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Citrullinated inter-alpha-trypsin inhibitor heavy chain 4 in arthritic joints and its potential effect in the neutrophil migration

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Summary

The citrullinated inter-alpha-trypsin inhibitor heavy chain 4 (cit-ITIH4) was identified as its blood level was associated with the arthritis score in peptide glucose-6-phosphate-isomerase-induced arthritis (pGIA) mice and the disease activity in patients with rheumatoid arthritis (RA). This study aimed to clarify its citrullination pathway and function as related to neutrophils. In pGIA-afflicted joints, ITIH4 and cit-ITIH4 levels were examined by immunohistochemistry (IHC), immunoprecipitation (IP) and Western blotting (WB), while peptidylarginine deiminase (PAD) expression was measured by reverse transcription–quantitative polymerase chain reaction (RT–qPCR), IHC and immunofluorescent methods. The pGIA mice received anti-lymphocyte antigen 6 complex locus G6D (Ly6G) antibodies to deplete neutrophils and the expression of cit-ITIH4 was investigated by WB. The amounts of ITIH4 and cit-ITIH4 in synovial fluid (SF) from RA and osteoarthritis (OA) patients were examined by I.P. and W.B. Recombinant ITIH4 and cit-ITIH4 were incubated with sera from healthy volunteers before its chemotactic ability and C5a level were evaluated using Boyden's chamber assay and enzyme-linked immunosorbent assay (ELISA). During peak arthritic phase, ITIH4 and cit-ITIH4 were increased in joints while PAD4 was over-expressed, especially in the infiltrating neutrophils of pGIA mice. Levels of cit-ITIH4 in plasma and joints significantly decreased upon neutrophil depletion. ITIH4 was specifically citrullinated in SF from RA patients compared with OA patients. Native ITIH4 inhibited neutrophilic migration and decreased C5a levels, while cit-ITIH4 increased its migration and C5a levels significantly. Cit-ITIH4 is generated mainly in inflamed joints by neutrophils via PAD4. Citrullination of ITIH4 may change its function to up-regulate neutrophilic migration by activating the complement cascade, exacerbating arthritis.

Keywords: citrullinated proteins, inter-alpha-trypsin inhibitor heavy chain 4, peptidylarginine deiminase 4, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation in synovial joints. Several factors have been reported in the pathogenesis of RA, including environmental, genetic and hormonal factors [1,2]. One trademark of the immune response in RA is the production of anti-citrullinated protein antibodies (ACPAs) [3], which can be present several years prior to the onset of the disease [4] and are stimulated by numerous citrullinated proteins in the synovial tissue of RA patients that act as sources of autoantigens [5].

This citrullination is catalyzed by peptidylarginine deiminases (PADs), of which there are at least four related isozymes in mammals, with each isotype highly conserved among mammals (70–95% homology) [6]. In the immune system, PAD2 and 4 are expressed with PAD4 expression mainly restricted to neutrophils and monocytes, while PAD2 is ubiquitously expressed in systemic tissue other

than immune cells [6]. Unlike other isozymes, PAD4 uniquely regulates cell growth, gene translation and neutrophil extracellular trap (NETs) formation by the citrullination of transcriptional factors or nuclear histones [7–9]. Polymorphisms of PAD4 that increase PADI4-mRNA stability have thus been identified as a major risk factor for RA pathogenesis, as they lead to the over-abundance of citrullinated proteins [10].

Neutrophils comprise more than 90% of the infiltrating cell population in synovial fluid from patients with RA [11], are also abundant in the pannus-cartilage junction [11], participate in the destruction of cartilage by releasing tumor necrosis factor (TNF)-α [12] and stimulate synoviocytes and osteoclasts in RA [13,14]. Indeed, past studies in murine models revealed that neutrophil depletion by anti-granulocyte receptor-1 (Gr-1) antibody administration successfully inhibited the development of arthritis [15]. However, few studies have compared PAD expression in neutrophil populations within joints and other tissues, and reductions in protein citrullination by neutrophil depletion have not yet been demonstrated *in vivo*.

Glucose-6-phosphate isomerase (GPI) is a ubiquitous glycolytic enzyme and was identified as a pathogenic autoantigen in K/B×N arthritic mice [16]. Immunization of a specific peptide which is a major $CD4^+$ T cell epitope of GPI could induce arthritis in DBA/1 mice (peptide GPI-induced arthritis; pGIA) [17]. $CD4^+$ T cells play a critical role in GPI-induced arthritis, and the effect of biological agents is similar to RA patients [18,19]. This model overlaps some aspects of phenomena that were seen in patients with RA, especially the early induction phase. However, their transient arthritis was fundamentally different from RA.

Inter-alpha-trypsin-inhibitor heavy chain 4 (ITIH4) is a protein of 120 kD, first discovered in murine and human plasma, named after its homology to other ITIH family proteins [20,21]. It is mainly synthesized within and secreted from the liver and its expression is up-regulated due to acute inflammation [22,23]. In humans, the suppressive effect of phagocytosis in neutrophils has been reported [24], but details of the mechanism are scarce. We previously reported a specific increase of the citrullinated form of ITIH4 (cit-ITIH4) in the blood from peptide glucose-6-phosphate isomerase-induced arthritis (pGIA) mice and RA patients by mass spectrometry analysis. The blood levels of cit-ITIH4 fluctuated according to arthritis scores in pGIA mice and disease activity in RA patients, while antibodies to the cit-ITIH4 epitope were specifically observed in RA patients [25]. However, the mechanism of ITIH4 citrullination and its role in arthritis has not been clarified.

Here, we investigated the expression of cit-ITIH4 in pGIA mice and RA patients, mainly focusing on inflamed joints, and further investigated the expression of PADs. We found an increased expression of cit-ITIH4 and overexpression of PAD4 in neutrophils, specifically in joints. In addition, we observed a specific role of cit-ITIH4 in neutrophil migration.

Materials and methods

Mice

Male DBA/1 mice were purchased from Charles River Japan (Tokyo, Japan), and used at 6–10 weeks of age. All mice were maintained under specific pathogen-free conditions at the University of Tsukuba. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba (reference number: 19-416) and all animal experiments were conducted in accordance with institutional ethics guidelines. All surgeries were performed under isoflurane anesthesia and utmost care was taken to minimize suffering. Mice were maintained in a standard specific pathogen-free facility at University of Tsukuba. Mice were housed with three to seven mice per cage and had access to water and food *ad libitum*.

Human sampling

Blood samples (n = 65, mean age = 52·2 years, range = 20–73 years, females = 81·5%) and synovial fluid (n = 7, mean age = 53·6 years, range = 32–72 years, females = 85·7%) were collected from Japanese patients with RA diagnosed by rheumatologists according to the 1987 American College of Rheumatology (ACR) classification criteria [26] or the 2010 ACR/European League Against Rheumatism (EULAR) classification criteria [27]. Control samples of blood and synovial fluid were collected from Japanese patients with osteoarthritis (OA) (n = 6, mean age = 60·3 years, range = 56–79 years, females = 83·3%). Blood samples were collected from healthy volunteers (HV) (n = 25, mean age = 48·7 years, range = 34–65 years, females = 80%). All samples were collected at the University of Tsukuba Hospital after informed consent was obtained. This study was reviewed and approved by the ethics committee of the University of Tsukuba (the reference number: H29-041).

Peptide GPI-induced arthritis

DBA/1 mice were immunized with 25 μg of peptide $GPI_{325–339}$ (pGPI) (Invitrogen, Carlsbad, CA, USA) in complete Freund's adjuvant (CFA) (Becton Dickinson, San Jose, CA, USA). Peptide GPI was either emulsified with CFA at a 1 : 1 ratio (volume/volume) or PBS + CFA was prepared as a vehicle control. For induction of arthritis in mice, 150 μl of the emulsion was injected intradermally at the base of the tail. Each mouse also received an injection of 200 ng of pertussis toxin

(Sigma-Aldrich, St Louis, MO, USA) intraperitoneally on days 0 and 2 after immunization to induce arthritis. Arthritis was assessed every other day and evaluated using a scale of 0–3 for swelling and redness of each paw. The clinical score was the sum of the scores for four paws, as described previously [17].

