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Critical role of p38 MAPK in IL-4-induced alternative activation of peritoneal macrophages

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Alternative activation of macrophages plays an important role in a range of physiological and pathological processes. This alternative phenotype, also known as M2 macrophages, is induced by type 2 cytokines such as IL-4. The binding of IL-4 to its receptor leads to activation of two major signaling pathways: STAT-6 and PI3K. However, recent studies have described that p38 MAPK might play a role in IL-4-dependent signaling in some cells, although its role in macrophages is still controversial. In this study, we investigated whether p38 MAPK plays a role in the polarization of macrophages in mice. Our results reveal that IL-4 induces phosphorylation of p38 MAPK in thioglycollate-elicited murine peritoneal macrophages, in addition to STAT-6 and PI3K activation. Furthermore, p38 MAPK inactivation, by gene silencing or pharmacological inhibition, suppressed IL-4-induced typical M2 markers, indicating the involvement of p38 MAPK in the signaling of IL-4 leading to M2-macrophage polarization. Moreover, p38 MAPK inhibition blocked phosphorylation of STAT-6 and Akt, suggesting that p38 MAPK is upstream of these signaling pathways. Finally, we show that in an in vivo model of chitin-induced M2 polarization, p38 MAPK inhibition also diminished activation of M2 markers. Taken together, our data establish a new role for p38 MAPK during IL-4-induced alternative activation of macrophages.

Keywords: Alternative activation · Chitin · IL-4 · JAK/STAT · Macrophages · p38 MAPK

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Introduction

Macrophages represent an essential cell population of innate immunity that plays a critical role in inflammation and host defense as well as in the maintenance of tissue homeostasis [1]. Two major macrophage phenotypes have been characterized according to the activation by different microenvironmental signals: the classically activated macrophages (M1) and the alternatively activated macrophages (M2) [1, 2]. M1 macrophages develop in response to proinflammatory stimuli like IFN-γ or IL-1β and bacterial products such as LPS. This phenotype has antimicrobial and cytotoxic functions and is characterized by enhanced production of proinflammatory cytokines, expression of MHC class II molecules, and generation of free radicals including nitric oxide (NO). In contrast, M2 macrophages differentiate in the presence of cytokines as IL-4 or IL-13. They have enhanced capacity for endocytosis but reduced motility and cytotoxic effects, showing both anti-inflammatory activities and a tissue-repair function. In addition, these M2 macrophages express a different subset of innate immunity molecules as compared to M1 macrophages [3, 4]. Expression of arginase-1 (Arg-1), the mannose receptor (MR), and genes involved in tissue remodeling such as chitinase 3-like 3

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(Ym-1) and resistin-like molecule α (RELM-α) also known as Found in Inflammatory Zone 1 (Fizz-1), have been associated with this M2 phenotype [1, 2, 5]. Furthermore, a set of genes conserved both in mouse and human have been recently described to distinguish M2 macrophages from the other phenotypes [6], including IFN regulatory factor 4 (IRF-4), signaling modulators such as Kruppel-like factor 4 (KLF-4), suppressor of cytokine signaling 1 (SOCS-1), and transglutaminase 2 (TGM2).

A hallmark of mouse M2 macrophages is the regulation of the L-arginine metabolism. Thus, M1 macrophages release NO via upregulation of iNOS using L-arginine as a substrate, whereas M2 macrophages convert arginine to ornithine and urea through the action of Arg-1 [3, 4].

Mechanistically, IL-4-induced Arg-1 expression has been described to be dependent on activation of STAT-6 [7, 8]. Binding of IL-4 to its receptor activates protein tyrosine kinases of the JAK family, leading to phosphorylation of STAT-6 and subsequent translocation into the nucleus, where it binds to specific regions of promoters of target genes. In addition, insulin receptor substrate (IRS)-1/2 can also become phosphorylated leading, in turn, to activation of PI3K and downstream kinases such as Akt [9].

Although most of the activities for IL-4 have been classically ascribed to the activation the JAK/STAT-6 pathway, more recent studies indicate that the involvement of other signaling molecules such as MAPK are also important [10–12].

Here, we report that IL-4 induces p38 MAPK activation in thioglycollate elicited murine peritoneal macrophages, leading to the subsequent phosphorylation of STAT-6. Furthermore, our results demonstrate that upregulation of typical M2 markers (Arg-1, Ym-1, Fizz-1, TGM2, IRF-4, or SOCS-1) upon IL-4 stimulation is dependent on p38 MAPK pathway, suggesting that activation of p38 MAPK plays a key role in the polarization toward a M2 phenotype. Finally, we report the role of p38 MAPK in controlling M2 macrophage polarization in a chitin challenge in vivo model.

Results

Characterization of the alternative activated phenotype of macrophages after IL-4 stimulation

To evaluate alternative activation of macrophages after IL-4 stimulation, we examined the levels of several M2 markers by quantitative PCR. Although it is difficult to establish a consensus set of markers for alternative activation of macrophages in mice and human; Arg-1, Ym-1, and Fizz-1 have been described to be upregulated in murine IL-4-stimulated macrophages, whereas MR, IRF-4, KLF-4, SOCS-1, and TGM2 have been shown to be overexpressed in both mice and human [6]. mRNA levels of Arg-1, Ym-1, Fizz-1, MR, KLF-4, IRF-4, TGM2, and SOCS-1 were markedly increased upon IL-4 treatment (Fig. 1A and Supporting Information Fig. 1A). Upregulation of Arg-1, Ym-1, Fizz-1, IRF-4, and TGM2 mRNA by IL-4 was time dependent with an optimal induction between 6 and 12 hours (Fig. 1B and Supporting Information Fig. 1B). In addition to quantitative PCR, regulation of Arg-1 and Ym-1 was also analyzed by immunoblot using specific antibodies. Protein levels of Arg-1 were detectable after 4 h of treatment with maximal induction at 6 and 8 h (Fig. 1C), whereas Ym-1 levels increased starting at 6 h (Supporting Information Fig. 1C). Moreover, in agreement with the upregulated levels of Arg-1, an increment in arginase activity using an urea-based assay was observed in IL-4 stimulated peritoneal macrophages (Fig. 1D).

Involvement of JAK/STAT signaling pathway in M2 markers expression

The classical IL-4 signaling pathway involves phosphorylation of STAT-6 by JAKs. Additionally, Arg-1 expression has been described to be STAT-6 dependent [7, 8]. Consistent with this idea, we observed increased phosphorylated levels of STAT-6 after IL-4 stimulation on peritoneal macrophages (Fig. 2A). To further investigate the role of JAK-STAT signaling in Arg-1 expression we used a general inhibitor of JAKs (JAK-I). Treatment with JAK-I abolished IL-4-mediated phosphorylation of STAT-6 (Fig. 2B). Moreover, Arg-1 mRNA (Fig. 2C) and protein levels (Fig. 2D) also decreased in presence of JAK-I. In addition, mRNA expression levels of Fizz-1 and Ym-1 were also inhibited in cells treated with IL-4 and JAK-I (Fig. 2E) as well as Ym-1 protein expression (Fig. 2F). As control was added equivalent percentage of DMSO (Vh) to the experiments performed in the presence of the inhibitors. These results confirm the relevance of JAK-STAT pathway on the expression of M2 markers.

