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Tissue-resident CD4⁺ T helper cells assist the development of protective respiratory B and CD8⁺ T cell memory responses

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Abstract

Much remains unknown about the roles of CD4⁺ T helper cells in shaping localized memory B cell and CD8⁺ T cell immunity in the mucosal tissues. Here, we report that lung T helper cells provide local assistance for the optimal development of tissue-resident memory B and CD8⁺ T cells after the resolution of primary influenza virus infection. We have identified a population of T cells in the lung that exhibit characteristics of both follicular T helper and T_{RM} cells, and we have termed these cells as resident helper T (T_{RH}) cells. Optimal T_{RH} cell formation was dependent on transcription factors involved in T follicular helper and resident memory T cell development including BCL6 and Bhlhe40. We show that T_{RH} cells deliver local help to CD8⁺ T cells through IL-21-dependent mechanisms. Our data have uncovered the presence of a tissue-resident helper T

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cell population in the lung that plays a critical role in promoting the development of protective B cell and CD8⁺ T cell responses.

INTRODUCTION

Tissue-resident memory B (B_{RM}) and T (T_{RM}) cells that reside in the mucosal sites have recently been identified and characterized (1–5). B_{RM} and T_{RM} cells are able to mount rapid recall responses in situ against invading pathogens before pathogen dissemination and, therefore, are thought to provide immediate and superior protection against secondary infections (1, 6–8). However, the mechanisms underlying the development and persistence of robust B_{RM} and T_{RM} cell responses in the respiratory tract are largely undefined. Furthermore, whether there are cellular and molecular pathways that can be targeted to simultaneously promote both B_{RM} and T_{RM} cell responses for maximal protection against pathogen reinfections remains unknown.

Influenza virus causes up to 56,000 deaths annually in the United States since 2010 (9). Influenza virus infection induces potent development of protective B_{RM} and CD8⁺ T_{RM} cell responses in the respiratory tract (5, 10, 11). Compared with memory B cells in the secondary lymphoid organs, lung B_{RM} cells have enhanced percentages of cells poised for cross-reactive memory repertoires and contribute to early plasmablast responses after influenza reinfection (1, 5, 12). Similarly, influenza-specific lung CD8⁺ T_{RM} cells can rapidly respond to heterologous influenza virus reinfection before the virus can replicate to high titers (13, 14). However, lung-protective CD8⁺ T_{RM} cell responses are short-lived in nature (15, 16), which poses a potential roadblock for developing T_{RM}-centered vaccination strategies. Therefore, better understanding the cellular and molecular mechanisms regulating the development and/or maintenance of lung-protective B_{RM} and/or CD8⁺ T_{RM} cell responses may aid the design of future influenza vaccines.

CD4⁺ T helper (T_H) cells are important in antiviral immune responses by providing essential “help” for the development of effector and memory CD8⁺ T and germinal center B (B_{GC}) cell responses (17–19). CD4⁺ T cell help, particularly during the priming stage, is vital for the development of circulating memory T (T_{MEM}) and CD103⁺ T_{RM} cells after primary influenza infection (20, 21). Similarly, CD4⁺ T cell help, mediated mainly through follicular helper T (T_{FH}) cells in the secondary lymphoid organs, is required for B cells to form GC and to produce high-affinity antibodies (Abs) during influenza infection (22–24). However, whether CD4⁺ T cells can assist local B and CD8⁺ T cell memory responses at the mucosal tissue after the resolution of primary infection is unknown. Several recent studies have also identified a population of PD-1^{Hi} “T_{FH}-like” cells in the peripheral tissues during autoimmunity (25–27). However, the developmental cues and the precise physiological function of these cells remain largely elusive. During influenza infection, the existence of a lung T_{FH}-like cell population that can potentially sustain lung B_{GC} cell responses has been recently demonstrated (26, 28, 29) but is still controversial (5, 27).

Here, we have identified a population of lung CD4⁺ T_H cells that are developed after influenza viral clearance and coexpress T_{FH} and T_{RM} features. On the basis of their gene expression, sessile features, and functional properties, we termed these cells tissue-resident

T helper (T_{RH}) cells. T_{RH} cells provide local help for the generation of optimal B_{GC} and B_{RM} cell responses, as well as a $CD8^+ T_{RM}$ population that is critical for the protection against heterologous influenza virus infection (30, 31). Our findings reveal a respiratory tissue-resident T_H cell population that is important in assisting local development of protective memory B and $CD8^+$ T cell responses.

RESULTS

Lung $CD4^+$ T cells provide “late” help for the formation of B_{GC} , B_{RM} , and $CD8^+ T_{RM}$ cell responses in situ

We sought to determine whether $CD4^+$ T cell help can assist local B and $CD8^+$ T cell memory responses at the mucosal tissue after the clearance of primary infection. We used a mouse model of influenza A virus PR8/34 (PR8) infection, in which viral clearance occurs within 10 days postinfection (d.p.i.) (32–34). We infected wild-type (WT) mice with PR8 virus and depleted $CD4^+$ T cells with α -CD4 (GK1.5) injections starting at 14 d.p.i. (Fig. 1A). $CD4^+$ T cells were largely depleted in the spleen and the lung (fig. S1A). At 42 d.p.i., we analyzed lung tissue B and T cell responses through intravenous (i.v.) injection of α -CD45 for 5 min before mouse sacrifice as reported (31, 35, 36). In this setting, $CD45_{i.v.}^-$ cells were within lung tissue, whereas $CD45_{i.v.}^+$ cells were in lung blood vessels. $CD4^+$ T cell depletion disrupted the formation of inducible bronchus-associated lymphoid tissue (iBALT), which contained B cell and $CD4^+$ T cell aggregates (28, 37, 38) (Fig. 1B and fig. S1B). $CD4^+$ T cell depletion also abrogated lung B_{GC} responses (fig. S1C). We then examined influenza hemagglutinin-specific B (HA-B) cell responses in the lung and spleen after $CD4^+$ T cell depletion using influenza HA-tetramer. Influenza PR8 HA tetramer mainly stained lung B cells of mice with prior PR8 but not X31 infection, which indicated the specificity of the HA-tetramer (fig. S1D). $CD4^+$ T cell depletion did not affect lung circulating ($CD45_{i.v.}^+$) nor splenic HA-B cell responses but drastically diminished total and HA-B cells in the lung tissue ($CD45_{i.v.}^-$) at the memory stage (Fig. 1C and fig. S1, E to G).

We next examined influenza-specific $CD8^+$ memory T cell responses in the lung and spleen after $CD4^+$ T cell depletion. To do so, we checked lung $CD8^+$ memory T cell responses against two dominant influenza major histocompatibility complex class I (MHC-I) H-2D^b-restricted epitopes, nucleoprotein (NP) peptide 366 to 374 ($NP_{366-374}$) and polymerase peptide 224 to 233 ($PA_{224-233}$) at 42 d.p.i. (31). It has been shown before that $CD8^+$ memory T cells against $NP_{366-374}$ or $PA_{224-233}$ epitope exhibit distinct phenotypic and recall properties (31, 39–41). Specifically, lung $CD8^+ NP_{366-374}$ memory T cells are highly protective and dominate over the $CD8^+ PA_{224-233}$ memory T cells in the secondary recall expansion upon rechallenge with heterotypic influenza virus (31, 39–41). Late $CD4^+$ T cell depletion did not affect lung circulating or parenchymal total $CD8^+$ or $PA_{224-233}$ memory T cell population (Fig. 1D and fig. S1, H to J) but caused a significant decrease of the magnitude of $CD8^+ NP_{366-374}$ memory T cells in the lung tissue compartment (Fig. 1D and fig. S1, H to I). The magnitudes of parenchymal $CD8^+ CD69^+ NP_{366-374} T_{RM}$ or $CD8^+ CD69^+ CD103^+ NP_{366-374} T_{RM}$ cells were also diminished after late $CD4^+$ T cell depletion (Fig. 1D and fig. S1K). Late $CD4^+$ T cell depletion did not affect the percentages of $CD103^+$ cells within the $CD8^+ CD69^+ NP_{366-374} T_{RM}$ population (fig. S1L). This finding is in

contrast with the results observed after CD4⁺ T cell depletion before influenza infection (21). Contrary to the diminished magnitude of lung CD8⁺ NP_{366–374} T_{RM} cells, late CD4⁺ T cell depletion did not decrease splenic CD8⁺ NP_{366–374} or CD8⁺ PA_{224–233} T_{MEM} cells (fig. S1M). These data suggest that continuous CD4⁺ T cell help after viral clearance is required for the persistence of optimal B and CD8⁺ T cell responses against a dominant protective epitope in the lung at the memory stage.

