Methotrexate-Loaded Nanoparticles Ameliorate Autoimmune Arthritis by Regulating the Balance of Interleukin-17-Producing T Cells and Regulatory T and B Cells

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Abstract

Background: Rheumatoid arthritis (RA) is a progressive systemic autoimmune disease that is characterized by infiltration of inflammatory cells into the hyperplastic synovial tissue, resulting in subsequent destruction of adjacent articular cartilage and bone. Methotrexate (MTX), the first conventional disease-modifying antirheumatic drug (DMARD), could alleviate articular damage in RA and is implicated in humoral and cellular immune responses. However, MTX has several side effects, so efficient delivery of low-dose MTX is important.

Methods: To investigate the efficacy of MTX-loaded nanoparticles (MTX-NPs) against RA, free MTX or MTX-NPs were administered as subcutaneous route to mice with collagen-induced arthritis (CIA) at 3 weeks after CII immunization. The levels of inflammatory factors in tissues were determined by immunohistochemistry, confocal microscopy, real-time PCR, and flow cytometry.

Results: MTX-NPs ameliorated arthritic severity and joint destruction in collagen-induced arthritis (CIA) mice compared to free MTX-treated CIA mice. The levels of inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor-α, and vascular endothelial growth factor, were reduced in MTX-NPs-treated mice. Numbers of phosphorylated STAT3-positive CD4+ cells and CD4+IL-17+ cells were reduced whereas the number of CD4+CD25+Foxp3+ cells increased in spleens from MTX-NPs-treated CIA mice compared to MTX-treated CIA mice. The frequency of CD19+CD25+Foxp3+ regulatory B cells increased in ex vivo splenocytes from MTX-loaded NPs-treated CIA mice compared to MTX-treated CIA mice.

Conclusion: The results suggest that MTX-loaded NPs have therapeutic potential for RA.

Background

Rheumatoid arthritis (RA) is a progressive autoimmune disease characterized by synovial inflammation, hyperplasia, and formation of rheumatoid pannus, resulting in destruction of the adjacent articular cartilage and bone structure. The development of RA involves a complex interplay between immune cells and inflammatory mediators including inflammatory cytokines, proteolytic enzymes, and prostanoids [1–3]. Although the causes of RA are unclear, infiltration of T and B cells into the joints leads to induction of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-17A and autoantibodies and drives the proliferation of fibroblast-like synoviocytes and destruction of bone [3, 4].

IL-17-producing T helper (Th17) cells, which produce IL-17A, IL-17F, IL-21, and TNF-α, are involved in the pathogenesis of various autoimmune diseases, including RA, multiple sclerosis, and psoriasis [5, 6]. IL-6 drives the differentiation of naïve T cells into Th17 cells by activating STAT3, a key transcription factor for Th17 cells [7, 8]. In RA synovium, IL-17-producing cells were observed in the T cell-rich area and IL-17 contributes to increased production of IL-6 and leukemia inhibitory factor. Moreover, it has synergistic effects with IL-1β or TNF-α on inflammation [9–11]. Rheumatoid synovial tissue contains abundant B cells, which secrete proinflammatory mediators and autoantibodies, including rheumatoid factor and
anti–citrullinated protein antibodies [3, 12]. Moreover, B cells act as antigen-presenting cells for autoreactive T cells [13].

Methotrexate (MTX) is a first-line disease-modifying antirheumatic drug (DMARD) that alleviates articular damage in RA [14]. It is an antifolate metabolite that inhibits folate-dependent enzymes in the de novo synthesis of purines and pyrimidines [15, 16]. MTX is used as monotherapy for patients with early RA [17] and as an anchor drug for combination therapy with other DMARDs or biologics in patients with established RA who are MTX-insufficient responders [18]. However, long-term use of MTX leads to drug resistance and causes severe side effects such as nausea, neutropenia, pulmonary fibrosis, and hepatitis [15, 19].

Nanoparticles (NPs) are promising therapeutics due to their ability to deliver and release drugs [20, 21]. Multiple NPs have been developed for drug delivery in various diseases. They inhibit loss due to fast excretion of drug from body by sustained release. NPs are typically administered by intravenous injection, oral feeding, or subcutaneous injection. Intravenously injected NPs are rapidly effective but also rapidly excreted and have a high risk of side effects. Oral feeding is the easiest and low risk, but the absorption rate is too low. Subcutaneous injection shows moderate effectiveness in relation to these methods, so has an advantage in occupying the middle ground. However, most NP studies for RA therapy have used intravenous injection and oral feeding.

