Therapeutic Targeting of Checkpoint Receptors within the DNAM1 Axis

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ABSTRACT
Therapeutic antibodies targeting the CTLA4/PD-1 pathways have revolutionized cancer immunotherapy by eliciting durable remission in patients with cancer. However, relapse following early response, attributable to primary and adaptive resistance, is frequently observed. Additional immunomodulatory pathways are being studied in patients with primary or acquired resistance to CTLA4 or PD-1 blockade. The DNAM1 axis is a potent coregulator of innate and adaptive immunity whose other components include the immunoglobulin receptors TIGIT, PVRIG, and CD96, and their nectin and nectin-like ligands. We review the basic biology and therapeutic relevance of this family, which has begun to show promise in cancer clinical trials.

Significance: Recent studies have outlined the immuno-oncologic ascendancy of coinhibitory receptors in the DNAM1 axis such as TIGIT and PVRIG and, to a lesser extent, CD96. Biological elucidation backed by ongoing clinical trials of single-agent therapy directed against TIGIT or PVRIG is beginning to provide the rationale for testing combination regimens of DNAM1 axis blockers in conjunction with anti-PD-1/PD-L1 agents.

INTRODUCTION
Immunostimulatory monoclonal antibodies (mAb) that block inhibitory checkpoint proteins such as CTLA4 or PD-1 and PD-L1 have made a paradigm shift in the development of anticancer therapies. Since the early studies targeting these checkpoints in the 2000s, roughly 10 additional coinhibitory molecules have been targeted clinically. The biology of these molecules is complex, in part because most T-cell regulatory pathways are multicomponent. CTLA4 (or CD152) is a homolog of the costimulatory receptor CD28, with both capable of binding the ligands CD80 and CD86. Reflecting its status as a key regulator of autoimmunity, CTLA4 displays an affinity for CD80 and CD86 that is several orders of magnitude greater than that of CD28 (1). CTLA4 outcompetes CD28 for access to their shared ligands and delivers inhibitory signals that dampen T-cell receptor (TCR; signal 1) and costimulatory signals (signal 2).

A similarly inducible inhibitory checkpoint receptor whose discovery postdates that of CTLA4 is PD-1, which upon binding to one of its two defined ligands, PD-L1 or PD-L2, restricts T-cell activation (2). In the clinic, several mAbs targeting either PD-1 or PD-L1 in solid tumors were tested before the approval of the first PD-1 blockers, nivolumab and pembrolizumab (3, 4). A mAb targeting CTLA4, three mAbs targeting PD-1, and three targeting PD-L1 have been approved by the FDA for treating 18 different malignancies (5, 6). Exploratory research is ongoing to discover novel checkpoints and eventually generate mAbs that could potentially augment the clinical results observed with PD-1 or CTLA4 blockade. With that in mind, additional T cell–specific inhibitory receptors have been studied extensively in preclinical cancer models and are being tested in the clinic either as single agents or in combination with other checkpoint blockers or chemotherapy. Among these, lymphocyte-activation gene 3 (LAG3), T-cell Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT), T-cell Ig and mucin domain 3 (TIM3), and B and T lymphocyte attenuator (BTLA4) have all been shown to overcome T-cell exhaustion in the tumor microenvironment (TME) and are considered to be promising druggable targets in the immuno-oncology space (7–10). Although blockers of each of these inhibitory checkpoints are in varying stages of clinical testing, none have secured FDA approval for use in cancer. Antibodies targeting TIM3 have shown encouraging preliminary antitumor effects in patients with hematologic malignancies, including myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML; ref. 11). The
DNAM1 antagonist antibody (20, 21). Overexpression of PVR or PVRL2 in the RMA mouse tumor cell line increased tumor rejection and host survival via T- and NK-mediated mechanisms, suggesting that each can act as costimulatory ligands in the TME, presumably via their activation of DNAM1. However, identification and characterization of the immune-checkpoint proteins TIGIT, CD96, and PVRL1 have generated a more complex picture of their function, because the interaction of PVR with TIGIT and CD96, and that of PVRL2 with PVRL1, can also mediate suppressive signaling through the co-inhibitory arms of the DNAM1 signaling axis, suggesting that they act to counterbalance DNAM1 costimulation. At the 30,000-foot level, this is analogous to CD28 and CTLA4 as counterbalancing costimulatory and co-inhibitory receptors, respectively, for CD80 and CD86; however, at a closer glance, there are significant differences that give the DNAM1 family its unique character. Although CD28 is expressed exclusively on T cells, DNAM1 axis members are expressed on both T and NK cells. In addition, CD28 is downregulated on T cells upon activation and differentiation, whereas DNAM1 maintains its expression also on differentiated effector cells (22, 23). Furthermore, CD80 and CD86 are restricted to immune cells, mostly antigen-presenting cells (APC), whereas PVR and PVRL2 are expressed on multiple cell types, including APC and tumor and endothelial cells. These differences in expression pattern suggest that whereas CD28 and CTLA4 probably play a dominant role in T-cell priming, DNAM1 axis members presumably regulate both priming and effector phase of T cells, resembling the PD-1 pathway. Nevertheless, similar to the higher-affinity CD80/86 interactions with CTLA4 versus CD28, TIGIT, CD96, and PVRL1 have higher affinities to their cognate ligands than DNAM1 and thus can inhibit T-cell and NK-cell activity through ligand competition (24–28). This was supported by several studies showing that the costimulatory effects driven by targeting the TIGIT/PVR interaction are dependent on the engagement of DNAM1 by PVR (27, 29). A recent study suggested that the infiltration of CD8\(^+\)DNAM1\(^{hi}\) T cells into TME may predict
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The efficacy of TIGIT blockade, as the presence of these cells is essential for the anti-TIGIT immunomodulatory activity (30). Additional complexity arises from the direct interruption of DNAM1 homodimerization by TIGIT, which results in its impaired functionality (29). Furthermore, DNAM1 expression on lymphocytes is modulated by membrane PVR that upon binding to DNAM1 induces its internalization and degradation, resulting in decreased antitumor function (31). Similarly, it was shown that PVRIG outcompetes DNAM1 for PVRL2 binding (ref. 32; Fig. 1).

