Brief Definitive Report

Perforin and Fas Killing by CD8$^+$ T Cells Limits Their Cytokine Synthesis and Proliferation

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Summary

During an immune response, effector CD8$^+$ T cells can kill infected cells by the perforin-dependent pathway. In comparison to CD4$^+$ T cells, which are major sources of cytokines, normal CD8$^+$ T cells produced less interleukin 2 and interferon γ, and proliferated less vigorously after antigenic stimulation. Killing of target cells was a major cause of these reduced responses, since perforin-deficient CD8$^+$ T cells showed substantially increased cytokine synthesis and proliferation. Cytotoxicity by the alternate Fas pathway also resulted in self-limitation of CD8$^+$ T cell cytokine synthesis. This relationship between cytotoxicity and cytokine synthesis may regulate CD8$^+$ T function in different phases of an immune response.

T cell activation results after interaction between a TCR and MHC protein containing a bound antigen peptide expressed on the surface of the APC. This is augmented by signals from nonantigen-specific costimulatory molecules (e.g., B7-CD28) resulting in T cell cytokine production and clonal expansion (1). For CD4$^+$ T cells, antigen recognition results in sustained interaction and mutual activation of T cells and APC (2, 3). Optimal activation of the T cell requires sustained interaction with an APC, since IL-specific RNA is rapidly lost after removal of the stimulating signal (4).

In addition to their cytotoxic ability, activated CD8$^+$ T cells can also secrete various cytokines that mediate proliferation, inflammation and other immune functions. The amount of cytokines such as IL-2 that is secreted by CD8$^+$ T cells, however, may be limited, since the proliferation of cytolytic CD8$^+$ T cells is often (5–9) but not always (10–12) dependent on IL-2 derived from other sources, normally CD4$^+$ T cells. CD8$^+$ T cells produce lower amounts of cytokines than CD4$^+$ T cells in response to APC (13), but similar amounts in response to polyclonal activation (13–15). Subsequent to activation, in contrast to CD4$^+$ T cells, CD8$^+$ T cells develop into highly cytolytic effectors and kill APCs rapidly (16). We therefore addressed the possibility that rapid killing of APCs by the CD8$^+$ T cells prevents continued interaction with the same or other T cells and therefore reduces synthesis of cytokines.

Materials and Methods

Cells. M12.4.1 cells expressing both class I and class II MHC proteins (17) were kindly provided from Dr. Laurie Glimcher (Department of Cancer Biology, Harvard Medical School, Boston, MA). L1210 cells and L1210 cells transfected with Fas (L1210Fas; 18) were kindly provided by Dr. Chris Bleackley (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada).

Cell Cultures. Allogeneic (H-2^b anti–H-2^d) CD4$^+$ Th1 and CD8$^+$ Tc1 cells were generated as described previously (19). Briefly, CD4$^+$ and CD8$^+$ T cells were purified from spleens of C57BL/6 mice that were maintained by the University of Alberta Health Sciences Laboratory Animal Services in accordance with Canadian Council on Animal Care (CCAC) guidelines. B cells, macrophages, and CD8 or CD4 T cells were removed on T cell columns (Biotex, Edmonton, Alberta, Canada). CD4 and CD8 T cells were then further purified by sorting on a cell sorter (EPICS Elite; Coulter Corp., Hialeah, FL) after staining with CD44-FITC and either CD4-PE or CD8-PE. CD44^{low} C57BL/6 CD4$^+$ and CD8$^+$ T cells were stimulated (10^4 per well) with irradiated (10,000 rads) allogeneic M12.4.1 cells (H-2^d, 2×10^4 per well) in 96-well flat-bottomed tissue culture plates in 200 μl RPMI plus 8% fetal bovine serum (FBS). Stimulation was carried out in the presence of IL-2 (1 ng/ml), IL-12 (80 pg/ml), and anti–IL-4 (50 μg/ml). Medium was changed every 3rd d. This stimulation resulted in differentiation and proliferation of effector cells of the Th1/Tc1 phenotypes (15). Synthesis of IL-2 and IFN-γ was confirmed by stimulation with Con A and testing of the supernatants by ELISA.

