

Prior Plasmodium infection establishes a monocyte-CD8⁺ T cell circuit for targeted, broad-spectrum respiratory antiviral immunity

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Title: Prior *Plasmodium* infection establishes a monocyte-CD8⁺ T cell circuit for targeted, broad-spectrum respiratory antiviral immunity

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Abstract

The principles governing how the immune system integrates distal infectious histories to orchestrate long-term, heterologous defense at remote mucosal barriers remain poorly defined. While trained immunity is well-characterized as a myeloid-mediated mechanism for antibacterial protection, the determinants of multi-lineage antiviral resistance are not established. Here, we show that recovery from systemic *Plasmodium yoelii* infection induces an integrated circuit between the respiratory epithelium, inflammatory monocytes, and CD8⁺ effector memory T cells (Tem) to provide broad protection against influenza A and respiratory syncytial viruses. In contrast to myeloid-dependent models, this resistance is strictly dependent on the synergy between both compartments; genetic or cellular ablation of either inflammatory monocytes or CD8⁺ T cells completely abolishes protection. Spatial and single-cell transcriptomics reveal that infected epithelial niches selectively recruit imprinted monocytes, which subsequently direct the site-specific accumulation and acute expansion of CD8⁺ Tem a Ccl4/Ccl5–Ccr5 axis. Within these localized foci, activated Tem executed rapid viral clearance through a T cell-intrinsic, INF- γ dependent manner. These findings define a multi-lineage "triad" synergy as an alternative arm of trained immunity, shifting the paradigm from cell-autonomous myeloid reprogramming toward an integrated innate–adaptive circuit for niche-restricted mucosal defense.

Keywords: CD8 effector memory T cell, Ccl4/Ccl5-Ccr5 axis, Trained immunity, Heterologous antiviral protection

Introduction

The COVID-19 pandemic has underscored the enduring vulnerability of human populations to emerging respiratory pathogens. While vaccination has significantly mitigated severe disease, the rapid waning of neutralizing antibodies and the continuous evolution of viral surface glycoproteins permit frequent breakthrough infections^{1,2}. These challenges emphasize the urgent need for "variant-tolerant" strategies that bolster broad, heterologous antiviral immunity directly at mucosal barriers³.

An intriguing epidemiological observation during the pandemic was the unexpectedly low COVID-19 morbidity and mortality reported in malaria-endemic regions of sub-Saharan Africa and Asia^{4,5}. While demographic factors, under-ascertainment, and climatic differences undoubtedly contribute^{6,7}, ecological analyses have identified a noticeable inverse correlation between *Plasmodium* prevalence and COVID-19 severity^{4,5}. These findings raise a provocative question: does prior exposure to systemic parasites fundamentally rewire the host's antiviral landscape? Despite the potential implications for global health, the immunological mechanisms underlying such cross-species protection remain speculative.

Over the past decade, the concept of trained immunity has redefined our understanding of host defense. Innate cells and their bone-marrow progenitors can acquire long-lasting functional adaptations following primary stimulation, a process driven by epigenetic and metabolic reprogramming that enables heightened responses to secondary, heterologous challenges^{8–10}. Classical models, such as BCG vaccination, demonstrate that reprogrammed monocytes and hematopoietic stem cells confer protection against diverse bacterial and viral pathogens^{11–14}. More recently, systemic BCG immunization was shown to protect against influenza A virus through an integrated axis involving IFN γ -producing CD4 T cell, either via CX3CR1^{hi} effector-memory CD4 T cells¹⁵, or in an integrated manner involving myeloid cells mediated activation and Th1 cell as effector cells¹⁶. However, while these vaccine models hint at inter-compartmental cooperation, whether natural, systemic parasitic infections mobilize localized, cytotoxic networks to secure distal mucosal defense remains an open question.

Plasmodium infection profoundly alters hematopoiesis and innate immunity. We and others recently demonstrated that a single *Plasmodium* episode leaves long-lived hemozoin deposits in the bone marrow niche^{17,18}, biases hematopoiesis toward myelopoiesis, and generates "trained" monocytes with enhanced bactericidal capacity, conferring protection against heterologous bacterial challenges such as cecal ligation and puncture-induced polymicrobial sepsis¹⁸. These findings established *Plasmodium*-induced trained immunity (PTI) as a distinct trained immunity program. Crucially, however, whether PTI could extend cross-species protection in the context of viral pathogens—particularly within the respiratory tract—has not been explored.

Here, we demonstrate that recovery from *Plasmodium yoelii* infection establishes a durable, broad-spectrum antiviral state in the lung, conferring resistance to both influenza A (IAV) and respiratory syncytial virus (RSV). We show that this protection is not a cell-autonomous trait of trained monocytes; rather, it emerges from a mandatory synergy between innate and adaptive compartments. Using single-cell and spatial transcriptomics, we identify a spatially organized "triad" comprising the infected respiratory epithelium, imprinted inflammatory monocytes, and pre-existing CD8⁺ effector memory T cells (Tem). Upon viral challenge, the infected epithelium selectively recruits monocytes, which in turn direct site-specific CD8⁺ Tem accumulation via a Ccl4/Ccl5–Ccr5 axis. Within these localized niches, Tem execute rapid, bystander viral control through an IFN γ -dependent program that operates independently of type I interferon and IL-15 signaling. By defining this multi-lineage circuit, our study expands the

paradigm of trained immunity from isolated myeloid reprogramming to an integrated, niche-restricted system of mucosal defense.

Results

***Plasmodium* infection induces durable heterologous protection against respiratory viruses**

We first investigated whether PTI confers long-term protection against respiratory viral challenge. Mice were infected with *Plasmodium yoelii* and allowed to fully clear the parasite. Six weeks post-infection—when the animals had returned to clinical baseline (memory phase)¹⁸—they were challenged intranasally with IAV (Fig. 1a).

Prior *Plasmodium* exposure fundamentally altered the clinical course of IAV infection. PTI mice exhibited significantly reduced weight loss, faster recovery, and markedly improved survival compared to PBS controls (Fig. 1b,c). This clinical resilience was mirrored by a striking preservation of pulmonary architecture; while controls suffered from widespread alveolar collapse and diffuse inflammatory infiltration, PTI mice maintained functional parenchymal integrity (Fig. 1d). Interestingly, while whole-lung qPCR analysis revealed only a modest reduction in total viral RNA copies, immunostaining for IAV NP protein showed a pronounced qualitative shift in viral topography (Fig. 1e, f). In contrast to the disseminated viral spread observed in controls, NP expression in PTI mice was confined to discrete epithelial regions. These observations suggest that PTI does not simply lower global viral load, but rather imposes a spatial constraint that prevents diffuse parenchymal dissemination.

When the IAV challenge was delayed until six months post-*Plasmodium* infection, trained mice still displayed improved weight maintenance and survival (Fig. 1g, h), a timescale comparable to the duration of BCG-induced trained immunity in humans¹⁹. Furthermore, PTI provided heterologous protection against a second, unrelated respiratory pathogen. Upon RSV challenge, trained mice showed lower lung viral titers and attenuated lung injury compared with naïve controls (Fig. 1i–k). Collectively, these results demonstrate that prior systemic *Plasmodium* infection establishes a durable, broad-spectrum antiviral state characterized by localized viral control and the maintenance of pulmonary organ function.

Monocytes are required but not sufficient for antiviral PTI

We previously established that PTI-mediated antibacterial defense relies on a myeloid-biased hematopoietic program and the generation of functionally reprogrammed monocytes with enhanced bactericidal capacity¹⁸. To determine if a similar monocyte-intrinsic mechanism orchestrates antiviral PTI, we first performed cellular depletion during IAV challenge. Depletion with anti-Gr1 antibody (targeting both monocytes and neutrophils) completely abolished the PTI-mediated survival advantage. To distinguish the relative contributions of these subsets, we selectively depleted neutrophils using an anti-Ly6G antibody. While Gr1⁺ cells depletion abrogated protection, Ly6G-specific depletion left PTI-mediated resistance intact (Fig. 1l, m), identifying monocytes as the essential innate mediators of the phenotype.

Consistent with a requirement for inflammatory monocyte recruitment²⁰, *Ccr2*^{-/-} mice—defective in bone marrow egression of inflammatory monocytes—failed to benefit prior *Plasmodium* exposure and remained highly susceptible to IAV (Supplementary Fig. 1a,b). This requirement for *Ccr2*-dependent recruitment mirrors the established role of monocytes in PTI-mediated antibacterial defense¹⁸.

We next asked whether trained monocytes are sufficient to transfer antiviral PTI. Ly6C⁺ monocytes from trained or naïve donors were adoptively transferred into naïve recipients before IAV challenge (Fig. 1n). In striking contrast to antibacterial PTI, where the transfer of

trained monocytes is sufficient to recapitulate protection¹⁸, adoptive monocyte transfer failed to improve weight loss or survival in the context of IAV infection (Fig. 1o,p). These results demonstrate that while inflammatory monocytes are an obligatory component of the antiviral response, monocyte-intrinsic training alone is insufficient to confer protection. This mechanistic divergence from antibacterial models suggests that antiviral PTI requires a more complex cooperation between trained monocytes and additional immune compartments.

PTI reshapes the lung immune landscape and expands antiviral CD8⁺ effector memory T cells

To map the systemic-to-mucosal immune landscape in an unbiased manner, we profiled lung-infiltrating CD45⁺ cells using single-cell RNA sequencing (scRNA-seq) at day 2 post-IAV infection. Unsupervised clustering and canonical marker annotation identified all major immune lineages, including inflammatory monocyte, monocyte, interstitial and alveolar macrophages, neutrophil, basophil, eosinophil, dendritic cells, NK cells, B cells, CD4 T cells, and CD8 T cells (Supplementary Fig. 2a; Fig. 2a). Consistent with our cellular depletion data and the established myeloid-centric model of PTI¹⁸, trained lungs exhibited a significant enrichment of inflammatory monocytes and interstitial macrophages (Fig. 2b). However, a prominent and unexpected feature of the PTI landscape was the marked expansion of the CD8 T cell compartment, whereas CD4 T cell and B cell frequencies remained largely comparable to controls (Fig. 2b).

To resolve the functional states of these cells, we performed high-resolution reclustering of the CD8 T cell population. Based on the expression of naïve/central memory (*Sell*, *Ccr7*, *Il7r*, *Tcf7*), activation and effector memory markers (*Cd44*, *Klrk1*), interferon-stimulated genes (*Isg15*, *Stat1*), cytotoxic molecules (*Prf1*, *Gzma*, *Nkg7*), and cell-cycle genes (*Mki67*), we identified five subsets: Naive/Tcm, Transitional memory, Activated_ISG⁺ Tem, Cytotoxic Tem, and Cycling Tem (Fig. 2c). Module scoring confirmed coherent segregation of naïve/Tcm, interferon-response, cytotoxic, and proliferative programs across these subsets (Fig. 2d, e; Supplementary Fig. 2b), consistent with established effector and memory CD8 T cell signature in viral infection²¹.

While Naive/Tcm-like cells predominated in controls, trained mice were significantly enriched for Activated_ISG⁺ Tem and Cytotoxic Tem (Fig. 2c). Gene Ontology (GO) analysis confirmed that these PTI-enriched clusters were functionally programmed for antiviral responses, vesicle-mediated secretion, and rapid cell-cycle progression (Fig. 2f). Pseudotime analysis using Monocle placed Naive/Tcm at the root of a trajectory that progressed through Transitional memory toward ISG-activated and cytotoxic effector memory states (Fig. 2g). Notably, by day 2 post-infection, CD8 T cells from PTI mice were already positioned significantly further along this differentiation trajectory than those from naïve animals, indicating that systemic parasitic imprinting induces accelerated adaptive effector differentiation upon mucosal viral challenge.

These data show that PTI not only biases the myeloid compartment, but also reshapes the CD8 T cell landscape, enriching for antiviral effector-memory states early during infection.

CD8 T cells are an obligatory cooperating arm for antiviral PTI

The rapid expansion and activation of CD8 T cells in PTI lungs suggested that CD8 T cells might be the missing partners required to complement monocyte-mediated defense. To test this, we performed systemic CD8 T cell depletion prior to IAV challenge (Fig. 2h). In striking contrast to isotype-treated PTI mice, those lacking CD8 T cells lost the survival advantage and exhibited weight loss kinetics comparable to naïve controls (Fig. 2i,j). Depletion efficiency was confirmed by flow cytometry (Supplementary Fig. 3). This requirement was genetically

confirmed using *Cd8^{-/-}* mice, which failed to benefit from prior *Plasmodium* infection (Fig. 2k,l).

Importantly, this mechanism is distinct from other models of heterologous respiratory immunity. For instance, recent studies of BCG-induced cross-protection against IAV emphasize a requirement for CX3CR1^{hi} CD4 effector T cells¹⁵. In our PTI model, however, CD4 T cell numbers and subset distributions did not differ significantly between groups at early time points (Fig. 2b and Fig 3b). Thus, the unbiased scRNA-seq profiling and loss-of-function experiments converge on a novel monocyte-CD8 T cell axis, defining an integrated innate-adaptive circuit that is indispensable for PTI-mediated antiviral protection.

PTI mobilizes a local reservoir of CD8 Tem that can transfer protection but still depend on monocytes in vivo

We next characterized the temporal dynamics of the CD8⁺ T cell response. Total lung CD8⁺ T cell numbers in PTI mice diverged from controls as early as day 2 post-infection, followed by a robust expansion through day 6 (Fig. 3a), whereas CD4⁺ T cell frequencies remained largely static (Fig. 3b). High-resolution phenotyping revealed that this expansion was restricted to the effector memory (Tem; CD44⁺CD62L⁻) compartment, while central memory (Tcm; CD44⁺CD62L⁺) populations remained unchanged (Fig. 3c; Supplementary Fig. 4). This pattern is consistent with effector memory and tissue-resident CD8 T cells acting as the main contributors to early local antiviral defense^{22,23}.

