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Minireview

Molecules and Cells

4-1BB: Still in the Midst of Darkness

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4-1BB is a member of the tumor necrosis factor receptor superfamily. The receptor functions mainly as a costimulatory molecule in T lymphocytes. In addition, several lines of evidence have shown that interactions between 4-1BB and its ligand are involved in the antigen presentation process and the generation of cytotoxic T cells. Recent studies, however, have demonstrated that 4-1BB plays more diverse roles: Signals through 4-1BB are important for long-term survival of $CD8^+$ T cells and the induction of helper T cell anergy. Clinically, there is great interest in 4-1BB, because T-cell activation induced by anti-4-1BB monoclonal antibodies is highly efficient in the eradication of established tumor cells in mice. Now, since mice deficient in 4-1BB or the 4-1BB ligand are available, subtle roles played by 4-1BB may be revealed in the near future.

Keywords: 4-1BB; 4-1BB Ligand; Antigen Presenting Cell; Costimulation; T Lymphocyte.

Introduction

The immune system responds rapidly to foreign antigens and expands effector cells to remove them from the body. Peptides derived from antigen presenting cells (APCs) are presented to T lymphocytes which respond specifically to those peptides in the context of major histocompatibility complex (MHC) molecules. However, the interaction between the T-cell receptor (TCR) on T lymphocytes and peptide-MHC complex on APCs is generally not sufficient to induce proliferation and differentiation of T lymphocytes into effector cells. For T lymphocytes to achieve full activation responses, the second signal should be provided by costimulatory molecules, which are surface molecules on APCs that bind to specific receptors on the T lymphocytes. Without the second signal at the time of antigen presentation, a T cell may be eliminated from the pool of antigen-responsive T lymphocytes either by promoting its death or by inducing anergy (Abbas *et al*., 1997)

Many molecules have been identified as having costimulatory activity in T cell activation (reviewed in Watts and DeBenedette, 1999). Of these, CD28 and its ligands, B7-1 and B7-2, have been considered as a critical costimulatory system for the initiation of T cell response. However, not all costimulatory signals are provided by CD28, i.e. some molecules are capable of replacing CD28 or having a synergistic effect on activating T cells. These molecules include other CD28 family members (CTLA-4, ICOS/B7RP-1, B7h, and B7-H1) (Dong *et al*., 1999; Hutloff *et al*., 1999; Swallow *et al*., 1999; Yoshinaga *et al*., 1999), cell adhesion molecules (LFA-1/ICAM1, CD2/LFA-3, and CDw150), heat-stable antigen (CD24), and members of the tumor necrosis factor receptor (TNFR) and ligand families (4-1BB/4-1BBL, OX40/OX40L, CD27/CD70, CD30/CD30L, HVEM/LIGHT, and other potential members). Recently, much attention has been paid to TNFR superfamily members due to their diverse roles in the immune system as well as their costimulatory function. Since its identification a decade ago, studies on 4-1BB have focused on its role as a costimulatory stimulus in T cells. A body of evidence is also accumulating that indicates that 4-1BB has more diverse roles than originally expected. In this review, we will review various aspects of 4-1BB that have been reported in the literature and discuss the future directions of 4-1BB studies.

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Abbreviations: APCs, antigen presenting cells; MHC, major histocompatibility complex; TCR, T-cell receptor.

