Involvement of histamine 4 receptor in the pathogenesis and progression of rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is one of the major autoimmune diseases with a global prevalence. Despite significant research into this disease, no drugs with acceptable safety profiles are yet available for its treatment. We investigated the possible anti-arthritic effects of the 4-methylhistamine (4-MeH) histamine 4 receptor (H4R) agonist and the JNJ77777120 (JNJ) H4R antagonist to explore the role of H4R in a mouse model of collagen antibody-induced arthritis (CAIA). Arthritis was induced via intravenous (tail vein) injection of Balb/c mice with a 5-clone cocktail of mAbs against collagen type II, followed by LPS, and the effects of treatment with 4-MeH or JNJ (30 mg kg⁻¹, i.p, twice daily) for 7 days (prophylactic or therapeutic regimens) were assessed. The results revealed increased paw edema, arthritic scores, joint histological inflammatory damage and matrix metalloproteinase-3 levels and high levels of T_h pro-inflammatory cytokine mRNA and serum proteins in CAIA mice or following H4R activation via 4-MeH. Additionally, 4-MeH efficiently increased expression levels of NF-κB p65. JNJ-treated mice showed a substantial reduction in all the previously mentioned effects, with a similar trend being observed under prophylactic and therapeutic treatment regimens. The results of the present work indicate that JNJ exhibits significant anti-inflammatory and anti-arthritic activities, demonstrating the clear involvement of H4R antagonism in the pathogenesis and progression of RA.

Keywords: 4-methylhistamine dihydrochloride, chemokines, collagen antibody-induced arthritis, cytokines, histamine 4 receptor, interleukin, JNJ77777120, mRNA expression, NF-κB p65, regulatory T cells, T-cell subsets

Introduction

Rheumatoid arthritis (RA) is a common destructive chronic inflammatory arthropathy for which there is currently no cure and that is associated with substantial personal, social and economic costs. RA is often considered the prototype disease for defining both the molecular and pathological bases of immune-mediated chronic inflammatory diseases and for validating targeted therapies (1).

The human histamine 4 receptor (H4R), discovered by Nguyen et al. (2), is the latest addition to the histamine receptor subfamily (3). This subfamily of G protein-coupled receptors consists of the H1, H2, H3 and H4 receptors, each of which exhibits distinct expression patterns and physiological functions, including playing roles in vasodilation, gastric acid secretion and neurotransmission (4). H4R has been shown to be involved in inflammatory processes, and the administration of H4R antagonists in preliminary in vivo models has been successful in counteracting the development of inflammation following a variety of stimuli (5). Although H4R has been detected in a variety of tissues at the mRNA level through RT–PCR, its overall expression is relatively low (6). However, H4R has been detected by immunohistochemical staining on monocytes, leukocytes, intra-epithelial cells and enterocytes in the gut (7). The expression of functional H4R has also been
demonstrated on human monocytes and neurons in native brain slices from the rat (7). H4R expression appears to be limited to specific subtypes of cells or specific local conditions in tissues, such as those associated with inflammatory processes. For example, up-regulation of H4R expression in diseased tissues has been postulated to occur in RA, where the expression level of H4R in synovial tissue taken from RA patients might be related to disease severity (8). Infiltration of macrophages and excessive formation of fibroblasts causes the secretion of a variety of cytokines from synovial membranes in RA patients, and these cytokines, in turn, stimulate osteolytic activities (9).

There is documented evidence of significant increases in histamine concentrations in synovial samples from RA patients (10). More convincingly, varying H4R expression levels have been observed in different cell types, including CD4+ T2, T2 cells, Langerhans dendritic cells and monocyte-derived dendritic cells, before and after immunological stimuli, thereby illustrating the up-regulation or down-regulation of H4R expression under specific pathophysiological conditions (6).

Morgan et al. (11) showed that H4R activation induced the recruitment of Foxp3+ Treg cells and inhibited allergic asthma in a murine model. Similarly, Cowden et al. (12) reported that therapeutic antagonism of H4R inhibited T-cell infiltration into the lungs and decreased the levels of T2 cytokines, IL-13, IL-5 and lung collagen. These data demonstrate that H4R antagonism can significantly ameliorate allergen-induced airway dysfunction. In addition, H4R agonists have been reported to induce eosinophil production in order to increase chemotactic responses (13). This action was completely inhibited by an H4R antagonist, JNJ (JNJ7777120) (14). These observations indicate potentially significant roles for H4R in the pathogenesis, progression and treatment of RA.

Many conventional anti-inflammatory and anti-rheumatic drugs, including glucocorticoids, aspirin, sodium salicylate, sulfasalazine and gold compounds, are inhibitors of NF-κB activation. The activation of members of NF-κB transcription family is proven to be a crucial factor for contributing to chronic inflammatory responses in the pathogenesis of RA (15–17). NF-κB is highly activated in synovial tissue of patients with RA (18, 19) and NF-κB binds to DNA sequence and further induces the transcription and production of cytokines, chemokines and inducible nitric oxide (20, 21). Besides, activation of NF-κB usually has great effect on other signal transduction proteins, many of which are relevant to RA, including iNOS, COX-2 and components of the MAPK pathway (22, 23). Thus, the modulation of NF-κB pathways may be an effective approach for the treatment of RA and other chronic inflammatory disorders.

