The long-term immunosuppressive effects of disulfide-linked HLA-G dimer in mice with collagen-induced arthritis

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Abstract

HLA-G, a natural immunosuppressant present in the human placenta during pregnancy, prevents fetal destruction by the maternal immune system. The immunosuppressive effect of HLA-G is mediated by the immune cell inhibitory receptors, LILRB1 and LILRB2. HLA-G forms disulfide-linked dimers by natural oxidation, and the dimer associates with LILRB1/B2 much more strongly than the monomer. Furthermore, the dimer formation remarkably enhanced the LILRB-mediated signaling. In this report, we studied the *in vivo* immunosuppressive effect of the HLA-G dimer, using the collagen-induced arthritis model mice. Mice were treated with the HLA-G monomer or dimer intracutaneously at the left foot joint, once or for 5 days, and the clinical severity was evaluated daily in a double-blind study. The HLA-G monomer and dimer both produced excellent anti-inflammatory effects with a single, local administration. Notably, as compared to the monomer, the dimer exhibited significant immunosuppressive effects at lower concentrations, which persisted for about two months. In accordance with this result, a binding study revealed that the HLA-G dimer binds PIR-B, the mouse homolog of the LILRBs, with higher affinity and avidity than the monomer. The HLA-G dimer is expected to be quite useful as an anti-rheumatoid arthritis agent, in small amounts with minimal side effects.

(199 words)

Abbreviations: Leukocyte immunoglobulin-like receptor (LILR), rheumatoid arthritis (RA), collagen-induced arthritis (CIA), Paired immunoglobulin-like receptor (PIR)

1. Introduction

HLA-G, a non-classical MHC class I (MHCI) molecule, is known as a natural immunosuppressant in the human placenta during pregnancy, in order for the fetus to evade the maternal immune responses. Several splice variants of HLA-G exist, including soluble and domain-deleted forms (HLA-G1-7), as well as a disulfide-linked dimer and a disulfide-linked β2m-free dimer *in vivo* [1]. HLA-G inhibits the cytotoxicity of NK cells and $CD8^+$ T cells, the allo-proliferation of $CD4^+$ T cells, and the maturation and function of dendritic cells [2], and also stimulates immunosuppressive regulatory T cells [3]. The reported receptors for HLA-G are leukocyte immunoglobulin-like receptor (LILR)B1 (also called as LIR1, ILT2, CD85j) and LILRB2 (LIR2, ILT4, CD85d) as well as killer-cell immunoglobulin-like receptor (KIR)2DL4. Among them, the accumulating evidence has indicated that the immunosuppressive effect of HLA-G is mediated by LILRB1 and LILRB2 on the maternal immune cells. While the expression of LILRB2 is restricted to antigen presenting cells, LILRB1 is more widely expressed on monocytes, dendritic cells, B cells, and subsets of natural killer (NK) and T cells. Thus, HLA-G plays a pivotal role in a broad range of immune suppression *in vivo*.

Clinically, HLA-G dysfunction causes pregnancy disorders, such as recurrent miscarriages and preeclampsia [4]. The pathological relevance of HLA-G was also found in transplantation, infectious diseases, autoimmune diseases, inflammatory diseases and cancers [5]. For example, in patients with rheumatoid arthritis (RA), asthma, or multiple sclerosis, the soluble HLA-G plasma levels and the HLA-G expression in monocytes are reduced [5]. The HLA-G molecule has been proposed as a new reagent, in combination with currently used immunosuppressive drugs, to improve allograft acceptance [6]. The genetically engineered form of HLA-G has been patented (EP1328290A2), and thus HLA-G could be administered to women with fertility problems. The administration of HLA-G negatively regulated the LILRB1-mediated

immune response, to suppress allo-immune responses in LILRB1 transgenic mice [7].

In a previous study, we found that a disulfide-linked HLA-G dimer mediates much more efficient signals than HLA-G monomers by binding to LILRB1 and LILRB2, thus enabling one HLA-G dimer to bind two receptors simultaneously [8]. Dimerization positions the intracellular domains of the receptors close to each other, thus supporting highly efficient signaling. Therefore, the recombinant HLA-G dimer is expected to function as a potent anti-inflammatory reagent.

