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*J Immunol* 1999; 163:1817-1826; ;
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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Strong TCR Ligation Without Costimulation Causes Rapid Onset of Fas-Dependent Apoptosis of Naive Murine CD4⁺ T Cells

Hidehiro Kishimoto and Jonathan Sprent

Activation-induced cell death of T cells typically occurs late in the primary response after a prior proliferative response. Here, we describe a novel form of cell death in which purified naive murine CD4⁺ cells undergo apoptosis within 18 h in vitro after strong TCR ligation. Such rapid-onset TCR-mediated death of T cells does not involve cell division and is Fas-dependent, inhibited by CD28 (and IL-6) costimulation and enhanced by IL-4 and IL-7; by contrast, spontaneous death of CD4⁺ cells cultured alone is Fas-independent and inhibited by IL-4 and IL-7. TCR-mediated Fas-dependent death of CD4⁺ cells is prevented by combined TCR/Fas ligation and by drugs that inhibit calcineurin-dependent signaling and mitogen-activated protein kinase MEK1 activation. The Journal of Immunology, 1999, 163: 1817–1826.

Under defined conditions, T proliferative responses to Ag can be followed by apoptosis (1–5). This phenomenon of activation-induced cell death (AICD) is largely Fas-mediated and reflects interaction of cell-surface Fas with Fas ligand (L), which is up-regulated on activated T cells (1–11). Fas-mediated AICD appears to be important in vivo because Fas-deficient T cells from lpr/lpr mice show delayed elimination of T cells after the primary response (6), which may explain the progressive lymphadenopathy seen in this strain (5).

The precise requirements for inducing AICD are unclear. In the case of preactivated T cell lines in vitro, AICD is promoted when T cells undergo TCR ligation in the presence of high concentrations of IL-2 or after prior exposure to IL-2 (12, 13). AICD in this situation is heavily Fas-dependent and cannot be prevented by costimulation, i.e., by CD28/B7 interaction. In other studies, however, exposing naive T cells to joint TCR/CD28 ligation in vitro without exogenous cytokines caused exponential growth of the cells for several weeks, even though the cells expressed high levels of Fas and FasL (14–17). The lack of Fas-mediated cell death in this situation is attributed to up-regulation of the anti-apoptotic molecule, Bcl-XL (16). However, other workers have failed to find evidence that Bcl-XL up-regulation prevents AICD (11).

AICD appears to be largely unrelated to the form of spontaneous apoptosis that occurs when normal naive T cells are cultured alone in vitro (18, 19). This type of death is Fas-independent and is prevented by combined TCR/CD28 ligation, but not by TCR ligation alone. The implication, therefore, is that, for mature T cells, Fas-dependent death is only demonstrable with primed T cells and not with naive T cells. However, following up our preliminary data on thymocyte subsets and lymph node (LN) cells (18), we show here that naive T cells are indeed susceptible to Fas-mediated death. This form of death occurs when naive T cells are subjected to strong TCR ligation in the absence of costimulation and is apparent after short-term (overnight) culture.

Materials and Methods

Mice

Adult C57BL/6 (B6), B6 Fas⁻/⁻ (B6lpr/+), B6 CD28⁻/⁻, and C3H/HeJ mice aged 6–10 wk were obtained from The Jackson Laboratory (Bar Harbor, ME).

Antibodies

Abs specific for the following markers were previously described (20): Thy1.2 (J1), rat IgM, CD4 (RL172, rat IgM), CD8 (S58, 8, rat IgM), CD25 (TD4, rat IgM), heat stable Ag (HSA) (J11D, rat IgM), and class II (M5/114, rat IgG). The following mAbs were purchased from PharMingen (San Diego, CA): anti-TCR-β (H57-597, hamster IgG), anti-CD28 (37.51, hamster IgG), anti-CD95 (Fas) (Jo2, hamster IgG), anti-CD95L (FasL) (MFL3, hamster IgG), anti-Bcl-2 (3F11, hamster IgG), anti-CD43 (87, rat IgG), anti-LFA-1 (M14/4, rat IgG), PE-conjugated anti-CD44 (IM7, rat IgG), PE-conjugated anti-CD5RB (23G2, rat IgG), PE-conjugated anti-CD95L, and CyChrome-conjugated anti-CD4 (H129.19, rat IgG). FITC-conjugated anti-bromodeoxyuridine (BrdU) (B44, mouse IgG) mAb was purchased from Becton Dickinson (San Jose, CA). Anti-Bcl-XL mAb (44, mouse IgG) was purchased from Transduction Laboratories (Lexington, KY).

