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Memory CD8+ T Cells Protect Dendritic Cells from CTL Killing[1]

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Abstract

CD8+ T cells have been shown to be capable of either suppressing or promoting immune responses. To reconcile these contrasting regulatory functions, we compared the ability of human effector and memory CD8+ T cells to regulate survival and functions of dendritic cells (DC). We report that, in sharp contrast to the effector cells (CTLs) that kill DCs in a granzyme B- and perforin-dependent mechanism, memory CD8+ T cells enhance the ability of DCs to produce IL-12 and to induce functional Th1 and CTL responses in naive CD4+ and CD8+ T cell populations. Moreover, memory CD8+ T cells that release the DC-activating factor TNF-α before the release of cytotoxic granules induce DC expression of an endogenous granzyme B inhibitor PI-9 and protect DCs from CTL killing with similar efficacy as CD4+ Th cells. The currently identified DC-protective function of memory CD8+ T cells helps to explain the phenomenon of CD8+ T cell memory, reduced dependence of recall responses on CD4+ T cell help, and the importance of delayed administration of booster doses of vaccines for the optimal outcome of immunization.

In addition to their function as CTLs capable of killing transformed or infected cells in a perforin- and FasL-dependent mechanism (1,2), CD8+ T cells have also been shown to play a regulatory role, being able to suppress Ag-specific immune responses (3,4). Their suppressor activity (5–7) involves the elimination of Ag-carrying dendritic cells (DCs)+ by effector CD8+ T cells (8) in a perforin-dependent mechanism (9). Activated CD8+ T cells have been shown to limit the CTL responses by restricting DC survival and the duration of Ag display in vivo in mice infected with *Listeria*, lymphocytic choriomeningitis virus, HSV, and malaria (10–12). This self-limiting nature of CD8+ T cell responses can be counteracted by CD4+ Th cells (10,13), known to be important for the establishment of CD8+ T cell memory and effective expansion of CTL precursors during recall responses (14–16). Although the mechanism of helper activity of CD4+ T cells was originally considered to involve Th cell-produced IL-2 (17) and the CD40L-mediated elevation of the stimulatory capacity of DCs (18–20), it was subsequently demonstrated that CD40L-expressing CD4+ T cells can induce in DCs

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endogenous granzyme B inhibitor (serpin SPI-6/PI-9) (13), rescuing the Ag-carrying DCs from killing by the same CTLs that they induce, and thus prolonging the time frame of effective stimulation of the expanding population of Ag-specific $CD8^+$ T cells (10,13).

In contrast to the widely studied suppressive activity of $CD8^+$ T cells, only recently was it demonstrated that $CD8^+$ T cells can also activate DCs and promote the Th1- and CTL-mediated type 1 immunity (21–27).

To reconcile the paradoxical ability of $CD8^+$ T cells to act as both suppressor and helper cells, we addressed the possibility that helper and suppressor functions are selectively displayed by $CD8^+$ T cells at different stages of activation. Using the models of peptide specific- and superantigen-driven activation of $CD8^+$ T cells isolated from blood and tissues and the in vitro preactivated $CD8^+$ T cells at different stages of activation, we show that the DC killing and DC-activating/protecting functions are exerted sequentially by human $CD8^+$ T cells. Although effector $CD8^+$ T cells kill DCs in a granzyme B- and perforin-dependent pathway, TNF-α-producing memory $CD8^+$ T cells display an equivalent activity to $CD4^+$ Th cells in protecting DCs from premature elimination by the effector cells, supporting the induction of functional Th1 and CTL responses.

Materials and Methods

Media, reagents, and cell lines

The cell cultures were performed using either IMDM (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone) or serum-free AIM-V medium (Invitrogen Life Technologies). Recombinant human (rhu) GM-CSF and IL-4 were gifts from Schering Plough. IL-2 was provided by Chiron. IFN-γ, TNF-α, and IL-1β were purchased from Strathmann Biotec. *Staphylococcus* enterotoxin B (SEB), used for priming a high number of naive $CD8^+$ T cells (24,28), was obtained from Toxin Technologies. CD40L-transfected J558 plasmacytoma cells were a gift from Dr. P. Lane (University of Birmingham, Birmingham, U.K.) and JY-1 cells were a gift from Dr. E. Wierenga (University of Amsterdam, Amsterdam, The Netherlands). Granzyme B inhibitors IETD-CHO and Z-IETD-fmk were obtained from Calbiochem.

Isolation of the naive, memory, and effector T cell subsets from peripheral blood and tissues

Mononuclear cells, obtained from the peripheral blood of healthy donors, were isolated by density gradient separation using Lymphocyte Separation Medium (CellgroMediatech). Naive $CD4^+CD45RA^+$ T cells and naive $CD8^+CD45RA^+$ T cells were isolated by negative selection with the StemSep CD4 and CD8 enrichment mixtures, respectively (StemCell Technologies). Biotinylated anti-CD45RO Ab was used in combination with enrichment mixtures for isolation of a naive population. The phenotype of the naive $CD8^+CD45RA^+CCR7^+$ T cell population was confirmed by flow cytometry. Tissue-type effector $CD8^+$ T cells were obtained from the liver-metastatic tumor tissue of colorectal cancer patients undergoing surgical resection, and cultured overnight in low-dose IL-2 to recover from the isolation-induced stress and possible effects of tumor-derived factors. The memory subset $CD8^+CD45RA^-CCR7^+$ T cells from peripheral blood was isolated using CD45RA-depleting/$CD8^+$ T cell enrichment mixture (StemCell Technologies).

Generation of DCs

Day 6 immature DCs (used as a readout of functional activity of $CD8^+$ T cells) were generated from peripheral blood monocytes cultured (5×10^5/ml) in IMDM/10% FBS supplemented with rhuIL-4 and rhuGM-CSF (both at 1000 U/ml) in 24-well plates (Falcon; BD Biosciences). Type 1-polarized mature DCs, used for the generation of effector- and memory-type $CD8^+$ T

cells in vitro, were obtained in serum-free AIM-V medium with IL-4 and GM-CSF, and matured (days 6–8) in the presence of TNF-α, IFN-γ, IL-1β, IFN-α, and poly I:C, as described (29).

