Enhancing regulatory T-cell function via inhibition of high mobility group box 1 protein signaling in immune thrombocytopenia

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Supplementary Appendix for

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Supplementary Methods

Strains and backgrounds of mice for the establishment of ITP murine models

Wild-type (WT) C57BL/6 mice (male, 8-12 weeks old) as platelet donors were obtained from the Centre for New Drug Evaluation of Shandong University (Jinan, China). C57BL/6 CD61-knockout mice (B6.129S2-Itgb3tm1Hyn/JSemJ, Stock No: 008819) were originally from Jackson Laboratory. Severe combined immunodeficient (SCID) mice with a C57BL/6 background (B6.Cg-Prkdc^{scid}/SzJ, Stock No: 001913, 6-8 weeks old) as spleen-cell transfer recipients were purchased from Jackson Laboratory (Bar Harbor, Maine, USA).

Treg cell-depletion in ITP murine models

$CD4^+$ $CD25^+$ T cells were first depleted from splenocytes of immunized CD61-knockout mice with $CD61^+$ platelets before splenocyte infusion to SCID mice using Magnetic cell sorting kits (Miltenyi Biotec, Bergisch Gladbach, Germany). The deletion efficiency was over 95% measured by flow cytometry. Splenocytes except Tregs were collected for infusion to establish the ITP murine models.

$CD8^+$ T cells or $CD19^+$ B cell-depletion in ITP murine models

Splenocytes from immunized CD61-knockout mice with $CD61^+$ platelets were first depleted of either $CD8^+$ T cells, or $CD19^+$ B cells before transfer by the procedures and reagents provided by the Magnetic cell sorting kits (Miltenyi Biotec, Bergisch Gladbach, Germany). The deletion efficiency was over 95% measured by flow cytometry. Splenocytes except $CD8^+$ T cells or $CD19^+$ B cells were collected for infusion to establish the ITP murine models.

Detection of serum anti-platelet CD61 specific antibodies in ITP mice

To determine the levels of anti-platelet antibodies, 5×10^6 platelets from C57BL/6 mice were incubated with 4 μL of undiluted serum from ITP mice(1) for 30 min at

room temperature. Next, 2 μL of PE-conjugated goat anti-mouse IgG (BETHYL; 1/200 final) was added and incubated in the dark for 30 min at room temperature, then for detection and analysis using flow cytometry.

Cytokine analysis of ITP mice

Serum samples of ITP mice at day 35 were tested for cytokines using the Meso Scale Discovery kit (Meso Scale Diagnostics, Rockville, MD, USA) following the manufacturer's instructions.

Cell isolation and culture from ITP patients and healthy controls

Peripheral blood mononuclear cells (PBMCs) from ITP patients and healthy controls were isolated using Ficoll–Hypaque centrifugation (Amersham Biosciences, Piscataway, NJ, USA). Isolation of circulating $CD4^+$ T cells and naïve $CD4^+$ T cells from patients with ITP or healthy controls was performed using anti-CD4 and anti-naïve CD4 magnetic beads separation (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated cells was found to be >90% by flow cytometry.

Cells were treated with either 18β-GA at various concentrations (12.5, 25, 50, and 100 μM), recombinant human HMGB1 protein (rhHMGB1, 100 ng/mL, Sigma-Aldrich, USA), or HSF1 inhibitor KRIBB11 (2μM) (2)for 3 days. DMSO (0.1 %) was used as the vehicle control.

The isolated cells were resuspended in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin and streptomycin (Solarbio, Beijing, China). PBMCs, (naïve) $CD4^+$ T cells and Tregs were cultured with recombinant human IL-2 (10 ng/mL, R&D Systems, Minneapolis, MN, USA), anti-human CD3 and anti-human CD28 antibodies (1 μg/mL; eBioscience, San Diego, CA, USA). Naïve $CD4^+$ T cells were supplemented with anti-TGFβ1 antibodies in addition (5 ng/ml, R&D Systems, Minneapolis, MN, USA).

