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Persistent Depots of Influenza Antigen Fail To Induce a Cytotoxic CD8 T Cell Response

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Encounter with Ag during chronic infections results in the generation of phenotypically and functionally heterogeneous subsets of Ag-specific CD8 T cells. Influenza, an acute infection, results in the generation of similar CD8 T cell heterogeneity, which may be attributed to long-lived depots of flu Ags that stimulate T cell proliferation well after virus clearance. We hypothesized that the heterogeneity of flu-specific CD8 T cells and maintenance of T cell memory required the recruitment of new CD8 T cells to persistent depots of flu Ag, as was the case for flu-specific CD4 T cell responses. However, robust expansion and generation of highly differentiated cytolytic effectors and memory T cells only occurred when naive CD8 T cells were primed during the first week of flu infection. Priming of new naive CD8 T cells after the first week of infection resulted in low numbers of poorly functional effectors, with little to no cytolytic activity, and a negligible contribution to the memory pool. Therefore, although the presentation of flu Ag during the late stages of infection may provide a mechanism for maintaining an activated population of CD8 T cells in the lung, few latecomer CD8 T cells are recruited into the functional memory T cell pool.


During immune responses to flu, CD8 effector cells can be heterogeneous with regard to their activation phenotype, ability to immediately secrete cytokines, and migration to the lung, where the virus replicates (1, 2). It is clear that the migration of highly differentiated IFN-γ-producing and cytotoxic CD8 T cells to the lung after flu infection is important for virus clearance and protection against reinfection (3–6). Yet, the Ag-dependent T cell stimulatory events required to generate a protective flu-specific CD8 CTL response are largely unknown.

Numerous studies have provided evidence that the efficient presentation of pathogen-derived Ag plays a critical role in the induction of effective T cell immunity to pathogens (7–12). A recent study by Zammit et al. (13) established that long-lived depots of flu Ag persist for at least 60 days beyond flu infection. They went on to demonstrate that persistent flu Ag functions to maintain an activated population of CD8 T cells in the lungs, which is predicted to provide immediate protection against reinfection (13). However, it is still unclear to what extent the late stages of Ag presentation induce the generation of functionally competent CD8 CTL responses during an acute infection, and whether new naive CD8 T cells stimulated by long-lived depots of flu Ag constitute a significant portion of the flu-specific memory population. Although current evidence suggests that the maintenance of CD8 memory T cells generated during acute infections is independent of Ag (14, 15), there is strong evidence that the maintenance of CD8 T cell memory during chronic infections is dependent on persistent Ag stimulation (16–19). Long-lived depots of flu Ag may also provide a mechanism for the generation and maintenance of T cell memory through the recruitment of new naive CD8 T cells into the memory pool by persistent Ag stimulation. This theory is supported by our own findings with CD4 T cells, which demonstrated that although high-dose Ag presentation, available during the first week of flu infection, resulted in the most efficient accumulation of IFN-γ-producing effectors in the lungs, persistent low-dose Ag presentation, available during the second and third weeks of flu infection, resulted in the most efficient CD4 memory T cell generation.

To define the role of the late stages of flu Ag presentation on the development of functionally competent CD8 effector and memory T cell populations, we analyzed CD8 T cell responses when naive CD8 T cells were introduced into mice at different times following flu infection. In this study, we show that only CD8 T cells primed during the first week of flu infection expand and differentiate into a large population of highly functional CTL effectors. Alternatively, naive CD8 T cells introduced after the first week of flu infection give rise to modest numbers of IFN-γ-producing effectors with little cytolytic activity. Our findings suggest that functionally heterogeneous CD8 T cell immunity is generated by exposure to flu Ag presentation during the acute phase of infection, and few latecomer CD8 T cells are recruited into the memory pool by long-lived depots of flu Ag.

Materials and Methods

Mice

Clone 4 Vβ8.2/Nα10 TCR-transgenic (Tg)3 mice (hemagglutinin protein Tg (HA Tg)) were backcrossed for at least eight generations to BALB/cBy. The CD8 T cells from HA Tg mice express TCRβ chains specific for the IYSTVASSL peptide Ag, encoded by amino acid residues 518–528 of the PR8 virus HA protein, and presented in the context of H-2Kb.

Abbreviations used in this paper: Tg, transgenic; HA Tg, hemagglutinin protein Tg; MDCK, Madin-Darby canine kidney; PA, polymerase; DLN, draining lymph node; WT, sham thymectomy; i.n., intranasal; Grb, granzyme B.

