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Sequential Activation of CD8+ T Cells in the Draining Lymph Nodes in Response to Pulmonary Virus Infection

Heesik Yoon,* Kevin L. Legge,§‡ Sun-sang J. Sung,§ and Thomas J. Braciale2*††

We have used a TCR-transgenic CD8+ T cell adoptive transfer model to examine the tempo of T cell activation and proliferation in the draining lymph nodes (DLN) in response to respiratory virus infection. The T cell response in the DLN differed for mice infected with different type A influenza strains with the onset of T cell activation/proliferation to the A/JAPAN virus infection preceding the A/PR8 response by 12–24 h. This difference in T cell activation/proliferation correlated with the tempo of accelerated respiratory DC (RDC) migration from the infected lungs to the DLN in response to influenza virus infection, with the migrant RDC responding to the A/JAPAN infection exhibiting a more rapid accumulation in the lymph nodes (i.e., peak migration for A/JAPAN at 18 h, A/PR8 at 24–36 h). Furthermore, in vivo administration of blocking anti-CD62L Ab at various time points before/after infection revealed that the virus-specific CD8+ T cells entered the DLN and activated in a sequential “conveyor belt”-like fashion. These results indicate that the tempo of CD8+ T cell activation/proliferation after viral infection is dependent on the tempo of RDC migration to the DLN and that T cell activation occurs in an ordered sequential fashion. The Journal of Immunology, 2007, 179: 391–399.

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aive T cells continually recirculate through the blood and lymph and, during recirculation, they enter the secondary lymphoid organs (SLO)§ (e.g., lymph nodes (LN), Peyer’s patches, and spleen) searching for cognate Ag delivered and displayed in the SLO by professional APC (e.g., dendritic cells (DC)) (1). Naive T cells enter the LN across high endothelial venules (HEV) by multistep adhesion cascades and, whether T cells encounter specific Ag there, these T cells activate/proliferate in the T cell-enriched LN (2, 3). This T cell/APC encounter is believed to occur primarily in the LN paracortex (where HEV are enriched). Intravital LN microscopy studies suggest that, upon entering the LN draining the peripheral sites of Ag deposition/infection (via the afferent lymphatics), the Ag-bearing DC migrate to and localize in the vicinity of the HEV (4, 5). This HEV localization event facilitates the APC encounter with naive Ag-specific T cells entering the LN from the blood. Once activated in the LN cortex, the responding T cells proliferate over several days, and they are thought to then migrate to the LN medulla and egress from the LN (via the efferent lymphatics) (5–7). Important information on the in vivo activation/proliferation tempo of naive T cells has come from studies using the adoptive transfer of T cells labeled with dilution-sensitive dyes (e.g., CFSE) into recipient mice subsequently challenged with Ag (5, 6, 8). Such studies have typically revealed evidence of extensive proliferation of transferred T cells in the SLO after Ag contact with considerable heterogeneity in the apparent tempo of T cell proliferation in situ (9–11).

The murine response to type A influenza infection has been extensively used as a model for the analysis of the requirements for CD8+ T cell induction in vivo (e.g., Refs. 12 and 13), the role of CD8+ T cells in virus clearance, and T cell effector mechanisms used in recovery from infection (14–16). More recently, this model has been used in the analysis of the response of respiratory DC (RDC) to pulmonary infection and of the role of RDC in CD8+ T cell induction in the draining LN (DLN) following intranasal infection (17). By using TCR-transgenic (Tg) mouse lines whose CD8+ (or CD4+) T cells express TCR directed to type A influenza epitopes, we (10, 18, 19) and others (20–23) have adapted the strategy of adoptively transferring naive TCR-Tg T cells into influenza-infected recipients to evaluate early events in the naive T cell response to Ag during natural infection. In this current report, we have used this strategy to analyze the in vivo CD8+ T cell response to murine infection with two different influenza strains. We demonstrate that the tempo of naive T cell activation/proliferation correlates with the tempo of RDC migration (from the respiratory tract to the DLN), and we demonstrate that the heterogeneity in the T cell proliferative response in the DLN reflects the sequential immigration of circulating naive Ag-specific CD8+ T cells into the LN followed by their subsequent activation/proliferation there.

Materials and Methods

Mice

The Clone-4 (CL-4) Tg mice and Thy1.1+ BALB/c mice were provided originally by Dr. R. W. Dutton (Trudeau Institute, Saranac Lake, NY) and Dr. R. I. Enelow (Yale University, New Haven, CT), respectively, and maintained under a pathogen-free environment.

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1 This work was supported by U.S. Public Health Service Grants AI-15608, HL-33391, and AI-37293 (to T.J.B.) and HL-70065 (to S.J.S.). K.L.L. was supported by 3 Abbreviations used in this paper: SLO, secondary lymphoid organ; DC, dendritic cell; LN, lymph node; HEV, high endothelial venule; RDC, respiratory DC; DLN, draining LN; Tg, transgenic; RT, room temperature; LYYVE-1, lymphatic endothelium hyaluronic acid receptor 1; i.n., intranasally; HA, hemagglutinin; p.i., postinfection; NDLN, nondraining LN; CL-4, Clone-4.
Virus and viral peptides

The preparation of influenza virus A/PR8/34 (H1N1) and mouse-adapted influenza virus A/JAPAN/305/57 (H2N2) were described elsewhere (10). Synthetic peptide HA518–526 of A/JAPAN (1YTVAGSL) and HA533–541 of A/PR8 (IYSTVASSL) were synthesized by the University of Virginia Biomolecular Research Facility. Their purity was confirmed by HPLC.