To determine whether this early accumulation required the recruitment of lymphocytes from secondary lymphoid organs, we treated mice with the S1P-receptor antagonist FTY720 to block lymphocyte egress. PTI-mediated protection was fully maintained under FTY720 treatment (Fig. 3d), demonstrating that the antiviral response relies on a pre-existing, local reservoir of Tem rather than de novo recruitment.

To assess the clonal dynamics of the CD8⁺ Tem pool, we sequenced the T cell receptor (TCR) repertoire in the lung. Tem from PTI mice exhibited higher TCR clonality and correspondingly lower Shannon entropy compared to PBS controls at both time points post IAV infection (Fig. 3e). This restricted diversity indicates that *Plasmodium* infection establishes a pre-existing, clonally expanded Tem pool in the lung prior to viral challenge. Analysis of high-frequency clonotypes confirmed a pronounced skewing in trained hosts: by day 2 post-infection, the top 10 clones comprised more than half of the total sequenced repertoire in PTI mice (Supplementary Fig. 5), reflecting the acute expansion of CD8⁺ Tem upon IAV infection.

To test the functional sufficiency of these cells, we adoptively transferred purified CD8⁺ Tem from PTI donors into naïve recipients (Fig. 3f). Recipients of PTI-Tem exhibited attenuated weight loss and improved survival compared to controls (Fig. 3g). CFSE-labeling confirmed that these transferred cells underwent robust *in vivo* proliferation within the lung (Fig. 3h, i). Notably, as neither donors nor recipients were previously exposed to IAV, this rapid expansion likely reflects cytokine-driven "bystander" activation. This competence was not a generic feature of Tem cells as OT-I Tem generated via OVA exposure failed to confer protection (Fig. 3j), suggesting that PTI-induced imprinting imparts a specific functional program to the Tem compartment.

Finally, we examined the obligatory synergy between these "trained" Tem and the innate compartment. While *Ccr2^{-/-}* mice generated CD8⁺ Tem populations following *Plasmodium* exposure, they remained entirely susceptible to IAV (Supplementary Fig. 1), indicating that CD8⁺ Tem is functionally incompetent in the absence of monocyte recruitment. To confirm this synergy, we transferred wild-type monocytes into trained *Ccr2^{-/-}* recipients (Fig. 3k). This correction fully restored protection, whereas monocyte transfer into untrained *Ccr2^{-/-}* mice had

negligible effect (Fig. 3l, m). These data define a mandatory innate–adaptive cooperation, where inflammatory monocytes "license" the localized activation and expansion of a pre-existing CD8⁺ Tem reservoir to secure the mucosal barrier from viral infection.

A spatially organized epithelium–monocyte–Tem triad is wired through Ccl4/Ccl5–Ccr5

To define the architectural logic of the monocyte-Tem synergy, we performed spatial transcriptomics on lung sections following IAV challenge. Viral transcripts mapped to discrete IAV⁺ epithelial spots (Fig. 4a). By applying cell-type signature scores, we identified monocyte-rich and CD8-rich spots, which in PTI lungs showed pronounced co-localization specifically within IAV⁺ regions (Fig. 4b,c). Non-parametric co-occurrence analysis confirmed a significant enrichment of monocyte-CD8 joint occupancy within these infected niches, a pattern largely absent in IAV⁻ regions or control lungs (Fig. 4d).

To resolve the molecular communication pathways within these hubs, we employed CellChat to infer intercellular signaling networks. In PTI lungs, inflammatory monocytes emerged as dominant signaling hub while CD8_Cytotoxic/Activated_ISG⁺ Tem were most prominent for incoming signaling compared to controls (Fig. 4e). Information flow analysis identified the CXCL and CCL chemokine families as the primary contributors to this network (Supplementary Fig. 6a), in line with the established role of chemokines in organizing antiviral T cell responses^{28,29}. Ligand-receptor probability mapping specifically highlighted several monocyte-Tem axes, including Cxcl9/10–Cxcr3, Cxcl16–Cxcr6, Ccl2/7/12–Ccr2, and Ccl3/4/5–Ccr5 with the latter showing the highest probability in PTI hosts (Fig. 4f).

ScRNA-seq and spatial mapping confirmed that inflammatory monocytes were the principal source of Ccl4 and Ccl5 (Supplementary Fig. 6b), while Ccr5 expression was selectively enriched in the Activated_ISG⁺ and Cytotoxic Tem subsets (Supplementary Fig. 6c). Notably, spots co-expressing these ligands and their receptor were preferentially sequestered within IAV⁺ niches (Fig. 4a,g,h; Supplementary Fig. 6d). Spots co-expressing *Ccl4* or *Ccl5* together with *Ccr5* were preferentially located within IAV⁺ niches (Fig. 4i; Supplementary Fig. 6e). This configuration is consistent with a model in which inflammatory monocytes at infected foci provide Ccl4/Ccl5 cues that recruit Ccr5⁺ CD8 Tem to the same microenvironment, in line with prior work implicating Ccr5 in the recruitment and positioning of effector CD8 T cells during respiratory virus infections²⁸.

To test the functional necessity of this axis, we pharmacologically inhibited Ccr5 using Maraviroc during IAV challenge. Ccr5 blockade completely abolished PTI-mediated protection, with trained mice exhibiting weight loss and mortality identical to naive controls (Fig. 4j). Furthermore, Maraviroc treatment significantly reduced the site-specific accumulation of CD8 Tem (Fig. 4k). These results demonstrate that the Ccl4/Ccl5–Ccr5 axis serves as the molecular bridge that couples monocyte recruitment to Tem deployment, securing the infected epithelial niche.

Finally, high-resolution confocal microscopy confirmed the physical co-localization of monocytes and CD8⁺ T cells within IAV-infected loci (Fig. 4l). Quantitative analysis revealed that while monocyte densities within IAV⁺ areas were comparable between PBS and PTI groups, the recruitment of CD8 T⁺ cells to these infected foci was significantly higher in PTI-trained hosts (Fig. 4m). Collectively, these findings establish that a spatially wired epithelium–monocyte–Tem triad, coordinated by Ccr5 signaling, is the structural basis for the enhanced antiviral defense observed in trained immunity.

IFN-γ program in CD8⁺ Tem is essential for PTI-mediated anti-viral effect

To identify the effector molecules driving PTI-mediated antiviral protection, we performed multiplex cytokine analysis of bronchoalveolar lavage (BAL) fluid. At day 2 post-infection,

PTI mice exhibited significantly elevated levels of *Ifn* α , *Il*-15, and *Il*-27, with notable increases in *Ifn* γ and *Il*-6 compared with controls (Fig. 5a; Supplementary Fig. 6a). Given that Type I interferons are central to defense against respiratory viruses, including IAV and RSV, acting on both infected and bystander cells³⁰, we first examined PTI in *Ifnar1*^{-/-} mice.

Despite the loss of canonical Type I IFN signaling, PTI-primed *Ifnar*^{-/-} mice remained significantly protected, exhibiting reduced weight loss and improved survival compared to their control counterparts (Fig. 5b, c). These data indicate that PTI provides a layer of antiviral resistance that operates independently of the host's Type I IFN response.

We next investigated the role of *Il*-15, a key driver of memory CD8⁺ T cell homeostasis and bystander activation³¹. While *Il*-15 induced robust CD8⁺ Tem proliferation in vitro (Supplementary Fig. 6b), PTI-mediated protection was largely preserved in *Il15*^{-/-} mice (Fig. 5d, e). Although the magnitude of protection was slightly attenuated compared to wild-type hosts, adoptive transfer of WT CD45.1⁺ Tem into *Il15*^{-/-} recipients revealed that donor cells expanded normally at days 2 and 6 post-infection (Fig. 5f). This suggests that while *Il*-15 may support the overall inflammatory environment, it is not an obligatory requirement for the expansion or function of the PTI-induced Tem reservoir.

In striking contrast, the protective effect of PTI was completely abrogated in *Ifngr*^{-/-} mice, which exhibited weight loss and survival rates indistinguishable from naive controls (Fig. 5g). Analysis of scRNA-seq data identified CD8⁺ T cells, specifically the Activated_ISG⁺ and Cytotoxic Tem subsets, as the primary source of increased *Ifng* expression in PTI lungs (Fig. 5h; Supplementary Fig. 6c). These subsets in PTI hosts further displayed a coordinated effector module characterized by the upregulation of *Stat1*, *Tbx21*, *Jak1*, *Zeb2*, and *Eomes* (Fig. 5i, j; Supplementary Fig. 6d-f). Collectively, these findings demonstrate that the PTI-mediated circuit culminates in a CD8⁺ Tem-intrinsic *Ifn* γ program that is essential for heterologous viral clearance, independent of Type I IFN and *Il*-15 signaling.

Discussion

This study shows that a prior *Plasmodium* infection establishes a durable state of heterologous resistance to respiratory viruses, extending *Plasmodium*-induced trained immunity beyond its previously described antibacterial effects¹⁸. PTI protected mice against both IAV and RSV months after parasite clearance. Protection required monocytes but could not be transferred by monocytes alone. Instead, PTI reshaped the lung immune landscape, expanding antiviral CD8 effector-memory T cells and accelerating their activation upon acute virus infection. Spatial and functional analyses revealed that virus-infected epithelium, inflammatory monocytes, and CD8⁺ Tem form a triad at infected niches, connected by a *Ccl4/Ccl5*–*Ccr5* axis. Within this triad, CD8⁺ Tem mediate rapid viral control through an *IFN* γ -dependent program that does not rely on type I IFN or *Il*-15 signaling in this context

These findings move trained immunity from a mainly myeloid-centered concept toward a cooperative circuit between innate and adaptive cells. Previous works have focused on monocytes/macrophages and hematopoietic progenitors as the main cell types of trained immunity, with enhanced cytokine production and microbial killing upon secondary challenge^{9,12–14}. Our previous work placed PTI in this framework by showing that hemozoin in the bone marrow drives a myelopoietic bias that generates trained monocytes protective against bacterial sepsis¹⁸. Other studies indicate that T cells can already participate at the induction stage of training³², and that *Ifn* γ -producing CD4⁺ T cells—either Cx3cr1⁺ effector cells or Th1 cells communicating with trained myeloid cells—are required for BCG-induced protection against respiratory viruses^{15,16}. The present data extend this concept to an endemic parasitic infection and show that the antiviral arm of PTI cannot be explained by monocyte-intrinsic

reprogramming alone. Instead, PTI organizes a circuit in which monocytes and a *Plasmodium*-primed CD8⁺ Tem compartment jointly mediate protection against the distinct respiratory viruses tested here. PTI thus aligns with recent BCG studies that place Ifn γ -producing T cells at the core of trained antiviral protection^{15,16}, while operating through a distinct cellular architecture that fits with the emerging idea of integrated organ immunity in the lung³³. Moreover, the early IFN γ production has been shown to mediate anti-virus infection via directly acting on epithelium cell via the acute Trm response, despite this effect was shown to be antigen-specific³⁴.

Mechanistically, PTI acts on both myeloid and T cell compartments. On the myeloid side, PTI increases inflammatory monocytes and interstitial macrophages in the lung upon viral infection, acting as sentinels sensing viral infection at epithelial surfaces. On the T cell side, PTI expands CD8⁺ Tem subsets enriched for interferon-stimulated and cytotoxic gene programs and shifts them further along an effector trajectory very early after infection. TCR sequencing indicates that PTI mobilizes pre-existing clonotypes rather than generating classical IAV-specific memory de novo, consistent with an innate-like Tem pool reminiscent of virtual memory and tissue-resident memory populations described in other settings^{24,31}. The persistence of protection under FTY720 treatment, which limits lymphocyte egress from lymphoid organs, supports the view that these *Plasmodium*-primed Tem are largely localized and rapidly activated in situ rather than being recruited de novo from the circulation.

The spatial analyses connect these changes at the tissue level. In trained lungs, monocytes and CD8⁺ T cells selectively co-accumulate at IAV-infected epithelial foci. Ligand–receptor analysis identifies Ccl4/Ccl5–Ccr5 as a dominant axis connecting inflammatory monocytes to Activated ISG⁺ and Cytotoxic Tem. Ccr5 has been implicated in the recruitment, positioning, and antiviral efficacy of effector CD8⁺ T cells in respiratory viral infections²⁸. Our data place Ccr5 at the heart of a trained immunity circuit as pharmacologic blockade of Ccr5 disrupts this axis and abolishes PTI-mediated protection, indicating that targeted chemokine–receptor wiring, exemplified here by Ccl4/Ccl5–Ccr5, is a critical element of how the triad is assembled in the lung.

On the effector side, our data point to a CD8⁺ Tem-intrinsic Ifn γ program as the key determinant of antiviral control. PTI lungs show broad induction of type I Ifns and Il-15, in line with an antiviral inflammatory milieu, but genetic loss of *Ifnar* or *Il-15* does not remove the protective effect, and Tem still proliferate in *Il-15*^{-/-} hosts. In contrast, disrupting IFN γ signaling completely abrogates PTI-mediated protection. Transcriptionally, Tem from trained mice upregulated *Ifng* together with *Stat1*, *Tbx21*, *Eomes*, and *Zeb2*^{30,35,36}, that fits with known regulators of IFN γ -high, terminally differentiated CD8⁺ T cells. These observations support a model in which PTI leaves behind an Ifn γ -centered Tem module that can be rapidly deployed at subsequent viral encounters, layered on top of canonical type I Ifn and Il-15 pathways.

The comparison with bystander activation is also informative. In acute hepatitis A, IL-15–driven, TCR-independent activation of memory CD8⁺ T cells leads to diffuse, innate-like cytotoxicity and hepatocellular injury³⁷. Related forms of bystander activation by memory or antigen-inexperienced CD8⁺ T cells have been linked to tissue damage in highly inflamed environments. In PTI, the response shares features of innate-like activation but is spatially constrained. Monocytes act as sentinels that sense viral infection in the epithelium, secrete chemokines to recruit Tem, and Tem deliver Ifn γ -dependent antiviral effector function in the immediate vicinity of infected cells. This triad configuration may allow the host to exploit the speed and potency of bystander-like CD8⁺ T cell activation while restricting effector function to virus-positive foci, thereby limiting collateral tissue damage.

