Selective dependence on IL-7 for antigen-specific CD8 T cell responses during airway influenza infection

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Interleukin-7 (IL-7) is a cytokine known for its importance in T cell development and survival. How IL-7 shapes CD8 T cell responses during an acute viral infection is less understood. We had previously shown that IL-7 signaling deficient mice have reduced accumulation of influenza-specific CD8 T cells following influenza infection. We sought to determine whether IL-7 affects early CD8 T cell expansion in the mediastinal lymph node and effector function in the lungs. Using IL-7Rα signaling deficient mice, we show that IL-7 is required for a normal sized mediastinal lymph node and the early clonal expansion of influenza-specific CD8 T cells therein. We show that IL-7 plays a cell-intrinsic role in the accumulation of NP366–374 and PA224–233-specific CD8 T cells in the lymph node. We also found that IL-7 shapes terminal differentiation, degranulation and cytokine production to a greater extent in PA224–233-specific than NP366–374-specific CD8 T cells. We further demonstrate that IL-7 is induced in the lung tissue by viral infection and we characterize multiple cellular sources that contribute to IL-7 production. Our findings on IL-7 and its effects on lower respiratory diseases will be important for expanding the utility of therapeutics that are currently available.

The influenza virus is an airway pathogen that infects lung epithelial cells and activates a network of immune cells. It causes seasonal and pandemic outbreaks with major global health and economic impacts. Seasonal variants of influenza can cause death in children, the elderly and immune-compromised individuals1. Vaccination is a cornerstone of the preventative measures taken towards influenza as it arms the adaptive immune system. Multiple cell types in the immune system are required during a response to influenza infection. However, we lack a complete understanding of the cellular aspects and intercellular signaling components that lead to efficient generation of functionally competent immune cells. At the center of immune responses are the cytokine signals that shape various aspects of immune cells2.

A hallmark of our immune response is its ability to develop memory to previously encountered pathogens—T cells are major players in this process. An ideal anti-viral response to influenza and other viruses, requires cytotoxic CD8 T cells for their swift and specific response. CD8 T cells employ multiple methods to kill infected cells and control viral replication, namely, granule-dependent (granzyme B and perforin) and ligand-dependent (Fas-FasL) means3.

In addition to TCR-MHC engagement (signal 1) and co-stimulation (signal 2), cytokine cues (signal 3) have great influence in activating and shaping CD8 T cell responses and their terminal differentiation. Once a CD8 T cell receives these signals, it is driven towards a robust clonal expansion phase whereby a single cell expands to ~ 10^5 cells4. The signal 3 cytokines that govern T cells are multifaceted and include interleukin-2 (IL-2), IL-6, IL-10, IL-12, IL-15 and others which dictate their terminal differentiation and inflammatory functions5,6.

The common gamma chain (γc) cytokine IL-7 is produced mainly by stromal cells in the bone marrow and thymus. At steady state, it plays an indispensable role in the development of both pre- and pro- B cells and T cells in mice7–10. IL-7 is important in the development and survival of T cells at specific stages of maturation in the thymus as the expression of IL-7Rα (CD127) is dynamically regulated11–13. IL-7 shares the IL-7Rα with...
thymic stromal lymphopoietin (TSLP), an alarmin cytokine that plays a major role in mucosal sites. In addition to its role in development, IL-7 also plays a canonical role in maintenance of memory T cells. The span of IL-7's function was further expanded in the past decade when it was shown to be able to shape the effector responses of cytotoxic CD8 T cells by enhancing their responses against tumors and bacterial infection, and reverse T cell exhaustion caused by chronic LCMV infection, thus, preventing liver pathology. However, the extent to which IL-7 regulates CD8 T cell response to acute viral infections is unknown. We had previously shown that IL-7 but not TSLP is important for the accumulation of influenza-specific CD8 T cells in the lungs but the mechanism by which this occurs is unclear. Since IL-7 is implicated in over 8 clinical trials for treatment of infections, solid tumors and other chronic conditions, the intricacies of IL-7 signaling in functional outcomes requires further inquiry.

In this study, we asked: what modulatory effects does IL-7 have on CD8 T cell priming and effector functions during an acute airway influenza infection? We used IL-7Ra knock-in mice (IL-7Ra449F), a hypomorphic model with targeted disruption of a signaling motif that permits development of naïve lymphocytes amenable in infection tests. We show that in the lung draining mediastinal LN (mdLNs), IL-7 is important for early priming and accumulation of CD8 T cells specific for influenza NP366–374 and PA224–233. Presented on H2Db in a cell intrinsic manner. We also show that IL-7 is important for the terminal differentiation and cytokine production in CD8 T cells. This study will aid in therapeutic development and vaccine adjuvant studies to design combinatorial therapeutic strategies.

