DISSERTATION

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INDUCTION AND PROPERTIES OF SOCS1 IN MYELOID CELLS BY ACTIVATION OF DECTIN-1

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List of abbreviations

Ab	antibody
APC	antigen presenting cell
APS	ammonium persulfate
AU	arbitrary units
Aqua dest	aqua destillata
αIL10	neutralizing IL10 antibody
Bcl10	B-cell lymphoma 10
BMDC	bone marrow-derived dendritic cell
BMM	bone marrow-derived macrophage
bp	base pairs
BSA	bovine serum albumin
C	celcius
CARD9	caspase recruitment domain-containing protein 9
CD	cluster of differentiation
cDNA	complimentary DNA
CIS	cytokine inducible SH2 domain containing protein
CLR	c-type lectin receptor
c_t	threshold cycle
C-terminus	carboxyterminus
DC	dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
Dectin-1	DC-associated c-type lectin-1
DMEM	dulbecco’s modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double-stranded
DTT	dithiotreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ERK	extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
FSC	forward scatter
fw	forward

Fig.	figure
GAPDH	Glycerinaldehyd-3-phosphate-dehydrogenase
GMCSF	Granulocyte macrophage-colony stimulating factor
Gp130	glycoprotein 130
h	hour(s)
h	human
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
IRAK	IL-1-receptor associated kinase
IRF	interferon regulatory factor
IκB	inhibitor of NF-κB
JAK	janus kinase
JNK	c-jun N-terminal kinase
kDa	kilo-dalton
KIR	kinase-inhibitory region
Ko	knockout
LF	Lipofectamine
LPS	Lipopolysaccharide
LTA	Lipotichoic acid
MACS	magnetic cell separation
MAL	MyD88 adaptor like protein
MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1
manLAM	mannosylated lipoarabinomannan
MAPK	mitogen-activated kinase
MDA5	Melanoma differentiation-associated gene 5
min	minute
mRNA	messenger RNA
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-dephenyl-2H-tetrazoliumbromide
MyD88	myeloid differentiation factor 88
NALP	NACHT-, LRR- and PYRIN-domain containing protein
NCBI	National Centre for Biotechnology Information
n.d.	not detected
NF-κB	nuclear factor kappa B
NLR	NOD (nucleotide-binding and oligomerization domain)-like receptor

n.s.	not significant
nt	nucleotides
ODN	oligodeoxynucleotide
PAGE	Polyacrylamide gel electrophoresis
Pam_3CSK_4	$Pam_3CysSerLys_4$ (N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-Phycoerythrin
PFA	Paraformaldehyde
Poly(IC)	Polynosinic-policytidylic acid
PRR	Pattern recognition receptor
PTO	Phosphotioate
Pyk2	Proline rich tyrosine kinase
qPCR	quantitative real time polymerase chain reaction
RAF1	rapidly growing fibrosarcoma
Ras (GAP)	rat sarcoma Virus (GTPase activating protein)
rE	relative expression
RIG-I	retinoic acid inducible gene I
RLR	RIG-I-like receptors
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	room temperature
rv	reverse
Src	sarcoma
SDS	sodiumdodecyl sulfate
sec	second(s)
SH2	src-homology 2 domain
SHP	SH2 containing phosphatase
SOCS	suppressor of cytokine signalling
ss	single stranded
SSC	sideward scatter
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase

TAE	Tris actetate EDTA
TAMRA	6-Carboxytetramethylrhodamin
TBS	Tris buffered saline
TE	Tris/EDTA
TEMED	Tetramethylethylendiamine
TICAM	TIR containing adapter molecule
Th-cell	T-helper cell
T-cell	T-lymphocyte
TGFβ	transforming growth factor beta
TIR	Toll/Interleukin 1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing protein inducing IFNβ
U	unit
v/v	volume per volume
w/v	weight per volume
WT	wild type

1. Summary

A hallmark of protective immunity during fungal infections is the regulated secretion of pro- and anti-inflammatory cytokines. Suppressor of cytokine signalling (SOCS) proteins constitute a class of key regulators of cytokine function. SOCS proteins are known as inducible negative feedback inhibitors of JAK/STAT (janus kinase/signal transducer and activator of transcription) pathways, but they can also be induced by Toll-like receptors. Dectin-1, another member of the PRR (pattern recognition receptor) family, is described to detect surface structures of fungal pathogens. However, induction and inhibition properties of SOCS proteins upon Dectin-1 stimulation have not been described yet.

In this study it is shown that SOCS1 mRNA and protein is induced in murine bone marrow-derived macrophages (BMMs) and dendritic cells (BMDCs) upon engagement of Dectin-1. SOCS1 was expressed independently of any TLR engagement as a direct target gene of the Dectin-1 ligand depleted Zymosan, which lacks TLR-binding capabilities. Additionally, the induction of SOCS1 by depleted Zymosan was completely independent of secreted type I interferon.

Induction of SOCS1 was mediated by a novel pathway that had not been described in conjunction with SOCS1 induction previously. In contrast to BMDCs, the transcription factor NF-κB was not responsible for SOCS1 expression in BMMs. This novel pathway encompassed the receptor proximal tyrosine kinases Src and Syk that activated the downstream tyrosine Proline-rich tyrosine kinase 2 (Pyk2). In BMMs, Pyk2 in turn caused activation of the MAPK (mitogen-activated kinase) ERK (extracellular signal-regulated kinase) thereby triggering SOCS1 induction. Inhibition of Pyk2 abolished Pyk2 and ERK phosphorylation as well as SOCS1 expression, whereas the activity of the MAPKs p38 and JNKII (c-jun N-terminal kinase) was completely unaffected.

Subsequently, a possible influence of Dectin-1 induced SOCS1 on Dectin-1 and TLR signalling was tested. SOCS1 did not modulate Dectin-1 signalling pathway but affected TLR signalling. Stimulation with depleted Zymosan led to an increased and prolonged NF-κB activation in SOCS1 knockout BMMs triggered by TLR9. Furthermore, IL12 and IL10 secretion induced upon stimulation via TLR9 was inhibited by Dectin-1 induced SOCS1.

In addition, it could be shown that IL17 producing T-helper (Th)-cells were increased by SOCS1 in BMMs. Therefore, BMMs from SOCS1 knockout as well as the respective controls were co-stimulated via TLR9 and Dectin-1 and co-cultured with wildtype $CD4^+$ Th-cells. Afterwards, a significant increase in IL17 production could be observed only in co-cultures with co-stimulated BMMs that still expressed endogenous SOCS1. Thus, SOCS1 is responsible for shaping the immune response towards an anti-fungal, IL17 producing Th-cell phenotype.

The results show that SOCS1 is expressed via a new pathway in Dectin-1 triggered myeloid cells. In BMMs, the SOCS1 expression is independent of NF-κB. SOCS1 induced via Dectin-1 influences TLR crosstalk and T-cell priming thus contributing to the appropriate immune regulation in fungal infections.

1. Zusammenfassung

Die präzise Regulation der Sekretion pro- und anti-inflammatorischer Zytokine stellt einen der wichtigsten Mechanismen bei der Immunantwort gegen pathogene Pilzinfektionen dar. Suppressor of Cytokine Signalling (SOCS) Proteine stellen eine Klasse von induzierbaren feedback Inhibitoren für den JAK/STAT (janus kinase/signal transducer and activator of transcription) Signalweg dar. Diese intrazellulären Proteine werden durch Zytokine, aber auch durch Toll-like Rezeptor (TLR) Liganden induziert. Neben TLRs ist Dectin-1 als weiteres Mitglied der PRRs (pattern recognition receptors) beschrieben. Dectin-1 erkennt Zellwandbestandteile pathogener Pilze. In dieser Arbeit sollte untersucht werden, ob Dectin-1 Aktivierung mit der Induktion von SOCS Proteinen und damit der Modulation von Zytokinantworten einhergeht.

Es kann gezeigt werden, dass SOCS1 (mRNA und Protein) nach Dectin-1 Stimulation muriner Makrophagen (BMMs) oder Dendritischer Zellen (BMDCs) induziert wird. Dabei erfolgte die SOCS1 Expression durch einen Dectin-1 Liganden, der keine TLR-Bindekapazität aufweist (modifiziertes Zymosan), direkt und TLR unabhängig.

Die SOCS1 Induktion wurde über einen Signalweg vermittelt, der in Zusammenhang mit der SOCS1 Expression noch nicht beschrieben war. Dabei war der Transkriptionsfaktor NF-κB in BMMs, im Gegensatz zu BMDCs, nicht für die SOCS1 Induktion verantwortlich. Nach Dectin-1 Stimulation wurden Tyrosin Kinasen der Src und Syk Familie aktiviert; diese stimulierten wiederum die nachgeschaltete Tyrosin Kinase Pyk2 (Proline rich tyrosine kinase 2). In BMMs induzierte phosphoryliertes Pyk2 die Aktivierung der MAPK (Mitogen-activated kinase) ERK (extracellular signal-regulated kinase). Phosphoryliertes ERK induzierte die NF-κB unabhängige Expression von SOCS1. Eine Inhibition der Pyk2 Aktivierung führte zu einer spezifischen Hemmung der ERK Phosphorylierung sowie der Induktion des SOCS1 Proteins. Die Aktivität der MAP Kinasen JNKII und p38 blieb unbeeinflusst.

SOCS1 wurde weiterhin auf seine Wirkung im Dectin-1 und TLR Signalweg untersucht. Es konnte eine inhibitorische Wirkung des SOCS1 Proteins auf den TLR Signalweg beobachtet werden, jedoch nicht auf den Dectin-1 Signalweg. Die Stimulation mit modifiziertem Zymosan führte zu einer verlängerten NF-κB Aktivierung in SOCS1 defizienten BMMs, die zusätzlich über TLR9 stimuliert wurden. Zudem war die TLR9 induzierte Sekretion der Zytokine IL12 und IL10 durch Dectin-1 induziertes SOCS1 Protein inhibiert. Zusätzlich konnte ein signifikanter Anstieg der IL17 Produktion in T-Helfer (Th) Zellen beobachtet werden, der abhängig von der Dectin-1 induzierten SOCS1 Expression in BMMs war. Dazu wurden BMMs aus SOCS1 knockout Mäusen und den entsprechenden Kontrollen via TLR9 und Dectin-1 kostimuliert und mit isolierten Wildtyp $CD4^+$ Th-Zellen kokultiviert. Eine erhöhte IL17 Produktion konnte nur in Kokulturen mit BMMs gezeigt werden, die noch endogenes SOCS1 Protein exprimierten. SOCS1 ist somit assoziiert mit einem Th-Zell Phänotyp mit erhöhter IL17 Produktion.

Die Ergebnisse zeigen, dass SOCS1 in Dectin-1 stimulierten myeloiden Zellen über einen neuartigen Signalweg induziert wird. In BMMs wird SOCS1 NF-κB unabhängig exprimiert. Darüber hinaus beeinflusst SOCS1 TLR-Signalwege und Th-Zell Antworten und wirkt somit an der Regulation von Immunantworten bei Pilzinfektionen mit.

2. Introduction

The immune system defends the host organism against invading microbes. Hence, the detection of a wide variety of different infectious microbes, such as bacteria, fungi and viruses is mandatory. This task implies that cells of the immune system have to discriminate between self and non-self structures.

In general, the immune system can be divided into innate and adaptive immunity. The innate immune system constitutes the first line of defense. It comprises cellular components, namely dendritic cells, macrophages, granulocytes and cellular factors, i.e. the complement system, that patrol constantly throughout the body to be in place when an infectious organism invades the body. Upon activation, innate immune cells in turn are capable of activating adaptive immunity. The mechanisms by which cells of the adaptive immune system respond to infections are decisively different from those of innate immunity. Lymphocytes share the common feature of somatical recombination of receptor molecules and clonal selection and thereby produce a large repertoire of individually different, high-affine receptors as postulated by Burnet. This allows the immune system to increase defense capabilities with further exposures of the host to the same pathogen (memory effects).

2.1. Innate immunity

The innate immune system represents a phylogenetically very old way to defeat pathogens at utmost efficiency. The innate immune response is the first line of host defense. It is responsible for immediate recognition and defense of microbial invasion. Thus, the components of the innate immune system often eliminate microbes even before adaptive immunity gets activated. Serving the need of rapid immune reactivity, the innate immune response is rather non-specific and does not provide long-lasting or protective immunity to the host organism. Epithelial cells build up a first physiological barrier to invading microbes and secret soluble cytokines and antimicrobial peptides, i.e. defensins. The cellular fraction of the innate immune system is composed of circulating or resting cells, i.e. dendritic cells (DCs), macrophages, neutrophils or natural killer cells that act as sentinels of innate immunity. Upon invasion of microbes that have penetrated the epithelial barrier, these cells get activated and start secreting cytokines and chemokines to recruit further immune cells to the site of infection. Innate immune cells combat pathogens by phagocytosis, secretion of antimicrobial factors and induction of an inflammatory reaction. Further, macrophages and dendritic cells are able to present microbial components on their cell surface, therefore acting as antigen presenting cells (APCs). APCs that were activated by contact with microbes in turn stimulate the adaptive immune system; these cells represent a pivotal interface between innate and adaptive immunity.

2.2. Recognition within the innate immune system

Ancient germline encoded receptors, the pattern-recognition receptors (PRRs), constitute the sensors by which innate immune cells recognize infectious microbes (1, 2). The respective ligands that bind to PRRs are highly conserved microbial molecules which were initially being termed pathogen associated molecular patterns (PAMPs). These patterns represent

molecules that are essential for microbial survival. Therefore, PAMPs are unlikely to mutate during evolution (3, 4). PRRs enable the host to detect a broad range of pathogens using only a limited number of receptors. Recognition is quick without the need of somatic recombination that is intrinsic to the lymphatic cells of the adaptive immune system (3, 5, 6). PRRs are present in the cytoplasm or on the cell surface of innate immune cells. In opposition to the concept that PRRs only recognize non-self structure, recent discoveries have led to the perception that PRRs are also able to detect host structures under certain conditions (4, 7).

2.3. Pattern recognition receptors

PRRs can broadly be divided into three groups based on their cellular location: (I) serum or tissue fluid, (II) surface or (III) cytoplasm and intracellular compartments like endosomes. They can be further subdivided into related families based on their molecular structure and/or function. Toll like receptors (TLRs), Retinoic acid inducible gene-I (RIG-I)-like receptors, Aim2-like receptors (absent in melanoma 2), Ifi (interferon inducible protein) and NOD (nucleotide-binding and oligomerization domain)-like receptors (NLRs) belong to these families (8-12). Further proteins with C-type lectin-binding domains (C-type lectin receptors, CLRs) have various functions but also serve as pattern recognition receptors in innate immunity (13). All of these receptors are composed of ligand binding domains and signalling domains. They are regulated either through dimerization and oligomerization or through conformational changes by enzymatic activities. Subsequently, signalling domains are activated and recruit adaptor proteins that initiate intracellular signalling.

2.3.1. Cytoplasmic receptors

Particular subgroups of PRRs reside in the cytosol and are expressed in a broad range of somatic cells, thus they are not restricted to professional innate immune cells like dendritic cells or macrophages. RIG-I-like receptors (RLRs) were originally described as RNA helicases (14). Beside this function, the family members RIG-I and MDA5 are able to recognize viral components, i.e. viral RNA, and consequently initiate an immune response (15, 16). NLRs represent the largest family of PRRs in humans (17). All NLRs possess a carboxy-terminal LRR domain that is responsible for ligand binding (11). NLRs are grouped in NODs and NALPs (11, 18). NODs mediate signaling upon ligand binding that leads to NFκB activation. NALPs activate caspase-1. Upon activation, NALPs undergo conformational changes and build up multimeric protein complexes which are termed inflammasomes (19, 20) (Fig. 2-1). The formation and activation of inflammasomes results in secretion of inflammatory cytokines, IL1β and IL18 after cleavage of their pro-forms by caspase-1 (21). In addition, Aim2-like receptors recognize foreign, cytoplasmatic dsDNA (12). Activation of Aim2-like receptors induces the recruitment of the adaptor protein ASC. Thereupon, Procaspase 1 is recruited to ASC via its CARD domain leading to the formation of an inflammasome. Within the NALP- as well as the Aim2-inflammasome, Procaspase 1 is cleaved to the active form, caspase 1, that results in the release of IL1-β (21-23). Ifi belongs to the same family as Aim2 and recognizes pathogenic dsDNA, leading to the induction of Type I IFNs (24, 25). In addition, several other DNA sensing receptors have been implicated,

including Lrrfip 1 (Leucin-reach repeat (in Flightless 1) interacting protein 1) and DExD/H box helicases DHX 9 and DHX 36 (12).

2.3.2. Surface receptors

Toll like receptors (TLRs)

The Toll protein was first described to play a pivotal role in the development of the fruit fly *Drosophila melanogaster* (26). However, loss-of-function mutations within the Toll protein also resulted in higher susceptibility of *Drosophila melanogaster* to fungal infection. This obsevation hinted towards another important function of Toll as a pathogen sensing receptor of the immune system (27, 28). The TLRs represent the mammalian homologes to the Toll-protein. TLRs are either expressed in the membranes of intracellular compartments, namely endosomes (TLR3, 7, 8 and 9) or on the cell surface (TLR1, 2, 4, 5, 6 and 10) (29-31). So far, 10 TLRs are described in humans (TLR 1-10) and 12 TLRs are described in mice (TLR 1-9 and 11-13) (32). Proximal events of activated TLRs are initiated by TIR-domain-containing adaptor molecules (TIRAP) or MyD88 adptor like proteins (MAL) (33). Most of the TLRs, like TLR2, TLR4, TLR6 or TLR9 employ the central adaptor protein myeloid differentiation factor 88 (MyD88), TLR 4 is capable of utilizing also TIR-domain- containing adaptor inducing IFN-β (TRIF; also known as TICAM1) and TLR3 only signals via TRIF (33-35). MyD88-dependent signalling cascades additionally use another adaptor molecule for the signal transduction, namely TIRAP. TLR-mediated MyD88 recruitment activates a conserved inflammatory pathway leading to activation of mitogen-activated protein kinases (MAPK) and translocation of active NF-κB into the nucleus (36-38). TRIF dependent signalling (i.e. TLR3) activates interferon regulatory factors (IRF) in a MyD88-independent manner. IRF activation links TLR-signalling to IFN secretion (39, 40). In mice, it is shown that TLRs not only activate NF-κB upon ligand binding, but are also able to activate other transcription factors like nuclear factor of activated T-cells (NFAT) (41).

Non-TLR PRRs

Although the secretion of cytokines and attraction of adaptive immune cells is mainly mediated by the TLRs, non-TLR PRRs can contribute to this response. They either present distinct PAMPs to the TLRs, such as described for CD36, which presents diacylglycerids to TLR2-TLR6 heterodimers, or CD14 that presents lipopolysaccharide (LPS) to the TLR4-MD2 complex (42, 43). On the other hand, non-TLR PRRs might also directly contribute to the inflammatory responses to invading microbes. Among the non-TLR PRRs, Dectin-1 is the only example that can act independently of TLRs, thereby inducing pro-and anti-inflammatory cytokines (44, 45). Dectin-1 is a membrane bound C-type lectin receptor that recognizes carbohydrate structures in the cell wall of different microbes, i.e. fungi. Figure 2-1 provides an overview of pathogen recognition by pattern-recognition receptors.

C-type lectins

The term C-type lectin was established to distinguish between Ca^{2+} dependent and Ca^{2+} independent carbohydrate binding lectins. C-type lectin receptors (CLRs) share at least one carbohydrate recognition domain that contains conserved residues and determine the

specificity of the respective CLR. The family of C-type lectin receptors consists of soluble and transmembrane forms (table 2-1) (46).

CLRs are central for fungal recognition but also contribute to recognition of other microbes. They interact with pathogens primarily through the recognition of fucose, mannose and glucan carbohydrate structures. Importantly, CLRs are highly specific for the glucan β-(1-3) linked backbone structures within these carbohydrates (45-47). In DCs, it is shown that recognition by CLRs leads to pathogen internalization and subsequent antigen presentation (48, 49). Several CLRs, such as DC-specific ICAM3-grabbing non-integrin (DC-SIGN) induce signalling cascades that modulate TLR-induced gene expression on the transcriptional or post-transcriptional level (46, 50). By contrast, other CLRs like Dectin-1 are also able to induce gene expression following carbohydrate recognition independently of other PRRs (51-54).

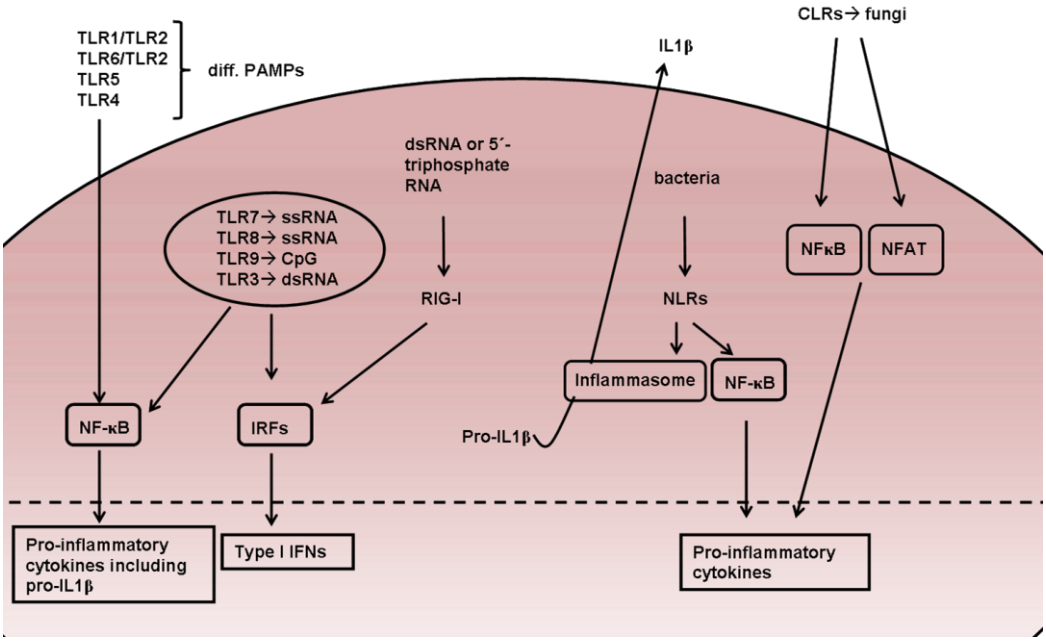

Fig. 2-1 Overview of some different pattern recognition receptors in myeloid cells

Stimulation of different PRRs entails the production of pro-.and anti-inflammatory cytokines, chemokines or interferons. TLR1, 2, 4, 5 and 6 are expressed on the cell surface and lead to activation of NF-κB upon stimulation, whereas other TLRs like TLR3, TLR7, TLR8 and TLR9 are situated within endosomes or are recruited after internalization of stimuli, i.e. CpG DNA (endocytotic pathway). These endosomal TLRs mainly activate IRF (interferon regulatory factor) transcription factors, for example after viral infection. Receptors located within the cytoplasm are able to recognize different PAMPs. dsRNA or 5´-triphosphate RNA, derived from viruses, are recognized by RIG-I like receptors (RLRs). These receptors are expressed in the cytoplasm of most cell-types, whereas other PRRs, like TLRs are only found in innate immune cells, i.e. DCs and macrophages. RLR binding leads to activation of IRF transcription factor. Further, NLRs (Nod like receptors) are also present in the cytoplasm of immune cells. Upon activation, NLRs form inflammasome complexes and activate NF-κB. Active caspase 1 within inflammasome complexes cleaves pro-IL1β to active IL1β. C-type lectin receptors (CLRs) are situated on the plasma membrane and are activated via fungal cell wall particles, viruses (HIV) or certain bacteria. Upon ligation, signalling cascades induce activation of transcription factors like NFAT (nuclear factor of activated T-cells) or NF-κB. Additionally, CLRs mediate phagocytosis of bound pathogens.

The CLR Dectin-1 is the onliest known PRR that is able to signal in a TLR-independent manner. On the other hand, it could be demonstrated that Dectin-1 signalling pathways can influence TLR signalling as depicted in more detail in later sections (2.2.1.2.1, 2.3.1) (46, 53, 55, 56). One important aim of this study was to clarify the question if Dectin-1, once stimulated, is able to negatively influence TLR signalling by inhibition, i.e. through inhibitory molecules like SOCS (suppressors of cytokine signalling). Therefore, Dectin-1 and its downstream signalling molecules are discussed in the following chapters in more detail.

Additionally, SOCS proteins and possible links between SOCS and Dectin-1 are described below.

Table 2-1 C-type lectin receptors and pathogen recognition (46)

CLR	Glycan PAMPs and *in vivo* ligands	Expression of CLR	Immunological outcome
Group I CLRs (mannose receptor family)			
Mannose receptor (CD206)	High mannose, fucose and sulphated sugars, *M. tuberculosis, M. kansasii, Francisella tularensis, Klebsiella pneumonia and Streptococcus pneumonia.* HIV-1 and dengue virus, *C. albicans, Cryptococcus neoformans* and *P. carinii. Leishmania spp.*	Myeloid DCs and macrophages	Phagocytosis and antigen presentation
Group II CLRs (asialoglycoprotein receptor family)			
Dectin2 (CLEC 6A)	High mannose, *M. tuberculosis, A. fumigatus, C.albicans,*	Myeloid DCs, pDCs, macrophages, B cells and neutrophils	TNF and IL6 production
Mincle (CLEC 4E)	α-mannose, *Malassezia spp.*	Myeloid DCs, monocytes and macrophages	Induction of TNF, IL10 and production of CXCL2
DCIR (CLEC4A)	HIV	Myeloid DCs, monocytes, macrophages, Bcells, neutrophils	Inhibition of IL8-induced TNF and IL12 production, inhibition of IL9 induced TNF and IFNα production
DC-SIGN (CD209)	High mannose and fucose, *M. tuberculosis, M. leprae, BCG, Helicobacter pylori, Lactobacilli spp.*	Myeloid DCs	Upregulation of TLR induced IL10 production, induction of Th1, Th2, Th17 and Treg differentiation, inhibition of Th1 cell differentiation, antigen presentation
Group II CLRs (asialoglycoprotein receptor family, Dectin-1 subfamily)			
Dectin-1 (CELC7A)	β-1,3-glucan, *M. tuberculosis, M. abscessus, C. albicans, Aspergillus fumigatus, Pneumocystis carinii, Penicillium marneffei, Coccidioides posadasii* and *Histoplasma capsulatum*	Myeloid DCs, monocytes, macrophages and B cells	Induction of Th1 and Th17 cell differentiation through induction of IL1β, IL6, IL23 and IL12, induction of TNF and CXCL2 production, phagocytosis (DCs), LTB_4 synthesis
MICL (CLEC12A)	ND	Myeloid DCs, monocytes, macrophagesand neutrophils	Inhibition of TLR4 induced IL12 production

Dectin-1

Dectin-1 is a type II transmembrane receptor that comprises a single extracellular carbohydrate recognition domain (CRD) connected to the intracellular domain via a stalk region of variable length. The cytoplasmatic tail of Dectin-1 is built up of an immunoreceptor tyrosine-based activation motif (ITAM)-like motif which is involved in signalling initiation (Fig. 2-2 A)(44, 52, 57). In contrast, other known CLRs like NK-cell-receptor-like C-type lectins (i.e. NKGD2 expressed on NK cells) associate with intracellular adaptor signalling molecules, i.e. DAP12, to carry out cellular activation (46). Dectin-1 was originally discovered to be restricted to dendritic cells; therefore, it was named “dendritic-cell-associated C-type lectin-1”. The receptor is now known to be expressed by many other cell types like macrophages or

monocytes as depicted in Table 2-1 (44, 46, 58, 59). Additionally, it was found that Dectin-1 is expressed in epithelial cells of the lung and the intestine which emphasizes its role in immune surveillance (58, 60). The molecular ligands for Dectin-1 are specified as β-(1-3)- and/or β-(1-6)-linked glucans, named carbohydrate polymers that have immunomodulatory activities. β-glucans are found, amongst others, in the cell wall of the yeast *Saccharomyces cerevisiae* where they are called Zymosan (44, 45, 54, 61).

Zymosan is composed of repeating glucose units connected by β-1,3-glycosidic linkages. Besides Dectin-1, Zymosan is described to bind to TLR2 or TLR6, thus acting in a collaborative way with TLRs (53, 55, 62, 63). Compared to other C-type lectins, Dectin-1 is an atypical C-type lectin receptor as the carbohydrate recognition takes place independent of metal-ions (45). Mutagenic analysis has indicated that at least two residues, Trp221 and His223, in the carbohydrate recognition domain are essential for carbohydrate binding (64). In addition to its β-glucan ligands, Dectin-1 is proposed to detect an endogenous T-cell ligand as it is described that Dectin-1 can bind to $CD4^+$ and $CD8^+$ Th-cells *in vitro* thereby increasing their proliferation (57). Therefore Dectin-1 has been suggested to act as a co-stimulatory molecule (57, 65).

Concerning downstream signalling in CLRs, Dectin-1 is the first example of non-TLR PRRs that can mediate intracellular signalling without the contribution of any other PRRs (46, 54). Upon ligand binding, Dectin-1 induces the production of various pro- and anti-inflammatory cytokines and chemokines, including tumor-necrosis factor, CXC-chemokine ligand 2 (CXCL2, also known as MIP2), IL2, IL10 and IL12 (63, 66-68). In addition, it can induce respiratory burst and antigen uptake via phagocytosis as described for DCs (69, 70). Importantly, Zymosan or other β-glucans are able to activate NF-κB in DCs but not in macrophages (71). The group of Goodridge *et al.* could show that Dectin-1 signals alone are insufficient for NF-κB activation and cytokine production. Whereas the adaptor molecules of Dectin-1 CARD9 (caspase recruitment domain-containing protein 9), Bcl10 (B-cell lymphoma 10) and MALT1 (mucosa associated lymphoid tissue lymphoma translocation gene 1) led to NF-κB activation in DCs, these proteins did not activate NF-κB in macrophages. CARD9 was rather recruited to phagosomes where it coordinated signalling to p38 MAPKs (71) (Fig. 2-2B).

On the one hand, Dectin-1 is sufficient to induce most of the described responses in DCs, but also collaborates with signals from TLR2 and TLR6 (i.e, TNF, IL12) (62, 63). The TLR ligand in Zymosan is unknown as TLRs do not bind to β-glucans. Signalling abilities of Dectin-1 depend on the intracellular ITAM like motif (Fig. 2-2 A). Sequences within this motif are also found in other activation molecules like DAP12 or Fc receptors (72-74). Upon ligand binding, YXXI/L (X is any amino acid) repeats within the ITAM like motif get phosphorylated by Src tyrosine kinases. ITAM phosphorylation leads to docking and phosphorylation of the SH2 domains of Spleen-tyrosine kinase (Syk), possibly by Dectin-1 dimerization (52, 70). The requirement for Syk in Dectin-1 signalling is cell type specific, as it is shown that in macrophages Syk is only needed for induction of the respiratory burst (69, 70); whereas in DCs, Syk is involved in cytokine production (i.e., IL10, IL2) (52). Activation of Syk leads to the recruitment of CARD9 (71, 75). Gross *et. al.* reported that CARD9 knockout mice are more susceptible to *Candida albicans* infections. Further, CARD9-deficient DCs are defective

in NF-κB activation upon ligand binding (75). In addition to CARD9, Zymosan stimulated DCs recruit Bcl10 and MALT1 to build up a complex with CARD9 (Fig. 2-2 B). The CARD9/Bcl10/MALT1 complex ultimately leads to release of NF-κB subunits from the NF-κB inhibitor IκBα, inducing nuclear transport of the subunits and subsequent activation of transcription of pro-and anti-inflammatory genes (Fig. 2-2 B) (44, 46, 75, 76).

Adjacent to Syk, Dectin-1 activation can lead to phosphorylation and activation of the serine/threonine kinase Raf1 by Ras, which drives phosphorylation of the NF-κB subunit p65 at Ser276 as shown for DCs (Fig. 2-2 B). Phosphorylated p65 serves as a docking site for the Histone acetyl transferase CREB binding protein (CBP) to acetylate p65 at different sites. This results in a prolonged and increased NF-κB activity (76, 77).

In addition to this canonical NF-κB pathway, a recent study has shown that activation of the Syk pathway by Dectin-1 also leads to the induction of the non-canonical NF-κB pathway, which mediates the processing of p100 subunits to p52. Thereupon, p52 couples with RelB and p52-RelB subunits enter the nucleus (75, 76). In addition, Dectin-1 was described to activate the transcription factor NFAT (nuclear factor of activated T-cells) very rapidly after stimulation as well (61) (Fig. 2-2). It is shown that NFAT contributes to IL10 induction in DCs in a Ca^{2+} dependent manner. Whether NFAT takes part in IL10 induction in macrophages is still controversially discussed (61, 66). On the one hand, it could be shown by Goodridge *et al.* that the transcription factors early growth response factor (egr) 2 and 3 are clearly induced upon Zymosan stimulation of Raw 264.7 cells (61). The family of egr transcription factors (egr1-egr3) is described to be induced by NFAT. Triggering of Dectin-1 led to the secretion of IL10 in a NFAT and egr dependent manner (61). In contrast, it had been shown by the group of Kelly *et al.* that other transcription factors may participate in IL10 production as well (66). They treated human macrophages with a NFAT inhibitor and observed only a modest decrease in IL10 production. It was rather found out that ERK as well as the transcription factor CREB mediate IL10 secretion upon Zymosan stimulation in these cells (66).

Upon engagement of Dectin-1 by pathogens like fungi or mycobacteria, downstream signalling pathways described above are activated and well described (44, 46). Importantly, the activation of signalling pathways within innate immune cells via receptor-bound cytokines or PRR ligands is only transient to prevent overwhelming immune reactions. Thus, a hallmark of protective immunity during infection is the regulated secretion of pro-and anti-inflammatory cytokines produced via these pathways (i.e. IL10, IL2, TNFα, IL12) (78). Several intracellular inhibitors are described. Among these, constitutive active tyrosine phosphatases (SHP1: SH2-containing phosphatase 1, PTP1B: protein tyrosine phosphatase 1 B, TCPTP: T-cell protein tyrosine phosphatase) and lipid-phosphatases (PTEN: phosphatase and tensin homolog deleted on chromosome ten, SHIP: SH2-containing 5'-inositol phosphatase) inactivate the respective activated receptors (79-82). The abrogation of the signalling is mediated by dephosphorylation of the tyrosine-phosphorylated intracellular ITAM motifs of the respective receptor. Further, proteins of the PIAS family (protein inhibitor of activated STAT) are described that interact with activated STAT molecules and thus inhibit binding of STAT transcription factors to the DNA (78, 83).

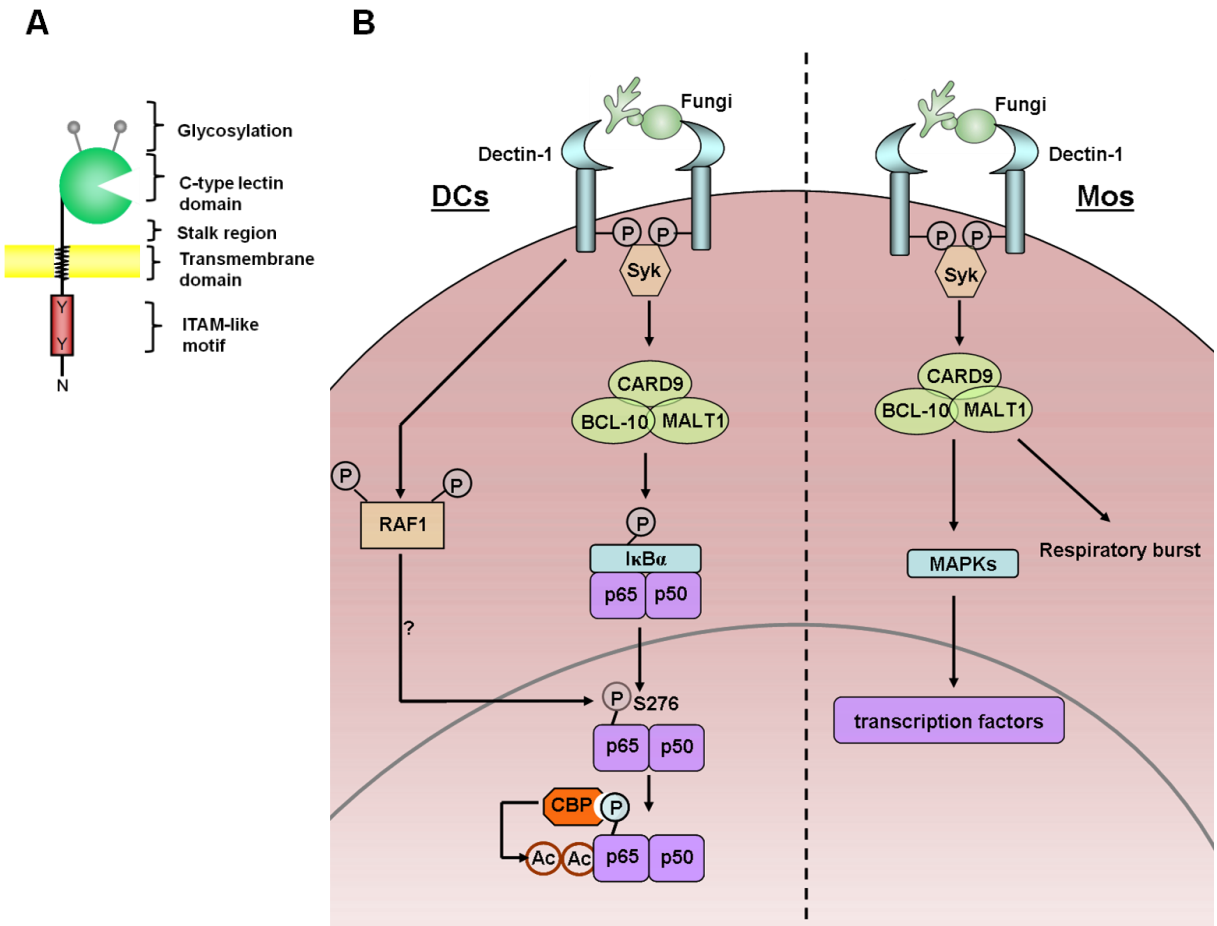

Fig. 2-2 Intracellular signalling by Dectin-1 (44)

(A), simplified schematic structure of Dectin-1 monomeric receptor. (B), Schematic overview of Dectin-1 signalling through Syk. Left site, dendritic cells (DCs): The binding of fungi to Dectin-1 induces phosphorylation of the YXXI/L motif in its cytoplasmic domain. Syk kinase is recruited to the dimerized phosphorylated receptor. Activated Syk (pSyk) leads to the formation of a complex composed of CARD9, Bcl10 and MALT1. This induces the activation of IκB kinase complex. IKKβ phosphorylates IκB, whereupon NF-κB subunits are released and translocate into the nucleus. In a Syk independent manner, Dectin-1 ligation also activates the serin/threonin kinase Raf1 via the Ras protein which leads to phosphorylation of p65 at S276 residue. Phosphorylated p65 acts as a docking site for CBP. The histone acetyltransferase CBP thereupon acetylates p65 resulting in a prolonged NF-κB activity. Depicted are only signalling events that lead to the activation of the non-canonical NF-κB pathway. Right site, macrophages (Mos): In contrast to DCs, macrophages do not activate NF-κB upon Dectin-1 ligand binding. Phosphorylation of Syk on the one hand is described to activate MAPKs. MAPKs thereupon recruit transcription factors, i.e. CREB, AP-1 or ATF1.On the other hand, pSYK induces respiratory burst.
Ac, acetylation; BCL-10, B cell lymphoma 10; CARD9, caspase recruitment domain family member 9; CBP, CREB binding protein; DCs, dendritic cells; Mos, macrophages; Syk, spleen tyrosine kinase; Raf1, rapidly growing fibrosarcoma 1.

In addition, SOCS (suppressor of cytokine signalling) proteins constitute a class of intracellular *feedback* inhibitors that are not induced until activating signals are transferred into the cell. It could be demonstrated that the CLR DC-SIGNR1 is able to induce SOCS1 upon infection of murine DCs with *Mycobacterium tuberculosis* (84). Additionally, the stimulation with manLAM, a specific ligand for DC-SIGNR1 led to induction of SOCS1 as well (84). However, if Dectin-1 is able to mediate SOCS1 expression is not known yet. One important aim of this study was to analyze if SOCS1 can be induced upon stimulation of Dectin-1 as well as the underlying pathway that leads to SOCS1 expression.

