Clinical Cancer Research

Molecular Pathways: Targeting CD96 and TIGIT for Cancer Immunotherapy

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Abstract

The receptors CD96 and TIGIT are expressed on the surface of T and natural killer (NK) cells, and recent studies suggest both play important inhibitory roles in immune function. CD96 has been shown to modulate immune cell activity in mice, with *Cd96*$^{-/-}$ mice displaying hypersensitive NK-cell responses to immune challenge and significant tumor resistance. TIGIT overexpression has been shown to reduce NK-cell–mediated cytotoxicity. TIGIT is also upregulated on T cells during cancer and chronic viral infection, with expression associated with effector T-cell exhaustion and increased regulatory T-cell suppression. The counterbalance between the putative inhibitory CD96 and TIGIT receptors and the activating receptor, CD226, offers unique strategies for immuno-oncology drug development. Blocking CD96 or TIGIT with mAbs has been shown to improve tumor control in mice, in particular when used in combination with PD-1/PD-L1 blockade. These results have highlighted these pathways as promising new targets for immune modulation. This review will examine the rationale behind targeting CD96 and TIGIT, and discuss the potential approaches in translating these preclinical findings into novel clinical agents. *Clin Cancer Res; 22(21); 5183–8. ©2016 AACR.*

Background

CD155, CD226, and TIGIT in immune regulation and cancer

The development of antibodies targeting immune checkpoint receptors PD-1 (1) and CTLA-4 (2) has been a monumental step forward in the clinical success of cancer immunotherapy. Although these inhibitors have been highly successful as monotherapies, more than a dozen alternate pathways exist that modulate immune responses (3), suggesting that combinatorial approaches may greatly augment response rates. This hypothesis is supported by a recent clinical trial, demonstrating improved objective responses when PD-1 and CTLA-4 inhibitors were used in combination (4). Although antibodies against CTLA-4 and PD-1 are thought to act predominately through T cells, another immune cell type, the natural killer (NK) cell, is gaining traction as a target for cancer immunotherapy, particularly for the control of metastases and blood cancers (5). NK cells are part of the innate lymphocyte family, and they play a critical role in viral and tumor immune surveillance. NK cells act by detecting and killing infected or cancerous cells via perforin-mediated cytotoxicity; they also regulate immune responses through the release of cytokines (6).

Candidate pathways for cancer immunotherapy include a cluster of immunoglobulin superfamily receptors that interact with nectin and nectin-like molecules (NECL), which are critical regulators in immune surveillance. Nectin and NECL family members were first characterized as adhesion molecules, mediating both homo- and heterophilic interactions (7). A diverse range of nectin and NECL protein–receptor interactions exist, with roles in immune regulation, virus entry to cells, and normal development (7, 8). Several nectin and NECL proteins have prominent roles in cancer surveillance, with CD155 (necl-5, PVR) the most well characterized. Although expression is low in normal tissue, CD155 is highly expressed on many cancer cell lines and primary tumors (9). CD155 has been linked with enhanced tumor proliferation (10) and migration (9). CD155 is also upregulated on many immune cells during inflammation (11) and on tumor-associated antigen-presenting cells (APC; ref. 12). CD155 expression is thought to modulate T- and NK-cell responses through CD226, TIGIT, and CD96 interactions (Table 1 and Fig. 1).

Engagement between CD155 and CD226 or TIGIT has been a major focus of research, with CD226 having dual roles as both an activating and adhesion receptor on NK cells (13, 14), whereas TIGIT acts as an inhibitory receptor and has been shown to reduce NK-cell cytokine production and cytotoxicity (15, 16). CD226 is also considered an activating receptor for CD8$^+$ T cells (17), with its downregulation observed in advanced cancer and associated with T-cell exhaustion (12, 18). The role of CD226 in tumor immune surveillance is supported by accelerated tumor growth in *Cd226*$^{-/-}$ mice (19, 20). Conversely, TIGIT is highly upregulated on both CD8$^+$ T cells and regulatory T cells (Treg) in many clinical tumor settings (12, 18, 21), with expression also correlating with other immune checkpoints such as PD-1. Across *in vitro* human assays, primate models, and mouse tumor models, TIGIT blockade has been shown to enhance T-cell function in particular, in

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Table 1. Biological roles of TIGIT and CD96 in lymphocyte function and outcomes of pathway inhibition relevant to immunotherapy

