

# Etanercept overcomes P-glycoprotein-induced drug resistance in lymphocytes of patients with intractable rheumatoid arthritis

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**Abstract** P-glycoprotein (P-gp) on activated lymphocytes is an adenosine triphosphate (ATP)-binding cassette transporter that causes drug resistance by exclusion of intracellular drugs in patients with active rheumatoid arthritis (RA). However, infliximab with methotrexate (MTX) can overcome P-gp-mediated drug resistance. We encounter patients who cannot continue infliximab or MTX. Here we tested how etanercept affected P-gp-mediated drug resistance in such intractable RA patients. Peripheral lymphocytes of 11 RA patients (3 switched from infliximab and 8 who could not be treated with MTX) were analyzed for P-gp expression by flow cytometry and for drug exclusion using radioisotope-labeled dexamethasone. Activated lymphocytes of RA patients overexpressed P-gp and coexpressed CD69. Incubation of these lymphocytes with dexamethasone *in vitro* reduced intracellular dexamethasone levels. Two-week etanercept therapy significantly reduced P-gp expression and eliminated such P-gp- and CD69-high-expressing subgroup. The reduction in P-gp resulted in recovery of intracellular dexamethasone levels in lymphocytes and improvement of disease activity, thus allowing tapering of corticosteroids. None of the patients experienced any severe adverse effects. Etanercept is useful for overcoming P-gp-mediated treatment resistance in intractable RA patients who have to discontinue infliximab or are intolerant to MTX.

**Keywords** Etanercept · Drug resistance · Lymphocytes · P-glycoprotein · Rheumatoid arthritis

## Introduction

The aims of treatment of rheumatoid arthritis (RA) with disease-modifying antirheumatic drugs (DMARDs) include control of abnormal immune system, erosive synovitis, and prevention of joint destruction. The main target of these DMARDs is the lymphocyte because activated lymphocytes are the main orchestrator of the pathogenic immune response, including enhancement of inflammation and destructive arthropathy [1]. However, some RA patients show drug resistance.

P-glycoprotein (P-gp) is an ATP-binding cassette transporter that can induce drug resistance in RA by excluding intracellularly targeted drugs [2]. Overexpression of P-gp results in reduction of intracellular concentrations of substrates involving DMARDs, including hydroxychloroquine, D-penicillamine, colchicines, bucillamine, sulfasalazine, and corticosteroids [3–6]. P-gp is highly expressed on activated lymphocytes in response to various stimuli, whereas normal resting lymphocytes express marginal levels of P-gp [7, 8]. It is thought that overexpression of P-gp on lymphocytes in patients with highly active RA [9] in response to lymphocytic stimuli such as cytokines could lead to P-gp-mediated multidrug resistance.

The highly effective tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) antagonists, etanercept and infliximab, have revolutionized treatment strategies for RA. However, we often encounter patients with intractable RA who are unable to continue infliximab or who are intolerant to methotrexate (MTX). Furthermore, such patients tend to depend on corticosteroids. Corticosteroid is a representative substrate and inducer of P-gp; therefore, treatment resistance is strongly enhanced in such corticosteroid-dependent patients by enhancing P-gp-mediated exclusion of drugs including

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corticosteroids and DMARDs from their pathogenic lymphocytes.

How to treat patients with intractable RA is an important clinical issue. The present study was designed to determine the mechanism by which etanercept modulates the expression of P-gp on lymphocytes and the exclusion of corticosteroid from lymphocytes. We also examined the clinical status in patients with intractable RA who were switched from infliximab or were intolerant to MTX.