Cell characterization and apoptosis

Tregs cultured from human, or Tregs from ITP mice's single-cell suspensions were stained and detected using anti-human or anti-mouse CD4, CD25, and Foxp3 conjugated-antibodies (eBioscience) as per manufacturer's instructions. Macrophages from ITP mice were stained using anti-mouse F4/80-, CD80-, CD86-conjugated antibodies (eBioscience). For apoptosis analysis, peripheral blood mononuclear cells (PBMCs) from ITP patients were stained with FITC-Annexin V and PI using a Cell Apoptosis Kit (Bestbio, Shanghai, China). Data analysis was carried out using a FACS Calibur cytometer equipped with Kaluza Flow Cytometry Analysis Software (Beckman Coulter).

Suppression capacity of Tregs on $CD4^+$ $CD25^-$ effector T cells in ITP patients

$CD4^+$ $CD25^-$ T cells (effector T cells) and $CD4^+$ $CD25^+$ $CD127^-$ Tregs were isolated from ITP patients using a $CD4^+$ $CD25^+$ $CD127^-$ Regulatory T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Tregs were incubated with 25 μmol/L of 18β-GA or 0.1 % DMSO for 3 days and then the drug was eluted. $CD4^+$ $CD25^-$ T cells were labeled with CFSE (5(6)-carboxyfluorescein diacetate N-succinimidyl ester, 5 μmol/L; Sigma-Aldrich), seeded at 2×10^5 cells/well and treated with DMSO or 18β-GA, and then co-cultured with or without Tregs at a ratio of 4:1. Samples were acquired for flow cytometry analysis after 5 days of co-culture. Data were analysed using the Flow-Jo software. Division index of effector T cells is the average number of cell divisions that a cell in the original population has undergone.(3) The lower the division index, the stronger the inhibition of Tregs on effector T cells.

Suppression capacity of Tregs on $CD8^+$ cytotoxic T lymphocytes induced platelet apoptosis in ITP patients

Inhibition of Tregs towards cytotoxic T lymphocytes-induced platelet apoptosis is detailed as previously described.(4) $CD8^+$ cytotoxic T lymphocytes (CTLs) were isolated from ITP patients using anti-CD8 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Autologous platelets were prepared and used as target cells. After isolated Tregs from ITP patients treated with 18β-GA (25μM) / DMSO for 3 days, Tregs and $CD8^+$ T cells were co-cultured at a ratio of 1:4 for 5 days. Then $CD8^+$ T cells and platelets were adjusted to 10^5/mL:10^6/mL and co-cultured for 4 hours, and the cells were harvested to analyse the platelet apoptosis. Platelets were stained with JC-1 using mitochondrial membrane potential assay kit (Beyotime, Nantong, China). JC-1 aggregates exhibiting red fluorescence represented the live platelets and JC-1 monomer showing green fluorescence represented the apoptotic platelets. The percentage of JC-1 monomer on platelets was defined as the apoptosis rate. The level of spontaneous platelet apoptosis was determined in the tubes holding only culture media and platelets.

Lentiviral interference of the expression of *HMGB1* in Tregs of ITP patients

Tregs from ITP patients were isolated and transduced with an mCherry-tagged lentivirus encoding the human HMGB1 short hairpin RNA (shRNA) target sequence: TTCCTCTTCTGCTCTGAGTAT (Genechem, Shanghai, China). The transduced cells were sorted by Moflo Astrios EQ (Beckman Coulter). HMGB1 downregulation was measured using RT-PCR analysis. HMGB1/control shRNA–transfected Tregs treated with 18β-GA or 0.1% DMSO for 3 days were then co-culture with $CD4^+$ $CD25^-$ T cells for 5 days. Division index was analysed as described before.

Quantitative real time-PCR

The mRNA expression of Foxp3 and HMGB1 of patients and mice was measured by real-time reverse transcription polymerase chain reaction (RT-PCR) (Supplementary Table 3). Relative gene expressional levels were finally measured on a LightCycler®

480 System (Roche Applied Science, Mannheim, Germany). GAPDH was used as the internal standard. Gene expression was calculated by the $2^{-\triangle Ct}$ method.

