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Bacterial superantigens reactivate antigen-specific CD8$^+$ memory T cells

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Abstract

Superantigens stimulate naive CD4$^+$ and CD8$^+$ T cells in a TCR V$_\beta$-specific manner. However, it has been reported that memory T cells are unresponsive to superantigen stimulation. In this study, we show that staphylococcal enterotoxins (SE) can activate influenza virus-specific CD8$^+$ memory cytotoxic T cells. *In vivo* SEB challenge of mice that had recovered from influenza virus infection (memory mice) resulted in the generation of vigorous influenza-specific cytotoxic T lymphocyte (CTL) activity and *in vitro* SEA or SEB stimulation of splenic T cells from memory mice, but not naive mice, also induced influenza-specific CTL. Analysis of the mechanism of activation suggested that although there may be a component of cytokine-mediated bystander activation, the CTL activity is largely generated in response to direct TCR engagement by superantigen. Moreover, influenza-specific CTL could be generated from purified CD8$^+$CD62L^{lo}CD44^{hi} (memory phenotype) T cells cultured in the presence of T cell-depleted splenic antigen-presenting cells and SE. Purified CD8$^+$ memory T cells also secreted lymphokines and synthesized DNA in response to superantigen. These results definitively demonstrate that CD8$^+$ memory T cells respond to SE stimulation by proliferating and developing appropriate effector function. Furthermore, the data raise the possibility that otherwise inconsequential exposure to bacterial superantigens may perturb the CD8$^+$ T cell memory pool.

Introduction

Superantigens are molecules expressed by microorganisms such as bacteria and viruses that stimulate T cells in a V$_\beta$-specific manner (1,2). For example, staphylococcal enterotoxins (SE), such as SEA and SEB, are potent superantigens that stimulate predominantly V$_\beta$1$^+$ and V$_\beta$3$^+$ murine T cells and V$_\beta$7$^+$ and V$_\beta$8$^+$ murine T cells respectively (3,4). Bacterial superantigens bind in a native, unprocessed form to non-polymorphic regions of class II MHC molecules outside of the peptide binding groove (1,2,5). As a consequence, superantigen recognition is not classically MHC restricted, and both CD4$^+$ and CD8$^+$ T cells respond (6–10). Thus, proliferation, cytokine production and cytotoxicity are readily detected following *in vivo* or *in vitro* treatment with SE (8,11,12).

The *in vivo* proliferative response to SE is followed by deletion of a substantial proportion of the responding T cells (13–15) and several groups have reported that the superantigen-responsive cells which survive the deletion process become anergic (16–22). Most studies of SE-induced anergy have focused on CD4$^+$ T cells, which show markedly reduced proliferation and cytokine production upon re-stimulation with SE, although the generality of these findings has recently been challenged. For example, SEB-induced anergic T cells can be fully responsive *in vitro* if the antigen-presenting cells (APC) present a high density of superantigen and appropriate co-stimulation (23). Moreover, T cells which appear anergic *in vitro* can be functional *in vivo* (24) and one group has reported that SEB induces a potent secondary response rather than anergy (25). There are also reports of proliferative anergy to SE among CD8$^+$ T cells (17,19,26), but while some groups observed reduced cytokine production (27), others showed increased IFN-γ production (19). However, the lytic effector function of CD8$^+$ T cells was not compromised by SE exposure, except in one study where low doses (0.01 μg) of SEA down-regulated cytotoxicity, while higher doses (10 μg) did not (17).

Because the frequency of superantigen-reactive T cells is much higher than the frequency of T cells that can recognize

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a conventional peptide antigen, SE activation of naive T cells is easily demonstrated (1). Since primary T cell responses generally have more stringent activation requirements than secondary responses (28–33), it was quite surprising when it was reported that previously activated T cells (34) and T cells with a memory phenotype (35) are non-responsive to bacterial superantigens. It has since become widely accepted that memory T cells are 'anergic' to superantigenic stimulation, despite the fact that these studies did not directly examine the response of superantigen-reactive, antigen-specific memory T cells. Although one of these groups did demonstrate a failure of SEB to stimulate keyhole limpet hemacyanin (KLH)-specific B cell help in an *in vitro* assay, there is only indirect evidence that any of the KLH-specific memory T_h cells were SEB reactive (35). Other studies with model protein antigens have yielded conflicting results (36,37).

In order to directly assess the response of antigen-specific memory T cells to superantigenic stimulation, we chose to examine the effects of SE on established memory in a well-characterized model system, namely influenza virus pneumonia in mice. Following recovery from influenza virus infection, long-term CD8$^+$ T cell memory is maintained (38–40). *In vitro* culture of splenic CD8$^+$ T cells in the presence of viral antigen results in the generation of specific CTL, and secondary virus challenge *in vivo* results in an accelerated CTL response (41) and more rapid viral clearance compared to a primary infection (42). We report here that bacterial superantigens, in the absence of antigen, stimulate the generation of secondary influenza-specific CTL *in vivo* and *in vitro*, and that the superantigen-mediated activation of virus-specific memory T cells is not mediated solely by bystander effects or lymphokines, but instead involves direct TCR ligation.

Methods

Mice

V_β8.1 TCR β chain transgenic mice backcrossed onto the CBA/CaJ background, termed CBA.βTG, have been previously described (43) and were bred at St Jude Children's Research Hospital. CBA/CaJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or bred at St Jude Children's Research Hospital. Mice were housed under specific pathogen-free conditions prior to infection with influenza virus.

Viral infections

The recombinant influenza A/HKx31 (H3N2) virus (44) was used throughout these studies and was prepared as previously described (45). Mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol (avertin) prior to intranasal infection with 50 hemagglutinating units (HAU) in a volume of 30 μl PBS. Mice were rested for a minimum of 8 weeks after infection before use in T cell memory experiments.