## Patients and methods

### Patients

Eleven patients with RA were included in this study and were treated with etanercept. The diagnosis of RA was based on the American College of Rheumatology (ACR) revised criteria for RA. Etanercept was administered subcutaneously 25 mg twice per week during the first 2 weeks; corticosteroids, MTX, and other DMARD dosages were kept unchanged. The clinical activity of RA was assessed by the disease activity score (DAS)28, calculated by using tender joint counts, swollen joint counts, erythrocyte sedimentation rate, and the patient's overall self-assessment of well-being [10]. The 11 patients with refractory RA fulfilled all of the following criteria: (1) DAS28 >5.1; (2) previously treated with at least 2 DMARDs or one DMARD and corticosteroids; (3) treated for RA for at least 2 years; and (4) received stable background DMARDs or corticosteroids for at least 2 months before the first administration of etanercept. Table 1 summarizes the clinical features of the 11 refractory RA patients. Cases 1 and 2 switched to etanercept after secondary loss of infliximab efficacy. Case 3 developed pancytopenia while being treated with infliximab and this was attributed to the loading of MTX with infliximab; the latter was discontinued and the dose of MTX was reduced. Cases 4–9 discontinued MTX treatment due to adverse effects such as alopecia, severe nausea, interstitial pneumonia, and renal dysfunction. Cases 10 and 11 had never been treated with MTX, due to RA-related complications; case 10 had interstitial pneumonia and case 11 had renal dysfunction.

The ethics committee of our institution approved the study, and informed consent was obtained from all patients enrolled in the study.

### Isolation of peripheral blood mononuclear cells (PBMCs)

Blood samples were obtained from 10 normal volunteers and 11 patients with refractory RA at weeks 0 and 2 of etanercept therapy. PBMCs were isolated by density

gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden), as described previously [9].

### Flow cytometry

Staining and flow cytometric analysis of PBMCs were conducted by standard procedures as described previously using a FACScan (Becton–Dickinson, Mountain View, CA) [9, 11, 12]. Briefly, PBMCs ( $2 \times 10^5$  cells/well) were initially incubated with polyclonal  $\gamma$ -globulin (10  $\mu$ g/mL, Yoshitomi Pharmaceutical Co.) to block Fc-receptors and then incubated with MRK-16, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) Ab in fluorescence-activated cell sorting (FACS) medium containing phosphate-buffered saline, 0.5% human serum albumin (HSA), and 0.2%  $\text{NaN}_3$  (Sigma-Aldrich Japan). For two-color analysis, PBMCs were incubated with cy-chrome-conjugated CD4 monoclonal antibody (mAb) or CD19 mAb and phycoerythrin (PE)-conjugated CD69 mAb after blocking free anti-mouse IgG-binding sites with irrelevant antibodies. Monoclonal antibodies-two-color-stained cells were detected by electronic gating based on their CD4, CD19 or CD69 expression using a FACScan. Amplification of mAb binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface antigens on one cell was performed using QIFIKIT beads (Dako, Kyoto, Japan), as reported previously [11, 12]. The data were used to construct a calibration curve of mean fluorescence intensity versus antibody-binding capacity. The cell specimen was analyzed on the FACScan and the antibody-binding capacity calculated by interpolation on the calibration curve. When the green-fluorescence laser detection was set at 500 nm in the FACScan, the antibody-binding capacity was equal to  $[202.98 \times \exp(0.0092 \times \text{mean fluorescence intensity})]$ , ( $R^2 = 0.9995$ ). Subsequently, the specific antibody-binding capacity was obtained after correcting for the background, and apparent antibody-binding capacity of the negative control anti-mouse IgG Ab. The specific antibody-binding capacity represented the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

### Measurement of intracellular accumulation of dexamethasone

Accumulation of dexamethasone intracellularly in PBMCs in vitro was analyzed by radioassay, as described previously [9] using a scintillation counter,  $^3\text{H}$ -labeled dexamethasone (Perkin, Boston, MA), and  $^{14}\text{C}$ -labeled *n*-butanol (Toho Biochemical, Tokyo). The cell-to-medium ratio (*C/M* ratio), which relates intracellular and extracellular dexamethasone

concentrations, was calculated using the following formula:  $C/M = [(^3\text{H in cell fraction}/^{14}\text{C in cell fraction})/(^3\text{H in medium fraction}/^{14}\text{C in medium fraction})]$ .

