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MKP-1 Promotes Anti-inflammatory M(IL-4/IL-13) Macrophage Phenotype and Mediates the Anti-inflammatory Effects of Glucocorticoids

Running title: MKP-1, macrophage polarization and glucocorticoids

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Abstract

Macrophage polarization refers to the ability of these cells to adopt different functional phenotypes according to their environment. Mitogen-activated protein kinase phosphatase-1 (MKP-1) is known to regulate the classical lipopolysaccharide (LPS) -induced proinflammatory macrophage activation and the inflammatory response. Here we investigated the effects of MKP-1 on the anti-inflammatory and healing-promoting macrophage phenotype induced by cytokines IL-4 and IL-13 and examined the potential mediator role of MKP-1 in glucocorticoid effects on the two macrophage phenotypes.

In MKP-1-deficient macrophages treated with IL-4 and IL-13 to induce the anti-inflammatory phenotype, the expression of phenotypic markers arginase 1, Ym-1 and FGF2 was reduced as compared to wild-type cells. In contrast, LPS-induced expression of the proinflammatory factors IL-6 and iNOS was significantly higher in MKP-1 deficient macrophages. Dexamethasone suppressed the proinflammatory phenotype and enhanced the anti-inflammatory phenotype. Interestingly, both of these glucocorticoid effects were attenuated in macrophages from MKP-1 deficient mice. Accordingly, dexamethasone increased MKP-1 expression in both LPS- and IL4+13-treated wild-type cells.

In conclusion, the findings support MKP-1 as an endogenous mechanism able to shift macrophage activation from the classical proinflammatory state towards the anti-inflammatory and healing-promoting phenotype. In addition, MKP-1 was found to mediate the anti-inflammatory effects of dexamethasone in a dualistic manner: by suppressing the proinflammatory macrophage activation and by enhancing the healing-promoting macrophage phenotype.

Introduction

Macrophages are important immune effector cells responsible for several physiological functions related to homeostasis and immunity [1]. They clear cellular debris and participate in recycling of nutrients (e.g. iron) [2,3]. In infections, macrophages phagocytize pathogens and present antigens to lymphocytes [4]. They also regulate inflammation by secreting pro-inflammatory and anti-inflammatory cytokines and other soluble factors. It is nowadays accepted that the macrophages performing different tasks actually display distinct phenotypes [5,6], a phenomenon known as macrophage polarization.

Macrophage phenotypes were initially modeled after the Th1/Th2 paradigm for T lymphocytes. This scheme has "classically activated" or M1 macrophages, induced by microbial products and proinflammatory stimuli, on one end. On the other end are macrophages that do not promote immune responses but support resolution of inflammation and tissue healing. These cells are collectively called "alternatively activated" or M2 macrophages and are often subdivided according to the phenotype-inducing stimulus and / or supposed function related to the phenotype. More recently, it has been increasingly recognized that grouping such diverse cells into two predetermined categories is not optimal; the characteristic set of features caused by each "polarizing" factor have been recommended to be considered as a distinct phenotype, especially concerning alternatively activated macrophages [7].

The macrophage phenotype induced by activators of toll-like receptor 4 (TLR4), such as bacterial lipopolysaccharide (LPS), along with that induced by the proinflammatory cytokine interferon gamma (INFγ), is a well-characterized form of "classical macrophage activation". These cells promote inflammation and effectively destroy intracellular pathogens in a setting of infection [8]. They express inducible nitric oxide synthase (iNOS), which converts L-arginine into L-citrulline

simultaneously releasing nitric oxide (NO), a potent vasodilator and cytotoxic effector molecule. Other characteristic inflammatory factors produced by classically activated macrophages include interleukin 6 (IL-6) and tumor necrosis factor (TNF) [9]. While these cells play a critical role in controlling infection, excessive or dysregulated classical activation has been implicated in the development of autoimmune and degenerative diseases [2] as well as atherosclerosis [10], obesity and insulin resistance [11].

A relatively well-established example of "alternative macrophage activation" is the phenotype induced by the major Th2 cytokines IL-4 and IL-13. These IL-4 / IL-13 -activated macrophages are associated with phagocytosis of tissue debris, as well as with the resolution of inflammation and wound healing [12], and are characterized by upregulation of factors involved in fibroblast activation and extracellular matrix synthesis, such as Ym-1, arginase 1 and fibroblast growth factor 2 (FGF2) [13,14]. While proinflammatory macrophages produce NO from arginine, macrophages activated by IL-4 and IL-13 express arginase 1 at high levels. This enzyme converts L-arginine into L-ornithine, which in turn acts as a precursor of many extracellular matrix components supporting wound healing [15,16]. Macrophage activation by IL-4 and IL-13 is critical in the resolution of inflammation and wound healing, but excessive and / or dysregulated forms of it have been linked to aberrant fibroblast activation and pathogenesis of fibrotic diseases. Accordingly, macrophages displaying an alternative phenotype stimulated by IL-4 and IL-13 have been detected in fibrotic lungs [17], kidneys [18] and liver [15].

Mitogen activated protein kinase phosphatases (MKPs), also known as dual specificity phosphatases (DUSPs), are enzymes that dephosphorylate mitogen activated protein kinases (MAKPs) and thus inactivate signaling cascades mediated through these pathways [19–21]. MAPKs regulate the activity of several enzymes and transcription factors involved in inflammatory responses [22,23]. MKP-1, which preferentially dephosphorylates p38 and JNK, is the best known MAPK phosphatase

[20,21,24]. MKP-1 expression is upregulated in response to many extracellular signals and cellular stress, such as LPS, cytokines and heat shock [23,25]. MKP-1 deficiency has been associated with an enhanced inflammatory response, and increased expression of proinflammatory genes has been observed both in macrophages isolated from MKP-1 knockout mice and in cells treated with MKP-1 targeting siRNAs [26–29]. Accordingly, MKP-1 deficient mice develop more severe disease in models of septic shock or collagen-induced arthritis [30,31]. Intriguingly, MKP-1 expression has been shown to be increased by certain anti-inflammatory drugs, i.e. glucocorticoids [32–34], the anti-rheumatic drug aurothiomalate [35] and the PDE4 inhibitor rolipram [36], and this property is thought to mediate many of their anti-inflammatory effects.