Cell purification

Purification of CD4⁺ LN T cells was performed as previously described (18). Briefly, pooled LN cells were treated with mAbs specific for HSA and CD8 plus guinea pig complement (C) for 45 min at 37°C, positively panned with anti-CD4 (RL172) mAb, then negatively panned with anti-class II (M5/114) mAb. Purification of CD4⁻ CD44⁻ LN T cells was performed by treating pooled LN cells with mAbs specific for CD45RB (23G2), HSA, and CD8 plus guinea pig C and rabbit C at 37°C, positively panned with anti-CD4 (RL172) mAb, then negatively panned with anti-class II (M5/114) mAb. Purification of splenic T B⁺ APC was performed by treating whole...
spleen cells with mAbs specific for CD4, CD8, Thy1.2, and HSA plus guinea pig C for 45 min at 37°C.

**Culture conditions**

Purified CD4+ cells (3 × 10^5) were cultured in 0.2 ml of RPMI medium supplemented with 5 × 10^{-5} M 2-mercaptoethanol, 1-glutamine, and 10% FCS in 96-well tissue-culture plates coated with anti-TCR (H57-597) ± anti-CD28 (37.51) mAbs or medium alone. To block CD28-B7 interaction, CTLA4-Ig (21) was added at 10 μg/ml. Where indicated, IL-2, IL-4, IL-7, IFN-γ (22, 23), and IL-6 (Genetics Institute, Cambridge, MA) were added to the cultures at 100 U/ml; IL-1 (R&D Systems, Minneapolis, MN), IL-12 (Genetics Institute), and TNF-α (R&D Systems) were added at 10 ng/ml. IFN-β (Access Biomedical, San Diego CA) was added at 1000 U/ml. For in vitro BrdU incorporation, purified CD4+ cells were cultured with 25 μg/ml of BrdU for 20 h. For inhibition of T cell stimulation, cyclosporine A (CSA) was added to the cultures at 10 μg/ml. Where indicated, 2N HCl for 30 min, and West Grove, PA) or rabbit anti-mouse IgG-FITC, respectively. For BrdU followed by rabbit anti-hamster IgG-FITC (Jackson ImmunoResearch, (25), cells were fixed with PBS containing 1% paraformaldehyde, 0.1% glutaralddehyde, and 0.02% Tween 20. After fixation, cells were incubated with anti-TCR (H57-597), anti-CD44 or anti-FasL and Cychrome-conjugated anti-CD4 (Access Biomedical, San Diego CA) was added at 1000 U/ml. For in vitro experiments described below, T cell apoptosis was routinely measured after overnight culture (18 h), i.e., well before the onset of T cell proliferation. Unless stated otherwise, purified B6 LN CD4+ cells were cultured in vitro in tissue culture wells precoated with anti-TCR mAb ± other mAbs. In all situations mAbs were presented in cross-linked (plate-bound) rather than soluble form.

**TCR ligation alone induces rapid apoptosis of mature CD4+ cells**

Apoptosis of cells cultured in medium alone vs culture in wells coated with anti-TCR mAb is shown in Fig. 1A. Despite the use of high-quality tissue-culture medium (RPMI 1640) and FCS pre-screened for lack of toxicity for mouse lymphoid cells, overnight culture of purified B6 CD4+ cells in medium alone led to substantial onset of “spontaneous” apoptosis, as defined by TUNEL staining. In this and many other experiments, apoptosis following culture of B6 CD4+ cells in medium alone overnight was around 30%. Viability of the cells before culture was 95%. When B6 CD4+ cells were cultured overnight in wells coated with high concentrations of anti-TCR mAb (10 μg/ml), apoptosis increased significantly, relative to cells cultured in medium alone (Fig. 1, A and B). In terms of Δ apoptosis, i.e., [% TUNEL- cells after culturing CD4+ cells with anti-TCR mAb] – [% TUNEL- cells for cells cultured in medium alone], TCR ligation induced specific apoptosis of around 15–20% of B6 CD4+ cells (Fig. 1C). Such TCR-mediated apoptosis after overnight culture was an invariant finding, seen in >20 experiments, and required high concentrations of cross-linked anti-TCR mAb (≥10 μg/ml) and thorough depletion of APC (see below); soluble anti-TCR mAb was ineffective (data not shown).

**Results**

As discussed earlier, AICD of mature T cells in vitro typically occurs late in culture after a prior proliferative response. For the experiments described below, T cell apoptosis was routinely measured after overnight culture (18 h), i.e., well before the onset of T cell proliferation. Unless stated otherwise, purified B6 LN CD4+ cells were cultured in vitro in tissue culture wells precoated with anti-TCR mAb ± other mAbs. In all situations mAbs were presented in cross-linked (plate-bound) rather than soluble form.

**Costimulation inhibits TCR-mediated apoptosis**

In marked contrast to TCR ligation alone, culturing normal B6 CD4+ cells in wells coated with a mixture of anti-TCR mAb (10 μg/ml) and anti-CD28 mAb (10 μg/ml) failed to cause apoptosis (Fig. 2, A and B, upper panels). In fact, levels of apoptosis in this situation were considerably below the level for cells cultured alone, thus leading to negative values for Δ apoptosis (Fig. 2B, upper panels); CD28 ligation alone had no effect. The lack of apoptosis following combined TCR/CD28 ligation correlated with marked enlargement of the cells as defined by forward scatter (FSC) (Fig. 2A). This contrasted with cells subjected to TCR ligation alone, where most of the cells remained small (gating on the surviving viable TUNEL- cells).

