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Binding of HLA-G to ITIM-Bearing Ig-like Transcript 2 Receptor Suppresses B Cell Responses

Abderrahim Naji,*,† Catherine Menier,*,† Fabio Morandi,‡ Sophie Agaugué,*,† Guitta Maki,*‡ Elisa Ferretti,§ Sylvie Bruel,*‡ Vito Pistoia,‡ Edgardo D. Carosella,*‡,†,1 and Nathalie Rouas-Freiss*,†,1

Inhibition of B cells constitutes a rational approach for treating B cell–mediated disorders. We demonstrate in this article that the engagement of the surface Ig-like transcript 2 (ILT2) inhibitory receptor with its preferential ligand HLA-G is critical to inhibit B cell functions. Indeed, ILT2–HLA-G interaction impedes both naive and memory B cell functions in vitro and in vivo. Particularly, HLA-G inhibits B cell proliferation, differentiation, and Ig secretion in both T cell–dependent and –independent models of B cell activation. HLA-G mediates phenotypic and functional downregulation of CXCR4 and CXCR5 chemokine receptors on germinal center B cells. In-depth analysis of the molecular mechanisms mediated by ILT2–HLA-G interaction showed a G0/G1 cell cycle arrest through dephosphorylation of AKT, GSK-3β, c-Raf, and Foxo proteins. Crucially, we provide in vivo evidence that HLA-G acts as a negative B cell regulator in modulating B cell Ab secretion in a xenograft mouse model. This B cell regulatory mechanism involving ILT2–HLA-G interaction brings important insight to design future B cell–targeted therapies aimed at reducing inappropriate immune reaction in allotransplantation and autoimmune diseases. The Journal of Immunology, 2014, 192: 1536–1546.

B cells represent an important arm of adaptive immune responses through Ag presentation, as well as production and secretion of Abs. This is the result of a complex process of differentiation that starts when B cells encounter Ag through engagement with the B cell Ag receptor that mediates the internalization, processing, and presentation of Ags to T cells, an important requirement for a successful immune response (1, 2). This engagement initiates a signaling cascade leading to B cell activation (3), which takes place after either T cell–dependent or –independent pathways. B cells subsequently differentiate into plasma cells or memory B cells (4). Failure in this tightly regulated process causes deleterious effects as exemplified by: 1) the autoimmune process via autoantibodies, autoantigen presentation to and activation of autoreactive T cells (5); and 2) graft rejection through the production of Abs against donor MHC Ags, alloantigen presentation to and priming of alloreactive T cells (6, 7). Inhibition of B cells constitutes, therefore, a rational approach for treating B cell–mediated disorders. One such treatment modality is rituximab, a B cell–depleting mAb against CD20 currently used in the treatment of B cell malignancies (8) and autoimmune disorders (5). Nevertheless, efforts to identify novel B cell–targeted therapies remain essential (9). To this end, understanding B cell functions and their regulatory pathways is of critical importance.

Like NK cells, T cells, macrophages, and dendritic cells, B cells express ITIM-bearing receptors such as the Ig-like transcript 2 (ILT2)/LLRB1/C0D8j (10). ILT2 binds to the third domain of HLA class I molecules associated with β2-microglobulin, with highest affinity for the nonclassical HLA class I molecule HLA-G (11). Notably, the effect of HLA-G interaction with its receptor ILT2 depends on its multimerization state because HLA-G multimers bind ILT2 with higher affinity and slower dissociation rates than monomers (12). ILT2–HLA-G interaction mediates inhibition of NK and CD8+ T cell cytolyis function (13), CD4+ T cell proliferation (14), generation of suppressor T cells (15, 16), and maturation of dendritic cells (17). However, no data are currently available on the role of ILT2–HLA-G I interaction in regulating B cell responses.

In contrast with classical HLA class I, HLA-G shows low polymorphism and is expressed in a limited number of healthy tissues such as cytotrophoblast, thymus, cornea, and erythroblasts (18). HLA-G can be expressed as seven different isoforms including four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) proteins through alternative splicing of the primary HLA-G transcript. HLA-G expression is tightly regulated by environmental factors, such as stress, cytokines, hormones, nutrient deprivation, and hypoxia (19). Therefore, although HLA-G protein expression is highly restricted in healthy tissues, it can be detected in many tissues under pathologic conditions such as allografts, autoimmune processes, and tumor lesions (18).
The biological functions of HLA-G have been originally described in maternal–fetal tolerance (20), and more recently in allograft acceptance and tumor escape (18). HLA-G is particularly relevant in the clinical setting because increased levels of soluble HLA-G have been detected in biological fluids from patients undergoing allotransplantation (15, 16), or suffering from inflammatory and autoimmune disorders (21) or solid tumors (22). In these pathological situations, HLA-G levels were associated with better allograft survival, reduced autoimmune activity, or tumor escape from immunosurveillance (18). In an attempt to identify novel B cell regulatory pathways, we have investigated the inhibitory effects mediated by the interaction between HLA-G and ITIM-bearing Ig-like receptor ITL2 in B cell biology.

