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Citrullinated human fibrinogen triggers arthritis through an inflammatory response mediated by IL-23/IL-17 immune axis --Manuscript Draft--

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Corresponding Author:	Jose C. Alves-Filho University of São Paulo Ribeirao Preto, Brazil
First Author:	André Lopes Saraiva, PhD
Order of Authors:	André Lopes Saraiva, PhD
	Raphael Sanches Peres, PhD
	Flávio Protasio Veras, PhD
	Jhimmy Talbot, PhD
	Kalil Alves de Lima, PhD
	João Paulo Mesquita Luiz, PhD
	Thiago Mattar Cunha, Professor
	Paulo Louzada-Junior Louzada-Junior, Professor
	Fernando Queiroz Cunha, Professor
Abstract:	Jose C. Alves-Filho
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Suggested Reviewers:	Isabelle Couillin Orleans University: Universite d'Orleans isabelle.couillin@cnrs-orleans.fr
	Hui-Rong Jiang University of Strathclyde huirong.jiang@strath.ac.uk
	Ari Waisman Mainz University: Johannes Gutenberg Universitat Mainz waisman@uni-mainz.de
	Ricardo Machado Xavier Federal University of Rio Grande do Sul: Universidade Federal do Rio Grande do Sul rxavier10@gmail.com

	Yan Lu Affiliated Hospital of Nanjing University of Chinese Medicine yfy0046@njucm.edu.cn
	Richard O Williams University of Oxford richard.williams@kennedy.ox.ac.uk
Response to Reviewers:	

November 08th 2021

Dr. Hajo Haase
Associate Editor
International Immunopharmacology

Manuscript Number: **INTIMP-D-21-02062R1**

Dear editor,

Please find enclosed the revised version of our manuscript entitled “*Citrullinated human fibrinogen triggers arthritis through inflammatory response mediated by IL-23/IL-17 immune axis*” by Saraiva et al. We greatly appreciate your interest and the opportunity that we have been given to revise the manuscript, providing a fair and constructive peer-review process.

We have addressed all the reviewers’ concerns. Please find a detailed point-by-point reply to the reviewer's comments attached to this revision.

Thank you again for your thoughtful consideration of our manuscript. We hope you now find this revised version acceptable for publication.

Sincerely yours,



José Carlos Alves-Filho

November 08th 2021

Hajo Haase
Associate Editor
International Immunopharmacology

Manuscript Number: **INTIMP-D-21-02062R1**

Dear Dr. Haase,

Please find attached the revised version of our manuscript entitled “*Citrullinated human fibrinogen triggers arthritis through inflammatory response mediated by IL-23/IL-17 immune axis*” by Saraiva et al. We greatly appreciate your interest in our manuscript.

We have detailed below our point-by-point response to the reviewers’ specific concerns and the changes/inclusions that we have been made to address them.

Reviewer #1:

All raised issues have been properly addressed.

We thank the referee for his/her constructive comments.

Reviewer #2:

1. In the sentence which you add (394-416), there are two description "spontaneous arthritis after immunization with xxx ". would it be appropriate? Because in this case arthritis is induced by antigen but not genetically spontaneous onset of disease. This word "spontaneous" may be better to not be used to avoid the misunderstanding.

This is a fair comment, and we have now revised the manuscript, removing the word “spontaneous” from the text.

Highlights

Immunization with citrullinated human fibrinogen induces ACPA antibody production

Joint injection of human fibrinogen causes arthritis in previously immunized mice

Human fibrinogen-induced arthritis depends on IL-17/IL-23 immune axis response

Citrullinated human fibrinogen triggers arthritis through an inflammatory response mediated by IL-23/IL-17 immune axis

André Lopes Saraiva^{a,1}, Raphael Sanches Peres^{b,2}, Flávio Protasio Veras^a; Jhimmy Talbot^a, Kalil Alves de Lima^b, João Paulo Mesquita Luiz^b, Thiago Mattar Cunha^{a,b}, Paulo Louzada-Junior.^{b,2}, Fernando Queiroz Cunha^{a, b}, José Carlos Alves-Filho^{a, b, *}

^a Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes 3900, Ribeirão Preto, São Paulo 14049-900, Brazil.

^b Department of Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes 3900, Ribeirão Preto, São Paulo 14049-900, Brazil.

² Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Headington Oxford, OX3 7FY, United Kingdom.

*** Corresponding author**

Prof. José Carlos Alves-Filho

Department of Pharmacology and Department of Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil. Email: jcafilho@usp.br

¹ Present Adress: Federal University of Uberlândia, Biotechnology Institute, Rua Acre S/N, 39400-902, Uberlândia, Minas Gerais, Brazil.

² Present Adress: Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Headington Oxford, OX3 7FY, United Kingdom.

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that causes joint destruction. Although its etiology remains unknown, citrullinated proteins have been considered as an auto-antigen able to trigger an inflammatory response in RA. Herein, we modified the classical antigen-induced arthritis (AIA) model by using citrullinated human plasma fibrinogen (hFIB) as an immunogen to investigate the mechanism of inflammation-driven joint damage by citrullinated hFIB in C57BL/6 mice. We found that hFIB-immunized mice showed high serum levels of anti-citrullinated peptides antibodies (ACPAs). Moreover, hFIB immunized mice showed increased mechanical hyperalgesia, massive leukocyte infiltration, high levels of inflammatory mediators, and progressive joint damage after the intra-articular challenge with citrullinated hFIB. Interestingly, hFIB-induced arthritis was dependent on IL-23/IL-17 immune axis-mediated inflammatory responses since leukocyte infiltration and mechanical hyperalgesia were abrogated in *Il17ra*^{-/-} and *Il23a*^{-/-} mice. Thus, we have characterized a novel model of experimental arthritis suitable to investigate the contribution of ACPAs and Th17 cell-mediated immune response in the pathogenesis of RA.

Keywords: rheumatoid arthritis, fibrinogen, citrullinated peptides, ACPAs, IL-17, IL23,

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disorder that affects around 0.5-1% of the worldwide population and leads to chronic inflammation in the joints [1]. Although RA etiology remains unknown, several studies have shown an interplay among genetic and environmental factors, which contribute to the auto-inflammatory process initiation [2]. RA has been associated with the presence of circulating auto-antibodies such as rheumatoid factor (RF), anti-citrullinated peptide/protein auto-antibodies (ACPAs), and more recently identified, anti-carbamylated and anti-acetylated peptides auto-antibodies [3–6].

Particularly, ACPAs have been the focus of extensive research. ACPAs are a class of antibodies that recognize proteins containing citrulline residues [7]. The generation of citrullinated peptides occurs through the activity of peptidyl arginine deiminases (PADs), a family of calcium-dependent enzymes, which mediate the replacement of a protein-bound arginine by citrulline [8]. These post-translational modifications termed citrullination or deamination are described by generating potential self-antigenic epitopes [7]. In the context of RA, it is widely accepted that the detection of ACPAs is a powerful tool for diagnostic and prognostic prediction [9,10]. In fact, most of the ACPAs-positive patients are also positive for RF, and there is currently a consensus between the American College of Rheumatology and the European League Against Rheumatism recommending ACPAs plus RF titers detection as one of the parameters employed for RA diagnosis [11].

A range of proteins has been described to be modified by the citrullination process, such as vimentin, collagen-II, α -enolase, and fibrinogen [12]. Among these proteins, citrullinated fibrinogen has been considered as an auto-antigen able to trigger an

inflammatory response. Humanized transgenic DR4-IE mice, which express RA-associated to MHC-II molecule DRB1*0401, develop arthritis when immunized with citrullinated human fibrinogen (hFIB) [13,14]. However, the susceptibility of wild-type mice to develop arthritis after immunization with citrullinated hFIB depends on the strain, especially DBA/1J and SJL/J, and immunization protocol [15,16]. This hampers the use of gene-deficient mice to understand the immunopathologic events that lead to inflammation-driven joint damage by hFIB as they are usually available in the C57BL/6 background. Thus, developing a suitable animal model for hFIB-induced arthritis may help us to gain a deeper insight into the inflammation-driven joint damage by ACPAs.

In the present study, we modified the classical antigen-induced arthritis (AIA) model [17] by using citrullinated hFIB as an immunogen to investigate the mechanism of inflammation-driven joint damage by citrullinated hFIB in C57BL/6 mice. Our results demonstrate that this adapted experimental arthritis model may serve as a helpful tool for pre-clinical studies aiming to discover new potential therapeutic targets for RA.

