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The MAPK phosphatase DUSP1: An IL-10-induced negative regulator of macrophage activation

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Abbreviations

°C	Centigrade
µFD	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar
AP-1	Activator protein 1
APC	Antigen-presenting cell
APS	Ammonium persulphat
ARE	AU-rich element
ATF-2	Activating transcription factor 2
ATP	Adenosine tri-phosphate
B-ATF	Basic leucine zipper transcription factor ATF-like
BCL-3	B-cell lymphoma 3
BCR	B cell receptor
BMM	Bone marrow-derived macrophage
bp	Base pair
BSA	Bovine serum albumin
CASP	Colon ascendens stent peritonitis
CD	Cluster of differentiation
cDNA	Complementary DNA
CHX	Cycloheximide
CNS	Central nervous system
CpG	Cytosine-guanosine oligonucleotide
CTL	Cytotoxic T cells
CTP	Cytosine tri-phosphate
d	Days
DC	Dendritic cell
DD	Death domain
ddH2O	Double distilled water
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri-phosphate
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
DUSP	Dual-specificity phosphatase
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EHV2	Equine herpes virus type 2
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
ESC	Embryonic stem cells
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Foetal bovine serum
fw	Forward
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIPL	Glycoinositolphospholipid
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSK	Glycogen synthase kinase
h	Hours
HET	Heterozygous
HRP	Horseradish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
IFN	Interferon
IKK	Inhibitor of κB kinase
IL	Interleukin
IL-10R	IL-10 receptor
IL-18BP	IL-18 binding protein
IL-1Ra	IL-1 receptor antagonist
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
IVT	*In vitro* transcription
JAK	Janus kinase

JNK	C-JUN N-terminal kinase
kb	Kilobase
kDa	Kilodalton
KO	Knockout
l	Litre
LCCM	L-cell-conditioned medium
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LXR	Liver X receptors
mA	Milliampere
MAL	MyD88-adaptor-like
MALP	Macrophage-activating lipopeptide
MAPK	Mitogen-activated protein kinase
MAPKAPK2	MAPK-activated protein kinase 2
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBq	Megabecquerel
MCP-1	Monocytes chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
min	Minutes
MK2	MAPK-activated protein kinase 2
MKB	MAPK binding domain
MKP	MAPK phosphatase
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	messenger RNA
mV	Millivolt
MyD88	Myeloid differentiation primary response Gene 88
NFIL3	Nuclear factor interleukin 3-regulated
NF-κB	Nuclear factor-κB
ng	Nanogram
nm	Nanometre

NOD	Nucleotide oligomerization domain
NP-40	Nonidet P-40
NT	N-terminal
ODN	Oligodeoxynucleotide
PAGE	Polyacrylamid gel electrophoresis
Pam3Cys	Tripalmitoyl-S-glyceryl cysteine
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEST	Domain rich in prolins, glutamates, serines and threonines
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PTP	Protein tyrosine phosphatase
rev	Reverse
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
rpm	Rounds per minute
RSK	Ribosomal protein S6 kinase
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
s	Seconds
SD	Standard deviation
SDS	Sodiumdodecylsulfate
SH2	Src homology 2 domain
SHIP	SH2 containing inositol phosphotase
SIGIRR	Single immunoglobulin IL-1R-related protein
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
sTLR	Soluble TLR

TAB	TGF-β binding protein
TAK	Transforming growth factor-β-activated kinase 1
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	N, N, N', N'-Tetramethylethyleneamine
TGF	Transforming growth factor
Th	T helper
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor molecule
TLR	Toll-like receptor
TMB	Tetramethylbezine
TNF-α	Tumor necrosis factor-α
TOLLIP	Toll-interacting protein
TRAF	Tumor necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TREM	Triggering receptor expressed on myeloid cells
TRIF	TIR domain-containing adaptor inducing interferon-β
TYK	Tyrosine kinase
UTR	Untranslated region
V	Volt
WT	Wild-type

1 Introduction

1.1 Innate and adaptive immunity

The most important function of the immune system is the protection of the body from a wide variety of pathogenic infectious agents with vastly differing natures, i.e. viruses, bacteria, fungi, protozoa and parasitic worms. This protection is mainly accomplished by the interplay of two closely linked systems, the innate immunity that is rather unspecific and can also be found in invertebrates and the antigen-specific adaptive immunity of higher vertebrates with its specific immunological memory (Fearon and Locksley, 1996).

The innate immune system is the first line of host response to pathogen invasion. It consists of cellular components like macrophages, monocytes, dendritic cells (DCs), natural killer (NK) cells, neutrophils and mast cells, but also of soluble factors as for example the complement system or defensins (Ganz, 2002; Janeway and Medzhitov, 2002). Whereas some cells can kill infected cells, like NK cells, others can phagocytose pathogens and are crucial for presentation of foreign antigen to cells of the adaptive immune system (Medzhitov and Janeway, 1998). The recognition of pathogens by cells of the innate immune system depends on germ line–encoded receptors that have evolved to recognize highly conserved pathogen-associated molecular patterns (PAMPs) therefore they have been termed pattern recognition receptors (PRRs). Examples are the membrane-bound toll-like receptors (TLRs), the intracellular nucleotide oligomerization domain (NOD) proteins or soluble factors like pentraxins and collectines (Basset et al., 2003). Signalling through these receptors induces the expression of a variety of immune-response genes, including cytokines that promote inflammation and help to activate adaptive immune response (Medzhitov et al., 1997).

Compared to the innate, the adaptive immunity responds rather late, but more specific and therefore more powerful to infections by pathogens. Adaptive immunity is based on clonal selection (Burnet, 1959) from a repertoire of cells, namely lymphocytes, bearing highly diverse antigen-specific receptors that enable the immune system to recognize any foreign antigen. The two main effector cells of the adaptive immune system are the B and T cells. When a B cell recognizes its target antigen by the B cell receptor (BCR) it is internalized and processed. Subsequently, the B cells move to specialized regions of the secondary lymphatic organs where they undergo differentiation and proliferation to become antibody producing plasma cells, a process called clonal expansion (Janeway et al., 2001). After secretion and binding to their specific antigens, these antibodies perform various effector functions,

including neutralizing antigens, activating complement, and promoting phagocytosis and destruction of microbes (Abbas and Lichtman, 2004). The other main branch of adaptive immunity is composed of the T cells. Depending on the expression of their co-receptor, they can be subdivided into two major functional subsets, the $CD4^+$ T helper (Th) cells and the $CD8^+$ cytotoxic T cells (CTLs). Unlike B cells, T cells are not capable of recognizing free antigens, instead they depend on other cells that process foreign proteins and present the resulting peptide fragments on self major histocompatibility complex (MHC) molecules on their surface where T cells with the appropriate antigen-specific T cell receptor (TCR) can bind to this receptor-peptide complex. Two major classes of MHC molecules exist. MHC class I molecules can be found on all nucleated cells, bind peptides derived from cytosolic proteins, and are recognized by CTLs. Class II molecules are expressed exclusively by professional antigen-presenting cells (APCs), like dendritic cells and macrophages. They bind peptides derived from endocytosed proteins, and are recognized by Th cells. In addition to the recognition of the antigen alone, a second signal through co-stimulatory molecules is necessary for activation and differentiation of the naïve T cell (Abbas and Lichtman, 2004). Once T cells have received both signals they expand and differentiate into effector T cells. The main function of fully activated CTLs is the killing of infected target cells by their lytic activity. Based on the type of antigen and the predominant cytokines, T helper cells differentiate into two major subtypes of cells known as Th1 and Th2 cells (Rogers et al., 1998). These subtypes are defined on the basis of the specific cytokines they produce. Th1 cells produce mainly interferon-gamma (IFN-γ) (O'Garra and Murphy, 1996), which acts on macrophages and B cells, stimulating phagocyte-mediated defence against infections, especially with intracellular pathogens. Th2 cells on the other hand produce interleukin (IL)-4, IL-5, IL-10 and IL-13 (Medzhitov and Janeway, 1998; Morel and Oriss, 1998), cytokines important to fight infections with parasites and for the down-regulation of Th1 responses. Specific elimination of pathogens is not the only important function of the adaptive immune system, it also generates differentiated memory cells (Metchnikoff, 1905; Carroll and Prodeus, 1998), which allow a more rapid and effective response upon re-infection.

1.2 Toll-like receptors

One main mechanism by which the innate immune system senses the invasion of pathogens is through Toll-like receptors (TLRs) (Akira and Takeda, 2004). The founding member of this receptor family is the *Drosophila melanogaster* protein Toll. It was initially identified as

being important for the establishment of dorso-ventral polarity during embryogenesis (Hashimoto et al., 1988). Only later, studies revealed its additional role for an effective immune response against fungal infection in adult flies (Lemaitre et al., 1996). The year after the discovery of the function of Drosophila Toll in immunity, the first mammalian Toll homologue, namely TLR4, was identified (Medzhitov et al., 1997). It could be shown that lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, is the natural ligand of TLR4, definitively linking it to innate immune responses (Poltorak et al., 1998). After this hallmark finding, more TLRs were identified by database searches, and thus far, 13 mammalian TLRs, 13 in mice and 10 in humans are known (Beutler, 2004). TLRs 1-9 are conserved between mice and humans, whereas TLR10 can be found exclusively in humans and TLR11 is only functionally active in mice (Chuang and Ulevitch, 2001; Zhang et al., 2004). Moreover, the biological role of TLRs 10, 12, and 13 still needs to be identified.

All TLRs are type 1 transmembrane receptors bearing an extracellular leucine-rich repeat (LRR) domain and a conserved intracellular Toll/IL-1R gene homology (TIR) domain. It is thought that the LRRs are directly involved in the recognition of the respective ligands of the TLRs, also known as pathogen-associated molecular patterns (PAMPs) (Akira and Takeda, 2004). PAMPs are conserved molecular motifs predominantly found in a wide range of microorganisms, including structural molecules like LPS, flagellin or double-stranded RNA (Figure 1). Additionally to these exogenous ligands, recent studies suggest that TLRs can also sense endogenous non-pathogenic molecules, derived from dead or dying cells (Guillot et al., 2002; Termeer et al., 2002; Vabulas et al., 2002). According to the “danger model” of immune activation the recognition of such self-molecules by TLRs is necessary to fully induce an immune response (Matzinger, 2002). Most TLRs are widely expressed in different tissues and cell types, including non-haematopoietic epithelial and endothelial cells (Hopkins and Sriskandan, 2005). Whereas many cell types express only a subset of TLRs, macrophages, dendritic cells and neutrophils as the main sentinel cells of the innate immune system express most of them. TLR distribution differs not only among different types of cells, but also on the cell itself. TLR1, 2, 4, 5 and 6 are expressed at the cell membrane, TLR3, 7, 8 and 9 however, can be found in intracellular compartments, like the endosomes.

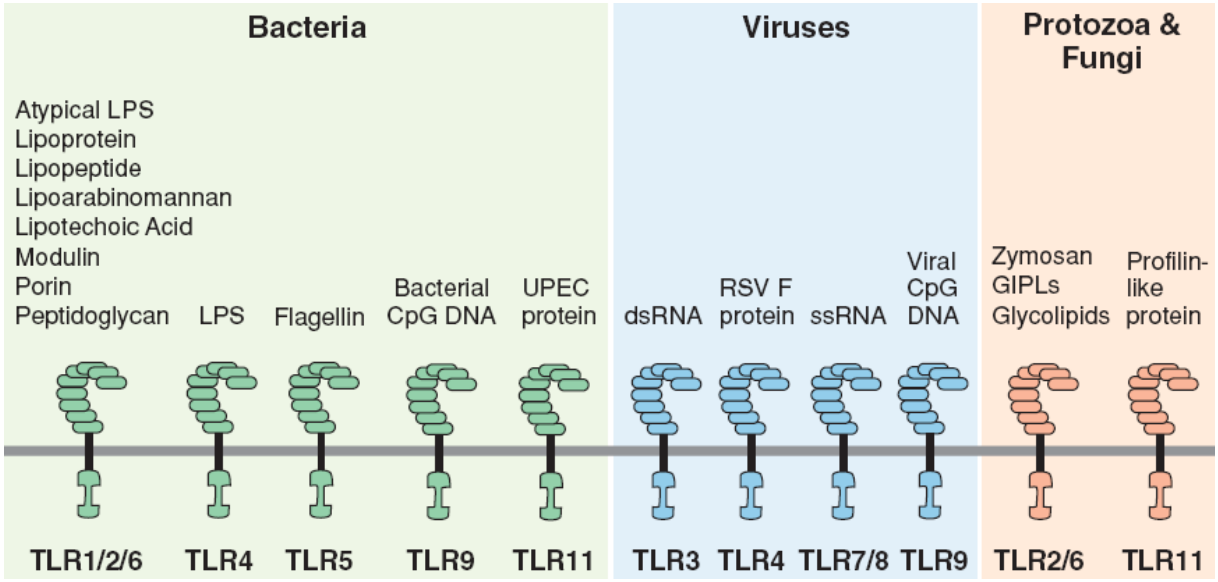


Figure 1. TLR ligand specificities.
TLRs recognize a diverse array of pathogen-derived products. Bacteria can be sensed by six TLRs. Heterodimeric TLR2/1 binds triacyl lipopeptides, whereas TLR2/6 dimers bind diacyl lipopeptides, lipoteichoic acid and glycoinositolphospholipids. TLR2 homodimers bind atypical LPS and peptidoglycan. Furthermore, TLR4 binds LPS, TLR5 binds flagellin, and TLR9 binds CpG motifs. TLR11 is expressed only in mice and senses uropathogenic bacteria. Viral dsRNA, RSV F protein, ssRNA, and unmethylated CpG motifs are recognized by TLRs 3, 4, 7/8, and 9, respectively. For antifungal responses, a TLR2/6 dimer senses zymosan, as well as glycolipids and glycoproteins from *Trypanosoma cruzi*. Finally, TLR11 can also recognize profilin-like protein from *Toxoplasma gondii* (adapted from West et al., 2006).

