



# Diverse mechanisms regulate the surface expression of immunotherapeutic target CTLA-4

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T-cell co-receptor cytotoxic T-cell antigen-4 (CTLA-4) is a critical inhibitory regulator of T-cell immunity and antibody blockade of the co-receptor has been shown to be effective in tumor immunotherapy. Paradoxically, the majority of CTLA-4 is located in intracellular compartments from where it is transported to the cell surface and rapidly internalized. The intracellular trafficking pathways that control transport of the co-receptor to the cell surface ensures the appropriate balance of negative and positive signaling for a productive immune response with minimal autoimmune disorders. It will also influence the degree of inhibition and the potency of antibody checkpoint blockade in cancer immunotherapy. Current evidence indicates that the mechanisms of CTLA-4 transport to the cell surface and its residency are multifactorial involving a combination of immune cell-specific adapters such as TRIM and LAX, the small GTPase Rab8 as well as generic components such as ARF-1, phospholipase D, and the heterotetrameric AP1/2 complex. This review covers the recent developments in our understanding of the processes that control the expression of this important co-inhibitory receptor for the modulation of T-cell immunity. Interference with the processes that regulate CTLA-4 surface expression could provide an alternate therapeutic approach in the treatment of cancer and autoimmunity.

**Keywords:** CTLA-4, trafficking, TRIM, LAX, Rab8

## INTRODUCTION

The co-receptor cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) is a central inhibitory regulator of T-cell proliferation and expansion (1–5). Its dampening effect on the activation process limits and terminates T-cell responses, and as such is important for regulating peripheral T-cell tolerance and autoimmunity. A negative role for the co-receptor in the control of proliferation and autoimmunity was initially observed in the striking phenotype of the *Ctla4*<sup>-/-</sup> mouse (6, 7). These mice show polyclonal T-cell activation or autoprolieration that leads to massive tissue infiltration and early lethality. An additional linkage of single-nucleotide polymorphisms (SNPs) in the region of CTLA-4 were subsequently found associated with a variety of autoimmune disorders that include type 1 diabetes, coeliac disease, myasthenia gravis, Hashimoto's thyroiditis, systemic lupus erythematosus (SLE), and Wegener's granulomatosis (8–12). Immune dysregulation in human subjects has also been reported recently with heterozygous germline mutations in CTLA-4 (13). This plurality of associated autoimmune disorders in human beings has pointed to a central role for the co-inhibitory receptor as a general regulator of the threshold signals needed for T-cell activation. Under normal conditions, the inhibition of signaling events protects against responses to lower affinity self-antigen while allowing responses to higher affinity foreign antigen. In this sense, minor changes in the surface expression of the co-receptor are thought to have significant effects on responses to autoantigen. Ipilimumab, a humanized anti-CTLA-4 checkpoint blockade antibody, has also been found impressively effective in the treatment of various tumors such as melanoma and small cell lung carcinomas (14,

15). Combined therapy with antibodies against another negative co-receptor PD-1 (programmed cell death-1) has been found to co-operate with anti-CTLA-4 to induce even more striking response rates (16).

Given that minor changes in the surface expression of the co-receptor are expected to have significant effects on responses to autoantigen and in cancer immunotherapy, it is important to understand the mechanisms that determine the expression of CTLA-4 on T-cells. This includes the intracellular pathways that determine the transport or trafficking of CTLA-4 to the cell surface as well as events that regulate its residency on the surface and endocytosis. Paradoxically, CTLA-4 is primarily located in intracellular compartments from where it is rapidly recycled to the cell surface. Only small amounts of the co-receptor can be detected on the cell surface at any given time, even when optimally expressed following T-cell activation. This review covers the recent developments in our understanding of the events that control the transport and expression of CTLA-4 to the cell surface for the modulation of T-cell immunity.

## STRUCTURE AND FUNCTION OF CTLA-4

CTLA-4 was one of the first and most extensively investigated co-inhibitory receptor of the immune system (17). The CTLA-4 gene consists of four exons: exon 1 contains the leader peptide sequence, exon 2 the ligand binding site, exon 3 encodes the transmembrane region, and exon 4 the cytoplasmic tail (18). Differential splicing of the CTLA-4 transcript results in a full-length transmembrane form (exons 1–4), soluble CTLA-4 (lacking exon 3), and a transcript encoding only for exons 1 and 4 (19,

20). Murine T-cells also express a ligand-independent CTLA-4 (liCTLA-4) containing exons 1, 3, and 4 (12). Although liCTLA-4 lacks the MYPPPY ligand binding domain, it strongly inhibits T-cell responses and, compared to full-length CTLA-4, its expression is elevated in regulatory and memory T-cells from diabetes resistant NOD mice (21).

