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To cite this version:

João Paulo M Luiz, Juliana E Toller-Kawahisa, Paula R Viacava, Daniele C Nascimento, Priscilla T Pereira, et al.. MEK5/ERK5 signaling mediates IL-4-induced M2 macrophage differentiation through regulation of c-Myc expression. Journal of Leukocyte Biology, 2020, 108 (4), pp.1215-1223. 10.1002/JLB.1MA0520-016R . hal-03008402

HAL Id: hal-03008402

https://hal.science/hal-03008402v1

Submitted on 16 Dec 2020

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MEK5/ERK5 signaling mediates IL-4-induced M2 macrophage differentiation through regulation of c-Myc expression

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Running title: ERK5 regulates IL-4-induced M2 polarization.

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ABSTRACT

Macrophages play a critical role in the regulation of immune responses. They are highly plastic cells, responding to diverse environmental stimuli to acquire different functional phenotypes. Intracellular signaling through mitogen-activated protein kinases (MAPKs) has been reported to regulate the differentiation of macrophages, but the role of the atypical MAPK ERK5 signaling in IL-4-mediated M2 macrophage differentiation is still unclear. Here, we showed that the ERK5 signaling pathway plays a critical role in IL-4-induced M2 macrophage differentiation. We found that pharmacological inhibition of MEK5, an immediate upstream activator of ERK5, with BIX markedly reduced the expression of classical M2 markers, such as *Arg-1*, *Ym-1*, and *Fizz-1*, as well as the production of M2-related chemokines and cytokines, CCL22, CCL17 and IGF-1 in IL-4-stimulated macrophages. Moreover, pharmacological inhibition of ERK5 with XMD8-92 also decreased the expression of several M2 markers induced by IL-4. In accordance, myeloid cell-specific *Erk5* depletion ($ERK5^{\Delta mye}$), using $LysM^{cre}/Erk5^{f/f}$ mice, confirmed the involvement of ERK5 signaling in IL-4-induced M2 polarization. Mechanistically, we found that pharmacological inhibition of ERK5 did not affect STAT6 phosphorylation, suggesting that ERK5 signaling regulates M2 differentiation in a STAT6-independent manner. However, we found that genetic deficiency or pharmacological inhibition of the MEK5/ERK5 pathway blocked the expression of c-Myc in IL-4-activated macrophages, which is a critical transcription factor involved in M2 differentiation. Taken together, our results reveal that activation of the MEK5/ERK5 signaling pathway is crucial in IL-4-induced M2 macrophage differentiation through the induction of c-Myc expression.

Keywords: Macrophage polarization, M2 macrophages, IL-4, MEK5, ERK5

INTRODUCTION

Macrophages are specialized innate immune cells that can acquire distinct functional phenotypes in response to a wide range of microenvironmental stimuli[1]. Conventionally, macrophages are classified in two well-established differentiated phenotypes: the classically activated (M1) and the alternatively activated (M2)[2]. M1 macrophages are typically activated by bacterial lipopolysaccharide (LPS) and IFN-γ, playing a key role in host defense against pathogens. On the other hand, M2 macrophages are involved in immunoregulation, tissue remodeling, and tumor promotion[1,3]. Based on the stimuli and the transcriptional profile, M2 macrophages can also be divided into different subtypes, from M2a to M2d. Among them, M2a macrophage – the subtype activated by type-2 cytokines interleukin (IL)-4 and IL-13 – is the most studied for its important role in allergic inflammation, helminth infections, wound-healing, and fibrosis. The interaction of IL-4 with its receptor IL-4R, comprised by the IL-4Rα and common γchain (γc), signals through the JAK-STAT6 pathway, which upregulates the expression of molecules associated with an anti-inflammatory response, including arginase 1 (*Arg1*), resistin-like-α (*Retnla*) and chitinase 3-like 3 (*Ym1*)[4,5]. However, the mechanism that regulates the M2a polarization induced by the IL-4/IL-4R signaling pathway is complex and not completely understood.

Previous studies have proposed that, in addition to the canonical pathways activated by IL-4, mitogen-activated protein kinases (MAPK) might play a role in the IL-4/IL-4R signaling pathway in macrophages[6,7]. In mammals, there are four conventional MAPKs subfamilies: ERK1/2, JNKs, p38 MAPKs, and ERK5. ERK5 is the most recently discovered and one of the least studied members of the MAPK family[8,9]. It is known that ERK5 is twice as large as classical MAPKs, containing an N-terminal kinase

domain with high homology with ERK1/2 and a unique and large C-terminal domain that controls its cellular localization and allows its association with and activation of several transcription factors[10]. Also, ERK5 activation generally involves sequential activation of different upstream MAPKs. Several extracellular stimuli, including cytokines, growth factors, and oxidative and osmotic stress, activate ERK5 through dual phosphorylation at Thr^{218}/Tyr^{220} residues in the N-terminal domain via activated MEK5[9,11]. Once activated, ERK5 undergoes autophosphorylation in multiple sites in the C-terminal domain, enhancing its transcriptional activity. Upon activation, ERK5 translocates from the cytosol into the nucleus and phosphorylates downstream targets, including other kinases and transcription factors[11,12].

It is established that the MAPK ERK5 pathway plays a role in many functions of myeloid cells. For instance, macrophage colony-stimulating factor (M-CSF) induces monocyte/macrophage differentiation and proliferation through the activation of ERK5 signaling[13]. ERK5 has been implicated in the regulation of M-CSF receptor (M-CSFR) expression, which is critical for the acquisition of functional macrophage phenotype mediated by M-CSF[14]. Although controversial, previous studies suggest that ERK5 exerts anti-inflammatory and immunoregulatory functions. For instance, in macrophages, ERK5 is involved in the efferocytosis of apoptotic cells and prevents inflammation and plaque formation in an experimental model of atherosclerosis[15].