The triad architecture has implications for how broad antiviral strategies might be designed. Work on BCG-induced integrated organ immunity shows that vaccines can imprint long-lived antiviral resistance by functionally coupling adaptive T cells to tissue myeloid and epithelial cells in the lung^{16,33}. Our findings indicate that a parasitic infection can reach a related end point via a different cellular module in which monocytes and CD8⁺ Tem are the main cooperating partners. In principle, vaccines, adjuvants, or other trained-immunity inducers could be tailored to reproduce key elements of this arrangement: (i) programming tissue myeloid cells toward chemokine-biased responses (for example, enhanced Ccl4/Ccl5 production), (ii) establishing or boosting an antiviral CD8 Tem pool in the lung with rapid, cytokine-responsive behavior, and (iii) reinforcing chemokine–receptor axes such as Ccl4/Ccl5–Ccr5 that bring monocytes and Tem together at infected epithelium. Such approaches would not replace classical neutralizing-antibody-based vaccines but could add an early, partially pathogen-agnostic layer of mucosal protection against respiratory viruses. At the same time, the link between bystander activation and immunopathology^{25–27}, underlines that any attempt to engineer such responses will need to balance speed and strength against the risk of tissue injury.

Several limitations point to future work. First, our experimental viruses were IAV and RSV; whether PTI similarly affects other respiratory viruses, including coronaviruses such as SARS-CoV-2, remains to be determined. Second, although adoptive transfer of Tem from PTI donors conferred protection whereas OVA-primed OT-I Tem did not, the antigen specificity and ontogeny of the PTI-dependent Tem pool remain incompletely defined: our data argue against classical IAV-specific memory and generic OVA-specific OT-I Tem as mediators, but whether these cells represent cross-reactive clones, microbiota-driven memory, or bona fide “virtual memory” requires further study. Third, we show that Ccr5 is required for Tem recruitment to infected niches, but the signals that drive Tem proliferation and full activation are less clear. Il-15 can promote Tem activation and proliferation, yet adoptively transferred Tem expanded to a similar degree in *Il15*^{-/-} and wild-type recipients, suggesting that additional cytokines or costimulatory pathways are involved and will need to be defined. Finally, our scRNA-seq and spatial analyses focus on an early time point; longer kinetic studies will be important to determine how long the triad is maintained and how it evolves during viral clearance and tissue repair.

In summary, PTI broadens the concept of trained immunity into the antiviral realm by organizing an integrated triad of infected epithelium, monocytes, and CD8⁺ Tem. Through Ccl4/Ccl5–Ccr5-dependent recruitment and an Ifn γ -driven effector program, this triad confers durable protection against distinct respiratory viruses in mice and outlines principles that could inform future trained-immunity-based strategies to strengthen antiviral defense at mucosal sites.

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Author contributions

S-C.C. conceived the study. J.Z., C.C., and S-C.C. designed the methodology. J.Z., C.C., Y.W., W.L., M.T. (Mingyu Tang), Y.Z., Q.G., M.L., Z.L., Y.C. (Yu Cheng), and M.T. (Mengsha Tong) performed the experiments. J.Z., C.C., Y.W., W.L., M.T. (Mingyu Tang), Y.Z., Q.G., M.L., Z.L.,

Y.C. (Yu Cheng), K.M., Y.C. (Yixin Chen), Z.Z., and S-C.C. analyzed the data. J.Z., C.C., and Y.W. wrote the initial draft. All authors reviewed, edited, and approved the final manuscript.

Conflict of Interest

The authors declare no potential conflicts of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors utilized ChatGPT to assist in the refinement of the manuscript's language and to enhance the clarity of the research findings. The use of the tool was limited to linguistic editing and stylistic optimization. All scientific interpretations, data analysis, and the final narrative logic were produced by the human authors, who remain fully responsible for the accuracy and integrity of the work.

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Materials and Methods

Mice

Six- to eight-week-old C57BL/6 mice were directly purchased from Xiamen University Laboratory Animal Center. *Il15^{-/-}* (T011657) mice were purchased from GemPharmatech Co., Ltd. CD45.1 and CD8 KO mice were kindly gifts from professor W.-H. Liu (Xiamen University). *Rag1^{-/-}*, *Ifngr^{-/-}* and *Ifnar^{-/-}* mice were generously provided by professor K. Mao (Xiamen University), and *Ccr2^{-/-}* mice were obtained from professor Y. Liang (Xinxiang Medical University). All strains were maintained under specific pathogen-free conditions at the Xiamen University Laboratory Animal Center, with ad libitum access to water and a standard diet (65% carbohydrate, 11% fat, 24% protein), at 21–24 °C, 40–70% relative humidity, and a 12-h light–dark cycle. Male and female mice, sex- and age-matched, were randomly assigned to experimental groups. All procedures were conducted in accordance with protocols approved by the Xiamen University Institutional Animal Care and Use Committee.

Plasmodium Infection

The nonlethal *Plasmodium yoelii* 17XNL (*Py*) strain was kindly provided by professor Jing Yuan (Xiamen University). To generate viable *Py*-infected red blood cells (iRBCs) for experimental inoculation, frozen stocks with >1% parasitemia were passaged through wild-type C57BL/6 mice. Briefly, thawed stocks were diluted to 5×10^5 iRBCs per mL, and 200 μ L was administered intraperitoneally. Parasitemia was monitored daily by Giemsa-stained thin blood smears from day 10 post-inoculation. When parasitemia exceeded 1%, whole blood was collected, diluted in PBS to 5×10^5 iRBCs per mL and stored at -80 °C until use. For experimental infections, 200 μ L of the prepared inoculum was administered intraperitoneally into recipient mice.

Respiratory viral infections

Influenza A virus (IAV, A/Puerto Rico/8/1934 [H1N1], PR8) was generously provided by Y. Chen (Xiamen University). Mice were intranasally inoculated with 40 μ L of IAV (1×10^5 PFU), and body weight and survival were monitored daily. For respiratory syncytial virus (RSV) challenge, mice were intranasally inoculated with 1×10^6 PFU. Five days post-infection, mice were euthanized, and the lung and nasal tissues were collected for viral load quantification and pathological assessment.

Monoclonal antibody treatment

Mice were intraperitoneally injected with 150 μ g per mouse of anti-CD8 (Bioxcell, clone 2.43), anti-Ly6G (Bioxcell, clone 1A8), or anti-GR-1 (Bioxcell, clone RB6-8C5) every 48 hours for 10 days. Control mice received corresponding isotype antibodies (Bioxcell, rat IgG2a or rat IgG2b) on the same schedule.

Isolation of cells from BALF and lung tissues

Mice were anesthetized with sodium pentobarbital (50 mg per kg, intraperitoneally) and perfused with PBS. Bronchoalveolar lavage fluid (BALF) was collected by inserting a catheter into the trachea, securing it with suture, and flushing the lungs five times with 1 mL of ice-cold sterile PBS. Lung lobes were subsequently excised, minced, and digested in RPMI-1640 medium containing 2% FBS, 5 mg collagenase type IV (Worthington, Cat. No. LS004189) and 0.5 mg DNase I (Sangon Biotech, Cat. No. A510099) at 37 °C for 20 min with agitation at 130 rpm. Digested tissue was passed through a 70-µm strainer to generate single-cell suspensions. Cells from both BALF and lung suspensions were pelleted, lysed with 1× ACK buffer, washed with PBS, and resuspended in FACS buffer (PBS, 2% FBS, 0.5 mM EDTA·2Na) for flow cytometry analyses.

Flow cytometry

Single-cell suspensions from BALF and lungs (3×10^6) were plated in U-bottom 96-well plates in FACS buffer. Cells were incubated with anti-CD16/32 antibody (BioLegend, Cat. No. 101320; clone 93; RRID: AB_1574975) to block Fc receptor–mediated nonspecific binding for 20 min at 4 °C, then washed twice with FACS buffer.

For immune cell staining, cells were incubated simultaneously with eFluor 780 viability dye (eBioscience, Cat. No. 65-0865-14) and the following antibody panel for 30 min at 4 °C: anti-CD45 BV711 (Biolegend, Cat. No. 103147; clone 30-F11; PRID: AB_2564383), anti-CD11b eFluor™ 450 (eBioscience, Cat. No. 48-0112-82; clone M1/70; PRID: AB_1582236), anti-Ly6G PE (eBioscience, Cat. No. 12-9668-82; clone 1A8-Ly6g; PRID: AB_2572720), anti-Ly6C PE-Dazzle594 (Biolegend, Cat. No. 128044; clone HK1.4; PRID: AB_2566577), anti-Siglec-F AF647 (BD Biosciences, Cat. No. 562680; clone E50-2440; RRID: AB_2687570), anti-CD11c BV510 (Biolegend, Cat. No. 117337; clone N418; PRID: AB_2562010), anti-MHC Class II PE-Cy5 (eBioscience, Cat. No. 15-5321-82; clone M5/114.15.2; PRID: AB_468800), anti-CD8a FITC (eBioscience, Cat. No. 11-0081-82; clone 53-6.7; PRID: AB_464915), anti-CD4 BV605 (Biolegend, Cat. No. 100451; clone GK1.5; PRID: AB_2564591), anti-NK1.1 PE-Cy7 (eBioscience, Cat. No. 25-5941-82; clone PK136; PRID: AB_469665). Cells were subsequently washed twice with FACS buffer.

For T cell function staining, cells were incubated simultaneously with eFluor 780 viability dye (eBioscience, Cat. No. 65-0865-14) and the following antibody panel for 30 min at 4 °C: anti-CD45 BV711 (Biolegend, Cat. No. 103147; clone 30-F11; PRID: AB_2564383), anti-CD8a FITC (eBioscience, Cat. No. 11-0081-82; clone 53-6.7; PRID: AB_464915), anti-CD4 BV605 (Biolegend, Cat. No. 100451; clone GK1.5; PRID: AB_2564591), anti-CD44 PE (Biolegend, Cat. No. 103008; clone IM7; PRID: AB_312959), anti-CD62L APC (eBioscience, Cat. No. 17-0621-82; clone MEL-14; PRID: AB_469410), anti-CX3CR1 AF647 (BD Biosciences, Cat. No. 567805; clone Z8-50; RRID: AB_2916740). Cells were subsequently washed twice with FACS buffer.

Flow cytometry was performed using SONY ID7000. Analysis was performed using FlowJo version 10.10.0.

Adoptive transfer CD8⁺ effector memory T cells (CD8⁺ Tem)

Spleen and peripheral lymph nodes from *Py*-TI mice were processed into single-cell suspensions, followed by centrifugation and red blood cell lysis with $1\times$ ACK buffer. Cell pellets were incubated with a biotin-conjugated antibody cocktail (anti-CD11b, BioLegend, Cat. No. 101204, clone M1/70, PRID: AB_312787; anti-CD4, BioLegend, Cat. No. 100404, clone GK1.5, PRID: AB_312689; anti-CD19, BioLegend, Cat. No. 115504, clone 6D5, PRID: AB_313639) for 20 min at 4 °C with gentle mixing. Streptavidin-coated magnetic beads (MedChemExpress, Cat. No. HY-K0208) were subsequently added and incubated for 10 min at 4 °C, and bead-bound cells were depleted by magnetic separation. The unbound fraction was stained with anti-CD8–BV421 (Biolegend, Cat. No. 100737; clone 53-6.7; PRID: AB_10897101), anti-CD44–PE, and anti-CD62L–APC for 30 min at 4 °C and sorted on a BD Aria III cell sorter. CD8⁺ effector memory T cells (CD8⁺CD44⁺CD62L⁻) were collected, washed twice in PBS, and resuspended at 1×10^6 cells/mL. A total of 2×10^5 cells in 200 μ L were transferred intravenously into recipient mice, which were simultaneously inoculated with IAV. Body weight and survival were monitored thereafter.

Adoptive transfer monocytes

Bone marrow cells were isolated from the femurs of mice treated with PBS or *Py*-TI and subjected to red blood cell lysis using ACK buffer. The resulting cell pellets were incubated with a biotin-conjugated antibody cocktail (anti-CD19, anti-Ly6G [BioLegend, Cat. No. 127604, clone 1A8, RRID: AB_1089180], anti-CD3 [BioLegend, Cat. No. 100303, clone 145-2C11, RRID: AB_312669], anti-NK1.1 [BioLegend, Cat. No. 108704, clone PK136, RRID: AB_313391]) for 20 min at 4 °C with gentle mixing. Streptavidin-coated magnetic beads were added and incubated for 10 min at 4 °C, followed by magnetic depletion of bead-bound cells. The unbound fraction was stained with anti-CD45–PerCP-Cy5.5 (eBioscience, Cat. No. 45-0451-82, clone 30-F11, RRID: AB_1107002), anti-CD11b–FITC (eBioscience, Cat. No. 11-0112-82, clone M1/70, RRID: AB_464935), and anti-Ly6C–BV421 (eBioscience, Cat. No. 48-5932-82, clone HK1.4, RRID: AB_10805519) for 30 min at 4 °C. Monocytes (CD45⁺CD11b⁺Ly6C^{high}) were sorted on a BD FACS Aria III cell sorter. A total of 2×10^5 sorted cells in 200 μ L were transferred intravenously into recipient mice concurrently inoculated with IAV, and body weight and survival were monitored thereafter.

In Vitro and In Vivo proliferation of CD8⁺ effector memory T cells

Single-cell suspensions were prepared from spleens and peripheral lymph nodes of CD45.1⁺ *Py*-TI mice. Following ACK-mediated erythrocyte lysis, CD11b⁺, CD4⁺, and CD19⁺ cells were removed by negative selection using biotin-conjugated antibodies and streptavidin-coated magnetic beads. The remaining cells were resuspended at 1×10^7 cells/mL, labeled with 1 μ M CFSE for 10 min at 37 °C, and washed three times with complete medium. Cells were then stained with anti-CD45.1-PE (eBioscience, Cat. No. 12-0453-82, clone A20, PRID: AB_465675), anti-CD8-BV421, anti-CD44-PE-Cy7 (Biolegend, Cat. No. 103030; clone IM7; PRID: AB_830787), and anti-CD62L-APC antibodies, and CD8⁺ effector memory T cells (CD8⁺CD44⁺CD62L⁻) were sorted on a BD Aria Fusion cell sorter. Purified CFSE-labeled CD8⁺ Tem was washed and resuspended at 1×10^6 cells/mL.

