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$CD8^+$ T Cells Responding to Influenza Infection Reach and Persist at Higher Numbers Than $CD4^+$ T Cells Independently of Precursor Frequency

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Abstract

The activation, localization, phenotypic changes, and function of CFSE-labeled naive influenza-specific $CD8^+$ and $CD4^+$ T cells following influenza infection were examined. Response of adoptively transferred $CD8^+$ T cells was seen earliest in draining lymph node. Highly activated cells were found later in the lung, airways, and spleen, were cytolytic, and expressed IFN-γ upon restimulation. Similar amounts of division at early time points, but higher numbers of $CD8^+$ T cells, were detected at 9 and 30 days postinfection after cotransfer of $CD4^+$ and $CD8^+$ T cells followed by infection. Transfer of much smaller numbers of $CD4^+$ and $CD8^+$ T cells led to more extensive expansion but the same difference in final number between the two cell types. These studies demonstrate how $CD8^+$ and $CD4^+$ T cells respond to influenza at early time points postinfection and the differential kinetics of antigen-specific $CD4^+$ and $CD8^+$ T cells.

Keywords: virus infection, T lymphocytes, cellular activation, cellular proliferation, cell surface molecules

Abbreviations: BAL, bronchoalveolar lavage; DLN, draining lymph nodes; NDLN, nondraining lymph nodes; HA, hemagglutinin

Introduction

Naive T cells become activated upon encounter with peptide antigen on the surface of antigen presenting cells in association with MHC molecules and then proliferate and differentiate to become effector cells [1, 2]. Most effectors die, but a minority become long-lived memory cells [3]. The surface phenotype changes as $CD4^+$ and $CD8^+$ T cells progress from naive to activated effectors and memory cells, with the gain of CD25, CD44, and CD43 and loss of CD62L (reviewed in [4, 5]). Some activated T cells can gain cytotoxic activity and secrete cytokines such as IFN-γ and TNF-α [6, 7], while others gain the ability to provide B cell help [4, 8, 9]. The actual events that regulate these processes are not fully understood, and much of our knowledge of T cell activation has been gained from in vitro studies and may not be fully applicable in vivo.

Following influenza infection in vivo, both $CD8^+$ and $CD4^+$ T cells are activated (reviewed in [10]), and the use of tetramers has improved the tracking of antigen-specific populations but is limited in the early phase of the response by the low precursor frequency of responding cells [11–13]. Adoptive transfer of antigen-specific lymphocytes counters this weakness and has been used to monitor the T cell response to viruses such as LCMV [14], VSV [15], influenza [16–18], and also responses to ovalbumin [19, 20]. The respective roles of $CD4^+$ and $CD8^+$ cells during infection by influenza have been investigated using knockout or depletion approaches [21–26], but these studies did not examine respective rates of activation or division of the $CD4^+$ and $CD8^+$ T cells. The analysis of $CD4^+$ and $CD8^+$ T cell responses side by side during pathogen infection has not been attempted until recently [27, 28].

Previous work from our laboratory showed that Tc1 and Tc2 effector $CD8^+$ lymphocytes were able to protect mice from a lethal influenza infection [16], and we compared the efficacy of protection by naive, effector, and memory populations [17]. In previous studies, we transferred large numbers (5×10^6) of CFSE-labeled naive influenza-specific TCR transgenic $CD4^+$ from HNT mice

and showed that after influenza infection, activated donor cells were detected in the spleen, lung, and bronchoalveolar lavage (BAL) in large numbers. The phenotypic changes increased with each division, and only cells that had gone through multiple divisions and expressed an activated phenotype were found in the lung and BAL [18].

Our primary goal here was to document the response of $CD8^+$ T cells in response to sublethal influenza infection and to compare the response of $CD4^+$ and $CD8^+$ T cells. We demonstrate, using adoptive transfers of influenza-specific $CD8^+$ and $CD4^+$ T cells that both $CD8^+$ and $CD4^+$ T cells were found to divide first in the draining lymph nodes (DLN) and were then subsequently found in lung, BAL, and spleen. Cotransfer of $CD4^+$ and $CD8^+$ T cells demonstrated that the $CD8^+$ T cells expanded to and were maintained for 30 days at higher numbers than $CD4^+$ T cells. CFSE-monitored division was not significantly different in $CD8^+$ T cells compared with $CD4^+$ T cells. Adoptive transfer of smaller numbers of $CD8^+$ and $CD4^+$ T cells resulted in similar peak numbers of cells as with larger transfer doses, confirming that the two cell types are regulated differently. Uniquely, adoptive transfer allows the analysis of the early phase of the response where T cell division and phenotypic change can be examined in the DLN and allows manipulation of the number of input cells.

Materials and methods

Mice

BALB/c $Thy1.1^+$ (gift from Dr. C. Suhr, Scripps Institute, La Jolla, CA), BALB/c ($Thy1.2^+$) hemaglutinin (HA)-specific clone-4 TCR transgenic (gift from Dr. L. Sherman, Scripps Institute), BALB/c HNT $CD4^+$ TCR transgenic mice (gift from Dr. D. Lo, Scripps Institute), (BALB/c HNT × $Thy1.1^+$) F1, and (BALB/c HA × $Thy1.1^+$) F1 mice were bred at the Trudeau Institute animal breeding facility. HA clone-4 mice recognize the peptide (IYSTVASSL) residues 518-26 from the hemagglutinin gene of influenza virus PR8 presented on H2-K^d [29]. BALB/c HNT mice are $CD4^+$ transgenic mice that recognize peptide (HNTNGVTAACSHE), residues 126-138, from HA in the context of I-A^d [30]. BALB/c $Thy1.2^+$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal procedures were done in accordance with guidelines from the Institute Animal Care and Use Committee (IACUC) at Trudeau Institute.

Cell culture medium

Cells were washed in RPMI with 1% FCS, 1 mM sodium pyruvate, 200 U/ml penicillin, 200 μg/ml streptomycin, 0.4 mM glutamine, 0.1 mM nonessential amino acid solution (Gibco Invitrogen Corporation, Grand Island, NY). Tissue culture medium (TCM) was wash media with 5% FCS and 4.5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). P815 cells for cytotoxicity assays were maintained in DMEM with high glucose (Irvine Scientific, Santa Ana, CA) supplemented with penicillin/streptomycin and 10% FCS [16].

