Proliferation-competent Tcf1+ CD8 T cells in dysfunctional populations are CD4 T cell help independent

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T cell maintenance in chronic infection and cancer follows a hierarchical order. Short-lived effector CD8 T cells are constitutively replaced from a proliferation-competent Tcf1-expressing progenitor population. This occurs spontaneously at low levels and increases in magnitude upon blocking PD-1 signaling. We explore how CD4 T cell help controls transition and survival of the progenitors and their progeny by utilizing single-cell RNA sequencing. Unexpectedly, absence of CD4 help caused reductions in cell numbers only among terminally differentiated cells while proliferation-competent progenitor cells remained unaffected with regard to their numbers and their overall phenotype. In fact, upon restoration of a functional CD4 compartment, the progenitors began to regenerate the effector CD8 T cells. Thus, unlike memory T cells for which secondary expansion requires CD4 T cell help, this is not a necessity for proliferation-competent progenitor cells in dysfunctional populations. Our data therefore reveals that proliferation-competent cells in dysfunctional populations show a previously unrecognized uncoupling of CD4 T cell help that is otherwise required by conventional memory T cells.

CD4 help | CD8 T cells | chronic infection | single-cell RNA sequencing | Tcf1

CD8 T cells are major players in the adaptive immune defense against intracellular pathogens, where in the context of acute infections caused by influenza or rhinovirus, antigen-specific CD8 T cells differentiate into highly functional effector and memory cells (1–3). In contrast, some viruses like HBV, HCV, and HIV in humans as well as certain strains of Lymphocytic choriomeningitis virus (LCMV) in mice establish chronic infections associated with the formation of effector CD8 T cells, which in particular in the latter two infections often show diminished functional activity—a phenomenon known as T cell exhaustion or T cell dysfunction (4). Yet, it was recently recognized that the maintenance of dysfunctional or exhausted terminally differentiated T cell populations requires a proliferation competent, T cell-specific transcription factor 1 (Tcf1) expressing progenitor population, which through de novo generation of terminally differentiated effector cells sustains the immune response in chronic infections (5–10). We therefore refer to these cells as effector progenitors. Moreover, this effector-progenitor Tcf1+ population is crucial for T cell expansion and enhanced effector function occurring after inhibitory receptor blockade (5, 11). Given the central role of this effector-progenitor population, it is of key importance to identify the factors defining its maintenance and functionality.

CD4 T cells have a unique role in antiviral immunity by supporting CD8 T cell function and differentiation, humoral immunity as well as via direct antiviral effector function (12). In the context of acutely resolved infections, CD4 help for CD8 T cells is redundant for the formation of primary immune response in case of sufficient inflammatory stimuli, but irreplaceable for the

generation of functional and long-lasting memory (13–15). In chronic infection, numbers and phenotype of antigen-specific CD8 T cell immune responses strictly depend on the presence of antigen-specific CD4 T cells. In humans, stronger antigen-specific CD4 responses are associated with lower HIV-1 plasma viral loads and, if occurring at the early phase of HCV infection, with subsequent control of it (16, 17). Absence of CD4 help in LCMV clone 13 is associated with progressive functional and numerical deterioration of the LCMV-specific CD8 T cell response, accompanied by reduced viral control (18–20). However, the reason for CD4 dependence of CD8 T cell in chronic infections remains so far unclear. Despite the aggravated chronic phenotype and reduced functionality, little is known about CD8 T cell differentiation in absence of CD4 help.

Based on the aforementioned observations, we initially hypothesized that CD4 T cell help is necessary for the proper maintenance and function of the Tcf1-expressing effector progenitors in chronic infection, however our observations largely contrasted this view. Using a combination of classical assessments of T cell dynamics coupled with high resolution single-cell resolved next generation RNA sequencing, we uncovered a shift

Significance

Effector CD8 T cell responses in chronic infection and cancer are maintained by proliferation-competent progenitors, which share features with the progenitors of memory T cells in acutely resolved infections. Combining classical assessment of population dynamics with single-cell RNA sequencing, we discovered a switch in the need for CD4 help between related populations in acute and chronic infection. While in acute infection, memory T cells but not effector T cells are CD4 help dependent, and we found the opposite in chronic infection. Thus, progenitor cells in chronic infection acquire a level of autonomous function that is not seen among their counterparts in acute infection. Vice versa, the dependence of the effector cells highlight why a functional antigen-specific CD4 T cell compartment is critical in therapeutic settings.