TIGIT	CD96
T cells	
• Increased effector T-cell function in *tigit*$^{-/-}$ knockout or anti-TIGIT mAb–treated mice, increased human effector T-cell function following antibody treatment or shRNA knockdown (12, 21, 22, 41, 50)	• Role of CD96 in T-cell function currently unknown
• Reduced suppressive function of Treg cells; reduced suppression of antitumor immune responses by *tigit*$^{-/-}$ Tregs; increased suppression of Th1, Th17 immune responses by TIGIT^{+} Tregs compared with TIGIT^{-} Tregs in mice (23, 51)	• Surface expression of CD96 upregulated on activated human T cells (26)
• Associated with markers of T-cell exhaustion in tumors or chronic viral infections, blockade improves effector T-cell function (12, 18, 21, 22, 43)	• CD96 mRNA expression increased and associated with a T-cell signature in nonsquamous non–small cell lung cancer cohort (21)
	• Reduced expression on CD8^{+} T cells from chronic HIV-infected patients compared with healthy controls (44)
NK cells	
• Blockade of TIGIT–CD112/CD155 interactions increases human and mouse NK-cell cytotoxicity and IFNγ production (15, 16, 46)	• Putative adhesion molecule of mouse and human NK cells (29, 31, 32)
• *Tigit*$^{-/-}$ mice do not have increased protection from lung metastases (11, 35)	• Putative activating receptor for human NK cells (31)
	• *Cd96*$^{-/-}$ mice or blockade with anti-CD96 mAb increases NK production of IFNγ (11, 35)
	• *Cd96*$^{-/-}$ mice or blockade with anti-CD96 mAb increases control of NK-cell–dependent tumors and metastases (11, 35)

combination with other checkpoints such as PD-1 (12), PD-L1 (21, 22), and TIM-3 (23). Although CD155 is considered the dominant ligand for CD226 and TIGIT, CD226 can also interact with CD112 (24), and TIGIT can interact with CD112 and CD113 (25).

CD96 expression, ligand interactions, and putative signaling pathways

CD96 (TACTILE) was first identified as an Ig superfamily receptor (26); however, it is now known to be a member of the extended nectin/NECL family, and its role in immune function has received little attention until recently. CD96 expression is broadly similar between mice and humans, and is present on a proportion of hematopoietic stem cells, αβ and γδ T cells, NK cells, and a subpopulation of B cells in humans (27–31) and present on αβ and γδ T cells, NK cells, and NKT cells in mice (11, 32). CD96 is not expressed on other immune cells, and expression is generally low or absent in organs without lymphocyte infiltrate (29). Interestingly, CD96 is expressed at far higher levels in mice than in humans, with almost all cells positive for the receptor at resting state, whereas basal expression is lower in humans (26). CD96 has been shown to be highly expressed in acute myeloid leukemia (AML), T-cell acute lymphoblastic leukemia (T-ALL; ref. 29) and myelodysplastic syndromes (33). CD96 has additionally been proposed as a cancer stem cell marker in leukemia (30, 33).

Akin to DNAM and TIGIT, the main ligand for CD96 is CD155, to which it binds with an affinity stronger than CD226, but weaker than TIGIT. Human CD96, CD226, and TIGIT bind to CD155 with dissociation constants (K_d) of 37.6 nmol/L, 119 nmol/L, and 3.15 nmol/L, respectively (25). Of note, mouse CD96 (mCD96), but not human CD96 (hCD96), has been shown to bind to CD111 (nectin-1; ref. 32). Other key differences also exist between human and mouse. For example, hCD96 exists as two splice variants that confer different binding affinities to CD155 (29). The sequence of hCD96, but not mCD96, contains a potential SH2-domain binding site within the cytoplasmic tail in the form of a YXXM motif (29), similar to that found in activating receptors. Both human and mouse CD96 sequences contain immunoreceptor tyrosine-based inhibitory motifs (ITIM; ref. 34) that putatively may provide inhibitory signals to lymphocytes following ligation. However, downstream signaling of the CD96 receptor has not been evaluated in detail. Given that CD155 contains cytoplasmic signaling motifs, it will be of interest to determine whether CD96 engagement triggers reverse CD155 signaling. Indeed, TIGIT/CD155 ligation has been shown to modulate the function of CD155-expressing dendritic cells (DC), inducing IL10 secretion (25).