For in vivo proliferation assays, 2×10^5 CFSE-labeled CD8⁺ Tem in 200 μ L PBS were intravenously transferred into CD45.2 wild-type or *Il15*^{-/-} recipient mice at the time of IAV infection. Donor cell expansion was assessed in the lungs on days 2, 4 and 6 post-inoculations by flow cytometric analysis of CFSE dilution.

For in vitro proliferation assays, 1.5×10^5 CFSE-labeled CD8⁺ Tem was cultured in 200 μ L complete RPMI-1640 medium with or without recombinant IL-15 (Pepro Tech, Cat. No. 210-15, 100 ng/mL) in V-bottom 96-well plates. Cells were harvested on days 3 and 6, and proliferative activity was quantified by CFSE dilution.

DynaSpatial library construction and sequencing

The DynaSpatial Spatial Gene Expression Slides and Reagent Kits (Dynamic Biosystems Inc.; Cat. No. 23043504, 23044704) were used for this study. Following the manufacturer's protocol, tissue sections were applied to the gene expression slide to generate spatially barcoded cDNA. Each slide contains a barcoded DNA array with 2,500 spots within a 5×5 mm capture area. Each spot is functionalized with a unique barcoded oligonucleotide and poly(dT)VN. The spot diameter is 50 μ m, with a center-to-center spacing of 100 μ m between adjacent spots. A fiducial frame is printed around the capture area to assist with spatial orientation during imaging and analysis.

Polyadenylated mRNAs released from the overlying cells were captured by the poly(dT)VN on the array spots. Subsequently, reverse transcription was performed in situ to synthesize spatially barcoded cDNA. The cDNA was then denatured, eluted from the slide into a collection tube, and amplified by PCR to generate sufficient material for library preparation. Sequencing libraries were constructed from the amplified cDNA according to the DynaSpatial library preparation protocol (Dynamic Biosystems, Suzhou, China). Briefly, the cDNA amplicons were fragmented enzymatically and size-selected to optimize the insert distribution. The fragments then underwent end repair, A-tailing, and adapter ligation to append the P5, P7, and unique i7 sample index sequences. A final PCR amplification was performed to generate the finished libraries, which contain the complete P5 and P7 primer sites for cluster generation. The constructed libraries were sequenced on an Illumina NovaSeq platform using a 150 bp paired-end configuration.

Spatial transcriptomics procession

In the spatial transcriptomics data processing, we used the Dynamic ST to process the raw sequencing fastq files and the corresponding H&E-stained image. The raw sequencing reads were aligned to the Influenza A virus (A/Puerto Rico/8/1934(H1N1) reference genome (NCBI Taxonomy ID: 211044) and the mouse reference genome GRCm39 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.20/), respectively, to distinguish host and pathogen transcripts. Quality control was performed using the tool's default parameters. This processing pipeline ultimately generated two gene expression matrices for the host and H1N1 for downstream analysis.

Spatially Informed Cell Type Deconvolution with CARD

Given that DynaSpatial spots capturing a heterogeneous mixture of multiple cell types, we conducted cell type deconvolution analysis using the standard pipeline supplied by R package CARD (<https://github.com/YingMa0107/CARD>). We used the previously-constructed and annotated single-cell RNA sequencing dataset as a reference, and then extracted the gene expression matrix and metadata with cell type labels. For spatial data, we input the gene expression matrix and spatial coordinate information of each spot. The conditional autoregressive-based deconvolution (CARD) model was initialized via the create CARD Object function with parameters min Count Gene = 0 and min Count Spot = 0 (retaining all genes/spots). Cell type proportions per spot were estimated through the CARD deconvolution function based on a non-negative matrix factorization model and a conditional autoregressive model.

Quantitative colocalization analysis of CD8⁺ T cells and monocytes in H1N1-infected regions using MISTy

We employed MISTy (Multiview Intercellular SpaTial modeling framework) to investigate co-localization patterns between CD8⁺ T cells and monocytes within or without H1N1-infected regions. MISTy (<https://saezlab.github.io/mistyR>) was designed to test whether the abundance of a certain cell type can be predicted by the spatial context characterized by the composition of neighboring cell types. H1N1-infected regions were defined as spots containing >300 H1N1 transcripts. Within these regions, MISTy models multi-view spatial relationships through two primary perspectives: an intrinsic view, capturing cell type compositions within each spot, and a juxta view, modeling second-order nearest-neighbor interactions. Cell type abundance features derived from deconvoluted spatial data were incorporated into both views. The analysis yielded the dependence of the spatial distribution of each cell type, quantifying the extent to which the abundance of monocytes can be predicted based on CD8⁺ T cell spatial context across multiple spatial scales.

Single-cell RNA seq(scRNA-seq)

CD45⁺ immune cells from the lungs of mice on the second day of IAV infection were sorted by BD Arial III for scRNA-seq, samples from three mice were pooled together and analyzed per condition. These cells were loaded on a 10X Genomics GemCode Single-cell instrument to generate single-cell Gel Beads-In-EMulsion (GEMs). Libraries were generated and sequenced from the cDNAs with Chromium Next GEM Single Cell 3' Reagent Kits v3.1 according to manufacturer's instructions.

scRNA-seq data processing and analysis

Raw sequencing data was processed and aligned mm10 mouse reference genome with CellRanger (10X Genomics) v 7.2.0. Downstream analysis was applied with the Seurat package (v5.3.0). Quality control was performed to create Seurat object with min features > 200 and removal of cells having < 200 or > 8000 expressed genes or > 5% mitochondrial counts. The raw gene expression matrix after quality control was cell-normalized over total expression. Then FindVariableFeatures (using RNA and vst as an assay and selection method as parameters) and normalization (method = "LogNormalize", scale factor = 10000) were

performed. After variable features using RunPCA, first 20 PCs were chosen to RunUMAP and FindNeighbors. The clusters were performed using resolution = 0.5 as parameters for further analysis and visualization. Pseudotime analysis were based on the Mono/Marco subset data using the monocle3.

Cell types were annotated using FindAllMarkers based on the expression of canonical signature genes: B cells (*Ms4a1*, *Cd19*, *Cd79a*, *Cd79b*); Neutrophils (*S100a8*, *S100a9*, *Ly6g*, *Retnlg*, *Ngp*); Alveolar macrophages (*Siglecf*, *Car4*, *Itgax*, *Chil3*, *Ear2*); Monocytes (*Fcgr4*, *Ccr2*, *Fcgr1*, *Lyz2*, *Ly6c2*, *Cx3cr1*); Interstitial Macrophages (*Itgam*, *Cd68*, *Adgre1*, *Cd80*, *Ccr5*, *Tnf*, *Il6*); Eosinophils (*Cd274*, *Cd24a*, *Cxcl2*, *Acod1*, *Il1rn*); CD4⁺ T cells (*Cd3e*, *Cd4*); CD8⁺ T cells (*Cd3e*, *Cd8a*); NK cells (*Klrb1c*, *Nkg7*, *Cd247*, *Cd160*); Basophils (*Fcer1a*, *Mcpt8*, *Ccl3*, *Cd200r3*); and Dendritic cells (*Itgax*, *Clec9a*, *Zbtb46*, *Siglech*, *Tcf4*).

To resolve the functional states of CD8⁺ T cells, the subset function in Seurat was used to isolate CD8⁺ clusters. These cells underwent a secondary round of scaling, PCA, and UMAP reduction. Five distinct CD8⁺ T cell subsets (Naive/Tcm, Transitional memory, Activated_ISG⁺ Tem, Cytotoxic Tem, and Cycling Tem) were annotated based on the specific expression patterns of memory (*Sell*, *Ccr7*, *Il7r*, *Tcf7*), activation (*Cd44*, *Klrl1*), interferon-response (*Isg15*, *Stat1*), cytotoxic (*Prfl*, *Gzma*, *Nkg7*), and proliferation (*Mki67*) genes.

To evaluate the transcriptional programs of CD8⁺ T cell subsets, the AddModuleScore function in Seurat was utilized to calculate specific pathway scores (Naive/Tcm, Effector, Interferon, Cytotoxic, and Proliferation) based on curated gene sets. For functional enrichment, DEGs upregulated in specific clusters were identified using FindMarkers (logfc.threshold = 0.25, min.pct = 0.25). Biological processes were analyzed using the clusterProfiler R package, and the top enriched GO terms were visualized using dot plots.

Pseudotime trajectory analysis of CD8⁺ T cell subsets was performed using the R package Monocle3. The Seurat object was converted into a CellDataSet (CDS). Following preprocessing and UMAP reduction within Monocle3, cells were clustered and the principal graph was constructed using the learn_graph function. The CD8⁺ Naive/Tcm cluster was designated as the root node to calculate pseudotime values, illustrating the developmental progression toward effector and cytotoxic states.

To infer the intercellular communication networks between monocytes and CD8⁺ T cell subsets, the R package CellChat (v2) was employed. The normalized scRNA-seq expression matrices from PBS and PTI groups were input into CellChat. Over-expressed genes and interactions were identified using the CellChatDB mouse reference. Communication probabilities were computed, and the signaling pathways were analyzed for information flow. The netVisual_bubble function was used to visualize the interaction strengths of specific ligand-receptor pairs sent from monocytes to CD8⁺ Tem receivers.

Drug administration

A CCR5 inhibitor maraviroc (MedChemExpress, HY-13004) or sphingosine 1-phosphate (S1P) antagonist Fingolimod (FTY720) hydrochloride (MedChemExpress, HY-12005) were dissolved in 10% DMSO + 40% PEG300 + 5% Tween-80 + 45% saline. Mice received daily

intraperitoneal injections of maraviroc (50 mg/kg) or FTY720 (1 mg/kg) in PBS- or *Py-TI*-treated groups.

PR8 viral RNA quantification

PR8 viral RNA levels in the lungs of challenged mice were quantified by quantitative RT-PCR. At the indicated time points after infection, lungs were harvested and homogenized in TRIzol reagent for total RNA extraction according to the manufacturer's instructions. RNA was reverse transcribed using random primers to generate cDNA. qRT-PCR was performed using primers targeting the influenza A virus *M* gene, and viral RNA levels were normalized to *Actb*. Relative expression was calculated using the $2^{-\Delta C_t}$ method. Primer sequences were as follows: *M* forward, 5'-CATGGAATGGCTAAAGACAAGACC-3'; *M* reverse, 5'-CCATTAAGGGCATTTTGGACA-3'; *Actb* forward, 5'-GTGACGTTGACATCCGTAAAGA-3'; and *Actb* reverse, 5'-GCCGGACTCATCGTACTCC-3'.

H&E staining

Tissue samples were collected and fixed in 4% paraformaldehyde for 24 h. Subsequently, tissues were dehydrated and cleared using a Leica TP1020 automated processor and embedded in paraffin. Finally, 5- μ m-thick sections were prepared and stained with hematoxylin and eosin (H&E).

Immunofluorescence Staining and Confocal Imaging

Lung tissues were fixed with BD Cytofix/Cytoperm solution (BD Biosciences, Cat# 554722; diluted 1:2 in PBS) overnight at 4°C, dehydrated in 30% sucrose for 16–20 h, and embedded in OCT compound (Sakura Finetek, Cat# 4583). Cryosections (25 μ m) were prepared using a CM1905 cryostat (Leica) and mounted on positively charged slides (Premiere). Sections were rehydrated with PBS and blocked with a solution containing 1% normal serum (mouse, rat, and rabbit), 1% bovine serum albumin (BSA), and 0.2% Triton X-100 in PBS. Subsequently, sections were incubated directly with a mixture of fluorophore-conjugated primary antibodies, including BV480 rat anti-mouse CD8a (clone ALY7, BD Biosciences, Cat# 566096), GFP Polyclonal Antibody, Alexa Fluor 555 (Invitrogen, Cat# A-31851), and Influenza A NP Monoclonal Antibody (clone D67J, FITC, Invitrogen, Cat# MA1-7322), for 24 h at room temperature in a dark humidified chamber. The sections were then stained with Hoechst 33342 solution for 10 min at room temperature, followed by five 10-min washes with PBS, coverslipped with Fluoromount-G (SouthernBiotech, Cat# 0100-01), and imaged using a Leica TCS SP8 confocal microscope. Three-dimensional reconstructions and image analysis were performed using Imaris software (Bitplane).

Immunofluorescence quantification

For immunofluorescence quantification, lung sections were classified into IAV-negative (IAV⁻) and IAV-positive (IAV⁺) areas based on influenza A virus nucleoprotein (NP) staining, which was used as a marker of viral infiltration. For each region type, six to ten randomly selected fields were acquired from different anatomical locations of each lung section.

Peritracheal regions with dense immune cell aggregation were excluded to avoid anatomical bias.

For each field of view, a grid-based approach was applied by dividing the image into 16 equal subregions to enable systematic and unbiased quantification and to minimize edge-related counting bias. CD8⁺ and CCR2⁺ cells were counted across all subregions and summed to obtain total cell numbers per field. Cell numbers were normalized to the imaged tissue area based on microscope calibration and expressed as cells per cm². For each mouse, mean cell densities were calculated separately for IAV⁺ and IAV⁻ regions.

