Duration of Antigen Availability Influences the Expansion and Memory Differentiation of T Cells

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The initial engagement of the TCR through interaction with cognate peptide–MHC is a requisite for T cell activation, and confers Ag specificity. Although this is a key event in T cell activation, the duration of these interactions may affect the proliferative capacity and differentiation of the activated cells. In this study, we developed a system to evaluate the temporal requirements for antigenic stimulation during an immune response in vivo. Using Abs that target specific Ags in the context of MHC, we were able to manipulate the duration of Ag availability to both CD4 and CD8 T cells during an active infection. During the primary immune response, the magnitude of the CD4 and CD8 T cell response was dependent on the duration of Ag availability. Both CD4 and CD8 T cells required sustained antigenic stimulation for maximal expansion. Memory cell differentiation was also dependent on the duration of Ag exposure, albeit to a lesser extent. However, memory development did not correlate with the magnitude of the primary response, suggesting that the requirements for continued expansion of T cells and memory differentiation are distinct. Finally, a shortened period of Ag exposure was sufficient to achieve optimal expansion of both CD4 and CD8 T cells during a recall response. It was also revealed that limiting exposure to Ag late during the response may enhance the CD4 T cell memory pool. Collectively, these data indicated that Ag remains a critical component of the T cell response after the initial APC–T cell interaction. The Journal of Immunology, 2011, 187: 2310–2321.

Antigen recognition by the TCR expressed by CD4 and CD8 T cells is the initial step in T cell activation that leads to clonal expansion and acquisition of effector function. Following the expansion phase, 90–95% of the T cells at the peak of the response undergo apoptosis (1). The remaining population of cells differentiates to become memory T cells that provide long-lasting protection to the host. Formation of an immunological synapse allows signal integration to occur through TCR engagement with peptide–MHC complexes, leading to T cell activation (2, 3). Although TCR triggering is required for the initial activation of a T cell, the role that the duration of the antigenic stimulus plays in the differentiation of a T cell during an immune response remains less clear.

CD8 T cells require only transient stimulation with Ag to initiate proliferation (4–6), whereas CD4 T cells appear to require a longer encounter with Ag for division to occur (7). Nonetheless, both CD8 and CD4 T cells require sustained periods of stimulation to differentiate into effector and memory T cells (7–9). In vivo studies suggest that 36–60 h of Ag availability is sufficient for T cells to undergo the programming necessary for the acquisition of effector function and differentiation to memory cells (6, 10–12). However, more prolonged Ag exposure may be required for optimal expansion and effector differentiation (13, 14). Moreover, successive T cell encounters with dendritic cells (DCs) following the initial priming event correlate with the induction of effector function (15). In contrast, other data indicate that antigenic stimulation beyond 2 d results in a diminished population of effector CD4 T cells (16, 17). Thus, there remains ambiguity in terms of the durational requirements of Ag recognition by T cells for mounting an effective immune response. We have previously shown that minimally limiting the amount of Ag during the priming of CD4 T cells allows maximal expansion but results in defective effector differentiation and memory development (18). Together, these studies indicate that T cell programming occurs very early during the T cell response and that Ag, even beyond the initial APC–T cell interaction, may continue to influence the process. In support of this, it has also been demonstrated that the contraction phase of the immune response is programmed early on during the response (19, 20).

Although the aforementioned studies have revealed the relevance of Ag presence beyond initial T cell activation during the immune response on T cell programming, further investigation is warranted. For example, *Listeria monocytogenes* infection followed by anti-biotic treatment has been used as an in vivo model to assess the role of Ag duration on T cell programming (10–12, 21). This results in the clearance of the pathogen, thereby eliminating the source of the Ag. One caveat of these studies is that the duration of Ag availability is only addressed indirectly by altering the duration of the infection. Thus, although bacterial clearance occurs rapidly following treatment, the potential for residual Ag, which
can continue to stimulate T cells, exists. Additionally, antibiotic treatment also curtails the inflammatory response. Inflammation in and of itself can influence the T cell response (22–26). Thus, it is difficult to discriminate the effects of Ag diminution from the reduction in inflammation. To circumvent these issues, an elegant study assessed the duration of Ag and T cell programming in vivo using an alternative approach (6) in which Ag-bearing DCs could be selectively depleted. The results indicate that a period of 6–12 h of antigenic stimulation is required for the differentiation of effector cells and development of memory cells, at least for the high-avidity TCR-transgenic CD8 T cell OT-I. In contrast, the magnitude of the CD8 T cell response correlated with the duration of Ag availability. However, this study did not address the in vivo endogenous response to infection.

In an effort to better understand the temporal requirements for Ag on T cell differentiation during an immune response, we set out to develop a model in which we could modulate Ag availability to CD4 and CD8 T cells in vivo while leaving all other parameters intact. To this end, we took advantage of two MHC–peptide-specific blocking Abs to examine the effects of limiting the duration of Ag availability to both CD4 and CD8 T cells during the primary and recall responses to pathogen. Through the Ab-mediated regulation of Ag during the course of infection, we were able to directly evaluate the temporal effects of Ag on T cells throughout the course of the immune response. We show that both CD4 and CD8 T cells share similar requirements for Ag availability during the primary immune response. However, Ag is only briefly required to drive the secondary response to infection, although differences between CD4 and CD8 T cells were noted. These findings hold implications with regard to the development of Ag-based immunotherapies with respect to the requirements of Ag duration necessary for adequate T cell responses and the development of memory T cells.

