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CXCR3 antagonist AMG487 suppresses rheumatoid arthritis pathogenesis and progression by shifting the Th17/Treg cell balance

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by uncontrolled joint inflammation and damage to bone and cartilage. Previous studies have shown that chemokine receptors have important roles in RA development, and that blocking these receptors effectively inhibits RA progression. Our study was undertaken to investigate the role of AMG487, a selective CXCR3 antagonist, in DBA/1J mice bearing collagen-induced arthritis (CIA). Following induction of CIA, animals were treated with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 until day 41 and evaluated for clinical score, and histological hallmarks of arthritic inflammation. We further investigated the effect of AMG487 on Th1 (T-bet), Th17 (IL-17A, RORγt, STAT3), Th22 (IL-22), and T regulatory (Treg; Foxp3 and IL-10) cells in splenic CXCR3$^+$ and CD4$^+$ T cells using flow cytometry. We also assessed the effect of AMG487 on T-bet, RORγt, IL-17A, IL-22, Foxp3, and IL-10 at both mRNA and protein levels using RT-PCR and Western blot analyses of knee samples. The severity of clinical scores, and histological inflammatory damage decreased significantly in AMG487-treated compared with CIA control mice. Moreover, the percentage of Th1, Th17, and Th22 cells decreased significantly and that of Treg cells increased in AMG487-treated mice. We further observed that AMG487-treatment downregulated T-bet, IL-17A, RORγt, and IL-22, whereas it upregulated Foxp3 and IL-10 mRNA and protein levels. This study demonstrates the antiarthritic effects of AMG487 in CIA animal model and supports the development of CXCR3 antagonists as a novel strategy for the treatment of inflammatory and arthritic conditions.

1. Introduction

Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disease that causes joint disability in 0.5%–1% of the global population [58]. The disease is characterized by inflammatory cell infiltration and damage to bone and cartilage in joints [17,57]. Similar to other immune disorders and pathological inflammatory responses, the innate and adaptive immune responses are involved in the development and pathogenesis of RA [8]. Faced with the complex etiology of RA, the administration of anti-rheumatic drugs to restrict inflammation and retard disease progression has been widely used in the clinic [21]. However, since many patients do not respond to such treatment, new therapeutic alternatives are needed for achieving disease modification. This requires a better understanding of the immunologic mechanisms associated with joint inflammation and disease progression.

The chemokine receptor CXCR3 is a G-protein-coupled chemokine receptor that has been shown to play a crucial role in several immunological and inflammatory responses [29]. Cumulative evidence indicates that CXCR3 is closely associated with inflammation and autoimmune diseases [2]. A previous study showed that CXCR3 is significantly expressed in peripheral blood and synovium of RA patients [59]. More recently, it was reported that the role of CXCR3 in RA pathogenesis includes the regulation T cells recruitment through downstream mediators, such as Ras/ERK, Src, and PI3K/Akt [39]. The blockade of CXCR3 pathway inhibits T cell recruitment in inflamed joints, which reduces the severity of arthritis [43]. Indeed, CXCR3-deficient mice show reduced arthritis symptoms [39].

AMG487 is a selective CXCR3 antagonist that exhibits biological activity in preclinical models of cellular recruitment [23,28]. At least two CXCR3 antagonists have shown efficacy in preclinical studies on

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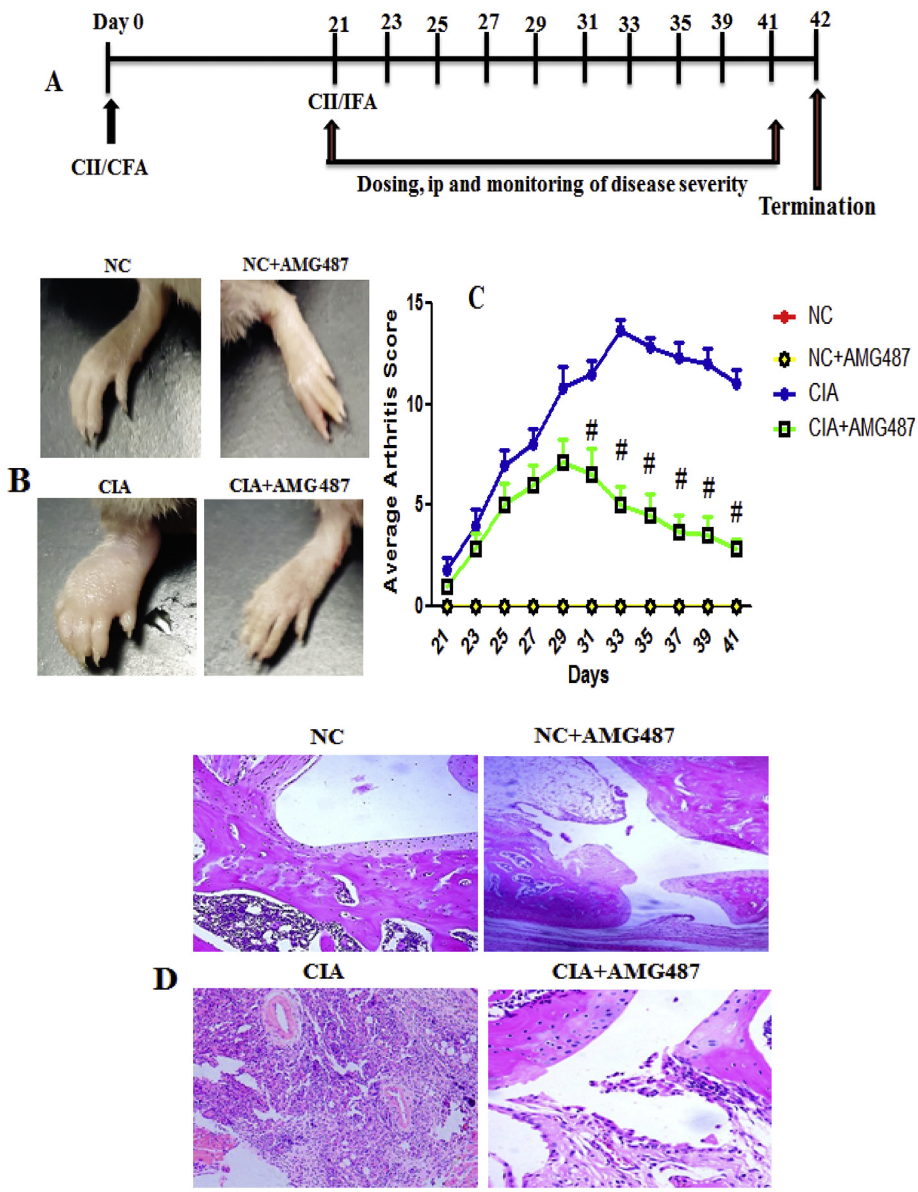

