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Direct Analysis of the Dynamics of the Intestinal Mucosa CD8 T Cell Response to Systemic Virus Infection[1]

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The CD8 T cell response to vesicular stomatitis virus infection was characterized in the spleen and intestinal mucosa using MHC tetramers. Surprisingly, the primary response persisted in the lamina propria long after the splenic response had declined. Furthermore, the response was characterized by a protracted effector phase in which cytolytic activity in the lamina propria, but not in the spleen, was maintained. The appearance of Ag-specific cells in the intestinal mucosa was largely, though not exclusively, a result of β_7 integrin-mediated migration. Infection with *Listeria monocytogenes* or with vaccinia virus also led to sustained mucosal responses. After reinfection of vesicular stomatitis virus-primed mice with a serotypically distinct virus, a sustained recall response was detected in all tissues. In CD40$^{-/-}$ mice, the mucosal, but not the splenic, response was compromised, resulting in diminished mucosal memory. The recall response was CD40 independent and correlated with memory levels, indicating that the mucosal and systemic responses operated independently. These findings illustrated the integrated yet distinct nature of systemic vs mucosal immune responses. ***The Journal of Immunology*****, 2001, 166: 2348–2356.**

The intestinal mucosa serves the essential function of nutrient absorption and consequently has an immense surface area. As this surface can serve as a target or entry point for myriad pathogens, it places enormous demands on the immune system. In fact, intestinal tissue is densely populated by lymphocytes and accessory cells, including CD4 and CD8 T cells, naive and memory B cells, dendritic cells, and IgA-secreting plasma cells. However, we do not yet have an adequate understanding of these tissue-specific populations with respect to function, regulation, origin, or Ag specificity. Within the mucosal effector sites (i.e., the lamina propria (LP)[3] and intraepithelial lymphocyte (IEL) compartments), intestinal lymphocytes are phenotypically unique compared with their counterparts in the secondary lymphoid tissue (i.e., lymph nodes (LN), Peyer's patches (PP), and spleen). There are several unusual T cell subsets, and most express surface markers or functions indicating an activated or memory status (1–3). For example, the CD8$\alpha\beta$ TCR$\alpha\beta$ and CD8$\alpha\alpha$ TCR$\gamma\delta$ cells at mucosal effector sites are constitutively cytolytic (4, 5). Our recent demonstration that Ag-specific intestinal TCR$\alpha\beta$ memory cells are also constitutively cytolytic may explain this function for at least some of the CD8$\alpha\beta$ IEL (6).

In vivo priming experiments demonstrate that intestinal primary and memory CD8 T cells can be induced by virus infection. Ag-specific CTL are detected within the LP and IEL compartments, as well as in secondary lymphoid tissue of mice following oral rotavirus infection (7). However, CTL are present in the LP but not in the IEL following footpad immunization, indicating discordance of the response even within mucosal effector sites. Additionally, memory CTL precursors (CTLp), as determined by limiting dilution analysis (LDA), are detected in the LP up to 21 days after oral infection, but could not be detected in the IEL at any time. The latter result points out the inherent problems with in vitro culture of mucosal T cells because IEL lytic activity is present at a time point at which CTLp are not found. Oral infection with reovirus also induces virus-specific primary and at least short-term memory IEL (up to 28 days) (8, 9). Berzofsky et al. analyzed CTL induction in response to an HIV combinatorial peptide and showed that intrarectal immunization led to primary and memory CTL within the LP, IEL, and spleen (10). In contrast, and unlike the results with s.c. rotavirus infection (7), s.c. immunization with the peptide led to CTL activity in spleen but not mucosa (10). Overall, these studies imply that induction of mucosal primary and memory CTL can depend on the route of infection as well as the form of the immunogen and provide a rationale for a more quantitative and long-term assessment of intestinal effector and memory T cell responses.

Most of the aforementioned studies, while informative, suffer from the requirement for measurement of lytic activity after in vitro reactivation or the use of LDA. Such studies are also complicated by the relative inability of mucosal T cells to be activated to proliferate in vitro (11–13). Furthermore, it is now clear from analyses using Ag-specific MHC class I tetramers and enzyme-linked immunospot assays that LDA underestimates the size of Ag-specific CD8 T cell responses by ~50-fold in primary responses and 10-fold during memory responses (14, 15). In mice, the splenic response to systemic infection with LCMV or *Listeria monocytogenes* has been studied in detail using MHC tetramers, and the results reveal that long-term memory is stable and correlates with the magnitude of the primary response (14, 16). Analysis of the anti-influenza CD8 response in bronchoalveolar lavage fluid after intranasal infection showed that a substantial primary or recall response was focused in the lung (17, 18). However, after clearance of the infection, the lung mucosa contains few memory-phenotype T cells, emphasizing the specialized nature of the intestinal mucosa, which contains many such cells. Kundig

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[3] Abbreviations used in this paper: LP, lamina propria; IEL, intraepithelial lymphocyte; LN, lymph node; PP, Peyer's patch; CTLp, CTL precursor; LDA, limiting dilution assay; VSV, vesicular stomatitis virus; N, nucleoprotein; APC, allophycocyanin; rLM, recombinant *Listeria monocytogenes*; CD40L, CD40 ligand; LCMV, lymphocytic choriomeningitis virus.

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et al. have argued that long-term CD8 T cell memory in the absence of persisting Ag is only protective to internal secondary lymphoid organs (19). This conclusion is based on the ability of LCMV-specific CD8 memory cells several weeks after the initial infection to clear LCMV from the spleen after systemic challenge but not from the footpad after infection at that site (19). These results imply that CD8 memory cells may not persist in the skin but require reactivation in draining LN before reappearance in the skin. Whether the intestinal LP and epithelium represent a similar system or harbor endogenous memory cells able to mount a rapid response without contributions from secondary lymphoid tissue has yet to be directly tested. The mechanisms for maintaining immunological memory are quite controversial. It has been proposed that memory T cells reflect an ongoing immune response to low levels of retained Ag (19, 20), although recent evidence indicates that the presence of Ag or even MHC is not required for memory T cell maintenance in secondary lymphoid tissues (21, 22). Whether such rules apply to memory cells in tertiary sites such as the intestinal mucosa is unknown. In light of these issues, it is important to identify the requirements for induction and maintenance of T cell memory within the specialized microenvironment of the intestinal mucosa.

As of yet, pathogen-specific intestinal CD8 T cell responses have not been studied in detail using MHC tetramers. Our initial studies using tetramers of H-2K^b-vesicular stomatitis virus (VSV) nucleoprotein (N)-derived peptide showed that a primary response was detectable in the intestinal LP and the epithelium and that this response was CD40 dependent (23). We have also used the adoptive transfer of OVA-specific TCR-transgenic CD8 T cells to intact hosts to visualize in vivo intestinal CD8 immune responses. This method allowed the identification of primary and memory donor cells within the LP and IEL compartments following systemic viral infection (6). Adoptive transfer is the only means to accurately track a response beginning with naive cells (24), because frequencies of endogenous Ag-specific naive cells are too low to detect with MHC tetramers. However, the primary response in this system is unlikely to quantitatively parallel the dynamics of an endogenous immune response due to the unnatural number of CTLp. Furthermore, the response of adoptively transferred cells is dictated in part by their original source (e.g., LN) and so may not accurately reflect tissue-specific responses. To circumvent these potential pitfalls, we have now used MHC tetramers to quantitate the overall CD8 T cell response to systemic viral and bacterial infections in the secondary lymphoid tissues as well as the intestinal mucosa. The results demonstrated surprising distinctions between the splenic and mucosal responses with regard to magnitude, longevity, and costimulatory requirements.

