IL-15 supports the generation of protective lung-resident memory CD4 T cells

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Tissue-resident memory T cells (T_{RM}) provide optimal defense at the sites of infection, but signals regulating their development are unclear, especially for CD4 T cells. Here we identify two distinct pathways that lead to the generation of CD4 T_{RM} in the lungs following influenza infection. The T_{RM} are transcriptionally distinct from conventional memory CD4 T cells and share a gene signature with CD8 T_{RM}. The CD4 T_{RM} are superior cytokine producers compared with conventional memory cells, can protect otherwise naive mice against a lethal influenza challenge, and display functional specialization by inducing enhanced inflammatory responses from dendritic cells compared with conventional memory cells. Finally, we demonstrate than an interleukin (IL)-2-dependent and a novel IL-2-independent but IL-15-dependent pathway support the generation of cohorts of lung T_{RM}.

INTRODUCTION

Memory CD4 T cells provide strong protection against viruses through multiple pathways. They are especially important for clearing pathogens such as influenza A virus (IAV) against which neutralizing antibody (Ab) alone cannot confer long-term immunity.[1] Several CD4 memory subsets have been described based on phenotypic, functional, and migratory properties,[2] and optimal protection often involves multiple specialized populations acting in concert.[3] A challenge for developing T-cell-based vaccines is thus identifying the kinds of memory cells needed for optimal clearance of individual pathogens and elucidating the signals required for the generation of each particular subset.

Tissue-resident memory T cells (T_{RM}) cells provide a first line of defense due to their location at the sites of infection and display distinct functional attributes vs. circulating memory subsets.[2,4–6] CD8 T_{RM} formation is complex and appears to differ between tissue sites and the pathogen or priming model employed. The generation of CD8 T_{RM} primed by IAV, and in many other models, requires transforming growth factor (TGF)-β-mediated upregulation of CD103 (a subunit of $\alpha_E\beta_7$ integrin).[7–13] In contrast to CD8 T_{RM}, elevated CD103 expression is not usually observed on CD4 T_{RM} in either animal models or in humans.[14,15] Thus it is likely that distinct cytokine cues and perhaps other signals differentially regulate CD8 and CD4 T_{RM} generation and maintenance.

We recently found that interleukin (IL)-2 signals to effector CD4 T cells between 5 and 7 days postinfection (dpi) with IAV are crucial to direct memory development. IL-2 signals enhance IL-7 receptor expression on CD4 T cells, thereby improving their access to IL-7, which is essential for the transition of effector cells into memory and for their survival thereafter. IL-2 also acts during this brief window of the CD4 T-cell response to downregulate pro-apoptotic molecules, especially Bim, to promote short-term survival of effectors and to thus enable a greater cohort of cells for memory development.[16,17] Almost all memory CD4 T cells primed by IAV in the spleen and draining lymph node (dLN) are dependent upon the receipt of IL-2 signals, as is a subset in the lung.[16] However, our studies also clearly indicate that a subset of memory cells in the lungs of IAV-primed mice, the primary site of infection, is IL-2 independent.[16]

Here we investigate the distinct subsets of IAV-primed memory CD4 T cells found in the lung and the role of IL-2 and other cytokine signals in their generation. We find that T_{RM} comprise the majority of these memory cells and, surprisingly, that they include both an IL-2-dependent and an IL-2-independent subset. The IL-2-dependent and -independent CD4 T_{RM} share a surface phenotype that is distinct from

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conventional memory CD4 T cells present in the lung and spleen and express a distinct transcriptional profile including preferential expression of many of the genes found to differentiate CD8 T_{RM} from circulating memory CD8 T cells. The CD4 T_{RM} are characterized by enhanced cytokine production and an increased capacity to induce rapid inflammatory responses from dendritic cells (DCs) as compared with conventional memory cells of the same specificity. Most importantly, CD4 T_{RM} are potent mediators of protection against IAV upon transfer to unprimed mice. Finally, we demonstrate that direct IL-15 signals to CD4 T-cell effectors are required to generate the IL-2-independent CD4 T_{RM} subset but that continued IL-15 is not required for its long-term maintenance. Our studies thus indicate that viral infection generates functionally specialized CD4 T_{RM} through distinct pathways, contributing to the heterogeneity of protective lung CD4 T-cell memory.

RESULTS

IAV primes IL-2-dependent and -independent lung-restricted CD4 T_{RM}

To investigate the role of IL-2 signaling in the generation of CD4 T_{RM}, we first transferred OT-II.Thy1.1 TcR Tg CD4 T cells to unprimed B6 mice and challenged with a low dose of A/PR8-OVA_{II}, recognized by the OT-II TcR.[18] We transferred 1×10^6 cells, a minimal number required to reliably track donor responses into the memory phase in this model of IAV infection. We used donor cells that had been primed in the presence of IL-2 *in vitro* prior to transfer in order to provide the requisite early-acting IL-2 signal needed to generate optimal CD4 T-cell effector responses against IAV.[16] Groups of mice were treated with an isotype control Ab or with IL-2-neutralizing Abs from 1 to 7 dpi to block conventional memory generation.[16] This IL-2-blocking regime faithfully replicates key aspects of the response of *Il2*$^{-/-}$ CD4 T cells against IAV in the lung and secondary lymphoid organs.[16] In agreement with our previous findings in a similar adoptive transfer model in BALB/c hosts,[16] peak effector expansion was equivalent in mice treated with IL-2-neutralizing or isotype Ab (not shown). However, IL-2 neutralization prevented virtually all donor cell recovery in the spleen and dLN by 28 dpi but left a population of readily detectable IL-2-independent memory cells in the lungs (**Figure 1a**).

To determine whether the IL-2-dependent and -independent memory cells detected in the lungs are T_{RM} or a subset of circulating memory cells, we administered fluorescent anti-CD4 Ab intravenously (i.v.) to B6 hosts at 28 dpi and analyzed labeling of donor cells in the lung after 3–5 min. This technique can readily discriminate blood-borne cells present in the circulation, which become labeled with the i.v. administered Ab, vs. those cells that are tissue-localized and thus protected from Ab labeling.[19] Roughly 80–90% of donor cells were not labeled (i.v.^{shielded}) in mice treated with isotype control Ab (**Figure 1b**), in agreement with previous studies demonstrating that the majority of lung memory CD4 T cells primed by IAV are not accessible to the vasculature.[20] Strikingly, all donor cells in mice treated with IL-2-neutralizing Ab are i.v.^{shielded} (**Figure 1b**). These i.v.^{shielded} donor cells in the lung fit criteria used to identify T_{RM}.[19] To determine whether the i.v.^{shielded} cells reside primarily in lung airways or the parenchyma, we separately analyzed donor cells recovered from the bronchial alveolar lavage or the lung proper. Few i.v.^{shielded} donor cells were recovered by bronchiolar lavage (**Figure 1c**), indicating preferential parenchymal vs. airway location of the i.v.^{shielded} memory cells in mice treated with either IL-2-neutralizing or control Ab.

Lung-resident memory cells express a T_{RM}-associated phenotype

To define the relationship between the IL-2-dependent and -independent i.v.^{shielded} memory cells primed by IAV and T_{RM} described in other models, we analyzed key surface markers CD103, CD69, and CD127. We also compared the phenotypes of i.v.^{sheilded} memory cells in the lungs with those of i.v.^{labeled} donor cells isolated from the lung. Although IAV-primed CD8 T_{RM} express high levels of CD103, which is known to be TGF-β dependent,[13, 21] all CD4 memory cells in the lung, whether i.v.^{labeled} or i.v.^{shielded}, express low CD103 (**Figure 1d,e**). Furthermore, in separate experiments, we observed no change in donor cell recovery from the lungs at 28 dpi in mice treated with either TGF-β-neutralizing Ab alone or with TGF-β-neutralizing Ab in conjunction with IL-2-neutralizing Ab from 1 to 7 dpi (data not shown). These findings suggest that CD4 T_{RM} develop through a pathway distinct from that supporting IAV-specific CD8$^+$ CD103$^+$ TGF-β-dependent T_{RM}.

