Therapeutic Targeting of Checkpoint Receptors within the DNAM-1 Axis

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Abstract

Therapeutic antibodies targeting the CTLA-4/PD-1 pathways have revolutionized cancer immunotherapy by eliciting durable remission in cancer patients. 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 CTLA-4 or PD-1 blockade. The DNAM-1 axis is a potent co-regulator 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 co-inhibitory receptors in the DNAM-1 axis such as TIGIT and PVRIG, and to a lesser extent, CD96. Biologic 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 DNAM-1 axis blockers in conjunction with anti-PD-(L)1 agents.
Introduction

Immuno-stimulatory monoclonal antibodies (mAbs) that block inhibitory checkpoint proteins such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) or programmed death 1 (PD-1), and programmed death-ligand 1 (PD-L1) have made a paradigm shift in the development of anti-cancer therapies. Since the early studies targeting these checkpoints in the 2000s, roughly ten additional co-inhibitory molecules have been targeted clinically. The biology of these molecules is complex, in part because most T cell regulatory pathways are multicomponent. CTLA-4 (or CD152) is a homolog of the co-stimulatory receptor CD28, with both capable of binding the ligands CD80 and CD86. Reflecting its status as a key regulator of autoimmunity, CTLA-4 displays an affinity for CD80 and CD86 that is several orders of magnitude greater than that of CD28 (1). CTLA-4 outcompetes CD28 for access to their shared ligands and delivers inhibitory signals that dampen TCR (signal 1) and co-stimulatory signals (signal 2). A similarly inducible inhibitory checkpoint receptor whose discovery postdates that of CTLA-4 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 its PD-L1 ligand in solid tumors were tested before the approval of the first PD-1 blockers – Nivolumab and Pembrolizumab (3,4). A mAb targeting CTLA-4, three mAbs targeting PD-1, and three targeting PD-L1 have been approved by the U.S. Food and Drug Administration (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 CTLA-4 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 (LAG-3), T cell Ig and immunoreceptor tyrosine-based inhibitory motif [ITIM] domain) (TIGIT), T cell Ig and mucin domain-3 (TIM-3), 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 TIM-3 have shown encouraging...
preliminary anti-tumor effects in patients with hematologic malignancies, including Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) (11). The promising candidates directed against LAG-3 and TIGIT are currently in Phase 3 clinical trials in combination with antibodies blocking the PD-1 pathway. For LAG3 alone, ten agents are being tested in clinical trials for a variety of cancers. Multiple anti-TIGIT antibodies are being evaluated in different clinical trials as a single-agent or in combination with PD-(L)1 blockers, as will be discussed in this review (12). Moreover, we will discuss the biology and immunotherapeutic significance of all constituents of the DNAM-1 (DNAAX accessory molecule 1, CD226) axis including the co-inhibitory receptors, TIGIT, poliovirus receptor-related Ig domain-containing protein (PVRIG), and CD96, their respective primary ligands, poliovirus receptor (PVR, CD155) and PVR-related 2 (PVRL2, Nectin-2, CD112), and the eponymous co-stimulatory receptor, DNAM-1 (Figure 1).

**DNAM-1: the central co-stimulatory molecule in an interacting immune regulatory network in the TME**

DNAM-1 is the central node that links together four receptors (DNAM-1 itself, TIGIT, PVRIG, and CD96) and two ligands (PVR and PVRL2) into one family. It is a 65 kilodalton (kD) member of the Ig superfamily that contains two extracellular Ig-like domains, an intracellular immunoreceptor tyrosine-based activation motif (ITAM) and is constitutively expressed on the surface of natural killer (NK) cells, T lymphocytes, platelets, monocytes and a subset of B cells. DNAM-1 was identified in a screen for cell surface molecules that mediate T cell cytotoxicity and was subsequently shown to act as a co-stimulatory molecule on NK cells as well as T cells (13). Consistent with a co-stimulatory function on T and NK cells, it was shown to mediate anti-tumor immune responses as demonstrated by increased tumor growth in DNAM-1 knockout mice (14,15).

