The Role of mTORC1 in Immune Adaptation to Inflammatory Stress

Dissertation

zur Erlangung des akademischen Grades

„doctor rerum naturalium“ (Dr. rer. nat)

vorgelegt dem Rat der Medizinischen Fakultät

der Friedrich-Schiller-Universität Jena

seit 1558

von *M. Sc. Kristin Ludwig*

geboren am *11.09.1990* **in** *Jena*

Gutachter

1. Apl. Prof. Dr. Ignacio Rubio, Uniklinikum Jena

2. Apl. Prof. Dr. Frank-D. Böhmer, Uniklinikum Jena

3. Prof. Dr. Matthias P. Wymann, Universität Basel

Tag der öffentlichen Verteidigung: 18.06.2019

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LIST OF ABBREVATIONS

4E-BP1	4E binding protein 1
AKT	Protein kinase B
AML	Angiomyolipomas
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid
BCAA	Branched chain amino acids
BCG	Bacille Calmette-Guérin
BSA	Bovine serum albumin
CARS	Compensatory anti-inflammatory response syndrome
CBA	Cytometric bead array
CCL	CC chemokine ligand
CHAK	CC chemokine-activated killer
CD	Cluster of Differentiation
CrP	C-reactive protein
CSIF	Cytokine synthesis inhibitory factor
CXCL	Chemokine C-X-C motif ligand
DEPTOR	DEP-domain-containing mTOR interacting protein
eIF-4E	Eukaryotic initiation factor 4E
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular signal-related kinase
FACS	Fluorescent-activated cell sorting
FKBP12	FK506 binding protein of 12 kDa
FRB	FK506-Ramapycin binding
GAP	GTPase-Activating protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK3	Glycogen synthase kinase 3
GTPase	Guanosine triphosphatase
HGPS	Hutchinson-Gilford progeria syndrome
HIF-1α	Hypoxia-inducible factor 1α
ICU	Intensive care unit
IFN	Interferon
IκB	Inhibitor of nuclear factor κB
IKKβ	Inhibitor of IκB kinases
IL	Interleukin
IRAK 1/4	Interleukin-1 receptor-associated kinase 1/4
IRF3	Interferon regulatory factor 3
LAM	Lymphangioleiomyomatosis
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotaxis protein 1
MEF	Mouse embryonic fibroblast
MFI	Mean fluorescence intensity

MIP-1α/β	Macrophage inflammatory protein 1α/β
MD2	Myeloid differentiation factor 2
mLST8	Mammalian lethal with Sec13 protein 8
mSIN1	Mammalian stress-activated protein kinase interacting protein
mTOR	mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
MyD88	Myeloid differentiation primary response 88
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide - hydrogen
NFκB	Nuclear factor κB
NK cells	Natural killer cells
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCG	Poly-(1-6)-B-D-glucopyranosyl-(1,3)-B-D-glucopyranose
PE	Phycoerythrin
PI3K	Phosphatidylinositol-4,5-biphosphate 3 kinase
PKC	Protein kinase C
PRAS40	Proline-rich AKT substrate 40 kDa
PVDF	Polyvinylidene fluoride
Protor-1	Protein observed with rictor- 1
PRR	Pathogen recognition receptor
RAL	Renal angiomyolipomas
RANTES	Regulated in activation, normal T cell expressed and secreted
Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
S6K	S6 kinase
S6P	S6 protein
SAR	Systemic acquired resistance
SEGA	Subependymal giant cell astrocytoma
SDS	Sodium dodecyl sulfate
SIRS	Systemic inflammatory response syndrome
TBC1D7	TBC 1 domain family member 7
TIR	Toll-interleukin-1 receptor
TIRAP	TIR-domain-containing adapter protein
TLR4	Toll-like receptor 4
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF6	TIR-domain-containing adapter-inducing IFN-β
TNFα	Tumor necrosis factor α
TSC	Tuberous sclerosis complex

SUMMARY

Innate immune cells, such as monocytes, were shown to be able to adapt to different inflammatory stimuli in distinct ways to protect the organism against recurrent infections by different mechanisms. Endotoxin tolerance can be induced by different pathogen-associated molecular pattern (PAMP) such as the lipopolysaccharide (LPS) and is characterized by a decreased inflammatory response when challenged with a second infection. Thus, endotoxin tolerance protects against a hyperinflammatory response and associated tissue damages. In contrast to LPS, the fungal cell wall compartment β-glucan induces trained immunity to protect cells and organisms through an increased pro-inflammatory response upon re-infection. Previous studies reported that these adaptation processes involve epigenetic changes based on a metabolic switch from oxidative phosphorylation to aerobic glycolysis, the so-called Warburg effect. mTORC1 as a key metabolic regulator, balances nutrient supply and energy demand of the cell and is therefore thought to play an important role in these adaptation processes. However, little is known about the exact underlying mechanisms, as well as the interaction of cellular metabolism and innate immune cell adaptation.

The role of mTORC1 was investigated within the scope of a clinical study comprising healthy volunteers as well as patients with Tuberous sclerosis complex (TSC). This genetic disorder is characterized by hyperactivation of mTORC1 caused by loss-of-function mutations in the main upstream inhibitor complex of mTORC1.

The present data demonstrate that primary human monocytes are able to adapt to inflammatory stressors, but these processes do not depend on mTOR activity. Induction of endotoxin tolerance is not affected in cells from TSC patients and not altered by chemical inhibition of mTOR signaling, as evidenced by cytokine production. Inflammatory priming with LPS or β-glucan likewise does not trigger alterations in mTORC1 activity or cellular metabolism. Signaling analysis were extended by the employment of primary human monocytes from healthy voluntary blood donors allowing to further investigate different inflammatory pathways. Examination of LPS primed monocytes reveal a distinct cytokine regulation, rather than a complete lack of inflammatory response in tolerant cells. Flow cytometric analysis and cytokine investigations confirm that induction of tolerance is not mediated by the abundance of cell surface receptor or the release of pro-inflammatory cytokines. In addition, adapting monocytes do not depend on a particular inflammatory signaling cascade, as examined by immunoblotting experiments. However, p38 as well as AKT signaling are downregulated and might be important mediators for the suppression of pro-inflammatory TNFα production in tolerant cells.

ZUSAMMENFASSUNG

Zellen des angeborenen Immunsystems, wie beispielweise Monozyten, sind in der Lage sich auf verschiedene Arten an inflammatorische Stimuli anzupassen, um den Organismus vor erneuten Infektionen zu schützen. Endotoxin Toleranz kann durch verschiedene Pathogen-assoziierte molekulare Strukturen (PAMP), wie Lipopolysaccharid (LPS), induziert werden und ist durch die verminderte Immunantwort während einer wiederkehrenden Konfrontation gekennzeichnet. Dabei schützt Endotoxin Toleranz vor einer gesteigerten Entzündungsreaktion und damit verbundenen Gewebeschäden. Im Gegensatz zu LPS induziert β-Glucan, ein Bestandteil der Zellwand von Pilzen, ein trainiertes Immunsystem, um Zellen und den Organismus über eine gesteigerte Entzündungsreaktion vor einer erneuten Infektion zu schützen. Frühere Studien zeigen, dass diese Adaptationsprozesse mit epigenetischen Veränderungen auf der Basis einer metabolischen Umstellung, von oxidativer Phosphorylierung hin zu aerober Glykolyse, dem sogenannten Warburg Effekt, einhergehen. mTORC1 besitzt eine Schlüsselfunktion in der Regulierung des Metabolismus, in dem es Nährstoffverfügbarkeiten und den Energiebedarf der Zelle im Gleichgewicht hält. Daher wird vermutet, dass mTOR eine wichtige Rolle in diesen Adaptationsprozessen einnimmt. Es ist jedoch nur wenig über die genauen zugrunde liegenden Mechanismen, sowie das Zusammenspiel von zellulärem Metabolismus und Anpassung der angeborenen Immunzellen bekannt.

Die Rolle von mTORC1 wurde in Rahmen einer klinischen Studie mit gesunden Probanden und Patienten mit Tuberöser Sklerose (TSC) untersucht. Diese genetische Erkrankung wird durch eine Hyperaktivität von mTORC1 gekennzeichnet, welche durch eine Funktionsverlustmutation des vorangestellten Hauptinhibitors von mTORC1 ausgelöst wird.

Die vorliegenden Ergebnisse zeigen, dass primäre humane Monozyten in der Lage sind sich inflammatorischen Stressoren anzupassen, jedoch sind diese Adaptationsprozesse nicht abhängig von der Aktivität von mTOR. Die Analyse der Zytokinproduktion beweist, dass die Induktion von Endotoxin Toleranz in TSC Patienten nicht beeinflusst ist und durch die chemische Inhibierung von mTOR nicht verändert wird. Inflammatorische Vorbehandlung durch LPS oder β-Glucan führt ebenso zu keinen Änderungen in der Aktivität von mTORC1 oder des zellulären Metabolismus. Die Untersuchungen der Signalübertragung wurde durch die Verwendung von gesunden, freiwilligen Blutspendern erweitert, um weitere inflammatorische Signalwege zu erforschen. Die Betrachtung von LPS vorbehandelten Monozyten hat aufgedeckt, dass in toleranten Zellen statt eines kompletten Verlustes der Entzündungsantwort, eine gezielte

Zytokinregulation zugrunde liegt. Durchflusszytometrische Analysen und die Untersuchung der Zytokine bestätigen, dass die Induktion von Toleranz nicht durch die Menge an Oberfllächenrezeptor oder inflammatorischen Zytokinen vermittelt wird. Des Weiteren sind adaptierende Monozyten nicht von einem bestimmten Signalweg abhängig, wie mittels Protein-Immun-Blots festgestellt werden konnte. Allerdings sind der p38 und der AKT Signalweg in toleranten Zellen runterreguliert und könnten damit wichtige Vermittler in der Unterdrückung der inflammatorischen TNFα Produktion darstellen.

1. INTRODUCTION

1.1 Immune adaptation processes

Classically the immune system is divided into innate immune responses, which react quick but in a non-specific manner regarding the encountering pathogen, and the adaptive immune system, reacting slower but specific as well as able to develop immunological memory. However, after first observations in the 1940´s, a memory-like state of innate immune cells like monocytes and macrophages has raised increased interest within the last decade. This is based on studies of plants and invertebrates, which lack an adaptive immune system but still can develop a systemic acquired resistance (SAR), protecting plants from recurring infections even by non-related pathogens [1]. Innate immunological memory seems to be a possible explanation for unspecific protection of a variety of vaccines against infections other than target diseases. An increasing body of evidence indicates for adaptation processes of cells of the innate immune system against diverse stimuli also in humans. Two of these putative protecting adaptation mechanisms are endotoxin tolerance and trained immunity. Though both processes trigger opposing immunological responses, after priming with distinct Pathogen associated molecular pattern (PAMP), they are reported to be protective against otherwise lethal infections with the same or a different PAMPs.

1.1.1 Endotoxin tolerance and LPS

Endotoxin tolerance is defined as a temporary hyporesponsiveness characterized by a decreased pro-inflammatory response of innate immune cells upon re-stimulation with endotoxin, to regulate acute pro-inflammatory response and protect from inflammation-induced injury. First observations of endotoxin tolerance were described over 70 years ago, when Paul Beeson reported, that humans, which recovered from malaria or typhoid fever revealed reduced fever when re-challenged with endotoxin [2]. However, the exact molecular mechanism underlying this protective effect of the innate immune system are still not fully understood.

In the classical model this endotoxin is Lipopolysaccharide (LPS), a major compartment of the outer membrane of gram-negative bacteria like *Escherichia coli*. Thereby, LPS increases the negative charge of the bacterial cell membrane to protect from chemical attacks and stabilizes the membrane structure [3]. It is composed of Lipid A, which anchors LPS into the bacterial membrane, a core domain and the so-called O-antigen, a polysaccharide chain with diverse compositions, depending on the bacterial strain [4].

The O-antigen is the very outer part of the bacteria and therefore can be recognized by host antibodies[5]. However, lipid A is responsible for the induction of inflammatory immune cell response upon receptor binding [6].

TLR4 signaling cascade

LPS can bind to the Toll-like receptor 4 (TLR4) in complex with CD14 (Cluster of differentiation 14) and MD2 (Myeloid differentiation factor 2) on different innate immune cells like monocytes, macrophages and dendritic cells, triggering intracellular TLR4 signaling cascades [7-9]. Upon dimerization of the receptor, the cytoplasmic tail of TLR4, containing a TIR (toll-interleukin-1 receptor) domain, recruits the adapter proteins MyD88, TIRAP, TRIF and TRAM (TRIF-related adapter molecule) (Fig. 4) [10, 11]. MyD88 (Myeloid differentiation primary response 88) and TIRAP (TIR domain containing adapter protein) subsequently recruit IRAK-1 or IRAK-4 (Interleukin-1 receptor-associated kinase-1/4), death domain containing kinases, which auto-phosphorylate and dissociate from the receptor to bind TRAF6 (TNF receptor-associated factor 6), followed by other signaling targets such as IKK (I kappa B kinases), Mitogen-activated protein (MAP) kinases or the activator protein-1 (AP-1) [11]. In contrast, recruitment of TRIF (TIR-domain-containing adapter inducing IFN-β) requires internalization of TLR4 complex to endosomes, where it activates the transcription factor IRF3 (IFN regulatory factor 3) and initiates the transcription of interferon (IFN) and IFN-inducible genes [12, 13]. Therefore, TLR4 can activate diverse signaling cascades including NFκB, the MAP kinases ERK (Extracellular signal-related kinase) and p38, c-Jun or AP1 and hence initiate pro-inflammatory cytokine production [11].

With LPS induced TLR4 signaling, immune cells try to counteract the putative bacterial infection with gram-negative bacteria by production of pro-inflammatory cytokines and mediators. This can result in an uncontrolled immune response and hence an endotoxic shock, along with tissue or organ damage. Therefore, the immune system developed defense strategies against endotoxic shock or associated high immune responses. One of these strategies is endotoxin tolerance. During initial exposure to LPS, cells produce an increased amount of TNFα (Tumor necrosis factor α), IL-1 (Interleukin 1), IL-6 and IL-8, among others [14]. However, macrophages primed with LPS, produce attenuated amounts of pro-inflammatory cytokines, such as TNFα, IL-6 and IL-12, associated with an increase of anti-inflammatory IL-10 secretion and reduced antigen presentation upon re-exposure [10, 15]. In addition to prevent exaggerated immune response and associated damage, endotoxin tolerance can protect from subsequent lethal infections. Mice rendered tolerant with lower amounts of LPS, displayed enhanced survival against high, otherwise lethal doses of LPS or induction of sepsis [16, 17].

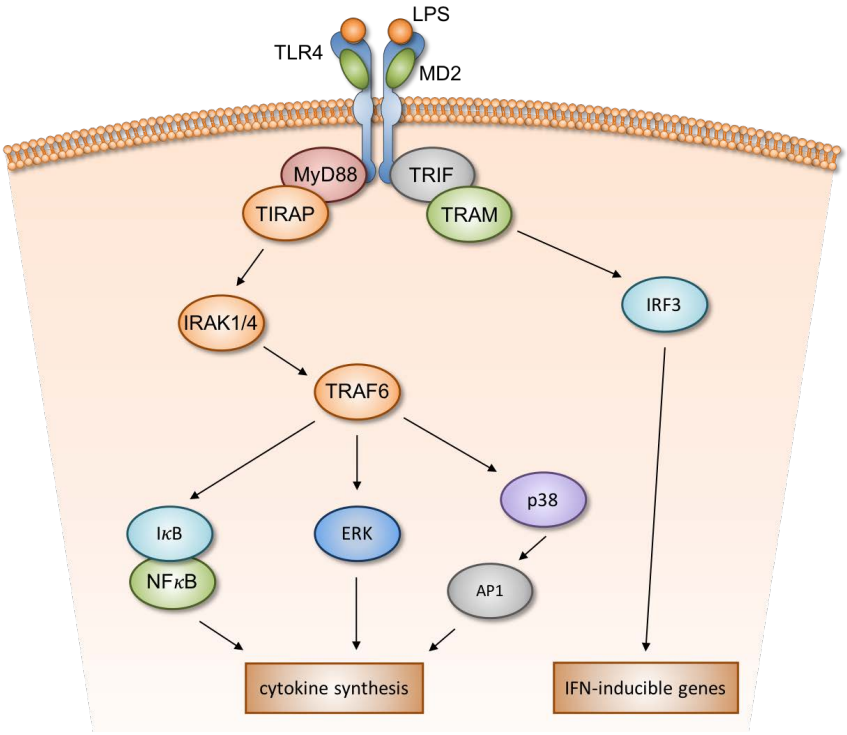


Fig. 1: Simplified scheme of the TLR4 signaling pathway.

Signaling cascade of TLR4 after binding of LPS. Oligomerization of the TLR4/MD2 complex activates 2 distinct signaling pathways. Binding of MyD88 and TIRAP to the TIR-domain of the receptor subsequently recruits IRAK1/4. Autophosphorylation of IRAK1/4 leads to dissociation from the receptor and binding to TRAF6. Active TRAF6 can promote the activation of different downstream signaling cascades. Phosphorylation of IκB leads to the dissociation of the inhibitor from the transcription factor NFκB, which can subsequently translocate to the nucleus. Activation of p38 activates the transcription factor AP1. Together with ERK signaling, these pathways activate the expression of cytokines to initiate an immune response. On the other hand, internalization of the TLR4 complex into endosomes results in the recruitment of TRIF and TRAM and the subsequent activation of the transcription factor IRF3, which promotes interferon (IFN)-inducible genes and promotes survival.

Cross-tolerance

Tolerance induction is not solely reported for LPS priming and LPS re-stimulation (homo-tolerance), but also cross-tolerance (hetero-tolerance) against bacterial or even fungal infections have been observed. Mice pre-treated with LPS were shown to have increased anti-bacterial activity against subsequent infections of *Salmonella enterica* and improved survival when challenged with *Cryptococcus neoformans* infection [18, 19]. Further, ligands of other TLRs such as PamCysSK4 as well as compounds of gram-positive bacteria like LTA (Lipoteichoic acid) were found to induce endotoxin tolerance, although much weaker compared to homo-tolerance [20].

In addition to tolerance induction by TLR ligands, viral infections were shown to decrease LPS-induced mortality via reduction of TNFα [21]. TNFα is a key inflammatory cytokine, which is mainly expressed by monocytes and macrophages, and exaggerated TNFα production was shown to have lethal effects [22]. TNFα is able to coordinate

immune response by inducing inflammatory signaling such as NFκB and MAPK, as well as promoting other pro-inflammatory cytokines [23]. Monocytes and macrophages, stimulated with TNFα, were confirmed to become tolerant against a subsequent TNFα stimulation and TNFα tolerant mice exhibit decreased mortality against otherwise lethal injections of TNFα [22, 24, 25]. Moreover, TNFα was reported to induce tolerance to gram-negative bacteria, LPS and bacteria-derived LPS-containing lipophilic outer membrane vesicles [26-28]. Thus, endotoxin, as well as TNFα tolerance, are protecting mechanisms to prevent deleterious consequences of inflammatory events, such as excessive or chronic inflammation.

Sepsis as clinical model for endotoxin tolerance

LPS triggers inflammation and promotes immunosuppression, both processes can increase disease mortality. Thus, endotoxin tolerance seems to be a diverse, but highly regulated, complex process with sepsis as main clinical model. Blood monocytes serve as the first line of host defense but their contribution in human sepsis is poorly understood. Processing of bacteria within phagocytic immune cells can lead to the release of endotoxin to the bloodstream, a condition called endotoxemia, which can trigger an uncontrolled immune system activation resulting in septic shock. TLR4 signaling after LPS binding was demonstrated to play a central role in sepsis induced by gram-negative bacteria [29]. Moreover, sepsis is characterized by an initial phase of excessive pro-inflammatory response, the systemic inflammatory response syndrome (SIRS), which can result in tissue damage and organ failure, followed by a second immune-compromised state. This immunosuppression is called compensatory anti-inflammatory response syndrome (CARS) and can cause an increased susceptibility to secondary infections [29]. During this post-septic immunoparalysis innate immune cells are in an endotoxin tolerant-like state [15, 30].

Monocytes derived from blood of sepsis patients displayed a decreased production of pro-inflammatory cytokines like TNFα and IL-6 when re-stimulated with LPS *in vitro*, whereas anti-inflammatory cytokines like IL-10 were increased [15, 31, 32]. Further, these monocytes displayed enhanced phagocytic activity and decreased antigen-presentation, supporting their protective and anti-inflammatory state [15, 33]. Decreased antigen-presentation suppresses T cell proliferation and therefore inflammation progress, while enhanced phagocytosis improves immune defense. The anti-inflammatory IL-10 was also increased, when cells were challenged with the TLR2 agonist Pam3CysSK4, indicating for cross-tolerance induction [15]. In addition to the immunosuppressive state, monocytes from sepsis patients or LPS pre-treated mice were

shown to enhance protective functions such as phagocytosis, anti-microbial activity and tissue remodeling [17, 34].

In addition to monocytes/macrophages, also other cells can be affected by endotoxin tolerance such as dendritic cells, neutrophils and T cells [35, 36]. Tolerant dendritic cells display a similar cytokine profile as macrophages along with increased endocytosis [35]. In contrast, neutrophils, rendered tolerant by endotoxin retain their pro-inflammatory cytokine response, however, loose cell surface expression of TLR4 [36]. Though endotoxin tolerance was initially described several decades ago, underlying molecular mechanisms are not yet fully understood, since its protective effect probably involves different parts of the immune system throughout the whole organism.

1.1.2 Trained immunity

Endotoxin tolerance mediates its protective effect, by suppression of immune system activation to prevent deleterious inflammation-associated tissue damage. Another assumedly protective adaptation process of innate immune cells is characterized by exactly the opposite. This adaptation process has been called trained immunity or innate immune memory and has raised increased interest during the last years. Trained immunity is characterized by an increased pro-inflammatory response of especially β-glucan primed, innate immune cells such as monocytes and macrophages upon re-stimulation with different PAMPs [37, 38]. This enhanced immune response was reported to protect against otherwise lethal infections with LPS, *Candida albicans* or *Staphylococcus aureus in vivo* [37, 39, 40]. In addition, different adaptation processes of innate immune cells after priming, also with other pathogens or PAMPs, including LPS-induced endotoxin tolerance, are meanwhile sometimes referred to as trained immunity or innate immune memory. The concept of trained immunity arose, among others, from observations of the tuberculosis vaccine BCG (Bacille Calmette-Guérin), which showed protective effects against infection-associated mortality beyond protecting against tuberculosis [41]. Further *in vivo* studies showed that priming with BCG decreased mortality of mice by a subsequent *Candida albicans* infection even in the absence of T and B cells [42]. Proof-of-principal trials of BCG revealed protecting effects against infections in healthy adults as well as new born children [41, 43]. In addition, beside the infectious disease malaria, which can trigger a hyperresponsive state, also infections with other parasitic or viral pathogens were reported to induce trained immunity [44-47]. However, the most prominent training compound is β-glucan.

β-1, 3(D)-glucan (β-glucan) represents a cell wall compartment of fungi such as *Candida albicans* and can be bound by dectin-1 on phagocytes [48]. Initial stimulation of innate

immune cells with β-glucan does not trigger elevated pro-inflammatory cytokine production. However, priming or training of monocytes with β-glucan results in an increased pro-inflammatory response upon re-stimulation with PAMPs such as LPS, characterized by enhanced pro-inflammatory cytokine levels like TNFα and IL-6 even after 7 days [43, 49, 50]. During the resting period between β-glucan training and re-stimulation, monocytes are suggested to evolve metabolic changes mediated by underlying transcriptional and epigenetic re-programming, to enable the production of increased cytokine amounts, pro-inflammatory mediators and receptors as well as to persist in a primed and hyperresponsive state [50]. These epigenetic changes involve chromatin organization and accessibility to the transcription machinery as well as histone modifications [38, 50].

Epigenetic changes in trained immunity

During the differentiation of monocytes to macrophages, cells undergo epigenetic changes such as histone modifications of promotors and distal elements [50]. This is supported by other data reporting that macrophages, which are transferred from one tissue to another undergo extensive re-programming of enhancers [51]. Priming with β-glucan alters histone modifications, characteristic of enhancers, such as H3K4me3 (histone 3 lysine 4 tri-methylation, marks promotors), H3K4me1 (marks distal regulatory elements/enhancers) and H3K27ac (marks active promotors and enhancers), with acetylation of H3K27 being the most dynamic (Fig. 5) [37, 50]. Further, whole-genome epigenetic profiling of trained cells revealed an increase especially in promotors of glycolytic genes and compounds of the mTOR pathway, as well as a genetic upregulation of hexokinase and pyruvate kinase, genes involved in glycolysis, by RNA sequencing *in vitro* and *in vivo* [40]. It is suggested that these epigenetic changes are also transferred to bone marrow-localized progenitor cells, to enable a longer protecting effect, since monocytes display a short half-live in circulation, a process which has already been observed for other innate immune cells [52-54].

An additional conceivable mediator of trained immunity are microRNAs, which were reported to have a long half-life and therefore could facilitate persistence of the trained state of innate immune cells, which have just limited proliferative capacity [55]. Additional data revealed a role of microRNAs also for the tolerant state of macrophages [56-58].

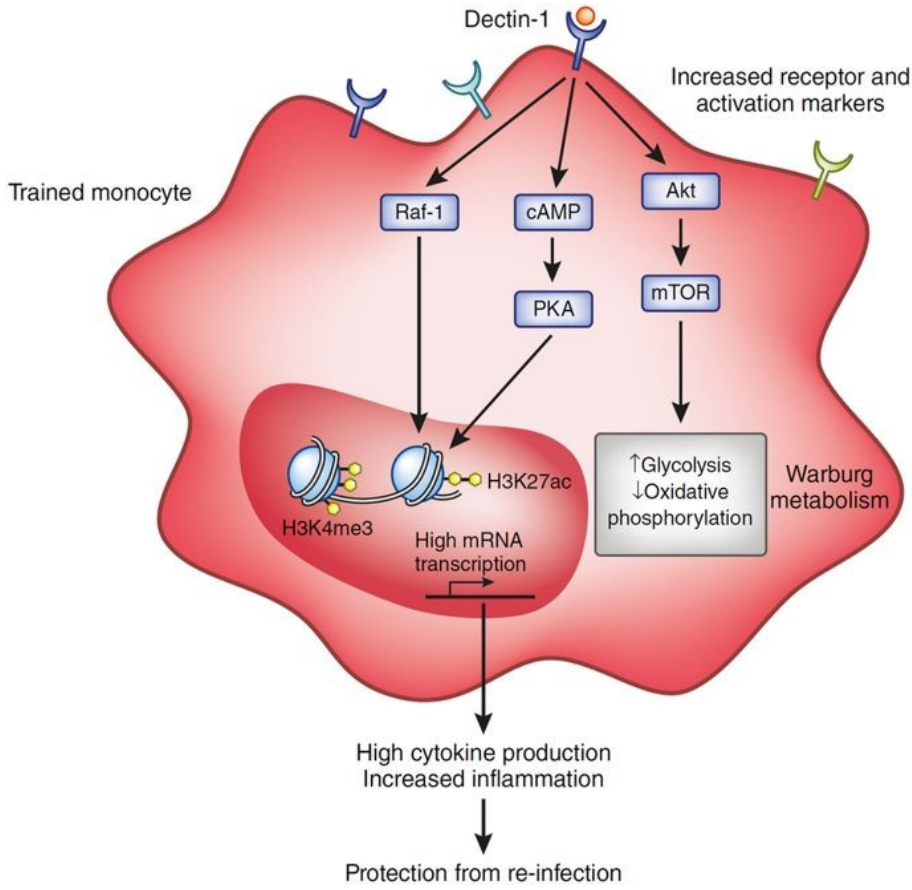


Fig. 2: Epigenetic and metabolic re-programming after β-glucan training.

Binding of β-glucan on dectin-1 triggers epigenetic changes, including histone modifications, and metabolic reprogramming of monocytes to induce training effect. Trained monocytes are thought to be in a hyperresponsive and protective state to improve survival upon re-challenging of the cells. Underlying metabolic re-programming is supposed to be mTOR dependent. Transferred with permission from Netea, M.G., et al., *Innate immune memory: a paradigm shift in understanding host defense.* Nat Immunol, 2015. **16**(7): p. 675-9 [59].

Epigenetic changes in adapting innate immune cells have also been described for endotoxin tolerance induction [60]. Cells rendered tolerant by LPS displayed gene-specific chromatin modifications associated with the silencing of genes, especially for inflammatory molecules [60, 61]. Further, it was reported that on a translational level, proteome remodeling is the underlying mechanism of LPS induced metabolic adaptation in macrophages [62]. However, β-glucan was reported to reverse an endotoxin tolerant state induced by LPS in human volunteers. Subsequent *in vitro* incubation of isolated monocytes with β-glucan triggers the reversal of epigenetic alterations along with the decreased cytokine production in tolerant cells. β-glucan treated cells return to a responsive state including transcriptional reactivation of unresponsive genes and histone modification alterations [61]. Hence, trained immunity could be a helpful tool for the treatment of different kinds of diseases such as impaired host defense in post-septic immune paralysis or cancer, as well as exaggerated immune responses in

autoinflammatory diseases and could contribute to new generation vaccines. However, the exact underlying mechanisms leading to these adaptational changes are still not known.

1.1.4 The Warburg effect in adapting monocytes

Normal cells produce energy by mitochondrial oxidative phosphorylation, since it represents the best yield of adenosine triphosphate (ATP). However, many cancer cells produce their energy predominantly by glycolysis and the resulting amounts of pyruvate are not completely oxidized but reduced to lactate even in the presence of abundant oxygen, a process which is called aerobic glycolysis. This metabolic switch from oxidative phosphorylation towards increased glycolysis is called Warburg effect and was first described by Otto Warburg in the beginning of the last century for neoplastic cells [63].