### Statistical analysis

Results are expressed as mean  $\pm$  standard deviation (SD). Student's *t* test was used to compare data of 2 groups. One-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) for multi-comparison was used to compare data among 3 or more groups. *P* values less than 0.05 were considered statistically significant.

## Results

### Effects of etanercept on P-gp expression

The expression levels of P-gp was significantly high on peripheral CD4+ and CD19+ lymphocytes in 11 intractable RA patients with high disease activity (Fig. 1a, b). In a previous study, we measured intracellular dexamethasone levels (IDLs) in lymphocytes of 50 RA patients and 10 normal volunteers and found that IDLs decreased in proportion to the expression levels of P-gp in lymphocytes [9]. IDLs correlated negatively with the levels of P-gp expression on either CD4 or CD19 lymphocytes. IDLs of PBMCs were significantly lower in the 11 RA patients, as determined by the *C/M* ratio, compared with the normal subjects, indicating that intracellular dexamethasone is exported through P-gp on lymphocytes (Fig. 1c).

However, the first 2 weeks of etanercept therapy significantly reduced P-gp expression on CD4+ and CD19+ lymphocytes in all 11 patients, including 2 patients who experienced a secondary loss of infliximab efficacy and 8 who did not use MTX (Fig. 1a, b). Furthermore, the reduced IDLs in PBMCs of these RA patients improved in parallel with the reduction in P-gp levels on the PBMCs. These results imply that etanercept therapy reduces P-gp levels and subsequently maintains IDLs in lymphocytes (Fig. 1d).

Furthermore, antibody staining for CD69, a well-defined early-activation surface marker of lymphocytes [13] used to monitor lymphocyte activation, showed P-gp expression on CD4+ and CD19+ lymphocytes with strong coexpression of CD69 (Fig. 2). We also reported previously that P-gp expression on in vitro-stimulated lymphocytes by fragmented hyaluronan, which is increased in the RA synovium and synovial fluid [8, 14, 15], was preferentially increased in cells with high CD69 expression. This result implies the existence of a P-gp-high-expressing subgroup