1.2.1 TLR signalling pathways

The recognition of PAMPs leads to the activation of downstream signalling cascades, ultimately resulting in the expression of multitudinous cytokines and chemokines as well as up-regulation of co-stimulatory molecules necessary for the activation of the adaptive immune response. TLRs dimerize after binding of their ligand. Albeit the majority of TLRs form homodimers, TLR2 can function as heterodimer with TLR1 or TLR6, each dimer responding to different ligands (Ozinsky et al., 2000). Signal transduction by the TLRs is achieved through the recruitment of adaptor molecules to the cytoplasmic region of the receptor containing the TIR domains (Takeda and Akira, 2005). Different TLRs use different adaptor molecules to propagate a signal, leading to different expression profiles. For example, activation of TLR3 and 4 induces a set of cytokines known as type I interferons (IFNs), whereas TLR2 and 5 do not. Although TLR7, 8 and 9 also induce type I interferons, the employed pathway is distinct from TRL3 and 4. To date, four TIR domain-containing adaptors are known, namely myeloid differentiation primary response Gene 88 (MyD88), TIR domain-containing adaptor molecule (TIRAP) also called MyD88-adaptor-like (MAL), TIR domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule

(TRAM) (Athman and Philpott, 2004; Yamamoto et al., 2004). The different signalling cascades are divided into MyD88-dependent and MyD88-independent pathways (Takeda and Akira, 2005; West et al., 2006).

1.2.1.1 MyD88-dependent pathways

MyD88 was initially identified as myeloid differentiation primary response gene induced by IL-6 in $M1D^+$ myeloid precursors (Lord et al., 1990). The encoded protein consists of a C-terminal TIR domain that can interact with the TIR domain of TLRs and an N-terminal death domain (DD), which binds to the death domain of the interleukin-1 receptor-associated kinase (IRAK) family (Muzio et al., 1997). Upon stimulation, MyD88 binds to the TLR and then recruits IRAK4 to this complex through interaction of the death domains of both molecules. IRAK4 recruits and phosphorylates IRAK1, which in turn auto-phosphorylates itself before associating with tumor necrosis factor receptor-associated factor 6 (TRAF6) (Cao et al., 1996; Arch et al., 1998). Next, IRAK1 and TRAF6 dissociate from this complex and form another complex composed of the transforming growth factor-β-activated kinase 1 (TAK1), the two adaptor proteins TGF-β binding protein (TAB) 1 and TAB2, and the ubiquitin-conjugating enzymes UBC13 and UEV1A (Shibuya et al., 1996; Deng et al., 2000; Takaesu et al., 2000; Chen, 2005). TAK1 belongs to the family of mitogen-activated protein kinase kinase kinases (MAPKKKs) and its phosphorylation initiates activation of two distinct signalling pathways. First, TAK1 activates the inhibitor of κB kinase (IKK) complex, leading to nuclear factor-κB (NF-κB) activation and translocation to the nucleus. Second, TAK1 is able to phosphorylate mitogen-activated protein kinase kinases (MAPKKs), which in turn phosphorylate mitogen-activated kinases (MAPKs) p38 and c-JUN N-terminal kinase (JNK) leading to activation of transcription factors like activator protein 1 (AP-1) and activating transcription factor 2 (ATF-2) (Wang et al., 2001). Both pathways ultimately result in the transcription of pro-inflammatory chemokines and cytokines like tumor necrosis factor-α (TNF-α), IL-6, IL-1β, IL-12 and CXCL8 (Hayden and Ghosh, 2004; Takeda and Akira, 2005). If MyD88 is absent, as in MyD88-deficient mice, the production of these cytokines after stimulation with the different TLR ligands is abolished, demonstrating that MyD88 is essential for inflammatory cytokine production through all TLRs, except TLR3 (Kawai et al., 1999).

1.2.1.2 MyD88-independent pathways

Even though activation of MAPK and NF-κB through most TLRs is impeded in MyD88-deficient cells, they can still be activated by TLR4 after binding of LPS, albeit with decreased kinetics (Kawai et al., 1999). Moreover, up-regulation of co-stimulatory molecules in

MyD88-deficient dendritic cells is normal as well as production of IFN-β after stimulation of TLR3 and 4 (Kaisho et al., 2001). These observations suggested the existence of pathways independent of MyD88 and led to the identification of additional adaptor proteins. TIRAP is an alternative adaptor specific for TLR2 and 4 that acts rather in combination with than independent of MyD88 (Yamamoto et al., 2002). The adaptor responsible for the MyD88-independent pathway, however, is TRIF. Evidence for this purpose was provided by the generation of TRIF-mutant mice that are defective in IFN-β production (Hoebe et al., 2003; Yamamoto et al., 2003). The current model for the MyD88-independent pathway suggests that stimulation of TLR3 or 4 recruits TRIF to their TIR domains resulting in the activation of the transcription factor interferon regulatory factor (IRF)-3 *via* an IKK-like kinase termed TBK-1 (Fitzgerald et al., 2003) and the delayed-phase NF-κB activation. IRF-3 induces transcription of IFN-β, which can itself induce several IFN-inducible genes, like CXCL10, GARG-16, or IRG-1 leading to an effective antiviral response (Kawai et al., 2001; Toshchakov et al., 2002; Yamamoto et al., 2002; Oshiumi et al., 2003).

1.2.2 Negative regulation of TLR signalling

The recognition of invading pathogens by TLRs is essential for mounting an effective immune response, however, the production of pro-inflammatory cytokines and chemokines can be a double-edged sword for the host. Excessive or uncontrolled responses can lead to hyper-inflammatory conditions like bacterial sepsis, chronic inflammatory diseases and autoimmune disorders. Therefore, it is not surprising that organisms have evolved mechanisms to modulate TLR-induced immune responses in order to maintain the immunological balance (Liew et al., 2005).

Several regulatory molecules acting on multiple levels of TLR signalling have been identified so far. The most direct attenuation is achieved by soluble decoy receptors. Until now, soluble receptors for TLR2 (sTLR2) and TLR4 (sTLR4) have been identified (Iwami et al., 2000; LeBouder et al., 2003). The exact mechanisms are yet not fully understood, but most likely the soluble forms block the interaction of the TLRs and their co-receptors, especially MD2 and CD14, resulting in the termination of TLR2 and TLR4 signalling, respectively. Other negative regulators interfere intracellularly with the TLR signal cascades. For example, IRAK-M is up-regulated upon stimulation of TLRs in monocytes and macrophages, indicating a negative feedback loop (Wesche et al., 1999). Although IRAK-M lacks the typical kinase activity of other family members, it prevents the formation of the IRAK1-TRAF6 complex by inhibiting dissociation of the IRAK1-IRAK4 complex from MyD88,

which results in the interruption of signalling to NF-κB and MAPK (Kobayashi et al., 2002). Suppressor of cytokine signalling (SOCS) 1 is a negative regulatory molecule of cytokine signalling (Alexander and Hilton, 2004). However, whether SOCS1 exerts a direct effect on TLR signalling is still controversial, but one potential mechanism might be the inhibition of IRAK1 activation (Kinjyo et al., 2002; Nakagawa et al., 2002; Baetz et al., 2004; Gingras et al., 2004). Other negative regulators are the adaptor molecule Toll-interacting protein (TOLLIP), which also inhibits IRAK1 activation, probably by decreasing its autophosphorylation (Zhang and Ghosh, 2002) and MyD88s a splice variant of MyD88 that is unable to bind to IRAK4 and to promote phosphorylation of IRAK1 (Burns et al., 2003). Finally, transmembrane proteins were also implicated in modulating TLR signalling. Examples are the orphan receptors single immunoglobulin IL-1R-related protein (SIGIRR) and ST2. Amongst others, they are recruited to TLR4 where SIGIRR sequesters IRAK1 and TRAF6, whereas ST2 sequesters MyD88 and TIRAP, thereby inhibiting proper signal transduction (Wald et al., 2003; Brint et al., 2004).

Of course not all TLRs are affected in the same way and magnitude by the different molecules, but the interplay of these regulators provides a potent mechanism to balance the TLR-mediated immune activation in order to avoid detrimental and inappropriate inflammatory responses.

1.3 Function and activation of macrophages

Macrophages originate from haematopoietic stem cells in the bone marrow. The differentiation of these common myeloid precursors into macrophages largely depends on cytokines like macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Lopez et al., 1992; Hamilton, 1993), but also on interactions with the stroma cells of the haematopoietic organs. Other important factors for the development of macrophages are IL-3, the tyrosine kinase KIT, molecules of the TNF family, TNF-receptor-related proteins, as well as transcription factors like PU.1 and other members of the ETS-family (Scott et al., 1994; McKercher et al., 1996; Valledor et al., 1998). The myeloid precursors develop into monocytes, which circulate in the bloodstream. From there, they migrate into the peripheral tissues and differentiate into specific tissue macrophages (Figure 2). The different macrophage population, like Kupfer cells (liver), osteoclasts (bones) or alveolar macrophages (lung), adapt their phenotypes to the respective environment (Gordon, 2003; Gordon and Taylor, 2005). Signals from surface as well as

secretory proteins of neighbouring cells and the extracellular matrix are responsible for these different phenotypes. The migration of the cells from the blood, and through the endothelial and epithelial barriers is controlled by adhesion molecules. These include β_1 and β_2 integrins, epidermal growth factor seven-transmembrane spanning (EGF-TM7)-type receptors, selectins and immunoglobulin-superfamily molecules such as CD31. Moreover, cytokines like transforming growth factor-β (TGF-β), chemokines and tissue-specific growth factors also influence the development of specific macrophage population.

