Pharmacology

Research Article

Pharmacology
DOI: 10.1159/000528780

Received: August 16, 2022
Accepted: December 12, 2022
Published online: January 25, 2023

Modulation of Neutrophil Function by Recombinant Human IgG1 Fc Hexamer in the Endogenous K/BxN Mouse Model of Rheumatoid Arthritis

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Keywords
Arthritis · Rheumatoid arthritis · Inflammation · Interleukin 1 · Neutrophil · Mouse model

Abstract

Introduction: Neutrophils are a pivotal cell type in the K/BxN mouse model of rheumatoid arthritis and play an essential role in the progression of the arthritis. They are readily activated by immune complexes (ICs) via their FcγRs to release IL-1β in addition to other cytokines, which are inducing cartilage destruction. Neutrophils also release neutrophil-active chemokines to recruit themselves in an autocrine manner to perpetuate tissue destruction. FcγR-expression on neutrophils is of crucial importance for the recognition of ICs. ***Methods:*** In this study, due to its high avidity for binding to FcγRs, we investigated the potential anti-inflammatory effect of a recombinant IgG1 Fc hexamer (rFc-μTP-L309C) on neutrophils in the K/BxN mouse model of endogenously generated chronic arthritis. 200 mg/kg rFc-μTP-L309C and human serum albumin (HSA), used as controls, were administered subcutaneously every other day. Mouse ankle joints were monitored daily to generate a clinical score. Immunohistology was used to evaluate neutrophil infiltration and TUNEL to assess apoptosis. ELISA was used to measure IL-1β. ***Results:*** Treatment with rFc-μTP-L309C, but not HSA, was able to significantly ameliorate the arthritis in the K/BxN mice. Significant neutrophil infiltration into the ankle joint was found, but treatment with rFc-μTP-L309C resulted in significantly less neutrophil infiltration. There was no significant influence of rFc-μTP-L309C on neutrophil death or apoptosis. Less neutrophil infiltration could not be correlated to chemokine-mediated migration. Significantly less IL-1β was measured in mice treated with rFc-μTP-L309C. ***Conclusion:*** In the endogenous K/BxN mouse model of rheumatoid arthritis, amelioration can be explained in part by inhibition of neutrophil infiltration into the joints as well as inhibition of IL-1β production. Given the observed inhibitory proper-

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ties on neutrophils, rFc-μTP-L309C may be a potential therapeutic candidate to treat autoimmune and inflammatory conditions in which neutrophils are the predominant cell type involved in pathogenesis.

Introduction

Neutrophils are arguably one of the most important effector cells in the induction of arthritis in the K/BxN serum transfer model [1]. This has been supported by numerous studies that have shown that neutrophil depletion or neutrophil-deficient mice are resistant to arthritis [2–4]. FcγR mediated activation of neutrophils is necessary for the initiation and progression of arthritis because it mediates the release of, e.g., IL-1β. IL-1β is a crucial driver of cartilage destruction in the K/BxN serum transfer model [5, 6]. When IL-1β is released into the joint, it promotes the release of neutrophil-activating chemokines such as CXCL1, CXCL5, and CCL9 from resident tissue cells in the synovium [7]. This causes neutrophils to recruit themselves in an autocrine manner to perpetuate tissue destruction [7–9]. Given the fact that FcγR engagement with immune complexes (ICs) is crucial for mediating arthritis development, we decided to investigate the effect(s) on neutrophils of a recombinant IgG1 Fc with a modified IgM tail piece having a mutation of lysine to cysteine (rFc-μTP-L309C), given its high avidity binding to FcγR [10].

Use of recombinant human Fc multimers to replace IVIG for treatment of autoimmune/inflammatory conditions is of growing interest [10, 11]. rFc-μTP-L309C is a hexameric recombinant Fc multimer. It was produced by fusing the 18-aa IgM tailpiece to the *C*-terminus of a variant human IgG1 Fc with a point mutation at position 309, and this point mutation stabilizes the hexamer through the formation of disulfide bridges [10]. We have previously shown that rFc-μTP-L309C has high binding avidity for Fc receptors and could suppress arthritis in the collagen-induced arthritis, collagen-Ab-induced arthritis, and the K/BxN mouse models [10, 12]. We investigated whether the observed mechanism of rFc-μTP-L309C in the endogenous K/BxN model was due to modulation of neutrophil function.

We observed that K/BxN mice treated with rFc-μTP-L309C had significantly fewer neutrophils in their ankle joints. However, we could not demonstrate an effect on neutrophil chemotaxis.

Next, we decided to investigate whether rFc-μTP-L309C induced neutrophil death in the joints of K/BxN mice. It has been shown that IVIG can regulate the survival of human but not mouse neutrophils [13]; thus, rFc-μTP-L309C may exhibit a similar effect as it is composed of the human Fc portion of IgG1. Using the TUNEL labeling assay, we could not demonstrate any significant increase in neutrophil cell death in the joints of K/BxN mice treated with rFc-μTP-L309C in comparison to K/BxN mice with severe arthritis in control groups treated with human serum albumin (HSA). A complementary assay was done using flow cytometry, and a similar pattern emerged.

When we investigated the functional effects of rFc-μTP-L309C on neutrophils, we found that rFc-μTP-L309C binds to FcγRIII (CD16) and prevents release of IL-1β. rFc-μTP-L309C could be a potential therapeutic candidate for autoimmune and inflammatory conditions where neutrophils are the predominant cell type involved in pathogenesis.

Materials and Methods

Mice

K/BxN mice spontaneously develop severe, chronic arthritis resembling human rheumatoid arthritis [12, 14, 15]. This occurs due to crossing KRN mice, having a TCR transgene that recognizes a peptide of glucose 6-phosphate isomerase (GPI), with mice having the MHC class II molecule I-Ag7. These F1 mice spontaneously produce anti-GPI which can then interact with the GPI surrounding the joints and cause severe inflammation and arthritis [12, 14, 15]. KRN mice on a C57BL/6 background were obtained from The Jackson Laboratory, a kind gift from C. Benoist. NOD/Lt mice were purchased from The Jackson Laboratory. Arthritic mice were obtained by crossing KRN mice (F, 6 weeks old) with NOD/Lt (M, 6 weeks old) mice to produce K/BxN mice expressing both the TCR transgene KRN and the MHC class II molecule I-Ag7. BALB/c (F, 6 weeks old) mice were purchased from The Jackson Laboratory. Mice were kept under a natural light-dark cycle, maintained at 22 ± 4°C, and fed with standard diet and water ad libitum. All experiments were performed after the animal use protocols (AUP 1788) were approved by the University Health Network Animal Research Committee in Toronto.