CTLA-4 is structurally related to CD28 with some 30% sequence homology (22). It was first described as the product of the *Ctla4* gene located at chromosome 1 (mouse) or 2 (human being) and is preferentially expressed in activated cytolytic T-cells (17). Subsequently, it was found to be expressed in all activated T-cells and used as an early activation marker. mRNA for CTLA-4 can be detected as early as 1 h post-activation with maximum expression between 24 and 36 h, the time when CTLA-4 is detectable on the cell surface (23, 24). In contrast to full-length CTLA-4, ligand-independent CTLA-4 is expressed in resting cells, but downregulated during early activation (21). Like CD28, CTLA-4 binds to ligands CD80 and CD86 but with greater avidity (25, 26). The same signature MYPPPY motif for binding is found in both co-receptors (27). The higher avidity of CTLA-4 for CD80 is due to the binding of one CTLA-4 homodimer to two CD80 molecules (28, 29) resulting in the formation of a stable CTLA-4/CD80 lattice structure in the immunological synapse (IS). This interaction may disturb the assembly of key signaling proteins needed for CD28 co-stimulation.

As mentioned, the importance of CTLA-4 in maintaining peripheral tolerance and homeostasis was first demonstrated with the autoimmune phenotype of CTLA-4-deficient mice. These mice show polyclonal T-cell activation leading to massive tissue infiltration and early lethality (6, 7). Further, SNPs of the human CTLA-4 gene have been implicated in the susceptibility to autoimmune disorders such as type I diabetes, rheumatoid arthritis, and multiple sclerosis (12). However, it is still unknown how and whether SNPs affect CTLA-4 function (i.e., intracellular trafficking, surface expression, dimerization). The soluble form of CTLA-4 has been linked to autoimmune diseases. High concentrations of soluble CTLA-4 can be detected in patients with various autoimmune diseases (30–32).

Unlike in the case of conventional T-cells (Tconv), suppressive regulatory T-cells (Tregs) express CTLA-4 constitutively on the cell surface. In fact, the pool of intracellular CTLA-4 seen in activated Tconv is less apparent in Tregs, a finding that may account for its constitutively high level of surface expression (33). Given this fact, it is not surprising that CTLA-4 is intimately linked to the regulation of Treg suppressor function (34, 35). Mechanisms that have been reported to account for Treg function include the secretion of the suppressive cytokines IL-10, IL-35, and TGF- $\beta$  (36), secretion of cytolytic granules containing granzyme and perforin as well as competition with conventional responder T-cells for CD80 and CD86 on antigen-presenting cells (APCs) (37, 38). Given its higher avidity for binding to CD80/86, CTLA-4 would block the availability of CD80 and CD86 for an interaction with Tconv. While CTLA-4 on Tconv induces their motility and limits their contact time with APCs, resulting in hypoactivation of these cells, CTLA-4 on Tregs does not influence their dwell times and, therefore, would allow the co-receptor to interfere with CD80/86 presentation to CD28 (39).

## CTLA-4 AND TUMOR IMMUNOTHERAPY

An exciting development over the past few years has been the use of anti-CTLA-4 in so-called checkpoint blockade in the treatment of cancers. These human studies originated from earlier mouse tumor models, which demonstrate that blockade of CTLA-4-mediated inhibition leads to enhancement of T-cell responses in tumor immunotherapy (40). Early human studies with limited numbers of patients (41–44) were expanded to larger phase III studies showing response rates as high as 30% on melanoma, small cell lung, and renal carcinoma (14–16). These studies led to the generation of antibodies to human CTLA-4, ipilimumab, and tremelimumab (45). Ipilimumab has been approved as monotherapy for the treatment of advanced melanoma. They have shown synergistic anti-tumor activity when utilized with vaccines, chemotherapy, and radiation (14). CTLA-4 antibodies have also induced a reversible occurrence of immune-related adverse events (IRAE) such as colitis, dermatitis, or endocrinopathies (46). The exact mechanism by which anti-CTLA-4 mediates enhanced anti-tumor reactivity is not clear, but may involve a combination of effects involving the lowering of the threshold needed to activate T-cells, a reduction in the number of Tregs, the reduced release of the suppressive factor indoleamine 2,3-dioxygenase (IDO) as well as broadening the peripheral T-cell receptor repertoire (47, 48). In certain instances, co-operation with interleukin-2 treatment has also been observed (49). More recently, antibodies against PD-1, another inhibitory co-receptor, have also demonstrated remarkable clinical anti-tumor activity against melanoma and other solid tumors (50). Further, the combination of anti-CTLA-4 and PD-1 antibodies achieved an even more effective anti-tumor response (16, 51). CTLA-4 engagement with CD80/CD86 attenuates the early activation of naive and memory T-cell, whereas PD-1 is mainly thought to modulate T-cell effector functions in peripheral tissues via binding to PD-L1 and PD-L2 (52). Since CTLA-4 and PD-1 regulate immune responses in a non-redundant fashion, combined blockade of both pathways may achieve more effective anti-tumor activity.

