

Increased multidrug resistance-associated protein activity in mononuclear cells of patients with systemic lupus erythematosus

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Abstract

Multidrug resistance-associated proteins (MRPs, ATP binding cassette sub-family C), P-glycoprotein (P-gp) and ATP binding cassette (ABC) sub-family G member 2 (ABCG2) are important drug efflux pumps emerging after long-term medications. We intended to detect whether these molecules are expressed in immune-related cells of patients with systemic lupus erythematosus (SLE) on long-term immunosuppressants.

Methods

Mono nuclear cells (MNC) and polymorphonuclear neutrophils (PMN) were isolated from healthy volunteers and SLE patients. The MPR-mediated transport activity of these cells was measured by using carboxy-2',7'-dichlorofluorescein diacetate (CFDA) efflux assay. P-gp-mediated transport activity of cells was detected by rhodamine 123 efflux assay. ABCG2-mediated transport assay was evaluated by mitoxantrone efflux assay. The intracellular expression of MRP₁, MRP₂, and MRP₃ molecules in MNC was detected by flow cytometry. The results were compared between MNC and PMN derived from normal and SLE groups.

Results

The specific dye-efflux function of MRPs in SLE-MNC is significantly higher than normal MNC. However, the expression of MRP₁, MRP₂, and MRP₃ molecules in SLE-MNC was not different from normal MNC. We also noted that only the duration of corticosteroid treatment in different clinical/laboratory parameters was significantly correlated with the increased activity of MRPs in SLE-MNC.

Conclusions

These results suggest that increased activity of MRPs in SLE-MNC is elicited by long-term corticosteroid therapy.

Key words

Multidrug resistance, multidrug resistance-associated proteins, P-glycoprotein, systemic lupus erythematosus, mononuclear cells.

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Abbreviations:

MDR:	multidrug resistance
MRPs:	multidrug resistance-associated proteins
P-gp:	P-glycoprotein
ABC:	ATP binding cassette
ABCG2:	ATP binding cassette subfamily G member 2
CFDA:	carboxy-2',7'-dichlorofluorescein diacetate
SLE:	systemic lupus erythematosus
MNC:	mononuclear cells
PMN:	polymorphonuclear neutrophils
DMARDs:	disease-modifying anti-rheumatic drugs

Competing interests: none declared.

Introduction

Systemic lupus erythematosus (SLE) is an archetype of systemic autoimmune disorder characterized by the production of diverse autoantibodies by autoreactive B and T cells. The main treatment strategy is to suppress the autoreactivity by using glucocorticosteroids and/or other immunosuppressants such as hydroxychloroquine, cyclosporine A, cyclophosphamide and azathioprine. However, long-term use of immunosuppressants may lead to drug resistance with gradual loss of drug efficacy in these patients (1).

It is conceivable that the occurrence of drug resistance is a crucial cause of treatment failure in patients with cancer (2, 3) and infectious diseases (4, 5). The molecular mechanisms underlying multi-drug resistance (MDR) are related to the over-activity of drug efflux pumps that belong to ATP binding cassette (ABC) super-family. These molecules are capable of mediating cellular extrusion of large variety of therapeutic drugs and are referred as MDR proteins. Three categories of MDR molecules including multi-drug resistance-associated proteins (MRPs, belong to ATP binding cassette sub-family C), P-glycoprotein (P-gp), and ATP binding cassette sub-family G member 2 (ABCG2) are the most important drug transporters to induce MDR in cancer cell lines (6). Over-expression of these molecules leads to reduced intracellular concentration of xenobiotics or different drugs such as corticosteroids, hydroxychloroquine, azathioprine and cyclosporine (7). Thus, these MDR molecules act as double-edged swords in either protection from cytotoxicity or development of resistance to offensive chemicals. Many authors demonstrated that over-expression of P-gp is related to poor response to disease-modifying anti-rheumatic drugs (DMARDs) in the treatment of patients with rheumatoid arthritis (RA) (8-12). However, the role of other important drug efflux molecules such as MRPs and ABCG2 in drug resistance of SLE remains unclear. In the present study, the expression and function of MRP1-3, P-gp and ABCG2 molecules in mononuclear cells (MNC) and polymorphonuclear neutrophils

(PMN) were compared between healthy volunteers and patients with SLE. We found that increased activity, but not the expression, of MRPs in SLE-MNC is related to multi-drug resistance after long-term use of corticosteroids.