Biological Reagents

Recombinant IgG1 Fc hexamer (rFc-μTP-L309C) was obtained from CSL Behring AG, Switzerland. HSA was obtained from the Canadian Blood Services, and its composition controlled by the US Food and Drug Administration.

Arthritis Treatment

Prophylactic Treatment

K/BxN mice were given treatment starting immediately after weaning to determine if rFc-μTP-L309C could prevent the arthri-

tis as previously described [12]. Mice were given 11 s.c. injections (one injection every other day) of 200 mg/kg rFc-μTP-L309C or HSA. HSA was used as a protein control.

Endogenous Treatment

As previously described [12], K/BxN mice with high clinical scores of 9 or greater were treated by s.c. injections of 200 mg/kg rFc-μTP-L309C or HSA on days 1, 3, 5, 7, 9, and 11. HSA was used as a protein control.

Arthritis Scoring

The clinical scores of the mice were monitored daily over the course of each experiment as previously published [12, 16]. Although the assessments were not blinded, the same individual was responsible for monitoring the clinical scores to minimize variation in the subjective assessment. The development of arthritis was assessed daily, and the severity of arthritis was scored for each paw on a 3-point scale, in which 0 = normal appearance, 1 = localized edema/erythema over one surface of the paw, 2 = edema/erythema involving more than one surface of the paw, and 3 = marked edema/erythema involving the whole paw. The scores of all four paws were added for a composite score, with a maximum score of 12 per mouse.

Neutrophil Histology

Joints were processed as previously described with slight modifications [16]. Briefly, dissected ankle joints from K/BxN mice that received 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C were embedded in OCT, frozen in liquid nitrogen, and mounted on a cryomicrotome support at −25°C. Mice treated with HSA and untreated BALB/c mice were used as controls. Sagittal sections (6–8 μm thick) were cut and transferred to an adhesive coated slide. Slides were stored at −80°C until use and then acetone-fixed for 1 min and air dried for 30 min. Sections were incubated with the primary antibody to mouse Ly6G and Ly6C (clone NIMP-R14, LifeSpan Biosciences, Seattle, WA) in 1% BSA/PBS overnight at 4°C. The slides were rinsed with PBS and incubated with the goat anti-rat IgG secondary antibody conjugated to HRP (Thermo Fisher Scientific) in 1% BSA/PBS for 1 h at room temperature. The slides were rinsed with PBS, and the neutrophils were visualized by incubation with 0.015% H_2O_2/0.05% 3,3′-diaminobenzidine (DAB) (Thermo Fisher Scientific) for 10 min at room temperature. Sections were counterstained with Mayers' hematoxylin (Sigma Aldrich). Representative images were taken using the LeicaSP8/STED Confocal Microscope, and the images were analyzed using QuPath.

Neutrophil Transwell Migration Assay

Based on a previously described method [17], bone marrow cells were collected into complete medium at 5×10^6 cells/mL, and these cell suspensions (200 μL) were added to the top of a polycarbonate transwell filter with a 3 μm pore (MilliporeSigma, Burlington, MA) inserted into a 24-well plate (Costar, Acton, MA) containing 300 μL of the above buffer with or without chemoattractant. The chemoattractant used was recombinant mouse CXCL1 (BioLegend, San Diego, CA). The plates were incubated at 37°C with or without 100 μg/mL of rFc-μTP-L309C added into the top chamber. After 1 h, the filter inserts were removed, and the cells from the upper and lower chambers were removed and retained separately. The cells recovered from each of the wells and were cytospun onto slides to be analyzed by immunofluorescence. The neutrophils were counted using a primary antibody to mouse Ly6G and Ly6C and goat anti-rat IgG secondary antibody conjugated to AlexaFluor 555 (Thermo Fisher Scientific). Neutrophils were counted using ImageJ. The results are expressed as the mean ± standard deviation (SD) of the chemotactic index (CI) for triplicate wells. The CI represents the fold change in the number of untreated cells that migrated in response to the chemoattractant divided by the basal migration of untreated cells that migrated in response to control medium.

TUNEL Assay

Dissected ankle joints from K/BxN mice that received 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C were embedded in OCT, frozen in liquid nitrogen and mounted on a cryomicrotome support at −25°C. Mice treated with HSA and untreated BALB/c mice were used as controls. Sagittal sections (6–8 μm thick) were cut and transferred to an adhesive-coated slide. Slides were stored at −80°C until use, then acetone-fixed for 1 min, and air-dried for 30 min. To analyze neutrophil cell death in the ankle joint, neutrophils were stained with a primary antibody to mouse Ly6G and Ly6C and goat anti-rat IgG secondary antibody conjugated to AlexaFluor 555. DNA fragmentation was examined by TUNEL according to the manufacturer's instructions using the In-Situ Cell Death Detection Kit (Sigma Aldrich). Briefly, a deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture, which incorporates fluorescein dUTP at DNA strand breaks, was used to detect apoptotic neutrophils. Nuclei were counterstained with DAPI (Thermo Fisher Scientific). Representative images were taken using the LeicaSP8/STED Confocal Microscope and the images were analyzed using QuPath.

Determination of Cell Death and Apoptosis

As previously described [13], bone marrow cells were collected into complete medium, and neutrophils were enriched using the EasySep mouse neutrophil enrichment kit (Stemcell Technologies). Neutrophils were incubated at 37°C for 4 h, 14 h, and 24 h with medium or with 100 μg/mL of rFc-μTP-L309C. To analyze neutrophil cell death, neutrophils were stained with Ly6G conjugated to APC (clone 1A8, BioLegend). Dead cells and apoptotic cells were labeled using an AnnexinV/Dead Cell Apoptosis Kit (Thermo Fisher Scientific). Cells were incubated at 37°C for 4 h, 14 h, and 24 h with mouse recombinant TNFα (R&D Systems) as a positive control and with mouse recombinant GM-CSF (R&D Sytstems) as a negative control. Staining was analyzed by flow cytometry on a BD LSR Fortessa (BD Biosciences), and the data were analyzed by using FlowJo software (Ashland, OR).

Neutrophil Respiratory Burst

For luminometry, bone marrow cells were collected into complete medium, and neutrophils were enriched using the EasySep Mouse Neutrophil Enrichment Kit [10]. Neutrophils were added to the wells of a microtiter plate containing 100 μL of luminol solution (Sigma Aldrich), and medium or 1 μg/mL diphenyleneiodonium chloride (BioShop, Burlington, ON) (negative controls), 1 μg/mL of mouse IgG and goat anti-mouse IgG immobilized on the plate (immobilized immune complexes [ilICs]) (Sigma Aldrich) (positive control), or 100 μg/mL of rFc-μTP-L309C were added. Chemiluminescence was recorded at 37°C for 30 min on a TECAN Infinite 200 PRO microplate reader (Tecan, Mannedorf, Switzer-

land). The area under the signal-to-time curve was calculated, and the results were expressed as relative light units.