Adaptive transfer of clone 4 CD8+ T cells

Naïve CD8+ T cells were purified from the spleen by positive magnetic bead selection (MACS; Miltenyi Biotec) as described earlier (18). More than 90% purified CD8+ T cells were then washed with serum-free DMEM and labeled with 5 μM CFSE (Molecular Probes) for 10 min at RT. After extensive washing, 10^7 labeled cells were transferred into Thy1.1+ BALB/c mice. Mice were rested at least for 24 h, then intranasally infected with a 0.1LD50 of influenza virus. CFSE-labeled CL-4 T cells were also used in in vitro cultures.

In vitro culture

2 x 10^6 of purified and CFSE-labeled CL-4 CD8+ T cells were incubated with 2 x 10^4 of peptide-loaded or virus-infected Thy1.1+ BALB/c spleenocytes in IMEM supplemented with 10% FBS, 10 U/ml penicillin G, 10 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 0.05% 2-ME without IL-2. Spleenocytes were pulsed with 100 nM peptides for 1 h at RT or infected with either 1000 TCID50 of influenza A/PR8 or 0.003 TCID50 of A/JAPAN. Free unbound peptides or viruses were washed away before adding the splenocytes to the coculture.

Virus titers

Virus titers were determined as described previously (24). Briefly, homogenated lung suspensions of influenza virus-infected mice were plated on Mardin-Darby canine kidney cells in flat-bottom 96-well plates with multiple dilutions. After 3 days of incubation in a humidified 7% CO2 incubator at 37°C, the supernatant of each well was mixed with fresh 1% chicken erythrocytes. Hemagglutination results were analyzed after 1 h of incubation at RT and TCID50 was calculated.

Detection of migrating RDC

Migrating RDC in the respiratory DLN were enumerated as previously described (17). Briefly, 50 μl of a 8 nM CFSE stock was intranasally administered to mice to label RDC and, at 6 h after CFSE labeling, the mice were then infected with influenza virus. At specified time points after viral infection, the DLN were harvested and the numbers of CD11c+ and CFSE+ cells were counted by flow cytometry.

Preparation of tissue lymphocytes and flow cytometry

At the time of collection, the LN or lungs were removed and placed in ice-cold FACS staining buffer (PBS with 2% FBS and 0.02% Na3VO4). LN or lung tissues were then subsequently disrupted and passed through a cell strainer (70 μm; BD Falcon). Approximately 10^7 cells were incubated with anti-CD16/32 (2.4G2) to block nonspecific FcR binding, stained with anti-CD8a (53-6.7), anti-Thy1.2 (53-2.1), and anti-CD69 (H1.2F3; BD Pharmingen) and analyzed with FlowJo software (Tree Star).

Blocking entry of T cells into LN by MEL-14 Abs

Anti-CD62L mAb (MEL-14) was produced and purified by the University of Virginia Lymphocyte Culture Center. To block further entry of T cells into LN, 100 μg of whole IgG2a molecules was injected i.v. into the tail vein of CFSE+CD8+ CL-4 transferred Thy1.1+ BALB/c mice at various times before or after influenza virus infection. The persistence, specificity, and duration of Ab binding to CD8+ CL-4 T cells were confirmed until 4 days after administration by flow cytometry (data not shown).

Enrichment of CL-4 T cells for flow cytometry

CL-4 T cells of low frequency were enriched by pull-down assay (25). Briefly, cells from homogenated and pooled LN were incubated with anti-CD90.2 magnetic beads (Miltenyi Biotec) for 15 min. After purification, CL-4 T cells of low frequency were enriched by pull-down assay (25).

Immunohistochemistry of LN

LN sections were blocked with 2.4G2 anti-Fc-RII/RIII mAb and serum and stained with Alexa Fluor 555- or Alexa Fluor 647-conjugated anti-lineage mAb (Caltag Laboratories), anti-Thy1.2 mAb, anti-HEV mAb MEC-79 (BD Pharmingen), or rabbit anti-lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1) Ab (AngioBio). Anti-Thy1.2, MEC-79, and LYVE-1 were conjugated to the Alexa Flours by mAb labeling kits according to the supplier’s (Molecular Probes) protocol. Three-color confocal microscopy was performed on a Zeiss LSM 510 assembly and the data were analyzed using the manufacturer’s Image Browser software.

Results

Kinetics of the in vivo CD8+ T cell response to influenza virus infection in the DLN

