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The Role of B7 Costimulation in CD4/CD8 T Cell Homeostasis

Xiang Yu,* Sylvie Fournier,† James P. Allison,‡ Arlene H. Sharpe,§ and Richard J. Hodes*

The effect of B7-mediated costimulation on T cell homeostasis was examined in studies of B7-1 (CD80) and B7-2 (CD86) transgenic as well as B7-deficient mice. B7 overexpression in transgenic mice resulted in marked polyclonal peripheral T cell hyperplasia accompanied by skewing toward an increased proportion of CD8 single-positive cells and a decreased proportion of CD4 single-positive cells in thymus and more markedly in peripheral T cells. B7-induced T cell expansion was dependent on both CD28 and TCR expression. Transgenic overexpression of B7-1 or B7-2 resulted in down-regulation of cell surface CD28 on thymocytes and peripheral T cells through a mechanism mediated by intercellular interaction. Mice deficient in B7-1 and B7-2 exhibited changes that were the reciprocal of those observed in B7-overexpressing transgenics: a marked increase in the CD4/CD8 ratio in peripheral T cells and an increase in cell surface CD28 in thymus and peripheral T cells. These reciprocal effects of genetically engineered increase or decrease in B7 expression indicate that B7 costimulation plays a physiological role in the regulation of CD4+ and CD8+ T cell homeostasis. The Journal of Immunology, 2000, 164: 3543–3553.

T cell activation is dependent upon both a primary signal delivered through the Ag-specific TCR and costimulatory signals, the best characterized of which are delivered through the CD28 receptor on T cells by the costimulatory ligands B7-1 (CD80) and B7-2 (CD86) (for review, see Refs. (1 and 2). The role of B7-mediated costimulation in T cell responses has been demonstrated by the ability of anti-B7 Abs or soluble fusion proteins of CTLA-4, a receptor with high affinity for B7-1 and B7-2, to affect in vitro or in vivo T cell responses (3–7).

More recently, costimulation has been studied in genetically engineered mice, including mice rendered deficient in CD28 (8), CTLA-4 (9, 10), B7-1, and/or B7-2 by homologous recombination (11). T cell differentiation appears to be normal in CD28-deficient mice, but mature T cells are functionally impaired, proliferating poorly in vitro in response to allogeneic cells, and failing to respond to costimulation by B7-expressing cells, indicating that CD28 is the primary costimulatory receptor in these responses (8). CTLA-4-deficient mice display a contrasting and striking phenotype, marked by profound in vivo lymphoproliferation with early lethality, indicating that CTLA-4 normally acts as a negative regulator of T cell activation (9, 10). Studies of mice rendered deficient in B7-1 and B7-2 have indicated an essential role for B7 costimulation in responses, including Ig class switching and germinal center formation (11). Transgenic mice overexpressing B7-1 or B7-2 have also been used as probes for the in vivo function of these costimulatory ligands. Constitutive expression of B7-1 or B7-2 transgenes on B and T cells resulted in decreased B cell numbers in the periphery and a lower B cell precursor frequency in bone marrow, an effect that was T cell mediated and CD28 dependent (12–14). Defects in T cell-dependent B cell function were also identified in transgenic (Tg)2 mice constitutively expressing B7-1 on B cells (12, 14).

In contrast to the strong consensus of data indicating a critical role for CD28-B7 costimulatory signaling in Ag-specific activation of mature T cells, the role of B7-CD28 interaction in thymic and post-thymic T cell development and homeostasis is less clear. CD28 is normally expressed on essentially all murine thymocytes, and B7-1 and B7-2 are also expressed in thymus, notably on dendritic and medullary epithelial cells, consistent with a possible role of CD28-B7 interaction in thymic development. Punt et al., in fact, observed that Ab cross-linking of surface CD28 significantly increased apoptotic death induced in thymic CD4+CD8+ cells by anti-CD3, suggesting that CD28 signaling might play a role in regulating negative selection in this population (15, 16). Consistent with a role for CD28 in thymic negative selection, Noel and coworkers reported that CD28-deficient mice have increased numbers of thymocytes and that these cells are resistant to anti-CD3-induced deletion in vivo (17). A possible role in thymic selection for another B7 receptor, CTLA-4, was suggested by Cilio et al. (18), who reported that anti-CTLA-4 can prevent anti-CD3-induced thymocyte deletion. In contrast, other studies of Ab-induced negative selection and of CD28-deficient mice have concluded that B7 costimulation is not required for TCR-mediated deletion of CD4+CD8+ thymocytes (19, 20).

To further analyze the role of B7-dependent interactions in T cell homeostasis we have analyzed the effects of B7-1 and B7-2 Tg overexpression or B7-1/B7-2 deletion on CD4+ and CD8+ T cell populations. Alterations of B7 expression were found to have profound effects on homeostasis of thymic and peripheral CD4 + and CD8+ T cells, indicating a substantial functional role of interactions between B7 and CD28/CTLA-4 costimulatory receptors in regulation of CD4+ and CD8+ T cell differentiation and homeostasis.

Materials and Methods

Reagents

Anti-B7-2 Ab (GL1) was prepared in this laboratory (3). Anti-CD4, CD8, B220, CD28, CTLA-4, B7-1, CD45.1 (Ly5.2), CD25, CD69, and TCR Vy Abs were purchased from PharMingen (San Diego, CA).