Molecular Biology of 4-1BB and 4-1BB Ligand

4-1BB was originally isolated by a differential screening procedure from activated mouse T-cell clones (Kwon and Weissman, 1989). The extracellular domain of 4-1BB contained an unusually large number of cysteine residues. Later, cysteine-rich pseudorepeat domains, also called the TNFR motifs, were defined from receptors for TNF-α, nerve growth factor (NGF), FasL, and other emerging molecules, and were grouped into the TNFR superfamily. 4-1BB contains four TNFR motifs, with the first one being partial, a characteristic shared by two soluble members of the TNFR superfamily, OPG (TR1) and DcR3 (TR6) (Pitti *et al*., 1998; Simonet *et al*., 1997; Yu *et al*., 1999) (Fig. 1A). The gene for the 4-1BB receptor resides on chromosome 1p36, in a cluster of related genes including those for CD30, OX40, TNFRII, HVEM (TR2), AITR (GITR), DR3 (Apo3) (Gruss and Dower, 1995; Gurney *et al*., 1999, Kwon *et al*., 1997; Marsters *et al*., 1996). This suggests that all of the genes evolved through a localized gene duplication event. Another interesting point is that except for DR3, all the genes have a costimulatory capacity in T lymphocytes in response to cognate ligand binding. In contrast to the extracellular domain, the cytoplasmic domain of TNFR members does not have any consensus sequence homology, with the exception of the "death domain", which is involved in the induction of apoptosis mediated by molecules such as Fas, and receptors for TRAIL. Interestingly, however, there is high sequence homology in the cytoplasmic domain among 4-1BB, CD27 and AITR (Kwon and Kwon, 1999; Kwon *et al*., 1999a; 1999b; Nocentini *et al*., 1997) (Fig. 1B). The *in vivo* significance for this finding is yet to be defined.

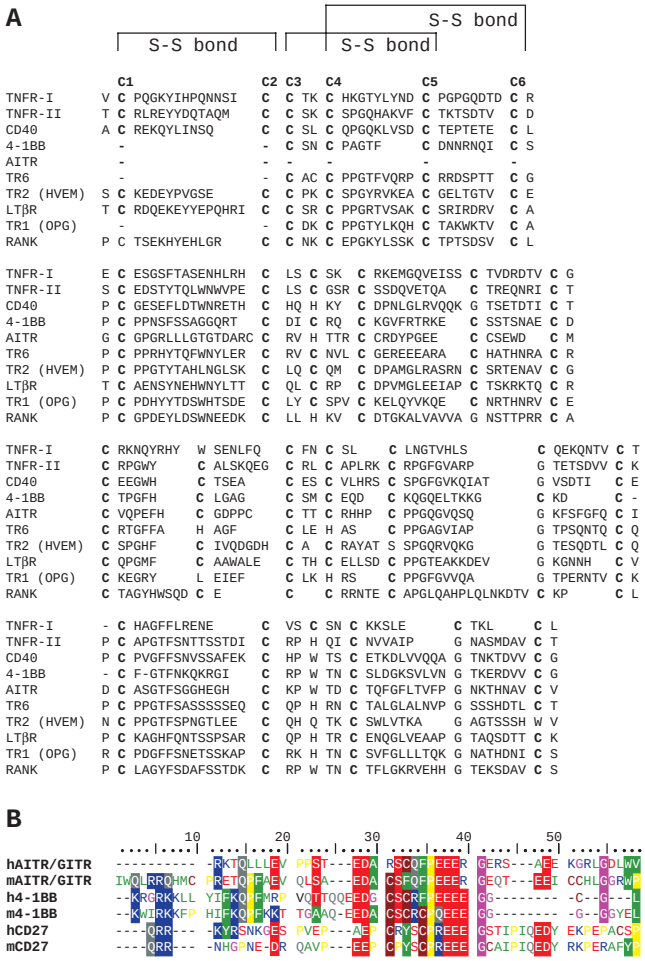

Fig. 1. A. Amino acid sequence alignment of the extracellular domain of 4-1BB with that of other TNFR members. Each of the four TNFR motifs is compared and conserved cysteine residues are shown in bold. Disulfide bonds also are shown on C1-C2, C3-C5, and C4-C6. **B.** Homology among the cytoplasmic domains of murine and human 4-1BB, AITR/GITR, and CD27.

The 4-1BB ligand (4-1BBL) is a member of the TNF ligand superfamily. In comparison with other members of the ligand superfamily, the human and mouse 4-1BBL sequences are poorly conserved (36% identity at the amino acid level). Like the receptor, the gene for 4-1BBL is clustered with those belonging to the TNF ligand superfamily such as CD27L and CD40L in the region of 19p13 (Gruss and Dower, 1995).

