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MEK1 Dependent and Independent ERK Activation Regulates IL-10 and IL-12 Production in Bone Marrow Derived Macrophages

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Abstract

The mitogen activated protein kinases ERK1/2 play an important role in response to toll like receptor (TLR) activation and cytokine production, including IL-10 and IL-12. Here, we examined the role of MEK1 in ERK1/2 activation in response to TLR4 agonist by using bone marrow-derived macrophages (BMDMs) from wild type (WT) and *Mek1^{d/d}Sox2^{Cre}* mice. Our data demonstrates that MEK1 is essential for ERK1/2 activation in response to LPS. Furthermore, stimulation of the TLR4 receptor of BMDMs derived from *Mek1^{d/d}Sox2^{Cre}* mice showed enhanced STAT4 phosphorylation and increased IL-12 secretion, but exhibited a significantly lower IL-10 production as compared to WT macrophages. Most interestingly, TLR ligation in the presence of recombinant IL-10 (rIL-10) or retinoic acid (RA) led to ERK1/2 activation independent of MEK1 in BMDMs derived from *Mek1^{d/d}Sox2^{Cre}* mice and led to inhibition of STAT4 and decreased IL-12 levels. Collectively, these data suggest that MEK1 is required for TLR4 mediated ERK activation and in turn regulates production of IL-10 and IL-12. It also indicates that ERK1/2 can be activated independent of MEK1 in the presence of IL-10 and RA and this activation negatively regulates IL-12, but positively regulates IL-10 production. These findings may have significant implications for the development of drugs that modulate MEK1 activity in the treatment of inflammatory, autoimmune and proliferative diseases such as cancer.

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Keywords

Macrophages; ERK; MEK1; MEK2; STAT/JAK; IL-12; IL-10

1. Introduction

Antigen presenting cells such as macrophages play a crucial role in both the innate and adaptive immunity through an early recognition of microbial pathogens. Mammalian immune cells recognize pathogens through conserved pattern recognition receptors (PRR), such as toll like receptors (TLRs). LPS, a cell wall component of gram negative bacteria, is recognized by TLR4 [1]. TLR4 stimulation activates various signaling pathways such as mitogen-activated protein (MAP) kinases and signal transducer and activator of transcription (STAT) pathways [2]. Such activation leads to release of soluble cytokines and chemokines such as TNF- α , IL-6, IL-10, IL-12 and Nitric oxide (NO) that subsequently facilitate the eradication of pathogens.

Cytokines produced by macrophages play a crucial role dichotomizing the fate of T helper (Th) cells towards Th1, Th2 and Th17. It is widely accepted that production of IL-12 by monocytes and dendritic cells results in Th1 differentiation, whereas IL-10 production leads to Th2 polarization [3, 4]. IL-12 plays as a bridge between the innate resistance and the adaptive immune response through the regulation of Th1 response [5, 6]. While IL-12 is important for clearance of bacteria and parasites like toxoplasmosis [6-9], excessive production of IL-12 may lead to autoimmunity [10]. On the other hand, IL-10, an anti-inflammatory cytokine, has been shown to be a strong inhibitor of many functions of macrophages, including the production of pro-inflammatory cytokines including, IL-12, TNF- α , and IL-6 [5, 11, 12]. The ratio of IL-10 and IL-12 appears to be important in shaping subsets of immune cells and maintaining immune homeostasis [13-17]. Although, both cytokines have been extensively studied, the molecular mechanisms that mediate the balance between IL-10 and IL-12 production have not been identified.

MAPKs are a family of serine threonine kinases that mediate signals in response to stress, microbial products, cytokines and TLR ligands in a variety of cell types [18]. Several studies have shown that MAPKs, including extracellular signal regulated kinase (ERK), c-Jun NH(2)-terminal kinase (JNK), and the p38 kinase play important roles in regulating both IL-10 and IL-12 production in response to TLR ligands [13-17]. For instance, both ERK1/2 and p38 activation have been shown to regulate IL-12 and IL-10 production [3, 19]. Interestingly, it has been shown that ERK1-deficient mice display a bias towards Th1 response[20].

There are two distinct gene isoforms of mitogen activated kinase kinase (*Mek*) present in mammals, *Mek1* and *Mek2*. The deletion of the *Mek1* gene leads to embryonic lethality whereas interruption of *Mek2* is compatible with live [21, 22]. MEK1 and 2 isoforms are directly upstream of ERK kinase [23] and each isoform has a unique role in ERK activation. For instance, MEK1 is capable of stimulating epidermal proliferation and in fibroblasts, MEK1 has a regulating function in cell migration [22, 24]. MEK1 deficient mice exhibit a lupus-like syndrome [25]. The physiological role of MEK1 in macrophages in response to

microbial stimuli is not well-defined, although, it is known that TLR activation leads to phosphorylation of MEK1/2 and ERK1/2. Here, we tested the hypothesis that activation of the MEK1 isoform plays a critical role in ERK1/2 activation in response to LPS and regulates differential IL-12 and IL-10 production.