Most T_{RM} express high surface CD69, which promotes tissue retention through repression of the sphingosine 1 phosphate receptor.[22–24] The i.v.^{shielded} donor cells in mice treated with IL-2-neutralizing or control Ab were uniformly CD69^{high} compared with i.v.^{labeled} donors (**Figure 1d,e**). To determine whether increased CD69 expression by the i.v.^{shielded} memory cells is driven by recent TcR stimulation, we transferred OT-II cells from donor mice with green fluorescent protein (GFP) driven by the Nur77 protein locus (*Nr4a1*^{eGFP}) that express GFP transiently after antigen stimulation.[17,25] We compared GFP signal from i.v.^{labeled} and i.v.^{shielded} donor cells at 28 dpi to that from donor cells in unprimed hosts. All donor cells expressed low, uniform levels of GFP (**Figure 1f**), indicating that continuing cognate TcR stimulation is not responsible for maintaining the CD69 expression on either the IL-2-dependent or -dependent i.v.^{shielded} cells, and arguing against a requirement for residual IAV antigen depots[26] in sustaining the CD69$^+$ memory cells.

We also analyzed IL-7 receptor expression (CD127), which is upregulated on CD4 T cells when they produce and respond to the autocrine IL-2 that facilitates effectors to transition to memory.[16] Intriguingly, the i.v.^{shielded} memory cells in mice treated with either control or IL-2-neutralizing Abs expressed lower CD127 than did i.v.^{labeled} memory cells (**Figure 1d,e**). This implies that the i.v.^{shielded} subset may be less dependent on

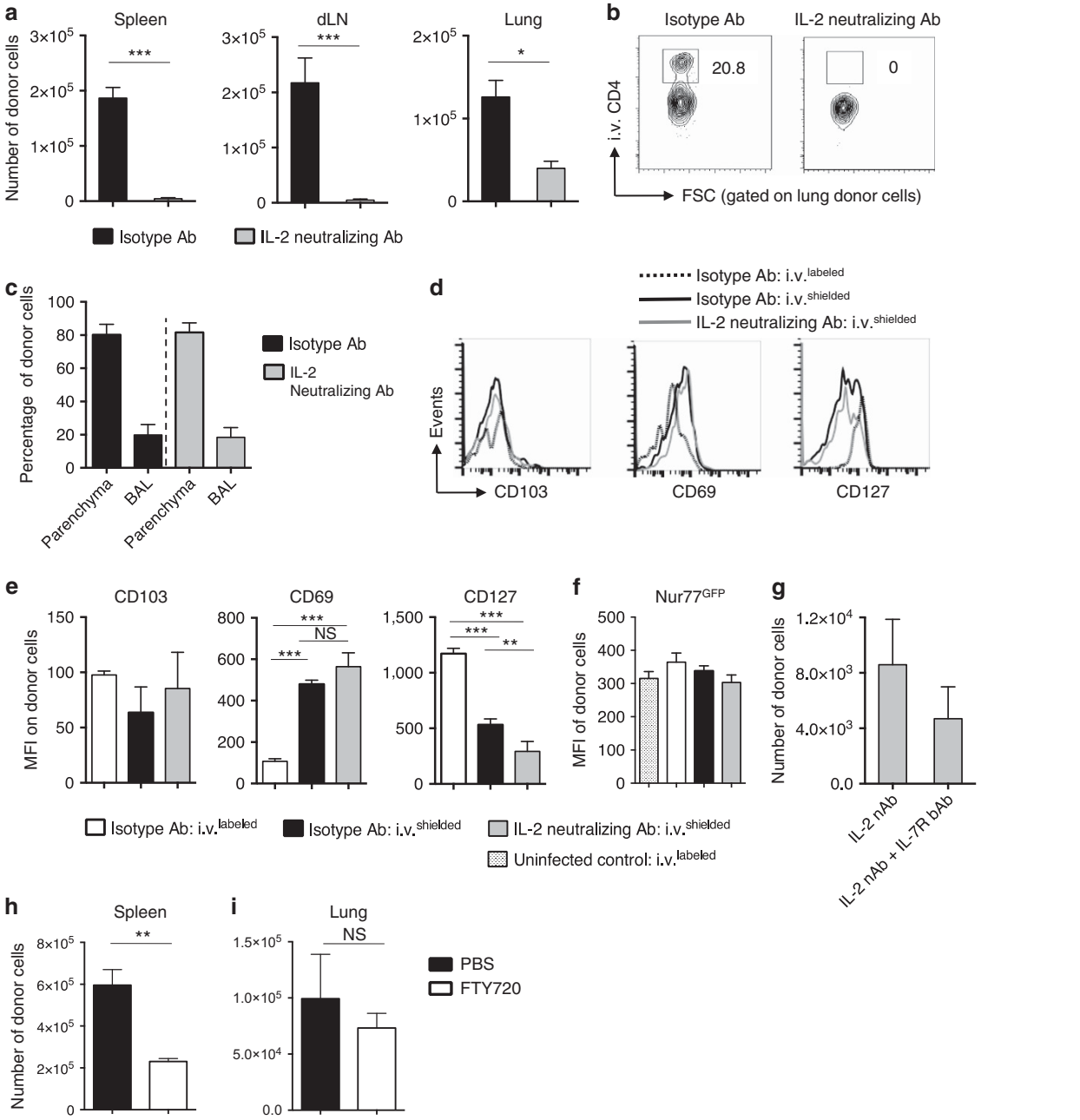

Figure 1 A tissue-resident memory T cell–associated phenotype is expressed by i.v.^{shielded} lung memory cells. Unprimed B6 mice received 1×10^6 congenic donor cells followed by priming with influenza A virus (IAV) and treatment from 1 to 7 dpi with interleukin (IL)-2-neutralizing antibodies (Abs) or isotype control Ab. (**a**) Donor cells were enumerated at 28 days postinfection (dpi) in the stated organs (four mice per group; one of the three similar experiments). (**b**) At 28 dpi, recipient mice were injected intravenously (i.v.) with fluorescent Ab specific for CD4 and the frequency of donor cells stained (i.v.^{labeled}) or not (i.v.^{shielded}) was determined (representative staining). (**c**) The percentage of donor cells at 28 dpi recovered either in the bronchial alveolar lavage (BAL) or from the lung parenchyma (three mice per group; one of the two experiments). (**d**) Representative staining and (**e**) mean fluorescence intensity (MFI) analysis for donor cell for CD103, CD69, and CD127. (**f**) Nur77^{GFP} OT-II donors were analyzed for green fluorescent protein expression at 28 dpi discriminated based on their ability to be labeled by i.v. administered CD4 Ab (three mice per group; one of the two experiments). (**g**) Mice receiving donor cells were treated with IL-2-neutralizing Ab (IL-2n Ab) from 1to 7 dpi, followed by treatment with phosphate-buffered saline (PBS) or with IL-7 receptor blocking Ab every other day from 10 to 26 dpi. The number of donor cells recovered from the lungs at 28 dpi is shown (four mice per group; one of the two experiments). Mice receiving donor cells and IAV priming were treated or not with FTY720 for 5 consecutive days beginning on 23 dpi. On 28 dpi, (**h**) spleens and (**i**) lungs were analyzed for total donor cells (three mice per group; one of the two experiments). dLN, draining lymph node; FSC, forward scatter; NS, not significant.

IL-7 survival signals compared with conventional memory CD4 T cells.[27] To test this, mice receiving donor cells and IL-2-neutralizing Ab were also treated with an IL-7 receptor blocking Ab starting at 14 dpi (when the virus is cleared) using a regime that in our previous studies reduced recovery of conventional CD4 memory cells primed by IAV by at least one log in the spleen and dLN and to a lesser but significant degree in the lung.[16] IL-7 receptor blockade did not significantly reduce the

recovery of the IL-2-independent lung memory cells (**Figure 1g**), which suggests that alternative survival factors regulate the maintenance of the i.v.^{shielded} memory cells in the lung.

Finally, we sought to confirm that the i.v.^{shielded} donor cells reside in the lung long term rather than representing a transient subpopulation of circulating cells. We thus treated IAV-primed mice that had received donor cells with FTY720, which blocks egress from the lymph nodes and results in a dramatic loss of circulating T cells after short-term treatment.[28] Treatment for 5 days beginning at 23 dpi reduced the number of donor cells detected in the spleen by about threefold (**Figure 1h**) but had no impact on the number of donor cells detected in the lungs (**Figure 1i**), indicating that the i.v.^{shielded} memory population in the lung is distinct from the circulating lymphocyte pool.

