

## The Path to Reactivation of Antitumor Immunity and Checkpoint Immunotherapy

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### Abstract

Cancer immunology has recently made major therapeutic inroads that represent clinical application of basic insights into mechanisms that govern immunity against tumors. Research into fundamental elements of T-cell and natural killer-cell biology, including the basis of antigen recognition, activation, proliferation, and survival, has informed the design of new therapeutic approaches to augment the body's natural anticancer immune response. Here, we describe some of the key steps that have provided the foundation for current strategies of immunotherapy. *Cancer Immunol Res*; 2(10); 926–36. ©2014 AACR.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### CME Staff Planners' Disclosures

The members of the planning committee have no real or apparent conflicts of interest to disclose.

### Learning Objectives

Research on the fundamental elements of immunity has informed the design of new therapeutic approaches leading to the recent success of cancer immunotherapy. One of the proposed mechanisms of checkpoint immunotherapy is to awaken the immune system. Upon completion of this activity, the participant should gain a basic knowledge of the key elements of lymphocyte biology that govern host immunity against tumors.

### Acknowledgment of Financial or Other Support

This activity was supported by a gift from the LeRoy Schecter Research Foundation.

### Introduction

The idea that the immune system, designed primarily to protect the body from invading pathogens, might also detect and destroy transformed cells implies that defects in immunity might result in an increased incidence of tumors (1, 2). Although early experiments failed to demonstrate increased tumor incidence in mutant *nu/nu* mice that harbored severe, but incomplete defects in T-cell development (3, 4), more stringent testing of this hypothesis using improved mouse models, including *Rag2*<sup>-/-</sup>,  $\alpha\beta$  T cell<sup>-/-</sup>, *Prf1*<sup>-/-</sup>, and *IFN $\gamma$* <sup>-/-</sup> mice, supported the immunosurveillance hypothesis (5–9).

One assumption of the immunosurveillance hypothesis was that cancer cells might be qualitatively different from normal

cells and recognized by T cells as foreign. This concept received support from the molecular definition of T-cell antigens as MHC-peptide complexes and observations that expression of T-cell antigen by tumor cells was enhanced by oncogenic mutations (10, 11). These findings led to a renewed focus on the potential impact of T-cell responses on tumor growth and the ability of T-cell subsets to mount protective antitumor responses. These efforts were accelerated by a series of hallmark studies that documented a strong predictive correlation between the intensity and type of effector T-cell infiltration into tumors with subsequent tumor progression and clinical outcome (12–14).

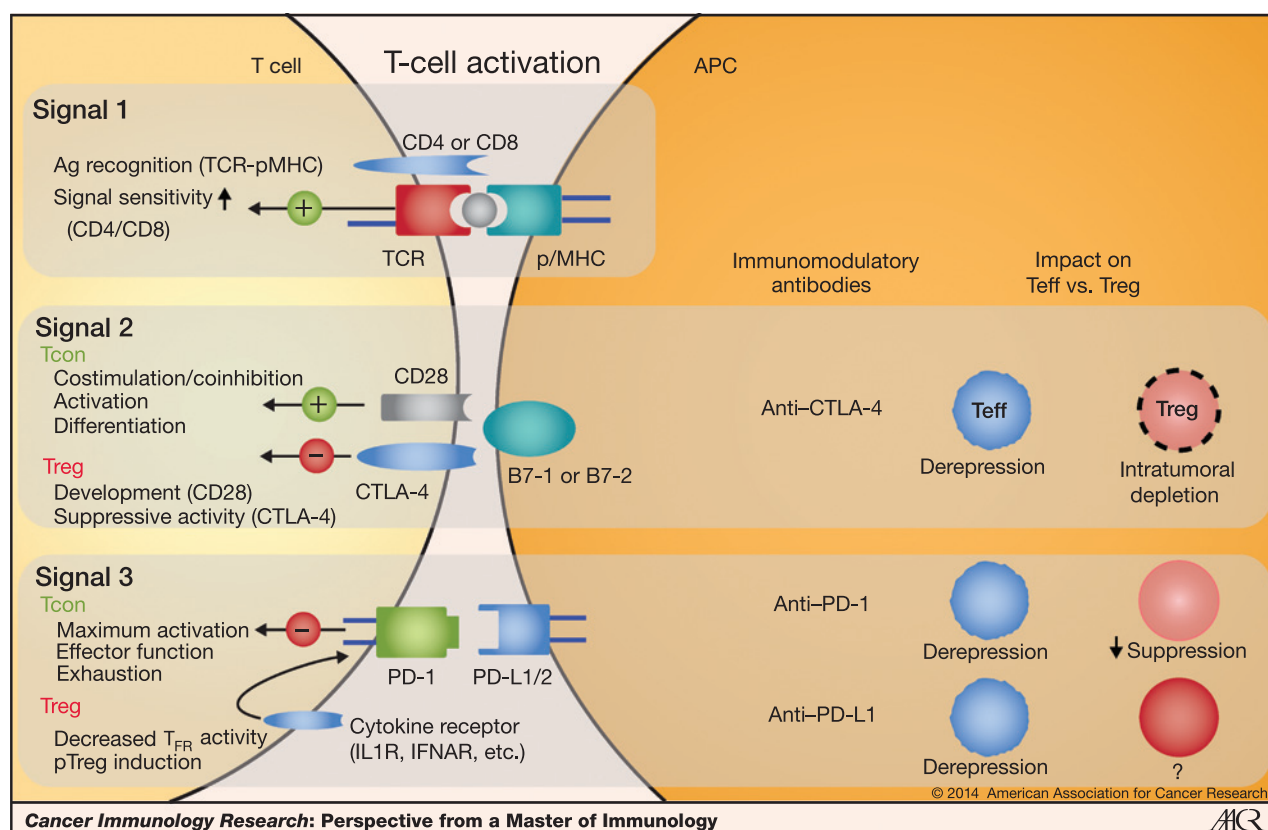
Major advances in defining the T-cell response to tumor antigens also came from increased understanding of signaling pathways that regulate T-cell activation, expansion, and differentiation. Engagement of the T-cell receptor (TCR) by MHC-peptide ligands transmitted a signal ("signal one") that was shown to be insufficient to promote an effective T-cell response. T-cell recognition is further refined by the engagement of CD4 and CD8 coreceptors expressed by the two major T-cell lineages. Interaction between the CD4 coreceptor expressed by T-helper cells and class II MHC expressed by macrophages, dendritic cells

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doi: 10.1158/2326-6066.CIR-14-0153

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**Figure 1.** T-cell activation and modulation of T-cell activity. Each T cell expresses a unique TCR that recognizes a specific antigen in the context of a specific MHC (signal 1). CD4 and CD8 coreceptors increase the sensitivity of antigen recognition by TCR. Optimal T-cell expansion and acquisition of effector function require signals transduced by costimulatory receptors (signal 2). CD28–B7-1/B7-2 interaction delivers an activation signal, whereas CTLA-4–B7-1/B7-2 interaction inhibits T-cell activation. Signaling via CD28 and CTLA-4 is also critical for the development and function of CD4 Treg. Inflammatory signals often induce upregulation of surface cytokine receptors and other receptors, including PD-1 (signal 3). Expression of PD-1 is associated with acquisition of an exhausted phenotype in T cells during infection and cancer. PD-1–PD-L1 interaction is involved in the inhibition of T<sub>FR</sub> activity and has also been implicated in pTreg generation. Preclinical and clinical data with checkpoint blockade using anti-CTLA-4, anti-PD-1, and anti-PD-L1 Abs suggest that increased antitumor immunity may be achieved by the combined effects of enhanced Teff activity and depletion or reduced suppression by CD4 Treg.