CD4⁺ T cells could possibly provide help to B or CD8⁺ T cells either in the circulation or in the lung. To determine whether lung tissue CD4⁺ T cells can provide local help, we infected WT mice with PR8 virus and depleted CD4⁺ T cells at 14 d.p.i. in the presence of FTY720 (Fig. 1E), a compound that blocks lymphocyte migration (42). FTY720 treatment drastically diminished T and B cell circulation in the blood (fig. S2A). The depletion of CD4⁺ T cells abolished B_{GC} cell development, total HA-B cells, and HA-specific B_{RM} (HA-B_{RM}) cells (identified as CD45_{i.v.}[−] B220⁺ GL7[−] IgD[−] IgM[−] CD38⁺ HA⁺) in the lung (Fig. 1, F to H, and fig. S2B) even in the presence of FTY720. Influenza-specific immunoglobulin G (IgG) in bronchoalveolar lavage fluid was also significantly decreased after CD4⁺ T cell depletion (fig. S2C). Further, CD4⁺ T cell depletion diminished lung tissue CD8⁺ NP_{366–374}-specific T_{MEM}, CD8⁺ CD69⁺ T_{RM}, and CD8⁺ CD69⁺ CD103⁺ T_{RM} cells but did not affect the number of total CD8⁺ T_{RM} cells (Fig. 1I and fig. S2D). Similar data were observed after CD4⁺ T cell depletion in the presence of FTY720 during H3N2 X31 influenza virus infection (fig. S2, E and F). Thus, lung CD4⁺ T cells can provide in situ help to local B and CD8⁺ T cells for the optimal development of B_{GC}, B_{RM}, and CD8⁺ NP_{366–374}-specific T_{RM} cells after viral clearance.

We next sought to examine whether lung tissue CD4⁺ T cells are sufficient for the generation of B_{GC}, B_{RM}, and CD8⁺ NP_{366–374}-specific T_{RM} cell responses after the clearance of the primary infection, in the absence of circulating CD4⁺ T cells. We injected low or high doses of α-CD4 into PR8-infected WT mice at 14 d.p.i. Low dose of α-CD4 treatment largely ablated CD4⁺ T cells in the blood but not in the lung parenchyma, whereas the high-dose α-CD4 injection depleted both circulating and lung parenchymal CD4⁺ T cells (Fig. 1J and fig. S2, G and H). High dose, but not low dose, of α-CD4 treatment diminished the magnitude of lung B_{GC}, influenza HA, or NP-specific B_{RM} (NP-B_{RM}) and CD8⁺ NP_{366–374} T_{RM} (including CD69⁺ T_{RM} or CD69⁺CD103⁺ T_{RM}) cells (Fig. 1, K and L). Together, our data suggest that lung tissue CD4⁺ T cells, rather than CD4⁺ T cells in the circulation, provide late “local help” for the optimal generation of B_{GC}, B_{RM}, and CD8⁺ T_{RM} responses after influenza infection.

Identification of a population of T_{FH}-like cells in the lung

We next sought to identify the lung CD4⁺ T cell populations that may mediate the local help to B and/or CD8⁺ T cells. To do so, we performed single-cell RNA sequencing (scRNA-seq) on CD45_{i.v.}[−] lung parenchymal CD4⁺ T cells at 28 d.p.i. Hierarchical clustering analysis identified five separated CD4⁺ T cell populations within the lung parenchyma (Fig. 2A and fig. S3, A and B). These cell populations included T_H1 effector/memory-like cells [expressing high levels of chemokine receptor *Cxcr6* and T_H1 transcription factor *Tbx21* (Tbet); cluster 0], T cells recently entering the lung or circulating effector memory T cells

[expressing the transcription factor *Klf2* and sphingosine 1-phosphate receptor 1 (*S1pr1*), which mediate lymphocyte migration; cluster 1], regulatory T (T_{reg}) cells (expressing T_{reg} lineage-specific transcription factor *Foxp3*; cluster 2), T_H17 -like (expressing T_H17 signature genes *Il17/Ccr6/Rora*; cluster 4), and a cluster of T cells exhibiting features of T_{FH} cells (expressing T_{FH} transcription factor *Bcl6* and signature cytokine *Il21*; cluster 3) (Fig. 2B and fig. S3C). Cluster 3 $CD4^+$ T cells also expressed higher levels of T_{FH} -associated surface molecules *Pdcd1* (PD-1), *Cxcr5*, and *Izumo1r* (FR4) (Fig. 2B).

Flow cytometry analysis identified a population of total $CD4^+$ or influenza-specific lung $CD4^+$ NP_{311–325} PD-1^{Hi}FR4^{Hi} T cells that expressed T_{FH} -associated markers including B-cell lymphoma 6 (BCL6), inducible T-cell costimulator (ICOS), and P2X purinoceptor 7 (P2RX7) but relatively lower levels of CXCR5 compared with splenic T_{FH} cells (Fig. 2, C and D, and fig. S3, D and E). Similar to splenic T_{FH} cells, lung $CD4^+$ PD-1^{Hi}FR4^{Hi} T cells expressed low levels of interferon- γ (IFN- γ) and interleukin-17 (IL-17; Fig. 2E and fig. S3F). However, splenic T_{FH} cells expressed significantly higher IL-4 than lung $CD4^+$ PD-1^{Hi}FR4^{Hi} T cells (Fig. 2E and fig. S3F). Using IL-21-vivid Verde fluorescent protein (VFP) reporter mice (43), we found that lung $CD4^+$ PD-1^{Hi}FR4^{Hi} T cells expressed high levels of IL-21, almost comparable with those of splenic T_{FH} cells (Fig. 2F). These data suggest that there is a lung tissue $CD4^+$ T cell population transcriptionally and phenotypically resembling T_{FH} cells in the secondary lymphoid organs.

Transcriptional profiling reveals lung T_{FH} -like cells exhibit T_{RM} gene signature

To gain more insights into the phenotype and identity of lung T_{FH} -like cells, we sought to compare the transcriptional signatures of lung T_{FH} -like cells to splenic T_{FH} , non- T_{FH} , and lung non- T_{FH} -like cell signatures. $Foxp3^+$ T_{reg} cells expressed folate receptor 4 (FR4), glucocorticoid-induced TNFR-related protein (GITR), and programmed cell death protein 1 (PD-1) (albeit their PD-1 levels were lower than T_{FH} -like cells) (Figs. 2B and 3A and fig. S3C). To minimize the potential contribution of T_{reg} cells on the transcriptional profiles of lung T_{FH} -like cells, we excluded splenic or lung $GITR^+$ $CD4^+$ T cells, which were mostly $Foxp3^+$ cells, in our sorting (fig. S4A). We then sorted splenic non- T_{FH} ($CD4^+CD44^{Hi}GITR^-CXCR5^{Low}PD-1^{Low}$), splenic T_{FH} ($CD4^+CD44^{Hi}GITR^-CXCR5^{Hi}PD-1^{Hi}$), lung non- T_{FH} -like ($CD45_{i.v.}^-CD4^+CD44^{Hi}GITR^-PD-1^{Low}FR4^{Low}$), and lung T_{FH} -like cells ($CD45_{i.v.}^-CD4^+CD44^{Hi}GITR^-PD-1^{Hi}FR4^{Hi}$) and performed RNA-seq analysis.