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2 Abbreviations used in this paper: Tg, transgenic; B6, C57BL/6; BALB, BALB/c; MFI, mean fluorescence intensity.

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Mice

BALB/c, C57BL/6 (B6), and congenic B6 Ly5.2 mice were obtained from Frederick Cancer Research Facility (Frederick, MD) and were maintained at Bioqual (Rockville, MD). All mice were used at 6–12 wk of age. B7-2 (line 7), B7-1T, and B7-1B Tg lines were previously characterized (12–14). CD28−/− mice on a B6 genetic background were generated by backcrossing CD28−/− mice (8) to B6 for five generations. Do11.10 TCR Tg mice were generated and bred on a BALB/c background by Murphy et al. (21) and maintained at Bioqual. All B7 Tg mice were maintained by successive backcross to B6 mice for at least six generations, and transgene-negative littermates were employed as controls in all experiments. Mice rendered deficient in both B7-1 and B7-2 by homologous recombination were previously described and were maintained after backcrossing to a BALB/c background (11).

Flow cytometric analysis

Single-cell suspensions were prepared from thymus, spleen, and brachial, axillary, and inguinal lymph nodes, and erythrocytes were removed by treatment with ACK lysing buffer (BioFluid, Rockville, MD). Anti-FcR mAb 24G2 was added to prevent FcR-mediated binding of mAb to the cells, and cells were then incubated with FITC-labeled mAb, biotinylated mAb, PE-labeled mAb, and streptavidin-Cy-Chrome conjugate (PharMingen) sequentially. For cytoplasmic staining of CTLA-4, cells were first stained for cell surface markers as described above. Cells were then fixed and permeabilized using Cytofix/Cytoperm solution (PharMingen), washed in Perm/Wash (PharMingen), and incubated with anti-CTLA-4-PE Ab (PharMingen). Viable cells (2–5 × 10^4) were analyzed by FACScan (Becton Dickinson, San Jose, CA) as previously described (22).

Northern blot analysis

Total RNA was isolated from lymphocytes using the Qiagen (Santa Clarita, CA) RNeasy kit and separated on 1.2% agarose gels. RNA was transferred onto nylon membranes (Schleicher & Schuell, Keene, NH) with 20× SSC using a turbo transfer apparatus (Schleicher & Schuell) and was UV cross-linked to membranes. Membranes were prehybridized with Quick-Hyb (Stratagene, La Jolla, CA) for 60 min at 65°C. A full-length CD28 cDNA probe (provided by Dr. Philip Lucas, National Institutes of Health, Bethesda, MD) was random primer labeled. After 2-h hybridization at 65°C, membranes were washed twice with 2× SSC/0.1% SDS and once with 0.1× SSC/0.1% SDS at 60°C. Membranes were stripped by boiling in 0.5% SDS solution, washed, and reprobed with a labeled actin probe (cDNA amplified using PCR primer pairs purchased from CLONTECH, Palo Alto, CA).

Mixed bone marrow chimeras

Radiation bone marrow chimeras were prepared as previously described (23). Recipient mice were lethally irradiated with 1000 rad and reconstituted with 10^7 T-depleted bone marrow cells. Analysis of chimeras was performed 5 wk after reconstitution.

Results

B7 expression in B7 Tg mice

Three lines of B7 transgenic mice were used to assess the effect of B7 expression on T cell homeostasis. B7-2 line 7 expresses mouse B7-2 under the control of the H-2Kb promoter and Ig μ-chain enhancer (13). Consistent with previous characterization, cell surface B7-2 expression in line 7 was detected on peripheral B cells, T cells, and thymocytes, with T cells exhibiting stronger B7-2 staining than B cells (Fig. 1) and equivalent expression on CD4+ and CD8+ T cells (data not shown). Both B7-1T and B7-1B Tg lines express B7-1 under the control of the Ig μ enhancer and promoter elements (12). In B7-1T Tg mice, B7-1 was expressed in thymus and on peripheral T cells, but not on B cells (Fig. 1). In B7-1B Tg mice, B7-1 was expressed on peripheral B cells, but not on peripheral T cells, with a low level of B7-1 detected on B7-1B thymocytes (Fig. 1). Non-Tg littermates express little B7-1 or B7-2 in thymus and a low level of B7-2 on spleen T cells.

FIGURE 1. B7 expression in B7 Tg mice. Thymocytes and splenocytes from B7 Tg mice were analyzed for B7 expression (B7-2 on B7-2 Tg mice, B7-1 on B7-1T and B7-1B Tg mice). Filled curve, transgene positive; bold line, control littermates; thin line, negative Ab control. Mean fluorescence intensity (MFI) is indicated for each B7-stained population. In B7-2 Tg mice, B7-2 was expressed on peripheral B cells, T cells, and thymocytes, with T cells exhibiting stronger B7-2 staining than B cells; in B7-1T Tg mice, B7-1 was expressed in thymus and on peripheral T, but not B, cells; in B7-1B Tg mice, B7-1 was expressed on peripheral B, but not peripheral T, cells, with a low level of B7-1 on thymocytes; non-Tg littermates express little B7-1 or B7-2 in thymus and a low level of B7-2 on spleen T cells.
B7-2 induces peripheral lymphocyte expansion in B7-2 Tg mice
The effect of Tg B7 overexpression on T cell development and differentiation was analyzed in thymus, spleen, and peripheral lymph nodes from Tg and non-Tg littermates. When B7-2 Tg and non-Tg B6 littermates were compared, a significant peripheral lymphocyte expansion was observed in B7-2 Tg spleen and peripheral lymph nodes, with the number of splenocytes increased by 40% and lymph node cells increased 6-fold over littermate controls (Fig. 2A). When B7-2 Tg mice were analyzed on a (BALB/c × B6)F₁ background, a more profound peripheral lymphocyte expansion was seen in both spleen and lymph nodes, with the number of splenocytes increased 200% and lymph node cells increased 10-fold over littermate controls (Fig. 2A). The number of thymocytes was not significantly different in Tg and control mice (data not shown). Thus, B7-2 transgene expression results in striking hyperplasia of peripheral lymphoid organs, and the genetic background (B6 vs (BALB × B6)F₁) appears to affect the magnitude of this lymphoid expansion. In B7-2 Tg spleen, there were consistent changes in cellular composition, including a decreased proportion of B cells and increases in T cells and non-T/non-B cells (Fig. 2B). In addition to increased cell numbers in secondary lymphoid organs such as spleen and lymph node, B7-2 Tg mice exhibited extensive lymphoid infiltration of organs, including lung and liver (data not shown).