However, further studies revealed that also immune cells such as T cells increase glycolysis upon activation [64, 65]. Activated or proliferating immune cells exhibit an increased demand of nutrients and hence increase glucose uptake and glycolysis [66]. More than 50 years ago, activated monocytes were described to increase aerobic glycolysis [67]. Upon activation, innate immune cells like monocytes, migrate to inflammatory sites which comprises enhanced actin assembly and thus, high ATP consumption. Although being less efficient, in regard to ATP generation, glycolysis can be upregulated multiple folds, resulting in a faster production of ATP than oxidative phosphorylation [68]. Aerobic glycolysis further enables immune cells to migrate into deep wounds, where oxygen is lacking. A metabolic switch from oxidative phosphorylation to glycolysis has already been observed in activated macrophages, dendritic cells and effector T helper lymphocytes and seems to be important for rapid immune response [69-71]. Moreover, stimulation with TLR ligands also induces enhanced glycolysis and, in most conditions, Warburg metabolism [69]. However, other data suggest that priming with LPS triggers an acute but transient increase in glycolysis, followed by a switch to oxidative phosphorylation or fatty acid oxidation [72]. Recent data, including a transcriptional and metabolic profiling of sepsis patients, revealed a shift from oxidative phosphorylation to aerobic glycolysis as an important component for the initial activation of host defense in leukocytes [73]. However, isolated leukocytes of sepsis patients as well as leukocytes rendered tolerant *in vitro,* displayed a generalized metabolic defect in glycolysis as well as oxidative metabolism [73].

Monocytes trained with β-glucan exhibit enhanced glycolytic activity along with increased glucose consumption and lactate production, as well as increased NAD^+/NADH ratio and reduced oxygen consumption [40]. This process is suggested to be the underlying

mechanism of trained immunity and the protective immunological effects triggered by β-glucan (Fig. 6), as blocking metabolic pathways increased mortality to a systemic fungal infection in mice [40, 73]. Since mTOR is a major regulator of cellular metabolism, including glycolysis, different studies suggest an important role for mTOR signaling in this metabolic switch by activating the transcription factor hypoxia-induced factor 1α (HIF-1α) [40, 50]. Moreover, mouse bone marrow-derived dendritic cells display an initial increase of glycolysis after TLR stimulation in an mTOR-independent manner, followed by an mTOR/HIF1α-dependent metabolic switch from oxidative phosphorylation to increased glycolysis [74-77]. However, whether epigenetic changes promote metabolic re-programming of the cells or metabolic adaptations produces metabolites, which could function as cofactors for enzymes involved in epigenetic modulation has still to be unveiled.

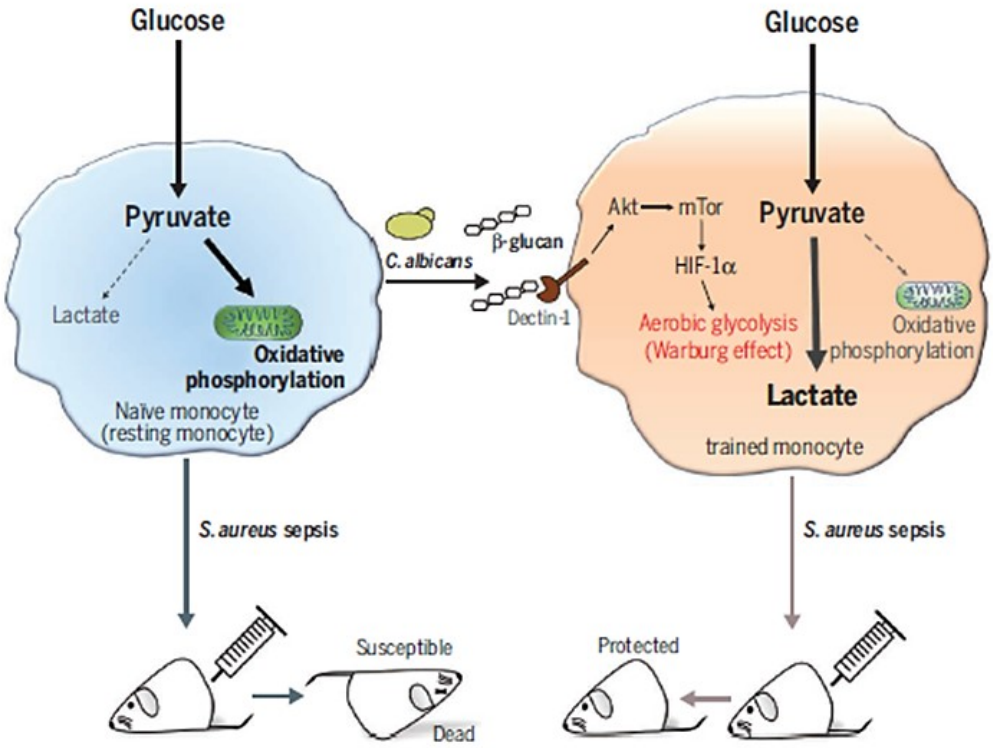


Fig. 3: Induction of Warburg metabolism in ß-glucan trained mice revealed a protective effect against re-infection.

Naive immune cells like monocytes rely their cellular metabolism on oxidative phosphorylation to achieve the maximal yield of ATP from every molecule of glucose. However, upon stimulation with *Candida albicans* or *Candida albicans*-derived β-glucan and its binding to the surface receptor Dectin-1, downstream signaling cascades including AKT, the metabolic key regulator mTOR and the Hypoxia-inducible factor 1α (HIF-1α) are activated, promoting the induction of Warburg metabolism, followed by enhanced glycolysis characterized by increased lactate production. This metabolic switch in trained monocytes is supposed to protect mice against subsequent sepsis induction by *Staphylococcus aureus*, which is otherwise lethal to mice with naive monocytes. Transferred with permission from Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science, 2014. **345**(6204): p. 1251086. [50].

1.2 mTOR signaling

1.2.1 mTOR complexes, signaling and functions

The mechanistic/mammalian target of rapamycin (mTOR) is an evolutionarily highly conserved serine/threonine kinase and belongs to the family of phosphatidylinositol 3-kinase (PI3K)-related kinases. As its name implies, it was discovered as the target molecule of rapamycin, an anti-fungal macrolide produced by the bacterium *Streptomyces hygroscopicus* and originally isolated from the soil of Easter Island in the 1970´s [78]. mTOR is an important signaling node within the cell, controlling cellular metabolism, survival, growth, protein and lipid synthesis as well as autophagy. Therefore, dysregulated mTOR activity is associated with diverse human diseases such as cancers, proliferative disorders, autism spectrum disorder and type 2 diabetes [79-81]. In addition, there is rising evidence that mTOR is also an important regulator of aging processes and age-related diseases [82, 83]. mTOR can be found in two functionally distinct multiprotein complexes mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), containing both unique and shared components [84].

mTOR complex 1

Besides mTOR as catalytic subunit, mTORC1 is composed of the scaffold protein regulatory-associated protein of mTOR (Raptor), the proline-rich AKT substrate 40 kDa (PRAS40), the mammalian lethal with Sec13 protein 8 (mLST8) and the regulatory protein DEP-domain-containing mTOR interacting protein (DEPTOR) (Fig. 1) and can further be found as a dimer [85-89]. Complex assembly enables mTORC1 to phosphorylate and activate downstream targets such as the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) or the p70 ribosomal S6 kinase (S6K) [90]. The latter regulates protein translation, cell size and cell proliferation by regulation of different substrates such as the ribosomal protein S6 (S6P), a component of the 40S ribosomal subunit [91]. Under growth-promoting conditions, S6P gets phosphorylated by S6K causing increased protein synthesis, while phosphorylation of 4E-BP1 by mTORC1 leads to dissociation of eukaryotic initiation factor 4E (eIF4E) and thereby protein translation [91-93].

mTORC1 integrates diverse upstream signals including amino acids, growth factors as well as genotoxic stress, oxygen levels and energy status of the cell [79, 94]. Thereby it balances anabolic and catabolic processes. Hence, mTOR signals whether intra- and extra-cellular conditions are favorable for anabolic processes such as cell growth, cell cycle progression and cell proliferation, and induces appropriate cellular responses.

mTORC1 can regulate metabolic pathways on transcriptional, translational and post-translational levels, and transgenic mice lacking S6K1 or 4E-BP1 exhibit severe metabolic phenotypes [95-100].

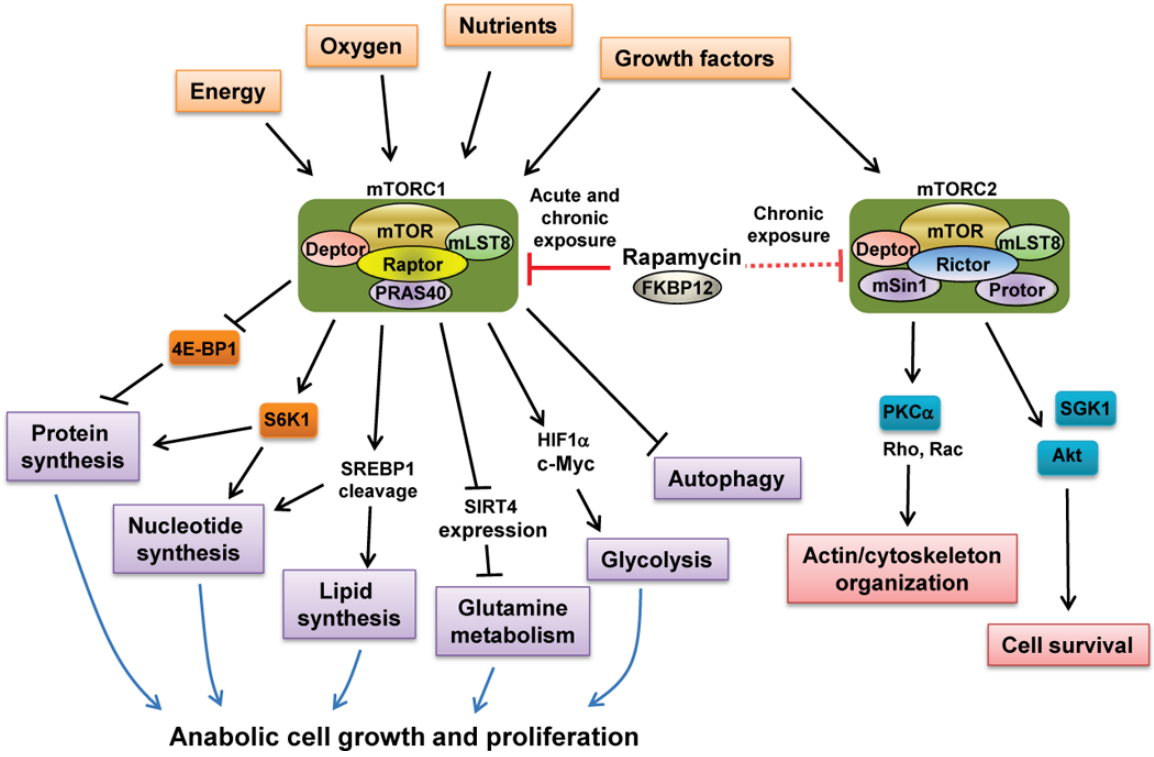


Fig. 4: Overview scheme of mTOR complex 1 and mTOR complex 2.

mTOR complex 1 and 2 are functionally distinct multiprotein complexes with shared and separate compounds. mTORC1 senses multiple environmental signals such as the cellular energy supply to balance energy-demanding processed like protein synthesis or cell growth through different downstream pathways. Functions of mTORC2 are not yet fully understood, but it was associated to the regulation cytoskeleton organization and promotion of survival. While mTORC1 is highly sensitive to rapamycin, mTORC2 functions seem to be impaired only with chronic exposure. Transferred with permission from Li, J., S.G. Kim, and J. Blenis, *Rapamycin: one drug, many effects.* Cell Metab, 2014. **19**(3): p. 373-9. [101].

A main function of mTORC1 is sensing the availability of especially branched-chain amino acids (BCAAs). Therefore, mTORC1 needs to be localized at the lysosomes, which serve as a platform, bringing amino acid recycling and mTORC1 in close proximity. A lack of amino acids results in dephosphorylation of S6K1 and 4E-BP1, since mTOR is not localized at the lysosomal membrane [102]. Sensing of amino acids, as well as the subcellular localization of mTOR can be mediated by raptor or Ras-related (Rag)-GTPases, depending on the specific amino acid [103, 104]. PRAS40 and DEPTOR display negative regulator of mTORC1 [89, 105]. In mammals, the small Rag-GTPases consist of RagA, RagB, RagC and RagD, with RagA/RagB GTP-bound and RagC/RagD GDP-bound representing the active complex, which anchors mTORC1 to the lysosome, where it is activated by the Ras homolog enriched in brain (Rheb)

[104]. Activated mTORC1 is able to directly phosphorylate PRAS40 and DEPTOR and therefore suppressing their physical interaction with mTORC1, resulting in an auto suppression [89, 105]. Moreover, PRAS40 dissociates from mTORC1 upon insulin stimulation, revealing its negative effect [87]. In addition, mTORC1 is mainly regulated and suppressed by the TSC1/TSC2 tumor suppressor complex (Fig. 5) [106]. Thereby, TSC1/2 (Tuberous sclerosis complex 1/2) is binding to and inhibiting Rheb, which is necessary to activate mTORC1 [107].

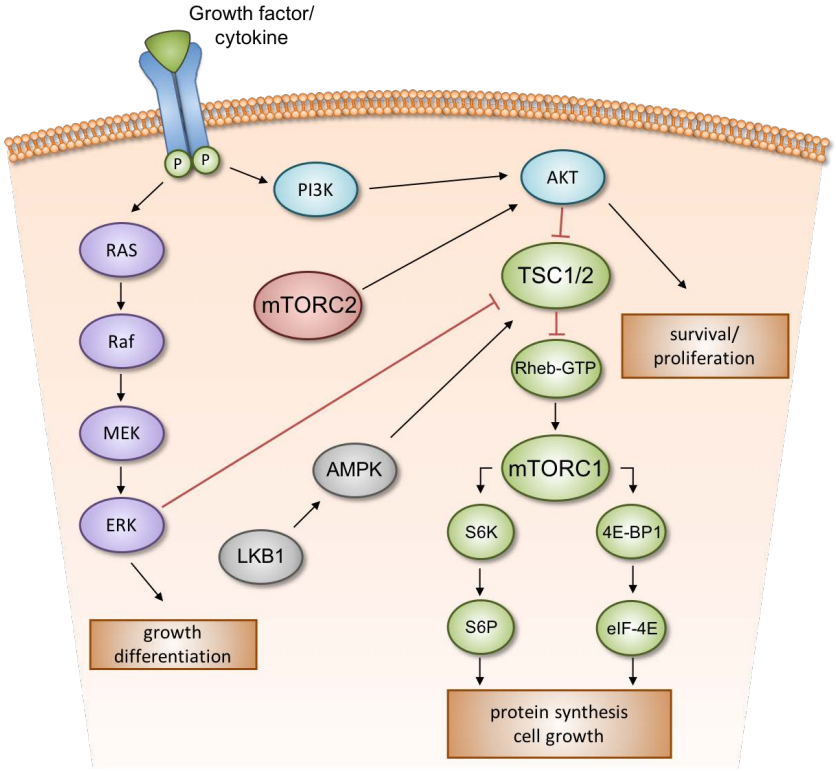


Fig. 5: Simplified scheme of mTOR signaling.

The mTOR complex 1 (mTORC1) mainly signals through the PI3K/AKT-pathway. AKT inhibits the main upstream inhibitor of mTORC1, the TSC1/2 tumor suppressor complex. Upon activation, TSC1/2 acts as a GAP to convert the small Ras-related GTPase Rheb from its inactive GDP-bound form to the active GTP-bound state, which subsequently activates mTORC1. In addition to AKT, other signaling pathways, such as ERK or AMPK regulate mTORC1 activity by an activating or inhibiting modulation of TSC1/2. Main downstream targets of mTOR are the S6 kinase (S6K), which subsequently phosphorylates S6 protein (S6P), a component of the 40S ribosomal subunit, or the 4E binding protein 1 (4E-BP1), which activates the eukaryotic initiation factor-4E (eIF-4E). Both pathways are used to activate protein biosynthesis and thus cell growth.

Sensing of growth factors like insulin by mTOR is mediated via the PI3K-AKT signaling pathway [108]. The serine/threonine kinase AKT phosphorylates and inactivates TSC2 leading to mTORC1 activation [108, 109]. Energy or oxygen levels of the cell are perceived by mTOR via the AMP-activated protein kinase (AMPK), which either also phosphorylates TSC2 to block mTORC1 activity or directly phosphorylates raptor [110-

113]. Thus, if energy levels are high, mTORC1 gets activated, whereas low energy levels result in mTORC1 inactivation. AMPK is a key metabolic regulator itself, making it a direct opponent of mTOR also in response to other cellular signals like genotoxic stress [114].

Further, mTOR signaling is associated with metabolically mediated extension of lifespan and delay of age-related diseases. Rapamycin treatment of old mice leads to an increased lifespan, restored self-renewal of hematopoietic stem cells and can delay the onset of age-related diseases such as cancer and Alzheimer´s disease [115-119]. On the other hand, mTORC1 signaling has been found to be hyperactive in many age-related diseases like cancer, and maybe also the Hutchinson-Gilford progeria syndrome (HGPS) [120].

mTOR complex 2

mTOR complex 2 comprises mLST8 and DEPTOR as well and additionally, the scaffold protein rapamycin-insensitive companion of mTOR (Rictor) the mammalian stress-activated protein kinase interacting protein (mSIN1) and the protein observed with rictor-1 (Protor-1) (Fig. 4) [121-123]. In mice, deletion of mTORC2 components leads to early embryonic lethality [124]. mTORC2 is primarily characterized as a downstream target of the insulin/IGF-1 signaling pathway and can be activated by phosphorylation of Serine 2481, regulating metabolism, proliferation, cytoskeletal organization and cell survival [125, 126]. It can also phosphorylate members of the Protein kinase C (PKC) family and thereby regulates cell proliferation, differentiation, apoptosis and telomere activities [127, 128]. Another main downstream target of mTORC2 is AKT, since phosphorylation of AKT at Serine 473 by mTORC2 is required for its full activation [129]. It is suggested that this activation proceeds via TSC1 and TSC2, linking mTORC2 activity to the regulation of mTORC1, because AKT directly controls mTORC1 activity through PRAS40 or the inhibitory phosphorylation of TSC1 and TSC2 [130-132]. Furthermore, the mTORC1 substrate S6K has been demonstrated to phosphorylate Rictor, revealing another way of cross-talk between both complexes [133].

1.2.2 mTOR inhibitors

Since a long time, it is known that mTORC1 is largely rapamycin sensitive which is used to treat solid tumors, organ rejection after transplantation, rheumatoid arthritis or coronary restenosis. Moreover, it enables intensive studies on functions of mTORC1 signaling within the cell. Rapamycin inhibits mTORC1 by binding to the FK506-binding protein of 12 kDa (FKBP12) to form a gain-of-function complex and interacts with the FKBP12-rapamycin binding domain (FRB) of mTOR as an allosteric inhibitor [134, 135].

It´s a matter of common knowledge, that rapamycin exclusively inhibits mTORC1, while mTORC2 is rapamycin-insensitive. The reasons for rapamycin-insensitivity of mTORC2 still remain elusive, as its molecular structure is still poorly understood. In yeast, it was reported that in its homologous TORC2, the Rictor homolog AVO3 masks the rapamycin-interacting domain of TOR [136]. Nevertheless, recent findings indicate, that mTORC2 can also be inhibited by chronic administration of rapamycin *in vivo* in diverse tissues, also by binding of rapamycin to FKBP12 and therefore inhibiting complex assembly of mTORC2 [137, 138].

Rapamycin is used in the clinics for decades, particularly because of its immunosuppressive functions, including treatment to prevent organ rejection after transplantations [139, 140]. Together, rapamycin and its analogs (rapalogs) have been approved for the treatment of various types of cancer, because of their anti-proliferative properties, including advanced renal cancer carcinoma, advanced neuroendocrine carcinoma or advanced or recurrent endometrial cancer [101]. However, as rapamycin has more cytostatic than cytotoxic properties, several studies showed that rapamycin-associated improvements lasted just as long as treatment [141-144]. In addition, prolonged rapamycin administration has been linked to diverse side effects, especially because of its immunosuppressive function, which can lead to the development of life-threatening viral or fungal infections, thrombocytopenia, hyperlipidemia, impaired wound healing, nephrotoxicity or altered insulin sensitivity [141, 145-147]. Further, rapamycin treatment has achieved only modest effects in the clinics, most likely because of the large number of mTORC1-regulated negative feedback loops [143, 144, 148].

Therefore, there is a need for additional mTOR inhibitors, such as the ATP-competitive mTOR inhibitor Torin1, which has been shown to inhibit both mTOR complexes, while not affecting their stability. Besides being highly specific, it is more capable to inhibit rapamycin-sensitive functions like protein synthesis or induction of autophagy than rapamycin. Further, it has the capability to additionally inhibit rapamycin-resistant functions of mTORC1 [149]. Therefore, ATP-competitive inhibitors as Torin1 are promising compounds for further clinical studies.

1.2.3 Tuberous Sclerosis Complex (TSC)

As stated above, mTORC1 is mainly inhibited by the TSC1/TSC2 tumor suppressor complex. A loss-of-function mutation of either TSC1 (also known as hamartin) or TSC2 (tuberin) is cause of Tuberous sclerosis complex (TSC), an autosomal dominant genetic disorder [150]. However, two-thirds of TSC mutations appear to occur *de novo* [151]. TSC is characterized by the formation of hamartomas, benign and non-invasive

tumor-like lesions in various important organ systems such as brain, heart, skin, kidney, lung and liver. The estimated birth incidence of TSC is approximately 1 in 6000 [150, 152].

TSC displays a variety of disease manifestations including facial angiofibromas and hypomelanotic macules, renal cysts or angiomyolipomas (RAL), pulmonary lymphangioleiomyomatosis (LAM), cardiac rhabdomyomas, retinal hamartomas and hepatic angiomas [150]. However, most severe manifestations of TSC comprise the central nervous system, 85 – 90 % of TSC patients exhibit neurological comorbidities. These include epilepsy in up to 90 % as well as subependymal nodules (90 – 100 %) and subependymal giant cell astrocytomas (SEGA) [114]. Among these, although SEGAs, slow growing tumors of mixed cellular lineage, affect solely 5 – 20 % of TSC patients, they display a significant cause of morbidity and mortality of the disease, because of the risk of sudden death caused by acute hydrocephalus [153]. Further, mental delay affects about 44 – 64 % of TSC patients with comprehensive impairment in about 30 % [150]. Thereby, the severity is associated among others with epilepsy and seizures as well as certain mutations in TSC2.

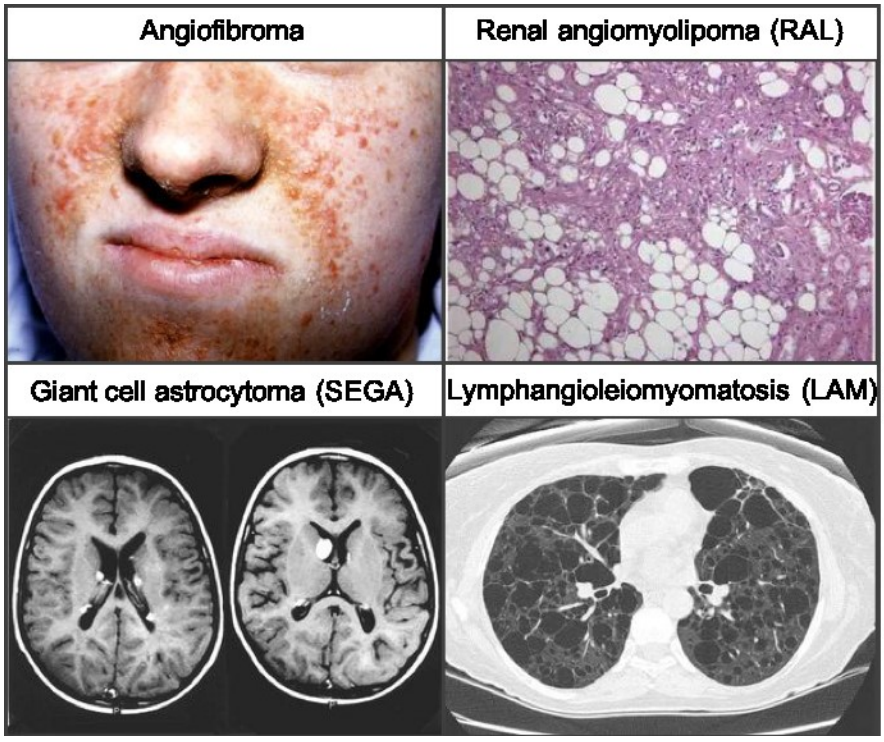


Fig. 6: Variety of different TSC-associated symptoms.

Tuberous sclerosis complex (TSC) comprises the formation of hamartomas. These benign malformations can affect all organs except from spinal cord, peripheral nervous system and skeletal muscles. Common disorder manifestations are angiofibroma of the skin, renal angiomyolipoma (RAL), subependymal giant cell astrocytomas (SEGA) or Lymphangioleiomyomatosis (LAM) of the lung. Translated and modified with permission from TSC Deutschland eV (www.tsdev.org).

Moreover, heterozygous mutations of TSC1 or TSC2 are associated to Autism spectrum disorder and epilepsy, possibly because TSC1 and TSC2 were shown to be important for axon formation and growth in mice [154]. Additionally, mice heterozygous for TSC2 mutations exhibited learning and memory defects, which partly could be reversed by rapamycin treatment, indicating to an mTORC1 dependent phenotype [155].

TSC1 and TSC2 form the tuberous sclerosis complex (TSC), together with TBC1D7 [156]. Thereby, TSC1 acts as a scaffold protein and stabilizes TSC2, which hast catalytic activity [157]. Binding of TSC1 to TSC2 prevents its ubiquitination and thereby degradation [158]. A loss of TSC1 or TSC2, as well as hyperactive growth factor signaling, leads to inappropriate mTORC1 activity, which causes several diseases in addition to TSC, including cancer, neuronal dysfunction or cardiac hypertrophy [79, 159]. Upon phosphorylation of TSC, its repressive function is reversible [107]. As a result, the TSC complex functions as a GTPase-activating protein (GAP), converting the small Ras-related GTPase Rheb from its inactive GDP-bound state to the active GTP-bound form, which in turn activates mTORC1 [106, 160]. Therefore, it was shown that TSC, like mTORC1, is also present at the lysosomal surface and dissociates in response to insulin and AKT-dependent phosphorylation of TSC2 [161]. Thus, Rheb-GTP activates mTORC1. Beside AKT, different other kinases such as ERK, AMPK or GSK3 (Glycogen synthase kinase 3) are able to phosphorylate TSC1 or TSC2 at distinct residues [110, 162-164]. Moreover, TSC2 was found to physically interact with mTORC2, suggesting that the TSC complex could regulate both mTOR complexes [130].

In patients with TSC, rapamycin analogs are approved for the treatment of subependymal giant cell astrocytoma (SEGA) and under investigation as therapeutics for angiomyolipomas (AML) and renal angiomyolipomas (RAL) as well as lymphangiomyomatosis (LAM) [101, 143, 144]. However, long-term administration of rapamycin or rapalogs could benefit other TSC-associated disease manifestations and elicits severe side effects.

1.2.4 Role of mTOR in innate immunity

mTORC1 has been reported to regulate the function of diverse immune cells including dendritic cells, macrophages, neutrophils, mast cells and natural killer cells, as well as T and B cells [158, 165-167]. Thereby mTORC1 has inhibitory and stimulatory functions. mTOR was shown to play a crucial role in T cell homeostasis, survival and quiescence and inhibition of mTORC1 by rapamycin impairs maturation of dendritic cells and inhibits clonal T cell expansion and T cell proliferation [142, 158, 168, 169]. Additionally, it was reported that deletion of Rheb, which activates mTORC1, inhibits the transition of monocytes to macrophages [170]. Inhibition of mTORC1 by rapamycin protects mice

against an otherwise lethal infection with *Listeria monocytogenes* [171]. Dysregulation of mTORC1 is associated with several types of cancer as well as autoimmune diseases and sepsis, further supporting its immunological function [172, 173].

In addition to immune system-specific organs, cells of the immune system have to be present in different tissues throughout the body. Thus, these cells need to adjust to a variety of different environments. Furthermore, many immune cells are able to exist in a resting state and can shift to an activated state upon stimulation. Especially activated immune cells produce large amounts of cytokines, chemokine and lipid mediators, accomplish drastic morphologic changes and migrate into tissues. These metabolically challenging processes require a certain flexibility of cellular metabolism. In addition, it is suggested that innate immune cells, as adaptive immune cells, can actively control their metabolic processes linking the availability of nutrients to their metabolic needs [69]. mTOR, as metabolic signaling node, is integrating extracellular signals like growth factors and hormones as well as intracellular cues such as nutrient supply and therefore plays an important role in regulating these processes.

Monocytes and macrophages, as part of the innate immune system, are mononuclear phagocytes and occupy important fundamental functions such as inflammatory cytokine production, pathogen clearance and tissue repair. In contrast to adaptive immune cells, innate immune cells do not express antigen-specific receptors, but cytokine receptors and pattern recognition receptors (PRR). The latter recognize pathogens, PAMPs and damage-associated molecular pattern (DAMP) and can induce the production of pro-inflammatory cytokines like TNFα, IL-6 or IL-12, as well as anti-inflammatory cytokines such as IL-10. Induction of cytokines can be mediated at the transcriptional and translational level. Since cytokines are highly controlled and have to be upregulated fast after pathogen recognition, they are likely to be regulated on a translational level. mTORC1 and its downstream targets S6P and 4E-BP1 thereby are strong regulator of protein biosynthesis.

LPS binding was demonstrated to trigger mTORC1 activity via ERK or PI3K and TLR4 signaling through MyD88, IRAK4 and the PI3K/mTORC1 pathway was shown to induce tolerance [163, 174]. The PI3Kγ/AKT/mTORC1 pathway was reported to promote the expression of immunosuppressive proteins like IL-10 or Arginase 1, upon LPS stimulation and mTOR also limits the production of pro-inflammatory cytokines by inhibiting the transcription factor NFκB [171, 175]. Inhibition of the pathway, otherwise, increases the phosphorylation of IκB kinase β (IKKβ) and thereby NFκB activity [175]. Toll-like receptor (TLR) antagonists can activate mTORC1 and mTORC2 in dendritic cells and neutrophils, as well as monocytes and macrophages of human and mice [162, 171, 176]. In addition, genetic or pharmacological inhibition of mTORC1 reduces the

production of the anti-inflammatory IL-10 in human and murine dendritic cells and monocytes and loss of TSC2 function was found to increase IL-10 production in monocyte-derived macrophages [171, 177, 178]. Moreover, also the production of inflammation-associated TNFα can activate IKKβ, which can interact with and inactivate TSC1, resulting in an activation of mTORC1 as negative feedback regulation [179]. On the other hand, TSC1 deletion in bone marrow-derived macrophages enhances inflammatory activation induced by LPS through constitutive active mTORC1 and NFκB activation [148, 180]. Thus, mTORC1 can promote or inhibit inflammation.

