Distinct Costimulatory Molecules Are Required for the Induction of Effector and Memory Cytotoxic T Lymphocytes

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Summary
A successful T cell immune response has two major products: effector T cells which directly or indirectly remove the antigens, and memory T cells, which allow a faster and more efficient recall response when challenged by related antigens. An important issue is whether costimulatory molecules on the antigen-presenting cells are involved in determining whether T cells will differentiate into effector or memory cells after antigenic stimulation. To address this issue, we have produced mice with targeted mutations of either the heat-stable antigen (HSA), or both HSA and CD28. We show that CD28/B7 and HSA provide two alternative costimulatory pathways for induction of immunological memory to influenza virus. Furthermore, our results revealed that B7 is essential for the generation of effector T cells from either naive or memory T cells, while HSA is not necessary for the generation of effector T cells. Our results demonstrate that the induction of memory T cells and effector T cells can utilize distinct costimulatory molecules. These results have important implications on lineage relationship between effector and memory T cells.

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Cellular adhesion molecule-1 (32, 33). Given the fact that effector cells and memory cells can be induced by the same antigen, we were interested in asking whether distinct costimulatory molecules are utilized for the induction of these two populations of cells.

Most in vitro studies suggest a major role for costimulatory molecules, particularly that of the B7 family members, B7-1 and B7-2, in the induction of effector T cells from naive T cells in vitro (34, 35). However, it is not clear whether B7 is required for the induction of effector T cells from memory T cells. Whereas the induction of effector T cells in vivo has not been systematically studied with regards to the requirement for costimulatory molecules, potent effects of CTLA4-Ig, a fusion protein with a high affinity for B7-1 and B7-2 (36), in blocking the rejection of allogeneic (37) or xenogeneic grafts (38) supports a major role of B7 in the induction of effector T cells in vivo. However, it is unknown whether induction of memory T cells requires costimulation. Several recent studies suggest that T cell priming can be achieved in the absence of a B7:CD28/CTLA4 interaction (39–41). Some of the studies were interpreted as evidence that antigen alone, if appropriately localized, would be sufficient to prime T cells (41). However, this interpretation contradicts a large collection of studies (10, 11, 42, 43) which demonstrate that engagement of T cell receptors in the absence of costimulation leads to the induction of T cell tolerance rather than immunity. An alternative possibility is that other costimulatory molecules, such
as HSA, are sufficient to induce immunological memory from naive T cells.

HSA is a GPI-anchored protein (44–46), and it is expressed on multiple lineages of hemapoietic and neuronal origin (47, 48). It was implicated as a costimulatory molecule when a mAb which blocks T cell proliferation and induces T cell unresponsiveness in vitro, was shown to bind HSA by expression cloning (25). Indeed, gene-transfection experiments indicated that HSA can transfer costimulatory activity to CHO cells (25). In addition, accumulating evidence supports a role of HSA in costimulating T cells in a variety of experimental models involving several different types of antigen-presenting cells (APC). On B cells, HSA and B7 appear to act synergistically in inducing clonal expansion of T cells (24–26, 29). HSA expressed on Langerhans cells is involved in both the induction of clonal expansion and prevention of clonal anergy of Th1 clones (27). More recently, HSA was shown to be induced during macrophage phagocytosis and to be critical for in vitro priming of naive CD8 T cells (30). Furthermore, tumors transfected with HSA induce better priming of anti-tumor CTL responses (28).

To address the requirement for costimulatory molecules in the induction of effector and memory CTL, we have produced mice with a targeted mutation of the HSA gene. We have also bred HSA-deficient mice with CD28-deficient mice (39) and thereby generated mice deficient in both HSA and CD28. We have chosen influenza virus as the antigen because both effector and memory CTL responses can be easily detected in vivo (1). Our results demonstrate that induction of effector and memory CTL utilizes distinct costimulatory molecules.

Materials and Methods

Antibodies, Cell Lines, Synthetic Peptide and Experimental Animals

Anti-B7-1 mAb 3A12 (18), anti-B7-2 mAb GL-1 (20), anti-CD28 mAb 37N (41), and anti-HSA mAb antibody 20C9 (25) were used in these studies. All mAbs were purified from hybridoma supernatants using a protein G column. A mixture of affinity-purified normal IgG from rat and hamsters were used as control. The thymoma cell line EL4 (H-2b), and mastocytomas cell line P815 (H-2k) were used as target cells for cytotoxicity assays.

