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Forum

HLA-G/LILRBs: A Cancer Immunotherapy Challenge

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Despite some success, many patients do not benefit from immunotherapy. New strategies to improve clinical efficacy include identification of novel immune-checkpoint (IC) targets or a combination of immunotherapy with antiangiogenic treatments. Here, we propose the therapeutic use of IC, HLA-G/LILRB, and explore its enhanced synergistic antitumor activity when combined with antiangiogenic therapies.

The IC HLA-G Involved in Tumor Escape

Throughout tumor progression, several mechanisms to escape immune destruction are induced. Nascent transformed cells lose their antigenicity and/or immunogenicity. They then acquire the expression of inhibitory molecules regulating T cell activation, termed ICs, and secrete suppressive cytokines, which promote an immunosuppressive microenvironment. Overall, these mechanisms lead to tumor escape via T cell exhaustion and suppressed cell induction.

A breakthrough in cancer therapy to control tumor progression and to re-establish immune function has been the development of immunotherapies, mainly based on antibodies directed at ICs or their ligands. Administration of these antibodies is clinically successful in restoring T cell co-stimulatory signals and reactivating antitumor T cell responses [1]. Despite the success of these therapies, a considerable proportion of patients remains unresponsive. To overcome this challenge, it is necessary to identify new IC targets or synergistic combinations that might restore an immune-supportive microenvironment, reactivate antitumor immunity, and improve the success of cancer immunotherapy.

HLA-G, a nonclassical MHC class I molecule that plays a crucial role in fetal–maternal tolerance, is an IC molecule [2]. HLA-G via interaction of the LILRB1 (ILT2) and LILRB2 (ILT4) receptors (Figure 1 and Table 1) inhibits cytotoxic T cells, natural killer (NK) cells, and B cells, induces T cell anergy, modulates myeloid cells, and promotes T regulatory cells (Tregs). Moreover, HLA-G expressed on antigen-presenting cells (APCs), such as myeloid-derived suppressor cells (MDSCs) or tolerogenic dendritic cells (DCs), promotes T cell hyporesponsiveness and induces Treg differentiation [2]. LILRB1 is expressed on different leukemia and solid tumors. While LILRB1 prevents primary cutaneous T cell lymphoma cell death and enhances gastric tumor growth, HLA-G/LILRB1 interaction inhibits neoplastic B cell proliferation. Blocking LILRB1 on myeloma or lymphoblastic cells does not prevent NK-mediated lysis [3]. LILRB2 is expressed in various solid tumors such as clear cell renal carcinoma (ccRCC) and stroma cells within the tumor microenvironment [4]. LILRB2 expression correlates with poor cell differentiation and advanced metastasis. One underlying mechanism

