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J Immunol 2008; 180:171-178; doi: 10.4049/jimmunol.180.1.171
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Loss of IL-7R and IL-15R Expression Is Associated with Disappearance of Memory T Cells in Respiratory Tract following Influenza Infection

Ching-Hung Shen,* Qing Ge,* Oezcan Talay,* Herman N. Eisen,* Adolfo García-Sastre,† and Jianzhu Chen2*

Following influenza virus infection, memory CD8 T cells are found in both lymphoid and nonlymphoid organs, where they exhibit striking differences in survival. We have assessed persistence, phenotype, and function of memory CD8 T cells expressing the same TCR in the airways, lung parenchyma, and spleen following influenza virus infection in mice. In contrast to memory CD8 T cells in the spleen, those residing in the airways gradually lost expression of IL-7R and IL-15R, did not respond to IL-7 and/or IL-15, and exhibited poor survival both in vivo and in vitro. Following adoptive transfer into the airways, splenic memory CD8 T cells also down-regulated IL-7R and IL-15R expression and failed to undergo homeostatic proliferation. Thus, although cytokines IL-7 and IL-15 play an essential role in memory CD8 T cell homeostasis in lymphoid organs, the levels of IL-7R and IL-15R expression likely set a threshold for the homeostatic regulation of memory CD8 T cells in the airways. These findings provide a molecular explanation for the gradual loss of airway memory CD8 T cells and heterosubtypic immunity following influenza infection. The Journal of Immunology, 2008, 180: 171–178.

Influenza A viruses cause epidemics and pandemics in human populations, inflicting enormous suffering and economical loss. The most commonly used influenza vaccines, formalin-inactivated viruses, induce neutralizing Abs against viral coat proteins hemagglutinin and neuraminidase. However, these vaccines need to be reformulated every year because hemagglutinin and neuraminidase often undergo sequence changes due to antigenic drift and shift. It has been proposed that inducing cellular immunity, particularly CD8 T cell responses, against conserved viral epitopes may confer long-term protection against different strains of influenza viruses (heterosubtypic immunity) (1, 2).

Following activation by appropriate Ags in lymphoid organs, CD8 T cells acquire effector functions and migratory properties that lead them to enter nonlymphoid tissues (3). Most of the effector CD8 T cells die by apoptosis after Ags are cleared, and only a small fraction of the cells survive and differentiate into memory cells, which reside in both lymphoid and nonlymphoid organs and provide long-term protection against reinfection by the same pathogen. However, the ability of memory CD8 T cells to confer heterosubtypic immunity against the influenza virus appears to be short lived and declines within a few months (1). This decline is shown to result from a gradual loss of virus-specific memory CD8 T cells in the airways (4). This loss is puzzling in view of the fact that significant numbers of memory CD8 T cells with the same Ag specificity persist in the spleen for almost the entire lifetime of a mouse (5, 6). The question that arises is why do memory CD8 T cells disappear rapidly from the airways, or more broadly, what mechanisms control the differences in survival of memory CD8 T cells in various tissues.

Cytokines IL-7 and IL-15 play an essential role in regulating the generation and maintenance of memory T cells (7). IL-7 promotes the survival and differentiation of memory CD8 T cells whereas IL-15 promotes their homeostatic proliferation (8–10). In the absence of IL-15, memory CD8 T cells gradually decline in numbers (11), which can be restored by overexpression of IL-7 (8). These advances in understanding memory T cell homeostasis have been based on studies of cells from lymphoid organs. If and how these cytokines regulate memory CD8 T cell homeostasis in nonlymphoid tissues, such as the lung airways, are unknown.

We have developed a mouse model of influenza virus infection where CD8 T cell responses to the virus can be monitored in any organ at any time. Using this model, we investigated the molecular basis underlying the differences in persistence of memory CD8 T cells expressing the same TCR in the airways, lung parenchyma, and spleen. Our data suggest that the differences in IL-7R and IL-15R expression on memory T cells in the airways and the spleen likely contribute to the differences in persistence of memory CD8 T cells between the two locations. These findings provide a molecular explanation for the gradual loss of airway memory CD8 T cells and cellular immunity against subsequent heterosubtypic influenza virus infection.

Materials and Methods

Mice and viruses

The 2C TCR transgenic mice on the RAG1–/– and C57BL/6 (B6-Thy1.2) background (2C+ RAG–/–) (12) were maintained in the animal facility at the Massachusetts Institute of Technology (MIT). These mice express a

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transgenic TCR on CD8 T cells recognizing SIYRYYGL peptide (SIY)3 in association with MHC class I K* molecule and were used as donors. Male B6 mice (Taconic Farms) and B6.PL-Thy1.1/CyJ mice (The Jackson Laboratory) expressing the congenic Thy1.1 marker were used as recipients at 8–12 wk of age. The use of animals was in compliance with the guidelines set by the MIT Committee on Animal Care. The recombinant WSN-SIY influenza virus encoding the SIY epitope in the neuraminidase stalk was constructed by plasmid-based reverse genetics (13) and grown in Madin-Darby canine kidney cells.

