SUPPLEMENTARY APPENDIX

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

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Supplemental Materials

Supplemental Method

Patients and controls

Plasma samples from 50 newly diagnosed ITP patients (26 females and 24 males; age range 16-76 years old, median 37 years old; platelet count 1-29 × 10^9/L, median 7 × 10^9/L) were collected for the detection of sHLA-G with ELISA. Among them, PBMCs from 17 ITP patients (7 females and 10 males; age range 16-76 years, median 43 years; platelet count range 1-21 × 10^9/L, median 6 × 10^9/L) were isolated for the *in vitro* investigation of mHLA-G and ILTs (**Table 1 and 2**). Samples from 15 healthy volunteers (7 females, 8 males; age range 18-50 years old, median 37 years old; platelet count 152-269 × 10^9 /L, median 214 × 10^9/L) were collected and used as controls.

The diagnosis of ITP was based on established practice guidelines (1): the age ≥ 16 years, platelet count < 30 × 10^9/L, and free from glucocorticosteroids or intravenous immunoglobulin G therapy for at least 3 months. Cases with complications, such as diabetes, hypertension, serious cardiovascular diseases, pregnancy, active infection, or other autoimmune diseases, were excluded for the study. As shown in **Table 2**, 14 patients with autoantibody (9 females and 5 males; age range 18-62 years, median 39.5 years; platelet count range 1-17 × 10^9/L, median 9 × 10^9/L) received standard ITP treatment high-dose dexamethasone (HD-DXM; 40 mg/d for 4 consecutive days). The initial response to HD-DXM was evaluated 2 weeks after HD-DXM treatment. Response (R) was defined as a platelet count ≥ 30 × 10^9/L, with at least a 2-fold increase from baseline, and an absence of bleeding. None response (NR) was defined as a platelet count of < 30 × 10^9/L, a less two-fold increase from baseline, or bleeding. Plasma and PBMCs were collected for the investigation of sHLA-G and ILTs in ITP patients received HD-DXM treatment.

Preparation of plasma and cells

Whole blood was isolated from ITP patients and healthy controls by venipuncture into ethylenediaminetetraacetic acid (EDTA). Platelets samples were separated by centrifugation at 200*g* for 10 min. Fresh platelets and platelet-poor plasma (PPP) were obtained by subsequent centrifugation at 850*g* for 5 min. PBMCs were isolated by

Ficoll density gradient separation.

$CD4^+$ T cells or $CD14^+$ monocytes were isolated by positive selection with anti-CD4 or anti-CD14 microbeads (Miltenyi Biotec) from PBMCs. The purity for $CD4^+$ and for $CD14^+$ cells was > 98% according to flow cytometry.

Cell culture

For rhHLA-G-modulated PBMC (or $CD14^+$ cells) generation, PBMCs ($CD14^+$ cells) were plated in 24-well culture plates (Corning, Corning, NY) in RPMI 1640 culture medium (Invitrogen, Grand Island, NY) with 10% FCS, 600 ng/mL rhHLA-G for 3 days.

To generate dendritic cells, $CD14^+$ cells were plated in twelve-well culture plates (Corning) in RPMI 1640 culture medium (Invitrogen) with 10% fetal calf serum (FCS), 1000 U/mL granulocyte-macrophage colony stimulating factor (GM-CSF; R&D) and 1000 U/mL interleukin-4 (IL-4; R&D) for 5 days. To activate dendritic cells, lipopolysaccharide (1 μg/mL; Sigma-Aldrich) was added with or without 600 ng/mL rhHLA-G and cells were incubated at 37 °C and 5% CO2.

Modified monoclonal antibody-specific immobilization of platelet antigen (MAIPA)

Platelet antibody testing was tested routinely in our hospital with the modified MAIPA, which was carried out as previously described in detail by Hou *et al* (2). Briefly, Washed platelets (1×10^8) obtained from healthy control blood (type O) were incubated with 110 μL of patient platelet eluate for 60 min at room temperature, washed three times with PBS/EDTA, and solubilized in 100 μL of Tris-buffered saline (TBS) containing 1% Triton X-100. Leupeptin (f.c. 100 μg/ mL) was added prior to platelet solubilization. After 30 min incubation at 4°C, insoluble material was removed by centrifugation and the supernatant was diluted 1:4 with TBS/0.5%Triton X-100/0.05% Tween-20. Fifty microliters of the monoclonal antibodies (mAbs) P2 IgG (Immunotech S.A., Marseille, France), specific for GPIIb/IIIa, or mAb SZ2 IgG (Jiangsu Institute of Hematology, Suzhou, China), specific for GPIb (final concentration 4 μg/mL) in PBS/EDTA/1%BSA were added to each well of a microtiter plate (Maxisorp; NUNC, Roskilde, Denmark), that had been coated with affinity-purified goat anti-mouse IgG (Immunotech S.A.,