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Fig. 1. Selective loss of Tcf1[−] but not Tcf1⁺ P14 T cells without CD4 help. (A–D) C57BL/6 mice (CD45.2⁺) were engrafted with transgenic gp33-specific P14 T cells (CD45.1⁺) prior to infection with LCMV c13 WT (A–D) or LCMV c13 WT or Mix (D). CD4 depletion (A, B, and D) and CD40L blockade (C) were performed as described in *Materials and Methods*. Representative CD8⁺CD45.1⁺ gated flow cytometry plots and corresponding data graphs are shown. P14 T cell numbers in spleens were determined 4 wk after infection and stained for the indicated markers (A and C). The numbers of P14 Tcf1⁺ and Tcf1[−] cells in spleens were determined 8 d (B), 14 d (B), and 4 wk (B and D) after infection. Symbols in data plots represent individual mice, small horizontal lines indicate the mean. ***P < 0.001; **P < 0.01; *P < 0.05 based on unpaired *t* test. Abbreviations: ctrl, control; w/o CD4, CD4 depleted; endo CD8, endogenous CD8.

of the requirement for CD4 T cell help from the need to support proliferation competent cells in acute infection toward a selective need for supporting the maintenance of terminally differentiated cells in chronic infection. In fact, we found that proliferation-competent Tcf1-expressing CD8 cells remained unaffected and functional in the absence of CD4 T cell help. Overall, we reveal a previously unrecognized CD4 independence of the Tcf1 positive progenitors and a specific requirement of CD4 help for the maintenance and proper differentiation of the effector compartment.

Results and Discussion

Selective Loss of Tcf1[−] but Not Tcf1⁺ P14 T-Cells without CD4 Help. To verify our original hypothesis that CD4 T cells are needed for the proper maintenance and function of the Tcf1-expressing effector-progenitor population, we transferred T cell receptor (TCR) transgenic, CD45 congenic, gp33-specific P14 T cells into control (ctrl) and CD4-depleted (without CD4) C57BL/6 (B6) hosts, followed by inoculation with wild-type LCMV clone 13 (c13 WT) strain, which establishes a chronic infection. As reported previously (18–20), anti-CD4 antibody treated hosts had a 4.5-fold reduced numbers of splenic P14s at 4 wk post infection (Fig. 1A). This was accompanied by an exacerbated dysfunctional phenotype characterized by increased expression of programmed cell death 1 (PD-1), thymocyte selection associated high mobility group box (Tox), and Helios Ikaros Family Zinc Finger 2 (Helios). In strong contrast to our initial hypothesis, we saw that the Tcf1-expressing progenitor population remained stable while the Tcf1-negative differentiated effector cells declined selectively in frequency and absolute numbers (Fig. 1B). Thus, only antigen-specific Tcf1-negative T cells are from a numerical perspective CD4 T cell help dependent but not the progenitor population. This was a surprising outcome, given the importance of CD4

help for the generation of functional and long-lasting memory in acute infection (13). Yet, absence of CD4 T cells increased PD-1 expression on both Tcf1⁺ and residual Tcf1[−] T cells, albeit at higher magnitude in the latter population (*SI Appendix, Fig. S1*). To test if the provision of CD4 help involves signaling through CD40/CD40L, we injected a CD40L-blocking antibody into clone-13 infected mice. Interestingly, this treatment increased PD-1 expression to similar levels as seen after depleting CD4 T cells but P14 numbers and their dynamics remained unchanged (Fig. 1C). Thus, absence of CD40 ligation only partially recapitulates the unhelped phenotype.

Next, we wanted to determine if the loss of the Tcf1-negative population occurs only when T cells have an exhausted phenotype or if it applies similarly to cells, which retain a normal phenotype in chronic infection. To address this, we took advantage of a previously established well-controlled clone-13 based experimental system (21) in which we can prevent T cell dysfunction by reducing the amount of antigen to which T cells are exposed. We achieve this by mixing wild-type clone-13 virus with a mutant bearing a point mutation in the gp33 peptide, which results in an H-2Db binding incompetent altered gp33 peptide ligand. As shown previously, a ~5:1 mix of mutant to wild-type virus results in an infection that is as chronic as a normal clone-13 infection, but in which endogenous gp33-specific and P14 TCR transgenic T cells are exposed to low gp33 quantities. As a consequence, gp33-specific but not T cells of other antigen specificities retain in this setup an acute effector phenotype over a period of at least 4 wk without showing signs of transition into conventional memory T cells. When looking at the CD4 dependence of this population we surprisingly observed a clear and roughly ~8-fold decline in the numbers Tcf1-positive T cells. A similar 8-fold reduction was seen for Tcf1-negative cells (Fig. 1D), which was not accompanied by increases in PD-1 expression. Taking the progenitor–progeny