CD4/CD8 ratios are skewed in B7 Tg mice
To determine the effects of B7 overexpression on CD4⁺ and CD8⁺ T cell differentiation and homeostasis, thymic and peripheral T cell populations were analyzed. A small, but statistically significant, increase in the percentage of CD8 single positive thymocytes and a similarly small but significant decrease in CD4 single-positive thymocytes was observed in B7-2 Tg mice compared with transgene-negative littermates (Table I). This pattern was best illustrated by comparison of CD4/CD8 ratios (percentage of CD4⁺CD8⁻/percentage of CD4⁻CD8⁺) as shown in Fig. 3A. In B7-2 Tg as well as B7-1T Tg lines, thymic CD4/CD8 ratios were significantly lower than those of transgene-negative littermates.
We next examined mature peripheral CD4⁺ and CD8⁺ cells in B7 Tg mice. Consistent with the observations made in thymus, B7 Tg mice have a higher percentage of splenic CD8⁺ cells and a lower percentage CD4⁺ cells than transgene-negative littermates (Table I). This skewing toward CD8⁺ cells is again most evident as a reduction in the CD4/CD8 ratio in spleen (Fig. 3A) and was consistently more marked in spleen than in thymus. Significant CD8 skewing was observed in spleen cells of B7-1B, B7-1T, and B7-2 Tg mice and was most marked in B7-2 Tg mice, in which there was a reversal of the CD4/CD8 ratio seen in control mice (Table I and Fig. 3A). Similar CD8 skewing was also observed in peripheral blood lymphocytes in B7-2 Tg mice and was present, but less marked, in lymph nodes (data not shown). These observations indicate that overexpression of B7 can lead to a shift in the

Table I. % CD4 and CD8 populations in lymphoid tissues of B7 transgenic mice

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<th>Tg⁺</th>
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* Values obtained from the analysis of n pairs of transgenic and control littermates. Average, arithmetic average. pp, Represents where Tg⁺ value is statistically different from Tg⁻ value by t test (p < 0.05).

**FIGURE 3.** CD4/CD8 ratios in B7 Tg and control mice. A, Ratios of CD4/CD8 single-positive cells in thymus and spleen of Tg and wild-type littermate controls are graphed. B7-2, B7-2 Tg mice; B7-1T, B7-1 Tg mice; B7-1B, B7-1B Tg mice (n = 3–5; see Table I for details). B, Ratios of CD4/CD8 single-positive cells in thymus and spleen of B7-2 Tg⁺CD28⁻/⁻ and control mice are graphed (n = 3). * and **, Tg⁺ value is statistically different from Tg⁻ value by t test (*, p < 0.05; **, p < 0.01). Gates used to define CD4 and CD8 cells were selected based on levels of staining with isotype-matched negative control Abs, and identical gates were used in analysis of all populations.
CD4/CD8 balance in thymus and, more markedly, in peripheral T cell compartments, favoring an increased proportion of CD8⁺ T cells.

**B7-induced lymphoid hyperplasia and CD8 skewing are CD28 dependent**

One pathway through which B7 overexpression might lead to peripheral lymphocyte expansion is the engagement of CD28. To determine whether CD28 is required for lymphocyte expansion in B7-2 Tg mice, the effect of B7-2 transgene expression was assessed in CD28-deficient mice. In contrast to B7-2 Tg mice with wild-type CD28 expression, there was no lymphocyte expansion observed in B7-2 Tg CD28⁻/⁻ mice (data not shown). CD4/CD8 T cell ratios were not skewed toward CD8⁺ T cells in either thymus or periphery (Fig. 3B). Thus, both lymphocyte expansion and CD8 skewing observed in B7-2 Tg mice appear to be dependent on CD28/B7-2 interaction.

**B7-induced lymphoid hyperplasia is influenced by TCR expression**

To determine whether TCR signaling plays a role in B7-2-induced lymphocyte expansion, the B7-2 transgene was expressed in mice that also express the Do11.10 TCR transgene specific for OVA peptide. Do11 TCR Tg thymocytes are positively selected and differentiate into mature T cells that are highly skewed toward CD4⁺ T cells (21), but it appears that peripheral T cells do not normally encounter a functional ligand and therefore maintain a naive phenotype (24). Heterozygous B7-2 Tg B6 mice were crossed with heterozygous Do11.10 TCR Tg BALB/c mice to generate (B6 × BALB/c)F₁ mice that were double Tg (B7-2 Tg "Do11⁻"), single Tg (B7-2 Tg "Do11⁻" or B7-2 Tg "Do11⁺"), or non-Tg (B7-2 Tg "Do11⁻").

