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Relevance of P-glycoprotein on CXCR4⁺ B cells to organ manifestation in highly active rheumatoid arthritis

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Abstract

Introduction: In rheumatoid arthritis (RA), P-glycoprotein (P-gp) expression on activated B cells is associated with active efflux of intracellular drugs, resulting in drug resistance. CXCR4 is associated with migration of B cells. This study was designed to elucidate the relevance of P-gp expression on CXCR4⁺ B cells to clinical manifestations in refractory RA.

Methods: CD19⁺ B cells were analyzed using flow cytometry and immunohistochemistry.

Results: P-gp was highly expressed especially on CXCR4⁺CD19⁺ B cells in RA. The proportion of P-gp-expressing CXCR4⁺ B cells correlated with disease activity, estimated by Simplified Disease Activity Index (SDAI), and showed marked expansion in RA patients with high SDAI and extra-articular involvement. In highly active RA, massive infiltration of P-gp⁺CXCR4⁺CD19⁺ B cells was noted in CXCL12-expressing inflammatory lesions of RA synovitis and RA-associated interstitial pneumonitis. In RA patient with active extra-articular involvement, intracellular dexamethasone level (IDL) in lymphocytes diminished with expansion of P-gp⁺CXCR4⁺CD19⁺ B cells. Adalimumab reduced P-gp⁺CXCR4⁺CD19⁺ B cells, increased IDL in lymphocytes, and improved the clinical manifestation and allowed tapering of concomitant medications.

Conclusions: Expansion of P-gp⁺CXCR4⁺ B cells seems to be associated with drug resistance, disease activity and progressive destructive arthritis with extra-articular involvement in RA.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by abundant accumulation of inflammatory cells, including activated B lymphocytes, in synovial tissues with enhanced inflammatory and destructive polyarthritis [1,2]. Activated B cells produce rheumatoid factor [3] and various inflammatory cytokines, such as tumor necrosis factor (TNF) [4] and interleukin (IL)-6 [5], infiltrate the synovial tissues, and present antigens to T cells [6]. Therefore, activation and infiltration of B cells play important roles in the pathological process of RA.

We reported previously that B cells are activated by cytokines and extracellular matrix adhesion, which constitutes part of the pathobiology of RA, and that such process could result in P-glycoprotein (P-gp)-mediated multidrug-resistance [7–9]. P-gp is a 170-kDa product of the multidrug resistance-1 (MDR-1), a member of the ATP-binding cassette (ABC) transporter superfamily of genes. P-gp functions as an energy-dependent transmembrane efflux pump. Overexpression of P-gp results in reduction of intracellular concentrations of drugs of P-gp substrates, such as colchicine, cyclosporine, and glucocorticoids, including cortisol and dexamethasone [9–14]. We have also reported that high expression of P-gp on B cells, which correlates with disease activity, can lead to the development of drug resistance and failure to control disease activity in RA patients with highly active disease [15,16]. However, the role of P-gp-overexpressing B cells in the inflammatory pathogenesis of RA has not been completely delineated.

Chemokines play a pivotal role in the activation and recruitment of immune cells at sites of inflammation [17]. CXCR4, expressed on B cells, stimulates their proliferation, and plays an important role in their migration and production of various cytokines [18–20]. Experimental and clinical evidence suggests the involvement of P-gp and CXCR4 in the migration of various inflammatory and cancer cells [17,21–24]. For example, over-expression of CXCR4 is associated with metastasis of breast cancer cells and high expression of P-gp is associated with poor prognosis in such patients [22,23]. Furthermore, P-gp-specific inhibitors reduce in vitro migration of breast cancer cells [24], and CXCR4-specific inhibitors also inhibit CXCL12-induced migration of breast cancer cells [17]. The expression of CXCR4 on B cells of patients with chronic lymphocytic leukemia correlates with disease progression and poor prognosis [25,26] and P-gp expression on leukemia cells correlates with disease aggressiveness and enhanced invasiveness [27,28]. Furthermore, there is sufficient evidence that confirms the involvement of the CXCL12/CXCR4 axis in various inflammatory conditions, including RA and pulmonary fibrosis [17,18,29]. The concentrations of CXCL12 in the...
serum and synovial fluid were higher in patients with RA than those with osteoarthritis [30]. Serum CXCL12 levels correlated positively with bone erosion score in RA [31]. However, the relevance of the CXCL12/CXCR4 axis to P-gp expression on B cells and its involvement in the pathological processes of RA remain unclear.

