CANCER IMMUNOLOGY

Inhibitory immune checkpoints in cancer immunotherapy

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Monoclonal antibodies and other agents that inactivate immune checkpoints like PD-1 and CTLA-4 have been effective against only certain types of cancer and have had highly variable efficacy in patients. These limitations have hastened investigations of additional checkpoints that can serve as therapeutic targets. Nevertheless, no other approach has yet reached the effectiveness of PD-1 and CTLA-4 inactivation. Recent studies have shown that experimental inhibitory immune checkpoints and the drugs targeting them display unexpected or undesirable mechanisms of action or regulation, thus highlighting previously underappreciated complexities of immune checkpoint–based therapies. Understanding these nuances is crucial for developing more effective and safer therapies. This Review explores the intricacies surrounding inhibitory immune checkpoints and offers insights for improved therapeutic strategies in the future.

INTRODUCTION

The body uses a complex array of mechanisms to regulate the development, differentiation, proliferation, and effector functions of immune cells (*1*, *2*). This regulation relies in part on balancing the interactions between activating and inhibitory receptors, the so-called "immune checkpoints," which ensure that the immune response is sufficient to defend against pathogens and cancer cells, while avoiding autoimmunity and inflammation (*1*–*4*). In general, a dominant activating receptor response leads to immune cell activation, whereas a shift toward inhibitory receptor engagement limits immune cell activation (Fig. 1).

Therapies targeting inhibitory immune checkpoints, such as monoclonal antibodies (mAbs), fusion proteins, or small molecules, have transformed the landscape of cancer treatment over the past 2 decades by harnessing the body's immune system, in particular T cells, to destroy malignant cells (*5*, *6*). The leading therapeutics have functioned as antagonizing molecules that target programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4). They have demonstrated remarkable clinical efficacy against cancers such as melanoma and renal cell carcinoma and have become standard parts of the anticancer armamentarium.

However, not all cancer types react to targeting of these checkpoints, and not all patients with a susceptible cancer type show a response (*7*–*9*). This drawback has accelerated attempts at identifying "second-generation" inhibitory checkpoints that can be targeted for cancer immunotherapy (*7*, *10*). Nonetheless, clinical studies targeting these alternative checkpoints have generally been disappointing because of insufficient patient benefit or high toxicity (*11*–*13*).

Traditional views on inhibitory checkpoints: PD-1 and CTLA-4 in T cells

Much work has been done toward understanding the biology of PD-1 and CTLA-4, which were the "first-generation" inhibitory immune checkpoints (Table 1). These receptors are broadly expressed at the surface of activated T cells, including cytotoxic $CD8^+$ T cells involved in antitumor immunity (*14*, *15*). They were noted to be more highly expressed on "exhausted" $CD8^+$ T cells, suggesting a possible role in the T cell exhaustion seen in many patients with cancer (*14*, *15*). The ligands of PD-1 were identified as programmed death-ligand 1 (PD-L1) and PD-L2, whereas those of CTLA-4 were shown to be the B7 molecules CD80 and CD86, all of which can be highly expressed on tumor cells or other cells in the tumor environment (*9*). PD-L1 is more broadly expressed in tumors compared with PD-L2, and functional and biochemical studies showed that engagement of PD-1 by PD-L1 suppressed the activating signals induced by engagement of the T cell antigen receptor (TCR) and the costimulatory receptor CD28, thereby preventing T cell activation (*16*, *17*). Triggering of CTLA-4 by its ligands also inhibited T cell activation because of competition of CTLA-4 with CD28 for binding to B7 molecules, which have a much higher affinity for CTLA-4 compared with CD28 (*18*).

On the basis of these characterizations, agents targeting PD-1 and CTLA-4 were developed for cancer immunotherapy and have shown compelling clinical effectiveness, including apparent cures (*19*, *20*) (Table 1). These drugs have received regulatory approval in multiple jurisdictions for a range of cancer types (*10*). For instance, agents inactivating PD-1 or PD-L1, including PD-1 mAbs nivolumab and pembrolizumab, as well as the PD-L1 mAb atezolizumab, have effectiveness against melanoma, renal cell carcinoma, non–small cell lung cancer, and head and neck squamous cell carcinoma, among others (*21*). Conversely, drugs targeting CTLA-4, such as the mAb ipilimumab, have activity against melanoma, renal cell carcinoma, hepatocellular carcinoma, colorectal carcinoma, and esophageal carcinoma (*22*).

Looking for additional inhibitory immune checkpoints to target

The somewhat restricted efficacy of agents targeting PD-1 or CTLA-4 has led to the accelerated preclinical and clinical evaluations of other inhibitory checkpoints expressed on $CD8^+$ T cells, including lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin (Ig) and mucin-domain containing-3 (TIM-3), and T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (TIGIT) (*23*–*30*) (Table 1). These second-generation checkpoints have a demonstrated capacity to inhibit T cell activation in vitro,

