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Soluble Antigen and CD40 Triggering Are Sufficient to Induce Primary and Memory Cytotoxic T Cells

Leo Lefrançois, John D. Altman, Kristina Williams, and Sara Olson

The signals directing induction of tolerance rather than immunity are largely unknown. The CD8 T cell response to soluble Ags generally results in deletional tolerance following transient, costimulation-dependent activation. We demonstrated that CD40 signaling reversed the outcome of this response. Adoptive transfer of OVA-specific CD8 T cells followed by soluble OVA immunization resulted in induction of lytic activity and optimal clonal expansion only when CD40 was triggered via an agonistic mAb. Activation of CD8 T cells by CD40 signaling was indirect, because CD40 expression by host cells was required. CD40 signaling along with soluble Ag immunization also induced expansion of secondary lymphoid and intestinal mucosal endogenous OVA-specific CD8 T cells as detected by MHC tetramer reactivity. When CD40 activation was included, long-lived secondary lymphoid and mucosal memory CD8 cells were generated from adoptively transferred and endogenous CD8 T cells. Mucosal and peripheral CD8 memory cells exhibited constitutive Ag-specific lytic activity, with mucosal memory cells being 10-fold more lytic than splenic or lymph node memory cells. These results demonstrated that CD40 signaling during a response to a poorly immunogenic soluble Ag was necessary and sufficient for CTL and memory T cell induction. The Journal of Immunology, 2000, 164: 725–732.

The long-term outcome of an immune response is determined by multiple factors, some of which remain undefined. Whether a T cell response results in induction of immune memory or in deletion or tolerance of T or B cells is determined in part by the form of Ag used for immunization (1, 2). In the case of CD8 CTL, viral infections generally result in production of long-term memory cells residing in the secondary lymphoid tissues, such as the spleen and lymph nodes (LN) (3, 4). In contrast, when the cognate antigenic peptide is used for immunization, a primary proliferative response is followed by deletion or anergy of Ag-specific CD8 T cells (5, 6). One of the factors governing the distinction between a productive CTL response induced by viral infection and a nonproductive response induced by tolerogenic forms of Ag may be the type or level of costimulation received during primary Ag recognition (7). Professional APC such as dendritic cells (DC) constitutively express the costimulatory molecules CD40 and B7 (8). DC activation, such as may occur in viral or bacterial infections, results in up-regulation of these molecules, which may be required for induction of a productive immune response (9). Processing by DC of noninflammatory forms of Ag, as in the case of soluble proteins, may not result in modulation of costimulators and therefore does not induce complete immunity.

Recently, the concept of effective help for CD8 T cell responses has been explored with regard to the involvement of CD40-CD40L interactions (10–14). CD40 engagement induces a prolonged CD8 T cell response in a model of graft-vs-host disease (14) and also delays superantigen-mediated deletion of CD4 and CD8 cells (15). In addition, in certain CD8 T cell responses that require CD4 T cells to prime for CTL induction, CD40-CD40L interactions are involved. CD40L is up-regulated following activation of CD4 (16–18) and at least some CD8 T cells (19–21). In this scenario, an Ag-specific CD4 T cell interacts with a DC and delivers a signal via CD40L to the DC, which allows that APC to become competent to drive CTL responses. A subsequent encounter of an Ag-specific CD8 T cell with the empowered DC will result in CTL priming (11). The factors that empower the DC are unknown, but it is known that CD40 triggering can up-regulate costimulatory molecules and inflammatory cytokines such as IL-12 (22).