TCRβ repertoire sequencing library construction

Lung tissues and peripheral blood were collected from PTI- and control-treated mice. Lung tissues were processed to obtain single-cell suspensions as described above. Peripheral blood was subjected to red blood cell lysis using ACK buffer. Single-cell suspensions from both tissues were stained with eFluor 780 viability dye (eBioscience, Cat. No. 65-0865-14), anti-CD45-PerCP-Cy5.5 (eBioscience, Cat. No. 45-0451-82, clone 30-F11, PRID: AB_1107002), anti-CD11b-PE-Cy7 (eBioscience, Cat. No. 25-0112-82, clone M1/70, PRID: AB_469588), anti-CD3-AF488 (Biolegend, Cat. No. 100210, clone 17A2, PRID: AB_389301), anti-CD44 PE (Biolegend, Cat. No. 103008; clone IM7; PRID: AB_312959), anti-CD62L APC (eBioscience, Cat. No. 17-0621-82; clone MEL-14; PRID: AB_469410), and anti-CD8-BV421 (Biolegend, Cat. No. 100737; clone 53-6.7; PRID: AB_10897101). CD8⁺ effector memory T cells (CD44⁺CD62L⁻) were sorted using a BD Aria III cell sorter, and 5 × 10³ cells per sample were collected into 96-well plates.

Sorted cells were centrifuged at 800 × g for 5 min at 4 °C and resuspended in RNase-free storage buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5 U/μL RNase inhibitor). Cells were subjected to direct lysis followed by reverse transcription using a template-switching strategy. First-strand cDNA was generated using a 2× Reverse Transcription reaction system, and cDNA quality was assessed by qRT-PCR for *Cd8a* expression.

Second-strand cDNA amplification was performed using template-switching oligo (TSO)-mediated PCR for 30 cycles. After each amplification step, products were purified using VAHTS DNA Clean Beads to remove residual primers and short fragments.

TCRβ libraries were generated by multiplex PCR using pooled TRBV family-specific primers and a TRBC reverse primer for 18 cycles. Amplified products were size-selected (300–400 bp) by agarose gel electrophoresis and purified using a gel extraction kit.

Purified libraries were quantified using Qubit and subjected to a second PCR to add Illumina sequencing adapters and sample-specific indices. Final libraries were purified using magnetic beads and sequenced on an Illumina NovaSeq platform (paired-end 250 bp) with a depth of approximately 1 × 10⁶ reads per sample.

TCRβ repertoire analysis

Raw paired-end reads from TCR β sequencing were processed using MiXCR (v3.0.13; -s mmu) for germline V(D)J alignment, clonotype assembly, and error correction. Downstream analyses were performed in R (v4.2.2) using the immunarch (v0.9.0) and vegan (v2.6-4) packages.

TCR diversity was assessed using Chao1 richness, Shannon entropy, and Gini–Simpson indices. Clonal expansion was evaluated by quantifying the relative frequency of dominant clonotypes. TRBV and TRBJ gene usage and CDR3 β length distributions were analyzed based on productive rearrangements.

To assess potential disease relevance, CDR3 β amino acid sequences were queried against the McPAS-TCR database using exact sequence matching, and the frequency of matched pathology-associated clonotypes was quantified.

Statistical analysis

Data are presented as mean $\pm$ SD. Comparisons between two groups were performed using unpaired two-tailed Student's t-test. Weight loss curves were analyzed by two-way ANOVA with Bonferroni multiple-comparison test. Survival curves were analyzed by the Kaplan–Meier method and compared using the log-rank (Mantel–Cox) test. Statistical significance is indicated as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

Figure Legends

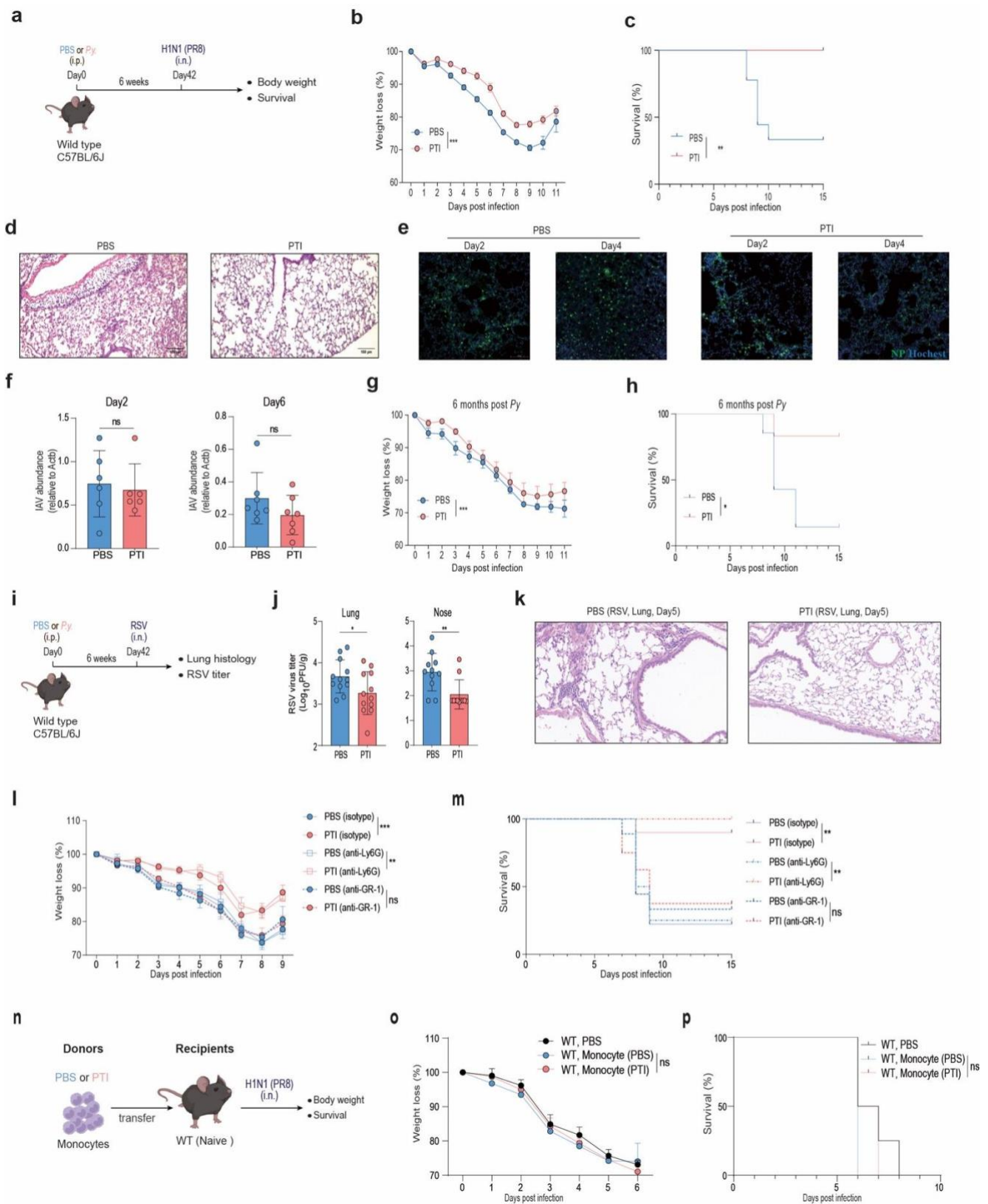


Fig. 1 PTI provides cross-protection against respiratory virus challenge. **a**, Experimental schema of *Plasmodium* infection followed by intranasal inoculation with IAV. **b**, **c**, Weight

loss (**b**) and survival (**c**) of mice challenged with PR8 (1×10^5 PFUs; $n \geq 14$ per group). **d**, Representative hematoxylin and eosin (H&E)–stained lung sections from PBS- or PTI–treated mice at day 4 post-IAV infection ($n = 5$ per group). **e**, Representative fluorescence images of IAV nucleoprotein (NP) in lung sections collected at day 2 and day 4 after inoculation in PBS- or PTI–treated mice ($n = 5$ per group). Scale bar, 100 μm . **f**, IAV abundance was measured at day 2 and day 6 post IAV challenge ($n \geq 6$ per group). **g**, **h**, Mice infected with *Plasmodium* and rest for 6 months were challenged with IAV, and weight loss (**g**) and survival (**h**) were monitored ($n > 10$ per group). **i**, Schematic of *Plasmodium* infection followed by intranasal inoculation with RSV. **j**, Viral load in lung and nasal tissues at day 5 post-RSV infection ($n > 10$ per group). **k**, Representative hematoxylin and eosin (H&E)–stained lung sections from PBS- or PTI–treated mice at day 5 post-RSV infection ($n > 10$ per group). **l**, **m**, Weight loss (**l**) and survival (**m**) were assessed in IAV-infected mice following administration of Ly6G- or GR-1–depleting antibodies ($n \geq 8$ per group). **n**, Schematic of adoptive transfer of monocytes isolated from PBS- or PTI-treated mice into naïve wild-type recipients. **o**, **p**, Weight loss (**o**) and survival (**p**) of recipient mice following IAV challenge ($n = 8$ per group).

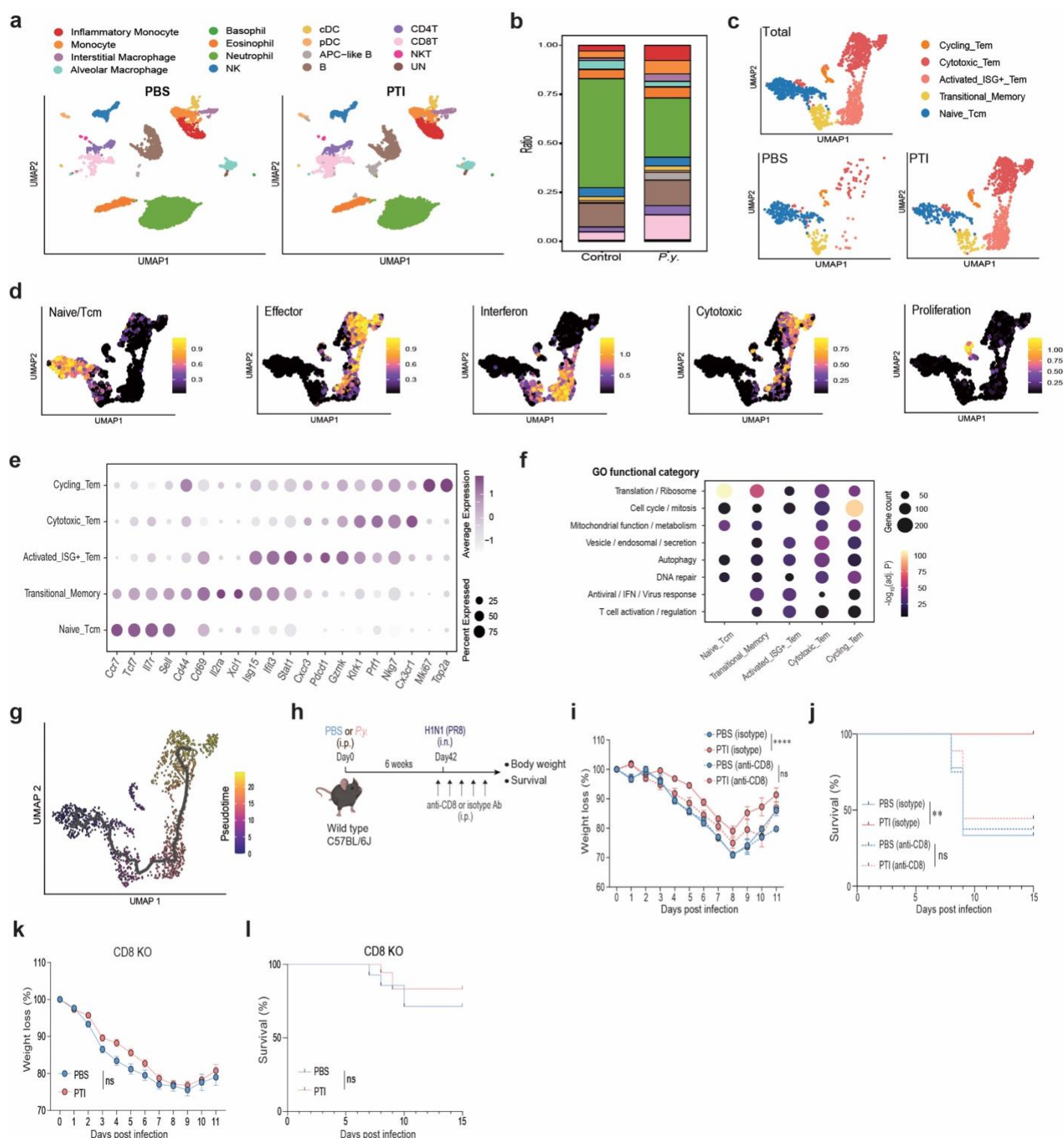


Fig. 2 CD8 T cells are indispensable for PTI-provided antiviral protection. **a**, UMAP visualization of scRNA-seq profiles of CD45⁺ cells isolated from lung tissue of PBS- and PTI-treated mice at day 2 after IAV challenge. **b**, Stacked bar plots indicating the relative abundance of the annotated immune cell lineages in the lung microenvironment of PBS and PTI groups. **c**, UMAP visualization of the reclustered CD8⁺ T cell population, displaying the five identified subsets across all cells (Total) and separated by treatment (PBS vs. PTI). **d**, UMAP feature plots displaying module scores for naive/central memory, effector, interferon

response, cytotoxic, and proliferation transcriptional programs across the CD8⁺ T cell subsets. **e**, Dot plot showing the expression of key defining genes across the five CD8⁺ T cell subsets. Node size indicates the percentage of cells expressing the gene, and color intensity represents the average scaled expression. **f**, GO functional enrichment analysis of biological processes upregulated in the specified CD8⁺ T cell subsets. **g**, Pseudotime trajectory analysis constructed using Monocle, illustrating the developmental progression of CD8⁺ T cell subsets from a naive/Tcm root toward effector memory states. **h**, The mouse model of CD8⁺ T cells depletion. **i, j**, PBS- and PTI-treated mice depleted of CD8⁺ T cells were challenged with IAV, weight loss (**i**) and survival (**j**) were monitored (n≥9 per group). **k, l**, Weight loss (**k**) and survival (**l**) were monitored in CD8 knock-out mice with or without prior *Plasmodium* infection following IAV challenge (n≥14 per group).