Adoptive transfer, infection, and tissue preparation
Donor cells (1–2 × 106 total cells containing 2.5–5 × 105 CD8+ 2C cells) from lymph nodes and spleen of 2C+ RAG—/- mice were injected i.v. into B6 or B6-Thy1.1 recipient mice. One day later, recipient mice were anesthetized with avertin (Sigma-Aldrich) and infected intranasally with 100 PFU of WSN-SIY virus. To analyze 2C T cell response, airway cells were obtained by bronchial lavage using 1 ml of HBSS each time for three times. After airway cells were collected, lungs were harvested and ground through a cell strainer, followed by incubation with 2 ml of milk digestion buffer (RPMI 1640 medium containing 3 mg of collagenase A (Roche) /ml, 5% FCS, and 10 mM HEPES (pH 7.4)) at 37°C for 1 h. Splenocytes were collected in 8 ml of HBSS through glass slides and filtering the cell suspension through 80-μm nylon filters. Numbers of total live cells from different samples were counted by trypan blue exclusion after the lysis of RBC. In some experiments, cells from several mice (usually 4–6, but as many as 20) were pooled as indicated to obtain sufficient numbers of cells for analysis. In some experiments, donor cells (1 × 106/ml) were labeled with CFSE (5 μM) at room temperature for 10 min before adoptive transfer.

For intratracheal transfer of splenic memory 2C cells, splenocytes were pooled from four infected B6 recipient mice 30 days postinfection (dpi). CD8+ cells were depleted with the CD8+ T cell isolation kit (Miltenyi Biotec). The remaining cells (enriched for CD8+ T cells) were labeled with CFSE (0.5 μM) or left untreated, and transferred into naive B6 mice intratracheally by flushing the spleen with frosted glass slides and filtering the cell suspension through 80-μm nylon filters. Numbers of total live cells from different samples were counted by trypan blue exclusion after the lysis of RBC. In some experiments, cells from several mice (usually 4–6, but as many as 20) were pooled as indicated to obtain sufficient numbers of cells for analysis.

Abs and flow cytometry
Abs to IL-7R, γc, TCRβ, CD8, CD62L, Thy1.2, mouse IgG1 (BD Biosciences), IL-7Ra, and human Fcγ (eBioscience) were used as conjugates with PE, FITC, or allophycocyanin. CCR7 was detected by CCL19-Fc fusion protein (eBioscience) followed by Abs to human Fcγ. SIY-specific CD8 T cells were identified by H-2Kb-Ig fusion protein (BD Biosciences) followed by Abs to mouse IgG1 and CD8. Clonotypic Ab 1B2 specific for the association with MHC class I Kb molecule and were used as donors. Male B6 mice (Taconic Farms) and B6.PL-Thy1.1/CyJ mice (The Jackson Laboratory) expressing the congenic Thy1.1 marker were used as recipients at 8–12 wk of age. The use of animals was in compliance with the guidelines set by the MIT Committee on Animal Care. The recombinant WSN-SIY influenza virus encoding the SIY epitope in the neuraminidase stalk was constructed by plasmid-based reverse genetics (13) and grown in Madin-Darby canine kidney cells.

In vitro proliferation analysis
Pooled samples from B6 recipient mice 30 dpi were collected. Aliquots were labeled with CFSE and cultured with IL-7 (20 ng/ml) and/or IL-15 (40 ng/ml) or medium alone for 5 days. To measure cell numbers before and after culture, aliquots were seeded and absolute numbers of cells were obtained by incorporating a fixed number of fluorescent beads (1 × 106 particles; BD Biosciences) in each sample during flow cytometry analysis. The number of live 2C cells in each aliquot was normalized by the number of 2C cells seeded in the culture. For samples treated with anti-CD3e Ab, cells were labeled with CFSE and incubated with either immobilized anti-CD3e Ab or medium alone for 2 days. Cells were then transferred to a new flask and cultured for 3 more days in the presence of IL-2 (10 U/ml). For analysis of cell division 7 or more days following the virus infection, cells (2 × 106 from airways or lung parenchyma and 1 × 106 from spleen) from infected mice were mixed with splenocytes (1 × 106) from naive B6 mice. The cells were labeled with CFSE and cultured for 2 days in the presence of IL-2 (10 ng/ml).

Quantitative RT-PCR and ELISA
To measure the levels of IL-7 and IL-15 transcripts in the tissues, first-strand cDNAs (100 ng) from mouse tissues (MTC Multiple Tissue cDNA Panels, Clontech Laboratories) were added to 25 μl of PCR premix containing SYBR Green and a primer set for the IL-7, IL-15, or β-actin gene (SuperArray Bioscience), followed by incubation at 95°C for 15 min and at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 50 cycles using an ABI 7000 real-time PCR cycler (Applied Biosystems). To measure the levels of IL-7R, IL-15R, γc, or β-actin transcripts in 2C T cells, 2 × 106 naive or memory 2C cells (2C TCR+ and CD8−) from the airways, lung parenchyma, and spleen were sorted by a MoFlow cell sorter (BD Biosciences), and the first-strand cDNAs were obtained with the CellsDirect qRT-PCR kit, according to the manufacturer’s instructions (In vitrogen Life Technologies). The PCR condition was the same as described above except a primer set for the IL-7R, IL-15R, γc, or β-actin gene (SuperArray Bioscience) was used. The condition described above, a single band with the expected size was detected for each primer set, and the standard curves had a minimal regression coefficient of 0.99.

Results
A model system to study CD8 T cell responses to influenza virus
Endogenous CD8 T cells specific for immunodominant epitopes of influenza viruses have been assayed during T cell responses to influenza infection (14). However, they are usually below the detection limit in most tissues before clonal expansion and 2 mo after infection. In addition, expression of diverse TCR by responding polyclonal CD8 T cells complicates direct comparison of their persistence in different organs. Thus, we have developed a mouse model of influenza virus infection in which a cohort of CD8 T cells specific for the same epitope can be monitored at any time in any organ.

