High-dose dexamethasone shifts the balance of stimulatory and inhibitory Fcγ receptors on monocytes in patients with primary immune thrombocytopenia

Running title: HD-DXM regulates FcγRs in ITP

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Abstract

The human Fcγ receptor (FcγR) system is composed of two opposing families, the activating FcγRI, IIa, III, and the inhibitory FcγRIIb. The disturbed balance of the activating and inhibitory FcγRs has been implicated in the pathogenesis of many autoimmune diseases. In this study the expression of FcγRs on monocytes was determined in 23 patients with primary immune thrombocytopenia (ITP) prior to and after high-dose dexamethasone (HD-DXM) treatment. The FcγRI expression was significantly higher in ITP patients, and decreased following HD-DXM treatment. The ratio of FcγRIIa/IIb mRNA expression on monocytes was significantly higher in untreated patients than in normal controls. After HD-DXM therapy the ratio decreased, and the increased expression of FcγRIIb mRNA and protein was in parallel with a remarkable decrease in expression of FcγRIIa, FcγRI, and monocytes’ phagocytic capacity. There was no significant difference in FcγRIII expression on monocytes between patients and controls. In vitro cell culture experiments showed that DXM could induce FcγRIIa and FcγRIIb expression in monocytes from ITP patients, with FcγRIIb at higher amplitudes. These findings suggested that the disturbed FcγR balance might play a role in the pathogenesis of ITP, and HD-DXM therapy could shift monocyte FcγR balance towards the inhibitory FcγRIIb in patients with ITP.
Introduction

The human Fcγ receptor (FcγR) system is composed of two opposing families, the activating FcγRs I, IIa, III and the inhibitory FcγRIIb, the balance of which determines the magnitude of the inflammatory response. FcγRIIb is the only FcγR that has an inhibitory function, and is expressed by a variety of immune cells, including B cells, monocytes, macrophages, dendritic cells (DCs), mast cells and basophils. FcγRIIb can decrease antibody production by raising the activation threshold of B cells if cross-linked to the B cell receptor (BCR).\textsuperscript{1,2} It inhibits the function of activating FcγRs, such as phagocytosis and pro-inflammatory cytokine release by monocytes and DCs. The disturbed balance of the activating and inhibitory FcγRs has been found in many autoimmune diseases such as system lupus erythematosus (SLE)\textsuperscript{3,4} and rheumatoid arthritis (RA)\textsuperscript{5,6}. Therapeutic response in RA and SLE was often associated with the restoration of the balance between activating and inhibitory FcγRs.\textsuperscript{7,8}

Primary immune thrombocytopenia (ITP) is an immune-mediated bleeding disorder in which platelets are opsonized by autoantibodies directed against platelet surface membrane glycoproteins (GPs), and prematurely cleared by FcγR-bearing macrophages in the reticuloendothelial system.\textsuperscript{9,10} Previous studies have shown that leukocyte IgG-FcγR, such as FcγRII and III, played an important role in the phagocytosis of autoantibody coated platelets.\textsuperscript{11} McKenzie et al. demonstrated that human FcγRIIa was necessary for antibody-mediated platelet clearance by using a murine model transgenic for human FcγRIIa and lacking murine FcγRI and FcγRIII.\textsuperscript{12} Samuelsson et al. showed that decreased expression of murine inhibitory FcγRIIb was associated with increased platelet destruction.\textsuperscript{13} In a multivariate logistic regression analysis of data from 60 children with ITP, the presence of the FcγRIIb\textsuperscript{T232} variant predicted a chronic disease course.\textsuperscript{14} Recently, it was reported that \textit{Helicobacter pylori} (\textit{H. pylori}) eradication could induce a significant platelet count increase in a subset of \textit{H. pylori} infected ITP patients, and that effect was shown to be mediated by shifting monocyte Fcγ receptor balance toward the inhibitory FcγRIIb.\textsuperscript{15} Whether there was difference in the expression of FcγRs in ITP patients compared with normal controls was unknown.
The therapeutic regimens for ITP include glucorticosteroids (GCs), intravenous immunoglobulin (IVIG), splenectomy, thrombopoietin receptor agonists, and other immunosuppressive drugs. IVIG could induce a beneficial response in ITP patients via up-regulating FcγRIIb on monocytes/macrophages, and the same results were showed in mouse models of ITP. High-dose dexamethasone (HD-DXM) have been widely recognized as the first-line therapy for patients who need to be managed. However, the effects of HD-DXM on FcγR regulation remain unelucidated in ITP patients. In the present study, we demonstrated decreased expression of FcγRIIb, and elevated expression of FcγRIIa as well as FcγRIII on monocytes in ITP patients before HD-DXM therapy. The HD-DXM treatment could induce a change of FcγR balance towards the inhibitory FcγRIIb, thus providing new insights into the mechanism of HD-DXM in the treatment of ITP.
Materials and methods

