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Induction and Visualization of Mucosal Memory CD8 T Cells Following Systemic Virus Infection

Sung-Kwon Kim, Kimberly S. Schluns, and Leo Lefrançois

Whether CD8 T cell memory exists outside secondary lymphoid organs is unclear. Using an adoptive transfer system that enables tracking of OVA-specific CD8 T cells, we explored the antigenic requirements for inducing CD8 T cell memory and identified intestinal mucosa memory cells. Although systemic immunization with soluble OVA induced clonal expansion, memory CD8 cells were not produced. In contrast, infection with virus-encoding OVA induced memory CD8 cells in the periphery and the lamina propria and intraepithelial compartments of the intestinal mucosa. Mucosal memory cells expressed a distinct array of adhesion molecules as compared with secondary lymphoid memory cells, suggesting that there may be separate mucosal and systemic memory pools. Mucosal CD8 memory cells rapidly produced IFN-γ after Ag stimulation. Reactivation of memory cells by Ag feeding resulted in increased cell size and up-regulation of CD28 and CD11c. CD8 mucosal memory cells exhibited ex vivo lytic activity that was up-regulated dramatically following Ag reencounter in vivo. Interestingly, reactivation of memory cells did not require CD28-mediated co-stimulation. The ability of the intestinal mucosa to maintain CD8 memory cells provides a potential mechanism for effective mucosal vaccination. The Journal of Immunology, 1999, 163: 4125–4132.

Immunological memory is one of the main features of adaptive immunity and is characterized by a more rapid and intense response upon reexposure to the initial immunogen. By definition, memory lymphocytes are long-lived cells with heightened reactivity to Ag, and are distinguishable from effector cells that have a significantly shorter life span. However, recent studies on the humoral and cellular response to viral infection have revealed new aspects regarding the nature of memory lymphocytes. For memory CTL, a significant portion are in cell cycle (1, 2), and moreover, freshly isolated memory spleen CD8 T cells from LCMV-infected mice can kill target cells directly ex vivo (2, 3). CD8 memory to bacterial Ags can also be induced, as in the case of infection with Listeria and other intracellular bacteria (4–6). To date, little is known regarding mucosal memory responses as a consequence of bacterial infection. In the case of B cell memory, there has been some question as to whether long-lived effector cells maintain memory in the humoral response. In the LCMV system, plasma cells, which are fully differentiated effector cells, survive long-term in the bone marrow, where they apparently continuously produce Abs (7). The presence of neutralizing serum Abs is thought to be the most effective mechanism of protection against systemic reinfection with some viruses. Therefore, long-lived humoral protective immunity could be achieved with effector cells reserved in a specialized microenvironment, i.e., bone marrow for plasma cells. Such plasma cells may be analogues of memory CTL, which retain lytic capabilities, albeit at lower levels than that of effector cells. Whether there are distinguishable subsets of memory T cells in vivo and whether they may have distinct anatomic niches for long-term survival is not known.

The cellular and molecular basis of T cell memory remains poorly understood, and progress has been hampered by the inability to trace long-term Ag-specific memory T cells in vivo. Recently, the use of adoptive transfer of TCR transgenic T cells to normal mice (2, 8) and the production of tetrameric major histo-incompatibility Ag-peptide complexes (4, 9, 10) have allowed visualization of the fate of Ag-specific T cells in vivo. These systems provide the means for clarifying controversial issues regarding T cell activation and memory generation. In a few studies, Ag-specific memory T cells were defined and characterized based on their long-lived nature (2, 4, 10). The majority of those in vivo studies focused on memory T cells found in secondary lymphoid tissues, such as lymph nodes (LN) or spleen, but did not examine tertiary sites, such as the mucosa-associated lymphoid tissues. In one case, the presence of protective memory cells was not detected in the footpad of LCMV-infected mice after virus challenge at that site (11). This finding was used as evidence that persisting Ag is needed to maintain memory outside of secondary lymphoid tissues. However, the mucosa present unique anatomical and functional attributes as compared with other tissues. Because many pathogens gain access to the host through mucosal tissues, this could be an important site in which to focus immune memory responses. In fact, as previously suggested (12), in cases of mucosal infection, local clinical symptoms caused by infectious agents may occur rapidly such that reactivation of memory T cells in draining secondary lymphoid tissues and eventual recruitment of effector cells to the mucosa may be ineffective in controlling the initial infection. Thus, from the standpoint of protective immunity, memory T cell responses may be critical outside the secondary lymphoid tissues.