Materials and Methods

**Mice**

C57BL/6j (CD45.1 and CD45.2) mice were purchased from Charles River Laboratories/National Cancer Institute (Wilmington, MA). TEs TCR-transgenic mice (27) for which CD4 T cells recognize the E α peptide (aa 52–68) from the I-E α MHC class II molecule in the context of I-A b were generously provided by R. Noelle (Dartmouth Medical School, Lebanon, NH) and bred and maintained on a RAG-deficient background. For adoptive transfer experiments, 1 × 10^7 naive TEs-RAG-2CD4 T cells were injected i.v. into congenic recipients. In some instances, the TEs cells were CFSE-labeled as previously described (18). Animal protocols were approved by the University of Connecticut Health Center Animal Care Committee.

**Isolation of lymphocytes and flow cytometry**

Single-cell suspensions were prepared from spleens and lymph nodes (LN) by mechanical disruption of the tissues between frosted glass slides. Cells were washed twice with PBS (Invitrogen Life Technologies) and pelleted by centrifugation. Following treatment with Tris-ammonium chloride to lyse RBCs, cells were washed and resuspended in HBSS. For isolation of lymphocytes from lung tissue, mice were anesthetized and perfused with PBS-heparin prior to tissue harvest to clear blood from the tissues. Lymphocytes were then isolated by cutting the tissue into small pieces, followed by digestion in collagenase for 1 h at 37°C. Following collagenase digestion, the tissue was passed over a 40-μm cell strainer. For staining, cells were resuspended in 0.2% BSA, 0.01% NaN₃ in PBS (FACS Buffer) at a concentration of ~1 × 10⁶ cells/ml and stained with indicated Abs. All Abs were obtained from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA), BioLegend (San Diego, CA), or Caltag Laboratories (Burlingame, CA). Following staining, cells were washed in FACS Buffer and fixed with 2% paraformaldehyde/PBS. Multiparameter flow cytometry was performed with a FACSCalibur or LSR II (BD Biosciences, San Jose, CA) cytometer, and data were analyzed using FlowJo software (Tree Star, Ashland, OR). In some experiments, peptide–MHC class I tetramers were used for identification of endogenous populations of Ag-specific CD8⁺ T cells. Single-cell suspensions were incubated for 1 h at room temperature with the tetramer, Fc block, and Abs against indicated cell-surface proteins. H-2Kiad tetramers containing the OVA-derived peptide SIINFEKL or the vesicular stomatitis virus (VSV) nucleoprotein-derived peptide RGYVYQQL were produced as previously described (28, 29).

**Infections**

Recombinant VSV (rVSV) containing the DNA sequence for enhanced GFP, the SIINFEKL peptide of OVA, and the Eα peptide (VSV-GSE) was generated as previously described (30). Mice were infected i.v. with 1 × 10⁷ PFU VSV-GSE or VSV-OVA (31), where indicated. For recall experiments, mice were infected with the Indiana strain of VSV followed by a recall challenge with the New Jersey strain of VSV or vice versa. For influenza virus infections, mice were inoculated intranasally with 1 × 10⁷ PFU WSN-ova1 (32).

**Peptide immunization**

In some experiments, memory TEs CD4 T cells were generated via peptide immunization as previously described (33). Briefly, 1 × 10⁵ TEs CD4 T cells were adoptively transferred into congenic hosts, and the following day, 100 μg Eα peptide (aa 52–68) (Invitrogen Life Technologies) along with 50 μg anti-OX40 (OX6 clone) was injected i.p. Eighteen hours later, mice were injected with 50 μg Salmonella typhimurium LPS (Sigma-Aldrich).

**In vivo inhibition of Ag presentation**

Purified Y-Ae mAb (34) was purchased from the National Cell Culture Center (NCCC) (Minneapolis, MN) and diluted in PBS. To evaluate the effect of Ag availability during the course of the immune response, 100–500 μg Y-Ae was administered i.p. at a concentration of 1 mg/ml at the indicated time points. For CD8 T cell-blocking experiments, 500 μg 25-D1.16 mAb (35) (NCCC) was administered at the indicated times. Mouse IgG2b and IgG1 (NCCC) were used as isotype controls for Y-Ae and 25-D1.16, respectively.

**Intracellular cytokine staining**

Lymphocytes were isolated from the spleen 5 d postinfection and restimulated in vitro with 10 μg/ml Eα peptide in the presence of GolgiStop (BD Pharmingen) for 5 h at 37°C. Following in vitro restimulation, lymphocytes were stained for surface markers, fixed, and permeabilized in PermWash (BD Pharmingen) and stained with anti–IL-2, anti–IFN-γ, or isotype control mAb (BD Pharmingen).

**Results**

**Duration of Ag availability affects the magnitude of the primary CD8 T cell response to systemic virus infection**

To investigate the temporal requirements for Ag in the activation of CD8 and CD4 T cells, we developed a model in which Ag availability following infection could be manipulated in vivo. To regulate the duration of Ag exposure to CD8 T cells, we used the 25-D1.16 mAb, which recognizes the OVA-derived peptide SIINFEKL bound to H2-K α (35). For CD4 T cell studies, we used the Y-Ae mAb that inhibits Ag presentation to Eα-specific CD4 T cells in vivo (18). Because we wished only to block Ag presentation to a subset of responding CD8 T cells, we verified that 25-D1.16 treatment in vivo did not deplete Ag-bearing cells (Supplemental Fig. 1). Spleen cells were coated with the SIINFEKL peptide and transferred to new hosts that were then treated with 500 μg 25-D1.16 or control mAb. Three days later, the survival of the cells was measured, and the results indicated that 25-D1.16 mAb treatment did not result in any loss of transferred cells (Supplemental Fig. 1). Thus, any effects observed should be due to inhibition of Ag recognition. Our previous findings also show that the Y-Ae mAb does not deplete APC or inhibit concomitant bystander responses (18).

