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# Modulation of Fas-Dependent Apoptosis: A Dynamic Process Controlling Both the Persistence and Death of CD4 Regulatory T Cells and Effector T Cells[1] FREE

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# Modulation of Fas-Dependent Apoptosis: A Dynamic Process Controlling Both the Persistence and Death of CD4 Regulatory T Cells and Effector T Cells[1]

**Alice Banz, Christiane Pontoux, and Martine Papiernik[2]**

**We have previously shown that regulatory $CD25^+CD4^+$ T cells are resistant to clonal deletion induced by viral superantigen in vivo. In this work we report that isolated $CD25^+CD4^+$ T cells activated in vitro by anti-CD3 Ab are resistant to Fas-induced apoptosis, in contrast to their $CD25^-CD4^+$ counterparts. Resistance of $CD25^+CD4^+$ T cells to Fas-dependent activation-induced cell death is not linked to their inability to produce IL-2 or to their ability to produce IL-10. The sensitivity of both populations to Fas-induced apoptosis can be modulated in vitro by changing the $CD25^+CD4^+$:$CD25^-CD4^+$ T cell ratio. The sensitivity of $CD25^-CD4^+$ T cells to apoptosis can be reduced, while the sensitivity of $CD25^+CD4^+$ T cells can be enhanced. Modulation of Fas-dependent apoptosis is associated with changes in cytokine production. However, while $CD25^-CD4^+$ T cell apoptosis is highly dependent on IL-2 (production of which is inhibited by $CD25^+CD4^+$ T cells in coculture), modulation of $CD25^+CD4^+$ T cell apoptosis is IL-2 independent. Taken together, these results suggest that $CD25^+CD4^+$ and $CD25^-CD4^+$ T cell sensitivity to Fas-dependent apoptosis is dynamically modulated during immune responses; this modulation appears to help maintain a permanent population of regulatory T cells required to control effector T cells.** ***The Journal of Immunology*****, 2002, 169: 750–757.**

Abnormal control of immune responses can lead to autoimmune and inflammatory diseases. Three mechanisms control autoreactive and activated T cells, namely clonal deletion, clonal anergy, and active suppression by regulatory $CD4^+$ T cell subsets (1–5). $CD4^+$ T cells, which constitutively express the IL-2R $\alpha$-chain (IL-2R$\alpha$ or CD25), participate in this regulation. Indeed, elimination or inactivation of these regulatory T cells is associated with the onset of autoimmune and inflammatory diseases and with abnormal peripheral T cell homeostasis (3–7).

Regulatory $CD25^+CD4^+$ T cells differ functionally from $CD25^-CD4^+$ T cells, although the latter can acquire CD25 upon activation (8, 9). In particular, $CD25^+CD4^+$ T cells differ from their $CD25^-$ counterparts by their cytokine profile. $CD25^+CD4^+$ T cells produce IL-10, an anti-inflammatory cytokine (10), but do not produce IL-2, making them dependent on exogenous IL-2 for expansion in vivo and in vitro (8, 9, 11–13). Conversely, $CD25^-CD4^+$ T cells produce IL-2 but not IL-10. Regulatory $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells also differ by their susceptibility to clonal deletion. We have previously shown that $CD25^+CD4^+$ T cells are resistant to clonal deletion induced by viral superantigen (vSAG)[3] in vivo (11). Although both $CD25^+CD4^+$ and $CD25^-CD4^+$ superantigen (SAG)-specific T cells were activated by vSAG, clonal deletion occurred at the expense of the $CD25^-CD4^+$ subset while the $CD25^+CD4^+$ T cell subset was spared.

$CD25^+CD4^+$ T cell resistance to clonal deletion may be important in maintaining a permanent population of regulatory T cells that can control and eventually suppress autoreactive T cells or harmful T cells activated during normal immune responses. Several experimental studies have shown that this regulatory population is needed to avoid autoimmune and inflammatory diseases. Mice rendered deficient in $CD25^+CD4^+$ T cells by neonatal thymectomy and nude mice reconstituted with $CD25^+$-depleted T cells develop autoimmune diseases. Transfer of $CD25^+CD4^+$ T cells from normal mice can avoid the onset of autoimmune disease in these models (14, 15). $CD4^+$ T cells expressing the $CD45RB^{low}$ phenotype (which overlap with $CD25^+CD4^+$ T cells) control the inflammatory diseases that occur in mice transferred with $CD45RB^{high}CD4^+$ T cells (16). IL-2R$\alpha$ knockout (KO) and IL-2 KO mice, which cannot support $CD25^+CD4^+$ T cell expansion (12), develop inflammatory bowel disease and lymphadenopathy, suggesting that regulatory $CD25^+CD4^+$ T cells may also participate in the control of peripheral T cell homeostasis (17, 18). It was recently shown that regulatory $CD45RB^{low}CD4^+$ T cells can control the expansion of $CD45RB^{high}CD4^+$ T cells in vivo (19). The mechanisms by which $CD25^+CD4^+$ T cells control immune responses are unclear. They may differ according to the model and the functions analyzed, and also according to the $CD25^+CD4^+$ T cell subset engaged. Various cytokines are also involved, of which IL-10 and TGF-$\beta$ seem to be the most efficient (2, 20–23). However, cytokines may participate to different degrees in different models and may also modulate different functions. IL-10 KO mice raised in a conventional environment develop inflammatory bowel disease but not lymphadenopathy, suggesting that IL-10 is involved not in T cell homeostasis but in the control of inflammatory reactions. The role of IL-10 in the suppression of T cell proliferation in vitro has not been demonstrated, but cell-cell interactions are known to be required (8, 13), and these may involve local cytokine production.

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[3] Abbreviations used in this paper: vSAG, viral superantigen; SAG, superantigen; MMTV, mouse mammary tumor virus; AICD, activation-induced cell death; KO, knockout.



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If $CD4^+$ regulatory T cells are mandatory to avoid autoimmune and inflammatory diseases, and are also necessary to control T cell homeostasis, their resistance to apoptosis may underlie their persistence during ontogenesis and immune responses. By using polyclonal activation of purified regulatory $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells, we found that the $CD25^+$ subset is more resistant than the $CD25^-$ subset to Fas-induced apoptosis. This is in keeping with our previous observation that $CD25^+CD4^+$ vSAG-specific T cells are resistant to clonal deletion in vivo. We then investigated whether $CD25^+CD4^+$ T cells could be sensitized to Fas-mediated apoptosis by contact with IL-2-producing $CD25^-CD4^+$ T cells in vitro. We found that sensitivity to apoptosis could be modulated in both subsets. This was associated with modulation of cytokine production. However, whereas $CD25^-CD4^+$ T cell apoptosis is highly dependent on the IL-2, the response of $CD25^+CD4^+$ T cells to Fas-induced apoptosis in vitro is IL-2 independent. The results suggest that regulation of apoptosis is a dynamic process that depends on the balance between $CD25^+CD4^+$ and $CD25^-CD4^+$ T cell numbers and is required for regulatory and effector T cell homeostasis.