In vitro induction of effector- and memory-type CD8$^+$ T cells

Naive CD8$^+$CD45RA$^+$CCR7^{high} T cells (5×10^5 cells/well) were activated with SEB-pulsed DCs (5×10^4 cells/well) in the presence of soluble (s) CD40L (sCD40L; Alexis). Although CD8$^+$CD45RA$^+$ T cells may contain low frequencies of primed cells, our previous studies showed a lack of differences between such cells isolated from adult or cord blood, when using polyclonal models of activation (24). IL-2 (50 U/ml) and IL-7 (5 ng/ml) were added on day 3. Subsequently, culture medium was replenished with fresh medium and cytokines every 2 days. Priming for 8 days resulted in CD8$^+$ T cells with a high content of the cytotoxic granule components granzyme B and perforin, referred to as effector-type CD8$^+$ T cells in the current study. CD8$^+$ T cells primed and cultured for 15 days yielded a functional phenotype of memory cells with low granzyme B and perforin content. The HLA-A2-restricted CD8$^+$ T cell clone (24) recognizing melanoma Ag gp100$_{209–217}$ was cloned from the TIL 1520 cell line provided by Drs. S. Rosenberg and J. Wunderlich (National Cancer Institute, Bethesda, MD), and used either 4 days after Ag-specific restimulation, or after prolonged culture (>4 wk) in IL-2 (100 U/ml), in the absence of stimulation.

Modulation of DC function by CD8$^+$ T cells

CD8$^+$ T cells (5×10^4 cells) were added to day 6 immature DC cultures with or without Ag SEB (or gp100 peptide). After 48 h, cells were harvested, washed, and analyzed by flow cytometry or stimulated with CD40L-transfected J558 cells (24) for 24 h. For DC protection studies, memory-type CD8$^+$ T cells (5×10^4 cells/ml) were added to immature DC cultures 6–8 h before the addition of effector-type CD8$^+$ T cells (5×10^4 cells/ml). When indicated, day 8-primed CD8$^+$ T cells were pretreated with the perforin inhibitor (24) concanamycin A (CMA; 100 nM) for 2 h and then added to immature DC cultures. The survival of DCs was assessed by staining with nonyl acridine orange dye (NAO; Sigma-Aldrich), as a marker of apoptosis (loss of mitochondrial potential: NAO which binds to mitochondrial cardiolipin in a membrane potential-dependent manner) (30). Light scatter properties and annexin V staining have been used, yielding similar results (see Fig. 3 and data not shown). Briefly, DCs were stained with 0.2 μM NAO in culture medium for 15 min at 37°C. The cells were washed and immediately analyzed by flow cytometry. For blocking TNF-α function in day 14 memory-type CD8$^+$ T cells, recombinant human soluble TNFRI (R&D Systems) and anti-human TNF-α Ab infliximab (a gift from Dr. C. Hilkens, University of Newcastle, Newcastle upon Tyne, United Kingdom) were added to culture wells with DCs and day 14 memory-type CD8$^+$ T cells.

Flow cytometry

Cell surface phenotype was analyzed by flow cytometry using Beckman Coulter XL. The FITC- and PE-labeled isotype controls (mouse IgG1 and IgG2a), anti-human CD86, anti-human perforin were obtained from BD Pharmingen. CD83 mAb was purchased from Immunotech and PE-labeled granzyme B Ab was obtained from Cell Sciences. Goat anti-mouse IgG, FITC conjugated, was obtained from Caltag Laboratories. For detection of intracellular PI-9, we used PI-9-specific mouse monoclonal IgG1 Ab as described (31). Briefly, for intracellular staining of PI-9, DCs were washed and then blocked with human Ig for 10 min at room temperature. Subsequently, cells were permeabilized with 300 μl of Permiflow (Invirion) for 60 min at room temperature and then washed. The cells were stained with unconjugated anti-human PI-9 Ab for 20 min at room temperature followed by staining with FITC-conjugated goat anti-mouse IgG Ab. Granzyme B and perforin staining were performed according to the manufacturer's protocol, using Permiflow as the permeabilization reagent.

Cytokine detection

Concentrations of IL-12, TNF-α, IL-5, and IFN-γ were determined using specific ELISA, using matched Ab pairs from Endogen. Granzyme B was detected in the supernatants by ELISA (Diaclone).

Microscopy

For TNF-α, granzyme B, and PI-9 visualization experiments, DCs were cultured on collagen-coated cover glass (size 12RD, thinness 1; Propper Manufacturing) placed in 24-well plates (Falcon). Before imaging, $CD8^+$ T cells, labeled with either CFSE (2.5 μM) or Calcein blue AM (10 μM; Molecular Probes) according to the manufacturer's protocol, were added to SEB-pulsed DCs (day 6) and incubated for 2 h at 37°C to allow conjugate formation. Following incubation, cells were fixed with 2% paraformaldehyde, permeabilized with Triton X-100, and were blocked with normal goat serum (Sigma-Aldrich). The primary Abs were mouse anti-human granzyme B (Caltag Laboratories), rat anti-human TNF-α (Serotec), mouse anti-human CD11c-Cy5 (BD Pharmingen), and mouse anti-human PI-9. The secondary Abs were goat anti-mouse Cy3 Fab 1 and goat anti-rat Cy3 (Jackson ImmunoResearch Laboratories). All the Abs were used at a final concentration of 5 μg/ml. Fixed and stained DC-CD8 conjugates were imaged with BX51 upright epifluorescence microscope (Olympus) with a ×60 objective and image capture was performed using Magnafire software (Optoronix). For PI-9 localization, DC-CD8 conjugates were imaged with an Olympus 500 scanning confocal microscope (Olympus) with a ×60 objective using Fluoview software. All image files were digitally processed sing Metamorph or Adobe Photoshop.

Results

$CD8^+$ T cells at different stages of activation selectively kill or activate DC

To evaluate the DC-modulating functions of freshly isolated effector and memory $CD8^+$ T cell subsets and in vitro-differentiated naive $CD8^+$ T cells at different stages of activation, we have used a superantigen (SEB) model (24,32,33). Similar to TCR-transgenic mice, this model allows the activation of a high proportion of $CD8^+$ T cells (24,28) without the need for prior cloning. In accordance with the observations from the in vivo TCR-transgenic mouse models (9,10), we observed that tissue-isolated effector $CD8^+$ T cells (but not memory or naive $CD8^+$ T cells) rapidly killed immature DCs (Fig. 1*A*, *left*). In sharp contrast, blood-isolated memory $CD8^+$ T cells did not kill DCs, but instead activated the DCs, increasing their expression of the maturation-associated costimulatory molecules (Fig. 1, *A*, *right*, and *B*). Similar to the IFN-γ-dependent ability of naive $CD8^+$ T cells to elevate the IL-12 production in DCs (24), but in contrast to the effector cells, memory $CD8^+$ T cells primed DCs for high production of IL-12p70 upon subsequent stimulation with CD40L (Fig. 1*B*, *right*).

