantly, 77.9% ±1.7 of these cells were eYFP− (Fig. 1e, f). Collectively, this provides the first evidence that a distinct population of GI Th22 cells develop independently of Th17 cells during *Cro* infection. Further, we demonstrate considerable Th17 cell phenotypic flexibility toward a Th22-like phenotype during infection, particularly within the CLP.

Th22 cells develop independently of IL-17A expression/Th17 differentiation during respiratory *Chlamydia muridarum* infection

Although IL-22 is important for host protection against a range of pulmonary bacterial pathogens,[20,37–39] the cellular source of IL-22 during respiratory infection is poorly defined, and no studies have examined Th22-cell development in this context. Thus, we performed flow cytometry of lung cells from dual-reporter (*Il17a*^eGFP*Il22*^tdTomato) mice (to allow additional cell surface marker staining of relevant lung immune cell subsets) in a model of experimental bacterial pneumonia using the obligate intracellular mouse pathogen *Chlamydia muridarum* (Cmu).

Following *Cmu* infection, ILCs (Lineage-CD90.2+CD127+), γδ T cells (CD3+CD4-γδTCR+) and Th cells were all detected among CD45+ lung cells. Numbers of ILCs and γδ T cells (early responding cells) peaked, then contracted, by 8 dpi (Fig. 2a, b). By contrast, Th cells (generation of the adaptive response) peaked at 8 dpi and remained elevated at 14 dpi (Fig. 2a, b). While IL-17A-producing γδ T cells were present at all time points, these cells did not contribute significantly to IL-22 production (Fig. 2a, c) (nor did NK cells, CD45+NK1.1+, data not shown). Between 4 and 14 dpi, both ILCs and Th cells produced IL-22, however, Th cells were the more abundant source of IL-22 (Fig. 2a, c). Th cells present 8 dpi, included 4.9% ±0.8 IL-17A+IL-22−, 2.9% ±0.7 IL-17A+IL-22+ and 4.0% ±0.9 IL-17A-IL-22+ cells (Fig. 2a, c). At 14 dpi, Th22-cell

T-helper 22 cells develop as a distinct lineage from Th17 cells during…
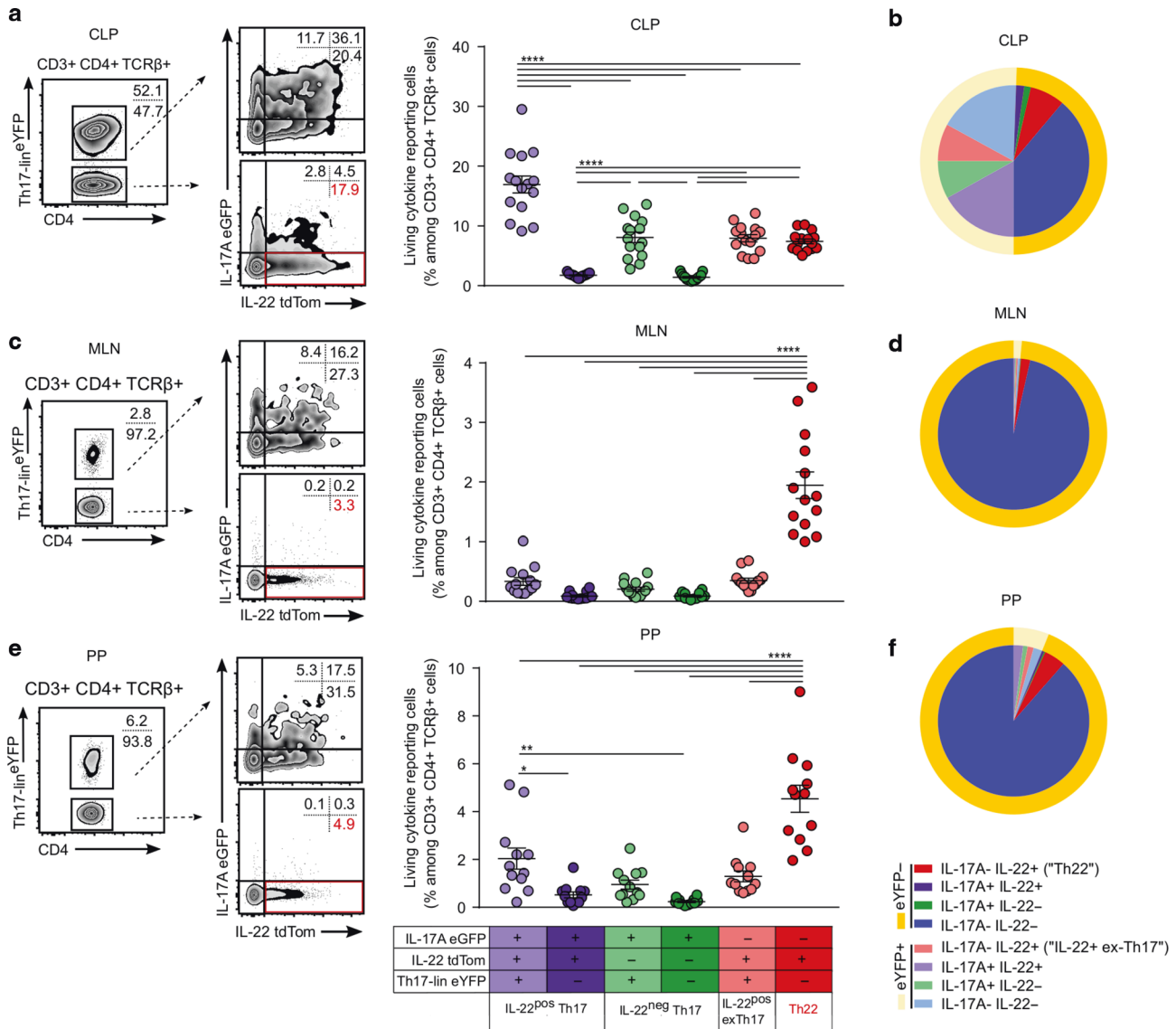JL Barnes et al.

1079

Fig. 1 Distinct Th22 cells are present during gastrointestinal *Citrobacter rodentium* infection which do not transition *via* an IL-17A-expressing stage. Triple-reporter ($Il17a^{Cre}Rosa26^{eYFP}Il17a^{eGFP}Il22^{tdTom}$) mice were orally gavaged with 1×10^9 colony-forming units (cfu) *Citrobacter rodentium*, then live Th cells (CD3+CD4+TCRβ+) isolated at 14 days post infection (dpi) from colon lamina propria (CLP; **a** n = 15), mesenteric lymph nodes (MLN; **c** n = 14) and Peyer's patches (PP; **e** n = 12) and analyzed by flow cytometry. Shown is representative reporter for Th17-lineage (eYFP; **a, c, e**; left-panels) or IL-17A (eGFP) and IL-22 (tdTom; **a, c, e**; right panels) and corresponding quantifications. Red gate indicates putative Th22 cells (eYFP-eGFP-tdTom+). Numbers in plots indicate relative percentages of parent-gate inside gate/quadrant. Graphs indicate mean ± SEM and each symbol represents one mouse. Results pooled from three independent experiments. **b, d, f** Relative proportion of cytokine-reporting cells among total Th cells in **a, c,** and **e** within the CLP (**b**), MLN (**d**) and PP (**f**). Outside ring indicates eYFP, inside pie indicates eGFP and tdTom. Data indicate absolute mean. * represents $p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ by One-way ANOVA with Tukey correction. See also Fig. S2.

frequency markedly decreased (2.0% ±.3; Fig. 2a, c). Thus, while multiple immune cell subsets contribute to lung IL-22 production during *Cmu* infection, most IL-22 production was attributable to Th22 cells.