Materials and Methods

Mice

C57BL/6J (Ly5.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-CD40$^{-/-}$ mice (25) were generously provided by Dr. Hitoshi Kikutani (Osaka University, Osaka, Japan) via Dr. Nancy Philips (University of Massachusetts Medical Center, Worcester, MA). C57BL/6-$\beta_7{}^{-/-}$ mice (26) were generously provided by Drs. Muller and Wagner (Institute for Genetics, University of Cologne, Cologne, Germany).

Infections and detection of Ag-specific CD8 T cells with MHC tetramers

Mice were infected by i.v. injection of 1×10^6 PFU of VSV, Indiana (Ind) serotype, or 1×10^7 PFU of vaccinia-N (27). Vaccinia-N stocks were generously provided by Jon Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD). For recall responses, mice initially infected with VSV-New Jersey (NJ) were infected with 1×10^6 PFU VSV-Ind. At the indicated times later, lymphocytes were isolated and VSV-N-specific CD8 T cells were detected using H-2K^b tetramers containing the N protein-derived peptide RGYVYQGL or as a control the OVA-derived peptide SIINFEKL (Research Genetics, Huntsville, AL) (28). Recombinant *L. monocytogenes* producing soluble OVA was produced as previously described (29, 30). Mice were infected with recombinant *L. monocytogenes* (rLM)-OVA by i.v. injection of 2×10^3 CFU, and CD8 T cell responses were detected using H-2K^b tetramers containing the OVA peptide. MHC tetramers were produced essentially as previously described (14, 31). Briefly, H-2K^b containing the BirA-dependent biotinylation substrate sequence was folded in the presence of human β_2-microglobulin and the N or OVA peptide. Biotinylation was performed with biotin-protein ligase (Avidity, Denver, CO). Tetramers were then produced from biotinylated HPLC-purified monomers by addition of streptavidin-allophycocyanin (APC; Molecular Probes, Eugene, OR). The modified H-2K^b cDNA and the β_2-microglobulin constructs were generously provided by J. Altman (Emory University, Atlanta, GA).

Intracellular detection of IFN-γ

Lymphocytes were cultured in DMEM/5% FCS/10% Nu Serum (Life Technologies, Grand Island, NY) with added HEPES, 2-ME, and antibiotics at a density of 1×10^6 cells per ml in a 24-well dish at 37°C with or without the addition of 1 μg/ml of the VSV-N protein-derived RGYVYQGL peptide. Golgiplug (containing brefeldin A; BD PharMingen, San Diego, CA) was added to unstimulated and stimulated cultures at a dilution of 1 μl/ml. Cells were harvested after 5 h and stained for cell surface Ags. Cells were then fixed in 4% paraformaldehyde/PBS for 20 min at 4°C, washed twice, and stored overnight at 4°C. The next day, the cells were permeabilized by incubating in Perm/Wash solution (BD PharMingen) for 20 min. The permeabilized cells were incubated with anti-IFN-γ-FITC (XMG1.2, 5 μg/ml; BD PharMingen) or control rat IgG1-FITC (R3-34, 5 μg/ml; BD PharMingen) for 30 min at 4°C and washed twice in Perm/Wash solution. The fluorescence intensities were immediately measured on a FACSCalibur (BD Bioscience, San Jose, CA).

Production of bone marrow chimeras

Lethally irradiated mice (1000 rad from a ^{137}Cs source) were injected i.v. with 5×10^6 bone marrow cells. B6-Ly5.2 mice were reconstituted with a 1:1 mixture of C57BL/6 (Ly5.1/5.2) and C57BL/6-$\beta_7{}^{-/-}$ (Ly5.1) bone marrow cells. Twelve weeks later, mice were infected with 1×10^6 PFU of VSV-Ind. Lymphocytes were isolated from infected and uninfected control mice 6 days later and analyzed by fluorescence flow cytometry. Donor and host lymphocytes were distinguished by staining with mAb specific for Ly5.1 and Ly5.2 (32).

Isolation of lymphocyte populations

IEL and LP cells were isolated as described previously (33, 34) and were derived from the entire small intestine. For cytotoxicity assays, panning of Percoll-fractionated IEL on anti-CD8 mAb-coated plates was performed to remove contaminating epithelial cells. Spleens were removed, and single-cell suspensions were prepared using a tissue homogenizer. The resulting preparation was filtered through Nitex nylon mesh (Tetko, Kansas City, MO), and the filtrate was centrifuged to pellet the cells.

Immunofluorescence analysis

Lymphocytes were resuspended in PBS/0.2% BSA/0.1% NaN_3 (PBS/BSA/NaN_3) at a concentration of 1×10^6–1×10^7 cells/ml followed by incubation at 4°C for 30 min with 100 μl of properly diluted mAb. The mAbs used were: anti-Ly5.1 or 5.2 (32), anti-CD11a (2D7), anti-CD8β (H35-17-2) (both from BD PharMingen), and anti-CD8α (CT-CD8a; Caltag, Burlingame, CA). The mAbs were either directly labeled with FITC, PE, Cy5, APC, or were biotinylated. For the latter, avidin-PE-Cy7 (Caltag) was used as a secondary reagent for detection. After staining, the cells were washed twice with PBS/BSA/NaN_3 and fixed in 3% paraformaldehyde in PBS. Relative fluorescence intensities were then measured with a FACSCalibur (BD Biosciences). Data were analyzed using WinMDI software (Joseph Trotter, Scripps Clinic, La Jolla, CA). For tetramer staining, cells were first incubated at 4°C with anti-CD8-PE (Caltag) at 0.1 μg/sample followed by washing and staining for 1 h at room temperature with MHC/tetramers. No difference in specific tetramer staining was noted with or without the inclusion of anti-CD8.

Measurement of cytolytic activity

Cytolytic activity was measured using [^{51}Cr]sodium chromate-labeled EL4 cells (an H-2^b thymoma) with or without the addition of 10 μg/ml of the VSV-N protein-derived peptide RGYVYQGL. Serial dilutions of effector cells were incubated in 96-well round-bottom microtiter plates with 2.5×10^3 target cells for 5 h at 37°C. Percent specific lysis was calculated as:

$100 \times$ [(cpm released with effectors − cpm released alone)]/[(cpm released by detergent − cpm released alone)].