Materials and methods

Patients and controls

Patients fulfilling the 1982 revised American College of Rheumatology criteria for the classification of SLE were recruited. The same number of sex- and age-matched healthy volunteers were the controls. Patients currently (within 4 weeks) on verapamil, diltiazem, cyclosporine and anti-arrhythmic medications (potential P-glycoprotein inhibitors) were excluded from this study. All of the participants signed the informed consents approved by Local Internal Review Board and Ethics Committee, National Taiwan University Hospital, Taipei, Taiwan. The disease activity of the SLE patients was judged by SLEDAI score. The complete blood routine, serum levels of albumin, creatinine, and complement C3 and C4, and anti-dsDNA and medications were recorded for correlation analysis. Because no gold-standard guideline to define corticosteroid resistance is available, we used simple surrogate criteria to define steroid hypo-responsiveness instead of steroid resistance. In clinical practice, we usually prescribed prednisolone and hydroxychloroquine as the first line medications for SLE treatment according to their disease activity. Once patients did not respond to the first line therapy (prednisolone high up to 1mg/kg/day+hydroxychloroquine 400mg/day), we then shifted the medications to more potent immunosuppressants such as cyclophosphamide or azathioprine. Accordingly, patients with SLE receiving more potent immunosuppressants other than prednisolone and hydroxychloroquine were considered corticosteroid resistance.

Preparation of MNC and PMN from peripheral blood of normal and SLE patients

Heparinized venous blood obtained from healthy volunteers or SLE patients was mixed with one-fourth volume of

2% dextran solution (molecular weight 464,000 Daltons) (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and incubated at room temperature for 30 min. Leukocyte-enriched supernatant was collected and layered over Ficoll-Hypaque density gradient solution (specific gravity 1.077) (Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 250g for 25 minutes, MNC were aspirated from the interface, whereas PMN were obtained from the bottom. The residual RBC in PMN suspension were lysed by incubation in chilled 0.83% ammonium chloride solution at 4°C for 10 min. The purity of PMN reached 98% confirmed by flow cytometry after stained with anti-CD16. The MNC suspension contained 90% lymphocytes, 5-8% monocytes and 2-5% other cell populations confirmed by Giemsa stain. The cell concentration of MNC and PMN was adjusted to 1×10^6 cells/ml suspended in 10% fetal bovine serum (FBS) in RPMI-1640 (10%FBS-RPMI). The viability of MNC and PMN was greater than 95% confirmed by trypan blue dye exclusion.

Measurement of MRP-mediated transport activity by carboxy-2', 7'-dichlorofluorescein diacetate (CFDA) efflux assay

MRP-mediated transport activity was measured by using CFDA (Sigma-Aldrich) efflux assay as reported by Laupèze *et al.* (15). CFDA, a non-polar non-fluorescent compound, can freely diffuse into cells and is cleaved by cytoplasmic esterase to become fluorescent CF, a substrate for MRP1, MRP2 and MRP3 extrusion. Briefly, 1×10^6 cells/ml were first loaded with 2mM CFDA for 30 min. To test the validity of the dye-efflux assay, the cells were loaded with 2mM of CFDA and 50μM of MK571 (a specific antagonist for MRPs) (Alexis Biochemicals, Lausen, Switzerland) or 2.5 mM probenecid (a non-specific MRPs inhibitor; Sigma-Aldrich) concomitantly for 30 min. We confirmed that the dye-efflux by MRPs was completely abolished by these inhibitors. Cells were then washed three times with ice-cold PBS, pH7.2, and were re-incubated in PBS containing 11 mM glucose at 37°C for 120 min.