The aim of this study was to investigate the effect of 4-methylhistamine (MeH, a potent, high-affinity H4 receptor agonist that displays >100-fold selectivity over other human H1, H2 and H3 receptor subtypes) or JNJ (an H4R antagonist) on murine collagen antibody-induced arthritis (CAIA), which shares both immunological and pathological features with human RA. To illustrate the role of H4R in this model of RA, we determined the following end-points of the RA response:

- paw edema, arthritis score, and the levels of T-cell subsets (CD4+, CD25+ or Foxp3+ cells and CD4-CD25-Foxp3+ Treg cells), CD4+ T cells secreting the intracellular cytokine IL-4 and glucocorticoid-induced TNFR (GITR)-expressing cells, which were investigated using flow cytometry in heparinized blood. In addition, the levels of the IL-17A, T1 and T2 cytokines and matrix metalloproteinase-3 (MMP-3) were examined through ELISA in serum samples following both prophylactic and therapeutic treatment, and we investigated the effects of 4-MeH or JNJ on pro-inflammatory IL-2, IL-1β, IL-6, IFN-γ, TNF-α, IL-17A, NF-κB p65, intercellular adhesion molecule-1 (ICAM-1) and anti-inflammatory IL-10, TGF-β1 and Foxp3 mRNA expression in ankle tissues via RT–PCR. Furthermore, we quantified the mRNA level of GATA-binding protein-3 (GATA-3), a key transcription factor associated with T2 development. In parallel, we determined the gene expression levels of the MCP-1 and macrophage inflammatory protein-2 (MIP-2) chemokines under prophylactic and therapeutic treatments. The effects of the applied treatments were confirmed by histological investigation of ankle tissue.

Methods

Animals

Female adult Balb/c mice, 6–7 weeks old and weighing 20–22 g, were obtained from the animal house of the College of Pharmacy of King Saud University, Riyadh, Kingdom of Saudi Arabia. The mice were maintained at 22 ± 2°C with a 12-h light/dark cycle, housed in a specific pathogen-free environment and fed standard rodent chow and given water ad libitum. All procedures were performed with the approval of the Institutional Animal Care and Use Committee.

Chemicals

The compounds 4-MeH dihydrochloride and JNJ were obtained from Axon-Medchem (The Netherlands). JNJ was dissolved in DMSO, whereas 4-MeH was dissolved in normal saline. A FITC-labeled mAb against mouse T-cell surface antigen CD4, a mAb against mouse Foxp3, an allophycocyanin (APC)-labeled mAb against mouse CD25, PE-labeled mAbs against mouse IL-4 and GITR, Fc receptor blocking reagent and fixation/permeabilization and permeabilizing solutions were obtained from Mitlenyi Biotech (Germany). Heparin was purchased from Sigma–Aldrich (USA). A Monoclonal Antibody Cocktail was obtained from Chondrex, Inc. (USA), and mouse MMP-3 and T1, T2 and T17 ELISA kits were obtained from Ray Biotech, Inc. (USA). The primers used in this study for gene expression were purchased from Applied Bio Systems (UK).

Induction of CAIA

A 5-clone cocktail of mAbs (Chondrex, Inc., USA) was administered via intravenous (IV) injection (tail vein) of Balb/c mice on day zero (0) at a dose of 1.5 mg per mouse, after which 25 µg per mouse of LPS was administered via intra-peritoneal injection on day 3. Arthritis was induced on days 3 and 4 and peaked on approximately days 7–10 (24). An injection of LPS on day 8 was administered to re-stimulate inflammatory
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arthritis. Macroscopic scoring of CAIA (arthritis) and the progression of CAIA was evaluated by macroscopic scoring of the paw every day for the entire experimental period. The measurement of the paw edema was performed using a volume displacement plethysmometer (Ugo Basile, Italy), which is the most commonly employed method to measure the paw edema (25).

Prophylactic and therapeutic treatment

To investigate the effect of JNJ and 4-MeH on the pathogenesis or progression of RA, we adapted prophylactic and therapeutic treatment regimens involving 4-MeH and JNJ in the CAIA mice model. In prophylactic treatment, Arthritis was induced via IV injection of Balb/c mice with a 5-clone cocktail of mAbs at a dose of 1.5 mg per mouse on day 0. JNJ or 4-MeH were administrated intra-peritoneally at a dose of 30 mg kg⁻¹, twice a day for seven consecutive days (days 0–7), followed by an intra-peritoneal injection of LPS (25 μg per mouse) on day 3, 1 h after regular JNJ or 4-MeH exposure to stimulate inflammatory arthritis. In therapeutic treatment, mice were induced with mAb at a dose of 1.5 mg per mouse on day 0; on days 3 and 8, mice were intra-peritoneally injected with 25 μg per mouse of LPS to initiate two episodes of arthritis. JNJ and 4-MeH were administrated intra-peritoneally at a dose of 30 mg kg⁻¹, twice a day for seven consecutive days after the second dose injection of LPS (days 8–14). The doses of JNJ and 4-MeH were selected based on the results of the previous in vivo study (5). Mice were sacrificed on day 8 (in prophylactic treatment) or day 14 (in therapeutic treatment) for blood and ankle tissue isolation. Control groups for prophylactic and therapeutic experiments received: saline group = saline only, DMSO group = DMSO only, LPS group = LPS only, mAb group = mAb only and arthritic control (AC) group = LPS + mAb.

Measurement of the severity of arthritis

The mice were observed daily after the injection of mAb for the development of arthritis until day 8 (in prophylactic treatment) or day 14 (in therapeutic treatment). The severity of arthritis was blindly scored on a 0–4 scale/paw to avoid scoring biases: 0 = normal; 1 = mild erythema or swelling of the wrist or ankle or erythema and swelling of any severity for 1 digit; 2 = more than three inflamed digits or moderate erythema and swelling of the ankle or wrist; 3 = severe erythema and swelling of inflammation of wrist or ankle; and 4 = complete erythema and swelling of the wrist and ankle, including all digits.