Fig. 1. AMG487 treatment ameliorated inflammation in collagen-induced arthritis (CIA) mice. (A and B) The CIA model in DBA 1/J mice was successfully established. AMG487 decreased the redness and swelling of joints in CIA mice. (C) Arthritis score index was used to assess the severity of arthritis. AMG487-treated CIA mice had decreased arthritis scores compared with CIA control group. (D) AMG487-treated mouse showed significant improvement in the inflammatory process with less damage to the joint space. Normal control (NC) mice received 1% (*v*/v) DMSO in saline intraperitoneally. Treated CIA mice were injected with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 to day 41. The level of significance was set at $^{*}p < .05$ compared with the NC group; $^{\#}p < .05$ compared with the CIA control group. Data are presented as mean ± SEM (*n* = 6 in each group).

inflammatory disease, and one CXCR3 antagonist has been assessed in clinical trials [7]. AMG487 has been extensively used in different animal models to inhibit the effects of chemokines and cytokines [24,62,69]. However, the effects of AMG487 on RA development, especially with respect to pathogenic and immune regulatory mechanisms, have not been elucidated yet. One approach to address this issue is the use of collagen-induced arthritis (CIA) animal models, which have been extensively investigated to elucidate pathogenic mechanisms related to human RA and to identify potential targets for therapeutic intervention [14].

In this context, the imbalance between different T cells e.g., T-helper cells 17 (Th17) versus regulatory T cells (Treg) is critical for the progression and development of RA, and it is strongly associated with the onset of autoimmunity and damage to the joint [9]. T-box transcription factor (T-bet) has been shown to be important for promoting Th1 cell differentiation while suppressing differentiation of other T cell lineages [1]. Cytokines secreted by Th1 cells are pro-inflammatory and have been associated to the pathogenesis of RA and other inflammatory disorders [15]. Similarly, Th17 cells contribute to inflammatory responses and have been associated in the pathogenesis of RA [30]. The development of Th17 cells is strongly affected by the STAT3/RORγt pathway [26]. On the other hand, regulatory T cells (Treg cells) are immunosuppressors and downregulate the effector functions of Th1/Th17 cells in autoimmune disorders. An imbalance in the ratio between Th17 and Treg cells towards an increase in Th17 and/or a decrease in Treg cells has been observed in RA patients [49]. Moreover, Treg cell activation plays an essential role in the prevention of autoimmunity [16]. In this respect, role of CXCR3 signaling on Th17/Treg balance has not been explored earlier in CIA model. Therefore, our study focused on exploring the mechanisms that regulate Th17/Treg cell balance during arthritis using CIA mouse model. Further, we assessed whether CXCR3 antagonist AMG487 has the potential to ameliorate the progression and severity of arthritis in through regulation of Th17/Treg cells.

2. Material and methods

2.1. Animals

Male DBA/1J mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and maintained in a specific pathogen-free animal facility at the King Saud University, where they had access to food and water ad libitum. All animal experiments were approved by