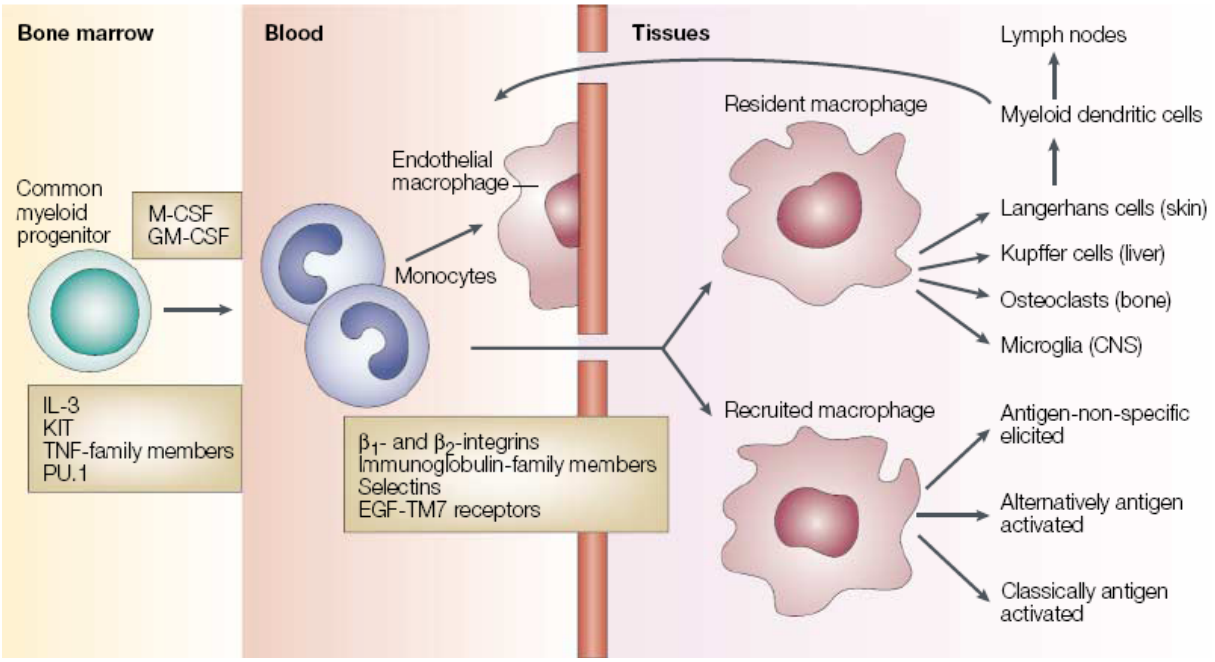


Figure 2. Differentiation, distribution and activation of macrophages *in vivo.*
(from Gordon, 2003)

The main function of macrophages is the endocytosis and phagocytosis of pathogens and cellular debris, thereby allowing its clearance from the body, its intracellular degradation, and processing for antigen presentation to T cells (Griffin et al., 1975; Unanue and Allen, 1987). Macrophages possess various membrane receptors that can bind opsonized or non-opsonized pathogens and dying cells. Examples are Fc receptors, lectins and scavenger receptors. As described above, TLRs can also recognize pathogenic compounds, but are unable to initiate phagocytosis. The pathogens are ingested into phagosomes, which fuse with lysosomes, where the pathogens are killed by lysosomal enzymes, reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) produced in these phagolysosomes. Macrophages also play a role in wound healing and in the regulation of haemo- and lymphopoiesis (Leibovich and Wiseman, 1988). Furthermore, activated macrophages secrete a number of

important proteins central to development of immune responses, including a collection of pro- and anti-inflammatory cytokines, such as IL-1, TNF-α, IL-6 and IL-10. But activation of macrophages is not only important for the production of these proteins, it also enhances the processing and presentation of antigens, phagocytosis and the production of antimicrobial and cytotoxic substances. Figure 3 illustrates the various possibilities of macrophage activation that can be induced by a number of exogenous and endogenous stimuli.

Innate activation (Figure 3a) is mediated by microbial stimuli recognized by PRRs, like TLRs, CD14/LPS-binding protein and various non-opsonic receptors. These stimuli induce the production of cytokines, like IFN-α/β, reactive oxygen species (ROS) and nitric oxide (NO). Humoral activation and phagocytosis is achieved by Fc- and complement receptors (Figure 3b). The priming stimulus IFN-γ, which is produced by NK- and T cells, initiates the classical activation (Figure 3c). However, for full activation a subsequent microbial trigger, such as LPS is required (Dalton et al., 1993). Alternative activation by IL-4 and IL-13 plays an important role in the induction of Th2-type immune response against parasites (Figure 3d) (Stein et al., 1992).

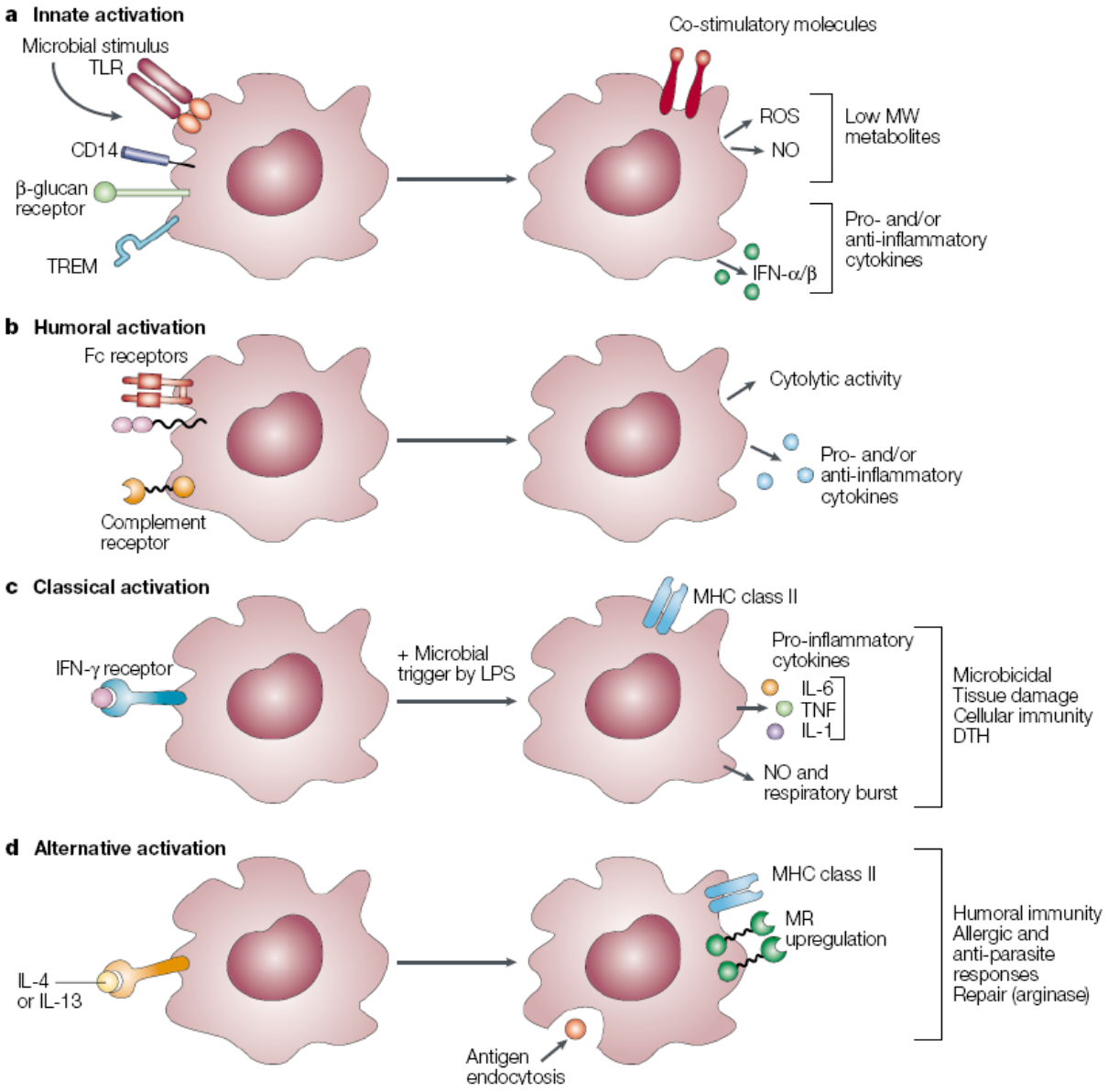


Figure 3. Different possibilities of macrophage activation.
DTH, delayed-type hypersensitivity; TREM, triggering receptor expressed on myeloid cells; ROS, reactive oxygen species (from Gordon, 2003).

1.3.1 Deactivation of macrophages

The activation of macrophages makes them a very powerful weapon in the fight against pathogens, however, it has to be tightly controlled to achieve effective anti-pathogen responses and at the same time prevent immunopathology. Down-regulation of macrophage activity is mediated by multiple mechanisms (Figure 4). Defects in these regulatory pathways can lead to autoimmunity and hyper-inflammation. On the other hand, there is evidence that pathogens, especially those adapted to intracellular life within macrophages, exploit these endogenous inducers of deactivation (Redpath et al., 2001). One way to downregulate macrophages is the inhibition of activating signalling pathways, like the TLR cascades.

Beside the ones already discussed above, this can be achieved by a mechanism called LPS tolerance, a transient state of cellular hypo-responsiveness to subsequent stimulation with LPS induced by prior exposure to TLR ligands (Mathison et al., 1990; West and Heagy, 2002). It protects the host from septic shock caused by hyper-activation of macrophages with persisting bacteria and LPS. To date, only few molecules mediating LPS tolerance are identified. Among them are the phosphatase SHIP (Sly et al., 2004) and IRAK-M (Li et al., 2000), which was already described above. However, the exact mechanisms underlying this phenomenon have not been fully elucidated.

A different possibility to deactivate macrophages is the interference with proinflammatory cytokine signalling. For instance, cytokine antagonists, like IL-18 binding protein (IL-18BP) (Novick et al., 1999) or IL-1 receptor antagonist (IL-1Ra) (Hirsch et al., 1996), which simply inhibit the signalling of the activating cytokine by retaining it in the cytosol and blocking of its receptor, respectively. Another group is the SOCS-family of proteins. They mainly function as regulators of Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) signalling pathways, which most cytokines use to transduce their signal, but as already mentioned, they may also directly interfere with TLR signalling (Starr et al., 1997; Kubo et al., 2003; Alexander and Hilton, 2004).

Pro-inflammatory cytokines are the driving force in the initiation of an effective immune response, however there are also anti-inflammatory cytokines with counterbalancing properties, such as IL-4, IL-10, IL-13, IFN-α and TGF-β. The main effect of these cytokines and their transcriptional targets is to inhibit the production of pro-inflammatory cytokines and other mediators by activated macrophages (Duffield, 2003). Other molecules with similar effects are the glucocorticoids, which are a class of steroid hormones. Beside their involvement in glucose metabolism, they are long known for their immunosuppressive capacities (Flower et al., 1986; Newton, 2000). As a consequence, synthetic glucocorticoids, like dexamethasone (DEX), are widely used as drugs to treat inflammatory and autoimmune diseases. Glucocorticoids bind to their receptor, a ligand-activated transcription factor present in the cytoplasm. This complex is then translocated into the nucleus, where it modulates transcription either positively (transactivation) or negatively (transrepression) (Clark and Lasa, 2003). Transactivation is largely mediated by binding of the complex to its DNA response element, inducing the expression of anti-inflammatory proteins, like IκBα, lipocortin-1 and DUSP1 (De Caterina et al., 1993; Auphan et al., 1995; Scheinman et al., 1995; Kassel et al., 2001; Chen et al., 2002). On the other hand, negative regulation of pro-inflammatory genes is achieved by means of transrepression. The ligated glucocorticoid

receptor can directly tether to other transcription factors, like AP-1 and NF-κB, thereby impairing their ability to induce gene expression (Gottlicher et al., 1998; De Bosscher et al., 2000; Newton, 2000). Many additional modulators of macrophage activity exist. Examples are the peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) that are involved in lipid metabolism, and like the glucocorticoid receptor inhibit activating transcription factors (Gilroy et al., 2004; Valledor and Ricote, 2004). The purine nucleoside adenosine has long been known for its implication in the control of inflammation. Recent studies could now show that the G protein-coupled adenosine receptor A_{2B} mediates anti-inflammatory effects, such as down-regulation of TNF-α production (Hasko et al., 2006; Yang et al., 2006). Although apoptotic cells act as a pro-inflammatory stimulus in the early phase of macrophage activation, there is evidence that at later time points they play an important role in the resolution of inflammation. Apoptotic cell death then switches off production of pro-inflammatory mediators and stimulates production of anti-inflammatory cytokines such as TGF-β from recruited macrophages that have internalized apoptotic cells (Lucas et al., 2003).

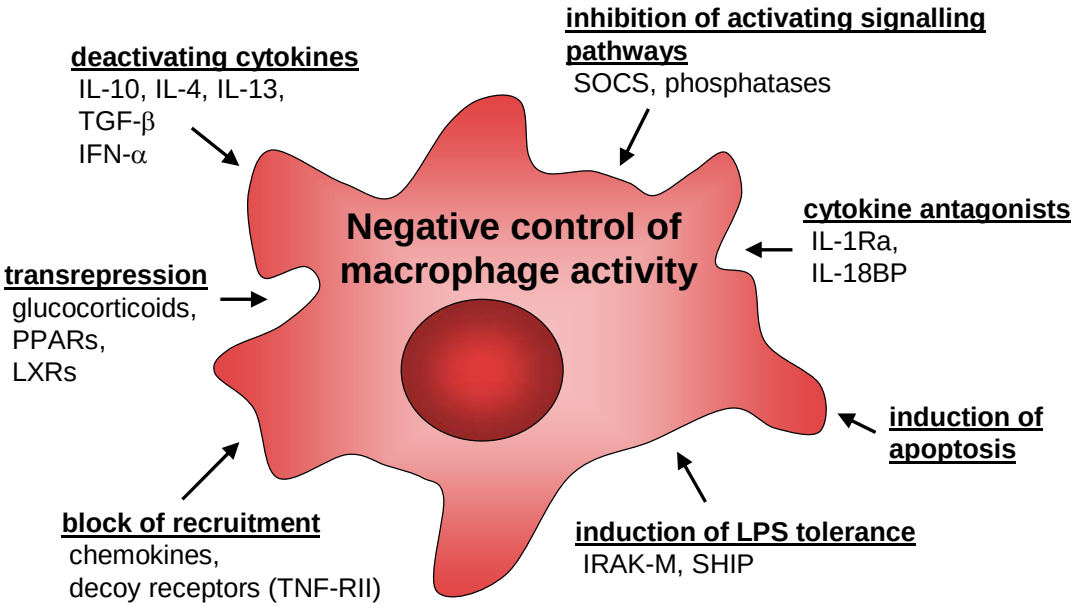


Figure 4. Overview of the mechanisms that can cause macrophage deactivation.