Superantigens

Recombinant SEA and SEB obtained from Toxin Technology (Sarasota, FL) were prepared and tested as previously described (46). *In vivo* treatment was by i.p. injection of 100 μg in a volume of 0.5 ml PBS. *In vitro* stimulations were with a final concentration of 10 μg/ml.

Spleen cell preparations

For direct *ex vivo* CTL assays and secondary *in vitro* cultures, single-cell suspensions were prepared from the spleens of memory mice. After red blood cells were lysed with Gey's solution, class II MHC$^+$ cells and CD4$^+$ T cells were depleted by negative panning first on plates coated with a mixture of mAb 10-3.6.2 [anti-I-A^k (47)] and 14-4-4s [anti-I-E (48)], and then on plates coated with mAb GK1.5 [anti-CD4 (49)]. The resulting cell suspensions typically contained $>$75% CD8$^+$, αβ TCR$^+$ cells and $<$5% CD4$^+$ cells (determined by FACS analysis). APC were prepared as described (50) by depleting Thy-1.2$^+$ cells from spleen cell suspensions with the IgM mAb AT83 (51) and a cocktail of rabbit and guinea pig complement (Cedarlane, Hornby, Ontario, Canada). The APC preparations contained $<$3% αβ TCR$^+$ cells. For *in vitro* stimulation with influenza virus, APC were infected with 4000 HAU A/HKx31 virus/10^7 cells for 2–4 h. APC were X-irradiated (7000 rad) prior to culture.

Immunofluorescent staining and cell sorting

Conjugated antibodies were purchased from PharMingen (San Diego, CA). CD8$^+$ T cell-enriched spleen cell suspensions were blocked with normal mouse serum (Pel-Freez, Rogers, AK) and stained first with biotinylated mAb IM-7 [anti-CD44, Pgp-1 (52)], and then with FITC-conjugated mAb 53.6.72 [anti-CD8 (53)], phycoerythrin (PE)-conjugated Mel-14 [anti-CD62L, L-selectin (54)] and Cy-Chrome-conjugated streptavidin. CD8$^+$CD62L^{lo}CD44^{hi} cells were then sorted on a FACStar Plus equipped with a high-speed sorting module (Becton Dickinson, Mountain View, CA). In the experiments described here, cells with this phenotype represented 2–5% of CD8$^+$ T cells prior to sorting. The purity of the sorted population, confirmed by FACS analysis, was ~95%. For clonal analysis, single CD8$^+$CD62L^{lo}CD44^{hi} cells were sorted directly into individual wells of 96-well plates using an automated cell delivery unit (Becton Dickinson).

Bulk culture of CD8$^+$ T cells

Cultures were set up in 24-well plates (Sarstedt, Newton, NC) with 3×10^6 CD8$^+$ T cell-enriched responders and 5×10^6 T cell-depleted APC/well in a total volume of 2 ml complete medium. In some experiments, recombinant human IL-2 (rhIL-2; Amgen, Thousand Oaks, CA) was added to a final concentration of 10 units/ml. In the co-culture experiments (Fig. 3), the wells contained equal numbers of each CD8$^+$ T cell-enriched population, for a total of 3×10^6 responders/well. In all experiments, control cultures referred to as 'mock-stimulated' were identical to experimental cultures except that no antigen was added. Sorted CD8$^+$ memory T cells were cultured at 5×10^3/well in a total volume of 0.2 ml in 96-well round-bottom plates (Sarstedt) with 1×10^5 APC/well. Cultures were incubated at 37°C in 10% CO_2 for 4–5 days prior to assay.

Cytotoxicity assays

For measurement of influenza-specific CTL, 1×10^6 L929 cells/well were labeled overnight with 100–200 μCi $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) in 1.5 ml complete medium

in six-well tissue culture plates (Sarstedt). The adherent cells were washed once with HBSS and infected with 4000 HAU influenza A/HKx31 virus for 2–4 h. The cells were washed again with HBSS, removed from the plates with trypsin–EDTA, washed twice more and plated with various dilutions of effector cells at 2×10^3/well in a final volume of 0.2 ml in 96-well V-bottom plates (Sarstedt). The I-E^k-transfected L929 cell line DCEKHi-7 (here termed L/I-E^k), kindly provided by Dr R. Germain (55), was ^{51}Cr-labeled as above and used for measurement of superantigen-dependent cellular cytotoxicity (SDCC) (8). SE were included at a final concentration of 1 μg/ml. Assays were incubated for 4–6 h at 37°C in 10% CO_2. Then, 100 μl of supernatant was collected and counted in a Packard Cobra gamma counter. Maximum ^{51}Cr release was determined by incubation of targets with 1% Triton X-100 and spontaneous release by incubation in medium alone. Percent specific lysis = [(experimental release – spontaneous release)/(maximum release – spontaneous release)]$\times$100.

Limiting dilution analysis (LDA)

CTL precursor (CTLp) frequencies were estimated as previously described (56–59). Briefly, sorted CD8$^+$ memory T cells were plated at various dilutions in 96-well round-bottom plates with 1×10^5 APC/well and rhIL-2 at 10 units/ml. Mock-stimulated control microcultures contained the same numbers of responders and APC and the same concentration of rhIL2, but no antigen. Cultures were incubated for 7 days before assay. The cultures were washed prior to assay. The individual wells were tested in 6 h ^{51}Cr-release assays against 1.5×10^3 targets. Wells were considered positive if specific ^{51}Cr release was greater than the mean + 3 SD of 32–48 wells of antigen and APC cultured with rhIL-2, but no responder T cells. Minimal estimates of CTLp frequencies were obtained by assay of 24–32 wells at each responder cell dilution and using the Poisson equation of the slope of the line relating frequency of negative wells (F_0) to the number of responder cells plated. The slope of the line was determined by χ^2 analysis yielding minimum frequency estimates, 95% confidence intervals of the frequency estimate and χ^2 estimates of the probability in which significance is indicated by $P >$ 0.05% (60). In some experiments, such as the one shown in Fig. 4(A), split-well analysis was performed to determine whether the microcultures would lyse uninfected target cells. The number of wells positive for lysis of negative control targets was consistently very low, so that reliable estimates of the frequency of non-specific CTLp were not possible. Moreover, exclusion of wells that lysed negative control targets from the analyses had no significant effect on the frequency estimates for influenza-specific CTLp.

