Growth factor midkine promotes NFAT-regulated T cell activation and T_H1 cell differentiation in lupus nephritis

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Abstract

Activated T cells play crucial roles in the pathogenesis of autoimmune diseases, including lupus nephritis (LN). The activation of calcineurin/nuclear factor of activated T cells (NFAT) and signal transducers and activator of transcription (STAT) 4 signaling is essential for T cells to carry out various effector functions. Here, we identified the growth factor midkine (MK; gene name, *Mdk*) as a novel regulator in the pathogenesis of TMPD-induced LN via activation of NFAT and interleukin (IL)-12/STAT4 signaling. Wild-type ($Mdk^{+/+}$) mice showed more severe glomerular injury than MK-deficient ($Mdk^{-/-}$) mice, as demonstrated by mesangial hypercellularity and matrix expansion, and glomerular capillary loops with immune-complex deposition. Compared to $Mdk^{-/-}$ mice, the frequency of splenic $CD69^+$ T cells and T helper (T_H) 1 cells, but not of regulatory T cells, was augmented in $Mdk^{+/+}$ mice in proportion to LN disease activity, and was accompanied by skewed cytokine production. MK expression was also enhanced in activated $CD4^+$ T cells *in vivo* and *in vitro*. MK induced activated $CD4^+$ T cells expressing CD69 through nuclear activation of NFAT transcription, and selectively increased *in vitro* differentiation of naïve $CD4^+$ T cells into T_H1 cells by promoting IL-12/STAT4 signaling. These results suggest that MK serves an indispensable role in the NFAT-regulated activation of $CD4^+$ T cells and T_H1 cell differentiation, eventually leading to the exacerbation of LN.

Introduction

Lupus nephritis (LN) is one of the critical determinants for mortality and morbidity of systemic lupus erythematosus (SLE) [1, 2]. T lymphocytes, which play crucial roles in adaptive immune responses, contribute to the initiation and perpetuation of autoimmunity in SLE [3, 4]. In this setting, a variety of cytokines and chemokines that they produce are involved in the pathogenesis of LN [5]. During T cell activation, these genes are regulated by calcium signaling calcineurin (Cn)-dependent transcription factor, nuclear factor of activated T cells (NFAT) together with other signaling pathways [6-9]. Given the prominent role of NFAT proteins in the regulation of T cell activation, NFAT has been considered to be an optimal target for therapeutic approaches to autoimmune diseases. Once self-tolerance is broken, the skewed profile of cytokine expression causes activation of T helper (T_H) cell subsets including T_H1, T_H17 and regulatory T cells (Tregs), through the dysfunction of signal transducers and activator of transcription (STAT) signaling, leading to the disruption of their reciprocal relationship in autoimmunity [1, 10]. Therefore, an enhanced understanding of adaptive immune activation and regulation of tolerance is required for novel therapeutic strategies.

The growth factor Midkine (MK; gene name, *Mdk*) has been suggested to have major biologic roles, including roles in promoting cell proliferation, cell survival, and the migration of various cells, and it also displays anti-apoptotic activity [11, 12]. Its major actions can be categorized into three areas: the nervous system, cancer and inflammation [13]. To date, the neuronal protective effects of MK have been investigated in *in vivo* studies such as in cerebral infarction and ischemia-induced neuronal death [14, 15]. In a series of prior studies, we generated mice deficient in the *Mdk* gene ($Mdk^{-/-}$) and used them to demonstrate that MK is involved in inflammation through cytokine

induction and modulation of the migration of neutrophils and macrophages in arterial restenosis, rheumatoid arthritis, ischemic renal injury and diabetic nephropathy [16-20]. Besides their elicitation via chemotactic activity, MK also serves as a negative immune modulator of Treg in peripheral lymph nodes. Inhibition of MK caused enhanced Treg expansion and subsequently suppressed auto-reactive T_H1 cell populations, eventually leading to attenuation of the severity of experimental autoimmune encephalomyelitis (EAE) similar to multiple sclerosis [21, 22]. It is well recognized that interleukin (IL)-2 signaling is essential for the development and function of Treg, a potent regulator of T cells subset. While IL-2 is induced in the development of EAE, the exacerbation of LN shows a reduction in IL-2 transcription with dysfunction of Tregs [23, 24]. The involvement of MK in the molecular mechanism of LN has not yet been elucidated in detail.

We therefore conducted the present study to investigate the role of MK in the pathogenesis of LN. Notably, MK expressed in splenic $CD4^+$ T cells positively regulated Treg-independent differentiation into T_H1 cells in active LN. To understand these phenomena, a detailed explanation of how the activation and differentiation of T cell subsets in LN are orchestrated by MK is required. We demonstrated that activation of $CD4^+$ T cells accompanies the secretion of MK that is responsible for skewed cytokine production, which, in turn, enhances splenic activated $CD4^+$ T cells expressing CD69 through the induction of NFAT-mediated gene transcription. In this setting, the altered cytokine profile further promotes the expansion and differentiation of T_H1 cells through IL-12/STAT4 signaling. The obtained results enabled an understanding of the potent role of MK in the activation of T cells in autoimmune diseases through amplification of a physiological vicious loop.

Materials and Methods

Animals and experimental design

Mdk$^{-/-}$ mice were generated as described previously [25]. After backcrossing of *Mdk*$^{+/-}$ mice for 20 generations with 129/SV mice, *Mdk*$^{+/-}$ mice were mated with each other to generate *Mdk*$^{+/+}$ and *Mdk*$^{-/-}$ mice that were used in this study. Experiments were performed using 8- to 12-week-old female mice weighing 20 to 25 g that were housed under controlled environmental conditions and maintained with standard food and water. LN was induced in *Mdk*$^{+/+}$ and *Mdk*$^{-/-}$ mice with an intraperitoneal injection of TMPD (pristane; 2,6,10,14-tetramethylpentadecane; 0.5ml/mouse) as described previously [26]. Mice were sacrificed at 6 months after treatment. MRL/*lpr* female mice were purchased from the Japan SLC, Inc. (Shizuoka, Japan), and then were sacrificed at 3 months after birth. Kidneys and the spleen were removed for examination. All of the animal experiments were performed in accordance with the animal experimentation guidelines of Nagoya University School of Medicine.

Histology

Kidney tissues were fixed in 10% formalin, embedded in paraffin and then cut into 2-μm-thick sections. Sections stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) were used for morphometric analysis of the activity indices of human LN as described previously [27, 28]. In brief, the extent of kidney injury was determined by assessing histological features reflective of LN activity such as endocapillary hypercellularity, leukocyte infiltration, subendothelial hyaline deposits, fibrinoid necrosis/karyorrhexis, cellular crescent and interstitial inflammation. All of the

quantifications were performed in a blinded manner by two independent expert nephropathologists.

Parts of the kidney tissues were snap-frozen in liquid nitrogen. Sections (4-μm thick) were cut with a cryostat and fixed with acetone. The sections were stained with rat anti-mouse IgG antibody (Ab) (Life technologies, Carlsbad, CA), C3 Ab (Abcam, Cambridge, UK), C1q mouse Ab (Abcam) or CD68 Ab (AbD Serotec, Oxford, UK), followed by detection with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG Ab (Zymed Laboratories, San Francisco, CA). The sections were stained with anti-CD3 Ab (Abcam) or rat anti-mouse CD4 Ab (Abcam), followed by biotin-conjugated rabbit anti-rat Ab (Nichirei, Tokyo, Japan). Staining was visualized with 3, 3'-diaminobenzidine (Dako, Carpinteria, CA), which produced a brown color. Negative controls involved replacement of the primary Abs with species-matched Abs. Leukocytes positive for CD3, CD4 or CD68 in the glomerulus of all renal regions were counted under a microscope in a blind manner. For electron microscopic analysis, kidneys were fixed in formalin, embedded in epoxy resin, and stained with uranyl acetate and lead citrate.

Biochemical examination, autoantibody analysis and IL-2 measurement

The ratio of albumin to creatinine in urine was measured as described previously [29]. The hallmarks of LN activity were determined using enzyme-linked immunosorbent assay (ELISA) methods, according to the manufacturers' instructions (IgG1, G2a and G2b, R&D Systems Inc., Minneapolis, MN; anti-ssDNA Ab and anti-dsDNA Ab, Shibayagi, Gunma, Japan). The IL-2 levels in the supernatant of cultured T splenocytes were measured with an ELISA kit (Thermo Fisher Scientific Inc., Waltham, MA).

