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p38 regulates the mTOR-mediated innate immune response

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Summary

The mitogen-activated protein kinase p38 plays a crucial role in the onset of inflammation. Due to their potent anti-inflammatory effects, p38-inhibitors have been evaluated in clinical studies for the treatment of inflammatory diseases, such as rheumatoid arthritis or Crohn's disease. However, most clinical trials failed to show clinical efficacy, and moreover, there was an unexpected appearance of inflammatory events under p38-inhibitor treatment in some patients. However, the underlying mechanism how inhibition of p38 may promote inflammation is largely unknown. I now show that the p38 inhibitors BIRB0796 and SB203580 augment the expression of the proinflammatory cytokine IL-12 in human monocytes or murine macrophages after stimulation with Toll-like receptor (TLR) ligands, while the production of the anti-inflammatory cytokine IL-10 is abrogated. On the molecular level, inhibition of p38 blocked the TLR-induced activation of the serine/threonine kinase mTOR, a recently identified critical regulator of IL-12/IL-10 production in the innate immune system. In agreement, direct inhibition of mTOR increased IL-12 production, while IL-10 was profoundly blocked. Activation of p38 with anisomycin or UV induced mTOR signaling, and accordingly, blocked IL-12 but enhanced IL-10 production of LPS-stimulated monocytes. Moreover, p38-mediated activation of mTOR in monocytes was dependent on TSC2, as deletion of TSC2 abolished the inhibitory effects of BIRB0796 and SB203580 on mTOR. Additionally, I observed that the isoform p38α is responsible for the activation of mTOR, as deletion of p38α in mouse macrophages blocked mTOR activation after TLR stimulation. *In vivo*, macrophage-specific knockout of p38α blocked mTOR signaling in the spleen of mice challenged with LPS and raised the IL-12 serum levels, while the levels of IL-10 were attenuated. On the functional level, inhibition of p38 or mTOR in monocytes strongly promoted the differentiation of CD4+ Th1 cells. In conclusion, we provide evidence that inhibition of p38 has proinflammatory effects in human monocytes as well as mouse macrophages and identify a novel link from p38 to mTOR that is central for the regulation of the innate immune response.

Zusammenfassung

Die Mitogen-aktivierte Proteinkinase p38 spielt eine entscheidende Rolle bei der Entstehung einer Entzündungsreaktion. Inhibitoren von p38 wurden aufgrund ihrer entzündungshemmenden Eigenschaften in klinischen Studien eingesetzt, um ihre Wirkung in Krankheiten, wie zum Beispiel Arthritis oder Morbus Crohn, zu untersuchen. Viele dieser klinischen Studien zeigten jedoch keine Wirksamkeit der Inhibitoren und darüber hinaus konnte sogar das Auftreten von Entzündungen beobachtet werden. Der grundlegende Mechanismus, der dazu führt, dass durch die Inhibierung von p38 Entzündungen auftreten können, ist bislang unbekannt. Wir zeigen hier, dass die Hemmung von p38 durch die Inhibitoren BIRB0796 und SB203580 in humanen Monozyten dazu führt, dass nach Stimulierung von Toll-like Rezeptoren (TLR) die Produktion des entzündungsfördernden IL-12 gesteigert wird, während die Expression des entzündungshemmenden IL-10 vermindert wird. Auf der molekularen Ebene kann man durch Hemmung von p38 die Aktivierung der Serin/Threonin Kinase mammalian target of rapamycin (mTOR) verhindern, deren Funktion als wichtiger Regulator der IL-12/IL-10 Produktion in der angeborenen Immunität erst vor kurzem entdeckt wurde. Direkte Inhibierung von mTOR durch Rapamycin steigert ebenfalls die Produktion von IL-12 und senkt die Expression von IL-10. Gleichzeitige Aktivierung von p38 und mTOR in Monozyten durch UV Licht und LPS senkt die Freisetzung von IL-12, während IL-10 vermehrt exprimiert wird. Darüber hinaus konnten wir zeigen, dass die Aktivierung von mTOR durch p38 über TSC2 verläuft, da in Zellen, bei denen TSC2 deletiert ist, die p38 Inhibitoren BIRB0796 und SB203580 ihre inhibierende Wirkung auf mTOR nicht mehr ausüben konnten. Darüber hinaus konnten wir durch die Verwendung von Knockout-Mausmakrophagen feststellen, dass die Untereinheit p38α für die Aktivierung von mTOR verantwortlich ist. Durch die Inhibierung von p38 oder mTOR in humanen Monozyten differenzierten CD4+Helferzellen, die zusammen mit den Monozyten inkubiert wurden, verstärkt zu Th1 Zellen. *In vivo* Experimente zeigten, dass der Makrophagen-spezifische Knockout von p38 den Gehalt von IL-12 im Serum von Mäusen steigerte, nachdem sie mit LPS infiziert wurden, während der Gehalt an IL-10 zurückging. Zusammenfassend konnten wir nachweisen, dass die Inhibierung von p38 in humanen Monozyten entzündungsfördernde Auswirkungen zur Folge hat und dass es eine bislang unbekannte Verbindung zwischen p38 und mTOR gibt, welche wichtig für die Regulation der angeborenen Immunantwort ist.

1 Introduction

1.1 The immune system

1.1.1 The innate immune system

The innate immune system is crucial for the defense against infectious microbes. It has two major functions. Firstly, it responds initially to a microbe and prevents infection of the host and secondly, it activates the adaptive immune system. The components of the innate immune system are epithelial barriers (e.g. skin), effector cells (e.g. neutrophils, macrophages, dendritic cells), cytokines, and the complement system.

The innate immune system recognizes microbes via so-called pattern recognition receptors (e.g. Toll-like receptors, Nod-like receptors, scavenger receptors) that are expressed in a wide variety of cell types. These pattern recognition receptors are stimulated by pathogen-associated molecular patterns (PAMPs), which represent characteristic microbial pathogens and are shared by classes of microbes. After receptor stimulation, an intracellular signaling pathway is started that leads to the expression of molecules important for the defense against microbes and onset of inflammation.

The first steps in the process of inflammation include the recruitment of leukocytes such as neutrophils and granulocytes to the site of infection. This is mediated by adhesion molecules expressed on endothelial cells that lead to the tethering and rolling of the leukocytes on the endothelium. Subsequently, adherent leukocytes are stimulated by chemokines to migrate through the endothelium and accumulate in tissues.

The recognition of the PAMPs by the pattern recognition receptors also promotes phagocytosis of the microbes. This process is mediated by neutrophils or macrophages. After recognition by the receptor, the plasma membrane encloses the microbe and forms an intracellular vesicle, named phagosome. After activation of the phagocytes by cell surface receptors, the phagosome fuses with the lysosome to form the phagolysosome and the phagocytosed microbes are killed by the enzymes ROS (reactive oxygen species) and NO (nitric oxide). At the same time, the activated macrophages perform two other important functions. Firstly, they secrete cytokines (TNF, IL-12 or IL-1) and secondly, they increase the expression of MHC (major histocompatibility complex) molecules and costimulators on their cell surface. Those are important steps in the onset of cell-mediated immunity (explained in detail below) [1].

1.1.2 Adaptive immune system

In contrast to the innate immune system, which recognizes many but a limited number of microbial products, the adaptive immune system recognizes a vast number of foreign substances. It is more specific than the innate immune system, has a memory function and provides a more sustained and stronger response. Two types of adaptive immunity are to be distinguished: humoral- and cell-mediated immunity.

Humoral immunity is mediated by B lymphocytes, which recognize extracellular antigens and differentiate into plasma cells to produce antibodies. These antibodies can neutralize microbes, opsonize them to promote their phagocytosis or activate the complement system. Cell-mediated immunity is mediated by T lymphocytes. One subpopulation of these T lymphocytes are the CD4+ T lymphocytes. Depending on the microbe and the cytokines produced by the APCs (antigen-presenting cells), naive CD4+ T lymphocytes may differentiate either into Th1, Th2 or Th17 cells, which all have distinct effector functions.

The response to microbes that reside within the phagosome is mediated by Th1 cells. Those are activated by APCs, which display processed antigens of endocytosed microbes on their class II MHC molecules and also provide the necessary second signals (costimulatory molecules (e.g.CD86) and cytokines) for T-cell activation. The APC can either be a dendritic cell (it presents antigen to naive T-cells during the recognition phase), a macrophage or a B lymphocyte (they present antigen to differentiated T-cells during the effector phase). APCs are important linkers of innate and adaptive immune system. One of the most important functions of an activated Th1 cell is to activate macrophages to kill phagocytosed microbes more efficiently. The activation is mediated by the secretion of the cytokine IFN-γ (Interferon-γ) and contact-mediated signals (CD40L-CD40 interaction) [1].

1.1.3 Pro- versus anti-inflammatory cytokines

Cytokines are proteins that can be produced by nearly all cells in response to microbes and other antigens. They play an important role in the regulation of inflammation, immune response and infection, as they serve to communicate information among inflammatory cells. Cytokines can be roughly divided into two classes: pro- and anti-inflammatory cytokines. While the proinflammatory cytokines (e.g. TNF, IL-1, IFN-γ, IL-12) can make a disease even worse, anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-13) can suppress the production of the proinflammatory cytokines and therefore lead to healing of the disease. TNF-α and IL-1 mediate the acute host inflammatory response. They recruit monocytes and neutrophils to the site

of infection and enable their attachment by inducing the expression of adhesive molecules on vascular endothelial cells. IL-12 links innate and adaptive immune response, being produced by dendritic cells and macrophages in response to intracellular microbes and stimulating production of IFN-γ by T-cells and NK-cells. IFN-γ in turn activates macrophages to kill phagocytosed microbes. In contrast, IL-10 inhibits host immune response or terminates the response after eradication of microbial infection by inhibiting macrophages and dendritic cells. It inhibits the production of IL-12 and down-regulates the expression of class II MHC molecules and costimulators. Thus, the ratio between pro- and anti-inflammatory cytokines affects the progress of a disease and a balance between them is a necessity in healthy organisms [1-2].

1.2 The p38MAPK

1.2.1 MAPK cascade

The MAPK (mitogen-activated protein kinase) cascade consists of three protein kinases (MAPK, MAPKK (MAPK-kinase) and MAPKKK (MAPKK-kinase)) and transmits extracellular signals to regulate intracellular processes (Figure 1) [3-4].

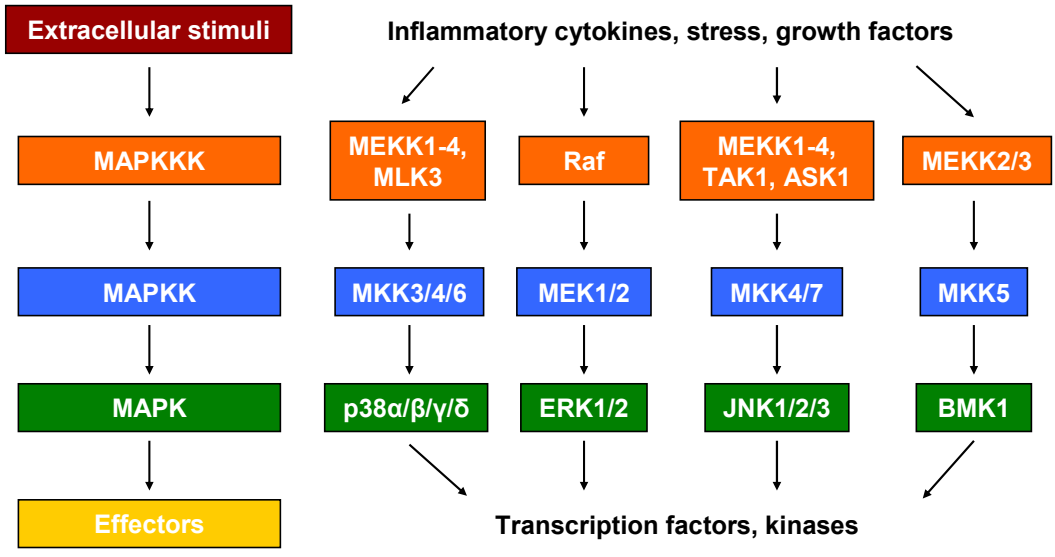

Figure 1. MAPK signaling cascade
MAPKKK can be activated by various extracellular stimuli. Afterwards, they phosphorylate MAPKK, which in turn activate a MAPK. The downstream effectors of MAPK are transcription factors and further kinases. Figure adapted from Thalhamer et al [5].

The MAPK family consists of four members, which are all serine/threonine kinases: p38, ERK (extracellular signal-related kinase), JNK (c-jun N-terminal kinase) and BMK1

(big MAPK 1) [6-7]. The MAPKs all share a dual phosphorylation motif Thr-X-Tyr and are phosphorylated and activated by their upstream MAPKKs [4, 8]. These MAPKKs can either be MEK1/2 (phosphorylate ERK), MKK3/6 (phosphorylate p38), MKK4/7 (phosphorylate JNK) or MEK5 (phosphorylates BMK1) [8]. The amino acid placed between threonine and tyrosine in the tri-peptide phosphorylation motif plays an important role in the recognition of the MAPKs by their MAPKKs [6]. ERK and BMK1 have a Thr-Glu-Tyr motif, p38 has a Thr-Gly-Tyr motif and JNK has a Thr-Pro-Tyr motif in its activation loop [8]. The MAPKKs also have a dual phosphorylation motif and are phosphorylated and activated by their specific MAPKKKs [8]. The MAPK cascade plays an important role in many cellular functions, such as cell differentiation, cell proliferation, apoptosis and cell migration [6, 8].

1.2.2 p38 MAPK subunits and signaling

There exist four different isoforms of p38, namely p38α [9], p38β [10], p38γ [11] and p38δ [12]. They have a sequence homology of more than 60 % to each other. While p38α and p38β are expressed ubiquitously, p38γ and p38δ seem to be expressed more restricted. p38γ is exclusively expressed in lung, pancreas, kidney, testis, and epidermis, while p38δ is expressed in skeletal muscle, heart, lung, and thymus. While the functions of p38α and p38β include stress response, differentiation, inflammation and proliferation, p38δ and p38γ seem to have more specialized functions [3].

The p38 family members can be activated by osmotic shock, heat, ultraviolet light, endotoxin, hormones, inflammatory cytokines and other stresses [4, 8]. The type of activation of p38 varies in different cell lines and depends on the stimulus [7]. Beside MKK3 and MKK6, MKK4 was also described to activate the p38 MAPK [12]. Another possible mode of activation is the autophosphorylation via TAB1 (transforming growth factor-β-activated protein kinase 1 - binding protein 1) [7, 13].

The four p38 isoforms have different downstream substrates and are thought to be expressed in different compartments of the same cell [3, 14-15]. The two main substrates regulated by p38 are transcription factors and protein kinases [3, 7]. MK2 (MAPK-activated protein kinase 2) was one of the first p38MAPK substrates identified [7, 16]. It plays an important role in the activation of HSP27 (small heat shock protein 27) [17] and LSP1 (lymphocyte-specific protein1) [7, 18]. Furthermore, MK2 is important for the phosphorylation and inactivation of TTP (tristetraprolin), which is an mRNA destabilizer [19-20]. In its unphosphorylated form, TTP binds to AU-rich elements of mRNAs and leads to their degradation, but when phosphorylated, TTP does not bind, resulting in stable and translatable mRNAs [19-20]. Other protein

kinases regulated by p38MAPK are PRAK (p38 regulated/activated kinase) [21], MSK1 (mitogen- and stress-activated protein kinase 1) [22] and MNK1 (MAPK signal–integrating kinase-1) [7, 23-24]. Transcription factors regulated by p38 MAPK include ATF1/2/6 (activating transcription factor 1/2/6), p53, MEF2 (myocyte enhance factor 2) and ELK1 (E-26 like protein 1) [4, 7].

1.2.3 Role of p38 MAPK in inflammation

A lot of evidence points to a central role of the p38 pathway in inflammatory processes. Most of the literature examined the role of p38α, while the other isoforms seem to play a more minor role for inflammation [14-15]. p38 is pivotal in the production of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6 [14-15, 25]. The regulation of these cytokines by p38 occurs at transcriptional and post-transcriptional level. While transcription factors regulated by p38 are responsible for the transcriptional regulation, the p38/MK2/TTP axis seems to control the post-transcriptional regulation of these cytokines by inhibiting the mRNA turnover [4, 14, 26]. Moreover, p38 designates responsible for the regulation of COX-2 (cyclooxygenase-2), a crucial enzyme during the first steps of prostaglandin biosynthesis. Prostaglandins are involved in many inflammatory reactions [27].

In addition, p38 is also involved in the expression of VCAM-1 (vascular cell adhesion molecule-1) and NO (nitric oxide) that are both important inflammatory molecules. VCAM-1 is a cell surface and adhesion molecule and it mediates the recruitment of inflammatory cells, such as lymphocytes and monocytes, from the blood to the tissue [1, 28]. p38 was shown to be involved in the post-transcriptional regulation of this molecule [28]. NO is generated by the enzyme iNOS (inducible NO synthase) in response to TNF-α and IL-1. It was shown that the p38 pathway regulates the expression of this enzyme [15, 29].

1.2.4 p38MAPK inhibitors

The p38 pathway plays an essential role in many inflammatory diseases, such as rheumatoid arthritis, psoriasis or Crohn’s disease. Due to its involvement in these diseases, the p38 MAPK has been thought to be a favorable target for anti-inflammatory drug therapy and a lot of effort has been made to generate p38 inhibitors [3-4, 30].

There exist two types of p38 inhibitors that differ in their way of binding to p38 (Figure 2): pyridinyl-imidazoles as well as diaryl urea compounds. While the pyridinyl-imidazoles, such as SB203580 ([4-(4´-fluorophenyl)-2-(4´-methylsulfinylphenyl)-5-(4´-

pyridyl) imidazole]), are ATP competitive inhibitors [31], the diaryl compounds, such as BIRB0796 (1-(5-tert-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy) naphthalen-1-yl]urea) are allosteric inhibitors [32].

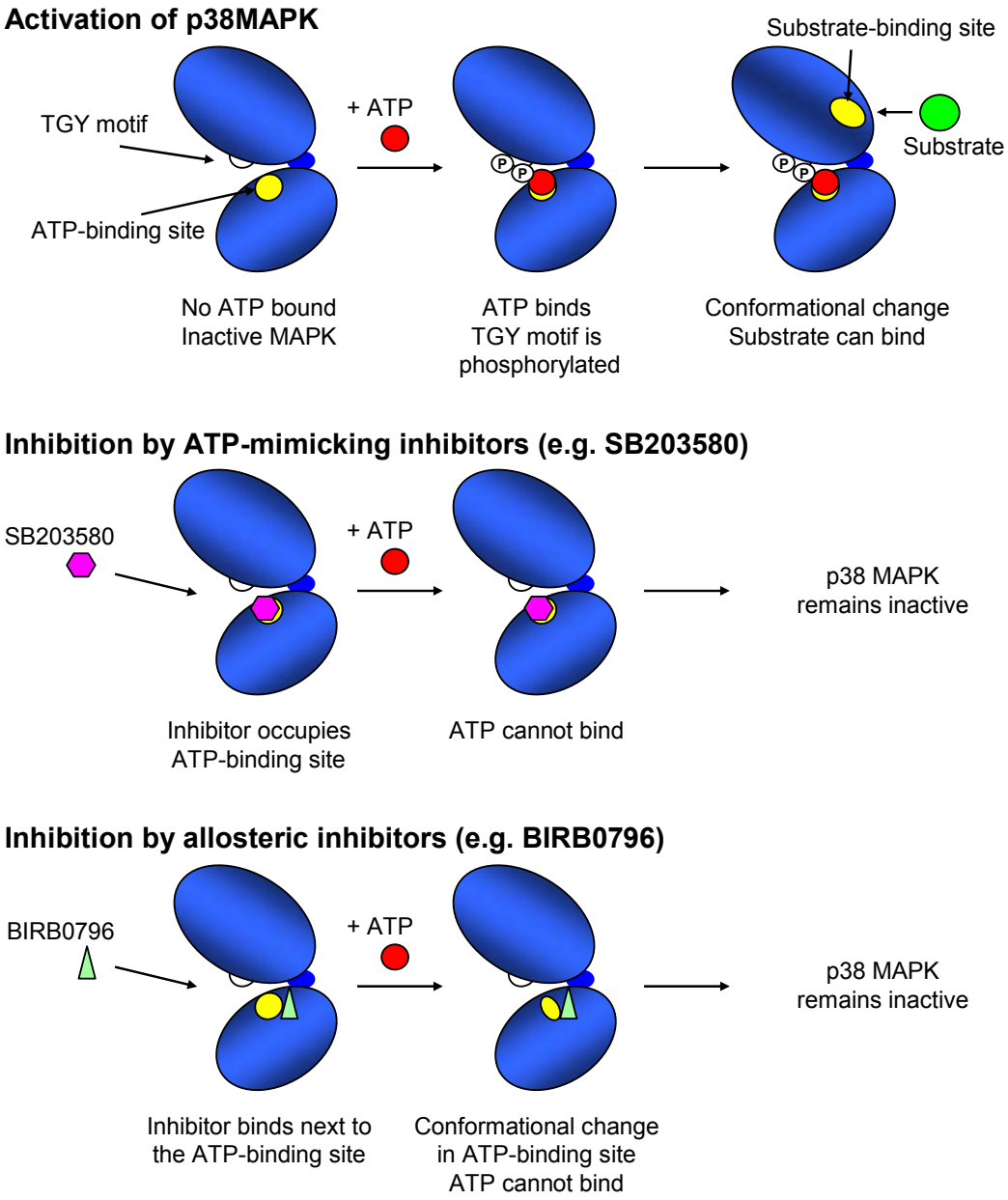

Figure 2. Mechanisms of p38MAPK activation and inhibition
ATP binds to the ATP-binding site and phosphorylates the TGY motif. This causes a conformational change, which enables the substrate to bind. SB203580 binds to the ATP-binding site and prevents binding of ATP. In contrast, BIRB0796 binds allosterically to the ATP-binding site. This induces a conformational change, which inhibits the binding of ATP. Therefore, p38 remains inactive. Figure adapted from Coulthard et al [3].

