CD80, CD86 and CD40 Provide Accessory Signals in a Multiple-Step T-Cell Activation Model

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INTRODUCTION

In this article, we summarize our contributions to the knowledge of the costimulatory network involving CD40-CD40L and B7-1/B7-2-CD28 interactions. Our findings and those of many other groups suggest a model in which multiple (rather than one) accessory signals, some of them in an ordered and interdependent fashion, support T-cell activation.

THE CONCEPT OF ACCESSORY SIGNALING

The concept of 'two signals' was developed by Bretscher & Cohn (1970) and was originally derived from studies with B cells. The essence of the two-signal model of B-cell activation was that antigen receptor occupancy alone induced paralysis, whereas antigen receptor occupancy plus a second signal, delivered by another cell type, induced activation. The two-signal model was subsequently also applied

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Abbreviations: APC, antigen-presenting cell; FcγR, Fc-gamma receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; LFA, leukocyte function-associated antigen; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; PBMC, peripheral blood mononuclear cells; PGE₂, prostaglandin E₂; PTK, protein tyrosine kinase; TCR, T-cell receptor; TNF, tumor necrosis factor; CyA, cyclosporin-A; CTL, cytokine T lymphocyte.

to T-cell activation by Lafferty & Cunningham (1975). For T cells, 'signal one' is now defined as occupancy of the T-cell receptor (TCR) by a complex formed between antigenic peptide and major histocompatibility complex (MHC) molecules on the antigen-presenting cell (APC) surface. 'Signal two' is delivered by soluble costimulatory factors or by a ligand molecule on the APC surface, which binds to a distinct receptor on the T-cell surface. According to the two-signal model, T cells triggered through the TCR (signal 1) in the absence of costimulation (signal 2) become anergic (Schwartz 1990). Manipulation of costimulatory signals for T cell activation can therefore potentially be used either to induce antigen-specific non-responsiveness against cellular or molecular targets, or to enhance or induce the immunogenicity of malignant cells or micro-organisms (Schwartz 1992, Lanzavecchia 1993, Boussiotis et al. 1994b). The relative importance of several molecules involved in costimulation of T cells, is however, still debated (van Seventer 1991, Jenkins & Johnson 1993). In some *in vitro* models, costimulation can be provided by soluble ligands such as interleukin-1 β (IL-1 β) (Kuhweide et al. 1990) or IL-6 (Ceuppens et al. 1988, Lorré et al. 1990). However, the most important costimulatory signals for T-cell activation are probably cell-cell interactions through membrane-bound ligand-receptor pairs (Steinman & Young 1991). These two mechanisms might sometimes co-operate, as close proximity can be required to deliver signals by labile soluble ligands, whereas soluble ligands can act indirectly by increasing the avidity of adhesion molecules on interacting cells. Another problem arises in defining the different mechanisms by which a costimulatory activity can operate during a cell-cell interaction. There can be a functional or physical interaction at the level of intracellular signal transduction between the costimulatory receptor and the TCR, resulting in an enhancement of the TCR/CD3 complex-transduced signals. This is probably the mechanism of the costimulatory action of the CD2 receptor (Springer 1990). CD5-mediated signal transduction also strengthens the TCR/CD3-induced signalling (Ceuppens & Baroja 1986, Imboden et al. 1990, Vandenberghe & Ceuppens 1991, Osman et al. 1993). Cell adhesion molecules can also intensify the contact between T cells and the APC, and thereby allow for low avidity TCR-antigen contacts. The integrin interactions are examples of this type of costimulatory mechanism (Springer 1990, Hogg & Landis 1993). Finally, the ligand-receptor interaction can initiate its own second messenger cascade, which synergizes with the TCR signals at the level of gene activation. Of this mechanism, the interaction between B7-1 (CD80) and B7-2 (CD86) on professional APC with CD28 on T cells is the most elaborated example (reviewed by Linsley & Ledbetter 1993, June et al. 1994a, June et al. 1994b, Allison 1994). B7-1 (B7/BB1, CD80) and B7-2 (CD86) both belong to the immunoglobulin superfamily and are monomeric transmembrane glycoproteins, coded for on human chromosome 3q13.3-3q21. Human B7-1 and B7-2 have 26% identity. A second receptor for B7-1 and B7-2, closely related to CD28 (chromosome 2q33-34), has been identified as

CTLA-4 (chromosome 2q33-34), which is expressed only on activated T cells (Linsley et al. 1992a). In contrast with CD28, the function of CTLA-4 is probably to down-regulate T cell activity (Waterhouse et al. 1995), although this hypothesis is still debated (Linsley 1995). A soluble fusion protein, CTLA-4-Ig, binds the B7-1/B7-2 counterreceptors with an avidity 20-fold higher than an analogous CD28-Ig (Linsley et al. 1991, Lane et al. 1993) and has therefore been used by several groups to block interactions between CD28 or CTLA-4 and their natural ligands.

In the following discussion, we will address the question of whether this signal from B7 molecules really represents a unique second signal, and whether it is therefore a good potential target for immunomodulation. Further, we will show the relation between the CD28 and the CD40 receptors, and present a multiple-step T-cell activation model.

TRIGGERING OF THE CD28 RECEPTOR PROVIDES AN EFFECTIVE COSTIMULUS FOR T-CELL ACTIVATION *IN VITRO*

The role of CD28 as a costimulatory signal receptor was evident long before the identification of its natural ligands (Hara et al. 1985, Lesslauer et al. 1986), and has been studied by many research groups first with anti-CD28 monoclonal antibody (mAb) (reviewed by June et al. 1990), and later with B7-1 and B7-2 transfected cells as artificial APC (reviewed by Linsley & Ledbetter 1993, June et al. 1994a, June et al. 1994b, Allison 1994). The findings generally indicate that CD28 triggering provides a potent costimulus for cytokine production. The costimulatory effect of CD28 signalling results in stabilization of mRNA for several cytokine genes (Lindsten et al. 1989). CD28-derived costimulatory signals also have regulatory effects at the transcriptional level of several cytokine genes (Thompson et al. 1989). Interestingly, T-cell proliferation and IL-2 production mediated by CD28 have been reported to be relatively resistant to inhibition with cyclosporin A (CsA) (June et al. 1987). CD28 triggering provides a unique signal as evidenced by the findings of June et al., that for maximal induction of IL-2 gene expression in human T lymphocytes the involvement of three distinct intracellular signals is required (June et al. 1989). In addition to an increased intracellular calcium concentration and protein kinase C (PKC) activation (both of which can be induced by TCR triggering), a third signal derived from CD28 triggering is needed. CD28 can couple to several signal transduction pathways, depending on the valency of stimulation and on the activation state of the T cell (June et al. 1994a). We and others have shown that triggering of CD28 with mAb or with the natural ligands CD80 or CD86 induces tyrosine phosphorylation of a set of substrates which is different from those induced by the TCR (Vandenberghe et al. 1992, Lu et al. 1992, Nunès et al. 1996). Protein tyrosine kinases (PTK) that have been suggested to be involved include the src kinases p56lck and p59fyn (Hutchcroft &

Bierer 1994) and the Tec family kinase ITK/EMT (August et al. 1994, Raab et al. 1995). Various recent studies have focused on the involvement of PI3-kinase in CD28 signalling, given the presence of a YNMN motif in the CD28 cytoplasmic tail that can associate with the p85 subunit of PI3-kinase (Ward et al. 1993, Prasad et al. 1994, Crooks et al. 1995, Truitt et al. 1995, Cai et al. 1995, Ueda et al. 1995). Finally, the development of acidic sphingomyelinase has been suggested (Boucher et al. 1995). The roles of and strict requirements for these various signal transduction cascades in mediating CD28-triggered signalling at various stages of T-cell activation remain incompletely resolved at the present time.

To address the functional effects of CD28 triggering, our experimental *in vitro* system used highly purified human T cells stimulated with a polyclonal activator, and either a mAb to CD28 (Baroja et al. 1989) or B7 molecules transfected into mouse cells (de Boer et al. 1992) as accessory signals. The use of T-cell preparations completely devoid of activated T cells, and of other cell types that potentially might provide helper signals, made it possible to study the effect of CD28 triggering as the sole accessory signal. From these studies, triggering of the CD28 receptor has emerged as an important and efficient costimulatory event in TCR- or CD2- induced activation.

