



HAL
open science

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

João Paulo M Luiz, Juliana E Toller-Kawahisa, Paula R Viacava, Daniele C Nascimento, Priscilla T Pereira, André L Saraiva, Douglas S Prado, Marc Lebert, Emanuele Giurisato, Cathy Tournier, et al.

► **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

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3

4 João Paulo M. Luiz¹, Juliana E. Toller-Kawahisa¹, Paula R. Viacava¹, Daniele C.
5 Nascimento¹, Priscilla T. Pereira¹, André L. Saraiva¹, Douglas S. Prado¹, Marc
6 LeBert^{2,3}, Emmanuele Giurisato⁴, Cathy Tournier⁴, Thiago M. Cunha¹, Fernando Q.
7 Cunha¹, Valerie Quesniaux^{2,3}, Bernhard Ryffel^{2,3}, José C. Alves-Filho^{1,*}

8

9 ¹ Department of Pharmacology, Center for Research in Inflammatory Diseases, Ribeirão
10 Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil.

11 ² CNRS, UMR7355, Orléans 45071, France;

12 ³ Experimental and Molecular Immunology and Neurogenetics, University of Orléans,
13 Orléans 45071, France.

14 ⁴Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine
15 and Health, University of Manchester, Manchester, United Kingdom

16

17

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

19

20 *** Corresponding author**

21 Prof. Jose C. Alves-Filho. Department of Pharmacology, Ribeirão Preto Medical
22 School, University of São Paulo, Av. Bandeirantes 3900, Monte Alegre 14049-900,
23 Ribeirão Preto, Brazil; Tel. +55 16 3602 3287 Email: jcafilho@usp.br

1 **ABSTRACT**

2 Macrophages play a critical role in the regulation of immune responses. They are highly
3 plastic cells, responding to diverse environmental stimuli to acquire different functional
4 phenotypes. Intracellular signaling through mitogen-activated protein kinases (MAPKs)
5 has been reported to regulate the differentiation of macrophages, but the role of the
6 atypical MAPK ERK5 signaling in IL-4-mediated M2 macrophage differentiation is
7 still unclear. Here, we showed that the ERK5 signaling pathway plays a critical role in
8 IL-4-induced M2 macrophage differentiation. We found that pharmacological inhibition
9 of MEK5, an immediate upstream activator of ERK5, with BIX markedly reduced the
10 expression of classical M2 markers, such as *Arg-1*, *Ym-1*, and *Fizz-1*, as well as the
11 production of M2-related chemokines and cytokines, CCL22, CCL17 and IGF-1 in IL-
12 4-stimulated macrophages. Moreover, pharmacological inhibition of ERK5 with
13 XMD8-92 also decreased the expression of several M2 markers induced by IL-4. In
14 accordance, myeloid cell-specific *Erk5* depletion (*ERK5^{Δmye}*), using *LysM^{cre}/Erk5^{fl/fl}*
15 mice, confirmed the involvement of ERK5 signaling in IL-4-induced M2 polarization.
16 Mechanistically, we found that pharmacological inhibition of ERK5 did not affect
17 STAT6 phosphorylation, suggesting that ERK5 signaling regulates M2 differentiation
18 in a STAT6-independent manner. However, we found that genetic deficiency or
19 pharmacological inhibition of the MEK5/ERK5 pathway blocked the expression of c-
20 Myc in IL-4-activated macrophages, which is a critical transcription factor involved in
21 M2 differentiation. Taken together, our results reveal that activation of the
22 MEK5/ERK5 signaling pathway is crucial in IL-4-induced M2 macrophage
23 differentiation through the induction of c-Myc expression.

24

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

1 INTRODUCTION

2 Macrophages are specialized innate immune cells that can acquire distinct functional
3 phenotypes in response to a wide range of microenvironmental stimuli¹.
4 Conventionally, macrophages are classified in two well-established differentiated
5 phenotypes: the classically activated (M1) and the alternatively activated (M2)². M1
6 macrophages are typically activated by bacterial lipopolysaccharide (LPS) and IFN- γ ,
7 playing a key role in host defense against pathogens. On the other hand, M2
8 macrophages are involved in immunoregulation, tissue remodeling, and tumor
9 promotion^{1,3}. Based on the stimuli and the transcriptional profile, M2 macrophages can
10 also be divided into different subtypes, from M2a to M2d. Among them, M2a
11 macrophage – the subtype activated by type-2 cytokines interleukin (IL)-4 and IL-13 –
12 is the most studied for its important role in allergic inflammation, helminth infections,
13 wound-healing, and fibrosis. The interaction of IL-4 with its receptor IL-4R, comprised
14 by the IL-4R α and common γ chain (γ c), signals through the JAK-STAT6 pathway,
15 which upregulates the expression of molecules associated with an anti-inflammatory
16 response, including arginase 1 (*Arg1*), resistin-like- α (*Retnla*) and chitinase 3-like 3
17 (*Ym1*)^{4,5}. However, the mechanism that regulates the M2a polarization induced by the
18 IL-4/IL-4R signaling pathway is complex and not completely understood.

19
20 Previous studies have proposed that, in addition to the canonical pathways activated by
21 IL-4, mitogen-activated protein kinases (MAPK) might play a role in the IL-4/IL-4R
22 signaling pathway in macrophages^{6,7}. In mammals, there are four conventional MAPKs
23 subfamilies: ERK1/2, JNKs, p38 MAPKs, and ERK5. ERK5 is the most recently
24 discovered and one of the least studied members of the MAPK family^{8,9}. It is known
25 that ERK5 is twice as large as classical MAPKs, containing an N-terminal kinase

1 domain with high homology with ERK1/2 and a unique and large C-terminal domain
2 that controls its cellular localization and allows its association with and activation of
3 several transcription factors¹⁰. Also, ERK5 activation generally involves sequential
4 activation of different upstream MAPKs. Several extracellular stimuli, including
5 cytokines, growth factors, and oxidative and osmotic stress, activate ERK5 through dual
6 phosphorylation at Thr²¹⁸/Tyr²²⁰ residues in the N-terminal domain via activated
7 MEK5^{9,11}. Once activated, ERK5 undergoes autophosphorylation in multiple sites in the
8 C-terminal domain, enhancing its transcriptional activity. Upon activation, ERK5
9 translocates from the cytosol into the nucleus and phosphorylates downstream targets,
10 including other kinases and transcription factors^{11,12}.

