Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation

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The kinetics of presentation of influenza virus–derived antigens (Ags), resulting in CD4 T cell effector and memory generation, remains undefined. Naive influenza-specific CD4 T cells were transferred into mice at various times after influenza infection to determine the duration and impact of virus-derived Ag presentation. Ag-specific T cell responses were generated even when the donor T cells were transferred 3–4 wk after viral clearance. Transfer of naive CD4 T cells during early phases of infection resulted in a robust expansion of highly differentiated effectors, which then contracted to a small number of memory T cells. Importantly, T cell transfer during later phases of infection resulted in a modest expansion of effectors with intermediate phenotypes, which were capable of persisting as memory with high efficiency. Thus, distinct stages of pathogen-derived Ag presentation may provide a mechanism by which T cell heterogeneity is generated and diverse memory subsets are maintained.

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Abbreviations used: Ag, antigen; CFSE, carboxyfluorescein diacetate succinimidyl ester; DLN, draining LN; HA, hemagglutinin; Tg, transgenic.

Efficient presentation of pathogen-derived antigen (Ag), resulting in T cell priming, is essential for effective immunity to pathogens. Several recent studies have suggested that presentation of pathogen-derived Ag by DCs is short lived and is rapidly down-regulated before Ag or pathogen clearance (1–3). Alternatively, other studies have favored the concept that Ag presentation continues for as long as the pathogen survives and perhaps even long after viable microorganisms have been eliminated (4, 5). During immune responses to influenza viruses, CD4 effectors can be heterogeneous with regards to their activation phenotype, ability to immediately secrete cytokines, and their migration to the lung where the virus replicates (6, 7). The prevailing concept is that there are progressive stages of T cell differentiation and that only the most differentiated effectors can migrate to tertiary sites of infection (6) and provide optimum protection against infection (8–10). However, these studies did not follow primary CD4 T cell responses through memory persistence, so it is not clear from these previous studies what impact different stages of pathogen-derived Ag presentation may have on primary effector differentiation and what impact this may have on the formation of a persistent memory population.

To define the role of different stages of pathogen-derived Ag presentation on the development of CD4 effector and memory populations, we analyzed CD4 T cell responses when naive CD4 T cells were introduced at different times after an acute, sublethal influenza virus infection. Influenza infection induces robust T cell responses, and although live virus is cleared within the first 10 d of infection (6, 11, 12), we have determined that influenza-derived Ag presentation to CD4 T cells persists for ≥3 wk after virus clearance. Our results also show that highly efficient Ag presentation, resulting in the generation of large numbers of highly differentiated IFN-γ–producing CD4 effectors that migrate to both lymphoid sites and the lung, is restricted to naive CD4 T cells that are recruited into the response within the first week of infection. However, influenza-derived Ag presentation persists for several weeks after viral clearance and is capable of generating modest numbers of highly divided but progressively less differentiated effectors. Importantly, the recruitment of naive CD4 T cells into the response after viral clearance appears to be necessary in order to generate the greatest numbers of persisting memory T cells.

RESULTS

Persistence of influenza-derived Ag presentation

After influenza virus infection in mice, viral replication was extremely rapid and resulted in

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$\geq 10^6$ PFU in the lungs and 10^4 PFU in the draining LNs (DLNs) in the period of 2–5 d after infection (Fig. 1 A). Viral titers fell thereafter, and live virus was completely cleared by days 12–14 of infection (Fig. 1 A). Other methods of determining viral presence, including a sensitive PCR-based test, also found no viral RNA in the lungs after day 12 of infection (unpublished data).

To evaluate whether influenza-derived Ag could be presented to CD4 T cells after virus clearance, we established an adoptive transfer model in which naive CD4 T cells were transferred into mice at various times after infection. Fig. 1 B illustrates the model system in which primary CD4 T cells are transferred (i) during a period of active virus replication, (ii) during a period when virus is being cleared, or (iii) after live virus is cleared. Our adoptive transfer model system used naive CD4 T cells from HNT TCR transgenic (Tg) mice, which respond to HNT peptide, derived from the PR8 strain of influenza A virus, presented by MHC class II (13, 14). Carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled naive HNT TCR Tg donor T cells were transferred into intact mice at various times after a sublethal infection with live PR8 virus and were analyzed 7 d after T cell transfer at the peak of the effector response (6).

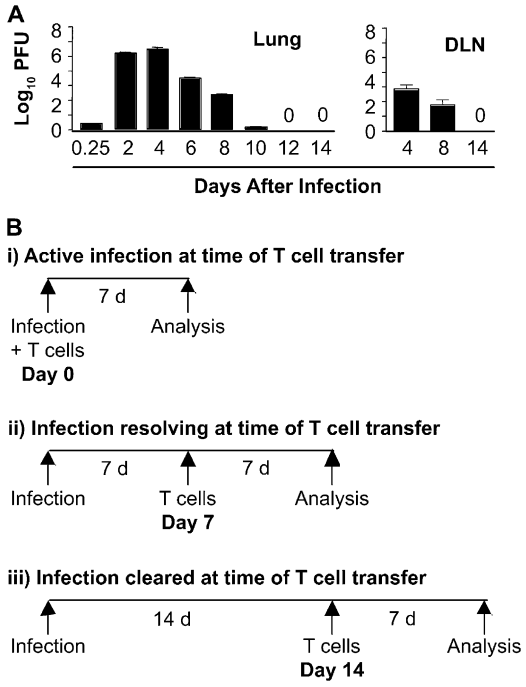

Figure 1. Rapid expansion and clearance of virus within 2 wk of influenza infection. (A) BALB/c mice were infected with PR8 influenza virus, and lungs and DLNs were assayed for live virus by plaque assay at the indicated times after infection. Values represent the mean ± SD of three mice. (B) Experimental setup to examine the responses of naive CD4 T cells to (i) active viral infection, (ii) resolving viral infection, or (iii) influenza-derived Ag presentation after virus clearance can be assayed by transferring CFSE-labeled naive CD4 T cells at various times after influenza infection.

The CFSE profiles of donor T cells indicated that substantial proliferation occurred even when donor T cells were transferred as late as 21 d after infection, and a small fraction of donor T cells had divided even when transferred as late as 35 d after infection (Fig. 2 A). The peak of influenza-derived Ag presentation, resulting in the greatest amount of cell division, was limited to the first 14 d after infection because the fraction of donor T cells that failed to divide within the first 7 d after transfer increased with time when transferred after live virus clearance (Fig. 2 A). Maximum recovery of responding donor T cells was seen in all organs when naive HNT TCR Tg CD4 T cells were transferred when live virus was still detectable (day 0 or 7 after infection; Fig. 2 B). Most notably, considerable donor T cell accumulation was only observed in the lung when naive T cells were transferred on the same day of infection.