Together, these results support the hypothesis that, after their initial priming, two distinct pathways, one IL-2 dependent and another that is IL-2 independent, support the generation of CD4 T_{RM} in the lung. The fact that T_{RM} generated in the presence and absence of IL-2 signaling share a common location and CD103^{low} CD69^{high} phenotype supports the hypothesis that alternate pathways exist to produce closely related subsets of T_{RM} that are physiologically relevant.

A conserved T_{RM} molecular signature

CD8 T_{RM} are distinguished from circulating CD8 memory cells in mice and humans by a unique gene expression signature.[7,29,30] To determine whether differences in transcriptional regulation similarly distinguish CD4 T_{RM} and conventional memory cells primed by IAV, we performed whole-genome microarray analysis comparing sort-purified donor T_{RM} (i.v.^{shielded} CD69^{high}) from the lung mice against i.v.^{labeled} CD69^{low} donors obtained from the lung and spleen. At a threshold of >1.5 and *P*-value of 0.05, 79 transcripts representing 69 known genes were identified as being differentially expressed between the T_{RM} and conventional memory cells (**Figure 2a** and **Supplementary Table SI** online). Differential surface expression of four of these genes, Slamf6, Tnfs11 (RankL), Klrg1, and Cdh1(CD324), was validated by flow cytometry (**Figure 2b,c**). The 69 genes clustered into 6 significant DAVID functional annotation clusters with the most enriched being "cell adhesion" (**Figure 2d** and **Supplementary Table SI**).

We next compared the 69 genes distinguishing lung CD4 T_{RM} with the transcriptional signatures of CD103$^+$ CD8 T_{RM} isolated from various mouse tissues in other studies, including lung CD103$^+$ T_{RM} primed by IAV infection.[7,29] Interestingly, nearly one-quarter (23%; 16 of the 69 genes) of the genes identified as uniquely regulated in CD4 T_{RM} overlap with the gene signature found to distinguish CD8 T_{RM} from conventional memory CD8 T cells (**Table 1**). Functional annotation clustering analysis of these 16 shared genes revealed a 12-fold enrichment in the "GTPase regulator and activator" pathway (*P*-value 0.003) while those genes unique to CD4$^+$ T_{RM} clustered in the "cell adhesion" pathway (*P*-value 0.009). Remarkably, the CD4 T_{RM} isolated from mice treated with IL-2-neutralizing Ab shared preferential expression of 15 of these 16 genes (see **Supplementary Table SII**). This analysis suggests that a key set of signature T_{RM} genes, perhaps involved in maintenance or function, are shared between CD4 and CD8 T_{RM} subsets and between CD4 T_{RM} generated by IL-2-dependent and -independent pathways but that distinct cellular interactions in the lung may support the retention of CD4$^+$ vs. CD8$^+$ T_{RM}.

CD4 T_{RM} are functionally specialized and protective

The unique gene expression by CD4 T_{RM} compared with conventional memory CD4 T cells suggests that the T_{RM} may possess distinct functional qualities. To evaluate their respective functions, we first compared the cytokine production potential of the IL-2-dependent and -independent T_{RM} with that of conventional i.v.^{labeled} memory cells. The T_{RM} generated in the presence or absence of IL-2 contained similar frequencies of interferon (IFN)-γ^+ and dual IFN-γ^+/IL-2$^+$ cells that were significantly greater than that of the conventional memory cells (**Figure 3a–c**). This enhanced potential to produce IFN-γ suggests that the T_{RM} may be better able to mediate protective responses against IAV, some of which depend on IFN-γ.[31]

We have previously shown that memory CD4 T cells isolated from the spleen and dLN of IAV-primed mice can protect otherwise naive mice against a lethal dose of IAV upon adoptive transfer.[31] To test whether the CD4 T_{RM} are also protective, we isolated donor cells from lungs of IAV-primed mice that were treated with IL-2-neutralizing Abs (in which all of the donor cells fit criteria of T_{RM}) and transferred them to new hosts. The "take" of T_{RM} was poor when the cells were transferred i.v. as compared with the recovery of an equal number of i.v.^{labeled} memory cells (**Figure 3d**), a result similar to that seen in studies of adoptive transfer of CD8 T_{RM} cells that reported limited survival of T_{RM} when introduced into the circulation.[32] Furthermore, those T_{RM} that could be re-isolated were largely confined to the lung vs. the i.v.^{labeled} donors that were found predominantly in the spleen (**Figure 3e**). We thus transferred the T_{RM} using the intranasal (i.n.) route. The "take" of T_{RM} in the lung 1 day following i.n. transfer was about 3%, resulting in about 7.1×10^4 ($\pm 1.07 \times 10^4$) cells detected in the lungs after the transfer of 2.5×10^6 donor T_{RM}. No donor cells were detected in the spleen or dLN. This number of lung donor cells is in line with the physiological number of donor T_{RM} detected in IAV-primed mice at 28 dpi (see **Figure 1**). When the mice were challenged with a lethal dose of A/PR8-OVA$_{II}$, recipients of 4×10^6 or 2.5×10^6 T_{RM} were protected while recipients of 1×10^6 cells did not survive (**Figure 3f**). Protection mediated by the T_{RM} was associated with dramatically reduced viral titers vs. those detected in control animals, which all succumbed to infection (**Figure 3g**, left). Protection mediated by T_{RM} transfer was also antigen dependent as the OVA-specific OT-II T_{RM} do not reduce viral titers in mice challenged with PR8 (lacking OVA$_{II}$ peptide expression; **Figure 3g**, right).

The earliest and perhaps most crucial function of CD4 T_{RM} in orchestrating protective responses is likely their ability to rapidly induce the production of inflammatory cytokines and