Cytokine Synthesis. For cytokine synthesis, day 10 effector CD4$^+$ Th1 and CD8$^+$ Tc1 cells were stimulated (5×10^4 per well) with varying numbers of irradiated (10,000 rads) allogeneic APC in 96-well round-bottomed tissue culture plates in 200 μl RPMI plus 8% FBS. Supernatants were collected at the indicated intervals, and cytokines were tested by ELISA as described previously (20).

Proliferation Assays. For proliferation, day 10 effector CD4$^+$ Th1 and CD8$^+$ Tc1 cells from perforin-deficient or control mice were stimulated (5×10^4 per well) with varying numbers of irradiated (10,000 rads) allogeneic M12.4.1 cells (H-2^d) in 96-well flat-bottomed tissue culture plates in 200 μl RPMI plus 8% FBS. Radiolabeled thymidine was added (1 μCi/well) at 48, 72, and 96 h. Cells were harvested 24 h after the thymidine pulse. Radioactive incorporation was determined by liquid scintillation counting.

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Cytotoxicity Assays. Target cells were labeled by incubating 10^7 cells in 50 μl RPMI plus 8% FBS with 100 μCi of ^{51}Cr for 45 min. Labeled targets were washed extensively before use in cytotoxicity assays. Day 10 effector $CD4^+$ Th1 and $CD8^+$ Tc1 cells were incubated at various ratios with ^{51}Cr-labeled M12.4.1 or EL4 target cells for 4, 6, or 8 h. Supernatants were collected, and radioactivity was detected by γ counting. Means and SDs of triplicate cultures are shown. The percent of cytotoxicity was calculated using the formula:

$$100 \times \frac{(cpm\ experimental - cpm\ spontaneous)}{(cpm\ total\ -\ cpm\ spontaneous)}$$

Results

Reduced Cytokine Synthesis by $CD8^+$ T Cells. We prepared alloantigen-specific (H-2^b anti–H-2^d) populations of CD4 Th1 and CD8 Tc1 cells, both expressing the Th1 cytokine pattern that includes IL-2 and IFN-γ (15, 21). Cytotoxicity and cytokine production of these cells were tested against M12.4.1, an H-2^d B lymphoma cell line that express both class I and class II MHC proteins (17). CD8 Tc1 cells, but not CD4 Th1 cells, were highly cytolytic towards the antigen-bearing M12.4.1 cells in both 4- and 8-h assays (Fig. 1 *A*).

Cytokine secretion by $CD4^+$ Th1 and $CD8^+$ Tc1 cells was inversely related to cytotoxicity (Fig. 1, *B* and *C*). Th1 cells produced substantially more IL-2 and IFN-γ than Tc1 cells when stimulated with low numbers of allogeneic M12.4.1 cells. At higher APC numbers, IFN-γ production by Tc1 cells increased to levels comparable to those secreted by the Th1 cell population. Secretion of IL-2 by Tc1 cells also increased with increasing APC number, although the levels were substantially below Th1 levels at all APC numbers. The reciprocality of killing and cytokine synthesis by $CD4^+$ Th1 and $CD8^+$ Tc1 cells suggests that APC killing could be the cause of reduced cytokine synthesis. Since effector $CD8^+$ T cells kill targets mainly by the perforin-dependent pathway, we next determined whether the presence or absence of perforin would influence cytokine production by $CD8^+$ T cells.