The model system takes advantage of CD8 T cells expressing a transgenic TCR called 2C (12) and a recombinant influenza virus that expresses the SIY epitope recognized by the 2C TCR, similar to the use of transgenic T cells expressing the OT-I TCR and SIINFEKL-expressing influenza virus (15). In a typical experiment, naive 2C cells from 2C TCR transgenic mice on the RAG1−/− and C57BL/6 (B6) background were adoptively transferred into B6 or congenic B6-Thy1.1 mice (2.5–5 × 105 2C TCR+ CD8− 2C cells per recipient). On the following day, recipient mice were infected intranasally with a sublethal dose (100 PFU) of the WSN strain of influenza A virus that expresses the SIY epitope (referred to as WSN-SIY). On different days following infection, the number and phenotype of 2C cells in various organs were monitored by cell counting plus flow cytometry, using the clonotypic Ab 1B2 to identify 2C cells (12).

References
3 Abbreviations used in this paper: SIY, SIYRYYGL peptide; dpi, days post-infection; MLN, mediastinal lymph node; FSC, forward scatter; pStat5, phosphorylated Stat5.
Using this model system, we examined the initial CD8 T cell response and subsequent development of memory CD8 T cells to influenza virus. As indicated by down-regulation of TCR and activation of CD69 expression (Fig. 1A), 2C cells (2C TCR\(^{+}\) CD8\(^{+}\)) first became activated 2 days postinfection (dpi) in the mediastinal lymph node (MLN) that drains the lung, but not in peripheral lymph nodes and spleen. Three dpi, 2C cells in the MLN were activated to express CD25 and CD44 and started to proliferate, and rapid proliferation occurred between 4 to 5 dpi (Fig. 1B). Following activation and proliferation, effector 2C cells became detectable in the airways (bronchial lavage) and the lung parenchyma around day 4 or 5 and the largest numbers of 2C cells were observed 7 dpi (Fig. 1B and data not shown). Unlike newly activated effector CD8 T cells (7 dpi), which were large (high forward scatter (FSC) values) and proliferating, by 21 dpi the persisting 2C cells in the airways, lung parenchyma, and spleen were as small as naive T cells (Fig. 1C) and had stopped Ag-induced proliferation (shown as CFSE\(^{hi}\) cells, Fig. 1D), indicating acquisition of memory phenotype. Similar to memory CD8 T cells induced by other infections, memory 2C cells induced by influenza virus were heterogeneous (16, 17). In the spleen, some exhibited the characteristic phenotype of central memory T cells (CD62L\(^{hi}\) CCR7\(^{hi}\)) and some intermediate memory T cells (CD62L\(^{lo}\) CCR7\(^{hi}\)) (Fig. 1E, F). In the airways and lung parenchyma, persisting 2C cells exhibited the phenotype of effector memory T cells (CD62L\(^{lo}\) CCR7\(^{lo}\)). As described below, memory 2C cells had a similar expression pattern of CD62L and homeostatic cytokine receptors as endogenous SIY-specific CD8 T cells from the same organs (Fig. 6). Together, these results suggest that 2C cell responses to influenza infection in this model system are comparable to those of previously characterized endogenous and TCR transgenic CD8 T cells in similar systems (14, 15, 18).

**Contraction and persistence of 2C cells in different organs**

To investigate the dynamics of T cell responses to influenza infection, we measured the number and percentage of 2C cells in the spleen, the lung parenchyma, and the airways 7 to 225 dpi. At the peak of the response (7 dpi), ~3.4% of cells in the spleen were 2C cells, accounting for a total of ~1 \times 10^7 cells (Fig. 2, A and B). By 30 dpi, 2C cells in the spleen have contracted three times and thereafter, stable numbers of 2C cells were maintained (3–4 \times 10^5 cells per spleen, Fig. 2B). Seven dpi, ~27% of cells recovered from the lung parenchyma were 2C cells and the total number was similar to those in the spleen. However, 2C cells in the lung parenchyma contracted more extensively and reached a steady state by 30 dpi with smaller stable numbers (1–2 \times 10^5 cells per mouse). Seven dpi, almost 50% of cells in the airways were 2C cells, and the total number was as large as in the spleen or as in the lung parenchyma. In contrast, 2C cells in the airways contracted even more extensively by 30 dpi and in addition the numbers continued to decline as time progressed. By 225 dpi, there were only a few hundred 2C cells remaining. These results show that although similar numbers of effector 2C cells are present in the spleen, the