Ag specificity of naive CD4 T cell responses

We used two separate model systems to establish that donor CD4 T cell responses were in fact Ag specific and not a consequence of ongoing inflammatory responses. CD4 T cells from HNT TCR Tg mice respond specifically to MHC class II (I-A^d) presentation of the HNT peptide encoded in residues 126–138 (HNTNGVTAACSHE) of the PR8 influenza A virus hemagglutinin (HA) protein (13,

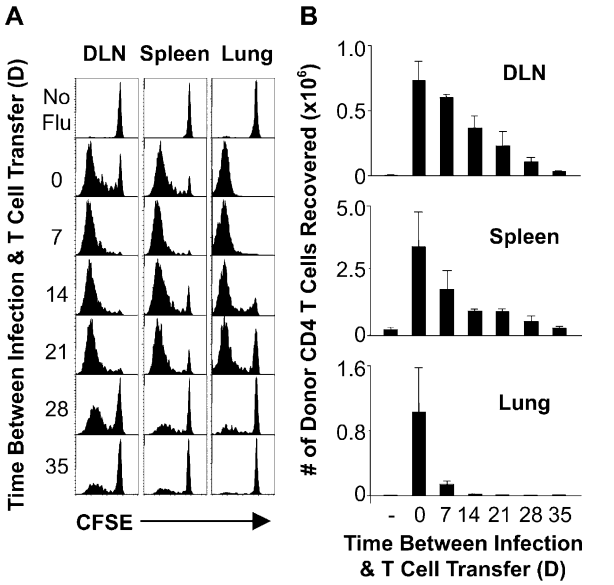

Figure 2. Influenza-derived Ag presentation stimulates naive CD4 T cells for ≥5 wk after infection. (A) 2×10^6 CFSE-labeled naive Thy1.1 HNT TCR Tg CD4 T cells were transferred i.v. into BALB/c mice previously infected with PR8 virus. 7 d after T cell transfer, DLNs, spleens, and lungs were harvested, and donor T cells were analyzed for CFSE dilution. Histograms were gated on donor Thy1.1 CD4 T cells and are representative of three individual mice in all experiments performed in this study. (B) Donor T cell numbers were calculated by multiplying the total number of live lymphocytes by the frequency of Thy1.1 CD4 T cells. Values represent the mean ± SD of three mice. Data are representative of three independent experiments.

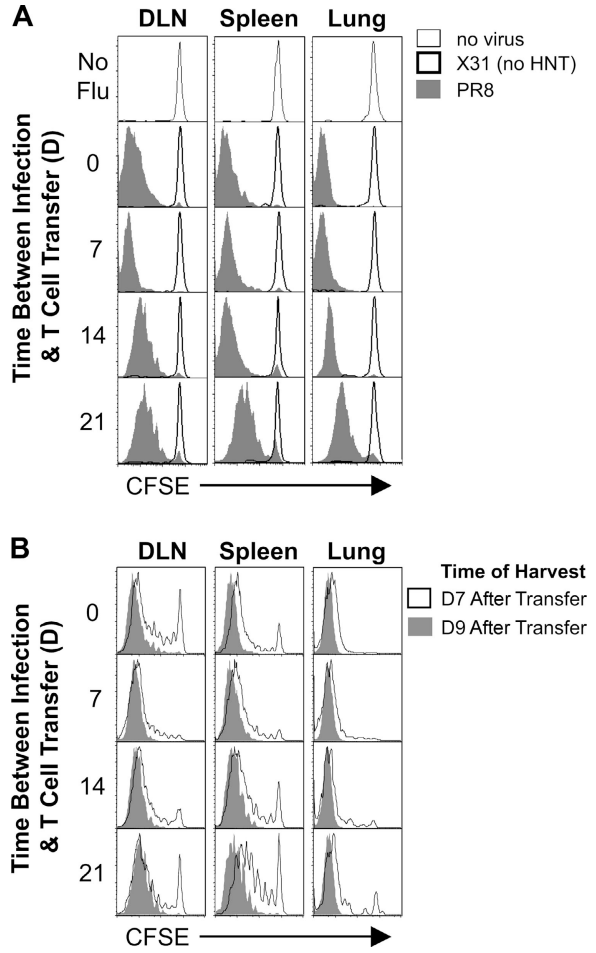

Figure 3. Proliferation of naive CD4 T cells to influenza-derived Ag presentation is Ag specific. CFSE-labeled naive Thy1.1 HNT TCR Tg CD4 T cells were transferred i.v. into BALB/c mice previously infected with the PR8 strain or the X31 strain of influenza virus, or into noninfected mice. (A) Donor T cells were analyzed 7 d after transfer for CFSE dilution. (B) Donor T cell responses to PR8 influenza virus infection were assayed 7 and 9 d after transfer. Data are representative of two independent experiments.

14). The X31 strain of influenza A virus does not contain this specific peptide sequence in its HA protein (15, 16), but it does induce a robust inflammatory response as indicated by lymphadenopathy and increased numbers of activated host T cells (unpublished data) (17). Naive HNT TCR Tg CD4 T cells were transferred into intact mice at various times after infection with a sublethal dose of either PR8 or X31 influenza A virus, and donor T cells were analyzed for cell division 7 d after transfer. The donor HNT TCR Tg CD4 T cells remained undivided and comparable with those transferred into uninfected mice despite the inflammation caused by the X31 virus infection (Fig. 3 A, open bold histograms). Only PR8 virus infection induced division of the transferred HNT TCR Tg CD4 T cells (Fig. 3 A, shaded histograms). This indicated that although the X31 strain of influenza virus induced a strong inflammatory response, presentation of PR8-specific HNT peptide was necessary to drive donor HNT-specific T cell division.

To further establish that division of donor T cells was Ag specific, we used a second model system in which ovalbumin-specific Tg CD4 T cells, purified from DO11.10 Tg mice (18), were transferred into intact BALB/c mice infected with a sublethal dose of PR8 influenza virus. In these experiments, the ovalbumin-specific donor T cells did not divide or accumulate in response to PR8 influenza infection (unpublished data). These two experimental systems indicated that the proliferation of influenza-specific T cells was Ag dependent and specific.

Because we observed that a fraction of the donor T cells (i.e., $CFSE^{hi}$) had not yet begun to proliferate in response to influenza-derived Ag presentation within the first 7 d after T cell transfer, we also examined donor CFSE profiles at later times after T cell transfer to determine if all of the transferred donor T cells would eventually divide. Fig. 3 B shows that

