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Human leukocyte antigen-G upregulates immunoglobulin-like transcripts and corrects dysfunction of immune cells in immune thrombocytopenia

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ABSTRACT

Human leukocyte antigen-G (HLA-G) is a non-classical major histocompatibility complex class I antigen with potent immune-inhibitory function. HLA-G benefit patients in allotransplantation and autoimmune diseases by interacting with its receptors, immunoglobulin-like transcripts. Here we observed significantly less HLA-G in plasma from immune thrombocytopenia (ITP) patients positive for anti-platelet autoantibodies compared with autoantibodies-negative patients or healthy controls, while we found that HLA-G is positively correlated with platelet counts in both patients and healthy controls. We also found less membrane-bound HLA-G and immunoglobulin-like transcripts on $CD4^+$ and $CD14^+$ cells in patients. Recombinant HLA-G upregulated immunoglobulin-like transcript 2 expression on $CD4^+$ and immunoglobulin-like transcript 4 on $CD14^+$ cells. HLA-G upregulated IL-4 and IL-10, and downregulated tumor necrosis factor-α, IL-12 and IL-17 secreted by patient peripheral blood mononuclear cells, suggesting a stimulation of Th2 differentiation and downregulation of Th1 and Th17 immune response. HLA-G-modulated dendritic cells from ITP patients showed decreased expression of CD80 and CD86, and suppressed $CD4^+$ T-cell proliferation compared to unmodulated cells. Moreover, HLA-G-modulated cells from patients induced less platelet apoptosis. HLA-G administration also significantly alleviated thrombocytopenia in a murine model of ITP. In conclusion, our data demonstrated that impaired expression of HLA-G and immunoglobulin-like transcripts is involved in the pathogenesis of ITP; recombinant HLA-G can correct this abnormality via upregulation of immunoglobulin-like transcripts, indicating that HLA-G can be a diagnostic marker and a therapeutic option for ITP.

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Introduction

Immune thrombocytopenia (ITP) is a common autoimmune bleeding disorder with decreased platelet counts.[1-4] The therapeutic regimens for ITP patients, including glucocorticosteroids, intravenous immunoglobulin, thrombopoietin receptor agonists, rituximab and other immunoregulatory drugs, are not always effective,

and only about one-third of ITP patients can achieve long-term remission.[5-7] Autoantibodies targeting platelets enhanced T-helper cell activity, initiated abnormal activation of cytotoxic T lymphocytes and disturbed cytokine profiles to accelerate platelet clearance and suppress platelet production in ITP patients.[1-4,8] Moreover, the deficiency and dysfunction of immunoregulatory cells (i.e., regulatory T cells [Tregs]),[9-11] tolerogenic dendritic cells[12] and myeloid-derived suppressor cells[13] also contribute to the loss of immune tolerance in ITP.

HLA-G is a non-classical major histocompatibility complex class I antigen found to be expressed in placental trophoblast cells in 1990s.[14] There are four membrane-bound (mbHLA-G1 to -G4) and three soluble (sHLA-G5 to -G7) isoforms generated by alternative splicing of the primary transcript.[15] It was initially believed that HLA-G in placental cells plays an important role in maternal-fetal immunotolerance by interacting with inhibitory receptors (immunoglobulin-like transcript 2 [ILT-2/LILRB1/CD85j], immunoglobulin-like transcript 4 [ILT-4/LILRB2/CD85d] and KIR2DL4 receptor) on leukocytes, which leads to CTL apoptosis, natural killer (NK) cells immobilization, mononuclear cells inhibition and suppressive cytokine environment.[16,17] Subsequently HLA-G was found not be restricted to placenta cells, but it is also expressed in human peripheral blood mononuclear cell (PBMC) and plasma.[18,19] Additionally to the immune inhibitory capability mentioned above, HLA-G-ILT2/ILT4 interaction suppressed lymphocyte proliferation[20,21] and inhibited antibody secretion of activated B cells.[21] Ideas that HLA-G protein facilitates allotransplantation by preventing immune rejection via these actions on immune cells was established. After liver and kidney transplantation, an increased HLA-G level was detected in patients with a reduced incidence of rejection.[22-24]

Recently, HLA-G-induced immune tolerance has been intensively investigated. The expression of HLA-G (and its cognate receptors, ILT) was correlated with immune escape, which suggests unfavorable outcomes in cancer patients.[25,26] On the other hand, HLA-G-induced tolerance can benefit patients with autoimmune diseases. Low soluble HLA-G (sHLA-G) level was found in patients with rheumatoid arthritis (RA) and multiple sclerosis, and was associated with disease progression in both cases.[27,28] Moreover, the decrease of the number and/or function of ILT2 and ILT4 was involved in the pathogenesis of systemic lupus erythematosus (SLE).[29,30] Another study by Kim *et al.*[31] showed that functional HLA-G 14-bp polymorphism was associated with susceptibility to RA and SLE. However, the role of HLA-G in ITP remains obscure.

LeMaoult *et al.*[32] reported that both membrane-bound HLA-G (mHLA-G) and secreted HLA-G5 (sHLA-G5) upregulated ILT2/ILT4 expression on APC and $CD4^+$ T cells without the stimulation from exogenous antigens.[32] This encouraged the development of recombinant human HLA-G protein (rhHLA-G). Further investigation demonstrated that *in vitro* synthetic HLA-G protein induced the tolerance of skin grafts *in vivo* successfully.[33] Nevertheless, little has been defined about the effect of rhHLA-G in autoimmune disease.

In this study, we investigated whether there is aberrant HLA/ILT expression and their contribution to the loss of immune tolerance in ITP. Our data showed that the expression of HLA-G and ILT were both decreased in ITP patients, which may result in impaired function of PBMC and dendritic cells (DC). rhHLA-G upregulated ILT on $CD4^+$ T cells and monocytes, and corrected dysfunction of immune cells. These findings indicate that HLA-G has the potential as a diagnostic marker and therapeutic option for ITP.

Methods

Patients and controls

Plasma samples from 50 newly diagnosed ITP patients were collected for the detection of sHLA-G with ELISA. Among them, PBMC from 17 ITP patients were isolated for the *in vitro* investigation of mHLA-G and ILT (Table 1-2). Samples from 15 healthy volunteers were used as controls. The diagnosis of ITP was based on established practice guidelines.[1] Fourteen patients with autoantibody received standard ITP treatment high-dose dexamethasone (HD-DXM; 40 mg/day for 4 consecutive days), as described in the *Online Supplementary Appendix* and shown in Table 2. Enrollment took place between March 2013 and December 2018. The study was approved by the Medical Ethical Committee of Qilu Hospital, Shandong University. Informed consent was obtained from all patients before collecting samples in accordance with the Declaration of Helsinki.

Reagents

RhHLA-G (Abcam, Cambridge, UK) was dissolved in PBS to prepare a stock solution of 60 g/mL, and stored at -80 °C, according to the manufacturer's instructions.

In vitro platelet apoptosis assay

PBMC were cultured with 600 ng/mL rhHLA-G for 3 days. Freshly isolated human platelets were adjusted to $1x10^8$/mL with 5 M prostaglandin E1. rhHLA-G-modulated PBMC ($1×10^6$) were then co-cultured with autologous or allogeneic platelets ($1×10^7$) as effector cells and target cells, respectively, and incubated at 37°C for 4 hours (h). Platelet apoptosis was detected using a mitochondrial membrane potential assay kit (Beyotime Biotechnology, Shanghai, China).

