JLB

BASIC-TRANSLATIONAL REVIEW

At the Bench: Preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy

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RECEIVED DECEMBER 5, 2012; REVISED MARCH 24, 2013; ACCEPTED MARCH 26, 2013. DOI: 10.1189/jlb.1212621

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ABSTRACT

Tumors can avoid immune surveillance by stimulating immune inhibitory receptors that function to turn off established immune responses. By blocking the ability of tumors to stimulate inhibitory receptors on T cells, sustained, anti-tumor immune responses can be generated in animals. Thus, therapeutic blockade of immune inhibitory checkpoints provides a potential method to boost anti-tumor immunity. The CTLA-4 and PD-1Rs represent two T cell-inhibitory receptors with independent mechanisms of action. Preclinical investigations revealed that CTLA-4 enforces an activation threshold and attenuates proliferation of tumor-specific T lymphocytes. In contrast, PD-1 functions primarily as a stop signal that limits T cell effector function within a tumor. The unique mechanisms and sites of action of CTLA-4 and PD-1 suggest that although blockade of either has the potential to promote anti-tumor immune responses, combined blockade of both might offer even more potent anti-tumor activity. See related review At the Bedside: CTLA-4 and PD-1 blocking antibodies in cancer immunotherapy. *J. Leukoc. Biol.* 94: 25–39; 2013.

Introduction

T lymphocytes play essential roles as orchestrators and effectors of the immune system. The power of T cells to defend against invading pathogens must be counterbalanced by mechanisms that prevent destructive immune responses targeted against self-tissues. Although thymic selection eliminates the majority of self-reactive T cells, a fraction escapes this process of central tolerance and retains the potential to inflict destructive autoimmune pathology against the self. The immune system evolved numerous strategies to restrain autoreactive T cells and maintain peripheral tolerance. In particular, a complex network of stimulatory and inhibitory receptors and ligands delivers cell-to-cell signals that dictate the outcome of T cell encounters with cognate antigen. Moreover, Tregs exert extrinsic control over autoreactive T cells, which is mediated, at least in part, by expression of many of the same stimulatory and inhibitory receptors.

The potential of T cells to attack and destroy tumors has long been recognized [1]. Moreover, recent experimental evidence has demonstrated that T cell surveillance throughout the life of an organism functions to prevent tumor development [2–4]. Nonetheless, T cells face an uphill battle in their attempts to recognize and eliminate tumors, as many of the same mechanisms that prevent autoimmunity also impair T cell responses to the "altered self" of tumors. In addition, it has become apparent that tumors are capable of selectively co-opting many of the inhibitory mechanisms that prevent sustained T cell responses to self-tissue. Selective blockade of these natural inhibitory checkpoints might provide a means of releasing the brakes on T cell activation and promoting potent anti-tumor responses. Indeed, antibodies that block the inhibitory receptors CTLA-4 and PD-1 have demonstrated an ability to improve outcomes for patients with at least some cancers [5–8].

CTLA-4 and PD-1 represent the two immune inhibitory receptors for which blocking agents have progressed furthest through clinical development as anti-cancer therapeutics. This review will highlight the distinctive biology of CTLA-4 and PD-1, including their unique molecular structures, expression patterns of receptors and ligands, and mechanisms of cell-intrinsic and cell-extrinsic activity. The distinct molecular and cellular mechanisms whereby CTLA-4 and PD-1 operate suggest that combined therapeutic manipulation of these pathways may be synergistic for cancer immunotherapy. The accompanying review by Callahan and Wolchok (At the bedside: CTLA-4 and PD-1 blocking antibodies in cancer immunotherapy)will focus on the clinical manipulation of CTLA-4 and PD-1, highlighting the future potential for combinatorial therapy in cancer patients.

Abbreviations: ALK=anaplastic lymphoma kinase, CTLA-4=cytotoxic T lymphocyte activation antigen 4, Foxp3=forkhead box p3, ITSM=immunoreceptor tyrosine-based switch motif, PD-1=programmed death 1, PD-L1/2=programmed death ligand 1/2, PP2A=protein phosphatase 2A, SHP-1/2=Src homology 2-containing tyrosine phosphatase 1/2, Treg=regulatory T cell, Y165/182=tyrosine 165/182

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CTLA-4

Discovery

Decades of research resulted in elucidation of the mechanisms, whereby T cells encounter antigen and undergo activation. It became apparent that TCR engagement is not in itself sufficient to enact clonal expansion and differentiation. Stimulation through the TCR alone results in an unresponsive, anergic state or activation-induced cell death, which are hypothesized to represent distinct mechanisms of peripheral self-tolerance [9, 10]. Indeed, theories of self/nonself-discrimination, put forward by Bretchser and Cohn [11] and Lafferty and Cunningham [12], had predicted the theoretical requirement for a second signal to drive lymphocyte clonal expansion. It was hypothesized that the first signal through the antigen receptor should provide specificity to the response, whereas an undefined, second signal should confirm that the specificity is directed against a nonself-antigen.

TCR: *Each T cell expresses on its cell surface a TCR with unique specificity. Engagement of the TCR with cognate peptide presented in the context of MHC molecules provides the initial signal ("signal one") for T cell activation.*

The two-signal theory of T cell activation received molecular confirmation by the discovery of stimulatory coreceptors, of which CD28 represented the founding member [13]. Coligation of the TCR, along with CD28, promoted T cell activation, proliferation, survival, and activation of effector function (**Table 1**)[14, 15]. In the absence of CD28 or its B7 ligands, T cell proliferation in response to TCR ligation was severely impaired [16, 17]. Important functions of CD28 costimulation were ultimately determined to include amplification of TCR signaling, stimulation of IL-2 production via stabilization of IL-2 mRNA, induction of the antiapoptotic molecule Bcl-XL, and activation of glucose uptake via signaling through the PI3K/Akt pathway, thereby providing the nutrients necessary for cell growth and proliferation [18–21].

CD28: *Stimulatory coreceptor expressed on the surface of T cells. Interactions between CD28 and B7 ligands expressed on the surface of APCs provide a second signal ("signal two") required for productive T cell activation, clonal expansion, and acquisition of effector functions.*

After the discovery and characterization of CD28, research efforts focused on identifying additional stimulatory coreceptors that could contribute to the activation of T cells. CTLA-4, also known at CD152, was identified initially as a gene expressed by activated cytotoxic T cells [22]. CTLA-4 shared significant homology with CD28 and bound to the same B7 ligands [23, 24]. Initial functional studies suggested that antibodies directed against CTLA-4 could enhance T cell activation in concert with antibodies directed against CD28, prompting the conclusion that CTLA-4 functioned as a costimulatory molecule [25]. However, it was later discovered that CTLA-4 was actually being inhibited under the conditions used in these experiments.

CTLA-4: *Inhibitory coreceptor expressed on the surface of activated T cells. Interactions between CTLA-4 and B7 ligands expressed on the surface of APCs counteract CD28-mediated costimulatory signals and impair the activation of T cells.*

Further investigation established that CTLA-4 functioned as an inhibitor of T cell activation [16, 26, 27]. Optimal crosslinking of the CTLA-4 receptor in the presence of TCR and CD28 stimulation resulted in suppression of IL-2 production and proliferative arrest, without induction of apoptosis [28, 29]. Engagement of CTLA-4 could induce an anergic phenotype similar to that observed in response to TCR stimulation alone [30], establishing the notion that CTLA-4 might function to counteract CD28-mediated costimulation.