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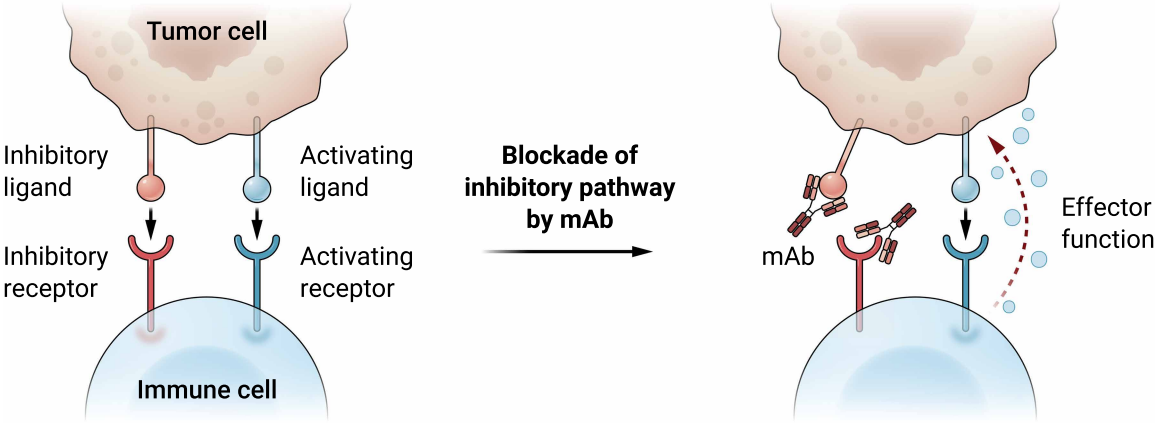

Fig. 1. Conventional model of immune checkpoint functions in cancer immunotherapy. The functions of immune cells are controlled by inhibitory and activating immune checkpoint receptors. When agents such as PD-1, PD-L1, and CTLA-4 mAbs block the interaction of inhibitory receptors with their ligands, the functions and signals induced by activating receptors are increased to eliminate tumor cells. Release of granules by cytotoxic lymphocytes is shown here as an example of effector function.

and their targeting has resulted in encouraging preclinical data in mice (*24*, *31*). They have also generally shown a good safety profile in phase 1 clinical trials. Thus far, drugs targeting these checkpoints have not broadly succeeded in advanced clinical trials (*32*). Only the LAG-3 mAb relatlimab has achieved regulatory approval, in the very specific case of patients with melanoma, and in combination with the PD-1 mAb nivolumab (*33*) (Table 1). Another LAG-3 mAb, favezelimab, did not meet its primary end point in a phase 3 clinical study (NCT05064059).

The limited success of targeting second-generation T cell checkpoints has led to a renewed appreciation that other immune cells beyond $CD8^+$ T cells, such as natural killer (NK) cells, macrophages, and neutrophils, may have inhibitory checkpoints that could also be targets for cancer immunotherapy (*34*–*40*). NK cells mediate cytotoxicity, including antibody-dependent cellular cytotoxicity (ADCC), upon engagement of the Fc receptor (FcR) FcγRIII (CD16) by the Fc portion of mAbs, which are bound to antigens on tumor cells (*41*). Favorable results have been obtained in preclinical and phase 1 clinical trials by targeting the NK cell inhibitory receptors NK group 2 member A (NKG2A) and TIGIT, although evidence of efficacy has not been or has yet to be confirmed in phase 3 clinical trials (*42*, *43*) (Table 1). These checkpoints are also expressed on $CD8^+$ T cells, suggesting a dual mechanism of action involving NK cells and $CD8^+$ T cells (*44*).

One caveat of targeting inhibitory checkpoints on NK cells is that these receptors, including NKG2A and TIGIT, not only inhibit NK cell activation when engaged by their ligands on cancer cells but also promote a developmental process known as NK cell education, which enhances NK cell activation when cancer cells lack the ligands for the inhibitory receptors (*45*, *46*). Thus, inhibitory receptors such as NKG2A and TIGIT can both inhibit and promote NK cell activation, and this duality of effects may lead to reduced efficacy during their therapeutic targeting.

Macrophages and neutrophils have the capacity to engulf and destroy cancer cells (*35*, *37*, *47*, *48*). Macrophages can incorporate entire tumor cells through phagocytosis, including antibody-dependent cellular phagocytosis (ADCP), after engagement of the Fc receptor FcγRI (CD64) by the Fc segment of mAbs directed against tumor cell antigens, whereas neutrophils are involved in a process called trogocytosis induced by FcγRI engagement, whereby they nibble off small fragments of tumor cells until cell death occurs (*48*, *49*). These processes are suppressed by inhibitory receptors such as signal-regulatory protein α (SIRPα), which recognizes the ligand CD47, which is often overexpressed on tumor cells (*50*–*52*) (Table 1). Promising data with SIRPα-CD47 blockade have been obtained in preclinical studies and initial phase 1 and 2 clinical trials against cancers such as lymphomas, although more recent studies have been less encouraging (*53*–*55*).

These disappointing results with second-generation checkpoints may be due to a multitude of factors, including low patient numbers or poor patient selection in the clinical trials. A disproportionate number of patients may lack relevant immune cells in the tumor microenvironment, for instance having "cold" tumors that are less susceptible to immunotherapy, in particular solid tumors. In other cases, patients may exhibit excessive toxicities, especially heavily pretreated individuals, who are usually the focus of phase 1 clinical trials.

However, the lackluster outcomes may also reflect a lack of sufficient insights into how these checkpoints and the drugs that target them operate. Often, the expression patterns, ligands, signaling mechanisms, and functions of second-generation checkpoints have not been well established before initiation of clinical trials. Likewise, therapeutic agents may be used without a full understanding of how they work.

