# Chapter 1

# HLA-G as an Inhibitor of Immune Responses

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## Abstract

HLA-G is a nonclassical human leukocyte antigen (HLA) class I molecule which plays important tolerogenic functions in various physiological and pathological situations such as fetus and transplant acceptance, and immune escaping of virus-infected and malignant cells. Here we describe a method, which allows for studying cell surface expression of HLA-G using specific antibodies with flow cytometry analysis.

**Key words** HLA-G, Flow cytometry, Antibody

## 1 Introduction

Human leukocyte antigen G (HLA-G) is a nonclassic HLA class I molecule that was initially observed to be restricted to the fetal-maternal interface on the extravillous cytotrophoblasts [1]. Unlike classical HLA class I antigens, HLA-G featured with limited polymorphism, restricted tissue distribution, slow turnover, limited peptide diversity, immunosuppressive properties, and seven isoforms, which include four membrane-bound (HLA-G1, -G2, -G3, and -G4) and three soluble isoforms (HLA-G5, -G6, and -G7) [2, 3].

The biological function of HLA-G is through binding to its receptors including ILT-2/CD85j, ILT-4/CD85d, KIR2DL4/CD158d, CD8, and CD160 expressed on different types of cells [4]. KIR2DL4 is expressed on NK cells, ILT-2 is expressed on B lymphocytes, some T lymphocytes and NK cells, and all dendritic cells and monocytes, ILT-4 is expressed by monocytes, dendritic cells, and recently reported in neutrophils. The CD8 is predominantly expressed on the surface of cytotoxic T cells, but can also be found on natural killer cells, CD160 is expressed by cytotoxic CD8+ T cells and NK cells, and a small proportion of CD4+ T cells [5]. By binding receptors expressed on various cells and the pathway of trogocytosis, HLA-G could inhibit the cytolytic function of NK cells and T lymphocytes, the alloproliferative response of CD4+ T cells, the ongoing proliferation of T cells and NK cells,

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the maturation of dendritic cells, the Ig secretion of B lymphocyte and phagocytosis of neutrophils, and induces regulatory cells [6–9]. In the context of clinical aspects, the significance of HLA-G expression has been extensively investigated in the fetal-maternal immune tolerance, the acceptance of solid organ transplants, and the immune escape of tumors and virus-infected cells [10].

In this chapter, we provide the protocol for a sensitive, quantitative, and simple method to measure cell surface expression of HLA-G on human cells with flow cytometry (or fluorescence-activated cell sorting, FACS). Please note that this protocol will allow detecting the certain types of HLA-G isoforms only according to the specificity of anti-HLA-G antibodies [11]. Other methods for the detection of HLA-G and HLA-G isoforms are available, such as quantitative RT-PCR, immunohistochemistry and Western blotting; however, these are not covered in this chapter.

## 2 Materials

Prepare all solutions using ultrapure water (prepared by deionized water to attain an electrical resistivity of 18 MΩ cm at 25 °C) and analytical or higher grade reagents. Store all reagents at 4 °C (unless indicated otherwise). Follow institutional guidelines for waste disposal.

### 2.1 Buffers

1. Cell detaching buffer: PBS (1×) containing 10 mM EDTA pH 8.0. We do not recommend using trypsin to detach adherent cells because it may strip/cleave the molecules expressed on the cell surface.
2. FACS buffer: PBS (1×) containing 1 % BSA and 10 mM EDTA (PBS-BSA) pH 8.0. Sterile filter solution and store at 4 °C or aliquot and freeze at –20 °C.
3. Cell fixing buffer: PBS (1×) containing 0.5 % paraformaldehyde (PBS-PFA). Store at room temperature.

### 2.2 Antibodies

1. We use anti-human HLA-G mouse monoclonal antibody MEM-G/9 (Exbio, Prague, The Czech Republic). This antibody targets native form of human HLA-G1 on the cell surface as well as with soluble HLA-G5 isoform in its beta2-microglobulin-associated form. For more information on the differences between anti-HLA-G antibodies please *see* **Note 1**.
2. If non-labeled primary antibody is to be used, subsequent staining with an appropriate fluorochrome-conjugated secondary antibody is required.