Cell preparation and adoptive transfer

Single cell suspensions of $CD8^+$ cells were prepared from cervical, inguinal, axillary, and brachial lymph nodes and spleens from clone-4 TCR transgenic $Thy1.2^+$ mice. Cells were purified by positive selection with anti-CD8α beads using MACS columns according to manufacturer's protocol (Miltenyi Biotech, Auburn, CA) and then centrifuged on a four-layer Percoll (Sigma) gradient to remove activated cells [16]. $CD4^+$ cells were prepared from HNT mice using antibody and complement depletion followed by a Percoll gradient [31] or by positive selection on MACS columns followed by centrifugation on Percoll as for CD8. Naive $CD8^+$ and $CD4^+$ T cells were over 90% pure and were not contaminated by significant numbers of other T cells or B cells. They expressed high levels of CD62L, low levels of CD25, and intermediate levels of CD44 (data not shown). Cells were counted using an ACT10 Coulter counter (Becton Coulter, Brea, CA) or trypan blue dye exclusion. Injected intravenously into the lateral tail vein of BALB/c $Thy1.1^+$ recipients were 5×10^6 CFSE-labeled $Thy1.2^+$ $CD8^+$ cells in 500 μl PBS (Gibco). For cotransfer experiments, 3×10^6 $Thy1.2^+$ HA mixed with 3×10^6 $Thy1.2^+$ HNT CFSE-labeled cells in 250 μl PBS were injected into $Thy1.1^+$ BALB/c mice. In some experiments, cells from HA ($Thy1.1^+ \times 1.2^+$) F1 or HNT ($Thy1.1^+ \times 1.2^+$) F1 mice were injected into BALB/c $Thy1.2^+$ recipients. No significant differences were seen between transferring Thy1.2 into Thy1.1 and Thy1.2 × Thy1.1 into Thy1.2 mice.

CFSE labeling

CFSE (Molecular Probes, Eugene, OR) was stored at −20°C in DMSO (Sigma) at a concentration of 10 mM. Cells were labeled with a 1/4000 dilution of the stock diluted 1:1 with cells at 2×10^7/ml in PBS and incubated for 8 min at RT. Cells were then quenched with FBS and washed three times with PBS [32].

Influenza virus and infections

Influenza Puerto Rico (PR8) (H1N1) virus (a gift from Drs. D. Morgan and L. Sherman) was grown in the allantoic fluid of embryonated hens eggs as described in Refs. [18 and 33]. Mice were infected with influenza by intranasal inoculation of 50 or 100 μl of virus in PBS. A sublethal dose of 15000 EIU (0.5 LD_{50} determined in BALB/c mice) or 6000 EIU (0.2 LD_{50}) was routinely given, 16–20 h after adoptive cell transfer. Control mice were given 50 μl PBS intranasally.

Tissue sampling and flow cytometry of surface markers

Mice were sacrificed by exsanguination under deep isofluorane inhalation anesthesia at various time points after influenza infection, and spleen, the nondraining axillary, and brachial and inguinal lymph nodes (NDLN) were taken. Bronchoalveolar lavage (BAL) was collected by washing the airways four times with 0.5 ml 10 mM EDTA (Sigma) in PBS. Lungs were removed following perfusion with 5 ml PBS via the left ventricle of the heart. Finally, the draining mediastinal and parathymic draining lymph nodes (DLN) were collected. Single cell suspensions were prepared using 70-μm nylon filters (Falcon, Franklin Lakes, NJ). The cells were washed and resuspended in FACS buffer [PBS with 2% (w/v) BSA (Intergen, Purchase, NY) and 0.1% (w/v) sodium azide (Sigma)] then incubated with 1 μg anti-FcR (clone 2.4G2, produced by Trudeau Institute) followed by anti-CD8-PerCP (53-6.7), or anti-CD4-perCP (RM4-5) anti-Thy1.2-APC (53-2.1), and one of a number of PE-labeled conjugates: anti-CD62L (MEL-14), anti-CCR5 (C34-3448), anti-CD44 (IM7), anti-CD25 (PC61, Caltag, Burlingame, CA), anti-CD43 (1B11), anti-CD69 (H1-2F3) anti-Vβ8.1/8.2 (MR5-2), and anti-Ly6C (HK1-4), all obtained from BD Biosciences, San Diego, CA, except where noted. Cells were then washed and analyzed on a four-color FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software. Profiles were analyzed using FlowJo (Treestar, San Carlos, CA) by determining the percentage of $CD8^+$ $Thy1.2^+$ lymphocytes. The number of donor cells was calculated by multiplying the percentage $CD8^+Thy1.2^+$ cells in the FlowJo live gate by the percentage of live cells and by the number of lymphocytes calculated by the Coulter counter.

Intracellular cytokine staining

Single cell suspensions from various organs were cultured in TCM with 11 μM HA peptide (W. Alton Jones Cell Science Center, Lake Placid, NY) overnight with 10 μg/ml Brefeldin A (Sigma) added after the first 4 h. The cells were harvested and incubated with anti-IFN-γ-PE (XMG1.2) or anti-TNF-α-PE (MP6-XT22), both from BD Biosciences, in saponin buffer (0.1% saponin from Quillaja Bark, Sigma) in PBS [34]. The cells were then processed as above for surface staining of Thy1.2 and CD8 and analyzed by flow cytometry.

Virus titer

Mice injected with $CD8^+$ T cells and influenza-infected were euthanized at various times postinfection by cervical dislocation. The lungs were removed, snap-frozen in liquid N_2, and stored at −70°C. The lungs were thawed, homogenized, and the influenza titer determined using the Madine Darby Canine Kidney (MDCK) cell plaque assay as detailed previously [17]. Plaque-forming units (PFU) are expressed per lung.

Cytotoxicity assay

Single cell suspensions were prepared from mice adoptively transferred with $CD8^+$ T cells and infected with influenza. B cells were depleted from spleen with anti-B220 beads using MACS columns according to manufacturer's instructions (Miltenyi Biotech). Lung cells were enriched for lymphocytes by centrifugation on Lympholyte-poly (Cedarlane, Hornby, Canada) and then incubated with anti-Thy1.2 beads. $Thy1.2^+$ and $Thy1.2^-$ populations were obtained from these cells using MACS columns (Miltenyi Biotech). ^{51}Cr-labeled P815 cells (Perkin Elmer NEN, Boston, MA), 1×10^4, pulsed or unpulsed with HA peptide were incubated with $Thy1.2^+$ or $Thy1.2^-$ lung cells, B cell-depleted splenocytes, DLN, or NDLN cell populations for 4 h as described previously [16]. Maximum lysis was determined by incubation with 0.1% Triton X 100 (Sigma) and spontaneous lysis by incubation in medium alone. The percentage lysis was calculated by (experimental–spontaneous lysis/maximum lysis–spontaneous lysis) × 100. Spontaneous lysis of unpulsed P815 cells was <20% in all wells.