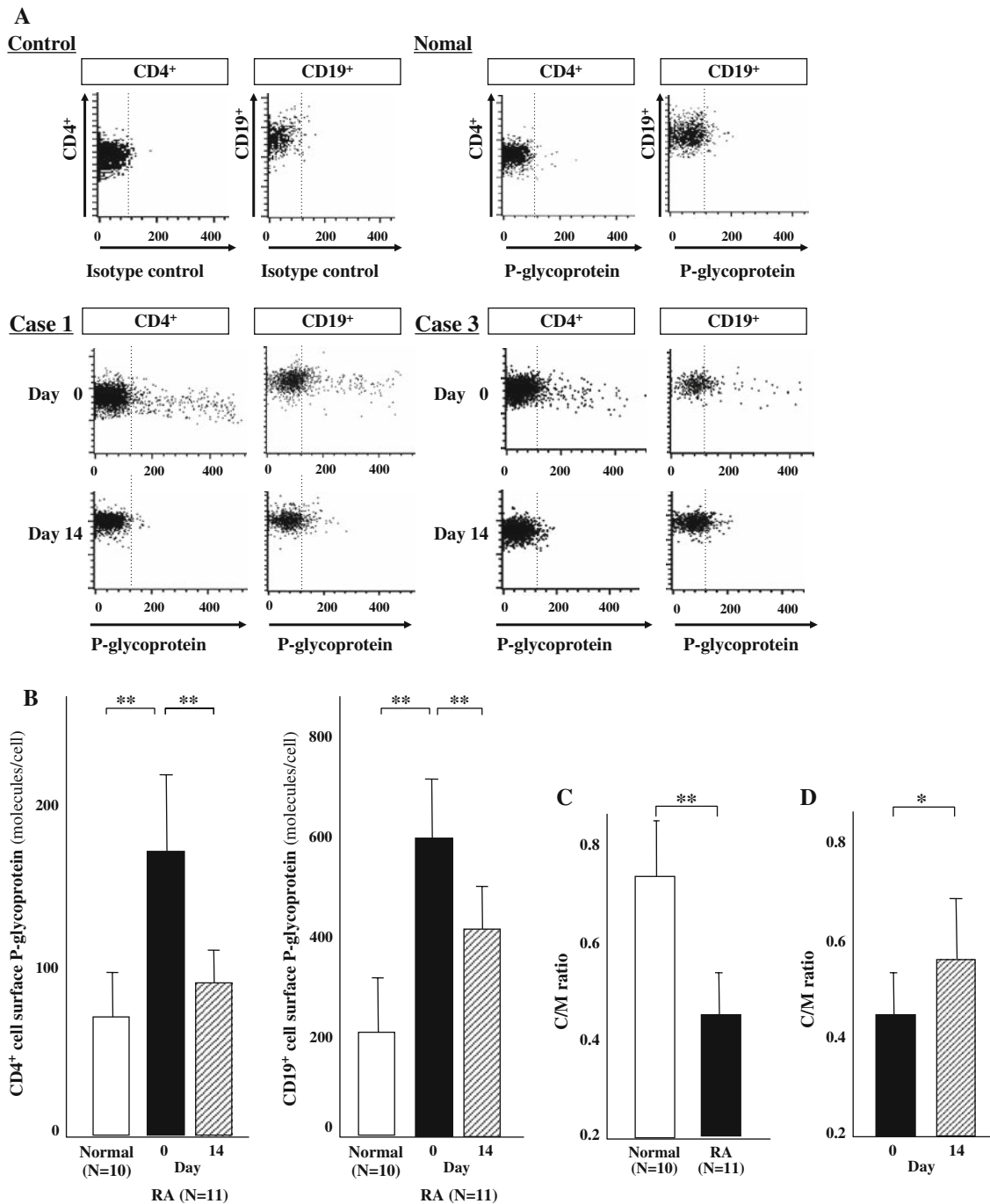
of activated lymphocytes with high CD69 coexpression. However, etanercept therapy resulted in almost complete disappearance of the P-gp- and CD69-high-expressing subgroups of CD4+ and CD19+ lymphocytes, with consequent significant reductions in the percentages of P-gp and CD69 high-expressing subgroups (Fig. 2).

Disease activity, evaluated by DAS28, improved within 2 weeks of etanercept therapy. This improvement was sustained for at least 6 months. Nine of the 11 patients were corticosteroid users before etanercept therapy, then none of the corticosteroid users needed to increase dose of corticosteroids. In 6 of the corticosteroid users, including 4 MTX nonusers, corticosteroids could be tapered or eliminated (Table 1). None of the 11 patients experienced any severe adverse effects related to etanercept therapy.

