

## Six-transmembrane epithelial antigen of prostate 4 (STEAP4) is expressed on monocytes/neutrophils, and is regulated by TNF antagonist in patients with rheumatoid arthritis

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### ABSTRACT

**Objective.** Human six-transmembrane epithelial antigen of prostate 4 (STEAP4) is one of the STEAP family as a homologue of mouse tumour necrosis factor- $\alpha$ -induced adipose-related protein (TIARP). Recently, we reported that the TIARP gene expression was remarkably increased in spleen and joints of glucose-6-phosphate isomerase (GPI)-induced arthritis model, suggesting pivotal association to arthritis. The aim of the present study was to assess the expression, localisation and function of STEAP4 in peripheral blood of patients with rheumatoid arthritis (RA).

**Methods.** Peripheral blood was obtained from seven patients with RA, the surface expression of STEAP4 was detected by flow cytometry. The number of neutrophils was compared with the expression of STEAP4 mRNA derived from peripheral blood of patients with RA. Neutrophils were introduced by HL60 with retinoic acid, and were transfected with GFP-STEAP4 plasmid DNA, then the migration of neutrophil-like HL60 was determined by transwell assay. In addition, the fluctuation of STEAP4 mRNA was analysed before and after treatment with infliximab in 40 patients with RA.

**Results.** STEAP4 was expressed on monocytes and neutrophils in peripheral blood in RA. The number of neutrophils and expression of STEAP4 mRNA was positively correlated. Migration of neutrophil-like HL60 was downregulated by overexpression of STEAP4. Expression of STEAP4 mRNA was significantly decreased after infliximab treatment in patients with RA, especially in good responders.

**Conclusions.** STEAP4 is expressed on monocytes and neutrophils in peripheral blood, regulates cell migration, is downregulated by TNF antagonist, and might be a possible predictor of response to TNF antagonist.

### Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder, characterised by inflammation and destruction of multiple joints. Prognosis for RA patients has significantly improved with

the recent use of biologics targeting tumour necrosis factor alpha (TNF- $\alpha$ ) (1, 2). Glucose-6-phosphate isomerase (GPI)-induced arthritis is a relatively new arthritis model with clear therapeutic benefits from TNF antagonists (3, 4). Using GeneChip analysis, we reported recently upregulation of TNF- $\alpha$ -induced adipose-related protein (TIARP) in GPI-induced arthritis (5, 6). TIARP is a transmembrane protein that is highly regulated by TNF- $\alpha$  and IL-6 in adipocytes (7, 8). In arthritic mice, TIARP was expressed in CD11b<sup>+</sup> splenocytes and clearly downregulated by treatment with anti-TNF- $\alpha$  (6).

Its human counterpart such as human six-transmembrane epithelial antigen of prostate 4 (STEAP4) was reported highly expressed in the bone marrow, placenta, and foetal liver (9), and STEAP4 was induced by TNF- $\alpha$  in human adipose tissue (10). Our previous study showed that it was also detected in peripheral blood and synovium from patients with RA (6), however its expression and role of patients with RA is still obscure.

To elucidate such a role of STEAP4 in RA, we first determined STEAP4 expression in peripheral blood. The role of STEAP4 in neutrophils was also examined using STEAP4 transfectant. After analysing the localisation of STEAP4 in the monocytes and neutrophils of RA patients, we investigated the fluctuation of expression to treatment with TNF antagonist.

### Materials and methods

#### Patients

Peripheral bloods were derived from 7 RA patients for analysis of flow cytometry. All RA patients satisfied the classification criteria of the American College of Rheumatology (11). Peripheral bloods were also collected from 40 patients with RA receiving infliximab (IFX) before and after 2 weeks of therapy. All subjects provided written informed consent, and the ethics review committee of the University of Tsukuba approved the study. Clinical response to treatment was determined using the European League Against Rheumatism (EULAR) criteria based on the DAS28 index after six months of the treatment.

### Flow cytometry

Peripheral blood was isolated from patients with RA washed with PBS containing 2% FBS and incubated with anti-human STEAP2 Abs (R&D Systems, Minneapolis, MN, USA) conjugated with Zenon Alexa Fluor 488 (Invitrogen, Eugene, OR, USA) and PE anti-human CD16 (Biolegend, San Diego, CA, USA) for 30 min. Different fluorescent cell populations were determined using an EPICS XL (Beckman Coulter, Fullerton, CA, USA).