Although it is known that the biological importance of ERK5 in macrophage activation, the functional role of ERK5 in M2 macrophage polarization induced by IL-4/IL-4R signaling is still unknown. In the present study, we investigated the role of the MEK5/ERK5 pathway in the regulation of M2 polarization induced by IL-4. We

demonstrate that genetic deficiency or pharmacological inhibition of ERK5 impairs IL-4-induced differentiation of M2 macrophages mediated, at least in part, through regulation of c-Myc transcriptional expression.

MATERIALS AND METHODS

Animals. C57BL/6, BALB/c, and STAT6-deficient mice (STAT6$^{-/-}$) were purchased from Jackson Mice. YARG knock-in mice, the reporter mice that express yellow fluorescent protein under control of the Arginase-1 promoter (Arg1-YFP)[16], were purchased from Jackson Mice. Myeloid cell-specific ERK5 conditional knockout mice (*ERK5*$^{\Delta mye}$) were obtained by crossing *ERK5*$^{flox/flox}$ with mice expressing Cre recombinase under the control of the Lysozyme promotor. *ERK5*$^{flox/flox}$ littermates were used as control. *ERK5*$^{flox/flox}$ and *ERK5*$^{\Delta mye}$ mice were kindly provided by Dr. Bernhard Ryffel (CNRS-University of Orleans, France). All experiments were carried out with six-to-eight-week old male and female mice according to the guidelines of the Animal Welfare Committee of the Ribeirão Preto Medical School, University of São Paulo (protocol number: 109/2015). All mice were kept under controlled environmental conditions with food and water *ad libitum* and maintained in a specific pathogen-free facility.

Cell culture. Bone marrow-derived macrophages (BMDM) were obtained using L929 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF), as previously described[17]. Briefly, bone marrow cells were obtained by flushing both mice femurs. After centrifugation, cells were resuspended in RPMI-1640 supplemented with 20 % of L929 cell-conditioned medium, 10 % of fetal bovine serum (Gibco), L-glutamine (2 mM), penicillin (100 U/mL) and fungizone (2.5 μg/mL). Bone marrow cells were seeded in four non-treated Petri dishes (Corning, 430591) and

incubated at 37 ºC and 5 % CO_2. Four days after seeding, supplemented RPMI-1640 medium was added, and then cells were incubated for an additional 3 days. At the end of this period, BMDM were harvested and plated in tissue culture dishes for subsequent experiments. For M2 differentiation, BMDMs were plated in tissue culture dishes and stimulated with interleukin-4 (R&D Systems) at 10 ng/mL for the indicated times.

Reagents. In some experiments, macrophages were pretreated with the following inhibitors: BIX 02189 (Tocris), a selective inhibitor of MEK5; XMD 8-92 (Tocris), an inhibitor of ERK5; and 10058-F4 (Tocris), a c-Myc-Max dimerization inhibitor. Optimal doses of inhibitors were determined for each experiment. Mock pretreatment was performed with vehicle alone (DMSO) at a maximum concentration of 0.05%.

Enzyme-linked immunosorbent sandwich assay (ELISA). The concentrations of CCL17, CCL22, and IGF-1 (R&D Systems) were determined in the supernatant from cultures by ELISA according to the manufacturer's instructions. Absorbance was read at 450 nm on a spectrophotometer (SpectraMax 190 Microplate Reader, Molecular Devices) using the SoftMax Pro software.

Quantitative Real-Time PCR. Total RNA was extracted from *in vitro* cultured macrophages using the RNeasy Mini Kit (Qiagen). The reverse transcription was synthesized with High Capacity (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in the StepOnePlus detection system (Applied Biosystems). The fold difference in mRNA expression between groups was determined by the ΔΔCt method. The relative expression levels of each gene were normalized by the expression

levels of the housekeeping gene *Gapdh*. The syber primer utilized for real-time RT-PCR were: *Arg1* (forward: CCA GAA GAA TGG AAG AGT CAG TGT, reverse: GCA GAT ATG CAG GGA GTC ACC), *Retnla* (forward: CCT GAG ATT CTG CCC CAG GAT, reverse: TTC ACT GGG ACC ATC AGC TGG), *Ym1* (forward: CAA GTT GAA GGC TCA GTG GCT C, reverse: CAA ATC ATT GTG TAA AGC TCC TCT C), *Mmp9* (forward: GCG TGT CTG GAG ATT CGA CTT, reverse: TAT CCA CGC GAA TGA CGC T), *Myc* (forward: TTG AAG GCT GGA TTT CCT TTG GGC, reverse: TCG TCG CAG ATG AAA TAG GGC TGT), *Gapdh* (forward: GGG TGT GAA CCA CGA GAA AT, reverse: CCT TCC ACA ATG CCA AAG TT).

Flow Cytometry. Arginase-1 protein expression was determined by using macrophages derived from YARG (Arg1-YFP) reporter mice. The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated F4/80 (11480185, BM8; eBioscience); Allophycocyanin (APC)-conjugated F4/80 (17480182, BM8; eBioscience) and Alexa Fluor 647 (AF647)-conjugated p-STAT6 (558242, J71-773.58.11; BD Biosciences). To determine STAT6 phosphorylation, BMDM were fixed, permeabilized, and intracellular stained. BMDMs were identified using a forward- and right-angle light scatter. The relative number of positive BMDM and the median fluorescence intensity (MFI) for each antibody were measured as an indicator of the receptor density. The fluorescence histograms were obtained on a logarithmic scale. The isotype control antibodies were used to set the background levels. Data were acquired in FACSVerse (Becton Dickinson, CA, USA) and analyzed with FlowJo software version 10 (Tree Star).