Recent learnings about inhibitory immune checkpoints

Our understanding of how inhibitory checkpoints work has evolved markedly in recent years, especially regarding the first-generation T cell checkpoints PD-1 and CTLA-4. In addition, analyses of the second-generation checkpoint SIRPα have provided valuable insights about the function of inhibitory checkpoints in innate immune cells. Collectively, the concepts derived from these studies likely apply to other, if not all, inhibitory immune checkpoints, including the second-generation checkpoint LAG-3, against which at least one mAb has achieved regulatory approval.

Inhibitory checkpoints have multiple molecular and cellular mechanisms of action

The mechanisms of action of PD-1, CTLA-4, and SIRPα are much more convoluted than previously believed. In all three cases, multiple molecular pathways and cell types are involved.

Table 1. Mechanisms and clinical contexts of representative immune checkpoints. Note: Many recent clinical trial results for immune checkpoint inhibitors are currently available only on official trial registries or company websites. MHCII, major histocompatibility complex II; FGL-1, fibrinogen-like protein 1; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; HLA-E, human leukocyte antigen-E; DNAM-1, DNAX accessory molecule-1.

Receptor	Proposed ligands	Ligand validation	Cytoplasmic signaling motifs	Mechanism(s) of action	Representative agents (and most advanced status)	Success in phase 3 clinical trials	Status	Refs
PD-1	PD-L1, PD-L2	Strong	ITIM, ITSM, other	Inhibition of T cell activation	Pembrolizumab (approved), nivolumab (approved), atezolizumab (approved)	Yes	Approved	(*9*, *16*, *17*, *60*, *61*, *66*, *99*)
CTLA-4	B7–1, B7–2	Strong	ITIM(?)	Competition with CD28; inhibition of T cell activation (?)	Ipilimumab (approved)	Yes	Approved	(*9*, *18*, *19*, *78*, *79*, *101*)
LAG-3	MHCII, galectin-3, FGL-1, others	Partial	KIEELE motif	Competition with CD4; inhibition of T cell activation (?)	Relatlimab (approved), fazelelimab (phase 3)	Yes	Limited approval	(*23*, *24*, *33*)
TIM-3	Galectin-9, CEACAM-1, others	Partial	Tyrosines	Poorly understood	Sabatolimab (phase 3), cobolimab (phase 3)	No	Some programs terminated; others ongoing	(*24*, *29*, *30*, *137*)
NKG2A	HLA-E	Strong	ITIM	Inhibition of T cell and NK cell activation; NK cell education (increased NK cell functions)	Monalizumab (phase 3)	No	Some trials ended; others ongoing	(*42*, *44*, *46*, *138*, *139*)
TIGIT	CD155, CD112, CD113	Strong	ITIM	Competition with DNAM-1; inhibition of T cell and NK cell activation; NK cell education (increased NK cell functions)	Tiragolumab (phase 3), belrestotug (phase 3)	No	Some programs terminated; others ongoing	(*24*, *25*, *32*, *41*, *43*)
SIRPα	CD47	Strong	ITIM	Inhibition of macrophage activation	Magrolimab (phase 3), ontorpacept (phase 2), evorpacept (phase 3), CC-95251 (phase 1)	No	Some programs terminated; others ongoing	(*35*, *52–54*, *89*, *90*, *102*, *109*, *111*, *112*, *140–142*)

PD-1

It has been reported that, upon ligand engagement, PD-1 recruits the Src homology 2 (SH2) protein tyrosine phosphatases (SHPs) SHP-2 and SHP-1, or both, via its cytoplasmic ITIM (*16*, *17*). These phosphatases are suppressors of T cell activation. In addition, a critical role for another cytoplasmic motif, the immunoreceptor tyrosine-based switch motif (ITSM), has also been described, although its exact mechanism of action is not known (*56*). In other receptors, such as signaling lymphocytic activation molecule (SLAM) family receptors, ITSMs can couple to inhibitory SH2 domain–containing molecules like SHP-1 and the lipid phosphatase SHIP-1 (*57*–*59*). PD-1 was also reported to inhibit T cell activation independently of these signaling motifs, but the precise mode of action was again not clarified (*60*–*62*).

Although PD-1 is often depicted as a monomer, a recent report revealed that PD-1 formed homodimers, by way of the PD-1 transmembrane domain (*60*). Prevention of homodimerization reduced the inhibitory function of PD-1, suggesting that dimerization is critical for PD-1 function and may be a useful target to inactivate PD-1. Other inhibitory checkpoints, including CTLA-4 and SIRPα, also formed homodimers that may be critical for their function, although the precise molecular mechanisms involved were not clarified (*63*, *64*). Agents that both block receptor-ligand interactions and prevent dimerization may be most efficient at interfering with the function of inhibitory checkpoints.

In addition to having a more complex molecular mechanism of action than initially believed, the T cell populations mediating the activity of PD-1 have been the topic of extensive debate, as reviewed by Patsoukis *et al.* (*65*). $CD8^+$ T cells are likely a key effector. Given that PD-1 is not expressed on naïve T cells, this latter subset is unlikely to be involved. However, PD-1 is present on effector (T_{eff}), memory (T_m), exhausted (T_{ex}), and on the more recently identified stem-like or precursors of exhausted (T_{pex}) $CD8^+$ T cells (*66*). Various studies have shown that the function of exhausted $CD8^+$ T cells could not be rescued by PD-1 inactivation, because their commitment to the exhausted phenotype may be irreversible (*67*, *68*). Rather, compelling data have indicated that PD-1 targeting can act by enhancing the functions of T_{eff} and T_{pex} cells, which can lead to the generation of new pools of activated tumor-specific $CD8^+$ T cells (*69*, *70*).