Differential gene expression and principal components analysis revealed that those four different $CD4^+$ T cell populations have distinct gene expression patterns, although lung $CD4^+$ PD-1^{Hi}FR4^{Hi} cells were more distinct from splenic non- T_{FH} cells relative to splenic T_{FH} or lung $CD4^+$ PD-1^{Low}FR4^{Low} cells (Fig. 3, A and B, and fig. S4B). When directly compared with lung $CD4^+$ PD-1^{Low}FR4^{Low} cells, lung $CD4^+$ PD-1^{Hi}FR4^{Hi} cells highly expressed T_{FH} -associated genes including *Il21*, *Tox2*, and *Pdcd1* (Fig. 3C). Gene Set Enrichment Analysis (GSEA) showed that lung $CD4^+$ PD-1^{Hi}FR4^{Hi} cells had an enrichment of T_{FH} -associated genes (44) relative to $CD4^+$ PD-1^{Low}FR4^{Low} cells (Fig. 3D). Conversely, lung $CD4^+$ PD-1^{Low}FR4^{Low} cells expressed higher levels of *Ly6c* and *Il7r* and showed enhanced enrichment of genes in transforming growth factor- β , hypoxia, and Notch signaling relative to PD-1^{Hi}FR4^{Hi} cells (Fig. 3C and fig. S4C). When compared with splenic

T_{FH} cells, lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells showed increased expression of genes associated with tissue migration, retention, and function including *Ccr2*, *Bhlhe40*, and *Cxcr6* (Fig. 3E) (45, 46). GSEA revealed that lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells had significant enrichment of T_{RM}-associated genes relative to splenic T_{FH} cells (Fig. 3F) (47). Lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells also had higher expression of genes associated with IL-2/signal transducers and activators of transcription 5 (STAT5), nuclear factor κ B (NF- κ B), and interferons (IFN) signaling, whereas splenic T_{FH} cells had higher enrichment of Myc and phosphatidylinositol 3-kinase (PI3K)–mammalian target of rapamycin (mTOR) signaling (fig. S4D). Thus, these RNA-seq analyses indicate that lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells exhibit transcriptional signatures of both T_{FH} cells and T_{RM} cells.

To confirm these observations, we sorted splenic T_{FH}, lung CD4⁺ PD-1^{Hi}FR4^{Hi}, or lung CD4⁺ PD-1^{Low}FR4^{Low} cells and performed Nanostring analysis of 560 immune-associated genes (31). Compared with lung CD4⁺ PD-1^{Low}FR4^{Low} cells, splenic T_{FH} and lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells expressed higher levels of T_{FH}-associated genes including *Bcl6*, *Sh2d1a*, and *Tcf7* (Fig. 3G). Compared with splenic T_{FH} cells, both PD-1^{Hi}FR4^{Hi} and PD-1^{Low}FR4^{Low} lung CD4⁺ T cell populations had enhanced expression of genes associated with tissue migration and residency but diminished expression of lymphoid migration or retention molecules *Sell* (CD62L) and *Ccr7* (Fig. 3H). These data suggest that lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells exhibit a “hybrid” gene signature of both conventional T_{FH} cells and T_{RM} cells.

Lung CD4⁺ PD-1^{Hi}FR4^{Hi} T cells are tissue resident

PD-1^{Hi}FR4^{Hi} cells expressed higher levels of CD69, CXCR6, and *Bhlhe40* molecules associated with T cell migration, retention, and function in the respiratory mucosal tissue, compared with splenic T_{FH} cells (Fig. 4A). To confirm their tissue residency, we performed parabiosis experiments and joined the circulation of PR8-infected CD45.1⁺ and CD45.1⁺ CD45.2⁺ congenic mice at 21 d.p.i. (Fig. 4B). We examined CD4⁺ T cell exchange between the two parabionts after 2 weeks of parabiosis. Close to 40 to 60% of splenic CD4⁺ T cells in parabiont hosts were derived from their parabiont pair (Fig. 4C). Within lung intravenous CD45 Ab protected tissue CD4⁺ T cell compartment, PD-1^{Hi}FR4^{Hi} total CD4⁺ or antigen (Ag)–specific CD4⁺ NP_{311–325} T cells exhibited limited exchange between the two parabionts (Fig. 4, D and E), suggesting that these cells are mostly tissue resident. Most of Ag-specific CD4⁺ NP_{311–325} T cells are tissue resident (Fig. 4E), whereas total lung CD4⁺ PD-1^{Low}FR4^{Low} T cells showed a higher circulating rate than those of CD4⁺ PD-1^{Hi}FR4^{Hi} cells (Fig. 4D). Thus, lung CD4⁺ PD-1^{Hi}FR4^{Hi} cells are lung tissue–resident T cells exhibiting both T_{FH} and T_{RM} features. On the basis of their gene signature, protein expression, cytokine production, and tissue residency property, we termed these cells as T_{RH} cells.

Optimal T_{RH} responses depend on both T_{FH} and T_{RM} transcription factors

We next sought to investigate the molecular mechanisms regulating lung T_{RH} cell development. We first examined whether lung T_{RH} cell development is dependent on the master transcription factor of T_{FH} cells, BCL6 (48, 49). We infected WT (*Bcl6*^{f/f}) or *Bcl6*^{-/-} mice with PR8 virus and examined total and influenza-specific T_{RH} or non-T_{RH} cells

in the lung tissue at 28 d.p.i. T cell-specific BCL6 deficiency greatly diminished both the frequencies and the magnitude of lung T_{RH} responses but not those of non-T_{RH} responses (Fig. 5, A to C). Consistent with prior work (50, 51), T cell-specific BCL6 deficiency also diminished splenic T_{FH} responses (Fig. 5D).

Bhlhe40 is critical for the development of tissue-resident CD8⁺ T cell responses (46). Because lung T_{RH} and non-T_{RH} cells expressed high levels of *Bhlhe40* (Fig 4A), we investigated the roles of Bhlhe40 in lung T_{RH} responses relative to splenic T_{FH} cells. Lung T_{RH} cells were enriched with *Bhlhe40*-associated genes (46) compared with splenic T_{FH} cells (Fig. 5E). T cell-specific *Bhlhe40* deficiency (*Bhlhe40*^{-/-}) modestly increased the frequencies of T_{RH} cells relative to non-T_{RH} cells within the influenza-specific NP_{311–325} CD4⁺ T cell population but not in the total lung CD4⁺ T cell population (Fig. 5, F and G, and fig. S5, A and B). *Bhlhe40* deficiency in T cells significantly diminished total and influenza-specific CD4⁺ T cells in the lung tissue but not in the spleen (fig. S5, C and D). These data suggest that *Bhlhe40* is required for the establishment of the overall lung-resident CD4⁺ T cell population after influenza infection, which was also observed with the lung-resident CD8⁺ T cells (46). As a result, the magnitude of both T_{RH} and non-T_{RH} cells in the lung was significantly decreased (Fig. 5G and fig. S5B). In contrast, *Bhlhe40* deficiency did not alter either the frequencies or the magnitude of the splenic T_{FH} response (fig. S5, A, B, E, and F). Previously, we have reported that *Bhlhe40* is required for the survival of CD8⁺ T cells in nonlymphoid tissues (46). Consistent with that observation, *Bhlhe40* deficiency resulted in enhanced cellular apoptosis in both lung T_{RH} and non-T_{RH} cells (Fig. 5H and fig. S5G). These data indicate that *Bhlhe40* is likely not important for the acquisition of T_{FH}-like features in T_{RH} cells but is vital in sustaining T_{RH} cell survival in respiratory mucosal tissues. Thus, the optimal formation of lung T_{RH} cells requires transcription factors involved in both T_{FH} (BCL6) and T_{RM} (Bhlhe40) development. Conversely, the formation of splenic T_{FH} cells is dependent on BCL6 but not Bhlhe40, and the development of lung PD-1^{Low}FR4^{Low} cells (probably consist of conventional T_{RM} cells) is dependent on Bhlhe40 but not BCL6.

T_{RH} cells assist the formation of protective B_{GC}, B_{RM}, and CD8⁺ T_{RM} cell responses

We hypothesized that T_{RH} cells are responsible for mediating the effects of CD4⁺ T cell help on lung local B and CD8⁺ T cells. Consistent with that hypothesis, T cell-specific BCL6 deficiency led to diminished B_{GC}, B_{RM}, and CD8⁺ NP_{366–374} T_{RM} cell responses (fig. S6, A to E). Furthermore, T cell-specific *Bhlhe40* deficiency resulted in diminished lung tissue CD8⁺ T, B, B_{RM}, and CD8⁺ T_{RM} cell responses, although it is possible that *Bhlhe40* deficiency in CD8⁺ T cells directly contributes to the diminished CD8⁺ T_{RM} cell phenotype in these mice as previously shown (fig. S7, A to C) (46).