In addition to MAPKs activation, activation of janus kinases (JAKs) and signal transducer and activator of transcription (STAT) pathways is crucial for the production of IL-12 and IL-10 [3, 26, 27]. In the human genome, there are four isoforms of *JAKs* (*JAK1*, 2, 3, and *Tyk2*) and seven *STATs* (*STAT1*, 2, 3, 4, 5A, 5B, and 6). Different groups of cytokine receptors signal via specific combinations of JAK and STAT isoforms. While *STAT4* activation is associated predominantly with IL-12 production, *STAT3* and *STAT1* have been shown to be important both in IL-12 and IL-10 production [3, 28-31]. Here, using a genetic approach, we show that TLR4 stimulation of BMDMs lacking MEK1 exhibit a significant increase in IL-12 and a decrease IL-10 production. Our results suggest that MEK1 influences the cytokine profile of BMDMs by an ERK and *STAT4*-dependent mechanism. Interestingly, the IL-12 phenotype in response to TLR ligation in MEK1 deficient BMDMs could be rescued in the presence of either rIL-10 or RA.

2. Materials and Methods

2.1. Chemicals and antibodies

LPS was purchased from Invivogen (San Diego, CA). U0126 was purchased from Calbiochem (San Diego, CA). Retinoic acid was purchased from Sigma Chemical (St. Louis MO). Recombinant IL-10 was purchased from R&D Systems (Minneapolis, MN). Phospho-specific antibodies against phospho-MEK1/2, ERK1/2, p38, JNK, *STAT1*, *STAT3*, *STAT4* as well as total ERK1/2, JNK, *STAT1*, *STAT3*, MEK1, MEK2, and β -actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against total p38 and total *STAT4* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Cell Signaling Technology, and horseradish peroxidase (HRP)-conjugated anti-goat antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Mice and isolation of bone marrow derived macrophages (BMDMs)

WILD-TYPE (WT) AND *MEK1*^{D/D} *SOX2*^{Cre/+} MICE WERE GENERATED ONTO THE 129 CC BACKGROUND AS PREVIOUSLY DESCRIBED [21, 32]. WT AND *MEK1*^{D/D} *SOX2*^{Cre/+} mice were maintained at animal facilities at the center de recherche sur le cancer de l'UNIVERSITÉ LAVAL (QUÉBEC, CA). ANIMAL STUDIES WERE APPROVED BY THE UNIVERSITY COMMITTEES ON USE AND CARE OF ANIMALS. BMDMs from mice were prepared as described previously [33]. Briefly, femurs and tibias from 6- to 12-week-old mice were dissected, and the bone marrow was flushed out. Macrophages were cultured with IMDM media supplemented with 30% L929 supernatant containing macrophage-stimulating factor, glutamine, sodium pyruvate, 10% heat-inactivated fetal FBS, and antibiotics for 5-7 days. BMDMs were re-plated at a density of 2×10^6 cells/well the day before the experiment.

2.3. Protein extraction and immunoblotting

After the appropriate treatments, cells were washed with PBS, and harvested in RIPA buffer (Millipore, Massachusetts) containing protease inhibitor and anti-phosphatase cocktails, as previously described [34]. Equal amounts of proteins (15µg) were mixed with the same volume of 2x sample buffer, separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene di-fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) at 20 V for 30 min using a semi dry transfer cell (Bio-Rad) as previously described [34]. The PVDF membrane was blocked with 5% dry milk in TBST (Tris-buffered saline with 0.1% Tween-20), rinsed, and incubated with primary antibody overnight. The blots were washed and incubated with HRP-conjugated secondary anti-IgG antibody. Membranes were washed and immunoreactive bands were visualized using a chemiluminescent substrate (ECL-Plus, GE Healthcare, Pittsburgh, PA). Images were captured on Hyblot CL film (Denville, Scientific Inc, Metuchen, NJ). Optical density analysis of signals was performed using ImageQuant software (version 5, Molecular Dynamics).

2.4. Nitrite determination by Griess reaction

NO levels were assessed by nitrite quantification as previously described [34].

2.5. Enzyme linked immunosorbent assay (ELISA)

Murine and human TNF- α , IL-10, IL-12 (p70), interferon- γ and IL-6 cytokine levels in cell culture supernatants were measured using ELISA DuoKits (R&D Systems) as previously described [35].

2.6. Statistical analyses

Statistical analyses were performed using SPSS software, version 21.0 (SPSS Inc; Chicago, IL). One way analysis of variance (ANOVA) test and *post hoc* repeated measure comparisons (least significant difference (LSD)) were performed to identify differences between groups. *ELISA* results were expressed as mean $\pm$ SEM. For all analyses, two-tailed p-values of less than 0.05 were considered significant.