### Representative case reports

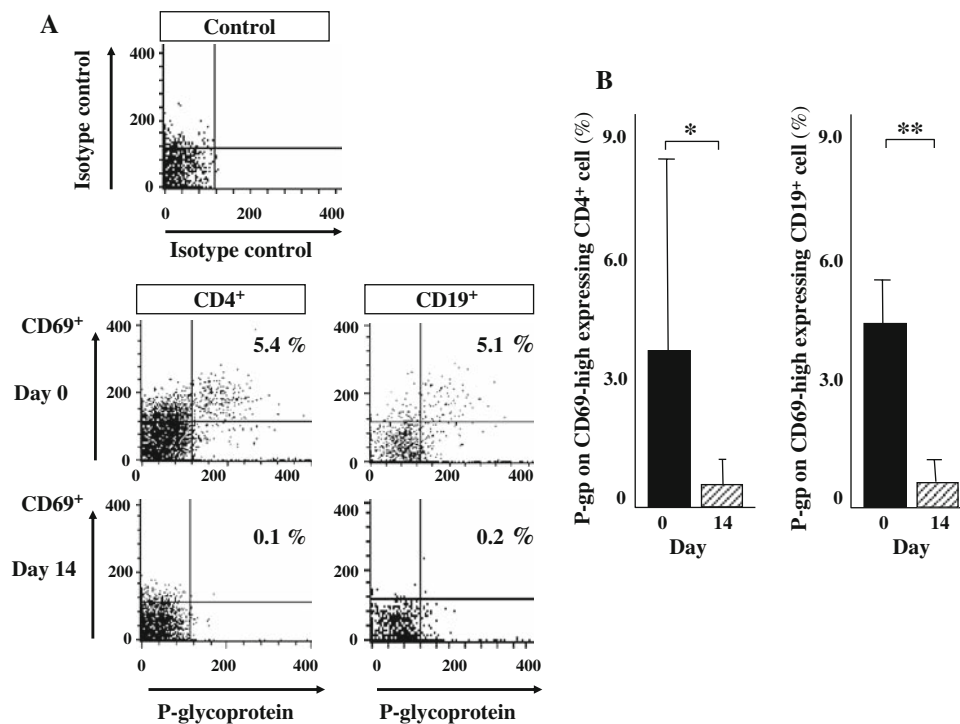
Case 1 (Table 1) switched from infliximab to etanercept after secondary loss of infliximab efficacy. Despite treatment with MTX, sulfasalazine, D-penicillamine, and corticosteroids, RA disease activity flared several times during a period of 2 years. A single infliximab infusion markedly improved RA disease activity from DAS28 of 7.1 to 2.4 within 14 weeks. However, arthritis exacerbated again after 38 weeks of infliximab infusion and flared continually despite dose escalation of MTX. Two years after starting infliximab, DAS28 was 5.2 and disease activity remained high. Peripheral lymphocytes also showed high P-gp expression (Fig. 1a). Furthermore, antibody staining for CD69 showed P-gp expression on CD4+ and CD19+ lymphocytes with strong coexpression of CD69 (Fig. 2a). The patient was therefore switched to etanercept. Two weeks later, the P-gp and CD69 high-expressing subgroups of CD4+ and CD19+ lymphocytes had disappeared (Fig. 2a). Disease activity improved and the patient entered clinical remission within 2 months. The concomitant prednisolone could be tapered from 7.5 to 2.5 mg/day within 6 months, and clinical remission was maintained for at least 2 years under etanercept.

Case 5 (Table 1; Fig. 3) had rheumatoid vasculitis with amyloidosis. Despite treatment with MTX, cyclophosphamide, azathioprine, and corticosteroids, disease activity increased continually during a period of 10 years, resulting in failed corticosteroid tapering and increased radiological progression. Renal dysfunction due to amyloidosis was aggravated with increasing serum amyloid A protein level and the patient became intolerant to MTX. Peripheral lymphocytes showed high levels of P-gp expression despite treatment with azathioprine (Fig. 3b). Two weeks after the first administration of etanercept, the P-gp high-expressing subgroups of CD4+ lymphocytes were eliminated, and



**Fig. 1** Effects of etanercept infusion on P-glycoprotein (P-gp) expression and intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from patients with rheumatoid arthritis (RA) and high disease activity. **a** Typical P-gp expression on CD4<sup>+</sup> and CD19<sup>+</sup> peripheral blood lymphocytes from normal subjects and patients with highly active RA (cases 1 and 3, Table 1), whose DAS28 were more than 5.1 points, before (day 0) and 14 days after (day 14) initial etanercept administration. The dotted line represents the gate set to discriminate negative- from positive-stained cells as determined by control FITC-conjugated anti-mouse IgG Ab. **b** P-gp expression on peripheral CD4<sup>+</sup> and CD19<sup>+</sup> peripheral blood lymphocytes from 10 normal subjects (*open bars*) and 11 patients

(Table 1) before (*closed bars*) and 14 days after (*hatched bars*) initial etanercept administration. Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Values are mean  $\pm$  SD of independent experiments. **\*\*** $P < 0.01$  by *t* test. **c** Intracellular dexamethasone levels were evaluated by determining the cell-to-medium (C/M) ratio in PBMCs from 10 normal subjects and 11 patients (Table 1) before initial etanercept administration. **\*\*** $P < 0.01$  by unpaired *t* test. **d** Intracellular dexamethasone levels were evaluated by determining the C/M ratio in PBMCs from the same patients before (*closed bars*) and 14 days after (*hatched bars*) initial etanercept administration. Values are mean  $\pm$  SD of 11 independent experiments. **\*** $P < 0.05$  by paired *t* test



