

1 Natural killer T cells ameliorate antibody-induced arthritis in macrophage migration inhibitory factor transgenic mice

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Abstract. Macrophage migration inhibitory factor (MIF) plays an important role in inflammatory diseases. It has been reported that anti-MIF treatment and *mif*-gene disruption ameliorate joint inflammation in a mouse model of arthritis induced by anti-type II collagen monoclonal antibodies and lipopolysaccharide (anti-IIC mAb/LPS). In the present study, using the anti-IIC mAb/LPS system, we have analyzed arthritis in MIF-transgenic (MIF^{Tg}) and wild-type C57BL/6 (WT) mice. We found that MIF^{Tg} mice developed more severe arthritis than WT mice. The histopathological scores were significantly higher in MIF^{Tg} mice and significantly increased numbers of CD69⁺ T cells were detected in the spleens of these arthritic MIF^{Tg} mice, compared with WT mice. Natural killer T (NKT) cells from MIF^{Tg} mice, compared with WT mice, produced reduced amounts of IL-4 upon stimulation with α -galactosylceramide (α -GalCer). Further, repeated administration of α -GalCer to MIF^{Tg} mice resulted in a profound reduction of both clinical and histopathological scores of arthritis, with a significant decrease in IL-6. The present findings demonstrate that overexpression of MIF exacerbates inflammation in this arthritis model and that NKT cells play an ameliorating role upon stimulation with α -GalCer in the inflammatory process in MIF^{Tg} mice.

Introduction

Macrophage migration inhibitory factor (MIF) was one of the first cytokines identified as a T cell-derived factor that was

able to inhibit the random migration of macrophages *in vitro* (1). After the molecular cloning of the *mif* gene (2), it has become increasingly clear that MIF is a unique cytokine with various actions on many target cells (3,4). Recent studies, using genetically engineered animals such as MIF knockout (5,6) and MIF transgenic (MIF^{Tg}) (7,8) mice, have revealed the importance of MIF in inflammatory diseases.

Collagen-induced arthritis (CIA) is an experimental model of rheumatoid arthritis (RA) that is induced by immunization of mice from susceptible strains with cartilage type II collagen (CII) (9). Administration of an arthritogenic cocktail of anti-CII monoclonal antibodies (mAb) followed by lipopolysaccharide (LPS) injection (anti-IIC mAb/LPS) can be utilized as an alternative way of inducing arthritis, irrespective of the genetic background of the animals (10,11). It has been reported that administration of neutralizing anti-MIF antibody (Ab) protects mice from CIA (12) and anti-IIC mAb/LPS-induced arthritis (13). In the CIA model, administration of OCH, a sphingosine-truncated analogue of α -galactosylceramide (α -GalCer) that functions as a prototypical ligand for natural killer T (NKT) cells, to C57BL/6 (B6) mice inhibited arthritis (14). This finding suggests a protective role of glycolipid-activated NKT cells in arthritis. It has also been reported that NKT cells play protective roles in autoimmune diseases in human and mouse (15,16). However, in other reports, it has been suggested that NKT cells can promote inflammation in the same or similar arthritis model in mice (17,18). Thus, the role of NKT cells in arthritis remains controversial.

NKT cells represent a novel lymphocyte lineage distinct from conventional T, B, and NK cells. The majority of human NKT cells express a unique T cell receptor (TCR), a single invariant V α 24-J α 18 chain paired preferentially with V β 11. Mouse NKT cells express mainly a semi-invariant V α 14-J α 18 chain paired preferentially with V β 8.2 (19). Upon activation, NKT cells rapidly produce large amounts of interleukin-4 (IL-4) and interferon- γ (IFN- γ) (20,21), which may play critical roles in the regulation of autoimmune diseases. It has been reported that RA patients have abnormalities in the number of V α 24⁺V β 11⁺ NKT cells in peripheral blood lymphocytes (15). We have also reported that NKT cells in patients with Wegener's granulomatosis and relapsing polychondritis exhibit a Th1-biased cytokine production profile (22).

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In the present study, we induced arthritis in MIF^{Tg} and wild-type (WT) mice with anti-IIC mAb/LPS to determine the impact of MIF on joint inflammation. We then examined whether multiple administration of α -GalCer influenced arthritis in MIF^{Tg} mice. Our results provide evidence that NKT cells can control joint inflammation in the context of high levels of MIF.