2.4. Suppressors of cytokine signaling (SOCS)

One group of proteins that negatively regulates intracellular signaling is termed *suppressors of cytokine signaling (SOCS)* (85). SOCS proteins are expressed in many cell types, i.e. dendritic cells, macrophages and also in T-cell subsets (86-88)
SOCS proteins were found to be induced via type I and type II cytokine receptors in a Jak/STAT dependent manner. Upon activation, they exert their inhibitory function on the activated receptor complex. Thus, they inhibit further signalling and therefore can be classified as negative feedback inhibitors. The SOCS family is composed of eight structurally related proteins, namely SOCS1-SOCS7 and CIS (cytokine inducible SH2 domain containing protein). SOCS1, SOCS2, SOCS3 and CIS are the best characterized members of the SOCS family (89). Each of the SOCS proteins exhibits a central SH2 (SRC homology 2) domain that is responsible for the binding to phosphorylated tyrosine residues (90, 91), an amino-terminal domain of variable length and divergent sequence (92) and a carboxy-terminal 40-amino-acid domain (known as the SOCS-box). This SOCS-box is conserved throughout the SOCS family members. The SOCS-box interacts with elongin B and elongin C, cullin-5 and RING-box-2 (RBX2), which recruits E2 ubiquitin transferase. Thus, CIS-SOCS-family proteins act as E3 ubiquitin ligase and mediate protein degradation (93, 94). In addition, SOCS1 and SOCS3 can directly inhibit JAK tyrosine kinase activity through their kinase inhibitory region (KIR) by acting as a pseudosubstrate (90, 95). The central SH2 domain determines the target of each SOCS or CIS protein. The SH2 domain of SOCS2, SOCS3 and CIS binds to phosphorylated tyrosines on activated cytokine receptors (89-91). SOCS3 binds to gp130-related cytokine receptors (96). The SH2 domain of SOCS1 directly attaches to Y1007 within the activation loop of JAK 2 (90, 97). Additionally, SOCS1 has been shown to bind directly to the type I IFN receptor (IFNAR) and the IFNγ receptor (IFNGR) (98, 99). As an inhibitor, SOCS1 regulates IFNAR1 specific signalling and abrogates phosphorylation of STAT1 (signal transducer and activator of transcription) transcription factor. Importantly, SOCS1 first binds to the activated IFN receptors and thereupon it interacts with JAK2 (89, 99). It was demonstrated that beside type I and type II interferons, SOCS proteins are induced by many different ligands in a JAK/STAT independent manner. For example, insulin or different TLR ligands have been proposed to induce SOCS1, SOCS2, SOCS3 and CIS (100, 101).

2.4.1. The role of SOCS1 in the immune system

Recently, it has been proposed that SOCS 1 and other SOCS proteins, e.g. SOCS3 are not only induced by cytokines like IFNs but also by TLR ligands such as LPS or CpG-DNA in a JAK/STAT independent manner (100, 102-104). Dalpke *et.al.* could show that this induction was fast and independent of any paracrine factor (103). Dendritic cells isolated from type 1 receptor deficient (IFNAR1$^{-/-}$) mice were still able to induce SOCS1 to the same level as compared to WT cells upon stimulation by different TLR ligands (103). Additionally, stimulation of the TNF-receptor led to an induction of SOCS1 in a JAK/STAT independent way (100). Further, Ramana *et al.* could show that upon stimulation with IFNγ, SOCS2 and SOCS3 were induced via a JAK1-dependent, but STAT1-independent pathway (105).

SOCS1 deficient mice are hyperresponsive to LPS, leading to an increase in TNF and IL12 production. Mice that are deleted for SOCS1 die within the first weeks after birth because of a multi-inflammatory syndrome caused by an infiltration of multiple organs with inflammatory cells (86, 106, 107). The increased production of IFNγ is indicted to be a crucial mediator of this multiorgan inflammation (108). However, as IFN$\gamma^{-/-}$/SOCS1$^{-/-}$ are still very sensitive to LPS induced shock, IFNγ independent mechanisms may exist (109, 110). Macrophages and DCs from SOCS1$^{-/-}$ mice produce increased levels of pro-inflammatory cytokines, such as TNF, IL12 and IFNγ upon ligation of TLRs (111). Several mechanisms have been proposed for the suppression of TLR induced cytokines by SOCS1. A direct effect of SOCS1 on the TLR-NFκB pathway could be shown. SOCS1 directly bound to p65 subunits of NF-κB, thereby facilitating ubiquitination and degradation of p65 (112-114). SOCS1 also bound tyrosine phosphorylated MAL (MyD88-adaptor like protein) through its interaction with Bruton´s tyrosine kinase (BTK) and induced ubiquitination and subsequent proteasomal degradation of MAL (115). Another inhibitory function of SOCS1 on IFNγ/JAK/STAT independent pathways could be shown by different groups. JAK/STAT independently induced SOCS1 was able to bind to IRAK, thereby inhibiting IL4 signalling (109, 110). Thus, SOCS1 could be responsible for LPS tolerance, probably in an INFγ dependent as well as independent way as described above. In addition to the NF-κB pathway, SOCS1 might regulate the stress-activated mitogen-activated protein kinases (MAPKs) JNK (c-JUN N-terminal kinase) and p38 by binding to ASK1 (apoptosis signal-regulating kinase 1), which is an upstream activator of both JNK and p38 (116). These data suggest that SOCS1 could interfere also with TLR signalling.

Also, Dectin-1 was shown to influence TLR signalling as presented for TLR2, TLR4 and to some extent for TLR 9 (63). Dennehy *et al.* demonstrated that co-ligation of Dectin-1 with TLR2 or TLR4 synergistically triggered IL10, IL23, TNF and IL6 production. On the other hand, IL12 secretion was suppressed upon co-ligation of Dectin-1 and TLR2 or TLR4. These results are similar to recent findings demonstrating that human DCs down-regulate IL12 expression when stimulated via TLR2 and TLR7/8 simultaneously (117). Additionally, it has been described that Dectin-1 inhibits IL12 expression induced by a number of different TLRs beside TLR2, such as TLR9 or TLR7 (55, 63). On the other hand, it has been shown that Dectin-1 is only able to down-regulate TLR2 induced IL12 secretion (118). Thus, it had to be clarified within this study if the observed reciprocal regulation of IL12 and IL10, IL6, IL23 and TNFα is relevant for co-ligation of Dectin-1 and different TLRs as well.

However, the signaling mechanism by which IL12 is downregulated following co-ligation of Dectin-1 and TLR2 has not been defined yet. As already described, SOCS1 seems to additionally modulate the intracellular network in TLR triggered cells; independently of JAK/STAT dependent pathways. Therefore, the question was raised whether SOCS1 could be induced in innate immune cells like dendritic cells and macrophages via engagement of Dectin-1, independently of TLRs. Furthermore, a possible influence of Dectin-1 induced SOCS1 on TLR stimulated cells had to be investigated in this study.

Other studies focused on the properties of SOCS1 expressed in macrophages and dendritic cells on T-cell lineages since Th17 cells are proposed to be crucial in protection against fungal pathogens.

2.5. T cell responses to fungi

Dendritic cells and macrophages provide an important link between innate and adaptive immunity. They are characterized by a high capacity of antigen processing, migration to lymphoid organs and expression of co-stimulatory molecules for lymphocyte activation. DCs have been proposed to direct the type of T helper (Th)-cell differentiation through their cytokine profile. For example, high amounts of IL12 produced by innate immune cells promote Th1-cell differentiation. Otherwise, TGFβ, IL6 and IL23 promote Th17 differentiation (119-121).

Recognition of β-glucan structures presented by fungi like *Candida albicans* to Dectin-1 on innate immune cells results in protective anti-fungal immunity (120, 122). Whole pathogens, i.e. *Candida albicans,* activate a panel of different PRRs on APCs (46). Thus, interactions between intracellular pathways influence the later T-cell response, e.g. via induction of Th17-cells and concomitant repression of other Th-cell types like Th2-cells (123, 124).

Th17-cell activation mainly occurs through the Dectin-1-Syk-CARD9 and mannose receptor signalling pathway in dendritic cells and macrophages, leading to IL17 production of $CD4^+$ Th-cells (125). Activation of this pathway in DCs leads to the secretion of pro-inflammatory cytokines, including IL6, IL23 and TNFα but less IL12 (55, 63, 125). Fungi or fungal particles not only consist of Dectin-1 ligands, but also bind to TLRs, as shown for TLR2 and TLR6 (44). This co-ligation of TLRs and Dectin-1 by fungal particles may be responsible for the low IL12 expression, as described above (2.3.1).

In conjunction with these findings, it has been described *in vitro* that the induction of Th17-cells by Dectin-1 triggered DCs is mediated through high amounts of IL6 and IL23 (125). *In vivo,* infection with *Candida albicans* promotes the differentiation of Th1 and Th17 responses (126, 127). Importantly, mice deficient in components of the Dectin-1 signalling pathway, i.e. CARD9, were not able to induce Th17 responses and were more susceptible to infections with *C. albicans* (128, 129). The different regulation of Th17 inducing cytokines (high amounts of IL6, IL23, TGFβ) and Th1 inducing cytokines (low levels of IL12) upon fungal infections suggests a collaboration between Dectin-1 and TLRs or other PRRs.

Again, the question arose whether SOCS proteins induced in innate immune cells could play a role as modulators in this cross-talk between Dectin-1 and TLRs. This would link SOCS proteins that are expressed in innate immune cells to the different Th-cell responses. Indeed, SOCS proteins produced by DCs were linked to DC activation followed by T-cell differentiation (89, 130). It could be shown that the $CD11c^+CD8\alpha^+$ DC subset produced larger amounts of IL12 and IFNγ and expressed lower levels of SOCS1 (130, 131). Additionally, SOCS1-siRNA treated DCs produced enormous amounts of IL12, the key cytokine to induce Th1 cells (84). SOCS3 also seems to manipulate Th-cell responses. SOCS3-transduced DCs have been shown to be highly effective inducers of Th2-cell differentiation *in vitro* and *in vivo* (132). Injection of WT mice with SOCS3 transduced DCs suppressed the development of *experimental autoimmune encephalomyelitis (EAE),* a Th17-cell mediated autoimmune disease. Suppression of EAE by SOCS3-transduced DCs might be due to the reduced Th17-cell differentiation as a result of reduced IL23 production (132). Thus, the expression of SOCS proteins in DCs or macrophages might have a crucial role in the balance between cytokines inducing different Th-cell subsets, i.e. Th17 and Th1 or Th2 (133). Furthermore,

SOCS proteins have been shown to be expressed in Th-cells as well. It has been described that SOCS1 has an important role in Th17-cell development as it is highly expressed in differentiated (memory) Th17-cells (89, 134) . The antagonistic effect of IFNγ on Th17-cell differentiation is tightly regulated by SOCS1 (130, 134). Thus, it was suspected that SOCS1 expressed by memory Th17-cells could be a mechanism for IFNγ resistance of memory Th17-cells.

However, a direct link between SOCS1 produced in APCs and the Th-17 cell response upon infection with pathogens like *Candida albicans* or other fungi was not described at the beginning of this study.

2.6. Objectives of this work

As illustrated in the chapters above, immune reactions upon infection, i.e. with fungi, have to be controlled very precisely in order to prevent overwhelming immune reactions. Thus, the interplay between different types of pattern recognition receptors seems to be crucial. Intracellular signalling following ligand binding and subsequent cytokine release is regulated very accurately by negative regulation, for example through suppressors of cytokine signalling (SOCS) proteins. The aim of this work was to analyze the induction of SOCS1 in Dectin-1 triggered myeloid cells and to study the impact of SOCS1 on different signalling pathways.

First of all, it should be analyzed whether murine bone marrow derived dendritic cells (BMDCs) and macrophages (BMMs) are capable of inducing SOCS1 upon stimulation of Dectin-1. SOCS1 expression was measured on mRNA and protein level after stimulating Dectin-1 with different concentrations of a specific ligand (depleted Zymosan).

In this context, the downstream signalling pathway leading to SOCS1 production should be examined in detail and the possible differences in BMDCs compared to BMMs should be identified, i.e. the contribution of NF-κB transcription factor. As MAPKs are described in conjunction with SOCS induction as well as Dectin-1 downstream signalling, one issue was to identify possible candidates involved in SOCS1 expression.

Finally, the outcome of SOCS1 expressed in BMMs or BMDCs should be examined. Therefore, on the one hand, myeloid cells from SOCS1$^{-/-}$ mice should be analyzed to ascertain if SOCS1 could function as negative feedback inhibitor in Dectin-1 signalling pathway. On the other hand, the impact of SOCS1 induced via Dectin-1 as a cross-regulator for TLR signalling should be analyzed. Further, a possible link between innate and adaptive immune cells should be analyzed. Therefore, it should be assessed whether SOCS1 knockout myeloid cells could influence T-cell priming in a co-culture setting.

Clarifying of these issues should help to disclose a role for Dectin-1 induced SOCS1 as an inhibitor within PRR signalling pathways.

3. Materials and Methods

3.1. Materials

3.1.1. Instrumentation

Instruments used throughout this study are listed in table 3-1.

Table 3-1 List of instruments and suppliers

AutoMACS	Miltenyi Biotec, Bergisch-Gladbach
Balance	EW600-2M, Kern & Sohn GmbH, Balingen
Blotting chamber	PerfectBlue™ Tank-Elektroblotter Web S, Peqlab, Erlangen
Centrifuges	1. Biofuge stratos, Multifuge 3S-R, Hereaus Instruments, Hanau 3. Biofuge fresco, Heraeus Instruments, Hanau 4. Biofuge pico, Heraeus Instruments, Hanau
Counting chamber	Neubauer 0.00025 mm2/0.1 mm, Brand GmbH, Schwerin
Electrophoresis chamber	PerfectBlue Mini ExM, Gelsize 14.4 x 10.2 cm, Peqlab, Erlangen
Flow Cytometry	BD FACS Canto (2-laser, 6colour) BD Biosciences, Heidelberg
Fluorescence-Inverse Microscope	Leica DMI 6000B, Leica Microsystems GmbH, Wetzlar
Gel Documentation-Sytstem (Chemiluminsecence detection	CN-3000.WL, Peqlab, Erlangen
Inverse Microscope	Axiovert 25 CFL, Carl Zeiss Jena GmbH, Jena
Heating Block	Thermomixer comfort, Eppendorf AG, Hamburg
Incubator	BBD6226, Heraus Instruments, Hanau
Magnetic shaking device	IKA® RCT basic, IKA®-Labortechnik, Staufen i.Br.
Microtiterplate Photometer	SUNRISE Absorbance Reader, Tecan, Salzburg, Austria
Orbital Shaker	1. Mini Rocker MR-1, Peqlab, Erlangen 2. Rocky®3D, Labortechnik Fröbel GmbH, Lindau 3. RS-24, Lab4You GmbH, Berlin
pH-Meter	Seven Easy, Mettler Toledo, Gießen
Power Supply	PowerSupply 1000/500, Bio-Rad, München
Reflected Light Microscope	Leica DMLS, Leica Microsystems GmbH, Wetzlar
Safety cabinet	Herasafe, Class II, Heraus Instruments, Hanau
SDS-PAGE System	PerfectBlue™ Twin S, Peqlab, Erlangen
Thermocycler	1.Quantitative RT-PCR: 7900HT Fast Real-Time PCR System, Applied Biosystems, Darmstadt 2. Primus 25 advanced®, Peqlab, Erlangen 3. Primus 96 advanced® Gradient, Peqlab, Erlangen
Spectrophotometer	NanoDrop®ND-1000 Spectrophotometer, Peqlab, Erlangen
UV platform	ECX-26M, Peqlab, Erlangen
Vortexer	MS 1, IKA® Inc., Wilmington, USA

3.1.2. Consumables

Single-use material is listed in table 3-2.

Table 3-2 List of consumables and suppliers

Blot membrane, Nitrocellulose	Immobilon-P Transfer, 0.45 µm pore size, MilliporeBillerica, USA
Blotting paper	Whatman® GB003, Whatman GmbH, Dassel
Caps	Eppendorf-caps 0.5 ml; 1.5 ml; 2 ml; Eppendorf AG, Hamburg
Cell culture bottles	Cellstar® 25 cm^2 / 75 cm^2 / 175 cm^2, Greiner Bio-One GmbH, Frickenhausen
Cell culture plates (flat bottom)	Cellstar® 6-/24-/96-well plates, Greiner Bio-One GmbH, Frickenhausen
Cell culture dishes	58 cm^2;145 cm^2, Nunc GmbH & Co. KG, Wiesbaden
Cell scraper	Disposable Cell Scraper, Greiner Bio-One GmbH, Frickenhausen
Cell strainer	100 µm, 70 µm cell strainer, BD Biosciences, Heidelberg
Cryo tubes	Nunc GmbH & Co. KG, Wiesbaden
ELISA plates	96 well, Greiner Bio-One GmbH, Frickenhausen
FACS tubes	BD Falcon™ 5 ml, BD Biosciences, Heidelberg
Gauge needle	MicrolanceTM 3, 27G, 0.4 mm x 19 mm, BD Biosciences, Heidelberg
High performance chemiluminescence film	Amersham, GE Healthcare, Buckinghamshire, UK
PCR plates	96 well plates, semi-skimed, Steinbrenner, Wiesenbach
PCR tubes	PP-PCR-tubes, Nat. 0.2 ml, Greiner Bio-One GmbH, Frickenhausen
Petri dishes	145 mm x 20 mm Greiner Bio-One GmbH, Frickenhausen
Pipet tips	1. TipOne® (0,1-100 µl; 1-200 µl; 100-1000 µl), Pipet tips, Starlab GmbH Ahrensburg 2. TipOne® (0,1-100 µl; 1-200 µl; 100-1000 µl), Filter tips, Starlab GmbH Ahrensburg
Protective Sheets	Absolute QPCR Seal, ABgene House, Epsom, UK
Sterile filter	Millex®-GS 0,22 µm, Millipore, Billerica, USA
Syringe	BD Discardit II 27G, 5 ml and 10ml, BD Biosciences, Heidelberg
Transwell inserts/plates	Costar® 6,5 mm Transwell®, pore size 0,4 µm, clear, 24 well, Corning
Tubes	Cellstar Tubes, (50 ml, 15 ml), Greiner Bio-One, Frickenhausen

3.1.3. Chemicals and reagents

If not stated otherwise, all solvents and chemicals were purchased from the following companies: Merck, Calbiochem (Darmstadt) and Sigma-Aldrich (Taufkirchen) with a purity of “pro analysi”. General reagents are listed in table 3-3.

Table 3-3 List of essential materials with suppliers used in this study

Aceton	Riedel-de Haën AG, Seelze
Acrylamid Rotiphenol	Roth, Karlsruhe
Agarose ultra pure	Gibco BRL, Life TechnologiesTM, Eggenstein
Ammoniumpersulfat (APS)	Sigma-Aldrich, Taufkirchen
Aprotinin	Sigma-Aldrich, Taufkirchen
Aqua ad iniectabilia (10 ml, 1000 ml)	Braun, Melsungen
Bovine Serumalbumin (BSA)	Sigma-Aldrich, Taufkirchen
Bromphenol blue	Sigma-Aldrich, Taufkirchen

Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Taufkirchen
Dithiothreitol	Sigma-Aldrich, Taufkirchen
dNTPs	MBI Fermentas, St. Leon-Rot
Dulbecco´s Modified Eagle Medium (DMEM, 1x)	Biochrom AG, Berlin
Acetic acid (100%)	Merck, Darmstadt
Ethanol	Riedel-de Haën AG, Seelze
Ethidiumbromide	Merck, Darmstadt
FACSClean™	BD Biosciences, Heidelberg
FACSFlow™	BD Biosciences, Heidelberg
Fetale Calf Serum (FCS)	Biowest, Nuaillé, Frankreich
Gel-loading buffer (6x) for agarose gels	MBI Fermentas, St. Leon-Rot
Glycerol	Sigma-Aldrich, Taufkirchen
Glycin	Sigma-Aldrich, Taufkirchen
Hydrochloric acid (HCl) (37%)	Merck, Darmstadt
IGEPAL® CA-630	Sigma-Aldrich, Taufkirchen
Isopropanol	Riedel-de Haën AG, Seelze
Kanamycin	InvitrogenTM, Karlsruhe
Leupeptin (-hydrochloride)	Sigma-Aldrich, Taufkirchen
L-Glutamin, 200mM (100x)	Biochrom AG, Berlin
Lipofectamin™ 2000	InvitrogenTM, Karlsruhe
Lipofectamin™ 2000 RNAiMax	InvitrogenTM, Karlsruhe
Magnesiumchloride-Hexahydrate ($MgCl_2x6H_2O$)	Merck, Darmstadt
Methylthiazoltetrazolium (MTT)	Sigma-Aldrich, Taufkirchen
ß-Mercaptoethanol	Sigma-Aldrich, Taufkirchen
Methanol	Riedel-de Haën AG, Seelze
Opti-MEM® I Reduced Serum Medium with GlutaMAX™ I	Invitrogen™, Karlsruhe
Paraformaldehyde	Merck, Darmstadt
PBS (1x) for cell culture	PAA Laboratories, Pasching, Austria
Penicillin/Streptomycin solution (100x)	PAA Laboratories, Pasching, Austria
Pepstatin A	Sigma-Aldrich, Taufkirchen
Percoll (p = 1.09 g/ml)	Biochrome AG, Berlin
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich, Taufkirchen
Phosphoric acid (H_3PO_4)	Merck, Darmstadt
Poly-D-Lysin	Sigma-Aldrich, Taufkirchen
Restore™ Western Blot stripping buffer	ThermoScientific, Karlsruhe
RPMI 1640 Medium (1x)	Biochrom AG, Berlin
Sodiumchloride (NaCl)	AppliChem GmbH, Darmstadt
Sodiumdodecylsulfate (SDS)	AppliChem GmbH, Darmstadt
Sodiumfluoride (NaF)	Roth, Karlsruhe
Sodiumorthovanadate (Na_3VO_4)	Sigma-Aldrich, Taufkirchen
Sodiumhydroxide (1 M)	Sigma-Aldrich, Taufkirchen
N,N,N´,N´-Tetramethylethylethylendiamine (TEMED)	Sigma-Aldrich, Taufkirchen
Tris-Base	Sigma-Aldrich, Taufkirchen
Triton X-100	Sigma-Aldrich, Taufkirchen
Trypan blue	Sigma-Aldrich, Taufkirchen
Trypsin/EDTA (10x)	PAA Laboratories, Pasching, Austria
Tween 20	Sigma-Aldrich, Taufkirchen

3.1.4. Water

To avoid microbial contaminations, all buffers, solutions and media for cell culture and stimulation experiments were prepared with pyrogen-free water (Braun, Melsungen, Germany), if not indicated otherwise.

3.1.5. Buffers and solutions

Selected buffers and solutions for Western blot, cell culture, ELISA, nucleic acid work and EMSA are listed in Table 3-4, Table 3-5, Table 3-6, Table 3-7 and Table 3-8, respectively.

Table 3-4 List of commonly used buffers and solutions used for western blot

RIPA lysis buffer	50 mM Tris-HCl, pH 7.4; 1 % (v/v) Igepal; 0.25 % (v/v) Natriumdeoxycholat; 150 mM NaCl; 1mM EDTA Ad before use: 1 µg/ml of Aprotinin; Leupeptin; Pepstatin and 1 mM Na_3VO_4, NaF and PMSF.
Acrylamid stock solution	30 % (v/v) acrylamid and bisacrylamid solved 29:1
SDS running buffer	25 mM Tris-base, pH 8.3; 192 mM Glycin; 0.1 % SDS (w/v)
SDS sample buffer (4x)	200 mM Tris-HCl, pH 6.8; 20 % (v/v) β-Mercaptoethanol; 8 % (w/v) SDS; 40 % (v/v) Glycerol; 0.04 % (w/v) Bromphenoleblue 1 % (w/v)
SDS blot buffer	25 mM Tris-base, pH 8.3; 192 mM Glycin, 10 % (v/v) Methanol
Stripping buffer	62.5 mM Tris-HCl, pH 6.7; 100 mM β-Mercaptoethanol; 2 % (w/v) SDS
SDS stacking gel buffer (2x)	250 mM Tris-HCl, 0.2 % (w/v) SDS; degassed before use
SDS separating gel buffer (3x)	1.125 M Tris-HCl, pH 8.8; 0.3 % (w/v) SDS; degassed before use
Blocking buffer	1x TBS; 0.1 % (v/v) Tween20; 5 % (w/v) milk powder For SOCS1 antibody: 1x PBS; 0.05% (v/v) Tween20; 3% (w/v) milk powder
Antibody dilution buffer	1x TBS; 0.1 % (v/v) Tween20; 5 % (w/v) BSA For SOCS1 antibody: 1x PBS; 0.05% (v/v) Tween20; 3% (w/v) milk powder
Wash buffer	1x TBS; 0.1 % (v/v) Tween20
10x TBS	100 mM Tris-HCl, pH 8.0; 1.5 M NaCl

Table 3-5 List of commonly used buffers and solutions used for cell culture

Trypsin solution	0.05 % (v/v) trypsin; 0.02 % (v/v) EDTA in 1x PBS
Trypan blue solution	2 mg/ml trypan blue in 1x PBS
Penicillin/Streptomycin (100x)	10000 U/ml penicillin G; 10 mg/ml streptomycin; 0.09 % (w/v) NaCl
10x PBS	80 mM di-sodiumhydrogenphosphate; 20 mM sodium-di-hydrogenphosphate; 1.4 M NaCl; pH 7.4

Table 3-6 List of commonly used buffers and solutions for ELISA

Coating buffer (pH 9.5)	0.1 M Sodiumcarbonate: 8.4 g $NaHCO_3$; 3.6 g Na_2CO_3; adjust to pH 9.5; ad 1000 ml H_2O
Coating buffer (pH 6.5)	0.2 M Sodiumphosphate: 11.8 g Na_2HPO_4; 16.1 g NaH_2PO_4; adjust to pH 6.5; ad 1000 ml H_2O
Blocking buffer	1x PBS; 10 % (v/v) FCS
Wash buffer	1x PBS; 0.05 % (v/v) Tween20

Table 3-7 List of commonly used buffers and solutions used for nucleic acid work

TAE (50x)	2 M Tris-Acetat, pH 8.0; 100 mM EDTA
TE	10 mM Tris-HCl, pH 8.0; 1 mM EDTA
Ethidium-bromide solution	1µg/ml ethidium bromide in H_2O_{dest}

Table 3-8 List of used buffers and solutions for EMSA

Lysis buffer A	10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl
Lysis buffer C	20 mM HEPES-KOH, pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA, pH 8.0; 25 % (v/v) Glycerol
TBE (10x)	0.089 M Tris; 0.089 M Boric acid; 20 mM EDTA; pH 8.0
Native gel	9 % (v/v) Acrylamid 19:1, 1x TBE buffer; 13 % (v/v) Glycerol; 0.06 % (v/v) TEMED; 0.06 % (v/v) APS

Binding buffer (5x)	100 mM HEPES, pH 8.0; 250 mM KCl; 2.5 mM DTT; 0.25 mM EDTA; 5 mM $MgCl_2$; 25 % (v/v) Glycerol
Loading buffer (20x)	0.25 % (w/v) Bromphenol blue; 50 % (w/v) Xylem cyano; 40% (w/v) Sucrose
Reaction mixture (premix)	1x binding buffer; 1x Poly (IC); 0.1 % (v/v) IGEPAL; 40 fmol biotinylated probe (egr1), 9 µg nuclear protein extract

3.1.6. Kits

Purchased kits used for this study are listed in table 3-9.

Table 3-9 List of purchased kits and suppliers

Absolute QPCR ROX®Mix	Thermo Scientific, Warrington, UK
BCA™ Protein Assay Reagent Kit	Pierce Biotechnology, Rockford, USA
BD OptEIA™ ELISA Set (IL12p40, IL10, IL6, TNFα)	BD Biosciences Pharminogen, Heidelberg
CD4+ T cell isolation kit II	Miltenyi Biotec, Bergisch Gladbach
eBioscience ELISA (IFNγ, IL23, IL4, TGFß)	eBioscience, Frankfurt
ELISA R&D Systems (IL17, KC)	R&D Systems, Abindon, UK
High Pure RNA Isolation Kit	Roche, Mannheim
High Speed Maxi Kit	Qiagen, Hilden
High-capacity cDNA transcription kit	Applied Biosystems, Life Technologies, Darmstadt
Immobilon™ Western Chemilumineszenz HRP Substrate	Millipore, Schwalbach
Nuclear extract kit	Active Motif, La Hulpe, Belgium
PowerSYBR®Green PCR Master Mix	Applied Biosystems, Life Technologies, Darmstad
peqGOLD total RNA kit	peqlab, Erlangen
QIAamp®DNA Blood Mini kit	Qiagen, Hilden
TransAM® NF-κB Transcription Factor Assay Kit	Active Motif, La Hulpe, Belgium

3.1.7. Primer

Probe for Electrophoretic Mobility Shift Assay (EMSA)

Table 3-10 Sequence of probes used for EMSA

Target gene	Sequence 5'-3'	Transcription factor
SOCS1.2 fw (promoter region)	Cy3-AAGACTGGCGCAGGGGCGGGCGCC	egr1
SOCS1.2 rv (promoter region)	Cy3-GGCGCCCGCCCCTGCGCCAGTCTT	egr1

Primer for quantitative real-time (RT)-PCR

Primers were designed with the software Primer3 (http://frodo.wi.mit.edu). Where possible, primers were placed on exon/intron boundaries to avoid amplification of genomic DNA. For this reason, only monoexonic genes were sensitive to amplification from genomic DNA (Table 3-11). Lyophilized Primers (fw: forward primer; rv: reverse Primer; 0.01 µmol, salt

free) and probes (pr) were purchaised from Eurofins MWG Operon, adjusted to 50 pmol/µl with $aqua_{dest}$. Primer aliquots were stored at -20°C.

Table 3-11 Primer sequences for the amplification of murine transcripts

Gene		Sequence 5'-3'	Product (cDNA)	Product (gen. DNA)[a]	Amplicon position	Data base[b]
β-actin	fw	CCCTGTGCTGCTCACCGA			383-400	
	rv	ACAGTGTGGGTGACCCCGTC	186 bp	-	568-549	NM_007393
	pr	CCCCTGAACCCTAAGGCCAACCG			405-427	
SOCS1	fw	CACCTTCTTGGTGCGCG			418-434	
	rv	CGCCACGTAGTGCTCCAG	189 bp	189 bp	606-589	U88325
	pr	TCGCCAACGGAACTGCTTCTTCG			439-461	
SOCS3	fw	ACCCACAGCAAGTTTCCCG			24-41	
	rv	AGTAGAATCCGCTCTCCTGCAG	136 bp	136 bp	159-138	U88328
	pr	TTGCGCACGGCGTTCACCAC			127-146	
CIS	fw	GAACCGAAGGTGCTAGACCCT			313-351	
	rv	TGTACCCTCCGGCATCTTCT	159 bp	159 bp	471-452	D31943
	pr	ATCTGCTGTGCATAGCCAAGACGTTCTCC			359-387	
egr1	fw	GCCTCGTGAGCATGACCAAT	77 bp	-	751-770	NM_007913.5
	rv	GCAGAGGAAGACGATGAAGCA			827-807	
egr2	fw	GTGTCGGATCTGCATGCGAAACTT	94 bp	-	1517-1540	NM_010118.3
	rv	GCAAACTTGCGGCCACAATAGTCA			1610-1587	
IRF7	fw	CGAGTGCTGTTTGGAGACTG	163 bp	-	468-487	NM_001252601.1
	rv	AGCCCAGGCCTTGAAGAT			630-613	
IL17	fw	CAAAACCAGGGCATTTCTGT	164 bp	-	115-134	NM1_45856.2
	rv	ATGGTGCTGTCTTCCTGACC			278-259	
IFNγ	fw	TCAAGTGGCATAGATGTGGAAGAA	92 bp	-	222-245	NM_008337.3
	rv	TGGCTCTGCAGGATTTTCATG			313-293	
IL23p19	fw	CCAGCGGGACATATGAATCT	180 bp	-	260-279	NM_031252
	rv	GATGTCAGAGTCAAGCAGGTG			459-439	
Pyk2	fw	ACCAGTGGATGTGGAGAAGG	145 bp	-	154-173	NM_011203.2
	rv	ACTCAGGAGGATGGAGGTGA			298-279	
IL10	fw	GGTTGCCAAGCCTTATCGGA	193 bp	-	300-319	NM_010548.2
	rv	ACCTGCTCCACTGCCTTGCT			492-473	
IFN-ß	fw	CCCTATGGAGATGACGGAG	161 bp	-	197-216	NM_010510
	rv	CTGTCTGCTGGTGGAGTTCA			357-338	
RORγT	fw	AATGTGGCCTACTCCTGCAC	152 bp	-	259-278	NM_011281
	rv	TCCCTCTGCTTCTTGGACAT			429-410	
Tbet	fw	GGTGTCTGGGAAGCTGAGAG	173 bp	-	545-564	NM_019507
	rv	CCACATCCACAAACATCCTG			736-717	

[a] no amplification of genomic DNA due to extended size by exon/intron structure
[b] National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov)

Primer for the genotyping of conditional SOCS1 ko mice

Table 3-12 Primer sequences for genotyping of SOCS1 ko mice

Gene locus		Sequence 5'-3'	Product size
Cre recombinase (LysMCre)	fw rv	CACCAAAATTTGCCTGCATT CCCAGGCTAAGTGCCTTCTC	641 bp
SOCS1 ko	fw rv	CCTCCAGCTGGCCCCTCGAGTAGGATG CATTCGCCATTCAGGCTGCGCAACTGTT	160 bp
SOCS1 wt	fw rv	GCATCCCTCTTAACCCGGTAC AAATGAAGCCAGAGACCCTCC	340 bp
LoxP ("hCD4 floxed")	fw rv	TTAGGCACTTGCTTCTGGTGC TTCTGGAAAGCTAGCACCACG	600 bp

3.1.8. Markers

Molecular markers used for electrophoresis are listed in table 3-13

Table 3-13 Markers used in nucleic acid – and protein – work

GeneRuler™DNA Ladder Mix (100-10000 bp)	Fermentas, St. Leon-Rot
PageRuler™Prestained Protein Ladder Plus	Fermentas, St. Leon-Rot

3.1.9. siRNAs

All siRNAs were purchased in HPLC purity from Qiagen (Hilden). Sequences shown in table 3-14 confer to the respective passenger-strand of the siRNA.

Table 3-14 List of relevant siRNAs used in this study

Name	Sequence 5'-3'	Target gene
Mm_Ptk2b_4	UAAAUUCCAAUGUACACUGTA	murine Pyk2 (Ptk2b)
siCon	GCAAGCUGACCCUGAAGUUCAU	no target

3.1.10. Enzymes

Enzymes (provided with reaction buffers) used throughout the study are listed in table 3-15.

Table 3-15 List of essential enzymes and suppliers

AccuTaq™ LA DNA Polymerase (5 U/µl)	Sigma-Aldrich, Taufkirchen
DNase I	MBI Fermentas, St. Leon-Rot
Pfu Polymerase	MBI Fermentas, St. Leon-Rot
Proteinase K	MBI Fermentas, St. Leon-Rot
Taq DNA Polymerase	MBI Fermentas, St. Leon-Rot

3.1.11. Antibodies

Antibodies used for the indicated applications are listed in table 3-16 to 3-18.

Table 3-16 List of antibodies for flow-cytometry

Species reactivity	Supplier	Fluorescence label	Antigen	Clone
mouse	AbD Serotec (Düsseldorf)	FITC	Beta Glucan	101210
mouse	BD (Heidelberg)	APC-Cy7	CD11b	M1/70
mouse	BD (Heidelberg)	APC	CD11c	HL3
mouse	BD (Heidelberg	FITC	CD3ε	66275
mouse	Miltenyi Biotec (Bergisch Gladbach)	PE	CD4	5051025059
mouse	BD (Heidelberg)	FITC	CD40	HM-40-3
mouse	BD (Heidelberg)	FITC	CD80	16-10A1
mouse	BD (Heidelberg)	FITC	CD86	GL1
mouse	BioLegend (Uithoorn, Netherlands)	PE-Cy7	F480	BM8
mouse	BD (Heidelberg)	PE	I-A/I-E class MHCII	2G9/M5/114.15.2
mouse	AbD Serotec (Düsseldorf)	FITC	IgG_2b	101210
human	BD (Heidelberg)	PE	CD4	5090120098

Table 3-17 List of antibodies used for Western blot analysis

Species	Source	Antigen	Phosphorylation sites	Final dilution
Rabbit	Cell Signaling Technology (Frankfurt)	β−actin	-	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	p42/p44 (ERK)	-	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	IκBα	-	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pp42/p44	Tyr202/Tyr204	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pIκBα	Ser32	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pJNK/SAPK	Thr183/Tyr185	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pp38	Thr180/Tyr182	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pPyk2	Tyr402	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	Pyk2	-	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pSyk	Tyr519/Tyr520	1 : 1000
Rabbit	Cell Signaling Technology (Frankfurt)	pSTAT1	Tyr701	1 : 1000
Mouse	Millipore (Schwalbach)	SOCS1	-	1 : 500
Anti-mouse	Cell Signaling Technology (Frankfurt)	Mouse IgG (HRP coupled)	-	1 : 4000
Anti-rabbit	Cell Signaling Technology (Frankfurt)	Rabbit IgG (HRP coupled)	-	1 : 4000

Table 3-18 List of antibodies used for *in vitro* T-cell assays and IL10 blocking experiments

Species	Source	Antigen
Anti-mouse	BD (Heidelberg)	CD28
Anti-mouse	BD (Heidelberg)	CD3ε
Anti-mouse	PeproTech (Düsseldorf)	IFNγ
Anti-mouse	eBioscience (Frankfurt)	IL10
Anti-mouse	eBioscience (Frankfurt)	IL4

3.1.12. Stimulating agents and inhibitors

Stimuli

Stimuli used in this study are listed in table 3-19.

Table 3-19 List of stimuli and sources

Stimulus	Source	Stock concentration
CpG-ODN 1668-PTO (5´-TCCATGACGTTCCTGATGCT-3´) fully PTO modified	MolBiol (Berlin)	250 µM (in Aqua dest.)
Depleted Zymosan	InvivoGen (Toulouse, France)	5 mg/ml (in Aqua dest.)
Ionomycin from *Streptomyces conglobatus*	Merck, Calbiochem (Darmstadt)	1 mg/ml (in DMSO)
Lipopolysaccharid (LPS) from *Salmonella minnesota* (smooth form)	Provided by U. Seydel (Borstel)	100 µg/ml (in Aqua dest.)
Pam_3Cys-Ser-$(Lys)_4$ [Pam_3CSK_4]	EMC Microcollections GmbH (Tübingen)	1 mg/ml (in Aqua dest.)
Phorbol-12-myristate-13-acetate (PMA)	Merck, Calbiochem (Darmstadt)	1 mg/ml (in DMSO)
Polyinosinic-polycytidylic acid [Poly(I:C)]	Sigma-Aldrich (Taufkirchen)	5 mg/ml (in Aqua dest.)
R848 (resiquimod)	InvivoGen (Toulouse, France)	1 mg/ml (in Aqua dest.)
Recombinant murine IFNγ	PeproTech (Hamburg)	1 mg/ml (in Aqua dest.)
Recombinant murine IL10	Immunotools (Friesoythe)	100 µg/ml (in 0.1% BSA w/v,Aqua dest.)
Recombinant murine IL12	PeproTech (Hamburg)	100 µg/ml (in Aqua dest.)
Recombinant murine IL4	PeproTech (Hamburg)	100 µg/ml (in Aqua dest.)
Recombinant murine IL6	Peprotech (Hamburg)	20 µg/ml (in 0.1% BSA w/v, PBS)
Recombinant human TGFß	Peprotech (Hamburg)	10 µg/ml (in 0.1% BSA w/v, PBS)

Inhibitors

Inhibitors used in this study are listed in table 3-20.

Table 3-20 List of inhibitors and sources

Inhibitor	Source	Stock concentration
AG17 (Pyk2 inhibitor)	Merck, Calbiochem (Darmstadt)	10 mM (in DMSO)
BAPTA (Calcium chelator)	Merck, Calbiochem (Darmstadt)	10 mM (in DMSO)

Bay 11-7082 (NF-κB inhibitor)	Merck, Calbiochem (Darmstadt)	10 mM (in DMSO)
Bay 61-3606 (Syk inhibitor IV)	Merck, Calbiochem (Darmstadt)	10 mM (In DMSO)
Cycloheximid (inhibitor of protein translation	Merck, Calbiochem (Darmstadt)	- (freshly prepared)
KN93 (CaMK inhibitor)	Merck, Calbiochem (Darmstadt)	10 mM (in DMSO)
PP1 (Src inhibitor)	Merck, Calbiochem (Darmstadt)	10 mM (in DMSO)

3.1.13. Mouse strains

C57BL/6 mice were purchased from Charles River Laboratories (Willmington, MA USA) and bred under specific pathogen free (SPF) conditions. Breeding and monthly control of infections were performed according to the list of GV-SOLAS 1995. Killing and dissection were approved and experiments were properly recorded and reported to the regional commission Tübingen.

Knockout mice

All knockout mice were on C57BL/6 background. IFN-α/β receptor deficient mice (IFNAR1$^{-/-}$) were kindly provided by Rainer Zawatzky (Heidelberg, Germany), MyD88/TRIF double deficient mice by U. Kalinke (Düsseldorf, Germany). Dectin1$^{-/-}$ mice were received from G. Brown (Aberdeen, UK), IFNγ$^{-/-}$ and SOCS1$^{-/-}$/IFNγ$^{-/-}$ were obtained from Martin Rottenberg (Stockholm, Sweden). Conditional SOCS1 knockout mice were received from T. Kay (Melbourne, Australia).