### 2.3 Other Materials and Equipment

1. 96-Well plates (U- or V-bottom) with lids or adhesive film.
2. Variable volume pipettes.

3. Sterile tips.
4. Centrifuge.
5. Flow cytometer.
6. Ice.

## 3 Methods

Grow cells as per protocol and treat cells as required for your experiment (*see* **Note 2**). We do not recommend using cells that are overgrown or exhibit excessive cell death (*see* **Note 3**). This protocol allows for detection of HLA-G expression on both adherent (such as JEG-3 cells) and on non-adherent cells (such as K562 cells). If adherent cells are used for the analysis of HLA-G expression, begin with **step 1** of the protocol and if non-adherent cells are used, omit **steps 1** and **2** below and begin directly with **step 3**. Prepare all buffers and solutions before starting the experiment. To be able to analyze the data obtained one needs to prepare and assess controls along with the experimental staining. The controls we routinely use are listed in Table 1.

1. Wash cells once in sterile 1× PBS by adding 10 ml sterile PBS to T75 flask and gently tap the flask. Be careful not to detach the cells at this point. Aspirate the PBS by decanting or by removing with a pipet.
2. After removing the PBS used for washing, add 5–10 ml of the detaching solution to T75 flask of cells, swirl slowly around so that all cells are covered with the detaching solution, and incubate for 5–10 min at 37 °C in incubator until the solution becomes cloudy.
3. Collect the detached cells or the non-adherent cells using a pipet and transfer into a fresh tube, such as a 15 or 50 ml conical centrifuge tube. Some cells may not detached completely just by incubation in detaching solution; detachment can then

**Table 1**
**Suggested negative/positive controls for the detection of HLA-G expression by flow cytometry**

| Type of control | Notes |
|---|---|
| Unstained cells | To check for dead cells using FCS/SSC plots |
| Cells stained with secondary antibody or isotype control antibody | Control for nonspecific binding of secondary or isotype controls to cells of interest |
| Cells that does not express HLA-G (if desired) | Control for nonspecific binding of anti-HLA-G antibody |
| Cells that express HLA-G (if desired) | Control for specific binding of anti-HLA-G antibody |

often be supported by gently tapping the side of the culture flask against one hand several times.

4. Resuspend cells with ice-cold PBS-BSA and adjust cell concentration to $2 \times 10^6$ cells/ml using hemocytometer or other suitable method.
5. Transfer 50 μl cell suspension per well in 96-well plate (U- or V-bottom). Prepare as many wells as you need to perform the experiment including all non-stained and/or isotype controls and/or secondary antibody only and/or positive controls (*see* Table 1).
6. Wash the cells once in 200 μl ice-cold 1× PBS by centrifugation at 300 × *g* for 5 min at 4 °C. Remove the supernatant after each wash by quickly flicking the plate upside down over a sink, and then carefully tapping it on clean paper towels to remove the remaining liquid.
7. While washing, prepare antibody solution by mixing ice-cold FACS buffer with the FITC-MEM-G/9 and isotype control (5 μg/ml final concentration) (*see* **Notes 4** and **5**).
8. Add 100 μl of the diluted antibody to each well and incubate with cells for 30–60 min at 4 °C. The final volume of the mix we typically use is 150 μl per well, but this could be scaled to from 50 to 250 μl if desired. *Do not* add the antibody to cells that are to be used as secondary and unstained cells control. Controls included negative (no stain added), isotype control (with similarly labeled, nonspecific primary antibody), and positive cell controls.
9. Pellet the cells by centrifugation (spin at 300 × *g* for 5 min at 4 °C).
10. Wash twice as in **step 6**. The samples are now ready for FACS analysis and you can proceed immediately to **steps 15** and **16**.
11. If you have used a non-labeled anti-HLA-G antibody in **steps 7** and **8** (indirect staining), dilute the secondary FITC-labeled anti-mouse IgG antibody to 0.25 μg/ml (or 1/100 from the stock) in FACS buffer. Vortex to mix.
12. Add the secondary FITC-labeled antibody to wells containing cells stained with the anti-HLA-G antibody and washed twice with 1× PBS. Also add the secondary FITC-labeled antibody to at least two wells containing isotype control-incubated and non-stained cells; these will be used as isotype control and a secondary antibody only control staining. *Do not* forget to leave some wells without any antibody addition as non-stained cell control wells.
13. Incubate for 20 min in the dark at 4 °C.
14. Wash twice as in **step 6**.