MTX-loaded nanoparticles (MTX-NPs) ameliorated RA after subcutaneous injection. They were formulated with hydrophobic poly (D, L lactide-co-glycolide) (PLGA) and amphiphilic polyvinyl alcohol (PVA). MTX was stably loaded into the NPs. MTX-NPs attenuated the severity of autoimmune arthritis and reciprocally regulated Th17 and regulatory T and B cells in vivo.

**Methods**

**Materials**

Resomer RG 502 H PLGA and PVA (MW 30,000–70,000) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide, 99.0% (methyl sulfoxide, DMSO) was purchased from Samchun (Seoul, Gangnam-gu, Korea).

**Preparation and characterization of MTX loaded nanoparticles**

PLGA (50 mg) and methotrexate (5 mg) were dissolved in DMSO at 60°C. The solution was added dropwise to an aqueous solution of 1% PVA (w/v). The solution was homogenized at 7000 RPM for 2 min using a homogenizer (Ultra Turrax® T-25 homogenizer; IKA®-Werke, Staufen, Germany). After homogenization, non-encapsulated substances were removed by dialysis in distilled water for 1 h through a 14,000 molecular weight cut-off membrane. The size distribution of MTX-NP was measured in PBS using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) at 25°C. Encapsulated MTX was quantified by assaying the absorbance of MTX at 370 nm and encapsulation efficiency was calculated.
by the formula: \( \text{(amount of encapsulated drug/amount of added drug)} \times 100\% \). MTX-NP was placed in a dialysis bag and immersed in a container containing 50 mL of PBS to analyze the drug release profile. At predetermined time points, 200 μL of external PBS were removed and the absorbance at 370 nm of MTX was measured using Microplate Reader Synergy H1 (Bio-Tek, USA).

**CIA induction and treatment with nanoparticles**

Six-week-old male DBA/1J mice were purchased from Orient Bio Inc. (Seongnam, Korea). To induce CIA in mice, CII was dissolved overnight in 0.1 N acetic acid (4 mg/mL) with gentle rotation at 4°C. DBA/1J mice were injected intradermally at the base of the tail with 100 mg of CII emulsified in Freund's adjuvant (Chondrex). Two weeks later, 100 μg of type II collagen dissolved and emulsified 1:1 with incomplete Freund's adjuvant (Difco) was administered to the hind leg of mice as a booster injection. On day 24 after the first immunization, mice were injected subcutaneously with 2.5 mg/kg MTX or MTX-NPs twice weekly. Animals were maintained under specific pathogen-free conditions at the Institute of Medical Science of the Catholic University of Korea and were fed standard mouse chow and water. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea; the procedure conformed to all National Institutes of Health of the United States guidelines (Permit number: 2020-0067-01).

**Histology**

Mouse joint tissues were fixed in 10% neutral-buffered formalin, decalcified in a decalcifying agent (National Diagnostics, Atlanta, GA, USA), embedded in paraffin, and sectioned. The sections (5 μm thick) were stained with hematoxylin and eosin (H&E) and scored as described previously [22]. Immunohistochemical analysis of IL-1β, TNF-α, and vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Dallas, TX, USA) was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections were examined by light microscopy (Olympus, Tokyo, Japan). Positive cells were enumerated visually by four individuals, and the mean values were calculated.

**Isolation of splenocytes**

Mouse spleens were ground using sterilized glass slides with frosted ends and red blood cells were lysed in hypotonic ACK buffer (0.15 mM \( \text{NH}_4\text{Cl} \), 1 mM \( \text{KCO}_3 \), and 0.1 mM EDTA, pH 7.4). The remaining splenocytes were filtered through a 40 μm cell strainer (Falcon, Durham, NC) and maintained in RPMI 1640 medium containing 5% fetal bovine serum (ThermoFisher Scientific, MA, USA).

**Flow cytometry**

To determine the frequency of germinal center (GC) B cells, splenocytes were immunostained with Alexa Fluor® 488-conjugated anti-GL7 (BioLegend, San Diego, CA, USA), PE-conjugated anti-Fas, and APC-conjugated anti-B220 (both ThermoFisher Scientific) antibodies. For regulatory B cells, splenocytes were immunostained with Percp-Cy 5.5 conjugated anti-CD19 (ThermoFisher Scientific), APC-conjugated anti-
CD25 (BioLegend), and PE-conjugated anti-Foxp3 (ThermoFisher Scientific) antibodies. Cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer set (ThermoFisher Scientific) according to the manufacturer's instructions. Events were collected using the FACS Calibur (BD Biosciences) or CytoFLEX (Beckman Coulter), and the data were analyzed using Flow Jo software, v. 7.6 (Treestar, Ashland, OR, USA).

