Therapeutic Effect of the Immunomodulator, FTY720 in Rheumatoid Arthritis Rat Model: Sphingolipids Perspective (FTY720 in Rheumatoid Arthritis, FTY720 on RA)

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Abstract

Introduction: Rheumatoid Arthritis (RA) is the most common autoimmune inflammatory arthritis. Fingolimod (FTY-720) is a Sphingosine 1-Phosphate (SIP) receptor modulator, reported to have a therapeutic effect in autoimmune disease models.

Aim: To evaluate the potential therapeutic effect of fingolimod on established rat model of RA and its possible underlying mechanisms.

Experimental Protocol: The arthritic model was established by subcutaneous immunization of female albino rats with Muramyl Dipeptide (MDP) and bovine type II collagen (C-II) (MCIA model). Rats were boosted with a second collagen-MDP emulsion 60 days after the first immunization. Rats were evaluated for arthritis severity using a macroscopic scoring system.

From the day of onset of arthritis symptom, the MDP/CII challenged rats, with similar arthritis scores, were randomly divided into three groups; arthritic control, FTY-treated and prednisolone (pred, was chosen as the positive drug) treated arthritic groups. Treatment was continued up to day 90. With non immunized rats were taken as non-arthritic negative control group.

Assessment of RA was performed by measuring serum rheumatoid factor-Immunoglobulin M (RF-IgM), Cartilage Oligomeric Matrix Protein (COMP) as specific rheumatoid biomarkers, Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) as inflammatory biomarkers to asses disease severity and activity. Blood and Synovial Fluid (SF) total and differential leukocytes were counted. Spleen and thymus indices were recorded while SIP levels were assayed in blood, spleen and thymus. Paws Cyclooxygenase-2 (COX-2) and Prostaglandin-E2 (PG-E2) proteins were quantified.

Results: Compared to normal control group, the arthritic rats displayed dramatic increase in blood inflammatory cells with massive joint cellular infiltration, increased arthritis scores, spleen and thymus indices, flaring in disease activity with cartilage erosion, upregulated paw COX-2/PGE2 protein levels together with increased SIP levels and high SIP gradient between blood and thymus, spleen.

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The anti-inflammatory and chondroprotective effects of FTY-720, similar to pred, was associated with decreased arthritis scores, recruitment of leukocytes into the inflamed joint, paw COX-2/PGE2 levels, and spleen index. Unlike the non significant effect of FTY-720 on thymus index, it was decreased under pred treatment. There were non significant change in SIP level and gradient in pred treated group while SIP level was increased with obliterated gradient with FTY 720 treatment, compared to the MCIA challenged female albino rats.

Conclusion: FTY720 was as potent as pred for suppressing arthritis, mostly through modulation of SIP signaling and COX-2/PGE2 axis suggesting FTY 720 may be an effective candidate drug for treating human with RA.

Key Words: Rheumatoid arthritis – MCIA – FTY720 – Fingolimod – Cyclooxygenase – Rheumatoid factor – IgM – Cartilage oligomeric matrix protein.

Introduction

RHEUMATOID Arthritis (RA) is a chronic, inflammatory, autoimmune, systemic disease, which primarily involves the joints, leading to inflammation, swelling, pain, stiffness, with inevitable progressive functional disability in up to one third of RA patients if not early and promptly treated [1].

Although the exact pathogenesis of RA is not fully understood, the immune and inflammatory systems are intimately linked. During the course of RA, an extensive array of inflammatory and immune cells, whether cell-mediated as well as humoral immune effectors are recruited to the synovial tissues. Each of these cell subsets acts at distinct stages in the course of the disease, to participate in the complex network of cell-cell interactions that governs RA initiation and progression, through the production of produce large amounts of chemokines, proinflammatory cytokines and release of inflammatory mediators [2].
The massive cellular infiltration of the synovium is controlled at a molecular level and enhanced by the expression of adhesion molecules, selectins and their ligands, integrins and chemokines and their receptors that govern regulation of cellular trafficking to the inflamed joint [3].

Ongoing inflammation in RA involves positive feedback loops between activated T cells, B cells, Dendritic Cells (DCs), macrophages, and their products, with destructive consequences for parenchymal cells and erosion of joint [4].

Prednisolone (Pred) and other Glucocorticoids (GCs) are very potent immuno suppressive and anti-inflammatory compounds that are among the top 10 most prescribed drugs in RA and other autoimmune diseases, although its long term use is associated with severe side effects. So current therapeutic intervention with high efficacy and preserved safety remains challenging [3].

The rat Collagen-Induced Arthritis (CIA) is a widely used and a well-established model of arthritis that displays several immunological and pathological features of human RA [4].

The immunomodulator Fingolimod (FTY720), is a chemical derivative of the fungal metabolite, myriocin. While FTY720 shared structural similarities with sphingosine, the unphosphorylated precursor of Sphingosine 1-Phosphate (S1P), it was predominantly activated by phosphorylation in vivo by Sphingosine Kinases-2 (SphK2), the major S1P producing enzymes, to the fingolimod phosphate (FTY-P), which led to more than 100-fold higher FTY-P concentrations in tissues than in blood [5]. FTY-P acts as a potent agonist on four of the five G protein-coupled receptors specific for S1P, S1P receptors (S1PR), S1PR1, S1PR3, S1PR4, and S1PR5 especially S1PR1. FTY720-P strongly induces internalization and degradation of (S 1P-Rs), acts as a functional antagonist at S 1P-Rs. S1P is a natural lysophospholipid and potent bioactive messenger known to regulate a wide range of fundamental functions and may play a role in RA pathology. Indeed, SphK1, SphK2 and S1P levels are elevated in the synovium of RA patients [8]. S1PRs were found to be expressed in synovial lining cells, vascular endothelial cells, and inflammatory mononuclear cells of RA synovium. Moreover S1P signaling via S1PR1 enhances synoviocyte proliferation, inflammatory cytokine-induced COX-2 expression, and PG-E2 production [5].

