

CTLA-4, an Essential Immune-Checkpoint for T-Cell Activation

Shunsuke Chikuma

Abstract The response of peripheral T lymphocytes (T cell) is controlled by multiple checkpoints to avoid unwanted activation against self-tissues. Two opposing costimulatory receptors, CD28 and CTLA-4, on T cells bind to the same ligands (CD80 and CD86) on antigen-presenting cells (APCs), and provide positive and negative feedback for T-cell activation, respectively. Early studies suggested that CTLA-4 is induced on activated T cells and binds to CD80/CD86 with much stronger affinity than CD28, providing a competitive inhibition. Subsequent studies by many researchers revealed the more complex mode of T-cell inhibition by CTLA-4. After T-cell activation, CTLA-4 is stored in the intracellular vesicles, and recruited to the immunological synapse formed between T cells and APCs, and inhibits further activation of T cells by blocking signals initiated by T-cell receptors and CD28. CTLA-4-positive cells can also provide cell-extrinsic regulation on other autoreactive T cells, and are considered to provide an essential regulatory mechanism for FoxP3+ regulatory T cells. Genetic deficiency of CTLA-4 leads to CD28-mediated severe autoimmunity in mice and humans, suggesting its function as a fundamental brake that restrains the expansion and activation of self-reactive T cells. In cancer, therapeutic approaches targeting CTLA-4 by humanized blocking antibodies has been demonstrated to be an effective immunotherapy by reversing T-cell tolerance against tumors. This chapter introduces CTLA-4 biology, including its discovery and mechanism of action, and discusses questions related to CTLA-4.

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1 Introduction: CD28 and CTLA-4 as Checkpoints

The activation and function of the peripheral T lymphocyte (T cell) reaction depends primarily on recognition of the antigen presented on the major histocompatibility complex (MHC) by the T-cell antigen receptor (TCR). This recognition, at the time of priming (the priming phase), causes the clonal expansion of T cells and the differentiation into effector T cells in secondary lymphoid organs. Recognition at the site of inflammation (the effector phase) results in an attack against infected tissue. The activation of self-reactive T cells can lead to an attack on self-tissue, leading to autoimmunity. To prevent this, there are many systems that have developed to keep self-reactive T cells in check.

The first signal, provided by TCR during recognition, does not cause activation of the T cells on its own. Full T-cell activation requires a second set of signals, called “costimulation,” which is mainly provided by activated antigen-presenting cells (APCs). The best-characterized costimulatory system is the CD28 receptor on T cells, triggered by its ligands, CD80 or CD86 (previously called B7-1 and B7-2, respectively) on activated professional APCs (dendritic cells, macrophages, and B cells). CD80 and CD86 are upregulated on activated APCs by microbial “danger signals” so APCs presenting microbial antigens can efficiently stimulate T-cell activation (Banchereau and Steinman 1998; Akira et al. 2001). Successful engagement of CD28 leads to IL-2 production (June et al. 1987), induction of anti-apoptotic protein Bcl-xL (Boise et al. 1995), and stimulates glucose uptake by inducing glucose transporter and glycolysis (Frauwirth et al. 2002), as well as the cell-cycle progression necessary for the massive clonal expansion of antigen-specific T cells. In contrast, resting APCs do not express a high level of CD80/CD86. T cells that are stimulated in the absence of the CD28 signal fall into an unresponsive state called “clonal anergy” and become refractory to further stimulation by the same antigen (Schwartz 2003). CTLA-4, another receptor that is structurally similar to CD28, is induced on activated T cells and binds to CD80 and CD86 with greater avidity than CD28. All CTLA-4 KO mice showed massive

lymphocyte proliferation in the lymph nodes and spleen, followed by an autoimmune attack against virtually all tissues by leucocytes, and premature death (Tivol et al. 1995; Waterhouse et al. 1995; Chambers et al. 1997b). The lethal phenotype is prevented by treating young CTLA-4 KO mice with antibody-depleting CD4+, but not CD8+ T cells (Chambers et al. 1997b). The conditional deletion of CTLA-4 only on CD4+ T-cells phenocopied germline CTLA-4 KO (Klocke et al. 2016), suggesting that the disease is CD4+ “helper T cell”-dependent. A recombinant soluble CTLA-4 (CTLA-4 Ig) blocking CD28 engagement by CD80/86 (Chambers et al. 1997b), CD28/CTLA-4 double knockout mice (Mandelbrot et al. 2001), or breeding CTLA-4 KO into CD80/CD86 double knockout mice (Triple KO; Mandelbrot et al. 1999) completely prevented lymphoproliferation and autoimmunity. Also, CTLA-4 KO remains healthy in mice genetically lacking autoreactive T cells (Waterhouse et al. 1997; Chambers et al. 1999; Bachmann et al. 2001; Greenwald et al. 2001; Gajewski et al. 2001). Conversely, by limiting the complexity of the T-cell repertoire by overexpressing TCR β chain as a transgene, it was shown that CTLA-4 knockout disease is totally self-antigen-dependent (Ise et al. 2009). These data clearly suggest that CTLA-4 provides negative feedback on the CD28-mediated activation of polyclonal, self-reactive CD4 helper T cells.

Thus, T-cell activation against self is fundamentally prevented by two “check-points”: 1. T cells in the absence of the CD28 signal (in the absence of CD80 and CD86 on APCs) become unresponsive. 2. Upon activation, CTLA-4 is induced, interacts with CD80/CD86, and prevents further activation of self-reactive T cells. After this discovery, several pairs of ligands and receptors were shown to have unique and functions in the immune system. It is now accepted that PD-1 and CTLA-4 blocks the T cell-mediated immune system at different levels, at the cell type and location (Chikuma 2016). CTLA-4 was also shown to be important for regulatory function. The dramatic phenotypes of CTLA-4 null mice attracted many researchers in immunology. It is now of particular importance that the artificial blockade of CTLA-4 was shown to break tolerance in the tumor setting, providing efficient immunotherapy against tumors (Leach et al. 1996). It turned out that the basis of T-cell inhibition by CTLA-4 was far more complex than previously expected. In this chapter, the history of discovery and basic research on CTLA-4 in terms of its biological roles and the mechanism of T-cell inactivation will be introduced, and the currently unsolved questions will be discussed.