1.2.5 Role of mTOR in trained immunity

mTOR has been revealed as the major regulator of glycolysis in activated lymphocytes [181, 182]. Further, RNA sequencing analysis revealed alterations in the mTORC1 pathway also in adapting monocytes as underlying mechanism [40]. It is suggested that adapting monocytes switch to Warburg metabolism upon induction of trained immunity in an mTORC1-dependent manner. Training of monocytes with β-glucan was reported to induce AKT phosphorylation, which is dependent on the dectin-1 receptor, as phospho-AKT levels are lost in cells derived from dectin-1 deficient patients [40]. Additionally, the training effect of β-glucan on TNFα cytokine levels is blocked by the PI3K/AKT inhibitor wortmannin. Since the PI3K/AKT pathway is an upstream regulator of mTORC1, also activation of mTORC1, induced by β-glucan stimulation, was diminished in dectin-1 deficient cells, along with decreased TNFα cytokine production [40]. Furthermore, rapamycin treatment leads to a dose-dependent inhibition of the training effect by β-glucan stimulation regarding TNFα levels [171].

An increasing line of evidence suggest that mTORC1 activates the transcription factor HIF-1α, which senses oxygen levels in the cell and can promote induction of glycolysis, as well as glycolytic enzymes [40, 183]. Induction of trained immunity results in an upregulation of the mTOR/HIF-1α pathway as the suggested key signaling path to increase glycolysis, also in the presence of oxygen. Activation of HIF-1α was enhanced in β-glucan trained monocytes in an mTORC1-dependent manner, since it was suppressed by mTORC1 inhibition through rapamycin [40]. Induction of HIF-1α has also been associated with high glycolytic activity and Warburg metabolism in cancer as well as functional re-programming of monocytes during sepsis [34, 184]. Disruption of these pathways on a genetic basis or by chemical inhibition leads to impaired training induction by β-glucan [40]. The HIF-1α inhibitor ascorbate blocks trained immunity in a dose-dependent manner with regard to TNFα levels and the protective effect of β-glucan training of mice subsequently infected with *Staphylococcus aureus* is lost in HIF-1α

deficient mice [40, 185]. In addition, HIF-1α was shown to activate TSC and in turn mTORC1 [186]. These data indicate a role of the mTOR/HIF-1α signaling pathway in adapting monocytes by regulating cellular metabolism.

2. OBJECTIVES

Adaptation processes of innate immune cells display a potential target for therapies against different kinds of diseases such as augmented inflammatory response and septic shock as well as post-septic immunoparalysis, autoimmune deficiency and autoimmune disorders, together with different types of cancer. Understanding the underlying molecular mechanisms could reveal possible targets for disease therapy.

Accumulating evidence hint towards an important role for underlying metabolic changes in adapting innate immune cells. Induction of trained immunity by β-glucan increases the glycolytic activity of human monocytes, resulting in increased glucose consumption and enhanced lactate production. With mTORC1 being a major metabolic regulator, RNA sequencing revealed an upregulation of mTORC1 signaling along with HIF-1α and other glycolytic enzymes in β-glucan stimulated cells. Chemical or genetic inhibition of either one pathway results in impaired training induction. Further, also the immune activation in response to LPS was shown to be dependent on metabolic changes within the cells. These data suggest a central role of mTORC1 signaling in the regulation of underlying metabolic changes to enable an appropriate immune response and immunological memory-like functions such as endotoxin tolerance or trained immunity in innate immune cells such as monocytes.

The aim of this study was to investigate the role of mTORC1 signaling in different adaptation processes such as endotoxin tolerance and β-glucan training, as well as in the underlying metabolic changes in adapting primary human monocytes. For these investigations, in addition to chemical inhibition of mTOR with rapamycin and torin, cells from patients with Tuberous sclerosis complex (TSC), displaying a genetic hyperactivation of mTORC1, should be analyzed for adaptation capacity. Further, molecular mechanisms of innate immune adaptation in concern to mTOR signaling should be revealed using healthy voluntary blood donors.

To achieve these aims, the following main objectives were addressed in this thesis.

- To investigate the effects of altered mTORC1 activity, cells from healthy donors and patients with TSC were challenged with LPS and β-glucan in the presence and absence of mTOR inhibitors.
- For studying mTORC1 signaling, upstream and downstream targets were investigated. Inhibitors against mTOR and common inflammatory-associated signaling molecules were used to determine altered immune adaptation.

- Investigations of endotoxin tolerance induction, including analysis of TLR4 surface availability and the effect of pro-inflammatory cytokines on the induction of tolerance were performed.

3. MATERIAL AND METHODS

3.1 MATERIAL

3.1.1 Chemicals and reagents

Accutase	Capricorn Scientific
Acetic acid	Carl Roth GmbH & Co. KG
Ammonium persulfate (APS)	SERVA Electrophoresis GmbH
Acrylamide/Bis-acrylamide (Rothiphorese Gel 30)	Carl Roth GmbH & Co. KG
β-glucan	kindly provided by Prof. David L. Williams, ETSU
β-mercaptoethanol	Fluka Biochemika
Bromophenol blue	Carl Roth GmbH & Co. KG
Bovine serum albumin (BSA, endotoxin free)	Carl Roth GmbH & Co. KG
Brefeldin A	Sigma-Aldrich Chemie GmbH
Cytofix™	BD Biosciences
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	Carl Roth GmbH & Co. KG
Gentamicin	Sigma-Aldrich Chemie GmbH
GlutaMax	Thermo Fisher Scientific Inc.
Human serum	Sigma-Aldrich Chemie GmbH
rhIL-8	Immunotools
Leupeptin-hemisulphate	AppliChem GmbH
LPS (O55:B5, #L2880)	Sigma-Aldrich Chemie GmbH
LY294002	Enzo Life Science GmbH
Methanol	Carl Roth GmbH & Co. KG
PBS w/ Mg^{2+}/Ca^{2+}	LONZA
PBS w/o Mg^{2+}/Ca^{2+}	LONZA
Pefabloc SC	AppliChem GmbH
Pepstatin A	AppliChem GmbH
Phosflow™ Perm Buffer III	BD Biosciences
Phosphatase Inhibitor cocktail	Sigma-Aldrich Chemie GmbH
Prestained Page Ruler	Thermo Fisher Scientific Inc.
Rapamycin	Calbiochem
RPMI 1640 Dutch Modification	Sigma-Aldrich Chemie GmbH

SB202190	Sigma-Aldrich Chemie GmbH
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich Chemie GmbH
Sodium pyruvate	Sigma-Aldrich Chemie GmbH
N,N,N',N'-Tetramethylethylenediamine (TEMED)	SERVA Electrophoresis GmbH
Torin	Tocris
Tris ultrapure	AppliChem GmbH
Tween 20®	SERVA Electrophoresis GmbH
UO126	Enzo Life Science GmbH

3.1.2 Kits

Micro BCA Protein Assay Kit	Thermo Fisher Scientific Inc.
ELISA Standard TNFα	Biolegend Inc.
CBA Flex Set	BD Biosciences
Proteome Profiler Human Cytokine Array Kit	R&D Systems

3.1.3 Antibodies

Table 1: Antibodies for Western blot analysis

name	clone	species	dilution	serial number	company
S6Kinase		rabbit	1:1 000	9202	Cell Signaling
Phospho-S6Kinase		rabbit	1:1 000	9205	Cell Signaling
S6-Protein	5G10	rabbit	1:1 000	2217	Cell Signaling
Phospho-S6-Protein		rabbit	1:1 000	2211	Cell Signaling
4EBP1		rabbit	1:1 000	9452	Cell Signaling
Phospho-4EBP1	T70	rabbit	1:1 000	9455	Cell Signaling
AKT		rabbit	1:1 000	9272	Cell Signaling
Phospho-AKT	D9E	rabbit	1:1 000	4060	Cell Signaling
ERK	137F5	rabbit	1:2 000	4695	Cell Signaling
Phospho-ERK	E10	rabbit	1:1 000	9106	Cell Signaling
p38		mouse	1:1 000	51-9002050	BD Transduction
Phospho-p38		rabbit	1:1 000	51-9002043	Cell Signaling
TSC1/Hamartin	D43E2	rabbit	1:1 000	6935	Cell Signaling
TSC2/Tuberin		rabbit	1:1 000	3612	Cell Signaling
HRP anti-mouse		goat	1:10 000	5210-0183	KPL
HRP anti-rabbit		goat	1:10 000	5220-0336	KPL

Table 2: Antibodies for flow cytometry

name	clone	fluorophore	dilution	serial number	company
Anti-human CD3		APC	1:10	21850036	Immunotools
anti-human CD14	61D3	Pacific blue	1:20	48-0149-42	eBioscience
Anti-human CD14		FITC	1:10	21620143	Immunotools
Anti-human pS6	Cupk43K	APC	1:20	17-9007-42	eBioscience
Anti-human TLR4	HTA125	Alexa fluor 488	1:10	53-9917-42	eBioscience
Anti-human TLR4	HTA125	APC	1:10	17-9917-42	eBioscience
Anti-human TNFa		PE	1:10	21453014	Immunotools

3.1.4 Buffers

Table 3: Buffers for Isolation and cell culture

Culture Media	RPMI 1640 Dutch modification 1 % Gentamicin 1 % sodium pyruvate 1 % GlutaMax
Isolation Buffer	PBS w/o Mg^{2+}/Ca^{2+} 1 % BSA 2 mM EDTA

Table 4: Buffers for SDS Page/Western blotting

Blocking Buffer	1 % BSA in 1 x TBS-Tween®
10x SDS PAGE Running Buffer	250 mM Tris 2 M Glycine 35 mM SDS
RIPA Buffer	50 mM HEPES (pH 7,5) 150 mM NaCl 5 mM MgCl 100 mM EGTA 1 % Nonidet P-40 0.5 % Deoxycholate 0.1 % SDS
5 x Sample Buffer	0.25 M Tris 50 % Glycine 10 % SDS 10 % β-mercaptoethanol 0.25 % Bromophenol blue

10% Separation Gel	2 M Tris (pH 8.8) 10 % acrylamide 0.01 % SDS 0.01 % APS 0.04 % TEMED
5% Stacking Gel	0.5 M Tris (pH 6.8) 5 % acrylamide 0.01 % SDS 0.01 % ammonium persulfate 0.1 % TEMED
Stripping Buffer (pH 6,7)	100 mM β-mercaptoethanol 62.5 mM Tris 2 % SDS
10 x TBS-Tween (pH 7,6)	0.1 M Tris 1 M NaCl 1 % Tween20®
Transfer Buffer (pH 10)	48 mM Tris 39 mM Glycine 0.037 % SDS 15 % Methanol

Table 5: Buffers for ELISA and Flow cytometry

ELISA Stop Solution	2 N H_2SO_4
ELISA Wash Buffer	1x PBS 1 % BSA 1 % Tween20®
FACS Buffer	1 x PBS 1 % BSA 2 mM EDTA
10 x PBS (pH 7.5)	1,37 M NaCl 27 mM KCl 100 mM Na_2HPO_4 18 mM KH_2PO_4

Solutions for stimulation

Lipopolysaccharide (LPS) from *Escherichia coli* (strain O55:B5) was solved in sterile water at a stock concentration of 1 mg/ml, aliquoted and stored at -20 °C. A fresh aliquot was thawed for each stimulation and pre-diluted in culture media. β-glucan was kindly provides by Professor David L. Williams from the East Tennessee State University as 5 mg/ml solution and stored at 4 °C. Rapamycin and torin were solved in DMSO at stock concentrations of 20 µM and 10 µM, respectively, aliquoted and stored at -20 °C.

3.2 METHODS

3.2.1 Design of the study

This clinical study was a prospective, explorative association study in cooperation with the Children´s Hospital of the University Hospital Jena. 19 patients, with diagnosed TSC, and 25 healthy volunteers with matched age and sex were included after written informed consent of participants or their legal representatives. A study protocol was developed in cooperation with the Center for clinical studies of the University hospital Jena. Before the study started a positive ethical vote from the central ethical board of Thuringia was available. A sample of peripheral blood was drawn with volumes being adapted according to the age of the participant (2.0 – 27.0 ml). Controls (with no clinical hint for TSC) were age and sex matched whenever possible. Patient and control samples were masked by an ID on site, transported to the laboratory within 4 h and processed immediately. According to the study protocol all participants were free of acute infectious disease as evidenced by CrP (C-reactive protein) levels under 10 mg/l and did not receive immunosuppressants others than everolimus.

3.2.2 Isolation of primary human monocytes from whole blood

For the isolation of primary human monocytes, blood samples from TSC patients and age and sex matched controls were drawn in the Children´s Hospital in Jena. Respectively 9 – 18 ml whole EDTA blood was diluted in isolation buffer to a final volume of 30 ml. The diluted blood was then carefully layered upon 15 ml of Histopaque®-1077 and centrifuged for 20 min at 800 g, without brake. The layer of Peripheral blood mononuclear cells (PBMCs) was harvested and washed two times with cold isolation buffer. Purified PBMCs were seeded at a concentration of 5 x 10^6 /ml in culture media and set for 1 h at 37 °C. After monocytes attached to the surface, non-adherent cells were washed out 3 times with warm PBS. Afterwards, culture media supplemented with 10 % human serum was added. Purity of preparations was verified via flow cytometry using a CD14 surface marker.

3.2.3 Isolation of primary human monocytes from Buffy Coats

To yield more cells for signaling analysis, Buffy Coats from the Institute for Transfusion medicine of the University Hospital Jena were purchased. Isolation of PBMCs was achieved as described for TSC patients and controls. Every 6 ml of Buffy Coat were diluted in 24 ml of Isolation buffer and layered onto 15 ml Histopaque®-1077. After centrifugation at 800 g for 20 min without break, the PBMCs were harvested and washed

additional two times with cold isolation buffer. Purified PBMCs were seeded at a concentration of 5 – 10 x 10^6 /ml and incubated for 1 h at 37 °C for attachment. Non-adherent cells were washed out 3 times with warm PBS and culture media supplemented with 10 % human serum was added.

3.2.4 Stimulation of primary human monocytes

To investigate adaptation processes of primary human monocytes, isolated cells were stimulated with two subsequent stressors. Unless otherwise stated, cells were first primed with either 100 ng/ml LPS or 3 µg/ml β-glucan for 24 h. To study the direct effect of mTOR inhibition, cells were additionally treated with 20 ng/ml rapamycin and 10 ng/ml torin for 30 min prior to and during the first 24 h of LPS or β-glucan treatment. Afterwards, supernatants were harvested, and cells were washed with warm PBS followed by re-stimulation with 10 ng/ml LPS for additional 24 h. Control cells were either treated with cell culture media or 10 ng/ml LPS alone in the absence of the priming step, at the same time as re-stimulation of primed cells. Media exchange and washing steps were performed exactly like with pre-treated cells.

For analysis of different inflammatory pathways cells were additionally treated with different inhibitors for 1 h prior to and during 24 h of LPS priming. Rapamycin and torin, for mTOR inhibition, were used in the same concentrations as stated above, SB202190 for inhibition of the p38 pathway was used at a concentration of 10 µM, UO126 for ERK inhibition at 50 µM and LY294002 for PI3K at 10 µM. Subsequently, cells were re-stimulated with 10 ng/ml LPS for additional 24 h in the absence of inhibitors.

To investigate paracrine inflammatory factors, cells were treated with conditioned media from monocytes to. Therefore, isolated monocytes were stimulated with 100 ng/ml LPS for 1 h, washed with warm PBS to remove the majority of remaining LPS and fresh culture media, supplemented with 10 % human serum, was added and cells were incubated for additional 23 h at 37 °C. Afterwards conditioned media, containing secreted cytokines, was harvested and centrifuged for 10 min at 10 000 g to pellet cells and cell debris. The media was further used to prime cells as described above with LPS for 24 h at 37 °C followed by a washing step and subsequent re-stimulation with 10 ng/ml LPS for additional 24 h at 37 °C.

3.2.5 Surface staining for flow cytometry

Isolated monocytes were harvested by Accutase-detachment and scraping, washed with FACS buffer and centrifuged at 600 g for 10 min at 4°C. Supernatant was removed, and cells were stained in 50 µl FACS buffer with the indicated antibody concentrations for

20 – 30 min on ice and in the dark. Afterwards, cells were washed with 500 µl FACS buffer, centrifuged and suspended in 300 µl FACS buffer. Cells were analyzed using a FACSCanto™ II (BD Biosciences) and Flow Jo software (TreeStar Inc.).

3.2.6 Intracellular staining for flow cytometry

To stain intracellular proteins following surface staining, cells were again centrifuged and fixed in 350 µl warm Cytofix™ Buffer (BD Biosciences) for 15 min in the dark. For analysis of intracellular cytokine production after LPS or β-glucan treatment, cells were additionally treated with 10 µg/ml Brefeldin A to block secretion of proteins. Cells were washed again and subsequently permeabilized with 350 µl ice-cold Phosflow Perm Buffer III (BD Biosciences), added dropwise, for 30 min on ice in the dark. Samples were washed two times with 1 ml FACS buffer and suspended in 50 µl FACS buffer with the indicated antibody concentrations and incubated for 30 min on ice and in the dark for intracellular staining. Afterwards cells were washed with FACS buffer, centrifuged and re-suspended in 300 µl FACS buffer. Analysis of the samples were performed using an LSRFortessa™ (BD Biosciences) and Flow Jo software (TreeStar Inc.).

3.2.7 Enzyme-linked Immunosorbent Assay (ELISA)

Analysis of cytokine levels in the cell culture supernatants was done using an TNFα ELISA Kit (Biolegend Inc.) following manufacturer´s instructions. Therefore, supernatants were harvested and centrifuged for 10 min at 10 000 g to clear from cells and cell debris. Antibodies against cytokines were immobilized on a 96-well plate before samples were added and bound. Afterwards antibodies against the specific cytokine and Horse-reddish peroxidase (HRP) were bound and HRP enzyme substrate was added. Depending on the concentration of cytokines bound and consequent amounts of HRP, substrate solution was metabolized indicated by color change of the substrate. Cytokine concentrations were calculated using a known TNFα standard cytokine range measured.

Capture antibodies were incubated over night at 4 °C on a shaker. Afterwards plates were washed 4 times with 300 µl wash buffer, blocked with 1x Assay Diluent for 1 h while shaking at room temperature (RT) and washed again 4 times. Cleared supernatants were diluted 1:7 in Assay diluent and 100 µl of sample or standard were added in duplicate to the plate and incubated for 2 h on a shaker. After 4 additional wash steps, 100 µl detection antibody were added and incubated for 1 h while shaking followed by washing steps. Avidin-HRP solution was added, incubated for 30 min and washed 5 times. Finally, 100 µl HRP substrate solution were added, incubated for

15 min without shaking and reaction was stopped by adding 100 µl of Stop solution. Color change was measured by absorbance at 450 nm using a Micro Plate reader (VersaMax) and was analyzed using SOFTmax Pro software (Molecular Devices).

3.2.8 Cytometric Bead Array (CBA)

To determine content of multiple cytokines simultaneously from cell culture supernatants the Cytometric bead array (BD Biosciences) was performed following manufacturer´s instructions. To enable the measurement of up to 30 cytokines at once, samples are mixed with a composite of beads coated with a capture antibody to one specific cytokine and a unique florescence intensity. Thereby each bead population, representing one cytokine, can be identified by a specific position in a grid of two fluorescent dyes. To assess the cytokine concentrations a mix of detection antibodies coupled to a reporter molecule (PE) is added to each sample (Fig. 7).

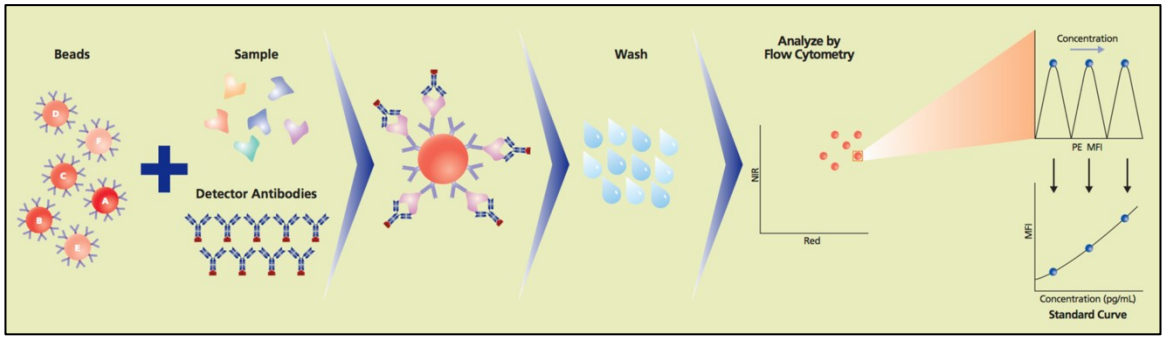


Fig. 7: Assay principle of the Cytometric bead array.
For the cytometric beads array a mixture of beads coupled to two distinct flow cytometric dyes with specific coupled antibodies against different cytokines are incubated with cleared cell culture supernatants to bind to cytokines. Detector antibodies against the same cytokines, coupled to another flow cytometric dye (PE), are added afterwards to bind the bead-cytokine complexes, followed by washing and analyzing using a flow cytometer. Distinct proportion of the two dyes coupled to the beads define its position and identification within a grid of up to 30 different bead populations. Afterwards every bead population, representing one specific cytokine, is analyzed for its mean fluorescent intensity (MFI) of PE to verify its concentration by use of a known standard mix and calculated standard curves. Transferred with permission from BDbiosciences.de.

Cell culture supernatants for cytokine measurements were centrifuged for 10 min at 10.000 g to pellet cells and cell debris and were stored at -20 °C until use, for short time storage. To bind cytokines 0,5 µl of each cytokine bead solution were mixed and diluted in capture bead diluent buffer to a final volume of 50 µl per sample and added to 50 µl cleared cell culture supernatant. Samples were carefully mixed and incubated for 1 h at RT. In parallel, 0,5 µl of each PE detection antibody was diluted in PE detection diluent to a final volume of 50 µl per sample. 50 µl of PE mix were added to the samples, mixed carefully and incubated for 2 h at RT in the dark. Afterwards, 1 ml of wash buffer was

added to each sample and centrifuged for 5 min at 200 g. Supernatant was aspirated and beads were suspended in 300 µl wash buffer. Recombinant standards with known concentrations were used to quantify the PE intensity of each sample. Beads were analyzed using a FACSCanto™ II (BD Biosciences) and Flow Jo software (TreeStar Inc.). Calculated cytokine concentrations were further normalized by protein content or cell count.

3.2.9 Proteome Profiler Human Cytokine Array

An overview of 36 different inflammatory cytokines, including those measured with CBA, were analyzed using the Proteome profiler human cytokine array (R&D systems). Thereby antibodies against 36 different cytokines are immobilized in duplicates on a small nitrocellulose membrane (Fig. 8). Following manufacturer´s instructions, up to 1,5 ml cleared cell culture supernatant were incubated with 15 µl secondary antibody mix for 1 h at room temperature, while membranes were blocked with included assay buffer 4 for 1 h, shaking at RT. Afterwards membranes were incubated with supernatants including secondary antibodies against all measured cytokines overnight at 4 °C on a shaker. Membranes were washed 3 times with 20 ml of included wash buffer for 10 min each and subsequently incubated with 2 ml of diluted Streptavidin-HRP solution per membrane for 30 min at RT shaking. After 3 additional washing steps, membranes were placed into a plastic sheet protector and 1 ml of mixed Chemi reagent mix per membrane were added. After 1 min incubation in the dark excess Chemi reagent mix was gently smoothed out of the plastic sheet protector and membranes were placed into an autoradiography cassette, x-ray films were placed on top of the membranes and incubated for different times as indicated.

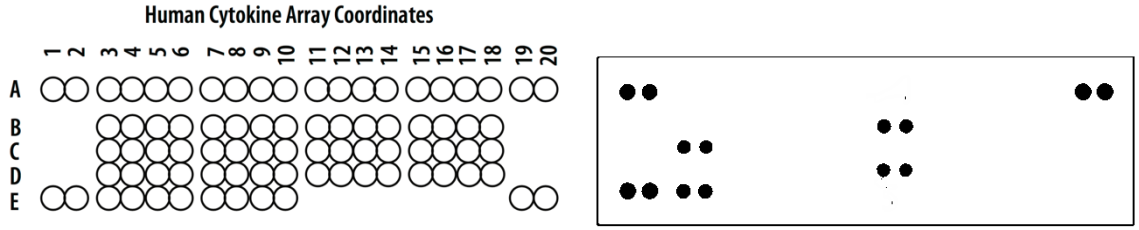


Fig. 8: Proteome Profiler Human Cytokine Array.

Template for evaluation of the Proteome profiler human cytokine array membranes (left) and a representative analyzed membrane (right) with 4 visible proven cytokines and additionally positive controls at the positions A1-A2, A19-A20 and E1-E2, as well as a negative control at E19-E20. Transferred and modified with permission from R&D Systems.

3.2.10 Bicinchoninic Acid Protein Quantification Assay (BCA)

Protein concentration of whole cell RIPA buffer lysates was measured using the Pierce Micro BCA Protein Assay Kit following manufacturer´s instructions. In short, 5 – 50 µl of each sample (containing a pre-estimated amount in the range of the standard curve) were diluted in water to a final volume of 300 µl. 150 µl of each sample was added in duplicate to a 96 well plate and 150 µl of BCA reagent was added per well. Samples were incubated for 2 hours at 37 °C and analyzed using a plate reader Infinite® 200 (TECAN) and i-control™ (METTLER TOLEDO) software to measure absorption rate at 570 nm. For quantification of the samples a known standard BSA concentration range was used.

3.2.11 Preparation of protein lysates

Preparation of protein lysates for protein content analysis or SDS Page was performed using RIPA lysis buffer on ice. After addition of lysis buffer, cells were scraped, incubated for 10 min on ice and then centrifuged for 30 min at 10 000 g and 4 °C to clear the lysates from cell debris. Supernatant was transferred to a new vial and the cell pellet was discarded. For protein analysis via Western blotting, lysates were mixed with appropriate amounts of 5x sample buffer and boiled at 96 °C for 5 min.

3.2.12 SDS PAGE and Western blotting

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed using 7.5 or 10 % polyacrylamide gels, depending on expected molecular sizes. Proteins were separated by size at a constant current of 35 mA per gel.

To transfer proteins from the gel to a PVDF membrane a Trans-Blot Cell Tank system (Bio-Rad™) for wet blotting was used. Proteins were transferred onto a 0.2 or 0.45 µm pore-size PVDF membrane, depending on protein size. Membranes were pre-activated by short term methanol incubation, followed by washing with distilled water and equilibration in TBS-Tween® for 10 min. Proteins were blotted by constant current of 1.5 A in a cooled Tank system. Subsequently, membranes were blocked for 1 hour at room temperature in blocking buffer. Blots were incubated with primary antibodies overnight at 4 °C under continuously shaking. Afterwards membranes were washed 3 times with TBS-Tween® and incubated with secondary antibodies for 45 min shaking at RT. Following 3 further washing steps, membranes were covered with Western Lightning Enhanced Chemiluminescent (ECL) Substrate for 1 min before analyzing bioluminescent signals using a LAS-2000 (Fujifilm) and Multi Gauge (PLX Devices) software.

3.2.13 Measuring metabolic parameters

Cell culture supernatant concentration of glucose and lactate were analyzed by the Institute for Clinical Chemistry of the University Hospital Jena. 150 µl of cleared supernatant were aliquoted, frozen and stored at - 80 °C until analysis.

3.2.14 Statistical analysis

GraphPad Prism 5 and 6 were used for statistical analysis. A Mann-Whitney test was performed to determine significances between different treatments within one experimental group. The Two-way ANOVA with Bonferroni post-test was used to determine significances between two experimental groups. * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$

4. RESULTS

4.1 Clinical study with TSC patients

4.1.1 Patient cohort

For this study in cooperation with the Children's Hospital Jena, 19 patients with diagnosed TSC who matched the inclusion criteria were enrolled after written consent. Patients comprised 13 male and 6 female individuals from 0 to 38 years of age. Within this cohort 4 TSC patients were analyzed two or more times in a distance of 6 - 16 months. In the following these measurements were included as individual experiments. Controls were age and sex matched whenever possible and handled in parallel. All participants met inclusion criteria.

Table 6: Patient cohort of clinical study

Male	13
Female	6
Age (mean and median)	12
Everolimus treatment	7
TSC1 mutation	1
TSC2 mutation	11
Mutation unknown	7

4.1.2 Establishment of monocyte adaptation

Trained immunity has raised increased interest in recent years and different fields of research and was supposed to be part of this study since it is suggested to be mediated by mTOR signaling. Therefore, verification of training effects of β-glucan in primary human monocytes showed by the research group of Mihai Netea was attempted [37, 40, 49, 50]. Since it was not possible to reproduce the training effect after a 7 days training period, different experimental conditions were adapted according to latest publications [61, 187]. Among this, different isolation protocols were used with or without subsequent Percoll® purification of PBMCs isolated with Histopaque®-1077, distinct cell counts were seeded and also the time points of β-glucan stimulation were adjusted. In addition, different β-glucans were used to induce training effects. Moreover, different serums, commercially available as well as donor specific serum, were tested to increase viability of the cells during long incubation times and the re-stimulation concentration of LPS was

reduced from 100 ng/ml to 10 ng/ml to ensure that strong LPS-induced cytokine induction does not mask a training effect. Further, additionally to the reported stimulation period of 7 days, including 5 days of resting between β-glucan priming and LPS re-stimulation, subsequent LPS re-stimulation was performed.

Induction of endotoxin tolerance with LPS, at the level of TNFα production, was stably induced in the all tested conditions. However, a reproducible training effect of β-glucan priming could not be observed after the 7 days stimulation period, irrespectively of the tested conditions (Fig. 9). Although cytokine measurements of TNFα by ELISA seemed to be increased in β-glucan primed cells after 7 days, this effect was lost after normalization of measurements to protein amount or cell count. Nevertheless, when cells were subsequently re-stimulated with LPS there was a training effect of β-glucan even after normalization, despite not reaching statistical significance. Thus, a subsequent stimulation protocol was used for further experiments.