To test the temporal requirements for Ag during the CD8 T cell response, we infected mice with rVSV containing the gene encoding chicken OVA (VSV-ova), and the response was measured using H-2Kiad-OVA tetramers. Mice were treated with 500 μg 25-D1.16 mAb, at days 0, 3, 4, 5, or 6 postinfection, and the OVA-
specific CD8 T cell response was measured in the spleen at the peak of the response on day 7 postinfection (Fig. 1A). The response was inhibited to the greatest extent (~80%) when mice were treated at the time of infection with the blocking Ab (Fig. 1). In addition, the expansion of OVA-specific CD8 T cells was reduced to the same extent in mice treated with the blocking Ab at days 1 and 2 (data not shown) or at days 3 and 4 postinfection compared with control-treated mice (Fig. 1A). Interestingly, there was no statistically significant difference between the extent of blocking when mAb was given on day 0 or at 4 d postinfection. In contrast to this, blocking Ag presentation at 5 or 6 d postinfection had no effect on the magnitude of the CD8 T cell response compared with control Ab-treated mice. 25-D1.16 mAb treatment had no effect on the concomitant CD8 T cell response to the VSV nucleoprotein, indicating the specificity of the inhibition as well as the lack of any general effects on APC function (Fig. 1C). Total numbers of OVA-specific CD8 T cells were also reduced, suggesting an effect on proliferation (Fig. 1D). These results indicated that the presence of Ag is required for up to 5 d postinfection for maximal expansion of Ag-specific CD8 T cells.

Following expansion, T cells undergo a period of contraction in which 90–95% of the T cells undergo apoptosis, whereas the remaining population goes on to develop into long-lived memory cells (1). To determine if Ag availability had an impact on the contraction of the responding T cells, mice were bled at 9, 11, and 15 d postinfection. At day 9 postinfection, OVA-specific CD8 T cells in the blood from mice treated at days 0 or 3 postinfection were significantly lower than control-treated mice (Fig. 1B), whereas at days 11 and 15 postinfection, only mice that were treated at the time of infection with the blocking Ab had significantly lower numbers of OVA-specific CD8 T cells (Fig. 1B). Although the percentage of OVA-specific CD8 T cells in the blood from mice that were treated at day 3 postinfection was lower compared with the control-treated mice, the difference did not reach significance. Thus, although expansion of responding CD8 T cells was reduced when the duration of Ag was limited, the number of OVA-specific CD8 T cells during the contraction phase normalized in the mice, suggesting that the duration of Ag availability during the priming of T cells affects the magnitude of the CD8 T cell response but also may affect the overall survival of the responding cells.

**Ag is required late after influenza virus infection for optimal CD8 T cell expansion**

We also analyzed the temporal requirements for Ag after intranasal infection with influenza virus expressing the OVA-derived SIINFEKL epitope (WSN-ova). Treatment with 25-D1.16 at the time of infection decreased the peak response at day 10 postinfection 10-fold in the lungs, the mediastinal LN (MLN), and the spleen (Fig. 2A, 2B). Remarkably, mAb treatment on days 2–7 inhibited the response 75–90% in the lung and MLN, with the spleen response being more variable. In fact, little difference was noted between blocking on day 2 as compared with day 7, suggesting either that Ag presentation began late or that later CD8 T cell–APC interactions subsequent to initial activation were important for continued expansion of CD8 T cells. We believe the latter to be true because T cell activation could be detected within the first 2 d postinfection (data not shown). It should be noted that mAb treatment did not affect virus replication in the lungs (Supplemental Table I).

Our results showed that there was a reduction in the magnitude of the OVA-specific CD8 T cell population at the peak of the immune response if Ag presentation was blocked within 7 d of influenza virus infection. This effect may be due to inhibition of further expansion due to a lack of Ag. However, Ag presentation is required at later times to maintain the CD8 T cell response. We conclude that Ag presentation is required late after influenza virus infection for optimal CD8 T cell expansion.

**FIGURE 1.** Ag is required for several days for optimal CD8 T cell response to virus infection. Mice were infected with 10^5 PFU VSV-ova-NJ and treated with 500 μg blocking 25-D1.16 mAb at the indicated times. Control mice received mouse IgG1 isotype mAb. Mice were then bled at days 7, 9, 11, and 15 postinfection. OVA-specific CD8 T cells were quantitated by flow cytometry using an OVA–Kb tetramer. A, Blood analysis at day 7. B, Kinetics of the OVA-specific CD8 T cell response. C, Analysis of OVA-specific and VSV-nucleoprotein-specific CD8 T cells after Ab treatment at day 0. D, Total numbers of OVA-specific splenic CD8 T cells 7 d postinfection and treatment with the indicated mAbs. The x-axis in A and B indicates the time postinfection that mice were treated with control or blocking Ab. Graphs represent the mean ± SEM of OVA-tetramer–positive cells as a percentage of the entire CD8 T cell population from three to four mice per group. These data are representative of three similar experiments. *p < 0.01.
expansion of responding cells at the time of mAb administration or due to the early contraction of the Ag-specific population due to reduced Ag presentation subsequent to Ab administration. To distinguish between these possibilities, we compared the size of the virus-specific CD8 T cell response at days 6 and 9 (approximate peak) postinfection (Fig. 3). In the lung and spleen, very few tetramer+ cells were present 6 d postinfection (day 6 PBS) likely due to insufficient time having passed to allow migration of activated CD8 T cells to the tissues. Whether the mice were treated with 25-D1.16 on days 3 or 6 postinfection, by day 9 postinfection, an increase in tetramer+ cells in the spleen and lung was evident, but did not reach maximum (PBS). However, in the MLN, the magnitude of the OVA-specific response on day 6 (day 6 PBS) was similar to the magnitude at day 9, when 25-D1.16 mAb was administered at either days 3 or 6 (Fig. 3). This result suggested that, upon administration at later times postinfection, 25-D1.16 mAb reduced the expansion of previously primed SIINFEKL-specific CD8 T cells and did not cause the depletion or contraction of the responding CD8 T cell population.