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The HA Tg mice were used at 4–6 wk of age. Thy.1+ BALB/cBy mice were bred at the Trudeau Institute’s Animal Breeding Facilities and were used at 8–15 wk of age. Thymi were surgically removed under nembutal anesthesia (Abbott Laboratories), and mice were allowed to recover for 10 days before flu infection. All experimental procedures involving mice were approved by the Trudeau Institute’s Institutional Animal Care and Use Committee.

### Virus infections

The PR8 flu virus was grown in the allantoic fluid of 10-day-old embryonated chicken eggs and characterized by a core facility at the Trudeau Institute. Mice were inoculated intranasally (i.n.) with 0.1 LD₅₀ (500 PFU) of PR8 flu virus in 100 μl of PBS (Sigma-Aldrich) during light isoflurane anesthesia (Webster Veterinary Supply). The x31 flu virus was provided by Dr. D. Woodland (Trudeau Institute). Mice were inoculated i.n. with 0.1 LD₅₀ (300 × 50% egg infectious units) of x31 flu virus in 100 μl of PBS.

### Virus quantification

Infectious viral titers (viral PFU) were determined using a modified Madin-Darby canine kidney (MDCK) cell plaque assay as previously published (9, 22). At various times after flu infection, lungs were harvested into 1 ml of DMEM (Invitrogen Life Technologies), homogenized, serially diluted, and added to duplicate wells of 6-well plates containing confluent monolayers of MDCK cells for 1 h at 37°C. One milliliter of agar overlay was added to each well (DMEM plus 0.2% BSA, 2 mg/ml NaHCO₃, 2 mM HEPES, 0.5% agar, 0.01% DEAE-dextran, and 0.5 μg/ml trypsin Sigma-Aldrich). After 2–3 days of incubation at 37°C, MDCK cells were fixed with 0.5 ml of Carnoy’s fixative and stained with 2% crystal violet in 20% ethanol (Sigma-Aldrich). Average PFU per lung = (mean number of plaques/0.1) × (1/dilution factor of tissue homogenate).

Alternatively, the number of viral RNA copies per lung were determined by quantitative RT-PCR. Viral RNA was isolated from prepared lung homogenates using TRIzol (Life Technologies) and 2.5 μg of RNA was reverse transcribed into cDNA using random hexamer primers and SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). Quantitative PCR was performed to amplify the polymerase (PA) gene of the PR8 flu virus. The following primers and probe were used to amplify the PR8 flu PA gene: forward primer, 5'-CG GTCCAAATTCCTGCTGA-3'; reverse primer, 5'-CATTGGGTTCTCTCTCA TCCA-3'; probe, 5'-FAM-CCAAGTCATGAAGGAGAGGGAATACCGC CG-3' (using an Applied Biosystems Prism 7700 Sequence Detector (Applied Biosystems) with 50 ng of sample cDNA per reaction. Data were analyzed using Sequence Detector version 1.7a software (Applied Biosystems), and the number of copies of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid of known concentration as a standard. Multiplying by dilution factors allows an estimation of the number of copies of PA gene per lung, thereby allowing for quantification of virus genetic material.

### Naïve CD8 T cell isolation and T cell transfers

Naïve CD8 T cells were enriched from spleens and peripheral lymph node lymphocytes by MACS using murine CD8α (Ly-2) MicroBeads and LS columns according to the manufacturer’s protocols (Miltenyi Biotec). Discontinuous Percoll (Sigma-Aldrich) gradient separation was used to enrich for small resting cells (23). The purified T cells were routinely used with greater than 95% purity using a FACSVantage SE flow cytometer and seeded in 96-well plates at 1 × 10⁶ cells/well. Irradiated splenocytes were pulsed with H-2Kd-restricted PR8-specific CD8 CTL peptide HATG126–134 and probe were used to amplify the PR8 flu virus gene: forward primer, 5'-PA GTCCAAATTCCTGCTGA-3'; reverse primer, 5'-PA GTCCAAATTCCTGCTGA-3'; probe, 5'-PA GTCCAAATTCCTGCTGA-3'; probe, 5'-FAM-CCAAGTCATGAAGGAGAGGGAATACCGC CG-3' (using an Applied Biosystems Prism 7700 Sequence Detector (Applied Biosystems) with 50 ng of sample cDNA per reaction. Data were analyzed using Sequence Detector version 1.7a software (Applied Biosystems), and the number of copies of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid of known concentration as a standard. Multiplying by dilution factors allows an estimation of the number of copies of PA gene per lung, thereby allowing for quantification of virus genetic material.