One approach to boost antitumor immunity by modulating the DNAM1 axis could arise from agonizing DNAM1, which has the potential to show therapeutic benefit in patients with cancer due to its potent T- and NK-cell coactivatory function. However, a caveat for this approach is the broad expression of DNAM1 on different cell types, including on platelets, where it was shown to stimulate activation and cell adhesion (33). Indeed, an agonist antibody directed against DNAM1 (LY3435151) was clinically evaluated in solid malignancies; however, the study (NCT04099277) was recently terminated for undisclosed reasons.

Another interesting approach could be the direct targeting of PVR or PVRL2 with antagonist antibodies. This may result in direct targeting may result in interference of DNAM1 costimulatory signaling, as well as in the induction of ADCC against APCs. Finally, PVR and PVRL2 are broadly expressed in peripheral normal tissues, creating a large target sink and increasing the risk of developing on-target, off-tumor toxicities. Indeed, thrombocytopenia was identified in monkeys administered an anti-PVRL2 mAb (36).

Altogether, costimulatory DNAM1 activity in T and NK cells is tightly regulated at the expression level and by its interaction with other axis members; understanding the biology of these interactions is crucial for the success of immuno-therapies targeting this axis.

TIGIT Blockade: A Promising New Clinical Modality for Treating Solid and Hematologic Malignancies

TIGIT was initially identified as an inhibitory receptor on T and NK cells based on shared gene and protein structure with known immune checkpoints (8, 24). TIGIT is also expressed on regulatory T cells (Treg) where it enhances their activation and suppressive activity (Fig. 2A). Similar to other immune checkpoints, TIGIT contains an extracellular Ig variable domain, a transmembrane domain, and an ITIM in the intracellular signaling domain (8, 24). The dominant ligand for TIGIT is PVR with a binding affinity (KD) reported as 1–3 nmol/L (Fig. 1; ref. 24). A recent study described Nectin4 (PVRL4) as a functional ligand of TIGIT with an affinity similar to that of TIGIT/PVR (37). PVRL2 and PVRL3 (CD113) have also been reported to bind TIGIT albeit at much weaker affinities (8, 19, 24). PVR expression was shown to positively correlate with an unfavorable prognosis in multiple malignancies such as lung adenocarcinoma (38) and breast (39),

Figure 2. The effects of DNAM1 axis modulation on immune cells. DNAM1 axis molecules and nectin and nectin-like ligands are expressed on various cells, and their interactions modulate immune function. A, TIGIT and DNAM1 exert opposite effects in Tregs upon PVR binding. TIGIT further stimulates Treg immunomodulatory activity, whereas DNAM1 disrupts Treg suppression. B, Although DNAM1 stimulates effector T- and NK-cell function, TIGIT and PVRIG induce their suppression. PVR engagement on dendritic cells by TIGIT induces a tolerogenic phenotype and reduces proinflammatory cytokine secretion.
panoramic melanoma, PVR expression on tumor cells was associated with resistance to anti–PD-1 immunotherapy (41). As mentioned above, TIGIT’s higher binding affinity for PVR compared with DNAM1 allows it to outcompete DNAM1 for ligand binding, similar to CTLA4 versus CD28 (21, 24, 27, 28). Although the expression of both CTLA4 and TIGIT is induced upon activation, CD28 is mainly expressed on naive T cells, whereas DNAM1 has broad expression on multiple T-cell subsets (23). Moreover, unlike CTLA4, TIGIT has been shown to directly interact with DNAM1, reducing the ability of DNAM1 to homodimerize and enhance immune activation (27, 29, 42). Recently, it was demonstrated that the TIGIT/PVR interaction induces dephosphorylation of DNAM1 at tyrosine 322 and interferes with its costimulatory responses on T cells (30). In addition to counteracting the stimulatory function of DNAM1 via direct binding and competition for their common PVR ligand, TIGIT can directly inhibit T- and NK-cell effector function by signaling through an ITIM domain (8, 43). TIGIT also indirectly regulates CD8+ T-cell priming and proliferation by engaging PVR on dendritic cells, which reportedly induces a tolerogenic phenotype and reduces their proinflammatory cytokine production via “reverse signaling” (Fig. 2B; ref. 24).