The difference of CF-linked fluorescence retained in the cells before and after re-incubation, reflects MRP-related transport activity. The percentage and mean fluorescence intensity (MFI) were analyzed by FACScalibur cytometry (Becton-Dickinson, San Jose, CA, USA) using Lysis II software.

Measurement of P-gp-mediated transport activity by rhodamine 123 efflux assay

P-gp-mediated transport activity was measured by rhodamine 123 efflux assay (16). Briefly, 1×10^6 cells/ml were first loaded with 500 ng/ml rhodamine 123 (Sigma-Aldrich) for 30 min at 37°C. After washing with ice-cold PBS, cells were re-incubated in PBS containing 11 mM glucose at 37°C for 90 minutes followed by FACScalibur flow cytometric analysis (Becton-Dickinson) as mentioned above. To test the validity of rhodamine 123 dye-efflux assay, a specific inhibitor of P-gp, verapamil 50μM (Sigma-Aldrich), was added to the cell suspension concomitantly with the dye for 30 min. We confirmed that the dye-efflux by P-gp was completely abolished in the presence of verapamil.

Measurement of ABCG2-mediated transport activity by mitoxantrone efflux assay

ABCG2-mediated transport activity was measured by mitoxantrone efflux assay (17). Briefly, 1×10^6 cells/ml were first loaded with 3μM mitoxantrone (Sigma-Aldrich) for 60 min at 37°C. After washes with ice-cold PBS, cells were re-incubated in PBS containing 11 mM glucose at 37°C for 90 min followed by flow cytometric analysis as mentioned above. To test the validity of mitoxantrone dye-efflux assay, a specific inhibitor of ABCG2, 0.1μM of fumitremorgin C (Alexis Biochemicals), was added to the cell suspension concomitantly with the dye for 30 min. We confirmed that the dye-efflux by ABCG2 was completely abolished in the presence of fumitremorgin C.

Analysis of efflux data and generation of D value

We used the method reported by Leith *et al.* (18) and Kolmogorov-Smirnov

(KS) statistic test (19) to compare the difference of fluorescence retained in the cells before and after dye efflux mediated by respective drug efflux pumping protein. The method reported by Beck *et al.* (20) was highly recommended by the first International MDR1 Methods Detection Workshop. The KS statistic test measures the difference between two distribution functions and generates a D value ranging between 0 and 1. Higher D value indicates greater difference between the distribution functions. This method allows accurate identification of small change of fluorescence in cells before and after efflux by MDR protein-mediated active transport. The D value is used as a continuous variable for further statistical analysis.

Detection of intracellular MRP1, MRP2 and MRP3 expression in MNC by flow cytometry

One hundred microliters of heparinized venous blood was added to 2ml FACS lysing solution (Becton-Dickinson) for lysis of RBCs. The residual fixed and permeabilized leukocytes were then incubated with 0.1μg/ml of mouse monoclonal antibody against human MRP-1 (catalog number 18873), rabbit polyclonal antibody against human MRP-2 (catalog number 20766), or rabbit polyclonal antibody against human MRP-3 (catalog number 20766) in ice-bath (0°C) for one hour. These monoclonal and polyclonal antibodies were purchased from Santa Cruz Biotechnology, (CA, USA.). After several washes with PBS, pH7.2, the cells were incubated with FITC-conjugated donkey anti-mouse IgG or donkey anti-rabbit IgG in 1:2000X dilutions as secondary antibodies for one hour in ice-bath. The cells only stained with secondary antibodies were the negative control (positive rate <5%). The percentage of positively stained MNC was detected by FACS-can flow cytometry (Becton Dickinson) after gating the MNC populations and analyzed by Lysis II software.

Statistical analysis

The data were represented by mean \pm SD in the whole study. Statistical significance was assessed by non-parametric

Wilcoxon rank-sum test for comparing qualitative variables in the different groups. Multiple linear regression test was applied to test the correlation and significance among different parameters. $P < 0.05$ was considered statistically significant.