## Materials and Methods

### *Mice*

C57BL/6 (H-$2^b$, Thy 1.2) mice were from CERJ (Le Genest, St. Isles, France), IL-2 KO (H-$2^b$, Thy 1.2) (24) and IL-10 KO (25) mice were from Transgenic Alliance (L'arbresle, France), and C57BL/Ba ($H2^b$, Thy 1.1) mice were from our own facilities. All mice were 6–10 wk old when used.

### *mAbs and flow cytometry*

The following Abs were used for flow cytometry and cell culture. Purified anti-CD8 (clone 53-6.7), purified anti-CD3 (clone 145-2C11), biotinylated anti-CD25 (clone PC61), biotinylated anti-CD4 (clone RM4-5), and FITC anti-TCR (clone H57-597) were prepared and coupled in our laboratory. Rabbit anti-hamster IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). PerCP-anti-CD4 (clone RM4-5), PE-anti-CD69 (clone H1.2F3), PE-anti-Fas (clone $JO_2$), PE-anti-Thy1.2 (clone 53-2-1), purified anti-Fas, and purified hamster IgG were from BD PharMingen (San Diego, CA). Flow cytometry was performed using a FACSCalibur device (BD Biosciences, Mountain View, CA) and data were analyzed with CellQuest software (BD Biosciences).

### *T cell purification*

Lymphocyte suspensions were prepared from peripheral (inguinal, axillary, cervical, and popliteal) lymph nodes. $CD4^+$ T cells were prepared by negative selection. Briefly, lymph node cells were incubated with purified anti-CD8 and then with goat anti-rat Ig-coated magnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway). B cells were depleted with goat anti-mouse Ig-coated Dynabeads. $CD8^+$ T cells and B cells coated with beads were removed with a magnet. $CD25^+CD4^+$ T cells were positively selected. Briefly, the enriched $CD4^+$ T cells were coated with biotinylated anti-CD25 and mixed with MACS Streptavidin Microbeads (Miltenyi Biotec, Paris, France). Cells coated with microbeads were selected by the magnetic field of a MACS separator (Miltenyi Biotec). Unlabeled ($CD25^-$) cells were negatively selected and $CD25^+$ cells were then eluted from the column after removing the magnetic field. The $CD25^+CD4^+$ and $CD25^-CD4^+$ populations were 90 and 95% pure, respectively.

### *In vitro activation*

Purified $CD25^+CD4^+$ and $CD25^-CD4^+$ lymph node T cells ($2 \times 10^5$ cells per well) were cultured for 70 h at 37°C in 5% $CO_2$-air in anti-CD3-coated 96-well culture plates, with or without 50 U/ml mouse rIL-2 (BD Biosciences). Plates were coated overnight with anti-CD3 (10 $\mu$g/ml; 50 $\mu$l per well). Plate-bound anti-CD3 was used for activation in the absence of APC, as APC was shown not to be mandatory for regulatory T cell function (26). All cells were grown in 200 $\mu$l of RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Grand Island, NY), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 1% sodium pyruvate (Biomedia, Foster City, CA), 5 mM HEPES (Life Technologies), and $10^{-5}$ M 2-ME. At the end of the culture period, the absolute number of living cells per well was measured with the trypan blue exclusion test. In some experiments, indicated numbers of $CD25^+CD4^+$ T cells from C57BL/6 (B6) or C57BL/Ba (Ba) mice were cultured alone or with $2 \times 10^5$ purified $CD25^-CD4^+$ T cells from Ba or IL-2 KO mice. The two subsets were distinguished by their different Thy-1 markers. Pooled supernatants from several wells in each group were stored at −20°C for cytokine assay.

### *Apoptosis assay*

T cells ($1 \times 10^6$) activated in vitro were incubated for 30 min at 37°C in 5% $CO_2$-air with 1 $\mu$g/ml hamster anti-Fas Ab or hamster IgG as control. Cells were then washed and cultured for 1, 2, 4, or 20 h in 96-well plates ($2 \times 10^5$ cells per well) with 1 $\mu$g/ml goat anti-hamster IgG. At each time point, anti-Fas-induced apoptosis was measured with the annexin V test (Boehringer Ingelheim, Gagny, France). Cells were counted, washed in PBS/10% FCS, and stained with biotinylated anti-CD4, PE-anti-CD25, and streptavidin-allophycocyanin. Cells were then washed twice and stained with FITC-labeled annexin V according to the manufacturer's instructions. The rate of apoptosis was determined by flow cytometry. Dead cells were excluded after labeling with 25 $\mu$g/ml propidium iodide, and annexin-positive cells were detected among $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells. In mixed cultures of $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells, the two subsets were distinguished by labeling with PE-conjugated anti-Thy1.2. In some experiments, hypodiploid nuclei (sub-$G_1$ peak) were detected by propidium iodide staining (27). Briefly, $2 \times 10^5$ cells were fixed in 70% cold ethanol for 30 min, washed, and incubated with 10 $\mu$g/ml propidium iodide and subjected to flow cytometry analysis.

### *Cytokine assays*

IL-2, IFN-$\gamma$, TNF-$\alpha$, and IL-10 were assayed in culture supernatants with ELISA kits (R&D Systems, Abingdon, U.K.) according to the manufacturer's recommendations. Results are expressed as picograms per milliliter of supernatant.

### *Statistical analysis*

Data are expressed as means ± SEM. Comparisons were made using Student's $t$ test and differences were considered significant if $p < 0.05$.