Western blotting

Isolated $CD4^+$ T cells from ITP patients and healthy controls cultured for 3 days were extracted. Immunoblots were performed using monoclonal rabbit anti-human GAPDH, monoclonal rabbit anti-human NF-κB (p65) / phosphor-NF-κB (p65) and rabbit anti-human HSF1 (Cell Signaling Technology, USA), rabbit anti-HSF1 (phospho S326) antibody and polyclonal rabbit anti-human HMGB1(Abcam, USA). Semi-quantitative evaluation of total protein level was analysed by calculating the ratio of the band of HMGB1 / NF-κB / phosphor-NF-κB to the housekeeping GAPDH signal.

Determination of acetylation level of plasma HMGB1 in ITP patients

Plasma of ITP patients were immunoprecipitated with the HMGB1 polyclonal antibody or control IgG (Proteintech, USA) using protein A/G-magnetic beads (MedChemExpress, USA). Samples were then used for western blotting to detect the acetylation level of HMGB1 with the polyclonal rabbit anti-human HMGB1 antibody (Abcam, USA) and acetylated-lysine antibody (Cell Signaling Technology, USA).

Preparation of fully reduced or oxidized HMGB1

Plasma of ITP patients and healthy controls were immunoprecipitated with the HMGB1 polyclonal antibody as described above with DSS crosslinker (MedChemExpress, USA). HMGB1 from ITP patients was exposed to either 5 mM DTT (MedChemExpress, USA) or 100 mM H_2O_2 for 1 hours to get fully reduced or oxidized HMGB1, and dialyzed with 96-well microdialysis plates (Thermo Fisher, USA) before the addition to $CD4^+$ T cells isolated from ITP patients(5).

Enzyme-linked immunosorbent assay (ELISA)

The content of HMGB1 in human and mouse plasma was determined using HMGB1 ELISA kits (OM533113, OM450958, OmnimAbs, USA) following the manufacturer's instructions.

Phagocytosis of macrophages in ITP patients and mice

For ITP patients, antibody-mediated phagocytosis assays were performed as described(6) with slight modifications. $CD14^+$ monocytes from ITP patients are isolated and induced to macrophages by phorbol 12-myristate 13-acetate (PMA, Multisciences, China) for 1 hour, and stimulated with 18β-GA or 0.1% DMSO for 24 hours. Platelets from healthy controls were incubated with CD41 monoclonal antibody or an isotype control antibody (eBioscience, USA), and labeled with 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen). Then monocyte-derived macrophages were incubated with CD41 or isotype control antibody-opsonized platelets at 37 ˚C for 1 hour at a 1: 10 ratio (macrophages: platelets). APC-anti human CD61-conjugated antibody was used to label the platelets that were not devoured by macrophages. The phagocytic index was calculated as the mean fluorescence intensity (MFI) of CMFDA obtained in the group of CD41-sensitized platelets divided by the MFI of CMFDA in the group of isotype control antibody-sensitized platelets by flow cytometry. Furthermore, the platelet retention could be visualized by immunofluorescence imaging using the EVOS™ FL Auto 2 Imaging System (Thermo Fisher) under 200× magnification, and the value of endocytic platelet numbers divided by macrophage numbers was counted using Imagepro Plus 6.0 software.

For ITP mice, spleens and livers were removed to prepare single cell suspensions at day 35. Cells from spleen and liver were incubated with CD41 antibody or an isotype control antibody (eBioscience, USA)-opsonized platelets at 37 ˚C for 1 hour at a 1: 10 ratio (macrophages: platelets). APC-anti mouse CD61 antibody was used to label the platelets that were not devoured by macrophages. The phagocytic index of spleen and liver in ITP mice was calculated as the MFI of CMFDA in the group of CD41-

opsonized platelets divided by the that in the group of an isotype control antibody-opsonized platelets by flow cytometry.

Lentiviral interference of *HMGB1* in monocyte-derived macrophages of ITP patients

Macrophages from ITP patients were transfected in vitro with lentivirus encoding the human HMGB1 shRNA and sorted by Moflo Astrios EQ. HMGB1 downregulation was measured using RT-PCR analysis. HMGB1/control shRNA–transfected macrophages treated with 18β-GA or 0.1% DMSO for 24 hours were then co-culture with CD41-sensitized platelets. Phagocytic index was analysed as described above.