To specifically determine whether T_{RH} cells are required for the development of memory B and CD8⁺ T cells in the lung, we generated CD4⁺ T cell-specific inducible BCL6-deficient mice (*Bcl6*^{CD4^{ERT2}}). Tamoxifen treatment efficiently caused gene recombination in CD4⁺ T cells but only minimally in other lymphocytes in *Bcl6*^{CD4^{ERT2}} mice (fig. S8, A to C). We then infected WT (*Bcl6*^{fl/fl}) or *Bcl6*^{CD4^{ERT2}} mice with PR8 and administered tamoxifen daily from 12 to 16 d.p.i. (five times) to specifically ablate BCL6 in CD4⁺ T cells

after influenza infection (Fig. 6A). To exclude the contribution of lymphoid organ T_{RH} cells in providing the “late” help to lung B (52) and $CD8^+$ T cells, we treated the mice with FTY720 daily to block T and B cell migration starting at 11 d.p.i. At the lung, the magnitude of $CD45_{i.v.}^- CD4^+$ T and B cells was decreased in the inducible BCL6-ablated group (fig. S8D). As with the constitutive BCL6 deficiency in T cells, inducible $CD4^+$ T cell-specific BCL6 ablation resulted in diminished T_{RH} but not non- T_{RH} responses in the lung (fig. S8E). The ablation of lung T_{RH} responses significantly diminished B_{GC} , influenza HA- B_{RM} , and NP- B_{RM} responses in the lung (Fig. 6, B to D). $CD8^+$ NP_{366–374} T_{RM} responses were also significantly impaired after T_{RH} ablation (Fig. 6E and fig. S8F). These data suggest that lung T_{RH} cells are important in assisting the development of local B and $CD8^+$ T cell memory responses in situ.

To examine the roles of T_{RH} cells in the maintenance of lung tissue B and $CD8^+$ T cell responses, we treated WT or *Bcl6*^{CD4^{ERT2}} mice with tamoxifen starting from 21 to 25 d.p.i. to ablate T_{RH} cell responses at the memory stage (Fig. 6F). We then examined B_{GC} , B_{RM} , and $CD8^+$ T_{RM} cell responses at 42 d.p.i. (Fig. 6, G to J). T_{RH} cell ablation at the memory stage did not lead to significant decrease of B_{RM} cell magnitude but significantly impaired B_{GC} cell responses at 6 weeks postinfection (Fig. 6, G to I). These data suggest that T_{RH} cells help program lung B_{RM} cell development but may not be directly required for their maintenance at the memory stage. However, T_{RH} cells are continuously needed for sustaining lung B_{GC} cell responses (Fig. 6G). These data are consistent with the data showing that B_{GC} cell responses are important for B_{RM} cell development in the first 3 weeks but may not notably contribute to lung B_{RM} cells after 20 d.p.i. (5). T_{RH} cell ablation at the memory stage also diminished $CD8^+$ NP_{366–374} T_{RM} cell responses, suggesting that lung T_{RH} cells continuously provide local help for the maintenance of $CD8^+$ NP_{366–374} T_{RM} cells (Fig. 6J). Together, our data indicate that T_{RH} cells are vital for programming lung B_{RM} cell development before 20 d.p.i. and are necessary for both the optimal development and maintenance of B_{GC} and $CD8^+$ NP_{366–374} T_{RM} cell responses at the memory stage.

Lung memory B cells generated from local B_{GC} cells harbored high portions of cross-reactive B cells to H3N2 A/Uruguay/716/07 strain (Urg) after influenza X31 (H3N2) virus infection (12). To examine whether T_{RH} cells help to generate those cross-reactive B_{RM} cells, we infected WT or *Bcl6*^{CDERT2} mice with X31 virus and injected the mice with tamoxifen in the presence of FTY720 as shown in fig. S8A. We then checked X31 strain-specific HA- B_{RM} and cross-reactive HA- B_{RM} cells against H3N2 Urg (12) using flow cytometry. T_{RH} ablation resulted in diminished strain-specific (X31 HA⁺ and Urg HA⁻) and cross-reactive (both X31 HA⁺ and Urg HA⁺) B_{RM} cell responses (Fig. 6K). These data indicate potential roles of T_{RH} cells in the development of both strain-specific and cross-reactive B cell immunity, although the function of those cross-reactive B cells in the protective immunity remains to be determined.

Because $CD8^+$ T_{RM} [particularly NP_{366–374} T_{RM} (31)] and possibly B_{RM} cells are important in mediating host heterologous protection (5), we next sought to determine whether the ablation of T_{RH} cells impairs host protective immunity against heterologous virus infection. To do so, we used a heterologous infection and challenge model in which X31 virus was used as the primary infection and lethal PR8 virus was used as the secondary challenge (46).

PR8 and X31 viruses differ in the viral surface proteins but share internal viral proteins such as NP (53). Hence, CD8⁺ T_{RM} and possibly B_{RM} cells against internal viral epitopes (mainly against viral NP protein) can provide heterologous protection (5, 31). We infected WT or *Bcl6*^{CD4ERT2} mice with X31 virus and treated the mice with tamoxifen. We confirmed that T_{RH} ablation affects CD8⁺ NP_{366–374} T_{RM}, B_{GC}, and B_{RM} cell development in the X31 model at 35 d.p.i. (fig. S8G). We then rechallenged the mice with a lethal dose of PR8 in the presence of FTY720 to block the contribution of circulating memory CD8⁺ T and B cells at 42 d.p.i. (Fig. 6L). Close to 40% of *Bcl6*^{CD4ERT2} mice succumbed, whereas WT mice were fully protected with lethal PR8 infection (Fig. 6M). Consistent with the increased mortality, lung from *Bcl6*^{CD4ERT2} mice exhibited increased infectious virus titer and elevated viral gene (NP and M2) expression compared with those of control mice (Fig. 6, N and O). Thus, T_{RH} cells are required for optimal protection against secondary heterologous infection, most likely through their help for the development of protective CD8⁺ T_{RM} and B_{RM} response.

Identification of the factors mediating T_{RH}-derived “local” help

CD40-CD40L interaction is critical for T cell help of B_{GC} cell responses (5, 51). Lung influenza-specific T_{RH} cells express modestly higher levels of CD40L than non-T_{RH} cells (fig. S9, A and B). CD40L blockade diminished lung B_{GC} formation and the magnitude of HA-B_{RM} responses in the lung tissue (fig. S9, C and D). These data indicate that CD40L may be necessary for T_{RH} cells to facilitate B_{GC} and B_{RM} cell responses, but further studies are required to firmly establish this idea.

The vast majority of lung IL-21-VFP⁺ cells were CD4⁺ T cells after influenza infection at 28 d.p.i. (fig. S10A). IL-21-VFP^{Hi} cells, which expressed the highest levels of IL-21 than those IL-21-VFP^{Low} cells (fig. S10B), were mainly T_{RH} cells (Fig. 7A). In addition, T cell-specific BCL6 deficiency impaired *Il21* expression in the lung (fig. S10C). These data suggest that T_{RH} cells are the main IL-21 producers in the lung after influenza virus infection. Because IL-21 is an important cytokine that has been implicated in facilitating B_{GC}, memory B and CD8⁺ effector and memory T cell responses (54), we blocked IL-21 signaling starting at 14 d.p.i. through intraperitoneal injection of α-IL-21R in the presence of FTY720 (Fig. 7B). IL-21 signaling blockade did not impair B_{GC} or influenza HA-B_{RM} cell responses in the lung tissue (Fig. 7, C and D). However, IL-21 signaling blockade significantly diminished total CD8⁺ and NP_{366–374} T_{RM} cell responses, suggesting that T_{RH} cells help CD8⁺ NP_{366–374} T_{RM} cells establishment and/or maintenance through IL-21 (Fig. 7E and fig. S10D). IL-21R blockade did not significantly diminish PA_{224–233}- or PB1_{703–711}-specific T_{RM} cell responses (fig. S10E), indicating that the roles of IL-21 in T_{RM} cell maintenance may be epitope specific. Lung local blockade of IL-21 signaling (intranasal delivery of α-IL-21R) also diminished lung CD8⁺ NP_{366–374} T_{RM} magnitude (Fig. 7F) but not splenic memory T cells (Fig. 7, G and H, and fig. S10F) nor lung parenchymal B_{GC} or HA-B cell responses (fig. S10G).