Costimulation of CD28 receptors induces enhanced IL-2 production and T-cell proliferation

Using the natural CD28 ligands, B7-1 or B7-2, transfected in mouse fibroblasts (3T6), we showed that CD45RO⁺ cells (but not CD45RA⁺ cells) could be activated with anti-CD3 presented on 3T6/FcγRII cells and remote costimulation via separate B7-1⁺ cells. A more stringent requirement for B7-mediated costimulation in the naive T-cell population was evident, since they required anti-CD3 and B7 to be presented on the same cell (van de Velde et al. 1993). Since B7 expression is restricted to professional APC, this finding indicates that naive T cells will be totally dependent on professional APC, and suggests a mechanism to control initiation of T-cell responses. Memory T cells, on the other hand, most probably also can be activated by antigen on non-professional APC, as might be required in secondary responses to infectious agents.

A proportion of the naive T-cell population could be stimulated when anti-CD5 instead of anti-CD3 was cross-linked on 3T6/FcγRII/B7-1 cells (Verwilghen et al. 1993). T-cell proliferation and IL-2 production could also be induced with anti-CD2 mAb (9-1+9.6) in the presence of B7-1-expressing 3T6 cells, but not with control 3T6 cells (Van Gool et al. 1993, Van Gool et al. 1995).

B7 provides a costimulatory signal for Th1- and Th2-type responses in vitro

Using *in vivo* disease models in mice, it has been suggested recently that B7-1 and B7-2 have different functional effects, in the sense that they differently influence

the induction of T-helper 1 (Th1)- or Th2-mediated immune responses (Kuchroo et al. 1995, Lenschow et al. 1995). We have addressed the potential differences between B7-1 and B7-2 by using transfected mouse cells as helper cells for human T-cell stimulation. When anti-CD3 mAb was used as polyclonal stimulator, cross-linked on 3T6/Fc γ RII/B7-1 cells, T cells produced Th1- as well as Th2-type cytokines IL-2, IL-4, IL-5, IL-6, IFN- γ , GM-CSF and TNF- α (de Boer et al. 1993, Lorré et al. 1994, Walter et al. 1994). Culture of T cells with anti-CD2 mAb (9-1+9.6) in the presence of 3T6/B7-1 cells also induced production of both Th1- and Th2-type cytokines: IL-2, IL-4, IFN- γ , IL-10 and TNF- α (Van Gool et al. 1995). Similar results were found when B7-2-transfected cells were used to provide costimulation through CD28 (unpublished observations). As shown in Figure 1, we thus did not find any difference between B7-1 and B7-2 in their capacity to help the generation of a Th1- or Th2-type response. To exclude differences in the level of expression of both B7 molecules, different concentrations of the transfected cells were used in this experiment. These results are similar to those reported by Lanier et al. (1995) and by Levine et al. (1995).

CD28-transduced effects in T cells are modulated by CsA

As already mentioned above, the CD28 costimulation pathway is largely resistant to the effects of CsA (June et al. 1987). The latter is of special interest to clinical medicine, as signalling via CD28 *in vivo* might be responsible for CsA-resistant T-cell activation during acute rejection episodes, and for the occurrence of acute graft versus host disease (GVHD) despite CsA treatment (Vogelsang & Morris 1993).

We studied the blocking capacity of CsA on the cytokine production by T cells stimulated with anti-CD3 and B7 molecules. As expected, the production of IL-2 was partially resistant to CsA. Similarly, the production of IL-4 and IL-10 was decreased but not completely blocked by CsA, while the production of IL-5 and IFN- γ was actually increased after adding CsA (unpublished results). A similar enhancement of IL-5 production by CsA under conditions of B7 costimulation has also been reported by Schandené et al. (1994). The mechanism of the divergent influence by CsA on the cytokine production of T cells costimulated through CD28 is still unclear. However, the CsA-mediated increase in the production of some Th1 and Th2 cytokines may be of considerable importance for the clinical application of CsA in immune responses when B7 molecules act as the major costimulators.

In an older study, at the time when we used anti-CD28 mAb 9.3 as costimulator, we also studied if CD28-mediated costimulation made T cells resistant against inhibition with calcitriol (Vanham et al. 1989). We demonstrated that phytohaemagglutinin (PHA)-induced T-cell activation in the presence of monocyte culture supernatants or a combination of monocyte-derived IL-1 β and IL-6 was completely blocked by calcitriol, while no inhibition was found in conditions when T cells were costimulated with mAb 9.3.

Figure 1. B7-1 and B7-2 provide costimulation for Th1 and Th2 responses. Human peripheral blood resting T cells (0.5×10^6) were stimulated with anti-CD3 mAb UCHT1 ($1 \mu\text{g/ml}$) in the presence of increasing numbers of mouse P815 cells transfected with human CD80 or CD86. Supernatants were collected after 48 h and IFN- γ and IL-4 were determined with an ELISA.

CD28 triggering induces T-cell responsiveness to IL-2

Addition of mAb 9.3 enhanced IL-2R expression on freshly isolated highly purified T cells when stimulated with PHA or cross-linked anti-CD3 mAb, and

it induced IL-2R expression on Con A-stimulated T cells (Baroja et al. 1989). CD25 expression could also be induced with anti-CD2 mAb (9-1+9.6) in the presence of 3T6/B7-1 cells, but not with control 3T6 cells (Van Gool et al. 1995). Moreover, Cerdan et al. (1995) have shown that CD28-mediated costimulation results in prolonged IL-2R expression. The induced IL-2 responsiveness is probably explained by the CD28-mediated long-term CD122 (IL-2R β) expression, which is regulated both at the transcriptional and posttranscriptional level (Cerdan et al. 1995).

Triggering of CD28 in the absence of a primary signal directly influences the T-cell responsiveness to IL-2. We first proved that cross-linking of CD28 with mAb 9.3 in the presence of IL-6 and IL-2 induced functional IL-2R on T cells (Baroja et al. 1988). In the presence of IL-2 and B7-1- or B7-2-expressing cells, a subpopulation of T cells was activated without any primary stimulus, as evidenced by the enhancements in T-cell proliferation, IFN- γ production and CD69 and CD25 expression (unpublished data). IL-1 β and IL-6 enhanced IL-2/B7-dependent T-cell activation. The induced responsiveness to IL-2 in the absence of a primary stimulus indicates that CD28 triggering does have functional effects by itself, and is not merely reinforcing the TCR-transduced signal. To explain the observed phenomena, we suggest that the B7-dependent T-cell responses to IL-2 result from IL-2 binding to the constitutively expressed IL-2R β - γ chain on resting T cells, and that B7 lowers the triggering threshold of this intermediate affinity receptor. CsA partially inhibited the T-cell proliferation induced by IL-2 and B7-CD28 interaction. Since the signal mediated through the IL-2R is probably totally CsA-resistant (Taniguchi & Minami 1993), these experiments demonstrate that the CsA-sensitive arm of the CD28-mediated intracellular signalling pathway is responsible for IL-2 responsiveness, as previously suggested by Ledbetter et al. (1990). From a functional point of view, the findings might explain how bystander T cells become non-specifically involved in an ongoing cellular immune reaction through interaction with APC. In inflammatory reactions, the local circumstances with monocytes producing IL-1 and expressing both B7-1 and B7-2 will create the conditions favoring non-specific T-cell responses to IL-2.