In contrast to the relatively well-established role of MKP-1 in classical macrophage activation, its effects on alternative macrophage activation remain mostly unexplored. The aim of the present study was to investigate the regulatory role of MKP-1 in IL-4 and IL-13 -induced alternative macrophage activation in comparison to its effects on TLR4 (LPS) -induced classical macrophage activation, and to evaluate if MKP-1 has a role in mediating the anti-inflammatory effects of the glucocorticoid dexamethasone in macrophages. In the nomenclature we adhere to the recent consensus paper [7] and refer to TLR4 activator -induced classically activated proinflammatory macrophage phenotype as M(LPS) cells and to IL-4 and IL-13 -induced alternatively activated anti-inflammatory and healing-promoting phenotype as M(IL-4/IL-13) cells.

Materials and methods

Cell cultures and macrophage polarization

Murine J774 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were cultured in 24-well plates for RNA extraction, ELISA measurements, and preparation of whole-cell lysates for Western blotting. Confluent cultures were exposed to fresh culture medium containing the compounds of interest.

Isolation and culturing of mouse peritoneal macrophages were carried out as described earlier [36]. In brief, male C57BL/6 MKP-1(–/–) mice (originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute [37]) were bred in the University of Tampere School of Medicine animal facilities. These mice, along with their wild-type (WT) controls, were housed under standard conditions of light (12-12 h light–dark cycle), temperature (22 ± 1°C) and humidity (50–60%). Food and water were provided *ad libitum*. In each experiment, cells from six WT and six knockout (KO) mice were pooled to give n=4.

In collection of peritoneal macrophages, the experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU), and the study was approved by The National Animal Experiment Board. Mice were sacrificed by suffocation with CO_2, followed by an immediate cervical dislocation. Primary peritoneal macrophages were obtained by intraperitoneal lavage with sterile phosphate-buffered saline (PBS) supplemented with

0.2 mM EDTA. Cells were washed, resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, and seeded on 24-well plates. The cells were incubated overnight and washed with medium to remove non-adherent cells before the experiments.

In order to study the type of TLR4 activation -induced classical macrophage activation, the cells were incubated with 10 ng/ml (J774) or 100 ng/ml (peritoneal macrophages) of a known activator of TLR4, bacterial LPS (Escherichia coli, 0111:B4, product number E-4391, Sigma) for 24 h (J774) or 4 h (peritoneal macrophages). This classical phenotype is henceforth called M(LPS). In experiments examining alternative activation, a combination of 10 ng/ml of IL-4 and IL-13 (both from R&D Systems, Minneapolis, MN, USA) was used to induce an alternative phenotype henceforth called M(IL-4/IL-13). When relevant, 1 μM dexamethasone was used in addition to LPS or IL-4 and IL-13. The concentrations of the chemical stimuli (LPS and IL-4 + IL-13) were chosen following our previous studies and literature, and the time points used in the experiments were selected based on time series in J774 murine macrophage studies (see Figs. 1 and 6) and on our preliminary experiments with peritoneal macrophages. The dexamethasone concentrations used in the experiments were chosen based on our previously published dose-response data [38].

Cell viability after treatment with the investigated compounds was excluded by modified XTT test (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Preparation of protein extracts

At predetermined time points, the culture medium was carefully removed and stored at -20°C for later analysis. The cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in cold lysis buffer containing 10 mM Tris base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 minutes, lysates were centrifuged (12,000 g, 4°C, 10 min), supernatants were collected and mixed 3:1 with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analyzed. An aliquot of the supernatant was used to determine protein concentration using the Coomassie blue method [39].

Western blotting

Prior to Western blotting, samples were boiled for 10 min and 20 μg of protein was loaded per lane on 10% or 12% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

After the transfer, the membrane was blocked with TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk (arginase 1 and β-actin) or 5% bovine serum albumin (MKP-1) at room temperature for one hour and incubated with the primary antibody in the blocking solution for one hour at room temperature (arginase 1) or at 4°C overnight (MKP-1 and β-actin). The membrane was washed with TBS/T, incubated with the secondary antibody in the blocking solution at room temperature for one hour and washed. Bound antibody was detected using

SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and ImageQuant LAS 4000 mini imaging system (GE Healthcare). The chemiluminescent signal was quantified with ImageQuant TL 7.0 image analysis software.

Rabbit polyclonal arginase 1, MKP-1 and β-actin (loading control) antibodies (primary antibodies), and goat HRP-conjugated anti-rabbit polyclonal antibody (secondary antibody) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

ELISA

The concentration of IL-6 in culture media was determined by ELISA, using reagents from R&D Systems Europe Ltd., Abingdon, UK (Catalog DY406), adhering to the protocol detailed by the manufacturer. The detection limit was 0.3 pg/ml. All samples were assayed in duplicate.

Nitrite measurement with Griess method

Nitric oxide (NO) production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in the aqueous environment, into the culture medium by the Griess reaction [40]. All samples were assayed in duplicate.

RNA isolation and quantitative RT-PCR

At predetermined time points, culture medium was removed, and total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.). Total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers

(Applied Biosystems, Foster City, CA, USA). cDNA was diluted 1:20 with RNase-free water and subjected to quantitative RT-PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes for murine IL-6, iNOS, MKP-1, arginase 1, Ym-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. The mRNA levels of basic fibroblast growth factor (FGF2) were determined by using TaqMan® Gene Expression Assay Mm00433287_m1 (Applied Biosystems). The PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Each sample was assayed in duplicate. The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2. For the FGF2 Gene Expression Assay, the relative mRNA levels were determined with the ΔΔCT method.

Statistics

Results are expressed as mean + standard error of mean (SEM). When indicated, statistical significance was calculated by ANOVA followed by Dunnett's or Bonferroni's multiple comparisons test where appropriate. Differences were considered significant at $p < 0.05$. For determining area under curve, trapezoidal integration was used. The statistical software GraphPad InStat3 was used for the analyses. The study was conducted in accordance with the BCPT policy for experimental and clinical studies [41].

Results

Markers of classical M(LPS) phenotype are increased while those of alternative M(IL-4/IL-13) phenotype are decreased in macrophages from MKP-1 deficient mice

The levels of interleukin 6 (IL-6) and inducible nitric oxide synthase (iNOS) expressions were investigated as typical markers of classical M(LPS) activation in J774 macrophages. LPS significantly increased the expression of both of these inflammatory factors, with mRNA levels peaking at 4-6 h and returning to basal levels at 24 h following exposure to LPS (Figures 1A-B). Accordingly, the levels of arginase 1 and Ym-1 were measured as typical markers of alternative M(IL-4/IL-13) activation. The combination of IL-4 and IL-13 increased the expression of arginase 1 and Ym-1 but the effect was rather slow in onset and mRNA levels continued to increase up to 12-24 h following IL-4/IL-13 stimulation. (Figures 1C-D).