The essential costimulatory requirements for generation of T cell memory are not defined. Although CD28-B7 interactions are necessary for primary T cell activation (7, 23, 24), and therefore generation of memory, the signals that distinguish nonproductive primary activation from induction of long-term immunity remain unclear. For example, primary activation of OVA-specific TCR transgenic CD8 T cells by soluble Ag requires CD28/B7-2 interaction, yet this reaction does not result in production of memory T cells (23). Similar results have been obtained in studies of activation of CD4 T cells (25, 26). Thus, additional costimulatory signals appear to be requisite for memory T cell induction. Considering the importance of CD40-CD40L interactions in development of B cell memory (17, 18), we wished to determine whether CD40 signaling was effective in induction of CD8 T cell memory. Using a T cell adoptive transfer system as well as visualization of endogenous Ag-specific T cells using MHC/peptide tetramer reagents, we demonstrate in this study that CD40 triggering along with soluble Ag immunization is sufficient for induction of CD8 memory T cells in secondary lymphoid tissues and in mucosal effector sites.

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1 Abbreviations used in this paper: LN, lymph node; DC, dendritic cell; CD40L, CD40 ligand; IEL, intraepithelial lymphocyte; LP, lamina propria; MLN, mesenteric lymph node; PLN, peripheral lymph node; sOVA, soluble OVA; APC, allophycocyanin.
Materials and Methods

Mice

C57BL/6j (Ly-5.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), C57BL/6Tac/Bc(KO)Ab2 mice (27) were purchased from Taconic (Germantown, NY). C57BL/6-Ly-5.2 mice were obtained from Charles River (Wilmington, MA) through the National Cancer Institute animal program. The OT-I mouse line was generously provided by W. R. Heath (WEHI, Parkville, Australia) and F. Carbone (Monash Medical School, Prahran, Victoria, Australia) (28) and was maintained as a C57BL/6-Ly-5.2 or C57BL/6-Ly-5.1 line on a Rag-1- background. C57BL/6-CD40+ mice (29) were generously provided by Dr. Hitoshi Kikutani (Osaka University, Osaka, Japan) via Dr. Nancy Philips (University of Massachusetts Medical Center, Worcester, MA).

Adoptive transfer

This method was adopted from Kearney et al. (30). A total of 2 × 10⁶ pooled CD8 LN cells from OT-I-RAG+ (Ly-5.1 or Ly-5.2) mice were injected i.v. into C57BL/6 (Ly-5.1 or Ly-5.2) mice. Two days later, 5 mg of OVA (grade VI, Sigma, St. Louis, MO) was administered by i.p. injection. Lymphocytes were isolated at the indicated times and analyzed for the presence of transferred cells by flow-cytometric detection of Ly-5 differences. Ab treatments were performed by i.p. injection of 100 μg of anti-CD40 mAb (clone 3/23) (31) or rat Ig as control. Each experiment was performed a minimum of three times.

Detection of OVA-specific primary and memory CD8 T cells with MHC tetramers

Mice were immunized by i.p. injection of 5 mg OVA with 100 μg anti-CD40 mAb or 100 μg control rat Ig. At the indicated times, lymphocytes were isolated and OVA-specific CD8 T cells were detected using H-2Kb tetramers containing the OVA protein-derived peptide SIINFEKL (32) or the vesicular stomatitis virus N protein-derived peptide RGYVYQGL. MHC tetramers were produced essentially as previously described (33, 34).

Isolation of lymphocyte populations

EL and LP cells were isolated as described previously (35, 36). For cytotoxicity assays, panning of Percoll-fractionated EIL 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 Nitiex, 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 were either directly labeled with FITC, PE, Cy5, APC, or were biotinylated. For the latter, avidin-PE-Cy7 (Caltag Laboratories, Burlingame, CA) was used as a secondary reagent for detection. For tetramer staining, cells were first reacted with PE-labeled anti-CD8α (Caltag Laboratories) and FITC-labeled anti-CD11a (PharMingen, San Diego, CA). 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 WinMDI software (Joseph Trotter; Scripps Clinic, La Jolla, CA).

Measurement of cytolytic activity

Cytolytic activity was measured using ⁵¹Cr sodium chromate-labeled EL4 cells (an H-2b 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 × 10⁴ 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).

