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RESEARCH ARTICLE

Inhibition of human Vγ9Vδ2 T-cell antitumoral activity through HLA-G: implications for immunotherapy of cancer

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Abstract Vγ9Vδ2 T cells play a crucial role in the anti-tumoral immune response through cytokine production and cytotoxicity. Although the expression of the immunomodulatory molecule HLA-G has been found in diverse tumors, its impact on Vγ9Vδ2 T-cell functions remains unknown. Here we showed that soluble HLA-G inhibits Vγ9Vδ2 T-cell proliferation without inducing apoptosis. Moreover, soluble HLA-G inhibited the Vγ9Vδ2 T-cell production of IFN-γ induced by phosphoantigen stimulation. The reduction in Vγ9Vδ2 T-cell IFN-γ production was also induced by membrane-bound or soluble HLA-G expressed by tumor cell lines. Finally, primary tumor cells inhibited Vγ9Vδ2 T-cell proliferation and IFN-γ production through HLA-G. In this context, HLA-G impaired Vγ9Vδ2 T-cell cytotoxicity by interacting with ILT2 inhibitory receptor. These data demonstrate that HLA-G inhibits the anti-tumoral functions of Vγ9Vδ2 T cells and imply that treatments targeting HLA-G could optimize Vγ9Vδ2 T-cell-mediated immunotherapy of cancer.

Keywords γδ T cell · HLA-G · IFN-γ · Cytotoxicity · ILT2 inhibitory receptor

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Introduction

Human peripheral blood γδ T cells are a subset of lymphocytes involved in immune responses against tumors and infections [1, 2]. The majority expresses a cell surface Vγ9Vδ2 encoded T-cell receptor (TCR). The Vγ9Vδ2 T cells recognize via their TCR some small nonpeptidic molecules termed phosphoantigens expressed at the surface of tumor or infected cells [3]. The activation of Vγ9Vδ2 T cells by phosphoantigens induces signaling pathways leading to proliferation and to production of proinflammatory cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) which are essential for boosting adaptive immune responses against tumors [4]. Besides their ability to proliferate and produce cytokines upon activation, the major function of Vγ9Vδ2 T cells is to kill tumor target cells or infected cells through the release of lytic granules. The cytolytic activity of Vγ9Vδ2 T cells has been shown to be dependent on γδ TCR triggering and is enhanced by the engagement of the NKG2D receptor with ligands such as MHC class I chain-related proteins A and B (MICA/B) or UL16 binding proteins (ULBP) [5, 6].

The ability of Vγ9Vδ2 T cells to directly kill tumor cells and to enhance adaptive antitumoral immune responses

through IFN-γ or TNF-α cytokine production demonstrates their participation in the immune response against cancers. In this regard, clinical trials including Vγ9Vδ2 T cells which were carried out in lymphoid malignancies and in patients with solid tumors have revealed disease stabilization and partial tumor regression in some treated patients [7–12]. Although the differences observed in the efficiency of treatments implicating Vγ9Vδ2 T cells have not yet been elucidated, they could result from specific tumor expression profiles of the molecules involved in tumor immune escape mechanisms [13].

Among the various strategies developed by tumors to escape the immune response, induction of the expression of the nonclassical human leukocyte antigen (HLA) class I molecule HLA-G has been proposed as an efficient way to turn-off antitumoral immune responses [14–17]. The nonclassical HLA class I molecule HLA-G differs from classical HLA class I molecules by having low allelic polymorphism and seven alternatively spliced isoforms which can lead to four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three soluble (HLA-G5, -G6 and -G7) isoforms [18, 19]. The most well-characterized isoforms are the membrane-bound HLA-G1 and soluble HLA-G5 isoforms which are composed of a heavy chain noncovalently associated with beta-2-microglobulin (B2M). In contrast to classical HLA class I molecules, the expression of HLA-G is highly restricted to specific tissues. Indeed, in physiological conditions HLA-G has been shown to be expressed in trophoblasts, thymus, mesenchymal stem cells, cornea and erythroblasts. However, its expression can be induced in some pathologies such as cancers, accepted transplants, inflammatory disorders and viral infections [18, 19].

The immunomodulatory function of HLA-G occurs through interaction with inhibitory receptors. To date, three receptors for HLA-G, immunoglobulin-like transcript 2 (ILT2/CD85j/LILRB1), ILT4 (CD85d/LILRB2) and KIR2DL4 (CD158d), have been characterized. These receptors have been shown to be selectively expressed in certain immune cell populations. The inhibition of immune effector cells through these receptors is ensured by intracytoplasmic immunotyrosine-based inhibition motifs (ITIM) which upon phosphorylation become docking sites for SHP-1 and SHP-2 phosphatases which in turn inhibit activatory signals.

Analysis of tumor biopsies from patients has revealed that HLA-G is expressed by tumors such as melanoma, renal carcinoma, some hemopathic malignancies, and ovarian cancers for which $\gamma\delta$ T-cell based immunotherapy protocols are currently under development [20]. Even though previous studies showing that HLA-G expression by tumor cell lines can inhibit $CD8^+$ T cell and NK cell cytotoxicity suggest a possible role of HLA-G on other cytolytic effector cells, the effect of HLA-G on Vγ9Vδ2 T cell antitumoral functions remains unknown [21–23].

In this study, we investigated the impact of HLA-G expression by tumors on the activity of Vγ9Vδ2 T cells. We report that both soluble HLA-G5 and membrane-bound HLA-G1 isoforms inhibited Vγ9Vδ2 T-cell functions, accounting for their antitumoral activity.

Materials and methods

Cells and culture

Vγ9Vδ2 T lymphocyte polyclonal cells were generated from peripheral blood mononuclear cells (PBMC) from healthy donors (1×10^6 cells/ml) as previously described [24]. On day 0, PBMC were activated with 3 μM bromohydrin pyrophosphate (BrHPP/Phosphostim; Innate Pharma, Marseille, France) in RPMI-1640 (Invitrogen) supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 100 IU/ml IL-2 (Pro-leukin, Innate Pharma) and were further incubated for 14 days. IL-2 was added to the medium every 2–3 days from day 4 at a final concentration of 100 IU/ml. The specific expansion of Vγ9Vδ2 T cells was followed by flow cytometry and the final population was tested in functional assays only if composed of at least 90% TCR$\gamma\delta^+$ cells. For all experiments Vγ9Vδ2 T cells were used only when at least 25% of the whole population expressed ILT2 on the surface (Table 1).

The M8 melanoma cell lines transfected with the pcDNA3.1 vector (Invitrogen) either alone (M8-pcDNA) or containing the HLA-G1 cDNA (M8-HLA-G1) or the HLA-G5 cDNA (M8-HLA-G5) were obtained as previously described [23].

FON is a human melanoma primary cell line derived from a patient's melanoma lesion as previously described [25]. A distinction is made between FON cells expressing endogenous HLA-G1 (referred to as FON HLA-G^+) and FON cells which have lost HLA-G1 and B2M expression after long-term in vitro culture (referred to as FON HLA-G^- $B2M^-$). FON HLA-G^- $B2M^-$ have been transduced with a B2M lentiviral construct in order to obtain FON HLA-G^- $B2M^+$ cells expressing HLA class I molecules but not HLA-G on their surface.

All cell lines were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Antibodies and flow cytometry

For flow cytometry, Vγ9Vδ2 T lymphocyte Fc receptors were blocked using 20 μg/ml of immunoglobulins from human serum (Sigma) prior to staining. All steps were performed on ice, and isotype-matched control antibodies

Table 1 Phenotype of BrHPP-expanded PBMC populations. PBMC from six donors were stimulated with BrHPP. Following 14 days of in vitro expansion, cells were stained with FITC-conjugated anti-TCRγδ mAb and PE-Cy5-conjugated anti-ILT2 mAb, or with anti-CD2, anti-LFA-1, or anti-NKG2D mAbs

	TCRγδ	ILT2	NKG2D	LFA-1	CD2
Mean ± SD ($n = 6$ donors)	91 ± 2%	33 ± 8%	89 ± 10%	100%	100%

were systematically used. Anti-TCR-γδ FITC (clone IMMU 510), anti-NKG2D PE (clone ON72), anti-CD2 FITC (clone 39C1.5), anti-ILT4 PE (clone 42D1) monoclonal antibodies (mAbs) were from Beckman Coulter; anti-LFA-1 FITC (clone MEM-25) and anti-CD3 PE (clone MEM-57) were from Exbio; anti-KIR2DL4 (clone 18170) was from RD Systems; anti-ILT2 PE-Cy5 (clone GHI/75) and anti-IFN-γ FITC (clone B27) mAbs were from BD Pharmingen; and anti-Ki-67 rabbit polyclonal antibody was from Chemicon International. For measurement of HLA-G and HLA -A, -B, -C and -E expression levels on FON tumor cells, we used anti-HLA-A, -B, -C and -E (clone TP25.99), kindly provided by Dr. S. Ferrone (University of Pittsburgh Cancer Institute, Pittsburgh, PA) and anti-HLA-G1 (Clone MEMG/09) from Exbio. Flow cytometry analyses were performed on an Epics XL cytometer (Beckman Coulter) using EXPO32 software (Beckman Coulter).

