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Title: Therapeutic application of human leukocyte antigen-G1 improves atopic dermatitis-like skin lesions in mice

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22

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24

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Abstract

Human leukocyte antigen (HLA)-G is an immune checkpoint molecule that plays critical roles in immune response and in triggering inhibitory signaling to immune cells such as T cells, natural killer cells, and antigen-presenting cells. Thus, the application of HLA-G can be considered for treating immune response-related inflammatory disorders. We have previously reported that treatment with HLA-G1 and HLA-G2 ameliorates the joint swelling associated with collagen-induced arthritis of DBA/1 mice, an animal model for rheumatoid arthritis. In this study, we further investigated the effects of HLA-G1 on atopic dermatitis (AD), the most common inflammatory skin disorder. AD-like lesions were induced with the extract of the house dust mite *Dermatophagoides farinae* in NC/Nga mice. Continuous administration of HLA-G1 ameliorated the AD-like skin lesions in the mice. Furthermore, production of immunoglobulin E, interleukin (IL)-13, and IL-17A was significantly reduced in HLA-G1-treated mice, suggesting a Th2/Th17-mediated immune-inhibitory function of HLA-G1 *in vivo*. Our studies shed light on novel therapeutic strategies with recombinant HLA-G proteins for immune reaction-mediated chronic inflammatory disorders.

1. Introduction

Human leukocyte antigen (HLA)-G is a non-classical HLA class I molecule [1]. It is well known that HLA-G consists of 7 spliced isoforms; HLA-G1 to G4 are membrane-bound forms and HLA-G5 to G7 are soluble forms [2]. The structure of HLA class I consists of a heavy chain with α1, α2, and α3 domains; beta-2 microglobulin (β2m); and a processed peptide to be presented [3]. HLA-G expression is restricted to the fetal–maternal interface, but accumulated evidence has shown its expression in the pancreas, thymus, and cornea [4]. Thus far, several cell surface molecules have been reported to function as HLA-G receptors. The leukocyte immunoglobulin (Ig)-like receptor (LILR)B1 (also known as Ig-like transcript 2 (ILT2) or CD85j), ubiquitously expressed on a variety of immune cells, and LILRB2 (also known as ILT4 or CD85d), expressed on monocytic cell lineages, function as inhibitory receptors [3].

HLA-G has been reported to be involved in a variety of diseases [5]. Membrane-bound HLA-G preferably interacts with its inhibitory receptors to suppress signaling [6]. On the other hand, the soluble form of HLA-G (sHLA-G) has been reported to be involved in patients with rheumatoid arthritis (RA) [7,8] and atopic dermatitis (AD) [9], indicating its utility as an important biomarker of these diseases [10]. There are several roles of HLA-G in immune responses *in vivo*: HLA-G inhibits the proliferation of T cells

and B cells [11], Ig secretion from activated B cells [12], and the induction of cytotoxicity by NK cells and cytotoxic T lymphocytes via interaction with LILRB1 [13]. In addition, HLA-G impairs dendritic cell (DC) maturation and antigen presentation to T cells by interacting with LILRB2 [14]. Therefore, HLA-G is currently thought to possess immunosuppressive properties and exert a tolerogenic (immune tolerance) status in immune reaction-based diseases and infections.

On the basis of these established clinical and immunological data, we hypothesized that the suppression of immune responses by using HLA-G proteins might be applicable to address chronic inflammatory disorders. To investigate the effects of the HLA-G1 monomer on AD *in vivo*, we performed experiments using *Dermatophagoides farinae* extract-induced AD model in NC/Nga mice [15,16]. It is noteworthy that clinical and immunological symptoms in *Dermatophagoides farinae* body (Dfb)-induced AD mice are quite similar to those in human AD, characterized by pruritic rash, scaling, and excoriation [17]. Here, we present evidence that HLA-G1 monomer exhibits the therapeutic effects on AD.

2. Materials and methods

2.1. Expression and purification of the recombinant HLA-G1 monomer

The recombinant human HLA-G heavy chain bearing the cysteine-to-serine mutation at 42 (C42S), which has 5 nonsynonymous substitutions to improve expression efficiency, was used for the preparation of the HLA-G monomer [18]. The HLA-G C42S heavy chain and β2m were produced in inclusion bodies using *Escherichia coli* strain BL21(DE3)pLysS competent cells (Merk Millipore, Darmstadt, Germany). The soluble HLA-G monomer was refolded from inclusion bodies and a peptide (RIIPRHLQL), and purified by chromatography on gel filtration (Superdex75 26/60, GE Healthcare, Chicago, IL, USA) and anion exchange columns (Resource Q, GE Healthcare, Chicago, IL, USA) as described previously [18,19] (Fig. 1A and B). The purified HLA-G1 monomer was replaced with phosphate-buffered saline (PBS) by dialysis, and endotoxins were removed by passaging them through the Detoxi-Gel endotoxin removing column (Thermo Fisher Scientific, Waltham, MA, USA). Eight micrograms of purified proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing and non-reducing conditions using a 15% acrylamide gel followed by Coomassie Brilliant Blue staining (Fig. 1C). Before loading on the gel, each sample was mixed with loading buffer (25 mM Tris-HCl (pH6.5), 5% glycerol, 1% SDS, and 0.05% bromophenol blue) with or without reducing agent 1% β-mercaptoethanol, and boiled at 95 °C for 5 min.

