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

Ami Takahashi[a], Kimiko Kuroki[a*], Yuki Okabe[a], Yoshiyuki Kasai[a], Naoki Matsumoto[b], Chisato Yamada[a], Toshiyuki Takai[c], Toyoyuki Ose[a], Shigeyuki Kon[b], Tadashi Matsuda[b], Katsumi Maenaka[a,d*]

[a]Laboratory of Biomolecular Science Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

[c]Department of Immunology, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

[c]Department of Experimental Immunology, IDAC, Tohoku University, Sendai, Japan

[d]Center for Research and Education on Drug Discovery, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

***Corresponding author at:**

Laboratory of Biomolecular Science, Faculty of Pharmaceutical Sciences, Hokkaido

University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

TEL: +81-11-706-3970, FAX: +81-11-706-4986

E-mail addresses:

k-kimiko@pharm.hokudai.ac.jp (K. Kuroki), maenaka@pharm.hokudai.ac.jp (K. Maenaka)

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Abbreviated title: Immunosuppressive effect of HLA-G2 dimer

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 β2m-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.

Abbreviations

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

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 α1, α2 and α3 domains, a peptide and beta-2 microglobulin (β2m). HLA-G2, and its soluble form HLA-G6, only contain α2 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 α1 domain, or a β2m-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 cytolysis [6, 7], $CD8^+$ T cell-mediated cytotoxic T-lymphocyte (CTL) responses [8], the alloproliferation of

86 $CD4^+$ T cells [9], the differentiation and Ig secretion of B cells [10] and the
87 maturation and function of DCs against antigens [11]. These studies suggested that
88 HLA-G1 exhibits a wide range of tolerogenic functions. Moreover, HLA-G induced
89 the development of tolerogenic cells such as HLA-G^+ regulatory T (Treg) cells [12],
90 $CD4^{low}$ and $CD8^{low}$ suppressor T cells [13], type 1 T regulatory (Tr1) cells and
91 interleukin (IL)-10-differentiated DCs (DC-10) [14]. In contrast, HLA-G2 has not
92 been well studied. The HLA-G*0105N null allele, which has a frameshift deletion of
93 cytosine at codon 130 (1597delC) in exon 3 (α2 domain) presumably results in a
94 lack of full-length functional HLA-G1 but the existence of HLA-G2. Therefore, the
95 identification of healthy subjects homozygous for HLA-G*0105N null allele
96 suggested that α2-domain-deleted HLA-G2 could perform the HLA-G1 function
97 [15]. Recently, it was reported that HLA-G2 could bind to LILRB2, but not to
98 LILRB1, and prolong the survival of skin allografts in both non-transgenic (PIR-B
99 was expected to function as a receptor) and LILRB2-transgenic mice [16, 17].
100 Inhibition of NKL, Raji, KG-1 and U937 tumor cell line proliferation by HLA-G2
101 was also demonstrated [17].
102 We have established a refolding method for the preparation of recombinant
103 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 (α1-α3 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 Na_2HPO_4 and 1.5 mM KH_2PO_4). 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 His^6 tag and a

129 FLAG tag in tandem at the C-terminal was generated by HEK293T cells. The gene
130 coding from Ser25 to Ser608 of PIR-B cDNA was cloned into the modified pCAGGS
131 plasmid vector. When HEK293T cells reached 80% confluence in D-MEM
132 supplemented with 10% inactivated fetal bovine serum (FBS) (HyClone Laboratories),
133 at 37°C with 5% CO_2 they were transfected with PIR-B plasmid and polyethylenimine
134 (PEI) max (Polysciences) and then cultured in D-MEM supplemented with 1% FBS at
135 37°C with 5% CO_2 for 72 hours. The supernatant containing recombinant PIR-B
136 proteins was collected and purified by His trap FF 5 mL (GE Healthcare) and a
137 Superdex 200 10/300 GL column (GE Healthcare). The recombinant PIR-B was
138 concentrated using an Amicon ultra-15 centrifugal filter unit (Millipore), and
139 biotinylated with BirA enzyme for 1 h at 30°C. The biotinylated protein was finally
140 purified by Superdex200 10/300 GL (GE Healthcare).

