

# Interferon $\gamma$ Plays a Critical Role in Induced Cell Death of Effector T Cell: A Possible Third Mechanism of Self-tolerance

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## Summary

We have used anti-T cell monoclonal antibodies (mAbs) as mimic ligands to study the effects of T cell receptor (TCR) ligation of cloned T helper type 1 cells in the presence or absence of accessory cells. Our results demonstrate that ligation of the TCR in the absence of accessory cells rapidly induces cell death. Cell death can be prevented by addition of spleen adherent cells, leading to strong clonal expansion. Induced cell death is inhibited by cyclosporin A and by anti-interferon  $\gamma$  (IFN- $\gamma$ ), and is restored by adding exogenous recombinant IFN- $\gamma$  to cyclosporin A-treated cells. These results demonstrate that IFN- $\gamma$  plays a critical role in cell death induced by anti-TCR mAbs in the absence of costimulatory cells. We propose that induced cell death of active effector T cells provides a third mechanism of tolerance in addition to intrathymic deletion of developing autoreactive T cells and peripheral inactivation of mature, naive T cells.

Engagement of the TCR can lead to a variety of consequences, such as cell proliferation, clonal anergy (1–3), and programmed cell death (4, 5). These consequences are critical for clonal selection, providing responses to nonself while preserving self-tolerance. Thus, the mechanisms that determine which of these responses will result are an essential component of self-nonself discrimination by T cells. In this report, we show that anti-TCR/CD3 stimulation in the absence of costimulatory signals leads to cell death of Th1 clones, and that IFN- $\gamma$  plays a critical role in this process.

## Materials and Methods

**Mice, Antibodies, and Cells.** BALB/cByJ mice were purchased from The Jackson laboratory (Bar Harbor, ME). Adult mice of >6 wk of age were used. mAbs specific for Thy-1 (Y19) and CD3  $\epsilon$  chain (YCD3-1) were prepared in this lab and have been described previously (6, 7). mAbs specific for all TCR- $\alpha/\beta$  (H57-597) were kindly provided by Dr. Ralph Kubo (8) (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). mAbs to IL-2 (S4B6.34) (9) and IFN- $\gamma$  (XMG 1.2) (10) were kindly provided by Dr. Tim Mosmann (University of Alberta, Edmonton, Canada). All antibodies were purified from hybridoma supernatant through a protein G-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ).

The cloned Th1 cells 5.9 were prepared in this laboratory and have been described (11). This clone was derived from BALB/cByJ mice and recognizes OVA presented by I-A<sup>d</sup>.

**Proliferation Response of T Cell Clones to IL-2 in the Presence of Crosslinked Anti-TCR/CD3 mAb** mAbs were diluted in PBS and incubated in 96-well flat-bottomed tissue culture plates (40  $\mu$ l/well)

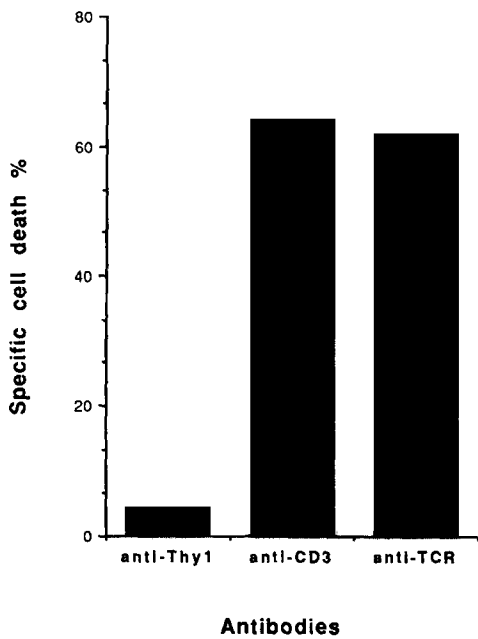
for 2 h at 37°C. At the end of the incubation, the plates were washed three times with Click's Eagle's Hank's amino acid (EHAA; Irvine Scientific, Santa Ana, CA) medium containing 5% FCS. Cloned T cells ( $2 \times 10^4$ /well) were cultured in the presence of murine rIL-2 (50 U/ml) for 42 h. These cultures were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]TdR for an additional 6 h and [<sup>3</sup>H]TdR incorporation was determined.