To verify that such reciprocal DC-modulating activities of tissue-isolated effector- and blood-isolated memory $CD8^+$ T cells indeed reflect their different stages of differentiation, we have used a SEB-based model of priming of blood-isolated $CD8^+CD45RA^+$ T cells (24), allowing us to study the regulatory functions of the same $CD8^+$ T cell cultures at different time points after activation. As shown in Fig. 2*A* (*left*, see the *insets*), similar to mouse in vivo models of $CD8^+$ T cell differentiation (34), $CD8^+$ T cells expanded for 6–8 days acquired a granzyme B^{high}, $perforin^{high}$ phenotype, typical of cytotoxic effector cells, and acquired the ability to kill SEB-loaded tumor cells (data not shown). Similar to the tissue-isolated effector cells, such in vitro-activated day 6–8(effector-type) $CD8^+$ T cells effectively killed DCs, as assessed by NAO staining (Fig. 2*A*, *left*) or annexin V (data not shown).

Compared with the effector-type $CD8^+$ T cells, the cells activated for >14 days (memory-type) expressed reduced levels of granzyme B and perforin. In contrast to the effector cells, such

memory-type $CD8^+$ T cells no longer killed DCs (Fig. 2*A*, *right*). Instead, similar to the blood-circulating memory cells, memory-type $CD8^+$ T cells induced DC maturation (manifested by up-regulation of CD86 and CD83) and primed DCs for high production of IL-12p70, the key Th1-and CTL-activating cytokine (35) (Fig. 2*C*).

Similar (stage of activation-dependent) differences were observed in case of $CD8^+$ T cells activated with a HLA-A2-restricted peptide Ag, $gp100_{209–217}$. As shown in Fig. 2, *B* and *D*, human gp100-specific $CD8^+$ T cell clone rapidly killed immature DCs when being preactivated with Ag-loaded DCs for 4 days, but lost such DC-killing function following their prolonged culture in the absence of Ag. Similar to the blood-isolated memory $CD8^+$ T cells and day 14 SEB-activated memory-type cells, such "resting" clonal gp100 $CD8^+$ T cells efficiently induced DC maturation and primed DCs for high IL-12 production (Fig. 2*D*).

The inability of the memory-type $CD8^+$ T cells to kill DCs did not result from any intrinsic defect resulting from long-term cultures, because they regained the ability to kill DCs upon short-term restimulation (Fig. 2*E*). These data, in conjunction with the results obtained using blood-isolated memory cells (Fig. 1*A*), indicate that after a transient period of DC-killing activity, activated $CD8^+$ T cells enter a "helper phase" of their activation cycle. Similar termination of the suppressor phase of activity has also been observed in case of tissue-isolated effector cells (data not shown).

Exogenous inhibitors of perforin and granzyme B restore DC-activating function of effector $CD8^+$ T cells

Prompted by the results of recent studies highlighting the role of perforin/granzyme B and Fas/FasL pathways in CTL-mediated elimination of DCs in mice (9,36), we have analyzed the role of the perforin/granzyme B- and Fas/FasL-mediated cytotoxic pathways in the killing of human DCs by effector $CD8^+$ T cells. As shown in Fig. 3*A*, DC killing was completely eliminated by the addition of EGTA or CMA, the inhibitors of the perforin-dependent (but not Fas/FasL-dependent) pathway of CTL-mediated killing (37,38). In contrast, no inhibition of DC killing was observed in the presence of the FasL antagonist (data not shown).

Taking advantage of the relative stability of CMA-induced perforin inhibition without affecting the secretion of $CD8^+$ T cell-produced TNF-α and IFN-γ (data not shown), we analyzed the outcome of the interaction of the CMA-pretreated effector cells with immature DCs. As shown in Fig. 3, *B* and *C*, pretreatment of the effector cells with CMA abrogated their DC-killing ability, resulting in the induction of phenotypic maturation of the DCs (TNF-α was used as a control for DC maturation) and their priming for high IL-12p70 production. Similar to the blocking of the perforin pathway, we also observed that pretreatment of DCs with the specific granzyme B inhibitors IETD-CHO or Z-IETD-fmk (39), abrogated CTL-induced DC death (Fig. 3*D*).

These results indicate that the perforin- and granzyme B-mediated cytolytic pathway is the principal mode of DC elimination by human effector $CD8^+$ T cells, and that, in its absence, the effector cells no longer suppress DC activity, but support it. These data also suggest that pharmacologic modulation of the perforin- or granzyme-mediated killing can be used to enhance the effectiveness of active and adoptive immunotherapies performed in the setting of existing disease, where Ag-specific effector $CD8^+$ T cells predominate.

Memory $CD8^+$ T cells protect DCs from CTL-mediated killing: equivalent induction of the endogenous granzyme B inhibitor, PI-9, by memory $CD8^+$ and $CD4^+$ Th cells

The inability of memory-type $CD8^+$ T cells to kill DCs even after 48-h cocultures was particularly intriguing in face of our observations that their killing function can be restored

following reactivation (Fig. 2*E*), and the data from mouse models that 30–96 h reactivated effector-memory and central-memory cells reacquire their DC-killing potential (8,40,41). These data suggested that the initial interaction of DCs with memory-type $CD8^+$ T cells may protect DC from the eventually acquired CTL activity of the same cells. To test this possibility, we have sequentially exposed DCs, first to memory-type $CD8^+$ T cells, followed by coculture with effector-type $CD8^+$ T cells.

As shown in Fig. 4*A*, the DCs exposed to memory-type $CD8^+$ T cells became resistant to subsequent killing by CTLs. This protective effect of the memory-type $CD8^+$ T cells was similar to that exerted by activated $CD4^+$ T cells, the classical "Th cells" (Fig. 4*A*), which have been proposed to mediate their helper function by DC protection (10,13). In further support of the similarity between the helper functions of $CD4^+$ T cells and $CD8^+$ memory T cells, DCs that interacted with either of these T cell subsets expressed similar levels of an endogenous granzyme B inhibitor—PI-9 (Fig. 4*B*), a human equivalent of murine serine protease inhibitor (SPI-6) (42)—shown to mediate the protection of mouse DCs from CTL-mediated killing (13). The analysis of PI-9 expression at the per cell basis, using confocal microscopy, revealed that PI-9 is massively up-regulated in DCs within 2 h following their interaction with memory-type $CD8^+$ T cells, with PI-9 expression being detectable in the individual DCs interacting with T cells already within 15 min (Fig. 4*C*). Similar kinetics of PI-9 induction was observed in DCs exposed to rTNF-α (Fig. 4*D*).