141 *2. 3. Surface plasmon resonance (SPR) analysis*

142 Either the C-terminal biotinylated PIR-B or chemically biotinylated BSA (as a control)
143 were immobilized on the surface of sensor chip CM5 (GE Healthcare) covalently
144 coupled with streptavidin. The purified recombinant HLA-G2 in HBS-EP buffer (GE
145 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 μg 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

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

2. 8. Statistical analyses

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

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 μM, 0.78 μM, 0.39 μM, 0.20 μM and 0.10 μM) 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.*,

206 unpublished data). HLA-G2 recognized the immobilized PIR-B ectodomain with slow
207 dissociation (1.4×10^{-3} s^{-1}). The apparent K_D, calculated using the 1:1 Langmuir binding
208 model fitting, was 130 nM, indicating the HLA-G2 homodimer bound to PIR-B with a
209 higher affinity than HLA-G1 monomer did (μM order). Therefore, HLA-G2 has the
210 potential to transmit effective signals through the inhibitory receptor, PIR-B, in mice.
211
212 *The HLA-G2 dimer immunosuppressive effect was maintained over one month*
213 Next, we assessed the function of HLA-G2 *in vivo* using CIA mice, which were
214 previously used for the study of HLA-G1 [5]. Mice were sensitized with bovine type II
215 collagen twice to induce arthritis as described in the materials and methods. Because a
216 clear anti-inflammatory effect was not observed when HLA-G2 was administered to
217 mice that developed initial arthritis symptoms (RA score > 3) (data not shown), we
218 examined the effect of HLA-G2 before the onset of disease. In our experiments, mice
219 began to develop arthritis approximately 12 days after the secondary sensitization.
220 Seven days after the second sensitization, non-progressive mice were selected, and
221 randomly divided into three groups. These mice were administered HLA-G2 protein
222 solution (high and low dose) or PBS once in the left hind footpad. The day of HLA-G2
223 administration was defined as “day 0”, and the clinical severity of arthritis (RA score)

224 and their weights were recorded.

225 Figures 2 and 3 show the representative data of 5 independent experiments.
226 Mice were administered 1.4 μg (high dose) or 0.14 μg (low dose) HLA-G2 in PBS, or
227 PBS alone as a negative control. The high dose group showed consistently lower RA
228 scores than the control group for more than one month (Fig. 2A and B). Of note, at the
229 last 3 days of the experimental period, differences in RA score between the high dose
230 and control groups was statistically significant (Fig. 2A). In contrast, the low dose
231 group did not show any significant anti-inflammatory effect although some inhibition of
232 arthritis in the early period was observed. A comparison of the incidence of arthritis (RA
233 score ≥3) indicated the day of onset of disease in the high dose treatment mice was
234 delayed (Fig. 2C). The incidence of disease at the end point was 75% in the high dose
235 group compared with 92% in the control group and 100% in the low dose group; a
236 decrease of 18% and 25%, respectively (Fig. 2C). The day at which the incidence was
237 50% was delayed in the high dose group (day 29) compared with the low dose group
238 (day 23) and the control group (day 24). The onset of disease in the low dose group was
239 suppressed only in the early period (Fig. 2C). A comparison of the RA scores of each
240 limb indicated that although mice were administered HLA-G2 locally, it had a
241 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]. Mclntire *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 μg) because the administration of 1.5 μg 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

287 contains cysteine 42, which forms an intermolecular disulfide bond that dimerizes with
288 HLA-G1. Therefore, some of the HLA-G2 homodimer could form a tetramer (dimer of
289 a homodimer), or a multimer. The purified HLA-G2 protein used in this study showed
290 partial polydisperse by dynamic light scattering (data not shown), which suggests that
291 the administered HLA-G2 solution includes tetramer and multimer populations. The
292 multimerization of HLA-G2 is likely contribute to a strong long-term effect through
293 the accumulation of PIR-B receptors. In accord, LeMaoult *et al*. reported that beads
294 coated with a synthetic $(\alpha1\text{-}\alpha3)_2$ polypeptide prolonged graft survival (the median
295 graft survival time was increased by 11 days compared to controls (18 days)) even
296 after 1 injection [17]; thus, the bead-coated HLA-G2 multimer was efficacious for
297 almost one month *in vivo*.
298 Taken together, our data suggests that HLA-G2/G6 functions as a tolerogenic
299 molecule similar to HLA-G1/G5 through its specific binding to LILRB2 in humans
300 and PIR-B in mice. Therefore, the HLA-G2/G6 proteins might be a useful
301 biopharmaceutical for the treatment of rheumatoid arthritis.
302
303