In brief, syngeneic H-2d EL4 cells were pulsed with (EL4-NP)\textsubscript{366-374} (18). Aliquots of (EL4-NP)\textsubscript{366-374} (18) were added to 10\textsuperscript{4} cells, 37°C, 1 h, irradiated syngeneic spleen cells for 5 d (Responders: stimulator ratio 5:1, with responder cells at a density of 10\textsuperscript{6}/ml). The end of culturing, viable cells were harvested and the cytotoxicity of these cells was determined by a 6 h \textsuperscript{51}Cr-release assay.

Evaluation of Memory CTL Responses

Three different assays were used to evaluate the activity of memory T cells.

Recall Response in 96-Well Microplate Culture without Exogenous Growth Factors. Varying numbers of A/JAP-primed spleen cells were restimulated in vitro with irradiated A/JAP-infected syngenic spleen cells (3 × 10\textsuperscript{3}/well) for 5 d at 37°C in Click’s EHAA medium containing 5% FCS. At the end of culturing, the cells were washed twice with medium, the \textsuperscript{51}Cr-labeled target cells were added and CTL activity was determined by \textsuperscript{51}Cr-release.

Bulk Culture. When CD28-deficient spleen cells are used, the recall CTL response in microplate culture is usually very weak, so the priming of CTL in experiments involving CD28-KO T cells were evaluated in bulk culture. In brief, pooled spleen cells from groups of 2–3 mice were harvested on day 7 after i.v. or i.p. injection of A/JAP (1,000 HAU) and were restimulated with A/JAP-infected (1,000 HAU/12 × 10\textsuperscript{3} cells, 37°C, 1 h), irradiated syngeneic spleen cells for 5 d (Responders: stimulator ratio 5:1, with responder cells at densities of 10\textsuperscript{6}/ml). At the end of culturing, viable cells were harvested and the cytotoxicity of these cells was determined by a 6 h \textsuperscript{51}Cr-release assay.

Limiting Dilution Analysis. Spleen cells were harvested from either naive mice or mice that have been immunized with A/JAP virus 5–7 wk previously. Graded numbers of spleen cells (pooled from 2 mice per group, 24 wells replicates at each cell density) were stimulated with A/JAP-infected, irradiated spleen cells. Supernatants from PMA-activated EL4 cells were added at the beginning of the culture at a final concentration of 1%. After 6 d of culture, the plates were washed once with medium, \textsuperscript{51}Cr-labeled, NP\textsubscript{366-374}-pulsed EL4 cells were added at 10\textsuperscript{5}/well. The wells that have given equal or greater than mean plus 3 × SD of medium release were scored as positive. The precursor frequency is calculated based on Poisson distribution.

Evaluation of Effector T Cells

In brief, syngeneic H-2d EL4 cells were pulsed with (EL4-NP) or without (EL4) 10 µg/ml of synthetic peptides corresponding
to AA 365–380 of influenza virus A/JAP nucleoprotein and labeled with $^{51}$Cr for 1 h at 37°C. The labeled target cells were then incubated with the effector cells for 6 h, and the released $^{51}$Cr in the supernatants were determined. The specific lysis percentages were calculated by the following formula:

$$\text{Specific lysis} \%= \frac{(cpm_{\text{samples}} - cpm_{\text{medium}})}{(cpm_{\text{maximum}} - cpm_{\text{medium}})} \times 100.$$ 

Spleen cells freshly isolated from the animals 6–8 d after viral infection were defined as primary effectors, and the CTL generated from primed spleen cells were defined as secondary effectors.