2 Structure of CTLA-4

2.1 *Discovery and Primary Structure of CTLA-4*

CTLA-4 was originally isolated from a cDNA library derived from mouse cytotoxic T cells, hence the name cytotoxic T-cell-associated antigen-4 (Brunet et al. 1987). The CTLA-4 gene is encoded on human Ch2q33 and mouse Ch1C1. CTLA-4

consists of 4 exons and 3 introns, and encodes a protein of 223 amino acids (Ling et al. 1999). The protein is a type I transmembrane protein belonging to the immunoglobulin (Ig) superfamily, and it bears a single Ig-V (variable)-like domain on the extracellular portion. Like other receptors in the CD28 family, CTLA-4 is highly glycosylated at its extracellular domain. CTLA-4 forms a homodimer on activated T cells, which is mediated by the disulfide bond at the cysteine residue at amino acid position 120 (Linsley et al. 1995). There is a well-conserved motif (amino acid sequence: MYPPPY) on the extracellular domain that is shared by CD28, which is critical for binding its ligands CD80 and CD86 (Peach et al. 1994). CD80 and CD86 bind to CTLA-4 with stronger affinity than to CD28, which may account for CTLA-4 antagonizing to the activation signal. Dimerized CTLA-4 binds to two ligands (Linsley et al. 1995). Ostrov et al. (2000) demonstrated by solving the crystal structure of the extracellular portion that CTLA-4 shows a unique mode of dimerization with its ligand-binding domain distal to the dimerization interface. Stamper et al. (2001) and Schwartz et al. (2001) solved the crystal structures of CTLA-4 in complex with CD80 and CD86, respectively. Both groups concluded that each CTLA-4 dimer binds to two CD80 molecules, forming a lattice-like structure and providing very high avidity binding. The results suggest that CTLA-4 induction on the surface of activated T cells results in ligand sequestration of CD28. CTLA-4 also bears a short cytoplasmic tail that is almost 100% conserved among many species. The tail is reported to bind to various signaling molecules that mediate subcellular trafficking and the function of CTLA-4 (Fig. 1a, discussed later).

2.2 *Alternatively Spliced Isoforms of CTLA-4*

The splicing of messenger RNA of CTLA-4 might result in the expression of functionally important isoforms in the genetics of autoimmunity. For example, human resting blood lymphocytes express an alternatively spliced isoform of CTLA-4 lacking Exon3, encoding the entire transmembrane domain, resulting in a soluble isoform consisting solely of the extracellular domain (soluble CTLA-4: sCTLA-4; (Magistrelli et al. 1999; Oaks et al. 2000). Since the ectodomain of CTLA-4 can bind to its ligands (CD80 and CD86) with blocking activity, sCTLA-4 might be acting as a naturally occurring antagonist for CD28-mediated T-cell activation by CD80 and CD86. Indeed, Ueda et al. (2003) reported a CTLA-4 allelic variant associated with a lower mRNA level of sCTLA-4 linked with susceptibility to human autoimmune diseases. It was also reported that a naturally occurring CTLA-4 variant that is completely lacking its extracellular domain may determine autoimmune susceptibility in a particular mice strain. The “ligand-independent form” (referred to as liCTLA-4) can preferentially accumulate to the immunological synapse, and thereby provide a tonic inhibitory signal (Bour-Jordan et al. 2011 and Chikuma unpublished). Non-obese diabetes (NOD) mice that exhibit autoimmune juvenile diabetes express a reduced amount

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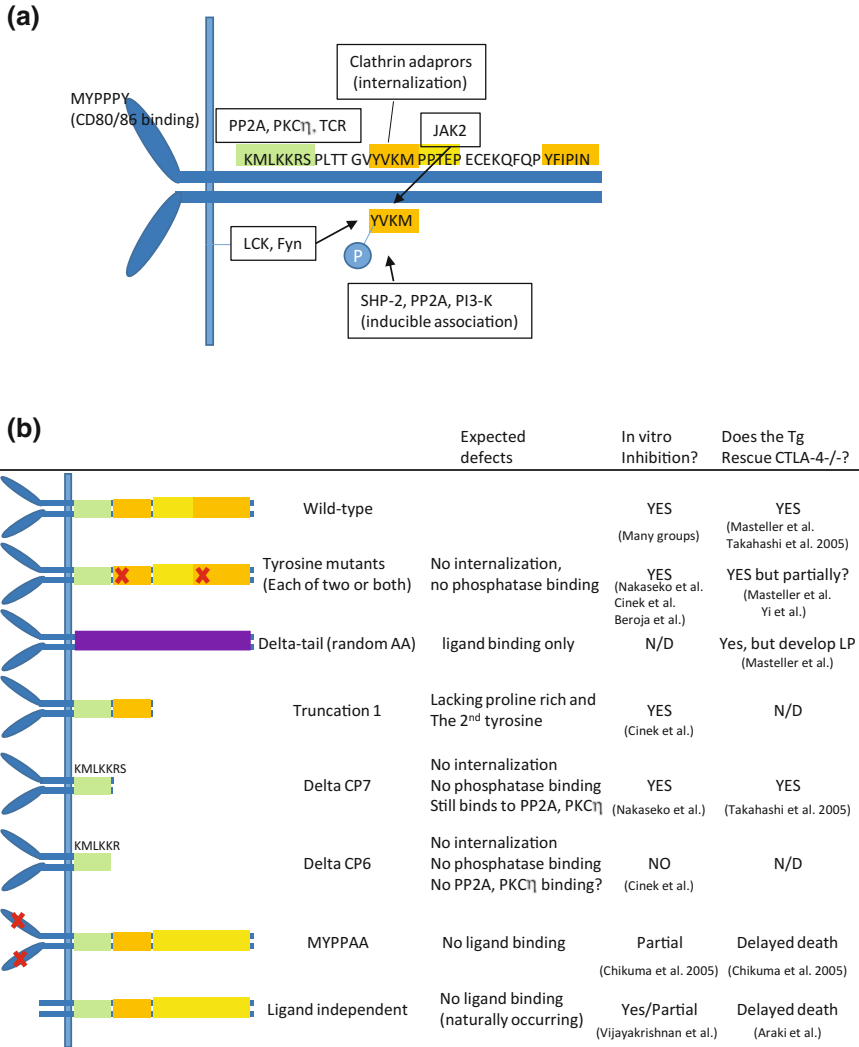


Fig. 1 A. schematic structure of CTLA-4. Note that the YVYVKM motif binds to the clathrin adaptor complex in its unphosphorylated form whereas the same motif binds to enzymes for signal transduction when phosphorylated, B. Summary of structure-functional studies. N/D: not done

of this isoform (Ueda et al. 2003), which accounts for their genetic susceptibility (Araki et al. 2009). A transgenic overexpression of liCTLA-4 or a mutant CTLA-4 that lacks the ligand-binding domain on T cells significantly retards the death of CTLA-4 KO mice, suggesting the function of a ligand-independent CTLA-4 signal (Vijayakrishnan et al. 2004; Chikuma et al. 2005; Araki et al. 2009). Interestingly, the activation status of T cells results in dynamic change in the ratio of transcripts encoding full-length, soluble, and ligand-independent forms of CTLA-4 transcripts,

suggesting the unique role of each spliced isoform in different T-cell subpopulations (Oaks et al. 2000; Vijayakrishnan et al. 2004). A super-short form of CTLA-4 that encodes only the signal peptide (Exon1) and part of the cytoplasmic tail (Exon4; out of its original translational frame) was reported to affect autoimmune mice when overexpressed (Liu et al. 2012; Ichinose et al. 2013). The expression of this super-short form may alter the mRNA of other isoforms, although the exact function of this isoform is not yet fully elucidated.