(DC), and B cells facilitated inflammatory and antibody responses to extracellular pathogens. On the other hand, interactions between the CD8 coreceptor expressed by CD8 cytotoxic T lymphocytes (CTL) favored recognition and elimination of intracellular parasites, including viruses, after infection of class I MHC<sup>+</sup> cells. However, coreceptor-dependent enhancement of TCR responses was not sufficient to provoke full T-cell activation. Integration of a complex set of signals delivered by costimulatory and coinhibitory receptors expressed by T cells is essential for robust and appropriate T-cell responses.

So far, this description of the T-cell response fits the clonal selection mechanism of recognition and response to microbial invasion envisioned by Talmage (15) and Burnet (16). According to this model, T-cell responses depend on the expansion of receptor-bearing clones as modulated by signals transduced from coreceptor and costimulatory receptors. According to this view, "tolerance is wholly a matter of the absence of the immunocyte" (15, 16). However, the discovery of the contribution of regulatory T cells (Treg)

to tolerance has forced a major revision of the clonal selection theory and its application to immunotherapy. Current therapeutic approaches to cancer immunotherapy have begun to incorporate advances in our understanding of signaling pathways that control activation and expansion of both effector T cells (Teff) and Treg. Here, we outline the seminal advances that have informed new and effective strategies to cancer immunotherapy.

### T-cell Activation

Each T cell expresses a unique TCR that can recognize a specific antigen in the context of specific MHC. Although engagement of the TCR by peptide-bound MHC (pMHC) at a relatively high affinity is essential for triggering T-cell signaling cascades (signal one), the nature of the T-cell response is determined by the sum of integrated signals—termed signals two and three—which originate from receptors that are differentially expressed by T cells under diverse immunologic conditions (Fig. 1).

## Signal One

### Effector T cells

Recognition of antigen by T cells had been convincingly documented (17–20) a decade before the genes that encode the TCR were identified. Successful TCR gene cloning depended on several critical experimental assumptions about somatic gene rearrangement in thymocytes and T cells (potentially similar to receptor rearrangement in B cells), leading to the isolation of T-cell-specific cDNA clones that specified proteins containing variable (V), diversity (D), joining (J), and constant (C) regions that were homologous to immunoglobulin (Ig) chains (21, 22). Antigen recognition, TCR assembly, and the structural features of the TCR–pMHC interaction indicated two distinct TCR types,  $\alpha\beta$  and  $\gamma\delta$ , with specificity for antigens carried by the V regions, similar to Ig (23). Studies of VDJ rearrangement mechanisms of the  $\beta$  and  $\delta$  TCR chains, and V and J elements of the  $\alpha$  and  $\gamma$  TCR chains indicated that they were sufficient to generate a very large TCR repertoire (23, 24). Although the genetic and structural diversity of TCRs is similar to that of B-cell receptors, they exhibit substantially lower binding affinity for their ligands than do antibodies. This reflects, in part, a lack of somatic hypermutation/selection of TCR genes and thymic deletion of T cells that express high-affinity TCRs for self-MHC–peptide complexes that include some tumor-associated antigens (25–28).

### T-cell ligands

The earliest definition of antigens came from studies of antibody responses that characterized them as mainly foreign proteins. Studies of the T-cell response revealed that, rather than proteins, T-cell antigens represented the now-familiar complex of MHC molecules and peptides (foreign or self). This core insight into T-cell recognition of antigen came from a decades-long search for genes that might control T-cell immune responses. George Snell (29) described murine genes that controlled expression of cell-surface antigens termed H antigens (for histocompatibility) responsible for tissue compatibility in transplantation. The significance of H antigens as genetically determined surface structures that regulated immune responses in addition to transplantation came from studies by Benacerraf and McDevitt (30), which identified a single autosomal-dominant genetic locus that controlled the response of immune cells to polypeptide antigens and regulated the interaction between macrophages and T cells that promoted T-cell activation (31). Ia (I region-associated) antigens, discovered by Unanue and colleagues (32) and Klein and colleagues (33), were considered the best candidate molecules responsible for this interaction. These seminal findings initiated several lines of investigation that led to the demonstration of pMHC–TCR interactions in multiple T-cell responses and a molecular definition of the ligand formed by MHC and peptide complexes recognized by T cells (34–40).

### CD4 and CD8 coreceptors

T cells are equipped with receptors (TCR) that directly recognize peptide–MHC complexes as well as additional

coreceptor molecules that increase the sensitivity of antigen recognition by the TCR. CD4 and CD8 molecules are expressed by distinct lineages of T lymphocytes that are genetically programmed to express helper function and class II restriction, or cytotoxic activity and class I restriction, respectively (41–43). The CD8 and CD4 cell-surface glycoproteins bind to the same individual MHC molecule as the TCR with different kinetics, ensuring that recognition is dominated by the TCR and secondarily enhanced by the coreceptors. The CD8 coreceptors interact with a nonpolymorphic region of the MHC class I  $\alpha 3$  domain on antigen-presenting cells (APC) and are expressed as disulfide-linked heterodimers (44). The CD4 coreceptor expressed by T-helper cells interacts with extremely low affinity with MHC class II binding (45), mainly during the early phase of TCR–MHC interactions, and functions as an early catalyst rather than providing stable support for the TCR–MHC interaction (46, 47). Although CD4-deficient mice can develop normal CD8<sup>+</sup> CTLs, T-helper cell activity is dramatically reduced (48). Conversely, CD8 T cells do not develop in CD8 $\alpha$ -deficient mice, which display defective cytotoxic but normal CD4 T-helper function (49). The critical contribution of coreceptors to the sensitivity of TCR recognition of pMHC comes from studies showing that coreceptor expression dramatically enhances the sensitivity of TCR-based detection of peptide ligands (50).

### TCR and Treg

T cells that express autoreactive TCRs may either undergo apoptosis or successfully differentiate into Treg in the thymus, based in part on the nature of TCR ligation by self-peptide–MHC complexes. TCR repertoire expressed by conventional T cells (Tcon) and Treg are distinct, with a small overlap with a higher proportion of self-reactive cells within the Treg pool (51, 52). Most studies support the idea that Treg development requires an interaction with self-antigens with a binding avidity that is intermediate between that required for the positive and negative selection of conventional MHC class II–restricted CD4 T cells (53). The decision between clonal deletion and Treg differentiation also depends on additional factors, including costimulation and cytokines, discussed below.