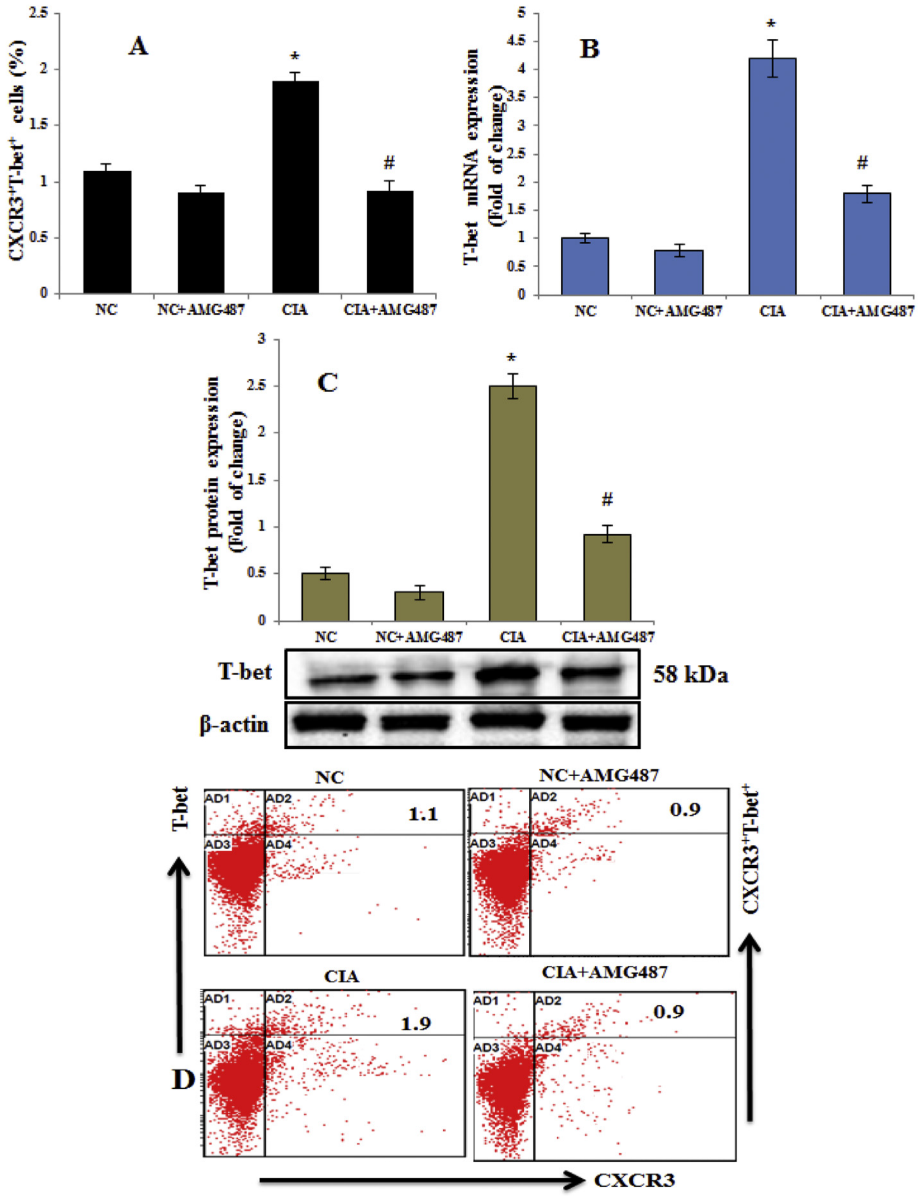

Fig. 2. Flow cytometry analyses of T-bet-producing CXCR3$^+$ spleen cells and RT-PCR and Western blot analyses of T-bet in knee tissues. (A) Effect of AMG487 on T-bet-producing CXCR3$^+$ cells analyzed by flow cytometry in the spleen cells. The cells were gated on forward and side scatter (FSC-SSC) dot plot, and then the lymphocytes were gated for analyzing the percentage of CXCR3$^+$T-bet$^+$ cells. (B) mRNA level of T-bet in knee tissues from mice treated with AMG487 analyzed by RT-PCR. (C) Protein level of T-bet in knee tissues from mice treated with AMG487 analyzed through Western blot. (D) Representative flow cytometry dot plot of one mouse from each group. Normal control (NC) mice received 1% (*v*/v) DMSO in saline intraperitoneally. Treated CIA mice were injected with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 to day 41. The level of significance was set at $^*p < .05$ compared with the NC group; $^\#p < .05$ compared with the CIA control group. Data are presented as mean ± SEM ($n = 6$ in each group).

the Institutional Animal Care and Use Committee. Mice were 9–11 weeks old at onset of the experiments.

2.2. CIA induction and AMG487 administration

Collagen induced arthritis was promoted as previously described [37]. Briefly, 100 μg of bovine type II collagen (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1 M acetic acid and emulsified with an equal volume of Freund's complete adjuvant (2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). The collagen emulsion was administered via intradermal injection at the base of tail into DBA/1 J mice on day 0. On day 21, another emulsion prepared with type II collagen and Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) was intradermally administered near the primary injection. Age-matched male DBA/1 J mice immunized with adjuvant alone were used as a control group. To block CXCR3 signaling, we used AMG487 (Tocris Bioscience, Bristol, UK), a potent and specific CXCR3 antagonist. Mice were intraperitoneally injected with AMG487 (5 mg/kg) or dimethyl sulfoxide (DMSO) every 48 h, starting from day 21 until day 41. Clinical scores were evaluated every second day after day 21.

2.3. Measurement of the severity of arthritis

Beginning on day 21, mice were scored for arthritis severity every other day as previously described [65]. Animals were evaluated by an observer unaware of the treatment regimens according to a macroscopic scoring system: 0 = no sign of arthritis, 1 = swelling and/or redness of the paw or one digit, 2 = involvement of two joints, 3 = involvement of more than two joints, and 4 = severe arthritis of the entire paw and digits. Arthritis index was calculated for each mouse by summing the scores for the individual paws [11].

2.4. Histological assessment of arthritis

Knee joints were removed and fixed for 7 days in 10% (*v*/v) formalin, decalcified in 5% (v/v) formic acid, embedded in paraffin, and sectioned (7 μm thickness). Hematoxylin and eosin staining was performed, and slides were photographed and analyzed by a histopathologist.

