

Role of P-glycoprotein on CD69⁺CD4⁺ cells in the pathogenesis of proliferative lupus nephritis and non-responsiveness to immunosuppressive therapy

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ABSTRACT

Introduction P-glycoprotein (P-gp) expression on activated lymphocytes in systemic lupus erythematosus (SLE) plays a role in active efflux of intracellular drugs, resulting in drug resistance. The role of P-gp-expressing lymphocytes in the pathogenesis of SLE remains unclear. The aim of this study was to determine the importance of P-gp⁺CD4⁺ cells in organ manifestations in refractory SLE.

Methods The proportion of P-gp⁺CD4⁺ cells was determined by flow cytometry in peripheral blood of patients with SLE (n=116) and healthy adults (n=10). Renal biopsy specimens were examined by immunohistochemistry for P-gp expression.

Results CD69 is a marker of CD4 cell activation. The proportion of both P-gp-expressing CD4⁺ cells and CD69-expressing CD4⁺ cells in peripheral blood was higher in SLE than control. The proportion of P-gp⁺CD69⁺CD4⁺ cells correlated with Systemic Lupus Erythematosus Disease Activity Index and was higher in poor responders to corticosteroids. Furthermore, the proportion of P-gp⁺CD69⁺CD4⁺ cells was significantly higher in proliferative lupus nephritis (LN) with poor response to corticosteroids. The efficacy of immunosuppressive therapy depended on the regulation of the proportion of P-gp⁺CD69⁺CD4⁺ cells. Marked accumulation of P-gp⁺CD4⁺ cells in renal interstitial tissue and high proportion of peripheral P-gp⁺CD69⁺CD4⁺ cells were noted in patients with proliferative LN.

Conclusions The results showed high proportion of P-gp⁺CD69⁺CD4⁺ cells in peripheral blood and their accumulation in renal tissue in patients with proliferative LN refractory to CS therapy, suggesting that P-gp expression on activated CD4⁺ T cells is a potentially useful marker for refractoriness to treatment and a novel target for treatment.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease with multiple systemic involvements. Both antibodies from activated B cells and autoreactive T cells play significant roles in the pathogenesis of SLE. The main goal of treatment is the control of immune-mediated organ manifestations with corticosteroids (CS) or other

Key messages

What is already known about this subject?

- ▶ One of the mechanisms of resistance to corticosteroids is overexpression of P-gp on lymphocytes in patients with SLE with high disease activity. Organ specificity of P-gp-expressing subset of lymphocytes remains unclear.

What does this study add?

- ▶ P-gp⁺CD69⁺CD4⁺ cells are the main orchestrators of proliferative lupus nephritis.
- ▶ High proportion of peripheral P-gp⁺CD69⁺CD4⁺ cells indicates resistance to intensive immunosuppressive therapy and renal damage.

How might this impact clinical practice?

- ▶ The control of P-gp⁺CD69⁺CD4⁺ cells overcomes refractory proliferative lupus nephritis.

immunosuppressants. However, we often experience patients with highly active SLE who do not respond to immunosuppressive therapies. In addition, repeated intensive immunosuppressive therapy, including high-dose CS, often causes treatment-related severe adverse effects, such as opportunistic infections. Therefore, for successful disease control with minimal adverse effects, it is important to identify the responsible molecules and cells that mediate the pathogenesis and treatment refractoriness to select treatments that target these molecules and cells.

We reported previously that CD4⁺ cells are activated by cytokines and that such process plays an important role in the pathobiology of SLE, and it can result in the acquisition of P-glycoprotein (P-gp)-mediated multidrug resistance.^{1,2} P-gp, a 170 kDa product of the multidrug resistance MDR-1 gene, is a member of the ATP-binding cassette transporter superfamily of genes and functions as an energy-dependent transmembrane efflux



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Table 1 Characteristics of the patients with SLE

	SLE patients (n=116)
Disease duration (years)*	5.2 (0.1–28)
BILAG score*	13.0 (0–48)
SLEDAI score*	10.5 (0–39)
Organ involvement	
n	100
Serositis	23
NPSLE	61
LN (proliferative LN)	68 (39)
Treatment with prednisolone (or equivalent)	
n	102
Dose (mg/day)*	40 (5–80)
No of patients on combination therapy at enrolment	
Cyclophosphamide	40
Azathioprine	6
Ciclosporin	7
Tacrolimus	1
Mizoribin	2
Disease activity and clinical response to corticosteroids	
Inactive disease	10
Responders	55
Low responders	51
Organ involvement in low responders	
n	49
Serositis	12
NPSLE	37
LN (proliferative LN)	38 (23)

*Data are median (range).