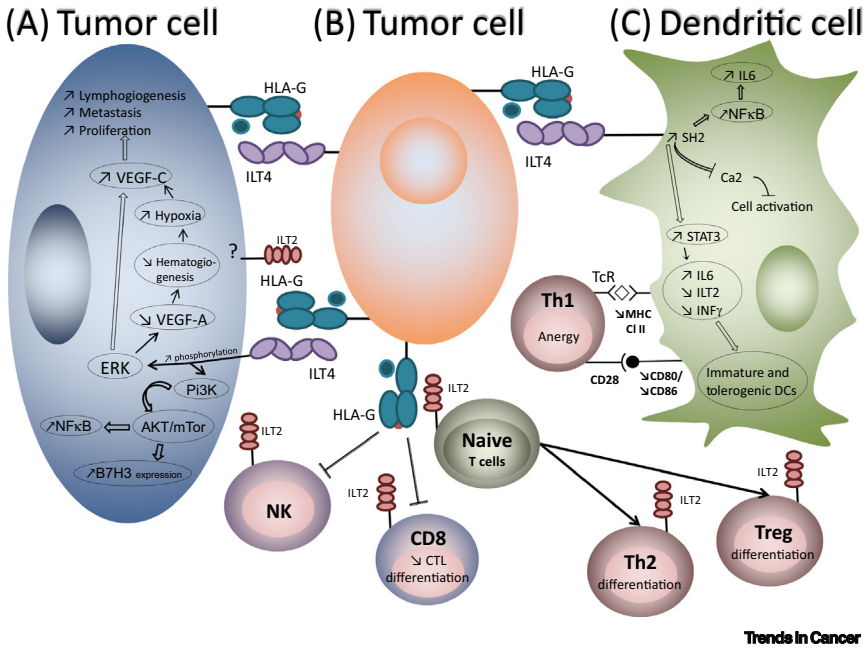

Figure 1. HLA-G-Induced Pathways Following Interaction with Its Receptors. (A) Interaction between LILRB2 expressed on solid tumor cells and HLA-G on neighboring tumor cells upregulates vascular endothelial growth factor-C (VEGF-C) expression via extracellular signal-regulated kinase (ERK) signaling pathway, leading to lymphangiogenesis, metastasis, and proliferation; upregulates B7-H3 expression via PI3K/AKT/mTOR signaling, improving tumor escape. (B) Interaction between HLA-G expressed on tumor cells and LILRB1-expressing natural killer (NK) and CD8$^+$ T cells promotes tumor escape by inhibiting cytotoxicity. LILRB1-expressing on naïve T cells induces their differentiation into Th2 cells and regulatory T cells (Tregs). (C) Interaction between HLA-G expressed on solid tumor cells and LILRB2-expressing dendritic cells (DCs) within the tumor microenvironment downregulates expression of nuclear factor (NF)-κ,B resulting in inhibition of interleukin (IL)-6 and calcium (Ca^{2+}) influx, thereby preventing cell activation. IL-6/STAT3 signaling prevents DC maturation, leading to their conversion into tolerogenic DCs. These modulated DCs promote Th1 cell anergy.

Table 1. HLA-G-Mediating Activities Following Interaction with Its Inhibitory Receptors LILRB1/LILRB2

Cell Type	LILRBs	Effect
Solid tumor cells	LILRB1/LILRB2	Proliferation
		Cell migration, thus cancer dissemination
		Secretion of VEGF by endothelial cells, favoring cancer dissemination
Endothelial cells	LILRB2	Proliferation, leading to neoangiogenesis
		Promote VEGF secretion and consequently vascular permeability
T cells	LILRB1	Inhibition of proliferation and cytotoxicity
		Induction of Tregs
		Prevent migration
NK cells	LILRB1	Inhibition of interferon-γ production and cytotoxicity
		Prevent migration
Macrophages	LILRB1/LILRB2	Polarization towards M2 phenotype
Neutrophils	LILRB2	Inhibition of reactive oxygen species
		Reduced phagocytosis
MDSCs	LILRB1/LILRB2	Expansion
		Induction of Tregs
DCs	LILRB1/LILRB2	Conversion into tolerogenic cells
		Prevention of maturation
		Modulation of T cell responses
		Induction of Tregs
DC-10s	LILRB2	Production of IL-10
		Inhibition of T cell responses
		Induction of Tr1 cells

is the upregulation of vascular endothelial growth factor (VEGF)-C [5], a well-characterized and efficient growth factor involved in lymphangiogenesis and lymphatic metastases.

In immunocompetent mice, HLA-G-expressing tumor cells proliferate, while in immunodeficient mice, administration of HLA-G-expressing tumor cells results in widespread metastasis, suggesting that HLA-G plays a role in immune escape in tumors. Moreover, *de novo* expression of HLA-G is found in most human tumors analyzed, but not in surrounding healthy tissues [2]. These findings are consistent with the involvement of the checkpoint HLA-G in tumor progression and invasiveness. Moreover, HLA-G/LILRBs affect a wider spectrum of immune cells compared to those modulated by cytotoxic T lymphocyte-associated antigen (CTLA)-4/B7 and programmed death (PD)-1/PD ligand-1; known targets of current IC blockade therapies. Therefore, we propose that the checkpoint HLA-G/LILRB should be explored as an additional target for cancer immunotherapy.

Effect of HLA-G/LILRB Checkpoint on Tumor-Infiltrating Cells

Tregs, NK cells, tolerogenic DCs, macrophages, and MDSCs are consistently found within tumors, where they cooperate in promoting tumor escape. HLA-G/LILRB pathways modulate these cell types by inhibiting DC maturation and differentiation, promoting macrophage differentiation into M2-like macrophages, and allowing MDSC expansion. There is also an increased frequency of HLA-G-expressing DC-10s [6] in peripheral blood of patients with high grade gastric cancer [7]. Since HLA-G expression in DC-10s is induced by interleukin (IL)-6 it can be assumed that the presence of IL-10 in the tumor microenvironment might promote the differentiation of T regulatory type 1 (Tr1) cells, an inducible subset of IL-10-producing Tregs characterized by co-expression of CD49b and lymphocyte-activation gene 3 protein (LAG-3) [8], which have been frequently associated with tumors [7], indicating that these cells may be involved in tumor escape.