1.4 Interleukin-10 – The 'macrophage deactivating factor'

Beside TGF-β, IL-10 is one of only two major anti-inflammatory cytokines. Discovered in 1989, it was initially described for its role in inhibition of IFN-γ production by Th1 T cells (Fiorentino et al., 1989). It soon became clear that IL-10 is a very potent inhibitor of pro-inflammatory cytokine production by APCs (Fiorentino et al., 1991). In fact, its ability to inhibit T cell activity is mainly mediated by the deactivation of APCs and the resulting lack of co-stimulatory signals (Ding and Shevach, 1992). These studies were soon extended and it could be shown that a broad spectrum of macrophage functions can be inhibited by IL-10, such as cytokine and chemokine synthesis, NO production, and expression of co-stimulatory molecules and class II MHC (Bogdan et al., 1991; Gazzinelli et al., 1992; Oswald et al., 1992; Ding et al., 1993). In recent years, IL-10 has additionally been implicated in the development and function of regulatory T cells (Belkaid et al., 2002). The importance of IL-10 was impressively demonstrated in IL-10-deficient mice. These mice develop chronic enterocolitis (Kuhn et al., 1993) and a lethal shock syndrome after injection of LPS or infection with *Toxoplasma gondii* (Berg et al., 1995; Gazzinelli et al., 1996). On the other hand, IL-10-deficient mice show elevated resistance to infections with pathogens, like mycobacteria (Murray and Young, 1999), leishmania (Kane and Mosser, 2001) and listeria (Dai et al., 1997), indicating that IL-10 can also interfere with the clearance of pathogens. Therefore, it is not surprising that these pathogens can induce IL-10 in order to circumvent effective clearance. Additionally, various latent viruses, including Epstein-Barr virus (EBV) and equine herpes virus type 2 (EHV2), encode IL-10 gene homologues as part of their immune-evading strategy (Redpath et al., 2001).

1.4.1 Structure and expression of IL-10

In mice and humans the *IL10* gene is located on the respective chromosome 1 and consists of five exons (Kim et al., 1992; Moore et al., 2001), which encode secreted proteins of ~178 amino acids and a molecular weight of 17-18 kDa. Mice and human sequences are rather well conserved, sharing ~73 % sequence homology. IL-10 belongs to the class II family of cytokines, which all have an α-helical bundle structure in common. In addition to IL-10, this family consists of various viral homologues, the interferons and eight recently discovered cellular cytokines, namely IL-19, IL-20, IL-22, IL-24, IL-26, IFN-λ1, IFN-λ2, IFN-λ3 (Pestka et al., 2004; Zdanov, 2004). Early studies suggested that IL-10 binds as a dimer to its receptor.

X-ray crystallographic analyses could later confirm this, and showed that IL-10 forms homodimers, similar to IFN-γ (Walter and Nagabhushan, 1995).

A wide variety of cell types have been reported to produce IL-10 *in vitro*, including activated macrophages, dendritic cells, several T cell subsets, NKT cells, but also B cells, mast cells and keratinocytes (Moore et al., 2001). Similarly, the receptors for IL-10 are also expressed on many of these cell types, suggesting that the IL-10 producers themselves can also be targets (Moore et al., 2001). Although a multitude of IL-10-producing cells were identified so far, only few reports exist about their contribution to the IL-10 production *in vivo*. One example is the work by Roers and colleagues who generated mice with a selective deficiency for IL-10 only in T cells to show that T cell-derived IL-10 is in fact important for the control of T cell responses (Roers et al., 2004). However, compared to mice with complete IL-10 deficiency they showed normal innate immune responses to endotoxic shock and skin irritation, indicating that these reactions are controlled by IL-10 from other cell types. A promising new approach to further identify IL-10-producing cells *in vivo* was recently reported by the group of Kamanaka (Kamanaka et al., 2006). Using an IL-10 reporter mouse, they could identify the intestine as a unique site for the induction of IL-10-producing T cells, which played a critical role in the regulation of inflammation in the gut.

Considering its importance in immune regulation, only little is known about the regulation of the *IL10* gene. In macrophages and DCs it seems that at least three different pathways can be employed upon stimulation with LPS, zymosan or TLR2 agonists, all resulting in transcriptional activation of *IL10*. These pathways depend on p38 mitogen-activated protein kinase and NF-κB activation, SYK activation or the activation of extracellular signal-regulated kinase (ERK), respectively (Dillon et al., 2004; Park et al., 2005; Rogers et al., 2005). The exact mechanisms of how these signal transducers induce *IL10* expression are incompletely understood. One possibility is the direct regulation of transcription. The *IL10* promotor contains several motifs known to play a role in transcription initiation, such as a cAMP response element, a glucocorticoid-response element, as well as binding sites for AP-1 and NF-κB (Wang et al., 1995; Platzer et al., 1999). In this context, a recent study could show that TLR2-induced IL-10 production is inhibited by IFN-γ *via* regulation of glycogen synthase kinase 3 (GSK3) and MAPKs, which resulted in concomitant suppression of CREB and AP-1 (Hu et al., 2006). Furthermore, many cell types constitutively express the transcription factors Sp1 and Sp3, which can positively regulate *IL10* transcription (Tone et al., 2000). However, the rapid induction of IL-10 synthesis does not only depend on the activation of transcription factors, but also on chromatin remodelling of the IL-10 promoter.

ERK activation causes a rapid and transient phosphorylation of histone H3 at specific regions of the IL-10 promoter, resulting in its transient exposure to the transcription factors that bind there (Zhang et al., 2006). Beside its transcriptional control, IL-10 expression is also regulated post-transcriptionally. This might account for the observation that many cell types indeed express IL-10 mRNA, but do not secret the protein (Powell et al., 2000).

1.4.2 IL-10 receptor and signalling

Soluble IL-10 mediates its action by binding to the membrane-bound IL-10 receptor (IL-10R). IL-10R is composed of two subunits, the ligand-binding IL-10R1 and the accessory subunit IL-10R2, which is shared with other members of the class II cytokine receptor family (Donnelly et al., 2004). Whereas IL-10R1 is expressed exclusively on haematopoietic cells and is up-regulated upon macrophage activation, IL-10R2 is ubiquitously expressed (Moore et al., 2001). Binding of IL-10 to IL-10R1 leads to the activation of JAK1, which in turn phosphorylates two tyrosine residues of the IL-10R1 chain (Rodig et al., 1998). These tyrosines are then able to dock the SH2 domains of the transcription factor STAT3 (Weber-Nordt et al., 1996). Another Janus kinase, namely tyrosine kinase 2 (TYK2) is associated with the IL-10R2 chain, however, it seems that it is dispensable for proper activation of downstream signalling (Karaghiosoff et al., 2000). In comparison, STAT3 is absolutely essential for proper signal transduction, since mice with tissue-specific deletion of STAT3 in myeloid cells lack the IL-10-mediated effects and develop chronic colitis similar to the IL-10-deficient mice (Takeda et al., 1999; Williams et al., 2004). However, there are few reports showing that other STAT family members might also be involved in transmission of the IL-10 signal to the nucleus (Finbloom and Winestock, 1995; Wehinger et al., 1996; O'Farrell et al., 1998).

As noted above, stunning evidence suggests that STAT3 is the only obligate factor for proper transduction of the IL-10-mediated anti-inflammatory effect, yet, this raises the question of pathway redundancy and specificity, since STAT3 is used by many cytokine receptors. For example, the IL-6R can activate STAT3, but unlike the IL-10R, it is not capable of eliciting an anti-inflammatory response. A possible explanation involves negative regulation by SOCS proteins. SOCS3 can bind to the IL-6R, but not to the IL-10R, thereby inhibiting signalling that might otherwise be mediated by STAT3 (Yasukawa et al., 2003). Additional proof was provided by mutating the SOCS3-binding site in the IL-6R, which allows the generation of an anti-inflammatory response (El Kasmi et al., 2006). Furthermore, using different chimeric receptor systems to generate signalling analogous to the IL-10R, the same study suggests that

the STAT3-mediated anti-inflammatory response can be established by any STAT3 activating cytokine receptor. A very recent study by the Foxwell group used a constitutively active STAT3 to show for the first time that STAT3 is the only signal required to convey the major anti-inflammatory activity of IL-10 within human myeloid cells (Williams et al., 2007). So far, it is not known how STAT3 mediates the anti-inflammatory effects of IL-10. Studies using the protein synthesis inhibitor cycloheximide could show that IL-10 is not able to suppress the expression of pro-inflammatory cytokines IL-12p40 and TNF-α in LPS-stimulated macrophages, demonstrating the necessity of new protein synthesis for IL-10 to exhibit its deactivating effects (Bogdan et al., 1992; Aste-Amezaga et al., 1998; Murray, 2005; Murray, 2006).

1.4.3 Target genes of IL-10

To date it is unclear, which IL-10-induced genes are operative in macrophage deactivation and which level of cytokine gene expression they may target (Murray, 2006). A potential mediator could act as a transcriptional repressor of pro-inflammatory gene transcription or post-translationally modify the activity of transcription factors at specific promotors. Furthermore, IL-10 could induce the expression of proteins that modify chromatin thereby silencing active inflammatory promotors, and finally, IL-10 could induce the sequestration or degradation of key transcriptional mediators at active promotors, as described for NF-κB (Saccani et al., 2004). Besides inhibiting the transcription of pro-inflammatory genes, a potential mediator of IL-10 might negatively regulate activating signal transduction pathways or influence the mRNA stability or translation of pro-inflammatory genes. Until recently, only few genes regulated by IL-10 in mononuclear cells were known, including FcR1 (Larner et al., 1993), monocytes chemoattractant protein-1 (MCP-1) (Yano et al., 1996), IL-1Ra (Jenkins et al., 1994) and SOCS3 (Ito et al., 1999). With the availability of the microarray technology, several profiling studies have greatly extended the list of IL-10-induced genes in murine, as wells as in human mononuclear cells (Lang et al., 2002; Williams et al., 2002; Kuwata et al., 2003; Jung et al., 2004; Perrier et al., 2004). In a study where IL-10-deficient macrophages were used to prevent effects from endogenous IL-10, roughly 15-20 % percent of LPS-induced genes were down-regulated in the presence of IL-10, including the historically recognized targets IL-12p40, TNF-α, IL-1β and IL-6 (Lang et al., 2002). These findings implicate that IL-10 does not affect LPS-induced gene expression *via* a general cellular mechanism but rather selectively inhibits the expression of a subset of inflammatory genes, while leaving others unaffected. Beside already known candidates, like SOCS3 and IL-1Ra,

the group of IL-10-induced genes comprised a number of newly identified genes, including Connexin43, several phosphatases, IL-4Rα, and many transcriptional regulators, like B-cell lymphoma 3 (BCL-3), nuclear factor interleukin 3-regulated (NFIL3) and basic leucine zipper transcription factor ATF-like (B-ATF) (Lang et al., 2002).

Studies by Kuwata and colleagues subsequently revealed that BCL-3 can only partially mediate the IL-10 effect. Although it inhibited TNF-α production, it did no affect IL-6, and mice deficient for BCL-3 lack the elevated cytokine levels observed in IL-10- and STAT3-deficient macrophages (Kuwata et al., 2003). This raises the question whether one master regulator of the IL-10 effect exists at all or if it is rather a collection of proteins, each controlling distinct parts of the pro-inflammatory response.