Significant lymphocyte expansion was observed in mice expressing only the B7-2 Tg (B7-2 Tg "Do11⁻") as documented by total cell yield of spleen (almost 3-fold increase) and lymph nodes (>10-fold increase; Fig. 4A). In contrast, mice expressing both Do11 TCR and B7-2 transgenes (B7-2 Tg "Do11⁺") showed significantly less expansion in spleen and no expansion in lymph node cell number. Mice expressing only the Do11 TCR Tg did not exhibit lymphocyte expansion over non-Tg controls. Taken together, these results indicate that lymphocyte expansion in B7-2 Tg mice is dependent upon TCR expression and is absent or diminished in mice with apparently reduced TCR signaling mediated by the OVA-specific Do11.10 TCR transgene.

**B7-2 Tg T cells express an activated phenotype**

Because lymphocyte expansion was observed in B7-2 Tg "Do11⁻" but not in B7-2 Tg "Do11⁺" double-Tg mice, we determined the activation status as reflected by the expression of early lymphocyte activation markers CD25 and CD69 in these animals. In parallel to lymphocyte expansion, only B7-2 Tg "Do11⁻" mice had substantially increased percentages of T cells expressing CD25 compared with B7-2 Tg "Do11⁺" double-Tg, Do11⁻ Tg, or wild-type non-Tg mice. B7-2 Tg "Do11⁻" peripheral lymphocytes were also large, as judged by forward light scatter (data not shown). In contrast, both B7-2 Tg "Do11⁻" and B7-2 Tg "Do11⁺" had increased proportions of CD69⁺ cells (Fig. 4B). These observations suggest that B7-CD28 interaction alone or in combination with reduced or absent TCR signaling can induce some early activation responses, such as expression of CD69, but cannot induce other in vivo activation events, including CD25 expression and T cell hyperplasia.

**Surface CD28 down-regulation in B7 Tg mice**

We next studied the effect of B7 transgenes on the expression of the B7 receptor CD28 in thymus and periphery. In B7-1T and B7-2 Tg lines, in which all thymocytes express a high level of B7-1 or
The observed down-regulation of cell surface CD28 expression could represent the consequence of B7 transgene expression by the same cells, e.g., by intracellular interaction of B7 and CD28, or alternatively might result from interaction of cell surface CD28 with cell surface B7 transgene products on other cells. To further probe these possibilities, mixed radiation bone marrow chimeras were made by reconstituting lethally irradiated B6 recipients with a mixture of bone marrow cells from B7-2 Tg and non-Tg B6 Ly5.2 congenic mice. The lymphocyte populations were analyzed by flow cytometry 1 mo after reconstitution. In these chimeras, cell surface CD28 was profoundly and comparably down-regulated on both transgene-positive and transgene-negative Ly5.2 cells (Fig. 6A), this down-regulation affecting CD4\(^+\)CD8\(^-\), CD4\(^-\)CD8\(^+\), CD4\(^+\)CD8\(^+\), and CD4\(^-\)CD8\(^-\) subpopulations (data not shown). The observed CD28 down-regulation on B7 transgene-negative thymocytes demonstrated that cell surface CD28 can be regulated through intercellular interaction with its B7 ligand.

To determine whether TCR expression affects B7 induced down-regulation of CD28 we examined CD28 surface expression in B7-2Tg × Do11TCR Tg mice. Equivalent CD28 down-regulation was observed in B7-2Tg+Do11+ and B7-2Tg+Do11− mice, where peripheral T cells do not appear to encounter an effective nominal Ag (Fig. 6B). B7-induced down-regulation of CD28 thus does not appear to require a high degree of TCR-specific signaling.

### CTLA-4 expression in B7 Tg mice

The expression of CTLA-4 (CD152), the second known receptor from wild-type mice expressed no detectable cell surface or cytoplasmic CTLA-4 (CD152), was also analyzed in wild-type and B7-2 Tg mice. Consistent with previous studies, freshly explanted splenic T cells from wild-type and B7-2 Tg mice were negative for surface CTLA-4 expression, and surface expression was induced equivalently on transgene-negative and transgene-positive T cells by in vitro activation (data not shown). In contrast, a significant difference between wild-type and B7-2 Tg mice was observed in cytoplasmic CTLA-4 expression by freshly explanted splenic T cells: 51.6% of B7-2 Tg CD4\(^+\) CD8\(^-\) cells vs 22.5% of wild-type CD4\(^+\) CD8\(^-\) cells, and 32.2% of B7-2 Tg CD8\(^+\) cells vs 5.2% of wild-type CD8\(^+\) cells were cytoplasmic CTLA-4 positive (Fig. 7). Thus, the B7-2 transgene results in markedly increased expression of cytoplasmic CTLA-4 expression by both CD4\(^+\) and CD8\(^+\) T cells. To determine whether cytoplasmic CTLA-4 expression is related to activation state of T cells, T cell subpopulations that are either positive or negative for the activation Ags CD25 or CD69 were...
analyzed. It was found that cytoplasmic CTLA-4 is preferentially expressed on CD25+ and CD69+ (activated) and on CD4+ and CD8+ T cells in both B7-2 Tg and non-Tg populations (data not shown).