Platelet retention analysis using living liver and spleen imaging of ITP mice

Platelets from wild-type C57BL/6 mice were incubated with 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide (DIR, Sigma-Aldrich, USA), and injected into two groups of ITP mice at day 35 described as before(7) via the caudal vein. Mice were euthanized, and their spleens and livers were removed 30 min after injection. Platelet retention analysis was assessed by total radiant efficiency (TRE) at the region of interest (ROI) with an in vivo living imaging system (PerkinElmer, Boston, US) of liver and spleen.

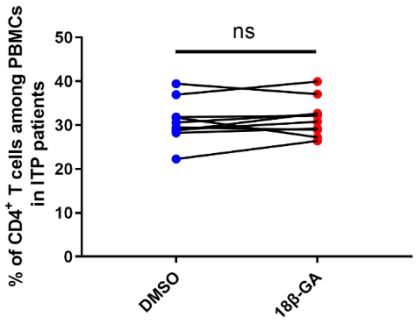
Statistical analysis

Data are reported as the mean ± SD or median with range. One-Way ANOVA, Two-Way ANOVA, and Friedman's test were performed for comparisons among the groups. Differences between the control and drug treatment groups were determined using Student's t test and Wilcoxon matched-pairs signed rank test. Correlation analysis was used to evaluate the relationship between platelets and HMGB1. Statistical analyses were performed using SPSS 23. A P values < 0.05 was considered statistically significant.

Supplementary Figure 1

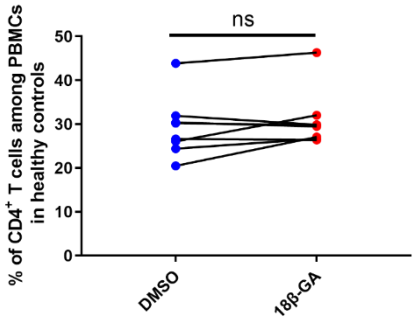
The percentages of CD4$^+$ T cells among the PBMCs remained unchanged after 18β-GA treatment in ITP patients and healthy controls.

A



The percentages of CD4$^+$ T cells among the PBMCs remained unchanged after treated with 18β-GA at the dose of 25 μM in ITP patients. Significance between the two groups of ITP patients was determined by Paired t test. n=10.

B

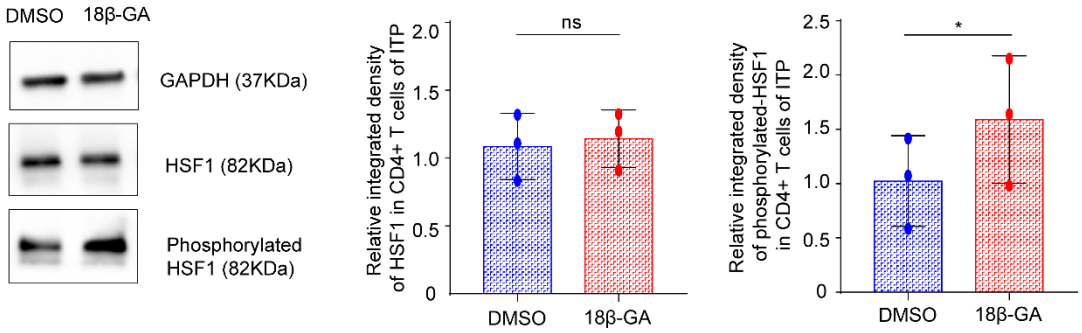


The percentages of CD4$^+$ T cells among the PBMCs remained unchanged after treated with 18β-GA at the dose of 25 μM in healthy controls. Significance between the two groups of healthy controls was determined by Wilcoxon matched-pairs signed rank test. n=8.

