

[ CASE REPORT ]

# Treatment with Methotrexate and Intravenous Cyclophosphamide Pulse Therapy Regulates the P-gp<sup>+</sup>CD4<sup>+</sup> Cell-related Pathogenesis in a Representative Patient with Refractory Proliferative Lupus Nephritis

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## Abstract:

Diffuse proliferative lupus nephritis (DPLN) is a serious organ complication. Drug resistance correlates with P-glycoprotein (P-gp) expression on activated lymphocytes. We encountered a refractory DPLN patient with expansion of peripheral CD69/CXCR3-co-expressing P-gp<sup>+</sup>CD4<sup>+</sup> cells producing IL-2 and IL-6. Treatment with high-dose corticosteroid combined with biweekly intravenous cyclophosphamide pulse therapy (IVCY) failed to reduce the population of activated P-gp<sup>+</sup>CD4<sup>+</sup> cells or control the disease activity. Methotrexate (MTX) with monthly IVCY reduced activated P-gp<sup>+</sup>CD4<sup>+</sup> cells and improved the clinical symptoms, resulting in long-term remission and tapering of corticosteroids. MTX-IVCY combination therapy, which down-regulates the activated P-gp<sup>+</sup>CD4<sup>+</sup> cell-mediated disease activity, may be useful for the treatment of refractory DPLN.

**Key words:** P-glycoprotein, diffuse proliferative lupus nephritis, CD4<sup>+</sup> cell, Methotrexate

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## Introduction

Lupus nephritis (LN) is one of the most serious organ complications in systemic lupus erythematosus (SLE) and shows various histologic patterns (1). In particular, diffuse proliferative lupus nephritis (DPLN), the International Society of Nephrology and the Renal Pathology Society (ISN/RPS) class IV, is a severe and significant form of LN. Patients with DPLN often show severe nephrotic syndrome and treatment resistance, resulting in progression to renal failure despite intensive immunosuppressive therapy (2).

One of the mechanisms underlying drug resistance to SLE treatment involves the extracellular excretion of drugs after their entry into the target cells through a process activated by the expression of P-glycoprotein (P-gp), which is present on the cell membrane (3). P-gp, a 170-kDa product of the multidrug resistance-1 (MDR-1) gene, is a member of the

ATP-binding cassette (ABC) transporter superfamily of genes and functions as an energy-dependent transmembrane efflux pump (4). Overexpression of P-gp results in a reduction in intracellular concentrations of xenobiotics, drugs, and poisons, such as vinca alkaloids, anthracyclines, antimalarials, colchicines, cyclosporine, and corticosteroids (CSs) (5).

The expression of P-gp on lymphocytes is induced by lymphocyte-activating stimuli, such as IL-2 (5). Overexpression of P-gp on lymphocytes along with lymphocyte activation results in the development of multi-drug resistance. In SLE patients with highly active disease, overexpression of P-gp on lymphocytes, along with lymphocyte activation, results in the development of multi-drug resistance (3).

CD69, a well-defined early-activation surface marker of lymphocytes, is a functional triggering molecule on activated CD4<sup>+</sup> cells. The CD69-signaling in CD4<sup>+</sup> cells mediates CD4<sup>+</sup> cell migration, the production of cytokines, and the proliferation of CD4<sup>+</sup> cells (6). We previously proposed that

P-gp-expressing CD4<sup>+</sup> cells, especially P-gp<sup>+</sup>CD69<sup>+</sup>CD4<sup>+</sup> cells, might be the main orchestrators of progressive DPLN through their direct infiltration into the kidney (7). In addition, CXCR3, a chemokine receptor, has been reported to be involved in recruiting CD4<sup>+</sup> T cells into the kidney of LN patients (8).