11

12 It is established that the MAPK ERK5 pathway plays a role in many functions of
13 myeloid cells. For instance, macrophage colony-stimulating factor (M-CSF) induces
14 monocyte/macrophage differentiation and proliferation through the activation of ERK5
15 signaling¹³. ERK5 has been implicated in the regulation of M-CSF receptor (M-CSFR)
16 expression, which is critical for the acquisition of functional macrophage phenotype
17 mediated by M-CSF¹⁴. Although controversial, previous studies suggest that ERK5
18 exerts anti-inflammatory and immunoregulatory functions. For instance, in
19 macrophages, ERK5 is involved in the efferocytosis of apoptotic cells and prevents
20 inflammation and plaque formation in an experimental model of atherosclerosis¹⁵.

21

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

1 demonstrate that genetic deficiency or pharmacological inhibition of ERK5 impairs IL-
2 4-induced differentiation of M2 macrophages mediated, at least in part, through
3 regulation of c-Myc transcriptional expression.

4

5 **MATERIALS AND METHODS**

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

19 **Cell culture.** Bone marrow-derived macrophages (BMDM) were obtained using L929
20 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-
21 CSF), as previously described¹⁷. Briefly, bone marrow cells were obtained by flushing
22 both mice femurs. After centrifugation, cells were resuspended in RPMI-1640
23 supplemented with 20 % of L929 cell-conditioned medium, 10 % of fetal bovine serum
24 (Gibco), L-glutamine (2 mM), penicillin (100 U/mL) and fungizone (2.5 μg/mL). Bone
25 marrow cells were seeded in four non-treated Petri dishes (Corning, 430591) and

1 incubated at 37 °C and 5 % CO₂. Four days after seeding, supplemented RPMI-1640
2 medium was added, and then cells were incubated for an additional 3 days. At the end
3 of this period, BMDM were harvested and plated in tissue culture dishes for subsequent
4 experiments. For M2 differentiation, BMDMs were plated in tissue culture dishes and
5 stimulated with interleukin-4 (R&D Systems) at 10 ng/mL for the indicated times.

6

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

12

13 **Enzyme-linked immunosorbent sandwich assay (ELISA).** The concentrations of
14 CCL17, CCL22, and IGF-1 (R&D Systems) were determined in the supernatant from
15 cultures by ELISA according to the manufacturer's instructions. Absorbance was read at
16 450 nm on a spectrophotometer (SpectraMax 190 Microplate Reader, Molecular
17 Devices) using the SoftMax Pro software.

18

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

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

10

11 **Flow Cytometry.** Arginase-1 protein expression was determined by using macrophages
12 derived from YARG (Arg1-YFP) reporter mice. The following monoclonal antibodies
13 were used: fluorescein isothiocyanate (FITC)-conjugated F4/80 (11480185, BM8;
14 eBioscience); Allophycocyanin (APC)-conjugated F4/80 (17480182, BM8;
15 eBioscience) and Alexa Fluor 647 (AF647)-conjugated p-STAT6 (558242, J71-
16 773.58.11; BD Biosciences). To determine STAT6 phosphorylation, BMDM were
17 fixed, permeabilized, and intracellular stained. BMDMs were identified using a
18 forward- and right-angle light scatter. The relative number of positive BMDM and the
19 median fluorescence intensity (MFI) for each antibody were measured as an indicator of
20 the receptor density. The fluorescence histograms were obtained on a logarithmic scale.
21 The isotype control antibodies were used to set the background levels. Data were
22 acquired in FACSVerse (Becton Dickinson, CA, USA) and analyzed with FlowJo
23 software version 10 (Tree Star).

24

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

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

6

7 **RESULTS**

8 **IL-4-induced M2 differentiation requires MEK5 signaling**

9 The activation of the ERK5 pathway involves the activation of ERK5 by MEK5. In
10 order to investigate whether MEK5 is involved in IL-4-induced M2 polarization, we
11 used the compound BIX02189 (BIX), a selective inhibitor of MEK5 signaling¹⁸.
12 Pharmacological inhibition of MEK5 reduced mRNA levels of genes encoding
13 arginase-1 (*Arg1*), chitinase 3-like 3 (*Ym1*), resistin-like alpha (*Retnla*), and matrix
14 metalloproteinase-9 (*Mmp9*), all hallmarks of M2 macrophages after stimulation with
15 IL-4 (Fig. 1a). Moreover, BIX inhibited the production of CCL17, CCL22, and the
16 growth factor IGF-1 by IL-4-activated macrophages (Fig. 1b). To extend our data, we
17 also used YARG mice containing a fluorescent eYFP reporter controlled by *arginase-1*
18 promoter gene¹⁶. Flow cytometry analysis revealed less than 1% of Arg1-YFP⁺ cells in
19 naive M0 (unstimulated/not-activated) macrophages. As expected, IL-4 substantially
20 increased the frequency of Arg1-YFP⁺ macrophages, which was markedly decreased by
21 BIX (Fig. 1c). Therefore, these data suggest that MEK5 signaling is involved in the IL-
22 4-induced M2 differentiation.

23

24 **ERK5 regulates IL-4-induced M2 macrophage differentiation**

1 To determine the role of the MAPK ERK5 pathway in IL-4-induced M2 polarization,
2 we initially used XMD8-92 (XMD), a small-molecule inhibitor of ERK5¹⁹, in
3 macrophages upon activation with IL-4. ERK5 inhibition with XMD reduced mRNA
4 levels of *Arg1*, *Retnla*, *Ym-1*, and *Mmp9* compared to IL-4-activated macrophages (Fig.
5 2a). Moreover, we found that ERK5 inhibition decreased the production of CCL17,
6 CCL22, and IGF-1 by IL-4-activated macrophages in a concentration-dependent manner
7 (Fig. 2b). Furthermore, pharmacological inhibition of ERK5 markedly reduced the
8 frequency of Arg1-YFP⁺ macrophages induced by IL-4 (Fig. 2c).