Killing by Perforin Limits Cytokine Synthesis and Proliferation of $CD8^+$ T Cells. Allospecific (H-2^b anti–H-2^d) $CD4^+$ and $CD8^+$ T cells were generated from normal mice or mice in which the perforin gene had been inactivated by homologous recombination (22). Fig. 2 *A* confirms that perforin-deficient $CD8^+$ Tc1 cells do not show the strong antigen-specific killing mediated by normal $CD8^+$ Tc1 cells. In contrast to this reduction in cytotoxicity, $CD8^+$ Tc1 cells from perforin-deficient mice produced substantially more IL-2 and IFN-γ relative to $CD8^+$ Tc1 cells from normal mice (Fig. 2, *B* and *C*). This difference was observed at all the time points tested, and was particularly evident when T cells were stimulated with lower numbers of APCs. In contrast to $CD8^+$ Tc1 cells, $CD4^+$ Th1 cells from normal or perforin-deficient mice synthesized similar amounts of cytokines. Thus, Fig. 2 demonstrates that perforin is important for $CD8^+$ T cell cytotoxicity, but substantially compromises their ability to produce cytokines.

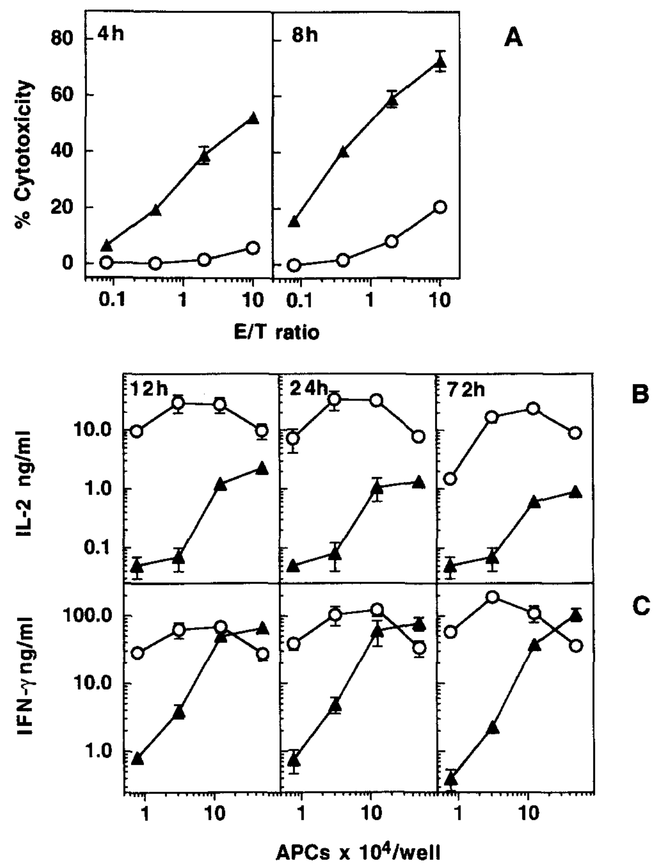

Figure 1. Cytokine synthesis and cytotoxicity is reciprocal in both CD4 and CD8 T cells. Allogeneic (H-2^b anti–H-2^d) $CD4^+$ Th1 and $CD8^+$ Tc1 cells cells were tested for their cytotoxicity (*A*), IL-2 secretion (*B*), and IFN-γ secretion (*C*) against M12.4.1 cells (H-2^d) on day 10. Means and SDs of triplicate cultures are shown. Similar results were obtained in two other experiments. -○-, CD4 Th1; -▲-, CD8 Tc1.

Effector $CD8^+$ Tc1 cells from perforin-deficient mice also proliferated more strongly than normal $CD8^+$ Tc1 cells in response to antigen stimulation at low to moderate APC numbers (Fig. 2 *D*). Proliferation of $CD8^+$ Tc1 cells from normal mice increased with increasing APC number, and at APC/T cell ratios of $>1{:}1$, proliferation of normal and perforin-deficient cells was similar. $CD8^+$ Tc1 cells proliferated (Fig. 2 *D*) even at APC/T cell ratios that induced low IL-2 production (Fig. 2 *B*) probably because IL-2 levels measured in the supernatant represent excess levels over and above the amounts required by the Tc1 cells during proliferation. $CD4^+$ Th1 cells proliferated more strongly than $CD8^+$ Tc1 cells under all conditions, and proliferation was not affected by inactivation of the perforin gene (Fig. 2 *D*). Thus, the presence of perforin in $CD8^+$ T cells not only compromises their cytokine synthesis, but also reduces their proliferative ability, possibly as a result of decreased IL-2 synthesis.