Immunohistochemistry

Ankle joint tissue samples were harvested at days 0, 7, 14 and 28 from mice immunized with pGPI or control. Samples were fixed in neutralized 10% formalin, embedded in paraffin and sectioned. To detect ITIH4 and PAD4 protein, the sections were incubated with rabbit anti-human ITIH4 antibodies (abcam, Cambridge, MA, USA) diluted 1 : 1000 and rabbit anti-PAD4 antibodies (Proteintech, Rosemont, IL, USA) diluted 1 : 200 as primary antibodies. A rabbit-specific IHC polymer detection kit horseradish peroxidase/3,3'-diaminobenzidine (HRP/DAB) (abcam) and HRP-conjugated goat anti-rabbit immunoglobulin (IgG) (H + L) (Bio-Rad) diluted 1 : 1000 were used as secondary antibodies. Hematoxylin or Giemsa were used as a counterstain.

Immunofluorescence staining

To detect PAD4 and neutrophil elastase (NE), the paraffin-embedded sections of murine joins were incubated for 90 min at room temperature with rabbit anti-PAD4 (Proteintech) diluted 1 : 200 and rat anti-NE antibodies (R&D Systems, Minneapolis, MN, USA) diluted 1 : 800 as primary antibodies. These samples were subsequently incubated with Alexa Fluor 488 anti-rabbit IgG (Invitrogen) or Alexa Fluor 546 anti-rat IgG (Invitrogen) for 1 h at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were analyzed by fluorescence microscopy (BZ-X710; Keyence, Osaka, Japan).

Immunocytochemistry

Cells from bone marrow, peripheral blood and joints from pGIA mice at day 14 were isolated and mounted on glass slides by Smear Gell (Genostaff, Toyko, Japan) before fixation in neutralized 10% formalin and specimens were preserved in 70% ethanol at 4°C. Ankle joints were washed with Hanks's balanced salt solution (HBSS for collecting cells. For immunofluorescence staining, specimens were incubated 1 h at room temperature with rabbit anti-PAD4 (Proteintech) at a dilution of 1 : 200 and with rat anti-Gr-1 antibody (Biolegend, San Diego, CA, USA) at a dilution of 1 : 200. Normal rabbit IgG (Invitrogen) and rat isotype IgG (Biolegend) were used as control staining. These samples were subsequently incubated with Alexa Fluor 488 anti-rabbit IgG (Invitrogen) or Alexa Fluor 647 anti-rat IgG (Invitrogen) for 1 at room temperature. Nuclei were counterstained with DAPI. Images were analyzed by fluorescence microscopy (BZ-X710; Keyence) with exposure time in which control IgG staining were not detected.

Western blot analysis

The murine tissue lysates were prepared by homogenization in modified radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0·5% sodium deoxycholate, 20 mM ethylenediamine tetraacetic acid (EDTA) and protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA)]. Proteins were separated by sodium dodecyl sulfate–polymerase chain gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. For Western blot of anti-modified citrulline (AMC) antibodies or anti-ITIH4 antibodies, 50- or 10-μg proteins were loaded per well, respectively. We prepared a modification buffer by mixing reagent A (20% H_2SO_4, 25% H_3PO_4 and 0·025% $FeCl_3$) and reagent B (1% diacetyl monoxime, 0·5% antipyrine, 1 M acetic acid) at a 2 : 1 ratio (volume/volume). This buffer was added to the blots before incubation in a light-proof container at 37°C for 2·5 h in order to modify citrulline residues. Blots were then washed with 0·05% Tween-20 in TBS (TBST), blocked with 5% milk in TBST and incubated overnight at 4°C with AMC antibodies (provided by the Tokyo Metropolitan Institute of Gerontology) diluted 1 : 3200 in 5% milk in TBST [28]. After washing, the blots were incubated with secondary antibodies consisting of HRP-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad, Hercules, CA, USA) diluted 1 : 5000 in 5% milk in TBST for 1 h at room temperature. For analysis of ITIH4 expression, Western blotting was performed as described above using rabbit anti-ITIH4 antibodies (abcam) diluted 1 : 3000. For immunoprecipitation, murine joint lysate and human synovial fluid were incubated with murine anti-ITIH4 antibody (provided by Showa University, Tokyo for human) or rabbit anti-ITIH4 antibody (Proteintech for mouse) overnight at 4°C and then incubated with Protein G agarose (Cell Signaling Technology) for 4 h at 4°C. Densitometric analysis was carried out using an ImageQuant LAS-4000 densitometer (GE Healthcare, Chicago, IL, USA) with band intensity determined by ImageQuant TL software (GE Healthcare) or Image J software (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription–quantitative polymerase chain reaction (PCR) analysis

We extracted total RNA from the ankle joints of pGIA or control mice using the Isogen (Nippon Gene, Tokyo,

Japan) extraction method according to the instructions provided by the manufacturer. The extracted RNA was reverse-transcribed to cDNA with random primers. We performed real-time–quantitative polymerase chain reaction (qPCR) using a TaqMan (Applied Biosystems, Carlsbad, CA, US) and SYBR Green (Takara Bio, Shiga, Japan) gene expression assay and Padi2 (Mm01341648_m1), PADI4 (Mm01341658_m1), ITIH4 (MA115999) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1, MA162228) primers. Real-time quantitative PCR was carried out using an ABI 7500 analyzer (Applied Biosystems). GAPDH expression of was used as the control.

Depletion of murine neutrophils *in vivo*

Mice were injected intraperitoneally with isotype control IgG or anti-lymphocyte antigen 6 complex locus G6D (Ly6G) mAb (1A8; Biolegend) on day 9 (1 mg), day 10 (2 mg) and day 12 (2 mg) after induction of arthritis. Two groups of four mice each were randomly assigned. Total white blood cells were counted with an automatic hemocytometer (Celltac α MEK6458; Nihon Koden, Tokyo, Japan), and the neutrophilic fraction was measured by fluorescence activated cell sorter (FACS)Verse (BD Biosciences) as $Gr1^{high}$ $CD11b^{+}$ after gating for $CD45^{+}$7-$AAD^{-}CD115^{-}$cells. Similarly, anti-Gr1 monoclonal antibodies (mAb) (RB6-8C5; Biolegend) was used for neutrophils depletion on day 7 (100 μg), day 9 (250 μg), day 11 (500 μg) and day 13 (500 μg).

Recombinant proteins

Recombinant full-length human fibrinogen and ITIH4 were provided by Fujirebio Inc. (Shanghai, China). An Expi293 Expression System (Gibco, Carlsbad, CA, USA) was used for recombinant proteins. Briefly, Expi 293F cells were transfected with the plasmid containing a hexa-His-tagged cDNA clone of *Homo sapiens* inter-alpha-trypsin inhibitor heavy chain and fibrinogen (α,β,γ). The transient transfected culture was then used for recombinant protein purification. The transfected cells were cultured according to the instructions supplied by the manufacturer and media were collected after 72 h of incubation. Protein purification was achieved by HisTrap HP (GE Healthcare) and purity was assayed by SDS-PAGE and WB. Citrullination was performed with rabbit skeletal muscle PAD (Sigma-Aldrich) with an enzyme–substrate molar ratio of 1: 50 in 0·1 mol/l Tris–HCl (pH 7.4), 3 mmol/l CaCl2 and 1 mmol/l dithiothreitol (DTT) for 1·5 h at 37°C. Buffers were changed to 0·1 M Tris–HCl with Amicon Ultra Centrifugal Filters (Merck, Kenilworth, NJ, USA; molecular weight cut-off (MWC) = 50 kD including PAD enzymes alone.