Determination of the number of CD4⁺, CD25⁺, Foxp3⁺ and CD4⁺CD25⁺Foxp3⁺ (T reg) cells in heparinized blood

Whole blood was collected in heparinized tubes from the retro-orbital plexus after the administration of light ether as an anesthetic for the assessment of CD4⁺ and CD25⁺ surface markers. FITC-labeled CD4 mAbs and APC-labeled CD25 mAbs were used to determine the percentage of CD4⁺ and CD25⁺ cell receptors. These antibodies were added directly to 100 μl of whole blood, protected from light, and the cells were lysed using a whole blood lysing reagent. Approximately 20 μl of the corresponding fluorescently labeled mAb was then added to the suspension of mononuclear leucocytes (CD4 FITC labeled and CD25 APC labeled (Miltenyi Biotech), followed by incubation at room temperature for 20 min, protected from light.

For staining of the intracellular marker Foxp3, the cells were permeabilized by consecutively adding working solutions of standard buffers (Mouse Foxp3 Buffer A and mouse Foxp3 Buffer C from the Miltenyi Biotech Mouse Foxp3 Buffer Set, Cat. No. 5110825091) to every test tube. The buffers were diluted according to the manufacturer’s instruction (Cat. No. 5110825091). The leucocytes were incubated for 30 min in the dark at room temperature and then washed twice with 2 ml of wash buffer. Next, a 20-μl aliquot of the PE-labeled antibodies against the intracellular marker Foxp3 was added to the re-suspended sediment. This solution was subsequently incubated for 30 min in the dark at room temperature, and the cells were washed twice with 2 ml of wash buffer. Measurements were performed on a flow cytometer (Beckman Coulter, USA). The analysis of the acquired data was accomplished using the Beckman Coulter CXP software application.

Assessment of an intracellular pro-inflammatory anti-inflammatory cytokine in CD4⁺ T cells

Whole blood (100 μl) was pipetted directly into a 12 × 75 mm fluorescence-activated cell-sorting tube containing 20 μl of mAb against CD4 (Miltenyi Biotech), followed by incubation at room temperature in the dark for 10 min. RBCs were lysed with 2 ml of ×1 lysis solution (BD Biosciences) for 10 min. After centrifugation at 300 × g for 5 min, the supernatant was discarded, and ×1 permeabilizing solution (500 μl, BD Biosciences, USA) was added to the pellet, followed by incubation for 10 min at room temperature in the dark. After washing with 3 ml of buffer (1% BSA and 0.1% NaN₃ in ×1 PBS), cytokine-specific antibodies (20 μl: IL-4; Miltenyi Biotech) were added to the cells, followed by incubation for 30 min at room temperature in the dark. After one final wash, the cells were suspended in 1% paraformaldehyde (500 μl) and stored at 4°C until flow cytometric analysis.

Determination of the number of GITR-expressing cells in heparinized blood

Samples of whole blood (100 μl) were pipetted directly into a 12 × 75 mm fluorescence-activated cell-sorting tube containing 20 μl of a monoclonal PE-labeled GITR antibody (Miltenyi Biotech), followed by incubation at room temperature in the dark for 20 min. RBCs were lysed using 3 ml of fluorescence-activated cell-sorting lysis solution (BD Biosciences) for 5 min. After centrifugation at 300 × g for 5 min, the cells were stored at 4°C until flow cytometry analysis.

Measurement of T₁, T₂, T₈, Tₐ and MMP-3 cytokine levels in the serum

Serum samples from all experimental animals and normal controls were collected and immediately prepared for the analysis of cytokine levels. In protocols, commercially
available kits were used with monoclonal, specific antibodies for each cytokine. Cytokine levels were measured via ELISA, using a kit from Ray Biotech, Inc. according to the manufacturer's instructions. Briefly, individual samples were added in triplicate to 96-well plates that had been coated with an anti-cytokine capture antibody. After overnight reaction, the plates were washed extensively, and biotinylated anti-cytokine detection antibodies were added, followed by incubation at room temperature for 2 h. Bound antibodies were detected using HRP-conjugated streptavidin and visualized using tetramethylbenzidine as a substrate, followed by measurement of the absorbance at 450 nm. Individual recombinant cytokines provided in the kits were used to establish standard curves.

RNA extraction and cDNA synthesis

All extraction procedures were performed on ice using ice-cold reagents. Total RNA from mouse ankle tissue was isolated from homogenates using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The obtained mRNA was quantified by measuring the absorbance at 260 nm, and its quality was determined by measuring the 260/280 ratio. cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mixture of 2.0 μl of ×10 reverse transcriptase buffer, 0.8 μl of ×25 dNTP mix (100 mM), 2.0 μl of ×10 reverse transcriptase random primers, 1.0 μl of multi-scribe reverse transcriptase and 3.2 μl of nuclease-free water. The final reaction mixture was held at 25°C for 10 min, then heated to 37°C for 120 min and 85°C for 5 s and, finally, cooled to 4°C.