We examined the activation kinetics of naïve TCR-Tg CD8+ T cells in vivo in response to intranasal (i.n.) influenza virus infection. In a CD8+ T cell/adoptive transfer model (10), CD8+ T cells were purified from the influenza hemagglutinin (HA)-specific TCR-Tg, CL-4 mouse line (10). These T cells recognize a site within the transmembrane domain of the A/PR8 (H1N1) HA corresponding to HA residues 533–541 (HA518–526) in the mature HA. The CL-4 T cells also recognize in a cross-reactive fashion, albeit less efficiently (R. M. Chu and T. J. Braciale, unpublished data), the corresponding HA amino acid sequence in the A/JAPAN (H2N2) HA (i.e., HA residues 529–537 which differs in two amino acids from the corresponding A/PR8 HA site). To examine the early phase of CD8+ T cell activation in vivo in response to infection, purified CL-4 T cells (Thy1.2+) were labeled in vitro with the dilution-sensitive dye CFSE before transfer into naïve Thy1.1+-congenic recipients. We routinely find that >98% of CL-4 T cells purified have a naïve phenotype; they have uniform expression of the surface markers ex vivo and do not produce effector cytokines at 24 h postinfection with cognate peptide in vitro (H. Yoon, unpublished data). Twenty-four hours later, recipient mice were infected i.n. with a sublethal dose of either A/PR8 or A/JAPAN virus. Over the ensuing 4–5 days, the spleen, lungs, peribronchial LN and mediastinal LN draining the respiratory tract and pooled nondonoring LN (NDLN) were excised from the infected recipient mice and examined for the localization, activation, and proliferation of the transferred CL-4 T cells. CL-4 T cell activation was assessed by the up-regulation of the early activation marker CD69, and T cell proliferation was evaluated by CFSE dye intensity. Fig. 1 shows the results of this analysis for the lung-draining peribronchial and mediastinal LN (DLN). CL-4 activation (i.e., CD69 up-regulation) was minimal in the DLN at day 1 postinfection (p.i.) with either virus (Fig. 1A) and comparable to CL-4 T cells transferred into uninfected recipients (Ref. 10 and data not shown). Over the next 24 h, up-regulation of CD69 was detected in the DLN of both A/PR8- and A/JAPAN-infected mice, with a larger fraction of CD69+ CL-4 T cells detected in the DLN of A/JAPAN-infected mice than in A/PR8-infected mice (i.e., ~50% vs ~25%, see Fig. 1A). Of particular note was the T cell proliferation tempo. In response to A/JAPAN infection, the CL-4 CD8+ T cells underwent extensive proliferation in the DLN between days 2 and 3 p.i., while in response to A/PR8, their proliferation was delayed by up to 24 h (i.e., onset between days 3 and 4 p.i.).

Also noteworthy is the finding that, after the onset of T cell division in the DLN (i.e., between days 2 and 3 for A/JAPAN infection and days 3 and 4 for A/PR8 infection), the activated CD8+ T cells had undergone up to five to six divisions in vivo over a 24-h period. This result suggests that the cell cycle time in vivo for the activated responding T cells may be extremely rapid. Of equal interest is the observation that CD69 expression levels on the responding CD8+ T cells decreases incrementally with each successive cell division (i.e., corresponding to the stepwise decrease in CFSE staining intensity). As previously reported (10), there is no significant activation/proliferation of naïve CL-4 T cells present.

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in the spleen, NDLN, or lungs of these infected mice at day 3 p.i. with A/JAPAN infection or at day 4 p.i. with A/PR8 infection (Fig. 1). Likewise, we do not detect any activation/proliferation of naive CL-4 T cells in the liver or bone marrow of A/PR8-infected mice and no evidence of activated/proliferating cells in the blood before day 4 of infection (H. Yoon, unpublished observations). These findings are consistent with the concept that the DLN are the primary site of CD8\(^{+}\) T cell activation in response to pulmonary type A influenza virus infection.

Consistent with recently published observations (26, 27), naive Tg CD8\(^{+}\) T cells can be transferred into infected recipients up to 5–6 days p.i. with A/PR8 virus and still undergo several rounds of proliferative expansion in the DLN, and low-level activation/proliferation of a small fraction of transferred Tg T cells can be seen in the spleen, NDLN, or lungs of these infected mice at day 3 p.i. with A/JAPAN infection or at day 4 p.i. with A/PR8 infection (Fig. 1B). Likewise, we do not detect any activation/proliferation of naive CL-4 T cells in the liver or bone marrow of A/PR8-infected mice and no evidence of activated/proliferating cells in the blood before day 4 of infection (H. Yoon, unpublished observations).

**FIGURE 1.** Tempo of naive CD8\(^{+}\) T cell activation in response to influenza virus infection. CL-4 TCR-Tg CD8\(^{+}\) T cells were isolated from the spleen/LN of donor mice, labeled with CFSE, then administered i.v. to recipient mice. The recipient mice were subsequently infected i.n. with either A/PR8 or A/JAPAN virus. A, Time course of CL-4 T cell activation (i.e., CD69 up-regulation) and proliferation (i.e., CFSE dye dilution) in the DLN at the indicated day p.i. B, CD69 expression and CFSE content for labeled CL-4 T cells in the spleen, lungs, and NDLN at day 4 p.i. (A/PR8) or day 3 p.i. (A/JAPAN). Data are representative of at least six independent experiments.

**FIGURE 2.** Response of CD8\(^{+}\) T cells to Ag-expressing APC in vitro. Purified CFSE dye-labeled CL-4 TCR-Tg CD8\(^{+}\) T cells were cocultured with A/PR8 peptide (PHA529)- or A/JAPAN peptide (JHA533)-pulsed splenocyte APC (A and B) or with A/PR8 or A/JAPAN virus-infected splenocyte APC (C and D). At the indicated times (days) after in vitro culture, the cultured CL-4 T cells were examined for activation marker (i.e., CD69) expression (A and C) and cell division/cell accumulation (i.e., CFSE dilution; B and D). Data are representative of four independent experiments.
observed when adoptive transfer is conducted up to 3–4 wk p.i. (C. W. Lawrence, H, Yoon, and T. J. Braciale, unpublished observations).