Signal Transduction Via 4-1BB and 4-1BBL

As is the case for other TNFR superfamily members, 4-1BB uses adaptor molecules called tumor necrosis factor receptor-associated factors (TRAFs) to transduce a downstream signal. The cytoplasmic domain of 4-1BB is able to associate specifically with TRAF1, TRAF2, and TRAF3 (Arch and Thompson, 1998; Jang *et al*., 1998; Saoulli *et al*., 1998). It possesses two universe TRAF2-binding consensus sequences, (P/S/A/T)X(Q/E)E (Ye *et al*., 1999) (Fig. 2A). Although it has not been clearly defined whether both sites are involved in the binding activity for TRAF2, point mutation analysis indicates that the C-terminal site ($PEEE_{246–250}$) is more critical for TRAF2 binding than the N-terminal site ($TTQE_{234–237}$) (Jang *et al*., 1998). Obviously, the former site is recognized by TRAF1 and TRAF3 (Jang *et al*., 1998), which suggests that all the three TRAF molecules compete to bind to that site. Therefore, there appears to exist a regulatory mechanism by which the biological actions exerted by 4-1BB are governed at this proximal step.

TRAF2 is required for the activation of the nuclear factor (NF)-κB, an important transcription factor for inflammation and for activation of another transcription factor, AP-1. In mouse T lymphocytes, ligation of the 4-1BB receptor by 4-1BBL induces association of TRAF1 and TRAF2 with the cytoplasmic domain of 4-1BB (Sauolli *et al*., 1998). However, T lymphocytes isolated from TRAF2-deficient mice or from mice overexpressing a dominant negative form of TRAF2 molecule fail to

augment IL-2 production in response to soluble 4-1BBL (Sauolli *et al.*, 1998). Current evidence suggests that the TRAF2-mediated IL-2 production by 4-1BB ligation is mediated by activation of JNK (c-Jun N-terminal kinase)/SAPK (stress-activated kinase) which is induced by apoptosis signal-regulating kinase-1 (ASK-1), a member of the mitogen-activated protein (MAP) kinase kinase kinase family (Cannons *et al.*, 1999) (See Fig. 2C). Thus, activation of JNK, rather than activation of NF-κB, appears to be essential for the costimulatory effects of 4-1BB. In fact, this conclusion is consistent with data obtained from TRAF2-deficient mice or mice expressing a dominant negative TRAF2 molecule (Lee *et al.*, 1997; Yeh *et al.*, 1997): TNF-mediated NF-κB activation is not affected in the thymocytes of these genetically engineered animals, whereas JNK activation is severely impaired.

Accumulating evidence indicates that there exists a signal transduction pathway via the 4-1BB ligand as well as via the 4-1BB receptor. Cross-linking of 4-1BBL with insect cells expressing 4-1BB induces strong B-cell proliferation synergistically with anti-IgM antibody (Pollok *et al.*, 1994). Similarly, cross-linking of 4-1BBL by soluble 4-1BB-Fc fusion protein induces monocyte activation such that the expression of IL-6, IL-8, TNF-α, and ICAM is up-regulated, whereas FcγRIII expression is down-regulated (Langstein *et al.*, 1998). Signals via 4-1BBL also lead to strong production of macrophage colony-stimulating factor (M-CSF) in monocytes, which in turn functions as a potent survival factor for monocytes (Langstein and Schwarz, 1999). In addition, the "reverse signaling" through 4-1BBL mediates monocyte proliferation by an autocrine mechanism that is as yet unknown (Langstein *et al.*, 1999). In T lymphocytes, T-cell proliferation induced by anti-CD3 antibody is inhibited by cross-linking of 4-1BBL (Schwarz *et al.*, 1996). Furthermore, this inhibition of T-cell proliferation is acompanied by programmed cell death (apoptosis). The apoptotic signal transmitted by 4-1BBL is independent of Fas, based on two observations (Michel *et al.*, 1999): (1) Antagonistic anti-Fas antibody fragments do not block 4-1BB-induced apoptosis, and (2) 4-1BB-Fc fusion protein (but not anti-Fas antibody) is capable of inducing apoptosis in resting T lymphocytes. In support of this, our observation shows that T lymphocytes isolated from 4-1BB knockout mice exhibit elevated capacity to proliferate in response to anti-CD3 antibody or mitogens, accompanied by increased resistance to activated-induced cell death, as compared to a wild type of mice. This phenomenon in 4-1BB knockout mice appears to be caused by the absence of inhibitory signals through 4-1BBL, since the elevated proliferation of T lymphocytes is abrogated by the triggering of 4-1BBL with insect cells overexpressing the 4-1BB receptor. Given these facts, it appears that 4-1BBL signaling mediates two opposite biological phenomena, i.e. cell survival and apoptosis, depending on cell types.

