B7–CD28 interaction is a late acting co-stimulatory signal for human T cell responses

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Abstract
The interaction of CD28 with one of the B7 molecules (CD80 and CD86) on professional antigen-presenting cells (APC) is generally considered as the most important co-stimulatory signal for T cell activation. APC in a resting condition express either no or only low levels of B7 molecules. These are up-regulated as a result of interactions with activated T cells, thus suggesting that B7–CD28 interaction is not required at initiation of T cell activation. To study this issue, we blocked B7–CD28 interaction at various time points after in vitro stimulation of peripheral blood T cells with allogeneic monocytes, Epstein–Barr virus-transformed B cells or soluble antigens. We observed that T cell proliferation and IL-2 production were inhibited by B7-blocking agents (CTLA-4–Ig or anti-B7 mAb) almost to the same degree when added either at initiation of culture or 24 h later. B7-blocking agents still resulted in significant inhibition of allogeneic T cell activation when added after 48 h. Furthermore, when CTLA-4–Ig was added at the start of an allogeneic T cell stimulation, addition of anti-CD28 mAb after 24 h of culture nearly fully restored T cell proliferation to control levels. Finally, we demonstrate that delayed addition of B7-blocking agents together with cyclosporin A 1 day after the onset of culture of T cells with allogeneic B cells is highly efficient to induce anergy as evaluated by lack of proliferation, cytotoxic T lymphocyte reactivity and IFN-γ or IL-5 production upon alloantigen rechallenge. Taken together, our data can explain why B7 expression on APC is not required at the time of initial APC–T cell contact, and suggest that the effect of the CD28 signal indeed consists in prolonging IL-2 production and amplifying T cell responses, rather than in providing a critical co-stimulatory signal at the time of initial TCR triggering.

Introduction
The interaction of CD28 on T cells with the B7 family (B7-1/CD80 and B7-2/CD86) molecules on professional antigen-presenting cells (APC) provides a crucial co-stimulatory signal for T cell activation (1,2). CD80 and CD86 are members of the Ig superfamily, and their expression is restricted to different types of professional APC (2,3). The CD28 molecule is expressed on the majority of T cells. Ligation of the CD28 by anti-CD28 mAb or by CD80 or CD86 co-stimulates T cell proliferation, cytokine production (in particular IL-2 production) and generation of cytotoxic activity (4–8). CTLA-4 is a second counter-receptor for both CD80 and CD86, and is expressed on activated T cells on which it functions as a negative signal receptor (9–12). A fusion protein, CTLA-4–Ig, composed of the extracellular domain of CTLA-4 and the Fc
part of a human IgG, binds CD80 and CD86 with high avidity (9), and has widely been used for blocking B7–CD28 interactions (1, 2). It has, for example, been demonstrated that CTLA-4-Ig "in vitro blocks alloantigen responses (13), and in vivo reduces specific antibody production, prolongs the survival of organ transplants (14–16) and suppresses several models of autoimmunity (1, 2).

Surprisingly in view of their important co-stimulatory activity, B7 molecules are either not expressed or have only low expression levels on professional APC. Peripheral blood dendritic cells (DC) are CD80+ and only weakly express CD86, but both molecules are rapidly induced during culture (17). On the other hand, DC generated in vitro from CD34 precursors express CD80+ earlier than CD86 in culture (3). Human peripheral blood monocytes constitutively express low levels of CD86 (7), while CD80 is expressed on monocytes only after activation with IFN-γ or granulocyte macrophage colony stimulating factor (18, 19). Resting human B cells are B7− and upon activation CD86 appears more rapidly than CD80+ (20). An important signal for induction or up-regulation of B7 molecules on all three types of professional APC is the interaction of CD40L on activated T cells with CD40 on the APC (21). The question therefore arises whether the CD28 signal should be critically delivered at the time of TCR triggering or whether it could also follow TCR triggering. The latter model would allow time for APC to up-regulate B7. In order to show at what stage of T cell activation the B7−CD28 signal exerts its action, we added CTLA-4-Ig or anti-B7 mAb at various time points of the culture to block B7−CD28 interactions in alloantigen-specific and in soluble antigen-specific T cell responses. Our experimental data indicate that B7 co-stimulation has a functional role in the late stages of T cell activation, both in naive and in memory T cells.