Killing by Fas Limits Cytokine Synthesis by $CD8^+$ T Cells. In addition to the perforin-dependent killing pathway, activated $CD8^+$ T cells express Fas ligand, which induces death in target cells that express Fas and are not protected by internal mechanisms (23–27). $CD8^+$ Tc1 cells from per-

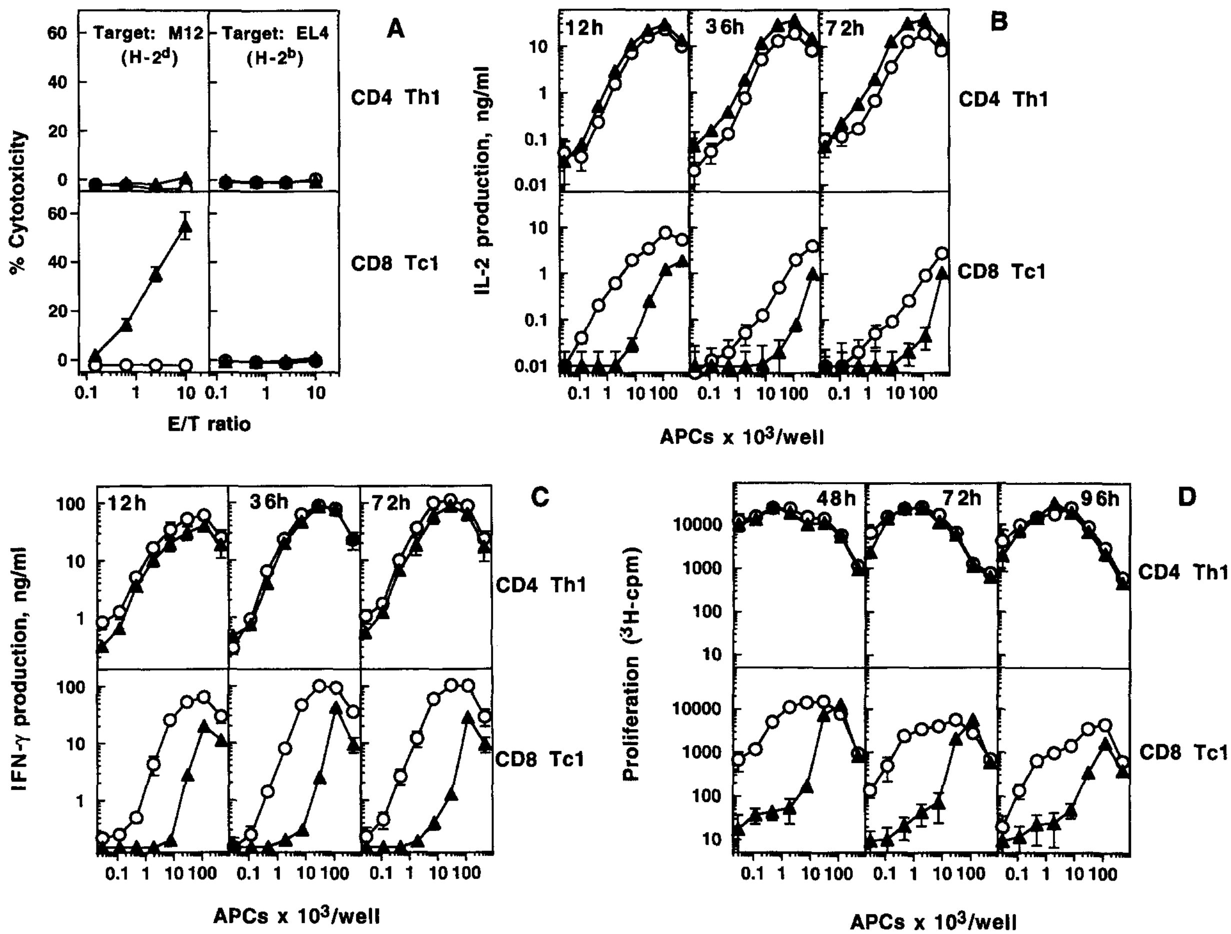

Figure 2. Killing of APCs by perforin inhibits the cytokine production and proliferation of $CD8^+$ but not $CD4^+$ T cells. Allogeneic ($H\text{-}2^b$ anti–$H\text{-}2^d$) $CD4^+$ Th1 and $CD8^+$ Tc1 cells from either perforin-deficient (C57BL/6) or control (C57BL/6) mice were tested for cytotoxicity in a 6-h assay (*A*), IL-2 secretion (*B*), IFN-γ secretion (*C*), and proliferative ability (*D*). The means and SDs of triplicate cultures are shown. Similar results were obtained in two other experiments. -○-, perforin −/−; -▲-, perforin +/+.

forin-deficient mice were therefore tested for cytotoxicity and cytokine production against allogeneic targets that expressed low (L1210) or high (L1210Fas) levels of Fas (18). As with perforin-dependent killing, Fas-dependent killing by Tc1 cells was also inversely related to cytokine production (Fig. 3). Perforin-deficient Tc1 cells killed L1210Fas cells very effectively (Fig. 3 *A*), but produced low amounts of IL-2 and IFN-γ (Fig. 3, *B* and *C*), whereas normal L1210 cells were killed less efficiently but induced higher levels of IL-2 and IFN-γ.

Discussion