PD-1 is also expressed on NK cells, innate-like lymphoid cells–2 (ILC2s), tumor-associated macrophages (TAMs), B cells, and some dendritic cells (DCs) (*71*–*75*). Interfering with the function of PD-1 in NK cells, ILC2s, or TAMs was reported to augment innate cell–mediated cytotoxicity and, secondarily, to facilitate the initiation of antitumor T cell responses (*71*, *75*). However, a recent study found that blockade of the PD-1–PD-L1 axis impaired antibody production by B cells, an effect that may be detrimental to antitumor immunity (*76*).

CTLA-4

CTLA-4 has a much greater affinity for B7 molecules than CD28 and, thus, is likely to operate by a competitive mechanism toward CD28. Nevertheless, CTLA-4 may also mediate inhibitory intracellular signals by recruiting the cytoplasmic phosphatases SHP-2 and protein phosphatase 2A (PP2A), which can inhibit TCR signaling (*77*–*79*). The relevance of these additional mechanisms to CTLA-4–mediated inhibition is unclear.

The precise T cell population(s) involved in the impact of CTLA-4 has also been studied. CTLA-4 is not expressed at the surface of naïve T cells, being instead found in intracellular compartments (*80*, *81*). After T cell activation, CTLA-4 is transported to the cell surface and becomes detectable on T_{eff}, T_m, T_{pex}, and T_{ex} $CD8^+$ T cells (*80*, *81*). Recent studies have shown that CTLA-4 mAbs enabled expansion of T_{pex} cells (*82*, *83*). CTLA-4 is also constitutively expressed at high levels on regulatory T (T_{reg}) cells and is required for their immune suppressive activity (*79*). There is solid evidence that a large part of the therapeutic effects of CTLA-4 mAbs is mediated by T_{reg} functional suppression or elimination (*84*).

SIRPα

In its cytoplasmic domain, SIRPα has ITIMs that can recruit SHP-1 and SHP-2, which would presumably suppress phagocytosis upon engagement of SIRPα by CD47 (*85*–*92*). Recent data indicated that SIRPα also used a CD47-independent mechanism to suppress antitumor immunity (*86*, *93*). The nature of this mechanism, which was shown to implicate binding of SIRPα to the integrin CD18 on macrophages, is discussed below.

In addition to macrophages, other innate immune cell types express SIRPα, namely, neutrophils, DCs, and NK cells, which may also be implicated in the impact of SIRPα-CD47 targeting. It was reported that inactivation of SIRPα-CD47 augmented the capacity of DCs to present tumor antigens to T cells (*94*, *95*). Conversely, in neutrophils, targeting of SIRPα-CD47 augmented trogocytosis of tumor cells, whereas, in NK cells, it enhanced NK cell–mediated cytotoxicity (*51*, *96*, *97*).

The growing appreciation of the complexity by which PD-1, CTLA-4, and SIRPα operate has highlighted the importance of exploring better how inhibitory checkpoints function. Blockade of all known and yet unknown mechanisms of inhibition by an inhibitory immune checkpoint may be needed for maximal therapeutic purposes, although it may also result in greater toxicities. As will now be discussed, mechanistic complexity also applies to the drugs targeting inhibitory checkpoints.

Drugs targeting inhibitory checkpoints have multiple mechanisms of action

Most therapeutic agents, whether they are mAbs, fusion proteins, or small molecules, are antagonists that block receptor-ligand interactions (Fig. 2A). This activity usually occurs because of direct binding to the interface involved in these interactions. It can also arise indirectly, because of conformational modification of the binding surface. Several mAbs are also efficient at inducing internalization of their molecular target, thereby preventing receptor expression at the cell surface and subsequent interactions with ligands. Bivalent mAbs and fusion proteins are particularly efficient at inducing internalization compared with monovalent agents (*98*). As highlighted, therapeutic agents can also interfere with receptor dimerization, a process needed for checkpoint function (*60*).

mAbs and Fc fusion proteins can also act by an "effector" mechanism, which is initiated through binding of their Fc domain to FcRs on the surface of innate immune cells, thus leading to their activation (Fig. 2B). The PD-L1 mAb avelumab is an IgG1 capable of strong FcR binding (*99*) and can induce ADCC and ADCP, which promote elimination of PD-L1–positive tumor cells (*100*). Likewise, the CTLA-4 mAb ipilimumab is another IgG1 that engages FcRs to deplete CTLA-4–expressing T_{reg} cells (*101*). Fc domains can also elicit complement-directed cytotoxicity (CDC), although this mechanism may not be widely used by mAbs targeting immune checkpoints.