For blocking experiments, Vγ9Vδ2 T lymphocytes were incubated for 30 min with a final concentration of 10 μg/ml isotype control mAb or blocking anti-ILT2 (clone GHI/75, BD Pharmingen) prior to use.

Preparation of HLA-G5 coated nanobeads

HLA-G5 recombinant protein was produced in SF9 insect cells infected with HLA-G5 baculovirus as previously described [26].

Magnetic goat anti-mouse IgG beads (Ademtech) were first incubated for 1 h at 4°C with anti-HLA-G5 mAb (clone 6A10). After washing, the 6A10-coated beads were incubated overnight with the SF9 cell lysates containing the recombinant HLA-G5 protein or with PBS 1×, 0.1% BSA alone as negative control. After extensive washes, beads were collected by magnetic separation and then used in functional assays.

Proliferation assays

Proliferation of Vγ9Vδ2 T cells was measured by ^{3}H-labeled thymidine incorporation. 10^5 Vγ9Vδ2 T cells were incubated for 48 h in the presence of 100 IU/ml IL-2 with either nanobeads or mitomycin C-treated FON cells in 96-well plates. Where indicated, plates were coated with 1 μg/ml of anti-CD3 antibody (OKT3). ^{3}H-labeled thymidine (Amersham Biosciences) was added (1 μCi per well) 18 h before harvesting. Incorporation of ^{3}H-labeled thymidine was measured on a β-counter (Wallac 1450, Amersham Biosciences).

Apoptosis assays

Vγ9Vδ2 T cells (5×10^5) were incubated for 48 h in the presence of nanobeads at a 1:10,000 cell/bead ratio. Early and late apoptosis were measured by staining Vγ9Vδ2 T cells with annexin V-FITC and propidium iodide (PI) following the manufacturer's instructions (Miltenyi Biotec). Early apoptotic Vγ9Vδ2 T cells were defined as annexin V-FITC-positive/PI-negative cells and late apoptotic Vγ9Vδ2 T cells as annexin V-FITC-positive/PI-positive cells. Changes in mitochondrial transmembrane potential were determined by loading Vγ9Vδ2 T cells with 40 nM $DiOC_6(3)$ (Molecular Probes) for 15 min at 37°C in an atmosphere containing 5% CO_2. The apoptotic Vγ9Vδ2 T cells were characterized by a low $DiOC_6(3)$ fluorescence.

Measurement of Ki-67 expression in Vγ9Vδ2 T cells

Vγ9Vδ2 T cells were incubated for 48 h with 100 IU/ml IL-2 and nanobeads at a 1:10,000 cell/bead ratio. For microscopy, 2×10^5 Vγ9Vδ2 T cells were laid on poly-L-lysine-coated slides for 5 min at 37°C. The cells were fixed with 3% paraformaldehyde for 10 min and permeabilized with 0.1% saponin and 0.1% Triton X-100 for 5 min. Staining was performed using Ki-67 rabbit polyclonal antibody (Chemicon International) followed by Alexa-488-conjugated secondary antibody (Invitrogen). The samples were mounted in Vectashield medium (Vector Laboratories) and examined on a Carl Zeiss microscope using a plan apochromat 63×/1.4 oil immersion objective. For flow cytometry analyses, 5×10^5 Vγ9Vδ2 T cells were fixed, permeabilized and stained with the indicated antibodies.

Measurement of intracellular Vγ9Vδ2 T-cell IFN-γ production by flow cytometry

Vγ9Vδ2 T cells (2×10^5) were incubated in the presence of brefeldin A at 10 μg/ml (Sigma) with either nanobeads (cell/bead ratio 1:10,000) and 60 nM BrHPP or stimulatory cells (E/T ratio 1:2) with or without 5 nM BrHPP. After 5 h of incubation at 37°C in an atmosphere containing 5% CO_2, conjugates were disrupted with ice-cold PBS 1× containing 10 mM EDTA. Cells were fixed with 3%

paraformaldehyde and permeabilized with 0.3% saponin. Intracellular staining was performed with IFN-γ FITC mAb (clone B27, BD Pharmingen).

ELISA

Vγ9Vδ2 T cells (1×10^5) were incubated overnight with either nanobeads (cell/bead ratio 1:10,000) and 60 nM BrHPP or stimulatory cells (E/T ratio 1:2) with or without 5 nM BrHPP. IFN-γ released into the supernatant was quantified following the manufacturer's instructions (ELISA MAX Set Deluxe, Biolegend).

Confocal microscopy

Vγ9Vδ2 T cells were conjugated with target cells which had been previously loaded for 15 min at 37°C with 2 µM CellTracker Orange CMTMR (Molecular Probes). After 150 min of incubation at 37°C, conjugates were laid on poly-L-lysine-coated slides for 5 min at 37°C. Cells were then fixed for 10 min with 3% paraformaldehyde and permeabilized with 0.1% saponin. Staining was performed with IFN-γ FITC mAb (clone B27, BD Pharmingen).

The samples were mounted in Vectashield medium (Vector Laboratories) and examined on a Carl Zeiss LSM 510 confocal microscope using a plan apochromat 63×/1.4 oil immersion objective.

Fluorescence intensities were analyzed over the entire Vγ9Vδ2 T cell membrane on single confocal sections using the "Profile" function of the Zeiss LSM 510 software. This function was used to draw a line across the Vγ9Vδ2 T cell, starting at an arbitrary point outside the Vγ9Vδ2 T cell and passing through the Vγ9Vδ2 T cell/tumor cell contact site. The software produces a graph of fluorescence intensity for each fluorophore at each point along the row versus the relative position of each point along the row.

Lentiviral construct and FON cell transduction

The human B2M cDNA was amplified by PCR with oligos 5′-AAAACGCGTATGTCTCGCTCCGTGGCC-3′ and 5′-AAAACTAGTTTACATGTCTCGATCCCACTT-3′. The PCR products were digested with *Mlu*I and *Spe*I restriction enzymes and cloned into pWPXL lentiviral vector (Addgene) from which GFP was removed with restriction enzymes *Mlu*I and *Spe*I.

Human B2M lentiviral particles were produced by triple-transfecting HEK293T cells by the calcium phosphate method with pWPXL-B2M, packaging plasmid psPAX2 and envelope plasmid pMD2.G (Addgene). The supernatant containing lentiviral particles was harvested 48 h after transfection. FON cells were transduced with lentiviral particles and the cell surface expression of HLA-class I molecules was checked by flow cytometry analysis 48 h after lentiviral transduction.

Cytotoxicity assays

The cytolytic activity of Vγ9Vδ2 T cells against ^{51}Cr-labeled target cells was assessed in a standard 4-h ^{51}Cr-release assay as previously reported [23]. For each experiment, triplicate samples were used.

Cell cycle analysis on Vγ9Vδ2 T cells

Vγ9Vδ2 T cells (1×10^6) were incubated for 48 h in the presence of nanobeads at a 1:10,000 cell/bead ratio in plates previously coated with 1 µg/ml anti-CD3 mAb. The cells were then fixed overnight at 4°C in 70% ethanol and stained with 40 µg/ml PI (Molecular Probes) in the presence of 100 µg/ml RNase A (Sigma). Analyses were performed on a BD FACSCanto II cytometer using BD FACSDiva software.

Statistical analyses

Data are presented as means ± standard error of the mean (SEM). Student's t test was used and P values lower than 0.05 were taken to be significant.

For correlation analysis, Vγ9Vδ2 T cells from ten different donors were used. After expansion, ILT2 levels on Vγ9Vδ2 T cell populations ranged from 6% to 60%. Vγ9Vδ2 T cells were stimulated and stained for IFN-γ as described above to determine the percentage of inhibition of IFN-γ production mediated by HLA-G. The association between the percentage of inhibition of IFN-γ production and the level of ILT2 expression on the Vγ9Vδ2 T cell population used was then determined by linear regression analysis. The coefficient of determination R^2 was calculated as the square of Pearson's correlation coefficient.

Results

ILT2 inhibitory receptor for HLA-G but not ILT4 nor KIR2DL4 is expressed at the surface of human Vγ9Vδ2 T cells

Previous studies have shown variable expression of the ILT2 inhibitory receptor for HLA-G at the surface of Vγ9Vδ2 T cells [27, 28]. Moreover, their expression of ILT4 or KIR2DL4 receptors remains unknown. Therefore, we first characterized the expression profile of HLA-G receptors present at the surface of Vγ9Vδ2 T cells. To this end, Vγ9Vδ2 T cells were amplified by stimulating PBMCs with phosphoantigens as previously described [24]. As

shown in Fig. 1, after 2 weeks of stimulation, more than 90% of phosphoantigen-expanded cells expressed a γδ TCR at their surface. Similar results were obtained when the phosphoantigen-expanded cells were stained with anti-TCRδ2 or anti-TCRγ9 mAb, indicating that this cell population corresponded to Vγ9Vδ2 T cells (data not shown). Additional cell-surface staining indicated that expanded Vγ9Vδ2 T cells expressed high levels of NKG2D activatory receptors but also LFA-1 or CD2 adhesion molecules as previously reported (Fig. 1 and Table 1).