Results

Comparison of MRPs, P-gp, and ABCG2-mediated transport activity of MNC and PMN from patients with SLE and normal individuals

CFDA, rhodamine 123 and mitoxantrone are fluorescence substances able to diffuse into viable cells. After entrance into the cell, the fluorescent dyes can be actively extruded out of cells by MRPs, P-gp, and ABCG2 drug-efflux molecules, respectively. The D value was applied for measuring the small difference of MDR protein-mediated dye-efflux. The representative CFDA efflux assay in MNC and PMN from one healthy volunteer and one SLE patient is demonstrated in Figure 1. The validity of CFDA dye-efflux was verified by the addition of non-specific CFDA inhibitor, probenecid (2.5mM) that could completely abolish the D value (data not shown). The D value of CFDA transport is significantly elevated in SLE-MNC compared to normal-MNC as shown in Figure 2A. However, the D values of rhodamine 123 and mitoxantrone in SLE-MNC are not different from normal-MNC (Fig. 2A). By contrast, there was no significant difference in the drug-efflux activities of the 3 molecules between normal and SLE-PMN (Fig. 2B). To further confirm the results in Figure 2A, specific MDR protein inhibitor was added to the individual drug-efflux assay to test the assay validity. The D values became negligible in 3 assays in the presence of specific antagonists (data not shown). Based on this, we further recruited another eight normal and 9 patients with active SLE for study. We found only the D value of CFDA-efflux of SLE-MNC remained significantly elevated compared to normal-MNC (Fig. 2C). Interestingly, the D value of P-gp in both normal and SLE-MNC was remarkably enhanced in this experiment compared to Figure 2A.