A critical attribute of the activity of CD47 mAb magrolimab (an IgG4) and SIRPα-Fc fusion protein ontorpacept (an IgG1; also known as TTI-621) is the ability to engage FcRs and trigger ADCP by

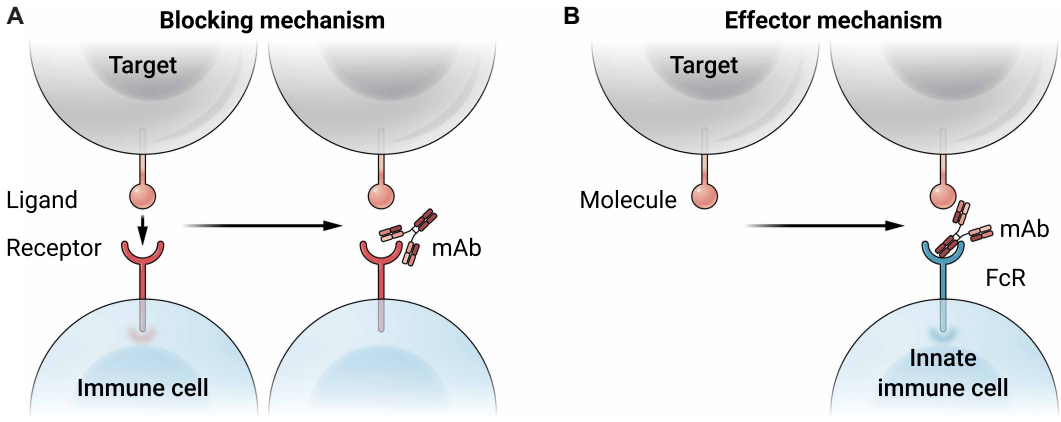

Fig. 2. mAbs can act by different mechanisms on immune cells. The two major mechanisms of action of therapeutic mAbs targeting inhibitory checkpoints are depicted. (**A**) Blocking mechanism. Most mAbs block the binding in trans of an inhibitory receptor to its ligand, thereby suppressing the function of the inhibitory receptor and promoting immune cell activation. Examples include PD-1 mAbs nivolumab and pembrolizumab. (**B**) Effector mechanism. Some mAbs that bind an antigen expressed on tumor cells or tumor-promoting immune cells trigger activation of innate immune cells by engaging activating FcRs via the mAb Fc segment. Examples include CTLA-4 mAb ipilimumab and PD-L1 mAb avelumab.

macrophages, in addition to blocking the SIRPα-CD47 interaction (*53*, *102*). Although IgG4 is typically weaker at engaging FcRs compared with IgG1, the concomitant blockade of SIRPα-CD47 likely can enhance the ability of these agents to trigger ADCP. Some CD47 mAbs were also reported to promote tumor cell death by a SIRPα-independent mechanism, although the importance of this mechanism in the clinical setting remains to be clarified (*103*, *104*).

Hence, in addition to antagonizing receptor-ligand interactions, either directly or indirectly, agents targeting immune checkpoints can trigger effector functions like ADCC and ADCP. Moreover, they may have direct effects on tumor cells, such as by promoting their death. New therapeutic agents could aim either to be selective in their mechanisms of action or to be multifunctional, depending on whether one wishes to influence all, or avoid some, of the mechanisms by which they can affect an inhibitory immune checkpoint.

Targeting inhibitory checkpoints can lead to avoidable toxicities

As one would anticipate, targeting PD-1 or CTLA-4 can lead to immune-related adverse events (irAEs) (*105*). Meta-analyses indicated that severe (grades 3 and 4) irAEs occurred in ~20% of patients, with risk factors including prior radiation therapy, lung disease, and combination therapies (*105*, *106*). Severe irAEs have been more prevalent with drugs targeting CTLA-4, compared with those targeting PD-1. Conditions such as colitis and rash occurred more frequently with CTLA-4 inhibitors, whereas pneumonitis and vitiligo were more common with PD-1 inhibitors (*106*).

The variations in irAEs between targeting of PD-1 and CTLA-4 are likely due to differences in the mechanisms of action of the checkpoints and their drugs (*9*). Although antagonists of PD-1 and CTLA-4 both augmented conventional T cell activation, targeting of CTLA-4 more prominently affected T_{reg} cells (*9*). Conversely, PD-1 blockade had additional effects on NK cells, ILC2s, macrophages, and B cells, which express PD-1 (*71*, *72*, *74*, *76*). Altered B cell functions during PD-1 blockade may also influence antibody-mediated irAEs (*74*, *107*).

Triggering of FcRs by mAbs or Fc fusion proteins can result in toxicities toward normal cells, especially if these cells highly express the target (*108*). It has been noted that the CD47 mAb magrolimab induced elimination of normal red blood cells and platelets, which abundantly express CD47, in all likelihood via an FcR-dependent mechanism (*47*, *109*). This effect resulted in frequent anemia and thrombocytopenia in clinical trials, and at times, required drug withholding. In some studies, magrolimab also caused lymphopenia or led to depletion of chimeric antigen receptor T (CAR T) cells when used in combination with CAR T cells (*110*). Unlike magrolimab, the SIRPα fusion protein ontorpacept was not associated with anemia; however, it also led to thrombocytopenia (*53*, *111*). The differential effects of magrolimab and ontorpacept on anemia were explained by the aptitude of magrolimab, but not of ontorpacept, to cluster CD47 on red blood cells (*112*).