Proliferation and cytokine assays

Sorted CD8$^+$CD62L^{lo}CD44^{hi} T cells (2×10^4) were cultured at 37°C in 96-well round-bottom plates with 2×10^5 irradiated, mitomycin C-treated, T cell-depleted APC in 0.2 ml/well complete medium in the presence or absence of 10 μg/ml SEB. After 45 h in culture, supernatants were removed and stored at –70°C for subsequent cytokine measurements. Fresh medium containing 1 μCi/ml [^{3}H]thymidine (Amersham) was added and the cultures incubated for an additional 24 h. The plates were harvested on a Tomtek cell harvester and the filters counted on a Packard Matrix 96 direct β counter. IL-2 and IFN-γ levels in culture supernatants were determined by sandwich ELISA as previously described (46). The sensitivities of the assays were 0.78 U/ml for IL-2 and 19.5 U/ml for IFN-γ.

Results

SEB reactivates influenza-specific memory CTL in vivo

To address the ability of SE to activate influenza virus-specific memory CD8$^+$ T cells, we took advantage of a TCR β chain transgenic mouse line. In these mice, a rearranged V_β8.1 transgene of no known antigen specificity is expressed in combination with a diverse repertoire of endogenous α chains. We have previously shown that CBA/CaJ mice which express this rearranged TCR β chain in >95% of peripheral T cells (CBA.βTG) are capable of mounting an influenza-specific CD8$^+$ CTL response (61,62). The responding virus-specific T cells were all found to express the transgenic TCR β chain and thus did not derive from the minor population of peripheral T cells expressing endogenous TCR V_β elements (62). This allowed us to study the effects of SEB on influenza-specific CD8$^+$ T cell memory in a situation where virtually all of the memory T cells should be superantigen reactive. In contrast, the influenza virus-specific CTL response of non-transgenic CBA/CaJ mice is highly diverse (62–66 and our own unpublished observations). Thus, only a fraction of the virus-specific memory pool in CBA/CaJ mice reacts with either SEB or SEA and we could examine SE effects on a subset of the total memory population.

To determine whether virus-specific memory cells respond to *in vivo* challenge with SE, CBA.βTG and CBA/CaJ mice that had been infected with influenza virus at least 8 weeks previously (memory mice) received a single i.p. injection of SEB or PBS. The mice were sacrificed 36–48 h later and CD8$^+$ T cell-enriched spleen cell suspensions were assayed for lysis of influenza-infected L929 cells. As shown in Fig. 1(A), SEB-treated CBA.βTG mice yielded influenza-specific CTL, while PBS-treated controls did not. A similar result was obtained when non-transgenic CBA/CaJ memory mice were used (Fig. 1B), although the level of cytolytic activity was, as expected, somewhat less. Little or no influenza-specific activity was detected in SEB-treated naive control animals (data not shown). However, substantial SDCC was observed (data not shown), as has been described by others (6,8,9,67). These results clearly show that antigen-specific CD8$^+$ memory T cells can be re-activated by *in vivo* exposure to bacterial superantigens.

SEA and SEB both stimulate secondary influenza-specific CTL in vitro

To further characterize the nature of the SE-induced activation of antigen-specific, CD8$^+$ memory T cells, we examined the effect of SE stimulation *in vitro*. As shown in Fig. 2(A), *in vitro* re-stimulation of splenic CD8$^+$ T cells from CBA.βTG memory mice with either influenza virus or SEB resulted in the generation of effector CTL capable of lysing influenza virus-infected L929 cells. This result was not dependent on the transgenic TCR β chain, as influenza-specific CTL activity was also generated following SEA or SEB stimulation of CD8$^+$ T cells

