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The immunosuppressive effect of domain-deleted dimer of HLA-G2 isoform in collagen-induced arthritis mice

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25 **Keywords:** HLA-G2, ILT4/LILRB2, PIR-B, CIA mice, immunosuppression

26 **Abbreviated title:** Immunosuppressive effect of HLA-G2 dimer

27

Abstract

HLA-G is involved in maternal-fetal immune tolerance and is reported to be a natural tolerogenic molecule. Seven-spliced isoforms including dimeric and β 2m-free forms have been identified. The major isoform, HLA-G1 (and its soluble type HLA-G5), binds to the inhibitory immune receptors, leukocyte immunoglobulin (Ig)-like receptor (LILR) B1 and LILRB2. We previously reported that HLA-G1 also binds to paired Ig-like receptor (PIR)-B, a mouse homolog of LILRBs, and had a significant immunosuppressive effect in collagen-induced arthritis (CIA) mice. Although HLA-G2 and its soluble form HLA-G6 bind specifically to LILRB2, its functional characteristics are largely unknown. In this study, we report the significant immunosuppressive effect of HLA-G2 dimer in CIA mice. Surface plasmon resonance analysis revealed a specific interaction of HLA-G2 with PIR-B. CIA mice were administered HLA-G2 protein subcutaneously once in the left footpad and clinical severity was evaluated in a double-blind study. A single administration of HLA-G2 maintained a suppressive effect for over 1 month. These results suggested that the HLA-G2 protein might be a useful biopharmaceutical for the treatment of rheumatoid arthritis by binding to inhibitory PIR-B.

46 **Abbreviations**

47 LILR, leukocyte immunoglobulin-like receptor; RA, rheumatoid arthritis; CIA,
48 collagen-induced arthritis; PIR, paired immunoglobulin-like receptor

49

1. Introduction

Human leukocyte antigen (HLA)-G is a non-classical HLA class I molecule, and functions as a tolerogenic molecule involved in pregnancy, transplantation, autoimmune diseases and some cancers [1]. It has three characteristics distinct from classical HLA class I molecules, including low polymorphisms, restricted tissue distribution and various isoforms. To date, seven spliced isoforms have been identified: HLA-G1 to -G4 are membrane-bound forms and HLA-G5 to -G7 are soluble forms. HLA-G1 and its soluble form HLA-G5 are the major isoforms. They have a typical HLA class I structure composed of a heavy chain including $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains, a peptide and $\beta 2m$ microglobulin ($\beta 2m$). HLA-G2, and its soluble form HLA-G6, only contain $\alpha 2$ domain-deleted heavy chains. Because the N-terminal ligand-binding domains of the membrane-bound and soluble forms are identical, hereafter HLA-G1 and HLA-G5 are simply termed HLA-G1, and HLA-G2 and HLA-G6 are termed HLA-G2. Furthermore, HLA-G1 exists as a disulfide-linked homodimer between Cys42 residues within the $\alpha 1$ domain, or a $\beta 2m$ -free monomer and homodimer [2].

Three cell surface receptors for HLA-G1 are expressed on immune cells: leukocyte immunoglobulin (Ig)-like receptor (LILR) B1 (also called leukocyte Ig-like Receptor (LIR) 1, Ig-like transcript (ILT) 2, CD85j), LILRB2 (LIR2, ILT4,

CD85d) and killer-cell immunoglobulin-like receptor (KIR) 2DL4. LILRB1 and B2 (LILRB1/2) contain immunoreceptor tyrosine-based inhibition motifs (ITIM) and function as immune inhibitory receptors. Although LILRB1/2 receptors also bind to classical HLA class I molecules, LILRB1/2 binds to HLA-G1 with a higher affinity than classical HLA class I molecules [3]. Therefore, it is thought that the tolerogenic effects of HLA-G are mainly induced by interactions with LILRB1/2. LILRB1 is widely expressed on immune cells including monocytes, dendritic cells (DCs), B cells and subsets of natural killer (NK) cells and T cells. However, the expression of LILRB2 is restricted to antigen presenting cells (APCs). The murine LILRB homolog, paired Ig-like receptor (PIR) -B, also binds to HLA-G [4]. While the LILR family has 5 inhibitory receptors (LILRB1-5) in humans, only one inhibitory PIR-B has been found in the mouse genome. PIR-B is expressed on murine APCs similar to LILRB2. In a previous study, we demonstrated that a disulfide-linked HLA-G1 dimer had a remarkable avidity for PIR-B binding, resulting in a higher affinity to immobilized PIR-B than its monomeric form [5].