B7 provides a costimulatory signal for CsA-resistant and partly IL-2-independent generation of cytotoxic activity

In a next series of experiments, the effect of CD28 triggering on cytotoxic T lymphocyte (CTL) generation was studied (Van Gool et al. 1993, Van Gool et al. 1995). No generation of CTL was achieved after triggering of CD28 alone, or when T cells were stimulated with anti-CD3 mAb presented on 3T6/Fc γ RII cells. When 3T6/Fc γ RII/B7-1 cells were used to provide an accessory signal, together with cross-linking anti-CD3 or soluble anti-CD2 mAb as primary sig-

nals, a strong generation of CTL could be demonstrated. The CTL generation with anti-CD3 or anti-CD2 and B7-1 was highly resistant to CsA. In later experiments, we found that B7-2 provides a similar costimulatory signal for the generation of CsA-resistant CTL activity (unpublished results). The CsA-resistant CTL generation under conditions of B7-1 costimulation was subsequently shown to be caused by persistent IL-2 production. Indeed, the combination of CsA and anti-IL-2R mAb completely blocked the generation of CTL. To study IL-2 dependency of CTL generation, we added blocking anti-IL-2R α/β mAb combined with a neutralizing anti-IL-2 mAb to cultures that were set up for generation of cytotoxic activity, induced by either anti-CD2 mAb and B7-1, or by anti-CD2 mAb and different concentrations of rIL-2. The blocking mAb completely inhibited IL-2-induced CTL generation, while B7-induced CTL generation was only partly inhibited. The results indicate the existence of an IL-2-dependent (CsA-resistant) and an IL-2-independent (CsA-sensitive) pathway for CTL generation under conditions of B7-1 costimulation. A potential role for other cytokines such as IL-4 or TNF- α cannot be excluded, but recent studies by Guerder et al. (1995) in mice also provide evidence for a CD28-transduced signal leading to IL-2-independent CTL generation (Guerder et al. 1995).

We also analysed the role of CD28-mediated costimulation for T-helper cell-independent activation of purified human CD8⁺ T cells (Van Gool et al. 1996). CD8⁺ T cells could be activated directly with anti-CD3 mAb bound on B7-1-transfected mouse cells, without the help of CD4⁺ cells (Figure 2). CTL activity was also generated in cultures of purified CD4⁺ cells when anti-CD3 was combined with an accessory signal from B7-1. The IL-2 production by CD8⁺ cells was much lower compared with the CD4⁺ cells (Figure 2A). On the other hand, the CD8⁺ T cells had a more pronounced cytotoxic activity (Figure 2B). The CsA-resistance of CTL generation in cultures of CD8⁺ or CD4⁺ cells could again most probably be explained by the fact that there was CsA-resistant IL-2 production mediated by CD28 cosignalling. The combination of anti-IL2R mAb and CsA indeed resulted in a complete inhibition of CTL generation. These data suggest that the CsA-resistant CTL generation was mediated by residual IL-2 production in CD8⁺ and CD4⁺ T cells, which could be further blocked by the anti-IL-2/anti-IL-2R mAb. Because the IL-2 level produced by CD8⁺ cells seemed critically low, this might explain the higher sensitivity of CD8⁺ cells to inhibition with CsA. On the other hand, it has been suggested that the intracellular CD28-mediated signalling pathway in mouse CD8⁺ cells might be different to that in CD4⁺ cells, which can then also explain a possible difference in CsA-sensitivity of T-cell activation (Abe et al. 1995). Until now, we have not tested the capacity of B7-2 for costimulation of human CD8⁺ T cells. Recently, it has been shown in the mouse system that B7-2 is not able to provide costimulation for highly purified CD8⁺ T cells, suggesting a difference

Figure 2. IL-2 production (A) and generation of CTL activity (B) with anti-CD3 mAb as the primary signal and with CD80 (B7-1) as an accessory signal in cultures of T cells and of their CD8⁺ and CD4⁺ subpopulations. Peripheral blood resting human T cells and negatively selected CD8⁺ and CD4⁺ cells were incubated with mitomycin-treated P815 or P815/CD80 cells (T cell/P815 ratio=4), and anti-CD3 mAb UCHT1 (1 μ g/ml). (A) Supernatants for the detection of IL-2 were collected after 2 days. The IL-2 concentrations were determined in a bioassay on CTLL cells. (B) The cytotoxic activity of the lymphocytes was analysed after 4 days using the anti-CD3-redirected cytotoxicity assay against P815 cells (E/T ratio=20). Results are expressed as percentage of total ⁵¹Cr release (TR%). CsA (400 ng/ml) was added as indicated from the beginning of the culture period.

in the B7-1- and B7-2-mediated CD28 triggering of CD8⁺ cells (Gajewski 1996).

CD28 triggering up-regulates T-helper cell functions

A further activity that can be enhanced by CD28 triggering is the helper function for B cells. Two well-defined elements in this activity are cytokine secretion and the interaction of CD40L on activated T cells with CD40 on B cells (Banchereau et al. 1994). TCR triggering alone can induce transient CD40L expression on T cells. However, anti-CD3 mAb cross-linked on 3T6/Fc γ RII/B7-1 cells further up-regulated and prolonged CD40L expression on CD4⁺ T cells, with a maximum expression between 36 and 48 h, compared with T cells stimulated with anti-CD3 alone (de Boer et al. 1993). B7 was also able to induce the production of IL-4 and IL-5. Efficient B-cell help was further demonstrated by measuring the production of immunoglobulins after adding resting B cells to the cultures. A blocking anti-CD40 mAb could partly inhibit the immunoglobulin production, indicating the functional relevance of the CD40-CD40L interaction for B-cell help. Thus, this study indicates that B7 induces Th2 cytokine production and enhances CD40L expression on T cells, which subsequently trigger CD40 so that the B cells start producing immunoglobulins.

DISTRIBUTION OF B7 MOLECULES AND REGULATION OF THEIR EXPRESSION

In this section we discuss our results on B7 expression in tissues and on peripheral blood cells. It was important to show, firstly, that B7 expression is restricted to professional APC, because only under these conditions could it be possible for an accessory signal to control immune activation. Secondly, regulation of B7 expression on APC is of potential importance for regulating immune responses, and provides an alternative target for immunomodulation.

In situ expression of B7-1 in normal tissues and in chronic inflammation

Our data on the in situ expression of B7-1 were obtained at a time when B7-2 was unknown, and provided evidence that the expression was almost exclusively found on professional APC (Vandenberghe et al. 1993). B7-1 expression was detected on cells from three distinct lineages. First, B7-1 was strongly expressed on cells of the dendritic cell system: Langerhans cells in skin, interdigitating dendritic cells in T-cell-dependent zones of lymph nodes and spleen and veiled cells in lymph node sinuses, and dendritic cells in the medulla and at the corticomedullary junction of fetal thymus. The observation of B7-1 on cells of the dendritic cell system in normal skin and spleen suggests that B7-1 may be constitutively ex-

pressed on interdigitating dendritic cells. Second, B7-1 was detected on infiltrating monocyte-derived cells in a context of chronic granulomatous inflammation, whereas resident macrophages, such as liver Kupffer cells and macrophages in normal spleen, were negative. Lastly, the finding of B7-1 on a subset of cells of the B-cell lineage, i.e. on follicle center B cells and in the lymph node pulp, confirms the expression of B7-1 on activated B cells as also found *in vitro*. From a functional viewpoint, it is interesting to note that follicle center B-cell development is T-cell-dependent. Accordingly, T lymphocytes are invariably found within the follicle center, as well as in the lymph node pulp. *In vivo*, B7-1 expression by follicle center B cells and B cells located in the pulp may thus function in bidirectional T-B co-operation, resulting in an efficient T-dependent humoral immunity. In a further study, we found that in the mucosa of patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis), both B7-1 and heat-shock protein 60 were strongly expressed by antigen-presenting mononuclear cells, which may play a role in the initiation or maintenance of the inflammatory process (Peetermans et al. 1995).

In vitro data from several groups have shown that B7-1 is expressed on activated B cells and monocytes (Yokochi et al. 1982, Freedman et al. 1982, Freedman et al. 1991). B7-2 (B70, CD86) follows a similar expression pattern, except that it is also expressed on resting monocytes and it is expressed earlier on B cells upon activation than B7-1 (Azuma et al. 1993a, Freeman et al. 1993). Data on the expression of B7-1 and B7-2 on dendritic cells are divergent: peripheral blood dendritic cells are B7-1⁻ and express B7-2 weakly, but both molecules are rapidly induced during culture (McLellan et al. 1995). On the other hand, dendritic cells generated from CD34⁺ precursors express B7-1 before they express B7-2 (Caux et al. 1994b).