A. 4-1BB cytoplasmic domain

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murine  KWIRKKFPHIFKQPFKKTTGAAQEEDACSCRCPQEEEGGGGG
human   KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCG
                             TRAF2-binding TRAF2-binding
                             motif 1       motif 2
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B. 4-1BB ligand cytoplasmic domain

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murine  –  DARHPAGTSCPSDAALLR
human         MEYASDASLDPEAP
                 Casein kinase I
                 Phosphorylation site
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Fig. 2. A. TRAF2 binding motifs in the cytoplasmic domains of human and murine 4-1BB. **B.** Caseine kinase I phosphorylation site in the cytoplasmic domain of 4-1BBL.

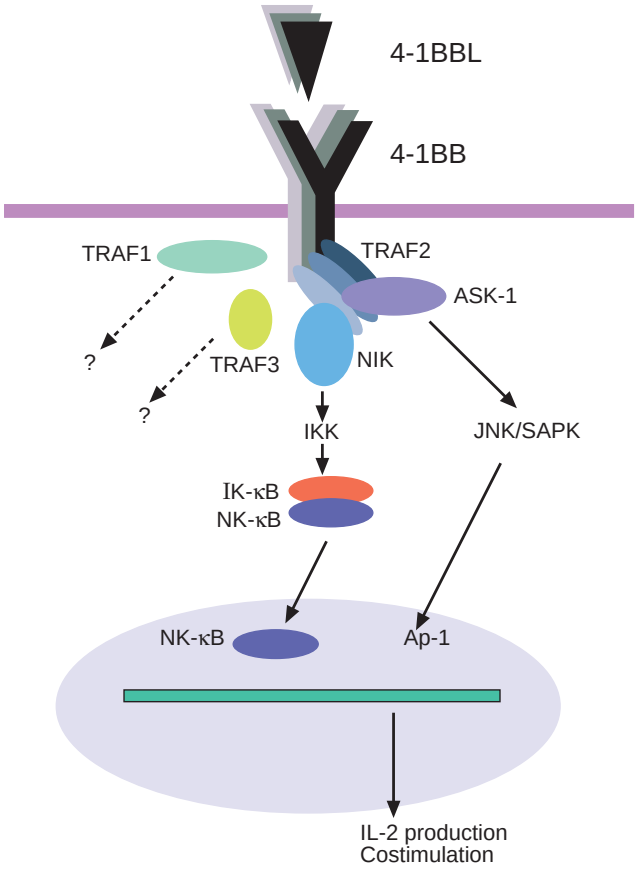

Fig. 3. Schematic diagram for the signal transduction pathways used by 4-1BB.

Even though reverse signaling through TNF ligand family members has been well documented (Grewal *et al.*, 1995; Stuber *et al.*, 1996; Suzuki and Fink, 1998; van Essen *et al.*, 1995; Wiley *et al.*, 1996), its molecular mechanism remains undiscovered. Interestingly, a phosphorylation site for casein kinase I (SXXS) is present in the cytoplasmic domains of all of the TNF ligand family

members known to utilize the reverse signaling — CD30L, 4-1BBL, CD40L, and FasL — except OX40 ligand (Watts *et al*., 1999) (Fig. 2B). In transmembrane TNF-α, this motif has been shown to be the phosphorylation target for casein kinase, and treatment of a macrophage cell line with dimeric soluble TNFR leads to the dephosphorylation of total cellular transmembrane TNF-α. One biochemical change following the dephosphorylation is an elevation of intracellular calcium levels (Watts *et al*., 1999).