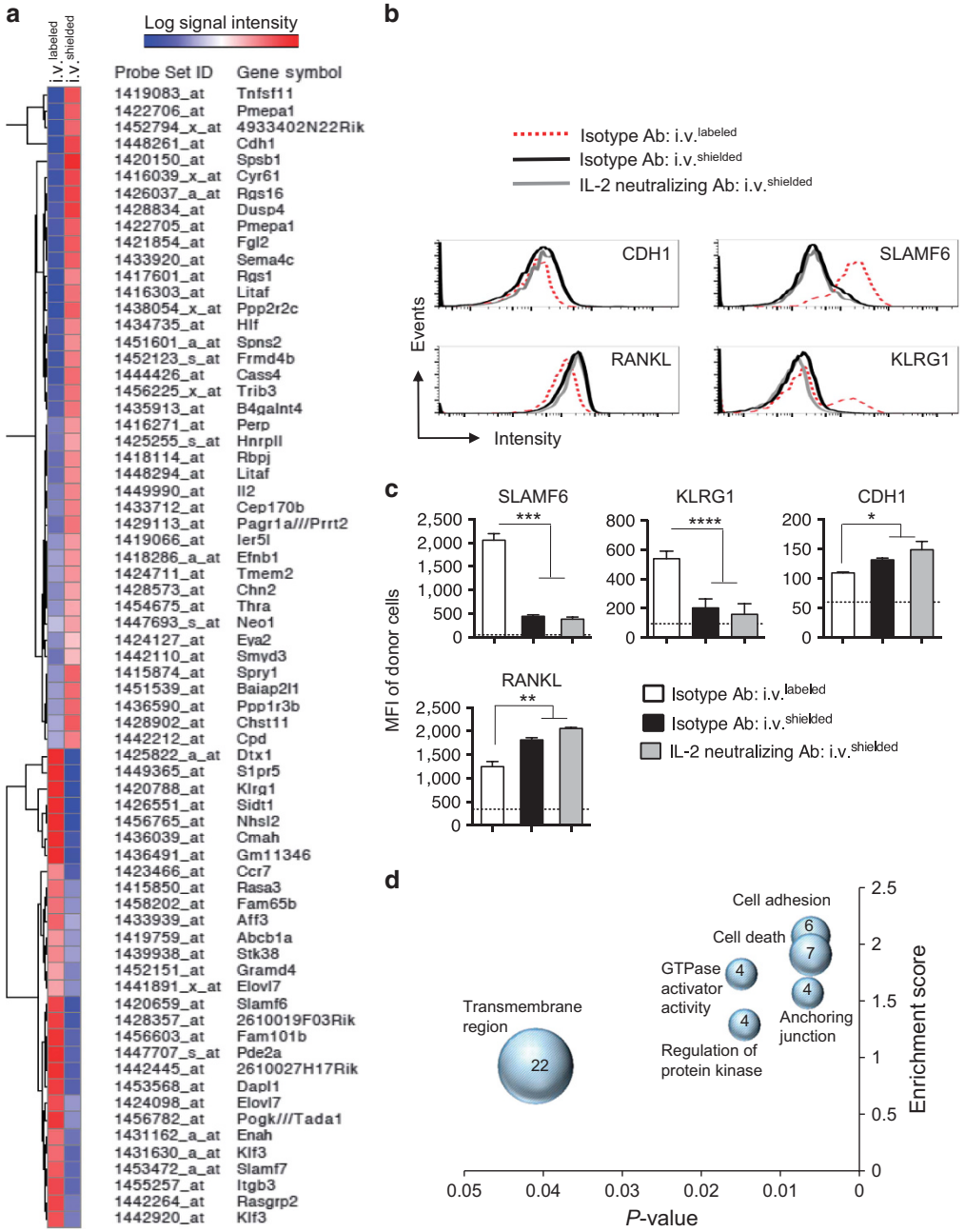

Figure 2 CD4 tissue-resident memory T cells express a unique transcriptional signature. OT-II cells were sort-purified from influenza A virus–primed hosts at 28 days postinfection. Whole-genome microarray was performed comparing i.v.^{shielded} lung donor cells to i.v.^{labeled} donor cells obtained from the lung and spleen. (**a**) Summary of genes differentially expressed by i.v.^{shielded} and i.v.^{labeled} donor cells at a threshold of >1.5-fold and *P*-value of 0.05. (**b**) Representative staining of validation of differential surface marker expression from stated subsets and (**c**) mean fluorescence intensity (MFI) analysis from three mice per group (dotted line in bar graphs represent FMO control staining for each marker; one of the three independent experiments). (**d**) DAVID functional annotation analysis was performed on the genes summarized in **Supplementary Table SI online** that were differentially expressed by i.v.^{shielded} and i.v.^{labeled} donor cells. The most significantly enriched pathways (*P*-value of 0.05) as well as the number of genes in each pathway are shown. Ab, antibody.

chemokines in the tissue site. Indeed, very low numbers of memory CD4 T cells in the lung mediate control of IAV titers prior to the influx of other adaptive immune cells by jump-starting inflammatory responses from DCs.[33,34] To evaluate the role of T_{RM} in this function, we first compared the ability of sort-purified OT-II T_{RM} cells to activate bone marrow–derived DCs pulsed with OVA_{II} peptide *in vitro* vs. an equal number of i.v.^{labeled} OT-II memory cells isolated from the spleen. Both T_{RM} and splenic memory cells similarly activated DCs to upregulate expression of CD40, CD86, and major histocompatibility complex-II by 40 h of culture (**Figure 4a**). However, significantly higher levels of several cytokines and

Table 1 Genes differentially expressed by lung CD4$^+$ T_{RM} generated by IAV challenge and published CD103$^+$ CD8$^+$ T_{RM} from various models and tissues

Gene	Product	↑/↓ T_{RM}	Reference
Cdh1	Cadherin 1	↑ in T_{RM}	7,29
Chn2	Chimerin 2	↑ in T_{RM}	7
Litaf	Lipopolysaccharide-induced tumor-necrosis factor	↑ in T_{RM}	7,29
Rgs1	Regulator of G-protein signaling 1	↑ in T_{RM}	7
Rgs16	Regulator of G-protein signaling 16	↑in T_{RM}	29
Cmah	Cytidine monophospho-*N*-acetylneuraminic acide hydroxylase	↓in T_{RM}	7
Elovl7	ELOVL family member 7, elongation of long chain fatty acids	↓in T_{RM}	7
Fam65b	Family with sequence similarity 65, member B	↓in T_{RM}	7
Rasgrp2	RAS, guanyl releasing protein 2	↓in T_{RM}	7
S1pr5	Shingosine 1-phosphate receptor 5	↓in T_{RM}	7
Sidt1	SID1 transmembrane family, member 1	↓in T_{RM}	7,29
Slamf6	SLAM family member 6	↓in T_{RM}	7,29
Klf3	Kruppel-like factor 3	↓in T_{RM}	29
Aff3	AF4/FMR2 family member 3	↓in T_{RM}	29
Ccr7	C-C chemokine receptor type 7	↓in T_{RM}	29
Dtx1	Protein deltex-1	↓in T_{RM}	29

IAV, influenza A virus; T_{RM}, tissue-resident memory T cells.

chemokines that we have previously correlated with protective inflammatory responses against IAV[33] were detected in co-cultures containing T_{RM} (**Figure 4b**).

We next asked whether enhanced inflammatory responses from DCs could be detected at early time points post-IAV challenge in the lungs of recipients of polyclonal CD4 T_{RM}. For these experiments, we sort-purified bulk i.v.^{shielded} CD4 T cells from IAV-primed mice at 28 dpi and transferred 5×10^5 i.n. to new hosts that were then primed with IAV. We observed more IL-6$^+$ and IL-12$^+$ CD11c$^+$ cells at 4 dpi in recipients of T_{RM} (**Figure 4c**), as well as higher numbers of natural killer cells and neutrophils (**Figure 4d,e**). These results indicate the rapid initiation of local inflammatory responses by T_{RM} following IAV challenge involving multiple innate immune populations.

IL-15 is required to generate but not maintain IL-2-independent T_{RM}

Finally, we sought to determine whether an alternative cytokine signal is required for the generation of CD4 T_{RM} through the IL-2-independent pathway. Given that IL-15 can support a degree of CD4 memory formation in some situations,[35] we analyzed IL-15 expression following IAV challenge. We found that IL-15 protein in the lungs steadily increased during the first week of infection (**Figure 5a**), which is consistent with previous studies analyzing gene expression.[36] In contrast, no IL-15 above background was detected in the serum (**Figure 5a**). The strong lung-restricted detection of IL-15 in response to IAV challenge suggests that it could be a central factor regulating CD4$^+$ T_{RM} generation.

To determine whether IL-15 can support CD4 T_{RM} generation, we first treated mice receiving donor cells with IL-2-neutralizing Ab alone, thus restricting donor fate to T_{RM} (**Figure 1**), or with IL-2-neutralizing Ab in conjunction with a blocking Ab against CD122 (IL2/IL-15 receptor β chain) from 1 to 7 dpi. Blocking CD122 efficiently disrupts IL-15 signals *in vivo*,[37–39] and because IL-2 is neutralized in these experiments, any impact of CD122 blockade can clearly be attributed to an impact on IL-15 and not IL-2 signaling. Efficient CD122 blockade was verified by fluorescence-activated cell sorter at 7 dpi on cells responding in the lungs (**Figure 5b,c**). The addition of anti-CD122 Ab did not reduce peak effector accumulation at 7 dpi (**Figure 5d**), consistent with reports that IL-15 does not affect CD4 T-cell expansion during IAV challenge.[40] Strikingly, in mice treated with CD122 blocking and IL-2-neutralizing Abs, virtually no donor cells could be recovered at 28 dpi (**Figure 5d**), suggesting that the IL-2-independent T_{RM} pathway requires IL-15. To confirm this finding, and to rule out a depleting effect of the CD122 blocking Ab, we transferred OT-II cells to wild-type (WT) or *Il15*$^{-/-}$ B6 mice, challenged with IAV, and treated all hosts with IL-2-neutralizing Ab from 1 to 7 dpi. No differences in peak donor expansion were seen at 7 dpi in WT or *Il15*$^{-/-}$ hosts, but donor cells were virtually absent in *Il15*$^{-/-}$ hosts at 28 dpi, while T_{RM} were readily detected in WT hosts (**Figure 5e**). Together, these results demonstrate the generation of a CD4 T_{RM} subset in the lung through an IL-2-independent pathway that requires critical IL-15 signals during the first week of infection.