Next *Cmu*-infected triple-reporter mice were used to determine the origin of Th22 cells in the lung compared to that in the GI tract following *Cro* infection. Flow-cytometric analysis revealed that among total lung Th cells at 8 and 14 dpi, only a small proportion of cells were eYFP+ (2.5% ±0.3 and 4.8% ±0.5, respectively; Fig. 2d, e). Some discordance between the two IL-17A reporter constructs was evident among putative lung-Th17 cells at 8 dpi, with 60.0% ±2.6 of cells actively expressing IL-17A being eYFP- (Fig. 2d, h). At 14 dpi, concordance between the IL-17A reporters improved, whereby 62.5% ±0.8 of GFP+ Th17 cells

co-expressed eYFP (Fig. 2e, h). Most putative lung Th22 cells at 8 dpi were eYFP- (93.9% ±0.4), which was sustained at 14 dpi (84.7% ±1.0; Fig. 2d, e, h). In the mediastinal lymph nodes (mLN), we noted a distinct lack of either current or previous IL-17A expression among Th cells. Further, most cytokine-reporting mLN Th cells exhibited a Th22 phenotype, without eYFP expression (Fig. 2f, g, h). These data show for the first time that Th22 cells are present in both lung and draining lymphoid tissues following *Cmu* infection, arising primarily *via* a pathway independent of current or previous IL-17A expression. Further, the pronounced Th17-to-Th22-cell phenotypic flexibility observed following bacterial infection in the GI tract appears to be absent in the respiratory compartment in response to *Cmu* infection.

T-helper 22 cells develop as a distinct lineage from Th17 cells during...
JL Barnes et al.

1080
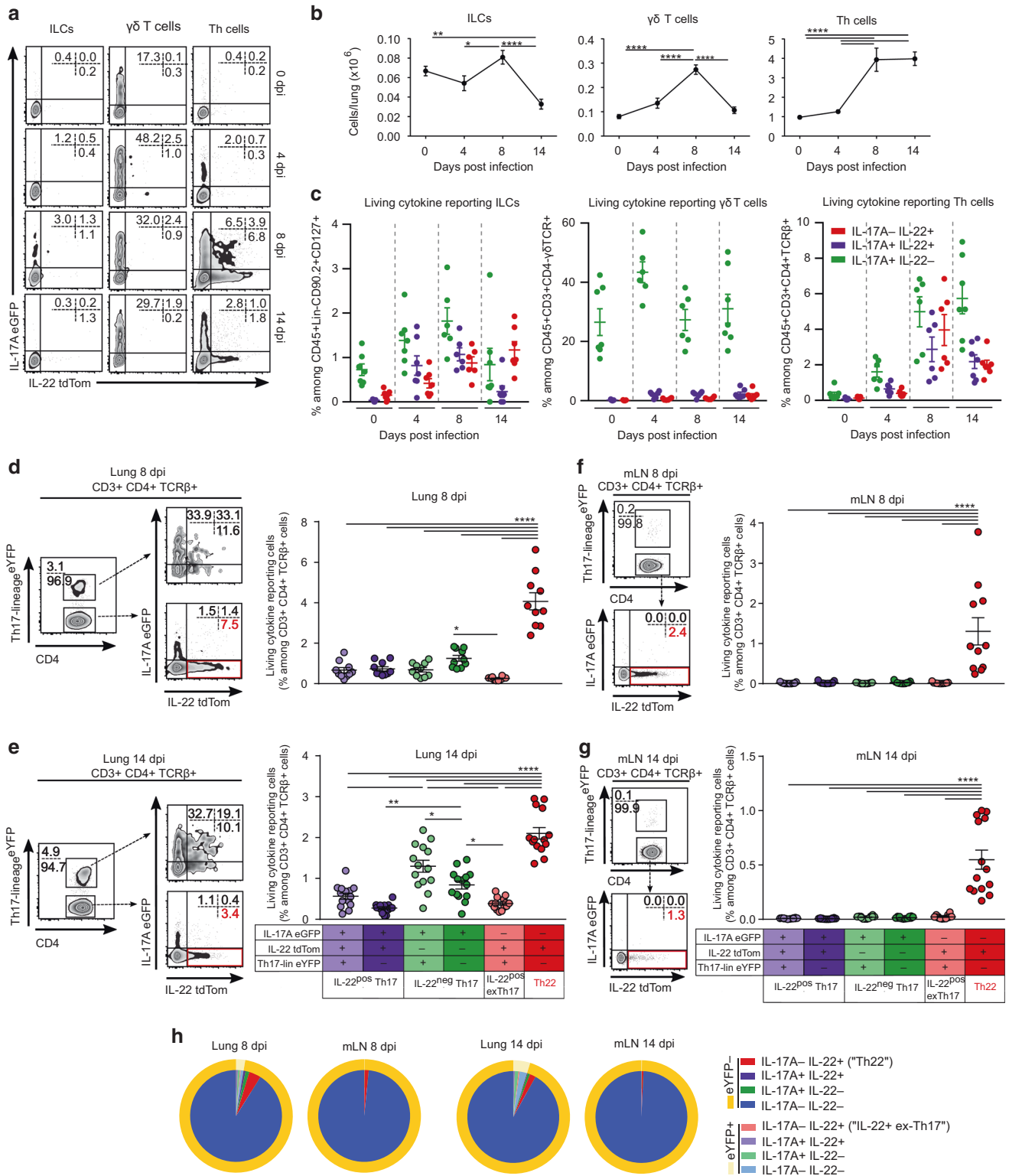
The transcription factor T-bet regulates Th17- and Th22-cell development in vivo

Next, to clarify the role of T-bet in regulating our distinct population of pulmonary Th22 cells that emerge following *Cmu* infection, T-bet-deficient (*Tbx21*$^{-/-}$*Il17a*^{eGFP}*Il22*^{tdTom}) and T-bet-competent (*Tbx21*$^{+/+}$*Il17a*^{eGFP}*Il22*^{tdTom})-dual-reporter mice were infected with *Cmu* and assessed 8 dpi. Elevated IL-17A and IL-22 protein levels (11.3- and 6.5-fold, respectively) were detected in

lung homogenates from T-bet-deficient mice, compared with T-bet-competent controls (Fig. 3a). Similarly, the frequency of IL-17A+ (eGFP+) and IL-22+ (tdTom+) lung cells was increased in T-bet-deficient mice (7.0- and 9.6-fold, respectively; Fig. 3d, e). Importantly, these T-bet-dependent differences were not observed in the absence of infection (data not shown). Higher frequencies of IL-17A (12.5-fold) and IL-22 (4.5-fold) expressing cells were also present in the mLN of T-bet-deficient mice (Fig. 3d,

T-helper 22 cells develop as a distinct lineage from Th17 cells during...
JL Barnes et al.