The aim of the present study was to elucidate the role of P-gp-expression on B cells in organ inflammation in refractory RA. For this purpose, we examined the expression of both CXCR4 and P-gp on peripheral B cells in both normal subjects and patients with RA, and also on infiltrated B cells at the inflammatory sites in RA, and determined the relation between the expression of CXCR4 and P-gp on B cells.

Patients and methods

Patients

The ethics committee of our institution approved the study and informed consent was obtained from all the control subjects and patients who were enrolled in the study. The study included 20 patients with RA (age: 35–84 years, median 66, females 17, males 3) and eight normal subjects (33–50 years, median 37, females 6, males 2). Blood samples were obtained from all healthy adult volunteers and the patients. All RA patients were admitted to our hospital between February 2010 and August 2011. Table 1 summarizes the clinical features of the RA patients. The diagnosis of RA was based on the American College of Rheumatology (ACR) revised criteria for RA. The clinical activity of RA was assessed by the Simplified Disease Activity Index (SDAI), calculated by using the 28-swollen joint count, 28-tender joint count, patient global assessment of disease activity (SDAI), calculated by using the 28-swollen joint count, 28-tender joint count, patient global assessment of disease activity on a 10-cm visual analog scale (VAS), global assessment of disease activity on a 10-cm VAS, and C-reactive protein level in mg/dl [32].

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the eight normal volunteers and 20 RA patients by density gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden), and the method described in detail previously [16].

Flow cytometry

Staining and flow cytometric analysis of PBMCs were conducted using standard procedures as described previously [16,33,34], on FACSscan (Becton Dickinson, Mountain View, CA). Briefly, PBMCs (2 × 10^5 cells/well) were initially incubated with polyclonal γ-globulin (10 μg/mL, Yoshitomi Pharmaceutical Co., Tokyo, Japan) to block Fc-receptors and then incubated with MRK-16 (a specific monoclonal antibody (mAb) against P-gp [35]; Kyowa Medex, Tokyo, Japan), followed by FITC-conjugated anti-mouse IgG antibody in FACS medium consisting of phosphate-buffered saline, 0.5% HSA, and 0.2% NaN₃ (Sigma Aldrich, Tokyo, Japan). For three-color analysis, PBMCs were incubated with cy-chrome-conjugated CD19 mAb and PE-conjugated CXCR4 mAb after blocking of free anti-mouse IgG-binding sites with irrelevant antibodies. Monoclonal antibodies-three-color-stained cells were detected by electronic gating based on their P-gp, CD19 or CXCR4 expression using FACSscan. Amplification of mAb-binding was provided by three-decade logarithmic amplifier.

Preparation of tissue samples from patients with rheumatoid arthritis

For this part of the study, we obtained synovial membranes that had been surgically resected by synovectomy or total knee replacement from RA patients (n = 4) and lung autopsy specimens (n = 2) that had been surgically resected from RA patients. We also obtained a control synovium specimen (free of major changes) that had been resected surgically from a patient with injury of the knee anterior cruciate ligament. The resected tissues had been stored at the Department of Pathology II, University of Occupational and Environmental Health, Kitakyushu, Japan. The resected tissues were fixed in 15% phosphate-buffered formalin and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) or other appropriate immunohistochemical stains.