Statistical analysis. All data are presented as the mean ± s.e.m. Statistical significance was estimated by unpaired Student's *t*-test for comparison between two groups. For

comparison between three or more groups, one-way ANOVA was used and followed by Bonferroni's posthoc test. Two-way ANOVA analysis was used for comparison among multiple groups. All statistical analyses were carried using GraphPad Prism 6.0 (GraphPad software). In all tests, a *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

IL-4-induced M2 differentiation requires MEK5 signaling

The activation of the ERK5 pathway involves the activation of ERK5 by MEK5. In order to investigate whether MEK5 is involved in IL-4-induced M2 polarization, we used the compound BIX02189 (BIX), a selective inhibitor of MEK5 signaling[18]. Pharmacological inhibition of MEK5 reduced mRNA levels of genes encoding arginase-1 (*Arg1*), chitinase 3-like 3 (*Ym1*), resistin-like alpha (*Retnla*), and matrix metalloproteinase-9 (*Mmp9*), all hallmarks of M2 macrophages after stimulation with IL-4 (Fig. 1a). Moreover, BIX inhibited the production of CCL17, CCL22, and the growth factor IGF-1 by IL-4-activated macrophages (Fig. 1b). To extend our data, we also used YARG mice containing a fluorescent eYFP reporter controlled by *arginase-1* promoter gene[16]. Flow cytometry analysis revealed less than 1% of Arg1-YFP$^+$ cells in naive M0 (unstimulated/not-activated) macrophages. As expected, IL-4 substantially increased the frequency of Arg1-YFP$^+$ macrophages, which was markedly decreased by BIX (Fig. 1c). Therefore, these data suggest that MEK5 signaling is involved in the IL-4-induced M2 differentiation.

ERK5 regulates IL-4-induced M2 macrophage differentiation

To determine the role of the MAPK ERK5 pathway in IL-4-induced M2 polarization, we initially used XMD8-92 (XMD), a small-molecule inhibitor of ERK5[19], in macrophages upon activation with IL-4. ERK5 inhibition with XMD reduced mRNA levels of *Arg1*, *Retnla*, *Ym-1*, and *Mmp9* compared to IL-4-activated macrophages (Fig. 2a). Moreover, we found that ERK5 inhibition decreased the production of CCL17, CCL22, and IGF-1 by IL-4-activated macrophages in a concentration-dependent manner (Fig. 2b). Furthermore, pharmacological inhibition of ERK5 markedly reduced the frequency of Arg1-YFP^{+} macrophages induced by IL-4 (Fig. 2c).

To further confirm the functional relevance of ERK5 in IL-4-induced M2 polarization and rule out off-target effects of pharmacological inhibitors, we used myeloid cell-specific ERK5 deficient mice (*ERK5*$^{\Delta mye}$). Consistent with our pharmacological data, ERK5-deficient macrophages showed reduced mRNA levels of M2 markers, such as *Arg1*, *Ym1*, and *Retnla* after stimulation with IL-4 (Fig. 3a). Furthermore, *ERK5*$^{\Delta mye}$ macrophages expressed lower levels of chemokines CCL17 and CL22 compared to IL-4-stimulated control (*ERK5*$^{flox/flox}$) macrophages (Fig. 3b). Altogether, these results indicate that ERK5 signaling is critical for IL-4-induced M2 differentiation.

ERK5 regulates M2 differentiation in a STAT6-independent manner

IL-4 binding to IL-4R-α phosphorylates STAT6, which is critical signaling for IL-4-induced M2 differentiation[4]. To explore the mechanism underlining the effect of ERK5 on IL-4-induced M2 differentiation, we next investigated whether inhibition of ERK5 could affect phosphorylation of STAT6. We initially validated the function of STAT6 in regulating IL-4-induced M2 differentiation by using BMDMs from STAT6-deficient mice. STAT6-deficient macrophages activated with IL-4 showed a substantial reduction

of CCL17, CCL22, and IGF-1 in comparison with WT macrophages (Fig. 4a). As expected, IL-4 increased phosphorylation of STAT6 at tyrosine 641 (Y641) in macrophages 1 h after stimulation. However, inhibition of ERK5 did not change the levels of IL-4-induced STAT6 phosphorylation in comparison to vehicle-control IL-4-activated macrophages (Fig. 4b). Thus, ERK5 signaling regulates M2 differentiation in a STAT6-independent manner.

MEK5/ERK5 pathway promotes M2 macrophage polarization through c-Myc

c-Myc is a transcription factor that has been proposed to be a marker of M2 macrophages and also acts as a regulator of M2 differentiation[20,21]. Indeed, we confirmed by quantitative RT-PCR that macrophages upregulate expression of Myc after stimulation with IL-4. We then evaluate the potential role of the MEK5/ERK5 axis in controlling IL-4-induced c-Myc expression in macrophages. Interestingly, pharmacological inhibition of the MEK5/ERK5 axis with BIX or XMD and genetic deficiency of ERK5 markedly reduced the mRNA expression of c-Myc induced by IL-4 in macrophages (Fig. 5a-b). Thus, these data suggest that the MEK5/ERK5 signaling pathway regulates the expression of c-Myc induced by IL-4 in macrophages.

To confirm the biological relevance of c-Myc to IL-4-mediated M2 differentiation, we used the compound 10058-F4, a small-molecule inhibitor of c-Myc[22]. We found that pharmacological inhibition of c-Myc reduced mRNA levels of *Retnla* and *Mmp9* and production of CCL22 and IGF-1 by macrophages after IL-4 stimulation (Fig. 5c-d). Finally, the 10058-F4 also decreased the frequency of Arginase-1-YFP-expressing macrophages upon exposure to IL-4 (Fig. 5e). Overall, these data suggest that the

MEK5/ERK5 signaling pathway controls IL-4-mediated M2 differentiation through modulation of c-Myc expression.

DISCUSSION

It is well described that IL-4 drives M2 polarization by interacting with IL-4R and activating JAK kinases (JAK-1 and JAK-3), which stimulates tyrosine phosphorylation of STAT6[4]. However, in addition to the canonical pathway activated by IL-4, it has been shown that MAPK signaling can also play a role in IL-4-induced M2 differentiation. Recent studies have demonstrated the role of c-Jun N-terminal kinase (JNK) and p38 MAPK pathways in M2 differenciation[6,7]. Here, we described the role of the MEK5/ERK5 signaling pathway in IL-4-induced M2 macrophage differentiation.