In contrast to conventional immunosuppressants, FTY720 does not impair T and B cell activation, proliferation, and effector function but interferes with cell trafficking between lymphoid organs and blood [1].

The intention of this study was to evaluate the immunomodulatory therapeutic approach of FTY-720 on clinically active arthritis, in a modified CIA model, MCIA, a relevant model of human RA [6] as an emerging target in lipid-based therapy of RA and its associated molecular mechanisms.

Material and Methods

Experimental animals:

Three-months-old female albino rats, with a mature immune system [7], were housed individually in cages, to minimize pain and distress, in a climate-controlled room with a 12-h light-dark cycle. The animals were allowed access to regular standard rats chow and water ad libitum, throughout the study. This study was conducted at Faculty of Medicine, Tanta University, on the period between February to May, 2016. Treatment and maintenance were conducted according to the guidelines established by the Research Ethical Committee of Faculty of Medicine, Tanta University, Egypt.

Induction of MDP/Collagen Induced Arthritis (MCIA):

After acclimation period of one-week, the RA model was evoked, where type-II collagen (CII), normally found in joint cartilage, was presented to the rats' immune system in the dermis and augmented by Muramyl Dipeptide (MDP) to reproducibly induce T cell activation, anti-CII autoantibody (autoAb) production, inflammation and severe arthritis with high incidence as previously reported by Koga T et al., [8].

MDP-collagen preparation: The native lyophilized bovine CII extracted from the nasal septum cartilage (Catalog #20041, Chondrex; LLC, Redman, WA, USA) was dissolved at 4mg/ml in 0.05 M acetic acid. Then the collagen was mixed with an equal volume of MDP (Sigma Aldrich) at a concentration of 2.5mg/ml. The MDP/CII solution was emulsified with an equal volume of Incomplete Freund's Adjuvant (IFA) (Catalog #7002, Sigma, St Louis, MO, USA) and was kept cold at 4°C and administered subcutaneously at the base of the tail within an hour of its preparation.

Induction of arthritis: The day of the first immunization was marked as day 0; each rat received subcutaneous (SC) injection of 0.2ml (200 mg of cold MDP/CII/IFA emulsion/rat). 60 days after the first immunization, the rats were boosted.
by a second injection of 0.1mL of the freshly prepared MDP/CII/IFA emulsion [6,8].

Clinical monitoring of RA: Animals were observed daily until the end of the third week and then every week till the end of the experimental period [9], to quantitatively evaluate the severity of the arthritis in each paw according to the degree and extent of erythema and edema of the periarticular tissue, as well as the swelling.

The arthritis severity was assessed by calculation of the Arthritis Index (AI) according to a macroscopic scoring system ranging from 0 to 16, as described by Kleinau S. et al., [10], as follows; each paw was scored on a graded scale from 0 to 4 as follows; grade 0 represents normal paws with no edema or swelling in the joint; grade 1 represents mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; grade 2 represents moderate redness and swelling of ankle or wrist; grade 3 represents severe redness and swelling of the entire paw including digits; grade 4 represents maximally inflamed limb with involvement of multiple joints.

Each paw was graded, and the four scores were summed to calculate the total score of each rat so that the maximum possible score per rat was 16 [11,12]. The arthritis grade was scored by the same observer blinded to the treatment group. Swollen digits were noted but paws were only considered arthritic when the entire paw was inflamed for 2 consecutive days [13].

Incidence percentage: Was calculated through dividing the number of animals with any clinical signs of arthritis over the total of the number animals in each group at specific time points [6].

The mean clinical severity score for each group was calculated as the mean of total arthritis scores of all rats within the group [6].

Grouping and treatment protocol:
Joint swelling and pain are the initial manifestations of RA [2]. Drugs administration were begun immediately at the onset of appearance of clinical signs of RA on day 15 post-immunization and continued until final assessment on day 90.

Experimental rats were randomly divided into 4 groups:

Non-arthritic control group (n=10): The rats were not immunized, but were injected with an equal volume of Phosphate-Buffer Saline (PBS) containing 0.05mmol/L acetic acid, at the same location. Served as a negative control for arthritis [4].

Arthritic control group (n=12): Arthritic animals were received an equal volume of normal saline.

FTY-treated arthritic group (n=11): Arthritic animals were daily received FTY720 (cat number #10006292, Cayman Chemical Company, Ann Arbor, MI, USA) (0.3mg/kg/IP) [15].

Prednisolone treated arthritic group (n=11): Arthritic animals were daily received Methylprednisolone (MP) acetate (10mg/kg/day, IP) [16] (DepoMedrol, Pharmacia and Upjohn, Belgium) is available as sterile aqueous suspension in the form of 1ml vial containing 40mg. Prednisolone was used as a reference.