9

10 To further confirm the functional relevance of ERK5 in IL-4-induced M2 polarization
11 and rule out off-target effects of pharmacological inhibitors, we used myeloid cell-
12 specific ERK5 deficient mice (*ERK5^{Δmye}*). Consistent with our pharmacological data,
13 ERK5-deficient macrophages showed reduced mRNA levels of M2 markers, such as
14 *Arg1*, *Ym1*, and *Retnla* after stimulation with IL-4 (Fig. 3a). Furthermore, *ERK5^{Δmye}*
15 macrophages expressed lower levels of chemokines CCL17 and CL22 compared to IL-
16 4-stimulated control (*ERK5^{lox/lox}*) macrophages (Fig. 3b). Altogether, these results
17 indicate that ERK5 signaling is critical for IL-4-induced M2 differentiation.

18

19 **ERK5 regulates M2 differentiation in a STAT6-independent manner**

20 IL-4 binding to IL-4R- α phosphorylates STAT6, which is critical signaling for IL-4-
21 induced M2 differentiation⁴. To explore the mechanism underlining the effect of ERK5
22 on IL-4-induced M2 differentiation, we next investigated whether inhibition of ERK5
23 could affect phosphorylation of STAT6. We initially validated the function of STAT6 in
24 regulating IL-4-induced M2 differentiation by using BMDMs from STAT6-deficient
25 mice. STAT6-deficient macrophages activated with IL-4 showed a substantial reduction

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

7

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

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

18

19 To confirm the biological relevance of c-Myc to IL-4-mediated M2 differentiation, we
20 used the compound 10058-F4, a small-molecule inhibitor of c-Myc²². We found that
21 pharmacological inhibition of c-Myc reduced mRNA levels of *Retnla* and *Mmp9* and
22 production of CCL22 and IGF-1 by macrophages after IL-4 stimulation (Fig. 5c-d).
23 Finally, the 10058-F4 also decreased the frequency of Arginase-1-YFP-expressing
24 macrophages upon exposure to IL-4 (Fig. 5e). Overall, these data suggest that the

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

1 **DISCUSSION**

2 It is well described that IL-4 drives M2 polarization by interacting with IL-4R and
3 activating JAK kinases (JAK-1 and JAK-3), which stimulates tyrosine phosphorylation
4 of STAT6⁴. However, in addition to the canonical pathway activated by IL-4, it has
5 been shown that MAPK signaling can also play a role in IL-4-induced M2
6 differentiation. Recent studies have demonstrated the role of c-Jun N-terminal kinase
7 (JNK) and p38 MAPK pathways in M2 differentiation^{6,7}. Here, we described the role of
8 the MEK5/ERK5 signaling pathway in IL-4-induced M2 macrophage differentiation.

9

10 MEK5 is the canonical upstream activator of ERK5. Upon activation by
11 MEKK2/MEKK3 via Ser³¹¹ and Thr³¹⁵ phosphorylation, MEK5 binds to the functional
12 domain of ERK5 (aa 78–139) and phosphorylates it in two residues in a Thr–Glu–Tyr
13 (TEY) sequence, which results in activation of ERK5¹¹. Very little is known about the
14 role of MEK5 in macrophages. It was recently shown that ectopic expression of
15 constitutive active mutant MEK5 in macrophages enhanced the secretion of M2 markers
16 by tumor-associated macrophage (TAM)-like cells differentiated in vitro with tumor
17 cell-conditioned medium or IL-6 and leukemia inhibitory factor (LIF)²³. Here we
18 provide evidence that MEK5 inhibition also decreases the expression of classical IL-4-
19 induced M2 markers and the production of chemokines produced by M2 macrophages.
20 Therefore, activation of MEK5 seems to be important to the induction of M2-related
21 genes by different stimuli.

22

23 Once activated by MEK5, ERK5 undergoes a conformational change that exposes a
24 nuclear localization signal in C-terminal, which facilitates the translocation of ERK5
25 into the nucleus⁹. Although recent works have described that ERK5 plays a role in

1 macrophage activation^{13,15}, its functional relevance for M2 polarization induced by IL-
2 4/IL-4R signaling pathway remained unknown. We found that pharmacological
3 inhibition of ERK5 suppresses the expression of typical M2 markers showing that
4 ERK5 is involved in the regulation of M2 macrophage differentiation. ERK5-null mice
5 are embryonic lethal due to cardiovascular defects²⁴. For this reason, we used a myeloid
6 cell-specific ERK5 deficiency to investigate its role in IL-4-induced M2 macrophages.
7 ERK5-deficient macrophages failed to polarize to an alternative phenotype after
8 stimulation with IL-4, confirming our pharmacological findings. These results also rule
9 out the possibility that defective M2 polarization after pharmacological inhibition of
10 ERK5 could be an off-target effect of XMD8-92. To our knowledge, this is the first
11 report about the involvement of the MEK5/ERK5 pathway in the regulation of M2
12 macrophage polarization induced by the IL-4/IL-4R signaling pathway.

13

14 STAT6 has an essential role in IL-4-induced M2 polarization⁴. STAT6 is activated upon
15 phosphorylation at Tyr641 by protein kinases from the JAK family, which allows its
16 translocation into the nucleus^{25,26}. The inhibition of ERK5 did not affect the
17 phosphorylation of STAT6 induced by IL-4, indicating that ERK5 and STAT6 may act
18 independently to induce M2 macrophage differentiation.