Only one exception to the concept of B7-1 restriction to professional APC has been found. Using immunohistochemical techniques, Verwilghen et al. (1994) observed the expression of B7-1 on synovial T cells in rheumatoid synovial tissues, whereas only a minority of T cells in osteoarthritic and normal synovial tissues expressed B7-1 (1994). Moreover, T cells in rheumatoid synovial tissues expressed activation markers such as HLA-DR, CD25 and CD69. B7-1 was not expressed on T cells derived from peripheral blood of patients suffering from rheumatic disease. These data suggest that a chronic activation state of T cells in tissues leads to a sustained B7-1 expression on these T cells. Also in patients with chronic liver diseases, *in situ* expression of B7-1 on T cells has been found by Barnaba et al. (1994). In the same study, *in vitro* data showed that activated T cells could act as professional APC, presenting recall antigens on MHC class II molecules, and inducing alloresponses. Several groups have reported that activated T-cell clones (Azuma et al. 1993c, Wyss-Coray et al. 1993) and T cells repeatedly activated *in vitro*, express functional B7 molecules (Sansom & Hall 1993). In our hands, B7 expression was also found on *in vitro* activated T cells. We,

however, did not find any B7-1 or B7-2 expression on peripheral blood T cells of patients with inflammatory diseases or HIV infection.

Modulation of B7 expression on monocytes

Only limited information is available on the regulation of the expression of B7-1 and B7-2. On analysis of several thousand peripheral blood samples of patients with a wide variety of diseases, peripheral blood monocytes were consistently found not to express B7-1. In contrast B7-2 was always expressed, and there was very little variation in the intensity of staining, whether the samples were from healthy controls or from patients with inflammatory diseases. Staining was done on whole blood samples, without any isolation procedure. We have then studied B7-regulation using freshly isolated peripheral blood mononuclear cells (PBMC) or monocytes (Barcy et al. 1995). As reported by Freedman et al. (1991), B7-1 was expressed on monocytes activated with IFN- γ . We found that both B7-1 and B7-2 were also markedly up-regulated after activation of monocytes with GM-CSF (Barcy et al. 1995). APC apparently can up-regulate GM-CSF production by T cells through IL-1 β or through CD28 triggering by B7 molecules (Kruger et al. 1996). Since GM-CSF itself up-regulates B7-1 expression and IL-1 β production by APC, a bi-directional regulatory feedback pathway between APC and T cells seems to modulate GM-CSF production. IL-2, IL-4 and TNF- α had no effect on the expression of both B7-1 and B7-2 on monocytes. On the other hand, cross-linking of the Fc γ R down-regulated B7 expression and, during activation of monocytes with IFN- γ or GM-CSF, prevented the up-regulation of B7-1 and B7-2. Although in these experimental conditions a large amount of TNF- α and of PGE₂ was secreted and also a minimal amount of IL-10, the lack of allo-antigenic T-cell stimulation by these monocytes was principally due to the lack of expression of B7-1 and B7-2, since anti-CD28 mAb could restore the allogeneic response. Willems et al. (1994) further showed that IL-10 could block the up-regulation of B7-1 on monocytes as induced by IFN- γ (1994), and at least part of the immunosuppressive effect of IL-10 could thus be due to inhibition of B7 expression.

In situ expression of B7-1 and B7-2 in malignant lymphoma

Many tumors express tumor-specific antigens, but it can be expected that naive T cells will not be activated but rather anergized, due to the 'non-professional' nature of the antigen presentation in the absence of critical costimulatory signals. Introduction of the genes encoding costimulatory molecules might enable antigenic tumor cells to function as professional APC. This has been supported by experiments in several animal models (Chen et al. 1992, Townsend & Allison 1993, Yang et al. 1995, Matulonis et al. 1996). Overall, the data on B7 transfection

support the two-signal hypothesis in that antigenic tumors are able to deliver signal 1 to T cells and, after transfection with the gene encoding B7, also signal 2, resulting in a tumor-specific T-cell response. By engineering tumor cells, making them act as 'professional' APC, naive T cells have been shown to differentiate into active effector cells that are also able to react with the tumor antigens on the non-transfected tumor cells throughout the body. It therefore seemed important to us to study B7 expression on human malignancies, derived from cells of the immune system. We concentrated on the *in situ* expression of B7-1 and B7-2 in malignant lymphoma. By immunohistochemical staining of tissue sections, B7-1 was found to be expressed by Reed-Sternberg cells of Hodgkin's disease, irrespective of the subtype, and by two Hodgkin's disease-derived cell lines, L428 and KM-H₂ (Delabie et al. 1993). Reed-Sternberg cells in all but one case of Hodgkin's disease studied also expressed B7-2. Reed-Sternberg cells might function as APC, explaining the large numbers of T cells present in the diseased tissues and also explaining the well-known phenomenon of T-cell rosetting around the Reed-Sternberg cells. Interestingly, B7-1 and mostly also B7-2 were also expressed by the malignant cells of anaplastic large cell lymphoma (so called Ki-1 lymphoma's). This finding extends the phenotypic similarities of anaplastic large cell lymphoma cells and Reed-Sternberg cells, which might both be derived from activated leukocytes (Stein et al. 1985, Stein et al. 1991). In Ki-1 lymphoma's, T cells are not numerous, indicating that the malignant cells do not function as APC, in spite of the expression of the accessory molecules. B7 was not expressed by the malignant cells of histiocyte-rich B-cell lymphoma (Delabie et al. 1992), a feature which distinguishes this entity from lymphocyte-predominant Hodgkin's disease or the paraganuloma subtype. Furthermore, B7 was also absent from the membrane of the malignant cells in T cell-rich B-cell lymphoma. B7-1, and in one case also B7-2, was expressed by a subset of malignant cells in follicular lymphoma but not by the malignant cells of diffuse B-cell lymphoma.

The presence of B7 on tumor cells apparently does not automatically lead to the eradication of the tumor by the immune system. The importance of tumor-specific antigens presented in the context of MHC class I and/or class II is underscored by the fact that the induction of expression of costimulatory molecules by weakly immunogenic tumors, including some tumors that do not express MHC molecules, did not result in restoration of their capacity to induce an immune response (Chen et al. 1994). Until now, all the studies on the latter have concerned murine tumor models, so it is not yet possible to extrapolate from these results what may happen in the case of spontaneous human tumors. In humans, the anatomical location, the tumor mass, the compromised immune system, and the long tumor/host relationship may contribute to making this approach less effective. Presentation of tumor antigens without sufficient costimulation by the tumor themselves likewise may induce peripheral T cells to be tolerant. However, it is still unclear whether anergy limits the effectiveness of an anti-tumor immune re-

sponse in cancer-bearing patients. A better characterization of the human spontaneous immune responses to tumors will help in finding the best therapeutical approach. A more complete understanding of which factors determine the effectiveness of costimulation-mediated therapy against different types of tumors will be necessary for the development of future applications. The question of which factors limit the effectiveness of B7 costimulatory activity in the generation of anti-tumor immunity is also exemplified by the fact that Reed-Sternberg cells in Hodgkin's disease express not only MHC antigens but also B7-1 and B7-2 molecules on their surface (Delabie et al. 1993).

DOES B7 PROVIDE THE ESSENTIAL SECOND SIGNAL FOR T-CELL ACTIVATION?

The experimental approach outlined as above allows the conclusion that CD28 ligation is an efficient costimulus for T-cell activation. It does not, however, yield information about whether CD28 ligation is absolutely required in the presence of other interactions between T cells and APC. In order to address this question, the effect of blocking the B7 molecules on professional APC was studied. If B7 is really unique, then we would expect a strong inhibitory effect of anti-B7 reagents. Further, according to the two-signal hypothesis, it should be possible to induce anergy, at least in primary immune responses against alloantigens and perhaps also during memory responses, after blocking the B7-CD28 interaction.