Having shown that the population of Vγ9Vδ2 T cells represented more than 90% of the expanded cells, we next assessed the levels of ILT2, ILT4 and KIR2DL4 surface receptors. As shown in Fig. 1 and Table 1, the Vγ9Vδ2 T cells from six donors had similar levels of expression of ILT2 inhibitory receptors (33 ± 8% of Vγ9Vδ2 T cells, $n = 6$). In contrast, staining for ILT4 or KIR2DL4 revealed that none of the Vγ9Vδ2 T cells from any donor tested expressed these receptors at their surface (Fig. 1, and data not shown).

These results indicate that in our experimental system, the expanded human Vγ9Vδ2 T cells always expressed the ILT2 inhibitory receptor for HLA-G but did not express ILT4 or KIR2DL4 receptors.

Soluble HLA-G5 isoform inhibits the proliferation of Vγ9Vδ2 T cells

The proliferation of Vγ9Vδ2 T cells could represent an important step for their antitumoral activity in vivo. Since HLA-G has been shown to inhibit the proliferation of $CD4^+$ T cells [29, 30], the impact of the soluble HLA-G5 isoform on Vγ9Vδ2 T-cell proliferation was investigated. For this purpose, nanobeads were precoated with soluble HLA-G5 isoform (Fig. 2a) in order to mimic HLA-G multimerization which is essential for HLA-G inhibitory function in vivo [31–34]. As shown in Fig. 2b, in the presence of IL-2, Vγ9Vδ2 T cells proliferated efficiently. However, when soluble HLA-G5 was added, proliferation was strongly inhibited despite the presence of IL-2. In contrast, control nanobeads did not significantly alter Vγ9Vδ2 T-cell proliferation.

Since soluble HLA-G has been reported to induce the apoptosis of T $CD8^+$ and NK $CD8^+$ cells [35, 36], the levels of apoptotic Vγ9Vδ2 T cells incubated with or without soluble HLA-G5 were compared by flow cytometry. As shown in Fig. 2c, the levels of either early or late apoptosis in Vγ9Vδ2 T cells remain similar in the presence or absence of soluble HLA-G5. In addition, Vγ9Vδ2 T cells showed comparable $DiOC_6(3)$ fluorescence profiles when incubated with control or HLA-G5-coated nanobeads, indicating that soluble HLA-G5 did not induce the apoptosis-related loss of mitochondrial transmembrane potential (see Supplementary material 1).

Given that the inhibition of Vγ9Vδ2 T-cell proliferation by soluble HLA-G5 was unlikely to result from apoptosis, we next investigated whether the inhibition was due to cell cycle blockade. For this purpose we assessed the expression of the protein Ki-67 which is present during all active phases of the cell cycle (G1, S, G2, and M), but is absent from resting cells (G0), thus making it an appropriate marker for cell cycling in a given cell population [37]. As shown in Fig. 2d, e, the expression of Ki-67 was detected in Vγ9Vδ2 T cells alone or coincubated with control beads. By contrast, Ki-67 expression was greatly reduced in Vγ9Vδ2 T cells coincubated with soluble HLA-G5.

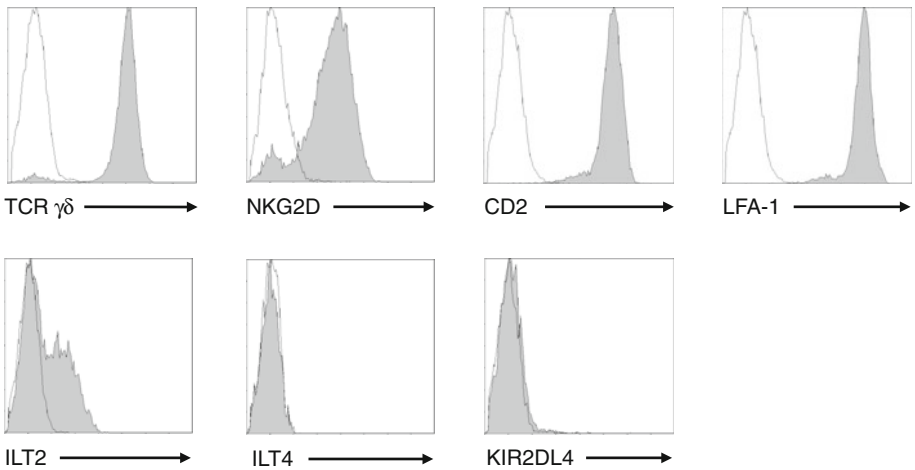


Fig. 1 ILT2 inhibitory receptor for HLA-G but not ILT4 and KIR2DL4 is expressed at the surface of Vγ9Vδ2 T cells. The cell surface expression levels of TCRγδ, CD2, LFA-1 and NKG2D markers in phosphoantigen-expanded PBMCs were analyzed by flow cytometry using specific mAbs (*gray-filled histograms*). ILT2, ILT4 and KIR2DL4 expression at the surface of Vγ9Vδ2 T cells was assessed by gating on the TCR $\gamma\delta^+$ cell population (*gray-filled histograms*). *Open histograms* show background staining obtained using isotype-matched control antibodies