Methods

Cells and cell lines
Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors by Ficoll-Hypaque density centrifugation and resuspended in complete medium: RPMI 1640 (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine, streptomycin (100 µg/ml), penicillin (100 U/ml) and 5% heat-inactivated autologous plasma. Monocytes were removed by cold agglutination and the T cells were further purified by treatment with Lympho-Kwik (One Lambda, Los Angeles, CA) supplemented with complement-fixing anti-NK and anti-monocyte mAb, as reported (22). Enriched monocyte preparations were prepared by rosetting of PBMC with AET-treated sheep red blood cells, removal of E-rosetting cells and grown in our laboratory. Humanized anti-CD25 (Tac) and anti-CD122 (mikβ-1) mAb were gifts from Dr Hakimi (Hofmann-La Roche, Nutley, NJ). A cell line secreting human CTLA-4–Ig fusion protein was a gift from Dr A. Lanzavecchia (Basel Institute of Immunology, Basel, Switzerland). CTLA-4–Ig was used at a final concentration of 10 µg/ml, which was shown in preliminary experiments to completely block co-stimulation with B7-transfected cells, without having non-specific effects in B7-independent culture systems. Tuberculin was purchased from Statens Serum Institute (Copenhagen, Denmark). Herpes simplex antigens and varicella antigens were from Hoechst (Marburg, Germany), and influenza virus antigen was from Solvay-Duphar (Weest, The Netherlands). Protein extracts of Dermatophagoides pteronyssimus (Dp) were prepared from lyophilized mite cultures (kindly provided by HAL, Haarlem, The Netherlands). Dp extracts were added to the cultures in order to block IL-2 consumption (23). 6 µCi [3H]thymidine (Amersham International, Amersham, UK) per well. Proliferation of monocytes. ARC cells, an Epstein–Barr virus (EBV)-transformed B cell line, was obtained from the ATCC (Rockville, MD) (HLA-DR8, alleles: DRB7*0801).

mAb and other reagents
All mAb for functional assays were used as purified antibodies. Anti-CD28 mAb 9.3 against a functional epitope of CD28 was a gift from Dr C. June (Naval Medical Research Institute, Bethesda, MD). B7-24 mAb is an anti-B7-1 (anti-CD80), obtained from a fusion of SP2/0 murine myeloma cells with splenocytes from a mouse immunized with SI9 insect cells expressing the human CD80 molecule (23, 24). The anti-B7-2 (anti-CD80) mAb IT2.2 was purchased from Pharmingen (San Diego, CA). A second mAb to CD86 (clone IG10) was a gift from K. Lorre (Innogenetics, Gent, Belgium). These anti-CD80 and anti-CD86 mAb have been shown to block receptor binding (7, 24, 25). Clone L243 producing anti-HLA-DR and clone OKM1, producing anti-CD11b were obtained from ATCC, and grown in our laboratory. Humanized anti-CD25 (Tac) and anti-CD122 (mikβ-1) mAb were gifts from Dr Hakimi (Hofmann-La Roche, Nutley, NJ). A cell line secreting human CTLA-4–Ig fusion protein was a gift from Dr A. Lanzavecchia (Basel Institute of Immunology, Basel, Switzerland). CTLA-4–Ig was used at a final concentration of 10 µg/ml, which was shown in preliminary experiments to completely block co-stimulation with B7-transfected cells, without having non-specific effects in B7-independent culture systems. Tuberculin was purchased from Statens Serum Institute (Copenhagen, Denmark). Herpes simplex antigens and varicella antigens were from Hoechst (Marburg, Germany), and influenza virus antigen was from Solvay-Duphar (Weest, The Netherlands). Protein extracts of Dermatophagoides pteronyssimus (Dp) were prepared from lyophilized mite cultures (kindly provided by HAL, Haarlem, The Netherlands). Dp extracts were added to the cultures in order to block IL-2 consumption (23). 6 µCi [3H]thymidine (Amersham International, Amersham, UK) per well. Proliferation of monocytes. ARC cells, an Epstein–Barr virus (EBV)-transformed B cell line, was obtained from the ATCC (Rockville, MD) (HLA-DR8, alleles: DRB7*0801).