BILAG, British Isles Lupus Assessment Group 2004 Index; LN, lupus nephritis; NPSLE, neuropsychiatric systemic lupus erythematosus; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

pump. Overexpression of P-gp results in reduction of intracellular concentrations of drugs of P-gp substrates, such as colchicine, cyclosporine and glucocorticoids.^{3–7} We have also reported that high expression of P-gp on CD4⁺ cells, which correlates with disease activity, can lead to the development of drug resistance and failure to control disease activity in patients with SLE who have highly active disease.^{2,8} In other words, overexpression of P-gp on lymphocytes along with lymphocyte activation results in the development of multidrug resistance.

CD69, a well-defined early-activation surface marker of lymphocytes, is a functional triggering molecule on activated CD4⁺ cells.⁹ CD69 expression is marginal on resting normal CD4⁺ cells but can be induced on these cells within hours on exposure to T cell activating stimuli.¹⁰ The CD69 signalling in CD4⁺ cells mediates CD4⁺

cell migration, in addition to production of cytokines and proliferation of CD4⁺ cells.¹¹ CD69 is persistently expressed on T cells infiltrating loci of inflammation in various chronic inflammatory diseases, such as rheumatoid arthritis (RA).¹² Furthermore, P-gp is predominantly induced on CD69⁺-active CD4⁺ cells *in vitro*¹⁰ and expressed on peripheral CD69⁺CD4⁺ cells in patients with RA.¹³ However, clinical validation of the relationship between CD69-expressing CD4⁺ cells and clinical pathogenesis of SLE remains unclear.

The aim of the present study was to elucidate the role of P-gp-expressing CD69⁺-active CD4⁺ cells in organ involvement in refractory SLE. For this purpose, we examined the expression of both P-gp and CD69 on peripheral CD4⁺ cells in both normal subjects and patients with SLE, and we investigated the relevance of P-gp⁺CD69⁺CD4⁺ cells to the clinical features of SLE. We also used immunohistochemistry to determine P-gp and CD4 expression on organ-infiltrating lymphocytes.

METHODS

Patients

The study was approved by the institution human research committee and informed consent was obtained from all the control subjects and patients who were enrolled in the study. The study included 116 patients with SLE (16–70 years old, median: 39 years, females: 108, males: 8) and 10 normal subjects (28–43 years old, median: 35 years, females: 8, males: 2). Blood samples were obtained from each patient and healthy adult volunteer. The diagnosis of SLE was based on the American College of Rheumatology revised criteria for SLE. All patients with SLE were admitted at hospital of the University of occupational and environmental health, Japan, between September, 2004 and August, 2011. The medical records of all 116 patients with SLE were checked for clinical disease activity, organ involvement (serositis, neuropsychiatric systemic lupus erythematosus (NPSLE) and lupus nephritis (LN)) and use of immunosuppressants and other therapies. SLE clinical disease activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)¹⁴ and British Isles Lupus Assessment Group 2004 Index (BILAG).¹⁵ Of 116 patients with SLE, 68 represented all patients diagnosed with LN from whom renal biopsies had been obtained previously and stored at the Department of Pathology II, University of Occupational and Environmental Health, Kitakyushu, Japan. We divided the patients with LN into two groups: the proliferative LN group (n=39), representing patients whose renal biopsies were classified as International Society of Nephrology/Renal Pathology Society 2003 class III and IV nephritis, and the non-proliferative LN group (n=29), representing all other patients with LN who were not diagnosed as proliferative LN. Moreover, we divided the patients with SLE into three groups by disease activity and clinical response to CS: (1) the inactive disease group (n=10), representing patients with clinical remission and

