Lymphocyte activation: **T-cell regulation by CTLA-4**

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Recent studies have shown that the CTLA-4 high avidity receptor for the B7 family of T-cell costimulatory molecules is also a powerful negative regulator of T-cell activation and autoreactivity.

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The initiation of immune responses usually requires interactions of T lymphocytes with antigen-presenting cells. The specificity of these interactions depends on the recognition by the T cell's antigen receptor of an antigenic peptide presented in a complex with molecules of the major histocompatibility complex (MHC). But a successful immune response requires additional costimulation of the T cell by molecules on the antigen-presenting cell. Notable amongst such costimulatory molecules are those of the B7 family, CD80 and CD86. These two molecules each bind to the T-cell receptors CD28 and CTLA-4 (cytotoxic T lymphocyte-associated molecule-4) and trigger cytokine production and activation of the T cell. Although the overall response to stimulation by the B7 family is strong activation, it has recently become apparent that triggering CTLA-4 has inherently different effects on T-cell activation than does triggering CD28 (reviewed in [1,2]). Here we discuss recent developments showing that CTLA-4 is an important negative regulator of T-cell activation.

CTLA-4 genetics and biochemistry

CTLA-4 was first identified in a search for genes expressed preferentially in cytotoxic T cells: its cDNA clone was detected in a library of cDNAs from a cytotoxic T-cell line following subtraction of cDNAs hybridizing to RNAs from a B-cell lymphoma [3]. Early studies showed that CTLA-4 shares amino-acid sequence homology with CD28, another molecule of the immunoglobulin superfamily that has only a single V-type domain. Both CTLA-4 and CD28 are homodimers with a single interchain disulfide bond. Moreover, the CTLA-4 and CD28 genes map in the same chromosomal regions in mouse and man, and are about 100 kilobases apart in the human genome. These findings suggest that the two genes are the products of a pre-speciation duplication event. CTLA-4 and CD28 have similar lymphocyte-restricted tissue distribution, but CTLA-4 is expressed only after lymphocyte activation whereas CD28 is also expressed on resting lymphocytes.

The amino-acid sequence of the cytoplasmic domain of CTLA-4 is completely conserved between man, mouse, rat and rabbit, strongly suggesting that the region has an important function. The cytoplasmic tail of CTLA-4 includes a binding site (-Tyr–Val–Lys–Met-) for the Src homology 2 (SH2) domains of the p85 subunit of phosphatidylinositol (PI) 3-kinase, which is commonly an element of intracellular signal transduction pathways. There is also a potential site for serine phosphorylation (-Arg–Ser–Pro–Leu-), which could be recognized by a proline-dependent serine/threonine protein kinase. One function of the cytoplasmic domain of CTLA-4 is to regulate its subcellular localization and cell-surface expression. Unlike CD28, CTLA-4 is primarily retained intracellularly in Golgi or post-Golgi compartment(s) [4]. The unique subcellular distribution of CTLA-4 is regulated by the sequence motif -Thr–Thr–Gly–Val–Tyr–Val–Lys–Met–Pro–Pro–Thr–Thr, which contains the PI 3-kinase binding site.

CD28 and CTLA-4 share common ligands, the B7-related molecules CD80 and CD86; CTLA-4 binds CD80 and CD86 with much higher avidity than does CD28. A soluble CTLA-4–immunoglobulin hybrid construct, CTLA4Ig, showed high avidity binding to CD80 and CD86 and has remarkable inhibitory effects on lymphocyte functions, both *in vitro* and *in vivo*. This indicates that molecules bound by the B7 ligands are involved in stimulating lymphocyte activation. However, because of the possible involvement in these interactions of both CD28 and CTLA-4, analysis of the individual role of CTLA-4 had to await the availability of specific reagents and/or procedures.

Studies of CTLA-4 function using monoclonal antibodies

Addition of anti-CTLA-4 monoclonal antibodies to *in vitro* model systems of T-cell activation generally leads to increased T-cell proliferation, but the mechanism by which this occurs has been controversial [1,2]. Early studies interpreted the effects as evidence that CTLA-4 is a costimulatory receptor, analogous to, but weaker than, CD28. However, the stimulatory effects of anti-CTLA-4 monoclonal antibodies have also been attributed to blocking of CTLA-4–B7 interactions, which have an inherently negative effect on T-cell activation [5] — by blocking an interaction that has inhibitory effects, T-cell activation is increased. This interpretation was supported by the observation that intact anti-CTLA-4 antibody had similar effects to those of Fab fragments of the antibody, even though Fab fragments were presumably unable to signal. Similar effects of anti-CTLA-4 antibodies were also seen in an *in*

vivo model of T-cell expansion [6]. Thus, two seemingly exclusive models predicted either positive or negative effects of CTLA-4 engagement during T-cell activation.

A new study has examined the effects of anti-CTLA-4 monoclonal antibodies on anti-tumor immunity [7]. Administration of anti-CTLA-4 antibodies to tumor-bearing animals led to striking tumor regression and immunity to rechallenge. These effects were seen even when animals bearing established tumors were treated with anti-CTLA-4 antibody. Surprisingly, administration of anti-CD28 antibodies did not have similar results; thus, anti-CTLA-4 antibodies specifically stimulated anti-tumor immunity. As with previous studies of the effects of anti-CTLA-4 monoclonal antibodies, the immune-stimulatory effect of the anti-CTLA-4 monoclonal antibodies could have resulted from direct stimulation of anti-tumor antigen-specific T cells, or from blocking negative effects of CTLA-4 on T-cell activation. Although the antibodies used for these experiments apparently block the binding of CTLA4Ig to B7 molecules, it is unclear whether the negative effects of CTLA-4 require its engagement by B7 molecules. Regardless of the mechanism, this study clearly establishes the potential benefits of anti-CTLA-4 monoclonal antibodies for stimulating anti-tumor immunity. It will be important to extend these studies to other tumor systems.

