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References

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Qualitative and Quantitative Effects of CD28/B7-Mediated Costimulation on Naive T Cells In Vitro

Shivanthi P. Manickasingham, Stephen M. Anderton, Christoph Burkhart, and David C. Wraith

The CD28/B7 system provides costimulatory signals necessary for optimal T cell activation. We have examined the effects of blocking B7.1 and/or B7.2 in an in vitro system using TCR transgenic T cells specific for myelin basic protein. Activation of naive T cells was found to be B7.2 dependent and not dependent on the presence of B7.1 molecules. However, increasing the strength of signal through the TCR using peptide analogues with higher affinity for MHC compensated for blockade of B7.2 molecules, suggesting that signal 1 alone can be sufficient for the activation of naive T cells. The role of B7 molecules in the differentiation of T cells was further investigated by restimulating T cells with fresh APC and peptide in B7-sufficient conditions. A down-regulation of IL-2 and IFN-γ production by T cells primed in the presence of anti-B7.2 mAb was partially overcome when high affinity peptide analogues were used to restimulate T cells. In contrast, a significant down-regulation of the differentiation of cells producing Th-2 cytokines was observed in the presence of anti-B7 Abs. Differentiation of IL-4-secreting cells was influenced by both B7.1 and B7.2, while IL-5 secretion was totally dependent on B7.2. These results suggest that B7-mediated costimulation is essential for the development of Th-2-associated cytokines, the absence of which cannot be overcome by increasing the strength of the signal through the TCR. The Journal of Immunology, 1998, 161: 3827–3835.

Full activation of naive CD4+ T cells is believed to require the engagement of the TCR by specific Ag in the context of MHC class II molecules in addition to an Ag-non-specific interaction referred to as costimulation. The best characterized costimulatory pathway involves the CD28-B7 family of costimulatory molecules. CD28 is expressed on the majority of naive and memory T cells (1), and signaling through this molecule is thought to permit the activation of Ag-specific T cells that would otherwise enter a state of anergy or nonresponsiveness (2). The B7 family of costimulatory molecules, B7.1 and B7.2, belong to the Ig superfamily and were initially thought to be only expressed on "professional" APC. Later studies have shown that B7 molecules are also expressed on murine activated T cells creating a highly complex system (3). B7.1 and B7.2 also bind to CTLA-4, a homologue of CD28 (4), although recent studies indicate that CTLA-4 delivers a negative signal to activated T cells (5).

The differential expression of B7.1 and B7.2 in addition to the fact that these molecules bind to distinct determinants on CD28 suggest that functional differences may also exist. In fact, murine B7.1 and B7.2 only share 25% amino acid homology and have marked differences in their cytoplasmic domains (17). In addition, murine B7.2 is expressed constitutively on professional APC and is rapidly induced in response to various stimuli (18, 19). B7.1, on the other hand, is expressed much later after activation and also at lower levels than B7.2 (19). However, studies into the role(s) of CD28/B7 molecules in the differentiation of Th cells have yielded several autoimmune diseases (8, 9). However, various studies using CD28-deficient mice have shown that although Ab responses were depressed, T cell proliferation could be induced after in vitro priming (10). In addition, recent studies using the nonobese diabetic (NOD) mouse as a spontaneous model of diabetes have shown that T cells from CD28−/− mice were capable of proliferating and producing IL-2 in response to the autoantigen GAD65 (9). These results indicate that cellular immunity was, to some extent, functional in CD28-deficient mice.

Recently, there has been considerable interest in whether B7.1 and B7.2 play distinct roles in the differentiation of Th subsets (11–13). CD4+ Th cells, upon antigenic stimulation, differentiate into subpopulations producing distinct spectra of cytokines and having separate effector functions (14). Th1 cells are characterized by IL-2, TNF-β, and IFN-γ production, thereby inducing a delayed-type hypersensitivity response in addition to IgG2a production (15, 16). Th2 responses, on the other hand, are characterized by IL-4, IL-5, and IL-10 production and provide effective help for humoral immunity (especially the production of IgE and IgG1 isotypes) and also possibly suppression of Th1-type responses (15, 16). Various recent reports suggest that B7.1 and B7.2 play distinct and differential roles in the induction of Th1 and Th2 responses. The differential expression of B7.1 and B7.2 in addition to the fact that these molecules bind to distinct determinants on CD28 suggest that functional differences may also exist. In fact, murine B7.1 and B7.2 only share 25% amino acid homology and have marked differences in their cytoplasmic domains (17). In addition, murine B7.2 is expressed constitutively on professional APC and is rapidly induced in response to various stimuli (18, 19). B7.1, on the other hand, is expressed much later after activation and also at lower levels than B7.2 (19). However, studies into the role(s) of CD28/B7 molecules in the differentiation of Th cells have yielded...
contradictory results. Studies by Kuchroo et al. have shown that blockade of B7.1 ameliorated experimental autoimmune encephalomyelitis (EAE), a Th1-mediated disease, whereas blockade of B7.2 exacerbated this disease (20). In contrast, anti-B7.2 treatment was seen to depress the development of diabetes, also a Th1-mediated disease, in the NOD mouse, whereas anti-B7.1 exacerbated disease (21). These findings suggest that the relationship between B7.1/B7.2 and cytokine development is not as straightforward as previously thought.