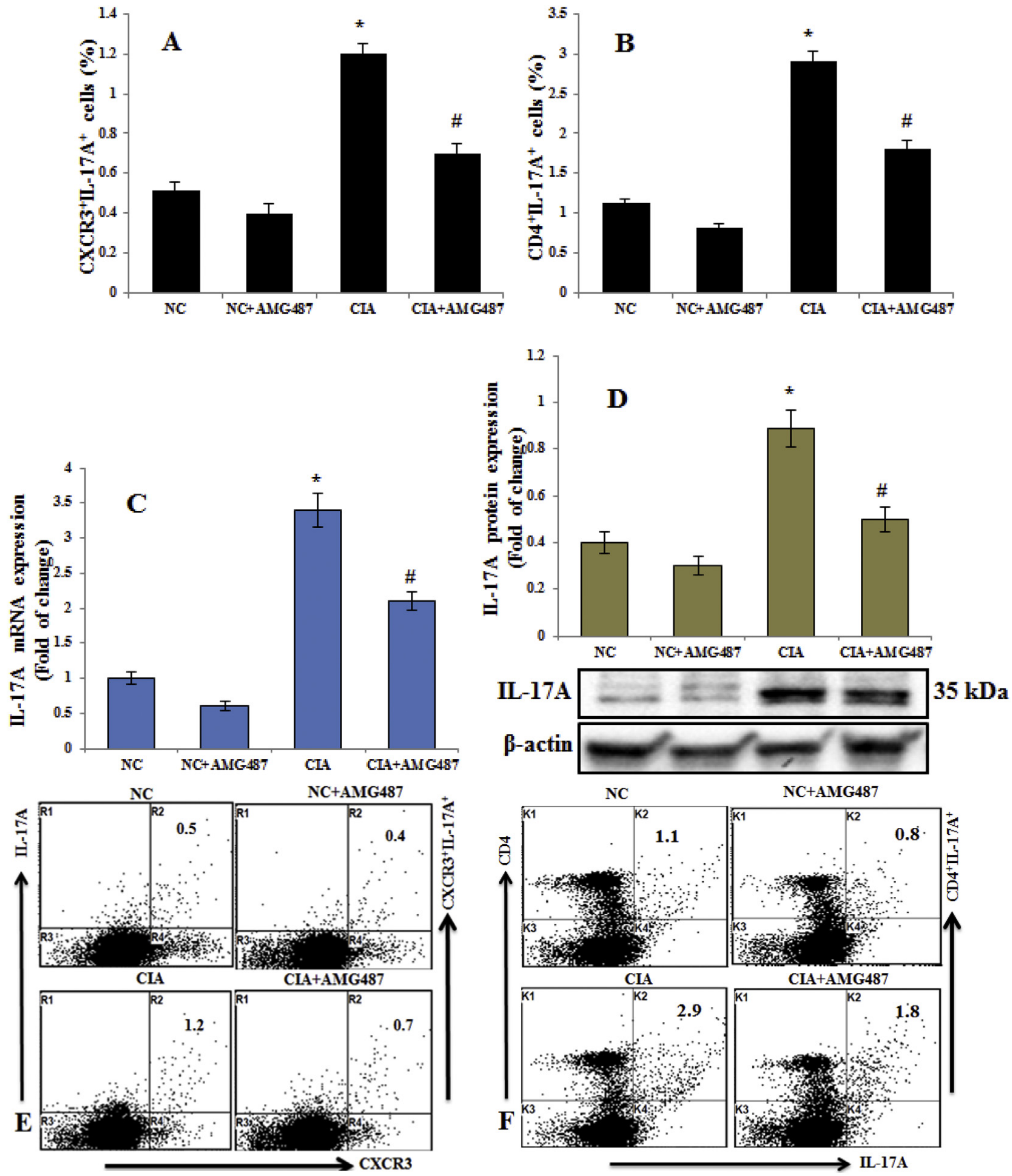

Fig. 3. Flow cytometry analyses of IL-17A-producing CXCR3$^+$ and CD4$^+$ spleen cells and RT-PCR and Western blot analyses of IL-17A in knee tissues. (A and B) Effect of AMG487 on the levels of IL-17A-producing CXCR3$^+$ and CD4$^+$ T spleen cells. The cells were gated on FSC-SSC dot plot, and then the lymphocytes were gated for analyzing the percentage of CXCR3$^+$IL-17A$^+$ and CD4$^+$IL-17A$^+$ cells. (C) mRNA level of IL-17A in knee tissues from mice treated with AMG487 analyzed by RT-PCR. (D) Protein level of IL-17A in knee tissues from mice treated with AMG487 analyzed through Western blot. (E and F) Representative flow cytometry dot plot of one mouse from each group. Normal control (NC) mice received 1% (v/v) DMSO in saline intraperitoneally. Treated CIA mice were injected with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 to day 41. The level of significance was set at $^*p < .05$ compared with the NC group; $^\#p < .05$ compared with the CIA control group. Data are presented as mean ± SEM (n = 6 in each group).

2.5. Flowcytometric analysis

Flow cytometry analyses were performed to assess T-bet, IL-17A, RORγt, STAT3, IL-10, IL-22, and Foxp3 production in CXCR3$^+$ and CD4$^+$ cells. Briefly, splenocytes were incubated with PMA (10 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (1 μg/ml; Sigma-Aldrich), and 1 μl/ml Golgi-plug (BD Biosciences, San Jose, CA, USA) for 4 h before staining as previously described [5]. Cells were washed, and surface staining of CXCR3$^+$ and CD4$^+$ (BioLegend, San Diego, CA, USA) was performed. After fixation and permeabilization (BioLegend), cells were stained with Th1 (anti-T-bet; BioLegend), Th17 (anti-IL-17A, anti-RORγt, and anti-STAT3; BioLegend), Th22 (anti-IL-22; BioLegend), and Treg (anti-Foxp3 and anti-IL-10; BioLegend) fluorescent antibodies. We acquired 10,000 cell events on flow cytometry (Beckman Coulter, Indianapolis, IN, USA) and analyzed the results using CXP software (Beckman Coulter).

2.6. RT-PCR analysis

Total RNA was extracted from knee tissues using TRIzol reagent (Life Technologies, Paisley, UK). cDNA was prepared using high-capacity cDNA reverse transcription (Applied Biosystems, Foster City, USA), followed by the real-time-PCR using SYBR® Green PCR master mix (Applied Biosystems), per manufacturer's instructions. The following primers were used in RT-PCR assays: CXCR3, F: 5′-ACAGCACCTCTCC CTACGAT-3′ and R: 5′-AATCTGGGAGGGCAAAGAGC-3′; T-bet, F:

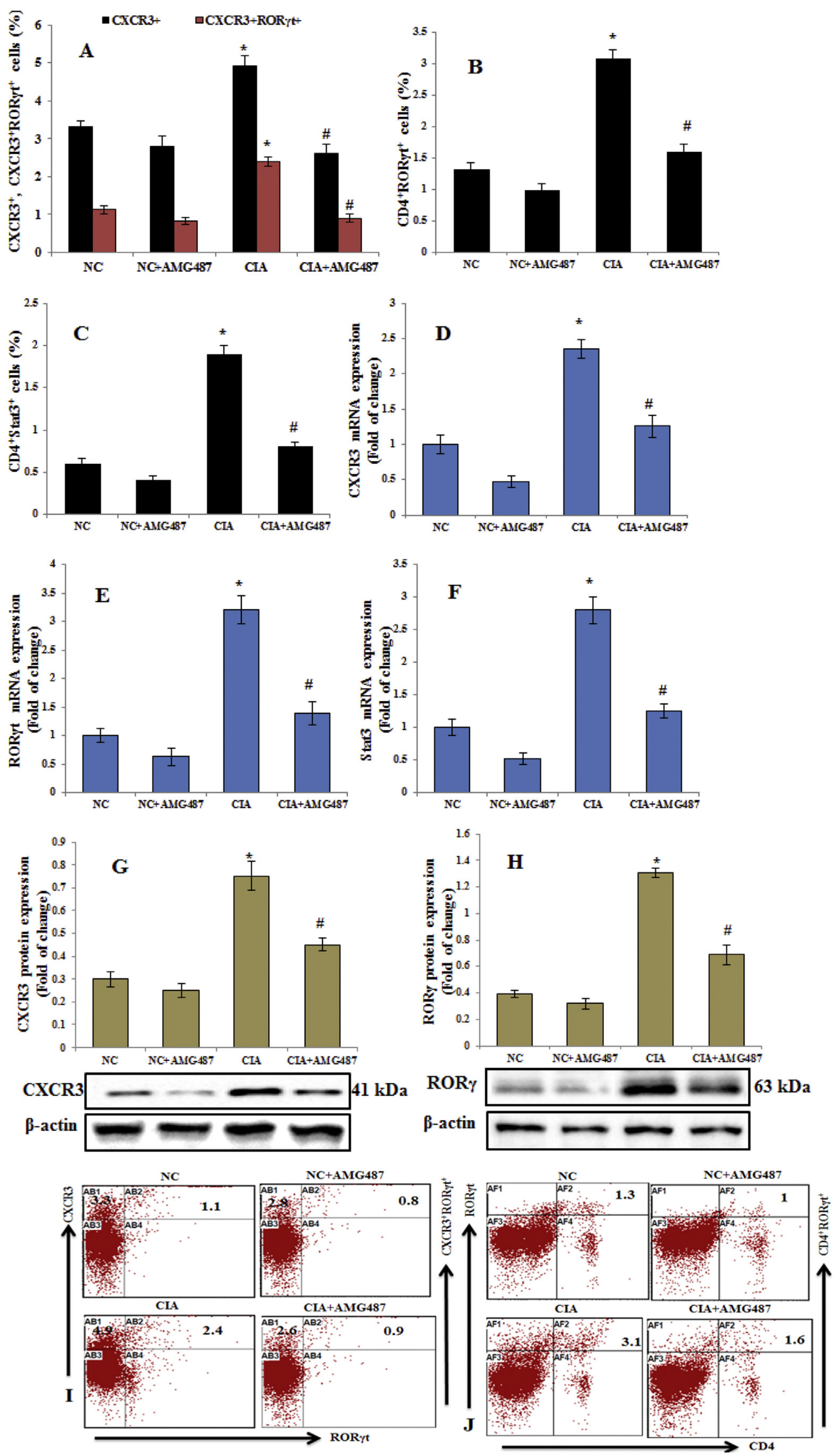

Fig. 4. Flow cytometry analyses of CXCR3-, RORγt-and STAT3-producing $CXCR3^+$ and $CD4^+$ T spleen cells and RT-PCR and Western blot analyses of CXCR3 and RORγt in knee tissues. (A–C) Effect of AMG487 on the levels of CXCR3, RORγt- and STAT3-producing $CXCR3^+$ and $CD4^+$ T cells analyzed by flow cytometry in spleen cells. The cells were gated on FSC-SSC dot plot, and then the lymphocytes were gated for analyzing the percentage of $CXCR3^+$, $CXCR3^+ROR\gamma t^+$, $CD4^+ROR\gamma t^+$ and $CD4^+STAT3^+$ cells. (D–F) mRNA level of CXCR3, RORγt, and STAT3 in knee tissues from mice treated with AMG487 analyzed by RT-PCR. (G and H) Protein level of CXCR3, and RORγt in knee tissues from mice treated with AMG487 analyzed through Western blot. (I and J) Representative flow cytometry dot plot of one mouse from each group. Normal control (NC) mice received 1% (v/v) DMSO in saline intraperitoneally. Treated CIA mice were injected with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 to day 41. The level of significance was set at $^*p < .05$ compared with the NC group; $^\#p < .05$ compared with the CIA control group. Data are presented as mean ± SEM (n = 6 in each group).

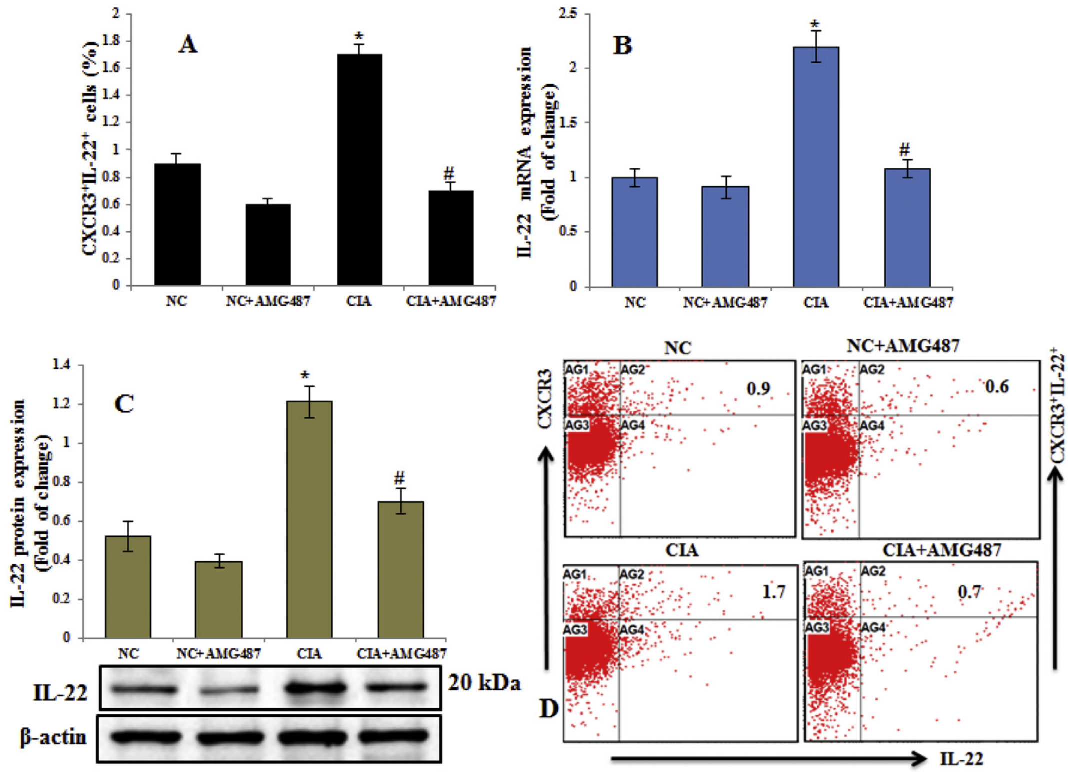

Fig. 5. Flow cytometry analyses of IL-22-producing CXCR3$^+$ spleen cells and RT-PCR and Western blot analyses of IL-22 in knee tissues. (A) Effect of AMG487 on the levels of IL-22-producing CXCR3$^+$ cells analyzed by flow cytometry in spleen cells. The cells were gated on FSC-SSC dot plot, and then the lymphocytes were gated for analyzing the percentage of CXCR3$^+$IL-22$^+$ cells. (B) mRNA level of IL-22 in knee tissues from mice treated with AMG487 analyzed by RT-PCR. (C) Protein level of IL-22 in knee tissue from mice treated with AMG487 analyzed through Western blot. (D) Representative flow cytometry dot plot of one mouse from each group. Normal control (NC) mice received 1% (v/v) DMSO in saline intraperitoneally. Treated CIA mice were injected with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 to day 41. The level of significance was set at $^*p < .05$ compared with the NC group; $^\#p < .05$ compared with the CIA control group. Data are presented as mean ± SEM (n = 6 in each group).