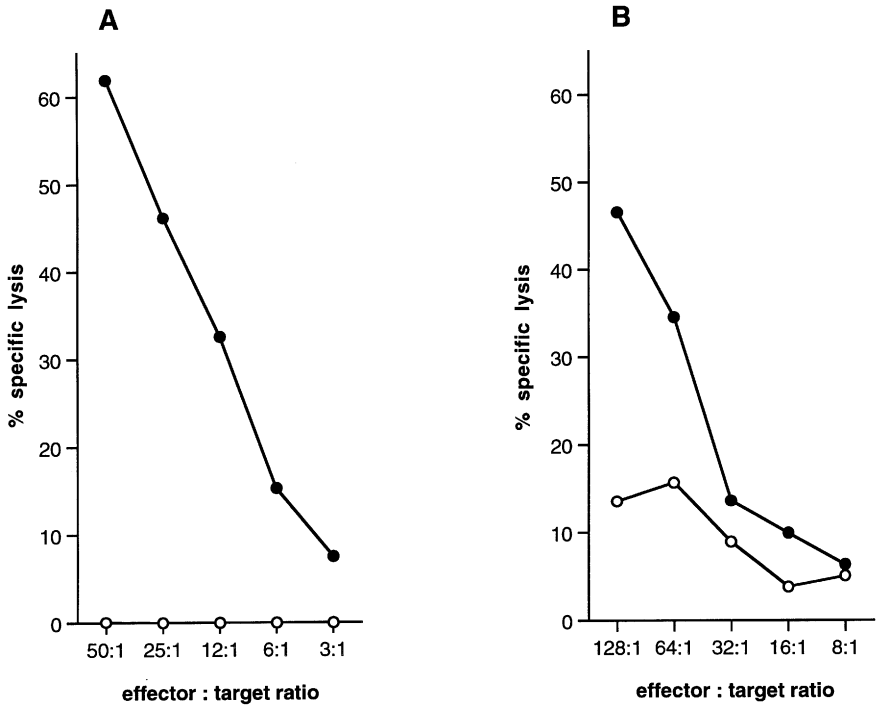

Fig. 1. *In vivo* administration of SEB leads to recovery of influenza-specific cytolytic effectors from the spleens of memory mice. Mice which had recovered from influenza infection were given 100 μg SEB in 0.5 ml PBS (closed symbols) or PBS alone (open symbols) i.p. 36–48 h before sacrifice. CD8$^+$ T cell-enriched spleen cell suspensions were tested for lysis of influenza virus-infected L929 cells in standard ^{51}Cr-release assays. (A) Spleen cells from CBA.βTG mice. (B) Spleen cells from CBA/CaJ mice. Lysis of uninfected L929 targets was <7% at the highest E:T tested. Each experiment was repeated three times with similar results.

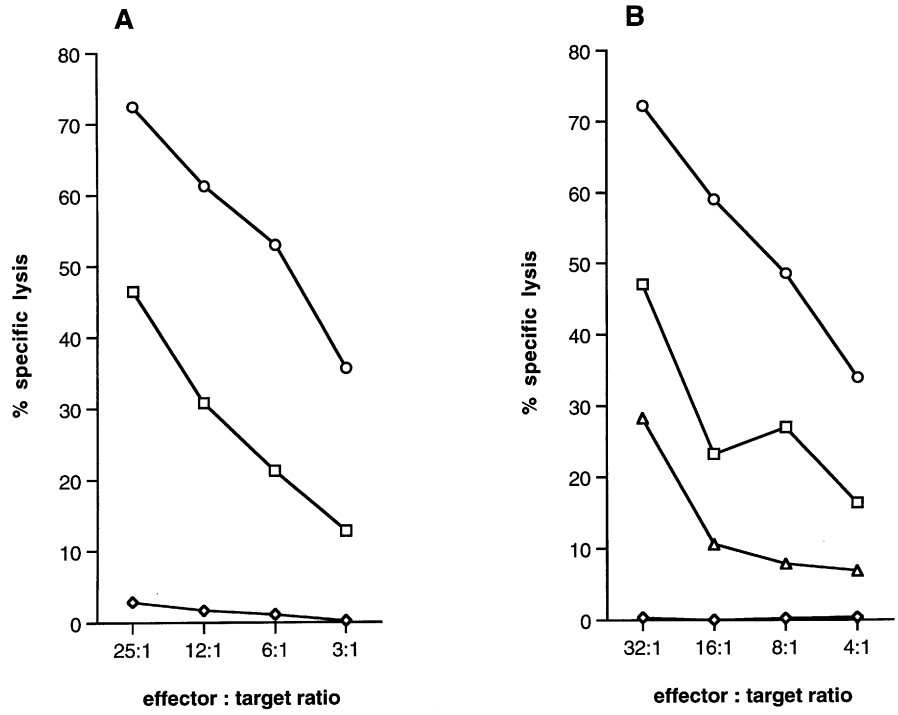

Fig. 2. SE stimulate the *in vitro* generation of influenza-specific CTL from memory mice. CD8$^+$ T cell-enriched spleen cells from memory mice were cultured with influenza-infected T cell-depleted normal splenic APC (circles), uninfected APC and 10 μg/ml SEB (squares), uninfected APC and 10 μg/ml SEA (triangles), or uninfected APC alone (diamonds). After 5 days, lysis of influenza virus-infected L929 cells was measured in a standard ^{51}Cr-release assay. (A) CBA.βTG responders. (B) CBA/CaJ responders. Lysis of uninfected L929 targets was 13% or less at the highest E:T tested. Each experiment was repeated twice with similar results.