Several studies have shown that TIGIT is a marker of T-cell dysfunction and is upregulated on human viral-specific CD8+ T cells, and human CD8+ and CD4+ T cells infiltrating a variety of solid and hematologic tumors. In the LCMV Clone 13 model of chronic viral infection, Johnston and colleagues demonstrated that TIGIT is highly expressed on CD8+ T cells that also express high levels of PD-1 (29), and that inhibition of these pathways increased viral clearance and T-cell effector function. Similarly, in murine models of colon and breast carcinomas, coblockade of TIGIT and PD-L1 resulted in tumor rejection and restoration of CD8+ T cells within the TME (29). Johnston and colleagues further demonstrated that mice treated with anti-TIGIT and anti–PD-L1 were unable to reject CT26 tumors when depleted of CD8+ T cells (29). Chauvin and colleagues also demonstrated that the TIGIT pathway blockade either alone or in combination with anti–PD-1 synergistically increased effector function of human NY-ESO-1–specific CD8+ tumor-infiltrating lymphocytes (TIL) from patients with melanoma (44). Collectively, these data demonstrate that TIGIT and PD-1/PD-L1 cloblockade acts through CD8+ T cells to generate an effective antitumor immune response. Interestingly, TIGIT was shown to be uniquely expressed by T memory stem (Tscm) cells, an emerging population of T cells, shown to be important for response to immunotherapy. Tscm cells display a functional state associated with enhanced self-renewal, have multipotency to generate memory and effector T-cell subsets, and were found to be associated with increased response to immunotherapy and to correlate significantly with PD-1 blockade therapeutic activity (45–47). Importantly, TIGIT and PD-1 are the dominant inhibitory T-cell checkpoints expressed on this memory cell subset (48).

Other studies reported that TIGIT predominantly suppresses antitumor CD8+ T-cell responses indirectly by enhancing Treg function in the TME (49–51). Kurtulüs and colleagues found that adoptively transferred TIGIT-deficient CD8+ T cells and wild-type CD4+ T cells into B16F10 melanoma tumor–bearing RAG-deficient mice did not alter tumor growth compared with transfer of wild-type TIGIT+ CD8+ T cells (49). In contrast, the transfer of TIGIT-deficient Tregs and wild-type CD4+FOXP3+ and CD8+ T cells significantly delayed B16F10 tumor growth compared with mice that received wild-type Tregs. These data suggest that in at least some tumor models, TIGIT+ Tregs play a more dominant role in suppressing antitumor immune responses compared with direct signaling of TIGIT on CD8+ T cells. Other studies have examined the mechanisms of TIGIT+ Treg-mediated immune suppression. Joller and colleagues showed that increased amounts of IL10 produced by TIGIT+ Tregs contributed to the generation of tolerogenic dendritic cells, thereby inhibiting the generation of effector T-cell responses (42, 51). Moreover, TIGIT blocking mAbs were shown, in addition to enhancing conventional T-cell activity, to reduce Treg secretion of IL10 (52). Finally, a recent study showed that Tregs in melanoma further upregulate TIGIT and downregulate DNAM1 expression, resulting in a higher TIGIT/DNAM1 ratio (53). This high TIGIT/DNAM1 ratio in Tregs regulates their suppressive function and stability and correlates with a poor clinical outcome following PD-1 and/or CTLA4 pathway blockade in patients with melanoma (44, 49, 53).

In addition to TIGIT’s role in T-cell biology, numerous groups have shown higher TIGIT expression on viral and tumor-infiltrating NK cells and that increased TIGIT levels are negatively correlated with the ability of NK cells to secrete proinflammatory cytokines and kill PVR-expressing tumor cells, suggesting that TIGIT abrogates NK-cell activation (Fig. 2B; refs. 8, 43, 54). In mouse models, lack of TIGIT+ NK cells was sufficient to slow B16 melanoma tumor growth, while antibody blockade of the TIGIT pathway reinvigorated the antitumor NK-cell response in multiple solid tumors and hematologic models (43, 55–57). Overall, survival was increased in these tumor models, indicating that NK cells may be critical for the therapeutic effects of TIGIT pathway–based immunotherapies (56, 57). Collectively, these studies demonstrate important roles for TIGIT-expressing CD8+ T cells, Tregs, and NK cells in regulating antitumor responses in mouse tumor models and human in vitro functional systems.