3. Results

3.1. Differential activations of ERK regulate IL-10/IL-12 production and STAT4 activation in response to TLR4 stimulation

Several studies suggested that IL-10 production is correlated to high antigen dose, in addition to STAT4 and sustained ERK activation [26, 31]. To further investigate the validity of these observations in our system, we isolated BMDMs and assessed cytokine production in response to various doses of LPS in the presence and absence of the MEK inhibitor U0126. BMDMs were pretreated for 30 min with U0126 (10 µM), and then treated with different doses of LPS for 24 hours. IL-10 and IL-12 were measured via ELISA in the conditioned media. In the absence of U0126 we detected a dose-dependent increase for IL-10 (Fig. 1A). In the presence of U0126 higher doses of LPS challenge did not evoke any substantial IL-10 production. In the absence of U0126, we detected a minimal amount of IL-12 production, in contrast the presence of U0126 and stimulation with higher doses of

LPS (100 and 500 ng/mL) led to a significant production of IL-12 (Fig. 1B). We assessed whether dose dependent IL-10 and IL-12 production is due to differences in phosphorylation of ERK. First, we asked whether stimulation of BMDMs with higher doses of LPS leads to higher ERK activation and is able to override the inhibitory effect of U0126. In the presence and absence of U0126, BMDMs were challenged with various doses of LPS. Figure 1C, shows that indeed 100 and 500 ng/mL LPS evoked a higher ERK phosphorylation (Thr202/Tyr204) compared to lower doses, but U0126 pretreatment completely blocked ERK phosphorylation in response to all doses of LPS (Fig. 1C and D). Several studies highlighted the regulatory roles of pSTAT1, pSTAT3 and pSTAT4 in IL-10/IL-12 production as well as Th1 and Th2 differentiation [36, 37]. Therefore, we assessed STAT1 (Tyr701), STAT3 (Tyr705) and STAT4 (Tyr693) phosphorylation in response to various doses of LPS in the presence and absence of U0126. Pretreatment with U0126 led to an increase in STAT4 phosphorylation even without LPS stimulation and it potentiated STAT4 phosphorylation in response to all LPS doses (Fig. 1E and F). The highest STAT4 activation was observed in response to 100 ng/mL LPS (Fig. 4E upper panel and Fig. 4F). Low doses of LPS (10 and 1 ng/mL) did not evoke any STAT3 phosphorylation in the presence or absence of U0126. Challenge with higher doses of LPS led to a comparable STAT3 phosphorylation in the presence and absence of U0126. We observed overall a tendency of decreased STAT1 phosphorylation in the presence of U0126. While this was significant in response to 10 ng/mL LPS, it did not reach statistical significance for higher doses of LPS (100 and 500 ng/mL). Pretreatment with U0126 did not affect JNK and p38 phosphorylation (data not shown). Collectively, these data indicate that ERK activation plays a key role in differential IL-10 and IL-12 production and lack of ERK activation increases STAT4 phosphorylation.

3.2. TLR4 mediated MEK phosphorylation is defective in MEK1 deficient BMDMs

Although, inhibitors of the MEK/ERK MAPK cascade including U0126 are quite selective, they do not differentiate between MEK1 and MEK2. The role of MEK1 in LPS mediated ERK activation and its effect on cytokine production in BMDMs is poorly understood. To gain insight into the role of MEK1 in macrophages in response to LPS, we applied a genetic approach by using BMDMs derived from *Mek1^{d/d} Sox2^{Cre}* mice. First, we confirmed that BMDMs derived from *Mek1^{d/d} Sox2^{Cre}* lacked expression of MEK1 protein by Western blot analysis (Fig. 2A). As shown, MEK1 deficient BMDMs show no expression of MEK1 protein. In contrast, MEK2 protein levels were comparable in both WT and MEK1 deficient macrophages (Fig. 2A). To further investigate the role of MEK1 in TLR4 (LPS) mediated MEK1/2 phosphorylation, murine BMDMs from WT and *Mek1^{d/d} Sox2^{Cre}* mice were cultured in the presence of LPS (100 ng/mL) for different periods of time. We choose this LPS dose as we observed the most robust cytokine response with this concentration (Fig. 1A and 1B). LPS treatment of WT BMDMs led to phosphorylation of MEK1/2 (Ser217/221), which persisted up to 3 hours (Figs. 2B and 2C). In contrast, *Mek1^{d/d} Sox2^{Cre}* BMDMs showed a lack of pMEK1/2. Only after prolonged exposure of the Western blot, we observed a delayed and minimal phospho MEK1/2 expression (Figs. 2B and 2C). The minimal phosphorylation seen in MEK1 deficient BMDMs is due to the presence of MEK2, as the antibody we used recognizes phosphorylated forms of both MEK1 and MEK2. We concluded that MEK1 is crucial in LPS mediated MEK1/2 phosphorylation.