During tumor development, antigen-specific T cells become dysfunctional under prolonged antigen stimulation, which leads to the induction of exhausted T cells. T cell exhaustion is characterized by poor effector functions and expression of PD-1, Tim-3, LAG-3, CTLA-4, and TIGIT [9]. Targeting CTLA-4 or PD-1 on T cells can be effective in several cancer settings. However, cancer-infiltrating T cells can become refractory to checkpoint-blockade-mediated reactivation. Recently, a population of tumor-infiltrating $CD8^+$ T cells expressing LILRB1, distinct from $CD8^+PD1^+$ T cells, was characterized in ccRCC patients [10]. The $CD8^+LILRB1^+$-mediated cytotoxicity is prevented by HLA-G expression and neutralized by anti-HLA-G antibodies, supporting the notion that targeting HLA-G can rejuvenate cancer-infiltrating exhausted T cells.

HLA-G Targeting for Cancer Immunotherapy

Two HLA-G-targeting approaches are under development for cancer immunotherapy: generation of anti-HLA-G neutralizing antibodies and chimeric antigen receptor (CAR) T cells specific for HLA-G. Better insight into the transcriptional regulation of *HLA-G* gene and the availability of appropriate anti-HLA-G antibodies is needed to implement these approaches. Alternative splicing of the primary HLA-G transcript might generate various isoforms, which lack the α1, α2, or α3 domains [11].

Whether these isoforms are differentially regulated or have different functions is not yet known. Currently available antibodies recognize only the α1 domain of the protein, preventing the detection of HLA-G expression in some cancer patients. Therefore, producing HLA-G antibodies that specifically recognize other domains, in particular an antibody directed to the HLA-G α3 domain, will be essential for developing future therapeutic approaches. The α3 domain is found in most HLA-G isoforms expressed in tumors and contains the epitope recognized by LILRB1 and LILRB2. Therefore, an α3 domain directed antibody will specifically block the interaction of HLA-G with LILRB1 and LILRB2 simultaneously. The use of such an antibody will also have a great advantage over using antibodies directed exclusively against one of the known HLA-G receptors LILRB1 or LILRB2, independently. Since LILRB1 and LILRB2 have different roles in immunity and are found in most immune cells, blocking these receptors might eliminate the complete immune response. In addition, HLA-G might act through other yet undescribed receptors besides LILRB1 or LILRB2, therefore, blocking LILRB1 or LILRB2 will not prevent the action of HLA-G that signals through this receptor.

HLA-G/LILRBs Pathways in Tumor Vascular Remodeling

Tumor vasculature and lymphatic endothelial cells have important roles in immune cell trafficking and constitute key elements that influence tumor behavior and treatment responses. When tumors cannot be sufficiently oxygenated, hypoxia occurs and triggers the upregulation of several genes, including VEGF and a variety of ICs, overall driving a shift towards an immunosuppressive environment. The persistent hypersecretion of proangiogenic factors within the tumor leads to the disorganization of nascent vessels, preventing T cell infiltration. Consequently, the tumor vasculature, mainly targeted by antiangiogenic drugs, has been considered a strategic challenge for antitumor treatment [12]. Although this approach would promote tumor vessel normalization, favor T cell infiltration, and enhance drug delivery to the tumor, antiangiogenic drugs offer only a modest survival benefit, suggesting that multiple nonredundant immunosuppressive mechanisms coexist within tumors.

The relationship of HLA-G and vascular remodeling has been shown in decidua, where embryo-derived HLA-G mediates tolerance by interacting with LILRB1 on maternal decidual (d)NK cells and promotes VEGF expression [13]. In the tumor, NK cells might promote tumor angiogenesis with a similar mechanism: transforming-growth-factor-β-mediated activation of tumor cells promotes VEGF secretion by NK cells supporting neoangiogenesis.

Combination of HLA-G/LILRB Blockade and Antiangiogenesis for a Synergistic Effect

The rationale underpinning the use of IC immunotherapy to enhance the efficacy of cancer antiangiogenic treatments comes from several studies. HLA-G/LILRB2 transcriptionally modifies the expression of VEGF-A/C in renal carcinoma cells derived from a highly vascularized tumor with immunogenic properties [5]. LILRB2 also induces the upregulation of VEGF-C in non-small cell lung cancer, promoting tumor progression [14]. In addition, pharmacological inhibition of VEGF-A leads to increased intratumoral $CD8^+$ T cells and decreased Treg recruitment, resulting in reduced tumor growth. Moreover, VEGF promotes the upregulation of PD-1 on T cells leading to T cell exhaustion [15]. Further studies that identify the molecular mechanisms underlying this two-way regulation would be valuable to understand the biology of the tumor and develop innovative therapeutic approaches.

