Interleukin-7, but Not Thymic Stromal Lymphopoietin, Plays a Key Role in the T Cell Response to Influenza A Virus

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Introduction

Influenza A virus is a common human pathogen which causes significant morbidity and mortality worldwide [1]. Seasonal strains that cause annual epidemics can cause severe disease in immunocompromised individuals, including the young and the elderly [2,3]. Novel reassortments of viral genes can occasionally result in highly pathogenic strains with the potential to cause severe disease in healthy adults [4–6]. Although a robust immune response is necessary to clear the virus, highly pathogenic strains can induce an overactive response that can significantly contribute to disease. Thus, a more detailed understanding of the determinants of the outcome of response to Influenza A infection might assist in preventative or therapeutic approaches.

Influenza A/PR/8/34 (H1N1) (referred herein as “PR8”) was derived from a human influenza strain and subsequently adapted to transmit from ferrets to mice [7]. This influenza A strain infects lung epithelial cells, forming a local infection that resolves in 10 days in wild-type mice [8]. Resolution of the primary infection is dependent on development of a well-defined influenza A specific T cell response. As a result, it is a clinically relevant model of a local respiratory virus infection.

Interleukin-7 (IL-7) has a critical role in the adaptive immune system and its response [9,10]. While IL-7 has been primarily characterized for its role in lymphocyte development, it has also been recently shown to boost the T cell response against chronic viral infections and tumors [11,12]. The role of the IL-7-related cytokine thymic stromal lymphopoietin (TSLP) has been more recently appreciated in the development of allergic and anti-parasite responses [13]. However, the role of these cytokines in acute viral infection is not clear.

IL-7 exerts its effects via a heterodimeric receptor of IL-7Rα and TSLP [14]. IL-7Rα also serves as a receptor for TSLP when it is paired with TSLPR [15]. IL-7Rα and TSLPR are expressed on T cells and dendritic cells, as well as other innate immune cells with most of these cells co-expressing the γc. IL-7 is currently in clinical trials to enhance the response of and expand T cells in patients infected with HIV as well as in cancer patients and after bone marrow transplants [16].

Abstract

The immune response to viral infection is ideally rapid and specific, resulting in viral clearance and establishment of immune memory. Some viruses such as HIV can evade such responses leading to chronic infection, while others like Influenza A can elicit a severe inflammatory response with immune-related complications including death. Cytokines play a major role in shaping the appropriate outcomes to infection. While Interleukin-7 (IL-7) has a critical role in T and B cell development, treatment with IL-7 has recently been shown to aid the adaptive T cell response in clearance of chronic viral infection. In contrast, the IL-7-related cytokine thymic stromal lymphopoietin (TSLP) has a limited role in lymphocyte development but is important in the immune response to parasitic worms and allergens. The role for these cytokines in the immune response to an acute viral infection is unclear. IL-7 and TSLP share IL-7Rα as part of their heterodimeric receptors with the gamma common chain (γc) and TSLPR, respectively. We investigated the role of IL-7 and TSLP in the primary immune response to influenza A infection using hypomorphic IL-7Rα (IL-7RαΔγc) and TSLPR−/− mice. We found that IL-7, but not TSLP, plays an important role in control of influenza A virus. We also showed that IL-7 signaling was necessary for the generation of a robust influenza A-specific CD4 and CD8 T cell response and that this requirement is intrinsic to CD8 T cells. These findings demonstrate a significant role for IL-7 during acute viral infection.

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Introduction

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IL-7 exerts its effects via a heterodimeric receptor of IL-7Rα and TSLP [14]. IL-7Rα also serves as a receptor for TSLP when it is paired with TSLPR [15]. IL-7Rα and TSLPR are expressed on T cells and dendritic cells, as well as other innate immune cells with most of these cells co-expressing the γc. IL-7 is currently in clinical trials to enhance the response of and expand T cells in patients infected with HIV as well as in cancer patients and after bone marrow transplants [16].
TSLP acts primarily in the generation of immune response against parasites, however, in contrast to IL-7, it has little role in the development of the immune system [17]. TSLP has effects on the adaptive immune system both directly on T cells and via innate immune cells, such as dendritic cells [18–20]. TSLP is over expressed in many atopic diseases in humans including asthma and dermatitis [21]. Its expression can also be induced in bronchial epithelial cells by viral molecular motifs, such as CpG, suggesting it may play a role in defense against viral infections [22].