Immunohistochemistry

Deparaffinized and rehydrated 5-μm-thick sections were antigen retrieved by heating in Target retrieval solution (Dako, Tokyo, Japan), and then incubated in 3% H₂O₂ for 5 min to block endogenous peroxidase activity, followed by rinsing. Immunostaining of paraffin sections of P-gp-positive tissues was reported to be excellent with JSB-1 among several primary antibodies against P-gp [36]. For single-staining immunohistochemistry, the sections were incubated with the

<table>
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<tr>
<th>Table 1. Characteristics of the study subjects.</th>
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<tr>
<td>RA patients (n = 20)</td>
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<tr>
<td>Age (years)</td>
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<td>Sex (females/male)</td>
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<td>Disease duration (years)</td>
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<td>RF</td>
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<td>SDAI</td>
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<td>Extra-articular organ involvement</td>
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<td>Interstitial pneumonia</td>
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<td>Felty’s syndrome</td>
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<td>Lymphadenopathy</td>
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<tr>
<td>Glomerulonephritis</td>
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<tr>
<td>Number of patients on treatment at enrolment</td>
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<tr>
<td>None</td>
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<tr>
<td>Prednisolone (or equivalent)</td>
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<tr>
<td>Dosage (median, range), mg/day</td>
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<tr>
<td>Methotrexate</td>
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<td>Dosage (median, range), mg/week</td>
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<tr>
<td>Sulfasalazine</td>
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<td>Tacrolimus</td>
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<td>Adalimumab</td>
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<td>Tocilizumab</td>
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*Data are median (range).
Accumulation of dexamethasone

14C-labelled n-butanol (1.61 mCi/mmol; Toho Biochemical, Tokyo, Japan) was diluted with unlabelled butanol (Sigma Aldrich, Tokyo, Japan) at a concentration of 0.5 MBq/mL. 3H-labelled dexamethasone (40.0 Ci/mmol; Perkin, Boston, MA) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Tokyo, Japan) at a concentration of 0.5 MBq/mL. A solution of 3,3’-diaminobenzidine (DAB) 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 in Vulcan Fast Red Chromogen Kit 2 (Biocare Medical, Pacheco, CA), counterstained with Meyer’s hematoxylin, and subsequently examined under a light microscope.

For double staining of CD19 and P-gp, the human tissue sections were incubated with LE-CD19 for 1 h at room temperature followed by MACH 2 Double stain 2 for 30 min at room temperature. Then, the sections were colorized for 10 min in a solution consisting of 20 mg of 3,3’-diaminobenzidine (DAB) 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 in Vulcan Fast Red Chromogen Kit 2 (Biocare Medical, Pacheco, CA), counterstained with Meyer’s hematoxylin, and subsequently examined under a light microscope.

**Accumulation of dexamethasone**

The expression level of CXCR4 on peripheral B cells was high in normal subjects, and varied among RA patients. On the other hand, P-gp expression was marginal on B cells of normal subjects, but high in patients with RA (Figure 1(a)). The expression level of P-gp correlated with CXCR4 expression level on B cells in patients with RA (Figure 1(b)). Furthermore, P-gp expression was preferentially high on CXCR4-expressing B cells (Figure 1(c)), and was significantly higher on CXCR4+B cells than that on CXCR4−B cells of RA patients (36.1%±24.6 vs. 13.7%±11.7; p < .0001). These results suggest the characteristic presence of P-gp+CXCR4+ B cells in the peripheral blood of RA patients.

**Relationship between synovial inflammation and P-gp+CXCR4+ B cells**

To elucidate the relevance of P-gp+CXCR4+ B cells to synovial inflammation, we analyzed the synovial tissues of RA patients.
with active synovitis. Figure 2 shows histopathological findings in a representative RA patient. This patient was 69-year-old female, with 10-year disease duration but free of organ involvement, treated with MTX 10 mg/week, and underwent synovectomy of the right wrist. Proliferation of synovial tissue and marked accumulation of inflammatory cells were noted in the synovial tissues of the inflamed joints (Figure 2(a)). The majority of these inflammatory cells were CD19+ B cells (Figure 2(b)). Immunohistochemical staining of serial sections obtained from the same specimen showed that the infiltrated CD19+ B cells also expressed both P-gp and CXCR4 (Figure 2(c,d)). Double staining for CD19 and P-gp confirmed the expression of P-gp on CD19+ B cells infiltrating the synovium (Figure 2(e)). Marked accumulation of P-gp and CXCR4 double-positive B cells (P-gp+CXCR4+ B cells) was noted in the active site of synovitis. To confirm the involvement of CXCL12/CXCR4 axis in the infiltration of P-gp+ B cells in active synovitis, staining for CXCL12 was performed. Fibroblast-like CXCL12+ cells were noted at the site of accumulation of inflammatory cells in the RA synovial tissue (Figure 2(f), RA). In contrast, both CXCL12+ cells and accumulation of inflammatory cells were absent in the synovial tissues of the control (Figure 2(f), NS).