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### Results

**Impact of persistent depots of flu Ag on CD8 T cell proliferation and expansion**

To determine the possible differences in Ag load when naïve CD8 T cells are introduced at various times after flu infection, we sublethally infected BALB/c mice i.n. with the highly pathogenic A/PR/8/34 (PR8) strain of influenza A virus, and determined virus titers at various times after infection using traditional plaque assay and quantitative PCR analysis of viral RNA (9). Viral replication was extremely rapid, with virus titers peaking in the lungs during the first 7 days of infection and falling to undetectable levels by day 12 of infection (Fig. 1A).

To confirm that long-lived depots of flu Ag could stimulate Ag-specific CD8 T cell proliferation beyond virus clearance, we used an adoptive transfer model similar to that of previous studies of T cell responses to flu infections (9, 13). Thy1.2+ naïve HA Tg CD8 T cells, expressing TCR specific for an antigenic peptide (IYSTVASSL) of the HA protein of PR8 flu virus presented by H-2Kd MHC class I (24), were transferred into intact BALB/c mice at various times after flu infection and allowed to respond to in vivo flu Ag presentation. Fig. 1 illustrates the model system where naïve flu-specific CD8 T cells are transferred (Fig. 1B) during a period of active virus replication with large Ag load, during a period when the virus is being cleared by the host immune response and there is an intermediate Ag load (Fig. 1C), or after live...
virus clearance and when there is low Ag load (Fig. 1D). As a control to establish that HA Tg CD8 T cell responses were Ag specific, and not a consequence of ongoing inflammatory responses, we infected an additional group of BALB/c mice with the x31 strain of influenza A virus, which does not contain the IYSTVASSL antigenic peptide sequence (20, 21), but does induce a robust inflammatory response (25). As expected, naive HA Tg CD8 T cells divided only in response to PR8 and not x31 flu infection, indicating that the PR8-specific IYSTVASSL antigenic peptide was necessary to drive donor HA Tg CD8 T cell responses (Fig. 2A; x31 infection).

The CFSE profiles of donor Thy1.2⁺ HA Tg CD8 effectors, recovered 7 days after T cell transfer, indicated that all naive HA Tg CD8 T cells proliferated when introduced at days 0 and 7 of PR8 flu infection, and substantial proliferation occurred even when donor HA Tg CD8 T cells were introduced at day 14 of infection (Fig. 2A). Transfer of naive HA Tg CD8 T cells at times as late as 60 days after PR8 flu infection resulted in detectable Ag-driven T cell proliferation, but at times beyond day 21 of infection resulted in progressively higher percentages of donor T cells that failed to proliferate in response to long-lived depots of flu Ag (data not shown). These results mirror our earlier finding with flu-specific CD4 T cells (9, 25–28). Therefore, it is possible that the recruitment of naive CD8 T cells by early flu Ag presentation establishes a population of highly differentiated memory T cells, suggesting that Ag presentation, even at early times after flu infection, may become limiting, due to the resolution of infection by the host immune response.

Although it has been established that long-lived depots of flu Ag function to maintain an activated population of flu-specific CD8 memory T cells in the lungs (13), it is not known whether the flu-specific CD8 memory population is comprised of CD8 T cells recruited into the antiviral response by early and/or late phases of Ag presentation. Persisting pools of memory T cells are often characterized by a substantial degree of heterogeneity in the T cell phenotype and function (9, 25–28). Therefore, it is possible that the recruitment of naive CD8 T cells by early flu Ag presentation establishes a population of highly differentiated memory T cells,
and latecomer naive CD8 T cells stimulated by persistent depots of flu Ag establish a population of intermediately differentiated memory T cells, thereby generating a combined population of heterogeneously differentiated CD8 memory T cells. To determine which stage of flu Ag presentation favors the generation of memory T cells, donor HA Tg CD8 T cells were analyzed as memory T cells 21 days after transfer, instead of as effectors 7 days after transfer. The CFSE profiles of the donor HA Tg CD8 T cells remained relatively unchanged between the effector and memory stages for T cells transferred on either day 0 or day 7 of flu infection (Fig. 2A, 7-day effectors vs Fig. 2C, 21-day memory). Interestingly, there were to be preferential survival of undivided donor T cells, compared with dividing donor T cells, during the transition to memory when the naive HA Tg CD8 T cells were transferred on day 14 after virus clearance. The actual number of surviving donor CD8 T cells revealed a dramatic impact of early vs late flu Ag presentation on the establishment of CD8 T cell memory. Naive CD8 T cells introduced on day 0 of flu infection had the greatest effector expansion (Fig. 2B), which translated to the largest number of CD8 memory T cells persisting 21 days after T cell transfer (Fig. 2D). Alternatively, naive CD8 T cells introduced after the first week of flu infection exhibited moderate to low effector expansions, which translated to low numbers of CD8 memory T cells. Therefore, it is likely that the flu-specific CD8 memory population is comprised mainly of CD8 T cells recruited into the antiviral response during the first week of flu infection, and naive CD8 T cells stimulated by long-lived depots of flu Ag do not constitute a significant portion of the total flu-specific CD8 memory T cell population.