Blood samples: 90 days after the first MDP/CII injection, all animals were sacrificed by cervical decapitation and blood was collected. Part of the fresh blood sample was used for the detection of the Erythrocyte Sedimentation Rate (ESR). The rest of the blood was collected and centrifuged for 10min at 3000rpm. Serum aliquots were then frozen at –20°C for further biochemical analysis.

ESR assay using the westergren method [17]: ESR was measured, as a better test for general severity of RA and as an indicator of chronic inflammatory disease state [18]. Tri-sodium citrate-treated blood was left to sediment for one hour in capillaries (TapvalTM tubes, Aquisel, Barcelona, Spain).

Serum C-Reactive Protein (CRP), as a better measure for acute phase responses, and RA activity [18], was determined using enzyme linked immunosorbent assay ELISA test kit obtained from (Catalog No. IB66103, IBL-America, Inc., USA).

Serum Rheumatoid Factor-Immunoglobulin M isotype (RF-IgM) concentration was assayed as an important biomarker and a potential diagnostic tool in rat model and humans with RA [4]. IgM-RF was analyzed by (ELISA) kit for RF-IgM quantitation (R & D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer’s instructions, as described previously [18,19].

Serum levels of Cartilage Oligomeric Matrix Protein (sCOMP): As a potential marker for cartilage involvement in experimental RA [20], was measured using the rat COMP ELISA (AnaMar Medical, Gothenburg, Sweden), according to manufacturer instructions, as described previously [21].
The total and differential leukocyte count in blood and synovial fluid:

The blood cytology: The WBC was determined automatically using a Coulter Counter JT hemocytometer (Hialeah, FL, USA) calibrated for rat blood, and the differential WBC was obtained by manual enumeration of May–Grünwald-Giemsa (MGG; Merck, Darmstadt, Germany)-stained blood cell smears.

Synovial lavage fluid cytology: In order to obtain SF, a small incision of the skin was made above the patella of knees followed by insertion of a needle into the synovial cavity and injection of 25 µls of saline containing EDTA into the synovial cavity. Five minutes later, the fluid was withdrawn by second needle that was inserted into the synovial cavity approximately 3mm from the infusion needle. The total cell count was determined by light microscopy. A differential cell count was also obtained using smears prepared from cell pellets and stained with Giemsa in accordance with the procedure of Salinas [22].

Organ index measurement: At the end of experimental period, the rats were weighed, sacrificed. Spleen and thymus were then promptly removed and weighed. The thymus and spleen indices were expressed as the ratio of the thymus or spleen wet weight respectively, divided by the body weight of the animal (mg/g) [23].

SIP concentration assay: Blood, spleen and thymus were obtained from all experimental groups. Lipids were extracted by chloroform/methanol/HCl solution and the SIP concentrations were determined using liquid chromatography tandem mass spectrometry as described previously [24]. Total protein concentrations were colorimetrically determined using Biuret reaction [25].

Paw sample preparation: After death, paws from all experimental animals were amputated above the ankle, and snap frozen in liquid nitrogen. Frozen paws were pulverized in a liquid nitrogen bath and divided into aliquots for extraction and analysis of PG and COX-2 protein levels. Samples were stored at −70°C until used.

Cyclooxygenase (COX)-2 and Prostaglandin (PGE2) proteins analysis:

COX-2 protein level was quantified by ELISA (Catalog no #760111, Cayman Chemicals, Ann Arbor, MI, USA) [26], and was expressed as nanograms COX-2 per milligram protein. PGE2 protein level was assayed by ELISA (Cayman Chemicals, Ann Arbor, MI, USA) [27]. Total protein content was determined using the Bio-Rad detergent compatible protein assay kit (Bio-Rad) according to the instructions. BSA (Sigma, Egypt) was used as standard.

Statistical analysis:

All data were expressed as mean ± Standard Deviation (S.D). Statistical analysis was carried out using GraphPad Prism version 6 (GraphPad Software, San Diego, California, USA). Difference between groups was evaluated by one-way analysis of variance (ANOVA). The Mann-Whitney U test was used to analyze differences between groups. p-values less than 0.05 were considered to be significant.

Results

Incidence and effect of FTY 720 on severity of arthritis in MCIA rats Fig. (1):

50 female albino rats were immunized with CIA/MDP. Only 48 animals presented with arthritic manifestation with high arthritic incidence (96% (48/50). According to the daily macroscopic evaluation, only thirty four arthritic animals with similar arthritis scores were preceded in our study. The rats that failed to develop arthritic lesion after immunization or those with different arthritic score were excluded from our study.

The first signs of arthritis were always observed in the hind limbs. The the affected limb became severely red and swollen in the ankle, tarsal, metatarsal and interphalangeal joints, this was reflected by increased arthritic score.

CIA/MDP challenged rats developed arthritis on day 14 post immunization with a peak severity on day 28 then the severity decreased gradually to minimum value on day 60 post arthritis induction.

With the second immunization (d 60), there were a dramatic increase in the severity of arthritis in the model group, where the arthritis score reaches the maximum value of (15.67 ± 1.7) on day (77), then started to decline again toward the end of study (d 90). By the end of our study, the swelling in the majority of affected animals was transformed to clear deformities of the paws.

