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*Review*

# Expression and Functional Significance of CTLA-4, a Negative Regulator of T Cell Activation

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**Abstract**. The generation of an effective immune response involves antigen-specific T cell expansion and differentiation of effector function. T cell activation requires at least two distinct signals, including signaling via the antigen-specific T cell receptor (TCR) and a costimulatory pathway. Antigen stimulation of T cells can lead either to a productive immune response, characterized by proliferation, differentiation, clonal expansion and effector function, or, in the absence of an appropriate costimulation, to a state of long-lasting unresponsiveness, termed anergy. Anergic T cells fail to proliferate and secrete cytokines in response to secondary stimulation. The interaction between the costimulatory molecule CD28 on T cells and members of the B7 family on antigen-presenting cell results in upregulation of T cell proliferation and cytokine production and induces the expression of the anti-apoptotic protein Bcl-xl. Based of these findings, the two-signal requirement model for T cell activation is generally accepted today. The negative regulatory mechanisms during T cell activation are not well understood, but they are crucial for the maintainance of lymphocyte homeostasis. For several years the functional role of the enigmatic CD28 homologue cytotoxic T lymphocyte antigen-4 (CTLA-4) in T cell activation has been both obscure and controversial. CTLA-4 was initially supposed to provide a costimulatory signal in conjunction with TCR/CD3 signaling. Today we know that CD28 and CTLA-4 molecules may have diametrically opposed functions: signaling via CD28, in conjunctive with TCR, is required for T cell activation, while signaling via CTLA-4 is a negative signal that inhibits T cell proliferation. How the T cell integrates signals through the TCR/CD3 complex, CD28 and CTLA-4 to initiate, maintain and terminate antigen-specific immune response is in fact not fully clarified. In this review, we will focus on the emerging role of CTLA-4 as a negative regulator of T lymphocyte activation and its role in the dynamic interplay of activatory and inhibitory signals.

**Key words:** CTLA-4; T cell activation; negative regulation.

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CTLA-4 (CD152) is a predicted T cell surface molecule which was discovered during a search for molecules involved in T cell cytotoxicity and was identified by differential screening of a subtracted cDNA library derived from a murine $CD8^+$ T cell line[4, 5] and human genomic DNA sequences[12]. LINDSTEN et

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al.[29] showed that within the T cell population CTLA-4 expression is restricted to the subset of T cells that also express cell surface CD28. CD28 and CTLA-4 have structural similarity and a number of common ligands, but their patterns of expression are quite distinct[21]. Unlike CD28, which is constitutively expressed by all murine T cells and the majority of human T cells[27], CTLA-4 appears to be induced upon activation of the T cell. On resting T cells, CTLA-4 protein levels are low or undetectable[50]. Transcription of the CTLA-4 gene increases rapidly following TCR/CD3 signaling and is enhanced by either CD28-mediated costimulation or IL-2[29, 43, 52]. However, DAMLE et al.[11] have shown that integrin-mediated costimulation also induces CTLA-4 surface expression. CTLA-4 molecules reach maximum levels on the cell surface 48–72 h after stimulation[32, 43, 52]. The maximum expression level of CTLA-4 is only 2–3% of the level of CD28 on the surface of the same cell[32]. WALUNAS et al. [52] report that greater levels of CTLA-4 were found on $CD8^+$ cells than $CD4^+$ cells, while others report equal amounts of CTLA-4 in activated human $CD8^+$ and $CD4^+$ T cells[31]. While CTLA-4 functions on the cell surface, recent studies[28, 30, 56] have revealed that, following T cell activation, the majority of CTLA-4 is localized intracellularly rather than on the cell surface. CTLA-4 is primarily detected in the perinuclear Golgi vesicules[28], and upon activation CTLA-4 transits from the internal stores to the cell surface and is rapidly reinternalized[30]. The polar orientation of the Golgi ensures a high local concentration of the CTLA-4 at the precise location of cell-cell contact (and TCR clustering) to promote rapid and efficient ligand binding. Although surface expression is low, activated T cells have an abundant store of CTLA-4, ready to be released to the cell surface.

