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Activation, Differentiation, and Migration of Naive Virus-Specific CD8⁺ T Cells during Pulmonary Influenza Virus Infection¹

Christopher W. Lawrence^{*†} and Thomas J. Braciale^{2*†‡}

The low precursor frequency of individual virus-specific CD8⁺ T cells in a naive host makes the early events of CD8⁺ T cell activation, proliferation, and differentiation in response to viral infection a challenge to identify. We have therefore examined the response of naive CD8⁺ T cells to pulmonary influenza virus infection with a murine adoptive transfer model using hemagglutinin-specific TCR transgenic CD8⁺ T cells. Initial activation of CD8⁺ T cells occurs during the first 3 days postinfection exclusively within the draining lymph nodes. Acquisition of CTL effector functions, including effector cytokine and granule-associated protease expression, occurs in the draining lymph nodes and differentially correlates with cell division. Division of activated CD8⁺ T cells within the draining lymph nodes occurs in an asynchronous manner between days 3 and 4 postinfection. Despite the presence of Ag for several days within the draining lymph nodes, dividing T cells do not appear to maintain contact with residual Ag. After multiple cell divisions, CD8⁺ T cells exit the draining lymph nodes and migrate to the infected lung. Activated CD8⁺ T cells also disseminate throughout lymphoid tissue including the spleen and distal lymph nodes following their emigration from draining lymph nodes. These results demonstrate an important role for draining lymph nodes in orchestrating T cell responses during a local infection of a discrete organ to generate effector CD8⁺ T cells capable of responding to infection and seeding peripheral lymphoid tissues. *The Journal of Immunology*, 2004, 173: 1209–1218.

The CD8⁺ T cell has been demonstrated to play an important role in recovery from experimental infection with viruses and certain bacteria (1–3). During systemic virus infection, the activation of naive CD8⁺ T cells results in the generation of antiviral effector T cells responsible for clearance of virus and virus-infected cells, as well as in the generation of memory CD8⁺ T cells capable of an accelerated response to subsequent virus infection (4–6). During the resolution phase of the response to systemic virus infection, memory CD8⁺ T cells have been reported to localize to both lymphoid and peripheral (nonlymphoid) tissues (7). Similarly, CD8⁺ T cells have been documented to play a critical role in recovery from experimental infection with viruses localized to a specific organ or tissues, e.g., influenza virus infection of the respiratory tract (8–10). Effector-memory CD8⁺ T cells likewise appear to be widely disseminated throughout the body during the induction and/or resolution of experimental pulmonary influenza virus infection (11).

After experimental intranasal (i.n.)³ influenza virus infection of mice, virus replication is restricted primarily to the respiratory ep-

ithelium (12) and is cleared within 10 days (13). Therefore, influenza virus infection of the respiratory tract should serve as a useful model for the analysis of early events in the activation/differentiation of CD8⁺ T cells responding to virus infection localized to a discrete peripheral site. Because the activation of naive T cells is believed to occur within organized secondary lymphoid tissues (14), e.g., the draining mediastinal lymph nodes (MLN) in the case of respiratory influenza virus infection, viral Ag must gain access to these lymphoid compartments and be presented to naive CD8⁺ T cells by professional APCs, e.g., dendritic cells (DC). Indeed, it has been reported that DC isolated from the MLN of influenza virus-infected mice are capable of stimulating virus-specific T cell responses (15). Recently, the migration of respiratory DC from the infected lungs to the draining regional lymph nodes in response to i.n. influenza virus infection has been documented (16).

The analysis of early events in the activation and differentiation of naive CD8⁺ T cells within secondary lymphoid tissues is hampered by the low frequency of Ag-specific CD8⁺ T cell precursors within the pool of naive T cells present in normal nonimmune animals (13). This limitation has made it difficult to precisely evaluate the tempo of naive CD8⁺ T cell activation/proliferation and the accumulation of effector T cells at the site of infection, as well as the relationship between T cell activation and cell division and the acquisition of effector functions. This limitation of naive T cell frequency has been overcome by the analysis of the response of naive TCR transgenic T cells to antigenic stimulation in vivo after adoptive transfer of the T cells into naive recipient mice (17–19). This approach has been successfully used to analyze the response of influenza-specific CD4⁺ T cells in vivo during pulmonary influenza virus infection (20).

To evaluate early steps in the activation, differentiation, and migration of CD8⁺ T cells responding to pulmonary influenza virus infection, we have analyzed the response of adoptively transferred CD8⁺ TCR transgenic clone 4 T cells in recipient mice to

*Beime B. Carter Center for Immunology Research, and Departments of [†]Microbiology and [‡]Pathology, University of Virginia Health Sciences Center, Charlottesville, VA 22908

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² Address correspondence and reprint requests to Dr. Thomas J. Braciale, Beime B. Carter Center for Immunology Research, University of Virginia Health Sciences Center, Medical Research Building 4, Health Sciences Center Box 801386, Charlottesville, VA 22908. E-mail address: tjb2r@virginia.edu

³ Abbreviations used in this paper: i.n., intranasal; MLN, mediastinal lymph node; NDLN, nondraining lymph node; HA, hemagglutinin; DC, dendritic cell; EID, egg infectious dose.

i.n. infection with the A/PR/8/34 (PR/8) influenza virus strain. This TCR transgenic T cell population, which is directed to a dominant epitope displayed by the PR/8 hemagglutinin (HA) (21), has been previously used to analyze aspects of CD8⁺ T cell tolerance or autoimmunity (21–24), tumor development (25), and CD8⁺ T cell mediated lung injury during infection (26).

In this report, we have examined the early phase of the CD8⁺ T cell response of transferred clone 4 T cells to influenza virus infection (days 2–7). We demonstrate that the induction of the CD8⁺ T cell response occurs primarily in the draining MLN. In the MLN, activated CD8⁺ T cells acquire effector activities. Analysis of the kinetics of the T cell response suggests that effector CD8⁺ T cells generated in the MLN not only migrate to the infected lungs but also seed other secondary lymphoid tissues, e.g., the spleen and non-draining peripheral lymph nodes (NDLN). Finally, we provide evidence that CD8⁺ T cell encounter with viral Ag in the draining MLN may be limited despite Ag persistence in the lymph nodes more than 5 days after infection. The implications of these findings are discussed.

Materials and Methods

Mice

Clone 4 TCR transgenic mice (H-2^d, Thy-1.2) were a generous gift from Dr. R. W. Dutton (Trudeau Institute, Saranac Lake, NY) (27). These mice were bred and housed in a pathogen-free environment and used at 8–11 wk of age for experiments. Thy-1.1^{+/+}-BALB/c mice were a kind gift from Dr. R. I. Enelow (Yale University, New Haven, CT). These mice were bred and housed in a pathogen-free environment and used at 10–13 wk of age for experiments.