To investigate the role of MKP-1 in classical M(LPS) and alternative M(IL-4/IL-13) macrophage activation, peritoneal macrophages were isolated from MKP-1 deficient and wild-type mice. After stimulation with LPS, IL-6 expression was 71% and iNOS expression 136% higher in macrophages from MKP-1 deficient mice compared to cells from wild-type mice (Figure 2A-B). In contrast, when the cells were stimulated with the combination of IL-4 and IL-13 to induce the alternative phenotype, the expression of arginase 1, Ym-1 and FGF2 was significantly lower (by 42%, 86% and 37%, respectively) in the macrophages from MKP-1 deficient mice than in those from wild-type animals (Figure 2C-E). These data suggest that MKP-1 is an endogenous factor regulating macrophage polarization from a proinflammatory phenotype towards an anti-inflammatory and tissue healing - promoting phenotype.

The effects of dexamethasone on M(LPS) and M(IL-4/IL-13) markers are attenuated in macrophages from MKP-1 deficient mice

Glucocorticoids are known to attenuate classical proinflammatory M(LPS) and to promote alternative inflammation-resolving M(IL-4/IL-13) macrophage activation, as also demonstrated here in J774 macrophages. When dexamethasone was added into the culture, it markedly inhibited IL-6 and iNOS mRNA expression (by 69% and 63%, respectively) and IL-6 and nitric oxide production (by 89% and 95%, respectively) in LPS-treated J774 macrophages (Figure 3). In contrast, dexamethasone increased the expression of arginase 1 (mRNA by 85%, protein by 71%) and Ym-1 (by 1630%) in macrophages exposed to the combination of IL-4 and IL-13 (Figure 4).

To study the role of MKP-1 in those glucocorticoid effects, we investigated the effects of dexamethasone in peritoneal macrophages from MKP-1 deficient and wild-type mice. As shown in Figures 5A and 5B, dexamethasone downregulated IL-6 and iNOS expression in both wild-type and MKP-1 deficient peritoneal macrophages exposed to LPS, but the effect in macrophages from MKP-1 deficient mice was attenuated in a statistically significant manner. In contrast, dexamethasone enhanced the expression of the M(IL-4/IL-13) markers arginase 1, Ym-1 and FGF2 in peritoneal macrophages from wild-type animals. Intriguingly, the effect of dexamethasone on the M(IL-4/IL-13) markers was significantly attenuated in macrophages from MKP-1 deficient mice (Figures 5C-E). These data suggest that MKP-1 mediates the anti-inflammatory effects of glucocorticoids via two mechanisms: by suppressing proinflammatory macrophage activation and promoting anti-inflammatory macrophage phenotype.

Dexamethasone enhances MKP-1 expression in murine macrophages

To support the role of MKP-1 in the observed glucocorticoid effects, we studied the effects of dexamethasone on MKP-1 expression. In the resting state, peritoneal macrophages from wild-type mice expressed MKP-1 at low levels. When dexamethasone was introduced to the culture, it increased MKP-1 expression up to five-fold. The MKP-1 increasing effect of dexamethasone was duplicated when it was added in combination with LPS or IL-4 and IL-13. (Figure 6A)

Similar effects were observed in J774 cells (Figures 6B-D). The time-course of MKP-1 expression in J774 macrophages (Figure 6B) showed a rapid increase which peaked at 1 h and was followed by another peak at 4 h possibly induced by pro-inflammatory cytokines trigged by LPS stimulation or feedback loops between MKP-1 and p38 kinase [42,43]. Dexamethasone enhanced MKP-1 expression in unstimulated J774 cells as well as in those exposed to LPS or IL-4 + IL-13, the combination of LPS and dexamethasone being able to induce the highest increase. The effects seen at mRNA level were also translated to protein levels (Figure 6D).

Discussion

In the present study, we show that both classical M(LPS) and alternative M(IL-4/IL-13) macrophage activation are significantly altered in cells from MKP-1 deficient mice. Opposite directions in the changes were observed: M(IL-4/IL-13) activation was attenuated whereas M(LPS) activation was augmented in macrophages from MKP-1 deficient animals. These findings display a central role of MKP-1 in the regulation of macrophage polarization towards the M(IL-4/IL-13) phenotype, which is critical in the resolution of inflammation and wound healing. More interestingly, the anti-inflammatory steroid dexamethasone was found to suppress M(LPS) activation and to enhance M(IL-4/IL-13) activation, and both of these effects were attenuated in cells from MKP-1 deficient animals. These findings together imply that MKP-1 is a significant mechanism in limiting inflammation and promoting tissue healing, and as such, can be considered a promising anti-inflammatory drug target.

The effects of MKP-1 on proinflammatory states, corresponding to M(LPS) activation, have been studied previously [21–23]. MKP-1 has been shown to be upregulated, possibly as a regulatory feedback mechanism, by pro-inflammatory stimuli such as IL-1 and bacterial LPS [26–29], and this was also confirmed in the present study. In addition, we found that the expression levels of M(LPS) markers iNOS and IL-6 were increased in macrophages from MKP-1 deficient mice as compared to cells from wild-type animals. These findings are supported by previous studies [28–30]. In animal models, MKP-1 deficiency has been shown to increase lethality in experimentally induced sepsis and endotoxic shock [30,41], as well as to cause increased inflammation and osteolysis in experimental arthritis and periodontitis [31,42]. Together these findings support the view that MKP-1 is a part of an endogenous feedback loop to control and limit inflammation mediated by proinflammatory classically activated macrophages [21,26].

In contrast to the well-established suppressive effects of MKP-1 on M(LPS) activation, very little is known about its possible effects on M(IL-4/IL-13) and other alternatively activated macrophage phenotypes. In connection to their studies on MKP-1 and macrophages in atherosclerosis, Kim and coworkers recently reported that MKP-1 deficiency suppressed IL-4-induced conversion of macrophages towards the M2 phenotype [43]. Our results corroborate these findings and extend them by showing that the enhancing effect of dexamethasone on M(IL-4/IL-13) activation is also mediated by MKP-1. Taken together, in primary peritoneal macrophages from MKP-1-deficient animals, the expression of the markers of inflammation-resolving M(IL-4/IL-13) phenotype in response to the respective stimuli was significantly attenuated, along with the increased expression of markers of the proinflammatory M(LPS) phenotype in response to the respective factor. These findings are plausible evidence that MKP-1 regulates the balance in functional macrophage phenotypes and indicate that MKP-1 shifts macrophage polarization towards an inflammation-alleviating phenotype. MKP-1 has also been shown to regulate the metabolic aspects of macrophage phenotype [43], factors that the present study was unable to address. Investigating these factors further is a promising avenue of future research.