Functions of CD96

Initial investigations of CD96 biology suggested a role in mediating human NK-cell adhesion to CD155-expressing target cells and was also proposed as a weak NK-cell–activating receptor (31). CD96 was also described as an adhesion molecule to CD155 and CD111 in mouse studies (29, 32). The first evidence that CD96 might be acting as an inhibitory receptor was shown in *Cd96*$^{-/-}$ mice, where NK cells produced greater IFNγ in response to LPS, IL12, or IL18 stimulation (11). This study also demonstrated a role for CD96 in cancer immune surveillance, with *Cd96*$^{-/-}$ mice showing robust resistance to experimental lung metastases and MCA-induced fibrosarcomas. The potential of targeting CD96 to enhance NK-cell control of metastases was highlighted in our recent article (35). In this study, we demonstrated that using mAbs against CD96 could reduce the number of lung metastases in a range of spontaneous and experimental models. The activity of anti-CD96 was dependent on NK cells, IFNγ, and CD226.

Clinical–Translational Advances

Therapeutic approaches

The complexity of interaction dynamics within the CD96/TIGIT/CD226/CD155 axis poses both opportunities and challenges for therapeutic translation in oncology. At the most fundamental level, the net inhibitory signals from either TIGIT or CD96 are counterbalanced via multiple mechanisms by the activating signal of CD226. Thus, an understanding of the dynamic regulation of CD226 expression and activity must be a core consideration when attempting to modulate TIGIT and CD96 activity. Therapeutic antibodies that reduce coinhibitory signaling via blockade of CD155 binding to CD96 and/or TIGIT have considerable experimental support in preclinical cancer models (21, 35). These data support a correlation between antibody blockade of the CD96/TIGIT/CD155 axis with enhanced anticancer activity through increased CD8^{+} T-cell or increased NK-cell function for anti-TIGIT and anti-CD96, respectively. As an alternative to blockade of ligand binding to coinhibitory receptors, antibodies that stabilize CD155 binding to CD226 might selectively potentiate an activating signal, and may serve as a

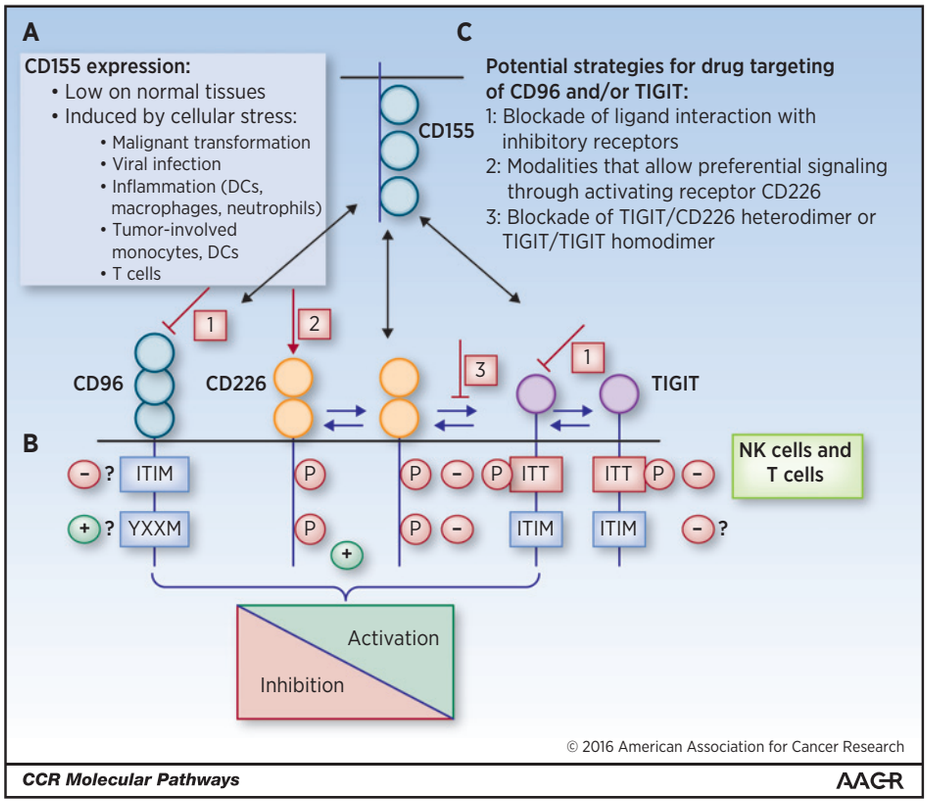

Figure 1.