The phenotype of CTLA-4-deficient mice confirmed its function as a crucial inhibitor of T cell activation. Mice with germline deletion of *Ctla4* developed a rapidly progressive, fatal lymphoproliferative disease, characterized by multiorgan T cell infiltration and death by 3–4 weeks of age [31, 32]. The auto-

TABLE 1. Summary of CD28, CTLA-4, and PD-1 functions on T cells

Receptor	Expression pattern	Ligand	Expression pattern	Functional outcome
CD28	Constitutive expression on T cells	B7-2 (CD86)	Constitutive expression on APCs Enhanced by APC activation	Promotes: • Productive activation • Clonal expansion • Acquisition of effector function
		B7-1 (CD80)	Activated APCs	
CTLA-4	Activated T cells Memory T cells Tregs	B7-1 (CD80)	Activated APCs	Raises activation threshold Attenuates clonal expansion
		B7-2 (CD86)	Constitutive expression on APCs Enhanced by APC activation	
PD-1	Activated T cells "Exhausted" T cells	PD-L1 (B7-H1)	Constitutive expression on APCs B and T cells Nonhematopoietic cells Enhanced by inflammatory cytokines	Restrains effector T cell function, primarily in nonlymphoid organs
		PD-L2 (B7-DC)	Constitutive expression on APCs Enhanced by APC activation	Restrains effector T cell function, primarily in lymphoid organs

Top row (CD28) highlights stimulatory, and middle (CTLA-4) and bottom (PD-1) rows highlight inhibitory receptor:ligand interactions, respectively. APCs include DCs, macrophages, and mast cells.

immune phenotype was not attributable to abnormal T cell development in the thymus [33, 34]; however, it was strictly dependent on antigen-specific stimulation through the TCR [34] and costimulatory signals delivered through CD28:B7 receptor:ligand interactions [35–37]. Thus, CTLA-4 emerged as a key negative regulator of T lymphocyte activation and enforcer of peripheral tolerance, appearing to operate primarily via antagonism of CD28-mediated costimulation.

Molecular structure

CTLA-4 is a type 1 transmembrane glycoprotein of the Ig superfamily, comprised of four domains, including a signal peptide, an extracellular cellular ligand-binding domain, a transmembrane domain, and a short cytoplasmic tail [22, 38–40]. CTLA-4 forms a covalently linked heterodimer that binds to oligomerized B7-1 (CD80) and B7-2 (CD86) ligands with higher affinity and avidity than CD28 [24, 41–45]. Although the cytoplasmic domain lacks any intrinsic enzymatic activity, it recruits various molecules involved in signaling and intracellular trafficking.

Multiple splice variants of CTLA-4 exist [23], including a soluble form in humans and a ligand-independent form in mice. Polymorphisms in the soluble version of CTLA-4 have been implicated in human autoimmune disorders, including Grave's disease, Hashimoto's thyroiditis, and type I diabetes [46]. Likewise, polymorphisms in the ligand-independent form of CTLA-4 may play a role in the pathogenesis of diabetes in the NOD mouse model [46, 47]. The ligand-independent isoform of CTLA-4 appears to suppress self-reactive T cells by producing tonic inhibitory signals that increase the threshold required for T cell activation [47]. The specific contributions of each of these splice isoforms to the overall biologic function of CTLA-4 remain unknown.

Clinical Questions: *Do CTLA-4 splice variants impair productive anti-tumor immunes responses in humans? Does CTLA-4 blockade mediate its anti-tumor effects, in part, by counteracting the functions of CTLA-4 splice variants?*

Expression pattern

Expression of CTLA-4 is primarily restricted to T cells (Table 1) [22], although expression on B cells and other cell types has been described [48]. In contrast to CD28, which is expressed on the surface of resting and activated T cells, CTLA-4 exhibits minimal expression in resting T cells (**Fig. 1**). CTLA-4 is induced at the mRNA and protein level in response to TCR activation [43]. Expression of CTLA-4 is enhanced by costimulation through CD28 and/or IL-2 [49]. Protein expression of CTLA-4 peaks at 24–48 h post-TCR stimulation and requires entry into the cell cycle [49, 50]. Antigen-experienced memory $CD4^+$ and $CD8^+$ T cells, as well as $CD4^+$ Tregs, maintain constitutive expression of CTLA-4 [51–54].

Of note, although $CD4^+$ and $CD8^+$ T cells express CTLA-4, the inhibitory functions of CTLA-4 on $CD4^+$ T cells appear to be relatively more important for the prevention of autoimmune pathology. CTLA-4-deficient $CD8^+$ T cells are incapable of inducing autoimmune pathology in the absence of CTLA-4-deficient $CD4^+$ T cells [35, 55]. This may be attributable, at least in part, to $CD4^+$ Tregs, which constitutively express high levels of CTLA-4 and depend on CTLA-4 for their suppressive functions [53, 54, 56]. Nonetheless, CTLA-4 does exert inhibitory activities on $CD8^+$ T cells and may be particularly important as a regulator of secondary responses by effector/memory $CD8^+$ T cells [51, 52, 57].

Clinical Question: *What are the relative contributions of effector and memory $CD4^+$ and $CD8^+$ T cells to the anti-tumor activity of CTLA-4 blockade in humans?*

Cellular localization

One of the most remarkable features of CTLA-4 biology is its pattern of intracellular localization and trafficking (**Fig. 2**). The majority of CTLA-4 resides within intracellular vesicles of the trans-Golgi network and endosomal compartments [49, 58, 59]. In resting T cells, a small amount of CTLA-4 protein cycles continuously from the Golgi apparatus to the cell surface, followed by rapid endocytosis and lysosomal degradation [49]. This intracellular trafficking pattern is mediated by association of the cytoplasmic tail of CTLA-4 with the clathrin-associated adaptor proteins AP-1 and AP-2/AP50 [60–64]. In addition, the interaction of CTLA-4 with a protein—TCR-interacting molecule (TRIM)—appears to be important for its proper intracellular localization and trafficking [65].

Clinical Questions: *Do CTLA-4 blocking antibodies undergo endocytosis and lysosomal degradation? If so, would inhibition of these processes enhance the anti-tumor efficacy of CTLA-4 blockade?*

Upon TCR engagement, CTLA-4 expression is induced, and intracellular vesicles containing CTLA-4 undergo relocalization to the immune synapse [58]. TCR-induced kinases Lck and ZAP70 phosphorylate the cytoplasmic tail of CTLA-4 at Y165, resulting in disruption of the CTLA-4:AP-2 interaction and retention of CTLA-4 at the cell surface in the immune synapse [62, 64, 66]. Of note, the stronger the TCR signal, the more CTLA-4 accumulates at the immune synapse, thus providing a dynamic and tunable inhibitory signal [67].

Receptor:ligand interactions

One of the primary mechanisms whereby CTLA-4 inhibits T cell function is via competition with CD28 for access to B7 ligands. CD28 and CTLA-4 share the ligands B7-1 (CD80) and B7-2 (CD86), whose expression is restricted to APCs, including DCs, B cells, and macrophages (Table 1) [24, 44, 45, 68–70]. Compared with CD28, CTLA-4 has higher affinity and avidity for B7 ligands, which has been attributed to homodimer formation by CTLA-4 that allows for bivalent binding of B7 molecules, in contrast to the monovalent binding of B7 ligands by CD28 [40, 71]. The stronger ligand-binding activity of CTLA-4 allows it to exert its inhibitory functions at a much lower surface density compared with that of CD28.