**Evaluation of Cell Death by Trypan Blue Exclusion and by Flow Cytometry with Propidium Iodide-Stained Cells.** T cell clones were incubated in 96-well plates coated with anti-T cell mAb (5  $\mu$ g/ml) or medium controls for 16–20 h. The cells were harvested by pipetting and diluted 1:2 with 0.1% trypan blue in PBS. The live and dead cells were counted in a hemocytometer. The percentage of specific cell death was calculated according to the following formula. Percent specific cell death =  $100 \times (\text{percent dead cells in sample} - \text{percent dead cells in medium}) / (100\% - \text{percent dead cells in medium})$ .

For FACS analysis of cell death, T cell clones were suspended in Click's EHAA medium containing 5% FCS at a density of  $3 \times 10^5$ /ml. This cell suspension was incubated in 24-well tissue culture plates (1 ml/well) that were precoated with anti-T cell mAb (5  $\mu$ g/ml) at 37°C in humidified air containing 5% CO<sub>2</sub>. Cells were harvested after a 16-h incubation, washed once with PBS, and suspended in PBS containing propidium iodide (2  $\mu$ g/ml). The fluorescence intensity was determined with a FACS 440 (Becton Dickinson & Co., Mountain View, CA).

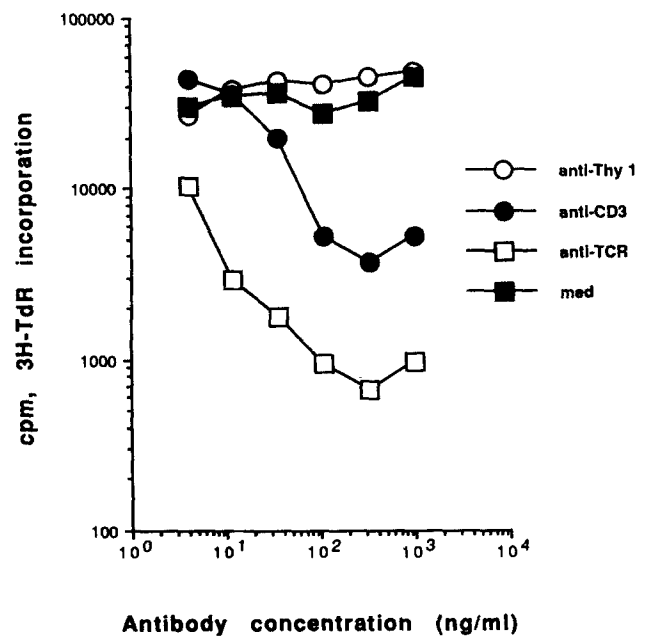
## Results and Discussions

**Crosslinking the TCR on Th1 Clones in the Absence of Costimulatory Signals Induces Cell Death.** Proliferation of clone 5.9 to anti-CD3/TCR mAbs requires both crosslinking antibodies and costimulatory signals from accessory cells.



**Figure 1.** Induction of cell death of 5.9 cells by anti-T cell mAbs. Data shown are percentages of specific cell death as determined by trypan blue exclusion of clone 5.9 cells 24 h after stimulation in plates coated with 5  $\mu\text{g/ml}$  of mAb. Similar data were obtained when T cells were stimulated with 0.05  $\mu\text{g/ml}$  of mAb.