For flow cytometry, 100 μg/mL of rFc-μTP-L309C, 1 μg/mL of mouse IgG and goat anti-mouse IgG (Sigma Aldrich), or medium (negative control) were added to neutrophils enriched from the bone marrow of K/BxN mice for 10 min at 37°C in a water bath. Respiratory burst activity was assessed using dihydrorhodamine-123 (Thermo Fisher Scientific). FITC staining was analyzed by flow cytometry on a BD LSR Fortessa, and the data were analyzed by using FlowJo software.

Binding of rFc-μTP-L309C to FcγRIII on Neutrophils

Neutrophils were enriched from the bone marrow of K/BxN mice using the EasySep mouse neutrophil enrichment kit. Neutrophils were incubated with or without 100 μg/mL of rFc-μTP-L309C for 30 min at 37°C and stained for Ly6G-APC, CD11b-FITC (BioLegend), and FcγRIII-PE (Thermo Fisher Scientific). FcγRIII staining was analyzed by flow cytometry on a BD LSR Fortessa, and the data were analyzed by using FlowJo software.

Il-1β ELISA

In vitro, neutrophils were enriched from the bone marrow of K/BxN mice using the EasySep mouse neutrophil enrichment kit. Neutrophils were preincubated with 100 μg/mL of rFc-μTP-L309C for 15 min at 37°C, followed by 1 μg/mL of mouse IgG and goat anti-mouse IgG for 16 h at 37°C. IL-1β levels were analyzed using the IL-1β ELISA Kit (R&D Systems, Minneapolis, MN, USA).

In vivo, IL-1β levels from the joints of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C were analyzed using the IL-1β ELISA Kit (R&D Systems). Mice treated with HSA were used as a control.

Joint Washes

Joint washes were performed as previously described [16]. Malleoli and surrounding soft tissue (excluding fat) were removed from both rear limbs and placed in RPMI 1640 + 5% FCS on ice for 60 min. The medium was then removed, centrifuged, and the supernatant (joint wash) stored at −20°C until subsequent analysis.

Safranin-O Stain

Mice were euthanized, and the left rear paws were fixed in 10% neutral-buffered formalin, decalcified, and embedded in paraffin. Sagittal tissue sections were stained with H&E and Safranin-O (HistoTox, Boulder, CO) [18]. Cartilage depletion was identified by the presence of diminished Safranin-O staining of the matrix and was indicated with an arrow.

Statistical Analysis

Statistical tests were performed using GraphPad Prism 8 for Windows software. Analyses of differences between sample groups were performed using the tests indicated in the figure legends. Different statistical analyses were used depending on the data and included Kruskal-Wallis with Dunn test, one-way ANOVA with Dunnett test, and Mann-Whitney test. Data shown are mean ± SD, unless otherwise stated. $p < 0.05$ was considered statistically significant.

Results

rFc-μTP-L309C Given Prophylactically Prevents Arthritis and Cartilage Damage

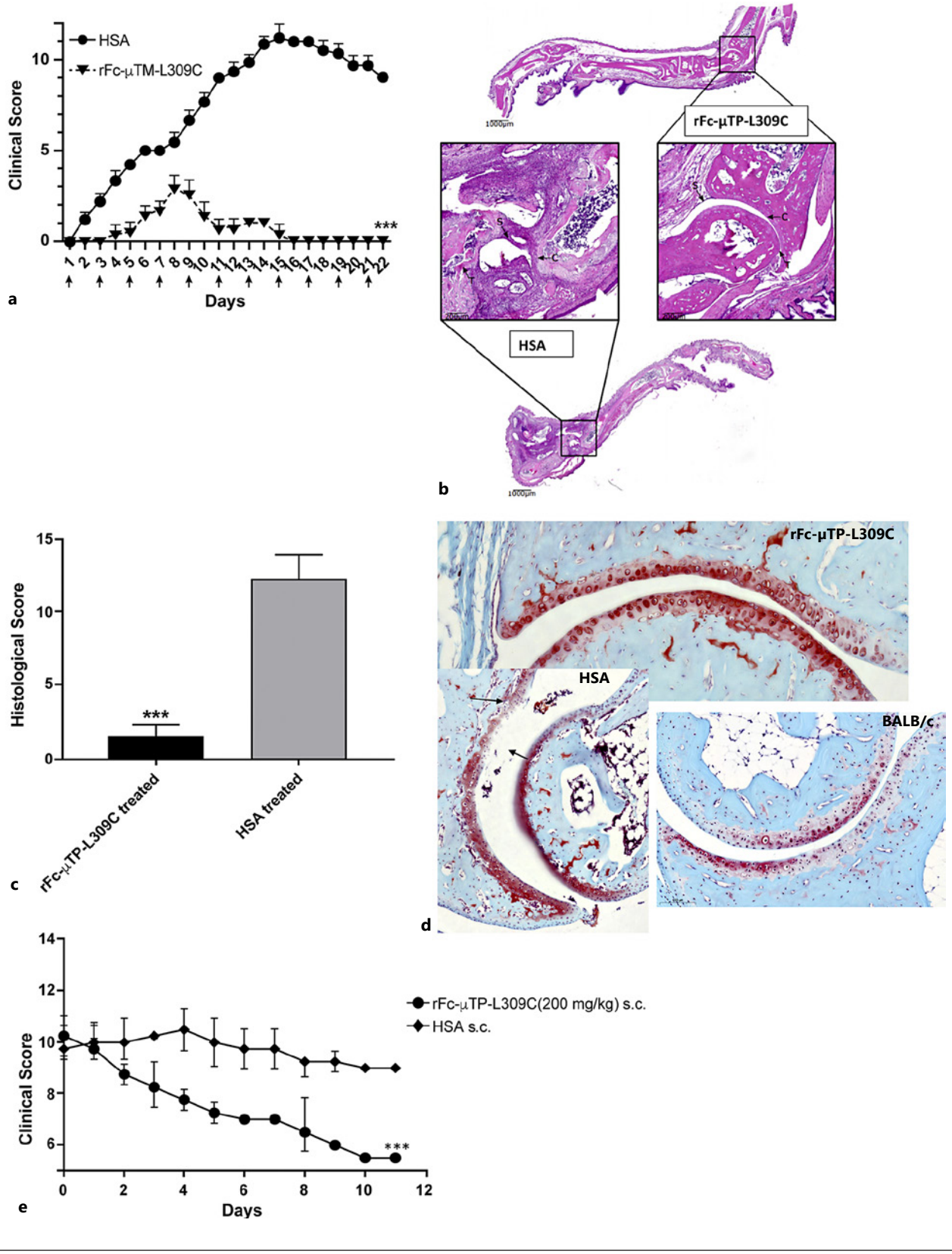
Mice given 11 s.c. injections of HSA developed severe arthritis, while mice given 200 mg/kg rFc-μTP-L309C failed to develop arthritis (Fig. 1a). Sagittal tissue sections from the left rear paws were stained with H&E and scored blinded to the treatment groups (Fig. 1b, c). Ankle joints were scored for exudate (presence of inflammatory cells within the joint space) (Fig. 1b). The histological score was significantly elevated for the HSA-treated mice compared to the rFc-μTP-L309C-treated mice (Fig. 1c). In addition, evaluation of cartilage damage in the joint using Safranin-O stain indicated that mice treated with HSA showed significant cartilage erosion (Fig. 1d) while mice treated with rFc-μTP-L309C showed no cartilage damage, like wild-type BALB/c mice.