The ATP competitive inhibitors only inhibit the p38α and p38β isoforms, while they fail to inhibit the p38γ and p38δ isoforms [3]. This is due to their requirement of

methionine 109 in the ATP-binding site that is lacking in p38γ and p38δ. This methionine residue is necessary to stabilize the interaction between the ATP-binding site and the inhibitor [3, 33].

Allosteric inhibitors, such as BIRB0796, inhibit all four isoforms of p38 by indirectly competing with ATP [34]. They bind next to the ATP-binding site and cause a conformational change that is incompatible with the binding of ATP [32].

Most of the clinically developed p38 inhibitors proved efficacy in blocking the production of proinflammatory cytokines *in vitro* and in animal models. Some of the inhibitors were evaluated in early phase clinical trials and only some of them reached phase III trials. Most of the clinical trials had to be stopped because of a lack of efficacy, safety issues, or unwanted side effects. Surprisingly, some of the inhibitors even showed an unexpected appearance of inflammatory events [3-4, 14, 35-36].

1.3 The mammalian target of rapamycin

1.3.1 Role of mTOR

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase with a molecular weight of 290 kDa. It belongs to the PIKK (phosphatidylinositol kinase-related kinase) family of proteins and has important functions in survival, metabolism, growth, proliferation, and aging of a cell [37-39].

1.3.2 mTOR signaling

The mTOR pathway can be activated by both extracellular and intracellular stimuli, including nutrients, growth factors, TLR ligands and varying energy levels. One of the best examples for the activation of mTOR is the stimulation of the insulin receptor that leads to the recruitment of PI3K (phosphoinositide 3-kinase) to the plasma membrane. Subsequently, PIP2 (phosphatidylinositol 4,5-bisphosphoate) is phosphorylated by PI3K, thereby generating PIP3 (phosphatidylinositol-3,4,5-trisphosphate) [37, 40]. Afterwards, PIP3 recruits the downstream Akt, which is phosphorylated by PDK1 (3-phosphoinositide-dependent protein kinase-1) [41]. Akt, also known as PKB (protein kinase B), is one of the most important survival kinases and exists in three isoforms (Akt1, Akt2 and Akt3). They are involved in the regulation of various cellular processes, such as growth, metabolism, apoptosis and proliferation [37, 39, 42]. Downstream of Akt is TSC2 (tuberous sclerosis complex protein 2), which is a negative regulator of mTOR. TSC2 is in a complex with TSC1 and has important tumor suppressive functions [37, 39]. The TSC1/TSC2 complex blocks Rheb (Ras homolog enriched in

brain), which is an important activator of mTOR [43]. After phosphorylation by Akt, TSC2 is inactivated, leading to a loss of suppression of Rheb. Subsequently, Rheb activates mTOR [37, 39, 44]. The two best characterized downstream effectors of mTOR are 4E-BP1 (eIF4E-bindig protein 1) and p70S6K (p70 ribosomal protein S6 kinase 1) (Figure 3). Phosphorylation by mTOR restrains 4E-BP1 from binding eIF4E (eukaryotic translation initiation factor 4E), which is important in the initiation of cap-dependent mRNA translation. Activation of p70S6K leads to the phosphorylation of the ribosomal protein S6. Thus, mTOR has a crucial function in protein translation [37, 39, 45].

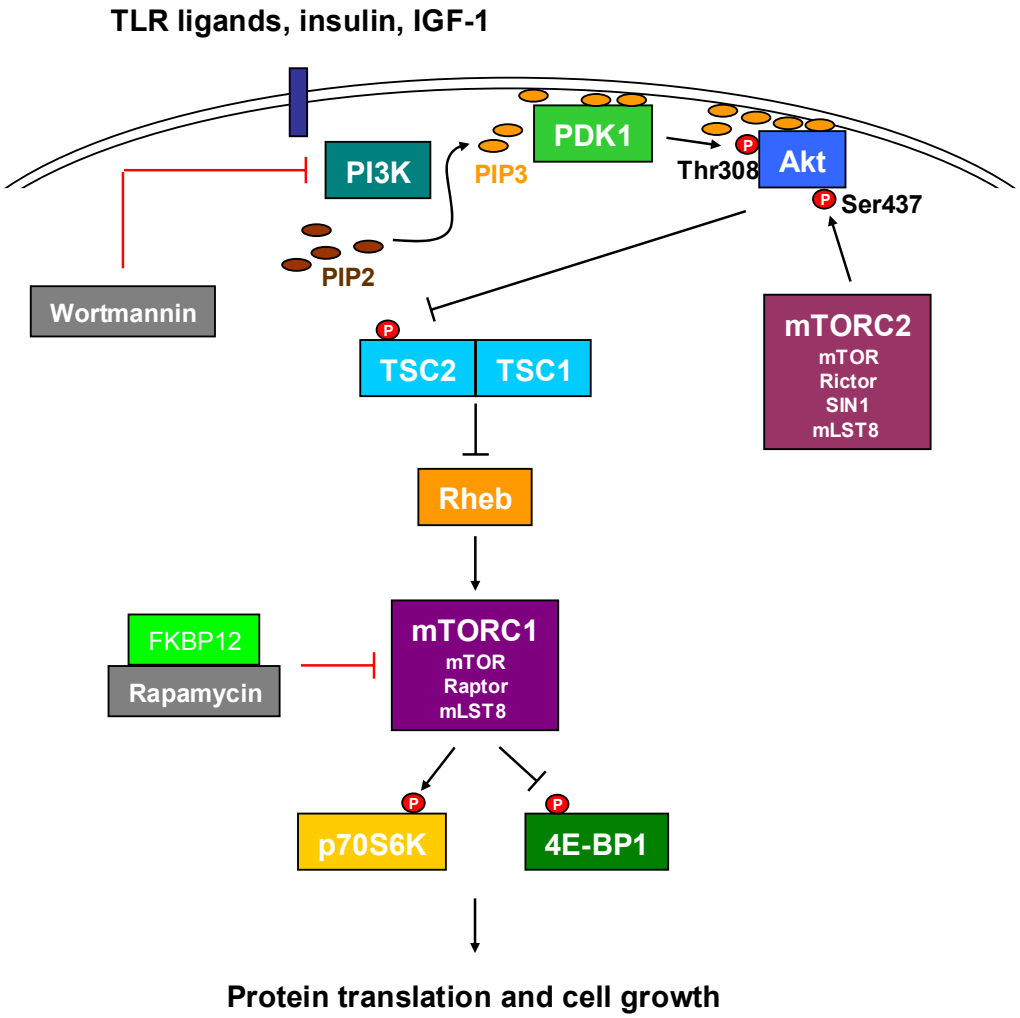

Figure 3. The PI3K/Akt/mTOR pathway
TLR ligands and growth factors stimulate their receptors, which leads to the recruitment of PI3K. This kinase converts PIP2 to PIP3 by phosphorylation. PDK1 is recruited and Akt is phosphorylated at Thr308. Akt can also be activated by mTORC2. Phosphorylated Akt phosphorylates TSC2, thus inhibiting the TSC1/TSC2 complex to activate Rheb. Rheb stimulates mTORC1 activity, which is sensitive to rapamycin. Downstream of mTORC1 are p70S6K and 4E-BP1. Activated mTORC1 is important for protein translation and cell growth. Figure adapted from Weichhart et al and Yang et al [37, 39].

1.3.3 Two complexes of mTOR

There exist two distinct complexes of mTOR that differ in their composition: mTORC1 (mTOR complex 1) and mTORC2. While mTORC1 is composed of mTOR, raptor (regulatory-associated protein of mTOR) and mLST8, mTORC2 is made up of mTOR, rictor (rapamycin-insensitive companion of mTOR), mLST8 and Sin1 [37, 46-47]. In contrast to mTORC2, mTORC1 is sensitive to rapamycin, which is thought to block the interaction between mTOR and raptor [37, 46, 48]. Subsequently, p70S6K and 4E-BP1 are not recruited and remain unphosphorylated. mTORC2 has recently been shown to phosphorylate Akt at Ser473 and is therefore thought to be upstream of mTORC1 [37, 49].

1.3.4 Inhibition of mTOR by rapamycin

In 1975, the bacterial strain *Streptomyces hygroscopicus* was isolated from a soil sample from the Easter Islands. This strain was found to produce a potent anti-fungal metabolite, which was named rapamycin, after Rapa Nui (Easter Island in the local language) [38, 39].

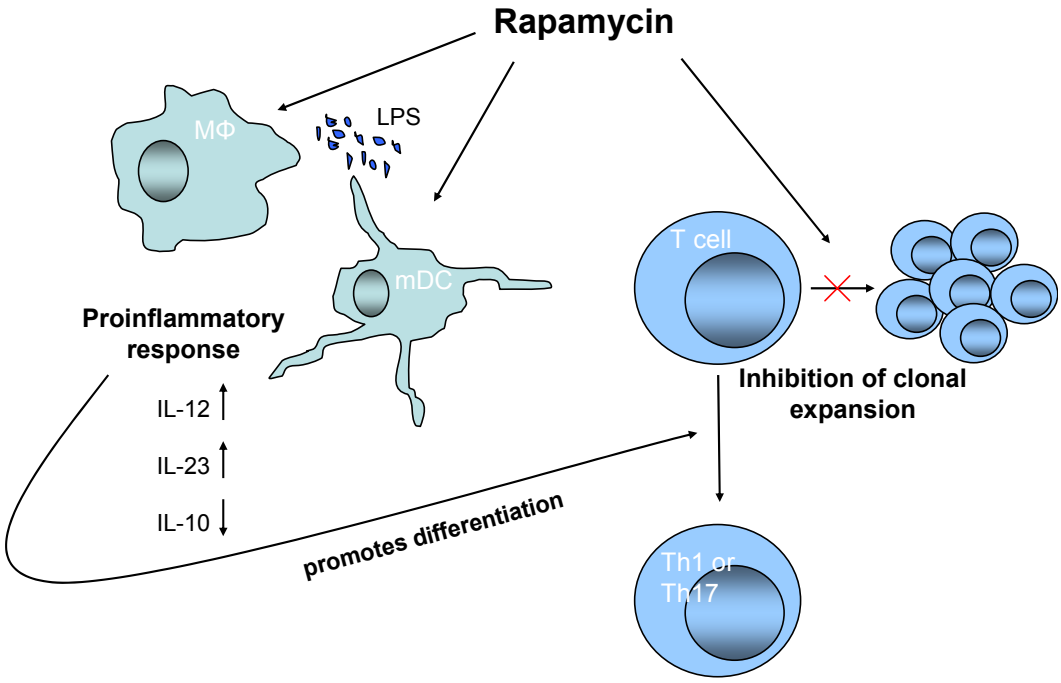

Figure 4. Effects of rapamycin on different cell types
In T-cells, rapamycin decreases the proliferation by inhibiting cell cycle progression. In macrophages and mDCs pretreatment with rapamycin and stimulation with LPS induces a proinflammatory immune response. Figure adapted from Janes et al [50].

Later on, rapamycin was found to have potent immunosuppressive and anti-proliferative activities and in 1999 it became an FDA (Food and Drug Administration)-approved drug [37, 51]. Rapamycin proved efficacious in organ transplantation to prevent allograft rejection [37]. Rapamycin and its analogs are also currently evaluated as anticancer agents [52].

Rapamycin acts on mTORC1 by forming a complex with FKBP12 (FK506-binding protein 12), thereby blocking the interaction between mTORC1 and Raptor [37, 39, 53-54]. In T lymphocytes stimulated with IL-2, rapamycin was found to decrease proliferation by inhibiting cell cycle progression [55]. This explains the immunosuppressive and anti-tumor activities of mTOR [56].

1.3.5 mTOR in inflammation

In contrast to the beneficial, immunosuppressive effects of rapamycin during transplantation, some side effects became apparent with the increased usage of rapamycin. Different inflammatory events occurred after usage of the agent [56-59]. This pointed to a role of mTOR in the regulation of inflammation.

Based on these reports, a lot of effort was made revealing mTOR as a central player in the regulation of the innate immune response [37, 50]. It was shown that inhibition of mTORC1 after TLR stimulation raises IL-12p40, IL-12p70 and IL-23 levels while impairing IL-10 levels in monocytes, macrophages and myeloid dendritic cells [60-61]. Weichhart et al. showed that the downregulation of IL-10 is mediated via the transcription factor STAT3 (signal transducer and activator of transcription 3) and the upregulation of IL-12p40 is regulated by NF-κB [61]. This proinflammatory action of rapamycin on APCs enhances their ability to stimulate T-cells. [37]. It was shown that rapamycin-treated monocytes promote the induction of Th1 and Th17 cells, indicated by the augmentation of IL-17 and IFN-γ cytokine levels after T-cell stimulation. In line with that, rapamycin protected *Listeria monocytogenes*-infected Balb/c mice from death by enhancing IL-12 and IFN-γ production [61].

2 Aim of the study

mTOR was recently found to be a critical regulator of IL-12/IL-10 production in the innate immune system. Inhibition of mTOR by rapamycin increases the levels of IL-12p40 in human monocytes, while it attenuates the levels of IL-10 [60-61].

The p38MAPK is involved in the regulation of IL-12p40. This regulation can either be positive or negative, depending on the cell type and stimulus [62]. Recently, it was shown that inhibition of p38 with SB203580 enhances the expression of IL-12p40 in primary human monocytes after stimulation with LPS, while it decreases the levels of IL-12p40 in THP-1 cells [63].

In face of the fact that p38 inhibitors evoked unexpected inflammatory side effects in clinical trials and due to the comparable effects of mTOR and p38 inhibition on the cytokine milieu in human monocytes, we assumed that p38 may play a role in regulating mTOR mediated immune response. As a molecular link of p38 and mTOR is unknown, we were interested in the consequences of inhibiting p38 or mTOR signaling for the inflammatory response and its connections. Finally, we wanted to understand the functional consequences of modulating mTOR and p38 in innate immunity for the subsequent effects on the adaptive immune response, both *in vitro* and *in vivo*. As p38 consists of four subunits, we were also interested in the question which subunit is responsible for a potential regulation of mTOR.

3 Material and methods

3.1 Media, buffers and solutions

RPMI + 10 % FCS + Glutamine + Pen/Strep

- RPMI 1640 (Gibco-BRL Life Technology)
- 2 mM L-glutamine (Gibco)
- 100 µg/ml streptomycin (Gibco)
- 100 U/ml penicillin (Gibco)
- 10 % FCS (Hyclone) (inactivated at 56°C for 30 min)

RPMI complete medium

- RPMI 1640 (Gibco-BRL Life Technology)
- 2 mM L-glutamine (Gibco)
- 100 µg/ml streptomycin (Gibco)
- 100 U/ml penicillin (Gibco)
- 10 % FCS (Hyclone) (inactivated at 56°C for 30 min)
- 50 µM 2-Mercaptoethanol (Sigma)
- 100 µM Non Essential Amino Acids (GIBCO)
- 1 mM Sodium Pyruvate (GIBCO)

DMEM + 10 % FCS + Glutamine + Pen/Strep

- Dulbecco's Modified Eagle Medium (Dulbecco)
- 4.5 g/L glucose (Sigma)
- 2 mM L-glutamine (Gibco)
- 100 µg/ml streptomycin (Gibco)
- 100 U/ml penicillin (Gibco)
- 10 % FCS (Hyclone) (inactivated at 56°C for 30 min)

10x Hemolysis buffer

- 90 g/L NH_4Cl (Merck)
- 10 g/L $KHCO_3$ (Merck)
- 0.37 g/L EDTA-Na_2 (Sigma)
- Dilute in 800 ml dH_2O, adjust pH to 7.3
- dH_2O ad 1000 ml
- Sterilize by filtration

1x Hemolysis buffer

- 100 ml 10x Hemolysis buffer
- 900 ml sterile H_2O

1x PBS

- 100 ml 10x PBS (without Ca2+ / Mg2+) (Lonza)
- 900 ml dH_2O

1x PBS-Tween (PBS-T)

- 100 ml 10x PBS
- 1 ml Tween 20 (Biorad)
- 899 ml dH_2O

50x Phosphatase inhibitor

- 500 mM NaF (Merck)
- 100 nM $Na_4P_2O_7$ (Merck)
- 100 mM Glycerophosphat (Sigma)
- 10 mM Na_3VO_4 (Sigma)
- store at 4°C

10x protease inhibitor

- 1 Protease inhibitor cocktail tablet (Roche)
- 2 ml dH_2O
- Freeze aliquots at -20°C

2x Hepes buffer

- 20 mM Hepes (Sigma)
- 140 mM NaCl (Merck)
- 2 mM EDTA (Sigma)
- pH7.9

Stripping Buffer

- 62.5 mM Tris-HCl pH 6.8
- 2 % SDS (GE-Healthcare)
- 100 mM 2-Mercaptoethanol (Sigma)

Tx-100 Lysis Buffer (10ml)

- 5 ml 2x Hepes buffer
- 1 ml 10x Protease Inhibitor
- 0.2 ml 50x Phosphatase Inhibitor
- 1 ml 10 %Tx-100 (Sigma)
- 2.8 ml dH_2O

4x Reducing Sample Buffer

- 250 mM Tris-HCl pH 6.8
- 40 % Glycerol (Sigma)
- 8 % SDS (GE-Healthcare)
- 400 mM Dithiothreitol (Sigma), added fresh
- Stain with bromphenolblue (Sigma)

5x Running Buffer

- 15.14 g/L Tris (Sigma)
- 72.07 g/L Glycin (Fluka)
- 5 g/L SDS (GE-Healthcare)
- dH_2O ad 1000 ml

1x Running Buffer

- 200 ml 5x Running Buffer
- 800 ml dH_2O

5x Transfer Buffer

- 15.14 g/L Tris (Sigma)
- 72.07 g/L Glycin (Fluka)
- dH_2O ad 1000 ml

1x Transfer Buffer

- 200 ml 5x Transfer Buffer
- 200 ml Methanol (Fisher chemical)
- 600 ml dH_2O

Acrylamide gels

Stack (10ml)	7.5 % (10ml)	10 % (10ml)	12 % (10ml)	
1.33 ml	2.50 ml	3.33 ml	4.00 ml	30 % AA/Bis (Biorad)
2.50 ml	-	-	-	0.5 M Tris-HCl, pH6.8
-	2.50 ml	2.50 ml	2.50 ml	1.5 M Tris-HCl, pH8.8
0.10 ml	0.10 ml	0.10 ml	0.10 ml	10 % SDS
6.07 ml	4.90 ml	4.07 ml	3.40 ml	dH_2O
10 µl	10 µl	10 µl	10 µl	TEMED (Biorad)
50 µl	50 µl	50 µl	50 µl	10 % APS (Amersham)

Table 1. Recipes for stacking and resolving gels used in SDS-PAGE.

4 % PFA in PBS

- 1.85 g Paraformaldehyde (Fluka)
- 3.5 ml dH_2O
- 10 µl 10 M NaOH (Merck)
- 65°C water bath
- Dilute in 41.25 ml PBS

LB^{Amp} medium

- 25 g Luria Broth (Sigma)
- 1 L dH_2O
- Autoclave at 121°C for 15 min
- Cool down to approximately 50°C
- Add 100 µg/ml Ampicillin (Sigma)

LB^{Amp} plates

- 32 g LB Agar (Lennox L Agar, Invitrogen)
- 1 L dH_2O
- Autoclave at 121°C for 15 min
- Cool down to approximately 50°C
- Add 100 µg/ml Ampicillin (Sigma)
- Pour into 10 cm petri dishes (BD Falcon)
- Store at 4°C

3.2 Methods

3.2.1 PBMC isolation and MACS

Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats by density gradient centrifugation using Ficoll-Paque^{TM} Plus (GE-Healthcare). Ficoll, a hydrophilic polysaccharide, has a density of 1.077 g/ml. This is bigger than the density of lymphocytes and monocytes, but smaller than the density of erythrocytes, granulocytes and thrombocytes. Therefore, the mononuclear cells are enriched in a layer above the ficoll layer, while the erythrocytes, granulocytes and thrombocytes can be found on the bottom of the tube, when being centrifuged.