**Tempo of CD8\(^+\) T cell activation in vitro**

The differences in the kinetics of CD8\(^+\) T cell activation in vivo in response to infection with these two viruses could be due to a difference in the efficiency of processing and presentation of the respective cognate PR8 HA\(_{533-541}\), or cross-reactive JAPAN HA\(_{329-537}\) epitope by APC, or a difference in the efficiency of T cell recognition (i.e., TCR engagement) of these two epitopes by CL-4 T cells might also account for these results. To investigate these possibilities, we examined the tempo of CL-4 CD8\(^+\) T cell activation in vitro after stimulation with splenic APC that had been either pulsed with one of the synthetic peptides (PHA\(_{533-541}\) or JHA\(_{429-537}\)) or infected with virus (A/PR8 or A/JAPAN). As Fig. 2 demonstrates, naive CD8\(^+\) T cells stimulated with either synthetic HA peptide rapidly activated (i.e., up-regulated CD69, by day 1 of in vitro culture; Fig. 2A) and underwent significant proliferation (i.e., two to three cell divisions; Fig. 2B) by day 2 post-stimulation. CD69 up-regulation on the naive T cells exposed to preprocessed peptide in vitro was uniform, suggesting that Ag encounter and the initial activation of the T cells was relatively synchronous. Indeed, the majority of the cultured T cells had undergone two to three divisions by day 2 of in vitro culture (Fig. 2B). The activation/proliferative responses to the preprocessed peptide epitopes was comparable at the peptide dose used to pulse APC (100 nM). At lower peptide doses (down to 1 nM or 100 pM), the cross-reactive JHA\(_{329-537}\) peptide was recognized with decreased (100 nM). At lower peptide doses (down to 1 nM or 100 pM), the cross-reactive JHA\(_{329-537}\) peptide was recognized with decreased responsiveness to the A/JAPAN-infected APC was less efficient by day 3 of stimulation than with A/PR8-infected APC (Fig. 2D).

**Kinetics of virus replication in the lungs and migration of RDC in vivo**

The above in vitro analysis revealed minimal if any difference in presentation of the two influenza HA epitopes and recognition by the T cells. We and others (17, 28, 29) have previously suggested that RDC migrating from the respiratory tract to the DLN will drive RDC maturation and subsequent migration, it was of interest to examine the time course of virus replication/accumulation in lungs of mice after i.n. infection with A/JAPAN or A/PR8 and the tempo of migration of the RDC to the DLN in response to infection. Fig. 3A shows the results of the lung virus titer analysis. For each of these mouse-adapted virus strains, virus replication was rapid with A/JAPAN virus reaching steady-state virus titers by 24 h p.i. and A/PR8 virus reaching steady-state titers by 48 h p.i. For each infection, mice were inoculated with an i.n. dose of infectious virus corresponding to 0.1 LD\(_{50}\) units. However, the i.n. inoculum dose for the more virulent A/PR8 virus (10 PFU) was ~300-fold lower than that for the A/JAPAN virus (3.2 \times 10^3 PFU). The difference in the initial rate of virus accumulation in the infected lungs between the two virus strains and the time difference before steady-state virus titers are achieved likely reflects this difference in initial inoculum size.

When we examined the time course of RDC migration to the DLN in response to infection, a corresponding difference was observed (Fig. 3B). RDC undergo a transient accelerated migration to the DLN (for both A/JAPAN and A/PR8) during the first 48 h p.i. However, consistent with the difference in the accumulation of infectious virus in the lungs, RDC migrating in response to A/JAPAN infection reached peak accumulation in the DLN more rapidly than RDC responding to the A/PR8 virus.

![Figure 3](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
**CD8<sup>+</sup> T cells activate in a synchronous sequential manner in the DLN**

The more rapid onset of RDC migration to the DLN following i.n. infection with A/JAPAN virus paralleled the higher percentage of CD69<sup>+</sup> cells and the more rapid onset of cell division by the CD8<sup>+</sup> T cells responding in the DLN to this strain (Fig. 1A). This finding suggested a link between the time course of RDC migration to the DLN and the response of virus-specific CD8<sup>+</sup> T cells trafficking through this site. Consistent with this notion, we detected minimal CD69 up-regulation on CD8<sup>+</sup> T cells present in the DLN at 24 h p.i. The pooled DLN from three mice for each treatment group were excised and analyzed for activation marker expression (CD69 level) and proliferation (CFSE dilution) at 3.5 days p.i. Numbers above subpopulations in each dot plot represent the number of cell divisions that the responding T cells have undergone in the DLN. Insets are the corresponding histogram plots of cell numbers (y-axis) vs CFSE dilution (x-axis) for each group at the time of DLN harvest. Data are representative of the following number of independent experiments: A:10; B/C; 2; D; 3; and E, 4.

**CD8<sup>+</sup> T cell recirculation through the SLO (1, 2), is that circulating naive virus-specific CD8<sup>+</sup> T cells continually enter the DLN, and, after RDC deposition, successive cohorts of T cell entering the DLN encounter Ag, activate, and then initiate the steps in cell division (all in a sequential manner reminiscent of a conveyor belt).

Accordingly, the first T cells to enter the DLN after Ag deposition would also be the first to activate/proliferate; and these cells would undergo the greatest number of cell divisions (i.e., display the lowest CFSE intensity) in the DLN at the time of analysis. Consistent with this concept of circulating naive virus-specific T cells which subsequently enter the DLN would also activate/proliferate; but they would undergo fewer divisions at the time of DLN sampling than the first cohort that had arrived in the DLN.

This conveyor belt effect would result in a pattern of apparent asynchronous cell division observed in the DLN in vivo after the onset of cell division (e.g., Fig. 1A).