**New naive CD8 T cells stimulated by depots of flu Ag are not required to generate memory**

We next wanted to determine whether new naive CD8 T cells, emerging from the thymus during the late phase of flu infection, were recruited into the CD8 memory pool by long-lived depots of flu Ag. Thus, we infected BALB/c mice with PR8 flu virus 10 days after thymus removal or sham thymectomy and assessed the endogenous effector and memory T cell generation by enumerating flu-specific, IFN-γ-producing host CD8 T cells. Thymectomy did not reduce the number of flu-specific, IFN-γ-producing CD8 T cells at either the effector (Fig. 3A) (7 days after infection) or memory (Fig. 3B) (37 days after infection) stages of the endogenous antiviral response, compared with sham thymectomy controls. In combination with our findings with delayed naive HA Tg CD8 T cell transfers (Fig. 2D), these results suggested that long-lived depots of flu Ag did not recruit significant numbers of new CD8 T cells into the memory T cell pool after live virus clearance.

**Late Ag presentation is inefficient at generating highly differentiated CD8 T cells**

To evaluate the level of differentiation of CD8 effectors generated during the first week of flu infection vs effectors generated during later stages of flu infection, we analyzed markers of T cell activation and cytokine function expressed by donor HA Tg CD8 effectors primed during the first, second, and third weeks of flu infection. We assessed CD62L expression, because high levels of CD62L expression have been used to define central memory-like T cells that migrate to and recirculate among peripheral lymphoid organs, whereas down-regulated expression of CD62L has been used to define effector memory-like T cells that migrate to nonlymphoid sites of infection, like the lung (29–34). We also assessed IFN-γ production as a marker of T cell function, suggested to correlate with protection against virus infections (6, 35–38).

Transfer of naïve HA Tg CD8 T cells into intact BALB/c mice on the same day as PR8 flu infection resulted in the generation of highly differentiated (CD62LlowIFN-γ+) donor CD8 effectors in the lungs, and the generation of intermediately differentiated (CD62Llow/midIFN-γ−/+) effectors in the DLN and spleen (Fig. 4A, day 0 transfer). Alternatively, transfer of naïve HA Tg CD8 T cells into intact BALB/c mice during the second (Fig. 4A, day 7 transfer) and third (Fig. 4A, day 14 transfer) weeks after flu infection resulted in the generation of progressively less-differentiated donor CD8 effectors, particularly in the DLN and spleen. The progressively less-differentiated phenotypes, resulting from delayed T cell transfer, were not due to an increase in the number of undivided or naïve donor CD8 T cells, because we excluded the CFSEhigh/undivided donor CD8 T cells from this phenotypic analysis. Additionally, we confirmed as little as one cell division resulted in a CD44high phenotype (data not shown); thus, all of the donor CD8 T cells analyzed for CD62L and IFN-γ expression had responded to flu Ag by dividing and up-regulating CD44 expression.

As the donor HA Tg CD8 effectors transitioned to memory, it became apparent that only naïve CD8 T cells transferred while there was live virus in the lungs of flu-infected mice (Fig. 4B, day 0 and 7 transfer), which resulted in highly differentiated memory T cells in the lung and intermediate differentiated memory T cells in the DLN and spleen. Naïve CD8 T cells transferred after virus clearance (Fig. 4B, day 14 transfer) established mostly a phenotypically and functionally undifferentiated population of memory T cells, with little to no CD62L down-regulation in the DLN and spleen, and little to no IFN-γ production.