MEK5 is the canonical upstream activator of ERK5. Upon activation by MEKK2/MEKK3 via Ser^{311} and Thr^{315} phosphorylation, MEK5 binds to the functional domain of ERK5 (aa 78–139) and phosphorylates it in two residues in a Thr–Glu–Tyr (TEY) sequence, which results in activation of ERK5[11]. Very little is known about the role of MEK5 in macrophages. It was recently shown that ectopic expression of constitutive active mutant MEK5 in macrophages enhanced the secretion of M2 markers by tumor-associated macrophage (TAM)-like cells differentiated in vitro with tumor cell-conditioned medium or IL-6 and leukemia inhibitory factor (LIF)[23]. Here we provide evidence that MEK5 inhibition also decreases the expression of classical IL-4-induced M2 markers and the production of chemokines produced by M2 macrophages. Therefore, activation of MEK5 seems to be important to the induction of M2-related genes by different stimuli.

Once activated by MEK5, ERK5 undergoes a conformational change that exposes a nuclear localization signal in C-terminal, which facilitates the translocation of ERK5 into the nucleus[9]. Although recent works have described that ERK5 plays a role in

macrophage activation[13,15], its functional relevance for M2 polarization induced by IL-4/IL-4R signaling pathway remained unknown. We found that pharmacological inhibition of ERK5 suppresses the expression of typical M2 markers showing that ERK5 is involved in the regulation of M2 macrophage differentiation. ERK5-null mice are embryonic lethal due to cardiovascular defects[24]. For this reason, we used a myeloid cell-specific ERK5 deficiency to investigate its role in IL-4-induced M2 macrophages. ERK5-deficient macrophages failed to polarize to an alternative phenotype after stimulation with IL-4, confirming our pharmacological findings. These results also rule out the possibility that defective M2 polarization after pharmacological inhibition of ERK5 could be an off-target effect of XMD8-92. To our knowledge, this is the first report about the involvement of the MEK5/ERK5 pathway in the regulation of M2 macrophage polarization induced by the IL-4/IL-4R signaling pathway.

STAT6 has an essential role in IL-4-induced M2 polarization[4]. STAT6 is activated upon phosphorylation at Tyr641 by protein kinases from the JAK family, which allows its translocation into the nucleus[25,26]. The inhibition of ERK5 did not affect the phosphorylation of STAT6 induced by IL-4, indicating that ERK5 and STAT6 may act independently to induce M2 macrophage differentiation.

In parallel to the well-known role of STAT6, previous studies reported that the transcription factor c-Myc is also involved in the regulation of M2 polarization[20,28]. It was shown that IL-4 up-regulates the expression of c-Myc and its translocation to the nucleus, which controls the expression of M2-related genes[20,21]. Interestingly, c-Myc was identified as a downstream substrate of activated ERK5[29]. Recent studies also have been demonstrated that c-Myc can be regulated at the transcriptional level by active

ERK5. Pharmacological inhibition or siRNA silencing of ERK5 downregulates gene and protein expression of c-Myc in different settings[32–36]. In the present study, the pharmacological inhibition of both ERK5 or MEK5 suppressed c-Myc expression in macrophages upon exposure to IL-4. Furthermore, c-Myc expression induced by IL-4 was reduced in ERK5-deficient macrophages. Consistently, inhibition of c-Myc-Max interaction decreased IL-4-induced M2-related genes. Thus, we associated the impairment in M2 polarization mediated by genetic deletion or pharmacological inhibition of ERK5 with marked downregulation of IL-4-mediated c-Myc expression. Interestingly, despite being essential in cell-cycle progression and apoptosis, inhibition of ERK5 did not affect viability and proliferation in IL-4-stimulated macrophages (data not shown). These results indicate that the MEK5/ERK5 pathway is essential to control the c-Myc-dependent transcriptional program in IL-4-induced M2 polarization.

In conclusion, our study highlights a critical role of the MEK5/ERK5 signaling pathway in controlling M2 macrophage polarization. We demonstrate that IL-4 engages ERK5 MAPK signaling to promote M2 response by controlling the expression of c-Myc. Indeed, given that macrophages are key players in diverse physiological responses, regulation of alternative macrophage activation by the ERK5 pathway may provide a potential target for macrophage-directed therapeutic strategies in many settings, including helminth infection, cancer, allergy and wound healing.

Conflict of Interest: The authors declare no potential conflicts of interest

Financial Support: The research leading to these results received funding from the São Paulo Research Foundation (FAPESP) under grant agreement nº 2013/08216-2 (Center for Research in Inflammatory Diseases) and from Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Acknowledgments: We are grateful to Ana Kátia dos Santos and Ieda Regina dos Santos for technical support.

Author contributions: Conceived and designed the experiments: JPML, and JCAF. Performed the experiments: JPML, PTP, PRV, DCN, and ALS. Analyzed the data: JPML, JETK, FQC, BR, and JCAF. Contributed reagents/materials/analysis tools: TMC, VQ, FQC, and BR. Wrote the paper: JPML, JETK, and JCAF.