Flow cytometric analysis

Splenocytes or renal cells were blocked with saturating amounts of anti-CD16/32 Abs (BD Biosciences, San Diego, CA) and were then stained with the following conjugated Abs: FITC/allophycocyanin (APC) rat anti-mouse CD3e, FITC/APC/phycoerythrin (PE)-Cy7 rat anti-mouse CD4, PE rat anti-mouse CD8a, IL-4, IL-17, CD45R/B220, IgM, CD44, and CD25; APC rat anti-mouse γ-interferon (IFN-γ), CD138, IgD, and CD62L; Alexa Fluor488 rat anti-mouse FoxP3, and APC/PE-Cy7 rat anti-mouse CD69 (BioLegend, San Diego, CA). Cells were acquired using a FACS Canto II flow cytometer (BD Biosciences).

Real-Time PCR

Mouse kidney tissues were snap-frozen in liquid nitrogen for total mRNA isolation as described previously [30]. Real-time PCR analysis was performed with an Applied Biosystems Prism 7500HT sequence detection system using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). TaqMan probes and primers for tumor necrosis factor (TNF)-α (*Celf2* Mm01336295_m1), IL-1β (*Il1β* Mm00434228_m1), IL-6 (*Il6st* Mm00439665_m1), IFN-γ (*Pglyrp2* Mm01348077_m1), monocyte chemoattractant protein (MCP)-1 (*Ccl2* Mm00441242_m1), and GAPDH (*Gapdh* Mm99999915_m1) were used. Amplification data were analyzed with Applied Biosystems Sequence Detection software version 1.3.1.

Western blot analysis

Mouse kidney and spleen tissues were snap-frozen in liquid nitrogen for protein

isolation and were then lysed in a radio-immunoprecipitation assay buffer (Santa Cruz Biotechnology, Dallas, TX). Lymphocytes from *in vitro* studies were lysed with the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (ThermoFisher Scientific Inc.). Western blot analysis was performed as described previously [31]. The blots were incubated with goat anti-mouse MK Ab [32], monoclonal anti-β-actin Ab (Sigma-Aldrich, St. Louis, MO), rabbit anti-mouse NFAT1 Ab, histone-H3 Ab, GAPDH Ab, STAT1 Ab, phospho-STAT1 Ab, STAT4 Ab (all from Cell signaling technology, Danvers, MA), and phospho-NFATc2 Ab (Santa Cruz Biotechnology); and mouse monoclonal anti-mouse phospho-STAT4 Ab (Santa Cruz Biotechnology), followed by incubation with peroxidase-conjugated anti-goat IgG, mouse IgG and rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA). The protein signals were visualized with an Amersham Imager 600 (GE Healthcare).

Intracellular staining and T cell differentiation assessment

Splenocytes were re-stimulated for 5 h with PMA (50 ng/ml) (Sigma-Aldrich St. Louis, MO) and ionomycin (1 μg/ml) (Sigma-Aldrich) along with brefeldin A (BD Biosciences). After surface staining with the indicated Abs, the cells were fixed with Fixation/Permeabilization Buffer (BD Biosciences), permeabilized with Perm/Wash Buffer (BD Biosciences), and stained with the following conjugated Abs: APC rat anti-mouse IFN-γ, PE rat anti-mouse IL-4, APC rat anti-mouse IL-17, PE rat anti-mouse IL-17, AF488 rat anti-mouse Foxp3 (BD Biosciences).

Naïve $CD4^+CD62L^+$ T cells were isolated from the spleen as described previously [26]. Purified cells were cultured in RPMI 1640 (Sigma Aldrich) supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and were then activated with

anti-CD3 and anti-CD28 Abs (Life technologies) for 3 days with the following cytokines and neutralizing Abs for the desired polarization: IL-12 (10 ng/ml) (R&D Systems) and anti-IL-4 (10 μg/ml) (BioLegend) for T_H1 cell polarization; IL-4 (50 ng/ml), IL-2 (200 units/ml) (R&D Systems), and anti-IFN-γ (10 μg/ml) (BioLegend) for T_H2 cell polarization; transforming growth factor (TGF)-β (1 ng/ml), IL-6 (50 ng/ml) (R&D Systems), IL-23 (5 ng/ml) (BioLegend), anti-IFN-γ (10 μg/ml), and anti–IL-4 (10 μg/ml) for T_H17 cell polarization; TGF-β (5 ng/ml), IL-2 (200 units/ml), and anti-IFN-γ (10 μg/ml) for Treg cell polarization.

Statistical Analysis

All values are expressed as means ± SEM. Statistical analyses were performed using an unpaired Student's *t*-test or the nonparametric Mann–Whitney U test for single comparisons or analysis of variance for multiple comparisons. Post hoc least significant difference tests were performed if the initial analysis of variance was significant. $P < 0.05$ was considered to indicate statistically significant differences.

Results

Midkine deficiency ameliorates glomerular injury in LN

In order to investigate the role of MK in the pathogenesis of LN, we evaluated the degree of glomerular injury in $Mdk^{+/+}$ and $Mdk^{-/-}$ mice at 6 months after TMPD treatment. No obvious differences between the two genotypes were found in kidney and body weight, or renal function (Table 1). The spleen weight (Table 1) and total numbers of splenocytes (data not shown) tended to be higher in $Mdk^{+/+}$ LN mice than in $Mdk^{-/-}$ mice. Basically, TMPD causes only mild glomerular damage, but not tubulointerstitial

injury. *Mdk*$^{+/+}$ mice showed more severe glomerular injury than *Mdk*$^{-/-}$ mice, as demonstrated by mesangial hypercellularity and matrix expansion, and glomerular capillary loops with immune-complex (IC) deposition (Figure 1A). In immunofluorescence analysis of the glomeruli, higher deposition of IgG, C3 and C1q was observed in the mesangial areas and partly in the capillary loop of *Mdk*$^{+/+}$ mice compared to *Mdk*$^{-/-}$ mice (Figure 1B). Consistent with the profiles of immunofluorescence analysis, increase of IC deposition was observed in *Mdk*$^{+/+}$ mice more than in *Mdk*$^{-/-}$ mice, using an electron microscopy (Figure 1B, C). Interestingly, the involvement of IC deposition in subepithelial areas was absent in *Mdk*$^{-/-}$ mice. The histological investigations shown in Figure 1A were complemented by morphological assessment of the activity index for human LN. The profile of the activity index of LN was consistent with the pathological findings (Figure 1A-D). The index was not enhanced in *Mdk*$^{-/-}$ mice at 6 months after treatment, compared with age-matched mice without treatment. Albuminuria was also greater in *Mdk*$^{+/+}$ mice than in *Mdk*$^{-/-}$ mice (Figure 1E). No obvious difference between *Mdk*$^{+/+}$ and *Mdk*$^{-/-}$ mice was observed in the tubular interstitium (data not shown). These results suggest that MK deficiency suppressed the degree of glomerular injury and LN disease activity after TMPD treatment.

Since enhanced humoral autoimmunity is required for the progression of LN, we also examined IgG values of the mice. TMPD treatment increased the titers of IgG subtypes in both genotypes, but the differences between the two genotypes were not significant (Figure 1F). Likewise, the profiles of autoantibody values specifically directed against ssDNA and dsDNA tended to be increased in lupus *Mdk*$^{+/+}$ mice, but the differences between the two genotypes were not significant (Figure 1G).

Infiltrating inflammatory cells are less marked in the glomeruli of Mdk$^{-/-}$ mice versus Mdk$^{+/+}$ mice

There is growing evidence for the participation of inflammatory cells in LN [1, 2] and for the chemotactic activity of MK [12]. To determine which subsets of leukocytes are induced by MK after TMPD treatment, we assessed the recruitment of inflammatory cells to the lupus kidneys. Infiltrating $CD3^+$, $CD4^+$, and $CD68^+$ cells were more prominent in the glomeruli of *Mdk*$^{+/+}$ mice than in the glomeruli of *Mdk*$^{-/-}$ mice (Figure 2A, B). TMPD treatment significantly augmented $CD3^+CD69^+$, $CD4^+CD69^+$ and $CD4^+CD45^+IFN\text{-}\gamma^+$ cells in the lupus kidneys of *Mdk*$^{+/+}$ mice compared to *Mdk*$^{-/-}$ mice, determined by flow cytometric technique (Figure 2C-G). These data suggest that effector T cells as well as M1 macrophages were more increased in the lupus kidney of *Mdk*$^{+/+}$ mice than in *Mdk*$^{-/-}$ mice. In support of these data and consistent with previous reports, high expression of inflammation-related molecules, including TNF-α, IL-1β, IL-6 and MCP-1, was observed in lupus *Mdk*$^{+/+}$ kidneys. The expression of these molecules was significantly higher in *Mdk*$^{+/+}$ than in *Mdk*$^{-/-}$ mice (Figure 2H). IFN-γ levels tended to be higher in lupus *Mdk*$^{+/+}$ mice than in *Mdk*$^{-/-}$ mice. No obvious differences between the two genotypes were found in the frequency of $CD4^+CD45^+IL\text{-}17^+$ cells (Figure 2G), Tregs or in the expression of IL-17 (data not shown).

