Anti-arthritic effect of E3 ubiquitin ligase, c-MIR, expression in the joints

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Abstract

Cellular modulator of immune recognition (c-MIR) is an E3 ubiquitin ligase that ubiquitinates MHC class II and CD86 for their endocytosis and subsequent lysosomal degradation. In accordance with their importance in antigen presentation, systemic c-MIR over-expression downmodulates adaptive immune responses. Rheumatoid arthritis (RA) is a chronic synovitis driven by autoimmunity in the joints. Since antigen-presenting cells, such as macrophages, dendritic cells (DCs) and rheumatoid factor-positive B cells are abundant in the rheumatoid synovial tissues, autoantigens released by tissue damage should be presented locally, leading to amplification of systemic arthritogenic immune responses. Assuming that inhibition of the antigen presentation in the synovial tissues should suppress systemic arthritis, we transferred the c-MIR gene to the hind leg synovial tissues from mice with type II collagen (CII)-induced arthritis, an animal model of RA. The gene was transferred adenovirally because adenoviruses can infect DC and macrophages in vivo. Unexpectedly, therapeutic effect was observed only in the treated joints. Splenocyte responses and serum antibodies against CII were not suppressed. Moreover, in vitro studies disclosed that c-MIR gene transfer suppressed IL-6 production from synovial fibroblasts stimulated with tumor necrosis factor (TNF)-α or IL-1β. Bone marrow-derived macrophages and DC from c-MIR transgenic mice were impaired in IL-6 and TNF-α production when stimulated with LPS. This suppression was controlled at the post-transcriptional level since their mRNA was not affected. These results have disclosed a new function of c-MIR, inhibition of inflammatory cytokine production. Induction of c-MIR in the joints could be a new therapeutic approach to the treatment of RA.

Keywords: arthritis, cytokine, inflammation

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects primarily the joints. The invasive granulomatous synovial tissues in the rheumatoid joints are called pannus and contain lymphocytes, dendritic cells (DCs), macrophages and synovial fibroblasts in an activated state. Since activated DCs, macrophages and rheumatoid factor-positive B cells can act as antigen-presenting cells (APC) (1–3), articular autoantigens could be presented to lymphocytes to induce their activation instead of anergy in the rheumatoid synovial tissues. Resulting joint tissue damage in turn promotes further release of the articular autoantigens, which should fuel the autoimmune circle of the arthritis. The T-cell infiltration in the rheumatoid synovial tissues and presence of autoreactive T cells against type II collagen (CII) (4) show that cellular immune responses against articular antigens indeed play a prominent role in RA.

Antigen presentation in the normal immune system is controlled by multiple regulatory components to avoid development of autoactivity. Cellular modulator of immune recognition (c-MIR) is an E3 ubiquitin ligase that ubiquitinates CD86 and MHC class II to induce their surface down-regulation by endocytosis and following lysosomal degradation (5, 6). The mRNA analyses of mouse splenocytes showed...
that c-MIR is expressed primarily by macrophages and DC (6). The c-MIR expressing cells were present in multiple organs, including heart, brain, placenta, kidney, liver, lung, muscle, pancreas, spleen, thymus, peripheral blood lymphocyte, lymph node, tonsil, fetal liver and bone marrow (BM) (5, 7). Nevertheless, transgenic c-MIR over-expression impaired development of CD4 T-cell generation in the thymus and in the periphery (6). The transgenic mice were resistant to induction of experimental autoimmune encephalomyelitis, showing that over-expression of c-MIR gene has an impact on in vivo immune reactions.