In this study we have examined the effects of blocking B7.1 and/or B7.2 on priming and subsequent Th cell differentiation in an in vitro system using TCR transgenic T cells specific for the myelin basic protein-derived peptide Ac1-9. We have tried to ascertain whether costimulation is only required under suboptimal levels of T cell activation by using altered peptide ligands of the Ag that bind with increasing affinity to class II MHC. The relative affinity of these analogues, which consisted of alanine (4A) or tyrosine (4Y) at position 4 instead of wild-type lysine, was analyzed by Fugger et al. (22). Their results showed that whereas Ac1-9 (4Y) had a relative binding affinity 50-100-fold greater than that of Ac1-9 (4A), the binding affinity of Ac1-9 was so weak that it was immeasurable. These APL have allowed us to examine whether the requirement for costimulation could be bypassed by increasing the strength of signaling to the T cell via the TCR. These studies reveal a differential requirement for costimulation for Th1 vs Th2 responses. There is a requirement for costimulation via B7.2 for Th1 responses that can be overcome by increasing the strength of the signal delivered via the TCR. Th2 responses are, on the other hand, more strictly dependent on costimulation via both B7.1 and B7.2.

Materials and Methods

Mice

Mice were bred and maintained at the Department of Pathology and Microbiology (Bristol, U.K.). Generation of the Tg4 TCR transgenic mouse, which expresses a TCR specific for the immunodominant Ac1-9 epitope of MBP, has been described previously (23). Transgenic T cells express the TCR-αβ (Vα4, Vβ2) of the Ac1-9-specific T cell hybridoma 1934.4 derived from the encephalitogenic T cell clone PJR-25 (24). These mice were used at 8 to 14 wk of age. Expression of the TCR was evaluated by two-color flow cytometry of peripheral blood using anti-CD4 (clone H129.19, Sigma) and an anti-Vβ8 mAb (F23.1). B10.PL mice were used as a source of I-A* expressing APC.

Peptide Ags

The acetylated N-terminal peptide of murine MBP (Ac1-9 AcASQKRPSQR) and the high affinity analogues with alanine and tyrosine at position 4 instead of wild-type lysine, was synthesized using F-moc chemistry on an AMS 422 multiple peptide synthesizer (Abimed, Lagenfeld, Germany).

Tissue culture medium and reagents

Cultures were maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Paisley, U.K.) supplemented with 5% FCS (Sigma, Poole, U.K.), 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 × 10^-5 M 2-ME (all from Life Technologies).

Primary stimulation of TCR transgenic splenocytes

Splenocytes from TCR transgenic mice were cultured in 96-well plates (Falcon, Becton Dickinson, Milton Keynes, U.K.) at 2 × 10^5 cells/well in 24-well plates (Falcon) with 4 µg/ml of Ac1-9 and biotinylated anti-B7 Abs or rat IgG2a (isotype control) at 20 µg/ml for 48 h. Cells were purified on a Nycoprep 1.077 g/ml animal gradient (Nycoprep) and washed, and subsequently binding of any biotinylated Ab was visualized with streptavidin-PE (Sigma). Cells were double stained with anti-CD3 (clone 29B, Sigma) and anti-1a (clone MRC OX-6, Serotec, Oxford), flow cytometric analysis was performed with a FACSScan flow cytometer (Becton Dickinson, Mountain View, CA), and data analyzed using CellQuest software (Becton Dickinson).

Results

The series of experiments described below were all performed at the same time and are therefore directly comparable. However, for the sake of clarity, results obtained with wild-type Ac1-9 will be discussed first followed by those generated with the Ac1-9 analogues 4A and 4Y.