Results

Systemic VSV infection induces a sustained Ag-specific mucosal CD8 response

VSV is an enveloped rhabdovirus that infects a broad range of cell types and transiently infects peripheral tissues of mice such that ~2 days after infection virus cannot be recovered (35, 36). The CD8 response to VSV in H-2^b mice is restricted to H-$2K^b$ and is specific primarily for the N from which one natural peptide has been identified (aa 52–59) (28, 37). Although splenic VSV-specific CTL responses have been characterized using in vitro CTL assays (27, 37), the magnitude of the response in terms of Ag-specific CD8 T cells is not known. Moreover, whether the endogenous primary or memory response extends to other sites such as the intestinal mucosa has not been tested. To compare the endogenous secondary lymphoid and mucosal anti-VSV CD8 response, we constructed H-$2K^b$-$N_{52–59}$ tetramers and quantitated N-specific CD8 T cells after systemic VSV infection (Fig. 1). No tetramer-reactive cells were detected in unimmunized mice (Fig. 1), and in infected mice no reactivity of an irrelevant K^b-OVA peptide tetramer was detected, confirming the specificity of the tetramer staining (data not shown). In the secondary lymphoid organs, the anti-VSV CD8 response was focused in the spleen, where 17% of CD8 cells were N specific on day 6 after infection, which was the peak of the splenic response (Figs. 1 and 2). This response was 4-fold greater than that observed in the mesenteric LN and 10-fold greater than the peripheral LN response (data not shown). The primary LP response was nearly twice as large as that of the spleen, in terms of the percentage of CD8 T cells. Thus, ~35% of LP CD8 T cells (9% of total lymphocytes) were N specific 6 days after infection. IEL also contained a substantial population of Ag-specific CD8$\alpha\beta$ T cells. Although ~5% of total CD8 IEL (which contain CD8$\alpha\alpha$ TCR$\gamma\delta$ cells as well as CD8$\alpha\alpha$ and CD8$\alpha\beta$ TCR$\alpha\beta$ cells) reacted with the N tetramer, all tetramer-positive cells at all time points were present in the CD8$\alpha\beta$ subset. Therefore, when the values were corrected for the proportion of IEL comprised by the CD8$\alpha\beta$ subset, the percentage of Ag-specific CD8$\alpha\beta$ IEL paralleled that found in the LP (Fig. 2).

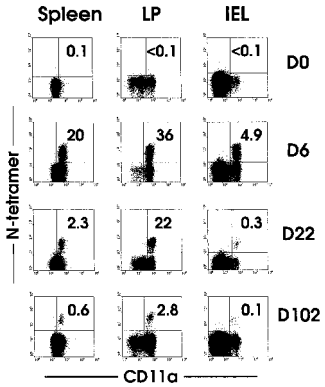

FIGURE 1. Infection with VSV leads to the appearance of virus-specific CD8 T cells in the spleen and intestinal mucosa. C57BL/6J mice were infected i.v. with 1×10^6 PFU VSV-Ind, and at the indicated time points lymphocytes were isolated from spleen, LP, or IEL, the percentage of Ag-specific CD8 T cells was assessed by staining with $N_{52–59}$-K^b tetramer, anti-CD8α, and anti-CD11a, and analysis was performed by fluorescence flow cytometry. Plots shown are gated on CD8α^+ lymphocytes, and values are percentages of $CD8^+$ T cells.

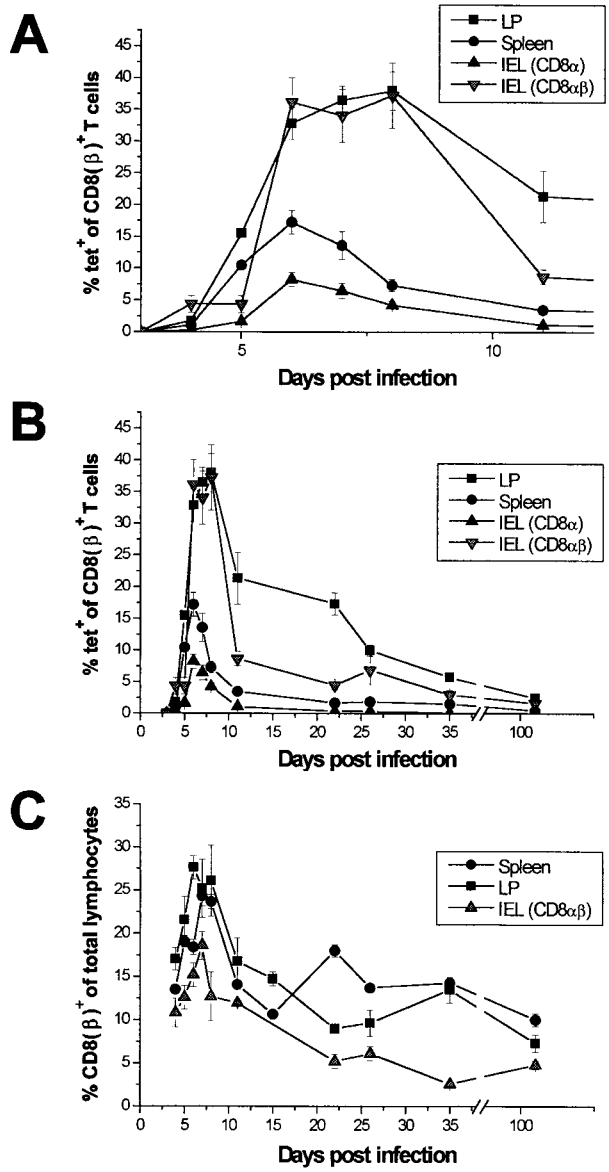

FIGURE 2. Tissue-specific kinetics of the anti-VSV CTL response. At the indicated days following infection, lymphocytes from four to eight mice were isolated and stained with tetramer, anti-CD8α or anti-CD8β, and anti-CD11a and analyzed by flow cytometry. *A*, Early phase showing sustained mucosal response, *B*, Overall response showing memory phase. *C*, Overall response showing the percentage of CD8$\alpha\beta^+$ lymphocytes in the spleen, LP, and IEL. Because IEL contains CD8$\alpha\alpha$ and CD8$\alpha\beta$ cells, the values for CD8α cells (representing the total CD8 cells in the IEL) vs CD8$\alpha\beta$ cells (representing conventional CD8 cells that contain all tetramer-positive cells) are shown for comparison. Values represent means ± SE.