IL-21R signaling blockade led to enhanced cellular apoptosis but not proliferation, specifically in CD8⁺ NP_{366–374} T_{RM} but not those of splenic memory T cells (Fig. 7I and fig. S10H). Compared with CD8⁺ PA_{224–233} T_{RM} cells, CD8⁺ NP_{366–374} T_{RM} cells

exhibited higher expression of genes associated with IL-21 signaling including *Batf* (fig. S11, A and B) (55). Furthermore, there was a higher expression of IL-21R in the CD8⁺ NP_{366–374} T_{RM} cells than CD8⁺ PA_{224–233} T_{RM} cells (fig. S11C). These data suggest that CD8⁺ NP_{366–374} T_{RM} cells, but not CD8⁺ PA_{224–233} T_{RM} cells, potentially receive IL-21 signaling in the lung at the memory stage after influenza virus infection. Consistent with the data, IL-21R blockade diminished basic leucine zipper transcription factor, ATF-like (BATF) expression in CD8⁺ NP_{366–374} T_{RM} but not CD8⁺ PA_{224–233} T_{RM} cells at 35 d.p.i. (Fig. 7J), indicating that T_{RH} cells provide local help to CD8⁺ NP_{366–374} T_{RM} cells in an IL-21–dependent manner. We then infected WT mice with PR8, treated the mice with α-IL-21R or control IgG in the presence of FTY720, and then challenged the mice with X31 at 6 weeks postinfection (Fig. 7K). Mice that received α-IL-21R treatment had enhanced infectious virus titers and elevated viral gene expression after X31 rechallenge (Fig. 7, L and M). Similar findings were observed in the model of primary X31 infection followed with PR8 virus rechallenge (fig. S11, D and E). Thus, IL-21–mediated T_{RH} cell help to CD8⁺ T cells promotes optimal heterologous immunity against influenza reinfection.

DISCUSSION

Here, we have identified a requirement for “in situ” CD4⁺ T cell help in the respiratory mucosa, which is mediated by what we have termed T_{RH} cells, in the development of localized protective memory responses after influenza virus infection. T_{RH} cells comanifest phenotypic and transcriptional hallmarks of both T_{FH} and T_{RM} cells. Tissue PD-1^{Hi}CXCR5^{Low}BCL6^{Int} T_{FH}-like cells have been previously observed (25–29). However, the cellular identity and developmental cues regulating their development have previously remained largely elusive. Furthermore, the physiological function of these cells beyond their help on the generation of BGC cells was unknown.

Using multiple lines of approaches including high or low doses of α-CD4 depletion, inducible CD4⁺ T cell–specific BCL6 ablation, and IL-21 blockade combined with long-term FTY720 treatment, we have provided comprehensive evidence that localized CD4⁺ T cell help, mediated by T_{RH} cells and IL-21, is required for the optimal generation of a population of protective T_{RM} cells (H-2D^b-restricted NP_{366–374} T_{RM} cells) after the resolution of primary infection. It was previously shown that continuous CD4⁺ T cell help beyond T cell priming (7 d.p.i.) was not required for the differentiation of CD103⁺ CD8⁺ T_{RM} cells, as measured by the levels of CD103 expression in GP_{33–41} T_{RM} cells after infection with recombinant influenza X31 virus expressing lymphocytic choriomeningitis virus GP_{33–41} peptide (21). Here, we found that persistent late CD4⁺ T cell help after viral clearance was required for the maintenance of the overall NP_{366–374} T_{RM} population (both CD103[–] CD69⁺ and CD103⁺ CD69⁺ T_{RM} cells). As a result, consistent with prior work (21), CD4⁺ T cell depletion after the resolution of primary infection did not affect the percentages of CD103⁺ T_{RM} cells nor CD103 expression level per cell within the total NP_{366–374} CD8⁺ CD69⁺ T_{RM} cell population. In addition, late CD4⁺ T cell help to Ag-specific T_{RM} cells appears to be epitope specific as the generation and/or the maintenance of PA_{224–233} or PB1_{703–711} T_{RM} cells are largely CD4⁺ T cell help or IL-21 independent.

An interesting question is why lung T_{RH} cell help, in the form of IL-21 provision, is specifically required for $CD8^+$ $NP_{366-374}$ T_{RM} cell responses. We have shown previously that $CD8^+$ $NP_{366-374}$ T_{RM} cells receive constant Ag engagement in the lung at the memory stage due to the chronic deposition of high levels of influenza NP Ag (31). Compared with conventional $CD8^+$ $PA_{224-233}$ T_{RM} cells, $CD8^+$ $NP_{366-374}$ T_{RM} cells express high levels of PD-1 and exhibit “exhausted-like” phenotypes similar to those of T cells receiving chronic Ag exposure during chronic viral infections (31, 56, 57). Consequently, the maintenance of these T_{RM} cells requires persistent MHC-I-dependent stimulation at the memory stage (31) similar to those of exhausted $CD8^+$ T cells (56). $CD4^+$ T cell help in the form of IL-21 has been recently demonstrated to sustain those PD-1–expressing $CD8^+$ T cells during chronic viral infection and tumor growth (58). Thus, $CD4^+$ T cell help and IL-21 signaling may be specifically required for maintaining the survival of lung $CD8^+$ T_{RM} cells receiving persistent low levels of in situ antigenic stimulation. Consistent with this idea, $NP_{366-374}$ T_{RM} cells express higher levels of molecules associated with IL-21 signaling, including IL-21R and BATF.

Influenza virus is able to undergo antigenic shift, drift, and reassortment to escape previously established host immunity. Current influenza vaccines require yearly update and only provide high levels of protection when influenza vaccine strains match exactly with the circulating strains. Much of the attention has been given to the development of potential universal influenza vaccines recently. The common goals of various universal influenza vaccine candidates are to induce broadly neutralizing influenza Ab, strong $CD8^+$ memory T cell responses against conserved epitopes and/or high levels of localized lung-resident mucosal immunity that can restrict viral spreading early (59–62). Because of the high mutation rates of influenza viruses, it is conceivable that the induction of “all inclusive” immune responses, i.e., induction of strong both B and $CD8^+$ T cell immunity in the mucosal tissue, may be required for the ultimate success of a universal influenza vaccine (62). Because of the distinct ability of T_{RH} cells in assisting local B_{RM} and T_{RM} cell development, it is tempting to speculate that the specific promotion of T_{RH} cells may simultaneously enhance both memory B and $CD8^+$ T cell immunity in the respiratory tract during vaccination, thereby providing rapid cross-reactive protection against broad-spectrum viruses.

In summary, our data have revealed a complex T-B cell interaction network that is programmed by lung T_{RH} cells for the maintenance of protective local respiratory immunity following acute influenza virus infection. Moving forward, it is of substantial interest to dissect the mechanisms modulating the development of T_{RH} cells and the precise function of T_{RH} cells in assisting the development of local B and $CD8^+$ T cell immunity during infection. The role of T_{RH} cells in vaccination and in other contexts remains to be examined.

METHODS

Study design

The goal of the study was to determine the roles of tissue-resident $CD4^+$ T_{TH} cells in coordinating protective tissue-resident $CD8^+$ and B cell responses after respiratory viral infection. We first examined lung $CD8^+$ T_{RM} , B_{GC} , and B_{RM} cell responses after the

depletion of CD4⁺ T cells after influenza viral clearance. We performed scRNA-seq and bulk RNA-seq, identified a tissue T_{FH}-like cell population coexhibiting both T_{FH} and T_{RM} gene features, and termed as T_{RH} cells. We used T cell-specific BCL6- or Bhlhe40-deficient mice to examine whether optimal T_{RH} cell development and/or maintenance were dependent on both T_{FH} and T_{RM} transcription factors. Using T cell-specific BCL6-deficient mice, we specifically ablated T_{RH} cells to measure lung CD8⁺ T_{RM} and B cell responses after primary influenza virus infection, as well as protection against a heterotypic influenza virus reinfection. Last, we used IL-21 reporter mice and an IL-21R blocking Ab to determine whether T_{RH}-dependent CD8⁺ T cell help is mediated by IL-21. Experiments were conducted in replicates as indicated in figure legends. No outliers were removed.