DC suppression assays

Freshly isolated $CD4^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (5 mM; Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes (min) at 37°C and quenched by adding 3 mL of cold fetal calf serum (FCS) for 5 min at 4°C. $CD4^+$ cells were washed with RPMI 1640 culture medium three times and seeded at 10^5 cells/well in U-bottom 96-well plates containing 1 μg/mL anti-human CD3 (Biolegend) and 50 U/mL recombinant human IL-2 (rhIL-2; R&D Systems) in triplicates. DC with or without rhHLA-G pre-modulation were added at a 5:1 ratio (T cells : DC). All cells were cultured for 5 days in humidified air with 5% CO_2 at 37°C, then collected for flow cytometry analysis.

Animal model and the treatment of HLA-G

Blood was drawn by retro-orbital bleeding. Platelets from C57BL/6 wild-type mice were prepared to immunize C57BL/6 CD61 knockout (KO) mice by transfusion 10 μL of 10^8 platelets weekly for 4 consecutive weeks.[34,35] The immunized CD61 KO mice were sacrificed, and their spleens were homogenized to prepare splenocyte suspension. On the day of transplantation, the C57BL/6 SCID mice were subjected to 200 cGy total body irradiation, and then injected with the indicated splenocytes ($5×10^4$) with or without HLA-G protein administration. Blood from saphenous veins was collected and platelet counts were detected weekly, as described in the *Online Supplementary Appendix*.[34,35]

Table 1. Clinical characteristics of untreated immune thrombocytopenia patients.

Patient No.	Sex/Age (yr)	Platelets counts (x10^9/L)	Bleeding symptoms	Autoantibodies	
				Anti-GPIb/IX	Anti-GPIIb/IIIa
1	M/76	2	EP	+	+
2	M/62	2	EP, GUH	+	+
3	M/62	10	PT, EC	+	+
4	F/56	8	EC, EP, GH	+	+
5	M/23	14	EC, GH	+	–
6	F/26	21	EC	+	–
7	F/50	4	None	+	–
8	M/18	4	EP, GUH	+	–
9	M/44	4	EC	–	+
10	F/33	1	EC, GH	–	+
11	F/43	6	GH, GUH	–	+
12	M/37	20	EC, GH, EP	–	+
13	M/50	1	GH, EC	–	–
14	M/21	6	EC, GUH	–	–
15	F/55	9	GH, EC	–	–
16	M/16	10	EC	–	–
17	F/30	11	EC, GH	–	–
18	M/73	1	PT, EC	+	+
19	F/33	16	GH, EC, GUH	+	+
20	F/76	2	EC, GH	+	–
21	M/23	29	EC	+	–
22	F/54	1	EC, PT	–	+
23	M/26	21	EC	–	+
24	F/66	5	EC, GH	–	+
25	F/24	7	EC, PT	–	+
26	M/47	19	EC	–	–
27	F/48	3	EC	–	–
28	M/16	6	EC	–	–
29	F/27	3	GH, EC	–	–
30	M/36	3	None	–	–
31	F/34	15	EC	–	–
32	F/47	7	EC, PT	–	–
33	M/32	2	EC, GH	–	–
34	M/27	20	None	–	–
35	M/28	5	EC, PT	–	–
36	F/39	10	None	–	–

No: number; F: female; M: male; yr: years; PT: petechiae; EC: ecchymoses; EP: epistaxis; GH: gingival hemorrhage; GUH: genitourinary hemorrhage. Plasma samples from patient No.1-50 were collected for the detection of secreted human leukocyte antigen-G (sHLA-G) with enzyme linked immunosorbent assay (ELISA). Peripheral blood mononuclear cell (PBMC) from patient No.1-17 were isolated for the *in vitro* investigation of membrane-bound HLA-G (mHLA-G) and immunoglobulin-like transcript (ILT) in immune cells.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between rhHLA-G modulated and unmodulated cells were determined by paired Student *t* tests. Correlative analysis was determined by nonparametric Pearson correlation. All the other statistics were analyzed with the Mann-Whitney U test. $P<0.05$ was considered a significant difference.

Results

HLA-G and ILT expression reduced in ITP patients

Platelet antibodies were identified by monoclonal antibody immobilization of platelet antigens (MAIPA) assay (shown in Table 1). In order to investigate the level of sHLA-G in ITP patients, platelet poor plasma (PPP) was isolated and sHLA-G was measured by enzyme linked immunosorbent assay (ELISA). Plasma HLA-G was reduced in ITP patients with anti-platelet antibodies compared to patients without autoantibodies and healthy controls. Among these patients, 11 were positive for anti-GPIIb/IIIa antibodies, 12 for anti-GPIb/IX antibodies, and 11 for both. However, no significant differences were found in the sHLA-G levels based on antibody type. ITP patients without anti-platelet antibodies showed no significant difference compared to healthy controls (Figure

Table 2. Clinical characteristics of immune thrombocytopenia patients treated with high-dose dexamethasone.

Patient No.	Sex/Age (yr)	Autoantibodies		Bleeding symptoms	Platelet counts ($x10^9$/L)	
		Anti-GPIb/IX	Anti-GPIb/IX		before	after
37	F/42	+	+	EP	7	78 R
38	F/22	+	+	GUH	9	23 NR
39	F/62	+	+	EC	2	166 R
40	F/56	+	+	EC, EP, GH	3	56 R
41	M/23	+	+	EC	1	86 R
42	F/26	+	–	EC	2	98 R
43	F/50	+	–	None	10	134 R
44	M/18	+	–	EP	9	86 R
45	M/44	+	–	EC, EP	1	40 R
46	F/33	+	–	EC, GH	17	107 R
47	F/43	+	–	EC, EP	13	214 R
48	M/37	–	+	GH, GUH	14	12 NR
49	M/50	–	+	EC, GH	15	52 R
50	F/21	–	+	EC, GUH	11	144 R

No: number; F: female; M: male; yr: years; PT: petechiae; EC: ecchymoses; EP: epistaxis; GH: gingival hemorrhage; GUH: genitourinary hemorrhage. Plasma samples from patient No.1-50 were collected for the detection of secreted human leukocyte antigen-G (sHLA-G) with enzyme linked immunosorbent assay (ELISA). Peripheral blood mononuclear cell (PBMC) from patient Plasma and PBMC from patient No. 37-50 were obtained for the investigation of sHLA-G and immunoglobulin-like transcript (ILT) in immune thrombocytopenia (ITP) patients received high dose demamethasone treatment.

1A-B). We then stratified ITP patients into platelet high (≥10×10^9/L) group and platelet low (<10×10^9/L) group and found that patients with high platelets showed significantly higher sHLA-G level compared to those with low platelets (Figure 1C). In order to assess whether the ITP group develops an increase in sHLA-G after normalization of their platelet counts due to standard ITP treatments, we tested sHLA-G level in 14 patients who received HD-DXM therapy. As shown in Table 2, 12 ITP patients responded to HD-DXM treatment and after treatment, the sHLA-G level in their plasma was significantly increased (Figure 1D). Thus, HLA-G might also be involved in the recovering process by HD-DXM treatments. Moreover, the level of sHLA-G positively correlated with platelet counts in patients with or without antiplatelet antibodies (r=0.602, P<0.01; r=0.584, P<0.05; Figure 1E-F). Interestingly, the sHLA-G level was also positively correlated with the platelet counts in healthy control (r=0.580, P<0.05, Figure 1G).

