Thrombopoietin receptor agonists shift the balance of Fcγ receptors towards inhibitory receptor IIb on monocytes in ITP

Supplemental Data

Supplemental methods

Preparation of peripheral blood mononuclear cells, monocytes, and plasma

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by gradient centrifugation (400 × g, 20 minutes) on Ficoll-Paque (Pharmacia Diagnostics), washed twice, and resuspended for magnetic separation and cell culture. Plasma was obtained from all subjects by centrifugation of heparinized peripheral blood and stored at -80°C for cytokine analysis.

Circulating CD14+ monocytes used to determine FcγRIIa and IIb mRNA expression were isolated from PBMCs as follows: PBMCs were resuspended in AutoMACS sample buffer and anti-CD14-coated magnetic beads (Miltenyi Biotec, 20 μL per 10^7 cells) for 25 minutes with constant rotation, then sorted in a miniMACS Separator. Flow cytometric analysis revealed that the purified fractions contained more than 95% CD14+ cells.

Flow cytometric analysis of FcγRs on circulating monocytes

Antibodies were purchased from eBioscience or BD Pharmingen. Heparinized whole blood (100 μL) was stained with peridinin chlorophyll protein complex (PerCP)-Cy5.5-conjugated anti-CD14 monoclonal antibodies (mAbs) (Clone 61D3), and in combination with phycoerythrin (PE)-conjugated anti-CD64/FcγRI (clone 10.1), fluorescein isothiocyanate (FITC)-conjugated anti-CD32/FcγRII (clone 3D3), or PE-conjugated anti-CD16/FcγRIII (clone 3G8) mAbs for 30 minutes in the dark. The same-species, same-isotype IgG was used as an isotype control. For staining of FcγRIIb, heparinized whole blood was incubated with PerCP-Cy5.5-conjugated anti-CD14 mAbs, permeabilized with commercialized Fixation/Permeabilization Concentrate and Diluent Kits (eBioscience), then stained with mAbs targeting the C-terminal portion of FcγRIIb (clone EP888Y, Abcam), and incubated with PE-labeled donkey anti-rabbit IgG (Santa Cruz
Biotechnology). After lysis of erythrocytes, cells were washed with phosphate-buffered saline (PBS)/1% bovine serum albumin/0.01% NaN3 and fixed in 1% paraformaldehyde in PBS. Analysis was performed with a FACSCalibur with CellQuest Pro software (BD Biosciences). Gates were set around monocytes based on their forward/sideward light scatter pattern and CD14 expression. Aside from the classical CD14+ monocytes, CD14dimCD16− subset, which was thought to be more mature and proinflammatory was also included and analyzed. The expression of FcγRI, FcγRII, FcγRIII, and FcγRIIb on gated monocytes were presented as geometric mean fluorescence intensity (MFI), which was calculated based on the intensity of the cells incubated with appropriate isotype-matched control IgG as a reference.

**Real-time RT-PCR analysis of FcγRIIa and FcγRIIb gene expression on human and murine monocytes**

TRIzol reagent (Invitrogen) was used for the extraction of total RNA from magnetically sorted human monocytes or splenocytes of ITP mice. RNA was converted into cDNA using the PrimeScript RT Master Mix (Perfect Real Time; Takara) according to the manufacturer’s instructions. Mutiplex real-time RT-PCR was performed using SYBR® Green Real-time PCR Master Mix (Toyobo) on a LightCycler® 480 instrument II (Roche Applied Science). Primers for all mRNA assays were intron spanning. Primer sequences for human FcγRIIa, FcγRIIb, and GAPDH as well as murine FcγRII, FcγRIII, and β-actin, and PCR cycling conditions were listed in Table 1.

The comparative threshold cycle (Ct) method with arithmetic formulas was used for relative quantification of FcγRIIa and FcγRIIb mRNA according to relative expression software tool (REST). The amplification efficiency between the target (FcγRIIa and FcγRIIb) and the reference control (GADPH) were compared by the delta Ct (△△Ct) calculation.

**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 °C, 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.\(^2\)\(^3\) Macrophages were 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. Unphagocytosed platelets were removed using 0.5 mM EDTA and 0.05% trypsin in PBS for 5 minutes at 37°C, then macrophages were detached using PBS/5 mM EDTA at 4°C. Extracellular fluorescence was then quenched by addition of 0.1% trypan blue. The mixture was centrifuged at 200 × g for 10 minutes at 4°C, and the pellet was incubated with DNA stain LDS-751 (FL3; Molecular probes). The macrophages were washed and resuspended for flow cytometry analysis. Intracellular FL1 GM-Green platelet fluorescence in the nucleated events was determined. The phagocytic index was calculated as the MFI obtained at 37°C divided by the MFI at 0°C.