Results
IL-7Ra signaling is required for accumulation of influenza-specific CD8 T cells. To assess the importance of IL-7 signaling in CD8 T cells following infection with influenza, we used mice expressing a mutated IL-7Ra whereby a cytoplasmic tyrosine important for signal transduction is substituted to a phenylalanine at residue 449 (IL-7Ra449F). We compared WT and IL-7Ra449F mice following infection with A/PR/8/34 (PR8) influenza virus and measured influenza-specific CD8 T cells by flow cytometry using MHCI tetramers (Suppl. Fig. 1a). We found that IL-7Ra449F mice have reduced proportions of NP366–374 and PA224–234-specific cells within CD8 T cells in the lungs 7 days post-infection (dpi) (Fig. 1a), which phenocopies past observation of this defect at 9 dpi in a new, embryo re-derivation based, specific pathogen free facility. Since the majority of influenza-specific T cells originate from tissue draining lymph nodes, we examined the mediastinal lymph nodes (mdLNs) of infected mice and found that IL-7Ra449F mice have reduced lymph node sizes, particularly the mdLN, compared to WT mice. Interestingly, unlike WT mice, there was little increase in mdLN size of IL-7Ra449F mice after influenza infection (Fig. 1b). Additionally, enumeration of the total influenza-specific cells in the mdLN at multiple days post infection revealed a consistent and substantial defect that is not due to a delay in expansion kinetics. (Fig. 1c). Consistent with lack of LN hyperplasia, the proportion of influenza-specific cells in IL-7Ra449F mdLN at 5 dpi was reduced indicating a defect in early priming of CD8 T cells (Fig. 1d).

Intrinsic requirement for IL-7Ra signaling in the accumulation of influenza-specific CD8 T cells in the mdLN. Previous reports have shown that IL-7 is required for the generation of lymph nodes independent of the peripheral lymphocyte pool. This likely contributed to the reduced lymph node sizes noted above. Therefore, it is possible that the reduction in influenza-specific CD8 T cell accumulation was due to factors extrinsic to T cells in the lymph node and that IL-7 was indirectly important for shaping the cellular and cytokine environment for optimal T cell activation. To address this, we created bone marrow (BM) chimeric mice whereby we grafted BM cells of wild type (WT) and IL-7Ra449F mice into lethally irradiated RAG-1-deficient hosts (Fig. 2a). Since WT lymphocytes outcompete IL-7Ra449F lymphocytes during development, we delivered a 1 to 10 ratio of WT to IL-7Ra449F cells respectively. Following engraftment and infection of the hosts, we noted a reversal of this ratio within the CD8 T cell compartment in the mdLN (Fig. 2b). More importantly, IL-7Ra449F CD8 T cells resulted in reduced NP366–374 and PA224–233—specific cells in proportion despite engraftment in a competent niche (Fig. 2b). These data suggest that IL-7Ra signaling plays an intrinsic role necessary for CD8 T cell expansion during influenza infection.

IL-7Ra plays a role in early priming of CD8 T cells independent of TCR and number of naive precursors in the mdLN. To determine if the reduction in influenza-specific CD8 T cells was due to reduced numbers of naive precursors or a result of gaps in TCR repertoire, we adoptively transferred SINIFEKLO OVA peptide-specific and MHCI restricted transgenic TCR CD8 T cells from OT-I mice crossed with IL-7Ra449F mice (CD45.2) in to BoyJ mice (CD45.1). We infected these mice with a modified version of the influenza PR8 virus that has the OVA (SINIFEKLO) peptide inserted into the stalk of the NA polypeptide (influenza PR8-OVA). Despite delivering equal number (1 × 10^6) of OT-I and OT-I;IL-7Ra449F CD8 T cells into distinct BoyJ (CD45.1) hosts, OT-I;IL-7Ra449F CD8 T cells did not expand to the same extent as wild type OT-I CD8 T cells 4 dpi (Fig. 3a) in the mdLN. This defect was observed as early as 3 dpi (Suppl. Fig. 1b). Interestingly, the expression of the early activation marker CD5 at 4 dpi was significantly reduced in OT-I;IL-7Ra449F CD8 T cells indicating a defect in priming (Fig. 3b,c). Furthermore, TCR expression on OT-I;IL-7Ra449F CD8 T cells showed a trend towards higher expression at 4 dpi albeit not significantly, further suggesting a possible defect in early priming (Fig. 3b,c).

Increased dendritic cell accumulation in the lungs of IL-7Ra449F mice. Evidence of IL-7 cell-intrinsic effects on CD8 T cells does not exclude cell-extrinsic effects. Antigen presenting cells, specifically dendritic cells (DCs) are key to activation of CD8 T cells and their subsequent response. Previous reports have demonstrated that IL-7Ra signaling plays an indirect role in the development of conventional DCs. Furthermore, IL-7
has been shown to regulate CD4 T cell proliferation in conditions of lymphopenia indirectly through DCs. We found that in the lungs of IL-7Rα449F mice, CD11b+ CD103− DCs but not CD11b− CD103+ DCs, accumulate up to 9 dpi, while in WT mice these DCs peak at 7 dpi and decrease in numbers at 9 dpi (Suppl. Figs. 1c and 2a). This increased accumulation could be due to cell-extrinsic factors such as increased viral load in the IL-7Rα449F mice as a result of lack of appropriate T cell response. Another plausible reason could be due to impaired lymph node homing signals from chemokines as a result of reduced draining lymph node size. Alternatively, IL-7 may have a direct effect on CD11b+ CD103+ DC maturation or migration. To test these hypotheses, we created 50:50 BM chimeras using WT:IL-7Rα449F or WT:WT BM cells (CD45.1:CD45.2) grafted into congenic BoyJ/WT hosts (CD45.1/2). We found that after infection, WT:IL-7Rα449F ratios of DC subsets were comparable to WT:WT

Figure 1. Accumulation of tetramer specific response in IL-7Rα449F is impaired following influenza infection. (a,d) Representative FACS plots and bar graphs of the frequency of NP366–374+ and PA224–233+ cells within CD8 T cells in (a) the lungs 7 dpi and (d) mdLN 5 dpi of WT and IL-7Rα449F mice. Gated within Live B220−, CD8+, (CD44+) cells. (b) Photograph images offering comparison of various mouse lymph nodes and (c) total number of NP366–374+ and PA224–233+ cells in the mdLN of WT and IL-7Rα449F mice at the indicated days post infection. Data are representative of 2–3 experiments with n = 4–7 per genotype. *P < 0.05 as determined by two-tailed Student’s t-test.
Ratios in both the mdLN and lungs for both DC subsets (Suppl. Fig. 2b,c). This suggests that the phenotypic elevation of DCs in IL-7Rα449F mice during influenza infection has a cell extrinsic cause.