3.3. MEK1 regulates cytokine production in BMDMs in response to LPS

Murine BMDMs from WT and *MeK1^{d/d} Sox2^{Cre}* mice were challenged with LPS for 24h. Murine TNF- α , IL-6, IL-10 and IL-12 cytokine levels in the conditioned medium were measured by ELISA. Challenge of BMDMs with LPS led to production of TNF- α , IL-6, and IL-10, however LPS challenge of MEK1 deficient BMDMs led to significantly reduced TNF- α (Fig. 3A), IL-6 (Fig. 3B), and IL-10 levels (Fig. 3C). Interestingly, BMDMs lacking MEK1 responded to LPS challenge with a significant increase in IL-12 production as compared to BMDMs from WT mice (Figs. 3D). LPS treatment led to a robust IL-10 production in WT macrophages, whereas BMDMs from MEK1 deficient mice responded to LPS with a significantly lower IL-10 production (10 fold decrease) as compared to WT (Fig. 3C). One of the main defense mechanisms of macrophages is their ability to produce nitric oxide (NO) in order to kill pathogens [38]. Hence, we investigated whether the ablation of MEK1 affects LPS mediated NO production. Nitric oxide production in conditioned media at 24h was measured using Griess reaction as an indirect measure of NO production. LPS challenge led to production of NO in both WT and MEK1 deficient BMDMs. Nitric oxide production was comparable in both genotypes (Figs. 3E). These results were in line with a previous study, which showed that ERK activation is not a requirement for NO production [39]. It is known that MEK/ERK activation is a positive regulator of several cytokines [30, 40], yet strikingly, we found that MEK1 deficiency led to an exuberant production of IL-12. We concluded that lack of MEK1 has little effect on NO production, whereas MEK1 plays an important role in regulation of IL-10 and IL-12 production in response to LPS.

3.4. MEK1 is required in LPS mediated ERK phosphorylation in BMDMs

Studies using MEK deficient fibroblasts suggested that MEK1 is dispensable for ERK phosphorylation. ERK phosphorylation is only abrogated when both, MEK1 and MEK2 were absent [24, 41]. In macrophages, however it is not known which MEK isoform is required for LPS mediated ERK1/2 phosphorylation [42]. To investigate this, BMDMs derived from WT or MEK1 deficient mice were cultured side by side under equal conditions and stimulated with LPS for different time points to assess MAP kinase activation. Cell lysates were subjected to immunoblotting using antibodies against the phosphorylated forms of ERK (Thr202/Tyr204), p38 (Thr180/Tyr182) and JNK (Thr183/Tyr185). Antibodies against corresponding total proteins were used to determine equal loading. As shown, LPS stimulation led to ERK1/2 phosphorylation in WT BMDMs, this phosphorylation peaked at 30 min and persisted up to 3 hours. In contrast, ERK1 and ERK2 phosphorylation was absent in BMDMs deficient in MEK1 (Fig. 4A). Mean densitometric analysis of 3 independent experiments is shown in Figure 4B. These data suggest that MEK1 is required to phosphorylate ERK1/2 in response to LPS. As shown in Figure 4C, LPS treatment led to a rapid phosphorylation of p38 in both WT BMDMs and MEK1 deficient cells. WT BMDMs exhibited slightly higher p38 phosphorylation as compared to MEK1 deficient BMDMs. The densitometric analysis of 3 independent experiments is shown in Figure 4D. We also detected JNK phosphorylation using phospho-specific antibody against JNK. Figure 4E shows that both WT and MEK1 deficient BMDMs responded with phosphorylation of JNK, but JNK phosphorylation persisted longer, up to 60 min, in BMDMs derived from MEK1 deficient mice (Figs. 4E and 4F). Taken together, our results suggest that MEK1 is

required in ERK phosphorylation in response to LPS in murine BMDMs, while p38 and JNK phosphorylation is independent of MEK1.