Supplementing CD4+ cells with APC provided further evidence that costimulation inhibited TCR-mediated apoptosis. Thus, when purified CD4+ cells were cultured in anti-TCR mAb-coated wells in the presence of syngeneic APC-enriched spleen cells (T−B+ spleen), addition of only 1% APC was sufficient to abolish Δ apoptosis (Fig. 2C); adding a higher proportion of APC augmented T cell viability (as with combined TCR/CD28 ligation) and led to negative values for Δ apoptosis (data not shown).

The capacity of APC to protect against TCR-mediated apoptosis could be largely or partly a reflection of CD28/B7 interaction. To examine this possibility, normal vs CD28+ spleen cells, i.e., cell populations containing CD4+ cells and large numbers of APC, were subjected to TCR ligation (Fig. 2D). As expected, TCR (or TCR/CD28) ligation of normal spleen cells led to negative values for Δ apoptosis of CD4+ cells, presumably indicating protection by APC (Fig. 2D, a). By contrast, TCR ligation of CD28−/−...
Spleen cells caused significant (10%) Δ apoptosis of CD4+ cells, indicating a lack of protection; similar findings applied when CTLA4-Ig was added to normal (CD28+/−) B6 spleen cells (Fig. 2D, a). Thus, the capacity of APC to protect CD4+ cells from TCR-mediated apoptosis seemed to be mainly a reflection of CD28/B7 interaction. This conclusion was based on enumerating the percent of TUNEL+ cells in the cultures. Counting total numbers of viable CD4+ cells (relative to the numbers initially cultured) gave similar results (Fig. 2D, b).

The notion that CD28/B7 interaction accounted for the protective function of APC predicted that, unlike CD28 ligation, ligation of other T cell molecules with costimulatory function, such as LFA-1 (26–28) and CD43 (29), would not protect against TCR-mediated apoptosis. As discussed later (see Fig. 6C), this was indeed the case.

**Role of Fas**

Significantly, the induction of TCR-mediated apoptosis was entirely Fas-dependent. Thus, with purified Fas-deficient B6+/−/+ CD4+ cells, TCR-mediated apoptosis was undetectable (Fig. 2, A and B, lower panels). In fact, subjecting B6+/−/+ cells to TCR ligation without costimulation was protective and led to negative values for Δ apoptosis. By contrast, the spontaneous apoptosis seen when cells were cultured in medium alone was no lower with B6+/−/+ CD4+ cells than with normal B6 CD4+ cells. Thus, only TCR-mediated apoptosis and not spontaneous apoptosis was Fas-dependent.

**Naive- vs memory-phenotype CD4+ cells**

The data presented above refer to normal unfractionated CD4+ cells. Since these cells comprise a mixture of naive- and memory-phenotype cells, TCR-mediated apoptosis might be restricted to memory-phenotype cells (which account for 10–20% of CD4+ cells in normal young B6 mice). This possibility seemed unlikely because, after TCR ligation of CD4+ cells overnight, the surviving cells did not show a selective depletion of memory-phenotype cells, e.g., CD45RBhi cells (Fig. 3A). Nevertheless, to examine this question directly, we prepared purified naive- vs memory-phenotype CD4+ cells from B6 mice. For preparing these cells, we avoided using FACS sorting because this procedure considerably augmented spontaneous apoptosis of cells cultured in medium alone (data not shown); we also avoided positive panning, where ligation with the mAb selected might influence cell survival. The approach used was to selectively deplete CD4+ cells of naive- or

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**FIGURE 2.** Role of costimulation and Fas on TCR-mediated apoptosis of B6 CD4+ cells. Cells were TUNEL stained after overnight culture. A, Representative TUNEL staining and FSC of normal B6 vs B6+/−/+ CD4+ cells cultured in wells containing medium alone or coated with anti-TCR mAb (10 μg/ml) or a mixture of anti-TCR and anti-CD28 mAb (10 μg/ml of each). As in A, but showing the data as Δ apoptosis and including control cultures with anti-CD28 mAb without anti-TCR mAb. B, Apoptosis of B6 CD4+ cells (3 × 10⁶/well) cultured on anti-TCR mAb-coated plates supplemented with graded numbers of APC (T B− B6 spleen); control cells were cultured with APC in the absence of TCR ligation. D, Δ apoptosis (a) and percent cell recovery (b) of CD4+ cells in spleen from B6 or B6/−/+ mice cultured on anti-TCR mAb-coated plates ± CTLA4-Ig (10 μg/ml); percent cell recovery was measured by counting total numbers of CD4+ cells before and after culture; to enrich for CD4+ cells, spleen cells were depleted of CD8− cells (with mAb + C) before culture. B and C, The data show mean values for triplicate cultures.