We have assessed the immunosuppressive effects of the HLA-G monomer and dimer, using collagen-induced arthritis (CIA) in DBA/1 mice, a typical animal model for studying the pathogenic mechanisms of RA. We found that the HLA-G monomer and dimer both exhibited excellent anti-inflammatory activity, with the dimer displaying much more significant anti-rheumatoid arthritis activity than the monomer, without apparent side effects. These effects were mediated through the mouse LILRB homologue, a paired immunoglobulin-like receptor (PIR)-B that binds to HLA-G, as previously reported [9, 10]. The present *in vitro* binding study revealed that the dimer shows a remarkable avidity effect for PIR-B binding, resulting in much higher affinity than the monomer, which ensures the strong anti-RA activity. From these findings, the HLA-G monomer or dimer might be useful as a new immunosuppressive agent, with small amounts resulting in long-term, persistent effects.

2. Materials and methods

2.1.Expression and purification of the HLA-G and PIR-B proteins

Site-directed mutagenesis of HLA-G was performed by PCR, using the forward primer HLA-GF (5′-ATGGGTAGTCATAGTATGCGTTAT-3′) and the reverse primer HLA-GR (5′-ATAACGCATACTATGACTACC-3′), which introduced nonsynonymous substitutions to improve expression efficiency. Recombinant HLA-G heavy chain and β2-microglobulin (β2m) were produced in inclusion bodies, using *Escherichia coli*

strain BL21(DE3)pLysS cells (Merck Millipore). The wild-type and C42S mutant HLA-G heavy chains were used for the preparation of the HLA-G dimer and monomer, respectively. The HLA-G heavy chain, β2m and a peptide (RIIPRHLQL) were refolded together and purified by chromatography on gel filtration (Superdex75 26/60, GE) and anion exchange columns (Resource Q, GE) as the monomer form. For the formation of the disulfide-bonded HLA-G dimer, the purified wild-type HLA-G was concentrated to 10 mg/mL with 5 mM dithiothreitol (DTT) and incubated for 4 days at 4 °C. The HLA-G dimer was purified by gel filtration chromatography (Superdex 200 10/300, GE) (Figure 1A, B). Endotoxins were removed by passage through a Detoxigel column (Pierce Biochemicals).

For the surface plasmon resonance analysis, the extracellular domain of PIR-B was expressed by HEK293T cells, using polyethyleneimine for transient expression. The supernatants were collected 72 h after transfection, and the His^6-tagged PIR-B protein was purified by chromatography on a Ni^{2+}-NTA affinity column (HisTrap FF, GE) and by gel filtration (Superdex 200 10/300, GE). The purified PIR-B protein was biotinylated with the BirA enzyme, and the buffer was replaced with HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) by gel filtration (Superdex 200 10/300, GE).

2.2.Mice

The male DBA/1J mice (6 weeks old) used in this study were purchased from Japan Charles River Breeding Laboratories (Yokohama, Japan), and were housed under specific pathogen-free conditions. All animal experiments were performed with the approvals of the Animal Care and Use Committee of Kyushu University and the Committee for Animal Research at Hokkaido University.

2.3.Induction of CIA

The bovine type-II collagen was dissolved in buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 8.0) at a concentration of 4 mg/ml, by stirring overnight at 4°C. The bovine type-II collagen was emulsified with an equal amount of Complete Freund's Adjuvant, for initial sensitization. For additional sensitization, the bovine type-II collagen was emulsified with an equal amount of Incomplete Freund's Adjuvant. On day 1, mice were injected intradermally at the base of the tail with 50 μl of the emulsion, containing 100 μg of bovine type-II collagen. On day 14, mice were injected intradermally at the base of the tail with 50 μl of the emulsion, containing 200 μg of bovine type-II collagen.

2.4.Treatment of CIA

The clinical severity of the mice was evaluated daily, after additional sensitization. The RA scores were judged according to the criteria based on the evidence of arthritis at the large joint of the limbs (0: no swelling, 3: detectable swelling, 4: non-severe swelling, 5: severe swelling), and at the small joints of the fingers (1 point for each finger). The maximum score per limb was 10, and that per mouse was 40. The body weight was measured daily, to consider the side effects. The treatment of the clinical score was evaluated daily, in a double-blind study.

2.5.Treatment with HLA-G proteins

In each experiment, 50 mice are sensitized with type-II collagen and mice which developed initial arthritis symptoms were selected to analyze. They were treated intracutaneously with the HLA-G monomer or dimer in PBS, at the left foot joint, either once or daily for 5 days. PBS was used as a negative control.

For sequential administration of HLA-G monomer, 20 mice (RA score; 3 to 9) were analyzed and for single administration of three doses of HLA-G monomer/dimer, 14 mice (RA score; 3) were analyzed.

2.6.Statistical analysis

The data are presented as the mean ± SD in Figure 2. Statistical analyses were done using StatView version 5.0 for Windows (Abacus Concepts Inc., Berkeley, CA, USA). $P < 0.05$ was considered to be statistically significant.