Polarization to alternative macrophages by IL-4 requires p38 MAPK and PI3K activation

In addition to STAT-6, JAK-mediated phosphorylation of the IL-4R also leads to activation of PI3K through the recruitment of IRS-1/2 [9]. Additionally, several recent studies have reported the involvement of p38 MAPK pathway in the activities of IL-4 [10–12]. To investigate the possible role of p38 MAPK and PI3K in Arg-1 activation, peritoneal macrophages were pretreated with vehicle (DMSO) or specific inhibitors previous to IL-4 stimulation. The specific inhibitors of p38 MAPKα and β, SB202190 (SB2) and SB203580 (SB3), markedly inhibited IL-4-induced Arg-1 mRNA expression, whereas the PI3K inhibitor LY294002 (LY2) partially suppressed Arg-1 expression (Fig. 3A). Furthermore, when we treated the IL-4-stimulated macrophages with LY303511 (LY3), an analog of LY294002 that is inactive toward PI3K [13], we have observed no significant inhibition of Arg-1 expression (Fig. 3A). Further analysis of the effects of p38 MAPK and PI3K inhibitors on the expression of the others M2 markers Ym-1, Fizz-1, TGM2, IRF-4, and SOCS-1, showed similar results (Fig. 3A and Supporting Information Fig. 2). Consistent with the effect of SB2 and LY2 on gene expression, arginase activity, and Arg-1, Ym-1, and Fizz-1 protein levels were also inhibited (Fig. 3B–D, and Supporting Information Fig. 2B). To further confirm the involvement of these

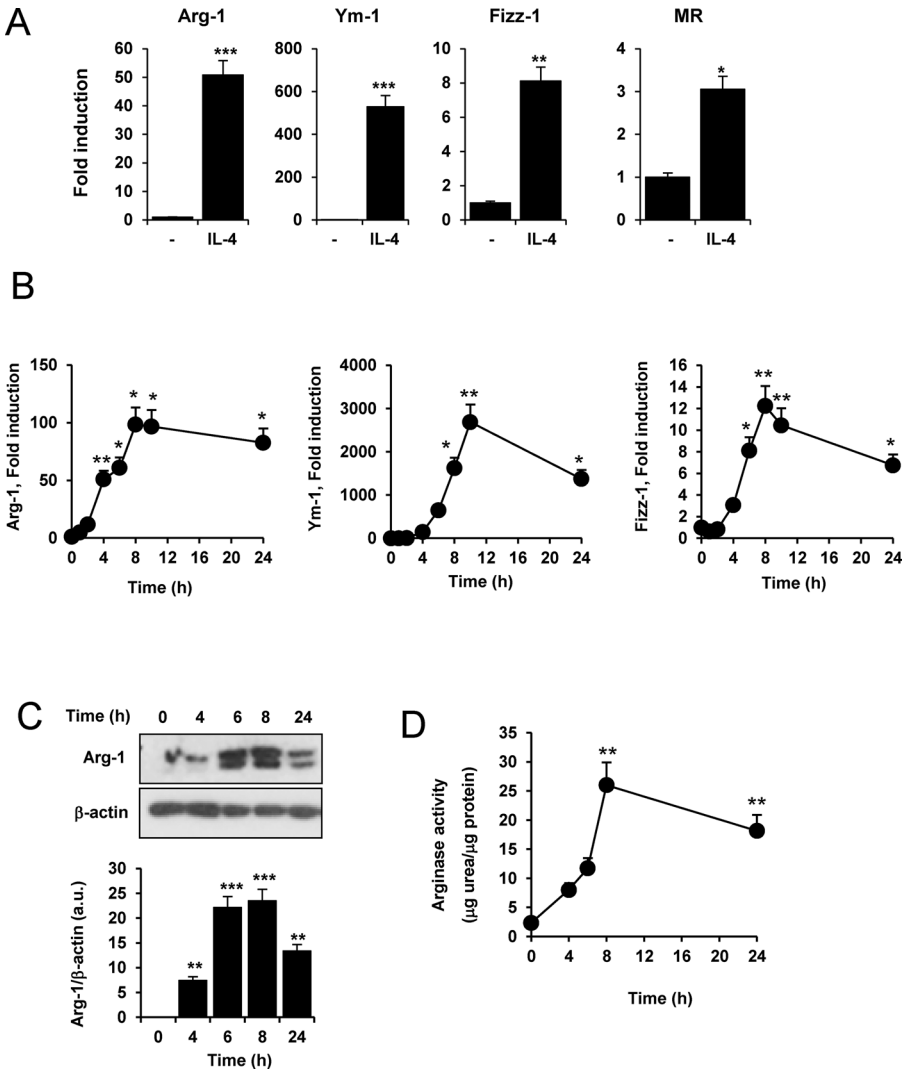

Figure 1. M2 markers expression in peritoneal macrophages after IL-4 stimulation. (A and B) Peritoneal macrophages from C57BL/6J mice were stimulated with IL-4 (20 ng/mL) for (A) 6 h or (B) 1, 2, 4, 6, 8, 12, and 24 h. The expression of Arg-1, Ym-1, Fizz-1, and MR was evaluated by quantitative PCR. Data are shown as mean + SD and are pooled from three independent experiments with $n = 3$ replicates. (C) Protein levels of Arg-1 were evaluated by Western blot after stimulation of peritoneal macrophages with IL-4 for the indicated times. Blot is representative of three independent experiments. Band intensity of Western blots was analyzed by densitometry, normalized to β-actin levels. Graph show the mean + SD of the fold change from control condition and are pooled from three independent experiments, performed in triplicate. (D) Peritoneal macrophages were stimulated with IL-4 for the indicated times. The Arg-1 activity was evaluated by the urea production. Data are shown as mean + SD and are pooled from three independent experiments with $n = 3$ replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ with respect to control condition; Student's t-test.

signaling pathways on the expression of M2 markers triggered by IL-4, we analyzed by Western blot the phosphorylation status of p38 MAPK and Akt, as a downstream target from PI3K activation. Consistently with the pharmacological sensitivity observed, p38 MAPK and Akt were substantially phosphorylated when stimulated with IL-4 (Fig. 3E and F). Phosphorylation levels of p38 MAPK in IL-4-stimulated cells were elevated after 15 min, whereas peak phosphorylation levels of Akt occurred after 60 min and decreased thereafter. In order to exclude the possibility that the inhibitory effects of SB2 may be a cell-type specific effect, we stimulated the murine macrophage cell line J744 with IL-4 in the presence or absence of p38 MAPK inhibitor SB2. mRNA expression levels of Arg-1, Ym-1, Fizz-1, and SOCS-1 were downregulated in IL-4 stimulated cells after SB2 treatment (Supporting Information Fig. 3A). As control, we observed p38 MAPK activation after IL-4 treatment on the J774 cell line (Supporting Information Fig. 3B).

Together with p38 MAPK activation, IL-4-stimulation has been described to induce ERK and JNK phosphorylation [10, 11]. To determine whether these signaling pathways are also involved in the M2 phenotype induction, we examined the effect of the ERK inhibitor PD98059 and JNK inhibitor SP600125. Neither PD98059 nor SP600125 had a significant effect on Arg-1 and Ym-1 expression in IL-4 stimulated macrophages (Supporting Information Fig. 4). These data indicate that p38 MAPK and PI3K signaling cascades are involved in the acquisition of M2 markers on peritoneal macrophages subjected to IL-4 stimulation.

p38 MAPK regulates STAT-6 and PI3K pathways

STAT-6 and PI3K pathways have been described to act independently, but in parallel to drive the transcription of STAT-6 responsive genes such as Arg-1 [14]. To further understand the mechanism of p38 MAPK in alternative activation of macrophages, we pretreated the cells with SB2 and LY2 following by IL-4 stimulation. As expected, inhibition of PI3K suppressed phosphorylation of Akt but did not prevent IL-4-induced STAT-6 phosphorylation (Fig. 4A), confirming the independence of both pathways. In addition, LY2 had not effect on p38 MAPK phosphorylation. Use of LY3 as a negative control of PI3K activation neither