These results show important differences in the regulation of cytokine production by $CD4^+$ and $CD8^+$ T cells. $CD4^+$ Th1 cells stimulated by antigen-bearing cells do not show short-term cytotoxicity (Fig. 1 *A*), and therefore interaction with even a single APC may be sufficient for sustained stimulation and/or costimulation, activation, and substantial cytokine production. In contrast, the CD8–APC interaction would be terminated rapidly because of Perforin- and Fas-mediated cytotoxicity. This may reduce cytokine production for two reasons: first, the antigen-bearing cell will not survive to present antigen to additional $CD8^+$ T cells; second, the brief period of interaction between the initial $CD8^+$ T cell and its target may not be sufficient for full activation and cytokine production. This may be analogous to the effect of reduced peptide antigen ligand concentrations on the APC, which stimulate cytotoxicity but not high cytokine production (28). Stimulation of $CD8^+$ Tc1 cells with higher numbers of APCs, however, induces strong cytokine production, probably because of repeated stimulation and/or stimulation of a greater proportion of $CD8^+$ T cells. Since normal $CD8^+$ T cells are strongly deficient in cytokine production, even at an E/T ratio of 1:1 (Fig. 2, *B* and *C*), multiple stimulation events may be required to fully activate each $CD8^+$ T cell. This may be a key regulatory mechanism whereby the presence of multiple infected targets will ensure the production of copious amounts of cytokines by $CD8^+$ Tc1 cells. This will promote the proliferation of effector Tc1 cells and hence the resolution of infection. On the contrary, when few targets are infected, it is not necessary for $CD8^+$ Tc1 cells to produce cytokines, since extensive proliferation or inflam-