5′-AACAAGGGGGCTTCCAACAA-3′ and R: 5′-CCACTGGAAGGATAGGGGGA-3′; IL-17A, F: 5′-ATCCCTCAAAGCTCAGCGTGTC-3′ and R: 5′-GGGTCTTCATTGCGGTGGAGAG-3′; RORγt, F: 5′-AGCTGTGGGGTAGATGGGAT-3′ and R:5′-ATCCGGTCCTCTGCTTCTCT-3′; STAT3, F: 5′-ATCCTAAGCACAAAGCCCCC-3′ and R:5′-TCCTCACATGGGGGAGGTAG-3′; IL-22, F: 5′-GGGGAGAAACTGTTCCGAGG-3′ and R:5′-GGCAGGAAGGAGCAGTTCTT-3′; Foxp3, F: 5′-GGTATATGCTCCCGGCAACT-3′ and R: 5-GATCATGGCTGGGTTGTC-3′; GAPDH, F: 5′-GGCAAATTCAACGGCACAGT-3′ and R: 5′-TGAAGTCGCAGGAGACAACC-3′. The real time-PCR data were analyzed using the relative gene expression method. The fold change in the target genes between treated and untreated cells, corrected by level of GAPDH, was determined using following equation: fold change = 2-Δ (ΔCt), where ΔCt = Ct(target-Ct(β-actin) and Δ (ΔCt) = ΔCt(treated) - ΔCt(untreated) [3,36].

2.7. Western blot analysis

Protein was extracted from knee tissues as previously described [53]. Briefly, knees were isolated, cut into small pieces, and homogenized in ice-cold protein lysis buffer followed by centrifugation at 12,000 rpm for 15 min [54]. Protein quantitation was performed using Direct Detect® Infrared Spectrometer (Merck, Darmstadt, Germany). Briefly, 25–50 μg of protein from each group was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, USA). Western blot analysis was performed using a previously described method [6]. Primary mouse monoclonal antibodies against T-bet, IL-17A, CXCR3, RORγt, IL-22, Foxp3, and IL-10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation for 2 h with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at room temperature. The bands corresponding to T-bet, IL-17A, CXCR3, RORγt, IL-22, Foxp3, and IL-10 were visualized using a Western blot detection chemiluminescence kit (Merck, Darmstadt, Germany), and quantified in relation to β-actin bands [4]. Western blot analysis was carried twice for each protein.

2.8. Statistical analysis

All data are presented as mean ± SEM, and six animals composed each group. Results were analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparisons tests. The level of statistical significance was set at $p < .05$. Analyses were performed in GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of AMG487 on arthritis development and joint histopathology

To investigate the potential role of AMG487 in CIA, we first evaluated clinical features in mice. Mice developed arthritis at approximately 21 days after the second collagen injection, and showed progressive paw swelling. After the onset of CIA, mice treated with AMG487 had decreased arthritis severity scores compared to those of control mice (Fig. 1A–C). A significant reduction in paw edema after AMG487 administration was also observed compared to that of CIA control mice (Fig. 1A–C). Untreated CIA control mice had obliterated joint spaces with signs of severe inflammation, whereas AMG487-treated mice showed a significant milder inflammatory process with reduced damage to the joint space (Fig. 1D).