CD28 and CTLA-4 are members of the immunoglobulin superfamily, and their closely related sequences suggest a common evolutionary origin. The genes for these molecules colocalize to the same chromosomal regions in humans (chromosome 2 at bands q33-q34)[25] and mice (chromosome 1)[15]. The two genes have an overall nucleotide sequence homology of about 75%. This strongly supports the hypothesis that the CTLA-4 and CD28 genes are the products of a prespeciation duplication event[15]. CTLA-4 surface expression is regulated at the posttranscriptional level[28, 30, 56] and a possibility of reciprocal regulation of CD28 and CTLA-4 at the mRNA level is suggested[29, 33]. Despite the structural organization and location similarity between CTLA-4 and CD28, these two genes are in fact an excellent example of functional divergence. The consequence of CD28 and CTLA-4 gene homology is the high degree of their structural similarity. CTLA-4 and CD28 share identity in extracellular and cytoplasmic regions (approximately 70 and 30%, respectively). Like CD28, CTLA-4 is a type I (extracellular N-terminus) transmembrane glycoprotein[5]. CTLA-4 consists of 186 aa residues, with 125 aa residues in the extracellular region and 37 aa residues in the cytoplasmic domain[15]. CD28 and CTLA-4 exist as disulfide-linked homodimers[34] and they share a hexapeptide-conserved MYPPPY motif (in single-letter acid code) in their variable domains necessary for binding to B7[41]. CTLA-4 is a receptor with an avidity for B7 ligands 10–50 times higher than that of CD28[31], with higher affinity for B7.1 and a slower off-rate than for B7.2[31, 39] due to unique residues in the CDR1 and CDR3-like domains of CTLA-4[41].

The mechanisms responsible for CTLA-4 signal transduction are still not fully clarified. It has been suggested by MARENGERE et al. [35] that tyrosine phosphatase Syp is involved in CTLA-4 signaling pathways, since examination of tyrosine phosphorylation levels in T lymphocytes from CTLA-$4^{-/-}$ mice revealed that a number of proteins, including CD3ζ, ZAP-70, p52SHC and protein tyrosine kinases – PTK (Fyn and Lck), are hyperphosphorylated. Their immunoprecipitation studies, using anti-p52SHC and anti-CD3ζ antibodies followed by anti-phosphotyrosines immunoblots, have shown that both p52SHC and CD3ζ are indeed hyperphosphorylated in the absence of CTLA-4. It has also been revealed that other signaling proteins, including Vav, PLCγ1 and CD3ε, are hyperphosphorylated on tyrosine residues in CTLA-$4^{-/-}$ T cells. Thus, in the absence of CTLA-4, PTK activity and tyrosine phosphorylation levels of signaling molecules are significantly increased in T cells. Tyrosine-phosphorylated CD3ζ recruits constitutive formation of Sos/Grb2/p52SHC hetero-complexes, by which the TCR in CTLA-$4^{-/-}$ T cells induces activation of Ras, playing a pivotal role in the activation of downstream effectors[16, 36]. The Ras signal transduction cascade activates the mitogen-activated protein kinases (MAPKs), which is constitutively activated in the absence of CTLA-4[35]. Thus, the Ras pathway activity is constitutively elevated in CTLA-$4^{-/-}$ T cells. The data suggest that CTLA-4 signaling may intersect with the Ras signaling pathway. On the other hand, the experiments provided by CALVO et al.[6] showed that engagement of CTLA-4 on activated T cells did not affect proximal TCR/CD3 signaling events, such as ZAP-70 and CD3ζ chain phosphorylation. It is suggested that the hyperphosphorylation of ZAP-70 and CD3ζ observed in T cells of CTLA-4-deficient mice may result from the hyperactivated state of the T cells rather than be a di-

rect consequence of the absence of the CTLA-4 receptor[49, 54].