Adoptive transfer experiments

Naive CD8⁺ T cells were purified from spleens of clone 4 mice by negative selection column separation techniques (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Within the purified population, >95% of CD8⁺ T cells exhibited a naive phenotype with respect to CD69, CD62L, and CD25 expression. Purified CD8⁺ T cells were labeled with 10 μ M carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Eugene, OR) for 10 min at room temperature. Labeled cells were washed with equal volumes of FBS (Atlanta Biologicals, Norcross, GA) and Iscove's media (Invitrogen, Carlsbad, CA), then again with serum-free IMEM. A total of 8.5×10^6 CFSE-labeled CD8⁺ T cells were i.v. injected into the tail vein of individual Thy-1.1^{+/+}-BALB/c recipients. Recipient mice were infected with influenza virus 24 h later.

Infection of mice

Influenza A/PR/8/34 (H1N1) was grown in day 10 chicken embryo allantoic cavities. Mouse-adapted influenza A/HK/1/68 (H3N2) was passaged several times in mouse lungs before a final preparation was grown in day 10 chicken embryo allantoic cavities. Mice were lightly anesthetized by Metofane inhalation (Janssen Pharmaceutical, Toronto, ON) and sublethally infected by i.n. inoculation (in 50 μ l) with either 350 egg infectious doses (EID) of influenza A/PR/8/34 or 24 EID of control influenza A/HK/1/68.

Preparation of tissue lymphocytes

At multiple days after influenza virus infection, mice were sacrificed by cervical dislocation. Lungs were perfused via the right ventricle of the heart with 5–10 ml of PBS containing 10 U/ml heparin (Sigma-Aldrich, St. Louis, MO) to remove blood lymphocytes from the lung vasculature. The lungs, spleen, and lymph nodes (draining MLN and non-draining popliteal, inguinal, mesenteric, and axillary lymph nodes) were dissected and placed into cold IMEM. Spleen and lung cell preparations were made by passing tissue through a steel screen. Lymph node cells were gently prepared by manual tissue disruption. Tissue debris was removed by centrifugation at $300 \times g$. Cells were counted and resuspended at appropriate concentrations for each particular experiment.

Peptide

Synthetic A/PR/8/34 HA peptide spanning residues 533–541 (HA_{533–541}) (IYSTVASSL) was synthesized by the University of Virginia Biomolecular Research Facility.

Ex vivo Ag sensor assay

To detect the presence of Ag in MLN at various times after infection, MLN were harvested from Thy-1.1^{+/+}-BALB/c mice at multiple days after infection with influenza A/PR/8/34 or 5 days after influenza A/HK/1/68 infection. MLN cells (1×10^6) were incubated in 96-well round-bottom plates (Corning, Corning, NY) with 2×10^5 purified naive CFSE-labeled clone 4 CD8⁺ T cells in IMEM medium supplemented with 10% FBS, 10 U/ml penicillin G, 10 μ g/ml streptomycin sulfate, 2 mM L-glutamine, and 0.05% 2-ME. In some experiments, 10 μ g/ml anti-H-2K^d Ab (clone SF1-1.1) or control IgG2a Ab (clone MPC-11) was added to the cultures. After 3 days of incubation at 37°C, the cells were labeled with Abs against CD8 α and Thy-1.2 and then analyzed for CFSE dilution by flow cytometry.

Ab labeling and flow cytometry

For cell surface labeling experiments, $\sim 2 \times 10^6$ cells were incubated with the following Abs in the presence of anti-CD16/32 (clone 2.4G2); anti-CD8 α (clone 53-6.7), anti-CD11a (clone M1/70), anti-CD25 (clone PC61), anti-CD49d (clone R1-2), anti-CD62L (clone MEL-14), anti-CD69 (clone H1.2F3), and anti-Thy-1.2 (clone 53-2.1) from BD Biosciences (San Diego, CA). Cells were labeled for 45 min at 4°C in staining buffer (PBS with 1% FBS, 0.02% NaN₃). The cells were then fixed and erythrocytes lysed in FACS Lysing Solution (BD Biosciences). For intracellular staining, cells were incubated for 5 h in IMEM supplemented with 10% FBS, 10 U/ml penicillin G, 10 μ g/ml streptomycin sulfate, 100 U/ml human IL-2, 2 mM L-glutamine 0.05% 2-ME, and 1 μ g/ml brefeldin-A (Sigma-Aldrich) in the presence or absence of 1 μ M HA_{533–541} peptide. After incubation, the cells were surface labeled with anti-CD8 α and anti-Thy-1.2 as described earlier. Fixed cells were permeabilized in staining buffer supplemented with 0.5% saponin (Sigma-Aldrich). Permeabilized cells were labeled with anti-granzyme B (clone GB12; Caltag Laboratories, Burlingame, CA), anti-IFN- γ (clone XMG1.2), or anti-TNF- α (clone MP6-XT22) (BD Biosciences). For analysis of CD69 expression by proliferating MLN and splenic T cells, cell suspensions of MLN and spleen tissue were prepared from adoptive transfer recipients after 5 days of infection. Cells ($1–2 \times 10^6$) were incubated with or without 50 nM HA_{533–541} peptide for 6 h in IMEM medium supplemented with 10% FBS, 10 U/ml penicillin G, 10 μ g/ml streptomycin sulfate, 2 mM L-glutamine, and 0.05% 2-ME. Cells were also stimulated with P815 mastocytoma cells (H-2^d) infected with A/PR/8/34 for 6 h. Cell surface labeling using Abs against CD8 α , CD69, and Thy-1.2 was performed as described earlier. Flow cytometry data for each of the experiments were acquired using a BD FACSCaliber (BD Immunocytometry Systems, San Diego, CA) and analyzed using CellQuest software (BD Biosciences).

Results

Kinetics of proliferation and tissue accumulation of responding influenza virus-specific CD8⁺ T cells

To analyze the kinetics of viral Ag-specific CD8⁺ T cell proliferation and recruitment during pulmonary virus infection in the murine model, we used an adoptive transfer approach using influenza A/PR/8/34 (PR/8) virus-specific TCR transgenic donor CD8⁺ T cells. Purified, naive transgenic clone 4 CD8⁺ T cells were labeled with the cell division-sensitive fluorescent vital dye CFSE to assess T cell proliferation in vivo by dye dilution in the standard flow cytometry based assay (28). Approximately 8.5×10^6 purified CFSE-labeled Thy-1.2⁺ clone 4 CD8⁺ T cells were adoptively transferred into Thy-1 disparate recipient mice (Thy-1.1^{+/+}-BALB/c), allowing early detection and quantitation of donor CD8⁺ T cells in vivo based on Thy-1.2 isoform expression. One day after cell transfer, recipient mice were inoculated i.n. with a sublethal dose of PR/8 virus. CFSE fluorescence intensity and Thy-1.2 expression were then monitored in the lungs and various lymphoid tissues of infected recipients over 24 h intervals between days 2 and 7 of infection to identify and assess the proliferative expansion and tissue localization of the responding CD8⁺ T cells.