B7-1 (CD80): *Ligand expressed on the surface of activated APCs. Although it can interact with CD28, B7-1 appears to be the primary ligand for CTLA-4, forming multivalent interactions that deliver inhibitory signals to T cells.*

The expression kinetics of B7-2 and B7-1 parallel the expression kinetics of CD28 and CTLA-4, respectively. B7-2 is consti-

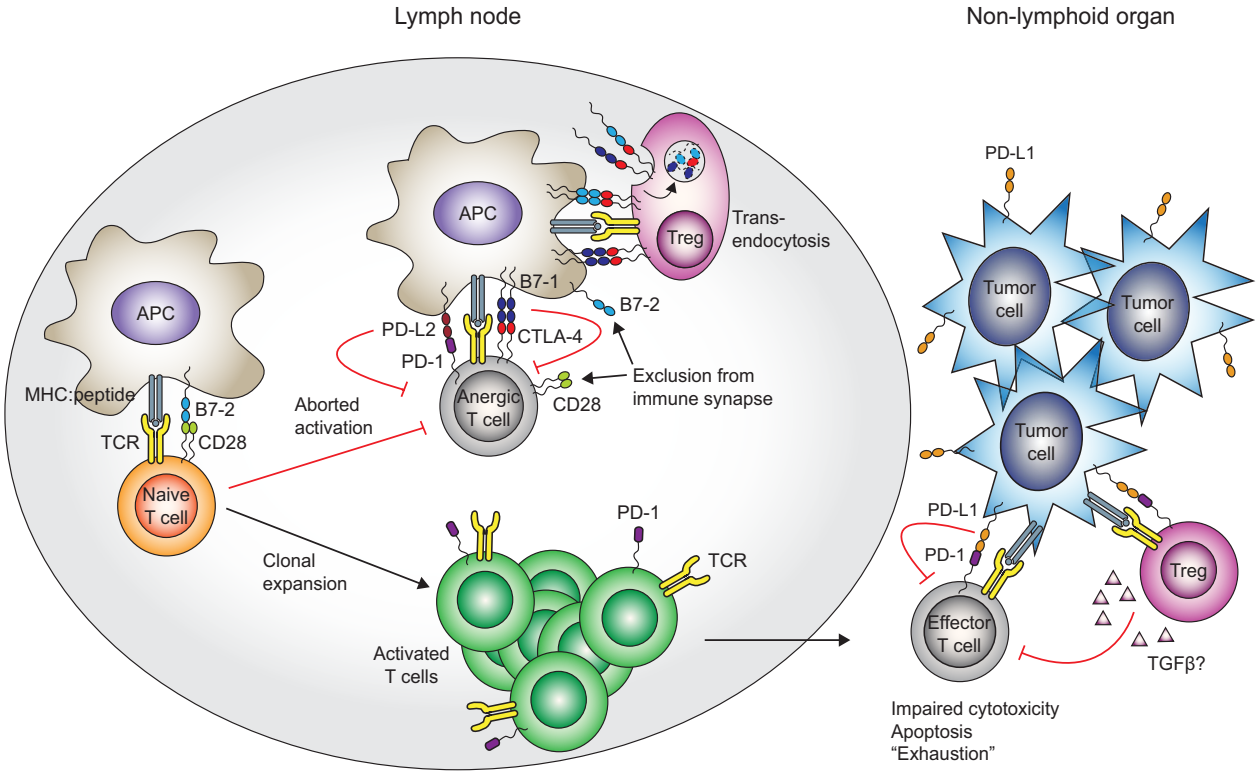

Figure 1. Unique spatiotemporal regulation of CTLA-4 and PD-1. Activation of a naïve T cell requires TCR-mediated signals and costimulatory signals, generated by CD28:B7 ligand interactions. Upon activation, T cells induce expression of the inhibitory receptors CTLA-4 and PD-1, and the relative balance of stimulatory and inhibitory signaling can dictate the outcome of the T cell response. When CTLA-4- and PD-1-mediated inhibitory signals dominate, T cell activation is aborted, resulting in an unresponsive anergic state. Tregs can tip the balance toward inhibitory signals by removing B7 ligands from the APC surface via transendocytosis, thus favoring B7 ligand sequestration by the higher-affinity CTLA-4 receptor. When TCR- and CD28-mediated stimulatory signals dominate, T cells undergo clonal expansion, acquisition of effector function, and trafficking through nonlymphoid tissues. Effector T cell function can be limited by PD-1 interaction, with PD-L1 expressed on the surface of nonhematopoietic cells, including many different tumors. Moreover, PD-1:PD-L1 interactions can enhance Treg function, resulting in an additional layer of effector T cell inhibition.

tutively expressed on resting APCs with further induction after activation, reaching a peak within 24 h. In contrast, B7-1 does not exhibit significant expression on resting APCs but is maximally induced ~48 h after APC activation [69, 70]. Thus, the current model suggests that CD28:B7-2 receptor:ligand interactions initiate T cell activation, whereas CTLA-4:B7-1 interactions can terminate the response in the later stages of activation [71]. Support for this model came from the observations that B7-2 stabilizes CD28 in the immune synapse, whereas B7-1 recruits CTLA-4 to the immune synapse at later stages of activation, resulting in the displacement of CD28 [72].

> **B7-2 (CD86):** *Ligand expressed constitutively on the surface of resting and activated APCs. Appears to be the primary ligand responsible for delivering costimulatory signals to T cells via monovalent interactions with CD28.*

Recent observations have demonstrated an interesting connection between CTLA-4:B7 receptor:ligand interactions and the unique cellular trafficking of the CTLA-4 molecule. Activated T cells expressing CTLA-4 were shown to acquire the ligands B7-1 and B7-2 from APCs by a process of transendocytosis [73]. The B7 molecules were endocytosed and degraded in the lysosomes of T cells in a CTLA-4-dependent manner (Fig. 1). As a consequence, CTLA-4-expressing T cells caused significant depletion of the B7 ligands from the surface of APCs. This provided a molecular mechanism for prior observations that the conditioning of activated APCs with CTLA-4-expressing Tregs resulted in the selective loss of B7 ligands from the surface of the APCs in a CTLA-4-dependent manner, thereby impairing the ability of the conditioned APCs to activate T cells [74, 75]. Thus, CTLA-4:B7 transendocytosis provides a mechanistic explanation of how CTLA-4 might inhibit T cell function in cis and in trans.

Intracellular signaling

In addition to competition for B7 ligands, CTLA-4 inhibits T cell activation through cell-intrinsic signaling cascades [76]. The importance of intracellular signaling via the cytoplasmic tail of CTLA-4 is demonstrated by the inability of CTLA-4 constructs lacking the cytoplasmic domain to suppress lymphoproliferative disease in CTLA-4-deficient mice [77]. On the other hand, CTLA-4 constructs incapable of binding to B7 ligands