The interaction between CTLA-4 and tyrosine phosphatase Syp is mediated by the Src-homology 2 (SH2) domains of Syp and the phosphotyrosine sequence YVKM (where Y – tyrosine, V – valine, K – lysine, M – methionine) within the CTLA-4 cytoplasmic tail[47] (Fig. 1). The CTLA-4-associated Syp has phosphatase activity towards the Ras regulator – p52SHC[35]. Dephosphorylated p52SHC can no longer associate with Grb2/Sos adaptor complex protein and abrogate the activation of the Ras pathway and inhibits T cell activation. Thus, the Ras signaling seems to be a target for the regulation by CTLA-4 of TCR/CD28-mediated T cell activation (Fig. 2). It has been showed by CALVO et al.[6] that CTLA-4 engagement selectively shuts off

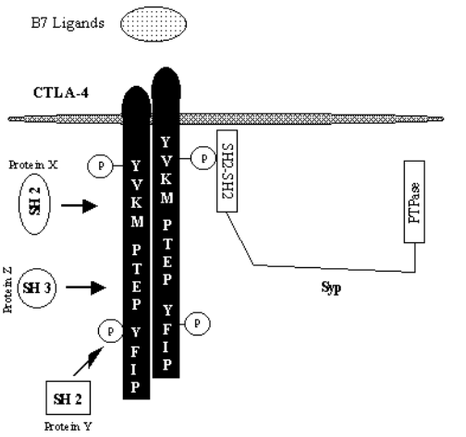


**Fig. 1.** Structure of CTLA-4/Syp complex (according to WATERHOUSE et al.[53])

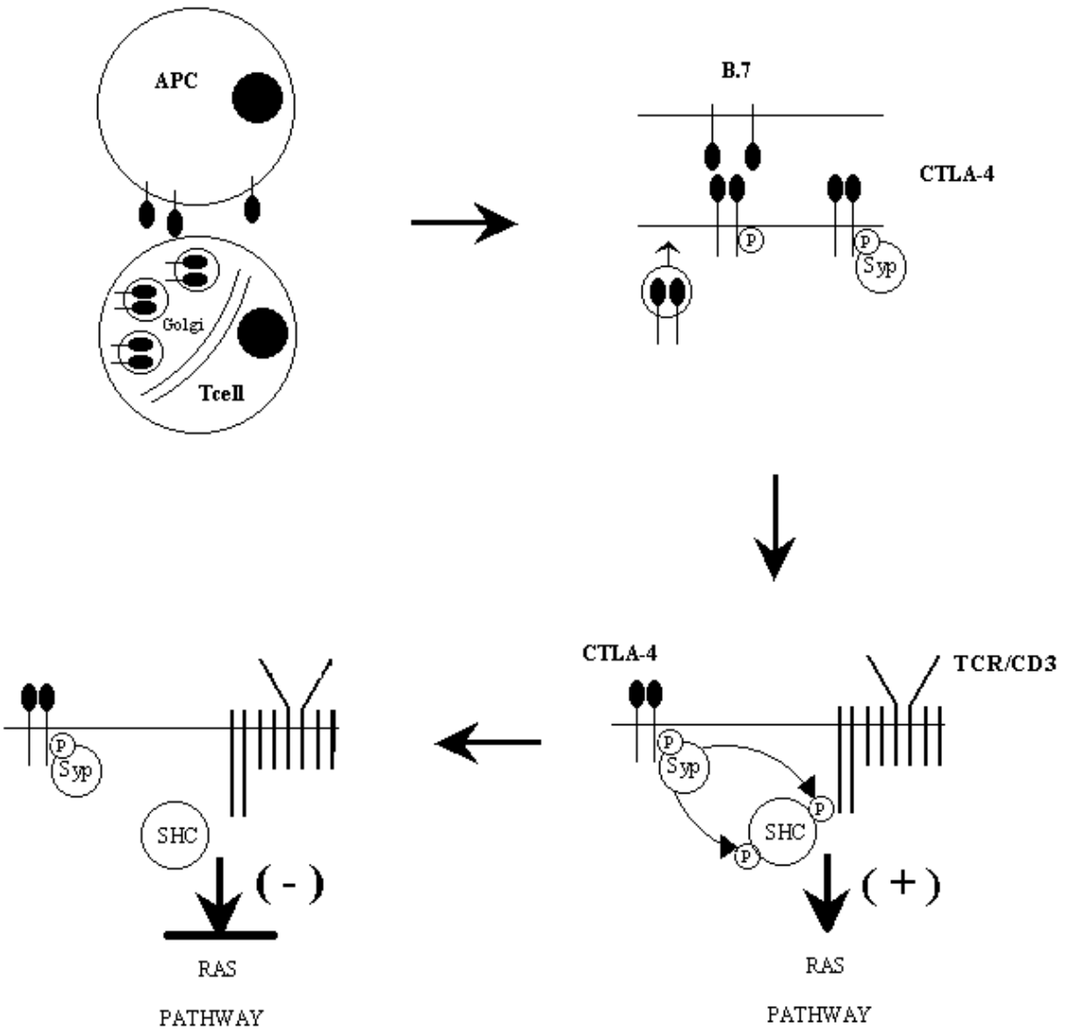


**Fig. 2.** CTLA-4-mediated regulation of Ras signaling pathway (according to WATERHOUSE et al.[53])

activation of downstream TCR/CD28 signaling events, especially MAPKs ERK (one of MAPKs)[36] and Jun-terminal kinase (JNK)[48]. Since activation of the ERK and JNK kinases is required for the stimulation of IL-2 and IL-2R gene transcription, the data provide a molecular explanation for the blocking of IL-2 production and T cell proliferation imposed by CTLA-4. However, WALUNAS et al.[50] reported also that the inhibition of proliferation and the inhibition of IL-2 secretion require distinct signaling pathways, since proliferation, but not IL-2 secretion, could be restored by addition of exogenous IL-2, suggesting that the inhibitory effect of CTLA-4 occured at the level of IL-2 gene induction rather than at the regulation of IL-2R (CD25) expression. The importance of levels of tyrosine phosphorylation for CTLA-4 signal transduction was also confirmed by CHAMBERS and ALLISON[7].