Results

CD40 triggering induces primary expansion of CD8 T cells

The adoptive transfer system allows the determination of the effect of CD40 triggering on Ag-specific clonal expansion and lytic activity on a per cell basis in vivo. Activation of OVA-specific OT-I TCR transgenic T cells with soluble OVA (sOVA) in the absence of adjuvant results in clonal expansion in the periphery (23). To determine whether CD40 signaling could affect this proliferation, we tracked OT-I T cells in PBL of cohorts of mice that received 100 μg of an agonistic anti-CD40 mAb (31) or a control Ab simultaneous with 5 mg sOVA by i.p. injection. PBL from nonimmunized mice contained on average 0.15% OT-I T cells. Interestingly, 24 h after immunization, irrespective of whether anti-CD40 mAb was administered, OT-I T cells disappeared from the circulation. This finding resembles a phenomenon in which alloreactive lymphocytes disappear from thoracic duct lymph after injection of allogeneic cells (37) and may reflect sequestration of Ag-specific cells in secondary lymphoid organs. Between 48 and 72 h, OT-I cells reappeared in the blood (Fig. 1A). At 72 h after immunization, percentages of OT-I cells had increased 20-fold in control mice (3.1 ± 1.1) and 31-fold in anti-CD40 mAb-treated mice (4.7 ± 0.9). However, by day 4, an exceptional increase in OT-I cells to 29.3 ± 6.6% of PBL had occurred in mice treated with anti-CD40 mAb as compared with control mice in which PBL contained 2.9 ± 1% OT-I cells (Fig. 1B). As little as 12.5 μg of anti-CD40 mAb was sufficient to induce this increase (data not shown). The proliferative response reached a peak on day 5, with 49.1 ± 5.6% of PBL made up by OT-I T cells. By day 6, the response in anti-CD40 mAb-treated and control mice had begun to decline and OT-I cells were essentially undetectable in control mice by day 18 (<0.1% of PBL). However, PBL from the anti-CD40 mAb-treated mice contained detectable OT-I cells for 30 days (4.4 ± 2.2%) and longer (see below). This response required the presence of Ag because, in the absence of sOVA, anti-CD40 mAb had no effect on OT-I T cells (data not shown).

Although we have observed no unusual effects of OT-I transfer and activation, it was possible that the large number of activated cells was influencing clonal expansion. To test whether CD40 triggering could induce growth of endogeneous OVA-specific CD8 T cells, we used MHC class I/OVA peptide tetramers to monitor the anti-OVA CD8 T cell response. Normal B6 mice were immunized with sOVA with or without the addition of anti-CD40 mAb. Lymphocytes were isolated 5 days later and stained with H-2Kb/SIINFEKL tetramers (Fig. 2). Immunization with sOVA did not result in the obvious appearance of tetramer+ CD8 T cells. It is possible that a very small number of cells were Ag specific, but the staining profile in this case was indistinguishable from that of unimmunized mice (Fig. 2 and data not shown). In contrast, including anti-CD40 mAb in the immunization protocol induced expansion of a significant population of tetramer-reactive cells that comprised 0.6% of total splenocytes. With anti-CD40 mAb included, appearance of tetramer+ cells was detectable at a dose of 0.5 mg OVA (data not shown). OVA-specific CD8 T cells also appeared in the intestinal mucosa, a site that normally contains high percentages of activated T cells. IEL and LP lymphocytes contained 0.9% and 1.9% tetramer+ cells, respectively. When CD8+ cells were gated and analyzed for CD11a expression, all of the Ag-specific cells expressed high levels of CD11a. Furthermore, this analysis indicated that ~10% of the CD8β+ cells in spleen and LP and ~5%
of the CD8<sup>+</sup> IEL were OVA specific. Interestingly, the endogenous response was reduced in the presence of transferred OT-I cells, presumably due to competition in the response by the much larger number of Ag-specific transferred cells (data not shown). These results along with those from the adoptive transfer studies indicated that CD40 signaling delivered a powerful proliferative signal to CD8 T cells.

