

Rescue of Thymocytes from Glucocorticoid-induced Cell Death Mediated by CD28/CTLA-4 Costimulatory Interactions with B7-1/B7-2

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Summary

During the differentiation of thymocytes to mature T cells the processes of positive and negative selection result in signals that either protect thymocytes from cell death, or delete, through apoptosis, thymocytes with self-reactive T cell receptors (TCR). Glucocorticoids have been shown to induce thymocyte apoptosis and are produced within the thymic microenvironment. Furthermore, steroid-induced apoptosis of thymocytes has been suggested as a potential mechanism for removal of nonselected thymocytes. In this report, we demonstrate that thymocytes can be rescued from glucocorticoid-induced apoptosis by incubation with cells that express high levels of B7-1 or B7-2. In addition, the ability to be rescued by B7-1 and/or B7-2 can precede expression of the TCR. We demonstrate that CD3⁺-depleted or CD3⁺/TCR-β⁺-doubly depleted thymocytes can be rescued from glucocorticoid-induced apoptosis through the interaction of CD28 or CTLA-4 on thymocytes with cells bearing high levels of B7-1 or B7-2. Furthermore, these transfected cells are major histocompatibility complex (MHC) class II negative and, while they may express MHC class I, there is no preferential rescue of CD8⁺ thymocytes in the presence of glucocorticoids. Together, these data suggest that the rescue of thymocytes from glucocorticoids can be independent of the TCR. We also demonstrate that, in addition to CD28, CTLA-4 is expressed on thymocytes, suggesting that rescue from glucocorticoid-induced cell death can be mediated by both CD28 and CTLA-4. A CTLA-4Ig fusion protein which binds to both B7-1 and B7-2 was shown to completely block the rescue of thymocytes from glucocorticoid-induced cell death. Therefore, we conclude that interactions between B7-1/B7-2 and CD28/CTLA-4 are sufficient and necessary for rescue of thymocytes from glucocorticoid-induced cell death.

TCR-β in association with pre-TCR-α is expressed at low levels on the CD4⁻CD8⁻ double negative (DN)¹ thymocyte population, and expression of this pre-TCR increases during the progression to the CD4⁺CD8⁺ double-positive (DP) stage (1). TCR-α genes rearrange during the DP stage of development, so that the thymocyte expresses a mature TCR-α/β together with the CD3 signaling complex (2). CD4⁺8⁺3⁺TCR-α/β⁺ thymocytes continue differentiating to the mature single positive (SP) CD4⁺8⁻3⁺ and CD4⁻8⁺3⁺ populations, which express TCR-α/β molecules capable of interacting with antigens associated

with MHC molecules. It is unclear how thymocytes progress through these maturational stages; however, it is well documented that approximately 95% of the thymocytes die during selection. As a potential mediator of the observed cell death, it has been reported that the thymic microenvironment produces glucocorticoids (3), and glucocorticoids induce thymocyte apoptosis (4, 5). Therefore, it is a reasonable assumption that glucocorticoids in the thymus are involved, at least in part, in the removal of thymocytes that are not selected to survive. Moreover, it has been demonstrated that rescue signals from glucocorticoid induced-cell death can be generated through the TCR complex (6). This would insure that T cells that express a functional TCR complex may be able to survive exposure to glucocorticoids.

TCR recognition of antigens associated with MHC

¹Abbreviations used in this paper: DN, double negative; DP, double positive; FSC^{hi} and ^{lo}, greater and lower forward scatter; ORF, open reading frame; SP, single positive; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase.

molecules preserves the antigen specificity of an immune response. However, for T cells to become activated, the interaction of CD28 or CTLA-4 on T cells with B7-1 or B7-2 expressed on APC is required (7). TCR engagement in the absence of costimulation results in anergy and/or death of T cells. CD28 is expressed on thymocytes (8), and participates in thymocyte development. In one study, substantial levels of apoptosis were detected when thymocytes were incubated with antibodies to both CD3 and CD28 (9). In another study, coligation of the anti-TCR antibodies in combination with anti-CD28 antibodies rescued thymocytes from apoptotic death (10). The differences between these studies likely reflect the effects of different antibodies, e.g., anti-CD3, anti-TCR, and anti-CD28, or different maturational stages of thymocytes used in each study. Nonetheless, both studies confirm that costimulatory molecules are involved in thymic development.

In this report, we confirm the role of costimulatory molecules in the thymus by demonstrating that the competence of thymocytes to survive glucocorticoid-induced apoptosis is mediated through CD28/CTLA-4 interactions with B7-1/B7-2. This process does not require, and may precede, acquisition of functional TCR.

Materials and Methods

Transfected Cells. The Ig λ 2-based vector VLPEA2.13 was constructed by inserting the V_L-V λ 2 intron just downstream of the 4.0-kb murine V λ 2 promoter derived from V λ CATXS (11) and flanked by a PmeI cloning site and a genomic segment, including at the C λ 2 polyadenylation, 3'-untranslated sequences, and 1-kb flanking sequences. The 1.65-kb E λ 2-4 enhancer fragment derived from V λ CATXS was inserted downstream of the transcriptional unit. The promoter-cDNA-enhancer insert can be released for transfection by digestion with Sall.