3 Function of CTLA-4

3.1 Cell-Intrinsic T-cell Inhibition by CTLA-4

It was first reported that the monoclonal antibodies (mAb) that block the binding of CTLA-4 to CD80/CD86 augment T-cell responses when added to the co-culture of T cells, APCs, and anti-CD3 antibody (which work as a TCR ligand) in a soluble form (Walunas et al. 1994). The F(ab)₂ form of the same mAb, which lacks the ability to bind Fc receptor had similar activity, suggesting that genuine blocking activity of CTLA-4-CD80/CD86 binding in this culture augmented the T-cell response. In contrast to the blocking experiment, anti-CTLA-4 immobilized on the surface of plastics works to crosslink/stimulate CTLA-4 and inhibits T-cell activation, as well as IL-2 production and proliferation (Krummel and Allison 1995; Walunas et al. 1994). This inhibition was associated with inhibition of the cell-cycle progression and expression of activation markers CD69 and CD25 (Krummel and Allison 1996). A membrane-bound single-chain variable fragment (scFv) of anti-CTLA-4 antibody engineered to be expressed on a fibroblast cell line can ligate CTLA-4 in vitro and inhibits activation of co-cultured T cells, triggered by anti-CD3 and anti-CD28 on the same cell (Griffin et al. 2000). A transgenic expression of CTLA-4 scFv on B cells ameliorated autoimmune diabetes in NOD mice (Fife et al. 2006) in vivo. Importantly, this amelioration was observed in NOD mice lacking CD80/CD86, suggesting inhibition of the activation of islet-reactive diabetogenic T cells by B cells expressing the artificial CTLA-4 ligand directly, but not through inhibition of CD28 ligation. The strain also shows almost no FoxP3 + Tregs due to the absence of the CD28 signal necessary for Treg development/survival (Salomon et al. 2000; Tang et al. 2003; Tai et al. 2005). Thus, the CTLA-4 ligand-mediated inhibition of autoimmune diabetes in Tg mice is, at least in part independent of Tregs (Fife et al. 2006). An ectopic expression of CTLA-4-deficient primary mouse T cells or cell lines with CTLA-4 or its mutants reconstitutes ligand-mediated T-cell inactivation by CTLA-4 in many systems (Nakaseko et al. 1999; Chikuma et al. 2005; Cinek et al. 2000; Baroja et al. 2000; Vijayakrishnan et al. 2004). On the basis of these experiments, CTLA-4 was suggested to be a negative regulator for T-cell activation. Overexpression of CTLA-4 or its mutants to cell lines lacking endogenous CTLA-4 can be seen to

mimic CTLA-4-mediated T-cell inhibition *in vitro* in many reports (Fig. 1b). Two groups reported that Jurkat cells (human-derived) expressing stable mouse CTLA-4 can be inhibited (in terms of IL-2 secretion) by immobilized anti-mouse CTLA-4 antibody. Subsequent mutational analysis showed that mutation of one or both tyrosine motifs to phenylalanine did not affect CTLA-4's ability to inhibit IL-2 production in the same system. Similar results were observed in a study, where CTLA-4-negative T cell clone was reconstituted with CTLA-4 by retroviral vectors (Nakaseko et al. 1999). Restimulated cells with wild-type CTLA-4 inhibited the activation, and the tyrosine mutants did not show any defects in the inhibition. In Nakaseko's study, CTLA-4 lacking almost all cytoplasmic regions but the membrane proximal to seven amino acids (KMLKKRS) showed suppressive activity. Although ligation of CTLA-4 alone does not cause changes in gene expression, it inhibits abundant gene expression by TCR/CD28 stimulation (Riley et al. 2002). This suppression is associated with inhibition of TCR-CD28-mediated biochemical events. It was reported that CTLA-4 ligation inhibits the phosphorylation of AKT (Parry et al. 2005), TCR ζ (Lee et al. 1998), Erk (Calvo et al. 1997; Baroja et al. 2000; Chikuma et al. 2005), Jnk (Calvo et al. 1997), and I κ b (Pioli et al. 1999), all of which are triggered by TCR-CD28. Interestingly, some reports suggested that CTLA-4 can inhibit T-cell activation even without ligand engagement. In mice, the transgenic expression of CTLA-4 mutants that lack either the entire extracellular domain (Vijaykrishnan et al. 2004; Araki et al. 2009) or an essential ligand-binding motif (MYPPPY; Chikuma et al. 2005) inhibited T-cell proliferation and rescued the CTLA-4 null phenotype significantly, but not fully. The study clearly indicated the T-cell-intrinsic inhibition by CTLA-4. CTLA-4 also mediates the induction of T-cell anergy, a form of tolerance. Greenwald et al. (Greenwald et al. 2001) used a genuine genetic model in which CTLA-4 was lacking on T cells bearing single TCR with known specificity to show this. The CTLA-4 KO rag2 KO TCR Tg (classII MHC-restricted; CD4+) showed resistance to anergy induction in the transferred host when challenged by the cognate antigen, whereas CTLA-4 WT rag2 KO TCR Tg were tolerized. In contrast, CTLA-4 KO CD8+ T cells in the analogous system showed no defects in anergy induction. In a similar system, CTLA-4 on CD8+ T cells did not regulate their anergy induction (Frauwirth et al. 2000, 2001). In contrast, PD-1 deficiency in the same system had effects on CD8+ T cells which led to the augmentation of CD8+ T-cell activation and resistance to anergy (Chikuma et al. 2009). These results suggest that CTLA-4 and PD-1 sets the threshold for CD4+ and CD8+ T cells, respectively.

3.2 Cell-Extrinsic T-Cell Inhibition by CTLA-4

In contrast to the direct inhibition of T cells, CTLA-4 (or T-cell-expressing CTLA-4) can indirectly inhibit the activation of other T cells. This non-cell-autonomous tolerance mechanism was observed in lethally irradiated wild-type mice receiving mixed bone marrow from CTLA-4 wild-type and KO

bone marrow when they did not develop the lymphoproliferative disease observed in CTLA-4 KO mice or recipients of CTLA-4 KO cells only (Bachmann et al. 1999). Similarly, Tivol and Gorski (2002) showed that the transfer of a mixture of Thy1+ splenocytes WT and CTLA-4 KO into Rag KO mice prevented inflammatory disease caused by the transfer of CTLA-4 KO cells only. In this setting, KO cells were eliminated *in vivo*, in the presence of WT T cells, suggesting that CTLA-4 on WT T cells can induce the deletion of KO T cells when the population coexist *in vivo*.