An open question in the TIGIT therapeutic antibody field is whether Fc receptor (FcR) coengagement is required to exploit the full clinical potential from TIGIT blockade. Multiple antibodies targeting TIGIT are currently in clinical development, some with FcR binding (typically human IgG1) and others with reduced binding to FcR (Table 1). Murine studies with anti-TIGIT mouse IgG2a antibodies (increased FcR-binding capacity) demonstrate monotherapy activity in vivo and increased potency compared with mouse IgG1 Abs (reduced FcR-binding capacity; refs. 58, 59). Recently, Yang and colleagues demonstrated that TIGIT blockade induced Fc-mediated depletion of Tregs that activate antitumor CD8+ T-cell responses, targeting tumor-shared antigens that are normally cryptic or suppressed by Tregs (60). In contrast, a mouse IgG1 anti-TIGIT antibody (low FcR-binding capacity) has been shown to significantly enhance the antitumor activity, particularly when combined with a blocking anti–PD-L1 antibody (53). Ultimately, the ability of mouse models to predict the importance of FcR binding by a therapeutic
Table 1. List of antagonistic antibodies targeting DNAM-1 axis family members that are under preclinical and clinical development

<table>
<thead>
<tr>
<th>Company</th>
<th>Compound</th>
<th>Target</th>
<th>Antibody isotype</th>
<th>Development stage</th>
<th>Combination therapy</th>
<th>Patient population</th>
<th>ClinicalTrials.gov identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roche</td>
<td>MTIG7192A tiragolumab</td>
<td>TIGIT</td>
<td>IgG1</td>
<td>Phase III</td>
<td>Atezolizumab, carboplatin + etoposide, rituximab, daratumumab, carboplatin + paclitaxel, cisplatin</td>
<td>NSCLC, cervical cancer, multiple myeloma, non-Hodgkin lymphoma, B-cell lymphoma, gastric adenocarcinoma, urothelial carcinoma, pancreatic adenocarcinoma</td>
<td>NCT04300647, NCT04294810, NCT04256421, NCT04045028, NCT03563716, NCT02794571, NCT03281369, NCT03869190, NCT03193190</td>
</tr>
<tr>
<td>Merck</td>
<td>MK-7684 vibostolimab</td>
<td>TIGIT</td>
<td>IgG1</td>
<td>Phase II</td>
<td>Pembrolizumab, carboplatin + paclitaxel, pemetrexed, MK-1308 (anti-CTLA4 mAb)</td>
<td>Advanced solid tumors, NSCLC, melanoma</td>
<td>NCT02964013, NCT04165070, NCT04305054, NCT04305041, NCT04303169</td>
</tr>
<tr>
<td>Arcus Biosciences</td>
<td>AB154</td>
<td>TIGIT</td>
<td>Inactive IgG1</td>
<td>Phase III</td>
<td>AB122 (anti-PD-1 mAb), AB928 (adenosine receptor antagonist) Durvalumab</td>
<td>Advanced solid tumors, NSCLC</td>
<td>NCT03628677, NCT04262856</td>
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<tr>
<td>BMS</td>
<td>BMS-986207</td>
<td>TIGIT</td>
<td>Inactive IgG1</td>
<td>Phase I/IIa</td>
<td>Nivolumab, pomalidomide + dexamethasone</td>
<td>Advanced solid tumors, multiple myeloma</td>
<td>NCT02913313, NCT04150965</td>
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<tr>
<td>Mereo</td>
<td>OMP-313M32 etigilimab</td>
<td>TIGIT</td>
<td>IgG1</td>
<td>Phase I</td>
<td>Nivolumab</td>
<td>Advanced solid tumors</td>
<td>NCT03119428</td>
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<tr>
<td>Compugen</td>
<td>COM902</td>
<td>TIGIT</td>
<td>IgG4</td>
<td>Phase I</td>
<td>N/A</td>
<td>Advanced malignancies</td>
<td>NCT04354246</td>
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<tr>
<td>iTeos</td>
<td>EOS884448</td>
<td>TIGIT</td>
<td>IgG1</td>
<td>Phase I</td>
<td>N/A</td>
<td>Advanced malignancies</td>
<td>NCT04335253</td>
</tr>
<tr>
<td>Innovent</td>
<td>IBI939</td>
<td>TIGIT</td>
<td>N/A</td>
<td>Phase I</td>
<td>Sintilimab (anti-PD-1 mAb)</td>
<td>Advanced malignancies</td>
<td>NCT04353830</td>
</tr>
<tr>
<td>Beigene</td>
<td>BGB-A1217</td>
<td>TIGIT</td>
<td>IgG1</td>
<td>Phase I</td>
<td>Tislelizumab (anti-PD-1 mAb)</td>
<td>Metastatic solid tumors</td>
<td>NCT04047862</td>
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<tr>
<td>Seattle Genetics</td>
<td>SGN-TGT</td>
<td>TIGIT</td>
<td>Effector-enhanced IgG1</td>
<td>Phase I</td>
<td>Pembrolizumab</td>
<td>Advanced malignancies</td>
<td>NCT04254107</td>
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<tr>
<td>EMD Serono</td>
<td>M6223</td>
<td>TIGIT</td>
<td>N/A</td>
<td>Phase I</td>
<td>Bintrafusp alfa (anti-PD-L1/TGFβ Trap)</td>
<td>Metastatic solid tumors</td>
<td>NCT04457778</td>
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<tr>
<td>Compugen</td>
<td>COM701</td>
<td>PVRIG</td>
<td>IgG4</td>
<td>Phase I</td>
<td>Nivolumab</td>
<td>Advanced solid tumors</td>
<td>NCT03667716</td>
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<td>GSK</td>
<td>GSK6097608 CD96</td>
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<td>Phase I</td>
<td>Dostarlimab</td>
<td>Neoplasms</td>
<td></td>
<td>NCT04446351</td>
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</table>