Materials and Methods

Cells

PBMCs were isolated from blood of healthy volunteer donors from the French Blood Establishment (EFS, Saint-Louis Hospital, Paris, France) by density-gradient centrifugation over Ficoll-Histopaque 1077 (Sigma). B cells were isolated from PBMCs using the Dynal undetouched-B cell isolation Kit according to the manufacturer’s instructions (Dynal). The purity of CD19+ cells was assessed by flow cytometry analysis and reached 95%. Besides, B cells were purified from human tonsil mononuclear cells undergoing routine tonsillectomy (Brest Medical School, Brest, France) as previously described (23). T3T cells line either wild type or transfected with the human CD40L were kindly provided by Dr. Nathalie Guriec (Brest Medical School). For chemokine receptor and migration experiments, mononuclear cells were isolated from tonsil from Ficoll-Histopaque density gradient and depleted of T lymphocytes by rosetting with neuraminidase-treated sheep erythrocytes. T cell–depleted fractions contained 95% CD19+ cells. B cells purified from tonsil were incubated with CD10 mAb (DAKO) and separated by immunomagnetic beads into CD10+ germinal center (GC) (24) and CD10− non-GC B cells at 4˚C to prevent spontaneous apoptosis of GC B cells. CD10+ GC B cells contained 90% CD38high cells, as assessed by flow cytometry (25). CD10− GC B cells were cultured 36 h with or without rCD40L (100 ng/ml; Immunotools). CD10+ non-GC B cells were cultured 36 h with or without rCD40L and goat anti-human Ig Abs (2 μg/ml; Jackson ImmunoResearch). Human T(3T) cells were purified from human tonsil mononuclear cells using anti-ICOS mAb (Santa Cruz) and immunomagnetic selection with anti-mouse IgG1-coated magnetic beads (Miltenyi). This positive selected cell fraction contained 95% CD4+ CXXCR4high T cells. The human FDC-like cell line HK1 is derived from follicular dendritic cells (FDCs) of the human tonsil and was kindly provided by Dr. Y.S. Choi (Ochner Clinic Foundation, New Orleans, LA). The human Burkitt lymphoma cell line Raji was obtained from the American Type Culture Collection. All cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 1% Fungizone, and gentamicin (Life Technologies). Surgically removed tonsils and normal peripheral blood samples were obtained after written informed consent was given; these consents were obtained from all patients according to the Helsinki Declaration, and the study was approved by our local ethics committee.

HLA-G aggregation onto nanoparticles

rHLA-G1 or rHLA-G5 was used as source of HLA-G proteins, and it is referred to in this article as HLA-G. rHLA-G1 was produced in the human lymphoblastoid cell line 721.221.HLA-G1 (kindly provided by Dr. Francesco Puppo, Department of Internal Medicine and Medical Specialties, University of Genoa, Genoa, Italy) by transfection of the 721.221 parental cell line with human HLA-G1 cDNA. Supernatants (SNs) were collected from the 721.221.HLA-G1 cell line after 72 h culture in RPMI 1640 10% FCS at 37˚C and 5% CO2, and subsequently used as source of HLA-G after being aggregated onto nanoparticles (15, 26). In brief, nanoparticles are monodisperse magnetic particles with a 300-nm diameter and are coated with goat anti-mouse IgG covalently bound to their surface (Bio-Adembeads monodispersed magnetic particles with a 300-nm diameter and are coated with goat anti-mouse IgG; Bio-Adembeads, Cowan strain I; Calbiochem) which was used as a direct B cell activator through binding to Ig and TLR2 at the final dilution 1/2000 (27). Tetanus toxoid (TT) from Pasteur Institute (Paris, France) was used at various concentrations to activate PBMCs. Tonsil B cells were activated during 5 d with 75 Gy–irradiated CD40L-transfected 3T6 fibroblast monolayer in presence of 50 U/ml IL-2 (Chiron) and 10 ng/ml IL-10 (Peprotech).