Patients and controls
Twenty-three newly diagnosed primary ITP patients (16 females and 7 males, age range 16-68 years, median 43 years) were enrolled in this study. Enrollment took place between March, 2009 and April, 2010 at the Department of Hematology, Qilu Hospital, Shandong University, Jinan, China. Patients were diagnosed according to the recently-published criteria including patients’ history, physical examination, complete blood count and peripheral blood smear examination that were consistent with ITP. The patients’ platelet count ranged between 2 and 19 × 10^9/L, with a median count of 10 × 10^9/L (Supplementary table 1). None of them had been treated with GCs prior to first sampling. Patients complicated with diabetes, hypertension, cardiovascular diseases, pregnancy, active infection, or connective tissue diseases, such as systemic lupus erythematosus, were excluded. In vitro monocyte culture was performed in fourteen newly-diagnosed ITP patients before HD-DXM treatment (9 females and 5 males, age range 21-58 years, median 40.5 years; platelet count range 3-18 × 10^9/L, median 11 × 10^9/L, Supplementary table 2), and samples from ten of them were also used for assays for the phagocytic capacity of monocyte-derived macrophages.

The normal control group consisted of 20 adult healthy volunteers (13 females and 7 males, age range 21-49 years, median 32 years). Platelet counts ranged from 162 to 274 × 10^9/L, with the median count of 196.5 × 10^9/L. The phagocytic capacity of monocytes was analyzed in additional ten healthy controls (7 females and 3 males, age range 21-52 years, median 38 years).

The study was approved by the Medical Ethical Committee of Qilu Hospital; Shandong University. Informed consent was obtained from all patients before enrollment in the study in accordance with the Declaration of Helsinki.

Treatment regimen
All patients received HD-DXM 40 mg/day for four consecutive days. Initial response evaluation was made at the end of the second week after treatment initiation. The response was evaluated
according to the following criteria: complete response (CR) was defined as platelet count ≥ 100 × 10^9/L and absence of bleeding; response (R) was defined as any platelet between 30 and 100 × 10^9/L and at least doubling of the baseline counts and absence of bleeding; no response (NR) was defined as any platelet count less than 30 × 10^9/L or less than doubling of the baseline counts or bleeding.

Preparation of peripheral blood mononuclear cells, monocytes, B cells, and plasma
Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by gradient centrifugation (400 × g, 20min) on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden), washed twice, resuspended for magnetic separation, and cell culture. Plasma was obtained from all subjects by centrifugation of heparinized peripheral blood and stored at -80°C until determination of cytokines.

Circulating CD14+ monocytes were isolated from PBMCs as follows: PBMCs were resuspended in AutoMACS sample buffer, 20 μL anti-CD14-coated magnetic beads (Miltenyi Biotec) per 10^7 cells for 25min with constant rotation before purification in a miniMACS Separator. The unlabeled cells were collected for further B cells isolation with anti-CD19-coated magnetic beads (Miltenyi Biotec).

Monocytes from additional fourteen newly-diagnosed ITP patients before treatment were adjusted to 1 × 10^6/ml in RPMI-1640 culture medium supplemented with 10% heat-inactivated human pooled AB serum at a density of 2 × 10^6 cells/well in a 6-well culture plate and incubated in humidified air in 5% CO₂ at 37°C in the presence of different concentration of DXM. After 16 h, the monocyte-derived macrophages were collected for real-time PCR analysis of FcγRIIa/IIb mRNA expression.

Cell surface staining for flow cytometric determination of FcγRs
Antibodies were purchased from BD Pharmingen (San Diego, USA) or JingMei Biotech (Shenzhen, China). Heparinized whole blood (100 μL) was stained with FITC-conjugated
anti-CD14 mAbs (clone M5E2), PE-Cy5-conjugated anti-CD14 (clone 4AW14), or FITC conjugated anti-CD19 (clone HIB19) mAbs, and in combination with FITC-conjugated anti-CD16/FcγRIII (clone 3G8), PE-conjugated anti-CD32/FcγRII (clone FLI8.26), or CD64/FcγRI (clone CT101) mAbs for 30 minutes in the dark. The same-species, same-isotype IgG was used as isotype control. The cross-reactivity between anti-CD16/FcγRIII mAb (clone 3G8) and anti-CD32/FcγRII mAb (clone FLI8.26) was determined, and shown to be lower than 8% (supplementary figure). Following lysis of erythrocytes, cells were washed with PBS/1% BSA/0.01% NaN₃ and fixed in 1% paraformaldehyde PBS. Analysis was performed using a FACSCalibur with CellQuest Pro software (BD Bioscience, Mountain View, USA). Gates were set around monocytes and B cells based on their forward/sideward light scatter pattern, CD14 or CD19 expression. Surface marker expression of FcγRI, FcγRII, FcγRIII on gated CD14+ monocytes and FcγRII on CD19+ B cells were expressed as geometrical mean fluorescence intensity (MFI), which was calculated based on the intensity of the cells incubated with appropriate isotype-matched control mAbs as a reference.

