In the past few years, the field of cancer immunotherapy has made great progress and is finally starting to change the way cancer is treated. We are now learning that multiple negative checkpoint regulators (NCR) restrict the ability of T-cell responses to effectively attack tumors. Releasing these brakes through antibody blockade, first with anti-CTLA4 and now followed by anti-PD1 and anti-PDL1, has emerged as an exciting strategy for cancer treatment. More recently, a new NCR has surfaced called V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation (VISTA). This NCR is predominantly expressed on hematopoietic cells, and in multiple murine cancer models is found at particularly high levels on myeloid cells that infiltrate the tumors. Preclinical studies with VISTA blockade have shown promising improvement in antitumor T-cell responses, leading to impeded tumor growth and improved survival. Clinical trials support combined anti-PD1 and anti-CTLA4 as safe and effective against late-stage melanoma. In the future, treatment may involve combination therapy to target the multiple cell types and stages at which NCRs, including VISTA, act during adaptive immune responses. Cancer Immunol Res; 2(6); 510–7. ©2014 AACR.
assignment of ligand and receptor ambiguous or irrelevant. As such, many of the so-called ligands transduce signals themselves. For the purpose and context of this Crossroads overview, "receptor" refers to the surface protein on CTLs and "ligand" is the surface protein on all other cell types that interact with CTLs. In addition to engaging CD28 and CTLA4, CD80 binds to the ligand PDL1, which then transduces a negative signal (Fig. 1; ref. 8). B-lymphocyte and T-lymphocyte attenuator (BTLA) signals negatively following interaction with herpesvirus entry mediator (HVEM; ref. 9), whereas HVEM itself has positive activity (10). Assignment of all family members within the super immunoglobulin (Ig) family has even been breached, as HVEM, a ligand for BTLA, is in the tumor necrosis factor receptor superfamily (TNFRSF). A further cross-family interaction occurs between B7-H6 and natural killer (NK) cell p30-related protein (Nkp30; ref. 11). The complexity of NCRs of the CD28-B7 family must be considered when targeting one or more of its members for therapy.

**CTLA4**

CTLA4 competes with CD28 for interaction with CD80 and CD86 (Fig. 1). Early after T-cell activation, CTLA4 expression on the cell surface is increased as a result of both increased mRNA expression and release from intracellular deposits. CTLA4 can bind CD80 and CD86 at higher affinity than CD28. This acts centrally to limit the extent of T-cell activation by competing with CD28 engagement and interrupting TCR signaling.

The first anti-CTLA4 human monoclonal antibody (mAb), ipilimumab, was approved in 2011 by the FDA for use in metastatic melanoma (12). Following activity in phase II clinical trials, success for ipilimumab was reported in a large phase III clinical trial involving treatment of 676 patients with metastatic melanoma, who had undergone previous failed treatment (13). Patients received 3 mg/kg of ipilimumab with or without a vaccine derived from the melanosomal protein glycoprotein 100 (gp100), or were treated with gp100 alone as an active control. Median overall survival was 10.0 months in the combination group ($P < 0.001$), and 10.1 months with ipilimumab alone ($P = 0.003$), as compared with 6.4 months in the control group. Furthermore, the 1- and 2-year survival rates increased from 25% and 14% in the controls to 46% and 24% with ipilimumab. It is of note that some patients exhibited delayed responses, sometimes with tumor progression before tumor regression. Although ipilimumab is generally believed to act by releasing the brakes on antitumor T-cell responses (14), we speculate that a major therapeutic mechanism of action may be ablation of regulatory T cells (Treg) within the tumor microenvironment (TME).

These results broke new ground as the first immunotherapeutic strategy showing improved survival in metastatic melanoma in a phase III trial. A subsequent phase III study...
compared a higher dose of ipilimumab (10 mg/kg) plus dacarbazine to dacarbazine alone in 502 patients with previously untreated metastatic melanoma (15). The results mirrored the first phase III trial, achieving longer survival times (11.2 vs. 9.1 months) and higher survival rates at 1 year (47.3% vs. 36.3%), 2 years (28.5% vs. 17.9%), and 3 years (20.8% vs. 12.2%). With the efficacy of CTLA4 blockade established, clinical trials are currently addressing ways to build on these results. As discussed below, combination therapies will likely improve the efficacy of immunomodulatory approaches and will be a driving concept of future studies in this field.