Fig. 2. Absence of CD4 help causes a major subset distribution shift in differentiated effector P14 cells without impacting the effector-progenitor population. (A–C) Single-cell RNA sequencing analysis of P14 T cells recovered at day 40 post LCMV c13 WT infection from ctrl and w/o CD4 hosts. (A and B) Each circle represents a single cell. All plots are generated with the use of a nonlinear dimensional reduction tSNE, placing similar cells in local neighborhoods in the high-dimensional space. (A) KNN cluster prediction (cell color) overlaid on the tSNE cell distribution (cell location). (B) tSNE distribution of control and unhelped P14 cells. Expression of key genes involved in CD8 T cell function and differentiation overlaid on the tSNE distribution. (C) Cluster distribution of ctrl and w/o CD4 P14 cells. Abbreviations are as follows: ctrl, control; w/o CD4, CD4 depleted; Eff, effector.

relation between Tcf1 positive and the downstream-located Tcf1-negative population into account, this observation suggests that the reduction of the latter is a consequence of the reduced number of the progenitors. Our data therefore underline that the CD4 independence of Tcf1-positive cells applies only to dysfunctional T cell populations and not to T cells that retain a non-dysfunctional phenotype in chronic infection. In fact, the latter show a similar CD4 dependence for maintaining long-term proliferative capacity as seen among memory T cells formed in acute LCMV Armstrong infection.

Absence of CD4 Help Causes a Major Subset Distribution Shift in Differentiated Effector P14 Cells without Impacting the Effector-Progenitor Population. We reasoned that the decline in differentiated Tcf1-negative T cells could either be a consequence of a direct action of helper T cells on this population or that the Tcf1-positive population, despite being maintained at comparable numbers without CD4 T cells, might be impaired in giving rise to differentiated Tcf1-negative T cells. To address the differentiation and dynamics with or without CD4 T cells at high resolution, we employed single-cell resolved RNA sequencing. The advantage

Fig. 3. CD8 T cells with pronounced effector and migration signatures are mainly affected by the lack of CD4 help. (A and B) Single-cell RNA-sequencing analysis of P14 T cells recovered at day 40 post LCMV c13 WT infection from ctrl and w/o CD4 hosts as explained in Fig. 2. (A, Upper) KNN predicted cluster 2 highlighted in tSNE. (A, Lower) Calculated absolute numbers of splenic P14 cells in ctrl and CD4-depleted animals that are similar to the cells in cluster 2. The calculation is based on the percentage representation of cluster 2 among total P14 T cells as explained in *Materials and Methods*, each symbol in the plot represent individual mouse and the bar the mean. (B) tSNE plots, gene expression of key and unique cluster 2 genes. Histograms, P14 T cells were isolated from control or CD4-depleted C57BL/6 mice host mice at 5 wk post infection and stained for Cx3cr1. Representative CD8+CD45.1+ gated flow cytometry histograms are shown, numbers indicate the fraction of cells with the indicated expression intensity. Abbreviations are as follows: ctrl, control; w/o CD4, CD4 depleted; Eff, effector.

Fig. 4. Unhelped effector cells undergo alternative differentiation characterized by functional reprogramming. Single-cell RNA-sequencing analysis of P14 T cells recovered at day ~40 post LCMV c13 WT infection from ctrl and w/o CD4 hosts. tSNE plots highlight the KNN-predicted cluster 3, 4, and 5 for illustrative purpose as explained in Fig. 2. Scatter plots show the predicted number of splenic cluster 3, 4, and 5-like P14 cells in ctrl and CD4-depleted animals (symbols represent data for individual mouse). Heatmap displays the characteristic for cluster 3, 4, and 5 gene signature. Abbreviations are as follows: ctrl, control; w/o CD4, CD4 depleted; EFF, effector.