Influence of costimulation on the primary response of transgenic T cells in vitro

Naïve spleen cells from the Tg4 transgenic mouse were stimulated in vitro with peptide Ac1-9 alone or in the presence of a control rat IgG2a, anti-B7.1, anti-B7.2, or a combination of both anti-B7 Abs. Results obtained with control rat IgG2a were equivalent to those generated without Ab. The concentration of Ab used was titrated and shown to be saturating (data not shown). T cell activation was measured by proliferation and cytokine production. There was no production of either IL-4 or IL-5, characteristic of the Th2 subset of cells (data not shown), following primary stimulation in vitro in the absence of anti-B7 mAbs. The level of proliferation appeared to correlate with IL-2 production, and both were dependent on costimulation via the B7.2 molecule (Fig. 1, A and B). Abs directed to B7.1 did not have a significant effect in isolation, but acted synergistically with anti-B7.2 Abs in reducing both IL-2 production and proliferation. IFN-γ was produced at normal levels in the presence of anti-B7.1 Abs (Fig. 1C). Production of this cytokine

Secondary stimulation of TCR transgenic T cells

Splenocytes were cultured in duplicate wells at 2 × 10^5 cells/well in 24-well plates (Falcon) with 4 µg/ml of Ac1-9 and anti-B7 Abs or rat IgG2a (isotype control) at 20 µg/ml for 48 h. Cells were purified on a Nycoprep 1.077 g/ml animal gradient (Nycoprep) and washed, and re-stimulated in the absence of B7 Abs with irradiated B10.PL APC and peptides Ac1-9 or Ac1-9 (4A or 4Y analogues) at a range of concentrations. Proliferation and cytokine production were assessed (daily over a 4-day period).

T cell proliferation

Twenty-four hours after the in vitro splenocyte assay was set up, cells were pulsed with 0.5 µCi of [3H]thymidine for 14 to 18 h. Thymidine incorporation was measured on a liquid scintillation beta counter (1450 Microbeta, Wallac, Milton Keynes, U.K.) and expressed as mean counts per minute.

Cytokine assay

Cytokine release was measured using a cell-based ELISA described by Beech et al. (25). The following cytokine-specific capture Abs were used to coat microtiter plates: JE56-1A12 (anti-IL-2), 11B11 (anti-IL-4), TRFK4 (anti-IL-5), or R4-6A2 (anti-IFN-γ). Splenocytes, previously stimulated with peptide and anti-B7 mAb, were added at approximately 1 × 10^5 cells/well and incubated for an additional 18 to 24 h. On the indicated days after culture, specifically bound cytokines were quantified using the following biotinylated secondary Abs: E56-1H4 (anti-IL-2), BVD6-24G2 (anti-IL-4), TRM25 (anti-IL-5), or XM2G1.2 (anti-IFN-γ; all from Pharmingen) followed by extravidin peroxidase (Sigma). The level of each cytokine was calculated using standard curves, obtained from known amounts of recombinant mouse cytokines (IL-4: Genzyme, Boston, MA; IL-2, IL-5, and IFN-γ: Pharmingen).

Flow cytometric analysis of B7 expression on T cells

Splenocytes were cultured as previously at 2 × 10^5 cells/well in 24-well plates (Falcon) with 4 µg/ml of Ac1-9 and biotinylated anti-B7 Abs or rat IgG2a (isotype control) at 20 µg/ml for 48 h. Cells were purified on a Nycoprep 1.077 g/ml animal gradient (Nycoprep) and washed, and subsequent binding of any biotinylated Ab was visualized with streptavidin-PE (Sigma). Cells were double stained with anti-CD3 (clone 29B, Sigma) and anti-1a (clone MRC OX-6, Serotec, Oxford), flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and data analyzed using CellQuest software (Becton Dickinson).
shown to play a role in the differentiation of IFN-γ-secreting cells at higher Ag concentrations. B7.1 was, however, completely inhibited by anti-B7.2 Abs. These results show clearly that the primary response of naive Tg4 cells in vitro is dependent on costimulation by B7.2 molecules.

**Influence of costimulation on the differentiation of Th subsets in vitro**

Tg4 spleen cells were cultured in the presence of both Ac1-9 and anti-B7 Abs and were subsequently restimulated with Ag and fresh APC after 2 to 3 days. It is clear from Figure 2, b and c, that the B7.1 molecule did not affect the differentiation of IL-2- or IFN-γ-secreting cells at higher Ag concentrations. B7.1 was, however, shown to play a role in the differentiation of IFN-γ-secreting cells at the lowest Ag dose tested and had a similar effect in two of three experiments. On the other hand, B7.2 contributed to the generation of both IL-2- and IFN-γ-secreting cells, and this effect was most marked at low doses of Ag. Despite the influence of B7.2 on IL-2 production, blockade of this molecule had only a marginal effect on proliferation (Fig. 2a). There was no evidence of synergy between B7.1 and B7.2 molecules, with, if anything, enhancement of IL-2 production and proliferation at certain Ag concentrations in the presence of anti-B7.1 Abs.