This non-cell-autonomous manner of CTLA-4-mediated inhibition is thought to be a major mechanism of tolerance by FoxP3+ Tregs. FoxP3+ Tregs are found to express high levels of CTLA-4 on the cell surface and intracellularly (Read et al. 2000; Takahashi et al. 2000; Salomon et al. 2000). FoxP3+ Tregs show a unique demethylation pattern on the CTLA-4 locus (Ohkura et al.), and FoxP3 can bind to CTLA-4 promoter (Wu et al. 2006) and drives its expression (Hori et al. 2003). FoxP3-deficient (germline KO or natural mutation; the so-called “scurfy” mutation) and CTLA-4 KO mice show a very similar phenotype with premature death by autoimmunity (Brunkow et al. 2001; Khattri et al. 2003; Fontenot et al. 2003). It has been, however difficult to determine if CTLA-4 expression on FoxP3+ Treg is critical for the regulatory function. For instance, FoxP3+ Tregs from CTLA-4 KO mice show suppression as efficiently as FoxP3+ Tregs from WT mice *in vitro* (Tang et al. 2004; Kataoka et al. 2005). To explore this question *in vivo*, Chikuma and Bluestone (2007) reconstituted RAG-1 KO recipients with bone marrow (BM) from CTLA-4KO (bred to a CD80/80 null background to avoid autoimmunity) mice and/or Foxp3-deficient mice. Control recipients, receiving either CTLA-4KO or Scurfy BM alone, rapidly developed interstitial pneumonitis, colitis, wasting, and dermatitis and died, which suggests that fatal autoimmunity occurred in the donor strain (Chikuma and Bluestone 2007). Next, the experiment was repeated using a 50:50 mixture of CTLA-4KO and Scurfy BM cells were tested to determine whether the addition of the CTLA-4+ FoxP3neg could overcome the pathology of and CTLA-4 or FoxP3-deficient cells in lymphocyte-null Rag1-KO recipients. Intriguingly, mixed BM chimeric recipients developed less severe autoimmunity and lived longer compared to CTLA-4KO or Scurfy BM alone, suggesting that the mixture of CTLA-4KO and Scurfy BM afforded some level of protection. The data suggest that CTLA-4 has a Treg-independent extrinsic function in the control of T-cell tolerance and homeostasis. Strikingly, the chimeric mice died with later kinetics, suggesting that CTLA-4 and Foxp3 must be expressed *in cis* on the same cell to fully prevent lethal autoimmunity and lymphoproliferation (Chikuma and Bluestone 2007). Wing et al. (2008) generated a mouse carrying a floxed CTLA-4 allele that allowed conditional knockout mice lacking CTLA-4 specifically on FoxP3+ Tregs when bred to mice expressing CRE under FoxP3 promoter. The resulting mice developed lethal autoimmunity, suggesting that the Treg-specific CTLA-4 expression is important for absolute maintenance of T-cell homeostasis. Again, mice showed much milder autoimmunity that affected limited organs and resulted in later death in comparison to the germline CTLA-4 KO (Wing et al. 2008). Conversely, Jain et al. (2010) showed that mice with CTLA-4 deleted only on activated conventional T cells (CTLA-4 on

FoxP3⁺ Tregs is intact) show significant, but not complete, protection from the lethal disease phenotype. Collectively, these results (Chikuma and Bluestone 2007; Wing et al. 2008; Jain et al. 2010) suggest that CTLA-4 on both activated conventional T cells and FoxP3⁺ Tregs is important for complete protection from death. As for the mechanism of cell-extrinsic regulation by CTLA-4, the wild-type T cells protecting autoimmunity appeared not to require inhibitory cytokines, such as TGF β or IL-10 (Friedline et al. 2009). Instead, the CTLA-4 regulation of APC activation and function was proposed. Using two-photon microscopy, Tang et al. (2006) showed that FoxP3⁺ Tregs do not directly interact with conventional autoreactive cells in an inflammatory setting. Instead, Tregs interact with APC to down-modulate their ability to activate autoreactive conventional T cells. Using an in vitro co-culture system of conventional T cells, Tregs, and APCs, Wing et al. (2008) showed that the Tregs outcompeted with conventional T cells for binding to APCs. APCs cultured with FoxP3⁺ Tregs are attenuated in the expression of activation markers, CD80 and CD86, and lost the ability to stimulate conventional T cells (Wing et al. 2008). Qureshi et al. (2011) reported that the downregulation of CD80/86 by CTLA-4-expressing cells is through the CTLA-4-mediated capture of CD80/86 on APC and trans-endocytosis into CTLA-4⁺ T cells. The trans-endocytosed CD80/86 is degraded within CTLA-4⁺ cells, resulting in a reduction of molecules on the APC (Qureshi et al. 2011). This trans-endocytosis model is attractive to explain cell-extrinsic regulation by CTLA-4, especially because the model works in a FoxP3 null setting in an antigen-specific system (Wang et al. 2012). On the other hand, Tai et al. (2012) showed that the internalization-defective CTLA-4 transgene on CTLA-4 KO Tregs was functional in vivo, suggesting that trans-endocytosis may not be important. CTLA-4 binding to CD80/86 is also suggested in the transmission of “reverse signaling” that induces the production of an enzyme indoleamine 2,3-dioxygenase (IDO) on APCs. IDO catalyzes the degradation of the amino acid tryptophan into the immune-inhibitory metabolite kynurenine, which is important in immuno-suppression in various immunologic settings, such as pregnancy, cancer, chronic infection, autoimmunity, and allergy (reviewed in Fallarino et al. 2003; Puccetti and Grohmann 2007). These hypotheses largely resulted from in vitro experiments using cells overexpressing CTLA-4 and/or CD80/CD86 so proof in the physiological system is less clear. CTLA-4-mediated cell-extrinsic inhibition is crucial in the maintenance of self-tolerance.

4 Mechanisms of CTLA-4-Mediated Immune Regulation

4.1 *The Biochemical Partners of CTLA-4*

The 33 amino-acid intracellular domain of CTLA-4 is 100% conserved among species, suggesting its importance in protection against lethal autoimmunity (Fig. 1a). The domain is composed of a lysine-rich membrane-proximal motif

(KMLKKRS) followed by two motifs containing tyrosine (YVKM and YFIP), with a proline-rich motif (PPTEP). The tail lacks a signaling module with enzymatic activity. Instead, the CTLA-4 tail interacts with many intracellular molecules, which control trafficking and signaling by CTLA-4.

4.1.1 SHP-2

Src-homology-containing tyrosine phosphatase-2 (SHP-2, also called Sh-PTP2 or Syp) is a cytosolic tyrosine phosphatase with a Src-homology 2 (SH2) domain. SHP-2 is involved in activation of the RAS-MAPK pathway, and transmits signals from cytokine receptor and co-inhibitory receptors (Lorenz 2009). SHP-2 interacts with CTLA-4 based on mutagenesis and immune coprecipitation studies. This association was suggested to be mediated by the SH2 of SHP-2 and the phosphotyrosine sequence YVKM within the CTLA-4 cytoplasmic tail when the motif is phosphorylated. This motif was not, however, a typical ITIM motif. The association of SHP-2 to CTLA-4 might be indirect or it may require other molecule(s) as adapter molecules (Schneider and Rudd 2000). The CTLA-4-SHP-2 interaction was proposed to cause tyrosine dephosphorylation of key signaling component required for T-cell activation (Marengere et al. 1996). For instance, SHP-2 phosphatase activity can dephosphorylate the TCR ζ chain (Lee et al. 1998) and the RAS regulator p52SHC (Marengere et al. 1996), and it has been suggested that the delivery of this enzyme by CTLA-4 to the specific region of the immunological synapse inhibits the TCR-CD3 complex-mediated signal transduction, leading to direct inhibition of T-cell activation (Lee et al. 1998). Similarly, Gab2, which binds directly to TCR ζ signaling complex, recruits SHP-2 there and blocks proximal TCR signaling (Yamasaki et al. 2001).

