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MAPKAP Kinase 2-Deficient Mice Are Resistant to Collagen-Induced Arthritis

Martin Hegen,1* Matthias Gaestel,† Cheryl L. Nickerson-Nutter,*, Lih-Ling Lin, and Jean-Baptiste Telliez1*

TNF-α is a pleiotropic cytokine considered a primary mediator of immune regulation and inflammatory response and has been shown to play a central role in rheumatoid arthritis (RA). MAPKAP kinase 2 (MK2) is a serine/threonine kinase that is regulated through direct phosphorylation by p38 MAPK, and has been shown to be an essential component in the inflammatory response that regulates the biosynthesis of TNF-α at a posttranscriptional level. The murine model of collagen-induced arthritis (CIA) is an established disease model to study pathogenic mechanisms relevant to RA. In this study, we report that deletion of the MK2 gene in DBA/1LacJ mice confers protection against CIA. Interestingly, the MK2 heterozygous mutants display an intermediate level of protection when compared with homozygous mutant and wild-type littermates. We show that MK2−/− and MK2+/− mice exhibit decreased disease incidence and severity in the CIA disease model and reduced TNF-α and IL-6 serum levels following LPS/n-Gal treatment compared with wild-type mice. Additionally, we show that levels of IL-6 mRNA in paws of mice with CIA correlate with the disease status. These findings suggest that an MK2 inhibitor could be of great therapeutic value to treat inflammatory diseases like RA.


Rheumatoid arthritis (RA) is an autoimmune, polygenic disease characterized by chronic inflammation of the synovial tissues primarily in peripheral joints (1, 2). This inflammation leads to cartilage and bone destruction, and ultimately, joint deformation. The etiopathogenesis of RA is poorly understood and is believed to be the result of environmental factors in combination with a genetic predisposition to the disease. The murine model of collagen-induced arthritis (CIA) (3) has been used extensively to improve our understanding of autoimmunemediated arthritis and to identify potential new therapeutic agents to treat RA and other inflammatory conditions. This disease model is an Ag-induced arthritis that is cartilage restricted. Type II collagen (CII) emulsified in CFA is injected. Immunization with the collagen emulsification induces an Ab response followed by clinical manifestations of arthritis.

MAPKAP kinase 2 (MK2) is a direct substrate of p38α and p38β MAPKs (4) and is responsible for many of the signaling events that follow the activation of these MAPKs (5). Physiological implications of MK2 activation are most clearly revealed by the targeted disruption of the MK2 gene in mice. MK2-deficient cells derived from mice have shown defects in motility, chemotaxis, and cytokine production (6–8). Several studies using MK2-deficient mice strongly support the central role of this kinase in the production of inflammatory cytokines such as TNF-α, IL-6, and IFN-γ (6, 9). AU-rich elements have been identified in the 3′ untranslated region of several cytokine mRNAs (10, 11) and are known to regulate mRNA stability (11, 12) and translation (13). In the case of TNF-α, IL-6, and IFN-γ it has been shown that MK2 regulates their expression by targeting their mRNA AU-rich elements (9, 14).

The MK2−/− mice have shown a severely reduced response to LPS-induced endotoxic shock due to a 90% reduction in TNF-α production (6). Given the key role of TNF-α in RA, this finding suggests that MK2 may be pivotal in the development of CIA. To assess the role of MK2 in RA, we established the MK2 deficiency in the DBA/1LacJ strain that is susceptible to CIA. Our results using MK2−/−, MK2+/−, and wild-type littermate mice indicate a crucial role of MK2 in the development of autoimmune CIA.

Materials and Methods

Mice

The Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. MK2-deficient DBA/1LacJ mice were obtained by crossing MK2-deficient C57BL/6 mice with the CIA-susceptible DBA/1LacJ mice for five generations using a marker-assisted accelerated backcrossing (MAX-BAX) scheme to obtain MK2−/−/DBA/1LacJ. The background in N5 was calculated at ~98% DBA/1LacJ using 64 microsatellite markers. DBA/1LacJ mice were originally obtained from The Jackson Laboratory. The MK2+/− DBA/1LacJ mice were then intercrossed to generate mice homozygous for the MK2 mutation (MK2−/−), heterozygous for the MK2 mutation (MK2+/−), and mice homozygous for the wild-type MK2 allele (MK2+/*). In CIA experiments, male mice 10–15 wk of age were used. The mice were maintained at a maximum of seven per cage and were fed mouse breeder chow and allowed access to water ad libitum. In each experiment, MK2−/−, MK2+/−, and wild-type mice were age matched. The mice were crossed and bred at Charles River Laboratories and maintained for the CIA experiments in the animal facility at Wyeth Research. All animals were routinely serologically tested and were negative for common pathogens.

