Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.13076

Clinical and functional significance of STEAP4-splice variant in CD14$^+$ monocytes in patients with rheumatoid arthritis

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Accepted for publication 24 October 2017

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Summary

Tumour necrosis factor alpha (TNF)-α-induced adipose-related protein (TIARP) is a negative regulator of inflammation in arthritis model mice. In humans, six-transmembrane epithelial antigen of prostate 4 (STEAP4) (human counterpart of TIARP) is also expressed in CD14$^+$ monocytes from patients with rheumatoid arthritis (RA). Recently, highly levels of exon 3-spliced variant STEAP4 (v-STEAP4) expression have been observed in porcine lung. The aim of this study is to elucidate the expression and functional role of v-STEAP4, comparing it with that of STEAP4, in the pathogenesis of arthritis. We identified v-STEAP4 in CD14$^+$ cells. The expression of STEAP4 and v-STEAP4 was higher in patients with RA than in healthy participants. We also found that STEAP4 and v-STEAP4 were correlated positively with C-reactive protein and that their expression was decreased after treatment with an interleukin (IL)-6 antagonist in patients with RA. To investigate further the role of STEAP4 and v-STEAP4, we produced STEAP4 and v-STEAP4 over-expressing human monocytic cell lines (THP-1) for functional analysis. In the v-STEAP4 over-expressing cells, the production of IL-6 was suppressed significantly, but TNF-α was increased significantly through lipopolysaccharide (LPS) stimulation. Immunoblot analysis revealed that phosphorylated (p-)nuclear factor kappa B (NF-κB) was increased after LPS stimulation and degradation of nuclear factor kappa B inhibitor alpha (IκBα) was sustained, whereas p-signal transducer and activator of transcription 3 (STAT-3) was decreased with v-STEAP4. We identified specific up-regulation of v-STEAP4 in RA monocytes. V-STEAP4 might play a crucial role in the production of TNF-α and IL-6 through NF-κB and STAT-3 pathways, resulting in the generation of RA.

Keywords: monocytes, rheumatoid arthritis, splice variant, STEAP4, TIARP

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease of the joints mediated primarily by proinflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-6. As these two cytokines play a pivotal role in the pathogenesis of RA, there are multiple reports of the efficacy of treatments that target such molecules [1–3].

Previously, we reported that the up-regulation of murine TNF-α-induced adipose-related protein (TIARP) [also known as six-transmembrane protein of prostate 2 (STAMP2) and tumour necrosis factor α-induced protein 9 (TNFAIP9)] in CD11b$^+$ primary splenocytes and joints of glucose-6-phosphate isomerase (GPI)-induced arthritis mice and could be suppressed with anti-TNF-α antagonists [4]. TIARP was, therefore, concluded to be a separate but similar key co-factor with TNF-α in the pathology of RA. Apart from TNF-α, however, TIARP expression is up-regulated additionally by other proinflammatory cytokines or mediators such as IL-6, IL-1β or lipopolysaccharide (LPS) in various cells, such as macrophages and hepatocytes [5–12]. Paradoxically, we previously reported that

TIARP$^{-}/^{-}$ mice develop polyarthritis spontaneously and are highly susceptible to collagen-induced arthritis and K/BxN serum transfer arthritis [13,14]. Furthermore, high expression levels of proinflammatory mediators [such as TNF-α, IL-6 and chemokine (C-X-C motif) ligand 2 (CXCL2)] and marked accumulation of neutrophils and macrophages have been described in the arthritic joints of TIARP$^{-}/^{-}$ mice [13]. In macrophages, TIARP can suppress the production of proinflammatory cytokines by inhibiting nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT-3) signalling pathways [13]. TIARP is therefore more complex than thought originally and might serve as a complex switching mechanism in inflammation associated with RA.

A human TIARP counterpart, known as six-transmembrane epithelial antigen of prostate 4 (STEAP4), is also expressed highly in CD14^{+} monocytes and synovial CD68^{+} cells in patients with RA [15,16]. STEAP4 mRNA expression was correlated positively with TNF-α in the synovium of RA [16]. Regarding the function of STEAP4, we have reported previously that over-expression of STEAP4 in human fibroblast-like synoviocyte (FLS) cell lines MH7A and HL60 forced differentiation to neutrophil-like cells after stimulation with *all-trans* retinoic acid [16,17]. STEAP4 suppresses the expression of IL-6 and IL-8 mRNA induced through TNF-α stimulation in MH7A and suppresses migration to RA synovial fluid in HL60 [16,17]. However, a functional analysis of STEAP4 in human monocytes has not yet been reported.

Recently, exon 3-spliced variant STEAP4 (v-STEAP4) has been found to be expressed highly in porcine lung and also in HepG2, which is a cell line derived from human liver carcinoma cells [18]. We found the same variant in GPI-induced arthritis (our preliminary observations). It is known that alternative splicing of pre-mRNA is a powerful and versatile regulatory mechanism affecting gene abundance and the functional diversity of proteins [19]. However, expression of v-STEAP4 in human monocytes and its function in RA remain unclear.

In the present study, to identify the significance of v-STEAP4 in monocytes from patients with RA, we compared the v-STEAP4 expression between patients with RA and healthy individuals (control group) and investigated the association between the expression level and clinical markers of RA. Furthermore, we generated a cell line that over-expresses v-STEAP4 and examined the effects of v-STEAP4 over-expression on various cytokine productions and intracellular pathways.

Methods

Study participants and blood samples

Peripheral blood samples were obtained from 14 healthy participants (control group), 19 patients with RA, and samples from an additional six patients with RA treated with tocilizumab were also obtained. Approval for this study was obtained from the local ethics committees of University of Tsukuba Hospital (reference number: H24–164). A signed informed consent to participate was obtained from each participant. Peripheral blood mononuclear cells (PBMC) were separated from whole blood using a Ficoll density gradient, according to the manufacturer's instructions. CD14^{+} cells from RA and control groups were purified from PBMC-enriched fractions using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and a magnetic cell separator, according to the manufacturer's instructions. Participant backgrounds are shown in Tables 1 and 2. The disease activity score (DAS) was calculated according to the response criteria of the European League Against Rheumatism [20]. All patients with RA fulfilled the American College of Rheumatology 1987 criteria for the classification of RA [21] and/or 2010 RA classification criteria [22].