Cell surface expression of mHLA-G was evaluated as shown by flow cytometry. Expression of mHLA-G on $CD4^+$ T cells and $CD14^+$ monocytes was significantly lower in ITP patients than in healthy controls. However, $CD8^+$ T cells and $CD19^+$ B cells did not show a significant difference in the expression of mHLA-G between ITP patients and healthy controls (Figure 1H-K).

We then analyzed ILT2 expression on $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD19^+$ B cells, as well as ILT4 on $CD14^+$ cells. Significantly lower ILT2 expression was found on $CD4^+$ T cells from ITP patients compared with those from healthy controls. By contrast, no significant difference was found on $CD8^+$, $CD14^+$, or $CD19^+$ cells. Decreased ILT4 expression was also observed on $CD14^+$ cells from ITP patients (Figure 2). These data indicate aberrant expression of mHLA-G and ILT in ITP patients. However, no correlation was found between mHLA-G/ILT and platelet count in our study (*Online Supplementary Figure S1*). The expression of ILT2 on $CD4^+$, $CD8^+$, $CD14^+$ or $CD19^+$ cells and the expression of ILT4 on $CD14^+$ cells showed no obvious changes after HD-DXM treatment (*Online Supplementary Figure S2*).

rhHLA-G upregulated ILT expression on $CD4^+$ and $CD14^+$ cells

In order to test whether exposure to exogenous HLA-G would lead to an upregulation of inhibitory receptors on PBMC, we exposed these cells to rhHLA-G and monitored ILT2/ILT4 expression. rhHLA-G upregulated ILT2 expression on $CD4^+$ T cells, as well as ILT4 on $CD14^+$ monocytes in both ITP patients and controls. However, rhHLA-G had no significant effect on ILT2 expression on $CD8^+$, $CD14^+$, and $CD19^+$ cells (Figure 2).

rhHLA-G reprogrammed PBMC cytokine secretion and regulated T-cell differentiation

Cytokines were measured in the supernatant of PBMC cultured with rhHLA-G. TNF-α, IL-12, and IL-17 levels were significantly reduced and IL-1β, IL-2, IL-4, IL-10, G-CSF, and GM-CSF levels were significantly elevated in rhHLA-G-treated systems compared with untreated systems. No significant difference in IFN-γ levels was found in the supernatant after rhHLA-G treatment (Figure 3A-J). IL-5, IL-6, IL-7, IL-8, IL-13, MCP-1, and MIP-1 levels secreted by ITP patient PBMC were below the detection limit.

In order to investigate whether rhHLA-G could enhance the expression of the Treg population, we also detected the percentage of $CD4^+CD25^+Foxp3^+$ Tregs. Tregs percentages in ITP patients were significantly lower than in healthy controls; However, rhHLA-G exposure did not expand Tregs in ITP patients (Figure 3K).

rhHLA-G exposure attenuated ITP patient PBMC-induced platelet apoptosis

Given that rhHLA-G upregulated ILT and reprogrammed cytokine secretion, we hypothesized that the inhibitory functions of PBMC were restored. In order to testify the hypothesis, we measured the ability of PBMC

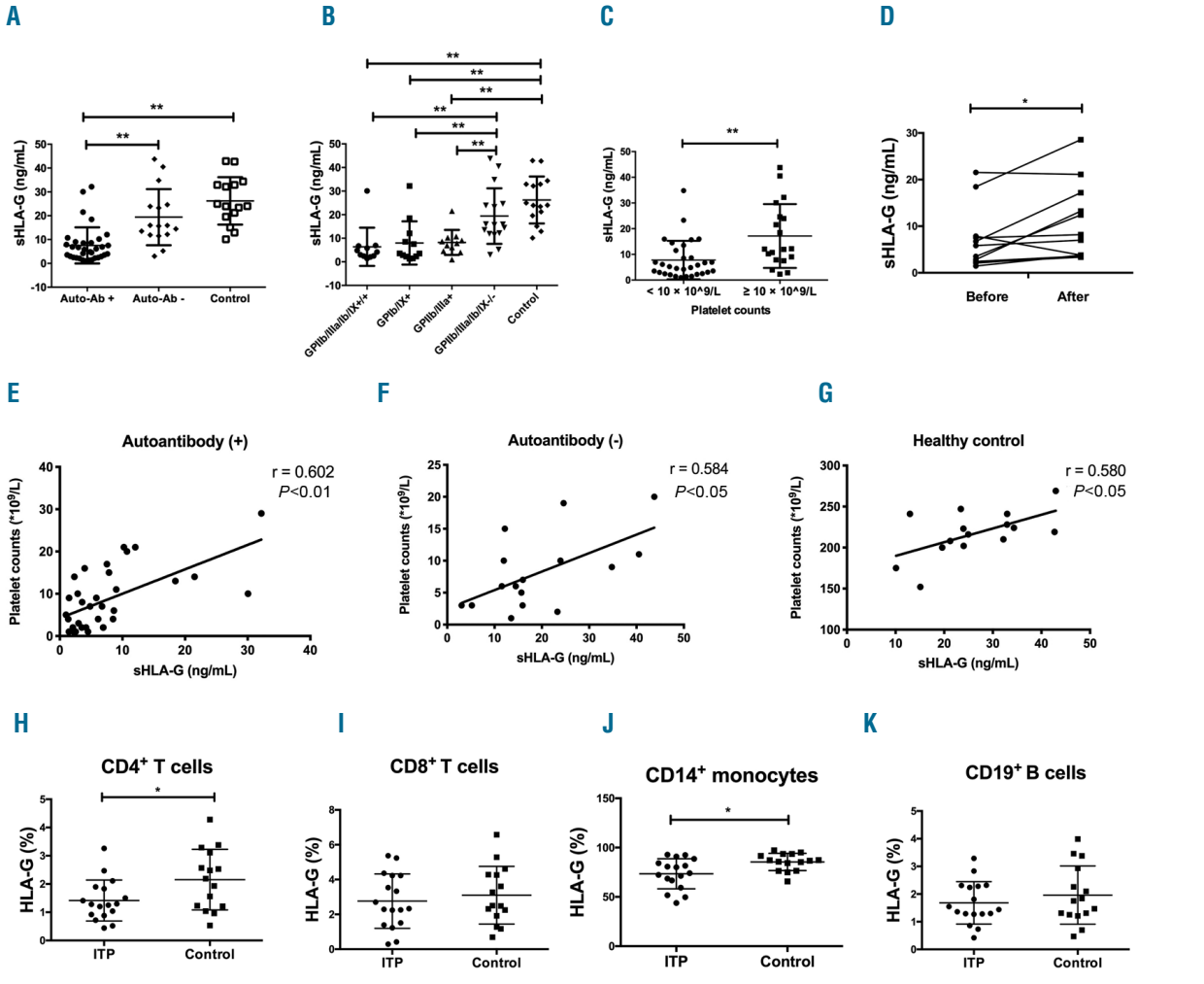

Figure 1. Reduced expression sHLA-G and mHLA-G in immune thrombocytopenia patient plasma. (A) The plasma concentration of secreted human leukocyte antigen-G (sHLA-G) was assayed by enzyme linked immunosorbent assay (ELISA). Immune thrombocytopenia (ITP) patients positive for anti-platelet autoantibodies (n=34) showed significantly reduced plasma sHLA-G compared to ITP plasma negative for anti-platelet autoantibody (n=16) and compared to healthy controls (n=15). (B) No significant differences in plasma sHLA-G were found between ITP patients with double positive (n=11), anti-GPIIb/IIIa positive (n=11), and anti-GPIb/IX positive (n=12) plasma. However, each of these groups showed reduced plasma HLA-G compared to double negative ITP plasma (n=16) and healthy controls (n=15). (C) sHLA-G level in plasma of patients with high platelets (≥10x10^9/L; n=19) was significantly higher than those with low platelets (<10x10^9/L; n=31). (D) Patients responded to high-dose dexamethasone (HD-DXM) treatments exhibited increased sHLA-G level (n=14). (E-G) Plasma concentration of sHLA-G correlated with platelet counts in autoantibody positive, negative patients and healthy controls. *P<0.05; **P<0.01. (H-K) Cell surface expression of membrane-bound HLA-G (mHLA-G) on CD4$^+$, CD8$^+$, CD14$^+$, and CD19$^+$ cells in ITP patients (n=17) and healthy controls (n=15).