Genotype analysis by PCR

The genotype of mice was determined by PCR using 100–200 ng of genomic DNA prepared from tail biopsies as template. The nucleotide sequence of the primers were 5′-tcggggggtgggggctcgtagggtgcg-3′ (MK2 forward (MK2F)), 5′-gttgctttcagctcagtggcaga-3′ (MK2 reverse (MK2R)), 5′-tgctcgcgatctaggctggcgc-3′ (NeoF); the primer set of NeoF and MK2 amplified a 800-bp fragment for the neo allele, and the set of MK2F and MK2R yielded a 500-bp fragment specific for the wild-type allele. The reaction was conducted using a Taq polymerase (Invitrogen Life Technologies) with one step of 1 min at 95°C followed by 35 cycles of amplification (1 min at 96°C, 1 min at 95°C, 1 min at 72°C) followed by one step at 72°C for 5 min.

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These procedures were performed in accordance with the Animal Welfare Act and the Innovative Animal Care and Use Committee (IACUC) approved the protocol. The animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1996).

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‡ Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, collagen type II; MK2, MAPKAP kinase 2.

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Induction and assessment of arthritis

Bovine collagen type II (CII) (Chondrex) was dissolved in 0.01 N acetic acid and emulsified in an equal volume of CFA containing 1 mg/ml heat-killed Mycobacterium tuberculosis (Sigma-Aldrich). Arthritis was induced by the initial immunization with 100 μg/100 μl emulsion by an intradermal injection in the base of the tail. Twenty-one days later after the initial immunization, the mice received a boost intradermal injection (base of the tail) of 100 μg/100 μl of bovine CII emulsified in IFA. Individual experiments contained at least 10 male MK2−/−, MK2+/−, and MK2+/+ DBA/1LacJ mice per group, and all experiments were performed four times. Mice were scored three times per week, beginning 3 wk after primary CII immunization, for signs of developing arthritis. The severity of the arthritis was assessed using a visual scoring system. Each paw was scored on a graded scale from 0 to 4: 0, normal paw; 1, swelling of one toe joint; 2, swelling of two or more toe joints, or increased swelling; 3, severe swelling; and 4, ankylosis throughout the entire paw. Each paw was graded and the four scores were added such that the maximal score per mouse was 16. Serum was collected at days 28, 35, 42, 49, 56, and 63 post immunization for anti-CII II ELISA testing. At the end of the studies, on day 63, paws were collected for histopathology.

**Histological techniques**

For histological processing, paws were fixed in phosphate buffer containing 10% formaldehyde and decalcified in sodium citrate. Paws were processed by routine methods to paraffin blocks. Specimens were sectioned at 6 μm and stained with H&E according to the manufacturer’s protocol (Sigma-Aldrich). The sections were evaluated for the degree of synovial hyperplasia, inflammatory cell infiltrate, cartilage damage, pannus formation, bone erosion, and ankylosis. The severity of the disease in the joint sections was graded using a scoring system from 0 to 4: 0, no abnormal findings (normal synovial membrane at one to three synoviocytes thick, absence of inflammatory cells, and smooth articulating cartilage surfaces); 1, minimal (synoviocyte hypertrophy, slight synovial membrane fibrosis, slight to mild inflammatory cell infiltrates into the synovial membrane/articular capsule and/or joint space); 2, mild (mild to moderate inflammatory cell infiltrates, pannus formation is minimal with superficial cartilage erosion); 3, marked (marked inflammatory cell infiltrates and fibrosis, mild to severe erosion of the cartilage extending into subchondral bone); 4, severe (loss of joint integrity through erosion or destruction with bone remodeling, massive inflammatory cell infiltrates, fibrosis and ankylosis). The severity score for a paw was weighted based on the number of joints within a paw receiving a specific score. Each paw was graded and the scores for four paws per mouse were averaged such that the maximal score was 4.