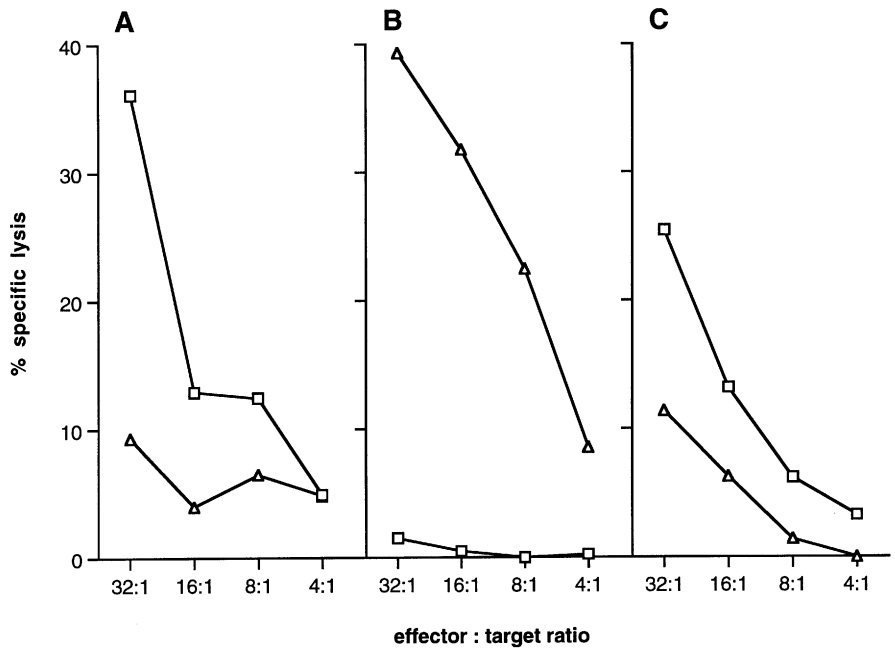

Fig. 3. The *in vitro* generation of influenza-specific CTL in response to superantigenic stimulation does not result from indirect activation of memory cells. CD8$^+$ T cell-enriched spleen cells from CBA.βTG memory mice and naive CBA/CaJ mice cultured together and stimulated with SEA (triangles) or SEB (squares) were assayed against influenza-infected L929 targets (A), SEA-coated L/I-E^k targets (B) or SEB-coated L/I-E^k targets (C). The data shown is representative of three similar experiments. Specific lysis of uninfected L929 cells or L/I-E^k cells in the absence of SE was $<$10%. Mock-stimulated cultures contained no influenza-specific lytic activity.

from non-transgenic CBA/CaJ mice (Fig. 2B). Addition of exogenous IL-2 to the cultures resulted in a slight increase in the levels of lysis, including background lysis of uninfected L929 cells, but otherwise had no effect on the results (data not shown). As expected, SDCC, but no influenza-specific lytic activity, was detected in SE-stimulated cultures of T cells from naive mice (data not shown). Thus, in keeping with the *in vivo* results, SEA and SEB stimulated memory CTL in secondary *in vitro* cultures.

SE stimulation of memory CTL appears to involve direct TCR recognition

The SE-induced activation of influenza-specific CTL observed *in vivo* and *in vitro* could have resulted either from a direct V_β-specific stimulation of CD8$^+$ memory T cells or from an indirect effect on memory CTL precursors mediated by superantigen-induced cytokines. We therefore tested the ability of superantigens to indirectly activate memory CTL *in vitro*. Naive CD8$^+$ T cells from non-transgenic CBA/CaJ mice were co-cultured with CD8$^+$ T cells from V_β8.1 transgenic memory mice in the presence of SEA. Generation of influenza-specific CTL in these cultures would be consistent with an indirect mechanism of activation, presumably involving cytokines produced by naive, non-transgenic T cells responding to SEA. Alternatively, if the effect depended on direct superantigen recognition by memory CTLp, these cultures should yield little or no influenza-specific CTL activity since only transgenic memory cells are present and V_β8.1$^+$ T cells do not react with SEA. As shown in Fig. 3(A), SEB, but not SEA, stimulated the generation of influenza-specific lytic activity in the mixed cultures. There was a vigorous superantigen-specific response to SEA, however, as indicated by the presence of substantial SDCC of SEA-coated targets following SEA stimulation (Fig. 3B). We also observed a low (and variable) level of SDCC of SEB-coated targets in the SEA-stimulated cultures (Fig. 3C), suggesting that there might be some cytokine-mediated, TCR-independent activation of CTLp under these conditions. However, the results of this experiment were consistent with the interpretation that most of the SE-stimulated influenza-specific lysis resulted from direct engagement of the TCR of SE-reactive CTLp by superantigen.

Superantigens stimulate CD8$^+$ T cells with a memory phenotype

A direct assessment of the ability of memory CTLp to respond to superantigenic stimulation in the absence of bystander effects requires the use of purified populations of memory cells. It has been shown that the vast majority of influenza-specific memory CTLp express low levels of L-selectin (CD62L) and high levels of Pgp-1 (CD44) (38–40,68). Therefore, sorted CD8$^+$CD62L^{lo}CD44^{hi} T cells from memory mice were cultured under limiting dilution conditions. As expected, in both transgenic (Fig. 4A) and non-transgenic (Fig. 4B) mice, a very high frequency of influenza-specific CTLp was detected among sorted CD8$^+$ T cells with this memory phenotype following culture with influenza-infected APC. In the transgenic mice, in which all of the influenza-specific CTLp express V_β8.1 and are therefore likely to be SEB-reactive, SEB was, if anything, more efficient than influenza virus in stimulating these memory cells (Fig. 4A). In the non-transgenic mice, SEB and SEA each stimulated a fraction of the influenza-specific CTLp (~1/4 and 1/40 respectively,