Results

The expansion and localization of adoptively transferred HA peptide-specific TCR transgenic naive $CD8^+$ T cells vary between lymphoid and nonlymphoid sites

We first assessed the magnitude of the response of naive HA peptide-specific $CD8^+$ TCR transgenic T cells to an influenza infection. Naive donor HA-specific $CD8^+$ T cells were transferred into BALB/c mice subsequently infected 16–20 h later with PR8 influenza. At various times postinfection, we analyzed single cell suspensions from five tissues: DLN (mediastinal and parathymic lymph nodes), NDLN (axillary, brachial, and inguinal lymph nodes), spleen, airways [bronchoalveolar lavage (BAL)], and lung. The absolute numbers of $CD8^+$ donor cells detected in each set of tissues are shown on a logarithmic scale in Figure 1A. The time of the peak $CD8^+$ response was similar in DLN, spleen, BAL, and lung, 8 to 9 days postinfection (Figure 1A and data not shown). Significant populations of donor cells were found in the DLN at Day 4 postinfection with no cells in lung and BAL at this time point. At Days 6 and 8 postinfection, high numbers of donor cells were found in lung and lower numbers in spleen and DLN. Numbers of donor cells declined in all organs by Day 29 postinfection with the exception of the NDLN. These data together indicated that the donor cell accumulation was focused near to the site of infection, the lung.

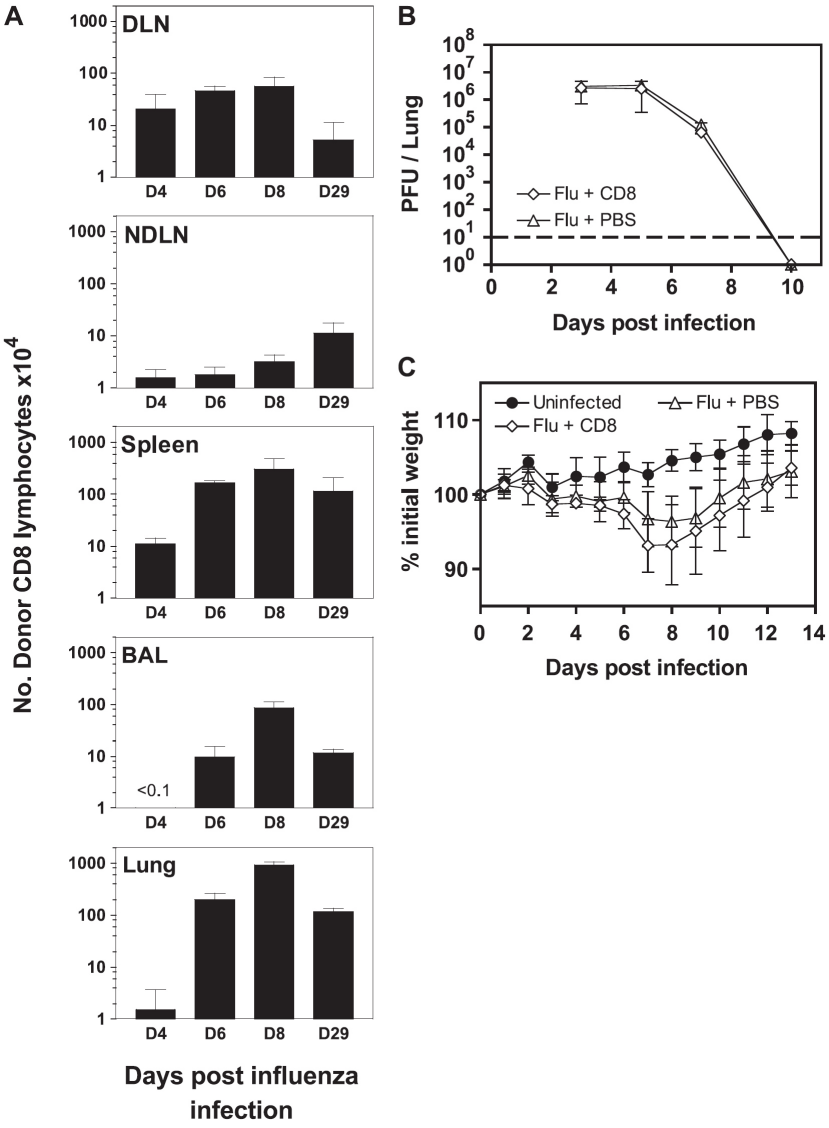

Figure 1. Adoptively transferred CD8$^+$ TCR transgenic T cells expand and then decline after influenza virus infection and mice clear the virus with concurrent loss and gain of body weight. (A) Thy1.2$^+$ CFSE-labeled naive clone-4 HA CD8$^+$ transgenic T cells, 5×10^6, were adoptively transferred into Thy1.1$^+$ syngeneic recipients infected intranasally 18 h later with 0.5 LD_{50} influenza. (A) Mice were sacrificed at indicated times, and the number of donor cells from draining lymph nodes (DLN), nondraining lymph nodes (NDLN), spleen, lung tissue, and brochoalveolar lavage (BAL) was determined by flow cytometry. Values shown are mean ± SD of three to six mice per group. These data are from a representative experiment repeated eight times with similar results. (B) Lung virus load (units shown are PFU per lung and dotted line indicates limit of detection) and (C) mouse weight with adoptive transfer of 7×10^5 naive CD8$^+$ T cells or PBS and 3000 EIU influenza infection. Results are from one representative experiment of three.

To compare the extent of proliferation in various tissues, we calculated the numbers of adoptively transferred CD8$^+$ T cells recovered from mice sublethally infected with influenza or mock-infected with PBS. To quantify the extent of the expansion of CD8$^+$ T cells brought about by influenza infection, we divided the number of adoptively transferred cells recovered from infected mice by the number of adoptively transferred cells recovered from uninfected controls. The result is shown in Table 1. Large numbers of cells are generated early in the response in the draining lymph node, and the action moves later to the lung and then to the BAL.