## Signal Two

### Costimulatory signals and T-effector cells

Engagement of TCR and coreceptor with pMHC on APC is not sufficient for complete T-cell activation. Optimal T-cell expansion and acquisition of effector function require signals transduced by surface molecules termed costimulatory receptors (Fig. 1). The requirement of a second signal for T-cell activation was initially suggested from an analysis of the allograft response that donor hematopoietic cells provided an essential signal for robust responses to grafted tissues (54, 55). After the description of MHC restriction by Zinkernagel and Doherty, the model was refined to include ligation of the TCR by pMHC as signal one and an APC-dependent inductive stimulus or signal two for full T-cell activation (56). Observations such as the failure of T cells to respond to

antigens expressed by chemically fixed APC unless viable splenocytes as APCs were provided (54, 57) implied that full T-cell activation depended on stimulatory signals provided by accessory cells. These findings suggested that T-cell responses might be inhibited by independently targeting costimulatory signals without knowing the exact nature of the antigen.

### CD28: a prototypic costimulatory receptor

CD28 is the best-characterized T-cell costimulatory molecule, first identified in the early 1980s as a T-cell surface receptor that enhanced TCR-induced proliferation and differentiation (58–60). These features suggested that CD28 might transduce signal two postulated by the two-signal hypothesis of lymphocyte activation.

The CD28 receptor, expressed by most T cells, homodimerizes via disulfide bonds between cysteine residues contained in the transmembrane regions (60). CD28 plays an essential role in T-cell activation and differentiation: TCR engagement in the absence of CD28 ligation results in either apoptosis or anergy characterized by impaired IL2 production and proliferation upon stimulation (61). Quantitative analysis of T-cell activation also suggested that the response of T-cell clones depends on a threshold number of ligated TCRs, which decreases substantially after provision of a costimulatory signal by professional APCs (62). This CD28-dependent reduction of threshold for T-cell activation reflects the formation of immunologic synapses that promote a lipid-associated increase in the local concentration of enzymes and adaptor molecules at the site of interaction between T cells and APCs (63).

### CD28 expression by Treg

In addition to its role as the primary costimulatory molecule for activation of T-effector cells, CD28 is essential for thymic development and peripheral homeostasis of FoxP3<sup>+</sup> CD4 Treg (64, 65). Selective deletion of CD28 expression in FoxP3<sup>+</sup> CD4 Treg resulted in systemic autoimmunity (66), whereas expansion of CD4 Treg with a superagonist anti-CD28 (CD28SA) antibody inhibited autoimmune disease (67–69). Although an initial clinical trial of i.v.-administered human CD28SA, TGN1412, provoked a life-threatening cytokine storm response (70), differential control of Treg and Tcon may be possible using much lower doses of CD28SA (71). These findings suggest that CD4 Treg may require weaker costimulatory CD28 signals than conventional T-effector cells due to their intrinsic self-reactivity, opening the possibility of potential therapeutic benefit.

### CTLA-4: a prototypic coinhibitory receptor

A screen of mouse cytolytic T-cell-derived cDNA libraries identified a gene called CTLA-4, which displayed a single V-like domain and a marked homology to CD28 (72). The proximity of the CTLA-4 gene to the CD28 locus suggested that they might represent products of gene duplication and mediate similar costimulatory activity (73, 74).

A key step in understanding CD28 and CTLA-4 came from cloning of the CD28 ligand, B7.1 (termed B7 at that time), from

human B-cell libraries (75, 76). An analysis of B7-deficient mice revealed a partial defect in the immune response, and equivalent binding of B7<sup>+</sup> and B7<sup>-</sup> activated B cells to CTLA-4-Ig, suggesting additional ligands for CTLA-4. Further studies led to the identification of B7.2 as an alternative ligand for CD28 and CTLA-4 (76, 77). Binding of CTLA-4 to B7 ligands inhibited T-cell proliferation and T-cell-dependent Ig responses (78, 79). Direct evidence that CTLA-4 and CD28 did not represent alternative costimulatory receptors came from findings that B7<sup>+</sup> APCs enhanced the response of CD28<sup>+</sup> but not CD28<sup>-</sup> T cells, i.e., CD28 represented the primary costimulatory receptor for B7-dependent T-cell costimulation (80). The inhibitory effects of CTLA-4 came from analyses of CTLA-4-deficient mice generated independently in 1995 (81, 82). These studies revealed that CTLA-4-deficient T cells were polyclonally activated and rapidly developed a severe and systemic autoimmune phenotype (81, 82). These findings established a functional asymmetry between the stimulatory CD28 coreceptor and the inhibitory CTLA-4 coreceptor (81). Cross-linking of CTLA-4 inhibited TCR/CD28-dependent T-cell activation (83), whereas experiments that varied the concentration of anti-CD28 and anti-CTLA-4 suggested that integration of signals from the CD28/CTLA-4 receptors regulated the TCR response (79). These and other observations suggested that levels of T-cell activation reflected a complex integration of signals from CD28 and CTLA-4, which in turn reflected the relative levels of B7.1 and B7.2 expressed by APCs (84–86).

These findings also suggested that CTLA-4 blockade might decrease coinhibitory signals, resulting in enhanced T-cell responses to microbial and tumor antigens. Treatment with anti-CTLA-4 Abs accelerated rejection of both B7-positive and B7-negative colon tumors, and enhanced antitumor responses upon subsequent challenge with B7<sup>-</sup> colon carcinoma cells (87). Enhanced responses to B7<sup>-</sup> tumors after anti-CTLA-4 Ab treatment reflected an interaction with CTLA-4<sup>+</sup> tumor-infiltrating immune cells, including T<sub>H</sub>1 cells, Treg, and possibly APCs. The efficacy of anti-CTLA-4 Ab treatment was initially attributed to the blockade of inhibitory signaling by CTLA-4<sup>+</sup> T<sub>H</sub>1 cells (88–90). More recent studies of the mechanism of anti-CTLA-4 therapy have suggested that FcγR-dependent elimination of CTLA-4<sup>hi</sup> tumor-infiltrating Treg by macrophages (rather than blockade) is the dominant mechanism that underpins the antitumor activity of CTLA-4 antibody treatment (91, 92).

### Expression of CTLA-4 by CD4<sup>+</sup> Treg

In contrast to T-effector cells, which require activation to upregulate CTLA-4, FoxP3<sup>+</sup> CD4 Treg constitutively express CTLA-4 (93, 94). In addition to functioning as a coinhibitory receptor on activated T cells, CTLA-4 contributes to immune suppression through its expression by CD4 Treg. The contribution of CTLA-4 to inhibitory activity of naturally occurring Treg (nTreg) came from findings that mice harboring CD4 Treg deficient in CTLA-4 secondary to FoxP3-Cre-mediated deletion developed a T-cell-mediated autoimmune disease, and increased antitumor immunity, that was associated with impaired CD4 Treg-suppressive activity (95, 96).

