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CTLA-4 trafficking and surface expression

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Abstract

The T-cell co-receptor cytotoxic T-cell antigen 4 (CTLA-4) has a strong inhibitory role as shown by the lymphoproliferative phenotype of CTLA-4-deficient mice. Despite its potent effects on T-cell function, CTLA-4 is primarily an intracellular antigen whose surface expression is tightly regulated by restricted trafficking to the cell surface and rapid internalisation. Recently, several signalling molecules such as Trim, PLD, ARF-1 and TIRC7 have been described to be involved in the transport of CTLA-4 to the cell surface. Minor changes in surface expression levels have major effects on the outcome of T-cell activation. Optimal regulation of CTLA-4 surface expression is crucial for the balance of stimulatory and inhibitory signals to maximize protective immune responses while maintaining immunological tolerance and preventing autoimmunity.

Introduction: cytotoxic T-cell antigen 4 – a multifaceted molecule

Optimal immune responses require an antigen-specific signal generated by the TcR ζ (T-cell receptor)–CD3 and CD4–CD8–p56lck complexes, followed by a second, co-stimulatory signal. There exists an array of different co-receptors on T cells including CD28, inducible T-cell co-stimulator (ICOS) and cytotoxic T-cell antigen 4 (CTLA-4), programmed death-1 (PD-1), B- and T-lymphocyte attenuator (BTLA) and T-cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3). These co-receptors not only provide critical positive second signals that promote and sustain T-cell responses, they can also downregulate immune responses. The balance of stimulatory and inhibitory signals is crucial to maximize protective immune responses while maintaining immunological tolerance and preventing autoimmunity.

CTLA-4 (CD152) is one of the most extensively studied inhibitory co-receptors. Like the related molecule CD28, CTLA-4 binds to B7-1 (CD80) and B7-2 (CD86), although with significantly higher avidity. A strong inhibitory role for CTLA-4 is demonstrated by the phenotype of CTLA-4-deficient mice, which develop a massive lymphoproliferative disorder characterized by polyclonal T-cell proliferation and early lethality [1,2]. The co-receptor modulates the threshold of signals needed for T-cell cytokine production and proliferation [3,4]. Furthermore, CTLA-4 ligation can lead to negative effects on the regulation of cell cycle and inhibits the transcription factors nuclear factor κ B (NF- κ B),

nuclear factor of activated T cells (NF-AT) and activator protein 1 (AP-1) [5,6]. CTLA-4 has also been implicated in the upregulation of FoxP3 expression induced by transforming growth factor β (TGF- β), leading to the generation of adaptive CD4⁺CD25⁺ regulatory T cells (Tregs) [7]. Furthermore, ligation of CTLA-4 may increase TGF- β production, which in turn induces FoxP3 expression [8-10]. Recently, it has been shown that enhanced CTLA-4 signalling promotes the generation of CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁻TGF- β 1⁺ adaptive Tregs [11]. Although CTLA-4^{-/-} mice still produce functional naturally arising Tregs, the induction of FoxP3 expression and generation of adaptive Tregs by TGF- β is abrogated in these mice, which might contribute to the severe lymphoproliferative phenotype [7]. Several mechanisms have been reported for CTLA-4 inhibition of T-cell activation such as ectodomain competition for CD28 binding to CD80 and CD86 [12], disruption of CD28 localization at the immunological synapse [13], modulation of TcR signalling by the phosphatases SH2 domain-containing protein tyrosine phosphatase (SHP-2) and the serine-threonine phosphatase PP2A [14-18], as well as interference with the expression or composition of lipid rafts on the surface of T cells [19-22]. In addition, CTLA-4 engagement of CD80 and CD86 on dendritic cells (DCs) can induce release of indoleamine 2,3-dioxygenase (IDO) [23,24], which has regulatory effects on T cells that result from tryptophan depletion. Several findings indicate that CTLA-4 not only sends negative but also positive signals. For example, in CD4-positive T cells from mice deficient in CD80 and CD86, extracellular signal-regulated kinase (ERK) activity was inhibited by CTLA-4, whereas at the same time, c-Jun N-terminal kinase (JNK) activity was increased [25]. Furthermore, CTLA-4 ligation activates the integrin regulator Rap-1 leading to upregulation of lymphocyte function-associated antigen-1 (LFA-1)-mediated adhesion [26,27]. CTLA-4 also can increase cell motility, and reverse the TcR-induced stop-signal needed for stable T cell-antigen-presenting cell (APC) interactions [28]. This latter observation provides an alternate mechanism to account for the dampening effect of CTLA-4 on T-cell proliferation.