99

100 *2.2. Mice*

101 Ten-week-old female NC/Nga mice were purchased from Japan SLC (Shizuoka, Japan)
102 and maintained under specific pathogen-free conditions. All experiments were approved
103 and performed in accordance with the guidelines of the Committee of Ethics on Animal
104 Experiments in Hokkaido University.

105

106 *2.3. Induction of dermatitis*

107 Dfb ointment was obtained from Biostir Inc. (Kobe, Japan) [15,16]. The postauricular
108 region of the skin of mice (n=4) was clipped using an electric clipper, and residual hair
109 was depilated using a hair removal cream. Hundred milligrams of the Dfb ointment was
110 applied on the shaved skin and the surface of both ears. From the second induction, 4%
111 SDS treatment to disrupt the mouse skin barrier was performed on the shaved skin and
112 the surface of both ears before Dfb ointment treatment, and SDS/Dfb ointment treatments
113 were repeated once daily every 3 days for 15 days. The design of this study is summarized
114 in Fig. 2. Four mice without dermatitis being induced by Dfb ointment were used as the
115 control group.

116

2.4. Treatment of NC/Nga mice with the HLA-G1 monomer

After Dfb ointment treatment, both ears were treated with 15 μg or 5 μg of purified recombinant HLA-G1 monomer; this treatment was repeated once daily every other day for 20 days (Fig. 2). PBS was used as a negative control. The ear thickness was evaluated using Dial Thickness Gauges (OZAKI MFG. Co., Ltd., Tokyo, Japan). Body weight changes were monitored twice a week.

2.5. Measurement of immunoglobulin E and cytokines by enzyme-linked immunosorbent assay

Blood was collected from NC/Nga mice on day 18, and serum samples were obtained by centrifugation (4,300 *g* for 5 min.). The total immunoglobulin E (IgE) concentration in serum was determined using the mouse IgE EIA kit (Yamasa Corp., Chiba, Japan), according to the manufacturer's instructions. Axial lymph nodes (LN) of each group of NC/Nga mice were removed, and lymphocytes from the LN were prepared 18 days after the first HLA-G1 treatment and stimulated with 1 μg/ml of immobilized anti-CD3 antibody (clone 145-2C11) and 5 μg/ml of soluble anti-CD28 antibody (clone 37.51) (BD Biosciences, San Jose, CA, USA) for 72 h. Supernatants were collected, and interferon (IFN)-γ, interleukin (IL)-13, and IL-17A concentrations were measured using the mouse

IFN-γ Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA), mouse IL-13 DuoSet ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA), and mouse IL-17A ELISA Kit (RayBiotech, Inc., Norcross, GA, USA), respectively, according to the manufacturer's instructions.

2.6. Immunohistochemical staining of ears

Ears were excised from mice, fixed with 4 % paraformaldehyde, and embedded in paraffin for hematoxylin and eosin (H&E) staining.

2.7. Statistical analysis

Statistically significant differences were calculated using Student's *t*-test and are indicated as *P* values. Differences of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Preparation of the recombinant HLA-G1 monomer

In an earlier study, we established the expression and purification methods for recombinant HLA-G1 monomer protein using the C42S mutant of the HLA-G heavy chain [18]. The HLA-G C42S heavy chain and β2m were produced as inclusion bodies,

153 and soluble HLA-G monomer was refolded from the inclusion bodies, followed by
154 purification with chromatography on gel filtration (Fig. 1A) and anion exchange columns
155 (Fig. 1B). The levels of purified HLA-G1 monomer were confirmed by SDS-PAGE (Fig.
156 1C). To use the HLA-G1 proteins *in vivo*, we removed endotoxins in the protein samples
157 using their specific columns (data not shown).
158
159 *3.2. Treatment with the HLA-G1 monomer ameliorated AD-like lesions induced by*
160 *Dermatophagoides farinae extracts in mice*
161 Among several reported mouse models for AD [20], we chose the Dfb extract-induced
162 AD-like model using NC/Nga mice, since *Dermatophagoides farinae* is well known as a
163 causative allergen in AD [21]. To study the effects of the HLA-G1 monomer on Dfb
164 ointment-induced AD in NC/Nga mice, we treated the mice with the HLA-G1 monomer
165 at 15 μg or 5 μg (Fig. 2). HLA-G1 was applied after AD was induced with Dfb ointment
166 to exclude the possibility that direct binding of Dfb ointment to HLA-G1 protein resulted
167 in any inhibitory effects on AD. At a macroscopic level, hemorrhage, scarring, and
168 dryness of the skin in HLA-G1-treated mice were markedly better than those in PBS-
169 treated mice (Fig. 3). On the basis of the evaluation of ear thickness, we found that
170 lesional skin of both ears was ameliorated by HLA-G1 treatment in a dose-dependent

manner, while the control mice did not show any changes of ear thickness (Fig. 4A and B). Importantly, the HLA-G1 treatment did not cause overt toxicity, as reflected by a lack of effect on body weight (Fig. 4C). Histological examinations of skin lesions by H&E staining of ear tissues revealed ulcers, crusts, and thickening of the epidermis, as well as elevated infiltration of inflammatory lymphocytes and hyperplasia of fibroblasts in the dermis from Dfb-induced AD mice (Fig. 5). In contrast, we found that ears from mice treated with HLA-G1 showed a marked decrease in epidermal hyperplasia and infiltration of inflammatory cells in the dermis (Fig. 5). These results suggested that the HLA-G1 monomer has the potential to be used as a therapeutic agent for AD.