The differentiation of IL-4-secreting cells was partially reduced in the presence of either B7.1 or B7.2 Abs (Fig. 2d). In addition, there were two notable observations. First, the effect of the B7.2 molecule was most marked at a low Ag concentration. Secondly, there was no evidence for a synergistic effect of the two anti-B7 Abs on the differentiation of IL-4-secreting cells.

Blockade of B7.1 molecules resulted in a modest reduction in the differentiation of IL-5-secreting cells. In marked contrast, the differentiation of these cells was completely blocked by anti-B7.2 Abs.

The combined results shown in Figure 2 clearly distinguish the influence of B7.1 and B7.2 on the differentiation of Th1 and Th2 cells. B7.1 blockade did not affect Th1 cell differentiation and yet consistently reduced the levels of Th2-associated cytokines on re-stimulation in the absence of anti-B7 Abs. B7.2 blockade reduced the levels of both Th1- and Th2-associated cytokines. Most notably, however, the differentiation of IL-5-producing cells as opposed to IL-4-producing cells was completely dependent on costimulation by B7.2.

**Antigenic peptides with increasing affinity for MHC influence the primary response of T cells in vitro**

The primary response of naive Tg4 T cells to peptide Ac1-9 was inhibited by coculture with an anti-B7.2 mAb (Fig. 1). The dependence of the primary response on costimulation was further tested through the use of peptides with higher affinity for MHC. The results shown in Figure 3 emphasize that the primary response of Tg4 T cells does not depend on B7.1. It is clear, however, that the inhibition of proliferation and both IL-2 and IFN-γ production by anti-B7 Abs in the primary response (Fig. 1, A–C) could be partially overcome by increasing the affinity of Ac1-9 analogues for their MHC restriction element (Fig. 3). Neither the intermediate (4A) nor the high affinity (4Y) analogue could, however, completely restore the ability of cells to fully respond to Ag in vitro. Interestingly, stimulation of cells with the higher affinity peptides failed to elicit secretion of IL-5, even in the absence of anti-B7 Abs. The highest concentration of the 4Y analogue stimulated secretion of IL-4, the levels of which were on the threshold of detection (data not shown). This low level of IL-4, however, was inhibited by the anti-B7.2 mAb (data not shown).

These results show that the primary response of naive Tg4 cells in vitro is predominantly of the Th1 type. Weak responses to antigenic stimuli were dependent on costimulation by B7.2, but were independent of B7.1. The dependence on costimulation via B7.2 was partially overcome through the use of more highly antigenic peptide Ags.

**T cells primed in the absence of B7 molecules reveal a differential pattern of dependence on costimulation for the secretion of Th1- and Th2-associated cytokines when restimulated using peptides with increasing affinity for MHC**

Both the primary response and differentiation of IL-2-secreting cells in the presence of Ac1-9 were B7.2 dependent. This dependence on B7.2 for differentiation of IL-2-secreting cells was, however, totally overcome when peptides with higher affinity for MHC (peptides 4A and 4Y) were used in the restimulation studies (Fig. 4, c and d). This is in contrast to what was seen in the presence of wild-type Ac1-9, where differentiation of IL-2-secreting cells was dependent on B7.2 (Fig. 2b). The addition of Abs to B7.1 and B7.2 led paradoxically to an increase in the level of IL-2 detected. This might be explained by less vigorous growth of these cultures. This is not supported, however, by the data shown in Figure 4, a and b, for B7.1. The differentiation of T cells capable of proliferating on
secondary stimulation with Ag and fresh APC was partially inhibited by primary culture only in the presence of anti-B7.2 and not the anti-B7.1 mAb.

The secretion of IFN-γ remained B7.2 dependent despite restimulating these cells using peptides with high affinity for MHC. Whereas the use of the higher affinity peptides caused only a slight increase in the level of IFN-γ produced by control cultures, a significant increase in cytokine levels was observed in the presence of anti-B7.2 mAb compared with the same concentration of wild-type peptide (Fig. 2c). There was no evidence for a role of B7.1 in this process, nor was there significant enhancement of the response in the presence of the anti-B7.1 mAb.

The secretion of IL-4 in control cultures was increased by the addition of a peptide, Ac1-9(4Y), with high affinity for MHC (Fig. 3b) compared with the wild-type peptide (Fig. 2d). The dependence on both B7.1 and B7.2 was not overcome by addition of high affinity peptides (Fig. 5, a and b); if anything, it was further enhanced.