MK deficiency suppresses the activation of splenic T lymphocytes in vivo

In general, T lymphocytes derived from the spleen play an important role in the development of LN [1, 3]. We therefore analyzed the expression of the T cell activation

markers CD69, CD62L and CD44 in $CD4^+$ splenocytes in TMPD-induced $Mdk^{+/+}$ and $Mdk^{-/-}$ mice. Compared to $Mdk^{-/-}$ mice, the percentage of splenic $CD4^+CD69^+$ and $CD4^+CD44^+CD62L^-$ cells in $Mdk^{+/+}$ mice was higher, whereas the percentage of naïve $CD4^+CD44^-CD62L^+$ T cells was decreased (Figure 3A-C). The profiles of activated T lymphocytes in the spleens were compatible with those of the kidneys. A striking increase in MK expression was found in $Mdk^{+/+}$ spleens at 6 months after TMPD treatment (Figure 3D, E). Flow cytometric profiles of $CD4^+$ and $CD8^+$ cells did not show any differences between the two genotypes (data not shown). In addition, T cell development in the thymus was unchanged by the presence of MK (data not shown). Since T cell subsets show inappropriate tissue homing in SLE [2, 33], the differentiation of T_H cell subsets in the lupus spleen was therefore examined. Consistent with the profiles of renal T lymphocytes, the frequency and the number of T_H1 cells were significantly enhanced in TMPD-induced $Mdk^{+/+}$ splenocytes compared to $Mdk^{-/-}$ splenocytes (Figure 2G, 3F, 3G). The number but not the frequency of T_H17 cells was also increased after TMPD treatment. There were no significant differences in the frequency and number of Tregs between TMPD-treated $Mdk^{+/+}$ and $Mdk^{-/-}$ spleens. No obvious differences were observed in the percentage of mature $CD138^+B220^+$, immature $IgD^+IgM^-B220^+$ and $IgD^+IgM^+B220^+$ B cells between the two genotypes (Figure3H). The collective data supported the idea that MK induction was associated with the activation of T cells in TMPD-induced LN mice, particularly of T_H1 cells, but was not associated with differentiation of Tregs.

MK promotes NFAT1-mediated T cell activation and T_H1 cell differentiation in splenocytes in vitro

To further clarify the involvement of MK in the activation and differentiation of T cells, we next assessed MK induction in activated T cells and the effect of MK on T_H1 cell differentiation *in vitro*, using naïve $CD4^+$ T cells from $Mdk^{+/+}$ or $Mdk^{-/-}$ spleens. Consistent with the profile of MK expression *in vivo* (Figure 3D, E), a gradual increase in MK production in the supernatant of cultured $CD4^+$ T lymphocytes from $Mdk^{+/+}$ mice was observed during their activation mediated by anti-CD3/CD28 Abs (Figure 4A). MK protein expression in the lysate of activated T cells showed a similar profile (data not shown). To determine whether the difference in the frequency of activated T cells between $Mdk^{+/+}$ and $Mdk^{-/-}$ mice is due to the presence or absence of MK, we next examined the effect of addition of the MK protein to $Mdk^{-/-}$ activated T cells. The frequency of $CD4^+CD69^+$ cells in $Mdk^{+/+}$ splenocytes exposed to anti-CD3/CD28 Abs was higher at 2 days after stimulation compared to $Mdk^{-/-}$ mice (Figure 4B, C). In $Mdk^{-/-}$ naïve $CD4^+$ T cells exposed to anti-CD3/CD28 Abs in the presence of 300 ng/ml MK, the frequency of activated $CD4^+CD69^+$ T cells was similar to that in $Mdk^{+/+}$ T lymphocytes exposed to anti-CD3/CD28 Abs. We further determined whether MK affects the dephosphorylation and translocation of NFAT into the nucleus, which is closely associated with T cell subset proliferation in SLE. After exposure to anti-CD3/CD28 Abs, nuclear translocation of NFAT was strikingly lower in $Mdk^{-/-}$ splenocytes compared with either $Mdk^{+/+}$ splenocytes or with $Mdk^{-/-}$ splenocytes treated with recombinant MK protein (Figure 4D, E). In support of these results, phosphorylation of NFAT in the cytoplasm was prominent in activated $Mdk^{-/-}$ T lymphocytes, whereas supplementation of the cells with recombinant MK protein strikingly inhibited this phenomenon (Figure 4D). The IL-2 promoter region includes binding elements for NFAT. In proportion to the nuclear activation of NFAT

transcription, therefore, IL-2 expression in the supernatant of cultured T lymphocytes was significantly lower in *Mdk*$^{-/-}$ splenocytes compared with *Mdk*$^{+/+}$, and *Mdk*$^{-/-}$ splenocytes treated with recombinant MK protein (Figure 4F). MK did not affect Cn expression during T cell activation (data not shown). These data suggest that MK induced activated CD4^{+} T cells expressing CD69 through the nuclear activation of NFAT transcription leading to the promotion of IL-2-associated T_H1 cell differentiation.

The molecular network of T_H1 cell differentiation involves various essential transcriptional factors, including members of the STAT family induced by various key cytokines. Under T_H1 cell differentiation conditions, the frequency of T_H1 cells derived from naïve CD4^{+} T cells of *Mdk*$^{+/+}$ spleens was significantly higher than that of T_H1 cells derived from naïve CD4^{+} T cells of *Mdk*$^{-/-}$ spleens (Figure 5A, B). Treatment of *Mdk*$^{-/-}$ naïve CD4^{+} T cells with the MK protein promoted T_H1 cell differentiation, resulting in a T_H1 profile similar to that found in *Mdk*$^{+/+}$ cells. In contrast, other T cell subsets such as T_H2, T_H17 cells and Tregs were not affected by the presence or absence of the MK protein in the mice under their respective differentiation conditions (data not shown). STAT1 and STAT4 are required for T_H1 cell differentiation derived from naïve T cells in response to IFN-γ (100 ng/ml) or IL-12 (10 ng/ml), respectively. We therefore examined the function of MK in the activation of STAT1 and STAT4 in this network. No obvious difference between the two genotypes in response to IFN-γ was found in phosphorylation of STAT1 (Figure 5C, D). Since STAT1 is required for the production of IgG autoantibodies in pristine-induced mouse model [34], this may cause no significant differences in the titers of IgG subtypes between the two genotypes. In contrast, treatment with IL-12 for T_H1 cell polarization promoted STAT4 phosphorylation in *Mdk*$^{+/+}$ T cells to a significantly higher degree than in *Mdk*$^{-/-}$ T cells (Figure 5E, F). The

combined data indicated that MK is induced in activated T cells and participates in T_H1 cell differentiation as an amplifying factor via promotion of the IL-12/STAT4 signaling (Figure 6).

Discussion

Activated T cells play critical roles in adaptive immune responses in diverse organs, which are categorized by the cytokines they produce [5, 35]. Various genetic approaches have been applied to demonstrate the importance of the NFAT signaling pathway in the regulation of T cell proliferation [4, 7, 9] and the essential role of members of the STAT family in T_H cell differentiation [10, 36]. The present study demonstrated that MK derived from $CD4^+$ T cells activates T splenocytes themselves and T_H1 cell differentiation, consequently leading to the exacerbation of glomerular nephritis in SLE. Spontaneous lupus-prone MRL/*lpr* mice also showed MK induction in the kidneys and spleens (Supplemental figure). MK induction in T cells was indeed found during activation of the T cells *in vitro*, and supplementation of $Mdk^{-/-}$ activated T cells with the MK protein induced the activation of NFAT signaling and CD69 expression in these cells with a profile similar to that in $Mdk^{+/+}$ activated T cells (Figure 6A). In addition, MK selectively regulates population and differentiation into T_H1 cells through IL-12/STAT4 signaling, which is independent of the Treg population (Figure 6B). Therefore, MK may be involved in a physiological vicious loop that acts as an inducer for expansion of activated T cells, including T_H1 cells, in LN.