3.1.14. Eukarytotic cells

All cells were cultured at 37 °C and 5 % CO_2 in a humidified environment. Composition of the respcetive cell media is decribed in chapter 3.1.15 and 3.2.1 (“generation and stimulation of bone marrow cerived dendritic cells and macrophages”).

<u>Murine BMDCs and BMMs</u>

Murine bone marrow derived dendritic cells (BMDCs) and macrophages (BMMs) were generated from bone marrow out of tibiae and femur as described in

<u>Murine T-cells</u>

Isolation of untouched murine CD4^{+} Th-cells from spleen was performed as described in 3.2.1.

3.1.15. Growth media

<u>Inactivation of fetal calf serum (FCS) (Biowest, France)</u>

Heat treatment of FCS inactivates the containing complement proteins. Sterile FCS was heat inactivated at 56 °C for 1 h, decanted into aliquots and stored at -20 °C.

<u>RPMI 1640</u>

Before use, the medium was complemented with 1 % (v/v) Penicillin / Streptomycin, 50 mM 2-Mercaptoethanol and with 10 % (v/v) or 2 % (v/v) heat inactivated FCS. During differentiation DCs were grown in medium with 10 % FCS but when harvested, the cells where starved in medium with 2 % FCS before stimulation.

DMEM
DMEM (Dulbecco's modified eagle media) was supplemented with 10 % (v/v) heat inactivated FCS (or 2 % in case of stimulation assays of BMMs) and 1 % Penicillin G (100 IU/ml) / Streptomycin (100 IU/ml).

3.2. Methods

3.2.1. Cell biology

Cell culture of mammalian cells

All eukaryotic cells were grown under 95 % humidity atmosphere at 37 °C and 5% CO_2 in an incubator. To determine the cell number of a cell suspension, 10 µl of cell suspension was mixed with 90 µl of trypan blue solution which stains exclusively dead cells. Cell numbers were determined with a Neubauer cell counting chamber.

Generation and stimulation of primary bone marrow-derived dendritic cells and macrophages

Generation of medium containing GMCSF or MCSF

LGM cells (as a source for GM-CSF) were grown in supplemented RPMI (see 3.1.4, RPMI) and L929 cells (as a source for M-CSF) were grown in supplemented DMEM (see 3.1.4, DMEM). Every third day, the cells were split. The old medium was removed and collected as a GM-CSF or M-CSF containing differentiation medium, respectively. The cells were incubated in 1x trypsin/ EDTA solution (Table 3-5) for 5 min at 37 °C, 5 % Co_2. The cells were detached from the flasks by gently pipetting up and down and split into new flasks at a ratio of 1:3 in fresh medium.

Generation of murine primary bone marrow-derived dendritic cells and macrophages

GM-CSF-derived dendritic cells (BMDCs) and M-CSF derived macrophages (BMMs) were prepared from 4- to 10-week-old mice as described by Inaba (135) with minor modifications. Briefly, mice were sacrificed by CO_2 asphyxia. Bone marrow was collected from femurs and tibiae by flushing with supplemented RPMI (BMDCS) or DMEM (BMMs) using a 27 G syringe. Afterwards, the cell suspension was centrifuged at 13.000 rpm for 5 min (Multifuge 3 SR). The pellet was resuspended in 20 ml of the respective medium (BMDCS in RPMI, BMMs in DMEM). Cells were placed in 145 cm^2 tissue-culture plates in 20 ml of the respective medium (BMDCS: RPMI 1640, 10 % (v/v) FCS, 1 % (v/v) Penicillin G / Streptomycin and 50 mM 2-mercaptoethanol; BMMs: DMEM, 10 % (v/v) FCS, 1 % (v/v) Penicillin G / Streptomycin). After 24 h non-adherent cells (fibroblasts) were collected, washed and counted. Further differentiation was done as follows.
BMDCs:
10^7 of non-adherent cells were seeded into 145 cm^2 tissue-culture plates in 20 ml of differentiation medium (RPMI 1640, 10 % (v/v) FCS, 1 % (v/v) Penicillin G / Streptomycin,

50 mM 2-mercaptoethanol and 4 % of GM-CSF containing supernatant). At day 5 fresh differentiation medium (20 ml) was added and at day 9 non-adherent, immature dendritic cells ($CD11c^+$, $B220^-$) were harvested by centrifugation (1300 rpm, 5min). Dendritic cells were plated for further stimulation assays. GM-CSF was derived from X-63 which are cytoplastoma cells secreting G-MCSF (LGM cells).

BMMs:

2–2.5 x 10^7 non-adherent cells were seeded into 145 cm^2 petri dishes (to avoid strong attachment of the cells) in 20 ml of differentiation medium (DMEM, 10% (v/v) FCS, 1% (v/v) penicillin G / Streptomycin and 30 % of M-CSF containing supernatant). At day 3, 20 ml fresh M-CSF containing supernatant (30 %) was added and at day 7, adherent BMMs were harvested. For this purpose, BMMs where washed with 1 x PBS and detached by 5 minutes incubation with 1xTrypsin/EDTA solution at 37 °C, 5% CO_2.Cells were washed with PBS / 2% FCS to inactivate trypsin. Cells were centrifuged (133 rpm, 5 min) and replated for stimulation.

Cell stimulation

Dendritic cells or macrophages were plated at $2x10^5$ in 96-well flat bottom format for ELISA. $5x10^5$ (BMDCs) or $3x10^5$ (BMMs) were plated in 24-well format for RNA preparation or $3x10^6$ and $6x10^6$ in 6-well format for protein lysates. Stimuli, inhibitors or blocking antibody (anti-IL10) were used as indicated in figure legends. For transwell-experiments, cells were placed in the lower and upper wells of a 24-transwell system (pore size: 0.4 µm, Ø 6.5 mm, clear inserts) and stimuli were added into the lower wells (BMDCs: $5x10^5$ in lower wells, $2x10^5$ in upper wells; BMMs: $3x10^5$ in lower wells, $1x10^5$ in upper wells). Cells in both chambers were analyzed by means of gene expression for transcript level as indicated.

In vitro T-cell stimulation assay

Isolation of $CD4^+$ T-cells

Splenic $CD4^+$ T-cells were purified from WT C57BL/6 mice and applying a protocol of the manufaturer. First, splenocytes were segregated from spleens by means of Percoll gradient. Briefly, organs are ruptured by a syringe punch and a cell strainer (pore size 100 µm) which served as a cell homogenizer. The cells were washed with ice-cold 1 x PBS and pelleted by centrifugation (1300 rpm, 10 min, 4 °C). Lymphocytes were isolated by means of Percoll (p = 1.09 g/ml) separating solution. After gradient centrifugation (4000 rpm, 20 min, 4 °C without brake), the Interphase between the two gradients contains the naïve T-cells and was taken off very gently. Cells were washed two times with ice-cold PBS (w/o) (1300 rpm, 10min, 4 °C). The pellet was resuspended in ice-cold PBS. Cells were counted and used as a source for naïve T-cells.

Magnetic-associated cell sorting

The immunomagnetic cell separation is based on magnetic antibody labeling (MACS-Microbeads). Labelled cells were separated by enrichment in a magnetic field and then eluted in a semi-automatic manner from the column via the AutoMACS device.

Purification of $CD4^+$ T-cells was performed using the $CD4^+$ T-cell isolation kit II according to manufacture's protocol. The cell pellet was reconstituted in 40 µl MACS buffer per 10^7 cells.
10µl/10^7 cells of a cocktail containing different biotin labeled antibodies against CD8a, CD11b, CD11c, CD19, CD45R, CD49b, CD105, anti-MHC class II and Ter 19 were used to separate $CD4^+$ T-cells (10 min, 4 °C). Afterwards an Anti-Biotin mix (20 µl/10^7 cells) was applied and the labelled cells were incubated for 15min at 4°C. After the labelling procedure, cells were washed with 2 ml/10^7 cells MACS buffer (1300 rpm, 10 min, 4 °C). Cells were resuspended in 500 µl MACS buffer per 10^8 cells. T-cells were then isolated by depletion of magnetically labelled cells via the AutoMACS Separator. $CD4^+$ T-cells were eluted in the negative fraction by means of the programm "Deplete". Thus, the negative fraction represents the enriched $CD4^+$ T-cells.

APC / T-cell co-culture

WT sorted $CD4^+$ T-cells (1x 10^5) were cultured together with BMMs or BMDCs (1 x 10^4) from $SOCS1^{-/-}$/$IFN\gamma^{-/-}$ or $IFN\gamma^{-/-}$ control mice. Alternatively, 1x10^5 $CD4^+$ T-cells were cultured with 1x10^4 BMMs from $SOCS1^{lox/-}$ Cre^+ (conditional SOCS1 knockout, described in 3.2.2.3) or $SOCS1^{lox/-}$ Cre^- mice as control. Co-cultures were carried out in 96 well-format. 2 µg/ml of plate-bound anti- CD3ε and 2 µg/ml of soluble anti-CD28 were added in order to activate T-cells. Therefore, 2 µg/ml anti- CD3ε antibody in 200 µl ice-cold PBS per well was added into 96-well plates and incubated at 4 °C for 16 h. At the next day, PBS was removed and sorted $CD4^+$ T-cells were added together with soluble anti-CD28 in medium. When T-cells were cultured in presence of BMMs, DMEM supplemented with 10 % (v/v) FCS and 1 % Penicillin G / Streptomycin was used. Co-cultures that consisted of T-cells and BMDCs were placed in RPMI, supplemented with 10 % (v/v) FCS, 1 % (v/v) Penicillin G / Streptomycin and 50 mM 2-mercaptoethanol. BMDCs or BMMs within the co-culture setting were stimulated with either 100 µg/ml depleted Zymosan, 1 µM CpG 1668 or a combination thereof. Th17 control were obtained from $CD4^+$ T-cells from WT C57BL/6 mice co-cultured with APCs from $SOCS^{-/-}$/$IFN\gamma^{-/-}$ or $IFN\gamma^{-/-}$ control mice or conditional SOCS1 ko BMMS and the respective control. To induce Th17 cells, 10 ng/ml TGFβ, 20 ng/ml IL6 and neutralizing antibodies against IFNγ (2 µg/ml) and IL4 (2 µg/ml) were added. Th1 control cells were induced by IL12 (2 ng/ml) and Th2 control wells by IL4 (10 ng/ml); both of them cultivated together either with APCs form $SOCS1^{-/-}$/$IFN\gamma^{-/-}$ mice or BMMs derived from conditional knockout mice.
On day 5, all cells were restimulated for 5 h (for ELISA) or 3 h (for RNA lysates) with PMA (50 ng/ml) and Ionomycin (750 ng/ml). Cytokines in the supernatants were analyzed by sandwich ELISA or cells were lysed to analyze gene expression by RT-PCR.
Instead of $SOCS1^{-/-}$/$IFN\gamma^{-/-}$ or $IFN\gamma^{-/-}$ cells macrophages from conditional SOCS1 deficient mice, that fail to express SOCS1 only in the myeloid compartment, and their appropriate controls were used for some experimental approaches.
As growth medium, DMEM supplemented with 10 % FCS and 1 % Penicillin G / Streptomycin was used.

Cell viability assay

Viability of stimulated cells was determined by MTT assay. MTT substrate is reduced by various mitochondrial dehydrogenases resulting in a change of substrate colour. After removing cell free supernatants for cytokine detection, MTT solution was prepared according to the manufacturer's recommendations and added to culture medium in a ratio of 1:20 (v/v). Cells were incubated for 30 min to 1 h at 37 °C and absorbance at 550 nm with 650 nm reference wave length was measured in a photometer.

Enzyme-linked immunosorbant assay (ELISA)

To determine the quantities of secreted cytokines or interferons cell free supernatants were analyzed by means of sandwich ELISA as indicated in the respective protocols of the manufacturers. In case of IL12p40, TNFα, IL10 and IL6 ELISA supernatants were diluted in 1 x PBS, supplemented with 10 % FCS (v/v) (blocking buffer).The dilution/blocking–buffer for IL17 and IL8 (KC) ELISA was composed of 1 x PBS, supplemented with 1 % BSA (w/v). Buffers for TGFβ, IFNγ, IL4 and IL23 ELISA were used according to the recommendations of eBioscience. Analyses were carried out in duplicates and all experiments were performed two to three times as indicated. Cytokines were detected by measuring the absorbance at 490 nm with 650 nm reference in a photometer (SUNRISE Absorbance reader, Tecan, Salzburg, Austria). Cytokine concentrations were calculated using a standard dilution of the respective recombinant cytokines. Finally, concentrations were calculated with the Magellan V 5.0 software (Tecan, Salzburg, Austria). Standard determination was performed according to the 4 parameter fit regression method.

Detection of NF-κB binding

All buffers used were provided by the manufacturer. For measurement of transcription factor activity, nuclear extracts were prepared 6 h after stimulation of SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ BMMs (9x10^{6}; therefore, 3 wells of 6-well format were combined, each well contained 3x10^{6} cells). Lysates were prepared using the Nuclear extract kit according to the protocol. In principle, media was aspirated and cells were washed with 2.5 ml per well ice-cold PBS supplemented with Phosphatase inhibitors. 1.5 ml PBS/Phosphatase inhibitors was added and cells were scraped from the dish. Thereupon, cells were centrifuge at 13.000 rpm, 10 min at 4 °C. Cell pellet from 3 respective wells was combined by resuspension in 750 µl 1x ice-cold hypotonic buffer. The cell suspension was transferred in a pre-chilled microcentrifuge tube and incubated for 20 min on ice. 37.5 µl detergent was added. Afterwards, the cell suspension was centrifuged at 13.000 rpm, 2 min at 4 °C. The nuclear pellet was resuspended in 50 µl complete lysis buffer by pipetting up and down and incubated for 30min on ice on a rocking platform (150 rpm). After centrifugation at 4 °C for 10 min, the supernatant containing the nuclear fraction (50 µl) was either stored at -80 °C or protein concentration was directly determined by means of BCA (3.2.3). For detection of NF-κB activity, the nuclear protein lysates were analyzed analyzed using the TransAM® NF-κB Transcription Factor Assay Kit. Analyses were performed according to manufacturer's protocol in duplicates and experiments were carried out two to three times as indicated. In a first step, 30 µl complete binding buffer was added to each well of a 96 well plate. The plates

are pre-coated with DNA oligonucleotides containing the NF-κB consensus sequence. Afterwards, 10 µg of nuclear fraction (in 20 µl complete lysis buffer) was added. The plate was incubated for 1 h at RT with mild agitation (100 rpm, rocking platform). Plates were washed 3 times with 200 µl 1 x wash buffer. Thereupon, 100 µl/well primary antibody diluted 1:1000 in 1 x antibody binding buffer was added. The provided antibodies recognize an epitope on the respective NF-κB subunit (p50, p65, p52, c-Rel, RelB). This epitope is accessible when NF-κB is activated. After 1h incubation at RT, the plate was washed 3 times with 1x wash buffer. An HRP-conjugated secondary antibody (100 µl/well, diluted 1:1000 in 1 x antibody binding buffer) detects the first antibody. Absorbance was measured at 490 nm with a reference wavelength of 650 nm in a photometer (SUNRISE Absorbance reader, Tecan, Salzburg, Austria).

Flow cytometry

In order to evaluate the purity of BM derived cells and to analyze the expression of co-stimulatory molecules on the surface, cells were marked with fluorescence-labeled antibodies (Table 3-16). Approximately $3x10^5$ cells were washed with 1 x PBS, supplemented with 2 % BSA (v/v). Afterwards, the supernatant was removed and cell suspension was adjusted to a volume of 100 µl with 1 x PBS / 2 % BSA and transferred into 5 ml-tubes. Cell suspension was incubated with the respective antibodies for 30 min at 4 °C and then washed two times with 1 x PBS / 2 % BSA. Afterwards, cells were analyzed in a flow cytometer. A first gate was set on viable cells as defined by forward (size) / sideward (granularity) scatter characteristics. Unlabeled and isotype stained cells served as negative control. Percentage of positive cells as well as mean fluorescence intensity (MFI) of positive cells was recorded and monitored using the FACS DIVA V 4.12 software.

siRNA transfection

For RNAi-mediated gene-silencing of endogenously expressed genes, cells were seeded in different formats. In the following, all denoted volumes correspond to one well. When cells were used for the preparation of RNA, 30 pmol siRNA was incubated in a volume of 100 µl of OptiMEM with 1.5 µl Lipofectamine2000 RNAiMAX within a 24-well format for 20 min. For preparation of protein lysates, 160 pmol siRNA was incubated in 6-well format with 7.5 µl of Lipofectamine2000 RNAiMAX. Afterwards, cells were applied directly to the reaction mixture (reverse transfection). For 24-well plates, 400 µl medium (DMEM without serum or antibiotics) containing $3x10^5$ cells was seeded. For 6-well plates, $2x10^6$ cells in a volume of 1.5 ml medium were plated. Cells were incubated at 37 °C. After 20 h, cells were stimulated as indicated in the respective figure legends. Knockdown efficiency was determined by Western blotting and quantitative RT-PCR.

3.2.2. Molecular biology

DNA sequencing

Sequencing was performed commercially by GATC (Konstanz). 50 ng/µl DNA (PCR product) was analyzed with standard primers (3.1.7). Results were validated by aligning with the theoretical sequences using the alignment program CLUSTALW2 (www.ebi.ac.uk/Tools/clustalw2).

Quantitative RT-PCR

RNA preparation

Total RNA from BMMs or BMDCS was isolated using the High Pure RNA Isolation Kit or additionally the peqGOLD Total RNA kit according to manufacturers' protocol. RNA preparation included a DNase I digestion to exclude amplification of genomic DNA during RT-PCR. RNA was either stored at -80 °C or directly used for cDNA synthesis.

cDNA synthesis

Synthesis of cDNA was performed with the High-capacity cDNA transcription kit or with the RevertAid™ First Strand cDNA Synthesis Kit. Messenger RNA was amplified using Oligo$(dT)_{18}$ primers. Briefly, 10 µl RNA lysate was added to 10 µl Master Mix containing dNTP's, Reverse transcriptase, RNAse inhibitors and Oligo$(dT)_{18}$ primer. The mixture was transcribed according to manufacturer's protocol. Finally, the cDNA (20 µl) was diluted in Aqua dest at a ration of 1:3.

Quantitative real-time (RT)-PCR

For quantification of cDNA, two approaches were used. First, the cyanid fluorescent dye SYBR-green that interferes with double-stranded DNA was used to monitor amplicon development. Second, a hybridization probe was used which shows increased specificity in detection. The probe constituted of a short complimentary oligonucleotide that binds to the respective internal target sequence of the amplicon. A fluorophore (5'-FAM) and a quencher (3'-TAMRA) were covalently attached to the probe. The emission of the reporter fluorophore is repressed via FRET (fluorescence resonance energy transfer) by the quencher. During PCR, primers and probe hybridize at their binding site within the target sequence. Upon amplification the probe is degraded through the 5'-3' exonuclease activity of the Taq polymerase. Thereupon, FRET is suppressed and the sequence specific signal can be detected.

In principle, 2 µl of template (cDNA) was added to 18 µl of MasterMix (table 3-20), respectively. Thereupon, the mix was distributed into 96-well format. The plates were sealed and gene expression was performed in the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Darmstadt). The program for amplification was carried out as follows: 95 °C 15 min; 40 x [95 °C, 15 sec; 60 °C, 1 min].

Typically, gene expression was measured in duplicates for each cDNA sample. Automatic detection of baseline and threshold values was used and the determined C_t values were

substracted from the C_t values of an endogenous constitutively expressed reference gene (β -actin) resulting in a ΔC_t for each target gene. Relative expression (rE) was calculated as $rE = 1/(2^{\Delta Ct})$. PCR reactions had been verified for similar efficiencies. Specificity of reactions was controlled by no-template as well as no-RT (no reverse transcriptase) samples. For SYBR-green detection, the dissociation curves of each amplicon were analyzed to determine the melting point. Respective test settings are listed in table 3-21.

Table 3-21 Test parameters for quantitative RT-PCR

Template/reaction	Primer (fw and rv)/reaction	Probe/reaction	Reaction mix
2 µl	25 pmol	3.7 pmol	10 µl of Absolute QPCR ROx Mix (Thermo Scientific, Warrington, UK)
2 µl	12.5 pmol	-	10 µl of PowerSYBR®Green PCR Master Mix (Applied Biosystems, Darmstadt)

Genotyping of mice

Conditional SOCS1

Tissue-specific SOCS1-deficient mice were generated using the Cre/LoxP system (136, 137). Therewith, *Socs1*$^{lox/lox}$ mice, that carry a *Socs1* allele flanked by two LoxP sites (Flox), are bred with Cre transgenic mice. The consequence of SOCS1 deficiency in myeloid cells (macrophages) was investigated by breeding *Socs1*$^{lox/lox}$ mice with *Socs1*$^{+/-}$ mice and mice expressing Cre under the promoter of endogenous Lysozyme M (*LysM-Cre*). We asked to obtain progeny that were *Socs1*$^{lox/-}$ and positive for *LysM-Cre*. Thus, the macrophages of these mice only have one floxed SOCS1 allele that is excised by the cre recombinase. Human CD4 (hCD4) containing a F43I mutation and intracellular truncation which abrogates its function was utilized as a reporter for effective Cre-mediated recombination. The presence of only one copy of *Socs1*^{lox} ensures the most efficient deletion (136, 137).

Purification of genomic DNA

DNA was isolated from tails of the respective knockout or control mice and tissue was digested with 18 units (895 units/ml) of Proteinase K at 56 °C over night. Thereupon, genomic DNA was isolated using the QIAamp®DNA Blood Mini kit (Qiagen, Hilton) according to manufacturer's protocol. In principle, mice tails were incubated in 180 µl buffer ATL with 20 µl (18 units) Proteinase K over night. Thereupon, 200 µl buffer AL was added to the sample and the components were mixed by vortexing. Afterwards, 200 µl ethanol (100 %) was added immediately and the sample was mixed again by vortexing. The mixture was added onto a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 min at RT: After washing with buffer AW1 and AW2, the DNA was eluted from the column membrane in 100 µl buffer AE. DNA was either used directly for PCR reaction or kept at -20 °C for long-term storage. Concentration of DNA was measured by means of the NanoDrop® ND-1000 UV / VIS Spectrophotometer. Aromatic rings of the DNA absorb UV light at λ=260 nm. The ratio of E_{260} / E_{280} (E = extinction coefficient) gives information about the purity of the prepared DNA.

Contamination with proteins is detected via the absorption at 280 nm. A value below 1.8 indicates significant protein contaminants.

Polymerase chain reaction (PCR)

PCR was used to exponentially amplify DNA that is synthesized *de novo* during a cyclic reaction process. PCR was performed according to general protocols using Accu Taq LA DNA polymerase. Annealing temperatures for standard cloning purposes were 5 °C under calculated annealing temperatures. Different PCR conditions are depicted in table 3-22.

Table 3-22 PCR conditions for genotyping of conditional SOCS1 knockout mice

Gene locus	Expected band size	Reaction conditions	PCR programm
Cre recombinase	650 bp	10x buffer: 2 µl dNTPs (10 mM each): 0.4 µl 5' Primer (50 µM): 0.2 µl 3' Primer (50 µM): 0.2 µl Taq Polymerase (125 units): 0.4 µl DMSO: 0.4 µl DNA: 10 – 200 ng ad H_2O_{des} to 20 µl	95 °C, 5min 95 °C, 1min 57 °C, 1min } 35x 72 °C, 1min 72 °C, 2min 4 °C, ∞
SOCS1 ko	160 bp	10x buffer: 2 µl dNTPs (10mM each): 0.4 µl 5' Primer (50 µM): 0.2 µl 3' Primer (50 µM): 0.2 µl Taq Polymerase (125 units): 0.4 µl DMSO: 0.4 µl DNA: 10 – 200 ng ad H_2O_{des} to 20 µl	95 °C, 5min 95 °C, 1min 57 °C, 1min } 35x 72 °C, 1min 72 °C, 2min 4 °C, ∞
Flox	600 bp	10x buffer: 5 µl dNTPs (10 mM each): 1 µl 5' Primer (50 µM): 0.5 µl 3' Primer (50 µM): 0.5 µl Taq Polymerase (125 units): 0.5 µl DMSO: 0.5 µl DNA: 10 – 200 ng ad H_2O_{des} to 50 µl	95 °C, 5min 95 °C, 1min 57 °C, 1.30 min } 41x 72 °C, 1,30 min 72 °C, 10 min 4 °C, ∞
WT	340 bp	10x buffer: 5 µl dNTPs (10 mM each): 1 µl 5' Primer (50 µM): 0.5 µl 3' Primer (50 µM): 0.5 µl Taq Polymerase (125 units): 0.5 µl DMSO: 0.5 µl DNA: 10 – 200 ng Ad H_2O_{dest} to 50 µl	95 °C, 5min 95 °C, 1min 53 °C, 1.30 min } 41x 72 °C, 1,30 min 72 °C, 10 min 4 °C, ∞

Gel electrophoresis

For DNA visualization standard agarose gel electrophoresis procedure was performed. In brief, DNA sample (50 µl or 20 µl, table 3-21) was mixed with 6 x loading dye. A volume of 20 µl of the DNA/loading dye mixture was loaded to 1 % (SOCS1ko, WT) or 2 % (Cre recombinase, Flox) 1 x TAE agarose gel electrophoresis. Gels were run at 100 V for 30 min – 1h. For UV-based detection, agarose gels were incubated for approximately 20 min in ethidiumbromide (1 µg/ml diluted in 1000 ml H_2O_{dest}). As a marker, GeneRuler™DNA Ladder Mix (100-10000 bp) was used.

3.2.3. Biochemistry

Preparation of Protein-lysates

According to the respective experiments, cells were seeded in 6-well cell culture plates. Following stimulation, the medium was removed and the cells were washed wit 2 ml/well ice-cold 1 x PBS supplemented with 1 mM NaF and Na_3VO_4. After centrifugation of the plates (800 rpm, 10 min, 4 °C) cells were lysed in 80 – 100 µl of freshly prepared RIPA lysis buffer (3.1.5) for 30 min at 4 °C within the wells of the plate. Incubation of the plates at -20 °C for 16h ensured proper cell-lysis. Thereupon, the cell suspensions were transferred into 1.5 ml tubes and cell debris were removed by centrifugation at 13.000 rpm for 20 min at 4 °C. The pellet was discarded and protein concentration in the supernatant was determined by means of BCA™ Protein Assay Reagent Kit. Lysates (80-100 µl) were stored at -20 °C.

Determination of protein concentration

Bicinchoninacid (BCA) assay kit was used to estimate protein concentrations in cell lysates. During the reaction process, compounds that consist of at least two peptide bonds form a complex with Cu^{2+}. Thereby Cu^{2+} is reduced to Cu^{+}. BCA and Cu^{+}-ions build a coloured complex that is measured at 540 nm (absorption maximum at 562 nm).
Samples (20 µl diluted 1:5 with H_2O_{dest}) were mixed with 180 µl of freshly prepared BCA / cupper sulphate solution in a 96-well plate and incubated at 37 °C for 30 min. BSA served as a standard at an initial concentration of 2 mg/ml. The colorimetric detection of proteins was performed using a photometer and concentrations were calculated with Magellan V 5.0 software. Standard determination was performed according to the 4 parameter fit regression method.

SDS – PAGE

SDS-PAGE (sodium dodecyl sulphate poly-acrylamid gel-electrophoresis) was used to separate proteins according to their respective molecular weight. SDS eliminates non-covalent interactions and charges the proteins negatively. Therefore they can be separated by gel electrophoresis exclusively through their molecular weight.
For protein analysis, cell-lysates (3.2.3) were mixed 4:1 with 4 x SDS-sample buffer (3.1.5) and denatured at 95 °C for 5 -10 min. The samples (25 µl – 55 µl) that were adjusted to similar protein concentration by BCA (3.2.3) were loaded on an 8 % or 12 % polyacrylamid gel and run at constant voltage for approximately 3 h. For PAGE a vertical gel chamber was used. A prestained protein marker served as a reference. Proteins from the gel were further analyzed by Western Blotting.

Western blot analysis

Proteins separated by SDS-PAGE were blotted on nitrocellulose membranes (Millipore, Billerica, USA) by semidry blotting procedure with 1.57 mA / cm^2 for 2 h. To block unspecific binding, membranes were incubated in blocking buffer (3.1.5) for 1 h on a horizontal shaker with mild agitation (180 rpm) at room temperature (RT). Afterwards, proteins were detected using specific primary antibodies diluted in the appropriate buffers (table 3-4, table 3-16).

After incubation at 4 °C overnight, membranes were washed three times with 1 x PBS supplemented with 0.05 % (v/v) Tween20 (3.1.5, table 3-4) and incubated with horseradish peroxidise (HRP) – conjugated secondary antibody diluted 1:1000 in the respective buffers (table 3-16) for 2 h at RT. After three further washing steps with 1 x PBS supplemented with 0.05 % (v/v) Tween20 bound antibodies were detected by chemiluminescence using enhanced luminol. Chemiluminescence was either detected with a cooled CCD-camera device or by means of High performance chemiluminescence films that were developed in a developing machine. Analysis was performed with the Chemi-Capt software.

Characterization of IFNγ$^{-/-}$/SOCS1$^{-/-}$ and IFNγ$^{-/-}$ BMMs or BMDCs

To determine knockout efficiencies of SOCS1 in BMDCs or BMMS isolated from of IFNγ $^{-/-}$/SOCS1$^{-/-}$ and IFNγ$^{-/-}$ mice (3.2.1). Thereupon, cells were harvested and seeded in 6-well format (3x10^6 cells per well in DMEM, 10 % (v/v) FCS, 1 % (v/v) Penicillin/Streptomycin) as described in 3.2.1.1, cell stimulation. After 16 h, medium was changed to DMEM, supplemented with 2 % (v/v) FCS, 1 % (v/v) Penicillin / Streptomycin.The cells were stimulated with LPS (100 ng/ml) for 4 h. Afterwards, cells were lysed and treated as described in 3.2.3. Expression of SOCS1 was analyzed by Western blot (3.2.3).

Stripping of nitrocellulose membranes

To detect further proteins on membranes already exposed to ECL, Restore™ Western Blot stripping buffer was used. The membranes were washed three times with 1 x PBS supplemented with 0.05 % (v/v) Tween20 (3.1.5, table 3-4) and afterwards incubated with 15ml Restore™ Western Blot stripping buffer for 15 min at RT on a horizontal shaker (180 rpm) to remove bound antibodies (stripping). Upon three washing steps membranes were re-subjected to the respective antibodies as described in 3.2.3 and table 3-17.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA constitutes a method for the detection of nuclear transcription factor activity. Within this assay, binding of nuclear transcription factors (i.e., egr1) to its respective labeled sequence can be analyzed. To detect binding of egr1 to the target sequence within the SOCS1 promoter region, Cy3 fluorescent probes were used (3.1.7, table 3-10). The migration of the probe which is bound by a transcription factor is retarded in the gel due to the higher molecular weight of this complex. Thus, an activated transcription factor is detected as a shifted band, whereas unbound probe rapidly migrates through the gel. EMSA was performed as described by Andrews and Faller (138) and Bode *et. al.* (139). In the following, the used protocol is explained in brief.

To detect binding of active egr1 to the SOCS1 promoter, 1,2x10^7 BMMs from WT C57/BL6 mice were seeded (4x10^6 cells per well in 6-well plates, 3 wells combined for lysis) and stimulated as indicated. Afterwards, cells were washed with ice-cold PBS supplemented with 1 mM PMSF and 1 mM Na_3VO_4. Subsequently, the cells were harvested in 1 ml ice-cold PBS / 1 mM PMSF / 1 mM Na_3VO_4 and 3 wells were combined for centrifugation (1300 rpm, 10 min, 4 °C). After removing the supernatant completely, nuclear extracts were prepared in 2 steps. First, cytoplasmatic fraction of stimulated cells was isolated by means of 400 µl

buffer A (3.1.5, table 3-7) for 20 min on ice. Nuclei were pelletd by centrifugation (1300 rpm, 5 min, 4 °C). Incubation of the nuclear pellet in 40 µl complete lysis buffer (buffer C, 3.1.5, table 3-7) for 30 min on ice resulted in nuclear extracts that were either stored at -80 °C or used directly for EMSA after determination of the protein concentration (3.2.3).
To achieve binding of transcription factors within the nuclear extract to the probes, binding reaction was set up as published (138). Briefly, 3 µl of nuclear extract (about 9 µg protein) were mixed with 50 fmol probe in a total reaction volume of 20 µl EMSA binding buffer (3.1.5, table 3-7) for 15 min at RT. Afterwards, the DNA-protein complexes were loaded on a native gel without the usage of bromphenol blue containing loading buffer. As a control for a proper run, one lane of the gel was loaded with loading buffer only. After approximately 3 h at 100 V, 4 °C, the gel was analyzed. Fluorescence was directly detected within the gels using the Typhoon Trio detector. Cy3 was excited at 550 nm and emission was detected at 570 nm.

3.2.4. Statistical analysis

All experiments were performed at least two times and the number of experiments is stated in the legends. Means + SD are shown. Significant differences were evaluated by analysis of variance (ANOVA) to compare three or more groups followed by Dunnett's test to compare selected groups. Data were analyzed by means of GraphPad prism 4.03 program (GraphPad Software, San Diego California, USA); $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) were considered significant. Quantification of bands from respective immunoblots was performed with the QuantiOne software from Bio-Rad.

4. Results

4.1. Dectin-1 engagement induces SOCS1 expression in myeloid cells

It has been shown that suppressor of cytokine signalling proteins (SOCS) 1 and 3 and cytokine-inducible SH2-domain containing protein (CIS) can be induced in innate immune cells including dendritic cells and macrophages where they regulate the cells' responsiveness to various cytokines (89, 140, 141). SOCS proteins are not only induced via the Jak-STAT pathway but can also be expressed when pattern recognition receptors (PRRs), especially TLRs, are engaged. This work addressed the question whether SOCS proteins, in particular SOCS1, can also be induced by the non-TLR PRR Dectin-1 in murine bone marrow derived dendritic cells (BMDCs) and macrophages (BMMs). Moreover, the pathways involved in Dectin-1 induced SOCS1 expression should be analyzed as well as possible functions of SOCS1 in Dectin-1 signalling.

4.1.1. Depleted Zymosan induces SOCS1 in BMMs and BMDCs

The initial step was to clarify the question whether Dectin-1 is able to trigger SOCS1 expression. It is known that Dectin-1 signalling partially interacts with signals from TLR2 or TLR6 (55, 63). This interaction results in an increase and prolongation of NF-κB activity and cytokine production upon receptor engagement as described for dendritic cells (76, 77). Therefore we made use of depleted Zymosan that should lack any TLR activating capacity (71). Depleted Zymosan is a cell wall preparation of the yeast *Saccharomyces cerevisiae* treated with hot alkali to remove all its TLR binding properties. Thus, depleted Zymosan acts as a Dectin-1 agonist but does not bind and activate TLRs. In contrast, untreated Zymosan is capable of binding to both, Dectin-1 as well as TLRs, especially TLR2.
Stimulation of bone marrow derived macrophages (BMMs) and dendritic cells (BMDCs) resulted in a robust induction of SOCS1 on transcriptional and protein level. SOCS1 mRNA was induced up to 40-fold in BMMs and 20 fold in BMDCs upon stimulation with depleted Zymosan and comparable to well-known SOCS1 inducers like LPS or IFNs (Fig. 4-1 [A, B]). LPS was always used as a positive control for proper SOCS1 induction. Additional priming of BMMs with IFNγ previous to stimulation with depleted Zymosan led to a further significant increase by 10-fold in SOCS1 mRNA (Fig. 4-1 [A]). Furthermore, triggering of Dectin-1 resulted in a dose-and time-dependent expression of SOCS1 as examined by qRT-PCR (Fig. 4-1 [C]). Whereas induction of SOCS1 mRNA displayed a peak-induction time at 4.5 h in BMMs, expression in BMDCs reached a plateau already after 2 h with constant expression even 16 h after initial stimulation. Importantly, LPS induced a constant expression of SOCS1 within the indicated time frame as well; SOCS1 mRNA was induced even up to 100-fold as compared to unstimulated control. In contrast, in previous reports LPS was described as a fast but short-termed inducer of SOCS mRNA. Protein levels of SOCS1 reflected mRNA induction. Depleted Zymosan clearly induced SOCS1 protein expression, even at lower concentrations (100 µg/ml). Similarly, LPS stimulation displayed induction of SOCS1 proteins at the used concentrations. However, LPS induced higher amounts of SOCS1 protein as compared to cells stimulated with depleted Zymosan (Fig. 4-1 [B]). This was expected since

LPS is published to be a strong inducer of SOCS1. Besides SOCS1, SOCS3 and CIS are well characterized members of the SOCS family. Thus, we analyzed BMDCs and BMMs for expression of the latter transcripts. Both cell types displayed an induction of SOCS3 and CIS upon engagement of Dectin-1 (Fig. 4-1 [D], [E]). In contrast to SOCS1, SOCS3 and CIS transcripts were induced up to 20-fold as compared to unstimulated control. Again, LPS served as positive control, leading to a strong expression of the displayed SOCS molecules.

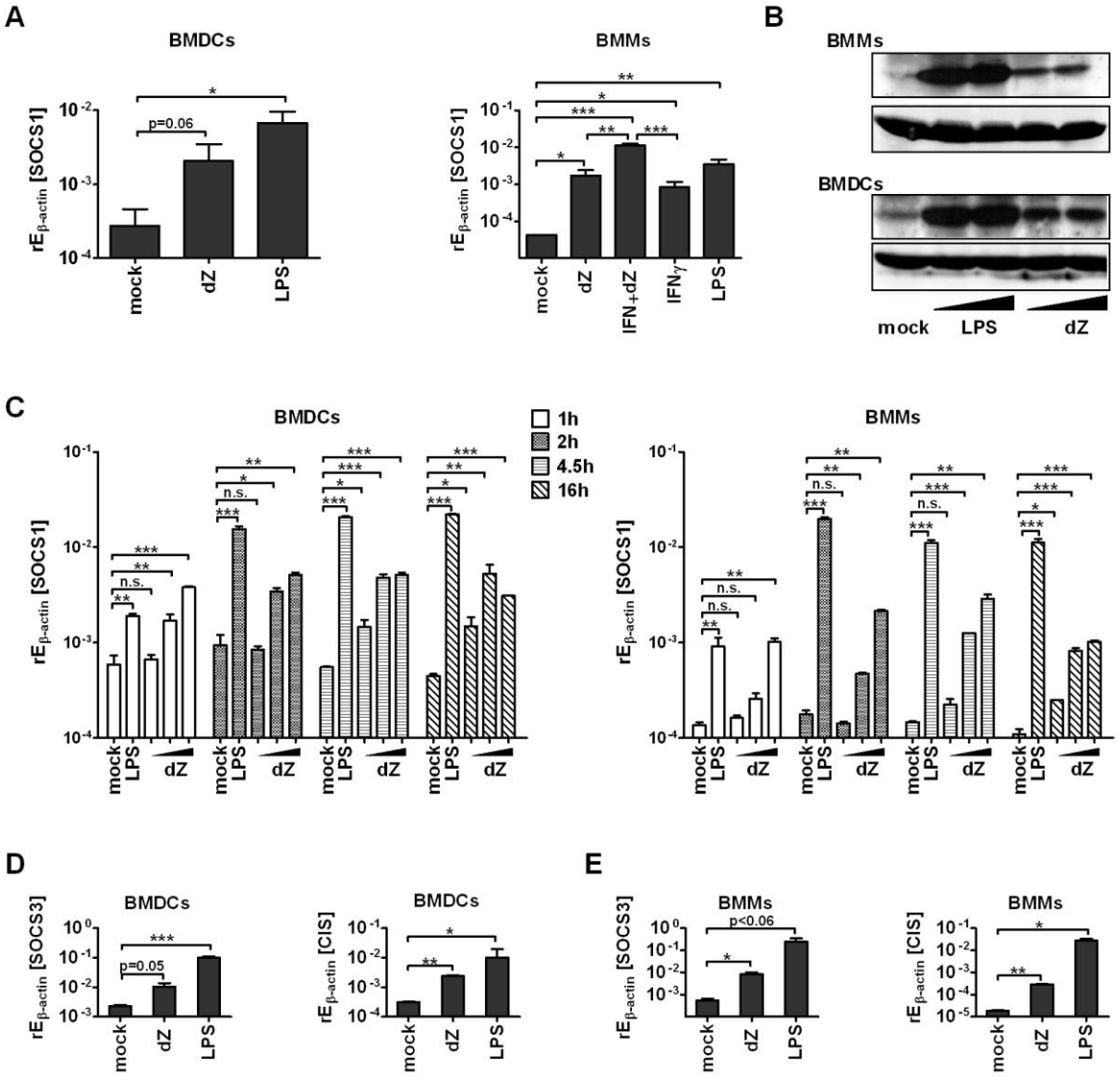

Fig. 4-1 Suppressors of cytokine signalling (SOCS) are induced upon engagement of Dectin 1

[A],[C], [D] and [E], RT-PCR analysis: BMDCs ($5x10^5$) or BMMs ($3x10^5$) from WT mice were stimulated with different concentrations of dZ ([A], [D] and [E], 100 µg/ml; [C], 10 µg/ml, 100 µg/ml, 500 µg/ml) or LPS (100 ng/ml)). [A, D and E], cells were lysed after 4.5 h of stimulation. [C], cells were stimulated for the indicated time-points. For priming experiments (BMMs, [A]), cells were pre-incubated with IFNγ (50 ng/ml) for 16 h. Cells were lysed and gene expression of SOCS1 [A, C], SOCS3 and CIS [D, E] was measured by quantitative RT-PCR. Depicted are expression levels normalized to the expression of the housekeeping gene β-actin. rE, relative expression. [B] BMDCs or BMMs ($18x10^6$) were stimulated with increasing concentrations of LPS (10 ng/ml to 100 ng/ml) or dZ (100 µg/ml to 500 µg/ml) for 6 h and lysates were analyzed by immunoblot with antibodies against SOCS1 or β-actin as control. [A], [D] and [E] n=3, mean + SD; [C] n=2, mean + SD, results in [B] are representative for 3 independent experiments. mock, unstimulated control

4.1.2. SOCS1 induction by depleted Zymosan is independent of TLRs

As mentioned above, Zymosan is capable of engaging also TLRs, but depleted Zymosan should only trigger Dectin-1. Therefore we analyzed BMDCs and BMMs isolated from wild type (WT) or MyD88/Trif$^{-/-}$ mice to ensure that depleted Zymosan induces SOCS1 independently of any TLR activity. All known TLRs use MyD88 or Trif (TLR3) as adaptor proteins for appropriate signalling. No reduction of SOCS1 mRNA levels could be observed when stimulating BMDCs and BMMs from knockout mice with depleted Zymosan as compared to cells from the WT control (Fig. 4-2 [A]). In contrast, LPS mediated expression of SOCS1 was completely abolished in cells from MyD88/Trif double deficient mice as expected from published data since LPS triggered TLR4 is able to signal via both, MyD88 and Trif (32, 34, 35). Compared to Fig. 4-1 [A] and [C], the SOCS1 expression in WT DCs induced by depleted Zymosan was attenuated. This could be due to variability in activation of primary cells. We observed in some DC preparations that the cells were pre-stimulated, resulting in a decreased induction of SOCS proteins as compared to other preparations. Consistent with the above observations that depleted Zymosan induced SOCS1 expression was not altered in MyD88/Trif$^{-/-}$ mice compared to WT mice, downstream signalling was unaffected in MyD88/Trif knockout BMDCs and BMMs. Phosphorylation of the MAPK ERK was not altered upon Dectin-1 triggering in WT compared to knockout cells (Fig.4-2 [B]). As expected, LPS induced ERK activation was completely abolished in the knockout cells. Importantly, BMDCs generated from MyD88/Trif double deficient mice were not able to develop a murine DC phenotype (low CD11c expression). Additionally, these cells showed no induction of ERK activation upon stimulation. This could be due to the knockout of MyD88 since this adaptor protein is an important myeloid differentiation factor (34). In contrast, BMMs displayed a normal phenotype (high CD11b, high F480), comparable to WT murine BMMs.