1081

Fig. 2 **Th22 cells develop independently of IL-17A expression/Th17 differentiation following respiratory *Chlamydia muridarum* infection.** Dual-reporter (*il17a*^{eGFP}*il22*^{tdTom}) mice received 300 inclusion-forming units (IFU) of *Chlamydia muridarum (Cmu)* intranasally (i.n.) then were assessed at 0 (uninfected), 4, 8 and 14 days post infection (dpi; $n = 6$/time-point). Lung cells were isolated, stained for surface markers then analyzed by flow cytometry. **a** Representative reporter for IL-17A (eGFP) and IL-22 (tdTom) within living innate lymphoid cells (ILCs; **a**, left-panels), γδ T cells (**a**, middle-panels) and Th cells (**a**, right panels). **b** Absolute numbers of lung- ILCs (**b**, left panel), γδ T cells (**b**, middle panel) and Th cells (**b**, right panel). Symbols indicate mean ± SEM. **c** Corresponding quantifications of cytokine-reporting cells in (**a**). Bars indicate mean ± SEM, each dot represents one mouse. Data represent two independent experiments. **d–g** Triple-reporter (*il17a*^{Cre}*Rosa26*^{eYFP}*il17a*$^{eGF-P}$*il22*^{tdTom}) mice received 300 IFU *Cmu* i.n. and live Th cells from lungs and mediastinal lymph nodes (mLN) at 8- ($n = 10$ and $n = 11$) and 14- ($n = 14$ and n = 14) dpi were analyzed by flow cytometry. Representative reporter for Th17-lineage (eYFP; **d**, **e**, left-panels; **f**, **g**, top-panels) or IL-17A (eGFP) and IL-22 (tdTom; **d**, **e**, right panels; **f**, **g**, bottom panels) in indicated populations and corresponding quantifications of cytokine-reporting cells. Red gate on flow-cytometry plots indicates putative Th22 cells (eYFP-eGFP-tdTom+). Bars in graphs indicate mean ± SEM pooled from three independent experiments where each dot represents one mouse. **h** Relative proportion of cytokine-reporting cells among total Th cells in (**d–e**). Outside ring indicates eYFP, inside pie indicates eGFP and tdTom. Data are absolute mean. Numbers in plots are relative percentage of parent-gate inside gate/quadrant. * represents $p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ by One-way ANOVA with Tukey correction.

f). Notably, there was a reduction, but not complete abrogation, of IFNγ in the lungs of infected T-bet-deficient mice (3-fold; Fig. 3a). IL-17A and IL-22 were also elevated in culture supernatants of T-cell specific (i.e., anti-CD3/CD28) restimulated lung (11.2- and 9.1-fold, respectively) and mLN (4.1- and 5.8-fold, respectively) cells isolated from T-bet-deficient mice, whilst IFNγ levels were reduced (2.6-fold in lung and 1.7-fold in mLN cells; Fig. 3b, c). These data suggest that during *Cmu* infection, T-bet acts to constrain T-cell-derived IL-17A and IL-22 production, alongside a well-recognized role in regulating IFNγ production.

Next, we specifically compared the frequency of IL-17A and IL-22 producing Th cells during *Cmu* infection. T-bet deficiency markedly increased the frequency of Th17 cells producing IL-17A alone (6.9% ±0.4 vs. 4.4% ±0.5), or co-producing IL-17A and IL-22 (43.3% ±3 vs. 2.9% ±0.5) and putative Th22 cells (13.7% ±0.7 vs. 4.4% ±0.4; Fig. 3g, h), compared with T-bet-competent mice. These three cell profiles were also increased in mLN of T-bet-deficient mice, with the most prominent effect observed in numbers of Th22 cells (4.2% ±0.4 vs. 1.2% ±0.2; Fig. 3g, i). Thus, elevated T-cell-derived IL-17A and IL-22 protein levels in infected T-bet-deficient mice were associated with increased frequencies of Th17 and Th22 cells. These findings provide new evidence that T-bet plays a role in negatively regulating Th22 lineage development in vivo and further evidence of an analogous role in Th17 cells.

T-bet regulates phenotypic stability and flexibility of Th17 and Th22 cells via a T-cell-intrinsic mechanism
The enhanced effect of T-bet deficiency on Th17/Th22-cell function during *Cmu* infection may be a consequence of a reduced IFNγ/type 1 environment, or due to an intrinsic Th-cell defect. To explore this, we isolated Th17 and Th22 cells from the lungs of *Cmu*-infected T-bet-competent or -deficient dual-reporter mice by FACS at 8 dpi, and transferred them into B6.CD45.1 congenic T-bet-competent recipients, which had been infected with *Cmu* 5 days earlier (Fig. 4a, b). We recovered donor CD4$^+$ T cells (CD4+CD45.2+) from recipient lungs 3 days post transfer and assessed their production of IL-17A and IL-22 reporters by flow cytometry (Fig. 4a, d). Purity of donor cells was routinely >90% (Fig. 4c).

Recovered T-bet-competent donor-Th17 cells largely maintained their expression of IL-17A alone (37.5% ±1.3), though 61.6% ±1.4 of cells lost IL-17A production and acquired a double-negative phenotype (Fig. 4d, e, g). Comparatively, 86.6% ±2.0 of recovered T-bet-deficient donor-Th17 cells maintained IL-17A production and only 12.5% ±1.6 acquired a double-negative phenotype, while 4.1% ±0.8 acquired IL-22 production (Fig. 4d, e, g), corroborating previous reports that T-bet acts to constrain mechanisms of IL-17A and IL-22 production by Th17 cells in vivo.[40,41] Critically, neither T-bet-sufficient nor -deficient-Th17 cells gave rise to Th22-like cells, directly supporting evidence from our fate-mapping experiments, showing that Th22 cells in the respiratory compartment are not derived from Th17p cells during bacterial infection.

Among recovered T-bet-competent donor-Th22 cells, 17.4% ±0.7 maintained their phenotype, exclusively producing IL-22. Thus, a proportion of the transferred Th22 cells exhibit lineage commitment and phenotypic stability (Fig. 4d, f, g). However, recovered T-bet-competent donor-Th22 cells also displayed instability after transfer, of which 78.1% ±0.7 acquired a double-negative phenotype (Fig. 4d, f, g) and 4.5% ±0.2 acquired IL-17A production, both with and without maintenance of IL-22 production, which indicated a degree of plasticity toward a Th17 phenotype (consistent with our in vitro findings). Compared to T-bet-competent donor-Th22 cells, this Th17-skewing was exaggerated amongst T-bet-deficient donor-Th22 cells, which gave rise to large populations of both IL-22-producing Th17 cells (22.0% ±1.4) and Th17 cells that had ceased IL-22 production (8.6% ±0.6; Fig. Figure 4d, f, g). Amongst T-bet-deficient donor-Th22 cells, over half (53.2% ±1.1) maintained IL-22 production, of which 58.5% ±2.6 maintained a Th22 phenotype (Fig. 4d, f, g). We noted that the transition of Th22 donor cells toward a double-negative phenotype was markedly reduced in T-bet-deficient donor-Th22 cells (38.17% ±1.3; Fig. 4d, f, g). Collectively, our data identify a major T-cell-intrinsic role for T-bet in influencing IL-17A and IL-22 production by Th17 and Th22 cells, in addition to regulating the frequency, phenotypic flexibility and stability of each lineage during respiratory infection.