T lymphocytes in the intestinal mucosa exhibit functional and phenotypic characteristics of activated or memory cells (13–17).

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3 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; IEL, intraepithelial lymphocyte; LP, lamina propria; MLN, mesenteric lymph node; PLN, peripheral lymph node; PP, Peyer’s patches; sOVA, soluble OVA; VSV, vesicular stomatitis virus.

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The phenotypic attributes of intestinal T cells include low expression of CD62L (18) and high level expression of activation markers such as CD11c (17). Moreover, CD8 T cells, when freshly isolated from the intraepithelial lymphocyte (IEL) or lamina propria (LP) compartments, show constitutive cytolytic activity (16, 19). Although the precise origin of these CTL remains unclear, recent data suggest that TCRγδ IEL mature extrathymically, whereas the production of most TCRαβ IEL requires the thymus (20, 21). Thus, although TCRγδ IEL may be activated in the intestinal epithelium, it is possible that thymus-derived TCRαβ IEL are activated outside of the epithelium and traffic to the intestinal mucosa. This was originally implied by the pioneering studies of Sprent (22), in which activated, allorreactive T cells were tracked in vivo. In recent studies, we have used an adoptive transfer model in which OVA-specific CD8 T cells can be tracked, and we demonstrated that activation of CD8 T cells outside of the mucosa was required for entry of these cells into the LP and the epithelium (23). OVA-specific CTL activity was detected in the intestinal mucosa and was attributable to the migrating transgenic donor cells. The latter was true whether immunization was via virus infection or through injection of soluble OVA (sOVA) without adjuvant. In contrast, CTL activity in secondary lymphoid tissue was generated only in response to virus infection and not to sOVA. Thus, the intestinal mucosa provides a potentiating environment for CTL responses. In the present study, we have examined whether this environment is conducive to maintenance of long-term CD8 T cell memory. We find that memory CTL are retained in intestinal tissues and that such cells can be clearly distinguished from peripheral memory T cells by phenotype and function. These findings help explain the constitutive lytic activity of TCRαβ IEL and also indicate that systemic immunization could provide mucosal protection against certain pathogens.

**Materials and Methods**

**Mice**

C57BL/6 (Ly-5.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-Ly-5.2 mice were obtained from Charles River (Wilmington, MA) through the National Cancer Institute animal program. C57BL/6J (Ly-5.2) mice were obtained from Charles River (Wilmington, MA). C57BL/6-Ly-5.1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-Ly-5.2 mice were obtained from Charles River (Wilmington, MA) through the National Cancer Institute animal program.

**Adoptive transfer and immunization**

This method was adopted from Kearney et al. (8). A total of 2.5 × 10⁶ pooled LN cells from OT-I-RAG²/² (Ly-5.2⁺) mice was injected i.v. into C57BL/6 (Ly-5.1) mice. Two days later, 5 mg of OVA (grade VI; Sigma, St. Louis, MO) was administered by i.p. injection, or 1 × 10⁶ PFU of VSV or VSV-OVA were injected i.v. The production of rVSV-OVA has been described previously (23). Lymphocytes were isolated at the indicated times and analyzed for the presence of transferred cells by flow-cytometric detection of Ly-5.2⁺ cells. Ab treatments were performed by i.p. injection of 100 µg of CTLA4-Ig or the CTLA4-Ig mutant 104 as control (25), which were generously provided by Philip Morton (G. D. Searle, St. Louis, MO). Mutant 104 does not bind to B7-1 or B7-2, but retains FcR binding. Injections were given daily starting on the day of immunization.

**Isolation of lymphocyte populations**

IEL and LP cells were isolated as described previously (26, 27). For cytotoxicity assays, panning of Percoll-fractionated IEL on anti-CD8 mAb-coated plates was performed to remove contaminating epithelial cells. LN and spleens were removed, and single cell suspensions were prepared using a tissue homogenizer. PLN included brachial, axillary, and superficial inguinal nodes. The resulting preparation was filtered through Nitex (Tetko Industries, Kansas City, MO), and the filtrate was centrifuged to pellet the cells.