Treatment with anti-CTLA-4 Ab (ipilimumab) in the context of tumor immunotherapy may thus have several effects that include derepression of Teff cells as well as reduction of CD4 Treg-dependent immune suppression. Recent studies indicated that, although anti-CTLA-4 treatment results in an increase of Teff and Treg numbers in lymph nodes, this treatment promotes depletion of intratumoral Treg via antibody-dependent cellular cytotoxicity (ADCC), resulting in an increase in the Teff:Treg ratio (91, 92, 97). Selective elimination of intratumoral Treg may depend in part on the upregulation of CTLA-4 expression by Treg within the tumor microenvironment, as well as the action of myeloid cells that express high levels of ADCC-competent Fc $\gamma$ Rs (91). These findings suggest that relative expression levels of CTLA-4 by Teff versus Treg, intratumoral levels of Treg, and expression of Fc $\gamma$ R by myeloid cells as well as anti-CTLA-4 antibody isotype may determine the net efficacy of immunomodulatory therapy.

### Signal Three

#### Inflammatory cytokine receptors and PD-1

A requirement for a third class of signals for optimal T-cell activation came from studies that suggested that inflammatory cytokines (e.g., IL1 for CD4 cells; IL12 and IFN $\alpha$ / $\beta$  for CD8 cells) were essential for optimal acute T-cell responses (98, 99). However, chronic and prolonged inflammatory stimuli frequently resulted in T-cell nonresponsiveness. Studies of the PD-1 receptor have begun to clarify the mechanism that underlies this form of unresponsiveness. Chronic viral infections that promote early T-cell activation followed by attenuated T-cell responses may reflect type-I IFN-dependent upregulation of PD-1 expression by antigen-specific T cells (100–102), whereas antibody-dependent blockade of the PD-1–PD-L1 inhibitory pathway can restore antiviral T-cell responses (102, 103; Fig. 1).

The PD-1 receptor was cloned in 1992 from T-cell hybridomas that underwent programmed cell death (104). The PD-1 receptor is expressed by T, B, and natural killer (NK) cells, but its biologic activity has been studied mainly in connection with T cells. Unlike CTLA-4 and other members of the CD28 family, PD-1 lacks a membrane-proximal cysteine residue required for homodimerization and is expressed as a cell-surface monomer (105–107). In contrast to the inhibitory CTLA-4 receptor, which is continuously endocytosed through its association with the adaptor complex AP-2, PD-1 is stably expressed on the cell surface (108) and delivers a negative signal that depends on tyrosine-based inhibitory motifs found in the PD-1 cytoplasmic domain.

The contribution of PD-1 to self-tolerance was initially apparent from the phenotype of PD-1-deficient mice (109). The interaction of PD-1 expressed by T cells with PD-1 ligands expressed by APCs delivered a negative signal (109, 110), and PD-1-deficient CD8 cells displayed augmented activation and proliferation (109). The high degree of homology between PD-1 and CTLA-4 extracellular domains suggested B7-like molecules as likely candidate ligands. Indeed, an interaction between PD-1 and B7-H1 (now PD-L1) and B7-DC (now PD-L2) inhibits TCR-dependent proliferation and cytokine

production (111–114). PD-L1 is broadly expressed by hematopoietic cells and nonhematopoietic cells, whereas expression of PD-L2 is restricted to hematopoietic cells, suggesting a potentially broad inhibitory impact of the PD-1–PD-L1 interaction on effector T-cell responses in many tissues (115).

More complicated interactions of B7–CD28 family members were predicted when it was discovered that B7.1 bound to PD-L1 and interacted with each other to inhibit T-cell activation (116) and may explain the greater effect of anti-PD-L1 (dual specific) blockade compared with anti-PD-1 or anti-PD-L2 blockade in mouse models of colitis and chronic viral infection (102, 117). Possibly, PD-1 ligands have evolved to engage in multiple binding interactions in addition to binding to their canonical receptor, PD-1. A recent study by the Freeman group revealed that a PD-L2 interaction with the repulsive guidance molecule b expressed in the nervous system and in macrophages may regulate respiratory tolerance in the lung and adds a layer of complexity to the molecular interactions of this inhibitory family and to therapeutic PD-1-based strategies (118).

Although PD-1-deficient mice develop a spectrum of autoimmune diseases, PD-L1-deficient mice display no obvious phenotype unless challenged with an infection or crossed onto an autoimmune-prone background (119). Findings that PD-1-deficient and PD-L1-deficient mice display a less severe phenotype than CTLA-4-deficient mice suggest a potential advantage for PD-1-based therapy in terms of side effects. This has so far been the case with fewer severe immune-related adverse events (IRAE) associated with anti-PD-1 Ab treatment compared with anti-CTLA-4 Ab treatment, although the overall incidence of IRAEs is similar.

#### PD-1 expression by Treg

PD-1 is expressed at high levels on both Treg and activated Teffs. Signaling through PD-1 promotes the development of induced Treg (120–122), whereas PD-L1-deficient APCs fail to efficiently convert naïve CD4 T cells into *in vitro*-induced Treg (iTreg), and PD-L1<sup>-/-</sup>PD-L2<sup>-/-</sup>Rag2<sup>-/-</sup> hosts reconstituted with naïve CD4 T cells rapidly develop a fatal inflammatory disorder associated with reduced peripherally derived Treg (pTreg) conversion (121). The PD-1–PD-L1 interaction also contributes to T follicular regulatory (T<sub>FR</sub>) cell function, because PD-1<sup>-/-</sup> mice display increased T follicular helper (T<sub>FH</sub>) cells and an enhanced suppressive activity on antibody production (123). Because T<sub>FR</sub> cells develop from thymus-derived Treg (tTreg), these studies suggest that PD-1 signaling may have distinct effects on the differentiation of induced and thymus-derived CD4 Treg.

#### PD-1 inhibitory mechanisms

Ligation of both CTLA-4 and PD-1 inhibits CD3/CD28-mediated upregulation of glucose metabolism and Akt activity and limits T-cell activation. This common outcome is achieved by distinct signaling mechanisms. CTLA-4 overrides costimulation by CD28 by virtue of its higher affinity to B7, and conversion to inhibitory pathways associated with SHP-2, PP2A, and AP-2, whereas PD-1 inactivates ZAP70, a major integrator of TCR-mediated signaling (124–127).

These nonoverlapping inhibitory pathways are consistent with an additive and synergistic inhibition of T-cell activation after blockade by both CTLA-4–B7 and PD-1–PD-L interactions and are consistent with preclinical studies suggesting that combined CTLA-4 and PD-1 blockade is more effective than single blockade in promoting rejection of B16 melanomas (128; Fig. 1).