These observations—together with the ability of CMA to abolish the DC-killing activity of CTLs without abrogating their ability to induce DC maturation, and our previous data that blocking of TNF-α-RI (known to be triggered by TNF-α and lymphotoxin (LT)-β) abrogates DC maturation (24)—prompted us to test whether the CTL-protecting helper activity of memory-type $CD8^+$ T cells is mediated by TNF-α and can be blocked by the addition of sTNFRI (blocking potential actions of TNF-α and LT) or TNF-α-specific Ab (infliximab; blocking TNF-α exclusively). In accordance with the key role of TNF-α (rather than LT), both reagents proved equally effective in converting the memory T cell-induced DC activation into memory T cell-induced DC death (Fig. 5*A*, data not shown for sTNFRI). As expected, these effects were accompanied by the prevention of the induction of PI-9 in DCs (Fig. 5*B*). In contrast, DCs exposed to exogenous TNF-α acquired resistance to CTL killing (Fig. 5*C*).

In accordance with the different outcome of interaction of memory vs effector T cells with DCs, we observed a significant release of TNF-α within 2 h of interaction of DCs with memory-type $CD8^+$ T cells, whereas the release of granzyme B by memory-type $CD8^+$ T cells was significant only at later time points. Effector T cells, however, simultaneously released both TNF-α and granzyme B (Fig. 5, *D* and *E*). Furthermore, the microscopic analysis of the DC-T cell interactions (at the 2-h time point, when PI-9 is induced in DCs: see Fig. 4, *C* and *D*) demonstrated equivalent mobilization of TNF-α in both memory-type and effector-type $CD8^+$ T cells (Fig. 5*F*), whereas, exclusively, the effector $CD8^+$ T cells, but not memory cells, directed granzyme B-containing cytotoxic granules toward the contact zone with DCs (Fig. 5*G*). This sequence of events indicates that the early TNF-α release by the memory $CD8^+$ T cells induces early PI-9 expression and protects DC from the subsequently released granzyme B.

"CD8 to CD8 help" and "CD8 to CD4 help": memory $CD8^+$ T cells support the de novo induction of CTLs and Th1 cells

In support of their ability to perform respective suppressor and helper functions during the de novo induction of type-1 immune cells, $CD8^+$ T cells at different stages of activation differentially regulated the expansion of naive $CD8^+$ and $CD4^+$ T cells and the development of their respective CTL and Th1 functions (Fig. 6). Importantly, in accordance with the role of memory $CD8^+$ T cells in regulating the survival and function of DCs (see Fig. 4), the helper

signals from memory $CD8^+$ T cells not only promoted the DC-driven functional differentiation of naive T cells but were also able to fully counteract the suppressive activity of effector $CD8^+$ T cells, resulting in the effective induction of functional CTL and Th1 responses even in the presence of effector $CD8^+$ T cells (Fig. 6).

Discussion

We show that in contrast to effector $CD8^+$ T cells which rapidly eliminate Ag-carrying DCs in a perforin- and granzyme B-dependent mechanism, human memory $CD8^+$ T cells protect DCs from CTL-mediated killing and exert DC-mediated helper function. These data indicate that the mechanism of $CD8^+$ T cell memory, in addition to the previously defined increased frequency of Ag-specific T cells and their rapid acquisition of effector functions, involves a novel $CD4^+$ T cell-like ability of memory $CD8^+$ T cells to prolong the life span of Ag-carrying DCs.

Our data support the following functional model of $CD8^+$ T cell memory: during primary immune responses, DC activation requires their exposure to factors representing pathogen-associated (PAMPs) or tissue damage-associated molecular patterns (DAMPs) (PAMPs vs "danger signals" or DAMPs) (43,44), implicating the need for a significant pathogen load and tissue damage. In contrast, the activation of DCs during secondary responses can benefit from the activating signals released by high numbers of pathogen-specific memory-type $CD8^+$ T cells, even before the destruction of the infected cells and the release of additional copies of the pathogen. Such memory $CD8^+$ T cell-dependent early DC activation helps the immune system to respond to the pathogen at much earlier stages of (re)infection, limiting its early spread, the extent of tissue damage, and the activation of innate defense mechanisms, thus preventing the onset of disease symptoms.

Moreover, because during the primary responses, the PAMP-and DAMP-mediated DC protection from newly arising CTLs is limited to the period of active infection and ongoing tissue damage, the optimal activation of the T cells recruited to the lymph nodes at later stages of primary responses (needed for the optimal development of memory cells (45,46)) is dependent on DC-protecting signals from $CD4^+$ Th cells (10). In contrast, during secondary immune responses, Ag-carrying DCs can also interact with the $CD8^+$ memory T cells that are gradually recruited to the sites of infection or to inflamed lymph nodes, preventing premature DC elimination by the arising effector cells and limiting their dependence on the pathogen-related or $CD4^+$ T cell-related survival signals.

In contrast to the CD40L-mediated induction of DC-associated PI-9 by $CD4^+$ T cells (13), our data demonstrate that the $CD8^+$ T cell-derived TNF-α plays a key role in inducing PI-9 expression and DC protection by memory $CD8^+$ T cells. Although recent observations suggest the presence of CD40L on mouse $CD8^+$ T cells (47), we did not find CD40L expression on human $CD8^+$ T cells (Ref. [24] and current data not shown). These data and the results of the TNF-α-neutralization experiments (Fig. 5, *A* and *B*) argue against a role of CD40L in PI-9 induction by human $CD8^+$ T cells. In addition to the currently identified DC protection by memory $CD8^+$ T cells, recall immunity may also benefit from the IFN-γ-dependent (24) ability of memory $CD8^+$ T cells to enhance the DC secretion of IL-12p70 (Figs. 1*B* and 2, *C* and *D*), jointly contributing to the superior magnitude and quality (Fig. 6) of recall immune responses.

Our study demonstrates the role of memory $CD8^+$ T cells in preventing CTL-mediated DC killing and promoting optimal de novo induction of functional CTLs and Th1 cells. These observations highlight the possibility of using this novel function of memory $CD8^+$ T cells while developing improved vaccination strategies in therapeutic settings. In accordance with such a possibility, our in vivo observations in mouse models (27) demonstrate that inclusion

of tumor-unrelated epitopes on DC vaccines can boost the immunologic and antitumor effects of vaccination against established tumors. Our data help to explain the high efficacy of prime-boost vaccination strategies (48) when the first and second doses of vaccine are delivered using antigenically distinct vectors (thus allowing the DCs that take up the second dose of vaccine to avoid exposure to the vector-specific T cells induced by the initial vaccine), and may help to further improve such strategies.

We are currently comparing the ability of different populations of memory cells to provide helper signals. Although our preliminary data suggest that blood-isolated central-memory and effector-memory $CD8^+$ T cells are both effective in protecting DCs from CTL killing (data not shown), we observed that the memory-type cells generated in our 2-wk-long cultures (in contrast to blood-isolated memory cells, expressing low levels of granzyme-B and perforin: see Figs. 1 and 2) can reacquire the DC-killing ability following short-term restimulation (Fig. 2*E*). These data, and the recently published observations that mouse effector-memory $CD8^+$ T cells and preactivated memory $CD8^+$ T cells can acquire DC-killing capacity in vivo within 30–96 h of exposure to Ag-loaded DCs (40,41), suggest that an optimal long-term DC protection in vivo may require the presence of central memory cells. Because mouse long-term memory cells have been shown to kill DC only following 72 h (but not 24 h) of antigenic challenge (8) and human central memory $CD8^+$ T cells have been shown to need up to 72 h of activation to reacquire the cytotoxic function (49), the DC protection by central memory T cells during secondary immune responses is likely to have a substantial time span.