To test this conveyor belt hypothesis of sequential T cell activation in response to infection, we conducted a series of experiments to inhibit the migration of circulating CD8<sup>+</sup> T cells from the blood into the DLN through the HEV by blocking CD62L function before/after i.n. infection by i.v. administration of the monoclonal anti-CD62L Ab Mel-14 (30). In the first series of experiments, CFSE-labeled CL-4 T cells were transferred into Thy1.1<sup>+</sup> recipients; then these T cell recipients received a single i.v. dose of anti-CD62L Ab (or a control, no Ab), and the DLN of the infected mice were harvested on the indicated days p.i., then analyzed for cell division (CFSE dilution). Numbers within panels indicate the number of CL-4 cell divisions as reflected in CFSE dye intensity. Data for each time point represent values for pooled DLN from three recipients for Ab-treated mice and one recipient at each time point for untreated controls. Data are representative of six independent experiments for each treatment group/time. 5 × 10<sup>4</sup> purified CL-4 T cells were adoptively transferred into A/PR8-infected control mice (B) or infected mice receiving anti-CD62L Ab at 24 h p.i. (C). DLN were isolated and pooled from control mice (n = 10) or Ab-treated mice (n = 7) at day 4 p.i. Donor cells were enriched for Thy1.2<sup>+</sup> cells by pull-down assay for flow cytometry.

**FIGURE 4.** Effect of anti-CD62L administration on CD8<sup>+</sup> T cell proliferation in the DLN. Naive CFSE-labeled CL-4 TCR-Tg CD8<sup>+</sup> T cells were transferred into recipient mice 24 h before i.n. infection with A/PR8 virus. Mice received no Ab (A) or a single i.v. dose of anti-CD62L mAb at the following times p.i.; B, at 24 h before infection (~24 h); C, at the time of infection (0 h); D, at 36 h; or E, at 60 h p.i. The pooled DLN from three mice for each treatment group were excised and analyzed for activation marker expression (CD69 level) and proliferation (CFSE dilution) at 3.5 days p.i. Numbers above subpopulations in each dot plot represent the number of cell divisions that the responding T cells have undergone in the DLN. Insets are the corresponding histogram plots of cell numbers (y-axis) vs CFSE dilution (x-axis) for each group at the time of DLN harvest. Data are representative of the following number of independent experiments: A:10; B/C; 2; D; 3; and E, 4.

**FIGURE 5.** Time course of CL-4 T cell proliferation in the DLN after anti-CD62L Ab administration. Naive CFSE-labeled CL-4 TCR-Tg CD8<sup>+</sup> T cells (10<sup>7</sup>) were transferred into recipient mice 24 h before infection with a sublethal i.n. dose of A/PR8 virus (A). At 24 h p.i., mice received a single i.v. dose of anti-CD62L Ab (or a control, no Ab), and the DLN of the infected mice were harvested on the indicated days p.i., then analyzed for cell division (CFSE dilution). Numbers within panels indicate the number of CL-4 cell divisions as reflected in CFSE dye intensity. Data for each time point represent values for pooled DLN from three recipients for Ab-treated mice and one recipient at each time point for untreated controls. Data are representative of six independent experiments for each treatment group/time: 5 × 10<sup>4</sup> purified CL-4 T cells were adoptively transferred into A/PR8-infected control mice (B) or infected mice receiving anti-CD62L Ab at 24 h p.i. (C). DLN were isolated and pooled from control mice (n = 10) or Ab-treated mice (n = 7) at day 4 p.i. Donor cells were enriched for Thy1.2<sup>+</sup> cells by pull-down assay for flow cytometry.
analyzed for CL-4 T cell localization/proliferation. Spleens and NDLN of the recipients were excised, sectioned, and subjected to immunofluorescence microscopy at day 1 (A) or day 4 (B–E) after i.n. A/PR8 infection. Day 4 DLN donors were untreated (B and C) or treated with a single i.v. dose of anti-CD62L Ab (D and E) at 24 h p.i. Day 1 infected LN shows transferred CFSE "high" CL-4 T cells with CFSE (green) and Thy1.2 (blue) colocalization and location of HEV, stained with MECA-79 (red). B, Staining of LN lymphatic endothelium (turquoise, short arrow) and HEV (red) of day 4 untreated DLN with DLN cortex (cortex identified). C, Distribution of CFSE "low" CL-4 T cells (blue) in the LN of day 4 p.i. control mice. D, Staining of lymphatic endothelium (turquoise) and HEV (red) in DLN of day 4 p.i. anti-CD62L-treated mice. Medullary sinuses are identified by MS. E, Localization of CFSE "low" Thy1.2 CL-4 cells (blue) to the medullary cords of DLN of day 4 p.i. anti-CD62L-treated mice.

As expected, administration of blocking anti-CD62L Ab at −24 h p.i. (Fig. 4B) or 0 h p.i. (Fig. 4C) resulted in almost complete depletion of transferred CL-4 T cells from the DLN with no evidence of significant CL-4 cell proliferation (CFSE dye dilution). The NDLN were likewise depleted of CL-4 T cells; but the transferred T cells were enriched in the spleen (data not shown), consistent with the reported CD62L-independent trafficking of circulating naive T cells to the murine spleen (30). In contrast, Ab administration at 60 h p.i. (before the onset of CL-4 T cell proliferation in the DLN to A/PR8 virus but after CD69 up-regulation on the T cells in the DLN) had minimal effect on the proliferative response of the transferred T cells to infection with at least five CL-4 T cell divisions detected in the DLN (Fig. 4E), a value comparable to untreated control CL-4 recipients infected (Fig. 4A). Ab administration at 36 h p.i. (when at least a portion of the circulating CL-4 T cells had entered the DLN and encountered Ag) resulted in a distinctly different CL-4 T cell proliferation profile (CFSE dilution). Inhibition of further naive CL-4 T cell migration into the DLN at 36 h p.i. produced a population of responding T cells displaying a restricted range of CFSE dye intensity, suggesting that the majority of T cells had undergone three to five divisions at the time of analysis (Fig. 4D). This finding was consistent with the hypothesis that a limited number of naive CL-4 T cells had sequentially entered the DLN and activated/proliferated in response to an Ag in a sequential manner. In this connection, it should be noted that identical results were obtained when F(ab')2 of the Mel-14 Ab were administered in vivo, indicating that the effect of the Ab was not due to Ab Fc-dependent sequestration of the transferred CL-4 T cells (data not shown).