2.7.Surface Plasmon Resonance (SPR).

SPR experiments were performed with a BIAcore3000 (GE). The biotinylated PIR-B protein and BSA (control) were immobilized on research-grade CM5 chips (GE), onto which streptavidin had been covalently coupled. The HLA-G monomer or dimer proteins in HBS-EP were injected over the immobilized PIR-B protein. The binding response at each concentration was calculated by subtracting the equilibrium response, measured in the control (BSA) flow cell, from the response in each sample flow cell. Kinetic constants were derived by using the curve-fitting function of the Biaevaluation 4.1.1 software (GE), to fit the rate equations derived from the simple 1:1 Langmuir binding model ($A + B \leftrightarrow AB$) or the bivalent model.

3. Results

3.1.Effects of HLA-G monomer on the CIA mice

A preliminary study using the antigen-induced dermatitis mice showed that swabs of 9μg of HLA-G monomer for 21 days exerted some anti-inflammatory effect (data not shown). In the present study, we administered the larger amount of HLA-G monomer (35μg) a day to mice for five days with expectations for more immediate and apparent immunosuppressive effects. The clinical arthritis score was evaluated in a double-blind study. At 13 days after the additional sensitization, 20 mice (RA scores: 3-9) were selected and grouped into the control (PBS) and HLA-G monomer groups (10 mice each, RA score average: 5.1 in each group). The mice were treated with the HLA-G

monomer or PBS once a day for 5 days. As shown in Figure 2A, the treatment with the HLA-G monomer inhibited the joint swelling in the mice for several days. Interestingly, the effect was observed for all limbs, despite the local administration at the left foot joint (Figure 2B).

3.2.The HLA-G dimer exerted stronger effects in the CIA mice

Next, we studied the *in vivo* anti-inflammatory effect of HLA-G dimer formation. As shown in Figure 2, the inhibition of RA symptoms was clearly observed after the sequential treatment with the 35μg/day of HLA-G monomer. We could expect that the HLA-G dimer possesses stronger effects from the in vitro study [8]. Therefore, we tried single local injections of HLA-G proteins, and tested the optimal dosage (150 μg, 15 μg, 1.5 μg per mouse).

In this comparison, the mice (7 individuals) that had an RA score of 3 at 13 days after the additional sensitization were classified as the "early onset group", and the mice (7 individuals) that had an RA score of 3 or 4 at 28 days after the additional sensitization were classified as the "late onset group". The following experiments were performed for these two groups.

In the early onset group, treatments with 150 μg of HLA-G monomer and dimer strongly exhibited the anti-RA effect for about two months (Figure 3A). As for the 15 μg treatment, the HLA-G dimer maintained a clear anti-rheumatoid arthritis effect comparable to that of 150 μg of dimer; however, the HLA-G monomer showed only a modest effect with a shorter duration than the dimer (Figure 3B). In the mice treated with 1.5 μg, neither the monomeric nor dimeric HLA-G showed any anti-RA symptoms (Figure 3C). The single administration of HLA-G sufficiently suppressed joint swelling, at a single dose of 15 μg for the dimer and 150 μg for the monomer in the early onset group, and these effects lasted for more than one month.

In the late onset group, the effective HLA-G dose for suppressing joint swelling was

less than that for the early onset group, which might possess a lower threshold of disease susceptibility. The HLA-G monomer and dimer suppressed joint swelling more effectively than the control (PBS) at doses of 150 μg/mouse and 15 μg/mouse (Figure 4A, B). The HLA-G dimer also had a joint swelling suppressing effect at a dose of 1.5 μg/mouse, whereas the HLA-G monomer did not show any suppressive effect at the same dose, as evidenced by the presence of joint swelling similar to that in the control (Figure 4C). These results thus demonstrated that the single administration of HLA-G can sufficiently suppress joint swelling, at a single dose of 1.5 μg for the dimer and 15 μg for the monomer in the rheumatoid arthritis late onset group, and these effects lasted for at least 50 days.

We showed that the HLA-G dimer provided a longer effect than the monomer, with a single HLA-G dose at concentrations at least several-fold lower than that of the monomer. The experiments also confirmed that the local intracutaneous administration of the HLA-G proteins to the left foot could suppress joint swelling in all four limbs (data not shown). Furthermore, the HLA-G monomer and dimer produced no side effects attributed to the HLA-G administration, as assessed by the common conditions and the body weights of the mice (data not shown). From these results, the single administration of HLA-G monomer and dimer at the appropriate time seemed to be enough to inhibit the initial symptoms of inflammation. These immunosuppressive effects were confirmed by multiple experiments when the HLA-G was administered at an early stage of RA score. However, when we administered the HLA-G proteins to more severe CIA mice, the clear immunosuppressive effects had not been observed (data not shown).