Supplementary Figure 2

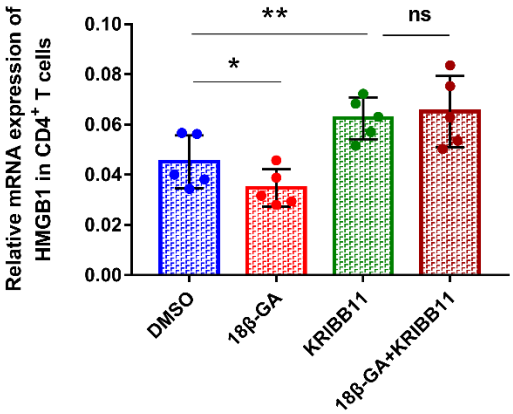
18β-GA inhibited the mRNA expression of HMGB1 via phosphorylation of HSF1.

A



The phosphorylation level of HSF1 in $CD4^+$ T cells of ITP patients was significantly increased after 18β-GA treatment for 3 days. Significance between the two groups was determined by Paired t test. n=3. *P=0.0286.

B

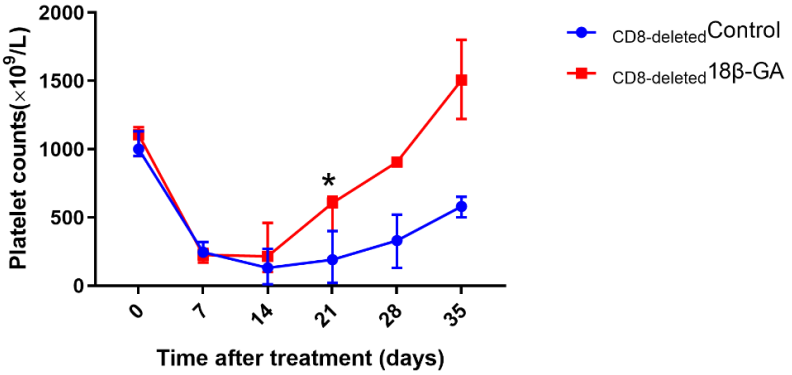


The mRNA expression of HMGB1 was significantly increased after the addition of HSF1 inhibitor KRIBB11 for 3 days, and the effect of KRIBB11 could not be reversed by 18β-GA. Significance between the two groups was determined by One-way ANOVA. n=5. **P=0.0055. Multiple comparisons: DMSO vs. 18β-GA, *P=0.0372; DMSO vs. KRIBB11, **P=0.0025.

Supplementary Figure 3

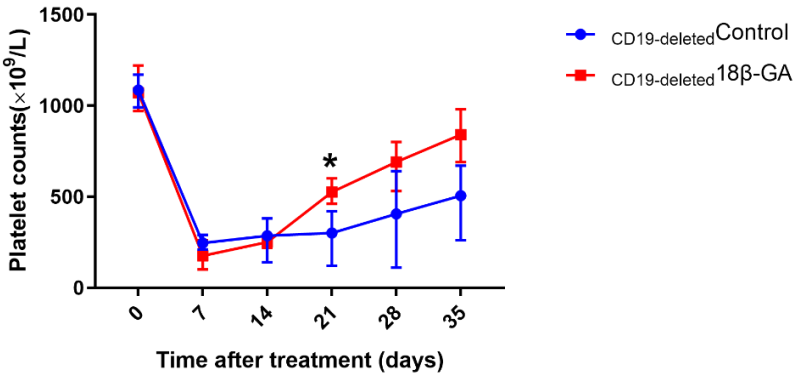
18β-GA was still capable to raise platelet counts in ITP mice after CD19$^+$ B / CD8$^+$ T cell-depletion.

A



Platelet counts increased in the 18β-GA group compared with those in the control group in mice receiving CD8$^+$ T cell-depleted splenocytes. Significant difference between groups emerged on day 21. The lines denoted the median (with range) in each group as the data is not normally distributed. Significance among groups was determined by two-way ANOVA. n=4 for each group. *P=0.0424.

B



Platelet counts increased in the 18β-GA group compared with those in the control group in mice receiving CD19$^+$ B cell-depleted splenocytes. Significant difference between

groups emerged on day 21. The lines denoted the median (with range) in each group as the data is not normally distributed. Significance among groups was determined by two-way ANOVA. n=4 for each group. *P=0.0440.

Supplementary Table 1. The key clinical information of ITP patients.