On administration of FTY720 on day (15), there were a drastic decrease in arthritic severity that were comparable to that of pred as reflected by marked decrease in mean arthritis score throughout the treatment period (5.3 ± 1.4 in FTY vs. 5.5 ± 1.3 in pred treated group). The improvement in arthritis
severity was apparent on the second day of drugs treatment.

**Effect of FTY 720 on the blood leukocyte count of MCIA rats (Table 1):**

Similar to RA, inflammation was not restricted solely to the joints of MCIA rats, but was systemic in nature. Immunization of female rats with CIA/MDP resulted in significant elevation of serum total inflammatory cells by about –3.4 fold and differential lymphocyte, monocyte and neutrophil counts by about 2.8, 3.1 and 8.5 respectively compared to the non-arthritic rats.

FTY720 drastically decreases blood total cell count by 66.6% to levels similar to the non-arthritic rats, while pred treatment decreased total cell count by about 51.1% compared to RA model rats.

FTY720 sharply decreased blood lymphocytes and neutrophils by 73% and 78.9% respectively in contrast to 57.8% and 61.8% decrease under pred treatment. Pred treatment failed to decrease monocyte count in MCIA rats while monocyte count decreased significantly with FTY 720 treatment.

**Effect of FTY 720 on the SF leukocyte count of MCIA rats (Table 1):**

Joint inflammation in arthritis is mediated by the migration of heterogeneous population of circulating inflammatory and immune cells into synovial tissues, where they subsequently release proinflammatory cytokines that not only perpetuate the inflammatory process but also increase bone resorption leading to disability [2]. The present results are consistent with these events. RA joint SF cellular infiltrate obtained from MCIA model revealed marked increased in both total and differential leucocyte count compared to the control non arthritic animals.

In this present study, we hypothesized fingolimod treatment would reduce the number of total and differential inflammatory cells migrating into synovium following experimental RA induction to greater extent than pred treatment particularly T lymphocytes thereby ameliorating joint inflammation and mitigate its destruction.

**Effect of FTY 720 on biomarkers of systemic disease activity in MCIA rats (Table 2):**

With induction and progression of arthritis in our model, there were marked increase in serum levels of CRP and ESR by about –8.3 and –5.6 fold respectively. CRP and ESR have been shown to correlate with disease activity and progression in different types of inflammatory arthritis [18]. CRP is released from liver in response to inflammatory mediators and improves antigen presentation and plays important role in the induction and maintenance of increased erythrocyte aggregation in the blood of RA patients [14].

FTY 720 exhibited apparent damping of disease activity as reflected from significant decrease in ESR and CRP to levels statistically similar to that of pred.

**Effect of FTY 720 on biomarkers of articular cartilage destruction, and (autoAbs) in MCIA rats (Table 2):**

COMP is used as an informative molecular marker for ongoing joint destruction [28], and elevated amounts of COMP can be found both in the SF and in the serum of patients with active (RA) [28]. Compared to control non arthritic animals, MCIA induced –3.8 fold increase in sCOMP levels. Reduced levels of COMP by FTY 720 and pred treatments indicate a chondroprotective effect through mitigation of the destructive process associated with RA.

Several (autoAbs) have been described in RA, but only RF, has shown sufficient sensitivity and specificity to be considered clinically useful in RA, where it is detectable in 80% of RA patients [29,30]. These (autoAbs), could appear early after immunization with CII, even before joint swelling was observed [31]. With induction of our RA model, there were marked increase in serum RF-IgM isotype by about –8.6 fold. This elevated level was greatly mitigated with FTY 720 and pred treatment.

**Effect of FTY 720 on organ indices in MCIA rats (Table 3):**

The thymus and spleen are the two major immune organs of body, and their relative weights are usually used to evaluate the immunoregulatory activity of drugs [30]. Both thymus and spleen indices in RA group were markedly increased compared with those in the control non arthritic rats.

FTY 720 treatment to arthritic animals resulted in significant reduction in spleen index but failed to affect thymus index compared to the MCIA group.

Compared with RA and FTY 720 treated RA groups, pred resulted in marked reduction in spleen index by (30.5% and 14.3%) respectively, and
dramatic reduction in the thymus index by (48.6% and 44.9%) respectively.

**Effect of FTY-720 on SIP levels in the plasma, spleen and thymus and the SIP gradient in MCIA rats** Fig. (2):

In RA group, the MCIA induced significant up regulation in SIP levels in plasma, spleen and thymus and widened the SIP gradient between blood and lymphoid organs. Pred treatment failed to modulate SIP levels or block SIP gradient. While FTY 720 treatment of RA animals resulted in up regulated SIP levels with blocked SIP gradient between thymus, spleen and blood.

**Effect of FTY-720 on serum COX-2 and PGE2 protein levels in MCIA rats** Fig. (3):

It was obvious from our results that MCIA induced significant up regulation of COX-2 protein level with subsequent increased PGE2 production. While fingolimod treatment resulted in marked reduction in both COX-2 and PGE2 to levels comparable to that of Pred treated RA group.

### Table (1): Effect of FTY 720 on the total and differential leukocyte count in blood (cell X 10^9/mm^3 of blood) and synovial fluid (cell X 10^9/mm^3 of synovial fluid) of MCIA rats.