To date, a number of studies of HLA-G1 have been published. HLA-G1 inhibited various immune responses including NK cell-mediated cytotoxicity [6, 7], CD8⁺ T cell-mediated cytotoxic T-lymphocyte (CTL) responses [8], the alloproliferation of

CD4⁺ T cells [9], the differentiation and Ig secretion of B cells [10] and the maturation and function of DCs against antigens [11]. These studies suggested that HLA-G1 exhibits a wide range of tolerogenic functions. Moreover, HLA-G induced the development of tolerogenic cells such as HLA-G⁺ regulatory T (Treg) cells [12], CD4^{low} and CD8^{low} suppressor T cells [13], type 1 T regulatory (Tr1) cells and interleukin (IL)-10-differentiated DCs (DC-10) [14]. In contrast, HLA-G2 has not been well studied. The HLA-G*0105N null allele, which has a frameshift deletion of cytosine at codon 130 (1597delC) in exon 3 (α 2 domain) presumably results in a lack of full-length functional HLA-G1 but the existence of HLA-G2. Therefore, the identification of healthy subjects homozygous for HLA-G*0105N null allele suggested that α 2-domain-deleted HLA-G2 could perform the HLA-G1 function [15]. Recently, it was reported that HLA-G2 could bind to LILRB2, but not to LILRB1, and prolong the survival of skin allografts in both non-transgenic (PIR-B was expected to function as a receptor) and LILRB2-transgenic mice [16, 17]. Inhibition of NKL, Raji, KG-1 and U937 tumor cell line proliferation by HLA-G2 was also demonstrated [17].

We have established a refolding method for the preparation of recombinant HLA-G2 proteins and showed its specific binding to LILRB2 (K. Kuroki *et al.*

unpublished). In this study, we first identified the specific binding of HLA-G2 to mouse LILRB homolog PIR-B by surface plasmon resonance (SPR) analysis. Next, we verified the *in vivo* effects of HLA-G2 using collagen-induced arthritis (CIA) mice. We found that HLA-G2 exhibited a significant anti-inflammatory effect that was maintained for a 1-month period with no apparent side effects. The immunosuppressive effect of HLA-G2 in CIA mice was likely to be mediated by interaction with PIR-B. These findings strongly suggest the potential future therapeutic application of HLA-G2.

2. Materials and methods

2. 1. Preparation of recombinant HLA-G2 proteins

The gene encoding ectodomain of HLA-G2 (α 1- α 3 domains, Gly1-Trp182), which is identical for its soluble form, HLA-G6, was ligated into a pGMT7 plasmid vector. The HLA-G2 ectodomain was expressed as inclusion bodies in BL21(DE3)pLysS (Merck Millipore) for *in vitro* experiments and ClearColi[®] BL21(DE3) competent cells (Lucigen) for *in vivo* experiments. The HLA-G2 inclusion bodies were refolded by a dilution method for three days and purified by size exclusion chromatography (SEC) using a HiLoad 26/60 Superdex 75 pg column (GE Healthcare) with SEC buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). For *in vivo* analyses, the buffer was exchanged by dialysis using phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄). Both HLA-G2 and PBS (control for *in vivo* analysis) prior to injection in mice were treated with a Detoxi-gel endotoxin removing column (Thermo Fisher Scientific).

2. 2. Preparation of recombinant PIR-B ectodomain.

The ectodomain of PIR-B (PIR-B) with a biotinylation tag, a His⁶ tag and a

FLAG tag in tandem at the C-terminal was generated by HEK293T cells. The gene coding from Ser25 to Ser608 of PIR-B cDNA was cloned into the modified pCAGGS plasmid vector. When HEK293T cells reached 80% confluence in D-MEM supplemented with 10% inactivated fetal bovine serum (FBS) (HyClone Laboratories), at 37°C with 5% CO₂ they were transfected with PIR-B plasmid and polyethylenimine (PEI) max (Polysciences) and then cultured in D-MEM supplemented with 1% FBS at 37°C with 5% CO₂ for 72 hours. The supernatant containing recombinant PIR-B proteins was collected and purified by His trap FF 5 mL (GE Healthcare) and a Superdex 200 10/300 GL column (GE Healthcare). The recombinant PIR-B was concentrated using an Amicon ultra-15 centrifugal filter unit (Millipore), and biotinylated with BirA enzyme for 1 h at 30°C. The biotinylated protein was finally purified by Superdex200 10/300 GL (GE Healthcare).