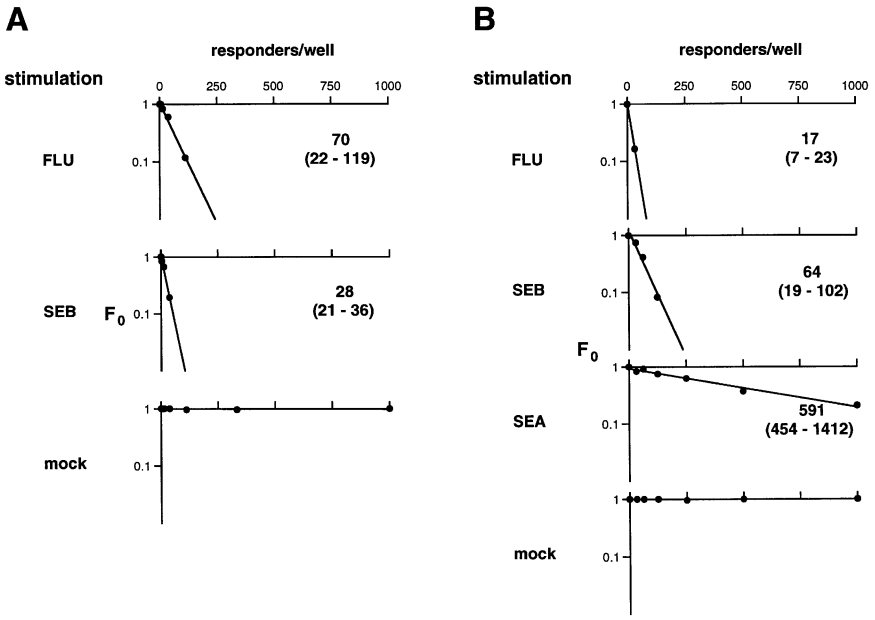

Fig. 4. SE stimulate CD8$^+$CD62L^{lo}CD44^{hi}, influenza-specific memory CTL precursors *in vitro*. CD8$^+$ T cell-enriched spleen cells from memory mice were stained and sorted as described in Methods. Sorted memory phenotype cells were cultured under limiting dilution conditions with irradiated T cell-depleted spleen cells, 10 units/ml IL-2 and the indicated stimulus for 7 days prior to assay against ^{51}Cr-labeled, influenza-infected L929 cells. The fraction of negative wells (F_0) at each dilution is plotted and the reciprocal of the CTLp frequency is shown (with 95% confidence limits in parentheses). (A) CBA.βTG responders. (B) CBA/CaJ responders.

Fig. 4B). Finally, even when single transgenic CD8$^+$ CD62L^{lo}CD44^{hi} memory T cells were sorted directly into individual wells, SEB presented on T cell-depleted APC stimulated the generation of influenza-specific CTL activity in 80/1152 wells (data not shown). These data clearly showed that influenza-specific CD8$^+$ memory T cells could directly respond to superantigenic stimulation.

Influenza-specific CD8$^+$ memory T cells respond to SEB in the absence of exogenous IL-2

It was possible that the memory CTLp detected under LDA conditions would have been anergic to SE in the absence of added IL-2. Therefore, it was important to assess the ability of CD8$^+$ T cells with a memory phenotype to respond to SE when cultured in the absence of exogenous lymphokines. Unfortunately, clonal growth of CTL precursors in the absence of exogenous IL-2 is not feasible and the extremely low frequency of CD62L^{lo}CD44^{hi} T cells in the spleens of memory mice (data not shown) did not allow recovery of sufficient cells for standard bulk CTL culture. We therefore tested the response of sorted memory cells to SE in a small-scale bulk CTL assay. Figure 5 shows that microcultures of transgenic CD8$^+$CD62L^{lo}CD44^{hi} T cells developed influenza-specific CTL activity in response to SEB presented on T cell-depleted APC without the addition of any exogenous cytokines. Similar cultures were set up to test the ability of CD8$^+$ memory T cells to proliferate and produce lymphokines. SEB stimulated modest thymidine incorporation (Fig. 6A) and secretion of substantial amounts of IFN-γ (Fig. 6B). Small quantities of IL-2 were also detected in the culture supernatants (~1 U/ml; data not shown). The use in these experiments of small numbers of sorted memory cells as responders, combined with the efficient depletion of T cells from the splenic APC population, served to minimize any possible contributions of lymphokines derived from naive T cells. Thus, memory CD8$^+$ T cells appear to be capable of producing sufficient quantities of cytokines in response to superantigenic stimulation to sustain their own proliferation and differentiation into cytolytic effectors.

Discussion

In general, memory T cells are more easily activated than naive cells, as reflected by less stringent requirements for co-stimulation, lymphokines or high antigen density on APC (28–33). It is also becoming clear that TCR recognition of peptide–MHC and superantigen–MHC complexes are comparable in many ways (69–72). Thus, reports that memory T cells are anergic to bacterial superantigens (34,35) were surprising and are somewhat at odds with our current understanding of T cell memory.

In this report, we have shown that influenza virus-specific CD8$^+$ memory T cells respond to *in vivo* or *in vitro* stimulation with bacterial superantigens by developing into functional effector CTL. Our analyses of sorted CD8$^+$CD62L^{lo}CD44^{hi} T cells clearly showed that individual memory cells could respond directly to superantigenic stimulation. The development of influenza-specific CTL in limiting dilution cultures (Fig. 4) and cultures of single sorted memory cells (data not shown) indicated that this response was independent of bystander T cell effects. To argue against the possibility that the memory cells were, in fact, anergic to SE stimulation, but that the addition of IL-2 to these cultures overcame the anergy, as shown in several *in vitro* models (73–77), we have also

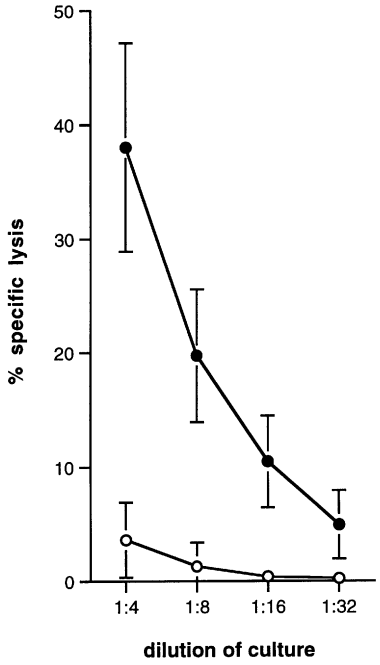

Fig. 5. SEB stimulates CD8$^+$CD62L^{lo}CD44^{hi}, influenza-specific memory CTL precursors *in vitro* in the absence of exogenous IL-2. CD8$^+$ T cell-enriched spleen cells from CBA.βTG memory mice were stained and sorted as described in Methods. Sorted memory phenotype cells (5×10^3) were plated with 1×10^5 irradiated T cell-depleted spleen cells in individual wells of 96-well round-bottom plates. After 5 days of culture with (filled symbols) or without (open symbols) 10 μg/ml SEB, the wells were washed, split, serially diluted and assayed against ^{51}Cr-labeled, influenza-infected L929 cells. The data are plotted as the mean ± SD of 12 wells at each dilution. Lysis of uninfected L929 cells was <5%. The experiment was repeated with similar results.

shown that the ability of SEB (presented on T cell-depleted APC) to stimulate influenza-specific CD8$^+$CD62L^{lo}CD44^{hi} T cells in bulk microcultures was not dependent on the addition of exogenous IL-2 (Fig. 5). Moreover, since SEB stimulated purified CD8$^+$ memory T cells to proliferate and secrete lymphokines, our experiments do not appear to be explained by partial or 'split' anergy, such as was described by Otten and Germain (74).