Role of 4-1BB in the Immune Responses

Expression of 4-1BB is inducible in T lymphocytes by various stimuli for T-cell activation (Kwon *et al*., 1989; Pollok *et al*., 1995; Schwarz *et al*., 1995). 4-1BB mRNA is detectable by 3 h and its surface expression peaks at 40–64 h and declines again by 110 h after stimulation (Vinay and Kwon, 1998). 4-1BB is expressed primarily on activated $CD4^+$ and $CD8^+$ T cells, activated natural killer (NK) cells (Melero *et al*., 1998a), and activated NK T cells. In constrast, 4-1BBL is expressed primarily on professional APCs such as mature dendritic cells (DeBenedette *et al*., 1997) as well as activated B cells and activated macrophages (Pollok *et al*., 1994), even though its message is detectable in activated T cells (Anderson *et al*., 1994) and NK cells (Kashii *et al*., 1999). This expression pattern suggests that pairing of 4-1BB and 4-1BBL is important for interactions between APCs and T cells.

Interestingly, 4-1BB is constitutively expressed on monocytes isolated from human peripheral blood (Kienzle and von Kempis, 2000). Signals through 4-1BB markedly induce the production of IL-8 and TNF-α and activate monocytes, which in turn is able to induce B-cell apoptosis by direct cell-cell contact.

The costimulatory capacity of 4-1BB has been well demonstrated in T lymphocytes using 4-1BBL-transfected cells, antibodies raised against 4-1BB, or a soluble form of the 4-1BB receptor. 4-1BB is able to stimulate both T-cell proliferation and high-level production of IL-2 by resting T cells when the T cells are provided with potent TCR signals simultaneously (Chu *et al*., 1997; DeBenedette *et al*., 1995; 1997; Saoulli *et al*., 1998). Even though signals through the TCR are a limiting factor for T-cell activation, 4-1BB costimulation is as effective as CD28 costimulation in the induction of IL-2 when TCR signals are strong enough (Saoulli *et al*., 1998) and in the proliferation of previously activated T cells (Hurtado, *et al*., 1995; 1997).

A number of *in vitro* studies on 4-1BB costimulation have been documented, as previously mentioned. However, *in vivo* studies on the role played by 4-1BB have just begun to be evoked, being greatly helped by availability of 4-1BB or 4-1BBL knockout mice. Shufford *et al*. (1997) have provided an important clue that signals delivered by 4-1BB involves the regulation of cytotoxic T cells in the cellular immune responses to antigen: Anti-4-1BB monoclonal antibodies induce preferential proliferation of $CD8^+$ T cells. This *in vitro* observation is further confirmed *in vivo* by showing that administration of anti-4-1BB antibodies enhances the generation of cytotoxic T cells in a murine model of acute graft versus host disease (GVHD) and enhances the rapidity of cardiac allograft or skin transplant rejection. Using 4-1BBL knockout mice, Tan *et al*. (1999) observed that there is a lower efficiency in generating lymphocytic choriomeningitis virus (LCMV)-specific cytotoxic T cells in the knockout mice than in a wild type of mice. This observation is consistent with data showing that when 4-1BB and 4-1BBL interaction is blocked, $CD34^+$ hematopoietic cell- or monocyte-derived dendritic cells have a lower capacity in priming antigen-specific cytotoxic T cells (Ferrazo *et al*., 1999). Taken together, these data suggest that interactions between 4-1BB on T cells and its ligand on dendritic cells play a role in the process of antigen presentation by dendritic cells to T cells.

