Opinion
CellPress

Confusing signals: Recent progress in CTLA-4 biology

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The mechanism of action of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) remains surprisingly unclear. Regulatory T (Treg) cells can use CTLA-4 to elicit suppression; however, CTLA-4 also operates in conventional T cells, reputedly by triggering inhibitory signals. Recently, interactions mediated via the CTLA-4 cytoplasmic domain have been shown to preferentially affect Treg cells, yet other evidence suggests that the extracellular domain of CTLA-4 is sufficient to elicit suppression. Here, we discuss these paradoxical findings in the context of CTLA-4-mediated ligand regulation. We propose that the function of CTLA-4 cytoplasmic domain is not to transmit inhibitory signals but to precisely control the turnover, cellular location, and membrane delivery of CTLA-4 to facilitate its central function: regulating the access of CD28 to their shared ligands.

The need for CTLA-4-based regulation

The generation of a large repertoire of T cell receptors is necessary to recognize a wide array of unknown pathogens throughout our lives. However, this approach to immune protection comes with drawbacks, most notably the need to control self-reactive T cells that are generated during this process. While the thymus provides some degree of selection against generation of self-reactive T cells, this process is by necessity incomplete, and self-reactive T cells populate our peripheral pool. CTLA-4 is a key player in the control of such cells, and mice genetically deficient in CTLA-4 have profound immune dysregulation and autoimmune disease [1,2]. Interestingly, the major cell type expressing CTLA-4 are Treg cells [3,4]. It is also clear that CTLA-4 does not operate in isolation, but that it directly antagonizes the costimulatory receptor CD28 (Figure 1). Accordingly, the fatal autoimmunity observed in CTLA-4-deficient mice is likely to be the result of excessive CD28 stimulation by its ligands CD80 and CD86. Indeed, blockade or deletion of either the ligands [5,6] or CD28 [7] prevents autoimmunity triggered by the loss of CTLA-4. Given that CTLA-4 binds the same ligands as CD28, but with higher affinity, it is also apparent that the system operates in an integrated fashion. Nonetheless, the molecular details of how CTLA-4 achieves its critical function have been widely debated. In this opinion article, rather than comprehensively review the CTLA-4 field, we consider some of the more recent findings in this area in the context of the literature, and suggest a new framework for their interpretation.

CTLA-4 inhibitory signals

How does CTLA-4 signal?

CTLA-4 has a 36-amino acid cytoplasmic tail that is devoid of intrinsic enzymatic activity and lacks a bona fide ITIM motif [8]. Unlike CD28, which is a surface receptor, CTLA-4 is highly endocytic, spending much of its time in intracellular vesicles (Box 1). It is constitutively present as a homodimer [9] and does not appear to undergo conformational change following ligand binding [10,11]. Numerous molecular interactions have been proposed to account for CTLA-4 inhibitory signaling in T cells. It is common practice to simply list these pathways as though all are equally validated, with their relative roles perhaps depending on cellular context. Yet, in our view, it is perhaps surprising that none of these proposed signaling pathways has been reproducibly demonstrated to the point where they provide anything approaching a robust platform, as has been achieved for other important signaling receptors.

A survey of the literature in this area highlights multiple contradictions. Some studies suggested that CTLA-4 altered phosphorylation of CD3ζ chains [12], but other studies found this not to be the case [13]. CTLA-4 was reported to disrupt the formation of ZAP-70 microclusters [14]; however, others found that CTLA-4 function did not interfere with ZAP-70 recruitment or phosphorylation [13,15]. Some reports showed that CTLA-4 interacted with phosphoinositide 3-kinase (*PI3K*) [16], although others found that the CTLA-4 cytoplasmic tail was unable to recruit this enzyme [17]. The tyrosine phosphatase SHP-2 (SYP) was originally reported to bind specifically to the tyrosine-phosphorylated YVKM sequence in the CTLA-4 cytoplasmic tail [18]; however, the requirement for tyrosine phosphorylation was later questioned [12]. Subsequent analysis concluded that there was not in fact a direct interaction between CTLA-4 and SHP-2 [19] and imaging approaches showed that neither SHP-1 nor SHP-2 were co-recruited with CTLA-4 to the immunological synapse [20]. CTLA-4 was reported to increase AKT activity [21], although others found decreased AKT activity following CTLA-4 engagement [22]. Similar to CD28, CTLA-4 has been shown to associate with the serine/threonine

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Keywords: CTLA-4; CD28; costimulation; T cell tolerance; T cell activation.