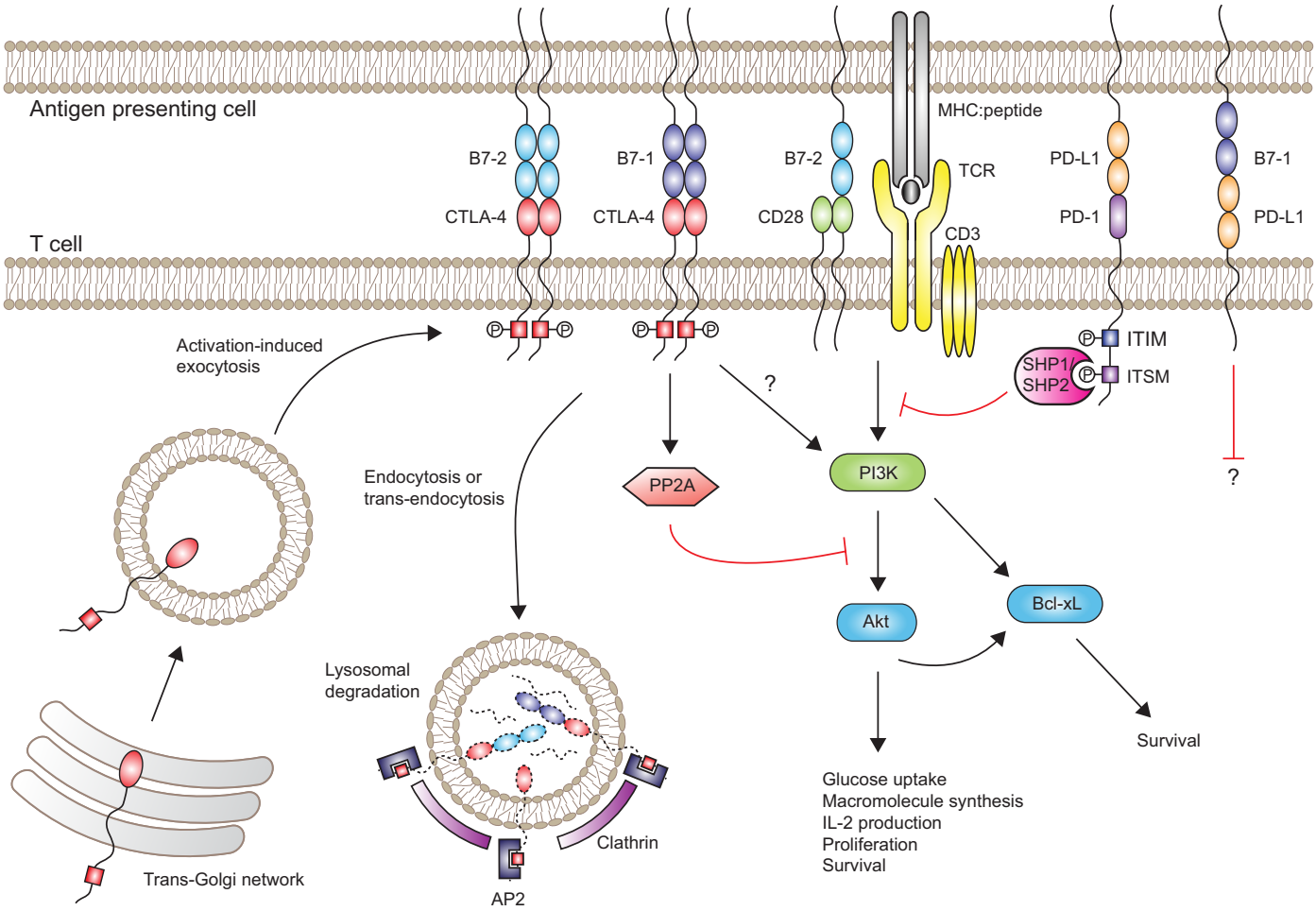

Figure 2. Distinct mechanisms of intracellular signaling by CTLA-4 and PD-1. Upon activation, T cells synthesize CTLA-4, and intracellular vesicles containing CTLA-4 undergo transport to the immune synapse. Phosphorylation of CTLA-4 at the YKVM motif (red box) promotes sustained expression of CTLA-4 at the cell surface by preventing AP2- and clathrin-mediated endocytosis. At the cell surface, CTLA-4 competes with CD28 for access to B7 ligands. CTLA-4-mediated transendocytosis of B7 ligands can further limit availability of B7 ligands for CD28. Ligation of CTLA-4 by B7-1 or B7-2 blocks TCR/CD28-mediated activation of Akt, in part, through CTLA-4-mediated activation of the serine/threonine phosphatase PP2A. However, CTLA-4 ligation does not interfere with PI3K-mediated induction of the antiapoptotic molecule Bcl-xL. In contrast, ligation of PD-1 by PD-L1 or PD-L2 (not shown) results in phosphorylation of ITSM (purple box) and ITIM (blue box) motifs within the cytoplasmic tail of PD-1. The phosphorylated ITSM motif recruits the tyrosine phosphatases SHP-1 and SHP-2, which in turn, block TCR/CD28-mediated activation of PI3K. Signals delivered via B7-1:PD-L1 interactions may also inhibit T cell function, although the signaling pathways have not been elucidated.

are capable of partially suppressing the autoimmune phenotype induced by CTLA-4-deficient T cells [78]. Thus, simple sequestration of B7 ligands by the extracellular domain of CTLA-4 is insufficient to explain its mechanism of inhibitory function. However, the requirement of the cytoplasmic domain of CTLA-4 may also be explained partly by its role in vesicular trafficking and transendocytosis.

The cytoplasmic tail of CTLA-4 interacts with a number of signaling molecules that inhibit proximal signaling via the TCR and CD28 (Fig. 2). TCR/CD28 signaling results in activation of numerous kinases, including Lck, Fyn, Lyn, Rlk, and Jak2, which are capable of phosphorylating Y165 and Y182 of the cytoplasmic domain [79–82]. Initial studies demonstrated that phosphorylation at Y165 creates a docking site for the protein tyrosine phosphatase SHP-2, which subsequently inhibits proximal TCR signaling via dephosphorylation of the TCRζ chain, linker for activation of T cells, and the Ras regulator p52SHC [83, 84]. CTLA-4 interferes with the formation of lipid rafts, TCR:ZAP70 microclusters, and the central supramolecular activation complex, each of which plays important roles in T cell activation [85–88]. In addition, CTLA-4 stabilizes expression of the ubiquitin ligase Cbl-b, an important negative regulator of signaling via the TCR and CD28 [89].

Several studies indicated that an important CTLA-4-interacting partner is the serine/threonine phosphatase PP2A [90, 91]. CTLA-4-mediated recruitment of PP2A results in inhibition of Akt, thereby inhibiting CD28-mediated induction of glucose uptake [19]. Importantly, CTLA-4 ligation does not affect upstream PI3K activation by CD28. In fact, the cytoplasmic tail of CTLA-4 may also interact directly with and activate PI3K [92, 93]. Unim-

peded PI3K activation permits induction of the antiapoptotic factor Bcl-xL, which facilitates survival of the anergic T cell after receiving inhibitory signals via CTLA-4 [93, 94]. However, T cell blastogenesis and proliferation cannot proceed in the absence of Akt-stimulated glucose uptake [93, 94].

PP2A: *Intracellular serine/threonine phosphatase whose functions include inhibition of Akt upon recruitment to the cytoplasmic tail of CTLA-4.*

Additional downstream consequences of CTLA-4 ligation include inhibition of IL-2 production and induction of cell cycle arrest [26–28]. The inhibition of IL-2 production appears to be a consequence of impaired nuclear accumulation of AP-1, NF of activated T cells (NFAT), and NF-κB [95, 96]. Cell cycle arrest results from CTLA-4-mediated inhibition of CDK4, CDK6, and cyclin D3, an effect that is partially independent of IL-2 [97].

Clinical Question: *Could pharmacologic inhibition of intracellular signaling molecules downstream of CTLA-4, such as PP2A, enhance and/or recapitulate the anti-tumor effects of CTLA-4 blockade in humans?*

Despite biochemical evidence that CTLA-4 might inhibit proximal TCR signaling, gene expression profiling suggests that the net effect of CTLA-4 ligation is blockade of CD28-mediated signals, without altering the number of transcripts activated downstream of the TCR [98]. The biologic function of CTLA-4 as a specific inhibitor of CD28 has been corroborated with numerous genetic approaches, most notably, with the demonstration that a single point mutation in CD28 is capable of completely abrogating the autoimmune phenotype in CTLA-4-deficient mice [37].

Role on Tregs

One striking observation regarding the phenotype of CTLA-4-deficient mice was that the defects observed were not cell-autonomous. T cell-deficient mice, reconstituted with a mixture of WT and CTLA-4-deficient T cells, remain healthy and do not develop the fatal autoimmune disorder [99, 100]. Moreover, CTLA-4-deficient T cells, present in mixed chimeric mice, respond normally to viral infection [101]. These findings do not exclude the presence of cell-autonomous mechanisms of CTLA-4 function but do suggest that they can be over-ridden by cell-extrinsic mechanisms.