**FIGURE 6.** Surface CD28 down-regulation in B7-2Tg/Ly5.2 mixed chimeras and B7-2Tg × Do11 mice. A, Surface CD28 down-regulation in B7-2Tg/Ly5.2 mixed chimeras. Thymocytes from mixed bone marrow chimeras were analyzed for surface CD28 expression. Two different chimeras were analyzed: B7-2Tg/Ly5.2 mixed chimeras were made from mixed B7-2Tg/Ly5.2 bone marrow transfer. CD28 expression was analyzed on gated Ly5.2+ and Ly5.2− thymocytes. B, Surface CD28 down-regulation in B7-2Tg × Do11 TCR mice. Thymocytes from B7-2Tg × Do11 TCR animals were analyzed for surface CD28 expression. The histograms are the overlaid curves of four different genotypes: 1) B7-2Tg/Do11 TCR+ (B7-2Tg/Do11+; filled curve), 2) B7-2Tg/Do11 TCR− (wild type; B7-2Tg/Do11−; dotted curve), 3) B7-2Tg/Do11 TCR+ (B7-2Tg/Do11+; thin curve), and 4) B7-2Tg/Do11 TCR− (B7-2Tg/Do11−; thick curve). The experiment shown is representative of three independent experiments.

The peripheral T cell expansion observed in B7-2Tg mice could be the result of oligoclonal or polyclonal expansion. When the TCR repertoire was assessed by analysis of Vβ expression in B7-2Tg mice, no significant change in TCR Vβ profile was observed in CD4 or CD8 single-positive thymocytes or splenic T cells (data not shown), with the exception of a modest, but significantly increased, proportion of Vβ5+ cells in both CD8+ single-positive thymocytes (12.2 ± 1.6% Vβ5+ in B7-2Tg+ and 8.6 ± 1.9% Vβ5+ in Tg−; p < 0.05) and CD4+ spleen T cells (5.3 ± 0.5% Vβ5+ in B7-2Tg+ and 3.3 ± 0.5% Vβ5+ in B7-2Tg−; p < 0.01). When TCR Vβ expression was assessed on activated (CD69+ or CD25+) T cells, the population of activated B7-2 Tg T cells was found to be broadly polyclonal (data not shown). T cell hyperplasia in B7-2 Tg mice is thus polyclonal and without gross distortion of the TCR Vβ repertoire.

**CD4/CD8 homeostasis in B7-1/B7-2-deficient mice**

If the effects of Tg B7 overexpression reflect an exaggeration of normal B7 function, it might be expected that a deficiency in B7 expression would have reciprocal effects. To test this hypothesis, T cell development was analyzed in mice that were rendered deficient in both B7-1 and B7-2 by homologous recombination. Peripheral T cells from B7-1/B7-2-deficient BALB/c and control mice were first analyzed. No significant differences were found in total cellularity of spleen and lymph nodes from deficient and control animals (data not shown). However, CD4/CD8 ratios in B7-1/B7-2-deficient mice were markedly increased in both spleen (4.1 ± 0.5 in B7-deficient mice, 2.2 ± 0.2 in controls) and lymph nodes (6.6 ± 0.6 in B7-deficient mice, 2.8 ± 0.2 in controls; Fig. 8); these differences were reciprocal to the decreased CD4/CD8 ratios observed in B7-overexpressing Tg mice. In thymus, total cell number was not different in B7-deficient and wild-type mice, but there were significant differences in the relative proportions of thymocyte subpopulations. The percentage of CD4+CD8− cells was significantly lower in B7-deficient mice (74.3 vs 83.8%; p < 0.03), whereas the percentage of CD4+ single-positive cells was higher in B7-deficient mice (17.2 vs 10.5%; p < 0.01); the ratio between CD4 and CD8 single-positive thymocytes tended to be higher in B7-deficient mice than in controls (6.0 in B7 deficient vs 4.2 in controls), although this difference was not statistically significant (p = 0.09) for the number of mice analyzed to date. When CD4/CD8 ratios were measured in B7-1 or B7-2 single-deficient mice, no skewer ratios were observed over those in littermate controls (data not shown). Taken together, the reciprocal effects of B7 overexpression and B7 deficiency strongly suggest that B7 plays a substantial physiologic role in regulating the development and homeostasis of mature CD4+ and CD8+ T cells.

**CD28 expression in B7-1/B7-2-deficient mice**

Studies of B7 Tg mice indicated that B7-1 or B7-2 overexpression results in down-regulation of cell surface CD28. To determine whether CD28-B7 interaction also plays a role in regulating CD28 expression under conditions of physiologic B7 expression, the effect of B7 deficiency on CD28 expression was examined. CD28 density on thymocytes from B7-1/B7-2-deficient mice (data not shown), with the exception of a modest, but significantly increased, proportion of Vβ5+ cells in both CD8+ single-positive thymocytes (12.2 ± 1.6% Vβ5+ in B7-2Tg+ and 8.6 ± 1.9% Vβ5+ in Tg−; p < 0.05) and CD4+ spleen T cells (5.3 ± 0.5% Vβ5+ in B7-2Tg+ and 3.3 ± 0.5% Vβ5+ in B7-2Tg−; p < 0.01). When TCR Vβ expression was assessed on activated (CD69+ or CD25+) T cells, the population of activated B7-2 Tg T cells was also found to be broadly polyclonal (data not shown). T cell hyperplasia in B7-2 Tg mice is thus polyclonal and without gross distortion of the TCR Vβ repertoire.