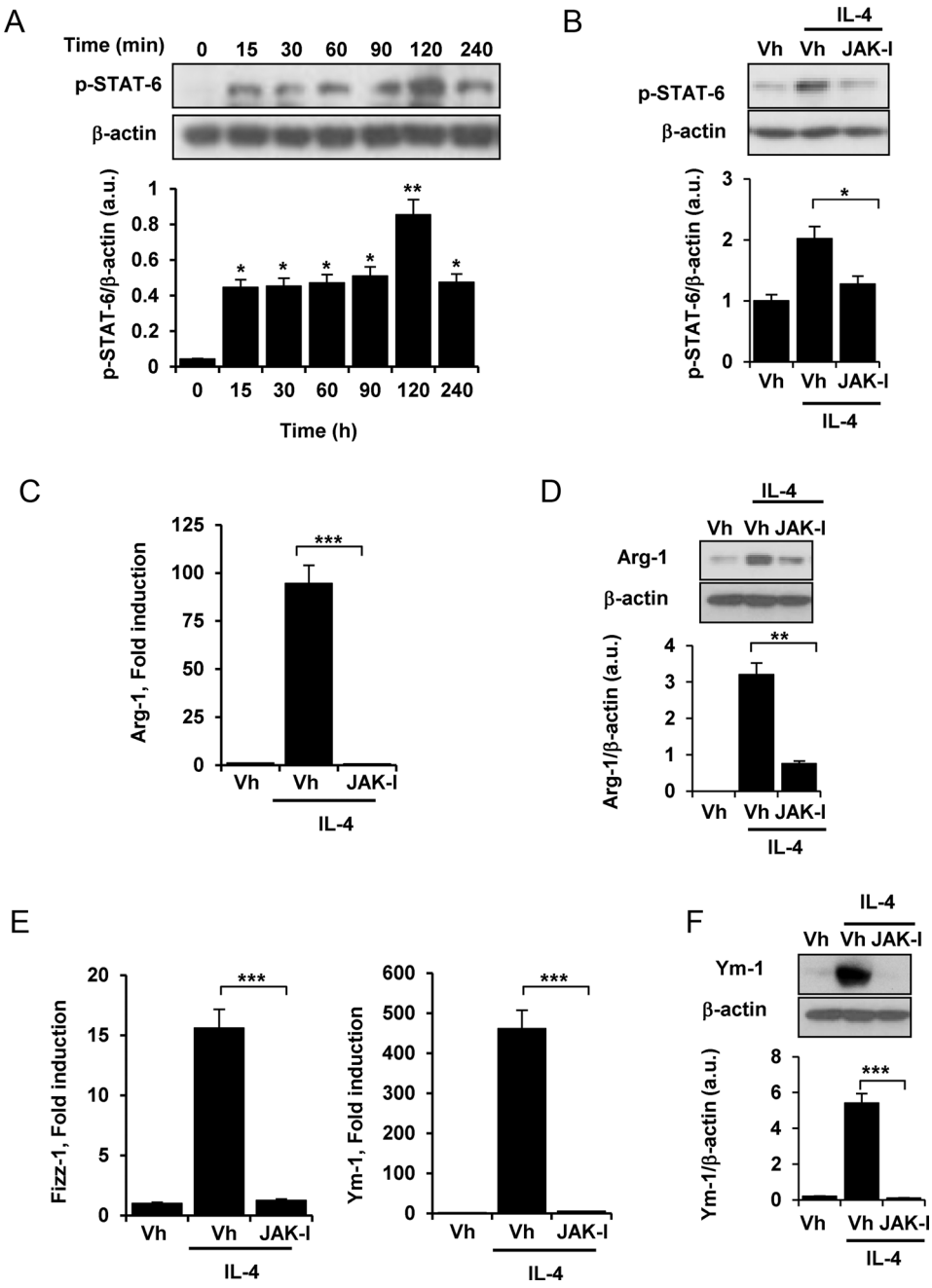

Figure 2. IL-4-induced M2 markers expression involves activation of JAK/STAT signaling pathways. (A and B) Peritoneal macrophages from C57BL/6J mice were treated (A) with IL-4 (20 ng/mL) for the indicated times or (B) with JAK-inhibitor I (JAK-I, 1 μM) for 30 min, followed by stimulation with IL-4 for 60 min. The phosphorylation STAT-6 (p-STAT-6) levels were evaluated by Western blot. (C) Peritoneal macrophages were treated as described in (B). Arg-1 expression was determined by quantitative PCR after 8 h of IL-4 treatment. (D) Protein levels of Arg-1 were evaluated by Western blot in peritoneal macrophages subjected to JAK inhibition followed by IL-4 stimulation for 24 h. (E) Fizz-1 and Ym-1 expression was determined by quantitative PCR on peritoneal macrophages preincubated with JAK-I and stimulated with IL-4 for 8 h. (F) Ym-1 protein levels were evaluated by Western blot in peritoneal macrophages subjected to JAK inhibition followed by IL-4 stimulation for 24 h. DMSO was used as vehicle (Vh) (<0.05%). (A, B, D, and F) Blots are representative of three independent experiments. Band intensity of Western blots was analyzed by densitometry, and normalized to β-actin levels. (A–F) Data are shown as mean + SD of the fold change from vehicle condition ($n = 3$) and are pooled from three independent experiments, carried out in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ with respect to vehicle condition or IL-4 treatment when indicate. Differences were analyzed by (A) t-test, (B–F) one-way ANOVA followed by Bonferroni posttest.

modified p38 MAPK phosphorylation (Supporting Information Fig. 5). Nevertheless, pretreatment of cells with SB2 inhibited Akt activation and totally abolished STAT-6 phosphorylation (Fig. 4A). The inhibitory effect of SB2 on STAT-6 phosphorylation was also assessed by confocal microscopy. Unstimulated macrophages showed resting STAT-6 levels and cytoplasmic distribution, whereas IL-4 treatment clearly induced STAT-6 phosphorylation and translocation to the nucleus, as showed by the colocalized signal. In contrast, pretreatment with SB2 inhibited phosphorylation and nuclear translocation of STAT-6 (Fig. 4B).

We next explored whether JAKs are upstream of IL-4-induced p38 MAPK activation. In hematopoietic cells, IL-4 mainly binds the type I receptor leading to activation of JAK-1 and JAK-3 [9, 15]. Cells were treated with JAK-I and AG490 (a specific JAK-2 inhibitor) previous to the stimulation with IL-4. As shown in Fig. 4C, JAK-I suppressed phosphorylation of p38 MAPK, whereas AG490 had no significant effects, suggesting that JAK-2 might be not essential for the IL-4-induced p38 MAPK phosphorylation. These results indicate that p38 MAPK activation occurs downstream JAK signaling and is involved on both STAT-6 and PI3K signaling.