IL-7 is inducible in lung tissues in response to influenza.

IL-7 is mainly produced by radio-resistant cells such as stromal and epithelial cells of the bone marrow and thymus, where it plays a major role in hematopoiesis and thymopoiesis. A few studies have demonstrated IL-7 expression in various tissues including liver, skin, intestines and lungs. While IL-7 is mainly produced in steady state lungs by lymphatic endothelial cells, its source during inflammation is unclear. To assess IL-7 expression dynamics in response to influenza,

Figure 2. Impairment in tetramer specific response seen in the mdLN of IL-7Rα449F mice is cell intrinsic. (a) Schematic of bone marrow chimera set-up. (b) Representative FACS plots and bar graphs of the frequency of NP366–374+ and PA224–233+ cells within CD8 T cells in the mdLN of WT and IL-7Rα449F chimeric mice 9 dpi. Gated within live, B220−, CD8+, CD44+, CD45.1+ or CD45.2+ cells. Data are representative of two experiments with n = 5-7 per genotype. *P < 0.05 as determined by two-tailed Student's t-test.

Figure 3. Expansion of adoptively transferred OTI-IL-7Rα449F CD8 T cells is impaired in the mdLN following influenza infection. (a) Scatter plot and representative bar graph of CD45.2+ Vα2+ CD8 T cells in BoyJ (CD45.1+) mice 4 dpi. Gated within live B220− CD8+ cells. (b) Histogram and (c) bar graph of median fluorescence intensity (MFI) of activation markers (CD5, TCR (Vα2), and CD69). Data are representative of two experiments. *P < 0.05 and **P < 0.01 as determined by two-tailed Student's t-test.
we first infected human type II epithelial cells (A549) with influenza and assessed Il7 mRNA using qRT-PCR. We found that IL-7 expression is induced within 24 h following influenza infection correlating with the antiviral response signified by IFN-β and viral replication demonstrated by M1 mRNA transcript expression (Fig. 4a).

To determine if IL-7 is induced in vivo, we used the IL-7εGFP/eGFP mice. We noted an increase in the number of cells expressing eGFP at 6 dpi (Fig. 4b). Interestingly, the majority of IL-7 εGFP + cells during infection are epithelial cells. The number of IL-7 εGFP + stromal cells also significantly increased during infection. However, IL-7 εGFP + lymphatic endothelial cells were reduced following infection (Fig. 4b). Altogether, in vitro and in vivo experiments suggest that lung epithelial cells are responsive to viral infection, and that during influenza infection, they become the primary source of IL-7.

**IL-7Rα449F CD8 T cells have reduced terminal differentiation.** The cytokine milieu that CD8 T cells are exposed to throughout the course of an immune response governs their terminal differentiation to effector cells and hence, their functional capabilities. Among the heterogeneous population of CD8 T cells that emerge during the expansion phase are the T-bet hi and granzyme B producing short-lived effector cells (SLECs) that are identified by their expression of the killer cell lectin-like receptor G1 (KLRG1) and low CD127 (IL-7Rα)6,31. Due to extra-physiological expression of the mutated IL-7Ra449F subunit (Suppl. Fig. 3), we limited our use of SLEC markers to KLRG1. Testing the expression of KLRG1 in influenza-specific cells revealed a reduced proportion of KLRG1+ cells in both NP366–374 and PA224–233-specific cells of IL-7Ra449F mice (Fig. 5). However, the difference in KLRG1 expression between WT and IL-7Ra449F mice was greater in PA224–233-specific compared to NP366–374-specific cells (Fig. 5). These data suggest that IL-7Ra signaling plays a role in the terminal differentiation of influenza-specific CD8 T cells.

**Reduced degranulation and cytokine production by IL-7Ra449F CD8 T cells.** Secretion of cytotoxic granules and inflammatory cytokines is a major event in the CD8 T cell effector response. Lysosome associated membrane protein-1 (LAMP-1) or CD107a is a membrane glycoprotein found in the lumen of granyme B and perforin containing vesicles. Detection of CD107a on the surface of CD8 T cells through flow cytometry after a short stimulation (4 h) with PMA/Ionomycin in the presence of CD107a detecting antibodies provides a direct method for identifying degranulating cells32. Using this method, we noted that CD8 T cells of IL-7Ra449F mice had reduced CD107a expression in antigen experienced cells (CD44+) and in tetramer-stained PA224–233-specific cells indicating decreased degranulation in these populations (Fig. 6a,b). Interestingly, this defect was notable in PA224–233-specific cells but not in NP366–374-specific cells. Furthermore, the proportion of cells expressing CD127 was notably higher in PA224–233-specific cells compared to NP366–374-specific cells in WT mice indicating increased influence of IL-7 on PA224–233-specific cells (Fig. 6c). As mentioned earlier, IL-7Ra449F cells have extra-physiological expression of CD127 (IL-7Ra), however, we found that WT and IL-7Ra449F influenza-specific CD8 T cells experience a similar fold decrease in expression of CD127 compared to naïve CD44+ CD8 T cells (Suppl. Fig. 3).