Neutrophil migration assay

Blood from healthy volunteers was drawn from the antecubital vein and neutrophils were isolated by density gradient centrifugation over Polymorphoprep (Abbott Diagnostics Technologies AS, Tokyo, Japan). Cells were stained with Diff-Quick (Sysmex, Kobe, Japan) to confirm that the purity of PMNs was greater than 90%. Neutrophil migration assays were performed in 96-well Transwell plates (3-μm pore size; Corning, New York, NY, USA). Purified human neutrophils were labeled with Hoechst 33342 for 10 min. After washing, 8×10^4 neutrophils in HBSS-0·1% bovine serum albumin (BSA) were added to the upper chamber and pooled, pooled normal human serum (diluted to 6% in 0·1MTris–HCl) or 0·1 MTris–HCl alone (control) was incubated with each protein or PAD enzymes alone for 30 min at 37°C, then diluted to 1 : 3 in HBSS-0·1% BSA and added to the lower chamber. The effect of PAD enzymes alone was evaluated by mixing in maximum estimated concentrations in the protein solutions. The migration assay was performed in a 5% CO_2 incubator for 45 min at 37°C. The migrated neutrophils were counted by fluorescence microscopy (BZ-X710; Keyence).

Enzyme-linked immunosorbent assay (ELISA)

Human ITIH4 levels in plasma and synovial fluid were measured using an enzyme-linked immunosorbent assay (ELISA) kit by R&D Systems while human C5a and C3a levels in incubated sera were measured with an ELISA kit by abcam and RayBiotech (Norcross, GA, UAS), according to the instructions supplied by the manufacturers, respectively.

Statistical analysis

All data are expressed as mean ± standard error of the mean (s.e.m.). Differences between groups were evaluated for statistical significance using the Mann–Whitney *U*-test and ordinary one-way analysis of variance (anova) with a *post-hoc* Tukey's test for multiple comparisons. *P*-values less than 0·05 were considered significant. Statistical analyses were performed using GraphPad Prism version 8.4.2.

Results

The increased amount of ITIH4 and its citrullination in pGIA joints

To clarify the mechanism of ITIH4 citrullination, we used a murine pGIA arthritis model in which DBA/1 mice were immunized with pGPI to induce polyarthritis. Ankle joint sections were then stained with anti-ITIH4 antibodies

by immunohistochemistry in both pGIA and control mice. ITIH4 was increased only in pGIA mice at day 14 (peak arthritic phase) and vanished on day 28 (post-arthritic phase) (Fig. 1a). There was no significant difference in ITIH4 mRNA expression in joints between pGIA and control mice at day 14 (Supporting information, Fig. S1). Joint lysate from pGIA mice in the arthritic phase was then assessed by immunoprecipitation followed by Western blot, which revealed the citrullination of ITIH4 (Fig. 1b). Compared to the tissue lysates and plasma from pGIA mice at day 14, the cit-ITIH4 levels were significantly increased in joints (Fig. 1c), suggesting that citrullination of ITIH4 mainly occurred in inflamed joints.

A possible contribution of PAD4 from neutrophils in the citrullination of ITIH4

We next investigated the expression of *Padi2* and *Padi4* mRNA in joints from pGIA mice by qPCR. At day 14, *Padi4* mRNA was significantly increased in pGIA joints compared with control mice (Fig. 2a). Immunohistochemistry with PAD4 antibodies revealed its expression at the protein level and morphological analysis revealed that 90·4 ± 0·25% of PAD4 expressing cells were neutrophils (Fig. 2b, Supporting information, Fig. S2). PAD4 protein also observed extracellularly around neutrophils, and ITIH4 existed in the close place in serial sections, indicative of ITIH4 citrullination via neutrophil-generated PAD4 (Fig. 2b). We then isolated infiltrating cells from pGIA joints at day 14, and immunocytochemistry assays using anti-Gr-1 antibodies were performed. We observed that 90·2 ± 1·4% of PAD4-positive cells were Gr-1-positive, suggesting that neutrophils are the main source of PAD4 in pGIA joints (Fig. 2c). Additionally, immunofluorescence staining in joints revealed that most of PAD4-expressing cells were co-stained with neutrophil elastase (Supporting information, Fig. S3)

Significant PAD4 expression in neutrophils localized to pGIA joints

To evaluate the difference of PAD4 expression in neutrophils localized to the joints, isolated cells from pGIA joints were compared with bone marrow and peripheral blood cells. These cells were examined by immunohistochemistry using anti-PAD4 and Gr-1 antibodies. Remarkably, PAD4 expression in Gr-1-positive cells derived from arthritic joints was significantly higher than other groups, both in positive ratio and intensity (Fig. 3a,b).

Neutrophil depletion attenuated the level of citrullinated protein in blood and joints of pGIA mice

To further investigate the association between neutrophils and citrullination, we attempted to deplete neutrophils in pGIA mice by intraperitoneal administration of anti-Ly6G antibodies. Anti-Ly6G antibodies decreased peripheral neutrophil counts and reduced the severity of arthritis compared with isotype controls (Fig. 4a,b). Histochemical examination revealed that the depletion of neutrophils in joints result in a marked decrease of local expression of PAD4 proteins (Fig. 4c). In addition, the expression of citrullinated proteins, including cit-ITIH4, was significantly attenuated by administration of anti-Ly6G antibodies in plasma and joints (Fig. 4d,e). Similar results were observed in neutrophils depletion using anti-Gr-1 antibodies (Supporting information, Fig. S4). These results indicate that neutrophils mediate the development of arthritis as well as the citrullination of proteins in pGIA mice.

Specific increase of citrullinated ITIH4 in synovial fluid from RA patients

To evaluate the association of citrullinated proteins in human joints, synovial fluid was subjected to Western blotting using AMC antibodies. We observed several citrullinated proteins, including 120 kD bands corresponding to the molecular weight of ITIH4, only in RA patients (Fig. 5a). Next, synovial fluid was analyzed via immunoprecipitation followed by Western blotting. ITIH4 precipitated in both OA and RA groups, but its citrullination was observed only in RA patients (Fig. 5b). We then compared the expression of the specific 120 kD citrullinated protein in plasma and synovial fluid from each patient by Western blotting. Among RA patients, the band intensities were significantly higher in synovial fluid than in plasma (Fig. 5c), suggesting that citrullination of ITIH4 mainly occurred in inflamed joints. ELISA revealed that total ITIH4 levels in plasma did not significantly differ among HV, OA and RA patients (Supporting information, Fig. S3a). In addition, ITIH4 levels in synovial fluid from RA patients were significantly higher than OA patients (Supporting information, Fig. S3b). The comparison of ITIH4 levels in each RA patient between in synovial fluid and plasma are shown in Supporting information, Fig. S3c.