3.3. Serum IgE and cytokine levels were reduced in NC/Nga mice treated with the HLA-G1 monomer

Since elevated levels of serum IgE are typically characteristic of patients with AD caused by *Dermatophagoides farinae* [22], we measured serum IgE levels derived from Dfb-induced AD mice after HLA-G1 treatment. We found that total IgE levels in the Dfb-induced AD mice treated with 15 μg of HLA-G1 were significantly lower than those in PBS-treated mice (Fig. 6A). In Dfb-induced AD mice, IL-13 and IL-17A levels were elevated; in contrast, the levels of these cytokines were significantly reduced in HLA-G1-

189 treated NC/Nga mice (Fig. 6C and D). IFN-γ production was also elevated in Dfb-induced
190 AD mice, but in contrast to IL-13 and IL-17A levels, IFN-γ level was significantly
191 increased by HLA-G1 treatment (Fig. 6B). These results suggested that HLA-G1 may
192 contribute to the therapeutic effects against AD by suppressing the excess allergic reaction
193 *in vivo*.
194
195 **4. Discussion**
196 Recent studies have been exploring immune checkpoint molecules as therapeutic targets
197 for a variety of disorders, especially cancer blockade via programmed death-1 (PD-1)/PD-
198 ligand 1 (PD-L1) pathways [23]. HLA-G is also thought to be an immune checkpoint
199 molecule expressed in tumor cells for blocking immune effectors via LILRB1 and/or
200 LILRB2 [2]. In our previous studies focusing on immune reaction-mediated
201 inflammatory disorders, HLA-G1 and G2 exhibited immunosuppressive effects on
202 collagen-induced arthritis (CIA) in DBA/1 mice, which is a typical murine model of
203 human RA [18,19]. Mouse antigen-presenting cells (APCs) such as DCs and monocytes
204 express paired Ig-like receptor (PIR)-B, a murine LILRB homolog that binds to human
205 HLA-G [18,19,24-26]. Thus, the behavior of PIR-B is similar to that of human LILRB2
206 in mice. In our previous studies, both the HLA-G monomer and dimer [18] and the HLA-

207 G2 dimer [19] showed significant therapeutic effects on CIA. In this study, we further
208 found that the HLA-G1 monomer was also effective against Dfb extract-induced AD-like
209 skin lesions in NC/Nga mice (Figs. 3-5). In addition, the effect of the HLA-G1 monomer
210 on AD-like lesions induced by the extract of another typical house dust mite,
211 *Dermatophagoides pteronyssinus*, in NC/Nga mice was also observed (data not shown),
212 suggesting that HLA-G1 can be a molecular therapeutic drug for house dust mite-induced
213 AD, whereas effects on AD induced with other allergens (e.g., metals, chemicals, or
214 drugs) remain to be investigated.

215 The role of HLA-G in the pathogenesis of AD remains ambiguous [27,28]. In
216 skin lesions with progressing AD, dermal DCs—especially Langerhans cells, a
217 specialized type of skin DCs—infiltrate to be activated by allergens. The activated DCs
218 induce the differentiation and proliferation of T-helper (Th) 2 cells producing IL-4 and
219 IL-13, which are typical pathogenic factors in AD [29]. Thus, Th2 polarization is a
220 preferable environment for AD induction and progression. In fact, we found that IL-13
221 level was increased in Dfb-induced AD mice (Fig. 6C). Because we also found reduced
222 levels of IL-13 in HLA-G1-treated mice (Fig. 6C), we believe that the result may support
223 the possibility that it was caused by the inactivation of PIR-B-expressing APCs by HLA-
224 G1, resulting in the inhibition of Th2 differentiation. It is also well known that IL-4 and

IL-13 are important factors that induce and amplify IgE production in AD [29]; thus, reduced levels of IL-13 might result in the decrease in IgE production in HLA-G1-treated mice (Fig. 6A). Evidence from the current study revealed that the mechanism of AD pathogenesis is more complicated, and Th17 cell (producing IL-17) activation is implicated [30,31]. Interestingly, we found that the IL-17A level was decreased in HLA-G1-treated mice (Fig. 6D). Th17 development is regulated by cytokines, especially the Th1 cytokine IFN-γ, which inhibits Th17 development *in vivo*. Thus, elevated IFN-γ levels in HLA-G1-treated mice (Fig. 6B) would explain the suppressive mechanism of AD by HLA-G1 via downregulating Th17 differentiation, indicating that IL-17 plays a pathogenic role in AD mice. The involvement of immune cells and associated cytokine/chemokine production remains to be identified to elucidate the mechanism of HLA-G1-induced suppression of AD. For instance, we speculate that B-cell function could be directly suppressed by HLA-G1, since B cells express PIR-B [32,33]. Khosrotehrani *et al*. reported that a major source of HLA-G in AD was derived from T cells [34]. Therefore, the function of T cell-derived HLA-G1 is of interest for further investigation.

Although we identified the therapeutic effects of HLA-G1 on AD-like lesions, moving further, efficient delivery systems should be considered to enhance an efficient

243 percutaneous introduction of HLA-G1 for successful therapeutic application. Moreover,
244 the improvement of the stability of HLA-G1 is practically important for *in vivo* clinical
245 application. This study provides novel insights on the function of HLA-G1, which can
246 provide clues on efficient therapeutic strategies for patients with inflammatory disorders,
247 to augment Th1 activation and downregulate Th2/Th17 differentiation by applying HLA-
248 G at inflammatory regions. Further investigation is required and will be important to
249 prove its effectiveness and precise mechanism for countering AD to develop strategies
250 for the application of the HLA-G1 monomer for other inflammatory disorders in the near
251 future.
252
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263

264 **Conflict of Interest**

265 The authors declare no conflict of interest.

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Figure legends

Figure 1 Purification of the recombinant HLA-G1 monomer.