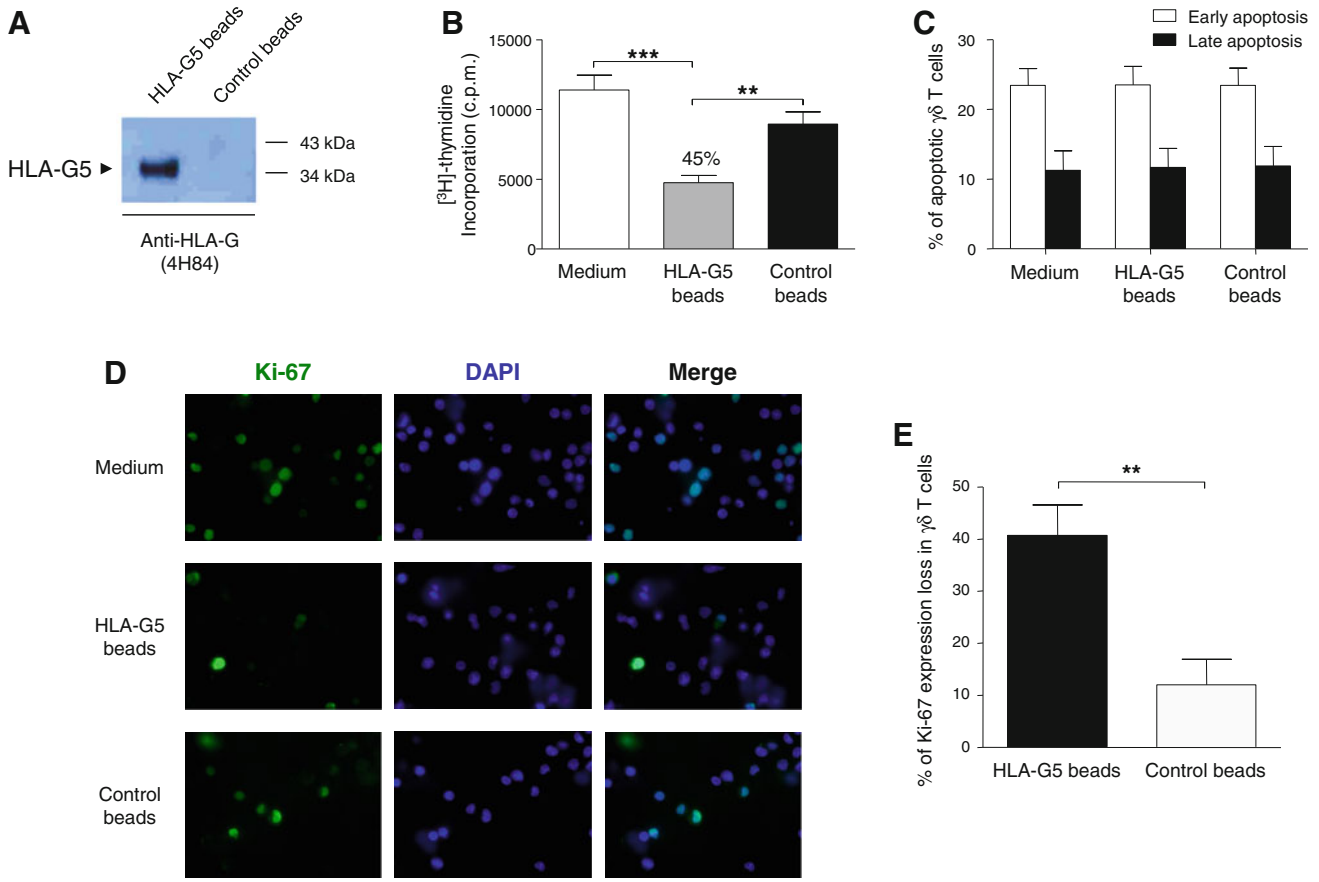


Fig. 2 Soluble HLA-G5 isoform inhibits the proliferation of Vγ9Vδ2 T cells. **a** Soluble HLA-G5 coating on nanobeads was checked by western blotting using anti-HLA-G mAb (clone 4H84). **b–e** Vγ9Vδ2 T cells were incubated for 48 h in the presence of IL-2 (100 IU/ml) alone, with control nanobeads or HLA-G5-coated nanobeads (10,000 beads per cell). **b** Proliferation of Vγ9Vδ2 T cells was evaluated by incorporation of ^{3}H-labeled thymidine during the last 18 h. The results are presented as the means ± SEM of seven independent experiments. ***$P = 0.0005$, **$P = 0.0029$. The percentage indicated represents the mean inhibition of proliferation observed with HLA-G5-coated nanobeads compared with control nanobeads. **c** The percentages of apoptotic Vγ9Vδ2 T cells were determined by flow cytometry after annexin V-FITC and PI double staining. The results are presented as the means ± SEM of five independent experiments. **d** Vγ9Vδ2 T cells were fixed, permeabilized, stained for Ki-67 (*green*) and mounted in medium containing a nuclear stain DAPI (*dark blue*) before analysis by fluorescence microscopy. **e** The percentages of Vγ9Vδ2 T cells expressing Ki-67 were determined by flow cytometry. The loss of Ki-67 expression observed in the presence of nanobeads was calculated as a percentage of control. The results are presented as the means ± SEM of three independent experiments. **$P = 0.0089$

These results indicate that the soluble HLA-G5 isoform can inhibit Vγ9Vδ2 T-cell proliferation by impairing cell cycle progression.

Soluble HLA-G5 isoform inhibits the phosphoantigen-induced production of IFN-γ by Vγ9Vδ2 T cells

Besides proliferation, recognition of phosphoantigens by Vγ9Vδ2 T cells induces their production of IFN-γ which is essential to enhance the efficiency of the adaptive immune responses against tumors. We next investigated the impact of soluble HLA-G5 on Vγ9Vδ2 T-cell IFN-γ production induced through phosphoantigen stimulation. As shown in Fig. 3a, b, measurement of intracellular IFN-γ levels by flow cytometry indicated that a 60 nM concentration of BrHPP phosphoantigen induced IFN-γ production in Vγ9Vδ2 T cells. However, when soluble HLA-G5 was added to Vγ9Vδ2 T cells, production of IFN-γ induced by BrHPP phosphoantigen was severely inhibited, whereas control nanobeads had very little impact.

Additional experiments with cells from donors expressing lower levels of ILT2 were performed and a linear regression was applied to determine if the expression levels of ILT2 in Vγ9Vδ2 T cell populations were significantly correlated with the HLA-G5-mediated inhibition of IFN-γ production. As shown in Fig. 3c, in Vγ9Vδ2 T cell populations expressing low levels of ILT2, soluble HLA-G5 poorly inhibited their production of IFN-γ. Conversely, in Vγ9Vδ2 T cell populations expressing elevated levels of ILT2, soluble HLA-G5 induced a markedly decreased IFN-γ production.

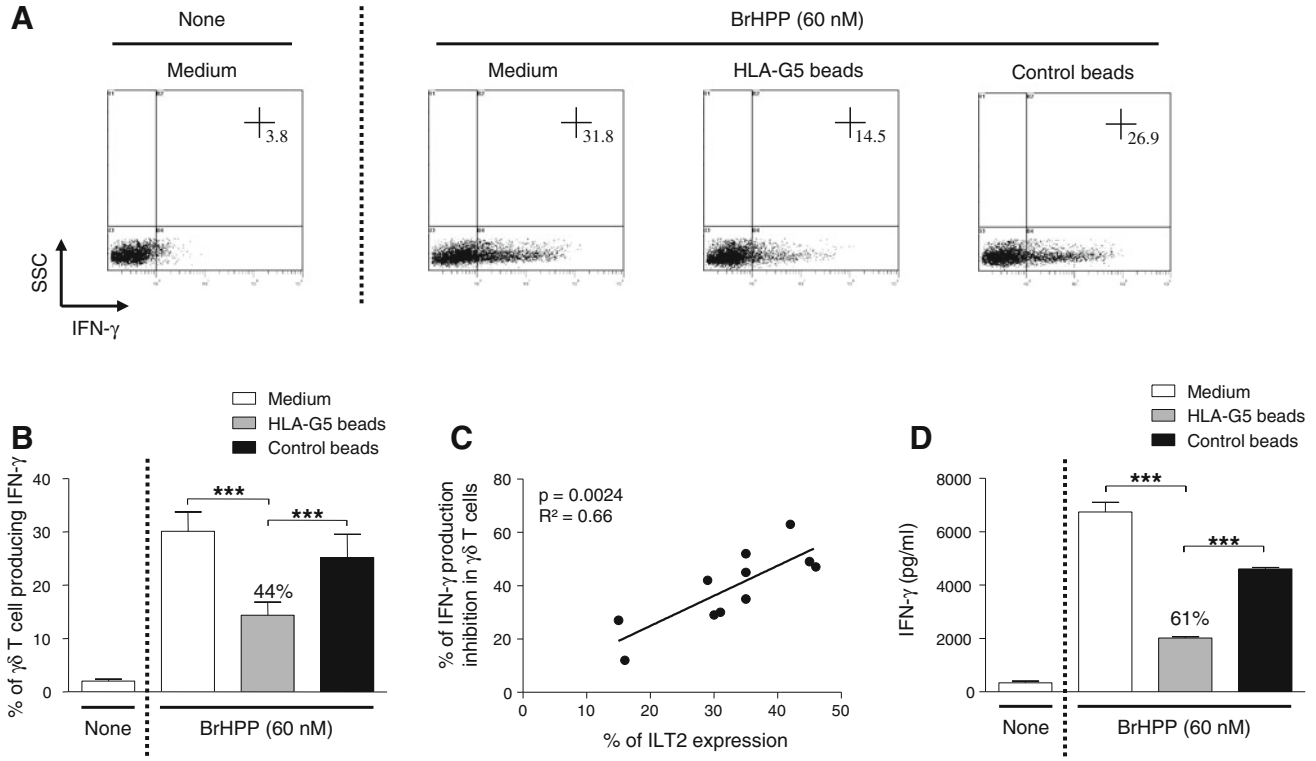


Fig. 3 Soluble HLA-G5 isoform inhibits the Vγ9Vδ2 T-cell production of IFN-γ induced through BrHPP phosphoantigen stimulation. Vγ9Vδ2 T cells were stimulated with BrHPP (60 nM) and incubated alone, with HLA-G5-coated nanobeads or with control nanobeads (10,000 beads per cell). **a–c** The percentages of Vγ9Vδ2 T cells producing IFN-γ after a 5-h incubation in the presence of brefeldin A (10 μg/ml) were assessed by flow cytometry following intracellular staining. **a** Dot plots from a representative experiment are shown. **b** The means ± SEM of nine independent experiments are shown. $***P < 0.0001$, $***P = 0.0008$. The percentage indicated is the mean inhibition observed with HLA-G5-coated nanobeads compared with control nanobeads. **c** Expression levels of ILT2 on Vγ9Vδ2 T cells and IFN-γ production inhibition mediated through HLA-G5 in 11 independent experiments are shown together with the corresponding regression line. $R^2 = 0.66$; $P = 0.0024$. **d** The levels of IFN-γ released by Vγ9Vδ2 T cells into the supernatant after overnight culture were quantified by ELISA. The results are presented as the means ± SEM of duplicate cultures and are representative of four independent experiments. $***P = 0.0007$; $***P < 0.0001$. The percentage indicated is the inhibition observed with HLA-G5-coated nanobeads compared with control nanobeads

The level of IFN-γ released by BrHPP-activated Vγ9Vδ2 T cells and the effect of soluble HLA-G5 on this release were also quantified by ELISA. As shown in Fig. 3d, Vγ9Vδ2 T cells released high levels of IFN-γ after an overnight incubation with BrHPP phosphoantigen. By contrast, soluble HLA-G5 efficiently inhibited the Vγ9Vδ2 T cell production of IFN-γ induced by BrHPP phosphoantigen stimulation (Fig. 3d).

These results indicate that HLA-G5 soluble isoform is able to sustain the inhibition of phosphoantigen-induced production of IFN-γ by Vγ9Vδ2 T cells, and that this inhibition is probably mediated through the interaction of HLA-G with the ILT2 inhibitory receptor.