Citrullinated ITIH4 with serum specifically induced neutrophil migration

Hitherto, the association of cit-ITIH4 and arthritis has not been investigated, but ITIH4 has a reported regulatory function on neutrophil phagocytosis [24]. We thus focused on the possible role of native and cit-ITIH4 in neutrophil migration. Recombinant human fibrinogen (control) and ITIH4 were incubated with or without rabbit skeletal PAD. Production of recombinant proteins and their citrullination are shown in Supporting information, Fig. S4. Briefly, control buffer or pooled normal human serum was incubated with each protein and added to the lower chamber of a Transwell plate. Neutrophils were isolated from the peripheral blood of

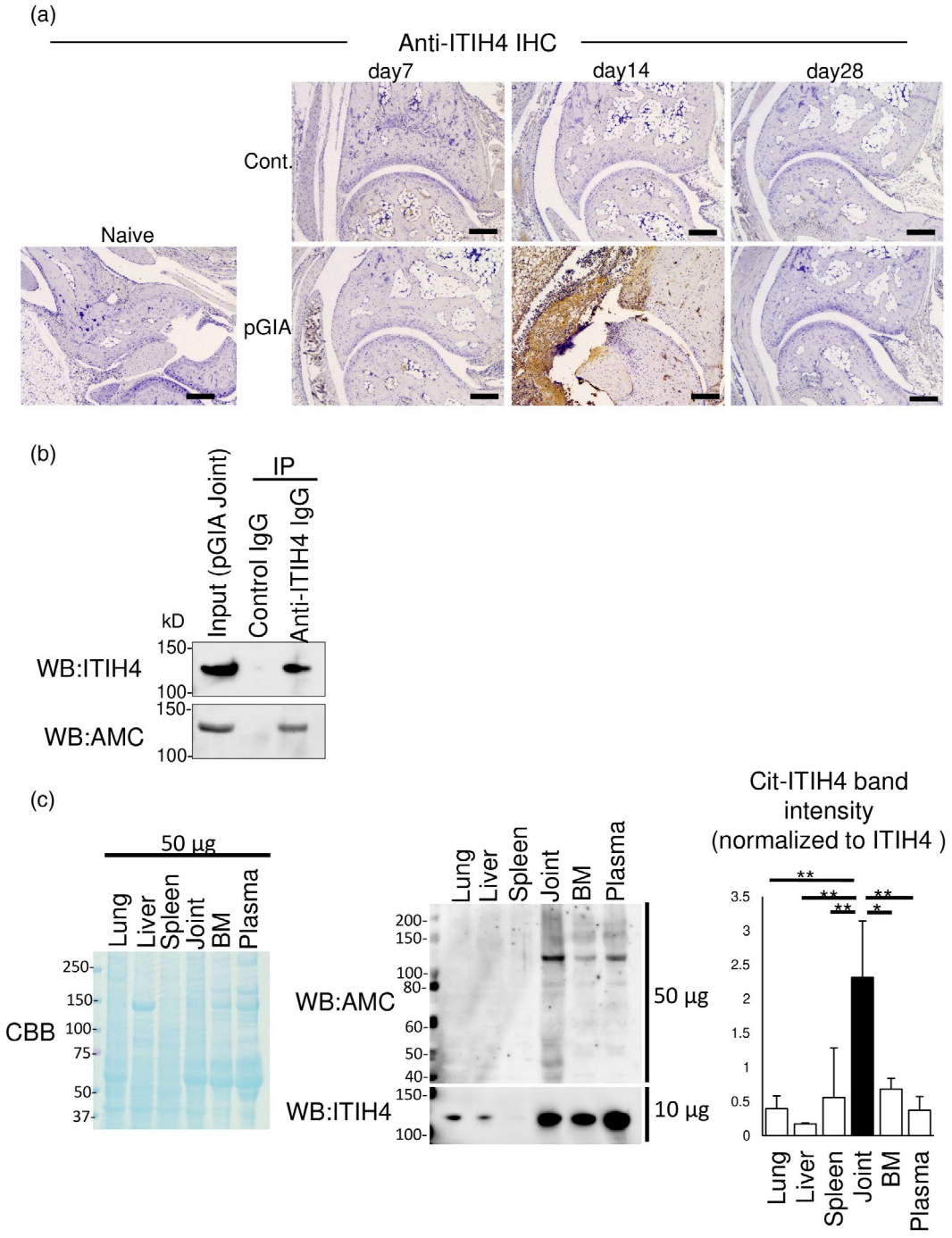

Fig. 1. The increase of inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) and citrullinated ITIH4 in arthritic joints of peptide glucose-6-phosphate isomerase-induced arthritis (pGIA) mice. (a) Joint sections were immunohistochemically stained with ITIH4 antibodies in pGIA or control mice. Scale bars: 200 µm. The experiments were performed on three different samples with similar results. Hematoxylin was used as a counterstain. (b) Joint lysate from pGIA mice at day 14 was subjected to immunoprecipitation using ITIH4 antibodies followed by Western blot analysis using ITIH4 and anti-modified citrulline antibodies (AMC) antibodies. (c) The tissue lysates [lung, liver, spleen, bone marrow (BM) and joint] and plasma from pGIA mice were analyzed by Western blot using AMC and ITIH4 antibodies independently. The amounts of loaded protein were labeled. Three mice were examined and *P*-values calculated using ordinary one-way analysis of variance (ANOVA) with a *post-hoc* Tukey's test for multiple comparisons; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