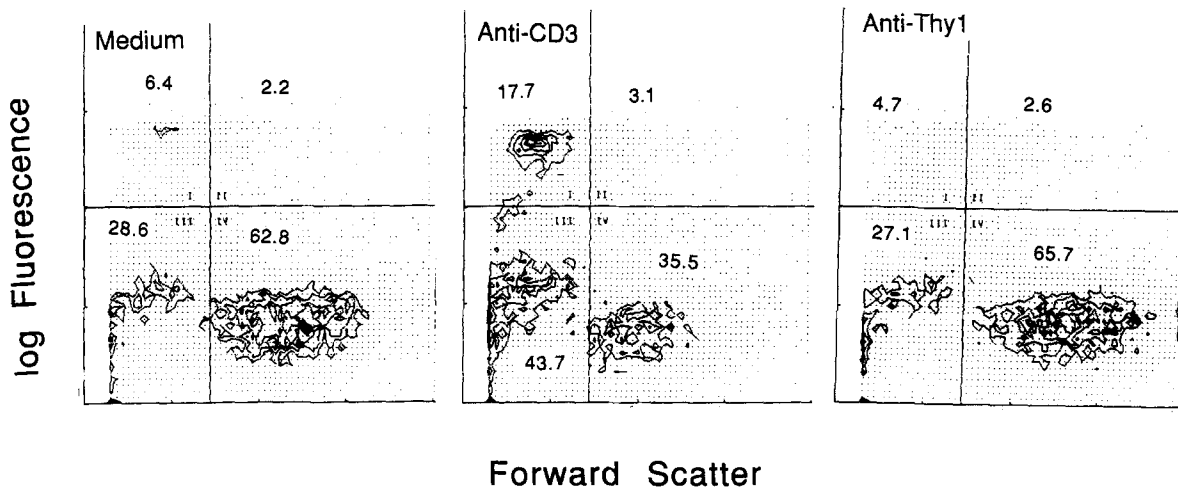
Plastic-adsorbed anti-TCR and anti-CD3 mAb do not induce proliferation in the absence of accessory cells (data not shown). However, these mAbs do induce death of clone 5.9 cells as determined by trypan blue dye exclusion after 16 h of culture (Fig. 1), fluorescence-activated cell sorter (FACS is a registered trademark of Becton Dickinson and Company) staining with propidium iodide (Fig. 2), and loss of responsiveness to IL-2 (Fig. 3). As shown in Fig. 1, ~65–70% of specific cell death was recorded by trypan blue dye exclusion. When



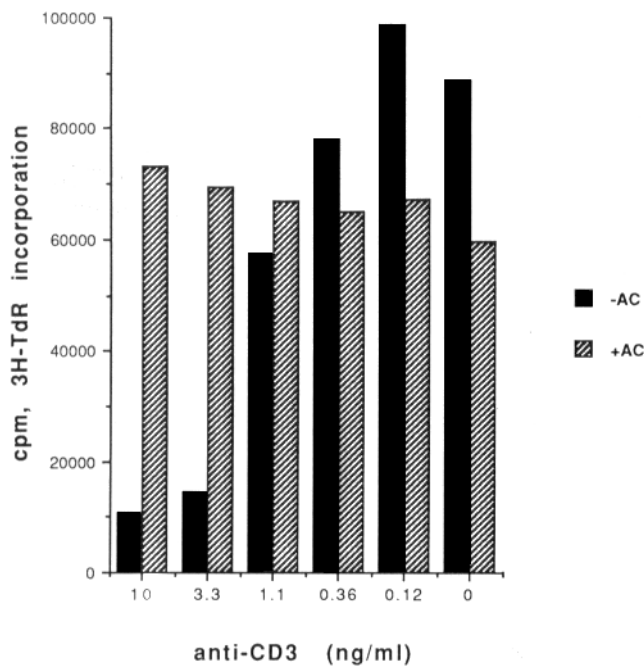
**Figure 3.** Inhibition of proliferation to IL-2 (50 U/ml) of 5.9 cells by various amounts of plate-bound anti-TCR/CD3 mAb. Data shown are means of duplicates from a 48-h assay.

5.9 cells were stained with propidium iodide, anti-CD3 mAb-treated cultures contained a significant proportion of brightly stained cells (~18%), while anti-Thy-1- or medium-treated cells contained <7% of brightly stained cells (Fig. 2). Furthermore, there is a marked reduction in the viable cells from anti-CD3-coated wells as determined by forward scatter, suggesting that some dead cells do not stain with propidium iodide.

Another manifestation of cell death is the loss of responsiveness to IL-2 (Fig. 3). T cells cultured with medium or