19

20 In parallel to the well-known role of STAT6, previous studies reported that the
21 transcription factor c-Myc is also involved in the regulation of M2 polarization^{20,28}. It
22 was shown that IL-4 up-regulates the expression of c-Myc and its translocation to the
23 nucleus, which controls the expression of M2-related genes^{20,21}. Interestingly, c-Myc
24 was identified as a downstream substrate of activated ERK5²⁹. Recent studies also have
25 been demonstrated that c-Myc can be regulated at the transcriptional level by active

1 ERK5. Pharmacological inhibition or siRNA silencing of ERK5 downregulates gene
2 and protein expression of c-Myc in different settings³²⁻³⁶. In the present study, the
3 pharmacological inhibition of both ERK5 or MEK5 suppressed c-Myc expression in
4 macrophages upon exposure to IL-4. Furthermore, c-Myc expression induced by IL-4
5 was reduced in ERK5-deficient macrophages. Consistently, inhibition of c-Myc-Max
6 interaction decreased IL-4-induced M2-related genes. Thus, we associated the
7 impairment in M2 polarization mediated by genetic deletion or pharmacological
8 inhibition of ERK5 with marked downregulation of IL-4-mediated c-Myc expression.
9 Interestingly, despite being essential in cell-cycle progression and apoptosis, inhibition
10 of ERK5 did not affect viability and proliferation in IL-4-stimulated macrophages (data
11 not shown). These results indicate that the MEK5/ERK5 pathway is essential to control
12 the c-Myc-dependent transcriptional program in IL-4-induced M2 polarization.

13

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

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

2

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

8

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

11

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

1 REFERENCES

- 2 1. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with
3 lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* **11**, 889–896 (2010).
- 4 2. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage
5 activation. *Nature Reviews Immunology* (2008). doi:10.1038/nri2448
- 6 3. Sica, A. & Mantovani, A. Macrophage plasticity and polarization: In vivo veritas.
7 *Journal of Clinical Investigation* (2012). doi:10.1172/JCI59643
- 8 4. Martinez, F. O., Helming, L. & Gordon, S. Alternative Activation of
9 Macrophages: An Immunologic Functional Perspective. *Annu. Rev. Immunol.*
10 (2009). doi:10.1146/annurev.immunol.021908.132532
- 11 5. Lawrence, T. & Natoli, G. Transcriptional regulation of macrophage polarization:
12 Enabling diversity with identity. *Nature Reviews Immunology* (2011).
13 doi:10.1038/nri3088
- 14 6. Jiménez-García, L., Herránz, S., Luque, A. & Hortelano, S. Critical role of p38
15 MAPK in IL-4-induced alternative activation of peritoneal macrophages. *Eur. J.*
16 *Immunol.* (2015). doi:10.1002/eji.201444806
- 17 7. Hao, J., Hu, Y., Li, Y., Zhou, Q. & Lv, X. Involvement of JNK signaling in IL4-
18 induced M2 macrophage polarization. *Exp. Cell Res.* (2017).
19 doi:10.1016/j.yexcr.2017.05.010
- 20 8. Simões, A. E. S., Rodrigues, C. M. P. & Borralho, P. M. The MEK5/ERK5
21 signalling pathway in cancer: a promising novel therapeutic target. *Drug*
22 *Discovery Today* (2016). doi:10.1016/j.drudis.2016.06.010
- 23 9. Nithianandarajah-Jones, G. N., Wilm, B., Goldring, C. E. P., Müller, J. & Cross,
24 M. J. ERK5: Structure, regulation and function. *Cellular Signalling* (2012).
25 doi:10.1016/j.cellsig.2012.07.007

- 1 10. Nishimoto, S. & Nishida, E. MAPK signalling: ERK5 versus ERK1/2. *EMBO*
2 *Reports* (2006). doi:10.1038/sj.embor.7400755
- 3 11. Drew, B. A., Burow, M. E. & Beckman, B. S. MEK5/ERK5 pathway: The first
4 fifteen years. *Biochimica et Biophysica Acta - Reviews on Cancer* (2012).
5 doi:10.1016/j.bbcan.2011.10.002
- 6 12. Stecca, B. & Rovida, E. Impact of ERK5 on the hallmarks of cancer.
7 *International Journal of Molecular Sciences* (2019). doi:10.3390/ijms20061426
- 8 13. Rovida, E. *et al.* ERK5/BMK1 Is Indispensable for Optimal Colony-Stimulating
9 Factor 1 (CSF-1)-Induced Proliferation in Macrophages in a Src-Dependent
10 Fashion. *J. Immunol.* (2008). doi:10.4049/jimmunol.180.6.4166
- 11 14. Wang, X. *et al.* The MAPK ERK5, but not ERK1/2, inhibits the progression of
12 monocytic phenotype to the functioning macrophage. *Exp. Cell Res.* (2015).
13 doi:10.1016/j.yexcr.2014.10.003
- 14 15. Heo, K. S. *et al.* ERK5 activation in macrophages promotes efferocytosis and
15 inhibits atherosclerosis. *Circulation* (2014).
16 doi:10.1161/CIRCULATIONAHA.113.005991
- 17 16. Reese, T. A. *et al.* Chitin induces accumulation in tissue of innate immune cells
18 associated with allergy. *Nature* (2007). doi:10.1038/nature05746
- 19 17. Kurowska-Stolarska, M. *et al.* IL-33 Amplifies the Polarization of Alternatively
20 Activated Macrophages That Contribute to Airway Inflammation. *J. Immunol.*
21 (2009). doi:10.4049/jimmunol.0901575
- 22 18. Tatake, R. J. *et al.* Identification of pharmacological inhibitors of the
23 MEK5/ERK5 pathway. *Biochem. Biophys. Res. Commun.* (2008).
24 doi:10.1016/j.bbrc.2008.09.087
- 25 19. Yang, Q. *et al.* Pharmacological inhibition of BMK1 suppresses tumor growth