The HLA-G1 monomer was purified by using (A) gel filtration chromatography on a HiLoad26/60 Superdex 75 column, followed by (B) ion exchange chromatography on a Resource Q 1 mL column. The dotted line indicates the concentration of NaCl in the elution buffer. (C) SDS-PAGE analysis of the HLA-G monomer under reducing and non-reducing conditions.

Figure 2 Time line of experiments.

Figure 3 Photographs of AD-like skin lesions of NC/Nga mice with or without HLA-G1 treatment.

Macroscopic features of AD-like skin lesions in NC/Nga mice treated with 15 μg of the HLA-G1 monomer or PBS on day 18. Four mice without dermatitis being induced by Dfb ointment were used as the control group.

Figure 4 Effect of the HLA-G1 monomer on Dfb ointment-induced AD in NC/Nga mice.

AD was induced with Dfb ointment treatment 6 times. The mice were treated with (A) 15 μg or (B) 5 μg of the HLA-G1 monomer every other day, and the thickness of the left and right ear was monitored every 4 days. (C) Body weight changes in NC/Nga mice treated with 15 μg of the HLA-G1 monomer or PBS were monitored twice a week. Bars indicate mean values ± SEM (n=4). Statistically significant differences are shown as *p* values (**p* < 0.05, ***p* < 0.01).

Figure 5 Histological analysis of AD-like skin lesions in NC/Nga mice.

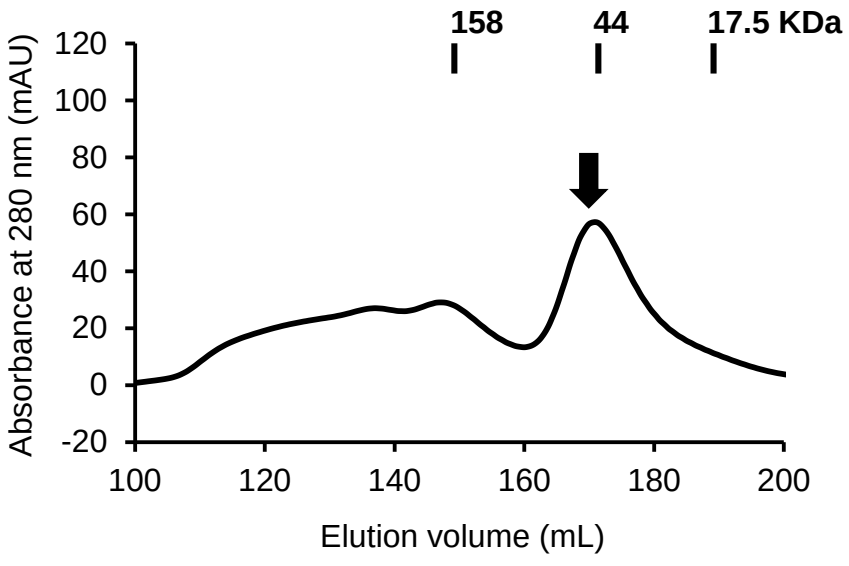
Excised ears of NC/Nga mice treated with 15 μg of the HLA-G1 monomer or PBS on day 18 were stained with H&E.

Figure 6 Serum levels of total IgE and cytokine levels from LN in NC/Nga mice.

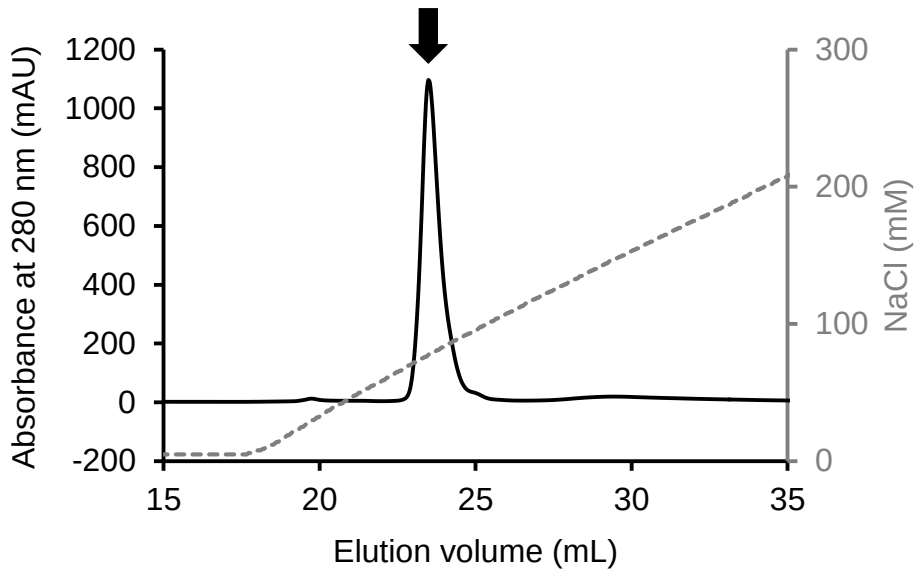
Serum levels of (A) IgE, and (B) IFN-γ, (C) IL-13, and (D) IL-17A levels in culture media from LN cells of NC/Nga mice treated with 15 μg of the HLA-G1 monomer or PBS were measured on day 18. Bars indicate mean values ± SEM (n=4). Statistically significant differences are shown as *p* values (**p* < 0.05, ***p* < 0.01). N.D., Not detected.