The regulation of inhibitory versus activation signals in NK cells and T cells by CD96/TIGIT/CD226 receptors is achieved by complex receptor/ligand and receptor/receptor counterbalancing mechanisms. CD155, CD96, TIGIT, and CD226 are all members of the Ig superfamily, and all share similar variable or constant Ig motifs in the extracellular domains, single-pass transmembrane regions, and short cytoplasmic domains. CD155 levels on different cells are upregulated as a result of cellular stress (**A**). In cancer, CD155 is increased on transformed cells and APCs within the tumor microenvironment and is sensed by the CD96/TIGIT/CD226 receptors to modulate antitumor immunity. Upon exposure to increasing levels of CD155, the net activation or inhibition of lymphocytes is fine-tuned by the integrated signaling of CD96, TIGIT, and CD226 (**B**). This net integration is dictated by the relative binding affinity of CD155 for different receptors, relative abundance of activating (CD226) versus inhibitory (CD96, TIGIT) receptors, the strength and quality of signal transduction by each receptor, and the modulation of ligand binding and biochemical signal transduction by homo- and heterodimerization of receptor complexes. The cytoplasmic domain of CD226 has a tyrosine (Y322) and a serine (S329), which become phosphorylated in a CD155-dependent manner. Phosphorylation of Y322 confers binding of CD226 to the SH2-domain containing protein Grb2 and downstream signaling. Serine 329 phosphorylation of CD226 mediates activation of protein kinase C and the association with lymphocyte function-associated antigen 1 (LFA1) as an intermediate for further signal transduction. TIGIT contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain and an immunoglobulin tail tyrosine (ITT) motif within the cytoplasmic tail. Upon ligand binding, both the ITT and ITIM domains of TIGIT are phosphorylated and recruit adaptor and signaling molecules. Although both mCD96 and hCD96 contain an ITIM-like domain, the human CD96 cytoplasmic domain uniquely also includes a YXXM motif. A detailed review of CD226, TIGIT, and CD96 signaling mechanisms is described in ref. 8. These molecular regulatory mechanisms provide distinct opportunities for drug development in immuno-oncology (**C**). Therapeutic antibodies that block CD155 binding to CD96 or TIGIT would reverse the inhibitory signaling by these receptors (**i**). Using either anti-CD96 or anti-TIGIT mAbs, this approach has received specific experimental support in mouse cancer models. Antibody or mutated ligand ("mutein") modalities that allow preferential signaling through the activating receptor CD226 might tip the balance between CD155-dependent activation and inhibition (**ii**). For instance, a mAb that recognizes and stabilizes the unique interaction complex of CD155/CD226 could enhance stimulatory signaling through CD226. Alternatively, engineered mutations in CD155 muteins that preferentially bind CD96 and/or TIGIT might competitively inhibit native CD155 binding and enhance lymphocyte activation. The blockade of the TIGIT/CD226 heterodimer or the TIGIT/TIGIT homodimer by drug modalities would potentially reduce TIGIT-dependent inhibitory signals (**iii**). DC, dendritic cell.

therapeutic strategy by providing a more robust counterbalance to TIGIT and CD96.

Functional TIGIT suppression of antitumor responses is both intrinsic to effector T cells (Teff) and indirect, via enhancement of Treg activity. The enrichment of TIGIT expression on tumor-infiltrating Tregs compared with peripheral Tregs (23) would suggest that maximal antitumor response could be achieved via modification of the mAb Fc region. Increased binding to activating FcR could mediate a selective depletion of TIGIT$^+$ Tregs at the tumor site through antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular-phagocytosis (ADCP), similar to what has been described for anti–CTLA-4 antibodies (36). The relatively greater TIGIT expression on Tregs versus Teffs within the tumor site would optimally enable this approach. Although CD96 is expressed on CD4$^+$ T cells (11), further work is necessary to determine whether CD96 influences the suppressive function of Tregs, and to define CD96 expression on Tregs and Teffs within the tumor microenvironment and periphery. Importantly, the ability of blocking a CD96 mAb to reduce B16F10 lung metastases was not

dependent on activating FcR (35), indicating that antitumor activity was not due to selective immune subset depletion.