Virus is cleared and weight loss is restored following the appearance of T cells in the lung and BAL

The presence of influenza virus in the lungs was measured using a plaque assay. Figure 1B shows that the viral titer in the lungs of mice after adoptive transfer of HA peptide-specific CD8$^+$ T cells followed by influenza infection is high from Days 2 to 6 postinfection and then decreases. The peak of cell numbers in the lung and BAL therefore correlates with clearance of virus from the lungs. Figures 1B and C show that the weight loss and viral clearance did not significantly differ in mice that

Table 1. Influenza infection leads to increased numbers of donor lymphocytes in DLN, lung, and BAL compared with uninfected controls

Days postinfection[a]	Organ sampled[b] DLN	NDLN	Spleen	Lung	BAL
Day	4 47.11[c]	0.25	0.28	0.84	6.22
Day 7	322.51	0.27	13.7	1651	17.062
Day 11	90.95	0.002	0.71	620	58.752

[a] Days postinfection with influenza.
[b] Organs were taken from euthanized mice and donor cell numbers determined using flow cytometry.
[c] The ratio of numbers of cells recovered from mice given 5 × 10^6 HA clone-4 naive transgenic T cells at Day –1, infected with influenza divided by mice given PBS intranasally. Numbers are the ratio of three infected animals per group divided by three uninfected animals. Representative experiment repeated three times.

did not receive donor cells, indicating that the adoptively transferred lymphocytes did not significantly accelerate viral clearance over the rate seen in the untreated host.

CFSE-labeled CD8+ T cells divided early during infection in the DLN and changed cell surface phenotype

The influenza-specific T cell precursor frequency is very high following the adoptive transfer of large numbers of antigen-specific cells allowing a detailed analysis of the early events that are below the level of detection using tetramer analysis. At Days 2 and 3 postinfection, all donor cells retained high levels of CFSE in all organs examined (data not shown) indicating that division had not yet begun. As shown in Figure 2A, donor cells have already divided up to seven times in the DLN by Day 4 postinfection; in contrast, low levels of cell division are seen in the spleen and no division in NDLN. The surface expression levels of CD43, CD44, and CD25 increased as the cells divided in the DLN but are still low in the spleen and NDLN at this time point. Adoptively transferred cells recovered from the DLN decrease cell surface expression of CD62L as the cells divide, but expression of this marker remains high on undivided cells recovered from NDLN and spleen. The Ly6C phenotype was low to intermediate in all tissues at Day 4. CD69 cell surface expression is increased initially on cells recovered from the DLN and then decreases as the cells divide (data not shown). The cell division and gain of an activated phenotype are seen first in DLN, and cells with an activated phenotype are found in other lymphoid and nonlymphoid organs at later time points.

Adoptively transferred CD8+ HA peptide-specific T cells possess an activated phenotype at the peak of cell accumulation

Figure 2B shows that virtually all of the adoptively transferred CD8+ T cells have divided by Day 8, as measured by loss of CFSE, and have a progressively activated phenotype with gain of activation-associated markers in the DLN, NDLN, and spleen. The cells that enter the lung and BAL all have low CFSE intensity, indicating that more than seven divisions have occurred, and possess high surface expression of CD43, CD44, CD25, and Ly6C and low CD62L. The level of CD25 on the donor cells found in the DLN at Day 8 postinfection is lower than that seen at Day 4, but other activation markers (CD43 and CD44) remain high at this time. The donor cells also expressed increased levels of CD54, CD11a, and CD49d upon activation (data not shown). In addition, the transgene TCR Vβ is expressed at high levels during the response, and there are a subset of cells present in the lung that express CCR5 (data not shown).

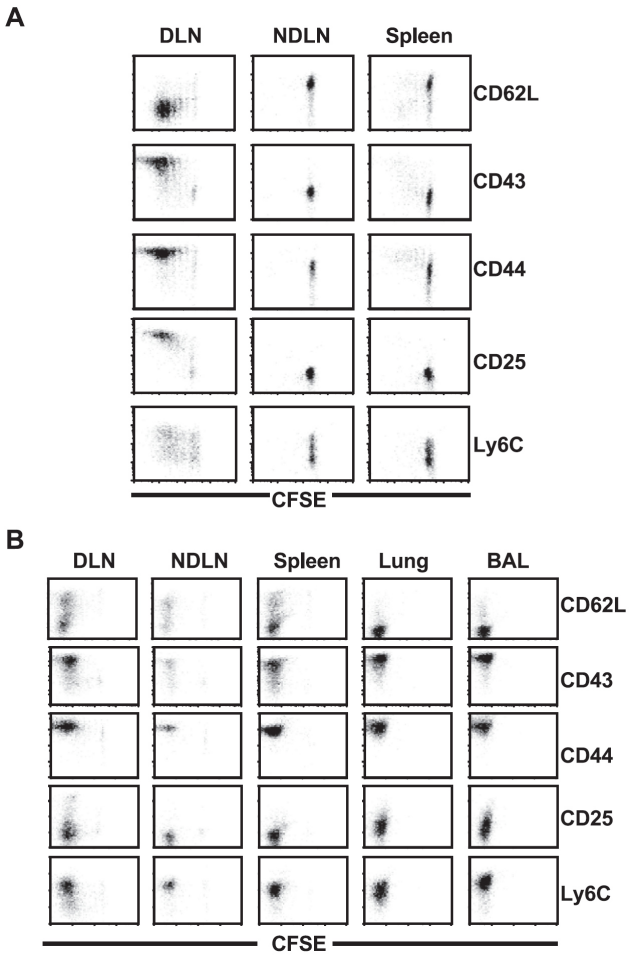

Figure 2. Adoptively transferred CD8+ T cells show division and gain of activation-associated molecules in the DLN at Day 4 postinfection and possess a highly activated phenotype at Day 8 postinfluenza. Cells were transferred and mice infected with influenza as described in Figure 1. (A) Four and (B) eight days later, mice were euthanized. Cells from DLN, NDLN, spleen, lung tissue, and BAL were analyzed by flow cytometry. Profiles shown are gated for cells that are CD8+Thy1.2+. Results are one mouse in a group of three from a representative experiment repeated eight times with similar results.