Figure 1

A


158
44
17.5 KDa
Absorbance at 280 nm (mAU)
120
100
80
60
40
20
0
-20
100
120
140
160
180
200
Elution volume (mL)

B


Absorbance at 280 nm (mAU)
1200
1000
800
600
400
200
0
-200
15
20
25
30
35
Elution volume (mL)
300
200
100
0
NaCl (mM)

C

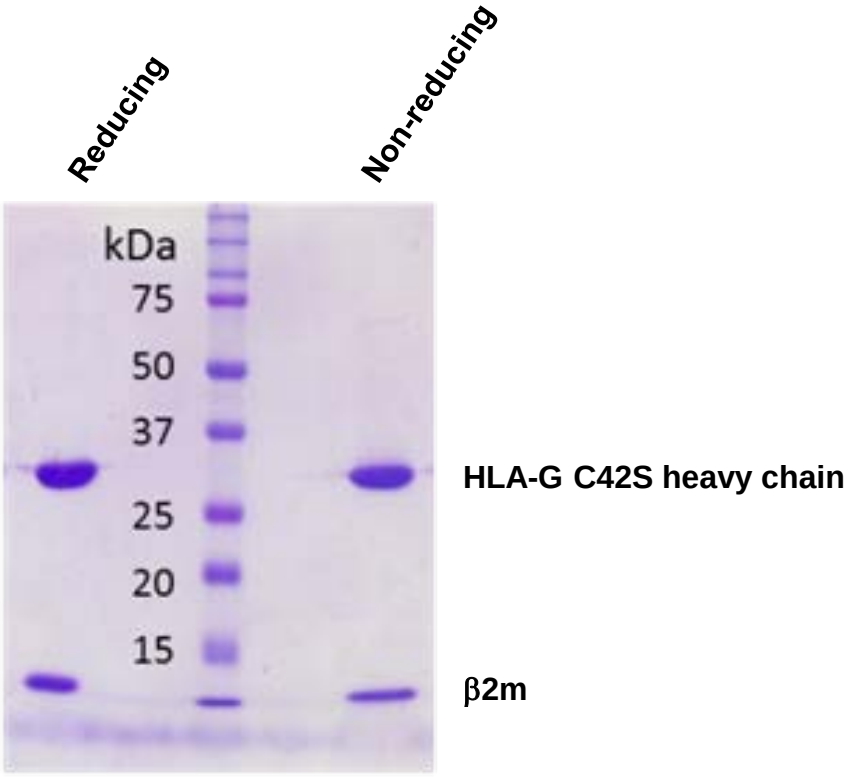

Reducing
Non-reducing
kDa
75
50
37
25
20
15
HLA-G C42S heavy chain
β2m

Figure 2

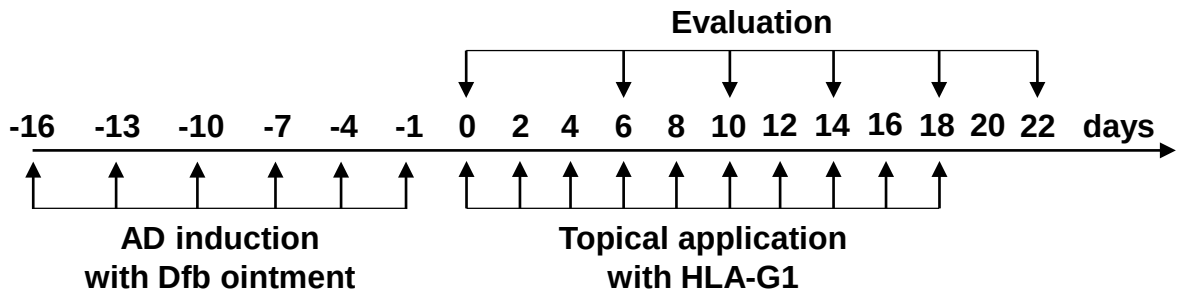

Evaluation
-16
-13
-10
-7
-4
-1
0
2
4
6
8
10
12
14
16
18
20
22
days
AD induction
with Dfb ointment
Topical application
with HLA-G1