Amplification of sequences encoding the open reading frame (ORF) of murine B7-1 was accomplished using PCR with oligonucleotides 5'-AGTACTATGGCTTGCAATTGTCAGT, and 5'-GTTTAAACTAAAGGAAGACGGTCTGTT, and plasmid pmB7-1 (provided by M.C. Crooks, University of California, San Diego, CA), as template DNA. The PCR fragment was ligated into pSG5 (12) to make pSG5-B7-1.14. The vector for expression of murine B7-1 in transfected cells was made by release of the B7-1 ORF from pSG5-B7-1.14 using ScaI and PmeI and ligation into the PmeI site of VLPEA2.13. Sequences encoding the ORF of murine B7-2 were amplified from CH12 lymphoma cDNA using PCR and oligonucleotides 5'-TGTAGACGTG-TTCCAGAACTTACGG and 5'-ACTGCCTTCACTCTG-CATTTGG and subcloned into Bluescript KS⁺ to make B7-2-2.5. The open reading frame was excised with AccI, blunted with Klenow fragment, and ligated into the PmeI site of VLPEA2.13.

J558L plasmacytoma cells were transfected with 2 μ g SV2-gpt and 10 μ g of either λ B7-1 or λ B7-2. Cells were electroporated using a GenePulser (Bio-Rad Laboratories, Richmond, CA) set at 280 V/960 μ fd and grown in selective medium. Viable clones were stained for expression of B7-1 and B7-2 using mAbs (PharMingen, San Diego, CA) and analyzed by FACScan[®] (Becton Dickinson and Co., Mountain View, CA).

Rescue from Glucocorticoid-mediated Cell Death. Thymocytes were harvested from either C57BL/6, AKR, or CBA/J strains of mice.

For each experiment, 2.5×10^6 cells were treated with dexamethasone at 10^{-6} M, or treated with dexamethasone in the presence of 8×10^5 fixed (1% paraformaldehyde) B7-1- or B7-2-transfected J558L plasmacytoma cells, or left untreated. After overnight incubation, the thymocytes were stained with a biotinylated anti-CD4, GK1.5, and a PE-conjugated anti-CD8 (PharMingen) followed by cychrome-conjugated streptavidin (PharMingen). Similar results were observed with thymocytes from each of the mouse strains.

Terminal Deoxynucleotidyl Transferase (TUNEL) Assay for Apoptosis Using Flow Cytometric Analysis. After cell surface staining, thymocytes were fixed in 1.5% paraformaldehyde and labeled with a fluorescein-conjugated dUTP using TUNEL (Gibco BRL, Grand Island, NY), which labels fragmented DNA. Cells were analyzed by flow cytometry on a FACScalibur[®] (Becton Dickinson and Co., San Jose, CA). Typically, two peaks were seen in the green fluorescence channel (FL-1), with the more intense green fluorescent peak representing apoptotic cells. Greater than 96% of the transfected cells were gated out of the analysis based on forward versus side scatter. B7-1 and B7-2 transfectants expressed low levels of CD4 and did not express CD8. For analysis of apoptosis in thymocytes, regions were set on CD4⁺CD8⁺ (DP), CD4⁺ or CD8⁺ (SP), and CD4⁻CD8⁻ (DN) populations. FL-1 histograms of fluorescence intensity, as a measure of apoptosis, were generated from each gated region. The DP population was gated, then additional gates for forward versus side light scatter were applied to assay for live versus apoptotic cells, as reported previously (13). Additional experiments were performed to address the generation of an intermediate peak in FL-1. Treated thymocytes were stained with propidium iodide, which indicated that the cells are not cycling (data not shown). Morphological data using Höchst dye and propidium iodide suggest that the cells are apoptotic (data not shown).

Depletion of CD3⁻ and TCR- β ⁺ Thymocytes. Thymocytes were incubated with an anti-CD3 mAb, 145-2C11, alone or in combination with a TCR- β -specific antibody, H57-597. Each antibody was used at 25 μ g/ml for 20 min on ice. Cells were then washed and incubated with a 1:50 dilution of baby rabbit complement (Accurate Chemical Co., Westbury, NY) at 37°C for 45 min. After depletion, cells were counted using trypan blue exclusion to determine viable cells, followed by staining for CD3 and TCR- β to verify depletion. Cells were then treated as described above.

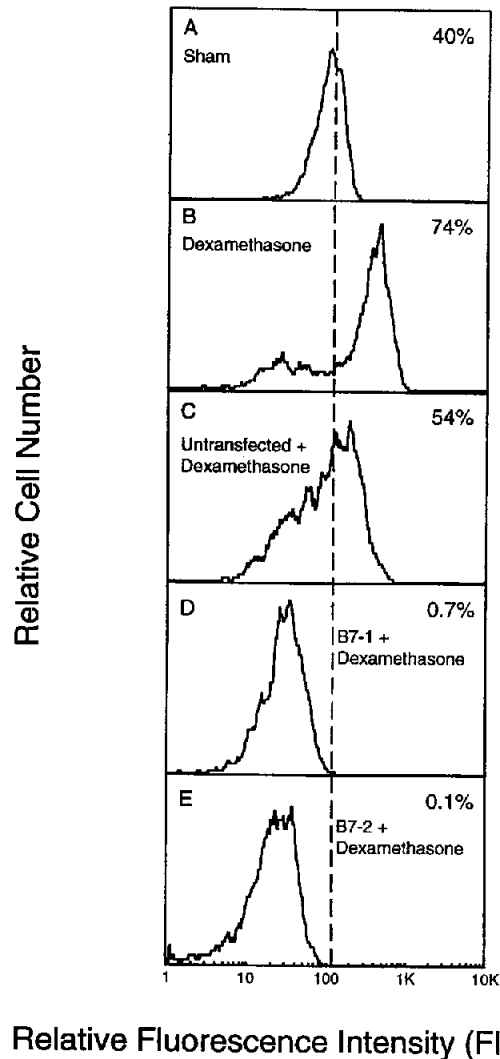
Blockade of Rescue from Glucocorticoid-mediated Cell Death. B7-1 and B7-2 transfectants were incubated with saturating levels of CTLA-4Ig fusion protein on ice for 20 min. Transfectants were then washed and immediately fixed in 1.0% paraformaldehyde as before. Both CTLA-4Ig treated and untreated transfectants were incubated with thymocytes in the presence of 10^{-6} M dexamethasone as described above. Alternatively, thymocytes were pretreated with anti-CD28 (PharMingen), or anti-CTLA-4 (provided by J. Allison, University of California, Berkeley, CA) at 25–50 μ g/ml, then incubated with transfectants and dexamethasone.