After transfer, donor T cells were detectable throughout the lymphoid tissue of recipient mice, including the lung-draining MLN, peripheral NDLN, and spleen. Few donor T cells were detected in the lungs of uninfected recipient mice at any time after cell transfer or in infected mice during the first 4 days of infection (Fig. 1). No

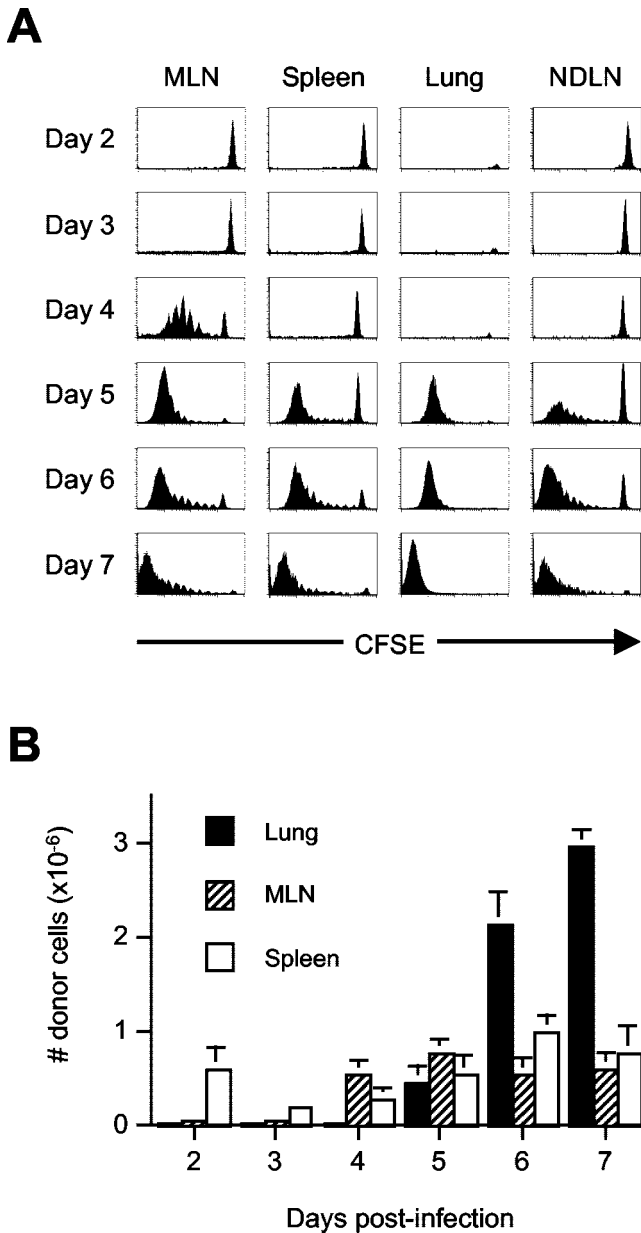


FIGURE 1. Kinetics and magnitude of in vivo clone 4 CD8⁺ T cell proliferation in influenza virus-infected mice. *A*, A total of 8.5×10^6 naive CFSE-labeled clone 4 CD8⁺ T cells (Thy-1.2) were transferred i.v. into Thy-1.1^{+/+}-BALB/c mice. The following day, recipient mice were i.n. inoculated with a 350 EID of influenza A/PR/8/34 (H1N1) virus. Spleen, lungs, MLN, and NDLN were harvested at days 2–7 after infection. Histograms of CFSE fluorescence are gated on Thy-1.2⁺ CD8⁺ cells. *B*, Absolute cell numbers of donor clone 4 T cells were calculated from organ cell counts and CD8⁺ Thy-1.2⁺ percentages.

evidence of cell division, i.e., CFSE dilution, was observed in any site until day 4 of infection when extensive proliferation was evident exclusively in the MLN (Fig. 1*A*). This proliferative response was associated with a 10-fold increase in total donor T cells in the draining MLN between days 3 and 4 of infection (Fig. 1*B*). The evidence of extensive proliferation, i.e., up to 7–8 cell divisions, (Fig. 1*A*) and donor T cell accumulation in the draining lymph nodes (Fig. 1*B*) between days 3 and 4 of infection suggests a division rate for these CD8⁺ T cells of ~3–6 h.

It is noteworthy that during the time of extensive T cell proliferation in the MLN at day 4 of infection, we found no evidence of

comparable proliferative expansion or T cell accumulation in the spleen, lungs, or distal NDLN (Fig. 1). This result changed dramatically at day 5 of infection, when the vast majority of donor Thy-1.2⁺ CD8⁺ T cells detected in the MLN appeared to have undergone at least seven divisions, with few of the donor T cells remaining undivided (Fig. 1*A*). Concurrently at day 5, donor CD8⁺ T cells that had undergone the same number of cell divisions (same CFSE intensity) as the proliferating T cells in the MLN had accumulated in the spleen, lungs, and NDLN (Fig. 1*A*). This was associated with a substantial increase in donor cells detected in the lungs and a modest increase in total donor cells detected in the spleen (Fig. 1*B*). Significant numbers of undivided CFSE^{high} donor T cells were present in the spleen and the NDLN, despite the accumulation of highly proliferating CFSE^{low} donor CD8⁺ T cells in these sites. Taken together, our kinetic data demonstrate that the likely and expected source of the proliferating CFSE^{low} donor T cells accumulating in the lungs at day 5 was the draining MLN, as previously suggested (29, 30). Our data also suggest that the MLN were the major source of the proliferating CFSE^{low} donor CD8⁺ T cells found in the peripheral lymphoid tissues (spleen and NDLN), at least at day 5 of infection.

At days 6–7 postinfection, there was extensive accumulation of CFSE^{low} donor CD8⁺ T cells in the infected lungs (~150-fold increase in total cells in the lungs between days 4 and 7; Fig. 1*B*). A further decrease of CFSE intensity in donor CD8⁺ T cells in the lungs occurred between days 5 and 7 of infection (Fig. 1*A*). This may reflect further proliferation of recruited effector CD8⁺ T cells in the infected lungs (see below), or could also reflect the contribution of donor T cells that had undergone greater than eight divisions in the draining MLN before their migration to the infected lungs during the latter stages of infection. In addition, at days 5–7 we repeatedly observed in both the MLN, and to a lesser extent in the spleen and the NDLN, the presence of a small but significant number of donor T cells which had undergone fewer than six divisions (CFSE^{moderate}) (Fig. 1*A*). These proliferating T cells could reflect the delayed proliferation of local CFSE^{high} T cells resident in the lymph nodes and/or spleen. Alternatively, they may represent the recruitment of recirculating naive donor T cells to the MLN, with the subsequent dissemination of the early activated T cells to these other lymphoid tissues.