Also, cells from TLR2 knockout mice displayed an unmodified expression of SOCS1 compared to WT cells when stimulated via Dectin-1 (Fig. 4-3 [A]). The TLR2 ligand Pam_3CSK_4 was capable of inducing SOCS1 expression in WT BMDCs and BMMs up to 40-fold compared to unstimulated cells whereas SOCS1 expression in TLR2 knockout mice was abrogated (Fig. 4-3 [A]). Pam_3CSK_4 served as a control for appropriate knockout. Accordingly, TLR2 that is described to be an important co-receptor of Dectin-1 is not involved in SOCS1 induction in Dectin-1 triggered cells. In addition, MyD88/Trif dependent pathways can be excluded, thus identifying Dectin-1 as a receptor that is able to induce SOCS1 upon stimulation with depleted Zymosan.

These findings were confirmed by stimulating BMMs isolated from Dectin1$^{-/-}$ mice with depleted Zymosan. As a result, SOCS1 expression was entirely abolished in the knockout BMMs in contrast to wildtype cells when stimulated with depleted Zymosan. LPS stimulation led to SOCS1 expression of more than 100-fold in WT BMMs at the same level as compared to Dectin1$^{-/-}$ cells (Fig.4-3 [B]). Thus, we could clearly demonstrate that SOCS1 induction upon triggering of cells with depleted Zymosan was exclusively mediated by Dectin-1.

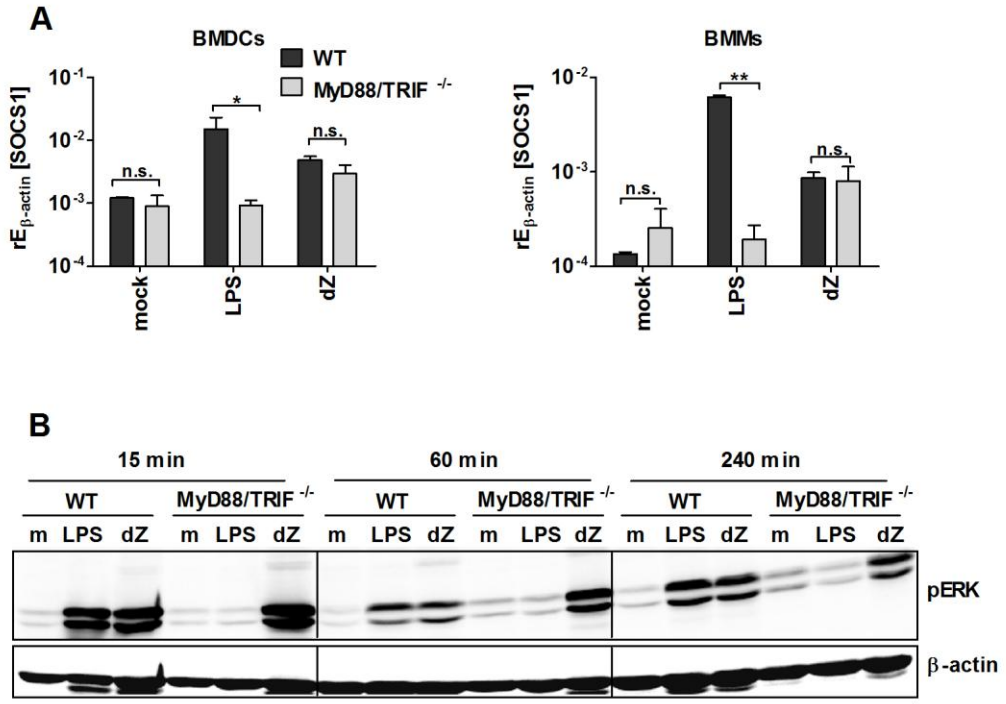

Fig. 4-2 Zymosan induced SOCS1 is expressed independently of TLR signaling

[A], BMDCs ($5x10^5$) or BMMs ($3x10^5$) from WT or MyD88/TRIF$^{-/-}$ mice were stimulated with either 500 µg/ml dZ or 100 ng/ml LPS for 4.5 h. Cells were lysed and gene expression of SOCS1 was analyzed by quantitive RT-PCR. Depicted are expression levels normalized to the expression of the housekeeping gene β-actin. rE, relative expression. [B], BMMs ($3x10^6$) from WT or Myd88/TRIF$^{-/-}$ mice were incubated with LPS (100 ng/ml) or dZ (500 µg/ml) as indicated. Lysates were analyzed by immunoblot. Blots were probed with antibodies specific for ERK 1/2 phosphorylated on Thr ^{202}/Tyr ^{204} or β-actin as control. [A] n=2, mean + SD, results in [B] are representative for 2 independent experiments

Our results show that SOCS1 is induced in both cell types, BMMs as well as BMDCs upon ligation of Dectin-1 with depleted Zymosan on transcript and protein level. In comparison to the used TLR stimuli (LPS, Pam_3CSK_4), Dectin-1 triggered SOCS1 expression was completely independent of any TLR activity as shown in MyD88/Trif knockout as well as TLR2 knockout cells. The next step was to clarify the question if SOCS1 is induced in a direct manner upon engagement of Dectin-1 without the need of any intermediate secreted or intracellular factors.

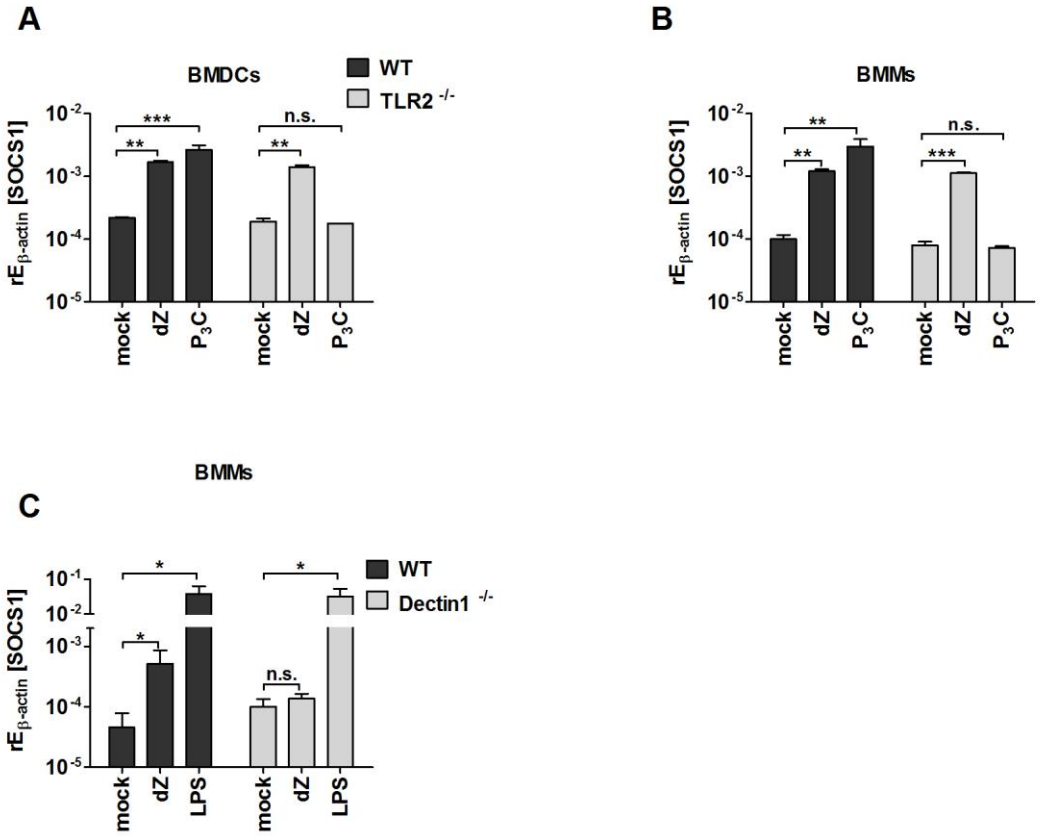

Fig. 4-3 SOCS1 induction is strictly dependent on Dectin-1

[A], [B] and [C], BMDCs ($5x10^5$) or BMMs ($3x10^5$) from WT [A, B, C], $TLR2^{-/-}$ [A, B] or Dectin-$1^{-/-}$ cells [C] were triggered with dZ (100 µg/ml) [A, B] or Pam_3CSK_4 (P_3C) (100 ng/ml) for 4.5 h. Expression of SOCS1 transcripts was measured by quantitative RT-PCR. Displayed are relative expression levels normalized to β-actin expression. [A] and [B] n=2, mean + SD.

4.1.3. SOCS1 is a direct target gene of Dectin-1

To further analyze the mode of induction of SOCS1 it was necessary to establish whether intermediate factors, i.e. secreted auto- or paracrine factors like type I IFNs, participate in SOCS1 expression. Foremost we made use of a transwell system in which BMDCs and BMMs were placed in both wells. Stimulation with depleted Zymosan or LPS was carried out in the lower wells only. The pore size of 0.4 µm of the transwell strainer excluded any diffusion of depleted Zymosan (average size of particles: 3 µm (142)) into the upper chambers which ensured a sole stimulation of cells in the lower wells. Accordingly, expression of SOCS1 in cells of the upper compartments would indicate that soluble, intermediate factors produced by depleted Zymosan stimulated BMMs or BMDCs in the lower wells participate in SOCS1 induction. Upon stimulation with depleted Zymosan expression of SOCS1 mRNA could only be observed within cells of the lower compartments (Fig. 4-4). Therein, SOCS1 transcripts were induced up to 20-fold as compared to the unstimulated control. In cells placed in the upper compartments, SOCS1 expression was not observed. In contrast, LPS that is able to enter the upper wells by diffusion was capable of inducing SOCS1 expression in BMMs or BMDCs in both chambers of the transwell (Fig. 4-4). Hence, LPS induced SOCS1 in both chambers to the same extent; up to 20-fold in BMDCs and 100-fold in BMMs as compared to the unstimulated control in the respective wells. These

results indicate that SOCS1 is expressed upon triggering of Dectin-1 in myeloid cells without the attendance of soluble factors.

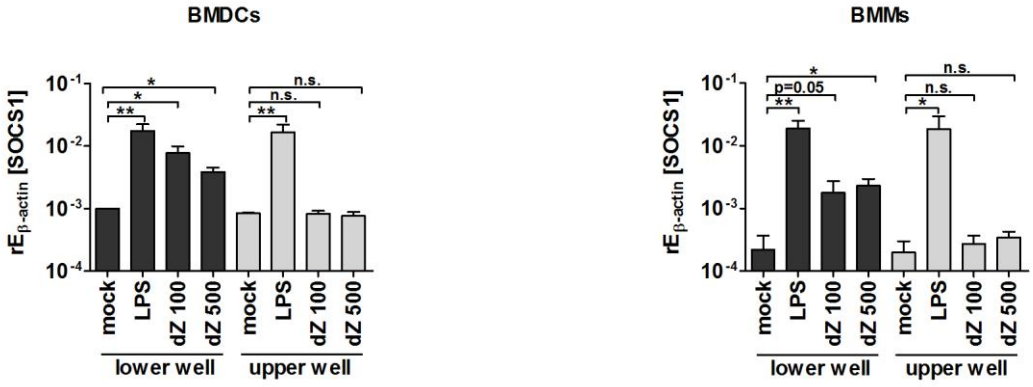

Fig. 4-4 Influence of soluble factors on SOCS 1 induction

BMDCs or BMMs were seeded in a transwell system (BMDCs: $5x10^5$ in lower wells, $2x10^5$ in upper wells; BMMs: $3x10^5$ in lower wells, $1x10^5$ in upper wells). Cells in the lower chambers were stimulated with dZ (100 µg/ml or 500 µg/ml) or LPS (100 ng/ml) or left unstimulated (mock). After 4.5 h, cell lysates of both wells were analyzed for SOCS1 expression by qRT-PCR; normalization was performed against β-actin as control. n=3, mean+SD

LPS is described to induce type I IFNs (143, 144). Thus, the expression of SOCS1 observed upon stimulation of cells within the upper chambers of the transwell could originate additionally from factors produced by LPS triggered cells.

IFNs produced by LPS or other TLR ligands subsequently activate the Jak/STAT pathway and therefore may be responsible for inducing SOCS1. In contrast, other publications excluded a contribution of TLR induced type I IFNs to induction of SOCS proteins (143). To investigate any possible role of secreted type I IFNs on the induction of SOCS1 after engagement of Dectin-1, cells from type I IFN receptor deficient ($IFNAR1^{-/-}$) mice were stimulated.

SOCS1 induction showed no differences in $IFNAR1^{-/-}$ cells compared to wildtype when stimulated with either LPS or depleted Zymosan (Fig. 4-5 [A]). These findings strengthen the results observed with the transwell approach in which SOCS1 was induced independently of secreted intermediate factors when cells were stimulated via Dectin-1. Importantly, cells from the knockout mice triggered with LPS displayed no difference in SOCS1 expression when compared to the wildtype control as well. Since it has been shown that TLR4 triggering induces SOCS in a direct and type I IFN-independent manner, we expected no difference in LPS induced SOCS1 mRNA level in $IFNAR1^{-/-}$ cells (Fig. 4-5 [A]).

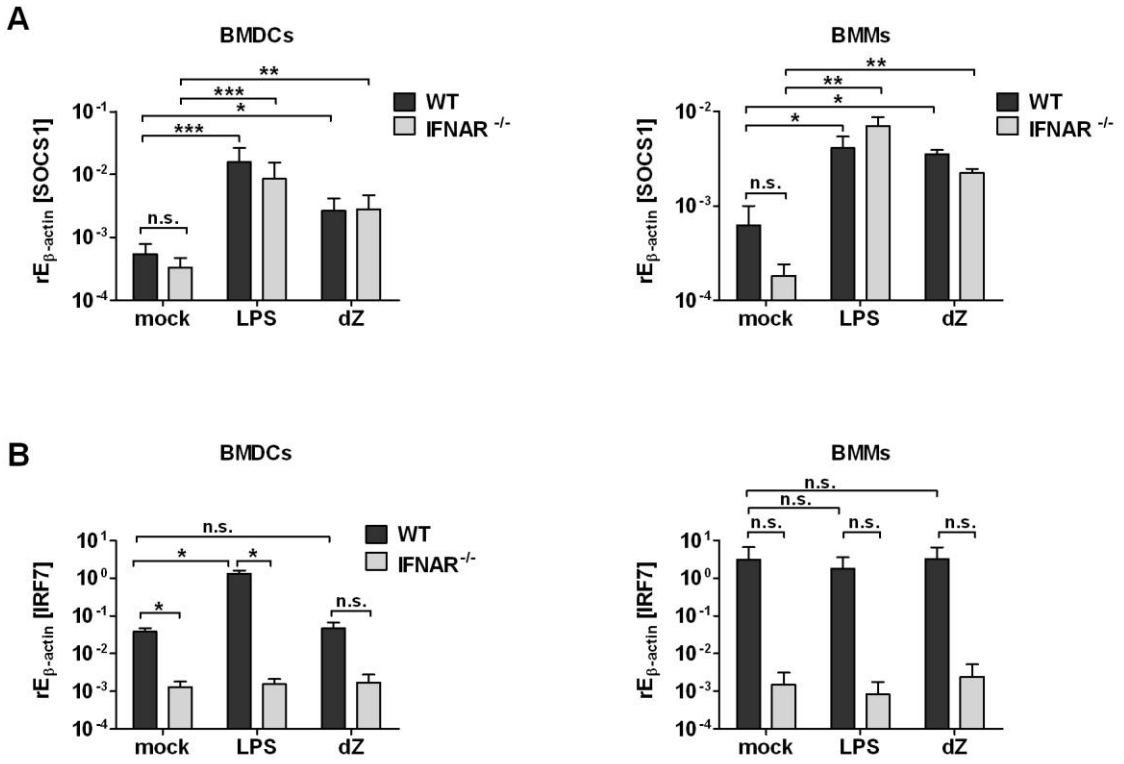

Fig. 4-5 No contribution of Type I IFNs to the expression of Dectin-1 induced SOCS1

[A] and [B], BMDCs ($5x10^5$) or BMMs ($3x10^5$) from WT or IFNAR1$^{-/-}$ (IFNAR$^{-/-}$) mice were stimulated as indicated with dZ (100 µg/ml) or LPS (100 ng/ml). After 4.5 h, cells were lysed and gene expression of SOCS1 [A] or IRF7 [B] as knockout control was analyzed by quantitative RT-PCR. Relative expression levels normalized to β-actin. [A] and [B], n=2; mean + SD

Expression of IRF7 (Interferon regulatory factor 7), which is a type I IFN dependent gene served as control. IRF7 is essential for the induction of IFN α/β dependent genes (39, 40, 145). Whereas IRF7 was induced by LPS up to 50-fold in WT DCs, IFNAR1$^{-/-}$ BMDCs expectedly showed loss of IRF7 induction as well as a reduced basal expression (Fig. 4-5 [B]). BMMS displayed no induction of IRF7 transcripts when triggered via LPS. Regarding treatment of myeloid cells with depleted Zymosan, neither BMDCs nor BMMs induced IRF7 mRNA. Importantly, the basal levels of IRF7 were reduced in IFNAR1$^{-/-}$ cells (Fig. 4-5 [B]). These findings suggest that BMDCs as well as BMMs can not produce type I IFNs when stimulated via Dectin-1 since IRF7 is a direct target gene of IFNα or IFNβ. However, many other type I IFN inducible genes are described beside IRF7. The transcription factor STAT1 is shown to be activated by phosphorylation upon stimulation with type I IFNs (146, 147). We therefore additionally analyzed STAT1 phosphorylation in BMMs and BMDCs upon stimulation with depleted Zymosan as well as LPS. Stimulation of BMMs as well as BMDCs with LPS led to a strong phosphorylation of STAT1 in a time-dependent manner. (Fig. 4-6 [A, B]) However, depleted Zymosan displayed a clear activation of STAT1 exclusively in BMDCs that was not detectable until 90 min after stimulation (Fig. 4-6 [B]). In BMMs, depleted Zymosan had no effect on STAT1 phosphorylation when compared to the unstimulated control (Fig. 4-6 [A]). Thus, these date rule out a contribution of the Jak/STAT pathway (indirect IFN signalling) to the induction of SOCS1.

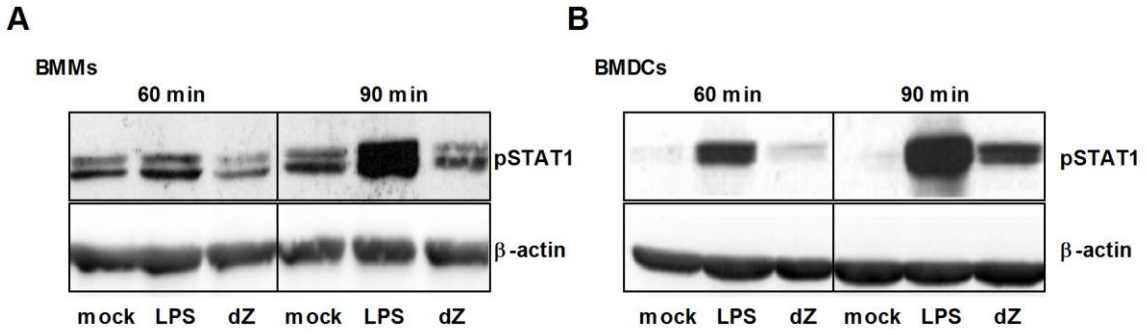

Fig. 4-6 STAT1 activation upon ligation of Dectin-1

[A], BMMs ($3x10^6$) or [B] BMDCs ($3x10^6$) from WT mice were triggered by dZ (500 µg/ml) or LPS (100 ng/ml) for the indicated time periods. Cell lysates were analyzed by immunoblot; blots were probed with antibodies directed against STAT 1 phosphorylated on Tyr701 and β-actin as loading control. Results show representative data from 2 independent experiments.

Based on the results obtained with the transwell experiments as well as the IFNAR1$^{-/-}$ cells, we concluded that auto- or paracrine, secreted factors, i.e. type I IFNs were not involved in expression of SOCS1 in depleted Zymosan stimulated BMMs as well as BMDCs.

To exclude that any intracellular intermediate protein may participate in SOCS1 expression, we next treated the cells with cycloheximid as an inhibitor of *de novo* protein biosynthesis. Therefore, mRNA is still expressed, whereas protein synthesis is inhibited. Thus, it can be analyzed if SOCS1 is transcribed directly without the need of any inducible intermediate protein. Application of cycloheximid prior to stimulation with depleted Zymosan or LPS had no effect on SOCS1 mRNA induction. Importantly, stimulation with depleted Zymosan following cycloheximid treatment even slightly increased SOCS1 mRNA induction (Fig. 4-7 [A, B]). Since cycloheximid is an inhibitor of protein synthesis, it can be hypothesized that also the protein expression of inhibitors of transcription factors is abrogated. This could explain an increase in mRNA levels of particular genes. To prove successful inhibition of protein synthesis, BMMs and BMDCs were stimulated with LPS and analyzed for secretion of IL12p40 and TNFα by ELISA. In case of the pre-treated cells, cytokine secretion was entirely abolished (Fig. 4-7 [C, D]). Our results suggest that SOCS1 is induced directly without any intermediate protein synthesis.

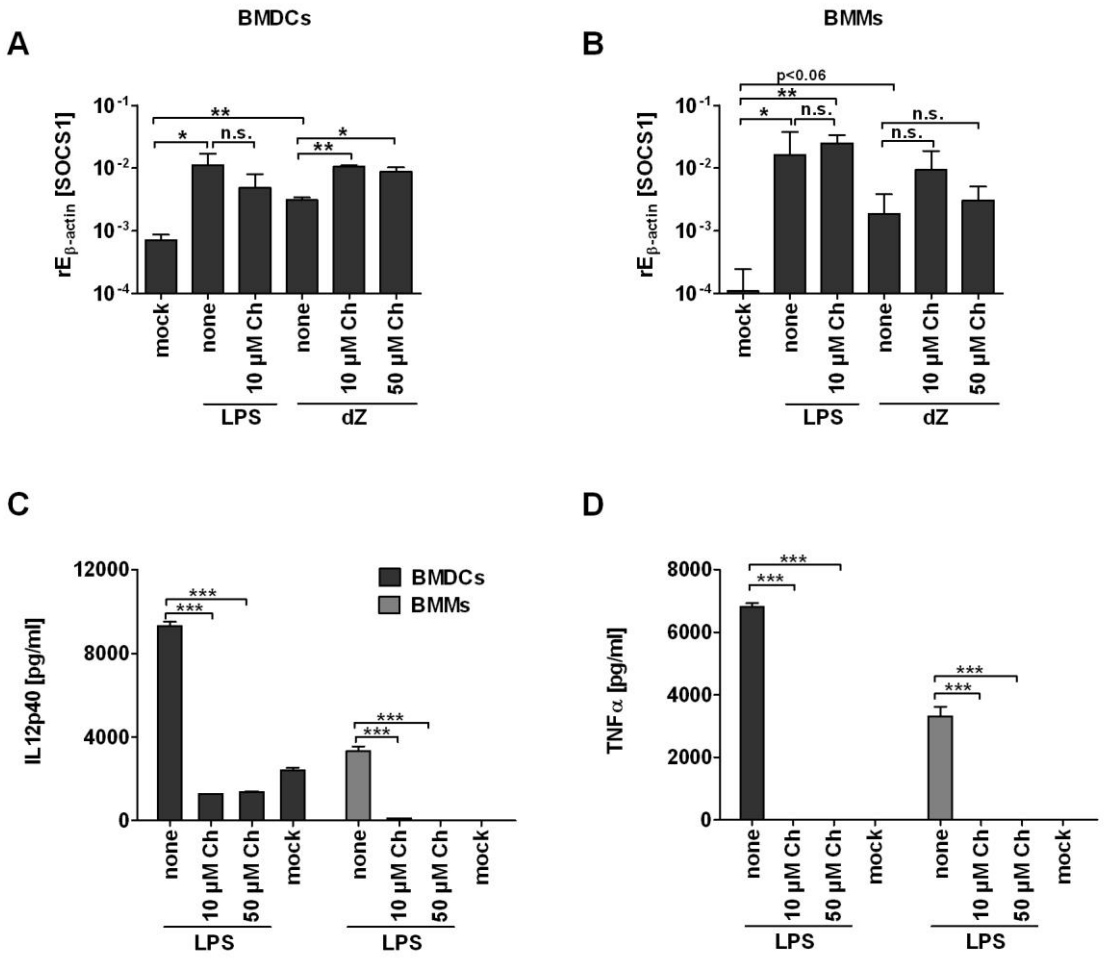

Fig. 4-7 SOCS1 induction is independent of intermediate protein biosynthesis

[A and B], BMDCs ($5x10^5$) or BMMs ($3x10^5$) pretreated for 1h with 10 or 50 µM Cycloheximid (Ch) were stimulated as indicated for 4.5 h. SOCS1 gene expression was measured by quantitative RT-PCR with β-actin as control reference. [C], BMDCs ($2x10^5$) or [D] BMMs ($2x10^5$) were pretreated with Ch as in [A]. Stimulation was performed with 100 ng/ml LPS for 16 h. IL12p40 and TNFα secretion in cell supernatants was determined by ELISA. none; without inhibitor; mock, unstimulated control. [A] n=3, mean+SD, [B and C] n=2; mean + SD.

Upon infection with fungi, myeloid cells generally produce high amounts of the anti-inflammatory cytokine IL10 (61, 120, 148). For this reason, we additionally wanted to exclude IL10 as a mediator of SOCS1 transcription in Dectin-1 triggered cells. Therefore, cells were incubated with an anti-IL10 antibody to block IL10 signalling prior to stimulation. Afterwards, expression of SOCS1 and SOCS3 mRNA were measured. Neither LPS- nor depleted Zymosan- induced SOCS1 could be inhibited by anti-IL10 antibody (Fig. 4-8 [A], [B]). These data verify our findings above of a direct SOCS1 expression. IL10 itself was not able to induce SOCS1 expression confirming published data (Fig. 4-8 [A]). SOCS3 is known to be effectively induced by IL10. Thus, we analyzed BMMs and BMDCs for expression of SOCS3 upon incubation with anti-IL10 antibody followed by stimulation with IL10. SOCS3 mRNA expression was effectively abolished in cells treated with the blocking antibody (Fig. 4-8 [B]). Hence, SOCS3 induction served as a control for functional antibody blockade.

These data further confirm that SOCS1 induction upon Dectin-1 ligation by depleted Zymosan is mediated in a direct way. Intracellular or soluble intermediates like type I IFNs or IL10 could be excluded.

Further, we assessed downstream signalling leading to SOCS1 induction in Dectin-1 triggered cells. Therefore, we analyzed if the transcription factor NF-κB could be responsible for SOCS1 induction.

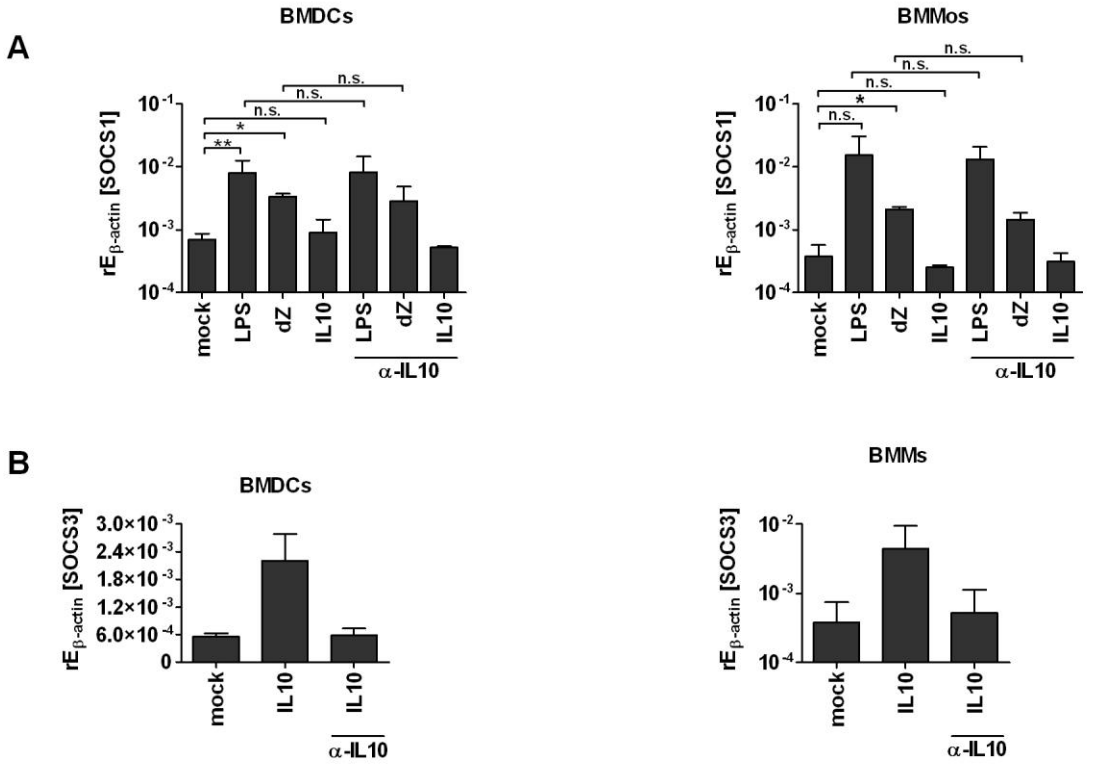

Fig. 4-8 IL10 does not play a role in Dectin-1-triggered SOCS1 induction

[A and B], BMDCs ($5x10^5$) or BMMs ($3x10^5$) were pretreated for 30 min with anti-IL10 antibody (α-IL10, 10 µg/ml) and stimulated with dZ (100 µg/ml), LPS (100 ng/ml) or IL10 (10 ng/ml) as control for another 4.5 h. Mock, unstimulated cells. Cells were lysed and analyzed for gene expression of SOCS1 [A] or SOCS3 [B] by quantitative RT-PCR. Displayed are relative expression levels normalized to β-actin. n=2; mean + SD

4.1.4. NF-κB is not involved in Zymosan induced SOCS1 induction in macrophages

Previously, Goodrige *et al.* showed that in BMMs, in contrast to BMDCs, NF-κB is not activated upon stimulation of Dectin-1 (71). We therefore investigated downstream signalling pathways of Dectin-1 in myeloid cells as our findings hint towards an unknown mode of transcriptional induction of SOCS1. First, we analyzed MAPK activation as well as activation of spleen tyrosine kinase (Syk) in Dectin-1 triggered cells to ensure activation of downstream signalling. Stimulation of BMDCs and BMMs led to a distinct phosphorylation and thereby activation of the MAPKs p38, JNKII and ERK as well as Syk. The latter is described to mediate proximal signalling of Dectin-1 in cells of the immune system, for example dendritic cells and macrophages, but also B-cells or T-cells (46, 52, 70). Importantly, Syk was not phosphorylated in BMMs stimulated via LPS (Fig. 4-9 [A]). These findings confirm that Dectin-1 triggered cells are capable of activating the MAPK p38, ERK and JNKII.

Next, a possible contribution of NF-κB to SOCS1 induction in Dectin-1 triggered cells was investigated. Activation of NF-κB can be demonstrated indirectly by phosphorylation of the NF-κB inhibitor IκBα. Phosphorylated IκBα is degraded and functional NF-κB subunits are

released. BMMs and BMDCs were stimulated by depleted Zymosan or LPS and analyzed for IκBα phosphorylation by immunoblot. LPS as well as depleted Zymosan led to a distinct phosphorylation of IκBα in BMDCs, yet, the unstimulated control already displayed slight phosphorylation. In contrast, BMMs showed phosphorylation of the NF-κB inhibitor only when stimulated by LPS (Fig. 4-9 [B]).

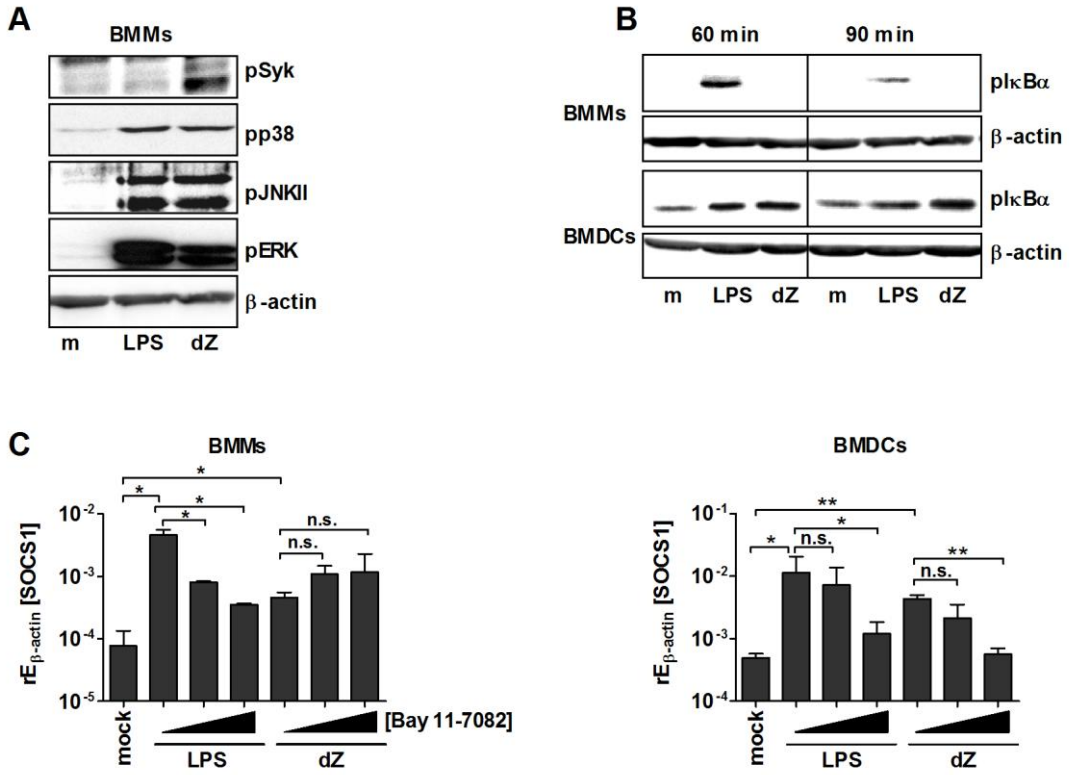

Fig. 4-9 NF-κB is not activated in BMMs upon Dectin-1 engagement

[A, B], Immunoblots of lysates of BMMs ($3x10^6$) or BMDCs ($3x10^6$). [A], cells were stimulated with LPS (100 ng/ml) or dZ (500 µg/ml) for 30 min. [B], cells were stimulated for the indicated time-periods with LPS (100 ng/ml) or dz (500 µg/ml). [A], blots were probed with Abs specific for ERK 1/2 phosphorylated on Thr ^{202}/Tyr ^{204}, JNKII phosphorylated on Thr^{183}/Tyr^{185}, p38 phosphorylated on Thr^{180}/Tyr^{182} and Syk phosphorylated on Tyr^{402}. [B], blots were probed with Abs against IκBα phosphorylated on Ser^{32}, [C], BMMs ($3x10^5$) or BMDCs ($5x10^5$) were pre-incubated for 1 h with 0, 10 or 50 µM of the NF-κB inhibitor Bay 11-7082 and stimulated with LPS (100 ng/ml) or dZ (100 µg/ml). Cells were lysed after 4.5 h and gene expression (SOCS1) was measured by quantitative RT-PCR. Shown are relative expression levels normalized against β-actin. Figures [A] and [C] show representative data for 3 experiments (mean + SD), results in [B] show representative data for 2 experiments. m, mock (unstimulated control)

These data indicate that NF-κB is not activated in BMMs upon stimulation with depleted Zymosan which had additionally been described in previous reports (71). To further confirm this observation, we made use of a highly specific NF-κB inhibitor (Bay 11-7082). BMMs and BMDCs were treated with 10 µM or 50 µM of the inhibitor 1 h prior to stimulation with LPS or depleted Zymosan. After 4.5 h of stimulation, cells were lysed and analyzed for SOCS1 mRNA expression. With respect to BMMs, none of the applied concentrations of the NF-κB inhibitor had an effect on SOCS1 induction. The induction was even slightly increased (Fig. 4-9 [C]). LPS stimulated cells displayed a decrease of SOCS1 transcripts when pre-treated with the inhibitor as expected, since TLR ligands are shown to activate NF-κB. Higher inhibitor concentrations down-regulated SOCS1 expression by more than 10-fold in LPS stimulated cells. In BMDCs, SOCS1 induction was diminished to basal levels upon the

application of the inhibitor followed by stimulation with depleted Zymosan as well as LPS (Fig. 4-9 [C]). Based on these results we claim that NF-κB plays a role in SOCS1 induction exclusively in BMDCs but not in BMMs when triggered via Dectin-1. The observed phosphorylation of IκBα upon stimulation with depleted Zymosan in BMDCs confirms a role of NF-κB only in these cells (Fig. 4-9 [B]). In contrast, LPS is capable of inducing SOCS1 in an NF-κB dependent manner in both cell types. Inhibition of NF-κB in both cells, BMDCs as well as BMMs led to a clear-cut inhibition of LPS induced SOCS1 expression. Additionally, phosphorylation of IκBα was observed in both cell types upon LPS stimulation.

As a NF-κB independent SOCS1 induction was never shown before, we carried out most of the following experiments in BMMs to reveal a possible new pathway to express SOCS1.

NFAT (nuclear factor of activated T-cells) is reported to be one of the first transcription factors that is activated upon recognition of Zymosan or live *C. albicans* yeast by Dectin-1 (61, 149). Since we excluded a contribution of NF-κB to SOCS1 induction in BMMs, we examined if NFAT could be a possible candidate. Therefore, we made use of a potent inhibitor of NFAT activity. Cyclosporin A (CsA) blocks nuclear translocation of activated NFAT and thus inhibits transcription of NFAT dependent genes. Early growth response factors (egr) are a family of transcription factors that are directly induced by NFAT. In BMDCs and BMMs, it has been shown that heat killed *Candida albicans* as well as Zymosan induce NFAT and egr1 and egr2 transcription. Thus, we first examined if egr1 and egr2 are expressed in BMMs that are stimulated via depleted Zymosan.