The above results suggested that, if any additional migration of circulating CL-4 T cells into the DLN were inhibited after the first cohort of T cells had entered the DLN in response to viral infection, then this initial cohort of T cells would proliferate in a synchronous manner, i.e., this early cohort of CL-4 T cells would respond as a single population displaying a single-cell division number (i.e., uniform CFSE dye dilution intensity) at the time of
DLN sampling. To examine this possibility, CL-4 T cell recipients received blocking Mel-14 Ab at 24 h.p.i. with A/PR8 virus, and the DLN of both Ab recipients and control (untreated) animals were analyzed at days 3–5 p.i. As Fig. 5A demonstrates, the single cohort of T cells that entered the DLN by 24 h.p.i. (i.e., after RDC migration from the lungs to DLN had been initiated) exhibited a remarkably restricted CFSE dye intensity which is consistent with the synchronous division of this first naive T cell cohort. Furthermore, at day 4 p.i., the T cells from the Ab-treated recipients had undergone five divisions (based on CFSE dye intensity), approximately the same number of divisions as the cells that had undergone the most extensive division (lowest dye intensity) in the untreated control CL-4 recipients (Fig. 5A). This finding suggests that the T cells in the control DLN which had undergone five rounds of division at day 4 p.i. represented the first cohort of naive CL-4 cells to respond to viral infection in the DLN.

In the above analysis, 10^7 CFSE-labeled TCR-Tg CL-4 T cells were adoptively transferred into recipient mice to allow for ready isolation of sufficient numbers of the responding T cells in the DLN of infected animals for subsequent analysis, particularly after Mel-14 Ab treatment. Both the pattern of sequential activation of the responding T cells in the DLN and the synchronization of the responding T cell cohort in the draining node after Mel-14 Ab treatment was observed when a 200-fold lower number of Tg T cells (i.e., 5 × 10^5 CL-4 T cells/recipient) was used in the adoptive transfer (Fig. 5, B and C).

Localization of synchronized CL-4 T cells in the DLN

If administration of anti-CD62L Ab at 24 h.p.i. restricted the responding T cells to a single early cohort of circulating CL-4 T cells entering the DLN, then this cell cohort might likewise traffic from the DLN cortex to the medulla in a synchronized manner (i.e., sequentially localize to distinct anatomical sites in the DLN during the course of naive T cell activation and proliferation in response to infection). To explore this possibility, we examined the localization of CFSE-labeled CL-4 T cells in the DLN of infected mice by immunofluorescence microscopy of DLN sections removed at day 1 p.i. when anti-CD62L was administered or at day 4 p.i. of Ab-treated or control (untreated)-infected CL-4 T cell recipients. Day 4 p.i. was chosen as the time for DLN sampling because our earlier studies (10) indicated that this was a time point when responding CL-4 T cells had undergone extensive proliferation in the DLN but had not as yet exited from the DLN and trafficked to the lungs. We used Abs specific for HEV (anti-peripheral LN addressin, MECA-79) and for LYVE-1 to distinguish the cortex and the medulla of the infected DLN. Dye-labeled CL-4 T cells were identified at day 1 p.i. by the intense CFSE dye fluorescence and at day 4 p.i. (after a loss of CFSE dye staining due to T cell proliferation) by staining with anti-Thy1.2 Abs to distinguish the transferred CL-4 T cells from recipient (Thy1.2^+^) T cells in the DLN.

As Fig. 6A demonstrates, at day 1 p.i., CFSE^{high} CL-4 T cells were readily detected in the cortex of the DLN largely, but not exclusively, in the vicinity of the HEV. The cell distribution pattern likely reflects the localization of both CL-4 T cells that had recently transited from the HEV into the DLN (and encountered viral Ag) as well as CL-4 T cells that had entered the DLN before the in flux of viral Ag bearing migratory RDC. At day 4 p.i. in untreated (control) infected CL-4 T cell recipients, lymphatic endothelial staining of harvested DLN (Fig. 6B) demarcated the cortex (and bordering subcapsular sinus) and the medulla (cords and sinuses). There was extensive accumulation of CFSE^{low}\'-Thy1.2^+^ CL-4 T cells uniformly throughout the DLN of these control animals (Fig. 6C), a pattern consistent with sequential influx and activation/proliferation of multiple successive cohorts of responding T cells dispersed throughout the DLN. In the DLN of infected mice receiving anti-CD62L Ab at 24 h.p.i., the medullary sinuses were likewise readily demarcated by lymphatic endothelial staining (Fig. 6D), but the Thy1.2^+^ (CFSE^{low}\') responding CL-4 T cells were, by contrast, predominately localized to a distinct region of the DLN in apposition to the lymphatic endothelial cells lining the LN medullary sinuses (Fig. 6E). The distinct anatomical localization of these CL-4 T cells within the DLN of treated animals supports the concept that a single initial cohort of CD8^+^ T cells has responded to infection by activating, proliferating, and subsequently migrating to the medullary cords of the DLN during the 4-day course of infection. In these Ab-treated day 4 DLN, the B and T cell regions of the LN cortex were identifiable, but relatively depleted of cells (compared to control DLN), with depletion of CD4^+^ T cells > B cells > CD8^+^ T cells and with the majority of CD3^-^Thy1.2^-^ cells in the medullary cords representing resident recipient CD8^+^ T cells (data not shown).