Superantigen reactivation of influenza-specific memory CTL was most readily demonstrable when CBA.βTG mice or cells were exposed to SEB (Figs 1A and 2A). However, in experiments with non-transgenic T cells, both SEB and SEA were clearly able to induce secondary influenza-specific CTL in bulk cultures (Fig. 2B). In the SEA stimulations in particular, only a small percentage of the virus-specific CTLp are superantigen reactive (see Fig. 4B) and only a small fraction of the SEA-reactive T cells are virus-specific CTLp. The generation of influenza-specific CTL effectors in these cultures is thus quite remarkable, given the massive proliferation of (presumably mostly naive) SEA-reactive, non-influenza-specific T cells under these conditions (e.g. see Fig. 3B). These data therefore suggest that memory cells are not at any particular proliferative disadvantage compared to naive cells in the bulk cultures. The LDA shown in Fig. 4(B) underscores this point and offers a plausible explanation for the less efficient generation of influenza-specific CTL following SEA stimulation in bulk culture (Fig. 2B), since SEA-reactive, influenza-specific memory CTLp were present at a 10-fold lower frequency than SEB-reactive precursors. We should also point out that Fig. 2 presents SE-stimulated, influenza-specific CTL activity on a 'per cell' basis, which actually understates the total specific lytic activity generated in these cultures compared to influenza virus-stimulated cultures, since there is much more extensive T cell proliferation in the superantigen-stimulated wells (data not shown). Thus, the naive CD8$^+$ T cells that respond to SE in these cultures effectively dilute the influenza-specific memory CTL.

We were unable to detect substantial influenza-specific CTL following SEA stimulation of mixed cultures of naive, non-transgenic T cells and V_β8.1 transgenic memory T cells (Fig. 3), suggesting that cytokine-mediated bystander activation of memory CTLp is not a major contributor to the SE-stimulation of virus-specific CTL shown in Figs 1 and 2. It is possible, of course, that the cytokine profiles induced by SEA and SEB in naive CD8$^+$ T cells (and APC) differ. For example, SEA might induce cytokines which suppress the influenza-specific CTL, or a key activating molecule may be produced in SEB-stimulated cultures, but not SEA-stimulated cultures. However, SEA clearly could activate influenza-specific CTL (Figs 2B and 4B), arguing against this possibility. Therefore, taken together with our inability to detect influenza-specific CTL following stimulation with lymphokines alone (data not shown), the data in Fig. 3 demonstrate that most of the observed influenza-specific CTL activity in SE-stimulated cultures resulted from direct superantigen recognition by memory CTLp. FACS analysis of V_β usage in the SE-stimulated cultures and cold target inhibition experiments were also consistent with this interpretation (data not shown). Moreover, we feel that the limitations of these experiments are obviated by the results obtained with purified memory T cells (Figs 4 and 5).

Despite some evidence to the contrary (25,32), the prevailing perception is that memory T cells do not respond to stimulation by superantigens. This was first shown for CD4$^+$CD45RB^{lo} T cells by Lee and Vitetta (35), who subsequently found that the 'anergic' memory cells did express activation markers upon SEB stimulation, although no proliferation or lymphokine secretion was observed (78). More recently, Hamel *et al.* (34) showed that previously activated T cells fail to respond to SEB *in vitro*, whether the initial activation was with SEB or with immobilized anti-TCR antibodies. These authors attributed their findings to intrinsic differences in TCR-mediated signal transduction in naive versus memory cells and concluded that non-responsiveness to superantigens is a 'general feature of memory T cells'. Our data argue strongly against this notion, at least for CD8$^+$ T cells.

The apparent disagreement between our results showing that CD8$^+$ memory T cells respond to superantigens and these recent reports of hypo-responsiveness or non-responsiveness of memory CD4$^+$ T cells to superantigens may reflect fundamental physiologic differences between CD4$^+$ and CD8$^+$ memory T cells, or could result from differences in the assays employed to measure CD4$^+$ and CD8$^+$ T cell responses. Most of our studies have focused on measurement