Confocal microscopy

Spleen tissues were snap-frozen in liquid nitrogen and stored at −70°C. Tissue sections (5 μm thick) were fixed in acetone. To stain IL-17+ or phosphorylated (p)-STAT3+ in CD4+ cells, Alexa Fluor® 488-labeled anti-CD4 (BioLegend), PE-labeled anti-IL-17 (eBioscience, San Diego, CA), and PE-labeled anti-p-STAT3 (pTyr705) (BD Biosciences) antibodies were used. To stain regulatory T (Treg) cells, Alexa Fluor® 488-labeled anti-CD4, PE-labeled anti-Foxp3, and APC-labeled anti-CD25 (BioLegend) antibodies were used. Sections were analyzed using the LSM 510 Meta Confocal Microscopy System (Carl Zeiss, Oberkochen, Germany). Positive cells were counted visually at high magnification by four investigators.

Real-time polymerase chain reaction

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and cDNA was synthesized with the Dyne First-Strand cDNA Synthesis Kit (Dyne Bio, Seongnam, Korea) according to the manufacturer's protocol. Gene expression was measured using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA) with SYBR premix (Bioline USA Inc. Taunton, MA). The following primers were used: IL-1β, 5′GGATGAGGACATGAGCACATTC3′ (sense) and 5′GGAAGACAGGCTTGTGCTCTGA3′ (antisense); IL-6, 5′AACGATGATGCACTTGCAGAAA3′ (sense) and 5′TCTGAAGGACTCTGCTTGTC3′ (antisense); IL-17A, 5′-TTTAACTCCCTTTGCCGCAAAA-3′ (sense) and 5′CTTTCCCTCCGCATTGACAC-3′ (antisense); and β-actin, 5′GTACGACCAGAGGCATACAGG3′ (sense) and 5′GATGACGATATCGCTGCGCTG3′ (antisense). mRNA levels were normalized to that of β-actin mRNA.

Statistical analysis

All statistical analyses were performed using Prism (v. 8 for Windows; GraphPad Software). P-values were calculated by two-tailed paired t-test and two-way analysis of variance (grouped). P < 0.05 was considered indicative of statistical significance.

Results

Preparation and characterization of MTX-NPs

We prepared PLGA NPs via a homogenization method using PVA as stabilizer. MTX was physically loaded into the NPs (Fig. 1a). MTX was stably encapsulated in a hydrophobic core (PLGA) with an encapsulation efficiency of 76.6 ± 4.9%. The size of MTX-NPs was 227.2 ± 1 nm and MTX-NPs were spherical by transmission electron microscopy (TEM) (Fig. 1b). The size is suitable for injection into body by syringes with small needle. The polydispersity index (Pdi) and zeta potential of MTX-NP were 0.139 ±
0.041 and \(-2.8 \pm 0.02\), respectively, showing a homogeneous formulation without aggregation and near-neutral surface, respectively. In PBS at pH 7.4, free MTX was slowly released from the PLGA core (Fig. 1c), indicating sustained release.

**MTX-NPs attenuate the severity of autoimmune arthritis**

To determine whether MTX-NPs could modulate the development of arthritis *in vivo*, free MTX or MTX-NPs were administered to mice with CIA at 3 weeks after CII immunization. Subcutaneous injection of MTX-NPs in arthritic mice significantly reduced the arthritis score and incidence compared with vehicle-treated CIA mice. Injection of free MTX also reduced the arthritis score and incidence in CIA mice, but statistical significance was not consistently achieved (Fig. 2a). Histologic examination of joints stained with H&E showed that the ankles of MTX-NPs-treated mice exhibited less severe inflammation, bone damage, and cartilage damage compared with vehicle-treated mice. Application of MTX-NPs, in particular, exerted a more profound inhibitory effect on joint destruction compared with free MTX (Fig. 2b). Furthermore, the levels of inflammatory mediators—including IL-1β, TNF-α, and VEGF—were significantly lower in the joint sections from MTX-NPs-treated mice compared with vehicle-treated mice (Fig. 3).