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memory-phenotype cells (and other cells) by negative selection, i.e., with mAb + C treatment. To prepare naive cells, CD4+ cells were depleted of CD44high cells with anti-CD44 mAb (Fig. 3B). Memory-phenotype CD4+ cells were prepared with anti-CD45RB mAb + C, thus generating CD45RBlow cells (Fig. 3B).

When purified naive-phenotype CD44low CD4+ cells were subjected to TCR ligation alone, Δ apoptosis levels were about the same as with unfractionated CD4+ cells (Fig. 3C). With memory-phenotype CD45RBlow cells, by contrast, Δ apoptosis levels were appreciably less than with naive cells or unfractionated cells. These findings indicated that TCR-mediated apoptosis of unfractionated CD4+ cells was largely (but not entirely) restricted to naive cells. In view of these findings, we used unfractionated cells for the remainder of the experiments.

**CD4+ cells from other mouse strains**

As mentioned above, values for Δ apoptosis when normal B6 CD4+ cells were subjected to TCR ligation alone overnight were relatively low, i.e., 15–20%. Similar Δ apoptosis levels were observed with CD4+ cells from C3H (Fig. 4A) and BALB/c (data not shown) mice. With these two strains, however, the levels of spontaneous apoptosis when CD4+ cells were cultured in medium alone (15–20%) were substantially lower than with B6 CD4+ cells (30%).

**Effects of cytokines**

Detecting TCR-mediated apoptosis of B6 CD4+ cells was clearly hindered by the high rate of spontaneous apoptosis when these cells were cultured in medium alone. As reported by others (19), certain cytokines, especially IL-4 and IL-7, are able to inhibit spontaneous death of naive T cells in vitro without inducing obvious signs of cell activation. Hence, lowering spontaneous apoptosis with these cytokines might augment TCR-mediated apoptosis. Conversely, adding these cytokines might have the opposite effect and prevent TCR-mediated apoptosis, i.e., by impairing Fas-mediated death.

Confirming the above report (19), culturing normal B6 CD4+ cells with IL-4 or IL-7 without TCR ligation substantially impaired spontaneous apoptosis (Fig. 4B); two other cytokines, IL-2 and IFN-γ, were less effective. When CD4+ cells were subjected to
TCR ligation, simultaneous addition of IL-4 or IL-7 had little effect on total percent apoptosis (Fig. 4C, upper panel). By lowering spontaneous death, however, addition of IL-4 or IL-7 led to a considerable (1.5- to 2-fold) increase in Dapoptosis levels, i.e., 25–30% (Fig. 4D, lower panel); this effect was not seen with IL-2. Similar results occurred with C3H CD4+ cells (Fig. 4D).

The above data imply that addition of IL-4 or IL-7 did not have an obvious effect on TCR-mediated apoptosis per se, indicating that the protective effect of these cytokines did not extend to Fas-mediated apoptosis. From a practical point of view, however, the capacity of IL-4 or IL-7 to reduce spontaneous apoptosis made TCR-mediated apoptosis much easier to detect, especially with B6 CD4+ cells. Thus, with addition of IL-4 or IL-7 to B6 CD4+ cells, percent apoptosis following TCR ligation was quite high (30 – 40%), relative to the low background apoptosis for cells cultured alone (5–10%) (Fig. 4C, upper panel). There was also an appreciable increase in Dapoptosis (Fig. 4C, lower panel).

We also tested several other cytokines, namely IL-1, IL-6, IL-12, IFN-β, and TNF-α (Fig. 4E). Like, IL-2 and IFN-γ (see above), none of these cytokines had more than a marginal effect in reducing spontaneous apoptosis. Interestingly, however, two of the cytokines, IL-6 and TNF-α, abolished TCR-mediated apoptosis. In fact, addition of IL-6 during TCR ligation closely mimicked the effect of CD28 costimulation in causing prominent blast transformation (data not shown) and reducing apoptosis to below background levels (Fig. 4E, lower panel). This effect was also seen to a lesser extent with TNF-α. The other cytokines were ineffective.

**FasL expression**

The finding that TCR-mediated apoptosis was entirely Fas-dependent (Fig. 2) implied that induction of apoptosis required up-regulation of FasL on the responding CD4+ cells, thus leading to death via Fas/FasL interaction. The observation that TCR-mediated apoptosis after overnight culture was relatively low suggested that FasL expression on the responding cells at this early stage of culture might be limited. In fact, surface staining of CD4+ cells after overnight TCR ligation revealed only a small shoulder of FasL expression (Fig. 5A); cells cultured in medium alone were negative. Interestingly, FasL expression was somewhat higher when TCR-stimulated cells were fixed before staining, indicating that FasL expression was largely intracellular (Fig. 5A). In control experiments, surface FasL expression after TCR ligation was clearly apparent on Fas-deficient B6 lpr/lpr CD4+ cells (Fig. 5A, lower panel). Hence, for normal B6 CD4+ cells, the paucity of surface FasL after TCR ligation may have been a direct consequence of Fas/FasL interaction, perhaps reflecting Fas-mediated blockade of serologically detectable sites on FasL. In support of this notion, addition of soluble anti-Fas mAb during TCR ligation of normal B6 CD4+ cells augmented surface FasL expression to the level seen with B6 lpr/lpr CD4+ cells (data not shown).