Discussion

In this report, we have used a TCR-Tg CD8^+^ T cell adoptive transfer model to examine the tempo of T cell activation/proliferation in the DLN in response to respiratory virus infection. We found that the onset of T cell proliferation in the DLN differed for mice infected with two different type A influenza strains. This difference in CD8^+^ T cell activation tempo was not attributable to an intrinsic difference in efficiency of TCR-Tg T cell recognition of the two viruses, since there was no difference in the onset of activation (i.e., CD69 up-regulation) or proliferation in vitro in response to either virus-infected or peptide-pulsed APC expressing the A/PR8 and A/JAPAN viral epitopes. However, the difference in the T cell activation/proliferation tempo (in response to the two viruses) did correlate with the onset and timing of accelerated RDC migration from the infected lung to the DLN, with the migratory RDC responding to the A/JAPAN infection exhibiting a more rapid tempo of accumulation in the DLN (i.e., peak migration of A/JAPAN at 18 h, A/PR8 at 24–36 h).

To better define the relationship between T cell migration into the DLN and the T cell proliferation tempo, we inhibited naive T cell influx into the DLN by i.v. administration of blocking anti-CD62L Ab at different times before/after infection. We found that anti-CD62L administration at 24 h.p.i. allowed only a single cohort of naive Tg CD8^+^ T cells to enter the DLN and undergo activation there. This cohort of T cells proliferated in a synchronous fashion within the DLN, as evidenced by the homogenous pattern of CFSE dilution (i.e., uniform or single-dye intensity) displayed by responding T cells at days 4 and 5 p.i. Histologic analysis of the DLN of mice treated with anti-CD62L at 24 h.p.i. revealed that this single cohort of synchronized cells was localized at day 4 p.i. within the DLN medulla aligned along the medullary lymphatic channels.

The induction of the adaptive (CD8^+^ T cell) response to murine respiratory influenza virus infection is primarily restricted to the LN draining the infection site (i.e., the respiratory tract) (31). This restriction of viral Ag presentation to the LN presumably reflects the fact that most influenza virus strains are restricted in their replication to the respiratory tract, with minimal systemic dissemination of the virus (32). Viral Ag transfer to the site of naive CD8^+^ T cell activation is believed to be mediated by professional APC, primarily DC including RDC, which have activated and migrated from the lungs to the DLN in response to infection (17, 28, 29), although low levels of infectious virus can be transiently detected in the DLN early in infection (19). Our analysis of the response of influenza HA-specific TCR-Tg CD8^+^ T cells to i.n. infection with two different type A influenza strains revealed a difference in the...
activation tempo of naive CD8+ T cells in the DLN (Fig. 1A). This difference in the timing of T cell activation/proliferation after infection with two viruses did correlate with the extent of accumulation of infectious virus in the lungs which occurred more rapidly after A/JAPAN inoculation than A/PR8 inoculation during the first 48 h p.i. The difference in infectious virus accumulation likely was not due to a difference in the rate of virus replication in vivo in the lungs but rather to the difference in the size of the initial i.n. inoculum used. T cell activation/proliferation tempo also correlated with the onset and timing of accelerated RDC migration from the infected lungs to the DLN with the migrant RDC responding to the A/JAPAN infection exhibiting a more rapid tempo of RDC accumulation in the DLN and a more rapid onset of the CD8+ T cell responses in the DLN. In this connection, we have found that increasing the i.n. inoculum dose of A/PR8 virus (to lethal inoculum dose levels) we can shorten the interval between the onset of infection and the initiation of CD8+ T cell activation/proliferation (H. Yoon, unpublished observations). We believe that the tempo of RDC migration from the respiratory tract to the DLN is not dictated by the i.n. inoculum dose of virus per se, but rather is dictated by the time required for replicating virus to reach peak steady-state virus load and this virus load provides the stimulus for RDC migration. Therefore, by increasing the i.n. inoculum dose of A/PR8 virus, more respiratory tract cells are initially infected and peak steady-state virus titers are achieved more rapidly. Overall, these results support the concept that migrating DC are the major (primary) transporter of viral Ag presented to naive CD8+ T cells in the DLN.

As reported by us (17) and observed here (Fig. 3), accelerated RDC migration in response to respiratory influenza virus infection occurs over a limited time frame, with RDC migration returning to basal levels by 48 h p.i. (with either virus). Thus, if migrant RDC serve as an important APC for activating naive virus-specific CD8+ T cells in the DLN, then viral Ag directly presented by RDC may not be available later in infection for presentation to naive CD8+ T cells entering the DLN at day 3 p.i. or beyond. In this connection, several lines of evidence suggest that LN-resident DC (LNDc), in particular CD8α− LNDc, which have acquired viral Ag (from migrant RDC) by cross-presentation are the major APC for the induction of naive CD8+ T cell responses in the DLN (33–35). The APC activity of the CD8α+ LNDc is reported to be maximal at day 3 p.i (36). Although this time point is after the onset of CD8+ T cell activation in the DLN in the studies described here (i.e., day 1–2 p.i.), differences in the CD8+ T cell activation tempo may reflect the virus strain used for infection and the inoculum dose of virus in a particular study. However, because migrant RDC were not detected in the DLN after 48 h p.i., naive CD8+ T cells that entered the DLN at 48 h p.i. or beyond were most likely stimulated by viral Ag displayed by LNDc, presumably through a cross-presentation mechanism. A determination of the relative contribution of migrant RDC and LNDc as APC in vivo for the CD8+ T cell response to influenza virus will require additional analysis.