Activation and adhesion marker expression by responding CD8⁺ T cells

To evaluate the phenotypic evolution of the donor CD8⁺ T cells during their activation and proliferation in response to virus infection, we analyzed the expression of several cell surface markers linked to T lymphocyte activation (CD69, CD25) and T lymphocyte adhesion/homing (CD62L, CD11a, CD49d) on the donor CD8⁺ T cells in the lymph nodes, spleen, and the lungs at various times after infection (Fig. 2). CD69 is an early marker of T cell activation whose expression is dependent on TCR engagement (31–35). In the draining MLN, we observed up-regulation of CD69 on a fraction (14%) of the CFSE^{high} undivided donor T cells at day 2 of infection, with the majority of the as yet undivided donor CD8⁺ T cells expressing CD69 by day 3 of infection (Fig. 2*A*). CD62L (L-selectin), the T cell lymph node homing receptor (36–38), is rapidly shed from the surface of T cells shortly after activation (39, 40). CD62L expression progressively fell on the MLN resident undivided donor CD8⁺ T cells between days 2 and 3 of infection in parallel with the increase in CD69 expression (Fig. 2*A*). CD62L expression remained low on the majority of the CFSE^{moderate/low} dividing donor T cell population through days 4–5. The α -chain of the high affinity IL-2R (CD25) was also up-regulated initially in undivided CFSE^{high} donor T cells within the

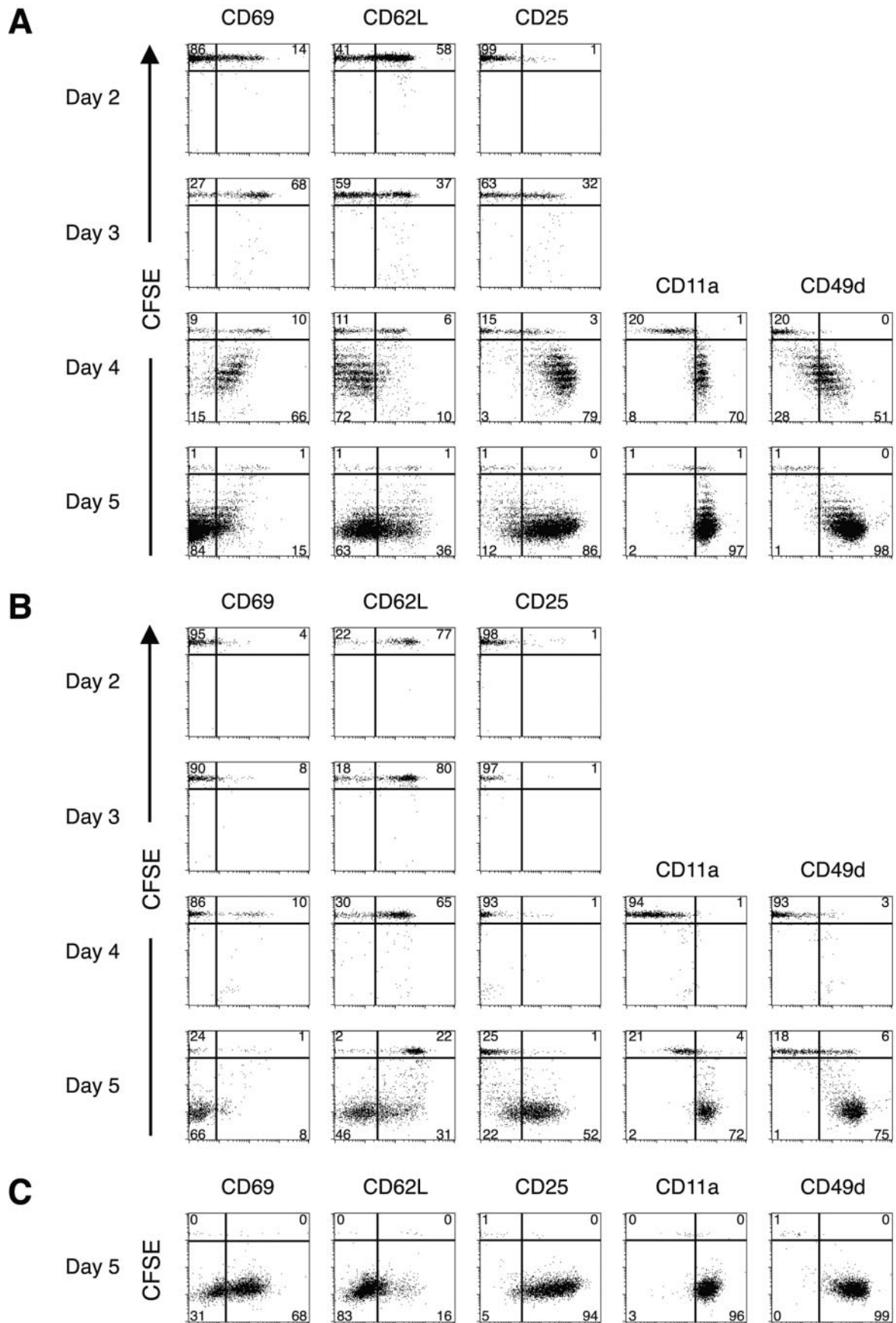


FIGURE 2. Cell surface phenotypic changes during division of donor T cells in influenza virus-infected mice. Using the same experimental transfer model described in Fig. 1, MLN (A), spleens (B), and lungs (C) were harvested at days 2–5 after infection. Dot plots are gated on Thy-1.2⁺CD8⁺ cells and analyzed for CFSE fluorescence as well as activation marker and adhesion molecule expression by staining with Abs against CD69, CD62L, CD25, CD11a, and CD49d. The percentage of gated cells in a given quadrant is shown in each corner of each respective quadrant.

draining MLN, and CD25 expression was sustained at a high level up to day 5 of infection among the dividing T cell population (Fig. 2A). The LFA-1 integrin α -chain, CD11a, and the VLA-4 integrin α -chain, CD49d, were expressed at low levels on undivided (CFSE^{high}) donor T cells and were increased in expression on dividing cells (Fig. 2A). These integrins are important for the interaction of activated T cells with vascular endothelium at sites of inflammation (41–43) and have been directly implicated in T cell recruitment to the lung (44–49).

In contrast to the phenotype of donor cells in the draining MLN, the pattern of cell surface marker expression in the spleen exhibited several notable differences (Fig. 2B). First and foremost, the undivided (CFSE^{high}) donor T cells that were present in the spleen predominantly retained the CD69⁻, CD62L^{high}, CD25⁻, CD11a^{low}/CD49d^{low} phenotype of naive (unactivated) CD8⁺ T cells through day 5 of infection (Fig. 2B). These data suggest that few donor T cells resident in the spleen had contacted Ag during the early phase of infection (day 0–3). Activation marker expression by undivided T cells in the spleen at day 4 suggested a potential modest, delayed response to Ag in the spleen relative to the activation kinetics in the MLN. In contrast, the CFSE^{low} proliferating CD8⁺ T cells first detected in the spleen at day 5 of infection (Fig. 1A) exhibited a highly activated phenotype (Fig. 2B) identical with that of the donor cells in the draining MLN (Fig. 2A).