**Real-time RT-PCR of FcγRIIa and FcγRIIb on monocytes and B cells**

The TRIzol reagent (Invitrogen, Carlsbad, USA) was used to isolate total RNA of monocytes or B cells. RNA was converted into cDNA using the PrimeScript® RT Reagent Kit (Perfect Real Time; Takara) according to the manufacturer’s instruction. Multiplex Real-time PCR was performed for FcγRIIa, FcγRIIb and the endogenous control (GAPDH) on an ABI PRISM_7500 Sequence Detection System (Applied Biosystem, Foster City, USA) by using SYBR Green (Applied Biosystems). The primers for all mRNA assay were intron spanning. The PCR reactions were cycled 40 times after initial denaturation and DNA Polymerase activation (95°C, 10 min) with the following parameters: denaturation 95°C, 15 s; annealing 62°C for FcγRIIa and 65°C for FcγRIIb, 15 s; extension 72°C, 45 s. The primers for FcγRIIa, FcγRIIb and GAPDH are as follows:

- **FcγRIIa-F**: ATCATTGTGGCTGTGGTTG; **FcγRIIa-R**: TGGTTCATAGTCATTGTTGGTTTCTTC; FcγRIIb-F: ATCCCACTAAATCTGTGAGGCTG; FcγRIIb-R, ACGGTTCTGTCATCCAGGCT; GAPDH-F, GCACCGTCAAGGCTGAGAAC; GAPDH-R, TGGTGAAGACGCCAGTGGA.
The comparative Ct method (using arithmetic formulae) 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 (GAPDH) were compared in order to use the delta Ct (△△Ct) calculation.

**Immunoprecipitation and Western blotting of FcγRIIa and FcγRIIb**

Monocytes (2 × 10^7 cells/sample) were solubilized in lysis buffer (1% Nonidet P-40, 10% glycerol, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 70 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.4 mM Na₃VO₄, 10 μg/ml each aprotinin, leupeptin, soybean trypsin inhibitor, and pepstatin A, and 500 μg/ml pefabloc, PH 7.4) for 1h at 4°C. The lysates were incubated overnight with a mixture of anti-FcγRII AT10 and FUN-2 antibodies (Sant Cruz Biotechnolgy, Sant Cruz, USA), and next 20 μL protein G-agarose beads (Beyotime Institute of Biotechnology, Nantong, China) were added and incubated for 2h at 4°C. The immunoprecipitates were analyzed by SDS-PAGE on polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked and incubated with goat anti-human FcγRIIa (R&D Systems, Minneapolis, USA) and FcγRIIb (abcam, Cambridge, USA), followed by polyclonal rabbit anti-goat Abs conjugated with HRP (ZSGB-BIO, Beijing, China). The reaction was developed by enhanced chemiluminescence. In addition, monocyte lysates were blotted with goat polyclonal antibodies specific for human β-actin (Sant Cruz Biotechnology, Sant Cruz, USA), as a protein loading control. Analysis of radiograms was performed by densitometry. Background values from each gel were subtracted to normalize measurements. Western blots were scored for relative densitometry normalized against β-actin.

**Enzyme-linked immunosorbent assays for IFN-γ and IL-4**

Plasma IFN-γ and IL-4 from ITP patients and healthy controls were also determined using a commercial ELISA kit (Jingmei, Beijing, China) according to manufacturer’s instructions. The detection limit for this assay was 2 pg/mL.
Isolation, CMFDA labeling and opsonization of platelets

Isolation and CMFDA labeling of platelets for phagocytic assays were done as previously described. Briefly, peripheral blood was obtained by venipuncture into trisodium citrate from the healthy volunteer. Platelet-rich plasma (PRP) was prepared, and platelets were adjusted to 10⁹/ml in the presence of 5 μM prostaglandin E1 (PGE1; Cayman, Ann Arbor, USA). CMFDA (GM-G; Invitrogen, Carlsbad, USA) was added to the platelets at a final concentration of 20 μM, and incubated in dark for 2 hours at 37°C, washed, and resuspended in PBS. For opsonization, GM-G-labeled platelets were incubated with 5 μg murine IgG2a anti-human major histocompatibility complex (MHC) class I monoclonal antibody (W6/32; abcam, Cambridge, USA) for 30 minutes at room temperature. Platelets were washed once, and used in phagocytic assay.