The sequences involved in CTLA-4 signaling also interact with distinct, but overlapping, motifs that direct its intracellular localization. Many studies[3, 10, 28, 30, 46, 56] have demonstrated that the unphosphorylated cytoplasmic tail of CTLA-4 is associated with AP50, the medium chain of the clathrin-associated coated pit adaptor protein complex AP2. Interaction with the AP50 subunit likely induces rapid internalization of CTLA-4 from the cell surface. Using the yeast two-hybrid method, it has been shown that sequences required for interaction of AP50 and CTLA-4 were localized to residues 161-TTGVY-165 in CTLA-4 (in single-letter amino acid code), which overlaps with the SH2-binding motif, 165-YVKM, involved in CTLA-4 signaling. There are other tyrosine residues involved in signaling and trafficking of CTLA-4, such as Y182 and Y201. It has also been found that AP50 binds to a CTLA-4 peptide containing unphosphorylated tyrosine residues (Y165, Y182 or Y201), but not to a peptide with phosphorylated tyrosine[3, 10, 38]. That was confirmed by ZHANG and ALLISON[56], who found that mutation of tyrosine Y165 or Y201 to phenyloalanine F165 or F201 in the YVKM motif of the human CTLA-4 cytoplasmic domain significantly diminished its binding to AP50 and resulted in loss of CTLA-4 intracellular localization by releasing CTLA-4 molecules to the cell surface. Conversely, CTLA-4 interacts with tyrosine phosphatase Syp (SHP-2) through the same motif involved in the interaction with AP50, except that interaction with Syp requires phosphorylated tyrosine Y165, Y182 or Y201. MIYATAKE et al.[38] searched for the tyrosine kinase responsible for the phosphorylation of CTLA-4. Their observations have shown that Src family tyrosine kinases (Fyn, Lck) associate with and phosphorylate tyrosine residues of CTLA-4 that are mainly responsible for interaction with tyrosine phosphatase Syp through SH2 domains. These results suggest that the phosphorylation state of a single tyrosine residue determines which effector molecules (AP50 or Syp) bind to the CTLA-4 cytoplasmic tail and whether CTLA-4 is transfered to the cell surface and, in consequence, delivers a negative signal or is internalized[38, 46, 56]. Therefore, the phosphorylation and dephosphorylation status of Y201, Y182 or Y165 in the CTLA-4 cytoplasmic tail determines the binding specifity of CTLA-4 and plays an important role in the signal transduction.