**Immunofluorescence analysis**

Lymphocytes were resuspended in PBS/0.2% BSA/0.1% NaN₃ (PBS/BSA/NaN₃) at a concentration of 1 × 10⁶–1 × 10⁷ cells/ml, followed by incubation at 4°C for 30 min with 100 µl of properly diluted mAb. The mAbs either were directly labeled with FITC, PE, or CyChrome, or were biotinylated. For the latter, avidin-Red 670 (Av-R670; Life Technologies, Gaithersburg, MD) was used as a secondary reagent for detection. After staining, the cells were washed twice with PBS/BSA/NaN₃, and fixed in 3% paraformaldehyde in PBS. Relative fluorescence intensities were then measured with a FACS Calibur (Becton Dickinson, San Jose, CA). Data were analyzed using Lysys II or WinMDI software.

**Measurement of cytolytic activity**

Cytolytic activity was measured using ³¹Cr sodium chromate-labeled EL4 cells (an H-2⁺ thymoma) with or without the addition of 10 µg/ml of the OVA-derived peptide SIINFEKL. Serial dilutions of effector cells were incubated in 96-well round-bottom microtiter plates with 2.5 × 10⁵ target cells for 6 h at 37°C. Percent specific lysis was calculated as: 100 × [{(cpm released with effectors) − (cpm released alone)}/(cpm released by detergent) − (cpm released alone)]

**Intracellular detection of IFN-γ**

Lymphocyte populations were isolated from unimmunized OT-I mice or from OT-I-transferred VSV-OVA-immunized mice 5 wk after infection. Cells were cultured in DMEM/5%/FCS/10% Nu Serum (Life Technologies, Gaithersburg, MD) was used as a secondary reagent for detection. After staining, cells were resuspended in PBS/0.2% BSA/0.1% NaN₃ (PBS/BSA/NaN₃) at a concentration of 1 × 10⁶–1 × 10⁷ cells/ml, followed by incubation at 4°C for 30 min with 100 µl of properly diluted mAb. The mAbs either were directly labeled with FITC, PE, or CyChrome, or were biotinylated. For the latter, avidin-Red 670 (Av-R670; Life Technologies, Gaithersburg, MD) was used as a secondary reagent for detection. After staining, cells were washed twice with PBS/BSA/NaN₃, and fixed in 3% paraformaldehyde in PBS. Relative fluorescence intensities were then measured with a FACS Calibur (Becton Dickinson, San Jose, CA). Data were analyzed using Lysys II or WinMDI software.

**Results**

**VSV-OVA immunization generates visible memory CD8 cells in the secondary lymphoid tissues and the intestinal mucosa**

To visualize Ag-specific CD8 T cell activation in a defined system, we used adoptive transfer of TCR transgenic CD8 T cells from the mouse line, OT-I. The majority of OT-I T cells express a TCR specific for an OVA-derived peptide in the context of H-2Kb (24). Donor cells are tracked by detection of differences in Ly-5.1 and Ly-5.2 expression between the donor and host. We previously showed that immunization of OT-I-transferred mice with either sOVA or VSV-OVA resulted in substantial clonal expansion in peripheral and mucosal tissues during primary responses (23). To determine the long-term outcome of the OT-I immune response to VSV-OVA or sOVA, animals were analyzed at late time points after adoptive transfer and immunization. Control mice were infected with wild-type VSV. During the primary response (day 6 in the experiment shown), Ag-specific clonal expansion of donor transgenic T cells occurs in PLN in response to VSV-OVA infection (Fig. 1) or sOVA immunization (23 and data not shown). Migration of donor OT-I T cells into the intestinal epithelium was only detectable in OVA-immunized mice and not in control mice. However, 35 days after immunization with sOVA or wild-type VSV, donor cells were not detectable in PLN and were barely detectable in IEL (Fig. 1). In contrast, OT-I cells were present in PLN and the intestinal mucosa, including the epithelium (Fig. 1) and LP (see below), of VSV-OVA-immunized mice. Phenotypic analysis demonstrated that donor cells detected in the LN and the intestinal mucosa expressed CD8ββ and the transgenic TCRαβ (data not shown). We defined these Ag-specific CD8 T cells...
been performed at least eight times with similar results. This experiment has been performed five times with comparable results. As indicated for day 35, left panels, few donor cells could be detected in mice receiving either wild-type VSV or sOVA. This experiment has been performed at least eight times with similar results.