Signaling downstream of IL-7Rα is largely dependent on phosphorylation of tyrosine 449, nested within a YxxM recruitment motif. Mice bearing a mutated receptor with homozygous knock-in replacement of this tyrosine with phenylalanine (IL-7Rα^{Y449F}) have impaired signaling in response to IL-7 [23]. Although IL-7Rα^{Y449F} mice have fewer mature T cells compared to WT mice, the lymphocyte compartment is significantly restored compared to IL-7Rα^{−/−} mice. This allows functional studies to be performed on these mice to investigate the role of IL-7Rα Tyr449 signaling. We showed that IL-7Rα^{Y449F} mice have a defective CD4 T cell response to Listeria monocytogenes infection, which suggested an unexpected role for IL-7-related cytokines in the primary T cell response [23]. Although IL-7 can boost T cell function during chronic inflammation, its role in the acute primary T cell response is unknown. Similarly, while TSLP has defined roles in the immune response to parasites, its role in viral infections has not been characterized.

We have investigated the role of IL-7 family cytokines during acute respiratory infection in mice infected with influenza A PR8. We found that IL-7, but not TSLP, plays an important role in viral clearance and protection from weight loss. We showed that IL-7, but not TSLP, plays an important role in viral clearance and protection from weight loss.

**Materials and Methods**

**Ethics Statement**  
Mice were housed at the UBC Centre for Disease Modelling facility, and all work was carried out according to University of British Columbia Animal Care and Biosafety Committee guidelines. Breeding and project protocols (A07–0415 and A07–0417) were approved by the institutional committee for this work. All efforts were made to minimize suffering, with minimally invasive procedures, and where warranted (viral infection), isoflurane and isoﬂurane and infected intranasally with 5 hemagglutinin units (HAU) of PR9 in 12.5 μL of sterile PBS.

**Cell Preparation and Flow Cytometry**

Bronchoalveolar lavage (BAL) fluid was obtained by inserting a tracheal catheter and washing the bronchoalveolar space four times with 1 mL of PBS with 10% FBS. Lymphocytes were extracted from the lungs of mice by mincing with scissors, digesting with 100 units/mL Collagenase IV for 1 hour at 37°C before filtering with a 70 μm filter to remove debris. Splenocytes were collected and forced through a 70 μm filter to obtain a single cell suspension.

Antibodies were purchased from BD Bioscience (San Diego, CA), eBioscience (San Diego, CA) or BioLegend (San Diego, CA). H2-Kb1 and I-Aq tetramers loaded with immunodominant NP365–374, PA224–233 and NP111–125 peptides from influenza A and labeled with PE or APC were made by the NIH Tetramer Core Facility (Atlanta, GA).

Tetramer staining was carried out at RT for 10 minutes before antibody staining at 4°C for 30 min. Data was acquired on a LSRII using FacsDiv software (BD Bioscience) and analysed using FlowJo software (TreeStar, Stanford, CA).

**Plaque Assays**

Lungs from infected mice were homogenized using a Fisher TissueMiser, diluted and incubated on confluent MDCK cells for 1 hour at RT. The cells were then washed and a solution of 0.7% agarose, 0.1% trypsin in ζ-MEM was applied. Samples were incubated at 37°C for 4 days before staining with crystal violet and counting of plaques.

**M1 RNA Detection**

Lung from infected mice were homogenized in Trizol (Invitrogen), RNA was extracted as per manufacturer’s instructions and cDNA was prepared. Levels of viral Matrix gene transcript were then detected using M1F AGATGATCTTCTAAACC-GAGGTCG, M1R TGCAAAAAACATCTCCAAGTCTCTG and an internal probe TCAGGCCCGCGAAAGC CG in duplicate using a Mx3005p PCR multiplex quantitative PCR instrument (Stratagene) with the Qiagen Quantitect qRT-PCR Kit (Qiagen). Total M1 copy numbers were determined by comparison to an in vitro transcribed standards of known concentration [24].