Blocking experiments

HLAG treatment and sensitization

In most experiments, cells were pretreated for at least 18 h with Ctrl or HLA-G. At the end of this sensitization period, cells were washed and then activated and used in experiments. Alternatively, cells were treated with Ctrl or HLA-G for 18 h then cultured simultaneously to the activation step. Nanoparticles were removed by magnetic depletion before flow cytometry analysis and in vitro migration assays. Cells were treated using 2.5 × 105 nanoparticles/cell, as previously described (26).

Cell proliferation

Pretreated cells with either HLA-G, Ctrl, or medium (Ø) were plated in triplicate wells at 104 cells/well, activated for 1–5 d, and pulsed with [3H]thymidine (1 μCi/well; Amersham, Biosciences). Cells were harvested 18 h later, and thymidine incorporation into DNA was quantified on a microbeta counter (Wallac 1450; Pharmacia).

Blocking experiments

To block HLA-G aggregated onto beads, we used the 87G mAb (Exbio). In brief, HLA-G were incubated for 2 h in PBS 0.1% BSA containing 50 μg/ml azide-free 87G mAb. The nanoparticles were then washed and used for experiments. To block the ILT2 receptor, we previously incubated cells with 50% human AB serum for 1 h at 37°C to ensure a total blockade of membrane-bound FcR; then we added 20 μg/ml anti-ILT2 (clone GHI-75; BD Pharmingen) for 2 h at 37°C.

Flow cytometry

Abs used for flow cytometric analyses were conjugated with either FITC, PE, ECD, or PC5 (Beckman Coulter, BD Pharmingen, Caltag Laboratories, or R&D Systems). In brief, cells were first incubated 30 min at 4˚C in 20% human serum and subsequently labeled with Abs. Irrelevant isotype-matched Abs were systematically used. Cells were analyzed on EPICS XL4 flow cytometer using Expo32 software (Beckman Coulter) or on FACSCalibur (BD) where at least 5 × 104 events were acquired and analyzed using the CellQuest software (BD). Results are expressed as percentage of positive cells or mean of relative fluorescence intensity obtained as follows: mean fluorescence intensity obtained with specific Ab × mean fluorescence intensity obtained with irrelevant isotype-matched Ab.

Immunofluorescence

Cells were pretreated with either HLA-G, Ctrl, or medium (Ø) and then activated. After 5 d, cells were harvested from the culture flasks and cyto- spins were prepared using SuperFrost/Plus slides (Merck) and a Cytospin 3 (Shandon). For staining, cells were fixed and permeabilized in 90% ethanol, causing disruption of the cell membrane, and then incubated for 30 min with FITC-labeled goat anti-human Ig (Beckman Coulter) recognizing the main isotypes (IgA, IgD, IgG, IgM) or FITC-labeled isotype-matched control at a HLA-G human control. Indeed, after cell fixation with ethanol, no surface Ig could be detected while allowing detection of intracytoplasmic Ig. Nuclei were labeled in red with propidium iodide (PI; Sigma). Slides were analyzed using an immunofluorescence microscope (Bio-Rad MRC1024; Bio-Rad). The percentage of cells positive for intracytoplasmic Ig was then determined by counting cells with fluorescent cytoplasm per 100 nuclei counted in a representative area.
Chemotaxis

Chemotaxis was investigated using 5-μm pore-size transwell plates (Costar) as described previously (28). Five hundred thousand cells were dispersed in the upper chamber, whereas chemokines or medium alone was added to the lower chamber. CXCL12 (Immunotools) and CXCL13 (Abnova) were tested at 300 ng/ml. Plates were incubated for 2 h at 37°C. Migrated cells were collected and counted, and migration index (MI) was calculated as following: (number of migrated cells/number of dispersed cells) × 100.

Biochemistry

Cells were incubated for various times in the presence of HLA-G or Ctrl. Total proteins were then extracted from cell lysates and quantified using BCA protein assay kit (Pierce). Total protein extracts (30 μg) were subjected to SDS-PAGE. Proteins were then resolved on 10% SDS-PAGE, except for 0.5% T polyreps (6% SDS-PAGE), and then transferred onto nitrocellulose membranes. Membranes were subjected to immunoblotting using Abs to proteins or phospho-proteins from AKT pathway and Foxo (Tebu-Bio). Immunoblotting of HLA-G was performed using 12% SDS-PAGE and the pathway kits (Cell Signaling). Membranes were subsequently probed with α-tubulin (Sigma). Quantification of blotted proteins was performed by densitometry of scanned films using the Fluochrom software (Alpha-Innotech). Immunoblotting of HLA-G was performed using 12% SDS-PAGE and the anti-HLA-G 4B4 mAb (15).