To determine if IL-7 signaling affects cytokine production, we treated whole lung single cell suspensions from infected mice with NP366–374 and PA224–233 peptides re and stained intracellular cytokines to detect IFNγ and TNFa using flow cytometry. NP366–374-specific cells generated low proportion of IFNγ+ TNFa+ cells compared to PA224–233-specific cells regardless of mouse genotype (Fig. 7a). However, WT PA224–233-specific cells generated abundant IFNγ+ TNFa+ cells, which were largely absent within IL-7Ra449F PA224–233-specific cells (Fig. 7b). We used TSLPR−/− mice as controls since IL-7Ra is required for both IL-7 and TSLP signaling. We found that TSLPR−/− CD8 T cells presented with reduced cytokine production as well, however, this effect did not follow the same pattern as with IL-7Ra449F mice (Fig. 7a,b).

To determine if IL-7 independently affects cytokine production, we used IL-7εGFP/eGFP mice that have an eGFP gene inserted disruptively into an Il7 exon thus serving as an IL-7 ligand knock-out in homozygotes29. Using this mouse model, we established that IL-7 is required for accumulation of IFNγ+ TNFa+ cells within PA224–233-specific but not NP366–374-specific cells (Fig. 7c,d).

**IL-7 signaling regulates expression of PD-1 in PA224–233 but not NP366–374 specific CD8 T cells.** Expression of inhibitory molecules such as PD-1 is known to be important to negatively regulate T cell activation and limit inflammation by T cells, however, sustained expression of these molecules can lead to dampening of protective immune responses33. To understand how IL-7 affects PA224–233-specific but not NP366–374-specific T cells, we evaluated the expression of the inhibitory receptor PD-1. We showed that IL-7Ra449F and IL-7εGFP/eGFP CD8 T cells have higher expression of this molecule (Fig. 8a,b). Specifically, the increase in PD-1 expression in IL-7Ra449F and IL-7εGFP/eGFP CD8 T cells was only evident in PA224–233-specific cells but not NP366–374-specific cells (Fig. 8a,b). In addition, TSLPR−/− CD8 T cells did not present with increased PD-1 expression (Fig. 8a). Together, this suggests that IL-7 plays distinct roles in CD8 T cell function depending on antigen specificity possibly by regulating PD-1 expression.

**Discussion**

Initial studies of IL-7 have described its role in B-cell lymphopoiesis and thymopoiesis34–36. The bone marrow and thymus are the best defined sources of IL-7 production consistent with such roles in primary lymphopoiesis38. Subsequent studies showed a role for IL-7 in memory cell development and maintenance, in effector response to viral infections and in enhancing T cell functions in chronic conditions4–11. CD8 T cell expansion and effector function depends on multiple factors including, but not limited to, the cytokine milieu. Previously, we demonstrated that IL-7 is required for the accumulation of tetramer positive CD4 and CD8 T cells during influenza...
Figure 4. IL-7 expression in lung tissues. (a) Quantitative PCR of IL-7, IFN-β and M1 in A549 cells at the indicated times post infection normalized against beta actin. M1 expression is further normalized to time point 0 h. Data is representative of 2 experiments n = 3 per experiment. (b) Expression of IL-7 in various CD45− lung cells using IL-7eGFP/WT mice. Epithelial cells (ECs) are CD45− EpCAM+, stromal cells (SCs) are CD45− EpCAM− CD31− and lymphatic endothelial cells (LECs) are CD45− EpCAM− and CD31+ GP38+. Data are representative of two experiments with n = 4 per genotype. *P < 0.05 as determined by two-tailed Student’s t-test.
Figure 5. CD8 T cells of IL-7Rα449F mice have reduced terminal differentiation. Scatter plots and bar graphs showing flow cytometric analysis of KLRG1 expression as a percentage within lung NP366–374+ and PA224–233+ CD8 T cells of WT and IL-7Rα449F mice 7–9 dpi. Data are representative of three experiments with n = 4–6 per genotype. *P < 0.05 as determined by two-tailed Student’s t-test.

Figure 6. Reduced degranulation of IL-7Rα449F lung CD8 T cells upon re-stimulation. (a, b) CD107a expression as median fluorescence intensity (MFI) in all antigen experienced (CD44+) NP366–374+ and PA224–233+ CD8 T cells of WT and IL-7Rα449F mice 7 dpi after PMA/Ionomycin re-stimulation. Data presented as (a) FACS plots and (b) bar graphs. Gated within Live B220−, CD8+, CD44+, NP366–374+ or PA224–233+ cells. Data are representative of two experiments with n = 4 per genotype. *P < 0.05 as determined by two-tailed Student’s t-test. (c) Scatter plots and Bar graphs showing flow cytometric analysis comparison of CD127 expression as a percentage within lung NP366–374+ and PA224–233+ CD8 T cells 7 dpi. Gated within Live B220−, CD8+, CD44+, NP366–374+ or PA224–233+ cells. Data are representative of three experiments with n = 4–5 per genotype. *P < 0.05 as determined by on-way ANOVA.
infection\textsuperscript{\textregistered}. The mechanism by which IL-7 accomplishes this and its role in other aspects of T cell response have yet to be elucidated.