3.3.The HLA-G dimer binds to PIR-B with an avidity effect

The binding of the HLA-G dimer to PIR-B was characterized by surface plasmon resonance (SPR). First, the HLA-G dimer was refolded as described in Materials and

Methods. The extracellular domain of PIR-B was expressed as a secreted form by transient expression of HEK293T cells. The HLA-G dimer or monomer was injected over sensor chip surfaces, on which biotinylated PIR-B or BSA (as a negative control) had been immobilized. The binding curves of the HLA-G monomer and dimer to PIR-B are shown in Figure 1C and D, respectively. The sensorgram of the HLA-G monomer showed a weak interaction with a fast association/dissociation rate (Figure 1C). The estimated K_d value was more than the μM order, as assessed by an equilibrium-binding analysis (data not shown). Matsushita *et al*. showed that the PIR-B-Fc fusion protein weakly binds to the HLA-G monomer (K_d = 2.3 μM) [10]. This affinity was derived from the avidity effect of the PIR-B dimerization. We found that the interaction between the HLA-G monomer and the PIR-B monomer was fast and weak (Figure 1C).

On the other hand, the HLA-G dimer showed an apparent avidity effect for PIR-B binding. Kinetic analyses of HLA-G dimer binding to the immobilized PIR-B revealed global fitting with the bivalent model (Figure 1D). The apparent K_d of the dimer for PIR-B was ~21 nM, and the dissociation rate was very slow (2.7×10^{-5} s-1), as determined by local fitting with the simple 1 : 1 (Langmuir) binding model. In comparison with the weak affinity of the HLA-G monomer, this binding feature was derived from bivalent binding, which was shown in the binding of the HLA-G dimer to LILRB1 [8]. Therefore, the binding of the HLA-G dimer to PIR-B clearly demonstrated the avidity effect, resulting in much higher overall affinity.

4. Discussion

RA is a chronic autoimmune inflammatory disease, affecting approximately 0.2 - 1% of the general population. As therapeutic agents for RA, anti-inflammatory drugs of the corticosteroid family, such as prednisone; cytotoxic drugs, such as azathioprine and cyclophosphamide; and fungal and bacterial derivatives, such as cyclosporin A and tacrolimus (FK506) have been used. Although these drugs are effective, their side

effects are significant, thus complicating the therapeutic approaches toward RA. The recent development of biologic agents that selectively block inflammatory cytokines (mainly TNF-targeting), such as etanercept, infliximab and adalimumab, represents a major advancement in therapeutic options. However, these reagents occasionally cause an anaphylactic reaction, as well as the production of anti-antibody antibodies and infections related to immunosuppression. Importantly, the anti-antibody antibodies neutralize the reagent, and thus repeated administrations will become less effective.

In this study, we showed that the recombinant HLA-G protein exhibited anti-inflammatory effects in CIA mice. PIR-B, the mouse orthologue of LILRB, is expressed in the myeloid cell lineage in mice and functions as an HLA-G receptor, in a similar manner to LILRB2 in humans. The recombinant HLA-G inhibited the maturation of murine dendritic cells *in vitro*, similar to the phenotype in HLA-G transgenic mice through the interaction with PIR-B [9]. *In vivo*, the HLA-G tetramer triggered the prolongation of allogenic graft survival, due to its immunosuppressive functions [9]. Our data revealed that the local administration of HLA-G proteins can reduce joint swelling throughout the four limbs, and the anti-RA activity persists for a relatively long time, even with a single, local intracutaneous administration at the left foot. In CIA mice, recombinant HLA-Gs would inhibit the DC maturation and the ability of antigen presentation might be down-regulated. The suppression of antigen presentation would lead to inhibition of systemic immune responses. The mechanism why the role of HLA-G could be sustained had not been clarified in this study, the inhibitin of DC maturation would affect a wide range of immune responses, thus immune tolerance could be prolonged. As expected, the HLA-G dimer exhibits stronger immunosuppressive effects than the monomer (Figures 3 and 4). We previously reported that the signal inhibition ability of the HLA-G dimer through LILRB1 was about 100-fold higher than that of the HLA-G monomer [8]. As for PIR-B signaling, a single administration sufficiently suppressed RA symptoms at doses of 15 μg for the dimer and

150 μg for the monomer in the RA early onset group (Figure 3), and 1.5 μg for the dimer and 15 μg for the monomer in the RA late onset group (Figure 4). Thus, the HLA-G dimer can be considered to exert about 10-fold stronger immunosuppression of PIR-B signaling, as compared to the monomer. These results also suggested that the appropriate doses of HLA-G for anti-RA effects are different for the early onset group, which possesses higher disease susceptibility, and for the late onset group, with lower disease susceptibility. When the HLA-G dimer is administered *in vivo*, the effective dose will differ depending on various factors, such as the symptoms of RA, age, sex, body weight, etc.