Proliferation assay
For allogeneic stimulation, T cells (0.5 × 10^6/ml) were mixed with allogeneic monocytes (0.05 × 10^6/ml) or with ARC cells (0.025 × 10^6/ml). For memory T cell activation, PBMC (1 × 10^5/ ml) were stimulated with tuberculin (20 U/ml), herpes simplex antigens (20 U/ml), varicella virus (100 µ/ml) or influenza virus (1/200 dilution) antigens. All cultures were performed in 96-well round-bottom culture microplates in 200 µl of complete culture medium. For blocking studies, either CTLA-4–Ig or anti-CD80 and/or anti-CD86 mAb were added at various time points of the culture. The cells were cultured at 37°C in an atmosphere of 5% CO_2 for 6 days. During the last 8 h of the culture period, the cells were pulsed with 1 µCi [3H]thymidine (Amersham International, Amersham, UK) per well. Proliferation of T cells was expressed as the mean c.p.m. of quadruplicate wells.

Cytokine production
T cells (1 × 10^6/ml) were mixed with allogeneic monocytes (0.1 × 10^6/ml) or with ARC cells (0.05 × 10^6/ml). Stimulation with ARC cells was performed in the presence of CsA (27). CTLA-4–Ig was added initially or at 24 h of the culture. For antigen-specific memory T cell responses, PBMC (1 × 10^6/ml) were stimulated with recall antigen in the presence or absence of CTLA-4–Ig initially or at 24 h of the culture. To measure IL-2 production, anti-Tac and mikβ-1 mAb (each at 2 µg/ml) were added to the cultures in order to block IL-2 consumption (28). Supernatants were collected after various culture
Prolonged B7–CD28 interaction

Periods. IL-2, IFN-γ and IL-5 were assayed by ELISA using pairs of mAb (anti-IL-2 Screening Line from Genzyme, Cambridge, MA, anti-IFN-γ Screening Line from Medgenix Diagnostics, Fleurus, Belgium and paired anti-IL-5 mAb from PharMingen). Ninety-six-well plastic plates (Nunc-Immuno plate) were coated overnight with anti-cytokine capture mAb. Culture supernatants or standard solutions were added together with the respective biotinylated anti-cytokine detecting mAb. The plates were incubated, washed, and subsequently peroxidase-conjugated streptavidin and tetramethylbenzidine chromogen solution were added. Optical density readings were done at 450 nm on an ELISA reader.

Anergy induction

The protocol to induce alloantigen-specific anergy has previously been published (29). In brief, T cells (1 × 10^6 cells) were mixed with ARC cells (0.05 × 10^6 cells) in 1 ml of complete culture medium on a 24-well culture plate. CsA (400 ng/ml) and anti-CD80 plus anti-CD86 mAb or CTLA-4–Ig were added at the beginning of the culture or 1 day later. After 5–6 days of incubation, the cells were washed, rested for 2 days and re-stimulated by original stimulator cells. The proliferative response and cytokine production upon alloantigen rechallenge were determined as explained above. CTL activity was measured in a 4 h target cell lysis assay using ARC cells as targets, as described (29).