2. Material and methods

2.1. Animals

Experiments were performed using male C57BL/6 mice (6-7 weeks of age) obtained from the Central Animal Facility of the University of São Paulo- Ribeirão Preto. In some experiments, age-matched knockout and wild-type mice were used. IFN γ (*Ifng*^{-/-}), IL-17 receptor A (*Il17ra*^{-/-}), and IL-23-p19 (*Il23a*^{-/-}) knockout mice (all in C57BL/6 background) were obtained from our Animal Facility. Animals were kept in a

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4 91 temperature-controlled room (20-22°C). Water and standard mice chow were
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6 92 provided *ad libitum*. During joint injection, animals were kept under isoflurane (2%)
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9 93 anesthesia. All experimental procedures followed the Animal Ethics Committee of
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11 94 the School of Medicine of Ribeirão Preto (protocol number 146/2011), University of
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15 16 96 17 18 97 **2.2. Fibrinogen-induced arthritis model**

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21 98 The schematic diagram of the immunization protocol is shown in Fig. 1B. C57BL/6
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23 99 mice were immunized with a subcutaneous (s.c.) administration of 0.2 mg of hFIB
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26 100 (fibrinogen from human plasma, Sigma-Aldrich, product number F3879) emulsified
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28 101 in 0.1 mL of Complete Freund's Adjuvant (CFA) and 0.1 mL of sterile phosphate-
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31 102 buffered saline (PBS). The hFIB was previously solubilized in PBS. Animals were
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33 103 boosted on days 7 and 14 with the same preparation, but CFA was replaced by
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35 104 Incomplete Freund's adjuvant (IFA). For the control (Ctrl) group, emulsion for the
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38 105 immunization was prepared with CFA and PBS without hFIB. On day 21, mice were
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41 106 challenged by intra-articular (i.a.) injection of hFIB (10 µg/ joint) into the right knee
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43 107 joint using a sterile 30-gauge syringe (BD Micro-Fine insulin syringes, 0.3 mL; BD
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45 108 Bioscience). Inflammatory parameters and mechanical hyperalgesia were
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48 109 determined seven hours after the challenge. Naïve control mice were injected with
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51 110 only 10µl of sterile PBS. Articular damage was assessed by histopathological
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53 111 analysis or proteoglycan loss assay. To this end, mice were immunized and then
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55 112 challenged twice with hFIB (10 µg/ joint) on days 21 and 28, and the knee joints were
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58 113 harvested on day 35 (Fig. 2A).

2.3. Western blot

Western blot analyses were performed to detect citrullinated residues from hFIB (30 µg, 50 µg, 70 µg; Sigma-Aldrich) and fresh bovine serum albumin (BSA). Briefly, citrullinated residues were detected using a mouse anti-peptidyl-citrulline monoclonal antibody (clone F95, Merk-Millipore, dilution 1:500) followed by incubation with HRP-conjugated goat anti-rabbit antibody (Pierce; dilution 1:10000). Blot was revealed using a kit containing chemiluminescence (ECL) reagent (GE Healthcare) in a Bio-Rad Chemidoc XRS device (Hercules, CA, USA).

2.4. Detection of anti-human fibrinogen IgG

Serum and intra-articular levels of total IgG anti-hFIB were measured according to a previous study with modifications [15]. Briefly, ELISA plates were coated overnight with hFIB (10 µg/mL) in PBS at 4°C. Next, plates were blocked with PBS/Tween 20 (0.05 % v/v) and FBS (0.3%) for two hours at room temperature. Plasma and articular lavage samples were diluted 1:50 following serial dilution (factor 2) and incubated for two hours at room temperature. Biotinylated goat anti-mouse IgG antibody (Vector Laboratories) was diluted 1:1000 and incubated for one hour at room temperature. Microplate reader Spectra Max-250 (Molecular Devices, Sao Jose, CA, USA) was used to detect changes in the oxidation of tetramethylbenzidine (TMB) at 630 nm that is associated with the levels of antibody.

2.5. Detection of ACPAs

To detect ACPAs, we employed a QUANTA Lite® CCP3 IgG ELISA kit, whose methodology was adapted to detect mice antibodies. This kit provides a sensitized

plate with synthetic citrullinated peptides. In this way, we incubated plates with mice serum and revealed through incubation with secondary biotinylated goat anti-mouse IgG antibody (Vector Laboratories; diluted at 1:5000) followed by incubations with streptavidin and TMB as a conventional ELISA assay.

2.6. Evaluation of mechanical hyperalgesia

Knee joint nociception was determined according to previously described [18]. Mice were housed in acrylic cages with a wire grid floor in a quiet and controlled temperature room. After environment adaptation, a gradual perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the knee joint. An electronic pressure-meter (IITC Inc., Life Science Instruments, Woodlands Hills, CA, USA) automatically records the force intensity applied until the reflex movement of the paw withdrawal. The withdrawal paw threshold was expressed in grams (g). Measurements were performed between 9:00 am and 5:00 pm. Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines.

2.7. *In vivo* bioluminescence imaging

Mice were anesthetized with isoflurane (2%) and injected intraperitoneally with XenoLight™ Rediject Inflammation Probe (100 mg/Kg; PerkinElmer®). Luminescence image acquisitions were performed using the *in vivo* image system (IVIS Spectrum; PerkinElmer®, Waltham, MA, USA) at 10 min post-injection of the probe. Images were captured and analyzed with Living Image Software (PerkinElmer®). Results were expressed as the intensity of radiance (p/sec/cm²/sr).

2.8. Determination of joint leukocyte infiltration

Leukocyte infiltration into the joints was assessed seven hours after intra-articular challenge with hFIB as previously described [18]. Briefly, articular infiltration of leukocytes was determined by washing the femur-tibial joint three times with 3.3 μ L PBS/ Ethylenediaminetetraacetic acid (EDTA 0.2 M), and subsequent cell counting was performed in a Neubauer chamber. The results were expressed as the numbers of leukocyte/ joint.

2.9. *Ex vivo* quantification of articular cytokines and myeloperoxidase (MPO) activity

MPO activity was quantified following the previously reported colorimetric method with modifications [19]. Mice were euthanized, and the knee joint was harvested. Joints were homogenized in 300 μ L of PBS containing protease inhibitor. Next, samples were centrifuged (10.000 g, 10 min), and the supernatant was removed for measurements of IL-17A and IL-1 β levels by ELISA (R&D Systems); results were expressed as pg of cytokine/joint. The remaining tissue pellet was then homogenized with NaCl (0.2%) and centrifuged (3.000 g; 10 min); the supernatant was discarded, and the pellet was homogenized with PBS/H-TAB (0.5% w/v) and centrifuged (10.000 g; 15 min). Measurements of MPO activity were carried out in the remaining supernatant. Enzymatic activity was determined by a colorimetric assay based on H₂O₂ consumption and consequent TMB oxidation that was monitored at 630 nm in microplate reader Spectra Max-250 (Molecular Devices, Sao Jose, CA, USA). Data were expressed as MPO activity/number of neutrophils.

2.10. Histopathological analysis and articular proteoglycan loss

Mice were euthanized, and their knee joint excised, fixed in buffered formaldehyde 4% (v/v), and decalcified with EDTA 10% (w/v). Next, samples were dehydrated in grade ethanol, embedded in paraffin, and sliced into sections for further hematoxylin-eosin staining. Proteoglycan amounts were determined using a previously reported protocol [20] with modifications. Briefly, the femur-tibial patella was carefully collected and fixed overnight with formaldehyde (4%). Next, the patella was incubated into a solution of formic acid (5%) for 4 h, and further incubated at 60°C for 16 h in free calcium and magnesium PBS containing papain (5 mg/mL), cysteine (5 mM), and EDTA (10 mM). Next, 50 µL of the supernatants from each sample was collected and transferred into a 96-well microtiter plate and revealed with 300 µL of 1,9-dimethyl methylene blue solution (DMMB, 50 ng/mL) at 525 nm. Values of proteoglycan content were determined using a standard curve of chondroitin sulfate, and proteoglycan loss was calculated by subtracting the values from injected and non-injected knees.