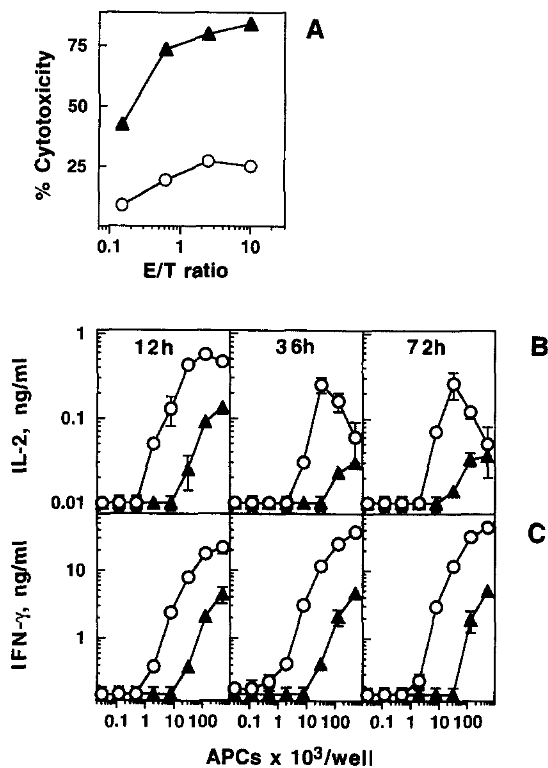

Figure 3. Killing of APCs by Fas inhibits the cytokine production by $CD8^+$ T cells. Fas-mediated cytotoxicity (*A*), IL-2 secretion (*B*), and IFN-γ secretion (*C*) by $CD8^+$ Tc1 cells (H-2^b anti–H-2^d) from perforin-deficient mice were tested against H-2^d targets expressing low (L1210) or high (L1210Fas) levels of Fas. Cytotoxicity was determined in an 8-h assay. The means and SDs of triplicate cultures are shown. Similar results were obtained in another experiment. APCs: -○-, L1210; -▲-, L1210 Fas.

mation are not required. Thus, the relationship between cytotoxicity and cytokine-producing ability of mature $CD8^+$ T cells may be a natural mechanism to allow resolution of the infection while curtailing unnecessary expansion of effectors and immune damage.

Perforin killing is directional, and the absence of perforin strongly enhances cytokine synthesis of $CD8^+$ T cells. This suggests that cytokine synthesis by normal $CD8^+$ T cells is inhibited as a result of APC death and the consequent absence of sustained stimulation rather than as a result of activation-induced T cell death.

Because of the potential damage caused by excessive $CD8^+$ T cell responses, involving both cell killing and inflammation, it is not surprising that there are multiple restraining mechanisms for $CD8^+$ responses. In addition to the self-limitation of $CD8^+$ T cell proliferation as a result of APC killing, IL-4 abrogates the ability of Tc1 cells to produce IL-2 and consequently proliferate (19).

Cytotoxic $CD8^+$ T cell effectors mediate immunity against various intracellular bacteria and viruses including listeria and LCMV (22, 29, 30). However, the proliferation and expansion of $CD8^+$ T cells may be aided by other cells and exogenous growth factors. $CD4^+$ T cells prevented rapid deletion of $CD8^+$ T cells after a transient response to antigen (31). Tolerance to transplantation antigens was induced by exposing $CD8^+$ T cells to tissue alloantigens in the absence of $CD4^+$ T cell help (32). Protective $CD8^+$ T cell responses during chronic but not acute LCMV infection required $CD4^+$ T cells (33). During the clearance of mouse hepatitis virus from the central nervous system, $CD8^+$ T cells appear to be the main effectors, but need $CD4^+$ T cell help (34). Our data offer an explanation for the dependence of some $CD8^+$ T cell responses on $CD4^+$ T cells.

The relationship between killing and cytokine production by $CD8^+$ T cells may help explain the regulation of $CD8^+$ T cell function during different phases of an immune response. Initially, the noncytolytic nature of naive $CD8^+$ T cells, in contrast to effector $CD8^+$ T cells, would ensure sustained stimulation of naive $CD8^+$ T cells after interaction with APCs. As the $CD8^+$ T cells mature into cytolytic effectors, cytokine production and proliferation may be sustained in the presence of large numbers of infected target cells or by cytokines contributed by $CD4^+$ T cells. The cytokines produced by $CD4^+$ and $CD8^+$ T cells will promote an inflammatory reaction, recruiting more effector cells to the site of infection. Finally, as the infection is brought under control, cytokine synthesis by $CD8^+$ T cells should decline as a result of target killing, while $CD8^+$ T cells remain fully cytolytic to kill the few remaining infected cells.

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