**FIGURE 2.** Restimulation of Tg4 T cells with wild-type Ac1-9. Splenocytes were primed in the presence or the absence of anti-B7 mAb and Ac1-9 (0.4 μg/ml). Viable cells, recovered 3 days after priming, were restimulated with a range of Ac1-9 concentrations and B10.PL APC in the absence of anti-B7 mAbs. Proliferation was measured (a) as described in Figure 1. Peak cytokine production, quantified by cell capture ELISA, was measured at 24 h (b and d), 48 h (c), and 72 h (e). The results are the means of duplicate wells and are representative of three experiments.

**FIGURE 3.** Primary proliferative T cell responses and cytokine production of Tg4 transgenic T cells following stimulation with Ac1-9 analogues. Whole splenic cells were stimulated as described in Figure 1. Cells were activated with a range of concentrations of Ac1-9 analogues, 4A (a, c, and e) and 4Y (b, d, and f). Proliferation and peak cytokine production were measured as described in Figure 1. The results are the means of duplicate wells and are representative of three experiments.
The partial dependence on B7.1 molecules, shown for peptides Ac1-9 (Fig. 2e) and Ac1-9(4A) (Fig. 5c), of IL-5 producing cells was totally overcome by using the highest affinity peptide Ac1-9(4Y) (Fig. 5d). In marked contrast to Th1 cells, the results shown in Figure 5, c and d, emphasize the B7.2 dependence of Th2 cell differentiation. Even with saturating doses of the highest affinity peptide (Ac1-9 4Y), the differentiation of IL-5-secreting cells remained totally B7.2 dependent.

These results reveal distinct patterns of dependence on costimulation for the differentiation of the Th1 and Th2 subsets of Tg4 T cells. Differentiation of cells secreting Th1 cytokines was B7.2 dependent and B7.1 independent. The B7.2 dependence for differentiation of IL-2-secreting cells could be overridden by peptides of higher affinity for MHC. In addition, B7.2 dependence for differentiation of IFN-γ-secreting cells could be partially overcome by higher affinity peptides. The results of these studies distinguish the costimulation dependence for differentiation of cells producing either IL-4 or IL-5. IL-4-producing cells were only partially dependent on either B7.1 or B7.2, and this could not be overcome with high affinity peptides. The differentiation of IL-5-producing cells was, by contrast, totally dependent on B7.2. Compared with wild-type Ac1-9, the high affinity peptides overcame the dependence on B7.1 for IL-5 secretion. However, there remains an absolute requirement for costimulation through B7.2 for IL-5 secretion, as evidenced by the fact that addition of the higher affinity peptide did not induce an overall increase in levels of IL-5 by T cells previously primed in the absence of B7.2 molecules.

Expression of B7 molecules on untreated and treated T cells

The expression of B7 molecules on activated murine T cells has been reported recently (3). It is possible that the effects we have observed on proliferation and cytokine induction in anti-B7-treated cultures may be due to direct signaling of T cells by these intact Abs rather than simply blockade of costimulatory molecules on
molecules on their own surfaces. These T cells had clearly up-regulated expression of B7 molecules compared with control, naive T cells, which displayed expression has also been reported to be markedly higher than that of T cells after treating whole splenic cells with biotinylated anti-B7 Abs displayed a mean channel number of 22.5 and 28.4 for expression of B7.1 and B7.2 molecules, respectively, on CD3+ cells (Table I). These T cells had clearly up-regulated expression of B7 molecules compared with control, naive T cells, which displayed mean channel numbers of 9.6 (B7.1) and 11.2 (B7.2). T cells that had been stimulated with Ag in the presence of anti-B7.1, anti-B7.2, or both Abs displayed mean channel numbers between 7.7 and 12.6. This shows that in our culture system, T cells required costimulation via B7.1 or B7.2 to up-regulate expression of B7 molecules on their own surfaces.

Discussion

We have examined the ability of B7.1 and B7.2 costimulatory molecules to affect the differentiation of Th1 and Th2 subsets in vitro. Our results reveal that the use of these costimulatory molecules was highly differential and depended on the following factors: 1) the composition of the starting T cell population (i.e., primary response of naive cells or their differentiation), 2) the particular cytokine assessed, and 3) the affinity of stimulating peptide.