A kinetic study was performed to compare the time course of the response and the longevity of the memory CD8 T cells in secondary lymphoid vs mucosal tissues. The peak of the primary response occurred in both sites at 4 days after immunization and was followed by rapid loss of donor cells in secondary lymphoid tissues and the intestinal mucosa, presumably due to activation-induced cell death (28). By day 16, the proportion of donor cells was 1–2% of total LN and spleen cells and 0.5 to 1% of the total IEL compartment, and these cells were either marginally larger or equal in size to naive OT-I cells (see below). These values represent 4–8% of CD8 T cells in LN and 3–6% of the CD8αβ IELs. After this time point, the percentage of OT-I memory cells remained relatively stable for at least 4 mo (Fig. 2), suggesting that ~2 wk after immunization, the residual cells could be classified as memory cells. Indeed, we have detected memory cells in the mucosa 1 yr after immunization (data not shown).

Intestinal mucosa-specific memory T cells are phenotypically distinguishable from secondary lymphoid memory T cells

Having defined memory CD8 populations in distinct anatomic locations, we wished to determine whether each population could be distinguished by phenotype. Therefore, we analyzed the expression of activation and adhesion molecules by OT-I memory T cells from the LN and the intestinal mucosa. Memory cells from LN or intestine expressed comparable levels of CD8αβ and TCR (data not shown). However, striking differences were noted in expression of certain adhesion molecules by LN vs intestinal mucosa-specific memory T cells (Fig. 3, A and B). CD44 was rapidly up-regulated on OT-I cells in gut, LN (Fig. 3A), and spleen (data not shown) after primary activation, and the level of CD44 expression remained high in LN-specific memory T cells (Fig. 3B). In contrast, memory OT-I T cells found in the IEL compartment exhibited more heterogenous expression of CD44 (Fig. 3B). OT-I memory cells in LP had CD44 levels only marginally lower than that of splenic and LN memory OT-I cells. Similar to naive host CD8 T cells, CD62L expression was heterogenous among naive PLN OT-I T cells (Fig. 3A). Upon primary activation, two distinct populations (CD62L high and CD62L low/intermediate) were detectable in PLN (Fig. 3A) and in the spleen, although in the latter CD62L low cells made up a larger portion of the cells as compared with those in LN (data not shown). Multiparameter analysis showed no difference in other activation Ag expression (CD44 and CD11a) between these two populations. In contrast, primary activated as well as memory mucosal OT-I cells for the most part lacked CD62L, although a small population of CD62L+ cells was present (Fig. 3). These phenotypes were identical with those of subsets of endogenous host IEL and LP populations.

The α8β7 integrin is expressed at high levels by most IEL and at lower levels by a subset of peripheral CD8 T cells in normal mice (29). Similar to the latter, naive OT-I LN cells expressed heterogenous levels of α8β7, with many cells lacking this integrin. Primary activated and memory LN OT-I cells expressed low levels of α8β7, indicating a loss of α8β7high cells from LN (Fig. 3, A and B). Interestingly, after primary activation, most LP OT-I cells...
lacked $\alpha_\beta_7$, while a distinct population of OT-I IEL had heterogeneous high levels of $\alpha_\beta_7$. This result suggested either that rapid up-regulation of $\alpha_\beta_7$ had occurred on OT-I IEL or that cells expressing $\alpha_\beta_7$ preferentially migrated to the epithelium. However, the majority of IEL-specific OT-I memory cells expressed high levels of $\alpha_\beta_7$ integrin, while LP memory cells contained discernible $\alpha_\beta_7$ high and $\alpha_\beta_7$ low populations, suggesting that up-regulation of $\alpha_\beta_7$ occurred after entry of cells into the mucosa. Overall, the memory T cells found in each location resembled subsets of the resident CD8 populations with regard to adhesion molecule expression.

Memory CTL exhibit lytic activity that can be up-regulated by reexposure to Ag

Memory T cells from LCMV-immune mice exhibit low levels of cytolytic activity (3). However, whether this is a generalizable attribute of CD8 memory cells is unclear. This is an important issue because more effective protection should be achieved if memory CD8$^+$ T cells can kill virus-infected cells immediately without a lengthy reactivation process. Therefore, we tested the lytic activity of mucosal and peripheral CD8 memory cells by performing cytotoxicity assays on ex vivo LN, spleen, and IEL populations containing OT-I memory T cells. Because the actual number of potential effectors was determined by flow cytometry, lytic activity on a per cell basis could be compared. At low E:T ratios (i.e., 1:1),
staining for IFN-γ with or without OVA peptide in vitro for 5 h, followed by intracellular staining for IFN-γ. No staining was observed with an isotype-matched control mAb under any conditions (data not shown). This experiment has been performed twice with similar results.