Numerous lines of evidence suggest that CTLA-4-expressing Tregs are responsible for the cell-extrinsic inhibitory effects on conventional T cells. As mentioned above, in distinction to conventional T cells, Tregs exhibit constitutive expression of CTLA-4 [53, 54], and CTLA-4 is a direct target of Foxp3, the lineage-specifying transcription factor for Tregs [102–104]. Antibody-mediated CTLA-4 blockade on Tregs abrogates their suppressive function in vivo [105].

Treg: *Subset of $CD4^+$ T cells defined by expression of the transcription factor Foxp3. Tregs play a critical role in preventing destructive immune responses, which may, in part, be mediated by their expression of CTLA-4.*

Elegant genetic studies demonstrated that expression of CTLA-4 by Tregs is critical for the cell-extrinsic control of conventional T cell activation. Conditional deletion of *Ctla4*, only in Tregs, resulted in fatal systemic autoimmune disease with similar features to that seen in mice with germline deletion of *Ctla4*, albeit with somewhat delayed kinetics [56]. The phenotype was not a result of impaired survival of Tregs but was associated with heightened expression of CD80 and CD86 on APCs in the mice with Treg-specific deletion of *Ctla4*. Thus, it was concluded that CTLA-4 expression by Tregs limits availability of CD80 and CD86 on APCs, thereby inhibiting conventional T cell activation in a cell-extrinsic fashion.

Clinical Question: *To what degree does antagonism of CTLA-4 function on Tregs contribute to the anti-tumor effects of CTLA-4 blocking agents in humans?*

Another mechanism whereby CTLA-4 might function on Tregs is via "reverse" B7 ligand-mediated signals transmitted to APCs [106]. Tregs can induce expression of the tryptophan-catabolizing enzyme IDO in DCs in a CTLA-4 dependent manner [107]. Moreover, certain suppressive functions mediated by Tregs are dependent on intact IDO function in DCs. The investigators concluded that CTLA-4-mediated signals delivered to DCs via CTLA-4:B7 reverse signaling induce expression of IDO and result in local depletion of the essential amino acid tryptophan, thereby inhibiting conventional T cell priming and clonal expansion. The relative importance of this mechanism of CTLA-4 inhibitory function requires further clarification.

Preclinical models of cancer immunotherapy

Shortly after the inhibitory functions of CTLA-4 were discovered, antibody-mediated blockade of CTLA-4 function was found to enhance the rejection of transplanted mouse colon carcinoma and fibrosarcoma tumors and additionally, delay growth of established tumors [108]. Moreover, anti-CTLA-4 treatment resulted in immune memory, such that previously challenged mice could reject subsequently implanted tumors without additional CTLA-4 blockade. The anti-tumor effects of CTLA-4 blockade have been extended to numerous other murine tumor models, including prostate carcinoma, renal cell carcinoma, and lymphoma [109]. Importantly, the antibody used in these studies was a nonstimulatory, bivalent antibody that did not induce optimal cross-linking of CTLA-4 but rather, blocked the interaction of CTLA-4 with B7 ligands without affecting the activity of CD28.

In less immunogenic murine tumor models, such as B16 melanoma or SM1 mammary carcinoma, CTLA-4 blockade did not demonstrate efficacy as a single agent [110, 111]. However, a combination of CTLA blockade with a GM-CSF-based tumor vaccine was highly effective in controlling these tumors. Likewise, in a murine model of primary prostate cancer, a combination of CTLA-4 blockade with an irradiated GM-CSF-expressing tumor vaccine was capable of controlling disease, whereas neither component was effective on its own [112]. Numerous other therapeutic strategies were found to be effective in combination with CTLA-4 blockade, including radiation, chemotherapy, and a variety of vaccination strategies with tumor antigens. The common theme that emerged was that any method of increasing tumor antigen availability could po-

tentially synergize with CTLA-4 blockade. Thus, CTLA-4 blockade in the setting of enhanced tumor antigen presentation likely functions to break T cell tolerance to tumor antigens and/or self-antigens expressed by tumors, thereby promoting effective anti-tumor immunity.

> **Clinical Question:** *What are the optimal strategies for enhancing tumor antigen delivery to synergize with CTLA-4 blockade as cancer immunotherapy, e.g., vaccination, cytokine delivery, radiation, cryoablation, or chemotherapy?*

Significant efforts were devoted to elucidating the cellular and molecular correlates for anti-tumor activity in the mouse models of CTLA-4 blockade. In the B16 melanoma model, measurements of total Tregs or tumor-specific cytotoxic T cells in the blood did not correlate with anti-tumor activity [113]. Surprisingly, CTLA-4 blockade increased the numbers of Tregs in tumor-draining LNs. The most powerful correlate of anti-tumor activity proved to be an increased ratio of effector T cells to Tregs within the tumor [113]. These findings raised the question of whether the anti-tumor activity was primarily a result of CTLA-4 blockade on conventional T cells or Tregs. An elegant reconstitution system demonstrated that isolated blockade of CTLA-4 on Tregs conferred no anti-tumor activity toward established B16 melanoma, whereas isolated blockade of CTLA-4 on conventional T cells conferred modest anti-tumor activity [114]. Importantly, however, combined blockade of CTLA-4 on conventional T cells and Tregs provided robust and synergistic anti-tumor activity.

These preclinical mouse models also provided the first glimpses of immune-related adverse events that might result from CTLA-4 blockade as an anti-cancer therapeutic strategy. In contrast to CTLA-4-deficient mice, transient CTLA-4 blockade did not result in widespread systemic autoimmunity. Instead, self-directed immune pathology was observed primarily in the setting of vaccination strategies using irradiated tumor cells combined with CTLA-4 blockade, specifically in the tissues from which the tumors were derived. In the B16 melanoma model, mice from the experimental group developed vitiligo [110], whereas in the transgenic adenocarcinoma of the mouse prostate cancer model, the experimental treatment resulted in prostatitis, even in mice that did not have tumors [112].

Summary of CTLA-4 function

Several models, which help to clarify the potential mechanisms of anti-tumor activity mediated by CTLA-4 blockade, have been proposed to explain the inhibitory function of CTLA-4 [109, 115]. The threshold model suggests that CTLA-4 enforces an activation threshold that limits productive TCR engagement by low-affinity, self-reactive T cells. This could be achieved by cell-extrinsic and cell-intrinsic mechanisms. Competition between CD28 and CTLA-4 for B7 ligands prevents activation of T cells by weak TCR signals in situations where B7 ligands are limiting. Inhibition of access to B7 ligands might occur in cis on the T cell engaging the APC or in trans by Treg-mediated depletion of B7 ligands from the APC surface. Likewise, intracellular recruitment of phosphatases sets an intracellular signaling threshold that must be overcome for productive TCR/CD28 engagement.

The attenuation model suggests that in situations where B7 ligands are not limiting, CTLA-4 functions to attenuate T cell proliferation and clonal expansion after activation. This model fits well with the spatial and temporal pattern of CTLA-4:B7-1 interactions that occur relatively late in T cell activation. CTLA-4-mediated attenuation of T cell clonal expansion might function to maintain a broad repertoire of TCR specificities responding to a given antigenic challenge. In the absence of CTLA-4, T cells with high-affinity TCR specificities would selectively dominate the immune response. As CTLA-4 is induced in direct proportion to the strength of TCR signals, the degree of attenuation may be tailored to the affinity of the TCR for antigen. As a corollary, domination of CTLA-4-mediated inhibitory signals over TCR/CD28-mediated stimulatory signals might abort proliferation prematurely, leading to T cell anergy.