<table>
<thead>
<tr>
<th></th>
<th>Control non-arthritic group</th>
<th>RA group</th>
<th>FTY720 treated RA group</th>
<th>Pred treated RA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood total leucocyte count</td>
<td>2.81±0.15</td>
<td>9.44±1.83</td>
<td>3.15±0.46</td>
<td>4.62±0.63</td>
</tr>
<tr>
<td>Blood lymphocyte count</td>
<td>2.2±0.54</td>
<td>5.66±0.58</td>
<td>1.53±0.52</td>
<td>2.39±0.62</td>
</tr>
<tr>
<td>Blood monocyte count</td>
<td>0.43±0.11</td>
<td>1.32±0.29</td>
<td>0.73±0.13</td>
<td>1.45±0.28</td>
</tr>
<tr>
<td>Blood neutrophil count</td>
<td>0.24±0.09</td>
<td>2.04±0.09</td>
<td>0.43±0.06</td>
<td>0.78±0.15</td>
</tr>
<tr>
<td>SF total cell count</td>
<td>1.91±0.21</td>
<td>9.35±0.96</td>
<td>2.44±0.33</td>
<td>4.29±0.44</td>
</tr>
<tr>
<td>SF lymphocyte count</td>
<td>0.66±0.24</td>
<td>4.20±0.45</td>
<td>0.47±0.11</td>
<td>1.55±0.20</td>
</tr>
<tr>
<td>SF monocyte count</td>
<td>0.94±0.13</td>
<td>4.48±0.51</td>
<td>1.43±0.34</td>
<td>2.58±0.48</td>
</tr>
<tr>
<td>SF neutrophil count</td>
<td>0.12±0.03</td>
<td>0.48±0.14</td>
<td>0.27±0.07</td>
<td>0.53±0.36</td>
</tr>
</tbody>
</table>

\(a: p<0.05\) versus control non-arthritic group. \(b: p<0.05\) versus RA model. \(c: p<0.05\) versus FTY720 treated RA group. Data are mean ± SD.

### Table (2): Effect of FTY 720 on biomarkers of articular cartilage destruction, systemic disease activity and autoAbs in MCIA rats.

<table>
<thead>
<tr>
<th></th>
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<th>RA group</th>
<th>FTY720 treated RA group</th>
<th>Pred treated RA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR (mm in one hour)</td>
<td>1.38±0.20</td>
<td>11.4±1.9</td>
<td>3.45±1.1</td>
<td>4.1±0.64</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>2.15±0.5</td>
<td>12.1±1.4</td>
<td>4.2±0.9</td>
<td>4.6±0.82</td>
</tr>
<tr>
<td>Serum RF-IgM isotype (IU/ml)</td>
<td>5.2±0.8</td>
<td>44.8±2.3</td>
<td>10.6±1.3</td>
<td>11.8±1.9</td>
</tr>
<tr>
<td>sCOMP (U/L)</td>
<td>3.04±0.2</td>
<td>11.4±1.4</td>
<td>4.2±0.5</td>
<td>4.4±0.6</td>
</tr>
</tbody>
</table>

\(a: p<0.05\) versus control non-arthritic group. \(b: p<0.05\) versus RA model. \(c: p<0.05\) versus FTY 720 treated RA group. Data are mean ± SD.

### Table (3): Effect of FTY 720 on organ indices in MCIA rats.

<table>
<thead>
<tr>
<th></th>
<th>Control non-arthritic group</th>
<th>RA group</th>
<th>FTY720 treated RA group</th>
<th>Pred treated RA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen index</td>
<td>3.1±0.3</td>
<td>5.18±0.65</td>
<td>4.2±0.37</td>
<td>3.6±0.47</td>
</tr>
<tr>
<td>Thymus index</td>
<td>0.94±0.12</td>
<td>2.2±0.28</td>
<td>2.05±0.14</td>
<td>1.13±0.13</td>
</tr>
</tbody>
</table>

\(a: p<0.05\) vs. control non-arthritic group. \(b: p<0.05\) vs. RA group. \(c: p<0.05\) vs. FTY 720 treated RA group. Data are mean ± SD.
Fig. (1): Effect of FTY-720 on arthritis severity assessed by the arthritis score, measured daily up to day 21 then weekly up to day 90, in MCIA rats. Data are expressed as mean ± SD.

*: p<0.05 versus control non arthritic group.  
#: p<0.05 versus RA model.  
@: p<0.05 versus FTY 720 treated RA group.

A: Day of 1st immunization (d 0).  
B: Day of onset of arthritis clinical manifestations (d14).  
C: Day of 2nd immunization (d 60).

Fig. (2): Effect of FTY-720 on S1P levels in the plasma, spleen and thymus and the S1P gradient in MCIA rats. Data are expressed as mean ± S.D.

*: p<0.05 versus control non arthritic group.  
#: p<0.05 versus RA model.  
@: p<0.05 versus FTY 720 treated RA group.  
SIP : Sphingosine 1-Phosphate.

Fig. (3): Effect of FTY-720 on serum COX-2 and PGE2 protein levels in MCIA rats. Data are expressed as mean ± S.D.