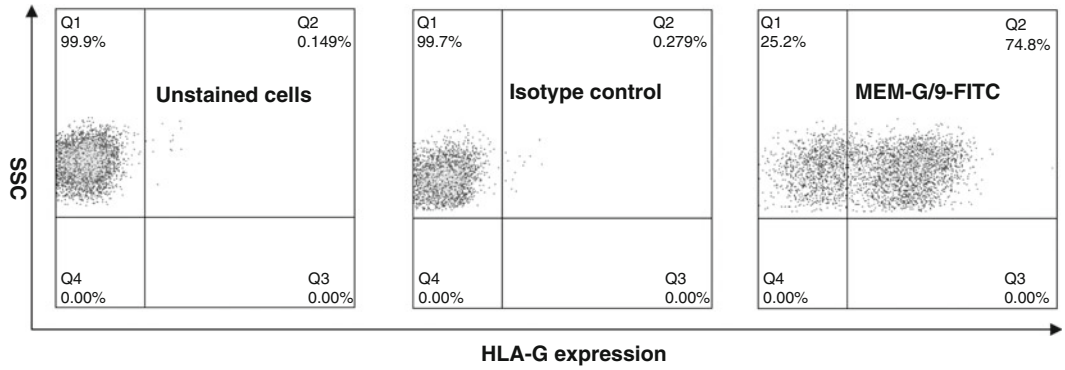


**Fig. 1** Flow cytometry analysis of HLA-G expression on the surface of K562-G1 cells

15. If cells are to be analyzed the same day they can be resuspended with 300 μl FACS buffer and transferred cells to Falcon tubes (5 ml polystyrene round-bottom tube, 12 × 75 mm) and kept at 4 °C in the dark. Alternatively cells can be fixed with cell fixing buffer and stored in the fridge for 2–3 days prior to analysis.
16. Analyze on a suitable flow cytometer (e.g., FACS Calibur, FACS Canto II) acquiring the non-stained cells control first, then the secondary/isotype antibody controls, and the HLA-G-stained cells. Example FACS plots for HLA-G expression in K562-G1 cells (exogenous HLA-G1 expression in K562 cells) [13], normal peripheral blood CD14+ monocytes, and CD3+ T lymphocytes are shown in Figs. 1 and 2, respectively (*see* **Note 6**).

## 4 Notes

1. Excellent anti-HLA-G antibodies with HLA-G isoform specificity, that generate strong signals during flow cytometry analyses, can be purchased from most major vendors such as Exbio, Abcam, BD Biosciences, etc. There are six HLA-G isoform-specific antibodies that are often used by researchers in flow cytometry analysis to address the expression and functional of HLA-G in various conditions.

   MEM-G/9 reacts with HLA-G1 on the cell surface as well as with soluble HLA-G5 isoform in its beta2-microglobulin-associated form. This antibody is the standard reagent thoroughly validated during 3rd International Conference on HLA-G (Paris, 2003). 01G (IgG1) and MEM-G/11 (IgG1) recognize HLA-G1, but not soluble forms. 2A12 (IgG1) and 5A6G7 (IgG1) recognize HLA-G5 and HLA-G6 isoforms but not HLA-G1 isoform. 87G (IgG2a) recognizes both membrane-bound and soluble forms of HLA-G (HLA-G1 and HLA-G5) and can block the interaction