1471-4906/
 http://dx.doi.org/10.1016/j.it.2014.12.001

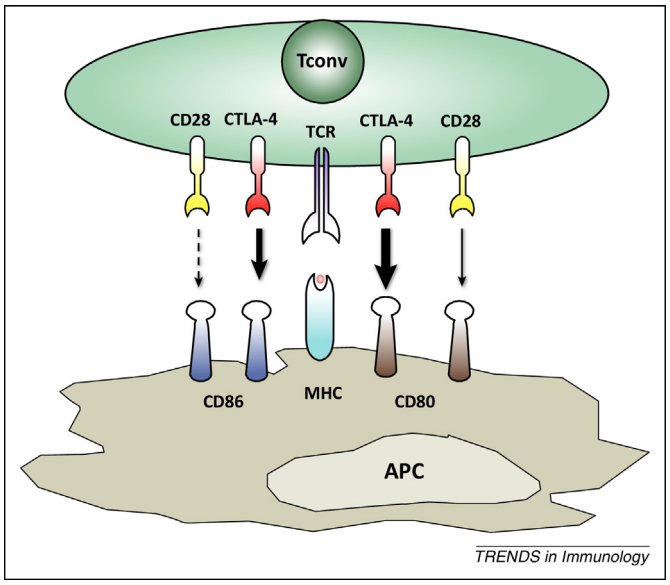

Figure 1. The CD28 and CTLA-4 receptors are connected by shared ligands. CD28 and CTLA-4 on the T cell bind to two ligands, CD80 and CD86, on antigen-presenting cells (APCs). The interactions take place with varying affinities (represented by thickness of the arrows). Given that CTLA-4 has higher affinity for both ligands, this sets up a competition between CTLA-4 and CD28 for ligand binding. The system is integrated in the sense that alteration of one component has the potential to affect the equilibrium of the other interactions. For example, blockade or deletion of CTLA-4 will lead to increased availability of ligands for CD28 binding and vice versa. Abbreviations: Tconv, conventional T cells; TCR, T cell receptor.

Box 1. CTLA-4 is a moving target

A key feature of CTLA-4 is its rapid and constitutive endocytosis from the plasma membrane resulting in approximately 90% of CTLA-4 being intracellular. Yeast two-hybrid interaction analysis revealed that the CTLA-4 cytoplasmic tail associates with the μ2 subunit of the adaptor complex AP-2 [89,90]. μ2 is known to bind tyrosine-containing YXXϕ motifs, where Y is tyrosine, X is any amino acid, and ϕ is an amino acid with a bulky hydrophobic side chain, such as the Y_{201}VKM sequence in CTLA-4. Indeed, substitution of Y_{201} for phenylalanine abolished the interaction with μ2 and inhibited endocytosis of CTLA-4 [89,90]. Strong evidence suggests that only the dephosphorylated form of YVKM can bind μ2 [89,91]. Collectively, these data formed the basis of a model in which nonphosphorylated CTLA-4 was internalized via its AP-2 association, whereas tyrosine phosphorylation stabilized it at the surface, permitting ligand engagement and consequent negative signals.

While this model is still widely accepted, several groups have reported that CTLA-4 inhibitory function does not require its tyrosine phosphorylation [92–94]. In addition, it is clear that primary human T cells continue to endocytose CTLA-4 following activation [81]. Given that CTLA-4 is reportedly phosphorylated by ZAP-70 or p56lck [91,92], it would be predicated that TCR activation of T cells would trigger CTLA-4 phosphorylation and surface retention. However, analysis of CTLA-4 expression in activated T cells showed that it was still predominantly intracellular and rates of CTLA-4 endocytosis remained high [81].

Given that early experiments relied on transfection of p56lck or p59fyn [91,95] or pervanadate treatment [89,95], it is possible that CTLA-4 remains largely dephosphorylated and, therefore, endocytic, during physiological T cell activation.

phosphatase PP2A [23], and it was suggested that activity of this phosphatase was responsible for CTLA-4-dependent AKT inhibition [22]. However, a CTLA-4 mutant lacking PP2A-binding sites appeared to show increased inhibitory function [24], suggesting that rather than eliciting CTLA-4 function, PP2A inhibited it.

We do not wish to discuss the findings of the above studies in detail. Rather, our intention is to highlight the conflicting nature of the literature, a fact frequently overlooked by those who suggest that negative signaling is a well-established paradigm. In our view, the favored approach of ascribing a long list of signaling mechanisms to CTLA-4 belies the true lack of consensus and progress in this area.