**MTX-NPs reciprocally regulate the Th17 cells and Treg cells in vivo**

To evaluate whether MTX-NPs suppress Th17 cells *in vivo*, the number of CD4 + IL-17 + Th17 cells in the spleens from CIA mice injected with MTX-NPs was investigated by confocal microscopy. The number of Th17 cells was lower in MTX-NPs- or free MTX-treated CIA mice compared with vehicle-treated CIA mice (Fig. 4a). STAT3 phosphorylation in CD4 + cells decreased in MTX-NPs- or free MTX-treated CIA mice compared to vehicle-treated CIA mice, but there was no statistical significance (Fig. 4b). To investigate whether MTX-NPs reciprocally regulate the population of Th17 and Treg cells *in vivo*, the number of CD4 + CD25 + Foxp3 + Treg cells in the spleen was investigated. The number of Treg cells in the spleen of MTX-NPs-injected mice significantly increased compared to vehicle-injected mice, and the increase was also significant compared with free MTX-injected mice (Fig. 4c). In addition, the mRNA levels of IL-6 and IL-17A, inflammatory cytokines related to Th17 cell development, significantly decreased in *ex vivo* splenocytes of mice injected with MTX-NPs compared to mice injected with vehicle (Fig. 4d).

**MTX-NPs increase regulatory B cells in vivo**

To evaluate whether MTX-NPs act on B-cell responses *in vivo*, we examined the number of *ex vivo* CD19 + B220 + GL-7 + Fas + GC B cells and CD19 + CD25 + Foxp3 + regulatory B (Breg) cells in splenocytes from CIA mice injected with MTX-NPs by flow cytometry. The number of GC B cells was lower whereas the number of regulatory B cells was higher in *ex vivo* splenocytes from MTX-NPs-injected CIA mice compared with vehicle-injected mice, but there was no statistical significance (Fig. 5).

**Discussion**
We investigated the therapeutic potential of MTX-NPs administered subcutaneously in a murine model of autoimmune arthritis. MTX-NPs significantly reduced the clinical and histologic severity of arthritis. MTX-NPs reduced the number of TNF-α- and VEGF-apositive cells and reciprocally regulated the number of Th17 and Treg cells in the spleen from MTX-NP-treated CIA mice. Moreover, injection of MTX-NPs showed a tendency to increase the number of regulatory B cells in \textit{ex vivo} splenocytes of CIA mice.

Synovial inflammation in RA involves infiltration of inflammatory cells, including monocytes/macrophages, B cells, and T cells, which are sources of chemokines and inflammatory cytokines \cite{23}. T cells are a key player in the inflamed joints of patients with RA \cite{24}. Although most subsets of CD4 \textplus{} T cells are involved in RA pathogenesis, Th17 cells in particular positively correlate with disease activity in RA \cite{25}. In CIA mice, deficiency of IL-17 suppressed the development of arthritis and reduced the production of type II collagen-specific immunoglobulin \cite{26}. Moreover, blockade of IL-17 ameliorated the severity of arthritis and prevented synovial inflammation and joint destruction in CIA mice \cite{27}. MTX exerts a potent therapeutic effect by modulating humoral and cellular immune responses in RA management \cite{14,28}. MTX increases the sensitivity of T cells to apoptosis \cite{16} and inhibits NF-\textkappaB activation in T cells via tetrahydrobiopterin 4 depletion and JNK activation \cite{29}. MTX suppresses the expression of IL-6 and IL-6-apositive proliferation of fibroblast-like synoviocytes from patients with RA \cite{30}. Thomas \textit{et al.} demonstrated that MTX suppressed the JAK/STAT pathway in the Drosophila system and human macrophage lines \cite{31}, but the effect of MTX on JAK/STAT signaling and Th17 and Treg frequencies in the CIA model are unclear. The number of STAT3 phosphorylated CD4 \textplus{} cells and of Th17 cells in the spleen of CIA mice were decreased by injection of free MTX, and the effect was increased by injection of MTX-NPs.

The importance of B cells in RA pathogenesis has been demonstrated. B-cell-deficient mice had impaired CIA development \cite{32}, and B-cell depletion by an anti-CD20 antibody delayed CIA development \cite{33}. Immunization with type II collagen induced the formation of germinal centers in lymph nodes, which was necessary for CIA development \cite{34}. Breg cells control autoimmune diseases by secreting anti-inflammatory cytokines (\textit{e.g.}, IL-10 and IL-35) and transforming growth factor-\beta \cite{35,36}. Moreover, Breg cells suppress the differentiation of proinflammatory lymphocytes (\textit{e.g.}, Th1 and Th17 cells) and dendritic cells \cite{37}. The frequency of Breg cells increased and that of Th17 cells and germinal center B cells decreased in \textit{ex vivo} splenocytes of MTX-NP-treated CIA mice.

