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Research Article

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Research Article

Role of inter-alpha-trypsin inhibitor heavy chain 4 and its citrullinated form in experimental arthritis murine models

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Abstract

Inter-α-trypsin inhibitor heavy chain 4 (ITIH4) is a major protein in serum and reported to be upregulated at the onset of rheumatoid arthritis (RA). Its citrullinated form, cit-ITIH4, is specifically found in the serum and synovial fluid of patients with RA. However, the detailed function of ITIH4 in arthritis remains unknown. The aim of this study was to clarify the role of ITIH4 and cit-ITIH4 using experimental arthritis models. ITIH4 and cit-ITIH4 expression was examined in steady-state mice and two different arthritis models, and their pathological effects were examined in *Itih4*-deficient mice. In naïve C57BL/6 (WT) mice, ITIH4 was expressed as mRNA in the liver and the lung and was expressed as protein in serum and hepatocytes. In K/BxN serum transferred arthritis (K/BxN-STA) and collagen-induced arthritis (CIA), ITIH4 and cit-ITIH4 in sera were increased before the onset of arthritis, and cit-ITIH4 was further increased at the peak of arthritis. In *Itih4*-deficient mice, citrullinated proteins in serum and joints, especially 120 kDa protein, were clearly diminished; however, there was no significant difference in arthritis severity between WT and *itih*$^{-/-}$ mice either in the K/BxN-STA or CIA model. CIA mice also exhibited pulmonary lesions and *itih4*$^{-/-}$ mice tended to show enhanced inflammatory cell aggregation compared to WT mice. Neutrophils in the lungs of *itih4*$^{-/-}$ mice were significantly increased compared to WT mice. In summary, ITIH4 itself did not alter the severity of arthritis but may inhibit autoimmune inflammation via suppression of neutrophil recruitment.

Keywords: arthritis, lung, chemotaxis/migration, neutrophils, autoimmunity

Abbreviations: ACPA: anti-cyclic citrullinated peptide antibody; AMC: anti-modified citrullinated protein; CFA: Freund's complete adjuvant; CIA: collagen-induced arthritis; GIA: GPI induced arthritis; ITIH4: inter-alpha-trypsin inhibitor heavy chain 4; mRNA: messenger RNA; PAD: peptidyl arginine deiminase; pGIA: peptide GPI induced arthritis; RA: rheumatoid arthritis; STA: serum transfer arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by polyarthritis with bone destruction and inflammation in particular organs. Anti-citrullinated protein antibodies (ACPAs) are known to be detected in RA, and, specifically, to precede disease onset [1]. ACPAs form immune complexes with citrullinated proteins and are involved in the exacerbation of arthritis by activating macrophages and promoting inflammatory cytokines production [2]. It has been reported that, in murine models, administration of anti-citrullinated fibrinogen antibodies aggravates arthritis [3]. A correlation with ACPAs has also been reported in interstitial lung disease (ILD), one of the extra-articular manifestations of RA [4, 5]. Based on these findings, ACPAs and their corresponding antigens, citrullinated proteins, are thought to be involved in the pathogenesis and exacerbation of RA.

Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) was discovered as human plasma protein PK120 with a molecular weight of 120 kDa [6]. It is mainly produced in the liver and is known as one of the acute inflammatory proteins whose expression is increased by infection and surgical invasion [7]. ITIH4 was reported to be elevated in serum after the onset of RA [8] and to correlate with RA disease activity [9]. We reported that citrullinated ITIH4 (cit-ITIH4) was specifically present in RA serum, likely had greater sensitivity and specificity than diagnostic markers such as ACPAs, and correlated with disease activity [10]. Regarding function, we have recently demonstrated native ITIH4 inhibits neutrophil chemotaxis via inhibition of the complement system, whereas cit-ITIH4 enhances neutrophil chemotaxis via complement activation [8, 11]. However, the role of ITIH4 in arthritis remains unclear.

Additionally, we have previously reported that cit-ITIH4 was increased in blood and joints of peptide glucose-6-phosphate

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isomerase-induced arthritis (pGIA) induced in DBA/1 mice [10]. pGIA is induced via a T-cell epitope peptide of GPI, identified as the target antigen in K/BxN models [12]. Regarding conventional C57BL/6 background, two different arthritis murine models are frequently used, K/BxN serum transferred arthritis (K/BxN-STA) and collagen-induced arthritis (CIA) [13–15]; however, ITIH4 and cit-ITIH4 have not been examined in such widely used arthritis models. Citrullinated proteins have been reported to be involved in developing inflammatory arthritis in CIA [16]. Additionally, ILD has also been reported to occur in CIA [17, 18] unlike other murine models [19]; yet, there is still no consensus regarding lung involvement in CIA.

Therefore, in the current study, to clarify the role of ITIH4 in experimental arthritis, we investigated the involvement of ITIH4 and cit-ITIH4 in two different arthritic murine models, K/BxN-STA and CIA. Furthermore, we generated Itih4-deficient (*Itih4*$^{-/-}$) mice to analyse arthritis and lung involvements in the two arthritis models.

Materials and methods

Mice

Age-matched male C57BL/6 and Itih4-deficient C57BL/6 (*Itih4*$^{-/-}$) mice (age: 8–11 weeks), DBA/1 mice, K/BxN mice, nonobese diabetic (NOD) mice, and KRN-transgenic mice were used for the experiments. *Itih4*$^{-/-}$ mice were generated using CRISPR-Cas9 at the Laboratory Animal Resource Center, University of Tsukuba. All experiments were performed under isoflurane anesthesia and euthanized via overdose anesthesia.

The University of Tsukuba approved the experimental protocol (Approval No. 170250) and the study was conducted strictly in accordance with the regulations of the Institutional Animal Experiment Committee of the University of Tsukuba, according to the Regulations for Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

All mice were bred under specific pathogen-free conditions at the University of Tsukuba, and C57BL/6 mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). KRN-transgenic mice were provided by Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and Dr. Koichiro Ohmura (Kobe City Medical Center General Hospital, Hyogo, Japan). Genotyping was performed using direct polymerase chain reaction (PCR). Mouse tails were harvested, and PCR templates were prepared using Direct PCR Lysis Reagent (Tail; Viagen Biotech, Los Angeles, USA). PCR Taq was prepared using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Carlsbad, CA, USA). Primers were obtained from Tsukuba Oligo Service (Ibaraki, Japan), and the sequences were: Fw: CAGTCCCTTCTCCGCATCAA and Rv: CTCCCAACTGTTTGGGGGAATC.