Materials and methods

Mice. WT (B6, Japan SLC, Hamamatsu, Japan) and MIF^{Tg} mice (Hokkaido University, Sapporo, Japan), (7) were used in this study. To detect the *mif* transgene, a pair of primers, CAGGS F1 5'-taatc aatta cgggg tcatt agttc atagc-3' and CAGGS B1 5'-tccca taagg tcatt tactg ggcac aatgc-3', were used for the amplification of a part of the vector sequence in the transgene (7). Polymerase chain reaction (PCR) amplification was performed with 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 1 cycle of 72°C for 7 min. A 302-bp band was amplified in MIF^{Tg} mice. All mice were maintained under specific pathogen-free conditions. All animal care and experimental procedures conformed to the regulations of the Committee of Hokkaido University on Animal Experimentation.

Experimental arthritis model. Arthritic mAb kits were purchased from Immuno-Biological Laboratories (Gunma, Japan), and arthritis was induced in 8- to 12-week-old mice according to the manufacturer's protocol. Briefly, mice were injected intravenously with a mixture of four anti-CII mAb (2 mg each), followed by intraperitoneal injection with 50 μ g LPS 3 days later. Mice were examined 3 times per week, and the joint inflammation of fore and hind paws was scored as follows: 0, no change; 1, focal redness of the limb or swelling and redness of 1 digit; 2, mild swelling and erythema of the limb or swelling of >2 digits; 3, marked swelling and erythema of the limb; 4, maximal swelling and redness of the limb or ankylosis. The clinical score was expressed as a cumulative value for all paws, with a maximum score of 16 per mouse.

Histopathologic study. Mice were sacrificed on day 14 after anti-CII mAb/LPS administration and their limbs were removed, fixed in 10% paraformaldehyde, decalcified in ethylenediaminetetraacetic acid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic analysis. Each joint was graded on a scale of 0-3: 0, normal; 1, thickening and proliferation of the synovial lining, with slight inflammatory cell infiltration; 2, grade 1 changes plus granulomatous lesions in the synovial sublining tissue; and 3, grade 2 changes plus pannus formation and bone destruction. The degree of arthritis was scored from 0 to 3, providing a maximum score of 12 per mouse.

Flow cytometric analysis. On day 14 after anti-CII mAb/LPS administration, spleen and lymph nodes (LN) were removed from arthritic mice. Both spleen and LN cells were incubated with 2.4G2 mAb (anti-Fc γ R) to block non-specific staining, and then stained with the following mAb conjugates: fluorescein isothiocyanate (FITC)-anti-TCR β (H57-597), -CD69 (H1.2 F3), -hamster IgG₁ isotype (A19-3), phycoerythrin (PE)-

anti-CD4 (RM4-5), -NK1.1 (PK136), or biotinylated-anti-rat IgG_{2a} (R35-95), followed by streptavidin-allophycocyanin (APC) (all from BD Biosciences, San Jose, CA). Propidium iodide (Sigma, St. Louis, MO) positive cells were electronically gated out from the analysis. The stained cells were analyzed on a FACScalibur flow cytometer (Becton-Dickinson, Mountain View, CA) with CellQuest software as described elsewhere (23).

T cell proliferation assay. Seven days after LPS injection, spleens were removed and prepared for *in vitro* culture. T cells were enriched with a magnetic-activated cell sorting (MACS) pan-T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. Enriched T cells were subsequently stimulated with irradiated (30 Gy) syngeneic splenocytes and denatured chick CII (10 or 50 μ g/ml; boiled for 10 min), or with concanavalin A (Con A) (5 μ g/ml) as a positive control, for 72 h. During the last 6 h of culture the cells were pulse-labeled with [³H]-thymidine and the incorporation was determined by direct β -counter Matrix 96 (Packard, Meriden, CT) (24).

NKT cell activation in vivo and quantification of cytokine production in vitro. MIF^{Tg} and WT mice were injected intravenously with 0.1 μ g/g body weight (BW) of α -GalCer (Kirin Brewery, Tokyo, Japan) or with 0.1% dimethylsulfoxide as a control. After 2 h, spleens were removed and single cell suspensions were prepared. Splenocytes were suspended in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 5 \times 10⁻⁵ M 2-mercaptoethanol (culture medium) and cultured in 24-well plates at 5 \times 10⁶ cells/ml for 90 min (23). Culture supernatants were harvested and quantified for IL-4 and IFN- γ using mouse IL-4 and IFN- γ enzyme-linked immunosorbent assay (ELISA) sets (Biosource) according to the manufacturer's protocol.