We next asked whether the IL-15 signals needed to generate the CD4 T_{RM} must be delivered through trans-presentation or if the CD4 T cells themselves need to express IL-15rα in order to mediate direct IL-15 signaling. To test this, we used WT (CD90.1$^+$/CD90.2$^+$) and conditional *IL15ra*$^{-/-}$ (CD90.1$^+$) OT-II cells transferred to the same or seperate WT (CD45.1$^+$)

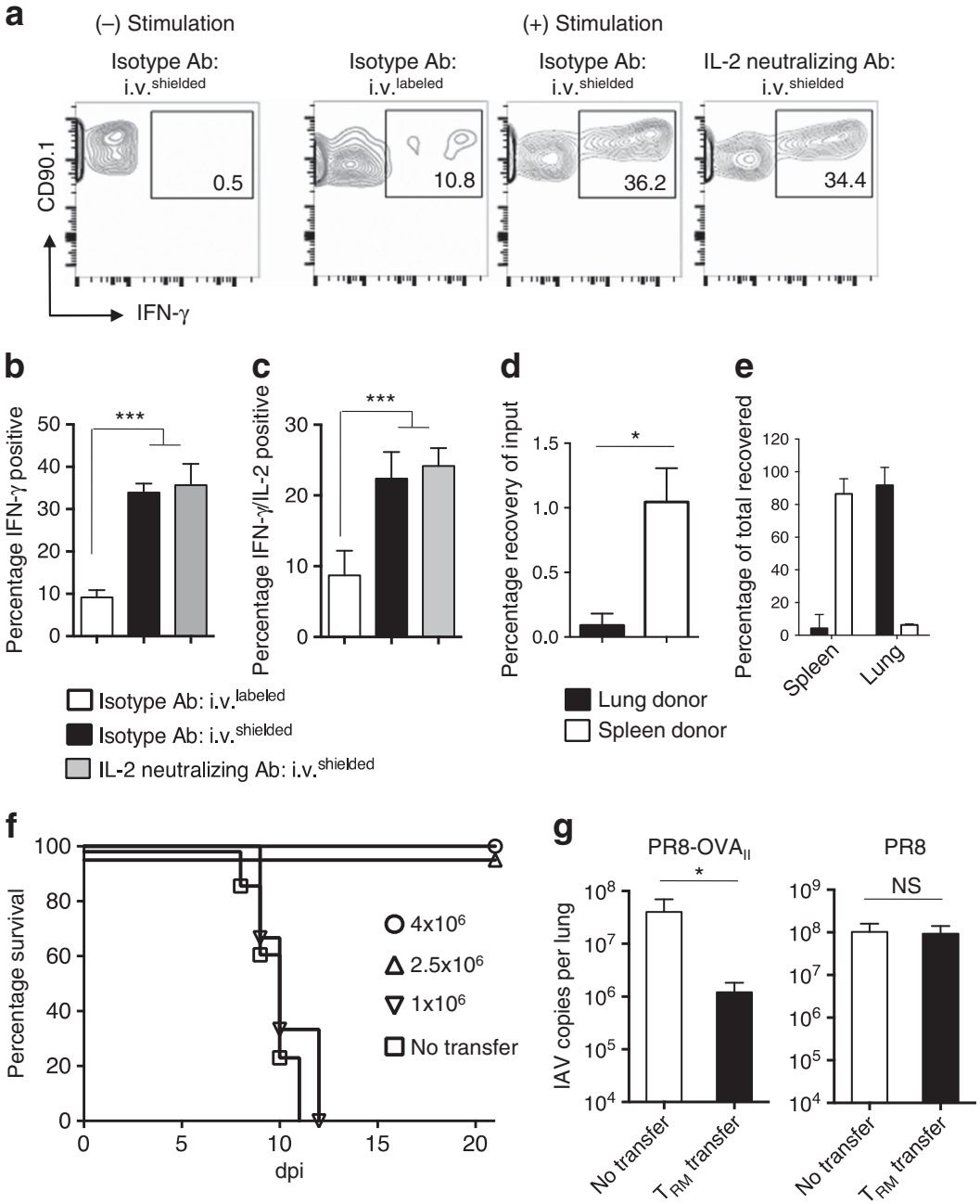

Figure 3 CD4 tissue-resident memory T cells (T_{RM}) display enhanced cytokine production capacity vs. conventional memory cells. At 28 days postinfection, donor cells in the lungs of influenza A virus (IAV)-primed mice were assessed for their ability to produce cytokines based on labeling with intravenous (i.v.) administered fluorescent CD4 antibody (Ab). (**a**) Representative staining of unstimulated (upper row) or stimulated i.v.^{labeled} and i.v.^{shielded} donor cells (gated on Thy1.1$^+$ cells) for interferon (IFN)-γ and summary of the frequency of (**b**) IFNγ-producing and (**c**) dual IFN-γ and IL-2-producing donor cells from three mice per group (one of the three experiments). An equal number of donor T_{RM} or splenic i.v.^{labeled} donor cells sort-purified from IAV-primed mice were transferred i.v. to new hosts in the absence of IAV infection. After 7 days, the total number of donor cells recovered from the spleen and lungs of adoptive hosts was determined. Shown is (**d**) the percentage of either donor population recovered relative to input and (**e**) the percentage of total donor cells recovered from either spleens or lungs of adoptive hosts (four mice per group). Donor T_{RM} isolated from IAV-primed mice and 5×10^6, 2.5×10^6, or 1×10^6 were transferred intranasally to unprimed mice followed by challenge with a lethal dose of PR8-OVA_{II}. (**f**) Survival of mice receiving T_{RM} vs. no transfer controls is shown (combined results from three separate experiments with three mice per group). (**g**) The viral titer from mice receiving 2.5×10^6 donor (OT-II) T_{RM} or not and challenged with either PR8-OVA_{II} (left) or PR8 (right) (3–4 mice per group). NS, not significant.

mice that were challenged with IAV and treated to block IL-2 from 1 to 7 dpi. Whether co-transferred or transferred to different hosts, the number of WT and *IL15ra*$^{-/-}$ donors was similar at 7 dpi, but at 28 dpi WT cells significantly outnumbered the *IL15ra*$^{-/-}$ donors (**Figure 5f–h**). This implies that effector cells require direct IL-15 signals within the first week of IAV infection to generate an IL-2-independent subset of CD4 T_{RM}.

Finally, we sought to determine whether, in addition to being required for the initial generation of the T_{RM}, IL-15 was also