- 1 through promyelocytic leukemia protein. *Cancer Cell* (2010).
2 doi:10.1016/j.ccr.2010.08.008
- 3 20. Pello, O. M. *et al.* Role of c-MYC in alternative activation of human
4 macrophages and tumor-associated macrophage biology. *Blood* **119**, 411–421
5 (2012).
- 6 21. Li, L. *et al.* A unique role for p53 in the regulation of M2 macrophage
7 polarization. *Cell Death Differ.* (2015). doi:10.1038/cdd.2014.212
- 8 22. Yin, X., Giap, C., Lazo, J. S. & Prochownik, E. V. Low molecular weight
9 inhibitors of Myc-Max interaction and function. *Oncogene* (2003).
10 doi:10.1038/sj.onc.1206641
- 11 23. Giurisato, E. *et al.* Myeloid ERK5 deficiency suppresses tumor growth by
12 blocking protumor macrophage polarization via STAT3 inhibition. *Proc. Natl.*
13 *Acad. Sci. U. S. A.* (2018). doi:10.1073/pnas.1707929115
- 14 24. Regan, C. P. *et al.* Erk5 null mice display multiple extraembryonic vascular and
15 embryonic cardiovascular defects. *Proc. Natl. Acad. Sci. U. S. A.* (2002).
16 doi:10.1073/pnas.142293999
- 17 25. Takeda, H. *et al.* Essential role of Stat6 in IL-4 signalling. *Nature* (1996).
18 doi:10.1038/380627a0
- 19 26. Gray, M. J., Poljakovic, M., Kepka-Lenhart, D. & Morris, S. M. Induction of
20 arginase I transcription by IL-4 requires a composite DNA response element for
21 STAT6 and C/EBP β . *Gene* (2005). doi:10.1016/j.gene.2005.04.004
- 22 27. Zhu, L. *et al.* TSC1 controls macrophage polarization to prevent inflammatory
23 disease. *Nat. Commun.* (2014). doi:10.1038/ncomms5696
- 24 28. Martinez, F. O. *et al.* Genetic programs expressed in resting and IL-4
25 alternatively activated mouse and human macrophages: Similarities and

- 1 differences. *Blood* (2013). doi:10.1182/blood-2012-06-436212
- 2 29. English, J. M., Pearson, G., Baer, R. & Cobb, M. H. Identification of substrates
3 and regulators of the mitogen-activated protein kinase ERK5 using chimeric
4 protein kinases. *J. Biol. Chem.* (1998). doi:10.1074/jbc.273.7.3854
- 5 30. Vaseva, A. V. *et al.* KRAS Suppression-Induced Degradation of MYC Is
6 Antagonized by a MEK5-ERK5 Compensatory Mechanism. *Cancer Cell* (2018).
7 doi:10.1016/j.ccell.2018.10.001
- 8 31. Pello, O. M. *et al.* In Vivo Inhibition of c-MYC in Myeloid Cells Impairs Tumor-
9 Associated Macrophage Maturation and Pro-Tumoral Activities. *PLoS One*
10 (2012). doi:10.1371/journal.pone.0045399
- 11 32. Perez-Madrugal, D., Finegan, K. G., Paramo, B. & Tournier, C. The extracellular-
12 regulated protein kinase 5 (ERK5) promotes cell proliferation through the down-
13 regulation of inhibitors of cyclin dependent protein kinases (CDKs). *Cell. Signal.*
14 (2012). doi:10.1016/j.cellsig.2012.08.001
- 15 33. Sureban, S. M. *et al.* XMD8-92 inhibits pancreatic tumor xenograft growth via a
16 DCLK1-dependent mechanism. *Cancer Lett.* (2014).
17 doi:10.1016/j.canlet.2014.05.011
- 18 34. De Jong, P. R. *et al.* ERK5 signalling rescues intestinal epithelial turnover and
19 tumour cell proliferation upon ERK1/2 abrogation. *Nat. Commun.* (2016).
20 doi:10.1038/ncomms11551
- 21 35. Kang, C., Kim, J. S., Kim, C. Y., Kim, E. Y. & Chung, H. M. The
22 pharmacological inhibition of ERK5 enhances apoptosis in acute myeloid
23 leukemia cells. *Int. J. Stem Cells* (2018). doi:10.15283/ijsc18053
- 24 36. Pereira, D. M., Gomes, S. E., Borralho, P. M. & Rodrigues, C. M. P.
25 MEK5/ERK5 activation regulates colon cancer stem-like cell properties. *Cell*

1 **FIGURE LEGENDS**

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

12

13 **Figure 2. M2 polarization induced by IL-4 is impaired by ERK5 inhibition.**
14 BMDMs were pretreated or not with ERK5 inhibitor, XMD8-92 (XMD, 10 μ M), for 30
15 min, and then stimulated with IL-4. (a) mRNA was obtained after 24 h, and gene
16 expression of *Arg1*, *Ym1*, *Retnla*, and *Mmp9* were determined by quantitative RT-PCR.
17 (b) Culture supernatant was collected after 48 h and CCL22, CCL17, and IGF-1 levels
18 were determined by ELISA. (c) Arginase-1-YFP expression was determined after 48 h
19 in Yarg BMDMs by flow cytometry. Data are shown as mean \pm s.e.m (n=4) and are
20 representative from 2 independent experiments. *p* values were determined using one-
21 way ANOVA followed by Bonferroni posthoc test (**p* < 0.05, ***p* < 0.01, ****p* <
22 0.001).