When donor cells first appeared in significant numbers in the lungs at day 5, the T cells were uniformly CFSE^{low}, suggesting that they had been derived from precursor cells undergoing greater than five divisions (Fig. 2C). These T cells exhibited the phenotype characteristic of activated effector CD8⁺ T cells (CD62L^{low}, CD25^{high}, CD11a^{high}, CD49d^{high}; Fig. 2C) that was shared by comparable CFSE^{low} T cells in the MLN and spleen (Fig. 2, A and C). Furthermore, the high expression levels of CD11a and CD49d may contribute to the enhanced migration efficiency of activated T cells into the lung compared with naive T cells (Figs. 1 and 2).

Effector activity of activated proliferating CD8⁺ T cells

The previously mentioned results suggested that during i.n. influenza virus infection, the draining MLN were the primary source of activated CD8⁺ T cells that homed to the infected lungs as well as the spleen, at least through day 5 of infection. Because the production of effector cytokines in response to antigenic stimulation and cell-associated killing activity is a hallmark of activated CTL with antiviral effector activity (1, 2), we examined the relationship between proliferation status (extent of cell division) and the expression of soluble effector molecules by proliferating donor CD8⁺ T cells in the draining MLN. To this end, we adoptively transferred naive CFSE-labeled clone 4 CD8⁺ T cells into Thy-1 congenic recipient mice as performed previously in Figs. 1 and 2. On day 4 following i.n. PR/8 virus infection, MLN cell suspensions were cultured for 5 h in vitro with or without the synthetic cognate PR/8 HA_{533–541} peptide. At the end of brief in vitro culture, the CD8⁺ T cells were examined by flow cytometry for intracellular expression of IFN- γ , TNF- α , and granzyme B.

As Fig. 3 demonstrates, we detected spontaneous IFN- γ synthesis from a fraction (36%) of the proliferating (CFSE^{moderate/low}) donor CD8⁺ T cells in the MLN at day 4 postinfection. This low level of spontaneous IFN- γ synthesis was restricted primarily to the dividing T cell population, as <10% of the undivided (CFSE^{high}) T cells produced the cytokine. After acute in vitro antigenic stimulation, the vast majority (>80%) of the dividing CD8⁺ T cells produced high levels of IFN- γ , and a significant fraction (42%) of the undivided (CFSE^{high}) T cells produced the cytokine in response to peptide stimulation. In contrast, there was minimal TNF- α production by the donor CD8⁺ T cells in the

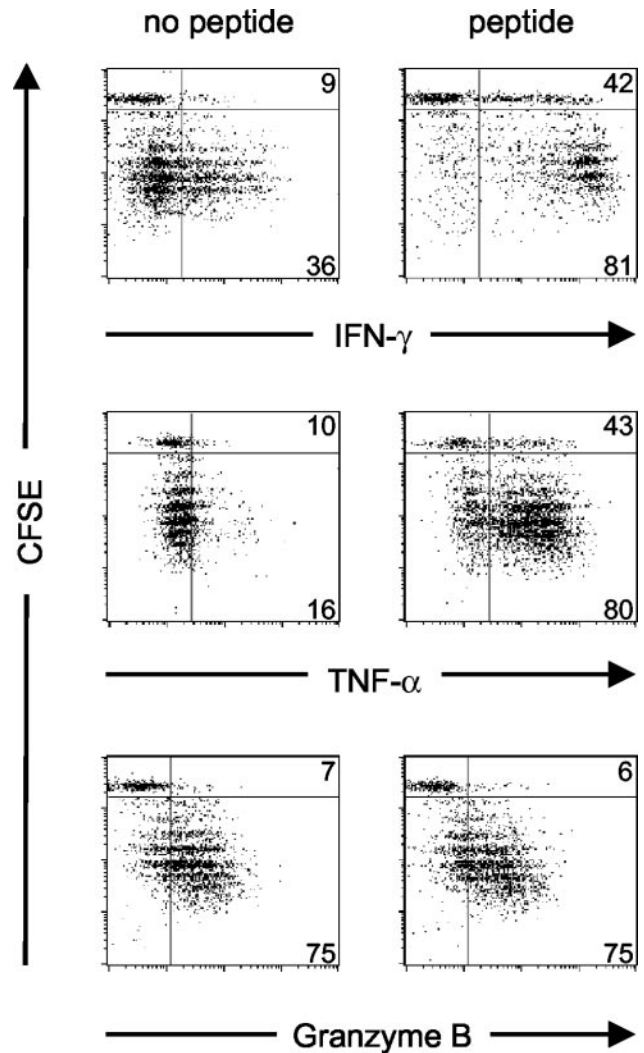


FIGURE 3. Effector molecule acquisition by dividing donor CD8⁺ T cells in the draining MLN. Using the same experimental transfer model described in Fig. 1, MLN were harvested from day 4 mice and cultured for 5 h with brefeldin A in the presence or absence of 1 μ M HA_{533–541} peptide. After incubation, cells were intracellularly stained with Abs against IFN- γ , TNF- α , and granzyme B. Dot plots shown are gated on Thy-1.2⁺ CD8⁺ cells. The percentage of positive cells within the undivided fraction is shown in the upper right quadrant. The percentage of positive cells within the dividing fraction is shown in the lower right quadrant.

absence of antigenic stimulation, but both dividing and undivided cells produced this cytokine in response to antigenic stimulation at frequencies comparable to that of IFN- γ (Fig. 3). The cytolytic granule-associated protease granzyme B was detected solely in the proliferating T cells. There was a trend toward higher levels of granzyme B expression with increasing cell division (Fig. 3), although the level of expression of this lytic molecule was not enhanced by specific acute in vitro antigenic stimulation. Overall, these data suggest that activated CD8⁺ T cells within the draining MLN are fully capable of expressing effector activity upon contact with Ag.

CD8⁺ T cell activation status and Ag availability in the MLN

The previously discussed results suggested that the proliferating CD8⁺ T cells in the draining MLN were the primary source of fully differentiated effector CD8⁺ T cells which emigrated from the lymph nodes and localized to the infected lungs and spleen.

Supporting this concept, we found that the expression of most activation and adhesion/homing markers was comparable for lung and spleen homing effector CD8⁺ T cells (Fig. 2). There was, however, one notable exception. CD69, a marker of early T cell activation/acute TCR engagement, was expressed on the majority of CD8⁺ T cells detected in the infected lungs on day 5 of infection (Fig. 2C). This finding is consistent with the concept that exposure of these effector T cells to viral Ag in the infected lungs would result in TCR engagement and acute up-regulation of CD69 on these T cells. By contrast, at day 5 of infection the vast majority of CFSE^{low} (greater than six cell divisions) CD8⁺ T cells present in the spleen did not express CD69 (Fig. 2B).

Analysis of CD69 expression on donor CD8⁺ T cells in the MLN at days 4–5 of infection revealed a much more complex pattern (Fig. 2A). At this site, high CD69 expression was evident on the undivided (CFSE^{high}) donor T cell subpopulation, but expression fell progressively over successive cell divisions (day 4). Cells that had undergone greater than five divisions (day 5) demonstrated the CD69⁻ phenotype comparable to effector CD8⁺ T cells in the spleen where viral Ag appears to be limited early after infection (Fig. 2, A and B). CD69 expression on T cells is linked to TCR engagement by Ag (31–35). In the current circumstance, CD69 levels fell progressively with succeeding cell divisions, suggesting that the majority of activated CD8⁺ T cells were no longer accessible to a reservoir of viral Ag still accessible to the undivided (CFSE^{high}) CD8⁺ T cells present in the MLN at this time.