rFc-μTP-L309C Ameliorates Severe, Chronic Arthritis

We next confirmed previous work [11] showing that rFc-μTP-L309C can ameliorate the severe arthritis that develops in the K/BxN mice (Fig. 1e). However, the mechanism of action of the recombinant protein is unknown. Therefore, herein, we focused our investigation on the neutrophil, as this granulocyte is known to be involved in arthritis [1–6] and is the likely cell infiltrate observed in Figure 1b.

Fig. 1. Prevention and amelioration of arthritis with Fc-μTP-L309C treatment in K/BxN mice. **a** The clinical scores are shown for mice treated with 11 injections of Fc-μTP-L309C or HSA before the development of arthritis in K/BxN mice. Injections were given on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 as indicated by arrows in the figures. Data shown as mean ± SD. ****$p < 0.0001$, 200 mg/kg Fc-μTP-L309C versus HSA. **b** The representative H&E-stained sections of ankle joints, where C indicates cell infiltration, T indicates tissue destruction, and S indicates sites of synovial inflammation. **c** The histological scores of Fc-μTP-L309C-treated mice and HSA-treated mice after 11 s.c. injections are shown here. Data show the mean ± SD histological scores of joints at day 21 of disease. ***$p < 0.001$, compared with HSA-treated mice. **d** The representative Safranin-O-stained sections of ankle joints of BALB/c mice, HSA-treated mice, and Fc-μTP-L309C-treated mice after 11 s.c. injections. Arrows indicate cartilage erosion here. **e** The clinical scores are shown for K/BxN mice given 6 s.c. injections of 200 mg/kg of Fc-μTP-L309C (mean ± SD) post the development of severe arthritis. ***$p < 0.001$ compared with HSA.

(For figure see next page.)


HSA
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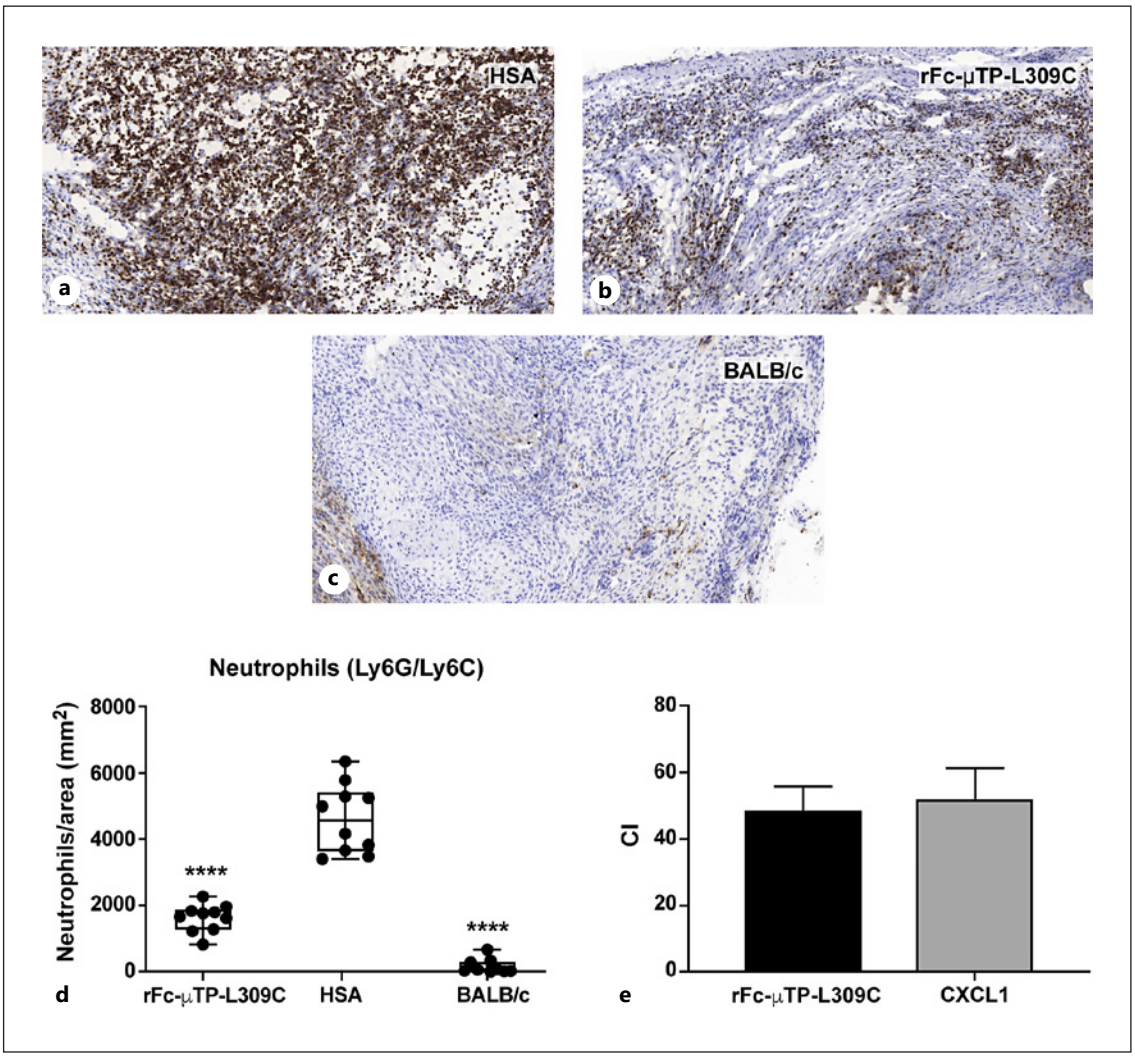