First, activation of naive T cells was not observed to be dependent on B7.1 molecules. Blocking these molecules had little effect on proliferation or IL-2 or IFN-γ production. It is not surprising that B7.1 blockade had a minimal effect on the primary T cell response to Ac1-9, since B7.1 expression is only found on activated, not on resting, APC (18). Activation of these cells, however, was highly B7.2 dependent, in agreement with previously published findings that B7.2 is the predominant CD28 ligand early in the immune response (18, 26). The greater dependence on B7.2 compared with B7.1 probably reflects the constitutive, albeit low, level of expression of the former on APC (26). The level of B7.2 expression has also been reported to be markedly higher than that of B7.1 during the course of a primary immune response in vitro (12). Interestingly, activation of naive T cells using peptide analogues exhibiting increasing affinity for MHC revealed that the dependence on B7.2 molecules observed with wild-type Ac1-9 was partially overcome using the Ac1-9 analogues 4A and 4Y. Therefore, a lack of costimulation during a primary immune response in vitro can be counteracted by increasing the strength of the activation stimulus through the TCR.

The activation of naive T cells using supraoptimal levels of TCR signaling in the absence of costimulation has important implications for T cell activation. Our findings are not consistent with the classical two-signal model for T cell activation, where signals 1 and 2 are thought to be essential for full T cell activation, whereas provision of signal 1 alone results in T cell anergy (27, 28). The latter model of T cell activation suggests that signals 1 and 2 are qualitatively different and act in synergy to activate T cells. We have found, however, that whereas low levels of TCR stimulation in the absence of CD28 costimulation led to a drastic down-regulation of IL-2 and IFN-γ production, an increase in the strength of signal through the TCR prevented the induction of T cell unresponsiveness. This suggests that the net result of CD28 costimulation is to lower the threshold required to trigger naive CD4+ T cells, such that these cells can be efficiently activated by low concentrations of Ag in the presence of CD28 costimulation. This is similar to the findings of Viola and Lanzavecchia (29) and Teh and Teh (30). In fact, in the former study it was shown that whereas approximately 8000 TCRs need to be engaged for T cell activation in the absence of CD28 costimulation, only approximately 1500 TCRs are required in the presence of CD28 costimulation (29). This is consistent with our results, where high levels of TCR engagement can compensate, to a certain degree, for a lack of costimulation for the production of IFN-γ and IL-2.

The ability of naive T cells, stimulated in the absence of costimulatory molecules to secrete both Th1- or Th2-associated cytokines upon subsequent restimulation was assessed using fresh APC (i.e., B7-sufficient conditions). Our results reveal an intriguing pattern of dependence on costimulation by both Th1- and Th2-associated cytokines. We consistently observed that a blockade of B7.1 molecules did not affect the ability of T cells to secrete IL-2 or IFN-γ. B7.2, however, had a major role in priming T cells for both cytokines, in particular IFN-γ. This is in agreement with a recent report that B7.2 molecules significantly contribute to the production of IFN-γ production (12). Even so, the dependence on B7.2 was not obligatory, since restimulating cells (previously exposed to wild-type Ac1-9 and anti-B7.2) using high affinity peptides could partially overcome the requirement for B7.2. Therefore, increasing the strength of the signal through the TCR during a secondary encounter with Ag appears to by-pass the initial requirement for costimulation to a certain degree. This enabled higher levels of cytokines to be produced in the absence of B7.2 when Ac1-9 analogues (4A and 4Y) were used instead of wild-type peptide.

The dependence on CD28/B7-mediated costimulation for differentiation of Th2 cytokine-secreting cells was very different from that observed for Th1 cells. Whereas B7.1 blockade had little or no effect on IFN-γ or IL-2 production in response to Ac1-9, both IL-4

### Table I. Median channel fluorescence intensity of staining showing expression of B7.1 and B7.2 molecules on CD3+ spleen cells

<table>
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<tr>
<th></th>
<th>Ac1-9 Only</th>
<th>Untreated</th>
<th>Ac1-9 + Anti-B7.1</th>
<th>Ac1-9 + Anti-B7.2</th>
<th>Ac1-9 + Anti-B7.1 + Anti-B7.2</th>
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<td>Expression of B7.1 on CD3+ cells</td>
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<td>11.2</td>
<td>11.7</td>
<td>12.6</td>
<td>8.9</td>
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</tbody>
</table>