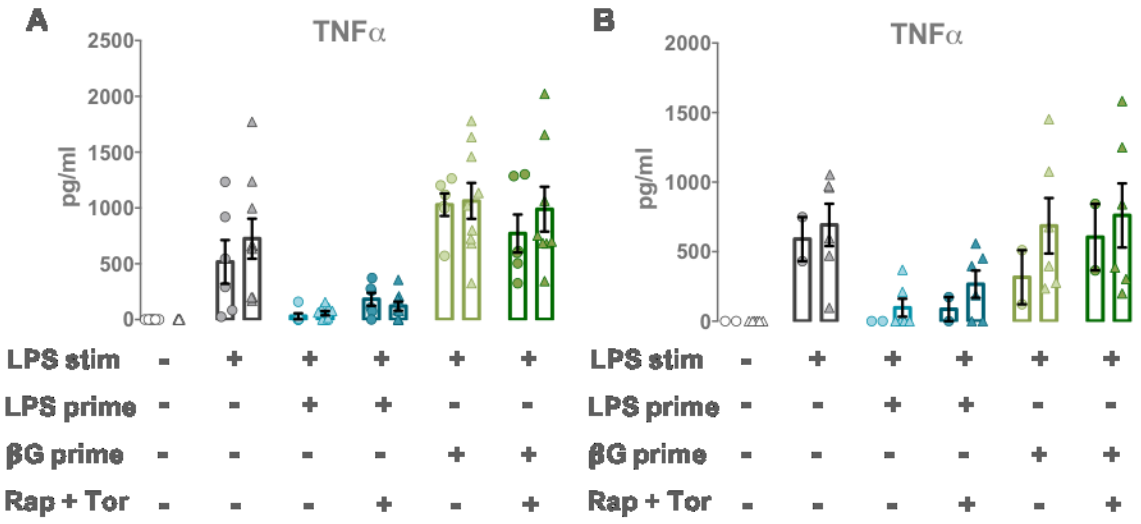


Fig. 9: Training effect of β-glucan is not reproducible after 7 days but present with subsequent stimulation.

Primary human monocytes of healthy controls (circle) and TSC patients (triangle) were primed with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) in the absence or presence of mTOR inhibitors rapamycin and torin (Rap + Tor) for 24 h. Afterwards cells were either (**A**) subsequently or (**B**) after a resting period of 5 days re-stimulated with 10 ng/ml LPS (LPS stim). Data-sets represent mean values of 2 – 9 independent experiments. Error bars are standard error of the mean (SEM). Mann-Whitney test (group comparison) and two-way ANOVA (pair-wise comparison) were used for statistical analysis.

4.1.3 Important pro and anti-inflammatory cytokines in this study

Innate immune cells like monocytes are the first line of host defense. Therefore, monocytes secrete a variety of pro- and anti-inflammatory cytokines, such as interleukins or chemokines and other mediators for autocrine, paracrine or endocrine communication and to recruit other immune cells for initiation and maintenance of a balanced inflammatory response. To cover a broad spectrum of cytokines a subset of pro- and anti-inflammatory cytokines was used to profile the response of monocytes at basal level, as well as after stimulation with LPS or β-glucan (Table 7). Among these, TNFα and Interleukin 6 (IL-6) display classical pro-inflammatory mediators of acute phase response. Together with IL-1β, TNFα and IL-6 represent endogenous pyrogens, which can induce fever as response to inflammation, as well as important mediators in inflammatory diseases such as sepsis. IL-1β was reported to enhance antigen-driven responses of T cells and can trigger the expression of IL-6 [188, 189]. IL-8, also known as neutrophil chemotactic factor, is an important mediator of neutrophil phagocytosis and migration and was shown to promote LPS-induced inflammation by IL-1β and IL-6 [190]. In addition to IL-8, the Monocyte chemoattractant protein 1 (MCP-1) recruits monocytes, but also other immune cells to inflammatory sites. MCP-1, the Macrophage inflammatory protein 1α/β (MIP-1α/β) and RANTES (regulated on activation, normal T cell expressed and secreted) belong to the CC chemokine family, which is involved in acute inflammatory phase and recruitment of granulocytes such as neutrophils. In addition, MIP-1α and MIP-1β can recruit monocytes and natural killer cells to inflammatory sites, induce fever and are known to interact with each other [191]. Moreover, the chemokine RANTES, in cooperation with T cells, triggers proliferation and activation of specific natural killer (NK) cells, CC chemokine-activated killer (CHAK) cells [192]. Further, RANTES and the relative chemokines MIP-1α/β have been identified as natural HIV-suppressive factor secreted by different immune cells [193].

Among the investigated cytokines, IL-10, also known as cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine, which was found to predominantly inhibit pro-inflammatory cytokines produced by LPS stimulation such as TNFα, IL-1β and IL-6 among with IL-8, MCP-1, MIP-1α and MIP-1β [194-197]. In addition, IL-10 inhibits proliferation and cytokine production of T cells, hence, limiting pro-inflammatory response and therefore was reported to protect against endotoxic shock in mice [196, 198-200]. Thus, IL-10 is an important mediator of immunoregulation and inflammation limitation.

Table 7: Important pro-inflammatory cytokines measured using CBA

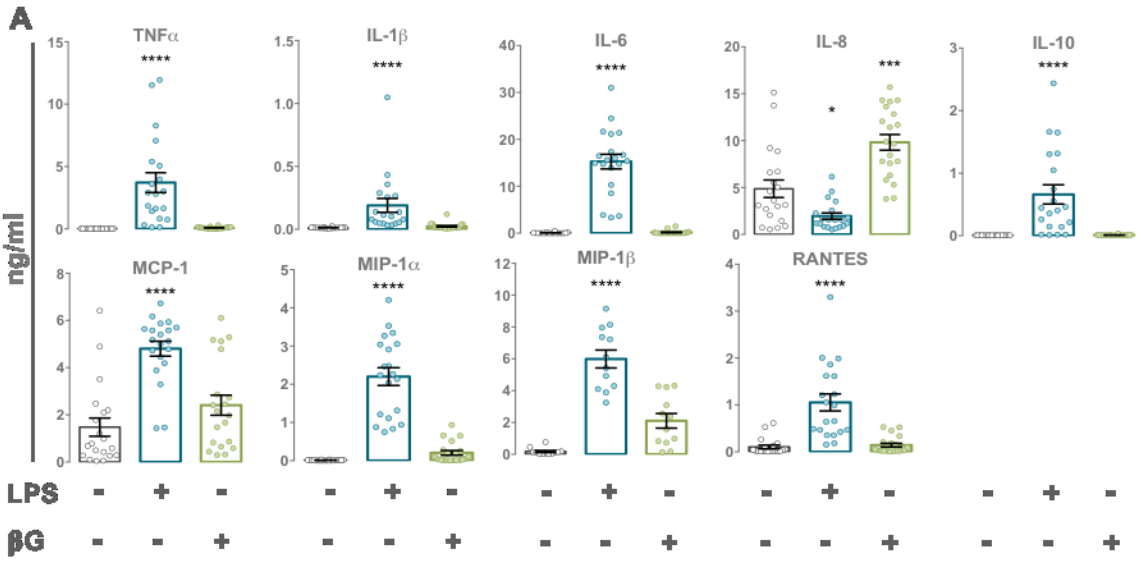
name	classification	pro-/anti-inflammatory	function
TNFα	Tumor necrosis factor	pro-inflammatory	• promotes phagocytosis • chemoattractant for neutrophils
IL-1β	interleukin	pro-inflammatory	• leukocytic pyrogen
IL-6	interleukin	pro-inflammatory	• triggers neutrophil production
IL-8 (CXCL8)	interleukin	pro-inflammatory	• neutrophil chemotactic factor • promotes phagocytosis and migration
IL-10	interleukin	anti-inflammatory	• counteracts pro-inflammatory cytokines • inhibits T cell proliferation
MCP-1 (CCL2)	chemokine	pro-inflammatory	• recruits monocytes, dendritic cells and memory T cells
MIP-1α (CCL3)	chemokine	pro-inflammatory	• chemoattractant for monocytes and granulocytes • promote pro-inflammatory cytokines as IL-1, IL-6, TNFα
MIP-1β (CCL4)	chemokine	pro-inflammatory	• chemoattractant for natural killer cells and monocytes
RANTES (CCL5)	chemokine	pro-inflammatory	• chemoattractant for T cells, eosinophils and basophils

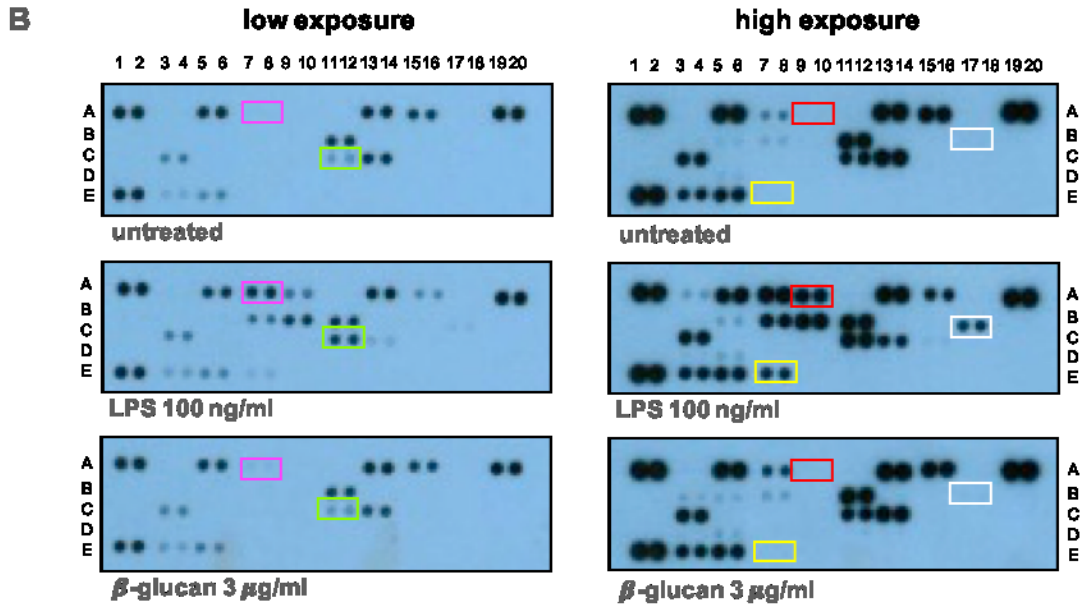
4.1.3 LPS induces pro-inflammatory cytokine release

Lipopolysaccharide (LPS) as bacterial cell wall compartment is a strong Pathogen-associated molecular pattern (PAMP) resulting in a high inflammatory immune response upon detection by innate immune cells like monocytes in the blood stream. β-glucan, another cell wall compartment, but from fungi, is not reported to trigger such a strong immunological response by cells of the innate immune system, but instead promoting an intracellular re-programming.

Thus, to verify the inflammatory state of isolated monocytes after stimulation with LPS or β-glucan, secreted cytokines were measured with the cytometric bead array (CBA), as well as a proteome profiling array, which displays the analysis of 36 different inflammatory cytokines. Stimulation of monocytes from healthy volunteers with 100 ng/ml LPS for 24 h significantly altered all cytokines measured by CBA (Fig. 10 A). While TNFα, IL-1β, IL-6, IL-10, MCP-1, MIP-1α, MIP-1β and RANTES were significantly increased, only levels of IL-8 showed a significant decrease by LPS. Cytokine proteome profiling confirmed the increase of TNFα, IL-1β, IL-6, IL-10, MIP-1α/β and RANTES and further revealed the induction of other pro-inflammatory cytokines such as Granulocyte

colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF) or CCL1 (CC chemokine ligand 1) (Fig. 10 B).





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position	cytokine	position	cytokine
A1, A2	positive control	B9, B10	GM-CSF
A3, A4	CCL1/I-309	B11, B12	ICAM-1
A5, A6	MCP-1	B17, B18	IL-1β
A7, A8	MIP-1α/β	C3, C4	IL-1rα
A9, A10	RANTES	C11, C12	IL-6
A13, A14	C5α	C13, C14	IL-8
A15, A16	CXCL1/GROα	D3, D4	IL-13
A17, A18	CXCL10/IP-10	E1, E2	positive control
A19, A20	positive control	E3, E4	MIF
B3, B4	CXCL11/I-TAC	E5, E6	Serpin E1
B5, B6	CXCL12/SDF1	E7, E8	TNFα
B7, B8	G-CSF	E19, E20	negative control

Fig. 10: LPS and β-glucan stimulation display distinct cytokine profiles.

Primary human monocytes were stimulated with either 100 ng/ml LPS or 3 µg/ml β-glucan or cell culture media for 24 h. Supernatants were collected and used for (**A**) cytometric bead array analysis or (**B**) a cytokine proteome profiling. Representative X-ray films of short-term exposure (2 min, left) and long-term exposure (30 min, right) as well as (**C**) a table with all cytokines identifiable and their specific position are depicted. For reasons of clarity, some important cytokines are color-coded. LPS (LPS 100 ng/ml) and β-glucan stimulation (β-glucan 3 µg/m) displayed distinct cytokine profiles. Stimulation with LPS induced secretion of a variety of 10 different inflammatory cytokines, while β-glucan stimulation was similar to untreated control.

On the other hand, stimulation with 3 µg/ml β-glucan did not induce increased cytokine production of TNFα, IL-1β, IL-6, IL-10, MIP-1α, or RANTES, but slightly increased MCP-1 and MIP-1β, and significantly enhanced IL-8, measured by CBA. In addition, cytokine profiling displayed similar cytokines produced by β-glucan stimulated cells as compared to untreated control cells. Only a slight increase MIP-1α/β could be observed, which confirmed CBA measurements.

4.1.3 TSC cells produce more pro-inflammatory cytokines upon LPS stimulation

To investigate the role of mTOR in the different adaptation processes and the adaptation capacity of primary monocytes from TSC patients and healthy controls to inflammatory stimuli, isolated cells were also primed with either 100 ng/ml LPS or 3 µg/ml β-glucan in the absence or presence of the mTOR inhibitors rapamycin and torin for 24 h. Afterwards cell culture supernatants were harvested, and different cytokines were measured using CBA and ELISA to verify the inflammatory state of the cells.

By analyzing 9 different cytokines (Table 7) it could be shown that primary human monocytes secrete various pro and anti-inflammatory cytokines after priming with LPS. The secretion levels of TNFα, IL-1β , IL-6, IL-10, MCP-1, MIP-1α, MIP-1β and RANTES were significantly increased after LPS treatment, both in control and TSC cells (Fig. 11, LPS prime). Only IL-8 displayed significantly decreased levels after LPS priming in the presence as well as absence of mTOR inhibitors compared to untreated controls also in cells from TSC patients. On the other hand, as seen for control cells, IL-8 was the only cytokine, which was significantly increased during β-glucan priming (βG prime) among with slight increases in MIP-1α and MIP-1β in TSC cells as well. All other cytokines were not affected by β-glucan. The anti-inflammatory cytokine IL-10 and the pro-inflammatory MCP-1 showed significantly decreased secretion levels upon mTOR inhibition with rapamycin and torin (Rap + Tor), while MIP-1β was significantly increased, giving a hint towards a dependency on mTOR activity.

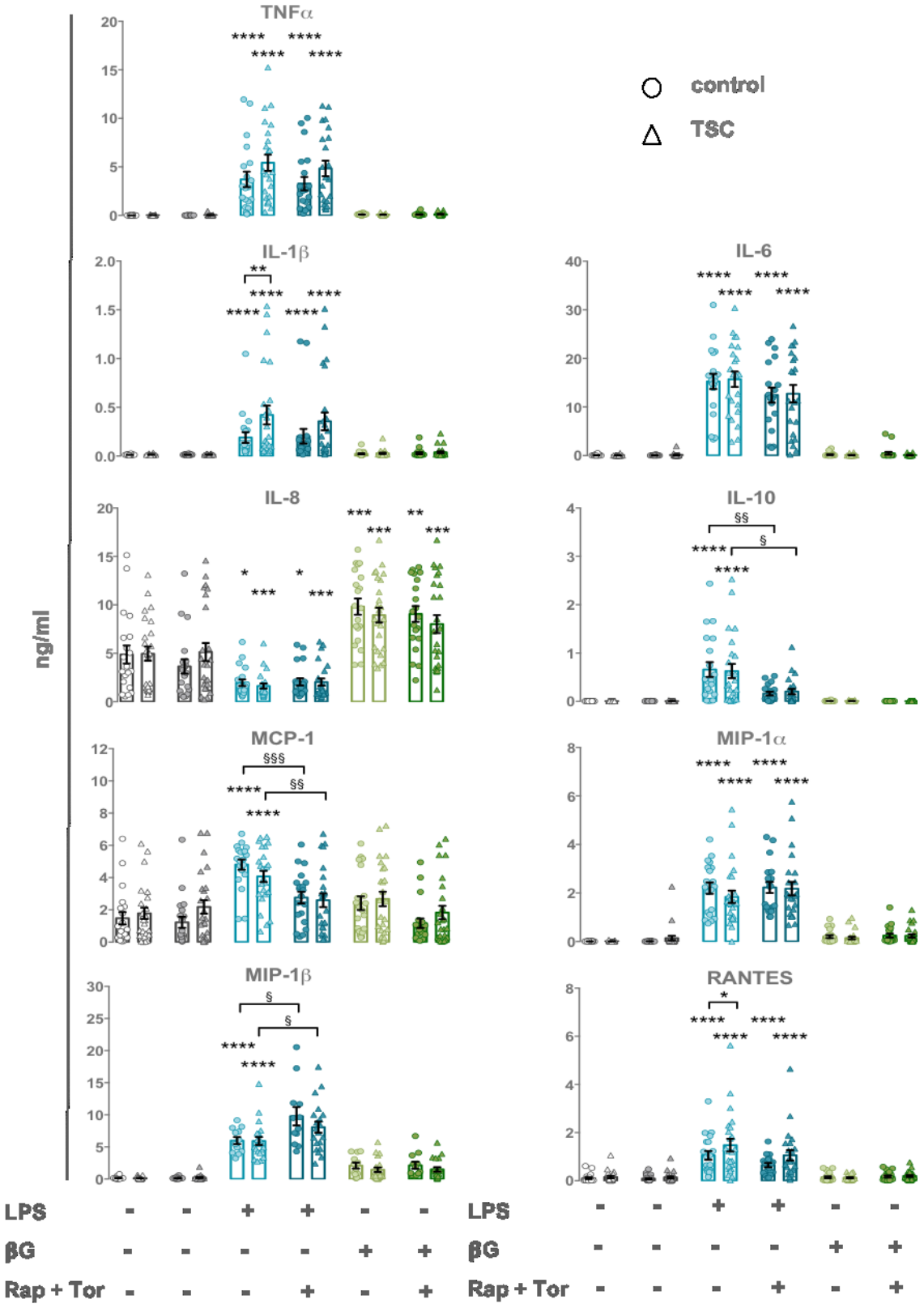

TNFα
IL-1β
IL-6
IL-8
IL-10
MCP-1
MIP-1α
MIP-1β
RANTES
control
TSC
ng/ml
LPS
βG
Rap + Tor

Fig. 11: LPS but not β-glucan induces more cytokine production.

CBA-based analysis of cytokine secretion of control cells (circle) or TSC cells (triangle) after 24 h of priming with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) in the absence or presence of mTOR inhibitors rapamycin and torin (Rap + Tor). For reasons of clarity 2 groups of untreated cells are depicted, as one will be in the following the only LPS stimulated (LPS stim) control (Fig. 12). Most cytokines were significantly increased after LPS priming compared to untreated control cells (*). Other cytokines showed significant reduction of secretion levels when primed in the presence of mTOR inhibitors compared to cells primed in the absence of inhibitors (§). Only IL-1β and RANTES displayed significant differences in cytokine production between control cells and TSC cells as indicated by brackets. Data-sets represent mean values of 13 – 20 control and 22 – 25 patient samples. Error bars are standard error of the mean (SEM). Mann-Whitney test (group comparison) and two-way ANOVA (pair-wise comparison) were used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Moreover, it could be demonstrated that monocytes from TSC patients, with hyperactive mTOR, produce more pro-inflammatory cytokines with respect to TNFα, IL-1β and also RANTES after priming with LPS independent of treatment with mTOR inhibitors. Secretion of IL-1β and RANTES were even significantly increased after priming with LPS alone in comparison to healthy control cells. An increase in TNFα production by TSC cells was also observed using ELISA measurements (data not shown).

4.1.4 Induction of endotoxin tolerance is not dependent on mTOR activity

Endotoxin tolerance is described as the lack of pro-inflammatory response to a second infection or appearance of LPS or other bacterial components. On the other hand, also β-glucan was reported to provoke an immunological memory-like response in cells of the innate immune system by training or priming the cells but resulting in an increased pro-inflammatory response upon re-exposure to LPS.

Stimulation of cells with of 10 ng/ml LPS alone (LPS stim) as a control, again displayed significantly increased levels of all addressed cytokines. Re-stimulation of LPS primed cells (LPS prime) revealed that monocytes developed a tolerant state as evidenced by the significantly decreased secretion of pro-inflammatory cytokines like TNFα, MIP-1β and RANTES, compared to control cells stimulated only once with LPS (LPS stim) (Fig. 12). Previous ELISA measurements additionally revealed induction of LPS tolerance with respect to TNFα levels (Fig. 9). The induction of tolerance on cytokine level was not altered by mTOR inhibitors.

Further the anti-inflammatory IL-10 displayed slightly decreased secretion levels in primed cells, while IL-1β was even significantly increased expressed by LPS primed cells compared to only LPS stimulated cells. β-glucan priming (βG prime) exclusively increased the secretion of TNFα to a significant level in control cells, which was

abolished by pre-treatment with mTOR inhibitors. Besides that, IL-10 and MCP-1 again showed significantly decreased secretion levels with mTOR inhibitor treatment and thus, a clear dependency on mTOR activity.

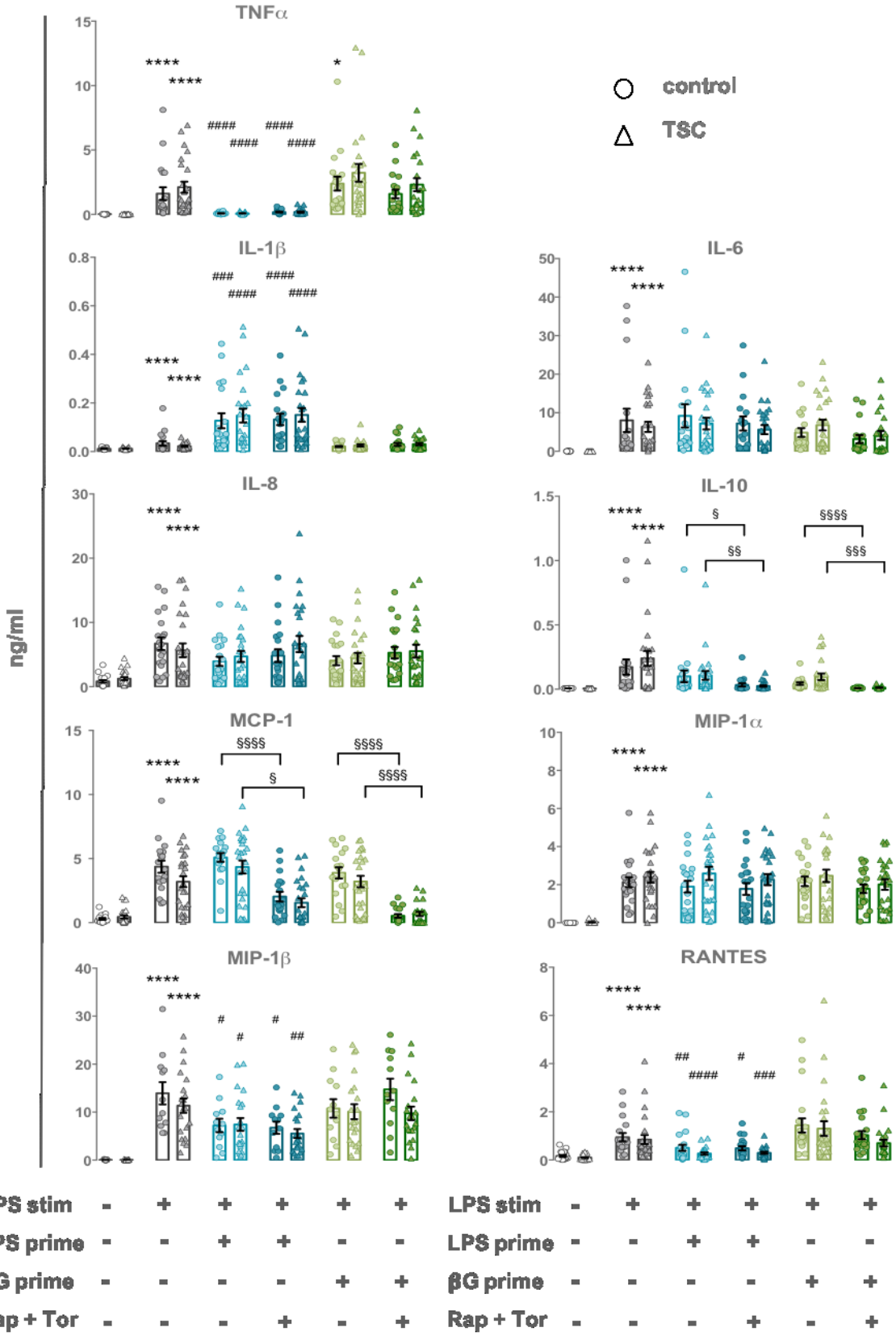


Fig. 12: LPS and β-glucan priming induce opposing adaptation processes in control and TSC cells.

CBA-based cytokine secretion of control cells (circle) or TSC cells (triangle) after 24 h of re-stimulation with 10 ng/ml LPS (LPS stim). Most cytokines were significantly increased after LPS stimulation compared to untreated control cells (*). Significant differences in cytokine secretion through LPS or β-glucan priming compared to only LPS stimulated cells are indicated (#), as well as significant alterations of secretion levels when primed in the presence of mTOR inhibitors compared to cells primed in the absence of inhibitors (§). For reasons of clarity only relevant significances are depicted. Data-sets represent mean values of 13 – 20 control and 22 – 25 patient samples. Error bars are standard error of the mean (SEM). Mann-Whitney test (group comparison) and two-way ANOVA (pair-wise comparison) were used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Other cytokines like IL-6, MIP-1α and IL-8 were not affected by priming of the cells. Furthermore, decreased IL-8 production after LPS priming could not be observed in the only LPS stimulated cells (LPS stim) compared to untreated controls. Also opposing IL-8 levels in the supernatants of LPS or β-glucan primed cells after the first 24 h period, seemed not to be crucial for IL-8 response to re-stimulation with LPS.

Additionally, monocytes from TSC patients did not exhibit different cytokine secretion profiles than control cells from healthy individuals. Upon re-stimulation with LPS, TNFα seemed to be the only cytokine which was still produced slightly more in TSC cells, like already observed upon priming with LPS. However, the induction of endotoxin tolerance by LPS at the level of TNFα and other cytokines was not affected, neither by different cytokine secretion levels during priming, nor by increased mTOR activity in TSC cells or mTOR inhibition by rapamycin and torin.

4.1.5 mTORC1 does not affect inflammatory priming

To further investigate the role of mTOR signaling in the different adaptation processes of endotoxin tolerance and β-glucan training, downstream targets of mTORC1 were determined. As blood amounts and thus, cell yield of TSC patients and matched controls were limited, signaling analysis via Western blotting was not possible. Therefore, one main downstream target of mTORC1, the S6 protein (S6P) was studied by intracellular staining for flow cytometry. Activated mTORC1 phosphorylates and activates the S6 protein kinase (S6K), which subsequently phosphorylates S6P. Phosphorylated S6P (pS6P) levels were measured by geometric mean fluorescence index (MFI) in CD14 positive monocytes. The effect of unspecific antibody binding due to the Fc region was verified using FcR blocking agents and was determined to have no effect on CD14 staining.

It was observed that single stimulation with LPS (LPS stim) lead to a significant increase of pS6P compared to untreated control cells (Fig. 13 B). Priming with LPS or β-glucan

further increased the amount of pS6P in control cells, though only significantly in control cells primed with β-glucan (βG prime). In TSC monocytes activation of S6P was almost constant compared to cells stimulated with only LPS. However, in both priming conditions pre-treatment with mTOR inhibitors rapamycin and torin resulted in a predominantly significant decrease of pS6P. Nevertheless, there were no significant differences between cells from TSC patients and healthy controls.

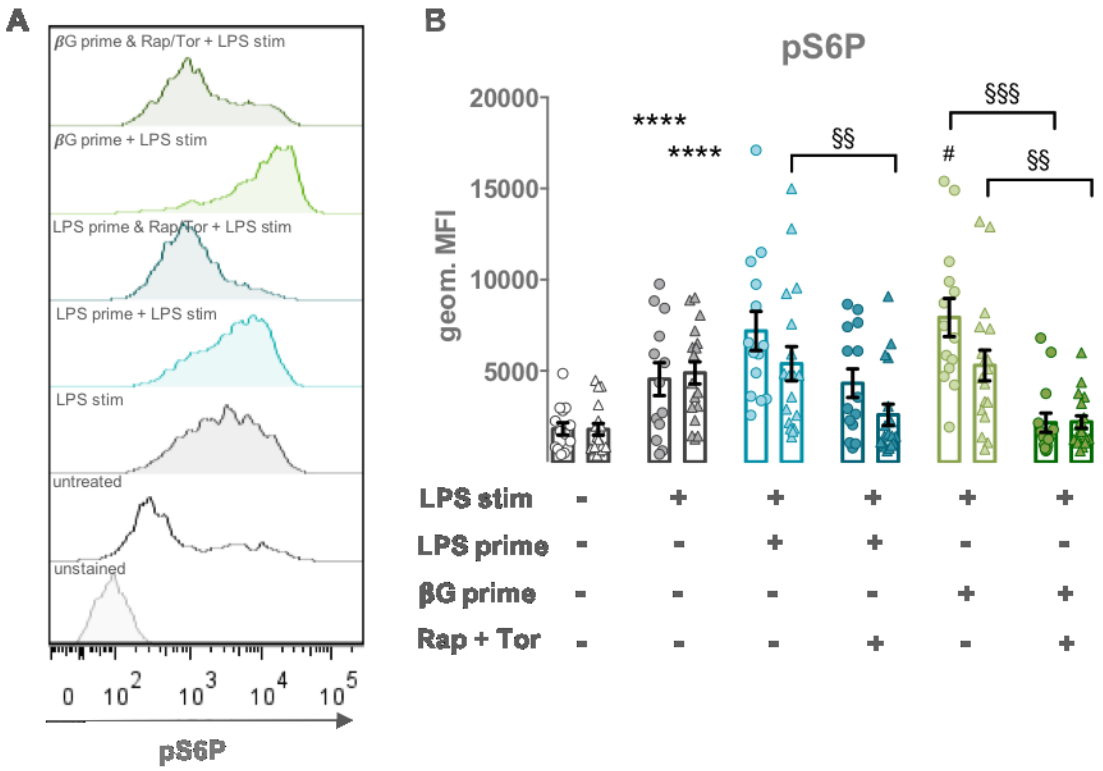


Fig. 13: Intracellular flow cytometric analysis of phosphorylated S6 protein reveals mTORC1 activation by LPS.