In vivo α -GalCer treatment. α -GalCer was used to treat mice with anti-IIC mAb/LPS-induced arthritis. Starting at day 3 after anti-IIC mAb/LPS treatment, mice were injected intraperitoneally twice per week with α -GalCer at a dose of 0.5 μ g/g BW (14). The control mice were injected with vehicle alone (0.05% polysorbate at final concentration) in phosphate-buffered saline (PBS).

Cytokine production. Sera from MIF^{Tg} mice (treated with α -GalCer or vehicle) were collected at day 14 after the induction of anti-IIC mAb/LPS-induced arthritis and frozen at -80°C. A panel of eight mouse cytokines [IL-12p70, IL-6, MCP1, IFN- γ , tumor necrosis factor (TNF)- α , IL-2, IL-4, IL-10] was measured simultaneously in a single sample (50 μ l) with cytokine bead array (CBA) kits according to the manufacturer's protocol. Transforming growth factor (TGF)- β was quantified using the QuantikineTM mouse TGF- β immunoassay kit (R&D Systems) according to the manufacturer's protocol.

Statistical analysis. Data were analyzed with either Mann-Whitney U-test or Student's t-test. Results were expressed as means \pm SE. All data analyses were performed using Statview software (Abacus Concept, Berkeley, CA). P<0.05 was considered statistically significant.