The alternative macrophage phenotype triggers resolution of inflammation and promotes wound healing [44–46]. Thus, one could speculate that defective activation of healing processes may be involved in the more severe inflammatory responses found in MKP-1 deficient animals in experimental models such as arthritis, inflammatory bone loss and sepsis [31,41,47]. The present results are also supported by the findings that muscle healing is impaired in MKP-1-deficient animals [48], and that MKP-1 is expressed at high levels in healing skin wounds [49]. This implies that during infection / inflammation, MKP-1 may serve as a factor maintaining balance between the acute inflammatory response and later wound healing / tissue repair processes.

MKP-1 expression is increased by glucocorticoids [33,50], which was also seen in the present study, and MKP-1 has been shown to mediate some of the anti-inflammatory effects of these compounds in macrophages and other cells exposed to proinflammatory stimuli [32,34]. In the present study, LPS-induced IL-6 and iNOS expression was downregulated by dexamethasone in peritoneal macrophages from wild-type animals, but this effect was impaired in macrophages from MKP-1 deficient mice, and these results are in line with the published data [29,51]. Dexamethasone was also found to increase the levels of markers of inflammation-resolving M(IL-4/IL-13) phenotype as shown previously [52,53]. Notably, the expression of M(IL-4/IL-13) markers induced by dexamethasone was impaired in macrophages from MKP-1 deficient mice as compared to those from wild-type animals. These data suggest that the dexamethasone-induced healing-promoting M(IL-4/IL-13) macrophage activation is at least partly mediated by MKP-1, which is a novel finding. Together these results suggest that MKP-1 is a central endogenous mediator of the anti-inflammatory effects of glucocorticoids, both by suppressing proinflammatory macrophage phenotypes and by shifting macrophage activation towards anti-inflammatory and healing-promoting phenotypes. These data support the importance of MKP-1 as an anti-inflammatory drug target, together with the earlier findings showing that the effects of compounds such as the anti-rheumatic drug aurothiomalate, PDE4 inhibitor rolipram, β_2 receptor agonist salbutamol and vitamin D are at least partly mediated by MKP-1 [35,36,54,55].

In conclusion, we found that the expression of M(LPS) markers (iNOS and IL-6) was suppressed by MKP-1, while the expression of M(IL-4/IL-13) markers (arginase 1, Ym-1 and FGF2) induced by the respective cytokines was enhanced by MKP-1. This suggests that MKP-1 limits the magnitude of the acute inflammatory response and stimulates the onset of tissue repair processes following inflammation and tissue injury. Furthermore, MKP-1 seems to be an important factor mediating the anti-inflammatory actions of glucocorticoids via two mechanisms: by suppressing proinflammatory

macrophage activation and by promoting the anti-inflammatory macrophage phenotypes that mediate healing processes.

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Figures

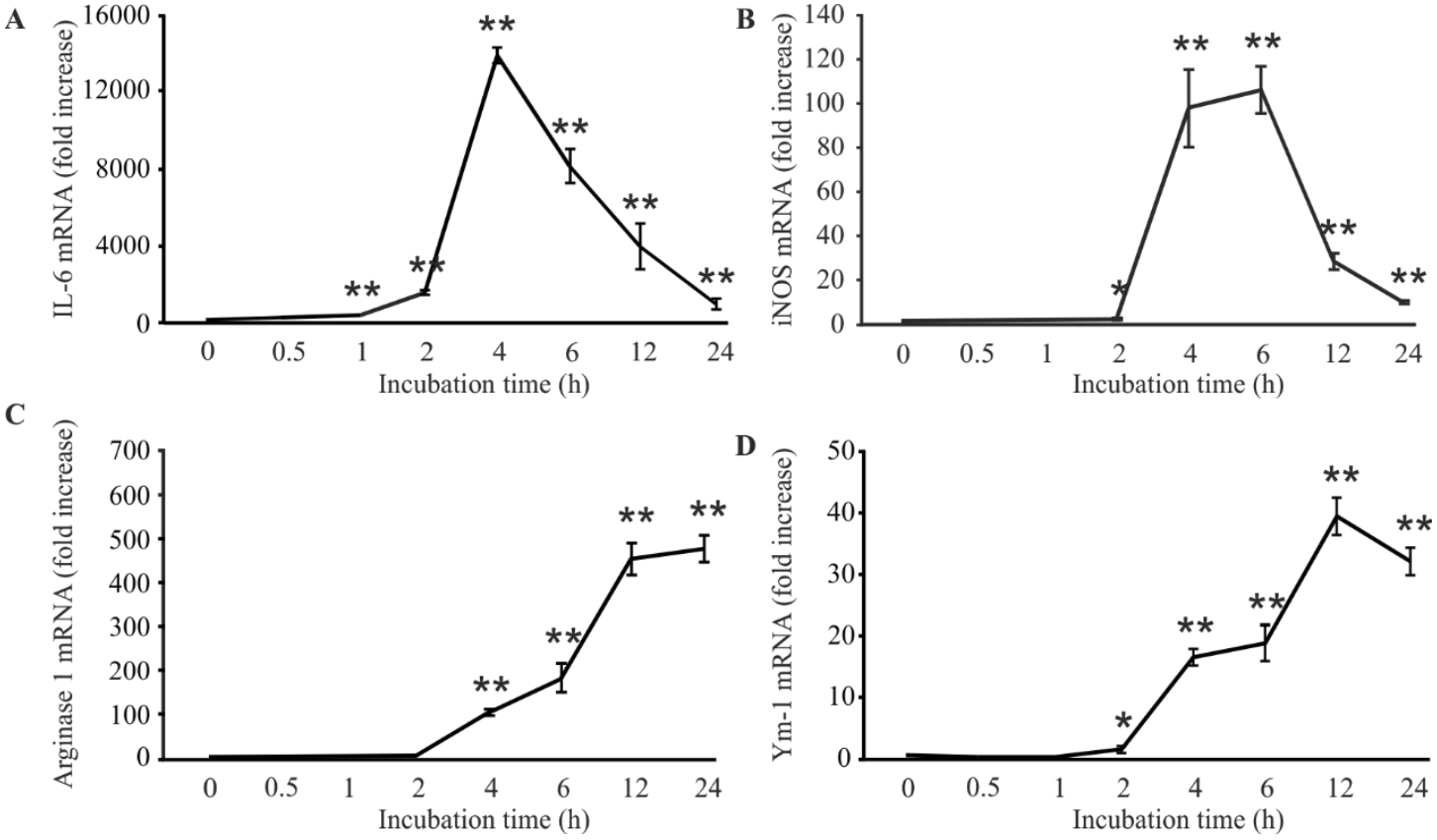

Figure 1: LPS and combination of IL-4 and IL-13 enhanced the expression of markers of the classical M(LPS) and alternative M(IL-4/IL-13) macrophage phenotypes. J774 macrophages were cultured with LPS (10 ng/ml) (A and B) or a combination of IL-4 and IL-13 (both 10 ng/ml) (C-D) for 0.5 – 24 h to induce the respective phenotypes. mRNA levels of the M(LPS) markers IL-6 (A) and inducible nitric oxide synthase (iNOS) (B), as well as M(IL-4/IL-13) markers arginase 1 (C) and Ym-1 (D) were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. The values were compared to the untreated control (0 h) which was set as 1. The results are expressed as mean ± SEM, n = 4. **: $p < 0.01$ and *: $p < 0.05$, compared to the 0 h time point.