### Tumors

Expression of PD-L1 by human cancers, including lung, ovary, colon carcinomas, and melanoma, is associated with apoptosis of tumor-reactive T cells, inhibition of T-cell activation, and reduced antitumor immune responses (129, 130). The mechanistic basis of PD-L1 expression by tumor cells is not well understood. Posttranscriptional expression of PD-L1 by human gliomas is increased after oncogenic mutation (e.g., loss of PTEN) and activation of the PI3K pathway, suggesting a tumor cell–intrinsic mechanism of immunoresistance and immune escape (131). Similarly, nucleophosmin/anaplastic lymphoma kinase oncoprotein was shown to induce PD-L1 expression via STAT3 enhancement in T-cell lymphomas (132). A recent analysis of membranous PD-L1 expression by melanoma cells and tumor-infiltrating lymphocytes (TIL) has suggested a significant correlation between TILs and IFN $\gamma$  production with PD-L1 expression by tumor cells (133) and provided a rationale for blockade of the PD-1–PD-L1 interaction to enhance endogenous T-cell responses to tumors.

### Perspectives

The hypothesis that the immune system might control tumor growth was put forward 60 years ago. The nature of T-cell antigens was defined 40 years ago. The TCR was cloned 30 years ago, and cellular mechanisms that regulate TCR-based activation have been extensively studied over the past three decades. Definitions of pathways that regulate T-cell activation, expansion, and survival have provided molecular targets to enhance T-cell responses or block immune inhibitory mechanisms. Recent clinical success of checkpoint blockade exemplifies the translational potential of these approaches.

### Combination immunotherapy

A large number of inhibitory receptors that regulate T-cell responses have been identified, including LAG3, 2B4, BTLA (B- and T-lymphocyte attenuator), IL10R, TIM3 (T-cell immunoglobulin and mucin 3), and NKG2A, and this list continues to grow. Preclinical and clinical testing of targeting of some of these inhibitory pathways in combination with CTLA-4 or PD-1 blockade are in progress, and some show promising results (134–136). Profiling of dominant inhibitory receptors expressed by different tumor types may be required to identify the most appropriate inhibitory surface receptors to target. For example, anti-LAG3/anti-PD-1–combined immunotherapy effectively clears established fibrosarcoma (Sa1N) and adenocarcinoma (MC38), but not B16 melanoma, which may reflect lower expression of LAG3/PD-1 expressed by TILs from B16 melanoma (135). Increased understanding and mechanistic

characterization of downstream signaling events involved in distinct inhibitory pathways are necessary to identify the most beneficial pairing of target molecules.

Emerging clinical data also show that not all patients are responsive to treatment with antagonistic Abs that block CTLA-4 and PD-1 (137–139). The challenge is to develop strategies to eradicate immunogenic tumors that are resistant to current Ab-mediated blockade of immune checkpoints. A recent study showing that tumors resistant to anti-PD-1 treatment could be eradicated by combining anti-PD-1 Abs with vaccines containing tumor-specific peptides with high MHC-binding affinity is instructive (140). Improved clinical outcomes may also depend on vaccines comprising high-affinity mutant peptides derived from exome sequencing and peptide-affinity algorithms (141).

Promotion of T-cell infiltration into tumors by eliciting local inflammation represents another option that might be combined with immune checkpoint blockade. Intratumoral administration of an oncolytic virus (Newcastle disease virus) resulted in an increase of TILs into local and distant tumors and rendered them more vulnerable to systemic anti-CTLA-4 blockade, leading to tumor rejection (142). Delivery of IFN $\beta$  into EGFR-expressing tumor tissue via administration of anti-EGFR–IFN $\beta$  elevated antigen cross-presentation by DCs and increased tumor regression; combined therapy with anti-PD-L1 Abs further enhanced the long-term efficacy of anti-EGFR–IFN $\beta$  by overcoming treatment-acquired resistance (143).

### Checkpoint blockade and NK cells

The general strategy of targeting inhibitory receptors to enhance effector T-cell responses can be applied to reactivate other effector cells that are equipped to eliminate tumors, e.g., NK cells. The response of NK effector cells is controlled by signals from activating (e.g., NKG2D) and inhibitory receptors that include the KIR family (Ly49 in mouse), leukocyte Ig-like receptor (LIR) family, and CD94/NKG2A. A therapeutic mAb specific for common inhibitory KIRs, IPH2101 (a human IgG4 mAb against KIR2DL-1, -2, and -3), that blocks inhibitory KIR signaling NK-cell responses, is currently in clinical trials for activity against multiple myeloma (144, 145).

Studies that have shown that interruption of the inhibitory interaction between NKG2A and Qa-1 (HLA-E in human) with blocking Abs enhances NK activity *in vivo* (146, 147) provide a framework for targeting NKG2A to enhance NK responses to tumors. Intratumoral NK cells display an unusual functional phenotype, including defective degranulation and IFN $\gamma$  production (148), and increased NKG2A expression (149). Possibly, increased expression of inhibitory receptors, including NKG2A, may contribute to the impaired functional phenotype of NK cells within tumor microenvironments (150). The finding that many tumors upregulate HLA-E also suggests that interruption of the HLA-E–NKG2A interaction may enhance antitumor responses of both NK cells and CD8<sup>+</sup> tumor-infiltrating CTLs (146, 151). Assessment of the levels of MHC class Ia and Ib (HLA-E) expressed by individual tumors may prove a useful guide for the selection of immunotherapeutic approaches. Recruitment and activation of the NK arm of the innate immune system combined with enhanced adaptive immunity

via checkpoint blockade may increase the proportion of patients that develop durable responses to immunotherapy.

Progress will also depend on continued research into basic NK biology, including insight into NK receptors and their ligands that regulate antitumor activity of NK cells. For example, very recent studies that have clarified the contribution of the CD96 receptor to regulation of the NK-cell response may form the basis for strategies that target CD96 to enhance antitumor immunity (152).

### Final common pathways

All of the above approaches depend on targeting known inhibitory receptors on effector cells or blocking inhibitory interactions between regulatory cells and effector cells. However, additional mechanisms that dampen intratumoral T-cell responses may be initiated by interactions with tumor cells, tumor-associated stromal cells that include fibroblasts, epithelial cells, macrophages, and regulatory T-cell subsets (153–155). Improving on the limited successes of cancer immunotherapy requires approaches that target intracellular inhibitory pathways that dampen the response of intratumoral effector T-cells.

Genes that inhibit expansion and activation of intratumoral CD8 T cells can be identified using an *in vivo* pooled shRNA screen in which shRNAs targeting inhibitory genes become enriched by releasing a block on expansion of intratumoral T-effector cells. After introduction of shRNAs into T cells, the subset of tagged shRNAs that restores intratumoral T-cell expansion can be used to further characterize individual candidate genes for functional activity. For example, targeting of one of these candidate genes, Ppp2r2d, resulted in reduction of T-cell apoptosis, enhancement of proliferation and effector cytokine production by tumor-specific CD8 T cells, and increased antitumor activity *in vivo* (156). Variations of this approach can be applied to interrogate other types of tumor-infiltrating effector cells, including intratumoral NK cells, and identify signaling pathways that suppress NK-cell antitumor responses (148, 156) and may define shared tumor-induced inhibitory pathways that dampen both NK and CD8 T-cell effector activities. Additional reporters that allow monitoring

of cytokines or cytotoxic molecules can be included for a more complete description of gene mechanisms that control critical T-cell effector functions in tumors.