Human blood was diluted with PBS in a 1:1 ratio and 35 ml of the suspension were laid over 14 ml Ficoll-Paque in a 50 ml conical tube. After centrifugation at 544 g and room temperature for 30 min, the mononuclear cell layer was transferred to a new 50 ml tube. After a washing step with MACS-buffer (Miltenyi Biotec) and centrifugation at 544 g and 4°C, red blood cells were lysed for 5 min on ice with 5 ml hemolysis buffer. After another two washing steps PBMCs were counted and either taken up in medium or prepared for CD14+ MACS magnetic cell sorting. When preparing for CD14+ MACS magnetic cell sorting, cells were again washed two times in MACS-buffer and incubated with 100 µl CD14 Microbeads (Miltenyi)/1x 10^8 cells in 800 µl MACS-buffer/1x 10^8 cells for 15 min at 4°C. After washing in MACS-buffer, cells were resuspended in 500 µl MACS-buffer/1x 10^8 cells and loaded on an LS-separation column (Miltenyi), which was first placed on a magnetic cell separator (quadro MACS, Miltenyi). After loading, the column was washed 3 times with 5 ml MACS-buffer each and flow-through was collected as the non-magnetic fraction. Then, CD14+ cells were isolated from the column by removing it from the magnetic cell separator and pushing a plug into the column. CD14+ cells were counted, washed twice in RPMI medium and taken up in RPMI + 10 % FCS + Glutamine + Pen/Strep. They were allowed to rest at 37°C and 5 % CO_2 for 30 min before stimulation.

In order to obtain myeloid dendritic cells (mDCs), the CD1c (BDCA-1) Dendritic Cell Isolation Kit (MACS, Miltenyi) was used. The flow-through of the CD14+ MACS was washed twice in MACS-buffer (centrifugation at 300 g, 4°C and 10 min) and the supernatant was removed. Cells were incubated with 100 µl FcR Blocking Reagent, 100 µl CD19 Microbeads and 100 µl CD1c Biotin Antibody in 200 µl MACS-buffer per 1x 10^8 cells. The incubation was performed for 15 min at 4°C. Afterwards, the cells were again washed twice as described and the supernatant was removed. The pellet was resuspended in 500 µl MACS-buffer/1x 10^8 cells and loaded on an LD-separation

column to deplete B-cells. The column was rinsed twice with 1 ml MACS-buffer and the flow-through was harvested. The cells in the flow-through were counted, washed in MACS-buffer as described and the supernatant was removed. Afterwards, the pellet was incubated with 100 µl Anti-Biotin Microbeads in 400 µl MACS-buffer per 1x 10^8 cells. The solution was incubated on ice for 15 min, followed by washing two times with MACS-buffer. The pellet was resuspended in 500 µl MACS-buffer per 1x 10^8 cells and loaded on an LS-separation column. The column was rinsed three times with 3 ml MACS-buffer. Afterwards, the column was removed from the cell separator and a plug was pushed into the column to obtain the positive selected cells (=$CD1c^+$). The cells were washed twice in RPMI medium and taken up in RPMI + 10 % FCS + Glutamine + Pen/Strep and further treated as indicated.

For the T-cell differentiation assay, the PBMCs without the positive selected CD14+ monocytes were used. Therefore, the flow-through of the CD14+ MACS-procedure was centrifuged at 500 g and 4°C for 7 min, washed twice in RPMI medium and taken up in RPMI complete medium. Until use, the cells were stored at a concentration of 2x10^6/ml in the fridge over night.

3.2.2 Cell culture

Cells were cultured in an incubator at 37°C and 5 % CO_2. CD14+ monocytes were isolated from whole blood as described and taken up in RPMI + 10 % FCS + Glutamine + Pen/Strep. Before stimulation, they were allowed to rest for 30 min. mDCs were treated the same way as monocytes.

In order to obtain monocyte derived dendritic cells (moDCs), 4x10^6 monocytes were seeded in a well of a 6-well plate (TPP) at a concentration of 1x10^6/ml and stimulated with 50 ng/ml GM-CSF (Peprotech) and 10 ng/ml IL-4 (Peprotech) for one week. After one week, the cells were splitted to 24-wells (Nunclon) and stimulated as indicated.

Mouse embryonic fibroblasts (MEFs) were cultured in DMEM + 10 % FCS + Glutamine + Pen/Strep. Tsc2+/+ p53-/- and Tsc2-/- p53-/- MEFs were a kind gift of David Kwiatkowski. Freezing stocks of these cells were thawed at 37°C and washed once in DMEM. After the procedure, the cells were seeded in a 10 cm tissue culture dish (Iwaki). The cells were passaged by trypsinization. For this, the medium was removed and cells were washed once with PBS. PBS was removed and 1 ml trypsin-EDTA was added to the plate. The plate was incubated at 37°C and 5 % CO_2 for 3 min. After that, the trypsinization was stopped by adding medium and cells were splitted 1:10 to new plates. Before stimulation of the MEFs, they were splitted as described to

12- or 24- wells, allowed to adhere and starved over night in DMEM + Glutamine + Pen/Strep without FCS.

Bone marrow of p38α fl/fl and p38α fl/fl LysMCre Δp38α mice was a kind gift of Jin Mo Park. The bone marrow was thawed at 37°C and cultured in RPMI complete medium + 10 % L929 culture supernatant in a petri dish (BD Falcon). After three days, supernatant was removed from the petri dish and bone marrow derived macrophages (BMDMs) were washed with PBS and new medium was added. The macrophages were then scraped from the plate and splitted to two new petri dishes. After another three days, cells were again washed with PBS and RPMI complete medium with 2 %, instead of 10 % FCS, was added. Cells were splitted to 24- or 6-wells and starved over night, before being stimulated as indicated.

3.2.3 Cell stimulation

Monocytes, moDCs, mDCs, macrophages and MEFs were pretreated for 90 min with the indicated concentrations of wortmannin (Sigma), SB203580 (Tocris Bioscience), BIRB0796 (Axon Medchem), SD169 (Calbiochem), rapamycin (Calbiochem), mithramycin (Sigma), DMSO (Sigma) or different combinations of them. After the pretreatment, cells were stimulated for the indicated times with lipopolysaccharide from Escherichia coli 0111:B4 (LPS), anisomycin from Streptomyces griseolus, zymosan A from saccharomyces cerevisiae (all from Sigma), IFN-γ (R&D), *Staphylococcus aureus* cells (SAC, Calbiochem), heat-killed *Listeria monocytogenes* (a kind gift of Dr. Caroline Lassnig), UV light or different combinations of them. In case of the UV light stimulation, the plates were placed on a 20x20 UV-transilluminator (MWG Biotech) and activated with UV light for 10 sec or 1 min. After the activation, cells were incubated at 37°C and 5 %CO_2 for the indicated times.

To investigate intracellular signaling, cells were stimulated for 30 min or 1 h and further treated as described. For determination of mRNA levels, cells were activated for 2 h, 4 h or 6 h and further proceeded as described. In order to measure cytokine levels, cells were stimulated for 24 h.

3.2.4 T-cell differentiation assay

In order to investigate the T-cell response elicited by differently pretreated monocytes, we adapted a T-cell differentiation assay protocol from Acosta-Rodriguez et al [64]. Monocytes were isolated from whole blood as described and seeded in 24-well plates in RPMI + 10 % FCS + Glutamine + Pen/Strep. They were allowed to rest for 30 min at 37°C, 5 % CO_2. Afterwards, monocytes were incubated with medium,

200 nM BIRB0796, 2 µM SB203580 or 100 nM rapamycin for 90 min and stimulated with 100 ng/ml LPS for 24 h. The cells were then washed with PBS and incubated with allogeneic T lymphocytes at a ratio of 1:1 in 24-well plates in RPMI complete medium. After one week, cell-free supernatants were taken off and IFN-γ, IL-17 and IL-4 production was determined by Luminex as described. The cells were activated for 5 h with 50 ng/ml phorbol-12-myristat-13-acetat (PMA) and 200 ng/ml ionomycin (both from Sigma) in the presence of 10 µg/ml brefeldin A (Sigma) for the last 3 h. Afterwards, cells were transferred to FACS-tubes and flow cytometry was performed as described.

3.2.5 Immunocytochemistry

Cells were cultured in 8-well Permanox^{TM} chamber slides (Lab-Tek Chamber Slide System) and pretreated as indicated. After stimulation, the culture medium was replaced with a freshly prepared solution of 4 % PFA in PBS and cells were fixed for 10 min at room temperature. The chambers were then removed from the slides and the cells were rinsed once with PBS. In order to quench the PFA, cells were incubated with 100 nM glycine in PBS for 15 min. Afterwards, cells were washed in PBS for 30 min. The next step was to permeabilize the cells by incubating them with methanol for 5 min at -20°C. After rinsing with PBS, slides were again washed with PBS for 30 min at room temperature. In order to block unspecific sites, cells were incubated with 1 % BSA in PBS for 1 h at room temperature. Afterwards, the primary antibody was added to the slides over night at 4°C in a humidity chamber.

Primary antibodies	Dilution	Origin	Company
p-MK2 (Thr334)	1:50 in PBS + 1%BSA + 0.02%NaN_3	rabbit	Cell signaling
p-S6 (240/244)	1:50 in PBS + 1%BSA + 0.02%NaN_3	rabbit	Cell signaling
Secondary antibodies			
Anti-rabbit Alexa Fluor 488	1:200 in PBS	goat	Invitrogen

Table 2. Primary and secondary antibodies used for immunofluorescence.

After removing the primary antibody dilution and rinsing the slides with PBS, cells were washed three times with PBS for 20 min. Following, cells were incubated with secondary antibody dilutions in PBS for 1 h at room temperature in the dark. After rinsing and washing the slides twice with PBS for 20 min, nuclei were stained with 0.1 µg/ml Hoechst-33342 (Invitrogen) in dH_2O. After 5 min of incubation, the slides were rinsed and washed with PBS for another 20 min. PBS was removed completely

and slides were mounted with mounting medium (Vectashield H-1000). Finally, slides were sealed with nail polish and images were taken on a fluorescence microscope (Olympus Provis AX-70) equipped with a digital camera (Olympus XC-50).

3.2.6 Preparation of Western Blot samples

Cells were pretreated in 6- or 12-well culture plates (Falcon, BD Biosciences) as indicated. After stimulation, cells were washed once with PBS and taken up either in 100 µl Triton X-100 Lysis Buffer or direct in 60 µl 1x SDS reducing sample buffer per well on ice. When taken up in 1xSDS reducing sample buffer, samples were kept on ice for 10 min and were then boiled for 5 min at 95°C. When cells were taken up in Triton X-100 Lysis Buffer, they were sonicated three times for 10 sec, 10 % Output (2 mm tip) and kept on ice for 30 min. Tubes were shaken regularly. After incubation on ice, cell debris was pelleted by centrifugation for 10 min, at 16000 g and 4°C. The supernatant was transferred to a new 1.5 ml tube and protein concentration was measured by using the BCA Protein Assay Kit (Thermo Scientific), according to the manufacturer's protocol. Western samples were prepared by adjusting the protein concentration with lysis buffer and adding 4xSDS reducing sample buffer. Samples were boiled for 5 min at 95°C and stored at -20°C until analysis by Western Blot.

3.2.7 RNA extraction and cDNA synthesis

Cells were pretreated as indicated. Total RNA was extracted in 500 µl TRIzol (Invitrogen). RNA was isolated as follows: 120 µl ddH_2O (Sigma) and 100 µl chloroform (Merck) were added to the 500 µl TRIzol phase in a 2 ml tube. After vortexing, the mixture was incubated at room temperature for 10 min, following centrifugation for 15 min at 13000 rpm and 4°C. The aqueous phase was transferred to a new 1.5 ml tube and 1 µl glycoblue (Ambion) was added. 280 µl cold isopropanol (Merck) were added, the tube was again vortexed and then incubated on ice for 30 min. Centrifugation for 30 min at 13000 rpm and 4°C followed. The supernatant was removed and the pellet was washed with 75 % EtOH (Merck). After drying the pellet, it was dissolved in 20 µl RNase free H_2O (Sigma) and cDNA was generated as follows: 8 µl 5xFirst Strand Buffer (Invitrogen), 4 µl 0.1 M DTT (Invitrogen), 2 µl 10 mM dNTPs (prepared from 100 mM dATP, dGTP, dCTP, dTTP (all from Invitrogen)), 2 µl 100 ng/ml Random Hexamers (Invitrogen) and 1 µl RNase OUT Inhibitor (Invitrogen) were added to the 20 µl H_2O and the tube was placed in a thermocycler at 65°C for 15 min. After the 15 min, 1 µl Superscript II (Invitrogen) was added and the reverse transcription was

started with the following program: 50 min at 42°C; 15 min at 70°C and cooling to 4°C. The cDNA was stored at -20°C until further use.

3.2.8 Flow cytometry

Cells were stimulated as indicated, transferred to FACS-tubes (Matrix) and kept on ice. After centrifugation at 300 g and 4°C for 5 min, supernatant was discarded and cells were washed with cold PBS. After another round of centrifugation and discarding the supernatant, cells were blocked with 1 µl beriglobin P (Behring) in 49 µl PBS for 10 min on ice. Following, tubes were filled with PBS and again centrifuged at 300 g and 4°C for 5 min. Afterwards, cells were incubated with 1 µl anti-CD4+ (BD Bioscience) in 49 µl PBS on ice for 30 min in the dark. Cells were again washed with PBS and centrifuged as described above. In order to fix and permeabilize the cells, we used the Fix & Perm Cell Permeabilization Kit (BD Bioscience) according to the manufacturer's protocol. Cells were incubated with 50 µl solution A (fixing solution)/sample for 15 min at room temperature, washed with PBS and then centrifuged as indicated. Afterwards, 1 µl of the respective antibody was dissolved in 49 µl solution B (permeabilizing solution) and added to the tube. The cells were incubated for 15 min at room temperature and washed with PBS. After centrifuging and discarding the supernatant, the cells were taken up in 50 µl PBS and analyzed on a FACSCanto II (BD Bioscience).

Antibodies	conjugate	Clone	Company
IFN-γ	FITC	4S.B3	BD Bioscience
IL-4	PE	8D4-8	BD Bioscience
CD86	PE	IT2.2	Pharmingen
HLA-DR	PerCP	L243	BioLegend
Lineage cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56)	FITC	SK7, 3G8, SJ25C1, L27, MφP9, NCAM16.2	BD Bioscience
CD11c	APC	3.9	BioLegend

Table 3. Antibodies used for flow cytometry.

3.2.9 Real-Time PCR

For the real-time PCR analysis, TaqMan Gene Expression Assays (Applied Biosystems) were used. 9 µl master mix, consisting of 5 µl TaqMan Universal PCR Master Mix 2x, 0.5 µl Assay Mix 20x and 3.5 µl ddH_2O, were mixed with 1 µl cDNA in a MicroAmp optical 96-well reaction plate (Applied Biosystems). Reactions were

performed in duplicates. After sealing the plate, it was transferred into an ABI Prism 7000 and the following program was performed: 10 min at 95°C; 40x [15 sec at 95°C; 1 min at 60°C].

Expression levels of target mRNAs were normalized to ubiquitin and are shown as fold increase to unstimulated control. The following PCR primers and TaqMan MGB probes (FAM-labeled) were used: IL-10 (human, assay ID: Hs00174086_m1); IL-12p40 (human, assay ID: Hs00233688_m1); IL-23p19 (human, assay ID: Hs00372324_m1); TNF-a (human, assay ID: Hs00174128_m1); UBC (human, assay ID: Hs00824723_m1); IL-12p40 (mouse, assay ID: Mm00439616_m1); IL-10 (mouse, assay ID: Mm01201237_m1) and UBC (mouse, Mm01288993_m1).

3.2.10 SDS-PAGE and Western Blot

Before protein samples were loaded and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was started, they were boiled for 5 min at 95°C and centrifuged at 16000 g and room temperature for 5 min. Next, the electrophoresis apparatus was set up and 10 µg protein per lane were loaded on a 7.5 %, 10 % or 12 % gel as desired. After electrophoresis, the gel was blotted on either a nitrocellulose (Whatman, Protran) or PVDF (Millipore) membrane. Before using the membranes, they were pre-wetted in 1x transfer buffer. The PVDF membrane was pre-wetted for 30 sec in methanol and then kept for 10 min in 1x Transfer buffer. Blotting was performed for 40 min at 15 V using the Trans-Blot SD semi-dry electrophoretic transfer cell (BIORAD). In order to investigate the blotting efficiency, membranes were rinsed with H_2O and stained with Ponceau S (Sigma) for 1 min. Ponceau S detects proteins unspecifically. The Ponceau staining was removed by washing for 1 min with PBS-T. Afterwards, the membrane was blocked in 4 % dry milk (BIORAD) in PBS-T for 1 h. The primary antibody was added over night at 4°C. Primary antibody dilutions have been used several times and stored at 4°C until use.

After washing three times for 10 min each with PBS-T, the membrane was incubated with the secondary antibody for 45 min at room temperature with 4 % dry milk in PBS-T. The secondary antibodies were conjugated with horseradish peroxidase (HRP). After another 3x 10 min wash step, Immobilon™ Western Chemiluminescent HRP Substrate was used to detect the antibodies. The reaction was visualized using Amersham Hyperfilm ECL (GE-Healthcare) and the AGFA Classic E.O.S. processor.

When a membrane was incubated with more than one primary antibody, it was washed twice with PBS-T for 5 min and stripped with Stripping Buffer for 15 min in a

50°C water bath. Afterwards, the membrane was blocked again in 4 % dry milk in PBS-T for 1 h at room temperature.

Primary antibodies	Dilution	Origin	Company
p38 MAPK	1:500 in PBS-T + 0.02%NaN_3	rabbit	Santa Cruz
p-p38 (Thr380/Tyr382)	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p38α MAPK	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p38β MAPK	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p38σ MAPK	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p-S6 (240/244)	1:2000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
S6-ribosomal protein	1:1000 in PBS-T + 0.02%NaN_3	mouse	Cell signaling
p-4E-BP1 (Thr37/46)	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
4E-BP1	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p-p70S6K (Thr389)	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p70S6K	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p-Erk (Tyr204)	1:500 in PBS-T + 0.02%NaN_3	mouse	Santa Cruz
GAPDH	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
c-Jun	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p-Akt (Ser437)	1:500 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p-MK2 (Thr334)	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
p-GSK3β (Ser9)	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
IkBα	1:500 in PBS-T + 0.02%NaN_3	rabbit	Santa Cruz
Histon H3	1:1000 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
Tuberin/TSC2	1:500 in PBS-T + 0.02%NaN_3	rabbit	Cell signaling
Secondary antibodies			
Anti-rabbit	1:3000 in PBS-T + 4% dry milk	donkey	GE Healthcare
Anti-mouse	1:10000 in PBS-T + 4% dry milk	goat	Bethyl Laboratories

Table 4. Primary and secondary antibodies used for Western Blot.

3.2.11 *Escherichia coli* transformation and plasmid preparation

Sp1-Luc was a kind gift of Amyn Habib. Competent *Escherichia coli* (*E. coli*) DH5α (Sigma) were thawed on ice. 90 µl of the thawed cells and 1 µl of the plasmid were mixed in a 1.5 ml tube and incubated on ice for 5 min. Afterwards, a heat shock was

performed (45 sec, 42°C). Immediately after the heat shock, cells were incubated on ice for 2 min, followed by addition of 900 µl LB medium. The suspension was incubated on the thermomixer (Thermomixer 5436, eppendorf) with vigorously shaking. 10 µl of the suspension were plated on LB^{Amp} plates and incubated at 37°C over night.

One colony of the grown *E. coli* was picked from the plate, inoculated into 10 ml LB^{Amp} medium and grown to mid log phase at 37°C. Afterwards, 2 ml of the suspension were inoculated into 250 ml LB^{Amp} medium and incubated at 37°C over night. The next step was to transfer the 250 ml of the E. coli suspension into five 50 ml conical tubes (Sarstedt) and centrifuge them at 4°C and 4500 g for 30 min. The supernatant was removed and the pellet was stored at -20°C until further use for plasmid preparation.

For the plasmid preparation, the EndoFree Plasmid Purification Maxi Kit (Quiagen) was used according to the manufacturer's instructions. In the last step, the plasmid was dissolved in 100 µl TE-buffer (provided with the Kit) and the DNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (peQLab). The concentration was adjusted to 1 mg/ml with TE-buffer and the plasmid was stored at -80°C until further use.

3.2.12 Reporter Gene Assay

MEFs were cultured as described, splitted to 24-well plates and allowed to adhere over night. For transfection of the MEFs, 1 µl Lipofectamine Reagent (Invitrogen) and 0.8 µg Sp1-Luc plasmid (1 mg/ml) were each diluted in 24 µl OPTI-MEM reduced serum medium (Gibco) and pooled afterwards. In all experiments 0.2 µg of a renilla luciferase construct (1 mg/ml) were cotransfected to normalize expression. The solution was incubated at room temperature for 15 min. Meanwhile, medium was removed from the 24-well plate and 250 µl DMEM + glutamine without FCS and antibiotics was added. After the 15 min incubation, the 50 µl of the solution were added to the 250 µl medium and MEFs were transfected for 3 h. Afterwards, the supernatant was removed and DMEM + glutamine + Pen/Strep + 10 %FCS was added over night. The next day, cells were pretreated as indicated and then stimulated for 24 h. After stimulation, cells were washed twice in PBS and taken up in 100 µl /well 1x Passive Lysis Buffer (Promega). The plate was shaken for 30 min at room temperature and then a luciferase assay (Promega Dual-Luciferase Reporter Assay System) was performed. For this, 10 µl of the cell lysate were transferred into a luminometer tube. The tube was inserted into the luminometer (Lumat LB9507, Berthold Biotechnologies) and the measurement was performed automatically by the instrument. First, the firefly luciferase activity was measured by the addition of LARII buffer. Through the addition

of the Stop/Glo buffer, the activity of the firefly luciferase was inhibited, while the renilla luciferase was activated. After measuring the activity of the renilla luciferase, the ratio between the activities of the firefly and the renilla luciferase was calculated by the luminometer.