Figure 3

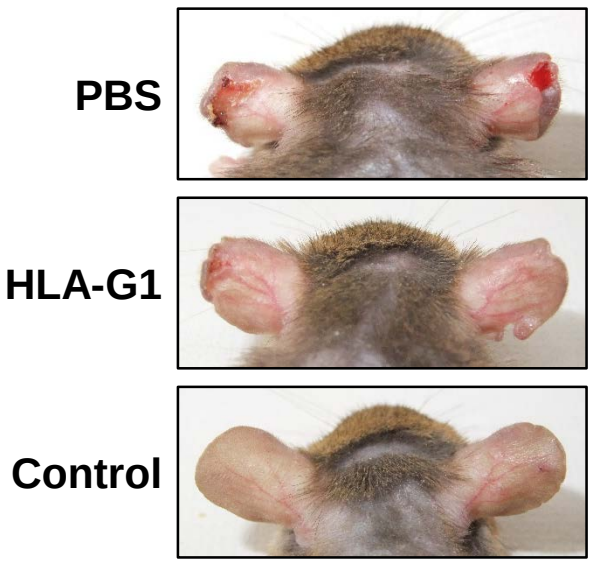
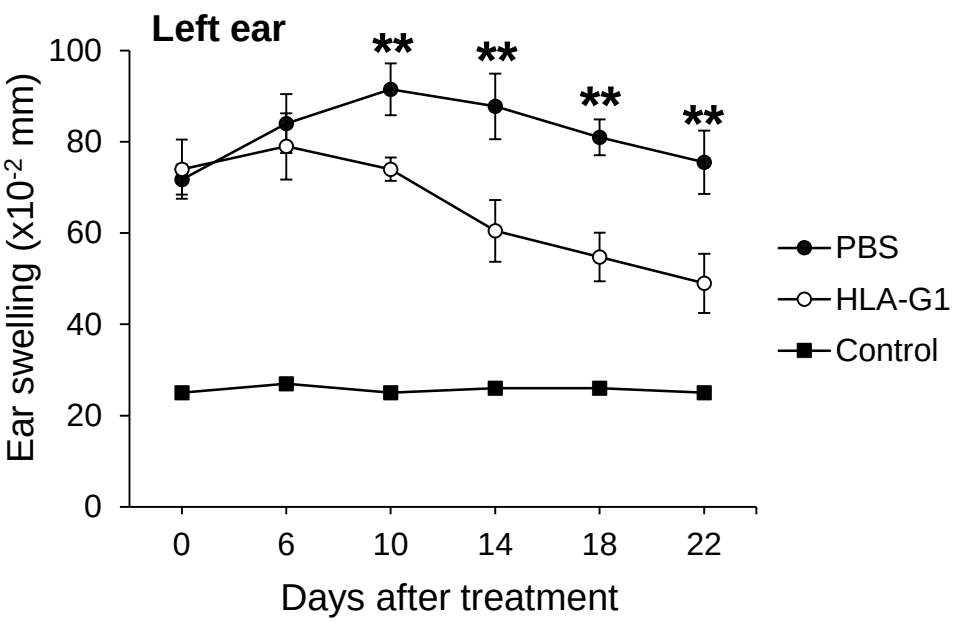
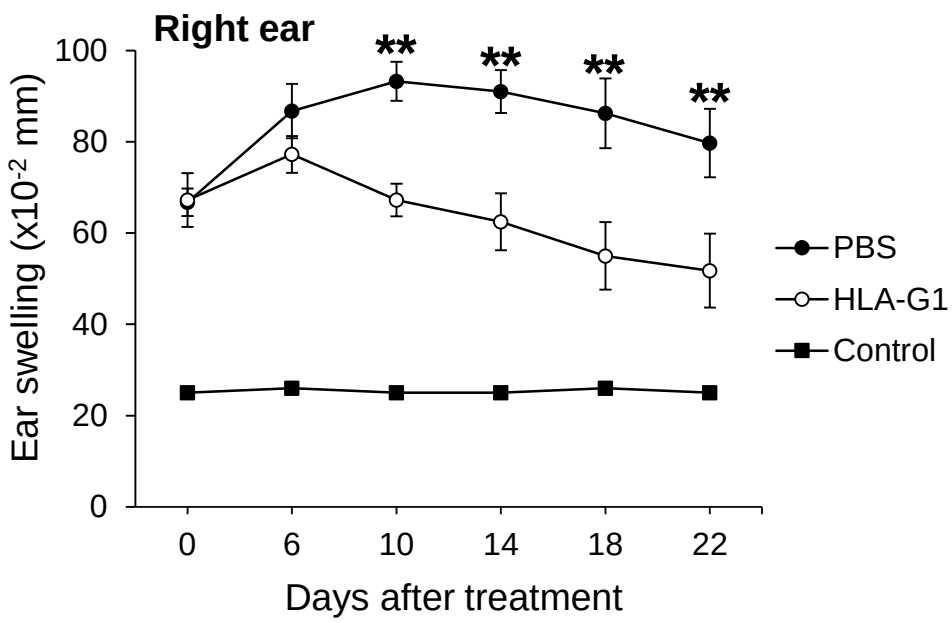