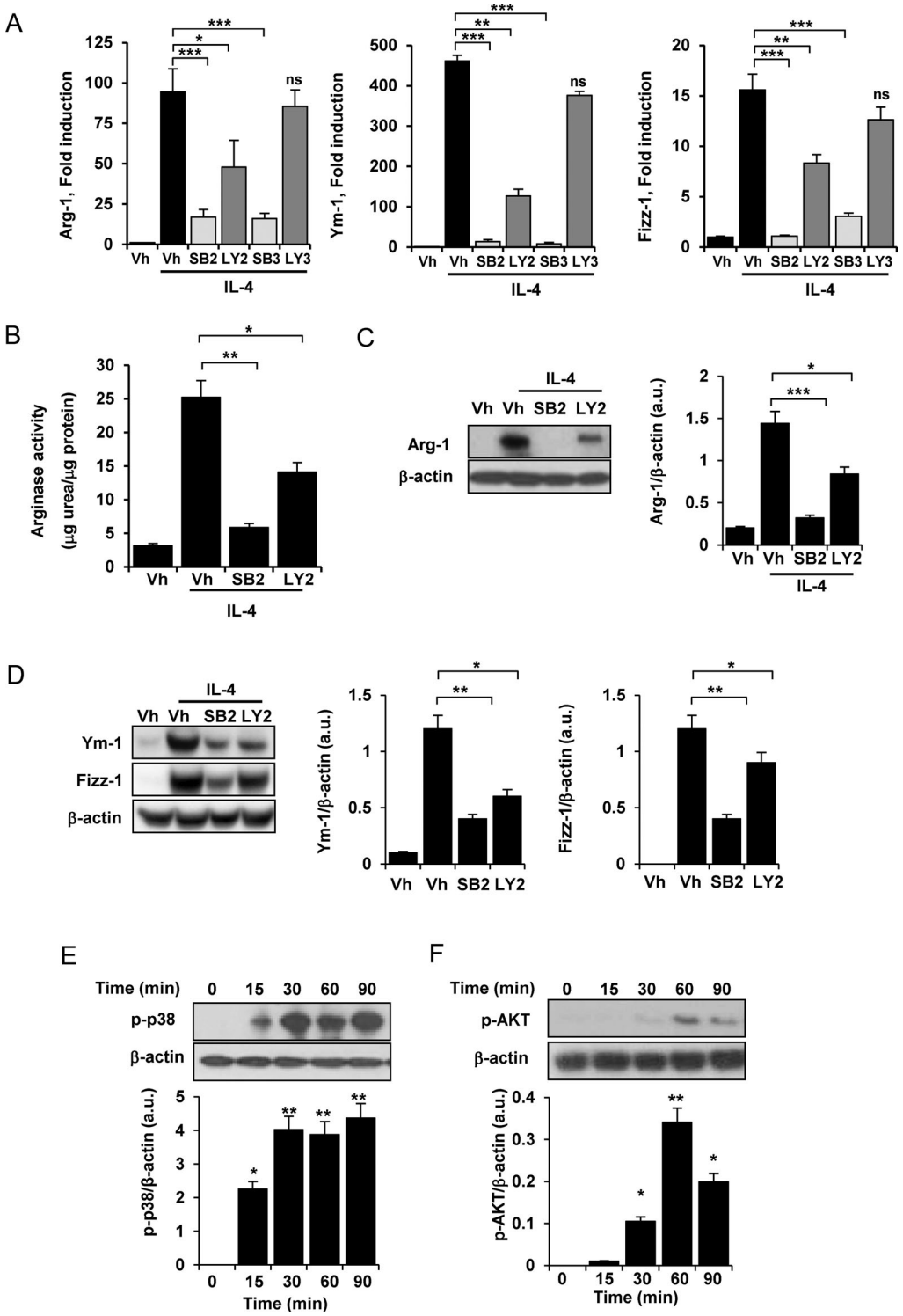

Figure 3. p38 MAPK and PI3K are involved in IL-4-induced M2 markers expression. (A) Peritoneal macrophages from C57BL/6J mice were preincubated with SB202190 (SB2, 10 μM), SB203580 (SB3, 10 μM), LY294002 (LY2, 10 μM), or LY303511 (LY3, 10 μM) for 30 min, following by stimulation with IL-4 (20 ng/mL) for 8 h. Arg-1, Fizz-1, and Ym-1 expression was determined by quantitative PCR. (B) Peritoneal macrophages were preincubated with SB2 or LY2 for 30 min and stimulated with IL-4 for 8 h. Arg-1 activity was evaluated by urea production. (C and D) Protein levels of (C) Arg-1 and (D) Ym-1 and Fizz-1 were evaluated by Western blot in peritoneal macrophages stimulated as in (B). (E and F) Phosphorylated levels of (E) p38 MAPK (p-p83) and (F) Akt (p-AKT) were evaluated by Western blot in peritoneal macrophages stimulated with IL-4 for the indicated times. DMSO was used as vehicle (Vh) (<0.05%). (C–F) Western blots are representative of three independent experiments. Band intensity of Western blots was analyzed by densitometry, normalized to β-actin levels. (A–F) Data are shown as mean + SD ($n = 3$) and are pooled from three independent experiments, performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.01$ with respect to vehicle condition or IL-4 treatment when indicate. Differences were analyzed by (A–D) one-way ANOVA followed by Bonferroni posttest; (E and F) t-test.

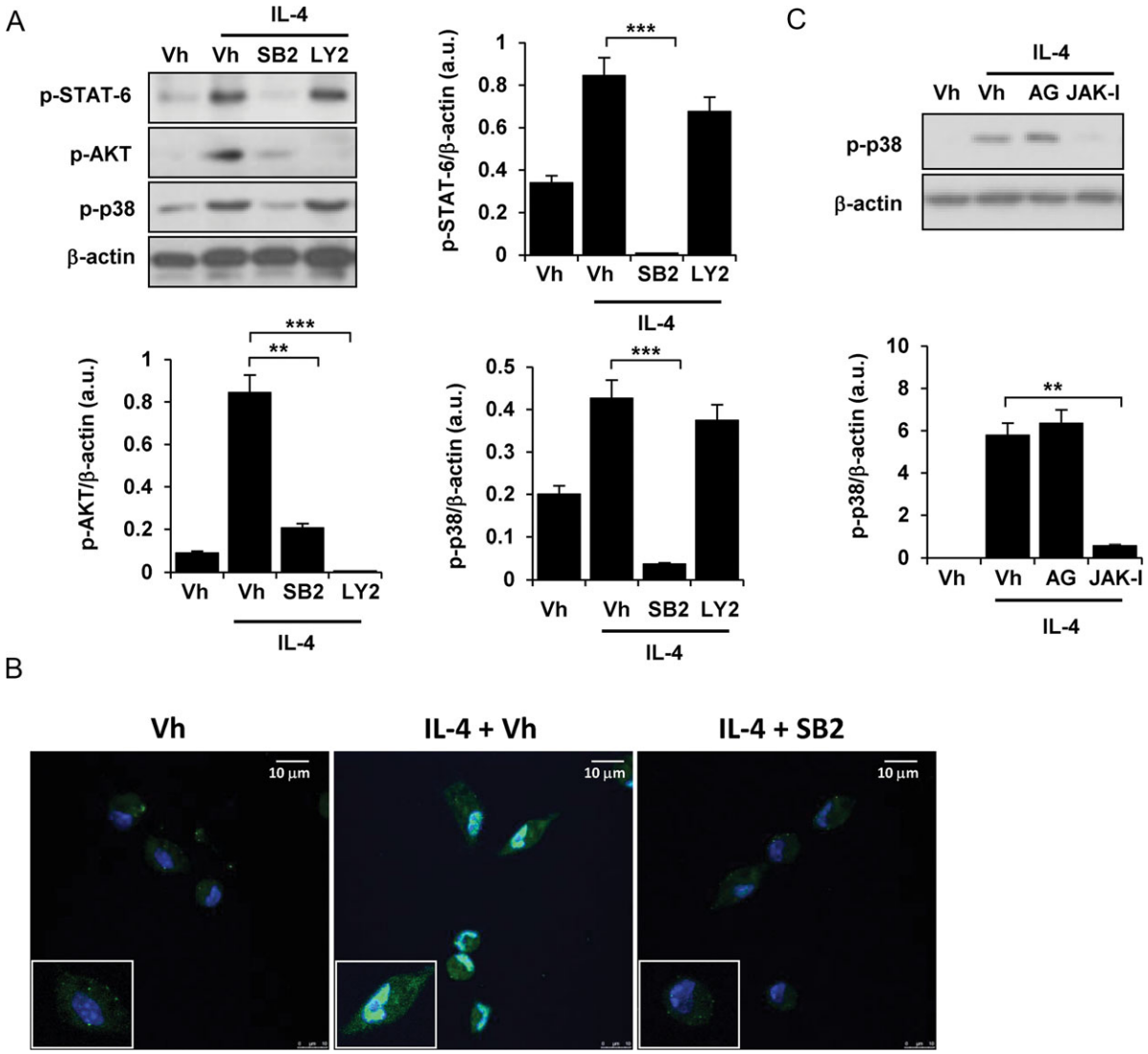

Figure 4. Phosphorylation of p38 MAPK occurs upstream STAT-6 and PI3K. (A) Peritoneal macrophages from C57BL/6J mice were preincubated with SB2 (10 μM) or LY2 (10 μM) for 30 min, following by stimulation with IL-4 (20 ng/mL) for 60 min. Phosphorylated levels of STAT-6 (p-STAT-6), Akt (p-Akt), and p38 MAPK (p-p38) were evaluated by Western blot. (B) Peritoneal macrophages were treated as in (A) and analyzed by confocal microscopy. p-STAT-6 expression and localization is shown in green and Hoescht 33 258 staining (blue) was used as a nuclear marker. Note overlapped nuclear signals on macrophages incubated with IL-4. One representative experiment out of three per group is shown. Scale bar = 10 μm. (C) Cells were preincubated with JAK-I (1 μM) and AG490 (25 μM) for 30 min, followed by stimulation with IL-4 (20 ng/mL) for 60 min. Phosphorylation of p38 MAPK (p-p38) was determined by Western blot. DMSO was used as vehicle (Vh) (<0.05%). (A and C) Western blots are representative of three independent experiments. The band intensity was analyzed by densitometry, normalized to β-actin levels. Data are shown as mean + SD of the fold change from vehicle condition, and are pooled from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.01$ with respect to IL-4 treatment. Differences were analyzed by one-way ANOVA followed by Bonferroni posttest.