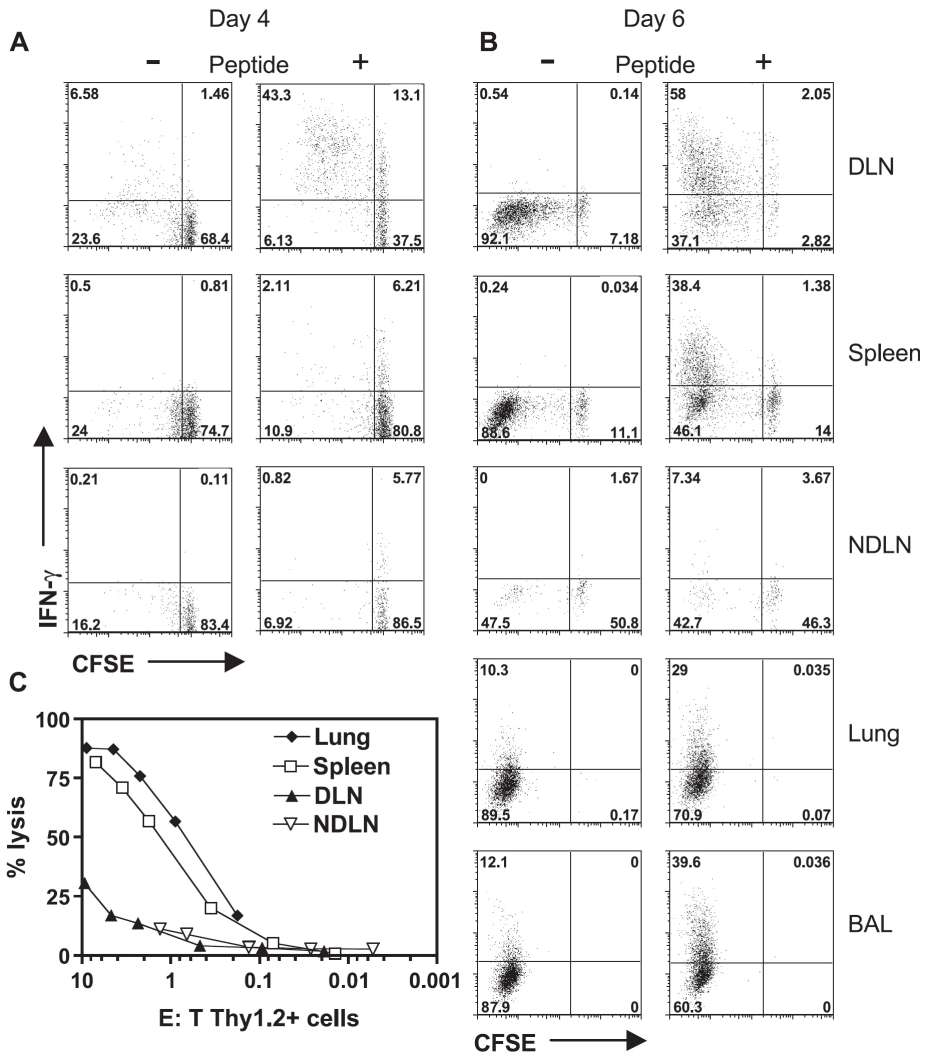

Figure 3. Adoptively transferred CD8$^+$ T cells are able to make IFN-γ upon restimulation and are immediately cytotoxic ex vivo. Thy1.2$^+$ CFSE-labeled naive clone-4 HA CD8$^+$ transgenic T cells were adoptively transferred into Thy1.1$^+$ recipients followed 18 h later by influenza infection. Mice were sacrificed 4 (A) or 6 (B) days postinfection, and cells from various organs were incubated with or without HA peptide overnight followed by intracellular cytokine staining. (C) Seven days postinfection, cytotoxicity of cells from various organs was tested in a ^{51}Cr release assay. E:T ratios are calculated on the number of Thy1.2 donor cells in the sample. Results shown are one representative experiment of (A, B) three and (C) two.

Adoptively transferred CD8$^+$ cells can produce IFN-γ in response to specific influenza peptide and are cytotoxic immediately ex vivo

Cytokine expression by donor CD8$^+$ CFSE-labeled T cells was examined ex vivo by flow cytometric intracellular cytokine staining to document the acquisition of effector function in the donor cells [35, 36]. IFN-γ expression by donor cells at Days 4 and 6 postinfection is shown in Figures 3A and B. Cells recovered at Day 4 showed significant IFN-γ expression in DLN, mostly from divided cells, but there was some IFN-γ expression in spleen and NDLN. At Day 6, cells from all organs examined produced intracellular IFN-γ in response to peptide, and the majority of these IFN-γ positive cells had divided many times (low CFSE intensity). The highly divided cells are also capable of producing TNF-α at Days 6 and 8 postinfection (data not shown).

To further characterize the effector function, we investigated whether donor cells were able to lyse peptide pulsed target cells in an ex vivo 4-h chromium release assay, isolated and tested without restimulation. As shown in Figure 3C, the immediate ex vivo HA peptide-specific CTL activity is similar for donor cells recovered from the lungs and spleen where high amounts of cytotoxicity were found. Cells recovered from the DLN were less cytotoxic, and cells from NDLN were without activity. Thus, the adoptively transferred CD8$^+$ T cells develop the surface phenotype and ex vivo function consistent with highly activated influenza-specific effectors in the influenza-infected host.

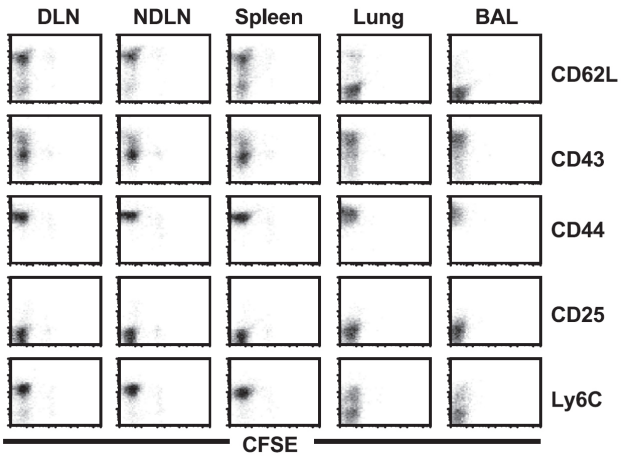

Figure 4. Adoptively transferred CD8+ lymphocytes possess a resting phenotype at Day 29 postinfection. Cells were transferred and mice infected with influenza as described in legend to Figure 1. After 29 days, cells from DLN, NDLN, spleen, lung, and BAL were analyzed by flow cytometry. Results are one mouse in a group of three from a representative experiment repeated eight times with similar results.