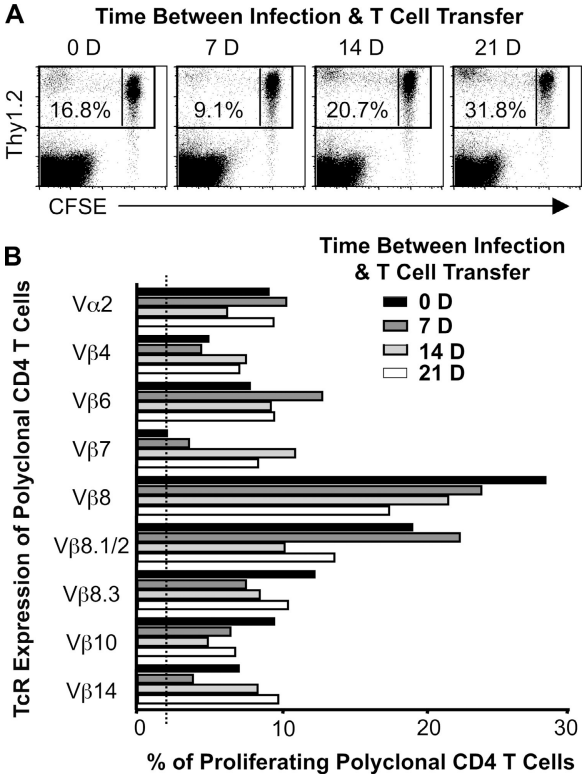

Figure 4. Long-term presentation of influenza-derived Ag stimulates polyclonal CD4 T cell responses. 10×10^6 CFSE-labeled naive, polyclonal Thy1.2 BALB/c CD4 T cells were transferred i.v. into Thy1.1xBALB/c mice previously infected with PR8 virus. 7 d after T cell transfer, DLNs, spleens, and lungs of three separate mice were pooled, and donor T cells were identified by gating on Thy1.2 T cells in the CD4 population (A). Numbers represent the percentage of donor T cells that had proliferated. (B) The percentage of proliferating polyclonal donor CD4 T cells expressing the indicated TCR Vβ or Vα gene product were determined by staining with 18 different anti-Vβ or anti-Vα antibodies and gating on the donor T cells that had divided (only the positive responders were graphed).

although a fraction of donor T cells recovered 7 d after T cell transfer were undivided (open histograms), by 9 d after T cell transfer (shaded histograms) >98% of the recovered donor T cells had divided.

Polyclonal CD4 T cells respond at late phases after infection

Natural processing and presentation of pathogen-derived Ag has been thought to be important for the generation of a broad repertoire of pathogen-specific T cells. To determine if multiple influenza-derived Ag epitopes were being presented after virus clearance, we transferred naive BALB/c polyclonal (non-Tg) CD4 T cells into intact Thy1.1xBALB/c mice at various times after infection with PR8 influenza virus. Polyclonal donor (Thy1.2$^+$) CD4 T cells that had divided, as indicated by the loss of CFSE, were analyzed 7 d after T cell transfer. Fig. 4 A shows that ≤30% of the recovered polyclonal donor T cells had divided, even when transfer of naive T cells was delayed until after virus clearance. Interestingly, delaying the polyclonal CD4 T cell transfer until day 7 after infection, which corresponded to the peak of the endogenous host T cell response, resulted in a suppression of the donor CD4 T cell response. To determine the diversity of the polyclonal response, we pooled lungs, DLNs, and spleens and analyzed the TCR Vβ and Vα gene expression on donor T cells that had undergone at least one round of proliferation. We found that polyclonal donor T cells expressing 9 different TCRs out of 18 Vβ and Vα chains screened had divided in response to influenza-derived Ag epitopes presented for ≥21 d after infection (Fig. 4 B). Strikingly, the diverse polyclonal donor T cell repertoire changed little as the delay between infection and T cell transfer increased, suggesting that the Ag-specific response remained broad even after live virus was cleared.

Late phases of influenza-derived Ag presentation can generate memory T cells

The persisting memory CD4 and CD8 T cell pool is characterized by a substantial degree of heterogeneity in phenotype and function, but the underlying mechanisms driving this heterogeneity remain unclear (17, 19–21). Recent results suggested that heterogeneity of different Ag-specific CD4 T cell responses are regulated by Ag exposure and load (22, 23). However, these studies only used telomere length and Ag-specific stimulation for 16 h as an indication of prior Ag exposure. We were able to more precisely define the time at which the naive CD4 T cells first encountered pathogen-derived Ag, and we have determined the impact exposure of naive CD4 T cells to vastly different levels and durations of influenza-derived Ag presentation on CD4 T cell persistence to memory.

To evaluate which conditions of influenza-derived Ag presentation favor the generation of memory T cells, naive HNT TCR Tg CD4 T cells (Fig. 5 A) or naive polyclonal CD4 T cells (Fig. 5 B) were transferred into mice at various times after influenza infection. Donor T cells were then harvested 21 (HNT TCR Tg) or 26 (polyclonal) d after transfer, corresponding with early memory stages of T cell differentiation. At day 21 after T cell transfer, only a small fraction of the HNT TCR Tg donor effectors persisted as memory, regardless of their time of transfer after infection (Fig. 5 A). Analysis of the polyclonal CD4 T cells that had divided in response to influenza-derived Ag presentation also indicated that primary T cell responses generated after live virus clearance were capable of persisting as memory (Fig. 5 B).

A clearer picture of the impact of early versus late influenza-derived Ag presentation can be seen in Fig. 5 C, in which the total number of donor T cells recovered from all organs is shown at the effector (day 7 after transfer) and the memory (days 21–26 after transfer) stages of the response. Naive CD4 T cells, either HNT TCR Tg or polyclonal, transferred on the same day as infection had the greatest expansion as effectors but underwent the most contraction to memory (Fig. 5 C, day 0 transfer). Alternatively, the donor T cells transferred after live virus had been cleared exhibited a moderate effector expansion but underwent a much smaller amount of contraction to memory (Fig. 5 C, day 14 and day 21 transfers). Interestingly, an average of $<2 \times 10^3$ of the total memory cells recovered from either the HNT

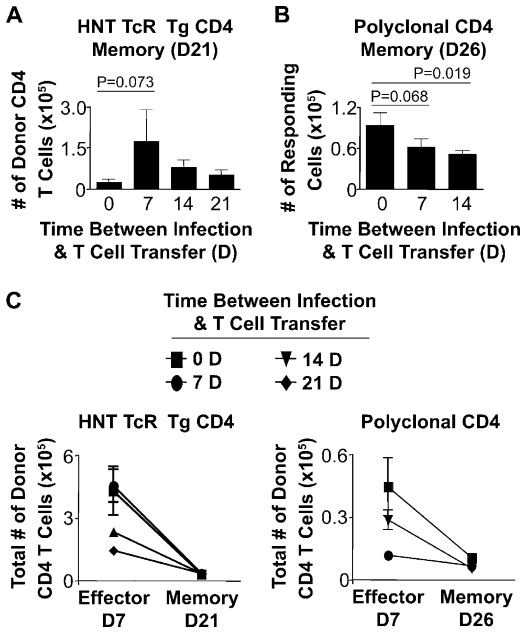

Figure 5. Late phase influenza-derived Ag presentation generates persistent CD4 T cells. CFSE-labeled naive Thy1.1 (A) HNT TCR Tg or (B) polyclonal CD4 T cells were transferred i.v. into BALB/c mice previously infected with PR8 virus. Total donor T cell numbers from pooled DLNs, spleens, and lungs were calculated at (A) 21 or (B) 26 d after T cell transfer. Values represent the mean ± SD of (A) 12 mice from four independent experiments or (B) 3 mice. (C) Total numbers of donor HNT TCR Tg or polyclonal CD4 T cells recovered at the effector and memory phases of the response were plotted. Data are representative of between two and four independent experiments.