Since the consensus that CTLA-4 plays a pivotal role in the regulation of T cell responses, it appears to be very important to define the stages of T cell activation at which CTLA-4 might function. Initial experiments suggested that CTLA-4 might function in the termination of T cell responses by inhibition of IL-2 production and proliferation. The mechanism of the termination was not based on apoptosis, allowing the survival of T cells differentiating into memory cells[19]. The other experiment showed that CTLA-4 might terminate T cell responses by induction of apoptotic cell death in the previously activated human T cell clone[14]. It was not elucidate in this report whether CTLA-4 engagement influences T cell apoptosis directly by affecting on bcl-xl expression or by inhibition of IL-2-secretion. SCHEIPERS and REISER[45] provided evidence that CTLA-4 ligation on the surface of activated murine $CD4^+$ T cells leads to the death of a substantial fraction of the cells, whereas in resting $CD4^+$ T cells the same stimulation conditions induce cell cycle arrest in G0/G1 without apoptosis. Cell death induced by CTLA-4 stimulation occurs independently of the Fas pathway, and, therefore, may involve a novel pathway[45]. CTLA-4-mediated apoptosis of activated T cells may be a means of terminating the function of previously stimulated T cells. Explanation of this mechanism may provide a therapeutic strategy to eliminate allo- and autoreactive T lymphocytes. Recent studies[1, 9, 22], however, suggest that CTLA-4 may function much earlier than was thought on the basis of maximal cell surface expression. KRUMMEL and ALLISON[22] have found that crosslinking of CTLA-4 inhibits several events that occur within the first 24 h of activation of naive T cells by anti-CD3 and anti-CD28 antibodies. CTLA-4 ligation effectively inhibits the proliferation response as a consequence of arrest the of G0/G1 stage of the cell cycle and also decreases the expression of early activation antigen CD69 as early as 24 h after stimulation. The block in the cell cycle progression is probably due to diminished IL-2 secretion and lower expression of the IL-2R $\alpha$ chain (CD25). These observations were also sup-