Evaluation of phagocytic capacity of monocyte-derived macrophages

In vitro phagocytosis of IgG-opsonized platelets by macrophages was carried out according to a previously described method with slight modification. The assay was performed in ten healthy controls, and ten patients before and after HD-DXM therapy. In short, PBMCs were obtained by Ficoll-Paque density gradient centrifugation, washed, and further purified by centrifugation on a hypotonic Percoll density gradient (1.129 g/ml; 400 × g, 30 min). Two interphases were found and the upper phase contained the enriched monocytes was collected. Monocytes were cultured in RPMI 1640 medium in a 5% CO₂/95% air atmosphere at 37°C for 2 hours, washed twice with PBS. The purity of the enriched monocytes was > 90% as assessed by flow cytometry analysis. The adherent cells were further cultured for 1 hour in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% heat-inactivated human pooled AB serum in the presence of 50 ng/ml phorbol 12-myristate 13-acetate. The cells were then washed twice with PBS, incubated with opsonized CMFDA-labeled platelets (macrophages: platelets, 1:5), centrifuged at 200 × g for 1 minute to established contact between macrophages and platelets, and further incubated for 1 hour on ice or at 37°C. To remove free platelets, the macrophages were treated with 0.5 mM EDTA and 0.05% trypsin in PBS for 5 minutes at 37°C, followed by
detachment of macrophages using PBS/5 mM EDTA at 4℃. Extracellular fluorescence was then quenched by additional of 0.1% trypan blue. The mixture was centrifuged at 200 × g for 10 minutes at 4℃, the supernatant discarded, and incubated with DNA stain LDS-751 (FL3; Molecular probes, Eugene, USA). 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℃ divided by the MFI at 0 ℃.

**Statistical analysis**

Data were expressed as mean ± SD. Statistical significance among patients prior to, after HD-DXM therapy, and normal groups was determined by ANOVA, and difference between two groups was determined by Student-Newman-Keuls test unless the data were not normally distributed, in which case the Kruskal-Wallis test and Nemenyi test, or Mann-Whitney U test was used. P value less than 0.05 was considered statistically significant.
Results

Clinical response to HD-DXM

Responses were reached in patients: CR in 16 (69.57%), R in 4 (17.39%), and NR in 3 (13.04%) of the 23 patients. Median platelet count 2 weeks after initial treatment was $137 \times 10^9/L$ (range 16-242, Supplementary table 1). No bleeding or other obvious complications were observed throughout the treatment.

Effect of HD-DXM treatment on the expression of FcγRI, FcγRII, and FcγRIII on monocytes and FcγRII on B cells in ITP patients

To investigate how 4-day HD-DXM administration act on FcγR regulation, the surface expression of FcγRs on circulating monocytes and B cells from ITP patients and healthy controls were determined. Blood samples for flow cytometry analysis of FcγRs were collected before and 2 weeks after HD-DXM initiation. Quantification of FcγRI expression on monocytes showed a significantly higher MFI in ITP patients (504.6 ± 58.6) compared with normal controls (309.8 ± 52.9, $P < 0.001$), and after 4-day HD-DXM therapy, FcγRI expression showed a significant decrease (317.5 ± 69.4, $P < 0.001$) compared with the levels before treatment (Fig. 1A, B).

FcγRII (IIa+IIb) expression on monocytes from ITP patients showed a slight increase after HD-DXM therapy compared with the levels before treatment (82.6 ± 16.0 vs. 74.5 ± 18.9), but this increase did not get statistically significant difference. Also there was no difference between ITP group and control individuals (83.2 ± 16.3, $P = 0.947$, ANOVA. Fig. 1A, C). After 4-day HD-DXM administration, monocytes from 17 patients (73.9%) showed increased FcγRII expression, and 6 patients (26.1%) had decreased FcγRII expression. There were no significant changes in FcγRIII expression on monocytes detected by FACS with anti-FcγRIII mAb after HD-DXM administration, and no difference was found among patients before, after therapy and the normal control group (9.9 ± 4.6, 8.9 ± 3.0, and 9.3 ± 3.2, respectively; $P = 0.694$, ANOVA. Fig. 1A, D).
The FcγR expression on B cells was also determined by flow cytometry in ITP patients and controls. As shown in Fig. 2A, B, the expression of FcγRII on B cells in ITP patients prior to HD-DXM administration was similar to normal controls (82.0 ± 13.0 vs. 83.2 ± 12.1), and HD-DXM treatment had no effect on B cell FcγRII expression (82.7 ± 11.8, \( P = 0.954 \), ANOVA. Fig. 2A, B).