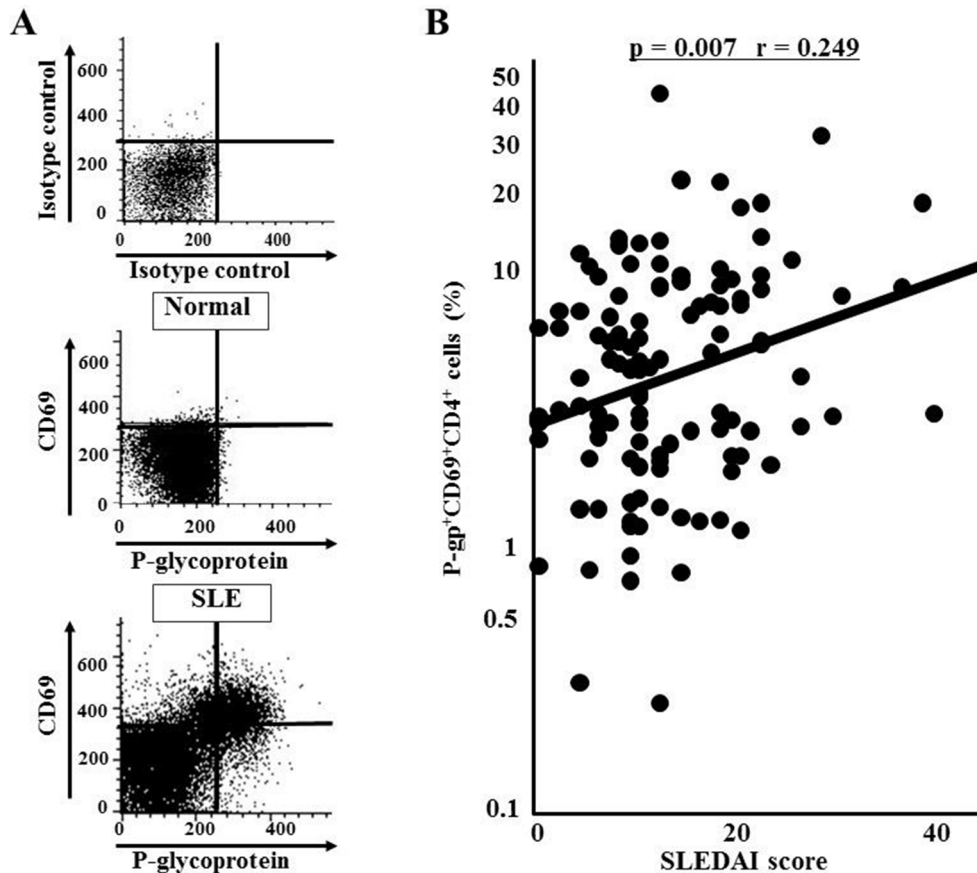


Figure 1 P-glycoprotein (P-gp) on CD69⁺CD4⁺ cells and relation with disease activity in patients with systemic lupus erythematosus (SLE). (A) Flow cytometric analysis of P-gp expression on CD69-positive and CD69-negative CD4⁺ cells in a representative normal subject and a representative patient with SLE. Appropriate isotype controls are shown. (B) Correlation of SLE disease activity score estimated by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) with proportion of peripheral P-gp⁺CD69⁺CD4⁺ cells in 116 patients with SLE. Pearson's correlation analysis.

SLEDAI of <3 points at the time of blood sampling; (2) the responders group (n=55), representing patients with SLEDAI score of <12 points and treatment intention was unnecessary, or those who responded well to 0.5–1.0 mg/kg body weight/day of prednisolone (PSL) equivalent and showed improvement in SLEDAI score to <12 points within 2 weeks of treatment; and (3) the low responders group (n=51), representing patients with SLEDAI score that was persistently >12 points despite treatment with 0.5–1.0 mg/kg body weight/day of PSL equivalent for >2 weeks. Table 1 summarises the clinical features of the patients with SLE. Low responders with proliferative LN received intensive immunosuppressive treatment in addition to high-dose CS therapy with 0.5–1.0 mg/kg body weight/day of PSL equivalent. Intravenous cyclophosphamide pulse therapy (IVCY) comprised intravenous infusion of cyclophosphamide (0.5–1 g/m²) once monthly. Biweekly IVCY (b-IVCY) used intravenous infusion of cyclophosphamide (500 mg) every 2 weeks. Methyl-PSL (mPSL) pulse therapy used intravenous infusion of mPSL (1 g/day for 3 days) every week. Rituximab (RTX) used twice intravenous infusion of RTX (1000 mg/body) every 2 weeks. Cyclosporine (CsA) used oral CsA 4–5 mg/kg body weight/day.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from the normal volunteers and patients with SLE by density gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden) and the method described in detail previously.¹⁶ Staining and flow cytometric analysis of PBMCs were conducted by standard procedures described previously,^{1,10,16} using FACScan (Becton Dickinson, Mountain View, CA). Briefly, PBMCs (2×10⁵ cells/well) were initially incubated with polyclonal γ -globulin (10 μ g/mL; Mitsubishi Welfarma, Osaka, Japan) to block Fc receptors. These cells were then incubated with MRK-16 (100 μ g/mL, Kyowa Medex, Tokyo, Japan), a specific monoclonal antibody (mAb) against P-gp,¹⁷ followed by the addition of fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (5 μ g/mL; Fujisawa, Osaka, Japan) in FACS medium containing phosphate-buffered saline, 0.5% human serum albumin (Mitsubishi Welfarma) and 0.2% Na₂S₂O₃ (Sigma Aldrich Japan, Tokyo) for 30 min at 4°C. For the three-colour analysis, we incubated PBMCs with cy-chrome-conjugated CD4 mAb (BD Biosciences Pharmingen) and PE-conjugated CD69 mAb (BD Biosciences Pharmingen) after blocking of free anti-mouse