T-cell-intrinsic T-bet suppresses transcription of Th22-promoting factors
Finally, we explored cell-intrinsic factors which may be involved in T-bet-mediated suppression of Th17- and Th22 responses during *Cmu* infection by comparing gene-expression signatures of Th17 and Th22 cells purified by FACS from the lungs of *Cmu*-infected T-bet-competent or deficient dual-reporter mice at 8 dpi. T-bet-deficient-Th17 cells exhibited increased expression of *Rorc*, *Il6ra* and *Il17a* whilst T-bet-deficient-Th22 cells had increased expression of *Rorc*, *Il23r*, *Il1r1* and *Il22*, compared to T-bet-competent cells (Fig. 5). This data suggests that T-cell-intrinsic T-bet mechanistically suppresses expression of factors known to promote Th17 and Th22-cell development, and directs divergent transcriptional programming between these two lineages.[15,24,33,40,41] Transcript levels of other key Th17/Th22-associated genes *Runx1*, *Maf* and *Ahr* were similar in both Th17- and Th22- cells and their expression was unaffected by the absence of T-bet (Fig. 5).

DISCUSSION
Th22 cells accumulate at sites of infection and in a range of chronic inflammatory disorders. However, the developmental origin and lineage classification of Th22 cells, their interrelationship with Th17 cells, role in total production of IL-22 and potential for phenotypic plasticity remain largely undefined. We have provided new evidence that Th22 cells generated in the mucosa and associated lymphoid tissues in response to bacterial infection
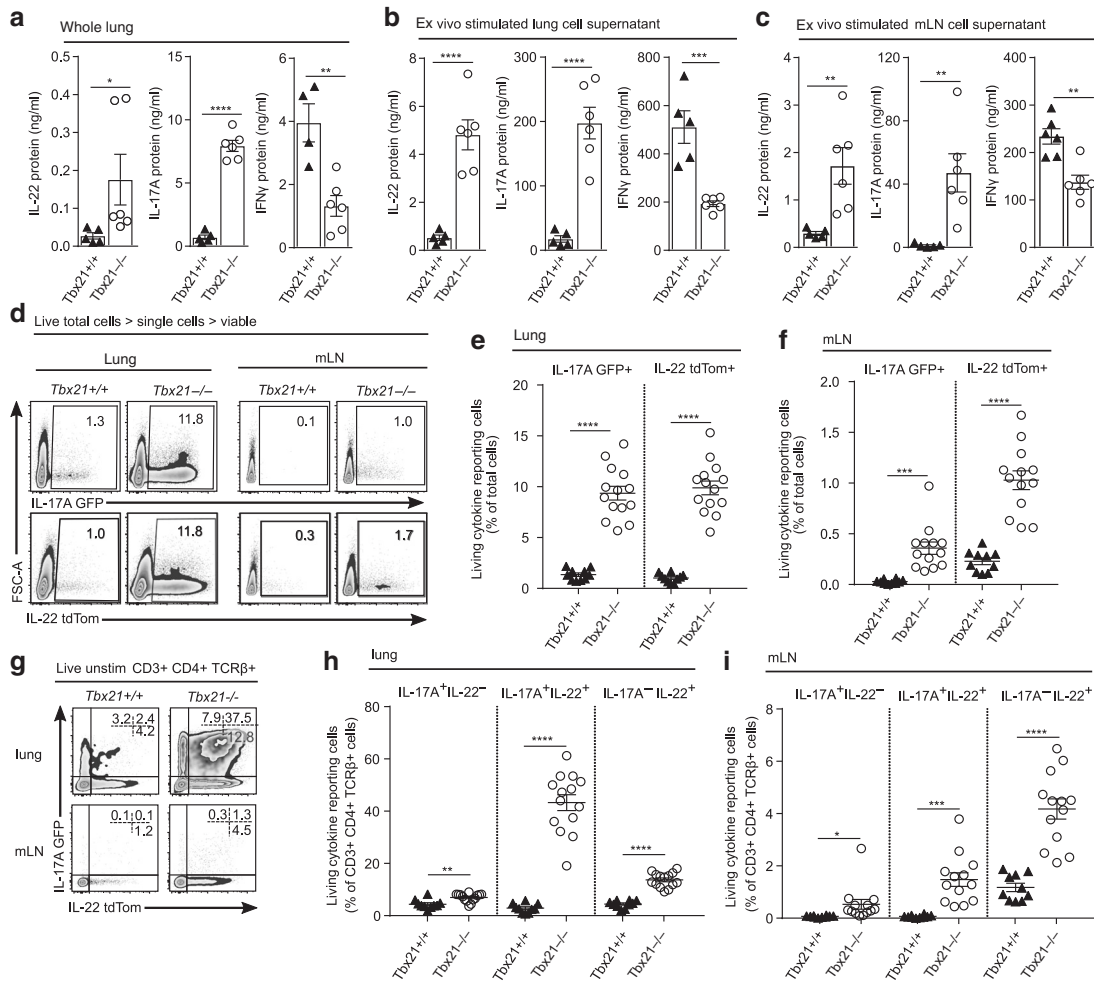
T-helper 22 cells develop as a distinct lineage from Th17 cells during...
JL Barnes et al.