Marseille, France), and incubated for 60 min at 4°C. After washes, 100µL of the diluted sensitized platelet lysate was added in duplicates to each well and incubated for another 60 min. After four subsequent washes, 100µL of alkaline phosphatase-conjugated goat anti-human IgG, either Fab-specific or Fc-specific (Sigma Chemical Co., Saint Louis, MI, USA), was added, and the plate incubated for 30 min at room temperature. After an additional six subsequent washes, substrate (p-nitrophenyl-phosphate) in 0.9 m diethanolamine buffer was added. The absorbance was recorded at 405 nm, with a reference wavelength of 492nm. Four normal PPPs were analyzed on each plate and an absorbance above the mean + 3 standard deviations of the controls was considered a positive reaction.

Enzyme-linked immunosorbent assay (ELISA) and Bio-plex Multiple Cytokine Test

The plasma concentration of soluble HLA-G was measured by ELISA (CUSABIO, Wuhan, China), according to manufacturer's instructions. The limit of detection was 1.25 ng/mL.

PBMCs (3×10^5) were cultured in 24-well plates with or without 600 ng/mL rhHLA-G. Three days later, culture supernatants were collected, and cytokine levels analyzed using a Bio-Plex Pro™ Human Cytokine 17-plex Assay (Bio-Rad, Hercules, CA, USA). With the Bio-Plex, we detected levels of granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, and tumor necrosis factor (TNF)-α, following the manufacturer's instructions. Median fluorescence intensities were collected and analyzed using a Bio-Plex 200 analyzer (Luminex, Austin, TX, USA).

Flow cytometry

For detection of mHLA-G and ILTs in PBMCs, cell surface staining was performed with phycoerythrin/Cy7 (PE/Cy7)-conjugated anti-HLA-G Abs (clone 87G), PE-conjugated anti-ILT2 Abs (clone GHI/75), fluorescein isothiocyanate (FITC)-conjugated anti-ILT4 Abs (clone 27D6), allophycocyanin (APC)-conjugated anti-CD4

Abs (clone RPA-T4), anti-CD8 Abs (clone RPA-T8), anti-CD14 Abs (clone M5E2), and/or anti-CD19 Abs (clone HIB19). Cells were incubated at room temperature for 30 min in the dark. Staining was analyzed on a Beckman Gallios^{TM} Flow Cytometer (Beckman Coulter) with Gallios^{TM} Cytometry List Mode Data Acquisition & Analysis Software (Beckman Coulter). Gates were set by their forward/side scatter light pattern to exclude the debris, and the positive expression of CD4, CD8, CD14, or CD19 based on the results of cells incubated with appropriate isotype-matched control IgG as a reference. Data are expressed as percentage (%) of labeled cells within the gates.

To analyze CD80 and CD86 expression in dendritic cells, cell surface staining was performed with FITC-conjugated anti-CD80 Abs (clone 2D10) and PE-conjugated anti-CD86 Abs (clone IT2.2). As shown in **supplemental Fig. 5**, gates were set by forward/side scatter light pattern. Apoptosis/dead cells were excluded and more than 99% of the cells were $CD14^-$ $CD83^+$ $CD80^+$ $CD86^+$ mDCs. Data are expressed as geometric mean fluorescence intensity (MFI) within the gates.

To measure the percentage of Tregs, cells were first stained with FITC-conjugated anti-CD25 Abs (clone BC96) and APC-conjugated anti-CD4 Abs (clone RPA-T4) for 30 min in the dark, permeabilized with Fixation/Permeabilization Concentrate and Diluent Kits (eBioscience), and then stained with PE-conjugated anti-Foxp3 Abs (clone 206D). Gates were set by CD4 expression and the proportion of $CD4^+$ $CD25^+$ $foxp3^+$ Tregs was calculated as previously described.