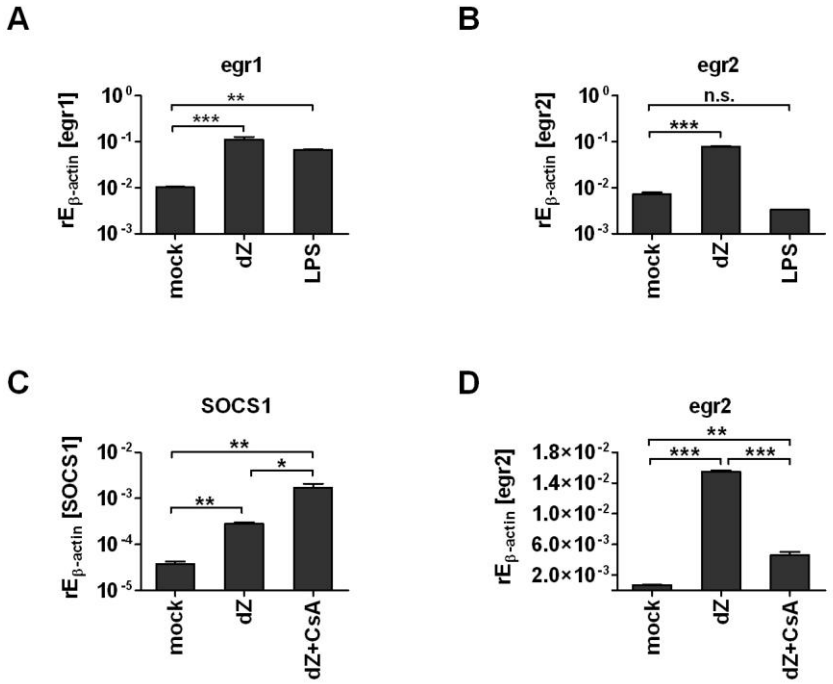

Fig. 4-10 egr1 and egr2 are induced in Dectin-1 triggered BMMs

BMMs ($3x10^5$) from WT mice were triggered by LPS (100 ng/ml) or dZ (100 µg/ml) for 1h. Cell lysates were analyzed by quantitative RT-PCR. Relative expression of [A] egr1, [B andD] egr2 and [C] SOCS1 is displayed normalized to β-actin. [C and D], $3x10^5$ BMMs were pre-treated with 10 µM CsA 1 h prior to stimulation. Cells were stimulated with 100 µg/ml depleted Zymosan and analyzed for expression after [C] 1 h or [D] 4.5 h. n=2; mean + SD

As illustrated in Fig. 4-10, egr1 [A] and egr2 [B] were definitely induced in BMMs by 10-fold as compared to unstimulated (mock) control following Detin-1 stimulation. Importantly, LPS showed a significant expression of egr1, but was not able to induce egr2 expression (Fig. 4-10 [A] and [B]). Afterwards, cells were incubated with 10 µM CsA for 1 h prior to stimulation with depleted Zymosan. Analysis of mRNA expression of depleted Zymosan induced SOCS1 revealed that CsA had no inhibitory effects on SOCS1 induction. The expression of SOCS1 was rather increased up to 10-fold as compared to stimulated cells that were not treated with the inhibitor (Fig. 4-10 [C]). In addition, induction of egr2 mRNA was examined to prove efficacy of the inhibitor. Cells were treated with 10 µM CsA prior to stimulation with depleted Zymosan. Thereupon, cells were analyzed for egr2 expression. Treatment of BMMs with CsA reduced the induction of egr2 transcripts by more than 70 % compared to stimulated cells (Fig 4-10 [D]). Based on these results, we concluded that NFAT activity is efficiently blocked by CsA since this inhibitor diminished egr2 expression. Otherwise, CsA even increased depleted Zymosan induced SOCS1 expression but did not at all block synthesis of SOCS1 mRNA. Thus, NFAT is not involved in SOCS1 induction. It even had anti-inhibitory effects.

We next attempted to confirm those findings and examined if egr transcription factors show binding activity to the SOCS1 promoter by EMSA. The consensus sequence of the egr factors has already been published (GCG(G/T)GGGCG) (150). Promoter elements of human SOCS1 to which murine egr could bind are described before (150). A probe was generated that comprised the sequence of the SOCS1 promoter with a Cy3-fluorophor coupled to the 5'-end (see 3.1.7 and Fig. 4-11 [B]). The binding sequence of egr1 differs in one base from the consensus sequence as depicted in Fig. 4-11 [B]. To test whether depleted Zymosan could induce binding of egr to the SOCS1 probe, cells were stimulated with depleted Zymosan, LPS or Zymosan and nuclear extracts were prepared after 6 h of stimulation. At earlier time-points, extracts from Zymosan stimulated cells did not show any binding activities. Binding activity of proteins from the nuclear extracts was assessed by EMSA. Detection of the probe was achieved via the coupled fluorophor Cy3. The EMSA results are depicted in Fig. 4-11 [A]. In all lanes, one band was detectable which might be assigned to an unspecific binding as it appears to be constant within all lanes. However, an additional band increased upon stimulation with LPS or Zymosan (Fig. 4-11 [A], indicated by an arrow). This band possibly corresponds to egr1. Supershift experiments with an antibody against egr1 could clarify these observations. Importantly, even unstimulated cells showed a weak binding of egr1 to the SOCS1 probe. Thus, the unstimulated control was already pre-activated.

Transcriptional function of egr for SOCS1 expression could not be verified in cells stimulated with depleted Zymosan. However, nuclear extracts from LPS as well as Zymosan stimulated cells showed an increase in binding activites. Since Zymosan also exhibits TLR binding activity, the observed binding of nuclear proteins extracted from Zymosan stimulated cells could originate from the TLR binding moiety.

Our results indicate that SOCS1 is induced in Dectin-1 triggered BMMs and BMDCs in a direct way. Additionally, BMDCs are shown to induce expression of SOCS1 in an NF-κB dependent manner. In BMMs, expression of SOCS1 by depleted Zymosan is independent of NF-κB as well as NFAT or egr.

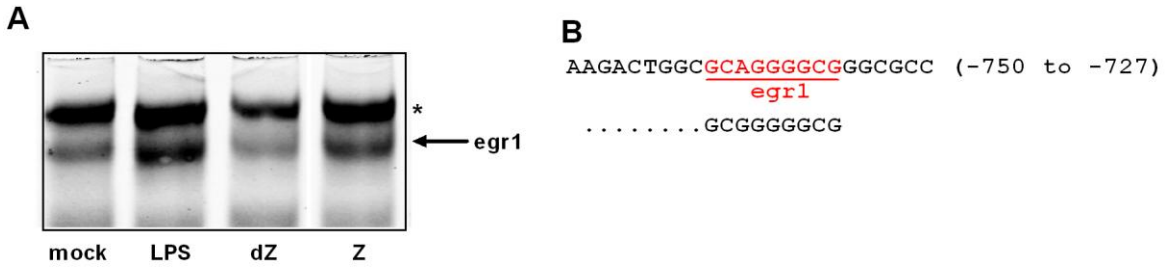

Fig. 4-11 Analysis of binding activities of nuclear extracts to an egr1 binding probe within the SOCS1 promoter
[A], BMMs ($9x10^6$) from WT mice were stimulated with LPS (100 ng/ml), dZ (500 µg/ml), Z (500 µg/ml) or left unstimulated (mock). After 6 h, nuclear extracts were prepared and added to the Cy3-labeled SOCS 1.2 probe containing an egr1 binding site. Samples were run in 6% acrylamide native gels and Cy3 fluorescence was detected. Asterisk indicates unspecific binding, arrow indicates a specific band. [B], Sequence of the probe including the binding site (red letters). Numbers indicate relative position from the A in the ATG initiation site in the human SOCS1 gene. Consensus sequence of egr1 is depicted below the probe. Results are representative for 2 independent experiments.

4.1.5. Pyk2 and ERK are key signalling molecules in SOCS1 induction in Dectin-1 triggered BMMs

On the basis of the above results, we next were interested in downstream molecules that could participate in Dectin-1 mediated expression of SOCS1. It has been proposed that the MAPK ERK acts downstream of C-type lectins, i.e. Dectin-1. Indeed, we easily observed ERK phosphorylation upon ligation with depleted Zymosan (Fig. 4-9 [A]).

Proline rich tyrosine kinase 2 (Pyk2) was recently identified as a member of cytoplasmatic protein tyrosine kinases that can be activated via Src and Syk kinases. Phosphorylated Pyk 2 itself is shown to activate downstream MAPK ERK (151, 152). To address the question whether Dectin-1 mediates Pyk2 / ERK activation in BMMs, cells were stimulated and activation of Pyk2 was determined. BMMs were stimulated with depleted Zymosan for different time-periods (15 min, 90 min and 240 min) and phosphorylation of Pyk2 was examined by means of immunoblot. Pyk2 phosphorylation could be detected in depleted Zymosan stimulated cells at any time point that was analyzed. After 240 min, phosphorylation of Pyk2 was even increased (Fig. 4-12 [A]). Quantification of the various bands is displayed as percentage of the unstimulated (mock) control directly above the respective blots. The unstimulated control (mock) was set 100 %. An increase in Pyk2 phosphorylation up to 325 % could be observed after 240 min of stimulation. After 15 or 90 min, intensities were increased up to 158 % (90 min) or 128 % (15 min) (Fig. 4-12 [A]). Since we observed SOCS1 peak induction after 4.5 h (Fig. 4-1 [C]), this late up-regulation of Pyk2 activity could be a hint for a possible contribution of Pyk2 to SOCS1 induction. Notably, Pyk2 displayed a basal activation in unstimulated cells. Utilization of inhibitors directed against Src (PP1) and Syk (Syki) kinases led to a strong reduction of Pyk2 phosphorylation almost to the levels of the unstimulated control. This places Pyk2 downstream of Src and Syk kinases. Again, band intensities are shown directly above the blots (Fig. 4-12 [A]).

Pyk2 also comprises a calcium binding motif by which it can be activated in a calcium dependent manner. Thus we used inhibitory reagents to block calcium signalling pathways. BAPTA is described as a chelator of intracellular calcium. Similarly, KN-93 is a potent and specific inhibitor of CaMK (calmodulin dependent protein kinase). When bound to Ca^{2+},

calmodulin activates CaMK. AG17 directly inhibits tyrosine phosphorylation of Pyk2. BMMs were treated with 10 µM of the respective inhibitors prior to stimulation with depleted Zymosan and analyzed for Pyk2 activation. Surprisingly, Pyk2 phosphorylation could still be detected in the presence of BAPTA or KN-93 (Fig. 4-12 [B]). In contrast, AG17 abolished activation of Pyk2 (Fig. 4-12 [B]) as shown by band intensities above the blot (78 % when compared to unstimulated control).

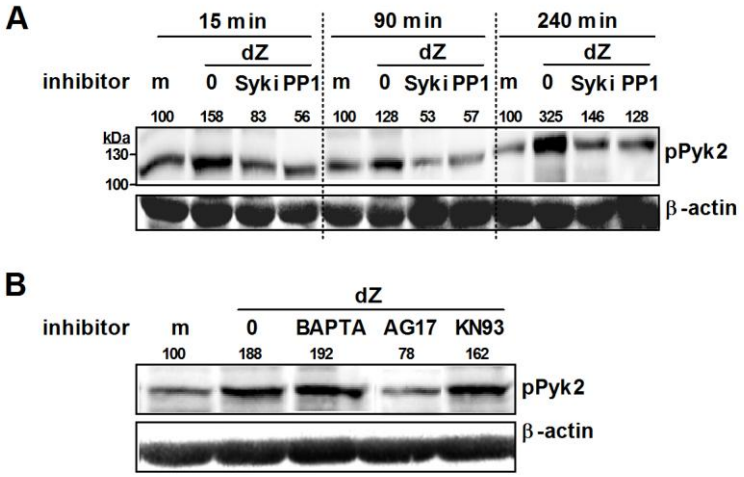

Fig. 4-12 Pyk2 is phosphorylated upon stimulation with dZ and is activated Src and Syk dependently

[A and B], WT BMMs ($3x10^6$) were pretreated for 1 h with 10 µM Syk inhibitor IV (Syki), 10 µM Src inhibitor (PP1) [A] or 10 µM BAPTA, AG17 and KN93 [B]. [A] Cells were stimulated for the indicated time periods with dZ (500 µg/ml). [B] Cells were stimulated with dZ (500µg/ml) for 30 min. Cells were lysed and probed by means of immunoblot with antibodies specific for Pyk2 phosphorylated on Tyr^{402} or β-actin. m, unstimulated control; 0, without inhibitor. AG17, Pyk2 inhibitor; BAPTA, calcium chelator; KN93, inhibitor of CaMK. Results are representative for 3 independent experiments. Densitometry data are stated directly above the blot (unstimulated control (mock) = 100%) as percentage of mock and are shown as bar graphs in the appendix.

Thus, Pyk2 is supposedly activated in a calcium independent way by Src and Syk kinases. Next, we assessed the activation of the MAPK ERK, JNKII and p38 after the application of calcium as well as Pyk2 inhibitors. Additionally, the effect of the inhibitors on the expression of SOCS1 protein had to be analyzed. BMMs were pre-treated with the respective inhibitors as described above. Phosphorylation of ERK, p38 and JNKII was detected by means of immunoblot after stimulation with depleted Zymosan. Importantly, ERK phosphorylation was completely blocked in cells pre-treated with AG17 and displayed activation at the levels of the mock control (as shown by intensities) (Fig. 4-13 [A]). In contrast, BAPTA and KN-93 had no effect on ERK phosphorylation; band intensities were similar to that of unstimulated probes. Concerning p38 and pJNKII, AG17 had no effect on the phosphorylation status. (Fig. 4-13 [B], [C]). Activation of p38 was even further increased by 67 % as compared to depleted Zymosan alone (Fig. 4-13 [B]) where cells were treated with AG17.

Accordingly, our data on the one hand show that the applied inhibitors BAPTA, KN-93 and AG17 are functional as they inhibited MAPK activation. Otherwise, AG17 was a potent inhibitor of Pyk as well as ERK phosphorylation, whereas it had no effect on p38 or JNKII activation. BAPTA and KN-93 were effective inhibitors of p38 and JNKII phosphorylation but did not affect ERK phosphorylation.

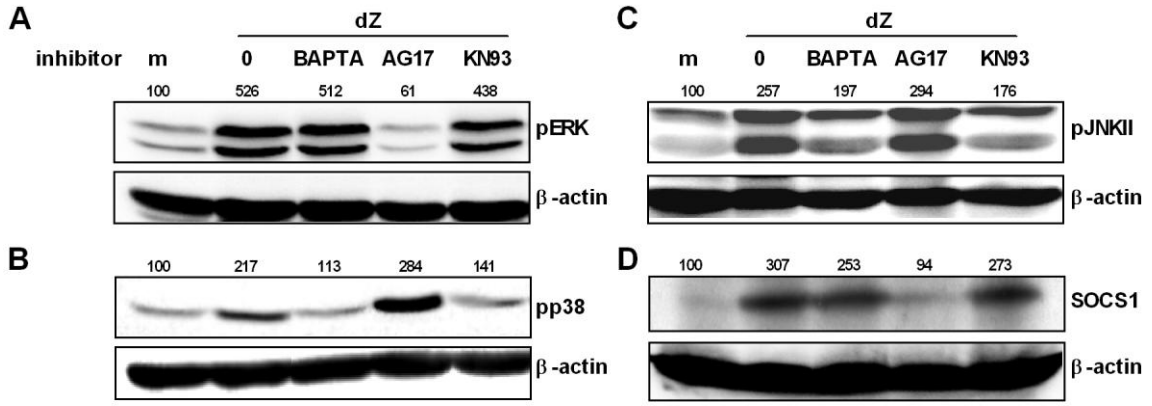

Fig. 4-13 Effects of Pyk2 inhibition on MAPK activity and SOCS1 expression

[A] – [C], WT BMMs ($3x10^6$) were pretreated for 1h with 10 µM BAPTA, AG17 or KN93. [A]-[C], cells were stimulated for 30min with dZ (500 µg/ml). Cell lysates were analyzed by immunoblot. Blots were probed with antibodies specific for [A] ERK 1/2 phosphorylated on Thr ^{202}/Tyr ^{204}, [B], p38 phosphorylated on Thr^{180}/Tyr^{182}, [C], JNKII phosphorylated on Thr^{183}/Tyr^{185} or β-actin. [D], WT BMMs ($18x10^6$) were pretreated as in [A] – [C]. Afterwards, cells were stimulated for 6 h with dZ (500 µg/ml). Cells were lysed and proteins were analyzed by western blot. Blots were probed with antibodies against SOCS1 or β-actin. Data are representative of 3 independent experiments. Band intensities are depicted directly above the blots (unstimulated control (mock) = 100%) as percentage of mock. Bar graphs of the quantitative data are shown in the appendix.

Subsequently the impact of the Pyk2/ERK pathway on the expression of SOCS1 had to be assessed. Therefore, we checked for SOCS1 induction by western blot after pre-incubation with Pyk2 and calcium inhibitors. In case of treatment with AG17, SOCS1 induction was completely abrogated whereas calcium inhibitors had no effect (Fig. 4-13 [D]). Thus, SOCS1 protein expression is probably independent of calcium signalling pathways as also observed for the activation of the MAPK ERK. Additionally, ERK as well as SOCS1 are most likely situated downstream of the tyrosine kinase Pyk2. We next had to analyze if ERK as well as SOCS1 are also placed downstream of Src and Syk kinases as already shown for Pyk2 (Fig. 4-12 [A]). BMMs were incubated for 1 h with 10 µM of PP1 (Src inhibitor) or Syki (Syk inhibitor), respectively. Afterwards, cells were stimulated with depleted Zymosan and analyzed for MAPK activation or induction of SOCS1. Application of Src and Syk inhibitors largely suppressed ERK activation as compared to stimulated cells. In contrast, pp38 and pJNKII were only affected by inhibiting Src (Fig. 4-14 [A, B, C]). Activation of p38 and JNKII was diminished to the level of unstimulated cells (mock). Src kinases also signal via the serin/threonin kinase Raf1. Raf1 is described to be an important activator of MAPKs. Therefore, it is possible that p38 for example is activated via a Src-Raf1 signalling pathway. SOCS1 expression induced by depleted Zymosan was entirely abolished by Src and Syk inhibitors as displayed by band intensities (Fig. 4-14 [D]). Thus, Syk kinase when activated, induces ERK phosphorylation as well as SOCS1 expression.

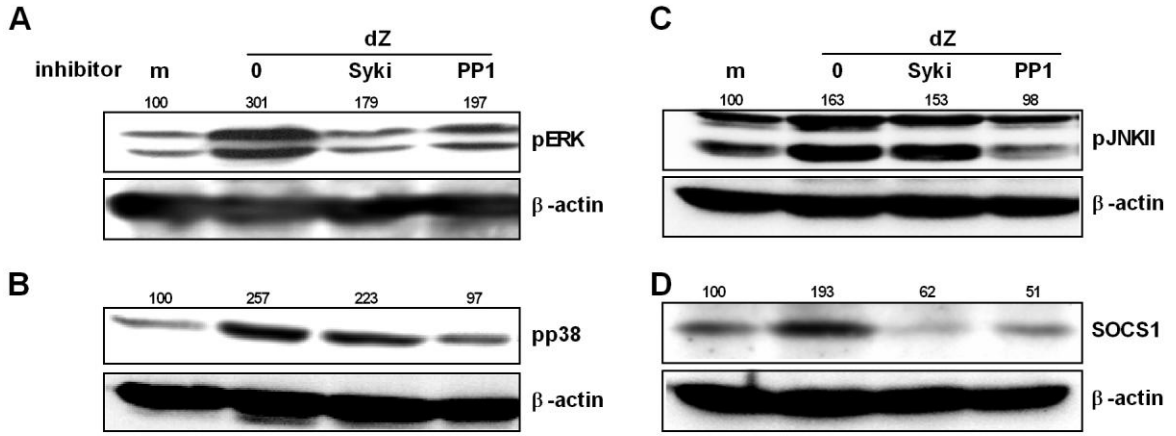

Fig. 4-14 Influence of Src and Syk inhibition on SOCS1 expression and MAPK phosphorylation

Immunoblots of BMM cell lysates pretreated for 1h with either 10 µM Syki or 10 µM PP1. [A]-[C], Cells ($3x10^6$) were stimulated with 500 µg/ml dZ for 30 min. Cells were lysed and blots were probed with Abs against [A] ERK 1/2 phosphorylated on Thr ^{202}/Tyr ^{204}, [B] p38 phosphorylated on Thr^{180}/Tyr^{182} [B], [C] JNKII phosphorylated on Thr^{183}/Tyr^{185}. [D], cells ($18x10^6$) were lysed and blots were probed with Ab against SOCS1. β-actin served as loading control. Displayed experiments show representative data for 3 independent experiments. Band intensities are depicted directly above the blots (unstimulated control (mock) = 100%) as percentage of mock. Bar graphs of the quantitative data are shown in the appendix.

Dectin-1 can also signal independently of Syk. Here, the serin/threonin kinase Raf1 is phosphorylated by Ras proteins. Ras proteins are activated by Src (52, 76). To test the relevance of Raf1 for the ERK dependent induction of SOCS1, we pre-treated BMMs with an inhibitor against Raf1.

BMMs were pre-incubated with 10 µM of PP1, Syki or Rafi (Raf1 inhibitor), respectively. The stimulated cells were analyzed for activation of ERK, p38 as well as for SOCS1 expression.

As displayed in Fig. 4-15, only the activation of p38 was diminished when Raf1 was inhibited, whereas phosphorylation of ERK and expression of SOCS1 were not affected. Thus, synthesis of SOCS1 was independent of Raf1 but is rather regulated by the tyrosine kinase Syk.

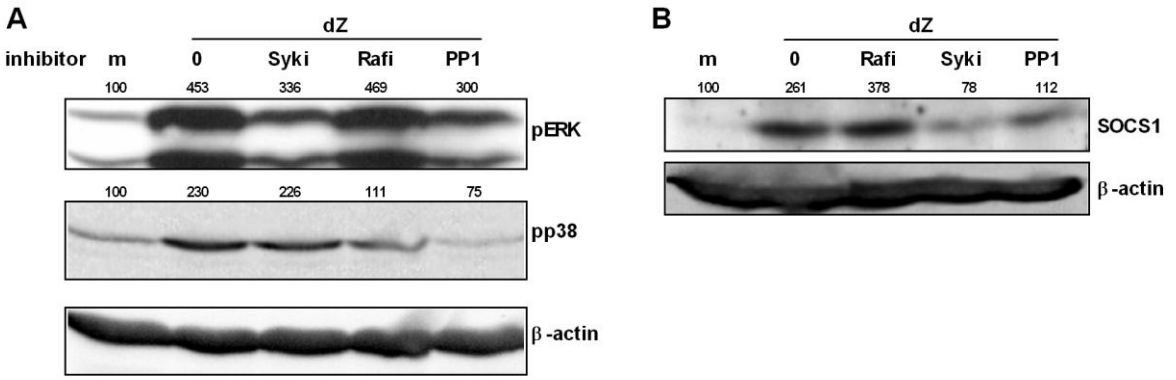

Fig. 4-15 SOCS1 is induced independently of Raf1 in Dectin-1 triggered cells

[A], BMMs ($3x10^6$) were triggered by dZ (500 µg/ml) for 30 min after a 1h incubation time with 10 µM Syki, Rafi or PP1. [B], BMMs ($18x10^6$) were pre treated with 10 µM Rafi, Syki or PP1 and afterwards stimulated with dZ (500 µg/ml). [A] and [B], cells were lysed and immunoblot was performed with Abs specific for ERK 1/2 phosphorylated on Thr ^{202}/Tyr ^{204}, p38 phosphorylated on Thr^{180}/Tyr^{182} [A], SOCS1 [B] or β-actin [A] and [B]. Data are representative for 2 independent experiments. Band intensities are directly depicted above the respective blots (unstimulated control (mock) =100%) as percentage of mock. Bar graphs of the quantification analysis are shown as bar graphs in the appendix.

So far, the signalling pathway to induce SOCS1 can be described as follows. Upon engagement of Dectin-1, the tyrosine kinase Src gets activated by phosphorylation. Therupon, the Spleen tyrosine kinases (Syk) is activated by Src kinase and subsequently phosphorylate the tyrosine kinase Pyk2. Via a yet unknown mechanism, Pyk2 activates the MAPK ERK. Thereupon, pERK induces expression of SOCS1 via an ERK dependent transcription factor.

To strengthen the observation that Pyk2 is involved in SOCS1 induction, we next analyzed Pyk2 as well as SOCS1 expression following knockdown of Pyk2. BMMs were transfected either with a siRNA directed against Pyk2 (siPyk2) or a control siRNA (siCon) with a non-genomic sequence to control sequence-specific RNA interference. After 20 h, cells were stimulated with depleted Zymosan or left unstimulated and analyzed for ERK phosphorylation and SOCS1 expression. Further, Pyk2 expression was analyzed in unstimulated cells to determine the knockdown efficiency.

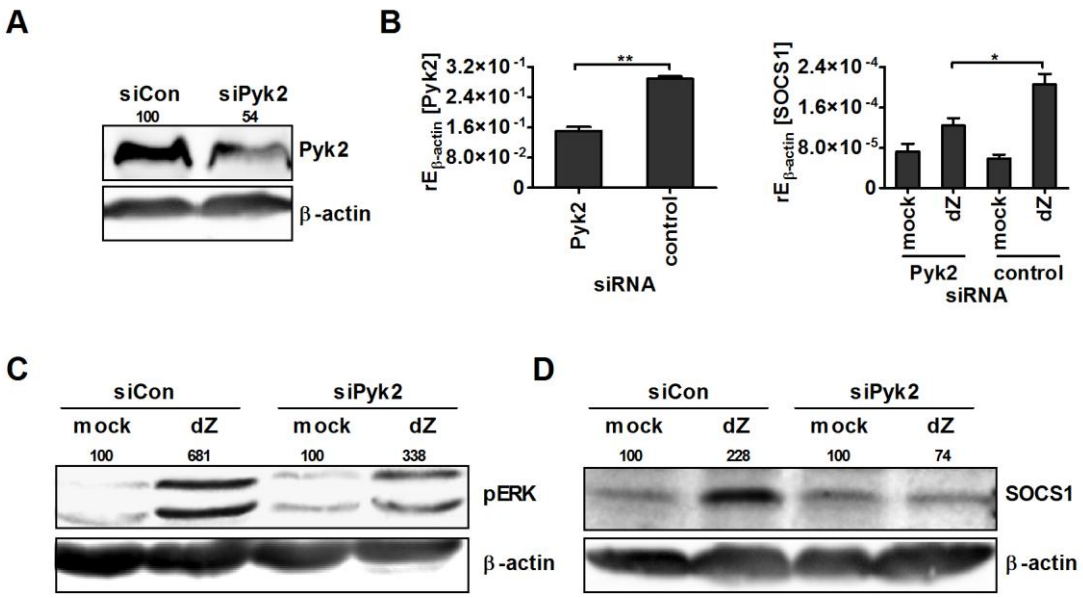

Fig. 4-16 Pyk2 knockdown reduces Dectin-1 mediated expression of SOCS1

[A]-[C] BMMs were transfected with 50 nM control or Pyk2-specific siRNA for 20 h. Afterwards, stimulation was performed for [B] 4.5 h, [D] 6 h or [C] 30 min. [A] Cells ($2x10^6$) were lysed and western blot was performed with Ab against Pyk2 to validate knockdown. [B] Cells ($3x10^5$) were lysed and analyzed for gene expression and knockdown by quantitative RT-PCR. Transcription was compared with control siRNA. [C], [D] Cells ($2x10^6$ [C] or $18x10^6$ [D]) were stimulated with dZ (500 µg/ml) and immunoblot was performed with Abs against ERK 1/2 phosphorylated on Thr ^{202}/Tyr ^{204} [C] or SOCS1 [D]. siCon; control siRNA. Results show representative data of two independent experiments (mean + SD). Band intensities are directly depicted above the respective blots (unstimulated control (mock) =100%) as percentage of mock. Bar graphs of the quantification analysis are shown as bar graphs in the appendix.

As depicted in Fig. 4-16 [A] and [B], siRNA directed against Pyk2 decreased Pyk2 expression by 50 % as compared to cells transfected with control siRNA. On protein level, densitometry analysis was carried out to determine the knockdown efficiencies. Stimulated cells were analyzed for SOCS1 expression on mRNA as well as protein level. SOCS1 transcripts were decreased by 50 % in stimulated cells transfected with the Pyk2 siRNA as compared to stimulated cells transfected with the control siRNA (Fig. 4-16 [B]). SOCS1 protein expression was almost completely abolished as analyzed by means of densitometry measurements. In BMMs transfected with the control siRNA, depleted Zymosan induced

SOCS1 expression up to 228 % compared to mock control (100 %) (Fig. 4-16 [D]). In contrast, in siPyk2 transfected cells, stimulation with depleted Zymosan did not increase SOCS1 expression over the level of the unstimulated control (Fig. 4-16 [D]). Similarly, ERK activity was blocked by Pyk2 siRNA. Stimulation of siPyk2-transfected cells with depleted Zymosan decreased ERK activation by 50 % as compared to stimulated cells that were transfected with the control siRNA (Fig. 4-16 [C]). ERK was phosphorylated by more than 500 % in siCon treated cells. Similarly, SOCS1 was induced by 120 %. The values are comparable to those observed in previous experiments with WT BMMs, i.e. in Fig. 4-13 [A] and [D] or Fig. 4-14 [D]. Thus, the expression of the respective genes seems to be unaffected by the control siRNA.

The data confirmed that SOCS1 induction in Dectin-1 triggered BMMs is mediated by Pyk2 and the downstream situated MAPK ERK. Accordingly, siRNA based downregulation of Pyk2 inhibited ERK activation and SOCS1 expression as shown for inhibition of Pyk2 via AG17.

Application of UO126 that specifically inhibits ERK phosphorylation verified these findings. Pre-incubation of BMMs with UO126 diminished SOCS1 expression by 40 % as compared to the control cells stimulated with depleted Zymosan (Fig. 4-17). However, we still could detect residual induction of SOCS1. ERK phosphorylation was completely abrogated upon incubation with UO126. UO126 is described as a potent inhibitor of MEK1 and MEK2, both of them activate ERK MAPK. In contrast, inhibitors directed against JNKII (JNKIIi) or p38 (SB203580) displayed no effect on SOCS1 expression (Fig. 4-17). Taken together, ERK is activated by Pyk2. ERK thereupon is responsible for SOCS1 induction as shown by inhibitor studies (inhibition of Syk, Src and Pyk2 kinases inhibited ERK activation and SOCS1 induction). Knockdown of Pyk2 inhibited phosphorylation of ERK and synthesis of SOCS1.

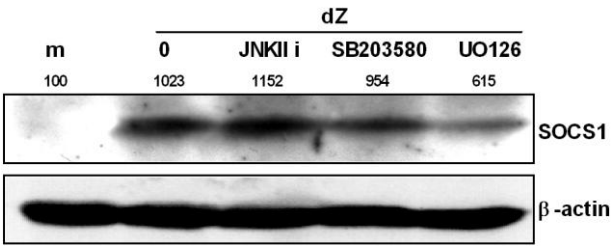

Fig. 4-17 The ERK inhibitor UO126 diminished Dectin-1 induced SOCS1 expression

Cells (18x10^6 BMMs) were treated with 10 µM of JNKIIi, SB203580 or UO126 prior to stimulation with 500 µg/ml dZ. Cells were stimulated for another 6h and proteins were measured in the lysates by immunoblot. Blots were probed with Abs specific for SOCS1 or β-actin. Results show representative data of 2 independent experiments. Band intensities are depicted directly above the blots (unstimulated control (mock) = 100%) as percentage of mock. Bar graphs of the quantitative data are shown in the appendix. JNKIIi, inhibitor of JNKII; SB203580, p38 inhibitor; UO126, ERK inhibitor

4.1.6. The signalling pathway leading to the induction of SOCS1 in BMDCs differ from that in BMMs

In BMDCs IκBα was clearly activated as shown by phosphorylation following engagement of Dectin-1. Upon inhibition of NF-κB, SOCS1 induction was decreased; thus it has to be concluded that NF-κB is involved in SOCS1 induction only in BMDCs but not in BMMs (Fig. 4-9). Consequently, phosphorylation status of IκBα was analyzed in cells treated with Pyk2 and calcium inhibitors (4.1.4) prior to stimulation with depleted Zymosan.

BAPTA and KN-93 led to diminished phosphorylation of IκBα by more than 50 % as compared to stimulated control. AG17 as a direct inhibitor of Pyk2 activity completely abolished it (Fig. 4-18 [A]). Hence, we checked for activation of Pyk2 in BMDCs with or without the application of the aforementioned inhibitors (Fig. 4-18 [B]). In contrast to BMMs, BMDCs showed a marked decrease of Pyk2 phosphorylation upon pre-incubation with BAPTA as well as KN-93 (about 60 %). In BMMs, Pyk2 phosphorylation was not affected by the calcium inhibitors. AG17 had similar effects in both cell types, BMMs as well as BMDCs. Pyk2 phosphorylation was abolished in BMDCs (Fig. 4-18 [B]) and BMMs (Fig. 4-12 [B]).To determine whether the Pyk2 as well as calcium inhibitors had the same effect on MAPK activation as observed in BMMs (Fig. 4-13), we analyzed MAPK phosphorylation. ERK phosphorylation was only diminished when Pyk2 was directly inhibited via AG17, whereas none of the inhibitors affected p38 phosphorylation. Otherwise, JNKII phosphorylation was completely blocked by BAPTA, KN-93 and AG17. Phosphorylation was even less as compared to mock control (Fig. 4-18 [A]). This led us to the assumption that pJNKII seems to play a role in the phosphorylation of IκBα. JNKII in turn is activated by pPyk2 (Fig. 4-18 [A]). Importantly, this pathway seems to be calcium dependent.

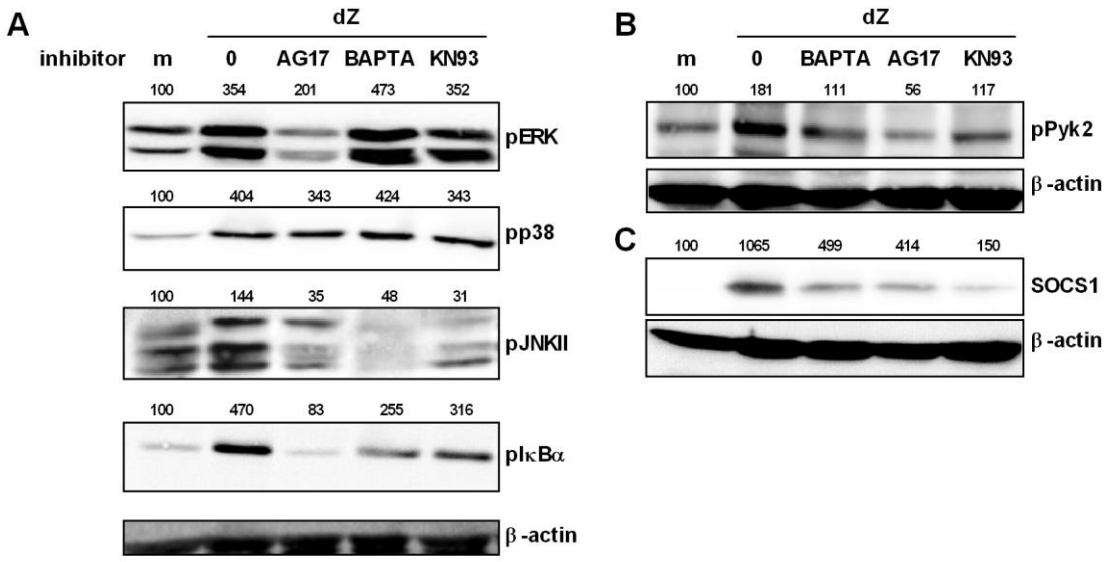

Fig. 4-18 SOCS1 protein expression is diminished in BMDCs upon treatment with Pyk2 and calcium inhibitors

[A] and [B], BMDCs ($3x10^6$) were pre-incubated for 1 h with 10 µM of the delineated inhibitors (BAPTA, AG17, KN93) and stimulated afterwards with dZ (500 µg/ml) for 90 min. Activation of MAPKs, Pyk2 and pIκBα was not observed until 90 min of stimulation. Cell lysates were analyzed by immunoblot with antibodies specific for ERK 1/2 phosphorylated on Thr^{202}/Tyr^{204}, p38 phosphorylated on Thr^{180}/Tyr^{182}, JNKII phosphorylated on Thr^{183}/Tyr^{185}, IκBα phosphorylated on Ser^{32} or β-actin. [C], BMDCs ($18x10^6$) were treated with inhibitors as described for [A] and [B] and stimulated for 6 h with dZ (500 µg/ml). Western blot of lysed cells was carried out with antibodies directed against SOCS1 or β-actin. Results show representative data for 2 independent experiments.

Regarding SOCS1 induction, Pyk2 and calcium inhibitors decreased SOCS1 protein expression levels by more than 50 %, respectively (Fig. 4-18 [C]). Notably, SOCS1 protein could be detected at the highest level after 6 h of stimulation as also observed for BMMs (Fig. 4-1 [B]). In conjunction with the fact that utilization of a NF-κB inhibitor down-regulated SOCS1 induction (Fig. 4-9 [B]), we speculate that in BMDCs, SOCS1 is induced in a calcium and NF-κB dependent way. The pathway leading to SOCS1 induction in BMDCs can probably be described as follows. Most likely, Src and Syk kinases are activated upon

binding of depleted Zymosan by Dectin-1. Syk kinase may activate Pyk2 kinase. IκBα is phoshorylated and thereupon, NF-κB induces transcription of SOCS1.
Whether NFAT plays a role in SOCS1 induction in BMDCs has to be further analyzed. Nevertheless, it is shown that calcium dependent, NFAT independent pathways are indicted to be responsible for downstream signalling of Dectin-1 (66).
As a NF-κB independent SOCS1 induction was never shown before, we carried out most of the following experiments in BMMs to reveal a possible new pathway to express SOCS1.

4.1.7. Summary of part 4.1

It could clearly be shown that the engagement of Dectin-1 by depleted Zymosan induced SOCS molecules, namely SOCS1, SOCS3 and CIS in murine BMDCs and BMMs. SOCS1 was induced without the attendance of any TLR as shown by the application of cells from MyD88/Trif double deficient mice. Further, SOCS1 was directly induced upon stimulation of BMDCs and BMMs with depleted Zymosan since no intermediate or secreted factor was needed. SOCS1 was still induced in the presence of cycloheximid or when cells from IFNAR1$^{-/-}$ mice were employed. Moreover, NF-κB as a possible transcription factor could be ruled out in BMMs. In BMMs, in contrast to BMDCs, the NF-κB inhibitor IκBα was not phosphorylated after Dectin-1 stimulation. Also, a NF-κB inhibitor only reduced Dectin-1 induced SOCS1 expression in BMDCs but not in BMMs. The transcription factors NFAT and egr1 as possible candidates to induce SOCS1 could be excluded. We further show evidence for a signalling pathway that involves the kinases Src and Syk as direct targets after Dectin-1 ligation. Importantly, Pyk2 is phosphorylated upon triggering of Dectin-1 by depleted Zymosan. Pyk2 is described to be activated in a Src dependent way.
In this study it is for the first time shown that Pyk2 is linked to SOCS1 induction. In BMMs, the following pathway can be proposed. Upon ligation of Dectin-1 by depleted Zymosan, Src and Syk kinaseas are activated by phosphorylation at the activated receptor. Thereupon, Pyk2 is phosphorylated and activates ERK. Activated ERK mediates the expression of SOCS1 via and ERK dependent transcription factor. NF-κB as well as NFAT are not involved in SOCS1 induction in BMMs. In contrast, we show that SOCS1 in BMDCs is induced via NF-κB. We next assessed possible properties of SOCS1 idnuced upon Dectin-1 engagement.

4.2. Influence of SOCS1 on Dectin-1 and TLR signalling pawthays

SOCS proteins have been initially identified as negative feedback inhibitors of type I and II IFNs. Since the Jak/STAT pathway is used by different cytokines, the properties of SOCS proteins are not only restricted to its inducing pathway (feedback), but also inhibit other Jak/STAT dependent cytokine signalling pathways (crosstalk). However, recent findings indicate that SOCS1 possibly also influences Jak/STAT independent signalling modules. Accordingly, a direct effect of SOCS1 on the TLR/NF-κB pathway has been proposed (109, 110). Therefore, it had to be studied whether SOCS1 might contribute to fine-tuning of Dectin-1 or TLR signalling.