Because receptor-binding domains are conserved in CD155 (25), therapeutic antibodies targeting this ligand are not likely to have selective effects on inhibitory (TIGIT and CD96) signals versus activating (CD226) pathways. However, the relatively higher binding affinity of CD155 to TIGIT or CD96 compared with the lower affinity CD226 interaction can theoretically be exploited for drug development. Greater selectivity toward CD226-dependent signaling and subsequent lymphocyte activation could be achieved using engineered variants (or "muteins") of CD155 or other nectins that retain TIGIT and/or CD96 binding, but cannot bind to CD226. By competing with native CD155, these modalities would functionally block TIGIT and/or CD96 inhibitory signaling, but retain the activation through CD226. The ability of TIGIT to form a signaling-competent homodimer (37) or, conversely, a heterocomplex with CD226 that impairs CD226 activation signaling (21), reveals the dynamic interactions of this receptor system and other potential anticancer drug approaches. Antibodies that target the interface between TIGIT homodimers might reduce inhibitory signaling, whereas targeting interactions between TIGIT and CD226 extracellular domains might conceivably block heterocomplex formation and relieve TIGIT-mediated inhibition of CD226 signaling.

Preclinical mechanistic data and known lymphocyte expression patterns suggest that combinatorial immunotherapy strategies targeting TIGIT and/or CD96 will have improved antitumor responses. For instance, combining anti-CD96 with either anti–CTLA-4 or anti–PD-1 mAbs resulted in a greater reduction in B16F10 lung metastasis compared with monotherapy treatment, an effect that is dependent on NK cells (35). Moreover, survival of mice with 4T1.2 spontaneous metastases was significantly increased by combining anti-CD96 and anti–CTLA-4 or anti–PD-1 compared with monotherapy. Similarly, treatment of CT26 tumors with a combination of anti-TIGIT and anti–PD-L1 dramatically improved antitumor responses compared with each as a monotherapy (21), whereas using an anti–TIM-3 mAb in *Tigit*$^{-/-}$ mice improved the control of B16F10 metastases and subcutaneous tumors (23). Evidence also exists that targeting TIGIT and CD96 in combination could be exploited as a therapeutic strategy. Although *Tigit*$^{-/-}$ mice showed enhanced immunity to either B16F10 melanoma grown as subcutaneous tumors (23), experimental B16F10 lung metastases were not reduced in *Tigit*$^{-/-}$ mice (11). However, treatment of *Tigit*$^{-/-}$ mice with an anti-CD96 mAb resulted in a greater reduction of B16F10 or EO771 lung metastasis than that observed with anti-CD96 mAb treatment in wild-type mice (35), suggesting that these pathways may instruct nonoverlapping lymphocyte subsets and/or distinct molecular mechanisms.

Although immunotherapy functions by increasing host antitumor immunity, the induction of immune-related adverse events (irAE) in patients can limit certain approaches. Clinically, CTLA-4 blockade is associated with more high-grade irAEs than PD-1 (4), and, in agreement, *Ctla-4*$^{-/-}$ mice develop a lethal lymphoproliferative disorder (38). However, *Pd-1*$^{-/-}$ mice can spontaneously develop a range of less severe immune pathologies (39, 40). To date, *Tigit*$^{-/-}$ (41) or *Cd96*$^{-/-}$ mice (11) have not shown spontaneous development of overt immune pathologies; however, *Tigit*$^{-/-}$ mice were more sensitive to the induction of experimental autoimmune encephalomyelitis (41), and TIGIT blockade was shown to increase experimental arthritis development (42). Although caution in the overinterpretation of animal studies is advised, these preclinical observations suggest that CD96- or TIGIT-blocking therapies might have favorable clinical toxicity profiles.

Open questions and challenges

Currently, the foremost challenge for translating TIGIT- or CD96-targeted therapies is to functionally validate blockade of these receptors in human lymphocytes. Two useful validation surrogates include the analysis of TIGIT and CD96 expression/function in patient tumor-infiltrating lymphocytes (TIL), or on T cells from individuals with chronic viral infections, and any correlation with exhaustion markers and/or phenotype. TIGIT was reportedly coexpressed with PD-1 on effector CD8$^+$ T cells during HIV or SIV infection, and increased TIGIT expression correlated with disease progression (22). TIGIT levels were elevated on CD4$^+$ and CD8$^+$ TILs and coexpressed with PD-1 on CD8$^+$ T cells in non–small cell lung cancer (NSCLC), colon cancer, and melanoma samples (12, 21), with similar expression seen on peripheral blood mononuclear cells (PBMC) from AML patients (18). Promisingly, treatment of HIV-specific CD8$^+$ T cells with an anti-TIGIT mAb increased IFNγ production (22). Similarly, treatment of tumor-specific melanoma CD8$^+$ TILs with an anti-TIGIT mAb augmented proliferation and IFNγ production (12, 43). These data are consistent with a coinhibitory function for TIGIT in the context of chronic antigen stimulation in humans and provide a sound rationale for further development of TIGIT blockade therapeutics.