Both HLA-G1 membrane-bound and HLA-G5 soluble isoforms expressed by M8 tumor cells inhibit the Vγ9Vδ2 T-cell production of IFN-γ

To assess whether HLA-G1 and HLA-G5 isoforms expressed by tumor cells had the same impact as soluble HLA-G5 on Vγ9Vδ2 T-cell IFN-γ production, M8 tumor cell transfectants expressing either the membrane-bound HLA-G1 isoform (M8-HLA-G1) or the secreted HLA-G5 isoform (M8-HLA-G5) or M8 cells transfected with the pcDNA3.1 vector alone (M8-pcDNA) were used for stimulation.

Figure 4a, b shows that incubation of Vγ9Vδ2 T-cells with either a low dose of BrHPP phosphoantigen or with M8-pcDNA tumor cells alone failed to stimulate their production of IFN-γ. However, when M8-pcDNA tumor cells were combined with a low dose of BrHPP phosphoantigen, the production of IFN-γ by Vγ9Vδ2 T cells was induced after 5 h of coincubation. Conversely, when Vγ9Vδ2 T cells were coincubated with M8-HLA-G1 tumor cells and a low dose of BrHPP phosphoantigen, the induction of IFN-γ production was highly inhibited (Fig. 4a, b).

Using diverse populations of Vγ9Vδ2 T cells expressing variable levels of ILT2 inhibitory receptor, a correlation between the inhibition of IFN-γ production induced by M8-HLA-G1 tumor cells and the expression levels of ILT2 on

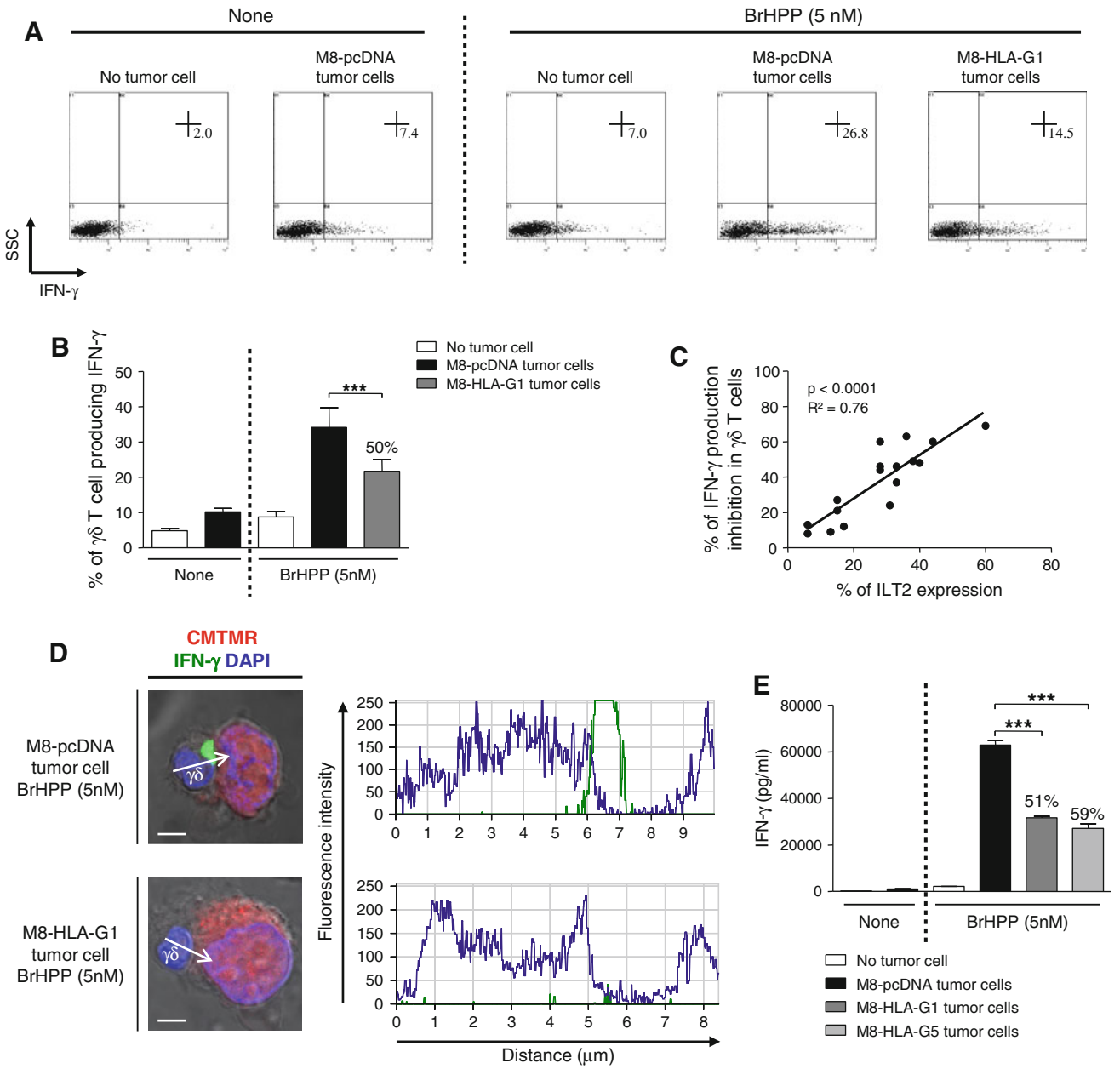


Fig. 4 M8 tumor cell lines expressing either membrane-bound HLA-G1 or soluble HLA-G5 isoforms inhibit the production of IFN-γ by Vγ9Vδ2 T cells. To study the impact of HLA-G1 and HLA-G5 isoforms expressed by tumor cell lines, IFN-γ production by Vγ9Vδ2 T cells was induced through incubation with M8 stimulatory tumor cells in the presence of BrHPP (5 nM). **a–c** After 5 h of incubation with M8-pcDNA or M8-HLA-G1 stimulatory tumor cells in the presence of brefeldin A (10 μg/ml) and BrHPP (5 nM), the percentage of Vγ9Vδ2 T cells producing IFN-γ was assessed by intracellular staining. **a** Dot plots from a representative experiment are shown. **b** The means ± SEM of 11 independent experiments are shown. ***$P = 0.0004$. The percentage indicated is the mean inhibition of the production of IFN-γ induced by M8-HLA-G1 tumor cells compared with M8-pcDNA tumor cells. **c** Expression levels of ILT2 on Vγ9Vδ2 T cells and IFN-γ production inhibition mediated through HLA-G1 in 17 independent experiments are shown together with the corresponding regression line. $R^2 = 0.76$; $P < 0.0001$. **d** The localization of the IFN-γ produced in Vγ9Vδ2 T cell/M8 tumor cell conjugates was analyzed by confocal microscopy. Vγ9Vδ2 T cells were conjugated with M8-pcDNA or M8-HLA-G1 stimulatory tumor cells (*red*) for 150 min and then stained for IFN-γ (*green*) and mounted in medium containing DAPI to color the nucleus (*dark blue*). *Scale bars* 5 μm. *Right panel* Intensities of green fluorescence (IFN-γ) and blue fluorescence (DAPI) along the arrows on the images. The images are representative of at least three independent experiments. **e** The levels of IFN-γ produced into the supernatants of Vγ9Vδ2 T cells alone or coincubated overnight with M8-pcDNA, M8-HLA-G1 or M8-HLA-G5 stimulatory tumor cells in the presence of BrHPP (5 nM) quantified by ELISA. The results are presented as the means ± SEM of triplicate cultures and are representative of three independent experiments. ***$P = 0.0001$; ***$P = 0.0002$. The percentage indicated is the inhibition of the IFN-γ production induced by M8-HLA-G1 or M8-HLA-G5 tumor cells compared with that observed for M8-pcDNA tumor cells

Vγ9Vδ2 T cell populations was established. As shown with soluble HLA-G5, inhibition of IFN-γ production induced by HLA-G1 was low in Vγ9Vδ2 T cell populations expressing low levels of ILT2 but was high in Vγ9Vδ2 T cell populations expressing elevated levels of ILT2 (Fig. 4c).

In order to visualize in situ the impact of HLA-G1 on IFN-γ production by Vγ9Vδ2 T cells conjugated with M8 tumor cells, staining for IFN-γ was performed in Vγ9Vδ2 T cell/M8-pcDNA tumor cell and Vγ9Vδ2 T cell/M8-HLA-G1 tumor cell conjugates and analyzed by confocal microscopy. As shown in Fig. 4d, production of high amounts of IFN-γ was detected in Vγ9Vδ2 T cells conjugated with tumor M8-pcDNA cells. Measurement of fluorescence intensity throughout the Vγ9Vδ2 T cells indicated that the production of IFN-γ was polarized at the contact site with the M8-pcDNA tumor cells. In agreement with flow cytometry experiments, IFN-γ production was not detected in the majority of Vγ9Vδ2 T cell/M8-HLA-G1 tumor cell conjugates analyzed by confocal microscopy.