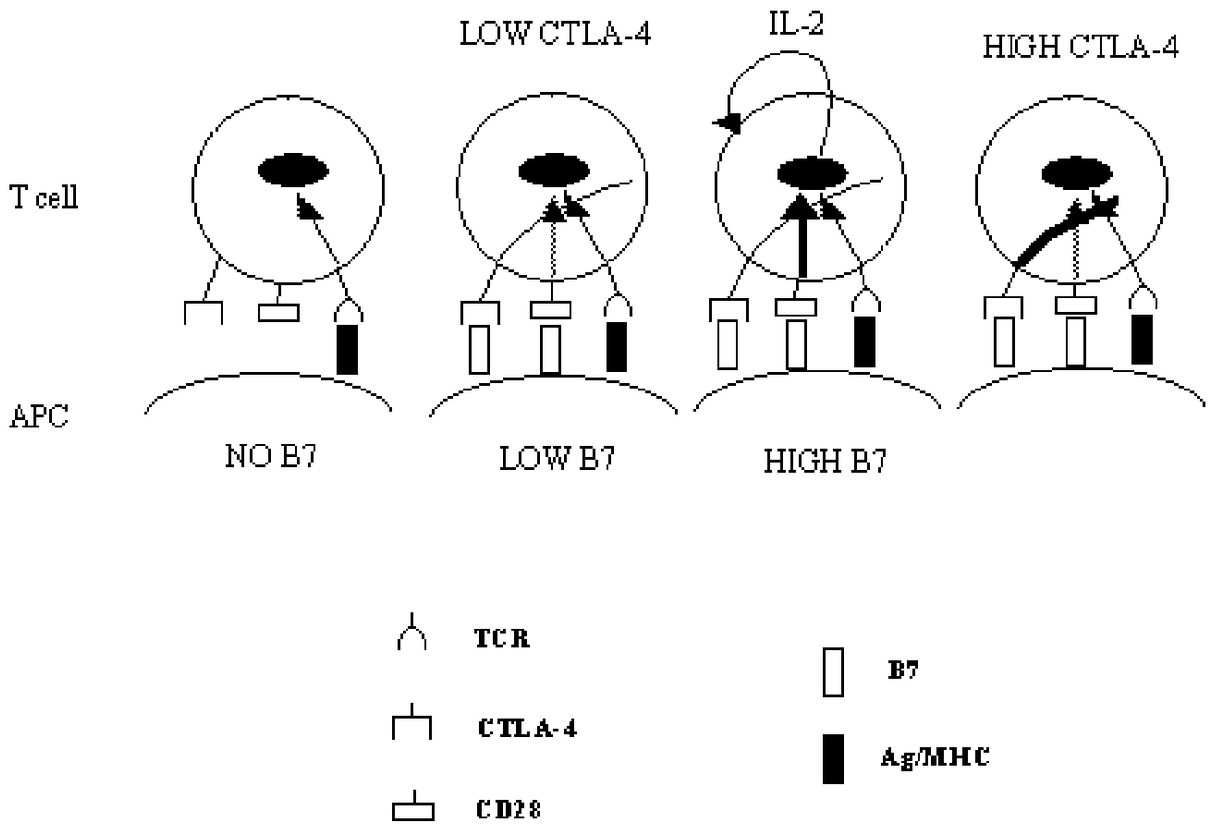


**Fig. 3.** Model of dynamic interplay between TCR/CD3, CD28 and CTLA-4 (according to CHAMBERS et al.[9])

ported by others[1, 45]. A possibility that CTLA-4 can restrict the transition of resting T cells to an activated state is suggested.

Direct evidence of a critical regulatory role for CTLA-4 comes from experimentally induced CTLA-4 deficiency in animal models. Mice lacking CTLA-4 developed severe lymphoproliferative disorder and autoimmune disease with massive lymphocytic infiltration and tissue destruction that resulted in death approximately 3 weeks after birth[49, 54]. Evidence suggested that the breakdown of lymphoid homeostasis in the CTLA-$4^{-/-}$ mice resulted from a failure in peripheral tolerance[8]. The potential of CTLA-4 as a negative regulatory receptor is also illustrated by the recent findings that inhibition of CTLA-4 signals prolongs T cell activation and expansion both *in vitro*[52] and *in vivo*[23]. In addition, the data taken on animal models have shown that blockade of CTLA-4-mediated signals retards the growth of an immunogenic tumor, implying augmented T cell-mediated antitumor immunity[18, 24, 26, 55]. Administration of anti-CTLA-4 mAb to mice concurrently with a tolerizing antigen regimen resulted in the appearance of antigen-reactive T cells [2, 42, 44, 51], indicating that CTLA-4 plays a role in setting the treshold for T cell activation. Moreover, a similar *in vivo* blockade of CTLA-4 has been found to markedly exacerbate disease in mice induced to develop experimental allergic encephalomyelitis[17, 20] and induce protective immunity to parasite infections[13, 37]. Thus, CTLA-4 is likely to play a critical role in the regulation of many aspects of the immune response.

In summary, the data presented above show that the expression of CTLA-4 is tightly controlled. Resting T cells express very low levels of CTLA-4. However, CTLA-4 expression is up-regulated strongly upon activation of the T cells. Because of its pattern of expression, it was originally thought that CTLA-4 might function exclusively in activated T lymphocytes. More recently, a new model has been proposed that suggests that CTLA-4 may be functional on resting as well as on activated T cells. According to this model (Fig. 3), presentation of antigenic peptide by an antigen-presenting cell (APC) that express a low level of B7 fails to stimulate resting T cells because of inhibition by

CTLA-4. Thus, CTLA-4 serves to attenuate responses and ensures T cell inactivity in the presence of low levels of costimulation or antigens. This attenuation may be responsible for maintaining peripheral tolerance to self-antigens which are presented in the presence of low levels of B7 on APC. The role of CTLA-4 in self--tolerance is thought to increase the threshold for activation above this level. On the other hand, CTLA-4--mediated inhibition can only be overcome if a resting T cell encounters an APC expressing high levels of B7 molecules, effectively resulting in CD28 stimulation. However, once the T cell has become fully activated, it expresses high levels of CTLA-4, which may be able to override signals transduced by CD28 even if the T cell encounters an APC that expresses high levels of antigen and B7 molecules. Therefore, CTLA-4 may serve two functions: on resting T cells it may serve to attenuate signals mediated by the TCR/CD3 and CD28 and thus contribute to peripheral tolerance. By contrast, on activated T cells CTLA-4 may serve to terminate T cell activation. Thus, CTLA-4 function is thought to ensure a balanced immune response by the down-regulation of early as well as late stages of T cell response. More information is needed to understand and exploit the regulatory effects of CTLA-4 and the reciprocal influence of TCR/CD3, CD28 and CTLA-4 signaling pathways.

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