## Results

### *Regulatory $CD25^+CD4^+$ T cells are resistant to Fas-dependent apoptosis and this resistance is not overcome by exogenous IL-2*

Purified $CD25^+CD4^+$ and $CD25^-CD4^+$ lymph node T cells were activated with anti-CD3 for 70 h in vitro, then washed and counted before incubation ($2 \times 10^5$ living cells per well) with hamster anti-Fas or an isotype control Ab for 30 min. The cells were incubated for 1–20 h with anti-hamster IgG to determine the time of maximum apoptosis. The percentage of apoptotic cells was measured by two different techniques: the FITC-labeled annexin V method, which detects cells at an early stage of the apoptotic process, and the intracellular propidium iodide staining, which measures hypodiploid nuclei (sub-$G_1$ peak). As shown in Fig. 1*A*, the highest percentage of annexin V-positive apoptotic $CD25^-CD4^+$ T cells was observed 1 h after Fas cross-linking (29% of $CD25^-CD4^+$ cells were annexin V positive and PI negative). When the Fas cross-linking period was increased from 1 to 20 h, the percentage of apoptotic $CD25^-CD4^+$ T cells fell gradually, possibly owing to gradual deletion of Fas-sensitive cells. In contrast, only 9% of $CD25^+CD4^+$ T cells were apoptotic 1 h after Fas cross-linking. No apoptotic cells were detected in this population when Fas cross-linking was extended up to 20 h. Results were confirmed using a second apoptosis test (Fig. 1*B*). The percentage of apoptotic $CD25^-CD4^+$ T cells with hypodiploid nuclei was clearly higher (37%) than the percentage of apoptotic $CD25^+CD4^+$ T cells (7%) 1 h after Fas cross-linking. The percentage of $CD25^-CD4^+$ T cells with hypodiploid nuclei remained high up to 6 h after Fas cross-linking (data not shown). In subsequent experiments, Fas-dependent apoptosis was tested 1 h after Fas cross-linking with the annexin V method.

Regulatory $CD25^+CD4^+$ T cells proliferate in response to anti-CD3 in vitro, but only if IL-2 is added (8, 9, 12, 13). Exogenous IL-2 is also crucial for programmed cell death, enhancing sensitivity to Fas-dependent activation-induced cell death (AICD) (17,

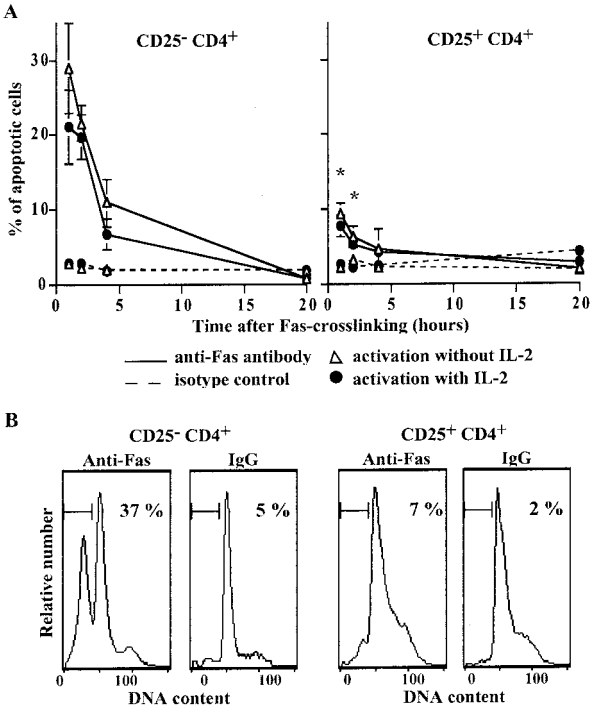


**FIGURE 1.** Activated $CD25^+CD4^+$ T cells are resistant to Fas-induced apoptosis. Purified $CD25^+CD4^+$ or $CD25^-CD4^+$ lymph node T cells were stimulated for 70 h with anti-CD3 in the absence (△) or presence (●) of IL-2. Following activation, cells were washed, collected, and incubated ($10^6$ viable cells) for 30 min at 37°C with hamster anti-Fas (solid lines) or an isotype control Ab (dotted line), and then cultured ($2 \times 10^5$ per well) for 1, 2, 4, or 20 h with anti-hamster IgG. The percentage of apoptotic cells was measured by two different techniques: the FITC-labeled annexin V method (*A*) and the intracellular propidium iodide staining (*B*). *A*, At various times after Fas cross-linking, the cells were collected, washed, and stained with FITC-labeled annexin V and propidium iodide. The percentage of annexin V-positive, PI-negative apoptotic cells among $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells is shown (means ± SEM of four independent experiments). *, $p < 0.01$, $CD25^+CD4^+$ vs $CD25^-CD4^+$ T cells cultured for 1 or 2 h with anti-Fas. *B*, After 1 h of Fas cross-linking, cells were fixed and DNA was stained with propidium iodide and analyzed by flow cytometry. The percentage of hypodiploid nuclei is indicated on each plot. Shown are representative data from one of two independent experiments.

28–32). Furthermore, apoptosis is linked to cell proliferation (33). However, the response to Fas cross-linking was unaffected when purified $CD25^+CD4^+$ and $CD25^-CD4^+$ lymph node T cells were first activated for 70 h with exogenous IL-2 (Fig. 1*A*).

Because the kinetics of Fas-dependent apoptosis could differ in the two subsets with the duration of the activation period, we studied susceptibility to apoptosis earlier during activation by anti-CD3, in the presence and absence of IL-2 (Fig. 2). When the activation period was increased from 24 to 70 h, the percentage of apoptosis increased among $CD25^-CD4^+$ cells 1 h after Fas cross-linking. In contrast, $CD25^+CD4^+$ T cells remained resistant to apoptosis. Similar results were obtained when IL-2 was added during the activation period.

Thus, most $CD25^+CD4^+$ T cells were resistant to Fas-dependent apoptosis, and exogenous IL-2, added during the activation period, did not overcome this resistance.

### *Both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells acquire an activated phenotype and express Fas after anti-CD3 stimulation*

Resistance to Fas-dependent apoptosis could be linked to a lack of activation or Fas expression following activation. Purified

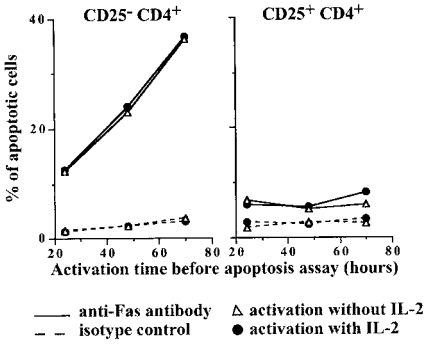


**FIGURE 2.** $CD25^+CD4^+$ T cells remain resistant to apoptosis whatever the duration of activation. Purified $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells were activated for 24, 48, or 70 h in the absence (△) or presence (●) of IL-2. Activated cells were collected and incubated with anti-Fas hamster Ab (solid lines) or an isotype control (dotted line) and were then cultured for 1 h with anti-hamster IgG. The percentage of annexin V-positive, PI-negative apoptotic cells among $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells 1 h after Fas cross-linking is represented. Shown are representative data from one of two independent experiments.