The elimination half-life of free MTX in human is 0.16 h for subcutaneous injection \cite{38}. Our NPs provided sustained release of MTX and increased the residence time. Although the release of MTX from NPs was faster than in prior reports, it affected the therapeutic results significantly. It is necessary to test and optimize further the release pattern of MTX by changing NP size or composition. In addition, MTX in NP could be taken up by T cells and B cells before release. This is because the NP surface was coated with PVA; we did not use polyethylene glycol groups to prevent fouling and unwanted uptake into immune cells. In addition, further modification with biological ligands will provide information for specific targeting of NPs to particular cell types.
Conclusion

In summary, our study showed a therapeutic efficacy of MTX-NPs in mice with CIA. Subcutaneous injection of MTX-NPs effectively alleviated the development of arthritis and suppressed the infiltration of inflammatory factors-expressing cells in the joint from CIA mice. Moreover, the number of pathogenic Th17 cells decreased while the number of regulatory T and B cells increased in the spleen of the MTX-NPs-injected group. These findings suggest that MTX-NPs have potential as a more advanced therapeutic strategy to overcome the limitations of MTX therapy.

Abbreviations

Rheumatoid arthritis: RA; tumor necrosis factor: TNF; interleukin: IL; IL-17-producing T helper: Th17; Methotrexate: MTX; disease-modifying antirheumatic drug: DMARD; Nanoparticle: NP; polyvinyl alcohol: PVA; Poly (D, L lactide-co-glycolide): PLGA; hematoxylin and eosin: H&E; vascular endothelial growth factor: VEGF; germinal center: GC; phosphorylated: p; regulatory T: Treg; transmission electron microscopy: TEM; polydispersity index: PDI; regulatory B: Breg.

Declarations

Ethics approval and consent to participate

All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea; the procedure conformed to all National Institutes of Health of the United States guidelines (Permit number: 2020-0067-01).

Consent for publication

Not applicable

Availability of data and material

All data are available in the manuscript or upon request to the authors.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contribution
JSP and DHL participated in the study design, data interpretation, and writing the manuscript. SCY, HSN, KHC and JWC carried out the animal experiments and analyzed data. CWY and SHP participated in the study design and data interpretation. HK, MLC and SHP conceived and designed the study, interpreted the data, and made critical revisions of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Characteristics of MTX-NPs. a Schematic of MTX-NP synthesis. b Size distribution, zeta-potential value, and TEM image of MTX-loaded nanoparticles. c MTX release profile of MTX-NPs.
Figure 2

MTX-NPs ameliorated the severity of collagen-induced arthritis. Beginning 3 weeks after the first immunization with type II collagen (CII), mice were injected subcutaneously with vehicle, free MTX, or MTX-NPs twice per week for 7 weeks (n = 5/group). a Arthritis score and incidence are shown for each group. b At 70 days after the first CII immunization, tissue sections from the paw and ankle joints of mice were stained with hematoxylin and eosin (original magnification × 40). Representative histological features are shown. Graphs present quantified levels of inflammation, bone damage, and cartilage damage. Values are means ± SEM. *, P < 0.05, **, P < 0.01, and ***, P < 0.001 vs. control group. Data are representative of two independent experiments.
MTX-NPs suppressed the levels of inflammatory mediators in vivo. Beginning 3 weeks after the first immunization with type II collagen (CII), mice were injected subcutaneously with vehicle, free MTX, or MTX-NPs twice per week for 7 weeks (n = 5/group). At 70 days after the first immunization with CII, sections of joint tissues (n = 5/group) were stained with antibodies against interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and VEGF. Graphs present numbers of antibody-positive cells for each cytokine. Data are means ± SEM of two independent experiments. *P < 0.05 vs. control group.

Figure 3
MTX-NPs regulate the number of Th17/Treg cells and expression of inflammatory cytokines in vivo. Beginning 3 weeks after the first immunization with type II collagen (CII), mice were injected subcutaneously with vehicle, free MTX or MTX-NPs twice per week for 7 weeks (n = 5/group). a-c At 70 days after the first immunization, spleen tissues were isolated and stained for CD4+IL-17+ (a), CD4+p-STAT3 (Y705)+ (b), and CD4+CD25+Foxp3+ (c) cells. Cell subsets were analyzed in four independent quadrants by confocal laser microscopy. The distributions of the cell populations are shown. d At 70 days after the first immunization, ex vivo splenocytes were isolated and the mRNA levels of IL-6, IL-1β, and IL-17 were determined by real-time PCR. Data are means ± SEM of two independent experiments. *P < 0.05 vs. control group.
MTX-NPs increased the number of regulatory B cells in vivo. Beginning 3 weeks after the first immunization with type II collagen (CII), mice were injected subcutaneously with vehicle, free MTX, or MTX-NPs twice per week for 7 weeks (n = 3/group). At 70 days after the first immunization, ex vivo splenocytes were isolated and the number of CD19+B220+GL-7+FAS+ and CD19+CD25+Foxp3+ cells determined by flow cytometry. Values are percentages of positive cells.