Fig. 2. rFc-μTP-L309C reduces neutrophils in the joints of K/BxN mice without chemotactic interference. **a–c** Representative images of ankle sections from K/BxN mice that were given 6 s.c. injections of 200 mg/kg of Fc-μTP-L309C (**a**) or treated with HSA (**b**; positive control). **c** BALB/c mice were used as negative controls. Ankle sections were stained for anti-Ly6G/anti-Ly6C (neutrophils) in brown and the tissue was counterstained with Hematoxylin. **d** The number of neutrophils/area (mm^2) is shown; error bars indicate the range of neutrophils/area (mean ± SD, $n = 10$). ****$p < 0.0001$ compared with HSA, Kruskal-Wallis with the Dunn test. **e** The cells that migrated to the bottom chamber in response to the chemotactic gradient were collected and enumerated by fluorescence microscopy. The results are expressed as the mean ± SD of the chemotactic index (CI) of triplicate cultures.

rFc-μTP-L309C Effect on Neutrophil Migration

K/BxN mice that were at high clinical scores of 9–12 were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of rFc-μTP-L309C, and HSA-treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and stained for neutrophils using immunofluorescence microscopy. The neutrophils were quantified using Qupath. There were significant numbers of neutrophils in the ankle joints of mice that were treated with HSA (Fig. 2a). There were significantly fewer neutrophils in the ankle joints of mice that were treated with 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C (Fig. 2b). There were no neutrophils in the ankle joints of BALB/c mice (Fig. 2c). The number of neutrophils in the joints of HSA- or rFc-μTP-L309C-treated mice compared to BALB/c control mice is quantified in Figure 2d.

This decrease in neutrophil infiltration was further investigated as possibly due to an effect on chemokine-mediated migration. This was investigated using the chemo-

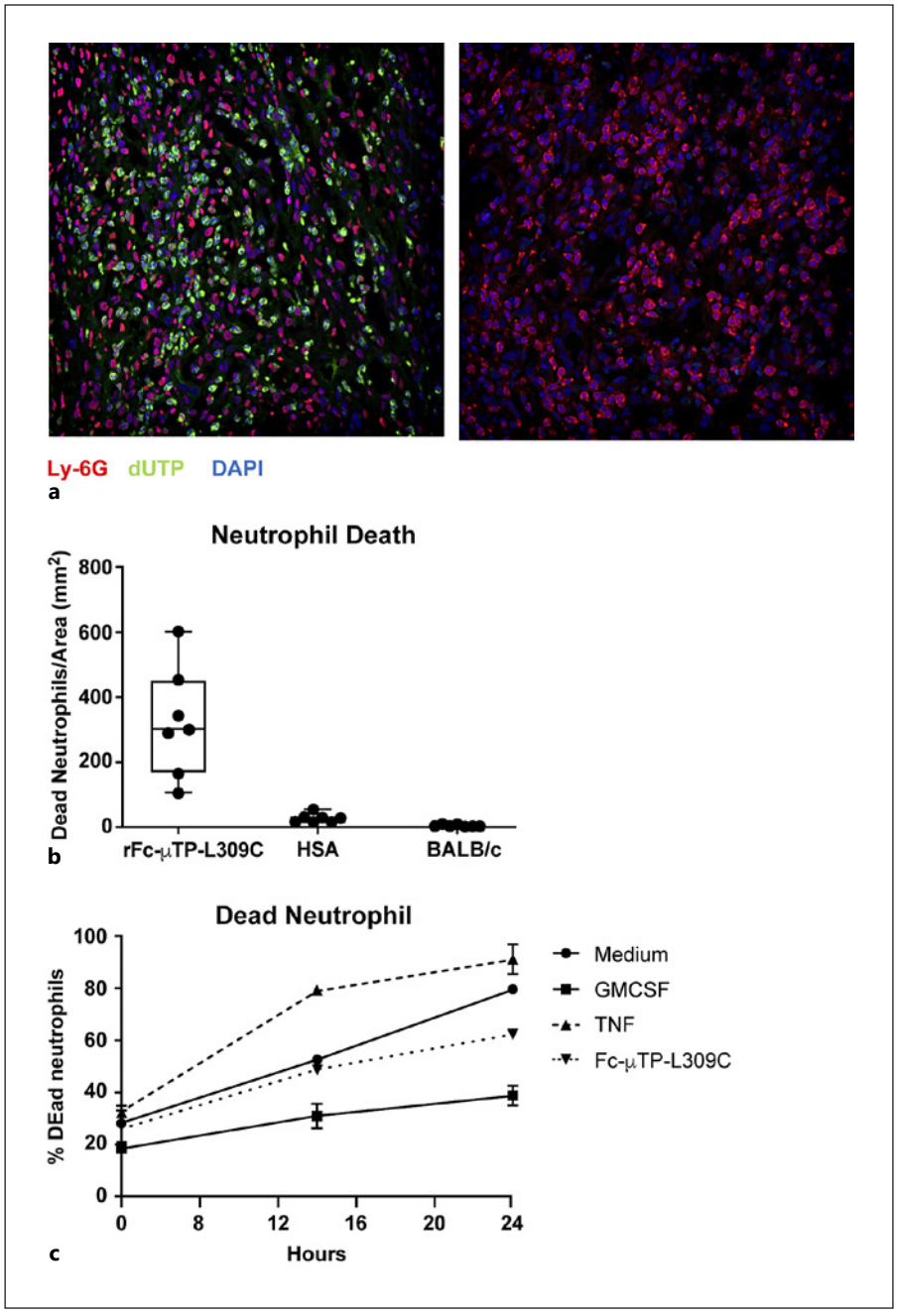

Fig. 3. The effects of rFc-μTP-L309C on neutrophil cell death. **a** Representative images of ankle sections from K/BxN mice that were given 6 s.c. injections of 200 mg/kg of Fc-μTP-L309C (left image). Mice treated with HSA (right image) and BALB/c (not shown) were used as controls. Ankle sections were stained for anti-Ly6G in red, dUTP in green, and DAPI in blue. **b** The number of dead neutrophils/area (mm^2) is shown; error bars indicate the range of neutrophils/area (mean ± SD, n = 10). **c** The number of dead neutrophils at 4, 14, and 24 h is shown; error bars indicate the range of dead neutrophils (mean ± SD, n = 10).

kine CXCL1 which is a primary chemokine involved in neutrophil migration. Using the transwell assay, we found that rFc-μTP-L309C did not inhibit CXCL1-promoted neutrophil chemotaxis (Fig. 2e). This result suggests that if there is an effect of rFc-μTP-L309C, it is not a direct effect on neutrophils but may require interaction with FcRs on other cell types to induce the inhibitory effect.