Missing in action: the elusive nature of CTLA-4 signaling

In addition to the lack of a detailed description of the CTLA-4 signaling components themselves, we note an increasing number of settings in which evidence of CTLA-4 signals might be expected, yet is not seen. Experiments attempting to identify the transcriptional response to antibody engagement of CTLA-4 revealed only subtle changes, mainly during the first 4 h of stimulation, a timeframe in which CTLA-4 expression is known to be low [25]. In Treg cells, which express the highest levels of CTLA-4, the effects of CTLA-4 engagement on transcript expression were described as 'weak or inexistent', while, by contrast, CD28 ligation had a robust transcriptional footprint [25]. Gene expression studies have also been performed using CTLA-4-sufficient or CTLA-4-deficient T cells isolated from bone marrow chimeric mice: these chimeras are healthy and provide the opportunity to examine CTLA-4-deficient T cells that have not been isolated from an animal with lymphoproliferative disease. These studies similarly failed to find evidence of suppressive signals in CTLA-4-expressing cells; the difference in terms of transcripts expressed in CTLA-4-deficient T cells, as compared with wild type cells, was described as 'minimal' [26]. Allison and colleagues used an adoptive transfer system to examine gene expression changes in CTLA-4-sufficient or CTLA-4-deficient T cells responding to cognate antigen *in vivo* [27]. Only ten independent genes were upregulated (more than twofold) in CTLA-4-expressing cells compared with their CTLA-4-deficient counterparts, one being CTLA-4 itself [27]. The authors concluded that there was 'no obvious signature of active negative regulation' in CTLA-4-bearing T cells.

Finally, an interesting study from Bluestone and colleagues examined a mouse expressing a mutant form of CTLA-4 in which the tyrosine residue at position 201 in the intracellular YVKM motif was replaced with valine (Y201V), thereby preventing phosphorylation of this motif [28]. One prediction of this experiment was that conventional T cells from these animals would be unable to transmit inhibitory signals via CTLA-4. While these mice developed more severe experimental autoimmune encephalomyelitis (EAE) following injection with MOG_{35-55} peptide in complete Freund's adjuvant (CFA) in conjunction with pertussis toxin, on closer examination it became apparent that this reflected a defect in the Treg rather than the conventional T cell population. Accordingly, conventional T cells from mice expressing Y201V-CTLA-4 were indistinguishable from wild type T cells in their capacity to induce EAE upon adoptive transfer, whereas Treg cells from these animals showed impaired disease regulation. While this could reflect a role for CTLA-4 in transducing activating signals in Treg cells, it provides little support for an inhibitory signaling model.

At its core, the concept of inhibitory signaling is built around recruitment of inhibitory apparatus by CTLA-4, which interferes with T cell receptor (TCR) and/or CD28 signaling, thereby thwarting early T cell activation. Such a concept is difficult to simply extend to Treg cells, given that in Treg cells both TCR signaling and CTLA-4 are required for suppression. Therefore, the function of CTLA-4 in Treg cells demands a different paradigm.

PKC-η: a new player in CTLA-4 signaling?

Perhaps the most enticing recent data relating to the concept of CTLA-4 signaling come from work performed in the Altman laboratory showing that CTLA-4 associates with the protein kinase C isoform PKC-η [29]. In these studies, Kong *et al.* revealed a physical association between CTLA-4 and PKC-η in Treg cells that is mediated by a membrane-proximal lysine motif in the CTLA-4 cytoplasmic tail. Notably, the interaction did not involve other motifs previously implicated in CTLA-4 signaling (e.g., YVKM). Importantly, Treg cells lacking PKC-η were defective in their capacity to suppress homeostatic T cell proliferation and antitumor responses.

Given the clear biochemical evidence of a molecular interaction between CTLA-4 and PKC-η, at first sight these data might appear to breath further life into the concept of CTLA-4 signaling. However, to our minds, it is less clear that a signaling event (i.e., ligand-triggered activation of a pathway) is occurring. Indeed, in hybridoma cells, the association of phosphorylated PKC-η with CTLA-4 appeared equivalent regardless of anti-CD3/CD86-Fc stimulation, suggesting that the association is constitutive [29]. Moreover, experiments with antibody stimulation (Box 2) only used anti-CD3 and anti-CTLA-4 together, with no comparison made with anti-CD3 alone; thus, as presented, there is no compelling evidence that a CTLA-4-triggered signaling event has occurred. This interpretation does not detract from the potential importance of the CTLA-4/PKC-η interaction. Indeed, the authors showed that this association mediates recruitment of a GIT2–αPIX–PAK complex that could have a key role in promoting cellular motility through focal adhesion disassembly [29]. Consistent with this notion, Treg cells lacking PKC-η interacted more strongly with antigen-presenting cells (APC), and were less efficient at serially engaging APC to elicit ligand downregulation (see below for discussion of ligand downregulation). Interestingly, GIT proteins are known to be involved in membrane recycling and endosomal dynamics [30], and can exhibit ADP-ribosylation factor GTPase-activating protein (ARF-GAP) activity towards ARF-1 [30], which has previously been implicated in CTLA-4 membrane transport [31]. αPIX is also implicated in recruitment of Lymphocyte function-associated antigen 1 (LFA1) to the immune synapse [32], which could be relevant given the importance of the LFA1/ICAM1 interaction in gluing Treg cells to their targets [33]. Thus, the PKC-η interaction with CTLA-4 could have several interesting roles in controlling CTLA-4 cell biology and function. However, in our opinion, the data presented do not invoke, or indeed provide support for, a ligand-driven inhibitory signal.