Along with an enhanced and accelerated early T cell response, activated splenic $CD4^+$ or $CD4^-CD8^-$ (DN) T cells directly invade organ tissues, including the kidneys, which provides aberrant help to B cells to induce various pathogenic

autoantibodies [4]. The existence of an impaired fine balance between lymphocyte survival and proliferation, and altered cytokine production are considered to be responsible for the pathogenesis of LN. In addition to the infiltration of macrophages into injured kidneys, MK indeed enhances the migration of splenic activated $CD4^+$ T cells in LN through the induction of various chemokines, and consequently contributes to augmentation of the severity of LN. In SLE T lymphocytes, NFAT plays a central role, not only in lymphocyte tolerance but also in productive activation of lymphocytes [4, 7]. Upon enlargement of the T cell receptor, activation of Cn induces dephosphorylation of NFAT in the cytoplasm. Thereafter, dephosphorylated NFAT rapidly translocates into the nucleus where it exerts crucial transcriptional activity to activate downstream targets in the pathogenesis of SLE. In this setting, NFAT also regulates transcription of the polarizing cytokines IFN-γ and IL-2 that drive T_H1 cell differentiation. In the present study, MK deficiency hampered NFAT nuclear translocation, whereas splenic MK induction facilitated the transcriptional activity of NFAT with the production of various immunomodulatory cytokines. In LN representing a variety of aspects and caused by multiple complex pathogeneses, it would be required for sustainability of the LN pathology to enhance and sustain the immune-specific $CD4^+$ T cells response/differentiation by the induction of potent cytokines in an autocrine or paracrine fashion. Our findings show that activated T cells and T_H1 cell differentiation were upregulated in an MK-dependent manner together with skewed cytokine production during pathological exacerbation of LN, revealing a positive amplification circuit for NFAT signaling.

Besides the critical roles of MK in chemotactic activity and subsequent inflammation [12], MK is also involved in immunologic responses in autoimmune

diseases [21]. MK suppresses IL-2-regulated STAT5 phosphorylation and dendritic cells-mediated $CD4^+CD25^+FoxP3^+$ T cells (Tregs) through the up-regulation of SH2 domain-containing protein tyrosine phosphatase (SHP2) leading to the exacerbation of EAE [21, 22]. Inhibition of SHP2 reduces the proliferation of DN T cells and decreases the production of IFN-γ and IL-17A in lupus-prone MRL/*lpr* mice [37]. In the present study, MK did not affect numbers of DN T cells and the production of these cytokines. Dysfunction of Tregs leads to the exacerbation of LN, with a reduction in IL-2 transcription in TMPD-induced LN and in lupus-prone MRL/*lpr* mice [23, 24, 38]. Patients with SLE also show suppression of Tregs and a negative correlation between Tregs and disease activity [2]. In addition, Amarilyo et al. demonstrate that IL-17 plays an important role for the development of TMPD-induced LN [39]. We therefore determined the involvement of Tregs and T_H17 cells in mice with TMPD-induced LN in the present study. Of note, however, no obvious difference in the populations of Tregs and T_H17 cells between the two genotypes was found irrespective of enhanced T_H1 cell differentiation in $Mdk^{+/+}$ LN mice. As demonstrated in studies of Suzumura et al. [21, 22], MK might regulate the development of Tregs in an IL-2 dependent manner. Since exacerbation of LN shows a striking reduction in IL-2 transcription [23, 24], MK might not affect Treg population and the subsequent T_H17 populations in the present study. In general, T_H1 cell differentiation in LN is accompanied by IFN-γ/STAT1 and IL-12/STAT4 signaling. Consequently, MK selectively phosphorylated IL-12/STAT4 signaling to promote T_H1 cell differentiation, but not phosphorylated IFN-γ/STAT1 signaling. Many basic and clinical studies to date have demonstrated that IFN-γ/STAT1 activation is also required for the pathogenesis of LN [40-44]. Given these facts, diverse mechanism of LN involves inappropriate tissue homing of T lymphocytes, including

T_H1, T_H17 and regulatory T cells (Tregs), through the dysfunction of STATs family signaling. The association of variations in the STAT4 gene in LN with severe renal insufficiency has been demonstrated [45]. While a STAT4-specific blockade ameliorates LN disease activity in MRL/*lpr* mice with advanced nephritis [46], transgenic STAT4 knockout mice showed more severe nephritis [47]. Knockout of STAT4 may cause either incomplete STAT inhibition or complementary activation of another pathway in the absence of STAT4 signaling. Therefore, direct or complete suppression of STAT4 signaling makes it difficult to design a therapeutic strategy without side effects. In addition, MK has also been demonstrated to serve as a regulator of mature B cell survival in B cell lymphoma [48]. In the present study, *Mdk*$^{-/-}$ mice didn't show any IC deposition in subepithelial areas, which was previously reported [49]. MK may affect humoral immunity thorough activation of T cells in TMPD-induced LN. Thus, MK may mediate STAT4-associated differentiation of T_H1 cells in the pathogenesis of LN, but may not affect Tregs.

To the best of our knowledge, a hyperactive Cn-NFAT pathway plays an essential role in such signaling for LN. Indeed, the Cn inhibitors, FK506 tacrolimus and cyclosporine A, are highly effective in blocking this signaling in SLE T cells. However, this therapeutic strategy has several side effects, including hypertension, hyperglycemia and renal toxicity. To date, MK blockade has been shown to improve hypertension in ischemic renal injury through regulation of the renin-angiotensin system [31, 32]. Accordingly, an MK-regulated device may lead to a reduction in toxicity from LN therapy. Herein, we demonstrated as a first-reported paper that MK induces NFAT-regulated activation of T cells and T_H1 cell differentiation through IL-12/STAT4 signaling leading to the exacerbation of LN (Figure 6). This study of MK thus provides

novel insights into LN that may open up new avenues and facilitate research on the cause and the development of therapeutics for LN.

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Table 1. General characteristics of the mice analyzed in this study

	Bwt	Kidney (g)	Spleen (g)
Mdk $^{+/+}$ (TMPD)	29.8±1.9	0.41±0.09	0.39±0.30
Mdk $^{-/-}$ (TMPD)	26.8±1.4	0.34±0.03	0.18±0.12
Mdk $^{+/+}$ (No Rx)	32.0±1.5	0.34±0.02	0.11±0.07
Mdk $^{-/-}$ (No Rx)	28.8±0.9	0.35±0.08	0.08±08

Data are expressed as means ± SEM (range).

Mdk $^{+/+}$, wild-type mice

Mdk $^{-/-}$, midkine gene-deficient mice

TMPD, 2,6,10,14-tetramethylpentadecane

No Rx, no treatment

Bwt, body weight

Figure legends

Figure 1. Midkine deficiency ameliorates lupus nephritis in vivo

(A) Representative photographs of PAS- and HE-stained glomeruli at 6 months in wild-type ($Mdk^{+/+}$) and in Midkine-deficient ($Mdk^{-/-}$) mice. No Rx, no TMPD treatment. Bar, 50 μm. (B) Immunofluorescence staining of mouse IgG (upper panels), mouse C3 (middle panels) and mouse C1q (lower panels) at 6 months after TMPD injection. Bar, 50 μm. (C) Representative photographs of the glomerulus at 6 months in $Mdk^{+/+}$ and in $Mdk^{-/-}$ mice, using an electron microscopy. Bar, 2.0 μm (left top, right bottom); 1.0 μm (right top, left bottom). Arrow, mesangial immune-complex (IC) deposition; arrow head, subepithelial IC deposition. (D) The lupus nephritis activity index (range 0–24) at 6 months after TMPD injection. Each TMPD treatment, n=11; No Rx, n=6. **p<0.01. Scatter plots display the data of individual mice and median values. (E) The urine albumin-creatinine ratio at 6 months. *p<0.05. (F) Serum IgG1, IgG2a and IgG2b titers. Black columns, $Mdk^{+/+}$; white columns, $Mdk^{-/-}$. Data are shown as means (columns) ± SEM (bars). n=6. (G) Serum anti ssDNA antibody (Ab) and anti dsDNA Ab titers at 6 months.

Figure 2. Midkine deficiency prevents the infiltration of inflammatory cells into the lupus kidney.

(A) Immunohistochemical staining of $CD3^+$, $CD4^+$ and immunofluorescence staining of $CD68^+$ cells in the TMPD-induced lupus kidney (left panels, $Mdk^{+/+}$ mice; right panels, $Mdk^{-/-}$ mice). Bar, 50 μm. (B) The numbers of $CD3^+$, $CD4^+$ and $CD68^+$ cells in glomeruli: c/gcs, count per glomerular cross section. Scatter plots display the data of individual mice and median values. **p<0.01; *p<0.05. n=8-10. (C) Representative

CD69 staining profiles of gated $CD3^+$ T cells in lupus mice assessed using flow cytometry. Values indicate percentages of $CD3^+CD69^+$ cells. (D) Percentage of renal $CD3^+CD69^+$ cells in **C**. Black columns, $Mdk^{+/+}$; white columns, $Mdk^{-/-}$. Data are means ± SEM. n=6. (E) Representative CD69 staining profiles of gated $CD4^+$ T cells in lupus mice. (F) Percentages of renal $CD4^+CD69^+$ cells in **E**. (G) Percentages of renal $CD4^+CD45^+$IFN-γ^+ (T_H1) and $CD4^+CD45^+IL17^+$ (T_H17) cells in lupus mice. Renal T cell subsets were determined by the profiles of intracellular cytokine expression, as described in the Materials and Methods. n=8-10. (H) The mRNA expression of inflammation-related molecules in lupus kidneys. n=6.