1.5 Dual-specificity MAPK phosphatases

MAPKs play an important role in various cellular processes, including proliferation, differentiation, stress, apoptosis and immune function (Chang and Karin, 2001; Johnson and Lapadat, 2002). As already described above, MAPKs are a crucial part of the TLR-signalling machinery activated during infections. The three major subfamilies that are expressed in the immune system are p38, ERK, and JNK (Dong et al., 2002). Their pathways are phosphorylation cascades, in which activation of the MAPKs is mediated by phosphorylation of their threonine and tyrosine residues through the respective upstream kinases. Downstream of the MAPKs, a large number of substrates that become serine/threonine phosphorylated have been defined, including transcription factors of the ATF/CREB and AP-1 family, kinases such as MAPKAPK2/MK2 (MAPK-activated protein kinase 2) and RSK (ribosomal protein S6 kinase), and proteins controlling mRNA stability and translation. Due to their central role in pro-inflammatory signalling events, MAPK activity has to be tightly controlled. Several mechanisms can control magnitude and duration of MAPK activation within the signalling pathways, however, one major point of control is the inactivation of the MAPKs by dephosphorylation of the threonine and tyrosine residues. Since both amino acid residues have to be phosphorylated for MAPK activity, dephosphorylation of either residue inactivates the enzyme. This can be achieved by members of different phosphatase families. MAPK phosphatases (MKPs) include the tyrosine-specific phosphatases PTPN5, PTPN7, and PTPRR, serine/threonine phosphatases (PP2A and PP2C), and members of the dual-specificity (threonine/tyrosine) phosphatase (DUSP) family (Keyse, 2000; Farooq and Zhou, 2004). So far, 30 protein-encoding DUSP genes have been found in the human genome, yet

only 11 members are *bona fide* MKPs with a MAPK binding domain (MKB) in addition to the DUSP domain (Table 1) (Lang et al., 2006). The 19 atypical DUSPs are much smaller and lack such MKBs, but may be still functional phosphatases as shown for DUSP14 (Marti et al., 2001), DUSP3 (Alonso et al., 2003) and DUSP22 (Alonso et al., 2002). The presence or absence of MKB, DUSP, and additional N- or C-terminal domains provides a reasonable basis for subdividing the DUSPs into four different groups (Table 1). Other properties, like substrate specificity and subcellular localization, have also been used to group the MKPs, however, these are not always well-defined and may depend on activation state, cell-type, and the presence of interacting proteins (Keyse, 2000; Theodosiou and Ashworth, 2002). Since most MKPs have overlapping substrate specificity it is relatively difficult to define specific physiological roles. Given the multitude of functions the MAPK execute, one can assume that the MKPs are important players in a broad range of different processes. For example, MKPs have been found to be implicated in stress responses, metabolic homeostasis, immune function, and they play important roles during early development (Dickinson and Keyse, 2006). In contrast to the MAPKs, which are ubiquitously expressed, many MKP genes show regulated expression during development, in a cell type-specific manner or in response to cellular activation. DUSP10 is the only MKP so far linked to the immune system. It is expressed in macrophages and T cells and can be induced by various TLR ligands, like LPS, peptidoglycan, and polyIC. Furthermore, DUSP10-deficient mice showed increased cytokine production, a decrease in T cell proliferation and were protected from experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis (Zhang et al., 2004).

Subgroup	Gene	Protein, Synonyms	Gene Structure
Type I	*DUSP3*	VHR	
	DUSP14	MKP-6	
	DUSP15	VHY	
	DUSP22	VHX, JSP1	
Type II	*DUSP1*	MKP-1	
	DUSP2	PAC-1	
	DUSP4	MKP-2	
	DUSP5	VH3	
	DUSP6	MKP-3, PYST1	
	DUSP7	MKP-X, PYST2	
	DUSP9	MKP-4, PYST3	
Type III	*DUSP10*	MKP-5	
Type IV	*DUSP8*	VH5	
	DUSP16	MKP-7, MKP-M	

Table 1. Classification of the dual-specificity MAPK phosphatases.
PTP, protein tyrosine phosphatase catalytical domain (DUSP domain) characterized by the phosphatase signature HCXXXXXR; MKB, MAPK binding domain; NT, N-terminal domain; PEST, domain rich in prolins, glutamates, serines and threonines.

1.5.1 Dual-specificity phosphatase 1

The dual-specificity phosphatase 1 (DUSP1, also known as MKP-1, erp, Ptpn16, 3CH134, CL100, and hVH1) is the prototypic member of the DUSP family. It was first described as an immediate early gene in serum-stimulated fibroblasts (Sun et al., 1993) and initially thought to preferentially dephosphorylate ERK. Only later it was shown that DUSP1 targets the MAPKs p38 and JNK, and to a much lesser extend ERK (Franklin and Kraft, 1997). DUSP1 is expressed in many different epithelial and haematopoietic cell types, and up-regulated in response to growth factors, oxidative and genotoxic stress (Keyse and Emslie, 1992; Liu et al., 1995), heat shock (Wong et al., 2005), hypoxia (Seta et al., 2001), and in various tumors. DUSP1 mRNA expression in macrophages was first reported in cells infected with *Listeria monocytogenes* (Kugler et al., 1997). Subsequent studies could then show that DUSP1 is induced in response to LPS and by ligation of the M-CSF receptor, and that both processes involve the action of protein kinase C (PKC) (Valledor et al., 1999; Valledor et al., 2000). Whether TLR-induced DUSP1 expression requires NF-κB activation has not been clarified yet, however, MAPKs control DUSP1 expression on various levels. Activation of the JNK

pathway for example resulted in increased amounts of DUSP1 mRNA, whereas ERK had no effect (Bokemeyer et al., 1996). Though, the effect of ERK on DUSP1 expression is controversial. In accordance with the previous study, the inhibition of ERK activation with the MEK-1 inhibitor PD98059 had no impact on LPS-induced DUSP1 expression (Valledor et al., 1999; Valledor et al., 2000). In contrast, the MEK-1 inhibitor U0129 was reported to block LPS-induced increases of DUSP1 at the mRNA and protein level (Chen et al., 2002). ERK-dependent signals also increase the stability of DUSP1 protein through phosphorylation. Furthermore, DUSP1 expression might be regulated on the post-transcriptional level, since its 3'-untranslated region (3'UTR) contains AU-rich elements (ARE), which are typical for mRNAs underlying complex regulation. A role for DUSP1 in macrophage deactivation was suggested by a number of studies, in which overexpression of DUSP1 inhibited MAPK phosphorylation and the production of cytokines IL-6 and TNF-α in response to various TLR stimuli (Chen et al., 2002; Shepherd et al., 2004; Zhao et al., 2005). Considering the negative regulatory effect of DUSP1 in macrophage activation, it is not surprising that the anti-inflammatory glucocorticoids are also reported to increase DUSP1 mRNA and protein levels in macrophages and mast cells (Kassel et al., 2001; Imasato et al., 2002; Zhao et al., 2005). Finally, the anti-inflammatory effect of the endocannabinoid anandamide on microglial cells after CNS damage could be recently correlated with an up-regulation of DUSP1 expression, which in turn switched off TLR-induced MAPK signal transduction in these cells (Eljaschewitsch et al., 2006).

2 Aims of the study

The anti-inflammatory cytokine IL-10 is essential for balancing the immune system. Negative control of activated macrophages thereby takes a centre stage of its regulatory action. Until now, it is only incompletely understood how IL-10 mediates its function on the molecular level. To address this question, a recently published microarray dataset generated in our laboratory was mined with the aim to identify potential mediators of the IL-10 effect (Lang et al., 2002). This analysis determined that the dual-specificity phosphatase 1 (DUSP1) was induced by IL-10. The first task therefore was to confirm these findings and was clarified using Northern blot techniques. Following this positive analysis I delved into identifying the mechanisms required for DUSP1 induction and these studies were performed using DUSP1 reporter constructs. Moreover it was elucidated how IL-10 cooperates with the glucocorticoid dexamethasone to induce DUSP1 and affects cytokine production by activated macrophages.
The second aim of this study was to determine the role of DUSP1 *in vivo*. For this purpose, the immune response of DUSP1-deficient mice was analyzed after eliciting innate immune responses. To further elucidate the regulatory role of DUSP1 in the innate immune system, transcription profiling experiments were performed to determine which induced genes are under the control of DUSP1. To complete the overall picture a further aim was to establish the necessity of DUSP1 during an ongoing infection.

3 Material and Methods

3.1 Material

3.1.1 Equipment

Analytical balance	Scaltec, Göttingen
Balance	Ohaus, USA
Centrifuge Biofuge Pico/Fresco	Heraeus, Hanau
Centrifuge Multifuge 3	Heraeus, Hanau
Curix 60 table-top processor	Agfa, Köln
Electrophoresis Apparatus PerfectBlue Duo	Peqlab, Erlangen
Electrophoresis Apparatus PerfectBlue Maxi	Peqlab, Erlangen
Electroporation device Gene Pulser	Bio-Rad, Munich
ELISA reader Sunrise	Tecan, Switzerland
FACSCalibur Flow Cytometer	Becton Dickinson, Heidelberg
Freezer -20°C	Siemens, Munich
Freezer -80°C	Thermo Scientific, USA
Fridge	Liebherr, Switzerland
Gel documentation Eagle Eye II	Stratagene, Heidelberg
GeneChip® Fluidics Station 450	Affymetrix, USA
GeneChip® Scanner 3000	Affymetrix, USA
Heatblock Dri-Block DB 2D	Techne, Burkhardtsdorf
Incubator Hera Cell 240	Heraeus, Hanau
MACS	Miltenyi, Bergisch-Gladbach
Microwave	Privileg, München
Microscope Zeiss Axiovert 40 C	Zeiss, Jena
Multichannel pipettes	ThermoLabsystems, USA
Multipipette plus	Eppendorf, Hamburg
Nanodrop®ND-1000 Spectrophotometer	Peqlab, Erlangen
Neubauer counting chamber	Roth, Karlsruhe
Nitrogen freezing tank Espace 300	Air Liquide, Düsseldorf
Orion Microplate Luminometer	Berthold, Pforzheim
pH-meter Multical	WTW, Weilheim

Phosphoimager Storm 840	Molecular Dynamics, USA
Pipettes	Gilson, USA
Pipetboy acu	Integra Biosciences, Fernwald
Plastic ware	Nunc, Wiesbaden; Falcon, USA
Power Supply Power Pac 200	Bio-Rad, Munich
Realtime PCR Systeme SDS7700	Applied Biosystems, USA
Radiographic cassette	Dr. Goos-Suprema, Heidelberg
Rotator OV3	Biometra, Göttingen
Sealing apparatus Folio	Severin, Sundern
Shaker	Peqlab, Erlangen
Sonicater UW 60	Bandelin Electronic, Berlin
Sterile bench	Heraeus, Hanau
Thermocycler Trio Thermoblock	Biometra, Göttingen
Thermomixer Comfort	Eppendorf, Hamburg
Ultra-Turrax T25	IKA Labortechnik, Staufen
UV-Crosslinker UV- Stratalinker® 2400	Stratagene, Heidelberg
Vortexer Genie 2	Scientific Industries, USA
Waterbath	Memmert, Schwabach
Western blot transfer tank	Peqlab, Erlangen

3.1.2 Consumable items, kits and enzymes

BCA™ Protein Assay Kit	Pierce, USA
Blood agar plates	Becton Dickinson, Heidelberg
Cell culture plates and Petri dishes	Falcon, USA; Greiner, Austria
Cell Scraper	TPP, Switzerland
Cell strainer 70 µM	Falcon, USA
Combitips	Eppendorf, Hamburg
Cryotubes 1 ml	Corning, USA
Dispenser	TPP, Switzerland
Electroporation cuvettes, 2mm	Bio-Rad, Munich
EndoFree® Plasmid Maxi Kit	Qiagen, Hilden
EndoFree® Plasmid Mini Kit	Qiagen, Hilden
ELISA-Kits	R&D Systems, USA
Ethilon thread	Ethicon, Norderstedt

Film Kodak BioMax Light	Sigma-Aldrich, Taufkirchen
Filter Tips	Kisker, Steinfurt
GeneChip® One Cycle Kit	Affymetrix, USA
GeneChip® MOE430A 2.0	Affymetrix, USA
HotStarTaq PCR Kit	Qiagen, Hilden
Insulin syringe 1 ml Sub Q	Becton Dickinson, Heidelberg
M-MuLV Reverse Transcription Kit	Peqlab, Erlangen
MACS LS^+ columns	Miltenyi, Bergisch-Gladbach
MaxiSorp 96-well ELISA plates	Nunc, Wiesbaden
Microcentifuge tubes 1.5 ml	Eppendorf, Hamburg
Microspin Columns	Amersham, Heidelberg
Microtainer SST	Becton-Dickinson, Heidelberg
Nitrocellulose membrane	Schleicher&Schuell, Dassel
Nylon membrane Hybond-XL	Amersham, Sweden
Optical 96-well plates	Applied Biosystems, USA
Parafilm®	Roth, Karlsruhe
PCR-plates 96-well	Peqlab, Erlangen
Platinum® *Pfx* DNA Platinum	Invitrogen, Karlsruhe
Prime Kit I	Stratagene, Heidelberg
QIAEX II Gel Extraction Kit	Qiagen, Hilden
Restriction enzymes	NEB, USA
Sterile injection needles Microlance™3	Becton Dickinson, Heidelberg
Syringe Discardit™ 10 ml	Becton Dickinson, Heidelberg
T4 DNA Ligase Kit	NEB, USA
Topo-TA-Cloning-kit	Invitrogen, Karlsruhe
Venous catheter 22 gauge	Venflon, Sweden
Western Lightning Chemiluminescence	PerkinElmer Life Sciences, USA
Whatman Paper	Schleicher&Schuell, Dassel