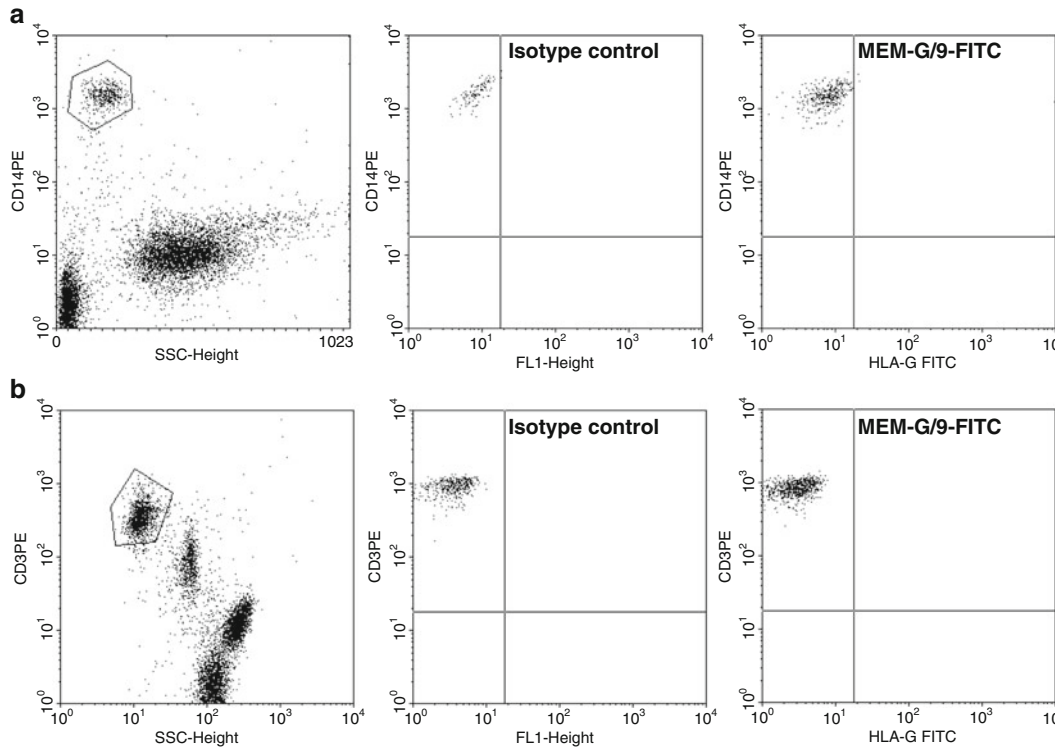


**Fig. 2** Flow cytometry analysis of HLA-G expression in normal peripheral blood cells. (**a**) A representative flow cytometry diagram shows CD14+ monocytes gated by CD14 and its HLA-G expression. (**b**) A representative flow cytometry diagram shows CD3+ T lymphocytes gated by CD3 and its HLA-G expression

of HLA-G with inhibitory receptors, and thus can be used in functional assays evaluating the role of HLA-G [11, 12].

2. Grow cells as per protocol. Replace the media on the day before experiment. If cells growing in suspension are used skip **steps 1** and **2**. If adherent cells are to be analyzed start from **step 1**.
3. Note that dead cells can bind antibodies nonspecifically and often give false-positive results in flow cytometry analysis. It is therefore advisable to use cultures with a high proportion of viable cells. Cell viability can be assessed by Trypan blue staining before cell staining. Further, the forward scatter (FSC) vs. sideward scatter (SSC) pattern of the cells during FACS analysis after the FACS measurement of HLA-Gexpression staining procedures will give a second clue about the condition of the cells (dead cells have a distinctive FSC/SSC pattern).
4. Both primary and secondary antibodies must be tittered on known HLA-G-positive (Choriocarcinoma cell line JEG-3, ATCC Number: HTB-36) and HLA-G-negative (Choriocarcinoma cell line JAR, ATCC Number: HTB-144) cell populations prior to use in actual experiments.

5. The final concentration of the staining antibody and the isotype control should be the same. Optimal antibody concentrations are determined by titration. Always run in parallel an isotype-matched control antibody.
6. For two- and three-color analysis, compensation controls must be run to compensate for spectral overlap. These consist of one sample each stained with each fluorescent reagent separately and a control containing both colors; for example if two-color analysis is performed with FITC and phycoerythrin (PE) then samples stained with FITC alone and PE alone and a sample certain to be positive for both colors should be run.

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