**Fig. 2** Etanercept eliminated P-glycoprotein (P-gp)-high-expressing subgroup of activated lymphocytes with high CD69 coexpression. **a** Flow cytometric analysis showing typical P-gp expression on CD69-high or -low-expressing CD4+ and CD19+ peripheral blood lymphocytes from case 1 (Table 1) before and 14 days after initial etanercept administration. Appropriate isotype controls are shown, and

percentages represent P-gp-positively stained CD69-high-expressing cells. **b** Percentages of P-gp-positively stained CD69-high-expressing CD4+ and CD19+ peripheral blood lymphocytes from 11 patients (Table 1) before (closed bars) and 14 days after (hatched bars) initial etanercept administration. Values are mean ± SD of 11 independent experiments. \**P* < 0.05, \*\**P* < 0.01 by paired *t* test

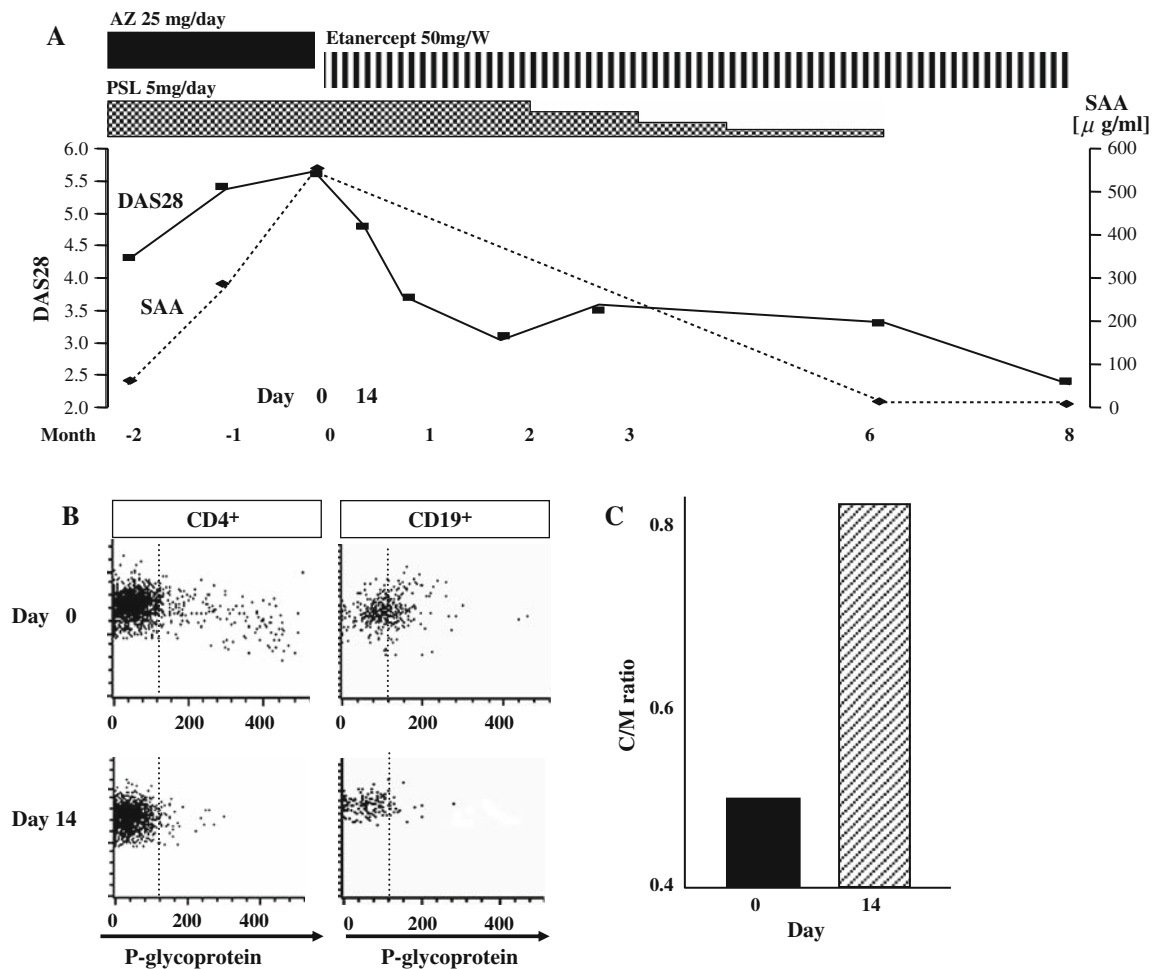
**Table 1** Clinical features of 11 patients with intractable rheumatoid arthritis treated with etanercept infusion