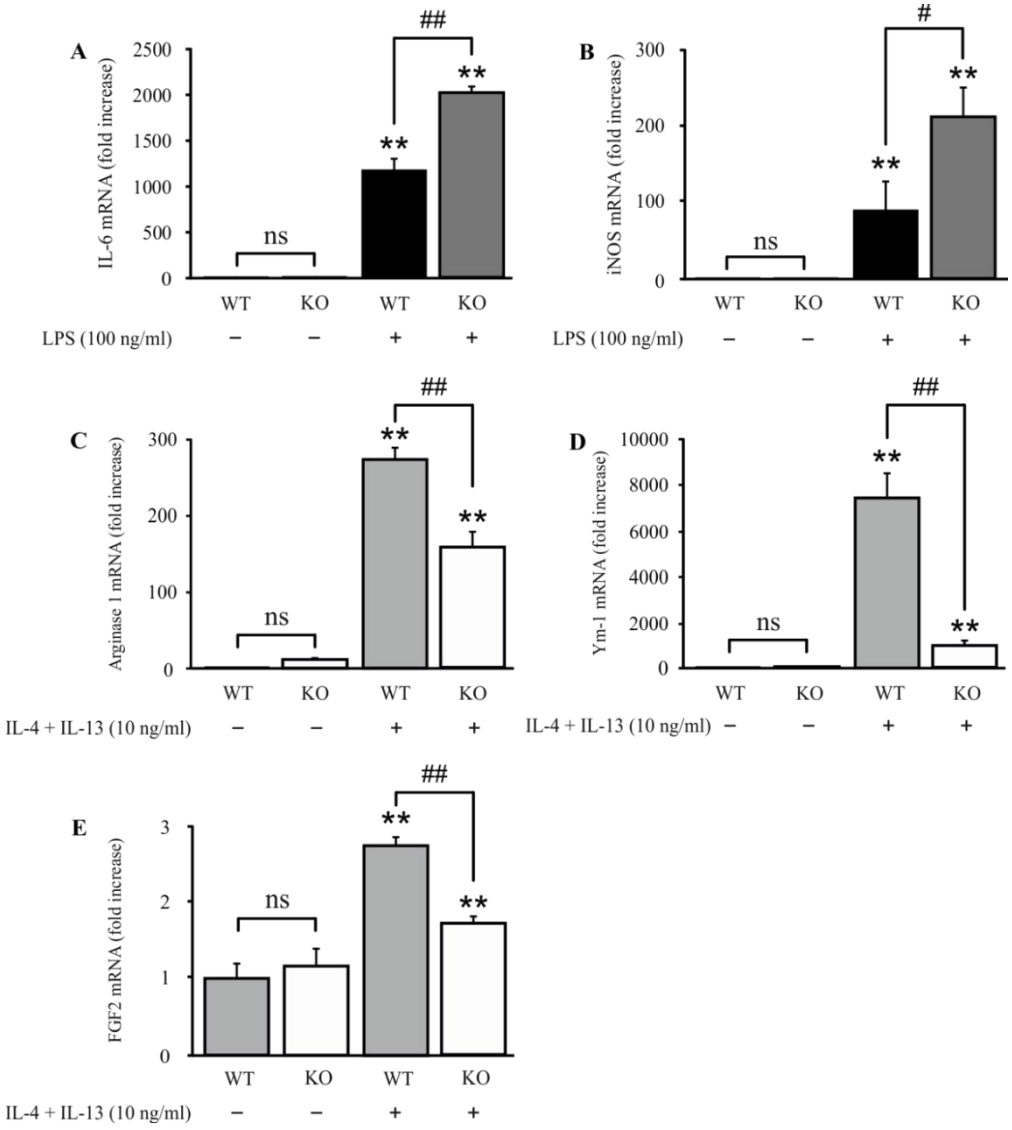

Figure 2. MKP-1 deficiency increased the expression of markers of classical M(LPS) phenotype and decreased the expression of markers of alternative M(IL-4/IL-13) phenotype in murine macrophages. Peritoneal macrophages from wild-type (WT) and MKP-1 deficient (knock-out, KO) mice were cultured with either LPS (100 ng/ml) for 4 h (A-B), or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 h (C-E). mRNA levels of the M(LPS) markers IL-6 (A) and inducible nitric oxide synthase (iNOS) (B), and M(IL-4/IL-13) markers arginase 1 (C), Ym-1 (D) and fibroblast growth factor 2 (FGF2) (E) were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. All values were compared to the WT control, which was set as 1. The results are expressed as mean + SEM, cells from six WT and six KO mice were pooled to give n = 4. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. The experiments were repeated three times with similar results.