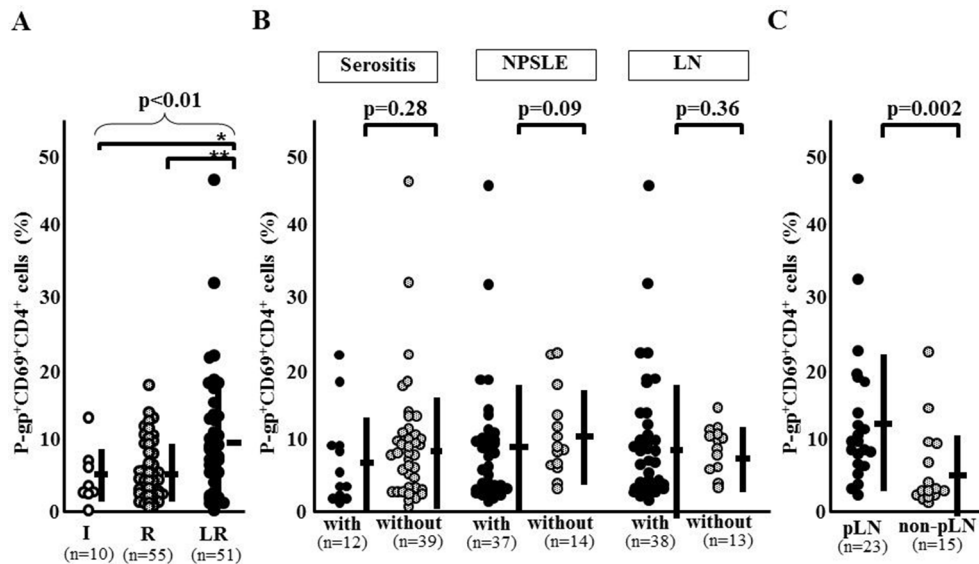


Figure 2 Proportion of peripheral P-gp⁺CD69⁺CD4⁺ cells and type of lupus nephritis (LN) in subgroups divided according to response to corticosteroids (CS) therapy. (A) Proportion of peripheral blood P-gp⁺CD69⁺CD4⁺ cells in patients with systemic lupus erythematosus with inactive disease, responders to CS and low responders to CS. Data of individual patients and group mean±SD. **p<0.01, by one-way analysis of variance. (B) Proportion of peripheral blood P-gp⁺CD69⁺CD4⁺ cells in low responders with and without serositis (left), neuropsychiatric systemic lupus erythematosus (NPSLE) (middle) or LN (right). Data are mean±SD. Non-paired t-test. (C) Proportion of peripheral blood P-gp⁺CD69⁺CD4⁺ cells in low responders with proliferative LN (pLN) or non-proliferative LN (non-pLN). Data are mean±SD. Non-paired t-test.

IgG-binding sites with irrelevant antibodies. mAb three-color-stained cells were detected by electronic gating based on their P-gp, CD4 or CD69 expression using FACScan. Amplification of mAb binding was provided by three-decade logarithmic amplifier.

Histopathological and immunohistochemical assessments

Surgically resected renal biopsy specimens were fixed in 15% phosphate-buffered formalin and embedded in paraffin. Five-micrometre-thick sections were stained with H&E or appropriate immunohistochemical stains. LN was diagnosed in all patients by histopathological examination of renal biopsies. Renal biopsy specimens were evaluated according to International Society of Nephrology/Renal Pathology Society 2003 classification of LN.¹⁸

For immunohistochemical staining, the deparaffinised and rehydrated 5-µm-thick renal sections were antigen retrieved by heating in Target Retrieval Solution (Dako, Tokyo) and then incubated in 3% H₂O₂ for 5 min 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), isotype-matched negative control antibody of P-gp (negative control mouse IgG1, dilution, 1:10; DAKO, Glostrup, Denmark) for 2 h or CD4 (LE-CD4, anti-human mouse mAb, dilution, 1:20; Dako, Glostrup, Denmark) for 1 h at room temperature followed by secondary antibody (MACH 2 Double Stain 1 or 2; Biocare Medical, Concord, CA) for 30 min at room temperature. Sections were stained for 10 min with a solution consisting of 20 mg of 3,3'-diaminobenzidine

tetrahydrochloride, 65 mg of sodium azide and 20 mL of 30% H₂O₂ in 100 mL of Tris/HCl (50 mmol/L; pH 7.6) or with Vulcan Fast Red Chromogen Kit 2 (Biocare Medical), counterstained with Meyer's hematoxylin and was then examined under a light microscope.

Statistical analysis

The characteristics of the study subjects were expressed as median and range. The proportions of peripheral CD4⁺ cells were expressed as mean±SD. The distribution of SLEDAI score exhibited Gauss distribution. The proportion of P-gp⁺CD69⁺CD4⁺ cells obtained in this study showed Gauss distribution following logarithmic transformation. We conducted the following statistical tests using these logarithmic values. The Student's t-test was used to compare data of two groups. One-way analysis of variance and Fisher's protected least significant difference were used to compare data of three groups. Correlations between two variables were examined by Pearson's correlation analysis. In the figures, the linear regression line is shown together with the Pearson's correlation coefficient (r) and the respective correlation p value. p Values less than 0.05 were considered statistically significant.