## MECHANISMS OF CTLA-4-MEDIATED INHIBITION

Despite the importance of CTLA-4 to autoimmunity and anti-tumor immunotherapy, the actual mechanisms responsible for its function are unknown. Much debate has focused on whether CTLA-4 inhibits T-cell responses by cell-extrinsic or -intrinsic mechanisms. Cell intrinsic mechanisms would reflect direct effects of the co-receptor on the expressing cell (i.e., signal transduction), while cell-extrinsic effects relate to the regulation of function via a distal cell or cytokine. Both mechanisms have been implicated in the *in vivo* function of CTLA-4 (53). A cell-extrinsic pathway for CTLA-4 was first described by Bachman and coworkers who found that Rag2-deficient mice reconstituted with a mixture of wild-type and CTLA-4-deficient bone marrow cells failed to develop autoimmune disease, while the transfer of *Ctla4*<sup>-/-</sup> bone marrow cells alone transferred disease (54). Cell-intrinsic and non-cell-autonomous (i.e., cell extrinsic) actions of CTLA-4 have been reported to operate to maintain T-cell tolerance to self-antigen (53). In agreement with this observation, Thompson and coworkers found that the loss of the cytoplasmic tail of CTLA-4 (i.e., cell intrinsic) affected the onset of disease as well as differences

in T-cell infiltration. These findings suggested possible differences for cell intrinsic versus extrinsic mechanisms in the autoproliferative versus migratory aspects of CTLA-4 inhibition (55). Others have emphasized the importance of cell-extrinsic mechanisms on both Tconv and Tregs, although this may vary with antigen dose and the model examined (56). It is possible that CTLA-4 utilizes different pathways for inhibition in different contexts or niches of the immune system.

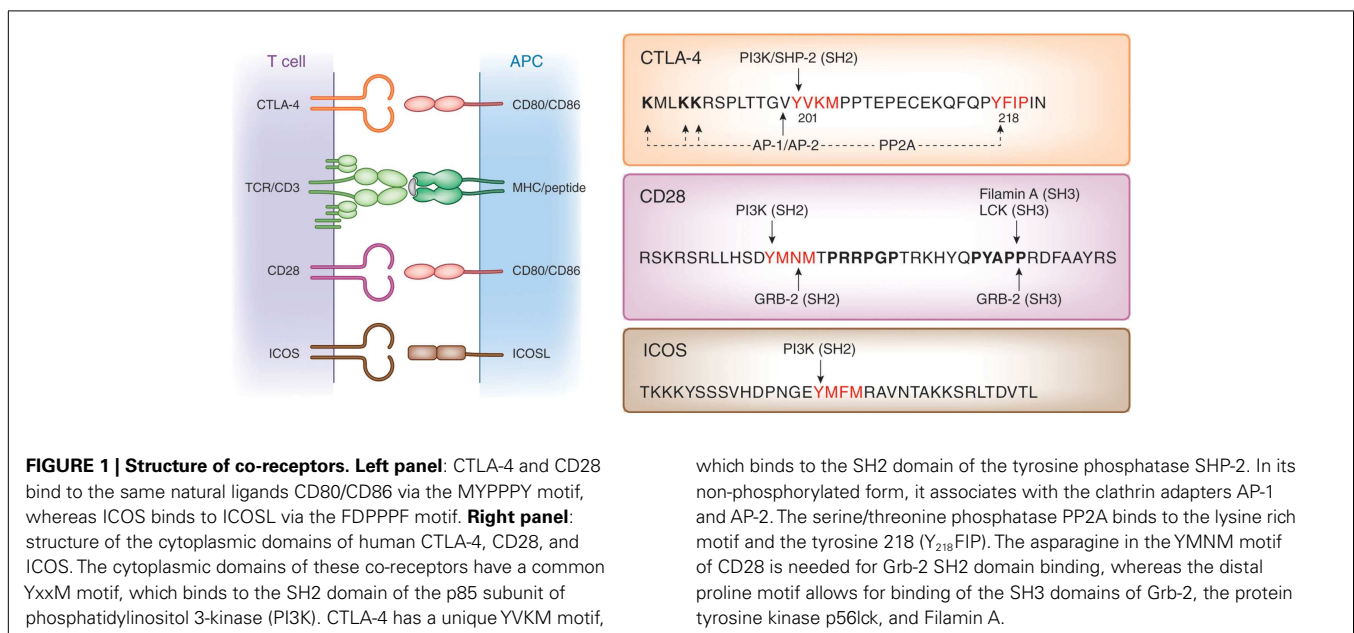
Cell intrinsic pathways include modulation of TCR signaling by phosphatases SHP-2 and PP2A (57), inhibition of ZAP-70 micro-cluster formation (58), and altered IS formation (59), as well as interference with the expression or composition of lipid rafts on the surface of T-cells (60–63). Like CD28 and ICOS, CTLA-4 possesses a small cytoplasmic tail containing, apart from its C-terminal YFIP motif, a YxxM consensus motif common of all three co-receptors (64) (Figure 1). Several intracellular proteins including the lipid kinase phosphatidylinositol 3-kinase (PI3K) (65), the phosphatase SHP-2 (4, 57, 66, 67) and clathrin adapter proteins AP1 and AP2 (68–70) have been reported to bind to the YVKM motif. The phosphatase PP2A has also been reported to interact with the cytoplasmic tail of CTLA-4 via the lysine rich motif and via the tyrosine residue at position 218 (71). CTLA-4-mediated phosphorylation of Akt is abrogated by the PP2A inhibitor okadaic acid (72). By contrast, PD-1 signaling inhibits Akt phosphorylation by preventing CD28-mediated activation of PI3K that is dependent on the immunoreceptor tyrosine-based switch motif (ITSM) located in its cytoplasmic tail (72).