4-1BB is able to replace CD28 in stimulating high-level IL-2 production by resting T cells in the absence of CD28 (Chu *et al*., 1997; DeBenedette *et al*., 1997; Saoulli *et al*., 1998). Since 4-1BB must be up-regulated before providing costimulatory signals for T cells, in contrast to CD28 which is expressed constitutively, 4-1BB may play a major role in the later stages of the immune response. *In vitro*, repetitive stimulation and rest down-regulate the expression of CD28 and induce unresponsiveness to constimulatory signals through CD28 but not to those through 4-1BB in $CD4^+$ T cells (Kim *et al*., 1998). Thus, it is plausible to hypothesize that CD28 and 4-1BB play sequentially differential roles in the stages of the immune response: CD28 is more important in the induction stages of the immune response and 4-1BB is more important in perpetuating the immune response. This task of 4-1BB may be achieved by providing a survival signal as well as a costimulatory signal for T cells (Hurtado *et al*., 1997; Takahashi *et al*., 1999).

One controversial question regarding 4-1BB is whether the receptor exerts its biological functions in a T helper-specific way. In human T cells, signals through 4-1BB re-direct CD28-mediated cytokine production from Th2 type into Th1 type (Kim *et al*., 1998). In contrast, signals through 4-1BB in mouse T cells promote a Th2 response (Chu *et al*., 1997). Consistent with this, allo-stimulated naive $CD4^+$ T cells, when cultured in the presence of cytokines favoring Th1 differentiation, evolved into Th1 subsets expressing low amounts of 4-1BB molecules (Vinay and Kwon, 1999). By contrast, allo-stimulated naive $CD4^+$ T cells enforced to differentiate into Th2 type evolved into Th2 cells expressing both 4-1BB and CD28. This expression pattern accounts for the ability of 4-1BB to trigger the proliferation and IL-4 production by Th2 cells

only and for the ability of CD28 to trigger proliferation and typical cytokine secretion by both Th1 and Th2 cells. The actual outcome of Th cell phenotype may depend on the context.

Novel functions of 4-1BB have been revealed from *in vivo* experiments using agonistic monoclonal antibodies to 4-1BB (Mittler *et al*., 1999; Takahashi *et al*., 1999). Staphylococcal enterotoxin A (SEA) injection rapidly induces 4-1BB expression in both $CD4^+$ and $CD8^+$ T cells (Takahashi *et al*., 1999). The kinetic of expression is faster and peak expression level is higher in $CD8^+$ T cells over $CD4^+$ T cells. When anti-4-1BB antibody is injected at the time of immunization SEA-specific $CD8^+$ T cells show a dramatically increased survival rate, suggesting that 4-1BB may be a long-term survival factor for activated $CD8^+$ T cells. *In vivo* activation of T cells using anti-4-1BB antibody when immunized with a T-cell-dependent antigen, sheep red blood cells (SRBC), abrogates humoral immune responses against the antigen (Mittler *et al*., 1999). The effect of anti-4-1BB antibody was not observed in mice immunized with a T-cell-independent antigen such as Trinintrophenol (TNP)-Ficoll. The abrogation of humoral immune responses induced by anti-4-1BB antibody is achieved through the induction of helper T cell anergy, in which process $CD8^+$ T cells do not seem to be required. It seems that anti-4-1BB antibody directly induces anergy of $CD4^+$ T cells. This interpretation is consistent with previous findings demonstrating that anti-4-1BB antibodies profoundly costimulated anti-CD3-activated $CD8^+$ T cells but marginally activated $CD4^+$ T cells (Shufford *et al*., 1997).