Structurally, c-MIR is among membrane-associated RING-CH (really interesting new gene-CH) (MARCH) gene family (7), which consists of 11 gene members, and also called MARCH-VIII. It was reported that c-MIR over-expression reduced expression of Fas, transferrin receptor, BM stromal cell antigen 2 (BST2; CD317) and syntaxin-4 in addition to CD86 and MHC class II (7, 8) although c-MIR-mediated ubiquitination was demonstrated only for CD86 and MHC class II. Over-expression of MARCH-I also down-regulated CD86, MHC class II, Fas and transferrin receptor (7, 9, 10). MARCH-II over-expression down-regulated CD86 and transferrin receptor while MARCH-IV and -IX over-expression down-regulated MHC class I and CD4 (7). Thus, members of the MARCH family appear to modulate antigen presentation in multiple aspects. However, it is still to be elucidated how these molecules cooperate to control the entire immune system.

We assumed that over-expression of c-MIR in synovial cells, especially APC in the arthritic joints, would suppress aberrant presentation of autantigens liberated by inflammation and suppress immune responses that amplify the arthritogenic immune circle. We addressed this question by transferring the c-MIR gene to the joints of mice with CII-induced arthritis (CIA), which is an animal model of RA. Adenoviral gene transfer was employed because recombinant adenovirus vectors can infect DC and macrophages as well as fibroblasts in vivo (11, 12). This local gene therapy failed to suppress systemic arthritic reactions. Instead, it exerted the local therapeutic effects, revealing a new anti-inflammatory function of c-MIR.

Methods

Reagents and cells

Antibodies against CD86 (BD Biosciences, San Jose, CA, USA), MHC class II I-Aβ-chain (clone KL295, ATCC) and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunoblotting. Recombinant mouse IFN-γ, TNF-α and human IL-1β were purchased from PeproTech (Rocky Hill, NJ, USA). Fibroblast-like synoviocytes (FLS) were isolated from the synovial tissues of mice with CIA and grown in DMEM supplemented with 10% fetal bovine serum (FBS) (13). BM cells were cultured for 6 days in RPMI 1640 supplemented with 10% FBS and 10% culture supernatant of macrophage colony-stimulating factor (M-CSF) producing CMG14-12 cells secreting M-CSF (14) to grow bone marrow-derived macrophages (BMM). Bone marrow-derived dendritic cells (BMDC) were prepared as described previ-ously (6). RAW264.7 cells were grown in RPMI 1640 supplemented with 10% FBS.

Transgenic mice

The c-MIR transgenic mice were bred on a C57BL/6 background. The c-MIR transgene is driven by an invariant chain promoter (6). Cells from male transgenic and non-transgenic littermate mice were used for in vitro experiments. In vivo experiments, the gender ratios were equal among the studied groups.

Recombinant adenoviruses

Recombinant adenoviruses containing a human cytomegalovirus (CMV) promoter, the intron A sequence (the largest intron of CMV), and a mouse c-MIR gene or a Escherichia coli lacZ gene were prepared with Adeno-X Expression System (Clontech Laboratories, Mountain View, CA, USA). The shuttle vector (15) was provided by Dr Mizuguchi (Osaka University, Japan). Recombinant adenoviruses were purified with cesium chloride density-gradient centrifugation (16). For in vivo gene transfer, the recombinant adenoviruses in saline at a concentration of 1 × 1011 infectious unit ml-1 were injected intra-articularly as described previously (17). Each mouse received bilateral intra-articular injection at the knee joints (10 μl per joint) and the ankle joints (5 μl per joint) and bilateral periarticular injection at the tarsal joints (5 μl per joint) of the hind legs at the same time. For in vitro gene transduction, FLS were infected with the recombinant adenoviruses at a minimal multiplicity of infection (MOI) that ensured 100% efficacy of infection (typically, 200-300 MOI).