Table 1. Demographic and clinical characteristics of patients with rheumatoid arthritis (RA) ($n = 19$)

Age	64·3 ± 10·2
Female, *n* (%)	13 (68%)
Disease duration (years)	14·7 ± 10·2
DAS28-CRP	3·1 ± 0·9
DAS28-ESR	3·4 ± 1·0
EDR (mm/h)	29·2 ± 20·2
CRP (mg/dl)	1·7 ± 1·8
Rheumatoid factor (IU/ml)	120·7 ± 168·5
Matrix metalloproteinase 3 (ng/ml)	243·5 ± 291·4
Anti-CCP antibodies positive, *n*	11 (unknown: 6)
Prednisolone use, *n* (%, mean dose)	11 (58%, 5·7 ± 0·6 mg/day)
Methotrexate use, *n* (%, mean dose)	11 (58%, 6·5 ± 0·7 mg/week)
Use of biological agents, *n* (%)	0 (0%)

Values are the mean ± standard deviation (s.d.). CCP = cyclic citrullinated peptide; CRP = C-reactive protein; DAS 28 = disease activity score in 28 joints; ESR = erythrocyte sedimentation rate.

Real-time quantitative reverse transcription–polymerase chain receptor (qRT–PCR)

Total RNA was extracted with Isogen (Nippon Gene, Toyama, Japan) using the protocol provided by the manufacturer, and cDNA was synthesized using a reverse transcription kit (Takara Bio, Otsu, Japan). qRT–PCR was performed using TaqMan probe method with the 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Data of the control group and patients with RA were analysed using prepared standard curves. All data were normalized to human glyceraldehyde-3-phosphate dehydrogenase endogenous control (VIC®/MGB Probe, Primer Limited; Applied Biosystems, Carlsbad, CA, USA). Primer and fluorescently tagged probe sets were produced

Table 2. Demographic and clinical characteristics of patients with rheumatoid arthritis (RA) with tocilizumab treatment ($n = 6$)

Age	53·7 ± 7·4
Female, *n* (%)	5 (83%)
Disease duration (years)	9·6 ± 5·7
Use of biological agents before tocilizumab, *n* (%)	2 (17%)
Anti-CCP antibodies positive, *n* (%)	5 (83%)
DAS28-CRP	3·5 ± 0·6
DAS28-ESR	3·7 ± 0·8
ESR (mm/h)	27·6 ± 9·9
CRP (mg/dl)	1·0 ± 0·50
Prednisolone use, *n* (%, mean dose)	6 (100%, 6·2 ± 1·2 mg/day)
Methotrexate use, *n* (%, mean dose)	3 (50%,10 ± 2·0 mg/week)

Values are mean ± standard deviation (s.d.). CCP = cyclic citrullinated peptide; CRP = C-reactive protein; DAS 28 = disease activity score in 28 joints; ESR = erythrocyte sedimentation rate.

that are specific to STEAP4 and v-STEAP4, respectively, as follows: STEAP4 probe [FAM]: 3′-TCTTTGCTTGGCTTTGCTGTCATTTCCACA-5′ [TAMRA], STEAP4 forward: 5′-GCAAGTCGGCAGGTGTTTG-3′, STEAP4 reverse: 3′-GTCCAAGATTACGAACAATATCCATCA-5′; v-STEAP4 probe [FAM] 3′-TTCTTGAGTATTGCCTGCCGACTTGCAT-5′ [TAMRA], v-STEAP4 forward: 5′-TGGGCTCTCCAGTCAGGAG-3′, v-STEAP4 reverse: 3′-CAGGCTGAGGAGGTGCTAAA-5′ (Sigma Aldrich, Tokyo, Japan).

Cloned vector DNA was verified to confirm the specificity (data not shown).

Cloning the DNA sequence of STEAP4 and v-STEAP4

The pLVSIN-IRES-ZsGreen1 Vector (Takara Bio, Shiga, Japan) was used to construct both STEAP4 and v-STEAP4 over-expressing cells. The vector expresses two proteins from a bicistronic mRNA transcript, allowing ZsGreen1 to be used as an indicator of transduction efficiency and a marker for selection using flow cytometry.

The DNA sequence of human STEAP4 (NM_024636.3) and its exon 3-spliced variant from initiation to termination codons obtained from human $CD14^+$ monocytes using the RT–PCR technique using PrimeSTAR Max DNA polymerase (Takara Bio) were inserted into the vector using the In-Fusion HD Cloning Kit (Takara Bio). The primers of STEAP4 and v-STEAP4 used for the RT–PCR are listed as follows: STEAP4/v-STEAP4 forward: 5′-ATGGAGAAAACTTGTATAGATGC-3′, STEAP4/v-STEAP4 reverse, 5′-CTAGTGTTTTGAGTTCCTTTCCC-3′. As a control, we also generated a lentiviral vector that expresses only ZsGreen1 (mock).

Lentiviruses were prepared by transfecting the plasmid into Lenti-X293T packaging cells which facilitate optimal lentivirus production. Lentiviral particles were obtained according to the manufacturer's instructions and were concentrated using a Lenti-X concentrator kit (Takara Bio).

Establishment of the STEAP4 over-expressing cell lines

The human acute monocytic leukaemia (THP-1) cell lines, which are a human monocytic cell line (RIKEN RBRC-RCB1189), were incubated at $7{\cdot}5 \times 10^4$ cells in 250 µl complete growth medium in each well for 24 h before transduction and then transduced with the lentiviral particles [multiplicity of infection (MOI) = 80] with 16 µg/ml of polybrene by centrifuge for 90 min at 1200 ***g*** at 32°C in 48-well plates. Sorting and enrichment of ZsGreen1-positive cells was performed using a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark).

In-vitro cell culture studies

THP-1 cells were cultured in RPMI-1640 (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich), 100 U/ml penicillin–streptomycin (GIBCO, Carlsbad, CA, USA) and 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) at 37°C in a humid atmosphere of 5% CO_2; 1×10^6 cells/ml each of STEAP4, v-STEAP4 over-expressing cells and mock were seeded in 48-well cell culture plates, incubated overnight for cell adhesion and stimulated by 1 µg/ml LPS in the presence or absence of S3I-201 and/or Bay 11–7082 (Sigma Aldrich).

The supernatants were collected after 24 h of LPS stimulation to measure the production of proinflammatory cytokines such as IL-6 and TNF-α using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) or cytokine bead array (BD Biosciences, San Jose, CA, USAS), according to the manufacturer's instructions. After 1 µg/ml of LPS stimulation for 0, 15, 30 or 60 min the remaining adherent cells were lysed in buffer [Tris-HCl pH 7·4, 50 mM, $MgCl_2$ 5 mM, phenylmethylsulphonyl fluoride (PMSF 2 mM, NP-40 0·5%] supplemented with protease/phophatase inhibitor (CST) for immunoblotting.

ELISA

ELISA kits (R&D Systems) were used to measure cytokines and chemokines in cell culture supernatants and also the serum concentrations of TNF-α and IL-6.

Cytometric bead array assay

The levels of the cytokines in the serum samples were measured using the BD cytometric bead array human inflammatory cytokines kit (BD Biosciences), which detects human cytokines simultaneously, including IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70. The assays were performed in accordance with the manufacturer's instructions. The samples were evaluated using a BD FACSVerse cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed using Flow

Cytometric Analysis Program (FCAP) Array software (BD Biosciences, San Jose, CA, USA).