For the staining of monocytic Fcγ-receptor (CD16), PBMCs with or without rhHLA-G pre-treatment were incubated with APC-conjugated anti-CD14 Abs (clone M5E2), APC-cy7-conjugated anti-CD16 (clone 3G8) Abs for 30 minutes in the dark. Gates were set by their forward/side scatter light pattern to exclude the debris, and the positive expression of CD14 based on the appropriate isotype IgG was gated. The expression of CD16 on $CD14^+$ cells were presented as geometric mean fluorescence intensity (MFI). All flow cytometry data were analyzed and presented as previously described (3).

Isolation, CMFDA labeling, and opsonization of platelets

Peripheral blood was obtained by venipuncture into trisodium citrate from the healthy

volunteer. Platelet-rich plasma (PRP) was prepared, and platelets were counted and adjusted to 10^9/ml in the presence of 5 μM prostaglandin E1 (PGE1; Cayman Chemical). CMFDA (GM-G; Invitrogen) was added to the platelets at a final concentration of 20 μM, and incubated in the dark for 2 hours at 37 ℃, washed, and resuspended in PBS. Opsonization of GM-G-labeled platelets was performed by incubating with 5 μg murine IgG2a anti-human major histocompatibility complex (MHC) class I monoclonal antibody (W6/32; Abcam) for 30 minutes at room temperature. Platelets were washed once and used in phagocytic assay.

In vitro phagocytosis assays

In vitro phagocytosis of IgG-opsonized platelets by macrophages was carried out according to a previously described method with a few modifications (4, 5). $CD14^+$ cells were separated from 8 randomly selected treatment naïve ITP patients and further cultured for 1 hour in Iscove modified Dulbecco medium supplemented with 10% heat-inactivated human pooled AB serum in the presence of 50 ng/mL of phorbol 12-myristate 13-acetate. The cells were then washed twice with PBS, and incubated with opsonized CMFDA-labeled platelets (macrophages : platelets, 1:5), centrifuged at 200 × g for 1 minute to establish contact between macrophages and platelets, and further incubated for 1 hour on ice or at 37°C. Macrophages were treated with 0.5mM EDTA and 0.05% trypsin in PBS for 5 minutes at 37°C to remove the free platelets and detached with cell scraper. Extracellular fluorescence was then quenched with 0.1% Trypan blue. The macrophages were washed and analyzed by flow cytometry. The phagocytic index was calculated as the MFI of GM-G obtained at 37°C divided by the MFI at 0°C.(6)

The coupling of HLA-G onto nanoparticles

rhHLA-G was used as source of HLA-G and aggregated onto gold nanoparticles (AuNP) as described before (7). All glassware was first washed with aqua regia and then rinsed with MilliQ water several times prior to synthesis. Briefly, 20ml of 1mM HAuCl was refluxed for 30min. Then 2ml of 1wt.% sodium citrate was quickly added and the color of solution changed from yellow to purple within 5 min. AuNPs were then incubated at 4℃ for 3 days with 60μg HLA-G proteins (HLA-G-AuNP). The control AuNP (Ctr-

AuNP) were obtained in similar manners using an eluate without HLA-G protein. The AuNPs were then washed, centrifuged at 15000 rpm for 10 minutes and resuspended in culture medium before use.

Generation of active ITP murine model and HLA-G-AuNP administration

The active ITP murine model was constructed according to a previously established method. Blood was drawn by retro-orbital bleeding. P Platelets from C57BL/6 wild-type mice were prepared to immunize C57BL/6 CD61 KO mice by transfusion 100 μL of 10^8 above platelets weekly for 4 consecutive weeks. 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 to inhibit innate immune responses and enhance engraftment. Within 3 hours of irradiation, all SCID mice were injected intraperitoneally with 100 μL of the indicated splenocyte preparations (at 5×10^4 final). The SCID mice were separated into 4 groups, treated with intraperitoneal injection of 7.5ug HLA-G AuNP, AuNP, HLA-G protein or PBS respectively. Blood from saphenous veins was collected and diluted 10 times in Ethylene Diamine Tetraacetie Acid (EDTA) anticoagulant weekly for platelet counts.