CIA and its assessment

CIA was induced in 7-week-old male DBA/1 mice (Japan Charles River Breeding Laboratories) as described previously (18). Twelve-week-old c-MIR transgenic mice were injected intra-dermally in two sites at the tail base with 200 μg of CII from chicken sternal cartilage (Sigma, St Louis, MO, USA) in CFA as described previously (19). To prepare the CFA, 100 mg of Mycobacterium tuberculosis H37 RA (BD Biosciences) was suspended in 20 ml of CFA (BD Biosciences). Arthritis in the limbs was assessed clinically using visual scoring from 0 to 4: 0, no swelling; 1, detectable swelling in one joint; 2, non-severe swelling in two or more joints; 3, severe swelling in two or more joints; 4, severe swelling in two or more joints including digital swelling (20). Hind paw thickness was measured with a micrometer (Mitutoyo Corporation, Kanagawa, Japan). For histological examination, hind paws were fixed in 10% buffered formalin, decalcified and embedded in paraffin. Sections were stained with hematoxylin and eosin or tartrate-resistant acid phosphatase (TRAP)/ALP Stain kit (Wako Pure Chemical Industries, Osaka, Japan). Severity of arthritis was graded from 0 to 3 as described previously (21), with minor modifications as follows: 0 = normal joint; 1 = mild arthritis: minimal synovitis and mild infiltration of mononuclear cells without cartilage/bone erosions; 2 = moderate arthritis: synovitis, moderate infiltration of mononuclear cells and erosions but joint architecture maintained; 3 = severe arthritis: synovitis, severe infiltration of mononuclear cells, erosions and loss
of joint integrity. TRAP-positive multinucleated cells containing more than two nuclei were assessed in TRAP-stained sections.

Serum levels of anti-CII antibodies and splenic T-cell responses to CII were quantified as described previously (22). CII-specific antibody units were determined using a reference serum created from pooled sera from arthritic mice. The 1:40 dilution of serum from the arthritic mice was assigned a value of 1000 units ml$^{-1}$.

ELISA and reverse transcription–PCR analysis

The amounts of TNF-$\alpha$, IL-6, IL-1$\beta$ and IL-10 in the culture supernatants of FLS, BMM and BMDC were quantified with specific ELISA of mouse TNF-$\alpha$, IL-6, IL-1$\beta$ and IL-10 (DuoSets; R&D Systems, Minneapolis, MN, USA).

RNA was isolated from the synovial tissues, FLS, BMM and BMDC using the ISOGEN II (NIPPN GENE, Tokyo, Japan) or the RNeasy mini RNA isolation protocol (Qiagen, Valencia, CA, USA). They were converted to cDNAs with SuperScript II RT (Invitrogen, Carlsbad, CA, USA). The Quantitect SYBR Green PCR kit (Qiagen) was used for the PCRs. In addition or the RNeasy mini RNA isolation protocol (Qiagen, Valencia, CA, USA). They were converted to cDNAs with SuperScript II RT (Invitrogen, Carlsbad, CA, USA). The Quantitect SYBR Green PCR kit (Qiagen) was used for the PCRs. In addition to the primers for $\beta$2-microglobulin (Gusb) (24) and Gapdh (25), those for Tnfa $5'$-GGACAAGGCTGCCG-GACTACG-3' (forward) and 5'-CTTGGGGGAGGGC -GACG-3' (reverse) were synthesized. Gusb or Gapdh expression was used for standardization of the amount of cDNA. Relative gene expression was determined with the $\Delta\Delta$Ct method.

Statistical analysis

Protein concentrations in the supernatants, levels of mRNA, paw thickness, number of TRAP-positive cells, antibody titers and $3^H$-thymidine incorporation were analyzed statistically with Student's paired $t$-test. The arthritis scores and histological scores were analyzed with Mann–Whitney $U$-test.

Results

c-MIR transgenic mice were resistant to CIA

To investigate functions of c-MIR in arthritis, we induced CIA in c-MIR transgenic and littermate mice. In contrast to the littermate mice, the c-MIR transgenic mice were resistant to development of arthritis (Fig. 1A). To assess immune responses to CII of these mice, serum levels of anti-CII antibodies were determined. The anti-CII-specific total IgG, IgG1 and IgG2a were lower in the c-MIR transgenic mice (Fig. 1B), implying that c-MIR gene suppressed antigen presentation.

c-MIR gene therapy exerted local therapeutic effects on CIA

Recombinant adenoviruses containing a mouse c-MIR gene and those containing a control lacZ gene were prepared. RAW264.7 cells were infected with these viruses and stimulated with IFN-$\gamma$ to induce expression of CD86 and MHC class II. Western blot analyses of the cell lysates showed decrease in the CD86 and MHC class II expression (Fig. 2A).