Monocytes from healthy controls (circle) or TSC patients (triangle) were primed with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) for 24 h and subsequently re-stimulated with 10 ng/ml LPS (LPS stim). mTOR inhibitors were added 30 min before and throughout 24 h of priming wherever indicated (Rap + Tor). Phosphorylation of S6 protein (pS6P) was analyzed by intracellular staining after 24 h of re-stimulation with LPS via geometric mean fluorescence index (MFI) in CD14 positive gated monocytes. Results are shown as (**A**) representative histogram of pS6P intensity and (**B**) quantitative analysis of pS6P. Activation of S6P was significantly increased after LPS stimulation alone as well as after both priming conditions in comparison to untreated cells (*). mTOR inhibition lead to a significant reduction in pS6P compared to cells primed without inhibitors wherever indicated by brackets (§). For reasons of clarity only relevant significances are depicted. Data-sets represent mean values of 14 control and 19 patient samples. Error bars are standard error of the mean (SEM). Mann-Whitney test (group comparison) and two-way ANOVA (pair-wise comparison) were used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

4.1.6 The metabolic state of primary human monocytes is not affected by inflammatory priming

Increased lactate production after inflammatory stimulation of primary monocytes

It is thought that priming with β-glucan is depending on a metabolic reprogramming from oxidative phosphorylation to increased glycolysis which would result in increased glucose consumption rates and lactate production. mTOR, as important cellular regulator of metabolic pathways, was reported to play an important role in the metabolic reprogramming of β-glucan primed monocytes. To investigate whether LPS or β-glucan primed cells undergo a change in their metabolic state, production of lactate was measured in the same cell culture supernatants used for cytokine analysis. This was done by the Institute for Clinical Chemistry of the University Hospital Jena.

In line with former results concerning cytokine release (Fig. 10), LPS priming lead to significantly increased lactate production with even more increase in cells from TSC patients compared to untreated control cells (Fig. 14, upper left panel). In comparison to LPS priming alone, pre-treatment with mTOR inhibitors rapamycin and torin displayed a decline of these effects. Both proofing that mTOR activation is involved in these metabolic changes. β-glucan treatment caused almost no differences In lactate concentration neither in the presence nor absence of mTOR inhibitors.

A significant increase in lactate production was also detected when cells were stimulated with LPS alone (LPS stim) at the time of re-stimulation (Fig. 14, upper right panel). Pre-treatment with LPS and β-glucan lead to an additional increase in lactate concentrations, however, these were revoked by rapamycin and torin pre-treatment. Taken together lactate levels in cell culture supernatants increased when mTOR is active as seen by intracellular flow cytometry of phosphorylated S6 protein (Fig. 13) as well as in cells from TSC patients, with hyperactive mTOR.

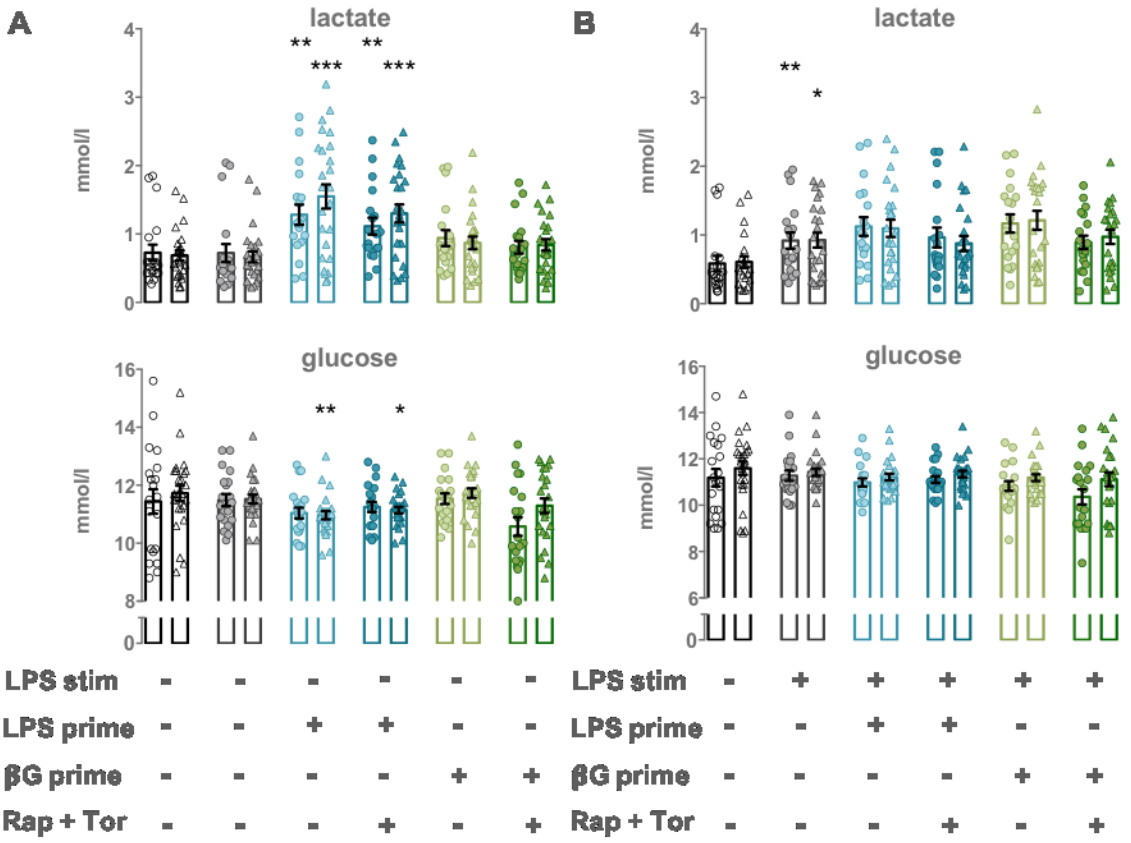


Fig. 14: Inflammatory priming does not alter the metabolic state of primary monocytes.

Analysis of lactate and glucose concentrations in cell culture supernatants of control (circle) or TSC (triangle) cells after (**A**) 24h of priming with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) in the absence or presence of mTOR inhibitors (Rap + Tor) or (**B**) 24 h of subsequent re-stimulation with 10 ng/ml LPS. LPS significantly increased lactate production compared to control cells (*). Data-sets represent mean values of 20 control and 25 patient samples. Error bars are standard error of the mean (SEM). Mann-Whitney test (group comparison) and two-way ANOVA (pair-wise comparison) were used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Increased glucose consumption of primary monocytes after inflammatory stimulation

As lactate is one main product of glycolysis, lactate production correlates with an increase in glucose consumption and therefore, decreased levels of glucose concentration in the cell culture supernatants should be detectable.

Upon priming of cells with LPS, glucose levels decreased, with more effect on TSC cells than healthy controls (Fig. 14, lower left panel). Pre-treatment with mTOR inhibitors, also demonstrated decreased lactate production, despite only reaching statistical significance in TSC cells. Additionally, β-glucan priming did not increase glucose consumption. As already observed for lactate production, glucose concentrations showed no alterations to the control and no differences between TSC cells and control cells. In contrast to β-glucan priming alone, additional pre-treatment with rapamycin and torin displayed increased glucose consumption with higher effects in control cells than in TSC cells.

Upon re-stimulation with LPS only (LPS stim), a decrease of glucose concentration and therefore an increase in glucose consumption could be observed (Fig. 14, lower right panel). This was further increased by cells primed with LPS or β-glucan. Pre-treatment with mTOR inhibitors slightly decreased glucose consumption upon LPS priming but did not have an impact on glucose consumption in β-glucan primed cells. Moreover, there were no significant differences between cells from TSC patients and healthy controls.

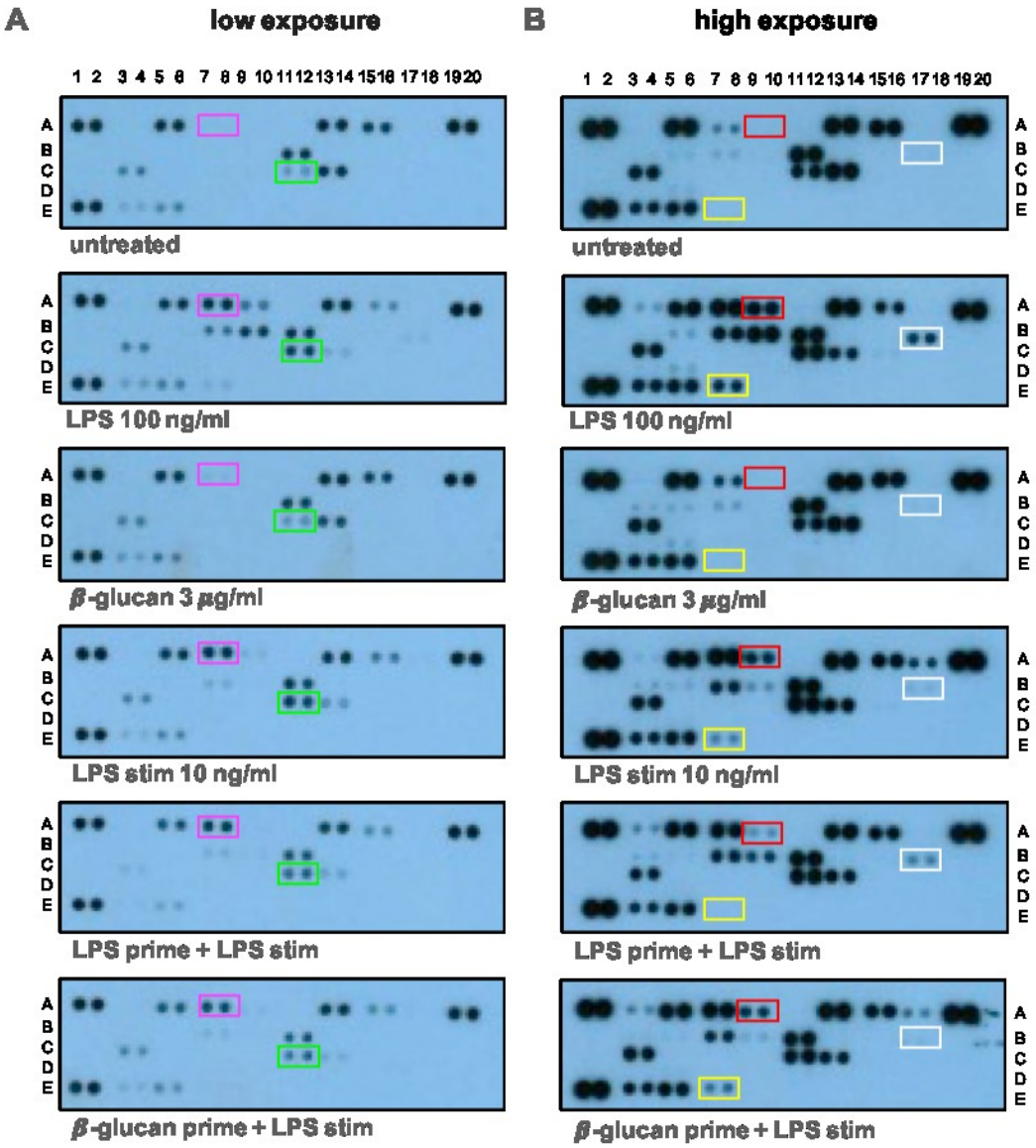
4.2 Buffy coats from healthy voluntary blood donors

TSC patients are mostly diagnosed in early childhood and can have a shortened expectancy of life depending on the degree of severity of the disease and comorbidities. In most cases patients reaching adulthood display a milder form of the disease and do not rely on constant medical check-ups. Therefore, this study was a cooperation with the Children´s Hospital of the University Hospital Jena, as most TSC patients are attended in the Children´s Hospital. For that reason, 84 % of enrolled patients were under the age of 18. Since the amount of blood drawn from patients and controls was in relation to the age of the individuals, yield of cells was limited. Hence, to complement the results obtained from TSC patients and matched controls, buffy coats from healthy voluntary blood donors, purchased from the Institute for Transfusion Medicine of the University Hospital Jena, were used to perform additional assays which required more cells than achieved from TSC patients.

4.2.1 LPS and β-glucan trigger distinct cytokine profiles

As performed for cells derived from TSC patients and matched controls, monocytes from healthy voluntary blood donors were primed with LPS and β-glucan, as described above, and then subsequently re-stimulated with LPS to verify their inflammatory state. Therefore, results from the proteome profiler array obtained during priming (Fig. 10) were complemented with the cytokine profile from re-stimulated cells. Thereby, comparison of cytokine profiles of cells stimulated with 100 ng/ml during priming and 10 ng/ml as LPS re-stimulation alone revealed that the induction of CCL1, IL-1β, G-CSF, GM-CSF, TNFα and RANTES were dose-dependent (Fig. 15, LPS 10 ng/ml vs. LPS 100 ng/ml). Re-stimulation of LPS primed cells further proofed, that tolerant cells do not completely block entire pro-inflammatory response but reduce distinct cytokines (Fig 15). According to previous cytokine measurements with TSC and control cells, secretion of TNFα and additionally CXCL10 were completely abolished, along with reduced levels of RANTES and MIP-1α/β. On the other hand, cells primed with β-glucan featured almost

the same cytokine profile as cells only re-stimulated with LPS. An increase in pro-inflammatory cytokines such as TNFα or IL-6 could not be observed.



C

position	cytokine	position	cytokine
A1, A2	positive control	B9, B10	GM-CSF
A3, A4	CCL1/I-309	B11, B12	ICAM-1
A5, A6	MCP-1	B17, B18	IL-1β ○
A7, A8	MIP-1α/β ●	C3, C4	IL-1rα
A9, A10	RANTES ●	C11, C12	IL-6 ●
A13, A14	C5α	C13, C14	IL-8
A15, A16	CXCL1/GROα	D3, D4	IL-13
A17, A18	CXCL10/IP-10	E1, E2	positive control
A19, A20	positive control	E3, E4	MIF
B3, B4	CXCL11/I-TAC	E5, E6	Serpin E1
B5, B6	CXCL12/SDF1	E7, E8	TNFα ●
B7, B8	G-CSF	E19, E20	negative control

Fig. 15: LPS and β-glucan stimulation display distinct cytokine profiles during priming and LPS re-stimulation.

Primary human monocytes were primed with either 100 ng/ml LPS or 3 µg/ml β-glucan or cell culture media for 24 h (transferred from Fig. 10) and were subsequently re-stimulated with 10 ng/ml LPS for additional 24 h. Supernatants were collected and used for a cytokine proteome profiling. Representative X-ray films of (**A**) short-term exposure (2 min) and (**B**) long-term exposure (30 min) are depicted. (**C**) Cytokines produced by monocytes stimulated with media (untreated), LPS or β-glucan. For reasons of clarity, some important cytokines are color-coded. LPS (LPS 100 ng/ml) and β-glucan priming (β-glucan 3 µg/m) displayed distinct cytokine profiles. Stimulation with LPS induced secretion of a variety of 9 different inflammatory cytokines, while β-glucan stimulation was similar to untreated control. Re-stimulation with 10 ng/ml LPS of untreated (LPS stim 10 ng/ml), LPS primed (LPS prime + LPS stim) or β-glucan primed (β-glucan prime + LPS stim) cells exhibited again distinct cytokine profiles. Cells primed with β-glucan did not display a different profile compared to only LPS stimulated cells, whereas LPS primed cells, diversely expressed a range of cytokines. While IL-1β and GM-CSF were increased, TNFα, MIP-1α/β, CXCL10 and RANTES were either reduced or completely blocked in tolerant cells.

4.2.2 mTORC1 signaling pathway is not altered by LPS priming

To analyze the impact of LPS or β-glucan priming on mTORC1 signaling, important downstream targets, particularly S6K and S6P, were analyzed using immunoblotting.

Stimulation of monocytes with LPS lead to a slight increase in S6K phosphorylation (pS6K) at the lower 70 kDa band within 30 and 60 min (LPS stim), which was lost after 24 h of stimulation (LPS prime) (Fig. 16 A). Re-stimulation with LPS after LPS priming caused similar activation of S6K, which was reduced when cells were primed in the presence of rapamycin and torin. Short time stimulation or priming with β-glucan likewise increased pS6K levels but β-glucan priming followed by LPS re-stimulation increased pS6K the most.

Phosphorylation of S6P displayed a similar pattern (Fig. 16 B), which is reasonable since S6P is the direct target of S6K. Short term LPS stimulation increased S6P phosphorylation, though is lost after 24 h. Priming with LPS did not affect S6P activation but treatment with mTOR inhibitors reduced S6P phosphorylation induced by LPS stimulation even after 24 h. Further, pS6P was slightly increased after short term β-glucan stimulation which decreased upon 24 h of priming. However, priming with β-glucan did not alter S6P phosphorylation by LPS re-stimulation, but decreased by combinatorial treatment with rapamycin and torin as well.

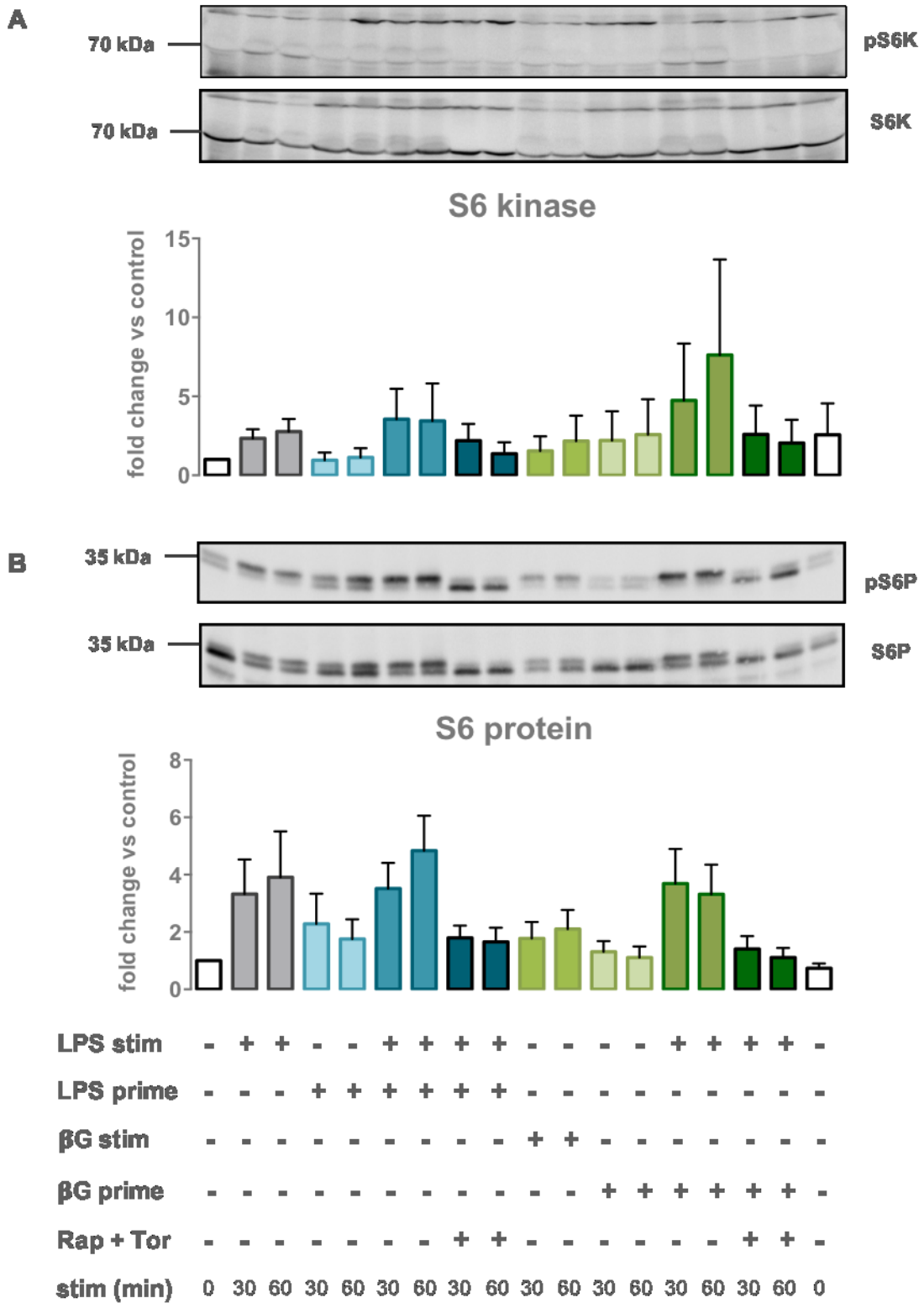


Fig. 16: Inflammatory priming does not affect mTORC1 signaling upon LPS re-stimulation.

Monocytes from healthy voluntary blood donors were primed with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) in the absence or presence of mTOR inhibitors (Rap + Tor) for 24 h or left untreated and were either re-stimulated with 10 ng/ml LPS (LPS stim) or stimulated with LPS or β-glucan alone (βG stim) for indicated times. Representative western blot of (**A**) phosphorylated (pS6K) an un-phosphorylated S6 kinase (S6K) (n=3) or (**B**) phosphorylated (pS6P) and un-phosphorylated S6 protein (S6P) (n=6). Graphs represent densitometric data and fold change versus un-phosphorylated controls. For S6K the lower band at 70 kDa was analyzed, while the upper band of S6P was used for quantification. Inhibition with mTOR inhibitors revealed

specificity of the signal. mTOR activity induced by LPS stimulation was not significantly changed by inflammatory priming with LPS or β-glucan. Data-sets represent mean values and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis.

4.2.3 Intracellular cytokine production of TNFα displays distinct cellular adaptation processes

Results from TSC patients and controls, as well as previous cytokine overview experiments, revealed that primary human monocytes developed different adaptation processes when primed with different immunological stressors. Priming with LPS induced a tolerant state characterized by the loss of pro-inflammatory cytokine release like TNFα, while β-glucan priming further increased TNFα levels in the supernatants. To exclude that differences in secreted TNFα levels are a consequence of alterations in the secretion process or related factors instead of altered cytokine production, intracellular TNFα levels were analyzed. Therefore, cells either primed or not with LPS or β-glucan for 24 h were subsequently treated with Brefeldin A, a secretion inhibitor that blocks transport of the cytokine to cellular membrane and its subsequent release, for 30 min before and during followed stimulation with LPS or β-glucan for 6 h to cover the time frame of maximum TNFα production.

As already seen for cytokine release to the supernatant, stimulation of cells with the priming dose of LPS resulted in a significant increase in intracellular TNFα production (LPS prime) (Fig. 17 B). When cells were primed with LPS and subsequently re-stimulated with LPS in the presence of Brefeldin A (LPS prime + LPS stim), there was no intracellular cytokine production compared to untreated control cells and a significant difference to only LPS primed cells. Thus, it can be conducted, that primed tolerant monocytes did not produce TNFα when challenged with a second appearance of LPS.

β-glucan priming resulted in slightly increased intracellular cytokine production compared to untreated control cells, despite not reaching statistical significance. If β-glucan primed cells were re-stimulated with LPS there was an enhanced increase in intracellular TNFα production compared to LPS priming alone and a significant increase in comparison to untreated control cells. Hence, both distinct adaptation processes could also be observed by analyzing intracellular cytokine production rather than just cytokine release to cell culture supernatant.

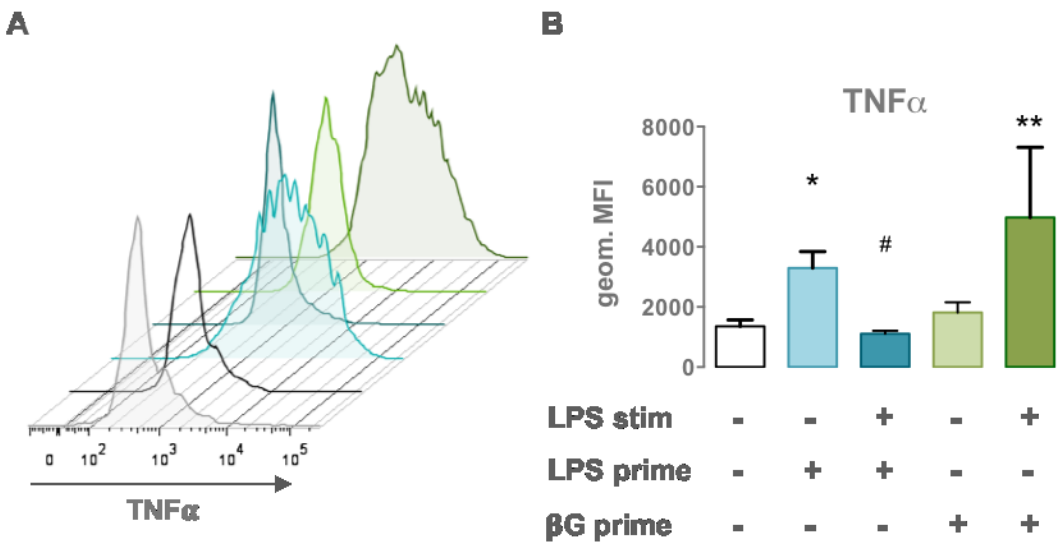


Fig. 17: LPS and β-glucan trigger opposing effects on intracellular TNFα levels.

For intracellular flow cytometry, cells were primed with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) for 24 h and were re-stimulated with 10 ng/ml LPS (LPS stim) in the presence of the secretion inhibitor Brefeldin A for 6 h to block TNFα release to cell culture supernatant. Cytokine production was analyzed by intracellular staining for flow cytometry. Cells were gated for CD14 and geometric mean fluorescence index (MFI) of TNFα was measured. (**A**) Representative histogram of intracellular TNFα staining. (**B**) Quantitative analysis of intracellular TNFα staining. LPS stimulation induced significant increased TNFα production compared to control cells (*) but was significantly blocked in comparison to LPS primed cells (#), whereas β-glucan did not trigger TNFα production upon priming alone but increased it after re-stimulation. Data-set represents mean values of 4 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$

4.2.4 TLR4 is internalized after LPS and β-glucan stimulation

In mammalian cells, LPS is recognized and bound to the cells by the Toll-like receptor 4 (TLR4). Further it was shown that binding of LPS to TLR4 leads to an induction of internalization of the receptor-complex to activate distinct signaling cascades as well as a decrease of receptor surface density. To investigate, whether a loss of surface receptors after increased internalization caused by priming of the cells is responsible for the decreased response during a second stimulation with LPS, TLR4 internalization was measured during LPS priming for up to 24 h. Additionally receptor internalization after β-glucan priming was monitored, as it was reported that β-glucan priming causes an increase in receptor surface density to increase the cellular response upon re-stimulation.

Priming of the cells with LPS did lead to an increase of receptor internalization starting at 1 h after stimulation (Fig. 18). Further, there was a continuous decrease of receptor surface density to a significant degree from 3 to 24 h of stimulation compared 0 min of stimulation. At this point, more than 50% of receptors were internalized. β-glucan priming did not increase surface abundance of TLR4 but rather decreased it.

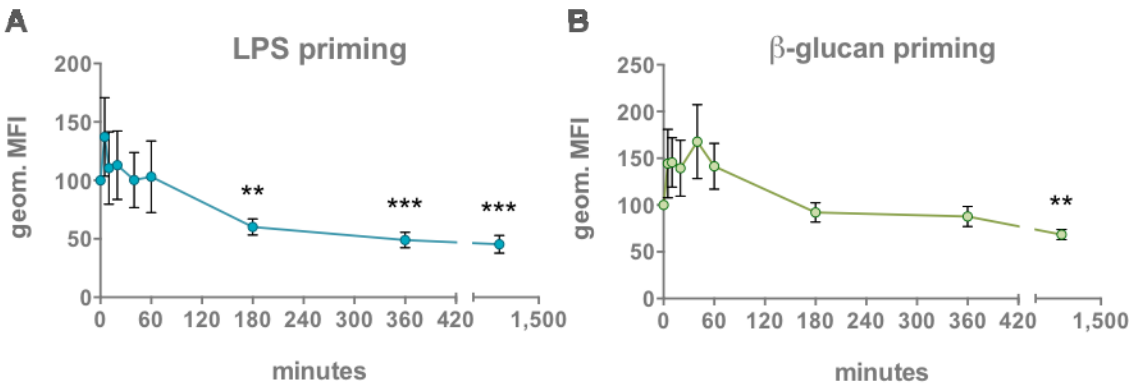


Fig. 18: Inflammatory priming triggers TLR4 internalization within 24 h.

Flow cytometric analysis of TLR4 surface staining after stimulation with (**A**) LPS or (**B**) β-glucan for indicated times. TLR4 cell surface abundance was significantly decreased during treatment with LPS compared to 0 min of stimulation (*). Data-set represents mean values of geometric mean fluorescence index (MFI) of 4 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4.2.5 Endotoxin tolerance is not mediated by ERK, PI3K or p38 signaling

Hyperactive mTOR in TSC cells and mTOR inhibition by rapamycin and torin did not affect tolerance induction and priming with LPS or β-glucan did not alter mTOR activity. Thus, other important inflammatory pathways, which were reported to be involved in immune cell signaling and related to mTOR, such as PI3K and the MAPK ERK and p38, were analyzed.

To investigate the impact of other inflammatory pathways, inhibitors against key molecules were used. Cells were pre-stimulated with rapamycin and torin for mTOR inhibition, SB202190 for inhibition of p38 MAPK (p38) signaling, UO126 for ERK signaling inhibition or LY294002 for PI3K inhibition for 1 h before and during 24 h of priming with LPS, followed by re-stimulation with LPS. In line with previous results, mTOR inhibition did not alter cytokine production after LPS priming except from IL-10 and MCP-1 (Fig. 19). Inhibition of p38 with SB202190 caused a decrease of pro-inflammatory cytokine production of TNFα, IL-1β, IL-6 and RANTES but also of the anti-inflammatory IL-10, reaching significance in TNFα, IL-1β and IL-10. MCP-1, IL-8, MIP-1α and RANTES were not affected. Treatment with the ERK inhibitor UO126 also inhibited the secretion of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and MCP-1, partly to a significant level, while MIP-1α and RANTES were not altered and IL-8 was even increased compared to only LPS primed cells.