Soduim dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting

Protein concentrations were calculated using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA) and an equal concentration was loaded into each well of SuperSep Ace 10–20% gels (Wako, Osaka, Japan). Anti-STEAP4 C-terminal region (Aviva Systems Biology, San Diego, CA, USA), which can recognize both STEAP4 and v-STEAP4, and anti-β-actin antibodies (Sigma-Aldrich) were diluted at 1 : 1000 and 1 : 3,000, respectively, with 10% Block Ace (Dainippon Pharmaceutical, Osaka, Japan). Downstream signalling proteins of Toll-like receptor 4 (TLR-4), such as NF-κB p65, phosphorylated (p-)NF-κB p65 (Ser536), NF-κB inhibitor alpha (IκBα), p-p38 (Thr180/Tyr182), p38,p-ERK1/2 (Thr202/Tyr204), extracellular-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), phospho-JNK (p-JNK) (Thr183/Tyr185), suppressor of cytokine signalling 3 (SOCS3), STAT-1, p-STAT-1 (Ser727), STAT-3 (Cell Signaling Technology, Danvers, MA, USA) and p-STAT3 (Ser727) (Assay Biotechnology Company, Sunnyvale, CA, USA) were diluted at 1 : 1000 with 10% Block Ace (Dainippon Pharmaceutical, Osaka, Japan). Anti-rabbit immunoglobulin (IgG)-horseradish peroxidase (HRP) conjugate secondary antibody (Bio-Rad, Hercules, CA, USA) was diluted with Solution 2 (Toyobo, Osaka, Japan) at 1 : 1000 for STEAP4, 1 : 2000 for the signalling proteins and 1 : 6000 for β-actin.

Statistical analysis

Data were shown as mean ± standard deviation (s.d.) or mean ± standard error of the mean (s.e.m.). Data regarding the STEAP4 over-expressing THP-1 cell lines were examined for statistical significance using the Tukey test after confirming $P > 0{\cdot}05$ by the Levene test. The Mann–Whitney *U*-test was used to test for differences in various parameters between the control and RA groups as well as for cytokine suppression assays with TLR-4 downstream signalling-specific inhibitors within THP-1 cell lines. Differences between before and after tocilizumab treatment groups were analysed by the Wilcoxon signed-rank test. Correlation between STEAP4 expression and clinical information was analysed by Spearma'sn test. *P*-values of less than 0·05 were considered statistically significant.

Results

Human monocytes contain the STEAP4 exon 3-spliced variant

To investigate variant expression in human primary monocytes, we examined STEAP4 by RT–PCR. We found not only a full-length STEAP4 band [1340 base pairs (bp)], but also lower molecular weight bands (812 bp) in three samples from control and RA, and the intensity of these 812 bp bands tended to be stronger in RA than control. Cloning and sequence of this DNA identified the heavy band as STEAP4 and the lighter band as its exon 3-spliced form (v-STEAP4) (812 bp) (Fig. 1a).

Both STEAP4 and v-STEAP4 mRNA expression levels and v-STEAP4/STEAP4 ratio are increased in RA *versus* control

We next performed qRT–PCR to analyse the mRNA expression levels of STEAP4 and v-STEAP4 in the $CD14^+$ monocytes of peripheral blood from 19 RA patients (RA group) and 14 healthy participants (control group) using specific primer sets. Both STEAP4 and v-STEAP4 expressions were significantly higher in the RA group compared with the control group ($P < 0{\cdot}05$) (Fig. 1b). A positive correlation between STEAP4 and v-STEAP4 was shown in the RA group only ($R = 0{\cdot}463$, $P = 0{\cdot}046$) (Fig.1c), and the ratio of variant to STEAP4 was significantly higher in the RA group than in the control group (Fig. 1d, $P < 0{\cdot}05$).

Correlation of STEAP4 and v-STEAP4 expression with clinical information in RA patients

In our previous reports, STEAP4 was induced by TNF-α or LPS stimulation in $CD14^+$ monocytes *in vitro* [15]. Another report noted that both STEAP4 and v-STEAP4 are induced by LPS in the HepG2 human hepatic cell line [18]. Indeed, both STEAP4 and v-STEAP4 expression in $CD14^+$ cells was significantly higher in the RA group than in the control group. However, the association between these inflammatory factors and their expressions in RA patients have not yet been validated. We investigated the correlation between the expression level of STEAP4 or v-STEAP4 and clinical information such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), matrix metalloproteinase 3 (MMP-3) or DAS in 28 joints (DAS28)-CRP/ESR, all of which are markers of clinical disease activity in patients with RA. Positive correlations were indicated in CRP ($R = 0{\cdot}619$, $P = 0{\cdot}005$) and ESR ($R = 0{\cdot}499$, $P = 0{\cdot}035$) with STEAP4, and in CRP ($R = 0{\cdot}549$, $P = 0{\cdot}015$) and RF ($R = 0{\cdot}485$, $P = 0{\cdot}035$) with v-STEAP4 (Fig. 2a and b), but MMP-3 and DAS-28-CRP/ESR were not correlated with either (Supporting information, Fig. S1a,b).

To elucidate the linear fluctuation between STEAP4 and v-STEAP4 mRNA resulting from treatment variation, we investigated the effects of tocilizumab (an IL-6 antagonist) on STEAP4 and v-STEAP4 mRNA expression levels. We have reported previously that STEAP4 expression was