Silencing of p38 MAPK suppress IL-4-induced M2 markers expression

Four isoforms of p38 MAPK (p38α, β, γ, δ) have been described, being p38α the predominant form express in monocytes and macrophages [16]. In order to confirm an essential role for p38 MAPK in the IL-4–induced polarization, we used specific siRNA to knock down p38 MAPKα in macrophages. Downregulation of p38 MAPK protein and consequently inhibition of p38 MAPK activation was verified by immunoblot analysis (Fig. 5A). Consistent with our previous results of chemical inhibition, knock down of p38 MAPK blocked Arg-1 gene expression (Fig. 5B) as well as protein amount (Fig. 5C). In all cases scrambled siRNA was used as control. In the same way, similar effects were observed for Ym-1 and Fizz-1 expression (Fig. 5D).

Deficient M2 polarization in chitin-treated mice after p38 MAPK inhibition

Finally, we asked whether p38 MAPK inhibition would impair M2 polarization in vivo. To corroborate the in vitro results, we

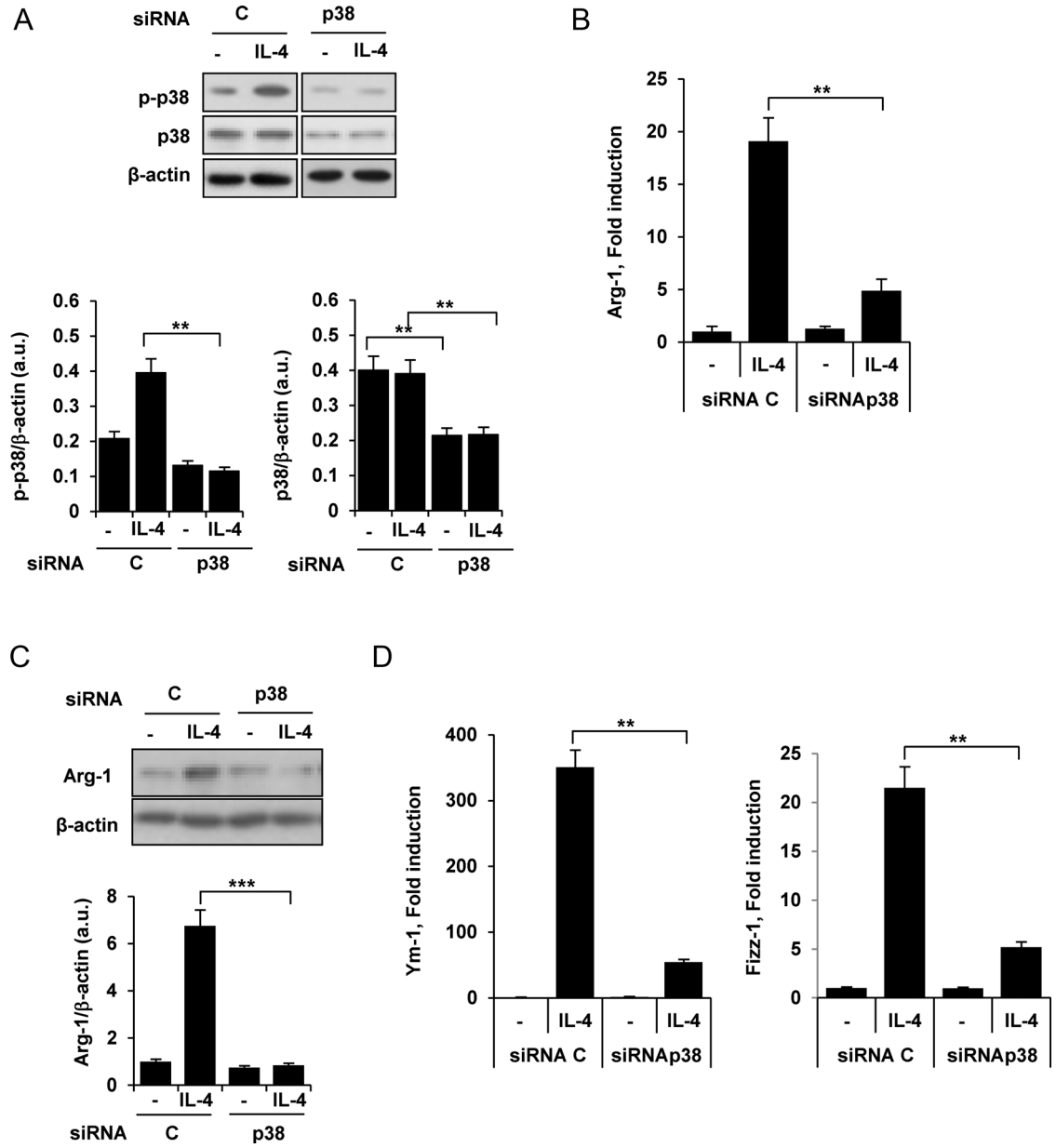

Figure 5. Silencing of p38 MAPK expression in macrophages attenuates IL-4-induced alternative polarization. (A) Peritoneal macrophages from C57BL/6J mice were transfected with control (C) or p38 MAPK siRNA and stimulated with IL-4 (20 ng/mL) for 60 min. The phosphorylated or total p38 MAPK was analyzed by Western blot. β-Actin was used as loading control. (B) Cells were transfected with control or p38 MAPK siRNA followed by stimulation with IL-4 (20 ng/mL) for 8 h. Arg-1 expression was determined by quantitative PCR. (C) Cells were treated with control or p38 MAPK siRNA, following by stimulation with IL-4 for 24 h. Protein levels of Arg-1 were determined by Western blot. (D) Cells were treated as in (B), and Ym-1 and Fizz-1 expression was determined by quantitative PCR. (A and C) Western blots are representative of three independent experiments. Band intensity of Western blots was analyzed by densitometry, normalized to β-actin levels. (A–D) Data are shown as mean + SD ($n = 3$) and are pooled from three independent experiments, each performed in triplicate. $^{**}p < 0.01$ and $^{***}p < 0.01$ with respect to control condition or IL-4 treatment when indicate. Differences were analyzed by one-way ANOVA followed by Bonferroni posttest.

used a model of M2 phenotype activation involving intraperitoneal administration of chitin that leads to the IL-4-dependent recruitment and polarization of M2 macrophages [17–19]. Chitin is a polymerized sugar and a structural component of helminths, arthropods and fungi [20]. Expression of M2 genes (Arg-1, IRF-4, KLF-4, and MR) was considerably lower in peritoneal exudate cells (PECs) from chitin mice treated with p38 MAPK inhibitor compared to controls treated with chitin alone (Fig. 6A). Furthermore, PECs analyzed by flow cytometry showed that the expression of MR on chitin-elicited macrophages ($F4/80^+$) treated with p38 MAPK inhibitor was also impaired (6.8% MR positive peritoneal macrophages on SB203580-pretreated chitin-mice versus 12% on chitin-challenged control mice) (Fig. 6B). Collectively, these findings support our model that p38 MAPK activation is important in the induction of alternative polarization of macrophages.