Quantification of mRNA expression in ankle tissue via RT–PCR

Quantitative analysis of target gene mRNA expression was performed via RT–PCR by subjecting the cDNA obtained from the above preparation methods to PCR amplification in 96-well optical reaction plates, using the ABI Prism 7500 System (Applied Bio systems). The 25-μl reaction mixture contained 0.1 μl of 10 μM forward primers and 0.1 μl of 10 μM reverse primers (40-μM final concentration of each primer), 12.5 μl of SYBR Green Universal Mastermix, 11.05 μl of nuclease-free water and 1.25 μl of the cDNA sample. The primers used in these assays were selected from PubMed and other databases, which are listed in Table 1. Assay controls were incorporated into the same plate, which consisted of no-template controls to test for contamination of any of the assay reagents. The real-time PCR data were analyzed using the relative gene expression (i.e., ΔΔCT) method, as described in Applied Biosystems User Bulletin No. 2. Briefly, the data are presented as the fold change in gene expression normalized to an endogenous reference gene [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] and relative to a calibrator.

Histopathological assessment of arthritis

The ankle joints were removed 7 or 14 days after the injection of mAb cocktail IV, then fixed for 4 days in 10% formalin, decalcified in decal solution (EDTA) in 5% formic acid, embedded in paraffin and sectioned (7-μm thickness). H&E staining was performed, and slides were photographed for analysis of joint pathology by a histopathologist.

Statistical analysis

All data are presented as the mean ± SEM and six animals are included in each group. The results were analyzed by ANOVA, followed by the Tukey–Kramer test. The level of statistical significance was set at P < 0.05 for differences between the pairs of compared groups.

Results

Effect of 4-MeH or JNJ on the macroscopic features of paw edema and the arthritis score

As shown in Fig. 1(A), dramatic increases in paw edema and arthritis scoring were observed in the AC mice following a single IV injection of mAb to induce CIAA. In addition, macroscopic redness and some edema were observed in many

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Direction and sequence</th>
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<tr>
<td>IL-2</td>
<td>F: 5'-TCCAGAACATGCGCGAGAAG-3' R: 5'-CTGTGCTGTCGCCAGACCAT-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: 5'-TCGTGTCTGCTGACCACATT-3' R: 5'-GTCTGTTGTTGCTTCCCTGTT-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: 5'-CCCGGAGAGAGACCTCACAG-3' R: 5'-GGAAATTTGGGTAGAAGAA-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F: 5'-TCTGGGCTTCTCCTCCTGCGG-3' R: 5'-GGGCGTGAGACCTCGTGTTG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5'-GGCGGAGTCGGGCAGAGGTA-3'</td>
</tr>
<tr>
<td>IL-17A</td>
<td>F: 5'-ACCACCGACGCCGAGAA-3' R: 5'-TGAGGGTGTGGAAGAA-3'</td>
</tr>
<tr>
<td>MIP-2</td>
<td>F: 5'-ACAAGACGATCTTCAAGAC-3' R: 5'-ACGTGTTGGAAGCA-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>F: 5'-ACCATGCCTGATAGCGCGC-3' R: 5'-GGTACCCCCCAGAGACCT-3'</td>
</tr>
<tr>
<td>NF-κB p65</td>
<td>F: 5'-ACATGCGCTGAACTCAGCG-3' R: 5'-GGGCGTGGTGGTGGGATG-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>F: 5'-GACGCGGAGGCTGAGCTGTA-3' R: 5'-GGGCGTGAGACCTCGTGTTG-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>F: 5'-CAGCGAGGAGGCTGAGCTGTA-3' R: 5'-GGGCGTGAGACCTCGTGTTG-3'</td>
</tr>
<tr>
<td>Foxp3</td>
<td>F: 5'-GCTATGCTGCTACCCGAGAATCT-3' R: 5'-GAGTGATGCTGGGTGG-3'</td>
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<tr>
<td>GATA-3</td>
<td>F: 5'-CTATCGAGCAAGGACCTGAG-3' R: 5'-GGGCGTGAGACCTCGTGTTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-CCAGACAAGGAGACTGAGCAGAAG-3' R: 5'-GGTCTGCGGATAATGTGAGG-3'</td>
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</table>
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of the other joints of the AC mice (Fig. 1B). Significant reduction in paw edema and arthritis scoring after prophylactic or therapeutic JNJ administration was observed compared with the 4-MeH or AC group (Fig. 1C).

Fig. 1. Effect of 4-MeH or JNJ on macroscopic features. (A) Paw edema. (B and C) Arthritic scoring. mAb = 5-clone cocktail of mAbs. Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. *P < 0.05 compared with the control groups (Saline, DMSO, LPS or mAb); **P < 0.05 compared with the AC group; ***P < 0.05 compared with the 4-MeH group. Each value indicates the mean ± SEM of six animals.

Effect of 4-MeH or JNJ on the number of T-cell subsets in the blood

The number of CD4+CD25+ cells exhibited a substantial increase in the AC group compared with other
Involvement of histamine 4 receptor in RA control (Saline, DMSO, LPS and mAb) groups (Fig. 2A). Treatment with 4-MeH induced a marked increase in both subsets in the prophylactic and therapeutic groups compared with the AC group, whereas JNJ caused a significant decrease in both subsets in both the prophylactic and therapeutic groups compared with the 4-MeH and AC groups (Fig. 2A).

Fig. 2(B) demonstrates that both AC and 4-MeH groups showed a significant decrease in the number of Foxp3+ and activated T<sub>reg</sub> cells (CD4+CD25+Foxp3+), compared with the control (Saline, DMSO, LPS and mAb) groups. The action of JNJ was completely different to the action of the 4-MeH or AC groups.

Effect of 4-MeH or JNJ on the number of GITR-expressing cells

GITR is considered a pro-inflammatory receptor. The number of cells expressing GITR was considerably increased in the AC and 4-MeH groups but significantly declined in the JNJ-treated group. This tendency was observed at equal levels in the prophylactic and therapeutic experiments (Fig. 3).