PD1

PD1 is a critical NCR, which mitigates inflammation to maintain peripheral tolerance (Fig. 1). Upon activation, T cells and B cells upregulate the NCR PD1, which becomes detectable from the cell surface by 24 hours (16, 17). PD1 then forms negative costimulatory microclusters associated with phosphatase SHP2 (Src homology 2 domain-containing tyrosine phosphatase 2) and TCRs, leading to the dephosphorylation of TCRs and suppression of T-cell functions (18). One of the PD1 ligands, PDL1, can be induced on dendritic cells (DC), monocytes, and macrophages (19). In contrast, the other PD1 ligand, PDL2, is broadly and constitutively expressed on immune cells and throughout the body (Fig. 1; ref. 19). PD1 expression can be increased by stimulation with type I or II interferons (IFN) on T cells, macrophages, and tumors (20, 21). Peripheral PD1 is thought to be important in dampening T cells to prevent autoimmunity or bystander damage in tissues, but it is also implicated in T-cell exhaustion and immune-cell evasion by tumors.

Many studies have described the correlation of PDL1 with invasiveness, metastasis, and poor prognosis. It appears well established that high PDL1 is generally associated with a poor outcome (22, 23). PDL1 expression is upregulated by IFNγ as a defense against collateral damage. It has been suggested that IFNγ produced by tumor-infiltrating lymphocytes (TIL) may be inducing PD1 expression, which in turn suppresses TIL activity, or leads to T-cell anergy or exhaustion (24). Thus, PD1 expression may be a result of TIL infiltration, or even an indicator of tumors for which useful antitumor responses may be generated.

Early clinical trials targeting the PD1 pathway with anti-PD1 or anti-PDL1 have generated much excitement. In phase I trials, compelling rates of objective responses have been achieved. Of particular note are three large phase I trials, which assessed Bristol-Myers Squibb’s anti-PD1 (BMS-936558, nivolumab) and anti-PDL1 (BMS-936559), and Merck’s anti-PD1 mAb (MK-3475, lambrolizumab). In the first trial, 296 patients with advanced melanoma, non–small-cell lung cancer (NSCLC), castration-resistant prostate cancer, renal cell cancer, or colorectal cancer received anti-PD1 over multiple escalation doses. Long-term durable responses were achieved in 18% to 28% in NSCLC, melanoma, and renal cell cancer (25). Interestingly, there was evidence of improved efficacy in patients whose tumors expressed PDL1 (objective responses in 0 of 17 PDL1” tumors and 9 of 25 PDL1” tumors). A similar finding was observed with patients treated concurrently with ipilimumab and nivolumab (26). If this correlation holds true, it remains to be seen whether PDL1 expression is a general marker of tumors with immune interaction that are sensitive to immunotherapy by any NCR, or whether this is a specific requirement for PD1 blockade. The concurrent dose-escalating phase I trial for blockade of PDL1 (BMS-936559) induced durable tumor regression at rates of 6% to 17% in NSCLC, melanoma, and renal cell cancer (27). A recent clinical trial tested another anti-PD1 formulation (lambrolizumab) in 135 patients with advanced melanoma at 10 mg/kg achieving a response rate of over 50% (28).

B7-H3

B7-H3 is another B7 family member that has been implicated as a regulator of tumor immunosurveillance (Fig. 1). Expression of B7-H3 mRNA is broad, detectable in most body tissues and some tumor cell lines (29), but protein expression is detected at relatively low levels. Peripheral blood leukocytes do not express B7-H3, but it can be induced on monocytes, DCs, and T cells by stimulation (30). Like PD1, the activities of B7-H3 have faced controversy. Both in mice and humans, there are reports that show B7-H3 acts as either a costimulatory molecule (29, 31) or a NCR (30, 32, 33). Contributing to the dilemma is the inability to credibly identify the receptor for B7-H3, a feature problematic to a number of members of the NCR family. However, there are data suggesting that the receptor for B7-H3 is expressed on activated T cells and NK cells (29). A receptor called triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2) has been proposed (34), as antibodies against either TLT-2 or B7-H3 inhibited contact hypersensitivity. However, a subsequent study did not find evidence of this interaction (33). The basis for these different findings is not clear; it is possible that different isoforms of B7-H3 may interact with more than one receptor.