**Anti-CII Ab ELISA**

IgG, IgG2a, IgG2b Ab levels against the immunogen were measured by standard ELISA methodology using peroxidase-conjugated secondary Ab and substrate ABTS. Serum dilutions, 1/1000, were chosen after preliminary assays. The OD was measured at 405 nm using a Spectramax Plus 384 plate reader (Molecular Devices). The anti-CII Ab concentrations were determined by reference to standard curves of murine IgG, IgG2a, or IgG2b for Fig. 3A (Southern Biotechnology Associates). In addition, anti-CII Ab concentrations were determined by reference to standard curves generated from 1/2 serial dilutions of a standard CII serum to calculate the Ab content (in arbitrary units per milliliter) for Fig. 3B.

**LPS/t-galactosamine-induced TNF-α and IL-6 production and determination of cytokine levels**

LPS from Escherichia coli, serotype O55:B5 (Sigma-Aldrich) and t-galactosamine (Sigma-Aldrich) were diluted in pyrogen-free saline. MK2−/−, MK2+/−, and wild-type littermate mice were injected i.p. with a combination of LPS (5 μg/kg body weight) and t-Gal (0.4 g/kg body weight). Ninety minutes after LPS/t-Gal injection, the mice were sacrificed via CO2 asphyxiation and bled by cardiac puncture. The serum samples were kept at −20°C before TNF-α and IL-6 analysis by ELISA. TNF-α and IL-6 levels in the serum samples were measured using a murine TNF-α ELISA (R&D Systems) and murine IL-6 ELISA (Assay Design, respectively).

**Extraction of paw RNA and TaqMan analysis of IL-6 and TNF-α expression**

RNA was extracted from paws, which were snap frozen in liquid nitrogen, using the Megachip RNA Mini kit (Qiagen). The IL-6 TaqMan probe 5'-ctttccttctcgaacgttcgccgagggag-3' and reverse primer 5'-aattaaccttcatctctcctcggcaggg-3' were used to amplify IL-6. The TNF-α TaqMan probe 5'-cttccacaccgctcagcgttacgagcc-3' was also labeled with the fluoroscent dye FAM. Forward primer 5'-ggctcgccgactcttg and reverse primer 5'-gacctccctggatatgtagataaa were used to amplify TNF-α. IL-6 and TNF-α mRNA levels were analyzed by quantitative real-time PCR on a 7900HT Sequence Detection System from Applied Biosystems. In each reaction, 50 ng of RNA was subjected to RT-PCR and TaqMan amplification in a 50-μl volume using the Reverse Transcriptase qPCR Master Mix (Eurogentec). The reverse transcriptase conditions were 48°C for 30 min followed by PCR with an initial denaturation step at 95°C for 10 min followed by 30 cycles of 95°C for 15 s followed by 60°C for 1 min.

**Statistical analysis**

Data are presented as the mean ± SE. Clinical and histopathological scores, and serum anti-CII IgG levels were analyzed with Student’s t test. Values of p < 0.05 were considered significant.

**Results**

MK2-knockout and MK2 heterozygote mice show reduced severity and incidence of CIA compared with wild-type littermate mice

To directly explore the pathophysiological role of MK2 in arthritis, we backcrossed MK2-deficient mice for five generations using an accelerated backcross breeding strategy (Max Bax) into the DBA/1LacJ mouse strain to obtain >98% DBA/1LacJ background. MK2−/−, MK2+/−, and wild-type DBA/1LacJ mice littermates were immunized with CII in CFA and boosted 3 wk later with CII in IFA. An observer unaware of the genotype examined the mice three times per week for signs of developing arthritis. Using a visual scoring system, the severity of the arthritis was assessed. A significant reduction of the disease severity scores was observed on day 49 postinoculation until the end of the study in the MK2−/− and MK2+/− mice compared with wild-type littermates (Fig. 1A). In addition, on day 58 postsinoculation, only 40% of the MK2−/− mice and 48% of the MK2+/− mice showed disease compared with 100% of the wild-type littermate mice (Fig. 1B). At the end of the study, on day 63, the mean disease severity score of wild-type littermate mice was 9, while MK2−/− and MK2+/− mice showed a reduced disease severity score of 2 and 5, respectively (Fig. 1C).