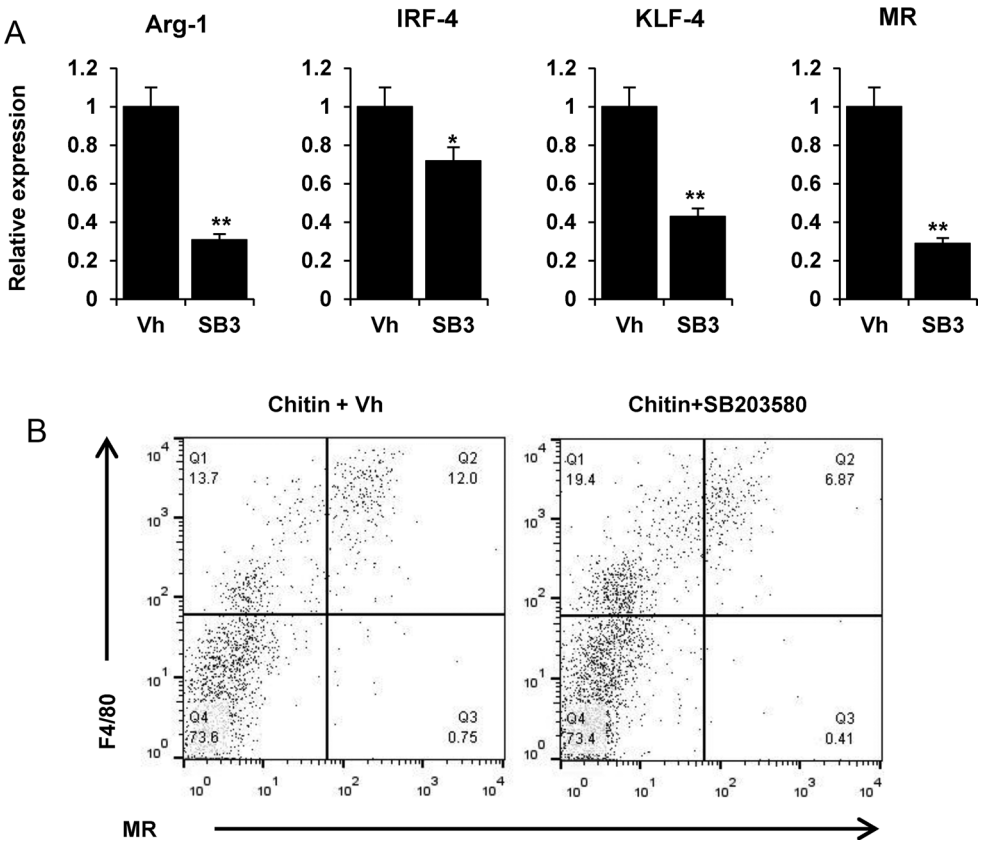

Figure 6. M2 polarization is impaired by p38 MAPK inhibition in vivo. C57BL/6J mice were subjected to 2 mg SB3 (SB203580) per kilogram body weight (b.w.) by i.p. injection in 1 mL sterile saline or equal amount of DMSO in 1 mL sterile saline for controls one hour before administration of chitin (800 ng, i.p.) (A) Peritoneal exudate cells (PECs) were collected by lavage after 48 h treatment and gene induction of M2-associated genes (Arg-1, IRF-4, KLF-4, and MR) was determined by quantitative PCR. Data are shown as mean + SD ($n = 3$) and are pooled from three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to chitin condition (t-test.) (B) Surface MR expression on F4/80^{+} cells was determined by flow cytometry. Numbers indicate the percentages of cells in each quadrant. In all cases, PECs were pooled after isolation and treatment groups consisted of four animals. Data are representative of three independent experiments.

Discussion

Macrophages are dynamic cells that can polarize to a proinflammatory phenotype M1 or an alternative M2 status showing different physiological functions [1, 2]. Whereas M1 macrophages produce proinflammatory cytokines (TNF-α, IL-6, or IL-1β) leading to a potent antimicrobial and cytotoxic phenotype, M2 macrophages are cells with strong anti-inflammatory effects involved in immune regulation, tissue remodeling, parasite killing, and tumor promotion [3, 4]. Maintenance of the balance M1 versus M2 macrophages is essential for homeostasis, as excessive presence or activity of one phenotype may lead to the development of different pathologies. Deciphering the mechanism by which these phenotypes are induced is, therefore, of fundamental importance.

In the current study, we described a role for p38 MAPK in IL-4-induced alternative activation of thioglycollate-elicited peritoneal macrophages. Using well-established markers for alternative activation, we provide evidence that IL-4-mediated upregulation of M2 markers is significantly inhibited in the presence of pharmacological p38 MAPK inhibitors (SB202190 and SB203580) or after p38 MAPK silencing. Moreover, IL-4 treatment of macrophages resulted in the phosphorylation of p38 MAPK. In addition, in vivo administration of p38 MAPK inhibitor to a model of chitin-induced M2 polarization diminished the expression of M2 markers on peritoneal macrophages, thus supporting the important role of this kinase in the activation of the M2 phenotype. Finally, our results demonstrate that p38 MAPK activation acts upstream of STAT-6 and Akt.

IL-4 plays an important role in the activation of an alternative phenotype of macrophages. Our results confirmed that in thyoglicollate-elicited peritoneal macrophages; IL-4 stimulation induced upregulation of classical M2 markers as Arg-1, Ym-1, and Fizz-1; but also demonstrated the induction of MR, KLF-4, IRF-4, SOCS-1, and TGM2, established as new M2 markers, both in mouse and human [6]. Induction of these genes was time dependent with a peak of activation between 6 and 12 h. These results stand in apparent contrast to those of Heller et al., and Weisser et al., [21, 22], that have shown a peak of Arg-1 induction later than 8 h in bone marrow derived macrophages (BMDM). However, according to our findings Broadhurst et al.

[23] have reported similar expression profile for Arg-1 on BMDM. These different findings may stem from the intrinsic phenotypic differences between peritoneal macrophages and BMDM based on their origins and specific conditions applied to generate the culture. Furthermore, our results are supported by the fact that IRF-4 and TGM2 also showed a similar pattern of expression.

Cellular responses to IL-4 are mediated by two different types of IL-4 receptor complex, type I and type II [15]. In hematopoietic cells, the type I receptor comprising the IL-4Rα and the common γ (γc) chain is mainly expressed. Indeed, analysis of the relative abundance of these receptors in peritoneal macrophages and J744 cells showed that IL-4Rα and γc were much higher expressed than IL-13Rα1 (data not shown). Binding of IL-4 to the type I IL-4R activates JAK kinases (JAK-1 and JAK-3), which then stimulate two different signaling pathways involving either STAT-6 or PI3K [9, 15]. Expression of the typical M2 markers (Arg-1, Ym-1, Fizz-1, and IRF-4) has been described to be dependent on STAT-6 activation [5, 24, 25]. Indeed, our study confirmed the involvement of type I receptor and the JAK1–3/STAT signaling pathway in the activation of these genes. Treatment of macrophages with JAK-I inhibitor blocked Arg-1, Ym-1, and Fizz-1 expression in IL-4-stimulated macrophages.

In addition to the classical pathways activated by IL-4, recent studies have proposed that MAPK might play a role in IL-4 signaling pathways. Indeed, it has been reported that both IL-4 and IL-13 induced ERK and JNK phosphorylation in human smooth muscle cells [10, 26]. Nevertheless, the role of p38 MAPK signaling seems to depend on the cell type [27]. While it has been described that IL-4 induces p38 MAPK activation in T and pro-B cells lines, epithelial cells, dermal fibroblast, and renal carcinoma cell lines [28–32], its role in monocytes/macrophages is still controversial. Thus, some authors have reported that IL-4 is unable to activate p38 MAPK in murine macrophages and human monocytes [27, 33, 34]. In contrast, others studies have demonstrated that IL-4 enhances the levels of p38 MAPK in the THP-1 human cell line and in the P388D1 mouse macrophage cell line [35, 36]. Our study showed that IL-4 induced p38 MAPK activation in thyoglicollate elicited peritoneal mouse macrophages and in the mouse macrophage cell line J744. In contrast, ERK and JNK inhibitors did not show significant effects on IL-4-induced M2 genes, suggesting that these kinases are not essential for M2 phenotype induction, at least in peritoneal macrophages. Furthermore, we have demonstrated that JAK signaling is important in IL-4-induced p38 MAPK activation as treatment with JAK-I inhibitor abolished phosphorylation of p38 MAPK. Although, this effect was not observed in the presence of the JAK-2 inhibitor (AG490), future experiments should be carried out in order to exclude the requirement of JAK-2 for p38 MAPK activation.