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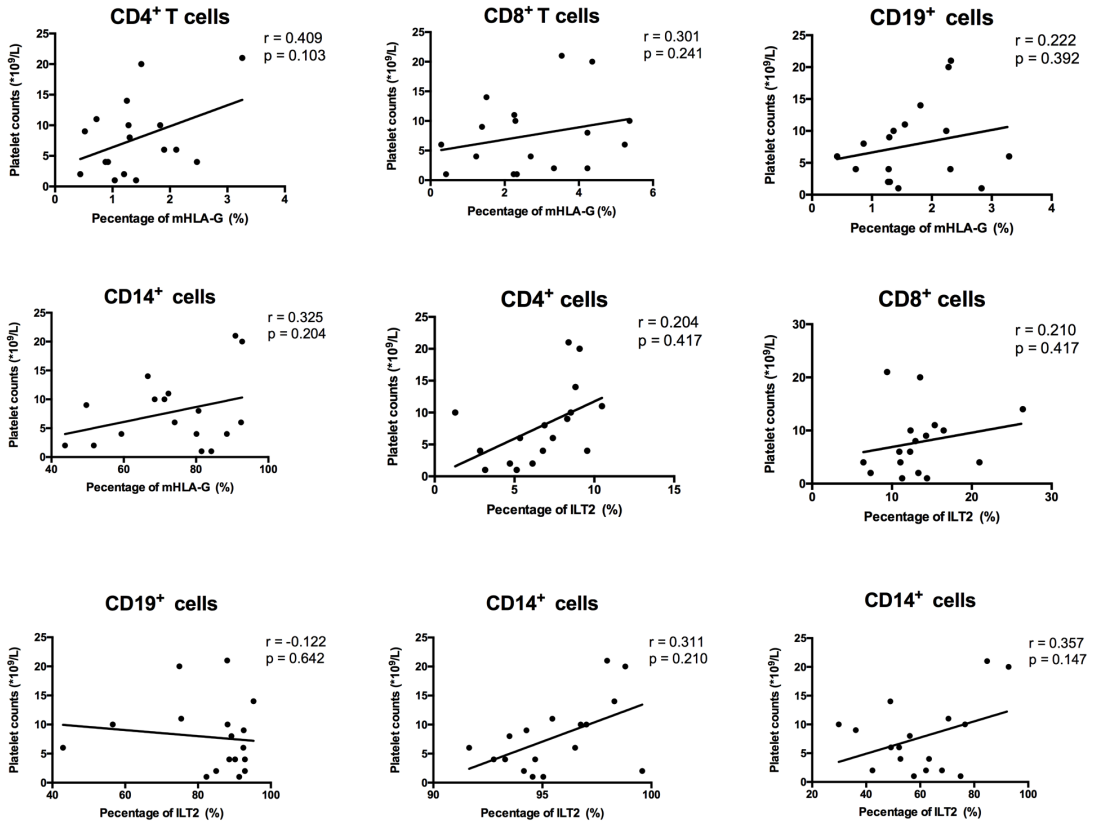
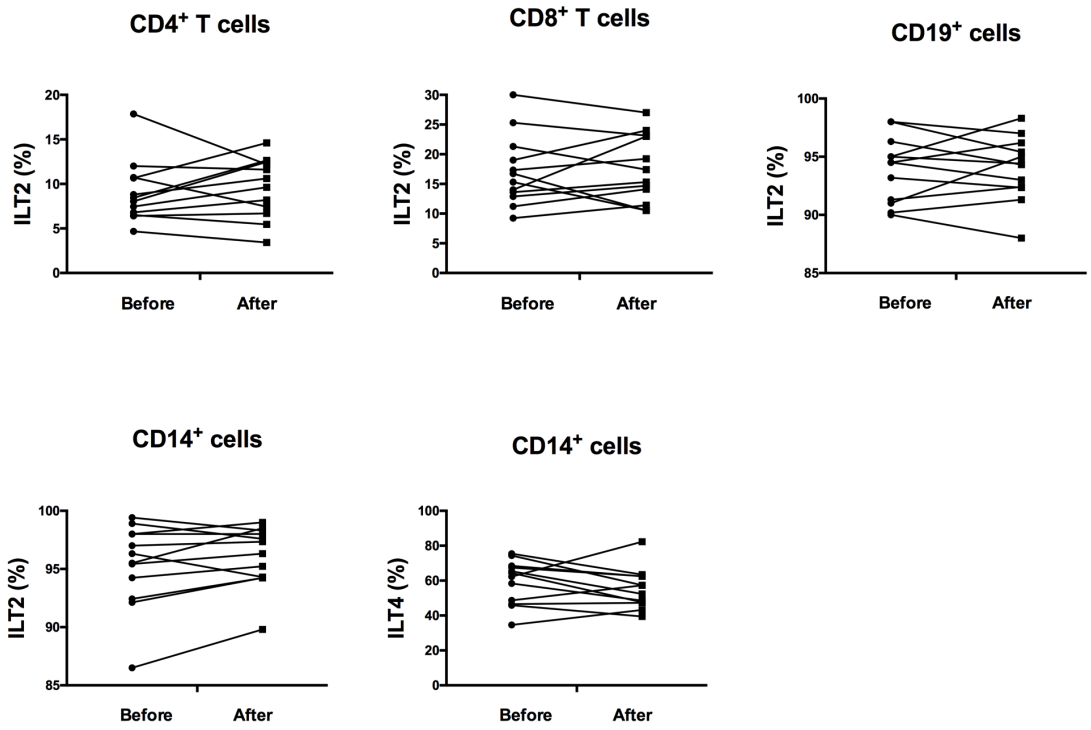
Supplemental Figures