**Gene and protein expression of FcγRIIa and IIb on monocytes**

Two types of FcγRII were expressed on monocytes: an activating receptor, FcγRIIa, and an inhibitory receptor, FcγRIIb.\(^{26}\) To evaluate the effect of HD-DXM on FcγRIIa/IIb mRNA expression of monocytes, real-time RT-PCR was performed on sorted monocytes before or after HD-DXM therapy using specific primers for FcγRIIa and IIb. The results showed that the ratio of FcγRIIa/IIb mRNA expression on monocytes was significantly higher in untreated ITP patients than normal controls (5.88 ± 5.12 vs. 0.31 ± 0.29, \( P < 0.001 \)). After HD-DXM therapy, the ratio of FcγRIIa/IIb mRNA decreased significantly (0.61 ± 0.86, \( P < 0.001 \)) in comparison with the level before treatment (Fig. 3A). Of the 23 patients, 22 showed decreased FcγRIIa mRNA expression, and 15 patients had increased FcγRIIb mRNA expression levels on monocytes after HD-DXM administration (Fig. 3B). Decreased ratios of FcγRIIa/IIb mRNA expression were observed in 18 of the 23 patients, whereas increased FcγRIIa/IIb ratios were found in 5 patients including 3 nonresponders and 2 responsive cases (Fig. 3C). However, no difference in FcγRIIb on B cells was found among patients prior to or post therapy, and normal controls (data not shown).

To further evaluate the effects of HD-DXM on protein expression levels of FcγRIIa and IIb, immunoprecipitation and western blotting were performed. The patients after therapy had a marked downregulation of FcγRIIa protein expression on monocytes compared with the levels before treatment (2.05 ± 0.81 vs. 1.07 ± 0.96, \( P < 0.001 \), Student’s \( t \) test, Fig. 4A). At the same time, the decreased FcγRIIa protein levels were associated the upregulated FcγRIIb levels. The relative densitometry of monocyte FcγRIIb in ITP group was 0.13 ± 0.31, which increased...
significantly after HD-DXM therapy (1.23 ± 1.25, \( P < 0.001 \), Mann-Whitney \( U \) test). 19 of the 23 patients had undetectable FcγRIIb expression before therapy, and 2 weeks after HD-DXM initiation, FcγRIIb expression was detectable in 14 patients (Fig. 4B). These results indicate that HD-DXM can modulate FcγRIIa/IIb expression, shifting the balance of the two opposing FcγR isoforms from an activating phenotype to an inhibitory phenotype on monocytes in patients with ITP.

**Effects of HD-DXM therapy on monocyte/macrophage's phagocytic capacity in ITP patients**

The balance of activating and inhibitory FcγRs determines the magnitude of the cellular response in monocytes. To evaluate whether modulation of FcγRs by HD-DXM, as shown above, has an effect on monocyte phagocytic capacity, monocytes from ITP patients before and after HD-DXM treatment were incubated with opsonized CMFDA-labeled platelets for FcγR-mediated phagocytosis assay. Monocytes from untreated ITP patients demonstrated higher phagocytic capacity compared with healthy controls (MFI ratio: 3.5 ± 0.7 vs. 2.2 ± 0.5, \( P < 0.05 \)). After HD-DXM therapy, phagocytic capacity of monocytes decreased significantly (2.6 ± 1.0, \( P < 0.05 \), Fig. 5A, B).

**Changes of plasma IFN-γ and IL-4 after HD-DXM therapy**

To examine the potential role of HD-DXM therapy on Th1/Th2 regulation, plasma IFN-γ and IL-4 levels in ITP patients and healthy controls were determined. In consistence with previous reports,\(^{27,28}\) significantly higher levels of plasma IFN-γ and lower IL-4 were found in untreated patients compared with healthy controls (IFN-γ: 62.7 ± 13.0 vs. 37.4 ± 4.8 pg/mL, \( P < 0.001 \); and IL-4: 8.9 ± 2.7 vs. 18.3 ± 4.7 pg/mL, \( P < 0.001 \), respectively). Plasma IFN-γ levels in untreated patients decreased significantly after HD-DXM administration (40.3 ± 11.0 pg/mL, \( P < 0.001 \)), while post-treatment levels of IL-4 increased significantly compared with the levels before treatment (18.5 ± 4.2 pg/mL, \( P < 0.001 \); Fig. 6).
**In vitro effects of DXM on regulation of FcγRIIa/IIb mRNA expression in cultured monocytes/macrophages**

Since the mRNA ratio of FcγRIIa/IIb was increased in untreated ITP patients, and reduced significantly after HD-DXM therapy, we investigated whether DXM, is able to act directly on monocytes/macrophages' FcγRIIa/IIb regulation. We cultured magnetically isolated monocytes from untreated ITP patients in the presence of different concentrations of DXM. After 16 h, the cells were harvested for real-time PCR. As shown in Fig. 7, DXM could increase the mRNA expression of both FcγRIIa and FcγRIIb, with FcγRIIb at higher amplitudes. This was a little different from the *in vivo* effect of HD-DXM (Fig. 3A, B), which demonstrated that most of ITP patients (22/23) showed downregulated FcγRIIa mRNA expression after HD-DXM administration.