A consistent feature of the in vivo analysis of the response of transferred CFSE-labeled naive CD8+ (and CD4+) T cells to Ag displayed in the SLO is the presence of activated T cells exhibiting heterogeneity in the number of cell divisions that they have undergone in the SLO (9–11). This heterogeneity in the extent of cell division could simply reflect differences in either the efficiency of the T cell/APC encounter, the strength of the signal imparted to the TCR on individual T cell precursors, or both, resulting in different onsets of T cell activation/proliferation or differences among naive T cells in the number of T cell divisions triggered by TCR engagement. Our findings suggest an alternative explanation, i.e., the time point at which naive T cell precursors leave the circulation and enter the Ag localization site (in the DLN) determines the onset of T cell activation and the subsequent programmed proliferation. Accordingly, the naive Ag-specific T cells that enter the DLN conveyer belt first would have undergone the greatest number of divisions (in this report, the lowest CFSE intensity). Therefore, these T cells would have also proceeded farthest along the differentiation pathway toward effector generation. This process of sequential T cell activation would be most readily demonstrated when (as in this report) Ag is localized to a peripheral site (e.g., the respiratory tract) and circulating T cells encounter Ag in the DLN of the site of Ag deposition/infection.

As previously reported (10), there is a progressive and incremental decrease in the intensity of CD69 expression on TCR-Tg CL-4 T cells responding to infection in vivo in the DLN, as the cells undergo successive rounds of cell division (Fig. 1). CD69 expression on T cells is controlled in part by Ag (through TCR engagement) and by cytokines (in particular type 1 IFN) produced in response to inflammatory stimuli/infection (37, 38). Because CD69 levels can be rapidly up-regulated on activated CD8+ T cells after TCR engagement (11, 39), the progressive (cell division-dependent) loss of CD69 expression on the responding CL-4 T cells in the DLN suggests that these T cells may be no longer in continuous contact with Ag after initial TCR engagement upon entering the DLN from the circulation. Our observations demonstrating the preferential anatomic localization of a single cohort of CL-4 T cells at day 4 p.i. to the medullary region of the DLN after synchronization by anti-CD62L Ab administration at 24 h p.i. (Fig. 6) is consistent with this idea and further suggests that, as the activated/proliferating T cells migrate from the LN cortex to the medullary, they may cease to encounter Ag, but may continue programmed divisions (40). The recent evidence linking CD69 expression with the control of T cell egress from LN also supports that idea (38).

It is also noteworthy that the initiation of cell proliferation by the transferred CL-4 T cells in the DLN appeared to diminish significantly by days 4 and 5 p.i., despite the fact that the numbers of naive circulating CL-4 T cells available to enter the DLN and activate (at this time) are not limiting. Several lines of evidence suggest that naive Ag-specific T cells can enter LN draining the site of infection at relatively late times (i.e., days 4 and 5 p.i.), undergo Ag-driven activation, and at least several rounds of proliferation without differentiating into mature effector cells (and may be destined for the memory T cell pool) (26, 27, 41). It is not clear whether this difference is due to a change in the quality of the antigenic stimulus for the T cells in the DLN or a modification in the architecture of the DLN themselves (42). However, the findings reported here reinforce the view that the time frame over which Ag-specific naive CD8+ T cells encounter Ag and activate after entering the DLN is likely to be limited, at least for those naive cells giving rise to mature effector T cells.

It is also important to note that, according to the conveyor belt model, the first cohort of T cells to initiate cell division in vivo (e.g., at 72 h p.i. for A/PR8; H. Yoon and T. J. Braciale, unpublished observations) will have undergone at least four to six divisions in the DLN over a 24-h period (i.e., by 96 h p.i.). This finding suggests that activated CD8+ T cells responding in vivo to an antigenic stimulus like virus infection may replicate with an extremely rapid initial division time (i.e., cell cycle time of ≤4 h). This would be a much faster division time than observed for naive T cell proliferation in vitro, and, if verified, suggests a novel mechanism for regulating T cell proliferative expansion in situ in the SLO. Furthermore, as suggested by the findings in Figs. 1 and 5, the responding T cells in the DLN appear to proliferate more rapidly (i.e., undergo more cell divisions per unit time at early points).
after the onset of cell division. Indeed, we found that the cell cycle time of the responding T cells increases significantly (more than doubles) after the cells have undergone more than five to six cell divisions (H. Yoon and T. J. Braciale, unpublished observations). The mechanism to explain the basis for the longer cell cycle time of the responding CD8\(^+\) T cells in vivo is currently unknown and under investigation.

Overall, our results demonstrate that naive CD8\(^+\) T cells responding to Ag deposition at a peripheral mucosal site (e.g., the respiratory tract) encounter Ag in the DLN, then activate/proliferate in an ordered sequential fashion reminiscent of a conveyor belt. The tempo of the T cell response is regulated by Ag availability in the DLN, which is, in turn, dependent on the tempo of Ag delivery to the DLN by migrant DC trafficking from the mucosal site. In the case of influenza infection of the respiratory tract, efficient Ag delivery to the DLN maybe limited. Thus, the continuous stream of circulating naive Ag-specific CD8\(^+\) T cells in the blood that will give rise to effector most likely enter the DLN, encounter Ag, and activate within a narrow time window. Findings reported here further suggest the division time of activated proliferating CD8\(^+\) T cells in the DLN is extremely rapid, thereby allowing for the rapid expansion of the number of responding T cells and the rapid generation of effectors. As these activation/differentiation events occur within the architectural constraints of the DLN, a deeper understanding of the interplay between structural and cellular elements of the SLO which regulate the host response to infection will be critical, both for elucidating the mechanism(s) of immune dysregulation mediated by microbes and for optimum vaccine design.

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