The total number of CD62Llow and IFN-γ+ donor CD8 effectors (Fig. 4C) and memory (Fig. 4D) T cells also revealed a dramatic impact of the early vs late flu Ag presentation on the establishment of CD8 T cell memory. Naïve HA Tg CD8 T cells transferred on day 0 of flu infection established a significant population of CD62LlowIFN-γ− effectors, which translated to a large number of CD62Llow/midIFN-γ− CD8 memory T cells persisting 42 days after T cell transfer. Alternatively, naïve CD8 T cells, introduced after the first week of flu infection (Fig. 4C and D, day 7 and 14 transfers), established progressively lower numbers of CD62LlowIFN-γ− effectors and memory T cells.

**Late phases of flu Ag presentation fail to induce the generation of cytotoxic CD8 T cells**

Although IFN-γ production and lung migration by CD8 T cells has been demonstrated as being important for protection against flu infection (6, 35–38), CD8 T cells that kill virus-infected cells through the acquisition of cytolytic functions are critical for the clearance of, and protection against, respiratory virus infections...
(3–5). Therefore, we assayed CD8 effector and memory T cells, generated by transfer at different times after PR8 flu infection, for their expression of molecules associated with a CTL phenotype. CD107a is expressed in the membrane of intracellular cytotoxic granules of CD8 CTL before Ag-induced stimulation, and is then transported to the surface of CD8 CTL following Ag-induced degranulation, a necessary precursor of cytolysis (39, 40). GrB is a potent cytotoxic mediator that has been widely assayed as an intracellular marker to identify Ag-specific CD8 CTL (10, 41–45). There is a strong correlate between CD8 CTL functional activity against Ag-bearing targets, intracellular GrB expression, and CD107a surface expression following Ag/APC-triggered degranulation (43). Therefore, we assayed donor CD8 HA Tg effector (Fig. 5A) and memory (Fig. 5B) populations, generated by transfer at various times following PR8 flu infection, for intracellular GrB expression and surface expression of CD107a following Ag/APC-triggered degranulation. The results revealed a hierarchy of potential CTL functionality, with donor CD8 T cells stimulated during the first week of flu infection and recovered from the lungs, having the most active CTL-associated phenotype (Fig. 5A), which was low but detectable at the memory stage of the response (Fig. 5B).

Our analysis of the CTL phenotype suggested that CD8 effectors recovered from the lungs following stimulation during the first week of flu infection were the most likely to be capable of cytotoxic activity against Ag-bearing targets. In fact, Johnson et al. (43) have reported that flu-specific CD8 T cells capable of cytotoxic activity could be detected in T cells freshly isolated during primary infection from the lung, but not from the DLN or spleens, even though activated CD8 T cells isolated from all organs expressed significant levels of IFN-γ, perforin, and GrB. They concluded that although some CD8 T cells in the DLN and spleen mature to express perforin and GrB, these T cells only reach frequencies capable of cytotoxic function when they accumulate in the infected lung. To determine whether the correlate between CTL-associated phenotype and cytotoxic activity held for our flu-specific CD8 effectors, we assayed donor CD8 HA Tg effectors, generated by naive T cell transfer on day 0 or 7 of flu infection for their ability to kill Ag-bearing APC targets directly ex vivo. To ensure that the target killing was mediated by our donor CD8 HA Tg effectors, and not by host T cells, we enriched the Thy1.2+/H11001 donor CD8 HA Tg effectors to 99% purity by FACS sorting of the lungs of Thy1.1+/H11001 host mice 7 days after naive CD8 HA Tg cell transfer. Fig. 5C shows that transfer of naive HA Tg CD8 T cells into intact BALB/c mice on day 0 or 7 of flu infection for their ability to kill Ag-bearing APC targets directly ex vivo. To ensure that the target killing was mediated by our donor CD8 HA Tg effectors, and not by host T cells, we enriched the Thy1.2+/H11001 donor CD8 HA Tg effectors to 99% purity by FACS sorting of the lungs of Thy1.1+/H11001 host mice 7 days after naive CD8 HA Tg cell transfer. Fig. 5C shows that transfer of naive HA Tg CD8 T cells into intact BALB/c mice on day 0 of PR8 flu infection generated donor CD8 effectors with high levels of cytotoxic function, whereas transfer of naive HA Tg CD8 T cells into intact BALB/c mice 7 days after flu infection generated donor CD8 effectors with minimal killing ability. Donor CD8 HA Tg effectors recovered from DLN and spleens, as well as those recovered from the lung but primed after virus clearance, had no ability to kill Ag-bearing targets (data not shown).
without restimulation (gray-filled histograms) with isotype control histograms indicated by black lines. APCs (gray-filled histograms), or APCs pulsed with irrelevant peptides as negative controls (black lines). Intracellular expression of GrB was assayed.