Clinical validation of the relationship of P-gp+CXCR4+ B cells to disease activity and active extra-articular involvement

We postulated that P-gp+CXCR4+ B cells play a role in the synovitis of RA. To test our hypothesis, we investigated the relationship between RA disease activity and the expression of P-gp and CXCR4 on peripheral B cells. There was no relation between the proportion of CXCR4+ B cells and RA disease activity, as assessed by SDAI ($r = 0.176, p = .4629$). However, the proportion of P-gp-expressing CXCR4+ B cells correlated significantly with SDAI in each patient ($r = 0.475, p = .0332$, Figure 3(a)).

We next investigated the relationship between P-gp+CXCR4+ B cells and extra-articular organ-involvement in RA patients with highly active disease. In highly active RA patients with SDAI $> 26.0$, P-gp expression level on CXCR4+ B cells was significantly higher in patients with extra-articular organ involvement compared with those without extra-articular organ involvement (Figure 3(b)). On the other hand, the SDAI score was not significantly different between the two groups (with: 38.6% ± 14.5, without: 39.0% ± 11.8; $p = .12$). Six patients had extra-articular involvement (one with interstitial pneumonia (IP), two with IP and rheumatoid vasculitis, one with Felty’s syndrome, one with amyloidosis, and...
one with lymphadenopathy). The smallest proportion of peripheral P-gp\textsuperscript{+}CXCR4\textsuperscript{+}B cells was 1.3% in patients without organ involvement, while the largest proportion was 90.0% in a patient with rheumatoid vasculitis (Figure 3(c)). The patient with rheumatoid vasculitis also had IP and the clinical and histopathological examinations confirmed RA-associated IP and excluded other IP, e.g. infection, silicosis, drug-induced IP, and malignancy-associated IP.

To elucidate the role of P-gp\textsuperscript{+}CXCR4\textsuperscript{+}B cells in active extra-articular organ inflammation, we analyzed a lung tissue from the patient with rheumatoid vasculitis. Interstitial fibrosis, marked accumulation of inflammatory cells and lymphoid follicle formation were noted in the thickened interstitial tissues of the injured lung (Figure 4(a)). Immunohistochemical staining of serial sections of the same specimen showed that the infiltrating CD19\textsuperscript{+} B cells in inflammatory lesions expressed both CXCR4 and P-gp.

**Figure 2.** Histopathological and immunohistochemical analyses of synovial membrane tissues from a representative patient with highly active RA. (a) Hematoxylin and eosin staining. (b) Immunostaining for CD19\textsuperscript{+} lymphocytes using anti-CD19 monoclonal antibody (mAb) with 3,3'-diaminobenzidine (DAB) (brown color). (c) Immunostaining for P-glycoprotein (P-gp) on lymphocytes using JSB-1 anti-P-gp mAb with DAB. (d) Immunostaining for CXCR4\textsuperscript{+} lymphocytes using anti-CXCR4 mAb with DAB. (e) Double-immunostaining for P-gp on CD19\textsuperscript{+} lymphocytes using JSB-1 anti-P-gp mAb with Vulcan Fast Red (FR) and anti-CD19 mAb with DAB. The membranous CD19-positive cells showed cytoplasmic staining for P-gp. (f) Immunostaining for CXCL12\textsuperscript{+} lymphocytes using anti-CXCL12 mAb with FR in the synovium of a representative RA patient (RA) and a healthy subject (NS). (g) Double-immunostaining for P-gp on lymphocytes using JSB-1 anti-P-gp mAb with Vulcan Fast Red (FR) and anti-CXCL12 mAb with DAB in the synovium of 4 RA patients including the representative RA patient (RA 1). CXCL12 expression was detected in some synovial fibroblasts. (b–g) Nuclear counterstaining with hematoxylin.
Fibroblast-like CXCL12\(^+\) cells were also noted at the site of accumulation of inflammatory cells to interstitial tissue of lung (Figure 4(e)). P-gp\(^+\) cells were noted around CXCL12\(^+\) cells. In contrast, both P-gp and CXCL12 positive cells were absent at the sites of almost-normal air vesicle structures free of inflammation (Figure 4(f)).