Further studies were needed to elucidate the discrepancy between *in vivo* and *in vitro* effects of DXM on FcγRII regulation.
Discussion

In this study, oral HD-DXM was used in a single 4-day course as the initial treatment schedule in previously untreated ITP patients with active diseases. As shown in Supplementary table 1, HD-DXM had a good initial response in ITP patients, which was in accordance with the previous reports.\textsuperscript{18-20} In the present study, FcγRs were investigated in ITP patients prior to and post the HD-DXM treatment. It was demonstrated that HD-DXM therapy could downregulate FcγRI expression on monocytes. The FcγRII and III expression showed no change after HD-DXM therapy. Since FcγRIIa could not be discriminated from the only inhibitory FcγRIIb by FACS, real-time RT-PCR and western immunoblotting were performed. The data demonstrated that the FcγRIIb mRNA and protein expression levels on monocytes increased significantly after HD-DXM treatment. Upregulation of FcγRIIb was in parallel with a remarkably decreased expression of FcγRIIa. However, the mRNA expression level of FcγRIIb on B cells was not affected by HD-DXM therapy.

The functional properties of monocytes or macrophages such as phagocytosis and antigen presentation were controlled by the balance of the activating FcγRs and the inhibitory receptor, FcγRIIb.\textsuperscript{29-31} Our results demonstrated that circulating monocytes from untreated ITP patients exhibited enhanced FcγR-mediated phagocytic capacity compared with healthy controls, and that trend was consistent with the upregulated FcγRI expression and increased FcγRIIa/IIb mRNA ratio. These abnormalities of FcγRs were corrected after HD-DXM therapy, suggesting the alteration of monocytes from an activating phenotype to an inhibitory phenotype. However, these changes were not found in nonresponders to HD-DXM and in 2 of 4 responders. Several studies demonstrated that IFN-γ, one of the Th1 cytokines, was upregulated,\textsuperscript{27} while IL-4 was downregulated in active ITP.\textsuperscript{28} Studies by Pricopl \textit{et al.} showed that IFN-γ, one of the Th1 cytokines, could downregulate FcγRIIb mRNA and cell surface expression on monocytes.\textsuperscript{26} On the contrary, IL-4, which belongs to Th2 cytokines, had the opposite function on monocyte FcγRIIb expression.\textsuperscript{26,32} In accordance with our previous report,\textsuperscript{28} the present data on plasma IFN-γ/IL-4 further suggested that Th1/Th2 balance in ITP was disturbed and HD-DXM could resume the balance. This prompted us to study whether HD-DXM's function on monocytes' FcγR regulation was a direct effect or a secondary one mediated by cytokines secreted by T cells. \textit{In
vitro monocytes culture experiments showed that DXM could upregulate mRNA expression of both FcγRIIa and FcγRIIb, which bore a little difference from Comber’s results. In their work, Comber et al. showed that treatment with DXM had no significant effect on the expression of FcγRII transcripts in in vitro cultured monocytes. Disparity in cell preparation procedures, or mRNA detecting methods might contribute to the discrepancy.

ITP is an autoimmune disorder in which platelets are opsonized by anti-platelet autoantibodies and phagocytosed by macrophages via FcγRs. Anti-GPIIb/IIIa antibodies are one of the primary autoantibodies found in ITP. It has been demonstrated that anti-GPIIb/IIIa autoantibodies are predominant of the IgG1 subclass, which has the highest affinity for FcγRII. FcγRs expressed on macrophages in the RES play two roles in ITP: platelet destruction and sustained autoimmune responses to platelet antigens. Increase in the expression of inhibitory FcγRIIb relative to the activating FcγRs could attenuate the dysfunction of macrophage in ITP. Recently, a single HD-DXM course was administered as first-line therapy in adult patients with ITP. HD-DXM could increase the number of CD4+Foxp3+ Treg cells. The Th1 cytokine dominance in ITP could also be corrected by HD-DXM. Our results showed that DXM could shift FcγR balance toward the inhibitory FcγRIIb on monocytes in ITP, and correct the enhanced phagocytic capacity of monocytes, thus providing a new mechanism for HD-DXM’s therapeutic effect on ITP.