$CD25^+CD4^+$ and $CD25^-CD4^+$ lymph node T cells were incubated with anti-CD3 for 70 h in vitro, with and without IL-2, before measuring cell size and CD69 and Fas expression. Before activation, the cell size of the two populations was identical. In contrast, more $CD25^+CD4^+$ T cells than $CD25^-CD4^+$ T cells expressed the CD69 activation marker (26 vs 12%, respectively; Fig. 3*A*). $CD25^+CD4^+$ T cells also expressed a slightly higher level of Fas protein on their surface. Cell size and CD69 expression increased in both subsets after 70 h of incubation with anti-CD3, although slightly less in $CD25^+$ than in $CD25^-$ cells (Fig. 3*B*). When IL-2 was added during the activation period, $CD25^+CD4^+$ T cells proliferated (12) and cell recovery increased (Fig. 3*C*) to the level observed with $CD25^-CD4^+$ T cells, which do not require exogenous IL-2 to proliferate. CD69 expression was identical when $CD25^-CD4^+$ T cells were activated with or without IL-2. CD69 expression was lower on $CD25^+CD4^+$ T cells activated with IL-2 than on $CD25^+CD4^+$ T cells activated without IL-2. However, the peak of CD69 expression on the $CD25^+$ subset was maximum 48 h after activation with IL-2 (data not shown) and was already down-regulated after 70 h. Fas expression increased on both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells after anti-CD3 stimulation, in both the absence and presence of IL-2, although to a lesser degree on the $CD25^+$ subset (Fig. 3*B*).

Thus, despite the different sensitivity of $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells to Fas-dependent apoptosis, both populations acquired an activated phenotype and expressed Fas on their surface after anti-CD3 stimulation.

### *IL-10 production by isolated $CD25^+CD4^+$ cells is not involved in their resistance to Fas-induced apoptosis in vitro*

$CD25^+CD4^+$ T cells constitutively express IL-10 mRNA (8, 11). It has been reported that IL-10 can prevent T cell apoptosis in vitro (34) and also protect T cells against AICD in vivo (35). Therefore, we investigated the possible involvement of IL-10 production by $CD25^+CD4^+$ cells in their resistance to Fas-induced apoptosis in vitro. Purified $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells from B6 mice and IL-10-deficient mice were activated by anti-CD3 for 70 h before testing Fas-dependent apoptosis after 1 h of Fas cross-linking. As shown in Fig. 4, $CD25^+CD4^+$ T cells from both B6 and IL-10 KO mice were resistant to apoptosis, contrary to $CD25^-CD4^+$ T cells. Thus, IL-10 production by $CD25^+CD4^+$ T

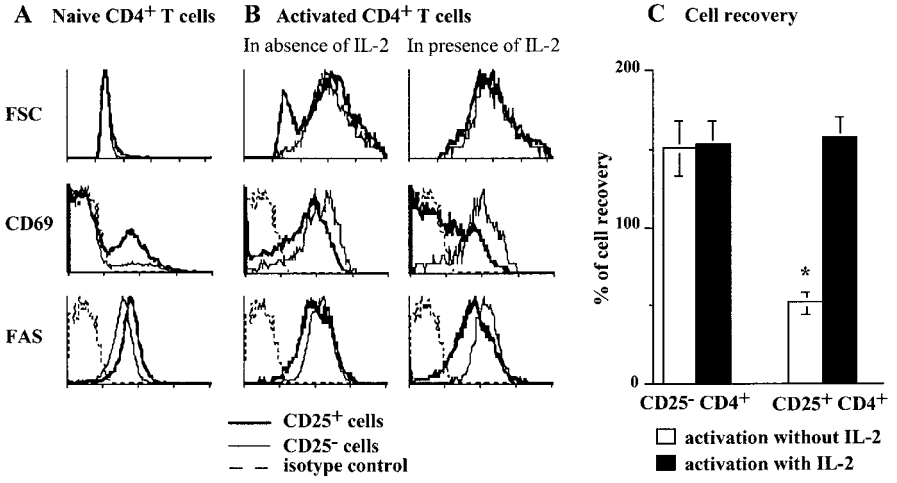


**FIGURE 3.** Both $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells acquire an activated phenotype and express Fas after CD3 cross-linking. Purified $CD25^+CD4^+$ and $CD25^-CD4^+$ lymph node T cells ($2 \times 10^5$ cells per well) were cultured for 70 h with anti-CD3 in the presence or absence of IL-2 and were stained with biotinylated anti-CD25, PerCP-anti-CD4, FITC-anti-TCR, PE-anti-CD69, or PE-anti-Fas, followed by allophycocyanin-streptavidin. The presence of blasts and the level of CD69 and Fas expression by $CD25^+CD4^+$ and $CD25^-CD4^+$ naive (*A*) and activated (*B*) T cells are shown as histograms. The bold line represents $CD25^+$ cells, the thin line represents $CD25^-$ cells, and the dotted line represents control staining. Shown are representative data from one of six independent experiments. *C*, Cells were cultured for 70 h with anti-CD3 in the absence (open bars) or presence (filled bars) of 50 U/ml IL-2. Viable T cells were counted by the trypan blue exclusion test. Results are expressed as the percentage of viable cells recovered after 3 days of culture relative to the initial number (means ± SEM of six independent experiments). *, $p < 0.001$ ($CD25^+CD4^+$ cells vs $CD25^-CD4^+$ T cells).

cells was not responsible for their resistance to Fas-dependent apoptosis in vitro.