**CD40 expression by host cells is required for CD8 T cell expansion**

Because CD8 T cells can express CD40 (31), it was important to determine whether the anti-CD40 mAb treatment had a direct or indirect effect on Ag-induced OT-I T cell expansion. To test this, OT-I cells were transferred to CD40<sup>−/−</sup> mice. Two days after transfer, the mice were immunized with sOVA with or without anti-CD40 mAb treatment. PBL and mesenteric LN (MLN) cells were then analyzed for the presence of OT-I cells. Without immunization in B6 mice, OT-I cells made up 0.4% and 0.1% of MLN cells and PBL, respectively (Fig. 3), and these percentages were similar in naive CD40<sup>−/−</sup> mice (data not shown). Naive OT-I cells expressed heterogenous amounts of CD44 (Fig. 3). Five days after immunization of B6 mice without addition of anti-CD40 mAb, OT-I cells comprised 1.2% and 1.4% of MLN cells and PBL, respectively, and all of the cells had high CD44 levels. However, much larger populations of OT-I cells were present in both sites when sOVA-immunized B6 mice were treated with anti-CD40 mAb with 11% of MLN cells and 50% of PBL bearing Ly-5.2 and high levels of CD44. In contrast, immunization with anti-CD40 mAb treatment of CD40<sup>−/−</sup> mice harboring OT-I cells did not result in an increase in OT-I cells as compared with immunization with sOVA alone. Therefore, the observed effect of anti-CD40 mAb on CD8 T cells was not due to direct effects of the mAb on the CD8 T cells, but rather on host cells, most likely Ag-bearing APC, as previously proposed (11–13).
CD40 triggering is sufficient to induce CTL against soluble Ag

In light of our results showing that activation of peripheral OT-I cells with sOVA induced proliferation but not lytic activity (23), we tested whether CD40 triggering would allow induction of CTL activity in this situation. As shown in Fig. 4, A and C, sOVA immunization resulted in expansion of OT-I cells in PLN, but minimal CTL induction by 3 days after immunization. E:T ratios are based on the actual number of OT-I cells. Even at later time points (4 to 10 days) after immunization, CTL activity was low in peripheral lymphoid organs (data not shown). Injection of the agonistic anti-CD40 mAb during immunization did not greatly affect the percentage of transferred OT-I cells at this time point (Fig. 4A). However, CD40 triggering resulted in a remarkable induction of CTL activity (Fig. 4C) that was attributable to the transferred OT-I cells. In addition, when immunization of unmanipulated mice with sOVA included anti-CD40 mAb, OVA-tetramer+ CD8 T cells were present in the spleen 6 days later, but were not detectable without anti-CD40 mAb treatment (Fig. 4B). These OVA-specific endogenous CD8 T cells were also potent effectors. On a per cell basis, endogenous OVA-specific CTL had lytic activity comparable with that of OT-I cells (Fig. 4C). Thus, CD40 activation functioned not only at the level of clonal expansion, but at the level of CTL differentiation.

Because CD4 T cells are required to provide help for some CD8 responses, we wished to determine whether CD4 cells were involved in the anti-CD40 mAb-induced activation of OT-I cells. To this end, OT-I cells were transferred to mice lacking MHC class II-restricted CD4 T cells by virtue of a lack of expression of the I-A\(^d\) protein. Two days later, mice were immunized with sOVA with or without the addition of anti-CD40 mAb. Three days later, lymphocyte populations were analyzed for donor cell numbers and for lytic activity. The absence of MHC class II had no effect on the expansion of OT-I cells in control mice or in mice treated with anti-CD40 mAb at this or later time points (data not shown). Similarly, high levels of lytic activity were detected in MLN cells from anti-CD40 mAb-treated B6 or MHC class II-deficient mice (Fig. 5). These results indicated that the induction of proliferation and lytic activity of primary activated OT-I cells by CD40 triggering did not require MHC class II-restricted CD4 T cells.