The regulation of FcγRIIb expression is complex and differs depending on the cell types. Besides the above mentioned cytokines IL-4 and IFN-γ, activation of complement may also affect FcγRIIb expression. Shushakova et al. demonstrated that the complement component C5a could directly upregulate FcγRIII and downregulate FcγRIIb expression by alveolar macrophage. ITP is a heterogeneous and complex autoimmune disorder. Besides our findings of HD-DXM on FcγRIIb regulation, recent studies by Pels suggested that the therapeutic effects of IVIG on ITP was possibly mediated by upregulation of FcγRIIb on macrophages. Asahi demonstrated that H. pylori eradication could induce the recovery of platelet counts in a certain part of H. pylori-positive ITP patients, which was associated with a change in FcγR balance toward the inhibitory FcγRIIb on monocytes. Thus it appeared that the FcγRIIb upregulation in ITP might be a common phenomenon in the recovering process induced by immune-modulating agents, and
the precise mechanism of FcγRIIb modulation in ITP await further investigation.

In summary, decreased FcγRIIb expression and increased FcγRIIa, and FcγRI expression in untreated ITP patients suggested the possible role of the disturbed FcγR balance in the pathogenesis of ITP. HD-DXM may reduce inflammation in ITP by restoring the balance of activating and inhibitory FcγRs, raising the threshold for monocyte activation and decreasing the phagocytic capacity of monocytes. The role of FcγRs remains to be elucidated, but they are possibly involved in the efficacy of HD-DXM in ITP treatment.
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Author Contributions

X.-G.L - designed research, performed research, analyzed data and wrote the paper; S.-H.M - performed research and analyzed data; J.-Z.S - performed research and analyzed data; J.R - performed research; Y.S - performed research; L.S - performed research and analyzed data; X.-Y.D - performed research and analyzed data; P.Q - performed research, C.-S.G - performed research and analyzed data; M.H - performed research and analyzed data, and J.P - designed research, performed research, analyzed data and wrote the paper.

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References


Figure Legends

Figure 1
Expression of FcγRs on monocytes from ITP patients before or after therapy and from normal controls.

(A) Representative scattergrams of surface expression of FcγRIII/CD16, FcγRII/CD32 and FcγRI/CD64 on CD14+ monocytes from a healthy control and an ITP patient before HD-DXM therapy. CD14+ monocytes were gated based on their forward/sideward scatter. (B-D) Expression (mean fluorescence intensity, MFI) of FcγRI/CD64 (B), FcγRII/CD32 (C), and FcγRIII/CD16 (D) on monocytes from ITP patients before and after HD-DXM therapy, and from normal controls. Significance among patients prior to, after HD-DXM therapy, and normal groups was determined by ANOVA, and differences between two groups were compared by Student-Newman-Keuls test. Bars represent SD. * P < 0.001.

Figure 2
Expression of FcγRII/CD32 on B cells from ITP patients before, after therapy and from normal controls.

(A) Representative scattergrams of surface expression of FcγRII/CD32 on CD19+ B cells from a healthy control and an ITP patient before HD-DXM therapy. CD19+ B cells were gated based on their forward/sideward scatter. (B) Expression of FcγRII/CD32 (MFI) on B cells from ITP patients before and after HD-DXM therapy, and from normal controls.

Figure 3
FcγRIIa/IIb mRNA ratio on monocytes from ITP patients and controls, and changes of FcγRIIa, IIb mRNA expression levels in ITP patients before and after treatment.

(A) FcγRIIa/IIb mRNA expression ratio on monocytes from ITP patients before and after treatment and controls was determined by quantitative PCR. The difference among these 3 groups was analyzed using Kruskal-Wallis test and difference between two groups was analyzed using Nemenyi test. * P < 0.001. (B) The FcγRIIa, IIb mRNA expression levels on monocytes from ITP patients were measured before and after HD-DXM treatment. * Patients who had decreased FcγRIIb mRNA expression after HD-DXM treatment. (C) After administration of HD-DXM, 18 of 23 patients had reduced FcγRIIa/IIb mRNA expression ratios, while the other 5 have similar or higher FcγRIIa/IIb mRNA expression ratios compared with those before treatment. * Patients who showed increased FcγRIIa/IIb mRNA ratios after HD-DXM treatment.
**Figure 4**  
Modulation of FcγRIIa, IIb protein expression on monocytes prior to treatment and 2 wk after initiation of HD-DXM therapy.  
(A) Representative Western blotting of monocyte lysates immunoprecipitated with anti-FcγRII cocktail and blotted with goat anti-human FcγRIIa, FcγRIIb and β-actin, were shown. (B) Changes of FcγRIIa and FcγRIIb protein expression on monocytes in patients after HD-DXM treatment. 14 of the 23 patients had increased FcγRIIb expression. * Patients who still showed no FcγRIIb expression after HD-DXM therapy.