3.2.13 Luminex

In order to determine cytokine levels, cell-free supernatants were taken off and stored at -20°C until analyzed by Luminex. The assays were performed by Margarethe Merio. Human cytokine levels were determined using following antibodies: IL-12p40, IL-12p70, IL-10, TNF-α, IL-6, IFN-γ, IL-1β and IL-17 (all R&D Systems). Mouse cytokine levels were measured by the use of the 6-plex Procarta Cytokine Assay Kit (Affymetrix) with antibodies to IL-12p40, IL-12p70, IL-10, TNF-α, IL-1β and IL-23.

3.2.14 Annexin V staining

$3x10^5$ monocytes were seeded on a 24-well plate and pretreated as indicated. After 24 h stimulation with LPS, the cells were transferred to a FACS-tube and washed twice with PBS. Cells were resuspended in 300 µl of 1x Annexin V Binding Buffer and 100 µl of the solution were transferred to a new FACS-tube. Cells were incubated for 15 min with 5 µl FITC-labelled Annexin V (Alexis) and 5 µl propidium iodid (Sigma) in the dark. Afterwards, 400 µl of 1x Annexin V Binding Buffer were added and cells were analyzed within one hour on a FACSCanto II (BD Bioscience).

3.2.15 Homogenisation of mouse tissue

Homogenisation of mouse tissue was performed by using the Precellys-ceramic kit 2.8 mm and the Precellys 24 tissue homogenizer (both from peQLab). Mouse tissue was dissected on dry ice and 30 mg were transferred to a 2 ml tube containing the beads. All the steps were performed without allowing the tissue to thaw. Afterwards, 1 ml Tx-100 Lysis Buffer was added and the tube was placed into the homogenizer. After the program (2x15 sec) was finished, the samples were incubated on ice for 30 min and centrifuged for 10 min at 4°C and 16000 g to pellet cell debris. Supernatant was transferred to a new tube and Western Blot samples were prepared as described earlier.

In order to isolate RNA from mouse tissue, 1 ml TRIzol instead of 1 ml Tx-100 Lysis Buffer was added and the same program as described above was performed on the tissue homogenizer. After the program was finished, we stored the samples at -20°C until isolating RNA as described earlier.

3.2.16 *Listeria monocytogenes* mouse model

Dr. Caroline Lassnig from the University of Veterinary Medicine Vienna performed the *in vivo* experiments. SB203580 hydrochloride (Cayman) was dissolved at 30 mg/ml in DMSO and further diluted to 1.5 mg/ml or 150 µg/ml with PBS for injection. Male C57BL/6 mice (10 weeks) were injected intraperitoneal with 30 µg/mouse SB203580 hydrochloride or solvent controls. 30 min later, mice were injected with $5x10^6$ *Listeria monocytogenes EGD* in PBS. Survival of the mice was monitored.

In a different experiment, mice were pretreated as indicated and injected with $5x10^6$ *Listeria monocytogenes* in PBS. After 6 h, mice were anesthetized and blood samples were taken for determining cytokine levels. Spleens were also isolated and homogenized as described.

3.2.17 p38α KO mice experiments

The experiments were carried out at the Cutaneous Biology Research Center, Charlestown, Massachusetts (group of Jin Mo Park). The mice were on a C57BL/6 background.

For a restimulation experiment, three p38α fl/fl mice and three p38α fl/fl mice expressing Cre under the control of LysM, were injected with 25 µg/mouse LPS and splenocytes were harvested 48 h later. $5x10^5$ splenocytes/well were restimulated with 100 ng/ml LPS or solvent control. After 48 h, supernatants were taken off and frozen.

For the isolation of organs, three p38α fl/fl mice and three p38α fl/fl mice expressing Cre under the control of LysM, were injected with 30 µg/mouse LPS. After 4 h, lungs and spleens from the mice were isolated and snap-frozen in 1.5 ml tubes. Sera samples were also taken from these mice.

The material was sent to us and we performed Luminex and tissue homogenization as described.

4 Results

4.1 Initial experiments

4.1.1 p38 subunit expression in human CD14+ monocytes

The p38 MAPK consists of the four subunits α, β, γ and δ. By measuring the mRNA and protein levels of the four subunits, Hale et al showed that p38α is the predominant form of p38 in human monocytes, followed by p38δ, whereas the expression of the p38β and γ subunits in monocytes is extremely low [65].

In order to extend these results and to investigate the effect of TLR stimulation on the expression of p38, we stimulated human monocytes with LPS and detected the expression of the subunits after 0, 1, 6 and 24 h by immunoblot. p38α was the dominant subunit expressed in human monocytes and its expression was not modulated by LPS stimulation. The second-highest expression levels were measured for p38δ. Again, there was no difference in the expression levels after TLR4 stimulation. Interestingly, p38β was induced following treatment with LPS after 1 h, and decreased again after 6 and 24 h (Figure 5). As Hale et al could not detect any p38γ in human monocytes and we had no antibody available, we did not measure p38γ levels. In summary, we showed that p38α is the predominant p38 subunit expressed in human monocytes, followed by p38δ, whereas p38β is induced after stimulation with LPS in a time-dependent manner.

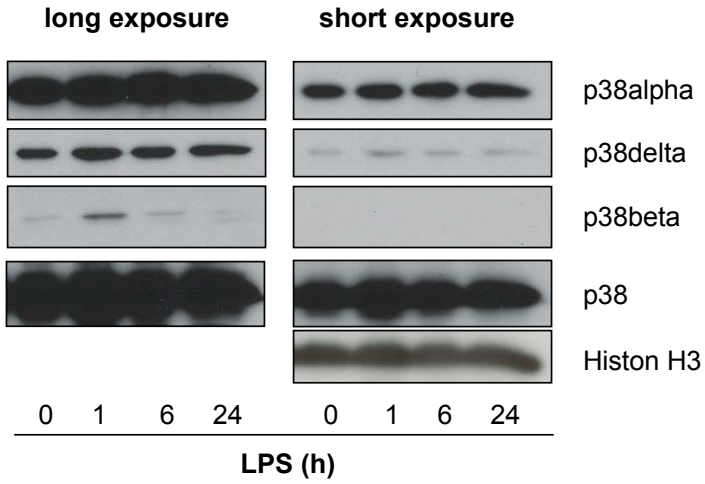

Figure 5. p38 subunit expression in human monocytes
Human CD14+ monocytes were stimulated with 100 ng/ml LPS for the indicated times. Whole-cell lysates were analyzed by immunoblot. Representative blots for two independent experiments are shown.

4.1.2 Rapamycin and p38 inhibitors do not evoke cell death

As it was important to know if the used inhibitors have an influence on cell viability, we performed Annexin V staining. The assay is based on the recognition of phosphatidylserine through Annexin V. Phosphatidylserine is normally distributed on the inner side of the cell membrane but translocates to the outer side when a cell undergoes apoptosis. The translocation also occurs during necrosis, with the difference that the cell wall becomes leaky during necrosis [66].

Human monocytes were pretreated as indicated and stimulated with LPS for 24 h. Afterwards, cells were stained with Annexin V and propidium iodide. Annexin V-positive cells were defined as being in early apoptosis, double-positive cells as being in late apoptosis or necrosis and double-negative cells as being living cells. Figure 6 shows that there was nearly no difference in the cell viability comparing the different pretreated monocytes. The highest number of apoptotic and necrotic cells was found in the unstimulated sample with less than 5 %.

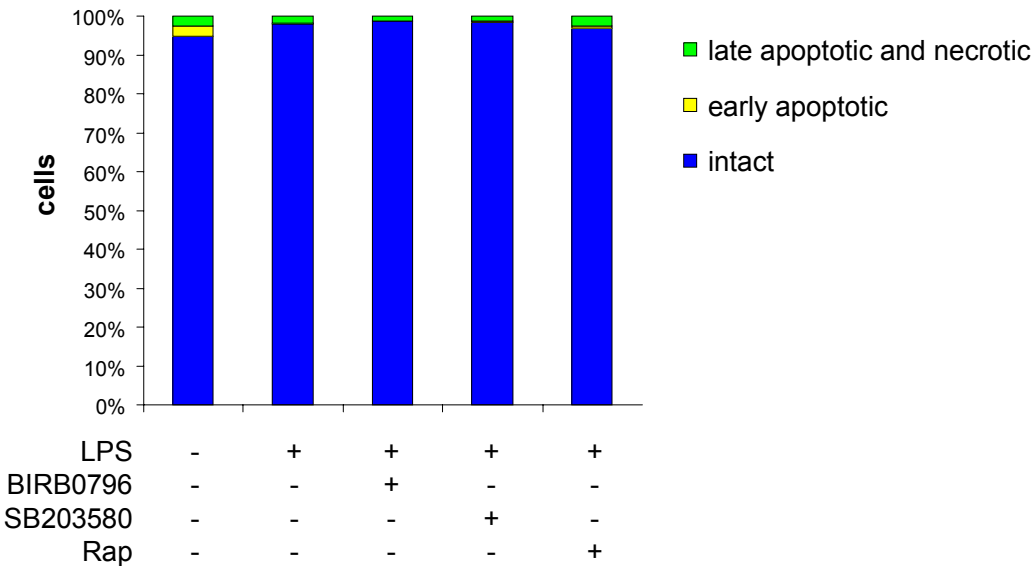

Figure 6. Influence of the used reagents on cell viability
Human monocytes were pretreated with medium, BIRB0796 (200 nm), SB203580 (2 µM) or rapamycin (100 nM) for 90 min and stimulated with LPS (100 ng/ml) for 20 h. Afterwards, cells were stained with Annexin V and propidium iodid. Annexin V-positive cells are defined as being in early apoptosis, double-positive cells as being in late apoptosis or necrosis and double-negative cells as being living cells.

4.2 p38 regulates the production of IL-12p40 and IL-10

4.2.1 p38 inhibition augments IL-12p40 and attenuates IL-10 cytokine levels

Next, we investigated the modulation of the innate immune response after p38 inhibition and TLR activation. Therefore, we pretreated freshly isolated human monocytes with the p38 inhibitors SB203580 or BIRB0796 and activated the cells with the TLR ligands LPS, *Staphylococcus aureus* (SAC), zymosan or *Listeria monocytogenes*. After stimulation for 24 h, we collected supernatants and determined cytokine levels. In order to compare the effects of p38 inhibition on cytokine production with the effects of mTOR inhibition, rapamycin was also used to manipulate the monocytes.

Inhibition of p38 with different concentrations of SB203580 or BIRB0796 led to an increase in the IL-12p40 levels after stimulation with LPS, while the IL-10 levels were attenuated compared to LPS stimulation alone (Figure 7A). The production of TNF-α was abrogated after inhibition of p38, which was already described earlier [25]. Pretreatment with rapamycin similarly decreased the production of IL-10 but promoted the production of IL-12p40. The expression of TNF-α was not significantly altered compared to the stimulated control (Figure 7A).

Inhibition of p38 followed by stimulation with the TLR2 ligand SAC and with *Listeria monocytogenes* (Figure 7B and 7E) showed similar results as the activation with LPS. Again, IL-12p40 levels raised and IL-10 levels decreased after pretreatment with BIRB0796 and rapamycin. As exception, inhibition of p38 with SB203580 did not augment IL-12p40, although IL-10 was still attenuated. In addition, the TNF-α levels were diminished after p38 inhibition, but did not change after rapamycin treatment.

Figures 7C and 7D show the cytokine levels after activation of the monocytes with two different concentrations of zymosan, another known ligand of the TLR2. Pretreatment of the monocytes with rapamycin promoted IL-12p40 production and inhibited IL-10, while the TNF-α production was not influenced. However, inhibition of p38 and stimulation of the cells with zymosan, which has been shown to activate p38 in peritoneal mouse macrophages [67], did not show a significant alteration in the IL-12p40, IL-10 and TNF-α production (Figure 7C and 7D). In all of these experiments, we also measured the IL-6 levels, but there was no significant alteration with p38 or mTOR inhibition (data not shown).

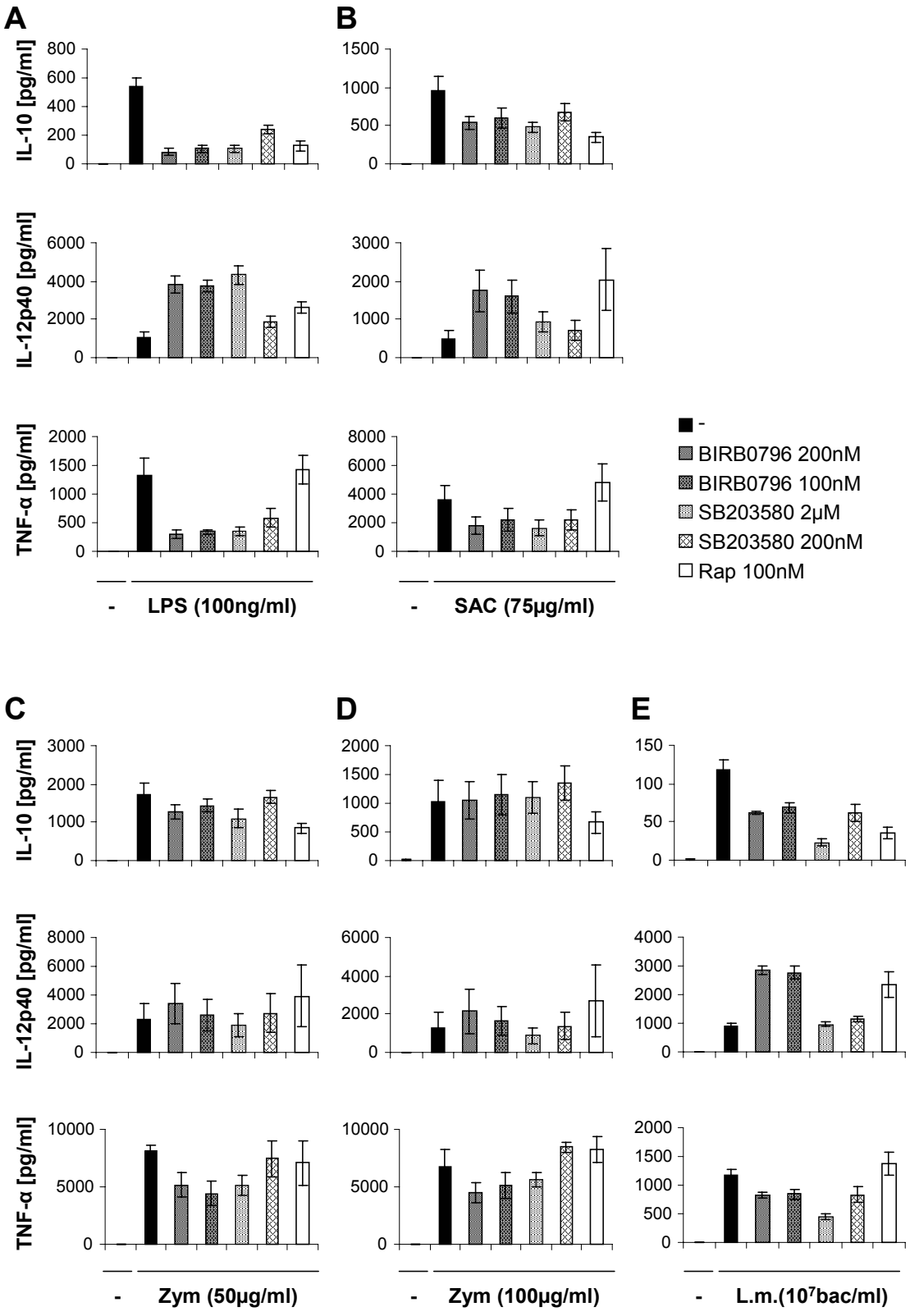

Figure 7. p38 regulates IL-12p40, IL-10 and TNF-α production after TLR stimulation
Human CD14+ monocytes were preincubated with medium, BIRB0796 (200 or 100 nM), SB203580 (2 µM or 200 nM) or rapamycin (100 nM) for 90 min and stimulated with either (A) LPS (100 ng/ml), (B) SAC (75 µg/ml), (C, D) zymosan (50 µg/ml or 100 µg/ml) or (E) *Listeria monocytogenes* (L.m.; 10^7 pfu/ml) for 20 h. IL-12p40, IL-10 and TNF-α in cell-free supernatants were measured by Luminex. Data represent the means ± SE of at least 3 donors.

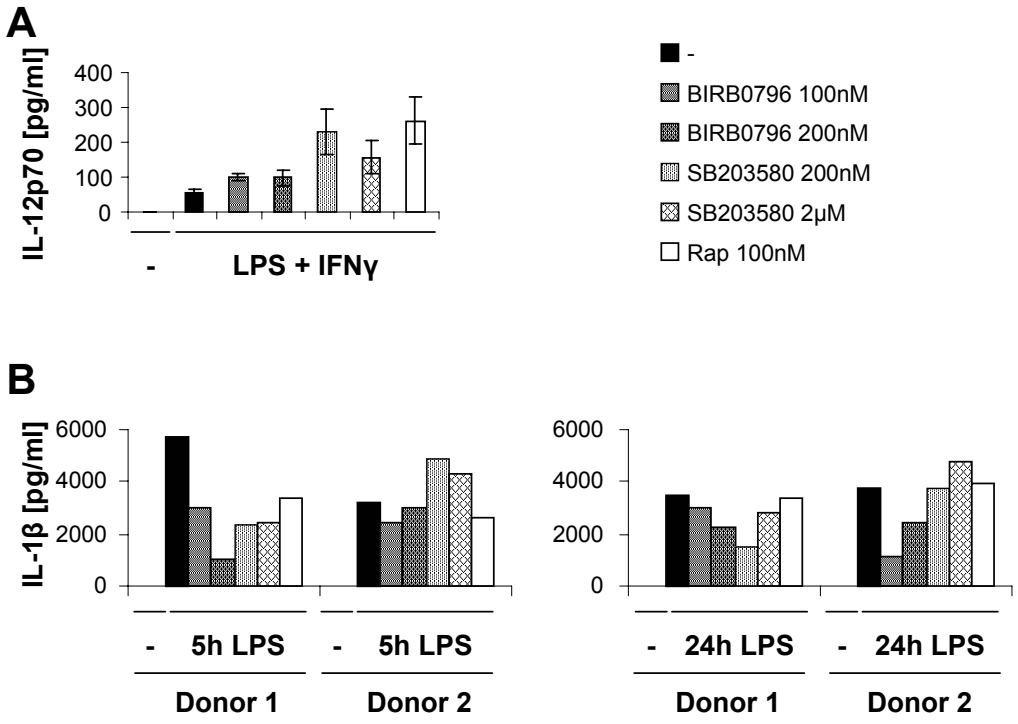

Figure 8. p38 regulates IL-12p70 and IL-1β production after TLR stimulation
Human CD14+ monocytes were preincubated with medium, BIRB0796 (200 or 100 nM), SB203580 (2 µM or 200 nM) or rapamycin (100 nM) for 90 min and stimulated with LPS (100 ng/ml) for 5 h or 20 h. For the measurement of (A) IL-12p70 production, cells were additionally stimulated with IFN-γ (30 ng/ml). For the measurement of (B) IL-1β, cells were additionally stimulated with 5 mM ATP for the final 45 min. Cytokine levels were determined by Luminex. IL-12p70 production is shown as the mean ± SE for at least 3 donors. IL-1β production is shown for two independent donors.

In a next step, we were interested if p38 also modulates the production of the inflammatory cytokines IL-1β and the biologically active heterodimer IL-12p70, which is composed of the subunits IL-12p40 and IL-12p35. In order to stimulate the production of IL-12p70 from human monocytes, we stimulated them with LPS in combination with IFN-γ. Similar to mTOR inhibition, inhibition of p38 increased the levels of IL-12p70 (Figure 8A). To gain insight into potential alterations of IL-1β production after p38 inhibition, cells were pretreated as described and activated with LPS in combination with ATP. The results for the two donors are quite different. While donor 1 showed impaired levels of IL-1β after p38 inhibition and TLR4 activation, donor 2 showed no alterations after treatment with BIRB0796 but higher levels of IL-1β after pretreatment with SB203580 (Figure 8B). Pretreatment with rapamycin and stimulation with LPS for 24 h had no influence on the production of IL-1β, while stimulation with LPS for 5 h diminished the levels of IL-1β.

Summarized, we show that inhibition of p38, similar to inhibition of mTOR, leads to a proinflammatory phenotype after activation with TLR ligands characterized by higher

levels of the proinflammatory IL-12p40 and lower levels of the anti-inflammatory IL-10. The production of the bioactive IL-12p70 heterodimer is also boosted after inhibition of p38 and activation with the TLR4 ligand LPS.

4.2.2 Different regulation of IL-12p40 and IL-10 in moDCs and mDCs

It was recently shown in our laboratory that mTOR plays a divergent role in mDCs and moDCs [68]. In mDCs, inhibition of mTOR increases proinflammatory cytokines and decreases the levels of IL-10 after TLR activation, while in moDCs this immunostimulatory phenotype is blocked [68]. We were now interested if inhibition of p38 shows similar effects as inhibition of mTOR in mDCs and moDCs.

moDCs and mDCs were generated as described, pretreated with p38 or mTOR inhibitors and stimulated with different TLR ligands. In moDCs, inhibition of p38 blocked IL-10 and IL-12p40 production after stimulation with LPS. Inhibition of mTOR with rapamycin also decreased the levels of IL-10, while there was no influence on the IL-12p40 production. TNF-α production did not change after inhibition of p38 or mTOR. Stimulation of moDCs with SAC did hardly lead to any IL-10 production and the levels did not change after blocking p38 or mTOR. IL-12 production was diminished after inhibition of p38 but enhanced after inhibition of mTOR (Figure 9A).