to induce platelet apoptosis. PBMC from ITP patients induced significantly higher apoptosis of both autologous and healthy control platelets compared with healthy controls and the addition of rhHLA-G attenuated platelet destruction by ITP PBMC. The decrease of platelet apoptosis demonstrates a protective effect of rhHLA-G in ITP. In order to determine whether the platelet apoptosis was mediated by PBMC cytotoxicity and by the platelet themselves, healthy control PBMC were cultured with autologous platelets and ITP platelets respectively. The results showed that rhHLA-G modulation did not change the ability of healthy control PBMC to induce both autologous and ITP platelet apoptosis (Figure 4).

Since Fcγ-receptor-mediated platelet phagocytosis is one of the most important mechanisms for antibody-mediated platelet destruction in ITP, we detected the Fcγ-receptor (CD16) expression and antibody-mediated platelet phagocytic capability by monocytes/macrophages. Our results showed that treatment of rhHLA-G had no significant effect on monocytic Fcγ-receptor (CD16) expression. Therefore, no difference was observed in the antibody-mediated platelet phagocytic capability between rhHLA-G modulated and unmodulated groups (*Online Supplementary Figure S3*), indicating that rhHLA-G protects ITP platelets through mechanisms other than by inhibiting platelet phagocytosis.

rhHLA-G suppressed DC maturation and T-cell proliferation *in vitro*

In order to assess whether rhHLA-G inhibits maturation of monocyte-derived DC, surface expression of CD80 and CD86 on DC was analyzed by flow cytometry. DC from ITP patients expressed higher levels of CD80 and CD86 than those from healthy controls. In the presence of rhHLA-G, CD80 and CD86 expression was markedly downregulated in both ITP patients and healthy controls (Figure 5A-D).

DC maturation, which increases the expression of cos-

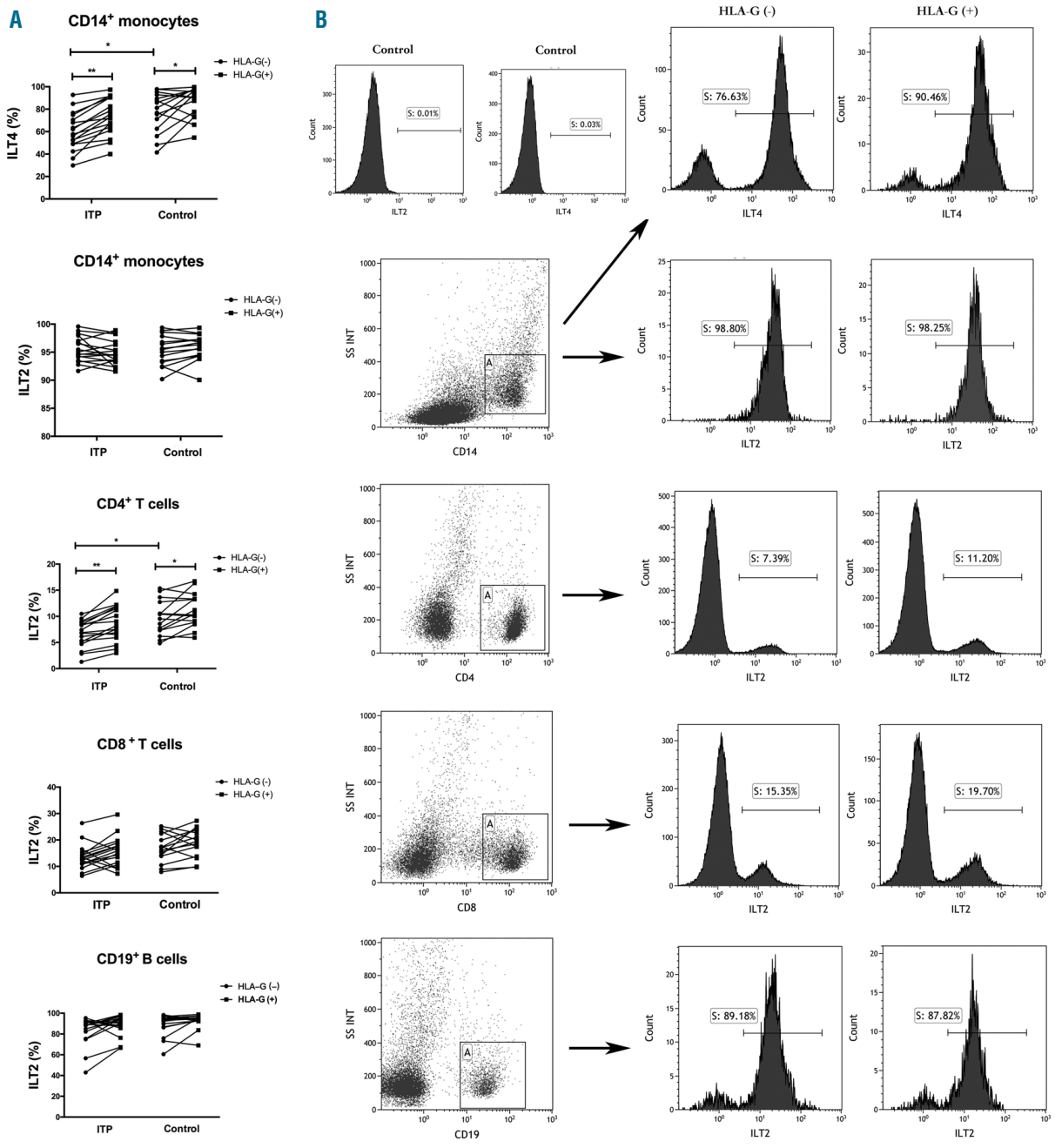

Figure 2. Impaired expression of immunoglobulin-like transcript in immune thrombocytopenia patients was recovered with exposure to rhHLA-G. (A) Cell surface expression of immunoglobulin-like transcript 2 (ILT2) on CD4$^+$, CD8$^+$, CD14$^+$, and CD19$^+$ cells and ILT4 on CD14$^+$ cells in immune thrombocytopenia (ITP) patients (n=17) and healthy controls (n=15) after culture with or without recombinant human leukocyte antigen-G (rhHLA-G) (600 ng/mL, 3 days). *P<0.05; **P<0.01. (B) Representative histograms of isotype control and ILT2 on CD4$^+$, CD8$^+$, CD14$^+$, and CD19$^+$ cells and ILT4 on CD14$^+$ cells in an ITP patient. Representative scattergrams of surface expression of CD14, CD4, CD8, CD19 from an ITP patient. Side scatter (SSC)^{low}CD14$^+$, SSC^{low}CD4$^+$, SSC^{low}CD8$^+$, SSC^{low}CD19$^+$ cells were gated for the following analysis.

timulatory molecules, such as CD80, CD86 and related cytokines, could contribute to T-cell proliferation and differentiation. In order to further evaluate DC-mediated T-cell proliferation, allogeneic CD4$^+$ T cells were cocultured with DC with or without rhHLA-G premodulation. DC from ITP patients stimulated significantly more CD4$^+$ T-cell proliferation compared with those from healthy controls *in vitro*. However, rhHLA-G-modulated DC from ITP patients lost their suppressive effect on CD4$^+$ T-cell proliferation compared to unmodulated cells (Figure 5E-F).