Mice and influenza viral infection

WT C57BL/6, CD45.1, and IL-21 VFP reporter mice were purchased from the Jackson Laboratory (JAX) and bred in-house. To generate CD45.1⁺ and CD45.2⁺ (CD45.1⁺/CD45.2⁺) mice, CD45.1 mice were crossed with C57BL/6 mice. *Bcl6*^{f/f} were generated as previously reported (50). *Bcl6*^T were generated by crossing with CD4-Cre transgenic. *Bcl6*^{CD4ERT2} were generated by crossing with CD4-ERT2 transgenic mice. *Bcl6*^{f/f} or *Bcl6*^{CD4ERT2} mice were additionally crossed with Rosa26 LSL-yellow fluorescent protein (JAX) mice for the determination of the efficiency of tamoxifen-induced gene recombination. *Bhlhe40*^{f/f} or *Bhlhe40*^T mice were generated as previously reported (46). All animal protocols were approved by the Institutional Animal Care and Use Committees (IACUC) of the Mayo Clinic (Rochester, MN). Sex- and age-matched 8- to 10-week-old mice of both sexes were used in the experiments. Influenza A/PR8/34 [~200 plaque-forming units (PFU) per mouse in the primary infection and ~1 × 10⁴ PFU/mouse in the secondary infection] and influenza A X31 (~800 PFU/mouse in the primary infection and ~1 × 10⁵ PFU/mouse in the secondary infection) were infected into the mice by intranasal under anesthesia as reported before (33). Both real death and humane sacrifice were counted as mouse mortality. According to the institute IACUC policy, we humanely euthanized infected mice when their weight was below 70% of original weight.

Ab administration in vivo

Influenza-infected WT mice were administrated with control IgG or various neutralizing or depleting Ab as described in the related Results sections. For CD4⁺ T cell depletion with high dose of α-CD4, mice were injected with 250 μg of α-CD4 weekly (clone: GK1.5, BioXCell) starting at 14 d.p.i. For circulating CD4⁺ T cell depletion, mice were intraperitoneally injected with 40 μg of α-CD4 for the first dose followed with 10 μg of α-CD4 weekly. CD40L blockade was achieved by the injection of 250 μg of α-CD40L (clone: MR-1, BioXCell) weekly, respectively. For systemic IL-21R blockade, 500 μg of α-IL21R (clone: 4A9, BioXCell) was injected intraperitoneally weekly starting at 14 d.p.i. For lung local IL-21R blockade, 50 μg of α-IL21R was injected through intranasal route weekly starting at 14 d.p.i. In some experiments, FTY720 (1 mg/kg; Cayman) was administrated via intraperitoneal injection daily from 13 d.p.i. to block lymphocyte migration until mouse euthanasia.

Tamoxifen treatment

To induce gene recombination in *Bcl6*^{CD4ERT2} mice, tamoxifen (Sigma-Aldrich) was diluted in warm sunflower oil (Sigma-Aldrich) and daily treated via intraperitoneal route for five consecutive times. Each application was 2 mg per mouse.

Immunohistochemistry and immunofluorescence

Left lobe of the whole lung was harvested and fixed in 10% formaldehyde (Thermo Fisher Scientific) until embedding. Fixed lung tissues were embedded in paraffin, sectioned at 10- μ m thickness. To identify tertiary lymphoid structure, the lung tissue slide was stained with hematoxylin and eosin by the Mayo Clinic Histology Core Laboratory (Scottsdale, AZ). To measure iBALT structure, and lung tissue sections were deparaffinized in CitriSolv (Thermo Fisher Scientific) for 30 min and then immersed in ethanol series from 100, 95, 85, and 75% to distilled H₂O for 5 min each for tissue hydration. For Ag retrieval, hydrated sections were steamed for 20 min in 1 mM EDTA (pH 8.0). Lung sections were blocked with Super Block Blocking buffer (Thermo Fisher Scientific) for 1 hour at room temperature. Anti-B220-efluor660 (clone: 4SM95, Invitrogen), anti-CD4-efluor570 (clone: RA3-6B2, Invitrogen), and/or anti-GL7-Alexa488 (clone: GL7, BioLegend) were stained on the lung tissue sections overnight at 4°C. After washing in 0.1% PBST [phosphate-buffered saline (PBS) with Tween 20], the slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and mounted. Tissue staining was reviewed, and representative images were acquired on a Zeiss LSM 780 confocal system (Carl Zeiss).

Parabiosis surgery

To examine tissue residency of lung T_{RH} or non-T_{RH} cells, parabiotic surgery was performed. CD45.1⁺ or CD45.1⁺/CD45.2⁺ mice were infected with influenza PR8. Three weeks later, mice were anesthetized with ketamine and xylazine. The lateral skin area was shaved and disinfected, and then a matching incision was made from the olecranon to the knee joint of each mouse. Matching area was opposed with continuous sutures. Parabionts were then allowed to rest for 14 days before analysis. Equilibration of parabionts was confirmed in the peripheral blood before tissue analysis.

Plaque assay

Monolayers of Madin-Darby canine kidney cells in a 12-well plate were washed twice with 1 $\times$ PBS. The cells were then inoculated with serial 10-fold dilutions of supernatants of the lung homogenate in serum-free medium. After incubation at 37°C for 1 hour, cells were washed twice with PBS and overlaid with minimum essential medium containing 0.5% agarose (Lonza) and TPCK-trypsin (0.5 gm/ml). After 3 days in culture, the cells were fixed with 4% of formaldehyde and stained with 0.1% crystal violet solution.

Single-cell RNA sequencing

Sorted CD45_{i.v.}⁺CD4⁺CD44^{Hi} T cells from pooled lung cells of mice (five mice) infected with influenza virus (28 d.p.i.) were loaded on the Chromium Controller (10 $\times$ Genomics). Single-cell libraries were prepared using the Chromium Single Cell 3' Reagent kit (10 $\times$ Genomics) following the manufacturer's instruction. Paired-end sequencing was performed

using an Illumina HiSeq 2500 in rapid-run mode. The CellRanger software package (10× Genomics) was used to align and quantify sequencing data from 10× Genomics. All scRNA-seq analyses were performed in R using the package Seurat (version 2.0) (63). The same number of cells from each group was used for log normalization, followed by identifying differentially expressed genes (DEGs) using variance-stabilizing transformation. DEGs in Fig. 2B were selected on the basis of the signature genes expressed by circulating memory T cells, T_H1 , T_H17 , T_{reg} , or T_{FH} cells.

Bulk RNA sequencing

Lung $CD45_{i.v.}^{-}CD4^{+}CD44^{Hi}GITR^{-}PD-1^{Hi}FR4^{Hi}$, $CD45_{i.v.}^{-}CD4^{+}CD44^{Hi}GITR^{-}PD-1^{Low}FR4^{Low}$, splenic $CD4^{+}CD44^{Hi}GITR^{-}PD-1^{Hi}CXCR5^{+}T_{FH}$, or splenic $CD4^{+}CD44^{Hi}GITR^{-}PD-1^{Low}CXCR5^{-}$ non- T_{FH} cells were sorted from a total of 15 mice that were infected with influenza virus (28 d.p.i.). RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) following the manufacture's recommendation. After quality control, high-quality [Agilent Bioanalyzer RNA Integrity Number (RIN) >7.0] total RNA was used to generate the RNA sequencing library. Complementary DNA synthesis, end repair, A-base addition, and ligation of the Illumina indexed adapters were performed according to the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). Paired-end libraries were sequenced on an Illumina HiSeq 4000 following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. Base calling was performed using Illumina's RTA software (version 2.5.2). Paired-end RNA-seq reads were aligned to the mouse reference genome (GRCm38/mm10) using RNA-seq spliced read mapper Tophat2 (v2.1.1). Pre- and postalignment quality controls, gene level raw read count, and normalized read count (i.e., fragments per kilobase million, FPKM) were performed using RSeQC package (v2.3.6) with National Center for Biotechnology Information (NCBI) mouse RefSeq gene model. DEGs were identified on the basis of the results of DESeq2 Wald tests. DEGs were presented in the form of volcano plots. For visualization, data were logarithmic transformed, and genes that exhibited $\log_2$ fold change values > 2 and $\log_{10} P > 25$ between compared groups were highlighted. For functional analysis, GSEA was used to identify enriched gene sets, from the hallmark collection of MSigDB, having up- and down-regulated genes, using a weighted enrichment statistic and a $\log_2$ ratio metric for ranking genes.