Cell-extrinsic mechanisms include CTLA-4 engagement of CD80/CD86 on dendritic cells (DCs) that can induce the release of IDO (73, 74). This enzyme catalyzes the degradation of the amino acid L-tryptophan to N-formylkynurenine leading to the depletion of tryptophan, which in turn can halt the growth of T-cells. Although IDO has been implicated in certain immune responses (75, 76), it is unlikely to solely account for the phenotype of the *Ctla4*<sup>-/-</sup> mouse since IDO-deficient mice fail to develop

autoimmunity (77). CTLA-4 has also been reported to increase the production of the immunosuppressive cytokine TGF- $\beta$  (78); however, TGF- $\beta$ -deficient mice differ from CTLA-4-deficient mice in the severity of the autoimmune phenotype (79). The multiorgan inflammatory syndrome can be inhibited by depletion of the activated CD4 positive T-cells leading to prolonged survival; however, the TGF- $\beta$ -deficient mice eventually die of myeloid hyperplasia (80).

Not unexpectedly, Tregs play a major role in cell-extrinsic regulation. Both CTLA-4-deficient and FoxP3-deficient mice exhibit a short life span due to massive lymphoproliferation (LP) and a systemic autoimmune-like syndrome (6, 7, 81). The conditional loss of CTLA-4 on FoxP3 expressing cells delayed the onset of disease to 7–10 weeks, rather than to 3–4 weeks observed in *Ctla4*<sup>-/-</sup> mice (82, 83). This indicated that Tregs help control the development of the *Ctla4*<sup>-/-</sup> phenotype and that both CTLA-4 and FoxP3 on the same cell subset are essential to fully prevent LP disease. However, while Tregs help to control the onset of disease, the fact that the mice still die suggests that another factor is causally responsible for the onset of the autoimmune-like syndrome.

The mechanism by which CTLA-4 facilitates Treg function is unclear but may involve the occupancy of CD80 and CD86 on DCs (82, 83). Trans-endocytosis or the removal of CD80 or CD86 from the surface of DCs may also occur (83, 84). Since both Tregs and Tconv can mediate this removal, it is uncertain whether this property can be the primary mechanism to account for Treg suppression. On the other hand, in certain models, some groups have claimed that the mere expression of CTLA-4 on either subset is sufficient to mediate cell-extrinsic suppression (33, 56). Tregs with higher CTLA-4 levels are able to be more effective in blocking or trans-endocytosis than Tconv cells. In this context, recent elegant work has shown that CTLA-4 can bind to the protein kinase C isoform  $\eta$  (PKC- $\eta$ ) in Tregs (and not Tconv cells) and that defective activation of CTLA-4-PKC- $\eta$  with another complex in PKC- $\eta$ -deficient cells correlates with the reduced depletion of