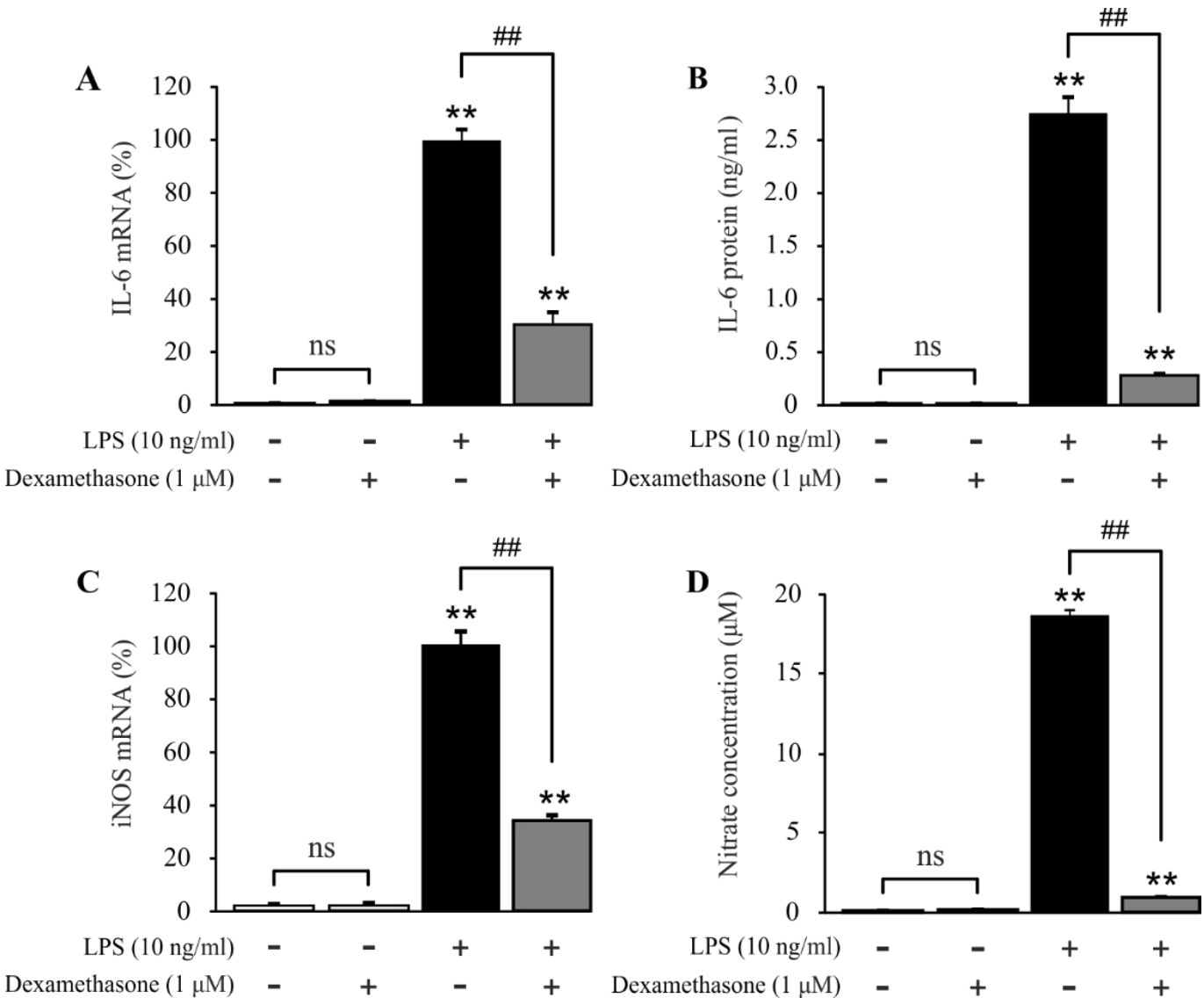

Figure 3: Dexamethasone decreased the expression of markers of proinflammatory M(LPS) activation in J774 macrophages. J774 cells were cultured with LPS (10 ng/ml) for 4 hours (A and C) or for 24 h (B and D), with or without dexamethasone (1 μM). IL-6 (A) and inducible nitric oxide (iNOS) (C) mRNA levels were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. The results were compared against cells treated with LPS alone, which was set as 100 %. IL-6 protein levels were determined with ELISA (B), and NO production was assessed by measuring the concentration of its stable metabolite nitrite by Griess method (D). The results are expressed as mean + SEM, n = 4. In B, LPS alone raised the IL-6 concentration to 2.8±0.2 ng/ml, and addition of dexamethasone reduced it to 0.3±0.01 ng/ml. In D, LPS alone raised nitrate concentration to 18.6±0.04 μM, and dexamethasone reduced it to 0.9±0.03 μM. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample.