Soluble Ag and CD40 activation are sufficient to generate CD8 memory T cells

The eventual outcome of activation of OT-I cells via immunization with sOVA in the absence of CD40 triggering was deletion of the
By day 14 after immunization, few, if any, OT-I cells were detectable in PBL (Fig. 1). However, when anti-CD40 mAb was included in the immunization regimen, OT-I cells were present in PBL after 30 days (Fig. 1). We tested whether CD40 triggering in the presence of Ag was sufficient to generate long-term CD8 memory cells in secondary lymphoid tissues (Fig. 6). We also determined whether OT-I memory cells were present in the intestinal mucosa because many cells in this site phenotypically resemble memory cells (38–41). At $10\text{ wk}$ after OT-I transfer and immunization with sOVA, donor cells were not detectable in the spleen or the IEL population. In striking contrast, a substantial population of OT-I cells was present in the spleen and IEL compartment of mice immunized with sOVA and treated with a single 100 $\mu\text{g}$ injection of anti-CD40 mAb (Fig. 6). In several mice tested, the percentage of OT-I memory cells varied from $0.8–5\%$ of spleen, LN, or intestinal LP lymphocytes, or of IEL (data not shown).

The homogenous avidity of a single TCR, as is the case with OT-I cells, could influence the outcome of the response against OVA. To determine whether CD40 signaling could drive a heterogeneous memory CD8 T cell response to OVA, we immunized normal B6 mice with OVA and simultaneously administered 100 $\mu\text{g}$ of agonistic anti-CD40 mAb. The presence of OVA-specific memory cells was then determined using H-2Kb-OVA peptide tetramers (Fig. 7). CD11a expression, which is increased on CD8 memory cells, was also analyzed. In mice that had been immunized with OVA and treated with control Ig, OVA-specific CD8 cells could not be found in spleen or LP. In contrast, a population of tetramer$^+$, CD11a$^\text{high}$, CD8 cells was readily detectable in spleen and LP of mice treated with anti-CD40 mAb and sOVA. The number of memory cells did not change appreciably from 30 to 142 days after immunization, indicating the stability and longevity of the memory pool. No staining was observed using a control tetramer containing a vesicular stomatitis virus N peptide (Fig. 7). Thus, CD40 activation alone was able to drive endogenous memory cell induction against a poorly immunogenic soluble protein.

To support the contention that the long-lived OT-I cells from CD40-activated mice were bona fide memory cells, we examined their phenotype and size. Although primary activated OT-I cells were significantly larger than naive OT-I cells, splenic OT-I cells $10\text{ wk}$ after immunization were essentially the same size as naive OT-I cells (Fig. 8). However, unlike naive OT-I cells, the long-lived OT-I cells expressed high levels of CD44, as did primary activated OT-I cells. Similar results were obtained from analysis of endogenous tetramer$^+$ memory cells (data not shown). These results indicated that CD40 triggering in the presence of soluble Ag resulted in induction of typical long-term CD8 memory cells in peripheral and mucosal tissues.

FIGURE 6. CD40 signaling and soluble Ag immunization induce OT-I memory cells. A total of 2 \times 10^6 CD8 OT-I LN cells (Ly-5.1) were transferred to B6 (Ly-5.2) mice. Two days later, mice were immunized with 5 mg OVA by i.p. injection with the inclusion of 100 $\mu\text{g}$ of anti-CD40 mAb or control rat Ig (sOVA panels). Ten weeks after immunization, spleen cells or IEL were analyzed for the presence of donor OT-I cells and CD8 expression by fluorescence flow cytometry.

FIGURE 7. CD40 signaling and soluble Ag immunization induce endogenous OVA-specific CD8 memory cells. B6 mice were immunized i.p. with 5 mg sOVA with the inclusion of 100 $\mu\text{g}$ of anti-CD40 mAb or control rat Ig (rIg). Lymphocytes were isolated at the indicated times from the spleen or LP, and three-color flow cytometry was performed using APC-labeled SIINFEKL/H-2Kb (OVA-tet-APC) or RGYVYQGL/H-2Kb (N-tet-APC) tetramers, anti-CD8a PE, and anti-CD11a FITC. The plots show analysis of gated CD8$^+$ cells.