**Trial of TNF inhibitor to overcome P-gp\(^+\) CXCR4\(^+\) B cells-related multidrug-resistance in refractory RA with extra-articular organ involvement**

The 69-year-old woman with Felty’s syndrome was treated with corticosteroid only during a period of 8 months but showed marked exacerbation of RA disease activity to SDAI of 40.6 with extra-articular organ involvements, including neutropenia and splenomegaly. She also showed massive accumulation of peripheral P-gp\(^+\) CXCR4\(^+\) B cells at 2 weeks before treatment (Figure 5(a), top panel; \(-2\) W). Despite intensive immunosuppressive therapy with methotrexate (MTX), tacrolimus, oral prednisolone (PSL) and methyl PSL pulse therapy, the proportion of peripheral P-gp\(^+\) CXCR4\(^+\) B cells remained high (Figure 5(a), top panel; 0 W), with concomitant flare of clinical symptoms and signs. Accordingly, treatment was switched to adalimumab, in addition to MTX and oral PSL. The new treatment resulted in significant fall in peripheral P-gp\(^+\) CXCR4\(^+\) B cells within 2 weeks (Figure 5(a), bottom panel; \(+2\) W), together with improvement of synovitis to SDAI of 13.0 and recovery of peripheral neutrophil count.

We also investigated the association between P-gp\(^+\) CXCR4\(^+\) B cells and drug-exclusion through P-gp. For this purpose, analysis of dexamethasone accumulation in PBMCs was conducted using radioisotope labelled dexamethasone. We reported previously that intracellular dexamethasone
level (IDL) in PBMCs of normal individuals (C/M ratio) was 0.73% ± 0.13%, mean ± SD [16]. The IDL in PBMCs before adalimumab treatment (Figure 5(b), 0 W) was markedly low. Reduction of P-gp⁺ CXCR4⁺ B cells by the addition of adalimumab resulted in recovery of IDL in PBMCs (Figure 5(b), +2 W).

Elimination of P-gp⁺ CXCR4⁺ B cells after treatment with adalimumab markedly reduced RA disease activity to SDAI of 3.5, maintained neutrophil count within the normal range, and eliminated the need for corticosteroids within 52 weeks (Figure 5(a), bottom panel; +52 W).

Another patient with highly active rheumatoid vasculitis including scleritis, skin vasculitis and active IP was treated with MTX combined with etanercept (ETN; dose of 25 mg, twice per week). MTX was increased to 12.5 mg/week, which resulted in improvement in disease activity and decrease in the proportion of P-gp-expressing CD19⁺ cells to 37.6%. However, ETN was stopped due to the development of septicemia following pyelonephritis. Treatment with antibiotics resulted in resolution of infection, but this was followed by re-activation of arthritis and IP. This was associated with marked increase in P-gp-expressing CD19⁺ cells with preferentially high percentage of P-gp-expressing CXCR4-expressing CD19⁺ cells (Figure 3(c), rheumatoid vasculitis). Despite resumption of ETN, IP remained active (Figure 4). While infection was under control, the patient died of respiratory failure 2 months after resumption of treatment with ETN.

**Discussion**

In this report, we demonstrated the potential relevance of P-gp⁺ CXCR4⁺ B cells in tissue damage in highly active RA, and that expansion of these cells in peripheral blood reflected serious organ involvement. This conclusion was based on the following main findings: (1) preferential expression of P-gp on CXCR4-expressing B cells in RA patients. (2) In patients with highly active RA, massive infiltration of P-gp⁺ CXCR4⁺ B cells was noted in RA synovial tissue positive for CXCL12 expression. (3) The level of P-gp expression on CXCR4⁺ B cells was closely related to RA disease activity. (4) Patients with highly active RA and extra-articular involvement showed marked expansion of peripheral P-gp⁺ CXCR4⁺ B cells. (5) Patients with highly active RA and IP showed both expansion of peripheral P-gp⁺ CXCR4⁺ B cells and accumulation of P-gp⁺ CXCR4⁺ B cells in the thickened interstitial tissues of the injured lung, together with CXCL12-expressing neovascular endothelial cells and fibroblasts. (6) Reduction in peripheral P-gp⁺ CXCR4⁺ B cells by combination therapy was associated with improvement in organ involvement in patients with highly active RA.