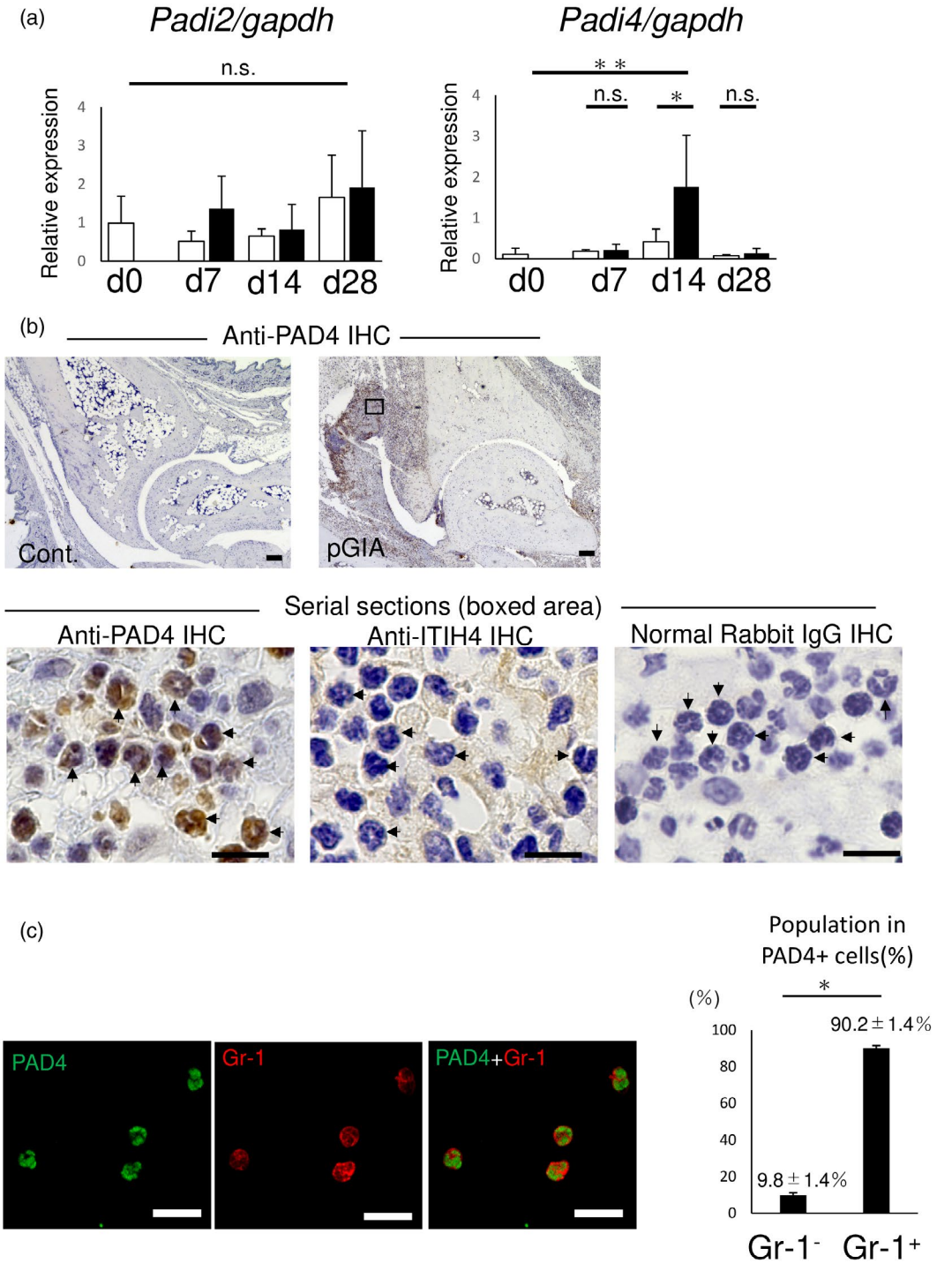

Fig. 2. Expression of peptidylarginine deiminase (PAD) gene and protein in neutrophils in arthritic joints of peptide glucose-6-phosphate isomerase-induced arthritis (pGIA) mice. (a) *Padi2* and *Padi4* mRNA levels in joints from control and pGIA mice. Data indicate means ± standard error of the mean (s.e.m.). *P*-values calculated using ordinary one-way analysis of variance (ANOVA) with a *post-hoc* Tukey's test for multiple comparisons. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$; $n = 6$ (d0), $n = 3$ (d7–28), per group. (b) Upper row: immunohistochemistry with PAD4 antibodies in ankle joints of control and pGIA mice at day 14. Scale bars: 50 μm. Lower row: serial joint sections (4 μm) of boxed area, stained with anti-PAD4, anti-inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) and control antibodies. Scale bars: 10 μm. Hematoxylin was used as a counterstain. These experiments were performed on three different samples with similar results. Arrows indicated neutrophils. (c) Immunocytochemistry using anti PAD4 and granulocyte receptor-1 (Gr-1) antibodies; three hundred cells were counted. Three mice were examined and *P*-values calculated using Mann–Whitney *U*-test; $^{*}P < 0{\cdot}05$. Scale bars: 20 μm.

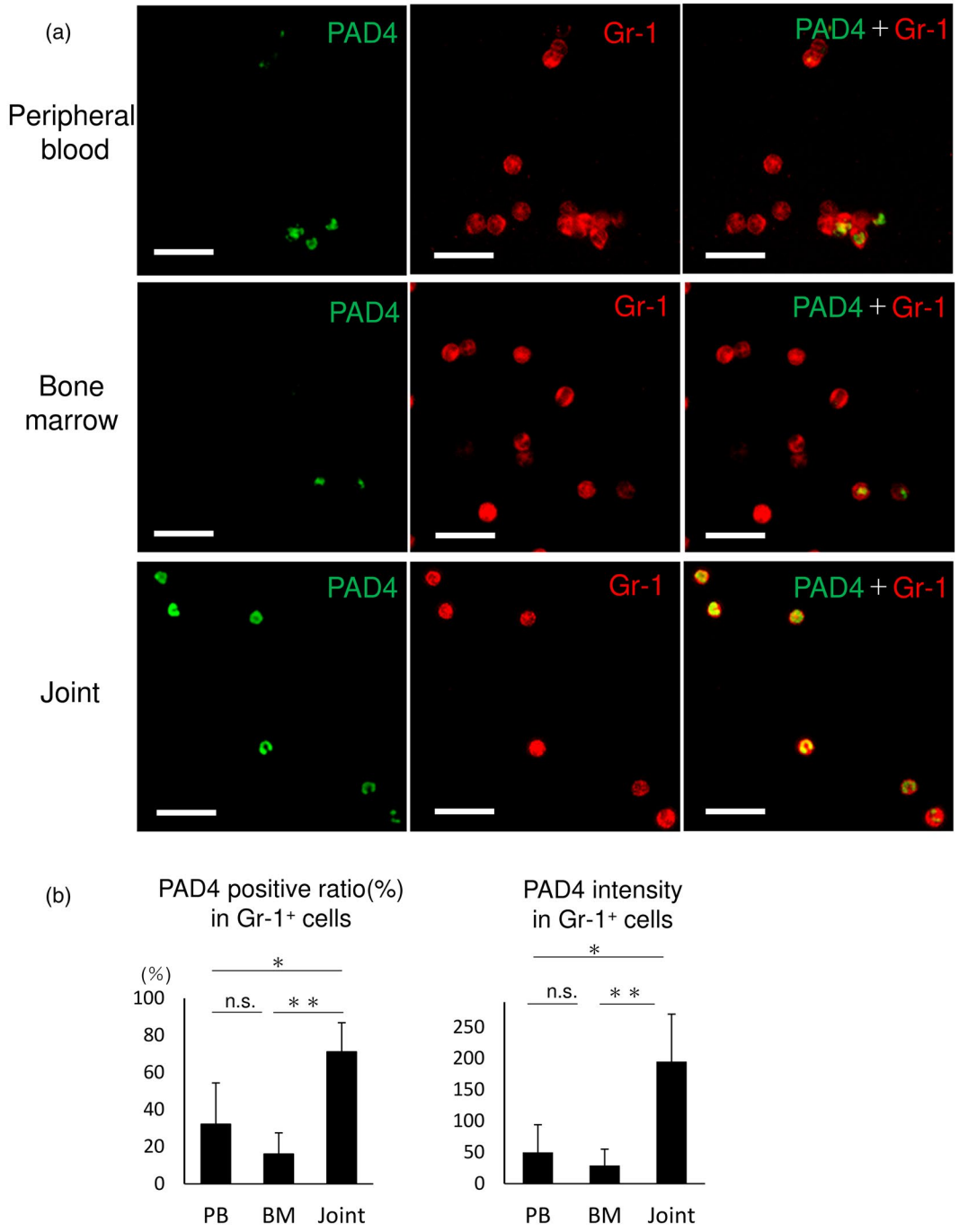

Fig. 3. PAD4 expression in peripheral blood, bone marrow and joints. (a) Immunocytochemistry using anti-PAD4 and granulocyte receptor-1 (Gr-1) antibodies in isolated cells from peripheral blood, bone marrow and joints of peptide glucose-6-phosphate isomerase-induced arthritis (pGIA) mice at day 14. (b) Positive ratio and intensity of PAD4 staining in Gr-1-positive cells. Data indicate means ± standard error of the mean (s.e.m.). Seven mice were examined and *P*-values calculated using ordinary one-way analysis of variance (ANOVA) with a *post-hoc* Tukey's test for multiple comparisons; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

healthy volunteers by density gradient centrifugation, and 8×10^4 neutrophils were added to the upper chamber (experimental schema in Fig. 6a). Interestingly, native ITIH4 with serum significantly attenuated the migration of neutrophils compared with native or citrullinated fibrinogen while the cit-ITIH4 with serum induced a significant migration (Fig. 6b). This finding suggests that ITIH4 might interact with other circulating proteins and