Abbreviation: N/A, not applicable.

antibody is limited, because murine FcRs do not mirror the structural diversity, expression, and Fc binding pattern of the human FcR (61). Moreover, given the overlap in TIGIT expression between CD8+ effector T cells and Tregs, a TIGIT antibody with potent effector function carries the risk of depleting the effector CD8+ TILs in addition to tumor Tregs. Studies on mechanisms of action of CTLA4 blockade emphasize caution in the direct extrapolation of FcR roles...
between mouse and human. Although previous murine studies showed that antitumor effects of a FcR-binding mouse IgG2a anti-CTLA4 were dependent on FcR expression and were associated with selective intratumoral depletion of Treg, ipilimumab, a human IgG1 that binds human FcR, failed to decrease intratumoral Treg density (62). Likewise, PD-L1 blockade was shown to require FcR engagement for mediating activity in vivo in mice (17), whereas in the human settings, anti–PD-L1 antibodies with silent Fc backbones exhibited clinical efficacy and are approved by the FDA. Therefore, it remains to be determined in patients which cell type plays a dominant role in generating antitumor responses during anti-TIGIT therapy and whether FcR engagement is a required feature in the clinic.

Another unanswered question being investigated in clinical trials is which treatments should be paired with anti-TIGIT therapy to generate robust and durable antitumor responses, especially in anti–PD-1/PD-L1-resistant patients. Although TIGIT is highly coexpressed with PD-1 on human TILs from different solid tumors and lymphomas, its expression also highly correlates with the expression of other checkpoint receptors, including LA13, TIM3, and BTLA4 (63–65). One study showed that TIM3 pathway blockade in TIGIT-deficient mice acts synergistically to reduce B16F10 melanoma tumor growth (49). Recently, we demonstrated that PVRL1/PVRL2 blockade induces TIGIT expression (26), and combining TIGIT with PVRL1/PVRL2 coblockade synergistically activates T cells in human functional assays which are translated into in vivo antitumor effects (66). Furthermore, TIGIT-blocking mAbs are being tested clinically in combination with other therapeutic modalities such as chemotherapy, IL15 cytokines, and myeloma-targeting antibodies (31).

Although the majority of anti-TIGIT antibodies are currently in early clinical investigation, several have progressed beyond phase I studies. These include tiragolumab (Roche-Genentech), which is currently recruiting patients in phase III studies in non–small cell lung cancer (NSCLC) and small cell lung cancer (SCLC); domnalamib (Arcus), initiating a phase III studies in NSCLC; and vibostolimab (Merck). Initial results have been presented from three clinical studies evaluating anti-TIGIT antibodies as a single agent and have demonstrated limited clinical activity, with overall response rates ranging from 0% to 3% (67, 68). Recently, data were reported from a randomized, phase II double-blind, placebo-controlled study of 135 patients with previously untreated NSCLC who were randomized 1:1 to tiragolumab plus atezolizumab versus placebo plus atezolizumab (67). The study met its coprimary endpoints of objective response rate (ORR) and progression-free survival for the combination versus atezolizumab alone in the intent-to-treat (ITT) population, demonstrating an improvement in ORR of 31.3% compared with 16.2%, and in median progression-free survival of 5.4 months compared with 3.6 months. Thus, this study provided the first proof of concept for TIGIT inhibition in a larger, randomized clinical trial. The clinical benefit derived from the combination was particularly effective in the PD-L1-positive ≥ 50% population, demonstrating a 66% ORR and a 70% reduction in the risk of disease worsening or death. This combination is currently being evaluated in a phase III study in PD-L1 ≥ 50% first-line NSCLC.

### CD96: A SECOND PVR-BINDING RECEPTOR WITH UNCLEAR FUNCTION IN HUMAN AND MOUSE

Originally described as T-cell activation, increased late expression (TACTILE), CD96 was discovered two decades ago as a member of the Ig superfamily that is highly upregulated on activated T and NK cells and mediates cell adhesion via interaction with its dominant ligand PVR (20, 69, 70). CD96 has an expression pattern similar but not identical to TIGIT, including on hematopoietic stem cells, αβ and γδ T cells, NK cells, and a subpopulation of B cells in humans (23, 71–75) and mice (23, 75, 76). It is highly expressed on human tumor-infiltrating CD8+ T cells, compared with normal tissues (23, 77). Moreover, its expression on T cells is increased upon stimulation, resembling the expression profile kinetics of DNAM1 (23). Like TIGIT, the high-affinity binding partner for CD96 is PVR. Although the binding affinity of CD96 to PVR is higher than that of DNAM1, it is lower than that of TIGIT (Fig. 1; ref. 24). CD96 also binds PVRL1 (CD111), but the functional relevance of the CD96/PVRL1 interaction has not been fully explored (75, 78).