Prolonged Ag presentation controls the magnitude of the CD4 T cell response

In vitro and in vivo data indicate that CD4 T cells require sustained antigenic stimulation for the programming of proliferation and effector differentiation (13). Few studies have evaluated the role of Ag duration for CD4 T cell differentiation in vivo during infection. At least for infection with L. monocytogenes, it appears that Ag duration may play a role in CD4 T cell differentiation (11, 12). To address the role of Ag duration on the differentiation of CD4 T cells, we used an adoptive transfer system and the I-Ab-Ea peptide-specific Y-Ae mAb (34, 36) to regulate Ag availability in vivo. We have previously shown that the Y-Ae mAb inhibits Ag

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**FIGURE 2.** Blocking Ag presentation with 25-D1.16 mAb after influenza virus infection inhibits the expansion of CD8 T cells. Mice were infected with 1000 PFU WSN-OVA1 and treated with 500 μg blocking 25-D1.16 mAb or IgG at the indicated times. On day 10 postinfection, cells from the lung, MLN, and spleen were stained with OVA–Kb tetramer and Abs to CD8 and CD11a, then analyzed by flow cytometry. A, Representative plots of gated MLN CD8+ lymphocytes showing staining for CD11a and OVA–Kb tetramer. Values indicate the percentages of tetramer+ cells among CD8+ T cells. B, Graph represents the total number of OVA-specific CD8+ cells in the indicated tissues; n = 3. This experiment was performed two additional times with similar results. Days indicate the day on which mice were treated with mAb.

**FIGURE 3.** Inhibition of the CD8 T cell response by 25-D1.16 mAb is through the abrogation of expansion of previously primed cells. Mice were infected with 1000 PFU WSN-OVA and treated with PBS or 500 μg blocking 25-D1.16 mAb at the indicated times postinfection. Mice from the different groups were sacrificed at the indicated times and lymphocytes isolated from the lung, MLN, and spleen, stained with OVA–Kb tetramer and Abs to CD8 and CD11a, then analyzed by flow cytometry. Graphs depict mean ± SEM of the total number of CD11a+tet+ CD8 cells in the indicated tissues of mice from the various groups; n = 4.
presentation to TCR-transgenic CD4 T cells (TEa) specific to the Eo peptide in vivo (18). Subsequent to adoptive transfer of $1 \times 10^5$ TEa TCR-transgenic T cells into congeneric mice, the mice were infected with VSV-GSE, a VSV recombinant expressing the Eo peptide. To regulate Ag exposure during infection, mice were treated with 100 µg Y-Ae mAb at different times following infection. Y-Ae treatment at the time of infection resulted in almost complete inhibition of the TEa-specific CD4 T cell response to VSV-GSE, with very few TEa-specific CD4 T cells detected in the blood 5 d postinfection (Fig. 4A, 4B). This correlated with an inhibition of proliferation at 3 and 5 d postinfection, as detected by the level of CFSE dilution (Supplemental Fig. 2). Moreover, in mice treated with the blocking Ab at either 2 or 3 d postinfection, the expansion of the TEa CD4 T cells was significantly reduced compared with control-treated mice at day 5 postinfection. Similarly, analysis of lymphoid and nonlymphoid tissues at 6 d postinfection also corresponded with reduced expansion of TEa CD4 T cells in mice in which Ag was limited up to and including 72 h postinfection (Fig. 4C). Thus, the CD4 T cells required at least 72 h of Ag availability to undergo expansion equivalent to that seen in control-treated mice, indicating that prolonged antigenic stimulation is necessary for maximal expansion of CD4 T cells responding to virus infection.

To assess whether the attenuated expansion of the TEa CD4 T cells in mice that had limited Ag exposure during the primary immune response altered effector function, we measured IL-2 and IFN-γ production by the TEa cells isolated from the spleen 5 d postinfection. As shown in Fig. 4D, cytokine production by TEa cells was dependent on the duration of Ag availability and strongly correlated with the magnitude of the response. Mice treated with Y-Ae at the time of infection, or 1 or 2 d following infection, exhibited decreased production of both IL-2 and the effector cytokine IFN-γ compared with TEa T cells that had access to Ag for longer periods of time. Thus, prolonged access to Ag by CD4 T cells resulted in increased capacity to produce cytokines, indicating that progressive differentiation of the CD4 T cells was dependent on the presence of Ag.

Collectively, these data demonstrated that during infection, both CD4 and CD8 T cells require sustained antigenic stimulation to undergo maximal expansion.