By 22 days after infection, the splenic response had decreased dramatically (~10-fold), while surprisingly the LP response had only declined ~2-fold. N-specific IEL comprised 0.3% of total IEL, which correlated to ~5% of CD8$\alpha\beta$ IEL, indicating that the magnitude of the IEL response at this time point did not correlate with the LP response. At 102 days after infection, memory cells could be detected in spleen, LP, and IEL and comprised 0.5, 2, and 0.5% of CD8$\alpha\beta$ cells, respectively (Fig. 1). At all time points after infection, VSV-specific CD8 T cells expressed high CD11a levels (Fig. 1). To assess the overall response in spleen vs mucosa, the percentage of N-specific CD8 T cells in the various tissues was plotted as a function of time (Fig. 2, *A* and *B*). For comparison, the percentage of lymphocytes

expressing CD8 within each tissue was measured throughout the response (Fig. 2*C*). The peak of tetramer-positive CD8 cells in the LP and IEL was sustained from day 6 to 8, while the splenic response peaked at day 6 and declined rapidly thereafter (Fig. 2*A*). The IEL response declined dramatically from day 8 to 12, while in contrast the LP response declined until day 11 and then was largely maintained at high levels until at least 22 days after infection (Fig. 2*B*). The total number of Ag-specific cells was ~10-fold greater in the spleen vs the small intestine LP at the peak of the response but only ~2- to 4-fold greater at 22 days postinfection, indicating that the response in the LP was more sustained as compared with the spleen (data not shown). By 35 days postinfection, all responses had declined substantially but N-specific CD8 cells in the LP, as compared with spleen and IEL, always made up a larger portion of the CD8$\alpha\beta$ compartment. Stable memory populations were maintained in the spleen and LP for at least 170 days, while beyond ~60 days, IEL memory cells were more inconsistently detectable (Figs. 1 and 2 and see below). The increase in the overall percentage of CD8 T cells during the primary response closely paralleled the increase in tetramer-positive cells in all tissues (Fig. 2*C*). However, at 22 days after infection when the percentage of tetramer-positive LP cells was significantly greater than that found in spleen, the total percentages of CD8$\alpha\beta$ cells in the LP and IEL as compared with the spleen had dropped below the CD8 levels before immunization. This result suggested that survival of Ag-specific as well as perhaps non-Ag-specific CD8$\alpha\beta$ cells in the mucosa following a virus infection may be differentially regulated as compared with splenic CD8 T cells.

The effector phase of the CD8 T cell anti-VSV response is sustained in the LP but not in the spleen

Considering the differences observed in the splenic vs the LP anti-VSV CD8 response, it was important to determine whether the mucosal tetramer-positive T cells were the functional equivalents of their splenic counterparts. To this end, the lytic activity of ex vivo N-specific CD8 T cells was measured without in vitro restimulation. Specific lysis at precise E:T ratios was determined by quantitating the percentage of tetramer-positive lymphocytes. As shown in Fig. 3*A*, 7 days following infection splenic and LP N-specific CD8 T cells had similar lytic activity on a per cell basis, as did IEL (data not shown). However, 20 days following infection when the LP response was still maintained at relatively high levels (Fig. 2*B*), virus-specific LP lymphocytes mediated substantial direct ex vivo lytic activity while their splenic counterparts did not (Fig. 3*B*).

Considering the difference in lytic activity between splenic and LP VSV-specific CD8 T cells, we examined another function of CD8 T cells, production of IFN-γ. Intracellular IFN-γ staining was performed 5 h after in vitro restimulation with N peptide (Fig. 3*C*). On days 7 and 20 following infection with VSV, the percentage of IFN-γ^+ CD8$^+$ splenocytes equaled 50% of the number of tetramer-positive cells. In contrast, 40 days following infection, the percentage of splenocytes producing IFN-γ correlated precisely with the percentage of tetramer-positive cells (98 $\pm$ 2%). In the LP, regardless of the time point examined following infection, roughly 75% of the tetramer-positive cells could be induced to produce IFN-γ. These results suggested that the absence of lytic activity in splenic N-specific T cells 20 days after infection was not indicative of a general nonresponsiveness because these cells could produce IFN-γ.

Distinct mucosal responses after infection with different pathogens

To determine whether the mucosal response to VSV was reflective of systemic infections with other pathogens, endogenous CD8 T cell responses were assessed following infection with either re-

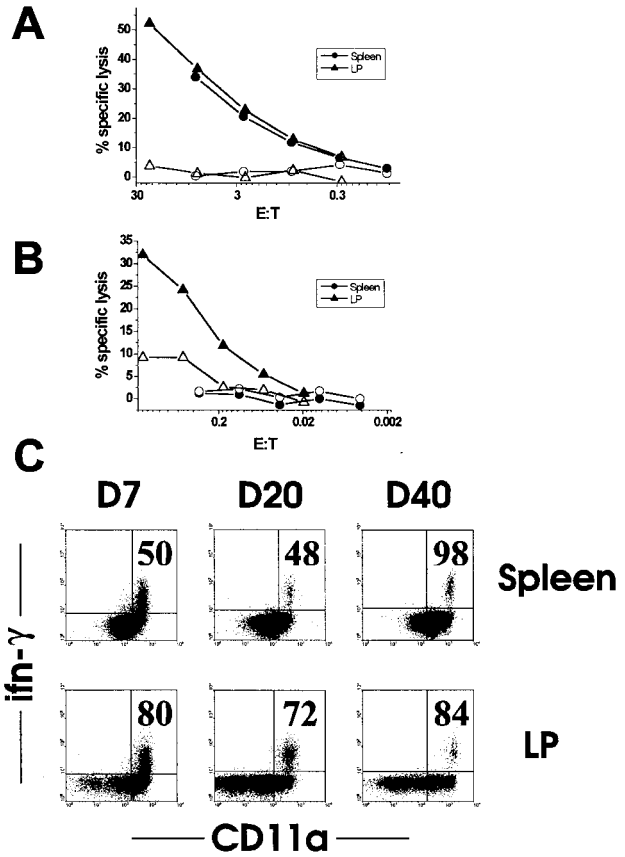

FIGURE 3. VSV infection leads to a prolonged cytolytic effector phase in the intestinal mucosa but not the spleen. Seven (*A*) or 20 (*B*) days following infection, isolated lymphocytes were incubated for 5 h with ^{51}Cr-labeled untreated EL4 target cells (open symbols) or target cells pulsed with $N_{52–59}$ peptide (filled symbols). E:T ratios were based on the actual number of tetramer-positive cells in the respective lymphocyte populations as determined by flow cytometry. Spontaneous ^{51}Cr release was <10%. This experiment has been performed on four separate occasions (a total of 18 mice) with comparable results. *C*, LP or splenic lymphocytes at the indicated times after infection were incubated for 5 h with $N_{52–59}$ peptide and brefeldin A, surface stained for CD8 and CD11a, permeabilized, and stained with anti-IFN-γ. Numbers shown represent the percentage of IFN-γ-producing cells as compared with the number of tetramer-positive cells when the same population was stained separately with CD8, CD11a, and K^b-N tetramer.

combinant vaccinia virus containing the VSV-N gene or rLM-OVA. Unlike the VSV response (Fig. 2*B*), the primary splenic and LP vaccinia N-specific responses were similar in terms of percentage of Ag-specific CD8 T cells at day 11 after infection (Fig. 4*A*). However, 9 days later (at day 20) the percentage of Ag-specific cells in spleen had dropped ~10-fold while the percentage of tetramer-positive cells in the LP had declined only ~2-fold. This difference was maintained throughout the memory phase.