Immunofluorescent Staining of Thymocytes for CD28 and CTLA-4 Expression. Single cell suspensions of thymocytes were stained with a biotin-conjugated anti-CD28 (PharMingen) followed by fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), or a fluorescein-conjugated anti-CTLA-4 (provided by J. Allison), and washed three times. Thymocytes were then stained with biotin-conjugated anti-CD4 (GK1.5), and PE-conjugated anti-CD8, followed by cychrome-conjugated streptavidin. Analysis was performed using a FACScalibur[®] flow cytometer. The isotype control was a hamster IgG Ab.

Results

Transfected Cells Expressing High Levels of Either B7-1 or B7-2 Rescue Immediately Ex Vivo Thymocytes from Glucocorticoid-mediated Cell Death. As previously described, apoptotic death can be measured by the TUNEL assay, i.e., labeling the ends of fragmented DNA with FITC-dUTP and nonlabeled nucleotides using terminal deoxynucleotidyl transferase (TdT) (14). We have adapted this approach to quantitate cells undergoing apoptosis using flow cytometry. For these experiments, immediately ex vivo thymocytes from various mouse strains were isolated, treated with dexamethasone in the presence or absence of paraformaldehyde-fixed B7-1 or B7-2 transfected cells, and incubated for 18 h. The transfected cells, which are larger and more granular than thymocytes, were gated out of the analysis based on forward versus side light scatter. Because paraformaldehyde-fixed transfected cells were used, only cell contact-mediated signals were provided to thymocytes. We experimentally defined "dead" cells to be those cells which demonstrated increased fluorescence from TdT-mediated uptake of FITC-dUTP after treatment with glucocorticoids. Furthermore, within each experiment the dead population was defined relative to "live" cells, which characteristically had a lower fluorescence profile also seen in live, untreated thymocytes. The use of internal controls to establish live versus dead populations is essential because of interexperiment variability in labeling with TdT/FITC-dUTP. In addition, the less fluorescent cells were classified as live by trypan blue exclusion, as well as by exclusion of propidium iodide, measured cytometrically. Cells referred to as DP were determined by electronically gating on the thymocytes that expressed both CD4 and CD8. All other thymocytes were gated out of the analysis. As shown (Fig. 1 A), after overnight culture without treatment (*Sham*), gated CD4⁺CD8⁺ (DP) thymocytes underwent spontaneous apoptosis (ranging from 23 to 40%), whereas immediately ex vivo thymocytes demonstrated <1% apoptotic cells (data not shown). We demonstrate here (Fig. 1 B) that dexamethasone induced substantial increases in apoptosis of DP thymocytes, as has been reported (4, 15). However, when either B7-1 or B7-2 transfected cells were included, dexamethasone-induced apoptosis was completely inhibited (Fig. 1, D and E, respectively). Interaction of the thymocytes with the nontransfected parental cells (Fig. 1 C) did not result in the rescue provided by cells transfected with either B7-1 or B7-2. These data support the conclusion that costimulatory interactions are involved in rescue from cell death. The modest inhibition of apoptosis seen with incubation with the parental cells most likely results from the low level of endogenous expression of B7-1 and B7-2 by these cells.

Protection from Glucocorticoid-induced Cell Death Does Not Require Expression of TCR. Given the reports that TCR can mediate rescue of thymocytes from glucocorticoids (8), we examined the question of whether costimulatory signals alone are sufficient to rescue thymocytes from glucocorticoid-induced cell death in the absence of TCR mediated



Relative Fluorescence Intensity (FITC-dUTP)

Figure 1. B7-1- and B7-2-transfected cells rescue thymocytes from glucocorticoid-induced cell death. Isolated thymocytes from C57BL/6 mice were treated with dexamethasone with and without B7-1- or B7-2-transfected cells for 18 h. After treatment, cells were stained for CD4 and CD8. Gates were set for CD4⁺8⁺ DP, DN, and CD4⁺ or CD8⁺ SP regions. TUNEL assays, TdT to label fragmented DNA of apoptotic cells with FITC-dUTP, were used to quantitate cell death. Analysis was restricted to the gated DP region. (A) Untreated thymocytes. (B) Dexamethasone, 10⁻⁶ M, treated thymocytes. (C) Thymocytes treated with 10⁻⁶ M dexamethasone and paraformaldehyde-fixed, untransfected parental J5581 cells (concentration of nontransfected cells:thymocytes; 1:5). (D) Thymocytes, 2 × 10⁶, treated with 10⁻⁶ M dexamethasone and incubated overnight with B7-1 transfectants, 4 × 10⁵ (concentration of transfectants:thymocytes; 1:5). (E) Thymocytes treated with 10⁻⁶ M dexamethasone and B7-2-transfected cells at a 1:5 (transfectant:thymocyte) concentration. Percent apoptotic cells (upper right corners of histograms), were determined by setting gates based on dexamethasone-induced increase in FL-1. Gates for the apoptotic population were set as represented by the dashed line. (Data shown are representative of at least three experiments.)