We herein report a DPLN case with P-gp-expressing CD4<sup>+</sup> cell-mediated multi-drug resistance, including resistance to intravenous cyclophosphamide pulse therapy (IVCY) and tacrolimus. We analyzed the phenotypes of P-gp<sup>+</sup>CD4<sup>+</sup> cells, including the co-expression of CD69 and CXCR3, and investigated the effect of treatment on subsets of P-gp<sup>+</sup>CD4<sup>+</sup> cells.

### Patient

The ethics committee of our institution approved the study, and informed consent was obtained from the patient enrolled in the study. The diagnosis of SLE was based on the American College of Rheumatology (ACR) revised criteria for SLE. The clinical disease activity of SLE was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). The diagnosis of LN was based on clinical features and laboratory tests and confirmed by a histopathological examination of a renal biopsy specimen. The LN diagnosis was made according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of LN.

### Interleukins production from CD4<sup>+</sup> cells

Peripheral blood mononuclear cells (PBMCs) from the SLE patient were isolated by density gradient centrifugation. CD4<sup>+</sup> cells were purified by negative selection using magnetic beads according to the recommended procedure supplied by the manufacturer (CD4 negative isolation kit; Dynal Biotech, Tokyo, Japan). The purity of the CD4<sup>+</sup> cells subset was determined by flow cytometry to be greater than 90%. Purified CD4<sup>+</sup> cells were plated onto a 12-well culture dish ( $2 \times 10^5$  cells/well) and incubated without stimulation for 6 hours at 37°C in RPMI 1,640 containing 5% FCS in the presence of 20 µg/mL brefeldin A (Sigma-Aldrich Japan, Tokyo, Japan). The CD4<sup>+</sup> cells were then treated with 4% formaldehyde (Sigma Aldrich Japan) in FACS medium consisting of phosphate-buffered saline (PBS), 0.5% human serum albumin (HSA; Mitsubishi Welfarma, Osaka, Japan), and 0.2% NaN<sub>3</sub> (Sigma Aldrich Japan) for 15 minutes and then with 0.1% saponin (Sigma Aldrich Japan) in FACS medium. A flow cytometric analysis was performed to assess the production of intracellular interleukins and the expression of P-gp on CD4<sup>+</sup> cells.

### Flow cytometry

Staining and a flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) and CD4<sup>+</sup> cells isolated from the SLE patient were conducted using standard procedures as described previously (9), by FACScan (Becton Dickinson, Mountain View, USA). In brief, PBMCs or CD4<sup>+</sup>

cells ( $2 \times 10^5$  cells/well) were first incubated with polyclonal  $\gamma$ -globulin (10 µg/mL; Mitsubishi Welfarma) to block Fc-receptors. These cells were then incubated with MRK-16 (100 µg/mL, Kyowa Medex, Tokyo, Japan), a specific monoclonal antibody (mAb) against P-gp (10), followed by the addition of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG Ab (5 µg/mL; Fujisawa, Osaka, Japan) in FACS medium for 30 minutes at 4°C. For the three-color analysis, we incubated the cells with cy-chrome-conjugated CD4 mAb (BD Biosciences Pharmingen, Tokyo, Japan) and PE-conjugated CD69, CXCR3, IL-2, IL-6, or IL-17 mAb (BD Biosciences Pharmingen) after blocking free anti-mouse IgG-binding sites with irrelevant antibodies. Monoclonal antibody-three-color-stained cells were detected by electronic gating based on their P-gp, CD4, CD69, CXCR3, IL-2, IL-6, or IL-17 expression using FACScan. Amplification of mAb-binding was induced using a three-decade logarithmic amplifier.