Intravascular labeling with α -CD45 and preparation of lung cell suspension

Mice were intravenously injected with 2 μ g of α -CD45 (clone: 30-F11; Tonbo Biosciences), which was diluted in 300 μ l of sterile PBS 5 min before sacrifice. To prepare single cells from lung tissue, lung was cut into small pieces, digested with type 2 collagenase (Worthington Biochemical), and dissociated in 37°C for 30 min with Gentle-MACS (Miltenyi). Cells were further ground through 70- μ m cell strainer (Falcon) and washed with plain Iscove's Modified Dulbecco's Medium (Gibco). After red blood cell lysis, cells were centrifuged and resuspended in cold fluorescence-activated cell sorting buffer (PBS, 2 mM EDTA, 2% fetal bovine serum, and 0.09% sodium azide) for flow cytometry analysis. Lung circulating immune cells are i.v. Ab^{+} , and lung tissue immune cells are defined by i.v. Ab^{-} . Lung T_{RM} cells were defined as $CD45_{i.v.}^{-}CD8^{+}$ tetramer $^{+}CD69^{+}$. Lung B_{RM} cells were defined as $CD45_{i.v.}^{-}B220^{+}GL7^{-}IgD^{-}IgM^{-}CD38^{+}$ influenza B cell Ag (HA or NP) $^{+}$.

B cell antigens

Influenza PR8-HA protein was a gift from M. C. Crank (National Institutes of Health). PR8-NP protein was purchased from Sino Biological. Purified Ags were biotinylated using an EZ-Link Sulfo-NHS-LCBiotinylation kit (Thermo Fisher Scientific) using a 1:1.3 M ratio of biotin to Ag. To make tetramers, biotinylated Ags were mixed with streptavidin–phycoerythrin (PE; PJ27S; ProZyme) at the ratio determined or at a 5:1 ratio using the biotin concentration provided by the manufacturer as described before (64). After a 30-min incubation on ice, unconjugated biotinylated Ag was often removed by several rounds of dilution and concentration using a 100-kDa Amicon Ultra (MilliporeSigma) or 300-kDa Nanosep centrifugal devices (Pall). Tetramers were stored at 1 μ M in 1 $\times$ Dulbecco's phosphate-buffered saline (DPBS) at 4°C before use. H3N2 X31-HA conjugated with allophycocyanin and H3N2 Urg (Uruguay)–HA conjugated with PE were previously reported (12).

Quantification and statistical analysis

Statistical analysis was done using GraphPad Prism 7.0 (GraphPad Software) and presented as means $\pm$ SEM. Unpaired or paired Student *t* tests (two group comparisons) and one-way or two-way analysis of variance (ANOVA) analysis (multiple group comparisons) were used in data analysis. Log-rank test was used for analysis of survival study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Lung CD4⁺ T cells deliver localized help to B and CD8⁺ T cells.

(A to D) WT mice were infected with PR8 strain of influenza virus and treated with control (Ctl) IgG or α-CD4 starting at 14 d.p.i. Mice were injected with α-CD45 intravenously 5 min before sacrifice at 42 d.p.i. (A) Experimental scheme. (B) Representative confocal images of iBALT in the lung. Lung sections were stained with α-CD4 (red), α-B220 (green), and DAPI (blue). (C) Frequencies and cell number of influenza HA-B cells in the lung tissue or blood vessels. (D) Lung tissue CD8⁺, CD8⁺CD69⁺, or CD8⁺CD69⁺CD103⁺ NP₃₆₆₋₃₇₄ or PA₂₂₄₋₂₃₃ T_{RM} cells were enumerated. (E to I) WT mice were infected with

PR8 and treated with Ctl IgG or α -CD4 (starting at 14 d.p.i.) in the presence of daily injection of FTY720 (starting at 13 d.p.i.). (E) Schematic of experimental design. (F) B_{GC}, (G) total HA-B (CD45_{i.v.}⁻HA-B), or (H) HA-B_{RM} cells were enumerated by flow cytometry. (I) Lung tissue CD8⁺, CD8⁺CD69⁺, or CD8⁺CD69⁺CD103⁺ NP₃₆₆₋₃₇₄ T_{RM} cells were enumerated. (J to L) WT mice were infected with PR8 and received Ctl IgG and high or low dose of α -CD4. (J) Experimental scheme. (K) Cell number of B_{GC}, HA-B_{RM}, or NP-B_{RM} cells in the lung tissue. (L) Lung parenchymal CD8⁺, CD8⁺CD69⁺, or CD8⁺CD69⁺CD103⁺ NP₃₆₆₋₃₇₄ T_{RM} cells were enumerated. In (B) to (D) were the representative data from at least two independent experiments (four to five mice per group). In (F), (G), and (I) to (L), data were pooled from two (F to G and I) or three (K and L) independent experiments (two to five mice per group). *P* values were calculated by unpaired two-tailed Student's *t* test in (C), (D), and (F) to (I). *P* values in (J) and (K) were analyzed by one-way ANOVA. Data are means $\pm$ SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. n.s., not significant.

Fig. 2. Identification of a population of T_{FH}-like cells in the lung tissue.

WT (A to E) or IL-21-VFP reporter (F) mice were infected with PR8. (A) t-Distributed stochastic neighbor embedding (tSNE) plot of scRNA-seq analysis of sorted lung CD45^{int}-CD4⁺CD44^{Hi} cells (pooled from five mice) at 28 d.p.i. (B) Heat map of indicated genes in each cluster from scRNA-seq data. (C) Kinetics of the percentages of PD-1^{Hi}FR4^{Hi} population in lung tissue, total CD4⁺, or influenza NP-specific (NP₃₁₁₋₃₂₅) CD4⁺ T cells. (D) Expression of T_{FH} cell-associated markers in lung total or influenza-specific PD-1^{Hi}FR4^{Hi}, PD-1^{Low}FR4^{Low}, or splenic T_{FH} (CD4⁺CD44^{Hi}PD-1⁺CXCR5⁺) cells at 28 d.p.i. (E) Frequency positive for IFN-γ, IL-17, or IL-4 by lung PD-1^{Hi}FR4^{Hi}, PD-1^{Low}FR4^{Low}, or splenic T_{FH} cells were identified by intracellular staining at 28 d.p.i. (F) IL-21-VFP expression in lung CD4⁺ PD-1^{Hi}FR4^{Hi}, PD-1^{Low}FR4^{Low}, or splenic T_{FH} at 28

d.p.i. In (C) to (F), representative plots, histograms, and graphs were collected from at least two independent experiments (two to four mice per group). *P* values in (E) and (F) were analyzed by one-way ANOVA. Data are means $\pm$ SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig. 3. Transcriptional profiling reveals PD-1^{Hi}FR4^{Hi} cells exhibit both T_{FH} and T_{RM} gene signatures.

(A to F) WT mice were infected with PR8. Lung PD-1^{Hi}FR4^{Hi} or PD-1^{Low}FR4^{Low} CD4⁺ T cells and splenic T_{FH} or non-T_{FH} cells were sorted after exclusion of GTR^{Hi} T_{reg} cells, and RNA-seq analysis was performed at 28 d.p.i. (A) Heatmap of DEGs among lung PD-1^{Hi}FR4^{Hi}, PD-1^{Low}FR4^{Low} CD4⁺ T cells, and splenic T_{FH} or non-T_{FH} cells. (B) Principal component analysis (PCA) of RNA-seq data of lung PD-1^{Hi}FR4^{Hi}, PD-1^{Low}FR4^{Low} CD4⁺ T cells, and splenic T_{FH} or non-T_{FH} cells. (C) Volcano plot of RNA-seq analysis of lung PD-1^{Hi}FR4^{Hi} or PD-1^{Low}FR4^{Low} CD4⁺ T cells. (D) GSEA of the core

T_{FH} signature genes in lung CD4⁺ PD-1^{Hi}FR4^{Hi} and CD4⁺ PD-1^{Low}FR4^{Low} cells. (E) Volcano plot of RNA-seq analysis on lung PD-1^{Hi}FR4^{Hi} CD4⁺ T and splenic T_{FH} cells. (F) GSEA of the core tissue residency signature genes of T_{RM} cells in lung PD-1^{Hi}FR4^{Hi} and splenic T_{FH} cells. (G and H) WT mice were infected with PR8. Lung PD-1^{Hi}FR4^{Hi} or PD-1^{Low}FR4^{Low} CD4⁺ T cells and splenic T_{FH} cells were sorted at 28 d.p.i. Nanostring analysis on 560 immune-associated genes was performed. The expression of T_{FH}-associated genes (G) or tissue residency-associated genes (H) in the three cell populations was depicted. For RNA-seq, data were from duplicates of pooled samples ($n = 15$). For nanostring analysis, data were from pooled samples ($n = 10$). ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate.

