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Multiplex bead-based immunoassay for the free soluble forms of the HLA-G receptors, ILT2 and ILT4

Ching-Lien Wu [a,b,1], Signe Goul Svendsen [c,1], Adrien Riviere [a,b,d], François Desgrandchamps [a,d], Edgardo D. Carosella [a,b], Joël LeMaoult [a,b,*]

[a] Hemato-Immunology Research Department, CEA, Hospital Saint Louis, 1 Avenue Claude Vellefaux, Bat Lailler, 75475 Paris Cedex 10, France
[b] University Paris Diderot, Sorbonne Paris Cité, UMR E_5 Institut Universitaire d'Hematologie, Saint-Louis Hospital, 1 Avenue Claude Vellefaux, 75475 Paris Cedex 10, France
[c] Department of Immunology and Microbiology, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark
[d] AP-HP, Saint-Louis Hospital, Department of Urology, 1 Avenue Claude Vellefaux, 75475 Paris Cedex 10, France

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ABSTRACT

Human leukocyte antigen (HLA)-G is an immune-inhibitory molecule that exerts its function via interaction with two main inhibitory receptors: ILT2 and ILT4. This interaction is considered to be an immune checkpoint. HLA-G can be found as a soluble molecule, but it is not known if its receptors can also be found as soluble molecules. In this work, we present a multiplex luminex-based assay to measure soluble ILT2 (sILT2) and soluble ILT4 (sILT4) molecules together. It is based on two antibody pairs, GHI/75 and HP-F1-PE for ILT2 and 27D6 and 42D1-PE for ILT4. The characterization of our method reveals that it specifically detects the free soluble forms of sILT2 and sILT4, and not those complexed to HLA Class I molecules such as their ligand of highest affinity HLA-G. A study on two small cohorts of cancer patients demonstrated that soluble ILT2 and ILT4 molecules were of low abundance in the plasma of healthy controls, but that elevated levels of plasmatic sILT2 were present in non-muscle-infiltrating bladder cancer patients. This demonstrated that the titration test is indeed working, and that soluble ILT2 molecules do exist in pathological contexts, which relevance may now be sought on larger cohorts and other pathologies.

1. Introduction

Human leukocyte antigen (HLA)-G (for review, see [1]) was initially identified as a molecule specifically expressed by the fetal cytotrophoblasts, at the fetal–maternal interface. In non-pathological conditions, HLA-G expression is tissue-restricted and expressed only in fetal extravillous cytotrophoblast, adult thymic epithelial cells, cornea, beta cells of the islets of Langerhans, and stem cells and precursor cells, which are, for most, tissues that are considered immunologically privileged. However, expression of HLA-G may be induced in other cell types during pathological processes which include cancer, inflammatory disorders, viral infections, and allotransplantation. HLA-G is an immune-inhibitory molecule which exerts its function via interaction with two main inhibitory receptors in the adult periphery: ILT2 (LILRB1/CD85j) and ILT4 (LILRB2/CD85d) [2,3]. The interaction between HLA-G and the ILT2/4 receptors is considered to be an immune checkpoint and the ILT-receptors are therefore of particular interest in the context of cancer [4,5]. ILT2 can be expressed by lymphoid and myeloid cells, whereas expression of ILT4 is myeloid-specific. Through these differentially expressed receptors, HLA-G can interact with B cells, T cells, NK cells, and antigen-presenting cells. ILT2 and ILT4 are similar in that they both have four tandem Ig-like extracellular domains and four and three immunoreceptor tyrosine-based inhibitory receptor motifs (ITIMs), respectively, in their cytoplasmic tails. ITIMs recruit the protein tyrosine phosphatase SHP-1 (Src homology 2 domain containing phosphatase 1), which is thought to inhibit early signaling events triggered by stimulatory receptors [6]. ILT2 and ILT4 have been shown to bind to classical (HLA-A and -B) and non-classical (HLA-G1, -E, and -F) MHC Class I molecules, however, studies indicate that they bind to HLA-G with a higher affinity than to classical MHC Class I molecules. Both receptors recognize HLA-G as dimer but not as monomers. HLA-G dimers being the active HLA-G forms;

* Corresponding author at: Hemato-Immunology Research Department, CEA, Hospital Saint Louis, 1 Avenue Claude Vellefaux, Bat Lailler, 75475 Paris Cedex 10, France.
E-mail address: joel.lemoult@cea.fr (J. LeMaoult).
[1] These two authors contributed equally to this work.