**Duration of Ag availability during the primary response affects the development of memory T cells**

Having established the temporal requirements of antigenic stimulation during the primary immune response, we next asked to what extent limiting Ag exposure early during the primary response affected the development of memory T cells. To this end, VSV-ova–infected mice were treated with the 25-D1.16 Ab during the primary infection. Ten weeks later, memory compartments in the spleen and lung were analyzed for the presence of OVA-specific CD8 T cells. Analysis of these tissues indicated that mice treated at the time of infection (D0) with 25-D1.16 mAb had an ~6-fold reduction in the number of memory cells isolated from the spleen and a 13-fold reduction in the lung, whereas mice treated at day 3 postinfection had an ~2- and 2.5-fold reduction in memory cells in the spleen and lungs, respectively (Fig. 5A). In addition, mice limited to Ag exposure for 4 d during the primary response, which resulted in reduced expansion at the peak of the primary response (Fig. 2), had a similar percentage of OVA-specific memory CD8 T cells compared with control-treated mice (Fig. 5A). We also examined memory development after 25-D1.16 mAb treatment of influenza virus-infected mice (Fig. 5B). Blockade from days 0–3 greatly reduced the number of memory cells generated by 31 d postinfection. A reduction was also noted when mAb was administered at day 6 after primary infection, but the difference did not reach statistical significance. Beyond that time, no difference in memory development was noted. Thus, although the proliferative capacity of CD8 T cells was strictly regulated by the continued presence of Ag during the primary phase of the immune response, the requirement for optimal memory cell differentiation was less stringent. Therefore, truncating the period of Ag availability to CD8 T cells during the primary response had overlapping yet independent effects on expansion and memory generation of CD8 T cells. These data demonstrate that for the generation of memory T cells, a threshold must be reached in regards to the duration of Ag exposure and that expansion does not absolutely correlate with the generation of memory cells.

Next, we determined how Ag availability during the primary response affected CD4 T cell differentiation to memory cells. To this end, $1 \times 10^5$ TEa CD4 T cells were adoptively transferred into congeneric B6 mice that were subsequently infected with VSV-GSE. In mice treated with an isotype control Ab at the time of infection, a small population of memory cells was detected in the spleen and lung at 4 wk postinfection (Fig. 6). In addition, reducing Ag availability for a period as short as 24 h postinfection did not inhibit the generation of memory CD4 T cells. Surprisingly, in mice in which antigenic stimulation was limited to 72 or 96 h (Day 3 or Day 4 in Fig. 6), the memory population in both the spleen and lung were greater than in control mice or when Ag was limited to 24 or 48 h of availability. Although the difference in the lung of mice treated 4 d postinfection were statistically significant compared with control treated mice, the difference did not reach statistical significance in the spleen. However, this trend was similar in independent experiments and suggested that for the differentiation of memory CD4 T cells, a lengthened period of antigenic stimulation during the primary immune response may be deleterious to memory generation. Furthermore, these data suggested that there was a window of antigenic opportunity, which resulted in optimal expansion, effector function, and differentiation to memory CD4 T cells.

**Reduced requirements for Ag availability during the recall response**

As compared with naive T cells, memory T cells are thought to require less overall stimulation to mount a response. To formally test this, mice were initially immunized with VSV-ova-NI and, 60 d later, were challenged with a different serotype of rVSV-ova (VSV-Ova-Ind) to avoid neutralizing Ab specific for the initial virus. Just prior to the recall infection, or at days 1–4 after the secondary challenge, mice were treated with 500 µg 25-D1.16 mAb. Five days after challenge, spleen and lung were analyzed for the expansion of OVA-specific CD8 T cells. In contrast to the primary response for which >4 d of Ag availability was necessary to drive optimal expansion, up to 24 h of antigenic stimulation was sufficient for OVA-specific memory CD8 T cells to generate a robust recall response to VSV-ova in both lymphoid and nonlymphoid tissues (Fig. 7). Reducing antigenic stimulation beyond 24 h had no effect on the magnitude of the recall response. These data indicated that the threshold for expansion was reached during the first 24 h of the recall response.

We also examined the TEa CD4 T cell response during secondary challenge with VSV. Seven months after adoptive transfer of TEa CD4 T cells and primary infection with VSV-GSE-Ind, mice were recalled with VSV-GSE-NI. Prior to secondary challenge, mice were bled to ensure even distribution of memory cells across the groups of mice that would be treated with the Y-Ae–blocking Ab. The percentage of Eo–specific memory CD4 T cells in the blood was similar in all groups (Fig. 8). Mice were then
FIGURE 4. Ag-specific CD4 T cells require sustained antigenic stimulation for optimal expansion and cytokine production. A, Kinetics of TTeA TCR-transgenic CD4 T cells in the blood in response to VSV-GSE at different times during the primary immune response under conditions of varying Ag availability. Total of $1 \times 10^6$ TTeA T cells was transferred 1 d before infection with $1 \times 10^5$ PFU VSV-GSE. Separate groups of mice received 500 μg Y-Ae Ab on different days of the response. At the time of infection, or at 24 h intervals postinfection for up to 5 d, mice were treated with Y-Ae. The populations of TTeA cells were analyzed at the indicated times by flow cytometry using a congenic marker. Data represent the mean ± SEM of four mice/group and are representative of several independent experiments. B, Individual time points from A. The x-axis indicates the time postinfection when mice were treated with the Y-Ae mAb. C, Magnitude of the TTeA response in spleen and lung at day 6 postinfection following Y-Ae treatment at days 1, 2, 3, or 4 postinfection. Mice were treated as in A, with the exception that they received 100 μg Ab. Data for A–C are derived from three to four mice per time point and treatment group and representative of three to four experiments. D, Limiting the duration of Ag availability results in reduced cytokine production by CD4 T cells. IL-2 and IFN-γ expression by TTeA cells in the spleen 5 d postinfection. Following adoptive transfer and infection as in A, mice were injected with Y-Ae or
challenged with $1 \times 10^6$ PFU VSV-GSE-NJ and treated with the blocking Ab at the indicated times for up to 4 d postinfection. Analysis of blood 4 d after secondary infection (Fig. 8) and spleen and lung 5 d after secondary infection (Fig. 8) revealed that mice treated with Y-Ae at the time of infection or 24 h postinfection resulted in attenuated expansion of the TEa T cells. Compared with the primary response, the period of antigenic stimulation required during the recall response was shortened by 2 d, as Ag was required for up to 4 d during the primary response and up to 2 d during the recall response. Thus, as with the CD8 T cell recall response, maximum CD4 T cell expansion occurred within a shortened period of antigenic stimulation.