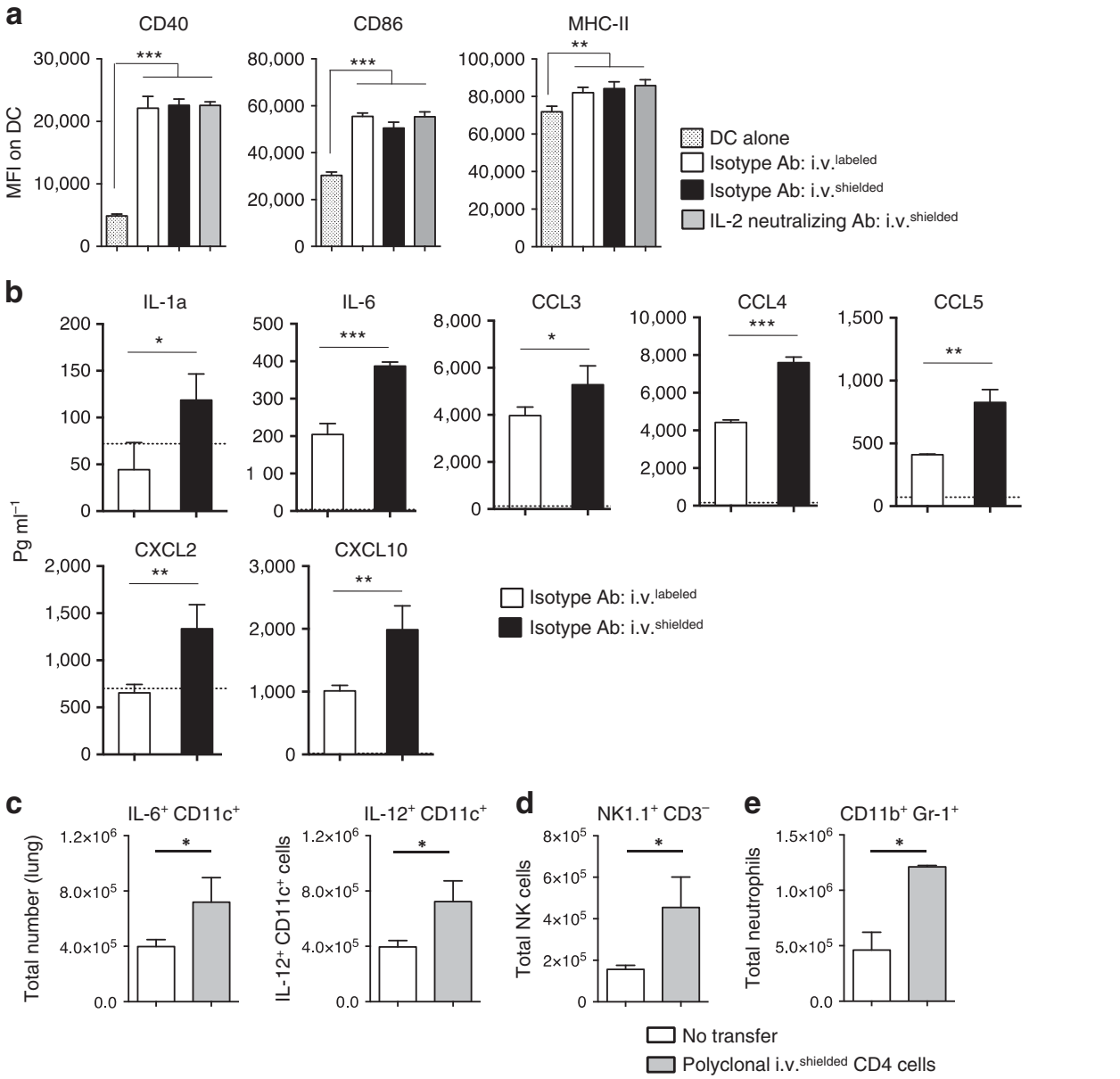

Figure 4 Lung tissue-resident memory T cells induce a more rapid inflammatory response from dendritic cells (DCs) than conventional memory CD4 T cells. Donor memory cells, 1×10^5, sort-purified from the lungs or spleens of influenza A virus (IAV)-primed mice on the basis of their ability to be labeled by CD4 Ab administered intravenously (i.v.) were cultured with an equal number of bone marrow–derived DC pulsed with OVA_{II} peptide. (**a**) After 40 h, DCs were analyzed for the expression of molecules associated with their activation (triplicate conditions; one of the two experiments). (**b**) Supernatants from cultures of DCs and memory cells were harvested at 40 h and analyzed for stated cytokines and chemokines by luminex. Dotted lines indicate the average level detected in supernatants from wells containing DC and peptide alone (triplicate conditions; one of the two experiments). Sort-purified polyclonal i.v.^{shielded} CD4 T cells from the lungs of IAV-primed mice were transferred to unprimed mice followed by PR8 challenge. At 4 days postinfection, lungs were harvested and analyzed for the presence of (**c**) IL-6$^+$ and IL-12$^+$ CD11c$^+$ cells, (**d**) natural killer (NK) cells, and (**e**) neutrophils and compared with mice not receiving donor cells (3–4 mice per group; 1 of the 2 experiments). Ab, antibody; CXCL, C-X-C motif chemokine ligand; MHC, major histocompatibility complex.

required for the long-term maintenance of this subset. We thus transferred donor cells to mice that were challenged with IAV and treated with IL-2-neutralizing Ab from 1 to 7 dpi. Beginning on day 14 postinfection, coinciding with viral clearance in this model, mice were treated with CD122-blocking Ab or an isotype control every second day up to 28 dpi and the presence of donor T_{RM} analyzed on 30 dpi. Although treatment with CD122-blocking Ab dramatically reduced the number of natural killer cells detected in the spleen (**Figure 5i**), a population known to be dependent on IL-15 for maintenance,[41] the number of donor T_{RM} was equivalent in mice treated with control vs. CD122-blocking Ab (**Figure 5j**). These results indicate that, while IL-15 signals received within the first week of infection support the development of lung CD4 T_{RM}, continuous IL-15 signals are not required for the survival of this subset. This requirement for IL-15 during the first week of infection is similar to the

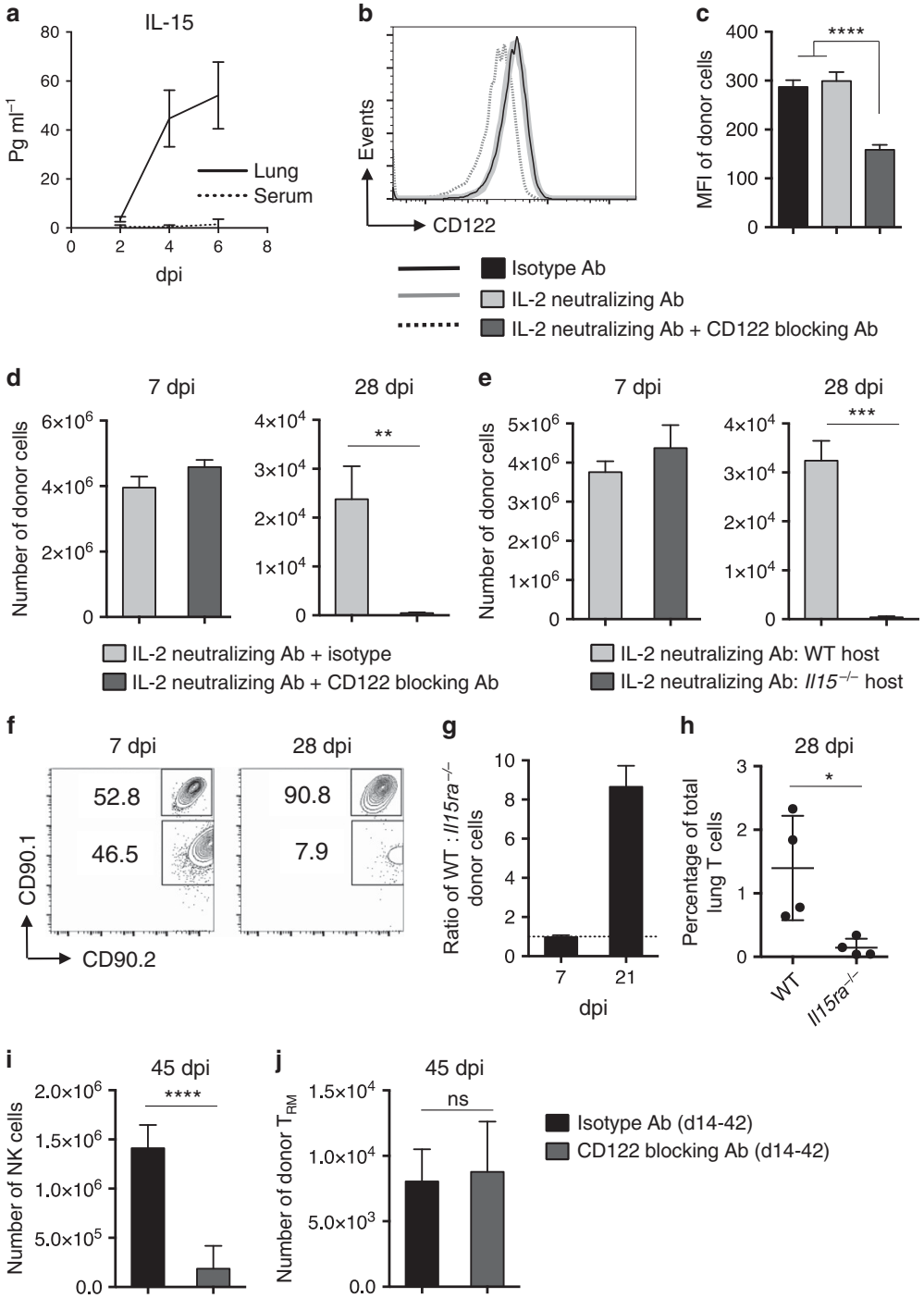

Figure 5 Interleukin (IL)-15 is required for the generation of lung CD4 tissue-resident memory T cells (T_{RM}). (**a**) Lung homogenate and serum was analyzed from influenza A virus (IAV)-primed mice on stated days for the presence of IL-15 protein (four mice per group; one of the four experiments). Mice receiving donor cells and IAV priming were treated either with IL-2-neutralizing antibody (Ab) alone or with IL-2-neutralizing Ab and CD122-blocking Abs from 1 to 7 days postinfection (dpi). (**b**, **c**) On 7 dpi, donor cells in the lungs were analyzed for the expression of CD122 (representative from one of the four mice; one of the three similar experiments). (**d**) The number of donor cells present in the lung at 7 and 28 dpi in mice treated as in **a** is shown (four mice per group). Donor cells were transferred to either wild-type (WT) or *Il15*$^{-/-}$ hosts that were then primed with IAV and treated with IL-2-neutralizing Ab from 1 to 7 dpi. (**e**) Donor cells in the lung were enumerated at 7 and 28 dpi (three mice per group; one of the three experiments). WT (Thy1.1/Thy1.2) and *Il15ra*$^{-/-}$ OT-II cells (Thy1.2) were transferred to WT CD45.1^{+} hosts that were primed with IAV and treated with IL-2-neutralizing Ab. (**f**) Representative staining for both donor populations at 7 and 28 dpi and (**g**) the ratio of WT : *Il15ra*$^{-/-}$ donor cells detected when co-transferred at 7 and 28 dpi and (**h**) the recovery of WT vs. *Il15ra*$^{-/-}$ donor cells when transferred to seperate hosts at 28 dpi (four mice per group; one of the two experiments). Mice receiving WT donor cells and challenged with IAV were treated with IL-2-neutralizing Abs from 1 to 7 dpi and with CD122-blocking Ab or an isotype control every second day from 14 to 44 dpi. (**i**) The number of natural killer (NK) cells recovered from the spleens of mice and (**j**) the number of donor T_{RM} recovered from the lung at 45 dpi was determined from 4 mice per group (1 of the 2 experiments).

requirement for IL-2 during the first week of infection to form the IL-2-dependent T_{RM} subset and supports the concept that these are alternate but parallel pathways.