We addressed these questions by using mice that express a hypomorphic IL-7Rα (IL-7Rα\textsuperscript{449F}) which leads to impaired IL-7 signaling by primarily abrogating STAT5 activation\textsuperscript{14}. This model provides a better alternative to using IL-7Rα\textsuperscript{-/-} mice since IL-7Rα\textsuperscript{449F} mice have defective signaling yet retain sufficient number of T cells to perform infection studies. We have previously used this mouse model to demonstrate an intact CD8 T cell effector response to intracellular \textit{Listeria monocytogenes} infection\textsuperscript{14}. In the current study, we found that defective IL-7Rα signaling led to reduced accumulation of influenza-specific CD8 T cells in the secondary lymphoid organ (mdLN) at early priming stages (5 dpi) which ultimately led to reduced accumulation of influenza-specific CD8 T cells.

**Figure 7.** Deregulated cytokine production in IL-7Rα\textsuperscript{449F} and TSLPR\textsuperscript{-/-} lung CD8 T cells. Representative scatter plots and bar charts of IFN-γ\textsuperscript{+} TNF-α\textsuperscript{-} or IFN-γ\textsuperscript{+} TNF-α\textsuperscript{+} CD8 T cells within (a) NP\textsubscript{366–374} and (b) PA\textsubscript{224–233} CD8 T cells 9 dpi and after peptide (NP or PA) re-stimulation. Representative scatter plots and bar charts of IFN-γ\textsuperscript{+} TNF-α\textsuperscript{-} or IFN-γ\textsuperscript{+} TNF-α\textsuperscript{+} CD8 T cells of IL-7\textit{eGFP/eGFP} mice within (c) NP\textsubscript{366–374} and (d) PA\textsubscript{224–233} CD8 T cells 9 dpi and after peptide (NP or PA) re-stimulation. Gated within Live B220\textsuperscript{-}, CD8\textsuperscript{+}, tetramer\textsuperscript{+} cells. Data is representative of three independent experiments with n = 3–5 mice per genotype. *P < 0.05 as determined by one-way ANOVA.
T cells in the lungs. Examination of IL-7Rα449F mdLN revealed a great reduction in its size. This is consistent with the fact that IL-7 is required for the development of lymphoid tissue inducer (LTi) cells that seed LN anlagens and drive the organogenesis of LNs19. Considering IL-7Rα 449F mice had reduced, albeit notable, numbers of influenza-specific CD8 T cells in their lungs, it is unclear where and how these CD8 T cells expand to significant numbers with an abnormal mdLN. It is possible that tertiary lymphoid organs in the lung tissues such as inducible bronchus-associated lymphoid tissue (iBALT) provide a suitable environment for the accumulation of de novo pathogen specific cells without requiring IL-7 or LTi cells37. We have shown that despite such extrinsic factors, IL-7Rα signaling is required cell intrinsically by CD8 T cells for early priming in the mdLN.

It is known that a population of CD8 T cells specific to a distinct peptide do not originate from a single naïve precursor but rather from tens to hundreds of precursors38–40. IL-7 signaling deficient mice have reduced thymic output of T cells, and this may result in a more stochastic or reduced chance of a T cell encountering a cognate MHC-peptide leading to reduced clonal expansion. In addition to these effects, IL-7 can play a role cell intrinsically by affecting TCR repertoire via VDJ recombination or TCR sensitivity8,41. We addressed this by adoptive transfer of CD8 T cells bearing a transgenic TCR (OT-I) in equal numbers (WT vs. IL-7Rα 449F) intravenously into congenic WT mice. Using this approach, we found that OT-I;IL-7Rα449F CD8 T cells expanded in response to infection with influenza PR8-OVA to a lower extent compared to OT-I CD8 T cells within total host CD8 T cells. Our findings show that IL-7 is intrinsically important for the accumulation of influenza-specific CD8 T cells during early priming phase in the mdLN independent of TCR specificity and the number of naïve T cell precursors. We have previously shown that IL-7Rα449F CD8 T cell form influenza-specific cells normally during systemic in vivo infection with L. monocytogenes yet do not proliferate well when exposed to suboptimal TCR stimulation in vitro in contrast to high dose TCR stimulation14. It is possible a low dose, local influenza infection recapitulates the low level TCR avidity model whereby IL-7 plays an essential role in CD8 T cells under low TCR avidity activation.

In addition to the intrinsic role that IL-7 plays in CD8 T cells, we found that IL-7Rα449F mice have continued accumulation of CD11b+ DCs in the lungs while in WT mice, the number of CD11b+ DCs peaks at 7 dpi then subsides. Previous studies using IL-7-/- and IL-7Rα-/- mice showed normal development of DC precursors in the BM, however these mice had reduced migratory DCs in secondary lymphoid organs31. Our BM chimera experiments showed that the effect of IL-7 in DC accumulation was indirect. Therefore, the accumulation of DCs we

Figure 8. Increased PD-1 expression in IL-7 signaling deficient CD8 T cells. Representative histogram plots and bar charts of PD-1 expression in antigen specific lung CD8 T cells of (a) WT versus IL-7Ra449F versus TSLPR-/- and (b) WT versus IL-7eGFP/eGFP. Gated within Live B220-, CD8+, tetramer+ cells. Data is representative of 2–3 independent experiments with n = 3–4 mice per genotype. *P<0.05 as determined by two-tailed Student's t-test.
noted in IL-7Ra<sup>449F</sup> mice was not due to a problem with migration or maturation and was likely due to the fact that viral clearance was impaired which led to continued recruitment of DCs to the lungs.