As might be expected from both stimulatory and inhibitory activities being detected for B7-H3, this molecule has been reported to have either immune-enhancing or immune-inhibitory impact in murine cancer models. Murine tumor cell lines transfected with B7-H3 result in increased CD8 responses, and therefore grow poorly in vivo or are rejected (35, 36). However, a large number of studies have shown that B7-H3 has detrimental effects in cancer, as it promotes tumor progression and metastasis, and reduces host survival (37). In humans, B7-H3 expression has been observed on many tumor types at varying frequencies (37). In gastric and pancreatic cancer, B7-H3 had a positive effect on survival and infiltration by CTL (38, 39). Interestingly, in clear cell renal cancer, B7-H3 expression was observed on the vasculature in 95% of specimens, but only 17% showed tumor-cell expression (40). Both vasculature and tumor-cell expression were associated with poor outcome. In NSCLC, the soluble form of B7-H3 was found to be a particularly useful biomarker for poor prognosis—better than even more traditional biomarkers such as carcinoembryonic antigen (CEA; ref. 41). As argued by Loos and colleagues (37), given the understanding that the level of expression of costimulatory molecules may influence their functional effect (42), both the intensity of staining, and the cell type involved should be taken into account in these kinds of studies.
B7-H4

Another promising NCR for cancer immunotherapy is B7-H4 (Fig. 1), whose binding partner remains unknown. B7-H4 is also known as B7x, B7 superfamily member 1 (B7-S1), and V-set domain–containing T-cell activation inhibitor 1 (VTCN1). B7-H4 is expressed on B cells and other antigen-presenting cells (APC). IL6 and IL10 increase its expression, while granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL4 reduce its expression (43). In murine models, B7-H4 has been shown to inhibit T-cell activation and cytokine production in vitro and in vivo, by antibody blockade, Fc fusion protein, and/or transfection on APCs (44–46). A role for B7-H4 on innate cells has been elucidated, as B7-H4 knockout mice are more resistant to Listeria monocytogenes infection, even in the absence of T cells in double knockout mice with recombination-activating gene-1 deficiency. The resistance to infection seems to be related to the elevated neutrophil numbers due to increased proliferation of neutrophil progenitors (47).

B7-H4 is an Ig protein with a well-established role as a biomarker for progression in cancers. B7-H4 is detected on a variety of tumors, including melanoma, NSCLC, prostate, ovary, stomach, pancreas, breast, esophagus, and kidney cancers, with expression on infiltrating myeloid cells, vasculature, and tumor cells (Fig. 1; refs. 43, 48). Most studies show a potential prognostic role correlating B7-H4 expression with tumor-stage grade biologic behavior, recurrence, and survival rate. The biologic activity, nonoverlapping expression pattern, and correlation with cancer progression are rationale for the development of B7-H4 as a new target for immunotherapy.

In addition to the CD28-B7 family of proteins, other Ig superfamily proteins may be targets for tumor immunotherapy. Two promising candidates are lymphocyte-activated gene-3 (LAG-3) and T-cell Ig- and mucin-domain–containing molecule-3 (TIM-3). LAG-3 is induced upon T-cell activation and binds major histocompatibility complex (MHC) class II molecules, leading to inhibition of TCR-induced calcium fluxes (49, 50). TIM-3 is expressed on activated T-helper 1 (Th1) cell subsets and interacts with Galectin-9, leading to suppression of macrophages and apoptosis of T cells (51, 52). Importantly, these molecules are expressed on exhausted T cells, and there is evidence that the activity of LAG-3 and TIM-3 may be synergistic with PD1 in this context (53–55).

VISTA—A New Immunomodulatory Target with Broad-Spectrum Activities

A new target for cancer immunotherapy is the Ig superfamily member V-domain Ig-containing suppressor of T-cell activation (VISTA, Entrez: 64115), also known as C10orf54, differentiation of ESC-1 (Dies1), platelet receptor GII4 precursor, or PD1 homolog (PD1H). The extracellular domain of VISTA is most similar to that of PDL1; however, VISTA possesses some unusual structural features. VISTA does not cluster with the CD28-B7 family at standard confidence limits, and therefore is rather weakly associated with the rest of the group (56). Although studies using Fc fusion proteins clearly show that VISTA has ligand activity (56, 57), receptor-like signaling activity has also been described (58). Indeed, the extracellular domain consists of a single IgV (variable region) domain, like the receptors in the family. Additionally, a recent study utilizing VISTA knockout mice demonstrated that endogenous VISTA has inhibitory effects both as a ligand on APCs and as a receptor on T cells (58). The binding partner(s) of VISTA responsible for these activities is currently unknown (Fig. 1).

