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) 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 insulate that T cells that express a functional TCR complex may be able to survive exposure to glucocorticoids.

TCR recognition of antigens associated with MHC
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 maturation states 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 Igk2-based vector VLPEk2.13 was constructed by inserting the Vk1-VA2 exon just downstream of the 4.0-kb murine VK2 promoter derived from VCATXS (11) and flanked by a BamHI cloning site and a genomic segment, including at the Ca2 polyadenylation, 3'-untranslated sequences, and 1-kb flanking sequences. The 1.65-kb EA2-4 enhancer fragment derived from VCATXS was inserted downstream of the transcriptional unit. The promoter-cDNA-enhancer insert can be released for transfection by digestion with SalI.

Amplification of sequences encoding the open reading frame (ORF) of murine B7-1 was accomplished using PCR with oligonucleotides 5'-AGTACTATGGCTTGCAATTGTCAGT, and 5'-GGCAGGCTCCCTCCTTAACTACA, 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 B7-2 was constructed using PCR with oligonucleotides 5'-AGTACTATGGCTTGCAATTGTCAGT, and 5'-GTGCATTACCGGTTCATTC, 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-B7-2-2.5. Thymocytes were transfected with either pSG5-B7-1 or pSG5-B7-2. The promoter-cDNA-enhancer insert can be released for transfection by digestion with SalI.

For each experiment, 2.5 × 10^6 cells were treated with dexamethasone at 10^-5 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 cyochrome-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 µg/ml for 30 min on ice. Cells were then washed and incubated with a 1:50 dilution of 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 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^-5 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 50 µg/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 a 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 signals. We removed the majority of CD3+ or CD3+/ TCR-β+ thymocyte populations and examined the ability of the B7 transfecants 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 × 10⁶/ml were treated with anti-CD3 (145-2C11) or anti-TCR (H57-597) at 20 µg/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 J558I 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 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.
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,
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
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 recognizing 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 Hoechst/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.

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