B7 provides a crucial second signal for T-cell activation with recall antigens

To study the requirements for costimulatory signals from B7 for memory T-cell responses against recall antigens, we used an *in vitro* culture of peripheral blood mononuclear cells in which autologous monocytes functioned as APC (Zhang et al. 1996). Tuberculin, influenza virus antigen, varicella antigen, mumps antigen, tetanus toxoid or candida albicans were used as recall antigen. Using blocking anti-B7-1 and anti-B7-2 mAb, we found that antigen-induced T-cell proliferation was highly dependent on B7-2. Costimulation through B7-1 could be demonstrated only under culture conditions where B7-2 had first been blocked. It should be noted that freshly isolated monocytes only express B7-2 but no B7-1. However, after culture in medium or in the presence of tuberculin, a transient up-regulation of both B7-2 and B7-1 was found, which could explain the costimulatory signaling of both. The fact that CTLA-4-Ig or anti-CD28 Fab inhibited antigen-specific T-cell activation to the same extent as the combination of anti-B7-1 and anti-B7-2 provides no evidence for a third costimulatory CD28 ligand other than B7-1 and B7-2. Experimental data on IL-2 and IFN- γ production correlated with the effects of anti-B7-1, anti-B7-2 and CTLA-4-Ig on T-cell proliferation, but the blocking effect of anti-B7-1 on IFN- γ production was even more clear than its

effect on proliferation. The maximal inhibitory rates for each antigen using the combination of both B7-blocking mAb were quite variable from donor to donor, but were never complete. This suggested to us that a subpopulation of the memory cells did not require B7 costimulation. To test this hypothesis, we studied the B7 dependence of (*in vitro*) preactivated T cells and of T-cell clones, using autologous monocytes as APC. In both situations, an almost total independence of B7 costimulation was found. Thus T-cell clones and recently activated memory cells do require costimulation, but costimulatory signals other than B7 are effective as well. This might have implications for treatment of autoimmune diseases, since it is unlikely that autoimmune T-cell clones that are actively involved in organ damage would still be inhibitable by B7-blocking agents.

Further evidence that T cells can become independent of CD28 triggering also comes from studies on CD57⁺ cells, which lose CD28 expression. The biological significance of these CD28⁻ cells is that they probably represent persistently activated cytotoxic memory cells (Azuma et al. 1993b). We investigated the responsiveness of T cells to costimulation with B7 in patients with HIV infection (Vingherhoets et al. 1995). We demonstrated in these patients an expanded population of CD28⁻ CD57⁺ CD8⁺ T cells that did not respond to B7-mediated costimulation, but which had *ex vivo* cytotoxic activity in an anti-CD3 redirected cytotoxicity assay. The presence of CD28⁻ CD57⁺ CD8⁺ T cells with *ex vivo* cytotoxic activity was similarly found in patients suffering from B-CLL (unpublished data).

The importance of prolonged B7-CD28-mediated costimulation for T-cell activation

We questioned whether the B7 signal was needed at the beginning of the T-cell activation process (as predicted from the two-signal model), or whether B7 also influenced the T-cell activation process at later stages. In experiments using PBMC and recall antigens, we found an inhibitory effect from anti-B7-1 mAb, although B7-1 was only clearly up-regulated on the monocytes after 24 h. This suggested a role of B7-1 in later stages of T-cell activation. Therefore, B7-blocking agents were added at various time-points either to *in vitro* cultures of human T cells mixed with allogeneic monocytes, or to cultures of PBMC stimulated with the soluble antigen tuberculin. We observed that addition of B7-blocking agents even at 24 h after initiation of culture dramatically inhibited T-cell proliferation and stopped IL-2 production. Further delayed addition of B7-blocking agents at 48 h still resulted in significant inhibition of proliferation. In contrast, anti-LFA-1 mAb could only block the MLR when added at the beginning of the culture. We therefore believe that prolonged B7-CD28 interaction is highly important and results in an enhancement of IL-2 production, leading to the prolongation of T-cell responses.

Induction of T-cell non-responsiveness against soluble antigens

The assessment of B7 as the most important costimulating ligand on APC has been made by looking to its capacity for blocking anergy induction. Tan et al. (1993), demonstrated that CTLA-4-Ig added to human MLR cultures induced alloantigen-specific hyporesponsiveness, as measured by proliferation upon restimulation with allogeneic monocytes in the absence of CTLA-4-Ig. Following a similar protocol, we have tried to induce non-responsiveness to recall antigens (unpublished data). Only in some donors did treatment with CTLA-4-Ig during *in vitro* stimulation of PBMC with tuberculin, influenza or mumps antigens induce non-responsiveness at the level of proliferation upon restimulation without CTLA-4-Ig. Antigen-specificity could be demonstrated using third-party recall antigens. Addition of IL-2 but not IL-4 during the culture with CTLA-4-Ig could prevent induction of non-responsiveness. We have no evidence yet whether non-responsiveness is really based on anergy induction. The failure to restore antigen-specific T-cell stimulation in the presence of IL-2 leaves open the possibility of other mechanisms, including clonal deletion. It is also not clear yet why non-responsiveness could be induced in PBMC cultures from some donors, but not from others. One possibility is that in some donors, memory T cells are less dependent on B7 costimulation for their activation. As explained above, this might result from recent antigen contact, a hypothesis which requires further investigation.

B7-blocking agents do not inhibit MLR against activated B cells

In an *in vitro* model, we then studied alloantigen-specific activation of freshly isolated human T cells by EBV-transformed B-cell lines, which highly express multiple adhesion molecules including CD11a-CD18, CD50, CD54, CD58, CD59, and the costimulatory molecules B7-1 and B7-2. By adding anti-B7-1 mAb, anti-B7-2 mAb or CTLA-4-Ig, which blocks both B7-1 and B7-2, we intended to identify the relative contribution of CD28-mediated costimulation for alloantigen-specific T-cell activation and the generation of antigen-specific CTL activity (Van Gool et al. 1994a). Anti-B7-1 mAb partially blocked the proliferative T-cell response against EBV-transformed B cells (CD80^{bright}CD86^{dim} ARC cells). Under these experimental conditions, the generation of cytotoxic T-cell activity was not blocked. By using CTLA-4-Ig or the combination of mAb against B7-1 and B7-2 in order to block all CD28 ligands, we did not obtain better inhibition of the T-cell activation. When CD80^{bright}CD86^{bright} RPMI-8866 cells were used as stimulators, CTLA-4-Ig or anti-B7-1 + anti-B7-2 mAb also did not block T cell activation.

Similar data were also obtained when highly purified CD8⁺ T cells were used as responders (Van Gool et al. 1996). Activation of CD8⁺ T cells by allogeneic

MHC class I on EBV-transformed B cells proceeded very efficiently (proliferation, cytotoxic activity and CD25 expression) without the help of CD4⁺ T cells, but was either not or only partially blocked by anti-B7-1 and anti-B7-2 mAb or CTLA-4-Ig. This indicates that other costimulatory signals are also effective, and that CD28 triggering is not absolutely required for initial T-cell activation by alloantigens on activated B cells. The relative importance of B7 molecules among multiple costimulatory signals might well depend on the type and activation state of the APC, rather than on the nature of the antigen (alloantigen versus soluble protein antigen).

Synergy between CsA and B7-1/B7-2-blocking agents for complete immunosuppression

Our data as summarized above thus argue against the unique role of B7 for initiating T-cell responses, and suggest instead that the B7-CD28 interaction functions to enhance and prolong T-cell activation. Many data from the literature also point in that direction. Blocking the B7-1/B7-2-CD28 interaction with CTLA-4-Ig was not sufficient for total inhibition of T-cell activation in allogeneic MLR (Tan et al. 1993), the *in vivo* effects of CTLA-4-Ig treatment were not uniformly efficacious for inhibition or prevention of immune responses (Linsley et al. 1992b, Turka et al. 1992, Lin et al. 1993, Pearson et al. 1994, Pescovitz et al. 1994, Blazar et al. 1994, Nishikawa et al. 1994), T-cell priming did not appear to be impaired in transgenic mice expressing a soluble form of CTLA-4 (Ronchese et al. 1994) and, finally, in CD28-deficient mice T-helper activity, although diminished, could still be demonstrated and delayed-type hypersensitivity and cytotoxic T-cell activity could still be induced (Shahinian et al. 1993, Green et al. 1994). On the other hand, one of the unique features of the B7-CD28 interaction is its CsA resistance. CsA can prevent T-cell activation only when the activation is totally dependent on calcium-induced calcineurin-mediated intracellular signalling (Bierer et al. 1993). The CD58/CD59-CD2 and CD54/CD50-CD11a-CD18 interactions result in increased intracellular calcium concentration and CsA-sensitive signal transduction (Sehajpal et al. 1989, van Seventer 1991, Springer 1990, Damle et al. 1993). Therefore, we have postulated that combining CsA, which affects the calcium-associated signal transduction, with agents that block the B7-1/B7-2-CD28 interaction would lead to a complete inhibition of T-cell activation.