In the setting of tumor immunotherapy, CTLA-4 blockade might lower the threshold of activation for low-affinity, tumor-specific T cells, allowing effective priming and clonal expansion. The additional blockade of CTLA-4-mediated attenuation might then allow more robust proliferation and clonal expansion of tumor-specific T cells. The additional effects of CTLA-4 blockade on Treg function could further enhance tumor-specific T cell effector activity within the tumor itself.

PD-1

Discovery

PD-1 was identified originally as a gene induced by a T cell hybridoma undergoing apoptotic cell death [116]. The phenotype of PD-1-deficient mice provided insights into its function as an inhibitor of T cell activation. In contrast to the rapid-onset systemic autoimmunity observed in CTLA-4-deficient mice, PD-1 deficiency results in delayed-onset, organ-specific autoimmunity with incomplete penetrance that varies depending on the genetic background of the mouse. After 6 months of age, PD-1-deficient C57BL/6 mice begin to develop a lupus-like syndrome, characterized by glomerulonephritis and arthritis with Ig and complement deposition in the affected tissues [117]. PD-1-deficient BALB/c develop an autoimmune-dilated cardiomyopathy, mediated by anti-troponin I antibodies [118, 119]. Moreover, PD-1 deficiency accelerates tissue-specific autoimmune disease in a variety of contexts, including the *lpr* model of lupus and NOD model of diabetes [117, 120]. Thus, genetic evidence suggested that PD-1 might function as an inhibitor of lymphocyte responses at peripheral tissues.

> **PD-1:** *Inhibitory receptor expressed on the surface of activated T cells. Interactions between PD-1 and its ligands, PD-L1 or PD-L2, restrain T cell function in nonlymphoid organs and lymphoid organs, respectively.*

Further insight into the role of PD-1 as a guardian against immune-mediated tissue destruction came from studies of PD-1 function in models of chronic infectious diseases. Considerable interest was generated by the observation that PD-1 is highly expressed by exhausted, dysfunctional T cells in the context of chronic infections [121, 122]. Antibody-mediated blockade of PD-L1, the major PD-L1, results in a restoration of T cell function and enhances control of viral replication. Im-

portantly, however, chronic viral infection in PD-L1-deficient mice results in fatal autoimmune pathology. Thus, the PD-1 pathway appears to protect the host from immune-mediated tissue destruction in the setting of chronic antigen stimulation.

Molecular structure

Like CTLA-4, PD-1 is a type 1 transmembrane protein of the Ig superfamily [116]. The protein structure of PD-1 includes an extracellular N-terminal IgV-like domain, a transmembrane domain, and a cytoplasmic tail [123]. In distinction to CTLA-4, the cytoplasmic tail of PD-1, contains an ITIM and an ITSM, which are involved in the transmission of inhibitory signals [124–126]. Unlike CTLA-4, PD-1 is monomeric on the cell surface, as it lacks the extracellular cysteine residue required for covalent dimerization [123]. Similar to CTLA-4, numerous splice variants of PD-1 have been identified in activated human T cells [127]. These splice variants have not been thoroughly studied, although a splice variant lacking the transmembrane domain has been proposed to represent a soluble factor involved in the pathogenesis of rheumatoid arthritis [128].

Expression pattern

PD-1 exhibits minimal expression on resting cells of the immune system; however, upon activation, PD-1 expression is broadly induced on T cells, B cells, NK cells, NKT cells, DCs, and macrophages [129] (Table 1 and Fig. 1). Induction of PD-1 in peripheral T cells occurs downstream of TCR signaling [130]. As mentioned above, PD-1 is highly expressed by dysfunctional T cells in the setting of chronic infections but is not expressed on resting memory T cells that arise after an acute infection [121, 122].

Ligand distribution

PD-1 binds two distinct ligands, known as PD-L1 (B7-H1) and PD-L2 (B7-DC), which are members of the B7 family of proteins (Table 1 and Fig. 1) [129]. PD-L1 is expressed constitutively on hematopoietic cells, including DCs, macrophages, mast cells, B cells, and T cells [131]. In contrast to B7 ligands, PD-L1 is also expressed on a wide range of nonhematopoietic cells, including endothelial cells and numerous types of epithelial cells [129, 132]. Inflammatory signals, including type I and type II IFNs and TNF-α, result in further induction of PD-L1 on hematopoietic and nonhematopoietic cells [132, 133]. Thus, the broad distribution of PD-L1 expression supports the importance of PD-1:PD-L1 interactions in regulating effector T cell responses in peripheral tissues, particularly at sites of inflammation.

PD-L1 (B7-H1): *PD-L1 broadly expressed by hematopoietic and nonhematopoietic cells. Numerous tumors express PD-L1, thereby inhibiting effective anti-tumor immune responses by PD-1-expressing T cells.*

In addition to its function as a ligand for PD-1, PD-L1 is reported to bind to B7-1 [134]. Experimental evidence suggests that B7-1:PD-L1 interactions inhibit T cell function independently of PD-1. In the NOD mouse model of diabetes, selective blockade of B7-1:PD-L1 interactions accelerates autoimmune destruction of the pancreas in already established diabetes but fails to precipitate disease in young mice [135]. Thus, the B7-1:PD-L1 interaction appears to function primarily in restraining the activity of effector T cells at sites of ongoing autoimmune pathology. This additional layer of complexity in the coinhibitory receptor/ligand system predicts possible differences in the therapeutic activities of antibodies that target PD-L1 compared with antibodies that target PD-1.

Clinical Question: *PD-L1 interacts with B7-1 and PD-1. Does this predict distinct clinical responses to PD-1 versus PD-L1 blockade as cancer immunotherapy?*

In contrast to PD-L1, PD-L2 exhibits a much more restricted expression pattern, with expression restricted to cells of the immune system, including DCs, macrophages, and mast cells [136, 137]. Like PD-L1, PD-L2 expression is enhanced by inflammatory signals, including IFN-γ, GM-CSF, and IL-4 [137]. The relative contributions of PD-L1 and PD-L2 to the inhibitory functions of PD-1 have been investigated through a variety of genetic approaches. Studies of PD-L1-deficient mice demonstrated that PD-L1 expression on hematopoietic cells inhibits T cell cytokine production in lymphoid tissues, whereas PD-L1 expression on nonhematopoietic cells plays a unique role in limiting pathologic immune responses in peripheral tissues [138]. The specific functions of PD-L2 are less clear, as PD-L2-deficient mice have variably been reported to have enhanced [138] or impaired T cell responses [139].

PD-L2 (B7-DC): *PD-L1 with expression restricted to APCs and certain hematopoietic malignancies. PD-1:PD-L2 interactions appear to restrain effector T cell function within lymphoid organs.*

Intracellular signaling

PD-1-mediated signals inhibit T lymphocyte glucose consumption, cytokine production, proliferation, and survival [129] (Fig. 2). Upon TCR stimulation, PD-1 undergoes phosphorylation of the tyrosine residues in the ITIM and ITSM motifs of the cytoplasmic tail, allowing recruitment of the phosphatases SHP-1 and SHP-2, which in turn, dephosphorylate proximal signaling molecules downstream of the TCR and CD28 [94, 132, 137]. Positional mutagenesis studies demonstrated that the ITSM motif is necessary for the inhibitory function of PD-1 [125, 126]. Moreover, PD-1 ligation and recruitment to the immune synapse appear to be necessary to mediate the inhibitory effects on proximal TCR signaling [126, 140].