* Spleenic T cells were incubated with Ac1-9, biotinylated anti-B7.1 (20 μg ml), or anti-B7.2 (20 μg ml) or both for 48 h. Dead cells were removed by density gradient centrifugation, and expression of B7.1 and B7.2 molecules on T cells were visualized by flow cytometry after staining with streptavidin-PE and anti-CD3-FITC. Results are representative of two experiments.
and IL-5 production were partially dependent on B7.1, the absence of which inhibited cytokine levels to approximately 40 to 60% of those observed in control untreated cells. Furthermore, a lack of B7.2 abrogated IL-5 secretion while significantly reducing levels of IL-4, suggesting a greater dependence on B7 molecules by Th2 subsets compared with Th1 subsets. Interestingly, whereas increasing stimulation through the TCR (using high affinity peptides) during restimulation overcame the requirement for costimulation by Th1 subsets, levels of IL-4 remained diminished even in the presence of high affinity peptides. In addition, IL-4 levels were significantly depressed in the presence of either B7.1 or B7.2 mAbs. However, the presence of both mAbs did not abrogate IL-4 production completely, leaving residual levels of cytokine secretion that were unaffected by a lack of CD28/B7-mediated costimulation. The question arises as to which cells were capable of producing low levels of IL-4 in a CD28/B7-independent fashion. Several possibilities exist. First, it is conceivable that some IL-4-secreting cells, possibly non-T cells, could be costimulation independent. For example, mast cells have been shown to be an important source of IL-4 and may be indirectly stimulated in these cultures. Alternatively, certain T cells may have a small but significant capacity to secrete IL-4 in a costimulation-independent manner. CD4+ NK1.1 cells or yd T cells, both capable of producing IL-4 (31, 32), may have produced small amounts in these cultures. Alternatively, all naive T cells may possess a limited capacity to secrete modest amounts of IL-4. In support of this, Flavell et al. generated a transgenic mouse expressing the thymidine kinase gene driven by the IL-4 promoter (33). When these transgenic mice were treated with ganciclovir, T cell function was profoundly affected, suggesting that the majority of naive T cells expressed the thymidine kinase protein and therefore IL-4 (33).

Whereas IL-4 secretion was shown to be partially dependent on both B7.1 and B7.2 molecules, analysis of IL-5 production, also a Th2-associated cytokine, revealed very different results. The dependence on B7.1, observed during wild-type Ag stimulation and to a lesser extent with Ac1-9(4A), was completely overcome using peptide Ac1-9(4Y), which displayed the highest affinity for MHC. The marked dependence on B7.2, however, was emphasized by the fact that blockade of B7.2 during priming abolished IL-5 production, even when cells were restimulated using supraoptimal levels of TCR stimulation.

These results indicate that the dependence and utilization of B7.1 and B7.2 molecules in the differentiation of naive T cells into Th1 and Th2 subsets differ considerably. In addition, analysis of cytokine production within these subsets suggests that additional heterogeneity is present with respect to B7 usage, particularly for the Th2 subset. These effects were mediated by a lack of CD28-mediated signaling during priming of naive T cells, although it is possible that the anti-B7 Abs may have been signaling directly by ligation of B7 molecules on T cells. However, this is highly unlikely, as T cells that had been primed in the absence of costimulation do not produce significant IL-2 or IFN-γ and therefore presumably exhibit a nonactivated/null phenotype with little or no B7 expression. This point was clarified by assessing the expression of B7 molecules on T cells after incubation with Ac1-9 and anti-B7 Abs. Biotinylated B7 Abs were used for these experiments, such that up-regulation of B7 expression on T cells during the culture period could be later visualized by flow cytometry using streptavidin-PE-linked Ab. T cells primed in the absence of anti-B7 Abs up-regulated the expression of both B7.1 and B7.2 molecules compared with that in control T cells incubated in the absence of both peptide and anti-B7 Abs. This supports earlier reports that T cells do express B7 molecules after activation (3), although more recent data indicate that T cell expression of B7.2 is found in an altered hypoglycosylated form that displays reduced binding to both CD28 and CTLA-4 (34). T cells that had been primed in the presence of anti-B7.1, anti-B7.2, or both Abs were, however, found to exhibit a median channel fluorescence intensity similar to that of nonactivated T cells. In fact, where both anti-B7 Abs were present, the T cells displayed B7 at lower levels than nonactivated T cells (Table I). It is surprising that splenic cultures incubated with anti-B7.1 Ab, while having little effect on proliferation and cytokine production during priming of naive T cells, did not up-regulate the expression of either B7.1 or B7.2 molecules on CD3+ cells. There results imply that interaction of T cells with both B7.1 and B7.2 is required for up-regulation of B7 molecules on T cells.