Box 2. Are agonistic anti-CTLA-4 Abs a valid experimental tool?

One significant pillar underpinning the concept of inhibitory signaling relates to the use of 'agonistic' anti-CTLA-4 Abs. The evidence that such Abs are indeed agonistic, and deliver inhibitory signals, stems from the practice of bead-coating anti-CD3 and anti-CD28 Ab to trigger T cell activation and cross-titrating anti-CTLA-4 Ab to identify a point where the latter elicits 'inhibition' of the T cell response. There are several potential issues with this approach. First, to some extent, these experiments are self-fulfilling prophecies, in that the ratios of Abs are chosen to give the desired result (i.e., lack of T cell proliferation). It is unlikely that these ratios in any way reflect the natural balance between CD28 and CTLA-4 engagement that occurs upon ligand binding in physiological settings. Indeed, the balance between CD28 and CTLA-4 engagement is pre-set by the natural ligand affinities and differs substantially between the two ligands [96]. A second issue relates to the fact that bead coating of stimulatory and inhibitory Abs can result in artifacts, whereby the inhibitory antibodies outcompete the activating Abs. Such coating bias has been reported for anti-CD3 Ab-driven T cell responses [97]. This issue is particularly problematic when the expected effects of CTLA-4 ligation are precisely mimicked by decreased CD3 or CD28 signaling. Thus, the same outcome could be achieved by CTLA-4 inhibitory signaling or by a decreased density of anti-CD3 or anti-CD28 Ab on the beads.

Notably, in many studies, the negative effects of 'agonistic' Ab are seen early, frequently before CTLA-4 is detectable by flow cytometry. This is typically explained by the possibility that CTLA-4 is functionally active while still being below our detection levels. While this is a theoretical possibility, alternatives worthy of consideration are that the antibody may nonspecifically disrupt early T cell activation, for example by diminishing CD3/CD28 clustering or by disrupting normal behavior of CTLA-4 following its induction.

In our personal experience, data generated using 'agonistic' Abs are simply not helpful in understanding CTLA-4 biology: predictions based on such data do not hold when tested in other systems (e.g., experiments involving ligand-driven responses or CTLA-4 gene-deficient mice). By contrast, disrupting CTLA-4 contact with its natural ligands, using antagonistic antibodies, appears to be generally reliable. Therefore, we would urge considerable caution in interpretation of experiments based on the concept of agonistic Abs.

Effects of CTLA-4 on motility

CTLA-4 has been proposed to increase T cell motility, thereby limiting contact time between T cells and APC. In this model, CTLA-4 ligation serves to reverse the 'stop signals' induced by productive TCR engagement and, therefore, to limit the formation of stable conjugates between T cells and APC [34]. In other words, it transmits a signal that prevents TCR induced adhesion. Similarly, a more recent study [35] concluded that CTLA-4 induced 'go' signals that make T cells more motile. Miska *et al.* reported that a blocking anti-CTLA-4 antibody (Ab) increased the motility of CD4 T effector (Teff) and Treg cells, but decreased the motility of CD8 Teff cells [36]. Taken at face value, this would suggest that CTLA-4 transmits a 'go' signal to increase motility in CD8 T cells but an 'arrest' signal in CD4 T cells. The latter is in contrast to the findings of Schneider *et al.*, who reported that CTLA-4 reversed the arrest signal in CD4 cells [34]. Furthermore, Miska *et al.* reported that CTLA-4 exerted the same effects on motility in CD4 Teff and CD4 Treg cells [36], whilst Lu *et al.* have argued that the CTLA-4 mediated reverse stop signal was largely limited to conventional T cells (Tconv) and not Treg cells [37]. Other studies have failed to find a role for CTLA-4 blockade in affecting T cell motility [38].