RESULTS

Expression of CD69 and P-gp on peripheral CD4⁺ cells in SLE

The expression level of P-gp and CD69 was marginal on peripheral CD4⁺ cells of normal subjects but was significantly higher in patients with SLE: P-gp⁺CD4⁺ cells (10.8%±8.0 vs 2.2%±1.1, p=0.001) and CD69⁺CD4⁺ cells

Table 2 Intensive immunosuppressive therapy to 12 low responders with highly active proliferative lupus nephritis

Case	Age (years)	Renal pathology				SLEDAI					
		Glomerular lesion (International Society of Nephrology/Renal Pathology Society 2003)		Interstitial lesion		*Intensive immunosuppressive therapies		After (observation period, weeks)			
		Age (years)	Pathology	Lymphocyte infiltration	Tubular atrophy	Dose of PSL (or equivalent) mg/kg bw/day	Vasculopathy	Before	Treatment response		
1	50	III(A)	+	+	-	1	-	b-IVCY (2)	26	2 (4)	I
2	16	IV-G(A/C)	+	+	+	0.8	+	IVCY (1)+pulse mPSL (3)+IA (1)	25	20 (4)	SF
3	44	IV-G(A/C)	+	+	-	1	-	b-IVCY (2)	19	14 (4)	SF
4	41	IV	?	?	?	0.8	?	IVCY (1)+pulse mPSL (3)+IA (1)+PE (1)	36	29 (4)	SF
5	44	IV-G(A/C)	+	+	-	0.8	-	IVCY (1)+MTX	14	10 (4)	I
6	34	III(A)	+	+	-	1	-	CsA	12	6 (2)	I
7	41	IV	?	?	?	0.8	?	RTX	30	38 (4)	SF
8	42	IV-S(A)	+	+	+	1	+	IVCY (1)	12	12 (4)	SF
9	16	IV-G(A/C)	+	+	+	0.8	+	b-IVCY (2)+IA (1)	20	10 (3)	I
10	42	IV-S(A)	+	+	+	0.8	+	IVCY (1)	12	8 (2)	I
11	38	IV-S(A)	+	+	-	0.8	-	IVCY (1)+IA (1)	22	16 (4)	SF
12	39	IV-G(A/C)	?	?	?	1	?	IVCY (1)+IA (1)	28	20 (2)	SF

*Numbers in parentheses represent the number of times of treatment in an observation period.

?, interstitial lesion could not be investigated in detail because renal biopsy was performed in other hospitals before transfer to our hospital; b-IVCY, biweekly IVCY; bw, body weight; CsA, ciclosporin; I, patient of the 'improvement group'; IA, immunopheresis; IVCY, monthly intravenous cyclophosphamide pulse therapy; MTX, methotrexate; PE, plasma exchange; PSL, prednisolone (or equivalent); pulse mPSL, methyl-prednisolone pulse therapy; RTX, rituximab; SF, patient of the 'severe flare group'; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

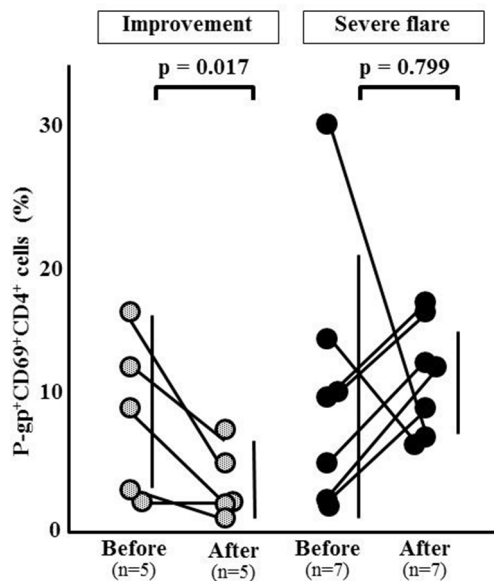


Figure 3 Relation of P-gp⁺CD69⁺CD4⁺ cells with outcome of intensive immunosuppressive therapy in patients with active proliferative lupus nephritis (LN). Proportion of P-gp⁺CD69⁺CD4⁺ cells in 12 low responders with highly active proliferative LN, including those who showed improvement and severe flare; before and after the administration of immunosuppressive therapies listed in table 2. Comparison of proportion of P-gp⁺CD69⁺CD4⁺ cells before and after immunosuppressive therapy in each group. Data are mean±SD. Paired t-test.