To more directly establish a link between CD69 expression and specific antigenic stimulation, we conducted adoptive transfers of naive donor CD8⁺ T cells into control mice subsequently infected i.n. with an antigenically distinct (H3N2) influenza A/HK/1/68 virus whose HA is not recognized by the TCR transgenic clone 4 T cells. At day 4 postinfection, lymphoid tissues were harvested and donor CD8⁺ T cells resident in these sites were examined for expression of CD69, CD62L, and CD25. As Table I demonstrates, donor CD8⁺ T cells harvested from the MLN, spleen, and NDLN exhibited a naive CD69⁻ phenotype shared by donor clone 4 CD8⁺ T cells harvested from uninfected recipient mice. Thus, the inflammatory stimulus produced by infection with a control influenza virus strain did not result in nonspecific CD69 expression on the CD8⁺ T cell population.

To further establish a direct link between CD69 up-regulation and TCR exposure to Ag, we examined the impact of short-term ex vivo stimulation with specific Ag on the expression of CD69 by transferred CD8⁺ T cells. To this end, we harvested MLN and spleens from recipients of CFSE-labeled CD8⁺ T cells 5 days after

Table I. Activation marker expression by CD8⁺ T cells transferred into control mice^a

	Percentage of Positive Cells		
	CD69 ⁺	CD62L ^{high}	CD25 ⁺
Uninfected			
Spleen	5	91	0
MLN	6	85	4
NDLN	4	90	1
A/HK/1/68			
Spleen	4	85	1
MLN	11	94	6
NDLN	6	67	1

^a A total of 8.5×10^6 naive CFSE-labeled clone 4 CD8⁺ T cells were transferred i.v. into Thy-1.1^{+/+}-BALB/c mice. Cells were analyzed from an uninfected recipient 1 day after transfer and from a control influenza A/HK/1/68 (H3N2) virus-infected recipient 4 days after infection. The percentages of Thy-1.2⁺ CD8⁺ T cells positive for the various activation markers are shown.

i.n. infection with PR/8 virus, when proliferating TCR transgenic donor CD8⁺ T cells in both the MLN and spleen exhibited a CD69⁻ phenotype. Cell suspensions from these tissues were subjected to brief in vitro culture in the presence or absence of the cognate HA_{533–541} peptide and then analyzed for CD69 expression. In the absence of antigenic stimulation (Fig. 4), donor CD8⁺ T cells isolated from either site retained the CD69⁻ phenotype. After a 6 h exposure to the cognate peptide, CD69 was rapidly expressed on the proliferating (CFSE^{low}) donor T cell populations in both the MLN and the spleen (Fig. 4). We have additionally observed CD69 re-expression by these cells as rapidly as 3 h after stimulation, and also when using PR/8-infected P815 mastocytoma cells as the specific stimulus (data not shown). Thus, these activated proliferating CD69⁻CD8⁺ T cells in the MLN were capable of up-regulating CD69 expression once Ag was accessible and the T cell Ag receptor was appropriately engaged. In this regard, it is also noteworthy that the undivided (CFSE^{high}) cell population in the spleen could also up-regulate CD69 in response to acute antigenic stimulation (Fig. 4).

Viral Ag persistence in the draining MLN

Because in vitro Ag exposure of the CD69⁻, CFSE^{moderate/low} T cells in the MLN resulted in the rapid expression of this early T cell activation marker, it seems likely that at days 4–5 of infection, these T cells were not in direct contact with Ag in vivo. However, as indicated above (Fig. 2A), we noted a direct correlation between CFSE content and the level of CD69 expression during this time period. This suggested that undivided T cells and T cells early in their proliferative cycle (fewer than three to four divisions) may still have been in contact with Ag still present in the MLN at day 5 of infection.

To determine the presence and extent of persistence of viral Ag in the draining MLN, we prepared MLN cell suspensions from Thy-1.1^{+/+}-BALB/c mice at different days after i.n. PR/8 virus infection. These cells were cocultured with purified naive CFSE-labeled clone 4 CD8⁺ T cells for 3 days, and T cell proliferation was determined by dye dilution. Controls for this analysis included

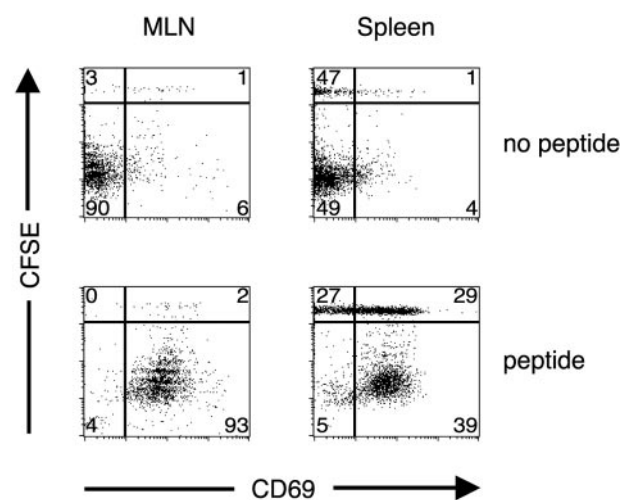


FIGURE 4. CD69 induction on day 5 MLN and splenic donor T cells by in vitro peptide stimulation. Using the same experimental transfer model described in Fig. 1, individual cell suspensions were prepared from the MLN and spleens of mice after 5 days of infection. Cultures were either stimulated with 50 nM HA_{533–541} peptide or incubated in medium for 6 h. Dot plots shown are gated on Thy-1.2⁺ CD8⁺ cells and analyzed for CFSE fluorescence and CD69 expression. The percentage of gated cells in a given quadrant is shown in the corner of each respective quadrant.

the culture of labeled naive T cells alone or with MLN cell suspensions from mice infected with the antigenically distinct influenza A/HK/1/68 virus strain. As Fig. 5A demonstrates, day 5 postinfection MLN cell suspensions from PR/8-infected mice were able to support proliferation of naive CD8⁺ T cells. Viral Ag persistence in the draining lymph nodes appeared to be of limited duration, as the proliferative response of the labeled CD8⁺ T cells rapidly declined to almost background levels when cocultured with whole MLN cell suspensions harvested at successive days up to day 12 of infection (Fig. 5A). The Ag specificity of the naive T cell proliferative response was indicated by the absence of significant proliferation by naive-labeled CD8⁺ T cells cultured alone or with MLN cell suspensions from lymph node donors infected with the control influenza A/HK/1/68 virus strain (Fig. 5A).