Since flow cytometry analysis of Vγ9Vδ2 T-cell IFN-γ production employs brefeldin A to impair secretion of soluble molecules, this method is not valid for the assessment of the role of soluble HLA-G5 produced by M8-HLA-G5 tumor cells on Vγ9Vδ2 T cell IFN-γ production. We therefore measured the impact of HLA-G5 and HLA-G1 expression by M8 tumor cells on Vγ9Vδ2 T cell IFN-γ production by ELISA. Figure 4e shows that overnight coculture of Vγ9Vδ2 T cells with M8-pcDNA tumor cells and a low dose of BrHPP phosphoantigen induced production of IFN-γ. In contrast, when Vγ9Vδ2 T cells were cocultured with M8-HLA-G1 or M8-HLA-G5 tumor cells and a low dose of phosphoantigen the levels of IFN-γ produced by Vγ9Vδ2 T cells were highly reduced.

Together, these results indicate that both membrane-bound HLA-G1 and secreted HLA-G5 isoforms expressed by M8 tumor cell transfectants inhibit the Vγ9Vδ2 T-cell production of IFN-γ.

Endogenous expression of HLA-G1 by FON human primary tumor cells inhibits Vγ9Vδ2 T-cell proliferation and IFN-γ production

Having shown that soluble HLA-G5 and M8 tumor cell transfectants expressing HLA-G1 or HLA-G5 isoforms inhibit proliferation and IFN-γ production of Vγ9Vδ2 T cells, we next investigated the impact of HLA-G1 endogenous expression by human primary tumor cells on these Vγ9Vδ2 T cell-mediated functions. For this purpose we took advantage of the FON primary tumor cells which endogenously express HLA-G1 and other HLA-class I molecules (FON HLA-G^+) at their surface but have lost the expression of HLA-G1 and B2M (FON HLA-G^- $B2M^-$) after long-term cell culture [25]. As shown in Fig. 5a, loss of B2M expression in FON HLA-G^- $B2M^-$ tumor cells impaired cell surface expression of HLA-class I molecules which were sequestered in the cytoplasm. Therefore, we transduced FON HLA-G^- $B2M^-$ tumor cells with a lentiviral construct containing human B2M in order to obtain FON HLA-G^- $B2M^+$ tumor cells expressing HLA class I molecules but not HLA-G1 at their surface (Fig. 5a). We used these FON tumor cells to determine the specific effect of HLA-G1 endogenous expression on Vγ9Vδ2 T-cell proliferation and IFN-γ production.

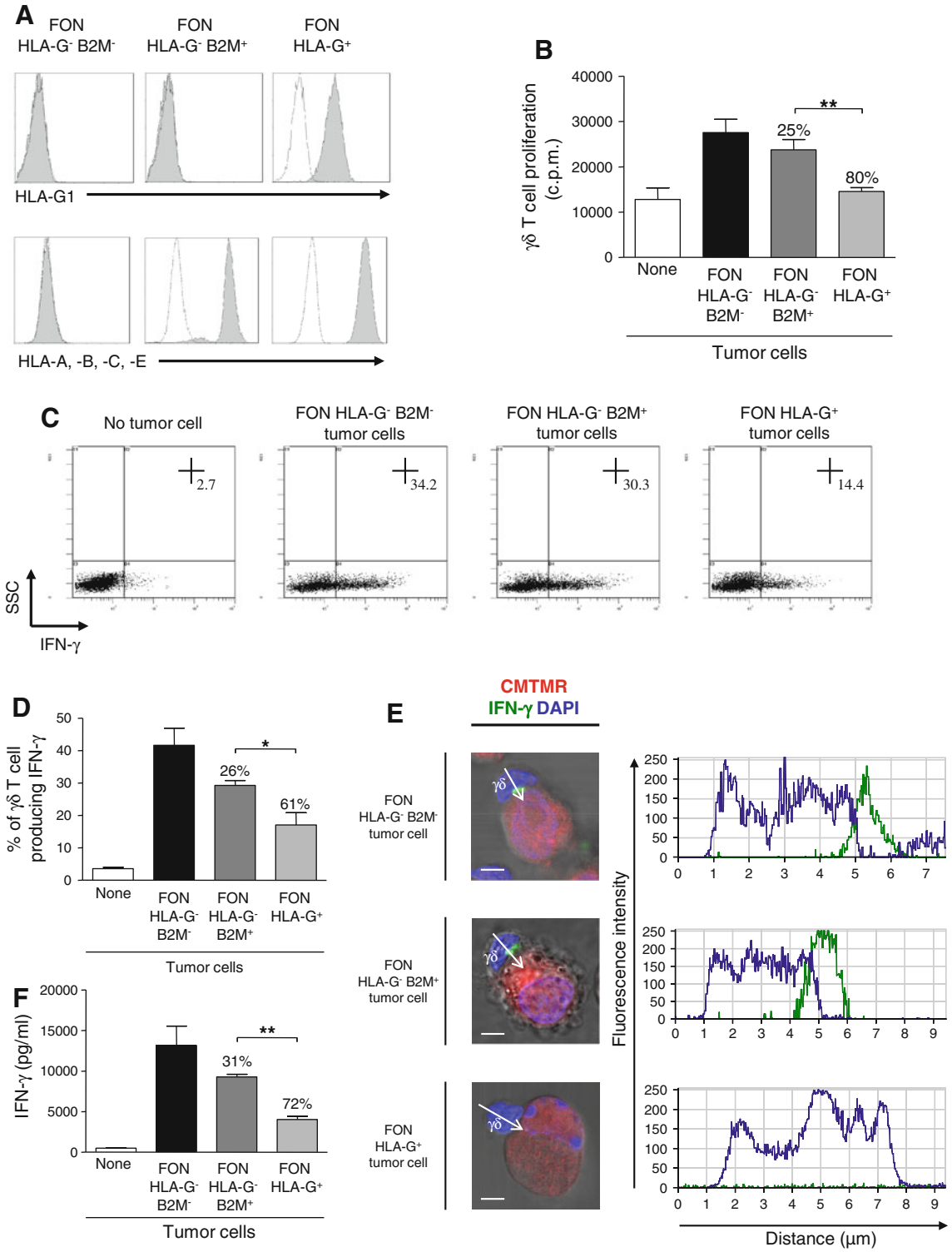
As shown in Fig. 5b, coincubation of Vγ9Vδ2 T cells with FON HLA-G^- $B2M^-$ tumor cells increased twofold their proliferation compared to that of Vγ9Vδ2 T cells alone. Similarly, when Vγ9Vδ2 T cells were co-incubated with FON HLA-G^- $B2M^+$ tumor cells, their proliferation was also increased. By contrast, FON HLA-G^+ tumor cells strongly inhibited the proliferation of Vγ9Vδ2 T cells.

We next determined if Vγ9Vδ2 T-cell production of IFN-γ was affected by FON HLA-G^+ tumor cells. As shown in Fig. 5c, d and f, coincubation of Vγ9Vδ2 T cells with FON HLA-G^- $B2M^-$ tumor cells or with FON HLA-G^- $B2M^+$ tumor cells induced production of IFN-γ which did not require addition of exogenous phosphoantigen. Moreover, confocal microscopy analysis of Vγ9Vδ2 T cell/FON HLA-G^- $B2M^-$ tumor cell or Vγ9Vδ2 T cell/FON HLA-G^- $B2M^+$ tumor cell conjugates showed that the Vγ9Vδ2 T cell production of IFN-γ was polarized at the area of contact (Fig. 5e). By contrast, when Vγ9Vδ2 T cells were coincubated with FON HLA-G^+ cells their production of IFN-γ was strongly inhibited and no IFN-γ was detected in the Vγ9Vδ2 T cell/FON HLA-G^+ tumor cell conjugates (Fig. 5c–f).

These data show that the endogenous surface expression of HLA-G1, but not of other HLA class I molecules, strongly inhibits the proliferation and the production of IFN-γ by Vγ9Vδ2 T cells.

Endogenous expression of HLA-G1 by FON tumor primary cells inhibits Vγ9Vδ2 T-cell cytolytic function through interaction with ILT2 inhibitory receptor

Since cytotoxicity is the main function of Vγ9Vδ2 T cells accounting for their antitumoral activity, we next investigated the impact of HLA-G1 expression by FON HLA-G^+ tumor cells on Vγ9Vδ2 T-cell cytolytic activity. As shown in Fig. 6, FON HLA-G^- $B2M^-$ and FON HLA-G^- $B2M^+$ tumor cells were efficiently lysed by Vγ9Vδ2 T cells. However, the cytolytic activity of Vγ9Vδ2 T cells was



highly inhibited when FON HLA-G$^+$ tumor cells were used as targets (Fig. 6). In order to determine whether ILT2 inhibitory receptors expressed at the surface of Vγ9Vδ2 T cells were involved in this inhibition, we performed blocking experiments using anti-ILT2 mAb. As shown in Fig. 6, cytolytic activity of Vγ9Vδ2 T cells against FON HLA-G$^+$ tumor cells was restored with blocking anti-ILT2 mAb whereas the isotype control mAb had no effect.