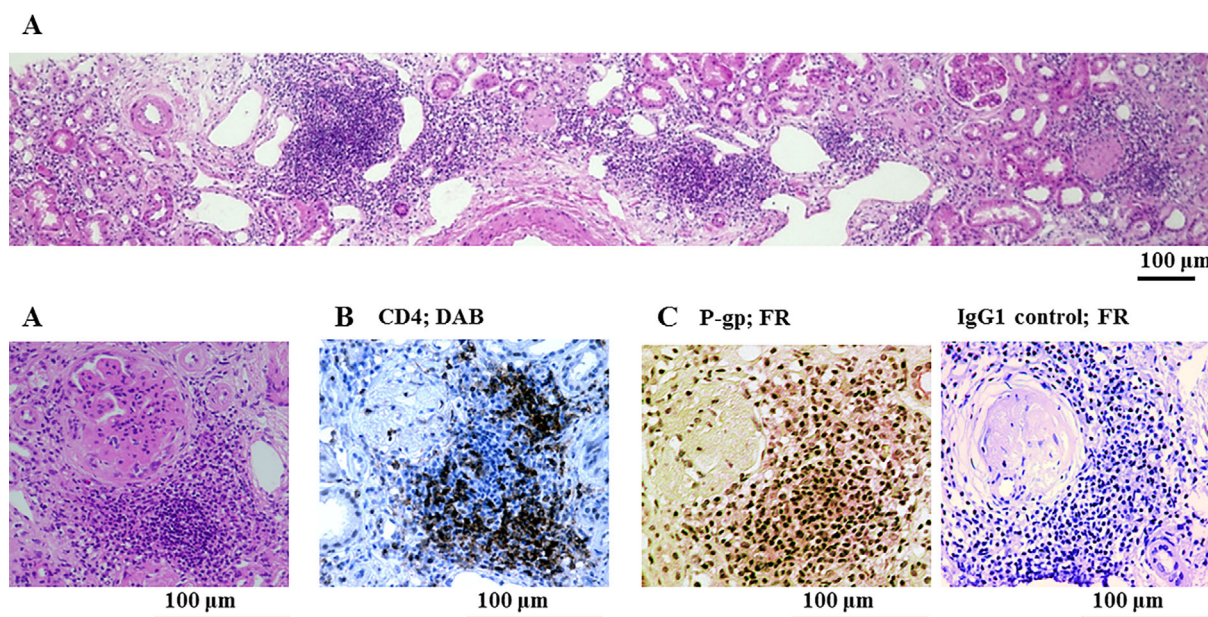
### Histopathological and immunohistochemical assessments

Resected renal biopsy specimens were fixed in 15% phosphate-buffered formalin and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) or appropriate immunohistochemical stains. LN was diagnosed in the patient by an examination of these renal biopsy specimens.

For immunohistochemical staining, the deparaffinized and rehydrated 5-µm-thick renal sections were antigen retrieved by heating in Target retrieval solution (Dako, Tokyo, Japan) and then incubated in 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes to block endogenous peroxidase activity, followed by rinsing. For single-staining immunohistochemistry, the sections were incubated with the primary antibody against P-gp (JSB-1, a murine mAb, dilution, 1:20; MONOSAN, Uden, the Netherlands) and isotype-matched negative control antibody of P-gp (negative control mouse IgG1, dilution, 1:10; DAKO, Glostrup, Denmark) for 2 hours or CD4 (LE-CD4, anti-human mouse mAb, dilution, 1:20, DAKO, Glostrup, Denmark) for 1 hour at room temperature followed by a secondary antibody (MACH 2 Double Stain 1 or 2, Biocare Medical, Concord, CA) for 30 minutes at room temperature. Sections were stained for 10 minutes with a solution consisting of 20 mg of 3,3'-diaminobenzidine (DAB) tetrahydrochloride, 65 mg of sodium azide, and 20 mL of 30% H<sub>2</sub>O<sub>2</sub> in 100 mL of Tri-HCl (50 mmol/L; pH 7.6), or with Vulcan Fast Red Chromogen Kit 2 (Biocare Medical), counterstained with Meyer's hematoxylin, and then examined under a light microscope.