Little OVA-specific killing activity was detected from MLN OT-I memory T cells (Fig. 4A), whereas at higher ratios (>10:1) MLN lytic activity could be detected (data not shown). In contrast, lytic activity of splenic and IEL OT-I memory cells was detectable at 1:1 E:T cell ratios, and higher ratios resulted in substantial lytic activity (Fig. 4A).

To determine the functional consequences of reactivation of memory OT-I T cells in the intestinal mucosa and secondary lymphoid organs, we fed VSV-OVA immune mice 10 mg of sOVA. Feeding was chosen because a potent neutralizing Ab response against VSV glycoprotein prevents reinfection, and because it was of interest to test the effect of oral administration of Ag on mucosal memory T cell responses. Two days after feeding, lymphocytes were isolated from PLN, MLN, spleen, and the intestinal mucosa and were analyzed for donor OT-I cell number. The size and lytic activity of the memory cells before and after secondary Ag encounter were assessed. At this time point, no significant increase in donor OT-I cell numbers was observed. However, memory cells were reactivated, as evidenced by a dramatic increase in cell size in all tissues (Fig. 4B). We then asked whether secondary challenge resulted in an increase in the cytotoxicity of memory CD8 T cells. As shown in Fig. 4A, oral OVA administration induced a substantial increase in the lytic activity of memory cells in MLN, spleen, and IEL. In the absence of OT-I T cell transfer, minimal lytic activity was detected in this or in the primary response at the E:T ratios used, presumably due to the low precursor frequency of endogenous OVA-specific T cells (data not shown). Thus, VSV-OVA-reactive OT-I memory cells in intestinal mucosa and in the periphery can be reactivated by oral administration of sOVA.

In addition to cytotoxic activity, we analyzed the production of IFN-γ following reactivation of memory OT-I cells in vitro (Fig. 5). After short-term culture (5 h) of naive or memory splenic or LP OT-I cells in the presence or absence of the antigenic peptide SIINFEKL, intracellular IFN-γ levels were measured by flow cytometry. Without the addition of peptide, neither naive nor memory OT-I cells produced detectable IFN-γ. Memory cells isolated from the spleen or from LP, but not naive splenic OT-I cells, rapidly up-regulated production of IFN-γ after restimulation with antigenic peptide. Although naive OT-I cells challenged with Ag did not produce detectable intracellular IFN-γ, >95% of memory OT-I cells contained significant levels of IFN-γ. Similar results were obtained using memory cells derived from LN or IEL (data not shown). These results indicate that mucosal and systemic memory CD8 T cells were functionally similar with regard to rapid up-regulation of lytic activity and IFN-γ production following Ag reencounter.

CD28-mediated costimulation is not required for reactivation of mucosal or peripheral memory CD8+ T cells