In mDCs, IL-10 was blocked after inhibition of p38 or mTOR and activation with the TLR ligands LPS, SAC or *Listeria monocytogenes*. The levels of IL-12p40 were augmented after pretreatment with p38 and mTOR inhibitors. TNF-α levels were unaltered after treatment with rapamycin, while they were diminished or also unaltered after inhibition of p38 (Figure 9B).

In summary, we extend our previous results with mTOR inhibitors and additionally show that inhibition of p38 differently regulates cytokine production in moDCs and mDCs.

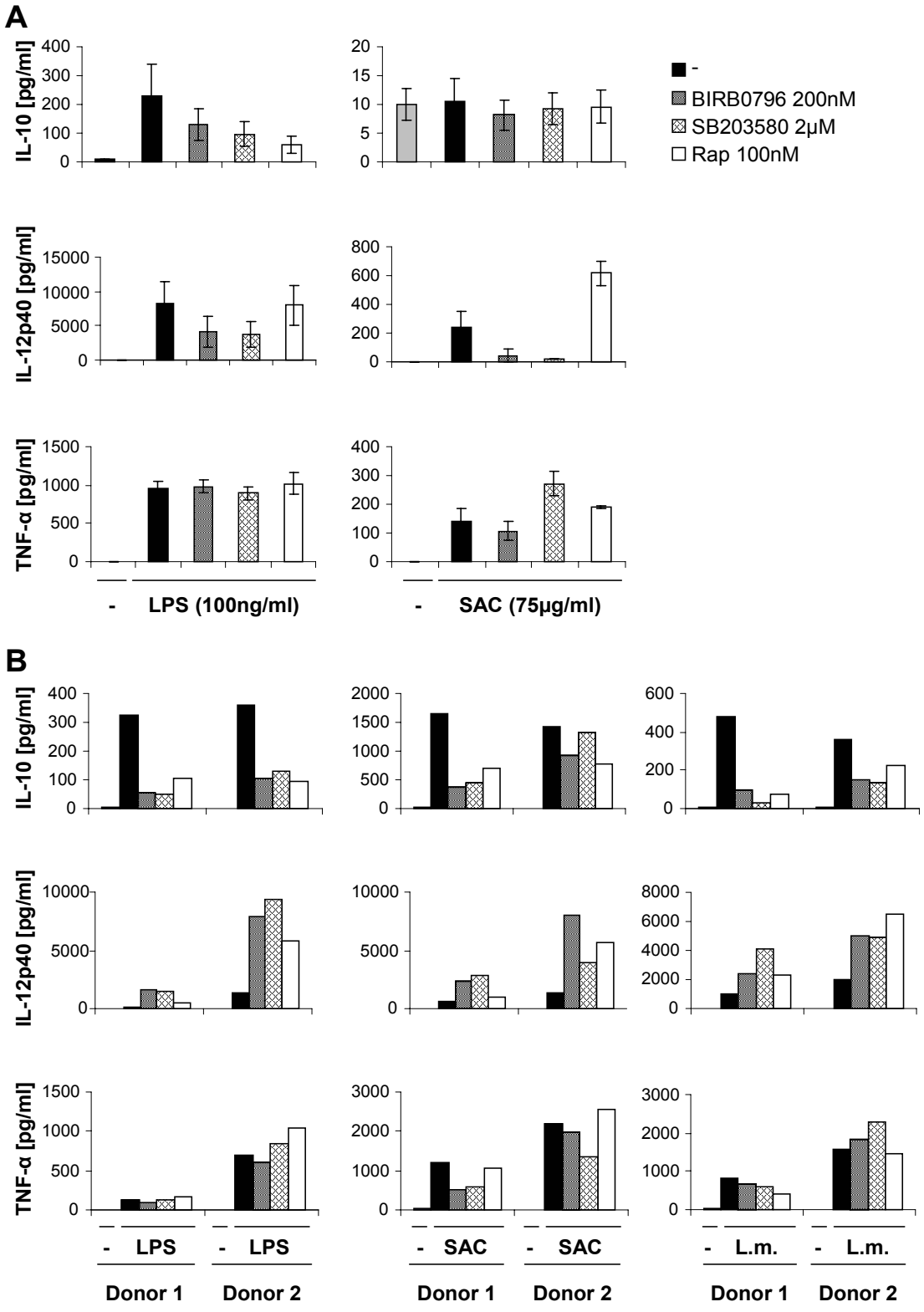

Figure 9. Different regulation of IL-12p40 and IL-10 levels in moDCs and mDCs
(A) Human moDCs or (B) mDCs were generated as described, preincubated with medium, BIRB0796 (200 nM), SB203580 (2 µM) or rapamycin (100 nM) for 90 min and stimulated with LPS (100 ng/ml), SAC (75 µg/ml) or *Listeria monocytogenes* (10^7 pfu/ml) for 20 h. IL-12p40, IL-10 and TNF-α levels in cell-free supernatants were assessed by Luminex. Cytokine levels of moDCs are shown as means ± SE for 3 donors. Cytokine levels of mDCs are shown for two independent donors.

4.2.3 p38 regulates IL-12p40 and IL-10 mRNA levels

The results so far indicate that blocking of p38 increases the protein levels of IL-12p40, while it decreases the levels of IL-10 after TLR stimulation. In order to investigate whether the production of these cytokines is regulated on the transcriptional level, we measured the mRNA levels at different time points of LPS stimulation. Figure 10 illustrates that the IL-12p40mRNA levels were augmented after pretreatment with BIRB0796, SB203580 or rapamycin, while the IL-10 levels were reduced. However, the mRNA levels of IL-23p19, which together with IL-12p40 forms the active IL-23, were diminished after p38 inhibition. After inhibition of mTOR, the mRNA levels of IL-23p19 increased (Figure 10).

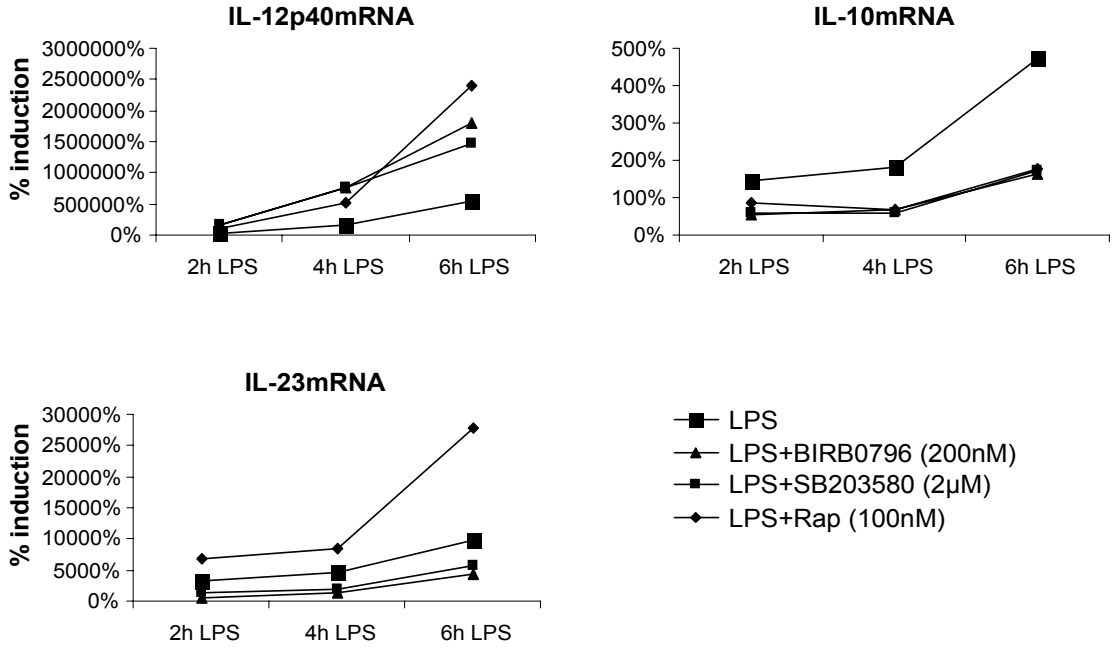

Figure 10. p38 regulates IL-12p40 and IL-10 mRNA production
Human CD14+ monocytes were preincubated with medium, BIRB0796 (200 nM), SB203580 (2 µM) or rapamycin (100 nM) for 90 min and stimulated with LPS (100 ng/ml) for 2, 4 or 6 h. IL-12p40, IL-10 and IL-23 mRNA levels were determined by RT-PCR. mRNA levels were normalized to ubiquitin and are shown as fold increases over the unstimulated controls. Representatives for two independent experiments are shown.

To further demonstrate the functional relevance of p38 for IL-12 production, we hyperactivated p38 with anisomycin or UV in the presence of LPS. Costimulation with LPS and UV diminished IL-12p40 levels (Figure 11A). Moreover, IL-12p40mRNA levels were strongly reduced when stimulating with LPS and UV or LPS and anisomycin simultaneously (Figure 11B).

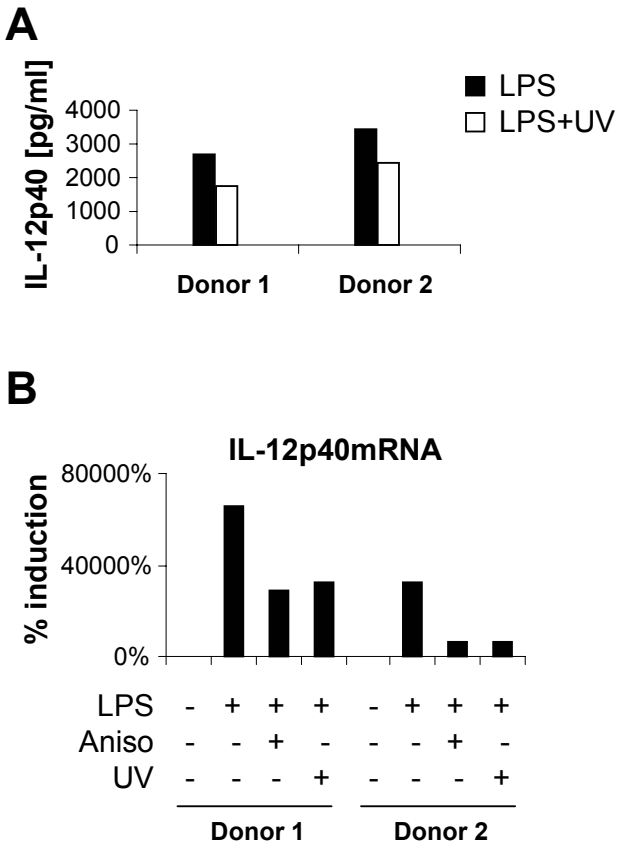

Figure 11. Hyperactivation of p38 diminishes IL-12p40
(A) Human monocytes were activated with LPS (100 ng/ml) and/or UV (as described in materials and methods) for 20 h. IL-12p40 levels in cell-free supernatants were determined by Luminex. Data of two different donors are shown. (B) Human monocytes were activated with different combinations of LPS (100 ng/ml), anisomycin (50 ng/ml) and UV for 2 h. IL-12p40 mRNA levels were measured by RT-PCR and normalized to ubiquitin. Fold increases over unstimulated controls are illustrated. Data of two independent donors are shown.

Sp1 (Specificity protein 1) has been shown to be a transcription factor important for the regulation of IL-12p40. Therefore, we determined the influence of mithramycin, a known inhibitor of Sp1, on IL-12p40 production. Pretreatment with mithramycin decreased the levels of IL-12p40 after LPS stimulation (Figure 12A). Importantly, the augmented production of IL-12p40 after BIRB0796 or rapamycin pretreatment could be reversed by concomitant treatment with 100 nM or 300 nM mithramycin (Figure 12A). These results suggest that inhibition of mTOR and p38 might enhance Sp1 activity to promote IL-12p40 production, which can be inhibited by the Sp1 inhibitor mithramycin.

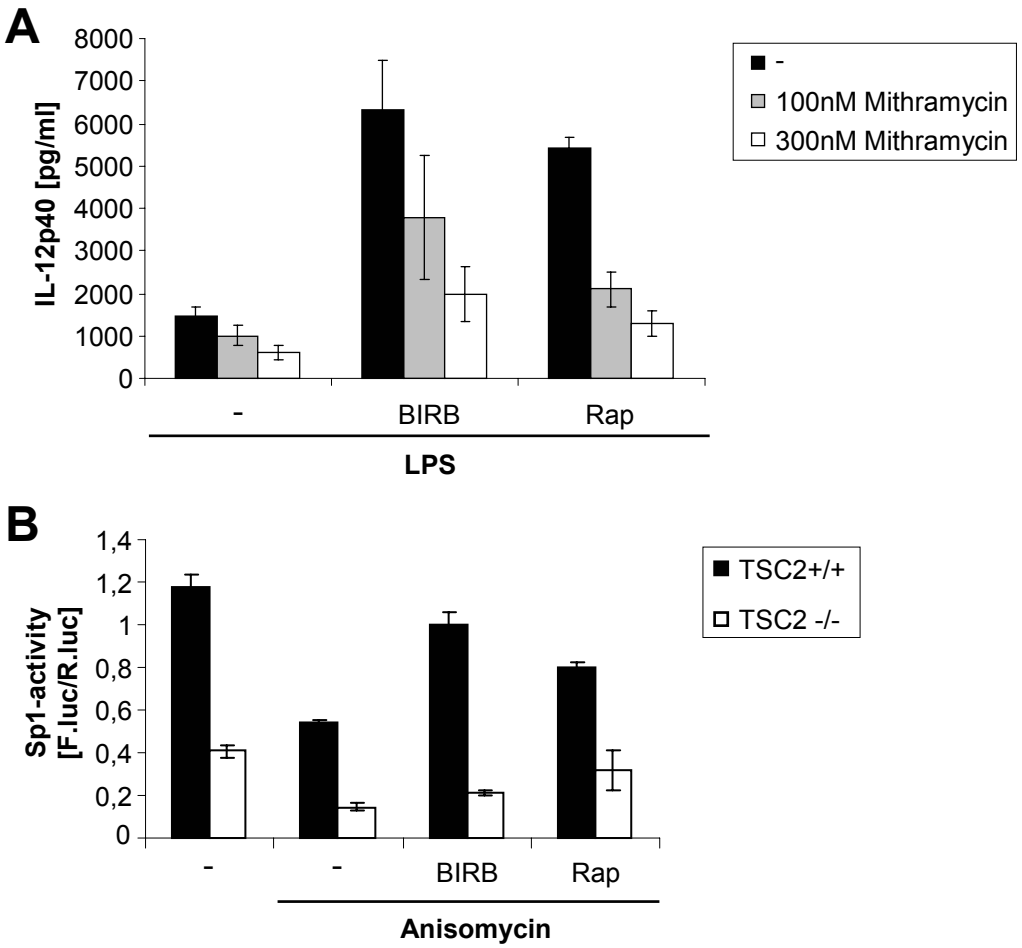

Figure 12. Possible role for Sp1 in regulating IL-12p40 production
(A) Human CD14+ monocytes were preincubated with medium, BIRB0796 (200 nM), rapamycin (100 nM) and/or mithramycin (100 nM or 300 nM) for 90 min and stimulated with LPS (100 ng/ml) for 24 h. IL-12p40 cytokine levels in cell-free supernatants were determined by Luminex. Data are shown as means ± SE for two donors. (B) TSC2+/+ and TSC2-/- MEFs were transfected with an Sp1-Luc plasmid as described. After transfection, cells were pretreated with medium, BIRB0796 (200 nM) or rapamycin (100 nM) and stimulated with anisomycin (100 ng/ml) for 24 h. Luciferase assay was performed as described. The ratio from the Sp1-firefly luciferase activity to renilla luciferase activity is shown. The experiment was performed in duplicates.

Moreover, transfection of an Sp1-plasmid into TSC2+/+ and TSC2-/- MEFs revealed that the activity of Sp1 was higher in TSC2+/+ MEFs compared to the TSC2-/- MEFs, suggesting that activation of mTOR inhibits Sp1 activity. In line, stimulation of p38 with anisomycin reduced the activity of Sp1 in TSC2+/+ MEFs compared to the unstimulated control. Pretreatment with BIRB0796 or rapamycin of anisomycin-stimulated TSC2+/+ cells restored the activity of Sp1 compared to anisomycin alone. Furthermore, stimulation with anisomycin blocked the activity of Sp1 in TSC2-/- MEFs, however, BIRB0796 failed to enhance the activity of Sp1 in these cells (Figure 12B).

These results indicate that activation of p38 via TSC2 and mTOR may reduce Sp1 activity, which is critical for IL-12p40 production.

4.3 A novel link from p38 to mTOR

4.3.1 Inhibition of p38 blocks mTOR

To analyze the molecular mechanisms of how the p38 and mTOR signaling might interact, we investigated if p38 is able to modulate mTOR signaling. Therefore, we pretreated human monocytes with different p38 inhibitors or rapamycin and stimulated them with LPS, anisomycin or UV, known activators of p38. We confirmed blocked p38 signaling by analyzing the phosphorylation status of MK2, a substrate of p38. While the inhibitors BIRB0796 and SB203580 blocked the phosphorylation of MK-2 after activation with LPS, SD-169 did not (Figure 13A). These results indicate that SD-169 does not block p38 activation in human monocytes *in vitro*. Strikingly, inhibition of p38 with BIRB0796 and SB203580 was able to block mTOR signaling after stimulation with LPS, anisomycin or UV. This was shown by the reduced phosphorylation of S6 and 4E-BP1 (Figures 13A, B, C). Additionally, the phosphorylation of p-p70S6K, a direct mTORC1 substrate and the kinase that phosphorylates p-S6, was totally blocked after treatment with BIRB0796 and SB203580 in LPS-stimulated monocytes (Figure 13A). As expected, the mTOR inhibitor rapamycin inhibited the phosphorylation of p70S6K, S6 and 4E-BP1. Moreover, the phosphorylation status of p38 was not affected by rapamycin (Figures 13A, B, C). p38 inhibition also led to an enhancement in the phosphorylation of ERK and c-jun, while rapamycin did not affect them. Interestingly, phosphorylation of Akt at Ser473 was blocked with the p38 inhibitors BIRB0796 and SB203580, while it was not blocked after pretreatment with rapamycin. The phosphorylation of GSK3 was not affected by p38 or mTOR inhibition (Figures 13A, C). Compared to the unstimulated control, the phosphorylation status of IκB-α was diminished with LPS stimulation, but there was no further modulation after pretreatment with p38 inhibitors or rapamycin (Figure 13A).

In conclusion, we demonstrate that inhibition of p38 strongly reduces mTOR signaling after stimulation with TLR-dependent and –independent stimuli indicating that p38 is a bona-fide activator of mTORC1 signaling.

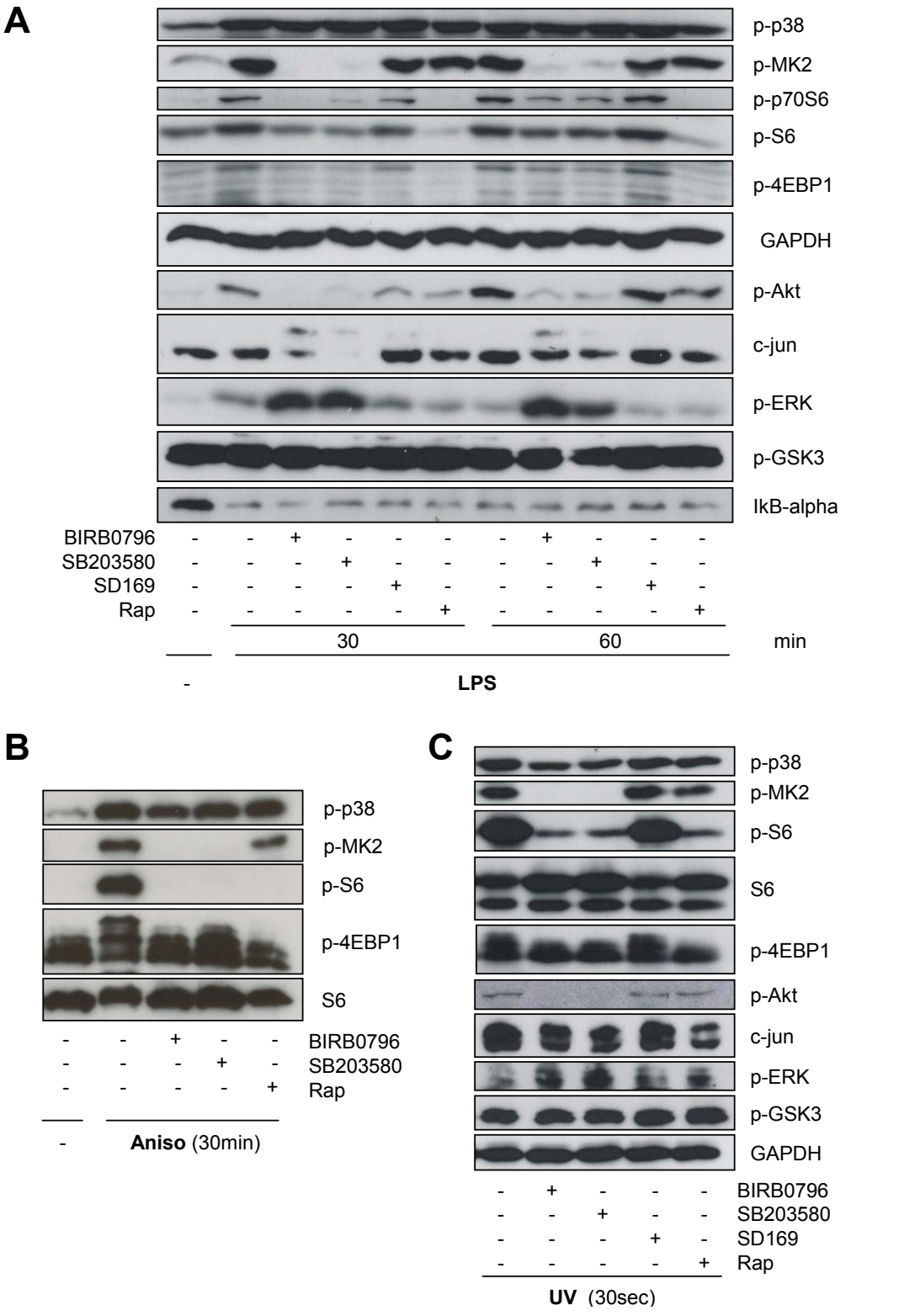

Figure 13. Inhibition of p38 blocks mTOR
Human monocytes were preincubated with medium, BIRB0796 (200 nM), SB203580 (2 µM), SD169 (200 nM) or rapamycin (100 nM) for 90 min and stimulated with (A) LPS (100 ng/ml), (B) anisomycin (50 ng/ml) or (C) UV (as described in methods) for the indicated times. Whole-cell lysates were analyzed by immunoblot. Representatives of at least two independent experiments are shown.