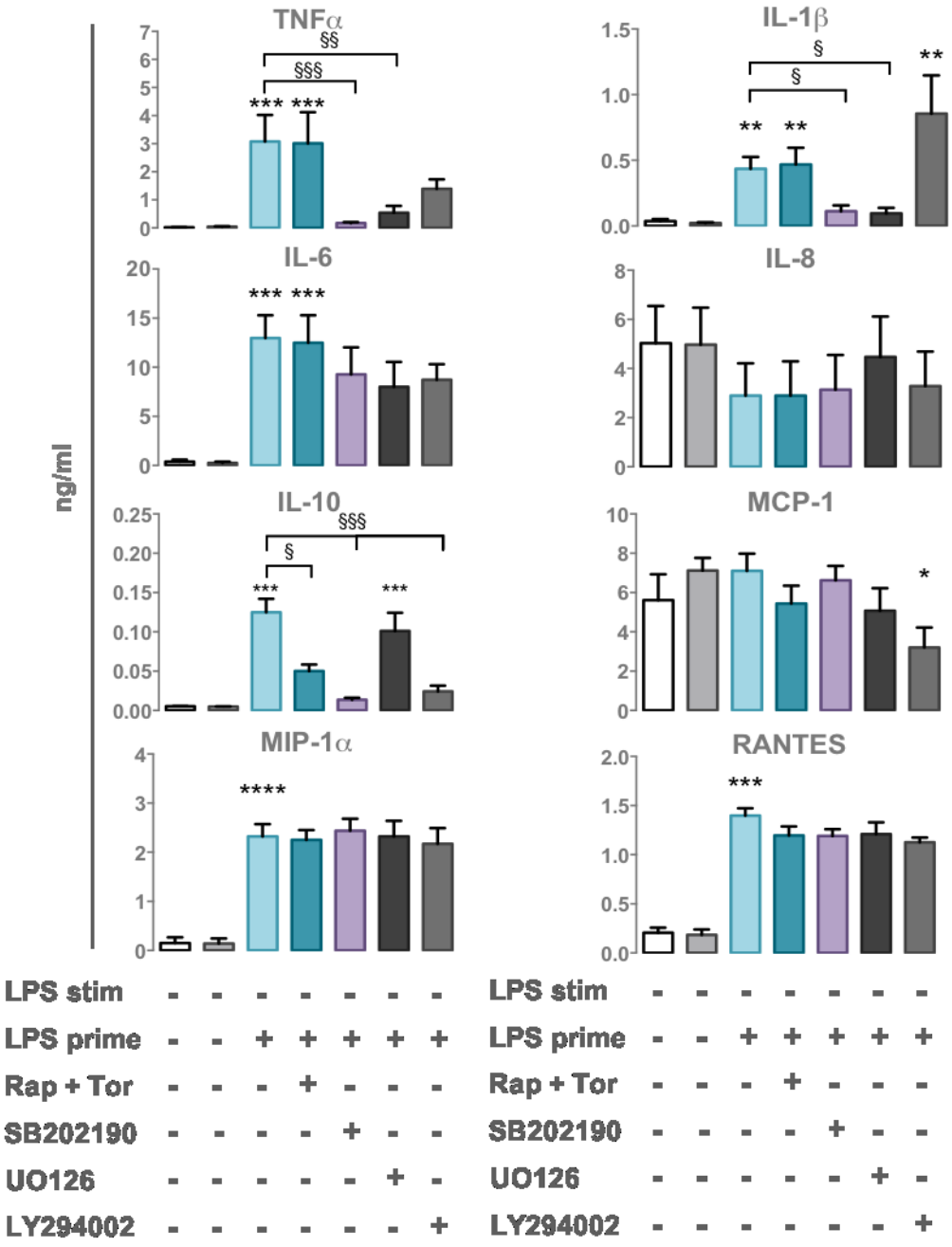


Fig. 19: Cytokine production upon LPS priming is depending on different inflammatory pathways.

Cells were primed with 100 ng/ml LPS (LPS prime) for 24 h in the presence of different inhibitors against key proteins of distinct inflammatory pathways. mTOR inhibitors (Rap + Tor), p38 inhibitor (SB202190), ERK1/2 inhibitor (UO126) and PI3K inhibitor (LY294002) were added 1 h before and during priming with LPS. Cytokine levels were analyzed using CBA. Significant increases compared to untreated control cells (*) or only LPS primed cells (§) are indicated. For reasons of clarity 2 groups of untreated cells are depicted as one will be in the following the only LPS stimulated (LPS stim) control (Fig. 20). Only relevant significances are shown. Data-sets represent mean values of 8 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * p≤0.05, ** p≤0.01, ***p≤0.001, ****p≤0.0001

Further, UO126 was the only signaling inhibitor, which did not inhibit IL-10 secretion. Blocking the activity of PI3K also caused a decrease of TNFα, but was not significantly reduced in comparison to cells stimulated with LPS alone. In addition, IL-6, IL-10, MCP-1 and RANTES levels were decreased with PI3K inhibition, with IL-10 reaching significance. Furthermore, LY294002 increased the pro-inflammatory cytokine IL-1β, while IL-8 and MIP-1α were not affected.

When cells were re-stimulated with LPS, LPS primed cells were rendered tolerant in case of TNFα evidenced by significantly reduced secretion compared to only LPS stimulated cells, irrespectively whether they were treated additionally with mTOR or PI3K inhibitors (Fig. 20). However, inhibition of p38 and ERK signaling during priming also reduced TNFα levels upon re-stimulation, despite not reaching statistical significance. The significant increase of RANTES induced by LPS stimulation was lost in all primed cells, independent of the inhibitor treatment. The p38 inhibitor SB202190 impaired the extent of TNFα tolerance induction the most and increased IL-6 secretion compared to only LPS primed cells. However, all other cytokines measured are unaffected by the inhibition of p38 signaling. TNFα levels were also slightly increased through ERK inhibition with UO126, whereas other pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, MCP-1 and MIP-1α were decreased in comparison to only LPS primed cells. Thereby reduction of IL-6 reached significance, along with a significant suppression of IL-10 compared to LPS stimulated cells. PI3K inhibitor LY294002 increased IL-1β among with IL-8 but also IL-10 in comparison to only LPS priming. Further, MCP-1 was decreased, while IL-6 and MIP-1α were not affected.

Due to these results it can be speculated that every signaling pathway is important for a different assembly of cytokines, but all distinct cytokine profiles of primed cells resulted to a greater or lesser extent in endotoxin tolerance induction by LPS on the level of TNFα. Therefore, as already seen for mTOR, the tested signaling pathways are not responsible for tolerance induction in monocytes.

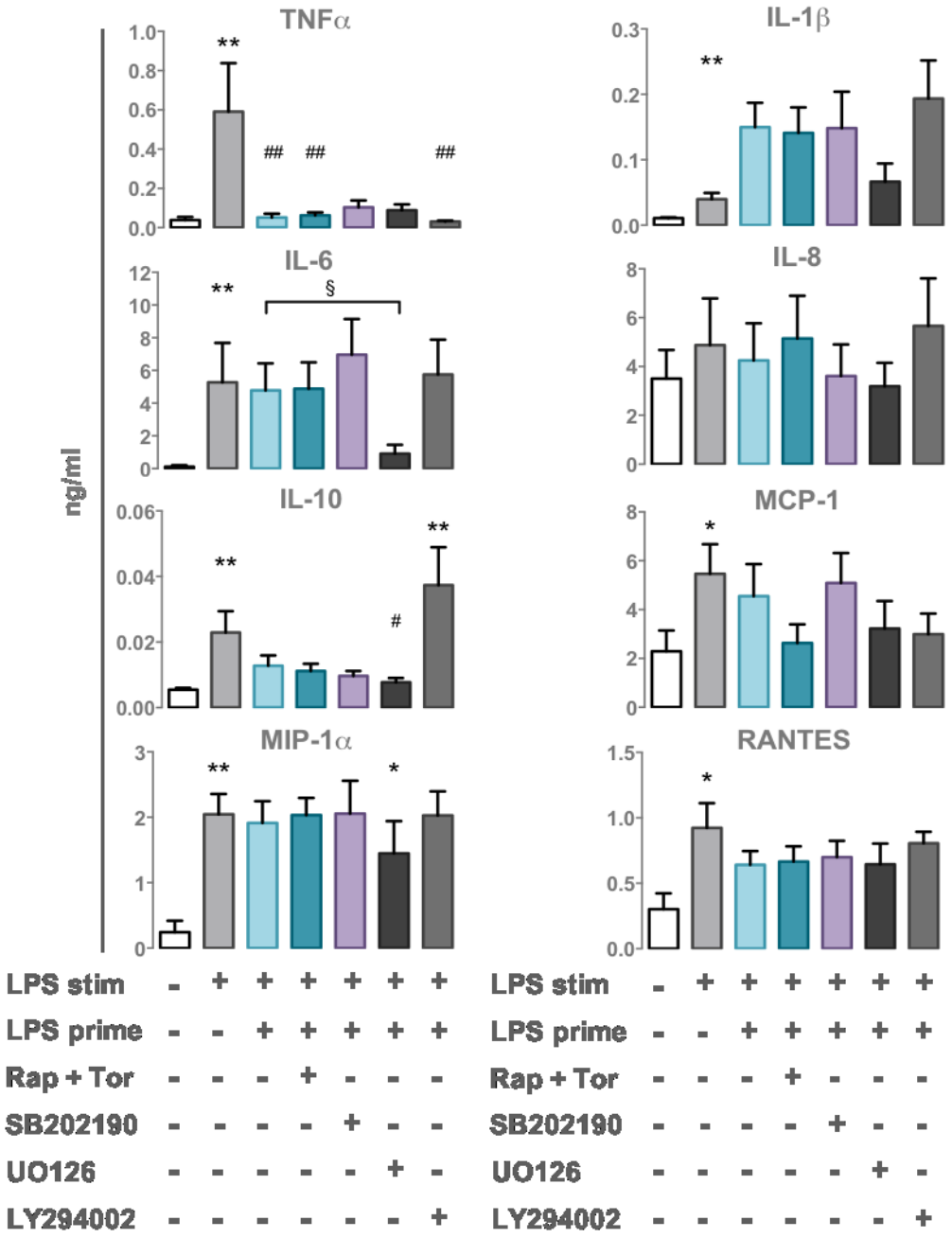
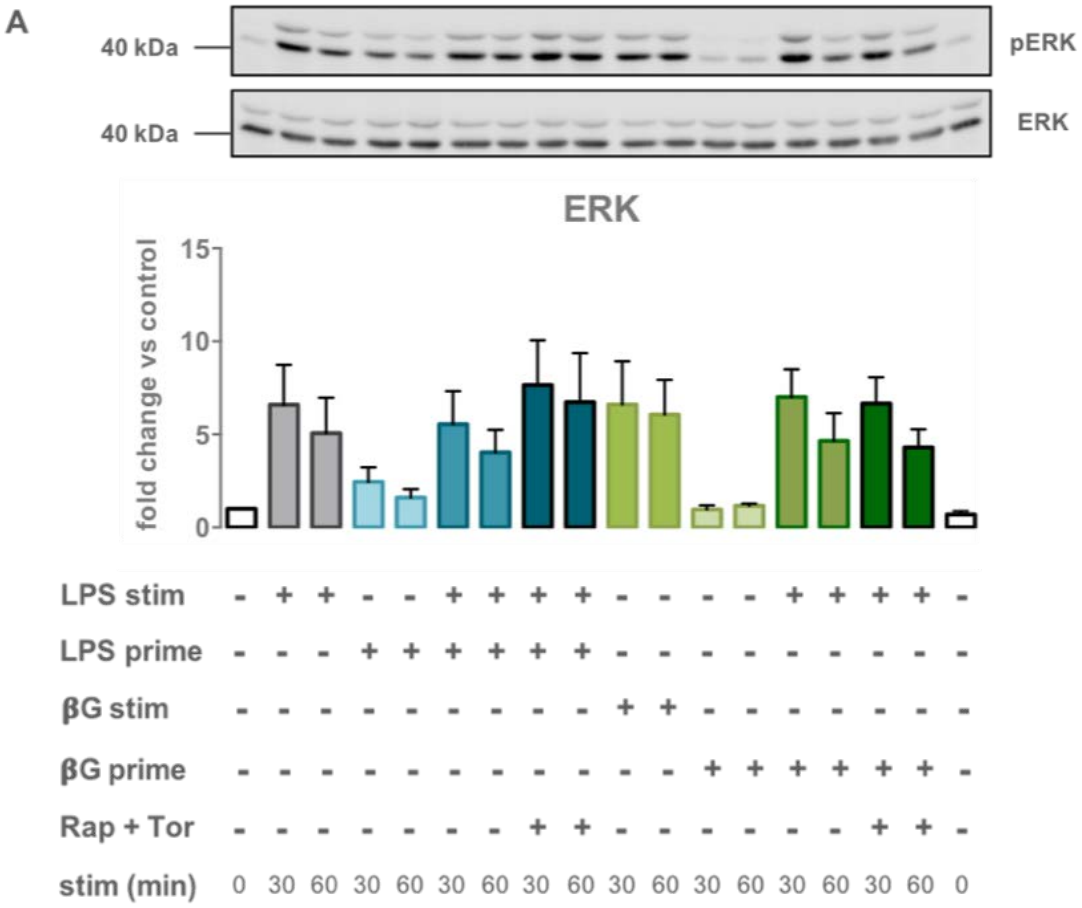


Fig. 20: Induction of endotoxin tolerance is not affected by inhibition of different inflammatory pathways.

Primed cells were re-stimulated with 10 ng/ml LPS for additional 24 h. Cytokine levels in cell culture supernatants were measured using CBA. LPS stimulation alone (LPS stim) triggered cytokine production of all cytokines measured, partly significantly (*). Inhibitors against p38 (SB202190), ERK (UO126) or PI3K (LY294002) were used. Every cytokine displayed distinct signaling pathway dependencies and not all primed cells evolved tolerance with respect to TNFα, evidenced by significantly decreased secretion compared to only LPS stimulated cells (#). Secretion of IL-6 was the only cytokine significantly decreased by UO126 in comparison to only LPS primed cells (§). For reasons of clarity only relevant significances are depicted. Data-sets represent mean values and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

In addition to cytokine profiles, intracellular signaling pathways were investigated using immunoblotting (Fig. 21 A). Similar to S6K phosphorylation levels (Fig. 16), ERK was activated by short-term stimulation with LPS (LPS stim) and β-glucan (βG stim). But priming did not alter ERK activation induced by LPS stimulation and ERK phosphorylation was further unaffected by mTOR inhibitors (Rap + Tor). Phosphorylation of p38 (p-p38) was increased upon short-term LPS stimulation (Fig. 21 B). Priming with LPS resulted in a decrease of p-p38 levels, irrespectively of mTOR inhibition. Thus, a slight induction of tolerance against the subsequent LPS stimulation was demonstrated. Short-term β-glucan stimulation did not activate p-p38, but β-glucan priming followed by LPS re-stimulation further elevated p-p38 compared to LPS stimulation alone. Hence, the p38 signaling pathway mirrored the effects of inflammatory priming seen for TNFα secretion. In addition to p38, short-term stimulation with LPS and β-glucan triggered also the induction of AKT phosphorylation (Fig. 21 C). But while β-glucan priming did not alter AKT activation, priming with LPS resulted in decreased pAKT levels, suggesting that AKT signaling is suppressed in tolerant cells.



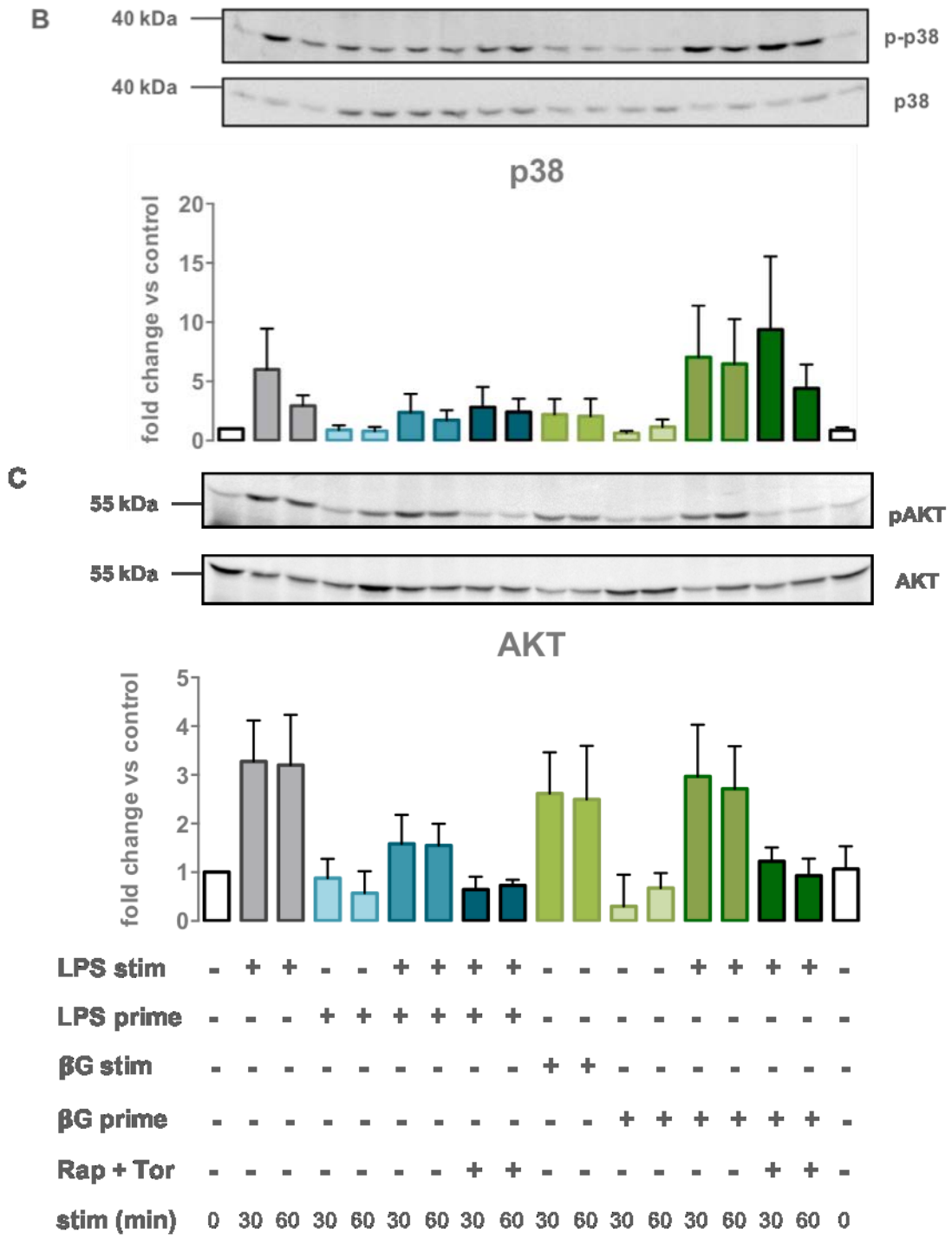


Fig. 21: Inflammatory priming does not affect ERK but inhibits p38 and AKT signaling in LPS tolerant cells.

Monocytes from healthy voluntary blood donors were primed with 100 ng/ml LPS (LPS prime) or 3 µg/ml β-glucan (βG prime) in the absence or presence of mTOR inhibitors (Rap + Tor) for 24 h or left untreated and were either additionally re-stimulated with 10 ng/ml LPS (LPS stim) or stimulated only with LPS or β-glucan (βG stim) for the indicated time. Representative western blot of (**A**) phosphorylated (pERK) and un-phosphorylated ERK (ERK) (n=7) or (**B**) phosphorylated (p-p38) and un-phosphorylated p38 (p38) (n=4) (**C**) phosphorylated AKT (pAKT) and un-phosphorylated AKT (AKT) (n=4) and quantitative analysis. Graphs represent densitometric data and fold change versus un-phosphorylated controls. ERK activity induced by LPS stimulation was not changed by inflammatory priming with LPS or β-glucan or by mTOR inhibitor treatment.

p38 and AKT displayed reduced phosphorylation in LPS primed cells. Data-sets represent mean values and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis.

4.2.6 Inhibition of p38 pathway does not alter LPS-induced TLR4 surface density

The p38 signaling pathway might be involved in the induction of endotoxin tolerance, as it got activated upon LPS stimulation but became tolerant in LPS primed cells. Furthermore, the inhibition of p38 determined almost a complete reduction of TNFα secretion during LPS priming and a decline of tolerance induction compared to LPS priming alone (Fig. 19). Therefore, it was investigated whether inhibition of p38 via SB202190 has an impact on the internalization of TLR4 after LPS priming. After 24 h of priming in the presence or absence of SB202190, internalization of TLR4 was not altered compared to only LPS treated cells (Fig. 22).

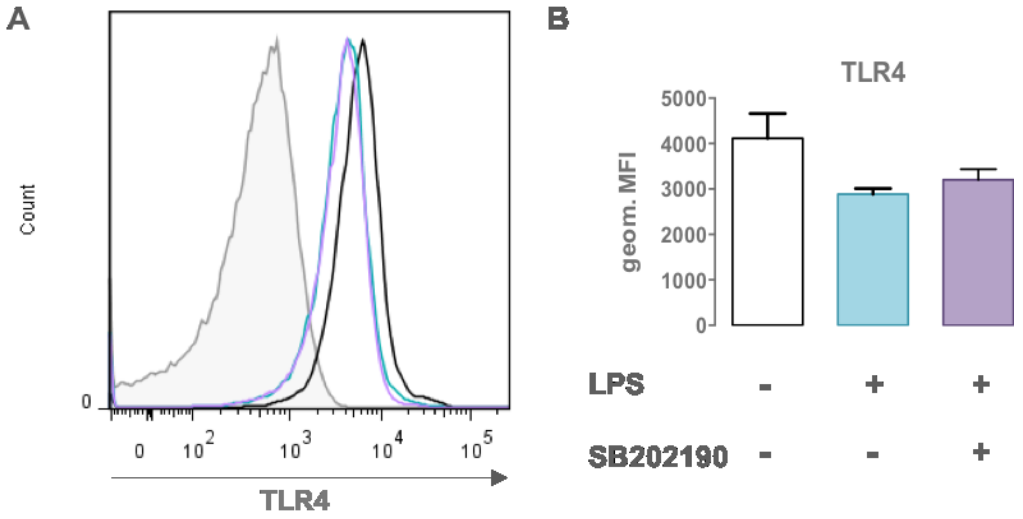


Fig. 22: p38 inhibition does not alter TLR4 internalization.

Monocytes from healthy voluntary blood donors were primed with 100 ng/ml LPS (LPS prime) in the presence or absence of p38 inhibitor SB202190 for 24 h. Cells were stained for TLR4 on cell surface and analyzed via flow cytometry. After pre-gating on CD14 for monocytes, geometric mean fluorescence index (MFI) of TLR4 staining was analyzed. (**A**) Representative histogram of surface TLR4 compared to unstained control (light grey) and (**B**) the quantitative analysis of 3 different experiments. Data-sets represent mean values and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis.

4.2.7 Cytokine production does not trigger endotoxin tolerance

Effects of LPS tolerance were most prominent in case of TNFα, which is established as a key readout. This pro-inflammatory cytokine was further described to induce tolerance itself, inhibiting pro-inflammatory gene expression induced by re-stimulation with TNFα (reviewed in [201]). Therefore, the question arose whether especially pro-inflammatory cytokines, produced during LPS priming, are mediating the tolerant state of monocytes.

To exclude the effect of LPS signaling, cells were primed with conditioned media, comprising secreted cytokines induced by 1 h of LPS stimulation, as described in the method section. This conditioned media comprised similar cytokine amounts as produced by 24 h of LPS stimulation (Fig. 23). Concentration levels of TNFα, IL-6, IL-8, IL-10, MCP-1 and RANTES were comparable to 24 h stimulation. Only IL-1β was significantly increased in conditioned media than in supernatants of cells stimulated with LPS for 24 h, while MIP-1α was significantly decreased. Thus, 1 h LPS treatment triggers similar cytokine secretion as 24 h of stimulation.

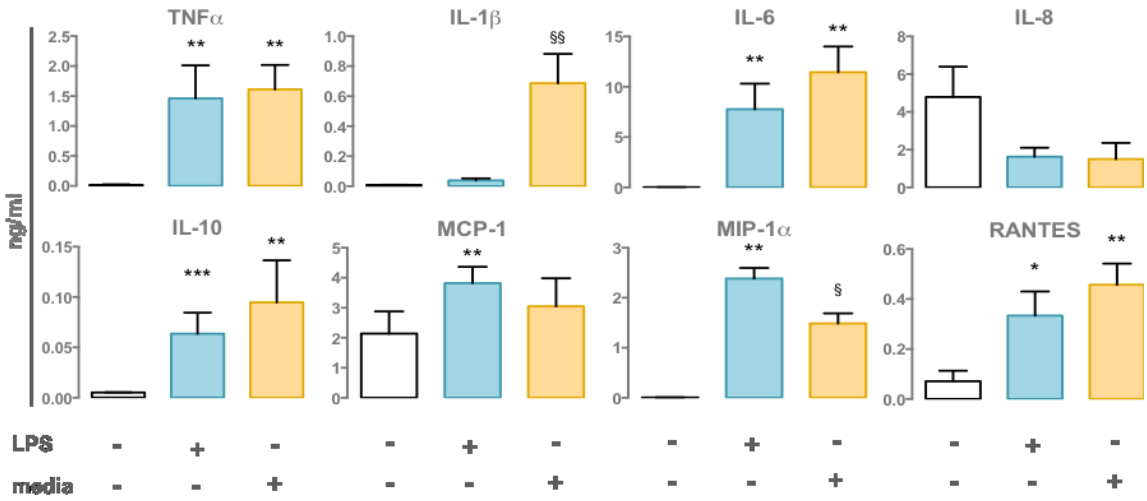


Fig. 23: 1 h LPS stimulation triggers a similar cytokine profile as 24 h of stimulation.
Conditioned media was produced by cells stimulated with 100 ng/ml LPS for 1 h followed by a subsequent media change and further 23 h of incubation without LPS. Conditioned media (media), comprising the secreted cytokines of 23 h of incubation, displayed a similar cytokine profile as produced during 24 h of LPS stimulation (LPS). Both LPS incubation periods significantly increased most cytokines compared to untreated control cells (*). Solely IL-1β and MIP-1α were significantly altered in conditioned media compared to 24 h of LPS stimulation (§). Data-sets represent mean values of 6 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Monocytes primed with conditioned media, comprising different inflammatory cytokines (Fig. 23), produced related amounts of cytokines compared to cells primed with LPS (Fig. 24). Concentration levels of TNFα, IL-8, MCP-1, MIP-1α and RANTES were similar to LPS priming, while IL-6 and the anti-inflammatory IL-10 were increased, when cells were primed with conditioned media. Only the secretion of IL-1β was significantly increased by cells primed with conditioned media compared to cells primed with LPS.

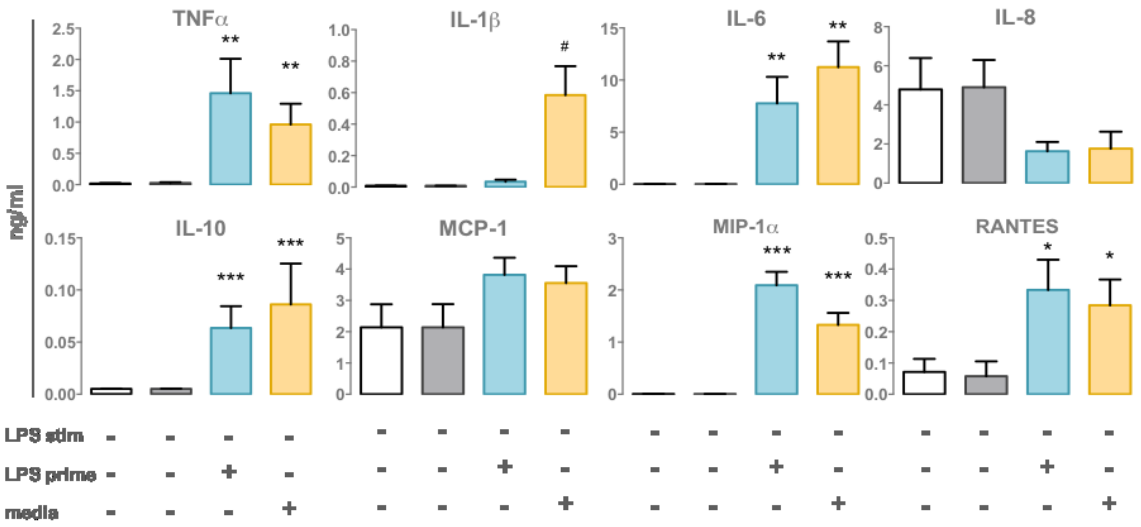


Fig. 24: Priming with conditioned media provokes an altered cytokine profile.

Cells were either primed with conditioned media or LPS for 24 h and secreted cytokine levels were measured using CBA. Both priming conditions lead to, partly significant, increases in cytokine secretion compared to unstimulated control cells (*). Priming with conditioned media resulted in similar cytokine levels compared to LPS priming, only IL-1β was significantly increased (#). For reasons of clarity 2 groups of untreated cells are depicted as one will be in the following the only LPS stimulated (LPS stim) control (Fig. 25). Data-sets represent mean values of 8 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Subsequent re-stimulation of LPS or media primed monocytes was used to investigate the induction of tolerance (Fig. 25). In regard to TNFα, LPS primed cells were rendered tolerant, as evidenced by significant decreased TNFα levels compared to only LPS stimulated cells. Though, TNFα levels of conditioned media primed cells were not diminished. Other cytokines such as IL-6 and MIP-1α were increased in supernatants of cells primed with conditioned media compared to LPS priming, despite not reaching statistical significance. Both priming conditions further significantly increased IL-1β, while IL-10 was significantly decreased. RANTES was also decreased by both priming conditions but did not reach significance. IL-8 and MCP-1 were not altered by priming of the cells. Thus, it seemed that presence of LPS during priming is necessary to induce endotoxin tolerance.