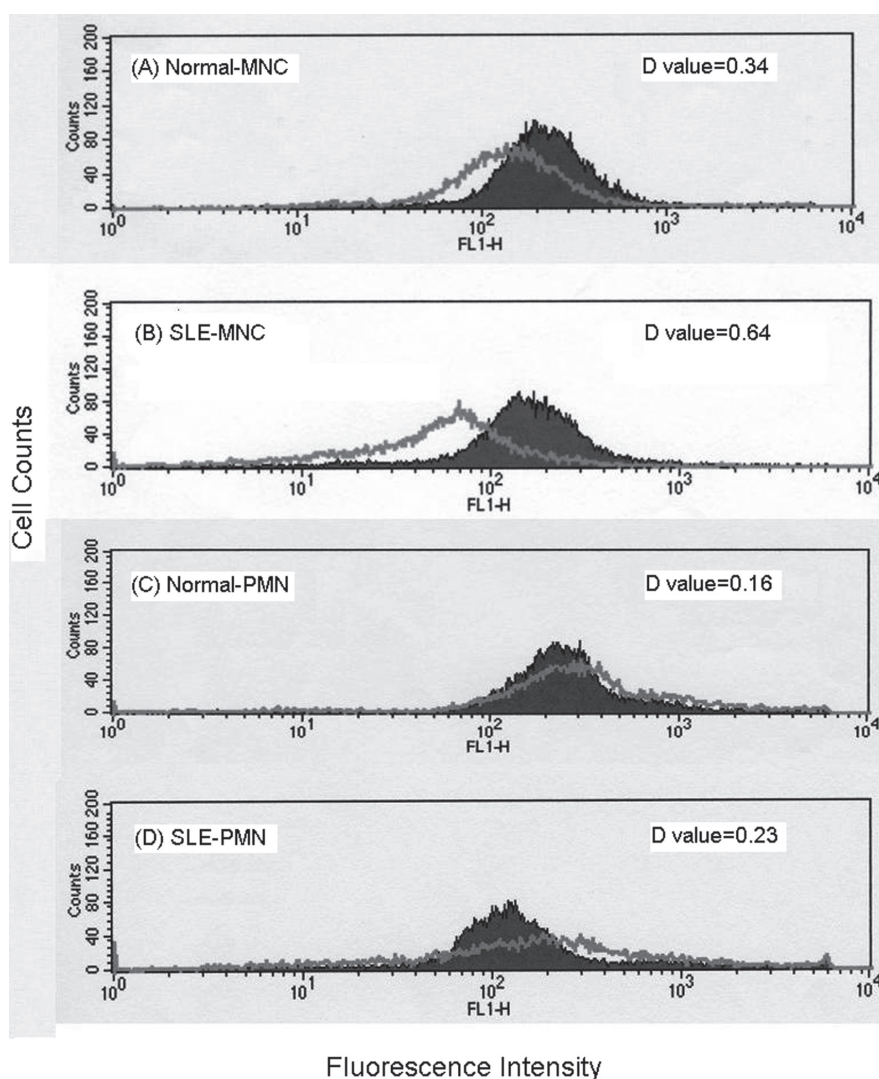


Fig. 1. A representative case demonstrating carboxy-2',7'-dichlorofluorescein diacetate (CFDA) dye-efflux activity of MNC and PMN in one healthy volunteer and one SLE patient detected by flow cytometry. The difference of esterase-cleaved fluorescent CF retained in the cytoplasm before (black area) and after (gray area) drug-efflux assay was denoted by D value. To test the validity of CFDA-efflux assay, a non-specific MRPs inhibitor, probenecid (2.4mM), was concomitantly incubated with cells loading with CFDA dye for 30 min. The inhibitor could completely abolish the dye-efflux by the cells. (A) Normal MNC, (B) SLE-MNC, (C) Normal PMN, (D) SLE-PMN

Comparison of intracellular MRP1, MRP2 and MRP3 expression in fixed and permeabilized normal and SLE-MNC by flow cytometry

Because CFDA dye-efflux was mainly mediated by MRP1, MRP2 and MRP3, we compared the intracellular expression of MRP1, MRP2 and MRP3 molecules in normal and SLE-MNC that was fixed and permeabilized by FACS lying buffer detected by flow cytometry. As shown in Figure 3, there is no significant difference in the percentage expression of MRP1, MRP2 and MRP3 in MNC between SLE and normal groups. Accordingly, the increased

CFDA efflux activity of SLE-MNC seems not due to increased MRP1-3 expression in the cells.

Correlation of clinical/laboratory parameters and medications with increased MRP-mediated efflux activity in SLE-MNC

The correlation of demographic data (age, sex), clinical parameters (disease duration, disease activity and glucocorticosteroid responsiveness), laboratory parameters (hematology, biochemistry and serology), and medications (prednisolone, hydroxychloroquine, cyclophosphamide and azathioprine)