Case	Age (years)	Disease duration (years)	Steinbrocker stage	Previous therapy	Concomitant PSL (mg/day)		DAS28	
					Initial	6 month	Initial	6 month
1	57	4	II	MTX, PSL, D-pen, SSZ, infliximab	7.5	2.5	5.2	3.0
2	55	9	IV	MTX, SSZ, infliximab	5.0	5.0	7.6	3.1
3	76	35	IV	MTX, PSL, SSZ, MZ, CsA, infliximab	5.0	5.0	6.9	3.4
4	54	8	II	MTX, SSZ, LEF, D-pen, CsA	—	—	7.2	5.0
5	31	10	IV	MTX, PSL, SSZ, BU, AZ, CPA	5.0	—	5.6	3.3
6	64	35	III	MTX, PSL, Gold, SSZ, BU, LEF, D-pen, CsA	6.0	3.0	6.9	5.2
7	58	3	III	MTX, PSL, SSZ, BU, LEF, AZ	5.0	5.0	6.3	3.4
8	66	38	IV	MTX, PSL, SSZ, BU	7.5	7.5	5.3	3.3
9	62	30	IV	MTX, SSZ, BU	—	—	5.1	3.3
10	56	2	II	PSL, CsA	7.5	4.5	6.5	5.5
11	62	9	III	PSL, Gold, BU	4.5	2.5	5.7	3.7

DMARD disease-modifying antirheumatic drugs, PSL prednisolone (or equivalent), 6 month after 6 months of initial etanercept administration, DAS28 disease activity score 28, MTX methotrexate, D-pen D-penicillamine, SSZ sulfasalazine, gold parenteral gold, LEF leflunomide, AZ azathioprine, CPA cyclophosphamide, BU bucillamine

most CD19+ lymphocytes expressed only low levels of P-gp, then the reduced IDLs in PBMCs recovered significantly (Fig. 3c). Administration of etanercept markedly

improved RA disease activity from DAS28 of 5.6 to 3.1 within 14 weeks, eliminated the need for corticosteroids, and markedly decreased serum amyloid A protein level



**Fig. 3** Clinical course and effects of etanercept infusion on P-glycoprotein (P-gp) expression and intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from case 5 (Table 1). **a** Clinical course of Case 5. *SAA* serum amyloid A protein, *PSL* prednisolone, *AZ* azathioprine. **b** P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from the same patient, before (day 0) and 14 days after (day 14) initial etanercept administration.

The *dotted line* represents the gate set to discriminate negatively from positively stained cells as determined by control FITC-conjugated anti-mouse IgG Ab. **c** Intracellular dexamethasone levels were evaluated by determining the *C/M* ratio in PBMCs from the same patient before (*closed bars*) and 14 days after (*hatched bars*) initial etanercept administration

from 553.8 to 13.3  $\mu$ g/mL within 6 months. Clinical remission was achieved within 8 months (Fig. 3a).

## Discussion

Our hypothesis stated that induction of P-gp on activated lymphocytes is involved in drug resistance in refractory RA patients and that reduction of P-gp levels is a prerequisite for overcoming the drug resistance. The present study demonstrated that etanercept reduced P-gp expression on lymphocytes, and increased IDLs in PBMCs of 11 patients with intractable RA within 2 weeks, similar to the effects observed in patients treated with infliximab. Etanercept successfully improved RA disease activity for at least 6 months and allowed tapering of corticosteroids.