3.1.3 Reagents

^{32}P-αdCTP 9.25 MBq	Amersham, Heidelberg
Actinomycin D	Sigma-Aldrich, Taufkirchen
Accutase	PAA, Cölbe
Agarose	Invitrogen, Karlsruhe

anti-IL-10 blocking antibody	Becton Dickinson, Heidelberg
ATP	Sigma-Aldrich, Taufkirchen
β-Mercaptoethanol	Gibco, Karlsruhe
BSA	Sigma-Aldrich, Taufkirchen
Coenzym A	PJK, Kleinblittersdorf
CpG1668	TIB MolBiol, Berlin
Cycloheximide	Calbiochem, Darmstadt
D-Luciferin	PJK, Kleinblittersdorf
Deoxynucleotide-triphosphates	Invitrogen, Karlsruhe
DEPC-H_2O	Ambion, USA
Dexamethasone	Sigma-Aldrich, Taufkirchen
DMSO	Sigma-Aldrich, Taufkirchen
DNA-Ladder 1 Kb	Invitrogen, Karlsruhe
DTT	Roche, Mannheim
Dulbecco's MEM	Biochrom, Berlin
Dulbecco's PBS 1x	Biochrom, Berlin
Ether	Hoechst, Frankfurt
Ethidiumbromide	Roth, Karlsruhe
Fetale Bovine Serum	Biochrom, Berlin
Geneticin (G-418 Sulphate)	Gibco, Karlsruhe
L-cell-conditioned medium	Own production
Loading Dye Solution 6x (Western)	Fermentas, St. Leon-Rot
LPS *E. coli* O55:B5	Sigma-Aldrich, Taufkirchen
MALP-2	EMC Microcollections, Tübingen
Milk powder	Roth, Karlsruhe
MOPS-EDTA-Sodium Acetate Buffer	Sigma-Aldrich, Taufkirchen
murine IFN-γ recombinant	tebu-bio, Offenbach
murine IL-6 recombinant	tebu-bio, Offenbach
murine IL-10 recombinant	tebu-bio, Offenbach
Narketan-xylopan	WDT, Garbsen
Oligo(dT) primer	Amersham, Heidelberg
Orange G	Sigma-Aldrich, Taufkirchen
$Pam3CysSer(Lys)_4$	EMC Microcollections, Tübingen
Penicillin/Streptomycin	Biochrom, Berlin

Poly I:C	Invivogen, France
Ponceau S	Sigma-Aldrich, Taufkirchen
Protease Inhibitor Cocktail	Roche, Mannheim
Proteinase K	Roche, Mannheim
R848	Invivogen, France
Random Hexamer	Amersham, Heidelberg
Reporter Lysis Buffer 5x	Promega, Mannheim
SB203580	Calbiochem, Darmstadt
SSC 20x	Invitrogen, Karlsruhe
SYBR-green	Molecular Probes, Karlsruhe
Streptavidin-HRP	R&D Systems, USA
TriFast	Peqlab, Erlangen
TMB tablets	Sigma-Aldrich, Taufkirchen
Tween20	Sigma-Aldrich, Taufkirchen
Absolute QPCR ROX Mix	ABgene, Hamburg
VLE-RPMI	Biochrom, Berlin

3.1.4 Primers and probes

Name	5'-3' sequence/Order Number	Description	Detector	Company
β-actin fw	ACCCACACTGTGCCCATCTAC	-	SYBR	Metabion
β-actin rev	AGCCAAGTCCAGACGCAGG	-	SYBR	Metabion
DUSP1	Mm_DUSP1_SG_1	QuantiTect Primer Assay	SYBR	Qiagen
F3	Mm_F3_SG_1	QuantiTect Primer Assay	SYBR	Qiagen
PTX3	Mm00477267_g1	Assay on Demand	FAM	Applied Biosystems
MEFV	Mm00490258_m1	Assay on Demand	FAM	Applied Biosystems

Table 2. Oligonucleotide primer and probes for Real-time PCR.

Name (Position relative to TSS)	5'-3' sequence (inserted restriction sites underlined)	restriction site
DUSP1 Promotor fw -1060 bp	ACGCGTTCGGTCTTCCCAGGTGTGATGAG TA	MluI
DUSP1 Promotor rev +83 bp	CTCGAGGTCGATCTTGTGCGGTGTCCTGT	XhoI
DUSP1 3'UTR fw +2194 bp	GCTCTAGAGGGCAACGGGAGGTGTGGGA GTTC	XbaI
DUSP1 3'UTR rev +2897 bp	CGGGATCCGAAAGCACGGAGAGCTCAAT AGAAACGTC	BamHI
STAT3 site #1 fw -7494 bp	CGACGCGTTTCCACTGCTGCTCCAGTCTG AGTGCTTGA	MluI
STAT3 site #1 rev -3295 bp	CGACGCGTTTCAGCAACCTTCTGTGACTC CCATCCACC	MluI
STAT3 site #2 fw -13137 bp	CGACGCGTCATACCCGTAACTTTCTGGGA AACCTCCAAAG	MluI
STAT3 site #2 rev -10549 bp	CGACGCGTTAGCATGGTGCTGTCAGGCAC TGCCAGACAA	MluI
STAT3 site #3 fw -21447 bp	CGACGCGTGTGGCCTGCAGCCTGCATCCA AATTCCTTA	MluI
STAT3 site #3 rev -19495 bp	CGACGCGTAAACCACAGCAGAGCCTCGG AGATACCACA	MluI

Table 3. Oligonucleotide primer for DUSP1 PCRs.
Primer for the amplification of the core promotor, the 3'UTR, and the three STAT3 sites upstream of the core promotor. All Primers were ordered from Metabion.

Gene	Clone ID	Backbone	restriction sites	Source
DUSP1	IRAKp961N2410Q2	pCMV-SPORT6	SalI/NotI	RZPD, Berlin
GAPDH	IMAGp998K2013887Q3	pCMV-SPORT6.1	EcoRV/NotI	RZPD, Berlin
IL-6	IMAGp998O176601Q3	pCMV-SPORT6	SalI/NotI	RZPD, Berlin
IL-10	IMAGp998L102501Q	pBluescript SK-	EcoRI/XhoI	RZPD, Berlin
NDR1	IRAVp968C0631D	DH10BTonA	SalI/NotI	RZPD, Berlin
SOCS3	IRAVp968B08111D	pYX-Asc	EcoRI/NotI	RZPD, Berlin
TNF-α	IMAG-p998A167735Q3	pT7T3D-Pacl	EcoRI/NotI	RZPD, Berlin
Luciferase	-	pGL3-basic	NcoI/XbaI	Promega

Table 4. Probes for Northern blot analysis.
Probes were prepared from plasmids obtained from Deutsches Ressourcenzentrum für Genomforschung (RZPD, Berlin), containing full-length cDNA, by digestion with the appropriate restriction enzymes. The probe for Luciferase was obtained from the reporter plasmid pGL3 (Promega).

3.1.5 Antibodies used for Western blot

Primary antibodies

Antigen	Isotype	Dilution	Blocking	Company
γ-tubulin	mouse, IgG1	1:5000	5 % milk	Sigma-Aldrich
DUSP1 (M-18)	rabbit	1:500	5 % milk	Santa Cruz
p38	rabbit	1:1000	5 % milk	Cell Signaling
phospho-p38 (Thr180/Tyr182)	rabbit	1:1000	5 % milk	Cell Signaling
phospho-ERK (Thr202/Tyr204)	rabbit	1:1000	5 % milk	Cell Signaling
phospho-JNK (Thr183/Tyr185)	rabbit	1:1000	5 % milk	Cell Signaling
phospho-MK2 (Thr334)	rabbit	1:1000	5 % BSA	Cell Signaling
phospho-ATF2 (Thr71)	rabbit	1:1000	5 % milk	Cell Signaling
IRF-1 (M-20)	rabbit	1:500	5 % milk	Santa Cruz
IKKβ (10AG2)	mouse, IgG1	1:1000	5 % BSA	Upstate
phospho-STAT1 (Ser727)	rabbit	1:500	5 % milk	Cell Signaling
phospho-STAT1 (Tyr701)	rabbit	1:1000	5 % milk	Cell Signaling
STAT1 (E-23)	rabbit	1:1000	5 % milk	Santa Cruz

Secondary antibodies

Antigen	Conjugate	Source	Dilution	Company
anti-mouse IgG F(ab')2-Fragment	HRP	goat	1:10000	Dianova
anti-rabbit IgG F(ab')2-Fragment	HRP	donkey	1:10000	Dianova

Table 5. Antibodies for Western blot analysis.

3.1.6 Antibodies used for flow cytometry

Antibodies

Antigen	Isotype	Conjugate	Dilution	Company
CD45.1	IgG2a	APC	1:100	eBioscience
CD45.2	IgG2a	Biotin	1:100	BD Bioscience
CD16/CD32	IgG2b	-	1:100	BD Bioscience

Conjugates

Antigen	Isotype	Conjugate	Dilution	Company
Biotin	Streptavidin	PE	1:100	Molecular Probes

Table 6. Antibodies and conjugates for flow cytometry.

3.2 Methods

3.2.1 Cell biology

3.2.1.1 Media used for eukaryotic cell culture

cRPMI:	500	ml RPMI 1640
	50	ml heat inactivated FBS
	5	ml Penicillin/Streptomycin
	500	µl β-mercaptoethanol 50 mM
cDMEM:	500	ml DMEM
	50	ml heat inactivated FBS
	5	ml Penicillin/Streptomycin
	500	µl β-mercaptoethanol 50 mM

3.2.1.2 Culture of RAW 264.7 macrophages

RAW 264.7 macrophages were cultured in cRPMI medium at 37°C, 5 % CO_2 and 85 % humidity. Cells were cultured to 60-70 % confluency. To split the cells, they were rinsed from the plate and centrifuged at 1200 rpm for 5 min. The cells were gently resuspended and 1/5th

to 1/10th of this solution were transferred to a new cell culture dish containing fresh medium. This procedure was carried out every two to three days.

3.2.1.3 Cryopreservation of RAW 264.7 macrophages

To cryopreserve RAW 267.4 macrophages, FBS substituted with 10 % DMSO was prepared. After detaching the cells, they were counted and centrifuged at 1200 rpm for 5 min. The cells were resuspended in 1 ml per $5x10^6$ cells, transferred to cryotubes and deep frozen at -20°C for 30 min before they were stored at -80°C overnight. Finally the frozen cells were transferred for long term storage in liquid nitrogen.
Cells were thawed very quickly at 37°C and then transferred in 10 ml of pre-warmed medium. After two washes with 1x PBS and spins at 1200 rpm cells were then resuspended in 5 to 10 ml of pre-warmed medium and transferred to a cell culture flask.

3.2.1.4 Stable transfection of RAW 264.7 macrophages

Cells were split the evening before the transfection. After harvesting the cells, they were counted and diluted in cRPMI containing 25 % FBS to $1x10^7$ cells/ml. For one transfection $5x10^7$ cells were mixed with 30 µg of the linearized plasmid DNA and then transferred to an electroporation tube. The transfection was carried out with the GenePulser at 300 mV and a capacity of 960 µFD. After incubating the cells 5 min at room temperature (RT) the cell solution was transferred into a Falcon tube containing 8 ml of cRPMI and spun down for 5 min at 1200rpm. Cells were grown overnight at 37°C in 2 ml cRPMI containing 25 % FBS on a 6-well plate. The next day medium was removed and fresh cRPMI containing Geniticin for selection of stably transfected lines was added. Cells were grown for 3 weeks, before the stable lines were analysed.

3.2.1.5 Generation of murine bone marrow-derived macrophages

The mice were sacrificed, the hind legs were removed and the bones were separated. Femora and tibiae were flushed with PBS using a syringe (best needle 27G) into a 10 ml petri dish. After erythrocyte lysis, bone marrow cells were taken up in cDMEM supplemented with 10 % L-cell-conditioned medium (LCCM) as a source of M-CSF and incubated overnight in 10-cm bacteriological plastic dishes. Non-adherent cells were counted and re-plated at a density of $0.5x10^6$ cells/ml in cDMEM with 10 % LCCM in 10-cm standard plastic dishes. At day four, 5 ml of fresh cDMEM with 10 % LCCM was added. After 6-7 days of differentiation, cultures were nearly confluent. Non-adherent cells were removed, the remaining cells washed once with 1x PBS and then incubated with 5 ml Accutase at 37°C for 5 min to detach the

cells. Accutase was then inhibited by the addition of 5 ml cDMEM. Cells were counted, re-plated at a density of $1x10^6$ cells/ml, and rested overnight before they were used for experiments.