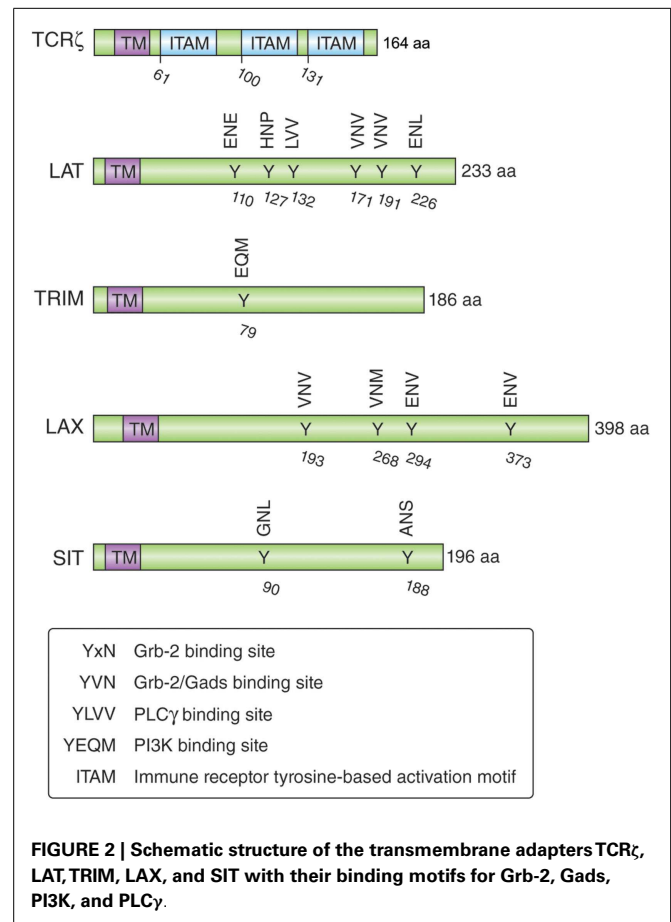
CD86 from APCs (85). CTLA-4-associated SHP-1/2 and PP2A are not recruited to the IS of Tregs (85, 86).

Another model involves a combination of cell-intrinsic and -extrinsic effects related to altered T-cell adhesion and motility (87, 88). We and others have shown that CTLA-4 ligation activates the small GTPase Rap-1 (89, 90). Rap1 is a key molecule involved in the activation of integrins such as lymphocyte function-associated antigen-1 (LFA-1). In this model, CTLA-4 is a motility activator and augments T-cells adhesion (88, 90). Significantly, anti-CTLA-4 alone was able to induce motility of primary T-cells and cell lines (58, 88). As a motility activator, CTLA-4 bypasses the TCR-mediated stop-signal that is needed for stable interactions between T-cells and APCs. This provided an alternate mechanism to account for the dampening effect of CTLA-4 on T-cell activation and has been confirmed in several different models (87, 88, 90–95). In this model, the cell intrinsic pathway involves activation of Rap1 and the ligation efficiency of the TCR on Tconvs, while the cell-extrinsic pathway involves the regulation of T-cell binding to APCs. The reversal of the stop-signal by CTLA-4 was exclusively seen on Tconv and not Tregs (39).

### CTLA-4 TRAFFICKING FROM THE TRANS-GOLGI NETWORK TO THE CELL SURFACE

Understanding the mechanisms by which CTLA-4 is transported to the cell surface will be the key to the development of novel strategies to increase or decrease its expression and functional effects. An ability to interfere with the trafficking pathways in T-cells would provide an alternate approach to the use of biologics such as anti-CTLA-4 antibodies. Previous studies have demonstrated the need of calcium for the release of CTLA-4 from the *Trans*-Golgi network (TGN) to the cell surface (69, 96), while other studies have implicated more generic processes involving the GTPase ADP ribolysation factor-1 (ARF-1) and phospholipase D (PLD) (97). However, these pathways are also involved in the transport of other non-lymphoid receptors and thus are not specific for CTLA-4. In this context, it has been demonstrated that TCRzeta (TCR $\zeta$ ) plays a central role in transporting the TCR to the cell surface (98, 99). TCR $\zeta$  is a member of the type III transmembrane adapter proteins (TRAPs), which possess a short extracellular domain, a single transmembrane domain, and a relatively long cytoplasmic tail with several tyrosine phosphorylation sites (100, 101) (Figure 2). Based on the TCRzeta model, we hypothesized that this family of transmembrane proteins might play a general role in the transport of surface receptors. Other members of the TRAP family include TRIM (T-cell receptor-interacting molecule), LAX (linker for activation of X cells), SIT (SHP2 interacting TRAP), and LAT (linker for activation of T-cells) (100, 101). As in the case of the TCR $\zeta$ , they are preferentially expressed in immune cells, but most of them lack the signaling effects seen with the TCRzeta chain. For example, they lack the immunoreceptor tyrosine-based activation motifs (ITAMs) needed for binding to the protein tyrosine kinase ZAP-70. Instead, they are enriched in binding sites for PI-3K and Grb-2/Gads (102, 103).