An intriguing aspect of the current findings is the possibility of manipulating the respective suppressive and helper functions of effector and memory $CD8^+$ T cells, using pharmacologic agents. We observed that blocking perforin or granzyme B activity results in the acquisition of helper functions by the effector cells, while blocking TNF-α interaction with TNFRI eliminates helper function of memory T cells. These data have direct pathologic and therapeutic implications for autoimmunity, chronic infections, and cancer. Although the blockade of TNF-α or TNFRI, using infliximab or eternacept, respectively, proved to be highly effective in the treatment of inflammatory bowel disease, rheumatoid arthritis, and other autoimmune diseases, a comparative evaluation of the role of these agents in limiting the DC-activating functions of $CD8^+$ T cells in these different disease settings may help to explain the differential efficacy of each of these agents in patients with different forms of autoimmunity, allowing further optimization of their treatment.

Although the inability of perforin-deficient $CD8^+$ T cells (and possibly NK cells) to control $CD8^+$ T cell expansion has been proposed to explain autoimmune damage in perforin knockout mice infected with lymphocytic choriomeningitis virus or *Listeria* (50), and the uncontrolled lymphoproliferation during viral infections in patients with perforin mutations (51), the current data provide the rationale for the evaluation of perforin or granzyme-targeting therapies as a tool to increase the efficacy of therapeutic vaccines in the settings of cancer and chronic infections, where terminally differentiated effector cells predominate (52–54). It also remains to be tested whether perforin- or granzyme-targeting approaches can be applied to enhance the long-term clinical effectiveness of adoptive immunotherapies with ex vivo-expanded tumor-infiltrating lymphocytes, where the long-term persistence of tumor-specific T cells and the positive clinical outcomes are tightly correlated with the frequencies of the adoptively transferred memory-type, expectedly non-DC-killing, $CD8^+$ T cells (55–57).

In summary, the current data help to reconcile the long-known paradoxical ability of $CD8^+$ T cells to play the reciprocal “suppressor” and “helper” roles, adding to our understanding of $CD8^+$ T cell memory, and facilitating the development of effective therapies of autoimmunity, cancer, and chronic infections.

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Abbreviations in this paper

DC	dendritic cell
rhu	recombinant human
SEB	*Staphylococcus* enterotoxin B
s	soluble
CMA	concanamycin A
NAO	nonyl acridine orange
LT	lymphotoxin
PAMP	pathogen-associated molecular pattern
DAMP	damage-associated molecular pattern

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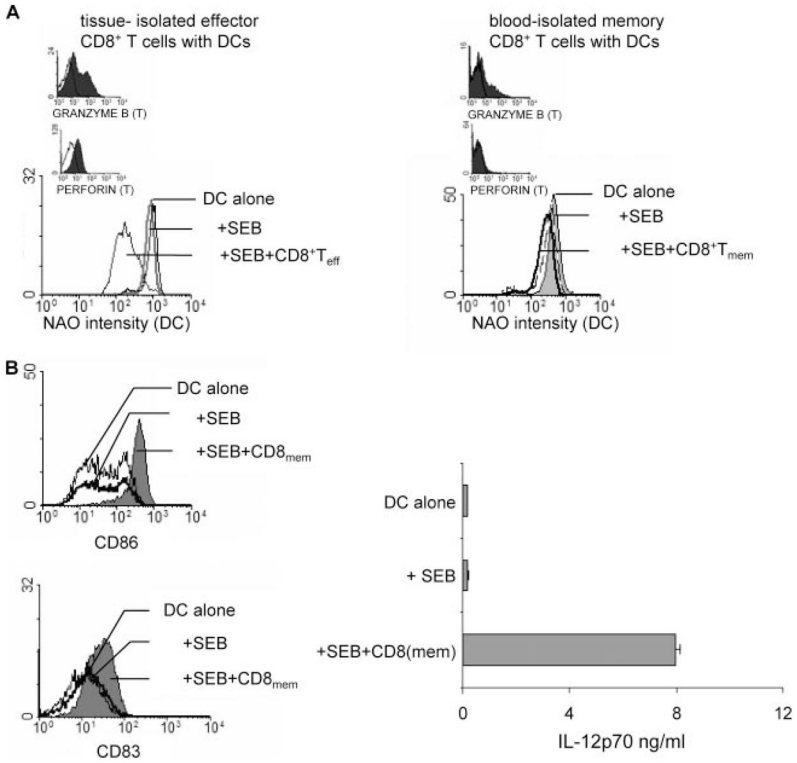

FIGURE 1.

DC-killing vs DC-activating function of tissue-isolated effector and blood-isolated memory $CD8^+$ T cells. *A* and *B*, Tissue-isolated effector $CD8^+$ T cells kill DCs while blood-isolated memory $CD8^+$ T cells induce DC maturation and prime them for high IL-12p70 production. SEB-pulsed immature DCs (day 6) were coincubated with blood-derived memory $CD8^+$ T cells or tissue-derived effector $CD8^+$ T cells for 48 h. *A*, DC viability was assessed by staining with NAO (30). *Left*, Tissue-derived effector $CD8^+$ T cells eliminate DCs, as indicated by decrease in NAO staining intensity, demonstrating the loss of mitochondrial integrity. *Right*, Blood-isolated memory $CD8^+$ T cells do not kill DCs as reflected by the maintained NAO-staining pattern of the DCs. *B*, *Left*, DC activation status was determined by flow cytometric analysis for surface expression of the costimulatory molecule CD86 and DC maturation-associated marker CD83. All data are gated on DCs, based on forward- and side-scatter profiles. *Right*, IL-12p70 production by DCs following stimulation with J588-CD40L, as measured by ELISA. Results (mean ± SD or triplicate cultures) are representative of three independent experiments.