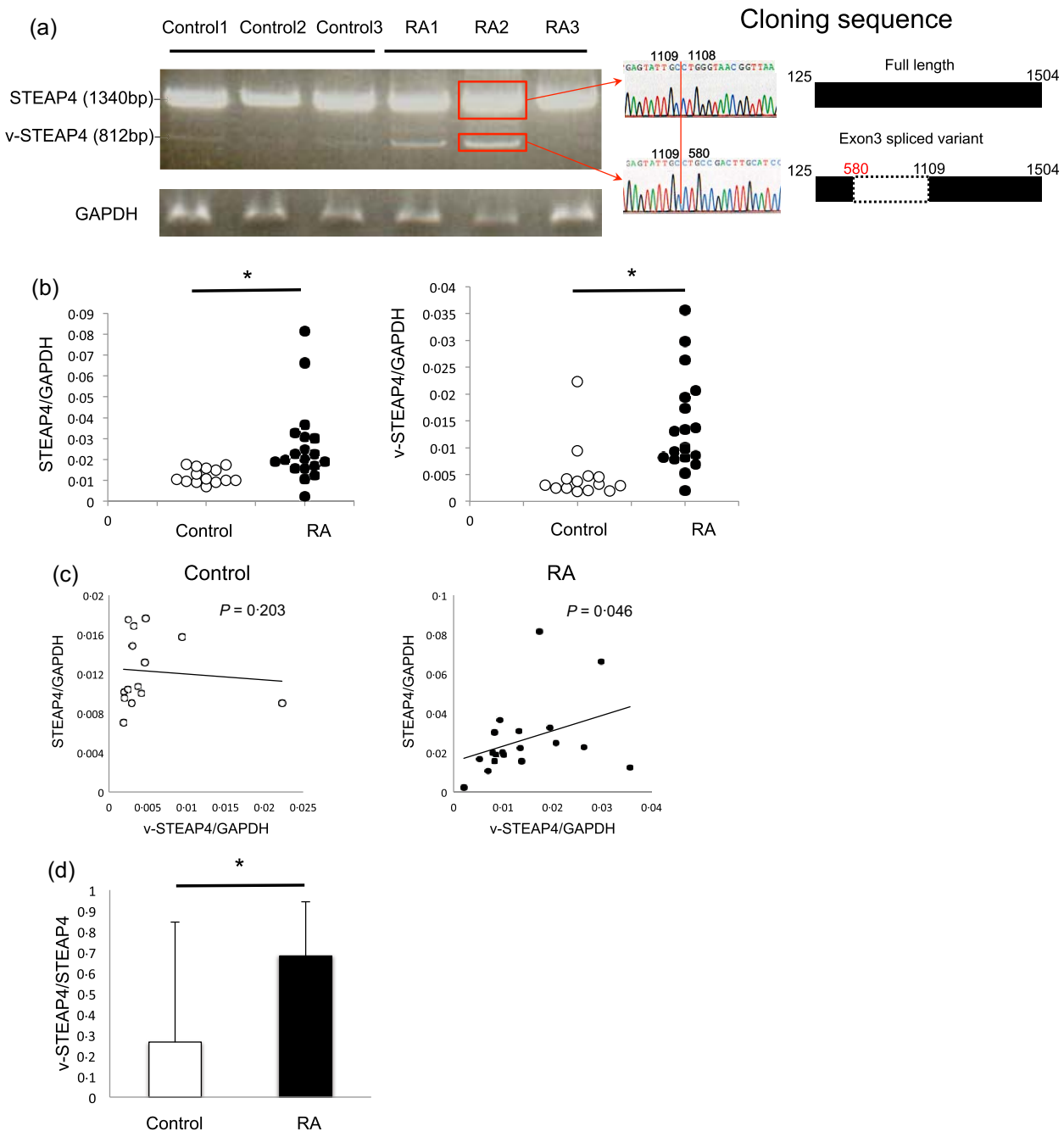

Fig. 1. Six-transmembrane epithelial antigen of prostate 4 (STEAP4) and its exon 3-spliced variant (v-STEAP4) mRNA expression levels in patients with RA and control. (a) Identification of STEAP4 and exon 3-spliced variant of STEAP4 (v-STEAP4) in $CD14^+$ monocytes (control, $n = 3$; RA, $n = 3$) using reverse transcription–polymerase chain reaction (RT–PCR) and the nucleotide sequences confirmed through cloning and sequence. (b) Comparison of STEAP4 (left) and v-STEAP4 (right) mRNA expression levels in peripheral blood $CD14^+$ monocytes between control ($n = 14$) and RA ($n = 19$) using qRT–PCR. $^*P < 0{\cdot}05$ by Mann–Whitney *U*-test (c) Analysis of correlation of mRNA expression by quantitative (q)RT–PCR between STEAP4 and v-STEAP4. *P*-values were calculated by Spearman's correlation. (d) Comparison of v-STEAP4/STEAP4 ratio by qRT–PCR in peripheral $CD14^+$ monocytes between control ($n = 14$) and RA ($n = 19$). Values are the mean ± standard deviation (s.d.). $^*P < 0{\cdot}05$ by Mann–Whitney *U*-test.

down-regulated by tocilizumab [15]. In the current study, the six patients treated with tocilizumab for 12 weeks and the tocilizumab-treated patients both showed significant decrease of STEAP4 and v-STEAP4 expression in $CD14^+$ monocytes without fluctuation in the ratio of v-STEAP4/STEAP4 (Table 2, Fig. 2c).