For both surface and internal staining of B6 CD4+ cells, significant up-regulation of FasL in the absence of costimulation required a high concentration of anti-TCR mAb (10 μg/ml) (Fig. 5A). Combined TCR/CD28 ligation clearly potentiated FasL up-regulation, but only with a low concentration of anti-TCR mAb
containing either medium alone or cross-linked anti-Fas mAb (10 μg/ml). Thus, with a high concentration of anti-TCR mAb, CD28 costimulation induced the same level of FasL up-regulation as TCR ligation alone.

The main conclusion from the above experiment is that the relatively low level of apoptosis induced by TCR ligation alone correlated with only low expression of FasL on the cell surface. Hence, the failure to see more substantial apoptosis after TCR ligation may reflect suboptimal up-regulation of FasL.

The data in Fig. 5A also make the point that FasL up-regulation alone was not sufficient to induce apoptosis. This is apparent from the finding that combined TCR/CD28 ligation induced FasL up-regulation but, unlike TCR ligation alone, failed to induce apoptosis (Fig. 2). One possibility here is that combined TCR/CD28 ligation favored up-regulation of antiapoptotic molecules, such as Bcl-XL (14–16). However, intracellular staining showed that CD4⁺ cells subjected to either strong TCR ligation alone or to combined TCR/CD28 ligation both showed an equivalent 10-fold up-regulation of Bcl-XL, relative to cells cultured in medium alone (Fig. 5B); levels of Bcl-2 remained unchanged.

**Apoptosis following direct Fas ligation with anti-Fas mAb**

As argued above, the observation that apoptosis following TCR ligation alone was only moderate rather than marked may have reflected suboptimal FasL up-regulation on the cell surface. If so, the prediction follows that bypassing Fas/FasL interaction, e.g., by subjecting TCR-stimulated cells to direct ligation via anti-Fas mAb, would accentuate apoptosis. To examine this question, CD4⁺ cells were subjected to Fas ligation before and after TCR stimulation.

It is well established that Fas expression is especially high on CD4⁺ thymocytes, but is also clearly detectable on mature LN T cells, including naive CD4⁺ cells (30). However, in confirmation of the results of others (30), culturing cells on plates coated with anti-Fas mAb induced marked apoptosis of CD4⁺ thymocytes but negligible apoptosis of LN CD4⁺ cells (Fig. 6A), even though Fas expression on normal LN CD4⁺ cells was clearly detectable (data not shown). Normal mature CD4⁺ cells were thus strongly resistant to Fas-mediated death.

The sensitivity of CD4⁺ cells to apoptosis induced by cross-linked anti-Fas mAb after prior TCR ligation is shown in Fig. 6B, upper panel. Here, B6 CD4⁺ cells were cultured in anti-TCR mAb-coated wells for 18 h and then, for an additional 6 h, in wells containing either medium alone or cross-linked anti-Fas mAb (10 μg/ml). It can be seen that apoptosis was substantially higher when, after transfer, the cells were exposed to anti-Fas mAb (35% Δ apoptosis) than when cultured alone (15% Δ apoptosis). This contrasted with the effects of combined TCR/CD28 ligation. Here, subsequent exposure of the cells to cross-linked anti-Fas mAb failed to cause apoptosis (Fig. 6B, lower panel). It should be noted that TCR and TCR/CD28 ligation both caused a similar small (2- to 3-fold) increase in Fas expression on CD4⁺ cells (data not shown).

The above experiment indicated that subjecting CD4⁺ cells to prior TCR ligation alone made the cells highly sensitive to apoptosis induced by direct Fas ligation. By contrast, like normal unstimulated T cells, CD4⁺ cells subjected to combined TCR/CD28 ligation remained resistant to Fas-mediated apoptosis.

As a control for the above experiment, fresh CD4⁺ cells were cultured overnight with anti-TCR mAb alone vs a mixture of anti-TCR and anti-Fas mAb. Surprisingly, such simultaneous ligation of TCR and Fas completely abolished apoptosis (Fig. 6C); this protective effect was not seen when the cells were cocultured with anti-TCR mAb plus either anti-LFA-1 or anti-CD43 mAb. The implication, therefore, is that subjecting resting CD4⁺ cells to continuous Fas ligation during TCR ligation prevented the cells from entering a Fas-susceptible state. The protective effect afforded by continuous Fas ligation thus mimicked the effect of CD28 costimulation.