**TCR Vβ repertoire in B7-2Tg mice**

The peripheral T cell expansion observed in B7-2Tg mice could be the result of oligoclonal or polyclonal expansion. When the TCR repertoire was assessed by analysis of Vβ expression in B7-2Tg mice, no significant change in TCR Vβ profile was observed in CD4 or CD8 single-positive thymocytes or splenic T cells (data not shown), with the exception of a modest, but significantly increased, proportion of Vβ5+ cells in both CD8+ single-positive thymocytes (12.2 ± 1.6% Vβ5+ in B7-2Tg+ and 8.6 ± 1.9% Vβ5+ in Tg−; p < 0.05) and CD4+ spleen T cells (5.3 ± 0.5% Vβ5+ in B7-2Tg+ and 3.3 ± 0.5% Vβ5+ in B7-2Tg−; p < 0.01). When TCR Vβ expression was assessed on activated (CD69+ or CD25+) T cells, the population of activated B7-2 Tg T cells was also found to be broadly polyclonal (data not shown). T cell hyperplasia in B7-2 Tg mice is thus polyclonal and without gross distortion of the TCR Vβ repertoire.
CD28-dependent increase in the proportion of CD8\(^+\)CD4/CD8 T cell balance, B7 overexpression giving rise to a substantial role in regulating CD4/CD8 T cell development and homeostasis. Specifically, the findings presented here demonstrate 1) B7 overexpression or deletion results in dramatic shifts in CD4/CD8 T cell balance, B7 overexpression giving rise to a CD28-dependent increase in the proportion of CD8\(^+\) cells and B7 deletion, resulting in an increased proportion of CD4\(^+\) cells relative to wild-type controls; 2) B7-2 overexpression results in a high degree of peripheral T cell hyperplasia, an effect that is dependent upon both CD28 and TCR; and 3) B7 expression modulates cell surface CD28 on both thymic and peripheral T cells.

Discussion

The mechanism by which the immune system regulates the homeostatic balance of peripheral CD4\(^+\) and CD8\(^+\) cells is poorly defined. The present studies assessed the possibility that interactions between B7 molecules and their receptors may play a role in CD4/CD8 T cell differentiation and homeostasis. Using genetically engineered B7-overexpressing or B7-deficient mice, this report provides evidence indicating that B7-dependent events play a substantial role in regulating CD4/CD8 T cell development and homeostasis. Specifically, the findings presented here demonstrate that 1) B7 overexpression or deletion results in dramatic shifts in CD4/CD8 T cell balance, B7 overexpression giving rise to a CD28-dependent increase in the proportion of CD8\(^+\) cells and B7 deletion, resulting in an increased proportion of CD4\(^+\) cells relative to wild-type controls; 2) B7-2 overexpression results in a high degree of peripheral T cell hyperplasia, an effect that is dependent upon both CD28 and TCR; and 3) B7 expression modulates cell surface CD28 on both thymic and peripheral T cells.

The observed effects of B7 expression on CD4 and CD8 lineages may be mediated at both thymic and post-thymic levels. In the thymus, commitment of precursors to the CD4 and CD8 lineages is determined by interaction between TCR complexes on thymocytes and Ag-presenting MHC class I or class II molecules expressed on thymic epithelial cells. In this regard, it is of interest that the TCR V\(\beta\) repertoire is not substantially different in B7-Tg and non-Tg mice, failing to identify altered TCR specificity as a mediator of altered CD4 and CD8 development or expansion. Expression of MHC class I and class II products, also capable of influencing CD4\(^+\) and CD8\(^+\) T cell development, was similarly unaltered in B7 Tg or -deficient mice (data not shown).

Interestingly, Tg mice expressing Bcl-2 or Notch IC have been reported to display an increase in the proportion of CD8 single-positive thymocytes similar to that observed in B7 Tg (25–27). The effect of Bcl-2 and Notch on CD4/CD8 lineage commitment may be related to the anti-apoptotic function of these two molecules on thymocytes (25, 28–30). Itano et al. (31) and Matechak et al. (32) postulated that relatively weak signaling through the TCR of CD4\(^+\)8\(^+\) precursors favors CD8 lineage commitment. In the context of this model, cells that would normally die by neglect because of the extremely low affinity of TCR for available self-Ag/MHC complexes may be rescued preferentially by anti-apoptotic signaling enhanced by Bcl-2 and Notch transgenes (30), favoring increased survival of cells committed to the CD8 lineage. The fact that B7-CD28 interaction can enhance T cell survival through the induction of anti-apoptotic genes such as bcl-x\(_L\) (33) and the observation that Bcl-2 is up-regulated during normal thymic development (26, 34) suggest that in B7 Tg mice, increased signaling through CD28 may affect CD4/CD8 lineage commitment by preferential Bcl-2-mediated rescue of CD8-committed thymocytes that would normally undergo death by neglect. In the absence of B7-CD28 signaling as seen in B7-1/B7-2-deficient thymus, physiologic levels of this rescue would not occur, resulting in decreased CD8 lineage commitment and favoring dominance of CD4.