TRIM is highly expressed in thymocytes and CD4 positive T-cells and forms a disulfide-linked homodimer (104). It possesses three tyrosine-based motifs in its cytoplasmic tail (two YxxL motifs

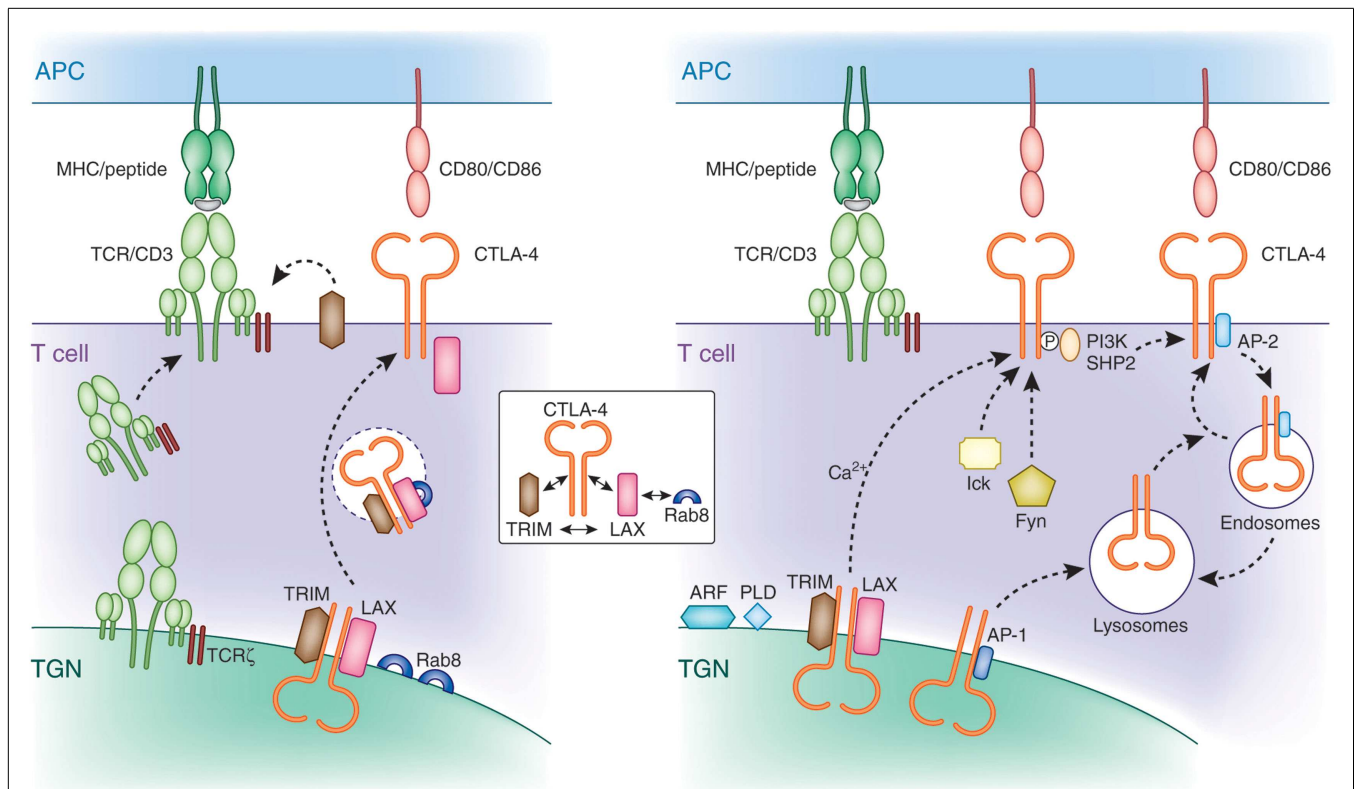


**FIGURE 2 | Schematic structure of the transmembrane adapters TCR $\zeta$ , LAT, TRIM, LAX, and SIT with their binding motifs for Grb-2, Gads, PI3K, and PLC $\gamma$ .**

and one YxxM motif), where the YxxM motif binds to the p85 subunit of PI3 kinase (102) (Figure 2). Initial TRIM overexpression studies in Jurkat T-cells suggested that TRIM upregulates the surface expression of the TCR and mediates increased calcium release after TCR ligation (105). However, T-cell development, TCR surface expression, and signaling events induced by TCR ligation are not impaired in TRIM-deficient mice (104). LAX is expressed as a monomer and possesses a longer cytoplasmic tail (398 aa versus 186 aa), which contains eight tyrosine-based motifs; five of them represent binding sites for Grb-2/Gads (103). LAX negatively impairs TCR signaling events as shown with LAX overexpression studies in Jurkat T-cells leading to inhibition of p38 and NFAT/AP-1 (106). Although LAX deficiency does not impair lymphocyte development, T- and B-cells are hyperresponsive upon TCR or BCR ligation and show increased cell survival (107). Mutation studies of the tyrosine-based motifs revealed the importance of the binding signaling proteins (Grb-2, Gads, and PI3K) in the inhibitory function of LAX (103).