In a recent paper from the Allison laboratory, the authors suggested that the effects of CTLA-4 Ab on T cell motility is not due to signaling but rather to the physical disruption of stable interactions between T cells and their targets [39]. The effects of anti-CTLA-4 Ab on intratumoral T cell motility required chronic treatment, leading the authors to speculate that indirect mechanisms, such as Treg cell depletion [40,41], may be contributing. Such a notion is consistent with the increased Teff cell motility observed after acute Treg cell depletion in one study [36]. Given the integrated nature of the CD28/CTLA-4 pathway (Figure 1), perturbation of one player invariably has an impact on the other components. Therefore, CTLA-4 blockade could alter T cell motility indirectly by augmenting CD28 stimulation. CD28 functions are good candidates for affecting motility. For example, CD28-driven PI3K activation has been shown to control T cell migration [42], and recent data demonstrate a critical role for CD28-driven activation of inducible T cell kinase (ITK) in T cell motility and tissue infiltration [43]. ITK is known to affect actin accumulation at the T cell–APC interface [44], and a role for the CD28-driven actin reorganization and T cell triggering is also emerging [45,46].

Thus, while CTLA-4 manipulation can alter T cell motility in certain settings, whether this involves intrinsic 'stop' or 'go' signals or whether the antibody works indirectly (e.g., by augmenting CD28 signaling or depleting and/or impairing Treg cells) is less certain. Critically, cell intrinsic effects of CTLA-4 should be discernable in bone marrow chimeric mice in which wild type and CTLA-4$^{-/-}$ cells are mixed. Thus, changes in motility and function involving an intrinsic signal through CTLA-4 would be predicted to affect CTLA-4-sufficient cells in a manner not observed for CTLA-4-deficient cells. The behavior of CTLA-4^{+} and CTLA-4^{-} cells in chimeric mice has been extensively examined and such differences have not been observed [47–50].

CTLA-4-mediated ligand downregulation

One area that has gathered considerable momentum in recent years is the idea that Treg cells expressing CTLA-4 can downregulate CD80 and CD86 on APC [51–53]. Work from the Sakaguchi group showed that Treg cells were able to downregulate both CD80 and CD86 [but not CD40 or MHC class II] on dendritic cells (DC) in a manner that was adhesion dependent [33]: downregulation was blocked by anti-CTLA-4 Ab and was abrogated if Treg cells were deficient in CTLA-4 [54]. The downregulation of costimulatory ligands by Treg cells or, conversely, their increased expression following Treg cell depletion, has been consistently observed by multiple groups both *in vitro* [55,56] and *in vivo* [57–60], suggesting that it is a core feature of Treg cell behavior. The use of CTLA-4 to remove ligands from APC in a cell-extrinsic manner is essentially an extension of the ability of CTLA-4 to compete with CD28 at the immune synapse in a cell intrinsic manner (Figure 2) [20,61].

We recently reported a mechanism that explains CTLA-4-dependent ligand downregulation [62]. In this model, CTLA-4 binds to, and physically removes, ligands from APC by transendocytosis, targeting them for lysosomal degradation. Intercellular transfer of proteins between cells in the immune system is not uncommon [63] and transendocytosis has previously been demonstrated for notch ligands [64] and ephrin-Bs [65]. Importantly, CTLA-4-mediated transendocytosis is driven by engagement of the TCR. This simple mechanism fits well with the known features of CTLA-4 cell biology as well as the TCR