These FcR-mediated toxicities can be avoided by using Fc-silent agents or by targeting SIRPα, rather than CD47. Nonetheless, targeting SIRPα with Fc-active mAbs led to cases of neutropenia in a phase 1 clinical study, given that neutrophils express SIRPα (*55*). Another report showed that the capacity of magrolimab to deplete CAR T cells was preventable by genetically modifying CD47 in the CAR T cells so that CD47 still engaged SIRPα but was no longer recognized by magrolimab (*110*).

Another consequence of inhibitory checkpoint targeting is hyperprogression diseases (HPDs) (*113*–*115*). HPDs are characterized by accelerated tumor growth upon treatment with inhibitory immune checkpoint drugs (*113*–*115*). They can lead to rapid clinical deterioration and death. HPDs have been reported in association with a variety of immune checkpoint–targeting agents and cancer types. In a meta-analysis, the frequency of HPDs was estimated to be up to ~40% (*114*). Although the mechanistic basis of HPDs is not well known, it may involve T cell anergy or T cell loss as a by-product of excessive T cell activation, or it may be due to hyperstimulation of FcR-expressing innate immune cells leading to creation of an immunosuppressive tumor microenvironment (*113*–*115*).

The increased awareness that agents engaging FcRs have effects beyond receptor-ligand antagonism has helped our understanding of why some adverse effects develop and how they can be prevented by inactivating FcR-binding (*101*, *116*). Although other irAEs such as autoimmunity may not be as fully avoidable, they can be treated by reducing drug dosage or with immunosuppressive drugs such as corticosteroids. They may also be improved by selectively directing the checkpoint-targeting agents to the tumor microenvironment,

for example, by engineering bispecific antibodies that bind dually to immune cells and to tumor cells.

Immune checkpoints are regulated not only in trans but also in cis

Accumulating data indicate that inhibitory immune receptors can interact with ligands not only in trans (i.e., with ligands displayed on another cell) but also in cis (i.e., with ligands present on the same cell) (*117*–*121*). Furthermore, there is evidence that the functional consequences of cis interactions can be opposite of those of trans interactions (*117*–*121*). Last, receptors can interact in cis with molecules that are not their canonical ligands, including ligands for other receptors or other receptors.

An example of cis interactions involves the recently appreciated interplay among PD-1, CTLA-4, and their ligands (*120*–*122*). In addition to the interaction of PD-L1 with PD-1 in trans, it was observed that PD-L1 on tumor cells bound with the ligands of CTLA-4, i.e., the B7 molecules, in cis (*121*, *123*) (Fig. 3A). Mutational analyses and structural data revealed that the PD-L1 sequences implicated in binding to PD-1 and B7 molecules were similar, implying that B7 interfered with the PD-1–PD-L1 interaction and, thereby, prevented the inhibitory function of PD-1 (*121*, *123*). Thus, blocking PD-L1 mAbs both prevented engagement of PD-1 and released PD-L1 from the B7 molecules. The implications of this dual mechanism on therapeutic efficacy remain to be fully clarified.

There are also examples of cis interactions involving inhibitory and activating checkpoints (*119*). A recent report showed that, in addition to binding of CD47 to SIRPα on macrophages, CD47 interacted in cis with the pro-phagocytic ligand SLAMF7 on tumor cells such as multiple myeloma and lymphoma (*119*) (Fig. 3B). This interaction prevented phagocytosis mediated by SLAMF7 (*119*). Because the binding sites on CD47 for SIRPα and SLAMF7 were similar, blocking CD47-targeting agents prevented not only the SIRPα-CD47 trans interaction but also the CD47-SLAMF7 cis interaction. SIRPα can also interact in cis with the integrin CD18 (a component of Mac-1) on macrophages (Fig. 3B). This association was independent of binding to CD47, involved a binding site distinct from that of CD47, and was needed for maximal suppression of phagocytosis (*93*). In preclinical studies, relief of the CD47-SLAMF7 interaction or the SIRPα-CD18 interaction seemed to be critical for the antitumor impact of CD47 mAbs and SIRPα mAbs, respectively. Thus, in