### Summary

Recent advances in our understanding of the mechanisms that regulate immune responses have led to the development of novel approaches to cancer immunotherapy. Current strategies that have made important inroads into the standard of care for patients with cancer are continuously being refined based on increased insight into antitumor immune responses. The validity of early concepts of cancer immunosurveillance has been confirmed and extended by current experimental approaches and technologies that allow increased insight into complex immunologic phenomena. These technological advances along with the development of high-throughput technologies and system-based approaches are continuing to provide a richer and more advanced understanding of the relationship between the immune system and cancer. Comprehensive gene expression profiling of the immune system (Immunological Genome Project) has led to more refined analyses of the genetic patterns associated with the development and function of immune-cell lineages (157, 158), whereas the recent development of the concept of "immune contexture" as a prognostic index in cancer represents an extension of this approach to clinical settings (159). These conceptual and experimental tools are rapidly increasing our ability to design more effective forms of immunotherapy aimed at a more precise modulation of tumor-associated T cells and other immune cells. Nonetheless, we have much to learn about T-cell and tumor biology before we will be able to fully and effectively harness the immune response to yield positive therapeutic outcomes.

### Acknowledgments

The authors thank A. Angel for manuscript preparation and designing figures.

Received August 18, 2014; accepted August 22, 2014; published online October 3, 2014.

### References

1. Burnet M. Cancer; a biological approach. I. The processes of control. *Br Med J* 1957;1:779–86.
2. Thomas L. On immunosurveillance in human cancer. *Yale J Biol Med* 1982;55:329–33.
3. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science* 1974;183:534–6.
4. Stutman O. Delayed tumour appearance and absence of regression in nude mice infected with murine sarcoma virus. *Nature* 1975;253:142–4.
5. Street SE, Cretney E, Smyth MJ. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* 2001;97:192–7.
6. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFN $\gamma$  and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 2001;410:1107–11.
7. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. Regulation of cutaneous malignancy by gammadelta T cells. *Science* 2001;294:605–9.
8. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoeediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002;3:991–8.
9. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* 2001;2:293–9.
10. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;254:1643–7.
11. Disis ML, Cheever MA. Oncogenic proteins as tumor antigens. *Curr Opin Immunol* 1996;8:637–42.
12. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells

- within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960–4.
13. Rusakiewicz S, Semeraro M, Sarabi M, Desbois M, Locher C, Mendez R, et al. Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. *Cancer Res* 2013;73:3499–510.
  14. Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* 2009;27:5944–51.
  15. Talmage DW. Allergy and immunology. *Annu Rev Med* 1957;8:239–56.
  16. Burnet FM. A modification of Jerne's theory of antibody production using the concept of clonal selection. *Aust J Sci* 1957;20:67–9.
  17. Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* 1968;14:181–96.
  18. Miller JF, Mitchell GF. Cell to cell interaction in the immune response. *Transplant Proc* 1969;1:535–8.
  19. Raff MC, Sternberg M, Taylor RB. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature* 1970;225:553–4.
  20. Schimpl A, Wecker E. Inhibition of in vitro immune response by treatment of spleen cell suspensions with anti-theta serum. *Nature* 1970;226:1258–9.
  21. Hedrick SM, Cohen DI, Nielsen EA, Davis MM. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* 1984;308:149–53.
  22. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature* 1984;308:145–9.
  23. Davis MM, Bjorkman PJ. T cell antigen receptor genes and T cell recognition. *Nature* 1988;334:395.
  24. Malissen M, Trucy J, Jouvin-Marche E, Cazenave PA, Scollay R, Malissen B. Regulation of TCR  $\alpha$  and  $\beta$  gene allelic exclusion during T-cell development. *Immunol Today* 1992;13:315–22.
  25. Holler PD, Holman PO, Shusta EV, O'Herrin S, Wittrup KD, Kranz DM. In vitro evolution of a T cell receptor with high affinity for peptide/MHC. *Proc Natl Acad Sci U S A* 2000;97:5387–92.
  26. Kisielow P, Bluthmann H, Staerz UD, Steinmetz M, von Boehmer H. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4<sup>+</sup>8<sup>+</sup> thymocytes. *Nature* 1988;333:742–6.
  27. Kreslavsky T, Kim HJ, Korolov SB, Ghitza D, Buch T, Vantor H, et al. Negative selection, not receptor editing, is a physiological response of autoreactive thymocytes. *J Exp Med* 2013;210:1911–8.
  28. Marrack P, Lo D, Brinster R, Palmer R, Burkly L, Flavell RH, et al. The effect of thymus environment on T cell development and tolerance. *Cell* 1988;53:627–34.
  29. Snell GD. Methods for the study of histocompatibility genes. *J Genet* 1948;49:87–108.
  30. Benacerraf B, McDevitt HO. Histocompatibility-linked immune response genes. *Science* 1972;175:273–9.
  31. Shevach EM, Rosenthal AS. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J Exp Med* 1973;138:1213–29.
  32. Unanue ER, Dorf ME, David CS, Benacerraf B. The presence of I-region-associated antigens on B cells in molecules distinct from immunoglobulin and H-2K and H-2D. *Proc Natl Acad Sci U S A* 1974;71:5014–6.
  33. Klein J, Figueroa F, David CS. H-2 haplotypes, genes and antigens: second listing. II. The H-2 complex. *Immunogenetics* 1983;17:553–96.
  34. Zinkernagel RM. Restriction by H-2 gene complex of transfer of cell-mediated immunity to *Listeria monocytogenes*. *Nature* 1974;251:230–3.
  35. Bevan MJ. In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature* 1977;269:417–8.
  36. von Boehmer H, Haas W, Jerne NK. Major histocompatibility complex-linked immune-responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high-responder mice. *Proc Natl Acad Sci U S A* 1978;75:2439–42.
  37. Sprent J, von Boehmer H, Nabholz M. Association of immunity and tolerance to host H-2 determinants in irradiated F1 hybrid mice reconstituted with bone marrow cells from one parental strain. *J Exp Med* 1975;142:321–31.
  38. Bjorkman PJ, Davis MM. Model for the interaction of T-cell receptors with peptide/MHC complexes. *Cold Spring Harb Symp Quant Biol* 1989;54 Pt 1:365–73.
  39. Chien YH, Davis MM. How alpha beta T-cell receptors 'see' peptide/MHC complexes. *Immunol Today* 1993;14:597–602.
  40. Katz DH, Katz LR, Bogowitz CA, Skidmore BJ. Adaptive differentiation of murine lymphocytes. II. The thymic microenvironment does not restrict the cooperative partner cell preference of helper T cells differentiating in F1 leads to F1 thymic chimeras. *J Exp Med* 1979;149:1360–70.
  41. Cantor H, Shen FW, Boyse EA. Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen: after immunization, antigen-specific suppressor and helper activities are mediated by distinct T-cell subclasses. *J Exp Med* 1976;143:1391–401.
  42. Reinherz EL, Kung PC, Goldstein G, Schlossman SF. Separation of functional subsets of human T cells by a monoclonal antibody. *Proc Natl Acad Sci U S A* 1979;76:4061–5.
  43. Swain SL. Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize class 1 major histocompatibility complex antigens regardless of their function. *Proc Natl Acad Sci U S A* 1981;78:7101–5.
  44. Parnes JR. Molecular biology and function of CD4 and CD8. *Adv Immunol* 1989;44:265–311.
  45. Weber S, Karjalainen K. Mouse CD4 binds MHC class II with extremely low affinity. *Int Immunol* 1993;5:695–8.
  46. Krummel MF, Sjaastad MD, Wulff C, Davis MM. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science* 2000;289:1349–52.
  47. Zal T, Zal MA, Gascoigne NR. Inhibition of T cell receptor-coreceptor interactions by antagonist ligands visualized by live FRET imaging of the T-hybridoma immunological synapse. *Immunity* 2002;16:521–34.
  48. Rahemtulla A, Fung-Leung WP, Schilham MW, Kundig TM, Sambhara SR, Narendran A, et al. Normal development and function of CD8<sup>+</sup> cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* 1991;353:180–4.
  49. Fung-Leung WP, Schilham MW, Rahemtulla A, Kundig TM, Vollenweider M, Potter J, et al. CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* 1991;65:443–9.
  50. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature* 2002;419:845–9.
  51. Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25<sup>+</sup> CD4<sup>+</sup> T cell receptors. *Immunity* 2004;21:267–77.
  52. Wong J, Obst R, Correia-Neves M, Losyev G, Mathis D, Benoist C. Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4<sup>+</sup> T cells. *J Immunol* 2007;178:7032–41.
  53. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2001;2:816–22.
  54. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* 1987;165:302–19.
  55. Lafferty KJ, Misko IS, Cooley MA. Allogeneic stimulation modulates the in vitro response of T cells to transplantation antigen. *Nature* 1974;249:275–6.
  56. Lafferty KJ, Woolnough J. The origin and mechanism of the allograft reaction. *Immunol Rev* 1977;35:231–62.
  57. Jenkins MK, Ashwell JD, Schwartz RH. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J Immunol* 1988;140:3324–30.
  58. Hara T, Fu SM. Human T cell activation. I. Monocyte-independent activation and proliferation induced by anti-T3 monoclonal antibodies in the presence of tumor promoter 12-o-tetradecanoyl phorbol-13 acetate. *J Exp Med* 1985;161:641–56.