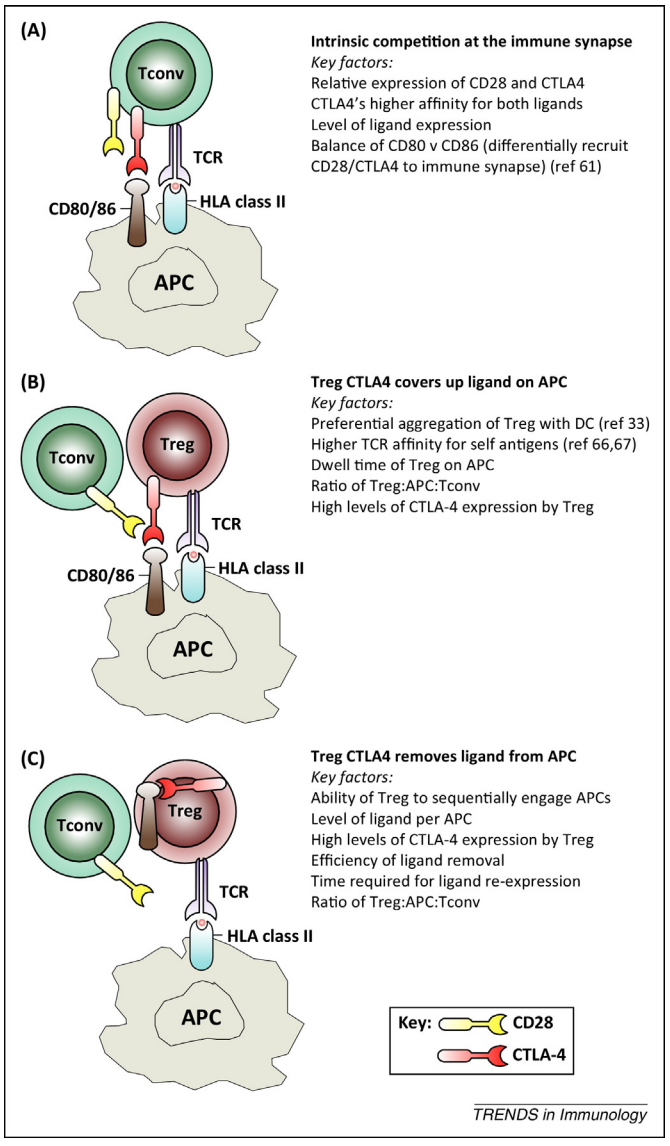

Figure 2. The various forms of ligand competition. The figure depicts three variations on the theme of competition between CD28 and CTLA-4 for access to ligand (drawn generically as CD80/86). **(A)** Activated T cells express both CD28 and CTLA-4, establishing a cell intrinsic competition for ligand access. Note that experiments from bone marrow chimeric mice suggest that this intrinsic role has a relatively minor role on T cell function *in vivo*. Nonetheless, this form of competition may have significant effects in some experimental settings. **(B)** Regulatory T cells (Treg) expressing CTLA-4 contact antigen-presenting cells (APC) and physically sequester ligands. This is a form of extrinsic ligand competition as well as cellular competition, because both Treg and conventional T cells (Tconv) are competing for the same APC simultaneously. **(C)** CTLA-4 removes ligands from the APC via transendocytosis. This is a form of cell extrinsic competition that is spatially and temporally separated from the activation of Tconv. In this model, CTLA-4-expressing Treg continually interact with APCs, remove ligands, and then detach. The APC is unable to provide CD28 costimulation until ligand re-expression occurs. Experiments from bone marrow chimeric mice suggest that cell extrinsic functions provide the major part of CTLA-4 function *in vivo*. Note that cell extrinsic regulation can be carried out by Tconv as well as by Treg, albeit with Treg having higher levels of CTLA-4 and providing more robust regulation [27,84]. Abbreviations: DC, dendritic cell; HLA, human leukocyte antigen; TCR, T cell receptor.

dependence of Treg cell function. Based on this model, it is possible to make several predictions concerning CTLA-4-dependent regulation. Most obviously, ligand removal is cell contact and time dependent (Hou and Sansom, unpublished 2014). Moreover, the expression level of CTLA-4 relative to the expression of its ligands will control the extent of downregulation. These quantitative considerations provide a simple conceptual framework and raise the notion of 'efficiency' in respect of CTLA-4-based ligand downregulation. In this regard, it is interesting that Treg cells lacking PKC-η were shown to have altered contact time with APC in a manner that limited their capacity to serially engage with cellular targets and achieve ligand depletion [29]. The concept that Treg cells have higher-affinity TCR recognition of self antigens [66,67] and show more stable interactions with APC compared with Tconv cells [33] is also consistent with them being well adapted to carry out transendocytosis. Notably, regulating the level of costimulatory molecules present on APCs would be a clear explanation for the role of CTLA-4 in peripheral tolerance, including in the linked suppression models studied by Waldmann and colleagues [68].

Is the major role of the CTLA-4 cytoplasmic domain to control cellular localization?

A surprising finding from several studies is that the extracellular domain of CTLA-4 is sufficient for substantial inhibitory function. Treg cells from CTLA-4$^{-/-}$ mice transgenically expressing only a membrane-anchored extracellular domain of CTLA-4 suppressed T cell responses as efficiently as those expressing wild type CTLA-4 [69]. Likewise, in other studies, expression of a tailless version of CTLA-4 was sufficient to confer suppressive capacity [70,71]. These data are in agreement with the original demonstration that expression of a tailless CTLA-4 molecule could prevent lethal pathology in CTLA-4$^{-/-}$ mice [72]. Collectively these studies are consistent with a model in which CTLA-4 can function without eliciting signal transduction through its cytoplasmic tail. Thus, the extracellular domain of CTLA-4 is capable of substantial immune regulation, albeit when overexpressed at the cell surface, as is the case in these mutants.