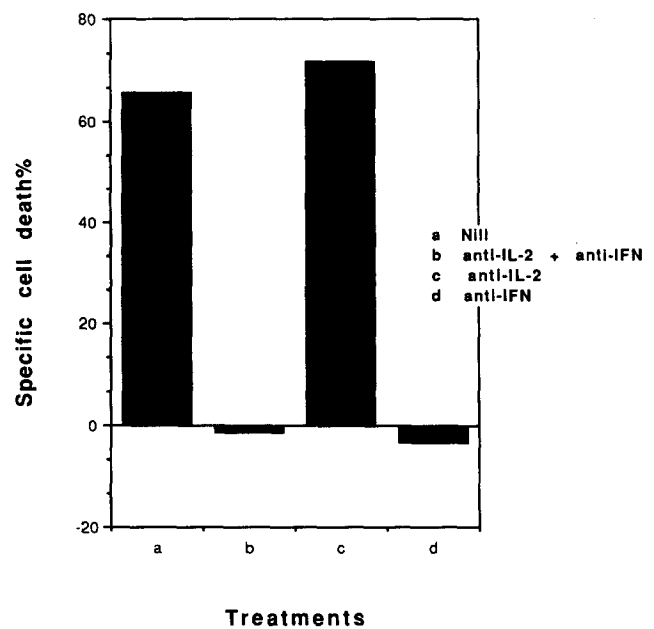
**Figure 2.** Analysis of cell death by flow cytometry. 5.9 cells were cultured with plate-coated anti-CD3 mAb (5  $\mu\text{g/ml}$ ) for 24 h, and cells recovered were stained with propidium iodide (2  $\mu\text{g/ml}$ ) to show dead cells.



**Figure 4.** Rescue of proliferation of clone 5.9 to IL-2 by accessory cells. The T cells ( $2 \times 10^4$ /well) were cultured with IL-2 (50 U/ml), given concentrations of plate-bound anti-CD3 mAb, in the presence (*shaded bars*) or absence (*solid bars*) of mitomycin C-treated, T-depleted spleen cells ( $10^5$ /well) for 48 h, and proliferation was determined by [ $^3$ H]TdR incorporation. Proliferation of mitomycin C-treated, T-depleted spleen cells is always  $<1,000$  cpm.

with plates coated with anti-Thy-1 mAb proliferate in response to exogenous IL-2, while such proliferative responses were significantly inhibited in the presence of plate-bound anti-CD3 or anti-TCR mAb. A similar level of cell death was observed in all of five other Th1 clones tested, but not in any of the Th2 clones tested (data not shown). Thus, the TCR ligation, which induces clonal expansion of cloned Th1 cells in the presence of accessory cells (data not shown), induces cell death when presented without accessory cells.

**Accessory Cells Prevent Induced Cell Death.** The loss of proliferation in response to IL-2 provides a second quantitative assay for the death of Th1 clones. To test if accessory

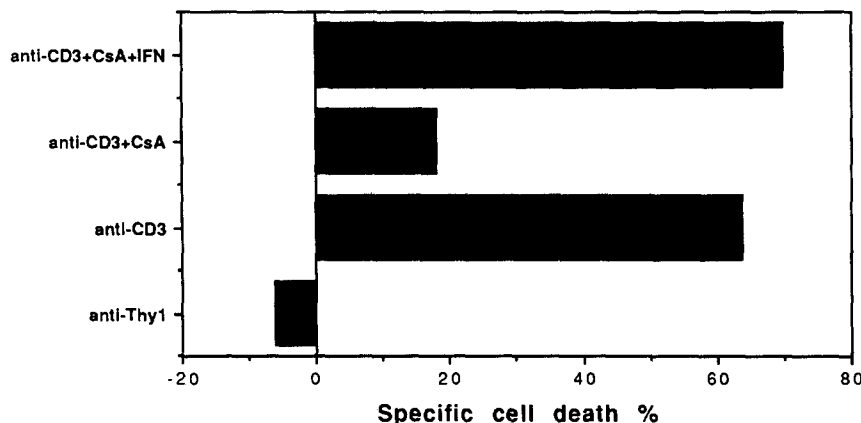


**Figure 6.** Inhibition of cell death by anti-IFN- $\gamma$  mAb. 5.9 cells ( $2 \times 10^4$ /well) were cultured with plate-bound anti-CD3 mAb (5  $\mu$ g/ml) in the presence of anti-IFN- $\gamma$  and anti-IL-2 mAbs (200  $\mu$ g/ml), and cell death was determined at 16 h by trypan blue exclusion.

cells could rescue the Th1 clones from death induced by anti-CD3 mAb, we measured the proliferation of the Th1 clone 5.9 to IL-2 in the presence or absence of anti-CD3 mAb and T-depleted spleen adherent cells. As shown in Fig. 4, the proliferative response of clone 5.9 to exogenous IL-2 was inhibited by plate-coated anti-CD3 mAb, and the degree of inhibition is proportional to the concentration of anti-CD3 mAb added. This inhibition is reversed by the addition of spleen-adherent cells.