The functional role of CD96 has been mainly investigated in murine NK cells. CD96 appears to act as an inhibitory receptor in murine NK cells, decreasing NK-cell activation. Chan and colleagues demonstrated that in CD96-deficient mice, NK cells produced greater IFNγ in response to LPS, IL12, or IL18 stimulation (76). A role for CD96 in resistance to experimental melanoma lung metastases, MCA-induced fibrosarcomas, and prostate carcinomas was also demonstrated in CD96-deficient mice or following anti-CD96 antibody treatment in wild-type mice. Combined blockade of CD96 and PD-1 or CTLA4 pathways increased cytokine secretion and survival in tumor-bearing mice in comparison with anti–PD-1 or anti-CTLA4 alone (76, 79). Furthermore, an additional report found that in mice that lack both PD-1 and CD96, or both TIGIT and CD96, there is enhanced tumor growth inhibition and/or complete response in dual receptor–deficient mice compared with mice lacking PD-1, TIGIT, or CD96 alone (80). Although CD96 was originally identified as an Ig receptor expressed on activated T cells, there are very few studies examining the functional role of CD96+ T cells. In addition to its inhibitory role in NK cells, recently, Mittal and colleagues showed that CD96 blockade suppressed tumor growth of several murine models in a CD8+ T cell–dependent manner (77). The antitumor efficacy of CD96 was mediated by increasing IFNγ secretion and was dependent on DNAM1, as CD96/DNAM1 cobioblockage abolished CD96 effect (77).

There are limited studies characterizing the functional role of CD96 in human NK cells, and the results are conflicting. Transcriptomic analysis comparing human hepatic CD96+ and CD96− NK-cell subsets suggests that CD96+ NK cells are functionally exhausted compared with CD96− NK cells and that CD96− cells are enriched with published gene signatures such as inhibition- and exhaustion-related genes that are correlated with the regulation of NK-mediated immunity (81). Fuchs and colleagues showed that the cytotoxic activity of human polyclonal NK cell lines was enhanced in the presence of a CD96 binding antibody (70), suggesting that human CD96 promotes NK-cell activation rather than inhibition. In
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costimulatory functions in human and mouse CD8+ T cells. or costimulatory receptor in different human in vitro assay explain why CD96 has been reported as either an inhibitory (72). The presence of a potential activating motif might explain why CD96 has been reported as either an inhibitory or costimulatory receptor in different human in vitro assay systems. Accordingly, it was recently reported that CD96 has costimulatory functions in human and mouse CD8+ T cells. It was shown in vitro and in vivo that deletion or disruption of CD96/PVR interaction results in impaired T-cell proliferation and inflammatory cytokine secretion (83).

Given these conflicting results, the role of CD96 in human T and NK cells is not fully understood, and collectively these studies indicate that CD96 pathway blockade may either enhance or inhibit lymphocyte killing of PVR+ tumor cells (Fig. 1). This will soon be tested clinically, as GlaxoSmithKline recently reported initiation of a phase I clinical trial evaluating a CD96-blocking antibody in patients with solid tumors, and that data should further inform the role and relevance of CD96 in the DNAM1 axis.

**PVRIG: THE RECENTLY DISCOVERED INHIBITORY RECEPTOR IN THE DNAM1 AXIS**

PVRIG was identified using in silico analyses designed to discover novel immunoreceptors that are involved in regulating lymphocyte function in the context of cancer (32, 84). Human and cynomolgus PVRIG share high sequence identity, and both proteins have a conserved ITIM in the intracellular signaling domain, which is lacking in the mouse homolog. PVRL2 is the cognate ligand for PVRIG, with a binding affinity of human PVRIG to human PVRL2 reported as 88 nmol/L (32). Studies investigating whether DNAM1 and TIGIT compete with PVRIG for binding to PVRL2 showed that TIGIT had little effect on interrupting the PVRIG/PVRL2 interaction, whereas DNAM1 was able to compete for this interaction, despite its reduced affinity to PVRL2 compared with PVRIG (26, 32). Although TIGIT is reported to have a very low-affinity interaction with PVRL2, its physiologic relevance is unclear, and our recent data suggest that PVRIG/PVRL2 serves as a distinct inhibitory signaling pathway in the DNAM1 axis and has a nonoverlapping function with the TIGIT/PVRL2 pair (Fig. 1; ref. 26).

Mouse PVRIG has a 59% protein identity with human PVRIG and a truncated intracellular signaling domain that contains phosphorylated tyrosine but lacks an ITIM, suggesting that mouse PVRIG may have a reduced role as a DNAM1 pathway checkpoint receptor compared with human PVRIG (85). At steady state, expression of murine PVRIG is detected on both T and NK cells, whereas in peripheral immune tissues, NK but not T cells express PVRIG. Upon activation, CD8+ T cells upregulate PVRIG expression, although at a much slower rate compared with related coinhibitory checkpoints. The relative expression of mouse PVRIG on TILs is lower than that seen in humans, and mouse PVRL2 in the TME and mouse tumor cell lines is lower than the expression detected in human TME, further supporting the idea that PVRIG plays a diminished role in mice compared with its role in humans (85). These findings, including the acquisition of an ITIM in human PVRIG and higher levels of human PVRIG and PVRL2 in the TME, suggest that greater effects of PVRIG inhibition may be seen in a human tumor setting relative to mouse preclinical models.