**Mixed Bone Marrow Chimeras**

CD45.1 mice were irradiated with two doses of 6.5 Gy four hours apart and then injected 24 hours later with a 9:1 mixture of IL-7Rα^{Y449F}:BoyJ or 1:1 mixture of TSLPR^{−/−}:BoyJ whole bone-marrow. Mice were infected with influenza A virus as above 6–8 weeks later.

**Statistical Analysis**

Data are presented as mean±SEM and analyzed by Student’s t test or one-way ANOVA with Tukey’s post-test as appropriate. Results giving a p-value of less than 0.05 were considered to be significant.

**Results**

IL-7 is Critical for Control of and Protection from Influenza A Virus Infection

To assess the role of IL-7Rα signaling in influenza A pathogenicity, WT, IL-7Rα^{Y449F}, TSLPR^{−/−} and IL-7Rα^{Y449F}
TSLPR<sup>−/−</sup> double-mutant (referred herein as “Dmu”) mice were infected with 5 HAU of influenza A PR8 by intranasal inoculation. This complement of genetic strains allowed us to distinguish the requirement for IL-7, or TSLP. We monitored mice for weight loss as an indicator of the severity of disease. While TSLPR<sup>−/−</sup> mice showed similar weight loss compared to WT mice, both IL-7R<sup>449F</sup> and Dmu mice lost significantly more weight from day 7 post-infection onwards compared to WT (Fig. 1a) suggesting that IL-7, not TSLP, was critical for protection from influenza A. We then determined whether the weight loss seen was due to a failure to clear the virus by examining the levels of infectious virus in the lungs of mice nine days after infection. While WT lung homogenates did not develop detectable plaques on MDCK cells and TSLPR<sup>−/−</sup> homogenates had low numbers of plaques, the lung homogenates from IL-7R<sup>449F</sup> and Dmu mice had elevated levels of infectious virus (Fig. 1b). These results suggested that while TSLP had no role in control of influenza A virus or protection from virus induced weight loss, IL-7 signaling through IL-7R<sup>449F</sup> was critical for both. Interestingly, viral M1 transcript levels were only elevated in Dmu mice (Figure S1), showing a discrepancy between the level of detectable infectious virus and viral transcripts in IL-7R<sup>449F</sup> mice for unknown reasons.

**IL-7 Signaling is Necessary for a Robust Specific T Cell Response against Influenza A Virus**

As IL-7R<sup>449F</sup> signaling is necessary for the formation of an effective primary CD4 T cell response and influences the response to TCR stimulation [23], we examined whether IL-7R<sup>449F</sup> was important for the formation of the T cell response to influenza A. We evaluated the CD4 and CD8 T cell response in WT, IL-7R<sup>449F</sup>, TSLPR<sup>−/−</sup> and Dmu mice infected with influenza A virus. The number of CD4 and CD8 T cells in the lung and BAL of IL-7R<sup>449F</sup> and Dmu mice was reduced compared to WT after infection, while TSLPR<sup>−/−</sup> mice had normal numbers of CD4 and CD8 T cells (Fig. S2). However, as both IL-7R<sup>449F</sup> and Dmu mice have reduced number of baseline CD4 and CD8 T cells in the spleen and lung prior to infection (Figure S3) we examined the frequency of CD8 T cells that recognize the virus with MHC class I tetramers containing immunodominant viral epitopes NP<sub>366-374</sub> and PA<sub>224-233</sub> [25,26]. At day 9 post-infection, the proportion of CD8 T cells that recognized both the NP<sub>366-374</sub> and PA<sub>224-233</sub> tetramers was significantly reduced in IL-7R<sup>449F</sup> compared to WT in both the BAL and the lung (Fig. 2a). IL-7R<sup>449F</sup> mice also had reduced frequency of CD4 T cells that recognize the NP<sub>311-325</sub> epitope (Fig. 2b). In contrast, TSLPR<sup>−/−</sup> mice had similar levels of tetramer-binding CD4 and CD8 T cells compared to WT mice and Dmu mice had a comparable T cell response to IL-7R<sup>449F</sup> mice. These results indicate that IL-7, but not TSLP, was important for the influenza A specific T cell response.