**FIGURE 1.** 2C cell responses to influenza virus infection. A, Cells from the MLN, peripheral lymph nodes, or spleen of infected recipient mice at the indicated times were analyzed for 2C TCR, CD8 plus CD69 or CD25 or CD44. The 2C TCR vs CD8 profile is shown for all live cells in the indicated tissues. Histograms shown are gated on 2C cells (2C TCR\(^{+}\) CD8\(^{+}\)). B, CFSE-labeled naive 2C cells were adoptively transferred into B6 mice followed by infection. Cells from the indicated tissues of the infected recipient mice were analyzed for cell proliferation. The 2C TCR vs CFSE profile is shown for CD8\(^{+}\) CFSE\(^{−}\) cells 2 and 3 dpi and 2C TCR\(^{+}\) CD8\(^{−}\) cells 4 and 5 dpi. C, Forward scatter (FSC) of 2C cells (2C TCR\(^{+}\) CD8\(^{+}\)) from the indicated organs of the infected recipient mice at the indicated dpi was measured by flow cytometry. Following normalization to the FSC value of naive 2C cells, the relative FSCVs dpi are plotted. Plots shown are representative data from one of three animals per time point. D, Cells (2 \times 10^5 from the airways and lung parenchyma and 1 \times 10^6 from the spleen) from the infected recipient mice at the indicated dpi were mixed with splenocytes (1 \times 10^6) from naive B6 mice. The mixed cells were labeled with CFSE and cultured for 2 days in the presence of IL-2. The CFSE intensity of 2C cells (2C TCR\(^{+}\) CD8\(^{+}\)) is shown (solid line). The shaded area represents the CFSE intensity of naive CD8 T cells from B6 mice. E, Cells were assayed for 2C TCR, CD8, and CD62L at the indicated dpi. Histograms show CD62L expression of 2C cells (2C TCR\(^{+}\) CD8\(^{+}\)). Solid line, 2C cells from the infected recipient mice; shaded area, naive 2C cells from 2C\(^{+}\) RAG\(^{-/}\) mice. F, CD8\(^{+}\) cells were enriched from the spleen and assayed for 2C TCR, CD62L, and CCR7. Cells from the airways and the lung parenchyma from infected recipients 30 dpi were assayed for 2C TCR, CD8, and CCR7. Histograms show CCR7 expression by 2C (2C TCR\(^{+}\) CD8\(^{+}\)) cells in the airways and the lung parenchyma and by CD62L\(^{hi}\) and CD62L\(^{lo}\) 2C TCR\(^{+}\) cells in the spleen. Solid line and shaded area are as in E. The numbers indicate relative mean fluorescence intensity of 2C cells from the infected mice following normalization to the MFI of naive 2C cells.

**FIGURE 2.** Persistence of 2C cells in different organs. Cells from the airways, the lung parenchyma, and the spleen at various dpi were analyzed. A, 2C TCR vs CD8 profiles. The numbers indicate mean percentages \(\pm\) SEM of 2C cells (2C TCR\(^{+}\) CD8\(^{+}\)) from the indicated organs of three mice. Plots shown are representative data from one of the three animals at each time point. B, Numbers of 2C cells in the airways ( ), the lung parenchyma ( ), and the spleen ( ). Error bars represent SEM from three mice.
A significant increase of the pStat5 level was detected in 2C cells from A). In contrast, no 3-fold in 2C cells from the spleen (Fig. 4).

IL-15 for 20 min, and the level of phosphorylated Stat5 (pStat5) was used as indicator of signal transduction. Because both IL-7R and IL-15R transduce signals through the associated Jak3/Jak1 kinases (21), phosphorylation of their common substrate, Stat5, was used as indicator of signal transduction. Memory 2C cells from the airways, lung parenchyma, and the spleen were stimulated with IL-7 and/or IL-15 for 20 min, followed by staining for 2C cells (Thy1.2 CD81) and intracellular staining for pStat5.

Data shown are gated on 2C cells. Solid line, 2C cells with cytokine treatment; shaded area, 2C cells in medium alone. The numbers are the fold increase of pStat5 in cytokine-treated cells over untreated cells. B. Cells from pooled samples of infected recipient mice 30 dpi were incubated with IL-7 and/or IL-15 for 5 days, followed by analysis for 2C cells (2C TCR1, CD81). The relative cell number, shown in percentage, is obtained by dividing the 2C cell number on day 5 by the input 2C cell number at the start of the culture. Plots shown are representative data from one of two experiments with similar results. C. Cells from pooled samples of infected recipient mice 30 dpi were labeled with CFSE and CFSE culture as in B, followed by analysis for 2C TCR1, CD81, and CFSE profiles. The CFSE intensity of 2C cells (2C TCR1 CD81) is shown on the same Y-axis scale. The numbers indicate percentages of dividing cells. Numbers are not shown for the airway 2C cells because too few cells survived.

Expression of cytokines IL-7 and IL-15 in different organs

The number of memory 2C cells in the airways is determined by the migration of cells in and out of the airways as well as the homeostatic regulation of resident cells. Because studies have shown that cells migrating into the airways and lung parenchyma become residents in these tissues and do not return to the vascular system (19), we investigated homeostatic regulation in the maintenance of memory T cells in the airways. IL-7 was readily detected by ELISA (>5 pg/ml) in the airways, the spleen supernatant, and plasma (Fig. 3A). Due to the dilution in obtaining bronchial lavage, the actual IL-7 level in the airways is likely to be significantly higher. To assay for IL-15, which binds to IL-15Rα on the cell surface with high affinity (20), we performed quantitative RT-PCR. The level of IL-15 transcripts in the lung was generally higher than that in the spleen (Fig. 3B). The levels of IL-7 transcripts in the lung and the spleen were similar. These results suggest that homeostatic cytokine IL-7 and probably IL-15 are present in the local airway environment.

Cytokine responses of memory 2C cells from different locations

That the numbers of memory 2C cells in the airways declined over time in the presence of homeostatic cytokines suggests a possibility that memory 2C cells in the local airway environment do not respond to IL-7 and IL-15. To examine this possibility, we measured the responses of 2C cells from different locations to cytokine stimulation. Because both IL-7R and IL-15R transduce signals through the associated Jak3/Jak1 kinases (21), phosphorylation of their common substrate, Stat5, was used as indicator of signal transduction. Memory 2C cells from the airways, the lung parenchyma, and the spleen 30 dpi were stimulated with IL-7 and/or IL-15 for 20 min, and the level of phosphorylated Stat5 (pStat5) was quantified by intracellular staining. Upon stimulation with IL-7, IL-15, or both, the levels of pStat5 increased by at least 3-fold in 2C cells from the spleen (Fig. 4A). In contrast, no significant increase of the pStat5 level was detected in 2C cells from the airways (1.4-fold) or the lung parenchyma (1.7-fold).