**Results**

Production of Mice Deficient for HSA and for both HSA and CD28. The disruption of the gene for the HSA in C57BL/6 ES line BL/6-III (50) was achieved by replacing the HSA promoter and the first exon with a neomycin-resistance expression cassette (Fig. 1a). Except for a somewhat reduced litter size, the mice homozygous for the targeted mutation (HSA-KO) (Fig. 1b) are indistinguishable from WT control in a conventional mouse facility. We have mated HSA-deficient mice with previously developed CD28-deficient mice and generated mice which are deficient in both HSA and CD28. As shown in Fig. 2, normal numbers of CD4 and CD8 T cells are generated in all mutant mice. Furthermore, the major types of APC, such as B cells, macrophages, and dendritic cells, are produced in normal numbers in all mutant mice. Thus, these mice can be used for studying the role of costimulatory molecules in the generation effector and memory T cells.

B7, but not HSA, Is Required for the Induction of Effector T Cells from Naive T Cells. C57BL/6/j mice infected with influenza virus mount a primary CTL response which is detectable on day 5, which peaks at day 7 or 8, and disappears within 2 wk after infection (1). In A/JAP (H2N2)-infected H-2b mice, the major antigenic epitope is NP365–380 of A/JAP virus (52); the minimal peptide was later identified as NP366–374 (53). We therefore used NP365–380-pulsed EL4 cells to detect the primary CTL response.

As shown in Fig. 3, WT and HSA-deficient mice mount a significant primary CTL response against NP-peptide-pulsed syngeneic EL4 target cells, but not against unpulsed EL-4, (Fig. 3, a and b), or peptide-pulsed allogeneic P815 (H-2b) targets (data not shown). The kinetics of the CTL response is similar in all mice (data not shown). Although in this experiment, the primary CTL response in HSA-deficient mice is somewhat lower than WT mice, in other experiments, HSA-KO mice appear to mount a higher primary CTL response (data not shown). Thus HSA is not required for the generation of primary CTL. In contrast, no virus-specific primary CTL response is detected in mice deficient for either CD28, or both CD28 and HSA, although in some experiments, a significant nonspecific cytotoxicity was detected in CD28-deficient mice, most likely due to NK cells. This lack of CTL response was not due to a change in the kinetics, since we have been unable to de-
tect primary responses against influenza virus between day 3 and day 14, in mice with a targeted mutation of CD28 (data not shown). Because CD28 is the major receptor for the costimulatory molecules B7-1/2, these results strongly suggest that B7 is necessary for the induction of primary CTL. This is more directly demonstrated by the results in Fig. 4, which show that a mixture of anti-B7-1/B7-2 completely eliminates the primary anti-influenza NP CTL re-

![Graphs showing CTL response against influenza virus](image)

Figure 3. Primary in vivo CTL response against influenza virus in WT mice (a), and mice with a targeted mutation of HSA (b), CD28 (c), or of both HSA and CD28 (d). Spleen cells were harvested from mice on day 7 after A/JAP-infection. The CTL activities were tested on either peptide (AA365-380 of the nucleoprotein from A/JAP virus)-pulsed (EL4-NP), or unpulsed EL4 cells (EL4). Representatives of three independent experiments using pooled spleen cells from 2–4 mice in each group are shown.

Figure 4. Anti-B7 mAbs completely block the production of effector CTL from naive T cells in both WT mice (a) or HSA-KO (b) mice. WT and mutant mice were infected with 1,000 HAU/mouse of influenza virus A/JAP by intraperitoneal injection on day 0. The mice were injected with either a mixture of normal rat and hamster IgG, or anti-B7-1 + anti-B7-2 mAbs (3A12+GL1) on days −1, 0, +1, at a dose of 100 µg/mouse/injection. Data presented are representative of three experiments using pooled spleen cells from 2–3 mice per group.
response in both WT and HSA-deficient mice. In contrast, anti-HSA mAb 20C9 does not block primary effector CTL responses (data not shown). These results also demonstrate that anti-B7 mAbs efficiently block the function of B7 in vivo.