3.2.2 Mice

All mice were kept under pathogen-free conditions at the animal facility of the Institute of Medical Microbiology, Immunology and Hygiene (Technical University, Munich, Germany). Animal experiments were approved and authorized by local government. Experiments were performed with 10-12 week old mice, unless otherwise stated. Prior to the experiment all the mice were genotyped according to standard laboratory protocol.

IL-10-deficient mice (Kuhn et al., 1993) backcrossed at least 10 generations onto a C3H background were bred at our own animal facility. $IKK\beta^{flox/flox}$/LysMCre and control mice were kindly provided by F. Greten from the Department of Internal Medicine II of the Technical University Munich (Greten et al., 2004). DUSP1-deficient mice on a mixed 129Sv x C57BL/6 background, initially generated at the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (Dorfman et al., 1996), were kindly provided by A. Cato from the Forschungszentrum Karlsruhe. For control experiments, mice backcrossed for seven generations onto C3H and 129Sv were used.

3.2.3 Animal experiments

3.2.3.1 Removal of organs

Mice were sacrificed by cervical dislocation and the respective organs were removed after the site of operation was disinfected with 70 % EtOH.

3.2.3.2 LPS-induced shock

Mice were used for LPS challenge at an age of 5-10 weeks. Experimental groups were matched for age and sex. *Escherichia coli* LPS was diluted in sterile PBS and injected intraperitoneally. In case of the survival experiments, the mice were monitored every 6 h.

3.2.3.3 Colon ascendens stent peritonitis (CASP)

The surgical procedure of CASP was performed as described before (Zantl et al., 1998). Briefly, for anesthesia, ether or narketan-xylopan was used. Prior to surgery, a venous catheter (18 or 20 gauge) was prepared by creating a notch at a distance of 3 mm from the orifice; 1 mm beyond, the catheter was circumferentially incised with a scalpel, sparing only a slim bar.

In complete anesthesia and after disinfection of the abdomen, the abdominal wall was opened through a 1-cm midline incision. After exposure of the ascending colon, the prepared catheter was stitched through the antimesenteric wall into the lumen of the ascending colon and then fixed with two stitches (7/0 Ethilon thread) placed approximately 10 mm from the ileocecal valve. Consecutively, the inner needle of the stent was removed and the stent was cut at the prepared site. To ensure proper intraluminal positioning of the stent, stool was milked from the cecum into the ascending colon and the stent until a small drop of stool appeared. Fluid resuscitation of animals was performed by flushing 0.5 ml of sterile saline solution into the peritoneal cavity before closure of the abdominal walls (two layers, muscle and skin; 4/0 Ethilon thread).

3.2.3.3.1 Bacterial counts

Peritoneal lavage fluids, lungs and livers were collected from mice sacrificed 12 h after CASP surgery. The organs were homogenized in 5 ml of PBS using an Ultra-Turrax device. Serial dilutions of lavage fluid or organ homogenates in PBS were plated on blood agar plates. CFU were counted after incubation at 37°C for 24 h and calculated per whole peritoneal cavity and organ, respectively.

3.2.3.4 Generation of bone marrow-chimeric mice

CD45.1/CD45.2 recipient C57Bl/6 mice were irradiated at 950 rad and allowed to rest overnight. The next day, bone marrow was prepared from DUSP1-deficient and heterozygous control mice and depleted of T cells using a magnetically-labelled Thy1.2-specific antibody as described. Then, $5x10^6$ cells per mouse were injected intravenously and the mice were allowed to rest for 4 weeks before the efficiency of reconstitution was examined by flow cytometry. For this purpose, mice were bled and cells were stained with antibodies specific for CD45.1 and CD45.2 as described.

3.2.4 Protein biochemistry

3.2.4.1 Buffers and solutions

RIPA buffer:	2	g SodiumDeoxycholate
	2	ml 10 % SDS
	0.8	ml 0.5 M EDTA
	0.42	g Sodium Fluoride
	2	ml NP-40
	ad 200	ml with 1x PBS
Lysis buffer:	10	ml RIPA buffer
	50	µl 200 mM Na-ortho-vanadate
	200	µl 50x Protease-inhibitor cocktail
4x Resolving gel buffer:	1.5	M Tris-HCl pH 8.8
4x Stacking gel buffer:	0.5	M Tris-HCl pH 6.8
10 % SDS:	10	% SDS in ddH_2O
10 % APS:	10	% Ammonium persulphat in ddH_2O
6x sample buffer:	7	ml stacking gel buffer
	1	g SDS
	3	ml Glycerol
	0.9	g DTT
	0.06	% Bromphenol blue
Tank buffer:	30.28	g Tris
	208.2	g Glycine
	50	ml 10 % SDS
	ad 10 l	with ddH_2O

Transfer buffer:	1.94	g Tris
	8,656	g Gylcine
	4	ml 10 % SDS
	200	ml Methanol
	ad 1000	ml with ddH_2O
10x TBS:	48.4	g Tris
	160	g NaCl
	ad 2000	ml ddH_2O
	adjust to pH 7.6	with HCl
1x TBS-T:	100	ml 10x TBS
	1	ml Tween20
	ad 1000	ml with ddH_2O
Luciferase substrate:	132	mg D-Luciferin
	210	mg Coenzym A
	5.14	g DTT
	292	mg ATP
	520	mg $(MgCO_3)_4Mg(OH)_2$ x $5H_2O$
	322	mg $MgSO_4$
	3.584	g Tricine
	37.2	mg EDTA
	ad 1000	ml with ddH_2O

3.2.4.2 Cell lysis

Cell lysis was carried out on ice. After stimulation the cells were washed twice with ice-cold 1x PBS and then lysed with 140 µl lysis buffer per $1.5x10^6$ cells for 45 min. The lysate was then collected and centrifuged at 13000 rpm for 5 min in a tabletop centrifuge to remove nuclei and debris. The supernatants were collected in a 1.5 ml tube, sonicated 15 s at maximum power and stored at -80°C. Protein concentration was determined using the BCA Protein Assay Reagent Kit according to manufacturer's guidelines.

3.2.4.3 SDS-polyacrylamid gel electrophoresis (PAGE) of proteins

SDS-PAGE was carried out using gels of 1.5 mm thickness. First the resolving gel (10 or 12.5 %) was poured and immediately covered with 2 ml isopropanol. After polymerisation of the gel, the isopropanol was removed and the gels were rinsed with water. The stacking gel (4 %) was poured and the comb was inserted. After complete polymerisation the gel was installed, overlayed with tank buffer and the combs were removed. Then the wells were flushed with tank buffer to remove residual acrylamid. The cell lysates were mixed 1:4 with 4x sample buffer and incubated for 5 min at 95°C. Depending on the comb 25 µl of the lysate were loaded for 20-well combs and 18 µl for 25-well combs, respectively. For the determination of protein size, 5 µl of a protein standard (Fermentas) were loaded on the gel. Gel electrophoresis took place at 300 V and 75 mA for 1-2 h.

	Resolving Gel		Stacking Gel
	10 %	12.5 %	4 %
Acrylamide solution	6.7 ml	8.3 ml	0.88 ml
4x Resolving gel buffer	5 ml	5 ml	-
4x Stacking gel buffer	-	-	1.66 ml
10 % SDS	0.2 ml	0.2 ml	66 µl
ddH_2O	8 ml	6.4 ml	4.06 ml
TEMED	6.7 µl	6.7 µl	3.3 µl
10 % APS	100 µl	100 µl	33.4 µl

Table 7. SDS-PAGE gel recipes

3.2.4.4 Western blot analysis

For the transfer of the proteins from the gels to a nitrocellulose membrane the tank blot method was carried out. Two Whatman papers, the nitrocellulose membrane and two sponges were equilibrated in transfer buffer together with the gel for 5 min. After assembling the complete set up in the transfer chamber, it was filled up with transfer buffer. If two gels were blotted, the transfer took place at 60 V and 210 mA for 2 h on a magnetic stirrer. After transfer, the membrane was incubated in water for 5 min on the shaker to remove remaining methanol. To ensure equal protein loadings for all lanes the membrane was stained with

Ponceau S solution for 5 min. To remove the staining afterwards the membrane was incubated in water again. To avoid unspecific binding the membrane was blocked for 1 h in 1x TBS-T with 5 % BSA or 5 % milk, depending on the first antibody used. This was followed by three washing steps in 1x TBS-T for 10 min. Incubation with the primary antibody (diluted in 1x TBS-T 5 % BSA or 5 % milk) took place at 4°C overnight. On the next day the membrane was washed three times for 10 min in 1x TBS-T and then incubated with the secondary antibody (diluted in 1x TBS-T 5 % BSA) for 1 h at room temperature on the shaker. After three washing steps for 20 min each, proteins were detected using Western Lightning Chemiluminescence Reagent.

If the membrane was subject to incubation with a different antibody, a stripping protocol was carried out to remove previous antibodies from the membrane. Briefly, the membrane was incubated in H_2O for 5 min, followed by incubation with pre-warmed 0.2 N NaOH for 20 to 40 min and a final wash in H_2O for 5 min. All steps were performed on a shaker. The membrane was then blocked again in 1x TBS-T with 5 % BSA or 5 % milk for 1 h.

3.2.4.5 Luciferase reporter gene assay

The linearized reporter constructs (25 µg) were transfected together with 5 µg of pcDNA3.1+ (Invitrogen), which carries the G418-resistance gene. Transfection and selection was carried out as described above. Reporter gene activation was analyzed after 2 weeks of G418 selection. For this purpose, cells were plated at $4x10^6$ cells/well in 96-well flat-bottom plates, rested overnight and stimulated as indicated for 8 h. Supernatants were removed and lysates prepared by adding 25 µl Reporter lysis buffer, followed by one freeze/thaw cycle. Luciferase activity was determined using 15 µl lysate with 50 µl luciferase substrate in a 96-well plate luminometer.

3.2.5 Molecular biology

3.2.5.1 Buffers and solutions

50x TAE:	242	g Tris Base
	500	ml ddH_2O
	57.1	ml Glacial acetic acid
	100	ml 0.5 M Na_2EDTA pH8.0
	ad 1000	ml with ddH_2O

4x DNA running buffer:	50	mg Orange G
	15	ml Glycerol
	0.5	ml 1 M Tris-HCl
	ad 50	ml with ddH_2O
6x RNA running buffer:	25	ml Glycerol
	1	ml 0.5 M EDTA pH 8.0
	125	µl Bromphenol blue
10x MOPS buffer:	1	Pack MOPS-EDTA-Sodium Acetate Buffer
	ad 1000	ml with ddH_2O
2x SSC washing buffer:	100	ml 20x SSC
	10	ml 10 % SDS
	ad 1000	ml with ddH_2O
0.2x SSC washing buffer:	10	ml 20x SSC
	10	ml 10 % SDS
	ad 1000	ml with ddH_2O
Church Buffer:	400	ml ddH_2O (~50°C)
	35	g SDS
	25.55	g Na_2HPO_4
	9.66	g NaH_2PO_4
	1	ml 0.5 M EDTA pH 8.0
	5	g BSA
	ad 500	ml with ddH_2O

3.2.5.2 Basic tools

Minipreps and Maxipreps were performed using the respective kits from QIAGEN following the manufacturer's guidelines. Kits from QIAGEN were also used for Gel extraction and DNA-purification. Ligations and restriction digests were done with the Quick Ligation Kit and restriction enzymes, respectively, from New England Biolabs following their instructions. If not described otherwise fragments were used in a 3:1 ratio of insert to vector. All plasmids

were transformed into OneShot TOP10 chemically competent *E. Coli* cells (Invitrogen) according to the manufacturer's guidelines.

3.2.5.3 Agarose gel electrophoresis

Agarose gels (1 %) were used to separate DNA fragments variable in size. TAE-buffer (1x) with EtBr (100 ng/ml) was used as electrophoresis and gel buffer. Samples were mixed 4:1 with sample buffer and separated at 80 V. For size determination of the fragments, 5 µl of a 1 kb ladder were used. DNA bands were visualized with UV light (254 nm).

3.2.5.4 PCR

If not stated otherwise, all PCRs were performed using the *Pfx* Platinum Polymerase (Invitrogen).