### Quantitative PCR

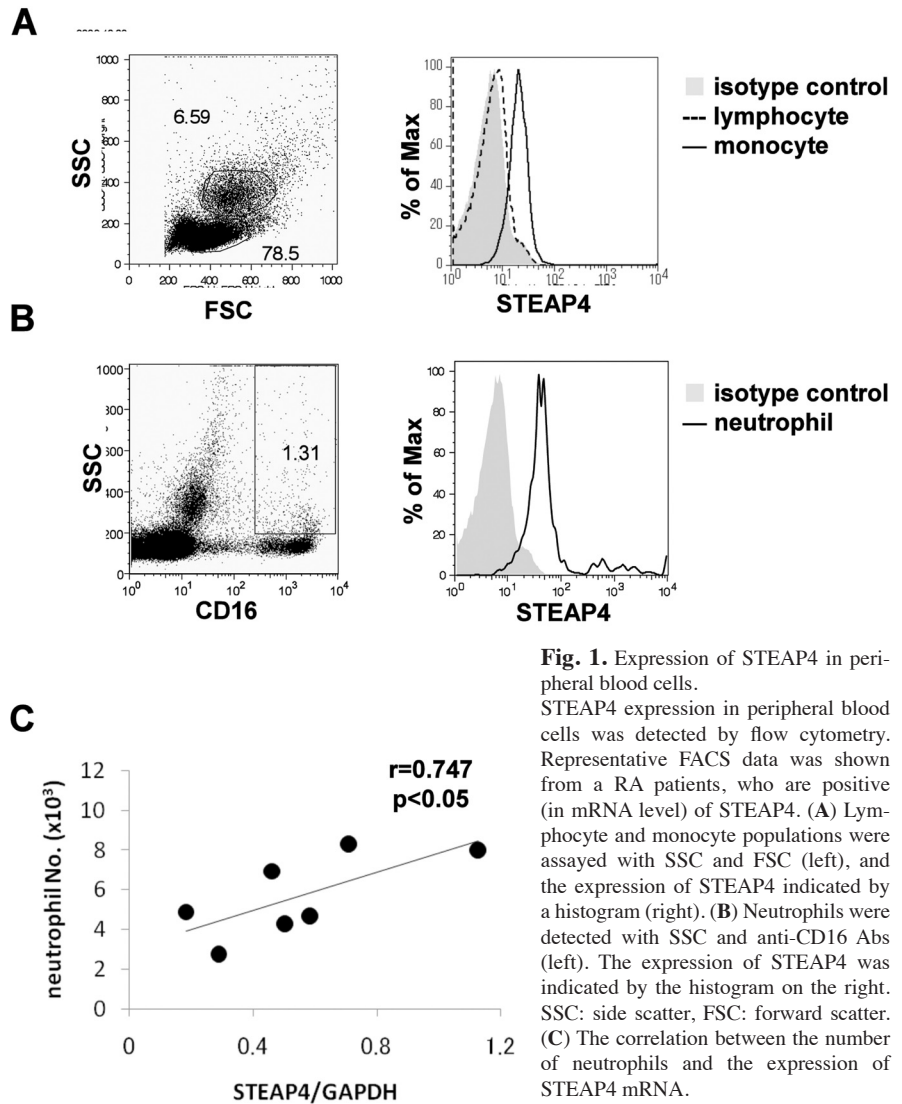
Total RNA was extracted with ISOGEN (Nippon Gene, Toyama, Japan) using the protocol provided by the manufacturer. cDNA was obtained by reverse transcription using a commercially available kit (Fermentas, Glen Burnie, ML, USA). Quantitative PCR was performed using a 7300 real time PCR system (Applied Biosystems, Foster City, CA) with qPCR Mastermix (Eurogentec, San Diego, CA, USA) and the following primers and probes: Human GAPDH (20 x, 4326317E, Applied Biosystems) and Gene Expression Assays for STEAP4 (Assay ID: Hs00226415\_m1, Applied Biosystems).

### Preparation of plasmid vector for transfection

Full-length STEAP4 cDNA was amplified from synovial tissues of patients with RA. The amplified cDNA product was cloned into pENTR TOPO vector (pENTR Directional TOPO Cloning Kits, Invitrogen) and sequenced. The full-length STEAP4 ORF from pENTR-TOPO-STEAP4 was fused in-frame to the C-terminus of GFP using the pcDNA-DEST47 Gateway Vector kit (Invitrogen) to generate GFP-STEAP4.

### Migration assay

HL60 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. HL60 was known to be induced to neutrophil by *all-trans* retinoic acid (ATRA). HL60 was cultured with 1  $\mu$ M ATRA for 72h and incubated with transfectional reagent (Lipofectamine LTX and PLUS Reagent, Invitrogen) containing GFP-empty or GFP-STEAP4 pDNA for 48h.