Two Costimulatory Pathways for Rapid Priming of Recall CTL Response In Vitro. Infection of mice with influenza virus primes a recall CTL response in vitro, detectable as early as 48 h after infection (our unpublished results). To test the requirement for costimulatory molecules in the priming of the recall CTL response, we infected WT and HSA-KO mice with influenza virus either in the presence or absence of antibodies to B7 or HSA. After 8 d, spleen cells were restimulated in vitro for 5–6 d with influenza virus-infected syngeneic spleen cells in the absence of mAbs and CTL activity was measured. Fig. 5a shows that the recall response requires in vivo priming. In the WT mice, the recall responses were not affected by treatment with a mixture of anti-B7-1 and anti-B7-2 antibodies during priming (Fig. 5b). In contrast, a mixture of anti-B7-1/B7-2 totally abolished the priming in the HSA-KO mice (Fig. 5b).

The efficacy of the anti-B7 mAbs in vivo is confirmed because such treatment completely eliminated primary CTL response in vivo in both WT and HSA-deficient mice (Fig. 4). Thus, costimulation by either B7 or HSA is required for the in vivo priming of the recall CTL response.

Several recent studies demonstrated that T cells can be primed in mice with a targeted mutation of CD28 (CD28-KO) (39), or transgenic for CTLA4-Ig (which very effectively blocks the interaction of B7-1 and B7-2 with CD28 and CTLA4) (40). To test whether costimulation by HSA accounts for this priming, we injected anti-HSA mAb into CD28KO mice. As shown in Fig. 6, the recall CTL response generated in CD28KO mice was inhibited almost completely by pre-treatment with anti-HSA during priming. In contrast, the priming to influenza virus in WT mice was not significantly affected by anti-HSA mAb. These results demonstrate that the targeted mutation of CD28 renders the CTL priming more dependent on costimulation by HSA.

To substantiate this conclusion, we have produced mice with targeted mutations of both the HSA and the CD28 genes (Fig. 2) and compared the recall CTL responses in mice with targeted mutations of HSA and/or CD28. Indeed, recall CTL response in mice deficient for both CD28 and HSA was ~100-fold lower than that in the WT mice (Fig. 6d). CD28-deficiency leads to ~3–5-fold reduction of recall CTL responses, while HSA-deficient mice mount normal recall CTL responses (Fig. 6d). The relative contribution of HSA– and CD28-mediated costimulation in T cell priming, however, cannot be determined by this experiment, because the CD28:B7 pathway is also required for optimal recall responses (see below). The observed re-
could also be attributed to a role of CD28 in the generation of effector from the primed T cells.

**Induction of Memory from Naive T Cells Requires Costimulation by Either B7 or HSA.** T cells responsible for long-term memory may be different from those for the early recall response measured here (54, 55). Since the above experiments used spleen cells that were primed only 8 d previously, we treated both WT and HSA-KO mice with anti-B7 mAbs for 3 wk and waited 100 d after immunization before harvesting spleen cells and assaying for memory cells in an in vitro recall culture. As shown in Fig. 7, while repeated treatment with anti-B7 mAbs reduced the recall CTL activity in the WT mice, there still were very potent recall CTL responses in such anti-B7-treated mice. In fact, the numbers of responder cells required for detectable recall responses in vitro were similar in both groups. In contrast, in HSA-KO mice, injection of a mixture of anti-B7 mAbs eliminated the generation of memory cells. These results demonstrate that costimulation by either B7 or HSA alone is sufficient for induction of memory T cells, regardless of when the memory activity is determined. The lack of long-term memory in anti-B7 treated HSA-KO mice rules out the possibility that memory cells are produced after decay of the antibodies in vivo. Moreover, the production of memory cells in anti-B7-treated WT mice depends on HSA. Thus, the induction of T cells responsible for rapid priming of recall CTL responses and long-term memory responses has a similar requirement for costimulatory molecules; either B7 or HSA can provide costimulation for induction of memory T cells.

We have also measured the frequency of the precursors for NP\textsubscript{366-374}-specific cytotoxic T cells by limiting dilutions. As shown in Table 1, naive WT, HSA-KO, CD28-KO, and CD28/HSA-KO mice have a similar number of NP-specific CTLp in the spleen. These results support the notion that T cell development is not grossly affected by targeted-mutation of HSA and/or CD28. 5–7 wk after viral infection, the CTLp is increased by 10–50-fold in WT mice and in mice deficient for either HSA or CD28, although targeted mutation of CD28 reduced the expansion of CTLp. Most importantly, no significant increase of CTLp can be detected after mice deficient for both HSA and CD28 are infected with influenza virus. These results demonstrated that one costimulatory pathway, mediated by either HSA or CD28, is necessary and sufficient for increase of the CTLp during induction of immunological memory.