4.2.1. No effect of SOCS1 on Dectin-1 signalling

First, we investigated the impact of SOCS1 as a feedback inhibitor of Dectin-1. For this purpose, BMMs from SOCS1$^{-/-}$ mice on IFNγ knockout background were used. Normally, SOCS1$^{-/-}$ develop a multi-inflammatory syndrome and die within 3 weeks after birth.
The neonatal defect is mainly based on hypersensitivity towards IFNγ. SOCS1/IFNγ double deficient mice display a diminished pathology and survive the neonatal period. Thus, we used SOCS1$^{-/-}$/IFNγ$^{-/-}$ cells to analyze phosphorylation of the kinases involved in SOCS1 expression in WT BMMs. As BMMs do not produce IFNγ, the IFNγ$^{-/-}$ background should have no impact when analyzing isolated cells. SOCS1$^{-/-}$/IFNγ$^{-/-}$ and the respective IFNγ$^{-/-}$ control cells were stimulated with depleted Zymosan for 15 min, 90 min or 240 min. Thereupon, phosphorylation of Syk, Pyk2 as well as the MAPK ERK was determined by means of immunoblot. In cells from the knockout as well as the control mice, phosphorylation of Syk and Pyk2 was observed after 90 min and was still detectable after 240 min of stimulation (Fig. 4-19 [A], [B]). These results were comparable to the phosphorylation status of WT BMMs stimulated by depleted Zymosan (Fig. 4-12, 4-13). ERK phosphorylation was induced in SOCS1$^{-/-}$/IFNγ$^{-/-}$ as well as IFNγ$^{-/-}$ BMMs already after 15 min and even could be measured after 240 min of stimulation. Interestingly, none of the kinases showed a difference in activation profile in cells from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice as compared to IFNγ$^{-/-}$ control cells (Fig. 4-19 [A], [B]).
As mentioned above, egr2 is one of the first genes directly activated by triggering of Dectin-1. Therefore, we next tested for a possible regulation of this transcription factor. Cells from double knockout and control mice were stimulated with depleted Zymosan and analyzed by RT-PCR for transcript levels of egr2. BMMs from the knockout as well as the control mice displayed a 10-fold induction, comparable to that of the WT cells (Fig. 4-10). No difference in egr2 expression could be observed (Fig. 4-19 [C]). NFAT and egr transcription factors are important for IL10 production. We additionally examined the secretion of this cytokine in SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ BMMs via an ELISA-based determination of the amount of IL10 in the supernatant of stimulated cells. However, IL10 secretion was not altered in SOCS1$^{-/-}$/ IFNγ$^{-/-}$ cells (Fig. 4-19 [B]).
These data indicate that SOCS1 is not a feedback inhibitor of the Dectin-1 pathway. No differences of the key kinases involved in Dectin-1 mediated SOCS1 induction, Syk, Pyk2 and ERK could be detected. Also, egr2 and IL10 as important molecules induced by Dectin-1 were not affected. Therefore, we next analyzed a possible impact of Dectin-1 induced SOCS1 on TLR signalling pathway.

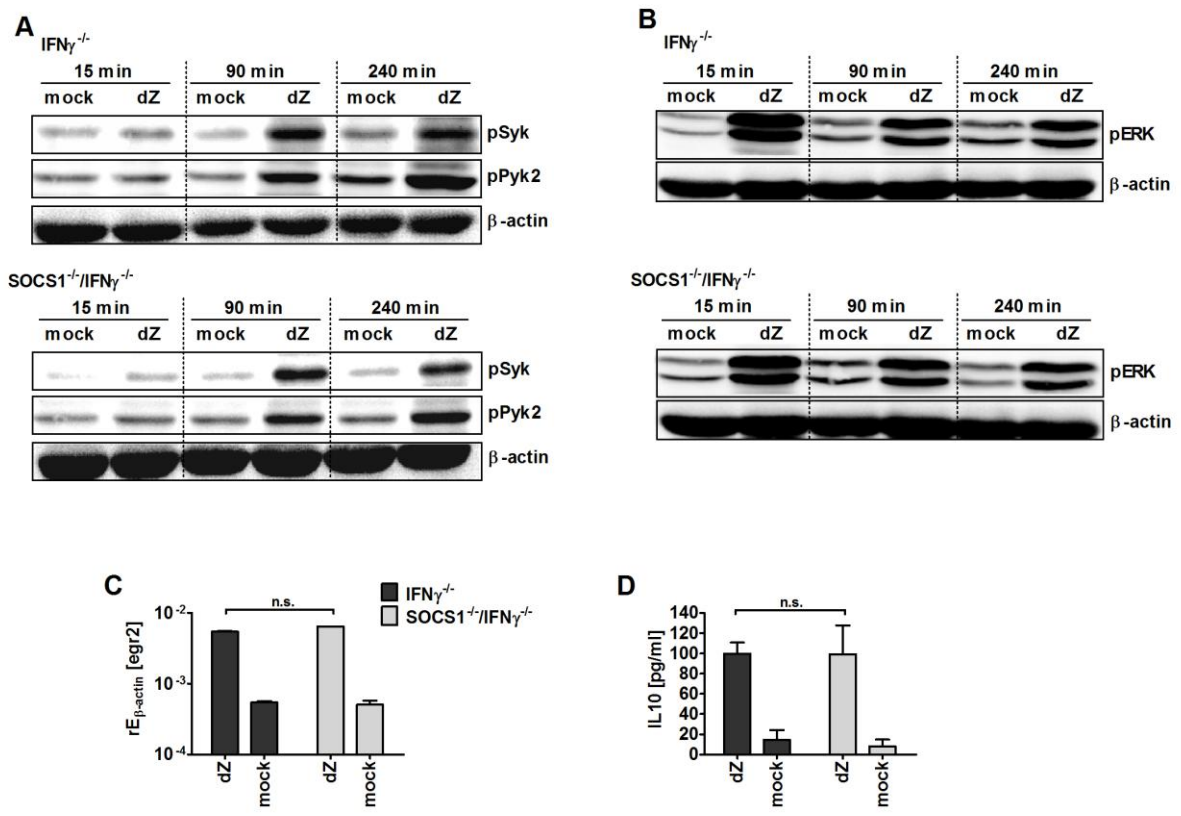

Fig. 4-19 SOCS1 is not a feedback inhibitor of Dectin-1 signalling

[A], [B] BMMs ($3x10^6$) from SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ mice were stimulated for the indicated time periods with dZ (500 µg/ml) or left unstimulated (mock). Cells were lysed and immunoblot was performed with Abs specific for [A], Pyk2 phosphorylated on Tyr^{402}, Syk phosphorylated on Tyr^{519}/Tyr^{520} and [B], ERK1/2 phosphorylated on Thr ^{202}/Tyr ^{204}. [C], [D] BMMS from SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ control mice were stimulated with dZ (100 µg/ml). [C] $3x10^5$ cells were lysed after 4.5h and analyzed for egr2 expression by quantitative RT-PCR. [D] Supernatants of $2x10^5$ cells were analyzed for the secretion of IL10 after 24h by ELISA. Results in [A and B] show representative data of 3 independent experiments. [C and D] n=3 (mean + SD).

4.2.2. TLR activation is modulated by Zymosan induced SOCS1

Besides its interaction with TLR2, Dectin-1 is able to modulate the responses of different TLRs, including TLR2/1, TLR2/6, and TLR4 (55, 63). In these cases, the production of IL12 was down-regulated by the addition of β-glucan what activates Dectin-1. As SOCS1 is induced through Dectin-1 but has no impact on this pathway, we assumed that it could act as a crosstalk inhibitor for TLR signalling. First of all, we analyzed whether WT BMMs triggered with depleted Zymosan could influence TLR signalling. Therefore, we stimulated cells with depleted Zymosan to trigger Dectin-1 solely or used it in a co-stimulatory approach with CpG-DNA or other pure TLR ligands (LPS, Pam_3CSK_4, Poly(IC) (PIC), R848). BMMs were pre-stimulated with the respective TLR ligand for 4h followed by stimulation with depleted Zymosan. Notably, pre-stimulation with depleted Zymosan prior to the application of the respective TLR ligands had the same effects on cytokine secretion.

Thereupon, secretion of IL12p40, TNFα and IL8 in supernatants of stimulated cells was determined. IL12p40 secretion was markedly reduced in BMMs and BMDCs upon co-activation of Dectin-1 and different TLRs when compared to stimulation with the sole TLR ligand, respectively (Fig. 4-20 [A]). Cells co-stimulated with CpG-DNA and depleted Zymosan showed a reduction of IL12p40 of about 50 %. Depleted Zymosan together with Pam_3CSK_4

even decreased IL12p40 cytokine production by almost 90 %. Interestingly, LPS was much less simulative than the other tested TLR ligands (CpG-DNA, Pam_3CSK_4, R848, PIC). Also, depleted Zymosan had almost no effect on LPS induced IL12p40 secretion. The amount of IL12p40 was only decreased by about 10 %. Notably, R848 production was unaltered in the co-stimulatory setting. In contrast, TNFα and IL8 cytokine secretion was up-regulated when cells were triggered via TLRs and Dectin-1 (Fig. 4-20 [B], [C]). Regarding TNFα, CpG-DNA and R848 turned out to be the most potent ligands in combination with depleted Zymosan. Cytokine secretion was increased by 90 %, whereas the production upon stimulation with LPS as well as Pam_3CSK_4 or PIC was only slightly up-regulated. Similarly, IL8 secretion was increased by co-stimulation. Cells stimulated by depleted Zymosan in combination with CpG-DNA, R848 or Pam_3CSK_4 displayed an increase in cytokine secretion of about 80-90 % when compared to TLR ligands alone (Fig. 4-20 [C]).

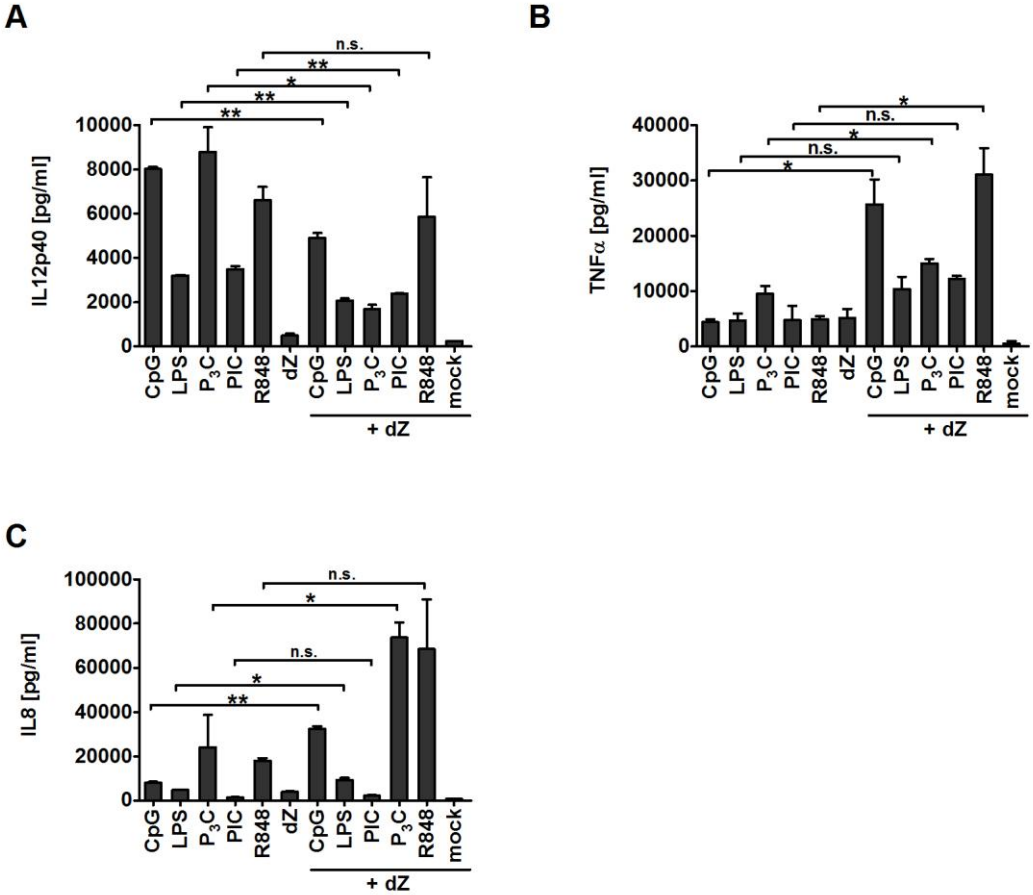

Fig. 4-20 Dectin-1 co-stimulation modulates TLR-induced cytokine profile

[A-C], WT cells ($2x10^5$ BMMs) were stimulated with dZ (100 µg/ml), CpG DNA (1 µM), LPS (100 ng/ml), P_3C (100 ng/ml), PIC (25 µg/ml) or R848 (500 ng/ml) for 16 h. In case of co-stimulation, cells were pre-stimulated with the respective TLR ligands for 4h following incubation with dZ (12 h). Secretion of [A] IL12p40, [B] TNFα or [C], IL8 was detected by ELISA. n=2 (mean + SD); n.s., not significant

Less is known about an influence of β-glucans like Zymosan on TLR9 signalling. Nonetheless, it was shown that the C-type lectin receptor DC-SIGNR1 is able to induce SOCS1 after an infection with mycobacteria which comprise a high CG-DNA content, thus triggering TLR9. Also, Dectin-1 was shown to be triggered by *M. tuberculosis* and

Mycobacterium abscessus (46, 153, 154). Due to these facts, we decided to analyze if SOCS1 could influence the crosstalk between TLR9 and Dectin-1. First, we examined BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$and IFNγ$^{-/-}$ mice for the production of cytokines when both Dectin-1 and TLR9 were triggered. As in wildtype cells, depleted Zymosan reduced the production of IL12p40 by CpG-DNA in IFNγ$^{-/-}$ cells significantly by 40 %. Notably, the induction of IL12p40 by CpG DNA was much higher in SOCS1$^{-/-}$/IFNγ$^{-/-}$and IFNγ$^{-/-}$ (Fig. 4-21 [A]) as compared to WT BMMs (Fig. 4-20 [A]). This could be attributed to the IFNγ$^{-/-}$ background. It is reported that IFNγ$^{-/-}$ mice are more sensitive to pathogenic stimuli (155-157).

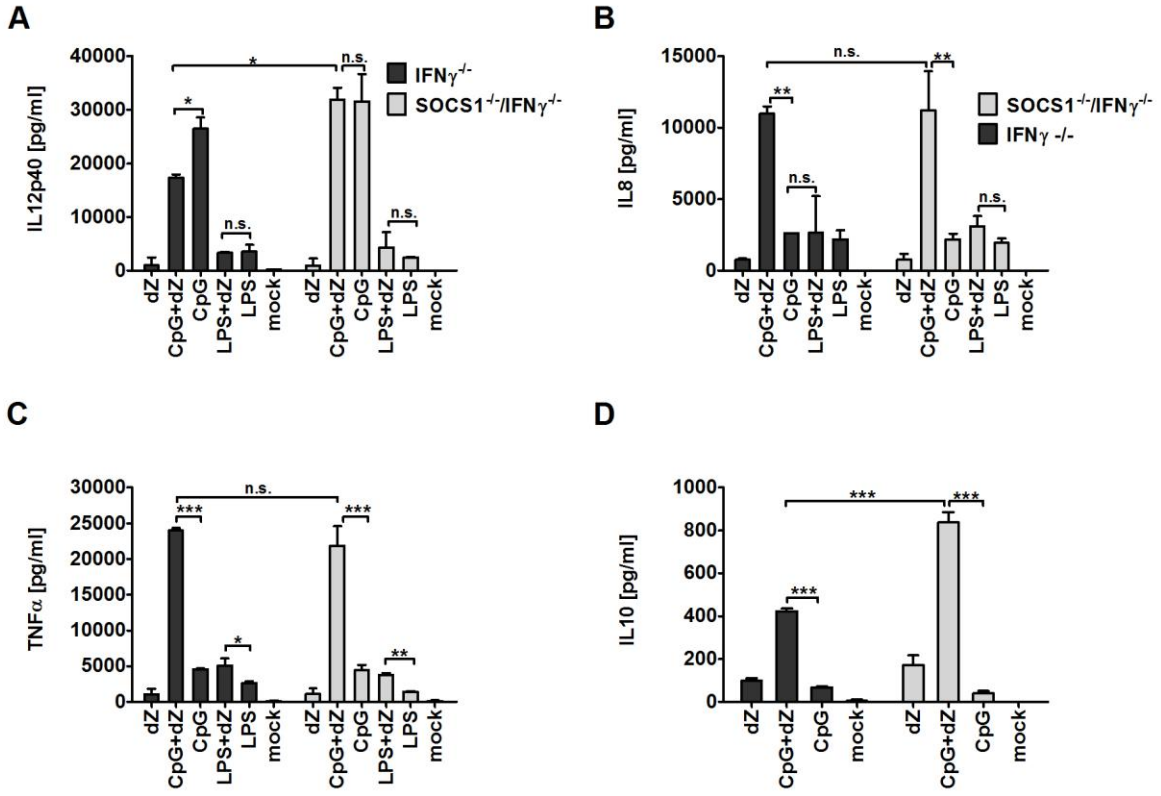

Fig. 4-21 SOCS1 expressed by depleted Zymosan differentially regulates TLR triggered cytokine production
[A]-[D] 2x10^5 BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ mice were stimulated with dZ, CpG-DNA (CpG), LPS or a combination thereof. [A] and [B], Cells (2x10^5) were stimulated with dZ (100 µg/ml), CpG (1 µM) or LPS (100 ng/ml) for 16 h. [C], 2x10^5 cells were stimulated as depicted for [A] and [B]. [D], BMMs (2x10^5) were stimulated with dZ (100 µg/ml) or CpG (1µM) for 20 h. For costimulation settings, cells were pre-stimulated for 4 h with CpG ([A]-[D]) or LPS ([A], [B]) following incubation with dZ (12 h [A-C]; 20 h [D]). Supernatants were analyzed for secretion of IL12p40, TNFα, IL8 or IL10 by ELISA.n=3 (mean + SD)

In comparison to IFNγ$^{-/-}$ cells, BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ displayed no decrease in IL12p40 secretion anymore when costimulated with CpG-DNA and depleted Zymosan. Cytokine secretion was even significantly enhanced up to nearly two fold as compared to IFNγ$^{-/-}$ cells (Fig. 4-21 [A]). This indicates that SOCS1 which is expressed upon engagement of Dectin-1 inhibits CpG-DNA induced IL12p40 production. When we focused on TNFα or IL8 secretion, we could not detect any difference in cytokine expression since TNFα and IL8 were up-regulated to the same extent upon co-stimulation in BMMs from knockout and control animals (Fig. 4-21 [B], [C]). Regarding IL10, cytokine secretion was markedly enhanced to more than two-fold in SOCS1$^{-/-}$/IFNγ$^{-/-}$ in comparison to the respective control (Fig. 4-21 [C]). LPS only slightly induced expression of IL12p40, TNFα or IL8.

Importantly, co-stimulation of SOCS1 knockout cells only had an influence on IL12p40 and IL10 secretion, but not on TNFα or IL8. Thus, Dectin-1 shapes the TLR9 cytokine profile, and this is partly dependent on SOCS1.

4.2.3. Increased activity of NF-κB subunits in SOCS1 knockout cells

It has been reported before that SOCS1 contributes to ubiquitination of NF-κB subunits resulting in subsequent degradation. Therefore, we wanted to examine whether the activation of NF-κB transcription factors is altered after co-stimulation with CpG-DNA and depleted Zymosan. We first verified if the level of SOCS1 protein differs in cells that were stimulated via TLR9 and Dectin-1. Therefore, we stimulated BMMs from WT mice with CpG-DNA or depleted Zymosan or a combination thereof and analyzed SOCS1 expression on protein level. As depicted in Fig. 4-22 [A], expression of SOCS1 could clearly be amplified after concomitant application of CpG-DNA and depleted Zymosan in comparison to cells stimulated with the individual ligands, respectively. Worth mentioning is the fact that depleted Zymosan alone had a higher potential to induce SOCS1 than CpG-DNA. The higher expression level of SOCS1 could explain the decrease of IL12p40 when TLR9 and Dectin-1 are triggered the same time.

To further address this issue, we tested for IκBα degradation and phosphorylation by immunoblot. BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ control mice were stimulated with depleted Zymosan, CpG-DNA or a combination of both ligands for different time periods (15 min, 90 min and 240 min). BMMs from the control mice showed no phosphorylation as well as degradation of IκBα when stimulated by depleted Zymosan. In contrast, CpG-DNA treated cells from the knockout as well as the control mice displayed a fast phosphorylation and subsequent degradation of IκBα that already vanished after 90 min of stimulation.

A prolonged and increased degradation as well as phosphorylation of IκBα was achieved via application of depleted Zymosan and CpG-DNA at the same time in cells from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice as compared to IFNγ$^{-/-}$ cells. After 90 min of stimulation, pIκBα could be detected and after 240 min, degradation of IκBα was measurable. The individual ligands alone also induced activation of IκBα in the knockout cells but with different kinetics. Whereas CpG-DNA induced IκBα degradation already after 15 min, the degradation of IκBα caused by depleted Zymosan developed after 90 min and 4 h; that would be in line with the clear increase in phosphorylation at the later time-points in a co-stimulatory setting (Fig. 4-22 [B]). Overall, the results suggest regulation of IκBα activation kinetics by SOCS1.

Accordingly, SOCS1 seems to influence the kinetics of IκBα phosphorylation and degradation and thus possibly also NF-κB activity. Reduced NF-κB activation could explain reduction of IL12p40 by Zymosan in IFNγ$^{-/-}$, which disappeared in cells from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice.

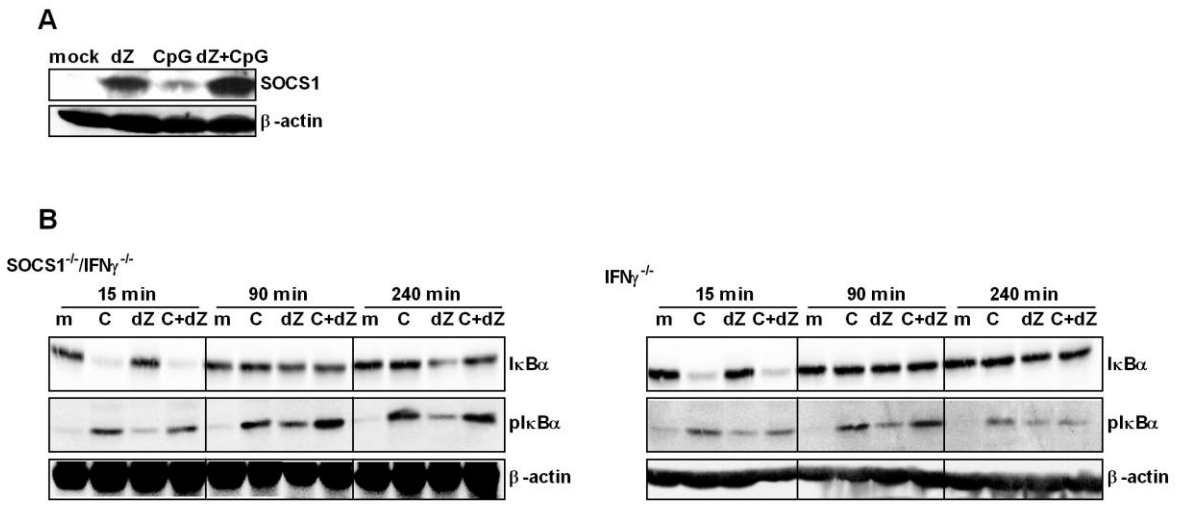

Fig. 4-22 Different kinetics of IκBα phosphorylation and degradation in cells from SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ mice
[A], WT cells (BMMs, 18x10^6) were incubated with dZ (500 µg/ml), CpG-DNA (1 µM) or dZ+CpG-DNA for 6h. Lysates were analyzed by immunoblot with Abs specific for SOCS1. [B], cells from IFNγ$^{-/-}$ or SOCS1$^{-/-}$/IFNγ$^{-/-}$ (3x10^6) were stimulated with dZ (500 µg/ml), CpG-DNA (1µM) or dZ+CpG for the indicated time periods, cells were lysed and immunoblot with Abs against IκBα or IκBα phosphorylated on Ser^{32} was performed. β-actin was used as loading control. Reults in [A] are representative for 2 independent experiments; data presented in [B] are representative for 3 independent experiments.

Next, we investigated transcriptional activity of NF-κB using an ELISA based system. Herein, the binding of different NF-κB subunits to their respective DNA elements was measured by an ELISA-based system (Trans-AM®). BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ mice were stimulated by depleted Zymosan, CpG-DNA or depleted Zymosan in combination with CpG-DNA. After 1 h and 6 h, cells were lysed and nuclear extracts were performed (3.2.1.5). Nuclear extracts were applied to 96 well plates pre-coated with a consensus binding site oligonucleotide of the transcription factor of interest (p50, p52). Similar to the method of ELISA, the transcription factors present in the nucleus upon activation are detected via a primary and a secondary, HRP-coupled antibody.

It is published that the NF-κB subunit p50 either as homodimer (p50/p50) or heterodimer (p50/p52) is responsible for the expression of IL12p40 and IL10 in BMMs (158, 159). Additionally, Dectin-1 is the only known CLR to induce the non-canonical NF-κB pathway, resulting in functional p52 subunits. Since we observed a significant up-regulation of IL12p40 and IL10 in SOCS1$^{-/-}$/IFNγ$^{-/-}$ when compared to IFNγ$^{-/-}$ cells, we decided to analyze p50 as well as p52 activation in these cells.

We observed a significant, time-dependent increase of NF-κB activity in BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice as compared to IFNγ$^{-/-}$ BMMs after co-ligation of Dectin-1 and TLR9 (Fig. 4-23). After 1h, only a slight increase in NF-κB activity was observed in SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs (Fig. 4.23 [A]). In contrast, at the later time-point (6 h), the activity of both subunits was much higher (Fig. 4-23 [B]). As compared to CpG-DNA or depleted Zymosan, we detected an increase of about 50% in SOCS1$^{-/-}$/IFNγ$^{-/-}$ cells that were co-stimulated. Even depleted Zymosan alone caused an increase in p50 and p52 activity in the double-knockout cells.

Our data indicate that SOCS1 induced by depleted Zymosan decreases NF-κB activation in CpG-DNA triggered cells and thereby modulates the latter activity. With regard to cytokine secretion, SOCS1 seems to influence IL12p40 and IL10, but not TNFα or IL8. Whereas

IL12p40 and IL10 are increased in the absence of SOCS1, TNFα and IL8 production was unmodified. IL12 as well as IL10 are delayed-type genes that showed late and prolonged NF -κB recruitment to the respective promoter (139).

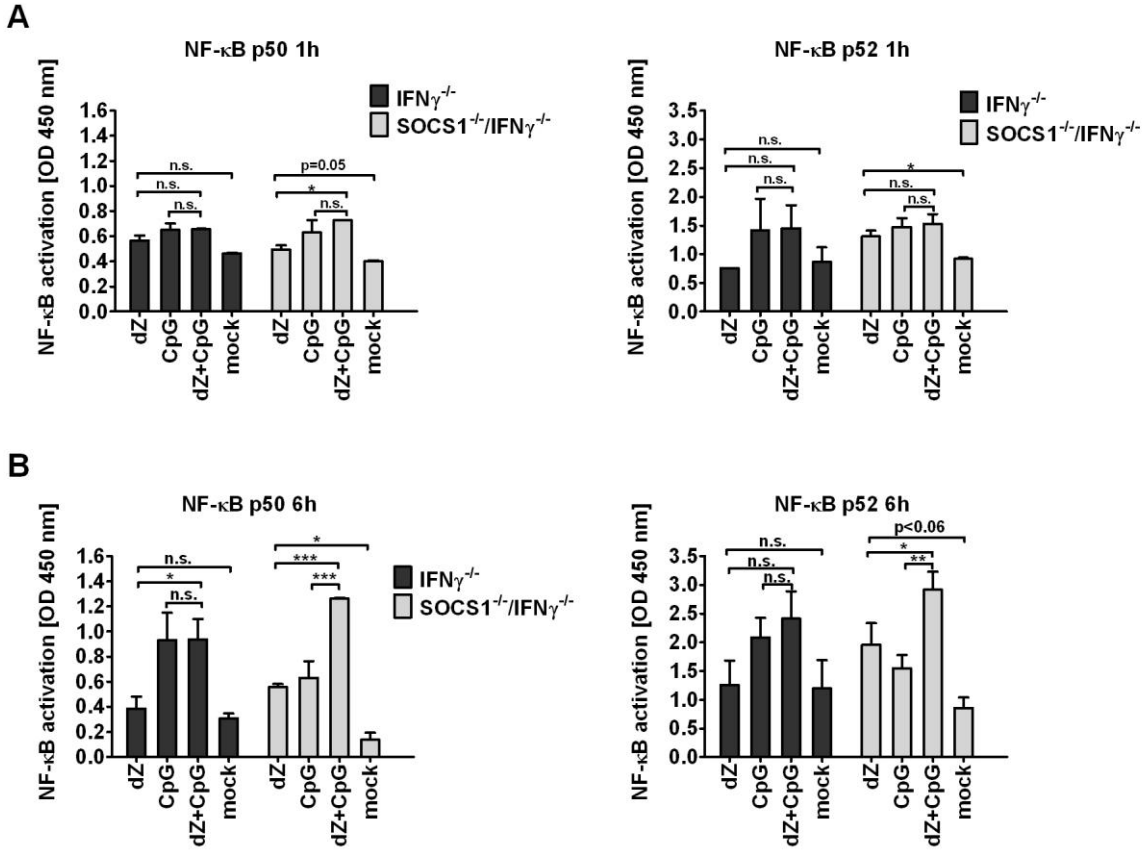

Fig. 4-23 Changes in NF-κB activity in SOCS1 deleted BMMs

BMMs ($9x10^6$) from SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ mice were incubated with dZ (100 µg/ml), CpG-DNA (1 µM) or dZ+CpG-DNA for [A] 1 h or [B] 6 h. Cells were lysed and nuclear extracts were analyzed for DNA binding activity of NF-κB subunits (p50 and p52) by TransAM® NF-κB Transcription Factor Assay Kit. n = 3 (mean + SD)

4.2.4. Summary of part 4.2

In the second part of the study it could be shown that SOCS1 induced via depleted Zymosan is not a feedback inhibitor of Dectin-1 signalling pathway. We observed no differences in the phosphorylation status of Syk, Pyk2 as well as ERK; kinases involved in SOCS1 induction (part 4.1). Thus, SOCS1 has no influence on its induction pathway. However, SOCS1 acts as a crosstalk inhibitor on TLR signalling. In BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice the secretion of IL12p40 was enhanced upon co-stimulation with CpG-DNA and depleted Zymosan, whereas IFNγ$^{-/-}$ cells produced less IL12p40 when co-stimulated. Similarly, IL10 secretion was significantly up-regulated more than two-fold in SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs as compared to cells from IFNγ$^{-/-}$ mice. This indicates an inhibitory role of SOCS1 in TLR/IL12p40 or IL10 regulation. The levels of TNFα, as well as IL8, were unaltered in knockout cells as compared to the control cells.

Co-stimulation of WT BMMs with depleted Zymosan and CpG-DNA resulted in an increase in SOCS1 expression what could explain the decrease in IL12p40 secretion in WT and IFNγ$^{-/-}$

BMMs. Thus, IκBα was analyzed for phosphorylation and subsequent degradation. Indeed, we detected an increased and extended phosphorylation and subsequent degradation of IκBα in BMMs from double knockout mice when co-stimulated via Dectin-1 and TLR9. Additionally, we analyzed NF-κB activity in SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ control BMMs. As it was shown that p50 and p52 are the NF- κB subunits responsible for IL12 and IL10 induction in BMMs, we determined the activation of these subunits. Indeed, we detected a significant increase in p50 and p52 activity in nuclear extracts from SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs as compared to IFNγ$^{-/-}$ cells after co-stimulation with depleted Zymosan and CpG-DNA. Thus, SOCS1 induced by depleted Zymosan supposedly inhibits the activity of p50 and p52. This inhibition may be responsible for the decrease in IL12p40 production observed in WT as well as IFNγ$^{-/-}$ BMMs.

4.3. Dectin-1 induced SOCS1 influences T-cell priming

The recently discovered Th-cell subset Th17 plays an important role in protection against invading fungal pathogens. It has been shown *in vitro* and *in vivo* that activation of the Dectin-1 pathway leads to the generation of Th17 cells (125-127). Moreover, it has been published that SOCS1 may be involved in T-cell functions, i.e. regulatory T-cells (160). As stimulation of BMMs led to a decrease in IL12p40 secretion upon co-engagement of TLR9 and Dectin-1 and since IL12 is one of the major cytokines that is responsible for Th1 polarization we wished to link our findings to adaptive immune responses.

4.3.1. Production of key cytokines required for Th17 specification

IL6 and TGFβ are reported to be the most important cytokines required for lineage commitment of Th17-cells whereas IL23 is a critical factor for sustaining these cells and for obtaining their pathogenic function *in vivo* (119, 161, 162).

Therefore, we first examined the secretion of these cytokines in BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ and the respective control (IFNγ$^{-/-}$) that were stimulated by depleted Zymosan together with CpG-DNA or the respective ligands alone. BMMs from IFNγ$^{-/-}$ mice displayed an elevated production of IL23, IL6 and TGFβ when co-stimulated by CpG-DNA and depleted Zymosan (Fig. 4-24 [A]-[C]). These cytokines were regulated the opposite way as compared to IL12p40. Whereas IL12p40 was decreased in IFNγ$^{-/-}$ BMMs, IL23, IL6 and TGFβ production was increased in the IFNγ$^{-/-}$ control cells as compared to the SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs. Regarding TGFβ, depleted Zymosan alone led to an increase. Though, in SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs secretion of these cytokines was neither up-regulated after stimulation via the sole ligand depleted Zymosan nor when TLR9 and Dectin-1 were triggered in parallel (Fig. 4-24 [A-C]). These data indicate that SOCS1 induced by Dectin-1 engagement influences the TLR9 cytokine profile. Therefore we conclude that SOCS1 might be responsible for the observed up-regulation of the cytokines required for Th17 induction, namely IL6, IL23 and TGFβ.

Thus, we further tested whether SOCS1 produced in BMMs by depleted Zymosan could shift T-cell response to IL17 producing cells.

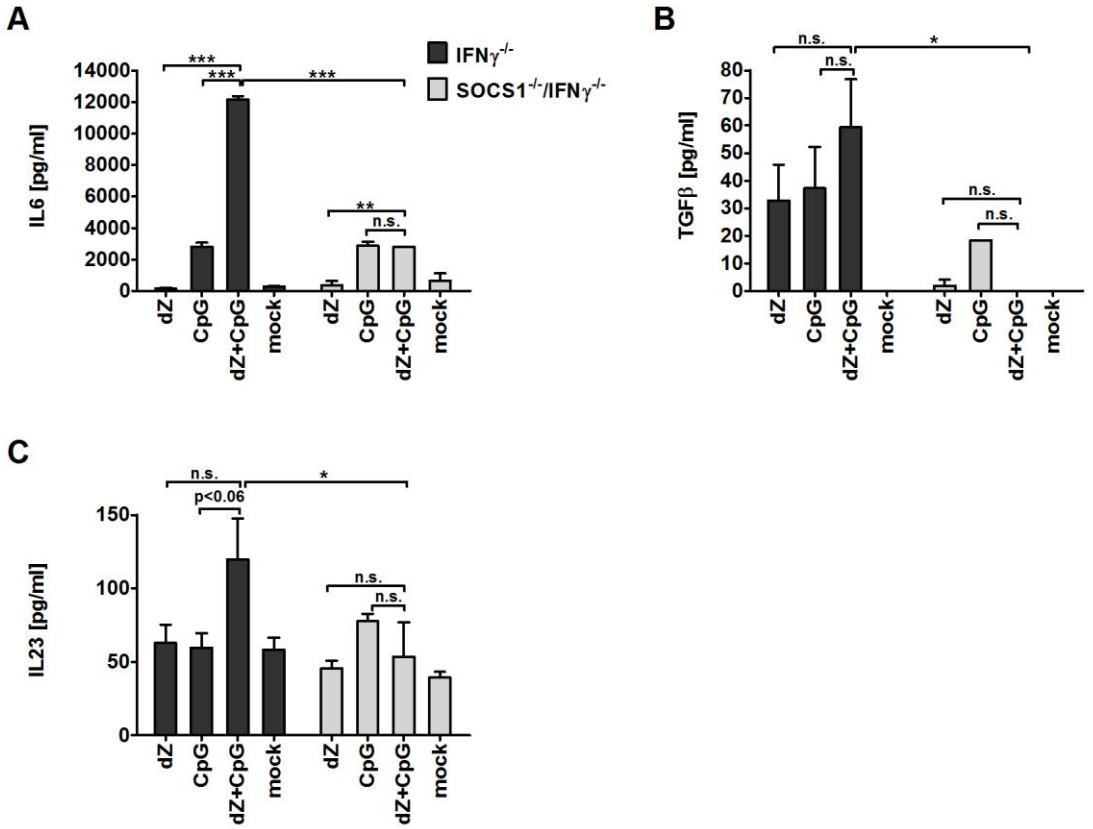

Fig. 4-24 Influence of SOCS1 on the secretion of cytokines required for induction of Th17-cells

[A]-[C], BMMs (2x10^5) from SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ mice were incubated with dZ (100 µg/ml), and CpG-DNA (1 µM) as indicated for 16 h [A] or 20 h [B]-[C]. In case of dZ+CpG, cells were pre-stimulated with CpG (4 h) following incubation with dZ (12 h [A]; 20 h [B], [C]). Secretion of IL6, TGFβ and IL23 in supernatants was measured by ELISA and experiments were carried out in duplicates. [A]-[C], n=2 (mean + SD)

4.3.2. SOCS1 produced via Dectin-1 increases CpG-DNA induced IL17 production from T-cells

Interplay between BMMs lacking endogenous SOCS1 and wildtype T-cells was studied by means of BMM/T-cell co-cultures. Co-culture experiments were set up in SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs (and BMDCs) or the respective control cells (IFNγ$^{-/-}$) and results were also compared to BMMs from conditional SOCS1 knockout mice. The latter mice are deficient in SOCS1 exclusively in cells from the myeloid lineage, especially macrophages (3.2.2). In the following, BMMs from knockout animals and the appropriated control were cultured together with isolated CD4$^+$ Th-cells from spleens from WT C57BL/6 mice as described in 3.2.1. After cultivation for 5 days, cytokine secretion was measured. First, we focused on SOCS1 knockout BMMs as well as BMDCs on an IFNγ$^{-/-}$ background. Therefore, BMMs or BMDCs from either SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ were stimulated with CpG-DNA, depleted Zymosan or a combination thereof. Thereupon, WT CD4$^+$ Th-cells were isolated and co-cultured with the respective cells as described in 3.2.1.

The key cytokine that is produced by Th17 cells is IL17 (124). After re-stimulation with PMA and ionomycin (3.2.1), we measured the secretion of IL17 within the co-cultures after 5 days of cultivation. Controls were set up as described in Fig. 4-25. Concerning Th17 positive control, cytokines to induce IL17-producing Th-cells were added extra. Thus, secretion of

IL17 should not differ between cultures with SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ cells. The same holds true for the respective Th1 and Th2 negative controls.
Secretion of IL17 in the Th17 positive-controls was markedly increased in cultures of WT Th-cells and IFNγ$^{-/-}$ as well as SOCS1$^{-/-}$/IFNγ$^{-/-}$ cells as compared to the respective unstimulated control (mock) (Fig. 4-25 [A], [B]). In contrast, co-cultures stimulated with Th1 or Th2 inducing cytokines (IL12 or IL4, respectively) showed no increase in IL17 secretion when compared to the unstimulated control. From these results we concluded that the isolated WT CD4^{+} Th-cells are able to become IL17 producers. Therefore, we next analyzed T-cells co-cultured with BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ that were stimulated via Dectin-1 and/or TLR9 for the production of IL17.
A clear-cut, significant increase in IL17 expression could be demonstrated when BMMs from IFNγ$^{-/-}$ animals were co-ligated via Dectin-1 and TLR9 compared to CpG-DNA or depleted Zymosan. In contrast, the concomitant administration of both stimuli to SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs did not change cytokine production when compared to CpG-DNA alone (Fig. 4-25 [A]). Depleted Zymosan alone led to an approximately 2-fold enhancement of IL17 secretion in WT T-cell/ SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMM co-culture. CpG-DNA stimulated BMMs, SOCS1$^{-/-}$/IFNγ$^{-/-}$ as well as IFNγ$^{-/-}$, displayed a significant increase in IL17 within the co-cultures. However, in co-cultures with CpG-DNA stimulated SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs, IL17 production was even increased as compared to those with CpG-DNA stimulated IFNγ$^{-/-}$ BMMs.
Similar results could be observed when BMDCs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ control mice were co-cultured with WT CD4^{+} Th-cells (Fig. 4-25 [B]). However, CpG-DNA alone had no effect on IL17 production, whereas depleted Zymosan increased cytokine secretion in cultures of IFNγ$^{-/-}$ BMDCs with WT Th-cells. Co-stimulation significantly increased IL17 production as compared to CpG-DNA or depleted Zymosan stimulated cultures. In contrast, co-cultures with SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMDCs displayed no increase in IL17 when BMDCs were stimulated by depleted Zymosan, CpG-DNA or both stimuli together as compared to the unstimulated control. Only the Th17 control displayed an increase in IL17 production. Th-cells cultured together with IFNγ$^{-/-}$ BMDCs even decreased IL17 secretion upon stimulation as compared to unstimulated control (mock) (Fig. 4-25 [B])
From the above results we conclude the following: SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs or BMDCs that are co-stimulated via depleted Zymosan and CpG-DNA are not able to further increase IL17 production in WT T-cells when compared to CpG-DNA or depleted Zymosan stimulated APCs. On the other hand, in the presence of SOCS1 (IFNγ$^{-/-}$ cells), BMDCs and BMMs induced a significant increase in IL17 secretion as compared to CpG-DNA or depleted Zymosan stimulated BMMs or BMDCs. Thus, SOCS1 that is induced via Dectin-1 in cells of the immune system (BMMs, BMDCs) leads to an increase in IL17 production in cells of the adaptive immune system (Th-cells). This could probably be mediated via cytokines that are responsible for the induction of the Th17 lineage, namely IL6, IL23 and TGFβ. These cytokines are markedly increased in BMMs from IFNγ$^{-/-}$ mice upon co-ligation of Dectin-1 and TLR9 when compared to BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice (Fig. 4-24). Therefore, IL6, IL23 and TGFβ are up-regulated in the presence of SOCS1 and supposably shift T-cell response towards a Th17 cell phenotype.