signals. We removed the majority of CD3⁺ or CD3⁺/TCR-β⁺ thymocyte populations and examined the ability of the B7 transfectants to inhibit dexamethasone-induced apoptosis. Experimentally, we used antibodies that recognize TCR or CD3, together with complement, to remove

Table 1. Depletion of CD3⁺ and TCR⁺ Thymocytes

Treatment	Percent reduction in total thymocytes*
Untreated	—
Complement	0%
anti-CD3	8%
anti-TCR	0%
anti-CD3 + complement	50%
anti-TCR + anti-CD3 + complement	87%

*Observations are representative of several experiments.

Thymocytes were isolated from mouse strains including C57BL/6. Cells at a concentration of 2×10^6 /ml were treated with anti-CD3 (145-2C11) or anti-TCR (H57-597) at 20 μ g/ml on ice for 30 min, followed by baby rabbit complement at a 1:50 dilution. Cells were incubated at 37° C for 1 h then washed three times with PBS. Cells were then stained with trypan blue and counted to determine the percent reduction in total thymocytes.

>50% of thymocytes (Table 1). Staining of cells after depletion revealed a marked loss of both CD3⁺ (Fig. 2 A) and TCR⁺ (Fig. 2 B) cells. As further support that TCR engagement is not necessary for rescue, the transfected J558L cells that express B7-1 or B7-2 do not express MHC class II (D.H. Wagner, unpublished result).

The B7 transfectants inhibited thymocyte apoptosis, as evidenced by loss of the apoptotic peak in TUNEL assays, but a population with intermediate fluorescence intensity was detected when B7-2 transfectants were used to inhibit dexamethasone induced death. (Fig. 3, D and H). To determine the nature of the cells in the intermediate peak, we set gates on forward versus side scatter of the previously gated DP thymocytes. It has recently been reported that cells with greater forward scatter (FSC^{hi}) are enriched for the live population, while those with lower forward scatter (FSC^{lo}) are apoptotic (13). This result is demonstrated in Fig. 3, A and E. The gated FSC^{hi} populations (solid lines, Fig. 3) of both CD3⁺-depleted and CD3⁺/TCR- β ⁻-depleted DP thymocytes were susceptible to dexamethasone-mediated apoptosis, but each contained a dexamethasone resistant subpopulation (Fig. 3, B and F). B7-1 transfectants completely inhibited dexamethasone-mediated apoptosis in both CD3⁺- and CD3⁺/TCR- β ⁺-depleted cells (Fig. 3, C and G, respectively) and furthermore, rescued the spontaneously apoptotic thymocytes (FSC^{lo} represented by dashed lines, Fig. 3, C and G), in the CD3⁺-depleted and the CD3⁺/TCR- β ⁺-depleted groups. At a ratio of 1:3 (transfectants:thymocytes), B7-2 transfectants rescued 15.5% of the CD3⁺-depleted cells and 20.6% of the CD3⁺/TCR- β ⁺-depleted cells from dexamethasone-mediated cell death, but still generated an intermediate peak. While the approach of scatter-based electronic gating did not allow us to determine the nature of the intermediate peak unequivocally, possible explanations for this peak include the labeling of breaks in DNA during cell cycle (not a reflection of

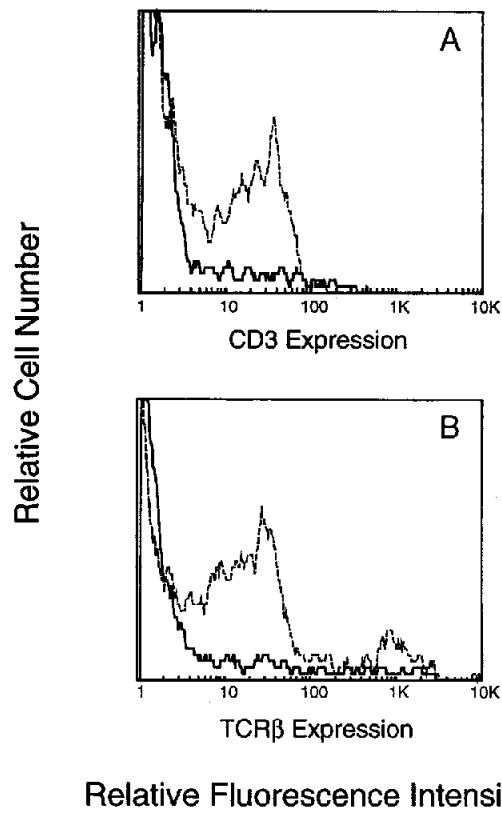


Figure 2. Depletion of CD3⁺ and TCR⁺ thymocytes. Whole thymocytes were treated with biotinylated 145-2C11 (anti-CD3 ϵ) and/or biotinylated H57-597 followed by baby rabbit complement as described in Materials and Methods. Immediately after depletion, cells were stained with biotinylated 145-2C11 and biotinylated H57-597 followed by FITC-streptavidin. Flow cytometric analysis was performed on a FACScalibur[®]. Histograms represent (A) CD3⁺ depletion, and (B) TCR- β ⁺/CD3⁺ depletion. Dashed lines are (A) CD3 or (B) TCR expression on untreated thymocytes and solid lines represent expression after depletion.

apoptotic death), or partial degradation during early stages of apoptosis.