Adoptively transferred CD8+ T cells still present at Day 30 postinfection are able to make cytokines upon restimulation in vitro

We had seen earlier that significant numbers of CD8+ lymphocytes persisted until at least Day 29 postinfection (Figure 1), and so the phenotype and CFSE intensity of these CD8 T cells were analyzed by flow cytometry as shown in Figure 4. The surface phenotype of these cells was significantly different from that seen during the effector phase. Resting CD8+ cells recovered from DLN, NDLN, and spleen expressed both high and low surface levels of CD62L, while CD8 T cells recovered from BAL and lung expressed low surface levels of CD62L. The cells in the lymphoid tissues had also decreased surface expression of CD43, but the majority of cells recovered from the lung maintained high surface expression of CD43. The expression of Ly6C was high to intermediate in lymphoid tissues and mixed medium and low in lung and BAL. The CD25 expression was low in all tissues examined, and CD44 remained high. The adoptively transferred CD8+ T cells persisting at Day 34 made IFN-γ and TNF-α when stimulated with HA peptide overnight, as shown in Figure 5. These cells resemble memory cells and produce cytokines after in vitro stimulation.

Cotransfer experiments showed that CD4+ T cells expanded less than CD8+ T cells and that the CD4+ T cells decline more rapidly

The numbers and accumulation kinetics we had found for CD8+ T cells differed from that previously found for CD4+ T cells [18]. It was therefore important to compare the recruitment and proliferation of adoptively transferred CD4+ and CD8+ T cells in the same animal to determine whether there were significant differences during influenza infection. We adoptively transferred 3 × 10^6 CFSE-labeled naive CD4+ HNT T cells and 3 × 10^6 CFSE-labeled naive CD8+ HA T cells into the same Thy-1 disparate host and enumerated these cells in the five sets of tissues at various times after influenza infection.

Neither CD4+ nor CD8+ T cells were present in the lungs or BAL in significant numbers on Day 4 (Figure 6A). At Day 7, both cell types appeared in the lung, and BAL and had downregulated CD62L surface expression (data not shown). The CD8+ donor cell numbers within the DLN were higher than that of CD4+ T cells, higher in the lung, and comparable in the spleen. The number of CD8+ T cells peaked 10-fold higher in the lung at Day 9, and the peak of the response was later than that seen for donor CD4+ cells. The subsequent decline of the CD4+ T cells was more pronounced than that of CD8+ T cells in the lung and BAL. In addition, there were many folds more CD8+ than CD4+ T cells in the lung and BAL at Day 30 postinfection, but more similar numbers of CD4+ and CD8+ T cells were seen in spleen, DLN, and NDLN. FACS profiles of CFSE-labeled donor cells recovered at Days 4 and 7 postinfection are shown in Figure 6B, demonstrating that CD4+ and CD8+ T cells divide similarly in response to influenza infection. (In the figure, CD4+ T cells are scored as CD8−, but the same picture was seen when cells were stained with anti-CD4, and CD8+ cells were scored as CD4−, data not shown). Both adoptively transferred CD4+ and CD8+ cells gained surface expression of CD43 and lost surface expression of CD62L concurrently with increasing number of divisions (data not shown). The cotransfer experiments confirmed that CD8+ T cells expanded more than CD4+ T cells, that the CD8+ T cell response was more prolonged, and that the higher number of CD8+ effectors at the peak was followed by higher numbers of persisting memory cells at Day 30 postinfection.

Adoptive cotransfer of decreasing numbers of CD8+ and CD4+ T cells shows that smaller numbers of CD8+ T cells expand to a greater extent than small numbers of CD4+ T cells

We were concerned that the high numbers of cells used in the adoptive transfers might lead to artificially high numbers of cells recovered and inefficient activation. In other studies, we found that adoptive transfer of small numbers (10^2) and large numbers (10^7) of naive transgenic CD8+ T cells with peptide pulsed dendritic cells resulted in similar numbers of antigen-specific cells recovered at Day 7 after transfer (Kemp et al., unpublished). We concluded that it should be possible to follow the later stages of the response in the current studies using much lower number of naive donor cells and determine whether kinetics of accumulation between CD4+ and CD8+ T cells were still different beginning with a lower number of cells.

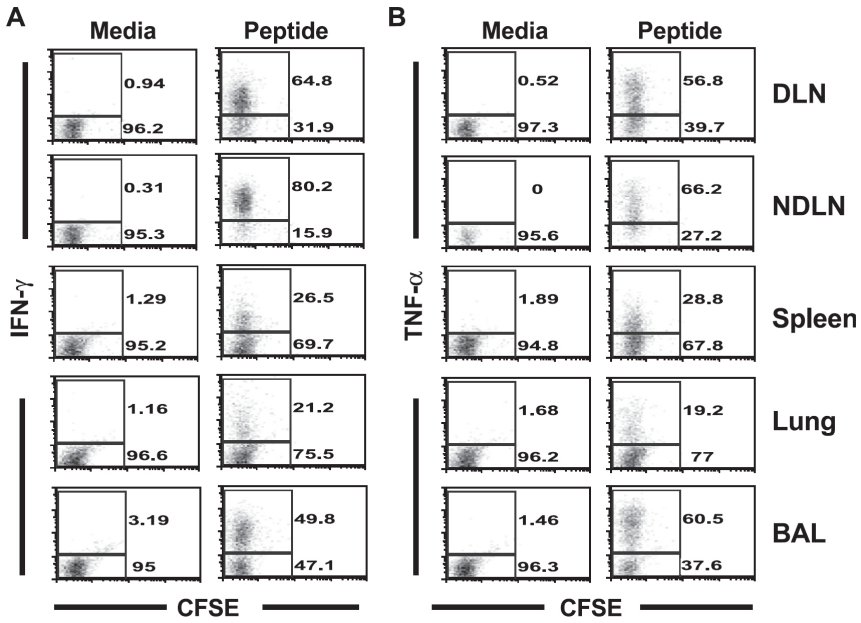

Figure 5. Adoptively transferred cells recovered at Day 34 postinfection are able to make IFN-γ and TNF-α upon in vitro stimulation. Cells were transferred and mice infected with influenza as described in legend to Figure 1. After 34 days, mice were sacrificed and cells from DLN, NDLN, spleen, lung, and BAL were incubated overnight followed by intracellular cytokine staining for (A) IFN-γ and (B) TNF-α. Results are one mouse in a group of two from a representative experiment repeated twice with similar results.