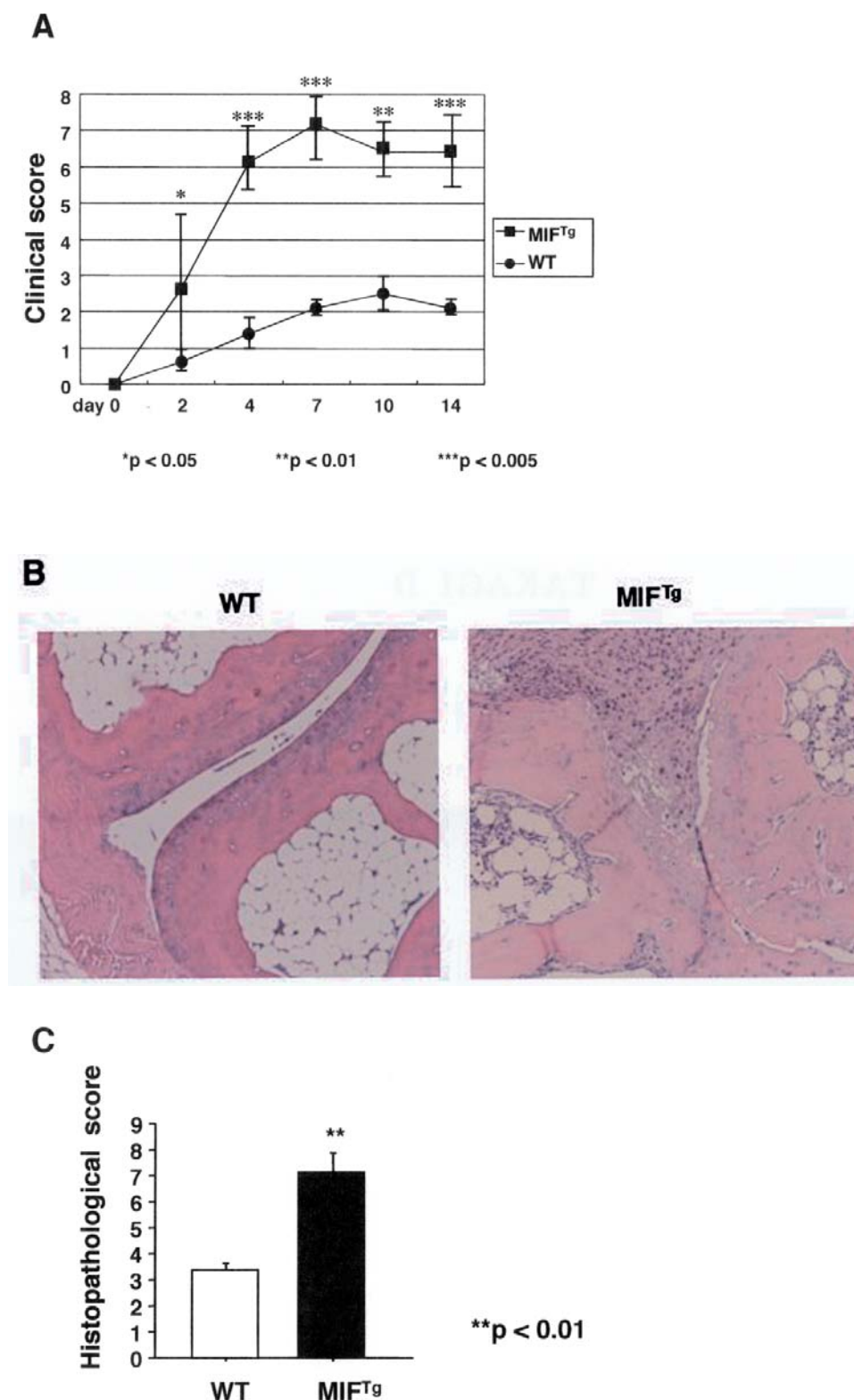


Figure 1. Clinical and histopathological scores of arthritis in WT and MIF^{Tg} mice. (A) Sequential change of clinical score. WT mice (n=8) (●) or MIF^{Tg} mice (n=7) (■) were treated with anti-IIC mAb (2 mg x 4/mouse)/LPS (50 µg/mouse) and the clinical score was analyzed during the following 2 weeks. (B) Representative histopathology of the ankle joint. The joint section of a WT and MIF^{Tg} mouse on day 14 after administration of anti-IIC mAb/LPS was stained with hematoxylin and eosin. (C) Histopathological arthritis scores of the joints. Means ± SE of the histopathological scores on day 14 in WT (n=5) and MIF^{Tg} mice (n=5) are shown.

Results

Exacerbated anti-IIC mAb/LPS-induced joint inflammation in MIF^{Tg} mice. To examine the impact of excess amounts of

MIF on the development of arthritis, we analyzed the joint swelling in WT and MIF^{Tg} mice after injection of anti-CII mAb/LPS. The MIF^{Tg} mice used in the present study demonstrated high MIF levels in their sera (>10 x that of WT) (8).

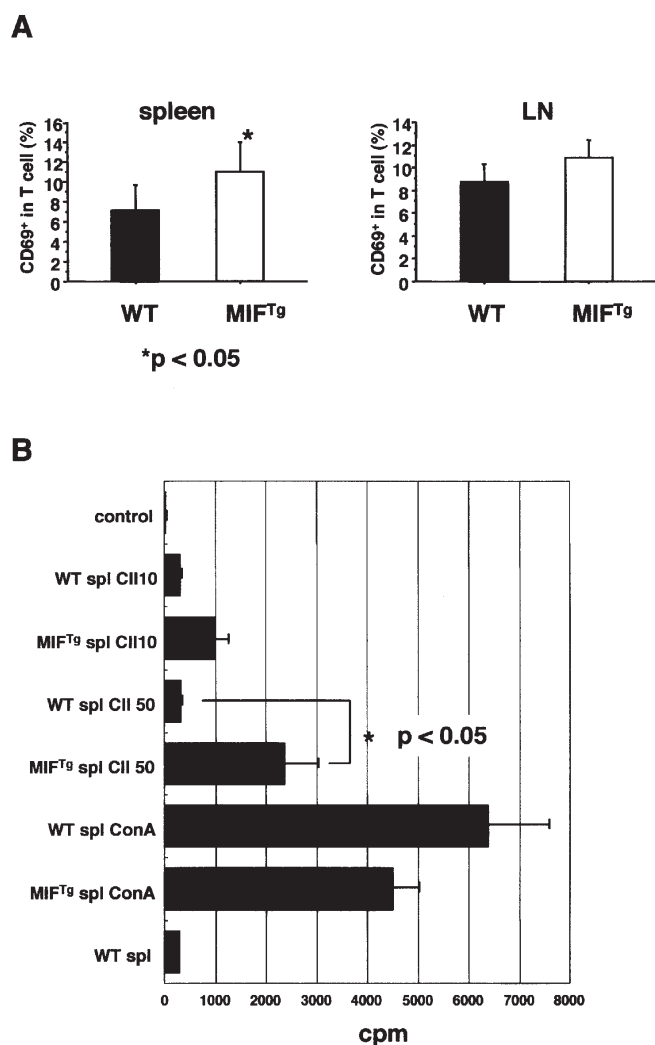


Figure 2. Proportion of CD69⁺ T cells and T cell proliferative responses to CII in WT and MIF^{Tg} mice. (A) Proportion of CD69⁺ cells. WT (n=5) and MIF^{Tg} mice (n=5) were treated with anti-IIC mAb/LPS and the proportion of CD69⁺ cells among the T cell population (TCRβ⁺) from spleen and LN was quantified as described in Materials and methods. Data are mean ± SE. (B) Proliferative responses of T cells. Seven days after anti-IIC mAb/LPS injection, MIF^{Tg} and WT mice were sacrificed and splenic T cells were cultured with irradiated splenocytes as APC in the presence or absence of chick CII (10 or 50 μg) or Con A. Control, no spleen cells; WT spl, splenocytes plus APC with no antigens. Data are mean ± SE.