**Cell cycle analysis**

To assess whether TCR-mediated apoptosis was associated with entry into cell cycle, CD4⁺ cells were cultured overnight on anti-TCR mAb-coated plates in the presence of BrdU and then, after fixation, double stained for BrdU vs PI incorporation (31, 32). For cells with subdiploid DNA, i.e., apoptotic cells, PI staining confirmed the results of TUNEL staining. Thus, the proportion of cells with subdiploid DNA was increased by TCR ligation alone (relative to control cultures where TCR/CD28 ligation both showed an equivalent 10-fold up-regulation of Bcl-XL, relative to cells cultured in medium alone (Fig. 5B); levels of Bcl-2 remained unchanged.

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Despite failing to undergo DNA synthesis, CD4\(^+\) cells subjected to TCR ligation showed clear signs of activation. Thus, relative to cells cultured alone, TCR ligation induced strong CD69 up-regulation and increased expression of CD44 (Fig. 7B); similar findings applied with purified CD44 low CD4\(^+\) cells (data not shown).

**Intracellular signaling**

To explore the signaling pathways involved in TCR-mediated apoptosis, we examined the effects of three drugs: 1) CSA, an inhibitor of calcineurin-dependent signaling (33), 2) PD98052 (PD), an inhibitor of MEK1 (a kinase controlling activation of MAPK ERK1) (34, 35), and 3) SB203580 (SB), an inhibitor of MAPKp38 (36, 37). As discussed below, two of these compounds, CSA and PD, blocked TCR-mediated apoptosis, whereas SB was largely ineffective.

When normal B6 CD4\(^+\) cells were subjected to TCR ligation alone, addition of CSA (10 \(\mu\)g/ml) reduced apoptosis by ~70\% (Fig. 8A, left panel) and abolished FasL up-regulation (tested on B6\(^{prpr}\) cells) (Fig. 8B). Hence, CSA appeared to inhibit TCR-mediated apoptosis by simply blocking up-regulation of FasL. Nevertheless, marked apoptosis occurred when B6 CD4\(^+\) cells were subjected to TCR ligation overnight in the presence of CSA and then cultured for another 6 h on anti-Fas mAb-coated plates (still in the presence of CSA) (Fig. 8C). Hence, despite preventing FasL up-regulation, addition of CSA during TCR ligation did not prevent CD4\(^+\) cells from acquiring sensitivity to Fas-mediated death. The implication, therefore, is that CSA did not totally abolish TCR-mediated signaling. In support of this view, CSA severely impaired TCR-mediated up-regulation of FasL, CD25, and CD69, but did not prevent up-regulation of CD44 (Fig. 8D). In fact, CD44 up-regulation was enhanced by CSA, even when purified CD44 low cells were used (data not shown). It should be noted that, at the concentration used, CSA had no effect on spontaneous apoptosis.

With combined TCR/CD28 ligation, the effects of adding CSA were more complex. Here, there were three salient findings. First, adding CSA during TCR/CD28 ligation blocked the protective effect of CD28 costimulation and induced (rather than prevented) apoptosis (Fig. 8A, left panel); such apoptosis was Fas-dependent because no apoptosis occurred in parallel experiments with B6\(^{prpr}\) cells (Fig. 8A, right panel). Second, with TCR/CD28 ligation, addition of CSA failed to block FasL up-regulation (a clear contrast to CSA plus TCR ligation alone) (Fig. 8B). Third, the resistance of TCR/CD28-stimulated CD4\(^+\) cells to death mediated by subsequent Fas ligation was abrogated by addition of CSA during initial culture (Fig. 8C). Based on these three sets of findings, the induction of Fas-mediated apoptosis following addition of CSA during combined TCR/CD28 ligation was a reflection of: 1) unimpaired up-regulation of FasL, combined with 2) abolition of the protection mediated by CD28 costimulation.

For CD4\(^+\) cells subjected to TCR ligation alone, the results with PD were essentially the same as with CSA. Thus, addition of PD totally abolished TCR-dependent apoptosis (Fig. 8E), markedly impaired FasL up-regulation (Fig. 8D), and had no effect on the acquisition of sensitivity to death via Fas ligation after initial TCR ligation (Fig. 8F). However, relative to the effects of CSA, there were two interesting differences with PD. First, despite suppressing FasL up-regulation, PD failed to impair TCR-mediated up-regulation of CD25, CD69, or CD44, indicating a specific effect of PD on FasL expression (Fig. 8D). Second, unlike CSA, PD did not interfere with the capacity of CD28 costimulation to prevent the onset of susceptibility to death via Fas ligation (Fig. 8F, compare with 8C).

In marked contrast to PD, adding SB to the cultures had little or no effect. Thus, addition of SB caused only a small (20\%) reduction in TCR-mediated apoptosis (Fig. 8E) and failed to impair up-regulation of surface markers (Fig. 8D).