In addition, we found that pharmacological inhibition of p38 MAPK by SB202190 and SB203580 or using specific siRNA suppressed the expression of typical M2 markers like Arg-1, Ym-1, Fizz-1, TGM2, IRF-4, or SOCS-1 after IL-4 treatment. In line with our findings, it has been previously described that adenosine alone or in combination with IL-4 increased p38 MAPK activation in macrophages leading to upregulation of Arg-1 expression. However, these authors reported no effect on p38 MAPK activation by IL-4 alone [34]. Moreover, a recent report demonstrated that p38 MAPK inhibition reduced the levels of the anti-inflammatory markers MR and Arg-1 on phosphatase calcineurin-deficient macrophages [37]. Despite these contributions, a link between IL-4, p38 MAPK and STAT-6 in M2 polarization of macrophages had not been well established. Our data not only demonstrated a decrease in STAT-6 phosphorylation and Arg-1 expression after silencing or chemical inhibition of p38 MAPK on macrophages activated with IL-4, but also showed the involvement of p38 MAPK in the regulation of other M2 markers such as Fizz-1 and Ym-1, and more interestingly in the activation of TGM2, IRF-4, or SOCS-1. Indeed, there is little data in the literature on the role of p38 MAPK in the regulation of these genes in macrophages. A previous study described that p38 MAPK inhibition suppressed the expression of IRF-3 mRNA in the macrophage-like cell line C7 [38], and recently it has been described that IRF-4 upregulation in CD4+ T cells was prevented by SB203580 [39], however to our knowledge, this is the first report about the involvement of p38 MAPK in the regulation of IRF-4 in macrophages.

In addition, when we used an in vivo model of M2 polarization via administration of chitin, we observed that p38 MAPK inhibition reduced M2 gene expression on peritoneal isolated macrophages, thus supporting the role of p38 MAPK in IL-4 signaling.

In our study, we also detected phosphorylation of Akt by IL-4 used as a target of PI3K activation. These results suggest that IL-4 can efficiently activate both STAT-6 and PI3K-dependent signaling pathways in peritoneal macrophages. Our data are in accordance with previous studies showing that activation of the PI3K pathway resulted in increased polarization of M2 macrophages [40]. However, the induction of Arg-1, Ym-1, and Fizz-1 expression was only partially affected by the PI3K inhibitor (LY294002), with no effects by the inactive analog LY303511 used as negative control. In addition, LY294002 also partially blocked expression of TGM2, IRF-4, and SOCS-1, indicating the relevance of PI3K in the activation of these genes. Moreover, inhibition of p38 MAPK activation by SB202190 completely blocked Akt phosphorylation. Based on these data, we hypothesize that activated p38 MAPK might act upstream of this pathway (Fig. 7). In accordance with this, we also observed that p38 MAPK inhibition blocked phosphorylation and nuclear translocation of STAT-6.

Although further studies are required to determine the detailed mechanism by which IL-4 targets p38 MAPK signals, our data clearly indicate that IL-4 induces upregulation of M2 markers in peritoneal macrophages through a mechanism that is dependent of p38 MAPK activation and subsequent phosphorylation of STAT-6. Consistent with our findings, Bhattacharjee et al. [41] have reported that in peripheral blood monocytes, IL-13 induces the activation of a Src family tyrosine kinase, namely Hck that regulates p38 MAPK tyrosine phosphorylation via the upstream kinases MKK3/6. In addition they found that this Src tyrosine kinase-mediated activation of p38 MAPK is important for serine phosphorylation of STAT-1 and 3 in alternative activated monocytes [42]. Although there is no conclusive data about the involvement of this Src tyrosine kinase in IL-4-stimulated monocytes or

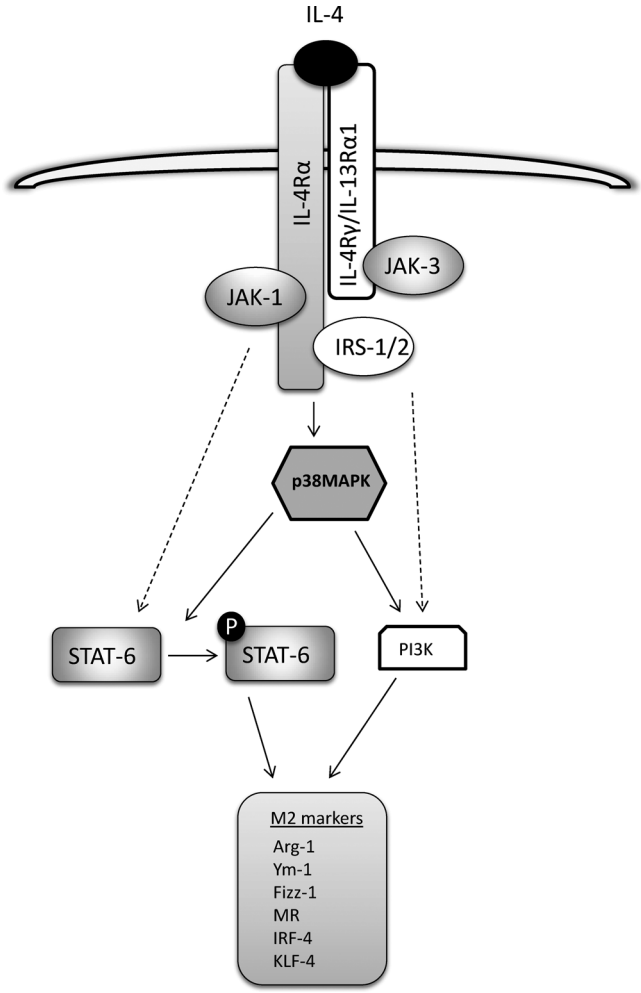

Figure 7. Proposed model for the role of p38 MAPK in IL-4-induced alternative activation of macrophages. IL-4 binds IL-4R leading to the activation of JAKs and phosphorylation of p38 MAPK, which stimulate two different signaling pathways (STAT-6 or PI3K). These signaling events lead to the macrophage polarization toward the M2 phenotype, which is characterized by the expression of the related markers Arg-1, Ym-1, Fizz-1, MR, KLF-4, and IRF-4. Dotted lines represent other hypothetical mechanisms of regulation reviewed in the Discussion section

STAT-6 activation, these authors suggest that this mechanism would be general within the alternative activation pathway. In addition, the p38 MAPK and Akt pathways have been proposed to interact at different levels [43]. Indeed, in line with our data, genetic ablation of p38 MAPKα or pharmacological inhibition with SB202190 suppressed Akt activation in mouse macrophages independently of PI3K [44]. In this report, the mechanism of p38 MAPK-Akt link involved MAPK-activated protein kinase-2 (MK2)-mediated activation of Akt via phosphorylation on Ser^{473} [45]. Interestingly, it has been previously reported that Akt can exist in a signal complex with p38 MAPK and MK2 [46] and inhibition of p38 MAPK suppressed MK2-mediated Akt phosphorylation in angiotensin II–treated smooth muscle cells [47]. Thus, the mechanisms proposed by these authors would explain the link between p38 MAPK and STAT-6/Akt, although involvement of Src kinases or MK2 in regulating IL-4-mediated alternative activation of monocytes/macrophages must be fully explored.

Finally, these results may provide evidence of a general mechanism for the IL-4 signaling, facilitating the future development of more effective therapeutic agents. Indeed, IL-4 and STAT-6 signaling are key components in the development of several respiratory pathologies including asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis, that show an excessive presence of M2 macrophages [48]. Moreover, recent evidence confirmed an increase in p38 MAPK activation in severe asthmatics and COPD patients [49, 50], suggesting that p38 MAPK might be a potential target for the treatment of pathologies with an excess of alternative macrophages.

Materials and methods

Animals

All procedures involving animals were carried out in accordance with European Union guidelines and the Declaration of Helsinki principles for the handling and use of laboratory animals. Studies were performed on male C57BL/6J mice. Mice were housed four per cage in a controlled environment (12 h light/dark cycle at 21°C) with free access to water and food.