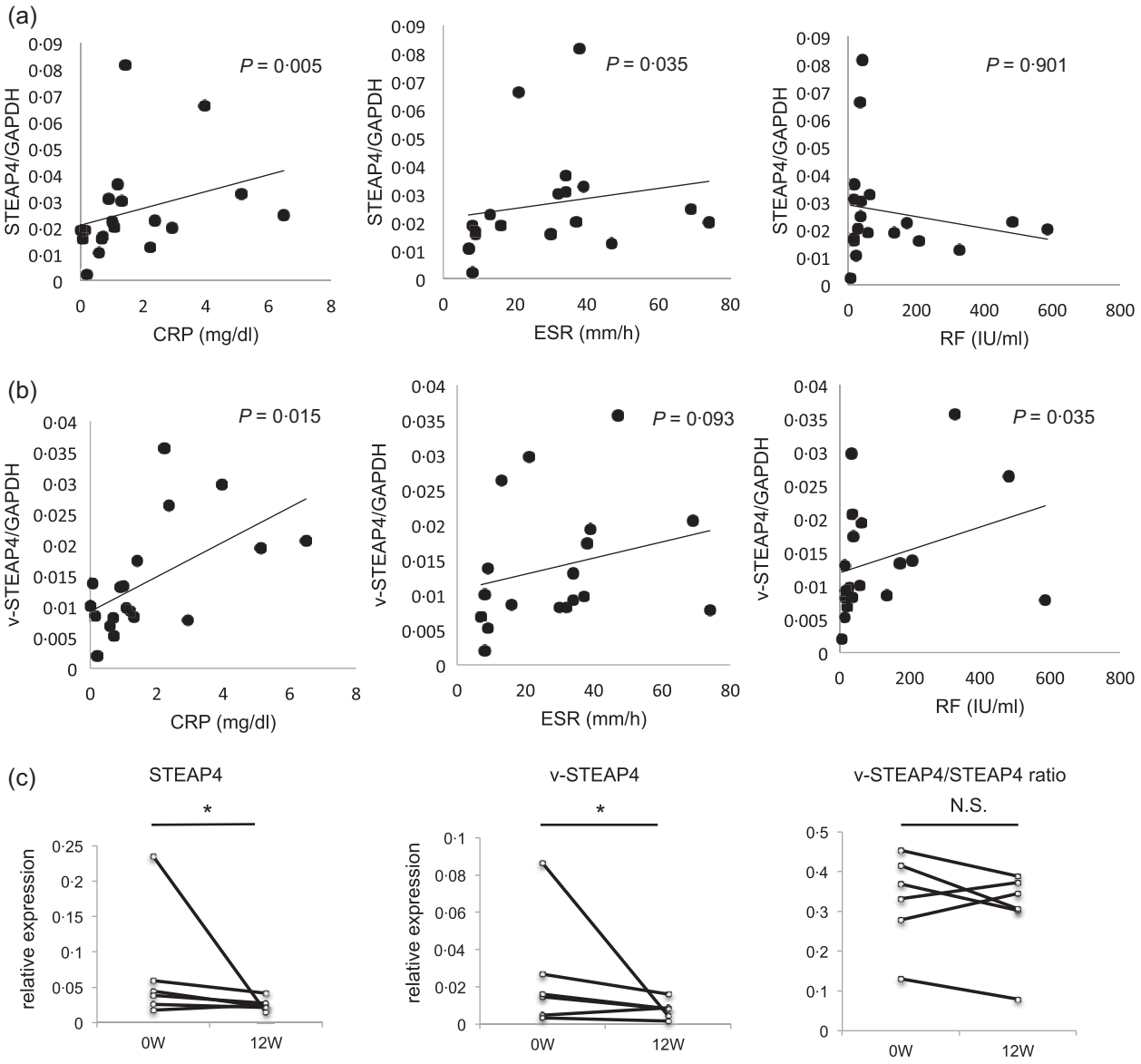

Fig. 2. Expression of six-transmembrane epithelial antigen of prostate 4 (STEAP4) is correlated with inflammatory parameters in RA. (a) Correlation between mRNA expression according to quantitative reverse transcription–polymerase chain reaction (qRT–PCR) of STEAP4 or (b) v-STEAP4 and serum clinical biomarkers titre such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) ($n = 18$) and/or rheumatoid factor (RF) ($n = 19$), respectively, in all RA patients. *P*-values were calculated using Spearman's correlation. (c) The expression of STEAP4 or v-STEAP4 in peripheral blood CD14$^+$ monocytes before and after RA treatment with tocilizumab for 12 weeks in six patients with reduced disease activity after therapy. *$P < 0{\cdot}05$ according to Wilcoxon's signed-rank test.

Establishment of STEAP4 and v-STEAP4 over-expressing monocytic cell lines (THP-1) for functional analysis

We have reported a previously functional analysis of STEAP4 via over-expression in the human FLS cell line MH7A. However, functional analyses of STEAP4 and v-STEAP4 in human monocytes have not yet been reported [16,17]. To analyse their relative functions, we produced STEAP4 and v-STEAP4 over-expressing cells. We selected THP-1 as host cells because they are derived from human monocytes and are reported more widely in the literature than other monocytic cell lines such as U-937.

Induction efficacy was checked using the reporter protein ZsGreen1, and each group of over-expressing cells was sorted fluorescently three times to enrich for transfected cells. After the third sorting the purity had increased to more than 95% (Fig. 3a). We next performed immunoblot analysis to confirm protein expression levels of STEAP4 and v-STEAP4 against actin as an internal control. The intensity of the full-length mRNA band (52kD) in STEAP4

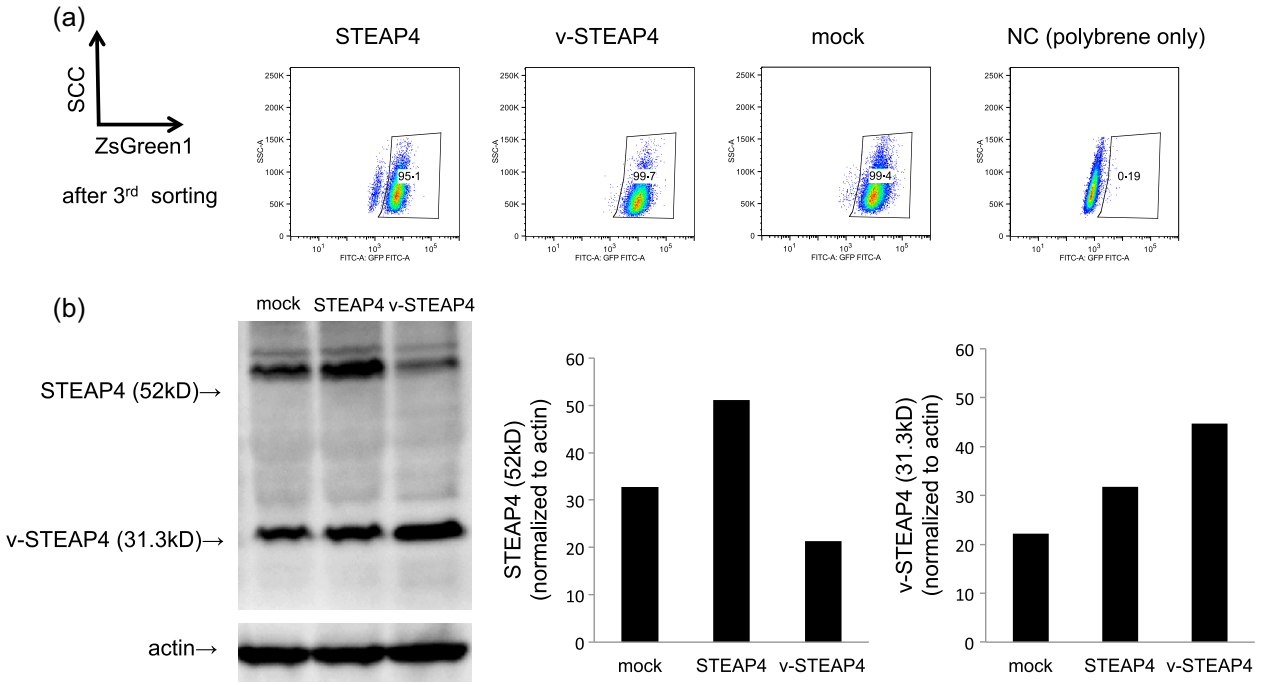

Fig. 3. Establishment of six-transmembrane epithelial antigen of prostate 4 (STEAP4) and v-STEAP4 over-expressing monocytic cell lines [human acute monocytic leukaemia cell line (THP-1)] for functional analysis (a) The efficacy of transduction of STEAP4, v-STEAP4 and empty vector as control (mock) was evaluated using reporter protein ZsGreen1. (b) STEAP4 over-expression in the experimental cell lines (STEAP4, v-STEAP4 and mock) was confirmed using immunoblot analysis. The relative protein expressions were divided by STEAP4 or v-STEAP4 with actin as internal control. [Colour figure can be viewed at wileyonlinelibrary.com]

over-expressing cells increased relative to mock, and the exon 3 deletion band (31·3 kD) was also increased in v-STEAP4 over-expressing cells (Fig. 3b).