of this approach is that it allows unbiased grouping of cells into clusters based on transcriptome similarities and then cluster-specific expression profiles can be determined. We therefore performed RNA sequencing of ~860 P14 T cells recovered from ctrl and ~860 from host without CD4 T cells at day 40 (d40) after a normal clone-13 infection (22). To visualize subpopulations which are shared or unique in both datasets, we first joined the data for ctrl and unhelped P14 T cells and used nonlinear dimensional reduction (tSNE), which aims to place cells with similar local neighborhoods in high-dimensional space together in low-dimensional space. This approach has the advantage that even cells, which are rare in one group and more abundant in the other, will cluster together and therefore can be identified in a merged cluster. Afterward, we used k-nearest neighbor (KNN) based on the Euclidean distance in PCA (principal component analysis) space to construct graph-based clusters (cell color), which colocalized with tSNE clusters (cell location; Fig. 24). With the merged dataset, we can then assess in which clusters unhelped cells are under- or overrepresented or maintained similarly.

We have identified 5 clusters in the merged dataset, 4 of which representing subsets within the differentiated effector compartment (as indicated by expression of *Gzma*, *Gzmb*, *Gzmk*, and *Fasl*) and 1 cluster that contains a higher frequency of *Tcf7* message positive T cells (the gene coding *Tcf1*). This cluster represents the less differentiated progenitor compartment of both groups (Fig. 2B), and we assigned it as the “shared effector-progenitors” cluster. Note that the reduced numbers of *Tcf1*-negative T cells in the depleted condition results in a higher percentage-wise representation of unhelped P14 T cells in the shared effector-progenitors cluster 1 (Fig. 2C) even though absolute numbers of *Tcf1*-positive cells per mouse were similar (Fig. 1B). We also quantified the distribution into the other clusters and found that ctrl effector cells preferentially distributed in cluster 2 and 3, while unhelped effector P14 cell were enriched in clusters 3, 4, and 5. Given this distribution, we assigned cluster 2 as help dependent cluster, 3 as shared effector cluster, 4 as cluster which preferentially harbors unhelped cells, and 5 as unique unhelped cluster. Despite these preferences, only cluster 5 was unique to the help-deficient group while all others clusters can be detected in both conditions. This indicates that absence of CD4 T cells help drives a major shift in the percentage-wise representation of cells in the differentiated effector clusters, without impacting the general capacity to form cells belonging to all of the clusters.

CD8 T-Cells with Pronounced Effector and Migration Signatures Are Mainly Affected by the Lack of CD4 Help.

The help-dependent effector cluster 2 showed the strongest decline in size (126-fold drop) in the absence of CD4 T cells (Fig. 3A). In the control population it comprises 1/3 of the total P14 cells (Fig. 2C). Differential gene expression (DGE) and gene set enrichment analysis (GSEA) indicate a superior effector state of this cluster (SI Appendix, Fig. S24). This is supported by the relatively increased expression of *Tbx21* (the gene coding T-bet) and decreased expression of molecules (*Pdcd1*, *Cd160*, *Cd244*, and *Entpd1*) reported to be linked to effector function deterioration (23–27). Of note, cluster 2 “help-dependent Eff” uniquely expressed a module of migratory molecules (Fig. 3B), among which are *S1pr1*, *S1pr5*, and *Cx3cr1*. The latter was confirmed at protein level. Interestingly, high expression of *Cx3cr1* was previously reported to correlate with cytotoxic function and decreased inhibitory receptor expression in tumor models (28). Similarly, *S1pr1* impacts lymphocyte aggress and is known to be controlled by zinc-finger transcription factor *Klf2* (29, 30), which is also up-regulated in this cluster. Given the particular expression profiles, we concluded that this cluster contains an effector subset, which exclusively expresses a set of migration-related molecules and is selectively lost without CD4 T cell help. Though further studies are needed, we take into consideration that *Cx3cr1* expression might serve as surrogate indicator for a superior effector cell state in chronic infections. The loss of the cluster 2 effector cells without CD4 help might at least in part explain the characteristic decline in viral control, without depreciating the role of diminished humoral immunity.

Unhelped Effector T-Cells Undergo Alternative Differentiation Characterized by Functional Reprogramming.