TCR Tg or polyclonal CD4 T cell populations were found in the lungs; rather, the majority of the memory T cells were recovered from the DLNs and spleens (unpublished data).

Early phases of influenza-derived Ag presentation result in high levels of differentiation

We previously reported that a high proportion of donor CD4 T cells that accumulated in the lungs of infected mice were $CFSE^{lo}$ and $CD62L^{lo}$ and that extensive division coupled with CD62L down-regulation correlated with the most highly activated IFN-γ–producing effectors at the peak of the CD4 T cell response to influenza infection (6). To determine whether different phases of influenza-derived Ag presentation promoted distinct patterns of differentiation, we analyzed CD62L expression and IFN-γ production by responding ($CFSE^{lo}$) donor HNT TCR Tg CD4 effector and memory T cells. The most highly differentiated 7-d donor effectors (92% $CFSE^{lo}/CD62L^{lo}$ and 56% IFN-γ^+) were those recovered from the lungs of mice infected on the same day as T cell transfer (Fig. 6 A). Donor T cells transferred at times after the first week of infection were progressively more heterogeneous in their differentiation (Fig. 6 A) and migrated in extremely low numbers to the lungs at the effector stage (Fig. 2 B).

Because the transferred donor T cells continued to divide between days 7 and 9 after transfer (Fig. 3 B), we examined whether this division resulted in further differentiation by the memory stage. Analysis of donor HNT TCR Tg CD4 memory T cells, harvested 21 d after transfer, suggested that the memory T cells remained remarkably unchanged (Fig. 6 B) compared with their level of differentiation as effectors (Fig. 6 A). Polyclonal donor memory T cells harvested from the lungs 26 d after T cell transfer also exhibited a similar heterogeneous pattern of differentiation as the HNT TCR Tg memory T cells (Fig. 6 C and not depicted). However, in contrast to the results seen with HNT TCR Tg memory T cells, highly differentiated IFN-γ–producing memory cells were also recovered from the DLNs and spleens regardless of the time of naive polyclonal T cell transfer (Fig. 6 C).

Late phases of influenza-derived Ag presentation contribute to memory

Naive CD4 T cells that were transferred after live virus clearance could become persisting memory (Figs. 5 A and 5B). Thus, we wanted to determine whether recruitment of naive CD4 T cells could contribute to memory. To determine if newly generated naive T cells that might enter the periphery after live virus clearance could contribute to the establishment or maintenance of memory CD4 T cells, we infected sham and thymectomized mice and tracked endogenous IFN-γ–producing CD4 T cell responses. Thymectomy did not reduce the number of IFN-γ–producing CD4 effector T cells assessed at day 7 after infection (Fig. 7 A), and there was a >50% reduction in the number of IFN-γ–producing CD4 memory T cells 26 d into the endogenous influenza-specific response of thymectomized mice (Fig. 7 B). These results indicate that newly generated naive CD4 T cells, responding to influenza-derived Ag presentation at times after virus clearance, were contributing to the memory pool.

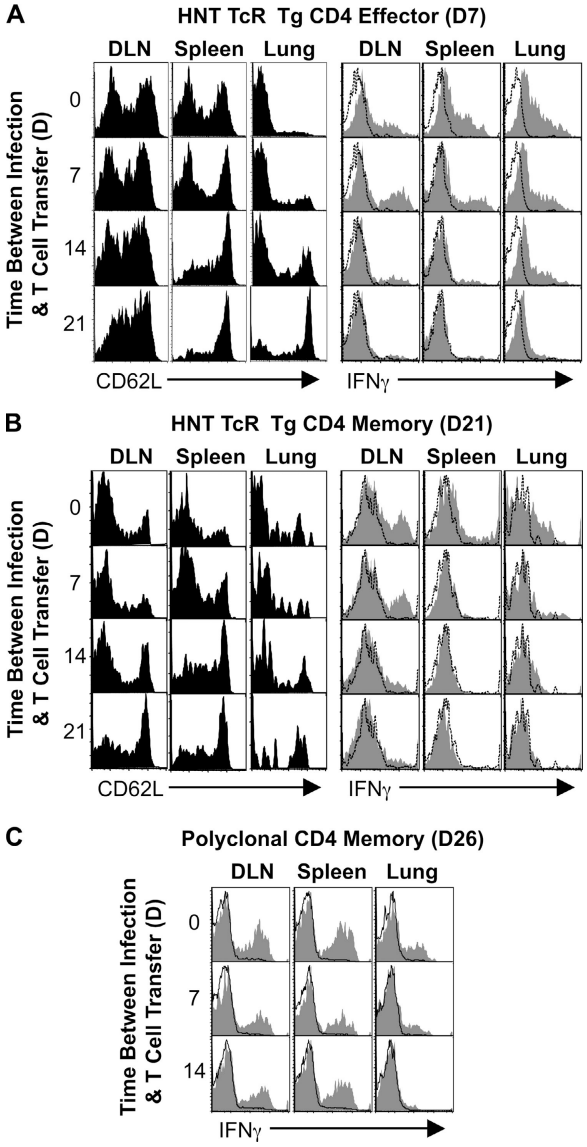

Figure 6. Highly efficient presentation of influenza Ag is limited to the early phase of infection. Naive Thy1.1 CD4 T cells were CFSE labeled and transferred i.v. into BALB/c mice previously infected with PR8 virus. (A) 7 and (B) 21 d after T cell transfer, responding ($CFSE^{lo}$) donor HNT TCR Tg CD4 T cells were assayed for CD62L expression and IFN-γ production. (C) IFN-γ production by responding ($CFSE^{lo}$) polyclonal donor CD4 T cells was assayed 26 d after T cell transfer. Data are representative of between two and four independent experiments.

DISCUSSION

It has been widely accepted that Ag localization, dose, and time of Ag/APC availability are each critical mediators in the regulation of immune responses (2, 3, 8, 10, 24–29).

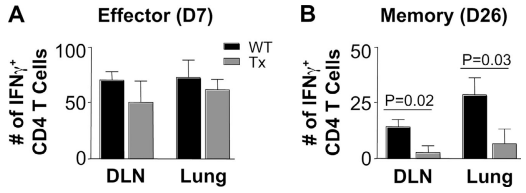

Figure 7. Late phases of influenza-derived Ag presentation significantly contribute to the generation of memory T cells. Sham or thymectomized BALB/c mice were infected with PR8 virus, and endogenous influenza-specific CD4 T cell responses were assayed by ELISPOT at (A) 7 and (B) 26 d after infection. Values represent the mean ± SD.