Cell cycle

Cells were pretreated with HLA-G, Ctrl, or medium (Ø). Cells were washed and fixed in 70% ethanol in PBS and incubated at 4°C overnight. Washed cells were incubated in PBS containing 40 μg/ml PI (Sigma) and 100 μg/ml DNase-free RNase A on ice for at least 10 min, as previously described (26). Cell cycle parameters were acquired using LSR flow cytometer and CellQuest software (Becton Dickinson). Cell cycle distribution was determined by automatic analysis using the flow cytometry analysis software FlowJo. The percentage of cells in each cell cycle phase, that is, G0/G1, S, and G2/M, is provided.

Apoptosis

Apoptosis induction was evaluated using Annexin VFITC/PI kit (Beckman Coulter) according to manufacturer’s instructions, and stained cells were analyzed using EPICS XL flow cytometer and Expo 32 software (Beckman Coulter). Cells treated with 100 ng/ml staurosporine (Sigma) were used as positive control.

HLA-G–specific ELISA

Concentration of soluble forms of HLA-G was evaluated on SNs from the human FDC line HK (kindly provided by Dr. Y.S. Choi) (29), and from human T94 cells that had been cultured for 48 h in RPMI 1640 10% FCS in presence or absence of 50 ng/ml IL-10 (Boehringer Ingelheim) or 100 pg/ml TGF-β (R&D Systems). HLA-G ELISA was performed using MaxiSorp Nunc-Immuno 96 microwell plates (Nunc) coated overnight at 4°C with mAb MEM-G/9 (Exbio) in 0.001 M PBS, pH 7.4. After three washes iSorp Nunc-Immuno 96 microwell plates (Nunc) coated overnight at 4°C and then incubated with 100 μl BSA for 30 min at room temperature (RT). 100 μl of samples or standard was added to each well and incubated at RT for 1 h. Plates were washed three times and then incubated with 100 μl biotinylated anti-β2-m mAb NAMB-1 at RT for 1 h (kindly gifted by Dr. Soldano Ferrone). After three washes, plates were incubated at RT for 1 h with streptavidin-horse radish peroxidase (GE Healthcare) 1:4000 in PBS 0.1% Tween 20, 0.1% BSA, for 1 h at RT. After three additional washes, plates were incubated with the TMB substrate (Sigma) for 5 min at RT. H2SO4 5 M was then added, and optical densities were measured at 450 nm. The assay’s lower threshold was 2 ng/ml HLA-G. Each sample was tested in duplicate.

Cytokines and Ig ELISA

SNs were harvested and used in ELISA to measure levels of cytokines (IL-2, IFN-γ, IL-4, and IL-10) or Ig (IgA, IgG, and IgM). IL-2, IFN-γ, IL-4, and IL-10 were measured by Th1/Th2 human ELISA Kit (eBioscience), according to the manufacturer’s instructions. Human Ig present in SN was measured using human IgA, IgG, and IgM ELISA quantitation kits (Bethyl), according to the manufacturer’s instructions. Plasma from mice were harvested after centrifugation of blood 10 min at 1800 rpm, aliquoted, and stored at −80°C until ELISA were performed. Total IgG was quantified using the mouse total IgG EasyTiter ELISA kit (Pierce). Mouse cytokines were quantified using the Th1/Th2/Th17 Multi-Analyte ELISAArray kit (Tebu-Bio).

Slot-blot analysis

A total of 5 × 106 M8-pcDNA cells were washed with cold PBS, and the pellet was lysed and boiled for 5 min. After centrifugation, the SN was blotted on a 10% SDS-PAGE, and proteins were transferred on Immobilon-P (Millipore, Bedford, MA). Blots were saturated with 5% fat-free milk/ PBS/0.2% Tween 20 for 1 h and then incubated with different mouse plasma samples diluted in PBS 1:100 using the mini-Protein II multiscannel apparatus (BioRad). Detection was performed using HRP-conjugated anti-mouse Ab (Sigma-Aldrich) with the ECL kit (Amersham). Incubations with mouse plasmas and secondary goat anti-mouse Abs were conducted in PBS 0.2% Tween 20, 0.5% BSA.

Mice and immunizations

Female BALB/c mice (6–10 wk of age) were obtained from Charles River Laboratories (L’Arbresle, France). Groups of mice were injected s.c. with 10 × 106 human melanoma M8-pcDNA (HLA-G+ xenogenic cells) or M8–HLA-G cells (HLA-G+ xenogenic cells) (13) resuspended in PBS. When indicated, the human cells were pretreated for 1 h with the anti-HLA-G 87G mAb at 20 μg/ml (Exbio) or the isotypic control (IgG2a; Exbio) before graft. The xenograft area was measured with digital calipers at indicated time points postgraft. Volume was estimated by the following formula (L × W2)/2, with L = length and W = width in millimeters. All experimental protocols were approved by the ethics review committee for animal experimentation of the Saint-Louis Hospital (Paris, France) and followed the guiding principles for the care and use of animals approved by our local committee.