These results indicate that memory 2C cells from the airways and lung parenchyma are impaired in immediate early signal transduction following IL-7 and IL-15 stimulation.

We also measured the survival and proliferation of memory 2C cells following cytokine stimulation. Memory 2C cells were labeled with CFSE and then cultured for 5 days with or without IL-7 and/or IL-15. Without adding cytokines, 54% of the input 2C cells from the spleen remained in the culture after 5 days (Fig. 4B). Because there was no significant dilution of CFSE intensity (Fig. 4C), these 2C cells survived for 5 days. Similarly, 40% of 2C cells from the lung parenchyma survived in the culture without proliferation. In contrast, very few 2C cells (<3%) from the airways survived the 5-day culture.

Importantly, 70 and 53% of 2C cells from the spleen proliferated in the presence of IL-7 or IL-15, respectively (Fig. 4C). A higher percentage of 2C cells (82%) proliferated in response to both IL-7 and IL-15 together. Correlating with proliferation, ~2.8, 3.6, and 6.8 times more 2C cells were recovered in the cultures in the presence of IL-7, IL-15, and both, respectively (Fig. 4B). In contrast, 2C cells from the lung parenchyma did not proliferate in the presence of IL-7. Although a similar fraction of the cells proliferated in the presence of IL-15 or IL-7 plus IL-15, as compared with 2C cells from the spleen, there was virtually no increase in the number of 2C cells, indicating that the proliferating cells from the lung parenchyma did not survive. Similarly, only very few 2C cells from the airways survived even in the presence of both cytokines.

FIGURE 3. Expression of IL-7 and IL-15 proteins and transcripts in different organs. A. The concentrations of soluble IL-7 in bronchial lavage (airways), spleen supernatant, and plasma were measured by ELISA. Error bars represent SEM from three naive B6 mice. B. The transcript levels of IL-7, IL-15, and β-actin in the indicated organs were determined by real-time PCR. The levels of IL-7 and IL-15 transcripts were normalized to that of β-actin transcripts from each organ and presented as relative levels. Error bars represent the range from duplicate samples.

FIGURE 4. Cytokine responses of memory 2C cells from different organs. A. Cells from pooled samples of infected B6-Thyl.1 recipient mice 30 dpi were incubated with IL-7 and/or IL-15 for 20 min, followed by staining for 2C cells (Thy1.2 CD81) and intracellular staining for pStat5. Data shown are gated on 2C cells. Solid line, 2C cells with cytokine treatment; shaded area, 2C cells in medium alone. The numbers are the fold increase of pStat5 in cytokine-treated cells over untreated cells. B. Cells from pooled samples of infected recipient mice 30 dpi were incubated with IL-7 and/or IL-15 for 5 days, followed by analysis for 2C cells (2C TCR1, CD81). The relative cell number, shown in percentage, is obtained by dividing the 2C cell number on day 5 by the input 2C cell number at the start of the culture. Plots shown are representative data from one of two experiments with similar results. C. Cells from pooled samples of infected recipient mice 30 dpi were labeled with CFSE and CFSE culture as in B, followed by analysis for 2C TCR1, CD81, and CFSE profiles. The CFSE intensity of 2C cells (2C TCR1 CD81) is shown on the same Y-axis scale. The numbers indicate percentages of dividing cells. Numbers are not shown for the airway 2C cells because too few cells survived.

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Together, these results suggest that airway memory cells do not survive well in vitro and do not respond to cytokines IL-7 or IL-15.

**Airway memory 2C cells are functional**

To rule out the possibility that memory 2C cells from the local lung environment (airways and lung parenchyma) are nonfunctional or incapable of proliferation, we assayed their proliferation and IFN-γ expression following TCR stimulation. Upon stimulation with immobilized anti-CD3 Abs followed by culturing in the presence of IL-2, memory 2C cells from the airways, the lung parenchyma, and the spleen all underwent multiple cell divisions, peaking at four to five divisions (Fig. 5A). Similarly, memory 2C cells from all three locations produced significant amounts of IFN-γ following SIY peptide stimulation (Fig. 5B). Although a small fraction of airway 2C cells produced some IFN-γ without peptide stimulation, these cells differ from the recently activated effector T cells by being as small as naive CD8 T cells (Fig. 1C), not upregulating IL-15Rβ (Fig. 6A, see below), and not proliferating.

**FIGURE 5.** Proliferation and IFN-γ production of memory 2C cells upon TCR engagement. A, Cells from pooled samples of infected recipient mice 30 dpi were labeled with CFSE and stimulated with anti-CD3. Solid line shows the CFSE intensity of 2C cells (2C TCR+ CD8+). The shaded area represents the CFSE intensity of undivided cells (no treatment). The asterisks indicate the peak numbers of cell division. B, Cells from pooled samples of infected recipient mice 30 dpi were cocultured with splenocytes (1 × 10⁶) from naive B6 mice in the presence (solid line) or absence (shaded area) of the SIY peptide for 4 h, followed by flow cytometry with anti-IFN-γ Abs. Data shown are gated on 2C cells (2C TCR+ CD8+). Representative data from two experiments are shown.