Effect of 4-MeH or JNJ on the secretion of anti-inflammatory cytokines by CD4+ T cells

The activation of H4R by 4-MeH markedly diminished the number of CD4+ T cells secreting IL-4, compared with the AC group. In contrast, JNJ increased the number of IL-4-secreting cells. A similar relationship was detected for both the 4-MeH and the JNJ prophylactic and therapeutic treatment groups (Fig. 4).

Effect of 4-MeH and JNJ on MMP-3 levels

Stimulation of arthritis induced a marked increase in MMP-3 levels in the AC group compared with control (Saline, DMSO, LPS and mAb) groups. Treatment with 4-MeH augmented this stimulation, whereas JNJ inhibited it equally in the prophylactic and/or therapeutic experiments (Fig. 5).

**Fig. 2.** Effect of 4-MeH or JNJ observed through flow cytometric analysis of the number of CD4+ and CD25+ (A); Foxp3+ and T<sub>reg</sub> cells in heparinized blood (B). mAb = 5-clone cocktail of mAbs. Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. *P < 0.05 compared with the control groups (Saline, DMSO, LPS or mAb); **P < 0.05 compared with the AC group; ***P < 0.05 compared with the 4-MeH group. Each value indicates the mean ± SEM of six animals.
Effects of 4-MeH and JNJ on the expression of pro-inflammatory cytokines

The AC group showed higher expression of IL-2, IL-1β and IL-6 in ankle joint tissues compared with their expression in the control (Saline, DMSO, LPS and mAb) groups. Treatment with 4-MeH intensified this expression, whereas JNJ antagonized it compared with the AC and 4-MeH groups in both the prophylactic and the therapeutic experiments (Fig. 6A). Moreover, the AC group exhibited a significant increase in IFN-γ, TNF-α and IL-17A expressions in ankle joint tissues, compared with the saline, DMSO, LPS and mAb groups. Treatment with 4-MeH stimulated the expression of these genes, whereas JNJ down-regulated their expression. The same pattern was observed in both the prophylactic and therapeutic experiments (Fig. 6B).

Effects of 4-MeH and JNJ on the expression levels of transcription factor NF-κB p65

As illustrated in Fig. 8, there was no significant difference in levels of the transcription factor NF-κB p65 between saline, DMSO, LPS and mAb groups. 4-MeH-treated and AC control groups markedly increased NF-κB p65, compared with saline, DMSO, LPS and mAb control groups. In the group treated with JNJ, the NF-κB p65 triggering was significantly...
Fig. 6. Effect of 4-MeH or JNJ on the expression of pro-inflammatory cytokine genes. mRNA expression was measured via quantitative RT–PCR of ankle tissue. mAb = 5-clone cocktail of mAbs. Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. *P < 0.05 compared with the control groups (Saline, DMSO, LPS or mAb); aP < 0.05 compared with the AC group; bP < 0.05 compared with the 4-MeH group. Each value indicates the mean ± SEM of six animals.

Fig. 7. Effect of 4-MeH or JNJ on the expression of pro-inflammatory mediator genes. mRNA expression was measured via quantitative RT–PCR of ankle tissue. mAb = 5-clone cocktail of mAbs. Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. *P < 0.05 compared with the control groups (Saline, DMSO, LPS or mAb); aP < 0.05 compared with the AC group; bP < 0.05 compared with the 4-MeH group. Each value indicates the mean ± SEM of six animals.
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inhibited, compared with 4-MeH-treated and AC control groups in both the prophylactic and the therapeutic treatments (Fig. 8).

Effects of 4-MeH or JNJ on the expression of anti-inflammatory cytokine genes

The arthritis animal model (AC group) and the 4-MeH-treated group exhibited marked down-regulation of IL-10 and TGF-β1, compared with the control (saline, DMSO, LPS and mAb) groups, whereas JNJ treatment up-regulated these genes following both prophylactic and therapeutic treatments (Fig. 9A).

Effects of 4-MeH or JNJ on the expression of Foxp3 and GATA-3

Fig. 9(B) illustrates one of the most important findings of this study, which is the down-regulation of AC and 4-MeH on the expression of the Foxp3 and GATA-3 genes compared with
their expression levels in the saline, DMSO, LPS and mAb groups. In contrast, JNJ induced the expression of both genes significantly in both the prophylactic and the therapeu-
tic groups (Fig. 9B).

Prophylactic effects of 4-MeH or JNJ on the serum expression levels of extracellular cytokines
Under the prophylactic treatment regimen, AC and 4-MeH markedly increased pro-inflammatory cytokine expression. In particular, IL-2, IL-6, IL-12, IL-17A, IL-23 and IFN-α were up-regulated compared with the control (Saline, DMSO, LPS and mAb) groups. AC and 4-MeH also markedly decreased the serum levels of the anti-inflammatory cytokines IL-4, IL-5, IL-10, IL-13 and TGF-β1. This inhibition was more prominent in the 4-MeH-treated group compared with the AC group. Treatment with JNJ caused a significant increased these effects compared with the AC and 4-MeH groups (Table 2).

Therapeutic effects of 4-MeH or JNJ on extracellular serum cytokine levels
Under the therapeutic treatment regimen, both AC and 4-MeH significantly increased the levels of IL-2, IL-6, IL-12, IL-17A, IL-23, IFN-γ and TNF-α compared with control (Saline, DMSO, LPS and mAb). Simultaneously, AC and 4-MeH markedly decreased the expression of the anti-inflammatory cytokines IL-4, IL-10, IL-13 and TGF-β. This down-regulation was further evident in the 4-MeH-treated group compared with the AC group. As observed above, JNJ almost completely reduced these effects (Table 3).