These results indicate that FON HLA-G$^+$ tumor cells expressing HLA-G1 at their surface inhibit the cytolytic

◀ **Fig. 5** Endogenous expression of HLA-G1 by human primary FON tumor cells inhibits Vγ9Vδ2 T-cell proliferation and IFN-γ production. **a** Expression of HLA-G and of the other HLA class-I molecules on the surface of FON HLA-G$^-$ B2M$^-$, FON HLA-G$^-$ B2M$^+$ and FON HLA-G$^+$ tumor cells were analyzed by flow cytometry using MEM-G/09 (anti-HLA-G1) and TP 25.99 (anti-HLA-A, -B, -C and -E) mAbs (*gray-filled histograms*). *Open histograms* show background staining obtained using isotype-matched control antibodies. **b** To assess the impact of FON tumor cells on Vγ9Vδ2 T-cell proliferation, incorporation of ^{3}H-labeled thymidine was measured after 48 h of incubation of Vγ9Vδ2 T cells either alone or with mitomycin-C-treated FON HLA-G$^-$ B2M$^-$, FON HLA-G$^-$ B2M$^+$ or FON HLA-G$^+$ tumor cells. The results are presented as the means ± SEM of four independent experiments. $^{**}P = 0.0018$. The percentages indicated are the mean inhibitions of proliferation induced by FON HLA-G$^-$ B2M$^+$ tumor cells or FON HLA-G$^+$ tumor cells compared with that induced by FON HLA-G$^-$ B2M$^-$ tumor cells. **c**, **d** After 5 h of incubation with FON HLA-G$^-$ B2M$^-$, FON HLA-G$^-$ B2M$^+$ or FON HLA-G$^+$ stimulatory tumor cells in the presence of brefeldin A (10 μg/ml), the percentage of Vγ9Vδ2 T cells producing IFN-γ was assessed by intracellular staining. **c** Dot plots from a representative experiment are shown. **d** The means ± SEM of six independent experiments are shown. $^{*}P = 0.0126$. The percentages indicated are the mean inhibitions of the production of IFN-γ induced by FON HLA-G$^-$ B2M$^+$ tumor cells or FON HLA-G$^+$ tumor cells compared with that induced by FON HLA-G$^-$ B2M$^-$ tumor cells. **e** Localization of IFN-γ produced in Vγ9Vδ2 T cells conjugated with FON tumor cells was analyzed by confocal microscopy. Vγ9Vδ2 T cells were conjugated with FON HLA-G$^-$ B2M$^-$, FON HLA-G$^-$ B2M$^+$ or FON HLA-G$^+$ tumor cells (*red*) for 150 min and then stained for IFN-γ (*green*) and mounted in medium containing DAPI to color the nucleus (*dark blue*). *Scale bars* 5 μm. *Right panel* Intensities of green fluorescence (IFN-γ) and blue fluorescence (DAPI) along the arrows drawn on the images. The images are representative of at least three independent experiments. **f** The levels of IFN-γ produced into the supernatants of Vγ9Vδ2 T cells alone or coincubated overnight with FON HLA-G$^-$ B2M$^-$, FON HLA-G$^-$ B2M$^+$ or FON HLA-G$^+$ tumor cells were quantified by ELISA. The results are presented as the means ± SEM of duplicate cultures and are representative of three independent experiments. $^{**}P = 0.0092$. The percentages indicated are the mean inhibitions of the production of IFN-γ induced by FON HLA-G$^-$ B2M$^+$ tumor cells or by FON HLA-G$^+$ tumor cells in comparison with that induced by FON HLA-G$^-$ B2M$^-$ tumor cells

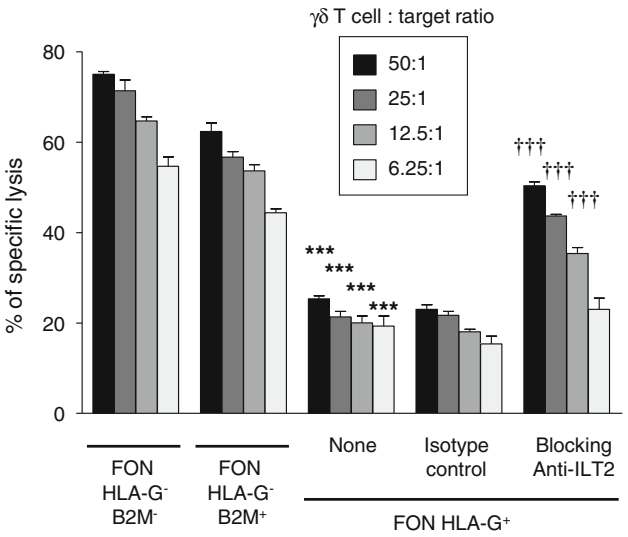


Fig. 6 Vγ9Vδ2 T-cell cytolytic function is specifically inhibited by FON HLA-G$^+$ tumor cells through ILT2 interaction. The cytolytic activity of Vγ9Vδ2 T cells was measured using a ^{51}Cr-release assay performed between Vγ9Vδ2 T cells and FON HLA-G$^-$ B2M$^-$, FON HLA-G$^-$ B2M$^+$ or FON HLA-G$^+$ tumor cells. Blocking anti-ILT2 mAb (clone GHI/75) or the corresponding isotype control mAb isotype (both 10 μg/ml final concentration) was added before coincubation and was present throughout the experiment. The data are presented as the means ± SEM of triplicate cultures from one representative of three experiments. $^{***}P < 0.0001$ for the ratios 50:1, 25:1 and 12.5:1, and $P = 0.0004$ for the ratio 6.25:1, as compared to FON HLA-G$^-$ B2M$^+$ tumor cells; $^{†††}P < 0.0001$ for the ratios 50:1 and 25:1, and $P = 0.0003$ for the ratio 12.5:1, as compared to FON HLA-G$^+$ tumor cells with the isotype control mAb

activity of Vγ9Vδ2 T cells through interaction with ILT2 inhibitory receptor.

Discussion

In this study we investigated the effect of the HLA-G immunomodulatory molecule on human Vγ9Vδ2 T-cell antitumoral functions. We demonstrated for the first time that HLA-G expression by tumor cells inhibits the proliferation, the production of proinflammatory cytokines and the cytolytic activity of Vγ9Vδ2 T cells by interacting with the ILT2 inhibitory receptor. The characterization of the known receptors for HLA-G at the surface of expanded Vγ9Vδ2 T cells allowed us to select donors expressing similar levels of ILT2. In addition, the recurrent negative staining for ILT4 and KIR2DL4 receptors on expanded Vγ9Vδ2 T cells ruled out any effect of these receptors in the follow-up of our study.

The role of HLA-G in the inhibition of CD8$^+$ T cell and CD4$^+$ T cell proliferation has been reported but the mechanisms involved remain controversial [30, 35, 36]. Indeed, studies by Solier et al. and Contini et al. [35, 36] have shown that HLA-G stops NK CD8$^+$ and CD8$^+$ T cell proliferation by inducing apoptosis. This is mediated by the interaction of HLA-G with CD8. However, Bahri et al. [30] have reported that HLA-G inhibits CD4$^+$ and CD8$^+$ T-cell proliferation by stopping cell cycle progression. In agreement with a previous report [38], staining of the Vγ9Vδ2 T cells used in our study indicated that they do not express CD8 at their surface (data not shown). Moreover, whereas soluble HLA-G5 isoform clearly inhibited the proliferation of Vγ9Vδ2 T cells induced by IL-2, it did not increase either early or late apoptosis. However, staining for Ki-67, a well-known marker of the active phases of the cell cycle, indicated that Ki-67 expression was inhibited in Vγ9Vδ2 T cells treated with HLA-G5. In addition, we found that Vγ9Vδ2 T-cell proliferation induced with anti-CD3 antibody was inhibited by soluble HLA-G. In this context, analysis of cell cycle phases using PI incorporation

indicated that soluble HLA-G inhibited cell cycle progression of Vγ9Vδ2 T cells (Supplementary material 2). Therefore, we propose that HLA-G interaction with Vγ9Vδ2 T cells inhibits their proliferation by impairing cell cycle progression rather than by inducing apoptosis. Previous studies have shown that γδ T-cell cycle progression can be stopped in G0/G1 phase of the cell cycle through ligation of the WC1 large type 1 transmembrane protein. In this case, the γδ T-cell growth arrest observed is correlated with (1) induction of the cyclin kinase inhibitor p27kip1 expression, (2) down-modulation of cyclins A, D2 and D3 expression, and (3) dephosphorylation of pocket proteins p107, p130 and retinoblastoma (pRb) [39–41].