Intriguingly, this spectrum of events is mediated by a receptor with a small cytoplasmic tail of only 37 amino acids [29] (Table 1). This domain contains two tyrosine-based motifs at position Y₂₀₁VKM and Y₂₁₈FIP (corresponding to Y165 and Y182, respectively, if the leader sequence is not included). Several intracellular proteins bind to the Y₂₀₁VKM sequence including the lipid kinase phosphatidylinositol 3-kinase (PI3K) [30], the phosphatases SHP-2 and PP2A [14-18] and clathrin adaptor proteins AP-1 and AP-2 [31-33] (Figure 1).

Despite its potent effects on T-cell function, CTLA-4 is primarily an intracellular antigen whose surface expression is tightly regulated by restricted trafficking to the cell surface and rapid internalisation [34,35]. This is important because minor changes in surface expression levels could have major effects on the outcome of T-cell activation. Furthermore, induction of CTLA-4 surface expression after stimulation is temporally restricted, and frequencies of CTLA-4 expressing cells do not increase in later generations as shown by Maszyna *et al.* [36].

Little is known regarding the regulation of intracellular trafficking pathways used by CTLA-4 to reach the plasma membrane. This review aims to summarize and discuss reported mechanisms that regulate CTLA-4 surface expression and hence T-cell function.

The *CTLA-4* gene and expression of different isoforms

Gene transcription

The *CTLA-4* gene consists of four exons and is located on chromosome 2 in humans and on chromosome 1 in mice. Exon 1 contains the leader peptide sequence and exon 2 the ligand binding site, which are both parts of the extracellular region of CTLA-4. Exon 3 encodes the transmembrane region, and exon 4 the cytoplasmic tail [37] (Figure 2). The transcriptional regulation of *CTLA-4* gene expression is only partially known. It may depend on NF-AT because modulation of NF-AT levels correlates directly with CTLA-4 expression [38]. In addition, inhibition of NF-AT activation by cyclosporine A causes a marked decrease in *CTLA-4* gene transcription [39]. Furthermore, it has been shown that NF-AT binds to the CTLA-4 promoter region [40]. CTLA-4 expression is also upregulated by cAMP, suggesting a cAMP-dependent regulation of *CTLA-4* gene transcription [41]. In T cells, CTLA-4 mRNA is first detected 1 h after TcR ligation and peaks around 24–36 h after stimulation when CTLA-4 is also detectable at the cell surface [42,43]. The stability of CTLA-4 mRNA is linked to TcR signalling and co-stimulation. CD28 coligation increases the half-life of CTLA-4 mRNA twofold compared with TcR engagement alone [39].

CTLA-4 isoforms

The resulting transcript of the *CTLA-4* gene can undergo alternative splicing, which is different between human and mouse CTLA-4. For human CTLA-4, there exists full-length mRNA containing exons 1–4 (fICTLA-4), a transcript coding for soluble CTLA-4 (sCTLA-4), which is detectable in human serum and does not contain exon 3 [44,45], and a transcript coding only for exons 1 and 4. The half-life of soluble CTLA-4 mRNA is shorter than that of full-length CTLA-4 mRNA [46]. Murine T cells can express an additional CTLA-4 transcript that is known as ligand-independent CTLA-4 (liCTLA-4), containing exons 1, 3 and 4 [47] (Figure 2). Despite the fact that liCTLA-4 lacks the ligand-binding domain (MYPPPY), it may associate with the TcR ζ chain and to be a more potent inhibitor of T-cell proliferation and cytokine production than fICTLA-4 [48]. Several gene polymorphisms have been described in the *CTLA-4* gene (Figure 2). Most extensively studied are a C to T transition at position –318 within the promoter region, a G to A transition at position 49 in exon 1, a dinucleotide repeat polymorphism (AT)_n and an A or G single nucleotide polymorphism (CT60) both in the 3' nontranslated region of exon 4. CTLA-4 polymorphisms have been linked to several autoimmune diseases such as Graves' disease, Hashimoto's thyroidism, Addison's disease, type 1 diabetes, coeliac disease, rheumatoid arthritis and multiple sclerosis [47,49]. Furthermore, an allelic variation of CTLA-4 that is correlated with reduced mRNA expression of the alternatively spliced soluble form of CTLA-4 has been associated with autoimmunity [47].