2.11. Flow cytometry

Cell suspensions (1×10^6 cells) from inguinal (draining) lymph nodes were stimulated for 4 h with phorbol 12-myristate 13-acetate (PMA 50 ng/ mL) and ionomycin (500 ng/ mL). Next, cells were stained with fluorochrome-conjugated monoclonal antibodies anti-mouse CD4 (clone RM4-5), anti-mouse IL-17A (clone TC11-18H10), and anti-mouse IFN- γ (XMG1.2), all acquired from BD Biosciences. Intracellular staining was performed using BD Cytofix/Cytoperm™ Fixation/Permeabilization

Solution kit (BD Biosciences) according to the manufacturer's instructions. FACS acquisition was conducted in flow cytometer BD FACSVerse (BD Biosciences, Sao Jose, CA, USA) and analyzed using FCS Express 4.0 (De Novo Software).

2.12. Recall experiments

Draining lymph node cell suspensions (inguinal, 1×10^5 cells) were stimulated in 96-well round-bottom plate with hFIB (100 $\mu\text{g}/\text{mL}$) or concanavalin A (5 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) for 96 h. Supernatants were harvested, and levels of IL-17A, IFN- γ , and IL-23 were determined by ELISA (R&D Systems).

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). Results were expressed as mean \pm standard error of the mean (SEM). Student's *t*-test or one-way ANOVA followed by Dunnett's post hoc test were employed depending on the number of experimental groups for each experiment. When Dunnett's test was performed, the mean of each group was compared with the mean of the Ctrl group. In all analyses, $P < 0.05$ was considered significant.

3. Results

3.1. Immunization of mice with citrullinated hFIB induces generation of ACPAs

We initially investigated whether hFIB purified from plasma contains citrullinated residues. Using a mouse anti-peptidyl-citrulline monoclonal antibody, we found a substantial amount of citrullinated residues on the hFIB (Fig. 1A). We did not detect

any citrullinated residues in BSA used as a control protein. To investigate whether hFIB can induce ACPAs in mice, we established an immunization protocol consisting of 3 rounds of immunization procedures with hFIB in adjuvant CFA/IFA (Fig. 1B). The Ctrl group received only CFA/IFA. We found very high titers of anti-hFIB specific total IgG antibody in the serum and articular lavage of hFIB-immunized mice on day 21 of the immunization protocol (Fig. 1C-D). Only residual signal of anti-hFIB specific reactivity was detected in the serum and articular lavage of Ctrl mice. Consistently, we also detected high serum levels of anti-CCP IgG antibodies in hFIB-immunized mice (Fig. 1E). These data suggest that hFIB is a citrullinated protein and promotes specific humoral responses in hFIB-immunized mice.

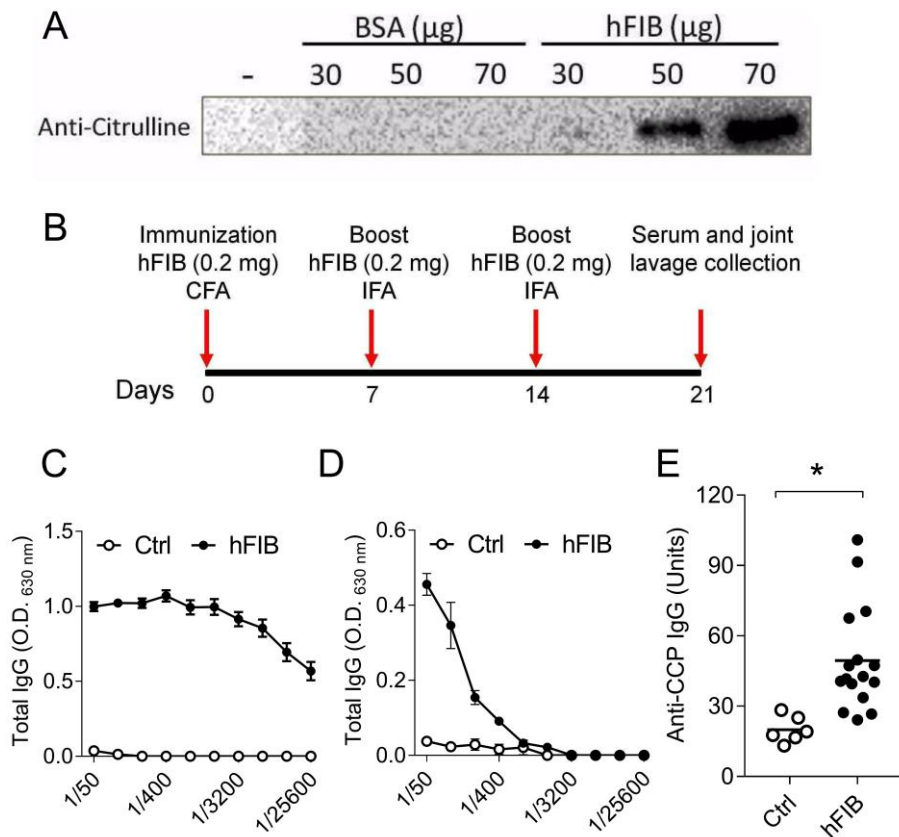


Figure 1. Purified hFIB has citrulline residues and induces the production of ACPAs. (A) Western blot analysis of citrullinated residues using an anti-peptidyl-citrulline monoclonal antibody.

Different amounts (30, 50, and 70 μ g) of hFIB and BSA were used to investigate the presence of citrulline modification. (B) Representative scheme of immunization protocol and sample collection. (C-D) Levels of anti-hFIB specific IgG antibodies quantified by ELISA in serum (C) and articular lavage (D) from Ctrl (open circle) or hFIB-immunized (close circle) mice (n=5). (E) Anti-CCP IgG antibodies quantified in the serum from Ctrl- or hFIB-immunized mice (n = 5-16). Data are represented by mean \pm S.E.M. * $P < 0.05$ vs. Ctrl group.

3.2. The articular challenge of immunized mice with hFIB induces features of rheumatoid arthritis

To investigate whether hFIB could mediate pro-arthritic effects in hFIB-immunized mice, we designed different experimental protocols that are represented in Fig. 2A. Mice were immunized either with hFIB and then challenged with knee joint injection of hFIB. Ctrl-immunized mice were also challenged with intra-articular injection of hFIB (10 μ g/joint), and naïve mice received intra-articular injection of PBS. After 7 h of the challenge, we found that hFIB promoted mechanical hyperalgesia in hFIB-immunized mice, independently of hFIB amounts used during the challenge (0.1, 1, and 10 μ g/joint) (Fig. 2B). This was accompanied by enhanced tissue injury in hFIB-immunized mice, evidenced by a loss of proteoglycan, and pronounced leukocyte infiltration determined by H&E stains of the knee joints (Fig. 2C-D). In fact, RA pathogenesis typically involves leukocyte infiltration into the joint, leading to cartilage destruction and bone erosion [21].