We previously reported that naive LN OT-I CD8+ T cells are heavily dependent on the costimulatory molecule B7-2 for primary in vivo activation (23). Although it has been implied that reactivation of memory cells may have less stringent requirements for CD28-mediated costimulation, little is known about the role of costimulation during in vivo reactivation of CD8 memory T cells. To test this, concomitant with feeding of OVA, mice containing OT-I memory cells were treated with CTLA4-Ig to block B7-1 and B7-2 interactions with CD28, or as a control a CTLA4-Ig mutant.
that does not bind B7-1 or B7-2. In the MLN, OT-I memory cells were reactivated after Ag feeding and CTLA4-Ig mutant treatment, as evidenced by a major increase in cell size and by up-regulation of CD28, CD11c (Fig. 6), and CD44 (data not shown). However, CTLA4-Ig treatment had little effect on the increase in cell size, indicating that CD28-mediated costimulation was not required for optimal reactivation (as measured by blastogenesis) of MLN CD8 memory cells by soluble Ag. In addition, CD28 up-regulation was not affected and CD11c induction was only partially inhibited by CTLA4-Ig treatment. This result suggested that the up-regulation of CD28 was TCR mediated and costimulatory molecule independent. Similar results were obtained from analysis of splenic OT-I memory cells (data not shown). Furthermore, intestinal mucosal memory CD8 cell reactivation was largely unaffected by CTLA4-Ig treatment (Fig. 7). The reactivation of IEL memory cells was unaffected, and reactivation of LP memory CD8 T cells was only marginally inhibited by CTLA4-Ig treatment, as measured by cell size increase. CD28 and CD11c up-regulation on LP and IEL OT-I memory cells was not inhibited by CTLA4-Ig treatment. The CTLA4-Ig preparations employed were active because they effectively inhibited primary OT-I activation and expansion in IEL as well as all other tissues examined after feeding OVA (Fig. 7, E and F, and data not shown). To determine whether the reactivation of lytic activity was affected by B7 blockade, we tested CTL activity in control and CTLA4-Ig-treated mice that had been fed Ag (Fig. 8). Although there was substantial up-regulation of lytic activity of splenic CD8 memory cells after Ag reencounter, no inhibition of this reactivation by CTLA4-Ig treatment was evident (Fig. 8). Similar results were obtained using cells from LN or IEL (data not shown). Thus, costimulation was not required for reexpression of phenotypic and functional modifications after secondary Ag encounter. These results indicated that the stringency of costimulatory requirements for memory CD8 T cell reactivation was distinct from that of naive CD8 T cells and enforced the concept of a rapid recall response by memory CTL.

Discussion

Our findings demonstrated that long-lived memory cells can be constituents of not only secondary lymphoid organs, but of the intestinal mucosa as well. Previous work has been equivocal on this point. Rotavirus-specific primary CTL, but not memory CTL, were detected in IEL, although memory CTL were found in the periphery (30). In contrast, antireovirus CTL precursors were present in IEL 4 wk after oral infection (31). In addition, mucosal immunization of BALB/c mice with an HIV multideterminant peptide resulted in long-term systemic and LP memory CTL, as assessed by in vitro reactivation and cytotoxicity assays (32). The possible presence of memory IEL was not tested. In another system, systemic or nasal immunization with recombinant adenovirus expressing the herpes simplex virus glycoprotein B resulted in induction of memory in systemic or genital-associated lymphoid tissue, depending on the route of immunization (33). These studies suffer from the inability to track Ag-specific T cells and obtain...
information on cell numbers, phenotype, and the relationship between peripheral and mucosal CTL. Furthermore, in some cases, the results are dependent on the in vitro reactivation and culture of CTL precursors from LP and IEL. These lymphocyte populations are notoriously poor responders to proliferative stimuli (34, 35). The adoptive transfer system has allowed us for the first time to visualize and localize Ag-specific mucosal memory CD8 T cells to the respective anatomic locales. Our studies demonstrate that a major portal of entry for pathogens, the intestinal mucosa, harbors a pool of CD8 memory cells at the ready for a recall response.

The issue of maintaining memory has been of great interest because it is critical to vaccine efficacy. The relative importance of intrinsic and extrinsic signals in memory T cell survival has not been fully delineated. Accumulating data suggest that memory T cells are not dormant, but in constant cycle (1, 2, 36), although the signals that induce memory T cell division are not entirely clear. Persistence of cognate or cross-reactive Ags, continuous engagement of the TCR via MHC, and nonspecific inflammatory cytokines have been proposed to be involved in the maintenance of CD8 memory (reviewed in Refs. 37 and 38). Current concepts suggest that long-term memory does not require persistence of Ag, but that continuous interaction with MHC class I molecules is essential to CD8 memory maintenance (38). Because CD8 memory cells are generally not found in nonmucosal tertiary tissues, except perhaps liver, our results suggest that the intestinal mucosa may provide organ-specific factors that aid in maintaining CD8 memory. This effect could be related to the inflammatory nature of the gut and the ability of the mucosa to induce CTL even when soluble Ag is used as immunogen. As we have previously shown, compared with peripheral T cell activation, costimulation via B7-1 is more important for CD8 T cell activation in the intestinal mucosa (23), and this and other constitutive costimulatory signals may be important in maintaining memory.