2. 3. Surface plasmon resonance (SPR) analysis

Either the C-terminal biotinylated PIR-B or chemically biotinylated BSA (as a control) were immobilized on the surface of sensor chip CM5 (GE Healthcare) covalently coupled with streptavidin. The purified recombinant HLA-G2 in HBS-EP buffer (GE Healthcare) was injected over each flow cell (Biacore 3000, GE Healthcare). The

binding responses were calculated by subtracting a response measured in the BSA immobilized control flow cell from that in the PIR-B flow cell. The kinetic parameters were calculated using a global fitting by the 1:1 Langmuir binding model ($A + B \leftrightarrow AB$) and bivalent binding model ($A + B \leftrightarrow AB$, $AB + B \leftrightarrow AB_2$) using BIAevaluation Software 4. 1. 1 (GE Healthcare).

2. 4. *Mice*

Six-week-old DBA/1J male mice were purchased from Japan Charles River Breeding Laboratories. All animal experimental procedures were in accordance with the Hokkaido University Manual for Implementing Animal Experimentation and were approved by the Committee for Animal Research at Hokkaido University.

2. 5. *Induction of CIA*

After 1 week of housing, mice were sensitized by subcutaneous injection in their tails of 100 μ g bovine type II collagen in an adjuvant solution for primary sensitization. The collagen adjuvant solution was prepared by the following method. Immunization grade bovine type II collagen (Chondrex) was dissolved at a concentration of 4 mg/mL in buffer (20 mM Tris-HCl, pH 8.0 and 150 mM NaCl) and stirred under dark conditions at 4°C overnight. The bovine type II collagen solution was

emulsified with an equal amount of complete Freund's adjuvant (CFA, 4 mg/ml, Chondrex), which was mixed on ice in cold room (4°C) for 15 min. Two weeks after primary sensitization, mice were secondary sensitized using the same injection procedure with 200 µg bovine type II collagen in adjuvant solution. Bovine type II collagen was dissolved at a concentration of 8 mg/mL in buffer (20 mM Tris-HCl, pH 8.0 and 150 mM NaCl) and stirred in the dark at 4°C overnight. The bovine type II collagen solution was emulsified with an equal amount of incomplete Freund's adjuvant (IFA, Chondrex), which was mixed on ice for 10 min.

2. 6. Evaluation of CIA

The clinical severity of arthritis (RA score) was recorded five times a week. Mice were scored according to the following criteria based on the evidence of arthritis at the joints: in limbs without fingers, 0 = no swelling, 3 = detectable swelling (including deformity without swelling), 4 = moderate swelling (sometimes with redness) and 5 = severe swelling (sometimes with bleeding from skin), and for limbs with fingers, 0 = no swelling and 1 = swelling. Thus, the maximum score per whole limb was 10, and per mouse was 40. This evaluation was performed double-blind.

2. 7. Treatment with HLA-G2

180 Seven days after the secondary sensitization, mice with no symptoms and with
181 normal body weight were randomly divided into three groups. Then, the mice in each
182 group were administered once with two serially 10-fold diluted HLA-G2 protein
183 solutions in PBS, or PBS alone (control), through the skin of their left hind footpads.

184 2. 8. *Statistical analyses*

185 JMP[®] 11 software (SAS Institute Inc., Cary, NC, USA) was used for statistical
186 analyses. Differences in RA scores were statistically analyzed between the high dose
187 HLA-G2 administered group and the control group using the Student's *t*-test.

188

3. Results

3. 1. The HLA-G2 dimer binds to PIR-B

We succeeded in preparing functional recombinant HLA-G2 proteins, which formed homodimers and functionally bound to LILRB2 (K. Kuroki, *et al.*, unpublished data). Briefly, the HLA-G2 was expressed as inclusion bodies in *Escherichia coli*, was refolded, and purified by size exclusion chromatography (Fig. 1A and B). In the current study, we characterized the binding of the HLA-G2 dimer to a mouse LILRB homolog PIR-B by SPR analysis. The HLA-G2 dimer was prepared by the refolding method. Purified biotinylated PIR-B and BSA as a negative control were immobilized on the surface of a CM5 sensor chip covalently coupled with streptavidin. The five serially two-fold diluted HLA-G2 proteins (1.56 μ M, 0.78 μ M, 0.39 μ M, 0.20 μ M and 0.10 μ M) were injected over the immobilized PIR-B (3000 RU) or BSA (2000 RU). The sensorgrams of specific binding were derived by subtracting the responses measured in the PIR-B immobilized flow cell from those measured in the BSA immobilized flow cell. The binding curve of HLA-G2 to the PIR-B is shown in Fig. 1C. The kinetic parameters were calculated by a bivalent fitting model, because the HLA-G2 formed a homodimer and had an apparent avidity for human receptor, LILRB2 (K. Kuroki, *et al.*,