However, how the efficiency of pathogen-derived Ag presentation in vivo shifts during an acute infection and whether distinct stages of pathogen-derived Ag presentation have different effects on primary CD4 effector and memory generation had not previously been directly demonstrated. This study revealed that distinct stages of influenza-derived Ag presentation dramatically affected the progression of naive CD4 T cells through a primary immune response and onto memory. Influenza-derived Ag was presented to naive CD4 T cells long after live virus clearance, and distinct phases of influenza-derived Ag presentation could dramatically affect the expansion, localization, differentiation, and persistence of Ag-specific CD4 T cells. Transfer of naive CD4 T cells during the early phase of influenza infection, which was characterized by high viral titers and inflammation, generated a robust primary CD4 effector response with high numbers of highly differentiated effectors migrating to the lungs within 7 d of T cell transfer. However, this robust response appeared to have driven a majority of the effectors to become terminally differentiated, as they contracted dramatically to low numbers of memory CD4 T cells. In contrast, the transfer of naive CD4 T cells at times during or after viral clearance generated more modest effector responses but underwent less dramatic contraction and resulted in a greater fraction of donor CD4 T cells capable of surviving to become memory CD4 T cells.

In accordance with previous observations, few memory CD4 T cells were ever recovered from the lungs, even though memory T cells were readily found in the periphery (21). This was not surprising considering that previous experiments by Swain et al. demonstrated that only the most highly activated and differentiated CD4 effectors have the appropriate combination of tissue homing adhesion molecules to traffic to tertiary sites, such as the lungs; but, as these cells progress to a resting memory state, they lose this pattern of tissue homing molecules, and their migration becomes more limited to lymphoid sites (30). Additionally, rapidly diminishing inflammation and Ag availability could also be mitigating factors in the exclusion of influenza-specific CD4 memory T cells from the lungs after viral clearance and resolution of infection.

Interestingly, analysis of polyclonal CD4 T cell responses to different stages of influenza-derived Ag presentation resulted in yet another level of dynamic regulation of the induction of Ag-specific CD4 effector responses, which was not apparent when analyzing HNT TCR Tg CD4 responses. Polyclonal CD4 T cells transferred into mice at the peak of the endogenous T cell response (day 7 after infection) exhibited a marked reduction in the percentage (Fig. 4 A) and absolute number (Fig. 5 C) of responding ($CFSE^{lo}$) effectors as compared with transfers at other times after infection. We believe that this may be a result of competition between very low numbers of naive influenza-specific CD4 precursors and a large number of highly activated endogenous influenza-specific CD4 effectors. Other investigators have observed that the burst size of a given virus-specific CD4 (31–34) or CD8 (35–39) T cell population is suggestive of competition between responding T cells of identical and different Ag specificity. In fact, the clonal burst size of CD4 T cells could be hypothesized to be extremely restricted, perhaps because of inefficient Ag presentation of MHC class II–restricted epitopes. Clonal competition for peptide–MHC class II is most likely to occur at the peak of the endogenous CD4 T cell response, which would prevent a small number of naive influenza-specific polyclonal CD4 T cells from gaining access to Ag/APCs. This would not be as evident when transferring large numbers of naive monoclonal TCR Tg CD4 T cells, as they would be better able to compete for access to Ag/APCs.

Previous experiments by Wong and Pamer presented evidence that Ag-specific priming of CD8 T cells during a systemic bacterial infection is transient and lasts only 72 h despite the persistence of viable bacteria (3). These authors hypothesized that there was a feedback mechanism that regulated the magnitude of CD8 T cell responses. This is compatible with the theory that naive T cells can be programmed with very short exposure to Ag/APCs, and further division and differentiation of T cells would be independent of additional Ag stimulation and would instead be driven by inflammatory mediators (8). However, several groups have shown that although a single brief exposure to Ag stimulation was sufficient to induce extensive proliferation of naive T cells, the acquisition of effector functions in vitro depended on more stringent Ag presentation requirements (9, 10, 40–42). In particular, Storni et al. reported that a single injection of lymphocytic choriomeningitis virus–derived Ag peptide was sufficient to stimulate Ag-specific CD8 T cells to undergo multiple rounds of proliferation in vivo, but that vaccination with peptide alone failed to generate IFN-γ–producing effectors. Only when peptide Ag was presented by activated APCs or was maintained in the host for several days did the Ag-specific T cells differentiate to produce IFN-γ (43). A recent report by Harari et al. went on to suggest that infectious pathogens that are cleared rapidly are associated with a lesser degree of CD4 T cell differentiation, that pathogen persistence resulting in low Ag levels generates an intermediate level of T cell differentiation, and that highly Th1 cell–polarized T cell differentiation requires pro-

longed pathogen persistence and high Ag levels (23). However, this report was limited by its inability to control for the duration and dose of Ag exposure and only used telomere length and Ag-specific stimulation for 16 h as an indication of prior Ag exposure. We were able to precisely define the time at which the T cells first encountered Ag in order to determine the impact that different stages of influenza-derived Ag presentation had on CD4 T cell expansion, differentiation, and persistence.

Our analyses suggest that although later phases of influenza-derived Ag presentation, and presumably limited exposure to Ag, are capable of driving multiple rounds of CD4 T cell division, prolonged exposure to Ag or Ag presented on highly activated APCs may be required for the generation of highly differentiated effector and memory CD4 T cells capable of producing high levels of IFN-γ at the site of infection. We suspect that the level of influenza-derived Ag presentation follow the rapid kinetics of viral expansion and contraction, except perhaps with a short lag to accommodate the time required for Ag uptake and processing by APCs. The early phase of influenza-derived Ag presentation most likely provided high levels of inflammation-induced costimulatory signals, access to live virus with the likelihood of higher Ag load, potentially few (if any) regulatory cells (44), and enough time for primary CD4 T cells to progress through many thresholds of proliferation and differentiation. As a result, a large proportion of highly differentiated effectors were likely to be terminally differentiated as they were continually exposed to long-lived Ag presentation and were thus not selected to enter the memory pool, possibly because of an inability to access and/or use survival signals. We would suggest that the slow acquisition of a medium to a highly differentiated phenotype, like that which can be accomplished when new naive CD4 T cells enter the response when high levels of inflammation and/or Ag presentation are subsiding, may allow a smaller number of CD4 effectors to better survive to memory. In fact, an earlier report by Wu et al. demonstrated that highly differentiated Th1 effectors, resulting from multiple in vitro stimulations, were short lived and did not persist efficiently into long-term memory, whereas activated effectors that had not yet progressed to an IFN-γ–secreting phenotype persisted into long-term memory (45). Our own results added support to this theory and suggested that new naive CD4 T cells exiting the thymus after viral clearance engage low levels of persisting influenza-derived Ag presentation and contribute to ≥50% of the endogenous memory population (Fig. 7). Additionally, we found that effectors capable of migrating efficiently to the lung early in the CD4 T cell response to influenza decayed rapidly over time, suggesting that the lung environment could have added to the elimination of highly differentiated effectors. Later phases of influenza-derived Ag presentation occurred when inflammation was diminishing, with no detectable access to live virus, and one might thus assume lower Ag load. It was also possible that regulatory cells were emerging to control the extent of the immune response after the pathogen had been eliminated (44).