3.5. MEK1 regulates phosphorylation of the JAK/STAT pathway

The JAK/STAT pathway plays an important role in regulation of IL-12 and IL-10, which in turn regulates Th1, Th2 and Th17 differentiation [43]. Several studies highlighted the regulatory roles of STAT1 and STAT3 in IL-10/IL-12 production as well as Th1 and Th2 differentiation [36, 37]. Therefore, we assessed STAT1 and STAT3 phosphorylation in response to LPS challenge. BMDMs derived from mice deficient in MEK1 or WT mice were cultured side by side under equal conditions and stimulated with LPS for different time points to assess STAT1 (Tyr701) and STAT3 (Tyr705) phosphorylation. BMDMs derived from both, WT and MEK1 deficient mice, showed comparable total STAT1 levels and responded similarly to LPS stimulation with an increase in STAT1 phosphorylation (Fig. 5A). Figure 5B shows densitometric values of 3 independent experiments. As shown in Figure 5C, WT and MEK1 deficient BMDMs responded to LPS challenge with STAT3 phosphorylation, but we observed a significantly increased phosphorylation of STAT3 in MEK1 deficient BMDMs as compared to WT (Fig. 5C). Figure 5D shows the densitometric quantification of 3 independent experiments. Activation of STAT4 also regulates IL-12 and IL-10 production [26], while IL-12 stimulation may lead to STAT4 phosphorylation, which in turn leads to increase IL-12 production in a positive feedback fashion [6]. Because, we observed an increase in IL-12 production in MEK1 deficient macrophages in response to LPS, we next determined the activation of STAT4 in response to LPS challenge in these cells. BMDMs derived from MEK1 deficient or WT mice were cultured side by side under equal conditions and challenged with LPS to assess STAT4 phosphorylation. Figure 5E shows phosphorylated STAT4 levels in WT BMDMs and MEK1 deficient macrophages. Macrophages from WT mice responded to LPS challenge with STAT4 phosphorylation. MEK1 deficient BMDMs exhibited constitutive activation of STAT4, both at baseline and in response to LPS. The STAT4 phosphorylation in MEK1 deficient BMDMs was sustained after 3h (Fig. 5E). To confirm that the increased pSTAT4 in MEK1 deficient BMDMs is specific, we detected phospho STAT3 and phospho ERK. As shown in Figure 5E (lane 2), MEK1 deficient macrophages exhibited no significant pSTAT3 at baseline but in LPS stimulation led to higher pSTAT3 as compared to WT macrophages. While WT macrophages responded to LPS with increased ERK phosphorylation, MEK1 deficient macrophages lack such a response.

3.6. Recombinant IL-10 and retinoic acid downregulate IL-12 production and activates ERK independent of MEK1

Because IL-10 is known to negatively regulate IL-12 production [3, 31], we tested whether exogenous recombinant IL-10 (rIL-10) has a similar inhibitory effect on IL-12 production in MEK1 deficient BMDMs. *Mek1^{d/d} Sox2^{Cre}* BMDMs were challenged with LPS in presence and absence of rIL-10 (10 ng/mL) for 24h. As shown in Figure 6A, exogenous rIL-10 completely blocked IL-12 production in MEK1 deficient BMDMs in response to LPS. Next, we asked whether LPS challenge in the presence of rIL-10 can lead to ERK activation independent of MEK1. As shown in Fig 6B, LPS challenge of MEK1 deficient BMDMs in the presence of exogenous rIL-10 led to ERK1/2 activation. Furthermore, we assessed

whether combination of exogenous rIL-10 and LPS can affect STAT4 activation. We found that rIL-10 decreased both the constitutive and LPS mediated STAT4 phosphorylation in *Mek1^{d/d} Sox2^{Cre}* BMDMs (Fig. 6C). These results collectively show that addition of rIL-10 blocks IL-12 production and this effect was associated with ERK activation independent of MEK1, as well as partial inhibition of STAT4 phosphorylation. To further prove the concept that ERK1/2 can be activated even in the absence of MEK1 and this is signal dependent, we tested the effect of retinoic acid (RA) on *Mek1^{d/d} Sox2^{Cre}* BMDMs. RA is a biologically active metabolite of vitamin A and evidence suggests that RA can negatively regulate Th1 type cytokines and favors production of Th2 type cytokines in APCs and T cells [44, 45]. Thus, we investigated whether the presence of RA can negatively regulate IL-12 production in MEK1 deficient BMDMs in response to LPS and similarly lead to ERK activation. MEK1 deficient BMDMs were stimulated with LPS in the presence or absence of RA (500 nM) for 24h and conditioned medium were assessed for IL-10 and IL-12 production. As shown in Fig. 6D, LPS challenge of MEK1 deficient BMDMs in the presence of RA led to significant reduction of IL-12 production as compared to stimulation with LPS alone. In contrast, the presence of RA led to a significant increase in IL-10 production as compared to LPS alone (Fig. 6E). Furthermore, MEK1 deficient BMDMs were stimulated with LPS in the presence or absence of RA to assess ERK activation. As shown in Figure 6F, LPS alone failed to induce ERK1/2 activation but LPS stimulation in the presence of RA led to ERK1/2 phosphorylation. Thus, these results collectively indicate that ERK 1/2 can be activated in a MEK1 dependent and independent fashion and such activation negatively regulates IL-12 production.