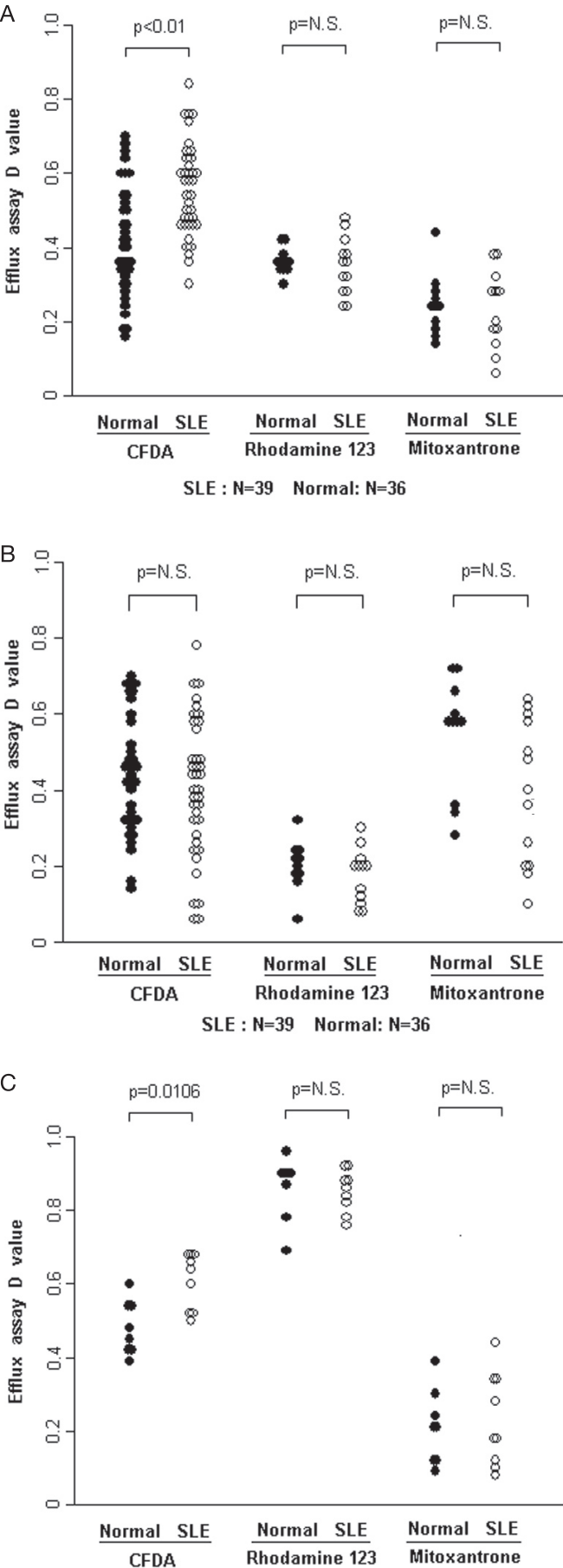


Fig. 2. Comparison of three dye-efflux activities in MNC and PMN of normal and SLE groups. Carboxy-2', 7'-dichlorofluorescein diacetate (CFDA) efflux assay is specific for MRP-mediated transport activity. Rhodamine 123 efflux assay is specific for P-gp-mediated transport activity. Mitoxantrone efflux assay is specific for ABCG2-mediated transport activity. (A) MNC (only a non-specific MRPs antagonist, probenecide 2.5mM, was used to test the validity of CFDA dye-efflux assay) (B) PMN (only a non-specific MRPs antagonist, probenecide 2.5mM, was used to test the validity of CFDA dye-efflux assay), and (C) MNC (Optimal concentration of three specific MDR inhibitors: 50 μ M of MK571 for MRPs; 50 μ M of verapamil for P-gp, and 0.1 μ M of fumitremorgin C for ABCG2, were used to test the validity of the respective assay. The specific antagonists could completely abolish the dye-efflux mediated by the three molecules).

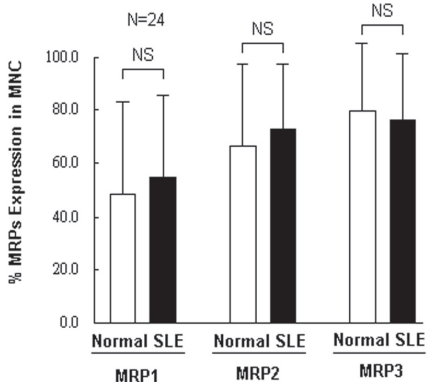


Fig. 3. Comparison of MRP1, MRP2 and MRP3 expression in MNC from healthy volunteers and SLE patients detected by flow cytometric determination.

with increased MRPs efflux activity in SLE-MNC is summarized in Table I. We found that leukocyte count and duration of prednisolone treatment are significantly correlated with increased MRPs activity in SLE-MNC. However, after adjusted by age and sex, only the duration of prednisolone treatment ($p=0.013$) is significantly correlated with increased MRP activity in SLE-MNC.