Studies in NOD mice, a murine model to study diabetes, have shown that the expression of liCTLA-4 but not fICTLA-4 was enhanced in memory/regulatory T cells of diabetes-

resistant mice compared with susceptible mice. These data suggest a protective role for liCTLA-4 in the development of autoimmune disease [48].

Protein expression level of CTLA-4 in various cell subsets

Dependent on the cell type and activation status, CTLA-4 is expressed at different levels. A characteristic of CTLA-4 is its elevated level in activated T cells compared with naïve T cells. In naïve human T cells, intracellular CTLA-4 is detectable only at low levels, whereas in murine naïve T cells, CTLA-4 is not expressed [46,50]. Human T cell clones and memory T cells show significant amounts of intracellular CTLA-4 [50]. In these cells, CTLA-4 is rapidly expressed on the cell surface upon activation compared with naïve T cells. Also, CTLA-4 is retained on the surface of memory T cells for a considerably longer time than in the case of activated naïve T cells [50].

fICTLA-4 and liCTLA-4 show a reciprocal expression pattern in murine T cells. While fICTLA-4 expression is rapidly induced after stimulation, liCTLA-4 is expressed in resting cells but downregulated during early activation [48]. In CD4⁺CD25⁺Foxp3⁺ Tregs, CTLA-4 is constitutively expressed at the cell surface with high amounts of intra-cellular CTLA-4 [43,51].

CTLA-4: subcellular localization

Although the total amount of CTLA-4 in activated peripheral T cells is comparable to CD28, it is hardly detectable at the cell surface. While CD28 resides mainly on the cell surface, CTLA-4 is primarily localized in intracellular compartments [52]. Intracellular CTLA-4 is found in the *trans* Golgi network (TGN) [52-54], as well as in endosomes, secretory granules [35] and lysosomal vesicles [55].

The localisation and translocation of CTLA-4 represents a highly dynamic process and is a prerequisite for its function. For example, CTLA-4 is present in enlarged, abnormal vesicles in Chediak Higashi syndrome, and T cells from patients suffering from this disease were shown to be defective in cycling CTLA-4 to the cell surface after stimulation [56]. In migrating cells, CTLA-4 is localized near the microtubule organising centre (MTOC), and both intracellular and surface CTLA-4 are released or focussed toward the site of TcR engagement [35,57], a process most probably mediated by TCR-induced MTOC reorientation. CTLA-4 localises to lipid rafts in the immunological synapse (IS) under conditions of APC-dependent TcR–CTLA-4 co-ligation and T-cell inactivation [20]. Its accumulation at the IS is regulated by the strength of TcR signalling. Differences in CTLA-4 localisation at the IS occur in the absence of an increase in total expression levels, suggesting that CTLA-4 translocation is regulated by a post-translational mechanism. The strength of the TcR signal therefore determines the extent of CTLA-4 surface expression [57].