Figure 3. $CD4^+$ T cells from the spleens of TMPD-induced mice

(A) Representative CD69 staining profiles of gated $CD4^+$ T cells in lupus mice. Values indicate percentages of $CD4^+CD69^+$ cells. (B) Percentages of splenic $CD4^+CD69^+$ cells in **A**. Black columns, $Mdk^{+/+}$; white columns, $Mdk^{-/-}$. Data are means ± SEM. *$p<0.05$. n=6. (C) Percentage of $CD62L^+CD44^-CD4^+$ and $CD62L^-CD44^+CD4^+$ T cells in the spleens of lupus $Mdk^{+/+}$ or $Mdk^{-/-}$ mice. **$p<0.01$. n=6-8. (D) Midkine (MK) expression in the spleen at 6 months determined by western blotting. (E) The intensities of the MK bands that were normalized to β-actin. Gray columns, $Mdk^{+/+}$ without TMPD treatment; black columns, $Mdk^{+/+}$ with TMPD treatment. n=6. (F) Percentages of splenic T_H1, T_H2, T_H17 and $CD25^+FoxP3^+$ Treg cells in $CD4^+$ T cells from mice treated with TMPD. n=6-8. (G) The number of splenic T_H1, T_H2, T_H17 and Treg cells. (H) Percentages of splenic $CD138^+$, IgD^+IgM^- and IgD^+IgM^+ cells of $B220^+$ cells in lupus splenocytes.

Figure 4. Midkine deficiency suppresses the NFAT-mediated activation of splenic T

lymphocytes treated with anti-CD3/CD28 antibodies in vitro

(A) Western blotting of midkine induction in the supernatant of cultured *Mdk*$^{+/+}$ CD4^{+} T cells stimulated by anti-CD3/CD28 antibodies (Abs). (B) Representative CD69 staining profile of gated CD4^{+} T cells in isolated *Mdk*$^{-/-}$ splenocytes with/without recombinant midkine (rhMK) after exposure to anti-CD3/CD28 Abs for 2 days. Values indicate percentages of CD4^{+}CD69^{+} cells. (C) Percentage of CD4^{+}CD69^{+} cells in isolated CD4^{+} T cells treated with/without rhMK, as described in **B**. Black columns, *Mdk*$^{+/+}$; white columns, *Mdk*$^{-/-}$; gray columns, *Mdk*$^{-/-}$ with rhMK. *p<0.05; **p<0.01. The results are means ± SEM. n=6-10 independent experiments. (D) Western blotting of nuclear NFAT1 and cytoplasmic phospho-NFATc2 expression in activated T splenocytes. (E) The intensity of nuclear NFAT1 bands was normalized as to Histone H3. n=6 independent experiments. (F) ELISA analysis of IL-2 expression in the supernatant of cultured CD4^{+} T cells after exposure to anti-CD3/CD28 Abs at 2 days. n=6 independent experiments.

Figure 5. Midkine selectively increases *in vitro* differentiation of T_H1 cells

(A) Representative CD4 and IFN-γ staining profile in isolated T cells from *Mdk*$^{+/+}$ mice, and *Mdk*$^{-/-}$ mice with/without recombinant midkine (rhMK) after exposure to anti-CD3/CD28 Abs for 3 days under T_H1 cell–polarizing condition. Values indicate percentages of CD4^{+}IFN-γ^{+} cells. (B) Percentage of CD4^{+}IFN-γ^{+} (T_H1) cells, as described in **A**. Black columns, *Mdk*$^{+/+}$; white columns, *Mdk*$^{-/-}$; gray columns, *Mdk*$^{-/-}$ with rhMK. *p<0.05. The results are means ± SEM. n=6 independent experiments. (C) Western blotting of the time course of phospho-STAT1 expression in anti-CD3/CD28 Abs-treated CD4^{+} T cells from *Mdk*$^{+/+}$ and *Mdk*$^{-/-}$ mice after their exposure to IFN-γ.

(D) Time course of phospho-STAT1 expression normalized to STAT1. Closed circles, *Mdk*$^{+/+}$; Open circles, *Mdk*$^{-/-}$. n=4 independent experiments. (E) Western blotting of the time course of phospho-STAT4 expression after their exposure to IL-12. (F) Time course of phospho-STAT4 expression. **p<0.05. n=6 independent experiments.

Figure 6. Schematic diagram showing the possible contribution of midkine to lupus nephritis

(A) Midkine-induced T cell expansion through activation of NFAT

Cn, calcineurin; IL, interleukin; NFAT, nuclear factor of activated T cells

(B) Midkine-regulated differentiation of T_H1 cells via the IL-12/STAT4 signaling in lupus nephritis

TCR, T cell receptor; APC, antigen presenting cell; STAT, signal transducers and activator of transcription; IFN, interferon

Supplemental figure legend

Supplemental figure. Midkine expression in MRL/*lpr* mice

(A) Midkine (MK) expression in the kidney of MRL/*lpr* mice at 3 months after birth, determined by western blotting. (B) The intensities of the MK bands that were normalized to β-actin. White column, control mice; black column, MRL/*lpr* mice. n=6. **p<0.01. (C) MK expression in the spleen of MRL/*lpr* mice. (D) Quantitative data were expressed as the relative ratio of MK to β-actin.