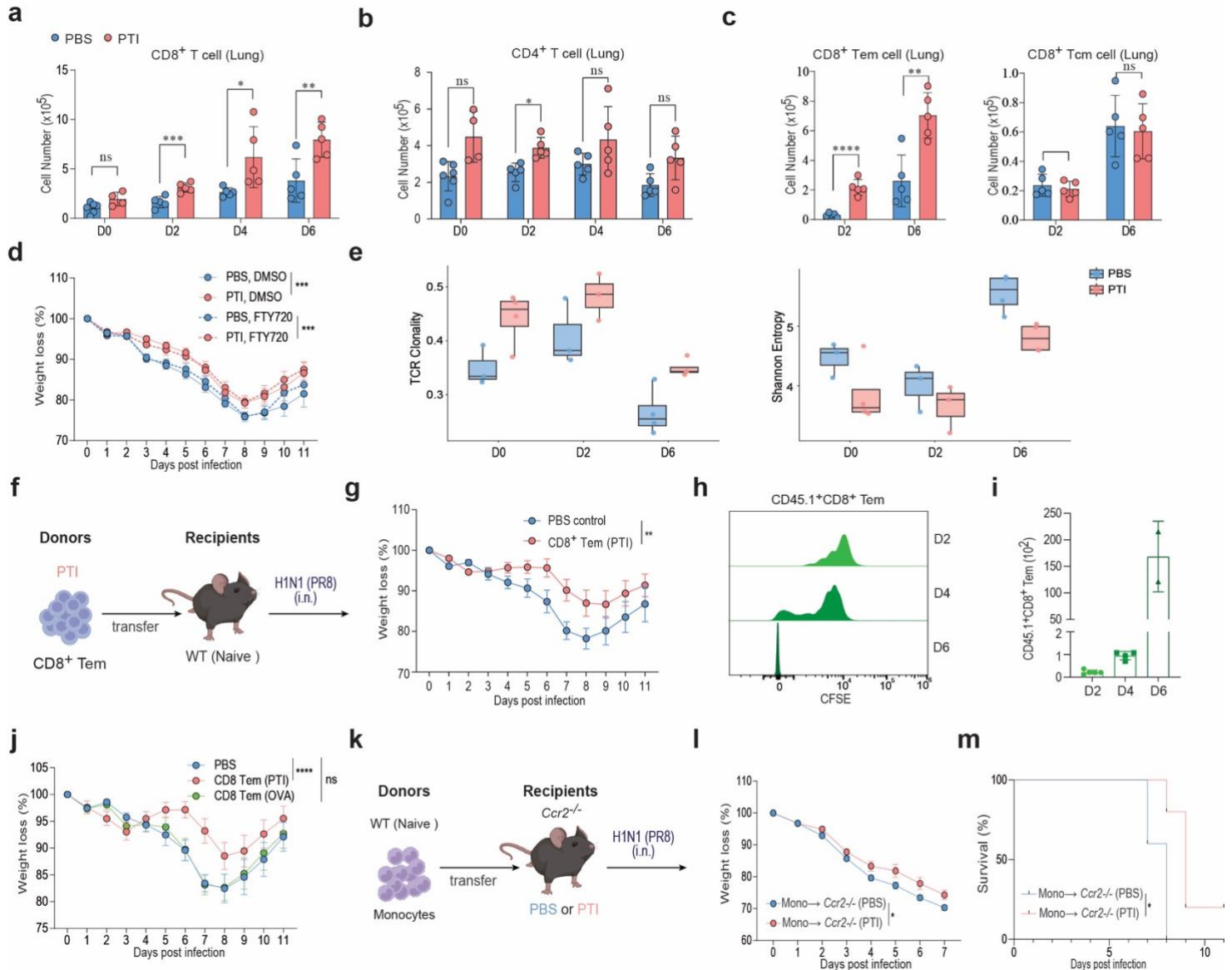


Fig. 3 Adoptive transfer of CD8 Tem from PTI mice confers antiviral protection. **a, b**, Flow cytometry analysis of lung CD8⁺ (**a**) and CD4⁺ (**b**) T cells from PBS- and PTI-treated wild-type mice at indicated time points post-IAV challenge. Absolute cell numbers were presented (n ≥ 4 per group). **c**, Absolute cell numbers of lung CD8⁺ Tem and CD8⁺ Tcm from PBS- and PTI-treated mice at day 2 and day 6 post-IAV challenge were presented (n = 5 per group). **d**, Weight loss of PBS- and PTI-treated mice with or without FTY720 administration following IAV challenge (n > 15 per group). **e**, Boxplots displaying TCR clonality (left) and Shannon entropy (right) of the CD8⁺ Tem pool in the lungs of PBS and PTI mice at day 2 and day 6 post-infection. **f**, Experimental schema of adoptive transfer of CD8⁺ Tem isolated from PTI mice into naïve wild-type recipients. **g**, Weight loss of PBS- and CD8⁺ Tem-recipient mice following IAV challenge (n > 10 per group). **h, i**, CFSE-labeled CD45.1⁺CD8⁺ Tem was adoptively transferred into naïve wildtype mice and analyzed at indicated time points post-IAV challenge. Proliferation was assessed by CFSE dilution using flow cytometry (**h**), and the absolute cell number of the expanded CD45.1⁺CD8⁺ Tem was determined (**i**) (n ≥ 2 per group). **j**, Weight loss of recipient mice after transfer of CD8⁺ Tem from PTI- or OVA-primed mice following IAV challenge (n ≥ 6 per group). **k**, Experimental schema of monocyte

adoptive transfer from naïve wild-type donors into PBS and PTI-treated *Ccr2*^{-/-} recipients. **l**, **m**, Weight loss (**l**) and survival (**m**) of *Ccr2*^{-/-} recipient mice following IAV challenge (n≥8 per group).

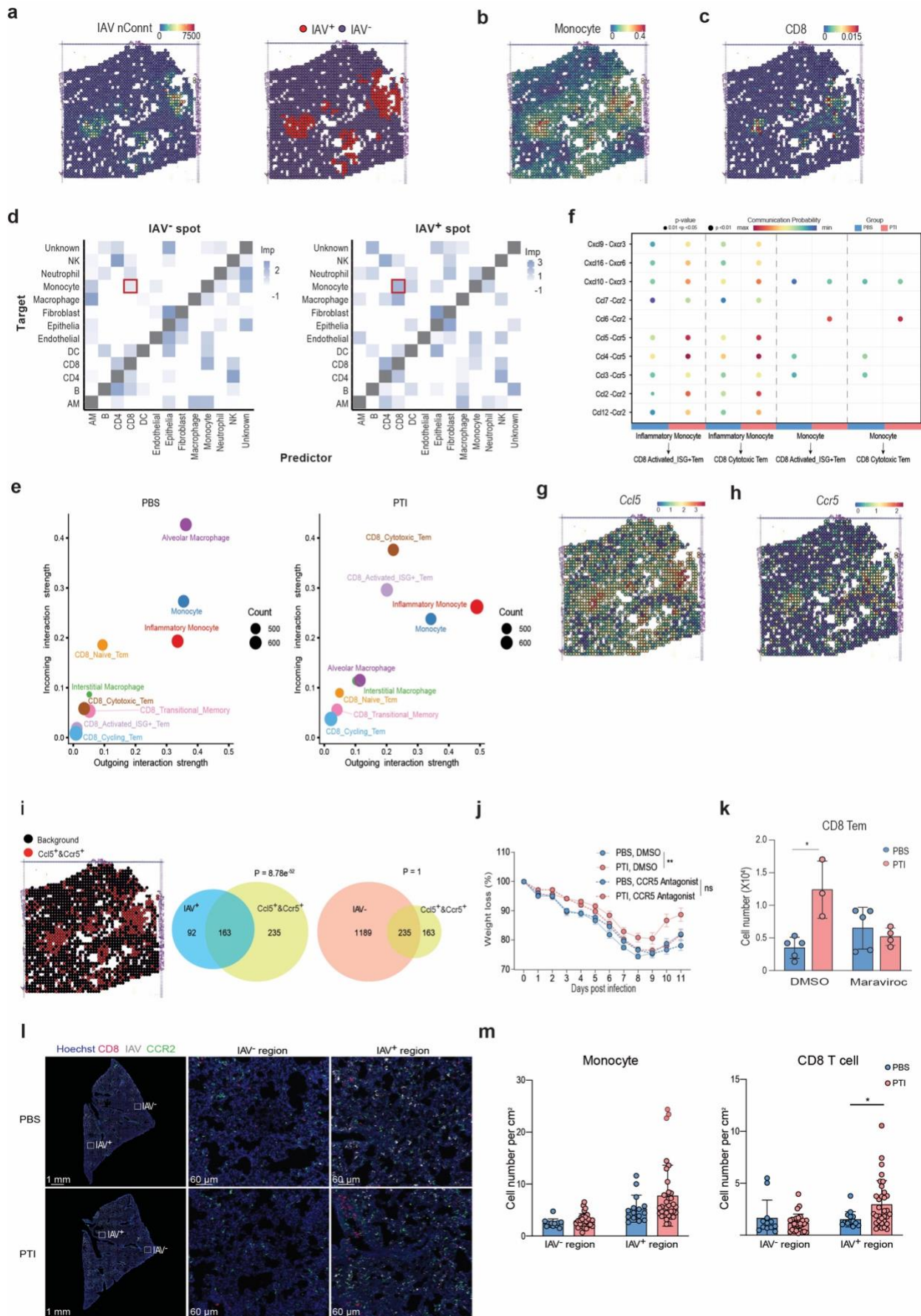


Fig. 4 Monocytes recruit CD8⁺ Tem through Ccl4/Ccl5–Ccr5 signaling in IAV-infected lungs of PTI mice. a, Spatial transcriptomics mapping of viral transcripts to define IAV-

infected (IAV⁺) regions in the lungs of PBS- and PTI-treated mice at day 2 post-challenge. **b**, **c**, Spatial feature plots displaying the signature scores for monocytes (**b**) and CD8⁺ T cells (**c**) across the lung sections. **d**, Enrichment and colocalization of cell type signature score in IAV⁻ and IAV⁺ regions. **e**, Scatter plots of outgoing and incoming intercellular interaction strengths for immune cell populations in PBS and PTI lungs, inferred by CellChat. **f**, Bubble plot showing the communication probabilities of predicted ligand–receptor pairs from monocytes/macrophages (senders) to CD8⁺ T cell subsets (receivers). **g**, **h**, Expression levels of Ccl5(**g**) and Ccr5(**h**) in regions. **i**, Colocalization of Ccr5 with Ccl5 signals, and overlap of IAV⁺ or IAV⁻ regions with Ccl5⁺Ccr5⁺ regions. **j**, Weight loss of PBS- and PTI-treated mice with or without CCR5 antagonist (Maraviroc) administration following IAV challenge (n>15 per group). **k**, Absolute cell numbers of BALF CD8⁺ Tem from of PBS- and PTI-treated mice with or without Maraviroc administration at day 4 post IAV challenge. **l**, Immunofluorescence images of lung tissues on the fourth day of IAV infection. **m**, The density of monocytes and CD8 cells in the lungs, as quantified from fig. 4l.