HLA-G treatment alleviated thrombocytopenia in a murine model of ITP

HLA-G capture by gold nanoparticle was observed by transmission electron microscopy (Figure 6A). The SCID mice engrafted with splenocytes from immunized CD61 KO mice exhibited profound thrombocytopenia within 28 days after transplantation. The SCID mice were separated into four groups. Group 1 received an intraperitoneal injection of 100 μL AuNP coated with 7.5ug HLA-G on the day of the splenocyte transplantation; group 2 received an intraperitoneal injection of same amount of control AuNP

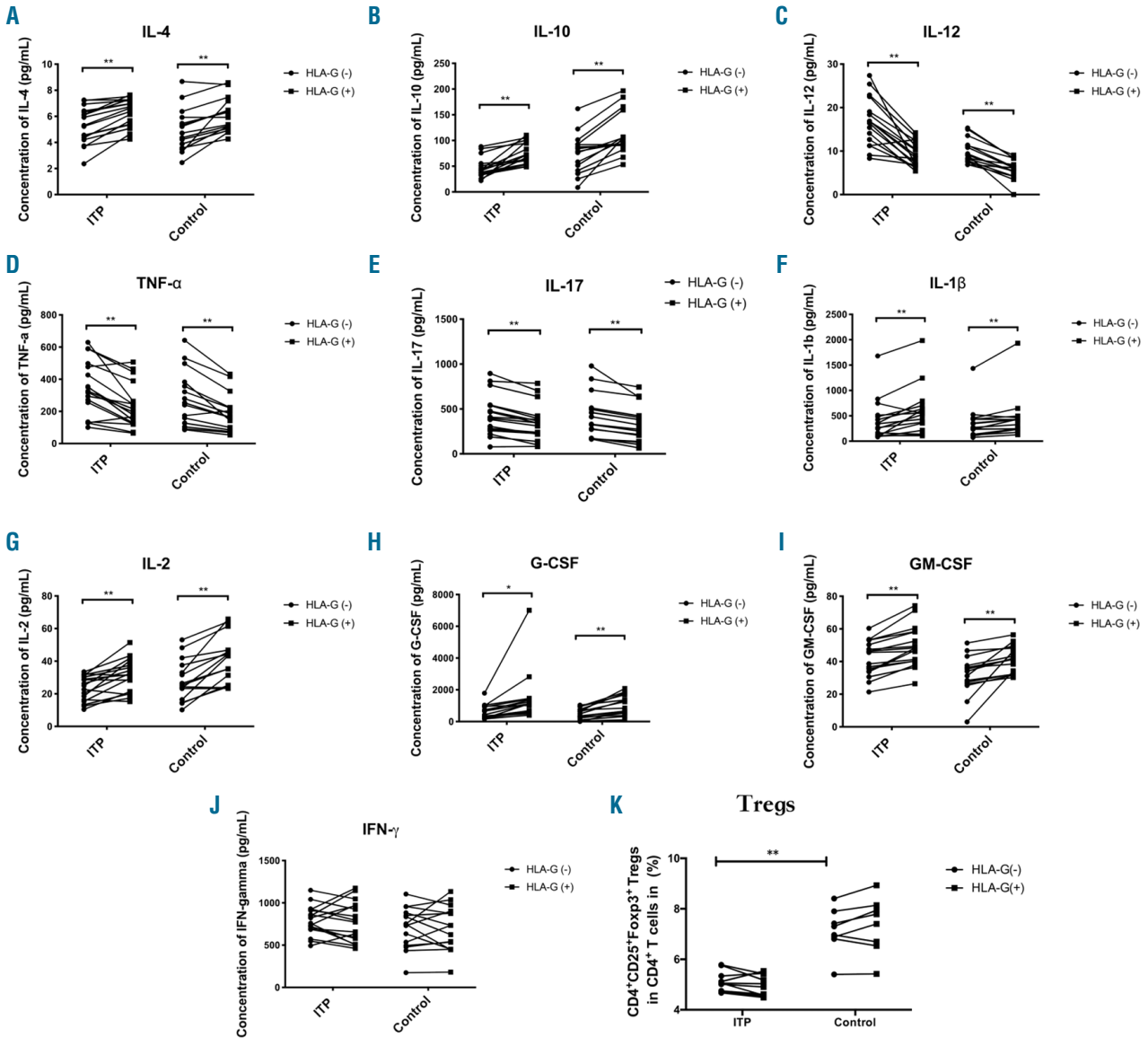

Figure 3. rhHLA-G reprogrammed cytokine profile in immune thrombocytopenia patients. Peripheral blood mononuclear cells (PBMC) were cultured with or without recombinant human leukocyte antigen-G (rhHLA-G) for 3 days and supernatants were collected. (A-J) Cytokine levels in the culture supernatant from 17 immune thrombocytopenia (ITP) patients and 15 healthy controls were determined by Bio-plex Multiple Cytokine Test. (K) Percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) in $CD4^+$ cells with or without rhHLA-G modulation from 10 randomly selected ITP patients and 8 healthy controls. *P<0.05; **P<0.01.

on the same day; group 3 received 7.5 ug HLA-G protein; group 4 received the same volume of phosphate-buffered saline as a blank control. According to routine monitored platelet counts, mice in group 1 showed significantly higher platelet counts than mice in group 2 at week 3 and 4 after irradiation. Mice in group 3 showed significantly higher platelet counts than mice in group 4, but lower platelet counts than mice in group 1 (Figure 6C). No significant difference in the survival rate among the four groups was observed in our study (Figure 6B).