4.1.2 PP2A

PP2A is a heterotrimeric serine-threonine phosphatase that dephosphorylates a wide variety of protein substrates involved in cellular activity. PP2A composes a very major fraction of cellular protein, and dephosphorylates many substrates (Lorenz 2009). In a yeast two-hybrid screen, Chuang et al. (2000) found that the catalytic subunit of PP2A associates with the cytoplasmic tail of CD28 and CTLA-4. Independently, Baroja et al. (2002) reported that the regulatory subunit (PP2AA) also interacted with CTLA-4. The catalytic and regulatory subunit of PP2A interacted with the lysine-rich KMLKKRS and YVKM motif within CTLA-4, respectively, suggesting tri-molecular interaction. Through mutagenesis of the KMLKKRS motif, Baroja et al. suggested that the PP2A interaction inhibits CTLA-4 suppressive activity. Parry et al. (Parry et al. 2005) showed that PP2A antagonizes phosphorylation-dependent AKT activation mediated by TCR and CD28, which was sensitive to ocadaic acid, an inhibitor of PP2A.

4.1.3 PKC- η (Eta)

Kong et al. (2014) reported that CTLA-4 associates with the η isoform of PKC. FoxP3⁺ Tregs contain a significant amount of phosphorylated PKC- η , which interacts with CTLA-4 at the immunological synapse. The mutation or truncation of the KMLKKRS motif greatly reduced PKC- η binding, suggesting the importance of this motif together with PP2A in CTLA-4 function. Germline PKC- η deficient mice demonstrated defects in conventional T-cell activations (Fu et al. 2011), but did not show any defects in the development of FoxP3⁺ Treg (Kong et al. 2014). They eventually exhibited lymphadenopathy, however, and their FoxP3⁺ Tregs were shown to have multiple defects in regulatory functions *in vitro* and *in vivo*. It appears that CTLA-4-PKC- η interaction is required for firm interaction of Tregs to APCs, by phosphorylation of PAK2, GIT2, two components of the focal adhesion complex, as well as a guanine nucleotide exchange factor, α PIX, at the immunological synapse. Mice with the Treg-specific deletion of PKC- η may result in Treg-specific defects in adhesion due to defective phosphorylation of the GIT2- α PIX-PAK complex.

4.1.4 Clathrin Adaptor Complex

Several groups reported that CTLA-4 interacts with AP1, and that AP2 subunits of the clathrin adaptor complex are primary components in the cell biology that induce the clathrin-mediated internalization of associated molecules (Chuang et al. 1997; Shiratori et al. 1997; Zhang and Allison 1997; Bradshaw et al. 1997; Schneider et al. 1999). AP1 and AP2 interact with the unphosphorylated form of Y201VKM, which may account for the preferential intracellular trafficking of CTLA-4.

4.1.5 Tyrosine Kinases

CTLA-4 phosphorylation and relocation to the immunological synapse are TCR-dependent. TCR-proximal kinase Lck and Fyn were shown to phosphorylate the Y201VKM motif (Chuang et al. 1999; Miyatake et al. 1998). Other tyrosine kinases, such as JAK2 (Chikuma et al. 2000) and Rlk (Schneider et al. 1998), were reported to directly bind to CTLA-4, and can phosphorylate Y201, suggesting that the broad extracellular signals that activate tyrosine kinases can stimulate CTLA-4 phosphorylation. Importantly, Lck and Fyn are membrane-associated src kinases, and their activity is TCR-dependent. The preferential recruitment of CTLA-4 to the IS may occur as a consequence of the trimolecular complex of LCK, CTLA-4, and TCR. The tyrosine motif is dispensable for CTLA-4-mediated inhibition *in vitro*. CTLA-4 KO mice overexpressing point-mutated CTLA-4 on tyrosine (YVKM \rightarrow VKMs) develop late lymphoproliferative disease, suggesting the importance of this residue in CTLA-4 activation and function (Yi et al. 2004).

4.2 Induction and Dynamic Localization of CTLA-4 to the Immunological Synapse

CTLA-4 expression is restricted to T cells, but it shows a unique expression pattern, which is important for the inhibitory function. (1) The expression of CTLA-4 is activation-dependent. (2) CTLA-4 preferentially localizes to the intracellular vesicle compartment, (3) recycles to the T cell surface upon engagement of TCR, where it participates at the contact site of T cells and APC, called the “immunological synapse,” (4) binds to CD80 and CD86, and is internalized from the immunological synapse to intracellular compartments. This unique pattern of CTLA-4 expression is critical for its specialized function as a negative regulatory molecule. Here, we focus on such dynamics of CTLA-4 mediated inhibition.

4.2.1 Induction of CTLA-4 on T Cells

In contrast to CD28's constitutive expression on the surface of all T cells, CTLA-4 expression is only found on activated T cells (Linsley et al. 1992). CTLA-4 on T cells is strongly induced by ConA and IL-2 (Brunet et al. 1987). TCR plus CD28 signals synergistically induce CTLA-4 expression by two mechanisms: enhanced transcription and an increase in the mRNA stability (Finn et al. 1997). A nucleotide sequence located within 335 bp upstream from the transcriptional start site of CTLA4 is sufficient for the induction (Perkins et al. 1996). FoxP3+ Tregs are poised to show demethylation on specific loci of CTLA-4, and an activated phenotype due to self-interactions during thymic development (Ohkura et al. 2012). CTLA-4 expression is sensitive to inhibition by cyclosporine, a calcineurin inhibitor. In addition, rapamycin, a small mTOR inhibitor that blocks the IL-2-mediated signaling cascade can control CTLA-4 expression. These results indicate that CTLA-4 upregulation is controlled by general T-cell activation signal. The effects of IL-2 and CD28 signaling were additive but independent, as the CD28 signal augmented CTLA4 expression in IL-2-deficient mice. In contrast, CTLA4 expression was not augmented by cytokines IL-4, IL-6, IL-7, or IL-12 (Alegre et al. 1996). Sodium butylate, an HDAC inhibitor, significantly augmented CTLA-4 expression, suggesting epigenetic silencing (Doyle et al. 2001). CD4+ CD25 + FoxP3+ Tregs showed the most abundant CTLA-4 expression in the steady-state condition, suggesting that these cells are constantly undergoing antigen recognition and activation (Takahashi et al. 2000; Read et al. 2000; Salomon et al. 2000). This concept is supported by the finding that Nur77, a proximal molecule expressed on recently activated cells, is expressed at its highest level (Zikherman et al. 2012).