Initial shRNA knockdown and overexpression studies demonstrated that TRIM facilitates the transport of CTLA-4 to the cell surface (108, 109). TRIM specifically co-precipitated CTLA-4, but not other T-cell co-receptors such as CD28. Overexpression of TRIM potentiated CTLA-4 expression due to increased release to the cell surface, which in turn led to increased suppression of T-cell activation. Subsequently, LAX was also found to bind, co-localize,





**FIGURE 3 | Mechanisms of CTLA-4 trafficking.** **Left panel:** CTLA-4 forms a multimeric complex composed of TRIM, LAX, and Rab8 for post-Golgi transport to the cell surface. TRIM and LAX bind to the cytoplasmic tail of CTLA-4, while LAX binds via its N-terminus to active GTP-Rab8 (inset). TRIM requires LAX for binding to Rab8 in a complex. LAX functions as a central coordinator by bridging Rab8 with the other LAX-associated proteins TRIM and CTLA-4. This multimeric complex facilitates the transport of newly translated CTLA-4 to the cell surface. The transmembrane adapters TRIM and LAX play a role for CTLA-4 transport similar to the TCRzeta chain, which resides in the TGN and surrounding vesicles and is needed for the association

with the hexameric TCR/CD3 in the TGN for proper and efficient cell surface expression. **Right panel:** non-phosphorylated CTLA-4 associates with the clathrin adapter complex AP-1 and AP-2. AP-1 regulates trafficking of CTLA-4 by shuttling the receptor from the TGN to lysosomes. In activated cells, newly synthesized CTLA-4 is transported to the cell surface, which is facilitated by the TRIM/LAX/Rab8 complex (left panel) and by more generic factors such as ARF-1, PLD, and calcium. Phosphorylation of CTLA-4 by Fyn and Lck recruits PI3K and SHP-2. Dephosphorylated CTLA-4 binds to AP-2 leading to the internalization of CTLA-4 into intracellular compartments such as endosomes and lysosomes from where it can recycle to the cell surface.

and facilitate CTLA-4 transport to the cell surface (110). CTLA-4 binding to TRIM and LAX was specific in that it did not associate with LAT. These data indicate that TRIM and LAX, both immune-specific type III proteins, bind to CTLA-4 to facilitate its transport to the cell surface (Figure 3).

Strikingly, downregulation of TRIM and LAX markedly reduced the presence of CTLA-4 expressing TGN proximal vesicles. This observation suggested a connection between CTLA-4 binding to TRIM/LAX and the budding of CTLA-4 positive vesicles from the TGN needed for transport to the cell surface. Further, these findings resemble that found for the transport of the TCR/CD3 complex to the cell surface (111). TCRzeta, which resides in the TGN and surrounding vesicles needs to associate with the hexameric TCR/CD3 in the TGN for proper and efficient cell surface expression. Rab proteins are members of the Ras superfamily and regulate protein transport of the secretory and endocytic pathway (112, 113). They are active in a GTP-bound state and become inactive with the conversion of GTP to GDP. Among the different members of the Rab family, Rab8 has been

shown to mediate the trafficking of newly synthesized proteins from the TGN to the plasma membrane (114), whereas other family members mediate transport of proteins among other intracellular organelles such as endoplasmic reticulum, endosomes, and lysosomes. In addition, Rab8 alters the reorganization of actin and microtubules, as well as directing membrane transport to cell surfaces (115, 116). Activation of Rab8 can lead to cell protrusions, whereas its depletion promotes the formation of actin stress fibers (117, 118). The  $\alpha_2\beta$  and  $\beta_2$ -adrenergic receptors have been described to bind Rab8 for transport to the plasma membrane (119). However, until recently, despite its high expression in T-cells, no immune cell-specific binding effectors of Rab8 have been identified.

A recent study by Banton et al. showed that the transmembrane adaptor LAX bound to the active form of Rab8, while at the same time also associated with CTLA-4 and TRIM (110). By contrast, CTLA-4 and TRIM failed to bind to Rab8. These findings demonstrate that CTLA-4 interacts with a protein complex in which TRIM and LAX bind to the co-receptor, TRIM

and LAX to each other, and LAX to Rab8 (**Figure 3**, see inset). Importantly, disruption of LAX-Rab8 binding profoundly reduced the formation of CTLA-4 containing vesicles proximal of the TGN as well as the expression of CTLA-4 on the cell surface. The reduction in CTLA-4 expression resulted in augmented immune responses. Overall, given the established role of Rab8 as a molecule that mediates intracellular trafficking of proteins from the TGN to the plasma membrane, its association with CTLA-4 provides a pathway for the control of CTLA-4 surface expression.