### *In vitro interaction of $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells modulates the sensitivity of both subsets to Fas-dependent apoptosis*

Cell-cell contacts are necessary for the suppressor activity of regulatory $CD25^+CD4^+$ T cells in vitro (8, 13). Thus, we investigated whether the sensitivity of the two populations to Fas-dependent apoptosis was modified by mixing $CD25^+$ and $CD25^-$ cells in vitro. $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were cultured alone or together at ratios of 1:10 (the physiological steady-state ratio in vivo) and 1:1. Cells were incubated with anti-CD3 for 70 h in vitro. Following activation, cells were collected, washed, counted, and submitted for 1 h to Fas cross-linking. Cells initially selected as $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells in $Thy1.2^+$ and $Thy1.2^-$ mice were detected in coculture by their respective expression of Thy1 (Fig. 5*A*). The percentage of apoptotic $CD25^-CD4^+$ cells was high (20%), whether the latter were cultured alone or together with $CD25^+CD4^+$ T cells at a ratio of 10 $CD25^-$ cells to 1 $CD25^+$ cell (Fig. 5*B*). In contrast, at a ratio of 1:1, most $CD25^-CD4^+$ T cells became resistant to Fas-induced apoptosis (7% of apoptotic cells). Thus, $CD25^+CD4^+$ T cells modulated the sensitivity of $CD25^-CD4^+$ T cells to apoptosis. At a 1:1 ratio, the percentage of apoptotic $CD25^+CD4^+$ T cells was not markedly different from that observed when $CD25^+CD4^+$ T cells were cultured alone (9 and 6%, respectively). However, at a ratio of one $CD25^+$ cell to 10 $CD25^-$ cells, the proportion of apoptotic $CD25^+CD4^+$ T cells increased to 23%. These results suggested that induction of Fas-dependent apoptosis could be modulated by interactions between the $CD25^+CD4^+$ and $CD25^-CD4^+$ T cell subsets, depending on the ratio between the two populations. To examine whether interactions between $CD25^-$ and $CD25^+$ cells during the activation phase were required to modulate Fas-induced apoptosis, each cell population was activated separately and then combined for 1 h during the apoptosis assay. Modulation of Fas-dependent apoptosis was no longer observed (Fig. 5*C*). At a 1:1 ratio, $CD25^-CD4^+$ T cells remained sensitive to apoptosis, whereas, at a 1:10 ratio, $CD25^+CD4^+$ T cells remained resistant, as when cultured and tested separately. These results showed that the two cell subsets had to be combined during the activation phase to modulate their sensitivity to Fas-dependent apoptosis.

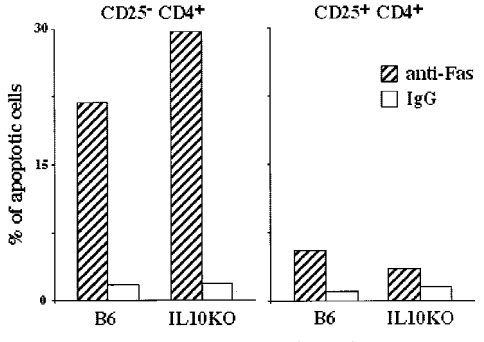


**FIGURE 4.** IL-10 does not render $CD25^+CD4^+$ T cells resistant to Fas-induced apoptosis. A total of $2 \times 10^5$ purified $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells from IL-10 KO and B6 mice were activated for 70 h with anti-CD3. Activated cells were collected and incubated with hamster anti-Fas (hatched bars) or an isotype control (open bars) and then cultured for 1 h with anti-hamster IgG. The percentage of apoptotic cells among $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells is represented 1 h after Fas cross-linking. Shown are representative data from one of three independent experiments.

The ratio of $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells during the activation phase might influence cytokine production and thus affect Fas-dependent apoptosis in vitro. Production of IL-2, which sensitizes CD4 T cells to Fas-dependent apoptosis (17, 28–32), is inhibited in a cell dose-dependent manner by interactions between $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells (8, 13). Thus, we measured cytokine production during the activation phase (Fig. 6). Production of TNF-$\alpha$ in vitro was not significantly modified in the different culture conditions, while IFN-$\gamma$ and IL-2 were decreased by cocultures in a cell dose-dependent manner, as previously shown (8, 36), compared with $CD25^-CD4^+$ T cells cultured