Figure 1

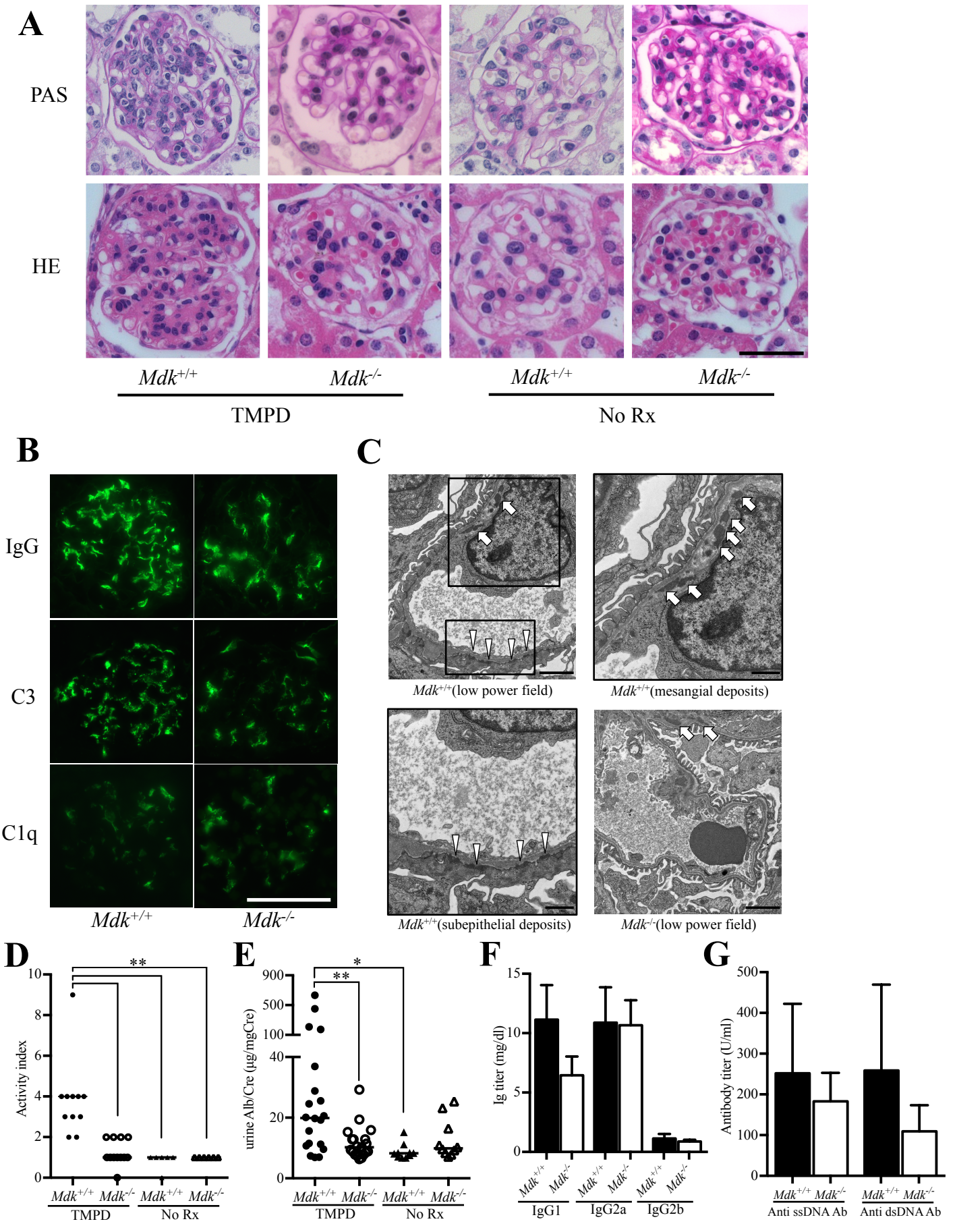

A
PAS
HE
Mdk+/+
Mdk-/-
Mdk+/+
Mdk-/-
TMPD
No Rx
B
IgG
C3
C1q
Mdk+/+
Mdk-/-
C
Mdk+/+(low power field)
Mdk+/+(mesangial deposits)
Mdk+/+(subepithelial deposits)
Mdk-/-(low power field)
D
Activity index
**
Mdk+/+ Mdk-/- Mdk+/+ Mdk-/-
TMPD
No Rx
E
urine Alb/Cre (μg/mgCre)
*
**
Mdk+/+ Mdk-/- Mdk+/+ Mdk-/-
TMPD
No Rx
F
Ig titer (mg/dl)
Mdk+/+ Mdk-/- Mdk+/+ Mdk-/- Mdk+/+ Mdk-/-
IgG1
IgG2a
IgG2b
G
Antibody titer (U/ml)
Mdk+/+ Mdk-/- Mdk+/+ Mdk-/-
Anti ssDNA Ab
Anti dsDNA Ab

Figure 2

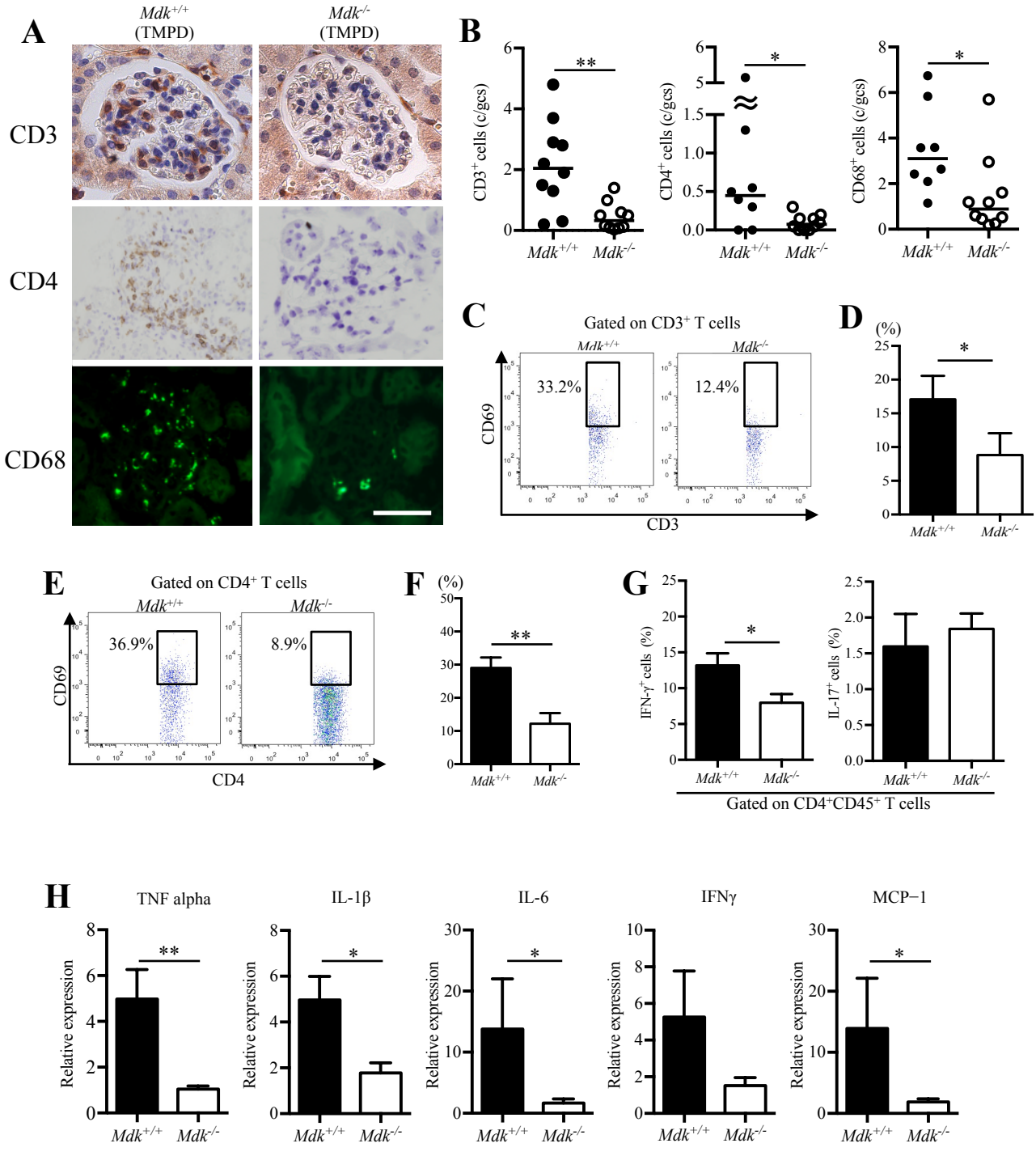

A
Mdk+/+ (TMPD)
Mdk-/- (TMPD)
CD3
CD4
CD68
B
CD3+ cells (c/gcs)
CD4+ cells (c/gcs)
CD68+ cells (c/gcs)
Mdk+/+
Mdk-/-
C
Gated on CD3+ T cells
33.2%
12.4%
CD69
CD3
D
(%)
E
Gated on CD4+ T cells
36.9%
8.9%
CD4
F
G
IFN-γ+ cells (%)
IL-17+ cells (%)
Gated on CD4+CD45+ T cells
H
TNF alpha
IL-1β
IL-6
IFNγ
MCP−1
Relative expression