For further comparison, the mucosal response to i.v. infection with *Listeria* was analyzed. Because no natural *Listeria*-derived H-2^b-restricted peptides have been defined, rLM-OVA was employed to allow visualization of OVA-specific CD8 T cells by staining with K^b-SIINFEKL tetramers. Infection with rLM-OVA led to the appearance of tetramer-positive CD8 T cells within the spleen and LP (Fig. 4*B*). The splenic response peaked at day 9 with ~5% of CD8 cells being OVA specific and quickly subsided almost to resting memory levels by day 13. In contrast, the primary LP response was much greater in magnitude and only gradually declined until ~22 days following infection. Again, this led to increased CD8 memory T cell percentages

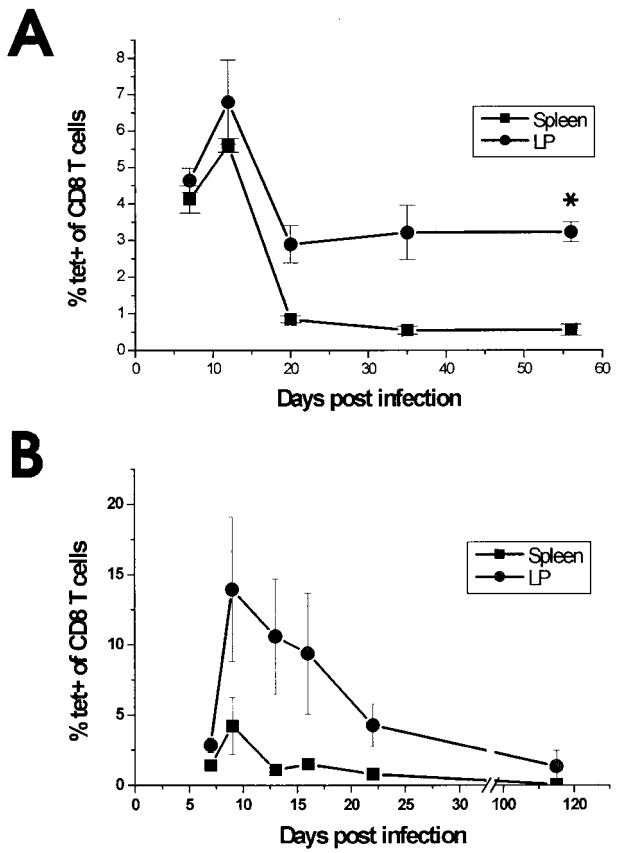

FIGURE 4. Distinct mucosal response profiles following infection with recombinant vaccinia-N or rLM-OVA. C57BL/6 mice were infected i.v. with 1×10^7 PFU recombinant vaccinia-N (*A*) or 2×10^3 CFU rLM-OVA (*B*). At the indicated days following infection, lymphocytes from three to seven mice were isolated and stained with anti-CD8, anti-CD11a, and K^b-N tetramer (*A*) or K^b-OVA tetramer (*B*). Values represent means ± SEs. *, An average of two mice at this time point.

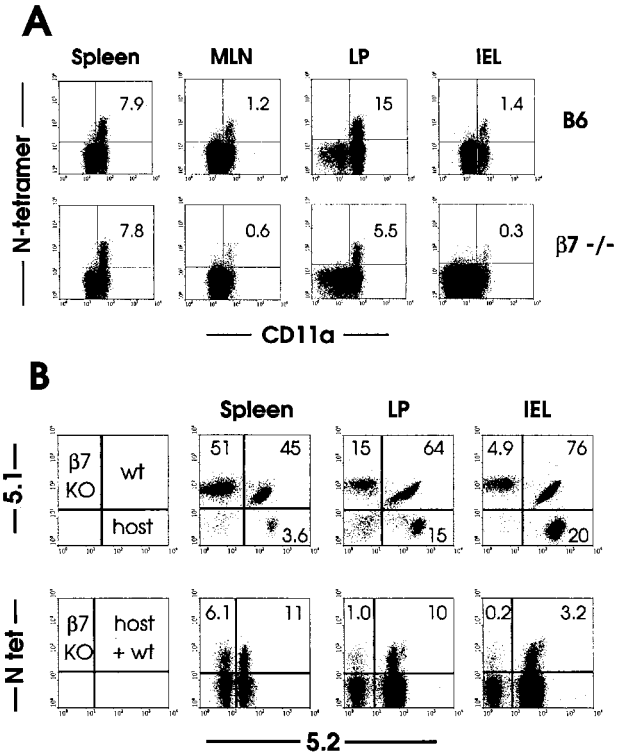

FIGURE 5. β_7 integrin is required for optimal CD8 response in the intestinal mucosa. *A*, $\beta_7^{-/-}$ mice were infected i.v. with 1×10^6 PFU VSV-Ind. Six days later, lymphocytes were isolated from various tissues, and the presence of Ag-specific CTL was assessed by flow cytometry after staining with tetramer, anti-CD8α, and anti-CD11a. Plots shown are gated on CD8α^+ lymphocytes. *B*, Chimeric mice were generated by lethal irradiation of Ly5.2$^+$ C57BL/6 mice followed by reconstitution with a 1:1 mixture of Ly5.1$^+$ $\beta_7^{-/-}$ and wild-type Ly5.1/5.2$^+$ C57BL/6 bone marrow cells. Ten weeks later, mice were infected i.v. with 1×10^6 PFU VSV-Ind. Six days following infection, lymphocytes were isolated from various tissues, and the presence of Ag-specific β_7^+ and β_7^- CD8 T cells was assessed by flow cytometry after staining with anti-Ly5, N_{52-59}-K^b tetramer, anti-CD8α, and anti-CD11a. Plots shown are gated on CD8α^+ lymphocytes, and values are percentages of CD8$^+$ T cells.

in the mucosa as compared with spleen (the response was detectable but minimal within the mesenteric and peripheral LNs, data not shown). It should also be noted that after oral infection with *Listeria* or intranasal or footpad infection with VSV, the response in the LP was prolonged similarly to that observed after i.v. infection (data not shown). Overall, the results indicated that the LP is generally the focus of CD8 T cell responses but that the kinetics and magnitude of the primary LP response as compared with that of the spleen may vary depending on the dynamics of the infection and the nature of the pathogen.

β_7 integrins are required for an optimal mucosal anti-VSV CD8 response

One interpretation of the finding that the peak of the LP anti-VSV and anti-*Listeria* responses was sustained beyond that of the spleen is that migration of N-specific T cells to the mucosa continues as the secondary lymphoid response wanes. In addition, some primary activation could occur in situ in the LP. Because the $\alpha_4\beta_7$ integrin is involved in the migration of activated CD8 lymphocytes into PP, LP, and IEL compartments (26, 38, 39), we assessed the requirement for β_7 integrins in generating mucosal CTL responses. $\beta_7^{-/-}$ mice were infected with VSV, and 6 days later various tissues were analyzed for the presence of Ag-specific CD8 T cells. While spleen (Fig. 5*A*) and peripheral LN (data not shown) responses were similar between control B6 and $\beta_7^{-/-}$ animals, a significant decrease in Ag-specific CD8 T cells was noted in mesenteric LN, LP, and the IEL compartment (Fig. 5*A*). The increase in total CD8 T cells was also reduced in $\beta_7^{-/-}$ mice but the Ag-specific mucosal T cells from normal or $\beta_7^{-/-}$ animals exhibited similar lytic activity on a per cell basis (data not shown). These data implied that in the context of a systemic VSV infection, at least some naive CD8 T cells were primed in secondary lymphoid tissue and migrated into the intestinal mucosa via a β_7-dependent mechanism.