DISCUSSION

We previously found that autocrine IL-2 signaling of CD4 T cells, which is induced by cognate antigen recognition, is needed during the effector stage of IAV infection to generate virtually all memory cells present in secondary lymphoid organs. The IL-2 prevents acute death of effector cells during the contraction phase and enhances their fitness to access IL-7 by upregulating sustained CD127 expression.[16,17] Here we show that IL-2 signals also support the generation of a subset of CD4 T_{RM} in the lung, which is consistent with findings of IL-2-dependent lung T_{RM} in an asthma model.[42] However, we also describe a novel IL-2-independent pathway in which direct IL-15 signals received by CD4 effector cells supports the generation of a separate but similar cohort of long-lived, highly functional and protective CD4 T_{RM} in the lung. As IL-2 and IL-15 both signal through the same CD122 receptor and downstream adaptor molecules, some of their respective impacts in promoting $CD4^+$ memory formation are likely similar. For example, similar to IL-2, IL-15 can mediate acute antiapoptotic effects in activated CD4 T cells *in vitro*.[43] However, treatment with subsaturating levels of IL-2 and IL-15 drives unique gene expression in T cells.[44] This suggests that key elements of the IL-15-dependent program promoting CD4 T_{RM} are likely distinct from the IL-2-dependent pathway, a hypothesis supported by the IL-7-independent survival of the IL-2-independent CD4 T_{RM}.

The remarkable degree of overlap in gene expression patterns distinguishing CD4 T_{RM} in this study and $CD8^+$ T_{RM} in other studies including those induced by similar IAV infection[7,29] is notable. This suggests that a conserved molecular program supports the maintenance and function of all T_{RM} (CD4 and CD8) in non-lymphoid tissues. The prominence of genes differentially expressed by both $CD4^+$ and $CD8^+$ T_{RM} associated with GTPase activity suggests a central role for these proteins in T_{RM} homeostasis, possibly through regulating cell shape as indicated by studies of skin CD8 T_{RM}.[45,46] On the other hand, that the genes we identified as uniquely expressed by lung $CD4^+$ T_{RM} were most enriched in cell adhesion pathways supports the hypothesis that different T_{RM} subsets (i.e. $CD4^+$ and $CD8^+$) within the same tissue may employ distinct mechanisms for local retention, perhaps indicating their residence in unique microenvironments. Recently, Hombrink *et al.*[30] have demonstrated remarkable conservation of gene expression between lung-resident CD8 T_{RM} in mice and humans. Further studies are required to determine to what extent the CD4 T_{RM}-specific gene expression patterns identified here translate into lung-resident human CD4 memory cells.

We found that the CD4 T_{RM} mediate robust protection against lethal IAV infection when transferred back to the lung. T_{RM}-mediated protection correlated with accelerated inflammatory responses in the lung marked by increased production of cytokines from DCs and elevated numbers of innate cells. In previous studies, this "jump-start" of innate immunity by memory CD4 T cells correlated with early viral control,[33,34] and we also saw impressive reduction of viral titers in recipients of CD4 T_{RM}. The rapid induction of inflammation likely also facilitates optimal CD8 T-cell trafficking by establishing early chemokine gradients[47] and may help the response of other CD4 T-cell specificities during heterosubtypic challenge.[48] Indeed, we found elevated levels of several chemokines in co-cultures of DCs and T_{RM} compared with co-cultures with conventional memory cells. Although all of these are factors are known to be produced by DCs, further studies are required to determine if the T_{RM} are also capable of specialized production of distinct chemokine signals. Our analysis suggests that the ability of CD4 T_{RM} to induce enhanced inflammatory responses may be related to their expression of surface molecules known to impact antigen-presenting cell function. For example, we found that Slamf6, which can interfere with T-cell adhesion to DCs,[49] to be expressed at lower levels by T_{RM} than by conventional memory cells, and RANKL (receptor activator of nuclear factor-κB ligand; TRANCE, TNFSF11), which can deliver an adjuvant-like signal to DCs,[50,51] to be expressed at higher levels by T_{RM}. It is important to point out that only minor differences in surface expression of RANKL, CDH1, and KLRG1, which may nevertheless be biologically significant, distinguish T_{RM} and conventional memory cells. As such, these markers are not as suitable for distinguishing CD4 T_{RM} and other memory subsets as are levels of CD127 and CD69. Further studies are needed to investigate the relative importance of these and other distinguishing attributes of T_{RM} in contributing to their local retention, survival, and protective function.

IL-15 has been proposed to act as a local "alarm" in tissues that promotes maximal Th1 and CD8 T-cell responses needed to clear viral pathogens.[52] Our findings provide support that this "alarm" can also act as a potent signal to induce long-lived CD4 T_{RM}. Although it is not clear from these studies that IL-15-production is strictly limited to the lungs, the lung-restricted detection of IL-15 following IAV infection supports the concept that T_{RM} generation is regulated through factors induced by infection at tissue sites. This is in sharp contrast to the CD4 T-cell-intrinsic autocrine IL-2 signals that are absolutely required to promote conventional memory CD4 T cells primed by IAV[16,17] and that also support a cohort of IAV-primed CD4 T_{RM}. Although our studies show near identical phenotype and function of the IL-2- and IL-15-dependent T_{RM}, further work is required to determine whether these subsets have distinct or overlapping roles in protective immunity upon reinfection. A recent study employing IL-15-adjuvanted vaccinia-based IAV vaccination found that memory $CD4^+$, but not $CD8^+$, T cells had a predominant role in the protection generated.[53] Although T_{RM} subsets were not assessed, the authors found CD4 T-cell responses in the lung were enhanced to a greater extent by IL-15 than those detected systemically.[53] This suggests that the incorporation of IL-15 into vaccination formulations, or strategies that lead to its local production, might boost the generation of protective, functionally specialized CD4 T_{RM} in the lung and perhaps other tissues.

METHODS

Mice. C57BL/6 (Taconic (Hudson, New York) or Jackson Laboratories, Bar Harbor, ME), B6.CD45.1 (Jackson), and *Il15*$^{-/-}$ mice on a C57BL/6 background (Taconic) mice were at least 8 weeks old at the time of infection. Donor CD4$^+$ T cells were obtained from 5-to-8-week-old OT-II.Thy1.1 mice that express a TcR recognizing aa 323-339 (ISQAVHAAHAEINEAGR) of chicken ovalbumin (OVA). OT-II.Thy1.1 mice, originally from Jackson Laboratories, were bred at the University of Massachusetts Medical School or at the University of Central Florida. *Nr4a1*^{eGFP}-expressing mice (Nur77 reporter mice) were originally obtained from Jackson Laboratories and used to breed *Nr4a1*^{eGFP} OT-II donor mice. *Il15ra*^{fl} mice, possessing loxP sites flanking exons 2–3 of the IL-15 receptor α chain (CD122) were obtained from Jackson Laboratories and bred to OT-II mice expressing Cre recombinase to generate conditional *Il15ra*$^{-/-}$ OT-II mice. All experimental animal procedures were conducted in accordance with University of Massachusetts Medical School's and University of Central Florida's Animal Care and Use Committee guidelines.