Figure 3

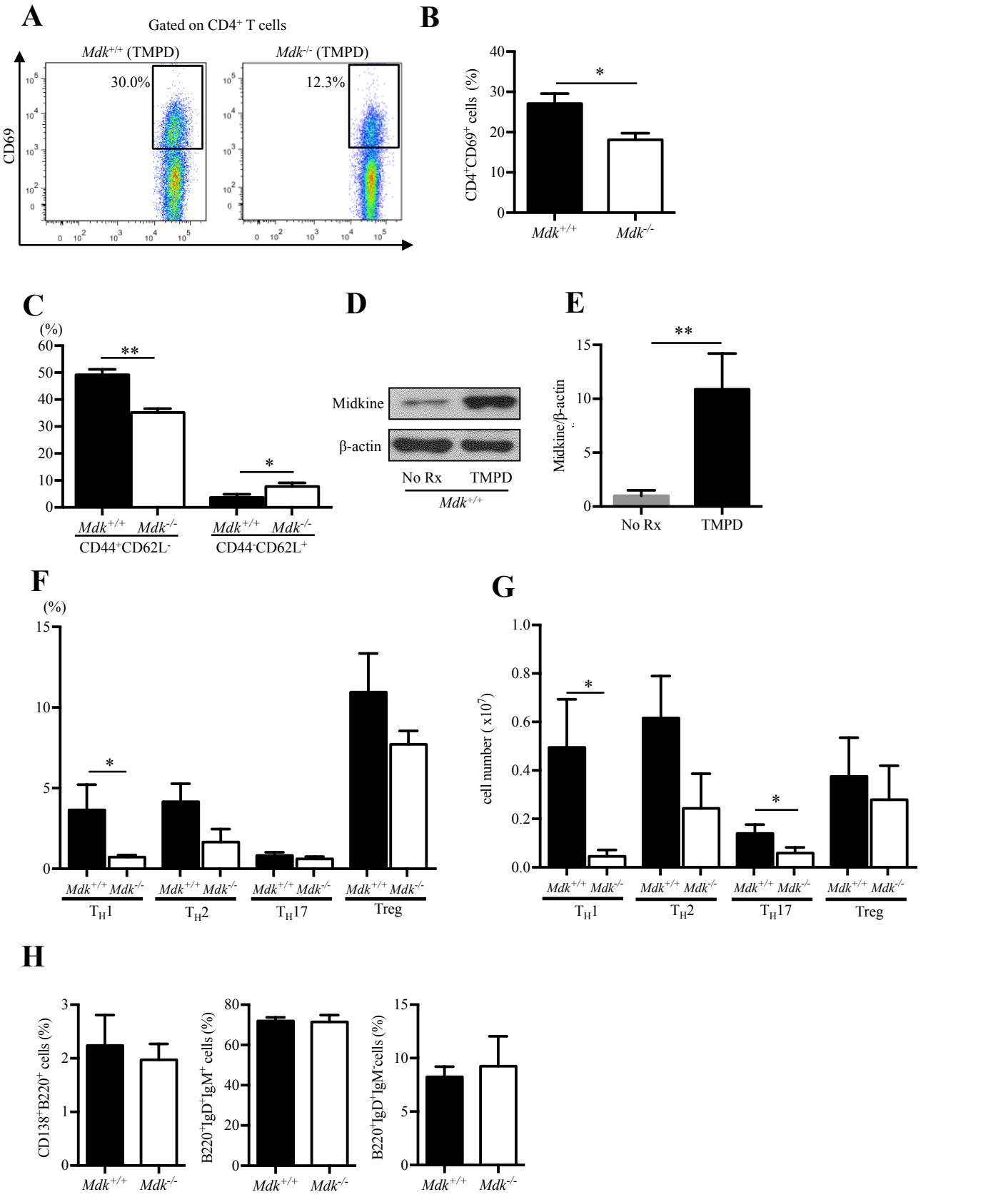

A
Gated on CD4+ T cells
Mdk+/+ (TMPD)
Mdk-/- (TMPD)
30.0%
12.3%
CD69
B
CD4+CD69+ cells (%)
Mdk+/+
Mdk-/-
C
(%)
Mdk+/+ Mdk-/-
CD44+CD62L-
Mdk+/+ Mdk-/-
CD44-CD62L+
D
Midkine
β-actin
No Rx
TMPD
Mdk+/+
E
Midkine/β-actin
No Rx
TMPD
F
(%)
Mdk+/+ Mdk-/-
TH1
TH2
TH17
Treg
G
cell number (x10^7)
TH1
TH2
TH17
Treg
H
CD138+B220+ cells (%)
B220+IgD+IgM+ cells (%)
B220+IgD+IgM cells (%)
Mdk+/+
Mdk-/-

Figure 4

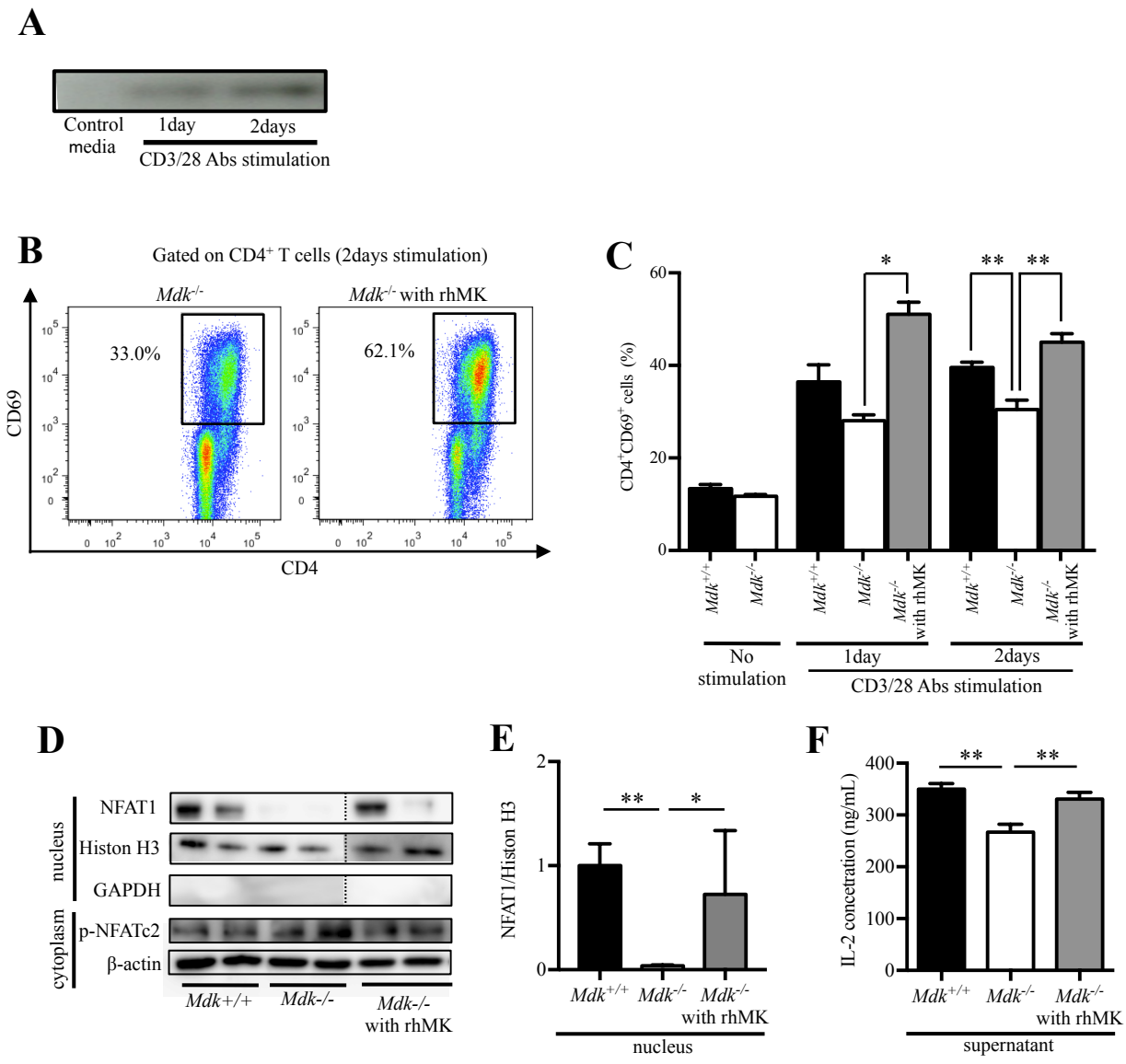

A
Control media
1day
2days
CD3/28 Abs stimulation
B
Gated on CD4+ T cells (2days stimulation)
Mdk-/-
Mdk-/- with rhMK
33.0%
62.1%
CD69
CD4
C
CD4+CD69+ cells (%)
No stimulation
1day
2days
CD3/28 Abs stimulation
D
nucleus
cytoplasm
NFAT1
Histon H3
GAPDH
p-NFATc2
β-actin
Mdk+/+
Mdk-/-
Mdk-/- with rhMK
E
NFAT1/Histon H3
nucleus
F
IL-2 concetration (ng/mL)
supernatant