Although a similar CD8 skewing phenotype is observed in the thymus of Bcl-2 Tg and B7 Tg mice, there are marked differences in the peripheral T cell populations of these two Tg models. In Bcl-2 Tg mice CD8 skewing is limited to the thymus and is not accompanied by increased export or peripheral expansion of

**FIGURE 7.** Cytoplasmic CTLA-4 expression by B7-2 Tg T cells. Freshly harvested CD4\(^+\) and CD8\(^+\) T cells from B7-2 Tg and control Tg mice were stained for cytoplasmic expression of CTLA-4. Filled curves correspond to anti-CTLA-4 cytoplasmic stained cells, and open curves correspond to staining with control Ab. The MFI is indicated for each anti-CTLA-4 stained cells, and open curves correspond to staining curves for cytoplasmic expression of CTLA-4. Filled T cells from B7-2 Tg and control Tg and CD8\(^+\) cells, 22.5% of wild-type CD4\(^+\) cells, 32.2% of B7-2 Tg CD8\(^+\) cells, and 5.2% of wild-type CD8\(^+\) cells.

**FIGURE 8.** Increased CD4/CD8 ratios in B7-1/B7-2 double-knockout mice. The CD4/CD8 ratios for single-positive cells in thymus, spleen, and lymph nodes are plotted for B7-1/B7-2-deficient and wild-type BALB/c mice (n = 3). **, The Tg\(^+\) value is statistically different from the Tg\(^-\) value by t test (p < 0.01).
CD8\(^+\) cells. In contrast, in B7 Tg mice CD8 skewing is observed in both thymus and periphery, accompanied by a striking peripheral lymphoid hyperplasia in B7-2 Tg. These differences may reflect the difference in tissue distribution of transgene expression in these two models, thymic restricted in the case of Bcl-2 driven by the proximal Lck promoter and more widely T and/or B cell expressed in the case of B7-1 and B7-2 Tg. It is also possible that B7 interactions with CD28 and/or CTLA-4 induce signaling events distinct from or in addition to those mediated by Bcl-2 or Notch.

In the periphery, transgene-mediated overexpression of either B7-1 or B7-2 resulted in CD28-dependent skewing toward CD8\(^+\) T cells, with a shift in peripheral CD4/CD8 ratio from ~2 in spleens of non-Tg mice to 0.5 in B7-2 Tg. It is notable that the degree of skewing observed in peripheral T cells is consistently greater than that observed in the thymus, suggesting the importance of peripheral factors in this altered homeostasis. Deficiency in B7-1 and B7-2 resulted in a reciprocal skewing toward CD4\(^+\) cells and alteration of CD4/CD8 ratio from 2 in control spleen to 4 in B7-deficient spleen. Genetically engineered overexpression and deficiency in B7 expression are thus capable of modulating the proportion of CD4\(^+\) and CD8\(^+\) peripheral T cells over approximately an 8-fold range. Moreover, the fact that either an increase or a decrease in B7 expression leads to altered CD4/CD8 ratios strongly suggests that B7 plays a role in determination of CD4/CD8 homeostasis under physiologic conditions. Peripheral lymphoid hyperplasia was most marked in B7-2 Tg (B6 × BALB/c)F\(_1\) mice, in which spleen cell numbers were 3-fold and lymph node numbers were 10-fold those in non-Tg controls. Hyperplasia reflected increased numbers of both T and non-T cells, and T cell expansion was polyclonal. When expressed in absolute cell numbers, the expansion of peripheral CD8\(^+\) cells is striking, with CD8\(^+\) cells in spleen and lymph nodes from B7-2 Tg mice increased as much as 4- and 15-fold, respectively. In addition to increased cell numbers in secondary lymphoid organs, B7-2 Tg mice exhibited abnormal lymphoid infiltration of organs, including liver and lung. Increased cell size and increased expression of CD25 and CD69 on Tg CD4\(^+\) and CD8\(^+\) T cells are consistent with extensive peripheral activation. Peripheral lymphoid expansion required CD28 and appeared to be TCR dependent as well, as reflected in the minimal lymphocyte expansion seen in TCR transgene-expressing Do11\(^+\) B7-2 Tg mice. It thus appears that increased CD28 signaling by overexpressed Tg B7 together with
physiologic levels of TCR signaling by ligands encountered in the periphery, results in abnormal T cell activation and expansion. Expression of either the B7-1 or B7-2 transgene had a similar effect in skewing the proportion of CD8\(^+\) T cells, indicating a similarity of B7-1 and B7-2 in this functional capacity. In contrast, marked overall lymphoid hyperplasia was observed only in B7-2 line 7 Tg. It is unclear whether this reflects a functional difference between B7-1 and B7-2 or a difference in quantitative expression or tissue distribution of Tg B7 products in these lines. The overall kinetics of cell division and cell death that contribute to the observed net T cell hyperplasia remain to be elucidated. It is of interest, however, that in preliminary studies, the proportion of apoptotic cells detected by either TUNEL or annexin staining is substantially greater in freshly harvested B7-2 Tg T cells than in littermate controls, indicating that expanded numbers of peripheral T cells are sustained in B7-2 Tg even in the face of increased cell death (data not shown).