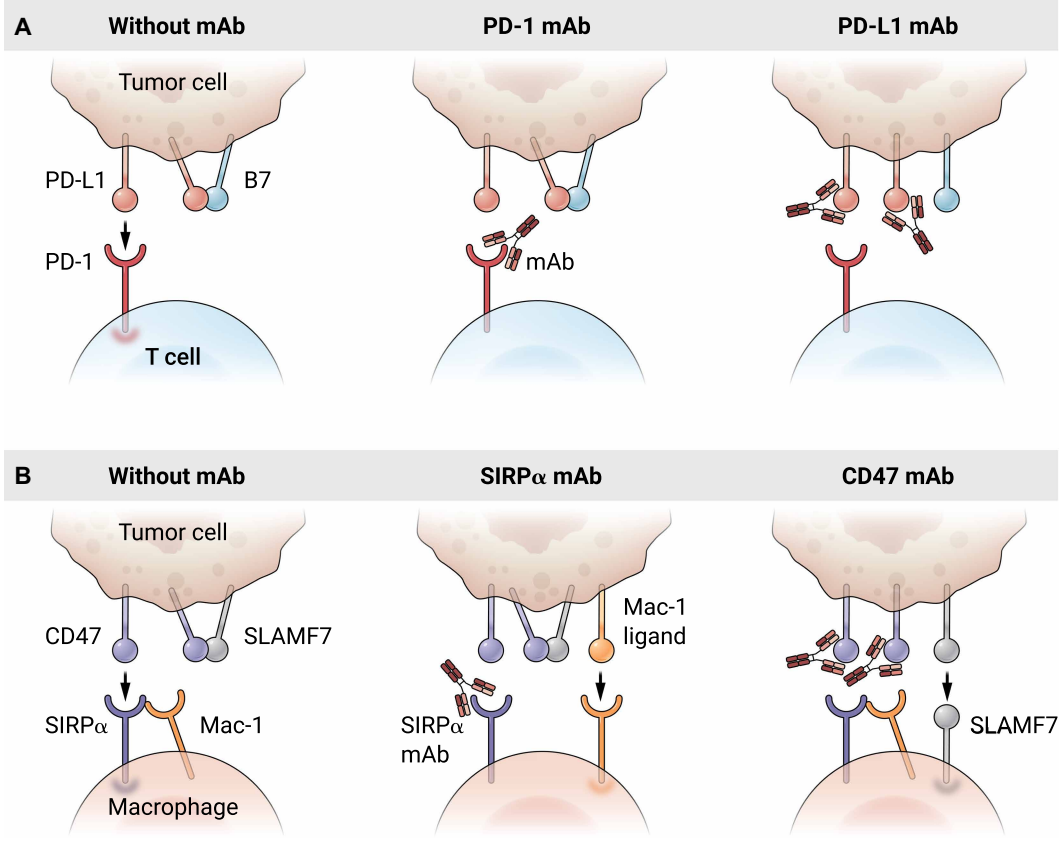

Fig. 3. Updated models of impact of targeting PD-1 and SIRPα pathways. (**A**) In addition to interacting in trans with PD-1 on T cells, PD-L1 on tumor cells interacts in cis with B7 molecules (CD80 and CD86) on tumor cells, thereby preventing the inhibitory function of PD-1. Blocking PD-L1 mAbs, but not PD-1 mAbs, not only disrupt the PD-1–PD-L1 interaction but also release B7 molecules from PD-L1. The impact of this phenomenon on T cell inhibition remains to be clarified. (**B**) Left: In addition to binding in trans to CD47 on tumor cells, SIRPα on macrophages interacts in cis with the pro-phagocytic receptor Mac-1 expressed on macrophages. Mac-1 is the CD11b/CD18 complex. CD11b is the binding subunit for presumed ligands on target cells, whereas CD18 binds in cis to SIRPα on macrophages. This dual mechanism of action augments the inhibitory effect of SIRPα toward phagocytosis. Conversely, CD47 on tumor cells not only binds in trans to SIRPα on macrophages but also interacts in cis with the homotypic pro-phagocytic ligand SLAMF7 on tumor cells, for greater suppression of phagocytosis. Middle: Some of the blocking SIRPα mAbs prevent the SIRPα-CD47 trans interaction and the SIRPα-CD18 cis interaction. They are most efficient at blocking the inhibitory function of SIRPα toward phagocytosis. Right: Blocking CD47 mAbs or SIRPα fusion proteins (not shown) not only prevent the SIRPα-CD47 trans interaction but also release SLAMF7 from CD47 in cis to enable SLAMF7-dependent phagocytosis.

addition to the better-known trans interactions with their canonical ligands, inhibitory checkpoints can be implicated in cis interactions with other ligands and other receptors that need to be considered for optimal therapeutic targeting.

Not all mAbs are created equal

mAbs directed at a cell surface molecule can vary widely in the impact on their target, depending on their binding site, their binding affinity, their capacity to trigger internalization, and their ability to induce, inhibit, or alter signaling (*35*, *99*, *116*). Disparities related to binding epitopes may be especially important in the clinical setting. For example, even though nivolumab and pembrolizumab both target the interface between PD-1 and PD-L1, they do so at nearly nonoverlapping epitopes and with different affinities (*99*). The clinical implications of these distinctions toward efficacy, toxicity, or both may be significant but have yet to be fully assessed (*66*, *99*).

The efficacy of mAbs can also be influenced by sequence polymorphisms in the target (*124*, *125*). SIRPα is highly polymorphic, and two major SIRPα variants exist in the human population: version 1 (V1) and V2 (*119*, *125*). V2 is particularly prevalent in Asian populations. Although both versions exhibit binding to CD47, some SIRPα mAbs bind exclusively to V1, preventing therapeutic effects in V1-negative patients (*119*). The existence of more subtle polymorphisms in other targets may also affect clinical efficacy, a notion that should be seriously assessed.