The restimulation studies shown in Figures 2, 4, and 5 suggest that whereas an increase in the strength of the signal through the TCR can partially compensate for a lack of costimulation during priming where Th1-associated cytokines are concerned, Th2-associated cytokines are far more sensitive to an absence of CD28/B7-mediated costimulation during priming. This may be due to the autocrine nature of IL-4 (35), which would cause the differentiation of IL-4-secreting cells to be more sensitive to the initial levels of T cell priming, including the presence or absence of B7 molecules. Although IL-4 was not detected during primary stimulation, it is possible that low levels may have been produced, essential for the differentiation of naive T cells into Th2 subsets. The presence of anti-B7 Abs in these primary cultures may inhibit this low level of IL-4 secretion, thereby drastically down-regulating the production of IL-4 and IL-5 by T cells upon subsequent encounter with Ag. In fact, low levels of IL-4 were detected in primary cultures stimulated with Ac1-9(4Y) and were inhibited in the presence of anti-B7.2 mAbs. In contrast, Th1-associated cytokines can be influenced a great deal by APC-derived stimuli, i.e., IL-12 production (36), such that the secretion of IL-2 and IFN-γ may be less dependent on the magnitude of the primary T cell immune response and the presence of costimulatory molecules.

Our data support a number of recent observations made with in vivo disease models where protective Th2-type immune responses were down-regulated in the absence of B7 molecules. Blockade of B7.1 and B7.2 down-regulated the mucosal immune response (characterized by IL-4 production and B cell switching to IgE production) following oral infection of mice with the nematode *Heligmosomoides polygyrus* (37). Likewise, blockade of B7.2 inhibited recruitment of eosinophils (dependent on IL-5 secretion) into the airway mucosa following allergen exposure (38), further supporting our findings that B7.2 blockade abrogated IL-5 production. In addition, T cells from CD28−/− mice, while capable of secreting levels of IFN-γ comparable to those in wild-type mice, were unable to produce IL-4 and IL-5 (39). Therefore, the differentiation of naive T cells toward a Th2 phenotype appears to be very much dependent on and regulated by CD28/B7 engagement.

Several groups have studied the consequences of selectively inhibiting B7.1 and B7.2 in the in vivo development of autoimmune diseases. Studies made in autoimmune diabetes and EAE demonstrate new levels of complexity regarding the roles of CD28 and B7 interaction in the development of autoimmune diseases. Kuchroo et al. showed that anti-B7.1 inhibited the development of EAE and blocked the pathogenic Th1 response, whereas anti-B7.2 exacerbated EAE and blocked the Th2 response (20). These observations raise the possibility that Th1-driven autoimmune diseases such as EAE require CD28-B7.1 interaction. Similar to EAE, murine diabetes, a spontaneous autoimmune disease, is believed to be promoted by a Th1 response. However, treatment of NOD mice...
with anti-B7.2 mAb blocked the development of diabetes, whereas anti-B7.1 accelerated disease (21). Because the pathology of both these diseases is mediated by autoimmune Th1 cells, these results are difficult to reconcile. There are, however, various possibilities that may account for these observations. First, differences in tissue distribution may reflect the opposing effects of anti-B7.1 and anti-B7.2 in these autoimmune disease models. While B7.2 is selectively up-regulated on islet cells in diabetic mice, B7.1 is preferentially expressed in SML mice during clinical relapses of EAE (40). Secondly, differences in temporal expression of B7.1 and B7.2 may modulate the ongoing immune response, contributing to the paradoxical effects seen in the above disease models. In addition, the contribution of other cell surface molecules that can increase TCR signal strength (e.g., CD2) may influence the pattern of dependence on B7.1 and B7.2 (41, 42). Lastly, our results have clearly demonstrated that the affinity of agonist peptide for MHC can also drastically influence B7 dependence and cytokine secretion. Studies using various Ag concentrations have shown that whereas high Ag doses favored the development of Th1 cells only, priming with low doses led to Th2-like responses (43, 44). In this study, increasing the dose and affinity of Ag led to an overall up-regulation of both Th1- and Th2-associated cytokines. However, while the lack of B7 molecules during priming had little effect on IL-2 and IFN-γ secretion by T cells stimulated with high affinity peptides, Th2-associated cytokines were drastically down-regulated in the absence of B7 molecules. Therefore, the secretion of Th2-associated cytokines requires signal 2 during priming, the absence of which cannot be compensated for by increasing the strength of signal 1 during restimulation. This suggests that signal transduction products generated by both TCR and CD28 ligation are both distinct and necessary for optimal Th2-associated cytokine production. Additionally, these experiments demonstrated that the state of differentiation at which T cells received CD28-mediated costimulation was crucial for the development of a Th2 response. This was evidenced by the fact that Tg4 T cells that had not received costimulation during priming, despite being restimulated under B7-sufficient conditions and in the presence of high affinity peptides, down-regulated their production of Th2-associated cytokines. This suggests that signaling via CD28/B7 ligation during priming of naive T cells may be essential for subsequent Th2 cytokine production.

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References


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