In both mice (56) and humans (57), VISTA is expressed predominantly on hematopoietic cells with the greatest densities on myeloid and granulocytic cells, and weaker expression on T cells. Similar to some members of the B7-CD28 family (e.g., PDL1; ref. 8), T cells both express and respond to VISTA. VISTA–Fc fusion protein and cellular overexpression of VISTA are suppressive to T-cell activation, proliferation, and cytokine production (56, 57). In vivo blockade of VISTA was found to enhance the T-cell response to OVA, and to exacerbate the development of experimental autoimmune encephalomyelitis (EAE; ref. 56). Interestingly, in a separate study, blockade with anti-VISTA and VISTA–Fc fusion protein was found to inhibit acute graft-versus-host disease (59). This immunosuppressive effect of VISTA may be due to the ability of specific anti-VISTA antibodies to deplete VISTA-bearing cells.

Both naive and antigen-experienced cells are sensitive to VISTA-induced suppression (56, 57), which suggests that in addition to constitutive expression of VISTA, the receptor may be constitutively expressed on resting T cells. Therefore, we speculate that VISTA may act as a rheostat to prevent promiscuous resting T-cell responses to self-antigens. In support of this notion, findings from Flies and colleagues (58) and our own studies show that mice lacking VISTA expression have elevated frequencies of activated T cells with a systemic proinflammatory phenotype.

VISTA in Cancer

The potential of VISTA to act as an NCR in the setting of cancer was first demonstrated by Wang and colleagues in a murine model of methylcholanthrene 105 (MCA105)–induced fibrosarcoma (56). MCA105 tumor cells engineered to overexpress VISTA–RFP were shown to grow in animals with immunity protective against the growth of control MCA105 cells. More recently, Le Mercier and colleagues described elevated VISTA expression on leukocytes within the TME and tumor-draining lymph nodes in murine cancer models (60). They also examined the use of an anti-VISTA mAb for intervention and found that VISTA blockade impaired tumor growth, with particularly dramatic results when used in combination with a tumor vaccine (60). Within the TME, a shift was generated toward antitumor immunity with elevated infiltration, proliferation, and T-cell effector function.

As compared with the activity of anti-CTLA4, anti-PD1, and anti-PDL1 in preclinical studies, the VISTA blockade data appear compelling (61–63). Similar to the findings of Wang and colleagues (56), Sorensen and colleagues reported melanoma regression and long-term survival rates of 30% to 40% in a study of CTLA4 blockade in combination with CD40 stimulation and the administration of an adenoviral vaccine (62).
Following the promising studies of VISTA in murine models of cancer, we initiated studies to evaluate VISTA expression in human cancers. As anticipated, we did not detect VISTA expression in a commercially available panel of cDNAs isolated from human nonhematopoietic tumor cell lines (data not shown; Human Cell Line MTC Panel; Clontech). The mouse data suggest that VISTA is exclusively expressed on leukocytes infiltrating the tumor. Therefore, VISTA expression was examined on colon and lung cancer lesions by fluorescence-based multiplex immunohistochemistry (IHC) as previously described using the GG8 clone of anti-human VISTA (57). When present, VISTA expression was confined predominantly to the infiltrating CD11b⁺ cells in the TME of colon cancer lesions, whereas infiltrating VISTA⁺ cells in some cases, but not others, expressed CD11b in lung cancer cells (Fig. 2). In all examined cases, we did not observe coexpression of VISTA and CD8 in infiltrating cells. However, lower VISTA expression levels on CD8⁺ T cells may be below the detection limits of this antibody by IHC staining. As the composition of the immune-cell infiltrates varies between tumors within the same cancer site, and in tumors of different cancer types (Fig. 2), we would predict that a frank myeloid infiltrate in tumor lesions would express high levels of VISTA. Future studies in larger cohorts of patients will be needed to identify tumor characteristics that may be associated with VISTA expression in the TME.

Two particularly interesting points for VISTA immunotherapy from observations in the mouse models are that VISTA blockade was effective without any detectable expression of VISTA on the tumor cells, and that VISTA blockade works even in the presence of high PDL1 expression (60). The lack of requirement for VISTA expression on the tumor suggests that VISTA blockade may have broad clinical applicability, and is potentially an advantage over PD1 or PDL1 blockade, which may require expression on the target tumor (25). Although PDL1 may shield tumor cells from immunosurveillance, VISTA blockade is still sufficient to allow antitumor activity to develop within the TME. These data suggest that VISTA activity in the TME is important for antitumor immunity, and that VISTA blockade may be a promising immunotherapy strategy. Because the VISTA and PD1 checkpoint pathways are independent, there may be potential synergy when they are targeted in combination.