Prophylactic effect of 4-MeH or JNJ on joint histopathology
The AC group shows a significantly obliterated joint space with moderate inflammatory cell infiltrate associated with soft tissue inflammation. 4-MeH-treated mice show aggravated severe inflammatory cell infiltrate with completely obliterated joint space associated with highly dense soft tissue inflammation and inflammatory giant cell formation. JNJ-treated mice show marked improvement in the inflammatory process with less obliteration of the joint space with remnant fibro-inflammatory cell infiltration (Fig. 10).

Therapeutic effects of 4-MeH or JNJ on joint histopathology
The AC group shows incompletely obliterated joint space with dense inflammatory cell infiltration surrounded by fibro-inflammatory soft tissue. 4-MeH-treated mice show highly aggravated dense inflammatory cell infiltration with completely obliterated joint space associated with severe soft tissue inflammation and fibrosis. JNJ-treated mice show improvement in the inflammatory process with less obliteration of the joint space by fewer fibro-inflammatory cells (Fig. 11).

Discussion
The involvement of histamine in the pathophysiological process of RA is controversial; however, many studies indicate that histamine may play a role in disease progression (26). An increase in the number of mast cells and in the concentra-
tion of histamine has been noted in the synovial tissue and fluid of patients with RA (10). H1R and H2R antagonists have been reported to exhibit inhibitory effects on rat adjuvant arthritis (27). In contrast, other studies have supported an anti-inflammatory role of histamine based on the finding that RA patients exhibit significantly lower levels of histamine in their circulation, compared with healthy controls.

H4R is also expressed in many organs in the human body, including the brain, heart, liver and lungs (3). However, particularly abundant expression of H4R has been reported in certain organs and cell types, especially in the spleen, leukocytes and thymus (28). Chemotaxis of mast cells and eosinophils is stimulated by histamine through H4R (14). IL-5 induces H4R-specific mRNA expression in HL-60, a human promyelocytic leukemia cell line (29). Additionally, H4R controls the release of IL-16 from CD8+ T lymphocytes (30). These observations suggest that H4R participates in immune reactions that underlie mechanisms of inflammation and allergy. RA is a disease arising from disturbances in the immune system, and as such, H4R is likely to play a significant role in this disease.

Anti-inflammatory effects of the H4R antagonist JNJ were evaluated in a model of acute skin inflammation (Cowden et al. (12)). The evidence from many laboratories suggests that histamine increases the secretion of T2 cytokines (IL-5, IL-10 and IL-13) and suppresses the secretion of T1 cytokines (IL-2, IFN-γ) (31–33). H4R inhibits IL-12 synthesis while augmenting IL-10 production, thus providing favorable conditions for the differentiation and development of T2 cells (34). The H4R is expressed on CD4+ T cells and up-regulated on T2 cells when it is stimulated with IL-4, a T2 cytokine (35). Moreover, it has been suggested that H4R plays a role in the activation of T cells. In this study, we explored the effects of the H4R agonist 4-MeH and the H4R antagonist JNJ in a mouse model of CAIA. Histological evaluation indicated that JNJ treatment diminished joint inflammation and reduced cartilage destruction, contrib-
uting to a defensive effect against CAIA. It thus appears that upon blocking the H4R, JNJ inhibits all the pro-inflammatory effects of histamine. Remarkably, JNJ exerted a more significant preventive effect against the early onset of CAIA compared with the AC and 4-MeH-treated groups. However, JNJ and 4-MeH exhibited converse effects after the onset of arthritis in both prophylactic and therapeutic experiments. These results indicate that JNJ can exert a distinct preventive effect during the course of autoimmune arthritis.

Both the arthritic and the 4-MeH-treated groups exhib-
ited prominent increases in the numbers of CD4+, CD25+ cells and those expressing GITR, whereas in the AC and 4-MeH-treated groups, the numbers of Foxp3+ cells and CD4+CD25+Foxp3+ Treg cells were markedly attenuated to an equal extent under the prophylactic and therapeutic treat-
ment regimens. JNJ up-regulated these effects in heparin-
ized whole blood. The AC group is a positive control group that received both the mAb cocktail and LPS injections to develop arthritis. The other control groups like saline or DMSO did not receive the above-mentioned treatments as
### Table 2. Prophylactic effect of 4-MeH or JNJ on extracellular pro-inflammatory and anti-inflammatory cytokines in serum determined using an ELISA array

<table>
<thead>
<tr>
<th></th>
<th>Pro-inflammatory cytokines (prophylactic effect)</th>
<th>Anti-inflammatory cytokines (prophylactic effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2 (pg ml⁻¹)</td>
<td>IL-6 (pg ml⁻¹)</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>35.2 ± 4.32</td>
<td>55.98 ± 4.87</td>
</tr>
<tr>
<td>LPS</td>
<td>42.11 ± 4.18</td>
<td>64.22 ± 4.25</td>
</tr>
<tr>
<td>mAb</td>
<td>44.02 ± 4.47</td>
<td>66.25 ± 4.02</td>
</tr>
<tr>
<td>AC</td>
<td>130.11 ± 4.02</td>
<td>110.09 ± 4.47</td>
</tr>
<tr>
<td>4-MeH</td>
<td>175.71 ± 4.30</td>
<td>178.91 ± 4.05</td>
</tr>
<tr>
<td>JNJ</td>
<td>71.87 ± 4.87</td>
<td>72.19 ± 4.01</td>
</tr>
</tbody>
</table>

Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. Each value indicates the mean ± SEM of six animals.