Pathological evaluation of organs

Organs collected from mice were fixed in 10% formalin buffer. Hematoxylin and eosin (HE) staining was performed at the University of Tsukuba, Institute of Medicine and Medical Sciences, Tissue Specimen Room. For lung tissue analysis, the trachea was filled with 10% formalin buffer and fixed.

K/BxN serum transfer arthritis

K/BxN mice were generated by crossing NOD mice with KRN TCR transgenic mice. Serum was collected from arthritic K/BxN mice and pooled until use. Arthritis was induced via intraperitoneal (i.p.) administration of the collected serum to C57BL/6 mice on day 0 and day 2. Arthritis severity was assessed by measuring joint thickness using calipers and visual scoring.

Arthritis was evaluated according to a previously described protocol [20].

Collagen-induced arthritis

Emulsions were prepared by dissolving 2 mg native chicken CII (Sigma-Aldrich, MO, USA) in 500 µl of 0.01 M acetic acid and emulsifying with complete Freund's adjuvant (CFA). CFA was added to 2.5 mg of heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories, MI, USA) and 500 µl Freund's incomplete adjuvant (Sigma-Aldrich).

Mice were anesthetized with isoflurane, and 100 µl of the emulsion was injected intradermally into the caudal region on days 0 and 21.

Arthritis was assessed visually and scored on a scale of 0–3 in each leg: 0 = normal, 1 = mild swelling or erythema, 2 = moderate swelling, and 3 = marked swelling and ankylosis. The scores for each limb were summed (maximum score = 12).

For histological evaluation, mice were sacrificed 54 days after the first CII immunization, and ankle joint tissue was collected. After fixation and embedding, ankle joint sections were HE stained.

For analysis of cell aggregation in the lungs of CIA, images were obtained and analysed using all-in-one fluorescence microscope BZ-X710 (Keyence, Osaka, Japan). The entire HE sections of the lungs were captured in connected images and the areas of cell aggregation and the entire lung were measured using the cell counting software of the microscope (Supplementary Fig. S3).

Real-time quantitative PCR

Total RNA was extracted from mouse tissues using the Isogen extraction method (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The extracted RNA was reverse transcribed using Prime Script RT Master Mix (TaKaRa, Tokyo, Japan) to obtain cDNA. Real-time quantitative PCR (qPCR) was performed using TaqMan (Applied Biosystems). As a primer, qPCR was performed using Itih4 (Mm00497648_m1; Applied Biosystems). qPCR was performed using QuantStudio 3 (Applied Biosystems). Expression levels were based on those of glyceraldehyde 3-phosphate dehydrogenase.

Western blot

Tissues collected from mice were homogenized and blotted in RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NP-40, 20 mM ethylenediaminetetraacetic acid [EDTA], and protease/phosphatase inhibitor cocktail [Cell Signaling Technology, Danvers, MA, USA]) to prepare the tissue lysate. After tissue lysis or serum, proteins were quantified using the TaKaRa Bradford Protein Assay Kit (Takara), developed

using sulfate-polymerase chain electrophoresis (SDS-PAGE), and analysed with transfer to polyvinylidene difluoride (PVDF) membranes. Western blot (WB) with anti-modified citrullinated (AMC) or anti-ITIH4 antibodies was performed using 50 µg (serum), 10 µg (serum and tissue), or 20 µg (serum) protein per well, respectively. The protein-transferred PVDF membranes were then reacted with citrulline modification buffer, which was prepared and light-protected at 37°C for 2.5 hours to modify citrulline residues. The citrulline modification buffer was prepared by mixing Reagent A (20% H2SO4, 25% H3PO4, and 0.025% FeCl3) and Reagent B (1% diacetyl monoxime, 0.5% antipyrine, and 1 M acetic acid) in a 2:1 ratio. The PVDF membrane was then washed with TBS (TBST) containing 0.1% Tween-20 and shaken with TBST containing 5% skim milk for blocking at room temperature for 1 hour. AMC antibody (provided by the Tokyo Metropolitan Institute of Gerontology) diluted 1:3200 in TBST containing 5% skim milk was used as the primary antibody reaction at 4°C overnight. After washing with TBST, the PVDF membrane was immersed in an horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L; Bio-Rad, Hercules, CA, USA) diluted 1:5000 in TBST containing 5% skim milk as the secondary antibody reaction and reacted at room temperature for 1 hour. For ITIH4 analysis, the primary antibody was changed to ITIH4 antibody (ab180139, Abcam), and WB was performed using the above procedure with chemical modifications. The reaction was then detected using enhanced chemi luminescence (cytiva, Tokyo, Japan) and photographed using Chemiluminescence, Fluorescence, Imaging Systems FUSION (FX 7. EDGE, Vilber Lourmat, Collégien, France).

Immunoprecipitation

Murine sera were incubated with rabbit anti-ITIH4 antibody (Proteintech, Rosemont, IL, US) overnight at 4°C and then incubated with Protein G agarose (Cell Signaling Technology) for 4 hours at 4°C. WB was performed using the procedure described above with chemical modifications.

Immunohistochemical staining

Ankle joint tissue samples were collected from CII-immunized mice on day 35. Samples were fixed in 10% formalin buffer (Wako) and paraffin-embedded in 2-µm sections. For citrullinated modification, ReagentA and ReagentB were mixed in the ratio of 2:1 and incubated at 37°C for 2.5 hours. After reaction with 2% BSA/PBS for 30 minutes to block, diluted AMC antibody (at a ratio of 1:3200) was used as the primary antibody to detect citrullinated proteins, together with rabbit isotype control monoclonal IgG (#3900, Cell Signaling Technology). HRP-conjugated goat anti-rabbit immunoglobulin (IgG; H + L; Bio-Rad) was used as a secondary antibody. DAB staining (simple stain DAB solution, Nichirei Bioscience) and hematoxylin staining were performed.

Flow cytometry

Lung tissue was harvested from mice, and lungs were isolated using the Gentle MACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and the Lung Dissociation Kit (Miltenyi Biotec). They were then processed using erythrocyte hemolysis buffer (eBioscience, San Diego, CA).