Concluding Remarks

Immunotherapy protocols based on HLA-G blockade with novel HLA-G antibodies, alone or in combination with antiangiogenesis protocols might constitute a promising strategy for breaking down tolerance in tumors and promote rejuvenation of exhausted tumor-infiltrating $CD8^+$ T cells. In addition, minor adverse effects could be produced by targeting HLA-G, since its expression under physiological conditions is highly restricted. In the future, an extended understanding of the crosstalk between tumor cells, endothelial cells, and immune cells could improve the effectiveness of protocols that may relieve endothelial anergy and enhance the abundance of activated T cells in tumors, ultimately improving outcomes for cancer patients.

Declaration of Interests

The authors declare no potential conflicts of interest.

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Forum

GSDME: A Potential Ally in Cancer Detection and Treatment

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Gasdermin E (GSDME) is an executioner of caspase-3 mediated regulated pyroptosis, and as such plays an important role in several cancers. Recent findings highlighted the potential of GSDME as a biomarker that can be implemented in cancer diagnosis, monitoring, and therapy.

GSDME and Cancer

The gasdermin E gene (*GSDME*), was originally characterized as a gene responsible for an autosomal dominant form of hereditary hearing loss. It is ubiquitously expressed in most organs [1]. The gene is epigenetically silenced in several cancers such as gastric, colorectal, and breast cancers, and is postulated to be a tumor suppressor gene. After cleavage by caspase-3, GSDME converts non-inflammatory apoptosis to necrosis by direct permeabilization of the plasma membrane through pore formation [2,3]. Despite the hypothesis that *GSDME* might be a tumor suppressor gene, no recurrent loss-of-function mutations in *GSDME* were found in tumor tissue initially. Instead, the gene seemed to be epigenetically inactivated through methylation which was first reported in gastric cancer. Soon after, additional reports of *GSDME* promoter hypermethylation in several other cancer types followed, corroborating that hypermethylation occurs pan-cancer and is the dominant form of silencing this gene [4,5]. Still, a clear link between hypermethylation and *GSDME* silencing is yet to be found [6,7], suggesting that this gene's regulation is complex and relies on the presence/absence of several cofactors that have not all been elucidated yet. Very recently however, some loss-of-function mutations were reported in *GSDME*-expressing cancers [8]. The current leading hypothesis is that cancer cells employ two strategies to evade tumor suppression by *GSDME*: epigenetic suppression most commonly, and loss-of-function mutations to a lesser extent. Recent studies on the function of the gasdermin gene family have further outlined the role of *GSDME* in regulated pyroptosis and its significance for cancer diagnosis, and immunotherapies.

Use of *GSDME* Methylation as a Detection Biomarker

Clinically useful detection biomarkers have to be highly sensitive, specific, and stable. Ideally, they should be capable of detecting cancers in early tumor stages. Such markers can be obtained from bodily fluids, solid tissues, or more importantly from circulating DNA in the bloodstream. Several studies have shown that *GSDME* methylation is a promising detection biomarker for cancer. Studies on breast [6] and colorectal [7] cancers, using genome wide methylation datasets from The Cancer Genome Atlas (TCGA), reported differential methylation between tumor and healthy tissues in all *GSDME* CpG sites, profiled by the Illumina 450K array. Moreover, in a more recent study, aberrant *GSDME* methylation was seen pan-cancer, with widespread hypermethylation in promoter CpGs and hypomethylation of gene body CpGs. Interestingly, these perturbed methylation patterns were used in a model that could identify cancer samples from normal samples and also predict the present tumor type, with high accuracy [5]. Currently, most biomarkers target only single cancers and few can simultaneously detect and localize multiple cancer types. The demonstrated performance of *GSDME* makes it an attractive candidate gene for further development as a cancer detection biomarker. Blood-based assays, such as liquid biopsies, are an ideal setting where DNA methylation could be measured through array or digital droplet PCR technologies, and used to detect tumors in a minimally-invasive manner. *GSDME* methylation could also be combined with other molecular determinants in a highly discriminative multi-analyte test, to achieve sensitivity. Methods to detect mutations have been known to struggle with early stage cancer patients due to detection limits, and in such cases methylation can serve as an alternative determinant in liquid biopsies. Moreover, age and disease stage were shown not to have measurable effects on sample identification, hence *GSDME* methylation could even be applicable in the early screening setting (Figure 1). (See Box 1.)

GSDME as a Prognostic Biomarker

Molecular changes that can be accurately measured and used to evaluate the course of a disease in absence of treatment are termed prognostic markers. With respect to *GSDME*, methylation as well as mRNA and protein expression were examined and proposed as prognostic cancer markers. *GSDME* in fact gets its original nomenclature, [i.e., inversely correlated with estrogen