*P < 0.05 compared with the control groups (saline, DMSO, LPS or mAb); **P < 0.05 compared with the AC group; ***P < 0.05 compared with the 4-MeH group.

### Table 3. Therapeutic effect of 4-MeH or JNJ on the serum contents of extracellular pro-inflammatory and anti-inflammatory cytokines determined with an ELISA array

<table>
<thead>
<tr>
<th></th>
<th>Pro-inflammatory cytokines (therapeutic effect)</th>
<th>Anti-inflammatory cytokines (therapeutic effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2 (pg ml⁻¹)</td>
<td>IL-6 (pg ml⁻¹)</td>
</tr>
<tr>
<td>Saline</td>
<td>36.01 ± 4.00</td>
<td>58.04 ± 4.01</td>
</tr>
<tr>
<td>DMSO</td>
<td>40.25 ± 4.03</td>
<td>63.20 ± 4.02</td>
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<tr>
<td>LPS</td>
<td>40.13 ± 3.88</td>
<td>70.24 ± 4.76</td>
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<tr>
<td>mAb</td>
<td>47.2 ± 4.05</td>
<td>71.02 ± 4.21</td>
</tr>
<tr>
<td>AC</td>
<td>158.04 ± 4.10</td>
<td>124.07 ± 4.01</td>
</tr>
<tr>
<td>4-MeH</td>
<td>205.54 ± 4.21</td>
<td>204.41 ± 4.02</td>
</tr>
<tr>
<td>JNJ</td>
<td>84.74 ± 4.01</td>
<td>87.94 ± 4.47</td>
</tr>
</tbody>
</table>

Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test.

*P < 0.05 compared with the control groups (saline, DMSO, LPS or mAb); **P < 0.05 compared with the AC group; ***P < 0.05 compared with the 4-MeH group. Each value indicates the mean ± SEM of six animals.
Fig. 10. Prophylactic effect of 4-MeH or JNJ on joint histopathology. Prophylactic indicates that 4-MeH and JNJ was administered directly prior to the injection (IV) of anti-collagen type II 5-clone cocktail of mAbs on day 0, and LPS was administered on day 3; the animals were observed up to day 8. (A) Representative section of joint histopathology is shown. (B) Pathology scores of each group were calculated and expressed as mean ± SEM of six animals. Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. *$P < 0.05$ compared with the control group (Saline); $aP < 0.05$ compared with the AC group; $bP < 0.05$ compared with the 4-MeH group.
Fig. 11. Therapeutic effects of 4-MeH or JNJ on joint histopathology. Therapeutic indicates that induction of the first episode of arthritis occurred when mAb was administered on day 0, and LPS was administered on days 3 and 8 to initiate a second episode of arthritis. 4-MeH or JNJ was administered directly prior to the second dose of LPS at day 8, and the animals were observed up to day 14. (A) Representative section of joint histopathology is shown. (B) Pathology scores of each group were calculated and expressed as mean ± SEM of six animals. Statistical analyses were performed through one-way ANOVA, followed by the Tukey–Kramer test. *$P < 0.05$ compared with the control group (Saline); $aP < 0.05$ compared with the AC group; $bP < 0.05$ compared with the 4-MeH group.
these are considered as naive control groups. Lymphocytes from the so-called T<sub>reg</sub> lineage, characterized by the expression of the surface molecule CD4<sup>+</sup> play a central role in the initiation and maintenance of arthritic processes. A major feature of T-cell activation is the production of cytokine mediators. T<sub>reg</sub> subpopulations can be distinguished on the basis of the set of cytokines they produce. T-cell stimulation leads to the up-regulation of several cell surface molecules, including CD25. Functionally, CD25 acts as the IL-2R α chain (36). Its expression is not specific to activated T cells; CD25 is also expressed by T<sub>reg</sub> cells and activated B cells (37).

Accumulating evidence supports the idea that CD4<sup>+</sup>CD25<sup>+</sup> cells play an essential role in controlling and preventing autoimmunity (38). During the last 5 years, somewhat paradoxically, several reports have described increased numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the peripheral blood (39) and synovial fluid (40) of RA patients. GITR is known to exhibit a co-accessory function in effector T-cell activation and may help to inhibit T<sub>reg</sub> cells function (41). GITR is activated by its ligand, GITRL, which is mainly expressed on antigen-presenting and endothelial cells (42). The effects of stimulation through GITR are generally thought to be mediated by attenuation of the immunosuppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> T cells (43) and by increased resistance of effector T cells to CD4<sup>+</sup>CD25<sup>+</sup> T-cell-induced suppression (44).

T<sub>reg</sub> cells express the transcription factor Foxp3 and are a key part of the immune system apparatus that controls inflammatory processes (45). T<sub>reg</sub> cells are crucial for the suppression of potentially harmful excessive immune responses (46) and have been identified as important mediators of peripheral immune tolerance. Defects in T<sub>reg</sub> cell function have been demonstrated to lead to failed tolerance in human autoimmune diseases, including RA (47). Moreover, T<sub>reg</sub> cells isolated from the synovial fluid or peripheral blood of RA patients have been reported to suppress the proliferation of autologous T cells in vitro (47).

Our results demonstrated that JNJ treatment up-regulated Foxp3 gene expression, which was correlated with increased numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in JNJ-treated mice, although the AC and 4-MeH-treated groups exhibited different responses under the prophylactic and therapeutic regimens. The pathogenesis of RA is classically viewed as involving deregulation of the T<sub>reg</sub>/T<sub>1</sub> balance, and the key players in this paradigm are the pro-inflammatory cytokines and the T<sub>reg</sub> subset of T cells (48). Evidence suggests that T-cell expansion and differentiation may occur within the synovial membrane as a result of a favorable cytokine environment characterized by the presence of IL-1β, IL-6, IL-23 and TGF-β (49). In humans, IL-1β, IL-6 and IL-23 can promote T<sub>reg</sub> differentiation, whereas TGF-β may inhibit T<sub>reg</sub> differentiation (50).