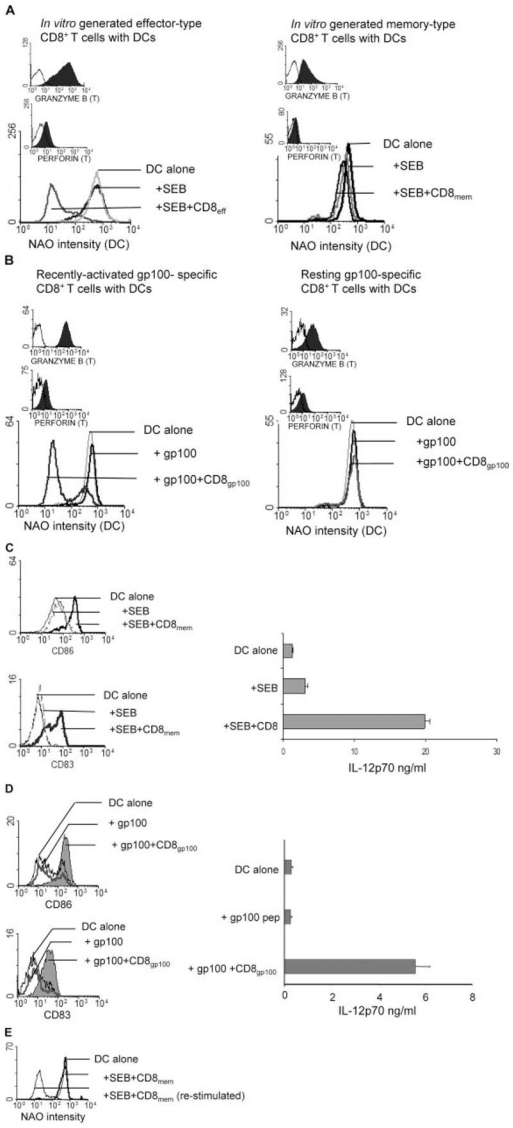

FIGURE 2.

DC-killing vs DC-activating functions of in vitro-generated effector-type $CD8^+$ T cells and memory-type $CD8^+$ T cells at different stages of activation. Interaction of DCs with the in vitro-generated effector (*left*)- or memory (*right*)-type $CD8^+$ T cells (*A* and *C*), or with melanoma (gp100)-specific $CD8^+$ T cells (*B* and *D*), at early or late stages of activation. *A* and *B, Left*, In vitro-generated granzyme B^{high}/$perforin^{high}$ effector-type $CD8^+$ T cells and activated gp100-specific $CD8^+$ T cells kill DCs as evident from decrease in NAO-staining intensity. *Right*, In vitro-generated granzyme B^{low}/$perforin^{low}$ memory-type $CD8^+$ T cells and resting gp100-specific $CD8^+$ T cells do not kill immature DCs as reflected by the maintained NAO-staining pattern of the DCs. *C* and *D*, Memory-type $CD8^+$ T cells at later stage of activation and resting melanoma (gp100)-specific $CD8^+$ T cells induce DC maturation and prime DC for enhanced IL-12 production. Day 6, immature HLA-$A2^+$ DCs were cocultured with melanoma gp100-specific HLA-A2-restricted $CD8^+$ T cells, in the presence of $gp100_{209–217}$ peptide (shaded histograms). Memory-type $CD8^+$ T cells were cocultured with SEB-loaded DCs for 48 h. *Left*, Activation status of DCs (CD86, CD83) was assessed by flow cytometry (data gated on DCs). *Right*, IL-12p70 was measured in supernatants after stimulation

of DCs with J588-CD40L. *E*, Short-time restimulated memory-type $CD8^+$ T cells reacquire the ability to kill immature DCs. Memory-type $CD8^+$ T cells (days 14–16 of culture) were prestimulated for 48 h (or not pre-stimulated) and cocultured with SEB-pulsed immature DCs (day 6) for 18–20 h. Results are representative of three independent experiments. DC killing and DC activation required the presence of Ag in all the above systems (data not shown).

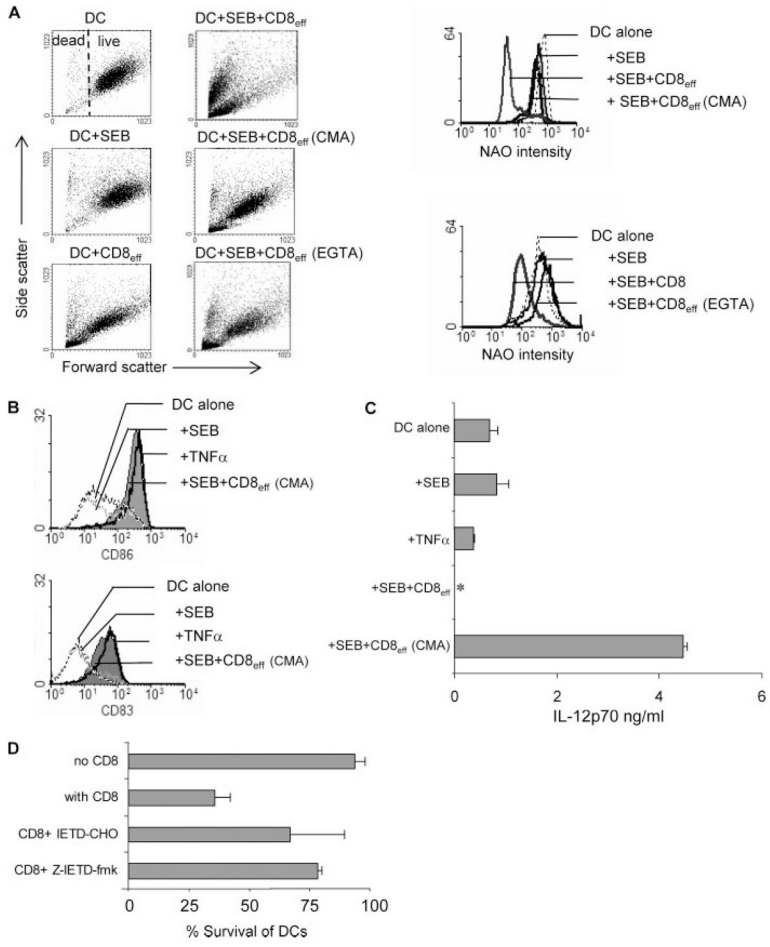

FIGURE 3.