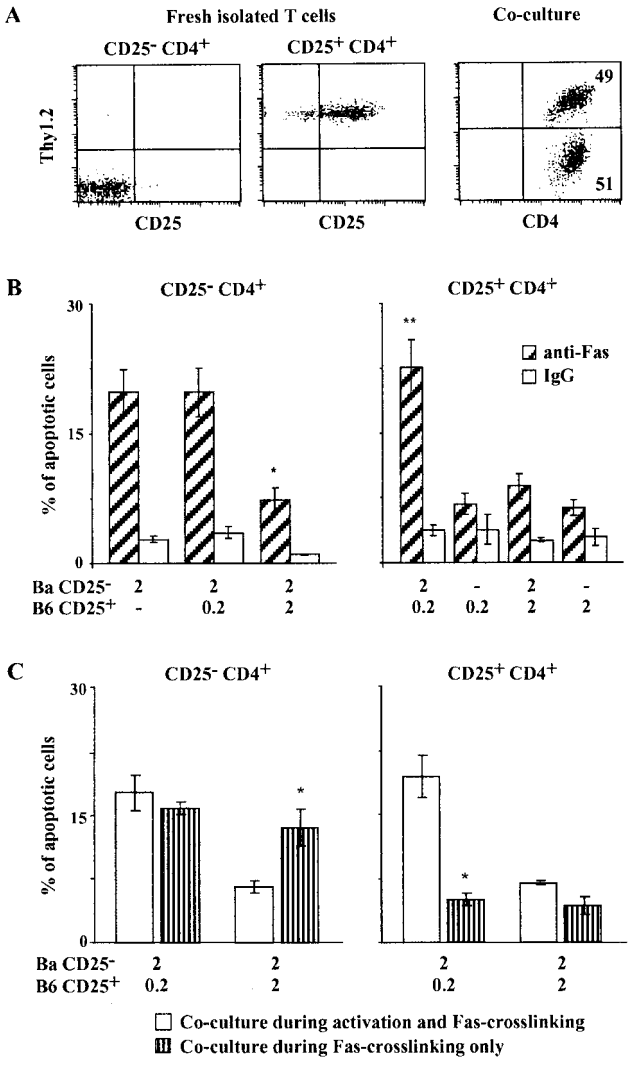


**FIGURE 5.** The sensitivity of $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells to Fas-induced apoptosis is modulated by mixing cells in vitro during the activation phase. CD4 T cells from Thy1.2$^-$ and Thy1.2$^+$ mice were selected on the basis of CD25 expression and then activated alone or in coculture. *A*, Fresh purified $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells before culture and their recognition by anti-Thy1.2 Ab after activation in coculture (1:1 ratio) are shown as dot plots. *B*, Indicated numbers ($\times 10^5$) of $CD25^+CD4^+$ T cells from B6 mice (Thy1.2$^+$) were cultured alone or with $2 \times 10^5$ $CD25^-CD4^+$ T cells from Ba mice (Thy1.2$^-$) and were stimulated with anti-CD3. Activated T cells were collected and incubated with anti-Fas (hatched bars) or an isotype control (open bars) and then cultured with anti-hamster IgG. The percentage of apoptotic cells among Thy1.2$^-$CD4$^+$ or Thy1.2$^+$CD4$^+$ cells is represented 1 h after Fas cross-linking (means ± SEM of seven independent experiments). *, $p < 0.02$ relative to $CD25^-CD4^+$ T cells cultured alone. **, $p < 0.01$ relative to $CD25^+CD4^+$ T cells cultured alone. *C*, Each cell population was stimulated together (open bars) or separately (striped bars) and then mixed and tested for Fas sensitivity. The percentage of apoptotic cells among Thy1.2$^-$CD4$^+$ and Thy1.2$^+$CD4$^+$ cells is represented 1 h after Fas cross-linking (means ± SEM of four independent experiments). *, $p < 0.008$ cells activated separately vs cells activated together.

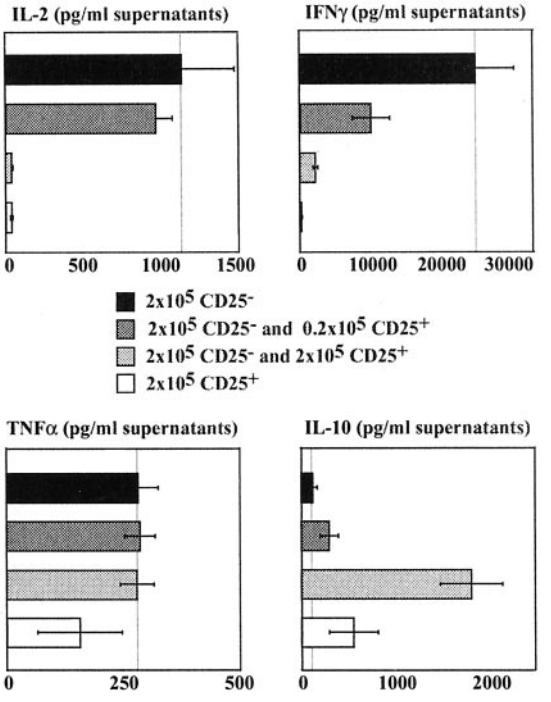


**FIGURE 6.** Modulation of apoptosis sensitivity is associated with modulation of cytokine production. IL-2, IFN-γ, TNF-α, and IL-10 were measured in supernatants of $CD25^+CD4^+$ T cells cultured alone or with $CD25^-CD4^+$ T cells, recovered 70 h after anti-CD3 stimulation. Results are expressed as cytokine levels (picograms per milliliter of supernatant). Means ± SEM of four individual experiments are shown.

alone. IL-10 production in the supernatants was increased by increasing the $CD25^+$:$CD25^-$ T cell ratio. However, we could not demonstrate the mandatory role of IL-10 in this system by using $CD25^+CD4^+$ T cells from IL-10 KO (data not shown).

To test the role of IL-2 in the increased sensitivity of $CD25^+CD4^+$ T cells to apoptosis, $CD25^-CD4^+$ T cells from IL-2 KO mice were cultured alone or with $CD25^+CD4^+$ T cells from normal Ba mice at the 10:1 ratio, then activated and submitted to Fas cross-linking. $CD25^-CD4^+$ cells from IL-2 KO mice were poorly sensitive to Fas-dependent apoptosis when cultured alone, as previously shown (28, 32). We then analyzed Fas-induced apoptosis in both subsets after mixed culture. As shown in Fig. 7*A*, 24% of $CD25^+CD4^+$ T cells in mixed culture were apoptotic, suggesting that $CD25^-CD4^+$ cells could induce Fas-dependent apoptosis in $CD25^+CD4^+$ T cells through an IL-2-independent mechanism. Interestingly, sensitivity of the $CD25^-CD4^+$ subset from IL-2 KO mice to Fas-dependent apoptosis was also enhanced by this interaction, increasing from 5 to 15%.

Cytokine production in these culture conditions was measured after activation (Fig. 7*B*). $CD25^-CD4^+$ T cells from IL-2 KO mice produced no measurable IL-2, significant amounts of TNF-α, and little IL-10 and IFN-γ. $CD25^+CD4^+$ T cells ($0.2 \times 10^5$) produced very low levels of each cytokine. The level of TNF-α was increased when $CD25^-CD4^+$ T cells from IL-2 KO mice were activated with normal $CD25^+CD4^+$ T cells at the 10:1 ratio ($p < 0.02$, activated together vs activated alone).

In conclusion, although modulation of apoptosis is associated with modulation of cytokine production, we cannot demonstrate the role of IL-10 or IL-2 in the modulation of the apoptosis sensitivity of $CD25^+CD4^+$ T cells.

## Discussion

Infectious mouse mammary tumor viruses (MMTV) encode a vSAG that activates vSAG-reactive T cells and then induces their clonal deletion. We have previously shown (37, 38) that the absolute number of vSAG-specific $V\beta6^+CD25^+CD4^+$ T cells remains constant during infection by MMTV (SW), while the number of $V\beta6^+CD25^-CD4^+$ T cells gradually falls. We concluded