**Personalizing Anti-VISTA Approaches in Cancer Immunotherapy**

Strategies for the use of specific NCR-based immunotherapy will be guided by the signature of NCRs that are observed...
within the TME (Fig. 2). This would involve screening of individual patients for markers that allow tailored blockade to their specific tumor—for example PD1 blockade for patients with tumors that are PDL1 rich. Likewise, greater efficacy may be achieved by introducing NCR blockade into a treatment regimen both within the period for the best therapeutic window—before tumors have undergone extensive immunodediting and lost immunogenicity—and to achieve the best interactions with conventional treatments. By their nature of targeting proliferating cells, standard cancer treatments are often immunosuppressive—however, locally, these effects can be immunostimulatory (64, 65). Release of antigen and damage-associated molecular patterns (DAMP) may recruit and activate TILs in the TME. In addition, homeostatic proliferation of leukocytes after depletion by chemotherapy may boost immune reactions stimulated concurrently. The timing of chemotheraphy may be important for combination with immune-stimulating strategies. A recent trial combined ipilimumab with paclitaxel and carboplatin, either as a phased regimen (two doses of chemotherapy followed by four doses of chemotherapy plus ipilimumab) or concurrent (four doses of chemotherapy plus ipilimumab followed by two doses of chemotherapy; ref. 66). Interestingly, the phased regimen was effective in increasing progression-free survival, but the concurrent treatment was not.

As described above, the signaling pathways for VISTA, PD1, and CTLA4 are quite distinct. This suggests that first, failure of one blockade strategy does not necessarily mean that a patient would fail in the other strategy, and second that multiple blockade agents could be combined synergistically (Fig. 2). Two phase I trials testing either Merck’s anti-PD1 antibody lambrolizumab or the Bristol-Myers Squibb’s anti-PD1 nivolumab did not find a difference between patients who had already undergone ipilimumab therapy (anti-CTLA4) as compared with those who had not (28, 67). Combinations of PD1, CTLA4, VISTA, and/or LAG-3 blockades in preclinical studies support synergy (refs. 53, 58; Wang and colleagues; manuscript in preparation). A recent trial testing concurrent treatment of ipilimumab and nivolumab achieved a 40% overall objective response rate, and 53% at the highest dose groups (26). This indicates that the combination of antibodies for NCR blockade is both safe and clinically effective. The combination of GM-CSF with ipilimumab has shown a trend toward improved treatment tolerance and a significant improvement in survival in an ongoing phase II trial (69). One of the advantages of VISTA blockade as an immunotherapeutic strategy is that VISTA expression is not required on the tumor cells. Instead, the relevant VISTA-expressing cells seem to be the myeloid infiltrate (60). A combinatorial approach may use some permutation of the following: vaccine and ipilimumab to generate new clonal T-cell expansion, VISTA blockade to counteract some of the suppressive activity of the infiltrating myeloid cells, PD1 or PDL1 blockade to release anergic CTLs, and either B7-H4 or PD1 blockade to release the immunogenicity of the tumor (Fig. 2). As we learn more about the signature of NCR expression in the TME across human cancers, better strategies for combinatorial therapeutic intervention may be devised. 

**Future Directions**

To improve the clinical effectiveness of NCR blockade, a clearer understanding of relevant biomarkers would be of great benefit. There is a need for markers both to predict how successful NCR blockade might be in a patient, and also to determine which NCR(s) should be targeted for tailored treatment. On the other end of the treatment process, biomarkers that indicate successful intervention are desirable. Treatment of cancers with NCR blockade can lead to delayed responses, sometimes even with tumor progression before tumor shrinkage. It has been proposed that RECIST criteria are potentially not the best criteria to use for evaluation of immunotherapeutic approaches, although the use of alternative criteria is controversial (1).

For VISTA, many questions still remain, not least being the identity of the receptor. As with B7-H3 and B7-H4, answering this question may be challenging, but would facilitate therapeutic development. Studies are under way examining how combination therapy of anti-VISTA with vaccines and/or blockade of other NCRs may be used to create synergistic responses. Although VISTA has been observed within the TME in human tumors, a comprehensive study of the correlation of VISTA expression with patient outcome in different tumor types is warranted. Antibodies targeting VISTA for cancer immunotherapy are already under development by Johnson & Johnson and VISTA may soon be a part of the immunotherapy revolution.

**Disclosure of Potential Conflicts of Interest**

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