Results

Addition of CTLA-4–Ig initially or at 24 h of the culture equally blocks T cell proliferation in response to allogeneic stimulation or stimulation with soluble antigen

CTLA-4–Ig was added at various time points to cultures of T cells with allogeneic monocytes. As expected, the proliferative T cell response was vigorously inhibited by CTLA-4–Ig added at the beginning of the culture, with a mean inhibition rate (± SEM) of 72 ± 10%. Interestingly, CTLA-4–Ig added at 16 or 24 h after initiation of the culture blocked the proliferation almost to the same extent as when added at culture initiation. CTLA-4–Ig still had inhibitory effects when added after 48 h of incubation (P < 0.03) (see Fig. 1A). We similarly tested whether memory T cell activation requires B7–CD28 interaction at an early stage of the activation process. To address this issue, PBMC were stimulated with recall antigen (tuberculin) in the presence or absence of CTLA-4–Ig added at various time points. As shown in Fig. 1(B), CTLA-4–Ig added at the start but also at 16 or at 24 h of the culture, significantly inhibited T cell proliferation in response to tuberculin.

Inhibition of T cell activation by delayed addition of anti-B7 mAb

Blocking mAb to B7-1 (CD80) and B7-2 (CD86) have subsequently been used to study the requirements for prolonged CD28 triggering during T cell activation. Effective blockade of allogeneic stimulation requires that both CD80 and CD86 molecules are blocked (7,25). As show in Table 1, mAb to CD80 and CD86 dose-dependently inhibited allogeneic T cell stimulation, similar to anti-HLA-DR. The data of Fig. 2 confirm

\[ \text{Table 1. Inhibition of T cell activation with mAb to CD80/CD86 in mixed leucocyte cultures}^{a} \]

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<th>mAb</th>
<th>Concentration (µg/ml)</th>
<th>Inhibition (%)</th>
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<td>Experiment 1</td>
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<td>Anti-CD86 +</td>
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<td>72</td>
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<tr>
<td>anti-CD80</td>
<td>2.5</td>
<td>75</td>
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<td>1.25</td>
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<td>Anti-CD11b</td>
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\[^{a}T} cells (10^5/well) were mixed with 10% allogeneic monocytes and cultured in 96-well flat-bottom culture plates for 6 days in the absence or presence of different concentrations of mAb as indicated. Thymidine incorporation was measured on day 6. Results for two experiments: percent inhibition.

Fig. 1. Effect of delayed addition of CTLA-4–Ig on T cell stimulation with allogeneic cells (A) and with tuberculin (B). In (A), CTLA-4–Ig (10 µg/ml) was added at various time points to cultures of T cells (0.5 × 10^6/ml) with allogeneic monocytes (0.05 × 10^6/ml). Proliferative responses were determined by measuring [3H]thymidine incorporation on day 6. The means (± SEM) of five experiments on different donors are presented. Mean thymidine incorporation was 1150 ± 256 c.p.m. for unstimulated T cell cultures. Statistical analyses show significant differences in proliferation when CTLA-4–Ig was added at time 0 (P = 0.03), at 16 h (P < 0.03) or 48 h (P < 0.03), compared to the control. In (B), CTLA-4–Ig (10 µg/ml) was added at various time points to cultures of PBMC (1 × 10^6/ml) with tuberculin (20 U/ml). The means (± SEM) of four experiments on different donors are presented. Mean thymidine incorporation (after 6 days) was 1354 ± 850 c.p.m. for unstimulated cultures. Statistical analyses show significant differences in proliferation when CTLA-4–Ig was added at time 0 (P < 0.04), or at 16 h (P < 0.04) or 24 h (P < 0.04), compared to the control.

![Image](image-url)
Fig. 2. Effect of anti-B7 mAb, added at different time points, on T cell activation induced by allogeneic monocytes (two independent experiments). T cells were cultured at a concentration of 0.5 × 10⁶ cells/ml with 10% allogeneic monocytes. mAb to CD80 and CD86 (2.5 µg/ml) or to CD11b (5 µg/ml) were added at different time points (0, 24, 48, 72 or 144 h). To the control cultures an equal value of medium was added. Proliferation was measured on day 6.