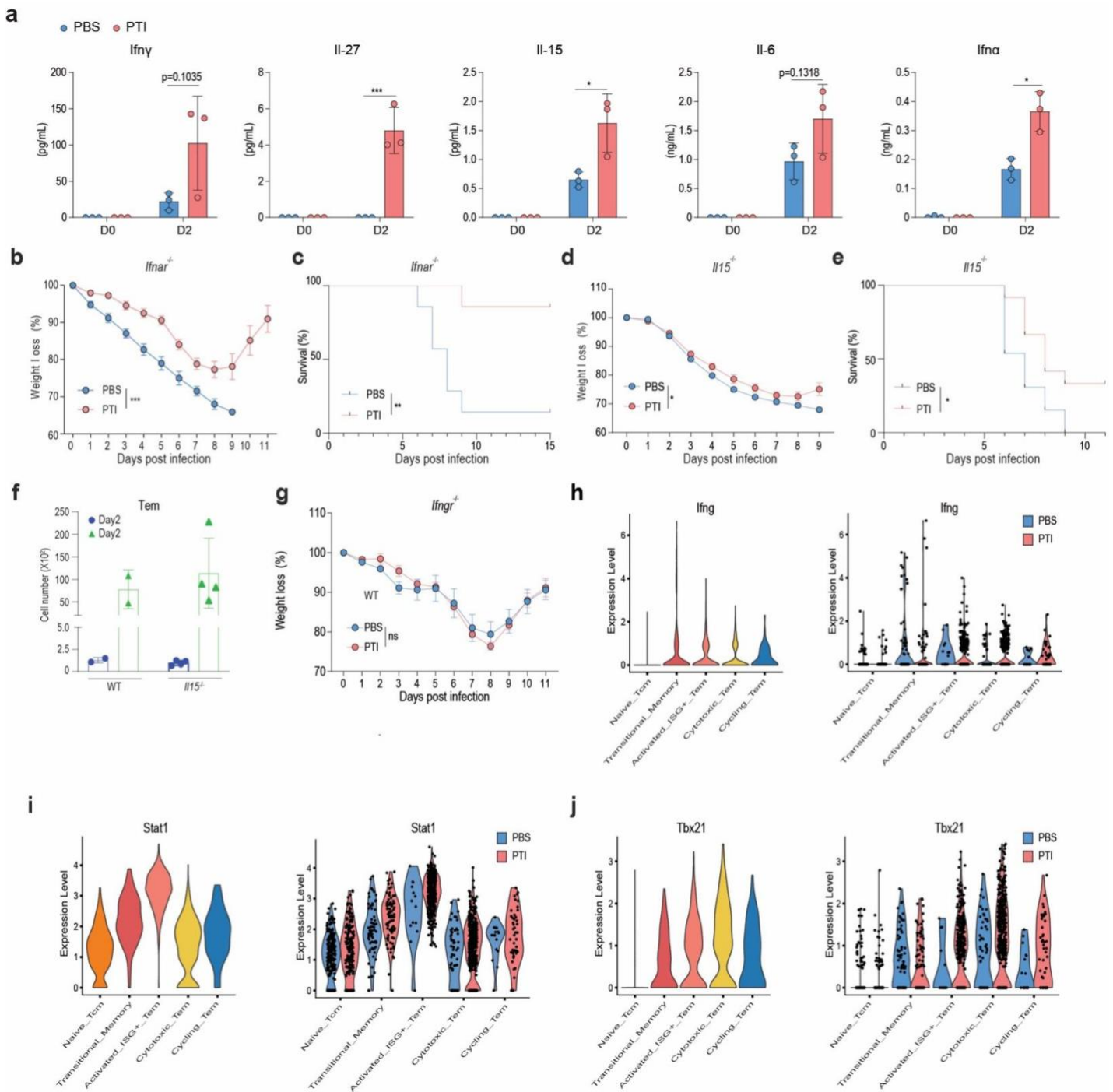
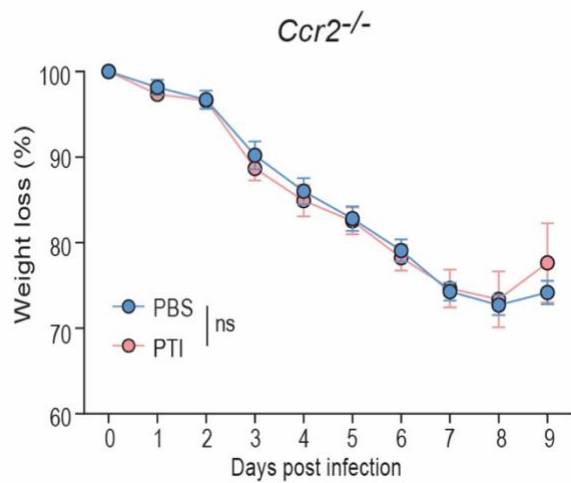
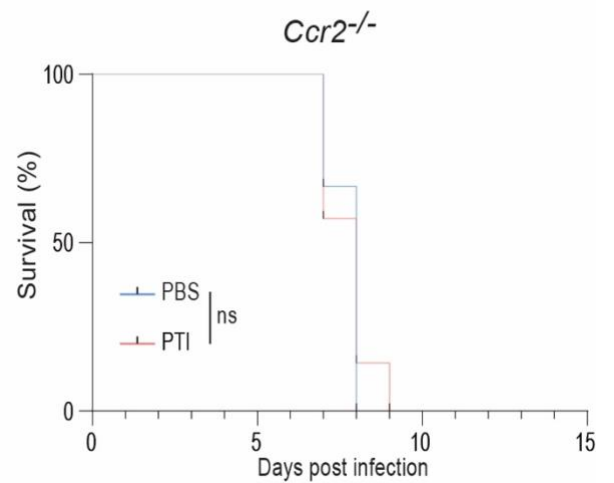


Fig. 5 CD8 Tem-mediated protection against IAV challenge depends on IFN- γ signaling.

a, Cytokine levels in bronchoalveolar lavage fluid were determined at day 2 post-IAV challenge (n=3 per group). **b**, **c**, Weight loss (**b**) and survival (**c**) of PBS- and PTI-treated *Ifnar*^{-/-} mice following IAV challenge (n=7 per group). **d**, **e**, Weight loss (**d**) and survival (**e**) of PBS- and PTI-treated *Il15*^{-/-} mice following IAV challenge (n $\geq$ 12 per group). **f**, Absolute cell numbers of enriched CD45.1⁺CD8⁺ Tem in wild-type and *Il15*^{-/-} mice were determined (n $\geq$ 2 per group). **g**, Weight loss of PBS- and PTI-treated *Ifngr*^{-/-} mice following IAV challenge (n>15 per group). (**h**-**j**) Violin plots displaying the single-cell expression levels of *Ifng* (**h**) and the downstream effector module genes *Stat1* (**i**) and *Tbx21* (**j**) across the five CD8⁺ T cell subsets. Expression profiles are presented for all cells collectively (left) and segregated by

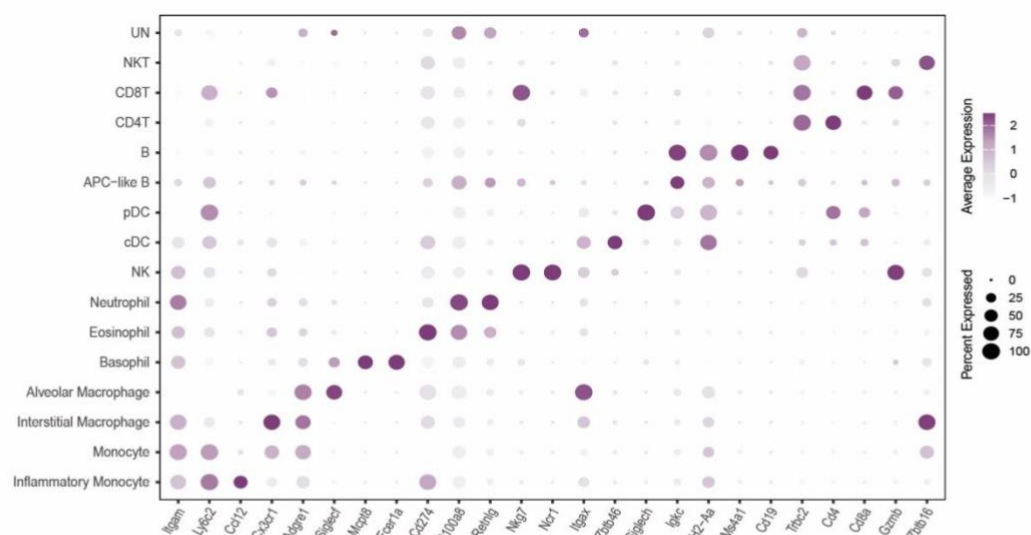
treatment group (PBS vs. PTI, right). Data in line graphs and bar charts are represented as mean $\pm$ SEM.

a**b**

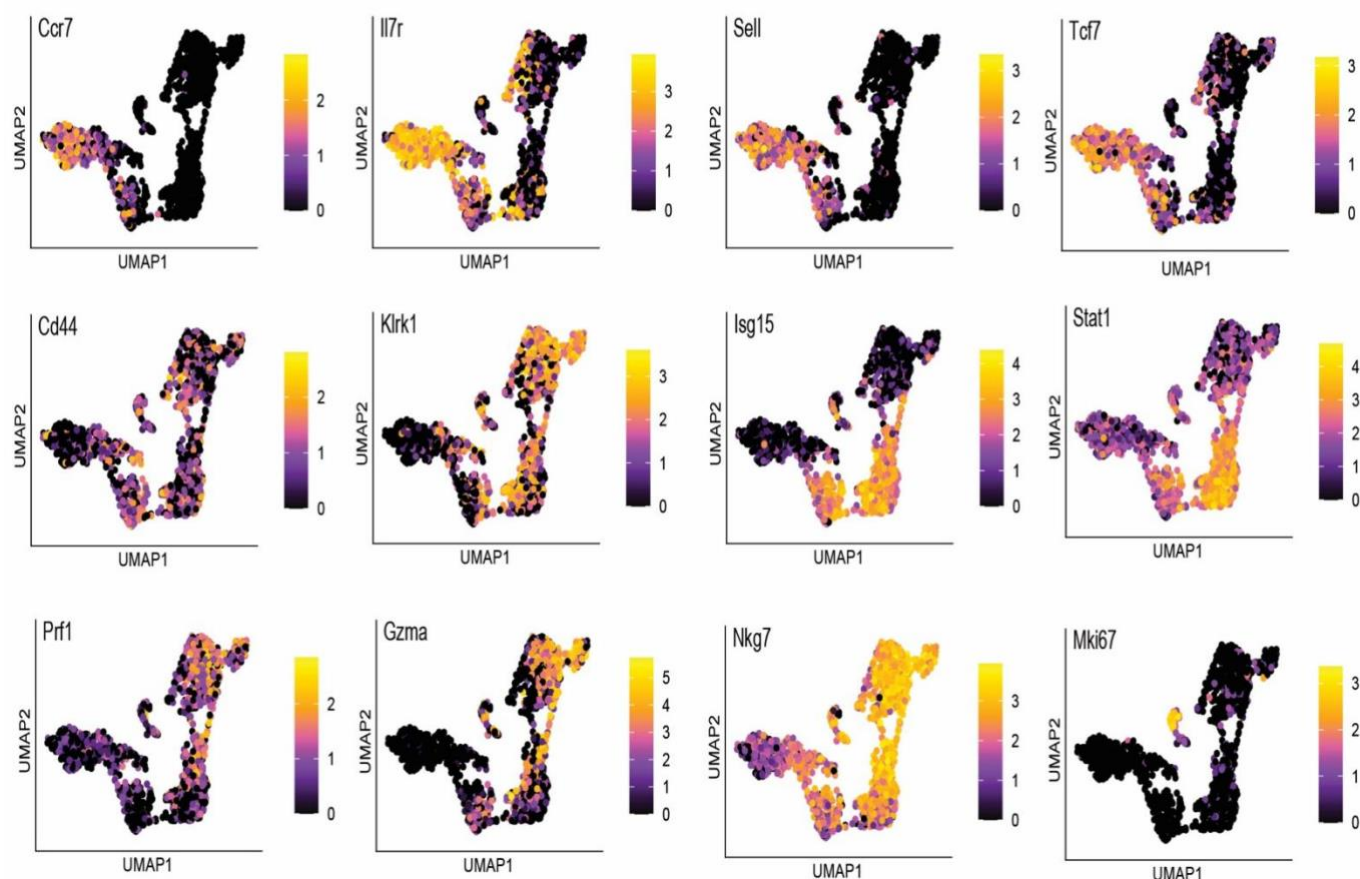
Supplemental Fig. 1 Monocytes are required for PTI-mediated protection against IAV.

a, b, Weight loss (**a**) and survival (**b**) were monitored in *Ccr2*^{-/-} mice with or without *Plasmodium* infection following IAV challenge (n≥11 per group).