For joint tissues, mouse ankle joints were excised, skinned, and treated with Liberase (Roche, Basel, Switzerland). The joint capsule was cut open and washed with culture medium (RPMI 1640 medium [Sigma-Aldrich] containing 10% fetal bovine serum [FBS], 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol), and the cells of the joint were harvested and stained.

Flow cytometry (FCM) analysis was performed using Fortessa (BD Biosciences), and data were analysed using FlowJo software (version 10: FlowJo, Tree star, OR, USA).

Fluorescent cell immunostaining (Immunocytochemistry/ Immunofluorescence)

Liver tissue was harvested from DBA/1 mice and homogenized to isolate liver cells. The cells were then fixed with 10% neutral formalin solution and stored in 70% EtOH. ITIH4 antibody (ab180139, Abcam), used as the primary antibody, was conjugated with AF488 (Life Technologies, Carlsbad, CA) for 5 minutes at room temperature. The reaction was allowed to stand for 1 hour at room temperature and the cells were then washed with PBS. They were reacted with DAPI for 3 minutes for nuclear staining and observed using an all-in-one fluorescence microscope (BZ-X710, Keyence).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 25 (IBM, NY, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Both analyses represent the mean ± SEM, and $P < 0.05$ was considered significant. Differences between groups were assessed using the Mann–Whitney U test, the log-rank test, and Welch's t test.

Results

Expression analysis of ITIH4

To investigate the expression of ITIH4 in mice under steady-state conditions, several organs were harvested from C57BL/6 mice and analysed using qPCR and WB. The results showed that ITIH4 mRNA was mainly expressed in the lung and liver, with the highest expression in the liver (Fig. 1A). ITIH4 was detected only in serum on WB (Fig. 1B). Although ITIH4 was not detected in the liver on WB, IFC revealed ITIH4 expression in hepatocytes (Fig. 1C). These results indicate that Itih4 is expressed as an mRNA in the liver and lung tissues. ITIH4 was present at the protein level in the liver and serum.

Citrullinated protein expression in two different arthritis models

We have previously observed the expression of 120 kDa citrullinated protein in the pGIA arthritis model. Therefore, in the current study, the expression of ITIH4 and cit-ITIH4 in arthritis models was investigated using the K/BxN-STA and CIA models, which have not been investigated prior. Serum was collected from K/BxN-STA and analysed using WB. ITIH4 expression was detected, and 120 kDa citrullinated protein (presumed to be cit-ITIH4) was specifically detected in serum from arthritic mice only (Fig. 2A). Then, to confirm course of expression over time in the CIA model, CIA was induced in C57BL/6 mice, and serum was collected at days 0, 20, 35, and 54 after initial immunization, and the expression of ITIH4 and citrullinated 120 kDa (cit-120kDa) protein was examined using WB. ITIH4 expression was highest on day 20 after CIA induction, and decreased with time (Fig. 2B). In contrast to

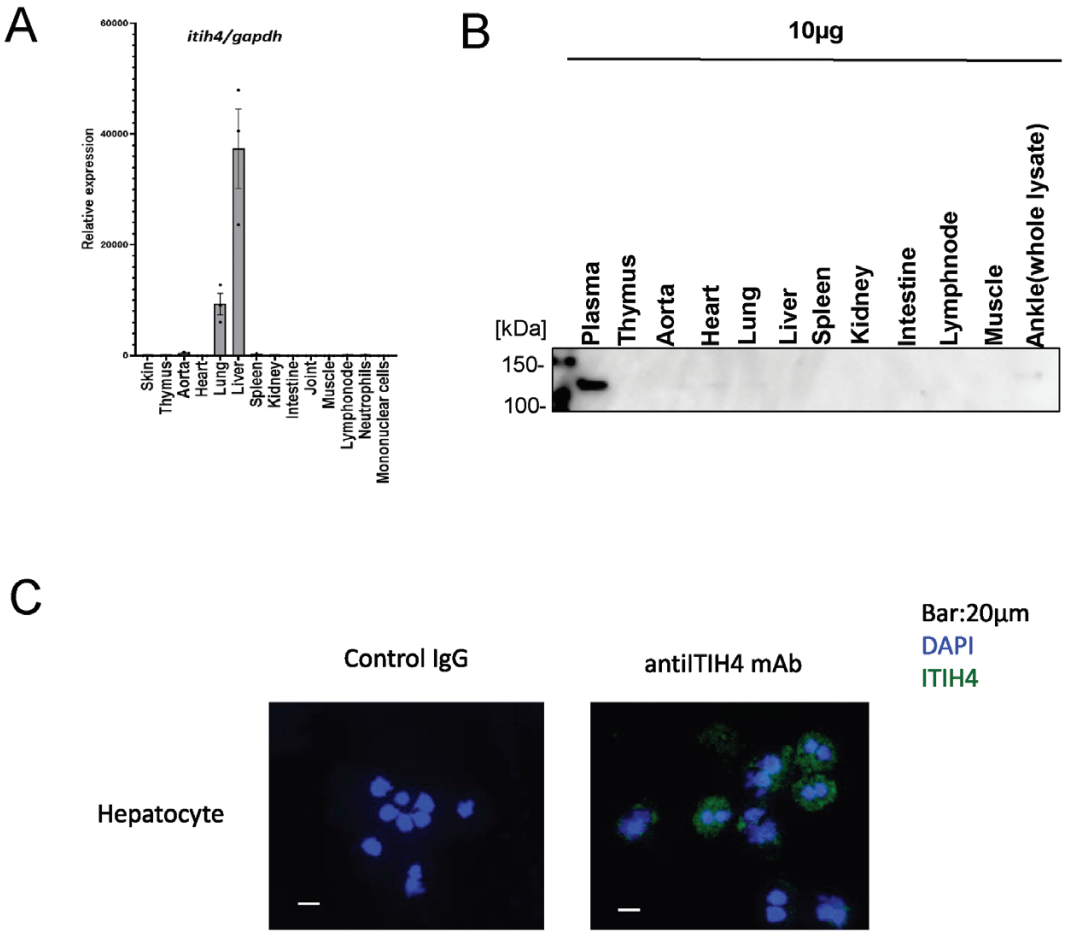

Figure 1. Expression analysis of ITIH4. (A) Itih4 mRNA expression in major tissues in WT mice (n = 3). (B) Expression of Itih4 as a protein in major tissues in WT mice (n = 1). (C) Expression of ITIH4 in liver cells of DBA/1 mice with IFC.