Using these viruses, we transferred the c-MIR gene to the joints of CIA mice. To induce CIA, DBA/1J mice were immunized twice with bovine CII. Three days after the second immunization, the mice were subjected to injection of the adenoviruses at the knees, the ankles and the tarsal joints of the hind legs. The same treatment was repeated after 4 and 11 days. In comparison with the control treatment with the saline or lacZ gene, the c-MIR gene transfer significantly suppressed clinical arthritis only in treated limbs (Fig. 2B–D). Histopathological examination of the control joints showed destructive pannus tissues massively infiltrated by mononuclear cells, cartilage destruction and bone erosion, which are characteristic of the RA pathology. These features were suppressed in the c-MIR-treated joints (Fig. 3A and B). TRAP staining of the sections showed that osteoclasts were decreased in the c-MIR-treated joints (Fig. 3C). Quantitative real-time PCR analysis of TNF-$\alpha$ and IL-6 mRNA expression in synovial tissues from c-MIR-treated and control mice demonstrated that TNF-$\alpha$ and IL-6 expression was both decreased in the c-MIR-treated joints (Fig. 3D). Although we had assumed that the ectopic c-MIR expression in the inflamed joints could attenuate systemic immune reactions against articular antigens and systemic arthritis, the therapeutic effect remained local.

Immune responses to CII were not affected by c-MIR gene transfer

The limited anti-arthritic effects of the c-MIR gene transfer implied that systemic anti-CII responses were not suppressed. To assess immune responses to CII of the treated mice, serum levels of anti-CII antibodies and in vitro proliferation of splenocytes in response to CII were quantified. Serum and splenocytes of CIA mice were collected 28 days after the second immunization. Anti-CII-specific antibodies of IgG1, IgG2a and IgG2b subclasses as well as proliferative responses of the splenocytes to CII were both comparable between the c-MIR-treated and control CIA mice (Fig. 4). Thus, c-MIR gene transfer failed to attenuate systemic auto-antigen-specific reactions of T and B lymphocytes.

C-MIR gene transfer suppressed in vitro IL-6 production from FLS stimulated with TNF-$\alpha$ and IL-1$\beta$

Since systemic antigen-specific autoimmune reactions were not affected, we assumed that local production of inflammatory cytokines in the joints was suppressed by the c-MIR

![Fig. 1. CIA in c-MIR transgenic mice. Arthritis score of c-MIR transgenic mice (c-MIR Tg) and the littermate mice (wild type, WT) was evaluated on the indicated days (A). The points and bars represent the mean ± SEM of seven mice. Forty-two days after the initial immunization, sera were collected for quantification of serum levels of anti-CII antibodies with total IgG, IgG1 and IgG2a. The concentrations were expressed with arbitrary units (B). Horizontal lines represent the mean of seven mice. **P < 0.01](image-url)
gene transfer. FLS isolated from the synovial tissues of the CIA mice were infected with the c-MIR or control lacZ adenoviruses and stimulated with TNF-α or IL-1β. IL-6 production was evaluated as their responses since it is one of the major inflammatory cytokines present in the rheumatoid and CIA joints (26, 27) and derives mostly from synovial fibroblasts (28, 29). When the c-MIR gene was transferred to FLS, it attenuated the IL-6 production in the response to TNF-α and IL-1β (Fig. 5A). mRNA quantitation of the treated cells showed that IL-6 mRNA levels of the stimulated FLS were not affected