The lack of a requirement for B7 in reactivation of CD8 memory cells may be explained at the level of the APC or the T cell. Our results indicated that secondary lymphoid memory cells could mount a rapid recall response in the face of B7-1,2 blockade, even though presumably professional APCs had processed and presented intact OVA. In the case of IEL memory cells, reactivation by oral administration of Ag implied, but did not prove, that intestinal epithelial cells can act as APC in vivo, at least for presentation to memory cells. This hypothesis was supported by the costimulation independence of IEL memory cells, because normal IEC do not express B7 molecules (39) and there is scant evidence to indicate that APC reside in the mouse epithelium. There is little available information on costimulatory requirements for CD8 memory T cell reactivation in vivo. Reactivation of alloreactive CTL does not require costimulation in vitro (40), but whether this holds true in vivo is not known. The form of the reactivating Ag may also be important for the long-term outcome of the recall response in that reactivation during microbial infections rather than via administration of purified protein may provide additional signaling. Nonetheless, our results open the possibility that boosting of a T cell memory response could be induced using oral Ag, which would be undesirable when attempting to induce oral tolerance. We are currently testing the long-term effects of oral Ag dosing on memory T cells.

Although it has been known for many years that the phenotype of LP and IEL CD4 and CD8 populations was suggestive of prior Ag exposure (13–17), the origin, life span, and Ag reactivity of such memory cells have not been defined. This has been particularly true for IEL, whose origin has been a matter of considerable debate (20, 21). In addition, the poor proliferative response and in some cases unique costimulatory requirements of these cells have distinguished them from more traditional activated or memory cells present in the secondary lymphoid organs (34, 35, 41–43). Our current and previous results indicate that a specific subset of CD8αβ TCRαβ IEL is derived from the peripheral T cell pool following activation (19, 23, 44). Thus, CD11a high, αβ low, CD62L high/intermediate cells appear to be recent arrivals in the intestinal mucosa. Our results would also suggest that a subset of recent immigrants generates long-lived memory cells that remain within the mucosa and express high levels of αβ integrin. Studies with parabiotic mice show that a small population of TCRαβ cells of unknown origin accumulates in the intestinal epithelium over time (45), and these may represent memory cells and/or recently activated T cells. However, the discrepancies in adhesion molecule expression between the majority of memory OT-I CD8+ T cells in the secondary lymphoid tissues and those in the intestinal mucosa suggested that these populations were distinct and were not part of a common pool. Comparison of the percentage of OT-I memory T cells in the LN or the spleen and the intestine in single mice supports this idea. That is, in some cases, highly disparate percentages of donor memory OT-I T cells were detected in the periphery as compared with the LP and IEL compartments, suggesting that these populations were separately maintained or had discordant life spans. Detailed trafficking studies will be needed to determine the precise relationship between peripheral and mucosal memory T cells.

The constitutive lytic activity of CD8 memory cells in the mucosa, as shown in this study, helps explain the constitutive lytic activity of IEL and LP CD8 T cells. Our original description of direct ex vivo lytic activity of TCRαβ and TCRγδ IEL utilized a redirected lysis assay that bypasses TCR specificity (16, 46). This assay is necessary to identify CTL in normal mucosa because the Ag specificity of these cells is unknown. The lytic activity of TCRαβ IEL is primarily contained within the Thy-1+ CD8αβ subset (34). Mucosal memory CTL generated in the OT-I transfer system retain Thy-1 and CD8αβ, suggesting that this population in normal mice contains substantial numbers of memory CTL. Induction of expansion of CD8αβ IEL and up-regulation of lytic activity are dependent on intestinal microbial flora (16, 47, 48). Thus, it appears likely that a subset of CD8αβ IEL and LP cells in healthy animals is comprised of bacterial Ag-specific primary effector cells and memory cells. In the face of systemic or mucosal infection or vaccination, Ag-specific effector cells would be generated in MLN, Peyer’s patches (PP), or spleen, resulting in migration to intestinal mucosa and generation of long-term memory. That some IEL are derived from PP has been a longstanding theory (49). Indeed, we observed activation of OT-I cells in PP after immunization, but the population of activated OT-I cells was always quantitatively larger in MLN than in PP (S.-K. Kim and L. Lefrançois, unpublished results). It is perhaps likely that CD8 cells in both of these sites contribute to the activated T cell pool following mucosal immunization. In any case, learning how to potentiate mucosal CD8 effector and memory responses will provide tools for improved vaccination against mucosal pathogens such as HIV.

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