In addition to DNAM-1’s direct co-stimulatory activity, a physical association with lymphocyte function-associated antigen 1 (LFA-1) has been demonstrated both in NK and activated T cells. This physical interaction was shown to promote the formation of stable target cell conjugates, and NK cells lacking LFA-1 expression are deficient in DNAM-1 driven cytotoxicity, suggesting...
a functional dependency between the two molecules (16). Further supporting the functional
interaction between the two molecules, crosslinking of LFA-1 results in Fyn protein tyrosine
kinase phosphorylation of DNAM-1 (16). LFA-1 and DNAM-1 demonstrate coordinated
expression and colocalization in the immune synapse of activated NK cells in the presence of
target cells (17), and DNAM-1 deficient CD8+ T cells have a decreased ability to recruit LFA-1
and lipid rafts to the immune synapse, while also showing reduced conjugation with target tumor
cells (18).

PVR and PVRL2, belonging to the superfamily of cell adhesion molecules, have been identified
as the ligands for DNAM-1 (Figure 1). PVR and PVRL2 mediate cell-cell adhesion by
generating heterotypic and homotypic (only PVLR2) interactions (19). High expression of these
molecules on tumor cells renders them susceptible to T and NK cell-mediated cytotoxicity,
which can be abrogated by an anti-DNAM-1 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 co-stimulatory ligands in
the TME, presumably via their activation of DNAM-1. However, identification and
characterization of the immune checkpoint proteins TIGIT, CD96, and PVRIG has generated a
more complex picture of their function, since the interaction of PVR with TIGIT and CD96, and
that of PVRL2 with PVRIG can also mediate suppressive signaling through the co-inhibitory
arms of the DNAM-1 signaling axis, suggesting that they act to counterbalance DNAM-1 co-
stimulation. At the 30,000-foot level, this is analogous to CD28 and CTLA-4 as
counterbalancing co-stimulatory and co-inhibitory receptors, respectively, for CD80 and CD86;
however, at a closer glance, there are significant differences that give the DNAM-1 family its
unique character. While CD28 is expressed exclusively on T cells, DNAM-1 axis members are
expressed on both T and NK cells. In addition, CD28 is downregulated on T cells upon
activation and differentiation while DNAM-1 maintains its expression also on differentiated
effector cells (22,23). Furthermore, CD80 and CD86 are restricted to immune cells, mostly
antigen presenting cells, while PVR and PVRL2 are expressed on multiple cell types, including
antigen presenting cells, tumor and endothelial cells. These differences in expression pattern
suggest that while CD28 and CTLA-4 probably play a dominant role in T cell priming, DNAM-1
axis members presumably regulate both priming and effector phase of T cells, resembling PD-1 pathway. Nevertheless, similar to the higher affinity CD80/86 interactions with CTLA-4 versus CD28, TIGIT, CD96, and PVRIG have higher affinities to their cognate ligands than DNAM-1 and thus can inhibit T and NK cell activity through ligand competition (24–28). This was supported by several studies showing that the co-stimulatory effects driven by targeting the TIGIT/PVR interaction are dependent on the engagement of DNAM-1 by PVR (27,29). A recent study suggested that the infiltration of CD8⁺DNAM-1^high T cells into TME may predict 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 DNAM-1 homodimerization by TIGIT which results in its impaired functionality (29). Furthermore, DNAM-1 expression on lymphocytes is modulated by membrane PVR that upon binding to DNAM-1 induces its internalization and degradation, resulting in decreased anti-tumor function (31). Similarly, it was shown that PVRIG outcompetes DNAM-1 for PVRL2 binding (32) (Figure 1).