**Fig. 1.** Expression of STEAP4 in peripheral blood cells.

STEAP4 expression in peripheral blood cells was detected by flow cytometry. Representative FACS data was shown from a RA patients, who are positive (in mRNA level) of STEAP4. (A) Lymphocyte and monocyte populations were assayed with SSC and FSC (left), and the expression of STEAP4 indicated by a histogram (right). (B) Neutrophils were detected with SSC and anti-CD16 Abs (left). The expression of STEAP4 was indicated by the histogram on the right. SSC: side scatter, FSC: forward scatter. (C) The correlation between the number of neutrophils and the expression of STEAP4 mRNA.

Neutrophil-like HL60 was suspended in a chemotactic chamber (Chemotaxi-cell, Kurabou, Osaka, Japan) on 24 well plate (CORNING, IWAKI GLASS, Tokyo, Japan) for 2 hours. Under medium was contained 5% synovium fluid derived from RA patients. Upper chamber was washed with PBS, and stained with Giemsa's Stain Solution (MUTO PURE CHEMICALS, Tokyo, Japan) after migration assay. Stained neutrophils were counted under microscope.

### Statistical analysis

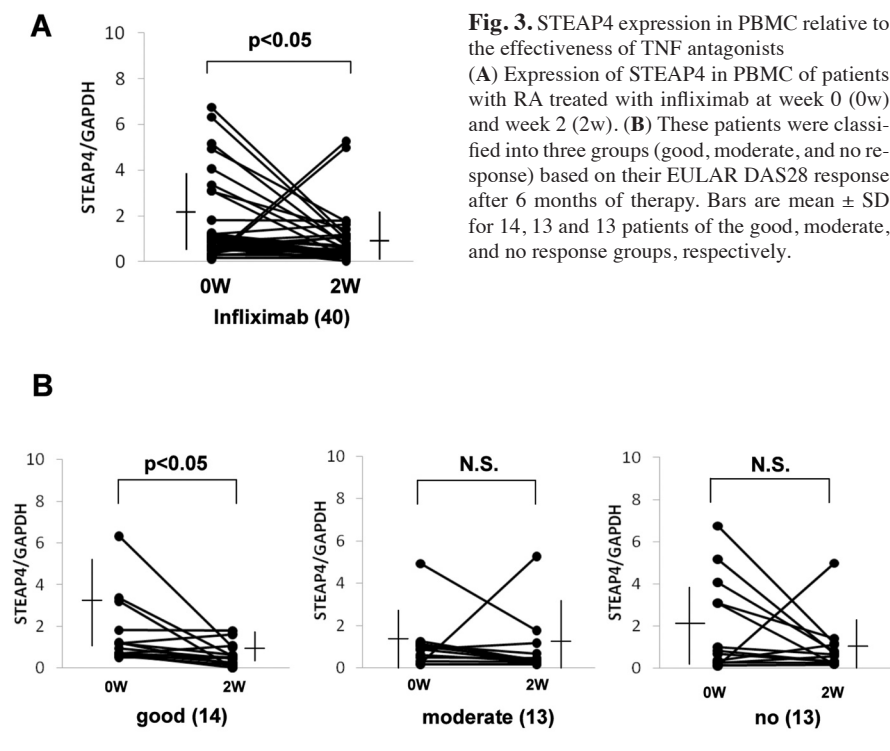
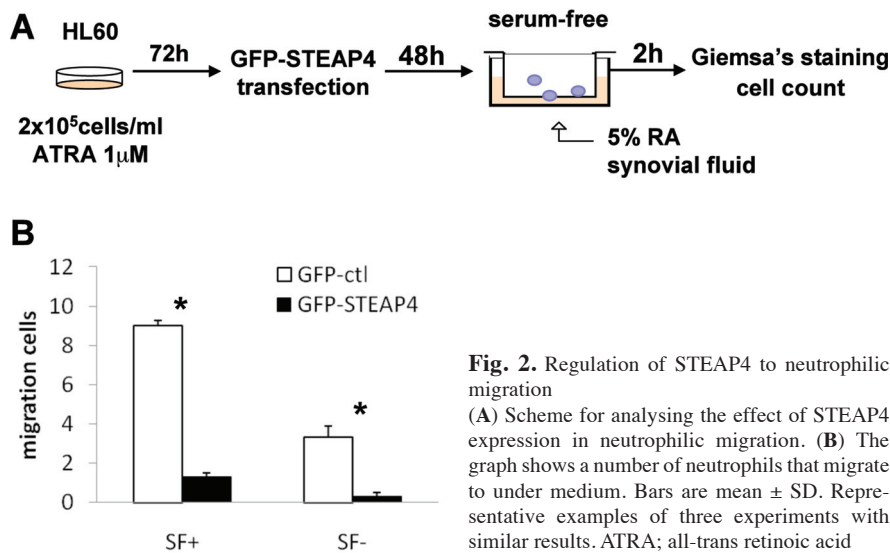
All data were expressed as mean  $\pm$  SD. The significance of correlations between variables was tested using Spearman's statistical test. The Mann-Whitney non-parametric two-sample rank test was used to compare the frequencies and concentrations of populations

in different groups. Wilcoxon's rank test was used to compare the expression levels of STEAP4 gene at weeks 0 and 2 based on the TNF antagonist analysis. A *p*-value less than 0.05 was considered significant.