CTLA-4 and PD-1 inhibit T cell activation of Akt and thereby, block CD28-mediated induction of glucose uptake; however, the level of the blockade is distinct [94]. PD-1-mediated recruitment of SHP-2 blocks proximal activation of PI3K, which prevents activation of Akt. In contrast, CTLA-4 ligation does not affect PI3K activation but instead, blocks Akt induction downstream of PI3K, in part, through the activity of PP2A. PD-1-mediated inhibition of PI3K prevents induction of Bcl-xL, thus potentially explaining the ability of PD-1 ligation to lead to apoptosis in some settings [94, 141]. In addition, suppression of IL-2 production may sensitize PD-1-expressing lymphocytes to cell death. PD-1-mediated inhibition of PKCθ and ERK pathway activation appear to be particularly important for suppression of IL-2 production [142, 143]. Of

note, exogenous IL-2 can over-ride PD-1 inhibitory signals [144, 145].

SHP-2: *Intracellular tyrosine phosphatase that is recruited to the cytoplasmic tail of PD-1, inhibiting T cell function by preventing activation of PI3K.*

Thus, partially overlapping but distinct intracellular signaling pathways are activated by ligation of CTLA-4 and PD-1. The more proximal blockade of TCR/CD28 by PD-1 is consistent with the more restrictive gene expression profile exhibited by activated T cells following ligation of PD-1 compared with ligation of CTLA-4 [94, 98]. The distinct mechanisms of inhibitory signaling by CTLA-4 and PD-1 suggest that combined inhibition of these pathways may result in therapeutic synergy.

Role on Tregs

In addition to its well-established role as a cell-intrinsic inhibitor of conventional T cell activation and function, PD-1 has been demonstrated to regulate the development, maintenance, and function of Tregs. Specifically, it was shown that PD-L1 expression by APCs plays a critical role in inducing differentiation of Tregs and maintaining their suppressive function [146]. Given the prominent expression of PD-L1 by tumors and the established role of Tregs as inhibitors of tumor-specific immune responses, PD-1:PD-L1-mediated generation of Tregs within the tumor microenvironment may represent an additional mechanism of immune evasion (Fig. 1). Thus, therapeutic blockade of PD-1 might mediate its effects via simultaneous disinhibition of effector T cells and inhibition of Treg development and function.

Clinical Question: *How does antagonism of PD-1 function on Tregs contribute to the anti-tumor activities of PD-1 blocking agents in humans?*

PD-1 blockade for cancer immunotherapy

The expression of PD-1 by tumor-infiltrating lymphocytes [147, 148] along with the expression of PD-L1 by numerous tumor types [149] suggest that the pathway might be involved in immune evasion by human cancers (Fig. 1). In particular, the broad expression pattern of PD-L1 on normal, nonhematopoietic cells is mirrored by PD-L1 expression on carcinomas of the lung, breast, colon, kidney, bladder, ovary, and cervix, as well as nonepithelial tumors, including melanoma, glioblastoma, multiple myeloma, T cell lymphoma, and various leukemias [150–155]. In addition, PD-L1 expression by myeloid cells within certain tumors correlates with impaired anti-tumor immunity [156, 157]. The prognostic significance of PD-L1 expression by tumors has been variable, correlating with inferior outcomes in some cases [151, 158–160] and superior outcomes in others [161]. In contrast to PD-L1, which exhibits aberrant expression in many tumors of epithelial origin, PD-L2 is highly expressed by a number of lymphoid malignancies [162], likely as a result of amplification of the chromosomal region encoding PD-L2 [163].

Clinical Question: *Does PD-L1 or PD-L2 expression on tumor cells predict efficacy of agents targeting the PD-1 pathway in cancer patients? This remains controversial.*

Various signaling mechanisms induce PD-L1 expression on malignant cells. Human glioblastoma specimens demonstrate increased expression of PD-L1 that is dependent on loss of phosphatase and tensin homolog and activation of the PI3K pathway [164]. Likewise, in ALK-positive T cell lymphoma, an ALK/STAT3 signaling pathway results in induction of high-level PD-L1 expression [165]. Expression of PD-L1 by multiple myeloma cells is induced by signaling via MEK/ERK/STAT1 and MYD88/TRAF6 pathways [153]. In numerous carcinomas, PD-L1 expression is induced and sustained by signaling through the IFN-γ/JAK2/IFN regulatory factor 1 pathway [150, 166]. Recent evidence demonstrates that tumor-infiltrating lymphocytes can directly induce PD-L1 expression on tumor cells via production of IFN-γ [161]. This feedback-inhibitory loop is analogous to that observed in chronic viral infection, whereby inflammatory cytokines produced by infiltrating T cells may induce tissue expression of PD-L1 to inhibit T cell function and limit tissue damage [121]. Thus, PD-L1 expression by tumors can result from activation of common oncogenic pathways or exposure to inflammatory cytokine produced by infiltrating immune cells.

Experimental manipulation of the PD-1:PD-L1 pathway confirms its importance in immune evasion by tumors. Overexpression of PD-L1 on a mouse mastocytoma tumor inhibits tumor-directed T cell cytotoxicity in vitro and promotes immune evasion leading to enhanced growth of the tumor in vivo [150, 154]. These effects are abrogated by antibody-mediated blockade of PD-L1, which promotes immune-mediated tumor rejection. Likewise, blockade of PD-L1 promotes immune-mediated destruction of tumors that naturally express PD-L1, including myeloma, melanoma, and mammary carcinoma [150, 167, 168]. In some settings, PD-L1 expression by the tumor promotes apoptosis of tumor-specific T cells [150], whereas in other systems, tumor expression of PD-L1 inhibits tumor cell lysis by cytotoxic T cells in the absence of apoptosis or anergy [167].

Of note, the effects of PD-1 blockade as an anti-cancer therapeutic may be mediated partially by immune cells other than T cells, including NK cells or B cells. Tumor-mediated production of IL-18 enhances PD-1 expression and inhibits the function of NK cells, another type of immune cell important for anti-tumor immunity [169]. Early studies demonstrated that BCR signaling induces PD-1 expression, which negatively regulates B cell proliferation and function [125, 170]. In fact, the lupus-like disease and dilated cardiomyopathy observed in PD-1-deficient mice may be, at least in part, a result of aberrant B cell function, given the predominant, antibody-mediated pathology [117, 118]. Blockade of PD-1 in chronic SIV infection enhanced proliferation of memory B cells and boosted anti-SIV antibody titers [171]. Thus, restoration of NK cell and B cell function might also play a role in the anti-tumor activity of PD-1 blockade. Although, the effects of PD-1 blockade on im-

TABLE 2. Summary of questions for future clinical-translational research

- Do CTLA-4 splice variants impair productive anti-tumor immune responses in humans? Does CTLA-4 blockade mediate its anti-tumor effects, in part, by counteracting the functions of CTLA-4 splice variants?
- What are the relative contributions of effector and memory $CD4^+$ and $CD8^+$ T cells to the anti-tumor activity of CTLA-4 blockade in humans?
- Do CTLA-4 blocking antibodies undergo endocytosis and lysosomal degradation? If so, would inhibition of these processes enhance the anti-tumor efficacy of CTLA-4 blockade?
- Could pharmacologic inhibition of intracellular signaling molecules downstream of CTLA-4, such as PP2A, enhance and/or recapitulate the anti-tumor effects of CTLA-4 blockade in humans?
- To what degree does antagonism of the CTLA-4 function on Tregs contribute to the anti-tumor effects of CTLA-4 blocking agents in humans?
- What are the optimal strategies for enhancing tumor antigen delivery to synergize with CTLA-4 blockade as cancer immunotherapy, e.g., vaccination, cytokine delivery, radiation, cryoablation, or chemotherapy?
- PD-L1 interacts with B7-1 and PD-1. Does this predict distinct clinical responses to PD-1 versus PD-L1 blockade as cancer immunotherapy?
- How does antagonism of the PD-1 function on Tregs contribute to the anti-tumor activities of PD-1 blocking agents in humans?
- Does PD-L1 or PD-L2 expression on tumor cells predict efficacy of agents targeting the PD-1 pathway in cancer patients? This remains controversial.
- What are the relative contributions of T cells, B cells, NK cells, NKT cells, DCs, and macrophages to the anti-cancer activity of PD-1 blockade, as PD-1 is expressed on all of these types of immune cells?
- Will combined blockade of CTLA-4 and PD-1 yield synergistic anti-tumor immunity, or will this strategy be limited by synergistic toxicity?

mune cells other than T cells are less well-studied, they may be relevant to manipulating the PD-1 pathway therapeutically.