Fig. 2. Effects of adenoviral c-MIR gene transfer of the CIA joints. RAW264.7 cells were infected with recombinant c-MIR (c-MIR) or lacZ (lacZ) adenoviruses. After 24 h, they were incubated with 200 ng ml⁻¹ IFN-γ for 24 h. The cell lysates were analyzed with western blotting using anti-CD86 antibody (CD86), anti-I-Aβ-chain antibody (MHCII) and anti-actin antibody (actin) (A). Mice with CIA were treated with injection of saline or recombinant adenoviruses expressing c-MIR or lacZ to the knees, the ankles and the tarsal joints of the hind legs 24, 28 and 35 days after the initial immunization. Arthritis score (B) and paw thickness (C) of the hind legs and arthritis score of the forelegs (D) were evaluated on the indicated days. The points and bars represent the mean ± SEM of six (lacZ) and five (c-MIR) mice. *P < 0.05, **P < 0.01, versus the lacZ control group.

Fig. 3. Histological assessment of the treated joints and local pro-inflammatory cytokine expression. Hind paws of saline-treated (saline) and c-MIR-treated (c-MIR) mice were collected 42 days after the initial immunization. Sections of the hind paws embedded in paraffin were stained with hematoxylin and eosin (A). Histological scores were compared between the two groups (B). The result represents the mean ± SEM of five (saline) and six (c-MIR) mice. Significant reduction of the histological findings was also observed in comparison with the lacZ-treated joints. Sections of the hind paws embedded in paraffin were TRAP stained. The TRAP-positive multinucleated cells on bone surface in the middle phalanx were enumerated in the two groups (C). The result represents the mean ± SEM of four (saline) and five (c-MIR) mice. The synovial tissues were collected at the same day, and RNA was extracted for quantification of TNF-α and IL-6 mRNA with real-time PCR (D). The mRNA levels were normalized to that of Gusb mRNA. RNA levels of the synovial tissues from the saline-treated mice were used to calculate a relative quantity of TNF-α and IL-6 mRNA. The results represent the mean ± SEM of five (saline) and six (c-MIR) mice. *P < 0.05, versus the saline control group.
These results implied that IL-6 production was down-regulated at the post-transcriptional level. c-MIR transgene suppressed in vitro inflammatory cytokine production from macrophages and DC stimulated with LPS.

Although it was reported that adenovirus vectors can infect macrophages in vivo (11), techniques for in vitro adenoviral gene transfer to primary macrophages have not been established. Instead, since we have established mutant mice with a c-MIR transgene driven by an invariant chain gene promoter (6), they were used to investigate the effects of c-MIR gene over-expression in primary macrophages and DC. For the transgene induction, some cells, including BMM, have to be treated with IFN-γ. BMM from the transgenic and wild-type mice were treated with IFN-γ and subsequently stimulated with LPS for inflammatory cytokine production. ELISA of the culture supernatants revealed that transgene suppressed production of TNF-α and IL-6 but not IL-1β (Fig. 6A). In the same manner, primary DCs were prepared from the BM of the transgenic and wild-type mice. These BMDC did not require the IFN-γ pre-treatment since BMDC express MHC class II as well as c-MIR transgene. When they were stimulated with LPS, expression of the c-MIR transgene suppressed production of TNF-α and IL-6 at the protein level (Fig. 6B) but not at the mRNA level (Fig. 6C). While production of these inflammatory cytokines was suppressed, that of IL-10 was not affected.