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**Figure 3.** Relation between P-gp⁺ CXCR4⁺ B cells and RA clinical features: scores of the Simplified Disease Activity Index (SDAI) and organ involvement. (a) P-gp expression on CXCR4⁺ CD19⁺ lymphocytes correlated closely with disease activity, as estimated by the SDAI score. Pearson’s correlation analysis was used to determine statistical significance. n = 20. (b) Flow cytometry showed P-gp⁺ CXCR4⁺ CD19⁺ lymphocytes in 11 RA patients with high disease activity (SDAI score > 26). The 11 RA patients included six with and five without organ involvement. Data represent the proportion of peripheral P-gp⁺-expressing CXCR4⁺ CD19⁺ lymphocytes. Data are mean ± SD. p Values were determined by non-paired t-test. (c) Flow cytometric analysis identified P-gp⁺-expressing CXCR4⁺-positive B cells in representative RA patients with rheumatoid vasculitis and patients without organ involvement (without involvement). Appropriate isotype controls are shown.
Several studies reported constitutive expression of CXCR4 on B cells in healthy subjects [18,37]. However, the expression of CXCR4 on B cells in RA is reported to be either similar [29] or lower [37] than that of healthy subjects. In the present study, the expression level of CXCR4 on peripheral B cells varied among RA patients, including only two treatment-free cases. CXCR4 expression on peripheral B cells was constitutive and P-gp expression was marginal in the control subjects, whereas P-gp expression increased with increases in CXCR4 expression on B cells in RA patients. Further examination is necessary; one of the mechanisms of down regulation of CXCR4+ B cells in RA could be the effect of treatment on P-gp+CXCR4+ B cells. In other words, down regulation of CXCR4+ B cells in RA might be related to the treatment.

Experimental and clinical evidence suggest the involvement of P-gp and CXCL12/CXCR4 axis in the migration of various inflammatory and cancer cells [16,20–23]. TNF-α is a clinically validated pathogenic factor in inflammatory erosive arthritis in RA [38] and is one of the inducers of P-gp expression on lymphocytes [9]. CXCL12 on RA fibroblast-like synoviocytes is regulated by TNF-α [39], and the CXCL12-binding capacity of RA synovial endothelium increases on exposure to TNF-α [40]. Plasma cells are localized in close proximity to CXCL12-expressing salivary gland epithelial cells in primary Sjögren syndrome [41]. P-gp expression on activated lymphocytes is directly regulated by activation of the Y-box binding protein-1 (YB-1) in response to immune stimuli, such as inflammatory cytokines [7,8]. The upstream of the CXCR4 gene contains putative consensus Y-box-binding site (inverted CCAAT box), but, it remains unknown whether activation of YB-1 is directly involved in the upregulation of CXCR4 gene [42]. P-gp expression had been reported to have no relationship to CXCR4 expression in ovarian cancer cells and to be independent prognostic factor from CXCR4 expression with regard to overall survival of patients with ovarian cancer [43]. Previous studies indicated that P-gp overexpression did not significantly alter the surface expression or distribution of CXCR4 on CD4+ cells [44]. However, our findings imply the novel relevance of P-gp expression to CXCR4 expression on B cells, and indicate that the high level of coexpression of P-gp with CXCR4 on B cells is associated with severe organ injury in patients with RA. Considered together, the present results suggest that P-gp+CXCR4+ B cells migrate into inflamed lesions and cause exacerbation of lesions.

In the present study, we also identified marked accumulation of P-gp+CXCR4+ B cells in the lungs of a patient with severe IP. The incidence of clinically significant RA-associated IP is nearly 10%, and is associated with poor survival.