The directed release of CTLA-4 to the cell surface occurs at the IS, where it may associate with TcR ζ within lipid rafts, leading to attenuation of the TcR signal by driving the TcR out of the lipid raft complex. Furthermore, it has been suggested that CTLA-4 increases the activation threshold by binding to non-raft TcR ζ and therefore inhibiting TcR association to

rafts [19]. Martin *et al.* [21] demonstrated that CTLA-4 co-ligation with either TcR or the combination of TcR and CD28 potentially inhibited surface expression of the glycosphingolipid GM1, a major component of lipid rafts. Blockade of GM1 release from intracellular stores was profound and closely mimicked the level of inhibition of proliferation and interleukin-2 (IL-2) production. The level of surface expression of GM1 after TcR–CTLA-4 coligation was only marginally greater than that observed for resting T cells. The effect was reversible, in that restimulation with anti-CD3 plus CD28 antibodies reversed the CTLA-4-mediated blockade by increasing GM1 expression at the cell surface. By blocking GM1 surface expression and therefore limiting lipid raft formation, CTLA-4 effectively prevents accumulation of key signalling proteins such as LAT (Linker for activation of T cells) that are required for TcR signalling.

Mechanisms involved in regulation of CTLA-4 surface expression

Despite the significance of CTLA-4 surface expression and cycling, little is known about the factors that regulate its overall expression. CTLA-4 levels rapidly decrease in the absence of new protein translation. Upon internalization, CTLA-4 is quickly degraded with a half-life of 2 h in activated T cells [31,32,34,55]. Inhibition of lysosomal degradation led to accumulation of CTLA-4. The rapid turnover rate of CTLA-4 ensures that the level of protein expression within the cell is closely linked to the rate of gene transcription and/or translation and therefore is a major factor in determining the precise kinetics of CTLA-4 function [55,57]. Several molecules and mechanisms have been described to be involved in either CTLA-4 internalisation or its release to the cell surface (externalisation), which taken together promote our understanding of CTLA-4 trafficking.

Membrane retention versus internalisation of CTLA-4

The best-described mechanism involved in regulating the surface expression of CTLA-4 is clathrin-dependent internalisation as shown in Figure 3. Leung *et al.* [52] first described an intracellular localisation motif in the cytoplasmic tail of CTLA-4. The transfer of an 11 amino acid sequence from the cytoplasmic tail of CTLA-4 to CD28 mediated intracellular localisation of CD28, which normally resides on the cell surface. This sequence contains the YVKM motif, which, when phosphorylated, represents a binding site for the p85 subunit of PI3K [30]. The function of the YVKM motif in the regulation of CTLA-4 surface expression was explained by binding of the clathrin adaptor complex AP-2 to plasma membrane-associated CTLA-4 [31,33,58,59]. Mutation of the AP-2 binding site (Y201F) increases the retention time on the cell surface of this CTLA-4 mutant [52]. AP-2 mediates the internalisation of CTLA-4 from the cell surface to endosomal and lysosomal compartments.

Binding of AP-2 to CTLA-4 occurs via its μ_2 chain and requires nonphosphorylated Y201 in the YVKM motif. On the other hand, the phosphorylation of Y201 by src kinases such as Lck or Fyn [15] allows for PI3K or SHP-2 binding and prevents the interaction with AP-2, which promotes CTLA-4 surface retention. Phosphorylation and dephosphorylation of Y201 thus regulates the balance between CTLA-4 trafficking and signalling [58,59]. Nevertheless, the Y201F mutant of CTLA-4 can still be detected intracellularly, suggesting that binding of AP-2 to the YVKM motif is not the only mechanism mediating internalisation of CTLA-4 [54,55].

In contrast to the plasma membrane–located co-receptor, Golgi-associated CTLA-4 binds to AP-1 via the same nonphosphorylated binding motif as to AP-2. Association of CTLA-4 with AP-1 mediates the shuttling from the TGN to endo- and lysosomal compartments for degradation. Therefore, AP-1 plays a role in regulating the overall amount of CTLA-4 [32]. Dysregulation of intracellular expression of CTLA-4 has been proposed to account for lymphoproliferative disease in Chediak-Higashi syndrome [56].