Nevertheless, although mouse PVRIG lacks an ITIM motif, PVRIG-deficient T cells do have increased function compared with wild-type T cells following antigen challenge. Moreover, in vivo syngeneic models utilizing MC38 colon carcinoma and B16 melanoma demonstrated that tumors grew slower in PVRIG-deficient compared with wild-type mice, and ex vivo analysis pointed to functional differences in T-cell responses. In the CT26 colon carcinoma model, reduced tumor growth and increased survival were observed after PVRIG and PD-1 cotargeting (85). This was further supported by increased antitumor responses in PVRIG/TIGIT double-knockout mice (66).

In the human setting, as in the mouse setting, results indicate that inhibition of this pathway may result in enhanced antitumor immunity (26). The activating effect of PVRIG blockade on CD8+ T cells has been demonstrated in vitro with antibodies that block the PVRIG/PVRL2 interaction. The ability of anti-PVRIG mAbs to promote T-cell responses, either individually or in combination with other immune checkpoints, was assessed using multiple human T-cell–based assays with both TILs and T cells from healthy donors. In all assay systems, PVRIG blockade increased T-cell proliferation, cytokine secretion, and cytotoxicity (26, 32). Although clinical monotherapy effects are rarely observed with inhibitory receptor antagonists other than anti–PD-1 (60, 86–89), numerous pathways modulate immune responses, suggesting that combinatorial approaches may increase rates of response (89–92). Compared with blockade of PVRIG, TIGIT, or PD-1 alone, the dual and triple combination of anti-PVRIG with anti–PD-1 or with anti-TIGIT further increased cytokine production and T cell–mediated killing of PVRL2/PVR+ tumor target cells (26).

In addition to PVRIG’s role in regulating T-cell responses, we have recently shown that the PVRIG blockade significantly enhances NK cell–mediated killing of PVRL2+ cancer cells (93), in line with other emerging data supporting the inhibitory functional role of PVRIG in these cells (94). Accordingly, Xu and colleagues demonstrated that blockade of PVRIG and TIGIT alone or in combination enhances trastuzumab-triggered antitumor response by human NK cells (95). Collectively, these data suggest that PVRIG and TIGIT receptors regulate NK-cell functions and that NK activation may be a determinant of clinical efficacy for inhibitors targeting each receptor (Fig. 2B).

In assessing tumor expression, hormonally regulated tumors, such as ovarian, endometrial, and breast cancers, along with kidney and lung tumors, demonstrated the highest PVRIG expression on T and NK cells. PVRIG expression in tumors was significantly increased on TILs compared with...
T cells infiltrating normal adjacent tissue, further highlighting its potential as a checkpoint receptor on lymphocytes. Moreover, PVRIG was coexpressed with PD-1 and TIGIT on TILs, peripheral memory T cells, and activated T cells. These data indicate that all three inhibitory molecules play a role in regulating the immune response and provide a rationale for the dual and triple blockade of these checkpoint receptors, depending on the ligand expression pattern in the TME (26).

PVRL2 is frequently expressed in various malignancies, and specifically PVRL2/PVR mRNA ratios are generally highest in hormonally regulated cancers, which was further validated by both IHC and flow cytometry. This, together with PVRIG levels in those tumors, suggests that these cancers are promising indications for PVRIG-blocking antibodies. In addition, increased RNA and protein PVRL2 expression levels were demonstrated in cancer relative to normal tissues, with expression seen in both PD-L1+ and PD-L1− patient samples across tumor types (26, 96). These analyses highlighted cancers in which PVRL2 might primarily regulate the immune response, and how this pathway may be relevant in patients who are PD-L1− or those who develop PD-1 resistance (96–98).

Based on the supportive preclinical data, an anti-PVRIG antibody was developed for clinical testing. COM701 is a humanized anti-PVRIG hinge stabilized IgG4 mAb that binds specifically to human and cynomolgus monkey PVRIG and disrupts the binding of PVRIG to PVRL2 (Table 1). Because PVRIG is predominantly expressed on CD8+ T and NK cells, and its expression is relatively low on Tregs (26), an IgG4 backbone was selected to avoid potential depletion of effector cells. COM701 inhibits the binding of PVRL2 to PVRIG in a dose-dependent manner with complete inhibition of PVRIG/PVRL2 interaction observed at saturating levels of COM701 (26). It is currently in phase I clinical testing, both as a monotherapy and in combination with the anti–PD-1 drug nivolumab (NCT03667716). In initial results presented from the dose-escalation arms of a phase I study, COM701 was shown to be well tolerated as a monotherapy and in combination with nivolumab, with no dose-limiting toxicities reported. Additionally, encouraging disease control rates of 69% (11/16) for the monotherapy and 75% (9/12) for the combination arm were observed in a heavily pretreated patient population. Moreover, two patients were reported with confirmed partial responses, one from the monotherapy arm (microsatellite-stable primary peritoneal cancer) and one from the combination arm (microsatellite-stable colorectal cancer, which has an extremely low response rate to anti–PD-1 alone). Future clinical trials involving triple blockade of PVRIG, TIGIT, and PD-1 are planned.