**FIGURE 6.** Expression of IL-7R and IL-15R by 2C cells in different organs over time. A, Cells from infected recipient mice were assayed for IL-7Rα, IL-15Rβ, γc, and 2C TCR expression. Data shown are gated on 2C cells (2C TCR+ CD8+). Solid line, 2C cells from the infected mice; shaded area, naive 2C cells from 2C RAG-/- mice. Numbers shown are relative percentages of the MFI of 2C cells from the infected mice following normalization to the MFI of naive 2C cells. B, CD8+ cells were depleted from the splenocytes of infected recipient mice 30 dpi and CD8+ cells were assayed for 2C TCR, CD62L, plus IL-7Rα, or IL-15Rβ, or γc chain. IL-7Rα, IL-15Rβ, or γc expression by CD62L(low) and CD62L(high) 2C TCR+ cells is shown. Histograms and numbers are shown as in A. C, B6 mice (no 2C transfer) were infected with WSN-SIY. Twenty-one and 30 dpi, cells were assayed for SIY-H-2Kb, CD8, and IL-7Rα or IL-15Rβ or γc. IL-7Rα, IL-15Rβ, or γc expression by CD62L(low) and CD62L(high) 2C TCR+ cells is shown. Solid line, SIY-specific CD8 T cells from the virus-infected mice; shaded area, TCRβ+ and CD8+ T cells from naive B6 mice. The numbers are relative percentages of the MFI of SIY-specific CD8 T cells from the infected mice following normalization to the MFI of naive CD8 T cells. D, 2C cells (2C TCR+ CD8+) were sorted from pooled samples from the indicated organs of infected recipient mice 30 dpi. Naive 2C cells were purified from a 2C RAG-/- mouse (indicated as naive). The transcript levels of IL-7Rα, IL-15Rβ, γc, and β-actin in sorted 2C cells were measured by real-time PCR. The levels of the indicated transcripts were normalized to that of β-actin transcripts. Error bars represent the range from duplicate samples.
without restimulation (Fig. 4). Thus, memory 2C cells from the airways and the lung parenchyma are as fully competent as memory 2C cells from the spleen to respond to Ag stimulation.

**Down-regulation of IL-7R and IL-15R by memory 2C cells in the airways**

Because phosphorylation of Stat5 occurs immediately following binding of IL-7 and IL-15 to their cognate receptors, lack of phosphorylated Stat5 in airway memory 2C cells upon cytokine stimulation (Fig. 4A) suggests a possibility that both IL-7R and IL-15R are down-regulated in airway memory 2C cells. To examine this possibility, we measured the expression of the IL-7Rα-chain, IL-15Rβ-chain, and γc chain by 2C cells at different time points after influenza infection. To be able to compare the receptor levels on 2C cells measured at different time points, the levels of IL-7Rα, IL-15Rβ, and γc on naive 2C cells were also measured each time and used as a base for normalization. Compared with naive 2C cells, 7 dpi, the levels of IL-7Rα were similarly down-regulated on 2C cells in the airways, the lung parenchyma, and the spleen (Fig. 6A), whereas the levels of IL-15Rβ were up-regulated. The levels of IL-7Rα and IL-15Rβ on 2C cells returned to the basal levels of naive T cells 14 dpi. However, different expression patterns were observed 21 dpi when 2C cells began to acquire memory phenotype. The levels of both IL-7Rα and IL-15Rβ on persisting 2C cells in the spleen progressively increased, whereas their levels on 2C cells in the airways progressively decreased (Fig. 6A). By 225 dpi, the levels of IL-7Rα and IL-15Rβ on 2C cells were ~9- and 6-fold higher, respectively, in the spleen than in the airways. Consistently, memory 2C cells expressed a higher level of γc in the spleen than in the airways. In addition, both CD62Lhigh and CD62Llow memory 2C cell subsets in the spleen expressed similarly high levels of IL-7Rα, IL-15Rβ, and γc chains (Fig. 6B). Because the levels of other molecules such as TCR remained quite constant over time (Fig. 6A), these results show that both IL-7R and IL-15R are specifically down-regulated in airway memory 2C cells.

Persisting 2C cells in the lung parenchyma appeared to exhibit an intermediate phenotype. Up to 60 dpi, the levels of IL-7Rα, IL-15Rβ, and γc on 2C cells in the lung parenchyma were similar to those on 2C cells in the airways, but by 225 dpi, the levels were approaching those on 2C cells in the spleen. Consistently, lung parenchymal 2C cells had a low level of CD62L expression up to 60 dpi (Fig. 1E), the same as the 2C cells in the airways. By 225 dpi, however, lung parenchymal 2C cells had a mixed low and high CD62L expression, identical with the pattern of splenic 2C cells. Because the lung was not perfused before lymphocyte isolation, the observed changes in parenchymal 2C cell phenotype may reflect contamination of the T cells by airway- and blood-derived (splenic) T cells.

To determine whether the differences in IL-7R and IL-15R expression by memory 2C cells in different organs also apply to endogenous polyclonal CD8 T cells, B6 mice without the 2C cell transfer were infected with WSN-SIY. Following the infection, cells from the airways, the lung parenchyma, and the spleen were assayed for SIY-H-2Kb, CD8, and IL-7Rα or IL-15Rβ or γc. As shown in Fig. 6C, 30 dpi endogenous SIY-specific CD8 T cells expressed significantly higher levels of IL-7Rα, IL-15Rβ, and γc in the spleen than in the airways and the lung parenchyma, indicating that the differences in homeostatic cytokine receptor expression by memory CD8 T cells in different organs are a general phenomenon.