As the p38MAPK consists of four different subunits (α, β, γ and δ), we wanted to know which of these subunits might be responsible for the activation of mTOR. The concentrations of the inhibitors used so far were in a range where they blocked specifically p38α and p38β and we also knew that the expression of p38β is very low in monocytes. Therefore, we made use of p38α fl/fl and p38α fl/fl LysMCre Δp38α bone marrow derived mouse macrophages to test the effect of p38α knockout on mTOR and on cytokine expression. On the molecular level, p38α was expressed in the p38α fl/fl, but not in the p38α fl/fl LysMCre Δp38α macrophages (Figures 14A, B). Interestingly, stimulation with LPS, anisomycin, UV or *Listeria monocytogenes* induced a strong phosphorylation of S6 in the p38α fl/fl macrophages, while the phosphorylation was blocked in the p38α fl/fl LysMCre Δp38α macrophages (Figure 14A, B). Stimulation with *Listeria monocytogenes* also activated 4EBP1 and p70S6K in the p38α fl/fl macrophages but failed to do so in the p38α fl/fl LysMCre Δp38α macrophages (Figure 14B). These results extend the findings in human cells and demonstrate that p38α activates mTORC1 in response to LPS, or stress-induced signals such as UV or anisomycin.

Next, we wanted to assess the functional importance of p38α in the bone marrow macrophages. The levels of IL-10 were attenuated in the p38α fl/fl LysMCre Δp38α macrophages, compared to the p38α fl/fl macrophages, after stimulation with LPS, SAC or *Listeria monocytogenes*, respectively. Moreover, the production of IL-12p40 and IL-12p70 was augmented in the p38α knockout macrophages after treatment with LPS or SAC, while there was no significant increase in the *Listeria monocytogenes*-treated knockout macrophages. The levels of IL-1β, IL-23 and TNF-α were not altered when comparing the p38α fl/f and p38α fl/fl LysMCre Δp38α macrophages after stimulation with LPS, SAC or *Listeria monocytogenes* (Figure 14C), demonstrating that p38α is a major regulator of the Il12/IL10 axis in murine macrophages.

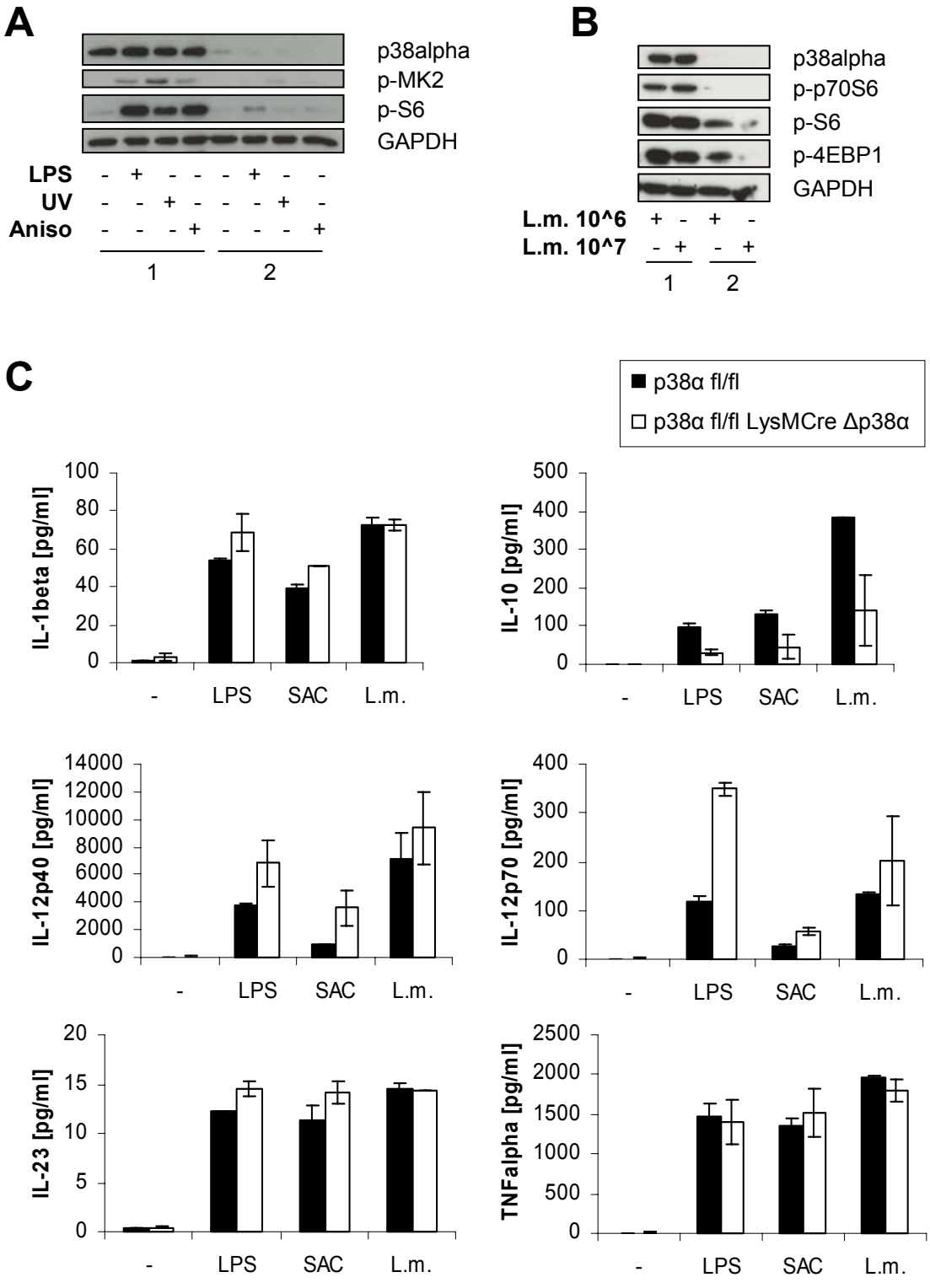

Figure 14. The subunit p38α activates mTOR
(A, B) p38α fl/fl (1) and p38α fl/fl LysMCreΔp38α (2) mouse macrophages were starved overnight and stimulated with medium, LPS (100 ng/ml), anisomycin (100 ng/ml) or UV (as described in materials and methods) for 1h, or with either 10^6 pfu/ml or 10^7 pfu/ml *Listeria monocytogenes*. Whole-cell lysates were analyzed by immunoblot. Representatives of two independent experiments are shown. (C) p38α fl/fl and p38α fl/fl LysMCreΔp38α mouse macrophages were starved overnight and stimulated with medium, LPS (100 ng/ml), SAC (75 µg/ml) or *Listeria monocytogenes* (10^7 pfu/ml) for 20 h. Afterwards, cytokine levels in the supernatants were determined by Luminex. Values are shown as means ± SE for two mice.

4.3.2 mTOR is activated via PI3K and p38 independently

Having established that p38α is a bona-fide activator of mTORC1, we wanted to further dissect the molecular signaling of p38 to mTOR. Therefore, we were now interested if p38 mediates its effect on mTOR signaling via the canonical PI3K pathway. Therefore, we treated human monocytes with the PI3K inhibitor wortmannin and/or the p38 inhibitor BIRB0796 and stimulated them afterwards with either LPS or anisomycin. Treatment with wortmannin alone did not affect the phosphorylation status of p38 and MK-2 after stimulation with LPS or anisomycin (Figures 15A, B). Both inhibitors were able to block phosphorylation of S6 partially (Figures 15A, B). Notably, simultaneous blockade of PI3K and p38 completely blocked the phosphorylation of S6 (Figures 15A, B). These results suggest that p38 is not downstream of PI3K, but serves as a second and parallel input signal to stimulate mTORC1 activity in human monocytes.

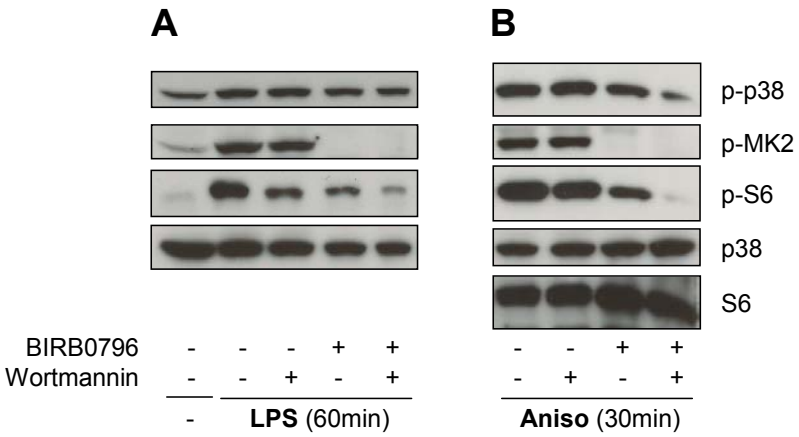

Figure 15. mTOR is activated via PI3K and p38 independently
Human monocytes were preincubated with medium, BIRB0796 (200 nM) and/or wortmannin (100 nM) for 90 min and stimulated with (A) LPS (100 ng/ml) or (B) anisomycin (50 ng/ml) for the indicated times. Whole-cell lysates were analyzed by immunoblot. Representatives for two independent experiments are shown.

In order to verify this dual input pathway for mTORC1 activation, we made use of the p38α fl/fl and p38α fl/fl LysMCre Δp38α bone marrow-derived mouse macrophages. As expected, in the p38α fl/fl LysMCre Δp38α macrophages, the p38 substrate MK-2 was not activated after stimulation with LPS (Figure 16A). In addition, the phosphorylation of MK-2 was blocked in the p38α fl/fl cells after pretreatment with SB203580 (Figure 16A). Nevertheless, wortmannin did not affect MK2 phosphorylation after stimulation with LPS (Figure 16A). The phosphorylation of p70S6K and S6 was reduced after pretreatment with wortmannin or SB203580 and stimulation with LPS in

the p38 fl/fl macrophages. Moreover, the phosphorylation of these two proteins was even more reduced after simultaneous usage of SB203580 and wortmannin, indicating that p38 and PI3K are two independent signal inputs for mTOR activation also in mouse macrophages. In support, the activation of S6 and p70S6K was attenuated in the p38α fl/fl LysMCre Δp38α macrophages compared to the p38α fl/fl macrophages and, as expected, SB203580 only modestly affected the phosphorylation of p70S6K and S6 in the p38 knockout macrophages, while the use of wortmannin could further block S6 and p70S6K. Concomitant treatment with SB203580 and wortmannin in the p38α fl/fl LysMCre Δp38α macrophages totally abrogated the phosphorylation of S6 and p70S6K (Figure 16A). Similar results were obtained when anisomycin was used to activate p38 and mTORC1 and BIRB0796 to inhibit p38 (Figure 16B).

To further confirm these results, we analyzed single cells by immunocytochemistry. Stimulation of p38α fl/fl macrophages with LPS activated S6, while this activation was attenuated in the p38α fl/fl LysMCre Δp38α macrophages. Moreover, inhibition of PI3K in the p38α fl/fl blocked phosphorylation of S6. Strikingly, inhibition of PI3K in the p38α fl/fl LysMCre Δp38α macrophages completely blocked S6 phosphorylation (Figure 17A). MK-2 was activated after treatment with LPS and pretreatment with wortmannin did not alter the activation status of this protein in the p38α fl/fl macrophages, whereas the phosphorylation of MK2 was inhibited in the p38α knockout macrophages (Figure 17B). These results establish that p38α activates mTOR signaling cooperatively with PI3K.

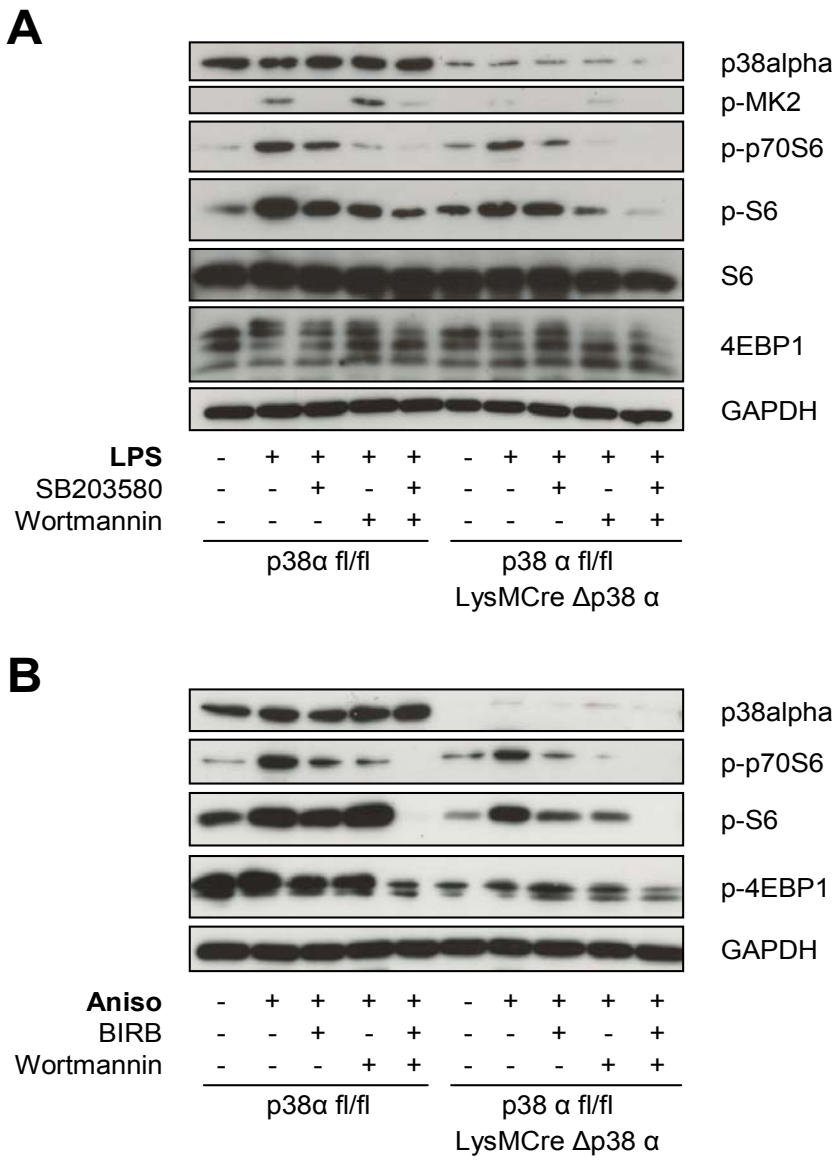

Figure 16. PI3K inhibition in p38α knockout macrophages completely blocks mTOR
p38α fl/fl and p38α fl/fl LysMCreΔp38α mouse macrophages were starved over night and pretreated with medium, BIRB0796 (200 nM), SB203580 (2 µM) and/or wortmannin (100 nM) for 90 min. Afterwards cells were stimulated with (A) LPS (100 ng/ml) or (B) anisomycin (100 ng/ml) for 1 h. Whole-cell lysates were analyzed by immunoblot. Representatives of two independent experiments are shown.

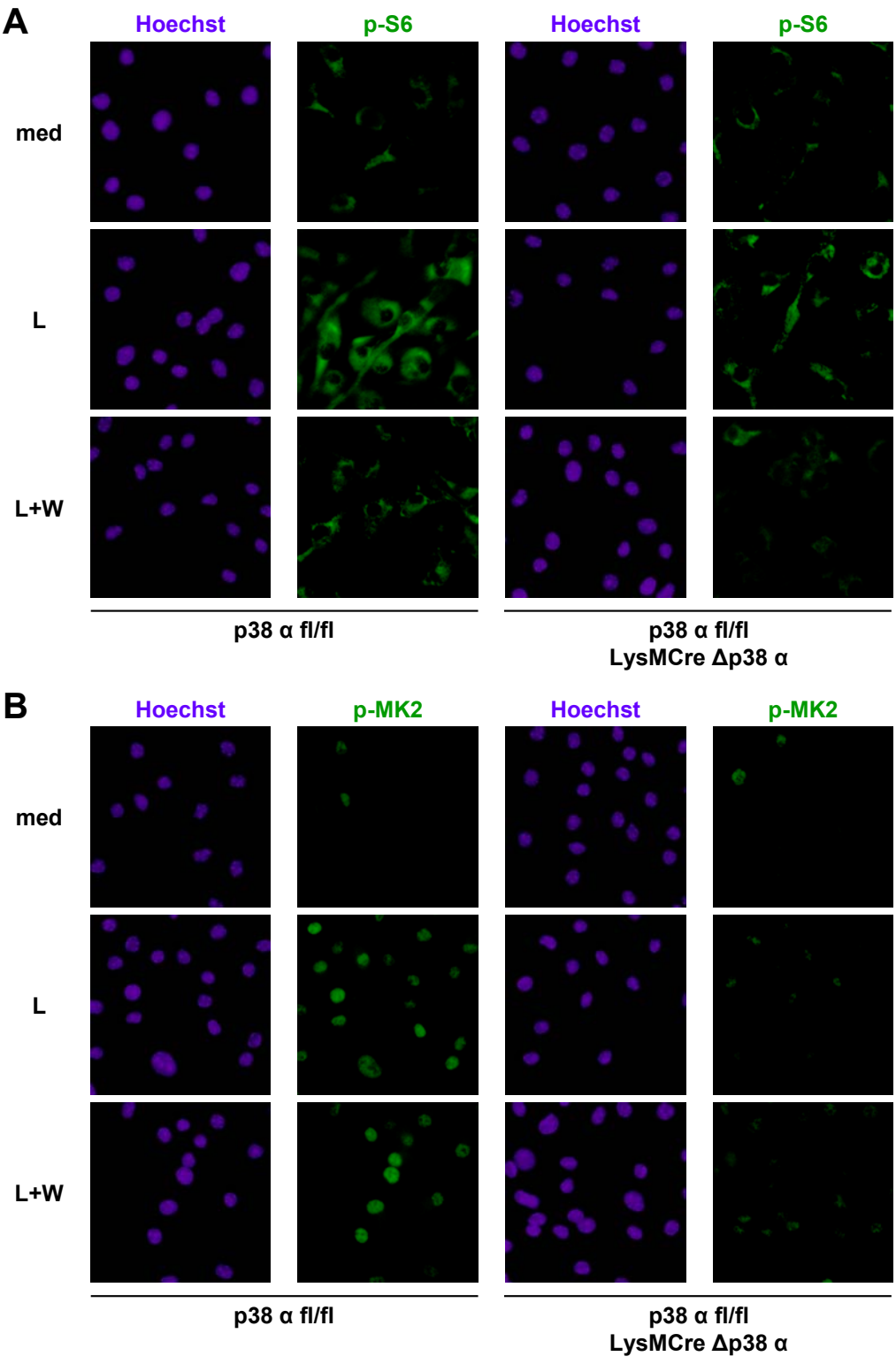

Figure 17. The subunit p38α and PI3K activate mTOR independently
p38α fl/fl and p38α fl/fl LysMCreΔp38α mouse macrophages were starved over night and pretreated with medium or wortmannin (100 nM) for 90 min. After stimulation with LPS (100 ng/ml) for 1 h, intracellular signaling was analyzed by immunocytochemistry. Antibodies against (A) p-S6 and (B) p-MK-2 were used. Nuclei were stained by using Hoechst 33342. Representatives for two independent experiments are shown.

4.3.3 p38 activates mTOR via TSC2

To further characterize the PI3K-indepdendent activation of mTOR by p38α, we made use of TSC2-/- murine embryonic fibroblasts (MEFs). TSC2 is in a complex with TSC1 and is a negative regulator of mTOR. After being phosphorylated by Akt, TSC2 becomes inactive and can no longer inhibit mTOR leading to constitutive mTOR activation [69].