(22.1%±14.5 vs 5.7%±1.8, $p < 0.001$). We also analysed the expression pattern of P-gp and CD69 on CD4⁺ cells and divided the study patients into four groups. As illustrated in figure 1A, a large P-gp⁺CD69⁺CD4⁺ cell subset was observed in patients with SLE but not in normal subjects. Furthermore, the proportion of P-gp⁺CD69⁺CD4⁺ cells correlated significantly with SLE disease activity (figure 1B), whereas the proportion of P-gp⁻CD69⁻CD4⁺ cells correlated negatively and significantly with SLE disease activity in each patient assessed for SLEDAI score ($p = 0.004$, $r = 0.265$). The proportions of P-gp⁻CD69⁺CD4⁺ cells and P-gp⁺CD69⁻CD4⁺ cells did not correlate significantly with the SLEDAI score ($p = 0.346$ and $p = 0.304$). These results suggest the characteristic presence of P-gp⁺CD69⁺CD4⁺ cells in the peripheral blood of patients with SLE, which reflects SLE disease activity.

Relationship between P-gp⁺CD69⁺CD4⁺ cells and organ involvement in SLE

We next analysed the relationship between P-gp⁺CD69⁺CD4⁺ cells and organ involvement in patients with SLE. There was no relation between the proportion of peripheral P-gp⁺CD69⁺CD4⁺ cells and the type of organ manifestation: serositis (with versus without, 4.5%±5.3 vs 6.4%±6.4; $p = 0.38$), NPSLE (6.7%±7.4 vs 5.3%±4.6; $p = 0.52$) and LN (6.7%±7.6 vs 5.1%±3.3; $p = 0.77$). Furthermore, because LN classification reflects the pathogenesis of various forms of LN, we analysed the relationship between the histopathological features of LN and the

proportion of P-gp⁺CD69⁺CD4⁺ cells. The proportion of P-gp⁺CD69⁺CD4⁺ cells was significantly higher in the proliferative LN group than non-proliferative LN group (8.5%±8.9 vs 4.3%±4.5; $p = 0.007$), whereas SLEDAI score was not significantly different between the two groups (16.0%±9.3 vs 12.6%±8.3; $p = 0.13$).

Relationship between P-gp⁺CD69⁺CD4⁺ cells and refractory proliferative LN

We also analysed the relation between clinical response to CS and the proportion of P-gp⁺CD69⁺CD4⁺ cells. The proportion of P-gp⁺CD69⁺CD4⁺ cells was markedly higher in the low responders group (figure 2A), but there was no significant difference in the proportion of P-gp⁺CD69⁺CD4⁺ cells between the inactive disease group and responders (figure 2A).

We also analysed peripheral P-gp⁺CD69⁺CD4⁺ cells in low responders according to the presence or absence of organ involvements. The presence or absence of serositis, NPSLE and LN had no effect on the proportion of P-gp⁺CD69⁺CD4⁺ cells (figure 2B). However, the proportion of P-gp⁺CD69⁺CD4⁺ cells was significantly higher in the proliferative LN group ($n = 23$) than non-proliferative LN group ($n = 15$) (figure 2C). SLEDAI score was not significantly different between the two groups (proliferative LN: 21.2%±7.4 vs non-proliferative LN: 17.4%±7.3; $p = 0.12$). These results suggest that P-gp⁺CD4⁺CD69⁺ cells were especially increased in refractory proliferative LN.

Roles of P-gp⁺CD69⁺CD4⁺ cells in poor responders to treatment and in pathogenesis of low responders with proliferative LN

We considered that P-gp⁺CD69⁺CD4⁺ cells play an important role in the pathogenesis of proliferative LN and contribute to the poor response to treatment. First, we assessed the relationship between the response to intensive immunosuppressive therapy and P-gp⁺CD69⁺CD4⁺ cells in low responders with proliferative LN. The proportion of P-gp⁺CD69⁺CD4⁺ cells could be analysed 2–4 weeks after the commencement of treatment in 12 of 23 low responders with proliferative LN who received intensive immunosuppressive treatment plus high-dose CS. Table 2 summarises the clinical and histopathological features of these 12 low responders. All patients had high disease activity (ie, high SLEDAI score of >12 points) despite the treatment with >0.5 mg/kg body weight/day of PSL followed by IVCY, oral CsA, RTX or mPSL pulse therapy.

The response to the aforementioned immunosuppressive therapy in the 12 low responders with proliferative LN was defined using change in disease activity estimated by SLEDAI score within the observation period. The 12 low responders with proliferative LN were further subdivided into two groups based on the treatment response: the severe flare group ($n = 7$) with a persistently high SLEDAI score (>12 points) despite the intensive immunosuppressive therapy and the improvement group ($n = 5$), who responded to the treatment and showed