Lipid kinases, but not PI3K, are involved in lysosomal sorting of CTLA-4 by promoting its internalisation and degradation. The treatment of cells with the lipid kinase inhibitor Wortmannin resulted in a higher surface and intracellular expression of CTLA-4, but not if the PI3K-specific inhibitor LY294002 was used. Wortmannin-mediated missorting of CTLA-4 is accompanied by partial inhibition of its endocytosis and decreased binding of the adaptin- β_2 subunit of AP-2 [60]. Although the μ_2 chain of AP-2 complexes can bind to CTLA-4, no binding to CD28 has been observed. PI3K plays a key role in regulating the endocytosis of CD28. Three distinct mutations in the YMN motif of CD28 (Y191, M194 and Y218) disrupted PI3K binding, resulting in inhibited endocytosis [61]. A correlation between levels of PI3K binding and internalization was observed. Growth factor receptors, such as the PDGF-R, which require PI3K at a postendocytotic step [62].

Release of intracellular CTLA-4 to the cell surface

TRIM as a chaperone

Another parameter in balancing CTLA-4 surface expression is the control of CTLA-4 externalisation. Several molecules have been reported to be involved in the release of CTLA-4 from intracellular compartments to the cell surface. TRIM is a 30-kDa type III transmembrane protein, which is expressed in human T cells and natural killer (NK) cells [63,64]. Its cytoplasmic tail contains several tyrosine motifs with the potential to bind to SH2 domains of signalling proteins. Upon TcR stimulation, TRIM is rapidly tyrosine phosphorylated and associates with the p85 subunit of PI3K [63]. Association of TRIM with the TcR ζ -CD3 complex has been shown in Jurkat T cells and human peripheral blood lymphocytes (PBLs) [63]. Overexpression of TRIM in Jurkat cells has been reported to induce an increase in TcR surface expression [64].

We recently observed intracellular binding of the transmembrane adaptor protein TRIM to CTLA-4 and co-localisation of both in the TGN [53]. The interaction was specific in that it was not observed with CD28. Furthermore, increased TRIM expression augmented surface CTLA-4 expression because of a more rapid release of CTLA-4 to the cell surface, leading to an increased ability to inhibit cytokine production and proliferation. After downregulation of TRIM expression by siRNA, CTLA-4 localisation was disturbed and restricted to the TGN. In primary T cells and hybridoma TRIM KD cells, a marked reduction of vesicles between the Golgi area and the plasma membrane was detected, consistent with impaired transport from the TGN. Therefore, the loss of TRIM expression results in an arrest of CTLA-4 trafficking in the TGN. This finding points to a role for TRIM in the generation of transport vesicles to shuttle CTLA-4 to the surface. Therefore, we propose that TRIM acts as a chaperone in regulating CTLA-4 expression and function by enhancing CTLA-4 transport to the surface of T cells [53].

The immune-specific interaction of TRIM and CTLA-4 presumably operates in conjunction with more general constituents involved in the trafficking process. How this complex interacts with generic events remains to be determined. The mild phenotype found in TRIM knockout mice suggests that other non-raft transmembrane adaptor proteins such as SIT or LAX may compensate for the absence of TRIM [65]. LAX-deficient T and B lymphocytes are hyper-responsive to anti-CD3- and anti-IgM-mediated stimulation [66], and SIT-deficient mice show an increased susceptibility to develop experimental autoimmune encephalomyelitis [67].

Other factors

Recently, it could be shown that surface and intracellular expression of CTLA-4 was reduced in activated T cells from TIRC7 (T-cell immune response cDNA 7)-deficient mice. Consistently, TIRC7^{-/-} T cells exhibited increased proliferation and expression of IL-2, interferon γ (IFN- γ) and IL-4 [68,69]. Co-localisation of TIRC7 and CTLA-4 occurred close to the site of TCR engagement, but no direct interaction of TIRC7 and CTLA-4 was shown [70]. Therefore, the activation-induced transmembrane protein TIRC7 might be involved in CTLA-4 expression via a yet unknown mechanism.

As in the case of many receptors, externalisation of CTLA-4 is also dependent on the activity of GTPase ADP ribosylation factor-1 (ARF-1) and phospholipase D (PLD). ARF family GTPases and PLD have been associated with the budding of vesicles at the Golgi apparatus [71-73]. PLD inhibitors or dominant negative mutants of ARF-1 or PLD could inhibit the release of CTLA-4 to the cell surface [54]. Presumably, other key regulators of trafficking (i. e. SNAREs) are also needed for CTLA-4 expression.