In contrast to the help-dependent effector cluster 2, the other differentiated effector clusters (3–5) showed elevated expression of inhibitory receptors including programmed cell death 1 (*Pdcd1*) coding PD-1, *Cd160*, and *Cd244* coding 2B4 (SI Appendix, Fig. S2B). Some expression of PD-1 and CD160 was noted on the progenitor cluster 1 which matches reports of expression of inhibitory receptor on the progenitor cells (5). The plasma membrane ecto-ATP diphosphohydrolase 1 (*Entpd1*) distinguished differentiated effectors from progenitor cells and had increased expression in clusters with more pronounced dysfunction. The *Cd244* coding 2B4 distinguished exhausted from both the progenitor cluster 1 and help-dependent effector cluster 2. While cells in cluster 3 declined in absence of CD4

help, those in cluster 4 increased despite at lower level (Fig. 4). Moreover, cluster 5 displaying the highest expression *Pdcd1* and *Entpd1* emerged selectively in help-deficient mice accounting for 7.8% of all unhelped P14s and related cells were not found in wild-type control mice. This cluster exhibit gene enrichment for oxidative phosphorylation, tricarboxylic acid (TCA) cycle, respiratory electron transport, and proteasome generation (*SI Appendix, Fig. S2C*). Altogether, our data identify substantial but discrete heterogeneity within dysfunctional T cells as we found evidence for the existence of 3 distinct dysfunctional clusters of which 1 is help dependent (cluster 3), 1 is help independent (4), and 1 becomes unleashed without help and displays the highest expression of *Pdcd1*.

Moreover, it underlines that not only the functional cells in cluster 2 but also cells in the dysfunctional clusters are qualitatively impacted by the absence of CD4 T cell help.

The Effector-Progenitor Phenotype Remains Unaltered without Help and Proliferative Capacity Can Be Restored by Subsequent Provision of Antigen-Specific CD4 T-Cell Help. The data obtained from the single-cell RNA sequencing did not provide evidence that the absence of CD4 T cells impairs the *Tcf1*-expressing progenitor population. In fact, DGE analysis revealed only minor differences with or without CD4 depletion (Fig. 5A). This included the known increases in PD-1 and reduced *Spg21* expression levels (31). This implies that the *Tcf1*-expressing progenitor cells are

Fig. 5. The effector-progenitor phenotype remains unaltered without help and proliferative capacity can be restored by subsequent provision of antigen-specific CD4 T cell help. (A) Single-cell RNA-sequencing analysis of P14 T cells recovered at day ~40 post LCMV c13 WT infection from ctrl and w/o CD4 hosts. (Upper) tSNE plot highlighting the KNN predicted cluster 1 for illustrative purpose. (Middle) shows the predicted number of cluster 1-like P14 cells in the spleens of ctrl and w/o CD4 animals used for single-cell RNA sequencing by projecting the percentage distribution over the total number of P14 cells observed in the respective animal. Lower heatmap, differentially expressed genes (DEG) between ctrl and w/o CD4 P14 cells colocalizing in cluster 1. (B–D) C57BL/6 mice (CD45.2+) were engrafted with transgenic gp33-specific P14 T cells (CD45.1+) prior to infection with LCMV c13 WT. Transgenic SMARTA CD4 T cells were transferred on day 25 post infection. Data were collected 35 d post infection. (B) Representative CD4+CD45.2+ gated (Left and Middle) and CD4+CD45.1+ (Right) flow cytometry plots and corresponding data graphs for all mice. (C) Representative CD8+CD45.1+ gated flow cytometry plots and corresponding data graphs for all mice. (D) Up, CD8+CD45.1+ gated flow cytometry plot. Down, enumeration of Ki-67 positive P14 T cells. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ based on unpaired t test. Abbreviations are as follows: ns, not significant ($P > 0.05$) based unpaired t test; ctrl, control; w/o CD4, CD4 depleted; prog, progenitors; DGE, differentially expressed genes.