59. Poggi A, Bottino C, Zocchi MR, Pantaleo G, Ciccone E, Mingari C, et al. CD3+ WT31- peripheral T lymphocytes lack T44 (CD28), a surface molecule involved in activation of T cells bearing the alpha/beta heterodimer. *Eur J Immunol* 1987;17:1065-8.
60. Aruffo A, Seed B. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc Natl Acad Sci U S A* 1987;84:8573-7.
61. Alegre ML, Frauwrith KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 2001;1:220-8.
62. Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 1996;273:104-6.
63. Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 1999;283:680-2.
64. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000;12:431-40.
65. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 2003;171:3348-52.
66. Zhang R, Huynh A, Whitcher G, Chang J, Maltzman JS, Turka LA. An obligate cell-intrinsic function for CD28 in Tregs. *J Clin Invest* 2013;123:580-93.
67. Lin CH, Hunig T. Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. *Eur J Immunol* 2003;33:626-38.
68. Beyersdorf N, Gaupp S, Balbach K, Schmidt J, Toyka KV, Lin CH, et al. Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. *J Exp Med* 2005;202:445-55.
69. Miyasato K, Takabatake Y, Kaimori J, Kimura T, Kitamura H, Kawachi H, et al. CD28 superagonist-induced regulatory T cell expansion ameliorates mesangioproliferative glomerulonephritis in rats. *Clin Exp Nephrol* 2011;15:50-7.
70. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* 2006;355:1018-28.
71. Tabares P, Berr S, Romer PS, Chuypilo S, Matskevich AA, Tyrsin D, et al. Human regulatory T cells are selectively activated by low-dose application of the CD28 superagonist TGN1412/TAB08. *Eur J Immunol* 2014;44:1225-36.
72. Brunet JF, Denizot F, Luciani MF, Roux-Dosseto M, Suzan M, Mattei MG, et al. A new member of the immunoglobulin superfamily—CTLA-4. *Nature* 1987;328:267-70.
73. Balzano C, Buonavista N, Rouvier E, Golstein P. CTLA-4 and CD28: similar proteins, neighbouring genes. *Int J Cancer Suppl* 1992;7:28-32.
74. Buonavista N, Balzano C, Pontarotti P, Le Paslier D, Golstein P. Molecular linkage of the human CTLA4 and CD28 Ig-superfamily genes in yeast artificial chromosomes. *Genomics* 1992;13:856-61.
75. Freeman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Nadler LM. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J Immunol* 1989;143:2714-22.
76. Freeman GJ, Gribben JG, Boussiotis VA, Ng JW, Restivo VA Jr, Lombard LA, et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science* 1993;262:909-11.
77. Freeman GJ, Borriello F, Hodes RJ, Reiser H, Hathcock KS, Laszlo G, et al. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science* 1993;262:907-9.
78. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* 1991;173:721-30.
79. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994;1:405-13.
80. Green JM, Noel PJ, Sperling AI, Walunas TL, Gray GS, Bluestone JA, et al. Absence of B7-dependent responses in CD28-deficient mice. *Immunity* 1994;1:501-8.
81. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in CtlA-4. *Science* 1995;270:985-8.
82. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995;3:541-7.
83. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995;182:459-65.
84. Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, et al. The interaction properties of costimulatory molecules revisited. *Immunity* 2002;17:201-10.
85. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* 1994;1:793-801.
86. Pentcheva-Hoang T, Egen JG, Wojnoonski K, Allison JP. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* 2004;21:401-13.
87. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 1996;271:1734-6.
88. Shrikant P, Khoruts A, Mescher MF. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity* 1999;11:483-93.
89. Quezada SA, Peggs KS, Curran MA, Allison JP. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J Clin Invest* 2006;116:1935-45.
90. Kavanagh B, O'Brien S, Lee D, Hou Y, Weinberg V, Rini B, et al. CTLA4 blockade expands FoxP3+ regulatory and activated effector CD4+ T cells in a dose-dependent fashion. *Blood* 2008;112:1175-83.
91. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* 2013;210:1695-710.
92. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res* 2013;1:32-42.
93. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2000;192:303-10.
94. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-61.
95. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008;322:271-5.
96. Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, et al. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. *J Immunol* 2006;177:4376-83.
97. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, et al. Activating Fc gamma receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *J Exp Med* 2013;210:1685-93.
98. Curtsinger JM, Lins DC, Mescher MF. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med* 2003;197:1141-51.
99. Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, et al. Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev* 2006;211:81-92.
100. Terawaki S, Chikuma S, Shibayama S, Hayashi T, Yoshida T, Okazaki T, et al. IFN-alpha directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. *J Immunol* 2011;186:2772-9.
101. Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their