We first used the *in vitro* model of T-cell stimulation with EBV-infected allogeneic B-cell lines, expressing multiple costimulatory molecules (Van Gool et al. 1994a). Anti-B7 or CsA each partially inhibited the induction of T-cell proliferation. CsA could not block alloantigen-induced generation of cytotoxic activity, probably as a result of CD28/CTLA-4 costimulation, which has previously been shown to induce complete CsA resistance of CTL precursors (Van Gool et al. 1993, Van Gool et al. 1995). By combining anti-B7-1 mAb with CsA, we were

able to inhibit T-cell proliferation completely induced by ARC cells (Figure 3A). Similarly, IL-2 production could be completely blocked by anti-B7-1 mAb and CsA together (Figure 3B). Furthermore, the combination of anti-B7-1 mAb and CsA could significantly block alloantigen-specific cytotoxic activity (Figure 3C). A synergistic activity of the combination of anti-B7-1 and CsA in blocking T-cell activation was found in primary as well as in secondary MLR. Although the stimulator ARC cells also express B7-2, we surprisingly found that anti-B7-1 with CsA was sufficient for complete inhibition of T-cell activation. In later experiments we found similar results with CTLA-4-Ig and CsA, or with anti-B7-1 and anti-B7-2 together with CsA, while anti-B7-2 and CsA were ineffective (unpublished data).

Similar data were obtained when highly purified CD8⁺ T cells were used as responders (Van Gool et al. 1996). CsA and B7-1/B7-2-blocking agents were synergistic in completely inhibiting activation of CD8⁺ cells in the MLR with allogeneic B-cell lines. Although professional APC thus apparently can provide multiple costimulatory signals for direct activation of CD8⁺ T cells, the signal derived from B7-1/B7-2 is unique in providing CsA resistance. Combining B7-blocking agents with CsA opens up new possibilities for treatment of GVHD and therapy-resistant autoimmune pathology.

THE COMBINATION OF CSA AND B7-1/B7-2-BLOCKING AGENTS: A NEW CONCEPT FOR ANERGY INDUCTION

As a consequence of these findings, we have investigated whether the presence of CsA and/or B7-1/B7-2-blocking agents during a primary MLR with EBV-infected B-cell lines as allogeneic stimulators, induces nonresponsiveness or anergy of human T cells, as tested upon restimulation in the absence of these agents (Van Gool et al. 1994b). T-cell non-responsiveness was primarily studied at the cytotoxic effector level, which was selected as being most relevant for transplant rejection and GVHD, and which was most difficult to achieve in other *in vitro* experiments (Otten & Germain 1991, Tan et al. 1993, Go et al. 1993). The combination

Figure 3. Anti-CD80 and CsA synergistically inhibit MLR-induced T-cell activation. Purified human T cells were stimulated in a primary MLR, using the EBV-transformed B-cell line ARC (HLA-DRw8; alleles: DRB1*0801) as stimulator (T-cell/B-cell ratio=4), with or without anti-CD80 mAb B7-24 (10 µg/ml) and/or CsA (400 ng/ml). (A) Cell proliferation, expressed as mean percentage of control ($\pm$ SEM, $n=10$), was measured by [³H]thymidine incorporation. (B) IL-2 production was quantified by a bioassay on CTLL, and is expressed as percentage of control. Supernatants were collected after 48 h. (C) CTL activity was measured in an alloantigen-specific cytotoxicity assay against the original stimulator cells, and is expressed as percentage of specific ⁵¹Cr release (SR%, mean $\pm$ SEM, $n=7$).

Figure 4. Anti-CD80 and CsA synergistically induce non-responsiveness of T cells to allo-antigen. Purified human T cells were stimulated during a primary MLR, using the EBV-transformed B-cell line ARC as stimulators (T-cell/B-cell ratio=4), without or with anti-CD80 mAb B7-24 (10 μ g/ml) and/or CsA (400 ng/ml). T cells were then washed and rested for 2 days and subsequently restimulated with ARC alone (T-cell/B-cell ratio=4). CTL activity after secondary MLR was measured in an anti-CD3-redirected cytotoxicity assay against P815 mastocytoma cells (A) and in an alloantigen-specific cytotoxicity assay against the original stimulator cells (B), and is expressed as SR% (mean $\pm$ SEM).

of anti-B7-1 mAb and CsA, but not each of them separately, prevented subsequent CTL generation (Figure 4A and B) and T-cell proliferation (Figure 4C) in a secondary MLR performed with the same stimulator cells (ARC) but in the absence of these blocking agents. Furthermore, the cells made non-responsive

Figure 4 (continued) (C) Cell proliferation in a secondary MLR, expressed as mean percentage of control ($\pm$ SEM), was measured by [3 H]thymidine incorporation. Addition of anti-CD80 and CsA to the primary MLR significantly reduced the generation of cytotoxic activity and proliferative response in the secondary MLR. Analysis of variance for A and B: $P < 10^{-4}$ and 0.02 respectively ($n=6$), analysis of variance for C: $P=0.03$ ($n=4$). (D) Cytokine production. Supernatants were collected after 48 h; IL-2 concentration was quantified by a bioassay on CTLL; IFN- γ and IL-4 were determined with an ELISA (representative experiment).

during the primary MLR with CsA and anti-B7-1 mAb also displayed no cytotoxic activity after a second restimulation (tertiary MLR), indicating the persistence of non-responsiveness (Van Gool et al. 1994b). Anti-B7-1 mAb combined with CsA also consistently induced non-responsiveness at the level of IL-2, IL-4

and IL-5 production, and hyporesponsiveness at the level of IFN- γ production (Figure 4D). Anti-B7-2 with CsA was not sufficient to induce non-responsiveness, but anti-B7-1 and anti-B7-2 together with CsA, or CTLA-4-Ig and CsA, yielded similar results as anti-B7-1 with CsA (unpublished results). A combination of blocking antibodies to adhesion molecules such as CD54 or CD58 with CsA, although immunosuppressive in the primary MLR, could not induce non-responsiveness (Van Gool et al. 1994b). Isolated CD4⁺ as well as CD8⁺ T cells primed in an MLR in the presence of both anti-B7-1 mAb (or CTLA-4-Ig) and CsA were nonresponsive upon restimulation with the same alloantigen (Van Gool et al. 1996). The alloantigen specificity of the non-responsiveness was demonstrated by using a third-party EBV-transformed B-cell line with a different MHC class II phenotype (Van Gool et al. 1994b). Furthermore, the induction of non-responsiveness was not due to clonal deletion of alloantigen-reactive T cells, since after stimulation with rIL-2 alone, anergic T cells regained strong antigen-specific cytotoxic activity (Figure 5). When anergic T cells were restimulated in a secondary MLR with B cells and rIL-2, minimal cytotoxic activity could be induced, compared to the cytotoxic activity after restimulation with rIL-2 alone, suggesting that anergy is maintained as long as the antigen is present. These data can be relevant for the *in vivo* situation, and are in accordance with findings by Ramsdell & Fowlkes (1992) on the importance of antigen persistence for maintaining a tolerant state (Ramsdell & Fowlkes 1992). Susceptibility of different organs to cellular rejection and variable ability of different organs to induce donorspecific non-reactivity is indeed reflected by their comparative content of migratory leukocytes, which cause a condition called chimerism (Starzl et al. 1993).