**Histological features of immunized MK2-deficient, MK2 heterozygote, and wild-type littermate mice**

An observer unaware of the genotype of the animals scored the histopathology of both front and rear paws. The severity of disease, as determined by the histological features, correlated with the observed visual scores (Fig. 2A). None of the MK2−/− mice that were classified as nonarthritic had any evidence of arthritis on histological examination; therefore, the histological analysis of the paws confirmed the visual scoring results. The joints of wild-type mice frequently showed severe pathology with cartilage and bone erosion, synovial inflammation, and formation of invasive pannus (Fig. 2B, a and d). None of the wild-type mice showed normal paws (Table I). In contrast, none of the MK2−/− mice were observed to have more than minimal pannus formation or fibrillation of the articular cartilage in the nonarthritic animal (Fig. 2B, c and f). In addition, in 80% of the MK2−/− mice, the joints were not affected (Table I). In MK2+/− intermediate disease severity was observed in the histological evaluation confirming again the visual scoring results with 54.4% of the joints not affected in these mice (Table I).

**Immune response against CII in MK2-deficient DBA/1LacJ mice**

High levels of anti-CII Abs accompany the development of the disease (15). To investigate whether the reduced severity and incidence of arthritis in MK2−/− and MK2+/− mice was due to the lack of an Ab response to type II collagen, the anti-CII-specific
levels of IgG, IgG2a, and IgG2b in the serum were measured at day 63 postimmunization. The anti-CII IgG levels did not correlate with disease severity showing that the reduced severity and incidence of arthritis in MK2 deficient mice is not due to a lack of Ab response (Fig. 3A). Furthermore, to assess whether there is any temporal difference between MK2−/− and MK2+/+ littermate in the production levels of anti-CII IgG Abs during the course of the study, serum was collected on days 28, 35, 42, 49, 56, and 63 postimmunization. No significant difference between MK2−/− and MK2+/+ mice was observed in the anti-CII IgG levels (Fig. 3B).

**TNF-α and IL-6 production in MK2−/−, MK2+/−, and MK2+/+ littermate mice**

MK2 plays a central role in cytokine production and TNF-α and IL-6 are key cytokines in the pathogenesis of CIA. Therefore, we evaluated the TNF-α and IL-6 serum levels in MK2−/−, MK2+/− and wild-type DBA/1LacJ littermate mice after injection of LPS-d-Gal. A comparison of the TNF-α serum level with the wild-type mice as a reference showed a 39% reduction in heterozygote mice and 95% reduction in MK2-deficient mice (Fig. 4A). Similar data were obtained with IL-6 showing a 37% reduction in heterozygous mice and 86% reduction in homozygous mutant mice when compared with the cytokine level in wild-type mice (Fig. 4B).

**TaqMan analysis of IL-6 and TNF-α expression in paws of mice with CIA**

TaqMan analysis of IL-6 and TNF-α mRNA was used to evaluate their level of expression in diseased and nondiseased paws. IL-6 and TNF-α mRNA levels from diseased (disease score 1, 2, or 3) and control paws (disease score 0) from MK2+/+, MK2−/−, and MK2−/− mice immunized and boosted with CII were quantified by TaqMan analysis after correction for GAPDH level in each sample (the data for IL-6 are shown in Fig. 5). Paws were harvested at different time points, between days 40 and 49 postimmunization, to be able to have at least one representative of each disease stage for the MK2+/+, MK2−/+ and MK2−/− mice. Because the MK2−/−
cohort develops CIA with a smaller incidence and lower severity when compared with the wild-type littermate group, fewer mice in the diseased group were obtained. Nevertheless, the IL-6 mRNA levels correlate with disease scores in all groups. One MK2−/− mouse in the diseased group had a disease score of 1 with a low IL-6 mRNA level and two MK2−/− mice had a disease score of 2 with comparable IL-6 mRNA to the disease score of 2 in the MK2+/− and MK2+/+ cohorts. Only one MK2−/− mouse with a disease score of 3 was available in this study and showed the highest level of IL-6 mRNA. There was no significant differences in TNF-α mRNA levels between MK2+/−, MK2+/+, and MK2−/− mice, as well as between diseased and nondiseased mice (data not shown).