**IL-7 and TSLP Regulate CXCR3 Expression on Virus Specific T Cells**

We then asked if the reduction in T cells seen in the lung of infected IL-7R<sup>449F</sup> mice was due to a failure of cells to migrate from the lymph nodes into the infected areas of the lung and airways. Several integrins and chemokine receptors, in particular CD11a [27], CD29, and CXCR3 [28] have been implicated in migration of T cells to the lung during influenza A infection. Although IL-7 has been reported to influence expression of the integrins CD11a and CD29 [29], we found no difference in the expression of these proteins on influenza specific T cells from IL-7R<sup>449F</sup>, TSLPR<sup>−/−</sup> or Dmu mice (Fig. 3a). However, there was a significant reduction in the expression of the chemokine receptor CXCR3 on influenza A specific CD8 T cells from the lungs of infected IL-7R<sup>449F</sup>, TSLPR<sup>−/−</sup> and Dmu mice. This suggested that IL-7 and TSLP may both regulate the expression of CXCR3 in mice infected with influenza A.

**Generation of Influenza A Specific T Cells Requires Cell Intrinsic IL-7 Signaling**

To investigate whether the requirement for IL-7R<sup>449F</sup> signaling was due to signaling within T cells, we made a series of mixed bone marrow chimeras to examine the response of congenically marked WT and mutant lymphoid cells within the same mouse. Irradiated RAG1<sup>−/−</sup> mice were reconstituted with a mixture of BoyJ (WT, CD45.1<sup>+</sup>) and IL-7R<sup>449F</sup> (CD45.2<sup>+</sup>) bone marrow. As IL-7R<sup>449F</sup> T cells performed poorly in competitive repopulation against WT during development [23] we transferred bone marrow at a ratio of 9:1 IL-7R<sup>449F</sup>:WT to obtain mice with a sufficiently large IL-7R<sup>449F</sup> CD8<sup>+</sup> T cell compartment to accurately evaluate their response. Reconstituted mice were then infected with influenza A six weeks after irradiation and transplantation and the frequency of influenza A specific CD8 T cells within either the
WT or IL-7Rα^{449F} derived cells was examined at day 9 post-infection. As expected, IL-7Rα^{449F} T cells were heavily outcompeted by WT T cells during development (Fig. 4a). As a result, IL-7Rα^{449F} T cells make up only 20% of the total T cell population in these mice, despite having been reconstituted at a 9:1 advantage. This output ratio is consistent with chimerism we have observed in uninfected mice (data not shown). Our data showed that within the same host, the frequency of NP366–374 specific cells among CD8 T cells in the lung was significantly reduced in the IL-7Rα^{449F} population compared to the WT population (Fig. 4a). The NP311–325 CD4 T cell response in IL-7Rα^{449F} cells also showed a trend towards impairment, but this was not a significant difference (Figure S4), perhaps due to the lack of a robust response in WT CD4 T cells in these chimeric mice. Taken together, our data clearly indicated a cell-intrinsic requirement for IL-7Rα signaling in the development of the specific CD8 T cell response to influenza A.