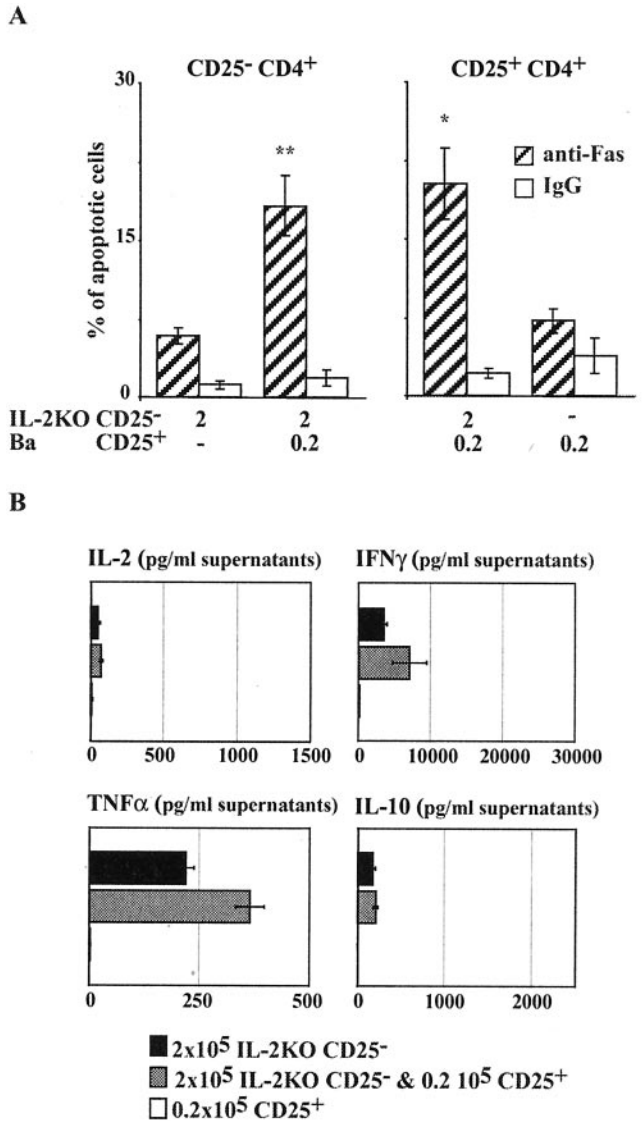


**FIGURE 7.** The enhanced susceptibility of $CD25^+CD4^+$ T cells to Fas-induced apoptosis is independent of IL-2 produced by $CD25^-CD4^+$ T cells. Indicated numbers ($\times 10^5$) of $CD25^+CD4^+$ T cells from Ba mice were cultured alone or with $2 \times 10^5$ $CD25^-CD4^+$ T cells from IL-2 KO mice. Cells were stimulated for 70 h with anti-CD3. *A*, Cells were collected and incubated with anti-Fas (hatched bars) or an isotype control (open bars) and then cultured with anti-hamster IgG. The percentage of apoptotic cells among $Thy1.2^-CD4^+$ or $Thy1.2^+CD4^+$ cells is represented 1 h after Fas cross-linking (means ± SEM of four individual experiments). *, $p < 0.01$ relative to $CD25^+CD4^+$ T cells cultured alone. **, $p < 0.01$ relative to $CD25^-CD4^+$ T cells from IL-2 KO mice cultured alone. Addition of normal $CD25^+CD4^+$ T cells to IL-2 KO $CD25^-CD4^+$ T cells restored Fas-dependent apoptosis of the latter. Response of $CD25^+CD4^+$ T cells to Fas-induced apoptosis was identical whether $CD25^-CD4^+$ T cells were from normal or IL-2 KO mice (compare to Fig. 5*B*). *B*, IL-2, IFN-$\gamma$, TNF-$\alpha$, and IL-10 were measured in supernatants recovered 70 h later. Results are expressed as cytokine levels (picograms per milliliter of supernatant). Means ± SEM of four individual experiments are shown.

that $CD25^+CD4^+$ T cells were resistant to clonal deletion induced by vSAG activation in vivo (11). Other reports show the differential susceptibility of T cell subsets to AICD. The Th1 and Th2 T cell subsets are functionally distinct and are defined on the basis of their cytokine profiles. In vitro, Th1 effectors, contrary to Th2 effectors, undergo rapid Fas/Fas ligand-mediated AICD upon reactivation with Ag (39, 40). Resistance of Th2 clones to AICD is not due to a lack of Fas expression. The susceptibility of memory T cells to AICD also differs from that of naive T cells (41, 42). Primed T cells are more resistant than naive T cells to Fas-mediated AICD in vitro and in vivo. In the present study, we found that isolated $CD25^+CD4^+$ T cells were more resistant than their $CD25^-CD4^+$ counterparts to apoptosis through polyclonal activation by anti-CD3 and triggering of the Fas pathway. This resistance of isolated $CD25^+CD4^+$ T cells was not due to an inability to be activated or to express Fas. Indeed, anti-CD3 cross-linking in the absence of IL-2 up-regulated CD69 and Fas expression and increased the cell size of both populations. Fas expression was similar in the two populations.

$CD25^+CD4^+$ T cells do not produce IL-2, even after activation (8, 11, 13), and do not proliferate in response to anti-CD3 in the absence of IL-2 (8, 9, 12). Furthermore, IL-2 is involved in cell death by enhancing sensitivity to Fas-dependent AICD (29). T cells activated in vivo are not susceptible to Fas-dependent apoptosis when IL-2/IL-2R signaling is disrupted by a lack of IL-2 or its receptor, although Fas is expressed on their surface (17, 28, 30–32). Additionally, there is a relationship between proliferation and apoptosis: no apoptosis is detected among nonproliferating T cells following Ag stimulation (33). However, while addition of IL-2 to cultured $CD25^+CD4^+$ T cells induces their proliferation (8, 9, 12) and enhances their numbers in vitro (this study), it did not modify resistance to Fas-dependent apoptosis. Isolated activated $CD25^+CD4^+$ T cells were thus resistant to Fas-induced apoptosis independently of IL-2.

IL-2 also promotes T cell survival (43). $CD25^+CD4^+$ T cells show poor proliferation (8, 9, 12, 13) and poor survival when incubated in the absence of IL-2. Like human $CD4^+$ T cells (44), these regulatory T cells are prone to nonspecific death in vitro, despite being resistant to specific Fas-mediated apoptosis. This susceptibility to cytokine privation in vitro may be explained by down-regulation of Bcl-2 or other members of the Bcl protein family (45, 46).

Resistance of $CD25^+CD4^+$ T cells to clonal deletion induced by vSAG in vivo (11) and to Fas-induced apoptosis after activation in vitro might also be dependent on IL-10, which is constitutively produced by these cells (8, 12). IL-10 protects T cells from apoptosis both in vitro, as shown for human T cells infected by EBV (34) and, in vivo, IL-10 KO mice injected with the bacterial SAG *Staphylococcus aureus* enterotoxin B display enhanced SAG-specific T cell clonal deletion in the thymus (35). However, we detected no difference between IL-10 KO and normal mice as regards Fas-induced apoptosis of isolated peripheral $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells, suggesting that IL-10 is not involved in resistance, at least in vitro. It is possible that peripheral T cells compensate for their IL-10 deficiency by using another cytokine for Fas-induced apoptosis in vitro. TGF-$\beta$ expressed by $CD25^+CD4^+$ T cells may be involved in protection from apoptosis, as both $CD4^+$ and $CD8^+$ T cell apoptosis is increased in mice lacking TGF-$\beta$ (47, 48).