largely spared from being impacted by the absence of CD4 T cells. To directly test this hypothesis, we reinstalled an LCMV-specific CD4 T cell compartment upon transfer of transgenic SMARTA CD4 T cells into CD4-depleted host mice on day 25 post infection. The CD4 depletion was discontinued on day 15 post infection to allow graft acceptance, which led to restoration of endogenous CD4 compartment including normal frequencies of regulatory T cells (Fig. 5B). This compartment was unable to reinvigorate the P14 response (Fig. 5C), probably because of the lack of antigen-specific CD4 T cells due to thymic tolerance in LCMV-infected hosts. In contrast, a SMARTA T cell transfer restored Ki-67 expression in about 90% of P14 T cells (Fig. 5D), and we observed similar numbers of total Ki-67 positive P14 T cells in control and reconstituted mice. Thus, retention of strong proliferative capacity in the progenitor population is not lost in the absence of CD4 T cell help. Of note, the addition of antigen-specific CD4 T cells aggravated immunopathology which limited the length of the possible observational period. We therefore had to terminate the experiment before absolute P14 numbers could reach normal levels (Fig. 5C). Of note, the transferred SMARTA cells remained free of Foxp3 expression (Fig. 5B). This supports the conclusion that conventional CD4 T cells maintain the effector compartment in chronic infection.

Altogether our data reveal a fundamental difference in the requirement for CD4 T cell help between dysfunctional and normal CD8 T cell populations. While the retention of proliferation competent T cells following an acute infection is critically linked to the provision of CD4 T cell help, either during or after the infection, proliferation-competent Tcf1-positive cells operate in dysfunctional populations independently from CD4 T cell help. CD4 T cells in such populations selectively support only the maintenance and prevent functional alterations in differentiated T cells. This differential requirement for CD4 T cell help correlated strictly with the T cell phenotype, but not with the infection type, as we made similar findings with T cell populations that retain an acute phenotype in otherwise persistent infection following low level of antigen exposure. We observed that blocking CD40L-CD40 signaling, one of the major pathways responsible for CD4 help provision, recapitulates only part of the phenotype seen upon CD4 depletion. This demonstrates the multimodal nature of the CD4 help provided to CD8 T cells. A question that remains untouched by our studies is whether CD4 T cell help in chronic infection is provided directly to antigen-specific CD8 T cells or upon accessory cells such as antigen-

presenting cells as it has been discussed for the provision of CD4 T cell help in acute infection (32).

Overall our findings underline that Tcf1-positive progenitor cells acquire a level of autonomous function in dysfunctional populations that is not seen among their counterparts in acute infection or not dysfunctional T cell populations. Vice versa, differentiated effector populations become more dependable and this might equip CD4 T cells specifically in chronic infection with the capacity to control the size and function of these cells.

Materials and Methods

Mice. C57BL/6 (B6) mice (CD45.2⁺) were obtained from Charles River (Germany), P14 TCR $\alpha\beta$ (CD45.1⁺) and SMARTA TCR $\alpha\beta$ (CD45.1⁺) transgenic mice were kindly provided by A. Oxenius. Mice were bred and maintained in modified specific pathogen-free (SPF) facilities initially at the University of Lausanne, Switzerland and later at the Technical University of Munich, Germany. Experiments were performed with at least 6-wk-old mice in compliance with the University of Lausanne and the Technical University of Munich institutional regulations and were legally approved by the veterinarian office of the canton Vaud, Switzerland and the regional veterinarian authority "Regierung of Oberbayern," Germany.

CD4 Depletion. In vivo depletion of CD4 lymphocytes was achieved by i.p. injection of 300 μ g anti-mouse CD4 antibody (clone GK1.5; Bio X Cell) diluted in PBS and injected on day -2, +1, and thereafter on each fifth day after infection. CD4 depletion was interrupted 10 d before SMARTA transgenic cell transfer to avoid depletion of the engrafted cells.

CD40L Blockade. In vivo CD40L blockade was achieved by i.p. injection of 300 μ g anti-mouse CD40L antibody (clone MR-1; Bio X Cell) diluted in PBS and injected on day -2, +1, and thereafter on each fifth day after infection.

Data Analyses. Scatter dot plots depict the mean. Statistical analyses were performed with Prism 7.0 (Graphpad Software). Unpaired *t* tests (two-tailed) were used. *P* values < 0.05 were considered significant (*P < 0.05; **P < 0.01; ***P < 0.001); *P* values > 0.05 were considered not significant (ns).

Data Availability. Refer to [SI Appendix](#) for: infections; purification of mouse T cells, adoptive cell transfers; surface and intracellular antibody staining and flow cytometry cell sorting of mouse cells; single-cell transcriptome amplification and RNA sequencing, and single-cell RNA-seq data preprocessing and analysis.

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