Functional analysis by LPS stimulation in STEAP4 and v-STEAP4 over-expressing cells

Next, to elucidate the function of STEAP4 and v-STEAP4, we attempted to detect proinflammatory cytokine production via LPS stimulation. To up-regulate the LPS receptor CD14, also known as TLR-4, we treated the THP-1 cells with phorbol myristate acetate (PMA) before LPS stimulation. TNF-α was clearly over-produced in v-STEAP4 over-expressing THP-1 macrophages (Fig. 4a, left), whereas the production of IL-6 was increased in STEAP4 over-expressing macrophages and decreased dramatically in the v-STEAP4 over-expressing macrophages (Fig. 4a, centre). Additionally, the fluctuation of IL-1β had a similar tendency to IL-6 (Fig. 4a, right).

We next investigated the fluctuation of downstream signalling proteins via LPS stimulation of the STEAP4 and v-STEAP4 over-expressing cells. First, we focused on NF-κB and STAT-3, because these cell-signalling molecules can be regulated using murine homologue TIARP [13]. In terms of NF-κB signalling, which is a major TLR-4 downstream signalling pathway to TNF-α production, p-NF-κB was increased at 15 and 30 min after LPS stimulation in the v-STEAP4 over-expressing cells, and degradation of the NF-κB inhibitory molecule IκBα was sustained at 30 and 60 min in the v-STEAP4 over-expressing cells (Fig. 4b). Conversely, over-expressing cells and, interestingly, degradation of total STAT-3 was also detected at 0, 15, 30 and 60 min in the v-STEAP4 over-expressing cells against actin as an internal control, whereas p-STAT-1 was almost comparable with the over-expressing cells. Moreover, SOCS3, known as a negative regulator of STAT-3, was also detected, but not induced through LPS stimulation, in the v-STEAP4 over-expressing cells at any time (Fig. 4c).

To confirm whether NF-κB or STAT-3 signalling is required for the production of TNFα or IL-6, respectively, we used a specific inhibitor for each in this experimental system. The production of TNF-α via LPS stimulation and phosphorylation of NF-κB and degradation of IκBα at 15 min after LPS simulation were suppressed by NF-κB inhibitor (Bay 11–7082) and IL-6 and phosphorylation of STAT-3 was suppressed by STAT-3 inhibitor (S3I-201) in uninfected THP-1 macrophages (Supporting information, Fig. S2a,b). This confirmed that NF-kB and STAT-3 are required for production of these cytokines. TNF-α production was suppressed by NF-κB inhibitor in mock and the v-STEAP4 over-expressing cells in a dose-dependent manner, but was not suppressed in the STEAP4 over-expressing

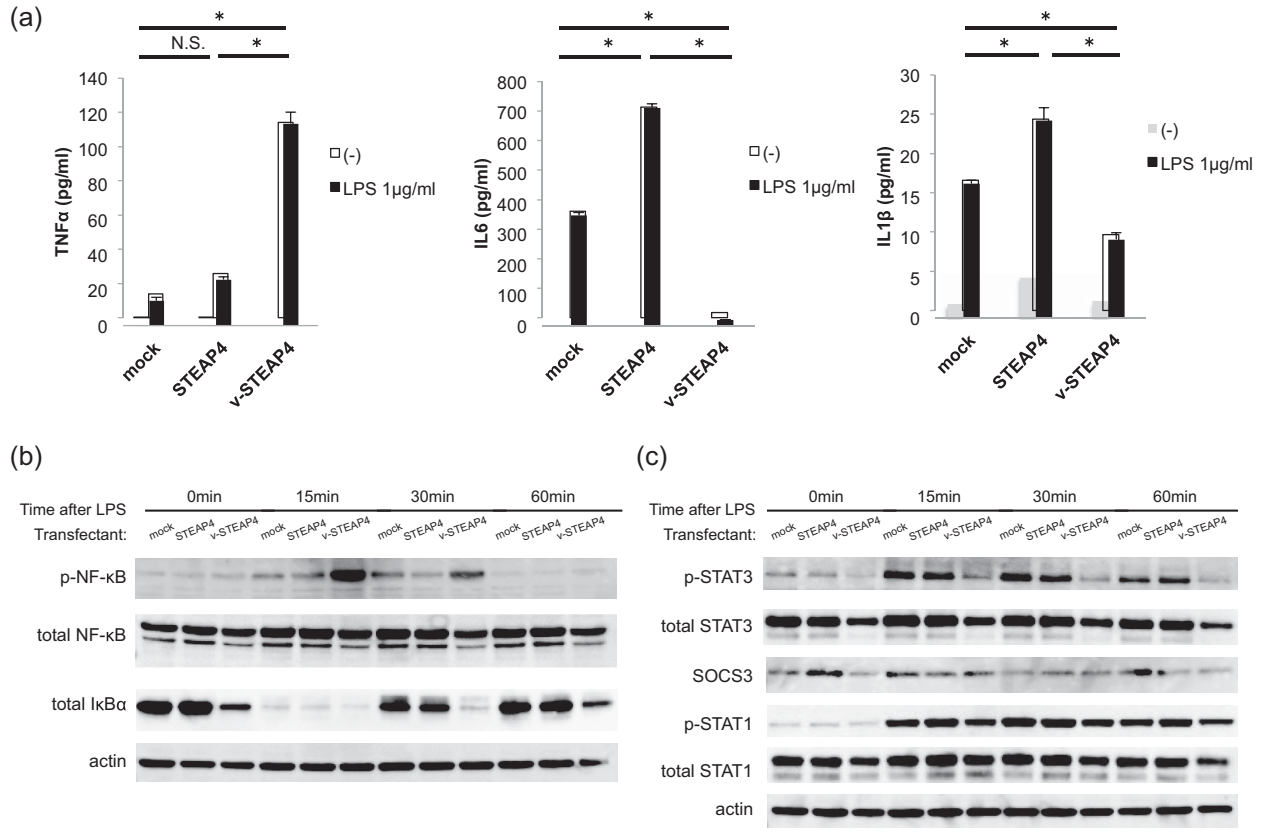

Fig. 4. Functional analysis through lipopolysaccharide (LPS) stimulation of six-transmembrane epithelial antigen of prostate 4 (STEAP4) and v-STEAP4 over-expressing cells. (a) The concentrations of tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in the culture supernatants were measured using cytometric bead array assay after 24 h with 1 μg/ml of lipopolysaccharide (LPS) stimulation from human acute monocytic leukaemia cell line (THP-1) macrophages after phorbol myristate acetate (PMA) treatment. The THP-1 macrophages were transfected with either plasmid expressing empty vector (mock) or the construct STEAP4, or v-STEAP4. Values are expressed as the mean ± standard error of the mean (s.e.m.) of three wells per group. $^*P < 0{\cdot}05$ by Tukey's test after confirming $P > 0{\cdot}05$ by Levene's test. (b) Levels of p-nuclear factor kappa B (NF-κB), total NF-κB, total nuclear factor kappa B inhibitor alpha (IκBα), actin, (c) p-signal transducer and activator of transcription 3 (STAT-3), total STAT-3, suppressor of cytokine signalling 3 (SOCS-3), total STAT-1, p-STAT-1 and actin in whole cell lysates from the transfected THP-1 macrophages with 1 μg/ml of LPS stimulation for 0, 15, 30 or 60 min were determined by immunoblot analysis ($n = 2$ wells per group).

cells (Supporting information, Fig. S2c, left). IL-6 production was suppressed by STAT-3 inhibitor in mock and the v-STEAP4 over-expressing cells, but was increased in the STEAP4 over-expressing cells (Supporting information, Fig. S2c, right).