Because of differences in the epitopes targeted, some but not all SIRPα mAbs also cross-react with SIRPβ and SIRPγ, two other members of the SIRP family (*119*, *126*). SIRPβ is an activating receptor expressed on macrophages that can trigger production of proinflammatory cytokines when engaged by mAbs (*127*). Although SIRPβ does not bind CD47, engagement of SIRPβ by some SIRPα mAbs enhanced antitumor immunity (*119*, *126*). SIRPγ is an activating receptor expressed on T cells that binds CD47, resulting in increased antitumor capacity upon engagement by CD47 (*128*). By blocking the SIRPγ-CD47 interaction, some SIRPα mAbs may cause unwanted dampening of T cell immunity.

mAbs can be modified to enhance efficacy or lower toxicity. These modifications include alterations of the Fc segment to influence binding to FcRs, consequently altering ADCC or ADCP or extending half-lives (*101*, *116*, *129*–*132*). For example, mutation of residues key for FcR-binding, like the so-called "LALAPG" mutation, can fully prevent binding to FcRs and disable ADCC and ADCP. Conversely, removal of fucosylation in the Fc region of mAbs such as the PD-L1 mAb avelumab, using engineered mutations or production in fucosylation-defective cells, enhanced binding to FcRs, thereby augmenting ADCC (*129*). The half-life of mAbs can be extended for greater therapeutic efficacy by enhancing binding to neonatal FcRs, which enable intracellular accumulation and slow extracellular release of mAbs (*133*). In addition, antibodies can be modified for selective targeting to tumor cells, coupling of chemotherapy drugs, or induction of concomitant T cell activation (*134*).

Mice are not very good at predicting outcomes in humans

Although studies of inhibitory checkpoint targeting in mice have provided valuable insights and are an essential component of preclinical testing, they are often not predictive of efficacy in humans (*135*). Multiple agents that were successful at eradicating cancer in mouse models failed in human clinical trials (*24*). The reasons for discrepancies are complex but likely include differences between mouse and humans in the immune system, tumor microenvironment, microbiome, and mAb properties.

There can also be dissimilarities in inhibitory immune checkpoint biology between the two species (*62*). Although there are limited studies about this issue, one recent report showed that mouse PD-1 was less efficient at inhibiting T cell activation compared with human PD-1 (*62*). This difference correlated with mouse PD-1 having a weaker interaction with its ligand and a less efficient capacity to recruit phosphatases compared with human PD-1. This feature may lead to an underappreciation of the therapeutic impact and toxicity of PD-1–PD-L1 targeting in mice.

To mitigate species discrepancies, researchers can conduct studies using multiple surrogate mAbs in the mouse. Another strategy involves the use of "humanized" mice, which are modified to express human molecules instead of their murine counterparts. This can be achieved by creating transgenic mice expressing a human inhibitory checkpoint instead of its mouse equivalent in mouse immune cells or by reconstituting immunodeficient mice with human immune cells and patient-derived tumor xenografts (*136*). Although these approaches hold promise, they are not without their drawbacks. There is a risk that human cells or human molecules present in mice do not interact properly with other mouse cells or with mouse molecular effectors and regulators, respectively, in the same way that they do in fully human settings (*136*).

Testing therapeutic agents in nonhuman primates, such as monkeys, is a viable alternative, especially for evaluating therapeutic safety (*136*). Many therapeutic agents designed for humans can cross-react with their equivalent molecular target in nonhuman primates, because of the evolutionary similarities in immune systems. However, the limited availability of nonhuman primate cancer models, as well as the ethical and logistical challenges associated with using primates in research, are deterrents. Considering the limitations of nonhuman animal models, either rodents or other primates, properly conducted clinical trials in humans remain a necessity.

Second-generation checkpoints need to be better understood

Compared with long-studied checkpoints like PD-1 and CTLA-4, second-generation checkpoints, such as TIM-3 and TIGIT, have not been explored as extensively from a fundamental research point of view (*24*). As shown for PD-1 and CTLA-4, the second-generation checkpoints likely serve diverse functions in different immune cells or stages of the immune response, contributing to more complex functions and regulation than initially thought and potentially leading to unwanted impacts of their therapeutic targeting (*13*, *32*). In this light, one may argue that the limited successes with targeting of second-generation checkpoints in the clinic may have been related in part to premature initiation of clinical trials with drugs that had not been ideally designed.

CONCLUSIONS

Like other molecular pathways, inhibitory immune checkpoints are far more complex than previously appreciated, necessitating a sustained appraisal of how they work and how they can be targeted for therapeutic purposes. Better drugs, including mAbs, can be designed with insights of their multifaceted mechanisms of action, the divergences in receptor and ligand roles, the existence of trans and cis interactions with ligands and partners, the differences between various agents against a given target, and the limitations of studies in mice.

Ideally, the therapeutic goal should be to suppress all mechanisms of action of an inhibitory checkpoint in all relevant cell types, while not negatively affecting other pathways or triggering immune

overstimulation, which can lead to toxicity. To this end, whether it is best to target a receptor or its ligand should be considered, because these two approaches can have different outcomes (*119*). Moreover, unless ADCC and ADCP are intended mechanisms of action, it is safer to use agents that do not bind to FcRs.

Clinical trials that are poorly designed, because of a limited understanding of checkpoint biology and the impact of drugs, or because of enrolling small numbers or suboptimally selected patients, are destined for a high failure rate and will be harmful to the field of immune checkpoint inhibitors. They can lead to the early dismissal of targets with high potential. Along these lines, publication of negative results from clinical studies should be encouraged and perhaps mandatory for approved clinical trials, because they provide important insights for future trials and research. Currently, many of the negative data about clinical trials are found in online news and not published in peer-reviewed journals (*137*–*142*).

Gaining a thorough understanding of how inhibitory immune checkpoints and their targeting agents function goes beyond just academic curiosity; it holds considerable importance for creating new therapeutic strategies and improving existing treatments against cancer. This knowledge will also facilitate the design of better combination therapies, which are and will continue to be the mainstay of most anticancer therapies.

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