Patient Number	Sex	Age (years)	Platelet counts ($\times 10^9$/L)	Symptoms	Antiplatelet antibodies		Bleeding score(8, 9)
					Anti-GPIIb/IIIa	Anti-GPIb/IX	
1	F	47	19	FT, PT, EC	-	-	1
2	F	59	4	GIH	+	+	3
3	F	27	30	EP, GH	-	-	2
4	F	46	30	EC	-	-	1
5	F	25	14	None	+	-	0
6	M	66	9	FT, EP, OMH	ND	ND	3
7	M	60	12	EC, GH	-	-	3
8	F	44	16	PT	-	-	1
9	F	54	5	PT, EC	ND	ND	1
10	F	48	38	HM	-	-	3
11	F	56	12	None	-	+	0
12	M	23	2	OMH	-	-	2
13	M	49	10	None	+	-	0
14	F	65	4	EC, GH	ND	ND	4
15	F	49	1	PT, EP, GH, GIH, HM	+	+	3
16	M	48	25	PT, EC	-	-	1
17	F	59	23	None	-	+	0
18	M	24	6	PT, GH	-	-	2
19	F	27	12	None	ND	ND	0
20	F	18	17	None	-	+	0
21	F	53	1	PT, EC	-	-	1
22	F	53	3	PT, EC	ND	ND	1
23	F	33	6	None	-	-	0
24	F	63	23	PT, EC, GH	-	-	3
25	M	40	31	PT	+	-	1
26	M	20	2	GH	-	-	2
27	M	23	21	PT, EP, GH	-	+	2
28	F	55	26	GH	-	-	2
29	F	52	1	PT, OMH	ND	ND	2

30	F	32	1	PT, EP, HM	+	+	3
31	M	64	11	EP, OMH	-	-	2
32	F	33	13	PT	+	+	2
33	M	62	21	EC, GH	ND	ND	2
34	M	53	31	None	+	-	0
35	F	52	20	EC, EP	-	-	2
36	M	45	13	EC	-	-	1
37	M	51	19	PT, GH	-	-	2
38	M	44	33	GH	ND	ND	2
39	M	40	32	PT, EC	-	-	1
40	F	39	6	EC	-	-	1
41	F	34	38	PT, GH, HM	+	-	3
42	M	26	3	PT, OMH	ND	ND	2

F, female; M, male; FT fatigue; PT petechiae; EC ecchymoses; GIH gastrointestinal hemorrhage; EP epistaxis; GH gingival hemorrhage; OMH oral mucosal hemorrhage; HM hypermenorrhea; ND, not determined;

Supplementary Table 2. Effect of 18β-GA on cytokines in ITP mice.

Cytokines (pg/ml)	Control group	18β-GA group	*P*
IFN-γ	3.02±0.29	1.64±0.92	0.0288*
IL-10	9.33±6.08	13.38±11.15	0.5469
IL-1β	2.54±0.72	3.09±0.91	0.3782
IL-6	202.59±20.94	74.04±55.11	0.0048*
TNF-α	25.02±6.56	14.96±4.17	0.0414*
IL-12/IL-23p40	4197.77±862.20	2444.46±991.72	0.0371*
IL-17a	0.62±0.53	0.54±0.47	0.8203
TGF-β	43,264.27±9,127.85	104,416.71±10,161.79	0.0001*

Data are expressed as mean±SD. Unpaired t test, n=4 in each group.

Supplementary Table 3. Quantitative real time-PCR

	Forward	Reverse
Human		
FOXP3	5′- GTGGCCCGGATGTGTGAAG-3′	5′-GGAGCCCTTGTCGGATGATG-3′
HMGB1	5′- AGCGAACAGCAGGGTTAGTG-3′	5′-TCTAAATTGCCTCCCACTTCCA-3′
GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′	5′-TGGTGAAGACGCCAGTGGA-3′
Mice		
HMGB1	5′-AGGCTGACAAGGCTCGTT-3′	5′-GATTTTGGGGCGGTACTCAGA-3′
GAPDH	5′-TGTCTCCTGCGACTTCAACA-3′	5′-GGTGGTCCAGGGTTTCTTACT-3′

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