1082

Fig. 3 **The transcription factor T-bet regulates Th17 and Th22-cell development in vivo. a–c** T-bet -competent (*Tbx21+/+*) or -deficient (*Tbx21−/−*), dual-reporter (IL-17A^eGFP IL-22^tdTom) mice received 300 inclusion-forming units (IFU) of *Chlamydia muridarum (Cmu)* intranasally, then were assessed at 8 days post infection. Cytokine concentration in whole-lung homogenates (**a** $n = 5–6$) and in supernatants of ex vivo T-cell-specific stimulated lung (**b** $n = 5–6$) and mediastinal lymph node (mLN; **c** $n = 5–6$) cultures as determined by ELISA. Bars indicate mean ± SEM and each symbol represents one mouse. Data are representative of two independent experiments. **d–i** Cells were isolated from lung and mLNs and analyzed by flow cytometry. Representative reporter for IL-17A (eGFP) and IL-22 (tdTom) in total living lung (**d**, left-panels) and mLN (**d**, right panels) cells and corresponding quantifications in (**e** $n = 10–14$, **f** $n = 10–13$). **g** Representative reporter for IL-17A and IL-22 in living lung (**g**, top-panels) and mLN (**g**, bottom panels) -Th cells (CD3+CD4+TCRβ+) and corresponding quantifications in (**h**; $n = 10–14$, **i**; $n = 10–13$). In the graphs bars indicate mean ± SEM and each symbol represents one mouse. Data are pooled from two independent experiments. Numbers in plots are relative percentages of parent-gate inside gate/quadrant. * represents $p < 0.05$, **$p < 0.01$, ***$p < 0.0001$, ****$p < 0.0001$ by Student's *t* test (**a–c**) or one-way ANOVA with Tukey correction (**e, f, h, i**).
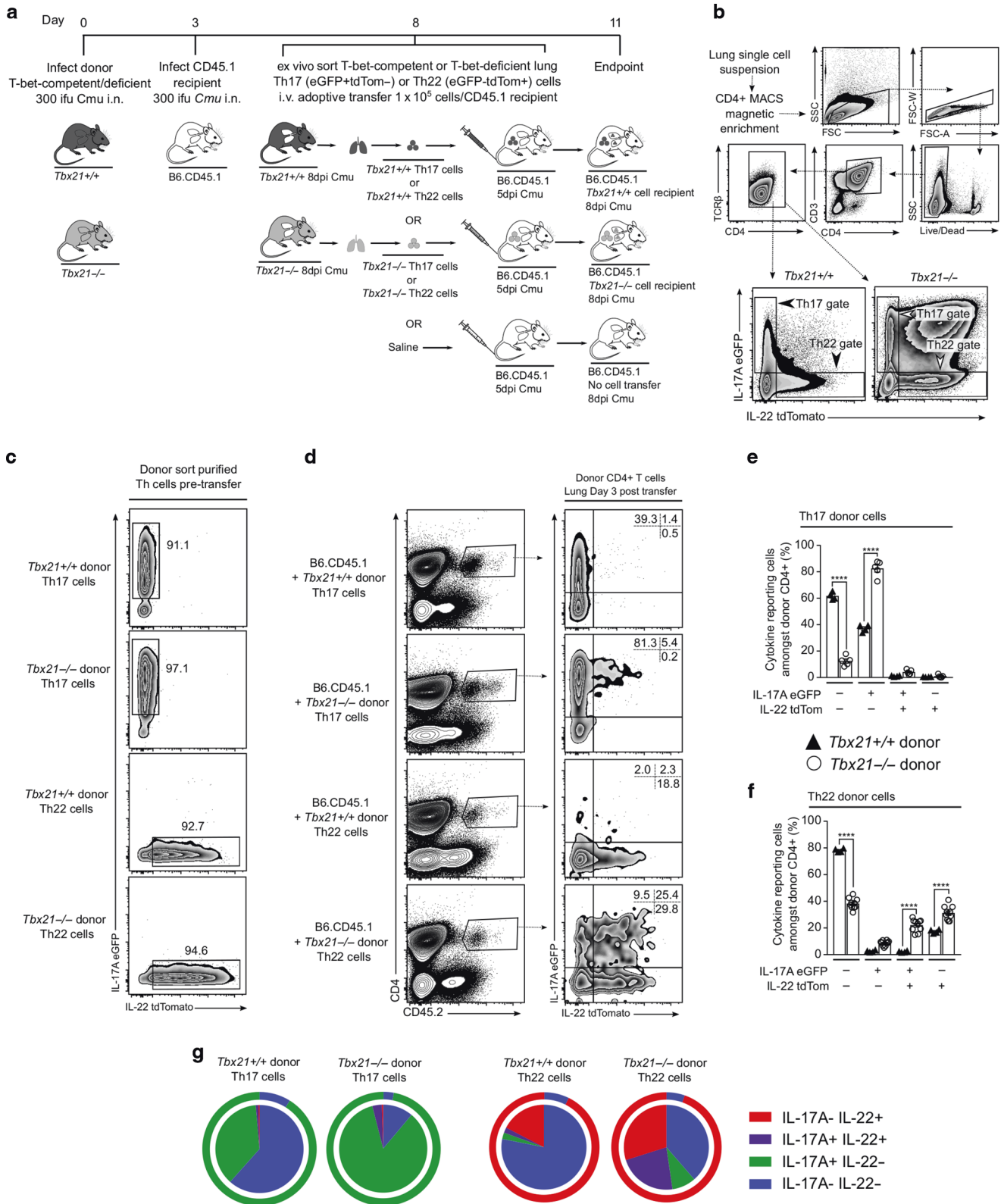
constitute a distinct cellular lineage and identify a previously unappreciated role for T-bet as a potential regulator of phenotypic stability and plasticity of these cells in vivo.

Several studies have reported distinct culture requirements for the generation of IL-17A+IL-22± (Th17) or IL-17A-IL-22+ (Th22) cells in vitro.[15,23,24,28] We have previously demonstrated that in vitro generated Th22 cells, like Th17 cells, are a distinct lineage that possess developmental plasticity.[24] Our next step was to determine if Th22 cells were a specific lineage and expanded in response to mucosal bacterial infection in vivo.

Until now, available evidence based on *Il22* fate-mapping studies has suggested that Th-derived IL-22 during infection may be transiently associated with Th17 cells,[21] and evidence of a dedicated Th22 lineage has been unsubstantiated. However, our data provide the first clear evidence that substantial IL-22 production in the lung, CLP and associated lymphoid tissues following bacterial infection can be ascribed to a dedicated Th22-cell subset, which develops via a lineage separate to Th17 cells in vivo. While this has not been previously reported in the context of infection, and more specifically

in mucosal tissues, our data are directly supported by two recent studies. Gartlan et al. used an established *Il17a* fate-mapping system in a model of GVHD to demonstrate that splenic Th22 cells, which arise from donor cells following allogeneic stem-cell transfer develop without transitioning via an IL-17A-expressing intermediate.[22] More recently, Perez, et al. used a similar fate-mapping triple-reporter system to show that intra-tumoral Th22 cells isolated during a model of colitis-associated colon cancer primarily developed independently of IL-17A.[23]

Interestingly, the CLP compartment represents a unique environment whereby, in response to *Cro* infection, two concurrent pathways exist for the expansion of Th22 cells. Outgrowth occurred equally via a distinct lineage but also from Th17p that had ceased production of IL-17A. It is unclear why two pathways exist in the CLP for the generation of Th22 cells, and why this notable Th17-to-Th22-cell plasticity was largely absent from the GALTs, lungs and mLN. Given the inherent propensity of GI-associated Th17 cells to divert to alternate Th programs (e.g., T-bet dependent transition to Th1-like cells),[30,42] one explanation could

T-helper 22 cells develop as a distinct lineage from Th17 cells during...
JL Barnes et al.