Statistical analysis

Statistical analysis has been performed using GraphPad Prism (GraphPad Software). Gaussian distribution of data was tested using Kolmogorov–Smirnov test. Mann–Whitney U test (2 groups) or Kruskal–Wallis test (≥3 groups) has been performed when Gaussian distribution was not assumed. One-way ANOVA test has been performed for experiment data assuming Gaussian distribution and comparing ≥3 groups at a single time point. Besides, a multiple-comparisons test has been performed to compare statistical significance between groups (Tukey’s multiple-comparisons test). In time-series experiments with data comparing >3 groups, we performed a repeated-measures (RM) two-way ANOVA test; then a multiple-comparisons test (Tukey’s multiple-comparisons test) has been performed. Significance was provided with the results assuming p < 0.05 as significant and were marked with asterisks in the figures.

Results

HLA-G inhibits T cell–dependent B cell response through interaction with ILT2

Among the HLA class I molecules, HLA-G constitutes the preferential ligand of ILT2, which is the sole known HLA-G receptor expressed by CD19+ B cells (Fig. 1A). To elucidate the effects of ILT2 engagement by soluble HLA-G, we first investigated the proliferative responses of PBMCs polyclonally activated with PWM, which triggers B cells via a T cell–dependent pathway. Because aggregation of HLA-G is critical for optimal interaction with ILTs (15, 17), PBMCs were activated in the absence or presence of HLA-G aggregated onto nanoparticles (i.e., HLA-G). As control, we used nanoparticles processed in the same manner as for HLA-G nanoparticles but using an HLA-G–free source (i.e., Ctrl). HLA-G capture by nanoparticles was assessed by immunoblot analysis (Fig. 1B, insert). Results show that HLA-G strongly inhibited PWM-induced PBMC proliferation compared with controls (Fig. 1B). In parallel to HLA-G aggregated onto nanoparticles, we used an HLA-G protein unbound to magnetic particles and found that, although similar, its effects were less potent than those mediated by aggregated HLA-G (data not shown). Of note, we have tested denatured HLA-G protein coated onto nanoparticles that does not longer inhibit B cell proliferation. Such denatured HLA-G protein is no longer capable of binding to ILT2 (data not shown).

The HLA-G–driven inhibition of PWM-activated PBMC proliferation was dose- and time-dependent (Fig. 1C) and could be
reversed by neutralizing ILT2–HLA-G interaction with mAbs against HLA-G (anti–HLA-G; Fig. 1D) or ILT2 (α-ILT2; Fig. 1E). The maximum inhibition was reached when HLA-G was added in the early steps of PWM activation (Fig. 1F) or upon preincubation of PBMCs with HLA-G for 18 h before PWM stimulation (Fig. 1G). Besides, stimulating B cells with anti-human Ig and CD40L, as well as CpG, has brought similar results showing that in those conditions of stimulation, ILT2–HLA-G interaction strongly inhibits B cell proliferation (data not shown). Moreover, cytokine assays carried out with PBMCs incubated in the presence of HLA-G showed: 1) reduced levels of IFN-γ and, to a lesser extent, IL-2 at day 5; and 2) increased IL-10 concentrations earlier at day 2 compared with control (Fig. 1H).

We then investigated the effects of HLA-G on PWM-induced B cell differentiation into Ab-secreting cells. A significantly reduced number of cells with intracytoplasmic Ig (Ig⁺ cells), as well as decreased levels of IgA, IgG, and IgM, were observed in the presence of HLA-G (Fig. 1I, 1J). Interestingly, both T and B cells are targets of the HLA-G–mediated inhibition with maximum effect observed when both cell populations had been pretreated as assessed by HLA-G–mediated proliferation inhibition (Fig. 2A). The secretion of IFN-γ and IL-2 (Fig. 2B), as well as of IgA, IgG, and IgM (Fig. 2C), was reduced when T and/or B cells were pretreated with HLA-G. In agreement with data obtained with PBMCs, T cell help proliferation and differentiation of CD19⁺ B cells isolated from tonsil were inhibited by HLA-G (Fig. 3A, 3B).