4.2.2 CTLA-4 Storage

CTLA-4 is not primarily localized on the T-cell surface, but instead resides intracellularly in a region that overlaps the Golgi apparatus (Leung et al. 1995) and/or endocytic compartment(s) with perforin-containing secretory granules (Linsley et al. 1996; Iida et al. 2000). The transfer of 11 cytoplasmic residues, TTVGVYVKMPPT, from the CTLA-4 cytoplasmic tail to CD28 conferred intracellular localization (Leung et al. 1995). Importantly, CTLA-4 expressed on the surface was also internalized, which explains its low levels of expression on the cell surface (Alegre et al. 1996). Consequently, on naïve, uninfected mice, CTLA-4 is hardly detected on the T-cell surface by highly sensitive multi-step fluorescent labeling and flow-cytometry detection, even in CD4⁺ CD25⁺ FoxP3⁺ Tregs, which have the most abundant CTLA-4 expression (Chikuma unpublished). CTLA-4 is likely transcribed and stored in the intracellular vesicle in the case of high-affinity antigen recognition.

4.2.3 CTLA-4 Localization and Inhibition at the Immunological Synapse

The dynamic movement of CTLA-4 to the contact site of APC and T cells immediately after transient calcium influx by TCR stimulation was first reported by Linsley et al. (1996). The immunological synapse has a structure composed of a central super-molecular cluster (c-SMAC) that includes TCR and signaling molecules, surrounded by peripheral SMAC containing adhesion molecules. Egen and Allison (2002) followed CTLA-4 recruitment to the immunological synapse by time-lapse microscopy. CTLA-4 is located at the uropod (the opposite site of T-cell movement), but rapidly relocated to the T-cell-APC contact site a few minutes after stimulation. Iida et al. (Iida et al. 2000) suggested that CTLA-4-containing secretory granules, also comprising perforin, rapidly relocates to the synapse upon TCR ligation. Chikuma et al. (2003) showed that CTLA-4, phosphorylated Lck, and TCR- ζ -chain form a trimolecular complex within the glycosphingolipid-enriched microdomain (also called the lipid raft) that is known to be enriched at the immunological synapse. The export of CTLA-4 to IS was dependent on TCR affinity, suggesting that CTLA-4 can preferentially inhibit CD4⁺ T cells that have higher affinity to antigens (Egen and Allison 2002). The CTLA-4 enrichment to the IS was dependent on CD80/CD86-binding (Pentcheva-Hoang et al. 2004), suggesting that the ligation of CTLA-4 by CD80/86 occurs before the formation of a mature immunological synapse. By observing the T-cell-APC contact surface using an artificial lipid bilayer as APC, Yokosuka et al. (2010) showed that CTLA-4 participated in the immunological synapse at a relatively later time point in mature synapse formation and sequestered CD28 out of the immunological synapse by competing for CD80/CD86 signaling. By collecting the lipid raft fraction by biochemical methods, Chikuma et al. (2003) showed that the TCR molecules within the raft decreased after the co-ligation of TCR and CTLA-4, suggesting that

CTLA-4 signal sequesters TCR from the lipid raft, enriched with Lck or other signaling molecules. The recruitment of phosphatases SHP-2 (Lee et al. 1998) and PP2A (Parry et al. 2005) to CTLA-4 causes dephosphorylation or the inhibition of phosphorylation of the TCR- ζ chain (Lee et al. 1998) and/or AKT (Parry et al. 2005), respectively; however, these phosphatases are not found to form a cluster with CTLA-4 to the immunological synapse (Yokosuka et al.). The association of phosphatases is likely very transient or happens at an earlier time point, when the CTLA-4 cluster is very small.

4.2.4 Internalization and Trans-Endocytosis

CTLA-4 binds to CD80/CD86 and brings them into the intracellular compartments for degradation (Qureshi et al. 2011), specifically at late time points of the T-APC interactions (Yokosuka et al. 2010). One key question is whether the CTLA-4 molecule trans-endocytosis and recycling is designed to move CTLA-4 into and out of the immunological synapse to regulate T-cell activation.

4.3 Structure and Functional Relationship

Given the importance of motifs used for molecular interaction (i.e., MYPPPY, KMLKKRS, YVKM) within CTLA-4, to date, many efforts have been made to understand their functional importance. Figure 2 summarizes many of the results. In vitro, CTLA-4-negative cells, reconstituted with mutants that are stably expressed on the plasma membrane show inhibitory activity when ligated by anti-CTLA-4 mAb or CD80/CD86. The critical point that makes it difficult to interpret the data is that CTLA-4 has two modes of inhibiting T-cell activation, namely, ligand competition and signaling. For example, deletion of the YVKM motif may abrogate phosphatase binding, but it augments cell surface expression and the inhibitory function of CTLA-4. Since the mutant CTLA-4 molecule lacking almost the entire cytoplasmic tail retains membrane-proximal KMLKKRS that is functionally able to protect the lethal phenotype of CTLA-4 KO mice. This motif binds to PP2A and PKC- η , which may be a minimal requirement of CTLA-4 function. Transgenic overexpression of this form of CTLA-4 completely (Takahashi et al. 2005) rescued the CTLA-4 KO phenotype whereas random amino acid substitution of the entire CTLA-4 tail only partially rescued it (Masteller et al. 2000) (see Fig. 2). Trans-endocytosis and degradation of CD80/86 by CTLA-4 is suggested to account for CTLA-4-mediated regulation (Qureshi et al.). Tailless CTLA-4 used in rescue experiments, which lacks the binding site for clathrin adaptors (YVKM), is functional when overexpressed on the T-cell compartment (Takahashi et al. 2005; Masteller et al. 2000) or the Treg compartment (Tai et al.), suggesting that endocytosis is not an absolute requirement for CTLA-4-mediated inhibition.