Taken together, these findings suggest that STEAP4 and v-STEAP4 over-expression influences several downstream cascades through LPS stimulation, and the main cascade in the v-STEAP4 over-expressing cells is TNF-α production via the NF-κB pathway and via IL-6/STAT-3 (summarized in Fig. 5).

Discussion

In this study, we first identified exon 3-spliced v-STEAP4 in human monocytes. STEAP4 and v-STEAP4 mRNA expression was higher in RA patients and was correlated positively to RA-mediated inflammatory markers such as CRP and/or ESR. In patients with RA, inflammatory cytokines such as TNF-α and IL-6 were over-produced. In our previous study, the mRNA expression of STEAP4 was induced following IL-6 increase by LPS or TNF-α stimulation in $CD14^+$ monocytes [15] and was decreased after treatment with an IL-6 or a TNF-α antagonist in PBMC from RA patients [15,17]. Another report noted that both STEAP4 and v-STEAP4 are induced by LPS in the HepG2 human hepatic cell line [18]. In this study, we found that the expression of v-STEAP4 was correlated positively with STEAP4 in $CD14^+$ monocytes from RA, and both STEAP4 and v-STEAP4 were decreased significantly by an IL-6 antagonist and were correlated positively with CRP. In contrast, there were no reports on over-expression of TNF-α and IL-6 from osteoarthritis (OA) patients. These findings suggested that STEAP4 and v-STEAP4 expression was up-regulated specifically in patients with RA, due probably to TNF-α or IL-6 stimulation. However, the mechanism of the specific regulation for v-STEAP4 has been unclear.

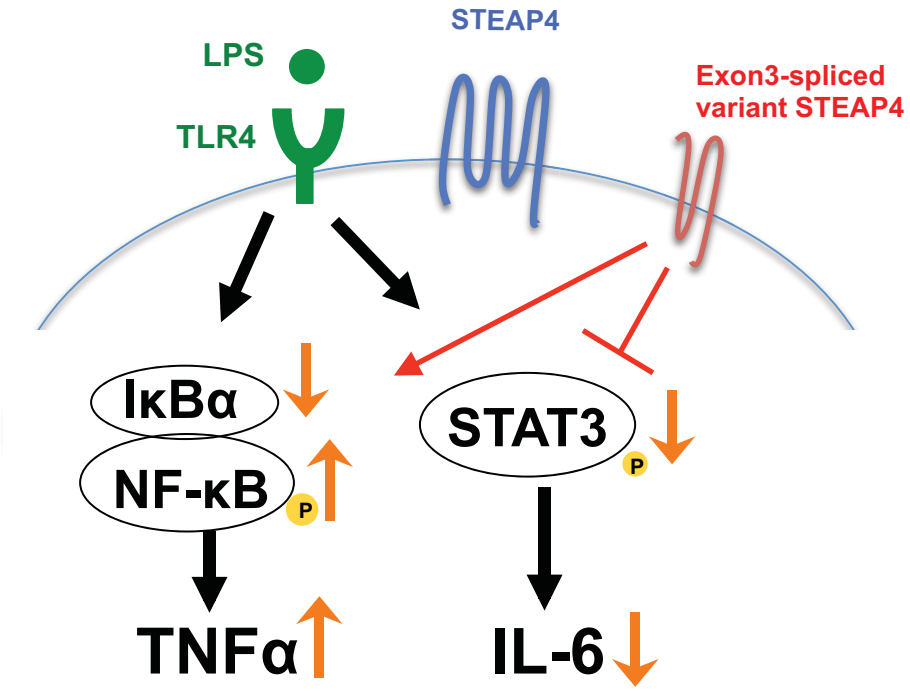

Fig. 5. Exon 3-spliced variant six-transmembrane epithelial antigen of prostate 4 (STEAP4) up-regulates the nuclear factor kappa B (NF-κB) pathway, but down-regulates the signal transducer and activator of transcription 3 (STAT-3) pathway. Experimental summary. Binding of the Toll-like receptor (TLR)-4 ligand lipopolysaccharide (LPS) to its receptor triggers phosphorylation and rapid degradation of nuclear factor kappa B inhibitor alpha (IκBα), allowing translocation of p-nuclear factor kappa B (NF-κB) to the nucleus and NF-kB-mediated up-regulation of TNFα, which triggers phosphorylation of p-signal transducer and activator of transcription 3 (STAT-3) and allows the translocation of p-STAT3 to the nucleus and STAT3-mediated up-regulation of interleukin (IL)-6.

To elucidate the functional difference between STEAP4 and v-STEAP4 in cytokine production and its intracellular signal cascade, we originally generated monocytic cell lines over-expressing either STEAP4 or v-STEAP4, and showed that the production of IL-6 and IL-1β via LPS stimulation was suppressed significantly in v-STEAP4, but TNF-α was increased in the v-STEAP4 over-expressing cells compared to mock. In contrast, STEAP4 increased production significantly of IL-6 and IL-1β, but TNF-α was not increased through LPS stimulation, suggesting that STEAP4 and v-STEAP4 have different pathways under LPS stimulation. STEAP4 was found in human monocytes, neutrophils and synovial fibroblasts in RA, and is thought to be a negative regulator in RA pathogenesis [15–17]. However, in human monocytes, over-expression of v-STEAP4 had an opposite effect on the production of TNF-α and IL-6 *in vitro*. The key result of this study is that over-expression of v-STEAP4 suppressed the production of IL-6 strongly. The mechanism of alternative splicing of STEAP4 is still unknown, but this functional difference of the LPS/IL-6 cascade sheds light on a predominant anti-inflammatory role of v-STEAP4.

In our mouse study, TIARP$^{-/-}$ mice (with knock-out of both TIARP and its variant) have high serum levels of IL-6 (but not TNF-α), were highly susceptible to collagen-induced arthritis and K/B × N serum transfer arthritis, and this was attenuated by an IL-6 antagonist [13,14]. Therefore, TIARP was also thought to be a negative regulator in arthritis – its function being more dependent upon the regulation of IL-6 than TNF-α *in vivo*. We also found the exon 3-spliced variant in glucose-6-phosphate isomerase (GPI)-induced arthritis progressing to severe arthritis (our preliminary observation). The variant of TIARP has the same function in arthritis models and is possibly associated with the suppression of IL-6.