Protection from Glucocorticoid-mediated Cell Death Can Be Blocked with CTLA-4Ig. To demonstrate that the rescue of thymocytes from glucocorticoids is mediated by B7-1 and/or B7-2, we performed the assay in the presence of a CTLA-4Ig fusion protein (16). CTLA-4Ig binds to both B7-1 and B7-2, thereby blocking interaction between the B7-1 and/or B7-2 and CD28/CTLA-4 molecules. As demonstrated in earlier experiments, B7-2 transfectants rescued gated, DP thymocytes from dexamethasone-induced cell death (Fig. 4 C). CTLA-4Ig binding to B7-1/B7-2 on transfected cells completely blocked inhibition of apoptosis (Fig. 4 D). Pretreatment of thymocytes with control antibodies, which included TCR- β -specific (H57-597), MHC class I-specific antibody (34-5-8), and MHC class II-specific antibody (MK-D6), did not block rescue (data not shown). Taken together, these data show that the signals for rescue of DP thymocytes from dexamethasone-induced apoptosis are provided by B7-1 or B7-2.

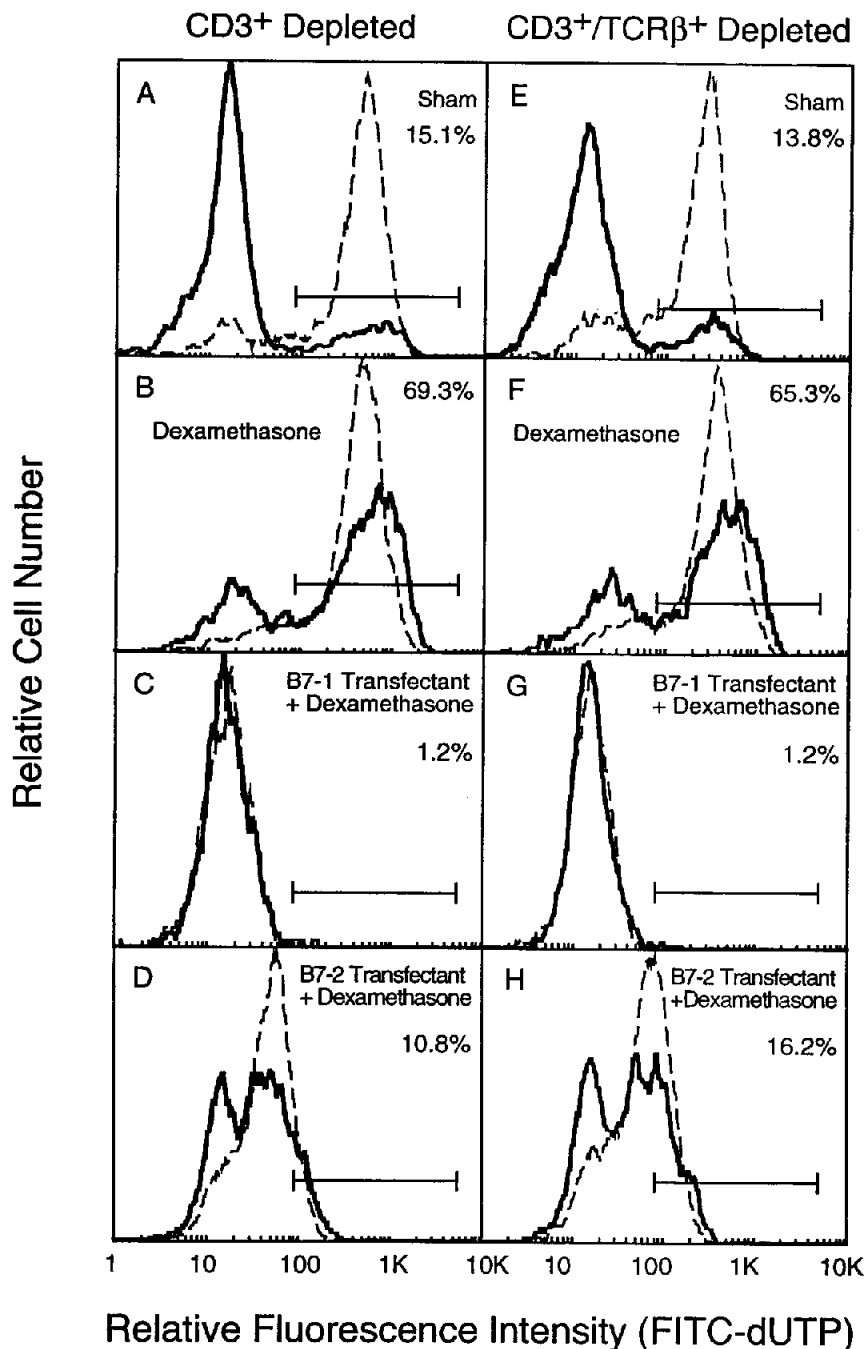


Figure 3. Rescue of CD3⁺- and TCR-β⁺-depleted thymocytes from glucocorticoid-induced cell death. Total thymocytes from C57/BL6 were treated as follows: (A–D) CD3⁺-depleted, (E–H) CD3⁺/TCR-β⁺-depleted. (A and E) Untreated thymocytes. (B and F) Thymocytes treated with 10⁻⁶ M dexamethasone. (C and G) Thymocytes treated with dexamethasone and B7-1 transfected cells [1:3 transfectant:thymocyte]. (D and H) Thymocytes treated with dexamethasone and B7-2-transfected cells [1:3]. After overnight incubation, cells were stained for CD4/CD8 expression and apoptosis levels were determined using the TUNEL assay read flow cytometrically. During analysis, gates were set for DP, DN, and CD4/CD8 SP regions. Additional gates for FSC^{hi} (most live population) and FSC^{lo} (most apoptotic population) were applied to the DP population. Analyses of apoptosis (increase in FL1) were performed on the FSC^{hi} (solid lines) and FSC^{lo} (dashed lines) gated regions of the DP population. Percent apoptosis (upper right corner) were determined by gating for apoptotic cells from the FSC^{hi} population only. (Data are representative of at least three experiments.)