4. Discussion

MEK1 and MEK2 are ubiquitously expressed in all cell types and share 85% amino acid homology [46]. Deregulation of MEK/ERK signalling is a hallmark of various types of cancers [47, 48]. MEK1 and MEK2 isoforms transmit different types of signal and play a differential role in ERK activation leading to distinct biological effects [49, 50]. Although, it is commonly assumed that these two MEK isoforms are functionally equivalent, several lines of evidence, including our current work suggests distinct roles of these two kinases [34, 46]. One study has shown that MEK1 deficient fibroblasts respond to EGF with sustained phosphorylation of ERK1/2, proposing a lack of negative feedback loop leading to this sustained ERK activation [46]. Another study found that MEK2 is the predominant isoform in human neutrophils, and MEK2 exhibited considerably higher activity than MEK1 in response to chemotactic peptide [51]. Previous studies have shown that MEK1 deficient mice develop autoimmune and lymphoproliferative diseases [25]. Interestingly, we found virtually no ERK1/2 phosphorylation in response to TLR4 in MEK1 deficient macrophages, suggesting an exclusive role for MEK1 kinase in activation of ERK1/2 in response to TLR4 ligands. However, rIL-10 and RA rescued LPS mediated ERK activation in MEK1 deficient macrophages. Our findings indicate that upstream signals differentially regulate MEK1 and MEK2 activation leading to various biological effects (TLR4 versus TLR4 in the presence of rIL-10 or RA stimulation). These results also suggest that LPS stimulation alone cannot activate MEK2 in the absence of MEK1 unless additional receptors are engaged (IL-10 receptor or the retinoic acid receptor), as neither rIL10 and RA alone nor TLR4 ligation

evoked ERK activation in MEK deficient macrophages. In contrast, LPS in the presence of RA or rIL-10 led to ERK activation independent of MEK1. Although several studies suggested that signal strength dictates ERK activation and its biological effect, no previous study evaluated the effect of rIL-10 and RA in MEK1 deficient macrophages and our study is the first study evaluating the role of MEK1 using MEK1 deficient BMDMs in response to TLR stimulation.

The innate immune response to pathogens is determined by cellular signaling and the cytokine microenvironment, which in turn determines the complex differentiation processes that lead to adaptive immune programming. Both IL-12 and IL-10 are important cytokines determining the cytotoxic versus the tolerogenic immune responses [3, 6]. In the early phase of the innate immune response, IL-12 acts on resting natural killer (NK) and T cells by inducing the production of several cytokines including IFN- γ . Together IL-12 and IFN- γ act on macrophages to optimize their cytotoxic, antimicrobial and antitumor activities [6, 52]. Because of the critical role of IL-12 in the immune system its production is tightly regulated. Previous studies suggested that IL-10 plays an important regulatory role that limits IL-12 production [26]. Indeed, the balance of IL-10 and IL-12 production regulates the immune homeostasis after pathogen exposure (13-17). Our results show that genetic ablation of MEK1 or chemical inhibition of the MEK pathway shift the balance towards IL-12 production.

Using BMDMs derived from MEK1 deficient and WT mice, we determined the underlying signaling pathway for increased IL-12 production. We show a comparable p38 and JNK activation but a lack of both ERK1 and ERK2 phosphorylation in MEK1 deficient cells as compared to WT BMDMs in response to LPS. Most interestingly, despite higher IL-12 production in MEK1 deficient BMDMs, we observed a lower TNF- α and IL-6 production in these cells.

The activity of a set of STAT proteins regulates diverse cytokine production, which can also be regulated by prior exposure of cells to one cytokine followed by the same or a different cytokine causing synergistic or antagonistic effects. For instance, priming of cells with IL-12 leads to STAT4 activation and production of IFN- γ and IL-12 [53], similarly, priming of dendritic or T cells with IFN- γ activates STAT4 and leads to production of IL-12 [54]. Homo- and heterodimerization of STAT proteins give the ability to this class of transcription factors to regulate a plethora of cytokine production [55]. While STAT4 is known to positively regulate IL-12 production in various immune cells, the role of STAT1 and STAT3 is not as straight forward. STAT3 can either act upstream and/or downstream of IL-10 to regulate the anti-inflammatory effect of IL-10 [56]. It can also regulate IL-12 production in a different context of immune responses [57]. Based on previous studies, we expected to have increased phosphorylation of STAT1 and decreased phosphorylation of STAT3 in TLR4 stimulated *Mek1^{d/d} Sox2^{Cre}* macrophages. In contrast, we observed an increase in STAT3 and STAT4 phosphorylation in response to LPS and a comparable STAT1 phosphorylation in MEK1 deficient BMDMs. A similar effect on STAT4 phosphorylation as well as IL-10 and IL-12 production was observed after U0126 pretreatment of WT BMDMs (Fig 1). The effect of U0126 pretreatment on phosphorylation of STAT1 and STAT3 was different, leading to slightly decreased phosphorylation of STAT1 and STAT3. We attributed this