Effect of rFc-μTP-L309C on Neutrophil Cell Death

K/BxN mice that were at high clinical scores of 9–12 were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of rFc-μTP-L309C, and HSA-treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12 and stained for dead neutrophils using TUNEL and visualized by immunofluorescence microscopy. The dead neutrophils were quan-

tified using Qupath. There were no dead neutrophils in the ankle joints of K/BxN mice treated with HSA or in the ankle joints of BALB/c mice (Fig. 3a, b). Although there was substantial TUNEL staining (green, indicating DNA fragmentation) in the joint section treated with 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C (Fig. 3a), only some neutrophils (Ly6G+) in the ankle joints of mice that were treated showed TUNEL staining; however, quantification of these results, although cell death was slightly increased (Fig. 3b), did not result in significance. We did not elucidate what other cells may have been TUNEL stained; however, because there was a trend for increased apoptosis of the neutrophils, we further examined rFc-μTP-L309C-induced neutrophil cell death using an in vitro assay.

In the in vitro assay, we investigated the number of dead neutrophils after 4, 14, and 24 h of exposure to medium, rFc-μTP-L309C, TNF-α, or GM-CSF using flow cytometry. Dead neutrophils were identified as cells that stained positive for Ly6G, annexin V, and propidium iodide. There was no significant difference in neutrophil death between neutrophils that were exposed to rFc-μTP-L309C or medium for 4, 14, or 24 h (Fig. 3c).

Effect of rFc-μTP-L309C on Respiratory Burst

The production of ROS by mouse neutrophils was detected over 30 min, and chemiluminescence was evaluated with a luminometer. Neutrophils treated with ilICs exhibited significant ROS production over time (Fig. 4a), as shown by an increase in relative light units. Whereas neutrophils treated with medium, diphenyleneiodonium

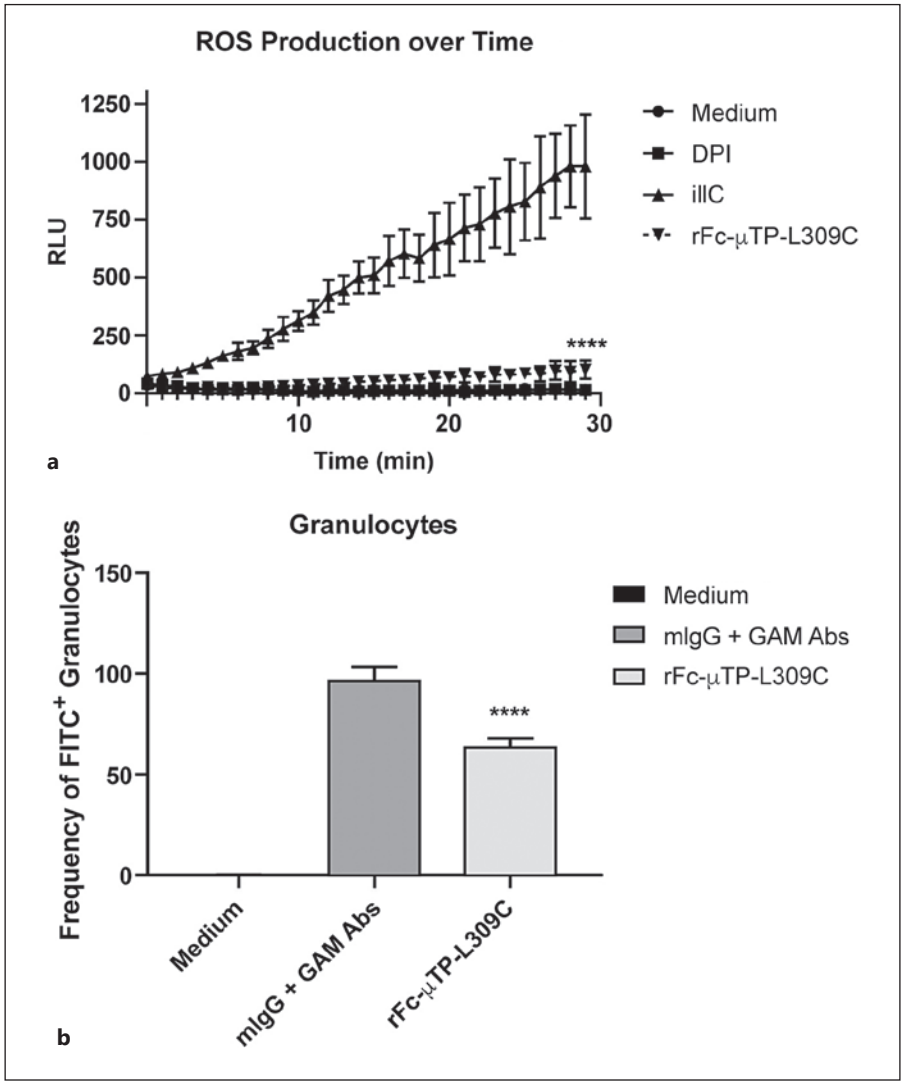

Fig. 4. The effects of rFc-μTP-309C on ROS production in neutrophils. **a** ROS production over time expressed as RLU; error bars indicate the range of RLU (mean ± SD, n = 6). ****$p < 0.0001$ for Fc-μTP-L309C compared with ilIC, one-way ANOVA with Dunnet test. **b** Frequency of FITC$^+$ cells as an indicator of respiratory burst; error bars indicate the range of frequency (mean ± SD, n = 8). ****$p < 0.0001$ for Fc-μTP-L309C compared with mIgG + GAM Abs, Kruskal-Wallis with the Dunn test.

chloride (a known inhibitor of NADPH oxidase), or rFc-μTP-L309C did not exhibit any ROS production over time (Fig. 4a).

However, when ROS production was assessed using dihydrorhodamine-123, which can be detected in the FITC channel when respiratory burst occurs, contradictory results were observed. Although the frequency of $FITC^+$ cells was significantly higher in neutrophils treated with mIgG + GAM in comparison to neutrophils treated with rFc-μTP-L309C (Fig. 4b), the frequency of $FITC^+$ cells was significantly higher in neutrophils treated with rFc-μTP-L309C in comparison to neutrophils treated with medium, which did not exhibit a respiratory burst (Fig. 4b).

rFc-μTP-L309C Reduces IL-1β Production

Neutrophils from the bone marrow of K/BxN mice were treated ex vivo with or without 100 μg/mL of rFc-μTP-L309C to analyze FcγRIII staining. 62% of neutrophils treated with rFc-μTP-L309C stained positive for FcγRIII compared to HSA-treated neutrophils (Fig. 5a), indicating that treatment with rFc-μTP-L309C can bind to FcγRIII.