Discussion

To the best of our knowledge, this is the first study to investigate the efflux activity of three major MDR proteins in immune-related cells of patients with SLE. The results showed that the efflux activity of MRPs family was significantly higher in SLE-MNC than normal MNC. Due to the extremely short half-life (6-8 hours *in vivo*) and life span (<3 days *in vitro*) of PMN, it is expected that the expression and function of MDR proteins in PMN do not change despite long-term exposure to different immunosuppressants. In addition, PMN do not express P-gp in the cells. Tsujimura *et al.* (14) demonstrated that P-gp molecule was expressed at significantly high levels as well as increased function in the peripheral blood lymphocytes of SLE patients in parallel with clinical steroid resistance. We did not compare the expression of P-gp molecule in normal and SLE-MNC because no functional change of P-gp between normal and SLE-MNC was found (Fig. 2A). It is possible that our SLE patients were

Table I. The correlation of different clinical/laboratory parameters and medications with D value of CFDA efflux activity in mononuclear cells of patients with SLE.

Clinical parameters	Mean	p-value
Age (years)	39.5 ± 16.6	0.247
Sex (F:M)	19:7	0.667
Duration of SLE (months)	82.7 ± 87.3	0.267
SLE-DAI score	9.7 ± 8.4	0.963
WBC (10 ⁶ /L)	7035 ± 3681	0.037**
Platelet (10 ⁶ /L)	228,000 ± 101,000	0.465
Albumin (g/dL)	3.7 ± 0.8	0.403
Creatinine (mg/ml)	1.3 ± 1.3	0.872
Anti-dsDNA (IU/ml)	101 ± 211	0.490
C3 (mg/dL)	79.4 ± 27.1	0.151
C4 (mg/dL)	21.6 ± 17.3	0.623
Prednisolone dosage (mg/day)	14.2 ± 15.1	0.199
Prednisolone duration (months)	40.5 ± 50.4	0.006**
Hydroxychloroquine usage (yes: no)	22:4	0.874
Cyclophosphamide usage (yes: no)	8:18	0.337
Azathioprine usage (yes: no)	4:22	0.251
Steroid responsiveness* (yes: no)	10:16	0.133

*Patients with SLE who received immunosuppressants more than prednisolone and hydroxychloroquine to control lupus disease activity were defined as steroid refractory.

**After analysis with multiple linear regression model adjusted by age and sex, only prednisolone duration ($p=0.013$), but not WBC count, is significantly correlated with the MRP-related transport activity.

less active than those of them (SLEDAI score 9.7 vs. 12) that would express less P-gp activity in their results but exhibit no difference in P-gp activity between SLE and normal MNC in our study. Instead, we noted that the enhanced MRP-mediated CFDA efflux is significantly correlated with long-term corticosteroid use (Table I). It is possible that P-gp contributes to drug resistance in highly active disease state while increased MRPs activity emerges in moderate disease activity after long-term corticosteroid treatment in SLE patients. Tazzari *et al.* (21) demonstrated that MRP1 expression is under the control of the PI3K/Akt signaling axis in AML blasts. Furthermore, PI3K/Akt/mTOR molecules were found hyper-expressed in the B cells of mouse lupus model (22). Accordingly, it is possible that increased MRP activity in SLE-MNC is due to hyper-expressed PI3K/Akt/mTOR pathway in these patients. Unfortunately, we failed to demonstrate that MRPs activity of SLE-MNC is higher in steroid refractory than in responsive patients in Table I ($p=0.133$). Due to cross-sectional analysis and small case numbers in this study, it is premature to conclude that the increased activity of MRPs in SLE-MNC is not related

to poor steroid responsiveness in the present study. A prospective study to recruit enough newly diagnosed SLE patients for correlating the corticosteroid responses and MRP activity of MNC is now under investigation.