We have previously reported that long-lived memory CD4 T cells are maintained in the absence of Ag or persistent pathogen. In fact, the adoptive transfer of effectors into MHC class II–deficient hosts indicated that CD4 memory T cells could be generated and maintained in the absence of additional Ag/MHC class II stimulation (46). One possible mechanism of the enhanced persistence of intermediately differentiated T cells could be that effectors generated by transfer of naive CD4 T cells after virus clearance or the emergence of new naive CD4 T cells from the thymus may be less likely to reencounter Ag as it is slowly disappearing and thus be less susceptible to programmed or activation-induced cell death (47).

The generation of intermediately differentiated effectors during late phases of influenza-derived Ag presentation, when Ag load is presumably very low, is consistent with the findings of Zaph et al. that, although *Leishmania major*–specific memory CD4 T cells can develop in the absence of pathogen, the memory T cells maintained in the absence of pathogen migrate to the LNs and do not produce IFN-γ (48). Our findings indicate that later phases of pathogen-derived Ag presentation are much less efficient at driving robust expansion and differentiation of primary effectors, yet result in persisting CD4 memory T cell populations. The results presented here provide the first direct in vivo evidence for the hypothesis put forth by Wang and Mosmann, as well as Lanzavecchia and Sallusto, that resting memory CD4 T cells may be generated from CD4 effectors at an intermediate stage of differentiation and that these partially primed T cells may constitute a pool of persisting Ag-specific T cells that provide extra flexibility for immune responses on subsequent pathogen challenge (49–52).

Collectively, these results suggest that extremely strong primary stimulation may not necessarily produce the best long-term CD4 T cell–mediated protection. Specifically, vaccines directed toward generating the most CD4 T cell memory should be designed to generate intermediate instead of highly differentiated effectors. We do not know the origin of the Ag presentation occurring during any of the phases of influenza infection, but it is possible that an undetected reservoir of virus exists, perhaps within the APC population. It is also possible that virus is completely cleared and a reservoir of Ag persists in protein or peptide form. The broad polyclonal CD4 T cell response was induced by both early and late phases of influenza-derived Ag presentation and seems most consistent with prolonged retention of whole virus or viral proteins. Because preliminary evaluation of APC populations in the lungs, DLNs, and spleens of influenza-infected mice during the early and late phases of influenza infection have not revealed any obvious alterations in APC populations (unpublished data), defining the APCs responsible for inducing late-phase Ag stimulation may provide critical insights necessary to develop the protocols that will optimally induce a persisting CD4 memory population.

Another question raised by these studies is how this persisting Ag presentation would affect an endogenous CD4 T cell response. We have shown evidence to suggest that as the immune system produces new naive T cells from the thymus, albeit at a reduced rate into adulthood (53), the new naive CD4 T cells arriving into the periphery after viral clearance become primed by long-lived influenza-derived Ag presentation in the weeks to months after infection. This late phase of naive CD4 T cell recruitment contributed considerably to the optimal generation of a persisting population of memory CD4 T cells. Our transfer model systems suggested that late phases of influenza-derived Ag presentation produced effectors of intermediate levels of differentiation. We would suggest that naive CD4 T cells that emerge from the thymus after viral clearance and encounter long-lived influenza-derived Ag presentation may provide a source of effectors that are not completely differentiated and may contribute to the generation of a memory subset whose function is to rapidly produce IL-2 and promote faster recall responses on reinfection. Alternatively, previous studies (7, 54), as well as our findings in this study, provide evidence that highly differentiated CD4 effector T cells, capable of making high levels of IFN-γ and very little IL-2 at the effector stage, regain the capacity to produce IL-2 as they progress to a resting memory state. The late recruitment of naive CD4 T cells to the response by prolonged but low levels of pathogen-derived Ag presentation may contribute to memory heterogeneity. After reinfection, either the intermediately differentiated memory T cells differentiate further, on strong Ag stimulation by activated APCs, to give rise to completely functional antiviral effectors, or the highly differentiated effectors that survive to memory rapidly reexpress high levels of IFN-γ in order to provide more immediate protection. Whatever mechanisms are involved in regulating the phases of pathogen-derived Ag presentation to CD4 T cells, understanding the late phases of Ag presentation during acute infections may be important in generating optimum CD4 T cell memory.

MATERIALS AND METHODS

Mice. HNT TCR Tg mice were backcrossed for ≥12 generations to BALB/c ByJ and crossed to Thy1.1xBALB/c mice to generate HNTxThy1.1 F1 mice. All TCR Tg mice were used at 4–6 wk of age. HNTxThy1.1 F1, BALB/c, and Thy1.1xBALB/c mice were bred at the Trudeau Institute's animal breeding facilities. Thymi were removed from BALB/c ByJ mice under nembutal anesthesia (Abbott) and allowed to recover for 10 d before use. All experimental procedures involving mice were approved by the Trudeau Institute's animal care and use committee.

Naive CD4 T cell isolations and CFSE labeling. Pooled spleen and LN cells were enriched for naive CD4 T cells by antibody and complement depletion and Percoll gradient separation as previously described (10). The purified cell populations were routinely >85% $CD4^+$ cells, 90–95% of which had a naive phenotype ($CD45RB^{hi}$, $CD62L^{hi}$, $CD44^{low}$, or $CD25^{low}$) and were TCR Tg. Purified naive CD4 T cells were stained, as indicated in the figure legends, with CFSE (Molecular Probes Inc.) by resuspending CD4 T cells in serum-free RPMI 1640 (GIBCO BRL) at 10×10^6 cells/ml, adding 1 μM CFSE, incubating at 37°C for 15 min, and washing cells before use.

Virus infections and T cell transfers. The A/PR/8/34 (PR8) strain of influenza A virus was produced in the allantoic fluid of 10-d-old embryonated chicken eggs and characterized by a core facility at the Trudeau Institute from an aliquot originally obtained from D. Morgan (The Scripps Research Institute, La Jolla, CA). Mice were inoculated intranasally during light isoflurane anesthesia (Webster Veterinary Supply, Inc.) with 0.1 LD_{50} (5×10^3 PFU/ml) of virus in 100 μl PBS. X31 influenza A virus was provided by D. Woodland (Trudeau Institute, Saranac Lake, NY), and mice were inoculated intranasally with 0.1 LD_{50} (300 EIU_{50}) of X31 virus in 100 μl PBS, as indicated in the figures. 2×10^6 naive Thy1.1 HNT TCR Tg CD4 T cells (6) or 10×10^6 naive Thy1.2 BALB/c polyclonal CD4 T cells were transferred in 200 μl PBS by i.v. injection into BALB/c or Thy1.1xBALB/c mice, respectively.

Viral plaque assay. Viral titers in lungs and DLNs (parathymic and mediastinal LNs) were determined using a modified 5×10^3 PFU/ml Madin Darby canine kidney (MDCK) cell plaque assay (55). At various times after infection, lungs and DLNs were harvested into 1 ml DMEM media (GIBCO BRL), homogenated, serially diluted, and added to duplicate confluent monolayers of MDCK cells for 1 h at 37°C. Each well was then covered with 1 ml of agar overlay (DMEM plus 0.2% BSA, 2 mg/ml $NaHCO_3$, 2 mM Hepes, PSG, 0.5% agar, 0.01% DEAE dextran, and 0.5 μg/ml trypsin; Sigma-Aldrich). After 2–3 d of incubation at 37°C, cells were fixed with 0.5 ml of carnoy's fixative and stained with 2% crystal violet in 20% ethanol (Sigma-Aldrich). PFU/ml = (mean number of plaques/0.1) × (1/dilution factor).