ITIH4, the cit-120kDa protein was not expressed at day 20 in the CIA model but was most strongly expressed at day 35, the extreme stage of arthritis. In addition, the expression levels of the cit-120kDa protein were low in mice with no observed arthritis and high in mice with arthritis. The cit-120KDa protein was confirmed to contain cit-ITIH4 by immunoprecipitation (Fig. 2C). These results suggest that, in the two different arthritic models, ITIH4 is upregulated in the early inflammatory phase, and that the cit-ITIH4 may be associated with severity of arthritis.

Generation of *Itih4*-deficient mice

To investigate the involvement of ITIH4 in the disease pathogenesis, *Itih4*$^{-/-}$ mice were generated by deleting an 1179 bp region, including exon3 to exon5 using CRISPR/Cas9. To confirm the depletion of Itih4, primers were generated by sandwiching the deleted region (Fig. 3A), and PCR and WB findings of serum confirmed the depletion of Itih4 at the gene and protein levels (Fig. 3B and C). To examine the effects on development and growth, the generated *itih4*$^{-/-}$ mice were matured until 25 weeks of age, before being sacrificed and several organs were harvested for histological evaluation. Neither developmental restrictions nor spontaneous organ damage including arthritis were observed (Fig. 3D). These findings indicate that ITIH4 did not affect the development and growth of the mice.

K/BxN-STA in *itih4*$^{-/-}$ mice

To investigate the involvement of ITIH4 in the pathogenesis of arthritis, K/BxN-STA was induced in C57BL/6(WT) and *itih4*$^{-/-}$ mice, and the severity of the arthritis was compared. The results showed no significant differences in the severity of arthritis (Fig. 4A–C). In addition, pathological examination of ankle joints on day 10 showed no significant differences in bone destruction and proliferation of synovium (Fig. 4D). The expression of ITIH4 in serum was confirmed in WT, and its expression was increased from steady state to the arthritis onset (Fig. 4E). Citrullinated protein (especially 120 kDa) was most highly expressed on day 6, and its expression decreased on day 9 when arthritis began to regress. Interestingly, in addition to ITIH4, the cit-120kDa band had almost disappeared in K/BxN-STA *itih4*$^{-/-}$ mice (Fig. 4E). These results suggest that ITIH4 does not affect the severity of arthritis in the K/BxN-STA model, but the cit-120kDa protein in this model was mainly cit-ITIH4.

CIA in *Itih4*$^{-/-}$ mice

To investigate another arthritis models further, CIA was induced in WT and *itih4*$^{-/-}$ mice, and the incidence and severity were compared. The results showed no significant difference in the severity and incidence of arthritis (Fig. 5A and B). In addition, pathological evaluation of ankle joints on day 54 showed no significant differences in inflammation in joints

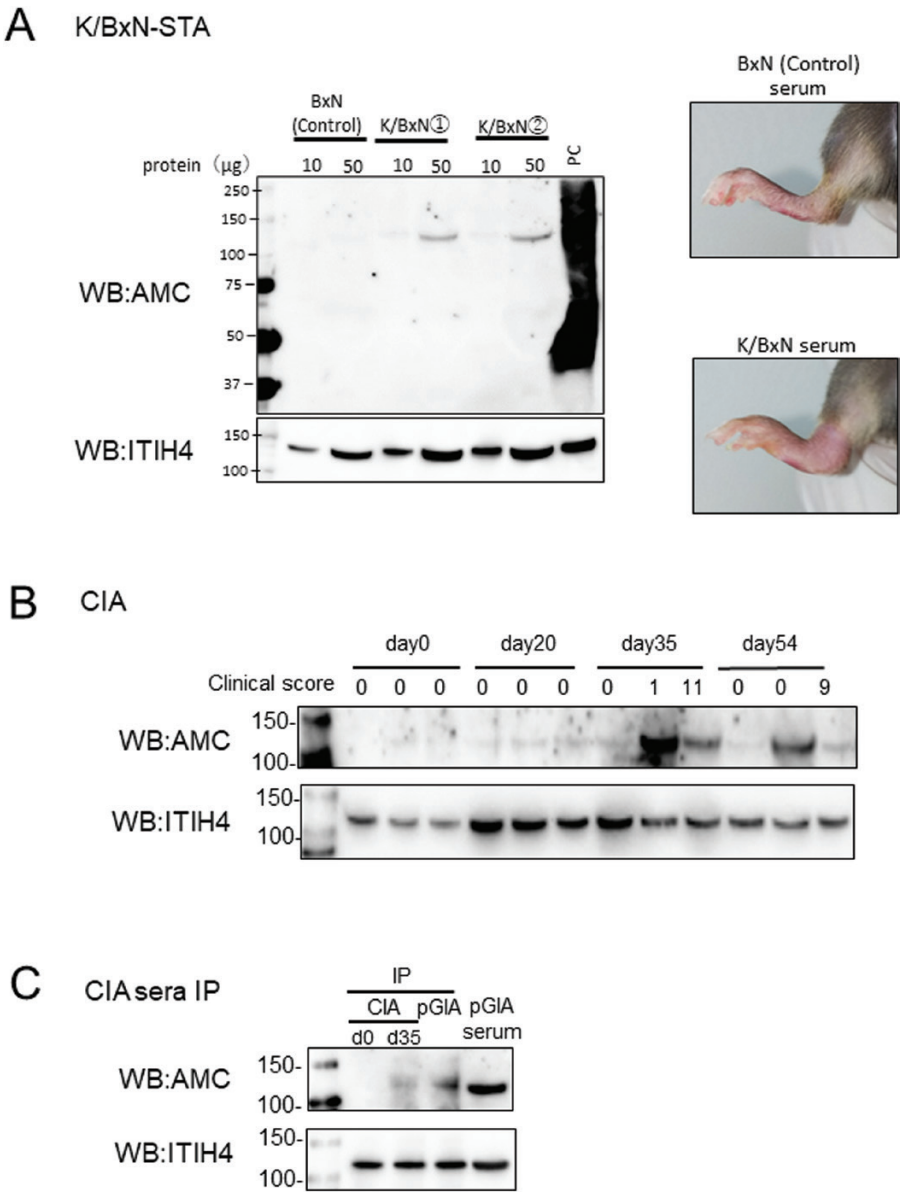

Figure 2. Citrullinated protein expression in two different arthritis models. (A) Serum from K/BxN mice was intraperitoneally administered to WT mice (n = 2) on days 0 and 2, and ITIH4 and citrullinated protein expression in serum was shown by WB with anti-ITIH4 and AMC antibodies. (B) WT mice (n = 3) were intradermally injected with CII emulsified with CFA on days 0 and 21. ITIH4 and citrullinated protein expression in serum on days 0, 20, 35, and 54 after initial immunization. (C) ITIH4 and citrullinated protein expression of CIA sera on days 0 and 35 immunoprecipitated with anti-ITIH4 antibody. Serum of pGIA was also immunoprecipitated with anti-ITIH4 antibody and used as a positive control.