DLN, spleens, and lungs were harvested, and donor T cells were analyzed for CD107a surface expression following restimulation with HA peptide-pulsed APCs (gray-filled histograms), or APCs pulsed with irrelevant peptides as negative controls (black lines). Intracellular expression of GrB was assayed without restimulation (gray-filled histograms) with isotype control histograms indicated by black lines. C, Donor Thy1.2 HA Tg CD8\(^+\) effectors were enriched to 99.9% purity from a pool of lungs 7 days after T cell transfer and assayed in vitro for the ability to kill target APCs pulsed with flu Ag vs irrelevant peptide. Data are expressed as the percentage of specific target lysis (average of triplicate wells). Data are representative of two independent experiments.

Discussion

These results suggest that persistent depots of flu Ag, although capable of stimulating naive T cells to divide long after live virus clearance, are inefficient at recruiting naive CD8 T cells into the antiviral immune response needed to clear the infectious virus (3, 4, 6, 43, 44). This is in striking contrast to our previous report that persistent depots of flu Ag are necessary for the generation and maintenance of CD4 memory T cells (9). We had also originally hypothesized that early and late presentation of flu Ag would cooperate to generate a population of phenotypically and functionally heterogeneous CD8 effectors that would persist to memory and provide functional flexibility during re-exposure to flu viruses. Although early presentation of flu Ag to naive CD8 T cells induces the generation of highly differentiated effectors, and late presentation of flu Ag induces intermediate differentiated effectors, our data fail to support this hypothesis, because few latecomer CD8 T cells expand and survive to memory compared with CD8 T cells primed during the first week of infection.

Because we used high precursor frequencies to track our donor HA Tg CD8 T cell populations, and high precursor frequencies have been linked to abnormal T cell differentiation and stability of memory T cells (46), we assayed for endogenous CD8 T cell responses to flu infection in thymicized mice. Thymectomy prevents the production of new naive T cells, leaving no naive T cells in the periphery to respond to long-lived flu Ag after all of the endogenous flu-specific precursors enter the antiviral response during the first week of infection. If recruitment of additional naive CD8 T cells by long-lived depots of flu Ag is required for the establishment of CD8 T cell memory to flu, there should have been a reduction in the total number of IFN-\(\gamma\)-producing CD8 memory T cells in thymectomized mice. We found no difference in the endogenous CD8 T cell responses to flu infection of thymectomized mice compared with sham treated mice. Therefore, although continued recruitment of naive CD4 T cells by long-lived depots of flu Ag is required for the maintenance of CD4 T cell memory to flu (9), recruitment of naive CD8 T cells by long-lived depots of flu Ag does not significantly contribute to the pool of CD8 memory T cells. It must be noted that thymectomy results in an accelerated decrease in the peripheral T cell pool over time and a low level of homeostatic T cell proliferation (47), which could result in a greater perturbation of peripheral CD8 T cell homeostasis compared with that of CD4 T cells. However, Dai and Lakkis (47) have shown that homeostatic proliferation of peripheral CD4 and CD8 T cells induced by thymectomy is not significantly different as long as secondary lymphoid organs are intact. Thus, differential homeostatic proliferation of CD4 and CD8 T cells does not account for the differences between CD4 and CD8 memory T cell generation in thymectomized mice. Additionally, we titrated the number of transferred donor HA Tg CD8 T cells from \(10^6\) to \(10^4\) flu-specific precursors, and found the highest level of donor CD8 T cell differentiation and establishment of memory when naive T cell transfer occurred on day 0 of flu infection, regardless of precursor frequency (data not shown). Therefore, we conclude that the recruitment of new naive CD8 T cells by long-lived depots of flu Ag is not required for the establishment of CD8 T cell memory to flu, while, at the same time, persistent depots of flu-Ag presentation may be functioning to maintain the activation state of CD8 T cells primed early after infection (13).