Fig. 3. Effects of mAb to CD80 or CD86, added at various time points, on T cell proliferation to several antigens. Anti-CD80 and anti-CD86 mAb (10 µg/ml), alone or in combination, were added at various time points to cultures of PBMC with tuberculin (20 U/ml), herpes simplex antigens (20 U/ml), varicella (100 mU/ml) or influenza (1/200) antigens. Proliferation was determined on day 6. Results are from one experiment.

Fig. 4. Effects of delayed addition of B7-blocking agents on allergen-induced T cell proliferation. PBMC from one allergic donor were stimulated with Dp antigens. A combination of anti-CD80 and anti-CD86 mAb (5 µg/ml) or anti-CD86 mAb alone (5 µg/ml) were added at 0, 24, 48 or 72 h after initiation of culture. One of two such experiments is shown. The proliferation was determined on day 6.

Delayed addition of anti-CD80 mAb 9.3 overcomes T cell inhibition by CTLA-4–Ig

In the next experiments, we studied whether the CD28 signal can be delayed for 24 h after TCR triggering. Purified T cells were mixed with allogeneic monocytes and CTLA-4–Ig was added at the beginning. Figure 5 shows that CTLA-4–Ig strongly inhibited T cell proliferation and that mAb 9.3 (anti-
CD28) added 1 day later overcame the inhibition. These data are in accordance with the inhibitory effects obtained by delayed addition of CTLA-4-Ig and further suggest that the CD28 signal has a functional role in late stages of T cell activation. As also shown in Fig. 5, the inhibition of the T cell proliferative response by CTLA-4-Ig was similarly reversed by addition of IL-2 after 24 h. These results suggest that late B7 co-stimulation is required for optimal IL-2 production.

CTLA-4-Ig stops IL-2 production when added 24 h after initiation of a mixed lymphocyte reaction

We therefore investigated the effect of delayed addition of CTLA-4-Ig on the production of IL-2 in cultures of T cells mixed with allogeneic monocytes or B cells. The supernatants were collected after different culture periods. It should be noted that in these cultures, IL-2 consumption was blocked with anti-IL-2R mAb. As Fig. 6(A) shows, addition of CTLA-4-Ig at the start of the experiment, but also when added 24 h after initiation of the culture, strongly reduced the production of IL-2 as compared to the control cultures. We then performed similar experiments by adding CTLA-4-Ig at the start or at 24 h of cultures of T cells mixed with an EBV-transformed B cell line (ARC) in the presence of CsA (Fig. 6B). The rationale of using CsA is that ARC cells are very strong stimulators and that the effect of CTLA-4-Ig or anti-B7 mAb on ARC-induced IL-2 production could only be observed in the presence of CsA (27,29). Under these circumstances CTLA-4-Ig again almost completely blocked IL-2 production when added at the beginning and it stopped production of IL-2 when added 24 h after the start of the culture.

Anergy can be induced by delayed addition of B7-blocking agents 24 h after initiation of the culture

Previously, we reported that the combination of anti-B7 mAb and CsA can induce reversible and alloantigen-specific T cell anergy against EBV-transformed B cells (29). If B7–CD28 interaction is mainly important in late stages of the T cell activation process, then delayed addition of B7-blocking agents together with CsA should still induce anergy. To prove this, T cells were mixed with ARC in the absence or presence of mAb to CD80 and CD86 or CTLA-4-Ig and CsA, added either at initiation or after 1 day (Fig. 7). The cells were cultured for 6 days, washed and rested for 2 days. Subsequently, a secondary stimulation was performed in the absence of blocking agents (29). Both procedures with addition of B7-blocking agents and CsA either at initiation or after 1 day were found to have the same efficiency for anergy induction, as tested at the level of T cell proliferation, of T cell cytotoxicity, and of both Th1 (IFN-γ) and Th2 (IL-5) type cytokine production (Fig. 7). Anergy could not be induced by early or delayed addition of mAb anti-CD80/anti-CD86, CTLA-4-Ig or CsA alone (not shown).
Fig. 7. Induction of anergy against alloantigens by delayed addition of B7-blocking agents and CsA. T cells (1 x 10^6/ml) from two different donors (donor 1, a and b; donor 2, c and d) were mixed with irradiated (3000 rad) ARC (0.05 x 10^6/ml) either alone (control), or in the presence of mAb to CD80 and CD86 (10 µg/ml) and CsA (400 ng/ml) (donor 1) or CTLA-4–Ig (10 µg/ml) and CsA (400 ng/ml) (donor 2), either at the start of the culture or after 1 day of the culture. The cells were subsequently washed, rested for 2 days and re-stimulated with ARC cells in the absence of blocking agents. Proliferation (a), cytotoxic activity against ARC (b), and the production of IFN-γ (c) and of IL-5 (d) on re-stimulation after 3 days (a and b) or 1 day (c and d) are shown. Cytotoxicity was measured as 51Cr release (% SR) from ARC target cells during a 4 h incubation. Cytokines were measured by ELISA.