The TCR transgenic clone 4 CD8⁺ T cells recognize processed viral HA in association with the H-2K^d molecule (21). When naive clone 4 CD8⁺ T cells were cocultured with day 4 MLN cells from PR/8 virus infected mice in the presence of a blocking anti-K^d Ab, the proliferative response of the T cells was abolished (Fig. 5B). This finding reinforces the specificity of this response, and coupled with the previously discussed data, suggests that the recognition of specific viral Ag-MHC class I complexes by TCR is required to stimulate T cell proliferation. These results strongly suggest that some viral Ag was present in the draining MLN for at least 5 days of infection.

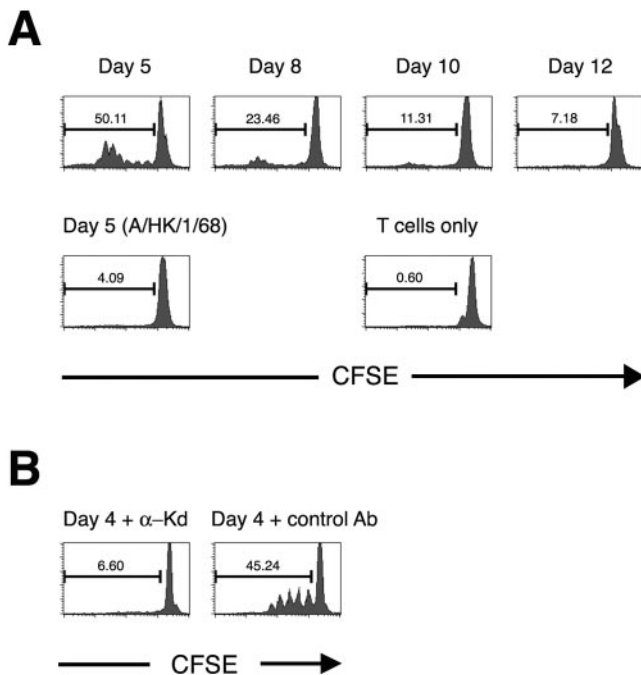


FIGURE 5. Persistence of viral Ag in MLN several days after influenza virus infection. Naive Thy-1.1^{+/+}-BALB/c mice were i.n. infected with A/PR/8/34 virus. At the indicated number of days after infection, individual cell suspensions of MLN were prepared and cultured with naive CFSE-labeled clone 4 CD8⁺ T cells. *A*, CFSE histograms are gated on Thy-1.2⁺ CD8⁺ cells. The percentage of divided cells in each culture is indicated within the respective histogram. Unstimulated naive CFSE-labeled clone 4 CD8⁺ T cells are shown as a reference for the CFSE intensity in undivided cells. Influenza virus A/HK/1/68 (H3N2) infected day 5 MLN cells were also used as a control for Ag-specific proliferation. *B*, Day 4 MLN cells from A/PR/8/34 virus-infected Thy-1.1^{+/+}-BALB/c mice were used to stimulate naive CFSE-labeled clone 4 CD8⁺ T cells in the presence or absence of H-2K^d blocking Ab.

Discussion

By adoptively transferring TCR transgenic CD8⁺ T cells into Thy-1 congenic recipient mice, we were able to track the Ag-specific response to pulmonary influenza virus infection for a homogeneous population of naive CD8⁺ T cells. This study reveals several important events that occur during the development of the CD8⁺ T cell response to influenza virus. Rapid T cell activation occurs exclusively in the draining MLN. By 2 days after infection, Ag-specific CD8⁺ T cells have undergone important phenotypic changes with respect to activation and adhesion molecule expression in the MLN, i.e., up-regulation of CD69 and loss of CD62L. Subsequently, upon activation the T cells differentiate into effector molecule producing cells and concomitantly rapidly undergo several divisions in the MLN. However, despite the presence of viral Ag in the MLN for several days following infection, dividing CD8⁺ T cells do not appear to be in constant contact with Ag after one to two divisions, suggesting sequestration of Ag from proliferating cells. The activated T cells then exit the MLN and traffic to the spleen and peripheral NDLN, whereas the most highly activated and divided population of cells traffic to the lung. These activated, effector competent T cells are thus primed to rapidly respond to local viral Ag in the lung and thereby control the virus infection.

After adoptive transfer of the TCR transgenic CD8⁺ T cells, donor cells were located throughout the lymphoid tissues of recipient mice (Fig. 1), as was expected for naive CD62L⁺ T cells (50). Donor T cells located in the MLN demonstrated activation by day 2 postinfection, as CD69 was rapidly up-regulated on donor T cells before their division within the MLN (Fig. 2A). CD25 expression and CD62L shedding was also rapidly induced on activating CD8⁺ T cells before their division in the MLN (Fig. 2A). Donor T cells divided rapidly between days 3–4 after infection exclusively in the MLN. This exclusive proliferation of T cells in the MLN early after infection correlates with the preferential accumulation of migrating airway DC to the draining regional lymph nodes during influenza virus infection, which occurs within 24 h after i.n. inoculation (16). Proliferation of naive clone 4 T cells in vitro is first evident 2 days after stimulation by cognate Ag (data not shown), suggesting naive T cells require 2 days for the cell division machinery to be fully activated to complete a cell division. Induction of T cell division in vivo was relatively delayed, however, due to the time required for airway DC to both deliver viral Ag from the lungs and initiate contact with T cells in the draining lymph nodes. As previously reported for TCR transgenic CD4⁺ T cells responding in vivo to influenza virus infection (20), division of the T cells in the MLN was asynchronous. Hence, we were able to detect T cells over a range of divisions at days 3–4 of infection by the laddering of CFSE intensities. We have preliminary evidence suggesting that the asynchronous T cell proliferation may reflect the continual asynchronous recruitment of circulating naive T cells into the MLN (H. Yoon and T.J. Braciale, unpublished observations). As such, T cells with fewer divisions likely migrated into the MLN slightly later and therefore activated more recently than earlier immigrant T cells in the MLN that have undergone several additional divisions.

In contrast to the proliferation of T cells in the MLN, there was no evidence of division of resident CD8⁺ T cells in any other tissues examined up to the fourth day after infection. We detected a small number of transferred, nondividing donor T cells resident in the lung over the first 4 days after infection (Fig. 1A). These resident lung CD8⁺ T cells were not stimulated to divide in situ during infection of the lung and remained CFSE^{high} despite extensive virus replication in the lungs (data not shown). This suggests

that the environment of the infected lung is unsuitable for naive T cell activation. This finding may be related to a variety of factors, including DC maturation state, site(s) of naive T cell localization, and local cytokine milieu in the infected lungs. Only after the activation and multiple divisions of cells in the MLN did we detect significant numbers of donor T cells accumulating in a synchronous manner in the lung by day 5 postinfection.