(Fig. 5C). To examine the effect of ITIH4 on infiltrating cells in the joint, ankles on day 54 were investigated, and infiltrating cells in the ankle joints were harvested for FCM analysis. The results showed no significant difference between WT and *Itih4*$^{-/-}$ mice (Fig. 5D and E). These results suggest that ITIH4 deficiency does not affect arthritis severity.

Lung lesions in *Itih4*$^{-/-}$ mice with CIA

Because ITIH4 mRNA expression was confirmed in the lungs (Fig. 1A), we speculated that ITIH4 deficiency affects the lung lesion of CIA and, therefore, examined lung lesions in CIA-induced *itih4*$^{-/-}$ mice. CIA is known to cause infiltration of inflammatory cells, mainly macrophages and neutrophils, in lung tissue as well [18]. Histologically, *Itih4*$^{-/-}$ mice tended to show increased inflammatory cell aggregation compared to WT mice (Fig. 6A and B). To examine the infiltrating cells in the lungs, FCM analysis was performed and it was found that only neutrophils in the lungs of the *itih4*$^{-/-}$ mice were significantly increased compared to WT mice (Fig. 6C and D). These results suggest that ITIH4 may be involved in suppressing neutrophil infiltration in the lung tissue in CIA.

Discussion

In the present study, using K/BxN-STA and CIA murine models, we showed that serum ITIH4 is increased prior to the onset of arthritis, and cit-120kDa protein (presumably cit-ITIH4) is subsequently increased during the extreme phase of arthritis, suggesting that 120 kDa citrullinated protein may be involved in the development of arthritis. However,

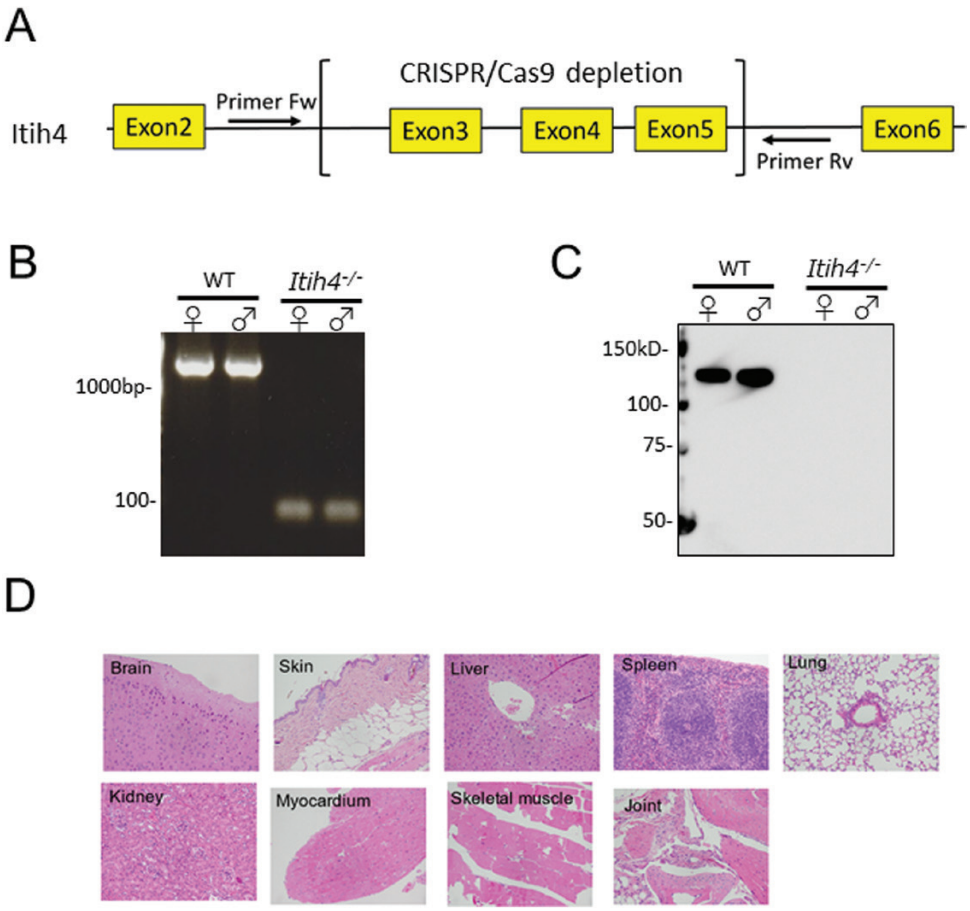

Figure 3. Generation of *Itih4*-deficient mice. (A) Schematic of primers used for confirmation of gene defects. (B) Confirmation of defects at the gene level of *itih4* using tail DNA. (C) Confirmation of defects at the protein level of ITIH4 in serum. (D) HE-stained images of major tissues in 25-week-old *itih4*$^{-/-}$ mice (n = 1). Original magnification ×200.

no significant differences in arthritis severity were observed between WT and *itih4*$^{-/-}$ mice in the two models. Cellular analysis of joint lesions in CIA mice revealed no significant differences between WT and *Itih4*$^{-/-}$mice, suggesting that ITIH4 did not directly alter inflammatory arthritis in these models.

Our previous study showed that ITIH4 inhibits neutrophil migration via suppression of the complement system [11]. Thus, ITIH4 deficiency may promote neutrophil migration into the joints. On the other hand, we have previously found that citrullination of ITIH4 altered its function to promote neutrophil migration with complement activation [11]. Because both ITIH4 and cit-ITIH4 have been shown to be present in the joints of RA and other mouse models [10, 11], and both ITIH4 and cit-ITIH4 are deficient in *itih4*$^{-/-}$ mice, it is possible that their competing effects on neutrophil migration are impaired, resulting no significant differences in arthritis severity.