HLA-G inhibits T cell–dependent Ag-specific memory B cell responses

To investigate the effects of HLA-G on specific B cell responses to a recall Ag, we stimulated PBMCs with TT. Proliferation of PBMCs...
in response to TT was strongly inhibited by HLA-G, and both cytokine and Ig secretions were affected by HLA-G with decreased secretion of IFN-γ, IL-2, IgA, IgG, and IgM (Fig. 3C–F). Decreased numbers of Ig+ cells were observed among PBMCs after 5-d activation with TT in the presence of HLA-G (Fig. 3G). Also, reduced expression levels of CD20 and CD69 were observed within the CD19+ cells at day 5 after TT stimulation (Fig. 3H, 3I).

**HLA-G inhibits T cell–independent B cell responses**

Next, we examined the effects of HLA-G on B cells activated by T-independent stimuli using pansorbin or SNs from PWM-activated PBMCs (PWM SN). HLA-G inhibited the proliferation of PBMCs, as well as of purified B cells, after pansorbin or PWM SN activation (Fig. 4A). This inhibitory effect was associated with: 1) reduced levels of IFN-γ and IL-2 together with enhanced IL-10 secretion when purified B cells were stimulated with pansorbin (Fig. 4B); 2) decreased numbers of intracytoplasmic IgG+ cells among activated PBMC or purified B cells (Fig. 4C); 3) decreased secretion of IgA, IgG, and IgM in culture SNs from purified B cells stimulated with pansorbin (Fig. 4D); and 4) reduced expression levels of CD20, CD69, and CD138 within the CD19+ cells when purified B cells were stimulated with pansorbin (Fig. 4E, 4F).

**HLA-G downregulates CXCR4 and CXCR5 chemokine receptor expression on GC B cells and dampens their chemotaxis to the respective ligands**

The chemokine receptors CXCR4 and CXCR5 regulate B cell trafficking within secondary lymphoid follicles (30). Therefore, we examined whether their expression on tonsil CD19+ B cell subsets was modulated by HLA-G. Results showed that CXCR4 expression was significantly downregulated in CD10− CD27− naïve memory B cells (Fig. 5A, 5C). Conversely, CXCR4 expression in CD10+ CD27+ naïve and CD10− CD27+ memory B cells was unaffected by HLA-G treatment (Fig. 5A, 5C). CXCR5 expression was significantly lower in tonsil GC, naïve, and memory B cells treated with HLA-G compared with control (Fig. 5B, 5D). When the same experiments were performed using peripheral blood naïve and memory B lymphocytes, no significant modulation of CXCR4 or CXCR5 expression by HLA-G was observed. GC B cells and non-GC B cells, that is, CD10+ and CD10− B cells, from tonsil were next subjected to in vitro migration assays, using CXCL12 and CXCL13 as chemoattractants. Migration to CXCL12 was significantly downregulated in GC B cells treated with HLA-G, whereas migration of non-GC B cells was unaffected by HLA-G (Fig. 5E, 5G). Similar results were obtained using CXCL13 as chemoattractant. MI was significantly lower in GC B cells treated with HLA-G compared with controls, whereas the MI of non-GC B cells was not altered by HLA-G (Fig. 5F, 5H).

**HLA-G is secreted by FDCs and Tfh cells**

We then examined whether the human FDCs cell line HK (29) and freshly isolated human Tfh cells that home in the follicular microenvironment of human tonsil secreted HLA-G in vitro with or without known HLA-G release inducers (19). HLA-G was detected in SNs from the HK cell line in all experimental conditions, whereas the MI of non-GC B cells was not altered by HLA-G (Fig. 5I, 5J).

**HLA-G induces G0/G1 cell cycle arrest and acts as a negative B cell regulator through AKT signaling pathway**

To address the precise mechanisms underlying HLA-G–mediated inhibition, we analyzed B cells for cell cycle progression and apoptosis. Because activated B cells proliferated slowly, the Raji
A cell line was used as a B cell model as previously described (31). Indeed, some features of Raji cells may resemble the state of activated B cells, and proliferation of Raji cells was inhibited by HLA-G in a dose- and time-dependent manner similar to normal B cells (32). HLA-G was found to alter cell cycle progression of Raji B cells (Fig. 6A, 6B), without any detectable apoptosis by using Annexin V/PI or DIOC6 staining (Fig. 6C). Supporting these data, additional experiments allowed us to exclude decreased cell viability because of the presence of HLA-G: 1) in our preliminary tests, we did not find any difference in cell viability with trypan blue staining whether B cells were activated in the presence or absence of HLA-G; 2) in blocking experiments using anti–HLA-G or anti–ILT2 mAb in the presence of HLA-G, PBMC proliferation could be restored, demonstrating that cells were still viable after HLA-G treatment (Fig. 1D, 1E); and 3) no inhibition of cell proliferation was observed when HLA-G was added in the later steps of PWM activation (96 h), showing in this study again that HLA-G treatment did not alter cell viability (Fig. 1F).