*: p<0.05 versus control non arthritic group.  
#: p<0.05 versus RA model.  
@: p<0.05 versus FTY 720 treated RA group.
Discussion

Despite enormous research being carried out for RA, it still remains a disorder which can be controlled and not treated. Moreover the severity of joint inflammation fluctuates, resulting in exacerbations and remissions with shortened life expectancy, and reduced quality of life. So the early intervention with efficacious safe treatments, aimed at tight control of the inflammatory process to prevent irreversible joint destruction and preserve function, remains elusive. Currently there is considerable interest in the potential of immunomodulatory therapies in the treatment of the immune based inflammatory diseases. In the current study we evaluate the effect of FTY 720, as novel therapeutic strategy, whose full spectrum of actions has not yet been uncovered, in MCIA rat model of RA.

The massive cellular influx especially lymphocytes, that were accounting for about 44.9% of cellular infiltrate, with MCIA induction and the marked improvement of arthritic rats with FTY 720 treatment, that was associated with reduction in cellular recruitment to RA joint implying that lymphocytes are important in driving the inflammatory process, and could be targeted in clinical therapy [31].

Consistent with our results, several reports recorded massive cellular influx in the inflamed joints in various animal models of RA [32,33] and in the SF of affected joints of RA patients [34,35].

Angyal et al., [31], suggested that antigen-specific T cells, which home to lymphoid organs and provide help to B cells for systemic autoAbs production, play a greater role in the development and progression of RA than the small population of T cells that migrate to the joints, but still have the power to drive chronic inflammation through antigen-independent mechanisms [32]. These T cell can activate monocytes/macrophages in a contact-dependent manner to induce the expression of inflammatory cytokines [32].

Consistent with this notion, FTY720 treatment of arthritic mice left the T-cell pool in the Primary Lymphoid Organs (PLOs) intact, but reduced T cells in both peripheral blood and joints [31].

FTY 720 treated arthritic rats displayed marked but not absolute lymphopenia in blood by about 73% compared to the MCIA group. It appears that the remaining cells in the circulation are more mature peripheral effector/memory cells, that generally lack homing receptors which are typically responsible for lymphocyte homing to Secondary Lymphoid Organs (SLOs) [36]. Pinschewer et al., [37], assumed that the remaining circulating T cells with fingolimod appear to be sufficient to mount systemic but not localized immune responses. Sawicka et al., [38], concluded that FTY720 has striking effects on lymphocyte migration and homing without apparent alteration in host defense.

Under effect of FTY720 treatment, not all lymphocyte subsets are affected equally. Previous studies [39,40] reported that although FTY720 significantly alters B cell numbers in the peripheral blood, the effects are far more profound on T cells. FTY720 also regulates B cell trafficking and migration from the bone marrow [41].

FTY720 as a high affinity agonist of S1PRs following its phosphorylation, downregulates and functionally antagonizes S1PRs on thymocytes and lymphocytes [39], creating a temporary S1P-null state, which renders these cells unresponsive to S1P, and depriving them from an obligatory S1P/S1PR-dependent signal to egress from PLOs, accompanied by an increase in lymphocyte trapping in peripheral lymph nodes and Peyer’s Patches (PP). As a consequence, lymphocytes are unable to recirculate to RA joint but remain functional in the lymphoid compartment. This is providing an explanation for the mechanism of FTY720-induced lymphocyte sequestration.

Beside blocking of the S1P-mediated lymphocyte chemotaxis, several lines of evidence pointed out that FTY720 engagement of endothelial S1P1 can increase tight junction formation [41], decreased the permeability of the endothelium of blood vessels [42] and lymphoid vascular sinuses that result in inhibited egression of lymphocytes from lymph nodes by closing egress portals [42], thereby preventing the migration of inflammatory cells toward sites of inflammation. Independent of S1PRs, FTY720 might act on the chemokine system involved in homing of T and B lymphocytes, altering their reaction to chemokine receptor rather than functions [41].

Beside its ability to block neutrophils, monocytes, and lymphocyte activation and migration [43], fingolimod specifically and effectively regulates the invasive migration of neutrophils and macrophages through endothelial layers at inflamed joint [42], through establishing the integrity of endothelial cell barriers and suppression chemotactic migration and phagokinetic activity of neutrophils and macrophages [44].

FTY720 treatment shift the activation states of macrophages from the classically activated pro
inflammatory, M1 phenotype that predominate in RA to alternatively activated anti-inflammatory, M2 phenotype, that enhance repair process [43], promote the balance in favor of an M2 phenotype [36].

FTY720 could induce its anti-inflammatory effect through preventing the leukocyte/monocyte-endothelial interactions by inhibiting the expression of adhesion molecules, which known to be up regulated in RA monocytes and suppressing the production of pro-inflammatory cytokines, therefore limits the leukocyte and monocyte number in SF of RA rats [43].

Our data indicate that modulation of S1PRs, through FTY720 treatment exhibited powerful anti-inflammatory effect, sufficient to induce a protective effect on autoimmunity in RA.

The protective effect of fingolimod recorded in our MCIA model is in accordance with previous results recorded in CIA-induced arthritis [45,46] and adjuvant-induced arthritis [18] in rats and SKG rats [1] which spontaneously develop T cell-mediated chronic autoimmune arthritis, where the FTY 720 treatment was associated with decrease edema, joint destruction and lymphocyte infiltration [31] and cytokine production [1].

Pred and other (GCs) have been used as a mainstay in the treatment of RA for five decades. They are effective anti-inflammatory agents with possible disease-modifying activity [47], they exert their clinical benefits from a complex spectrum of effects downstream of Glucocorticoid Receptor (GR) activation, which serves to modulate several thousand genes. This results in the down-regulation of many pro-inflammatory mediators including transcription of many cytokines and the expression of adhesion molecules on different cells [46], and up-regulation of anti-inflammatory and pro-resolving factor [47].