How then can tailless CTLA-4 elicit suppressive function, yet CTLA-4 with a mutated cytoplasmic tail [28] or an inability to bind PKC-η [29] be impaired? We propose a model in which suppressive function is mediated by the extracellular domain of CTLA-4; however, the cytoplasmic domain contributes to suppressive function by controlling the quantity, cellular localization, and timing of CTLA-4 expression at the membrane. According to this model, the role of the cytoplasmic domain is not to transmit inhibitory signals, but rather to direct the appropriate trafficking of the CTLA-4 molecule. In doing so, the cytoplasmic domain becomes a key modifier of the efficiency of CTLA-4 function.

The role of the cytoplasmic domain in regulating CTLA-4 expression patterns is well known. Specifically, the tyrosine-based YVKM motif mediates rapid endocytosis from the plasma membrane via interaction with the clathrin adaptor activating protein 2 (AP-2; Box 1). Endocytosis likely involves other motifs (Figure 3) because YVKM mutants are not completely defective in endocytosis [31,73,74]. Accordingly, the proline motif has been suggested to also contribute to AP-2 binding [75] and the C-terminal tyrosine sequence YFIPIN functions as an alternative (albeit weaker) endocytic adaptor [76]. AP-1 interactions have been linked with CTLA-4 degradation [77], while mutating the YVKM motif to YEKM has been suggested to influence CTLA-4 recycling [74]. The cytoplasmic domain of CTLA-4 reportedly controls its recruitment to lipid rafts [78] as well as mediating interactions with the scaffold proteins T cell receptor-interacting molecule (TRIM) and linker for activation of X cells (LAX), which influences CTLA-4 surface expression [79,80]. The recent study by Kong *et al.* also suggests an important role for the membrane proximal lysine motif in bringing CTLA-4 into complex with PKC-η. Together, these data argue for a sophisticated control of CTLA-4 expression that utilizes a variety of motifs in the cytoplasmic domain.

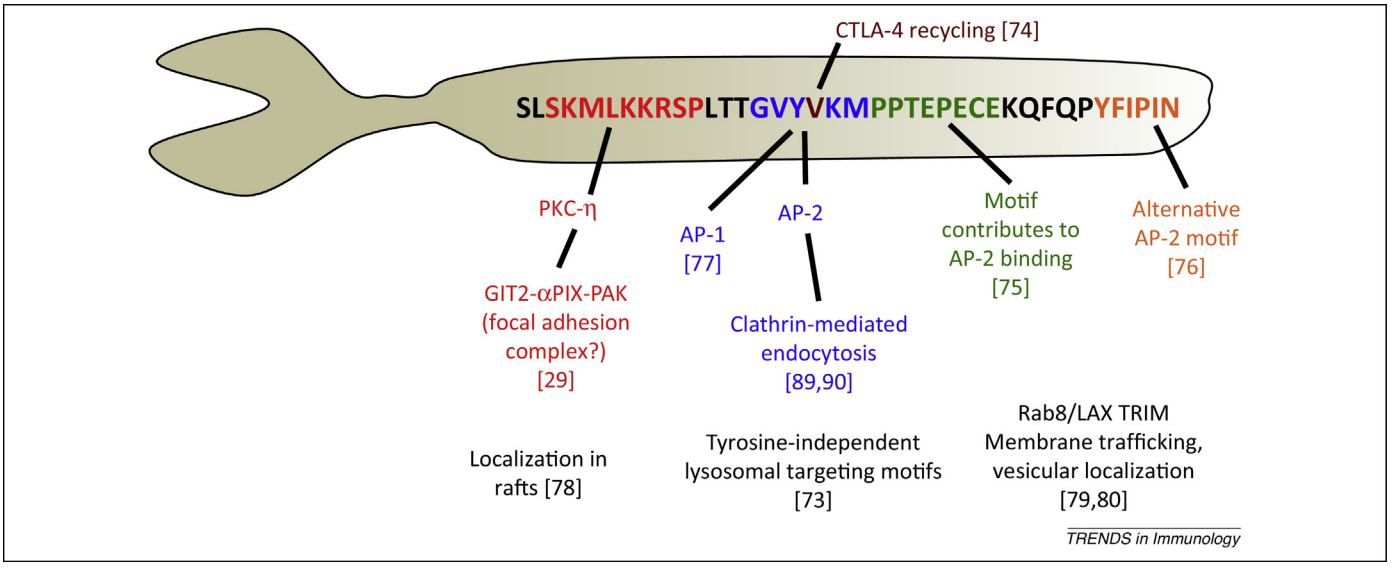

Figure 3. Identified cytoplasmic domain motifs involved in CTLA-4 trafficking and cellular localization. The amino acid sequence of the CTLA-4 cytoplasmic domain is shown in single-letter code. Reported motifs involved in cellular localization are shown in color, whereas associations and/or functions that require the CTLA-4 cytoplasmic domain but for which the specific motifs are not defined are shown in black. For definition of abbreviations, please see main text.