Exogenous inhibitors of perforin/granzyme B pathway convert effector-type $CD8^+$ T cells into helper $CD8^+$ T cells. *A*, Inhibition of perforin in effector-type $CD8^+$ T cells results in the survival of the interacting DCs. Release of functional perforin was blocked by either pretreatment of the effector-type $CD8^+$ T cells with CMA (100 nM) or by the addition of EGTA (4 mM) during DC-CD8 coculture. The survival of DCs was analyzed by change in the light scatter properties (as indicated by dot plots: *left*), and verified using the NAO staining (*right*). Broken line within the dot plot separates live and dead cell populations. NAO analysis (*right*) included both regions (live and dead DCs) while the $CD8^+$ T cell population was excluded. *B* and *C*, CMA-treated effector-type $CD8^+$ T cells were cocultured with SEB-loaded DCs for 48 h. *B*, CMA-treated (perforin-blocked) effector-type $CD8^+$ T cells enhance DC activation. TNF-α (50 ng/ml) induced DC maturation was used as positive control. *C*, CMA-treated (perforin-blocked) effector-type $CD8^+$ T cells induce type 1-polarized phenotype in DCs, characterized by enhanced ability to produce IL-12p70.*, Below detection limit. Results are representative of three independent experiments. *D*, Inhibition of granzyme B in effector-type $CD8^+$ T cells results in survival of interacting DCs. DCs pretreated (1 h) with IETD-CHO (200 μM) or Z-IETD-fmk (20 μM) were cocultured with effector-type $CD8^+$ T cells. Survival of DCs was analyzed after 10–12 h. The data from two experiments are expressed as percent survival of DCs (mean ± SEM).

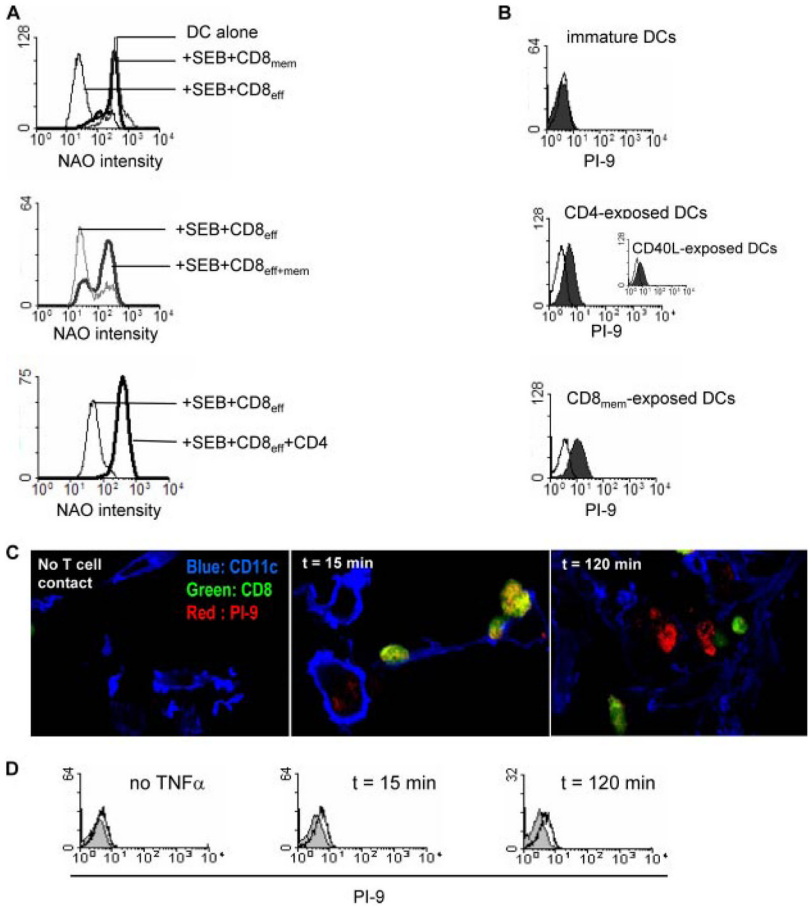

FIGURE 4.

Memory-type $CD8^+$ T cells and $CD4^+$ T cells induce DC expression of endogenous granzyme B inhibitor and protect DCs from CTL-mediated killing. *A*, Exposure of DCs to memory-type $CD8^+$ T cells confers protection from effector-type $CD8^+$ T cell-mediated death, analogous to $CD4^+$ T cell-mediated protection. Memory $CD8^+$ T cells or $CD4^+$ Th cells were cocultured with SEB-loaded DCs for 8–10 h, followed by the addition of effector $CD8^+$ T cells. DC viability was assessed by NAO staining at 24 h. *B*, Memory-type $CD8^+$ T cells induce uniform DCs expression of the endogenous granzyme B inhibitor: PI-9 (10 h coculture). DCs exposed to memory-type $CD8^+$ T cells, $CD4^+$ T cells (or CD40L: see the *inset*) were stained for intracellular PI-9. *C*, Rapid induction of PI-9 in DCs exposed to memory-type $CD8^+$ T cells, visualized by confocal microscopy: localization of PI-9 (red) in DCs (blue), following the interaction with memory-type $CD8^+$ T cells (green) for 15 min and 2 h and lack of PI-9 expression in isolated DCs. *D*, Rapid induction of PI-9 in DCs exposed to TNF-α. Intracellular expression of PI-9 (open profiles) in DCs has been analyzed by flow cytometry before and after (15 and 120 min) the exposure to rTNF-α (50 ng/ml).

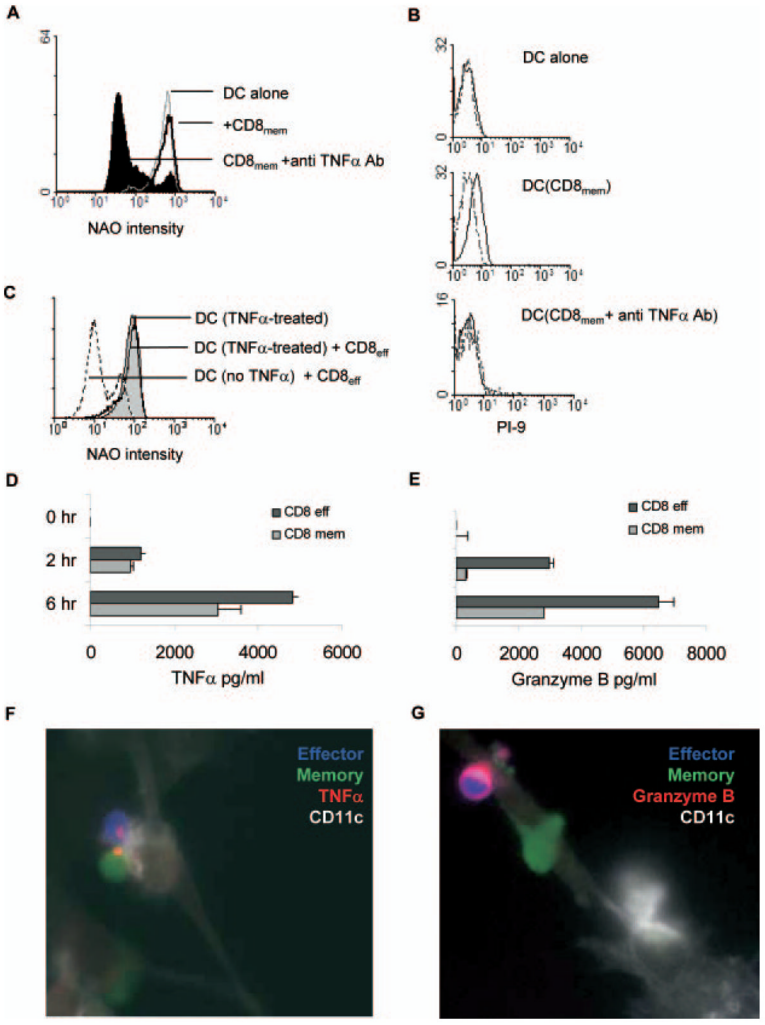

FIGURE 5.