## Case Report

A 44-year-old woman with highly active proliferative LN showed clinical flare repeatedly over a period of 18 years despite treatment with methyl-PSL pulse therapy, IVCY, azathioprine, mizoribine, rituximab (RTX), CsA, and



**Figure 1.** Histopathological and immunohistochemical analyses of renal tissues from the present patient with refractory progressive proliferative lupus nephritis. (A) Hematoxylin and Eosin staining. Bottom: magnified view of a section of the top image. (B) Immunostaining for CD4<sup>+</sup> lymphocytes using anti-CD4 monoclonal antibody (mAb) with 3,3'-diaminobenzidine (DAB) (brown). (C) Immunostaining for P-gp on lymphocytes using JSB-1 anti-P-gp mAb (P-gp) or isotype-matched negative control antibody immunoglobulin G1 (IgG1 control) with Vulcan Fast Red (FR) (red). (B, C) Nuclear counterstaining with hematoxylin.

tacrolimus (TAC). The refractory proliferative LN eventually progressed to nephrotic syndrome with severe hypoalbuminemia (serum albumin <2.0 g/mL) for 2 years. RTX was stopped due to the development of vasculitis, and TAC was also discontinued due to exacerbation of diabetes mellitus and hypertension, resulting in the development of nephrotic syndrome.

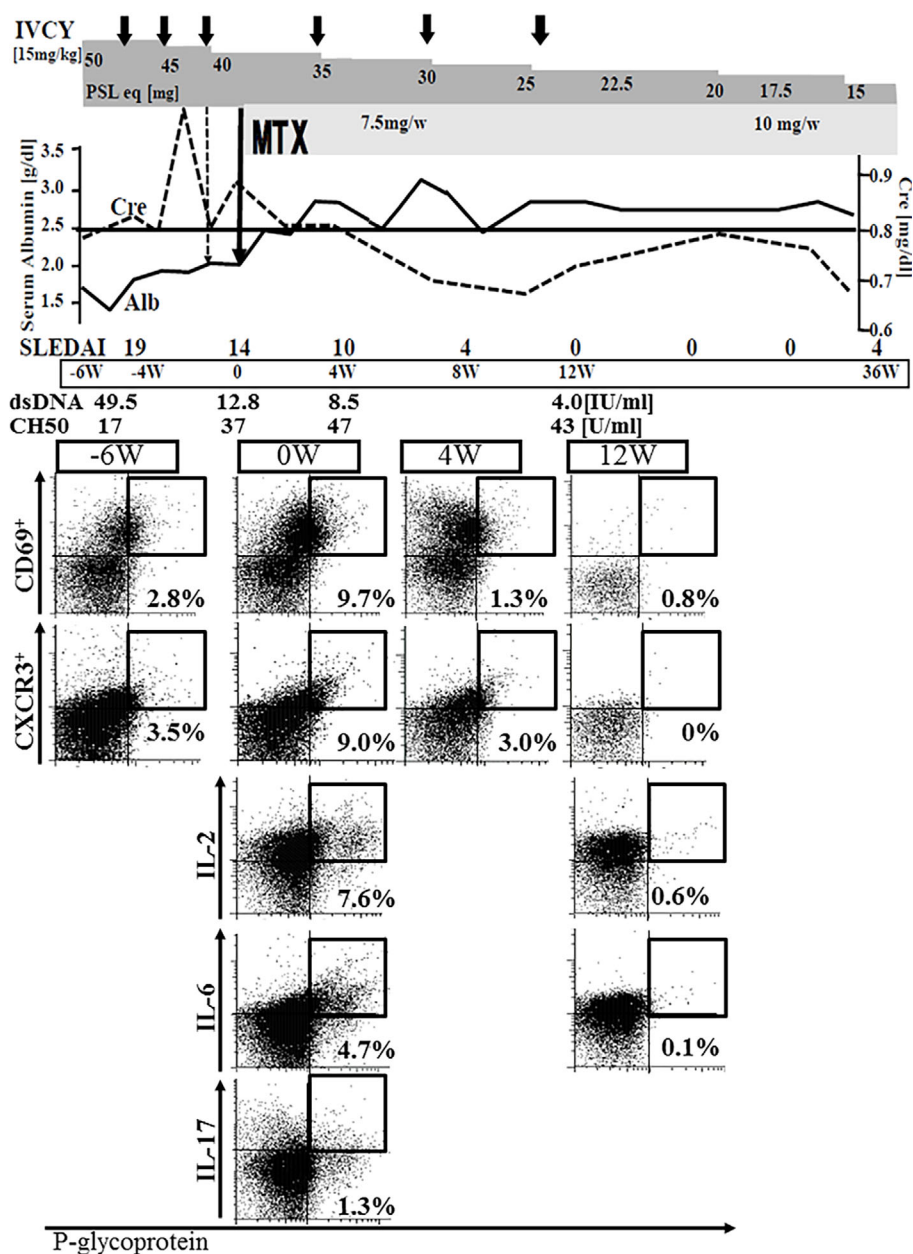