Supplemental Figure 1



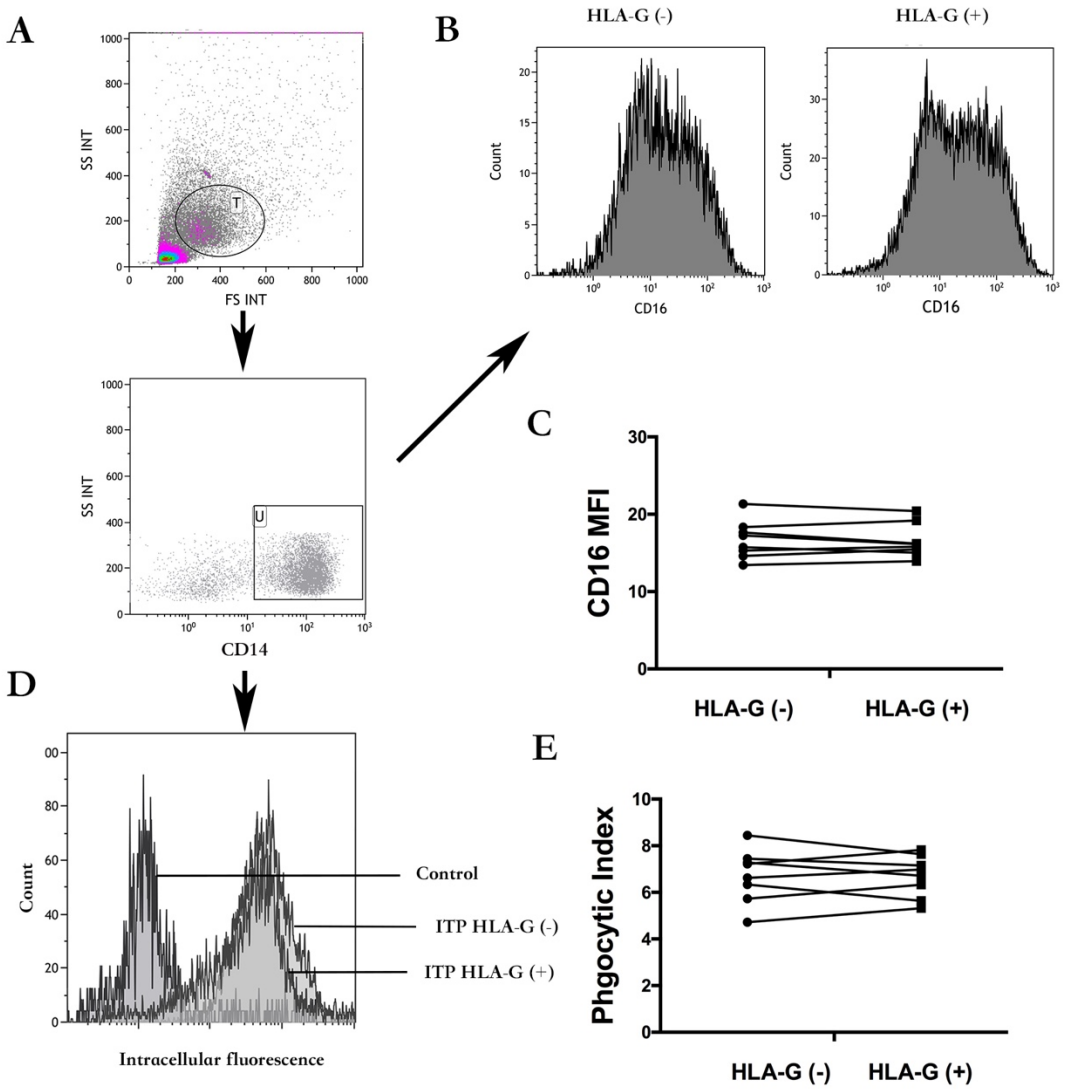
Supplemental Figure 1: Both mHLA-G and ILTs have no correlation with platelet counts. Correlation of mHLA-G or ILT2/4 expression on $CD4^+$, $CD8^+$, $CD14^+$, and $CD19^+$ cells and platelet counts in ITP patients (n = 17) and healthy controls (n = 15). Spearman correlation was used to analyze the expression of mHLA-G/ILTs and platelet counts.