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ILT2 and ILT4 thereby preferentially bind to active HLA-G forms [7,8]. Even though there are similarities between the two receptors, ILT2 and ILT4 differ at the level of the HLA-G structures they recognize: ILT2 is a receptor for HLA-G associated with β2-microglobulin, whereas ILT4 also recognizes HLA-G free heavy chains [7].

Interestingly, overexpression of cell-bound HLA-G receptors has been reported in the course of the same pathologies in which a *de novo* expression of HLA-G was observed. Indeed, ILT2 expression by T cells and ILT4 expression by monocytes were up-regulated in AIDS [9,10], ILT2 expression by breast carcinoma infiltrating cells was up-regulated concomitantly to HLA-G expression by the tumor [11]; ILT2 and HLA-G coexpression was found in primary cutaneous T cell lymphomas [12]; and both HLA-G-expressing macrophages and ILT2-expressing $CD4^+$ T cells were detected in lesions of psoriasis [13]. Thus a direct relationship might exist between up-regulation of HLA-G expression and up-regulation of its receptors. Of note, HLA-G was shown to up-regulate the expression of inhibitory receptors, including ILT2 and ILT4, in immune cells of various lineages [14].

The interaction between HLA-G and the ILT receptors is considered to be an immune checkpoint and we therefore argue for the necessity of measuring not only HLA-G in patient samples but also ILT2 and ILT4. This is important at the level of cell-surface expression, since ILT2 receptors are only expressed by a minority of $CD4^+$ T cells (<5%), $CD8^+$ T cells (10–15%), and peripheral NK cells (15–25%). Indeed, variations in membrane-bound ILT expression might significantly change HLA-G function; an increase in membrane-bound ILT2 expression might for instance increase HLA-G impact. Alternatively, non-cell-bound ILT molecules, secreted as soluble forms or released through shedding, would compete with membrane-bound ones for ligands such as HLA-G and decrease their functional impact. As yet, there is no method to detect and measure soluble ILT2 and ILT4 HLA-G receptors, nor is there any evidence that ILT2 and ILT4 can be found as soluble molecules.

Here, we describe a multiplex bead-based immunoassay to titrate the free soluble forms of the ILT2 and ILT4 inhibitory receptors. Furthermore, we show that free soluble ILT molecules are of low abundance in the plasma of healthy donors and that it is likely they complex with soluble HLA Class I molecules. Increased levels of free soluble ILT2/4 molecules could be detected in the plasma of cancer patients, validating our immunoassay. The impact of increased free soluble ILT2/4 levels could not be investigated due to the small size of our patient cohorts.

2. Materials and methods

2.1. Blood, patients and samples

EDTA-plasma was collected from untreated patients with B-cell tumors (B-cell chronic lymphocytic leukemia and lymphoma) or with non-muscle-infiltrating bladder cancer during their routine examination at the Department of Hematology, or the Department of Urology at the Saint-Louis Hospital in Paris, respectively. All patients gave informed consent to the study, which was approved by the local institutional board. Healthy blood donors from the blood donors unit of the Saint-Louis Hospital were systematically included. The plasma was frozen at −80 °C until use. Before the assay was run the samples were defrosted and spun at 2000 g for 10 min to remove precipitates.