Discussion

We have disclosed a new function of c-MIR, which was originally known to down-regulate expression of membrane-bound molecules, primarily associated with antigen presentation. It is inhibition of inflammatory cytokine production and potentially applicable to arthritis treatment. The suppressed cytokines included IL-6 from synovial fibroblasts, macrophages and DC and TNF-α from macrophages and DC. These cytokines are involved critically in the pathology of autoimmune arthritis (21, 30) and blocked as actual therapeutic intervention to treat RA patients. Other inflammatory mediators than these cytokines might be suppressed by c-MIR. However, since IL-1β production from macrophages and IL-10 production from DC were not affected, the suppression was not based on general inhibitory effect on production of secretory proteins. Also, reduction of the cytokine release was not attributable to diminution of mRNA. It implied that the down-regulation of IL-6 and TNF-α production was controlled at the post-transcriptional level and was not the secondary event to suppression of other cytokine production. Nevertheless, in vivo gene transfer experiments revealed that inflammatory cytokines were down-regulated at the mRNA level. This is most likely attributable to general suppression of arthritis. The post-transcriptional control might be mediated by reduced expression of syntaxin-4, which is one of the target molecules of c-MIR (8) and plays a crucial role in TNF-α exocytosis (31, 32). Precise molecular interaction how c-MIR interferes inflammatory cytokine production remains to be elucidated in the present study and needs further investigation. Although it was known that targets of c-MIR are primarily membrane-bound surface molecules, the present data demonstrated that c-MIR has an impact on other types of molecules in the cells. Actually, expression of membrane-bound type 2 tumor necrosis factor receptor (TNFR2) on
FLS was not altered by the c-MIR expression (data not shown). AIRE (autoimmune regulator) is an E3 ubiquitin ligase but also appears to act as a transcription factor (33). The newly defined function of c-MIR could be independent of E3 ubiquitin ligase activity.

Suppression of CIA in the c-MIR transgenic mice accompanied impaired immune reaction to CII. Thus, systemic expression of c-MIR can suppress antigen-specific immune reactions. In contrast, the intra-articular c-MIR gene delivery failed to suppress systemic immune responses to CII. The synovial tissues of the untreated joints might have continued to serve as a platform for autoantigen presentation. MHC class II down-regulation by c-MIR might not be profound enough to evade antigen presentation in the synovial tissues. Alternatively, although the inflamed synovial tissues contained activated APC as well as lymphocytes, primary sites for lymphocyte priming and activation remained outside of the synovial tissues.

Since adenoviral vectors cannot infect primary macrophages and DC in vitro, these cells derived from c-MIR transgenic mice were used to discern the effects of c-MIR. However, expression of the c-MIR transgene in macrophages had to be driven by addition of exogenous IFN-γ. We believe that further investigation on the cytokine down-regulation by c-MIR in macrophages should be carried out in other systems.

Adenoviral gene transfer was a tool to study the effects of c-MIR up-regulation in the synovial cells. Since viral vectors, especially adenoviruses, may provoke inflammation, quite a few problems have to be solved for actual clinical use. Direct transduction of recombinant c-MIR protein is an alternative means. Inclusion of c-MIR in liposome should enable tissue transduction of the c-MIR protein (34). Addition of protein transduction domains could be also considered (35). The ultimate approach should be pharmacological induction of the c-MIR gene expression in the joints although regulation of c-MIR gene expression is largely unknown.

The present study disclosed that up-regulation of the c-MIR expression in the synovial cells could be a new therapeutic approach to the arthritis treatment. The c-MIR gene

Fig. 6. Effects of the c-MIR transgene on BMM and BMDC. BMM from c-MIR transgenic mice (c-MIR Tg) and wild-type C57BL/6 mice (WT) were pre-treated with 30 ng ml⁻¹ IFN-γ for 24 h. After stimulation with LPS at the indicated concentrations for 24 h, TNF-α, IL-6 and IL-1β in the culture supernatants were quantified with ELISA (A). BMDC were stimulated with LPS without the pre-treatment. TNF-α, IL-6 and IL-10 in the culture supernatants were quantified with ELISA (B). Levels of TNF-α and IL-6 mRNA were quantified with real-time PCR using BMDC samples stimulated with LPS for 3 h (C). RNA levels of unstimulated cells from WT were used to calculate a relative quantity of TNF-α and IL-6 mRNA. **P < 0.01.
was our primary interest because an autoimmune disease model of multiple sclerosis was suppressed in the c-MIR transgenic mice (6). However, other proteins of MARCH family genes in arthritis will be the future target of the arthritis research.

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