The ability of CTLA-4Ig to completely block rescue demonstrates that the interaction between B7-1 or B7-2 with CD28/CTLA-4 on thymocytes is sufficient to prevent glucocorticoid-induced cell death. The question arises as to whether B7 molecules mediate rescue through interactions with CD28, CTLA4, or both. Pretreatment of thymocytes from the C57BL/6 strain with anti-CD28 completely blocks the rescue from glucocorticoid-mediated cell death provided by the transfectants. In contrast, pretreatment of thymocytes from BALB/c with anti-CD28 blocks 14% of the rescue signal, while pretreatment with anti-CTLA-4 completely blocks rescue (data not shown). These

data suggest that C57BL/6 mice predominantly use CD28 for thymocyte rescue from glucocorticoids, while BALB/c may use predominantly CTLA-4.

While CD28 is constitutively expressed on peripheral T cells and thymocytes (8) and CTLA-4 is induced on activated T cells (17) the expression of CTLA-4 on thymocytes had not been demonstrated. The expression by thymocytes of both CD28 and CTLA-4 is expected if engagement of these molecules can mediate rescue from glucocorticoids. Therefore, thymocytes were triply stained for CD4, CD8, and CTLA-4 or CD28. Gates were set for DP, CD4, CD8 SP, and DN thymocyte populations (Fig 5, A,

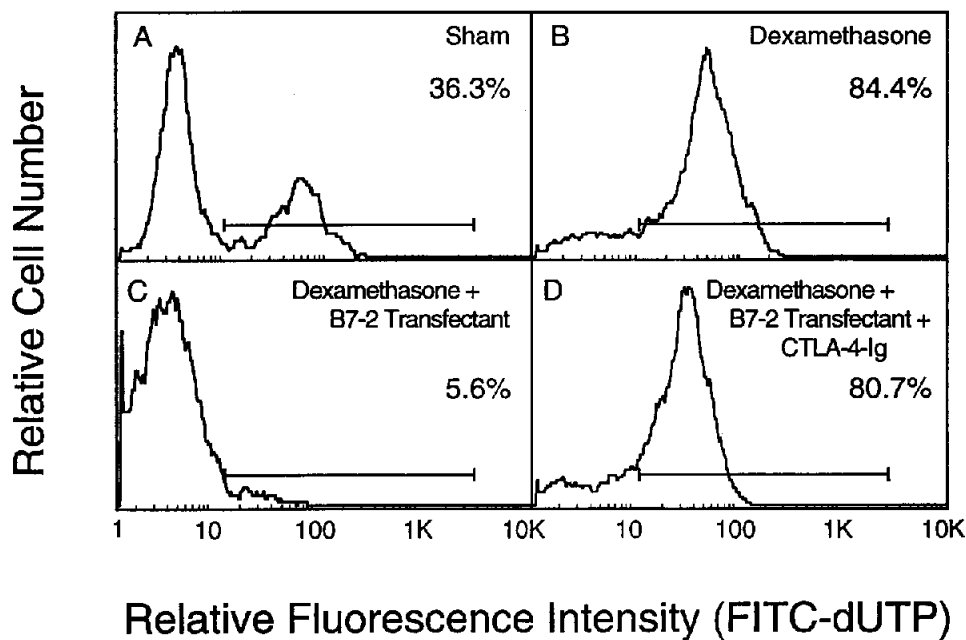


Figure 4. Blockade of B7-1/2-mediated rescue from glucocorticoid-induced cell death. Thymocytes, 2.5×10^6 , from BALB/c mice were CD3⁺ depleted as described in Materials and Methods. Apoptosis in the DP thymocyte population was determined by TUNEL assay as described in Fig. 1. (A) Untreated. (B) Thymocytes treated with 10^{-6} M dexamethasone. (C) Thymocytes treated with 10^{-6} M dexamethasone and 8×10^5 B7-2 transfectants. (D) Thymocytes treated with dexamethasone at 10^{-6} M and 8×10^5 B7-2 transfectants, which had been pretreated with 1 μ g/ml CTLA-4-Ig fusion protein. Control antibodies included anti-TCR β , H57-597, anti-MHC class I (H-2^d), 34-5-8, and anti-MHC class II (I-A^d), MK-D6. All antibodies were used at 25 μ g/ml.

B, C, and D, respectively). CTLA-4 expression was demonstrated in both SP populations, with CD4⁺ SP having higher expression than CD8⁺ SP cells. CD4⁺CD8⁺ DP thymocytes expressed CTLA-4, and the DN cells expressed low but detectable levels as compared with isotype controls. As has been previously reported (18), thymocytes also expressed CD28 (Fig. 6). These results support our observations that CD28 and CTLA-4 may each participate in the rescue of thymocytes from glucocorticoid-induced cell death.