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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/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 ± standard

413 deviation.

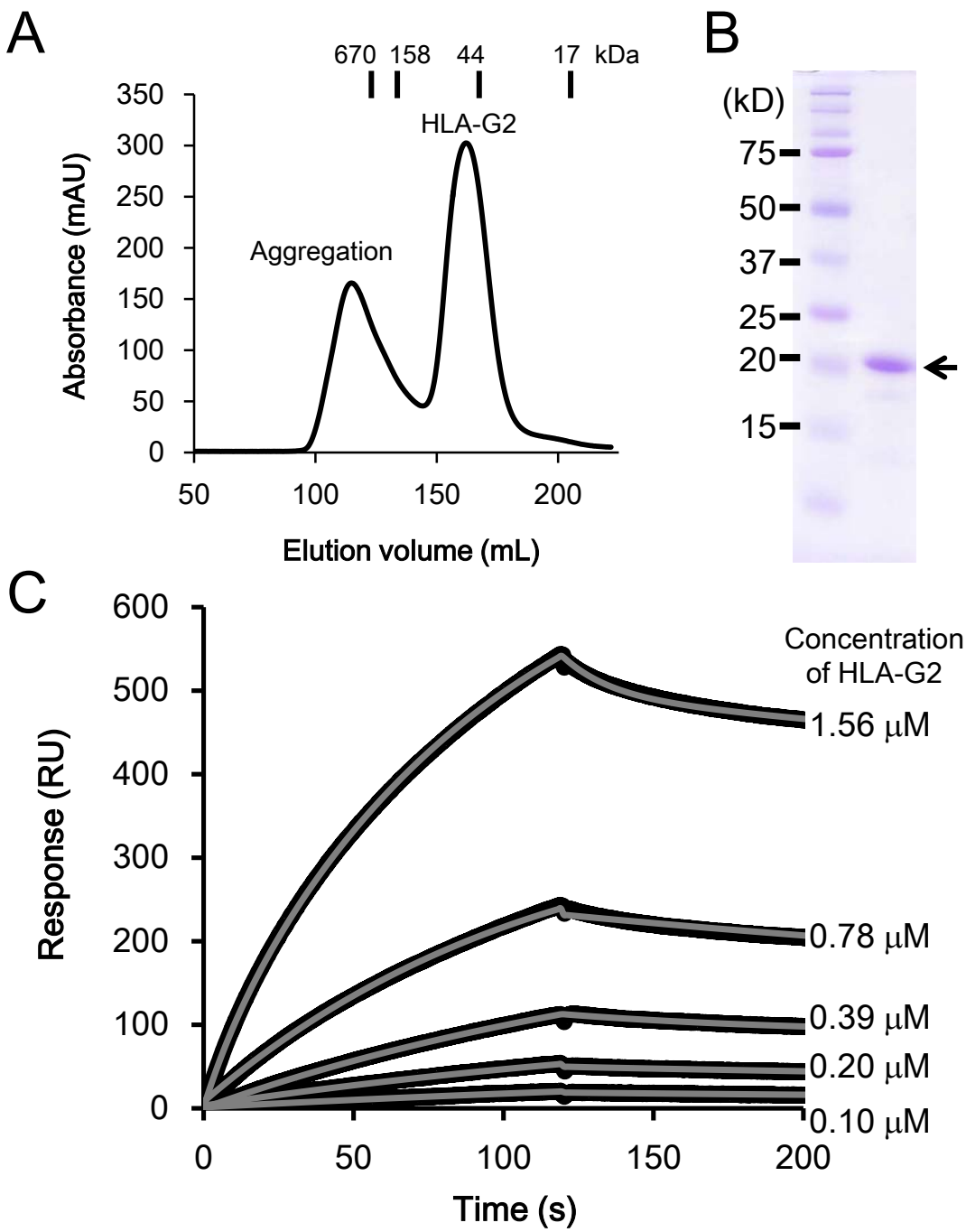
414 **Fig. 3.** The mean RA scores of each limb after treatment with high dose (1.4 μg, circles)