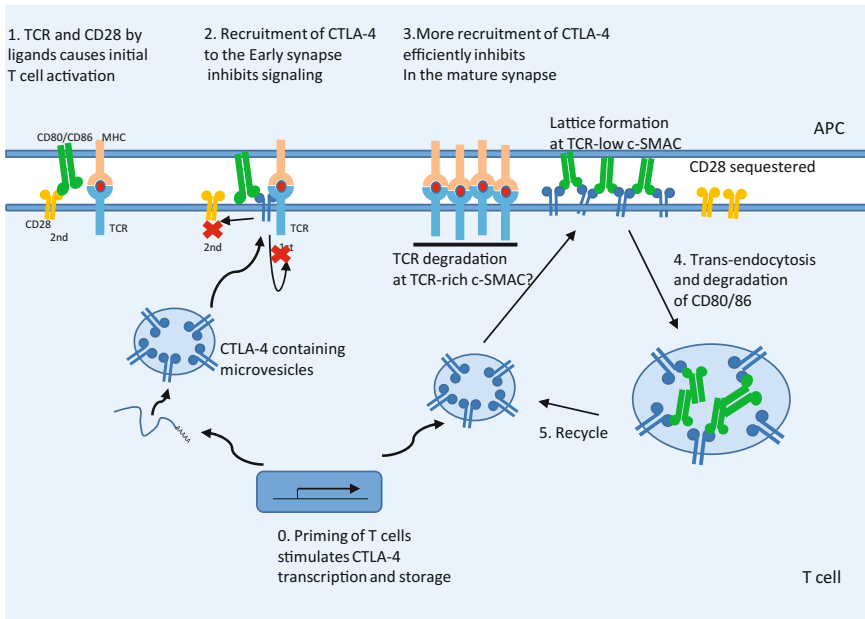


Fig. 2 A proposed model of spatiotemporal T-cell inhibition by CTLA-4

5 Biological Roles of CTLA-4

5.1 CTLA-4 Genetics in Autoimmunity: Mice to Humans

Germline CTLA-4 knockout mice show lymphoproliferation, and die from multi-organ failure and a cytokine storm, suggesting the indispensable role of CTLA-4 in regulating self-tolerance (Tivol et al. 1995; Waterhouse et al. 1995; Chambers et al. 1997a). In humans, single nucleotide polymorphisms in the regulatory region, Exon1, 3'untranslated region, have been suggested to be associated with various autoimmune diseases (Gough et al. 2005; Scalapino and Daikh 2008), including celiac disease (Djilali-Saiah et al. 1998), rheumatoid arthritis (Yanagawa et al. 2000), multiple sclerosis (Ligers et al. 1999), type I diabetes (Todd 1997), Graves' disease (Yanagawa et al. 1995), Hashimoto's thyroiditis, autoimmune Addison's disease (Donner et al. 1997), systemic lupus erythematosus (Hudson et al. 2002) to name a few. This clearly suggests that CTLA-4 insufficiency is a genetic factor in human autoimmunity. The alternative splicing of CTLA-4 mRNA is also suggested to determine autoimmune susceptibility. Ueda et al. (2003), through a comprehensive genetic association study, showed that the alternatively spliced form of the CTLA-4 locus that determines the relative amount of soluble CTLA-4 (sCTLA-4) ligand-independent liCTLA-4 versus ligand-binding CTLA-4

(lacking the entire part of the extracellular domain) determines autoimmune susceptibility in mice and humans.

There were no case reports of nonsense mutation of CTLA-4 resulting in human autoimmunity until recently. Two groups (Schubert et al. 2014; Kuehn et al. 2014) reported familial CTLA-4 deficiency from five families that show a common variable immunodeficiency (CVID) syndrome in which patients exhibit symptoms including recurrent infection, hypogammaglobulinemia, autoimmune cytopenia, cerebral infiltration, autoimmune hemolytic anemia, autoimmune enteropathy, and granulomatous lung disease. A heterozygous nonsense mutation of CTLA-4 in exon 1 is reported in autosomal-dominant immune disorders. The penetrance of identified individuals with the mutation was not 100%, but they show low expression of CTLA-4 (especially on Tregs) and defects in trans-endocytosis of CD80 and CD86. The study suggested that even haploinsufficiency (heterozygous loss) of CTLA-4 causes severe disease in humans. Another group reported the most extensive form of CTLA-4 deficiency in humans (Lo et al. 2015). The reported autoimmunity was not due to a mutation of the CTLA-4 protein itself, but was linked to a mutation in the LRBA gene (encoding the lipopolysaccharide-responsive and beige-like anchor protein), and caused a juvenile autoimmune manifestation similar to CTLA-4 deficiency (such as humoral immune deficiency and autoimmunity associated with lymphoproliferation) (Lo et al. 2015). LRBA co-localizes with CTLA-4 in the endosomal vesicles. Mutation/deficiency alters this functionality resulting in accelerated turnover of CTLA-4 by lysosomal degradation, leading to reduced CTLA-4 protein. Inhibiting the degradation by the lysosomal inhibitor chloroquine reduced CTLA-4 degradation and amelioration of autoimmunity. The authors concluded that chemicals that inhibit lysosomes (such as chloroquine) can be used as drugs to treat autoimmunity (by stabilizing CTLA-4 protein.) As described before, in mice, CD28-CTLA-4 double KO, CTLA-4/CD80/86 triple knockout, or CTLA-4 KO treated with CD28 antagonists (CTLA-4 Ig) do not develop autoimmunity, suggesting a key role of the CD28 signal. Patients with LRBA mutation who were treated with abatacept that blocks the CD28 signal show dramatic improvement in autoimmune symptoms (Lo et al. 2015), suggesting that excess CD28 signaling is the cause of the disease. Therefore, it would be beneficial to examine if individuals showing autoimmunity have mutation/polymorphism on the CTLA-4 locus, which should predict the effectiveness of abatacept therapy (Boussiotis 2014; Schubert et al. 2014).

5.2 *CTLA-4 in Infection*

Some reports suggest that CTLA-4 is critical for optimal T-cell response in antigen-specific immunity. CD4⁺ cells from CTLA-4 KO show spontaneous Th2-type skewing, even in the genetic absence of STAT6 (Bour-Jordan et al. 2003). Accordingly, CTLA-4 KO mice (partially rescued from lymphoproliferation by the tailless CTLA-4 transgene) show defects in controlling leishmania infection

(Masteller et al. 2000). Since CTLA-4 knockout mice experience early death by lymphoproliferation, it was difficult to experimentally address how CTLA-4 KO mice mount antigen-specific immunity to infection. To clarify this, CTLA-4 in infection was addressed using mice reconstituted by bone marrow from CTLA-4 KO and WT mice to protect them from autoimmunity-associated death (Bachmann et al. 1999; Homann et al. 2006). Bachmann et al. showed that although half of the T cells in these animals do not express CTLA-4 genetically, both CTLA-4 KO T cells and WT T cells responded to leishmania, lymphocytic choriomeningitis virus (LCMV), and mouse mammary tumor virus (MMTV) normally and contracted them equally. In addition, Homann et al. showed that both CTLA-4 KO T cells and WT T cells respond normally to invading viruses and decrease in number rapidly after elimination of the pathogen (Homann et al. 2006). These investigators proposed that CTLA-4 works mainly to inhibit autoreactive CD4+ helper T cells rather than by modulating/terminating ongoing immune responses against exogenous pathogens.

5.3 *CTLA-4 in Cancer*

The goal for cancer immune therapy is to enhance patients' immune systems in order to reject cancer. This concept was proposed by pioneer studies that showed that tumor cells mutated from healthy cells are recognized as "non-self" by T lymphocytes (De Plaen et al. 1988; Lurquin et al. 1989). The problem is that mutations in cancer cells may not always create a strong agonistic peptides epitope for T cells, and may instead stimulate tolerance rather than protective T cell responses. Therefore, enhancement of tumor recognition and activation by T cells through manipulation of the known pathway, such as costimulation, is beneficial. It was first shown that transfecting CD80, a CD28/CTLA-4 ligand, on a poorly immunogenic cancer cell line stimulated the mouse immune system for rejection upon tumor transplantation (Chen et al. 1992; Yang et al. 1995). The data demonstrated that recognition of antigens on a tumor can be augmented by additional signals mediated by CD28 and/or CTLA-4, leading to efficient T-cell activation and attack. Allison and colleagues demonstrated that a systemic administration with blocking anti-CTLA-4 mAb in mice boosted the anti-tumor response, resulting in the rejection of a transplanted tumors (Leach et al. 1996; Kwon et al. 1997). These reports established a milestone that the blockade of negative costimulatory molecules to their physiological ligand promotes tumor immunity. This and subsequent results led to the concept of immune-checkpoint blockade in cancer treatment. Subsequently, the CTLA-4 blockade was shown to be effective in combination with tumor vaccination in mice (van Elsas et al. 1999). Shrikant et al. (1999) used an antigen-specific tumor elimination mouse model to elucidate the mechanism of augmented tumor immunity by CTLA-4 blockade. They showed that tumor-specific CD8+ cells are generally anergic, but exhibit tumor attack upon administration of anti-CTLA-4 antibody in vivo. This re-activation of CD8+ T cells was dependent on CD4+ helper T cells and IL-2

produced by this population, suggesting that re-activation of the helper response indirectly boosts the killer-mediated anticancer responses (Shrikant et al. 1999). The result also supported the notion that CTLA-4 blockade not only directly augments effector CD8⁺ T cells, but also indirectly boosts immune responses by acting on helper T cells.