**Figure 3.** Functional interplay between the PD-1 pathway and the DNAM1 axis. A, T-cell inhibition is induced by the interaction of PVRIG and/or TIGIT with cognate ligands expressed on tumor cells or APCs. PD-1 stimulation by PD-L1 further suppresses T-cell function by dephosphorylating the DNAM1 signaling motif and reducing its expression. B, Full restoration of T-cell activation by coinhibition of PVRIG, TIGIT, and PD-1.
Targeting of Checkpoint Receptors within the DNAM1 Axis

was poorly understood. Wang and colleagues have provided some insight into the intersection of the DNAM1 and PD-1 pathways (58). Using a cell-free system, the authors demonstrated that DNAM1 is dephosphorylated by the PD-1/SHP2 complex, suggesting that it is a downstream target of PD-1 signaling (Fig. 3A). This was further reinforced by the observation that antigen-specific intratumoral CD8+ T cells isolated from MC38-OVA tumor–bearing mice treated with an anti–PD-1 antibody showed a high level of DNAM1 phosphorylation, whereas DNAM1 on TILs isolated from isotype control-treated mice showed a lack of phosphorylation.

Extending the above results, Wang and colleagues assessed the impact of DNAM1 blockade or ablation in an in vivo tumor model responsive to anti–PD-1 in combination with a glucocorticoid-induced tumor necrosis factor receptor (GITR) agonist antibody. The survival of tumor-bearing mice was dramatically increased by the combination treatment and could be reversed either by the addition of an antagonist DNAM1 antibody or upon treatment of tumors engrafted into DNAM1 knockout mice. The authors also demonstrated an effect of PD-1 inhibition on DNAM1 expression; increased expression was seen on antigen-reactive clones following anti–PD-1 treatment, and further increased with GITR agonism. Taken together, the results implicate derepression of DNAM1 costimulatory signaling in response to PD-1 inhibition, similar to the role

Figure 4. Clinical landscape of the DNAM1 axis.
that has been proposed for CD28 involvement in an anti-PD-1 response (99). Another study has similarly shown that DNAM1 is required for PD-1 blockade antitumor activity in preclinical mouse models (100). In accordance with these results, DNAM expression on TILs was shown to correlate positively with the response to PD-1 blockade in patients with melanoma (101). These data provide a molecular rationale for the combination effects observed with PD-1 inhibition when combined with blockade of coinhibitory molecules in the DNAM1 axis (Fig. 3B).

SUMMARY

Given recent advances in our understanding of DNAM1 regulation, including a possible connection with the PD-1 pathway and the broad and upregulated expression of the axis ligands PVR and PVRL2 in the TME, the coinhibitory receptors in the axis are increasingly attractive as targets for cancer immunotherapy. Preclinical and early clinical data in solid tumors have begun to emerge from clinical trials with TIGIT inhibitors, and additional clinical data are expected by the end of 2020 (Fig. 4). Interestingly, although initial studies in NSCLC indicate greater efficacy for TIGIT blockade in PD-L1hi patients, early clinical data for PVRIG blockade highlight activity in indications that are generally unresponsive to PD-1 therapy and that typically demonstrate low PD-L1 expression. Emerging clinical data for the PVRIG inhibitor COM701 and multiple TIGIT inhibitors should yield additional insight into functional interactions between the immune-checkpoint inhibitors in the DNAM1 axis, both with each other and in conjunction with modulation of the PD-1 signaling pathway.

Furthermore, defining the patient population most likely to respond to each therapeutic intervention is of great interest. As mentioned above, early data suggest that PD-L1 might be a potential biomarker of response to TIGIT blockade (67), at least when combined with PD-L1 blockers. However, the expression of other pathway members is also important. PVRIG and/or PVRL2 high-expressing tumors might support dominance of the PVRIG pathway and thus preferentially respond to PVRIG blockade (26). Likewise, patients with TIGIT and/or PVR high-expressing tumors could potentially benefit from TIGIT blockade. Finally, the expression levels of DNAM1, which is in the center of the axis, could also serve as a biomarker of response to combination blockade of the axis members.

Authors’ Disclosures

Z. Alteber reports personal fees from Compugen Ltd outside the submitted work; in addition, Z. Alteber has a patent for anti-PVRIG antibodies formulations and uses thereof pending. E. Ophir reports personal fees from Compugen Ltd outside the submitted work; in addition, E. Ophir has a patent for triple-combination antibody therapies pending and anti-PVRIG antibody–related provisional patent applications pending; and Compugen Ltd is developing anti-PVRIG and anti-TIGIT monoclonal antibodies being evaluated in phase 1 clinical trials. No disclosures were reported by the other authors.

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