Role of 4-1BB in Pathological Status

TNFR superfamily members play important roles in the body: Individuals with mutations in genes encoding for some of these molecules exhibit clinical defects. For example, mutations in Fas or FasL, and CD40L genes are the causes of autoimmune disease (Takahashi *et al*., 1994) and helper IgM syndrome (Aruffo *et al*., 1993) in man, respectively. In addition, individuals with autosomal dominant periodic fever syndromes, which are characterized by unexplained episodes of fever and severe localized inflammation, are associated with missense mutations on the TNFR1 gene (McDermott *et al*., 1999). As previously discussed, the gene for 4-1BB is in close proximity to many TNFR superfamily members. Deletions or translocation of this chromosomal region are associated with solid tumor and several hematopoietic malignancies (Schwarz *et al*., 1997). This may have an implication that malfunction of 4-1BB is a potential causative factor for some tumors. However, there are no phenotypic abnormalities found in mice deficient in 4-1BB or 4-1BBL (DeBenette *et al*., 1999; Tan *et al*., 1999). Here it is worth mentioning that a soluble form of 4-1BB is detected in the sera of patients with rheumatoid arthritis (Michel *et al*., 1998). It is derived from alternative splicing and released from activated T cells. It is not known whether a soluble form of 4-1BB functions as an antagonistic factor inhibiting membrane-bound 4-1BB from interacting with 4-1BBL.

Administration of anti-4-1BB antibodies has been shown to eradicate established large tumors in mice (Melero *et al*., 1997). Interestingly, anti-4-1BB-mediated tumor elimination is a complex process which requires $CD4^+$ T cells and NK cells as well as $CD8^+$ T cells (Melero *et al*., 1997; 1998a). Thus, it seems that the immune response induced by anti-4-1BB antibodies augment tumor-specific cytotoxic activity of $CD8^+$ T cells, which is regulated by $CD4^+$ T cells and NK cells. Similarly, the introduction of 4-1BBL into tumor cells confers full immunogenicity and thus enhances the amplification of an anti-tumor immune response (Guinn *et al*., 1999; Melero *et al*., 1998b). In this case, 4-1BBL and B7 molecules cooperatively contribute to the cytotoxic activity of $CD8^+$ T cells.

4-1BB expression is routinely detected in patients with acute infection. However, it appears that 4-1BB expression is not detectable in cancer patients and HIV-1-infected individuals (Wang *et al.*, 1998). Interestingly, the level of 4-1BB expression and the percentage of 4-1BB-expressing T cells is higher in HIV-1^+ individuals after T-cell stimulation than in HIV-1^- individuals (Wang *et al.*, 1998). This phenomenon is more remarkable in $CD8^+$ T cells than in $CD4^+$ T cells. In spite of this low response to stimulatory signals, $CD4^+$ T cells proliferate properly in response to anti-CD3 plus anti-4-1BB antibodies. In addition, signals through 4-1BB enhance HIV-1 replication in $CD4^+$ T cells presumably via NF-κB activation. The *in vivo* significance of this observation is not known.

Future Prospects

More than 20 members of the TNFR superfamily have been identified, many of which show costimulatory activity in T lymphocytes. Further, an increasing number of CD28 family members have been emerging. This raises a puzzling question: Why should there be so many costimulatory molecules? The most likely answer seems to be that each molecule serves different functions. This subtle difference among costimulatory molecules has been demonstrated. For example, stimulatory 4-1BB antibodies support preferential proliferation of $CD8^+$ T cells, while ligation of CD28 has a much larger proliferative effect on $CD4^+$ T cells than $CD8^+$ T cells (Watts and DeBenette, 1999). Similarly, CD28 is required for generating antiviral $CD4^+$ T cells responses, like CD40 and OX40, but is dispensible for $CD8^+$ T cells responses (Kopf *et al*., 1999; Whitmire *et al*., 1999). In contrast, 4-1BB is important for the generation of antiviral $CD8^+$ T cell responses (Tan *et al*., 1999). Obviously, many costimulatory molecules act

independently with overlapping functions. As previously mentioned, CD28 plays an important role in the induction stages of the immune response, whereas 4-1BB may be responsible for sustained $CD8^+$ T effector responses, as is seen in OX40 which maintains $CD8^+$ effector responses (Gramaglia *et al*., 1998). The availability of mice deficient in 4-1BB or 4-1BBL would help to clarify these two important questions.

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