At her sixth admission in November 2010 due to severe flare with erythema, massive edema, elevated levels of anti-double-stranded DNA antibody, and hypocomplementemia, an examination of a renal biopsy specimen showed both features of active proliferative LN classified as ISN/RPS IVG (A/C) and the marked accumulation of inflammatory cells in the renal interstitial tissue (Fig. 1A). To determine the cells involved in the pathogenesis and lack of response to treatment, we analyzed the P-gp expression on peripheral lymphocytes by flow cytometry. In this regard, we reported previously that only a small proportion of peripheral lymphocytes express P-gp in normal individuals [CD4<sup>+</sup> cells: 2.2%±1.1% (7), CD19<sup>+</sup> cells: 7.0%±2.7%, mean±standard deviation (SD) (11)]. In this patient, the proportions of P-gp-stained peripheral CD4<sup>+</sup> and CD19<sup>+</sup> cells were 4.6% and 5.4%, respectively. Thus, the P-gp expression was slightly increased on CD4<sup>+</sup> cells and marginal on B cells in our SLE patient compared with normal subjects. Immunohistochemical staining of serial 5-µm-thick sections from the same specimens showed that the majority of these inflammatory cells were CD4<sup>+</sup> cells (Fig. 1B) and P-gp-positive cells (Fig. 1C), suggesting that P-gp<sup>+</sup>CD4<sup>+</sup> cells had accumulated