Figure 5

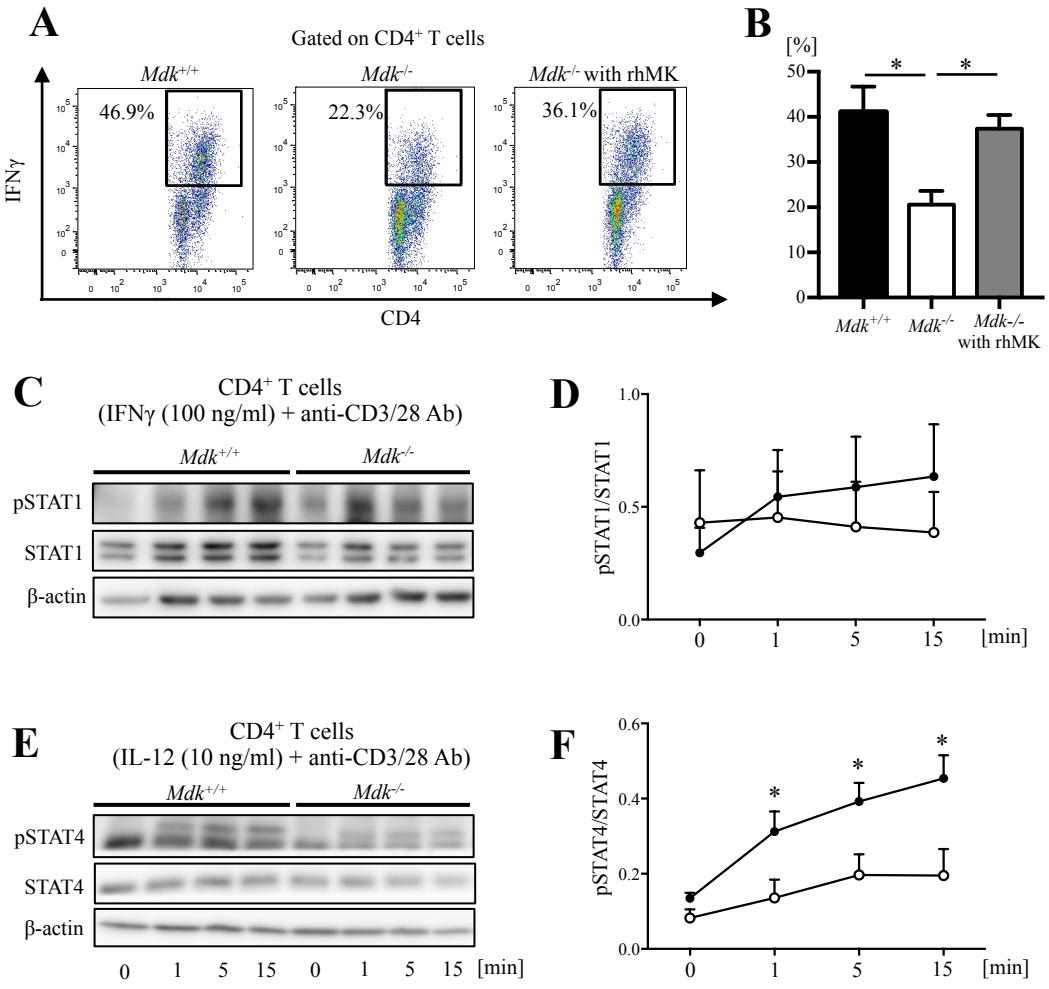
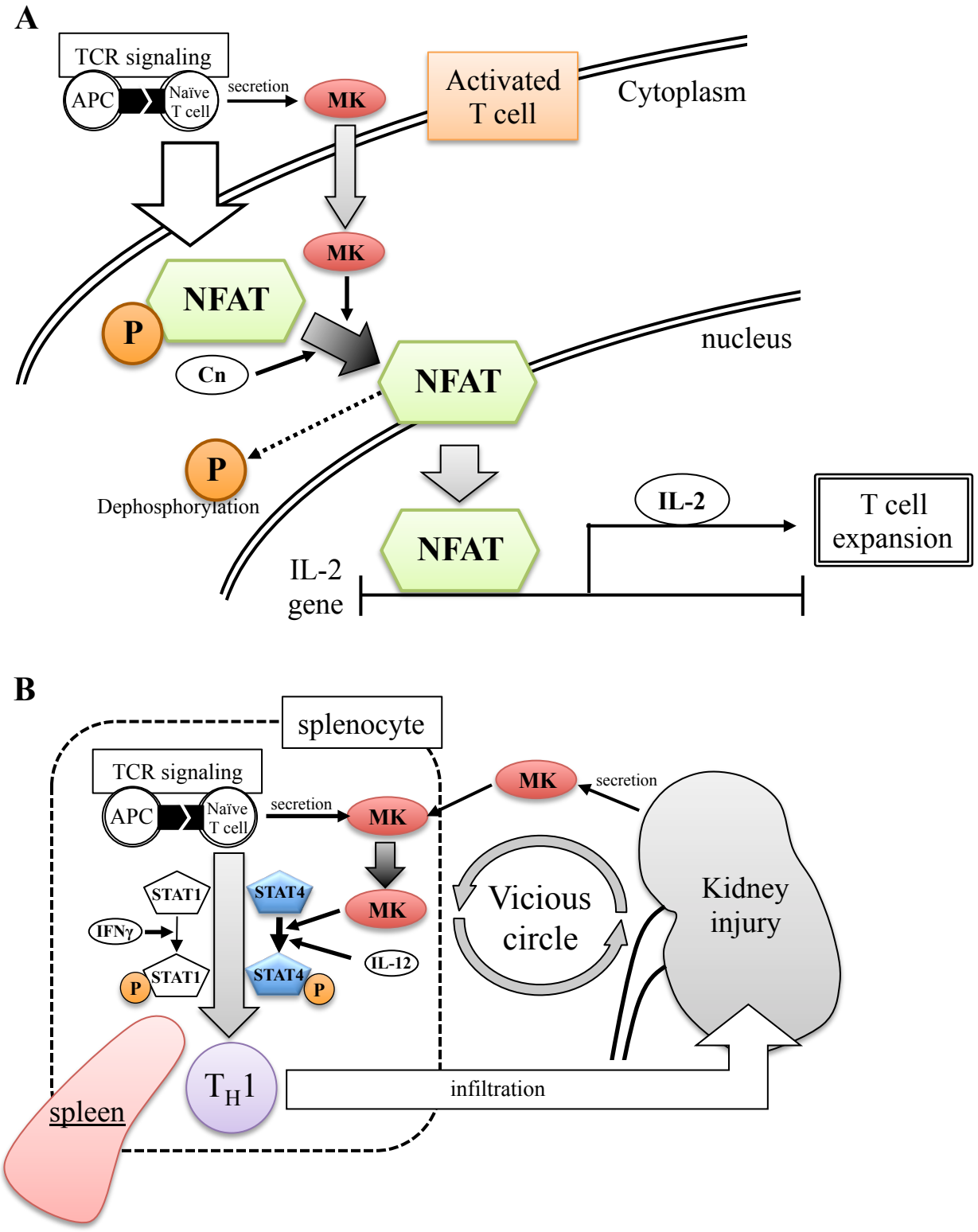
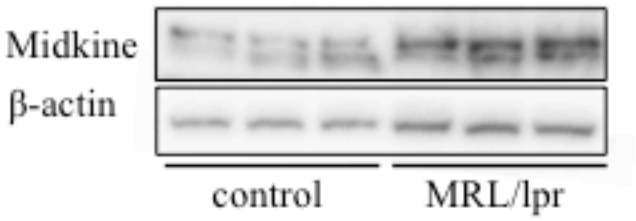

A
Gated on CD4+ T cells
Mdk+/+
Mdk-/-
Mdk-/- with rhMK
46.9%
22.3%
36.1%
IFNγ
CD4
B
[%]
50
40
30
20
10
0
*
*
Mdk+/+
Mdk-/-
Mdk-/- with rhMK
C
CD4+ T cells
(IFNγ (100 ng/ml) + anti-CD3/28 Ab)
Mdk+/+
Mdk-/-
pSTAT1
STAT1
β-actin
D
pSTAT1/STAT1
1.0
0.5
0.0
0
1
5
15
[min]
E
CD4+ T cells
(IL-12 (10 ng/ml) + anti-CD3/28 Ab)
Mdk+/+
Mdk-/-
pSTAT4
STAT4
β-actin
0
1
5
15
0
1
5
15
[min]
F
pSTAT4/STAT4
0.6
0.4
0.2
0.0
*
*
*
0
1
5
15
[min]

Figure 6


A
TCR signaling
APC
Naïve T cell
secretion
MK
Activated T cell
Cytoplasm
MK
NFAT
P
Cn
NFAT
nucleus
P
Dephosphorylation
NFAT
IL-2
T cell expansion
IL-2 gene
B
splenocyte
TCR signaling
APC
Naïve T cell
secretion
MK
MK
secretion
STAT1
STAT4
MK
IFNγ
IL-12
P
STAT1
STAT4
P
Vicious circle
Kidney injury
spleen
TH1
infiltration

Supplemental figure

A



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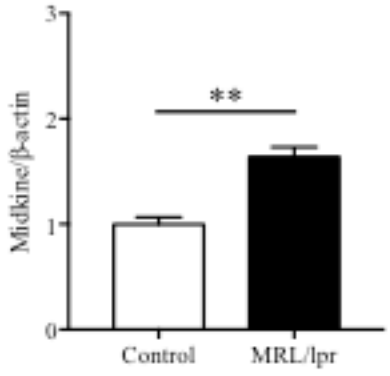

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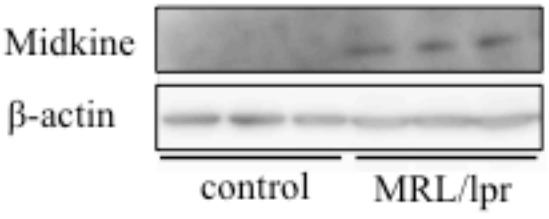

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