**Figure 5**  
Phagocytic capacity of monocyte-derived macrophages from ITP patients and normal controls.  
Platelets were isolated, labeled with the cell tracker CMFDA, opsonized with W6/32 antibody, and incubated with monocyte-derived macrophages for 1 hour, and analyzed on a flow cytometry. (A) Representative histogram of macrophage intracellular fluorescence from one ITP patient prior to- and post-treatment. Platelets were labeled with the cell tracker CMFDA, opsonized with W6/32, and coincubated with macrophages at 37°C for 1 hour. Negative controls were performed at 4°C for 1h. The phagocytic index was calculated as the MFI obtained at 37°C divided by the MFI at 0 °C. (B) Phagocytic capacity of macrophages from ITP patients before and after HD-DXM therapy, and from normal controls. * P < 0.05

**Figure 6**  
Plasma levels of IFN-γ and IL-4 in untreated ITP patients, patients after HD-DXM therapy, and healthy controls.  
Levels of plasma IFN-γ and IL-4 were determined by ELISA. Elevated plasma IFN-γ level, and decreased IL-4 level was detected in untreated ITP patients compared with patients after HD-DXM therapy (IFN-γ: 62.7 ± 13.0 vs. 40.3 ± 11.0 pg/mL, P < 0.001; and IL-4: 8.9 ± 2.7 vs. 18.5 ± 4.2 pg/ml, P < 0.001, respectively) or healthy controls (37.4 ± 4.8 pg/ml, P < 0.001; and 18.3 ± 4.7 pg/ml, P < 0.001, respectively). There was no significant difference in plasma IFN-γ or IL-4 levels between post-treatment ITP patients and healthy controls (P > 0.05). *P < 0.001.
Figure 7

**DXM upregulates the mRNA expression of FcγRIIa and IIb on *in vitro* cultured monocytes.**

Magnetically isolated monocytes from untreated ITP patients were cultured in the presence of different concentrations of DXM for 16 h, and harvested for real-time PCR. β-actin mRNA was used as controls. Relative mRNA level represents the FcγRIIa or IIb expression normalized by β-actin (FcγRIIa/β-actin, FcγRIIb/β-actin) in each cell culture with respect to the expression of FcγRIIa/β-actin or FcγRIIb/β-actin measured in untreated cells. *P < 0.05
Figure 1

A

Control

ITP

CD16

CD32

CD64

CD14
Figure 1

B

MFI of CD64 on monocytes

- patients before HD-DXM
- patients after HD-DXM
- controls

* indicates significant difference.
Figure 1

MFI of CD32 on monocytes

- ◼ patients before HD-DXM
- ▼ patients after HD-DXM
- ▲ controls
Figure 1

D

MFI of CD16 on monocytes

- patients before HD-DXM
- patients after HD-DXM
- controls
Figure 2

A) Control

CD32 vs. CD19

CD32: 0% to 90.54%
CD19: 0% to 90.09%

A) ITP

CD32 vs. CD19

CD32: 0% to 9.46%
CD19: 0% to 9.91%
Figure 2

MFI of CD32 on B cells

- 
- patients before HD-DXM
- patients after HD-DXM
- controls
Figure 3

A

- **patients before HD-DXM**
- **patients after HD-DXM**
- **controls**

FcyRllα/IIb ratio

- * denotes statistical significance.
Figure 3
Figure 3

C

FcyRIIa/Ilb mRNA ratio

before HD-DXM

after HD-DXM

patients

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Figure 4

A

Patients

1  2  3

- before HD-DXM
- after HD-DXM

1  2  3

FcγRIIa
FcγRIIb
β-actin

4  5  6

FcγRIIa
FcγRIIb
β-actin
Figure 4

B

- FcγRIIa before HD-DXM
- FcγRIIa after HD-DXM
- FcγRIIb before HD-DXM
- FcγRIIb after HD-DXM

relative densitometry

patients
Figure 5

- Before HD-DXM
- After HD-DXM
- Control

Intracellular fluorescence
Figure 6

Plasma cytokine levels (pg/ml) for IFN-γ and IL-4 before and after HD-DXM compared to control.

- IFN-γ before HD-DXM: High levels compared to control.
- IFN-γ after HD-DXM: Moderate levels compared to before.
- IL-4 before HD-DXM: Low levels compared to control.
- IL-4 after HD-DXM: No significant change.

Significance indicated by asterisks (*) for comparison.
Figure 7

The graph shows the relative quantitative mRNA levels for Fcγ RIIa and Fcγ RIIb at different concentrations of DEX (μM). The x-axis represents the concentration of DEX (0, 0.5, 1.0, 2.0 μM), and the y-axis represents the relative quantitative mRNA level. Significant differences are indicated by * symbols.
High-dose dexamethasone shifts the balance of stimulatory and inhibitory Fcγ receptors on monocytes in patients with primary immune thrombocytopenia

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