**B7, but not HSA, Is Required for the Generation of Effector CTL from Memory T Cells.** The activation of memory T cells gives rise to effector T cells. To test whether this process requires costimulatory molecules, we stimulated primed spleen cells in the presence of mAb against either B7 or HSA. As shown in Fig. 8 a, for spleen cells primed when both B7 and HSA-costimulation pathways are intact, the recall response requires costimulation by B7, but not the HSA. To test whether priming conditions affect the requirement for costimulatory molecules in eliciting effector...
CTL from memory T cells, spleen cells primed when B7 or HSA was blocked were restimulated in the presence of anti-HSA or anti-B7 mAbs. Again, T cells primed in the absence of costimulation by either B7 or HSA require co-stimulation by B7 for a recall response (Fig. 8, b and c). No recall CTL can be detected in microculture when both B7 and HSA are blocked during priming (Figs. 4 and 8 d). Thus, regardless of the priming conditions, B7 but not HSA is essential for eliciting effector from memory T cells.

**Discussion**

A critical issue pertinent to the basis of self-nonself discrimination in the immune system is whether induction of immunological memory requires costimulation, in addition to antigen. The two-signal theory of T cell activation (9–13) argues that stimulation of naive T cells in the absence of costimulation leads to tolerance rather than immunological memory. Classical experiments using either fixed APC (56) or transplantation of cultured allogeneic or xenogeneic endocrine grafts (57) indicate that immunological tolerance is induced when costimulatory activity is eliminated, either by depletion or by inactivation of resident APC. Recently, it has been documented that blocking the B7-CD28/CTLA4 interaction leads to tolerance rather than immunity to xenogeneic grafts (38). However, several more recent experiments designed to test the requirement of B7:CD28/CTLA4 interaction in the induction of memory T cells do not support an essential role of B7 family members in the induction of immunological memory. Thus, transgenic mice which constitutively express a high level of CTLA4Ig, a fusion protein consisting of the high affinity B7 receptor...
CTLA4 fused to the immunoglobulin Fc portion, show an even better priming than the littermate controls (40). In addition, fibroblasts transfected with viral glycoproteins induce successful priming in the absence of B7 expression (41). Because the B7:CD28 interaction is not essential, it has been proposed that induction of T cell memory does not require costimulation.

Given the fact that several costimulatory molecules have been discovered, we were interested in testing whether a lack of requirement for B7 in the induction of immunological memory was due to the existence of other redundant costimulatory pathways. We have used three different assays to measure the memory CTL response. The first is a microplate restimulation culture in the absence of exogenous cytokines. Such assay allows us to estimate the number of spleen cells necessary to generate a detectable CTL response (presumably including helper and CTLp). The second assay is a bulk culture involving a larger number of cells (10^6 to 10^7 cells in 10 ml). Whereas this assay is perceived to be less quantitative than the first assay, it is more sensitive and appears to be the only assay that allows detection of optimal recall CTL response in spleen cells from CD28-deficient mice without adding exogenous cytokines.

Most likely, this is due to the involvement of CD28 in eliciting CTL from memory cells. Thirdly, we have used a limiting dilution assay to measure the precursor frequency of NP366-374-specific CTL. This method is widely used to measure CTL memory, however, it should be noted that this assay only measures the persistency of expanded CTL. Utilization of three different assays allows a more comprehensive measurement of the memory CTL response.