TSLP has been shown to influence CD8 T cell homeostasis and survival in competition with WT cells although no defect was observed in TSLPR^{2/2} mice [30]. To determine if a role for TSLP in the generation of influenza A specific T cells could be observed in competition with WT cells, we transferred a 1:1 mixture of TSLPR^{−/−}:WT bone marrow into irradiated recipients. Reconstituted mice were then infected with influenza A six weeks after irradiation and transplantation, and the frequency of influenza A specific CD8 T cells within either the WT or TSLPR^{−/−} derived cells was examined at day 9 post-infection. WT and TSLPR^{−/−} T cells contributed equally to the influenza A

Figure 2. CD4 and CD8 T cell response to Influenza A requires IL-7Rα, but not TSLPR, signaling. A) Representative FACS plots and bar graphs of percent of total CD8 T cells specific for NP_{366-374} (top) or PA_{224-233} tetramer (bottom) in the BAL and lung. B) Representative FACS plots and bar graph of percent of total CD4 T cells specific for NP_{311-325} tetramer in the BAL and lung. Data shown is from one representative experiment of three experiments, n = 3-5. *p < 0.05 by ANOVA with Tukey’s post-test.

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specific CD8 T cell populations in the lung (Fig. 4b) and BAL (data not shown). Furthermore, the NP311–325 specific CD4 T cell response in the TSLPR−/− compartment also showed no impairment, consistent with direct infection of TSLPR−/− mice (Figure S4). Our data refute a role for cell intrinsic TSLP signaling in the T cell response to influenza A, suggesting that the observed defects in IL-7Rα449F cells in chimeric animals were due to the loss of IL-7 signals.

**Discussion**

IL-7 and TSLP have well described roles as modulators of T cell immunity. IL-7 has been shown to enhance the T cell response against chronic viral infections and tumours [11,12] and we have previously reported its role in the T cell response to systemic challenge by *Listeria monocytogenes*. TSLP has primarily been characterized for its role in Th2-mediated immune responses, however it can be strongly induced by a variety of viral ligands and has been shown to influence the T cell response to BCG [22,31]. The role of IL-7 in mature lymphocytes is difficult to study due to the severity of B and T cell defects in IL-7Rα−/− mice [32]. We previously generated IL-7Rα449F mice that are hypomorphic for IL-7Rα function. Unlike IL-7Rα−/− mice, these mice develop mature B and T cells allowing evaluation of the role of IL-7Rα signaling in mature lymphocyte function. By comparing IL-7Rα449F mice to WT and TSLPR−/− mice we are able to identify discrete roles for IL-7 or TSLP for an observed phenotype.

We showed that IL-7-mediated signaling was necessary for the generation of the primary CD4 and CD8 T cell responses against a physiologically relevant, acute influenza A mucosal challenge. The impaired CD4 T cell response to influenza A in IL-7Rα449F mice is consistent with our previous findings with a systemic *Listeria monocytogenes* challenge [23]. However, the defect in the primary CD8 T cell response in IL-7Rα449F mice to influenza A was surprising as no such defect was observed in the *Listeria* study. As both CD4 and CD8 IL-7Rα449F T cells showed a dose dependent...
CD45.1 BoyJ mice were reconstituted with (A) 9:1 IL-7R
signaling in CD8 T cell response to Influenza A.

Engagement by sub-optimal antigenic stimuli is enhanced by IL-7R
requirement for IL-7R signals. Our current model is that TCR
infection and antigenicity of pathogen challenge can determine the
defect in anti-CD3 induced division [23], we postulate the route of
infection and antigenicity of pathogen challenge can determine the
requirement for IL-7R signals. Our current model is that TCR
engagement by sub-optimal antigenic stimuli is enhanced by IL-
7R signals, is impaired in IL-7Rx449F T cells and that this can be
negated by strong T cell receptor signaling.

Our data showed no role for TSLP in the CD8 T cell response
to influenza A PR8 in contrast to recent findings [33]. As
alterations in the gut normal flora can influence the immune
response to influenza A [34], it is possible that differences in the
flora of the mice alter the contributions of the TSLP dependent
response in these mice. Alternatively, although both studies
examined low dose challenges of influenza A, the virus dose may
still contribute to the differences seen between the studies.

Previous studies have shown that depletion of lymphoid subsets
could raise the susceptibility of mice to influenza A infection [5].
However, these studies were performed in mice that lacked one or
more lymphoid subsets. Models of partial lymphopenia have not
been studied and thus it is unclear whether the reduced number of
CD4 and CD8 T cells in IL-7Rx449F mice contribute to the
increased severity of the influenza A infection in these mice.