The functional significance of such a specific expression pattern is gradually emerging. Intracellular trafficking of CTLA-4 to lysosomes is likely important in the degradation of ligands that are captured by transendocytosis [81]. Endocytosis may also be required to limit the amount of CTLA-4 at the plasma membrane, thereby allowing sufficient CD28 engagement, which is important for Treg cell homeostasis. The PKCeta interactions are also consistent with the positional control of CTLA-4 within focal contacts and with effects on Treg cell adhesion. The rapid delivery of CTLA-4 to the synapse following TCR signaling is facilitated by the existence of an intracellular pool that can be quickly mobilized, similarly to some cytokines and CTL granules [82,83]. Thus, it is clear that the cytoplasmic domain controls the amount, timing, and fate of CTLA-4 that comes to the cell surface and that quantitative alterations in these parameters can affect function. Accordingly, while the ectodomain is ultimately responsible for CTLA-4 function, the cytoplasmic domain contains the controls.

Concluding remarks

The debate over the molecular mechanism of CTLA-4 function is now well into its second decade. While inhibitory signaling has been a favored theme throughout this time, in our opinion there is still little cohesive evidence for such a pathway. Meanwhile, the growing appreciation that a major role of CTLA-4 is in Treg cells has emphasized the importance of alternative mechanisms of CTLA-4 function. This has resulted in something of a hybrid model, where it is argued that CTLA-4 mediates inhibitory signaling in Tconv cells but then behaves differently in Treg cells.

While it is possible that CTLA-4 performs different functions in different lymphocyte subsets, our view is that there is no compelling evidence for this, neither is there a need to invoke such a model. Imaging experiments have shown that cell intrinsic competition between CD28 and CTLA-4, based on affinity for ligands at the immune synapse, can operate in both Treg and Tconv cells [20]. Similarly, transendocytosis of ligands can be mediated by both Tconv and Treg cells [62], and it has been shown that *in vivo* CTLA-4 can function in a cell extrinsic manner in Tconv as well as Treg [27,84]. Moreover, CTLA-4 is capable of conferring suppressive function in the absence of forkhead box P3 (Foxp3) [85], and unconventional regulatory T cells that lack Foxp3 have been shown to downregulate ligands on DC in a CTLA-4-dependent manner [86]. Thus, ligand downregulation appears to be a hard-wired function of CTLA-4 regardless of cell type. Therefore, postulating fundamentally different functions for CTLA-4 in Treg and Tconv cells is unnecessary in our view. We propose that control of CD28 access to its ligands is likely to be the major, if not sole, function of CTLA-4. Given the increasing manipulation of the CD28/CTLA-4 system therapeutically, the need for clear and predictive models of CTLA-4 function is pressing, and important beyond simple academic interest.

One consequence of a ligand competition model is that quantitative efficiency becomes a key concept. A clear prediction of this model is that there will be settings where CTLA-4 is present and competes for ligand, yet has little or no influence on the ensuing T cell response. For example, in situations where there are large numbers of APCs expressing high amounts of ligand, CTLA-4 competition is numerically overwhelmed. A corollary of this concept is that changes in parameters that affect efficiency of CTLA-4 competition, such as cellular adhesion, dwell time, CTLA-4 recycling efficiency, and affinity for ligand, will affect the degree of CTLA-4-based regulation. Therefore, the 'window' of effective CTLA-4 control may shift following changes to the cytoplasmic tail, and yet the ectodomain alone may also appear completely effective in certain settings. In short, there is no absolute measure of CTLA-4 function. If 10% CTLA-4 efficiency is sufficient to control a given response, the function of a CTLA-4 mutant may appear intact. Conversely, if 70% efficiency is required to control a given response, a mutant with 60% efficiency will appear defective. This may explain why CTLA-4 molecules with mutated cytoplasmic domains can appear either defective or intact, depending on the context. This quantitative issue is amply demonstrated by the observation that founder mice bearing the same CTLA-4 mutation exhibited differing levels of CTLA-4-dependent control that correlated with expression level of the transgene [72].

Consistent with this concept, we and others have recently found that heterozygous mutations in human CTLA-4 can also lead to quantitative defects in regulation and the emergence of an immune dysregulation syndrome [87,88]. In our opinion, there is sufficient evidence to consider moving away from signaling concepts towards a quantitative model of ligand competition. We believe that this will act as a more robust framework for interpreting the impact of naturally arising mutations, which are increasingly likely to be identified as a result of next-generation sequencing programmes.

Acknowledgments

L.S.K.W. is funded by a Medical Research Council Senior Fellowship.

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