We have demonstrated for the first time that IL-7 is inducible in lung epithelial cells in response to viral infection in vivo. While the increase in total IL-7-eGFP<sup>+</sup> cells in the lung was modest, we noted a shift in the population that are positive for IL-7-eGFP. In naïve mice, the majority of IL-7-eGFP<sup>+</sup> cells were lymphatic endothelial cells (LECs) as previously reported<sup>29,30</sup>. However, following infection with influenza, IL-7-eGFP<sup>+</sup> epithelial cells (ECs) and to a lesser extent stromal cells (SCs) expanded while LECs decreased in frequency. Our results demonstrate that IL-7 can be produced by lung tissues and this could shape the function of CD127 expressing CD8 T cells locally. It is unclear to what extent IL-7 produced by epithelial cells influences nearby cells and the significance of the shift in sources of IL-7. In addition, more sensitive approaches are required to compare the levels of IL-7 expression between the different lung tissues.

Terminal differentiation of activated CD8 T cells is important for the generation of short-lived effector cells (SLECs) that express killer cell lectin-like receptor G1 (KLRG1) and low CD127<sup>6,31</sup>. KLRG1 expression is an early event during terminal differentiation to SLEC. The difference in KLRG1 expression between WT and IL-7Rα<sup>449F</sup> mice was greater within PA<sub>224–233</sub>-specific cells than in NP<sub>366–374</sub>-specific cells. We also noted a similar trend in CD107α expression between WT and IL-7Ra<sup>449F</sup> mice whereby the defect in CD107α expression was more pronounced in PA<sub>224–233</sub>-specific cells than in NP<sub>366–374</sub>-specific cells. Increased expression of CD127 by PA<sub>224–233</sub>-specific cells supports the hypothesis that PA<sub>224–233</sub>-specific cells have elevated dependence on IL-7 signaling. Furthermore, the increased expression of mutated CD127 in IL-7Ra<sup>449F</sup> mice did not compensate for the loss of Tyr449 signifying the importance of this signaling residue.

Pro-inflammatory cytokines such as IFNγ and TNFα are important during an anti-viral response to help with recruiting and activating other cells. Within NP<sub>366–374</sub>-specific cells, we did not observe any differences in expression of IFNγ or TNFα between WT and IL-7Ra<sup>449F</sup> or IL-7-eGFP/eGFP CD8 T cells. However, the large reduction in IFNγ<sup>+</sup> TNFα<sup>+</sup> population in PA<sub>224–233</sub>-specific cells of IL-7Ra<sup>449F</sup> or IL-7-eGFP/eGFP mice followed a similar pattern to our findings with CD107α<sup>+</sup>. NP<sub>366–374</sub>-specific cells did not generate IFNγ<sup>+</sup> TNFα<sup>+</sup> cells as notably as PA<sub>224–233</sub>-specific cells, consistent with other groups<sup>42,43</sup>. Our work further corroborates what previous studies have shown, that TSLP shapes effector T cell responses following influenza infection. However, these effects were shown to occur indirectly through programming of DCs<sup>44</sup>. Altogether, this suggests that IL-7 may differentially regulate the effector function of CD8 T cells based on their antigen-specificity. Further investigation into the downstream signaling is necessary to understand the mechanism that leads to this response.

Previous studies have shown hierarchical differences between NP<sub>366–374</sub> and PA<sub>224–233</sub>-specific CD8 T cells whereby tissue resident memory CD8 T cells that are NP<sub>366–374</sub>-specific expressed higher levels of inhibitory molecules including PD-1 at 30 dpi and beyond due to persistent antigen exposure and TCR stimulation<sup>45</sup>. IL-7 is also known to enhance cytokine production and reverse T cell exhaustion by repressing inhibitory pathways during chronic viral infections in mice<sup>15,16</sup>. It is well established that TCR signaling duration correlates positively with PD-1 expression but little is known about this phenomenon in the context of influenza-specific CD8 T cells<sup>46,47</sup>. In an acute hepatitis B virus infection, PD-1 expression in CD8 T cells is negatively correlated to CD127 expression, and blocking PD-1 in acute lymphocytic choriomeningitis virus infection increases the frequency of the CD127<sup>+</sup> population<sup>48,49</sup>. Our finding that PD-1 expression is higher within PA<sub>224–233</sub>-specific but not NP<sub>366–374</sub>-specific CD8 T cells with IL-7 signaling deficiency is indicative of an antigen-dependent role for IL-7 in regulating their function. This is suggestive of a negative regulatory role for IL-7 on PD-1 expression dependent on TCR specificity. This hypothesis is corroborated by a previous study where infection with a high pathogenicity influenza virus strain, such as PR8, resulted in elevated PD-1 expression in influenza-specific CD8 T cells compared to infection with the low pathogenicity influenza x31 strain<sup>43</sup>. This in turn inhibited effector function by specifically affecting development of IFNγ<sup>+</sup> TNFα<sup>+</sup> cells<sup>43</sup>. Our studies suggest that PA<sub>224–233</sub>-specific IL-7 signaling deficient CD8 T cells do not receive the necessary signals to down regulate PD-1. Further studies are required to define the relationship between IL-7 and PD-1 in an acute infection setting and the mechanism by which this specifically affects T cells in an antigen-specific manner.