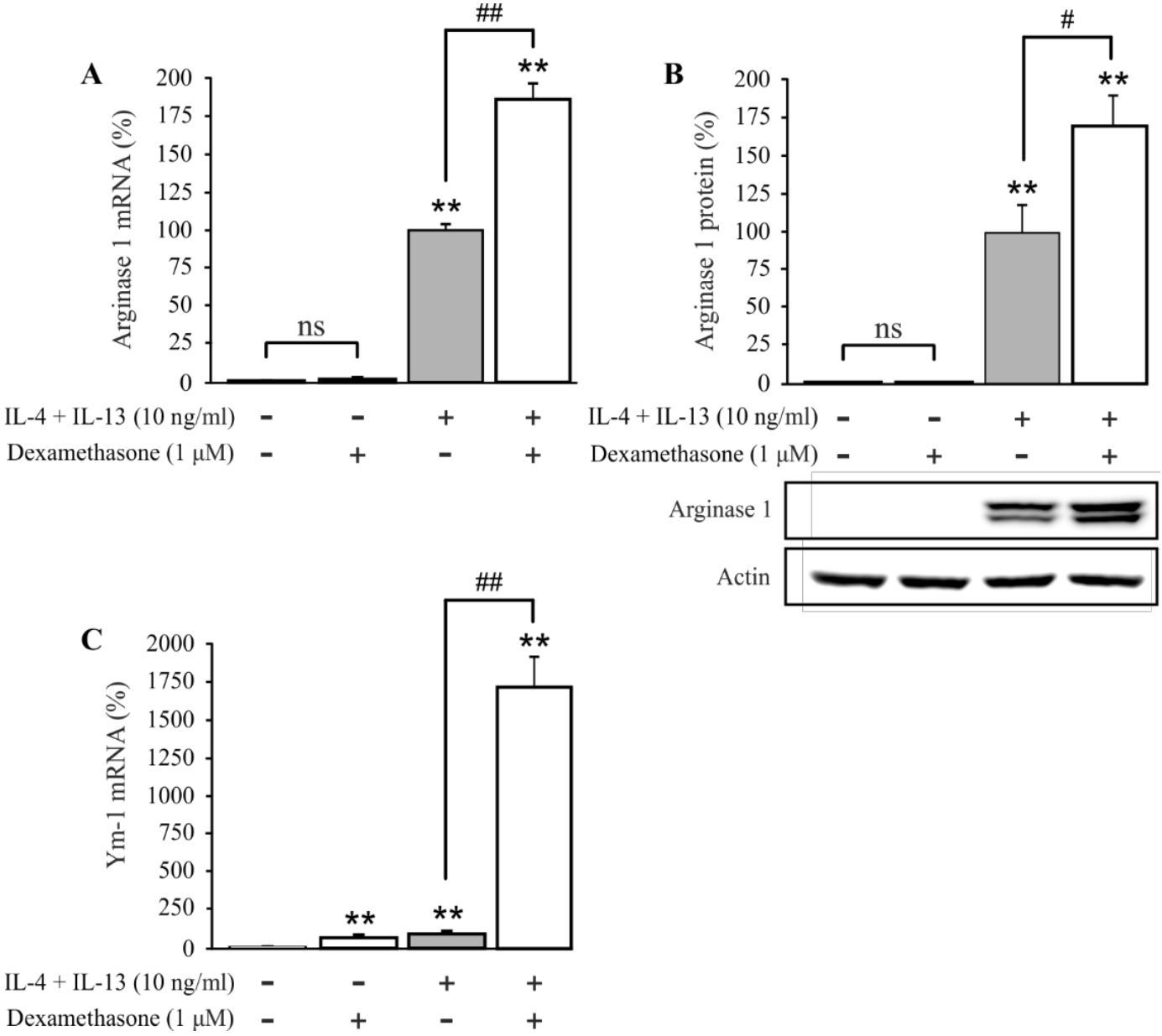

Figure 4: Dexamethasone increased the expression of markers of the anti-inflammatory and healing-promoting M(IL-4/IL-13) activation in J774 macrophages. J774 macrophages were cultured with a combination of IL- 4 and IL-13 (both 10 ng/ml) for 24 h, with or without dexamethasone (1 µM). Arginase 1 (A) and Ym-1 (C) mRNA levels were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. Arginase 1 protein levels were determined with Western blotting (B). All values were compared to the sample treated with IL-4 and IL-13 alone, which was set as 100 %. The results are expressed as mean + SEM, n = 4. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. In (B), shown is also a representative Western blotting gel of four with similar results.

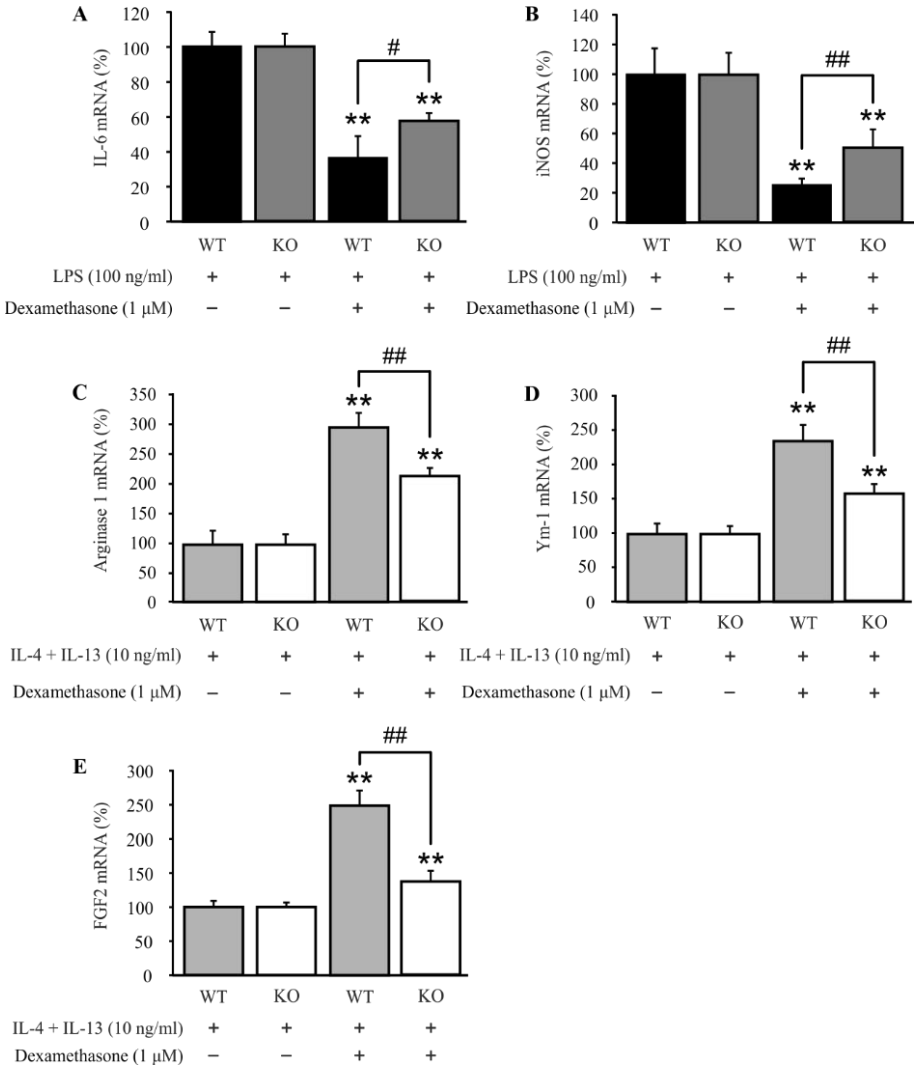

Figure 5. Dexamethasone decreased markers of M(LPS) activation and increased those of M(IL-4/IL-13) activation in macrophages from wild-type mice, and both effects were attenuated in macrophages from MKP-1 deficient mice. Peritoneal macrophages from wild-type (WT) and MKP-1 deficient (knock-out, KO) mice were cultured with either LPS (100 ng/ml) for 4 h (A and B) or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 h (C-E), in the absence or presence of dexamethasone (1 µM). mRNA levels of the M(LPS) markers IL-6 (A) and inducible nitric oxide synthase (iNOS) (B), and the M(IL-4/IL-13) markers arginase 1 (C), Ym-1 (D) and fibroblast growth factor 2 (FGF2) (E) were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. WT and KO values were compared separately to the respective samples without dexamethasone, which were set as 100 %. The results are expressed as mean + SEM, cells from six WT and six KO mice were pooled to give n = 4. **: $p < 0.01$, compared to the control treated with LPS or IL-4 + IL-13 alone; #: $p < 0.05$ and ##: $p < 0.01$, compared to the indicated sample. The experiments were repeated two times with similar results.