It is also possible that the loss of cit-ITIH4 was replaced by an increase in other citrullinated proteins, which may have affected the severity of arthritis in ITIH4-deficient mice. Indeed, citrullinated proteins in CIA joints were present in ITIH4-deficient mice as in WT (Supplementary Fig. S1). Since the citrullinated proteins have been reported to contribute to the pathogenicity of arthritis [2, 3], it is possible that citrullinated proteins other than cit-ITIH4 exacerbated arthritis and counteracted the effect of cit-ITIH4 deficiency.

In this study, we did not perform mass spectrometry to determine whether the 120 kDa citrullinated protein was cit-ITIH4. However, immunoprecipitation using anti-ITIH4 antibody confirmed the presence of a 120 kDa citrullinated protein as well as pGIA serum, which had previously been confirmed to contain cit-ITIH4 by mass spectrometry, indicating the presence of cit-ITIH4 in CIA sera [10].

Although it is known that CIA causes inflammatory cell infiltration in the lungs, there are few studies on extra-articular lesions in CIA, and the mechanism remains unclear. In the present study, we observed that lung regions in CIA and *itih4*$^{-/-}$ mice showed increased inflammatory cell infiltration with increased numbers of neutrophils in the lung tissue (Fig. 6C). As mentioned above, ITIH4 has been shown to inhibit neutrophil migration [11]. ITIH4 has been reported to be expressed in alveolar epithelial cells in humans and rats [21], and ITIH4 mRNA expression was observed in the lungs of WT mice in this study (Fig. 1A), Thus, it is possible that ITIH4 inhibits neutrophil migration in the lungs of CIA mice. Itih4 has also been shown to act as a target hub in the downstream regulatory network of MicroRNA-21, which is important for the regulation and development of lung disease [22]. In *itih4*$^{-/-}$ mice, losing this hub role may have only exacerbated inflammation in the lung tissue, with no effect on the arthritis.

A limitation of this study is that the *itih4*$^{-/-}$ mice that were generated are deficient in both ITIH4 and cit-ITIH4,

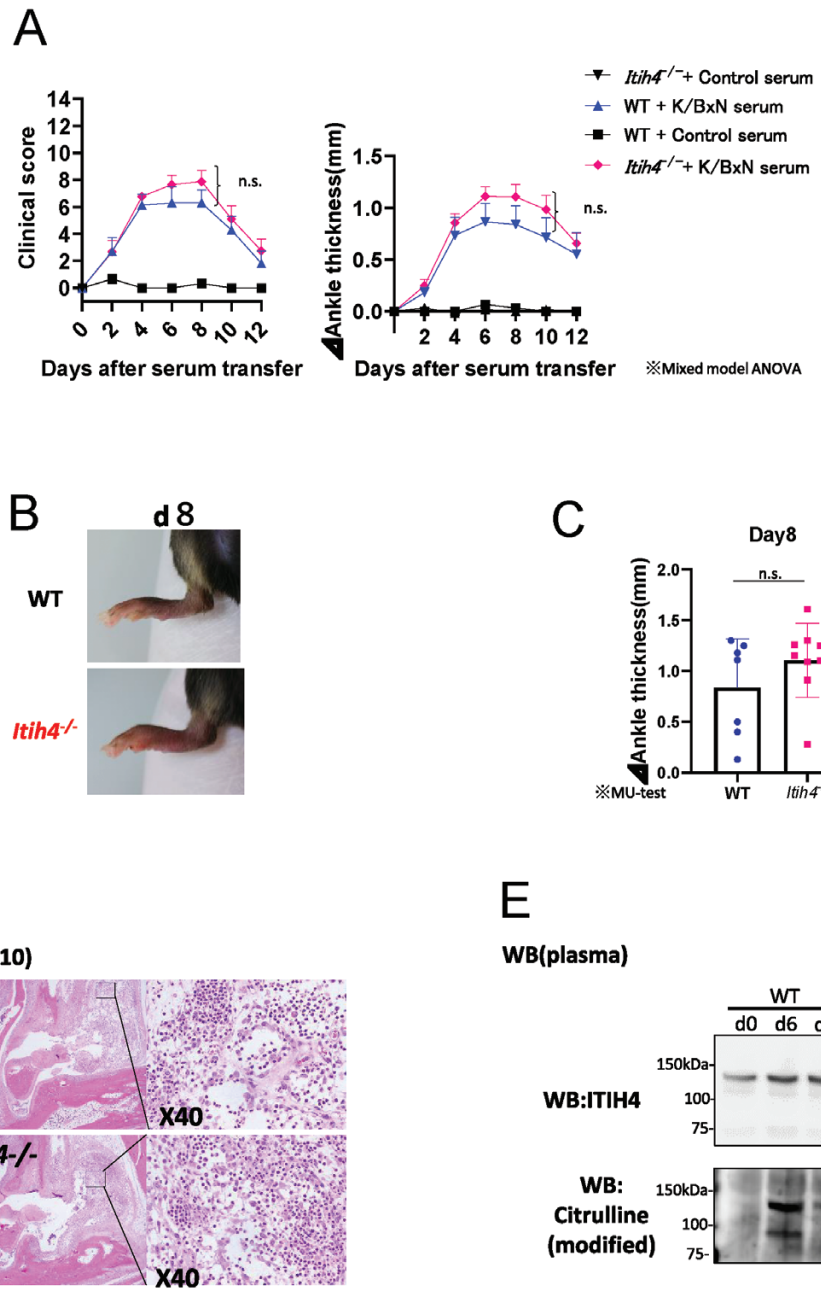

Figure 4. K/BxN-STA in *itih4*$^{-/-}$ mice. (A) WT (n = 7) and *itih4*$^{-/-}$ (n = 3) mice were intraperitoneal injection with K/BxN mice serum on days 0 and 2. As a control group, WT (n = 3) and *itih4*$^{-/-}$ (n = 9) mice were intraperitoneally injected with control serum. (B) Images of the ankle joints of both WT and *itih4*$^{-/-}$ mice were taken on day 8 following their initial immunization. (C) Comparison of ankle thickness on day 8 in WT and *itih4*$^{-/-}$ mice. (D) HE-stained images of the ankle joint at 10 days after initial immunization. (E) WT mice were intraperitoneally injected with K/BxN mice serum on days 0 and 2. ITIH4 and citrullinated protein expression in serum on days 0, 6, and 9 after initial immunization with ITIH4 and AMC antibodies. *P* values were calculated using mixed model ANOVA (A) and MU-test (C).