Altogether, the TRIM-LAX-Rab8 connection to CTLA-4 trafficking to the cell surface will operate in co-operation with other generic processes. The ADP ribosylation factor (ARF) family GTPases and PLD are needed for the budding of vesicles at the Golgi apparatus (120–122). As in the case of Rab8, ARFs are members of GTP binding proteins of the Ras superfamily. There are six conserved ARF proteins in mammalian cells and are well established regulators of vesicle trafficking and actin re-modeling. In particular, ARF1 is involved in the regulation of vesicle transport in the TGN and the activation of PLD. PLD hydrolyzes phosphatidylcholine generating phosphatidic acid (PA) and choline. Further, the hydrolyzation of PA generates diacylglycerol, which, in addition to its signaling role, has a functional role in membrane modulation (123, 124). Dominant negative mutants of ARF-1 or PLD inhibit the release of CTLA-4 to the cell surface (97). However, unlike Rab8, none have been reported to associate with CTLA-4. Most likely, other key regulators of trafficking (i.e., SNAREs) are also needed for CTLA-4 expression. These mechanisms have been described for many surface expressed receptors and are not specific for CTLA-4.

## RECYCLING OF CTLA-4

Following cell surface expression, CTLA-4 is rapidly internalized and again recycled to the plasma membrane of T-cells. This presumably occurs due to control of the inhibitory effects of CTLA-4 on the immune response (i.e., needs to be tightly regulated). The one exemption is Tregs, which show constitutively surface expressed CTLA-4 (24, 125). Rapid removal of CTLA-4 from the cell surface is mediated by the heterotetrameric adapter protein AP2 via clathrin-dependent internalization (68–70). AP-2 binding is regulated by the phosphorylation of the YVKM motif in the cytoplasmic tail of CTLA-4 (126, 127). Phosphorylation of CTLA-4 by protein tyrosine kinases p56lck, p59fyn, and Rlk (128, 129) promotes binding to PI3K or SHP-2 leading to the production of phosphatidylinositol (3,4,5)-triphosphate (D3 lipids) or dephosphorylation of tyrosine residues on substrates such as ITAMs in the TCR/CD3 complex and ZAP-70 (130). AP-2 binding to CTLA-4 is inhibited by the phosphorylation of the YVKM motif. Instead, once the recruitment and engagement of PI 3K is complete, CTLA-4 is dephosphorylated exposing the non-phosphorylated TGVYVKM motif. Binding of AP-2 generally involves the independently folded appendage domains of the large  $\alpha$  ( $\alpha_1$  or  $\alpha_2$  isoform) and  $\beta_2$  subunits, each separated from the heterotetrameric adapter core by a flexible hinge (131). Its binding to CTLA-4 mediates the internalization of the co-receptor from the cell surface to endosomal and lysosomal compartments. Golgi-associated CTLA-4 also binds to the heterotetrameric AP-1 via

the same motif (69) where it shuttles the receptor from the TGN to lysosomes. Further, upon TCR stimulation, CTLA-4 containing secretory lysosomal vesicles are released to the cell membrane resulting in increased CTLA-4 surface expression (132). Further, under conditions of T-cell inactivation, CTLA-4 colocalizes with the TCR to lipid rafts in the IS (61). The polarized release of CTLA-4 toward the site of TCR engagement has been correlated with a repositioning of the microtubule organizing center (MTOC) in T-cells (96, 133). The extent of CTLA-4 surface expression is determined by the strength of the TCR signal (133). In contrast to full-length CTLA-4, ligand-independent CTLA-4 (lacking exon 2 encoding the ectodomain including the MYPPPY motif needed for CD80/86 binding) expressed in resting mouse T-cells is downregulated during activation (21). Also, compared to activated effector T-cells, CTLA-4 is considerably longer retained on the surface of memory T-cells (134). The molecular basis for the different expression levels of CTLA-4 in both cell types remains to be established. Intracellular trafficking to the cell surface as well as endocytosis and recycling determine the overall level of CTLA-4 on the surface of T-cells.

## SUMMARY

Optimal regulation of CTLA-4 surface expression is crucial for the balance of stimulatory and inhibitory signals to elicit proper immune responses. Minor changes in surface expression levels could have major effects on the outcome of T-cell activation. Levels of surface expressed CTLA-4 are regulated by endocytosis, recycling, and newly synthesized CTLA-4. Besides generic factors (i.e., ARF-1, PLD, SNAREs) needed for transport of multiple receptors to the cell surface, the recently identified CTLA-4-TRIM-LAX-Rab8 complex is specific in facilitating CTLA-4 transport to the cell surface. This finding is of potential importance for the development of new therapeutics that will be designed to enhance anti-tumor immunity or to increase expression in the control of autoimmune disease. Cell permeable peptides (CPP) and/or siRNA targets of immune cell trafficking adapters TRIM or LAX could provide an alternate therapy especially for patients with severe IRAE during treatment with CTLA-4 antibodies. Further, a combination of anti-PD-1 antibodies and CPP could achieve an even more effective anti-tumor response.

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