23

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

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

7

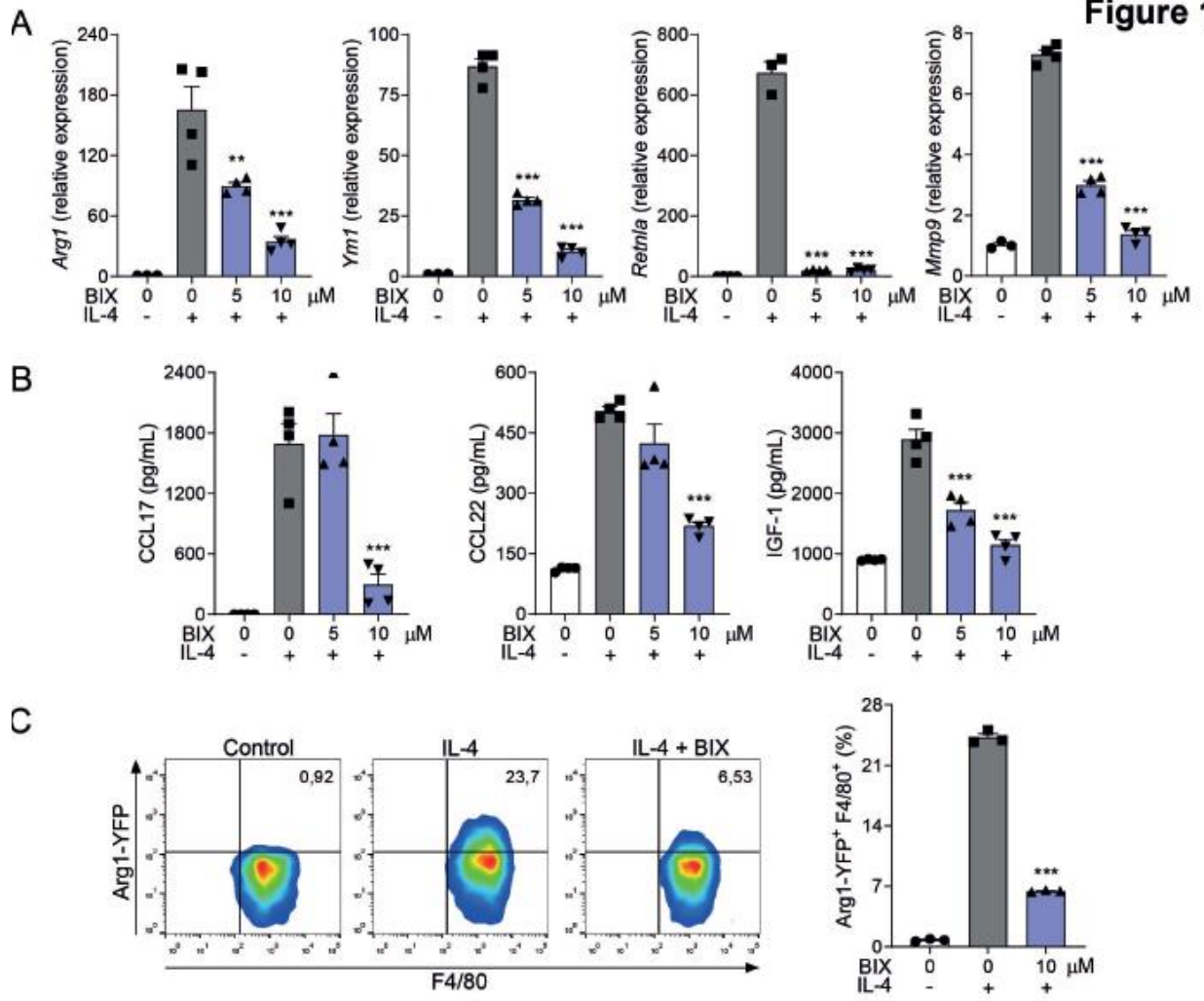
8 **Figure 4. IL-4 activates STAT6 signaling pathway in an ERK5-independent**
9 **manner.** (a) STAT6^{+/+} and STAT6^{-/-} BMDMs were stimulated or not with IL-4 (10
10 ng/mL). Culture supernatant was collected after 48 h, and CCL17, CCL22, and IGF-1
11 levels were determined by ELISA. (b) WT BMDMs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

17

18 **Figure 5. MEK5/ERK5 is crucial to c-Myc-mediated M2 polarization induced IL-**
19 **4.** (a) BMDMs were pretreated or not with MEK5 or ERK5 inhibitors, BIX (10 μ M),
20 and XMD (10 μ M), respectively, for 30 min and then stimulated with IL-4 (10 ng/mL),
21 when indicated. mRNA was obtained after 6 h, and gene expression of c-Myc was
22 evaluated by quantitative RT-PCR. (b) ERK5^{fl/fl} and ERK5 ^{Δ myc} BMDMs were
23 stimulated or not with IL-4 (10 ng/mL). mRNA was obtained after 6 h, and gene
24 expression of c-Myc was evaluated by quantitative RT-PCR. (c) WT BMDMs were
25 pretreated with the c-Myc inhibitor, 10058-F4 (F4, 30 μ M), for 30 min and them

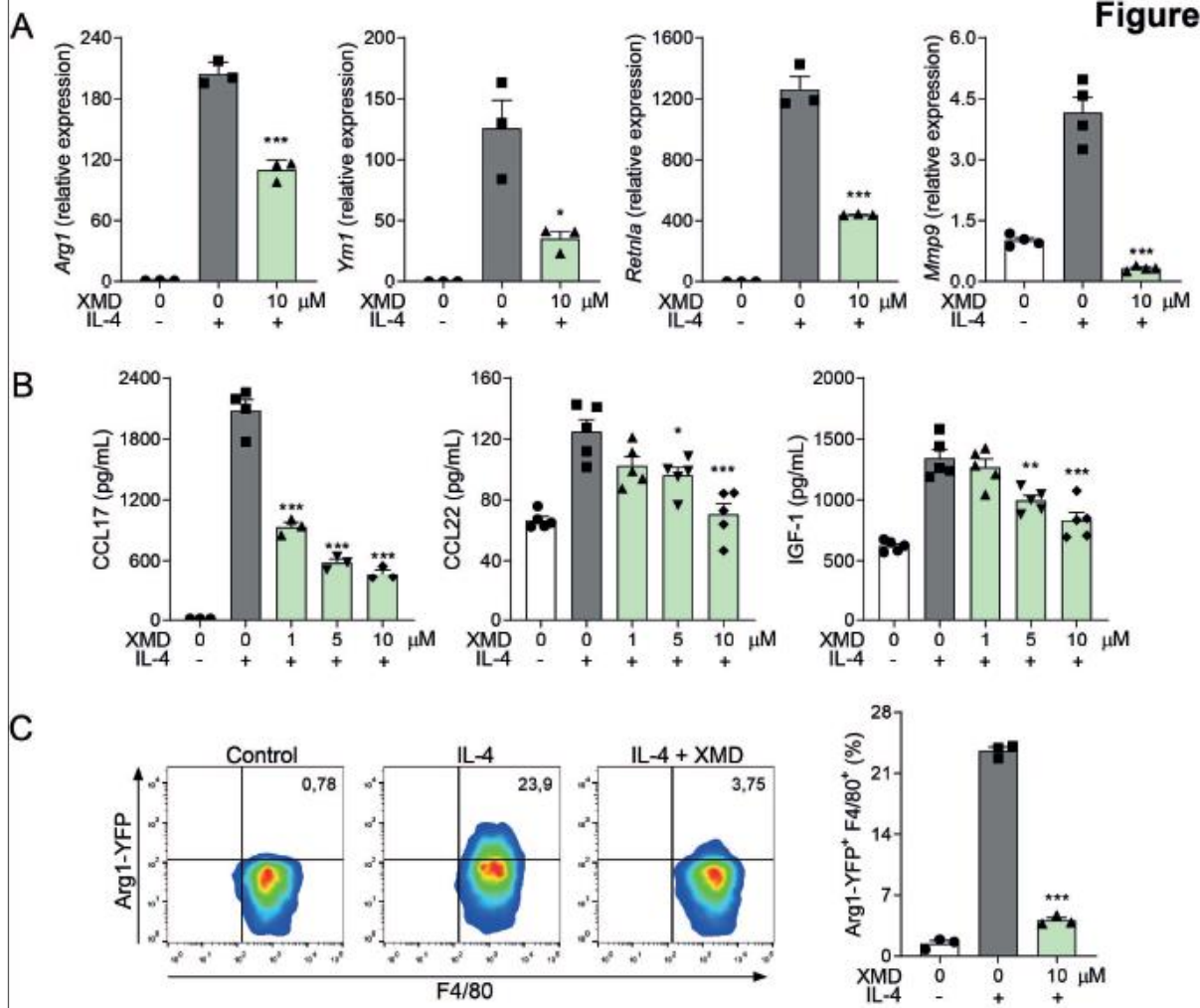
1 stimulated with IL-4. mRNA was obtained after 24 h and gene expression of M2
2 markers, *Retnla* and *Mmp9*, were evaluated by quantitative real-time PCR. (d) Culture
3 supernatant of BMDMs treated as in “c” was collected after 48 h, and CCL22, and IGF-
4 1 levels were measured by ELISA. (e) Yarg BMDMs were pretreated with F4 (30 μ M)
5 and then stimulated with IL-4 for 48 h. Arginase-1-YFP expression was assessed by
6 flow cytometry. Data are shown as mean \pm s.e.m (n=4) and are representative from 2
7 independent experiments. *p* values were determined using one-way and two-way
8 ANOVA followed by Bonferroni posthoc test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).
9