**Discussion**

Since naive T cells show a high rate of spontaneous apoptosis when cultured alone in vitro, it is perhaps not surprising that the...
additional cell death reported here, when T cells (CD4\(^+\) cells) are subjected to strong TCR ligation, is not seen routinely. In this respect, it should be emphasized that detecting specific apoptosis of CD4\(^+\) cells after overnight TCR ligation required stringent conditions: 1) the use of purified T cells rigorously depleted of APC, 2) the presence of high concentrations of anti-TCR mAb in cross-linked form, and 3) a sensitive assay, TUNEL, for detecting apoptotic cells. Departing from these conditions, e.g., by failing to deplete APC or using soluble rather than cross-linked anti-TCR mAbs, resulted in only very limited apoptosis (relative to cells cultured alone).

Although the level of TCR-mediated apoptosis was quite low, i.e., 15–20% \(\Delta\) apoptosis for B6 CD4\(^+\) cells, such apoptosis was undetectable with \(\text{lpr/lpr}\) CD4\(^+\) cells and was not seen when normal B6 CD4\(^+\) cells were subjected to combined TCR/CD28 ligation with APC. Thus, TCR-mediated apoptosis was entirely Fas-dependent and prevented by costimulation. By contrast, the spontaneous apoptosis seen when T cells were cultured alone was totally Fas-independent and not blocked by costimulation (Fig. 1B). Hence, spontaneous apoptosis and TCR-mediated apoptosis appeared to be essentially different.

Further evidence for the fundamental difference between spontaneous apoptosis and TCR-mediated apoptosis came from studies on the effects of adding IL-4 or IL-7. For spontaneous apoptosis, addition of IL-4 or IL-7 (but not other cytokines) was clearly protective for CD4\(^+\) cells and reduced apoptosis to low levels. By contrast, adding these cytokines during TCR ligation failed to impair apoptosis; indeed, \(\Delta\) apoptosis levels increased appreciably. Thus, IL-4 and IL-7 impeded spontaneous apoptosis, but enhanced TCR-mediated apoptosis.

The observation that TCR-mediated apoptosis after overnight culture was Fas-dependent implies that cell death reflected Fas/FasL interaction. Using FACS analysis, other workers have reported rapid up-regulation of FasL on the cell surface after TCR ligation (11). We confirmed this finding with B6 and \(\text{lpr/lpr}\) CD4\(^+\) cells, but not with normal cells. Thus, in our hands, cell-surface FasL up-regulation after TCR ligation of normal B6 CD4\(^+\) cells was very low at 18 h, although appreciable FasL up-regulation was apparent in permeabilized cells. Hence, the finding that TCR-mediated death after overnight culture was only moderate may have reflected suboptimal up-regulation of cell-surface FasL. In support of this notion, subjecting TCR-ligated T cells to cross-linked anti-Fas mAb in secondary culture, thereby bypassing the requirement for Fas/FasL interaction, considerably augmented apoptosis.

Confirming the findings of others (30), Fas ligation of fresh CD4\(^+\) cells failed to cause apoptosis, even though Fas expression on naive T cells was only slightly lower than on stimulated cells. This finding is in line with the view that the sensitivity of T cells...
to Fas-mediated death is controlled by Fas-associated inhibitory molecules, such as FLIP (38, 39). These inhibitory molecules presumably have to dissociate from Fas in order for T cells to become Fas-sensitive. If so, it is of interest that, in contrast to TCR ligation alone, combined TCR/Fas ligation prevented apoptosis. Indeed, operationally, TCR/Fas coligation had the same protective effect as TCR/CD28 coligation. However, unlike TCR/CD28 ligation, TCR/Fas ligation did not enhance blast transformation, implying that Fas ligation did not act simply by providing a surrogate form of costimulation (40). In future experiments, it will be of interest to determine whether, unlike TCR ligation alone, combined TCR/ Fas ligation prevents dissociation of inhibitors, such as FLIP.

The capacity of costimulation to prevent AICD is controversial. Thus, some workers find that costimulation is highly effective at inhibiting AICD (13–15, 41), whereas other do not (11). For the rapid onset TCR-mediated apoptosis of naive T cells reported here, costimulation clearly played a crucial role in preventing Fas-mediated death. In fact, providing costimulation via CD28 during TCR ligation reduced apoptosis to below the level for cells cultured alone, thus leading to negative values for Δ apoptosis. Moreover, unlike T cells exposed to TCR ligation alone, T cells subjected to combined TCR/CD28 ligation were resistant to apoptosis when subsequently exposed to cross-linked anti-Fas mAb (Fig. 6). This finding did not reflect differing levels of Fas on the cells because TCR and TCR/CD28 ligation both caused an equivalent mild increase in Fas expression. It is of interest that one of the cytokines tested, IL-6, closely resembled CD28 ligation in providing protection against apoptosis. This observation is in line with evidence that IL-6 can provide costimulation for naive T cells (42–44).