To identify the intracellular signaling cascades involved in ILT2/HLA-G–mediated inhibition, we analyzed the AKT signaling pathway, which is frequently dysregulated in B cell disorders (3). HLA-G decreased the PWM SN–induced phosphorylation of AKT at Ser 473 in primary B cells after 12-h activation (Fig. 6D) without modifying AKT protein levels (data not shown). An essential function of AKT signaling is to regulate cell cycle progression through phosphorylation and inactivation of GSK-3β, c-Raf, and...
Foxo proteins (33, 34). In this study, we found that HLA-G decreased the phosphorylation of these downstream signaling proteins GSK-3\(\beta\) (Fig. 6D), c-Raf (Fig. 6D), and Foxo1, Foxo3a, and Foxo4 (Fig. 6D).

**HLA-G promotes a Th2 response and inhibits B cell Ab secretion, therefore inducing in vivo immune tolerance in a murine xenograft model**

To provide in vivo evidence of the inhibitory effect mediated by HLA-G on B cells, we finally developed a xenograft model in mice (Fig. 7A). Although there is no murine homolog of HLA-G, this study was made possible by the fact that human HLA-G interacts with the murine receptor paired Ig-like receptor B (PIR-B), the homolog of human ILTs (35, 36). In this regard, tetramers of HLA-G were found to prolong skin allograft survival in mice by inducing a tolerogenic environment (37). In agreement with these data, we found that HLA-G\(^{+}\) xenograft displayed extended survival and growth compared with HLA-G\(^{-}\) counterpart, and that blocking HLA-G function by a specific Ab inhibited xenograft growth in immunocompetent mice (Fig. 7B). We then analyzed the Ab response from mice injected with HLA-G\(^{-}\) or HLA-G\(^{+}\) xenogeneic cells. Although the total amount of plasma IgG was similar between mice groups (Fig. 7C), we observed a dramatic decrease of anti-xenograft Abs present in the plasma of HLA-G\(^{+}\) xenograft-bearing mice compared with HLA-G\(^{-}\) counterpart at day 27 postgraft (Fig. 7D). To correlate with the slot-blot data (Fig. 7D), HLA-G\(^{+}\) versus HLA-G\(^{-}\) xenograft growth over time was as follows: 270 versus 40 mm\(^3\) at day 7, 70 versus 0 mm\(^3\) at day 13, 0 versus 0 mm\(^3\) at days 20 and 27. These results demonstrate that Ag-specific B cell response is drastically affected in HLA-G\(^{+}\) xenograft-bearing mice. Ab data were strengthened by flow cytometry experiments showing that the level of mouse IgG specific for
xenografted cells was quantitatively reduced in HLA-G+ xenograft mice compared with HLA-G− mice (data not shown).

Finally, we analyzed the presence of Th1 versus Th2 cytokines in mice plasma. Although IFN-γ and IL-2 levels were decreased (Fig. 7E–H), IL-4 and IL-10 were detected at high levels (Fig. 7G, 7H) in mice injected with HLA-G+ cells compared with HLA-G− cells. This cytokine environment in mice injected with HLA-G+ cells is strongly in favor of a Th2 immune response.

Discussion

Immune regulation is achieved by the integration of positive signals delivered by Ag receptors, costimulatory/adhesion molecules, and cytokine receptors, with negative signals provided by inhibitory receptors. Identifying these receptor–ligand interactions is important for understanding and elucidating mechanisms that prevent leukocyte overactivation that could potentially result in tissue damage/autoimmunity. These molecules also potentially represent targets for therapies directed at modulating immune responsiveness.

Although effects of the ILT2–HLA-G receptor–ligand interaction have been described extensively for myeloid and T cells, the only effect of ILT2 ligation on B cells shown previously was using an anti-ILT2 mAb (clone HP-F1) (10). In that study, pretreatment of an EBV-transformed B cell line with anti-ILT2 mAb inhibited BCR-induced Ca2+ flux. In that same study, the authors reported that anti-ILT2 mAb had a minimal effect on primary B cells, arguing that a higher order magnitude of ILT2 cross-linking may be necessary to have an effect on primary cells. Our study is the only one to date that demonstrates an effect of ILT2 via HLA-G on various primary B cell responses.