We have shown that the HLA-G dimer interacts with the extracellular domain of PIR-B, and exhibits much higher affinity than the monomer and an avidity effect (Figure 1C, D). This avidity effect is similar to those of the LILRBs. PIR-B has two additional Ig-like domains on its C-terminal side, resulting in six Ig-like domains (designated as D1-D6) in the extracellular domain, while the LILRBs have only four Ig-like domains. PIR-B D1D2 is reportedly the major MHCI binding domain, and D3-D6 supports MHCI (H-2 and HLA-G) binding by D1D2 [10]. Although PIR-B may be more flexible than the LILRBs, D1D2 adopts a suitable domain orientation for MHCI recognition. Our data suggested that HLA-G dimerization generates two PIR-B binding sites. The HLA-G dimer would bind to two PIR-B molecules with proper orientations and no structural hindrance or loss of interaction area, and with locations close enough to assemble on the cell surface for appropriate signaling, as shown for the interaction between the HLA-G dimer and LILRBs for sufficient signaling [8].

Based on these results, the HLA-G dimer is useful as an anti-inflammatory reagent that exerts persistent effects with small amounts. The HLA-G dimer is formed through an intermolecular disulfide bond that links Cys42 of the HLA-G heavy chain by natural oxidation [8], and thus it is a component naturally present *in vivo*. In the placenta, soluble HLA-Gs produced by either secretion or proteolytic shedding from the fetal cell

surface can suppress maternal immune cells effectively, through LILRBs. Thus HLA-G acts specifically, and has a distinct site of action for immune suppression, as compared to the aforementioned drugs. Therefore, the HLA-G dimer agent is expected to have fewer side effects and a better safety profile. To elucidate the immunosurpressive effects of HLA-G in RA, we tried to examine the proinflammatory (TNF-a, IL-1b, IL-6) and anti-inflammatory cytokine (IL-10, IL-4) levels in mice (data not shown). We did not fond significant difference of tipical cytokine levels between HLA-G and PBS-administered mice on 60 days after administration. In future, we should archive a change of inflammation- related cytokine levels with time.

In many immune disorders, such as autoimmune disease, transplant rejection, and allergy, powerful immunosuppressive drugs are currently used. The use of the HLA-G dimer will open up new pathways for the treatment of infertility, such as pre-eclampsia, as well as transplantation management and inflammatory and autoimmune disease treatments. It may be possible to reduce the amount of immunosuppressive drugs and minimize the side effects of these drugs by using HLA-G and immunosuppressive or biological drugs together. Therefore, the HLA-G monomer and dimer might be novel useful protein agents for medical use, in the prevention or treatment of RA. A relief from the burden on the patient's body would be expected by the combinatorial use of HLA-G with immunosuppressive drugs.

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Figure legends

Figure 1 Purification of soluble HLA-Gs and surface plasmon resonance analyses.

(A) Gel filtration chromatogram of the HLA-G wild type molecule on a Superdex 200 10/300 column after the formation of dimer. The dimer fraction was used as HLA-G dimer.

(B) SDS-PAGE of the HLA-G monomer and dimer under non-reducing (lanes 1, 2) and reducing (lanes 3, 4) conditions.

(C) Binding of the HLA-G monomer to PIR-B. The HLA-G monomer (90 μM) was injected for 60 sec through flow cells bound with control (BSA, dotted line) and PIR-B (solid line) proteins. Representative data are shown.

(D) Kinetic analyses of the HLA-G dimer with the immobilized PIR-B. The HLA-G dimer was injected at the indicated concentrations. Response curves (black lines) were fitted locally with the bivalent analyte model (grey line).

Figure 2 The changes in the RA scores of HLA-G monomer-treated mice.

The mice were continuously administrated HLA-G monomer (triangles) or PBS (circles) for 5 days (day 1-5), and the RA score was evaluated in a double-blind study.

The average ± SD values of the total RA scores of four limbs (A) and of each limb (B) are shown.

Figure 3 The changes in the RA scores of the early onset group.

Representative data of the changes in the RA scores of the early onset group with time, after single administrations of HLA-G monomer (triangles), dimer (circles) and PBS (squares). Each symbol means a mouse administered and the gap in the RA score is due to a vacation of staff.