One approach to boost anti-tumor immunity by modulating the DNAM-1 axis could arise from agonizing DNAM-1, which has the potential to show therapeutic benefit in cancer patients, due to its potent T and NK cells co-activatory function. However, a caveat for this approach is the broad expression of DNAM-1 on different cell types, including on platelets, where it was shown to stimulate activation and cell adhesion (33). Indeed, an agonist antibody directed against DNAM-1 (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 T and NK cell activation by interrupting the dominant interactions with TIGIT and PVRIG, respectively. When using effector function antibodies, an antibody-dependent cellular cytotoxicity (ADCC) against PVCR/PVRL2-expressing tumor cells could be applied, while avoiding the risk of potential ADCC against T cells. Moreover, in addition to direct effects on immune cells, interfering with non-immune tumor promoting effects of PVR or PVRL2 could provide additional benefit for this approach (34). Accordingly, an anti-PVRL2 antagonizing mAb developed by Takeda exhibited anti-tumor effects attributed mainly to ADCC.
against tumor cells (35). Potential drawbacks of anti-PVR or anti-PVRL2 mAbs are that direct targeting may result in interference of DNAM-1 co-stimulatory signaling, as well as in the induction of ADCC against antigen presenting cells. 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, co-stimulatory DNAM-1 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 immunotherapies targeting this axis.

**TIGIT blockade - a promising new clinical modality for treating solid and hematological 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 Treg cells where it enhances their activation and suppressive activity (Figure 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 ($K_D$) reported as 1-3 nM (Figure 1) (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), breast (39), pancreatic (25), and bladder (40) cancers. In metastatic melanoma patients, 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 to DNAM-1 allows it to outcompete DNAM-1 for ligand binding, similar to CTLA-4 versus CD28 (21,24,27,28). While the expression of both CTLA-4 and TIGIT is induced upon activation, CD28 is mainly expressed on naïve T cells, whereas DNAM-1 has broad expression on multiple T cell subsets (23). Moreover, unlike CTLA-4, TIGIT has been shown to directly interact with DNAM-1, reducing the ability of
DNA-1 to homodimerize and enhance immune activation (27,29,42). Recently, it was demonstrated that the TIGIT/PVR interaction induces de-phosphorylation of DNA-1 at tyrosine 322 and interferes with its co-stimulatory responses on T cell (43). In addition to counteracting the stimulatory function of DNA-1 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 (8,44). 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 pro-inflammatory cytokine production via “reverse signaling” (Figure 2B) (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 hematological tumors. In the LCMV Clone 13 model of chronic viral infection, Johnston et al. 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, co-blockade of TIGIT and PD-L1 resulted in tumor rejection and restoration of CD8+ T cells within the TME (29). Johnston et al. 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 et al. also demonstrated that the TIGIT pathway blockade either alone or in combination with anti-PD-L1 synergistically increased effector function of human NY-ESO-1-specific CD8+ tumor-infiltrating lymphocytes (TILs) from melanoma patients (45). Collectively, these data demonstrate that TIGIT and PD-1/PD-L1 co-blockade acts through CD8+ T cells to generate an effective anti-tumor 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, were found to be associated with increased response to immunotherapy, and to correlate significantly with PD-1 blockade therapeutic activity (46–48). Importantly, TIGIT and PD-1 are the dominant inhibitory T cell checkpoints expressed on this memory cell subset (49).
Other studies reported that TIGIT predominantly suppresses anti-tumor CD8+ T cell responses indirectly by enhancing Treg function in the TME (50–52). Kurtulus et al. 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 to transfer of wild type TIGIT+ CD8+ T cells (50). 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 anti-tumor immune responses compared to direct signaling of TIGIT on CD8+ T cells. Other studies have examined the mechanisms of TIGIT+ Treg-mediated immune suppression. Joller et al. showed that increased amounts of IL-10 produced by TIGIT+ Tregs contributed to the generation of tolerogenic dendritic cells, thereby inhibiting the generation of effector T cell responses (42,52). Moreover, TIGIT blocking mAbs were shown, in addition to enhancing conventional T cell activity, to reduce Tregs secretion of IL-10 (53). Finally, a recent study showed that Tregs in melanoma further upregulate TIGIT and downregulate DNAM-1 expression resulting in a higher TIGIT/DNAM-1 ratio (54). This high TIGIT/DNAM-1 ratio in Tregs regulates their suppressive function and stability and correlates with a poor clinical outcome following PD-1 and/or CTLA-4 pathway blockade in melanoma patients (45,50,54).