We demonstrate that HLA-G aggregated onto nanoparticles inhibits both T cell–dependent and –independent naive and memory B cell responses through ILT2 interaction. This inhibition targets B cell proliferation, as well as B cell differentiation, into Ab-secreting cells. The rationale of HLA-G aggregation onto nanoparticles lies in the fact that it mimics the active polymeric structure found in vivo (11). Indeed, both crystallography and biochemical studies have shown that ILT2 binds mostly cell-surface multimers in vitro and in vivo, and that multimers signal more efficiently than monomers (12, 38). Moreover, HLA-G aggregation has shown its effectiveness in previous functional studies (15, 26, 37).
Furthermore, HLA-G downregulates IL-2 and IFN-γ, whereas it increases IL-10 production by activated PBMCs and purified B cells, suggesting the occurrence of synergistic inhibitory activity of HLA-G and IL-10 that are interdependent in some experimental systems (39). Also, we found that HLA-G favors a Th2 cytokine profile at the expenses of Th1 cytokines in vitro and in vivo.

A striking finding was the ability of HLA-G to inhibit B cell Ab production as shown by reduced Ig secretion irrespective of the B cell activation protocols used. Such an HLA-G inhibitory role on B cell response is in accordance with clinical observations showing reduced HLA alloantibody levels in kidney transplant patients (40) or inhibition of the humoral response in heart transplant patients who express HLA-G (41).

We show that HLA-G dampens expression of CXCR4 and CXCR5 in GC B cells. Accordingly, the latter cells exhibited a dramatically reduced chemotaxis to CXCL12 and CXCL13. CXCR5 was significantly downregulated by HLA-G in naive and memory B cells from tonsil, but migration of these cell fractions to CXCL13 was unaffected. Taken together, our findings suggest that HLA-G participates in the control of B cell trafficking in the GC. This hypothesis is supported by our findings showing the secretion of HLA-G by the FDC HK cell line and purified tonsil T FH cells in vitro.

HLA-G reduces B cell proliferation by inducing a G0/G1 cell cycle arrest. We defined signaling events leading to HLA-G inhibitory effects through decreased phosphorylation of AKT, GSK-3β, c-Raf, and Foxo1, Foxo3a, and Foxo4 detected by immunoblot analysis with phosphospecific Abs as indicated. α-Tubulin was used to assess that same amounts of proteins were run in polyacrylamide gels. One representative experiment of three is shown. Green arrows indicate decreased phosphorylation. Numbers indicate the relative protein quantifications as mean ± SEM (n = 3) using Ctrl values as baseline. Significance was evaluated with Mann–Whitney U test. AKT protein does not vary with Ctrl or HLA-G. *p < 0.05.

Disruption of the delicate balance between activating and inhibitory signals regulating normal B cell activation can lead to the production of autoantibodies and autoimmunity (5), or alloantibodies responsible for allograft rejection (7). In these clinical settings, HLA-G is particularly relevant because increased levels of HLA-G have been described in plasma from solid organ transplant patients with better graft acceptance (15, 39), and in plasma and cerebrospinal fluid from multiple sclerosis patients with reduced disease activity (43). Notably, overexpression of ILT2 has been reported in the course of the same pathologies (44). Our study brings a new highlight on HLA-G–induced tolerance operating in these patients that is likely achieved by acting on the B cell response. Indeed, we show in this article the in vivo inhibitory role of HLA-G on B cells through the use of a xenograft model.
approach was made achievable because human HLA-G can bind and mediate an inhibitory signal via the murine receptor PIR-B, the homolog of human ILTs (36, 37). The substantial difference between the species (ILT-2 and PIR-B) is the absence of PIR-B expression on murine T and NK cells, whereas ILT-2 is expressed on both human subsets. On B cells, both ILT-2 and PIR-B are expressed and have been shown to bind to SHP-1 (10, 35). Thus, the differences in effect of HLA-G between human and murine B cell immunity might be because of the fact that HLA-G targets T and NK cells and might affect indirectly B cell functions in human.

Our data provided in this work show that when both T and B cells are sensitized by HLA-G, the effect on proliferation inhibition is more profound compared with controls. Furthermore, our results show a decreased Ab secretion directed against the xenoantigens in HLA-G+/xenograft-bearing mice compared with their HLA-G− xenograft-bearing counterparts. Presence of specific Abs against xenoantigens in the plasmas of the different groups of mice was analyzed at day 27 or in kinetic at days 7, 13, 20, and 27 by slot blot. The numbers indicated on the right correspond to the molecular mass (in kDa) of protein markers. Data from two representative experiments are shown. (E–H) Plasma concentrations of cytokines from Ctrl mice or mice injected with HLA-G+ xenograft-bearing cells were quantified by ELISA at day 7 postinoculation. Results are given as means (n = 3). Significance was evaluated with Kruskal–Wallis test. *p < 0.05.

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Disclosures

The authors have no financial conflicts of interest.
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