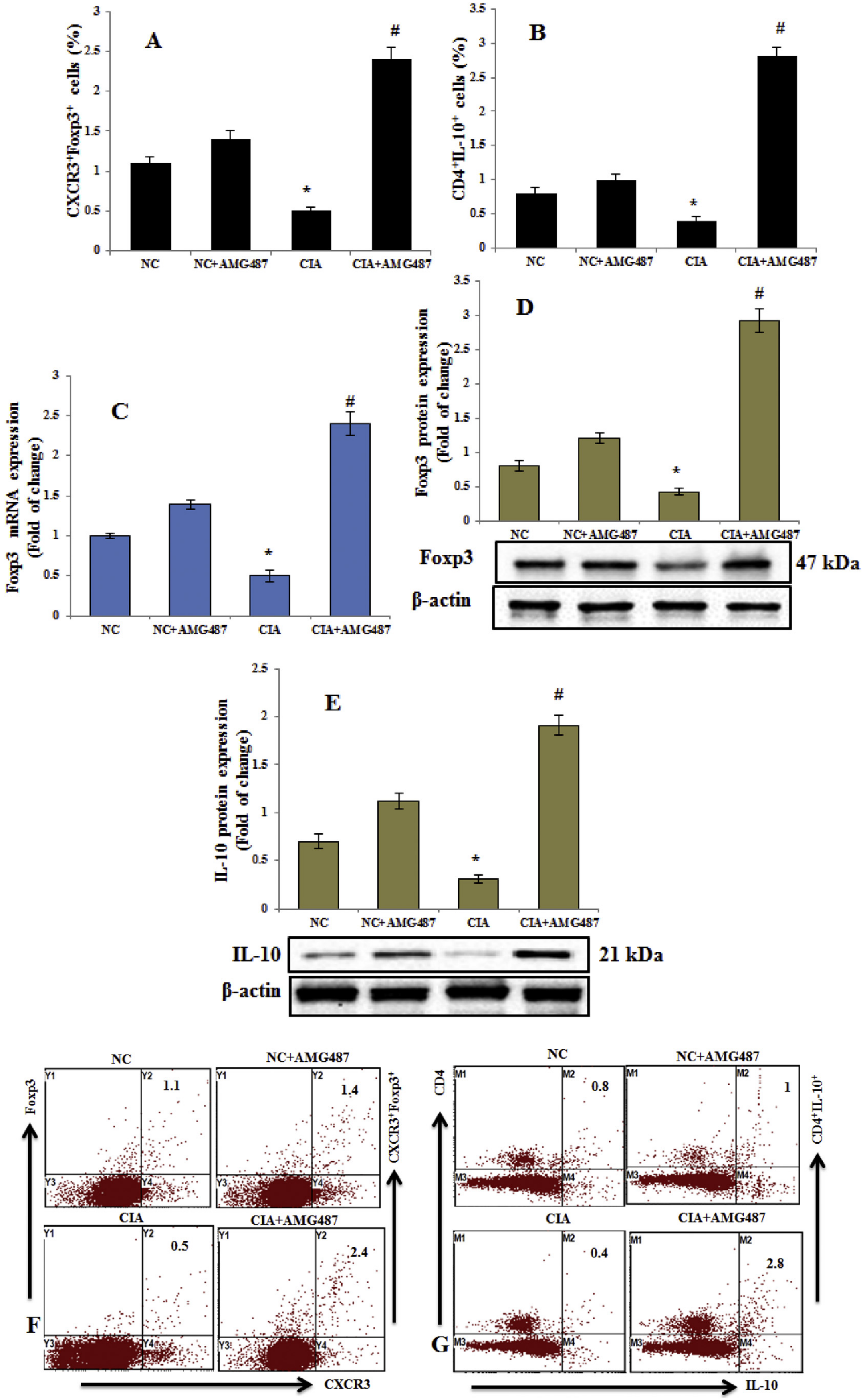

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Fig. 6. Flow cytometry analyses of IL-10- and FoxP3-producing $CXCR3^+$ and $CD4^+$ T spleen cells, RT-PCR analysis of FoxP3 in knee tissues, and Western blot analyses of IL-10 and FoxP3 in knee tissues. (A and B) Effect of AMG487 on the levels of IL-10- and FoxP3-producing $CXCR3^+$ and $CD4^+$ T cells in spleen analyzed by flow cytometry. The cells were gated on FSC-SSC dot plot, and then the lymphocytes were gated for analyzing the percentage of $CXCR3^+Foxp3^+$ and $CD4^+IL\text{-}0^+$ cells. (C) mRNA level of Foxp3 in knee tissues from mice treated with AMG487 analyzed by RT-PCR. (D and E) Protein level of IL-10 and FoxP3 in knee tissues from mice treated with AMG487 analyzed through Western blot. (F and G) Representative flow cytometry dot plot of one mouse from each group. Normal control (NC) mice received 1% (v/v) DMSO in saline intraperitoneally. Treated CIA mice were injected with 5 mg/kg AMG487 intraperitoneally every 48 h, starting from day 21 to day 41. The level of significance was set at $^*p < .05$ compared with the NC group; $^\#p < .05$ compared with the CIA control group. Data are presented as mean ± SEM (n = 6 in each group).

3.2. AMG487 inhibits CXCR3 and Th1-related transcription factor

Then, we further investigated the effect of AMG487 on $CXCR3^+$ cells. We found that the number of T-bet-producing $CXCR3^+$ cells increased in the spleen of CIA control mice as compared with that of normal control (NC) mice (Fig. 2A). AMG487-treated CIA mice showed a significant decrease in the levels of T-bet-producing $CXCR3^+$ cells compared with those of the CIA control mice (Fig. 2A). To further clarify the mechanism of AMG487, we used RT-PCR and Western blotting to examine changes in gene and protein levels of T-bet in knee tissues. The levels of T-bet mRNA and protein in CIA control mice were significantly higher than those in NC mice (Fig. 2B and C). AMG487 administration in CIA mice decreased T-bet mRNA and protein levels compared with those in CIA control mice (Fig. 2B and C). Therefore, our results demonstrated that CXCR3 antagonist administration decreases T-bet levels, which could represent a new target for the development of novel RA therapies.

3.3. AMG487 inhibits Th17/RORγt and STAT3 signaling pathway

Th17 cells are major players in the development and pathogenesis of RA. Flow cytometry was used to analyze the effect of AMG487 on the Th17/RORγt and STAT3 pathways in splenic cells. We first assessed the effect of AMG487 on IL-17A-producing $CXCR3^+$ and $CD4^+$ cells in CIA and normal mice. We found that the number of IL-17A-producing $CXCR3^+$ and $CD4^+$ cells was significantly increased in the CIA control mice as compared to NC mice (Fig. 3A and B). AMG487 administration in CIA mice caused a significant decrease in the levels of IL-17A-producing $CXCR3^+$ and $CD4^+$ cells as compared with CIA control mice (Fig. 3A and B). Moreover, CIA control mice showed a significant increase in IL-17A transcript and protein expression as compared to NC mice in knee tissues (Fig. 3C and D). The administration of AMG487 treatment decreased both mRNA and protein levels of IL-17A in knee tissues of CIA mice compared with CIA control mice (Fig. 3C and D). These results indicate that the AMG487 could attenuate development of RA progression through diminishing IL-17-producing cells.

Marked increases in the number of CXCR3- and RORγt-producing $CXCR3^+$ and $CD4^+$ T cells were observed in CIA control mice compared with NC mice (Fig. 4A and B). AMG487 treatment in CIA mice decreased the occurrence of $CXCR3^+$ and RORγt-producing $CXCR3^+$ and $CD4^+$ T cells in the spleen (Fig. 4A and B). We further examined the effect of AMG487 on STAT3-producing $CD4^+$ T cells in CIA mice and found that AMG487 treatment suppressed the number of STAT3-producing $CD4^+$ T cells (Fig. 4C). Simultaneously, CIA control mice significantly increased mRNA expressions of CXCR3, RORγt and STAT3 in knee tissues as compared with NC mice (Fig. 4D–F). AMG487 treatment of CIA mice significantly inhibited CXCR3, RORγt and STAT3 mRNA expression as compared with CIA control mice (Fig. 4D–F). CIA control mice also had increased CXCR3 and RORγt protein expressions, whereas AMG487 prevented this induction and significantly decreased CXCR3 and RORγt protein expressions in knee tissues (Fig. 4G and H). Our results indicate that AMG487 could serve as an anti-inflammatory agent in RA treatment.

3.4. AMG487 downregulates Th22 cells during arthritis

To understand how AMG487 inhibits arthritis pathogenesis in CIA mice, we examined the effects of AMG487 on IL-22-producing cells. AMG487 treatment significantly decreased the levels of IL-22-producing $CXCR3^+$ cells as compared to CIA control mice (Fig. 5A). We also assessed IL-22 mRNA and protein expression in knee tissues. Consistently, IL-22 mRNA and protein expression levels decreased in knee tissues of AMG487-treated CIA mice compared to CIA control mice (Fig. 5B and C). Thus, CXCR3 antagonist could be a promising treatment of autoimmune diseases including RA.

3.5. AMG487 upregulates Treg cells

As shown in Fig. 6A and B, AMG487 administration in CIA mice resulted in significant increases in the levels of IL-10- and Foxp3-producing $CD4^+$ and $CXCR3^+$ T cells as compared with the CIA control mice. To further examine the regulating effect of AMG487 on IL-10 and Foxp3 activity, mRNA and protein expressions were investigated in knee tissues (Fig. 6C–E). A significant increase in the mRNA and protein levels of IL-10 and Foxp3 was observed in the CIA mice treated with AMG487 as compared with the CIA control mice (Fig. 6C–E). Taken together, these results revealed that AMG487 upregulated Treg cells, which could retard RA progression.

4. Discussion

In the present study, we demonstrate that AMG487 has anti-inflammatory activity. We found that untreated CIA mice developed swelling earlier and reached the highest arthritis score. First, we investigated the effects of AMG487 on the pathogenesis of RA in a CIA model. Arthritis severity in AMG487-treated mice was significantly lower than in CIA control mice. Synovitis and bone erosion are the two important features of RA, so we further investigated the pathological findings in CIA mice. Interestingly, AMG487 administration improved the histopathological features, including inflammatory cell infiltration and bone destruction in the joint of CIA mice. Our data suggest that the CXCR3 antagonist has an essential role in the downregulating inflammatory responses during CIA.