Interestingly, the magnitude of the TEa recall response (Fig. 8) was not as robust as the primary response (Fig. 4). This was not due to precursor frequency, as memory TEa CD4 T cells were readily detectable 7 mo after primary infection (Fig. 8), whereas naïve T cells in the blood following adoptive transfer of $1 \times 10^4$ cells are virtually undetectable. In addition, the CD8 T cell response to VSV in the same mice was greater during the recall compared with the primary response (data not shown). Thus, it was possible that the memory TEa CD4 T cells were particularly refractory to the secondary infection due to the enhanced memory CD8 T cell response. Although perhaps unlikely, it is also possible that nonneutralizing cross-reactive Abs specific for VSV G protein could confer a limited level of protection (37). To eliminate the memory CD8 T cell response or any secondary Ab response against the virus during a recall challenge, we generated memory CD4 TEa T cells in mice by peptide/adjuvant immunization, as previously described (33). Four weeks after immunization, memory cells were barely detectable in the blood (data not shown). Mice were then infected with VSV-GSE-Ind to generate a TEa recall response. In this scenario, VSV-GSE will induce a primary CD8 T cell response, allowing the evaluation of the memory CD4 T cell response in the absence of a memory CD8 T cell response. Similar to the previous studies, mice were injected with the Y-Ae Ab at different times during the infection. Five days postinfection, lymphoid and nonlymphoid tissues were analyzed for expansion of the TEa memory population. Indeed, the magnitude of the response in control-treated mice was $\sim5$- and 20-fold greater in the spleen and lung, respectively, compared with control mice that were primed and recalled with VSV (Fig. 9, IgG2b; Fig. 8, Control). This experiment demonstrated that memory TEa CD4 T cells can, in fact, generate a robust recall response. The response was effectively blocked when Y-AE was administered at the time of infection (Fig. 9), but inhibition was rapidly lost as in the previous experiment (Fig. 8). Interestingly, in the peptide-immunized mice, treatment with the Y-Ae mAb 4 d after the recall response resulted in a significant increase in TEa effector memory cells in the spleen and peripheral LN relative to control Ab-treated mice (Fig. 9). This result was reminiscent of the increase in memory CD4 T cells observed in mice treated with the Y-Ae Ab 4 d after primary infection (Fig. 6), and suggested that mouse IgG2b control Ab at the indicated times postinfection. Five days postinfection, gated TEa cells were analyzed for IL-2 and IFN-γ production by intracellular staining after Eα peptide stimulation in vitro. Values represent the mean of two mice per group. *p < 0.05, **p < 0.01.
prolonged APC–CD4 T cell interactions could have a negative impact on the response. Thus, enhanced populations of CD4 T cells may be generated by defining the optimal period of Ag availability.

Discussion

The strength of stimulation following activation of a T cell is a critical component in determining the outcome of a T cell response (38). Sufficient stimulation results in proliferation and differentiation into effector and memory T cells, whereas insufficient stimulation results in an abortive response. Several factors including the Ag dose, TCR avidity (39–43), costimulation (44), and the duration of these interactions (7, 45) determine the overall strength of stimulation. Integration of these signals by the T cell initiates a program for proliferation and differentiation. In this study, we have looked in isolation at one component of this complex set of interactions, the duration of Ag availability, and how it affects the dynamics of the T cell response during infection.

These studies examined the temporal requirements of Ag availability for CD4 and CD8 T cell differentiation during an active infection. We developed a model in which the duration of Ag presentation could be manipulated via the administration of MHC–peptide-specific Ab. This model provides many advantages over previous studies. First, the blocking mAbs, 25-D1.16 (35) and Y-Ae (34) are specific for a single MHC–peptide and thus do not alter other T cell responses during the infection. Thus, the overall infectious cycle and level of inflammation remains intact. Lastly, manipulating the duration of Ag is easily controlled and can be tailored to the time frame investigated. In our system, it would appear that sufficient OVA epitope after VSV or influenza virus infections is available to drive robust CD8 T cell expansion. Moreover, the epitope is sustained late during the response as evidenced by our ability to inhibit the response at relatively late times postinfection. This, however, may not always be the case, as recently shown for the CD8 T cell response to certain influenza virus epitopes, in which the apparent lack of persistent epitope expression resulted in diminished CD8 T cell recruitment (46).