Discussion

Our results demonstrate that interactions of the costimulatory molecules CD28 and CTLA-4 with cells bearing B7-1 or B7-2 mediate inhibition of glucocorticoid-induced apoptosis in thymocytes. We suggest that thymocytes that do not express appropriately responsive CD28/CTLA-4 costimulatory molecules are deleted as part of the maturation process during thymocyte development. Supporting this interpretation are reports that CD28 knockout mice undergo both positive and negative selection (19). CD28

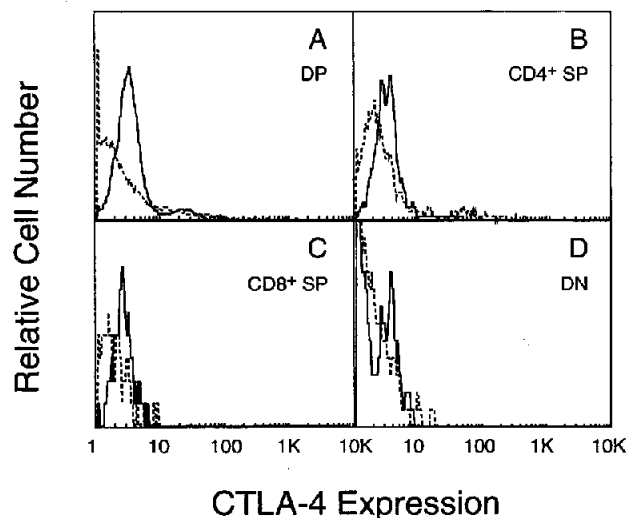


Figure 5. Expression of CTLA-4 on thymocytes. Profiles of thymocytes from C57BL/6 mice were generated by triple staining thymocytes for the expression of CD4, CD8, and CTLA-4 as described in Materials and Methods. CTLA-4 expression (solid lines) compared with isotype controls (dashed lines) on: (A) DP; (B) CD4⁺; (C) CD8⁺; and (D) DN, populations of thymocytes.

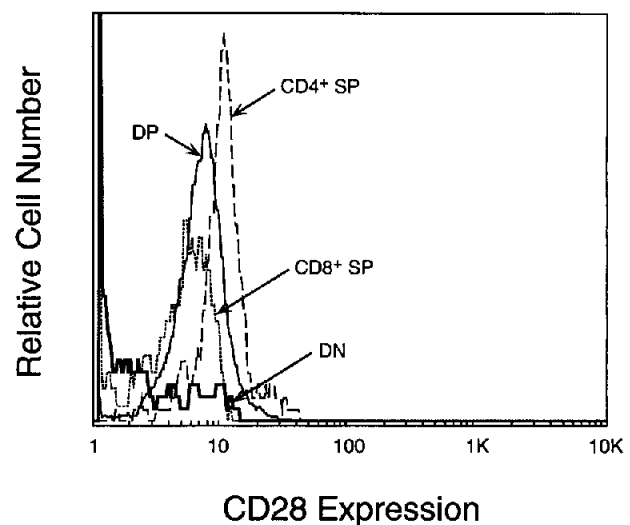


Figure 6. CD28 expression on thymocytes. Thymocytes were triple stained for CD4, CD8, and CD28 as described in Materials and Methods. CD28 expression was highest on CD4⁺ SP thymocytes (dashed line), and expression was detected on DP thymocytes (solid line) and CD8⁺ (dotted line) with some expression detected on DN thymocytes (solid gray line).

knockout animals may still have thymocytes expressing CTLA-4 which can interact with B-7 bearing APC and thus are protected from glucocorticoid-induced death. In contrast, CTLA-4 knockout mice have reduced numbers of DP thymocytes, accumulating DN and CD3⁺ SP cells, and exhibit massive lymphoproliferation resulting in autoreactive T cells (20, 21). This observation can be explained by the acute susceptibility of DP thymocytes to glucocorticoids in the absence of a rescue signal provided by costimulation. Without rescue, a relative decrease in the number of DP thymocytes would shift the thymic profile by increasing the proportion of DN thymocytes. The remaining DP population likely would be rescued by CD28 interactions. Interestingly, the CTLA-4 knockouts were generated on a C57BL/6 background and, as we have indicated, rescue of thymocytes from glucocorticoids in C57BL/6 can result from interactions between CD28 and the B7 bearing APC. This can be interpreted to mean that survival in the presence of glucocorticoids mediated by CD28 interactions in the absence of CTLA-4-mediated interactions results in the preferential expansion of autoaggressive T cells, which mediate dramatic self-destruction in the host. The rescue of T cells capable of being costimulated from "death by neglect" (22) provides the basis for subsequent costimulatory interactions required for T cell activation.

T cell activation requires recognition of antigens associated with MHC-encoded molecules (23) and a second signal provided by costimulatory interactions (24). Peripheral T cells must express a repertoire of TCR capable of recog-

nizing any antigen the T cell may encounter. Thus, the TCR preserves the antigen specificity in an immune response. Why then is costimulation necessary? If positive and negative selection in the thymus generated all possible T cell receptors capable of recognizing foreign antigens and eliminated all self-reactive T cells, costimulation would not be required. However, if all T cell receptors capable of recognizing self antigens were not removed in the thymus (25), one would predict the necessity for a control mechanism to prevent self destruction. The existence of peripheral self-reactive T cell receptors is well documented. Therefore, the expression of costimulatory molecules on self-tissue must be tightly regulated to avoid T cell-mediated self destruction. We propose that the requirement for T cell costimulation provides such control. That is, the ability of a T cell to be costimulated allows the T cell to function. Therefore it is reasonable that a mechanism exists to insure that T cells can be costimulated and this process likely occurs during development in the thymus.