Other post-translation events

As in the case of major histocompatibility (MHC) molecules and some G protein-coupled receptors that require glycosylation to ensure proper folding and surface expression [74], regulation of *CTLA-4* surface expression might also be influenced by its glycosylation. A common polymorphism in the CTLA-4 gene, leading to substitution of threonine 17 to alanine 17 in the signal peptide, is associated with susceptibility to autoimmune diseases such as Graves' disease and type 1 diabetes. T cells homozygous for the *Ala17* allele showed impaired inhibitory function and reduced upregulation of CTLA-4 [75,76]. CTLA-4 Ala17 was found in lower levels at the cell surface than CTLA-4 Thr17. The Thr17Ala substitution results in defective processing in the endoplasmic reticulum, leading to aberrant glycosylation of one of the N-glycosylation sites in the CTLA-4 molecule [77].

Lysosomal secretion

Besides the release of newly synthesized protein from the TGN to the plasma membrane, the surface expression of CTLA-4 is enhanced by activation-induced secretion of CTLA-4-enriched lysosomes [55]. Accumulation of CTLA-4 was detected in lysosomes in Th1 (T helper cell 1) clones and normal T cells. Upon TcR stimulation, CTLA-4-containing lysosomes are mobilised to the cell membrane, resulting in an increase of CTLA-4 surface expression together with the lysosomal glycoprotein 85. Taking into account that the half-life of CTLA-4 is fairly short [55,57], these observations favour a model in which newly

synthesized CTLA-4 is released to the plasma membrane, rapidly internalised and targeted to lysosomal compartments where it is degraded without further stimulus. Upon TCR signalling, CTLA-4-containing secretory lysosomal vesicles are released to the cell surface to the site of TcR engagement [55], which is consistent with Linsley *et al.* [35], who demonstrated directional transport of CTLA-4.

A model of CTLA-4 trafficking

CTLA-4 surface expression is a highly dynamic process, which has to be precisely timed, localized and quantitatively adequate. Its tight regulation is a prerequisite for an efficient and controlled immune response. Bringing together recent advances in the understanding of the mechanisms regulating CTLA-4 surface expression, we propose the model shown in Figure 3. Newly synthesized CTLA-4 resides in the TGN, where it associates with the transmembrane adaptor TRIM and/or the clathrin adaptor AP-1. Upon T-cell activation, CTLA-4 is translocated to the cell surface toward the site of TcR engagement. The presence of TRIM promotes the release of CTLA-4 to the cell surface. Externalisation of CTLA-4 to the cell surface is also dependent on PLD and GTPase ADP ribolysation factor-1 activity. Association with AP-1 in the TGN, on the other hand, mediates the transport of CTLA-4 to endosomal or lysosomal compartments. Surface-exposed CTLA-4 is stabilized on the cell surface by phosphorylation of the Y201 residue by src kinases, which also enables binding of SHP-2, PP2A and the p85 subunit of PI3K and signalling via CTLA-4. Subsequent dephosphorylation at Y201 allows the binding of the clathrin adaptor AP-2 and the rapid internalisation and transport of CTLA-4 to endosomal or lysosomal compartments. This process involves Wortmannin-sensitive lipid kinases but not PI3K [60]. Internalised CTLA-4 might be degraded quickly [58] or recycled to the cell surface via secretory lysosomes [55].

Concluding remarks

It is still unclear which is the main compartment of intracellular CTLA-4. Interconnectivity of exo- and endocytotic pathways might account for reports with different findings on CTLA-4 localisation within the cell. Several connections between intracellular compartments have been described such as retrograde transport from endosomes to the TGN [78] or direct transport from the TGN to lysosomal compartments as mediated by AP-1 [32]. Therefore, localisation of CTLA-4 in distinct intracellular compartments may depend on the type of stimulus, kinetics and cell type. The fact that CTLA-4 expression varies in different T-cell subsets leads to the assumption that the regulation of CTLA-4 cycling might have cell type specific elements. Furthermore, there is the intriguing idea of a special storage compartment for CTLA-4 in memory T cells and/or Tregs, which would allow for immediate availability of CTLA-4 in response to antigenic stimuli to exert its immunomodulatory function. Since CTLA-4 is known as one of the markers for Tregs, unique CTLA-4 trafficking might correlate with yet unknown particular functions of CTLA-4 in these cells. Further studies might reveal cell type-specific regulation of the CTLA-4 trafficking process, storage and surface release consistent with different aspects of CTLA-4 function.