Tissue preparation. Donor Thy1.1 HNT TCR Tg CD4 T cells or Thy1.2 polyclonal CD4 T cells were isolated from DLNs, spleens, and lungs of experimental mice at the effector (day 7) or memory (days 21–28) stages after transfer. Mice were exsanguinated under lethal i.p. avertin anesthesia by perforation of the abdominal aorta. Lungs were perfused with 10–20 ml PBS to remove blood lymphocytes. Cell suspensions were prepared by mechanical disruption of organs and passage through a nylon membrane. RBCs were lysed using RBS lysis buffer (BD Biosciences) according to the manufacturer's protocol.

Flow cytometry. Cell suspensions were incubated on ice with saturating concentrations of fluorochrome-labeled mAbs in FACS buffer (PBS plus 0.5% BSA and 0.02% NaN_3; Sigma-Aldrich). Allophycocyanin-labeled anti-CD4 and PerCP-labeled anti-Thy1.1 or anti-Thy1.2 (BD Biosciences) were used to identify donor T cells. FITC-labeled anti–L-selectin (CD62L), and isotype controls were used for phenotype analysis (BD Biosciences). FACS analysis was performed using FACSCalibur (BD Biosciences) and FlowJo (Tree Star, Inc.) software.

Intracellular cytokine staining. Donor T cells harvested from experimental mice were restimulated for 4 h with 10 ng/ml PMA and 50 ng/ml ionomycin (Sigma-Aldrich) or for 6 h with 10^6 HNT-pulsed A20 B cell lymphoma APCs at a concentration of 10^6 total cells/ml. 10 μg/ml Brefeldin A (Sigma-Aldrich) was added for the final 2 h of culture and maintained throughout the intracellular cytokine staining. Donor T cells were surface stained with anti-Thy1.1, or anti-Thy1.2, and anti-CD4 as described above, fixed in 100 μl 4% paraformaldehyde, and stained in saponin buffer (PBS plus 1% FBS, 0.1% NaN_3 and 0.1% saponin; Sigma-Aldrich) containing anti–IFN-γ–PE.

ELISPOT assay for IFN-γ–secreting cells. Multiscreen HA plates (Millipore) were coated overnight with anti–IFN-γ antibody (clone R4-6A2; BD Biosciences) at 5 μg/ml in PBS. Plates were washed with PBS/Tween 20 and blocked using complete RPMI 1640 media with 10% FBS. DLN or lung cells were added at 10^6 cells/well and serially diluted 1:2 in RPMI media. Irradiated naive splenocytes were added as APCs with or without MHC class II–restricted PR8 $HA_{126–138}$ and $nucleoprotein_{216–230}$ peptides. Plates were incubated overnight at 37°C, washed with PBS/

Tween 20, and incubated with biotinylated anti–IFN-γ antibody (clone XMG1.2; BD Biosciences) at 1 μg/ml overnight at 4°C. Plates were washed with PBS/Tween 20 and incubated with streptavidin–alkaline phosphatase (ExtraAvidin; Sigma-Aldrich) at 1:500 for 1 h at 25°C. Spots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Sigma-Aldrich) tablets dissolved in double-distilled H_2O at 1 mg/ml. The reaction was terminated with H_2O, the plates were dried, and spots were counted using a dissecting microscope (SMZ800; Nikon Instrument Inc.). Data is plotted as the number of IFN-γ spots per 1.2×10^5 (DLN and lung effectors and lung memory) or 2.5×10^5 (DLN memory) input cell number. Two-tailed Student's *t* test was used to determine statistical differences between groups.

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REFERENCES

1. Hermans, I.F., D.S. Ritchie, J. Yang, J.M. Roberts, and F. Ronchese. 2000. CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. *J. Immunol.* 164:3095–3101.
2. Hafalla, J.C., G. Sano, L.H. Carvalho, A. Morrot, and F. Zavala. 2002. Short-term antigen presentation and single clonal burst limit the magnitude of the CD8(+) T cell responses to malaria liver stages. *Proc. Natl. Acad. Sci. USA.* 99:11819–11824.
3. Wong, P., and E.G.P. Am. 2003. Feedback regulation of pathogen-specific T cell priming. *Immunity.* 18:499–511.
4. Miga, A.J., S.R. Masters, B.G. Durell, M. Gonzalez, M.K. Jenkins, C. Maliszewski, H. Kikutani, W.F. Wade, and R.J. Noelle. 2001. Dendritic cell longevity and T cell persistence is controlled by CD154-CD40 interactions. *Eur. J. Immunol.* 31:959–965.
5. Medema, J.P., D.H. Schuurhuis, D. Rea, J. van Tongeren, J. de Jong, S.A. Bres, S. Laban, R.E. Toes, M. Toebes, T.N. Schumacher, et al. 2001. Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte–induced apoptosis: differential modulation by T helper type 1 and type 2 cells. *J. Exp. Med.* 194: 657–667.
6. Roman, E., E. Miller, A. Harmsen, J. Wiley, U.H. Von Andrian, G. Huston, and S.L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J. Exp. Med.* 196:957–968.
7. Swain, S.L. 1999. Helper T cell differentiation. *Curr. Opin. Immunol.* 11:180–185.
8. van Stipdonk, M.J., E.E. Lemmens, and S.P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* 2:423–429.
9. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity.* 8:89–95.
10. Jelley-Gibbs, D.M., N.M. Lepak, M. Yen, and S.L. Swain. 2000. Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. *J. Immunol.* 165:5017–5026.
11. Graham, M.B., and T.J. Braciale. 1997. Resistance to and recovery from lethal influenza virus infection in B lymphocyte–deficient mice. *J. Exp. Med.* 186:2063–2068.
12. Brimnes, M.K., L. Bonifaz, R.M. Steinman, and T.M. Moran. 2003. Influenza virus–induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J. Exp. Med.* 198:133–144.
13. Lo, D., J. Freedman, S. Hesse, R.D. Palmiter, R.L. Brinster, and L.A. Sherman. 1992. Peripheral tolerance to an islet cell-specific hemagglutinin transgene affects both CD4+ and CD8+ T cells. *Eur. J. Immunol.* 22:1013–1022.
14. Hurwitz, J.L., E. Herber-Katz, C.J. Hackett, and W. Gerhard. 1984. Characterization of the murine TH response to influenza virus hemagglutinin: evidence for three major specificities. *J. Immunol.* 133:3371–3377.
15. Burt, D.S., K.H. Mills, J.J. Skehel, and D.B. Thomas. 1989. Diversity of the class II (I-Ak/I-Ek)–restricted T cell repertoire for influenza hemagglutinin and antigenic drift. Six nonoverlapping epitopes on the HA1 subunit are defined by synthetic peptides. *J. Exp. Med.* 170:383–397.
16. Verhoeyen, M., R. Fang, W.M. Jou, R. Devos, D. Huylebroeck, E. Saman, and W. Fiers. 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature.* 286:771–776.
17. Woodland, D.L., and R.W. Dutton. 2003. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr. Opin. Immunol.* 15:336–342.
18. Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science.* 250:1720–1723.
19. Kassiotis, G., and B. Stockinger. 2004. Anatomical heterogeneity of memory CD4+ T cells due to reversible adaptation to the microenvironment. *J. Immunol.* 173:7292–7298.
20. Ahmadzadeh, M., S.F. Hussain, and D.L. Farber. 2001. Heterogeneity of the memory CD4 T cell response: persisting effectors and resting memory T cells. *J. Immunol.* 166:926–935.
21. Swain, S.L., R.W. Dutton, and D.L. Woodland. 2004. T cell responses to influenza virus infection: effector and memory cells. *Viral Immunol.* 17:197–209.
22. Harari, A., F. Vallelian, and G. Pantaleo. 2004. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur. J. Immunol.* 34:3525–3533.
23. Harari, A., F. Vallelian, P.R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J. Immunol.* 174:1037–1045.
24. Mandel, T.E., R.P. Phipps, A. Abbot, and J.G. Tew. 1980. The follicular dendritic cell: long term antigen retention during immunity. *Immunol. Rev.* 53:29–59.
25. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med.* 182:1591–1596.
26. Constant, S., D. Sant'Angelo, T. Pasqualini, T. Taylor, D. Levin, R. Flavell, and K. Bottomly. 1995. Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of CD4+ T cells. *J. Immunol.* 154:4915–4923.
27. Croft, M., L.M. Bradley, and S.L. Swain. 1994. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J. Immunol.* 152:2675–2685.
28. Ingulli, E., A. Mondino, A. Khoruts, and M.K. Jenkins. 1997. In vivo detection of dendritic cell antigen presentation to $CD4^+$ T cells. *J. Exp. Med.* 185:2133–2141.
29. Langenkamp, A., G. Casorati, C. Garavaglia, P. Dellabona, A. Lanzavecchia, and F. Sallusto. 2002. T cell priming by dendritic cells: thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur. J. Immunol.* 32:2046–2054.
30. Swain, S.L., J.N. Agrewala, D.M. Brown, and E. Roman. 2002. Regulation of memory CD4 T cells: generation, localization and persistence. *Adv. Exp. Med. Biol.* 512:113–120.
31. Probst, H.C., T. Dumrese, and M.F. van den Broek. 2002. Cutting edge: competition for APC by CTLs of different specificities is not functionally important during induction of antiviral responses. *J. Immunol.* 168:5387–5391.
32. Harrington, L.E., R. van der Most, J.L. Whitton, and R. Ahmed. 2002. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. *J. Virol.* 76:3329–3337.
33. Topham, D.J., and P.C. Doherty. 1998. Longitudinal analysis of the acute Sendai virus-specific CD4+ T cell response and memory. *J. Immunol.* 161:4530–4535.