An interesting consequence of altered B7 expression was its effect on cell surface CD28 on thymic and peripheral T cells. Transgenic overexpression of either B7-1 or B7-2 resulted in substantially decreased cell surface CD28 on thymocytes and on CD4\(^+\) and CD8\(^+\) peripheral T cells. The observed reciprocal increase in CD28 expression in thymic and peripheral T cells from B7-deficient mice strongly suggests that expression of CD28 under physiologic conditions in wild-type mice is influenced by B7 engagement, possibly through receptor internalization or shedding. CD28 down-regulation in B7 Tg mice as well as CD28 up-regulation in B7-1/B7-2-deficient mice were observed in CD4\(^+\), CD4\(^+\)8\(^+\), CD4\(^+\)8\(^-\), and CD4\(^+\)8\(^-\) thymocyte populations, suggesting that CD28 on each of these populations is modulated by B7 ligation. Modulation of cell surface CD28 appeared to be mediated by intercellular interaction of CD28-expressing T cells with B7-1- or B7-2-expressing cells. In principle, this regulation of CD28 might serve as a compensatory mechanism to moderate the delivery of excessive CD28 costimulatory signals generated during thymic development and peripheral immune responses. However, the observed thymic and peripheral CD8 skewing together with the lymphoid hyperplasia and infiltration observed in B7-2 Tg mice indicate the limited efficacy of any compensatory mechanism mediated by CD28 down-regulation. Expression of the other known B7 receptor, CTLA-4, was also influenced by B7 transgene expression. Neither wild-type nor Tg T cells expressed detectable cell surface CTLA-4 unless activated in vitro. Cytoplasmic CTLA-4 was detected, however, and levels of expression were consistently greater in both CD4\(^+\) and CD8\(^+\) T cells from B7-2 Tg mice than in wild-type T cells. The higher levels of cytoplasmic CTLA-4 observed in B7-2 Tg T cells may reflect the increased activation state of these T cells. The functional implications of increased cytoplasmic CTLA-4 are not clear in the absence of detectable cell surface expression. It is possible that upon in vivo activation, B7-2 Tg T cells are capable of rapid transport of CTLA-4 to the cell surface, where CTLA-4-mediated signaling could contribute to preferential down-regulation of CD4\(^+\) T cell activation and the observed skewing toward CD8\(^+\) cells in these mice.

The findings reported here suggest a central role for B7-dependent signaling in regulating the development and homeostasis of CD4\(^+\) and CD8\(^+\) T cells, a role that is potentially mediated through the costimulatory receptors CD28 and CTLA-4. The observed CD8\(^+\) T cell skewing and expansion in B7Tg mice and the CD4\(^+\) T cell skewing in B7-deficient mice demonstrate that B7 expression can substantially influence the balance between CD4\(^+\) and CD8\(^+\) T cells. The fact that B7-dependent CD8 skewing and lymphoid expansion do not occur in B7-2 Tg CD28\(^{-/-}\) mice indicates that B7-2 interaction with the CD28 costimulatory receptor mediates these effects in the presence of transgenically increased B7 expression. The observation that CD28-deficient mice expressing physiologic levels of B7 display normal CD4/CD8 ratios and peripheral homeostasis (Ref. 8 and this study; data not shown) suggest that CD28 may not play an equivalent role in CD4/CD8 T cell homeostasis under all conditions of B7 expression. It has recently been reported that B7-dependent interactions with another receptor, CTLA-4, play an important role in T cell activation and proliferation. CTLA-4-deficient mice appear to undergo normal thymic development (35), but express a phenotype of profound peripheral lymphoproliferation in which CD4\(^+\) T cell expansion predominates, with enlarged lymphoid organs, an activated T cell phenotype, and extensive lymphoid infiltration of multiple organs, resulting in early death (9, 10). From these and other results, it has been concluded that CTLA-4 serves as a receptor that functions to negatively regulate T cell activation. The in vivo consequences of CTLA-4 deficiency are B7 dependent, suggesting that the lymphocyte expansion is mediated by B7 interactions with a receptor other than CTLA-4, probably with CD28 (36). The lymphoproliferative
CTLA-4-deficient phenotype is also dependent upon TCR signaling and is prevented by expression of the same Doi11.10 transgene that was demonstrated here to prevent the lymphoproliferation and CD8 skewing induced by B7-2 transgene expression.

Taken together, these results suggest that B7-dependent signaling acts through CD28 and CTLA-4 receptors to play a critical role in T cell homeostasis (Fig. 10). The overall effect of B7 costimulation is determined by interactions between B7 and two known receptors, CD28 and CTLA-4. The extensive expansion of CD4+ T cells that occurs in CTLA-4-deficient mice at physiologic levels of B7 and TCR signaling suggests that CTLA-4 preferentially inhibits activation and expansion of CD4+ cells under these conditions. The findings reported here suggest that CD28 signaling favors CD8+ T cell expansion, as reflected in the preferential CD28-dependent expansion of CD8+ cells in B7 Tg mice as well as the decreased proportion and absolute number of CD8+ T cells in B7-deficient mice. Relative to B7-deficient mice, both wild-type and Tg mice have a decreased CD4/CD8 ratio, and this effect is quantitatively greater in overexpressing B7 Tg mice, indicating that the CD4/CD8 ratio is inversely related to the quantitative level of expressed B7. Importantly, all these effects are strongly influenced by TCR expression, suggesting that the consequences of altered costimulatory signaling reflect the pre-existing activation state of a cell and the strength of its TCR signaling. The preferential effect of CD28 signaling on CD8+ commitment and expansion identified in the present studies suggests that CD4+ and CD8+ T cells differ, on the average, in their activation thresholds or in the strength of ambient TCR-mediated signaling of these populations under physiologic conditions.

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References