Acute-phase proteins particularly CRP has been widely studied in human rheumatology as biomarker of arthritic disease, used to assess the innate immune system's systemic response to infection and inflammation [14]. The reduction in the CRP and ESR brought about by FTY treatment support its antiarthritic effect, possibly enhanced by its anti-inflammatory activities with suppression of all stages of disease progression.

Arthritic Index (AI) includes the combined index of inflammation, formation of nodules, and extent of spread of the disease to other organs, which mirror the full picture of the disease [45]. AI increased markedly in arthritic rats reflecting evolving of disease activity and greatly mitigated with FTY720.

The elevated RF-IgM in our MCIA model support the autoimmune character and the importance of innate immunity in RA [31]. The systemic production of RF in RA involves synchronized activity of both B cell via toll-like receptors and autoreactive T cells, that are located in the SLOs and provide help to B cells for autoAbs secretion together with several genetic predispositions to arthritic diseases [32].

The suppressed production of RF-IgM induced by FTY-720 in arthritic rats reflect remarkable effect of FTY720 on humoral immune responses in our model, probably attributed to the dual inhibitory capacity on both B and T-helper functions [34].

We previously pointed that FTY720 treatment induced an accumulation of naive B cell in the bone marrow [41]. Fingolimod also diminishes germinal center formation [48] and reaction [49] so drastically curtails Abs production, mostly caused by a selective inhibitory effect on germinal center lymphocytes, either by inhibiting lymphocyte recruitment or retention to the germinal center/follicular DCs reticulum [49]. This effect is augmented by progressive reduction in the number of peripheral T cells, including T-helper, with time due to homing results in insufficient help signals for germinal center reaction.

Han S et al., [49], confirmed FTY720 evoked significant reduction in germinal center formation in the spleens, LNs, and PPs together with inhibited Abs production.

Similar to our results, FTY720 induced downregulation of Ab production in vivo [32] and in vitro [50].

The elevated sCOMP levels, a prominent component of articular cartilage, after onset of arthritis denotes ongoing joint destruction, confirmed the erosive changes and disabling nature in our experimental arthritis [20]. The elevated sCOMP levels mostly attributed to uncontrolled inflammatory process. In addition, activated synovial fibroblasts, chondrocytes and osteoclasts contribute to the underlying cartilage and bone damage [51]. The reduced levels of COMP by FTY720 and pred treatments, indicate a chondroprotective effect. This effect explained at least by the powerful anti inflammatory effect of FTY720 and pred.
Matching with our results, Lai WQ et al., [32], previously reported chondroprotective effect of FTY720 with decreased sCOMP in experimental arthritis.

While the thymus is a specialized PLO, devoted to T lymphocytes maturation, the spleen is the largest SLO and the largest peripheral B cell compartment in the body, specialized to present blood-borne antigens in an uniquely organized ways through combined the innate and adaptive immunity [49].

During the immune response, antigen-specific T and B lymphocytes are transiently retained within antigen-bearing lymphoid organs, proliferate severely, undergoing activation and clonal expansion and then exiting as effector cells. With formation of germinal center in the white pulp of spleen [39, 49]. Consistent with this fact the significantly increased thymus and spleen indices in MCIA rats.

Unlike, the marked reduction in spleen index and the non significant effect on thymus index with fingolimod treatment of arthritic animals, the pred evoked pronounced reduction in both spleen and thymus indices.

Our results has been supported by the previous findings obtained from several in vivo research studies [39,40,49] pointed to block the degree of naive lymphocyte from the thymus with sequestration of circulating mature lymphocytes into peripheral lymphoid organs by acceleration of lymphocyte homing and thereby decreases the number of lymphocytes in blood and spleen during the course of FTY720 treatment. This FTY720 effect enhanced the marked reduction in germinal center formation and reaction [48,49] as previously declared in our discussion.

Our results regarding organ indices could be clarified by the results evidenced previously from several studies that have been recorded marked atrophy of thymic cortex associated with lymphopenia with immunologically incompetent thymocytes [40] and suppressed thymus weight [47] with chronic pred dosing in experimental arthritic model. While FTY720 did not have any clear effect on the numbers of thymocytes or intrathymic differentiation of T cells [40]. Moreover FTY720 didn’t evoke corticosteroid induction in vivo [40].

The blood S 1P level is supplied by endothelium, platelets, but it is well-buffered by RBCs, which should ensure quite stable S1P values and metabolized by tissue cells via (re) phosphorylation by SphK2 and final degradation by the S1P-lyase. While tissue S 1P levels are tightly regulated by a balance among synthesis, release, and degradation [52].

A concentration gradient of S 1 P exists between plasma and interstitial fluids which is thought to be a major regulator of lymphocyte egress through binding to S1PRs. This process is finally adjusted not only by S1PRs surface expression but also by its internalization and desensitization [53].

In control normal group, there was apparent concentration gradient in SIP levels between blood and lymphoid organs with blood S 1P was relatively higher than that in lymphoid organs. With the RA induction this gradient was amplified/augmented with up regulated SIP levels compared to the normal group, implicating a clinically relevant role of S 1 P- S 1PR axis in RA, that was shown to be crucial for the lymphocyte trafficking into and out of the SLOs cells by maintaining their response threshold to diverse lymphotactic factors.