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 pro-inflammatory cytokines and kill PVR-expressing tumor cells, suggesting that TIGIT abrogates NK cell activation (Figure 2B) (8,44,55). 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 anti-tumor NK cell response in multiple solid tumors and hematological models (44,56–58). Overall, survival was increased in these tumor models, indicating that NK cells may be critical for the therapeutic effects of TIGIT pathway-based immunotherapies (57,58). Collectively, these studies demonstrate important roles for TIGIT-expressing CD8+ T cells, Tregs, and NK cells in regulating anti-tumor 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) co-engagement 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 to mouse IgG1 Abs (reduced FcR binding capacity) (59,60). Recently, Yang *et al.* demonstrated that TIGIT blockade induced Fc-mediated depletion of Tregs that activate anti-tumor CD8\(^+\) T cell responses, targeting tumor-shared antigens that are normally cryptic or suppressed by Tregs (61). 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 (62).

Ultimately, the ability of mouse models to predict the importance of FcR binding by a therapeutic antibody is limited, since murine FcRs do not mirror the structural diversity, expression, and Fc binding pattern of the human FcR (63). 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 very CD8\(^+\) TILs in addition to tumor Tregs. Studies on mechanisms of action of CTLA-4 blockade emphasize that caution in the direct extrapolation of FcR roles between mouse and human. While previous murine studies showed that anti-tumor effects of a FcR-binding mouse IgG2a anti-CTLA-4 were dependent on FcR expression and were associated with selective intra-tumoral depletion of Treg, Ipilimumab, a human IgG1 that binds human FcR, failed to decrease intra-tumoral Treg density (64). 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 FDA approved. Therefore, it remains to be determined in patients which cell type plays a dominant role in generating anti-tumor 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 anti-tumor responses, especially in anti-PD-1/PD-L1 resistant patients. While TIGIT is highly co-expressed 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 LAG-3, TIM-3, and BTLA4 (65–67). One study showed that TIM-3 pathway blockade in TIGIT deficient mice acts synergistically to reduce B16F10 melanoma tumor growth (50). Recently, we demonstrated that PVRIG/PVRL2 blockade induces TIGIT expression (26), and combining TIGIT with PVRIG co-blockade synergistically activates T cells in human functional assays which are translated into in-vivo anti-tumor effects (68). Furthermore, TIGIT blocking mAbs are being tested clinically in combination with other therapeutic modalities such as chemotherapy, IL-15 cytokines, and myeloma targeting antibodies (31).

Whilst the majority of anti-TIGIT antibodies are currently in early clinical investigation, several have progressed beyond Phase 1 studies. These include tiragolumab (Roche-Genentech), which is currently recruiting patients in Phase 3 studies in Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC), domvanalimab (Arcus) initiating a Phase 3 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 0-3% (69,70). Recently, data were reported from a randomized, Phase 2 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 (69). The study met its co-primary 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 objective response rate (ORR) of 31.3% compared to 16.2%, and in median progression-free survival (PFS) of 5.4 months compared to 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 3 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 (20,71,72). 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 (23,73–77) and mice (23,77,78). It is highly expressed on human tumor-infiltrating CD8+ T cells, compared to normal tissues (23,79). Moreover, its expression on T cells is increased upon stimulation, resembling the expression profile kinetics of DNAM-1 (23). Like TIGIT, the high-affinity binding partner for CD96 is PVR. While the binding affinity of CD96 to PVR is higher than that of DNAM-1, it is lower than that of TIGIT (Figure 1) (24). CD96 also binds PVRL1 (CD111), but the functional relevance of the CD96/PVRL1 interaction has not been fully explored (77,80).