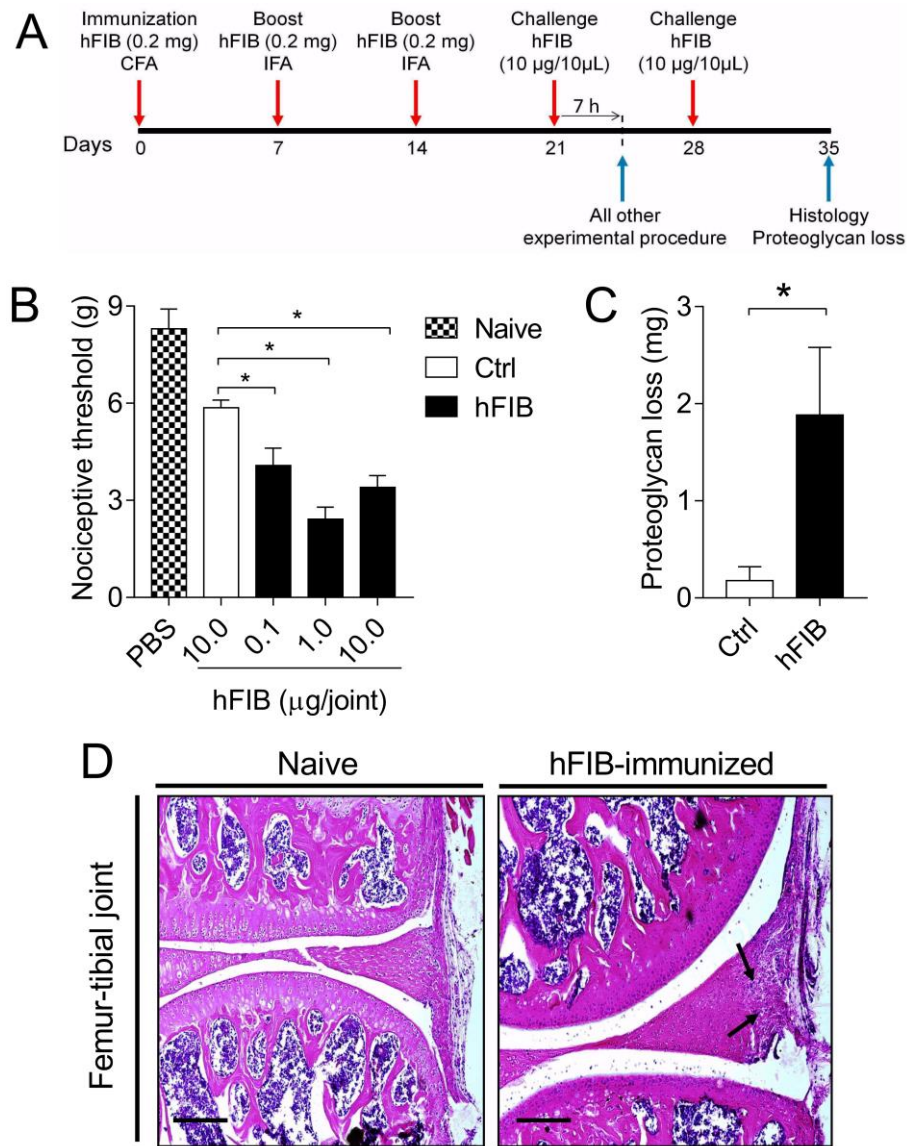


Figure 2. The articular challenge of immunized mice with hFIB leads to increased hyperalgesia and joint damage. (A) Representative scheme of immunization protocol for arthritis induction and joint damage evaluation. (B) Articular mechanical nociceptive response evaluated 7 h after intra-articular challenge with hFIB in different doses as indicated. Ctrl-immunized mice were challenged with hFIB (10 µg/joint), and naïve mice received PBS. (C) Proteoglycan loss was determined in patellar bone. (E) Hematoxylin-eosin staining of the knee joint section of naïve and hFIB-immunized mice. Black arrows indicate the region of the hyperplastic synovium. Scale bar indicates 200 µm. Data are represented by mean ± S.E.M (n = 5). * $P < 0.05$ vs Ctrl group.

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4 279 Importantly, at 7 h after the hFIB challenge, we observed that hFIB-immunized mice
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6 280 exhibited a massive leukocyte infiltration (Fig. 3A). Of note, 95% of infiltrating cells
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9 281 at this time point were granulocytes (data not shown). Additionally, we also examined
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11 282 *ex vivo* and *in vivo* MPO activity in the knee joints followed by the hFIB challenge.
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14 283 *Ex vivo* MPO activity in hFIB-immunized mice was significantly higher than vehicle-
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16 284 immunized group (Fig. 3B). Similar data were observed *in vivo* since there were
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19 285 higher bioluminescent signals for MPO activity in the knee joint from mice immunized
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21 286 and challenged with hFIB, now designated by FIBIA mice (Fig. 3C-D). Furthermore,
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24 287 we found increased levels of IL-17A and IL-1 β in the knee joint from hFIB-immunized
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26 288 mice compared to the vehicle group (Fig. 3E-F). These data collectively suggest that
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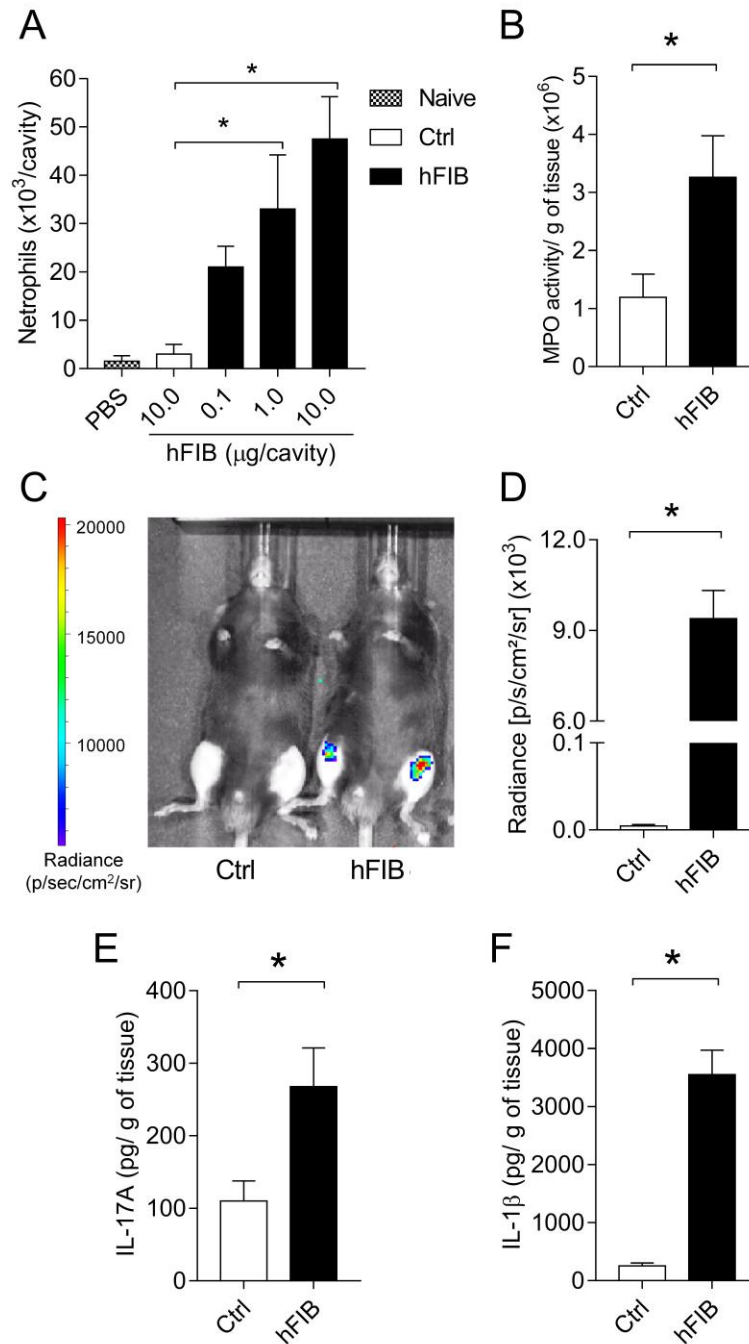


Figure 3. The articular challenge of immunized mice with hFIB leads to massive leukocyte infiltration and cytokine production. (A) Articular neutrophil infiltration evaluated 7 h after intra-articular challenge with hFIB in different doses as indicated. Ctrl-immunized mice were challenged with hFIB (10 µg/joint), and naïve mice received PBS. Neutrophil infiltration was determined by counting cells collected in joint cavity lavage. (B) MPO activity in knee joint homogenate was determined 7 h after the hFIB-challenge (10 µg/joint). (C-D) *In vivo* MPO activity was assessed as

bioluminescence emission resulting from the reaction between MPO and XenoLight™ RedJect Inflammation Probe. (C) Images were acquired using IVIS imaging system 10 minutes after probe injection (i.p.) and, (D) MPO activity was represented as radiance intensity. (E-F) Levels of IL-17 and IL-1 β in the supernatant of joint homogenates were quantified through ELISA assay. Data are represented by mean \pm S.E.M (n = 5). * $P < 0.05$ vs Ctrl group.

3.3. Th17 cells are required for the development of hFIB-induced arthritis

Th17 cells have a crucial role in RA pathogenesis [22,23]. Thus, we sought whether Th17 cells could have a role in the hFIB-induced arthritis model. Notably, we observed that hFIB-immunized mice had increased frequency of CD4⁺IL-17A⁺ cells in the draining (inguinal) lymph nodes (Fig. 4A top charts, and 4B). There were no changes in the frequency of CD4⁺IFN γ ⁺ cells (Fig. 4A bottom charts, and 4C). In addition, in an antigen-specific recall experiment, cells from lymph nodes of hFIB-immunized mice re-stimulated *in vitro* with hFIB showed enhanced secretion of IL-17A, IL-23, and IFN γ (Fig. 4D-F). There were no differences between groups in cells re-stimulated with non-specific stimuli (e.g., ConA; data not shown).