Next, neutrophils from the bone marrow of K/BxN mice were treated ex vivo with rFc-μTP-L309C, followed by mouse IgG and goat anti-mouse IgG, and IL-1β levels were measured by ELISA. Neutrophils that were pretreated with rFc-μTP-L309C exhibited significantly less IL-1β production after 16 h in comparison to neutrophils treated with mouse IgG and goat anti-mouse IgG (Fig. 5b).

K/BxN mice that were at high clinical scores of 9–12 were given 6 s.c. injections on days 1, 3, 5, 7, 9, and 11 of 200 mg/kg of rFc-μTP-L309C, and HSA-treated mice and BALB/c mice were used as controls. The ankle joints of these mice were dissected on day 12, and the synovial flu-

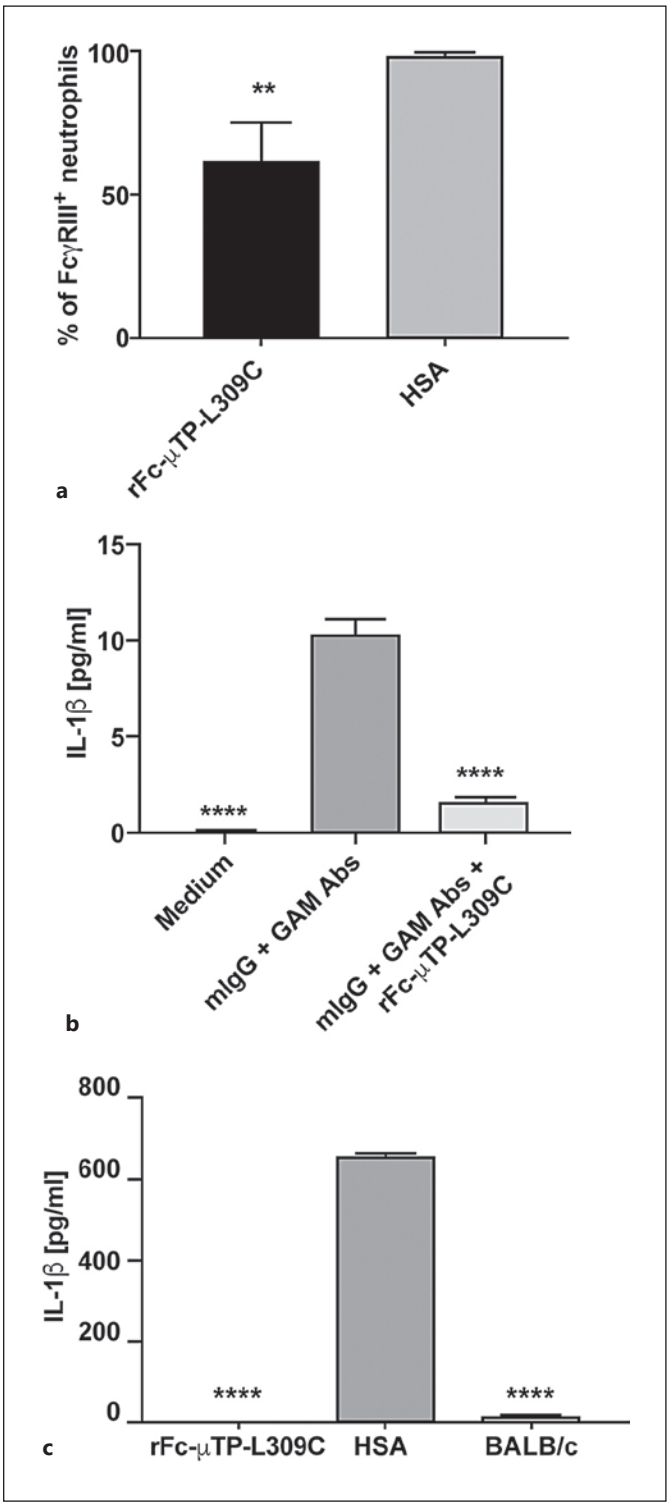

Fig. 5. rFc-μTP-L309C prevents IC-induced IL-1β release from neutrophils. **a** Percentages of neutrophils that stained positive for FcγRIII from the bone marrow of K/BxN mice that were treated ex vivo with 100 μg/mL of Fc-μTP-L309C or with HSA. Shown are the average percentages; error bars indicate range of percentages (mean ± SD, $n = 6$). $^{**}p < 0.01$, compared with HSA, Mann-Whitney test. **b** Concentrations of IL-1β from neutrophils treated ex vivo with mIgG and GAM Abs or with Fc-μTP-L309C and mIgG and GAM Abs. Shown are the average IL-1β concentrations measured in pg/mL; error bars indicate the range of concentrations (mean ± SD, $n = 8$). $^{****}p < 0.0001$, compared with HSA, Kruskal-Wallis with the Dunn test. Shown are the concentrations (**c**) of IL-1β in the synovial fluid of K/BxN mice that were treated with 6 s.c. injections of 200 mg/kg of Fc-μTP-L309C and HSA was used as a protein control. Injections were done on days 1, 3, 5, 7, 9, and 11, and synovial fluid collection was done on day 12. Shown are the average IL-1β concentrations measured in pg/mL; error bars indicate the range of concentrations (mean ± SD, $n = 8$). $^{****}p < 0.0001$, compared with HSA, Kruskal-Wallis with the Dunn test.

id was collected for an ELISA to measure IL-1 β levels. IL-1 β levels were significantly lower in mice given 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C in comparison to mice treated with HSA (Fig. 5c).

Discussion

We have previously shown that rFc-μTP-L309C is effective at treating K/BxN arthritis [11; and Fig. 1]. Because neutrophils have been shown to be the critical mediators of arthritis in this model and are present in high numbers in the synovium of RA patients, where they can promote significant joint inflammation and destruction [1, 19], we wanted to determine whether rFc-μTP-L309C affects neutrophils in the joints of K/BxN mice. First, we used DAB staining to detect neutrophils in the joints of K/BxN mice. There were significantly fewer neutrophils in mice that were given 6 s.c. injections of 200 mg/kg of rFc-μTP-L309C in comparison to mice treated with HSA (Fig. 2a, b). To better understand how rFc-μTP-L309C inhibits neutrophil infiltration into the joint tissue, we hypothesized that rFc-μTP-L309C could be affecting the neutrophils in K/BxN mice in three major ways, such that there would be fewer neutrophils in the joints.

First, we investigated whether rFc-μTP-L309C that has high avidity for FcγRs, could directly mitigate neutrophil migration by interaction with neutrophil FcγRIII, to which we had shown rFc-μTP-L309C can bind (Fig. 4a), using a transwell migration assay. FcγRs have been shown to maintain and enhance cytokine and chemokine responses that occur during the initiation phase of arthritis [20]. We used CXCL1 as a potent chemokine in this assay since it has been shown to coordinate leukocyte trafficking to the joint in the endogenous K/BxN model [8]. However, we found that rFc-μTP-L309C did not inhibit the CXCL1-promoted chemotaxis of neutrophils (Fig. 2e). Perhaps another chemotactic chemokine, such as CXCL5 or CCL9 [7], would have been inhibited, but we did not investigate these.