415 or low dose HLA-G2 (0.14 μg, triangles), or PBS alone (squares). These data are from

416 the same experiment as Fig. 2 (n = 12/group).

417

Fig. 1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670 158 44 17 kDa
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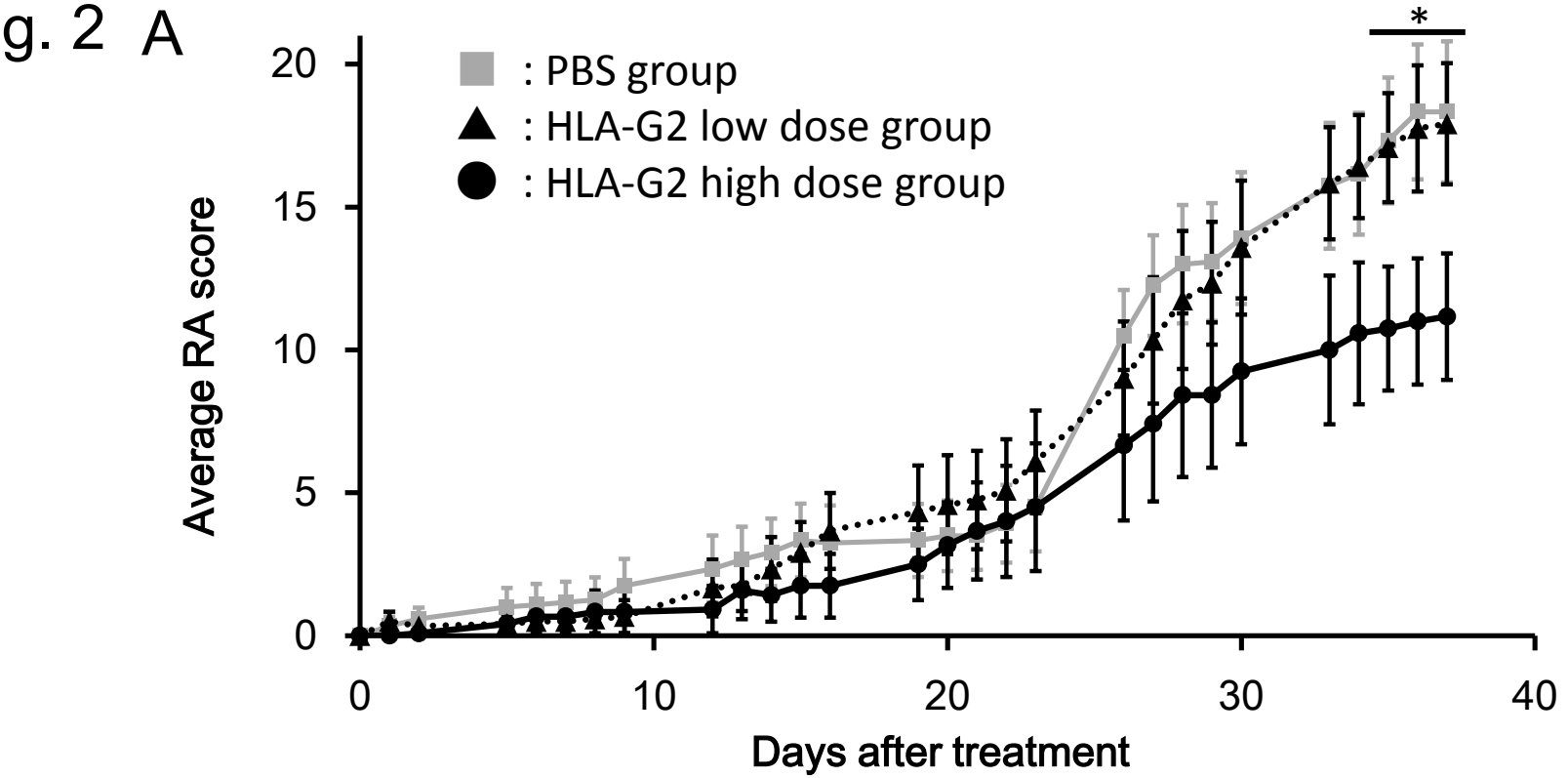
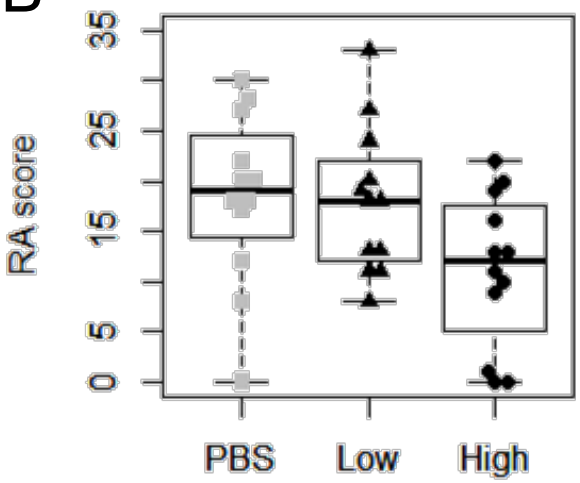
Concentration of HLA-G2
1.56 μM
0.78 μM
0.39 μM
0.20 μM
0.10 μM
Response (RU)
600
500
400
300
200
100
0
0
50
100
150
200
Time (s)