Fig. 1A shows that WT mice exhibited mild joint inflammation, whereas MIF^{Tg} mice showed considerable swelling starting from day 2-3, with a peak at 7-10 days. The clinical score of the MIF^{Tg} mice was significantly higher than that of WT mice at all stages after mAb/LPS injection. We next evaluated the histopathological arthritis score for each limb from MIF^{Tg} and WT mice on day 14 after mAb/LPS treatment. As shown in Fig. 1B, joints of MIF^{Tg} mice, as compared with WT mice, exhibited marked inflammation such as marked infiltration of inflammatory cells to the synovium and destruction of the joint cartilage. The mean histopathological arthritis score of MIF^{Tg} mice was significantly higher than that of WT mice (Fig. 1C).

T cells in anti-IIC mAb/LPS-induced arthritis in MIF^{Tg} mice. Although the arthritis analyzed in the present study has been

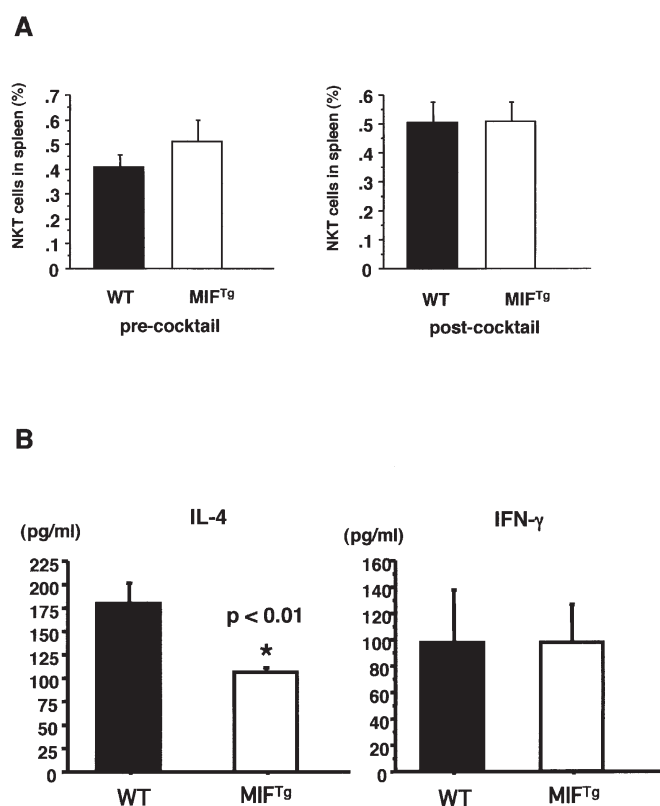


Figure 3. Prevalence of NKT cells in the spleen and cytokine production by the NKT cells. (A) The percentage of NKT cells (NK1.1⁺TCRβ⁺) from spleens of WT (n=5) and MIF^{Tg} mice (n=6). The proportion was analyzed before (pre-cocktail) and after (post-cocktail) administration of anti-IIC mAb/LPS. Data are mean ± SE. (B) α-GalCer-induced cytokine production by spleen cells. WT (n=5) and MIF^{Tg} mice (n=5) were administered α-GalCer and 2 h later spleens were removed and splenocytes were cultured as described in Materials and methods. IL-4 and IFN-γ were quantified by ELISA in the culture supernatants collected 90 min later. Data are mean ± SE.

primarily induced by direct effects of the anti-IIC mAb, T cells may be activated by the cartilaginous component, CII, following inflammation and destruction of the joints. To examine the potential role of T cells in the arthritis induced by mAb/LPS in MIF^{Tg} and WT mice, we analyzed the proportion of CD69⁺ (activated) T cells in the spleen and LN. After induction of arthritis, the proportion of CD69⁺ T cells increased in both MIF^{Tg} and WT mice (data not shown). When the proportion of CD69⁺ T cells was compared at 14 days after anti-IIC mAb/LPS administration, significantly higher proportions were detected in spleens of MIF^{Tg} mice than in WT mice (Fig. 2A). In LN, the proportion of activated T cells was also increased in MIF^{Tg} mice, although the difference was not statistically significant.