However, the inhibition was incomplete suggesting that additional mechanisms for lymphocyte homing to the gut may operate in the context of a complete absence of β_7 integrins. Indeed, in our hands, the $\beta_7^{-/-}$ mice contained only partially reduced numbers of intestinal lymphocytes (data not shown). To provide a more realistic gauge of the potential requirement for β_7 integrins in activated T cell homing to the mucosa, we generated mixed chimeras by reconstituting normal mice (B6-Ly5.2) with equal numbers of $\beta_7^{-/-}$ (B6-Ly5.1) and wild-type (B6-Ly5.1/5.2) bone marrow cells. In this way, approximately equal numbers of naive β_7^+ and β_7^- CTLp would be present in the secondary lymphoid tissue of the same animal and so competition for migration into the mucosa should occur. Both infected and uninfected animals showed a similar reconstitution profile. While secondary lymphoid organs contained roughly equal numbers of $\beta_7^{-/-}$ (Ly5.1$^+$)

and wild-type (Ly5.1/5.2$^+$) CD8 lymphocytes, mucosal tissues (mesenteric LN, PP, LP, and IEL) showed a greatly decreased population of $\beta_7^{-/-}$ Ly5.1$^+$ lymphocytes (Fig. 5*B* and data not shown). The Ly5 phenotype of tetramer-positive CTL in infected animals showed a similar pattern in that the ratio of $\beta_7^{-/-}$:$\beta7^{+/+}$ Ag-specific T cells was far lower in the mesenteric LN, LP, and IEL compartments as compared with the ratio in the spleen. Thus, there was at least a 10-fold difference between the ability of the $\beta_7^{-/-}$ cells and the wild-type Ag-specific CD8 T cells (which included some host radiation-resistant cells) to migrate to the LP and IEL (Fig. 5*B*). Furthermore, this defect was more severe than that observed in intact $\beta_7^{-/-}$ mice (Fig. 5*A*), indicating that those results underestimated the importance of β_7 integrins in mucosal CD8 T cell trafficking. Nevertheless, the requirement for β_7 integrins in appearance of activated CD8 cells in the intestinal mucosa was not absolute.

The VSV-specific recall response generates large numbers of long-lived memory cells.

Having established a system in which endogenous virus-specific primary CD8 T cells migrated to the intestinal mucosa and generated memory cells, we wished to determine whether the various anatomically distinct memory populations responded to secondary infection. Because serotype-specific neutralizing Ab prevents reinfection with the initial virus (40), we used a second VSV serotype, VSV-NJ, to prime mice. Because the cross-reactive anti-VSV CTL response is primarily directed toward N-protein epitopes (27, 37), we then immunized secondarily with VSV-Ind 4–6 mo later. In immune mice, N-tetramer-positive memory cells were readily detectable in spleen and LP but were barely detectable in the IEL compartment (Fig. 6*A*). At 2 days after secondary infection of immune mice, memory cells had disappeared from the spleen but were present in LP. The loss of Ag-reactive cells from the circulation soon after immunization has been previously observed in primary CD8 responses and may be due to sequestration of T cells with APCs (41, 42). Five days after reinfection of VSV-NJ immune mice with VSV-Ind, there was an explosive recall response in all tissues analyzed (Fig. 6*A*), including the intestinal epithelium. Even at day 3 after infection, a large increase in Ag-specific cells was noted in spleen and intestinal mucosa (Fig. 6*B* and data not shown). These results indicated that N-protein-specific cross-reactive CD8 T cells were activated in situ after secondary infection and/or had migrated to the mucosa after activation in the secondary lymphoid tissue. Interestingly, at 35 days after infection, secondary memory cells comprised a large proportion of CD8 cells in the spleen (19%), the LP (30%), and the epithelium (10% of CD8$\alpha\beta$ cells). When the overall response was examined over time (Fig. 6*B*), the remarkable sustenance of memory levels was evident in the splenic and mucosal CD8 T cell populations up to at least 54 days after secondary infection. At the peak of the recall response, >40% of the CD8 T cells in the LP were Ag specific, and this had declined only by ~50% nearly 2 mo later. The responses in the spleen and the epithelium were also large, but again by day 54 the overall decline represented only about half of the peak response.

Distinct requirements for CD40-mediated costimulation in the mucosal vs splenic anti-VSV CD8 T cell response

The distinct characteristics of the primary splenic and LP response suggested that the responses may be independently controlled. Our previous results suggested that the primary CD8 T cell LP response to VSV was inhibited in the absence of CD40-CD40 ligand (CD40L) interactions (23). However, it is not known whether the induction of VSV-specific memory CD8 T cells in any tissue is influenced by CD40/CD40L interactions. Therefore, we infected CD40-deficient mice (25) with VSV and measured the primary