- advantages for clinical application. *Nat Immunol* 2013;14:1212–8.
102. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006;439:682–7.
  103. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 2007;27:670–84.
  104. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992;11:3887–95.
  105. Zhang X, Schwartz JC, Guo X, Bhatia S, Cao E, Lorenz M, et al. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* 2004;20:337–47.
  106. Lazar-Molnar E, Gacser A, Freeman GJ, Almo SC, Nathenson SG, Nosanchuk JD. The PD-1/PD-L costimulatory pathway critically affects host resistance to the pathogenic fungus *Histoplasma capsulatum*. *Proc Natl Acad Sci U S A* 2008;105:2658–63.
  107. Lin DY, Tanaka Y, Iwasaki M, Gittis AG, Su HP, Mikami B, et al. The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. *Proc Natl Acad Sci U S A* 2008;105:3011–6.
  108. Shiratori T, Miyatake S, Ohno H, Nakaseko C, Isono K, Bonifacio JS, et al. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. *Immunity* 1997;6:583–9.
  109. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999;11:141–51.
  110. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 2001;291:319–22.
  111. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999;5:1365–9.
  112. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027–34.
  113. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261–8.
  114. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med* 2001;193:839–46.
  115. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;26:677–704.
  116. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 2007;27:111–22.
  117. Kanai T, Totsuka T, Uraushihara K, Makita S, Nakamura T, Koganei K, et al. Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. *J Immunol* 2003;171:4156–63.
  118. Xiao Y, Yu S, Zhu B, Bedoret D, Bu X, Francisco LM, et al. RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance. *J Exp Med* 2014;211:943–59.
  119. Dong H, Zhu G, Tamada K, Flies DB, van Deursen JM, Chen L. B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. *Immunity* 2004;20:327–36.
  120. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). *Int Immunol* 2007;19:337–43.
  121. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 2009;206:3015–29.
  122. Amarnath S, Mangus CW, Wang JC, Wei F, He A, Kapoor V, et al. The PDL1-PD1 axis converts human TH1 cells into regulatory T cells. *Sci Transl Med* 2011;3:111ra20.
  123. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* 2013;14:152–61.
  124. Parry RV, Chemnitz JM, Frauwrith KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 2005;25:9543–53.
  125. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A* 2001;98:13866–71.
  126. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 2004;173:945–54.
  127. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* 2012;209:1201–17.
  128. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* 2010;107:4275–80.
  129. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793–800.
  130. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 2005;65:1089–96.
  131. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* 2007;13:84–8.
  132. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc Natl Acad Sci U S A* 2008;105:20852–7.
  133. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 2012;4:127ra37.
  134. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 2010;207:2187–94.
  135. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* 2012;72:917–27.
  136. Fourcade J, Sun Z, Pagliano O, Chauvin JM, Sander C, Janjic B, et al. PD-1 and Tim-3 regulate the expansion of tumor antigen-specific CD8(+) T cells induced by melanoma vaccines. *Cancer Res* 2014;74:1045–55.
  137. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363:711–23.
  138. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2443–54.
  139. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* 2013;369:122–33.
  140. Binder DC, Engels B, Arina A, Yu P, Schlauch JM, Fu YX, et al. Antigen-specific bacterial vaccine combined with anti-PD-L1 rescues dysfunctional endogenous T cells to reject long-established cancer. *Cancer Immunol Res* 2013;1:123–33.

141. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* 2013;19:747–52.
142. Zamarin D, Holmgaard RB, Subudhi SK, Park JS, Mansour M, Palese P, et al. Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. *Sci Transl Med* 2014;6:226ra32.
143. Yang X, Zhang X, Fu ML, Weichselbaum RR, Gajewski TF, Guo Y, et al. Targeting the tumor microenvironment with interferon-beta bridges innate and adaptive immune responses. *Cancer Cell* 2014;25:37–48.
144. Romagne F, Andre P, Spee P, Zahn S, Anfossi N, Gauthier L, et al. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood* 2009;114:2667–77.
145. Benson DM Jr, Bakan CE, Zhang S, Collins SM, Liang J, Srivastava S, et al. IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood* 2011;118:6387–91.
146. Lu L, Ikizawa K, Hu D, Werneck MBF, Wucherpfennig KW, Cantor H. Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A pathway. *Immunity* 2007;26:593–604.
147. Leavenworth JW, Schellack C, Kim HJ, Lu L, Spee P, Cantor H. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')<sub>2</sub>. *Proc Natl Acad Sci U S A* 2010;107:2562–7.
148. Vitale M, Cantoni C, Pietra G, Mingari MC, Moretta L. Effect of tumor cells and tumor microenvironment on NK-cell function. *Eur J Immunol* 2014;44:1582–92.
149. Gillard-Bocquet M, Caer C, Cagnard N, Crozet L, Perez M, Fridman WH, et al. Lung tumor microenvironment induces specific gene expression signature in intratumoral NK cells. *Front Immunol* 2013;4:19.
150. Chretien AS, Le Roy A, Vey N, Prebet T, Blaise D, Fauriat C, et al. Cancer-induced alterations of NK-mediated target recognition: current and investigational pharmacological strategies aiming at restoring NK-mediated anti-tumor activity. *Front Immunol* 2014; 5:122.
151. Gooden M, Lampen M, Jordanova ES, Leffers N, Trimbois JB, van der Burg SH, et al. HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8(+) T lymphocytes. *Proc Natl Acad Sci U S A* 2011;108:10656–61.
152. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat Immunol* 2014; 15:431–8.
153. Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune micro-environments in solid tumors: new targets for therapy. *Genes Dev* 2011;25:2559–72.
154. Ostrand-Rosenberg S. Immune surveillance: a balance between pro-tumor and antitumor immunity. *Curr Opin Genet Dev* 2008;18:11–8.
155. Elpek KG, Cremasco V, Shen H, Harvey CJ, Wucherpfennig KW, Goldstein DR, et al. The tumor microenvironment shapes lineage, transcriptional, and functional diversity of infiltrating myeloid cells. *Cancer Immunol Res* 2014;2:655–67.
156. Zhou P, Shaffer DR, Alvarez Arias DA, Nakazaki Y, Pos W, Torres AJ, et al. In vivo discovery of immunotherapy targets in the tumour microenvironment. *Nature* 2014;506:52–7.
157. Heng TS, Painter MW. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* 2008;9:1091–4.
158. Benoist C, Lanier L, Merad M, Mathis D. Consortium biology in immunology: the perspective from the Immunological Genome Project. *Nat Rev Immunol* 2012;12:734–40.
159. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* 2013;39:11–26.