so we could not elucidate the functions of each in isolation. ITIH4 and cit-ITIH4 have opposite functions, with ITIH4 suppressing neutrophil migration by inhibiting the complement system and cit-ITIH4 inducing neutrophil migration by promoting the complementary system [11]. Because both are deficient, it is possible that the incidence and severity of the arthritis did not differ significantly. To suppress protein citrullination, we previously reported that treatment with the Cl-amidine, a PAD inhibitor, ameliorated arthritis in pGIA model and decreased serum citrullinated protein [23]. GIA models using *Padi4* knockout mice were also reported to ameliorate arthritis [24]. Thus, inhibition or depletion of PAD4, which mediates citrullination, reduces the production of citrullinated proteins and alleviates arthritis. However, inhibition of citrullination of ITIH4 alone has not been implemented. Therefore, in the future, to focus on each protein in isolation, it is necessary to analyse the function of ITIH4 and cit-ITIH4 independently by their independent transfer.

Also, in DBA/1 mice with CIA model, Cl-amidine administration ameliorated arthritis and decreased serum citrullinated protein levels [25]; and further, *Padi4* knockout mice showed decreased arthritis severity [26]. Thus, based

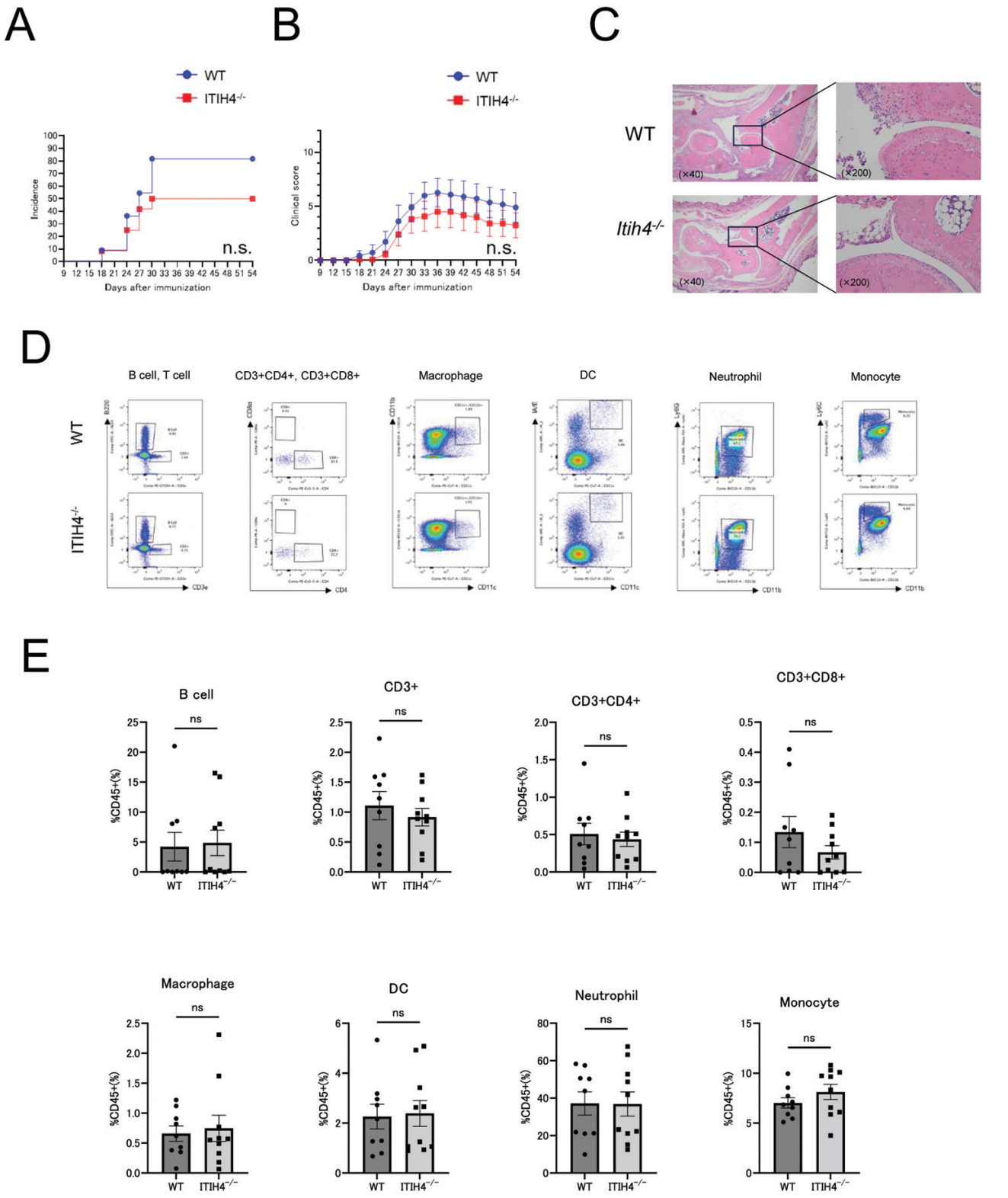

Figure 5. CIA in *Itih4*$^{-/-}$ mice. WT (n = 11) and *itih4*$^{-/-}$ (n = 12) mice were injected intradermally with CFA-emulsified CII on days 0 and 21 (A) incidence and (B) severity of CIA. (C) HE-stained images of the ankle joint at 54 days after initial immunization. (D) FCM analysis of ankle joint fluid 54 days after initial immunization, representative FACS image. (F) Percentage of each cell (WT, n = 9 *Itih4*$^{-/-}$, n = 10). Each data is presented as mean ± SEM. P values calculated using Mann–Whitney U test (B), log rank test (A), and Welch's t test (D, E).

on the above results, studies in DBA/1 mice have shown that inhibition or deletion of PAD4 ameliorates arthritis in several murine models. In contrast, no improvement in arthritis was observed in the K/BxN-STA model using *Padi4* knockout C57BL/6 mice, suggesting that PAD4 is dispensable for the development and maintenance of K/BxN-STA arthritis [27]. C57BL/6 mice are less susceptible to CIA than DBA/1 mice, and its severity is known to be lower and more variable [28].