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FIGURE LEGENDS

Figure 1. MEK5 is required for IL-4-induced M2 polarization. Bone marrow-derived macrophages (BMDMs) were pretreated or not with MEK5 inhibitor, BIX02189 (BIX, 5 or 10 µM), for 30 min and then stimulated with IL-4 (10 ng/mL) when indicated. (a) mRNA was obtained after 24 h, and gene expression of *Arg1*, *Ym1*, *Retnla*, and *Mmp9* was determined by quantitative RT-PCR. (b) Culture supernatant was collected after 48 h and CCL22, CCL17, and IGF-1 levels were determined ELISA. (c) Arginase-1-YFP expression was determined after 48 h in Yarg BMDMs by flow cytometry. Data are shown as mean ± s.e.m (n=4) and are representative from 2 independent experiments. *p* values were determined using one-way ANOVA followed by Bonferroni posthoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 2. M2 polarization induced by IL-4 is impaired by ERK5 inhibition. BMDMs were pretreated or not with ERK5 inhibitor, XMD8-92 (XMD, 10 µM), for 30 min, and then stimulated with IL-4. (a) mRNA was obtained after 24 h, and gene expression of *Arg1*, *Ym1*, *Retnla*, and *Mmp9* were determined by quantitative RT-PCR. (b) Culture supernatant was collected after 48 h and CCL22, CCL17, and IGF-1 levels were determined by ELISA. (c) Arginase-1-YFP expression was determined after 48 h in Yarg BMDMs by flow cytometry. Data are shown as mean ± s.e.m (n=4) and are representative from 2 independent experiments. *p* values were determined using one-way ANOVA followed by Bonferroni posthoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 3. ERK5-deficient macrophages are resistant to M2 polarization induced by IL-4. $ERK5^{fl/fl}$ and $ERK5^{\Delta mye}$ BMDMs were stimulated or not with IL-4 (10 ng/mL). (a)

mRNA was obtained after 24 h and gene expression of *Arg1*, *Ym1*, *Retnla* was determined by quantitative RT-PCR. (b) Culture supernatant was collected after 48 h, and CCL17 and CCL22 levels were determined by ELISA. Data are shown as mean ± s.e.m (n=4) and are representative from 2 independent experiments. *p* values were determined using two-way ANOVA followed by Bonferroni posthoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 4. IL-4 activates STAT6 signaling pathway in an ERK5-independent manner. (a) STAT6$^{+/+}$ and STAT6$^{-/-}$ BMDMs were stimulated or not with IL-4 (10 ng/mL). Culture supernatant was collected after 48 h, and CCL17, CCL22, and IGF-1 levels were determined by ELISA. (b) WT BMDMs were pretreated or not with ERK5 inhibitor, XMD8-92 (XMD, 10 µM), for 30 min and then stimulated with of IL-4 (10 ng/mL) for 60 min, when indicated. STAT6 phosphorylation was determined by flow cytometry. Data are shown as mean ± s.e.m (n=4) and are representative from 2 independent experiments. *p* values were determined using one-way and two-way ANOVA followed by Bonferroni posthoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 5. MEK5/ERK5 is crucial to c-Myc-mediated M2 polarization induced IL-4. (a) BMDMs were pretreated or not with MEK5 or ERK5 inhibitors, BIX (10 µM), and XMD (10 µM), respectively, for 30 min and then stimulated with IL-4 (10 ng/mL), when indicated. mRNA was obtained after 6 h, and gene expression of c-Myc was evaluated by quantitative RT-PCR. (b) ERK5$^{fl/fl}$ and ERK5$^{\Delta mye}$ BMDMs were stimulated or not with IL-4 (10 ng/mL). mRNA was obtained after 6 h, and gene expression of c-Myc was evaluated by quantitative RT-PCR. (c) WT BMDMs were pretreated with the c-Myc inhibitor, 10058-F4 (F4, 30 µM), for 30 min and them

stimulated with IL-4. mRNA was obtained after 24 h and gene expression of M2 markers, *Retnla* and *Mmp9*, were evaluated by quantitative real-time PCR. (d) Culture supernatant of BMDMs treated as in "c" was collected after 48 h, and CCL22, and IGF-1 levels were measured by ELISA. (e) Yarg BMDMs were pretreated with F4 (30 µM) and then stimulated with IL-4 for 48 h. Arginase-1-YFP expression was assessed by flow cytometry. Data are shown as mean ± s.e.m (n=4) and are representative from 2 independent experiments. *p* values were determined using one-way and two-way ANOVA followed by Bonferroni posthoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