> **Clinical Question:** *What are the relative contributions of T cells, B cells, NK cells, NKT cells, DCs, and macrophages to the anti-cancer activity of PD-1 blockade, as PD-1 is expressed on all of these types of immune cells?*

Importantly, there were no significant adverse, immune-related events reported in preclinical models of PD-1 or PD-L1 blockade. The lack of immune-mediated pathology is consistent with the lack of systemic autoimmunity observed in PD-1-deficient mice compared with CTLA-4-deficient mice. Nonetheless, the preclinical models predict that PD-1 or PD-L1 blockade could exacerbate pre-existing autoimmune disease or lead to organ damage in the setting of chronic infections.

Summary of PD-1 function

PD-1 can be conceived of as a dampener of effector T lymphocyte function in nonlymphoid organs. Genetic evidence indicates that in a healthy host, PD-1 does not play a critical role in regulating immune homeostasis or preventing widespread autoimmune pathology. However, there may be specific organs where PD-1 exerts inhibitory effects on self-reactive lymphocytes, as evidenced by the strain-specific autoimmunity observed in PD-1-deficient mice. In contrast to the healthy host, PD-1 plays a vital role in limiting immune-mediated tissue destruction at sites of ongoing infection or inflammation. The inflammatory environment induces expression of PD-L1 on involved tissues, which provides feedback inhibition via the PD-1 receptor and reduced responsiveness to antigen stimulation by lymphocytes at the site of inflammation. PD-L1 expression by inflamed tissues also promotes the differentiation of Tregs and maintenance of their suppressive function, thus providing an additional mechanism of cell-extrinsic inhibition of effector T cell function.

Tumors appear to use the PD-1 axis in a similar fashion to inflamed tissues, thereby shielding themselves from immune-mediated destruction. Tumor cells can activate expression of PD-L1 via common oncogenic signaling pathways. Alternatively, tumor-infiltrating lymphocytes can thwart their own efforts by producing inflammatory cytokines that induce PD-L1 on tumor cells and myeloid cells within the tumor. PD-1:PD-L1 interactions can transmit cell-intrinsic inhibitory signals that block tumor-directed cytolysis and promote T cell apoptosis. Moreover, PD-L1 expression by tumor cells promotes the development of suppressive function of Tregs, which offer an additional layer of protection to the tumor. Therapeutic blockade of PD-1 or PD-L1 might break these multiple layers of immune inhibition, allowing effective anti-tumor T cell responses that could additionally be assisted by enhanced B cell or NK cell function.

> **Clinical Question:** *Will combined blockade of CTLA-4 and PD-1 yield synergistic anti-tumor immunity, or will this strategy be limited by synergistic toxicity?*

CONCLUDING REMARKS

The mechanisms whereby CTLA-4 and PD-1 exert their inhibitory effects on T cell activation are multifaceted, including cell-intrinsic and cell-extrinsic mechanisms. CTLA-4 functions primarily to limit T cell activation and clonal expansion, whereas PD-1 functions primarily to limit effector T cell function in the peripheral tissues. Their distinct molecular structures, spatiotemporal regulation, intracellular signaling pathways, ligand distribution, and function on Tregs and other immune cells suggest that combined therapeutic blockade of CTLA-4 and PD-1 could synergize to mediate robust anti-tumor immunity. On the other hand, combined therapeutic

blockade of CTLA-4 and PD-1 might also result in synergistic immune-mediated toxicity.

Therapeutic synergy could potentially result from blockade of CTLA-4 and PD-1 on the same cell or different cells. CTLA-4 blockade might lower the threshold of activation, thereby allowing recruitment and activation of a low-affinity, tumor-specific T cell. The tumor-specific T cell would undergo enhanced clonal expansion as a result of blockade of CTLA-4-mediated attenuation of the proliferative response. Upon activation and clonal expansion, the tumor-specific T cell and its progeny would induce expression of PD-1. The tumor-specific T cell would then migrate to the site of the tumor, where PD-L1 might be highly expressed. Blockade of PD-1 interactions would allow the tumor-specific T cell to unleash its full armamentarium of effector function on the tumor cells. Alternatively, PD-1 blockade might function predominantly to restore the effector functions of pre-existing tumor-infiltrating lymphocytes within the tumor microenvironment that have been rendered impotent by PD-L1 expression on the tumor cells. In addition, CTLA-4 and PD-1 blockade would be expected to synergize in removing the cell-extrinsic, inhibitory effects of Tregs within the tumor microenvironment.

Initial preclinical models of combined CTLA-4 and PD-1 blockade have demonstrated promising results [172–174]. In the B16 model of melanoma, a Fms-like tyrosine kinase 3-based tumor cell vaccine was combined with blocking antibodies directed against CTLA-4, PD-1, and PD-L1 [172]. CTLA-4 blockade alone resulted in rejection of 10% of tumors, whereas PD-1 blockade alone resulted in rejection of 25% of tumors. However, mice treated with a combination of CTLA-4 plus PD-1 blockade rejected 50% of tumors, with a further increase to 65% tumor rejection when CTLA-4, PD-1, and PD-L1 were targeted simultaneously. Combined blockade induced highly favorable effector T cell:Treg ratios within the tumors and resulted in a significantly reduced proportion of suppressive myeloid cells. Interestingly, the tumor-infiltrating T lymphocytes, present in the setting of combined CTLA-4/ PD-1/PD-L1 blockade, exhibited significant coexpression of CTLA-4 and PD-1, thus implying that combined blockade of these pathways was unleashing the activity of tumor-specific T cells that would have otherwise been suppressed. Moreover, this observation suggests that therapeutic synergy results from combined blockade of CTLA-4 and PD-1 on the same tumor-specific T cell. A significant increase in toxicity from combined blockade was not reported.

It will be exciting to follow up these promising preclinical observations with combined blockade of CTLA-4 and PD-1/ PD-L1 in human subjects, particularly in the setting of advanced melanoma. However, the safety and feasibility of combined blockade will need to be investigated rigorously, given the poor correlation between adverse immune-related events in preclinical models and those observed in patients. In addition to CTLA-4 and PD-1, numerous other immune inhibitory receptors and ligands are being investigated as potential targets for cancer immunotherapy, including B and T lymphocyte attenuator, lymphocyte activation gene 3, and T cell membrane protein 3 [175]. The potential for combined blockade of immune checkpoints offers new hope for cancer patients. A sound understanding of the cellular and molecular mechanisms whereby these immune modulators function will be essential to guide their optimal use in the clinic (**Table 2**).

AUTHORSHIP

A.M.I. and C.B.T. wrote the review.

ACKNOWLEDGMENTS

The Thompson laboratory is supported by grants from the U.S. National Institutes of Health and National Cancer Institute. The authors thank Jedd Wolchok, Maggie Callahan, and members of the Thompson laboratory for helpful discussions.

DISCLOSURES

The authors declare no conflict of interest.

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KEY WORDS:
tumor · receptor · inhibitory · coinhibitory · costimulatory · costimulation · PD-L1 · ipilimumab