Supplemental Figure 2



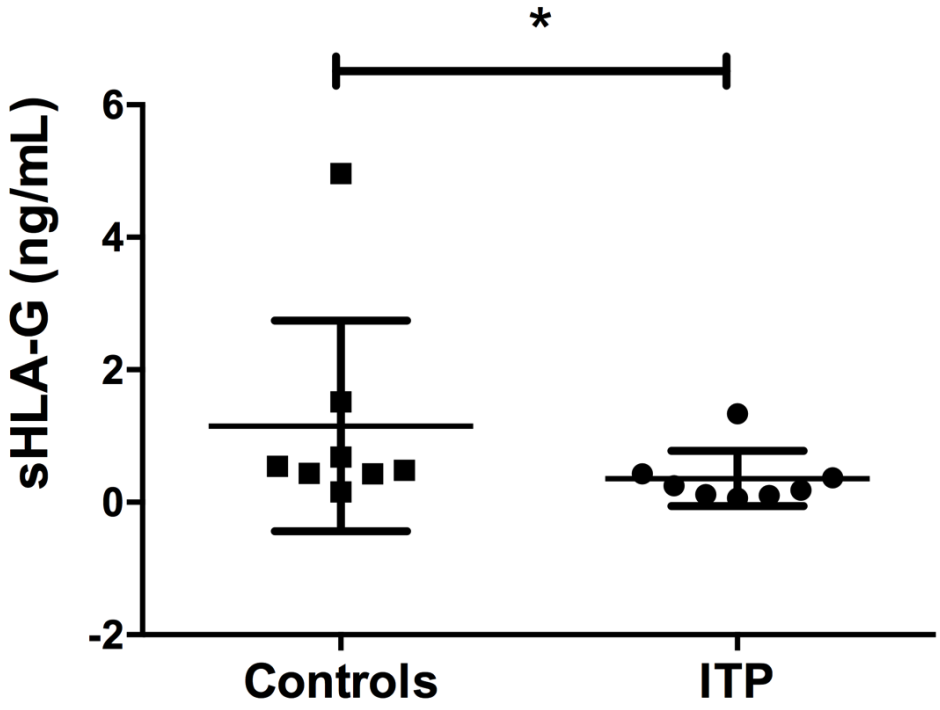
Supplemental Figure 2: The expression of ILT2 and ILT4 in patients responded to HD-DXM. Surface expression of ILT2 on $CD4^+$, $CD8^+$, $CD14^+$ and $CD19^+$ cells, and ILT4 on $CD14^+$ cells in 12 ITP patients responded to HD-DXM therapy. Differences before and after treatment were determined by paired Student t tests.