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Figure 1. Cytotoxic T-cell antigen 4 (CTLA-4) function at the T cell–antigen-presenting cell (APC) interface.

Both CD28 and the inhibitory co-receptor CTLA-4 bind to B7–1 (CD80) and B7–2 (CD86) molecules on APCs. The cytoplasmic tail of CTLA-4 contains two tyrosine-based motifs. The YVKM motif in the intracellular tail constitutes a binding site for the lipid kinase PI3K, the phosphatases PP2A and SHP2 and the clathrin adaptor proteins AP-1 and AP-2.

Figure 2. Cytotoxic T-cell antigen 4 (CTLA-4) structure.

(a) The CTLA-4 protein consists of three domains and a leader peptide. The leader peptide corresponds to exon 1 in the *CTLA-4* gene, the ligand binding domain to exon 2, the transmembrane region to exon 3 and the cytoplasmic tail to exon 4. Four different isoforms of CTLA-4 protein can be generated by differential splicing: full length CTLA-4 (exons 1, 2, 3 and 4), soluble CTLA-4 (exons 1, 2 and 4), ligand independent CTLA-4 (exons 1, 3 and 4, murine only) and an isoform consisting of exons 1 and 4 only. (b) The gene structure of CTLA-4 contains four exons, two untranslated regions preceding exon 1 and downstream of exon 4 and a promoter region up to -335 bp. Several gene polymorphisms described within the *CTLA-4* gene have been connected with autoimmune diseases. Some of these are indicated on the figure [e.g. in exon 1 A49G and (AT)_n in the -3' untranslated region (UTR)].

Figure 3. Model describing the regulation of cytotoxic T-cell antigen 4 (CTLA-4) surface expression.

This model summarizes the known mechanisms involved in CTLA-4 surface expression. Newly synthesized CTLA-4 binds to the transmembrane adaptor TRIM in the TGN promoting its release to the cell surface. CTLA-4 externalisation is also dependent on PLD and ARF-1 activity. Alternatively, binding to clathrin adaptor protein AP-1 mediates direct transport to lysosomal compartments. Surface residency of CTLA-4 is stabilized by phosphorylation of tyrosine 201 in the YVKM motif, which also allows for PI3K, SHP2 and PP2A binding and therefore CTLA-4 signalling. Clathrin adaptor AP-2 binds to dephosphorylated tyrosine 201 and mediates internalisation of CTLA-4 and its transport to endosomes and lysosomes. Upon T-cell activation, CTLA-4 enriched lysosomes are released to the cell surface. ARF-1, GTPase ADP ribolysation factor-1; PLD, phospholipase D; TRIM, T-cell receptor interacting molecule; TGN, *trans* Golgi network.

Table 1
Main CTLA-4 functions

CTLA-4	Refs
Increases threshold for cytokine production and proliferation	[3,4]
Inhibits cell cycle progression and transcription factors NF- κ B, NF-AT, AP-1	[5,6]
Induces indoleamine 2,3-dioxygenase release by dendritic cells	[23,24]
Modulates composition and cell surface expression of lipid rafts	[19-22]
Upregulates LFA-1-mediated adhesion via activation of Rap-1	[26,27]
Increases cell motility, reverses TcR-induced stop signal	[28]
Reduces T-cell-APC contact time	[28]

Abbreviations: AP-1, activator protein 1; APC, antigen-presenting cell; CTLA-4, cytotoxic T-cell antigen 4; LFA-1, lymphocyte function-associated antigen-1; NF- κ B, nuclear factor κ B; NF-AT, nuclear factor of activated T cells; TcR, T-cell receptor.