The suppressive function of regulatory $CD25^+CD4^+$ T cells is mediated in vitro by cell-cell contacts that lead to down-regulation of IL-2 production by the $CD25^-CD4^+$ subset (8, 13). Induction of Fas-mediated apoptosis might thus be regulated by such contacts. Interestingly, both the sensitivity of $CD25^-CD4^+$ T cells and the resistance of $CD25^+CD4^+$ T cells to Fas-dependent apoptosis were modified when the two populations were cocultured. The presence of both cell subsets during the activation phase is mandatory for this phenomenon, as mixing preactivated cells during the apoptotic assay alone is ineffective. Modulation depended on the ratio of the two cell subsets. At a ratio of 1:1, $CD25^-CD4^+$

T cells became resistant to apoptosis. $CD25^+CD4^+$ T cells regulated the activation and proliferation of $CD25^-$ cells during the activation phase (data not shown) and thereby reduced the sensitivity of the latter to AICD. This is in keeping with previous reports showing that $CD25^+CD4^+$ T cells suppress IL-2 production and thus the proliferation of polyclonally activated $CD25^-CD4^+$ T cells in vitro (8, 13). To elucidate the mechanism involved in the modulation of apoptosis, we tested the role of IL-10 and/or IL-2 in this modulation. We found that coculture of $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells at a 1:1 ratio totally inhibited both IL-2 and IFN-$\gamma$ production and enhanced IL-10 production. However, we found that IL-10 privation did not modify the sensitivity of isolated $CD25^-CD4^+$ or $CD25^+CD4^+$ T cells to Fas-mediated apoptosis. An attempt to demonstrate the mandatory role of IL-10 in apoptosis regulation by using $CD25^+CD4^+$ T cells from IL-10 KO mice was inconclusive (data not shown). IL-10 is not the only suppressive factor used by $CD25^+CD4^+$ T cells for regulation (6). Alternative mechanisms may be used in the absence of IL-10 to counteract the effect of proapoptotic factors. Engagement of membrane-linked cytokines may be one of these mechanisms. Indeed, it has been suggested that membrane-bound TGF-$\beta$, expressed by $CD25^+CD4^+$ T cells, is involved in their regulatory function (47). TGF-$\beta$ is also involved in apoptosis regulation (48), which may explain why cell to cell interaction is needed for both regulation and modulation of apoptosis (8, 13, 47).

Interestingly, these results are in keeping with our previous in vivo data showing that vSAG-induced clonal deletion spares not only the vSAG-specific $CD25^+CD4^+$ T cell subset but also a residual $CD25^-CD4^+$ T cell subset (11). The ratio of the two populations at the end of the clonal deletion process is 1:1. Resistance to apoptosis and its modulation may protect not only the regulatory suppressive population but also a small specific effector population, which may have a role in subsequent immune responses.

The question of why, when the ratio of the two subsets in coculture was 10 $CD25^-CD4^+$ T cells to 1 $CD25^+CD4^+$ T cell, the former remained sensitive and the latter became sensitive to apoptosis is intriguing. This inducible sensitivity of the regulatory T cell subset to apoptosis during immune responses may also be relevant to the general mechanisms controlling autoreactive T cells. Apoptosis of part of the regulatory population at some point of the immune response may be required to limit their control on the $CD25^-CD4^+$ T cell subset. Indeed, control of $CD25^-CD4^+$ clonal deletion by the $CD25^+CD4^+$ subpopulation has previously been shown in vivo (49). In mice infected at birth by MMTV (SW), depletion of $CD25^+CD4^+$ T cells by anti-CD25 Ab from birth onwards leads to more rapid and extensive death of vSAG-specific $CD25^-CD4^+$ T cells. This suggests that $CD25^+CD4^+$ T cells may hinder $CD25^-CD4^+$ T cell death in vivo. Deletion of autoreactive T cells might be able to proceed more rapidly when the number of $CD25^+CD4^+$ regulatory T cells is limited.

These results suggest that the sensitivity of both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells to Fas-dependent apoptosis can be modified during the course of an immune response, with the probable aim of maintaining an equilibrium between regulatory and effector T cells.

The mechanism by which $CD25^+CD4^+$ regulatory T cells become sensitive to apoptosis is not clear. $CD25^-CD4^+$ T cell IL-2 production did not explain the change in $CD25^+CD4^+$ T cell sensitivity to Fas-mediated apoptosis in coculture at a ratio of one $CD25^+$ cell to 10 $CD25^-$ cells, as $CD25^-CD4^+$ T cells from IL-2 KO mice had the same effect. Interestingly, $CD25^+CD4^+$ T cells correct the Fas-dependent cell death defect described in IL-2 KO mice (28, 32). The $CD25^+CD4^+$ T cell subset, which requires IL-2 to expand, is lacking in IL-2 KO mice (12). Thus, the abnormal AICD in these mice may be linked not only to the absence of IL-2 but also to the absence of IL-2-dependent $CD25^+CD4^+$ T cells. Interestingly, Wolf et al. (50) have shown that both the $CD25^+CD4^+$ and the $CD25^-CD4^+$ subsets control T cell activation and accumulation in IL-2-deficient mice when transferred in vivo. They suggested that the $CD25^+$ subset might act on the initial activation step, whereas the $CD25^-$ subset might act on the clonal contraction step mediated by AICD. Our results confirm that the two subsets interact to control AICD and reveal the existence of an additional, IL-2-independent mechanism.

Thus, $CD25^+CD4^+$ T cell resistance to Fas-dependent apoptosis induced by vSAG in vivo (11, 12) and to polyclonal activation in vitro can be modulated by environmental factors. Experimental interaction of regulatory $CD25^+CD4^+$ T cells and effector $CD25^-CD4^+$ T cells in vitro showed that sensitivity to Fas-dependent apoptosis depends on the ratio of these two populations during activation. During induction of tolerance to vSAG in vivo, the number of vSAG-specific $CD25^+CD4^+$ T cells remains identical to that in naive mice (12). This suggests that the modulation of sensitivity to apoptosis in vivo favors the persistence of the regulatory $CD25^+CD4^+$ T cell subset. Given the important role of $CD25^+CD4^+$ T cells in the control of immune responses, their resistance to apoptosis in vivo is of major interest.

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