Discussion

ITP is characterized by autoantibody-mediated platelet phagocytosis,[1-3] in which the number deficiency and/or dysfunction of immune regulatory cells, such as Tregs, tolerogenic DC, and MDSC, leads to the loss of immune tolerance in ITP patients. As an important immunoregulatory molecule, HLA-G executes its inhibitory immune function through suppression of T cells, B cells, and APC.[16,19]

In this study, the levels of sHLA-G in ITP plasma is correlated with the presence of anti-platelet autoantibodies, but no significant difference was observed between Ab-negative ITP patients and healthy controls, indicating the involvement of HLA-G in the pathogenesis of autoantibody-mediated platelet clearance. A previous study showed that HLA-G inhibited B-cell proliferation and antibody secretion,[21] which may explain why patients with low levels of sHLA-G tended to have high levels of anti-platelet antibodies. Furthermore, sHLA-G positively correlated with platelet counts in ITP patients and in healthy controls, suggesting that HLA-G may have other protective effects.

sHLA-G exerts its inhibitory immune function by inter-

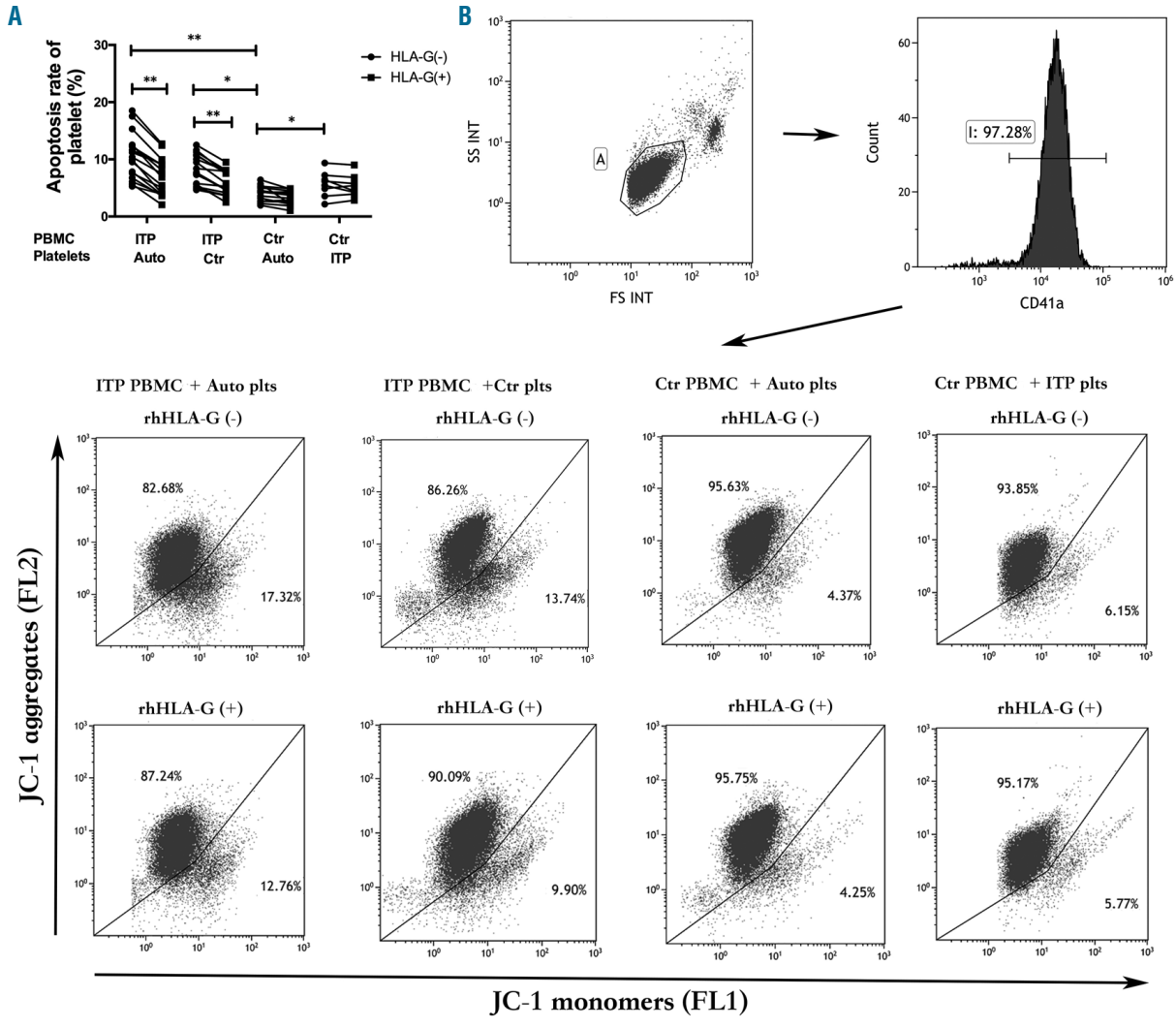

Figure 4. rhHLA-G attenuated immune thrombocytopenia patient peripheral blood mononuclear cell-induced platelet apoptosis. Recombinant human leukocyte antigen-G (rhHLA-G)-modulated peripheral blood mononuclear cells (PBMC) were cultured with autologous or allogeneic platelets for 4 hours, and platelet apoptosis was assayed with JC-1 mitochondrial potential test. (A) Apoptosis of autologous (Auto) or healthy control (Ctr) platelets cocultured with PBMC from immune thrombocytopenia (ITP) patients (n=17); and apoptosis of autologous and ITP platelets co-cultured with PBMC from healthy controls (n=15; n=8). *P<0.05; **P<0.01. (B) Representative scattergrams of JC-1 mitochondrial potential test for platelet apoptosis. JC-1 is a mitochondrial membrane potential-sensitive carbocyanine probe. JC-1 monomers emit green fluorescence whereas JC-1 aggregates emit orange-red fluorescence. Platelets were gated according to forward scatter (FSC), side scatter (SSC), and CD41a, then platelet apoptosis was analyzed. The dots in the right lower gate represent apoptotic platelets.

acting with receptors ILT2 and ILT4, which were differentially expressed on CD4$^+$, CD8$^+$, CD14$^+$, and CD19$^+$ cells. In ITP patients, we observed decreased expression of ILT2 on CD4$^+$ cells, as well as ILT4 on CD14$^+$ cells in comparision with healthy controls. Consistent with the previous study,[32] we found that rhHLA-G upregulated ILT4 expression in CD14$^+$ monocytes, and ILT2 expression in CD4$^+$ cells, but not on CD8$^+$, CD14$^+$, and CD19$^+$ cells in ITP patients, probably due to the relatively high baseline expression of ILT2 on CD14$^+$ and CD19$^+$ cells. Shiroishi *et al.*[36] reported that CD8 is also the receptor for HLA-G, and it can compete with ILT2 and ILT4 for HLA-G binding. sHLA-G triggers Fas/Fas-Ligand (FasL)-mediated apoptosis in CD8$^+$ T cells by interacting with CD8 molecules,[37] which could alleviate the suppression on megakaryocyte apoptosis and increase platelet production.[38] ILT upregulation by rhHLA-G indicates that sHLA-G could also regulate immune cells.

Cytokines are crucial factors in maintaining self-tolerance by controlling cell proliferation, differentiation, and migration. We and other groups demonstrated that type 1 (IL-2, IFN-α, IL-12, TNF-α), type 17 (IL-17) cytokines were significantly increased and type 2 (IL-4, IL-5, IL-10, IL-13) were decreased in ITP patients.[39-41] Patients whose platelet count responded to therapeutic regimens often experienced correction of a disturbed cytokine profile. Agaugué S *et al.*[42] found that HLA-G contributed to tumor escape by expanding MDSC and supporting Th2 *versus* Th1/Th17. Meanwhile, HLA-G could also help restore the balance of cytokines in ITP patients. We found that rhHLA-G upregulated IL-4 and IL-10 secretion, and downregulated TNF-α, IL-12, and IL-17 secretion by ITP patient PBMC, indicating a promotion of Th2 and inhibition of Th1 and Th17 by rhHLA-G. Our results demostrated rhHLA-G did not expand the CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs. We suspect that rhHLA-G might exert its suppressive function through other mechanisms. A novel subset of regulatory T cells expressing HLA-G was described, and it was proven

that CD4+ HLA-G+ Tregs suppressed T-cell proliferation by secreting IL-10 and sHLA-G.[43,44] A more recent study by Pankratz *et al.*[45] showed that CD4+ HLA-G+ Tregs are the key player in inflammation and can ameliorate graft-*versus*-host diseases *in vivo*. Here, the proportion of CD4+ HLA-G+ cells was significantly decreased in ITP patients, further establishing the role of HLA-G in reconstructing immune tolerance in ITP patients.