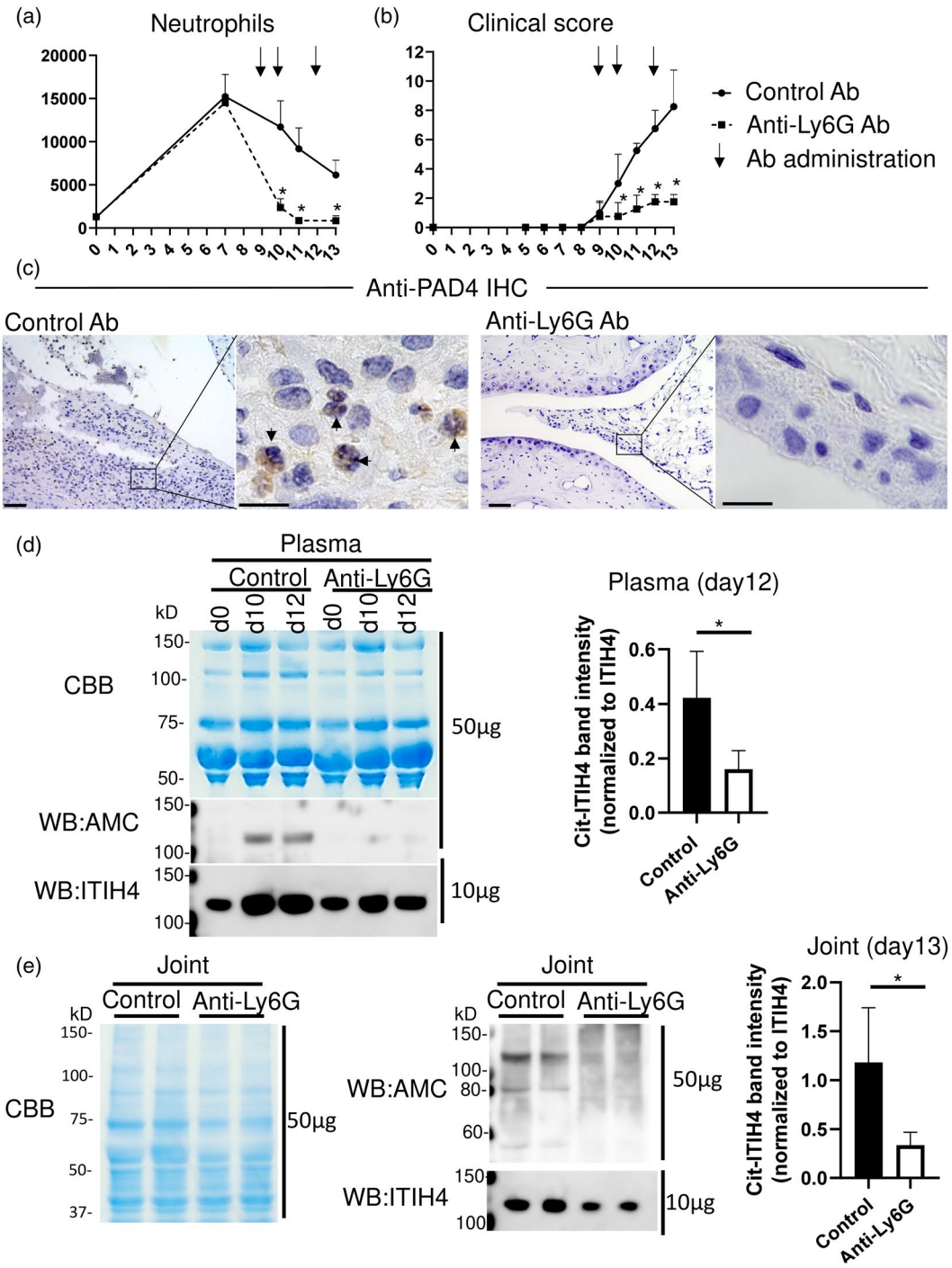

Fig. 4. Reduced peptide glucose-6-phosphate isomerase-induced arthritis (pGIA) and citrullinated protein expression in neutrophil-depleted mice. (a,b) Neutrophil counts in peripheral blood (a) and clinical score (b) in pGIA mice treated with control antibodies ($n = 4$, solid line) and anti-lymphocyte antigen 6 complex locus G6D (Ly6G) antibodies ($n = 4$, dash line). (c) Immunohistochemistry with PAD4 antibodies in ankle joint sections on day 13. The second or fourth column image is a magnified view of the boxed area in the first or third image, respectively. Arrows indicated neutrophils. Hematoxylin was used as a counterstain. Scale bars, first and third image: 50 μm, second image: 20 μm. (d,e) Expression of citrullinated proteins in plasma (d) and joints (e) were analyzed by Western blot using anti-modified citrulline antibodies (AMC) antibodies and anti-inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) antibodies. Western blot with ITIH4 antibody indicated total ITIH4. Joints from two mice of each treatment group were loaded as representative. Band intensities on days 12 (plasma) or 13 (joints) were compared in each group. The amounts of loaded protein were labeled. *P*-values calculated by Mann–Whitney *U*-test; $^*P < 0{\cdot}05$.

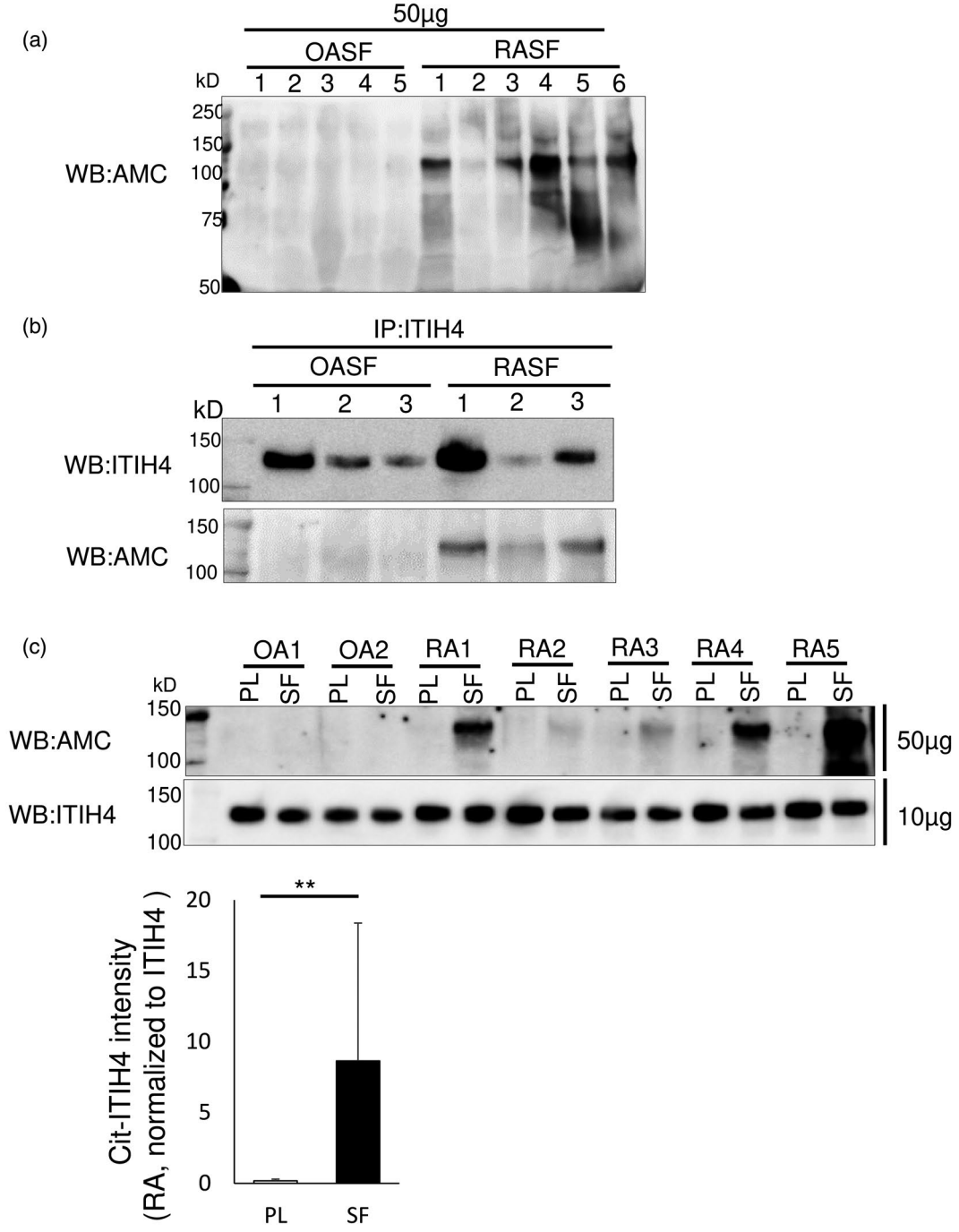

Fig. 5. Citrullinated inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) in the synovial fluid of rheumatoid arthritis (RA) patients. (a) Synovial fluid (SF) from RA ($n = 6$) and osteoarthritis (OA) ($n = 5$) patients was analyzed by Western blot using anti-modified citrulline antibodies (AMC) antibodies. (b) Immunoprecipitation of SF from RA ($n = 3$) and OA ($n = 3$) patients with anti-ITIH4 antibodies followed by Western blot using AMC antibodies. (c) A comparison of citrullinated protein at 120 kD between plasma (PL) and SF via Western blot using AMC and ITIH4 antibodies. Band intensities at 120 kD in RA ($n = 7$) patients were assessed. The amounts of loaded protein were labeled. *P*-values calculated by Mann–Whitney *U*-test; $^*P < 0{\cdot}05$.