a

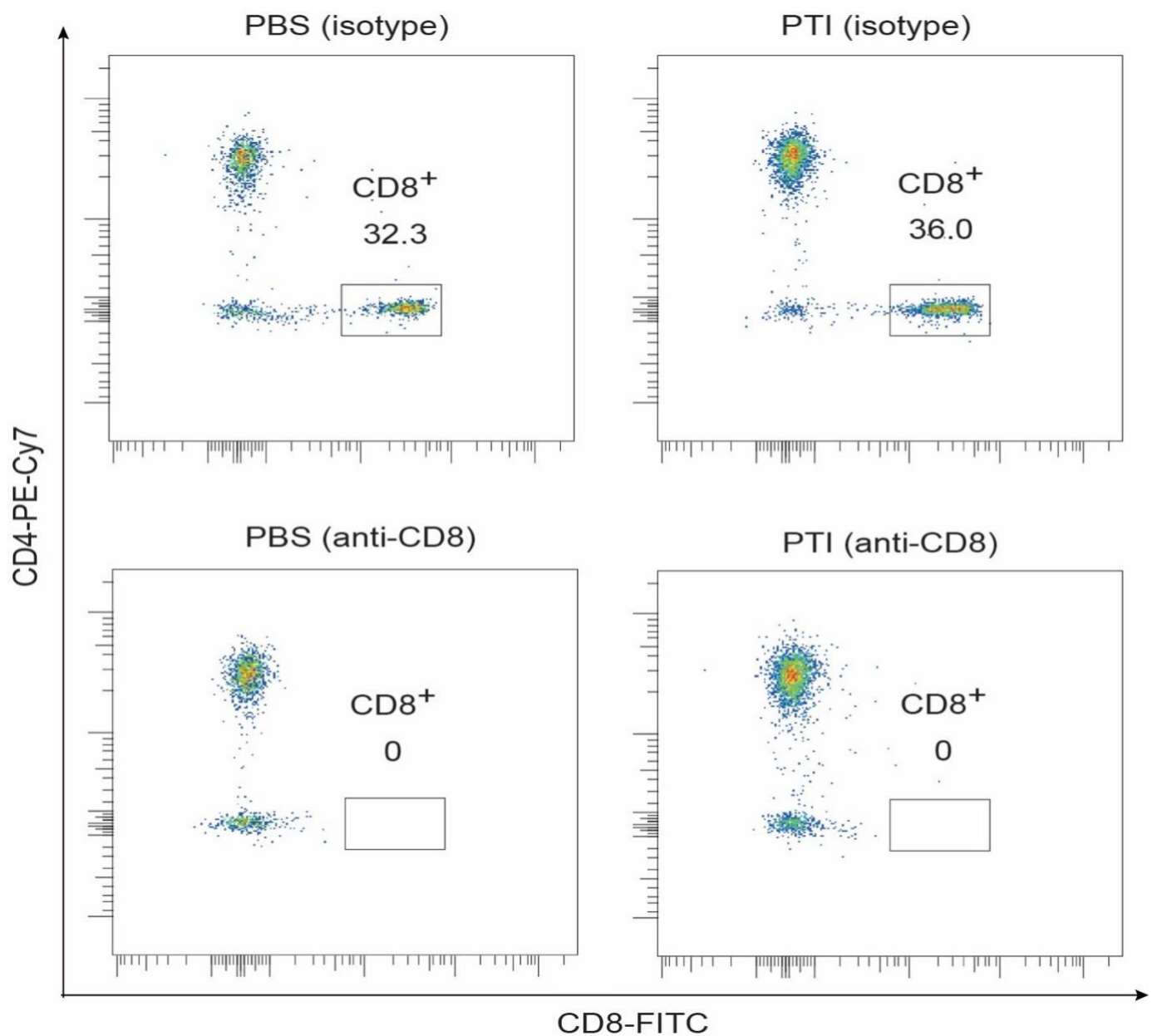


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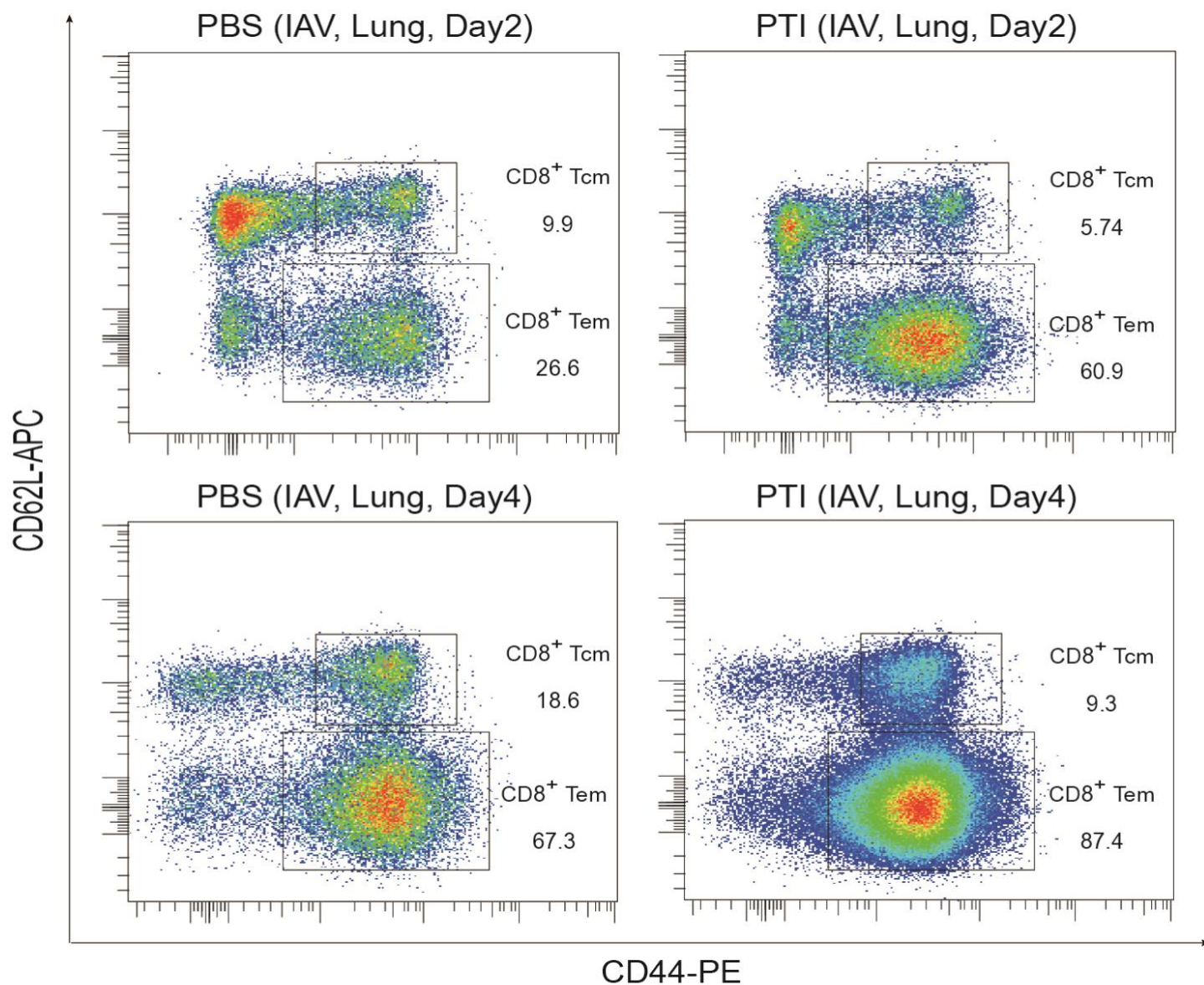


Supplemental Fig. 2 Transcriptional markers defining lung-infiltrating leukocyte populations and CD8⁺ T cell subsets. **a**, Dot plot detailing the expression of canonical cell type-specific marker genes used to identify the major CD45⁺ immune lineages. Node size corresponds to the proportion of cells expressing the marker, and color intensity indicates the average scaled expression within each lineage. **b**, UMAP feature plots showing the single-cell expression levels of selected naive/memory (*Ccr7*, *Il7r*, *Sell*, *Tcf7*), activation/effector (*Cd44*,

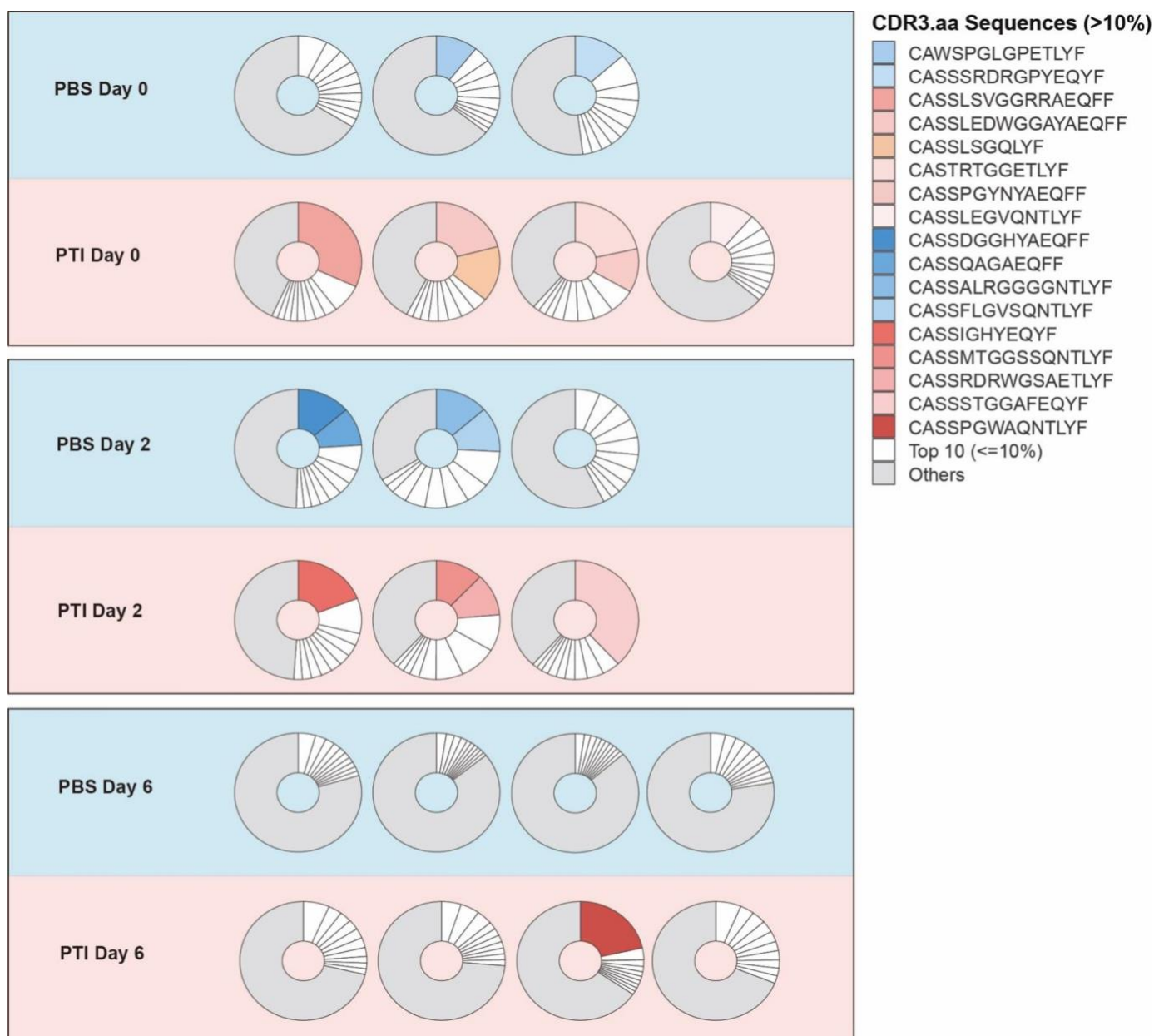
Klrk1), interferon-stimulated (*Isg15*, *Stat1*), cytotoxic (*Prfl*, *Gzma*, *Nkg7*), and cell-cycle (*Mki67*) genes within the reclustered CD8⁺ T cell population.



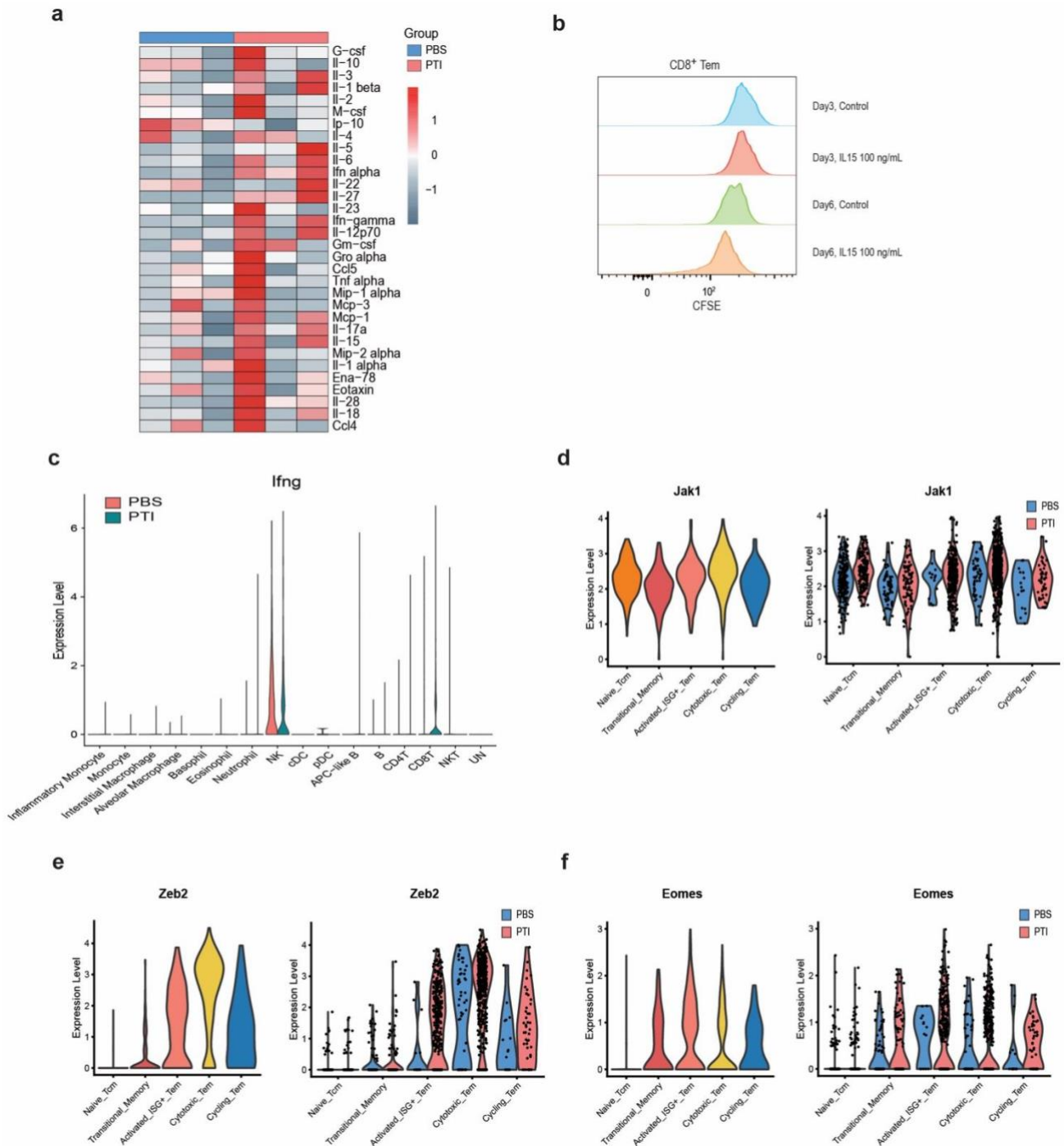
Supplemental Fig. 3 CD8⁺ T cell depletion efficiency. Representative flow cytometry plots showing the efficiency of CD8⁺ T cell depletion using a CD8-depleting antibody.



Supplemental Fig. 4 Flow cytometry analysis of CD8⁺ T cell subsets. Representative flow cytometry plots of lung CD8⁺ Tem and Tcm T cells in PBS- and PTI-treated mice at day 2 and day 4 post-IAV challenge.



Supplemental Fig. 5 Plasmodium imprinting induces rapid clonal expansion of the CD8⁺ Tem repertoire upon viral challenge. Pie charts illustrating the TCR repertoire diversity within the lungs of PBS- and PTI-treated mice at day 0, day 2, and day 6 post-IAV infection. Slices represent the proportion of individual high-frequency clonotypes (defined by unique CDR3.aa sequences).



Supplemental Fig. 7 Cytokine profiling and validation of the CD8⁺ Tem IFN-γ effector module. **a**, Heatmap illustrating the relative abundance of various cytokines in the BAL fluid from PBS- and PTI-treated mice at day 2 post-IAV challenge, measured by multiplex Luminex assay (n = 3 per group). **b**, Histograms depicting the in vitro proliferation of CFSE-labeled CD45.2⁺ CD8⁺ Tem cultured with or without IL-15 supplementation, assessed via CFSE dilution using flow cytometry (n ≥ 3 per group). **c**, Violin plot detailing *Ifng* expression levels across all annotated lung-infiltrating immune cell lineages from the scRNA-seq dataset. **d-f**, Violin plots displaying the single-cell expression levels of downstream IFN-γ effector module genes *Jak1* (**d**), *Zeb2* (**e**), and *Eomes* (**f**) within the reclustered CD8⁺ T cell subsets, presented for all cells (left) and split by PBS and PTI treatment groups (right).