Supplemental Figure 3



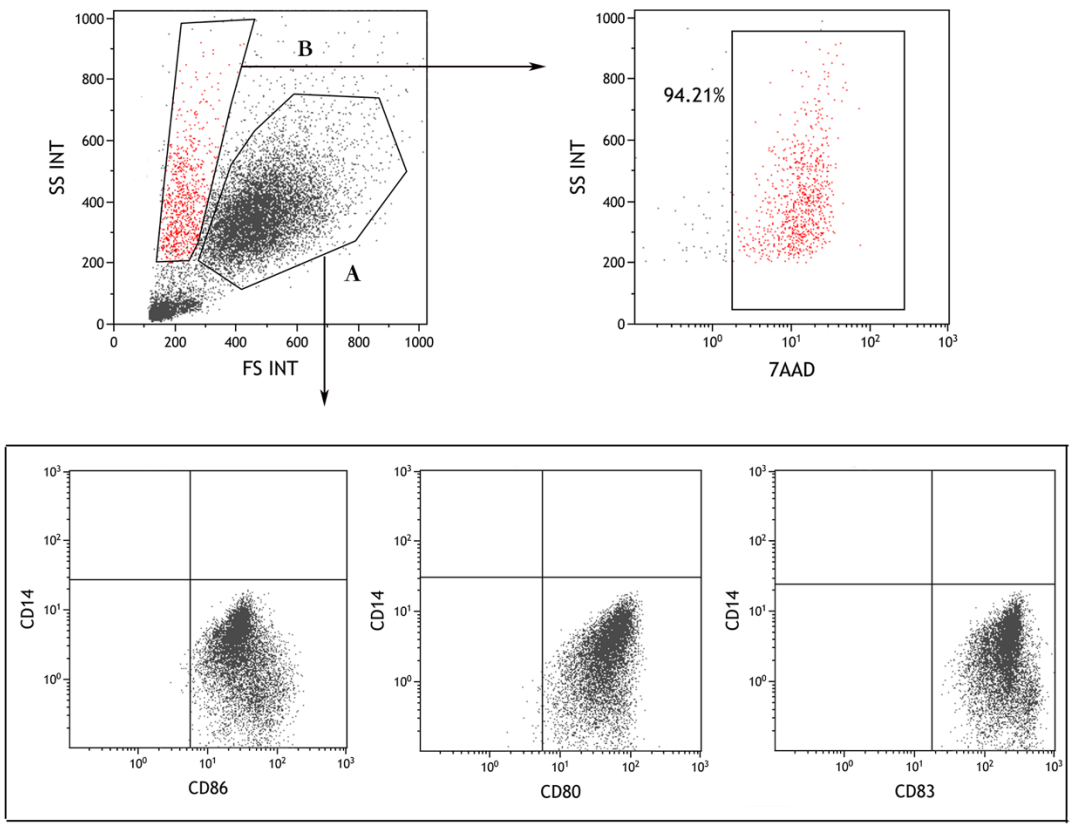
Supplemental Figure 3: The effect of rhHLA-G on CD16 expression in $CD14^+$ cells and capability to induce platelet phagocytosis of monocytes/macrophages. (A) Representative scattergrams of surface expression of CD14 from an ITP patient. Gate U represented $SSC^{low}CD14^+$ cells. (B) Representative histogram of MFI of CD16 on $CD14^+$ cells with rhHLA-G treatment and saline from an ITP patient. (C) MFI of CD16 on $CD14^+$ cells in 8 randomly selected ITP patients. (D) Representative histogram of monocytes/macrophages intracellular fluorescence with and without HLA-G treatment from an ITP patient. (E) Phagocytosis index of monocyte-derived macrophage with and without rhHLA-G treatment in 8 randomly selected ITP patients. Differences with and without rhHLA-G treatment were determined by paired Student t tests.

Supplemental Figure 4



Supplemental Figure 4: The sHLA-G level in culture system of CD14$^+$ monocytes from ITP patients and healthy controls. The concentration of sHLA-G in culture system of CD14$^+$ monocytes from 8 ITP patients and 8 healthy controls. Differences between ITP group and control group were determined by paired Student t tests. *P < 0.05; **P < 0.01.

Supplemental Figure 5



Supplemental Figure 5: Identification and gating strategy of monocyte-derived DCs.

Representative scattergrams of forward vs. side scatter of DCs from a healthy control. Gate A represented FSS^{high} SSC^{high} cells. More than 99% of the cells in Gate A were $CD14^{-}$ $CD86^{+}$ $CD80^{+}$ $CD83^{+}$ mDCs; Gate B represented FSC^{high} SSC^{low} apoptosis/dead cells. More than 90% of the cells were $7AAD^{+}$ apoptosis/dead cells. The MFI of CD80 and CD86 on mDCs in Gate A were evaluated.