contribute to the migration of neutrophils. After referring to a previous study in other ITIH family proteins [29], we focused on the complement system and measured the level of C5a, which is the major product of the cascade and acts as a chemotactic factor for neutrophils. As we expected, the concentration of C5a

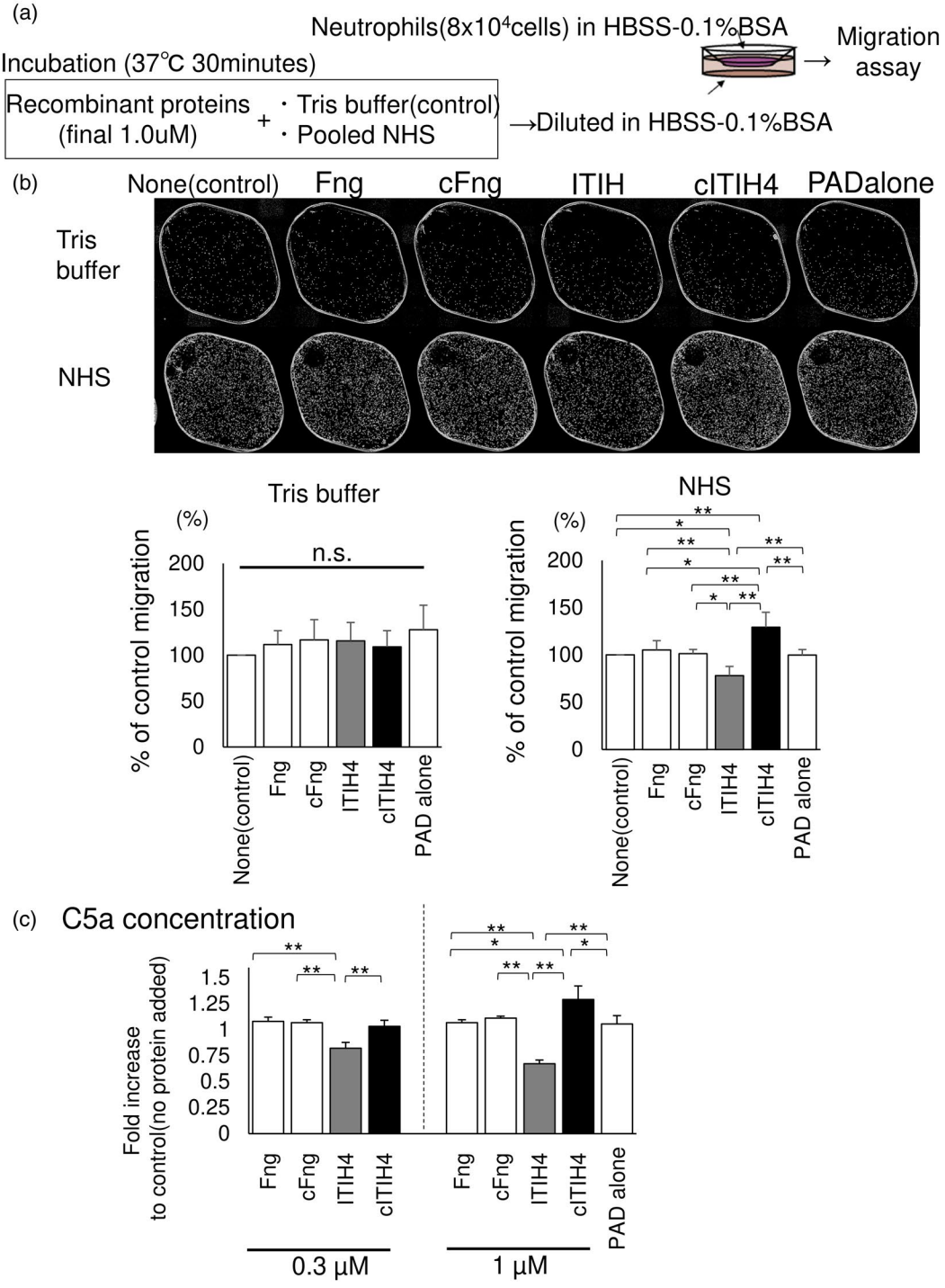

Fig. 6. The increased neutrophil migration in sera incubated with citrullinated inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4). (a) Experimental scheme. Pooled normal human sera (n = 5) or 0·1 M Tris–HCl (control) were incubated with each protein. The effect of PAD enzymes alone was evaluated by adding maximum estimated concentrations in protein solutions. (b) Migrated cells in control and incubated sera. The representative results of migration are displayed at the top. The number of migrated neutrophils is shown as a relative percentage compared to chambers without proteins. These results are representative of four independent experiments. (c) C5a concentration was analyzed in incubated sera with or without recombinant proteins (0·3, 1 μM). Values are shown as fold change *versus* control sample (no protein added). These results are representative of three independent experiments. *P*-values calculated using ordinary one-way analysis of variance (ANOVA) with a *post-hoc* Tukey's test for multiple comparisons compared to chambers without proteins; $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$.

was significantly decreased in sera with native ITIH4, suggesting the possibility of complement cascade inhibition *in vitro*, whereas cit-ITIH4 activated the cascade (Fig. 6c). The level of C3a was also decreased in sera with native ITIH4, which possibly indicated its inhibitory potential at the early stages of the cascade before the cleavage of C3 (Supporting information, Fig. S7)

Discussion

In the present study, we present clear evidence of the increased expression of cit-ITIH4 both in joints from pGIA mice and RA patients. Neutrophils with PAD4 in joints might contribute to this citrullination. This was shown by decreases in ITIH4 citrullination upon depletion of neutrophils by anti-Ly6G antibodies. In addition, we demonstrated that cit-ITIH4 enhances the migration of human neutrophils, supporting an association between cit-ITIH4 and the generation of arthritis.

In our study, we speculated that the over-expression of PAD4 enzyme in infiltrating neutrophils from pGIA mice would induce the citrullination of ITIH4 in joints. Considering the dominant distribution of cit-ITIH4 in arthritic joints both from pGIA mice and RA patients, it was reasonable to assume that cit-ITIH4 chiefly originates in inflamed joints and acts as a serum-specific serum biomarker of arthritis, as we have previously reported [25]. Additionally, as with other citrullinated proteins [30], abundant intraarticular cit-ITIH4 could be a major source of autoantigens and stimulate antibody production against ITIH4, as we have previously reported [25].

Concerning the up-regulation of PAD4 in joint neutrophils we propose three mechanisms, the first of which is NETs-dependent PAD4 production. During the NETs formation process, PAD4 is over-expressed both in murine and human neutrophils, then followed by histone citrullination and nuclear chromatin decondensation [31–33]. Several reports suggest NETs formation in joints during murine arthritis and RA [34,35], and it was reported that TNF-α, interleukin (IL)-17A, rheumatoid factor and ACPA stimulate neutrophils to initiate the process in humans [35]. Indeed, citrullinated histone-3, a common marker for NETs, was detected in joints from pGIA mice at day 14 (our preliminary observations), which supports such a scenario. The second possibility is that IL-6 signaling induced PAD4 production, as Yahagi *et al.* [36] reported that IL-6 stimulation to neutrophils could induce PAD4 production in mice independently from the NETs pathway. The third possibility is immune-mediated pore-forming pathways, since Romero *et al.* [37] revealed that perforin and the membrane attack complex activates PAD4 in neutrophils and hypercitrullination in the joints of RA patients. Further examinations of NETs, IL-6 and perforin production are, thus, necessary to elucidate the pathway of PAD4 generation in joint-localizing neutrophils. In addition, it was recently reported that PAD4 on the surfaces of neutrophils could increase by inflammatory stimuli, which could relate to extracellular citrullination of ITIH4 protein [38].