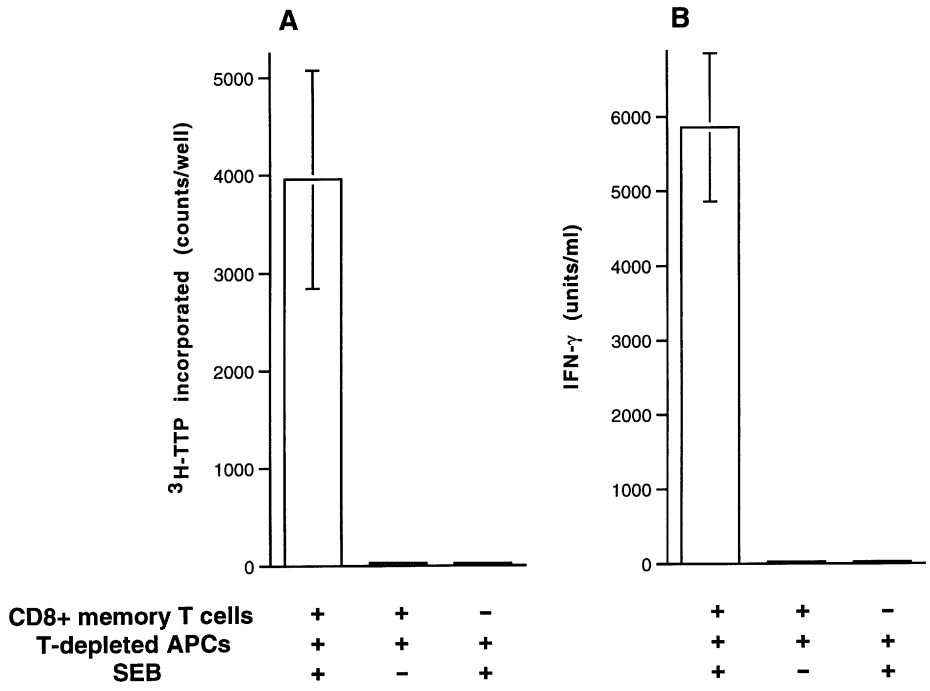

Fig. 6. SEB stimulates proliferation and cytokine production in memory T cells. CD8$^+$CD62L^{lo}CD44^{hi} T cells from CBA.βTG memory mice were purified as in Fig. 5. Sorted memory phenotype cells (2×10^4) were plated with 2×10^5 irradiated, mitomycin C-treated, T cell-depleted spleen cells in individual wells of 96-well round-bottom plates. (A) After 2 days in culture under the conditions indicated, 1 μCi [^{3}H]thymidine was added to each well and the plate incubated for an additional 24 h. Thymidine incorporation is plotted as the mean ± SD of 10 wells for each condition. (B) Supernatants from 2 day cultures were diluted 10-fold and assayed for IFN-γ by ELISA. Concentrations were determined by comparison with a standard curve of 2-fold dilutions of the recombinant cytokine. The data is plotted as the mean ± SD of 10 wells for each condition.

of virus-specific cytolytic effector function, but we have also shown that CD8$^+$ memory T cells proliferate and secrete cytokines in response to SEB. Although we detected only very small amounts of IL-2 in the cultures (data not shown), we have shown that SEB alone can drive the differentiation of purified CD8$^+$ memory cells into cytolytic effectors *in vitro*. It also seems likely that at least some of the IL-2 produced in these cultures was consumed by the proliferating T cells. Although the cytokine ELISAs and thymidine incorporation assays (Fig. 6) do not prove that the same cells mediating influenza-specific cytolysis are also proliferating or producing cytokines, they do show that SEB can elicit each of these functions from at least some memory cells. Taken together, our data clearly demonstrate that SEB induces CD8$^+$ memory cells to proliferate and differentiate, and strongly suggest that these cells also produce sufficient IL-2 to sustain their own proliferation and differentiation.

Our results could also be reconciled with the earlier studies if one considers that the reduced proliferative responses of CD4$^+$ memory cells to superantigenic stimulation (34,35,37,78) may not accurately reflect the capacity of these cells to mediate effector functions. In this regard, a recent study by Schultz *et al.* (25) is of interest. These authors show that secondary challenge with SEB induces a very potent anamnestic response which is markedly accelerated and of shorter duration when compared with a primary response, and most of the parameters typically measured to assess T cell activation returned to normal on day 2 of the secondary response. This is not unlike the situation in respiratory virus infections, where the response to secondary challenge is characterized by a more rapid mobilization of CTL into the lung, without the same dramatic increase in CTLp frequency that is seen in a primary infection (41,79,80). Thus, sustained proliferation may not be required for an effective CD8$^+$ memory response. Similarly, secondary CD4$^+$ T cell responses might be expected to emphasize rapid development of effector function rather than massive clonal expansion. If this were true, then the apparent anergy of memory T cells in several independent studies may reflect a failure to appreciate substantial differences in the kinetics of primary versus secondary responses at the cellular level. Indeed, data from some of the earlier studies are consistent with this interpretation, such as the early proliferative response of previously activated T cells to SEB noted by Hamel *et al.* (34). It is unfortunate that the term anergy has been assigned to a number of very different situations that bear little resemblance to what was originally described for T cells by Jenkins and Schwartz (81) or Lamb and co-workers (82,83). We feel that an overly broad definition of this term could lead to confusion between physiologically relevant changes in the magnitude or kinetics of a T cell response, such as might be expected in immunologic memory, and true non-responsiveness characteristic of a state of tolerance. Finally, it is important to consider the possibility of synergistic interactions between naive and memory responses *in vivo* (84) when extrapolating from *in vitro* results with purified T cell subsets.

To our knowledge, the present study represents the first report of superantigen effects on established T cell memory to a viral pathogen. The finding that bacterial superantigens can activate virus-specific memory cells may have pathophysiological relevance, since respiratory virus infections are frequently complicated by secondary bacterial infections (85). We have recently demonstrated a lethal synergism between SEB and influenza infection in both CBA/CaJ and CBA.βTG mice (46). Current studies in our laboratory are directed toward understanding the effects of superantigen exposure on the generation of the primary anti-viral immune response, as well as the development and maintenance of virus-specific memory responses. In light of the results presented here, it is possible that subclinical infections with superantigen-secreting bacteria might influence the long-term maintenance of T cell memory established during earlier viral infections.

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Abbreviations

APC	antigen-presenting cell
CTL	cytotoxic T lymphocyte
CTLp	CTL precursor
HAU	hemagglutinating unit
KLH	keyhole limpet hemacyanin
LDA	limiting dilution analysis
PE	phycoerythrin
rhIL-2	recombinant human IL-2
SDCC	superantigen-dependent cellular cytotoxicity
SE	staphylococcal enterotoxin

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