unpublished data). HLA-G2 recognized the immobilized PIR-B ectodomain with slow dissociation ($1.4 \times 10^{-3} \text{ s}^{-1}$). The apparent K_D , calculated using the 1:1 Langmuir binding model fitting, was 130 nM, indicating the HLA-G2 homodimer bound to PIR-B with a higher affinity than HLA-G1 monomer did (μM order). Therefore, HLA-G2 has the potential to transmit effective signals through the inhibitory receptor, PIR-B, in mice.

The HLA-G2 dimer immunosuppressive effect was maintained over one month

Next, we assessed the function of HLA-G2 *in vivo* using CIA mice, which were previously used for the study of HLA-G1 [5]. Mice were sensitized with bovine type II collagen twice to induce arthritis as described in the materials and methods. Because a clear anti-inflammatory effect was not observed when HLA-G2 was administered to mice that developed initial arthritis symptoms (RA score > 3) (data not shown), we examined the effect of HLA-G2 before the onset of disease. In our experiments, mice began to develop arthritis approximately 12 days after the secondary sensitization. Seven days after the second sensitization, non-progressive mice were selected, and randomly divided into three groups. These mice were administered HLA-G2 protein solution (high and low dose) or PBS once in the left hind footpad. The day of HLA-G2 administration was defined as “day 0”, and the clinical severity of arthritis (RA score)

and their weights were recorded.

Figures 2 and 3 show the representative data of 5 independent experiments.

Mice were administered 1.4 μ g (high dose) or 0.14 μ g (low dose) HLA-G2 in PBS, or

PBS alone as a negative control. The high dose group showed consistently lower RA

scores than the control group for more than one month (Fig. 2A and B). Of note, at the

last 3 days of the experimental period, differences in RA score between the high dose

and control groups was statistically significant (Fig. 2A). In contrast, the low dose

group did not show any significant anti-inflammatory effect although some inhibition of

arthritis in the early period was observed. A comparison of the incidence of arthritis (RA

score ≥ 3) indicated the day of onset of disease in the high dose treatment mice was

delayed (Fig. 2C). The incidence of disease at the end point was 75% in the high dose

group compared with 92% in the control group and 100% in the low dose group; a

decrease of 18% and 25%, respectively (Fig. 2C). The day at which the incidence was

50% was delayed in the high dose group (day 29) compared with the low dose group

(day 23) and the control group (day 24). The onset of disease in the low dose group was

suppressed only in the early period (Fig. 2C). A comparison of the RA scores of each

limb indicated that although mice were administered HLA-G2 locally, it had a

generalized systemic effect (Fig. 3). Even though the RA scores in the hind right paw of

the high dose of HLA-G2 administered and the control groups were not different, the RA scores of both front paws and the hind left paw were lower in the high dose groups compared with the control group (Fig. 3). Finally, the RA score of all four limbs in the high dose group was lower compared with the control group.

Therefore, these results indicate that HLA-G2 had an immunosuppressive effect that was dose-dependent in CIA mice. Any negative effects such as a phenotype other than arthritis and weight loss were not observed after the administration of HLA-G2 protein (Fig. 2D). The mean body weight in the control and low dose groups tended to be decreased compared with the high dose group because of severe arthritis.

4. Discussion

In this study, we demonstrated that purified HLA-G2 recombinant proteins prepared by the refolding method showed significant immunosuppressive effects in CIA mice. As in the case of HLA-G1 [5], HLA-G2 also showed long-term generalized effects from a single local administration (Figs. 2 and 3). Furthermore, we identified that HLA-G2 specifically bound to PIR-B, the murine homologue of human LILRB. Because PIR-B is only expressed by APCs, the effects of HLA-G2 in mice are expected to suppress antigen presentation. Advanced biologics that target specific molecules of immune responses have been effective in RA patients. Generally, inflammation-related cytokines and their receptors have been targeted by biologics including monoclonal antibodies and soluble Ig-fusion receptor proteins [18]. Recently, drugs against “immune checkpoint” targets such as CTLA-4 and PD-1/PD-L1 are expected to be developed, especially for cancer treatment [19]. PIR-B in mice and LILRB2 in humans are inhibitory cell-surface receptors possessing ITIM motifs, which might be new candidate targets for immune checkpoint biologics. Our study showed that the modulation of antigen presenting cells by these receptors might be useful for inducing a prolonged tolerogenic phenotype *in vivo*.