S1P has been shown to act as a chemotactic signal, guides lymphocytes and other various S1PRs expressing leukocyte subsets toward the high S1P concentration, possibly through overcoming retention signals, provide directional information, and/or promote reverse transmigration across blood or lymphatic vessels, promoting the rapid expansion of immune functions, this could explain the flared inflammation and disease activity of RA rats [54].

S1P concentrations inversely modulate S1PRs surface expression on lymphocytes [54] that turned out to be critical for maintaining lymphocyte circulation [53]. Schwab SR et al., [54], recorded disappearance of the surface S 1 P when the concentration of S1P in the lymphoid organs increases, to return back when SIP concentration dropped again.

Elevated SIP level observed in thymus and spleen in FTY720 treated RA rats may be a compensatory mechanism to aberrant FTY720 mediated S1PR internalization and down regulation in lymphoid organs [54] or accumulated after disruption in its metabolism by inhibition of SIP lyase activity with fingolimod treatment [55] or enhanced formation by SphKs [54].

S 1P accumulation that was predominantly seen in peripheral blood in RA treated with FTY720 could attributed mostly to marked FTY720 mediated inhibition of S1P lyase in tissue [55], the enzyme responsible for irreversible blood S1P
degradation [52], or as a compensatory mechanism to preserve SIP gradient between blood and lymphoid organs specially after upregulated SIP level in these organs with FTY treatment.

Similar to previous results [54,56], the SIP gradient between blood with high SIP levels and lymphoid organs with low SIP levels was annulled with FTY720 treatment. While pred had no significant effect on SIP compartmentalization.

The finding that inflammatory and immune cells egress requires the expression of SIPR on these cells and the presence of SIP gradient suggests points at which egress can be controlled and blocked by FTY720.

The established concept that PGE2 synthesis in inflammation is always COX-2-dependent [57], has been supported by significant MCIA-induced up regulated COX-2/PGE2 levels demonstrating a significant participation of PG derive via the COX.

The involvement of PG pathways in the pathogenesis of arthritis has been shown in animal models by using mice lacking genes, such as (COX-2), PGE synthase, that displayed decreased pain responses, decreased delayedtype hypersensitivity, and suppression of CIA [58]. Moreover, nonsteroidal anti-inflammatory drugs, which represent an effective therapy for treating RA, elicit their effects by inhibiting (COX) activity and blocking the downstream production of prostanoids, including (PGE2) [59]. In an animal model of polyarthritis, neutralizing PGE2 with monoclonal antibodies reduced both the signs and levels of inflammatory markers of the disease [59]. COX-2, is the product of an immediate early response gene in inflammatory cells.

PGE2 is at the apex of a proposed cascade of proinflammatory mediators in rheumatoid synovial tissue. (PGE2) contributes to pain and swelling during synovial inflammation through induction of hyperalgesia and increased vascular permeability [60]. PGE2 not only a dominant proarthritogenic cytokine, it also potentiates the effects of other mediators and modulates bone resorption through stimulation of osteoclast formation. Moreover PGE2s enhance the local expression of COX-2 in inflamed synovial tissue [27,59].

The results reported here provide specific insights on mechanisms by which novel sphingolipid signaling pathways are involved in the regulation of key rate-limiting steps, COX-2, which in turn are required for the production of the key inflammatory mediator PGE2 in response to proinflammatory cytokines, known to be involved in pathophysiology of RA. Moreover, inhibition of COX-2 also modulated local and systemic cytokine production in arthritic rats [27].

COX-2 derived PGE2 also plays a central role in the humoral responses, regulates B cell proliferation and activation as well as survival [60]. Since blocking this pathway substantially decreases Abs production. This could explain the protective mechanism of FTY720 in RA.

Moreover, the role of SIP in the modulation of COX-2 expression has been reported previously. Kitano M. et al., [61], has demonstrated that SIP mediates SIPR-dependent COX-2 induction and PGE2 production in several cell types. It is possible that the anti-inflammatory capacity of FTY720 attributed to blocking of this effect via down-regulation or internalization and lysosomal degradation, of SIPR induced by FTY720.

It has been assumed that at least a portion of the beneficial action of FTY720 is due to their inhibition of PGE2 production by eliminating the influence of SIP. Therefor SIP/SIPRs pathway seems to be required upstream of the induction of COX-2.

Crofford et al., [62], reported robust anti-inflammatory effects of pred possibly due to inhibition of Cox-2 mRNA through the destabilization of Cox-2 mRNA and/or suppression of Cox-2 transcription. Consistent with our results, the reports obtained previously from patients with active RA [63,64], that revealed dramatic improvement under GC therapy.

Conclusion:
The results from this study showed that FTY 720 was as potent as pred in suppressing arthritis, highlighted the divergent and independent roles of FTY720 in modulating a number of pathways central to the pathophysiology of RA. These results clearly suggest S 1 P-targeted therapy through FTY 720 could to be promising therapeutic strategy to combat RA, most probably through modulation of SIP signaling and COX-2/PGE2 axis, efficiently trapping of the inflammatory cells, dampening the inflammatory response, attenuating the severity, reversing structural changes and preventing the progression in our arthritis rat model.

Conflict of interest:
We have no conflict of interest to declare.
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