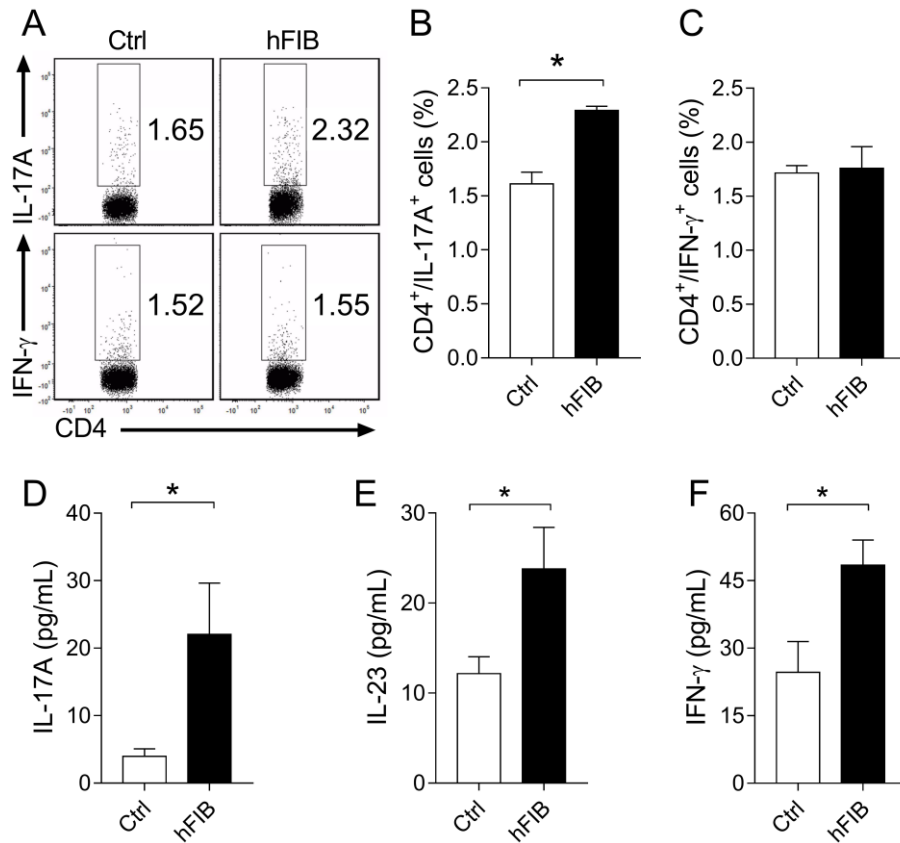


Figure 4. Citrullinated hFIB induces Th17 cell response. (A-C) Flow cytometry analysis of cells from inguinal lymph nodes. Representative images derived from cell acquisition in a flow cytometer (A) and frequencies of CD4⁺ cells producing IL17 (B) or IFN-γ (C). (D-F) Cytokine levels in the culture supernatant of draining lymph node cells from hFIB-immunized or vehicle-immunized mice. Cells were stimulated for 96 h with hFIB (100 μg/mL), and levels of IL-17A (D), IL-23 (E), and IFN-γ (F) were tested by ELISA. Data are represented by mean ± S.E.M (n = 5). * *P* < 0.05 vs Ctrl group.

Finally, to further support our *ex vivo* findings, hFIB-induced arthritis was induced in genetically-deficient mice for IL-17RA (*Il17ra*^{-/-}), IL-23p19 (*Il23a*^{-/-}), and IFN-γ (*Ifng*^{-/-}). Compared to WT mice, hypernociceptive response was significantly reduced in *Il17ra*^{-/-} and *Il23a*^{-/-} mice (Fig. 5A and 5B). Consistently, there was diminished leukocyte infiltration into the joint from both *Il17ra*^{-/-} and in *Il23a*^{-/-} mice (Fig. 5D and 5E). Besides, we also found that *Il17ra*^{-/-} mice have lower articular proteoglycan loss

than WT mice (supplementary Figure 1). In contrast, clinical and inflammatory parameters were similar between *Ifng*^{-/-} and WT mice (Fig. 5C and F). Taken together, these findings indicate that the IL-23/IL-17 immune axis is critical for arthritis development induced by hFIB.

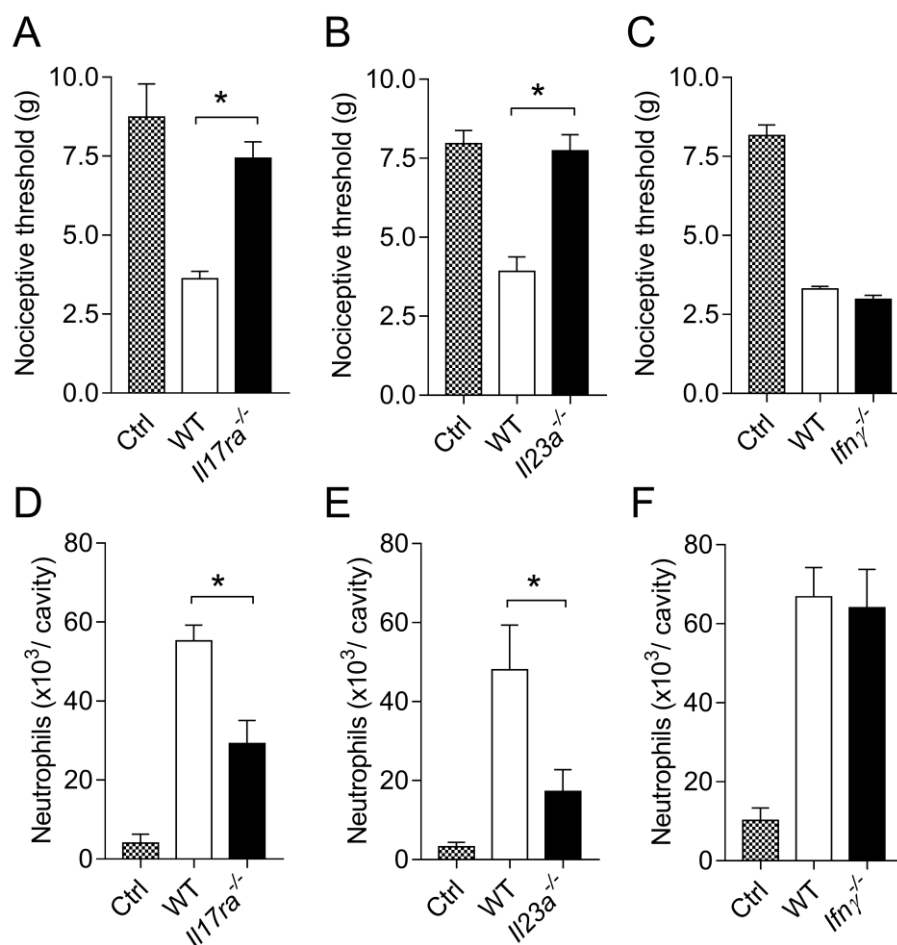


Figure 5. IL-17 and IL-23 but not IFN-γ are required to arthritogenic responses induced by citrullinated hFIB. (A-C) Knee joint hypernociception was evaluated by electronic Von Frey 7 h after the challenge with hFIB (10 μg/ joint). Responses of WT mice were compared to those of *Il17ra*^{-/-} (A), *Il23a*^{-/-} (B), and *Ifng*^{-/-} (C). Hypernociception was expressed as nociceptive threshold (g). (D-F) Neutrophil infiltration was quantified in articular lavage from hFib-immunized WT mice compared to

Il17ra^{-/-} (D), *Il23a*^{-/-} (E), and *Ifng*^{-/-} mice (F). Data are represented by mean ± S.E.M (n = 5). * *P* < 0.05 compared to WT mice.