**The Induction of Cell Death in Cloned Th1 Cells Requires Production of IFN- $\gamma$ .** To analyze the mechanism of anti-CD3/TCR-induced cell death, cyclosporin A (CsA) was added to cultures of clone 5.9 cells. As seen in Fig. 5, CsA significantly inhibits the death of cloned Th1 cells induced by anti-CD3.

Because CsA inhibits cell death induced by anti-CD3, a



**Figure 5.** Inhibition of anti-CD3-induced cell death by CsA and restoration of cell death by rIFN- $\gamma$ . 5.9 cells ( $2 \times 10^4$ /well) were cultured with plate-bound anti-CD3 mAb (5  $\mu$ g/ml) and CsA (100 ng/ml) for 24 h, and cell death was determined by trypan blue exclusion. IFN- $\gamma$  (1,000 U/ml) was added at the beginning of culture. Specific cell death of clone 5.9 is  $-0.4\%$  when treated with CsA (100 ng/ml) and IFN- $\gamma$  (1,000 U/ml) in the absence of anti-CD3.

possible role of cytokines in the induced death of cloned Th1 cells was examined. As we can detect a large amount of IL-2 (100 U/ml) and IFN- $\gamma$  (1,000 U/ml) in anti-CD3-induced clone 5.9 supernatants, we tested the ability of anti-IL-2 and anti-IFN- $\gamma$  mAbs to inhibit cell death. As shown in Fig. 6, anti-IFN- $\gamma$  mAb inhibits cell death induced by anti-CD3, while anti-IL-2 mAb has no effect. Similarly, anti-IFN- $\gamma$  mAb reversed the inhibition of IL-2 responsiveness induced by anti-CD3 mAb (data not shown). The role of IFN- $\gamma$  was confirmed by reconstitution experiments, which demonstrate that IFN- $\gamma$  restored cell death that had been inhibited by CsA (Fig. 5), and also reconstituted inhibition of IL-2 responsiveness (data not shown). Thus, IFN- $\gamma$  appears to be required for this response. Its removal by anti-IFN- $\gamma$  prevents cell death, and its provision to Th1 cells blocked by CSA restores cell death. However, IFN- $\gamma$  alone is not sufficient for cell death, since IFN- $\gamma$  treatment of unstimulated clone 5.9 cells is not cytotoxic, and IFN- $\gamma$  is produced in large amounts when clone 5.9 is stimulated by anti-TCR mAb and accessory cells, which does not cause cell death. Further experiments show that the missing factor is not a cytokine, as anti-CD3-stimulated 5.9 supernatant can induce death of CsA-treated 5.9 cells only in the presence of crosslinked anti-CD3 (data not shown).

In summary, our results demonstrate that TCR crosslinking

induces death of Th1 clones unless costimulatory cells are present. The mechanism of Th1 cell death may resemble that of T<sub>H</sub> hybridomas reported previously by Ashwell and coworkers (12, 13). There are, however, two clear differences in our results. First, one can not examine signals required for clonal expansion as opposed to cell death of T<sub>H</sub> hybridomas, and indeed, T cell hybrids are not rescued by addition of costimulatory cells. Second, the role of IFN- $\gamma$  has not been addressed in studies by Ashwell and coworkers (12, 13). As lack of costimulatory signals is a characteristic of nonlymphoid tissue cells (14), our finding that ligation of the TCR in the absence of costimulatory signals leads to cell death of effector T cells suggests that such processes may play a role in eliminating activated effector T cells specific for tissue antigens. Furthermore, the requirement for IFN- $\gamma$  in this process implies that this proposed self-tolerance mechanism is likely to apply to activated T cells rather than naive T cells. We speculate that the induced death of effector T cells reported here provides a third mechanism of immune tolerance, which, together with clonal deletion of developing T cells in the thymus (15) and clonal anergy of naive T cells in the periphery (1-3), serves to eliminate autoreactive T cells. These issues are discussed more fully elsewhere (16).

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