Fig. 4. Lung PD1^{Hi}FR4^{Hi} CD4⁺ T cells are tissue resident.

(A) WT mice were infected with PR8. The expression of CD69, CXCR6, and *Bhlhe40* in lung CD4⁺ PD-1^{Hi}FR4^{Hi}, CD4⁺ PD-1^{Low}FR4^{Low} NP₃₁₁₋₃₂₅ T cells, or splenic T_{FH} cells at 28 d.p.i. (B to E) CD45.1⁺ (host) or CD45.1⁺ CD45.2⁺ (partner) WT mice were infected with PR8. Parabiosis surgery was performed at 21 d.p.i. Mice were euthanized 14 days later for analysis. (B) Schematic of parabiosis experiments. (C) Composition of Host-derived or Partner-derived CD4⁺ T cells in the spleen of the parabionts. (D) Frequencies of Host-derived or Partner-derived cells in the lung PD-1^{Hi}FR4^{Hi} or PD-1^{Low}FR4^{Low} total CD4⁺ T cell compartment. (E) Frequencies of Host-derived or Partner-derived cells in influenza-specific lung CD4⁺ PD-1^{Hi}FR4^{Hi} or CD4⁺ PD-1^{Low}FR4^{Low} NP₃₁₁₋₃₂₅ T cell compartment. In (A), the representative histograms were from at least two independent experiments (three to four mice per group). In (C) to (E), parabiosis data were pooled from two different experiments. *P* values were calculated by one-way ANOVA in (D) and (E). Data are means ± SEM. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig. 5. Both BCL6 and Bhlhe40 are required for optimal lung T_{RH} cell responses.

(A to D) *Bcl6*^{fl/fl} or *Bcl6*^{ΔT} mice were infected with PR8. (A) Representative dot plot, (B) frequencies, and (C) cell numbers of T_{RH} or non-T_{RH} in lung CD45^{LV}- total CD4⁺ or CD45^{LV}- influenza-specific CD4⁺ NP₃₁₁₋₃₂₅ T cells at 28 d.p.i. (D) Frequencies (top) or cell numbers (bottom) of splenic total T_{FH} or NP₃₁₁₋₃₂₅ T_{FH} at 28 d.p.i. (E) GSEA of the *Bhlhe40*-associated genes in lung T_{RH} (PD-1^{Hi} FR4^{Hi}) and spleen T_{FH} cells. (F to H) *Bhlhe40*^{fl/fl} or *Bhlhe40*^{ΔT} mice were infected with PR8. (F) Representative dot plot, (G) frequencies (top), or cell numbers (bottom) of lung NP₃₁₁₋₃₂₅ T_{RH} or non-T_{RH} cells at 28 d.p.i. (H) Active caspase 3/7-fluorochrome-labeled inhibitors of caspases (FLICA)⁺ cells in lung NP₃₁₁₋₃₂₅ T_{RH} or non-T_{RH} cells at 28 d.p.i. In (A) to (D) and (F) to (G), data were pooled from two independent experiments (three to four mice per group). In (H), representative data were from at least two independent experiments (four mice per group). *P* values were calculated by unpaired two-tailed Student's *t* test. Data are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig. 6. TRH cells are required for the development of lung protective CD8⁺ T_{RM} and B cell immunity.

(A to J) *Bcl6^{fl/fl}* or *Bcl6^{ΔCD4ERT2}* mice were infected with PR8. (A to E) Tamoxifen was administered daily (12 to 16 d.p.i.) in the presence of daily FTY720 administration (11 to 34 d.p.i.). (A) Schematic of experimental design. Cell numbers of (B) *B_{GC}*, (C) *HA-B_{RM}*, (D) *NP-B_{RM}*, (E) *CD8⁺CD69⁺ NP_{366–374} T_{RM}*, or *CD8⁺CD69⁺ PA_{224–233} T_{RM}* at 35 d.p.i. (F to J) Tamoxifen was administered daily from 21 to 25 d.p.i. in the presence of daily FTY720 administration (20 to 41 d.p.i.). (F) Schematic of experimental design. Cell

numbers of (G) B_{GC}, (H) HA-B_{RM}, (I) NP-B_{RM}, (J) CD8⁺CD69⁺ NP_{366–374} T_{RM}, or CD8⁺CD69⁺ PA_{224–233} T_{RM} at 42 d.p.i. (K) *Bcl6*^{f1/f1} or *Bcl6*^{CD4ERT2} mice were infected with X31 strain (H3N2) of influenza. Tamoxifen was administrated daily (12 to 16 d.p.i.) in the presence of daily FTY720 administration (11 to 34 d.p.i.). Representative dot plot (left) and cell numbers (right) of X31 strain-specific B_{RM} or cross-reactive HA-B_{RM} (to H3N2 A/Uruguay/716/07 strain) at 35 d.p.i. (L and M) *Bcl6*^{f1/f1} (*n* = 11) or *Bcl6*^{CD4ERT2} (*n* = 15) mice were infected with X31 and administered tamoxifen from 12 to 16 d.p.i. Mice were rechallenged with PR8 at 42 d.p.i. in the presence of FTY720 (starting from 41 days). (L) Schematic of experimental design. (M) Host mortality following PR8 challenge was monitored. (N and O) *Bcl6*^{f1/f1} or *Bcl6*^{CD4ERT2} mice were infected with X31 and administered tamoxifen. Mice were challenged with PR8 at 40 d.p.i. in the presence of FTY720. Lung (N) infectious virus titers and (O) viral gene expression were measured at day 2 post rechallenge. In (A) to (H) and (J) to (K), all data were pooled from two (C, D, G, H, J, and K) or three (B and E) independent experiments (two to five mice per group). In (A) to (O), *P* values were calculated by unpaired two-tailed Student's *t* test. *P* value of survival study in (M) was calculated by log-rank test. Data are means ± SEM. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001.

Fig. 7. T_{RM} help to CD8⁺ T cells is IL-21 dependent.

(A) Representative dot plot of IL-21^{Hi} or IL-21^{Low} CD4⁺ T cells from the IL-21-VFP reporter mice infected with PR8 (28 d.p.i.). (B to E) WT mice were infected PR8 with or without IL-21R blockade through intraperitoneal (i.p.) route starting at 14 d.p.i. in the presence of FTY720 administration (13 to 34 d.p.i.). (B) Experimental scheme. Cell numbers of lung parenchymal (C) B_{GC}, (D) HA-B_{RM}, and (E) CD8⁺CD69⁺ NP₃₆₆₋₃₇₄, or CD8⁺CD69⁺ PA₂₂₄₋₂₃₃ T_{RM} cells. (F to J) WT mice were infected with PR8 with or without IL-21R blockade through intranasal (i.n.) route at 14 d.p.i. (F) Experimental scheme. (G) Frequencies or (H) cell numbers of lung tissue CD8⁺CD69⁺ NP₃₆₆₋₃₇₄ or

CD8⁺CD69⁺ PA_{224–233} T_{RM}. (I) Percentage of apoptotic cells were identified by active caspase 3/7-FLICA staining within lung CD8⁺ NP_{366–374} T_{RM} or splenic CD8⁺ NP_{366–374} T_{MEM} at 28 d.p.i. (J) Representative histogram of BATF expression in lung CD8⁺ NP_{366–374} or CD8⁺ PA_{224–233} T_{RM} of mice received with Ctl IgG or α-IL21R at 35 d.p.i. FMO, fluorescence minus one control. (K to M) WT mice were infected with PR8 with or without IL-21R blockade in the presence of FTY720. Mice were challenged with X31 at 40 d.p.i. (K) Experimental scheme. (L) Lung infectious virus titer or (M) viral gene expression were measured at day 2 after rechallenge. In (A) and (I) to (M), representative data were from at least two independent experiments (four to five mice per group). In (C), (E), and (G) to (H), data were pooled from two independent experiments (three to five mice per group). *P* values of all experiments were calculated by unpaired two-tailed Student's *t* test. Data are means ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.