HEK cells transfected with ILT2 or ILT4 were used in preliminary tests. The melanoma M8 cell line [15] was transfected with HLA-G5-GFP and used for the purpose of detecting ILT2/4-coupled HLA-G.

2.2. Antibodies and recombinant proteins

The candidate antibodies for ILT2 capture were anti-human CD85j clone GHI/75 (BD Pharmingen), clone 292319 (R&D Systems), and polyclonal goat anti-ILT2 (R&D Systems); ILT4 capture antibody candidates were anti-human CD85d clone 27D6 (eBioscience), clone 287219 (R&D Systems), and polyclonal goat anti-ILT4 (R&D Systems). The detection antibodies for ILT2 and ILT4 were CD85j-PE clone HP-F1 (Beckman Coulter) and CD85d-PE clone 42D1 (Beckman Coulter).

PE-conjugated goat anti-mouse (Beckman Coulter), and goat anti-human antibodies (Jackson ImmunoResearch) were used as secondary antibodies and for bead validation.

Recombinant human ILT2-Fc and ILT4-Fc chimeric proteins (R&D Systems) were used as standard proteins in the immunoassay. Anti-HLA-G G233 antibody was from Exbio, Praha.

2.3. Coupling of antibodies and proteins to beads

We prepared different sets of beads: some were coupled with recombinant ILT2-Fc or ILT4-Fc proteins and used for antibody assessment, and some were antibody-coupled beads used in the immunoassay. The coupling procedure was the same for both types of bead preparations: proteins or antibodies were coupled to Bio-Plex Pro Magnetic COOH Beads (Bio-Rad) using the Bio-Plex Amine Coupling kit (Bio-Rad) and according to the manufacturers protocol. Briefly, the stock solution of microbeads was vortexed thoroughly and sonicated for 15 s before 100 µl of monodispersed beads were transferred to a coupling reaction tube. The supernatant was discarded using the magnetic bead separator and the beads were washed in 100 µl wash buffer and vortexed for 30 s. The beads were then resuspended in 80 µl bead activation buffer and vortexed for 30 s. Solutions of N-hydroxysulfosuccinimide, S-NHS, (Sigma–Aldrich) and 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide, EDAC, (Merck-Millipore) were prepared in bead activation buffer to a concentration of 50 mg/ml immediately before use, and 10 µl of each solution were added to the beads. After 20 min of incubation on a shaker at room temperature, 150 µl PBS, pH 7.4 were added to the activated beads, and after vortexing the supernatant was discarded by using the magnetic bead separator. The PBS wash step was repeated and after removal of the supernatant the beads were resuspended in 100 µl PBS, pH 7.4, and vortexed again. 12 µg of antibody or 5 µg of recombinant protein were added to the beads and was added PBS, pH 7.4, to a final volume of 500 µl. The beads were incubated with agitation over night at 4 °C in the dark. After discarding the supernatant the beads were washed in 500 µl PBS, pH 7.4, and resuspended in 250 µl blocking buffer, vortexed, and incubated on a shaker for 30 min at room temperature. The supernatant was discarded and the beads were washed before the final addition of 500 µl storage buffer. Protein coupled beads were systematically validated by flow cytometry using PE-coupled goat anti-mouse, or goat anti-human antibodies depending on the primary antibody.

2.4. Bead-based immunoassay

MultiScreen filter plate (Millipore) was incubated with wash buffer (PBS, pH 7.4, with 0.05% Tween 20) for a 5 min before the supernatant was discarded and addition of 50 µl of antibody-coupled beads in assay buffer (at least 5000 of each region of beads) and 50 µl of sample. The assay buffer (10×) is composed of 500 mM phosphate, pH 7.0, containing 1 M NaCl, 1% NaN3 and 1% BSA and diluted to 1× in dH_2O, pH 7.4. For the standard curve a serial dilution of recombinant human ILT2/ILT4 Fc proteins were used (R&D). The filter plate was incubated on a shaker for 1 h at room temperature and then washed twice with wash buffer. After the second