1083

a — Experimental timeline: Day 0 Infect donor T-bet-competent/deficient 300 ifu Cmu i.n. (Tbx21+/+ and Tbx21−/−); Day 3 Infect CD45.1 recipient 300 ifu Cmu i.n. (B6.CD45.1); Day 8 ex vivo sort T-bet-competent or T-bet-deficient lung Th17 (eGFP+tdTom−) or Th22 (eGFP-tdTom+) cells i.v. adoptive transfer 1 × 10^5 cells/CD45.1 recipient; Day 11 Endpoint. Tbx21+/+ 8dpi Cmu → Tbx21+/+ Th17 cells or Tbx21+/+ Th22 cells → B6.CD45.1 5dpi Cmu → B6.CD45.1 Tbx21+/+ cell recipient 8dpi Cmu. OR Tbx21−/− 8dpi Cmu → Tbx21−/− Th17 cells or Tbx21−/− Th22 cells → B6.CD45.1 5dpi Cmu → B6.CD45.1 Tbx21−/− cell recipient 8dpi Cmu. OR Saline → B6.CD45.1 5dpi Cmu → B6.CD45.1 No cell transfer 8dpi Cmu.

b — Lung single cell suspension, CD4+ MACS magnetic enrichment gating (SSC/FSC, FSC-W/FSC-A, TCRβ/CD4, CD3/CD4, SSC/Live/Dead). Tbx21+/+ and Tbx21−/− plots of IL-17A eGFP vs IL-22 tdTomato showing Th17 gate and Th22 gate.

c — Donor sort purified Th cells pre-transfer. Tbx21+/+ donor Th17 cells 91.1; Tbx21−/− donor Th17 cells 97.1; Tbx21+/+ donor Th22 cells 92.7; Tbx21−/− donor Th22 cells 94.6. IL-17A eGFP vs IL-22 tdTomato.

d — Donor CD4+ T cells Lung Day 3 post transfer. B6.CD45.1 + Tbx21+/+ donor Th17 cells (39.3 | 1.4 / — | 0.5); B6.CD45.1 + Tbx21−/− donor Th17 cells (81.3 | 5.4 / — | 0.2); B6.CD45.1 + Tbx21+/+ donor Th22 cells (2.0 | 2.3 / — | 18.8); B6.CD45.1 + Tbx21−/− donor Th22 cells (9.5 | 25.4 / — | 29.8). CD4 vs CD45.2 and IL-17A eGFP vs IL-22 tdTomato.

e — Th17 donor cells: Cytokine reporting cells amongst donor CD4+ (%). IL-17A eGFP −/−/+/+; IL-22 tdTom −/−/+/+. ▲ Tbx21+/+ donor; ○ Tbx21−/− donor. ****

f — Th22 donor cells: Cytokine reporting cells amongst donor CD4+ (%). IL-17A eGFP −/+/+/−; IL-22 tdTom −/−/+/+. ****

g — Pie charts: Tbx21+/+ donor Th17 cells; Tbx21−/− donor Th17 cells; Tbx21+/+ donor Th22 cells; Tbx21−/− donor Th22 cells. Legend: IL-17A− IL-22+ (red); IL-17A+ IL-22+ (purple); IL-17A+ IL-22− (green); IL-17A− IL-22− (blue).

be that colonic Th17-lineage positive Th22 cells arise from resident intestinal-Th17 cells; providing a rapid local mechanism to amplify Th22 responses to *Cro*, rather than relying solely on de novo differentiation of putative Th22 cells in secondary lymphoid tissues. Further, our data suggest that previously observed heterogeneity within IL-22 producing colonic T cells[21,43] likely reflects a mixed population arising from these separate developmental pathways. In a recent study, drug-induced inhibition of lymphocyte egress from lymph nodes during *Cro* infection markedly reduced IL-22-producing Th-cell accumulation in the colon, suggesting that the developmental origin of most colonic Th22 cells is likely the MLN.[44]

T-helper 22 cells develop as a distinct lineage from Th17 cells during…
JL Barnes et al.

1084

Fig. 4 T-bet regulates plasticity and phenotypic stability of Th17 and Th22 cells via a T-cell-intrinsic mechanism. a Adoptive transfer protocol. T-bet-competent (*Tbx21*+/+) or -deficient (*Tbx21*−/−) dual-reporter (*Il17a*^eGFP^*Il22*^tdTom^) donor mice received 300 inclusion-forming units (IFU) of *Chlamydia muridarum* (*Cmu*) intranasally (i.n.; day 0), then 8 days post infection (dpi; day 8) Th17− (CD3+CD4+TCRβ+eGFP +tdTom-) or Th22− (CD3+CD4+TCRβ+eGFP-tdTom+) cells were purified from the lungs of donor mice by FACS. Pooled donor cells from 6–8 mice were transferred via intravenous injection into male T-bet-competent B6.CD45.1 congenic recipients (1×10^5 cell/mouse) that were infected 5 days earlier with 300 IFU (*i.n.*) *Cmu*. Living donor cells (CD45.2+CD4+) that homed to lungs of recipient mice were assessed 3 days post transfer/8 dpi (day 11). Gating strategy (**b**) and purity (**c**) of sorted donor Th cells before transfer (routinely > 90%). CD4+ T cells were magnetically enriched from lungs of recipient mice and analyzed by flow cytometry. **d–f** Representative plots of donor cells (CD4+CD45.2+; **d**, left-panels) among total recipient lung CD4+ T cells and reporter for IL-17A (eGFP) and IL-22 (tdTom; **d**, right panels) in donor cells and their corresponding quantifications in (**e**; *Tbx21*+/+ Th17 cells, n = 4; *Tbx21*−/− Th17 cells, n = 5) and (**f**; *Tbx21*+/+ Th22 cells, n = 4; *Tbx21*−/− Th22 cells, n = 10). Numbers in plots are relative percentage of parent-gate cells inside gate/quadrant. Bars in graphs indicate mean ± SEM combined from two independent experiments with 2–5 recipient mice/group where each symbol represents one recipient mouse. **g** Relative proportion of cytokine-reporting cells among donor Th cells from (**d–f**). Outside ring indicates pre-transfer phenotype, inside pie indicates phenotype following adoptive transfer. Absolute mean is shown.

The appreciation that some Th17 and Th22 cells have an interchangeable phenotype led us to investigate the role of the transcription factor T-bet as a promiscuous regulator of plasticity. There are equivocal reports regarding the role of T-bet in Th22 development. Basu et al. reported that T-bet deficiency results in decreased *Il22* expression and IL-22 production by Th22 cells in vitro, and reduces Th22-cell number (identified by intracellular cytokine staining) among mixed populations of colonic CD4+ T cells following *Cro* infection.[15] By contrast, we have previously demonstrated using IL-22 reporter mice that the development of Th22 cells and their production of IL-22 in vitro is suppressed by T-bet.[24] In the present investigation, we show that during active *Cmu* infection a striking increase in both IL-17A and IL-22 cytokine production and Th17- and Th22-cell frequency occurred in T-bet deficient reporter mice. It is possible that spatiotemporal localization of Th22 cells in conjunction with the inflammatory milieu regulates the expression of T-bet and the subsequent impact on Th22 function.