Figure 4 The changes in the RA scores of the late onset group.

Representative data of the changes in the RA scores of the late onset group with time, after single administrations of HLA-G monomer (triangles), dimer (circles) and PBS (squares). Each symbol means a mouse administered and the gap in the RA score is due to a vacation of staff.

Figure 1. Kuroki et al.

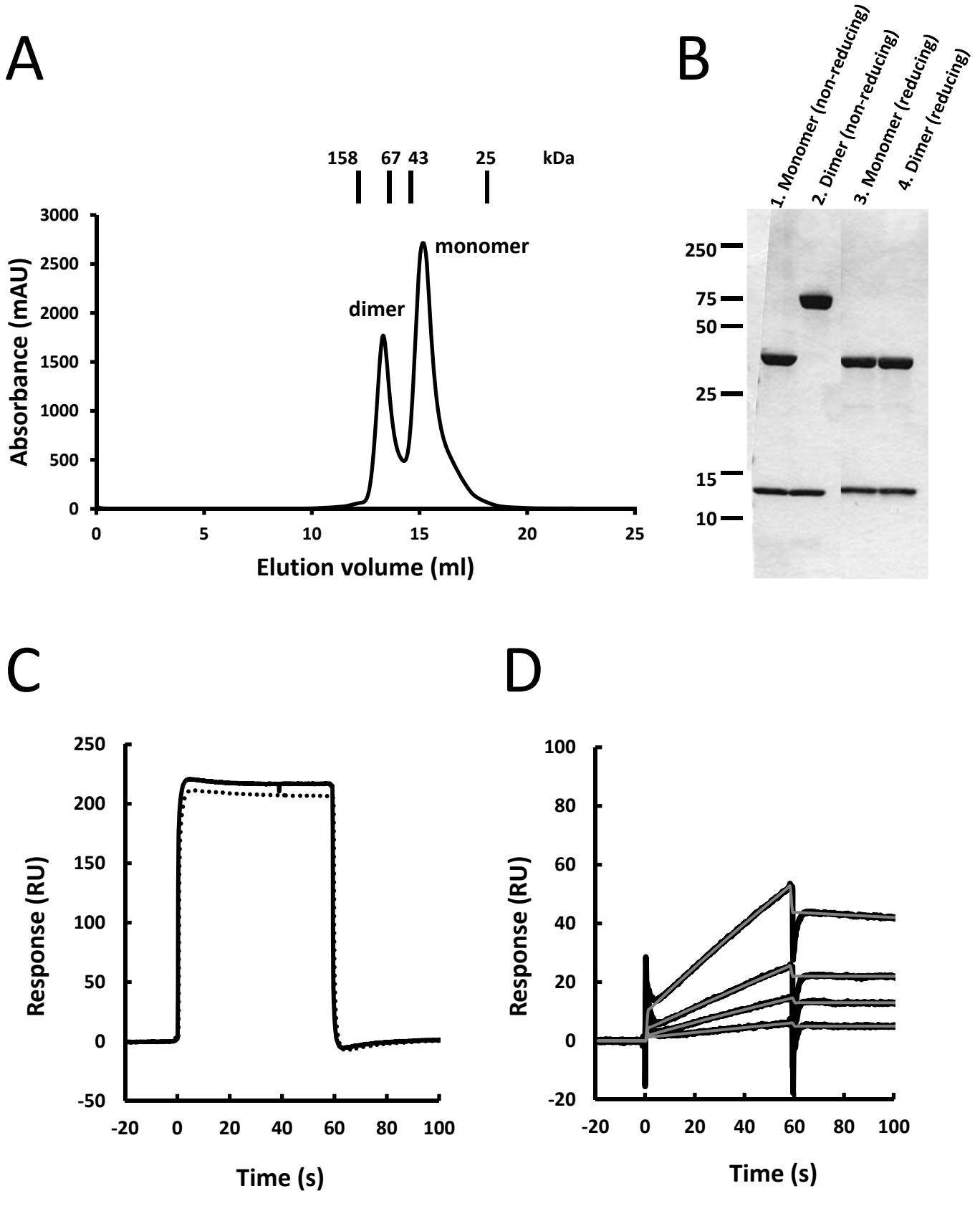

Figure 2. Kuroki et al.