FIGURE 8. CD40 activation generates typical CD8 memory cells. A total of 2 \times 10^6 CD8 OT-1 LN cells (Ly-5.1) were transferred to B6 (Ly-5.2) mice. Two days later, mice were left untreated (naive) or were immunized with 5 mg OVA by i.p. injection with the inclusion of 100 $\mu\text{g}$ of anti-CD40 mAb. Five days (naive or activated) or ten weeks (memory) after immunization, spleen cells were analyzed for the presence of donor OT-I cells and CD8 and CD44 expression by fluorescence flow cytometry. The data shown are from gated Ly-5.1$^-\text{CD8}^+$ cells. FSC, forward light scatter. Filled histograms, memory cells; bold open histograms, naive cells; thin-lined open histograms, activated cells.
days later, the lytic activity of spleen cells (\( \bigcirc \), filled symbols) or control rat Ig (open symbols). Thirty days later, the lytic activity of spleen cells (\( \bigcirc \)), IEL (\( \square \)), or MLN cells (\( \triangle \)) was measured by \(^{51}\)Cr release from SIINFEKL-pulsed EL4 (H-2\(^b\)) cells. E:T ratios are based on the percentage of OT-I cells in the respective populations. Specific lysis in the absence of immunization was <5%. Spontaneous \(^{51}\)Cr release was <10%.

Peripheral and mucosal memory CD8 T cells are directly cytolytic ex vivo

To examine the functional characteristics of OT-I memory cells generated via CD40 activation, we tested their lytic activity ex vivo without restimulation in culture (Fig. 9). The adoptive transfer system allows comparison of the lytic activity of the memory cells from different tissues on a per cell basis, because the number of OT-I donor cells can be used to calculate precise E:T ratios. Immunization with OVA in the absence of anti-CD40 mAb did not induce detectable lytic activity in spleen cells, MLN cells, or IEL. In contrast, memory cells from all tissues examined exerted substantial Ag-specific lytic activity (Fig. 9). Interestingly, splenic and MLN memory cells had similar lytic activity on a per cell basis, while IEL memory cells exhibited ~10-fold higher lytic activity, further supporting the concept that the intestinal mucosa is a permissive site for CTL development (23).

Discussion

The results presented defined the outcome of CD40 signaling on CD8 T cell activation. Using the T cell adoptive transfer system, we demonstrated that CD40 activation in the presence of a poorly immunogenic soluble Ag provided a powerful proliferative signal to CD8 T cells. Similarly, as assessed by MHC tetramer staining, an impressive increase in endogenous OVA-specific CD8 T cells was observed when immunization with sOVA included an agonistic anti-CD40 mAb. The effect of anti-CD40 mAb was indirect in that this treatment had no effect on OT-I cells transferred to CD40\(^{-/}\) mice. This is an important result because CD8 T cells express CD40 (21, 31), and further supports the concept that APC activation is essential to bring the CD8 T cell response to fruition. Significantly, CD40 activation also provided potent signals to drive induction of CD8 T cell cytotoxic activity. Thus, this system allowed direct visualization of the CD8 T cell response in the presence or absence of CD40 activation.

Our findings suggest a mechanism by which tolerance to soluble, noninflammatory Ags may be mediated. However, the mechanism by which sOVA enters the MHC class I Ag processing and presentation pathway is not clear. In other studies of CD8 priming by sOVA, whole spleen cells loaded with OVA were used (12, 42), and so it is possible that Ag was acquired by APC after phagocytosis of dying cells (43, 44). We have previously shown that the peripheral OT-I proliferative response to sOVA requires CD28-mediated costimulation via B7-2 to induce proliferation (23), indicating that professional APC are acquiring soluble Ag. Nevertheless, as shown in this study, this interaction does not lead to induction of lytic activity in secondary lymphoid tissues, but to deletion (23). Therefore, provision of the classical signal 2 (45, 46) results in tolerance via deletion in this example (23). However, the outcome of the response appears to be the result of a lack of CD40 triggering. Induction of anergy rather than deletion may also occur in the absence of CD40 ligation, although in the system described in this work, this was not observed. The necessity for inflammatory signals, such as CD40 activation, to drive productive CD4 and Ab responses has been previously demonstrated (47–49). In the case of those CD8 T cell responses that require CD4 T cell help, it is known that CD40-mediated activation of APC or virus infection of APC can bypass the CD4 T cell requirement (11–13). Indeed, the results presented (Fig. 5) showed that MHC class II-restricted CD8 T cells were not required to induce lytic activity or proliferation of transferred OT-I cells. Thus, although the in vivo signals for proliferation and induction of lytic activity can be separated in our system, both can be provided by CD40 activation. This finding provides the basis for considering CD40 agonists as potential adjuvants to amplify weak or nonproductive CD8 responses to soluble, poorly immunogenic compounds.