in the renal interstitial tissue.

Based on this finding, we next analyzed the phenotypes of peripheral P-gp<sup>+</sup>CD4<sup>+</sup> cells. A high proportion of peripheral CD69<sup>+</sup>CD4<sup>+</sup> cells was noted [23.2%, compared with 5.7%±1.8 in normal individuals (7)]. A further analysis showed that the CD69<sup>+</sup>CD4<sup>+</sup> cells included CD69<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells (Fig. 2, -6W). In addition, CXCR3<sup>+</sup>CD4<sup>+</sup> cells were detected in peripheral blood, and the majority of P-gp<sup>+</sup>CD4<sup>+</sup> cells expressed CXCR3 (Fig. 2, -6W).

Despite intensive immunosuppressive therapy with switching from CSs to PSL at 1 mg/kg/day followed biweekly IVCY 3 times, the proportion of peripheral CD69<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells and CXCR3<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells increased substantially with concomitant flare in clinical symptoms and signs. A further analysis of the cytokine production by peripheral P-gp<sup>+</sup>CD4<sup>+</sup> cells showed the production of interleukin (IL)-2 and IL-6 but not IL-17 (Fig. 2, 0W).

The above results indicate that the poor control of activated P-gp<sup>+</sup>CD4<sup>+</sup> cells, which show preferential renal infiltration and cytokine overproduction, induces drug resistance and continued flare of proliferative LN. Other groups have reported that MTX can control cellular immunity in SLE and that MTX combined with IVCY therapy can be effective against refractory proliferative LN (12, 13). Accordingly, treatment in the present patient was switched to weekly oral MTX with monthly IVCY in addition to CSs. This resulted in a significant decrease in peripheral CD69<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells within 4 weeks (Fig. 2, 4W), along with improvement in proliferative LN and recovery of the serum al-



**Figure 2.** The reduction in P-gp<sup>+</sup>CD4<sup>+</sup> lymphocytes following IVCY-MTX therapy was associated with an improvement in refractory proliferative lupus nephritis. A flow cytometric analysis showed P-gp expression on target molecule positive CD4<sup>+</sup> cells at 6 weeks before treatment (-6W), just at the start of treatment (0W), at 4 weeks after the commencement of treatment (4W), and at 12 weeks after the commencement of treatment (+12W). Percentages represent the proportion of P-gp and target molecule double-positive CD4<sup>+</sup> lymphocytes. Clinical course; PSL eq: prednisolone equivalent, IVCY: intravenous cyclophosphamide pulse therapy, MZ: mizoribine, MTX: methotrexate, Cre: creatine, Alb: albumin, SLEDAI: systemic lupus erythematosus disease activity index, ds DNA: anti-double-stranded (ds) DNA antibody, CH<sub>50</sub>: 50% hemolytic complement activity

bumin level to >2.5 g/dL. MTX combined with IVCY therapy eliminated CD69<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells, accompanied by the disappearance of CXCR3<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells and IL-2- and IL-6-producing P-gp<sup>+</sup>CD4<sup>+</sup> cells within 12 weeks (Fig. 2, 12W). The elimination of activated P-gp<sup>+</sup>CD4<sup>+</sup> cells was associated with SLE remission (SLEDAI score 0 point) within 12 weeks, with serum albumin levels maintained at >2.5 g/dL. The above response allowed tapering of CSs to 0.35 mg/kg/

day within 36 weeks. The patient did not experience any serious treatment-related side effects, such as serious infections, severe cytopenia, or severe liver dysfunction, during the observation period.

## Discussion

In our patient with refractory DPLN, poor control of

DPLN was associated with a high proportion of multidrug-resistant activated P-gp<sup>+</sup>CD4<sup>+</sup> cells, which overexpressed CD69 and CXCR3 and produced IL-2 and IL-6. In addition, active infiltration of lymphocytes and the accumulation of P-gp<sup>+</sup>CD4<sup>+</sup> cells in the renal interstitial tissue was noted. The infiltration of activated lymphocytes into tubulointerstitial lesions is associated with a decreased estimated glomerular filtration rate (eGFR) and treatment resistance, resulting in poor renal outcomes in LN cases (14, 15). Previous studies have shown the expansion, circulation, and homing of pathogenic lymphocytes to target organs in various systemic autoimmune diseases (7, 16, 17). For example, the numbers of CD25-expressing T cells were significantly increased in the peripheral blood of patients with active cutaneous lupus erythematosus as well as in a subset of skin-homing cutaneous lymphocyte antigen (CLA)<sup>+</sup>CD4<sup>+</sup> cells (16). Circulating V $\delta$ 1<sup>+</sup> T cells were found to be significantly increased in number and accumulated in perivascular areas of the skin in patients with systemic sclerosis (17). These data suggest that P-gp<sup>+</sup>CD4<sup>+</sup> cells can expand, enter the circulation and accumulate in renal interstitial tissue in proliferative LN. In this study, treatment resulted in the early elimination of CD69<sup>+</sup> P-gp<sup>+</sup>CD4<sup>+</sup> cells compared to CD69<sup>+</sup>CD4<sup>+</sup> cells. Therefore, CD69<sup>+</sup> P-gp<sup>+</sup>CD4<sup>+</sup> cells, among CD69<sup>+</sup>CD4<sup>+</sup> cells, are suggested to be involved in the pathogenesis and response to treatment of DPLN.