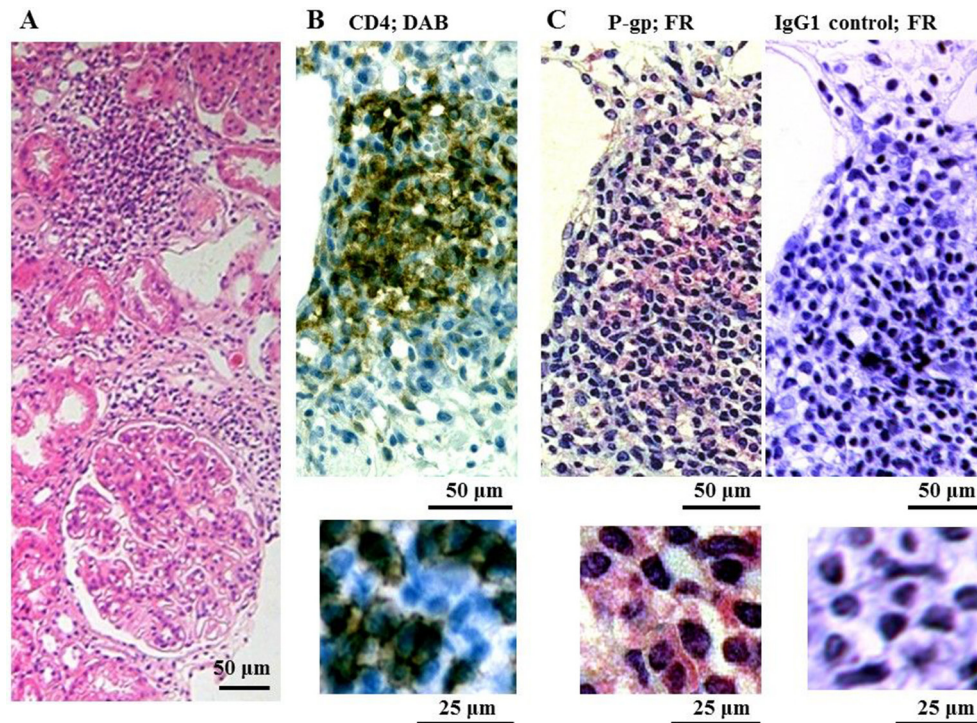


Figure 4 Histopathological and immunohistochemical analyses of renal tissues from a representative patient with refractory progressive proliferative lupus nephritis (LN). (A) H&E staining. (B) Immunostaining for CD4⁺ lymphocytes using anti-CD4 monoclonal antibody (mAb) with 3,3'-diaminobenzidine (DAB) (brown colour). (C) Immunostaining for P-glycoprotein (P-gp) on lymphocytes using JSB-1 anti-P-gp mAb (P-gp) or isotype-matched negative control antibody IgG₁ (IgG₁ control) with Vulcan Fast Red (FR) (red colour). (B–C) Nuclear counterstaining with hematoxylin. Bottom: magnified view of a section of each of the top image.

clinical improvement in response to intensive immunosuppressive treatment (based on a decrease in SLEDAI score to <12 points). Before the administration of immunosuppressants, the proportion of P-gp⁺CD69⁺CD4⁺ cells was similar in the two groups (severe flare: 11.2±10.1% vs improvement: 9.1%±6.1%; $p=0.88$). However, intensive immunosuppressive therapy significantly reduced the proportion of these cells in the improvement group compared with the severe flare group (severe flare: 12.1±4.3% vs improvement: 3.9%±2.8%; $p=0.003$). In fact, the proportion of P-gp⁺CD69⁺CD4⁺ cells decreased significantly in each of the five patients of the improvement group, whereas it increased in five of seven of the severe flares group despite the treatment (figure 3).

Next, we examined the pathogenic role of P-gp⁺CD69⁺CD4⁺ cells in proliferative LN by histopathological examination of the 12 low responders with proliferative LN. Histopathological examination confirmed renal interstitial lymphocyte infiltration in 9 of the 12 patients (table 2). Figure 4A shows sections, stained with H&E, of a representative patient with proliferative LN (Case 5, table 2) with marked accumulation of inflammatory cells in the renal interstitial tissue. Immunohistochemical staining of serial sections of 5 µm thickness from the same specimens showed that the majority of these inflammatory cells were CD4⁺ cells (figure 4B) and P-gp⁺ cells (figure 4C). These findings suggest accumulation of P-gp⁺CD4⁺ cells in the renal interstitial tissue, and these imply that P-gp⁺CD69⁺CD4⁺ cells infiltrate

renal interstitial tissue and cause inflammation, with close correlation with disease activity and resistance to treatment in patients with proliferative LN. The results suggest that improvement in disease activity in patients with highly active proliferative LN in response to intensive immunosuppressive treatment is mediated through reduction of P-gp⁺CD69⁺CD4⁺ cells.