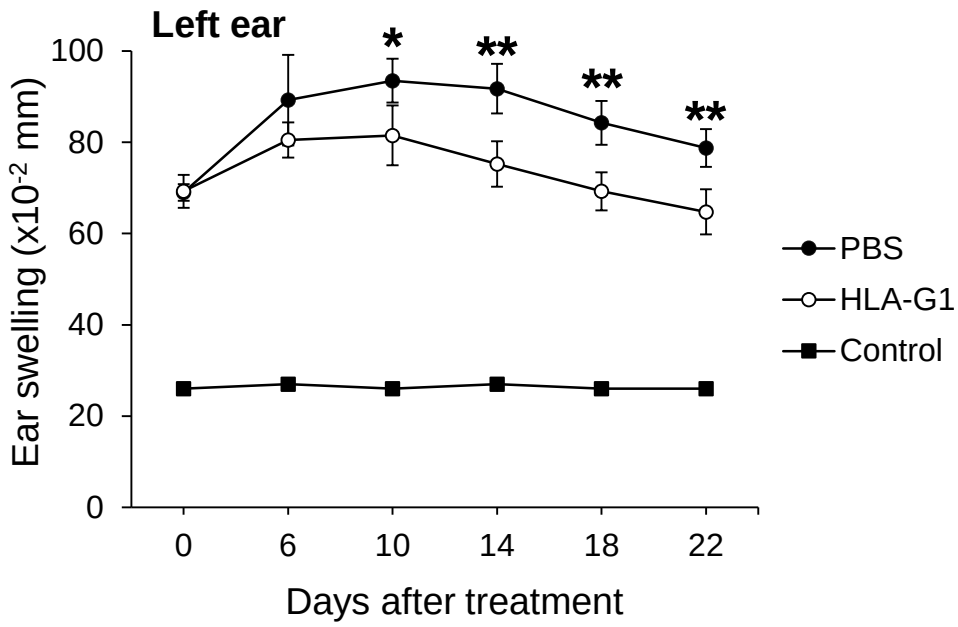
Figure 4

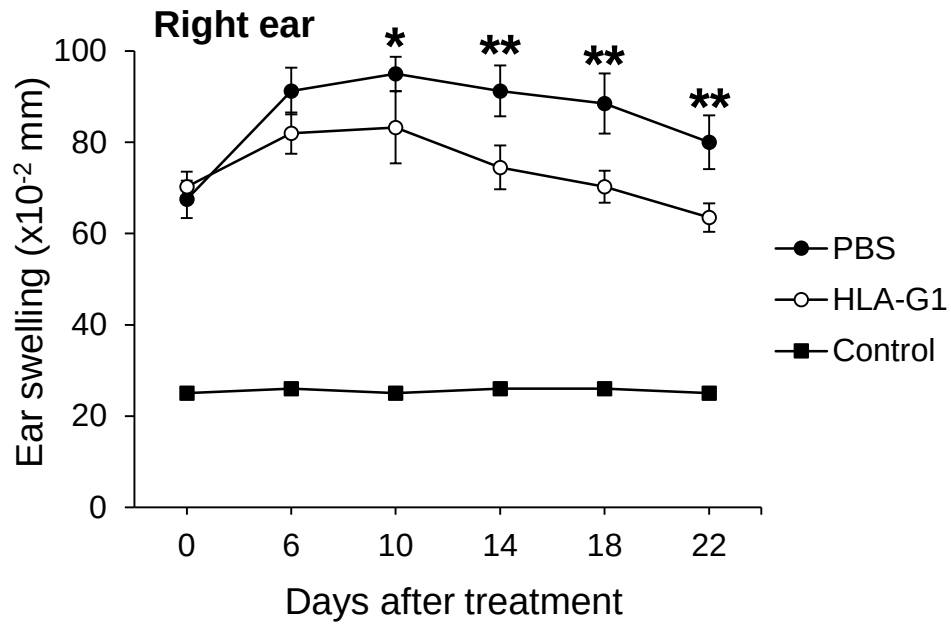
A


Left ear
Ear swelling (x10-2 mm)
100
80
60
40
20
0
0
6
10
14
18
22
Days after treatment
**
**
**
**
PBS
HLA-G1
Control


Right ear
Ear swelling (x10-2 mm)
100
80
60
40
20
0
0
6
10
14
18
22
Days after treatment
**
**
**
**
PBS
HLA-G1
Control

B


Left ear
Ear swelling (x10-2 mm)
100
80
60
40
20
0
0
6
10
14
18
22
Days after treatment
*
**
**
**
PBS
HLA-G1
Control


Right ear
Ear swelling (x10-2 mm)
100
80
60
40
20
0
0
6
10
14
18
22
Days after treatment
*
**
**
**
PBS
HLA-G1
Control

C

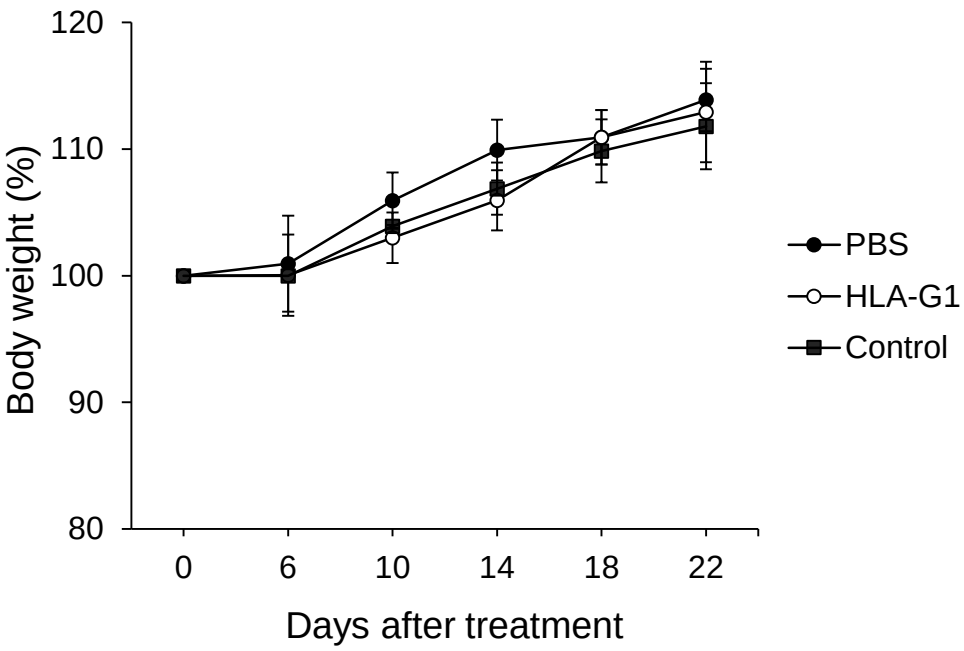

Body weight (%)
120
110
100
90
80
0
6
10
14
18
22
Days after treatment
PBS
HLA-G1
Control