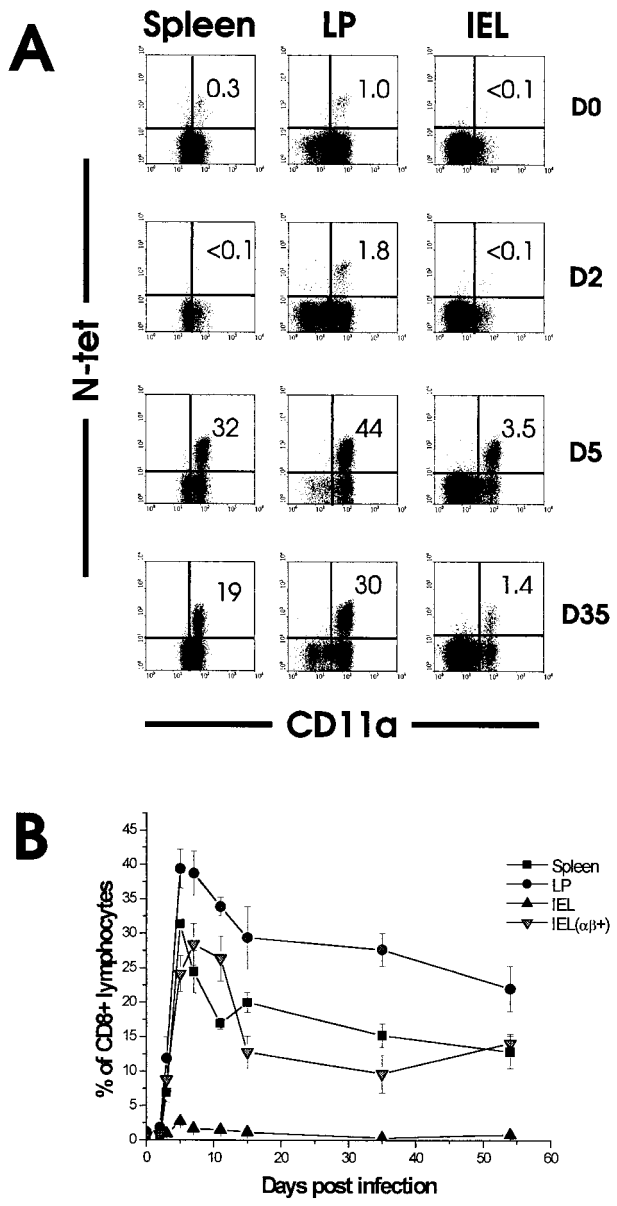

FIGURE 6. Secondary infection with a different VSV serotype elicits a potent recall response. C57BL/6 mice primed with 1×10^6 PFU VSV-NJ i.v. and rested for 4–6 mo were challenged by i.v. infection with 1×10^6 PFU VSV-Ind. At the indicated times, lymphocytes from spleen, LP, and IEL were analyzed by flow cytometry after staining with N_{52-59}-K^b tetramer, anti-CD8α or anti-CD8β, and anti-CD11a (*A*). *B*, Overall recall response in spleen and mucosa. Values represent means ± SE of data from four to six mice per time point.

response and the appearance of memory cells (Fig. 7, *A* and *B*). Six days after infection, no difference in the splenic CD8 response was observed in the absence of CD40. In contrast, substantial inhibition (3- to 5-fold) of the LPL and IEL response was observed in CD40-deficient mice. Despite the lower numbers of Ag-specific T cells and the general inhibition of the increase in CD8 T cells, lytic activity on a per cell basis was no different from control (data not shown). This dichotomy was maintained into the memory phase of the response such that at 80 days after infection splenic memory CD8 cell numbers were similar in control and CD40$^{-/-}$ mice while mucosal memory cells in the LP were drastically reduced (Fig. 7*B*). IEL memory cells could not be detected in either control or CD40$^{-/-}$ mice in this experiment. These findings provided

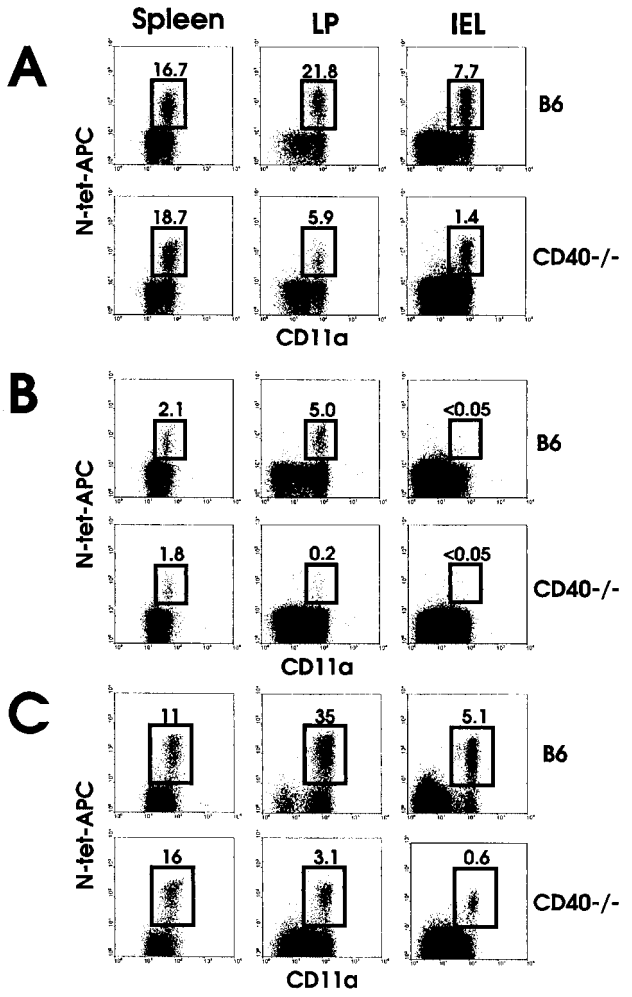

FIGURE 7. Defective mucosal but normal splenic anti-VSV CD8 T cell responses in the absence of CD40-CD40L interaction. *A*, Naive C57BL/6 and C57BL/6-CD40$^{-/-}$ mice were infected i.v. with 1×10^6 PFU VSV-Ind, and 6 days later lymphocytes were isolated from spleen, LP, or IEL and the percentage of Ag-specific CD8 T cells was assessed by flow cytometry after staining with N_{52-59}-K^b tetramer, anti-CD8α, and anti-CD11a. *B* and *C*, CD40 is required for generation of mucosal anti-VSV CD8 memory but not for mucosal or splenic recall responses. *B*, Naive C57BL/6 and CD40$^{-/-}$ mice were infected i.v. with 1×10^6 PFU VSV-Ind and were analyzed 80 days later as described above. *C*, Recall responses were generated by infection of mice with VSV-NJ 80 days after primary infection with VSV-Ind and were analyzed 3 days after secondary infection as described in *A*. Plots shown are gated on CD8α^+ lymphocytes, and values are percentages of CD8$^+$ T cells.

strong evidence that the costimulatory requirements for induction and/or maintenance of the mucosal CD8 T cell memory pool were distinct from those governing the generation of CD8 T cell memory in secondary lymphoid organs.

Given the surprising difference between splenic and mucosal CD8 memory development in CD40$^{-/-}$ mice, we tested the role of CD40-CD40L interactions in the recall response. VSV-Ind-immune normal or CD40$^{-/-}$ mice harboring N-specific memory cells (Fig. 7*C*) were infected with VSV-NJ, and 3 days later responses were measured by tetramer reactivity of splenic and mucosal lymphocytes. In normal mice, a robust recall response was evident in all tissues (Fig. 7*C*), and this was solely due to recall because the primary response cannot be detected at this time point (Fig. 2). In CD40$^{-/-}$ mice the recall response was also substantial. In the spleen, the response was similar to that of control animals. In the intestinal mucosa, based on the numbers of starting memory cells, the secondary response in CD40$^{-/-}$ mice was equal to or greater than that observed in control animals (Fig. 7*C*). However, despite a normal splenic response, the overall recall mucosal response of CD40$^{-/-}$ mice was significantly less than that of control animals, suggesting that migration from the spleen or LN was not contributing significantly to the mucosal recall response.

Discussion

This study provides an intriguing comparison of the splenic vs the mucosal endogenous CD8 T cell response to systemic virus infection. While the primary splenic response to VSV infection rapidly waxed and waned, the primary LP response was characterized by a sustained Ag-specific CD8 T cell population, which only gradually declined over a period of ~4 wk to a stable and substantial memory population. Moreover, during this prolonged response, LP but not splenic Ag-specific cells exhibited high levels of direct ex vivo lytic activity. This result supported the concept that the functional CD8 T cell response to VSV was independently controlled in mucosal vs secondary lymphoid tissue. In comparison, the dynamics of the LP response to vaccinia infection were distinct but the contraction of the LP response was again limited as compared with that of the spleen. The LP response to *L. monocytogenes* infection was also much greater and longer lasting than that detected in the spleen and more closely resembled the anti-VSV response. Overall, the data suggested that the LP provides a unique milieu for amplifying and sustaining CD8 T cell responses to microbial infection.