DISCUSSION

In this study, we demonstrated the potential relevance of P-gp⁺CD69⁺CD4⁺ cells in refractory proliferative LN. P-gp expression was preferentially high on CD69-overexpressing CD4⁺ cells of patients with SLE. The relationship between P-gp expression and CD69 signalling in CD4⁺ cells remains unclear. CD69 is a member of the C-type lectin-like receptor family¹⁷ and acts as a signal transducer that promotes activation of the MAPK/ERK pathway.¹⁹ The MAPK/ERK pathway induces YB-1 nuclear translocation,^{20 21} transcription of MDR-1²² by binding activated YB-1 and expression of P-gp, resulting in cell migration.^{23 24} Experimental and clinical evidence suggests the involvement of P-gp and CD69 in the migration of these cells and that CD69 signalling in T cells induces T cell migration.²⁵ Other studies showed that P-gp expression on leukaemia cells correlates with disease aggressiveness and enhanced invasiveness.^{26 27} Furthermore, overexpression of P-gp is associated with metastasis of breast cancer cells and poor prognosis.²⁸ Also, P-gp-specific inhibitors reduce *in vitro*

migration of breast cancer cells.²⁹ Considered together, CD69-mediated signalling could induce the expression of P-gp on CD4⁺ cells through the activation of the MAPK/ERK pathway, which might be associated with the increased invasive behaviour. Thus, P-gp⁺CD69⁺CD4⁺ cells seem to have high-migration capacity and can exacerbate pathological lesions.

Our results demonstrated increased proportion of P-gp⁺CD69⁺CD4⁺ cells, the majority of P-gp⁺CD4⁺ cells, in patients with proliferative LN and that proliferative LN with expansion of peripheral P-gp⁺CD69⁺CD4⁺ cells showed active infiltration of lymphocytes and accumulation of P-gp⁺CD4⁺ cells in the renal interstitial tissue. Previous studies showed expansion, circulation and homing of pathogenic lymphocytes to target organs in various systemic autoimmune diseases.^{30–33} For example, a significant increase of CXCR4-expressing B cells was reported in peripheral blood of patients with active SLE and in renal tissues of LN.³⁰ CXCR4 expression on circulating B cells was higher in both active LN and active NPSLE.³¹ Another study noted a significant increase in CD25-expressing T cells in peripheral blood of patients with active cutaneous lupus erythematosus as well as in a subset of skin homing.³² Circulating Vδ1⁺ T cells were significantly increased and accumulated in perivascular areas of the skin in patients with systemic sclerosis.³³ These data suggest that P-gp⁺CD69⁺CD4⁺ cells can expand, enter the circulation and accumulate in renal interstitial tissue in proliferative LN.

Our findings provided the first evidence for the renal preferential selection of P-gp and CD69 co-expressing CD4 cells. The relevance of P-gp or CD69 to renal preference has not been clear. Sfikakis *et al*³⁴ reported that the extent of decrease in CD69 expression depends significantly on the remission level of proliferative LN. Huls *et al*³⁵ reported that P-gp-deficient mice are protected against ischaemia-induced renal injury and that such protection was also observed after bone marrow transplantation from P-gp-deficient mice into irradiated wild type mice. However, the mechanism of induction of the desirable results in the kidney by deficiency of P-gp or by underexpression of CD69 remains unclear.

In the present study, expansion of P-gp⁺CD69⁺CD4⁺ cells was especially clear in low responders with proliferative LN. The NIH regimen and EULAR/ERA-EDTA recommendations stipulate initial therapy with high-dose CS combined with IVCY for proliferative LN.^{36–37} The majority of the 12 low responders with highly active proliferative LN in the present study were treated with high-dose CS and IVCY but some continued to show severe flare. We demonstrated in a previous study that reduction of P-gp (achieved by intensive immunosuppressive treatment) resulted in resolution of steroid resistance.^{2,38} However, in the present study, a few patients of the low responders group with proliferative LN responded inadequately to intensive immunosuppressive therapy combined with high-dose CS and this poor response was associated with increases in peripheral

P-gp⁺CD69⁺CD4⁺ cells. On the other hand, large reduction in peripheral P-gp⁺CD69⁺CD4⁺ cells following the combination therapy was associated with improvement in clinical features in refractory proliferative LN. Accordingly, we propose that P-gp⁺CD69⁺CD4⁺ cells could be the main orchestrators of progressive proliferative LN mediated through their direct infiltration in the kidney and that treatments that target these cells could potentially control disease activity in refractory proliferative LN.

CONCLUSIONS

P-gp⁺CD69⁺CD4⁺ cells infiltrate the kidney, resulting in renal tissue damage and resistance to therapy. The control of P-gp⁺CD69⁺CD4⁺ cells seems important for improvement of refractory proliferative LN. Overexpression of P-gp on peripheral CD69⁺CD4⁺ cells is a potentially useful marker of treatment resistance and of renal damage induced by direct infiltration of CD4⁺ cells. Measurement of the percentage of peripheral P-gp⁺CD69⁺CD4⁺ cells in patients with proliferative LN with highly active disease could help in the selection of appropriate treatment strategy including reinforcement of P-gp or CD4 cell-targeting therapy.

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