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 et al. demonstrated that in CD96-deficient mice, NK cells produced greater IFN-γ in response to LPS, IL-12, or IL-18 stimulation (78). 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 CTLA-4 pathways increased cytokine secretion and survival in tumor-bearing mice in comparison to anti-PD-1 or anti-CTLA-4 alone (78,81). 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 to mice lacking PD-1, TIGIT or CD96 alone (82). 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 et al. showed that CD96 blockade suppressed tumor growth of several murine models in a CD8+ T cell-dependent manner (79). The anti-tumor efficacy of CD96 was mediated by increasing IFN-γ secretion, and was dependent on DNAM-1, as CD96/DNAM-1 co-blockade abolished CD96 effect (79).
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 suggest that CD96\(^+\) NK cells are functionally exhausted comparing to 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 (83). Fuchs et al. showed that the cytotoxic activity of human polyclonal NK cell lines was enhanced in the presence of a CD96 binding antibody (72), suggesting that human CD96 promotes NK cell activation rather than inhibition. In contrast to this report, Carlsten et al. demonstrated that while DNAM-1 blockade significantly reduced NK degranulation and cytotoxicity, an anti-CD96 mAb did not affect tumor cell killing when NK cells were co-cultured with fresh ovarian carcinoma cells (84). Additionally, we have demonstrated that inhibition of the CD96/PVR interaction with two distinct, antagonistic anti-CD96 antibodies had no effect on IFN-\(\gamma\) secretion from primary human T cells (26). The cytoplasmic tail of both human and mouse CD96 has an ITIM-like motif for inhibitory signaling, but human CD96 also contains a YXXM motif, which is found within other known activating receptors including CD28 and NKG2D (74). The presence of a potential activating motif might explain why CD96 has been reported as either an inhibitory or co-stimulatory receptor in different human in vitro assay systems. Accordingly, it was recently reported that CD96 has co-stimulatory functions in human and mouse CD8\(^+\) T cells. It was shown in-vitro and in-vivo that depletion or disruption of CD96/PVR interaction results in impaired T cell proliferation and inflammatory cytokines secretion (85).

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 (Figure 1). This will soon be tested clinically, as GlaxoSmithKline recently reported initiation of Phase 1 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 DNAM-1 axis.

PVRIG – the recently discovered inhibitory receptor in the DNAM-1 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 (32,86). 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 nM (32). Studies investigating whether DNAM-1 and TIGIT compete with PVRIG for binding to PVRL2 showed that TIGIT had little effect on interrupting the PVRIG/PVRL2 interaction, whereas DNAM-1 was able to compete for this interaction, despite its reduced affinity to PVRL2 compared with PVRIG (26,32). While 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 DNAM-1 axis, and has a non-overlapping function with TIGIT/PVR pair (Figure 1) (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 DNAM-1 pathway checkpoint receptor compared to human PVRIG (87). 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 co-inhibitory 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 to its role in humans (87). 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 to 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 to 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 co-blockade (87). This was further supported by increased anti-tumor responses in PVRIG/TIGIT double knockout mice (68).

In the human setting, as in the mouse setting, results indicate that inhibition of this pathway may result in enhanced anti-tumor 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 (60,88–91), numerous pathways modulate immune responses, suggesting that combinatorial approaches may increase rates of response (91–94). Compared to 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 (95), in line with other emerging data supporting the inhibitory functional role of PVRIG in these cells (96). Accordingly, Xu *et al.*, demonstrated that blockade of PVRIG and TIGIT alone or in combination enhances trastuzumab-triggered anti-tumor response by human NK cells (97). Collectively, these data suggests that PVRIG and TIGIT receptors regulate NK cell functions and that NK activation may be a determinant of clinical efficacy for inhibitors targeting each (Figure 2B).

In assessing tumor expression, hormonally regulated tumors, such as ovarian, endometrial, 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 to T cells infiltrating normal adjacent tissue, further highlighting its potential as a checkpoint receptor on lymphocytes. Moreover, PVRIG was co-expressed 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 also by both IHC and flow cytometry. This, together with PVRIG levels in those tumors, suggest 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- patients samples across tumor types (26,98). 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 (98–100).

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). Since 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 1 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 pre-treated 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.