In this study, the administration of JNJ reduced the serum levels of the T<sub>1</sub> IL-2, IL-6, IL-12, IL-17A, IL-23, IFN-γ, TNF-α and T<sub>reg</sub> cytokines compared with their levels in the AC and 4-MeH-treated groups under both the prophylactic and the therapeutic treatments. These findings suggested that JNJ can down-regulate the T<sub>1</sub> cell response to mediate an anti-inflammatory function. The balance of cytokines produced by the T<sub>reg</sub>/T<sub>1</sub> subsets of T cells plays an important role in the development of RA (40). In this study, we observed that JNJ increased the production of IL-4 by T<sub>reg</sub> cells under both the prophylactic and therapeutic treatments. It is clear that JNJ can shift the cytokine balance from T<sub>reg</sub>1 cytokines towards a T<sub>reg</sub>2 cell response.

JNJ decreased the production of MMP-3 detected in the serum compared with both the arthritic and 4-MeH-treated groups under the prophylactic and therapeutic regimens. There is substantial evidence that MMPs are capable of mediating matrix degradation and joint destruction. Immunohistological and in situ hybridization analyses have revealed the abundant presence of MMP-3 in rheumatoid synovial membranes (51).

Inflammatory cytokines and chemokines are believed to be involved in the development of RA (52). Therefore, these cytokines are regarded as targets for RA treatment. In the arthritic model examined in this study, inflammatory cytokines such as IL-1β, IL-6 and TNF-α and chemokines such as MCP-1 were shown to contribute to the development of arthritis (53). Moreover, it is well known that chemokines play an important role in the regulation of arthritic inflammation, including the infiltration of lymphocytes, monocytes and neutrophils (54). In this study, the expression of IL-1β, TNF-α, MIP-2 and MCP-1 mRNA was markedly increased in the arthritic and 4-MeH-treated groups, whereas JNJ-treated mice showed a significant reduction under both the prophylactic and the therapeutic treatments.

Consistent with these findings, there are two additional potential explanations regarding the mechanism underlying the preventive effects of the JNJ and 4-MeH treatments. The levels of IL-2, IFN-γ and ICAM-1 mRNA expression were markedly increased in the AC and 4-MeH-treated groups. However, treatment with JNJ attenuated the expression of these genes over the course of the prophylactic and therapeutic treatments. ICAM-1, an important molecule involved in leukocyte recruitment during inflammation, is expressed at increasing levels in synovial tissue during arthritis, suggesting a role in the pathogenesis of RA (55).

IL-17A increases the activation of synoviocytes and the expression of other cytokines, contributing to cartilage and bone destruction (56). IL-17A is detected at higher levels within the joints of patients with RA, and in CAIA models, elevated levels of IL-17A are observed in inflamed synovia (57). In this study, the administration of JNJ reduced the mRNA expression levels of the IL-17A cytokines in ankle tissue, and JNJ inhibited IL-17A cytokine production in the serum to an equal extent under both the prophylactic and the therapeutic treatments.

In contrast, the levels of IL-10, TGF-β1, Foxp3 and GATA-3 mRNA expression were significantly down-regulated in the AC and 4-MeH-treated groups, whereas treatment with JNJ significantly up-regulated these genes to an equal degree under both the prophylactic and the therapeutic regimens. TGF-β is a pleiotropic cytokine that controls cell growth and differentiation (58). Within the immune system, TGF-β inhibits the growth of T cells, B cells and other hematopoietic cells and suppresses IFN-γ production (59).

Secreted products of activated T cells and direct cell–cell contacts induce activation of macrophages, the major producers of inflammatory cytokines in RA synovium. NF-κB controls the expression of cytokines IL-1β and TNF-α, the essential mediators of inflammation in RA. In addition, they modulate
Involvement of histamine 4 receptor in RA

various genes through both transcriptional and post-transcriptional mechanisms in RA and play key roles in adaptive immune inflammatory responses (60, 61). The functions of NF-κB suggest that the inhibition of NF-κB activity would reduce the production of pro-inflammatory cytokines and further modulate the related inflammatory reactions (62, 63). It is worthy of note that suppression of NF-κB inhibited expression of many pro-inflammatory molecules, including IL-1, TNF-α, IL-6, IL-8, ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), but had little, if any, effect on the expression of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (64-66). This suggests that NF-κB facilitates the impaired balance of pro-inflammatory and anti-inflammatory molecules in the arthritic joint. Therefore, an alternative targeting to the signal pathways of NF-κB was proposed (67).

GATA-3 is a transcription factor that is highly expressed in T cells and promotes T2, while inhibiting Th1 differentiation (68). Our results demonstrate that JNJ effectively modulates anti-inflammatory cytokines to accelerate the joint inflammation repair process and inhibits the infiltration of inflammatory cells into the damaged area through the down-regulation of chemokines and cytokines. In conclusion, our results provide a comprehensive assessment of the immunomodulatory pathways in which H4R may act in the pathogenesis and treatment of RA and suggest that JNJ may serve as a potent anti-inflammatory and anti-arthritic agent in RA.

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References


49 Raza, K., Falci, M., Curnow, S. J. et al. 2005. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. Arthritis Res. Ther. 7:R784.