Cell culture and preparation of elicited peritoneal macrophages

Peritoneal macrophages were elicited by intraperitoneal injection of 2.5 mL 3% thioglycollate (Becton-Dickinson) in distilled water and were prepared as previously described [51]. Cells were seeded at $1 \times 10^6/cm^2$ in RPMI containing 10% FBS. Nonadherent cells were removed 2 h after seeding by extensive washing with medium.

All pretreatment protocols were conducted at 37°C for 30 min before addition of 20 ng/mL of IL-4 (Peprotech). JAK-inhibitor I (Santa Cruz) was used to inhibit all JAKs at a concentration of 1 μM. The specific JAK-2 inhibitor tyrphostin-AG490 (Sigma) was used at a concentration of 25 μM. SB202190 and SB203580 as inhibitor of p38 MAPKα and β, and the PI3K inhibitor LY294002 (all from Sigma) were used at a concentration of 10 μM. LY303511 (10 μM) was used as a negative control of PI3K activation. Mock pretreatment was performed with vehicle alone (DMSO) (maximum concentration 0.05%) and indicated on the figures. Following pretreatment, cells were treated with IL-4 for the indicated times.

Arginase activity measurement

Arginase activity was assessed in cell lysates indirectly by measuring urea concentration generated by the arginase-dependent

hydrolysis of l-arginine [52]. Briefly, cells were lysed with 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100–containing protease inhibitor mixture (Sigma) for 30 min at room temperature. Standards were prepared by serially diluting a stock of urea (Sigma) in 50 mM Tris-HCl (pH 7.5) to yield a standard range from 25 to 1500 μg/mL. Lysates and standards (25 μL) were mixed with 25 μL of 10 mM $MnCl_2$ in 50 mM Tris-HCl (pH 7.5) in a 2 mL Eppendorf tube. Tubes were then incubated for 10 min at 55°C for activation. Next, arginine hydrolysis was conducted by incubating 50 μL of the lysates and standards with 50 μL of 0.5 M l-arginine at 37°C for 75 min, followed by the addition of 400 μL stopping solution ($H_2SO_4/H_3PO_4/H_2O$ = 1/3/7, v/v/v). To measure the amount of urea in each tube, 50 μL of 9% 1-phenyl-1,2-propanedione-2-oxime (Sigma) in 100% ethanol was added to each sample and standard, and tubes were incubated at 100°C for 60 min. Tubes were placed in the dark at 25°C for 30 min. Samples and standards (100 μL/well) were transferred in triplicate to a 96-well plate, and optical density was read at 540 nm with a 690 nm correction. Sample concentrations were determined from the standard curve and converted to Arginase Units using the following formula: (Urea Produced (μg/mL)/Total Protein (μg/mL)).

Total extracts and Western blot

Cells cultured in 6-well plates were lysed at 4°C with 0.2 mL buffer A per well (0.5% Chaps, 10 mM Tris pH 7.5, 1 mM Cl_2Mg, 1 mM EGTA, 10% Glycerol, 5 mM β-mercaptoethanol) and protease inhibitor cocktail (Sigma). Protein content was assayed with the Bio-Rad protein reagent. All cell fractionation steps were carried out at 4°C. Protein extracts were subjected to SDS-PAGE (10–15% gels) and blotted onto polyvinylidene difluoride membranes (GE Healthcare), which were incubated with the following antibodies: anti-Arg-1 (sc-20150), anti-p-STAT-6 (sc-71793) (Santa Cruz Biotechnology), anti-pAkt, anti-p-p38, anti-p38 (Cell signalling), anti-Ym-1 (Stem Cell), anti-Fizz-1 (Abcam) or anti-β-actin (Sigma). After incubation with HRP-conjugated secondary antibody, protein bands were revealed with an enhanced chemiluminescence kit (GE Healthcare). β-Actin was used as a loading control. After treatment with 100 mM β-mercaptoethanol, 2% SDS in TBS and heating at 60°C for 30 min, blots were sequentially reprobed with antibodies.

RNA isolation and quantitative PCR

Total RNA was isolated from cells with Trizol reagent (Invitrogen). Quantitative PCR (SYBR Green) analysis was performed with an ABI 7500 Fast sequence analyzer as described [53]. Each sample was run in duplicate, and all samples were analyzed in parallel for the expression of the housekeeping gene 36B4 (acidic ribosomal phosphoprotein P0), which was used as an endogenous control for normalization of the expression level of target genes. Fold induction was determined from mean replicate values.

Primer used for quantitative PCR sequences were as follows:

Arg-1: TGAGAGACCACGGGGACCTG,
GCACCACACTGACTCTTCCATTC;
Fizz-1: CCATAGAGAGATTATCGTGGA,
TGGTCGAGTCAACGAGTAAG;
Ym-1: TGGAATTGGTGCCCCTACAA,
AACTTGCACTGTGTATATTG;
MR: GTAGTACCGGAGGGTGCAGA,
TTTGCATCAGTGAAGGTGGA;
36B4: AGATGCAGCAGATCCGCAT,
GTTCTTGCCCATCAGCACC

Confocal microscopy analysis

Cells were grown on coverslips. After specific treatments, samples were fixed with –20°C methanol for 10 min. Fixed cells were stained with anti-mouse p-STAT-6 antibody (sc-71793) for 1 h at room temperature, followed by incubation with FITC-conjugated goat anti-mouse for 30 min (Jackson Immunoresearch, product code 115-095-006). Nuclei of the cells were visualized using Hoescht 33258. The stained cells were examined under a confocal laser-scanning microscope (Leica TCS SP5).

Cell transfection with siRNA for p38 MAPK

Cells were transiently transfected with p38 MAPK siRNA (sc-29434) consisting of a pool of three target-specific 19–25 nt siRNAs designed to knockdown gene expression of p38 MAPKα, or control siRNA (sc-37007) using a siRNA transfection reagent (sc-295228) at 1:1 (lipid/siRNA) according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). The transfection mixture was added to cells in siRNA transfection medium (sc-36868) and incubated at 37°C for 6 h. Then, 1 mL of 2X normal growth medium was added without remove the transfection mixture. Cultures were maintained for another 24 h. Then, cells were stimulated with IL-4 for the indicated times. To evaluate the siRNA transfection efficiency and viability of transfected cells, cells were transfected with a fluorescein-conjugate control siRNA, according to the manufacturer's instructions (Santa Cruz Biotechnology). The percentage of fluorescein positive cells was then assessed by flow cytometry corresponding to around 80%. Western blot analysis was performed to determine the knockdown effect of p38 MAPK. No cytotoxic effect of siRNA was observed on cells, as determined by trypan blue exclusion assay.

In vivo M2 activation

For in vivo experiments, C57BL/6J mice were divided in two experimental groups (n = 4 in each group). Mice were subjected to 2 mg SB303580 per kilogram body weight (b.w.) by i.p. injection in 1 mL sterile saline or equivalent amount of solvent control (DMSO) in 1 mL sterile saline one hour before administration of

chitin. Chitin (800 ng, Sigma) was injected i.p. to induce recruitment and polarization of M2 cells into the peritoneum as previously described [17, 18]. PECs were collected by lavage after 48 h and gene induction was determined by quantitative PCR. Additionally, PECs were stained with F4/80 antibody to detect macrophages, and surface MR expression on F4/80$^+$ cells was analyzed by standard techniques of flow cytometry (FACS Calibur).

Statistical analysis

The data presented are shown as means ± SD of three independent experiments. Statistical significance was estimated by two-tailed unpaired Student's *t* test for comparison between two groups. For comparison between two or more groups, one-way ANOVA, followed by Bonferroni's post hoc comparisons was used. Differences were considered significant at $^*p < 0.05$. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software). For Western blots, a linear correlation was observed between increasing amounts of input protein and signal intensity.

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Abbreviations: **Arg-1**: Arginase-1 · **Fizz-1**: Found in Inflammatory Zone 1 · **IRF-4**: IFN regulatory factor 4 · **KLF-4**: Kruppel-like factor 4 · **M1**: classically activated macrophage · **M2**: alternative activated macrophage · **PEC**: peritoneal exudate cell · **TGM2**: transglutaminase 2 · **Ym-1**: chitinase 3-like 3

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