In addition, rhHLA-G exerted their immunosuppressive effects by promoting IL-1β, IL-2, G-CSF, and GM-CSF production. A previous study demonstrated that GM-CSF suppresses the immune response by expanding

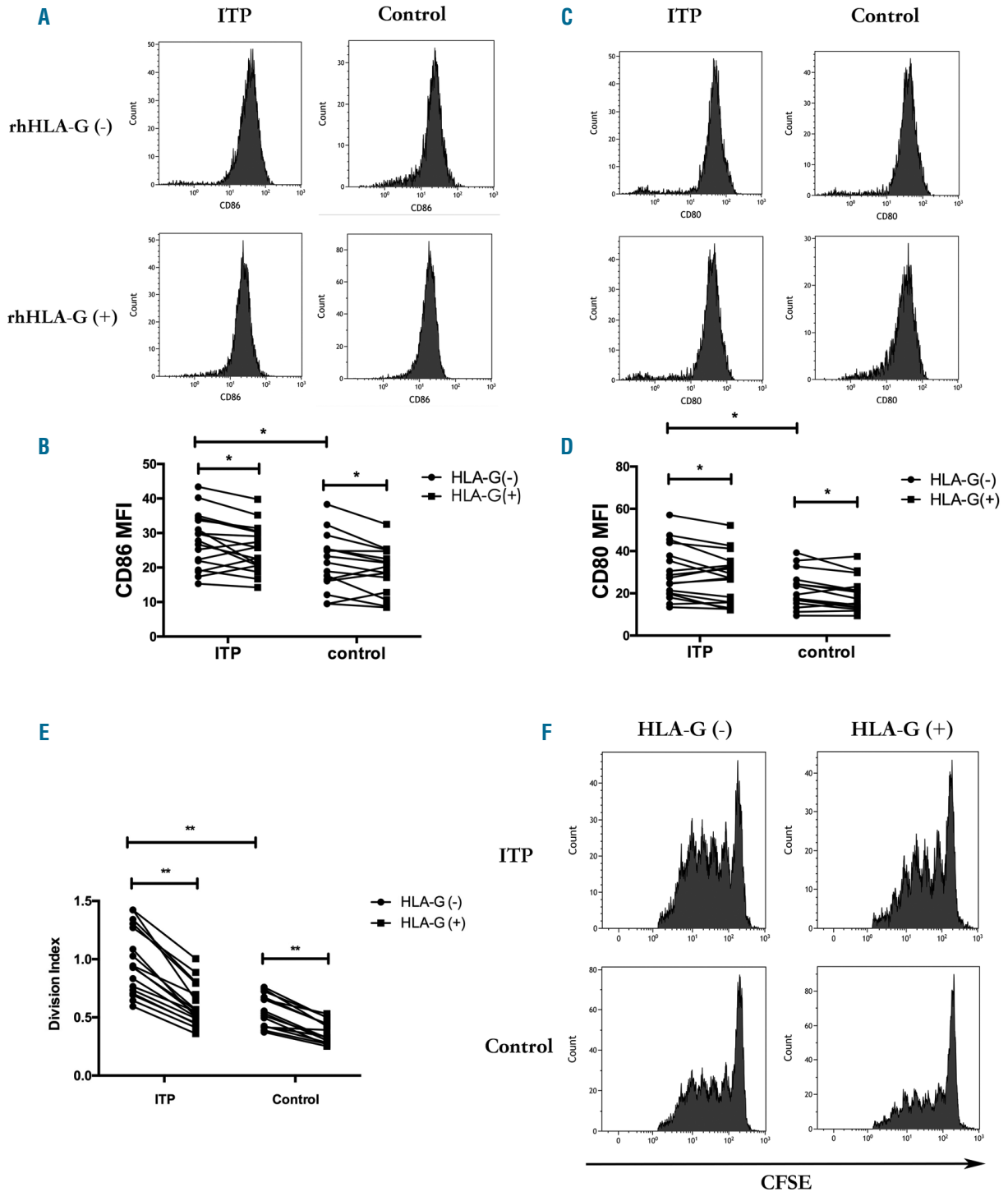

Figure 5. rhHLA-G modulation decreased expression of costimulation molecules on dendritic cells and inhibited CD4+ T-cell proliferation by dendritic cells. (A) Representative histograms and (B) analysis of CD86 MFI on dendritic cells from 17 immune thrombocytopenia (ITP) patients and 15 healthy controls. (C) Representative histograms and (D) analysis of CD80 MFI on dendritic cells from 17 ITP patients and 15 healthy controls. *P<0.05; **P<0.01. (E) Representative histograms and (F) analysis of CD4+ T-cell proliferation stimulated by *in vitro* generated dendritic cells with or without recombinant human leukocyte antigen-G (rhHLA-G) premodulation from 17 ITP patients and 15 healthy controls. *P<0.05; **P<0.01.

MDSC population, which may inhibit CD4+ T-cell proliferation and CTL-mediated platelet lysis.[46] Pickup *et al.*[47] found that G-CSF promoted differentiation of CD11b+ Ly6G+cells, increased IL-10 secretion from myeloid cells and inhibited T-cell proliferation by upregulating inducible nitric oxide synthase, arginase, IL-6, IL-10, IL-1β, and vascular endothelial growth factor, which are beneficial and protective in autoimmune diseases. On the other hand, IL-10 and IL-1β combined with GM-CSF and/or IL-2 are able to induce HLA-G protein expression.[48] This may initiate a possible positive feedback loop connecting cytokines and immune cells, and supporting immune tolerance.

Binding of HLA-G to its cognate receptor ILT may repair the disturbed cytokine profile and restore the immunosuppressive functions of various cells, such as T cells and DC. In our study, rhHLA-G could not affect platelet apoptosis directly, while rhHLA-G modulation attenuated platelets apoptosis when cocultured with PBMC from ITP patients, indicating the protective effect of HLA-G is through modulation of PBMC. sHLA-G interacts with CD8 molecules and induces apoptosis of cytotoxic T cells.[37] In addition, HLA-G can suppress the cytolytic function of CD8+ T cells through downregulation of granzyme B expression, and impair perforin granules polarization toward target cells,[49,50] which might explain its protective effect to platelets. Therefore, rhHLA-G may be a potential strategy to protect their own platelets from destruction in clinical practice.