Memory-type $CD8^+$ T cell-derived TNF-*α* plays a crucial role in helper function. *A*, Neutralization of TNF-*α* in the cocultures of SEB-loaded DCs and memory-type $CD8^+$ T cells using anti-TNF- *α* Ab, reduces DC survival (24 h cultures). *B*, Anti-TNF-*α* Ab, blocks the memory-type $CD8^+$ T cell-induced induction of PI-9 in DCs (10 h time point). *C*, Exogenous TNF-*α* protects immature DCs from CTL-mediated killing. Immature DCs were pretreated with rhuTNF-*α* (100 ng/ml; 24 h) before cocultures with effector $CD8^+$ T cells. *D* and *E*, Different relative kinetics of the TNF-*α*vs granzyme B release in memory and effector $CD8^+$ T cells during the interaction with DCs. Note that the release of TNF-*α* precedes granzyme B release in memory-type $CD8^+$ T cells, but not in effector cells. *F*, Four-color fluorescent microscopy demonstrating the presence of TNF-*α* (red) in both effector (blue) and memory (green) $CD8^+$ T cells interacting (2h) with DCs (grey). *G*, Presence of high amounts of granzyme B (red) in the effector $CD8^+$ T cells (blue) interacting (2h) with DCs (grey), but not in the memory $CD8^+$ T cells (green).

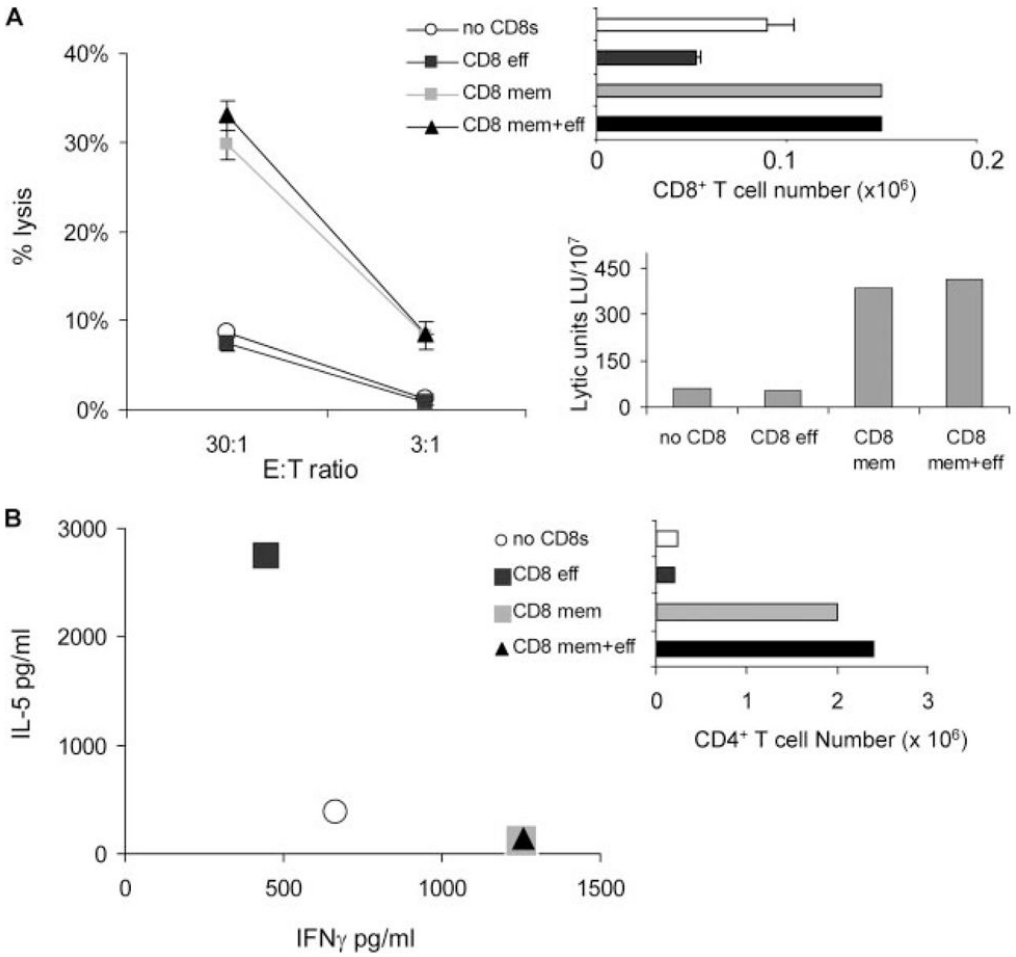

FIGURE 6.

Memory $CD8^+$ T cells support de novo induction of functional CTLs and Th1 cells. *A* and *B*, Memory and effector $CD8^+$ T cells have reciprocal impact on the DC-driven expansion of CTL and Th1 cell progenitors and the development of CTL and Th1 functions. Blood-isolated naive $CD8^+$ or $CD4^+$ T cells were primed with the SEB-loaded immature DCs in the absence or presence of gamma-irradiated memory-type or effector-type $CD8^+$ T cells. The expanding cultures of naive $CD8^+$ or $CD4^+$ T cells were harvested, respectively, at day 5 or 10, counted and tested for their functional activity, using a CTL assay or the analysis of their Th1/Th2 cytokine profiles. *A*, Memory $CD8^+$ T cells support the expansion of CTL precursors and their acquisition of functional activity. *Left*, CTL activity of $CD8^+$ T cell cultures performed in the presence or absence of memory or effector $CD8^+$T cells was assessed by ^{51}Cr-release assay, using SEB-loaded JY-1 cells as targets. Data from one of two independent experiments that both yielded similar results. *B*, Memory $CD8^+$ T cells support the expansion of Th1 cell precursors and the acquisition of Th1 cytokine production profiles. *Right*, Ability of memory-type $CD8^+$T cells, but not effector-type $CD8^+$T cells, to induce naive $CD4^+$ T cell proliferation. *Left*, Naive $CD4^+$ T cells primed in the presence of memory-type $CD8^+$ T cells develop a strongly polarized Th1 cytokine profile as determined by ELISA. Data from one of two independent experiments that both yielded similar results.