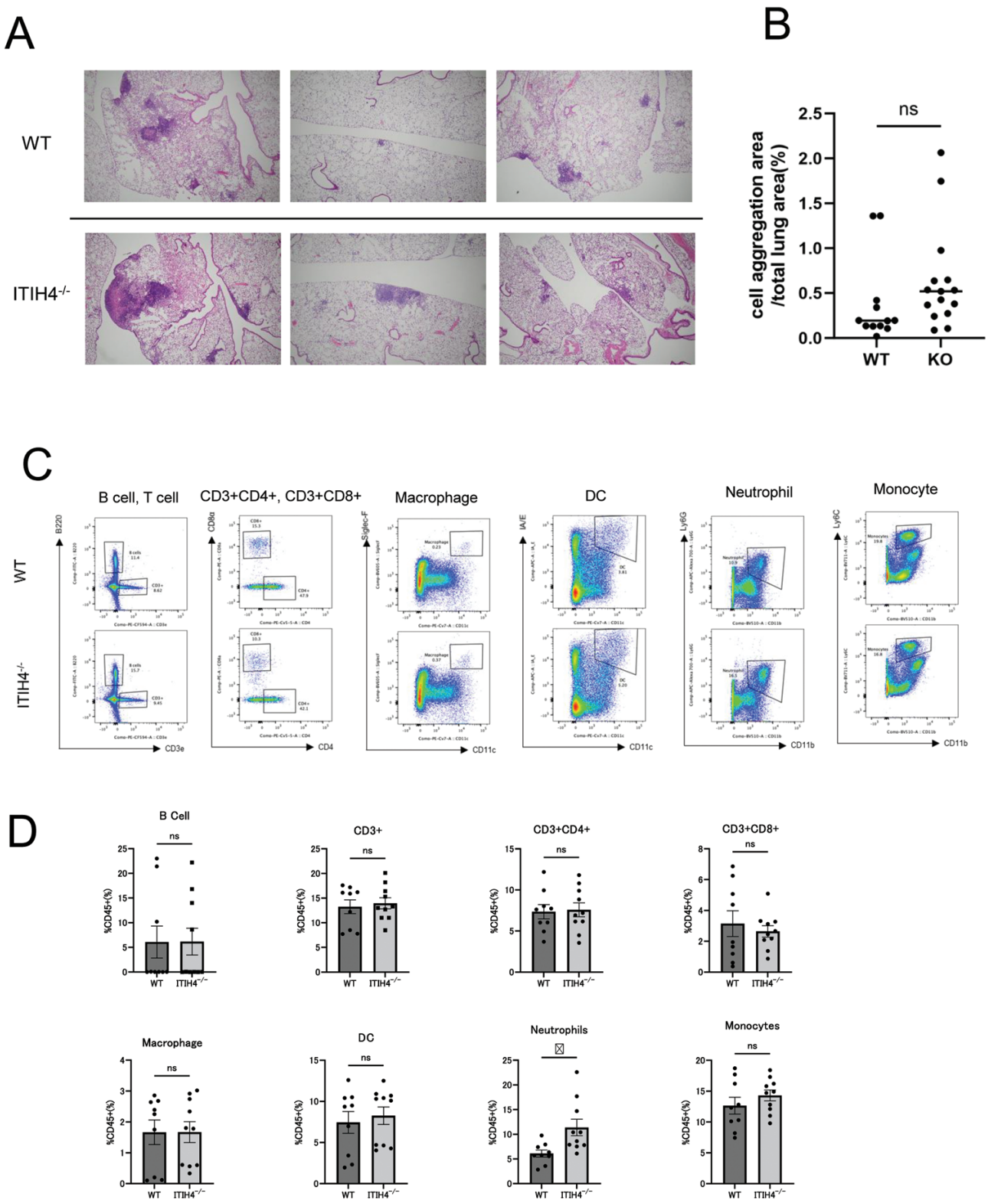

Figure 6. Lung Lesions in *Itih4*$^{-/-}$ Mice with CIA. WT and *itih4*$^{-/-}$ mice were injected intradermally with CFA-emulsified CII on days 0 and 21. (A) Representative HE-stained images of the lungs at 54 days after initial immunization. WT (n = 3) and *itih4*$^{-/-}$ mice (n = 3). Original magnification ×40. (B) Percentages of cell aggregation areas relative to total lung areas (WT, n = 12 *itih4*$^{-/-}$, n = 15). (C) FCM analysis of lungs 54 days after initial immunization, representative FACS image. (D) Percentage of each cell (WT, n = 9 *itih4*$^{-/-}$, n = 10). Each data is presented as mean ± SEM. P values were calculated using Welch's t test (B, C, D).

These findings suggest that PAD4-induced citrullination has different pathogenic roles in DBA/1 and C57BL/6 mice. Therefore, ITIH4/cit-ITIH4 in this study may also show different results depending on whether CIA or pGIA is induced in DBA/1 mice. Ideally, *itih4*$^{-/-}$ mice should be generated using DBA/1 mice to confirm differences in arthritis in the GIA model.

Taken together, ITIH4 deficiency did not alter the severity of arthritis in the arthritis model of the setting of this study, however, it did increase neutrophil infiltration in the lung lesions. For detailed functional analysis of ITIH4 and cit-ITIH4, it is necessary to consider protein transfer assays or analysis in other animal models.

Supplementary Data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

This study was approved by the Institutional Animal Care and Use Committee of the University of Tsukuba (protocol code 21-204, 22-227).

Conflict of interests

The authors declare that they have no competing interests.

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Data availability

Data are available from the corresponding author upon reasonable request.

Author contributions

Conceptualization, T.I., A.Oh., A.Os and I.M.; data curation, T.I., A.Oh. and A.Os., T.N., M.S., H.M., H.A., Y.K., H.T, S.M., S.T., A.I. and I.M.; formal analysis, T.I., A.Oh. and A.Os.; funding acquisition, I.M.; investigation, T.I., A.Oh. and A.Os.; methodology, T.I., A.Oh, A.Os., M.S., H.M., S.M., S.T., A.I. and I.M.; project administration, A.Oh. and I.M.; resources, S.M., S.T., A.I. and I.M.; supervision, I.M.; validation, T.I., A.Oh., A.Os and H.M.; visualization, T.I., A.Oh. and A.Os.; writing—original draft preparation, T.I., A.Oh. and I.M.; writing—review and editing, A.Oh and I.M. All authors have read and agreed to the published version of the manuscript.

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