Nonetheless, our bone marrow chimeras with IL-7Rx449F T cells
clearly showed in lymphoreplete mice that IL-7Rx signals are
necessary for an effective T cell response. Thus, while lympho-
penia may contribute to disease severity in IL-7Rx449F mice, it is
by no means the sole reason for the loss of viral control observed.

Migration of cells to the site of influenza infection is dependent
on expression of integrins and chemokine receptors. We found no
defect in the expression of the integrin subunits CD11a or CD29.
IL-7Rx449F and TSLPR−/− mice both showed reduced expres-
sion of CXCR3. However due to the lack of a defect in both the
proportion of tetramer positive cells or disease in TSLPR−/−
mice, we would argue that although interesting, this chemokine
receptor is not required for the T cell response to influenza A. The
contribution of a migratory defect to the T cell deficiency observed
in the lung could be determined by comparing the number of
influenza A specific T cells in the lung versus the draining lymph
nodes. If the response is normal in the mediastinal lymph node in
IL-7Rx449F mice, this would imply that defective migration of the
cells to the lung, and not initiation and amplification of the T cell
response is responsible for the reduction in influenza A specific T
cells observed in these mice.

IL-7 has a clear role in survival and homeostasis of T cells,
which is in part mediated by signaling through STAT5. STAT5
also has a critical role in maintaining the T cell response to LCMV
as expression of dominant-negative STAT5 in T cells results in a
reduction in cellular proliferation and survival [35]. IL-7Rx449F
cells do not phosphorylate STAT5 in response to IL-7, suggesting
that the defect seen in this mouse may mirror that seen in
dominant-negative STAT5 mutant cells.

We show that IL-7, and not TSLP, plays an important role in
the control of influenza A virus and in the generation of the
influenza A specific T cell response. These findings have
implications for shaping therapies directed at use of IL-7 in
immune reconstitution and inhibition of TSLP in atopic pathol-

Supporting Information

Figure S1 Quantification of M1 viral RNA in the lungs.
C57BL/6, IL-7Rx449F, TSLPR−/− and Dmu mice were infected
intranasally with 5HAU influenza A PR8. At day 9 post-

infection lungs were harvested, homogenized in Trizol and

absolute amount of influenza A M1 mRNA was determined by
qPCR. Data shown is pooled from three experiments.*p<0.05 by
ANOVA with Tukey’s post-test.

(EPS)
to identify WT or mutant derived cells. Each population was post-infection. CD4 T cells were stained for CD45.1 and CD45.2 and spleen from uninfected C57BL/6, IL-7R 

and Dmu mice. Data shown are from one representative experiment of three experiments, n = 3–4. *p<0.05 by ANOVA with Tukey’s post-test.

Baseline CD4 and CD8 T cell in the lung and spleen. Total number of (A) CD8 and (B) CD4 T cells in the lung and spleen from uninfected C57BL/6, IL-7R 

and Dmu mice. Data shown are from one representative experiment of three experiments, n = 3–4. *p<0.05 by ANOVA with Tukey’s post-test.

Cell-intrinsic requirement for IL-7R 

Lethally irradiated CD45.1 BoyJ mice were reconstituted with (A) 9:1 IL-7R 

BoyJ or (B) 1:1 TSLPR 

:BoyJ bone-marrow and allowed to recover for 6–8 weeks. Mice were infected with 5HAU of PR8 and the CD4 T cell response was analyzed at day 9 post-infection. CD4 T cells were stained for CD45.1 and CD45.2 to identify WT or mutant derived cells. Each population was analyzed for the percent of CD4 T cells that recognize the NP 

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Author Contributions

Conceived and designed the experiments: AWP E-KL JHS NA. Performed the experiments: AWP DTP JHS E-KL. Analyzed the data: AWP DTP E-KL NA. Contributed reagents/materials/analysis tools: FJ SFZ NA. Wrote the paper: AWP DTP NA.
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