effect to the inhibition of both MEK1 and 2 in the presence of U0126. These data suggest that increased STAT3 tyrosine phosphorylation is not sufficient to induce IL-10 production in *Mek1^{d/d} Sox2^{Cre}* in response to LPS alone. Interestingly, TLR4 stimulation in the presence of rIL-10 or RA led to a dramatic increase of STAT3 phosphorylation (data not shown) in addition to MEK1 independent ERK activation, leading to an increase in IL-10 and decrease in IL-12 production. Our results indicate that STAT3 phosphorylation alone is not sufficient to upregulate IL-10 and such regulation requires ERK activation. While JAK kinases are responsible for phosphorylation of tyrosine residues on STAT proteins, serine phosphorylation can occur without JAK. Several serine kinases have been implicated in the phosphorylation of STATs, including ERK and p38 MAP kinases [43]. However, we observed increased tyrosine phosphorylation on STAT4 in both in MEK1 deficient BMDM and when using MEK/ERK inhibitor. Further studies are needed to investigate the role of MEK1 in STAT4 phosphorylation. Both in mice and human cell systems, studies have shown that IL-10 production by Th1 cells requires a high antigen dose as well as IL-12 mediated STAT4 and sustained ERK activation [26, 31]. Our study is in agreement with prior studies that the IL-10 production correlates well with stronger signals, yet our study advanced prior knowledge that inhibition of MEK1/2 via U0126 or loss of MEK1 function potentiates the LPS effect on STAT4 activation and IL-12 production. Interestingly, in our study the challenge of macrophages from WT and MEK1 deficient mice with LPS evoked differential production of IL-10 and IL-12 (Fig. 3), suggesting that MEK1 plays a key role in macrophages to balance IL-10 and IL-12 homeostasis. This effect is mechanistically linked to its regulatory role of MEK1/ERK and STAT4 in response to LPS in macrophages. While MEK1 is a positive regulator of ERK which in turn increases IL-10 production, it negatively regulates STAT4 activation in response to LPS, as MEK1 deficiency led to an increase in STAT4 tyrosine phosphorylation and increased IL-12 production. This data is in line with previous observations that STAT4 is an important positive regulator of IL-12 and that ERK activation is important for IL-10 production [3, 17, 58]. Yet, our observation advances the field because it identifies the role of MEK1 but not MEK2 as the upstream kinase in regulation of ERK in response to LPS. Our results show a previously unreported, essential function for MEK1 in macrophages in regulating the ERK1/2 and STAT4 pathways in response to TLR4 activation. A lack of MEK1 activity shifts the balance from IL-10 towards IL-12 production. Interestingly, this paradigm could be shifted when MEK1 deficient cells were treated with RA and exogenous IL-10.

In the light of the critical role of IL-12 for triggering Th1 responses, our results raise an opportunity to design an inhibitor targeting specifically MEK1 to improve the cytotoxic immune response. Such inhibitor may have applications both as adjuvant for anticancer medication as well as in parasitic infection such as toxoplasmosis.

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Highlights

MEK1 is a critical regulator of diverse cellular functions.

TLR4 ligation of MEK1 deficient BMDMs failed to activate ERK1/2 but enhanced STAT4 phosphorylation and IL-12 production. Retinoic acid or rIL-10 rescued this phenotype.

MEK1 dependent and independent ERK activation regulates IL-12 and IL-10.

Figure 1. Inhibition of MEK/ERK pathway differentially regulates IL-10/IL-12 production and STAT4 activation

BMDMs derived from WT were cultured in the absence and presence of U0126 (30 min) and challenged with different doses of LPS (500, 100, 10, and 1 ng/mL) for 24 h. Conditioned media were analyzed for IL-10 (A) and IL-12 (B). C) BMDMs were isolated from WT mice and challenged with various doses of LPS in the presence and absence of U0126. Western blot analysis was performed using phospho ERK antibody and equal loading was confirmed using total ERK antibody. (D) Densitometric values expressed as fold increase of the ratio phosphorylated/total values of ERK. E) BMDMs were isolated from WT mice and challenged with various doses of LPS (500, 100, 10, and 1 ng/mL) in the presence and absence of U0126. Western blot analysis was performed using phospho-specific antibodies for STAT4, STAT1, and STAT3. Equal loading was confirmed using total STAT4, STAT3, and STAT1 antibodies. (F) Densitometric values expressed as fold increase of the ratio phosphorylated/total values of STAT4. Using ANOVA Mann-Whitney U test, a p value <0.05 was considered significant and error bars indicate SEM.

Figure 2. TLR4 mediated MEK1/2 phosphorylation is defective in MEK1 deficient BMDMs
 Murine BMDMs from WT and MEK1 deficient mice were cultured at a density of 2×10^6 cells/well 24h before stimulation. Cells were treated with LPS (100 ng/mL) for different time points as indicated. Whole cell extracts were prepared and 15 μ g of proteins were subjected to SDS-gel electrophoresis and Western blot analysis. (A) Detection of MEK1 and MEK2 isoforms. Whole cell extracts were prepared and 15 μ g of proteins were subjected to SDS-gel electrophoresis and Western blot analysis using antibodies against MEK1 and MEK2. Equal loading was confirmed using MEK2 and β -actin antibodies. As shown, *Mek1^{d/d} Sox2^{Cre}* BMDMs lack expression of MEK1. (B) LPS induced phosphorylation of MEK1/2 in WT and *Mek1^{d/d} Sox2^{Cre}* BMDMs. Murine BMDMs were challenged with LPS for different time points as indicated. Whole cell extracts were prepared and 15 μ g of proteins were subjected to SDS-gel electrophoresis and Western blot analysis was performed using antibodies against the phosphorylated forms of MEK1/2 (Ser217/221). Representative results are shown out of a total of 3 experiments. Equal loading was determined using antibodies against β -actin. (C) Densitometric values of pMEK1/2 and fold increase of pMEK1/2 to β -actin in response to LPS treatment at different time points. Results shown are representative of three independent experiments.