We show here that in mice with a targeted-mutation of the HSA gene, but not in the WT mice, CD28 is required for the induction of immunological memory. In contrast, in CD28-deficient mice, the HSA is necessary for T cell priming. Memory T cell response is hardly detectable in mice deficient for both HSA and CD28. Thus, induction of immunological memory requires costimulation, but either CD28 or CD28/CTLA4:B7 interactions are sufficient. It should be noted that while our studies have stressed the importance of costimulation mediated by either HSA or CD28, we cannot rule out contribution of other costimulatory molecules in the induction of memory T cells. In this regard, it is worth noting that blockade of both CD40L:CD40 and CD28/CTLA4:B7 interactions is more efficient than simply blocking either one in preventing clonal expansion of

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**Figure 8.** B7, but not HSA, plays an important role in the induction of effector T cells from memory T cells, regardless of the priming conditions: microplate culture in the absence of exogenous cytokines. Spleen cells from either WT (a and b) or HSA-deficient mice (c and d) pretreated with either normal Ig (a and c) or a mixture of anti-B7-1 and anti-B7-2 (b and d) were infected with A/JAP virus as detailed in the legend to Fig. 3. Increasing numbers of spleen cells were stimulated in vitro for 5 d with A/JAP-infected spleen cells, in the presence of either medium, or a mixture of anti-B7-1 and anti-B7-2, or anti-HSA mAb (final concentration at 5 μg/ml), and CTL activity was determined in a 6-h ^51Cr-release assay. Representative of three independent experiments are shown. Similar results were obtained when memory activity is measured in bulk cultures.
ICAM-1 (60) may participate in induction of memory T cells. Induced costimulatory molecules, such as CD44 (59) and self-reactive helper T cells (58). It is likely that CD40L-stimulatory molecules can be utilized in the induction of effector T cells. Induction of effector T cells from memory T cells requires B7, while the production of effector T cells utilizes B7 but not HSA. By definition, memory T cells give rise to effector T cells after further stimulation by antigen. However, effector T cells are unlikely to be mandatory precursors for memory T cells and distinct costimulatory molecules could be used at different phases of the immune response.

Figure 9. A model for the involvement of costimulatory signals in the generation of effector and memory T cells. Two major products, effector and memory T cells, are produced from naive T cells after viral infection. The production of memory T cells requires costimulation by either HSA or B7, while the production of effector T cells utilizes B7 but not HSA. By definition, memory T cells give rise to effector T cells after further stimulation by antigen. However, effector T cells are unlikely to be mandatory precursors for memory T cells and distinct costimulatory molecules could be used at different phases of the immune response.

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self-reactive helper T cells (58). It is likely that CD40L-induced costimulatory molecules, such as CD44 (59) and ICAM-1 (60) may participate in induction of memory T cells.

It is worth noting that targeted mutation of CD28 alone causes a three- to fivefold reduction in recall CTL response, while HSA-deficiency alone is not sufficient to affect immunological memory (Fig. 6). These results may indicate that CD28-dependent costimulatory pathway is more important than that of HSA for induction of memory CTL. However, since memory cells are quantitated by the effector T cells they produced, and since production of effector cells from memory cells requires B7 (Fig. 8), the reduction in recall CTL response may be due to lack of CD28 on the memory cells. In this regard, it is worth noting that transgenic expression of CTLA4Ig, which blocks B7:CD28/CTLA4 interaction, does not affect induction of immunological memory when it is assayed in the absence of CTLA4Ig (40). Similarly, priming in the presence of anti-B7 mAbs does not affect induction of recall response (Fig. 5). In contrast, since HSA is not involved in the induction of effector T cells from memory T cells (Fig. 8), a reduction in recall response caused by targeted mutation of HSA can be attributed to HSA involvement during priming.

An important conclusion of our study is that distinct costimulatory molecules can be utilized in the induction of effector and memory T cells. Induction of effector T cells depends strictly on costimulation by B7, while induction of memory T cells requires costimulation by either B7 or the HSA. These conclusions are based on three lines of evidence. First, a mixture of anti-B7-1 and anti-B7-2 mAb completely blocks the generation of primary CTL response in WT mice, yet it does not seem to significantly affect the generation of memory CTL responses. While the magnitude of the recall response was affected somewhat in the long-term memory response, the number of memory cells was not altered. Second, a targeted mutation of CD28 eliminates the antigen-specific primary CTL responses, yet it causes only a small reduction in the number of precursor cells at 5–7 wk after priming. Third, costimulation by HSA plays no role in the induction of effector T cells from either naive or memory T cells, yet it provides an alternative costimulatory pathway for the induction of memory T cells. That HSA enhances priming CTL responses is supported by a recent study in which transfection of HSA into tumor cells lead to enhanced memory CTL response (28).