Figure 1



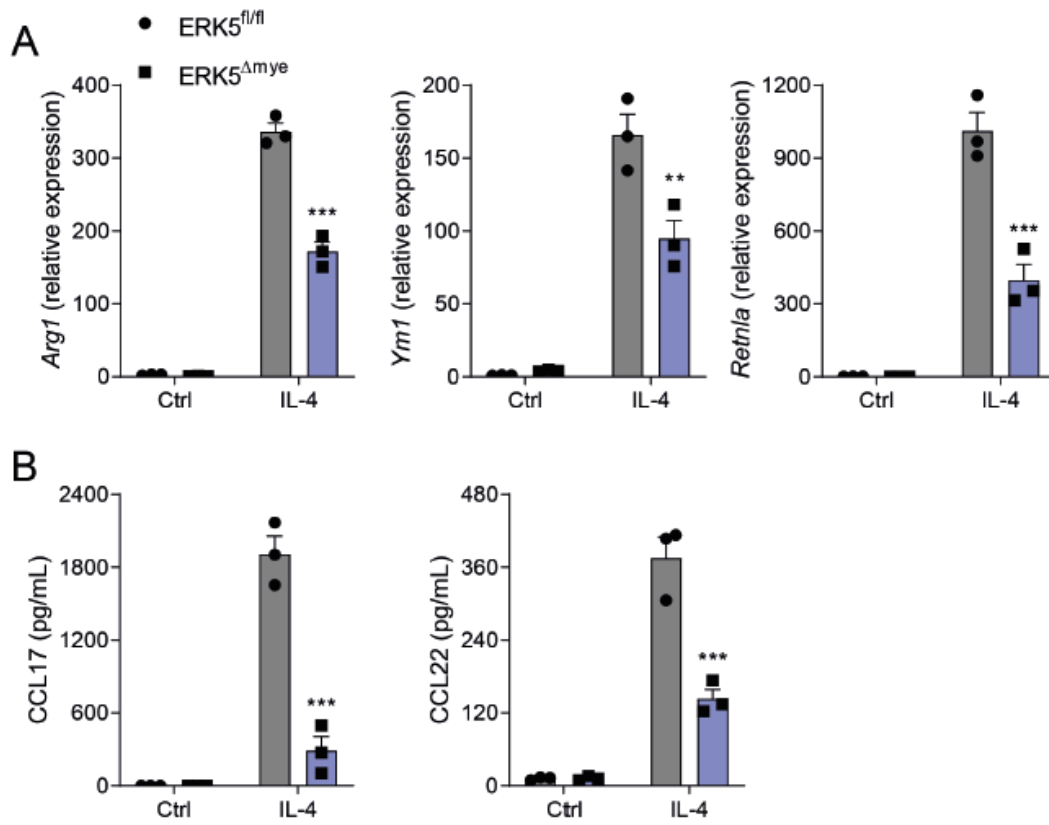
1
2

Figure 2



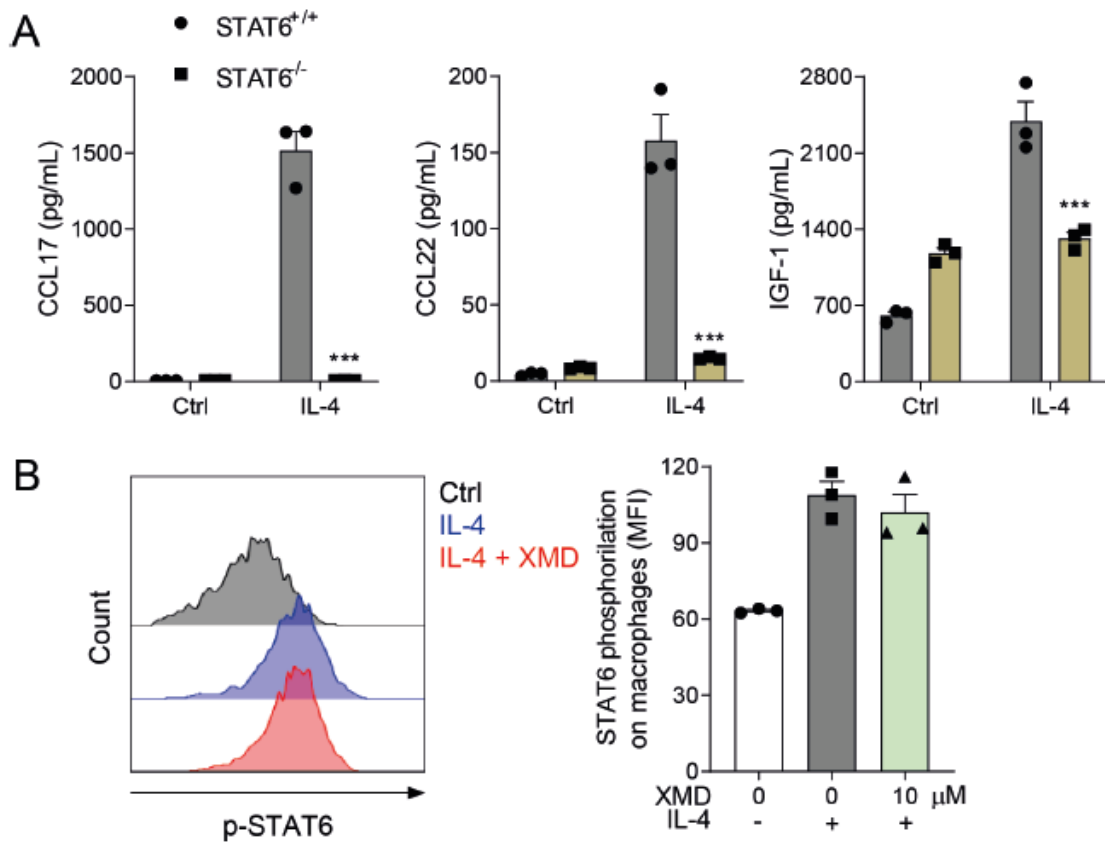