Furthermore, the majority of CXCR3<sup>+</sup>CD4<sup>+</sup> cells in our patient expressed P-gp during the active phase of DPLN. CXCR3<sup>+</sup> T cells are recruited into the inflamed renal tissues and are a valuable marker of disease activity in LN (8). In murine LN, CXCR3-deficient mice develop less kidney damage than do wild-type mice, emphasizing the pathogenic importance of CXCR3 (18). In this regard, patients with Crohn's disease exhibit resistance to CS in association with the accumulation of CXCR3<sup>+</sup>P-gp<sup>+</sup>Th17 cells in the gut (19). Pro-inflammatory human Th17 cells are restricted to a subset of CXCR3<sup>+</sup> cells that stably express P-gp and produce both Th17 and Th1 cytokines upon TCR stimulation (19). CXCR3<sup>+</sup>Th17 and CXCR3<sup>+</sup>Th1 as pathogenic effector T cells are involved in the development of murine LN based on their infiltration into the kidney (18). However, in our patient with refractory DPLN, P-gp<sup>+</sup>CD4<sup>+</sup> cells produced IL-2 and IL-6 but not IL-17. An analysis of the chemokine expression and cytokine production showed that CD69<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells are preferentially Th1 rather than Th17. The therapeutic effect of MTX on nephritis in BWF1 and MRL/lpr mice is due mainly to the suppression of Th1 rather than the suppression of humoral immunity (13). The response to MTX therapy in our patient with proliferative LN suggests that pathogenic CD69<sup>+</sup>P-gp<sup>+</sup>CD4<sup>+</sup> cells are indeed preferentially Th1 cells.

With regard to the treatment of proliferative LN, the NIH regimen and EULAR/ERA-EDTA recommend the initial use of high-dose CSs in combination with IVCY. Lehman et al. (12) reported that the combination of intravenous MTX and IVCY effectively controlled recurrent or refractory pro-

liferative LN in children with significant disease activity after treatment with IVCY alone. In our patient, DPLN clinical activity flare was noted persistently despite high-dose CS treatment combined with three sessions of biweekly IVCY, which suppressed humoral immunity. In addition, the clinical course of the patient showed that MTX with monthly IVCY therapy successfully controlled DPLN after biweekly IVCY alone and markedly reduced the population of multidrug-resistant active P-gp<sup>+</sup>CD4<sup>+</sup> cells, which expressed CD69 and CXCR3 and produced IL-2 and IL-6. The mechanisms underlying the down-regulation of P-gp expression with MTX in CD4<sup>+</sup> cells remain unclear. We previously proposed that activation of lymphocytes by IL-2 induced the nuclear translocation of YB-1 and transcription of the MDR-1 gene, thereby resulting in the expression of P-gp on lymphocytes (5). YB-1 nuclear translocation is induced by the activation of the ERK pathway (20). MTX has been reported to decrease soluble IL-2 receptor (21, 22) and reduce ERK activation in fibroblast-like synoviocytes (23). These data suggest that MTX may inhibit both lymphocyte activation according to IL-2 and signal transduction through the ERK pathway and that such processes may result in a reduction of the P-gp expression.

The lack of a response to treatment in proliferative LN patients with expansion of peripheral P-gp<sup>+</sup>CD4<sup>+</sup> cells that co-express CD69 and CXCR3 may be mediated by not only humoral immunity but also Th1 preferential cellular immunity; therefore, treatment that targets P-gp<sup>+</sup>CD4<sup>+</sup> cells might be important for resolving intractable proliferative LN. Our finding of the effectiveness and tolerability of the combination of oral weekly MTX and monthly IVCY, the efficacy against Th1 of which was reinforced with the use of MTX, needs to be confirmed in larger studies employing treatment regimens that target P-gp<sup>+</sup>CD4<sup>+</sup> cells in patients with intractable proliferative LN.

#### Author's disclosure of potential Conflicts of Interest (COI).

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