Because CD4 T cells can provide help for CD8 T cell responses in both priming and induction of effector from memory T cells, our results could be explained on the basis of a requirement for costimulation in the CD4 T cell compartment. However, previous studies from others and us have demonstrated that, in an anti-influenza CTL response, induction of memory T cells is independent of CD4 T cells (61, 62). Blocking the priming of CD4 T cells, therefore, cannot lead to a defect of CD8 T cell response as reported here. Furthermore, we demonstrate here that targeted mutation of both CD28 and HSA prevents clonal expansion of CTLp (Table 1).

A simple model can be proposed to explain the requirement for distinct but overlapping costimulatory molecules in the induction of effector and memory CTL. The strength of the total signals received by naive T cells could determine whether they will differentiate into effector or memory T cells. A stronger stimulation (integration of signals from TCR and costimulation) would lead to the production of effector cells, while a weaker stimulation would be sufficient to produce memory cells. HSA is a less potent costimulatory molecule for clonal expansion of T cells than B7 family members, so it can only be utilized for the induction of a memory T cell responses. B7, being a stronger costimulator, can be used for both memory and effector T cell responses, depending on the concentration of the molecule on the surface of APC and the strength of the TCR/antigen interaction. For example, dendritic cells, which express a high level of B7, may induce the production of effector T cells, while B cells, which express a lower level of B7, may be more likely to induce only memory T cells.

Our study may explain the interesting difference between viruses regarding the requirement for the costimulatory receptor CD28 in the induction of CTL in vivo. Shihanian et al. (39), showed that CD28 is not required for induction of CTL specific for lymphocytic choriomeningitis virus (LCMV) in vivo. Since LCMV causes a productive infection in mice, naive T cells can first be primed, and then these primed T cells can give rise to effector CTL after repeated stimulation. The influenza virus used in this
study, and the vesicular stomatitis virus used by Kündig et al., do not cause productive infection, and thus the CTL response in vivo become CD28-dependent after one round of infection (63).

A successful adaptive immune response has two major T cell products: effector T cells and memory T cells. The lineage relationship between these two types of cells has not been resolved. Because effector T cells and memory T cells share certain activation conditions (64) and express several identical activation markers (65), it has been proposed that memory T cells are derived from effector T cells (55, 64, 65). A critical prediction of this hypothesis is that memory T cell responses should be eliminated when the effector T cell responses are abrogated. Results presented in this study show that memory CTL responses are largely intact when effector T cell development is completely absent. These results are not consistent with the notion that effector T cells are mandatory precursors for memory T cells. Furthermore, we showed that costimulation by HSA does not contribute to the generation of effector cells, but it can and does contribute to the generation of memory cells. This qualitative difference in costimulatory molecules used for effector vs memory T cell responses strongly suggests that these two types of cells can be products of different activation pathways (see Fig. 9 for a model).

A critical test for the model is whether memory cells can be produced when effector T cells are ablated. A major obstacle to such a test is that memory and effector T cells are traditionally measured at different times after antigenic stimulation. Effector CTL disappears within two weeks after influenza viral infection, yet memory cells are generally measured long after that. This is necessary not because of the kinetics of production of memory, since elegant studies demonstrated that T cell memory is produced as early as three days after antigen challenge (66); rather, it is because of the need to differentiate memory from effector cells. Since blocking B7 or CD28 efficiently eliminates production of effector T cells, we can use the same spleen cells to test whether priming can take place when effector CTL are not generated. Such an analysis strongly supports the notion that the primed T cells are not derived from effector T cells. Experiments are under way to test whether the activity measured in this early recall assay represents true immunological memory.

In conclusion, we have produced mice with a targeted mutation of HSA, and mice deficient for both HSA and CD28. We have demonstrated that the induction of memory T cells requires costimulation by either B7 or HSA, while induction of effector T cells depends on B7 but not HSA. Our study firmly establishes that induction of immunological memory requires costimulation, and that either B7 or HSA is sufficient for this step. It also raises an interesting possibility that memory T cells can be induced without going through an effector phase.

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