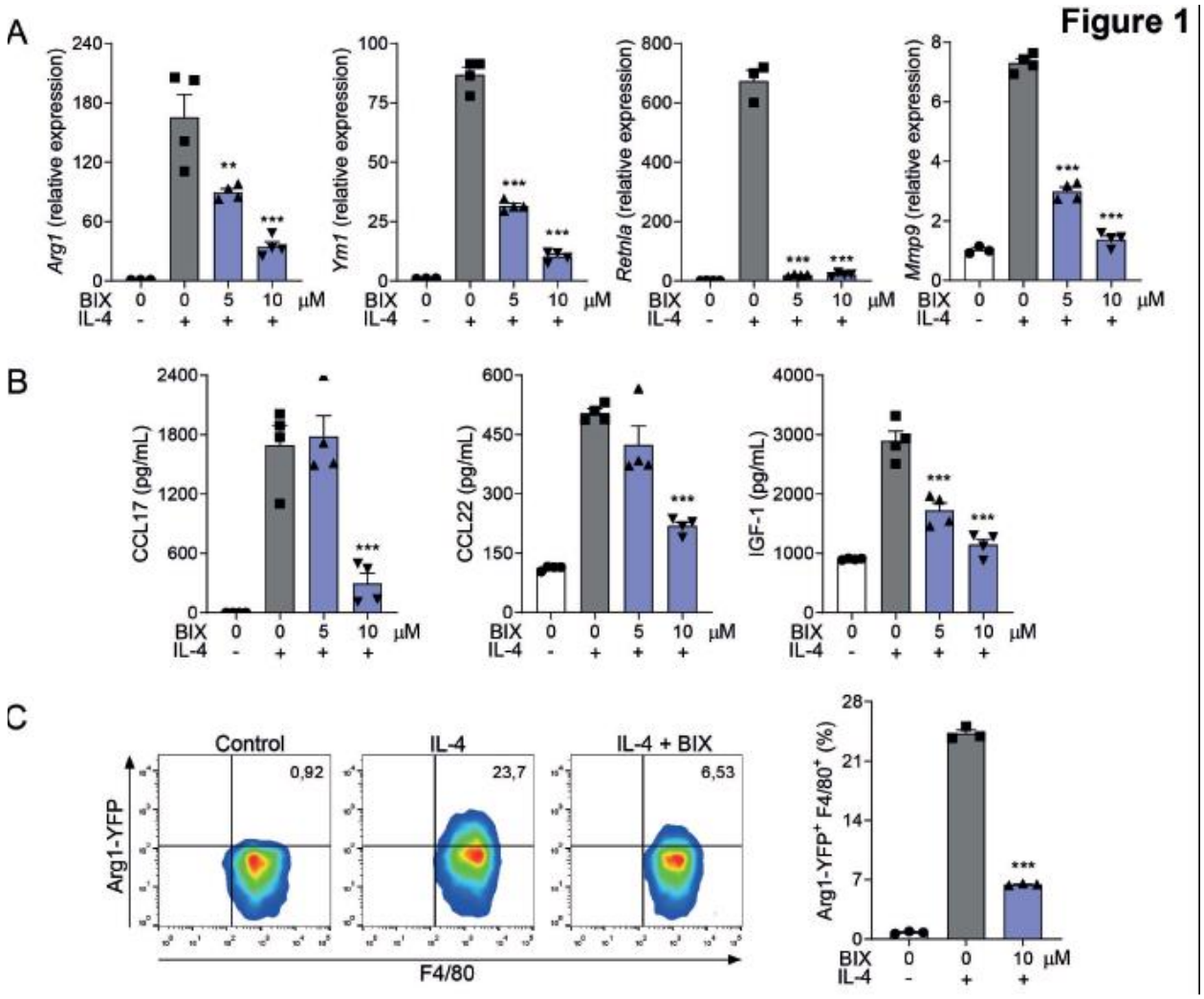


Figure 1
A
Arg1 (relative expression)
Ym1 (relative expression)
Retnla (relative expression)
Mmp9 (relative expression)
BIX 0 0 5 10 μM
IL-4 - + + +
B
CCL17 (pg/mL)
CCL22 (pg/mL)
IGF-1 (pg/mL)
C
Control
IL-4
IL-4 + BIX
0,92
23,7
6,53
Arg1-YFP
F4/80
Arg1-YFP+ F4/80+ (%)

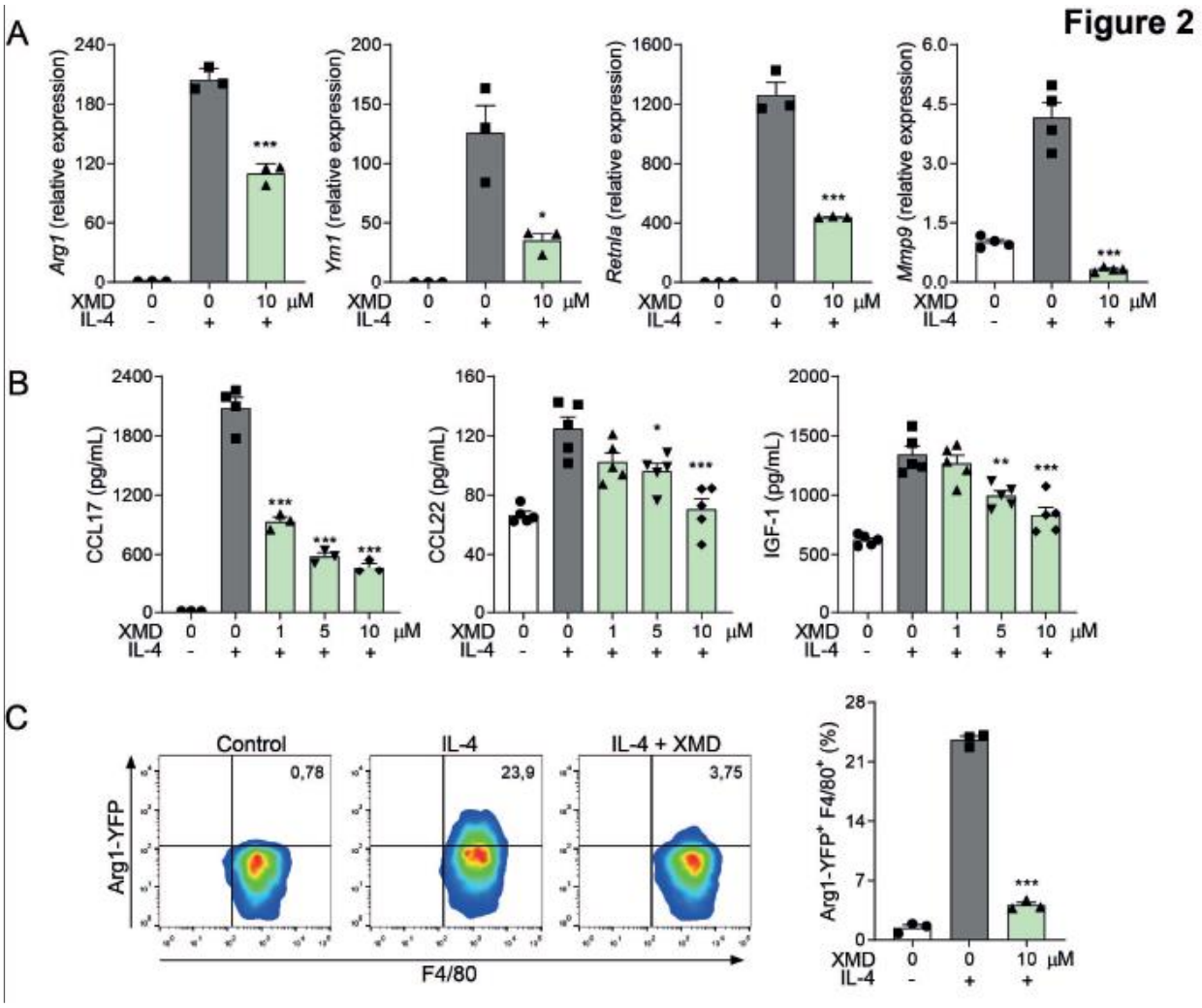


Figure 2
A
Arg1 (relative expression)
Ym1 (relative expression)
Retnla (relative expression)
Mmp9 (relative expression)
XMD 0 0 10 µM
IL-4 - + +
B
CCL17 (pg/mL)
CCL22 (pg/mL)
IGF-1 (pg/mL)
XMD 0 0 1 5 10 µM
IL-4 - + + + +
C
Control
IL-4
IL-4 + XMD
0,78
23,9
3,75
Arg1-YFP
F4/80
Arg1-YFP+ F4/80+ (%)