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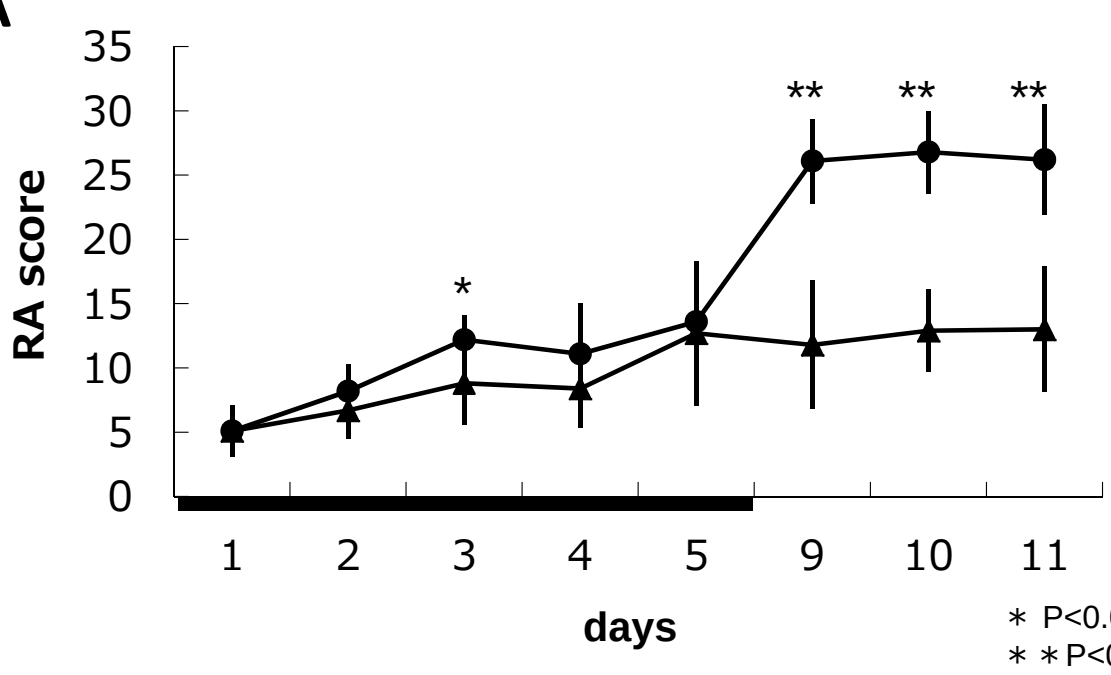

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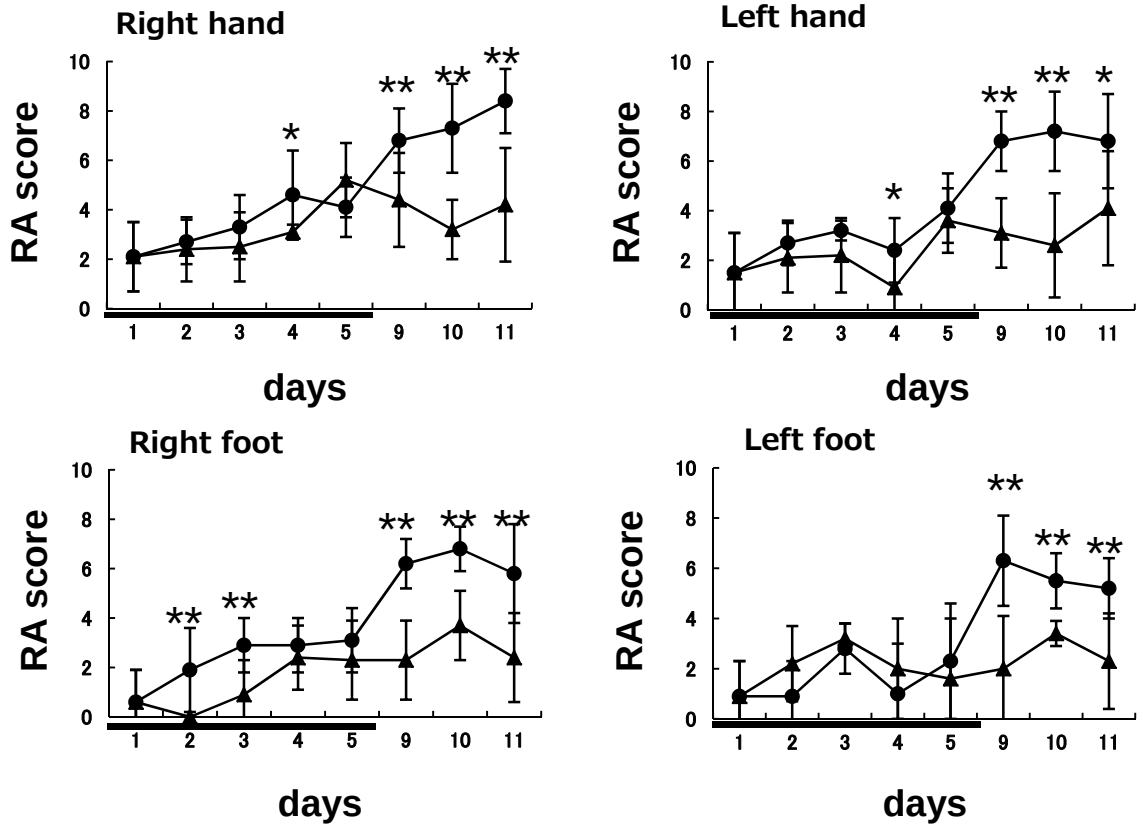