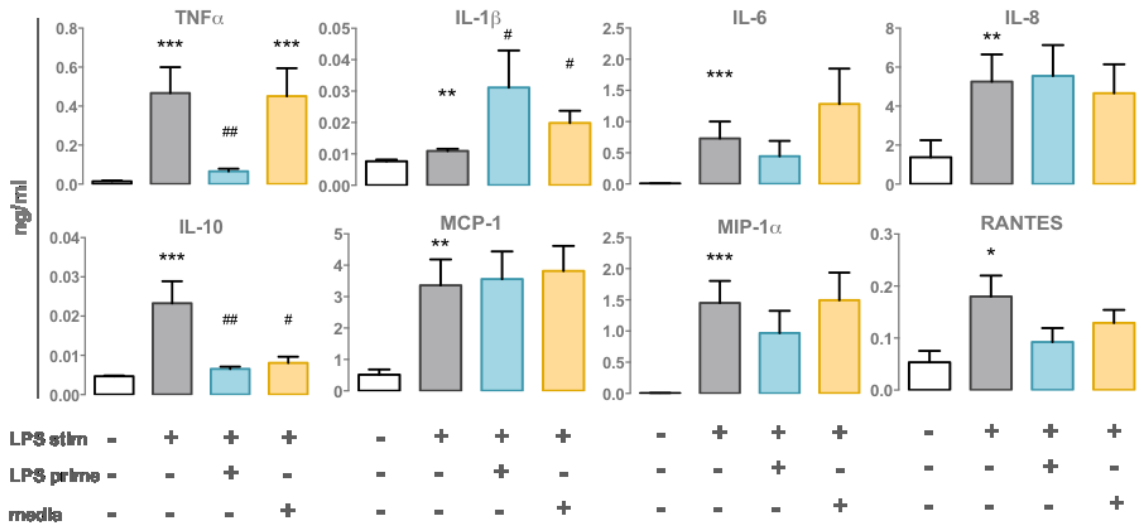


Fig. 25: Pro-inflammatory priming does not induce endotoxin tolerance.

Upon re-stimulation with 10 ng/ml LPS, cells primed with conditioned media displayed an altered cytokine profile by CBA analysis. Stimulation with LPS increased cytokine production significantly compared to unstimulated control cells (*). Priming with LPS triggered endotoxin tolerance as evidenced by significantly decreased TNFα secretion compared to only LPS stimulated cells (#). Conditioned media did not induce endotoxin tolerance in regard to TNFα. Data-sets represent mean values of 8 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4.2.8 IL-8 partly mimics β-glucan priming

Previous experiments with TSC patients and controls as well as Buffy Coats introduced IL-8 as the only cytokine which was increasingly produced by β-glucan training and even decreased through LPS priming. So far, the underlying molecular mechanisms of β-glucan priming or training are still not completely understood. To get a better knowledge, it was investigated whether priming with IL-8 exhibits same effects as β-glucan.

Monocytes primed with β-glucan increased the secretion of all cytokines measured, with a significant increase of TNFα, IL-8, MCP-1 and MIP-1α (Fig. 26). In contrast to β-glucan, cells primed with IL-8 did not secrete more cytokines than untreated control cells.

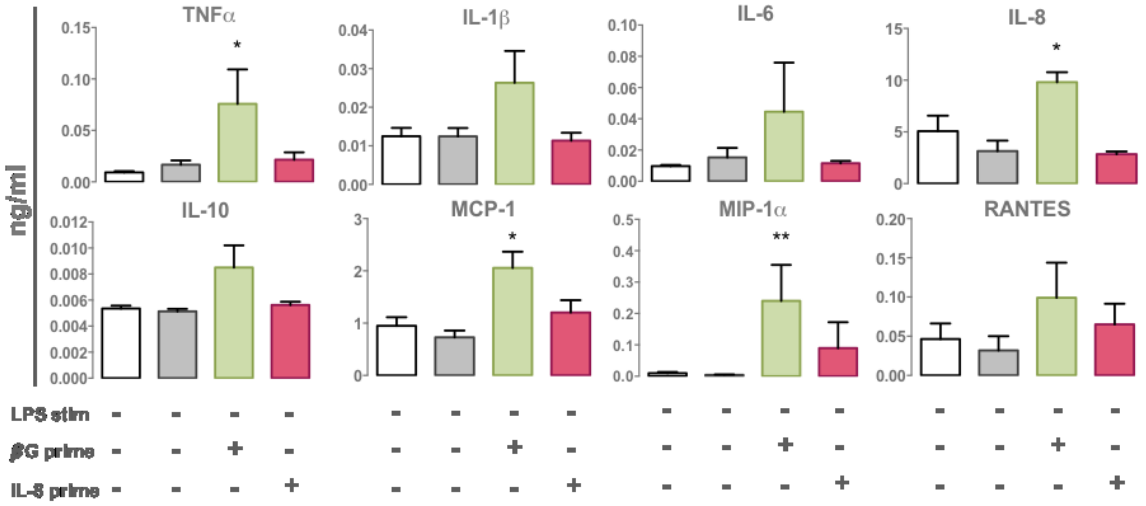


Fig. 26: IL-8 priming does not trigger cytokine production.

Purified monocytes were either left untreated or primed with 3 µg/ml β-glucan or 150 ng/ml IL-8 for 24 h. In contrast to β-glucan primed cells, priming with IL-8 did not provoke significantly increased cytokine production compared to untreated cells (*). For reasons of clarity 2 groups of untreated cells are depicted as one will be in the following the only LPS stimulated (LPS stim) control (Fig. 27). Data-sets represent mean values of 9 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$

Furthermore, when cells were re-stimulated with LPS, IL-8 primed cells displayed similar results then cells primed with β-glucan concerning IL-6, IL-8, IL-10, MCP-1, MIP-1α and RANTES (Fig. 27). Levels of TNFα and IL-1β were slightly reduced compared to β-glucan primed cells. However, both priming conditions did not exhibit significant alterations of pro-inflammatory cytokines in comparison to LPS stimulation alone.

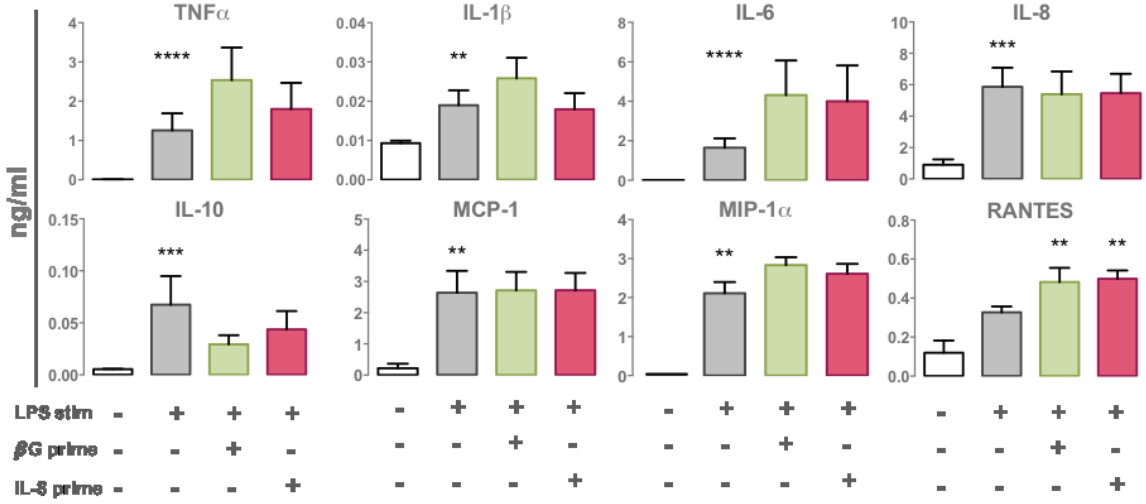


Fig. 27: β-glucan training is partly mimicked by IL-8 priming.

Re-stimulation of β-glucan or IL-8 primed cells with 10 ng/ml LPS for 24 h showed that some cytokines are equally secreted while others are distinct between both priming conditions. Significant increases in cytokine levels compared to an untreated control are indicated (*). For reasons of clarity only relevant significances are depicted. Data-sets represent mean values of 9 independent experiments and error bars are standard error of the mean (SEM). Mann-Whitney test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

5. DISCUSSION

5.1 Adaptation of primary human monocytes is not dependent on mTOR activity

Innate immune cells such as monocytes or macrophages represent the first line immune response by sensing microbial infections or tissue damage and subsequently the production of key inflammatory cytokines, which coordinate inflammatory response as well as adaptive immune cell activation. The adaptation of innate immune cells to pathogens or PAMPs is evolutionary conserved from plants and invertebrates to humans. Thereby cells and tissues can be protected against recurrent infections. However, among innate immune cell adaptation, different opposing processes are described. Endotoxin tolerance was first described in the 1940´s but is still part of extensive research. Tolerance is defined by hyposensitivity of cells or tissues towards a repeated stimulation or infection with the same or different stressor. LPS, the outer-membrane compartment of gram-negative bacteria, represents the prototypic inducer of endotoxin tolerance. A tolerant state is believed to protect cells against an overwhelming immune response and associated detrimental tissue damage, which potentially results in endotoxic or septic shock. However, LPS-induced enhanced inflammatory response as well as sepsis can promote immunological anergy, or immunoparalysis. This immune compromised state is characterized by endotoxin tolerance and predisposes patients for life-threatening secondary infections.

More recent data defined an opposing adaptation process, induced by fungal cell wall compartments [202]. This process was called innate immune memory or trained immunity and was initially described for the tuberculosis vaccine BCG [203]. Further studies demonstrated also a protective effect of pre-stimulation with β-glucan against otherwise lethal injections of LPS or *Staphylococcus aureus* in a murine sepsis model [50]. In contrast to endotoxin tolerance this protective effect is mediated by increased pro-inflammatory response. In the clinics, 50% of ICU (intensive care unit) patients develop a systemic inflammatory response syndrome (SIRS) and 25% of patients develop infectious complications after major surgery [204, 205]. Pre-treatment with the β-glucan PCG (Poly- (1-6)-B-D-glucopyranosyl-(1-3)-B-D-glucopyranose) has been demonstrated to significantly reduce these surgery-associated complications [206].

In the mentioned adaptation processes, cytokines are the major regulators of innate immune response. Thereby TNFα acts as a pro-inflammatory master cytokine. Its rapid induction is fundamental for the required immune response [207]. Further, it activates

important inflammatory signaling cascades such as NFκB and MAP kinase (MAPK) signaling and promotes the production of other pro-inflammatory cytokines like IL-6 [23, 208]. β-glucan trained monocytes were reported to enhance secretion of TNFα and IL-6, whereas endotoxin tolerant cells block the expression of these cytokines [28, 50]. Cytokines are highly regulated proteins, which have to be quickly upregulated. Therefore, they are often controlled at the translational level. The serine/threonine kinase mTORC1 is a major regulator of protein biosynthesis by phosphorylation of its main downstream targets S6 kinase and 4E-BP1. In addition, activation of immune cells as well as migration and phagocytosis largely increase the demand of energy and require a certain metabolic flexibility. Beside its translational regulatory functions, mTORC1 is also a key regulator of cellular metabolism by sensing nutrient abundance to balance anabolic and catabolic processes. In β-glucan trained monocytes, mTORC1 was shown to be important in the regulation of a metabolic re-programming by activation of HIF-1α [40]. This metabolic switch from oxidative phosphorylation towards aerobic glycolysis is called Warburg effect and has been well characterized for cancer cells and activated immune cells [63, 64, 209]. Enhanced glycolysis provides fast energy for immune cells for a rapid immune response and elevated cytokine production.

mTORC1 comprise signals of different pathways, but its main upstream inhibitor is the TSC1/TSC2 tumor suppressor complex. Patients with tuberous sclerosis complex (TSC) are characterized by loss-of-function mutations in either TSC1 or TSC2, which causes hyperactivation of mTORC1 signaling. Thus, cells from TSC patients represent a unique genetic model of hyperactive mTORC1. Most investigations concerning functions of mTOR were performed by chemical or genetic inhibition or by deletion of Raptor and Rictor, essential elements of the distinct complexes, associated with structural changes.

LPS induces pro-inflammatory cytokine production

Stimulation of primary human monocytes from TSC patients and controls revealed that monocytes exhibit opposing adaptation processes determined by the used PAMP (Fig. 12). LPS induced the secretion of almost all cytokines measured by a cytometric bead array of TSC patients and matched controls (Fig. 11). This is consistent with previous reports, that LPS triggers pro-inflammatory cytokine production to initiate immunological response [210]. Among these, TNFα represents the prototypical pro-inflammatory cytokine of the acute phase reaction. TNFα is mainly produced by activated monocytes and macrophages, along with other immune cells such as neutrophils, NK cells and CD4+ T cells. TNFα is an endogenous pyrogen, thus, able to induce fever, apoptosis and cell death and was identified as the key mediator of lethal endotoxemia and septic shock [211-213]. In addition, prolonged TNFα production can

induce a damaging pro-inflammatory response which can lead to mortality, chronic inflammation and diseases [22]. Injection of TNFα alone was sufficient to induce symptoms as seen in sepsis and septic shock [214]. However, anti-TNFα antibody treatment for sepsis just demonstrated poor results in clinical studies [215, 216]. As an immunological regulator, TNFα can promote the secretion of other acute phase cytokines such as IL-6, which is also associated with the induction of the potentially harmful cytokine storm and immune paralysis in sepsis. Blockade of IL-6 signaling was reported to improve survival in two murine sepsis models [217, 218]. High IL-6 serum levels are also associated with poor outcome in sepsis and other inflammatory diseases [219, 220]. In addition, IL-6 inhibition has been proven to be highly effective in the treatment of chronic inflammatory diseases such as rheumatoid arthritis or Crohn´s disease [221]. However, IL-6 also exhibits anti-inflammatory properties by promoting other anti-inflammatory cytokines and is able to block TNFα and IL-1β mediated immune response, acting as a negative feedback loop [222-225].

Within this study a strong induction of TNFα and IL-6 induced by LPS were observed in cells of TSC patients and matched controls (Fig. 11), as well as in cells of healthy voluntary donors (Fig. 10 & 15). Thereby LPS in priming concentration (100 ng/ml) as well as LPS re-stimulation concentration (10 ng/ml) induced a range of 9 different pro-inflammatory cytokines, including IL-6, MIP-1α/β, CCL1 or CXCL12 and in a dose-dependent manner, TNFα, IL-1β, RANTES, G-CSF and GM-CSF. These results were consistent with measured pro-inflammatory cytokines of TSC patients and controls.

LPS induces also anti-inflammatory cytokine production

In addition, the anti-inflammatory IL-10 was also upregulated after LPS treatment in cells from TSC patients and matched controls (Fig. 11). In general, IL-10 is a suppressive cytokine, acting as a paracrine feedback loop in macrophages by inhibiting the differentiation of neighboring cells [226]. IL-10 was originally described as secreted cytokine synthesis inhibitory factor (CSIF) and is reported to counteract especially pro-inflammatory cytokines induced by LPS, such as TNFα or IL-1β, by inhibiting their synthesis, but also IL-6 and IL-8 as well as the pro-inflammatory CC chemokines MCP-1, MIP-1α and MIP-1β and is able to mediate enhanced degradation of pro-inflammatory cytokine transcripts [194, 197, 227-229]. This process is thought to limit pro-inflammatory activation to prevent exaggerated immune response. Further, IL-10 was reported to regulate the TNFα converting enzyme as well as being able to block NFκB and therefore directly balancing pro-inflammatory response as autoregulatory factor [230]. Moreover, IL-10 deficient mice are highly sensitive to LPS, thus IL-10 is a key mediator to overcome endotoxic shock, including sensitization to LPS [231]. Other data reported that

LPS-induced IL-10 secretion was mediated by the PI3K/AKT/mTOR pathway and a loss of TSC2 results in increased IL-10 levels [175, 177]. IL-10 additionally revealed protecting effects in murine sepsis models and IL-10 producing bacteria were shown to counteract hyperactive immune response in a murine model of Crohn´s disease [232-235]. However, in humans, IL-10 additionally exhibits undesired pro-inflammatory effects, as described for endotoxemia, by enhancing the activity of NK cells and cytotoxic T cells [227]. IL-10 expression has its peak at later time points compared for instance to TNFα, which has an intracellular concentration peak within 2 h [236]. Therefore, the produced amounts of IL-10, which accumulated during the first 24 h priming period, could represent the first reaction of the cells to adapt to high LPS concentrations and the initial pro-inflammatory cytokine response towards endotoxin tolerance induction, including an anti-inflammatory phenotype.

LPS and β-glucan priming show opposing effects on IL-8

In contrast to all other investigated cytokines, IL-8 was decreased upon LPS stimulation and therefore seemed to be actively taken up or degraded by the cells. This was confirmed by cytokine profiling of healthy voluntary donors (Fig. 15). IL-8 is also known as the neutrophil chemotactic factor, as its main function is inducing chemotaxis in target cells. Once arrived at inflammatory sites, IL-8 can further promote phagocytosis. Moreover, IL-8 is able to regulate the secretion of pro-inflammatory cytokines like TNFα, IL-1β and IL-6 by NFκB and ERK signaling [190, 237]. Thus, decreased levels of IL-8 could also represent an anti-inflammatory phenotype initiation. β-glucan, on the other hand, did not trigger pro-inflammatory cytokine production which is in line with previous studies [61]. Nevertheless, only IL-8 was significantly enhanced by β-glucan in TSC and control cells (Fig. 10). For the first time, our results could show, that β-glucan initiates IL-8 production, which therefore could act as a possible mediator of β-glucan training. However, increased IL-8 released upon β-glucan was not observed by cytokine profiling of a healthy voluntary blood donor (Fig. 15) but was significantly increased by cytometric bead analysis of other healthy voluntary donors (Fig. 26). This observation might be caused by distinct sensitivity of these different methods or individual variances in cellular response, since cytokine profiling by proteome profiling array could be performed with only one donor.

Rapamycin inhibits the secretion of distinct cytokines

The inhibition of mTOR activity with rapamycin and torin did not affect cytokine production in general, but rather only specific cytokines. Most cytokines measured were not affected upon mTOR inhibition, however, MCP-1, IL-10 and MIP-1β were

significantly altered (Fig. 11). While IL-10 and MCP-1 secretion was significantly decreased, mTOR inhibition even increased levels of MIP-1β. This suggests a dependency of MCP-1, IL-10 and MIP-1β on mTOR signaling. A decrease of IL-10 production following rapamycin treatment has already been reported, but in part associated with decreased TNFα or IL-6 levels, which could not be confirmed here (Fig. 11) [171, 210, 238]. This could be due to the fact that in the present study rapamycin and torin were used in combination to inhibit whole mTOR signaling instead of rapamycin treatment alone. Moreover, there is contrary data on the effect of rapamycin on the expression of TNFα and IL-6 [180, 210]. The rapamycin analog sirolimus was shown to attenuate IL-10 production in human whole blood [239]. Further, TSC2 negative mouse embryonic fibroblast (MEF) cells, with consequential hyperactive mTORC1, produce more IL-10 mediated by mTORC1 and NFκB [177]. In consistence, rapamycin-induced mTORC1 suppression blocks NFκB and subsequent pro-inflammatory response in murine macrophages [171]. However, if mTOR inhibition decreased levels of IL-10 and MCP-1, cytokine production should be enhanced in cells of TSC patients with hyperactive mTOR, whereas MIP-1β should be decreased compared to control cells. Instead, MCP-1 levels were even decreased, while IL-10 and MIP-1β secretion was not affected by hyperactivation of mTOR in TSC cells (Fig. 11).

Monocytes from TSC patients increase pro-inflammatory cytokine release

The pro-inflammatory cytokines TNFα, IL-1β and RANTES were strongly upregulated with LPS stimulation in TSC cells, with significant alterations of IL-1β and RANTES compared to healthy control cells (Fig. 11). This finding is consistent with previous studies and might be attributed to increased protein biosynthesis in cells with hyperactive mTORC1 signaling. Mice lacking the upstream inhibitor TSC1 show an increased pro-inflammatory response when challenged with LPS [178, 180]. Induction of the PI3K/AKT/mTOR pathway is commonly assumed to promote an anti-inflammatory phenotype, however, loss of TSC1 was shown to enhance LPS-induced pro-inflammatory cytokine response in murine macrophages [178]. In addition, a previous study with cells from TSC patients reported an increase of the pro-inflammatory cytokines IL-1β and IL-6, but also the anti-inflammatory IL-10 in TSC cells compared to healthy controls, although not reaching statistical significance [240]. This is consistent with the suggested contrary effect of rapamycin and mTORC1 hyperactivation in TSC cells in regard to IL-10. However, an increase in IL-6 as well as IL-10 expression could not be confirmed in this study. Differences, in the achieved results, could be explained by different LPS concentrations and stimulation times, as well as the fact that in the present study secreted cytokines were profiled, instead of RNA expression analysis.

β-glucan priming did not induce a distinct training effect

When primed cells were re-exposed to LPS, opposing adaptation processes could be observed. As it was already seen in pre-experiments with ELISA measurements (Fig. 9), priming with LPS induced a tolerant state in regard to TNFα and further MIP-1β and RANTES, while β-glucan pre-treatment enhanced TNFα secretion (Fig. 12). In addition, hyperactivation of mTOR in TSC cells again displayed increased TNFα production upon LPS stimulation alone (LPS stim) compared to control cells, despite not reaching statistical significance, perhaps because of declined LPS concentration for re-stimulation. However, enhanced TNFα secretion by β-glucan training reached significance solely in healthy control cells and was lost with mTOR inhibition, which correlates with previous studies [37, 40, 49]. TNFα production was also not altered in cytokine profiling of healthy voluntary blood donors challenged with β-glucan (Fig. 15). This might confirm only weak effects of β-glucan on pro-inflammatory cytokine response. However, since cytometric bead analysis demonstrated enhanced TNFα levels, cytokine profiling with X-ray films might not be sensible enough to detect induced differences. In contrast to previous reports, other pro-inflammatory cytokines measured, such as IL-6, were not affected by priming with β-glucan [37, 50, 61]. In conclusion, only weak and inconsistent training effects could be observed in this study. Since initially monocyte stimulation with the originally recommended training protocol of 7 days did not exhibit a trained pro-inflammatory response, the stimulation protocol had to be adjusted. However, training effects poorly reached a significant level after normalization to cell count or protein amount. This might be accounted for β-glucan induced morphological changes or elevated monocyte to macrophage differentiation as reported by others, leading to increased cell size as well as enhanced attachment to the surface and improved survival compared to untreated control cells [49, 241]. These normalization steps are missing in some previous studies, eventually explaining differences in the results obtained [37, 49]. On the other hand, the number of healthy individuals and TSC patients included might not be sufficient to see a clear statistical effect as individual inflammatory response can vary widely. Since immune response is a complex system and till date it is not known which aspects could affect β-glucan training, there are presumably individuals which respond to a greater or lesser extent, described as responder or non-responder, which might explain distinct results of the present study compared to other data. Further, included individuals displayed a broad age difference, which can affect immunological response, since the immune system evolves during lifetime. In contrast to previous studies, which analyzed adult voluntary blood donors, the majority of TSC patients and controls in the present study were under the age of 18.

LPS-induced tolerant cells suppress specific pro-inflammatory cytokines

In general, upon re-stimulation of LPS primed monocytes, measured cytokines could be categorized in three different patterns. First, cytokines rendered tolerant such as TNFα, MIP-1β and RANTES, second, IL-10 and MCP-1, cytokines which were sensitive to mTOR inhibition and third, cytokines which were not affected by priming of the cells, such as IL-1β, IL-6, IL-8 and MIP-1α (Fig. 28).

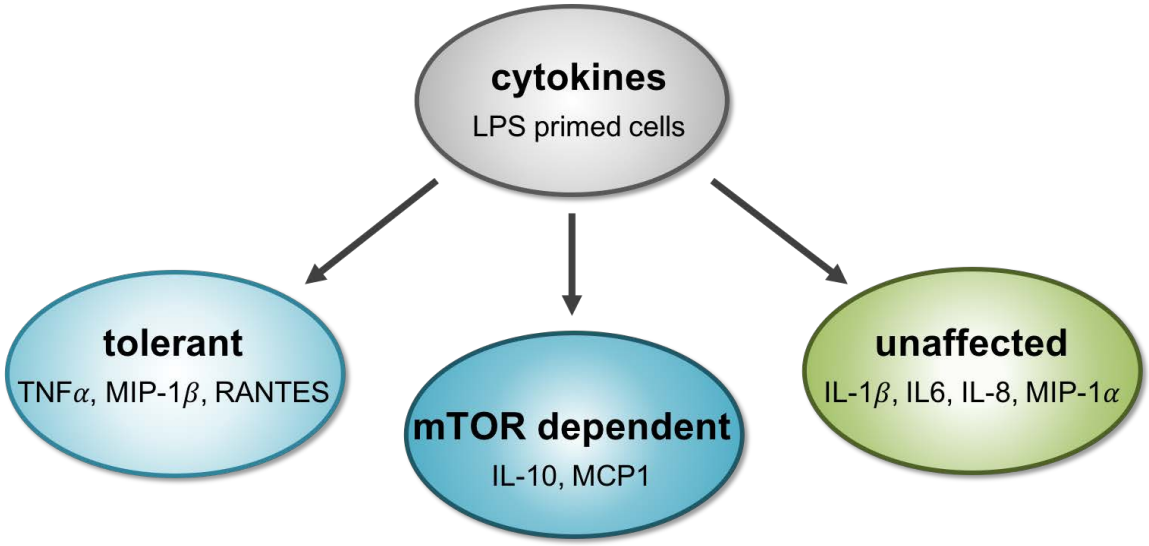


Fig. 28: Cytokines produced by LPS tolerant cells can be classified in 3 groups.
Cytokines secreted by cells rendered tolerant with LPS displayed distinct pattern and therefore can be divided into 3 different groups. The pro-inflammatory cytokines TNFα, MIP-1β and RANTES became tolerant, since their expression was suppressed or completely blocked in tolerant cells. The anti-inflammatory IL-10 and the pro-inflammatory MCP-1 showed a high dependency on mTOR activity, proofed by expression suppression through the mTOR inhibitors rapamycin and torin. And finally, other cytokines such as IL-1β, IL-6, IL-8 or MIP-1α were not altered in tolerant cells, approving that tolerance is not characterized by a switch from pro-inflammatory cytokine expression to an anti-inflammatory phenotype.

Induction of endotoxin tolerance by LPS significantly decreased secretion of TNFα, MIP-1β and RANTES upon re-stimulation in cells from TSC patients and matched controls (Fig. 12). A reduction of RANTES as well as MIP-1α/β was also observed with the cytokine profiler assay of healthy voluntary blood donors, along with a complete block of TNFα and CXCL10 (Fig. 15). CXCL10, also known as Interferonγ-induced protein-10 (IP-10), acts as a chemokine to promote inflammation. It has been reported to be induced upon LPS stimulation but suppressed in tolerant cells, mediated by TRIF and IRF3 signaling, which is consistent with present data [242].

TNFα represents the key readout in endotoxin tolerance and was shown to be greatly decreased in several studies [15, 30, 60, 242]. As discussed above TNFα is a strong trigger for inflammatory immune response and is able to induce sepsis like symptoms by itself [214]. Further, pre-treatment with TNFα can cause tolerance to subsequent, otherwise lethal doses of either TNFα or LPS *in vivo,* thus, defining TNFα as a robust readout for the occurrence of endotoxin tolerance [25, 28, 201, 243, 244]. On the other hand, prolonged TNFα secretion is also associated to chronic inflammatory diseases such as rheumatoid arthritis, indicating for the importance of a tightly regulated and restricted expression [245].

However, induction of tolerance was not affected neither by mTOR inhibitors, nor altered in TSC cells (Fig. 12). This does not support a role of mTOR in the process of endotoxin tolerance. In addition, other pro-inflammatory cytokines, such as IL-6, along with MCP-1 and MIP-1α, were not affected by LPS priming. In other studies, IL-6 was reported to be decreased in tolerant cells exactly like TNFα, which could not be confirmed here [24, 50]. IL-1β can trigger the release of IL-6 wherefore IL-6 expression might be caused by prolonged IL-1β secretion in tolerant cells [188]. Apart from that, since IL-6 also has anti-inflammatory properties, prolonged IL-6 secretion could represent an anti-inflammatory phenotype. Thus, endotoxin tolerance seems not to be a simple switch from pro-inflammatory towards anti-inflammatory response, but a selectively regulated process.

As seen for LPS and β-glucan priming, IL-10 and MCP-1 showed a repeated dependency on mTOR signaling, as combinatorial treatment with rapamycin and torin during priming still inhibited cytokine secretion upon LPS re-stimulation in the absence of inhibitors, partly to a significant level (Fig. 12). However, different cytokine levels upon priming did not affect tolerance induction in these cells, suggesting that IL-10 as well as MCP-1 levels were not important for induction of endotoxin tolerance. Moreover, IL-10 levels were higher in LPS re-stimulated cells compared to primed cells. This indicates, that a lack of TNFα production in tolerant cells, could inhibit induction of IL-10, which is contrary to other data claiming that high IL-10 levels are essential for tolerance induction [26]. However, the authors additionally state that rapamycin, as inhibitor of IL-10 secretion, prevents tolerance induction, which could also not be confirmed in the present study. Differing results might be explained by the use of *Porphyromonas gingivalis* instead of LPS for tolerance induction and re-stimulation or the usage of IL-10 neutralizing antibodies for a complete block of IL-10 signaling, as well as the application of rapamycin alone for mTOR inhibition. Furthermore, although mTOR inhibition alters cytokine levels, as already seen during priming of the cells, there are no significant

differences between TSC cells and healthy controls in regard to IL-10 and MCP-1 production. In addition, inhibition of mTOR did not completely block cytokine secretion, suggesting that there are also other signaling pathways important for these cytokines or at least able to compensate mTOR inhibition. This also demonstrates a complex regulation of cytokine production in tolerant innate immune cells.

mTORC1 activity is not altered in LPS or β-glucan primed cells

Flow cytometric analysis was performed to investigate mTORC1 activation by measuring phosphorylation levels of its downstream target S6 protein (S6P) in CD14 positive monocytes after re-stimulation of primed cells with LPS for 24 h (Fig. 13). Stimulation with LPS significantly induced S6P phosphorylation and consequently mTORC1 activity, which is consistent with previous findings [210]. Both priming conditions further increased pS6P in control cells, but not TSC cells, which could point towards negative feedback loops counteracting high mTORC1 activation in TSC patients on basal level as well as during stimulation. Inhibition with rapamycin and torin lead to a predominantly significant reduction of mTORC1 activity even after 48 h of incubation including 24 h without inhibitors. Hence, mTORC1 inhibition with rapamycin and torin was both efficient and long-lasting as well as specific, proofing that at the time of re-stimulation with LPS, mTORC1 activity was still suppressed. However, the obtained results also revealed that mTORC1 is active in tolerant cells but blocked by rapamycin and torin, though both conditions resulted in same cytokine levels, except from IL-10 and MCP-1 (Fig. 12). Especially TNFα levels were not altered in tolerant cells by mTOR inhibition. Therefore, mTORC1 activity seems not to be involved in induction or maintenance of a tolerant state, which has already been assumed before.