Figure 3

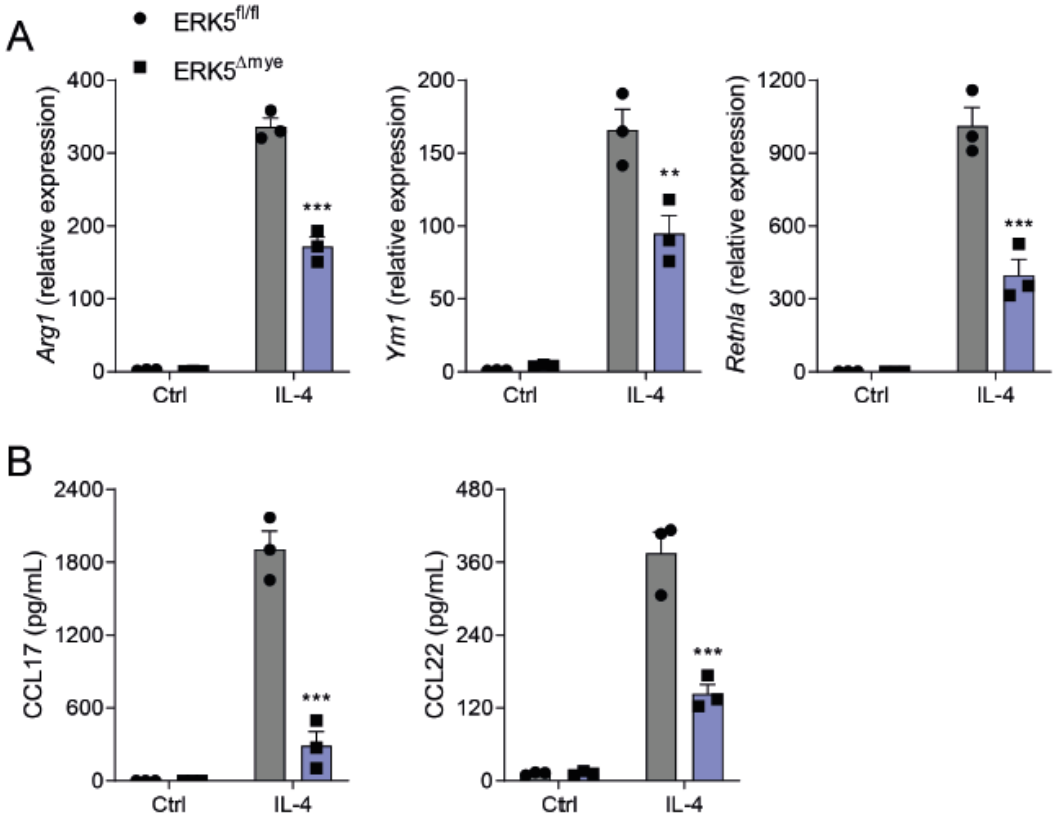
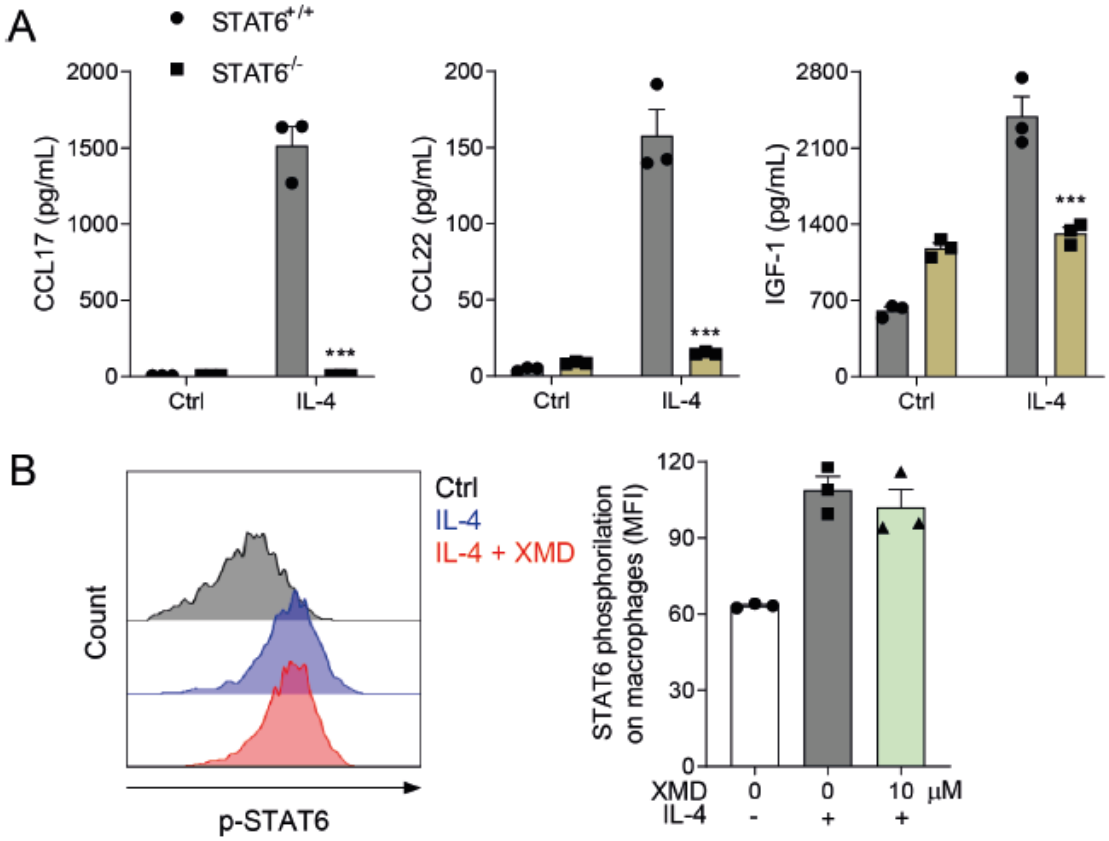