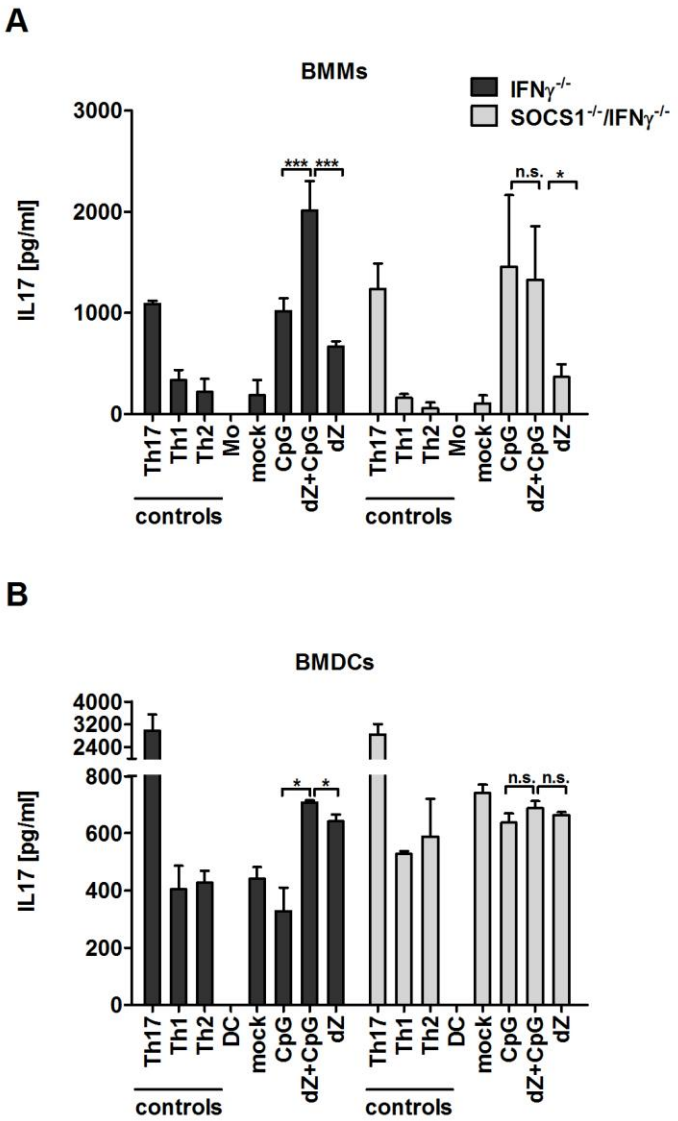

Fig. 4-25 SOCS1 expressed in BMMs and BMDCs via Dectin-1 leads to an increase in IL17 secretion in Tcells

$CD4^+$ T-cells ($1x10^5$) from C57BL/6 WT mice were co-cultured for 5 days with [A] BMMs ($1x10^4$) or [B] BMDCs ($1x10^4$) from $SOCS1^{-/-}/IFN\gamma^{-/-}$ or $IFN\gamma^{-/-}$ mice and anti-CD3ε and anti-CD28 in the presence or absence of depleted Zymosan (100 µg/ml), CpG-DNA (CpG) (1 µM) or a combination thereof. Positive controls (Th17) were treated with 10 ng/ml TGFβ, 20 ng/ml IL6 and anti-IL4 (2 µg/ml) and anti-IFNγ (2 µg/ml). Th1 and Th2 T-cells were induced via IL12 (2 ng/ml) and IL4 (10 ng/ml), respectively.
On day 5, cells were restimulated with PMA and Ionomycin for 4 h. IL17 in the supernatant was measured by ELISA. Mo; BMMs without any stimuli or T-cells, DC; BMDCs without any stimuli or Th-cells, mock; $CD4^+$ Th-cells + BMMs [A] or BMDCs [B], unstimulated. A], n=4 (mean + SD) [B], n=3 (mean + SD).

Since RORγT is one of the main transcription factors through which Th17 cells can be identified (163, 164), we next examined the expression levels of RORγT transcripts in T-cells from co-cultures. Experiments were set up as described for Fig. 4-25. After re-stimulation on day 5 for 4 h, cells were lysed and analyzed by means of quantitative RT-PCR for the expression of RORγT mRNA. Only in Th-cells cultured with BMMs from control mice ($IFN\gamma^{-/-}$), RORγT mRNA was markedly up-regulated by 10-fold upon engagement of Dectin-1 whereas depleted Zymosan had no effect on mRNA expression in the $SOCS1^{-/-}/IFN\gamma^{-/-}$ BMMs/ WT Th -cell cultures (Fig. 4-26). Surprisingly, co-stimulation only slightly increased the expression of the transcription factor by two-fold as compared with depleted Zymosan stimulated cells (Fig. 4-26).

Overall, the expression of RORγT is only increased in co-cultures with WT Th-cells cultured together with $IFN\gamma^{-/-}$ but not in cultures with $SOCS1^{-/-}/IFN\gamma^{-/-}$ BMMs. These results strengthen

the above findings that SOCS1 induced by Dectin-1 in BMMs is involved in the induction of Th17 cells.

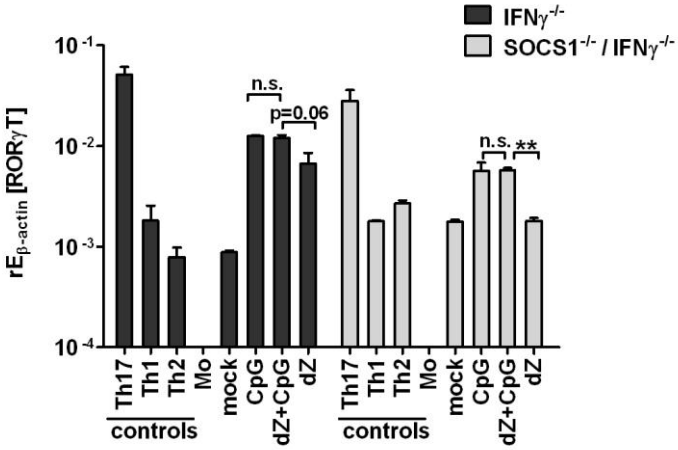

Fig. 4-26 Expression of RORγT transcription factor in BMM/Th-cell co-cultures

$CD4^+$ Th-cells ($1x10^5$) from C57BL/6 WT mice were co-cultured for 5 days with BMMs ($1x10^4$) from $SOCS1^{-/-}/IFN\gamma^{-/-}$ or $IFN\gamma^{-/-}$ mice and anti-CD3ε and anti-CD28 in the presence or absence of depleted Zymosan (100 µg/ml), CpG-DNA (CpG) (1 µM) or a combination thereof. Positive controls (Th17) were treated with 10 ng/ml TGFβ, 20 ng/ml IL6 and neutralizing antibody against IL4 (2 µg/ml) and IFNγ (2 µg/ml), respectively. Th1 and Th2 T-cells were induced via IL12 (2 ng/ml) and IL4 (10 ng/ml), respectively. On day 5, cells were restimulated with PMA and Ionomycin for 4 h. Cells were lysed and gene expression was determined by quantitative RT-PCR. Relative expression is depicted, normalized to β-actin. n=3 (mean + SD)

Next, we wanted to compare the above illustrated results with an approach where BMMs from conditional SOCS1 knockout animals were used.

First of all, cells were controlled by the appropriate genotype. LysM-Cre has previously been reported to mediate efficient loxP recombination in myeloid cells, i.e. BMMs. For an efficient deletion of SOCS1 in BMMs, mice with the genotype $SOCS1^{lox/-}$ were required. One copy of SOCS1 is flanked by two loxP sites (floxed) whereas the other SOCS1 allele is completely deleted. Additionally, cells have to be positive for the Cre recombinase gene- under the promoter of Lysozyme M (LysM)- to ensure an entire knockout (136, 137) (Fig. 4-27 [A]). The tyrosine recombinase Cre is known to catalyze site-specific recombination events. It recognizes loxP sites that flank a specific DNA sequence or gene. Upon recognition of the loxP sites, the Cre recombinase excises the DNA in between. Thus, mice with the genotype $SOCS1^{lox/-}$ that express the Cre recombinase are able to delete both alleles of SOCS1. The Lysozyme M is shown to be expressed especially in macrophages upon stimulation. The $SOCS1^{lox}$ allele is placed under the natural SOCS1 promoter and carries a truncated version of human CD4. When Cre mediated deletion of SOCS1 was effective, the human CD4 reporter is switched active. Thus, we measured hCD4 expression in BMMs via FACS analyzes. First, BMMs with the genotype $SOCS1^{lox/-}/Cre^+$ (#124, Fig. 4-27 [A]) as well as the respective control cells ($SOCS1^{lox/+}/Cre^-$) (#123, Fig. 4-27 [B]) were stimulated by IFNγ and LPS to induce SOCS1 expression. Thereupon, cells were harvested and analyzed by means of FACS (3.2.1, “Flow cytometry”). Successful knockout is indicated in case of up-regulation of hCD4 (PE-coupled antibody against human CD4 was used). Cells in P2 are gated on P1 in the forward /sideward scatter diagram and show cells stained positive for hCD4. BMMs with the genotype $SOCS1^{lox/+}$, Cre^- do not express the Cre recombinase. These cells have

one allele of WT SOCS1 and one allele is floxed. Thus, these mice do not have a SOCS1 knockout allele and behave like WT mice (136, 137). Accordingly, we did not observe any up-regulation of hCD4 in stimulated control BMMs. In contrast, SOCS1$^{lox/-}$, Cre^{+} BMMs were highly hCD4 positive (76,8 %) after stimulation (Fig. 4-27 [B],lower panel), yet a small population was hCD4 negative. This could be due to inefficient staining with anti-hCD4 or incomplete knockout.

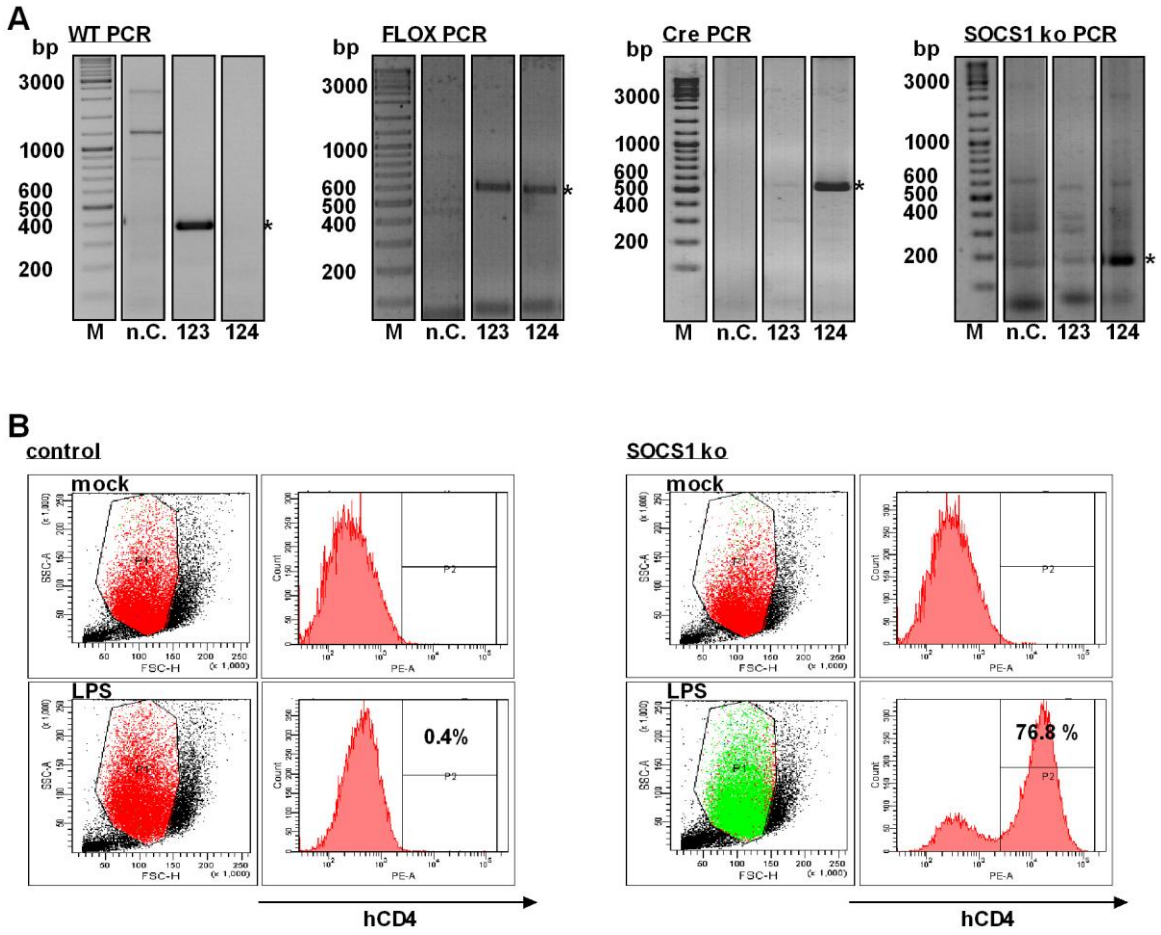

Fig. 4-27 Tissue specific SOCS1 deficiency

[A], Results of genotype PCR. Genomic DNA was isolated from the respective knockout or control mice as described in Materials and Methods "Purification of genomic DNA" in 3.2.2. PCR reaction conditions for the different PCRs are described in "Polymerase chain reaction" in 3.2.2. 10-200 ng DNA was loaded on a 1.5% (SOCS1 ko and Cre PCR) or 2% (FLOX and WT PCR) agarose gel and analyzed. 123, control (Fig. 4-28) that is negative for LysMCre (Cre^{-}). 124, SOCS1$^{flox/-}$ LysMCre^{+} (Cre^{+}). M, marker; n.C., negative control; ***,** specific bands: WT (340 bps), FLOX (600 bps), Cre (650 bps), SOCS1 ko (160 bps). [B], FACS analysis of hCD4 surface expression in SOCS1$^{flox/-}$ LysMCre^{+} BMMs (SOCS1ko) and SOCS1$^{flox/+}$ LysMCre^{-}Cells (appr. 4x10^{5}) were stimulated for 16h with 50 ng/ml IFNγ and 100 ng/ml LPS (lower panels) or left unstimulated for 16h (upper panels). Afterwards, cells were stained with PE labelled anti-hCD4 antibody and analyzed via FACS.

Consequently, those cells were used to investigate T-cell responses in a co-culture of SOCS1 knockout BMMs together with wildtype CD4^{+} Th-cells. Cells were incubated as described for SOCS1$^{-/-}$/IFNγ$^{-/-}$. After 5 days, supernatants were analyzed for cytokine secretion after re-stimulation for 4 h with PMA and ionomycin. Concerning Th17 markers, namely IL17 and RORγT, we measured a similar pattern as analyzed in case of SOCS1$^{-/-}$/IFNγ$^{-/-}$ and IFNγ$^{-/-}$ cells. IL17 secretion was significantly increased by 50 % when control (SOCS1$^{lox/-}$ Cre^{-}) BMMs within the co-culture setting were co-stimulated with depleted Zymosan and CpG-DNA as compared to ligation of Dectin-1 or TLR9, respectively

(Fig. 4-28 [A]). Within the Th17 control, IL17 secretion was markedly increased as compared to Th1 or Th2 controls.

These results could be reflected by RORγT expression (Fig. 4-28 [B]). Within the control BMMs/ WT Th-cell co-culture, stimulation of the control cells with depleted Zymosan, CpG-DNA or a combination of both stimuli induced an increase in mRNA expression up to 10-fold over mock. However, co-stimulation of the control BMMs via TLR9 and Dectin-1 within the co-culture did not further increase RORγT expression as compared to CpG-DNA or depleted Zymosan stimulated cells. In co-cultures of SOCS1 knockout BMMs and WT Th-cells, only the stimulation with CpG-DNA induced a slight but significant increase in RORγT mRNA expression in WT Th-cells. In contrast, neither the stimulation with depleted Zymosan nor the concomitant application of CpG-DNA and depleted Zymosan induced RORγT expression over the unstimulated control.

Consequently, we confirmed the results displayed for SOCS1$^{-/-}$/IFNγ$^{-/-}$ or IFNγ$^{-/-}$ cells. The advantage of the conditional knockout system is that the knockout of SOCS1 is inducible in particular cell types. When the cre recombinase is expressed under the promoter of Lysozyme M, a knockout is only achieved within cells of the myeloid lineage, i.e. macrophages.

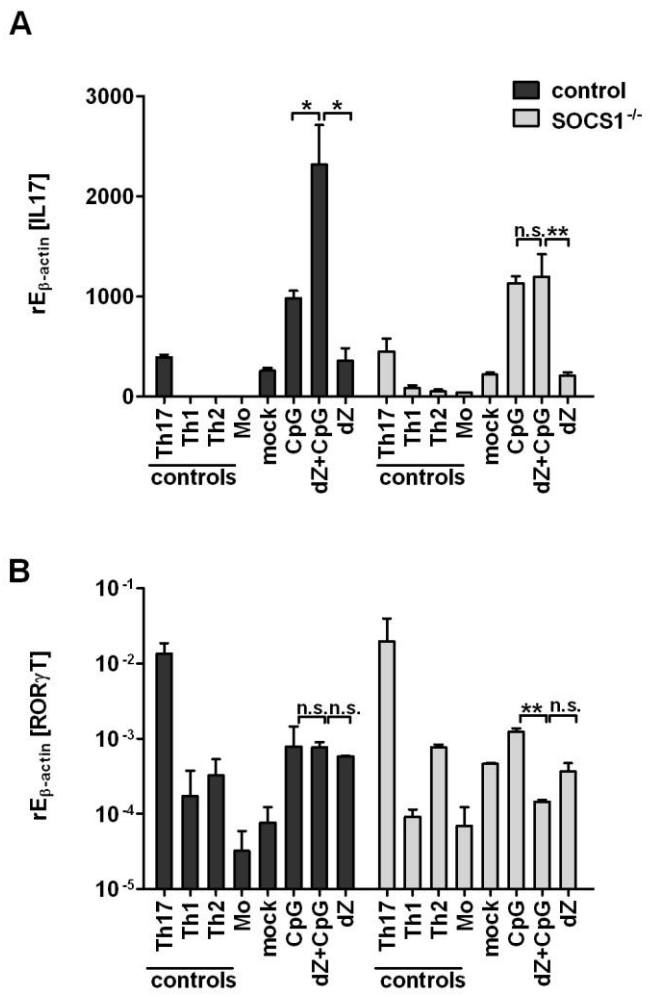

Fig. 4-28 IL17 secretion and expression of RORγT in T-cells cultured with conditional SOCS1 ko BMMs

CD4+ T-cells (1x105) from C57BL/6 WT mice were co-cultured for 5 days with BMMs (1x104) from SOCS1flox/- LysMCre+ mice or control mice (SOCS1flox/+ LysMCre-). Anti-CD3ε and anti-CD28 in the presence or absence of depleted Zymosan (100 µg/ml), CpG-DNA (1 µM) or a combination thereof was added. Positive controls (Th17) were treated with 10 ng/ml TGFβ, 20 ng/ml IL6 and neutralizing antibody against IL4 (2 µg/ml) and IFNγ (2 µg/ml), respectively. Th1 and Th2 T-cells were induced via IL12 (2 ng/ml) and IL4 (10 ng/ml), respectively. On day 5, cells were restimulated with PMA and Ionomycin for 4 h. Cells were lysed and gene expression was determined by quantitative RT-PCR. Relative expression is depicted, normalized to β-actin. Experiments were carried out in duplicate wells.

4.3.3. Summary of part 4.3

In the last part it could be demonstrated that SOCS1 induced by depleted Zymosan affects T-cell priming. SOCS1 influences the shift of $CD4^+$ Th-cells towards IL17 producing T-cells to some extent. The key cytokines that are claimed to be responsible for inducing Th17 cells, namely IL6, TGFβ and IL23 were markedly increased in $IFN\gamma^{-/-}$ BMMs upon co-stimulation with CpG-DNA and depleted Zymosan. In the absence of SOCS1 ($SOCS1^{-/-}/IFN\gamma^{-/-}$ BMMs), only triggering of TLR9 led to an up-regulation of these cytokines. Consequently, secretion of IL17 from WT Th-cells that were cultured with $IFN\gamma^{-/-}$ BMMs or BMDCs was significantly increased upon co-stimulation with depleted Zymosan and CpG-DNA of the innate immune cells. The same holds true for the hallmark transcription factor of Th17 T-cells, RORγT.
This contribution of SOCS1 expressed in BMMs to the induction of IL-17 producing Th-cells represents an important link between innate and adaptive immune responses.

5. Discussion

5.1. Direct induction of SOCS1 in myeloid cells upon ligation of Dectin-1

It had not been shown before that engagement of the PRR Dectin-1 could lead to the induction of SOCS proteins. Though, the here presented results show that SOCS1 mRNA and protein is expressed in BMDCs and BMMS when Dectin-1 is stimulated by depleted Zymosan.

In terms of SOCS1 mRNA expression, a time- and dose-dependent induction was achieved within BMDCs as well as BMMs when stimulated via depleted Zymosan. LPS led to a maximum SOCS1 induction already after 2 h of stimulation. With both stimuli, a steady-state SOCS1 induction was achieved that even remained constant 16 h after addition of LPS or higher concentrations of depleted Zymosan. In BMMs a slight decrease was observed after 16 h. However, previous reports described a fast but shortened SOCS1 expression that declined already 4 h after stimulation as shown for IL6 dependent induction of SOCS1 (104, 165). In general, the genes encoding for SOCS proteins are small and comprise only less introns (SOCS1 and SOCS3: one intron, SOCS2 and CIS: two introns).Thus, these genes display characteristics of immediate early genes (95, 141, 166, 167) and transcription can occur much faster than the expression of mRNA transcribed from poly cistronic genes. This could explain the rapid induction of SOCS1 mRNA upon stimulation of BMMs or BMDCs with LPS or depleted Zymosan. Otherwise, mRNA was stable even 16 h after stimulation. Gene expression can be regulated at different levels. On the one hand, mRNA translation can be inhibited by microRNAs. The double stranded miRNA is incorporated into a protein complex (RISC). The double strand is uraveled and the formed single-strand binds to its complementary target sequence within the respective mRNA. Thereupon, the microRNA/mRNA structure is either degraded by an endoribonuclease within the RISC complex or the complex is stable, thus leading to repression of translation (168). MicroRNA 155 (miRNA 155) is described to bind to SOCS1 mRNA; thereby SOCS1 mRNA is degraded (169, 170). Due to the durable stability of depleted Zymosan or LPS induced SOCS1 mRNA, SOCS1 in this case may be regulated differentially.

On the other hand, protein stability is regulated. It could be demonstrated that the SOCS1 protein has a short half-life time, and thus is degraded rapidly after its synthesis by the proteasom. Additionally, SOCS1 has been shown to be regulated via translational repression (171, 172). This regulatory mechanism is mediated by an additional start codon within the 5' non-translated region and possibly leads to the decreased SOCS1 expression that is observed in resting cells.

Further, priming of BMMs with IFNγ prior to stimulation had synergistic effects on SOCS1 expression. Macrophages respond to a range of different cytokines and chemokines during innate and adaptive immune responses. Among these, IFNγ (originally called macrophage-activating factor) is described to be the most important one. IFNγ orchestrates leukocyte attraction, growth and maturation of many cell types as well as enhancement of natural killer (NK) cell activity (173). It has been reported that treatment of macrophages with IFNγ followed by LPS stimulation alters intracellular signalling pathways in different ways. On the

one hand, pre-incubation of macrophages with IFNγ augmented DNA binding of NF-κB in response to LPS (174, 175); similar results were obtained when DCs were treated with Dectin-1 agonists in combination with TLR2 stimuli (53). Otherwise, LPS affected the IFNγ signalling pathway by increasing the activation of STAT1 binding to DNA. Finally, it could be demonstrated that interaction between LPS and IFNγ not only occurs at the level of signal transduction, but also may involve the induction of factors that act in an autocrine manner (174, 176, 177). One could claim that within BMMs that were stimulated via Dectin-1 following priming with IFNγ similar effects could lead to the observed up-regulation of SOCS1 transcripts. Either depleted Zymosan influences JAK/STAT activation or IFNγ affects activity of transcription factors responsible for SOCS1 induction in Zymosan triggered cells. Additionally, IFNγ itself is able to induce SOCS proteins (87, 88, 165). This could also explain the increased expression of SOCS1 observed by co-stimulation of BMMs with depleted Zymosan and IFNγ. Similarly, co-stimulation of TLR9 and Dectin-1 increased SOCS1 expression. Hence, co-stimulation of TLR9 and Dectin-1 synergistically influenced SOCS1 expression.

5.1.1. Dectin-1 induced SOCS1 is expressed TLR independently

Engagement of Dectin-1 by depleted Zymosan led to an induction of SOCS1 in a MyD88/Trif independent manner. This observation rules out any contribution of TLRs to SOCS expression.

MyD88 is utilized by all TLRs with the exception of TLR3 that activates downstream signalling through Trif (178). Importantly, LPS is capable of employing both, MyD88 and Trif. Depleted Zymosan was able to induce SOCS1 expression in both, WT and MyD88/Trif double deficient mice to the same extent. Also, downstream signalling as depicted for ERK activation was completely unaltered when cells were triggered via Dectin-1. In contrast, expression of SOCS1 mRNA as well as ERK phosphorylation were completely abrogated when knockout cells were triggered via TLR4, thus confirming the importance of MyD88 as well as Trif adaptor molecules for TLR4 signalling but not for Dectin-1 mediated downstream signalling. Therefore, a participation of MyD88 and/or Trif adaptor proteins in the Dectin-1 signalling pathway can be excluded as well as any TLR ligands that could be present as contaminants within depleted Zymosan. Zymosan is extracted from the cell wall of the yeast *Saccharomyces cerevisiae* that is shown to bind also to TLR2 and TLR9, yet the real TLR ligand within Zymosan is not identified (44, 46, 55, 63). Depleted Zymosan is generated out of Zymosan by treatment with hot alkali to remove any TLR binding moiety (61, 62). Thus, we corroborated that SOCS1 expression induced by depleted Zymosan is exclusively mediated by Dectin-1. This could be confirmed by stimulation of cells from Dectin1$^{-/-}$ mice where SOCS1 induction vanished completely.

5.1.2. Intermediate factors are not involved in Dectin-1 mediated SOCS1 induction

Next, we had to clarify the question whether the induction of SOCS1 occurs in a direct or indirect pathway dependent on para- or autocrine factors as described by different groups (103, 140, 143). Crespo *et al.* claimed that LPS is capable of inducing SOCS1 via type I IFNs (IFNα/β) as auto/paracrine acting factors. LPS-induced SOCS1 production was diminished in

an assay where neutralizing antibodies against IFNα/β were used. In contrast, Baetz *et al.* could clearly show that SOCS1 is induced directly upon stimulation with LPS, without the contribution of type I IFNs. Dendritic cells isolated from IFNAR1$^{-/-}$ mice were stimulated with different TLR ligands, i.e. LPS or LTA. SOCS1, SOCS3 and CIS mRNA induction showed no difference in the knockout cells as compared to WT control cells.

Here, Dectin-1 dependent SOCS1 induction was independent of any intermediate or secreted factors as examined by different methods. First, a transwell system was used to exclude any secreted factor that could be responsible for Dectin-1 mediated SOCS1 induction. BMMs as well as BMDCs were seeded in the two compartments of a transwell. Cells within the lower wells were stimulated and cells of both chambers were analyzed for SOCS1 mRNA expression. Depleted Zymosan particles have a size of about 3 µm (142) and thus are not able to enter the upper wells. We observed SOCS1 induction only in cells of the lower compartments upon stimulation with depleted Zymosan. Similar results could be obtained by the group of Crespo *et al.* Macrophages generated from LPS-responsive C3H/HeN-mice and LPS-none responsive C3H/HeJ mice were placed in a transwell system. Upon stimulation with LPS, no induction of the SOCS1-gene could be observed within macrophages generated from C3H/HeJ mice (140). Thus, an influence of an auto-or paracrine factor on the expression of SOCS1 could be excluded.

Therefore, we propose a direct induction of SOCS1 by depleted Zymosan, independent of secreted factors. These results could be verified with BMMs generated from IFNAR1 deficient mice. In these cells, SOCS1 could still be expressed upon engagement of Dectin-1 with depleted Zymosan. Interestingly, also LPS was still able to induce SOCS1 mRNA within the knockout cells. This could also be observed by Baetz *et. al.* TLR ligands like LPS, LTA or CpG-DNA induced SOCS1 molecules in a direct way, independent of IFNs (143).

It is described that TLR ligands induce type I IFNs, i.e. IFNβ and thereupon STAT1 activation (105, 147, 173, 179). TLR3 and TLR4 signalling pathways use Trif/TICAM-1 as adaptor molecules. Thereupon, Type I IFNs (i.e., IFNβ) are secreted and activate the expression of STAT1/2 dependent genes via the JAK/STAT pathway (146, 179). Thus, we analyzed BMDCs and BMMs for STAT1 phosphorylation and detected STAT1 phosphorylation in BMMs and BMDCs following LPS stimulation. Though, depleted Zymosan only led to STAT1 activation when applied to BMDCs but not BMMs. However, a contribution of IFN signalling to SOCS1 induction could be ruled out as examined by transwell as well as IFNAR1 knockout experiments.

Additionally, we analyzed SOCS1 expression after treatment of cells with a blocking antibody directed against IL10. IL10 is one of the first cytokines that is produced upon an infection with pathogenic fungi (66-68). Inhibition of IL10 receptor prior to stimulation with depleted Zymosan had no effect on SOCS1 induction in BMMs and BMDCs. The same effect could be observed when LPS was used as a trigger for SOCS1. On the other hand, SOCS3 expression was diminished in cells pre-treated with anti-IL10 following stimulation with IL10. Since IL10 was described as an inducer for SOCS3 but not for SOCS1, we therefore expected an influence of anti-IL10 only on SOCS3 expression (180, 181).

To foreclose a participation of intracellular intermediates, we used cycloheximid to block intermediate protein biosynthesis. Subsequently, SOCS1 mRNA was analyzed by

quantitative RT-PCR. SOCS1 transcripts could still be detected after stimulation with depleted Zymosan. Surprisingly, we observed a slight increase in SOCS1 transcripts. This may point towards other classes of signalling inhibitors. These inhibitors may have the ability to inhibit signalling cascades and thereby also SOCS1 expression (103, 109). Thus, the blockade of protein biosynthesis by cycloheximid possibly also abrogates the synthesis of these inhibitors, thereby increasing SOCS1 expression. Again, also LPS induced SOCS1 mRNA independently of any intermediates. Similar results were obtained by the group of Stoiber *et al.* It could be shown that LPS induced SOCS3 on mRNA level. The expression was resistant to cycloheximid treatment, yet it was even increased (102). Additionally, Nakagava *et al.* claimed that the induction of SOCS1 was independent of any intermediate protein (109). Raw264.7 macrophages were pre-treated with cycloheximid followed by stimulation with LPS for different time-periods. Afterwards, an induction of SOCS1 mRNA was already detected after 1 h of stimulation (109). Also, SOCS3 was described to be induced in a direct manner. Bode *et al.* could show that LPS as well as TNFα are capable of inducing SOCS3 mRNA directly, without the contribution of an intermediate factor (100). In this context, the induction of SOCS proteins was linked to MAPK activation. It could be shown that the MAPK p38 is involved in the induction of SOCS3 mRNA expression, especially upon stimulation with TNFα (100).Beside TNFα, IL1β was able to induce SOCS3 in a direct way as shown in liver cells (182). In addition, the direct activation of the MAPK pathway by PMA (phorbol myristate actetate) led to an induction of SOCS3 protein (183).

5.2. NF-κB does not serve as a transcription factor for SOCS1 induction in BMMs stimulated via Dectin-1

One important difference between BMDCs and BMMs triggered via Dectin-1 is the activation of the transcription factor NF-κB. In previous reports, it was demonstrated that zymosan-stimulated DCs activated NF-κB in a CARD9/Bcl10/MALT1 dependent manner (75). Indeed, we could confirm these findings with regard to IκBα. This inhibitor of NF-κB is phosphorylated and degraded by the proteasom upon stimulation of PRRs (184, 185). Ligation of Dectin-1 and TLR4 induced phosphorylation of IκBα in BMDCs. In contrast, IκBα was not activated in BMMs after stimulation of Dectin-1.

Previous studies have indicated that in macrophages, Dectin-1 signals collaborate with TLR2 signals to enhance TLR2-mediated NF-κB activation and proinflammatory cytokine production. In contrast, Dectin-1 signals alone appear to be insufficient for activation of the transcription factor and cytokine induction (71). It was rather discussed that CARD9 in macrophages is recruited to phagosomes where it activates p38 MAPK signalling without the attendance of NF-κB. However, in correlation with the data mentioned above, priming of BMMs with IFNγ or GM-CSF contributed to Dectin1-CARD9-induced TNFα production (71). These data again point to a cross-talk between different receptor signalling cascades, thereby modifying the outcome of a stimulated cell. Importantly, the contribution of Dectin-1 signals to pro-and anti-inflammatory cytokine induction seems to be variable in different macrophage or dendritic cell populations since Dectin-1 engagement directly triggers TNFα production by resident peritoneal cells and alveolar macrophages but not in Flt3L-derived DCs (71). We could confirm the above results through the application of an NF-κB inhibitor.

In BMDCs, the inhibition of NF-κB decreased SOCS1 expression levels when cells were stimulated by LPS or depleted Zymosan, whereas SOCS1 induction in BMMs was only abrogated when cells were triggered by LPS following incubation with the inhibitor. Thus, we excluded that SOCS1 induction occurs through NF-κB signalling in BMMs. However, in BMDCs, NF-κB seems to be involved in SOCS1 expression upon stimulation with depleted Zymosan.

Nuclear factor of activated T-cells (NFAT) is a transcription factor that is rapidly activated upon ligation of Dectin-1 (61, 67, 149). Thus, we speculated that NFAT might play a role in induction of SOCS1 in BMMs. NFAT is situated in the cytoplasm and is activated in a Ca^{2+} dependent manner (186). Calmodulin triggers dephosphorylation of NFAT via calcineurin, thereby promoting transport of the transcription factor into the nucleus. Within the nucleus, NFAT can cooperate with multiple transcriptional partners, including AP1 (186, 187). On the other hand, calcium pathways activate CaM (calmodulin) dependent kinase (CaMK) that contributes to MAPK activation (149). It is described that NFAT, when activated via Dectin-1 is responsible for the fast induction of early growth response factors (61), another family of transcription factors that in turn can bind to the IL10 promoter (188-190). Moreover, egr1 transcription factor has been found to bind to the SOCS1 promoter (150). However, we were not able to ascertain any contribution of NFAT to the expression of SOCS1, since CsA, a very specific NFAT inhibitor, was ineffective in inhibiting SOCS1 expression. Furthermore, we were not able to detect any binding activity of egr1 to the respective binding site within the SOCS1 promoter upon ligation with depleted Zymosan, although egr transcription factors (egr1 and egr2) were induced at the transcript level. Binding of egr to the SOCS1 promoter was analyzed by means of electromobility shift assay (EMSA) with a labelled probe that comprised the binding sequence of egr1 within the SOCS1 promoter. Importantly, only the stimulation with LPS or Zymosan induced binding of egr1 to the promoter. In contrast to its depleted form (depleted Zymosan), Zymosan also exhibits TLR2 and TLR6 binding activity (44, 55, 63). Thus, the slight increase in egr1 binding to the respective SOCS1 promoter sequence could be caused by stimulation of TLRs via Zymosan. As shown by other groups, egr1 induced by LPS regulates SOCS1 expression. Via ChIP analysis, it could be demonstrated that the SOCS1 promoter is bound to egr1 *in vivo* (150). Since IFNγ was shown to be a potent inducer of SOCS1 mRNA in a STAT dependent manner, the promoters of SOCS family members were analyzed for STAT binding. Indeed, the promoter region of SOCS3, CIS and SOCS1 displayed STAT binding elements. A deletion of the STAT1/STAT3 binding element within the SOCS3 promoter resulted in a defect of SOCS3 induction via LIF (leukemia inhibitory factor) (191). In addition, it was described that IRF1 (interferon regulatory factor1) is upregulated in a STAT1 dependent manner and binds to the AANNGAAA repeat sequence within the SOCS1 promoter (150, 192). Also, transcriptional repressors, GFI-1B and Krüppel-like factor 4, were able to regulate the SOCS1 promoter (192).

Interestingly, depleted Zymosan induced egr1 and egr2 expression in BMMs, whereas LPS only induced expression of egr1. As activation by dephosphorylation and translocation of NFAT into the nucleus is strictly calcium dependent and since we did not observe any contribution of CaMK on SOCS1 expression, this strongly supports our findings of a calcium independent induction of SOCS1.

5.3. Downstream signalling molecules involved in Dectin-1 mediated SOCS1 induction

5.3.1. Pyk2 (proline-rich kinase 2) plays a key role in Dectin-1 mediated SOCS1 induction

Besides the differences in contribution of NF-κB to SOCS1 expression, the upstream signalling pathways also displayed some differences in BMMs as compared to BMDCs. First, we focused on Dectin-1 signalling within murine BMMs. It has been shown that the non-receptor tyrosine kinase Pyk2 (proline-rich kinase 2) is activated upon triggering of Dectin-1 (66). Pyk-2 was responsible for the production of IL10 in human macrophages, but this was shown to be Ca^{2+} dependent as delineated above (66). So far, only little is known about the role of Pyk2 kinase in inflammation. Thus, different activation patterns of Pyk2, calcium dependent (66) as well as calcium independent are possible. Under homeostatic conditions, Pyk2 is important for cell morphology, motility and adhesion in a variety of cells including macrophages (151, 193). Additionally, Pyk2 has been shown to be activated in response to a broad range of stimuli, including extracellular signals that elevate intracellular calcium concentrations, agonists of G protein-coupled receptors and engagement of Ag receptors on T cells, B cells and mast cells (151, 193, 194).

In addition, it emerged that Syk (spleen tyrosine kinase) and Src kinases were situated upstream of Pyk. Src and Syk have been reported to be directly recruited to phosphorylated Dectin-1 receptor (44, 46, 195). Interestingly, also LPS was shown to induce IL8 expression via activation of Pyk2 as described for endothelial cells (196). As we clearly identified induction of SOCS1 in a Pyk2 dependent way upon ligation of Dectin-1, it would be crucial to analyze TLR stimulated cells of the immune system for a possible phosphorylation of Pyk2. Further, some data point towards a Pyk2 activation triggered by IFNγ (197). The group of Takaoka *et al.* could show that Pyk2 was phosphorylated by stimulation of murine embryonic fibroblasts with IFNγ. Furthermore, it could be observed that Pyk2 contributed to STAT1 activation within these cells. Thus, Pyk2 is described as a downstream molecule for different receptors beside Dectin-1 that are prone to induce SOCS1 as well, i.e. IFNγ/STAT1.

Interestingly, we observed baseline phosphorylation of Pyk2 in unstimulated BMMs, as also reported for human macrophages (198). This could be due to inhibitory phosphorylation of Src kinases as already observed in macrophages (199). Src gets phosphorylated at its negative regulatory phosphorylation site (Y507), possibly via autophosphorylation. This inhibitory phosphorylation seems to be important as macrophages that were deficient in the two phosphatases CD45 and CD148, which are described to remove inhibitory phosphate residues from Src kinases upon ligation of Dectin-1, were defective in Syk activation. This seemed to be specific for Dectin-1 ligands (200). Accordingly, Pyk2 could be regulated in a similar manner that inhibitory phospho-tyrosines have to be removed. On the other hand, the applied antibody is described to be directed against Pyk2 only when phosphorylated at Tyr402 that is required for activation of Pyk2. This may be contrary to the possible detection of an inhibitory phospho-tyrosine as displayed by immunoblot.