We propose that a mechanism which removes, by cell death, the majority of thymocytes is provided by the presence of glucocorticoids in the thymus. This constitutes a process of death by neglect (22), such that those thymocytes that fail to undergo any life-promoting interaction in the thymus, die. Hence, rescue from glucocorticoid-induced cell death by costimulatory interactions predictably would insure that thymocytes develop into T cells capable of being costimulated when responding to nonself antigens or damaged self antigens at sites of inflammation.

The authors gratefully acknowledge Drs. Philippa Marrack, J. John Cohen, and Uwe D. Staerz for critical review of the manuscript; Jennifer VanderWall for expert technical assistance; Dr. Richard Duke and Mary Schleicher for assistance with Höchst/propidium iodide staining; Dr. J.P. Allison for the kind gift of anti-CTLA-4; and the Bioprocess Group of Bristol-Meyers Squibb Pharmaceutical Institute for kindly providing the CTLA-4Ig.

This work was supported by grants AI22295, AI37523 (J.H. Freed); AI33470 (M.K. Newell and J.H. Freed) and training grant T32AI00048 (D.H. Wagner) from the National Institutes of Health.

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Received for publication 25 April 1996 and in revised form 28 June 1996.

References

1. Dillon, S.R., and P.J. Fink. 1995. Thymic selection events mediated by the pre-TCR do not depend upon a limiting ligand. *Int. Immunol.* 7:1363-1373.
2. von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell.* 76:219-228.
3. Vacchio, M.S., V. Papadopoulos, and J.D. Ashwell. 1994. Steroid production in the thymus: implications for thymocyte selection. *J. Exp. Med.* 179:1835-1846.
4. Wyllie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (Lond.)* 284:555-556.
5. Cohen, J.J., and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132:38-42.
6. Iwata, M., S. Hanaoka, and K. Sato. 1991. Rescue of thymocytes and T cell hybridomas from glucocorticoid-induced apoptosis by stimulation via the T cell receptor/CD3 complex: a possible in vitro model for positive selection of the T cell repertoire. *Eur. J. Immunol.* 21:643-648.
7. Allison, J., and L. Lanier. 1985. Identification of antigen receptor-associated structures on murine T cells. *Nature (Lond.)* 314:107-109.

8. Linsley, P.S., and J.A. Ledbetter. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* 11:191–212.
9. Punt, J.A., B.A. Osborne, Y. Takahama, S.O. Sharrow, and A. Singer. 1994. Negative selection of CD4⁺CD8⁺ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. *J. Exp. Med.* 179:709–713.
10. Shi, Y., L.G. Radvanyi, A. Sharma, P. Shaw, D.G. Green, R.G. Miller, and G.B. Mills. 1995. CD28-mediated signaling in vivo prevents activation-induced apoptosis in the thymus and alters peripheral lymphocyte homeostasis. *J. Immunol.* 155:1829–1837.
11. Hagman, J., C. Rudin, D. Haasch, D. Chaplin, and U. Storb. 1990. A novel enhancer in the immunoglobulin 1 locus is duplicated and functionally independent of NFκB. *Genes Dev.* 4:978–992.
12. Giovane, A., A. Pintzas, S.M. Maira, P. Sobieszczuk, and B. Wasyluk. 1994. Net, a new ets transcription factor that is activated by ras. *Genes Dev.* 8:1502–1513.
13. Renno, T., M. Hahne, J. Tschopp, and H.R. MacDonald. 1995. Peripheral T cells undergoing superantigen-induced apoptosis in vivo express B220 and upregulate fas and fas ligand. *J. Exp. Med.* 183:431–437.
14. Ortaldo, J.R., A.T. Mason, and J.J. O'Shea. 1995. Receptor-induced death in human natural killer cells: involvement of CD16. *J. Exp. Med.* 181:339–344.
15. Duke, R.C., R. Shervenak, and J.J. Cohen. 1983. Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc. Natl. Acad. Sci. USA.* 80:6361–6365.
16. Seder, R.A., R.N. Germain, P.S. Linsley, and W.E. Paul. 1994. CD28-mediated costimulation of interleukin-2 (IL-2) production plays a critical role in T cell priming for IL-4 and interferon gamma production. *J. Exp. Med.* 179:299–304.
17. Linsley, P.S., J. Greene, W. Brady, J. Bajorath, J.A. Ledbetter, and R. Peach. 1994. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA4. *Immunity.* 1:793–801.
18. Gross, J.A., E. Callas, and J.P. Allison. 1992. Identification and distribution of the costimulatory receptor CD28 in the mouse. *J. Immunol.* 492:380–388.
19. Shahinian, A., K. Pfeffer, K.P. Lee, T.M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P.S. Ohashi, C.B. Thompson, and T.W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science (Wash. DC).* 261:609–612.
20. Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* 3:541–547.
21. Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K.P. Lee, C.B. Thompson, H. Griesser, and T.W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science (Wash. DC).* 270:985–988.
22. King, L.B., M.S. Vacchio, K. Dixon, R. Hunziker, D.H. Margulies, and J.D. Ashwell. 1995. A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development. *Immunity.* 3:647–656.
23. Marrack, P., and J. Kappler. 1987. The T cell receptor. *Science (Wash. DC).* 238:1073–1079.
24. Linsley, P., and J.A. Ledbetter. 1993. Role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* 11:191–212.
25. Matzinger, P. 1994. Tolerance, danger and the extended family. *Annu. Rev. Immunol.* 12:991–1045.