A
ERK5fl/fl
ERK5Δmye
Arg1 (relative expression)
400
300
200
100
0
***
Ctrl
IL-4
Ym1 (relative expression)
200
150
100
50
0
**
Ctrl
IL-4
Retnla (relative expression)
1200
900
600
300
0
***
Ctrl
IL-4
B
CCL17 (pg/mL)
2400
1800
1200
600
0
***
Ctrl
IL-4
CCL22 (pg/mL)
480
360
240
120
0
***
Ctrl
IL-4

Figure 4


A
STAT6+/+
STAT6-/-
CCL17 (pg/mL)
2000
1500
1000
500
0
***
Ctrl
IL-4
CCL22 (pg/mL)
200
150
100
50
0
***
Ctrl
IL-4
IGF-1 (pg/mL)
2800
2100
1400
700
0
***
Ctrl
IL-4
B
Count
p-STAT6
Ctrl
IL-4
IL-4 + XMD
STAT6 phosphorilation on macrophages (MFI)
120
90
60
30
0
XMD 0 0 10 μM
IL-4 - + +

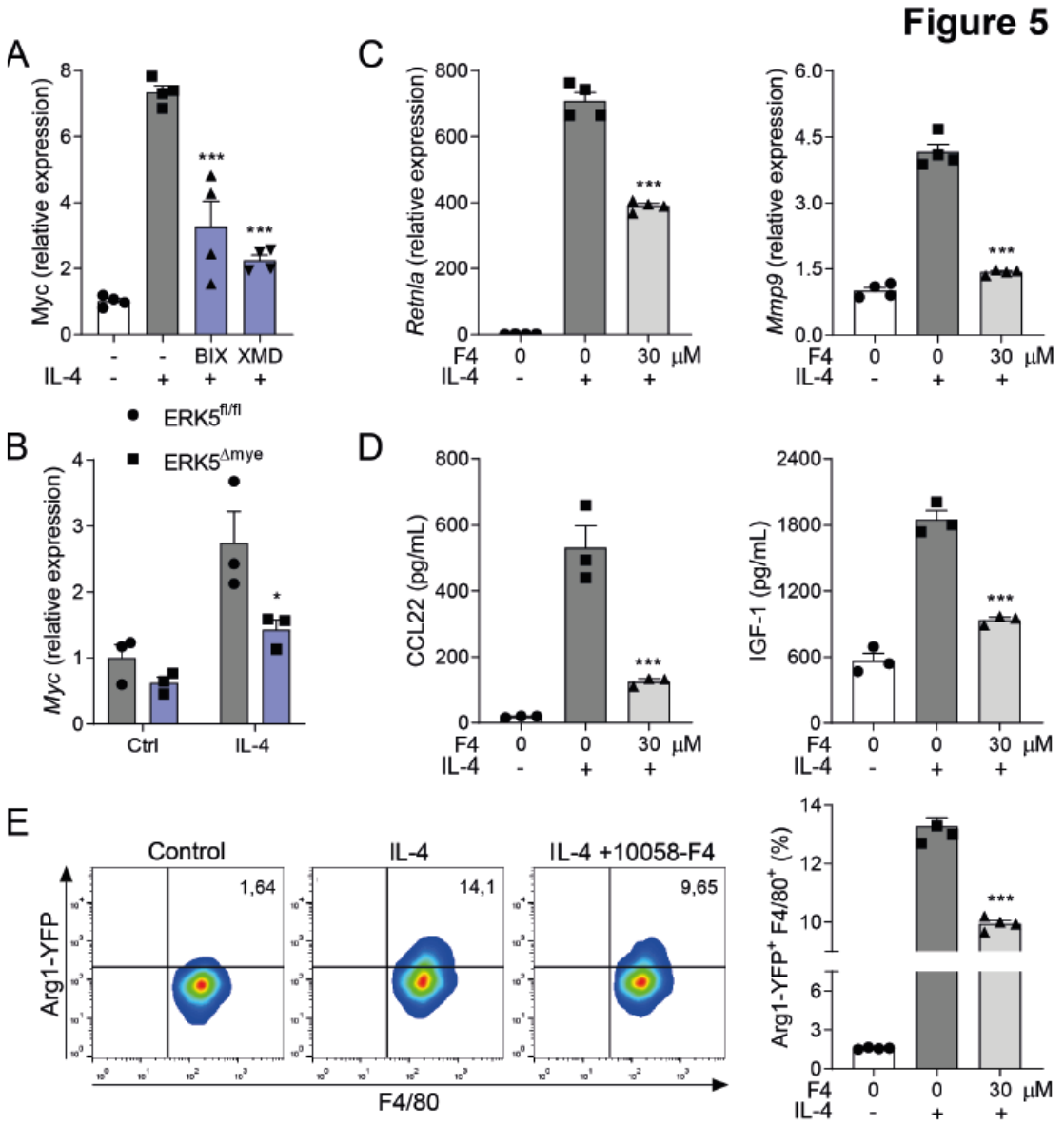


Figure 5
A
Myc (relative expression)
IL-4
BIX
XMD
ERK5fl/fl
ERK5Δmye
B
Myc (relative expression)
Ctrl
IL-4
C
Retnla (relative expression)
Mmp9 (relative expression)
F4
30 μM
D
CCL22 (pg/mL)
IGF-1 (pg/mL)
E
Control
IL-4
IL-4 +10058-F4
1,64
14,1
9,65
Arg1-YFP
F4/80
Arg1-YFP+ F4/80+ (%)

1