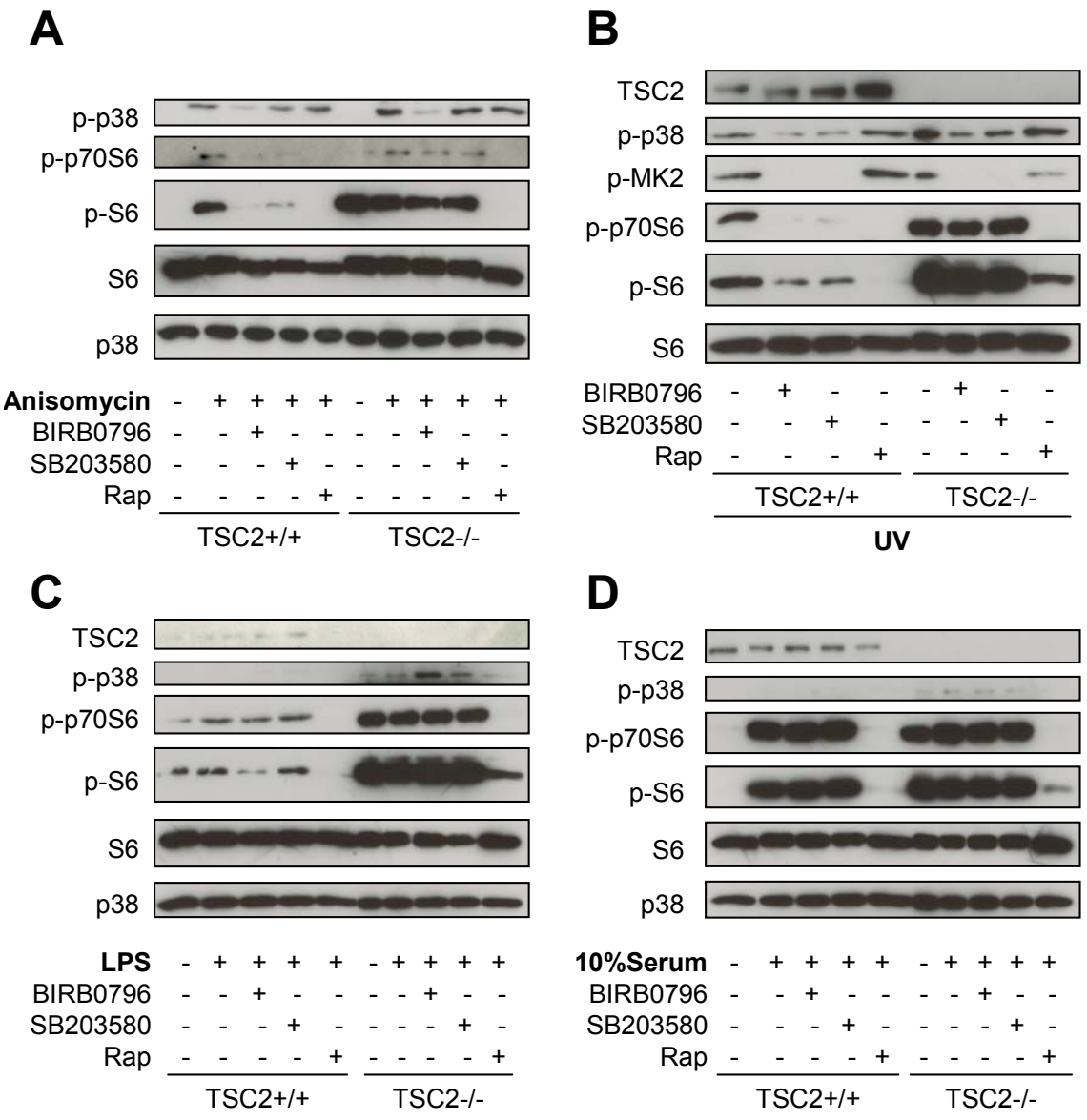

Figure 18. p38 activates mTOR via TSC2
TSC2+/+ p53-/- and TSC2-/- p53-/- MEFs were starved overnight and pretreated with medium, BIRB0796 (200 nM), SB203580 (2 µM) or rapamycin (100 nM) for 90 min. Afterwards, MEFs were stimulated with (A) anisomycin (100 ng/ml), (B) UV (as described in methods), (C) LPS (100 ng/ml) or (D) 10 % FCS for 60 min. Whole-cell lysates were analyzed by immunoblot.

When stimulating the TSC2+/+ and TSC2-/- MEFs with anisomycin or UV, the proteins p38, S6 and p70S6K were strongly activated (Figures 18A, B). Strikingly, the p38 inhibitors BIRB0796 and SB203580 were able to block the phosphorylation of S6 and p70S6K in the TSC2+/+ MEFs, while they failed to do so in the TSC2-/- MEFs (Figures 18A, B). Rapamycin was able to inhibit the phosphorylation of the mTOR substrates, both in the TSC2+/+ and TSC2-/- MEFs (Figures 18A, B) suggesting that p38 activates mTOR signaling by TSC2.

LPS is only a poor stimulator for MEFs. Hence, stimulation of TSC2+/+ MEFs with LPS did not activate p38 or mTOR (Figure 18C). Nevertheless, pretreatment with rapamycin blocked the phosphorylation of S6 and p70S6K (Figure 18C). The loss of TSC2 caused hyperactivation of mTOR, which could be blocked with rapamycin, but not with the p38 inhibitors (Figure 18C).

Activation of the MEFs with 10 % FCS led to a strong phosphorylation of p70S6K and S6, while p38 was not phosphorylated (Figure 18D). Again, the phosphorylation of p70S6K and S6 could only be blocked with rapamycin in the TSC2+/+ and TSC2-/- MEFs, respectively (Figure 18D). Pretreatment with BIRB0796 and SB203580 did not alter the phosphorylation status of S6 or p70S6K after stimulation with 10 % FCS (Figure 18D) indicating that serum activates mTOR in a p38-independent way.

In summary, LPS and 10 % FCS failed to activate p38 in the MEFs and therefore, p38 inhibitors had no effect on the phosphorylation status of the mTOR substrates. In contrast, stimulation with anisomycin and UV strongly activated p38. Inhibition with BIRB0796 and SB203580 blocked mTOR in the TSC2+/+ MEFs, but not in the TSC2-/- MEFs suggesting that p38 activates the mTOR pathway via TSC2. The lack of TSC2 had no effect on the inhibitory function of rapamycin due to the fact that rapamycin inhibits mTOR downstream of TSC2.

4.4 p38 inhibition induces Th1 response

4.4.1 Inhibition of p38 upregulates CD86 on mDCs

To investigate what consequences inhibition of p38 has on the development of an inflammatory immune response, we analyzed the regulation of CD86 (B7-2) on mDCs after treatment with p38 inhibitors. CD86 belongs together with CD80 (B7-1) to an important class of costimulators for T lymphocytes. These two molecules are expressed on macrophages, B lymphocytes and dendritic cells [1].

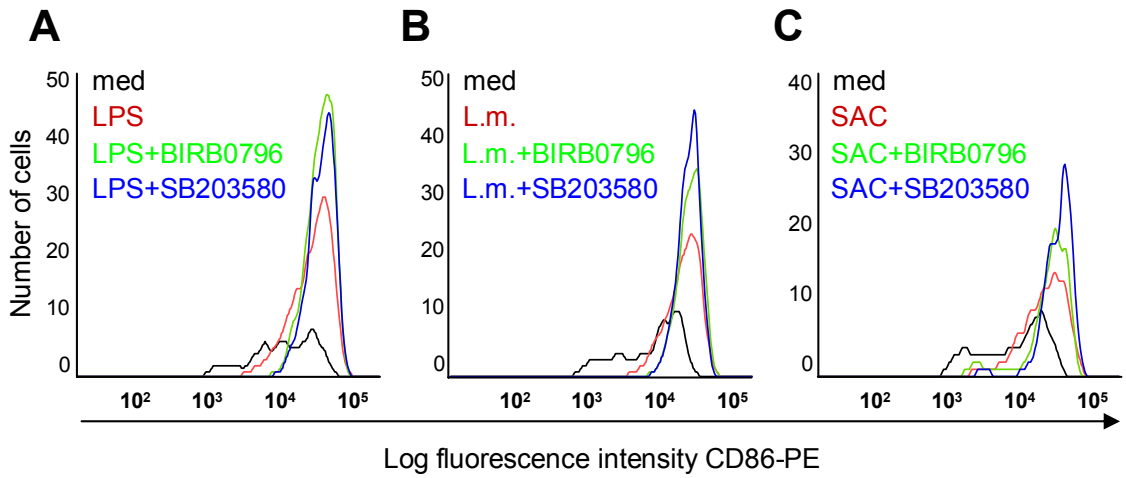

Figure 19. Inhibition of p38 upregulates CD86 on human mDCs
mDCs were generated as described and pretreated with medium, BIRB0796 (200 nM) or SB203580 (2 µM) for 90 min. Afterwards, cells were stimulated with (A) LPS (100 ng/ml), (B) *Listeria monocytogenes* (10^7 cfu/ml) or (C) SAC (75 µg/ml) for 24 h. Subsequently, mDCs were prepared for FACS analysis. Representatives for two independent experiments are shown.

After treating mDCs with medium, BIRB0796 or SB203580 cells were stimulated with LPS, SAC or *Listeria monocytogenes*. After gating the mDCs, we generated a histogram for the CD86 positive cells (Figures 19A, B, C). Figure 19A shows that stimulation with LPS upregulates CD86 on mDCs compared to the unstimulated control. Moreover, CD86 expression is even higher after pretreatment with the p38 inhibitors SB203580 and BIRB0796 (Figure 19A). The same results were achieved after stimulation with *Listeria monocytogenes* (Figure 19B) and SAC (Figure 19C). Thus, inhibition of p38 boosted the expression of CD86 on mDCs.

4.4.2 p38 inhibition in monocytes promotes Th1 differentiation

In order to investigate if inhibition of p38 or mTOR in monocytes can elicit different T-cell responses after LPS stimulation, we established a T-cell differentiation assay. Monocytes were isolated from whole blood as described and treated with medium, BIRB0796, SB203580 or rapamycin. After stimulation with LPS for 24 h, monocytes were washed and incubated with CD4+ T lymphocytes.

After one week of priming, supernatants were taken off. Rapamycin, BIRB0796 and SB203580 pretreated monocytes induced elevated production of IFN-γ after LPS stimulation, compared to LPS stimulation without pretreatment (Figure 20A). The IL-17 levels were not altered in the BIRB0796 and SB203580 treated monocytes, while they were reduced in the rapamycin treated cells (Figure 20A).

To further analyze a potential influence of p38 on T-cell differentiation, primed T-cells were activated for 5 h with PMA/Ionomycin. Figure 20B shows that there was nearly no IL-4 production after T-cell activation with PMA/Ionomycin. LPS-treated monocytes elicited an IFN-γ response in the CD4+ T-cells that was even higher in rapamycin, BIRB0796 and SB203580 pretreated monocytes (Figure 20B). These results indicate that the proinflammatory phenotype (high IL-12p40/ low IL-10), which was observed in the SB203580 and BIRB0796 treated monocytes, is able to promote Th1 responses. This is comparable with rapamycin-pretreated monocytes, which were also able to enhance Th1 responses.

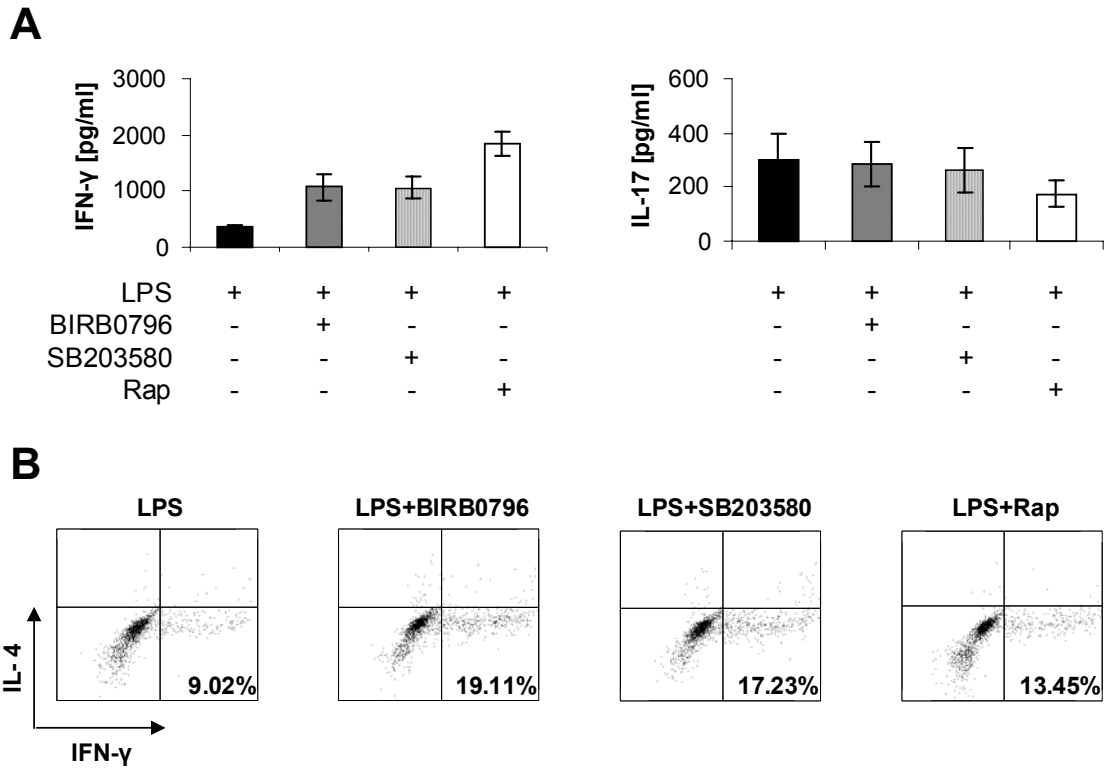

Figure 20. p38 inhibition in monocytes promotes Th1 differentiation
Human CD14+ monocytes were pretreated for 90 min with medium, BIRB0796 (200 nM), SB203580 (2 µM) or rapamycin (100 nM) and activated with LPS (100 ng/ml). After 20 h stimulation, allogeneic PBMCs were primed with those monocytes for one week. (A) IFN-γ and IL-17 levels in cell-free supernatants were determined by Luminex. Data represent means ± SE for at least three independent experiments. (B) Primed PBMCs were activated for 5 h with 50 ng/ml PMA and 200 ng/ml Ionomycin in the presence of 10 µg/ml Brefeldin A for the last 3 h. Intracellular cytokine staining for IL-4 and IFN-γ in CD-4 T-cells is illustrated. Representatives for at least three independent experiments are shown.

4.5 *In vivo* experiments

4.5.1 Macrophage specific p38α KO in mice alters mTOR pathway

Next, we wanted to corroborate our findings *in vivo* and tested the effects of p38 inhibition for cytokine production and activation of mTOR. Therefore, mice with a macrophage specific knockout of p38α were challenged with LPS. The IL-10 levels in the sera of p38α knockout mice were attenuated compared to the p38α fl/fl mice, while the levels of IL-12p40 and IL-12p70 were augmented (Figure 21A). However, the production of IL-23, TNF-α and IL-1β was not altered when comparing the p38α fl/f and p38α fl/fl LysMCre Δp38α mice (Figure 21A).

Moreover, reactivated splenocytes from p38α fl/fl LysMCre Δp38α mice displayed higher levels of IL-12p40 in the supernatants compared to the splenocytes of p38α fl/fl mice. The TNF-α production was not altered (Figure 21B). Figure 21C shows that p70S6K and 4EBP1 were activated in spleens from p38α fl/fl mice challenged with LPS, while the phosphorylation of these proteins was totally blocked in p38α fl/fl LysMCre Δp38α spleens.

In summary, these results indicate that p38α is important for the activation of mTOR in macrophages *in vivo* and modulates IL-10/IL-12 signaling.

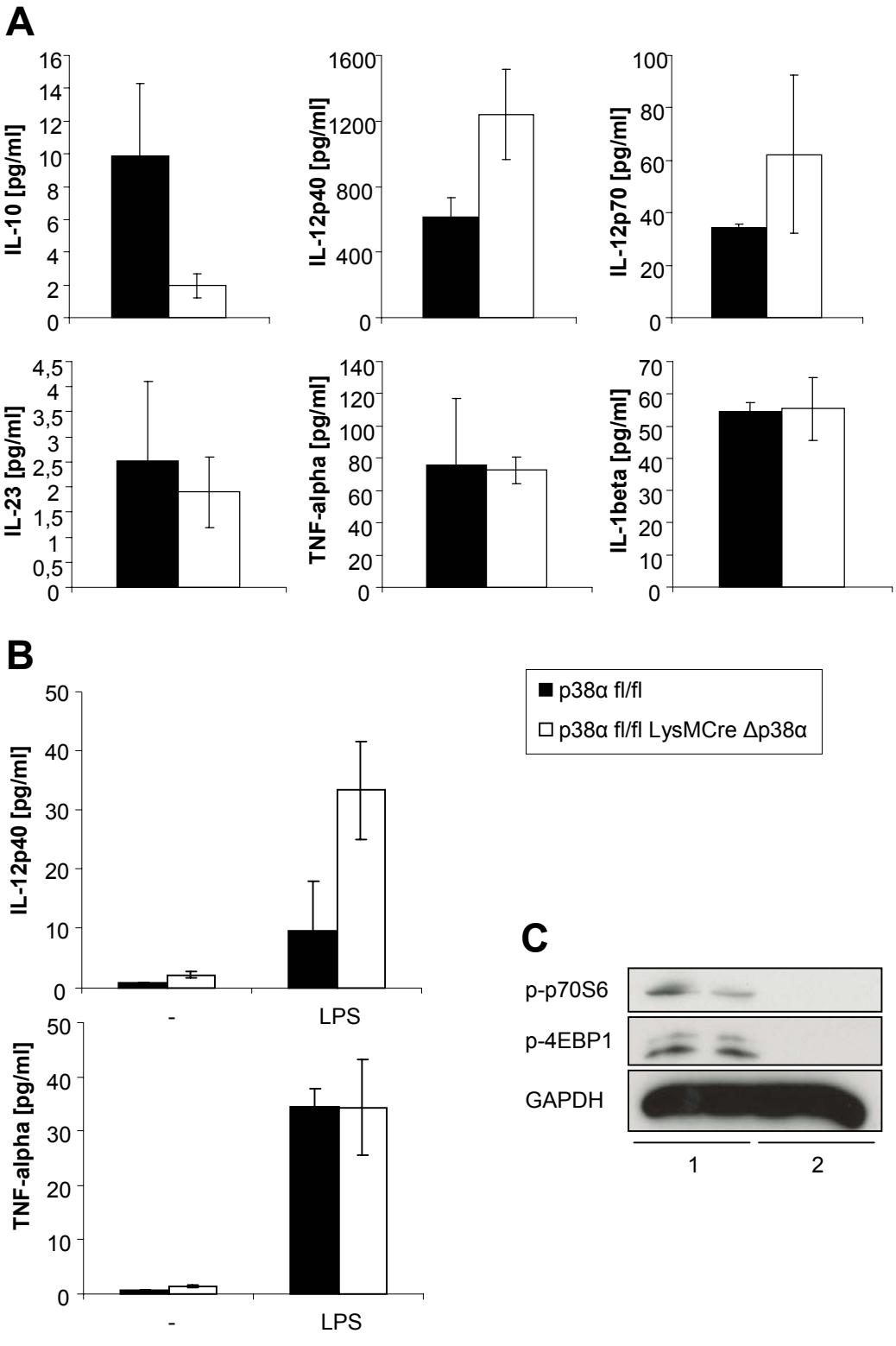

Figure 21. p38α knockout in mice alters the mTOR pathway
Three p38α fl/fl and three p38α fl/fl LysMCreΔp38α were injected with 30 µg/mouse LPS for 4 h. (A) Serum samples were taken and cytokine levels were determined by cytokine assay kit. (C) Spleens were isolated, homogenized and analyzed by immunoblot. The mTOR activation for two p38α fl/fl (1) and two p38α fl/fl LysMCreΔp38α (2) mice is shown. (B) Three p38α fl/fl and three p38α fl/fl LysMCreΔp38α were injected with 25 µg/mouse LPS for 4 h. Splenocytes were isolated and restimulated with 100 ng/ml LPS or solvent control. Cytokine levels in the cell free supernatant were determined by cytokine assay kit.

4.5.2 p38 inhibition in C57BL/6 mice has no influence on *Listeria* infection

Next, we wanted to find out whether inhibition of p38 with SB203580 displays the same inflammatory phenotype as the macrophage specific knockout of p38α in mice. Therefore, we made use of C57BL/6 mice and challenged them with *Listeria monocytogenes*.