Several reports confirmed the effectiveness of neutrophil depletion in murine arthritis models [15,39,40], and the mechanism was speculated to be decreasing in degranulation or proinflammatory cytokines, such as TNF-α, IL-1 or IL-6 [40]. Here, our novel data indicate that the depletion of neutrophils in our murine model attenuated the expression of citrullinated proteins in blood and joints, indicating the importance of neutrophils in promoting citrullination. However, as we could not rule out the possibility that the attenuation of citrullination was mediated by diminishing arthritis, studies that deplete macrophages or other effector cells in this model may provide clearer evidence. Additionally, further studies are needed to determine if this approach can ameliorate ACPA production by reducing citrullinated autoantigens in RA patients.

Regarding the increase of ITIH4 in arthritic joints, we propose two possible mechanisms. The first probability is that local production of ITIH4 occurs in arthritic joints *in situ*. However, elevated ITIH4-mRNA levels in joints from pGIA mice were not observed. The second probability is that ITIH4 diffused into the joints via a concentration gradient from plasma where ITIH4 naturally exists. Concentrations of ITIH4 in plasma were higher than in synovial fluid (Supporting information, Fig. S5c), suggestive of an influx into joints rather than local production. In addition, previous reports showed other human ITIH family proteins bound on the surface of human neutrophils [41]. It is therefore possible that ITIH4 tended to remain in inflamed joints containing populations of infiltrating neutrophils.

Choi-Miura *et al.* reported that ITIH4 suppressed a phagocytosis activity of neutrophils [24]. This effect was assumed to occur by the binding of ITIH4 to the cell surface actin of neutrophils [24]. There are no reports of ITIH4 protein on neutrophil adhesion and migration, but other ITIH family proteins have been reported to suppress its adhesion and the release of reactive oxygen species [41]. In our observation, ITIH4 or cit-ITIH4 protein itself was not chemotactic because ITIH4 or cit-ITIH4 protein alone did not induce the migration of neutrophils (Fig. 6b).

In RA patients, it is suggested that neutrophils play a crucial role in initiating the inflammatory response and the progression of the disease, and its migration to the joint is an essential initial step [42]. The assumption that cit-ITIH4 activates complement proteins may be a potential mechanism for the induction of neutrophil migration we observed. Okroj *et al.* [29] reported that other human ITIH family proteins bind to multiple complement proteins (C1q, C2,C3,C4,C5, properdin and factor D) to inhibit cascading. Similarly, the suppressive effects of native ITIH4

in the complement cascade was confirmed after observing the altered function of cit-ITIH4 in this study. After citrullination of a protein, drastic structural and functional changes may occur due to the reduction of its net positive charge [43]. It is thus possible that cit-ITIH4 lost its native binding ability but gained other interactivity with complement proteins or complement regulatory proteins. Experiments using ITIH4-deficient mice may support addressing the precise physiological role of ITIH4. It is unknown whether cit-ITIH4 alone initiates the cascades. However, cit-ITIH4 might have more potent effects on the cascades in joints than in plasma because it is more abundant in inflamed joints.

In conclusion, we have shown the accumulation of cit-ITIH4 in inflamed joints both in pGIA mice and in patients with RA. Neutrophils were observed to play a crucial role in citrullination via PAD4 in pGIA mice, and their cit-ITIH4 product may enhance neutrophilic inflammation by activation of the complement cascade. Regulation of neutrophil migration in light of this citrullination activity in joints could be a new strategy against autoimmune arthritic diseases such as RA.

Acknowledgements

A. Os., I. M. and T. S. designed the study. A. Os, N. M., A. Oh. and I. K. performed experiments and collected the data. Y. K., H. T. and I. M. analyzed the data. A. I. and Y. S. provided antibodies. T. A .and N. I. provided recombinant proteins. A. Os., I. M. and T. S. wrote the manuscript. All authors have read and approved the manuscript for publication. We thank Dr Bryan J. Mathis for the critical reading of the manuscript. This work was supported by a Research Grant from Fujirebio Inc. and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and Japan Society for the Promotion of Science (grant number 18K08403).

Disclosures

The authors declare that they have no competing interests.

Data Availability Statement

Data are available from the corresponding author on reasonable request.

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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 *Itih4* mRNA in joints from pGIA mice. *Itih4* mRNA level in joints from control (N = 5) and pGIA mice (N = 6) at day 14. Data indicate means ± SEM. P value calculated by Mann–Whitney U test.

Fig. S2. PAD4 expressing cells in pGIA joint. Immunocytochemistry using anti-PAD4antibodies in ankle joints pGIA mice at day 14. The second and third column is a magnified view of the boxed area in the first column. Scale bars: 200 μm (first column), 20 μm (second and third column). Giemsa was used as a counterstain. Histological evaluation was performed in a blinded manner by two dependent obsevers. A total of three hundred PAD4-positive cells were counted in at least five different high power fields. P values calculated using ordinary one-way ANOVA with a post hoc Dunnett's test for multiple comparisons. $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$.

Fig. S3. The expression of neutrophil elastase and PAD4 in pGIA joints. Immunofluorescence staining using anti PAD4 and neutrophil elastase (NE) antibodies in ankle joints pGIA mice at day 14. The middle and lower row are magnified views of the boxed area in the upper row. The experiments were performed on three different samples with similar results. Scale bars: 50 μm.

Fig. S4. Neutrophil-depletion by ant Gr-1 antibodies in pGIA mice. (a, b) Neutrophil counts in peripheral blood (a) and arthritis score (b) in pGIA mice treated with control Abs (N = 4, dash line) and anti-Gr1 Abs (N = 4, solid line). (c) Immunohistochemistry with PAD4 antibodies in ankle joint sections on day 13. The image in the second column is a magnified view of the boxed area in the first image. Arrows indicated neutrophils. Hematoxylin was used as a counterstain. Scale bars, first and third image: 50 μm, second image: 20 μm (d,e) Expression of citrullinated proteins in plasma (d) and joints (e) were analyzed by Western blot using AMC antibodies and anti-ITIH4 antibodies. Western blot with ITIH4 antibody indicated total ITIH4. Joints from two mice of each treatment group were loaded as representative. Band intensities on days 12 (plasma) or 13 (joints) were compared in each group. The amounts of loaded protein were labelled. P values calculated by Mann–Whitney U test. $^{*}P < 0{\cdot}05$. The neutrophilic fraction was measured by FACS verse (BD) as $Gr1^{high}$ $CD11b^{+}$ after gating for $CD45^{+}$7-AADcells.

Fig. S5. ITIH4 concentration in plasma and synovial fluid. ITIH4 concentration was analyzed in plasma (HS; n = 25, OA; n = 6, RA; n = 65) (a) and synovial fluid (OA; n = 6, RA; n = 6) (b) by ELISA. ITIH4 concentration was compared in each RA patients between synovial fluid and plasma (c). Each line showed each patient. Data indicate means ± SEM. P values calculated by Mann–Whitney U test and ordinary one-way ANOVA with a post hoc Tukey's test for multiple comparisons. $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$.

Fig. S6. Citrullination of proteins. Recombinant human ITIH4 and fibrinogen (Fng) were citrullinated using rabbit PAD and confirmed by AMC assay.

Fig. S7. The concentration of C3a in sera with native or citrullinated ITIH4. C3a concentration was analyzed in incubated sera with or without recombinant proteins. Values are shown as fold change versus control sample (no protein added). These results are representative of three independent experiments. *P* values calculated using ordinary one-way ANOVA with a post hoc Tukey's test for multiple comparisons compared to chambers without proteins. $^{**}P < 0{\cdot}0.01$, $^{*}P < 0{\cdot}05$.