We transferred varying numbers of $CD4^+$ and $CD8^+$ cells into the same animal and compared the numbers found in various organs after at different time points postinfection. As shown in Figure 7, we again found that the number of both $CD4^+$ and $CD8^+$ T cells recovered at later time points was not proportional to the numbers injected but converged towards the same peak level. The greater expansion and localization to the lung of $CD8^+$ T cells than $CD4^+$ were still apparent at the intermediate and lowest input dose. The overall kinetics of the two

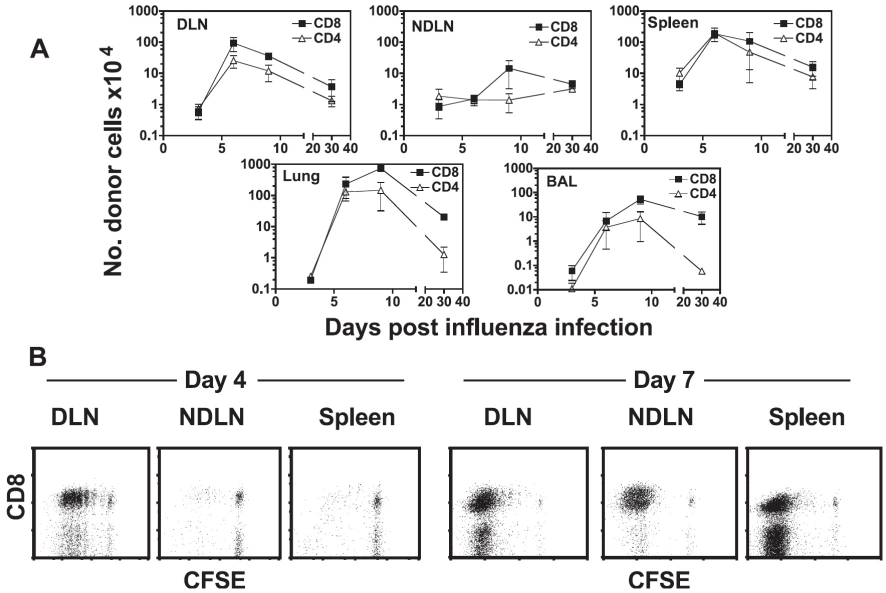

Figure 6. $CD8^+$ T cells possess more prolonged expansion and reach higher numbers than $CD4^+$ T cells, but division is similar after influenza infection. $Thy1.2^+$ CFSE-labeled naive HNT $CD4^+$ transgenic T cells, 3×10^6, and $Thy1.2^+$ CFSE-labeled naive clone-4 HA $CD8^+$ transgenic T cells, 3×10^6, were adoptively transferred into $Thy1.1^+$ recipients followed 18 h later by influenza infection. (A) Mice were sacrificed at indicated times and cells from DLN, NDLN, spleen, lung, and BAL were analyzed by flow cytometry and the number of cells in each organ calculated. Values shown are mean ± SD of three mice per group. (B) CFSE profiles versus $CD8^+$ staining in donor cells isolated at Days 4 and 7 postinfection. These are representative data from one experiment repeated five times with similar results.

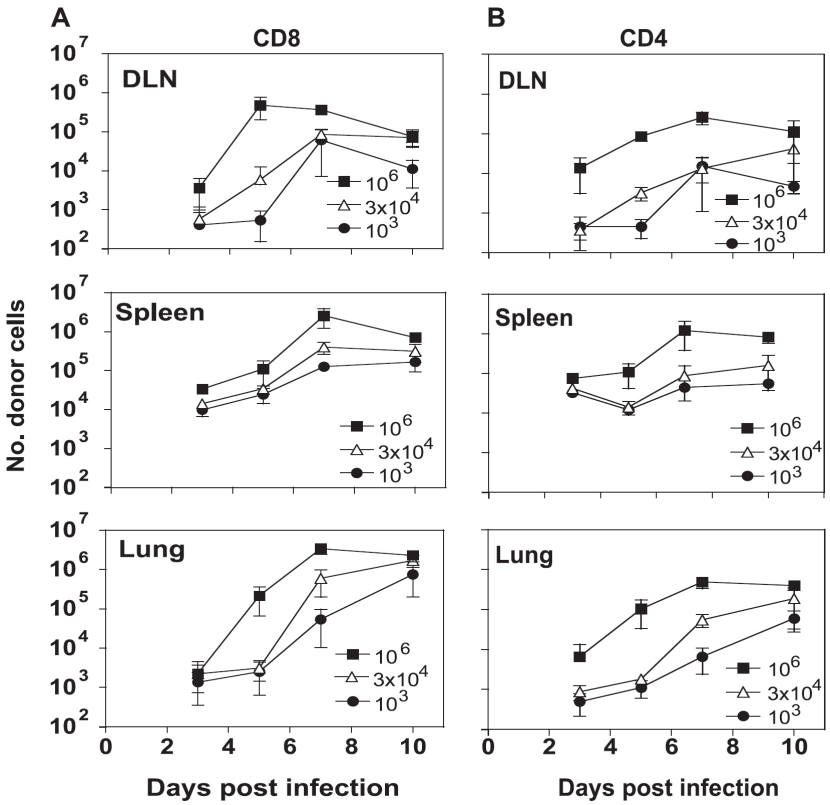

Figure 7. Titration of donor cells reveals similar dose-dependent kinetics between CD4+ and CD8+ T cell accumulation after influenza infection in vivo. Graded numbers of (Thy1.1+ × Thy1.2+) F1 CD4+ and CD8+ T cells were mixed and adoptively transferred into Thy1.2+ recipients subsequently infected with influenza (6000 EIU). The number of donor Thy1.1+ CD8+ (A) or CD4+ (B) T cells was calculated from DLN, spleen, and lung. Values shown are mean ± SD of four mice per group. These data are from a representative experiment repeated twice with similar results.

responses are similar for the CD4+ and CD8+ T cell populations. Therefore, we find that decreasing the numbers of adoptively transferred cells confirms the observation that CD8+ T cells reach a higher peak number than CD4+ T cells in the lung, and these increased numbers also occur with smaller numbers of input cells.

Discussion

We have shown here that adoptively transferred CFSE-labeled HA peptide-specific TCR transgenic CD8+ T cells are found to have begun to divide first and produce IFN-γ in the DLN, then at later time points are detected in large numbers in the lung, BAL, and spleen in response to influenza infection. Highly activated effectors recovered from the lung are able to kill peptide pulsed targets ex vivo and are able to produce cytokines upon in vitro restimulation. The surface phenotype of these cells differs in various tissues and changes as the cells divide. The expression of CD25, CD43, CD44, and several other adhesion molecules becomes elevated, while the expression of CD62L declines. When the activation of CD4+ and CD8+ T cells in response to influenza were compared directly in the same mouse, both cell types were initially found in large numbers in the DLN and at later time points in the lung, BAL, and spleen. The gain of effector phenotype and CFSE-monitored cell division was similar in both CD4+ and CD8+ T cells. CD8+ T cells expanded to higher numbers than CD4+ T cells, and the decline in number of CD8+ T cells after the peak was more gradual. The number of recovered CD8+ and CD4+ T cells drops dramatically in all organs sampled after virus has been cleared, but a residual population of adoptively transferred memory cells is detectable until at least Day 30 postinfection.