1
2

Figure 3



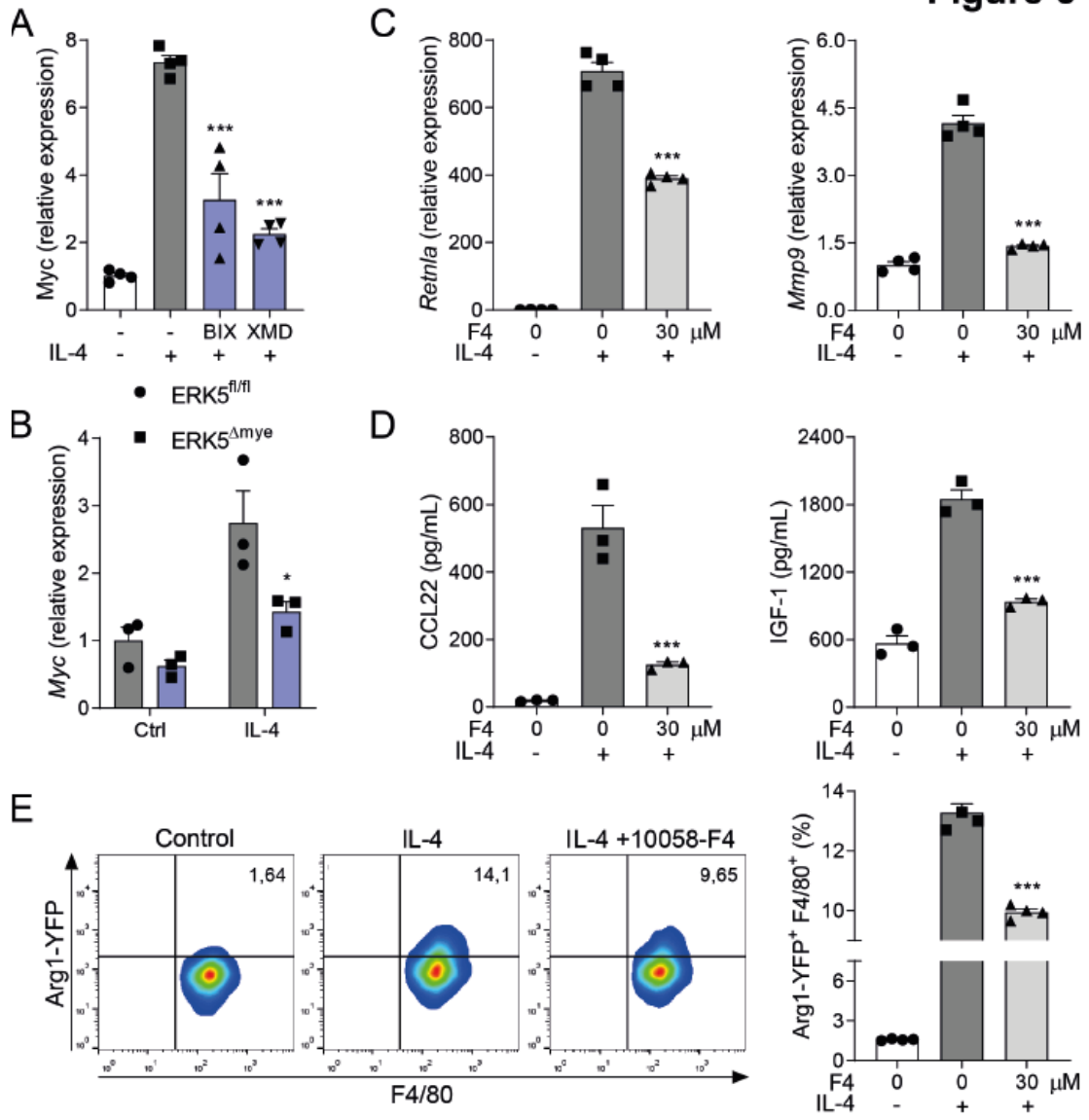
1
2

Figure 4



1
2

Figure 5



1