Discussion

In this study, we investigated the effect of blocking B7–CD28 interaction at different time points of primary (alloantigen-specific) or memory (soluble antigen-induced) T cell responses. We demonstrate that addition of B7-blocking agents at 24 h and in some instances even up to 72 h of culture still significantly inhibited T cell proliferation. Furthermore, we demonstrate that delayed addition of anti-CD28 mAb can restore the T cell proliferation in cultures in which B7–CD28 interaction has been blocked at the start of experiment. We interpret these results as a strong indication that B7 co-stimulation is not required for the early stage of T cell activation but that it has a role for optimizing T cell responses by enhancing IL-2 production after the first 24 h of T cell culture. Indeed, CTLA-4–Ig added at 24 h of the culture strongly reduced further IL-2 production, while addition of IL-2 24 h after initiation of the culture in the presence of CTLA-4–Ig totally restored T cell proliferation, confirming that lack of IL-2 is responsible for decreased T cell growth. It is interesting to note that mRNA for IL-2 can be detected 7 h after stimulation of PBMC with antigens (unpublished observation) and that thus mRNA for IL-2 is present at time 24 h when CTLA-4–Ig is added. Our finding that initial or delayed addition of CTLA-4–Ig almost equally affects IL-2 production as measured between 48 and 72 h could therefore most easily be explained by late effects of CD28 signaling, either on mRNA translation or on mRNA stability. Stabilization of mRNA for cytokines has indeed been shown to occur as a result of CD28 triggering (31) and might be the major effect of this co-stimulatory pathway. An alternative explanation is provided by the findings that CD28 triggering is required for Bcl-XL up-regulation and prevention of apoptosis (32). Further studies on Bcl-XL and on apoptosis after delayed blocking of B7 might resolve these issues. Moreover, both effects are not mutually exclusive and an excess of IL-2 in the absence of CD28 triggering might also prevent apoptosis.

In our experiments, addition of mAb anti-CD80 and anti-CD86 at different time points to cultures of PBMC with recall antigens revealed that a prolonged or late CD86 signal (and not the CD80 signal) is responsible for prolongation of T cell responses. Interestingly, we observed that anti-CD80 mAb had no effect on T cell responses, also not when added after 24 or 48 h. These findings thus do not support the hypothesis that CD80, as it slowly appears on APC, functions to augment late stages of T cell activation (7). As shown in our previous study on this matter (30), an inhibitory effect of anti-B7-1 mAb could, however, be demonstrated when anti-B7-2 was also added to the cultures. Thus, B7-2 is the major co-stimulator on monocytes, while low levels of B7-1 become relevant only when no B7-2 is available. This point in fact supports the conclusion in the present manuscript, because B7-1 was clearly detectable on monocytes after 24 h of culture but not in the beginning of the culture (unpublished observations), and still B7-1 was able to co-stimulate T cells as tested in the absence of B7-2 co-stimulation (30). An alternative hypothesis is that CD80 at lower levels of expression preferentially binds to the negative signal receptor CTLA-4 and also slowly dissociates from CTLA-4 (33), so that it can mainly function to deliver a negative signal for ongoing T cell activation (10–12).