We have reported previously that expression levels of P-gp on RA lymphocytes correlated closely with disease activity [9]. On the other hand, lymphocytes positive for CD69, which is one of the very early activation antigens, have been reported to be involved in the pathogenesis of RA, chronic inflammatory liver diseases, and asthma [13, 16]. Before administration of etanercept, P-gp was expressed on lymphocytes with a high CD69 expression. Etanercept could regulate both disease activity and treatment resistance by reduction of these pathogenic lymphocytes that had never been inhibited by prior treatments. We reported previously that intensive immunosuppressive therapies such as glucocorticoid (GC) pulse therapy and intravenous cyclophosphamide therapy overcame treatment unresponsiveness mediated by P-gp overexpression on lymphocytes in refractory active systemic lupus

erythematosus (SLE) [17]. Therefore, in RA patients, it is expected that various immunosuppressants can reduce P-gp expression and thus improve P-gp-mediated drug resistance. However, case 5 in the present study showed high levels of P-gp expression on lymphocytes despite treatment with azathioprine, cyclophosphamide, and GC. Although we do not have any evidences of other biological agents except TNF antagonists in RA patients, biological agents might be more efficient than other intensive immunosuppressive therapies in reducing P-gp expression on active lymphocytes in RA patients, because TNF is one of the major inflammatory mediators in the pathogenesis of RA.

We also described previously that infliximab combined with MTX overcame treatment unresponsiveness mediated by P-gp overexpression on active lymphocytes that could not otherwise be controlled with MTX [9]. However, patients with secondary loss of infliximab efficacy who were treated with etanercept in the present study improved clinically and showed marked reduction in P-gp expression on lymphocytes as effectively as patients treated with infliximab combined with MTX in previous studies [9]. Therefore, etanercept seems to inhibit P-gp expression by mechanisms different from those of infliximab. Previous studies examining therapy switches from infliximab to etanercept [18–20] also indicated that in cases of infliximab the switch to etanercept was successful [19, 20]. Etanercept and infliximab are different drugs from several aspects including route of administration (subcutaneous versus intravenous), pharmacodynamics, and pathophysiology such as regulation of lymphotoxin, interleukin (IL)-18, and bone biomarkers [21, 22]. In this study, high levels of P-gp on CD69-overexpressing activated lymphocytes were observed in 2 refractory patients despite treatment with infliximab. Infusion of infliximab concomitant with MTX could not suppress the pathogenic lymphocytes and might induce drug resistance. However, switching from infliximab to etanercept successfully eliminated the P-gp-CD69-high-expressing activated lymphocytes and therefore improved disease activity. Although further cases need to be tested, we suggest measuring P-gp expression as a potentially useful early predictor of response to etanercept after switching from infliximab.

We have previously demonstrated significantly higher P-gp expression level on CD19+ lymphocytes in highly active RA patients treated with corticosteroids than those without corticosteroids [9]. The present study demonstrated that 9 of 11 patients with intractable RA whose lymphocytes expressed high levels of P-gp were dependent on corticosteroids. These patients improved clinically without severe adverse effects and showed marked reduction in P-gp expression on lymphocytes. Furthermore, corticosteroids could be tapered in 5 of these patients. Therefore, etanercept therapy is a worthy strategy in such patients in

terms of efficacy, tolerability, and overcoming acquired P-gp-mediated treatment resistance.

Taken together, etanercept is useful for overcoming P-gp-mediated treatment resistance in patients with intractable RA. Etanercept markedly reduced P-gp on lymphocytes and improved disease activity not only in MTX-naive patients but also in refractory patients despite infliximab, as effectively as patients treated with infliximab combined with MTX reported in previous studies [9]. Therefore, we propose that P-gp expression on lymphocytes is a good marker for the response to biological agents and early prediction of drug efficacy.

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**Conflict of interest statement** None.

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