To study CD4 T cell responses in conjunction with the blocking mAb, we used an adoptive transfer system because the endogenous Ea-specific CD4 T cell response is essentially undetectable using conventional means (data not shown). Although this did not allow for a direct comparison of the endogenous CD4 and CD8 T cell responses, similarities were identified between the two subsets of cells. Both CD4 and CD8 T cells required sustained Ag availability during the primary immune response for optimal expansion (47). These results showed that Ag per se continues to drive the T cell response long after initial infection.
and T cell activation. These late APC encounters serve to enhance expansion as well as augment functionality because late interactions were required for optimal cytokine production by CD4 T cells. In the case of the CD8 T cell response to influenza virus infection, late costimulation through CD27 is also required for optimal expansion (48). Blocking CD27/CD70 interactions on days 6 or 8 after influenza virus infection results in a decrease in the number of Ag-specific CD8 T cells apparently through Fas-mediated apoptosis driven by CD4 T cells. In our system, any CD4 T cell response will remain intact, so the effects we observed were directly mediated through CD8 T cell–APC interactions.

For both CD4 and CD8 T cells, the recall response required a substantially shorter duration of Ag availability compared with the respective primary response. Thus, upon challenge, memory T cells initiated and sustained division with a substantially reduced duration of antigenic exposure as compared with naïve T cells. These studies formally established the duration of antigenic stimulation required for a recall response to occur. Our results agreed with the general tenet that memory T cells are more readily activated than naïve T cells. This is clearly true for effector functions, which are rapidly induced following memory T cell activation in the absence of Ag. Induction of proliferation could be considered the second phase of reactivation, resulting in generation of a new numerically increased cohort of secondary memory cells. In fact, previous work indicates that the rate of proliferation is similar for naïve versus memory CD8 T cells (49). Nevertheless, our results suggested that TCR triggering is either more sensitive to lower levels of Ag or that initial TCR triggering results in sustained activation in the absence of Ag. Previous studies support these concepts in that TCR-associated signal transduction components are modified in memory T cells as compared with naïve T cells (50–52). Nevertheless, despite heightened TCR responsiveness, costimulation is required for memory T cell reactivation in some cases. Blockade of CD28 greatly inhibits the CD8 and CD4 T cell recall response to influenza virus infection (53, 54), whereas CD40L blockade inhibits the secondary CD8 T cell response to VSV infection (55). In addition, the lack of expression of certain costimulators (e.g., CD70) by particular DC subsets may preclude efficient memory CD8 T cell reactivation (56). Thus, heightened TCR responsiveness in memory T cells may not necessarily correlate with costimulation independence. In part, this hypothesis may be explained by the increase in TCR avidity that is observed in polyclonal memory CD8 T cells (57, 58), whereas functional avidity maturation occurs in transgenic T cells expressing monoclonal TCRs (59).

For the CD4 recall response, the pre-existing memory E-specific CD4 T cell population expanded in response to secondary challenge, yet the response was not as robust as the memory CD8 T cell response. This finding is in agreement with recent reports in which memory CD4 T cells do not respond as well during secondary Ag encounter (60, 61) and may be due to reduced levels of IL-2 production in combination with increased levels of IFN-γ production (60). Although we did not measure cytokine production in the secondary response, we showed that the TEa recall response was greatly enhanced when mice were immunized with peptide/adjuvant and then challenged with VSV-expressing Eα. Although it is possible that the two forms of immunization
differentiation of cells may be asynchronous and continued
differentiation, but required additional interactions with DCs
CD8 T cells had received sufficient stimulation for programmed
shown). Thus, these data suggested that by day 4 postinfection, the
were largely unaffected by mAb treatment (47 and data not
tional properties of memory CD8 T cells after VSV infection. Although the CD8 T cell primary
and secondary responses to VSV infection are CD4 T cell in-
dependent (55), rapid viral clearance by memory CD8 T cells
could limit the secondary CD4 T cell response in part through
reduction of Ag. Of particular interest was the finding that limiting
Ag late in the primary T cell response to VSV infection resulted in an
increase in the CD4 T cell memory population. A similar result
was observed during VSV recall of memory cells generated by
peptide/adjuvant immunization. These data suggested that con-
tinued antigen stimulation had a negative impact on memory
development. These events could be related to induction of cell
death, which can result from sustained antigenic stimulation (62).
In contrast, our previous work shows that even a minor change in
Ag expression (through injection of 1ug of Y-Ae) early in the
response is sufficient to block memory development while not
affecting initial expansion (18). Thus, for effective generation of
T cell memory, there is a favorable window of activation related to
Ag levels. Determining the optimal period of antigenic stimulation
of a T cell may lead to more rational vaccine designs.

As stated above, for optimal expansion, endogenous CD8 T cells
required the presence of Ag for at least 4 d following VSV infection
and at least 7 d following influenza virus infection. This difference
can be attributed to the level of Ag expressed at different times
postinfection with the different viruses and may also be manifested in
the differences in the time required for the CD8 T cell responses to
reach apogee (~day 7 for VSV and ~day 10 for influenza virus).
Thus, priming for the influenza virus response appears more pro-
tracted perhaps due to the necessity for local viral replication
to occur in the lung epithelium, whereas VSV can productively
infect many cell types. Clearly, the overall quantity and the loca-
tion of Ag can affect response outcome, but nevertheless, lim-
iting Ag exposure resulted in an overall decrease in the magnitude of
the response. This is in contrast to earlier work showing that
CD8 T cell responses are unchanged after antibiotic treatment
24 h post-L. monocytogenes infection (10–12). These differences
can be accounted for by the reduced inflammation mediated by
antibiotic treatment and truncated infection. Additionally, it is
likely that residual Ag persists beyond bacterial clearance, further
complicating data interpretation. In our model, Ag availability was
modulated by inhibiting Ag presentation to the responding Ag-
specific T cells. All other parameters of the infection and immune
responses were maintained. Thus, in our model, we were able to
specifically examine the effects of Ag availability while main-
taining all other parameters. Although CD8 T cell expansion was
reduced when Ag duration was shortened, memory differentiation
was apparent. In agreement with other studies, the duration of Ag
availability affects the magnitude of the primary response (9, 13)
but does not necessarily correlate with the development of
memory (6, 20). Interestingly, whereas limiting the Ag duration
for up to 4 d resulted in attenuated expansion, there were divergent
effects on the memory populations generated. In mice treated at
day 4 postinfection, the magnitude of expansion was similar to
mice treated with blocking Ab 24 h earlier (Fig. 1). Remarkably,
the resulting memory population in the two groups was markedly
different (Fig. 5). This was not the result of changes in the ratio of
short-lived versus memory precursor effector populations, which
were largely unaffected by mAb treatment (47 and data not
shown). Thus, these data suggested that by day 4 postinfection, the
CD8 T cells had received sufficient stimulation for programmed
differentiation, but required additional interactions with DCs
for continued proliferation. This result also suggested that the
differentiation of cells may be asynchronous and continued
throughout the primary response. It will be interesting to de-
termine if there are functional differences in the memory popula-
tions generated under the varying durations of Ag exposure.
We would surmise that such memory cells would exhibit normal
functional capabilities because even in the absence of accumula-
tion of T cells during the primary response, functional memory
cells are generated (6).