Figure 3. MEK1 regulates cytokine production in BMDMs in response to LPS

BMDMs derived from WT and *Mek1*^{Δ/Δ} *Sox2*^{Cre} mice were cultured under similar conditions and treated with LPS (100 ng/mL) for 24 h. Conditioned media were analyzed for (A) TNF-α, (B) IL-6, (C) IL-10, and (D) IL-12 using ELISA. (E) The nitrite concentration in the culture medium was used as a measure of NO production. After stimulation/incubation, the generation of NO in the cell culture supernatants was determined by measuring nitrite accumulation in the medium using Griess reaction. Data are presented as mean of three independent experiments. Using ANOVA Mann-Whitney U test, a p value <0.05 was considered significant and error bars indicate SEM.

Figure 4. MEK1 is required in LPS mediated ERK phosphorylation

Murine BMDMs were treated with LPS (100 ng/mL) for different time points as indicated. Whole cell extracts were prepared and 15 μ g of proteins were subjected to SDS-gel electrophoresis and Western blot analysis with antibodies against the phosphorylated forms of (A) ERK (Thr202/Tyr204), (C) p38 (Thr180/Tyr182) and (E) SAPK/JNK (Thr183/Tyr185). Equal loading was determined using antibodies against total ERK, total p38 and total JNK. Representative results are shown out of a total of 3 independent experiments. Densitometric analysis expressed as fold increase of the ratio phosphorylated/total values of ERK (B), p38 (D) and JNK (F) in response to LPS treatment of 3 independent experiments. Using ANOVA Mann-Whitney U test, a p value <0.05 was considered significant and error bars indicate SEM.

Figure 5. MEK1 regulates phosphorylation of the JAK/STAT pathway in response to LPS treatment

Murine BMDMs derived from WT and *Mek1^{fl/fl} Sox2^{Cre}* were treated with LPS (100 ng/mL) for different time points as indicated. (A) Whole cell extracts were prepared and 15 µg total protein was subjected to SDS-PAGE and Western blot analysis using phospho-epitope-specific antibodies for STAT1 (Tyr701). Equal loading was confirmed total STAT1 antibody. (B) Densitometric values expressed as fold increase of the ratio phosphorylated/total values of STAT1. (C) Detection of phospho-STAT3 (Tyr705) in response to LPS treatment for different time points as indicated. (D) Densitometric values expressed as fold increase of the ratio phosphorylated/total values of STAT3. (E) BMDMs were isolated from WT or MEK1-deficient mice challenged with LPS for different time points as indicated. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using phosphoepitope-specific antibodies against STAT4, STAT3 and ERK. Equal loading was determined using antibodies against β-actin and total ERK. (F) Densitometric values expressed as fold increase of the ratio pSTAT4/β-actin. Using ANOVA Mann-Whitney U test, a p value <0.05 was considered significant and error bars indicate SEM.

Figure 6. Recombinant IL-10 and retinoic acid down regulate IL-12 production and activate ERK independent of MEK1

(A) BMDMs were isolated from MEK1-deficient mice, challenged with LPS (100 ng/mL) in the presence or absence of rIL-10 (10 ng/mL) for 24h. Conditioned media were analyzed for IL-12 using ELISA. (B and C) BMDMs derived from *Mek1^{d/d} Sox2^{Cre}* mice were cultured in the presence or absence of rIL-10 and treated with LPS (100 ng/mL) for different time points as indicated. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using phosphoepitope-specific antibodies against phospho ERK (B) and STAT4 (C). Equal loading was determined using antibodies against total ERK and STAT4. (D and E) BMDMs derived from *Mek1^{d/d} Sox2^{Cre}* mice were cultured in the presence and absence of RA (500 nM) and challenged with LPS (100 ng/mL) for 24 h. Conditioned medium were analyzed for IL-12 (D) and IL-10 (E) via ELISA. (F) BMDM derived from *Mek1^{d/d} Sox2^{Cre}* mice were cultured in the presence or absence of RA (500 nM) and treated with LPS for different time points as indicated. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using phosphoepitope-specific antibodies against ERK. Equal loading was determined using antibody against total ERK. Using ANOVA Mann-Whitney U test, a p value <0.05 was considered significant and error bars indicate SEM.