It is interesting to note that regardless of the time after flu infection when responding CD8 T cells were analyzed, it is the time...
of naive CD8 T cell transfer that has the dominant effect on the differentiation of the resulting effector and memory T cell populations. We could speculate that T cells transferred at early stages of flu infection are exposed to inflammatory mediators for a longer duration, compared with T cells transferred at late stages of infection, and thus take longer to equilibrate as a resting memory population. However, if we compare the data in the bottom row of Fig. 4A and the top row of Fig. 4B, where naive CD8 T cells are transferred on day 14 of flu infection and analyzed on day 21 of infection vs T cell transfer on day 0 of infection and analyzed on day 42 of infection, it is clear that early T cell transfer results in an expanded population of highly differentiated memory T cells (Fig. 4D). Conversely, naive CD8 T cells transferred after virus clearance and analyzed as activated effectors 7 days after transfer, 21 days after flu infection (Fig. 4A, day 14 transfer), do not down-regulate CD62L and express IFN-γ to the level observed in CD8 T cell populations responding following transfer on day 0 of infection. If time of infection when T cells were analyzed were the major determinant of phenotype, T cells analyzed 21 days after infection would have a greater level of CD62L down-regulation and IFN-γ expression, compared with T cells analyzed 42 days after infection, regardless of time of T cell transfer.

Regardless of the factors controlling CD62L and IFN-γ expression, it is clear that the acquisition of protective cytolytic function is restricted to those CD8 T cells primed during the first week of flu infection. This finding is compatible with reports that as little as a 2-fold change in perforin or GrB expression results in a dramatic difference in the ability of Ag-specific CD8 T cells to lyse targets (48), and virus-specific CD8 CTL can be detected in freshly isolated cells from the lung, but not from the DLN or spleen, during primary flu infection (43). We concur with these authors that the lack of CTL function outside the lung, as well as by CD8 T cells transferred after the first week of flu infection, is likely to be due to incomplete CD8 T cell maturation and low frequencies of CD8 T cells expressing the CTL phenotype. Therefore, we conclude that although some latecomer CD8 T cells mature into effectors capable of coexpressing GrB and surface CD107a, CD8 effectors only acquire significant cytotoxic function when they are primed during the first week of flu infection and accumulate in sufficient numbers in the infected lung.

Together our results are most compatible with a linear differentiation of flu-specific CD8 T cells, where acquisition of antiviral CTL function occurs at a stage of differentiation beyond that required for CD62L down-regulation and IFN-γ expression. Our data also support a model where the number of CD8 memory T cells persisting after primary stimulation are directly proportional to the number of effectors generated at the peak of the immune response (49, 50). The majority of flu-specific CD8 memory T cells generated by Ag-driven priming during the first week of flu infection, resulting in a heterogeneous population of CD8 memory T cells comprised of both central and effector memory lineages. However, a small number of naive CD8 T cells can be primed by long-lived depots of flu Ag and survive as a small memory population. It is interesting to speculate that those few latecomer CD8 T cells that survive to memory may constitute an independently generated and overwhelmingly central memory-like lineage (CD62L<sub>high</sub>,<sup>106</sup>) which may expand into a large effector memory population upon re-encounter with flu viruses.

These findings are not inconsistent with the model put forth by Wong and Pamer (12) that priming of naive CD8 T cells is transient due to a feedback mechanism that regulates the magnitude of CD8 T cell responses through selective killing of Ag-bearing APCs. In fact, we would suggest that the rapid decline of efficient CD8 T cell priming during late flu Ag presentation may in fact be due to the early generation of potent CTL that kill the majority of activated APCs presenting high levels of flu Ag, thereby leaving only a small number of immature APCs to present persistent Ag after virus is cleared and inflammation wanes. This would result in inefficient priming of latecomer CD8 T cells and fit with the findings of Storni et al. (51), who reported that although a brief Ag exposure is sufficient to stimulate multiple rounds of proliferation by CD8 T cells, Ag presentation without APC activation fails to induce the generation of highly differentiated CD8 T cell effectors.

We would suggest that T cell-mediated vaccines must be designed to mimic the natural course of flu infection. Such a vaccine would induce high levels of Ag presentation and inflammation to induce optimal CD8 T cell responses, while at the same time establishing a persistent depot of Ag presentation to induce optimal CD4 T cell responses.

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Disclosures
The authors have no financial conflict of interest.

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