In summary, we have found that IL-7 is required for an optimal response to acute influenza infection as it shapes the early priming stages of CD8 T cells. Moreover, IL-7 produced by lung tissues is important for the terminal differentiation and effector function selectively in antigen-specific CD8 T cells. Various cytokines have the ability to enhance CD8 T cell responses, however, rigorous testing is necessary to evaluate the adverse responses that these cytokines have on bystander cells. Using cytokines such as IL-7 to complement existing therapies may be beneficial given fewer off target effects due to the limited subset of cells that express CD127. IL-7 is currently in clinical trials for treatment of infections and tumors. Additional studies are necessary to expand the use of IL-7 in other conditions and to study its efficacy when delivered in combination with other agents.

**Materials and methods**

**Mice.** All mice were housed and used in the Center for Disease Modeling facility (CDM) at the University of British Columbia (UBC) and all work with animals was carried out with approval and in accordance with the ethical guidelines of the University of British Columbia Animal Care and Biosafety Committees. IL-7Ra<sup>449F</sup> mice were generated in-house as described<sup>14</sup>. Briefly, they express a mutant form of the IL-7Ra with a single amino acid mutation from Tyr to Phe at position 449. C57BL/6, BoyJ (B6SJL-Ptprca Pepcb/BoyJ) and C57BL/6-Tg (TcraTcrb) 1100Mjb/J (OT-I) mice were obtained from the Jackson Laboratory (Bar Harbour, ME, USA). IL-7-eGFP/eGFP mice were a gift from J.M. McCune (UCSF)<sup>29</sup>. In all cases, age-matched and sex-matched male and female mice between the ages of 6–12 weeks were used.
**Virus.** Influenza A/PR/8/34 (PR8) was purchased from Charles River Laboratories (Wilmington, MA). Influenza A/PR/8/34-OVA (PR8-OVA, kindly provided by Dr. Tania Watts, University of Toronto) was propagated in-house in chicken eggs as previously described. Mice were sub-lethally infected under anesthesia (isoflurane) with 5 Hemagglutinin Units (HAU) of influenza PR8 or 64 HAU of influenza PR8-OVA in 12.5 µL of sterile PBS intranasally.

**Tissue preparation.** Mice were anesthetized with 5% isoflurane in 1 L/min O₂ and euthanized by cervical dislocation and perfused with 10 ml cold PBS (+5%FBS, 2 mM EDTA). Lungs were excised and processed by mincing with scissors followed by enzymatic digestion using 180 units/ml collagenase IV and 20 µg/ml DNase I (Worthington biochemical) in 5 ml RPMI incubated at 37 °C for 30–45 min in a shaker incubator before filtering through 70 µm filters and lysing RBCs with ACK lysis buffer. To assess non-hematopoietic cells in the lungs, dispase (1 u/ml) was added to the enzyme cocktail. Mediastinal lymph nodes (mdLN) were collected and crushed through 70 µm filters and suspended as single cells in cold PBS (5%FBS, 2 mM EDTA).

**Antibodies and flow cytometry.** All cell surface staining was done at 4 °C for 30 min in the dark. Anti-CD8α [53–6.7] (APC-eFluor780), anti-B220 [RA3-6B2] (PE-eFluor610), anti-CD44 [IM7] (PE-Cy7), anti-IFN-γ [XMG1.2] (A488), anti- TNFα [MP6-XT22] (PE), anti-MHCII [M5/114.15.2] (FITC), anti-CD11b [M1/70] (PE-Cy7), anti-CD326/EpCAM [G8.8] (PE-Cy7) anti-CD107a [eBio1D4B] (PE) and Rat IgG2a kappa Isotype control [eBR2a] (PE) were purchased from Thermo Fisher (Waltham, Massachusetts). Anti-KLRG1 [M1A1] (APC), anti-CD127 [SB/199] (PE) anti-CD11c [N418] (biotin), anti-CD45 [30-F11] (Pacific Blue), anti-CD45.2 [104] (PerCP/Cy5.5 or BV421), anti-CD31 [MEC13.3] (biotin), anti-Gp38 [B.1] (PE) and anti-F4/80 [BM8] (PE-Cy7) were purchased from Biolegend (San Diego, California). Anti-CD103 [M290] (PE), anti-TCR Vα2 [B20.1] (PE) and anti-CD45.1 [A20] (APC) were purchased from BD Biosciences (Franklin Lakes, New Jersey). Anti-CD11c [N418] (Alexa fluor-647), anti-CD45.1 [A20] (A488), anti-B220 [RA-6B2] (FITC) and anti-F4/80 [BM8] (biotin) were purchased from AbLab (Vancouver, British Columbia).

Tetramer staining was done at room temperature for 30 min in the dark. H2-Kb tetramers loaded with immune-dominant NP366–374 and PA224–233 peptides from influenza and labeled with Brilliant Violet-421 or Alexa fluor-647 were manufactured and donated by the NIH Tetramer Core Facility (Atlanta, GA).

Viability staining [cat# L34957 and 65-0865-14] (Thermo Fisher) was used according to manufacturer’s instructions.