Dividing CD8⁺ T cells were also found to leave the MLN and not only populate the lungs, but also the spleen and peripheral lymph nodes. The activated CD8⁺ T cells that populated the spleen at day 5 of infection differed in cell surface phenotype from the migrating T cells trafficking to the lungs at day 5 of infection. In particular, CD69 was not expressed on the majority of migrant CD8⁺ T cells detected in the spleen at day 5, whereas the majority of corresponding T cells in the lungs were CD69⁺. Acute CD69 up-regulation on T cells is linked to TCR engagement by Ag (Refs. 30–34 and Fig. 4). When antigenic stimulation is withdrawn, CD69 expression subsequently declines (31, 35). It is therefore likely that the absence of TCR stimulation by Ag in the spleen (vs the presence of viral Ag in the infected day 5 lungs) accounts for the difference in CD69 expression between activated CD8⁺ T cells migrating to these sites.

We observed that the absolute number of responding CD8⁺ T cells in the lungs continued to increase up to day 7 of infection, whereas the division driven expansion of donor CD8⁺ T cells in the draining MLN and the accumulation of CFSE^{low} migrant CD8⁺ T cells in the spleen leveled off between days 5–7 of infection. This continued accumulation of activated CD8⁺ T cells in the lungs likely reflects the ongoing recruitment of activated T cells from the MLN to the infected lungs. We cannot, however, readily explain the absence of a similar continued accumulation on MLN-derived T cells in the spleen. It is possible that activated CD8⁺ T cells continue to traffic to the spleen, but in the absence of continued antigenic stimulation at this site, activated T cells in the spleen die from neglect at a rate comparable to the rate of accumulation. An alternate, perhaps more intriguing explanation for the selective continued accumulation of these activated T cells in the lungs is that the CD8⁺ T cells undergo one or more rounds of division (perhaps in response to viral Ag) after migration to the infected lungs. The progressive loss of CFSE intensity of donor Thy-1.2⁺ CD8⁺ T cells in the lungs between days 5 and 7 (Fig. 1A) is consistent with this interpretation.

Our findings on the response of transgenic CD8⁺ T cells to pulmonary influenza virus infection directly parallel results reported on the primary CD4⁺ T cell response to influenza with respect to the tempo of activation and tissue distribution of naive and effector cells (20). However, in contrast to the reported results for influenza virus-specific transgenic CD4⁺ T cells (20), in which direct proliferative expansion of resident T cells in the spleen in response to infection was suggested, we have been able to identify the draining MLN as the likely primary or at least initial cellular source of activated CD8⁺ T cells in the spleen. We have also similarly demonstrated the early dissemination of acute effectors from the draining lymph nodes into multiple NDLN. We believe the CD8⁺ T cells migrating from the MLN likely also contribute substantially to the virus-specific populations detected in peripheral lymph nodes much later after influenza virus infection (11). The dissemination of such activated T cells throughout peripheral lymphoid tissue could ultimately contribute to the pool of central memory CD8⁺ T cells generated in response to primary pulmonary influenza virus infection.

Important CD8⁺ T cell effector functions in response to viral infections include the secretion of cytokines and proapoptotic granzymes (2, 51). To determine whether acquisition of these ef-

factor functions was initiated before T cell migration to the lung, we examined the effector status of CD8⁺ T cells in the draining MLN of virus-infected mice. Our results indicate that CD8⁺ T cells rapidly acquire the ability to produce cytokines and granzyme B during their activation in the MLN (Fig. 3). We coupled our protein analyses with CFSE labeling to further explore the relationship between effector molecule expression and cell division. Granzyme B production was very tightly associated with cell division (Fig. 3). T cells initiated granzyme B expression after the first division, with increasing levels of the effector protease made following each successive division. In addition, granzyme B production was evident without *in vitro* stimulation, suggesting that accumulation of the cytolytic enzyme (i.e., within granules) is part of a developmental program following T cell activation. Several previous studies have suggested that cytokine expression depends on division (52–55). Indeed, after *in vitro* stimulation with specific peptide, T cells that had divided produced high levels of both IFN- γ and TNF- α (Fig. 3), with similar frequencies of divided T cells producing either cytokine (80–81%) or granzyme B (75%). However, we detected a significant fraction of undivided T cells that also produced both cytokines after *in vitro* peptide stimulation, consistent with reports of cytokine production by activated T cells before the onset of cell division (55–58). It remains possible that such cytokine producing cells have entered the cell cycle but not yet completed a cell division.

The expression dynamics of CD69 in the MLN provided a dramatic example of the environmental changes that CD8⁺ T cells experienced during their divisions there. CD69 up-regulation was detected on a fraction (14%) of donor CFSE^{high} CD8⁺ T cells in the MLN at day 2 of infection, with the majority of CFSE^{high} cells expressing CD69 on day 3 before cell division (Fig. 2A). With the onset of cell division between day 3 and day 4, CD69 expression was retained at high levels on at least 50% of undivided CFSE^{high} cells and was likewise high on early dividing cells (one to two divisions). At the same time, however, cells that had undergone three or more divisions demonstrated a gradient of decreasing CD69 expression with increasing cell division. This culminated at day 5, when the majority of CD8⁺ T cells that had undergone six or more divisions were CD69^{low/-}. Nevertheless, the few remaining cells that had undergone fewer than six divisions displayed the inverse relationship between the number of cell divisions and CD69 intensity, i.e., a direct correlation between CFSE and CD69 intensities. Because the CFSE^{low}CD69⁻ T cells in the MLN at days 4 and 5 rapidly up-regulated CD69 in response to *in vitro* antigenic stimulation (Fig. 4), these proliferating T cells were not refractory to Ag-induced CD69 expression. This observation suggests that these activated T cells were proliferating in the absence of continued exposure to Ag. Consistent with this view, a number of recent reports have demonstrated the capacity of CD8⁺ T cells to undergo multiple rounds of division in an Ag-independent manner (59–61).

We also detected the presence of viral Ag for >5 days in the draining MLN following virus infection (Fig. 5). This finding of Ag persistence in the MLN reveals an interesting dichotomy. The CD69^{low/-} phenotype of the multiply divided T cells in the MLN at days 4 and 5 implies that these CD8⁺ T cells are not exposed to Ag. Yet, because undivided and early division CD8⁺ T cells display a CD69^{high} phenotype, these T cells are most likely exposed to viral Ag in the MLN. To explain this dichotomy and account for the CD69^{low/-} phenotype of the CD8⁺ T cells undergoing Ag-independent proliferation, we suggest that after one to two divisions, activated CD8⁺ T cells are no longer in physical contact with viral Ag-presenting APC. A recent report (62) investigating the anatomy of Ag presentation to T cells within lymph nodes

supports this concept. In that model, Ag-bearing DC in protein-immunized mice were found to be sequestered in a specific area of the draining lymph nodes. These DC were preferentially localized near high endothelial venules in the outer paracortex, which placed Ag presentation immediately proximal to the T cell entry site for the lymph node. T cells were later found scattered deeper in the Ag-free paracortex at a time when T cells had undergone several divisions. Such a mechanism of Ag partitioning away from proliferating T cells after one to two divisions may occur within the draining lymph nodes of influenza virus-infected mice and account for the difference in CD69 expression between CFSE^{high} and CFSE^{low} donor CD8⁺ T cells.

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