Although the specific mechanism by which addition of the
blocking Abs is regulating the immune response was not defined,
several lines of evidence point to a likely explanation. T cell priming
occurs in three phases with multiple T cell–DC interactions taking
place during the first 48 h of the response (63). Other studies have
also shown that T cells undergo multiple T cell–DC interactions
during an immune response and that these interactions are important
in the differentiation of effector T cells (15). In situ analysis of the
CD8 T cell immune response reveals that Ag-specific CD8 T cells
form localized clusters with DC in the spleen at 5 d after L.
monocytogenes infection, and administration of 25-D1.16 prevents cluster
formation (64). Moreover, a recent study using real-time imaging
techniques shows that blocking MHC class II in vivo promoted the
dissociation of T cell–DC interactions (14). Therefore, the 25-D1.16
and Y-Ae mAbs are most likely disrupting stable T cell–DC
interactions and potentially preventing subsequent interactions from
developing. The precise DC subset and location of inhibition is
likely dependent on the infection type and the time of blocking
mAb administration. For example, early after influenza virus in-
festation, DC migrating from the lung to the draining MLN are
important for T cell priming. Thus, early administration of mAb could
result in inhibition of early T cell–DC interactions in the LN. Con-
versely, late postinfection activated CD8 T cells that have mi-
grated to the lung tissue may interact with Ag-bearing APC or with
infected parenchymal cells, resulting in further T cell expansion
(56, 65–70).

In summary, we have developed a model to assess the Ag re-
quirements for both CD4 and CD8 T cells in vivo during an active
infection. Prolonged Ag availability was required for maximal
T cell expansion, as shortening the period of Ag availability
resulted in a decrease in the magnitude of the primary response.
The duration of Ag availability also influenced memory T cell
development. However, the magnitude of the response did not
necessarily correlate with memory generation, suggesting that
independent mechanisms regulate T cell proliferation and differ-
entiation. For recall responses, the Ag requirements were less
compared with the primary response, as T cells required a shorter
period of Ag availability for maximal expansion to occur. Lastly,
optimal CD4 T cell populations may be generated by determin-
ing the optimal window of Ag availability. Understanding these
temporal requirements for Ag availability by T cells during an
immune response and its effect on effector and memory differ-
entiation will be critical for the design of future vaccines. The
period of Ag availability needs to be considered and may need
to be customized to individual CD4 and CD8 T cell responses to
elicit the maximum protective capacity of a vaccine.

Disclosures
The authors have no financial conflicts of interest.

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Supplemental figure 1. Treatment with 25D1.16 mAb does not cause the depletion of SIINFEKL presenting cells. CD45.1+ splenocytes were coated with SIINFEKL peptide then adoptively transferred into CD45.2 hosts which were treated with 500 μg of either rat IgG or 25D1.16 mAb. Three days after transfer the spleens were harvested and transferred cells detected by flow cytometry. (A) B220- CD11c-, CD11c+ and B220+ splenocytes all expressed surface MHC:SIINFEKL complexes as detected by Alexa 647 conjugated 25D1.16 mAb. (B) The percentage of transferred cells recovered from either IgG or 25D1.16 mAb treated mice is represented graphically.
Supplemental Figure 2. Y-Ae affects the response through inhibition of proliferation. 
1x10^5 CFSE-labeled CD45.2 T Ea cells were transferred and 24 hrs later the mice were injected with 100ug Y-Ae and infected with VSV-GSE. At 3 and 5 days after infection the expansion and CFSE dilution of the Tea cells was measured by flow cytometry.
Supplemental Table 1. Virus titers in the lung are not affected by 25D.1.16 treatment. Mice were infected with influenza virus (WSN-ova) and the next day were treated with 250ug of control IgG or 25D.1.16. Lungs were harvested 4 and 8 days after infection and virus titers were determined by plaque assay using MDCK cells. Values represent the average titers per whole lung +/- the SD of 3 mice per group. No statistically significant difference was detected between the groups.

<table>
<thead>
<tr>
<th>Day after infection</th>
<th>Control IgG</th>
<th>25D.1.16</th>
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<tbody>
<tr>
<td>4</td>
<td>2.7±0.3x10^4</td>
<td>3.0±0.8x10^4</td>
</tr>
<tr>
<td>8</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
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