Oerlemans *et al.* (23) in an *in vitro* study demonstrated that cells over-expressing MRP1 induced by long-term hydroxychloroquine exhibited high corticosteroid resistance by a mechanism of cross-provocation. This result suggests that MRPs family plays an important role in steroid resistance as well as P-gp through cross-provocation. Besides, a number of *in vitro* studies revealed that ABCG2 and MRP1 over-expression involved in DMARDs resistance. Interestingly, these T cells can extend resistance to other non-related DMARDs after long-term exposure to one agent by resistance spreading (23-25). Clinically, many immunosuppressive agents potentially induce MDR in patients with rheumatoid arthritis (7-12). Our study clearly demonstrates that long-term use of corticosteroids, but not hydroxychloroquine, cyclophosphamide or azathioprine, is the contributing factor for increased MRPs activity in SLE-MNC. In addition to RA and SLE, MDR gene expression was also observed in other

autoimmune diseases such as myasthenia gravis (26) and immune thrombocytopenic purpura (27). However, most authors only focused on P-gp but not on other drug efflux molecules expression. Since long-term immunosuppressive therapy is mandatory for autoimmune diseases, the emergency of MDR is quite important in clinical practice. It is equally interesting that cyclosporine suppresses lymphocyte activation via inhibition on both calcineurin and P-gp activity (28). Therefore, it is possible that calcineurin inhibitors such as cyclosporine and FK506 not only suppress autoimmune activity, but prevent multidrug resistance. Currently developed synthetic MDR reversing agents, including PSC-833, VX-710, S-9788, GF-120918, LY 33597 and XR9576, are now under clinical trial for improving the efficacy of cancer therapy (29-31). The combination of these compounds with traditional immunosuppressants would be a novel effective therapy for SLE patients in long-term corticosteroid therapy. The mechanisms for multidrug resistance are sophisticated that involve in impaired drug delivery to target cells, defective cellular uptake, alternations in intracellular drug activation, target inhibition, and unknown processes downstream of target inhibition (10). The combination therapy would be an important strategy to overcome drug resistance in the treatment of infectious disease (32) and rheumatic disorders for minimizing the drug resistance (10).

Three unsettled problems remained in the present study:

1. Inconsistency between increased activity and normal expression of MRP molecules in SLE-MNC. Several possibilities can be deduced including over-expression of less characterized MRP molecules other than MRP1-3, augmentation of MRPs activity by lupus disease activity *per se*, mutate MRP molecules, or even post-translational modification of MRP molecules, but not increased intracellular MRPs expression. However, more investigations are needed to solve it;
2. The molecular basis of corticosteroid-induced drug-resistance was not elucidated in the present study. It is

possible that the residual drug-resistant lymphocytes emerge after long-term contacting with offensive lymphocytotoxic corticosteroids. These selected lymphocytes expand by autoimmune mechanism of SLE leading to enhanced MRP activity in these patients; 3. Whether increased MRP activity in SLE-MNC is congenital or acquired was not determined in the present study. Our preliminary results reveal that the increased MRP activity in SLE-MNC restored to normal function after disease remission (data not shown). Accordingly, it is quite possible that MRP activity is correlated with lupus disease activity with abnormal signaling pathway in patients with SLE.

Besides the drug efflux pumping activity, P-gp molecules also mediate the transmembrane transport of IL-2, IFN- γ and IL-4 (33, 34). The block of P-gp inhibits T cell activation (33). However, the P-gp knock-out mice can equally transport these cytokines (35). It seems more likely that the P-gp molecule may involve in cytokine release from cells indirectly by exporting molecules that regulate the cytokine release. MRP1 was also demonstrated as transmembrane transporter of leukotrienes to mobilize dendritic cells entering into lymph nodes (36). On the other hand, many cytokines can stimulate MDR protein expression in different cells. Tsujimura *et al.* (37) demonstrated that IL-2 transcriptionally regulates P-gp expression in lymphocytes. Proinflammatory cytokines IL-6 and IL-1 can induce MRP family proteins expression in different tissue cells (38-40). These data suggest the possibility of mutual modulation among MDR proteins and cytokines. The increased MRP family efflux activity in SLE-MNC is probably affected by high serum levels of proinflammatory cytokines that involve lupus pathogenesis. Obviously, more investigations are needed to prove it. In conclusion, MRPs efflux function is enhanced in SLE-MNC that correlates with long-term steroid therapy. The addition of MDR inhibitors may be useful in preventing multidrug resistance in long-term corticosteroid therapy in patients with SLE.

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