T-bet is essential for optimal production of IFNγ, a cytokine that negatively regulates Th17 and Th22 differentiation.[18,24,42,45] However, our adoptive transfer studies clearly demonstrate that T-bet intrinsically regulates both Th cells and their respective production of IL-17A and IL-22 at the site of bacterial infection, not attributable to a reduction in IFNγ levels. Following transfer, T-bet-deficient-Th22 cells increased production of IL-22 and displayed marked Th17-like plasticity. By comparison, enhanced phenotypic stability and low levels of IL-22 induction were observed with T-bet-deficient-Th17 cells. Notably, a significant proportion of adoptively transferred T-bet-competent-Th22 cells maintained stability and phenotypic identity in T-bet-sufficient mice, which is contrary to our previous study whereby in vitro generated T-bet-sufficient-Th22 cells adoptively transferred into a similar IFNγ-high environment in mice infected with influenza A virus completely lost their ability to produce IL-22, whilst acquiring the ability to produce IFNγ.[24] This likely reflects an absence of key regulatory factor(s), that contribute to stabilization of the Th22 phenotype in vivo, from our defined Th22-polarizing conditions used for in vitro differentiation and/or the influenza induced inflammatory milieu.

The molecular mechanism by which T-bet influences Th22-cell outgrowth in vivo remains to be determined. To gain insights into potential regulatory factors we profiled genes previously implicated in Th22-cell development, and demonstrated that purified T-bet-deficient-Th22 cells have enhanced expression of *Rorc*, *Il23r* and *Il1r1*.[15,24,33] Recently, Runx1 was identified as a positive transcriptional regulator of *Il22* expression and IL-22 production by Th22 cells in vitro. Runx1 synergizes with Rorγt at the *Il22* enhancer, as well as positively regulating *Rorc* and *Il23r* expression, to further enhance IL-22 production.[33]

Whilst no difference in *Runx1* expression was observed in T-bet-deficient-Th17 or -Th22 cells, T-bet is known to directly interact with, and sequester, Runx1. The binding of T-bet to Runx1 suppresses the activation of a set of genes including *Rorc*, and Rorγt target gene *Il23r*, in Th17 cells and inhibits the Th17

developmental program.[40,46] Accordingly, colonic Th17 cells are reported to be hyper-responsive to IL-23 during colitogenic responses in T-bet-deficient mice, leading to high production of IL-17A and IL-22 and enhanced *Rorc*, *Il23r*, *Il1r1*, *Il17a*, and *Il22* expression.[41] In our study T-bet deficiency appeared to have distinct effects on the transcription of specific signature genes in Th17 cells compared to Th22 cells. Enhanced expression of *Il6ra* and *Il1r1* was unique to T-bet-deficient-Th17 and -Th22 cells, respectively. IL-1 signaling plays a critical role in Th17 cell differentiation, whereby IL-6 and IL-23 synergistically promote the production of IL-17 and IL-22.[47] We speculate that the acquisition of IL-17A- and increased IL-22- production by T-bet-deficient-Th22 cells may also reflect a regulatory mechanism downstream of dysregulated IL-1 receptor signaling. Collectively, our data provide in vivo evidence to support a divergent role for cell-intrinsic T-bet-mediated suppression of Th17- and Th22-cell differentiation and phenotypic flexibility during bacterial host defense responses. T-bet may limit Runx1-mediated gene activation, as well as modulate the IL-1 and IL-23 signaling axes. However, we recognize that T-bet regulation of Th22 cells is likely multifaceted and requires further investigation.

Cellular sources of IL-22 during infection and in chronic inflammatory conditions are diverse.[9,15,20,21,27] Th17 cells have been considered the primary source of IL-22 in many of these inflammatory settings and it is uncertain whether Th22 cells represent a critical or redundant source of this cytokine. *Cmu* infection of the lung increases IL-17A and IL-22 levels, and both cytokines are reported to contribute to host protection.[37,48] IL-17A production during the early and late phases of *Cmu* infection is primarily derived from γδ T cells and Th17 cells, respectively,[48] however the cellular sources of IL-22 have remained unclear. In the present study, we confirm previous reports on the cellular origin of IL-17A and identify that Th22 cells are the principal producers of IL-22 in the lung during *Cmu* infection. CD4+ T cells represent the primary source of IL-22 following infection with other intracellular bacterial pathogens such as *Mycobacterium tuberculosis*.[39] By contrast NK cells and γδ T cells are the primary cellular sources of IL-22 during respiratory infection with the extracellular bacterial pathogens *Klebsiella*-[49] and *Streptococcus-pneumoniae*,[38] respectively. However, we observed, only minimal IL-22 reporter activity in γδ T cells and none in NK cells following infection with *Cmu*, suggesting that IL-22 production by specific cells may depend on pathogen-specific factors.

In conclusion, our study demonstrates that a distinct subset of Th22 cells arises as a lineage separate from the Th17 differentiation program following mucosal infection of the lung and gut; although in some compartments alternative transition pathways exist. Th17 and Th22 cells demonstrate bidirectional phenotypic flexibility within infected tissues and their draining lymph nodes. T-bet acts as T-cell-intrinsic negative regulator of Th17 and Th22-cell development and their respective production of IL-17A and IL-22 at the site of bacterial infection. T-bet influences phenotypic flexibility of Th22 cells as a regulator of plasticity, and acts to
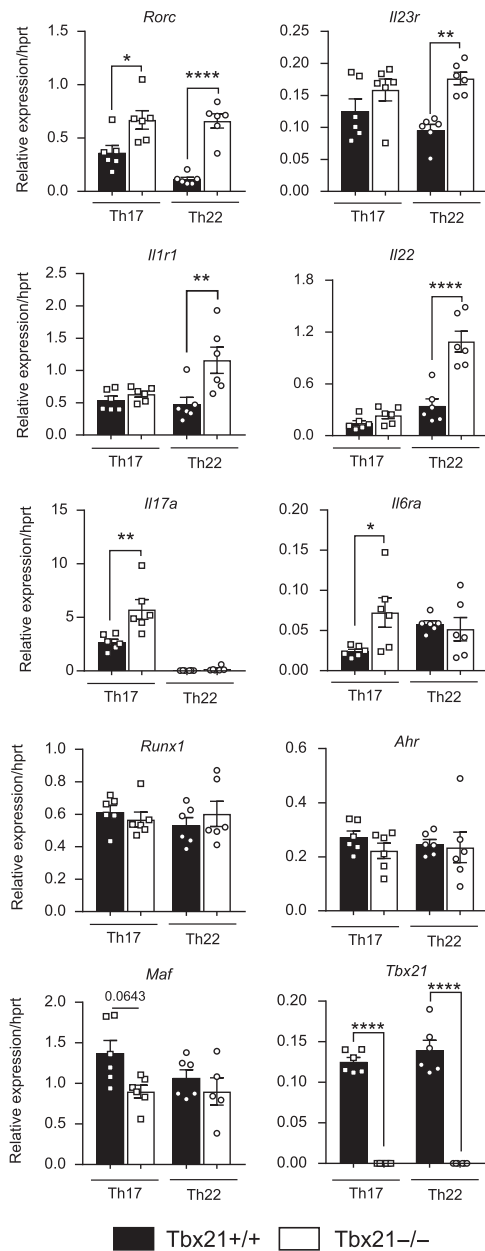
T-helper 22 cells develop as a distinct lineage from Th17 cells during…
JL Barnes et al.