**Discussion**

This study is the first to demonstrate the role of MK2 as a key player in the pathogenesis of CIA. MK2−/− mice show markedly reduced severity and incidence of disease in CIA (Fig. 1) and these results were confirmed by histopathological evaluation (Fig. 2). Interestingly, the MK2−/− mice showed an intermediate level of disease protection and severity when compared with their MK2+/+ and MK2−/− littermates. In addition, TNF-α levels are drastically reduced in MK2−/− mice when compared with wild-type littermates after LPS/β-Gal treatment, which is consistent with a previous report (6). In vivo IL-6 levels are also markedly reduced in the MK2 mice when compared with wild-type littermates. Additionally, MK2−/− mice display intermediate levels of TNF-α and IL-6 when compared with homozygous mutant and wild-type littermates indicating an MK2 gene dosage effect. Our data clearly demonstrate the role of MK2 in an arthritic pathology that is mediated by cytokines such as TNF-α and/or IL-6. Furthermore, no difference in the anti-collagen Ab response in MK2+/+, MK2+/−, and MK2−/− mice was observed (Fig. 3), suggesting that the reduced disease incidence and severity in MK2−/−, MK2+/− mice when compared with MK2+/+ mice cannot be attributed to an impaired immune response. In situ analysis of IL-6 mRNA levels in paws from nondiseased and diseased mice in the MK2+/+, MK2+/−, and MK2−/− cohorts showed that they correlate with the disease stage, regardless of the genotypic background (Fig. 5). Overall, IL-6 mRNA levels increased with disease severity in MK2+/+, MK2+/−, and MK2−/− mice. The fact that diseased MK2−/− mice show elevated IL-6 mRNA levels comparable to levels from wild-type littermates suggests that there is a compensatory mechanism leading to IL-6 production, otherwise deficient in most MK2−/− mice. In the case of TNF-α, we observed no significant differences in the mRNA levels between MK2+/+, MK2+/−, and MK2−/− mice (data not shown). This is consistent with the previous observation showing similar levels of TNF-α mRNA in LPS-treated spleen cells from MK2+/+ and MK2−/− mice (6). All together, this data shows that diseased MK2−/− mice exhibit similar mRNA cytokine profiles as diseased MK2+/− and MK2+/+ mice.

Although the etiology of RA is not yet fully understood, TNF-α has long been known to play a crucial role in the immunoinflammatory cascade leading to the development of the disease. In recent years, several anti-TNF-α agents (Enbrel, Remicade, and Humira) have been shown to be relatively safe and effective therapies for the treatment of various inflammatory conditions most notably for the treatment of RA (16). Deletion of MK2 by gene targeting

**TABLE I.** *Arthritis severity score analysis by groups*<sup>a</sup>

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male WT</th>
<th>Male HET</th>
<th>Male KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>11</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>Average Severity Grade</td>
<td>2.14</td>
<td>1.12</td>
<td>0.45</td>
</tr>
<tr>
<td>Percent of mice with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Four normal paws</td>
<td>0.00</td>
<td>34.78</td>
<td>60.00</td>
</tr>
<tr>
<td>One arthritic paw</td>
<td>9.09</td>
<td>4.35</td>
<td>20.00</td>
</tr>
<tr>
<td>Two arthritic paws</td>
<td>27.27</td>
<td>21.74</td>
<td>10.00</td>
</tr>
<tr>
<td>Three arthritic paws</td>
<td>9.09</td>
<td>21.74</td>
<td>0.00</td>
</tr>
<tr>
<td>Four arthritic paws</td>
<td>54.55</td>
<td>17.39</td>
<td>10.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>At the end of the CIA study, on day 63, the paws were removed, and fixed in 10% buffered formalin. Histological sections were obtained and stained with H&E. The sections were evaluated for hyperplasia, fibroplasia, inflammatory cell infiltration, pannus formation, bone and cartilage erosion, and ankylosis. The severity of the disease in the joint sections was graded using a scoring system from 0 to 4 (see *Materials and Methods*). WT, MK2+/+; HET, MK2+/−; KO, MK2−/−.

**FIGURE 3.** A, Anti-CII IgG, IgG2a, and IgG2b levels in serum of CII-immunized MK2+/+, MK2+/−, and MK2−/− mice at the end of the study (day 63). No significant difference between MK2+/+, MK2+/−, and MK2−/− was observed. B, Anti-CII Ab levels in serum of CII-immunized MK2+/+ and MK2−/− mice at day 28, 35, 42, 49, 56, and 63 of the study. No significant difference between MK2+/+ and MK2−/− was observed.
IL-6 therapy with an anti-IL-6 receptor mAb (MRA) ameliorates RA patients could have a positive therapeutic effect without requiring complete inhibition of MK2 activity. Consequently, MK2 is an ideal target for an inhibitor that could ameliorate numerous inflammatory diseases, including RA.

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Disclosures
The authors have no financial conflict of interest.

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