We then measured the levels of IL-7Rα, IL-15Rβ, and γc transcripts by quantitative RT-PCR using sorted 2C cells from different locations 30 dpi. As shown in Fig. 6D, while the γc transcript levels were similar in the persisting 2C cells from different locations, the level of IL-7Rα transcripts in 2C cells from the spleen was 2- and 8-fold higher than the levels in airway and lung 2C cells, respectively. Similarly, the level of IL-15Rβ transcripts in 2C cells from the spleen was 17- and 2-fold higher than the levels in airway and lung 2C cells, respectively. These results suggest that the down-regulation of IL-7R and IL-15R expression in the airway memory T cells is likely regulated at the transcriptional level.

**Down-regulation of IL-7R and IL-15R and diminished homeostatic proliferation by splenic memory cells in the airways**

To determine whether the down-regulation of IL-7R and IL-15R is an intrinsic property of airway memory CD8 T cells or is effected by the local airway environment, we transferred splenic memory 2C cells into naive B6 recipients intratracheally, recovered the transferred cells from the airways and 14 days later, and measured the levels of cytokine receptor expression. Although the levels of IL-7Rα, IL-15Rβ, and γc were uniformly high in splenic memory 2C cells 30 dpi (Fig. 6A), the levels of these receptors were significantly down-regulated on splenic memory 2C cells recovered from the airways (Fig. 7A). In particular, IL-7Rα continued to decrease from day 7 to 14 post transfer, whereas the TCR expression by the same 2C cells was not affected. Furthermore, 14 days after transfer, very few splenic memory 2C cells in the airways proliferated (Fig. 7B). In contrast, when splenic memory 2C cells were transferred into B6 recipients i.v., most of the 2C cells (>97%) recovered from the spleen underwent cell division. These results suggest that the local airway environment induces down-regulation of IL-7R and IL-15R expression in memory CD8 T cells.

**Discussion**

Studies have shown that the ability of memory CD8 T cells to confer heterosubtypic immunity against influenza virus declines...
within a few months (1). This decline was later found to result from a gradual loss of virus-specific memory CD8 T cells in the airways (4). Because it is difficult to obtain a sufficient number of memory cells from the airways following a polyclonal CD8 T cell response, the mechanisms underlying the loss of airway memory CD8 T cells have not been properly examined. Indeed, current knowledge about memory T cell homeostasis has been largely derived from the analysis of cells from lymphoid organs (7, 22–24), in which cytokines IL-7 and IL-15 are identified as key regulatory factors. Whether the same or different mechanisms regulate memory T cell persistence in nonlymphoid locations, such as in the airways, however, are unknown.

In this report, we examined this issue by following a cohort of TCR transgenic T cells following influenza virus infection. The use of TCR transgenic T cells made it possible to recover sufficient numbers of memory CD8 T cells from the airways for analysis as well as compare memory T cell persistence, phenotype, and function in different organs without the confounding effects of the TCR heterogeneity of a polyclonal T cell response. We found similar levels of IL-7 protein in bronchial lavage and spleen supernatant and similar levels of IL-7 and IL-15 transcripts in the lung and the spleen. However, IL-7R and IL-15R expression was gradually lost from persisting memory CD8 T cells in the airways but not in the spleen. The observed selective retention of memory T cells with low IL-7R and IL-15R in the airways could result from either gradual down-regulation of the cytokine receptor expression (a gradual change in phenotype over time) or selection for memory T cells that do not express the cytokine receptors (selection of cells with a particular phenotype over time). Although our results do not unequivocally distinguish the two possibilities, the existing data are more consistent with the former possibility. The loss of receptor expression is specific for IL-7R and IL-15R because TCR, CD8, Thy-1, and CD44 expression on the same cells is stably maintained. When memory 2C cells from the spleen were adaptively transferred into the airways, expression of the IL-7R and IL-15R was gradually down-regulated. Consistent with our results, a recent study showed that when purified CD44high IL-7Rα+ memory CD8 T cells from spleen of Sendai virus-infected mice were transferred into the airways of naïve mice, they also down-regulated the surface level of IL-7Rα as early as 18 h after the transfer (25). These findings suggest that the down-regulation is induced by the airway environment, not by some intrinsic differences between airway and splenic memory CD8 T cells.

How does the airway environment induce the down-regulation of IL-7R and IL-15R on memory T cells? One possibility is that IL-7R and IL-15R molecules are shed from memory CD8 T cells in the airways (26), perhaps via the action of a protease (27). Based on the results that the levels of IL-7R and IL-15R were high on 2C cells in the airways 14 dpi (Fig. 6A) and that normal levels of TCR, CD8, CD44, and Thy-1 were detected on airway memory 2C cells 30 dpi, this possibility seems unlikely. Furthermore, the levels of IL-7R and IL-15R transcripts in memory 2C cells were lower in the airways than in the spleen, suggesting that the down-regulation is at the transcriptional level. Cytokines, such as IL-2, IL-4, IL-6, IL-15, and even IL-7 itself, and signaling through TCR, have been shown to down-regulate IL-7R expression at the transcriptional level (28). The presence of these cytokines and other yet-to-be-identified molecules in the airway environment may have induced the down-regulation of IL-7R and IL-15R expression. Consistent with this possibility, IL-7 was readily detected in bronchial lavage and IL-15 transcripts were detected in the lung.