**PD-1: A player in the DNAM-1 axis?**

While several studies have demonstrated additive or synergistic effects of PD-1 pathway blockade with inhibition of TIGIT or PVRIG (26,29,32,45,57), the mechanism was poorly understood. Wang and colleagues have provided some insight into the intersection of the DNAM-1 and PD-1 pathways (59). Using a cell-free system, the authors demonstrated that DNAM-1 is dephosphorylated by the PD-1/SHP2 complex, suggesting that it is a downstream target of PD-1 signaling (Figure 3A). This was further reinforced by the observation that antigen-specific intra-tumoral CD8+ T cells isolated from MC38-OVA tumor-bearing mice treated with an anti–PD-1 antibody showed a high level of DNAM-1 phosphorylation, whereas DNAM-1 on TILs isolated from isotype control-treated mice showed a lack of phosphorylation.

Extending the above results, Wang and colleagues assessed the impact of DNAM-1 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 by either the addition of an antagonist DNAM-1 antibody or upon treatment of tumors engrafted into DNAM-1 knockout mice. The authors also demonstrated an effect of PD-1 inhibition on DNAM-1 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 de-repression of DNAM-1 co-stimulatory signaling in response to PD-1 inhibition, similar to the role that has been proposed for CD28 involvement in an anti-PD-1 response (101). Another study has similarly shown that DNAM-1 is required for PD-1 blockade anti-tumor activity in pre-clinical mouse models (102). In accordance with these results, DNAM expression on TILs was shown to correlate positively with the response to PD-1 blockade in melanoma patients (103). These data provide a molecular rationale for the combination effects observed with PD-1
inhibition when combined with blockade of coinhibitory molecules in the DNAM-1 axis (Figure 3B).

Summary

Given recent advances in our understanding of DNAM-1 regulation, including a possible connection with the PD-1 pathway and the broad and up-regulated expression of the axis ligands PVR and PVRL2 in the TME, the co-inhibitory 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 is expected by the end of 2020 (Figure 4). Interestingly, while initial studies in NSCLC indicate greater efficacy for TIGIT blockade in PD-L1\textsuperscript{high} patients, early clinical data for PVRIG blockade highlights 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 DNAM-1 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 suggests that PD-L1 might be a potential biomarker of response to TIGIT blockade (69), 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 better benefit from TIGIT blockade. Finally, the expression levels of DNAM-1, which is in the center of the axis, could also serve as a biomarker of response to combination blockade of the axis members.
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**Table 1.** List of antagonistic antibodies targeting DNAM-1 axis family members that are under preclinical and clinical development. N/A refers to not applicable.
Figure legends

Figure 1: TIGIT and PVRIG are parallel inhibitory pathways in the DNAM-1 Axis. Interactions between DNAM-1 axis molecules and their cognate ligands on tumor or antigen presenting cells (APCs) induces activation (+) or inhibition (-) of T and NK cells function. Arrow thickness represents the relative affinity between the interacting molecules.

Figure 2: The effects of DNAM-1 Axis modulation on immune cells. DNAM-1 axis molecules and nectin and nectin-like ligands are expressed on various cells, and their interactions modulate immune function. A) TIGIT and DNAM-1 exert opposite effects in Tregs upon PVR binding. TIGIT further stimulate Treg immuno-modulatory activity, whereas DNAM-1 disrupts Treg suppression. B) While DNAM-1 stimulates effector T and NK cell function, TIGIT and PVRIG induce their suppression. PVR engagement on DCs by TIGIT induces a tolerogenic phenotype and reduces pro-inflammatory cytokine secretion.

Figure 3: Functional interplay between the PD-1 pathway and DNAM-1 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 de-phosphorylating the DNAM-1 signaling motif and reducing its expression. B) Full restoration of T cell activation by co-inhibition of PVRIG, TIGIT, and PD-1.

Figure 4: Clinical landscape of the DNAM-1 Axis
Figure 1

[Diagram showing interactions between Tumor Cell or APC and T or NK Cell via PVRL2, PVR, LFA-1, DNAM-1, TIGIT, and CD96.]
Therapeutic Targeting of Checkpoint Receptors within the DNAM-1 Axis

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