CXCR3 is a phenotypic marker of Th1 cells and has been previously shown that Th1 cells are predominately found in the joints of RA patients [63]. CXCR3 is significantly expressed in the majority of T cells and is considered to play a critical role in RA [56,60]. It has also been shown that CXCR3 expression is associated with inflammatory reactions [55]. Previous studies have underlined the importance of Th cells transcription factors in the progression of RA. Moreover, the expression of T-bet has an immunomodulatory effect on the development of RA [38]. Although, another study suggests that T-bet could be a main culprit in disease initiation and progression [48]. We hypothesized that CXCR3 blockade would inhibit Th1 cells by inhibiting T-bet pathway. Our results indicated that treatment of CIA mice with AMG487 resulted in the inhibition of T-bet mRNA and protein expression, suggesting an anti-inflammatory mechanism of AMG487. Our results indicate that T-bet signaling plays a critical role in the joint destruction; therefore, it has an important role in RA and bone damage. Our findings specify that AMG487 has protective effect which could yield through the inhibition of T-bet signaling in RA. Previous studies also showed that blockade of CXCR3 pathway led to inhibition of T cell recruitment in inflamed joints, thereby reducing the severity of arthritis [43]. It was further confirmed in CXCR3-deficient mice that showed reduced arthritis symptoms [39].

Interleukin-17 plays an important role in the initiation and development of RA [31]. Several mice models of arthritis support the role of IL-17A in RA pathogenesis [47]. It has also been reported that the involvement of IL-17A pathway has a central role in cell migration to the inflammatory site, which precedes its role during pathogenesis [10]. A previous study confirmed that IL-17A is associated with joint destruction in RA patients [32]. IL-17A levels in serum and synovial fluid are significantly higher in RA patients, which corroborates clinical scores and cartilage degradation [44,66]. In contrast, IL-17A upregulates the expression of chemokine receptors, which are highly expressed in RA joint [22]. RORγt has a critical role in RA pathogenesis, and the attenuation of its expression could be used to control progression of RA [20,51]. STATs are the prominent actors in RA progression, and increased STAT3 expression signaling is known to contribute to immunopathogenesis [61,68]. STAT3 has been described to be closely associated with osteoclastogenesis, and its inhibition was effective in treating arthritis in CIA mouse model [45]. Our results demonstrated that the levels of IL-17A-, RORγt- and STAT3-producing $CXCR3^+$ and $CD4^+$ T cells were significantly decreased in the spleen cells of AGM487-treated CIA mice. These results were further confirmed by mRNA and protein expression levels in knee tissues. Our results revealed that AMG487 treatment in CIA mice decreased mRNA and protein expression levels of CXCR3, IL-17A, RORγt, and STAT3. Therefore, it can be concluded that the anti-arthritic effect of AMG487 is caused by the inhibitory action on IL-17A and by downregulating RORγt/STAT3 expression in the CIA model. These results suggest that the main effect of AMG487 on arthritis is supported by its activity on inflammatory signaling.

Interleukin-22 is a potent pro-inflammatory cytokine and its increased expression activates fibroblast-like synoviocytes in RA [13]. A previous report showed that IL-22 levels are significantly high in RA patients [67]. Increased expression of IL-22 was also found in the synovial tissue of RA patient [25]. It has been reported that blocking IL-22 could be beneficial in RA patients [12]. In this study, we showed that inhibiting IL-22 could retard the progression of arthritis in CIA model. We found that AMG487 treatment reduced the number of IL-22-producing $CD4^+$ T cells in the spleen of CIA mice. Our study also confirmed the anti-arthritic as well through the effect of AMG487 on mRNA and protein levels. Similarly, mRNA and protein levels of IL-22 were decreased by AMG487 treatment in knee tissues. Our data suggest that AMG487-mediated inhibition of IL-22 activation could contribute to its efficacy in CIA and potentially human RA.

It is known that the development of RA is associated with Th17/Tregs imbalance in patients with RA [50]. Earlier evidence also indicates the reciprocal role of Th17/Treg cell balance in autoimmune diseases [41]. More importantly, Th17/Tregs imbalance has been involved in the pathogenesis of RA [34]. Treg cells exert excellent preventive and therapeutic effects on CIA [33]. Several studies indicated that the percentage of Treg cells is reduced in RA [27,42]. Moreover, Treg cells also exert their suppressive effect through secreting inhibitory cytokines [52]. Interleukin-10 is a potent immunoregulatory cytokine that inhibits the expression of proinflammatory mediators [40]. Increased IL-10 expression ameliorates CIA via suppressing Th17 cells [64]. Our results demonstrated that AMG487 increased Foxp3- and IL-10-producing $CXCR3^+$ and $CD4^+$ T cells in the spleen. We also found that the mRNA and protein expression levels of Foxp3 and IL-10 were significantly increased in the knee tissues of AMG487-treated CIA mice. Thus the observed anti-inflammatory effects of AMG487 could be due to their ability to increase the levels of Foxp3/IL-10 cells, thereby regulating the Th17/Treg cell balance. This suggests that the chemokine receptor antagonist AMG487 has marked advantages as a new therapeutic agent for RA.

Recently, it has been reported that some Th subsets show dual nature, i.e. Th1-like Th17 cell in autoimmune disorders such as RA. These cells co-express both RORγt and Tbet and produce both IL-17A and IFN-γ [35]. Although we have not investigated dual expressing Th subsets in our study, it is plausible that Th1-like Th17 play an important role in RA pathogenesis in this CIA model as our data show increase in both Tbet and RORC. Future studies are needed to investigate this aspect as well as the effect of CXCR3 signaling on these cells.

In conclusion, our study showed that the chemokine receptor antagonist AMG487 ameliorates arthritis in the CIA model. Furthermore, in this experimental model, AMG487 downregulated IL-17A/RORγt signaling, which is one of the main factors in RA pathogenesis. We further revealed that the extent of suppression is positively correlated with the reversal of Th17/Treg cell imbalance. Therefore, it is expected that AMG487 treatment can ameliorate RA disease by regulating Th17/Treg function. Taken together, our findings showed that AMG487 is a potential therapeutic agent for the treatment of RA and other autoimmune disorders.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109395.

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