Since the EBV-transformed ARC cells that were used as stimulators are CD80^{bright} CD86^{dim}, we obtained indirect evidence for a major role for B7-1 compared with B7-2. Complete immunosuppression and anergy induction at the level of cytotoxicity and proliferation was indeed found after adding anti-B7-1 mAb and CsA to the MLR with EBV-transformed B cells as stimulators (Van Gool et al. 1994b). In these conditions, B7-2 apparently could not prevent anergy induction. Similar results were also obtained when RPMI-8866 cells (CD80^{bright} CD86^{dim}) were used as stimulator cells. Costimulation through B7-2, however, did occur because cells anergized by anti-B7-1 and CsA retained the capacity of residual IFN- γ production, which completely disappeared in the case of pretreatment with CTLA-4-Ig and CsA. Our finding that treatment with CTLA-4-Ig or anti-B7-1 plus anti-B7-2 in combination with CsA induced anergy, excludes the possibility that B7-2-mediated costimulation could have been required for the induction of anergy. The fact that experiments using anti-B7-1 + anti-B7-2 in combination with CsA yielded the same results as experiments using CTLA-4-Ig and CsA excludes costimulation through other B7-like molecules on EBV-transformed B cells. Comoli et al. (1995) recently confirmed our *in vitro* experimental data

Figure 5. Restimulation with IL-2 in the absence of alloantigen reverses anergy. T cells were primed with ARC (T-cell/B-cell ratio=4) or rendered non-responsive against ARC during a primary MLR with the combination of CsA (400 ng/ml) and anti-B7-1 mAb B7-24 (10 μ g/ml) as explained in Figure 4. Thereafter, cells were rested and subsequently restimulated with ARC cells and/or rIL-2 (10 U/ml). Antigen-specific CTL activity was measured after 4 days (E:T=20). The data are representative of four experiments. No LAK activity against ARC was found in control experiments with unprimed T cells cultured in IL-2 (10 U/ml) alone.

with MLR between peripheral blood mononuclear cells from unrelated donors, and demonstrated that the anergic cells act as suppressor cells.

The mechanism by which CsA in our model contributes to induction of non-responsiveness remains an intriguing problem and is in contradiction with some recent publications indicating that CsA prevents anergy induction (Sloan-Lancaster et al. 1993). As anergy induction is an active process requiring at least partial T-cell activation, it seems likely that a Ca^{2+} -independent limb of the TCR signalling pathway, unaffected by CsA, is required for induction of nonresponsiveness. As CsA affects late steps of intracellular signal transduction, changes in early signalling events after TCR triggering might be responsible for non-responsiveness (Cho et al. 1993). Lack of IL-2 is likely to play a major role in induction of anergy. IL-2 has indeed been shown to prevent the anergic state of T-cell clones, anergized by alloantigen stimulation in the presence of CTLA-4-Ig, and this effect apparently results from signalling through the common γ chain of the IL-2, IL-4

and IL-7 receptor (Boussiotis et al. 1994a). We suggest that lack of IL-2 was primarily responsible for anergy induction with anti-B7-1 and CsA (Van Gool et al. 1994b). Using both CsA and anti-B7-1 mAb, the residual IL2 production was only $\pm 2\%$ of the control level in the primary MLR (Van Gool et al. 1994a). Moreover, anergy induction could be prevented by addition of rIL-2 during the induction phase. On the other hand, blocking IL-2 activity with anti-Tac, Mik β 1 and B-G5 (anti-IL-2) mAb during the primary MLR was not sufficient for anergy induction (unpublished data). In the latter experiment, costimulation through other cytokines, which can trigger the common γ chain, might have prevented anergy induction (Boussiotis et al. 1994a), while the production of these alternative cytokines was also blocked with the combination of CsA and anti-B7.

Addition of CsA and B7-blocking agents after 24 h can still induce anergy

In a previous section we have explained that delayed addition of B7 blocking agents (up to 24 h after initial stimulation) was still effective in blocking IL-2 production. If lack of IL-2 after the initial activation phase was indeed responsible for anergy induction, then it should be possible to induce anergy with CsA and anti-B7 added 24 h after initiation of the culture. In the model with EBV-transformed B cells (ARC), we found that addition of CsA in combination with CTLA-4-Ig or anti-B7-1+anti-B7-2 mAb on day 1 of the primary MLR indeed induced anergy. Proliferation, IL-5 and IFN- γ production and alloantigen-specific cytotoxic activity were all inhibited to the same level, compared with the conditions in which the blocking agents were added at the beginning of the primary MLR. It should be taken into account that in these experiments at least some IL-2, IL-5 and IFN- γ were produced during the first 24 h of the culture. Apparently, low levels of these cytokines were not able to prevent subsequent anergy induction, most probably because they were not sufficient for IL-2R triggering. The results support the concept that anergy induction results from lack of IL-2R γ triggering after initial T-cell activation (Boussiotis et al. 1994a)

THE CD40-CD40L INTERACTION AS A COSTIMULATORY SIGNAL
FOR T CELLS

CD40 is a 45–50 kDa glycoprotein, which is a member of the tumor necrosis factor receptor superfamily, and is expressed on professional APC and on different other types of cells including tumor cells (Banchereau et al. 1994). CD40L, which is a member of the type-II transmembrane glycoprotein with homology to TNF- α , is transiently expressed on activated T cells. Crosslinking of CD40 on B cells is essential for B cell growth, isotype switching and Ig synthesis, and results in upregulation of B7 molecules (Banchereau et al. 1994). CD40 triggering on monocytes induces production of IL-12, TNF- α and IL-1 β (Alderson et al. 1993,

Shu et al. 1995, Kiener et al. 1995, Stüber et al. 1996). Moreover, B7 expression (and the ensuing antigen-presenting capacity) of monocytes and dendritic cells are up-regulated after CD40 signalling (Kiener et al. 1995, Caux et al. 1994a).

Triggering of CD40 on B-CLL tumor cells

Up-regulation of B7 expression as a result of CD40 triggering can potentially be used to enhance immunogenicity of B cell malignancies. A high B7 expression might indeed enhance the chances for recognition of these malignant cells by T cells. A recent study in our laboratory is dealing with the T-B-cell interaction and its functional consequence for the expression of B7-1 and B7-2 on freshly isolated malignant B cells (unpublished data). We could induce B7-1 and B7-2 expression in B cells from patients with B-CLL, using short term cocultures (22 h) with CD40L-transfected mouse fibroblasts. The immunogenicity of these B-CLL cells (>95% pure) was then investigated by *in vitro* primary and secondary MLR of autologous and allogeneic T cells against original and CD40-stimulated B-CLL tumor cells. Primary MLR of autologous and allogeneic T-cell subsets against unmanipulated B-CLL cells did not result in proliferation or induction of cytotoxic T cells. In contrast, CD40-stimulated B-CLL cells strongly up-regulated B7-1, B7-2 and CD54 molecules and elicited a strong proliferative response of allogeneic T cells. Moreover, allogeneic cytotoxic T cells were generated that were capable of lysing the original, unmanipulated tumor. This allogeneic response against CD40-stimulated B-CLL cells was partly inhibited by anti-B7-1 or anti-B7-2 antibodies and almost completely inhibited by CTLA-4-Ig fusion protein, demonstrating the primary role of B7-1 and B7-2 costimulatory molecules. We were not (yet?) able to induce autologous tumor-specific cytotoxic T cells. In conclusion, although B-CLL cells potentially may carry tumor-specific antigens, they clearly lack the potential to stimulate T cells that eventually recognize them. Induction of B7-1 and B7-2 costimulatory molecules strongly enhances the immunogenicity of the B-CLL tumor cells in allogeneic MLR. The failure to induce an autologous immune response may be the result of *in vivo* induction of anergy or deletion. Alternatively, inadequate presentation of putative tumor antigens in the context of MHC molecules may be the cause of non-responsiveness.

Collectively our studies indicate that under appropriate conditions, expression of costimulatory molecules of the B7-family on the tumor cells in association with MHC class I as well as with class II molecules, may lead to successful activation of both T-cell subpopulations resulting in antigen-specific immune responses and potentially therefore also in tumor rejection.