In addition to generation of a productive CD40-triggered peripheral primary immune response to soluble protein, a significant population of Ag-specific CD8 T cells was also detected in the intestinal mucosa. This response was visualized by reactivity with H-2K\(^b\)/SIINFEKL tetramers and provided direct identification for the first time of endogenous Ag-specific CD8 T cells in the LP and in the intestinal epithelium during an immune response in vivo. This finding indicated that systemic activation resulted in an ongoing CD8 T cell response in the mucosa and, most likely, in other tertiary tissues. This scenario makes good sense in that the goal of the immune response is to seek and destroy incoming pathogens and, in the absence of a localized Ag, the system takes a buckshot approach to mounting a response. Although it has been suggested that immunization via a mucosal route is needed to generate a mucosal response (50), as shown in this study, this is clearly not the case for all CD8 T cell responses.

The cellular and molecular factors required for generation of long-term immunological memory are largely unknown. As we and others have shown, costimulation-dependent primary CD4 or CD8 responses to soluble or noninflammatory Ags generate poor memory (23, 26). The addition of inflammatory signals in the form of microbial infection or adjuvants provides the necessary milieu to drive the response to completion and generate memory cells (51–53). Our results identified CD40 signaling as a means to generate long-term CD8 memory to poor immunogens. Memory in this case also extended to the intestinal mucosa. Secondary lymphoid memory CD8 cells generated via CD40 triggering physically (size) and phenotypically resembled memory cells generated via virus infection (3, and Lefranc¸ois, unpublished). Furthermore, CD8 memory cells induced by CD40 activation exhibited direct ex vivo lytic activity, as has been shown for antiviral CD8 memory cells (3, 54). However, memory cells in the intestinal epithelium exhibited ~10-fold higher levels of lytic activity than peripheral.

FIGURE 9. OT-I memory cells generated by CD40 triggering and sOVA are constitutively cytolytic. A total of 2 \( \times 10^6 \) CD8 OT-I LN cells (Ly-5.1) were transferred to B6 (Ly-5.2) mice. Two days later, mice were immunized with 5 mg OVA by i.p. injection with the inclusion of 100 \( \mu \)g of anti-CD40 mAb (filled symbols) or control rat Ig (open symbols). Thirty days later, the lytic activity of spleen cells (\( \bigcirc \)), IEL (\( \square \)), or MLN cells (\( \triangle \)) was measured by \(^{51}\)Cr release from SIINFEKL-pulsed EL4 (H-2\(^b\)) cells. E:T ratios are based on the percentage of OT-I cells in the respective populations. Specific lysis in the absence of immunization was <5%. Spontaneous \(^{51}\)Cr release was <10%.
memory cells, indicating that the intestinal mucosa is a proactive site for CD8 memory cells, which most likely make up a significant portion of CD8 cells in LP and IEL of normal mice and humans. Other studies have shown that in the absence of CD40 signaling, less CD8 memory to LCMV is induced, and this is apparently due to a reduced primary response (55–57). The primary LCMV immune response is CD28 independent, however (58, 59), so it is unclear whether this result can be generalized with regard to CD40 involvement in induction of CD8 memory. In any case, the ability to bypass tolerance induction via CD40 ligation will provide a system that will allow eventual definition of the factors required for memory CD8 T cell generation.

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