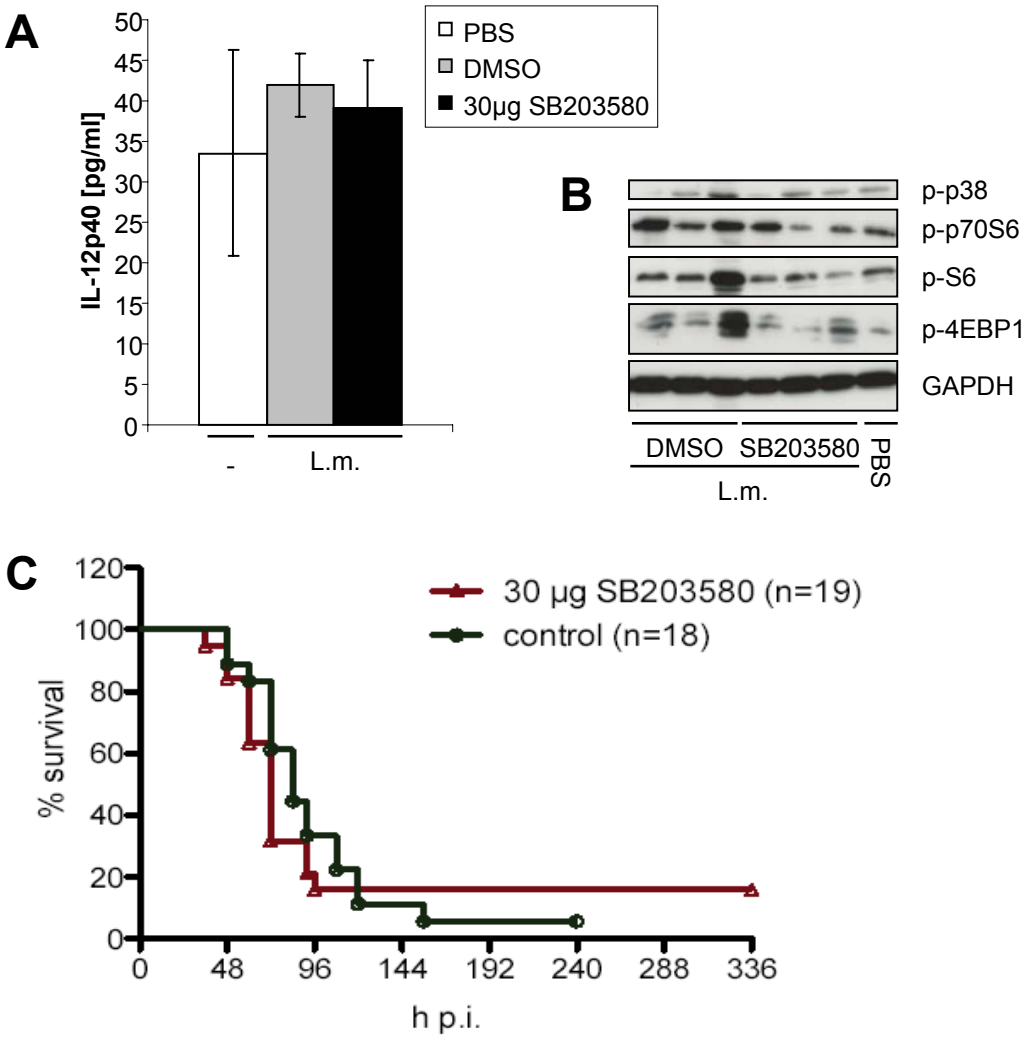

Figure 22. p38 inhibition in C57BL/6 mice has no influence on *Listeria infection*
(A) C57BL/6 mice were injected with solvent control, 30 µg/mouse SB203580 or PBS. 30 min later, mice were injected with $5x10^6$ *Listeria monocytogenes* or left untreated for 6 h. Serum samples were taken and cytokine levels were determined by cytokine assay kit. Cytokine levels are shown as means ± SE for at least three mice. (B) Spleens were isolated, homogenized and analyzed by immunoblot. Data from three DMSO treated, three SB203580 treated and one PBS treated mice, are shown. (C) C57BL/6 mice were injected with solvent control (n=18) or 30 µg/mouse SB203580 (n=19). 30 min later mice were injected with $5x10^6$ *Listeria monocytogenes* and survival of the mice was monitored. Summary of three independent experiments is shown.

Figure 22A shows that there was no induction of IL-12p40 production after *Listeria monocytogenes* infection. Moreover, pretreatment with SB203580 did not alter the IL-12p40 levels in the sera of C57BL/6 mice compared to solvent control-treated mice after *Listeria* infection (Figure 22A).

In order to find out whether mTOR was activated after *Listeria monocytogenes* infection, we homogenized the spleens of the mice and analyzed the cell signaling by immunoblot. Only one out of three mice that were pretreated with DMSO and infected with *Listeria* showed an induction of p-p38, p-4EBP1, p-S6 and p-p70S6K, compared to the PBS treated, uninfected mice. The other two mice, which were pretreated with DMSO, showed weak or no induction of these proteins compared to the uninfected mice. Pretreatment with SB203580 and infection with *Listeria monocytogenes* kept the phosphorylation status of p38, p70S6K, S6 and 4EBP1 at the level of the uninfected mice (Figure 22B).

Figure 22C illustrates that pretreatment with 30 µg/mouse SB203580 had no influence on the survival rate of C57BL/6 mice after infection with $5x10^6$ *Listeria monocytogenes*. Eighty percent of both, the solvent control and the SB203580-treated mice, were dead 96 hours after the *Listeria* infection.

Summarized, these results indicate that, p38 is not activated after *Listeria monocytogenes* infection and hence, does not contribute to IL-12p40 production in C57BL/6 mice *in vivo*.

5 Discussion

We show here that p38 in human monocytes negatively regulates IL-12 production, while it promotes IL-10 in myeloid innate immune cells after stimulation with different TLR ligands. The enhanced inflammatory response after p38 inhibition was manifested by the potential of these cells to strongly promote Th1 differentiation in CD4+ T-cells. Moreover, we provide evidence for a connection between the p38 and mTOR pathway that has not been shown so far in innate immune cells. The link of these two pathways seems to be at the level or upstream of TSC2, which is a negative regulator of mTOR. Moreover, we could also identify p38α to be the isoform responsible for the activation of mTOR and the potential to modulate IL-10/IL-12. These findings provide a basis for a better understanding of the signal transduction pathways that regulate innate immune response.

In the first steps of the work, we confirmed the findings from Hale et al and showed that p38α is the predominant subunit expressed in human monocytes [65]. The finding that p38β is time-dependently induced 1 hour after stimulation with LPS was quite interesting. This could indicate that this subunit has a role during the first steps of an immune response either to enhance these responses, or it may serve as a negative regulator. While p38δ was expressed second strongest in monocytes, we did not measure the levels of p38γ, as Hale et al showed that it is not expressed in human monocytes [65]. However, as most of the literature points to a role of p38α in inflammation, we moved on using p38α and p38β specific inhibitors in our experiments.

Next, we analyzed the cytokine levels expressed by monocytes after inhibition of p38 or mTOR and stimulation with different bacterial stimuli. We confirmed the already known fact that inhibition of p38 blocks TNF-α production, as TNF-α is translationally regulated via the p38 substrate MK2 and TTP [4, 14, 26]. We found that the levels of IL-12p40 and IL-12p70 were increased after inhibition of p38 and TLR stimulation in human monocytes. Recently, Boucher et al also found in murine macrophages that p38 negatively regulates IL-12 [63]. The finding was in line with the known augmentation of IL-12p40 and IL-12p70 expression after mTOR inhibition. Furthermore, the expression of the anti-inflammatory IL-10 was blocked with rapamycin pretreatment, as well as with inhibition of p38 after stimulation with different TLR ligands. Thus, inhibition of p38 or mTOR followed by TLR stimulation displayed similar alterations of the cytokine milieu, providing a first hint that the p38 and mTOR pathway might be linked.

Interestingly, cytokine levels were not altered after p38 inhibition and activation with zymosan. This might be due to lack of p38 activation with zymosan in human monocytes.

Furthermore, we were interested in the regulation of the IL-12p40 levels after inhibition of p38 in mDCs and moDCs, as it was recently shown in our lab that there is a divergent role in the differentiation of mDCs and moDCs. In mDCs, inhibition of mTOR increased proinflammatory cytokines and decreased the levels of IL-10 after TLR activation, while in moDCs this immunostimulatory phenotype was blocked [68]. We extended these results and investigated the role of p38 in mDCs versus moDCs. While there was an increase in the levels of IL-12p40 after p38 inhibition in the mDCs, there was a decrease in moDCs. The levels of IL-10 were blocked in both, the mDCs and moDCs after p38 inhibition and TLR activation.

The real-time PCR data pointed to a role for p38 in regulating the production of IL-10 and IL-12p40 at the transcriptional level. Comparable with direct inhibition of mTOR, the IL-12p40mRNA levels increased after inhibition of p38, while the IL-10mRNA levels were impaired. In contrast, the mRNA levels of IL-23p19 were attenuated after inhibition of p38, while they were enhanced after direct inhibition of mTOR. Further investigation revealed that treatment with mithramycin, which is an inhibitor of Sp1, could block the enhanced cytokine production of IL-12p40 after p38 inhibition. Additionally, an Sp1-Luc plasmid that was transfected into MEFs, showed higher activity after inhibition of p38 or mTOR. These findings were in line with some recently published works and point to a role of Sp1 in the transcriptional regulation of IL-12p40 after p38 or mTOR inhibition. Dobreva et al showed that a CTCTAA/GC polymorphism in the IL-12p40 gene (IL-12B) influences the effects of p38 inhibition on IL-12p40 production in PBMCs [70]. Shimokawa et al found that this polymorphism affects the transcriptional activity of the IL-12p40 promoter, by showing that the transcriptional activity of the GC allele is higher than that of the CTCTAA allele. Moreover, the transcription factor Sp1 binds with high affinity to a region including the GC sequence, while it fails to bind a region including the CTCTAA sequence [71]. Furthermore, Boucher et al showed that the binding of Sp1 to the IL-12p40 promoter is enhanced after inhibition of p38 by SB203580 [63]. Interestingly, Astrinidis et al recently identified Sp1 to interact with p70S6K in fibroblasts. Cells deficient in Sp1 show strong S6 phosphorylation indicating that Sp1 could be regulated via mTOR to regulate the transcription of IL-12p40 [72]. As there are several transcription factors involved in the

regulation of IL-12p40, further analysis, which is ongoing, will be required to more clearly understand the mechanisms of transcription factor activation and the relationship between them. As Sp1 seems to have an important role within the signaling of the mTORC1 substrates, it would be interesting to further dissect its role in signaling, the precise mechanisms of its activation and the associated function as a transcription factor for IL-12p40.

Flow cytometry analysis revealed that CD86, an important costimulator for T lymphocytes, was upregulated on mDCs after p38 inhibition and stimulation with LPS, *Listeria monocytogenes* or SAC. Together with the finding that IL-12p40, which is important for the differentiation of Th1 cells, was augmented in monocytes after pretreatment with SB203580 or BIRB0796, this suggested that inhibition of p38 in monocytes might modulate the differentiation pattern of CD4+ T-cells. Indeed, our results showed that inhibition of p38 or mTOR in human monocytes promoted Th1 differentiation. Recently, Yang et al showed that p38 inhibition in murine BMDM promotes Th1 differentiation [73].

Based on these findings, we wanted to gain insight into the molecular mechanisms of how p38 and mTOR signaling might be connected. As rapamycin treatment did not alter p38 phosphorylation after TLR activation, p38 was not downstream of mTOR. Most interestingly, p38 inhibition in human monocytes was able to block the substrates of mTOR after stimulation with LPS, anisomycin or UV, strongly suggesting that p38 activates mTOR. Interestingly, inhibition of p38 or PI3K could only partially block the mTOR substrates, while direct inhibition of mTOR with rapamycin could totally block the phosphorylation of S6 and 4EBP1. Further analysis showed that PI3K inhibition had no effect on the activation of p38 and that concomitant inhibition of PI3K and p38 was able to block mTOR totally. This pointed to a dual input to activate mTOR, on the one hand via the canonical PI3K pathway and on the other hand via p38 (Figure 23).

Subsequently, we made use of TSC2 knockout MEFs to elucidate if the link from p38 to mTOR is upstream or downstream of TSC2. Stimulation of the MEFs with UV or anisomycin revealed that inhibition of p38 blocked mTOR in the TSC2+/+ MEFs, but failed to do so in the TSC2-/- MEFs. Again, rapamycin blocked the phosphorylation of the mTOR substrates, both in the TSC2+/+ and TSC2-/- MEFs. Thus, it was obvious that the signal from p38 to mTOR is mediated via TSC2 in MEFs. Whether this is also true for innate immune cells, needs further investigation. We would recommend

knockdown experiments using TSC2 siRNA and inhibition of p38 with the used inhibitors. Interestingly, stimulation of MEFs with serum or LPS failed to activate p38 and therefore, the p38 inhibitors had no effect on the phosphorylation status of S6 and 4EBP1.

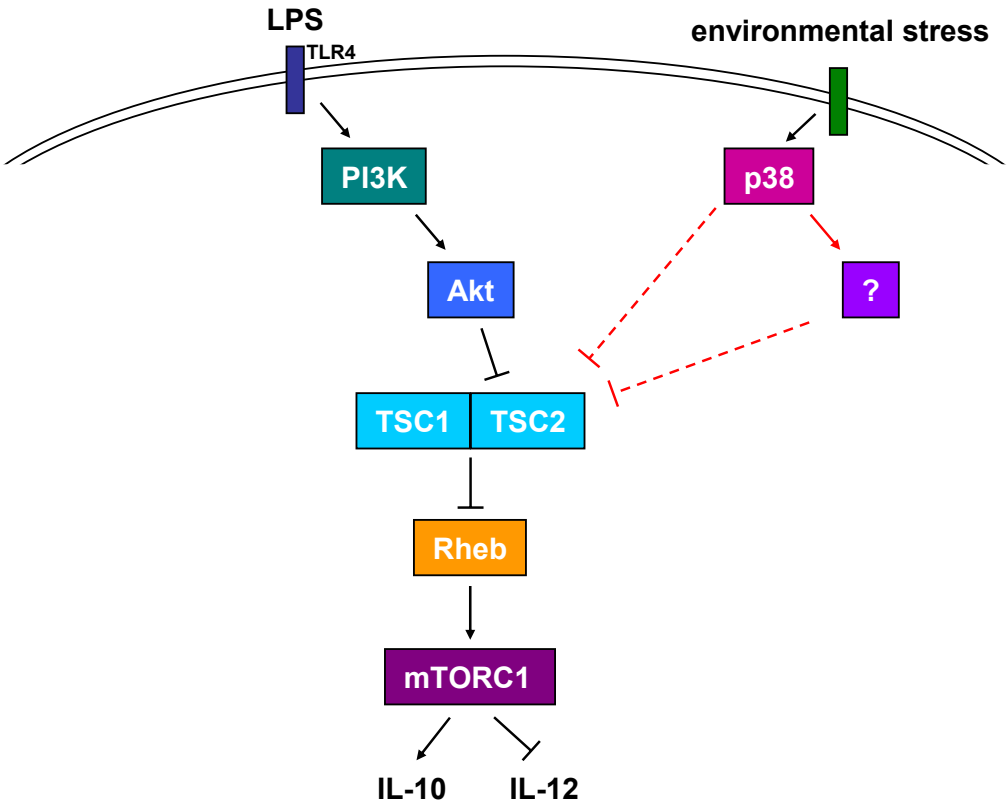

Figure 23. mTORC1 is activated via PI3K and p38 independently
On the one hand, mTORC1 is activated via the PI3K/Akt axis. Activation of Akt inhibits TSC2, a negative regulator of mTORC1. On the other hand, mTORC1 is activated by p38 promoted inactivation of TSC2. Whether p38 inhibits TSC2 directly or via a downstream substrate of p38 is not clear yet.

As there are four isoforms of p38, we wanted to know which of them is responsible for the activation of mTOR. As the p38 inhibitor concentrations used were in a range where they blocked specifically the subunits p38α and p38β, we investigated the role of p38α by using p38α knockout BMDM. Stimulation of the cells with LPS, anisomycin, UV and *Listeria monocytogenes* revealed that knockout of p38α resulted in inhibition of the mTOR substrates S6 and 4EBP1. Again, we could show that only treatment with wortmannin and therefore inhibiting the input signal via PI3K, could completely block mTOR signaling in the p38α knockout macrophages, compared to a partial inhibition in the p38α floxed macrophages. This was shown both by immunoblot and immunofluorescence. Again, this suggests that mTOR is activated via PI3K and p38

independently and that the subunit p38α is responsible for the activation of mTOR. Nevertheless, knocking down the other subunits of p38 in human monocytes could further reveal their function in mTOR activation in innate immune cells. On the functional level, knockout of p38α and stimulation with LPS or SAC increased the cytokine levels of IL-12p40 and IL-12p70, while the levels of IL-10 were abrogated. This enhancement in IL-12p40 production was also shown by Kim et al in 2008 after stimulation of p38α knockout BMDM with LPS [74]. Although in our experiments, mTOR was blocked in the p38α knockout macrophages after stimulation with *Listeria monocytogenes*, the levels of IL-12p40 were not altered. This could be due to different downstream mediators that are activated or blocked by *Listeria monocytogenes*, but not by LPS or SAC.

In order to investigate a link from p38 to mTOR *in vivo*, two different mouse models were used. Firstly, the immune response of p38α fl/fl LysMCreΔp38α mice after an LPS shock was analyzed. According to the results achieved *in vitro*, we found higher IL-12p40 serum levels in p38α fl/fl LysMCreΔp38α mice compared to the p38α fl/fl mice. Moreover, reactivated splenocytes of p38α fl/fl LysMCreΔp38α mice produced higher levels of IL-12p40, compared to the splenocytes of control mice. Immunoblot analysis of homogenized spleens revealed that mTOR was active in spleens of p38α fl/fl mice while it was blocked in spleens of p38α fl/fl LysMCreΔp38α mice. These *in vivo* results confirmed our observed findings, showing that p38α activates mTOR and that knockout of p38α leads to an inflammatory modulation of IL-12 and IL-10 after LPS shock.

In contrast to these results, the immune response of C57BL/6 mice to a *Listeria monocytogenes* infection was not altered after treatment with 30 µg/mouse SB203580. Neither the IL-12p40 serum levels, nor the mTOR pathway was altered after pretreatment with the p38 inhibitor. Moreover, monitoring the survival revealed no differences in the survival rates of *Listeria monocytogenes* infected mice and mice infected with *Listeria monocytogenes* after pretreatment with the p38 inhibitor. Additionally, the phosphorylation status of p38 and the mTOR substrates in the spleens was not altered comparing uninfected and *Listeria monocytogenes* infected mice. The IL-12p40 serum levels also did not change after *Listeria monocytogenes* infection compared to the uninfected mice. This could mean that p38 was not activated in this *in vivo* model and therefore inhibition of p38 had no effect on the survival of the mice. It

could also be possible that *Listeria monocytogenes* blocked or enhanced downstream proteins that influence the IL-12p40 production.

The primarily finding of our work was that p38 and the mTOR pathway are connected in innate immune cells. While I showed in this work that p38α enhances mTOR signaling in innate immune cells and MEFs, it was shown recently that p38β was involved in the suppression of mTORC1 in MEFs after energy starvation. This was mediated via activation of a pathway including PRAK (p38-regulated/activated kinase) and Rheb, which is a negative regulator of mTOR [75]. On the other hand, Li et al showed that MK2, a downstream substrate of p38, is responsible for the phosphorylation of TSC2 at Ser1210 in HEK293 cells. This phosphorylation enhanced the interaction of TSC2 with 14-3-3 and therefore led to an inhibition of TSC2 [76]. In Drosophila, p38 was shown to activate mTORC1 via an MK2 independent manner [77]. Further investigations will be required to reveal the exact function of the different p38 subunits in activating mTOR in respect of different extra- and intracellular stimuli. The participation of the downstream substrates of p38 in p38/mTOR signaling could also be part of future investigations.

Interestingly, some side findings came up when inhibiting p38 in human monocytes that could be possible targets for further investigations. One of these findings was that inhibition of p38 enhances the phosphorylation of ERK. Whether this is important in p38/mTOR signaling could be subject of further investigation, because increased ERK phosphorylation was also observed after p38 inhibition in a remnant kidney model. In this model, p38 inhibition was associated not with the expected improvement of renal disease, but with worsening [78]. In contrast, ERK was also found to phosphorylate and inactivate TSC2, thus activating mTOR [79]. In turn, this suggests that our observed downregulation of mTOR after p38 inhibition is not mediated via the activation of ERK. The Western Blot results also demonstrated that the phosphorylation of Akt at Ser473 is inhibited after treatment with p38 inhibitors, while it is not inhibited with rapamycin. As there are three different isoforms of Akt in mammalian cells [42], further research is necessary to reveal which isoform is involved in mTOR signaling. How far mTORC2, which is known to phosphorylate Akt at Ser473, is involved in the p38/mTORC1 signaling will also need further investigation. It would also be interesting to know if the signal is mediated directly from p38 to TSC2 or if it is transmitted via MK2, PRAK or MSK1/2 that are all substrates of p38. As already mentioned earlier, PRAK is involved in the inactivation of mTORC1 in energy-starved MEFs [75]. In contrast, MSK1/2

deficiency was shown to result in higher production of IL-12p40 and IL-12p70 [80]. Experiments with inhibitors of the downstream substrates or the use of knockout MEFs could reveal the importance of these substrates in our findings.

In summary, our results display a role for p38 in limiting proinflammatory cytokine production after TLR-dependent and -independent stimulation that is mediated via the activation of mTOR. Inhibition of p38 in innate immune cells boosts the inflammatory reaction in vitro and in vivo. While activation of p38 in innate immune cells reduces the levels of IL-12p40, it enhances the levels of IL-10. This could be an important finding for new therapies of inflammatory diseases in respect of the balance of pro- and anti-inflammatory cytokines.

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8 Curriculum vitae

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