A fully humanized chimeric antibody named ipilimumab has been shown to be effective in melanoma in monotherapy (Hodi et al. 2010), in combination with chemotherapy (Robert et al. 2011) or with anti PD-1 antibody (Wolchok et al. 2013). Although manipulation of this pathway is attractive in cancer therapies, the immune-related adverse effects are often problematic. It was initially reported that 50–60% of patients receiving anti-CTLA-4 therapy showed adverse events (Hodi et al. 2010; Robert et al. 2011) affecting various organs (reviewed in Michot et al. 2016). Analogous to mouse studies, these data suggest that there are many autoreactive T cells in the periphery that need to be under continuous control by CTLA-4. Currently, patients with immune-related adverse events higher than grade 3 are primarily treated by steroids (reviewed in Michot et al. 2016). In the future, adequate management if these side effects are likely and it is expected that there will be an abrogation of adverse events without avoiding anticancer response. However, in case of subacute, life-threatening autoimmunity, given the example of human CTLA-4 deficiency, abatacept targeting the CD28 pathway might be important.

Conclusions and perspectives

CTLA-4 is no doubt important in the maintenance of T-cell homeostasis. The longstanding basic question of why CTLA-4 deficiency results in a lethal autoimmune phenotype in mice has not, however, been solved. An interesting observation was made by two groups. An induced deletion of CTLA-4 at adulthood resulted in milder autoimmunity compared to the original germline KO (Klocke et al. 2016). Another group developed a similar system deleting CTLA-4 in adult mice and did not cause autoimmunity (Paterson et al. 2015). Although there is a technical argument regarding the efficiency of the deletion in both models, the mice did not die, unlike germline KO mice. Similarly, it was suggested that successful anti-CTLA-4 treatment on adult cancer patients, at least in part rely on Fc-receptor-mediated depletion of CTLA-4⁺ T cells (Simpson et al. 2013), however, this treatment does not result in overt lymphoproliferation. An assumption based on these data is that CTLA-4 expression is particularly important during the neonatal period when polyclonal T cells undergo lymphopenia-driven expansion to seed the body (Fig. 3a) (Min et al. 2003). This form of T-cell proliferation does not require high-affinity interaction of TCR with the MHC-self-antigen complex, but is driven by the CD28 signal (Hagen et al. 2004). The neonatal period is also a time for development of self-reactive FoxP3⁺ Tregs that prevents activation of other T cells (Itoh et al. 1999) (Yang et al. 2015). Therefore, the loss of CTLA-4 from birth causes a CD28-dependent massive expansion of nearly the entire repertoire of low-affinity self-reactive T cells without proper regulation by FoxP3⁺ Tregs, resulting in lymphoproliferation and death, due to a cytokine storm (Fig. 3a). After

CTLA-4, an Essential Immune-Checkpoint ...

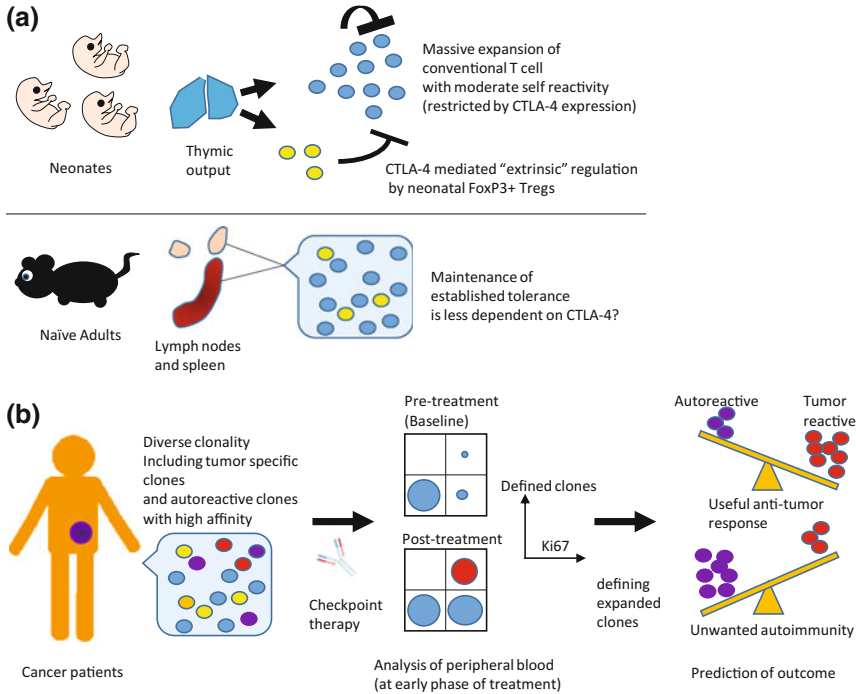


Fig. 3 A scheme representing the difference of CTLA-4-mediated inhibition between neonatal mice and naïve (uninfected) adult mice. A possible “immune monitoring” of cancer patients under checkpoint therapy to predict treatment outcome. The idea of Ki67-mediated detection of early-responding T-cell population is from (Huang et al. 2017) and (Kamphorst et al. 2017)

the initial wave of lymphocyte expansion ceases, CTLA-4-mediated inhibition is less important for maintaining the homeostatic condition of T cells (Klocke et al. 2016; Paterson et al. 2015).

In the cancer setting, high-affinity T cells receive the advantages of CTLA-4 blockade and regain their ability to fight, because these T cells recruit more CTLA-4 to the immunological synapse and are preferentially inhibited by CTLA-4 (Egen and Allison 2002). The problem is that the autoreactive memory T cells present in patients are also activated by this treatment. The balance and affinity of existing autoreactive/anticancer memory T cells will differ from person to person, which may determine the outcome of the treatment. Monitoring individuals for these clones will thus provide more efficient anticancer immunity with fewer treatment side effects (Fig. 3b). These approaches are currently in development, combining Ki67 (a proliferation marker) to monitor T cell re-activation before and after immune checkpoint therapy using patient peripheral blood samples (Huang et al. 2017; Kamphorst et al. 2017). It will hopefully become possible to detect and follow the activation of cancer/self-specific T-cell clones in the periphery during anti-CTLA-4 therapy. This will provide useful information when considering future cancer immune therapy (Fig. 3b).

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