More recently, Bahri et al. [30] showed that soluble HLA-G can induce cell cycle arrest of $CD4^+$ and $CD8^+$ T cells by preventing the phosphorylation of pRb. In addition, p27kip1 accumulates while the expression of cyclins A, B, D2 and E is down-modulated in T cells treated with HLA-G. Since ILT2 was defined as the HLA-G receptor mediating T cell cycle arrest, we may propose that in our case the cell cycle arrest of Vγ9Vδ2 T cells mediated through HLA-G/ILT2 interaction occurs through a similar induction of p27kip1 expression accompanied by the dephosphorylation of pRb and the down-modulation of diverse cyclins. In this model, the interaction of HLA-G with ILT2 would induce the activation of phosphatases which would attenuate the γδ T-cell signaling pathways leading to their proliferation. However, given the numerous signaling components and the complexity of the relationship between the diverse transduction pathways implicated in the induction of γδ T-cell proliferation, further investigations are required to clearly characterize the biochemical events targeted during the HLA-G-mediated γδ T-cell cycle arrest.

Vγ9Vδ2 T cells are considered high IFN-γ producers. IFN-γ is a critical cytokine for the efficiency of the anti-tumoral adaptive immune response [42]. Our data showed that the HLA-G5 soluble isoform alone could inhibit Vγ9Vδ2 T-cell production of IFN-γ induced by phosphoantigen stimulation. Moreover, M8 tumor cell transfectants expressing HLA-G1 or HLA-G5 were able to inhibit Vγ9Vδ2 T-cell production of IFN-γ. Despite high levels of HLA class I molecules on the surface of M8-pcDNA control tumor cells, no inhibition of Vγ9Vδ2 T-cell IFN-γ production was observed in this case. The ability of HLA-G1 to strongly inhibit the production of IFN-γ by Vγ9Vδ2 T cell compared to other HLA class I molecules was confirmed using FON tumor cells. The discrepancy between the lack of inhibition of Vγ9Vδ2 T-cell IFN-γ production by HLA-G-negative tumor cells and the high expression level of HLA class I molecules emphasizes the important role of HLA-G in the inhibition of IFN-γ production by Vγ9Vδ2 T cells. Previous reports have demonstrated that the production of IFN-γ by NK cells could be inhibited through the interaction of HLA-G with the inhibitory receptor ILT2 [43, 44]. In the present study, we observed that the inhibition of IFN-γ production mediated by HLA-G was significantly correlated with the levels of ILT2 detected on the surface of Vγ9Vδ2 T cells. Together these data strongly suggest that the interaction of HLA-G with ILT2 is responsible for the inhibition of IFN-γ production in Vγ9Vδ2 T cells.

In our experimental model, the induction of Vγ9Vδ2 T cell IFN-γ production by M8 tumor cells required the presence of suboptimal doses of exogenous phosphoantigens while FON primary tumor cells alone induce the production of high amount of IFN-γ. Since flow cytometry did not reveal any major differences in the expression of costimulatory molecules between FON and M8 tumor cells, it is possible that this discrepancy results from differential levels of endogenous phosphoantigens as previously reported by Gober et al. [45]. This mechanism would involve dysregulation of the mevalonate pathway in FON cells leading to accumulation of metabolites such as phosphoantigens and thus to the activation of Vγ9Vδ2 T cells.

Previous studies have shown that the interaction of HLA-E expressed at the surface of tumor cells with CD94/NKG2A inhibitory receptor could inhibit Vγ9Vδ2 T-cell cytolytic function [46–48]. Here, the use of FON HLA-G^- $BM2^+$ tumor cells expressing HLA-A, -B, -C and -E versus FON HLA-G^+ tumor cells expressing HLA-A, -B, -C and -E and HLA-G1 ruled out any involvement of HLA-E or other HLA molecules in the inhibition of Vγ9Vδ2 T-cell cytolytic function. Moreover, Vγ9Vδ2 T-cell cytolytic function was restored with the use of the blocking ILT2 antibody indicating that the inhibition of Vγ9Vδ2 T-cell cytolytic activity was mediated through the interaction of the inhibitory receptor ILT2 with HLA-G1. These results are in agreement with surface plasmon resonance studies showing that ILT2 binds to HLA-G with a three- to four-fold higher affinity than to classical HLA class I molecules [49]. Together, these data suggest that HLA-G is more efficient than other HLA class I molecules in inhibiting Vγ9Vδ2 T cell function, through interaction with ILT2 inhibitory receptor.

The surface expression of ILT2 on $CD8^+$ and γδ T cell subsets has been associated with high cytolytic activity and production of Th1 cytokines [27, 28, 50, 51]. The expression of the inhibitory receptor ILT2 at the surface of the most cytolytic T cells has been proposed to represent a regulation mechanism which would provide a safety mechanism to control potentially harmful cells. These studies are consistent with our data since the killing of FON HLA-G^+ tumor cells by Vγ9Vδ2 T cells was almost fully restored with the use of the blocking ILT2 mAb despite an ILT2 surface expression of 40% in the total

Vγ9Vδ2 T cell population. Thus, the elevated antitumoral potential of the ILT2$^+$ Vγ9Vδ2 T cells associated with the tumoral expression of HLA-G, the preferred ligand for ILT2, indicate that this interaction might play a major role in the inhibitory mechanism leading to the attenuation of Vγ9Vδ2 T-cell antitumoral functions.

Besides the expression of HLA-G1 on the surface of tumor cells, the expression of soluble HLA-G by diverse tumor cells and elevated soluble HLA-G levels in the plasma from patients with renal cell carcinoma (RCC), ovarian carcinoma and chronic lymphocytic leukemia have been reported [52–54]. In addition, soluble HLA-G either alone or produced by tumor cells is able to impair NK cell cytolytic activity [55–58]. Consistent with these findings, we found that M8 tumor cells expressing soluble HLA-G5 isoform were able to inhibit the cytolytic activity of Vγ9Vδ2 T cells as effectively as M8-HLA-G1 tumor cells (data not shown). These results suggest that even if a low amount of tumor cells express HLA-G, their permanent production of soluble HLA-G into the microenvironment could help the tumor to escape the antitumoral functions of Vγ9Vδ2 T cells.

Previous studies have shown that NK cell and CD8$^+$ T cell cytotoxicity is inhibited by the HLA-G1 isoform, and also by the HLA-G2, -G3 and -G4 isoforms [22, 23, 59]. Therefore, we performed cytotoxic assays using Vγ9Vδ2 T cells and tumor transfectant cells expressing HLA-G2, HLA-G3 or HLA-G4 isoforms. However, in our experiments, no inhibition of Vγ9Vδ2 T-cell cytolytic activity was observed (data not shown). These differences may have been a result of the expression of KIR2DL4 on NK cells and a subset of CD8$^+$ T cells. Indeed, since KIR2LD4 recognizes the alpha-1 domain of HLA-G common to all isoforms, it is a good candidate to explain this difference in sensitivity to HLA-G.

Even though the structure of the Vγ9Vδ2 T cell immunological synapse has been characterized, the effect of inhibitory signaling on this molecular organization remains unknown [60]. HLA-G1 or HLA-G5 expression by transfected tumor cells has been shown to inhibit NK cell cytoskeletal reorganization at the immunological synapse upon interaction with ILT2 [44, 57]. In addition, the inhibitory signal induced through ILT2/HLA-G interaction was shown to impair the polarization of NK cell lytic granules toward the target cell [44, 57]. Since the activation of Vγ9Vδ2 T cells depends on both TCR triggering and costimulatory signals, comparison of the molecular events occurring at the γδ T-cell synapse formed with tumor cells expressing or not HLA-G1 might help to understand the molecular mechanisms governing the killing of tumor target cells by Vγ9Vδ2 T cells.

The reactivity of Vγ9Vδ2 T cells against a broad range of tumors, including solid tumors such as melanoma, squamous cell carcinoma, colon and renal carcinoma and hematopoietic tumors such as B cell lymphomas, has been shown [61]. These observations, together with the engineering of synthetic phosphoantigens for clinical applications, have lead to the development of clinical trials based on the use of phosphoantigen-activated Vγ9Vδ2 T cells [62]. Nevertheless, it is now well established that HLA-G is expressed by several types of tumor. For instance, HLA-G expression has been frequently found in melanoma, RCC lesions and on B-cell lymphomas [63–65]. Vγ9Vδ2 T cells are known to be reactive against such tumors and phosphoantigen-expanded Vγ9Vδ2 T cells have been tested in clinical trials involving RCC. However, our results clearly indicate that constitutive expression of HLA-G by tumor cells inhibited Vγ9Vδ2 T-cell cytolytic activity which represents their main antitumoral function. Moreover, inhibition of Vγ9Vδ2 T-cell IFN-γ production through the tumoral expression of HLA-G also acts as an efficient mechanism to dampen the activation of the effector cells involved in the adaptive antitumoral immune response. Future studies addressing whether the antitumoral activity of Vγ9Vδ2 T cells is abrogated by RCC cell or B-cell lymphoma expressing HLA-G in an autologous experimental system will therefore provide useful information.

In conclusion, our study indentified HLA-G as a potential tumoral target since blocking its expression or inhibitory function in tumors could improve the Vγ9Vδ2 T cell-based immunotherapy of cancers.

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