Moreover, there were no significant or pronounced differences in cytokine secretion between healthy control cells and cells from TSC patients (Fig. 12). This could imply that, although some cytokines are dependent on mTORC1 signaling, mTORC1 is not important in the overall adaptation of primary human monocytes *in vitro*. This is in accordance to a lack of general differences between cells treated in the presence or absence of mTOR inhibitors. Otherwise, it is possible that cells from TSC patients already adapted to some degree to increased mTORC1 activity by negative feedback loops, to diminish associated negative effects. As for instance the mTORC1 downstream target S6K has been demonstrated to directly inhibit growth factor-induced PI3K/AKT signaling via IRS1 (insulin receptor substrate 1), the molecular intermediate between insulin receptor and PI3K [148]. In addition to S6K, mTORC1 itself is also able to suppress IRS1 as well as PRAS40 or DEPTOR, compartments of its own complex, and also AKT, an upstream regulator [89, 105, 246, 247]. Inhibition of mTOR by rapamycin

can also induce the Ras/ERK signaling kinase [248]. In consistence with that, cells from TSC patients seemed to be less sensitive to further mTOR inhibition by rapamycin and torin and TSC patients are not considered to be immunologically affected in general.

5.2 Adaptation of primary human monocytes is not dependent on the metabolic status

Activation of immune cells and cytokine production increases energy demand of the cells. β-glucan trained as well as LPS tolerant cells are thought to undergo metabolic re-programming, as the basis for immune cell adaptation [40, 73]. Thereby the metabolic switch from oxidative phosphorylation to aerobic glycolysis, called Warburg effect, is suggested to rapidly provide required energy for immune response. This enhanced glycolysis is characterized by an increase in lactate production and glucose consumption. As key metabolic regulator, mTORC1 was reported to play an important role in this metabolic-reprogramming [40].

As seen for mTORC1 activity, LPS also significantly increased lactate production as well as partly glucose consumption and thus, glycolytic activity of stimulated cells (Fig. 14, upper panel), which is consistent with previous studies [209, 214]. Increased metabolic activity could be caused by enhanced activation levels and increased pro-inflammatory cytokine production, observed in LPS treated cells. Lactate production was significantly increased in both, controls and TSC cells, but to a higher degree in cells of TSC patients, associated with a significant reduction in glucose concentration. It can be assumed that increased glucose consumption rates in TSC patients, which indicate for enhanced metabolic activity, could be caused by hyperactive mTORC1. Increased mTORC1 activity, induced by the loss of TSC2, was demonstrated to increase aerobic glycolysis mediated by enhanced NFκB activity and GLUT-3 (glucose transporter type-3) expression in MEF cells [249]. Nevertheless, a lack of significant differences in glucose consumption of control cells could be based on high glucose concentrations in cell culture media masking otherwise pronounced alterations. In contrast to LPS, lactate levels were not significantly increased by β-glucan priming and just slightly altered. It is suggested, that β-glucan training of monocytes increases metabolic activity and induces Warburg metabolism to promote epigenetic changes and a constitutively active state [40]. This metabolic switch could not be observed in control cells as well as cells from TSC patients. However, most studies refer to the 7 days stimulation period to induce Warburg metabolism [38, 40, 73]. Nevertheless, β-glucan trained cells displayed significantly increased TNFα production in healthy control cells also upon subsequent

LPS stimulation, indicating for a trained state, though cells did not switch to increased glycolysis before. It can be concluded that metabolic re-programming may not be important in trained cells. Instead the increase in glycolysis represents the enhanced activation state of the cells.

LPS and β-glucan primed monocytes did not alter metabolic activity

Re-stimulation of the cells with LPS again significantly increased lactate production in non-primed cells, although to a slighter extent than during LPS priming, which could be caused by decreased LPS concentration for re-stimulation compared to LPS priming (Fig. 14, lower panel). This may also constitute to less differences between TSC cells and controls. Priming of cells with either LPS or β-glucan further increased lactate production and glucose consumption but did not cause significant alterations. These effects were reversed by rapamycin and torin treatment during priming. This further confirms, that suppressed mTOR activation affects cellular metabolism, even after additional 24 h without inhibition. However, production of lactate was not completely abolished by mTOR inhibitors, demonstrating for a complex cellular regulation and mTOR-independent pathways, which may counteract decreased mTOR activity. Moreover, increased lactate levels also in tolerant cells, illustrates that less metabolic activity is not the basis of decreased pro-inflammatory cytokine production in endotoxin tolerant cells. As seen in CBA measurements of TSC cells and controls, as well as with cytokine proteome profiling analysis of healthy voluntary blood donors, instead of a complete suppression of all produced cytokines, tolerant cells block only specific pro-inflammatory cytokines such as TNFα or RANTES. Other inflammatory cytokines like IL-1β or GM-CSF are still increasingly secreted, which consequently involves metabolic activity and in turn sustained lactate production (Fig. 15).

Taken together, results from cells isolated from patients with TSC, which are suggested to have hyperactive mTORC1 signaling because of loss-of-function mutations of upstream inhibitors, as well as healthy controls, did not indicate for a role of mTORC1 in different adaptation processes of innate immune cells like monocytes. Neither hyperactivity of mTORC1 in TSC patients, nor the inhibition of mTOR by a combination of rapamycin and torin during priming of control or TSC cells did affect induction of endotoxin tolerance or β-glucan training, as evidenced by cytokine secretion. In addition, inhibition of mTORC1, which has been demonstrated via flow cytometric analysis of the downstream target S6 protein, also inhibited glycolytic activity. However, tolerant as well as trained cells displayed the same mTORC1 and glycolytic activity, though different cytokine profiles. Thus, mTORC1 signaling as well as the metabolic state of the cells do not seem to be important in adaptation processes of innate immune cells.

5.3 Endotoxin tolerance is not mediated via receptor internalization or pro-inflammatory cytokine release

Previous results demonstrate that cytokine release is a tightly controlled process. The LPS-induced release of important cytokines such as TNFα or IL-6, was shown to be regulated on the translational level, with IL-6 in a mTOR-dependent manner [210, 250]. Inhibition of mTORC1 with rapamycin was shown to reduce IL-6 secretion but did not alter mRNA levels [210]. Thereby, LPS induces activation of mTORC1 and subsequent phosphorylation of the downstream target 4E-BP1 [210, 251]. Upon phosphorylation, 4E-BP1 dissociates from the eukaryotic initiation factor 4E (eIF-4E) and thus, enables protein synthesis. In addition to IL-6, IL-10 was also reported to be translationally regulated by mTORC1, whereas TNFα was not altered by mTOR inhibition [210]. This selective regulation of LPS-induced cytokine expression could be confirmed in the present study. For further analysis of mTORC1 signaling, the activation of other downstream targets was examined. The second direct downstream target of mTORC1 is S6K which in turn activates S6P. S6P is part of the 40S ribosomal subunit and therefore also controlling protein translation.

mTORC1 signaling is not altered in primed monocytes

Western blot analysis of healthy voluntary blood donors confirmed intracellular flow cytometric measurements of downstream mTORC1 signaling with regard to phosphorylated S6P. Monocyte stimulation with LPS induced phosphorylation of S6K (pS6K) and subsequently S6 protein (pS6P) (Fig. 16). mTORC1 was activated within 30 min and slightly increased after 1 h but decreased again within 24 h, to almost basal level with regard to S6K. S6P was still activated after 24 h, suggesting that activation of S6P is more enduring or stable than pS6K, which is consistent with flow cytometric analysis of pS6P in TSC and control cells, where S6P phosphorylation was still significantly increased after 24 h. At the same time, stimulation with β-glucan also slightly activated S6K phosphorylation within 1 h and even lasted up to 24 h of incubation, whereas S6P was less activated by short-time β-glucan stimulation and decreased to almost control level in 24 h. These contrary activation levels indicate for a tightly regulated response to different PAMPs within downstream signaling cascades, instead of flipping a switch for mTOR activation.

Pre-treatment with LPS or β-glucan before re-stimulation slightly increase pS6K levels, which were markedly decreased by mTOR inhibition within the first 24 h of priming. When monocytes were primed with β-glucan, activation levels of S6K were increased most, however, along with higher variances. Increased variations of measurements,

between different individuals primed with β-glucan, were also observed in other experiments as secretion levels of some cytokines and also with intracellular flow cytometric TNFα measurements (Fig. 17). As stated above, an enhanced variety from one individual to another could indicate for widely individual differences in the response to inflammatory stimuli such as β-glucan, including responder and non-responder. Since this study has several limitations and the relatively small number of healthy volunteers included, especially for immunoblotting experiments (3-7), might not be enough to exhibit clear statistical effects. This would increase the importance of donor selection, along with the need for increased individual numbers, to discriminate different responding groups as well as reaching significant differences to control conditions.

Further, activation of S6P by β-glucan was more short-lived than S6K and almost on control level after 24h. In contrast to S6K, no further activation of S6P through priming could be observed. Thus, higher activation of S6K is not forwarded to downstream S6P. Moreover, it could be possible that S6P may reached maximal activity measurable by western blotting, as flow cytometric analysis in TSC and control cells displayed slightly increased S6P activation by priming with LPS and β-glucan (Fig. 13). mTOR inhibition with rapamycin and torin during priming reduced both signals to almost control level. These findings are consistent with pS6P analysis by flow cytometry, confirming the specific effect of these two inhibitors to mTORC1, proofing, that inhibition of IL-10 and MCP-1 secretion upon stimulation is mTORC1 dependent.

However, as confirmed by flow cytometric analysis, there was no induction of mTORC1 inhibition in tolerant cells, meaning that mTORC1 signaling is still active in endotoxin tolerant cells. Thus, either mTORC1 signaling is not associated with induction and maintenance of a tolerant state or mTORC1 signaling is continuously regulated and thereby not important for distinct inflammatory cytokine production, but maybe intracellular re-programming. This might also explain less differences between cells from patients with TSC and healthy controls.

LPS tolerant monocytes do not lack surface abundance of TLR4

In parallel to mTOR signaling, other possible factors of endotoxin tolerance induction were investigated. It is reported, that binding of LPS to TLR4 forces internalization of the receptor within a few minutes. However, internalization was only followed for up to 90 min [12]. Tolerant neutrophils were shown to lose surface abundance of the receptor [36]. In addition, there are contrary data published, with some data indicating no differences in cell surface abundance of TLR4 after LPS stimulation, while further studies even suggested an increase in TLR4 after 48 h of LPS incubation [252-254]. However, none of the mentioned studies was investigating primary human monocytes.

Furthermore, receptor internalization is still suggested to be a mediator of endotoxin tolerance, but there is no data about receptor surface abundance after 24 h of LPS stimulation. In the present study, it has been shown that in fact, LPS induces internalization of TLR4 in primary human monocytes, reaching significant reduction after 3 h (Fig. 18). These results slightly deviate from previous studies of murine macrophages, which could be accounted for, besides using different species, by high variances during the first hour of stimulation in this study. These variances might be initiated by increased stress for the cells, caused by short resting periods between media change for stimulation and harvesting of the cells. However, TLR4 is continuously internalized reaching minimal surface abundance of approximately 40% after 24 h. At this time point, cells were normally re-stimulated with LPS resulting in a complete block of TNFα secretion. Thus, the complete lack of TNFα production, seen in cytometric analysis of intracellular TNFα (Fig. 17) cannot be explained by the loss of surface receptors and abolished LPS binding. Further, not all pro-inflammatory cytokines were blocked by tolerance induction with LPS such as IL-1β, MCP-1 or MIP-1α (Fig. 12). Hence, regulation of endotoxin tolerance and associated cytokine expression seems to be a tightly controlled process mediated by downstream signaling of TLR4, rather than by loss of the receptor on the cellular surface to inhibit ligand binding.

Different inflammatory pathways mediate cytokine production

Nevertheless, mTORC1 signaling was not affected in tolerant cells, suggesting that mTORC1 is not a direct downstream target of TLR4 or its activation is strictly regulated in regard to specific cytokine productions. Therefore, other pro-inflammatory pathways, reported to be associated with TLR4 signaling such as PI3K and the MAP kinases ERK and p38 were analyzed [255]. By chemical inhibition of the key molecules, it could be demonstrated, that every cytokine depends either on more than 2 different inflammatory pathways or was completely unaffected as seen for MIP-1α (Fig. 19). This finding indicates for the contribution of several signaling pathways to trigger the secretion of distinct cytokines and complex regulated processes. In addition to mTOR, IL-10 secretion was even more dependent on p38 and PI3K signaling, while inhibition of ERK did not have any effect. The PI3Kγ/AKT pathway is a direct upstream inhibitor of mTOR thus, decreased levels of IL-10, as well as MCP-1, by LY204002 could, at least partly, be mediated by subsequent downstream inhibition of mTOR. In addition, LY294002 was shown to inhibit LPS-induced phosphorylation of mTOR as well as its downstream target 4E-BP1 [210]. However, since the suppressive effect of PI3K inhibition was elevated in comparison to mTOR inhibition, the secretion of IL-10 and MCP-1 has to be additionally regulated by another downstream signaling path of PI3K, except from mTOR. In

addition, p38 has been reported to regulate the transcript stability of LPS-induced IL-10 by its downstream target MK2 (MAPK-activated kinase 2) in macrophages [256]. A dependency of IL-10 on p38 signaling could also be confirmed in the present study by the usage of the p38 inhibitor SB202190 (Fig. 19). On the other hand, IL-10 was reported to mediate the inhibition of p38 in an MK2-dependent manner as a negative feedback loop [257, 258].

Secretion of TNFα was diminished by all inhibitors, except from rapamycin and torin, which is in line with cytokine measurements of TSC and control cells and consistent with other studies [210]. Inhibition of ERK, and even more p38, reduced TNFα to a significant level compared to LPS stimulation alone, indicating that TNFα production is highly dependent on these pathways. Indeed, ERK signaling has been reported to be important for the nuclear transport of TNFα and therefore essential for the LPS-induced production of the cytokine [259]. p38 is also described as a critical factor in the regulation of inflammatory mediators such as TNFα in monocytes and macrophages *in vivo,* mainly by its α-isoform [260, 261]. Thereby p38 was shown to regulate TNFα production on the translational level [250]. Further, mice with p38-deficient macrophages were demonstrated to have impaired pro-inflammatory cytokine production, particularly in regard to TNFα, associated with being largely protected against an otherwise lethal LPS injection [262]. This pro-inflammatory effect is presumably mediated by the p38 downstream target MK2, since MK2-deficient mice are also largely resistant to lethal challenges with LPS, caused by an almost complete suppression of LPS-induced TNFα production [263]. In addition, also the production of other pro-inflammatory cytokines such as IL-6 or IL-β seem to be MK2-dependent in murine spleen cells [263]. These findings could be confirmed in the present study, with TNFα, IL-1β and IL-10 being significantly reduced by p38 inhibition.

All used inhibitors lead to a different cytokine profile and none was similar to LPS treatment alone, suggesting a contribution of all tested pathways in the induction of LPS-induced immune response. However, although all treatment conditions initiated a distinct cytokine profile, TNFα as well as RANTES production was diminished upon re-stimulation with LPS by all primed cells, despite not reaching statistical significance in all conditions (Fig. 20). Inhibition of p38 and ERK signaling, which caused significant decreased pro-inflammatory cytokine production during priming, did not induce significant endotoxin tolerance, as evidenced by TNFα levels, upon re-stimulation. Furthermore, IL-10 secretion was not affected by ERK inhibition during priming, but significantly decreased during re-stimulation. Since IL-10 was not directly dependent on ERK signaling, its expression might be abolished, because of significantly decreased

pro-inflammatory cytokine levels during LPS priming, such as TNFα, IL-1β or IL-6. This effect is consistent with previous studies claiming that IL-10 secretion is a direct consequence of pro-inflammatory cytokine production. p38 inhibition likewise decreased these cytokines during LPS priming and diminished IL-10 secretion upon re-stimulation, despite not significantly. These results indicate a role of produced pro-inflammatory cytokines, induced by LPS priming, for the induction of endotoxin tolerance. Thus, this hypothesis was tested in further experiments as discussed below. Although all pathways investigated seem to be crucial for distinct cytokines, the results demonstrated that not a particular pathway is responsible for TLR4 induced immune response, but rather a complex signaling network mediates an appropriate immune cell activation.

Further analysis of the impact of 38 on TLR4 internalization revealed that inhibition of p38 signaling with SB202190 did not affect internalization of TLR4 after 24 h of LPS stimulation (Fig. 22). Upon p38 inhibition receptor internalization was not altered, however, almost no TNFα was secreted in those cells (Fig. 19). Thus, supporting a role of p38 downstream of TLR4, consistent with previous results suggesting that enhanced receptor internalization alone cannot trigger the complete lack of TNFα production. Hence, intracellular signaling of p38 and other inflammatory pathways were investigated.

Tolerant monocytes suppress p38 and AKT signaling

Immunoblotting experiments revealed that phosphorylation of ERK displayed a similar pattern as the mTOR downstream target pS6P (Fig. 21). LPS induced short-term activation of ERK within 1 h but decreased again during 24 h of stimulation, which could also be observed by β-glucan treatment. In contrast to mTOR, rapamycin and torin treatment did not affect ERK activation, proofing specificity of both inhibitors to mTOR. However, priming of the cells neither resulted in increased EKR phosphorylation in β-glucan trained cells, nor diminished pERK levels in tolerant cells, suggesting that, similar to mTOR, ERK signaling was still active in tolerant cells. On the other hand, p38 and AKT signaling were also temporarily activated by LPS, and to a lesser extent by β-glucan, but phosphorylation was lost after 24 h of incubation. However, p38 and AKT were less active in LPS tolerant cells. Along with fewest TNFα production upon inhibition of p38 during priming, this also indicates for a role of p38 in the downstream cascade of TLR4-induced TNFα production. In addition to these findings, p38 also seemed to be important in endotoxin tolerant cells, since p38 inhibition diminished tolerance induction in regard to significantly decreased TNFα levels (Fig. 20). p38 and AKT signaling were found to be less active and thus, the only pathways affected in tolerant cells. Nevertheless, p38 inhibition neither completely blocked TNFα during LPS priming, which was additionally shown to be dependent also on other pathways such as PI3K and ERK,

nor further inhibited TNFα secretion upon LPS re-stimulation. These findings indicate for the activation of other TNFα-inducing pathways during prolonged p38 inhibition or after tolerance induction, such as ERK signaling (Fig. 29).

Additionally, a lesser extent of tolerance induction observed upon SB202190 (p38) as well as UO126 (ERK) treatment (Fig. 20) might be explained by the significant lack of pro-inflammatory cytokines such as TNFα and IL-1β during LPS priming. In contrast to this assumption, LY294002 (PI3K) treatment also decreased TNFα production upon priming, despite not significant, but displayed the strongest tolerance induction. However, PI3K inhibition instead caused an increased secretion of another pro-inflammatory cytokine, IL-1β, additionally indicating for a role of expressed pro-inflammatory cytokines in the induction of tolerance.

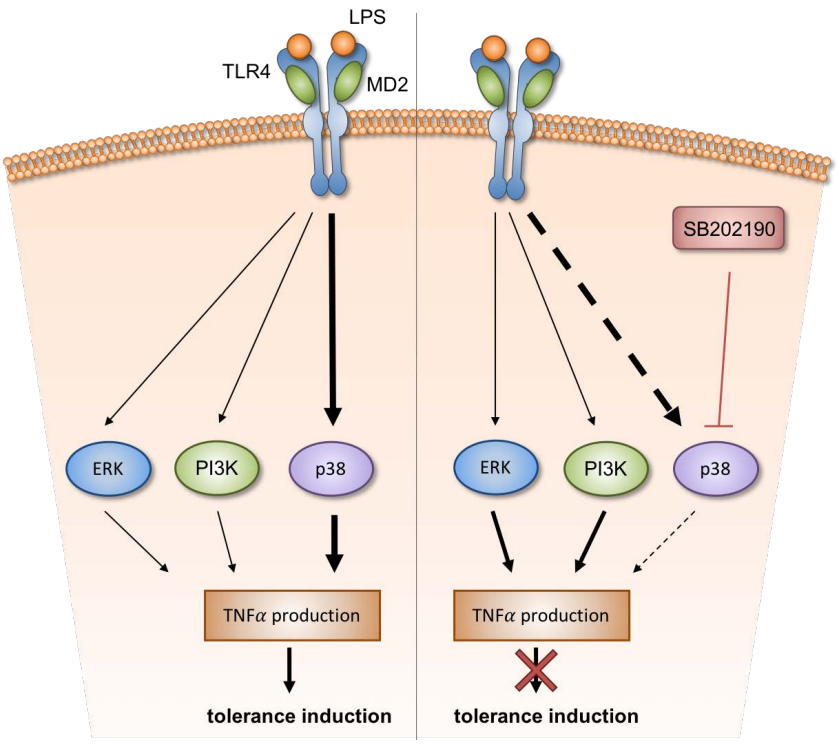


Fig. 29: TLR4 signaling in LPS stimulated monocytes in regard to TNFα production.
LPS-induced TNFα production seems to be highly dependent on p38 signaling but is also triggered by PI3K and ERK signaling in human monocytes. TLR4 downstream signaling cascades probably initiate the induction of endotoxin tolerance. When p38 is inhibited by SB202190, monocytes produce less TNFα when challenged with LPS and also exhibited a less pronounced LPS tolerance in regard to TNFα expression. This suggests that p38-induced TNFα secretion or other downstream targets of p38 are important in mediating a tolerant state after LPS stimulation.

Pro-inflammatory cytokines are not sufficient to induce endotoxin tolerance

The impact of secreted pro-inflammatory cytokines in the induction of endotoxin tolerance was examined using conditioned media from LPS-treated cells. Thereby, it could be shown, that stimulation of monocytes with LPS for 1 h secreted almost same amounts of cytokines as cells treated for 24 h (Fig. 23). Only MIP-1α was significantly decreased compared to 24 h stimulated cells, which could be caused by the withdrawal of already produced cytokines through media exchange after 1 h to eliminate remaining LPS. In contrast, IL-1β was the only cytokine significantly increased, which could be explained by an increased resumption rate or enhanced degradation during prolonged LPS treatment to promote an anti-inflammatory phenotype. This suggests, that 1 h LPS treatment is enough to induce pro-inflammatory immune response. Consistent with that, stimulation of monocytes with LPS for 1 h was demonstrated to be sufficient to induce endotoxin tolerance [15].

Further, cells primed with conditioned media secreted almost same amounts of cytokines than cells stimulated with LPS for 24 h, again except from IL-1β (Fig. 24). Since all cytokine levels were either the same or less compared to media contents before priming (Fig. 23), it is likely, that stimulation with conditioned media did not trigger any further cytokine secretion but rather displayed LPS-induced cytokine levels triggered by 1 h of LPS stimulation. This might also be the reason for repeated higher IL-1β levels upon media priming compared to LPS priming. Moreover, re-stimulation with LPS revealed that cells primed with conditioned media were not rendered tolerant, as evidenced by unchanged LPS-induced TNFα levels (Fig. 25). However, media-primed cells neither displayed the same cytokine profile than only LPS stimulated cells, nor the same as LPS primed cells. In addition to TNFα, levels of IL-1β and MIP-1α were similar to LPS stimulated cells, while IL-10 and RANTES were equal compared to tolerant cells. This suggests, that priming with cytokines did alter inflammatory cellular response, nevertheless, did not induce endotoxin tolerance. Therefore, presence of pro-inflammatory cytokines such as TNFα is not sufficient to trigger endotoxin tolerance in primary human monocytes. This is also supported by other experiments, as inhibition of different inflammatory pathways showed, that several distinct cytokine profiles can induce tolerance, also in the absence of secreted TNFα, despite not all reaching statistical significance. Further, mice injected with IL-1β, IL-6 or TNFα instead of LPS, also just partly displayed a tolerant-like state when re-challenged with LPS [16]. The authors suggested, that rather than cytokine production, surface receptor abundance or soluble binding proteins are responsible for endotoxin induction. In the present study, the loss of TLR4 on the cell surface of human monocytes could be excluded in charge of the

induction of endotoxin tolerance. However, other studies, investigating whole organisms, showed a protecting effect of TNFα pre-treatment towards endotoxemia and sepsis [27]. Thus, tolerance has to be regulated on the signaling level upstream and downstream of pro- and anti-inflammatory cytokine expression. This might explain the weak results of anti-TNFα antibody treatment in clinical trials of sepsis in the 1990´s. Since the immune response of monocytes and macrophages is more complex than the response to inflammatory cytokines, followed by a switch towards an anti-inflammatory phenotype, more insights in the exact contributing signaling cascades are needed for a better understanding and benefit from this protecting adaptation mechanism.

6. CONCLUSIONS

Innate immune memory, as an adaptation processes of innate immune cells such as monocytes and macrophages, could bear promising therapeutic targets of a variety of diseases, including hyperinflammation and sepsis, as well as immunoparalysis, chronic inflammation and autoimmune disorders. Understanding the underlying molecular mechanisms of endotoxin tolerance and β-glucan training will help improving therapies against exaggerated inflammation as well as immunosuppression and new-generation vaccines.

The present study suggests, that constitutive mTORC1 activity in monocytes from TSC patients, as well as the chemical inhibition of mTOR in control or TSC cells, does not alter immune cell adaptation capability. Monocytes with hyperactive mTORC1, together with cells subjected to mTOR inhibition, are not impaired in inducing endotoxin tolerance, whereas β-glucan training displays inconsistent results. Reproducible training effects with β-glucan priming could not be achieved and also enhanced glycolysis of trained cells was not detectable. Nevertheless, LPS induces the activation of mTORC1 signaling along with increased pro-inflammatory cytokine release and enhanced glycolytic activity, followed by an endotoxin tolerant state in healthy monocytes as well as cells from TSC patients. However, induction of tolerance is neither dependent on mTORC1 activity, nor on metabolic capacity, since tolerant cells still display mTORC1 signaling as well as enhanced glycolysis. Inhibition of mTORC1 with rapamycin and torin, verified by different signaling analysis, furthermore does not impair initiation of tolerance. In addition, endotoxin tolerance has to be mediated downstream of TLR4, since receptor internalization does not explain the complete block of TNFα expression seen by cytokine measurements of cell culture supernatants, as well as intracellular flow cytometry. The p38 MAPK signaling cascade seems to be an important mediator of LPS-induced TNFα release, since p38 inhibition reduced the induction of tolerance. However, also other inflammatory pathways are involved in the secretion of LPS-induced cytokines including, ERK, PI3K and mTORC1, although there is not a particular signaling cascade responsible for mediating tolerance induction. The release of pro-inflammatory cytokines such as TNFα are not sufficient to induce an endotoxin tolerant state, though triggering adaptation processes. These findings confirm endotoxin tolerance as a highly regulated, complex adaptation process involving different intracellular pathways for mediating an appropriate immune response. A better understanding of adaptational and memory-like functions of innate immune cells, like monocytes, could help modulating overwhelming inflammatory responses, as well as immunoparalysis, providing access to new therapeutic strategies.

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DANKSAGUNG

An erster Stelle gilt mein Dank meinem Doktorvater apl. Prof. Dr. Ignacio Rubio für seine wissenschaftliche und methodische Unterstützung und Anleitung während der gesamten Bearbeitungsphase meiner Dissertation und die stets aufbauenden Worte in schweren Phasen.

Weiterhin danke ich meinem Zweitgutachter apl. Prof. Dr. Frank.-D. Böhmer für die hilfsbereite wissenschaftliche Betreuung und die konstruktiven Anregungen. Sowie Prof. Dr. Matthias Wymann für die Erstellung des Gutachtens meiner Arbeit.

Ich möchte auch der RTG1715 und allen Verantwortlichen danken, die mir das Erstellen dieser Arbeit überhaupt erst ermöglicht haben.

Ebenfalls möchte ich mich besonders bei OA Dr. med. Ralf Husain von der Kinderklinik Jena bedanken, für dessen Mühen und Unterstützung zur Rekrutierung der Patienten und Probanden, sowie der Bereitstellung des Probenmaterials, ohne das diese Arbeit nicht möglich gewesen wäre.

Besonders danken möchte ich außerdem meinen lieben Kolleginnen, Anne Kresinsky, Melanie Kahl, Dr. Anne Hennig, Dr. Daniela Reich, Fabienne Haas und Martina Beretta, die mich während meiner Promotion begleitet haben, immer ein offenes Ohr für Probleme hatten und mir stets mit Rat und Tat zur Seite standen. Ohne euch wäre diese Zeit nicht zu so einer schönen Erfahrung geworden.

Danken möchte ich auch meinen Kommilitonen/innen aus der RTG1715 für die fachliche und vor allem moralische Unterstützung in jeder Phase unserer gemeinsamen Zeit, sowie das Teilen von Freud und Leid auf dem manchmal steinigen Weg.

Mein Dank gilt weiter allen Kollegen/innen, die ich während meiner Zeit am CMB kennenlernen durfte. Ihr alle habt dazu beigetragen, dass ich mich sehr wohl gefühlt habe und die Zeit immer in guter Erinnerung behalten werde.

Ein besonderer Dank gilt meinen Eltern, die mich auf meinem Weg durch das Studium und die Promotion begleitet und zu jeder Zeit unterstützt und ermutigt haben. Ohne euch wäre diese Arbeit nicht möglich gewesen.

Ebenso danke ich meinen Mädels, die mich auch abseits des wissenschaftlichen Alltags durch alle Höhen und Tiefen begleiten.

Mein ganz besonderer Dank gilt meinem Freund Stefan, der während allen Phasen meines Studiums unterstützend an meiner Seite stand und auch in schweren Zeiten mein Rückhalt ist.

LIST OF PUBLICATIONS

Pai GM, Zielinski A, Koalick D, **Ludwig K**, Wang ZQ, Borgmann K, Pospiech H, Rubio I. *TSC loss distorts DNA replication programme and sensitizes cells to genotoxic stress.* Oncotarget 2016, 7 (51) 85365-85380

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