**Generation of active ITP murine model and romiplostim administration**

Female C57BL/6 wild-type (WT) and C57BL/6 severe combined immunodeficient (SCID) mice, were used as platelet donors and splenocyte-transfer recipients, respectively. Female C57BL/6 CD61-knockout (KO) mice were used as platelet recipients and the source of immune splenocytes, Female C57BL/6 WT mice were obtained from the Center for New Drug Evaluation of Shandong University. C57BL/6 SCID mice were purchased from the Jackson Laboratory (Bar Habor, ME) and bred in Model Animal Research Center of Nanjing University. CD61 knockout (KO) C57BL/6 mice were kindly provided by professor Jun-ling Liu from the Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai, China. All animal studies were approved by the Animal Care and Use Committee of Shandong University.
Leukoreduced platelets from C57BL/6 wild-type mice were prepared as previously described. Briefly, blood was collected by retro-orbital eye bleeding from anesthetized donor mice into phosphate buffered saline (PBS) containing citrate/phosphate, dextrose, acetate (CPDA buffer), diluted with PBS/CPDA and centrifuged at 150 × g for 15 minutes. PRP was washed 3 times by centrifugation at 450 × g for 15 minutes. The washed platelets were then resuspended in PBS/CPDA and adjusted to 10^9/ml. For immunization, each C57BL/6 CD61-KO mouse was injected with 100 μL resuspended CD61^+ platelets (10^8 platelets) via vein weekly for 3 weeks. To detect the production of anti-CD61 antibodies in the transfused C57BL/6 CD61-KO mice, sera from immunized mice at a series of titrations from 1:100 to 1:12800 was incubated with 1 × 10^6 WT CD61^+ platelets, labeled with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich), and analyzed by flow cytometry. When the titers were found to be > 1:1600, the immunized mice were killed by cervical dislocation and their spleens were removed, homogenized in RPMI 1640, and washed twice by centrifugation at 300 × g for 10 minutes. Splenocyte suspensions were further treated with ammonium-chloride-potassium (ACK) lysing solution and washed to remove red blood cells (RBCs). The splenocytes were adjusted in PBS to a concentration of 5.0 × 10^5/mL. Non-immunized C57BL/6 mice were used as source of control splenocytes.

For induction of active ITP, C57BL/6 SCID mice were first γ-irradiated (200 cGy) to deplete NK cells on day 0. The splenocytes suspension (5 × 10^4 splenocytes) from immunized CD61-KO mice was transferred intraperitoneally into SCID mice within 3 hours. Platelet counts of SCID recipients were measured weekly. Percentage of immature platelet fraction (IPF) was detected by Sysmex XE-2100 autoanalyzer.

As Eltrombopag is an oral, small molecule, non-peptide thrombopoietin-receptor agonist. It binds only to the human and chimp TPO receptor (TPO-R). Histidine 499 in the transmembrane region of the TPO-R is vital for eltrombopag binding. This residue is a leucine in all species except humans and chimpanzees, which explains the strict species restriction. If the human TPO-R was changed at residue 499 from histidine to leucine, eltrombopag was no longer active. On the contrary, changing residue 499 from a leucine to a histidine in the murine TPO-R allowed eltrombopag to be active. The strict species restriction of eltrombopag makes it difficult to carry out effectiveness studies in preclinical models. By contrast, romiplostim is an Fc-peptide fusion protein which binds to TPO-R and activates the intracellular transcriptional pathway, leading to
increased platelet production. In mice, rhesus, and humans, romiplostim produced a
dose-dependent rise in platelet counts. Based on these above-mentioned data, we used
romiplostim to stimulate platelet production in the murine model of ITP.

To investigate the effect of TPO-RAs, SCID mice were weighed and romiplostim was
intrapertoneally injected at 100 μg/kg the same day as splenocytes transferred and every 3 days
thereafter for 28 days. Saline was administrated intraperitoneally similar to romiplostim as control.
Blood from saphenous veins was collected and diluted 10 times in Ethylene Diamine Tetraacetie
Acid (EDTA) anticoagulant weekly for platelet counts. All mice were anesthetized and sacrificed
by cervical dislocation after the 28-day protocol.

In vivo platelet clearance assay
Leukoreduced platelets C57BL/6 wild-type mice were prepared and labeled with 5μM of CMFDA
at 37 °C. After an hour, platelets were washed and adjusted to a concentration of 5 ×
10⁹/mL in PBS. Labelled platelets (100ul) were injected to 5 ITP mice with romiplostim
therapy and 5 with saline via tail vein. Whole blood was collected and the fraction of labeled
platelets was determined at 1 minutes (T1), 15 minutes and 30 minutes after injection by flow
cytometry. The data were calculated as the percentage of labelled platelet at 15 or 30 minutes
divided by the fraction at 1 minute.

Mouse anti-TPO antibody enzyme-linked immunosorbent assay
Whole blood were obtained by retro-orbital eye bleeding into dry tubes and centrifuged at 6000
rpm for 5 minutes. The serum was collected and stored at -80°C. The levels of anti-TPO antibody
were assayed by enzyme-linked immunosorbent assay (ELISA) (MyBioSource) following the
manufacturer’s recommendations. The detection range for mouse anti-TPO antibody was 10
ng/mL-0.156 ng/mL.
Supplemental results

The effect of romiplostim treatment on platelet count, percentage IPF and in vivo platelet clearance in ITP mice.