Further evidence that prolonged B7–CD28 interaction is required for the late stages of T cell activation comes from our findings on anergy induction. We used the anergy induction protocol as reported by us before (29). Addition of mAb to CD80 and CD86 or CTLA-4–Ig together with CsA to a primary mixed lymphocyte reaction with ARC as stimulators induces alloantigen-specific anergy. We now report that addition of these blocking agents can be delayed for 24 h after contact with alloantigen and still induce anergy with a similar efficiency. Anergy induction probably requires partial T cell activation interaction has been blocked at the start of experiment. We lack of IL-2 is indeed a determining factor for anergy induction during the ensuing stage of T cell expansion. Lack of IL-2 is indeed a determining factor for anergy induction during the ensuing stage of T cell expansion. Lack of IL-2 is thereby preventing T cells from entering the late stages of T cell activation even in the presence of B7-blocking agents. These partly activated T cells will thus require IL-2 in order to prevent anergy induction during the ensuing stage of T cell expansion. Lack of IL-2 is indeed a determining factor for anergy induction, as found by Boussiotis et al. (35). Blocking the B7–CD28 signal even after 24 h inhibits IL-2 production (this report) and addition of rIL-2 to the primary mixed lymphocyte reaction prevents anergy induction (29). Although some IL-2 was produced during the first 24 h, this low concentration of IL-2 apparently is unable to prevent anergy induction.

The fact that the blockade of the B7–CD28 interaction after TCR triggering and during the further process of T cell activation still can efficiently inhibit T cell responses may be
of importance for developing new therapeutic protocols for immunosuppression. One previous report demonstrated that delayed administration of CTLA-4–Ig up to 2 days after heart transplantation prolonged the survival of the graft (16). The explanation proposed by the authors is that the migration of DC to the graft site takes some time. According to our experimental results, an additional reason might be that, since CD28 co-stimulation mainly affects late stages of T cell responses, the most effective time for administration of B7-blocking agents in vivo will be at 24 h after antigen contact.

The concept of CD28 signaling as a late acting signal is also supported by recent in vivo data. The generation of CD28-deficient mice has revealed that, surprisingly, T cell responses to alloantigens and soluble antigens in these mice could still be induced (36,37). However, T cell responses in CD28-deficient mice did not reach optimal levels, also suggesting that CD28 signaling is not required for initiation of T cell activation, but rather for prolongation of T cell responses. CD28 knockout mice have also been used to confirm that the CD28 signal is important for regulating cell survival (through up-regulation of Bcl-xL) and not for early proliferation (38). Interestingly, it was also found with CD28 knockout mice that lack of CD28 co-stimulation can be compensated by a prolonged TCR stimulation, thus preventing anergy induction and leading to generation of functional T cell responses in vivo (39).

On the basis of our findings, we suggest that the major role of the B7–CD28 interaction is to enhance and to sustain IL-2 production after TCR triggering has occurred, resulting in amplification and prolongation of T cell responses. This in turn will be important for the prevention of anergy induction and possibly of apoptosis. The concept of a late acting signal provides a rationale for the fact that B7 molecules are induced or up-regulated on APC after interaction with activated T cells (17–20), largely through CD40L–CD40 interaction (21). TCR triggering alone is sufficient as an initial signal to induce CD40L on T cells (40). CD40L can then interact with CD40 on the APC and induce B7-1 or up-regulate B7-2 expression. It is thus logical that B7 is a late acting co-stimulatory signal, because otherwise, up-regulation on APC after initial TCR triggering would not be a useful event, except perhaps for interaction with CTLA-4. Moreover, the immunosuppressive effects of blocking CD40–CD40L interaction resulting in a lowering of B7 expression (21), become more understandable in the frame of the present findings.

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**Abbreviations**

APC antigen-presenting cell  
CsA cyclosporin A  
CTL cytotoxic T lymphocyte  
DC dendritic cell  
Dp *Dermatophagoides pteronisimus*  
EBV Epstein–Barr virus  
PBMC peripheral blood mononuclear cells

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