Samples were collected on either a FACSCanto, LSRII (BD Biosciences) or the Attune NxT (Thermo Fisher) and data were analyzed with FlowJo software Tree Star (Ashland, Oregon).

**Bone marrow chimeras.** Recipient mice were irradiated with 2 doses of 6.5 grey (Gy) or 650 rad at least 4 h apart. For the following 10 days, they were supplemented with antibiotics ad libitum (2 mg/ml neomycin sulfate). 24 h after radiation, femurs and tibias were collected from donor CD45.1 and CD45.2 mice (WT and IL-7Rα 449F respectively). RBCs were removed using sterile ACK lysis. For tetramer response experiments, a total of 5 × 10^5 donor BM cells were injected intravenously (I.V.) at a 1:1 ratio to deliver WT:IL-7Rα 449F into Rag1-/- hosts. For dendritic cell experiments, a total of 5 × 10^5 donor BM cells were injected I.V. into BoyJ (CD45.1/0.2) hosts. 6–8 weeks elapsed for reconstitution before challenge with influenza infection. After euthanasia, spleens and BMs were assessed for reconstitution efficiency and ratios.

**Adoptive transfer.** Single cell suspensions were prepared from multiple OT-I and OT-I;IL-7Rα 449F mice spleens and C8T cells were purified using the CD8 T cell negative selection kit (EasySep® Mouse CDB + T Cell Isolation Kit) from Stem Cell Technologies. 1 × 10^6 cells were transferred I.V. into BoyJ (CD45.1) hosts and 24 h later hosts are challenged with 64HAU PR8-OVA intranasally. MdLN was harvested at experimental endpoint and cells were stained for surface markers and analyzed by flow cytometry as described above.

**Cell culture and IL-7 RT-qPCR.** A549 (ATCC CCL-185) human type II alveolar epithelial cells were obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia). Cells were passaged and expanded in 10% FBS F-12 K Medium from ATCC (Cat No. 30-2004). For experimental use, 5 × 10^5 A549 cells were seeded into 6-well plates in media and expanded for 24 h to achieve confluence. After 1 h of serum starvation, cells were infected with 200 HAU PR8 in PBS and incubated for 2 h on a plate shaker to initiate infection. Virus containing PBS was aspirated and replaced with F-12K media containing 0.5% BSA and 0.5 µg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin. Cells were then incubated for the assigned experimental time points. Cells were lysed and RNA was extracted using PureLink RNA Mini Kit (Thermo Fisher). After treatment with amplification grade DNase I (Thermo Fisher), cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad) and cDNA quantification was performed using the SoFast EvaGreen Supermix kit (Bio-Rad). Primer sequences are as follows. β-actin Forward: GAC ATG GAG AAA ATC TG; β-actin Reverse: ATG ATC TGG GTC ATC TTC TC; Human IL-7 Forward: CCA GGT TAA AAG AAG AAA ACC; Human IL-7 Reverse: TTT CAG TGT TCT TTA GTG CC; Human IFN-β Forward: ACG CCG CAT TGA CCA CCT TAT; Human IFN-β Reverse: GTC TCA TTC CAG CCA GTG CTA; M1 Forward: AGA TGA GTG TTC TAA CCG AGG TCG; M1 Reverse: TGC AAA AAC ATC TTC AAG TCT CG. Measurements were acquired using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad).
**Ex vivo T cell re-stimulation.** Lungs from mice infected (7–9 days) with PR8 were excised and prepared as above. To measure CD107a, 5 × 10⁶ lung cells were re-stimulated for 4 h (37 °C, 5% CO₂) with 1% BSA RPMI containing 50 ng/mL PMA and 500 ng/mL ionomycin from Sigma-Aldrich (St. Louis, Missouri); Monensin from BD Biosciences (Franklin Lakes, New Jersey) used according to manufacturer’s instructions; and anti-CD107a [eBio1D4B] PE®. Following re-stimulation, cells were stained for viability and surface markers then analyzed by flow cytometry.

To measure IFNγ and TNFα, 5 × 10⁶ lung cells were re-stimulated for 3 h (37 °C, 5% CO₂) with 10 nM NPr060, 274 and PA232, 233 Peptides from Anspec (Fremont, California) in the presence of Brefeldin/A from BD Biosciences (Franklin Lakes, New Jersey) in 1% BSA RPMI. Following re-stimulation, cells were stained for viability and surface markers followed by intracellular cytokine staining using the Cytofix/Cytoperm kit from BD Biosciences (Franklin Lakes, New Jersey) then analyzed by flow cytometry.

**Statistical analysis.** All analyses were performed on GraphPad Prism 8 (La Jolla, CA, USA). Samples that were identified as outliers by the Gibbs tests were excluded from the analysis. Two-tailed t-tests and one-way ANOVA with Tukey’s post-test were performed as appropriate and P<0.05 was considered statistically significant. Unless specified, all error analysis in bar charts are presented as S.E.M.

**Ethics.** All experiments described using mice had ethical and procedural approval from the University of British Columbia Animal Care Committee (protocol A17-0249) as per guidelines set by the Canadian Council on Animal Care. No human studies were undertaken. All methods are reported in accordance with ARRIVE guidelines.

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Author contributions
A.S. conceived and designed the project, performed and analyzed experiments, and wrote the manuscript. J.J., H.B.S. and C.Y. performed and analyzed experiments and reviewed the manuscript. J.S. performed and analyzed experiments. N.A. conceived and designed the project and reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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