Figure 4. P-glycoprotein and CXCR4 expression on infiltrated CD19+ lymphocytes in the lungs of a patient with highly active rheumatoid vasculitis and severe interstitial pneumonia. (a) Hematoxylin and eosin staining. (b) Immunostaining for CD19+ lymphocytes using anti-CD19 monoclonal antibody (mAb) and 3,3’-diaminobenzidine (DAB). (c) Immunostaining for P-gp on lymphocytes using JSB-1 anti-P-gp mAb and Vulcan Fast Red (FR). (d) Immunostaining for CXCR4 on lymphocytes using anti-CXCR4 mAb and DAB. (e) Immunostaining for CXCL12+ lymphocytes using anti-CXCL12 mAb with FR. (f) Double-immunostaining for P-gp on lymphocytes using JSB-1 anti-P-gp mAb with Vulcan Fast Red (FR) and anti-CXCL12 mAb with DAB. CXCL12 expression was detected in some parts of the endothelium and synovial fibroblasts. (b–f) Nuclear counterstaining with hematoxylin.
and reflects more severe underlying disease [45,46]. Therefore, it is important to control clinically active IP. Other groups have reported that CXCR4 antagonists can significantly suppress pulmonary metastasis of breast cancer cells and melanoma cells in mice [17]. The use of CXCR4 antagonists could be a useful treatment strategy for RA-associated IP [47]. In a recent study, we reported the usefulness of tacrolimus, a P-gp inhibitor, in increasing IDLs in RA lymphocytes [16]. Other investigators reported the efficacy of tacrolimus in IP associated with autoimmune diseases, including RA [48,49]. Based on these findings, we propose that inhibition of CXCR4 and P-gp activities by these antagonists could be therapeutically effective in preventing organ damage and improve clinical outcome of RA patients.

A review of the clinical course of patients examined in the present study showed that treatment with a TNF antagonist can successfully control organ involvement, together with marked reduction in peripheral P-gp⁺CXCR4⁺ B cells. Previous studies demonstrated that lymphocyte-activating cytokines, such as IL-2 and TNF-α, can up-regulate the expression of P-gp on lymphocytes, while TNF antagonists, infliximab and etanercept, can reduce P-gp expression on B cells [7,9,15,16,50]. With regard to the effects of TNF on the CXCL12/CXCR4 axis, TNF is reported to inhibit CXCL12 production from bone marrow stromal cells [51]. How TNF antagonists reduce P-gp and CXCR4 expression on B cells remains obscure at present and further studies are necessary to explore this issue. Regardless of the mechanism of action, our results showed that treatment of refractory RA with organ involvement by TNF antagonists resulted in elimination of P-gp⁺CXCR4⁺ B cells with subsequent control of the pathological process.

**Conclusion**

We have demonstrated in the present study the infiltration of P-gp and CXCR4-overexpressing B cells in the inflamed tissues of RA patients with highly active disease and that such patients apparently acquire P-gp-mediated multidrug resistance against corticosteroids and probably certain disease modifying antirheumatic drugs (DMARDs), which are substrates of P-gp. It is possible that local accumulation of P-gp and CXCR4-overexpressing B cells in the inflamed tissues enhances articular and extra-articular tissue damage and that reduction of P-gp⁺CXCR4⁺ B cells through the administration of biological agents can overcome drug resistance and organ involvements in refractory RA. Accordingly, we propose that expansion of peripheral P-gp⁺CXCR4⁺ B cells is a potentially useful marker for drug resistance and progressive destructive arthritis with extra-articular involvement, and could help in decision making regarding the selection of treatment strategy including targeting preferentially P-gp⁺CXCR4⁺ B cells in RA patients with highly active disease.
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Conflict of interest

Y. Tanaka has received consulting fees, speaking fees, and/or honoraria from Abbvie, Chugai, Astellas, Takeda, Santen, Mitsubishi-Tanabe, Pfizer, Janssen, Eisai, Daiichi-Sankyo, UCB, GlaxoSmithKline, Bristol-Myers and has received research grants from Mitsubishi-Tanabe, Chugai, MSD, Astellas, and Novartis. All other authors declare no conflict of interest.

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