CD40L transduces a signal for T-cell activation

The interaction between CD40 and CD40L might also be important in the opposite direction. There are indeed some indications from the literature that

CD40L transduces an activating signal to the T cells (Cayabyab et al. 1994). Data on CD40 knock-out mice suggest an important signalling function of CD40L for T-cell activation (Van Essen et al. 1995). By using P815 mouse mastocytoma cells transfected with CD40, we studied the effect of CD40-mediated costimulation for polyclonal T-cell activation (Peng et al. 1996). We found that CD40- or B7-1-mediated costimulation both induced the production of Th1- and Th2-type cytokines (IL-2, IL-4, IL-5, IL-10, IFN- γ). The fact that B cells are CD40⁺, and that CD40-CD40L interaction induces Th2-type cytokine production, is thus compatible with the concept that B cells solicit their own help. In the next series of experiments, we then studied whether the presence of IL-12, a potent inducer of Th1 differentiation, would affect cytokine synthesis by T cells co-activated with CD40. CD40-CD40L interaction synergized with rIL-12 in inducing IFN- γ production. rIL-12 slightly decreased the CD40L-mediated IL-4 and IL-5 production. The mechanism of how CD40 co-operates with IL-12 to enhance IFN- γ production was independent of endogenous IL-2 production, because the synergistic activity was not found at the IL-2 level, and blocking experiments with anti-IL-2R mAb did not inhibit IFN- γ production. In this experimental set-up we also confirmed the synergy between rIL-12 and B7-1 in inducing IFN- γ production. Thus, signalling through CD40L costimulates T-cell activation without preference for Th1 or Th2 cytokines, and co-operates with IL-12 for T-cell differentiation into Th1-type T cells.

Preliminary data further indicate that anti-CD40 mAb can be used to inhibit MLR between allogeneic monocytes and T cells, further pointing to the importance of CD40-CD40L interactions for T-cell activation. Moreover synergistic effects between anti-CD40 and anti-B7 indicate the importance of both stimulatory pathways in T-cell activation.

GENERAL DISCUSSION: TOWARDS A MULTIPLE-STEP T-CELL ACTIVATION MODEL

Overwhelming evidence about the impact of costimulatory signals for complete T-cell activation has emerged and has allowed investigators to develop new strategies for immune intervention, both for suppression of the immune system (auto-immune pathology, transplant rejections and GVHD) and for stimulation of the immune system (vaccination and tumor immunology). Because the expression of B7-1 and B7-2 is restricted to 'professional' APC, while the expression of other adhesion molecules and MHC is widely distributed, because CD28 expression is restricted to T cells, and because the signal via CD28 is CsA-resistant, the B7-1/B7-2-CD28 interaction is unique. New data are in fact available which show that the classical two-signal model does not completely cover the complex process of T-cell activation. First of all, we have demonstrated that the B7-mediated costimulation, although unique in its CsA resistance, is not the only costimulating signal

for T-cell activation. Secondly, we have clearly demonstrated that B7 costimulation can be delayed for at least 24 h of T-cell activation, in contrast to the costimulation via other adhesion molecule pairs. Thirdly, the B7-CD28 interaction is closely linked to the CD40-CD40L interaction, which itself creates a cognate interaction between T cells and APC.

Our experiments confirm that the B7-CD28 interaction plays an important role in antigen- and alloantigen-induced T-cell activation. However, our results also suggest that the mere blockage of the B7-CD28 interaction during allotransplantation in humans would be insufficient to prevent transplant rejection. We assume that in our *in vitro* model, the primary costimulatory requirements are delivered mainly by CD40, CD54, CD50, CD11a-CD18, CD58, CD59 and possibly B7, and that the continued influence of the prolonged B7-CD28 interaction induces the processes (cytokine gene transcriptions and mRNA stabilization) necessary for optimal T-cell activation. Because costimulatory signals through ubiquitous adhesion molecules and/or through regulatory cytokines are apparently calcium-dependent and CsA-sensitive, the combination of CsA and agents which specifically block the B7-1/B7-2-CD28/CTLA-4 interaction, at least *in vitro*, results in complete immunosuppression and induction of alloantigen-specific anergy. The combination of B7-blocking agents and CsA was indeed highly effective and synergistic in blocking T-cell activation and inducing subsequent anergy. The idea of blocking the B7-CD28 interaction in combination with other blocking agents has led to some *in vivo* studies which confirm the concept: coblockade of the CD11a-CD18 - CD54 interaction together with blockage of the B7-CD28 interaction was highly effective in preventing acute GVHD (Blazer et al. 1995), and the combination of CsA and B7-CD28 blocking agents has been tried out in a rat heart transplantation model in which graft rejection in general is difficult to manipulate (Boiling et al. 1994).

The specific role of CD40-CD40L interaction both at the APC level and at the T-cell level, for activation as well as for differentiation, has also been studied. Conditions for prolonged costimulation through B7 are created partially by the positive feed-back mechanisms inducing prolonged CD40L expression on T cells, which not only costimulates T cells but which in return induces up-regulation or induction of B7 expression on APC.

The whole process of complete T cell activation in our view can be described in a multiple-step T-cell activation model. Based on our results and on data from the literature, we can assume that CD40L, which is rapidly induced by TCR/CD3 stimulation, binds to CD40 on APC. Subsequently, B7-1 and B7-2 expression are induced or enhanced on these APC, and monocytes or DC will produce more IL-12. B7 molecules in turn will enhance and prolong CD40L expression on T cells, and both CD28 and CD40L will serve further as efficient costimulatory receptors for B7 and CD40 to generate stronger T-cell activation. We speculate that the decision between Th1 or Th2 differentiation will not depend on B7- or CD40-

mediated costimulation at the T-cell level, but rather on the balance of cytokine productions at the APC level, which itself can be influenced by T-cell factors such as IFN- γ , GM-CSF and CD40L. When monocytes or DC are involved in the cognate T cell-APC interaction, CD40 triggering will induce IL-12 production, which co-operates with CD40L signalling for IFN- γ production. When B cells are involved in the cognate T cell-APC interaction, T cells in the absence of monocytic cytokines may start with the production of Th2 cytokines and CD40 triggering is required for the immunoglobulin heavy chain switch at the B-cell level. When dendritic cells are involved in the cognate T cell-APC interaction, CD40 triggering will induce B7 up-regulation and T cells will be activated by both CD40- and B7-mediated costimulation. The idea that B7 costimulates CD40L expression on T cells and that CD40 triggering itself induces up-regulation of B7 molecules, further argues that the B7-CD28 interaction is involved in prolonged activation of T cells. If the CD40-CD40L interaction influences the B7-CD28 interaction, theoretically we must be able to target the latter interaction by manipulating the former. These findings open new possibilities for immunotherapy, both at the level of immunosuppression and at the level of immunostimulation.

SUMMARY

In this review, a sequential multiple-step model for T-cell activation is proposed. In a series of *in vitro* studies, highly purified freshly isolated human peripheral blood T lymphocytes were stimulated through the CD28 receptor, with mAb or with natural ligands B7-1 or B7-2, along with TCR stimulation, in the absence of other costimulatory interactions. Ligation of the CD28 receptor, along with stimulation of the TCR, was found to up-regulate pleiotropic *in vitro* activities, including the secretion of both Th1 and Th2-type cytokines, B-cell help, and the development of cytotoxic activity. This costimulatory action involves CD4⁺ and CD8⁺ as well as naive and memory T-cell subsets. The expression of B7-1 and B7-2 on professional APC *in situ* in both normal and pathological tissues, and its up-regulation on monocytes by GM-CSF and IFN- γ is consistent with this role. Additional studies have addressed the contribution of interactions between CD28 and B7-1 and B7-2 in T-cell activation initiated by normal un-engineered APC, such as stimulation with recall antigens and primary MLR. Blockade of the interaction between CD28 and B7-1/B7-2 under these conditions failed to completely inhibit T-cell responses or to induce anergy. Complete inhibition and anergy were, however, induced with a combination of CsA, targeting downstream TCR-triggered signalling, as well as anti-B7-1- and anti-B7-2-directed reagents. Interestingly, and in contrast to anti-LFA-1 mAb, the addition of anti-B7-1 or anti-B7-2 reagents could be delayed until at least 48 h after the initiation of T-cell stimulation, indicating a requirement for a late interaction between CD28 and its counter-receptors. Interactions between CD40L on activated T cells and CD40

on APC may serve to sustain, enhance or prolong the presentation of B7-1 or B7-2 on the APC, and thus to prevent anergy induction, or ineffective or abortive T-cell stimulation. Based on these data a sequential multiple-step T-cell activation model is proposed, and novel strategies for immuno-intervention can be designed.

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