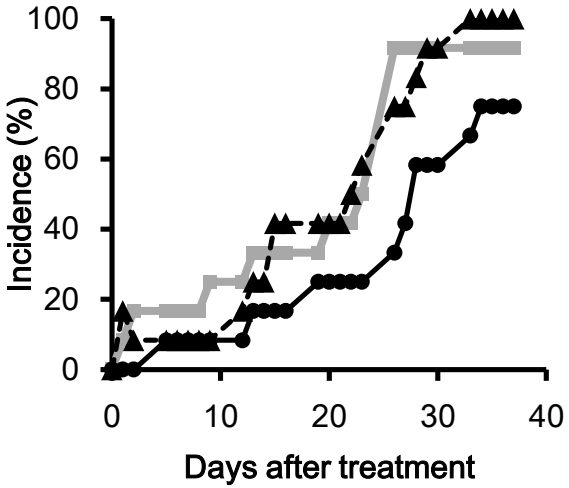
Fig. 2 A


: PBS group
: HLA-G2 low dose group
: HLA-G2 high dose group
Average RA score
Days after treatment
*

B


RA score
PBS
Low
High

C


Incidence (%)
Days after treatment

D

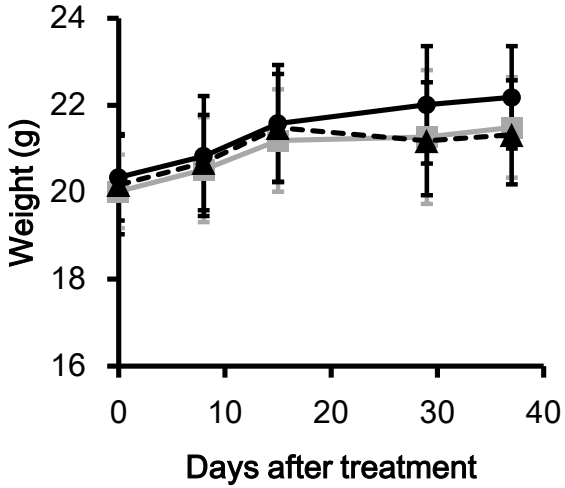
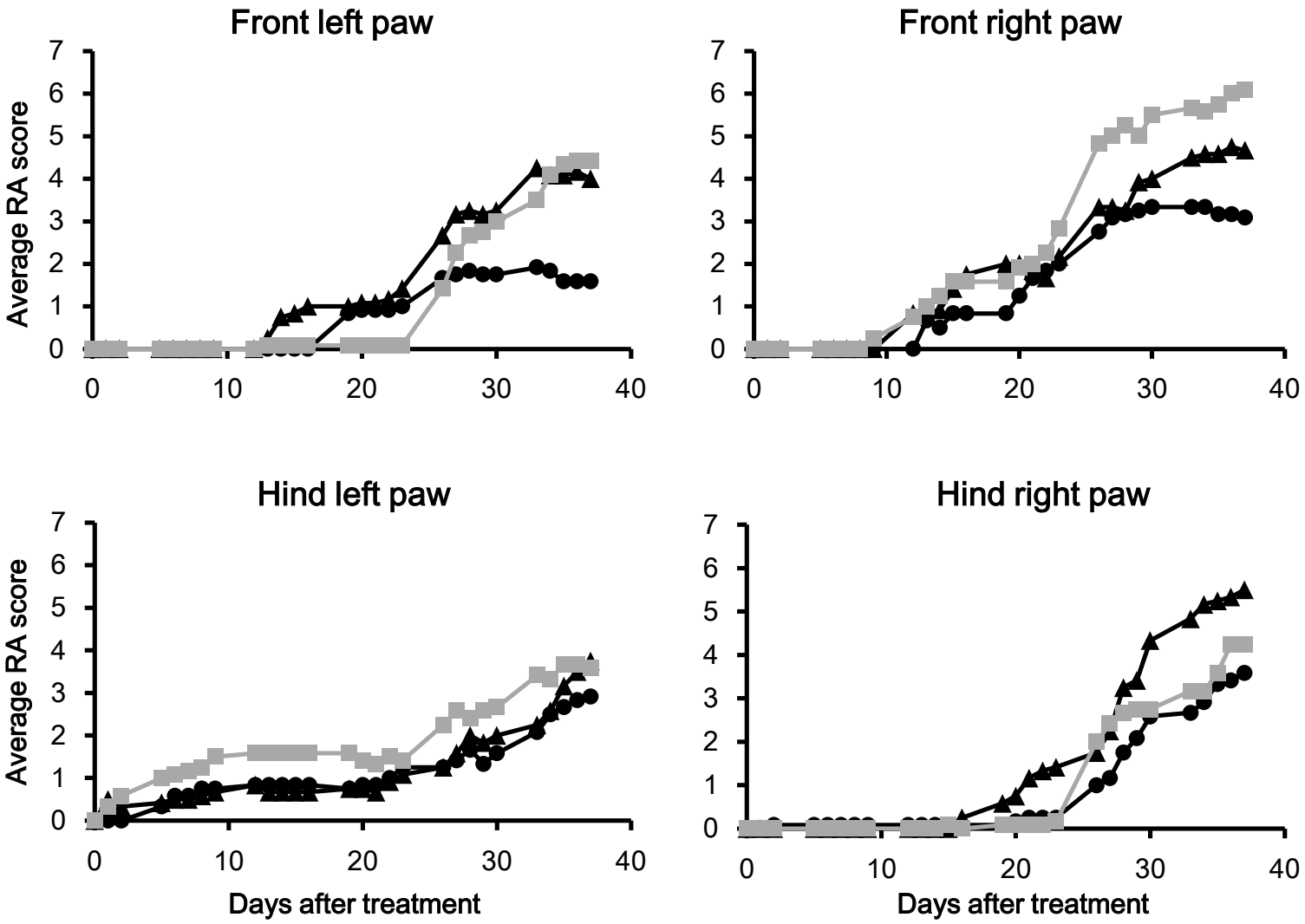

Weight (g)
Days after treatment

Fig. 3


Front left paw
Front right paw
Hind left paw
Hind right paw
Average RA score
Days after treatment
0
1
2
3
4
5
6
7
0
10
20
30
40