Adoptively transferred influenza HA-specific CD8+ T cells in the DLN divide and express increasingly high levels of CD25, CD43, and CD44 and lower levels of CD62L at 4 days postinfection. These changes are not seen in cells found in the spleen and NDLN at Day 4 suggesting that the HA-specific cells are initially activated in the draining lymph nodes which had been proposed before from cell accumulation data [21]. At later time points, the HA-specific cells possess an activated phenotype in all organs examined. The cells in the lung and BAL have higher levels of CD25 and CD43 and lower levels of CD62L correlating well with the higher cytotoxic activity of cells isolated from the lung. The different populations of cells in the two locations could represent different subsets of effectors or cells at different activation states.

Cell division is generally correlated with activation for both $CD4^+$ and $CD8^+$ T cells [6, 37, 38] but may not be the case with activation-induced tolerance [39]. We address this question directly using CFSE and surface activation markers and find that division correlates with activation in this infection (Figure 2 and Figure 3). Cytotoxic cells were recovered from the lungs and spleen, and these populations consisted an overwhelming majority of divided cells. Undivided cells express elevated levels of CD69 (data not shown) and can produce cytokines upon restimulation in vitro (Figure 3), suggesting that these cells are partially activated as recently reported by others [38].

CD43 expression declines in the lymphoid tissues but remains high in the lung and BAL at Day 30 and highlights differences between lymphocytes persisting in the lungs/BAL and lymphoid tissues [40 and 41]. Previous studies had only shown CD43 decline in the spleen [42]. The Ly6C expression on naive cells consists both a positive and negative population, the significance of which is unclear [3]. Here, we show an increased surface level of Ly6C during influenza infection which is similar to anti-CD3 activation in vivo [43]. Ly6C remains high on memory $CD8^+$ cells in peripheral lymphoid tissues and is mixed within the lung and BAL. Ly6C may play a role in the trafficking of $CD8^+$ lymphocytes [44], but the significance of the biphasic level of expression in the lung is not clear but could indicate differential function or migration ability.

The adoptively transferred CD8 T cells develop effector function, but this differs in cells from different locations. We found high levels of ex vivo CTL activity in cells from the lung and spleen, but little or no activity in cells from the DLN and NDLN. CTL activity in cells from the influenza-infected lung has been reported previously [13, 33]. This lack of CTL activity in DLN agrees with Johnson et al. [45], but they also found low CTL activity in the spleen. The higher numbers of CTL in the spleen after adoptive transfer and infection may reflect higher numbers of activated cells accumulating or that the adoptively transferred cells have better access to antigen and therefore are more highly activated. The adoptively transferred cells are able to make IFN-γ and TNF-α, as previously found using tetramers to identify influenza-specific $CD8^+$ T cells [7]. Our data indicate that IFN-γ is acquired early in the response before cells have divided showing that IFN-γ is switched on earlier than previously appreciated.

Recent suggestions have been made that $CD8^+$ T cells proliferate more rapidly than $CD4^+$ T cells [2, 27, 28]. In our experiments, we followed the expansion of CFSE-labeled $CD4^+$ T cells and $CD8^+$ T cells in the same mouse. Our data are in agreement with that found by Homann et. al. [27] who reported that $CD4^+$ and $CD8^+$ T cells expanded to a peak at a similar rate, but the number of $CD4^+$ cells decline more rapidly after the peak. We have also confirmed the same difference between the two cell types, using very much lower initial number of $CD4^+$ or $CD8^+$ T cells demonstrating that both cell types were capable of perhaps 10000-fold expansion, but total numbers were lower for $CD4^+$ T cells than $CD8^+$ T cells indicating that these two populations were regulated differentially after the first five to six divisions. The cells that persist 30 days after adoptive transfer express low levels of CD25 indicating resting memory cells [3]. The higher numbers of $CD8^+$ than $CD4^+$ we see at Day 30 are in agreement with recent studies that suggest that $CD8^+$ T cells are more stable as memory cells than $CD4^+$ T cells after respiratory infection [46]. The mechanism of differential survival of $CD4^+$ and $CD8^+$ T cells after influenza infection is unclear but could be due to differential expression of Bcl_2 as proposed for LCMV [27]. Other mechanisms could be that the cells are responding to different costimulatory signals such as OX40 [47] or CD27 [48] or different amounts of persisting antigen presented by MHC class I or II molecules [49]. The TCR transgenic cells may not reflect all populations of antigen-specific cells because immunodominance and antigen affinity may affect the relative kinetics of the two populations [50, 51]. Examination of host $CD8^+$ and $CD4^+$ T cell responses to influenza infection indicates that the $CD8^+$ T cells predominate in the lungs as seen with the adoptive transfer studies (data not shown). Also, these data shown here agree with studies analyzing antigen-specific $CD4^+$ and $CD8^+$ T cells after Sendai virus infection [46].

In summary, we have described an adoptive transfer system that allows the visualization of both the early and late stages of antigen-specific $CD4^+$ and $CD8^+$ T cell response during influenza infection in the same recipient. We have been able to demonstrate the relative kinetics of an antigen-specific population of both $CD4^+$ and $CD8^+$ T cells responding to influenza infection. The phenotypic changes that occur and localization of the cells into the lung and BAL are similar for both $CD4^+$ and $CD8^+$ T cells. Both $CD4^+$ and $CD8^+$ immune responses to influenza appear to be initiated in draining lymph nodes. The responding cells undergo similar changes in the expression of cell surface markers. The $CD4^+$ and $CD8^+$ T cells appear to divide at a similar rate, but $CD8^+$ T cells increase to 10-fold more than $CD4^+$ T cells. The $CD8^+$ T cells continue to expand after the $CD4^+$ T cells peak and then decline in numbers more slowly than $CD4^+$ T cells. Thus, $CD4^+$ and $CD8^+$ T cells are regulated differentially to perform distinct functions, and this adoptive transfer system is ideal to investigate these responses.

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