How costimulation via CD28 or IL-6 prevents Fas-mediated death after TCR ligation is unclear. The simplest idea is that costimulation promotes synthesis of various antiapoptotic molecules, such as Bcl-XL (14–16) and IGF-1 (45), or, alternatively, inactivation or decreased production of proapoptotic molecules, such as c-myc (46). With the relatively high concentration of anti-TCR mAb used, we could find no evidence that CD28 costimulation caused a significant increase in Bcl-XL, (or Bcl-2) expression. Costimulation could also antagonize TCR-mediated apoptosis by inducing NF-kB activation (47). Whatever the explanation, it is of interest that the protective effect of CD28 ligation was calcineurin (CSA)-dependent but MAPKK MEK1 (PD-) and MAPK p38 (SB-) independent. The precise significance of this finding will have to await further investigation.

Although typical AICD is preceded by a prior proliferative response, the rapid onset of TCR-mediated apoptosis described here did not involve cell division. Thus, cell cycle analysis indicated that, after overnight culture, the cells died either in G0 or early G1; very few of the surviving cells had entered S phase. Despite this finding, the cells showed clear signs of activation, e.g., up-regulation of CD69 and CD44, in addition to FasL. Indeed, the Fas dependency of TCR-mediated apoptosis indicated that cell death had to be preceded by cell activation, i.e., activation leading to up-regulation of FasL. Bearing in mind that resting T cells were resistant to Fas-mediated death (by cross-linked anti-Fas mAb), TCR-mediated death appeared to hinge on at least two signaling events: 1) induction of FasL up-regulation, and 2) acquisition of sensitivity to death via Fas ligation. Since TCR ligation elicits multiple signaling events, the signal transduction pathways controlling FasL up-regulation and Fas-mediated death may be essentially different. In support of this idea, CSA and PD both blocked FasL up-regulation following TCR ligation, but failed to inhibit the onset of susceptibility to death induced by Fas ligation. This finding is surprising because the targets of these two drugs, calcineurin and MEK1, are components of separate signaling pathways. This is exemplified by the different effects of these drugs on the expression of cell-surface molecules. Thus, although both drugs inhibited FasL up-regulation after TCR ligation, PD had no effect on the expression of CD25, CD69, and CD44, whereas CSA inhibited CD25 and CD69 expression but enhanced CD44 expression. These data imply that the signaling pathways controlling TCR-induced up-regulation of FasL, CD25/CD69, and CD44 are distinct. It is of interest that, unlike CSA, the inhibitory function of PD was restricted to FasL up-regulation. Since FasL up-regulation is under the control of c-myc function is being explored (48, 49). Unlike PD, SB showed no capacity to prevent FasL up-regulation.

In the case of CSA, it is of interest that, with combined TCR/ CD28 ligation, CSA blocked the protective (antiapoptotic) effect of costimulation, but did not prevent FasL up-regulation, thus promoting the rapid onset of Fas-mediated apoptosis (Fig. 8A).

This finding raises the intriguing possibility that the rapid (<36 h) Vβ-specific deletion of CD4+ cells seen in vivo after injecting mice with superantigens plus CSA (50, 51) could be Fas mediated.

The physiological significance of TCR-mediated apoptosis requires comment. Under in vivo conditions, the rapid onset of TCR-mediated apoptosis soon after T cell contact with a pathogenic microorganism would potentially be disastrous. Hence, evolutionary pressures have presumably shaped the immune system to avoid this problem: mature T cells have to be susceptible to deletion (to destroy unwanted effector cells), but only after the pathogen concerned has been eliminated. As shown here, at least in vitro, normal naive T cells have an innate susceptibility to rapid TCR-mediated apoptosis, but only when deprived of contact with APC. Hence, under normal in vivo conditions, the prevalence of APC in the lymphoid tissues may be vital for protecting T cells from TCR-mediated death.

A priori, the protective function of APC could be mediated via T cell interaction with a variety of cell-surface molecules on APC. However, testing mAbs specific for three different costimulatory/ accessory molecules on T cells, namely CD28, LFA-1, and CD43, revealed that only CD28 ligation induced protection. Although other molecules on T cells were not tested, it is notable that CD28+/– T cells were strongly resistant to the protective function of APC. Hence, the ability of APC to protect T cells against TCR-mediated apoptosis may be largely or solely a reflection of CD28/B7 interaction. If so, it would follow that CD28+/– mice should be prone to TCR-mediated T cell deletion soon after Ag injection. Although direct evidence on this question is sparse, it is of interest that injecting CD28+/– mice with soluble Ag not only failed to induce T cell expansion but appeared to cause significant T cell deletion by day 3 postinjection (52). Hence, the possibility emerges that, in addition to providing classic costimulation, CD28/B7 interaction in vivo may play a crucial role in keeping T cells alive, i.e., by preventing TCR-mediated apoptosis. This might explain why many different cell types are B7+.

Acknowledgments

We thank Barbara Marchand for typing the manuscript.

References