1085

Fig. 5 T-cell-intrinsic T-bet suppresses transcription of Th22-promoting factors. T-bet-competent (*Tbx21*+/+) or -deficient (*Tbx21*−/−) dual-reporter (*Il17a*[eGFP]*Il22*[tdTom]) mice received 300 inclusion-forming units of *Chlamydia muridarum* (*Cmu*) intranasally, then 8 days post infection, Th17− (CD3+CD4+TCRβ+eGFP+tdTom−) or Th22- (CD3+CD4+TCRβ+eGFP+tdTom+) cells were purified from the lungs by FACS (as in Fig. 4b, c). Shown is gene expression in sorted cells relative to *Hprt* expression. Bars indicate mean ± SEM from a single experiment, n = 6, each symbol represents one mouse. * represents $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by One-way ANOVA with Tukey correction.

suppress Th17 cell stability. Comparative analysis of Th22 cells in response to a range of pathogenic stimuli will provide future mechanistic insight into the precise factors that regulate the development of Th22 cells as both a distinct lineage and as a derivative of Th17p at mucosal barriers.

METHODS
The source of all reagents are specified in Supplementary Table 2.

Mice
Specific pathogen-free mice on a C56BL/6 background were used for this study and were bred at the Australian Bioresources facility (ABR; Moss Vale, NSW, Australia). C57Bl/6 and B6.SJL-*PtprcaPep3b*/BoyJARC mice were purchased from ABR. C57BL/6-*Il17a*tm1Bcgen/J-*IL22promTdtomato* (dual-reporter) and B6.129S6-*Tbx21*tm1Glm/J-C57BL/6-*Il17a*tm1Bcgen/J-*IL22promTdtomato* (T-bet-deficient dual-reporter) mice were generated previously as reported.[24] B6.129×1-Gt(ROSA)26Sor[tm1(EYFP)Cos]/J (*R26R*[eYFP]) and *Il17a*[tm1.1(icre)Stck]/J (*IL-17a*[Cre]) mice, obtained from Lab Animals Services at The University of Adelaide, Australia, were intercrossed with dual-reporter mice to generate *Il17a*[Cre/+] triple-reporter mice. All animals that entered experimental protocols were maintained in individually ventilated cages in approved containment facilities within the Bioresources Facility, the Hunter Medical Research Institute (Newcastle, NSW, Australia). Equal numbers of male and female littermates were randomly assigned to experimental groups in the age range of 6–10 weeks, unless otherwise stated with no influence of sex observed. Animal experiments were conducted in accordance with Australian Animal Research legislation.

Single-cell suspensions
Following dissection of solid tissues; spleen, mLN, MLN and PP were mechanically dissociated by forcing through a 70-µM nylon cell strainer. Lung tissue was roughly chopped into ~2 mm^3 pieces then digested in GentleMACS C-tubes according to manufacturers' instructions. CLP lymphocytes were isolated as described in ref. [50] Red blood cells in lung and spleen cell suspensions were lysed with ammonium chloride solution, then washed twice before subsequent staining.

Flow cytometry
All cells were incubated with anti-mouse CD16/32 (2.4G2; 20 µg/mL), prior to staining with combinations of the fluorochrome-conjugated Abs listed in Supplementary Table 2. Living cells were used in all analyses. SYTOX™ Blue Nucleic Acid Stain was used for discrimination of dead cells. Prior to sorting, CD4+ T cells were enriched from lung cell suspensions using CD4 (L3T4) MicroBeads and LS-columns. Multi-parameter analysis and cell sorting was performed on a BD FACSAria III cell sorter and analyzed with FlowJo software V10. Leukocyte subsets were identified using hierarchical gating. When three reporter parameters were considered for analysis, Boolean gating was used to determine relative frequency of cytokine-reporting cells among the parent population.

Th-cell differentiation assay
Naive splenic CD4+ T cells were differentiated as previously described.[24]

Infections
Adult mice were administered 300 inclusion-forming units of *Cmu* Mouse Pneumonitis strain Nigg II (ATCC® VR-123™) intranasally in 30 µl of sucrose phosphate glutamate buffer or orally gavaged with 1×10^9 colony-forming units of *Cro* strain DBS-100 (ATCC® 51459™) in 200 µl of sterile DPBS.

Ex vivo cell culture
Lung (1×10^6 cells/well) and mLN (5×10^5 cells/well) cells were stimulated with anti-CD3ε (2 µg/mL platebound) and anti-CD28 (1 µg/mL platebound; 4 µg/mL soluble) in complete IMDM. Cell-free supernatants (SN) were collected after 48 h and stored at −80 °C until subsequent protein assessment.

Protein assessment
Cytokine levels in lung homogenates, and lung and mLN culture SN, were measured by enzyme-linked immunosorbent assay (ELISA) as previously described.[24] All analytes were measured on a SpectraMax M5 Microplate Reader with SoftMax Pro v5.4.3 software.

T-helper 22 cells develop as a distinct lineage from Th17 cells during…
JL Barnes et al.

1086

Adoptive transfer model

Sort purified lung-Th17 or -Th22 donor cells were pooled from 8 (*Tbx21*+/+) or 6 (*Tbx21*−/−) animals and transferred (1×10^5 cells per recipient; 2–5 male recipients per group) in 100 µl of Normal Saline by intravenous tail-vein injection, into *Cmu*-infected B6. CD45.1 congenic recipients. Donor cells were recovered from recipient lungs by enrichment of total CD4$^+$ T cells by magnetic cell separation using CD4 (L3T4) MicroBeads and LS-columns followed by surface staining with fluorochrome-conjugated Abs specific for CD4 and the WT C57Bl/6 pan-leukocyte marker, CD45.2. Control recipients that did not undergo adoptive transfer were injected with equivalent volume of saline alone.

Real-time quantitative PCR

Cells were sorted directly into TRIzol™ LS reagent. Total RNA was DNase treated (TURBO DNA-*free* ™ kit; Ambion) and reverse-transcribed (SuperScript IV Reverse Transcriptase kit). cDNA served as a template for the amplification of genes of interest by RT-qPCR, using gene-specific oligonucleotides (see Supplementary Table 1), KAPA SYBR® FAST PCR Master Mix and the ViiA 7 Real-Time PCR System. Transcript level was determined using the ΔCt method (2^−(GOI Ct – Hprt Ct)), relative to *hprt* expression.

Statistical analysis

For comparisons between 2 groups, an unpaired two-way Student's *t* test was used, or between multiple groups, a one-way ANOVA was used, corrected for multiple comparisons using GraphPad Prism v7.03 software. *P* values <0.05 were considered statistically significant.

AUTHOR CONTRIBUTIONS

J.L.B. conceived, designed, performed, analyzed and prepared visual presentation of all experiments with technical assistance from L.R.S., A.L., and H.L.T and input from K.A., S.M., G.E.K., J.R.M. and R.Y.K. M.W.P. conceptualized generation of triple-reporter mice and designed *Cmu* time-course and fate-mapping experiments. S.K., J.C.H., G.B. and P.M.H. provided essential reagents and advice on infection models. K.A. and S.M. revised and edited the manuscript. P.S.F. conceived the project. P.S.F. and M.W.P. acquired funding. J.L.B. and P.S.F. wrote the manuscript.

ADDITIONAL INFORMATION

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T-helper 22 cells develop as a distinct lineage from Th17 cells during…
JL Barnes et al.

1087

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