By reprogramming the cytokine profile in ITP patients, HLA-G established an optimal environment for the impaired cell populations, such as DC, to regain their tolerogenic function. We found that lower expression of mHLA-G and ILT4 on CD14+ cells from ITP patients, confirming the loss of immune tolerance in ITP. However, the effect of HLA-G on DC is still controversial. Liang *et al.*[51] demonstrated that HLA-G-treated DC maintained a tolerogenic phenotype CD80low CD86low HLA-DRlow, and HLA-G inhibited DC maturation via IL-6 signaling pathway. However, Le Friec *et al.*[52] found that sHLA-G inhibits human DC mediated T-cell proliferation without altering DC maturation processes. In order to determine the function of DC in ITP patients as well as the protective role of HLA-G, we sorted CD14+ cells and isolated DC *in vitro*, and then modulated them with rhHLA-G. As expected, binding of HLA-G to ILT2/4 on DC suppressed the expression of costimulatory molecules and showed decreased ability to stimulate T-cell proliferation. These results indi-

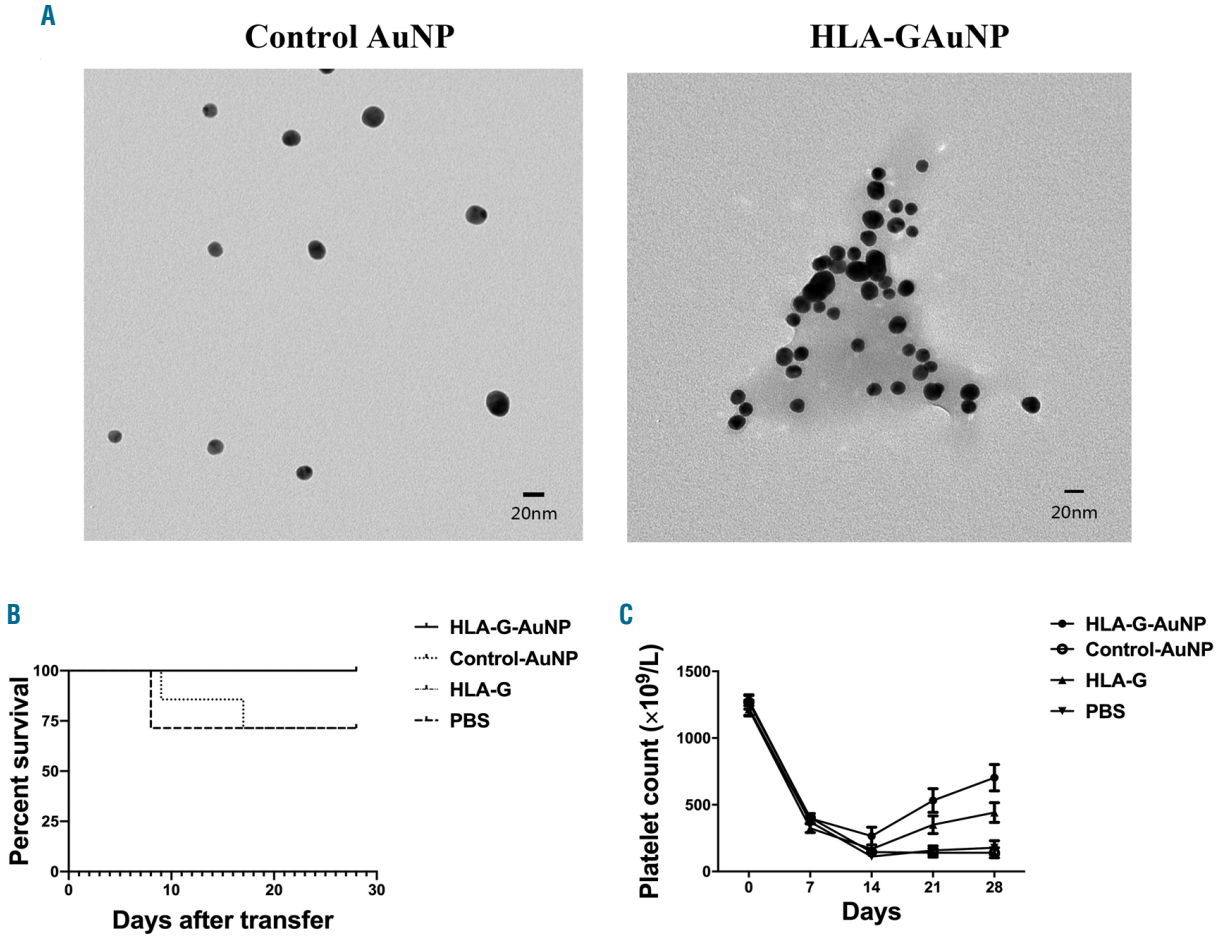

Figure 6. HLA-G treatment alleviated thrombocytopenia in a murine model of immune thrombocytopenia. (A) Capture of human leukocyte antigen-G (HLA-G) by AuNP compared with control AuNP is shown in the image from the electron micrograph. (B) Survival rate and platelet counts in a murine model of immune thrombocytopenia (ITP) from group 1-4 (n=7). (C) Platelet counts in a murine model of ITP from group 1-4 (n=7). The data are expressed as platelet counts (1x10^9/L) ± standard error of the mean over time (days).

cated that HLA-G could induce DC into tolerogenic DC.

As shown before, HLA-G exerts an immunosuppressive effect through interaction with ILT in humans. Interestingly, HLA-G could also interact with the murine receptor paired immunoglobulin-like inhibitory receptor (PIR-B), which is expressed on myeloid cell lineage and B lymphocytes, and triggers inhibitory signaling pathways. By binding with PIR-B, HLA-G aggregation could mimic the active polymeric structure found *in vivo* and has shown effectiveness in the context of allogeneic transplantation in mice.[42] Moreover, the inhibitory ability of HLA-G polymers was about 100-fold higher than HLA-G monomer in arthritis mouse model.[53] Here we demonstrated a single administration of HLA-G aggregated onto nanoparticles could alleviate thrombocytopenia in a murine model of ITP, further support the expectation of HLA-G as an immunosuppressive drug for autoimmune disease. PIR-B is absent on murine T and NK cells. Even though our *in vitro* study showed HLA-G could regulate T-cell differentiation, the HLA-G mediated effect on murine T and NK cells may be indirect.

Our data also demonstrated the safety of HLA-G protein (about 7.5 μg/per mouse) in the murine model of ITP. Liang *et al*.[51] showed the effectiveness of HLA-G in inducing transplantation tolerance at a dosage of 20 ng per mouse. Kuroki *et al*.[53] found HLA-G displayed significant anti-rheumatoid arthritis with a safety profile, even at a higher dose of 150 μg per mouse. Even though the optimal dosage of rhHLA-G used as a treatment option for ITP still needs to be further investigated, the recombinant HLA-G protein is expected to be safe *in vivo*.

Above all, the regulation of HLA-G and ILT expression is involved in the pathogenesis of ITP, and rhHLA-G could upregulate ILT expression and correct the dysfunction of immune cells. Several studies demonstrated that therapeutic reagents including dexamethasone and decitabine can induce HLA-G expression through different mechanisms.[54,55] Our results also showed increased sHLA-G expression in patients responding to HD-DXM (Figure 1D). Moreover, Pedersen and colleagues[56] found that dexamethasone combined with 1α, 25-Dihydroxyvitamin D3 induced ILT expression. Hence, whether HLA-G is involved in HD-DXM response in ITP is worthwhile investigating in the future.

Conclusions

Decreased expression of HLA-G and ILT are involved in the immunopathogenesis of ITP. rhHLA-G upregulated HLA-G and ILT expression and corrected the dysfunction of immune cells in patients with ITP. Our study sheds new light on prognosis and treatment targeting HLA-G in the management of ITP.

Disclosures

No conflicts of interest to disclose.

Contributions

QF and JM designed the experiments, analyzed the data, and wrote the manuscript; XL and ZS performed the experiments, analyzed the data, and prepared the manuscript; JP, MH, HN evaluated the data and prepared the manuscript; MX corrected the manuscript; YS, YW, ZZ, HL, LS, YZ performed the experiment and analyzed data; JY, CM and CG analyzed data, and contributed to the manuscript preparation. All authors read and approved the final manuscript.

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