Currently, the validation of hCD96 as a potential immunotherapy target is not as advanced as TIGIT. Although CD96 surface expression is increased on human T cells after activation (26, 44), a high percentage of resting CD8$^+$ T cells from healthy controls express CD96, and the fraction of CD96$^+$ CD8$^+$ T cells was reduced in individuals infected with HIV (44). Interestingly, serum levels of soluble CD96 were found to be elevated in patients with chronic viral hepatitis B infection (45), suggesting that persistent antigen exposure can increase CD96 levels and/or cell surface shedding. In human cancers, CD96 surface expression on TILs versus PBMCs has not been well characterized; however, CD96 mRNA expression, along with TIGIT, was highly expressed and associated with a T-cell signature in lung cancer (21). Current data from preclinical cancer models indicate an inhibitory function for CD96 on NK cells, but whether the same function exists on mouse T cells and human NK or T cells remains to be elucidated. The determination of CD96 expression in human T-cell subsets and TILs and a comprehensive validation of functional pathway activity in human immune cells is an area of active research and is a prerequisite for the development of CD96 blockade therapeutics.

Presently, the relationship between specific structural/biophysical attributes and optimal anticancer mechanisms of TIGIT- or CD96-targeted therapies is not well described. The rationale for blocking CD155/TIGIT binding to augment CD8$^+$ T-cell function as a cancer immunotherapy approach is sound. Although TIGIT robustly inhibits human NK-cell–mediated cytotoxicity *in vitro* (16, 46) in mice, TIGIT NK-cell–mediated tumor suppression is less pronounced than CD96 (11). Moreover, the potentially dominant contribution of TIGIT^{high} Treg cells in the anticancer immune response (23) suggests that a blocking antibody might also augment effector functions. These observations should be reconciled to understand the full, integrated contribution of TIGIT on distinct lymphocytes (e.g., CD8$^+$ T cells, CD4$^+$ T cells, and NK cells) for an optimal antitumor response.

The ability of CD96 or TIGIT to counterbalance activation mediated by CD226 appears to drive most of the anticancer activities of CD96- and TIGIT-targeted therapies. Indeed, a CD226-blocking antibody reversed suppression of CT26 tumors by an anti-TIGIT mAb (21). Similarly, CD96 suppression of B16F10 lung metastases, via genetic deletion or blocking antibody, was mostly dependent on intact CD226 function (11, 35). Within this paired inhibitory/activating axis, the magnitude and integrated quality (activation vs. inhibition) of lymphocyte signaling are dictated not only by the relative availability (and selective competitive binding) of certain ligands, but also by the kinetics and coordinated expression of receptor levels. To this end, CD226 was down regulated on $CD8^+$ T cells and NK cells in AML patients (18, 47), and low CD226 was detected on $CD8^+$ TILs from melanoma patients compared with PBMCs (12). CD226, TIGIT, and CD96 receptor expression was reportedly dynamically modulated by ligand exposure. CD155 interaction decreases surface expression of CD226 (48) and CD96 (31) on contacting cells. Conversely, increased levels of CD226 and CD96 have been observed in $Cd155^{-/-}$ mice, whereas TIGIT levels were unchanged (49). These dynamic alterations in activating and inhibitory receptor levels may tip the balance in net signaling output in a context-dependent manner and potentially alter responses to CD96- and TIGIT-targeted therapies.

Clearly, emerging preclinical evidence suggests there is much promise in modulating the CD96/TIGIT/DNAM/CD155 axis for immuno-oncology. A clearer understanding of the molecular and context-dependent mechanisms by which CD96 and TIGIT function in immunity will pave the way for their therapeutic application in cancer, either as monotherapies or in combination with other therapies. At this stage, the ultimate impact of fine-tuning the function of TIGIT and CD96 receptors on cancer patient outcomes is wholly unknown, but will be interesting to monitor as knowledge advances.

Disclosure of Potential Conflicts of Interest

J.J. Miles reports receiving a commercial research grant from Bristol-Myers Squibb. M.W.L. Teng reports receiving speakers bureau honoraria from Merck Sharpe & Dohme. M.J. Smyth reports receiving a commercial research grant from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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