Next, we analyzed the T cell proliferative responses to CII in MIF^{Tg} and WT mice. T cells from the spleens of MIF^{Tg} mice mounted significantly higher proliferative responses to CII (50 μg/ml) than splenic T cells from WT mice at 7 days after arthritis induction (Fig. 2B). Similar levels of T cell proliferation against Con A were observed in MIF^{Tg} and WT mice. These findings suggest that T cells in anti-IIC mAb/LPS-treated MIF^{Tg} mice are specifically and systemically more activated by CII antigens than those in WT mice.

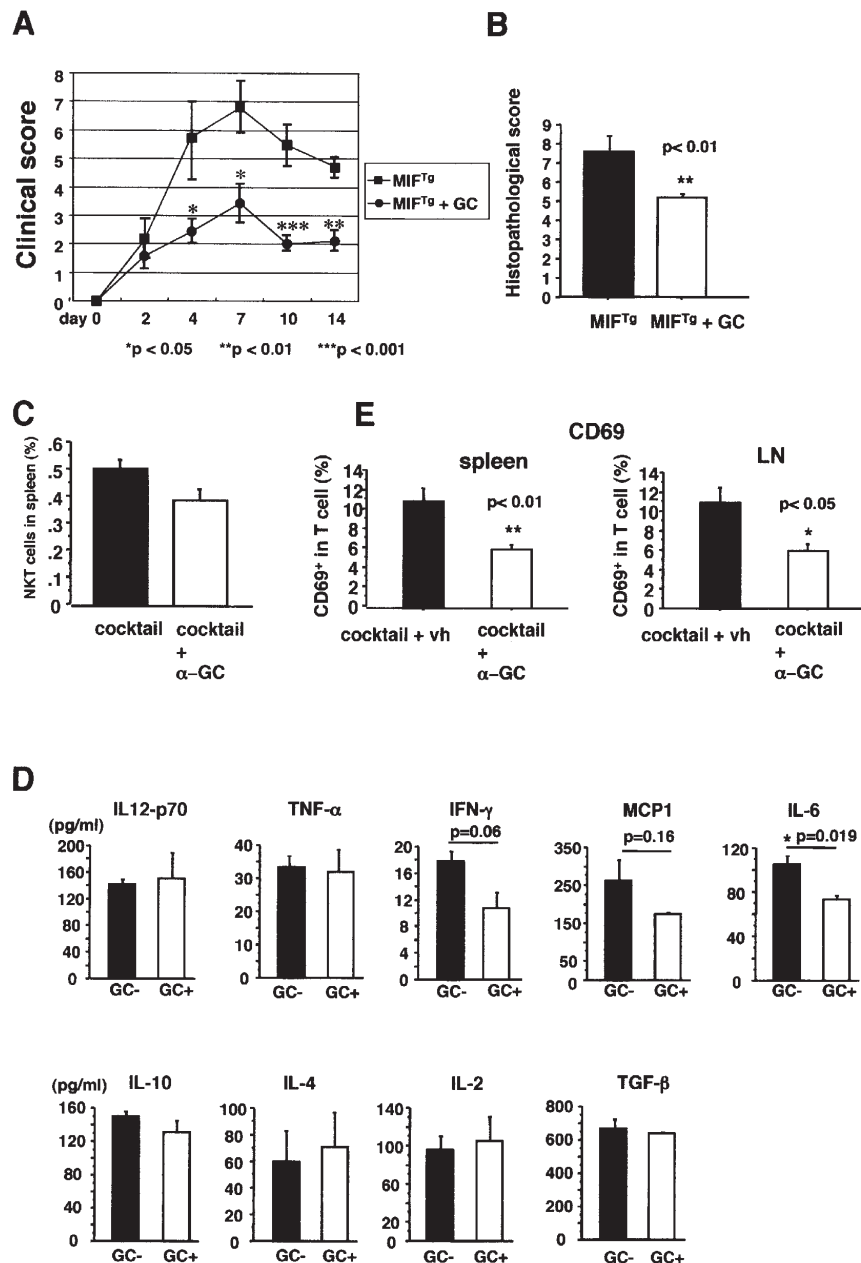


Figure 4. Effect of repeated α -GalCer administrations on anti-IIC mAb/LPS-induced arthritis in MIF^{Tg} mice. (A) Clinical arthritis scores. MIF^{Tg} mice were treated with 500 μ g/kg of α -GalCer (MIF + GC) (n=9) or vehicle (MIF) (n=6), twice per week starting from day 0 after administration of anti-IIC mAb/LPS. (B) Histopathological arthritis scores. Mean histopathological scores \pm SE of MIF^{Tg} mice treated with α -GalCer (MIF + GC) (n=6) or vehicle control (MIF) (n=5) are shown. (C) Percentage of NKT cells (NK1.1⁺TCR β ⁺). The mean proportions \pm SE of NKT cells in the spleen of anti-IIC mAb/LPS-injected MIF^{Tg} mice are shown. These mice were treated with α -GalCer (cocktail + α -GC) or vehicle alone (cocktail) as described in A. (D) Cytokine levels in sera. MIF^{Tg} mice with anti-IIC mAb/LPS-induced arthritis were treated with α -GalCer (GC+) or vehicle (GC-) as described in A. Sera from these mice were quantified for the levels of IL-12p70, IL-6, MCP1, IFN- γ , TNF- α , IL-2, IL-4, and IL-10 by CBA kit. Amounts of TGF- β were quantified as described in Materials and methods. Data are mean \pm SE. (E) Percentage of CD69⁺ cells. CD69⁺ cells among total T cells (TCR β ⁺) were deduced from spleen and LN cells from MIF^{Tg} mice with arthritis treated with vehicle (cocktail + vh) or α -GalCer (cocktail + α -GC). Data are mean \pm SE.

NKT cells in MIF^{Tg} mice with anti-IIC mAb/LPS-induced arthritis. It has been reported that NKT cells activated by the α -GalCer analogue OCH protect against arthritis by promoting Th2-biased immune deviation (14). To examine NKT cell-mediated immunomodulation in our experimental model, we first analyzed the size of the NKT population in spleens of MIF^{Tg} and WT mice, before and after administration of the anti-CII mAb/LPS. No significant differences were noted in

the proportion of NKT cells between MIF^{Tg} and WT mice before or after treatment with anti-CII mAb/LPS (Fig. 3A).

We then intravenously injected α -GalCer (0.1 μ g/g BW) into untreated MIF^{Tg} and WT mice, removed the spleens 2 h later and quantified cytokine production by splenocytes *ex vivo*, as described elsewhere (23). Upon α -GalCer administration, splenocytes from MIF^{Tg} mice produced significantly reduced levels of IL-4 in the supernatants (Fig. 3B). In contrast, no