Figure 3. Kuroki et al.

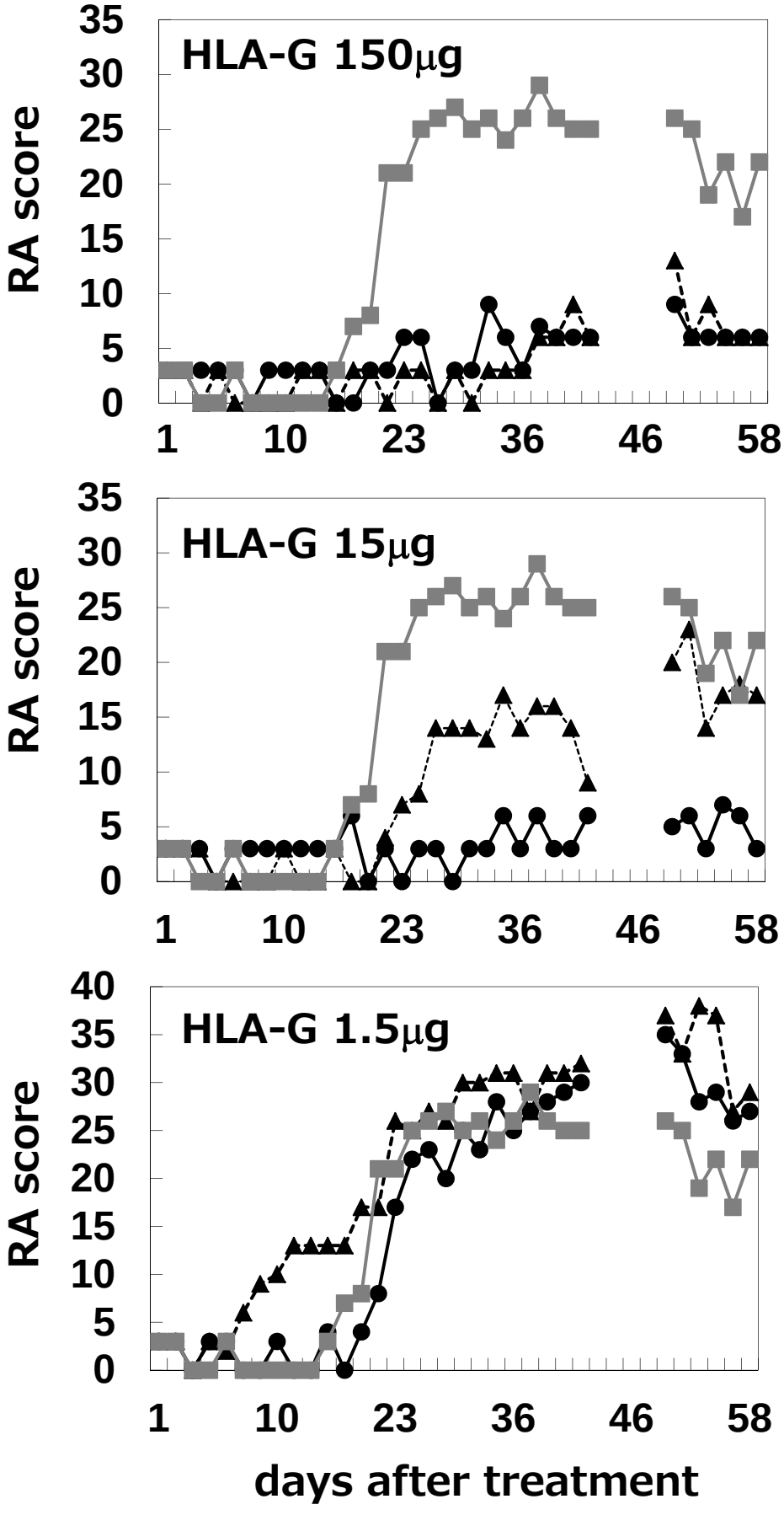

Figure 4. Kuroki et al.