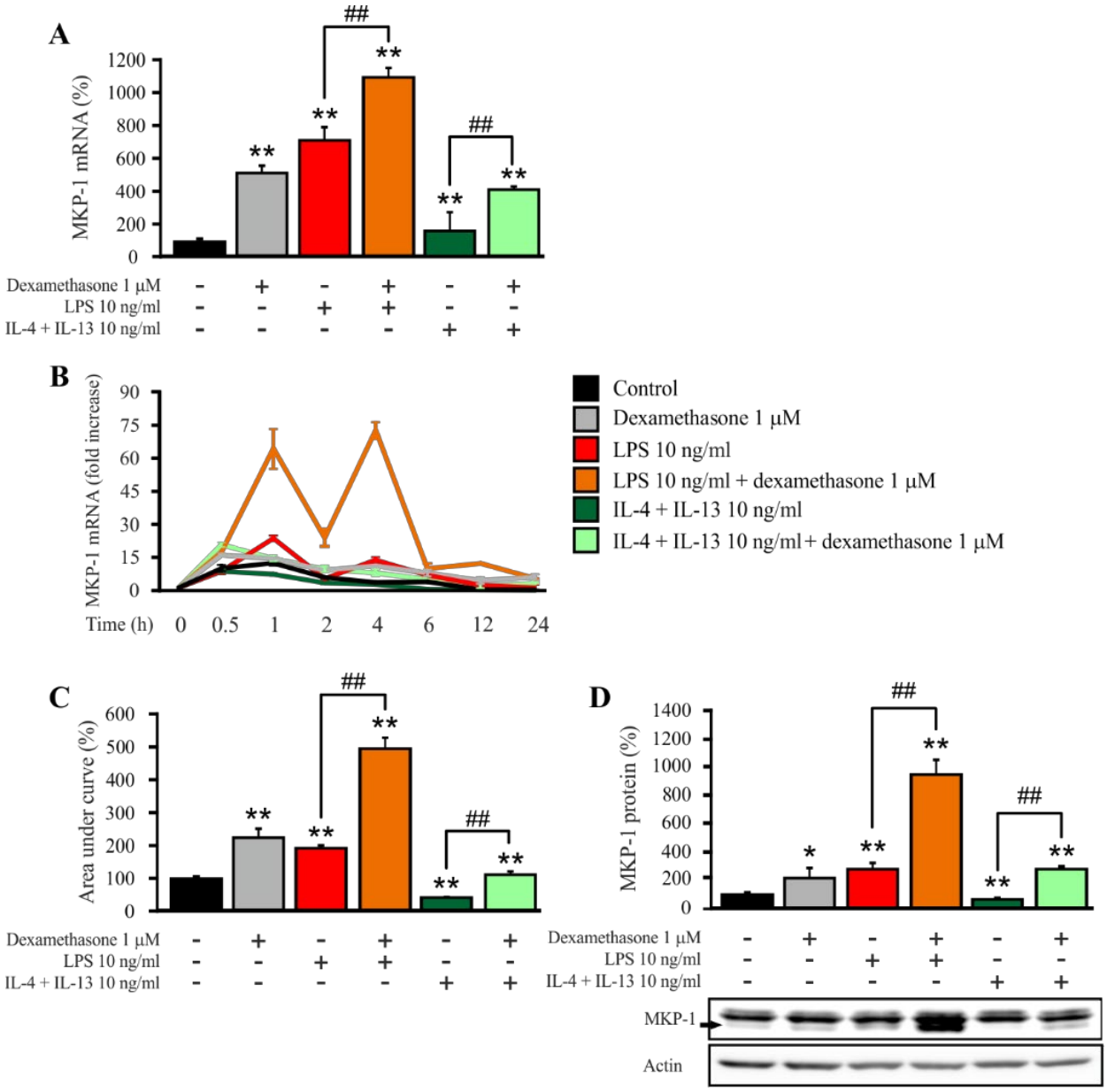

Figure 6. MKP-1 expression was increased by dexamethasone in classically activated M(LPS) and alternatively activated M(IL-4/IL-13) macrophages. (A): Peritoneal macrophages from wild-type mice were incubated for 1 h with dexamethasone (1 µM), with LPS (10 ng/ml), with IL-4 and IL-13 (10 ng/ml) or with their combinations as indicated, and MKP-1 mRNA was detected by quantitative RT-PCR. (B and C): J774 macrophages were incubated for 0.5 – 24 h with dexamethasone (1 uM), with LPS (10 ng/ml), with IL-4 and IL-13 (both 10 ng/ml) or with their combinations as indicated. MKP-1 mRNA was detected by quantitative RT-PCR and its expression levels were normalized against GAPDH. (B) shows the time-response curves and (C) indicates total area under the curves of (B), estimated by trapezoidal integration. (D): J774 macrophages were incubated for 1 h as described in (A), and MKP-1 protein levels were determined with Western blotting and actin was used as a loading control. The results are expressed as mean + SEM, n = 4.

**: $p < 0.01$ and*: $p < 0.05$, compared to the control, ##: $p < 0.01$ for indicated comparisons. In (D), shown is also a representative Western blotting gel of four with similar results.

Tables

Table 1: Primers and probes used for quantitative RT-PCR

Primer/probe	Sequence
mIL-6 forward	5′-TCGGAGGCTTAATTACACATGTTC-3′
mIL-6 reverse	5′-CAAGTGCATCATCGTTGTTCATAC-3′
mIL-6 probe	5′-Fam-CAGAATTGCCATTGCACAACTCTTTTCTCA-TAMRA-3′
miNOS forward	5′-CCTGGTACGGGCATTGCT-3′
miNOS reverse	5′-GCTCATGCGGCCTCCTT-3′
miNOS probe	5′-Fam-CAGCAGCGGCTCCATGACTCCC-TAMRA-3′
mMKP-1 forward	5′-CTCCTGGTTCAACGAGGCTATT-3′
mMKP-1 reverse	5′-TGCCGGCCTGGCAAT-3′
mMKP-1 probe	5′-Fam-CCATCAAGGATGCTGGAGGGAGAGTGTT-TAMRA-3′
mARG1 forward	5′-TCCAAGCCAAAGTCCTTAGAGATTAT-3′
mARG1 reverse	5′-CGTCATACTCTGTTTCTTTAAGTTTTTCC-3′
mARG1 probe	5′-Fam-CGCCTTTCTCAAAAGGACAGCCTCGA-TAMRA-3′
mYm-1 forward	5′-AGTGGGTTGGTTATGACAATGTCA-3′
mYm-1 reverse	5′-GACCACGGCACCTCCTAAATT-3′
mYm-1 probe	5′- Fam-AGCTTCAAGTTGAAGGCTCAGTGGCTCA-TAMRA-3′
mGAPDH forward	5′-GCATGGCCTTCCGTGTTC-3′
mGAPDH reverse	5′-GATGTCATCATACTTGGCAGGTTT-3′
mGAPDH probe	5′-Fam-TCGTGGATCTGACGTGCCGCC-TAMRA-3′