Knockout mice

Our view of CTLA-4 has been transformed by two studies describing CTLA-4 gene knockout mice [8,9]. In both cases, heterozygous (+/–) mice appeared healthy, but CTLA-4 null mutant (–/–) mice showed severe lymphoproliferative disorders and early lethality (within 3–4 weeks after birth). T-cell blasts (having an activated/memory cell-surface marker phenotype) accumulated in the peripheral lymphoid organs of CTLA-4-deficient mice, and serum immunoglobulin levels were elevated up to about 100-fold for some isotypes [8]. Mice from both laboratories suffered massive lymphocytic infiltration of several internal organs, including the heart and pancreas. In contrast, mice lacking CD28 had only relatively minor defects in peripheral immune responses.

At this juncture, we have learned that CTLA-4 and CD28 have inherently different properties and functions, although they have some sequence homology and share common ligands (Table 1). But many new questions come to mind. For example, what is the role of CTLA-4 during development? The early lethality seen in CTLA-4-deficient mice indicates that CTLA-4 is vitally important early in development of the immune system. Surprisingly, however, patterns of CTLA-4 expression during development are unknown; to our knowledge, there is no information available on the cellular expression of CTLA-4 around or before birth. And what is the effect of CTLA-4 on activation of mature T cells? Both the knockout mice and studies with monoclonal antibodies have suggested that the role of CTLA-4 is negative, but the early defects in the CTLA-4-deficient mice make it difficult to ascertain the effects of the absence of this molecule on mature T cells. Thus, it is unclear whether the negative regulatory effects of the CTLA-4 gene have the same cellular and molecular basis as the inhibitory effects of anti-CTLA-4 monoclonal antibodies. Also, many experiments have shown stimulatory effects of anti-CTLA-4 monoclonal antibodies. Might CTLA-4 have additional stimulatory effects in addition to its negative regulatory role? And why is there is a lack of lymphocyte control in CTLA-4-deficient mice? This could be due to a failure in development of thymic or peripheral tolerance in these mice. For example, CTLA-4 might be required for effective negative selection of autoreactive clones. The lack of peripheral control might also indicate increased activation in the periphery of normally selected T cells.

The results so far also raise questions about the possible relationship between CTLA-4 and cell death. Why, in CTLA-4-deficient mice is there no control of lymphocyte activation by normal mechanisms, such as the Fas/Fas-ligand (FasL) cell-death triggering system? Although peripheral T cells from CTLA-4-deficient mice were sensitive to cell death induced by crosslinking of the Fas receptor [8], these mice might nevertheless have alterations in the Fas/FasL system. Supporting this possibility was the detection in the livers of CTLA-4-deficient mice of aggregates of (most probably activated) mononuclear cells in the absence of tissue destruction. Liver cells are very sensitive to Fas-based killing; the infiltrating, presumably activated, lymphocytes should express the Fas ligand. Nonetheless, there was no obvious tissue destruction. Another hint of Fas/FasL alterations was the spontaneous proliferation *in vitro* of lymph-node cells from CTLA4-deficient mice. These cells should be killed, at

Table 1

Contrasting properties of T-cell receptors CD28 and CTLA-4

Property	CD28	CTLA-4
Binding to CD80 and CD86	Low avidity	High avidity
T lymphocyte expression:		
Type of expression	Constitutive	Inducible
Time during T-cell response	Continuous	Late
Levels on cell surface	Moderate	Very low
Primary subcellular localization	Cell surface	Intracellular
Effect on T-cell proliferation of:		
Intact antibodies	Strong stimulation	Weak stimulation
Crosslinked antibodies	Strong stimulation	Strong inhibition
Effect of gene deletion	Mild immuno-suppression	Lethal lympho-proliferation

least in part, by Fas–FasL interactions. Possibly CTLA-4 expression is required for Fas/FasL expression on activated T cells around birth. Alternatively, CTLA-4 might play another direct or indirect role in regulating death of T lymphocytes.

Finally, is there a role for CTLA-4 abnormalities in human disease? Mutations in many important lymphocyte molecules cause similar immunodeficiencies in mice and man. Now that the severe phenotype of CTLA-4 deficiency in mice has been determined, it will be important to determine whether CTLA-4 deficiencies cause a similar phenotype in humans. Perhaps CTLA-4 deficiencies will turn up in newborn or very young children with lymphoproliferative disorders. It is also possible that older people heterozygous for deficiencies in CTLA-4 may be more prone to autoimmune diseases. As the deficiencies in CTLA-4 homozygous null mice are so profound, gene-dosage effects on CTLA-4 expression may lead to intermediate phenotypes. Linkage between the *ctla-4* locus and human autoimmune disorders have been observed [10]. This finding, together with the recent data from CTLA-4-deficient mice, tells us of a possible role of CTLA-4 abnormalities in the pathogenesis of human disease.

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