Second, if rFc-μTP-L309C did not inhibit CXCL1 neutrophil migration, perhaps the lack of neutrophils in the joint was due to neutrophil death. In the TUNEL assay looking for apoptosis, we did observe a marginal increase in apoptosis in the remaining neutrophils in the joint tissue of K/BxN mice treated with 200 mg/kg of rFc-μTP-L309C in comparison to mice treated with HSA (Fig. 3a, b). However, when we examined ex vivo using flow cytometry to confirm the TUNEL results, we found that there was no difference in the number of dead neutrophils treated with rFc-μTP-L309C for 4, 14, or 24 h in comparison to neutrophils in medium for the same length of time (Fig. 2c). Based on previous publications using IVIG [12], it seems that human neutrophil apoptosis is induced by the Fab portion of the immunoglobulin, not the Fc portion. Furthermore, mouse neutrophils have been shown to be resistant to cell death using IVIG [13]. We have preliminary results that confirm that rFc-μTP-L309C cannot directly induce apoptosis in mouse neutrophils (unpublished). The observation that some other cell type may have been going through apoptosis in the ankle joint due to rFc-μTP-L309C treatment is of interest, but we did not investigate which cell type this may be. Our results may indicate an indirect mechanism of cell death, perhaps initiated by rFc-μTP-L309C binding to neutrophil or monocyte-macrophage FcγRs, that induces production of an apoptosis-inducing cytokine, such as TNFα.

As a third possible mechanism of reduced neutrophil infiltration in the joints and/or destruction of the joint tissue, we investigated if rFc-μTP-L309C induces a respiratory burst in neutrophils. The engagement of FcγRs on neutrophils with ICs can induce ROS production and furthermore, a respiratory burst [21–23]. Upon activation of neutrophils by ICs via FcγRs, the NADPH oxidase is assembled at cellular and granular membranes and becomes activated [24]. This activation results in extracellular ROS generation, which can be detected by dihydrorhodamine-123, or intracellular ROS generation, which can be detected by luminol [25]. Depending on the level of ROS produced by NADPH oxidase, neutrophils may die by apoptosis [26]. Crosslinking FcγRs on neutrophils with multimeric Fc proteins such as rFc-μTP-L309C could potentially have the same effect.

Using human neutrophils, Spirig et al., 2018 [10] showed that rFc-μTP-L309C did not induce ROS; however, it is not known what effects rFc-μTP-L309C has on ROS production in mouse neutrophils. We used luminometry to determine whether there was extracellular ROS production from neutrophils that were treated ex vivo with rFc-μTP-L309C. We found that there was significant extracellular ROS production from neutrophils treated with ilICs but that there was no extracellular ROS production from neutrophils treated with rFc-μTP-L309C (Fig. 4a). Our results are confounding, however, because we found that there was significant intracellular ROS production from neutrophils treated with ICs and rFc-μTP-L309C (Fig. 4b).

It has been shown that FcγR engagement, specifically FcγRIII, by ICs in neutrophils in K/BxN mice mediates IL-1β release [4, 5, 27, 28]. IL-1β is a crucial mediator of

cartilage destruction in K/BxN mice [8]. We decided to investigate whether rFc-μTP-L309C could prevent the release of IL-1β from neutrophils and thus provide some additional evidence as to how rFc-μTP-L309C is able to ameliorate the arthritis in K/BxN mice. First, we found that rFc-μTP-L309C binds to FcγRIII on neutrophils from K/BxN mice (Fig. 5a). Then we found that rFc-μTP-L309C prevents the IC-stimulated release of IL-1β from neutrophils ex vivo (Fig. 5b). Importantly, using synovial fluid of K/BxN mice that were successfully treated with rFc-μTP-L309C, we found that IL-1β was significantly reduced compared to HSA-treated mice (Fig. 5c). Taken together, rFc-μTP-L309C ameliorates arthritis in K/BxN mice, in part, through inhibition of production of damaging IL-1β.

Although the effects of rFc-μTP-L309C on mouse neutrophil cell death and respiratory burst need further investigation, it is evident from our studies reported herein that rFc-μTP-L309C ameliorates the arthritis in the endogenous K/BxN mice by its inhibitory effects on infiltration of neutrophils into the joints and its reducing production of IL-1β. Recombinant Fc multimers are being developed for use in a number of autoantibody/IC-driven autoimmune diseases such as ITP, NMO, MG, lupus, etc. [10, 11, 16]. Results from experimental mouse models have shown encouraging results that suggest rFc multimers could replace IVIG in the treatment of these disorders [10, 11, 16]. Our work shows that rFc-μTP-L309C could be a potential therapeutic candidate for autoimmune and inflammatory conditions in which neutrophils are the predominant cell type involved in pathogenesis, especially rheumatoid arthritis.

Acknowledgments

We thank the staff at the University Health Network animal facility at 67 College Street for their help in maintaining this arthritis mouse model. We also are grateful to Dr. Christophe Benoist for his gracious gift of KRN mice.

Statement of Ethics

The study did not involve human subjects. Animal studies were performed with the approval of the University Health Network Animal Care Committee, Animal Use Permit (AUP) 1788.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

The laboratory of S.V.G. is supported by grants from the Swiss National Science Foundation (310030_184757, 310030E_205559) and Swiss Cancer League/Swiss Cancer Research (KFS-4958-02–2020). R.J.A. and K.F.B. are recipients of a Canadian Blood Services (CBS) Postdoctoral Fellowship Award. B.J.B.L. is a recipient of a CBS Graduate Fellowship Program Award. D.R.B. is supported, in part, by Health Canada. However, the views expressed herein do not necessarily represent the views of the Canadian federal government.

Author Contributions

Ruqayyah J. Almizraq, Kayluz Frias Boligan, and Bonnie J.B. Lewis contributed to the experimental design, writing and editing of the manuscript, and integrity of the data. Selena Cen helped with the experimental work and collection of the synovial fluid. Heather Whetstone processed the bone for section preparation. Rolf Spirig and Fabian Kaesermann provided the recombinant protein for the study. Ian K. Campbell helped with instructions on the collection of synovial fluid and gave advice for working with an arthritis mouse model. Stephan Von Gunten helped revise the manuscript and provided advice for working with neutrophils. Donald R. Branch was the principal investigator, conceived of the experimental approach and design of the study, and wrote and edited the manuscript. All the authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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