5.3.2. The MAPK ERK is responsible for SOCS1 induction in BMMs but not in BMDCs

In this study it could be shown that Pyk2 is activated upon Dectin-1 signalling, yet Pyk2-mediated induction of SOCS1 occurred through ERK in a manner that was independent of calcium signalling pathways. Hence, we analyzed activation of MAPKs activated by stimulated Dectin-1, namely ERK (p42/p44), p38 and JNKII. All three kinases could be activated by phosphorylation as a result of engagement of Dectin-1 by depleted Zymosan. As observed for Pyk2 activation, only the application of AG17, an inhibitor specifically directed against Phospho-Pyk2, led to an inhibition of Pyk2 and ERK activation, whereas a calcium chelator (BAPTA) or an inhibitor of CaMK had no effect on Pyk2 and ERK phosphorylation. The missing activation of ERK upon Pyk2 inhibition by AG17 locates ERK downstream of Pyk2 (Fig. 5-1). Consequently, SOCS1 induction was lost as also observed by direct inhibition of ERK.

Regarding JNKII and p38 activation, AG17 displayed no effect, yet even slightly increased phosphorylation. Otherwise, inhibition of Ca^{2+} signalling pathways blocked phosphorylation of these MAPKs. Confirming these observations, MAPKs have been shown to trigger SOCS transcription (100, 143, 201, 202), although ERK specifically had not been analyzed in detail so far. The MAPK p38 was linked to SOCS1 induction, i.e. after infection of macrophages with *Toxoplasma gondii.* In this infection model it was proposed that SOCS1 gets activated via p38 (202). Otherwise, p38 was directly activated via TNF and thereby led to the induction of SOCS3 expression (183). Our data implicate that ERK is responsible for SOCS1 transcription and is situated downstream of Pyk2 (Fig. 5-1). Pyk2 itself is located downstream of Src and Syk kinases since inhibition of Src and Syk abolished Pyk2 as well as ERK activity. While the application of a Syk inhibitor specifically abolished ERK but not p38 of JNKII phosphorylation, the Src inhibitor PP1 also inhibited phosphorylation of p38 and JNKII. The downstream signalling of Dectin-1 additionally comprises the MAPKKK Raf1 (46, 52, 76) that is activated via the Src kinase as well. Raf1 activation does not require pSyk, but the Raf1 signalling still involves the ITAM-like motif of the Dectin-1 receptor (44, 203). Administration of an inhibitor directed against Raf1 largely suppressed phosphorylation of p38 and JNKII but not ERK, thus ruling out a contribution of Raf1 in ERK activation. Additionally, SOCS1 protein expression was only affected by inhibition of Syk or Src kinases, but not when Raf-1 activation was blocked. The exact mechanism by which Pyk2 regulates ERK and subsequent SOCS1 expression remains still unclear, but previous work has shown that in other cell types Syk and Pyk2 can activate ERK via the MAPKKK Tpl2 (151, 152, 204). Also, phosphorylated Pyk2 is described to activate ERK via different stimuli, i.e. IL2, PMA or MCSF (151, 193, 194) in different cell types (mast cells, monocytes, PBMCs).

The key role for the tyrosine kinase Pyk2 in ERK activation could be confirmed by means of siRNA mediated gene silencing of Pyk2. Knockdown of Pyk2 resulted in an inhibition of ERK phosphorylation and SOCS1 expression. Due to a relatively weak knockdown efficiency of about 50% it was difficult to attain a complete inhibition of SOCS1 or pERK.

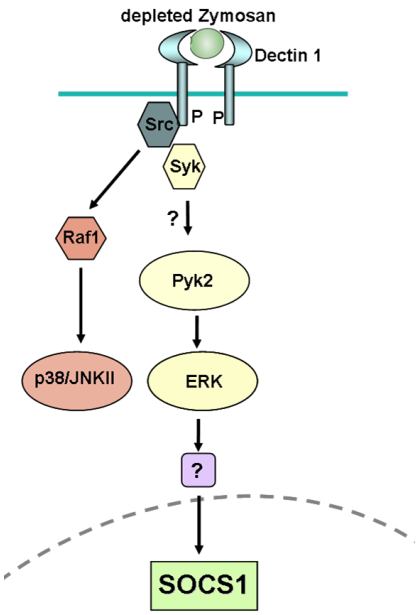

Fig. 5-1 Proposed SOCS1 induction pathway in macrophages upon stimulation of Dectin-1

Ligation of Dectin-1 by depleted Zymosan induces phosphorylation of the intracellular ITAM like motif. The tyrosine kinases of the Src family are recruited to the phosphorylated dimerized receptor. Thereupon, spleen tyrosine kinase (Syk) binds to active Src kinases and in turn gets phosphorylated. Proline-rich tyrosine kinase 2 (Pyk2) binds to Syk. Thereupon, Syk gets phosphorylated and induces ERK activation. Phosphorylated ERK induces SOCS1 expression. Src additionally activates Raf1 by phosphorylation (44, 46). Raf1 is activated by pSrc and induces phosphorylation of the MAPKs p38 and JNKII.

Our findings suggest the following SOCS1 induction pathway upon Dectin-1 stimulation (Fig. 5-1): Engagement of Dectin-1 via depleted Zymosan probably leads to dimerization of Dectin-1 monomers (as also depicted in Fig. 2-2) and subsequent cross-phosphorylation of intracellular ITAM-like motifs (195). Src kinases are recruited and activated and thereupon phosphorylate spleen tyrosine kinase (Syk). This is followed by phosphorylation and activation of Pyk2. MAPK ERK is stimulated and subsequently induces SOCS1 expression. ERK is described to activate various transcription factors upon stimulation, i.e. ATF1 or Fos, both of which are strongly induced by high levels of ERK activation (148, 205). As described in the following, the signalling pathway involved in SOCS1 induction in BMDCs emerged to differ from the situation in BMMs.

5.3.3. JNKII is involved in Dectin-1 mediated SOCS1 induction in BMDCs

As mentioned, BMDCs induced SOCS1 most likely through the transcription factor NF-κB. Thus, we analyzed possible candidates involved in SOCS1 expression in BMDCs. First, it was remarkable that these cells displayed less basal phosphorylation of Pyk2 as compared to BMMs; the phosphorylation status of Pyk2 might be cell type dependent and possibly also the downstream signalling cascades. Additionally, Pyk2 can be activated by stimuli that increase intracellular calcium levels. It is also activated in response to stress signals (such as TNFα or hyperosmotic shock), thereby inducing Jun N-terminal kinase (JNK) activation (151). Indeed, we observed calcium dependent activation of Pyk2 in BMDCs, since the application of inhibitors of the calcium signalling pathways abolished Pyk2 phosphorylation. In contrast, treatment with calcium inhibitors prior to stimulation with depleted Zymosan had no effect on Pyk2 activation in BMMs. The inhibition of CaMK and the chelation of Ca^{2+} as well as

blocking Pyk2 phosphorylation inhibited SOCS1 expression in BMDCs. Further, the observations within this study concerning downstream signalling in BMDCs represent another hint that NF-κB participates in SOCS1 induction. All utilized inhibitors (AG17, BAPTA and KN93) abrogated the phosphorylation of IκBα; thus NF-κB is suggested to be the main transcription factor for SOCS1 in these cells. As shown by different groups, Dectin-1 signals activated NF-κB directly in a CARD9/Bcl10 dependent manner (71, 75). Gross *et al.* demonstrated that CARD9-deficient BMDCs failed to produce TNFα upon stimulation with β-glucans (75). Further, Goodridge *et al.* could clearly show that depleted Zymosan stimulated BMDCs displayed a strong NF-κB binding activity in nuclear extracts as displayed by EMSA (71).

Regarding MAPK activation, JNKII phosphorylation was abrogated by all inhibitors, whereas none of the latter had an effect on pp38. Therefore, we suppose that SOCS1 induction in BMDCs is initiated by Pyk2, mediated via the MAPK JNKII and the transcription factor NF-κB. Worth mentioning is the observation that ERK activation was also abrogated by AG17 in BMDCs. Thus, ERK may somehow be involved in downstream signalling of Pyk2. Additionally, some groups could show that NF-κB can be activated in a MAPK dependent manner in dendritic cells (148), as depicted for pERK. JNKII MAPK is described to activate various different transcription factors, i.e. ATF family members or AP-1 (206). However, JNKII has not been associated with NF-κB activation till now.

5.4. Influence of Dectin-1 induced SOCS1 on the TLR9 pathway

Concerning the role of SOCS1 in the Dectin-1 pathway, we were not able to find evidence for an inhibitory role of SOCS1 as feedback inhibitor. Such a mode of action was proposed for SOCS1 regulation upon ligation of the CLR DC-SIGNR I (207). Innate immune cells (murine DCs and human PBMCs) were infected by *Mycobacterium tuberculosis,* a pathogenic ligand for both, Dectin-1 and DC-SIGNR1. Upon knockdown of SOCS1 via siRNA a significant reduction in *M. tuberculosis* CFU in both mouse DCs and human PBMCs was observed (207). In addition, DC-SIGNR1 bound to SOCS1 as shown by immunoprecipitation.

To circumvent lethality of SOCS1$^{-/-}$ mice, we used BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice to analyze a possible inhibitory potential of SOCS1 for Zymosan/Dectin-1 signalling. Since the severe phenotype and lethality of SOCS1 deficient mice mostly originates from an increased sensitivity to IFNγ, SOCS1 deficiency on an IFNγ$^{-/-}$ background counteracts the early onset of a multi-inflammatory syndrome. These mice survived the neonatal phase and displayed a reduced pathology (108, 111). First, we checked for activation of the kinases involved in SOCS1 induction in BMMs, namely Syk, Pyk2 and ERK. In cells from SOCS1$^{-/-}$/IFNγ$^{-/-}$ as well as IFNγ$^{-/-}$ mice, phosphorylation of the respective kinases could be observed. No differences in the phosphorylation profile of Syk, Pyk2 and ERK could be detected in SOCS1$^{-/-}$/IFNγ$^{-/-}$ cells as compared to IFNγ$^{-/-}$ control cells. Also, direct target genes of Dectin-1 like egr2 and IL10 were not differentially expressed in SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs as compared to IFNγ$^{-/-}$ control cells. Therefore, SOCS1 induced by Dectin-1 does not serve as an inhibitor on Dectin-1 signalling. Indeed, less is described about a possible inhibitory role of SOCS1 on JAK/STAT independent pathways. The main function of SOCS1 is described as an inhibitor of JAK/STAT signalling pathways.

Thus, a possible inhibitory potential of SOCS1 on other PRR signalling pathways, namely TLRs was analyzed. During an infection, fungi probably will stimulate TLRs (TLR2, TLR6, TLR9) in combination with C-type lectins (44, 51, 53, 55, 63). Therefore we speculated that SOCS1 might be important for cross-regulation of different receptors. Indeed, it has already been described that co-activation of Dectin-1 and TLRs changes the cells' cytokine profiles (55, 63). Co-ligation of Dectin-1 with β-glucans and TLR2 enhanced IL6 and IL23 production but downregulated IL12 expression. To address the question if also other TLR ligands differ in their cytokine secretion when they are administered together, we first used WT BMMs and co-stimulated them with different TLR ligands (Pam_3CSK_4, CpG-DNA, LPS, Poly(IC) and R848) and depleted Zymosan. We observed an inhibition of IL12 secretion, but an up-regulation in case of TNFα, IL10 or IL8 when Dectin-1 was stimulated parallel to TLR activation. Less is known about an influence of β-glucan on TLR9 signalling. It had been described that co-stimulation of human PBMCs or murine BMDCs by CpG-DNA and β-glucan induced collaborative TNF responses. TNF secretion was significantly increased upon co-stimulation as compared to CpG-DNA alone (55, 208). Additionally, Dectin-1 was described to bind to mycobacteria. Mycobacteria comprise a high CG-DNA content thereby being able to stimulate TLR9 (153, 154). Thus, we decided to focus on the possible properties of SOCS1 induced by depleted Zymosan on TLR9 signalling.

The inhibitory effect of depleted Zymosan on CpG-DNA induced IL12p40 was similar in BMMs from IFN$\gamma^{-/-}$ mice as compared to WT cells. However, in SOCS1$^{-/-}$/ IFN$\gamma^{-/-}$ cells, this inhibition was abrogated; secretion of IL12 was even enhanced by concomitant Dectin-1 stimulation as compared to CpG-DNA alone. In cells of WT mice, SOCS1 induction could clearly be enhanced when cells were co-treated with depleted Zymosan and CpG-DNA. From these results, we conclude that the inhibition of CpG-DNA induced IL12 by depleted Zymosan was dependent on SOCS1. In contrast, secretion of other cytokines like TNFα or IL8 was not modified in SOCS1$^{-/-}$/ IFN$\gamma^{-/-}$ cells. Similarly, ligation of Dectin-1 and TLR9 resulted in an up-regulation of IL10 production, as previously described for co-stimulation of BMDCs with Pam_3CSK_4 and β-glucans (63). The group of Dennehy *et al.* could show that co-stimulation of murine BMDCs with Pam_3CSK_4 markedly increased the secretion of IL10 in comparison to Pam_3CSK_4 stimulated cells (63).

Knockout of SOCS1 significantly increased the secretion of IL10 as compared to control cells. Thus, SOCS1 shapes the overall reaction pattern in situations where multiple, different PRRs, like TLRs and CLRs, are triggered in parallel (Fig. 5-2). Accordingly, the reciprocal regulation of distinct cytokines, i.e. IL12 and IL10 or IL6 in WT and IFN$\gamma^{-/-}$ control cells may explain how fungi regulate the production of these cytokines. The collaborative effects of Dectin-1 and TLR may provide a new approach for adjuvant development, as combinations of specific TLR ligands have been described as promising candidates for novel vaccines. It has been described that certain TLR-ligands can synergize with each other to enhance T-cell response through synergistic activation of DCs (209-211). It was shown by Zhu *et al.* that immunization with an HIV peptide vaccine in combination with the TLR-ligands MALP2, Poly(IC) and CpG induced a substantially higher response to viral load as compared to HIV peptide in combination with one TLR ligand alone (209). Additionally, a combination of TLR7/8 or TLR3 ligands resulted in a strong increase in secretion of pro-inflammatory

cytokines, i.e. IL12, and a more efficient $CD8^+$ Th-cell priming. Poly(IC) is already approved for clinical testing in humans, and can be used to mature DCs *ex vivo* prior to an administration *in vivo.* On the other hand, an inhibition of T-cell priming and cytokine production was observed when LPS and Poly(IC) or Zymosan and Poly(IC) were combined (210, 211).

The opposing regulation of TNF and IL12 or IL10 might be due to the discriminative characteristics of the binding of NF-κB subunits to their respective promoter regions. Delayed-type genes, i.e. IL12 and IL10 need a prolonged recruitment of NF-κB subunits to the promoter. These genes need a sustained binding of the transcription factor to their respective promoters and stimulus-dependent chromatin-modifications are required (139). In contrast to DCs, it is described that in macrophages, the transcription of IL12p40 and IL10 can be promoted by p50/p50 homodimers upon stimulation (158, 159). Analysis of interactions of NF-κB subunits (p65, p50, c-Rel and RelB) at the IL12p40 promoter *in vitro* revealed that p50/p50 homodimers and with an even higher affinity also p50/c-Rel heterodimers bind to the IL12p40 Rel site (159). In addition, it could be shown in macrophages that p50 is the onliest Rel family member to drive IL10 transcription upon stimulation with LPS (158).

Thus, in case of induction of the anti-inflammatory cytokine IL10, p50/p50 homodimers might play the major role, whereas IL12p40 is mainly transcribed by p50/cRel heterodimers (158, 159). This indicates a different regulation of pro-and anti-inflammatory cytokines by means of different NF-κB subunits. Importantly, Dectin-1 is the only known CLR to induce the non-canonical NF-κB pathway. In canonical NF-κB signalling, p105 is constitutively processed by the proteasom into active p50 but is maintained cytoplasmatically as a hetero- but also homo-dimer (with p65 or p50) by its interaction with inhibitory IκB proteins, such as IκBα (184, 185). In contrast, in the non-canonical NF-κB signaling pathway, p100 is processed to active p52 only when the pathway is activated (212, 213).

Therefore, we analyzed BMMs from $SOCS1^{-/-}/IFN\gamma^{-/-}$ as well as from the respective control ($IFN\gamma^{-/-}$ mice) for activation of NF-κB subunits. Indeed, activation of p50 and p52 was significantly up-regulated in $SOCS1^{-/-}/IFN\gamma^{-/-}$ cells co-stimulated with depleted Zymosan and CpG-DNA. It can be speculated that SOCS1 blocks activation of NF-κB p50 and p52 subunits when triggered via TLR9 and Dectin-1. It is published that SOCS1 induces degradation of bound p65 within the nucleus (112-115), most likely via its function as an E3 ubiquitin ligase (93, 94). Ryo *et al.* showed that SOCS1 associated with p65 and regulated its ubuquitin-mediated proteolysis (113). Mouse primary splenocytes were treated with LPS. After immunoprecipitation, p65 was shown to bind endogenous SOCS1. Further, it could be shown that SOCS1 was transported rapidly into the nucleus following stimulation. Thereupon, SOCS1 but not SOCS3 or CIS bound to p65 within the nuclear compartment. SOCS1 bound p65 is ubiquinated in a SOCS-box dependent manner (112).

Thus, we suggest that also other subunits, i.e. p50 and p52, could be possible targets of SOCS1 induced ubiquitination and degradation.

As mentioned, NF-κB dimers are maintained within the cytoplasm by their interaction with inhibitory IκB proteins. In TLR9/Dectin-1 co-stimulated $SOCS1^{-/-}/IFN\gamma^{-/-}$ cells an increase in IκBα phosphorylation as well as degradation was observed. The decomposition of IκBα

occurred at later time-points, compared to its phorphorylation. This could be explained by the fact that phosphorylation of IκB proteins targets the latter for degradation, thus, this event has to take place first (184, 185, 214). It has been described that SOCS1 is located within the nucleus as well as within the cytoplasm, as shown for SOCS1 transfected HEK cells (112). It might also interact with IκBα directly or with the bound NF-κB subunits as described for p65 (112, 114). Depleted Zymosan alone led to a subtle phosphorylation of IκBα when macrophages on IFNγ knockout background were used as opposed to WT macrophages. This might reflect subtle changes in the differentiation status of the cells and indeed missing NF-κB activation in macrophages by Zymosan has been discussed controversially (71, 215). On the one hand, it is described that Dectin-1 ligation was not able to trigger NF-κB dependent TNFα production in murine BMMs as well as thioglycollate-elicited peritoneal macrophages (71). Otherwise, Zymosan was capable of inducing NF-κB translocation into the nucleus and transcriptional activity within macrophages (215). This might indicate that only certain macrophage populations or cells at a particular differentiation status activate NF-κB transcription factors upon stimulation of Dectin-1.

We identified SOCS1 induced by depleted Zymosan to be a regulator of TLR9 signalling pathway, since it influenced TLR induced cytokine secretion (Fig. 5-2). IL12 secretion was decreased in IFNγ$^{-/-}$ BMMs in comparison to SOCS1$^{-/-}$/ IFNγ$^{-/-}$ BMMs. As IL12 is described to be the main cytokine responsible for the polarization of Th1-cells (124, 216), a decrease in IL12 may shift Th-cell response, i.e. towards Th17 cells (Fig. 5-2). Thus, SOCS1 expressed by Dectin-1 stimulation may secondary modulate Th-cell priming. Therefore, we analyzed Th-17 cell induction by SOCS1$^{-/-}$/ IFNγ$^{-/-}$ and IFNγ$^{-/-}$ BMMs upon co-stimulation of Dectin-1 and TLR9.

5.5. SOCS1 expressed in BMMs by Dectin-1 stimulation increases IL17 production of Th-cells

It has been shown that Dectin-1 when activated on dendritic cells is able to prime Th17 responses (44, 120, 125, 126). Upon interaction of activated DCs, i.e. by TLR ligands, with CD4^{+} Th-cells, the latter cells can differentiate into a variety of effector and regulatory subsets, including classical Th1- and Th2-cells, follicular helper Tcells, Tregs (regulatory Tcells) and Th17-cells (121, 122). The nature of the cytokines produced by APCs in response to various ligands defines the type of Th cell differentiation. For example, the production of IL23 along with IL6 and TGFβ leads to the generation of Th17-cells, while TGFβ in combination with IL2 induces regulatory T-cells (119-121, 217, 218). Dectin-1 signalling via Syk kinases primarily induces Th17 response (126). It was demonstrated that within WT DCs / WT CD4^{+} Th-cells, the stimulation of DCs with CpG-DNA induced a T-cell differentiation mainly towards the generation of IFNγ-producing Th1-cells, whereas the presence of curdlan as a Dectin-1 ligand led to the accumulation of IL17-producing Th17-cells (125). Additionally, stimulation of murine DCs via Dectin-1 led to the production of IL17 by CD4^{+}CD25^{+} regulatory T-cells (219). Isolated regulatory WT T-cells were cultured in presence of WT DCs that were stimulated via Dectin-1. Thereupon, an increase in IL17 producing cells could be observed (219). However, the effect of a combination of CpG-DNA and curdlan or another Dectin-1 ligand on T-cell differentiation has not been described.

Additionally, SOCS1 was linked to T-cell responses. T-cells from *Mycobacterium tuberculosis* infected mice displayed an increased association with the CD3 and CD28 T-cell receptors. Further, an increased association of SOCS1 with the IL12-receptor was observed in infected T-cells (207). The group of Takahashi *et al.* also described that SOCS1$^{-/-}$ Treg cells were easily converted into IFNγ producing Th1-like cells (160). Thus, SOCS1 plays an important role in T-cell differentiation.

Since it is shown in this study, that the overall reaction pattern in TLR9/Dectin-1 co-stimulated innate immune cells is regulated by SOCS1 we speculated that SOCS1 might also affect subsequent T cell priming. Therefore, we first of all analyzed SOCS1$^{-/-}$/IFNγ$^{-/-}$ BMMs as well as IFNγ$^{-/-}$ control cells for the secretion of IL6, IL23 and TGFβ. Interestingly all of these cytokines were markedly increased upon co-stimulation of TLR9 and Dectin-1 in IFNγ$^{-/-}$ cells, whereas in BMMs from SOCS1$^{-/-}$/IFNγ$^{-/-}$ mice the expression of IL6, IL23 and TGFβ was inhibited. In contrast to IL12p40, these cytokines were up-regulated in the control cells as compared to the SOCS1 knockout BMMs. Similarly, it has been demonstrated that IL12 suppressed IL23 and IL17 production in WT DC / WT Th-cells and, vice versa, IL23 and IL17 inhibited IL12 production by Th1-cells (126). This indicates a cross-regulation between the Th17 / IL23 and Th1 / IL12 pathway.

Thus, particular cytokines seem to be regulated differentially by SOCS1 in APCs. This in turn supposedly influences T-cell lineage commitment. We further show that BMMs or BMDCs triggered with depleted Zymosan and CpG-DNA in a co-culture setting induced IL17-producing Th-cells only in the presence of SOCS1. Additionally, we examined WT T-cells cultured together with BMMs derived from conditional SOCS1 knockout mice. These mice were deficient in SOCS1 only in cells from the myeloid lineage, especially macrophages. Only co-cultures with control BMMs and WT Tcells showed an elevated production of IL17 when APCs were stimulated with CpG-DNA and depleted Zymosan. RORγT (RAR related orphan receptor gamma) is the key transcription factor of Th17 cells. It renders T-cells susceptible for IL23 since it controls expression of the IL23 receptor and it amplifies and sustains the Th17 cell differentiation process (163, 164, 220). Thus, we also analyzed expression of this transcription factor in co-cultures of CD4^{+} WT T-cells that were either cultured together with BMMs from SOCS1 knockout mice on IFNγ knockout background or with BMMs from conditional SOCS1 knockout mice and the respective control. The results correlated with the findings observed for IL17; only T-cells cultured with control BMMs displayed increased induction of RORγ when BMMs were stimulated with CpG and depleted Zymosan or depleted Zymosan alone. Worth mentioning is the fact that within the co-stimulatory setting, no increase in RORγT expression as compared to stimulation with depleted Zymosan or CpG could be induced. This could be explained by the observation that also other transcription factors are responsible for the Th17 functions, i.e. STAT3 and RORalpha. It is shown that IL17 production is diminished but not abrogated in mice deficient of RORγT. A recent study reported that RORα could fulfil a similar function as RORγT (123, 164). In addition, STAT3 directly binds to IL17 promoter, indicating that STAT3 and RORγT cooperate (123, 221). Therefore, STAT3 or RORα transcription factors should be analyzed in T-cells co-cultured with myeloid cells that were triggered via TLR9 in combination with

Dectin-1. This would reveal if other transcription factors beside RORγT within the IL17 producing Th-cells are also regulated via SOCS1 produced upon co-stimulation of APCs.
We conclude that SOCS1 expressed upon Dectin-1 stimulation in myeloid cells plays an essential role in shifting T-cell responses towards IL17-producing Th-cells.

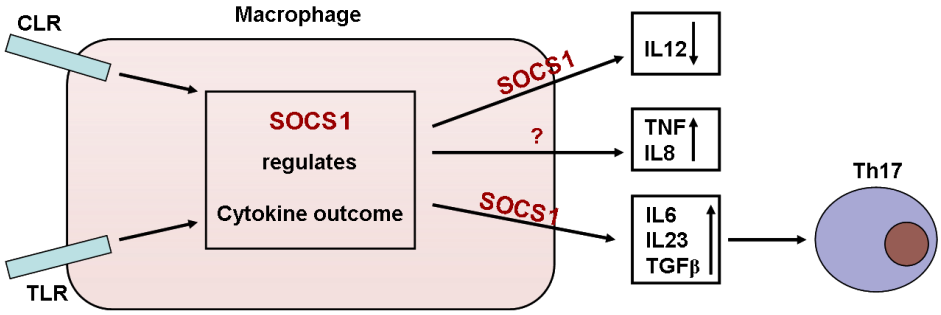

Fig. 5-2 Overview of the proposed Cytokine regulation in co-stimulated macrophages

Cytokines are regulated differentially upon co-stimulation of myeloid cells by TLR lignads, i.e. CpG-DNA and CLR ligands, i.e. depleted Zymosan. IL12 secretion is decreased upon co-stimulation, whereas TNF, IL8, IL6, IL23 and TGFβ production is increased. The regulation of IL12, IL6, IL23 and TGFβ is mediated by SOCS1 expressed upon ligation of CLR. Therefore, SOCS1 is supposedly involved in Th17-differentiation processes. On the other hand, SOCS1 does not participate in the increased IL8 and TNFα production.

Accordingly, the influence of SOCS1 expressed through Dectin-1 on the TLR 9 induced cytokine profile and subsequent T-cell response can be summarized as follows:
Upon an infection with microbial pathogens, not only one single PRR is engaged. Different PRRs are rather stimulated the same time to coordinate an immune response specific for the respective pathogens. Within this study, it could be shown that Dectin-1 and TLR9 differentially regulate T-cell response when co-stimulated on APCs. On the one hand, cytokine profile is shifted in co-stimulated cells when compared to CpG or depleted Zymosan triggered cells. IL12 secretion was decreased, whereas the production of IL6, IL23 and TGFβ was increased in the presence of SOCS1. On the other hand, $CD4^+$ Th-cell response was influnced by SOCS1 in co-stimulated APCs. IL17 production of $CD4^+$ Th-cells was increased when APCs isolated from $IFN\gamma^{-/-}$ were co-stimulated by depleted Zymosan and CpG-DNA as compared APCs stimulated with the single ligands. In contrast, $IFN\gamma^{-/-}/SOCS1^{-/-}$ APCs were not able to increase IL17 production in a co-stimulatory setting. Therefore, SOCS1 produced within dendritic cells or macrophages is an essential regulator of T-cell responses when multiple PRRs are engaged the same time, i.e. during fungal infection.

5.6. Outlook

This study demonstrates that SOCS1 is induced in BMMs and also BMDCs upon Dectin-1 stimulation in a direct manner without the contribution of auto- or paracrine factors or any intracellular intermediates. Pyk2 and ERK were mandatory for SOCS1 expression in BMMs, whereas JNKII and NF-κB were involved in SOCS1 transcriptional control in BMDCs. SOCS1 did not act as a direct feedback inhibitor, but cross-regulated TLR signalling by inhibiting IL12 expression. As a consequence SOCS1 regulated the activation profile of innate immune cells including subsequent stimulation of adaptive immunity. The contribution of SOCS1 expressed in BMMs and BMDCs to the induction of IL17 producing Th-cells represents an important link between innate and adaptive immune responses.

However, important questions remain to be solved. On the one hand, the transcription factor leading to the induction of SOCS1 in BMMs has not been identified. Thus, one should analyze possible candidates, i.e. AP1 that is shown to be activated via pERK. Further, the upstream pathway leading to activation of Pyk2 and thereupon ERK remains unclear. Syk is activated and responsible for SOCS1 induction. In addition, CARD9 is one important mediator of Syk downstream pathways. Thus, BMMs and BMDCs from CARD9 knockout mice could be examined to figure out if this adaptor protein is involved in the signalling pathway presented here. Importantly, a comparison of the Dectin-1 ligand depleted Zymosan and a live organism, i.e. *Candida albicans,* has to be carried out in order to examine an organism that binds to different PRRs beside Dectin-1.

Additionally, another important step would be to analyze IL17 producing T-cells in a co-culture setting with BMMs or BMDCs isolated form SOCS1 knockout or control mice and incubated with fungi like *Candida albicans.* In this context, the possible role of Th1 or Th2 cells has to be further analyzed. To evaluate the contribution of SOCS1 to the IL17 pathway *in vivo,* it is necessary to set up an infection model were conditional SOCS1 knockout mice are infected with different pathogenic strains of *Candida albicans*. These mice should then be assessed for survival, fungal growth and parameters of inflammatory and adaptive Th1/Th17 immunity to analyze if SOCS1 is relevant in Th-cell response *in vivo.* Additionally, the IL17 producing Th17 cells should be examined further in detail by means of intracellular FACS analysis.

Concerning the downstream signalling leading to the induction of SOCS1 in myeloid cells, BMDCs have to be further analyzed to find out if it is possible that Raf1 in this case could play a more prominent role in SOCS1 induction.

Thus, different cell types activate transcription of SOCS1 via different kinases.

6. Bibliography

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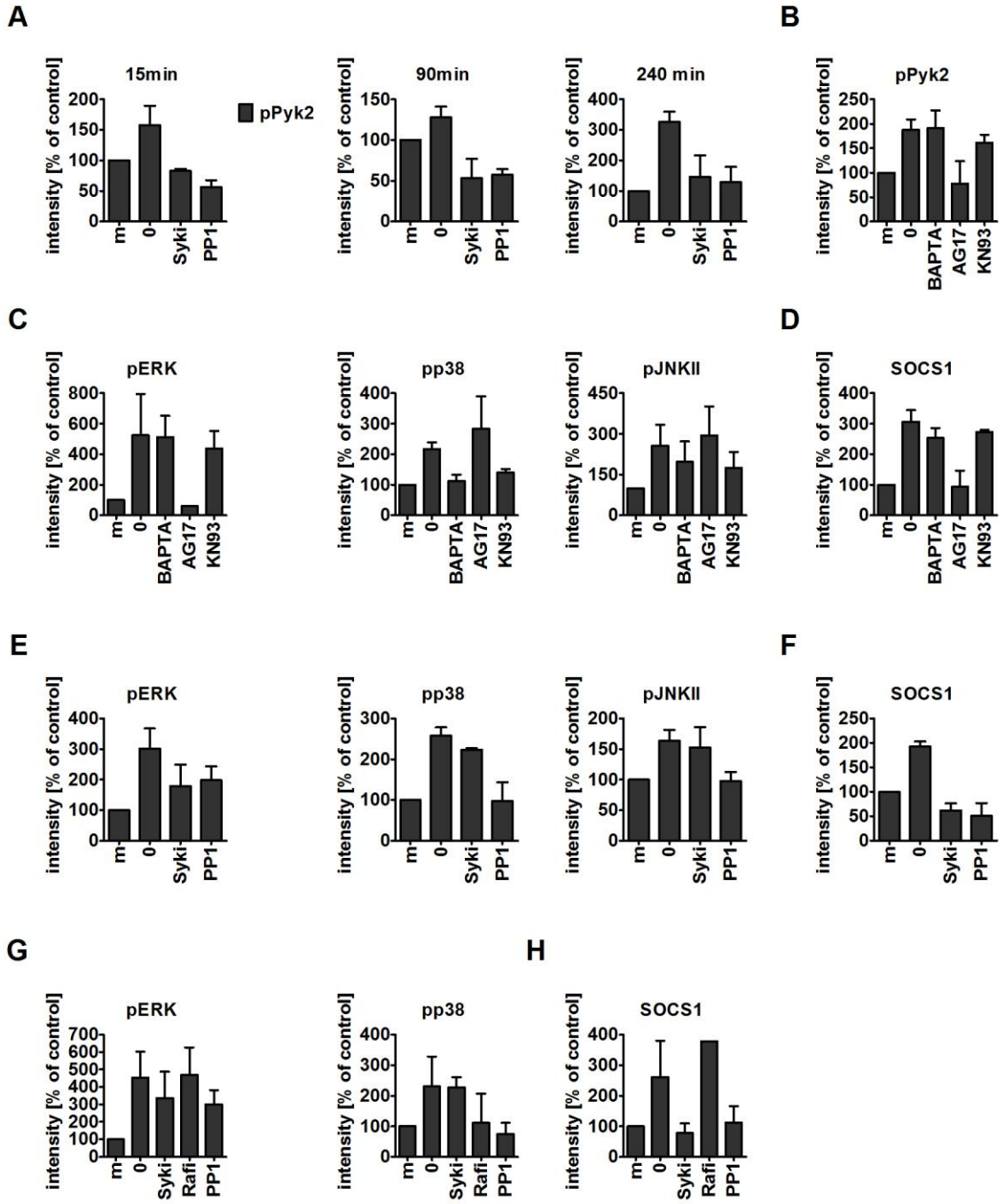
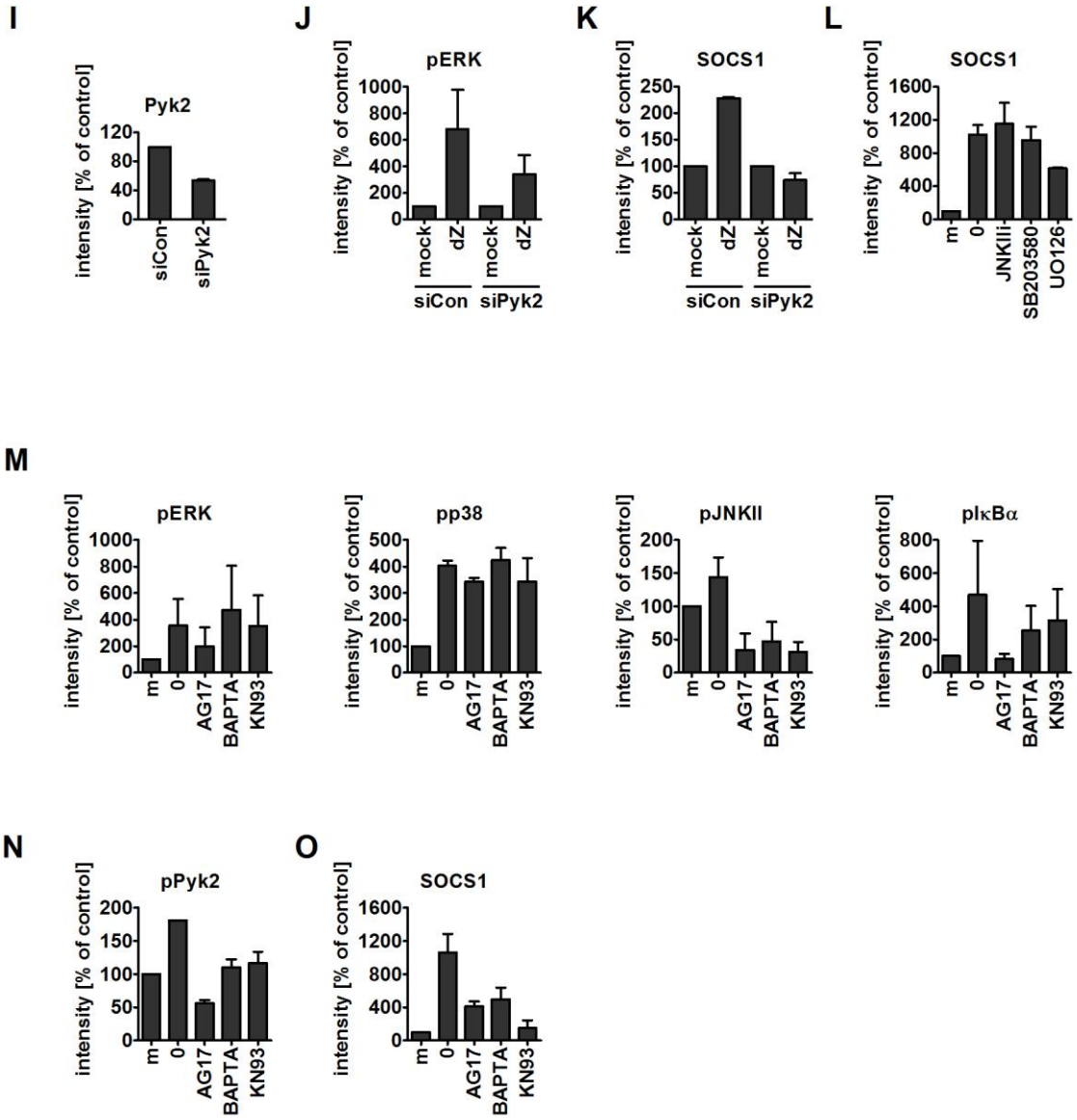
7. Appendix

Quantitative Analysis of western blots from Figure 4-12 to Figure 4-18. Data were quantified by densitometry and are depicted as percentage of unstimulated (mock) control. Mock was set 100%. Fig. 4-12: A, B; Fig. 4-13: C, D; Fig. 4-14: E, F; Fig. 4-15: G, H; Fig. 4-16: I, J, K; Fig. 4-17: L; Fig. 4-18: M, N, O.




I
Pyk2
intensity [% of control]
siCon
siPyk2
J
pERK
mock
dZ
siCon
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K
SOCS1
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0
JNKIi
SB203580
UO126
M
AG17
BAPTA
KN93
pp38
pJNKII
pIκBα
N
pPyk2
O

8. Publications and Presentations

8.1. Publications

Eberle ME, Dalpke, AH.

Dectin-1 stimulation induces suppressor of cytokine signalling 1 (SOCS1) thereby modulating TLR signalling and T-cell responses.

J Immunol. 2012, accepted

Gehrig S, **Eberle ME**, Botschen F, Rimbach K, Eberle F, Eigenbrod T, Kaiser S, Holmes WM, Erdmann VA, Sprinzl M, Bec G, Keith G, Dalpke AH, Helm M.

Identification of modifications in microbial, native tRNA that suppress immunostimulatory activity.

J Ex Med. 2012 Feb 13; 209(2):225-33

Stutz A, Kessler H, **Kaschel ME**, Meissner M, Dalpke AH.

Cell invasion and strain dependent induction of suppressor of cytokine signaling-1 by Toxoplasma gondii.

Immunobiology. 2012 Jan;217(1):28-36

Kurz J, Eberle F, Graumann T, **Kaschel ME**, Sähr A, Neumann F, Dalpke AH, Erdinger L.

Inactivation of LPS and RNase A on photocatalytically active surfaces.

Chemosphere. 2011 Aug;84(9):1188-93

8.2. Presentations

Talk: Induction and properties of SOCS1 in myeloid cells by activation of Dectin-1.

Mariel-Esther Eberle and Alexander Dalpke.

Mini-Symposium “Crossroads in immune signaling”, Heidelberg, Germany, November 2011

Poster: Induction of Suppressor of Cytokine Signaling (SOCS) molecules via Dectin-1 in macrophages and dendritic cells.

Mariel-Esther Kaschel and Alexander Dalpke.

Toll-meeting 2011 “Decoding innate immunity”, Riva del Garda, Italy, May 2011

Poster: Induction of Suppressor of Cytokine Signaling (SOCS) molecules via Dectin-1 in macrophages and dendritic cells.

Mariel-Esther Kaschel and Alexander Dalpke.

40th Annual meeting oft he Deutsche Gesellschaft für Immunologie (DGFI) Leipzig, Germany, September 2010

Poster: Induction of Suppressor of Cytokine Signaling (SOCS) molecules via Dectin-1 in macrophages and dendritic cells.

Mariel-Esther Kaschel, Ann-Kathrin Reuschl and Alexander Dalpke.

Spring School of Immunology, Ettal, Germany, February 2010

Poster: Induction of Suppressor of Cytokine Signaling (SOCS) molecules via Dectin-1 in macrophages and dendritic cells.

Mariel-Esther Kaschel, Ann-Kathrin Reuschl and Alexander Dalpke.

13th Joint Meeting of the Signal Transduction Society (STS), Weimar, Germany, November 2009

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