CD4 T-cell isolation and cell transfer. Naive CD4$^+$ T cells from OT-II mice were obtained from pooled spleen and lymph nodes. Single-cell suspensions were passed over nylon wool, followed by percoll gradient separation and positive MACS selection using CD4 microbeads (Miltenyi, Auburn, CA). Resulting cells were routinely >97% TCR$^+$ and expressed a characteristic naive phenotype (small size, CD62L^{hi}, CD44^{lo}, and CD25^{lo}). CD4 T cells were cultured under Th1-polarizing conditions including exogenous IL-2 as previously described.[16] The resulting effector cells were thoroughly washed and rested for at least 3 days in media free of antigen (Ag) from which live cells were purified by lympholyte separation (Cederlane, Burlington, NC). The resting, IL-2-primed donor cells were adoptively transferred to unprimed mice in 200 µl phosphate-buffered saline (PBS) by i.v. injection.

Donor CD4 T cells expressing CD90.1 were re-isolated from the lungs of IAV-primed CD90.2 B6 mice by first digesting and homogenizing lungs using GentleMACS (Miltenyi) followed by positive selection using MACS for CD90.1. The purity of donor cells was confirmed by fluorescence-activated cell sorter and 1–4 × 10^6 cells transferred i.n. to unprimed B6 hosts. In other experiments, CD4$^+$ i.v.^{shielded} cells were obtained from the lungs of donor B6 mice by cell sorting followed by i.n. transfer of 5 × 10^5 cells to unprimed recipients.

Viral infection and in vivo treatments. A/PR8 and A/PR8-OVA$_{II}$ (H1N1) (kindly provided by P. Doherty) was grown in the allantoic cavity of embryonated hen eggs and characterized at the Trudeau Institute. Mice were infected i.n. under light isoflurane anesthesia (Webster Veterinary Supply, Ocala, FL) with a sublethal (0.1 LD$_{50}$) dose in 50 µl PBS. Mice received adoptively transferred T cells in 200 µl of serum-free media via i.v. or retro-orbital injection and were infected on the same day.

In some experiments, mice were treated with 250 µg per day of the anti-IL-2 Abs S4B6 and JES6-1A12 (Rat IgG2a) from 1 to 7 dpi or with appropriate isotype control Ab (all from BioXcell, West Lebanon, NH). Some mice receiving anti-IL-2 Ab treatment were also given 250 µg of anti-CD122 Ab clone TM-β1 or isotype control (Rat IgG2b) from 1 to 7 dpi or an isotype control (Bioxcell). In other experiments, mice were treated with an IL-7 receptor-blocking Ab clone A7R34 (Bioxcell). All Ab was administered by i.p. injection in 200 µl of PBS.

In some experiments, IAV-primed mice were treated for 5 consecutive days with 2.5 mg kg^{-1} of FTY720 (Cayman Chemical, Ann Arbor, MI) dissolved in water by i.p. injection.

In vivo fluorescent Ab labeling, tissue preparation, and cell isolation. Anesthetized mice were injected i.v. with 3 µg of APC-labeled anti-CD4 Ab in 100 µl of PBS and killed 3–4 min later by cervical dislocation followed by exsanguination by perforation of the abdominal aorta. Lungs were perfused by injecting 10 ml of PBS in the left ventricle of the heart. Lungs, spleen, and dLNs were prepared into single-cell suspensions by mechanical disruption of organs and passage through a nylon membrane or by enzymatic digestion using GentleMACS (Miltenyi). In some experiments, donor cells from the lung or spleen were isolated using cell sorting based on the expression of Thy1.1, CD69 and the i.v. administered APC-labeled anti-CD4 Ab.

Flow cytometry and intracellular cytokine staining. Flow cytometry was performed using fluorochrome-labeled Abs for surface staining anti-CD90.1 (OX-7), anti-CD90.2 (53-2.1), anti-CD4 (RM4.5), anti-CD69 (H12F3), anti-CD127 (A7R34), anti-CD122 (TM-β1), anti-CD103 (B-Ly7), anti-KLRG1 (2F1), anti-CDH1 (DECMA-1), anti-SLAMF6 (13G3-19D), anti-RANKL (IK22/5), anti-CD40 (1C10), anti-CD86 (GL1), anti-major histocompatibility complex-II (M5/114.15.2), anti-CD11c (HL3), anti-NK1.1 (PK136), anti-CD11b (M1/70), and anti-CD3 (17A2) (BD Biosciences, San Jose, CA, eBioscience, San Diego, CA, or BioLegend, San Diego, CA). Intracellular cytokine staining was performed by stimulating cells for 16 h with OVA$_{II}$ peptide-pulsed APC. After 2 h, 10 µg ml^{-1} Brefeldin A (Sigma, St. Louis, MO) was added, and after a further 2 h, the cells were surface stained and fixed for 20 min in 4% paraformaldehyde. Intracellular staining was performed by permeabilizing the cells for 10 min in 0.1% saponin before staining for cytokine by the addition of anti-IFN-γ (XMG1.2), anti-IL-2 (JES-5H4), anti-IL-6 (MP5-20F3), anti-IL-10 (JES-16E3), anti-IL-12 (C17.8), and anti-IL-17 (eBio17B7) fluorescently labeled Abs. Analysis was performed using LSRII or Canto II instruments (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) analysis software.

T-cell and DC co-culture and detection of cytokines and chemokines. Sort-purified OT-II memory cells from the lung (CD69$^+$ i.v.^{shielded}) or spleen (CD69^{low} i.v.^{labeled}) 0.5 × 10^5 per well were co-cultured *in vitro* in Costar 24-well plates (Corning, Corning, NY) in the absence or presence of OVA$_{II}$ peptide with 1 × 10^5 syngeneic bone marrow-derived DCs prepared as previously described.[16] Levels of cytokines and chemokines in triplicate culture supernatants were determined using a Mouse Multi-Plex Luminex Kit (Millipore, Burlington, MA) read on a Luminex 200 reader (Luminex, Austin, TX). Luminex analysis was similarly used to detect IL-15 protein from lung homogenates and serum samples.

Microarray analysis. Prior to harvest, mice receiving donor cells and IAV challenge 28 days prior were administered fluorescently labeled anti-CD4 i.v. and the spleens and lungs harvested 3–5 min later. Donor cells were isolated by first enzymatically digesting the tissues using GentleMACS (Miltenyi) followed by cell sorting based on Thy expression (gating on donor Thy marker) and CD69 (used as a confirmatory marker for T$_{RM}$ in the lung).

Total mRNA was isolated (Qiagen, Hilden, Germany) and amplified (Ambion, Austin, TX) from sort-purified populations of OT-II memory cells isolated from the lungs or spleens of IAV-primed mice at 28 dpi. cRNAs were labeled and hybridized to M430 2.0 chips according to Affymetrix protocols in triplicate. Data were normalized with the Plier algorithm, log transformed, and analyzed with GeneSpring GX 11.0 (Agilent Technologies, Overland Park, KS). Significant genes were selected based on *P*-values <0.05 and fold change >1.5 after correction for false discovery.

Real-time PCR. Viral titers were determined by quantitation of viral RNA. RNA was prepared from whole lung homogenates using TRIzol (Sigma), and 2.5 µg of RNA was reverse transcribed into cDNA using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen, Waltham, MA). Quantitative PCR was performed to amplify the polymerase (PA) gene of A/PR8-OVA$_{II}$ using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) with 50 ng of cDNA per reaction and the following primers and probe: forward primer, 5′-CGGTCCAAATTCCTGCTGA-3′; reverse primer, 5′-CATTGGGTTCCTTCCATCCA-3′; and probe, 5′-6-FAM-CCAAGTCATGAAGGAGAGGGAATACCGCT-3′. Data were analyzed with Sequence Detector v1.7a (Applied Biosystems). The copy number of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid of known concentration as a standard.

Statistical analysis. Unpaired, two-tailed, Students *t*-tests, and $\alpha = 0.05$ were used to assess whether the means of two normally distributed groups differed significantly. One-way analysis of variance with Bonferroni's multiple comparison posttest was employed to compare multiple means. All error bars represent the s.d. Significance is indicated as $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Data availability. Microarray data that support the findings of this study will be deposited in the Gene Expression Omnibus with accession number GSE105257.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS

T.M.S., S.L.S., and K.K.M. designed experiments, analyzed data, and wrote the manuscript. T.M.S., K.D., C.F., J.H., C.C., and K.K.M. performed experiments.

DISCLOSURE

The authors declared no conflict of interest.

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