The transferred SCID mice exhibited a profound thrombocytopenia within 7 days. The transferred SCID mice received an intraperitoneally injection of romiplostim showed significantly higher platelet levels and percentage IPF than those injected saline after irradiation (supplemental Figure 3A and 3B). To investigate the effect of romiplostim on the platelet destruction, CMFDA-labelled platelets from WT mice were injected to the SCID mice at the 28th day via tail vein. Results showed that at both 15 minutes and 30 minutes after injection, the percentage of CMFDA-labelled platelets was significantly higher in mice with romiplostim treatment compared with untreated mice (supplemental Figure 4).

The levels of serum anti-TPO antibody after romiplostim treatment in ITP mice.

To evaluate whether the infused cells react against the human romiplostim that was injected every three days, the levels of anti-TPO antibodies in the mouse serum was measured. However, the concentration of anti-TPO antibody in the serum was below the minimum detectable level.

Effects of eltrombopag on CD14\textsuperscript{dim}\textsuperscript{CD16} monocytes

We also analyzed the CD14\textsuperscript{dim}\textsuperscript{CD16} monocytes, and found that the frequency and absolute numbers of the subset decreased significantly in responders after eltrombopag treatment (supplemental Figure 5A, B), whereas no difference in the frequency and absolute numbers of CD14\textsuperscript{dim}\textsuperscript{CD16} monocytes was observed in non-responders (supplemental Figure 5C, D).
References


Supplemental Figure 1

(A) Representative scattergrams of surface expression of CD14+ monocytes from an ITP patient. R1 represented CD14+ monocytes. Representative histogram of MFI of FcγRI/CD64, FcγRIIA/CD32, FcγRIIB/CD32b and FcγRIII/CD16 from an ITP patient. (B) Representative scattergrams of surface expression of CD14+ monocyte-derived macrophages from the same ITP patient. R2 represented CD14+ macrophages. (C) MFI of FcγRI/CD64, FcγRIIA/CD32, FcγRIIB/CD32b and FcγRIII/CD16 on monocyte-derived macrophages in 6 untreated ITP patients and 6 healthy control. Difference between ITP patients and healthy control was determined by Mann-Whitney U tests.
Supplemental Figure 2: Percentages of DCs (CD11b^+ CD11c^+), NK cells (CD11b^+ DX5^+), and macrophages (CD11b^+ F4/80^+) in the SSC^lowCD11b^+ cell population. Representative scattergrams of surface expression of CD11b^+ splenic macrophages from an ITP mouse. R1 represented SSC^lowCD11b^+ cells. Representative histogram of percentages of DCs (CD11b^+ CD11c^+) (2.90 ± 0.65, n = 5), NK cells (CD11b^+ DX5^+) (3.28 ± 0.94, n = 5), and macrophages (CD11b^+ F4/80^+) (90.37 ± 2.55, n = 5) in the SSC^lowCD11b^+ cell population from an ITP mouse.
Supplemental Figure 3: Platelet counts and percentage IPF of ITP mice with romiplostim therapy and saline. (A) Platelet counts in ITP mice with romiplostim therapy (n = 7) and saline (n = 6). The data are expressed as platelet counts ($\times 10^9$/L) ± SEM over time (days). (B) Percentage IPF in ITP mice with romiplostim therapy (n = 5) and saline (n = 5). Differences between 2 groups were determined by Mann-Whitney U tests.
Supplemental Figure 4

Supplemental Figure 4: In vivo platelet clearance of ITP mice with romiplostim therapy. (A) Representative scattergrams of forward vs. side scatter from whole blood of an ITP mouse. R1 was set by previous addition of a PerCP-CD41 marker and the fraction of CD41\(^+\) in this gate was above 97%. (B) Representative scattergrams of fraction of CMFDA-labelled platelet at 1, 15 and 30 minutes after injection from two ITP mice with romiplostim therapy and saline. (C) The fraction of CMFDA-labelled platelet at 1, 15 and 30 minutes after injection from ITP mice with romiplostim therapy (n = 5) and saline (n = 5). The data are expressed as percentage of labelled platelet (%) ± SEM over time (minutes) Differences between ITP mice with romiplostim therapy and saline were determined by Mann-Whitney U tests.
Supplemental Figure 5: Changes in the frequency and absolute numbers of circulating CD14<sup>dim</sup>CD16<sup>+</sup> monocytes after eltrombopag treatment. (A, B) The frequency and absolute numbers of circulating CD14<sup>dim</sup>CD16<sup>+</sup> monocytes decreased significantly in responders. (C, D) No changes in the frequency and absolute number of circulating CD14<sup>dim</sup>CD16<sup>+</sup> monocytes were observed in non-responders.