34. Christensen, J.P., and P.C. Doherty. 1999. Quantitative analysis of the acute and long-term CD4(+) T-cell response to a persistent gammaherpesvirus. *J. Virol.* 73:4279–4283.
35. Kedl, R.M., J.W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr. Opin. Immunol.* 15: 120–127.
36. Chen, W., L.C. Anton, J.R. Bennink, and J.W. Yewdell. 2000. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. *Immunity.* 12:83–93.
37. Kedl, R.M., W.A. Rees, D.A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, and P. Marrack. 2000. T cells compete for access to antigen-bearing antigen-presenting cells. *J. Exp. Med.* 192:1105–1113.
38. Butz, E.A., and M.J. Bevan. 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity.* 8:167–175.
39. Murali-Krishna, K., J.D. Altman, M. Suresh, D.J. Sourdive, A.J. Zajac, J.D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity.* 8:177–187.
40. Haynes, L., P.J. Linton, S.M. Eaton, S.L. Tonkonogy, and S.L. Swain. 1999. Interleukin 2, but not other common γ chain–binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *J. Exp. Med.* 190:1013–1024.
41. Bird, J.J., D.R. Brown, A.C. Mullen, N.H. Moskowitz, M.A. Mahowald, J.R. Sider, T.F. Gajewski, C.R. Wang, and S.L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity.* 9:229–237.
42. Rogers, P.R., G. Huston, and S.L. Swain. 1998. High antigen density and IL-2 are required for generation of CD4 effectors secreting Th1 rather than Th0 cytokines. *J. Immunol.* 161:3844–3852.
43. Storni, T., C. Ruedl, W.A. Renner, and M.F. Bachmann. 2003. Innate immunity together with duration of antigen persistence regulate effector T cell induction. *J. Immunol.* 171:795–801.
44. Mills, K.H. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* 4:841–855.
45. Wu, C.Y., J.R. Kirman, M.J. Rotte, D.F. Davey, S.P. Perfetto, E.G. Rhee, B.L. Freidag, B.J. Hill, D.C. Douek, and R.A. Seder. 2002. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat. Immunol.* 3:852–858.
46. Hu, H., G. Huston, D. Duso, N. Lepak, E. Roman, and S.L. Swain. 2001. CD4(+) T cell effectors can become memory cells with high efficiency and without further division. *Nat. Immunol.* 2:705–710.
47. Zhang, X., T. Brunner, L. Carter, R.W. Dutton, P. Rogers, L. Bradley, T. Sato, J.C. Reed, D. Green, and S.L. Swain. 1997. Unequal death in T helper cell (Th) 1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J. Exp. Med.* 185:1837–1849.
48. Zaph, C., J. Uzonna, S.M. Beverley, and P. Scott. 2004. Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. *Nat. Med.* 10:1104–1110.
49. Wang, X., and T. Mosmann. 2001. In vivo priming of CD4 T cells that produce interleukin (IL)-2 but not IL-4 or interferon (IFN)-γ and can subsequently differentiate into IL-4– or IFN-γ–secreting cells. *J. Exp. Med.* 194:1069–1080.
50. Lanzavecchia, A., and F. Sallusto. 2002. Progressive differentiation and selection of the fittest in the immune response. *Nat. Rev. Immunol.* 2:982–987.
51. Farber, D.L. 1998. Differential TCR signaling and the generation of memory T cells. *J. Immunol.* 160:535–539.
52. Kaech, S.M., E.J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2:251–262.
53. Steffens, C.M., L. Al-Harthi, S. Shott, R. Yogev, and A. Landay. 2000. Evaluation of thymopoiesis using T cell receptor excision circles (TRECs): differential correlation between adult and pediatric TRECs and naive phenotypes. *Clin. Immunol.* 97:95–101.
54. Carter, L.L., and S.L. Swain. 1998. From naive to memory. Development and regulation of CD4+ T cell responses. *Immunol. Res.* 18:1–13.
55. Tobita, K., A. Sugiura, C. Enomote, and M. Furuyama. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol. (Berl.).* 162:9–14.