4. Discussion

In the present study, using citrullinated hFIB as an immunogen, we modified the classical AIA model [17] to investigate the immunopathologic events that lead to inflammation-driven joint damage in C57BL/6 mice induced by citrullinated fibrinogen. To this end, mice were immunized with citrullinated hFIB and then subsequently challenged with intra-articular injection of citrullinated hFIB into the knee joint. Similar to the AIA model, we hypothesize that the challenge with hFIB would induce an immune complex formation in the joint that triggers a monoarthritis feature. Indeed, hFIB immunized mice showed increased mechanical hyperalgesia, massive leukocyte infiltration, and high levels of inflammatory mediators in the knee joint after the intra-articular challenge with citrullinated hFIB.

Autoantibodies to citrullinated fibrinogen are increased in the blood of RA patients [24,25], and the transfer of antibody to citrullinated fibrinogen into DBA/1J mice enhances tissue injury in collagen antibody-induced arthritis (CAIA) model [26]. Thus, citrullinated fibrinogen has been suggested as an auto-antigen able to trigger an inflammatory response in RA patients. In our study, we detected high levels of anti-ACPA IgG antibodies in the serum of hFIB-immunized mice. Therefore, we hypothesize that the presence of citrullinated residues in hFIB might be responsible for triggering, at least in part, the immune response observed during hFIB-induced arthritis. Indeed, citrullinated residues in hFib are necessary to trigger the immune response and promote arthritis. It was demonstrated that neither specific T cell

response nor auto-reactive antibody production is evidenced when unmodified hFib is used during the immunization process [14,27]. Moreover, there is a close correlation between the degree of citrullination of fibrinogen and disease activity in RA patients [28]. Thus, the citrullination of fibrinogen appears to govern its immunogenicity.

Th17 cells play a pivotal role in RA pathogenesis, interacting with synovial fibroblasts and mediating tissue injury [29]. Either genetic deficiency or antibody blockade of IL-17A reduces arthritis progression and joint damage [30,31]. Moreover, approaches that modulate Th17 cell responses have been proposed as potential targets for therapeutics [32,33]. Besides, IL-23 is an essential mediator involved with the differentiation and maintenance of the Th17 phenotype [34]. Consistently, we demonstrated that draining lymph node cells from hFIB-immunized mice showed higher Th17 cell frequencies and produced high levels of IL-17A and IL-23 in response to hFIB. Importantly, genetic ablation of IL-17RA or subunit IL23p19 resulted in a reduction of arthritis's clinical and inflammatory parameters and reduced joint damage. In accordance, peripheral blood mononuclear cells (PBMCs) from RA patients stimulated with citrullinated aggrecan, a component of articular cartilage, produce high levels of IL-17A [35]. Moreover, in the collagen-induced-arthritis (CIA) model, a subpopulation of CD4⁺IL-17A⁺ T cells has been reported to be responsive to citrullinated fibrinogen stimulation [36]. In contrast, we showed that deficiency of IFN- γ failed to reduce clinical and inflammatory parameters of arthritis, suggesting that Th1-mediated immune response is not required for hFIB-induced arthritis. Thus, our data suggest a central contribution of the IL-23/IL-17 immune axis for arthritis induced by citrullinated hFIB.

In summary, we developed a novel experimental arthritis model proposed here as fibrinogen-induced arthritis (FIBIA). The arthritis was associated with an efficient formation of ACPA autoantibodies, intense mechanical hyperalgesia, massive inflammatory cell infiltration, synovial hyperplasia, and Th17 cell-mediated immune response involvement, reflecting typical features of RA pathogenesis. Mouse models of arthritis using citrullinated fibrinogen have been previously reported. For instance, humanized transgenic DR4-IE mice, expressing RA-associated to MHC-II molecule DRB1*0401, develop arthritis when immunized with citrullinated hFIB [13,14]. Also, DBA/1J and SJL/J mice develop arthritis after immunization with citrullinated hFIB [15,16]. DBA/1 mice are widely used in collagen-induced arthritis (CIA) model because they also develop arthritis after immunization with type II collagen [37]. CIA shares similarities with human RA and is considered the gold standard of experimental models of RA [38]. However, the ability to study the effect of targeted gene deletion on immunopathologic events of CIA and hFIB-induced arthritis is problematic in DBA/1J and SJL/J mice as most transgenic mice are on the C57BL/6 background. The experimental design of the FIBIA model in C57BL/6 mice is relatively simple, shares many similarities with human RA, and has the advantage of allowing the use of gene-deficient mice in the C57BL/6 background, providing a suitable mouse model to investigate the contribution of ACPAs and Th17 cell-mediated immune response in the pathogenesis of RA. Moreover, the FIBIA model may serve as a valuable tool for pre-clinical studies aiming to discover new potential therapeutic targets for RA.

Abbreviations

ACPA: anti-citrullinated peptide/protein antibodies; BSA: bovine serum albumin; CFA: Complete Freud's Adjuvant; ConA: concanavalin A; DMMB: dimethyl methylene blue; hFIB: human fibrinogen; IFA: Incomplete Freund's Adjuvant; MPO: myeloperoxidase; PAD: peptidyl arginine deiminase; PBMC: peripheral blood mononuclear cell; PMA: phorbol-myristate-acetate; RA: rheumatoid arthritis; RF: rheumatoid factor; Th: T helper.

Competing interests

The authors declare that they have no competing interests.