To the best of our knowledge, this is the first report concerning the intracellular function of human v-STEAP4 in comparison with STEAP4. Immunoblot analysis confirmed a clear difference in p-NF-κB and p-STAT-3 in v-STEAP4 over-expressing cells (via LPS stimulation). The fluctuation in TNF-α production was compatible with activation of NF-κB signalling, and IL-6 was compatible with phosphorylation of STAT-3 in v-STEAP4 over-expressing cells. These findings suggest that over-expression of v-STEAP4 influences production of each cytokine through each of their respective signalling pathways.

What is the molecular mechanism on the enhancement of NF-κB and suppression of p-STAT-3? The precise mechanism of these opposing effects of v-STEAP4 for NF-κB and STAT-3 signalling pathways has not yet been

elucidated. Myeloid differentiation primary response 88 (MyD88) and its downstream signalling (TNF receptor-associated factor/TGF-β-activated kinase 1 (TRAF6/TAK1) complex are existed upstream of an IκBα/NF-κB complex as TLR-4 downstream signalling [23]. SOCS3 is one of the intrinsic negative regulators of TLR-4 downstream signalling, and was known as a negative regulator of the phosphorylation of STAT-3 and was also reported as a negative regulator of the TRAF6/TAK1 complex [24–26]. As a hypothesis, we thought v-STEAP4 might regulate the SOCS3 mediated pathway. However, in our study, SOCS3 was neither suppressed nor induced with LPS stimulation in the v-STEAP4 over-expressing cells. Therefore, the enhancement of NF-κB and suppression of STAT-3 phosphorylation by v-STEAP4 was not mediated by SOCS3.

In addition, we confirmed that NF-κB signalling was required for TNF-α production, whereas STAT-3 signalling was required for IL-6 production with LPS stimulation in uninfected THP-1 macrophages by using specific inhibitors such as Bay 11–7082 for NF-κB or S3I-201 for STAT-3. These inhibitors suppressed TNF-α and IL-6 production in mock and the v-STEAP4 over-expressing cells, but not in the STEAP4 over-expressing cells, which also suggests that STEAP4 might possibly regulate other TLR-4 signalling pathways apart from the NF-κB and STAT-3 cascades.

We also analysed other TLR-4-related signalling cascades such as the mitogen-activated protein kinase (MAPK) family (p38, ERK1/2 and JNK) (Supporting information, Fig. S3). STEAP4, but not v-STEAP4, decreased p-ERK1/2 and p-p38. Thus, STEAP4 might have only a partial influence on the MAPK cascade (perhaps localized to TNF-α production), because the fluctuation of cytokine production was not suppressed significantly along with these MAPK family members. Conversely, STEAP4 might increase p-STAT-1, which could be associated with increase of IL-6 production in STEAP4 – especially with a STAT-3-specific inhibitor.

The ligands for STEAP4 and v-STEAP4 are still unknown. STEAP4 was located in endosome in fibroblast by over-expressing cells [16], but their localization was not identified clearly, and probably different between expressing cells (e.g. monocytes, neutrophils, fibroblasts, adipocytes, etc.). In addition, although both molecules exist endogenously, there is no evidence concerning the interaction between STEAP4 and v-STEPA4. For further functional study in the future, their localization, ligands and interaction should be investigated.

In conclusion, our seminal results verify that v-STEAP4 is not only expressed more highly in RA patients, it also demonstrates a functional significance in the ability to specifically control inflammatory cytokine cascades induced by LPS that mimic RA-inflamed conditions. Our study provides new insights into the molecular mechanism of how STEAP4 and its variant control inflammation in human monocytes in RA, and future studies are needed to dissect the precise mechanism for further therapeutic exploitation.

Acknowledgements

We thank the Medical English Communications Center of the University of Tsukuba for the critical reading of the manuscript. H. E., I. M. and T. S. wrote the manuscripts. H. E., I. M., A. I. and T. S. designed the study. H. E. performed the experiments and coordinated statistical study. H. K., I. K., Y. T., Y. K. and H. T. participated in discussion. This work was supported by a Grant-in-Aid for Scientific Research [Grant-in-Aid (C) (grant number: 15K09518)] from the Ministry of Education, Culture, Sports, Science and Technology and Japan Society for the Promotion of Science.

Disclosure

None.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The expression of six-transmembrane epithelial antigen of prostate 4 (STEAP4) rheumatoid arthritis (RA) is not correlated with disease activity score in 28 joints-C-reactive protein (DAS28-CRP), DAS28-erythrocyte sedimentation rate (ESR) and matrix metalloproteinase 3 (MMP3) in RA. (a) Correlation between mRNA expression of STEAP4 or (b) v-STEAP4 and DAS28-CRP, DAS28-ESR ($n = 15$) or MMP3 ($n = 14$), respectively, in RA patients. *P*-values were calculated by Spearman's correlation.

Fig. S2. The inflammatory cytokine productions by lipopolysaccharide (LPS) were suppressed by Toll-like receptor (TLR)-4 downstream signalling specific inhibitors in transfected human acute monocytic leukaemia cell line (THP-1) macrophages. (a) Concentrations of tumour necrosis factor (TNF)-α and interleukin (IL)-6 in the culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) after 24 h with 1 μg/ml or 100 ng/ml of LPS stimulation and in the presence or absence of a specific inhibitor such as Bay 11-7082 for nuclear factor kappa B (NF-κB) or S3I-201 for signal transducer and activator of transcription 3 (STAT-3) from (a) untransfected or (c) transfected THP-1 macrophages after phorbol myristate acetate (PMA) treatment. Values are shown as the mean ± standard error of the mean (s.e.m.) of (a) three or (c) four wells per group. $^{*}P < 0{\cdot}05$ according to the Mann–Whitney *U*-test. (b) Levels of p-NF-κB, total NF-κB, total nuclear factor kappa B inhibitor alpha (IκBα), p-STAT-3 and total STAT-3 in whole cell lysates from THP-1 macrophages with or without 1 μg/ml of LPS stimulation for 15 min in the presence or absence of Bay 11-7082 or S3I-201 were determined by immunoblot analysis ($n = 2$ wells per group).

Fig. S3. The effects of six-transmembrane epithelial antigen of prostate 4 (STEAP4) rheumatoid arthritis (RA) and v-STEAP4 on phosphorylation of mitogen-activated protein kinase (MAPK) family [p38, extracellular regulated kinase (ERK)1/2 and c-Jun N-terminal kinases (JNK)]. P-p38, total p38, p-ERK1/2, total ERK1/2, p-JNK and total JNK in whole cell lysates from the transfected transfected human acute monocytic leukaemia cell line (THP-1) macrophages with 1 μg/ml of lipopolysaccharide (LPS) stimulation for 0, 15, 30 or 60 min were determined by immunoblot analysis ($n = 2$ wells per group).