PIR-B constitutively binds to mouse MHC class I molecules and inhibits immune cell reactions against self components. *In vivo* analyses using PIR-B deficient mice clearly demonstrated its critical role in B cell suppression, DC maturation, and balancing Th1/Th2 immune responses [20]. McIntire *et al.* reported that HLA-G6 (the soluble form of HLA-G2) induced TGF- β production in PMA/IFN- γ -treated U937 cells [21]. Our preliminary study indicated that IL-17A tended to be down regulated and that IL-10 and TGF- β tended to be up-regulated in lymph nodes in the early phase of disease after HLA-G2 administration (data not shown), although these differences were not statistically significant. Further experiments are required to reveal the mechanism of immune suppression induced by HLA-G2.

The specific binding affinity of HLA-G2 against PIR-B is stronger than that of the HLA-G1 monomer [22] but weaker than that of the HLA-G1 dimer [5]. However, the effective dose of HLA-G2 is low (1.4 μ g) because the administration of 1.5 μ g of HLA-G1 dimer did not have an immunosuppressive effect in CIA mice [5]. The recognition mode between PIR-B and HLA-G2 has not been clarified yet, but the HLA-G2 homodimer presumably possesses two PIR-B binding sites; thus, one HLA-G2 homodimer can bind to two PIR-B receptors similar to that of HLA-G2-LILRB2 binding (unpublished data). Moreover, the HLA-G2 molecule

contains cysteine 42, which forms an intermolecular disulfide bond that dimerizes with HLA-G1. Therefore, some of the HLA-G2 homodimer could form a tetramer (dimer of a homodimer), or a multimer. The purified HLA-G2 protein used in this study showed partial polydisperse by dynamic light scattering (data not shown), which suggests that the administered HLA-G2 solution includes tetramer and multimer populations. The multimerization of HLA-G2 is likely contribute to a strong long-term effect through the accumulation of PIR-B receptors. In accord, LeMaoult *et al.* reported that beads coated with a synthetic ($\alpha 1$ - $\alpha 3$)₂ polypeptide prolonged graft survival (the median graft survival time was increased by 11 days compared to controls (18 days)) even after 1 injection [17]; thus, the bead-coated HLA-G2 multimer was efficacious for almost one month *in vivo*.

Taken together, our data suggests that HLA-G2/G6 functions as a tolerogenic molecule similar to HLA-G1/G5 through its specific binding to LILRB2 in humans and PIR-B in mice. Therefore, the HLA-G2/G6 proteins might be a useful biopharmaceutical for the treatment of rheumatoid arthritis.

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Figure legends

Fig. 1. Purification and binding analyses of recombinant HLA-G2 protein. (A) Size exclusion chromatogram of HLA-G2 on a Superdex 75 26/60 prep grade column. (B) SDS-PAGE analyses of purified HLA-G2 under reducing conditions using a 15% acrylamide gel and Coomassie Brilliant Blue staining. (C) Kinetic analyses of HLA-G2 with PIR-B immobilized on the sensor chip. HLA-G2 was injected at the indicated concentrations. Response curves (black lines) were fitted locally with the bivalent analyte model (gray lines).

Fig. 2. Evaluation experiment of the HLA-G2 dimer in CIA mice. Results are representative of 5 independent experiments. (A) Mean RA scores and standard error of the control, low dose and high dose groups, indicated by gray squares, black triangles and black circles, respectively. Twelve mice were used per group ($n = 12$), and the low and high dose of HLA-G2 proteins was 0.14 μg and 1.4 μg , respectively. $*P < 0.05$ (Day 35: $P = 0.045$, Day 36: $P = 0.034$ and Day 37: $P = 0.042$. The high dose group was compared to the control group by Student's t -test. (B) RA scores of the last day of disease and their box plots are shown. Each dot represents a single mouse ($n = 12/\text{group}$). Boxes indicate the first and the third quartiles of each RA score. Lines inside the boxes indicate the median of each RA score. (C) The incidence of arthritis indicates

412 the rate of mice whose RA score was ≥ 3 . (D) Changes in body weight $\pm$ standard
413 deviation.

414 **Fig. 3.** The mean RA scores of each limb after treatment with high dose (1.4 μ g, circles)
415 or low dose HLA-G2 (0.14 μ g, triangles), or PBS alone (squares). These data are from
416 the same experiment as Fig. 2 (n = 12/group).
417

Fig. 1

Fig. 2 A

B

C

D

Fig. 3