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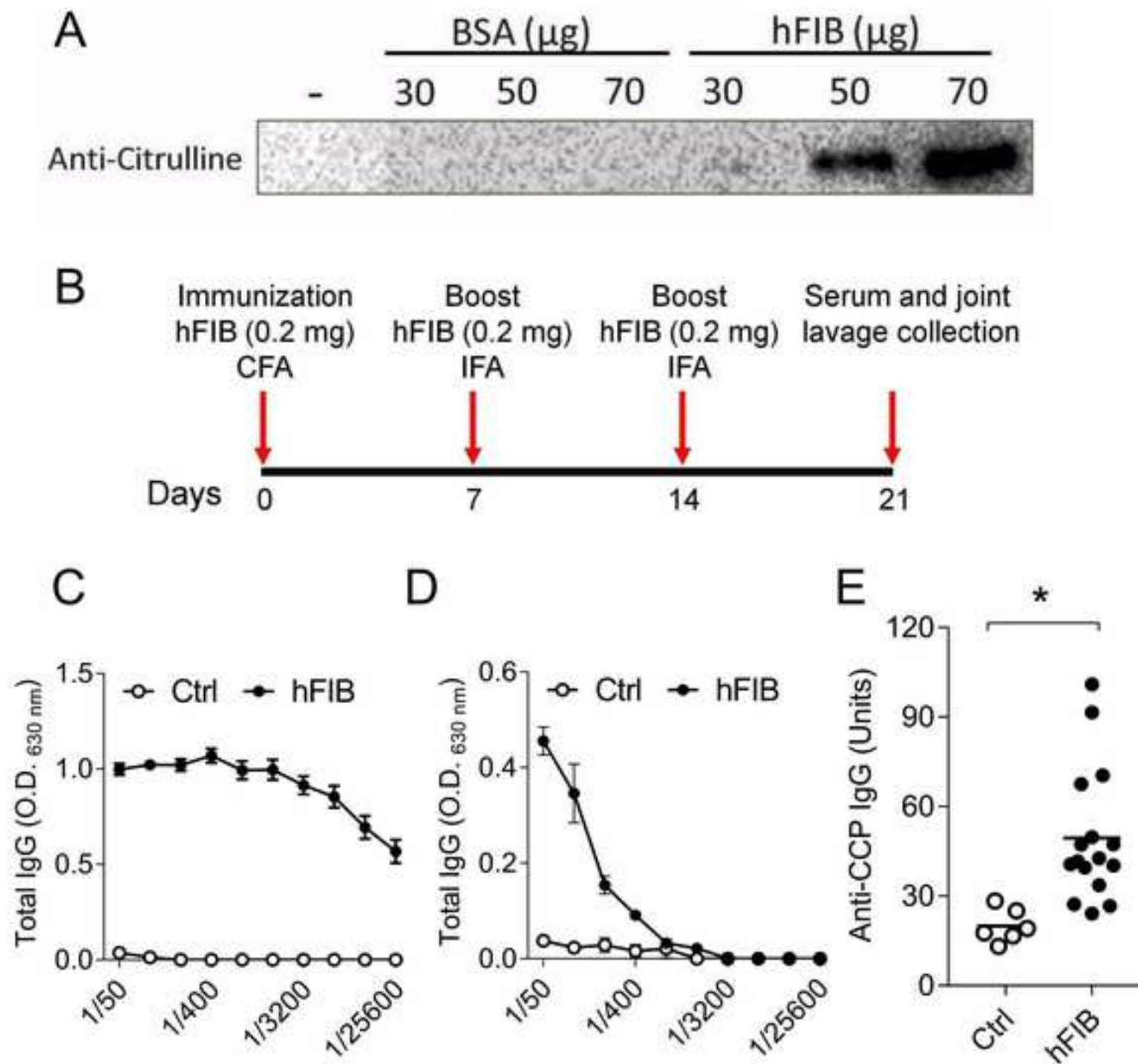
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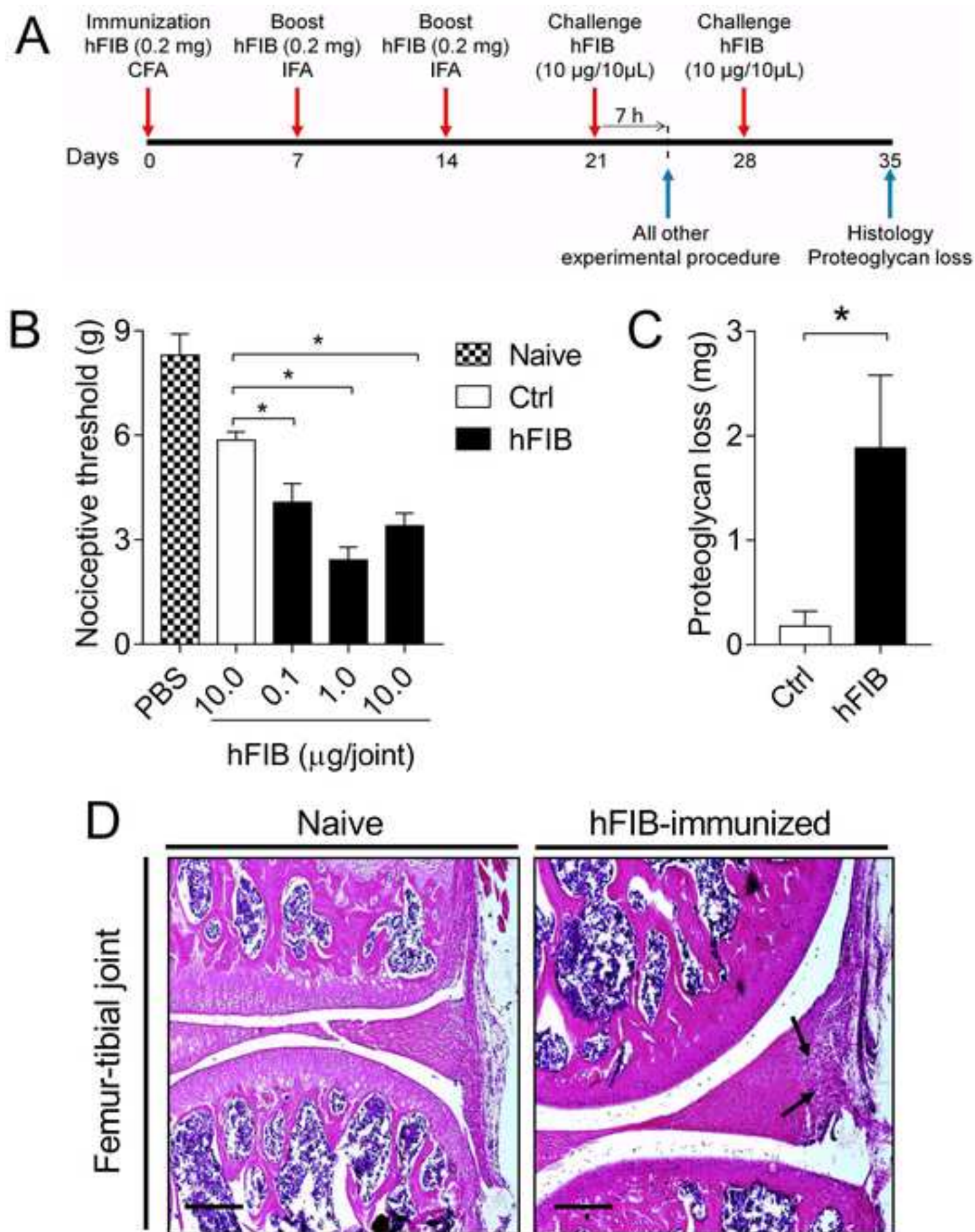
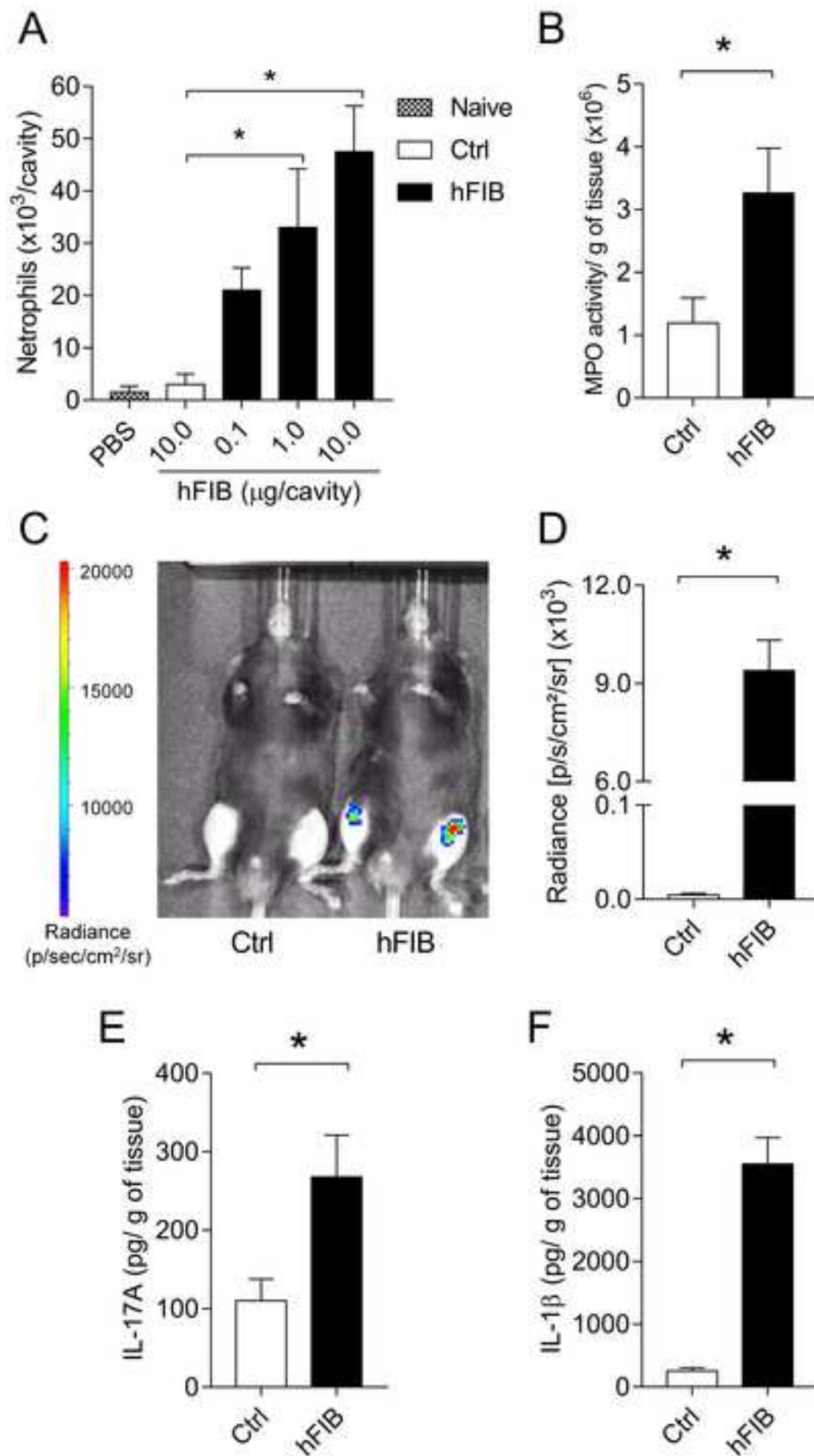
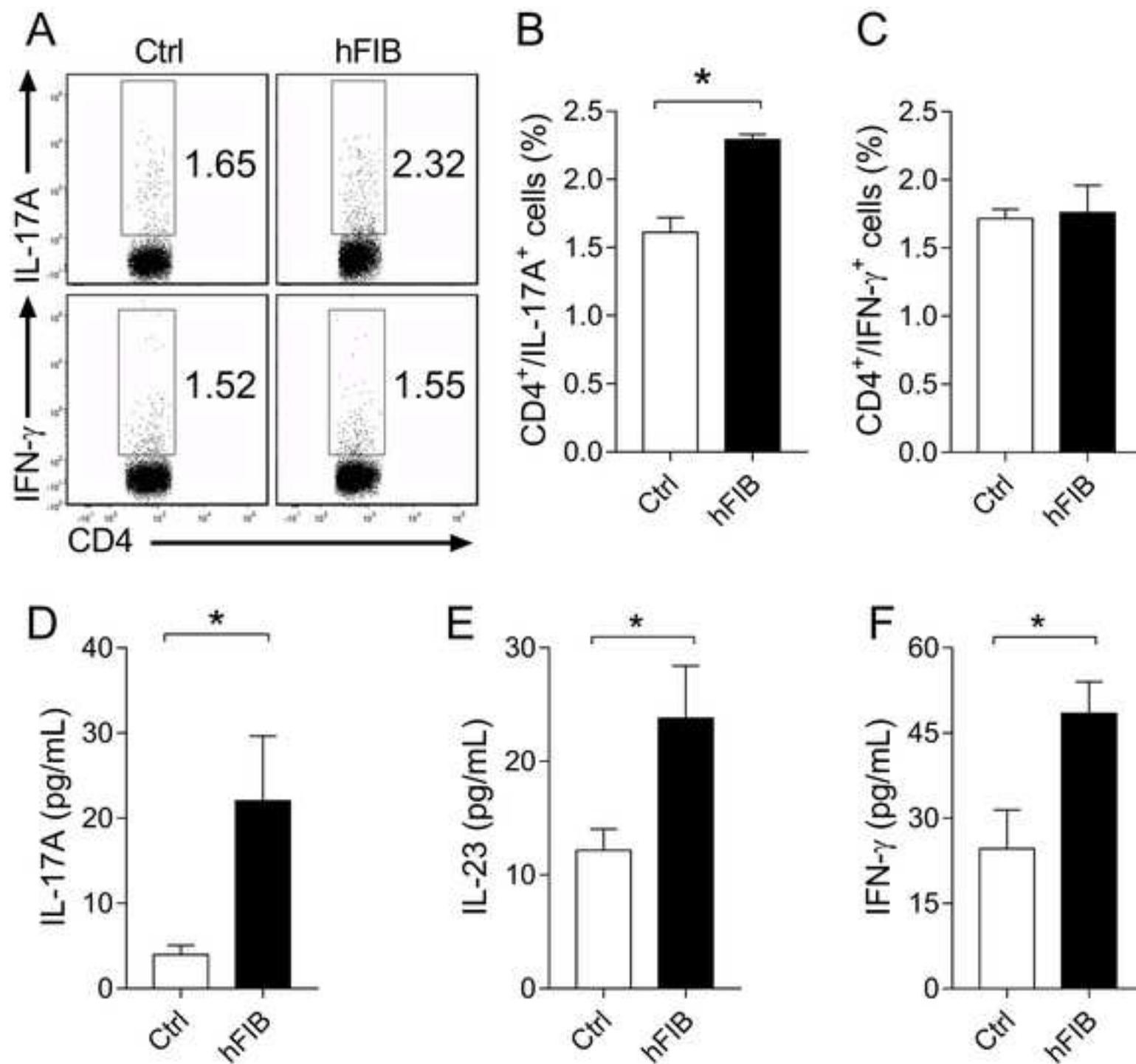
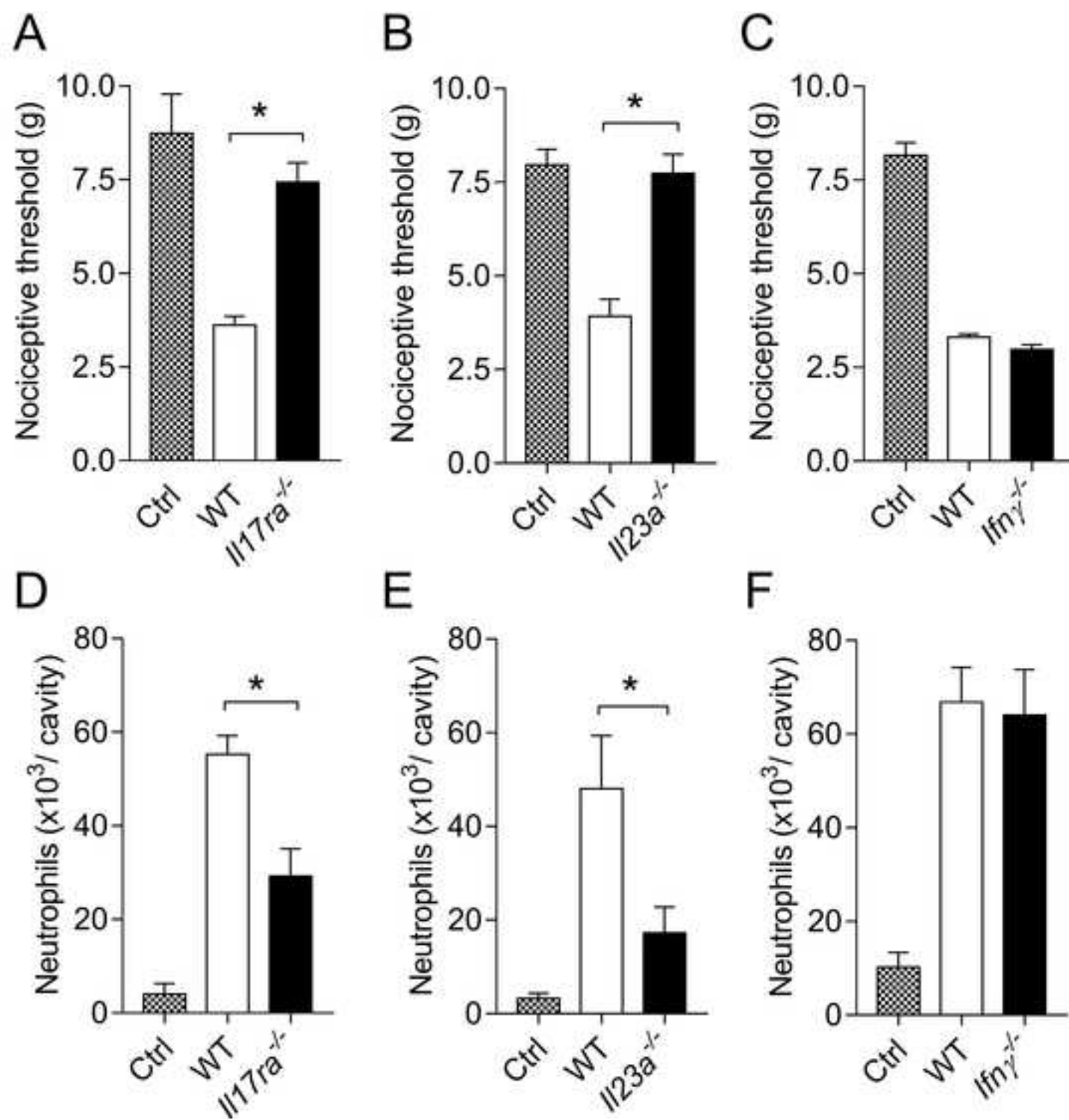


Figure 3







Author statement

ALS: Conceptualization, Methodology, Formal analysis Writing-Original draft.

RSP: Conceptualization, Methodology, Writing-Original draft. **FPV:**

Methodology, Investigation. **KAL:** Methodology, Investigation. **JT:** Methodology,

Investigation. **JPML:** Methodology, Investigation. **TMC:** Methodology,

Resources. **PLJ:** Methodology, Resources. **FQC:** Methodology, Resources.

JCAF: Methodology, Supervision, Writing-Review and Editing, Funding

Acquisition. All authors read, revised, and approved the manuscript.



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Supplementary Material

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