One consequence of IL-7R and IL-15R down-regulation is the loss of response of airway memory CD8 T cells to the corresponding cytokines. Stimulation of airway memory 2C cells with IL-7, IL-15, or both failed to induce Stat5 phosphorylation, survival, or proliferation, whereas upon the same stimulation, splenic memory 2C cells immediately underwent Stat5 phosphorylation, followed by proliferation and survival. Because airway and splenic memory 2C cells proliferated equally well to anti-CD3 stimulation and produced similar levels of IFN-γ in response to SIY peptide, the nonresponsiveness of airway memory CD8 T cells to IL-7 and/or IL-15 stimulation is specific, not due to a general defect, because these cells are dying in the airways.

What is the relationship between the down-regulation of IL-7R and IL-15R and gradual disappearance of memory T cells from the airways? One possibility is that the down-regulation is a consequence of memory T cells dying in the airways. Lung airways are exposed to the external environment and lined with surfactants, which inhibit T cell activation and survival (29). Molecules such as NO produced by alveolar macrophages in the airways are also known to inhibit Ag- or mitogen-induced T cell proliferation (30, 31). Despite these unfavorable conditions, some of the transferred splenic memory 2C cells were able to persist in the airways for at least 2 wk (Fig. 7), suggesting that the airway environment can support significant memory cell survival, consistent with the presence of IL-7 in the bronchial lavage (Fig. 3). Therefore, an active suppression mechanism such as down-regulation of the cytokine receptors is likely required to limit their survival in the airways.

Our findings suggest that the down-regulation of IL-7R and IL-15R expression is a significant contributing factor to the poor persistence of memory CD8 T cells in the airways. The gradual down-regulation of cytokine receptors is temporally correlated with the gradual decline of memory T cells in the airways following influenza virus infection. When splenic memory T cells were adaptively transferred into the airways, the receptor down-regulation was also associated with the cessation of homeostatic proliferation. Cytokines IL-7 and IL-15 are key factors that promote the survival of memory T cells in the lymphoid organs (1, 7). The down-regulation of IL-7R and IL-15R obviously prevents airway memory T cells from responding to these cytokines. As the levels of IL-7 and IL-15R transcripts in the lung were generally higher than those in the spleen (Fig. 3), airway memory T cells likely have access to both cytokines as memory T cells do in the spleen. However, even in the presence of excess amount of IL-7 or IL-15, such as in vitro, airway memory 2C cells did not survive or proliferate, whereas splenic memory 2C cells responded readily to the cytokines. These findings demonstrate the essential role of IL-7R and IL-15R expression in determining the cellular responses to these cytokines. However, they also raise the question what, if any, factors help to keep the persisting memory T cell alive in the airways. Although further studies are needed to answer this question, controlling memory T cell survival by regulating IL-7R and IL-15R expression likely provides a more stringent control than that by cytokines. The unresponsiveness of airway memory CD8 T cells to IL-7 and IL-15 not only has a critical effect on their persistence, but also likely blocks their ‘bystander’ proliferation (TCR independent) in response to subsequent unrelated infection in the airways (32, 33).

The difference in IL-7R and IL-15R expression by memory 2C cells in the airways and the spleen are unlikely due to the use of TCR transgenic T cells, which significantly increases the frequency of responding CD8 T cells (34). First, identical with the endogenous polyclonal SIY-specific memory CD8 T cells (Fig. 6C), memory 2C cells from the airways 30 dpi were all CD62Llow and CCR7low, suggesting that the frequency of responding CD8 T cells does not alter memory T cell development in the airways. Second, the ratios of CD62Lhigh to CD62Llow memory CD8 T cells in the spleen were similar between the transferred 2C cells and the
endogenous SIY-specific CD8 T cells, indicating that transferred
2C cells are not biased toward central memory cell differentiation.
Third, both the memory 2C cells and the endogenous SIY-specific
CD8 T cells exhibit similar surface phenotypic changes: high lev-
els of IL-7Rα, IL-15Rβ, and γc, in the spleen and low levels in the
airways. Fourth, transferring splenic memory 2C cells into the air-
ways of naive recipient mice led to the down-regulation of cyto-
kine receptors, indicating that down-regulation in the airways is
not a matter of initial frequencies of responding T cells, but an
active local process. Together, these observations suggest that the
use of TCR transgenic T cells are unlikely to have affected the
outcome of memory T cell persistence in the airways.

In summary, the down-regulation of IL-7R and IL-15R expres-
sion is likely a contributing factor for the poor survival of memory
CD8 T cells in the airways. These findings provide a molecular
explanation for the gradual loss of heterosubtypic immunity fol-
lowing influenza virus infection. Because the protective immunity
mediated by memory CD8 T cells also wanes rapidly following
other respiratory infection, such as respiratory syncytial virus (35),
the findings presented in this study may represent a general char-
acteristic of airway memory CD8 T cells. As the lung is a major
portal of infection, our findings also highlight the challenge to
vaccine strategies aimed at inducing long-lasting cellular immu-
ity against respiratory infections, such as influenza virus.

Acknowledgments

We thank Drs. Astrid Flandorfer and Peter Palese (Mount Sinai School
of Medicine, New York, NY) for constructing the WSN-SIY virus and advice,
and members of the Chen laboratory for advice and assistance.

Disclosures

The authors have no financial conflict of interest.

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