It is unclear what mechanisms control the distinct kinetics of tissue-specific CD8 T cell responses. One possibility was that the protracted LP responses were due to continual migration of CD8 T cells from the periphery. Indeed, the majority of the primary mucosal response was β_7 integrin dependent implying that many of the Ag-specific T cells had originated outside of the mucosa. This contention is supported by our previous demonstration that adoptively transferred CD8 T cells required activation before migrating to the intestinal mucosa in a β_7 integrin-dependent pathway (39). However, the transferred cells were derived from LN, so it was not possible to draw conclusions regarding the response of endogenous mucosal T cells. The extended LP response may also be related to a relative inability of the LP environment to induce apoptosis in responding CD8 T cells, but this remains speculative. What is clear is that the LP is not a depot for dying CD8 T cells (43), because the heightened LP response resulted in a larger long-term memory pool. Another possible explanation for the prolonged LP response is that Ag levels were sustained preferentially in the LP compartment. VSV has been shown in vitro to infect activated T and B cells, and the LP is rich in such cells (44–46). If true in vivo, this infection may provide interesting parallels to that mediated by viruses such as HIV and SIV, which focus in intestinal mucosa and infect activated lymphocytes (47–49). It has been difficult to isolate VSV from infected mice even early after infection, but a nonproductive infection in vivo, such as has been described in vitro for B cells, could provide an Ag depot that is slowly cleared by cytolytic CD8 T cells. These findings could lend credence, or alternatively indicate caution, to the suggestion that recombinant VSV be employed as a potential human vaccine (50, 51). In contrast to the primary response, the recall response led to high, sustained levels of memory cells in all tissues (Fig. 6). This result does not favor the theory that VSV Ag was harbored preferentially in the LP. In the case of *Listeria* infection, bacteria cannot be isolated from any tissues a few days after infection. Thus, it seems unlikely that the sustained mucosal response is due to persistent infection, but whether processed Ag is preferentially sequestered in mucosal or other nonlymphoid tissues is not known and difficult to assess.

One of the fascinating results of this work showed that the anti-VSV response in the IEL compartment was not coordinated with the LP response at all time points. Although the primary response of IEL was sustained, the collapse of this response was more rapid than that of the LP response. The results suggested that the IEL response can be regulated independently of the LP response. This assertion was best exemplified when mucosal memory responses were analyzed. While all mice examined contained VSV-specific CD8 memory T cells in the spleen and LP, the IEL memory response was unpredictable ~2 mo after infection, although IEL memory cells were present in some mice 6 mo after infection. This finding signifies that the LP and IEL memory pools could be distinct in that LP and IEL memory cells did not establish an apparently stable equilibrium. However, upon a secondary infection a rapid and sustained appearance of effector cells occurred in all tissues including the intestinal epithelium, indicating that the mucosal immune system is poised to mount a swift response at the immunological frontier.

Costimulatory requirements for generation of the mucosal response were also distinct from those needed for the splenic response. While the initiation of the anti-VSV response throughout the body was B7 dependent (Ref. 52 and data not shown), the intestinal, but not the splenic, response was dependent on CD40 for further amplification (Fig. 7). In contrast to our findings, a recent study in which splenic anti-VSV CD8 responses were quantitated by measuring intracellular IFN-γ production showed that CD40 and CD28, but not CD4 T cells, were required for generation of the primary response (36). However, in agreement with our findings, two previous studies did not find a requirement for CD40 in the splenic CD8 anti-VSV primary response using CTL activity as a measure (53, 54). The results of Andreasen et al. (36) also do not agree with several reports indicating that MHC class II-restricted CD4 T cells are essential for the splenic CD8 T cell response to VSV (54–56). It is difficult to reconcile a requirement for CD40 in the absence of a requirement for CD4 T cells given current hypotheses regarding CD8 T cell maturation to CTL (57–59). The reason for the discrepancy is not obvious but our results using MHC class I tetramers did not demonstrate a need for CD40 in mounting a splenic anti-VSV CTL response. In contrast, in the intestinal mucosa, although activated CD8 T cells appeared in the LP and IEL after infection of CD40$^{-/-}$ mice, the cells did not expand further to normal levels. This data suggested that the sustained response in the LP may not be due to continual migration from the periphery but due to additional costimulatory signals specific to the LP. As with VSV infection, the induction of primary splenic lymphocytic choriomeningitis virus (LCMV)-specific CD8 T cells in the absence of CD40-CD40L interactions is normal as measured by bulk CTL assays and enzyme-linked immunospot for IFN-γ (53, 60, 61). However, in one report, the primary CD8 T cell anti-LCMV response as measured by LDA was partially impaired in CD40L$^{-/-}$ mice (62) as was the generation of LCMV-specific memory CD8 cells (53, 62). This defect was likely due to impairment of CD4 help (53, 61, 62). In our studies, the reduced mucosal anti-VSV primary response in CD40$^{-/-}$ mice translated to severely diminished mucosal CD8 memory, while the normal splenic primary response resulted in normal splenic CD8 memory levels. We also observed a discordant recall response in the spleen vs the mucosa in CD40$^{-/-}$ mice (Fig. 7). Thus, the large recall response in the spleen did not result in a normal LP recall response. Taken together, these findings provided ample support for the hypothesis that the intestinal CD8 memory pool is maintained, at least in part, separately from the peripheral recirculating CD8 memory pool.

Although fewer memory cells were generated in the mucosa of CD40$^{-/-}$ mice, the fold increase of virus-specific CD8 cells within this site was equivalent to that in spleen following a secondary infection (Fig. 7). This result demonstrated that in comparison to Ag-specific LP CD8 T cells in the primary response, resident LP memory cells in vivo exhibited a significantly lower threshold of activation to secondary infection. The lack of a requirement for CD40 costimulation in the recall response may be related to the selection of a subset of clones with increased TCR affinity for MHC peptide (63, 64). Furthermore, this phenomenon may overcome the requirement for CD40-expressing APC and allow rapid reactivation outside of secondary lymphoid tissue. The ability of LP and IEL memory cells to be reactivated in situ perhaps by nonprofessional APC such as intestinal epithelial cells in the case of IEL makes teleological sense because this would allow a rapid secondary response to be mounted at a frequent site of pathogen entry. Our demonstration that numerous microbe-specific memory CD8 cells with lytic activity reside within the LP strengthens the argument that CD8 T cells at this site may serve a protective role against pathogen invasion and spread in the intestinal mucosa. This finding supports the hypothesis described for human CD4 T cells that central memory and effector memory subsets exist that may reside in distinct anatomical locations (65). Our findings also indicated the utility in pursuing CD8 T cell eliciting vaccines against enteric microbes, whether by oral or parenteral vaccination. This strategy might be especially useful against agents that have multiple serotypes that confound Ab-mediated protection. Future studies are needed to address the respective roles of systemic vs mucosal memory cells in antimicrobial immunity.

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