difference was observed for IFN- γ production between WT and MIF^{Tg} mice. Although NK cells also produce IFN- γ in these conditions, most of the early IFN- γ is produced by NKT cells (25). We therefore conclude that NKT cells exhibit a Th1-biased cytokine production profile in the presence of MIF.

Suppression of arthritis development by α -GalCer administration. It has been reported that persistent NKT cell activation results in the acquisition of a Th2 phenotype by mainstream T cells (26). To examine whether continuous NKT cell activation may modulate MIF-aggravated arthritis, we administered α -GalCer (0.5 μ g/g BW) or vehicle alone intraperitoneally to MIF^{Tg} mice twice per week after anti-IIC mAb/LPS injection. The mean clinical score of arthritis was profoundly reduced in α -GalCer-treated MIF^{Tg} mice compared to vehicle-treated MIF^{Tg} mice (Fig. 4A). In accordance, histological scores were significantly lower in α -GalCer-treated MIF^{Tg} mice compared to vehicle-treated (control) MIF^{Tg} mice on day 14 (Fig. 4B). As shown in Fig. 4C, the proportion of NKT cells was slightly reduced in α -GalCer-treated MIF^{Tg} mice compared with control mice. Quantification of various cytokines in the sera revealed a slight increase in IL-4 levels and a significant and moderate decrease in IL-6 and IFN- γ levels, respectively, in the α -GalCer-treated group (Fig. 4D). No differences were noted in the levels of other cytokines analyzed.

The proportion of CD69⁺ T cells within the T cell population of spleen and LN was significantly lower in the α -GalCer-treated group compared with that in the control group (Fig. 4E). These results demonstrate that multiple administrations of α -GalCer ameliorate anti-CII mAb/LPS-induced arthritis and suppress T cell activation by modifying NKT cell functions in MIF^{Tg} mice.

Discussion

MIF plays a number of distinct roles in the immune system. It has been reported that MIF enhances Th1 immune responses (8,27). Th1 cells secreting IFN- γ and TNF- α exacerbate RA, whereas Th2 cells producing IL-4 and IL-10 suppress arthritis (28,29). Consistent with these reports (8,27-29), administration of anti-MIF Ab during the induction phase of CIA leads to skewing of the immune response towards Th2 type cytokine production and suppression of arthritis onset (12). Ichiyama *et al* (13) also reported that disruption of the *mif* gene resulted in inhibition of joint inflammation in an arthritis model. On the other hand, MIF up-regulates the expression of MMP-13 that is involved in bone resorption (30). This finding indicates that MIF directly activates expression of proteolytic enzymes that induce tissue destruction. Thus, it seems that MIF aggravates arthritis through several pathways. We considered that it was important to investigate how MIF influenced the anti-IIC mAb/LPS-induced arthritis, a relatively new arthritis model.