Figure 5

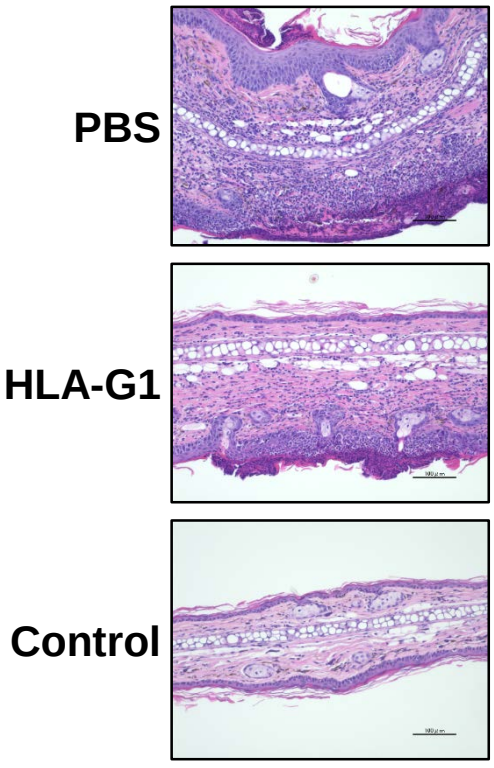
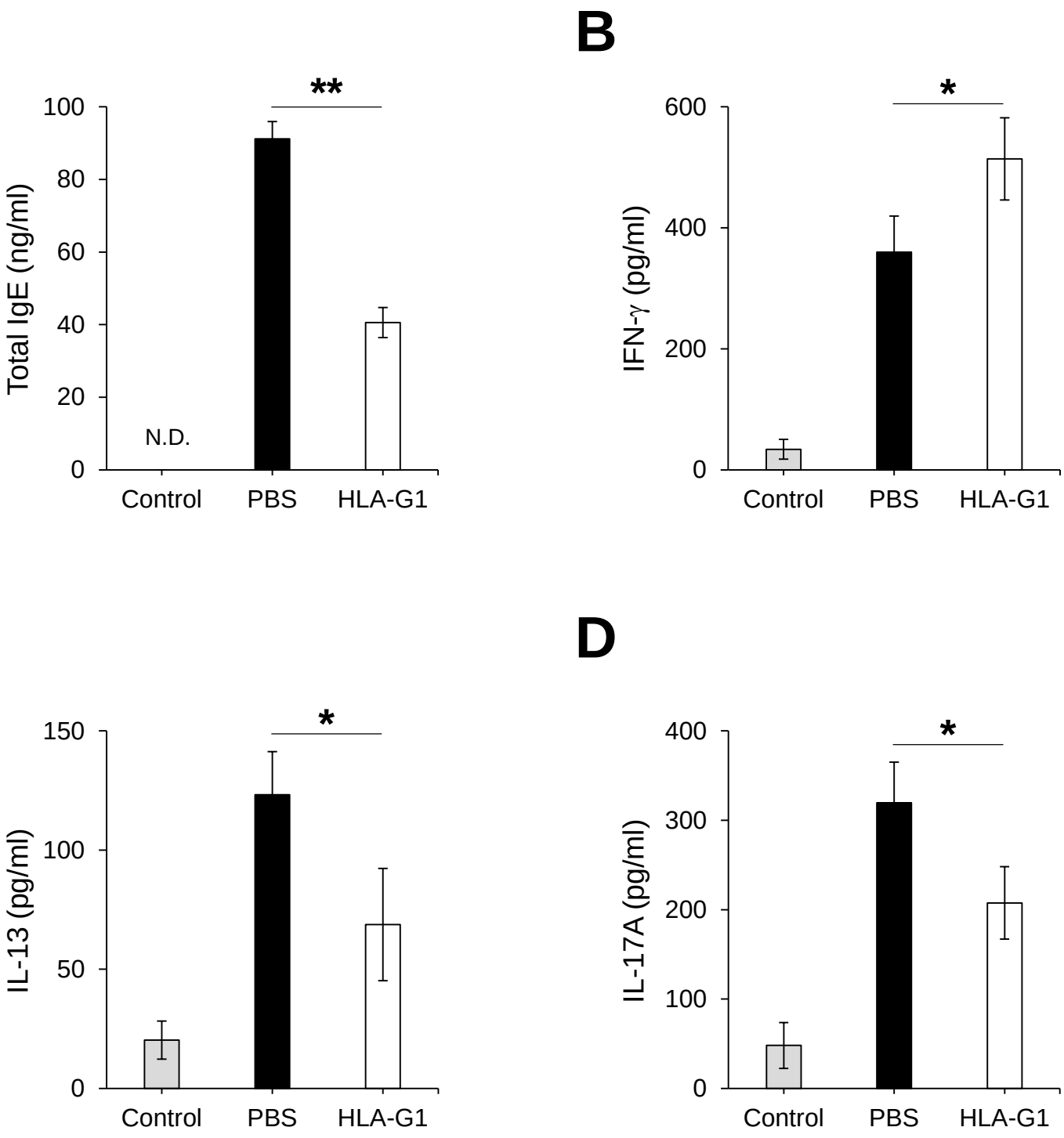


Figure 6


A
Total IgE (ng/ml)
100
80
60
40
20
0
N.D.
**
Control
PBS
HLA-G1
B
IFN-γ (pg/ml)
600
400
200
0
*
Control
PBS
HLA-G1
C
IL-13 (pg/ml)
150
100
50
0
*
Control
PBS
HLA-G1
D
IL-17A (pg/ml)
400
300
200
100
0
*
Control
PBS
HLA-G1