### Results

#### Monocytes and neutrophils are the main source of STEAP4 mRNA in peripheral blood

Our previous study detected STEAP4 mRNA in peripheral blood of patients with RA (8). To further characterise the TIARP-expressing cells in peripheral blood, we performed flow cytometry in the present study. STEAP4 was mainly expressed in monocytes compared to lymphocytes in peripheral blood (Fig. 1A), and was detectable in neutrophils (Fig. 1B). The expression of STEAP4



mRNA was clearly correlated with the number of neutrophils in peripheral blood from seven patients with RA (Fig. 1C,  $r=0.747$ ,  $p<0.05$ ).

*STEAP4 regulates migration of neutrophils*

To analysis the function of STEAP4 in neutrophils, we used HL60 cell line. HL60 is derived from human promyelocytic leukaemia and inducible to neutrophil by ATRA. The migration of HL60 was assessed by transwell assay after differentiation to neutrophil and

transfection with GFP-STEAP4 pDNA (simple method in Fig. 2A). The migration of HL60 transfected with GFP-STEAP4 was downregulated comparing to GFP-empty transfectant (Fig. 2B).

*STEAP4 expression is downregulated by TNF antagonists*

To analyse the relationship between therapy with TNF antagonists and STEAP4, we compared the expression of STEAP4 before and after 2-week treatment with TNF antagonists, such as IFX. IFX treatment was associated with

a significantly reduction of STEAP4 expression (Fig. 3A). We next divided the patients into three groups (good, moderate, and non responders) according to the clinical response to IFX at 6 months after IFX therapy using the EULAR criteria, which are based on the DAS28 index. Only good responders to the IFX ( $n=14$ ) showed a significant reduction in STEAP4 expression in PBMC (Fig. 3B).

**Discussion**

The STEAP protein family was first identified in human prostate cancers (12-14). TIARP-like proteins such as STEAP4 are highly expressed in the bone marrow, placenta, and foetal liver (9), and STEAP4 is induced by TNF- $\alpha$  in human adipose tissue (10). We also demonstrated recently that TIARP was upregulated in GPI-induced arthritis (5), and was mainly expressed on CD11b<sup>+</sup> splenocytes and hyperplastic synovium (6). STEAP4 was expressed on synoviocytes from RA patients and regulated IL-6, IL-8, and cell proliferation (15). The signal transduction pathway is still unclear. In this report, we identified the surface expression of STEAP4 in monocytes and neutrophils in peripheral blood (basically, CD11b<sup>+</sup> cells) of patients with RA.

The expression of STEAP4 mRNA was clearly correlated with the number of neutrophils in peripheral blood from seven patients with RA, thus we focused on neutrophils. To reveal the effect of STEAP4 in neutrophil migration, we performed a transfection analysis. Since isolated neutrophils have difficulty in surviving for a long time, and DNA transfection easily leads to apoptosis to them, we used HL60 cells for transfection. HL60 was transfected with GFP-STEAP4 or GFP-empty after differentiation to neutrophils by all-trans retinoic acid. The migration cell numbers to the bottom medium containing 5% RA synovial fluid were significantly reduced in GFP-STEAP4<sup>+</sup> HL60 cells, suggesting a diminished effect of migration capacity of STEAP4. We also screened chemokine receptors such as CXCR2 expression in HL60, which was comparable between STEAP4 and empty transfected cells (data not shown).

In the GPI-induced arthritis model, anti-TNF mAb was effective (4) and TIARP was clearly downregulated by anti-TNF- $\alpha$  mAb (6). In this study, TNF antagonist such as IFX clearly downregulates the expression of STEAP4 in peripheral blood of patients with RA, especially in good responders. However, since the mechanism of its downregulation by IFX is still unknown, further study is needed to clarify it.

In conclusion, STEAP4 is expressed on monocytes/neutrophils in peripheral blood, regulates cell migration, is downregulated by TNF antagonist, and might be a possible predictor of response to TNF antagonist.

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