In the present study, using the arthritis model induced by anti-CII mAb/LPS, we showed that high systemic expression of MIF enhanced joint inflammation. The number of activated (CD69⁺) T cells and the proliferative response of CII-specific T cells in spleens from MIF^{Tg} mice with arthritis were significantly increased. However, it appears unlikely that the CII-specific T cells are directly involved in initiation of the

disease in this passive transfer model of anti-IIC mAb/LPS-induced arthritis. Our findings suggest that MIF primarily enhances initiation of disease mediated by Ab/LPS, resulting in increased CII leakage from the damaged joints. In turn, increased release of CII may augment priming of CII-specific T cells and further exacerbate the arthritis. Thus, the responsiveness of the CII-specific T cells appeared to correlate with the enhanced arthritis score in MIF^{Tg} mice.

Our experiments demonstrate that, upon a single stimulation with α -GalCer, the rapid IL-4 secretion by spleen cells from non-arthritic MIF^{Tg} mice was reduced as compared with spleen cells from nontransgenic mice (Fig. 3B). This finding suggests that a constitutively high level of MIF shifts the cytokine balance produced by NKT cells to Th1 prior to arthritis induction. NKT cells rapidly secrete large amounts of Th2 cytokines such as IL-4 and IL-10 as well as Th1 cytokines such as IFN- γ (16,20,21). It, thus, seems possible that high concentrations of MIF in MIF^{Tg} mice directly or indirectly influence the high capability of NKT cells to produce large amounts of cytokines, and under this condition NKT cells from transgenic animals produce less IL-4 than those from WT mice. Although it remains unclear at the present time how MIF shifts NKT cells to Th1-biased immune deviation in MIF^{Tg} mice, it is likely that the Th1-biased NKT cell status is related to the aggravated arthritis in MIF^{Tg} mice.

A number of reports have indicated that NKT cells play a critical role in the regulation of autoimmune responses (31). Various abnormalities in the number and function of NKT cells have been observed in human autoimmune diseases (15,22,32,33) as well as in a variety of mouse strains that are genetically predisposed to the development of autoimmune diseases (34-39). However, the regulatory role of NKT cells in the development of autoimmune arthritis has not been completely explored. We demonstrated that aggravated inflammation in anti-IIC mAb/LPS-induced arthritis might be attributed to a Th1 shift of NKT cells in MIF^{Tg} mice. However, we also found that repeated stimulation of NKT cells with α -GalCer resulted in significant suppression of the inflammatory response in the joint tissues and reduced T cell activation in the spleens of these mice. In these experiments, MIF^{Tg} mice received several α -GalCer injections resulting in low levels of IFN- γ and IL-6 in the sera, as compared with levels of these cytokines observed after a single α -GalCer injection. It has been reported that the dose and timing of α -GalCer treatment influences the immunomodulatory activity of NKT cells (40). It appears that multiple injections of α -GalCer to the arthritic MIF^{Tg} mice altered NKT cell functions and shifted the cytokine balance from pathogenic Th1 to the non-destructive Th2 state. Similar findings have been reported by Singh *et al* (26,36). However, since differences in cytokine levels between α -GalCer- and vehicle-treated MIF^{Tg} mice were modest, it is likely that mechanisms other than a shift in the cytokine response (e.g., direct cell-to-cell interactions) may be involved in the NKT cell-mediated regulation of arthritis (41).

Recently, it has been reported that NKT cells exacerbate mAb/LPS-induced joint inflammation in another Ab-mediated arthritis model, the K/BxN serum transfer model, through suppression of TGF- β 1 production (18). However, in our arthritis model no difference was noted in the serum TGF- β levels between the α -GalCer-treated and control groups of



These contradictory findings for the functional roles of NKT cells in local inflammation led us to speculate that NKT cells may play distinct roles in different models of autoimmune diseases. This possibility should be pursued in further investigations.

We have demonstrated that multiple administration of α -GalCer to MIF^{Tg} mice results not only in a reduction of arthritis severity, but also in a slight decrease in serum IFN- γ and a slight increase in serum IL-4. We also found that the serum IL-6 level was significantly lower in α -GalCer-treated MIF^{Tg} mice than in untreated mice. These findings indicate that NKT cells stimulated in this way induce a shift from a pathogenic anti-joint Th1 response to a non-destructive Th2 response in the experimental model employed in the present study.

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