PCR mix for 50 µl:	
5	µl 10x buffer
2	µl 10 mM dNTPs
1	µl $MgSO_4$
0.5	µl 100 µM 5' primer
0.5	µl 100 µM 3' primer
0.5	µl DNA (equals 50 ng)
1	µl *Pfx* Platinum Polymerase
29.5	µl ddH_2O
10	µl Enhancer

In general, amplification occurred using the following protocol with modifications for annealing temperature (dependent on used primer combination) and extension time (dependent on the size of the amplified fragment):

95°C	5 min	
95°C	30 s	35-40 x
65°C	45 s	
68°C	4 min	
68°C	10 min	
4°C	pause	

3.2.5.5 Generation of DUSP1 reporter constructs

The *DUSP1* promotor fragment, the 3'UTR and the fragments containing the STAT3 sites were amplified by PCR using BAC clone RP23-138L4 (obtained from RZPD) as template. The construct referred to as DUSP1_luc was derived using primers amplifying the promotor from position -1060 to +83 relative to the transcriptional start site (Sommer et al., 2000) and then ligated into the MluI and XhoI sites of the vector pGL3-basic (Promega) carrying the luciferase reporter gene. The 3'UTR of *DUSP1* ranging from position +2194 to +2897 relative to the transcriptional start site was identified using the Eldorado Tool of GenomatixSuite (Genomatix). It was PCR amplified and ligated into the DUSP1_luc reporter constructs after removal of the SV40-based 3'UTR by cutting with XbaI and BamHI to generate the DUSP1_luc_dusp1 reporter construct. The fragments containing the STAT3 sites (positions indicated in Table 3) were identified using the MatInspector Tool of GenomatixSuite. After amplification by PCR they were ligated into the MluI site of the DUSP1_luc_dusp1 reporter construct to generate the STAT3_DUSP1_luc_dusp1 constructs.

3.2.5.6 Total RNA extraction

Up to $2x10^6$ cells per well of a 6-well plate were lysed in 1 ml of peqGOLD TriFast reagent for 5 min at RT. Cell lysates were stored at –80°C or directly used for total RNA extraction. Next, 200 µl of chloroform were added and each sample was shaken for 15 s and incubated for 2-3 min at RT. The samples were then centrifuged for 15 min at 13000 rpm at 4°C in a tabletop centrifuge to achieve a complete separation of the aqueous and phenol phases. The top aqueous phase containing the dissolved RNA was transferred to a RNase-free reaction tube and mixed with 500 µl isopropanol. Precipitation of the RNA occurred at RT for 10 min. The RNA was pelleted by centrifugation (13000 rpm, 4°C, 10 min) and the pellet was washed twice with 1 ml 70 % ethanol (13000 rpm, 4°C, 5 min). Then it was dried at RT and dissolved in 21 µl DEPC-treated H_2O for 15 min at 56°C on a shaker. Purity and concentration of the dissolved RNA was determined by measuring the absorption at 260 and 280 nm, respectively. The extracted RNA was stored at –80°C.

For the extraction of total RNA from organs, one half of the spleen and the lung or one third of the liver was homogenized in 2 ml of TriFast using an Ultra-Turrax device. Subsequent steps were done as described for cell solutions.

3.2.5.7 First strand cDNA synthesis

The synthesis of cDNA for real-time RT-PCR analysis was carried out using the peqGOLD M-MuLV Reverse Transcriptase. A reaction tube was prepared and 5 µl of DEPC-H_2O was added. Then 1 µl of a primer mix (equals 100 ng random hexameres + 250 ng Oligo(dT) primer) and 1 µg of total RNA were added. To denaturate the RNA the mixture was incubated for 5 min at 70°C and cooled on ice. Then the following solutions were added:

3	µl 10x buffer
2	µl 10 mM dNTPs
0.25	µl M-MuLV RT (50 U)
3	µl 50 mM DTT
ad 30	µl with DEPC-H_2O

The reaction mix was incubated for 1 h at 42°C, centrifuged shortly and stored at -20°C for further usage.

3.2.5.8 Relative quantitation of gene expression by real-time PCR analysis

Real-time PCR was used to determine the relative amount of produced mRNA encoding for specific genes. All PCRs were performed in a volume of 20 µl on the SDS7700 cycler (Applied Biosystems). The primers for β-actin, F3 and DUSP1 were designed using PrimerExpress Software (Applied Biosystems) and the SYBR-green master mix was used to detect accumulation of PCR product during cycling. For detection of MEFV and PTX3 pre-designed Assays-On-Demand (Applied Biosystems), consisting of the primers and a TaqMan probe were used.

PCR mix for 20 µl: **SYBR-green**

0.6	µl SYBR-green
0.18	µl 100 µM 5’ primer
0.18	µl 100 µM 3’ primer
10	µl 2x Universal Master Mix
7.04	µl DEPC-H_2O
2	µl cDNA

TaqMan probe

1 µl Primer-Ready Mix
10 µl 2x Universal Master Mix
7 µl DEPC-H_2O
2 µl cDNA

Thermal cycling was initiated by a first denaturation step at 95°C for 15 min, and continued with 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of target genes was normalized to β-actin and displayed as fold-change relative to the untreated sample used as the calibrator (set to 1).

3.2.5.9 Microarray analysis of gene expression

Affymetrix GeneChip® probe microarrays are manufactured using technology that combines photolithography and combinatorial chemistry. Up to 1.3 million different oligonucleotide probes are synthesized on each array. Each oligonucleotide is located in a specific area on the array called a probe cell. Each probe cell contains hundreds of thousands to millions of copies of a given oligonucleotide. Hybridization of fluorescent-labelled cRNAs from cells or tissues allows the quantification of expression of hundreds to tens of thousands genes in one experiment. The Affymetrix GeneChip® MOE430A 2.0 used here is a single array representing 22690 probe sets for approximately 14000 well-characterized mouse genes.
Total RNA of murine spleens was prepared as described and 5 µg of this RNA was further processed and hybridized to the murine expression array MOE430A 2.0 according to Affymetrix protocols. Briefly, total RNA is first reverse transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA is purified and serves as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction is carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labelling. The biotinylated cRNA targets are then cleaned up, fragmented, and hybridized to GeneChip® expression arrays. Three biological replicates per condition were analyzed. The microarrays were scanned and analyzed using Affymetrix Microarray Suite v5.0 software.
For global normalization and the generation of expression values, Affymetrix CEL files were imported into the software RMAExpress (Ikehara, 1998), where background correction algorithm and quantile normalization were used. The list of significantly regulated genes was obtained by applying the SAM multiclass algorithm (Tusher et al., 2001) of the samr package

for R. Further data preparation was performed with the Spotfire DescisionSite Software (Spotfire), and hierarchical clustering was performed using the program Genesis (Sturn et al., 2002).

3.2.5.10 Northern blot

RNA was isolated as described. The gel apparatus was incubated overnight in 3 % H_2O_2 to destroy ribonucleases. The following solutions were added to 5–10 µg of RNA:

3	µl 10x MOPS buffer
10	µl Formamide
6	µl Formaldehyde
ad 27	µl with DEPC-H_2O

The samples were incubated for 10 min at 70°C and cooled on ice. Next, 3 µl of the loading dye were added and the RNA was size-separated by gel electrophoresis at 140 V for 2 h on a formaldehyde denaturing agarose gel. To stain the RNA, the gel was incubated for 45 min in 1x MOPS buffer with EtBr (100 ng/ml), washed twice with ddH_2O for 1 h, and finally UV visualized for loading control. Then, RNA was transferred by the capillary transfer method onto a positively charged nylon membrane (Hybond-XL, Amersham) in 20x SSC buffer (Invitrogen). After the transfer, the RNA was cross-linked with the membrane using a UV-Crosslinker (Stratagene).

For probe preparation, clones containing partial or complete cDNA of the genes of interest were obtained from RZPD (Berlin). The insert was released by appropriate restriction enzyme digestion and gel purified. DNA (25 ng) was labelled with 5 µl of [^{32}P]-dCTP (Amersham) using the PrimeKit I (Stratagene) and purified over columns (Amersham) according to the manufacturer's protocols. The membrane was pre-hybridized with 20 ml of Church buffer for 2 h at 65°C on a rotator (Biometra). Then, the probes were hybridized to membranes in 8 ml of fresh Church buffer overnight. The membrane was washed twice with 2x SSC + 0.1 % SDS for 10 min at 65°C, followed by one wash with 0.2xSSC + 0.1 % SDS for 20 min at 65°C. For detection, the Phosphoimager System (Molecular Dynamics) was used.

If the membrane was subject to incubation with a different probe, a stripping protocol was carried out to remove the previous probe from the membrane. Briefly, the membrane was incubated in pre-boiled 0.1 % SDS for 30 min, followed by pre-hybridization with Church buffer.

3.2.6 Immunology

3.2.6.1 Enzyme linked immunosorbent assay (ELISA)

3.2.6.1.1 ELISA buffers and solutions

Blocking buffer:	1	x PBS
	1	% BSA
	5	% Sucrose
Reagent Diluent:	1	x PBS
	1	% BSA
Washing buffer:	1	x PBS
	0.05	% Tween 20
Phosphate-Citrat buffer:	25.7	ml 0.2 M Na_2HPO_4
	24.3	ml 0.1 M Citric Acid 1-hydrate (pH 5.0)
	50	ml ddH_2O
	adjust to pH 5.0	with HCl
Substrate reagent:	1	tablet Tetramethylbezine (TMB)
	10	ml 0.05 M Phosphate-Citrat buffer
	2	µl H_2O_2 30 %
Stop solution:	2	N H_2SO_4

3.2.6.1.2 Determination of cytokine and chemokine levels by ELISA

In ELISAs a capture antibody is linked to a polymeric matrix. By adding cell extracts or supernatants, antibody-antigen complexes are formed. These complexes can be detected by adding a detection antibody which recognizes a different epitop of the antigen. The detection antibody is tagged with an enzyme (e.g. peroxidase) which transforms a colourless substrate into a coloured product. By measuring the intensity of the colour the level of, for example cytokine production, can be determined.

All proteins were detected by DuoSet ELISA Development System (R&D Systems) following the manufacturer's protocol. Briefly, plates were coated with 100 µl per well of capture antibody (720 ng/ml) in 1x PBS and incubated overnight at 4°C. The next day the plate was

tapped dry and 250 µl per well of blocking buffer were added. The following incubation took place for 1 h at room temperature or at 4°C overnight. Afterwards, the plate was washed 3 times with 250 µl washing buffer, tapped dry and 100 µl per well of the samples in reagent buffer and standards were added. Incubation took place at room temperature for 2 h or at 4°C overnight and was followed by three washing steps. The detection antibody (36 µg/ml) was diluted in reagent diluent and added in a volume of 100 µl per well, the plate was incubated for 2 h and washed 3 times. This was followed by incubation with 100 µl per well of Streptavidin-HRP (50 µl in 10 ml reagent diluent). Finally the plate was washed 3 times and fresh substrate reagent was added (100 µl/well). The plate was incubated in the dark because the substrate reagent contains H_2O_2, which is known to be light sensitive. The incubation time ranged from 10-60 min, depending on the protein detected. To stop the reaction 50 µl per well of stop solution were added and the plate was analyzed in the ELISA reader at 450 nm (reference at 570 nm).

3.2.6.2 NO-Assay

The concentration of nitric oxide in the supernatant of the cultured cells was measured using the Griess-Reagent (Green et al., 1982).

Griess Reagent A:	0.4	g N-(1-Naphtyl) Ethlyendiamin Dihydrochlorid
	ad 200	ml ddH_2O
Griess Reagent B:	4	g Sulphanilamid
	10	g H_3PO_4
	ad 200	ml ddH_2O

The supernatant (50 µl) was mixed with 50 µl of a 1:1 mixture of Reagent A and B in an ELISA plate (Nunc). After incubation for 10 min at RT, the plate was measured in the ELISA reader at 540 nm. The NO concentration of the samples was calculated by comparing their absorbance to that of a range of standard concentrations of $NaNO_2$ on the same plate.

3.2.6.3 Flow cytometry

3.2.6.3.1 Flow cytometry buffers

FACS buffer:	1	% BSA in 1 x PBS

Erythrocyte lysis buffer:	8.29	g NH_4Cl
	1	g $KHCO_3$
	37.2	mg Na_2EDTA
	adjust to pH 7.2-7.4	with 1 N HCl
	ad 1000	ml ddH_2O

3.2.6.3.2 Analysis of cell surface antigens by flow cytometry

Up to $1x10^6$ cells per staining were centrifuged at 2000 rpm at 4°C for 1 min in a 1.5 ml tube. The supernatant was discarded and the cells were washed twice with 1 ml FACS buffer (centrifugation at 2000 rpm, 4°C, 1 min) and resuspended in 1 ml FACS buffer. To stain dead cells, 1 µl of Ethidium monoazide (0.5 mg/ml) was added and cells were incubated 10 min on ice in darkness, followed by 10 min in direct light. After washing the cells once, cell surface Fc-receptors were blocked by incubating the cells with unlabeled anti-CD16/CD32 antibody for 10 min at 4°C. Meanwhile the appropriate staining solutions were prepared. After blocking Fc-receptors the cells were centrifuged one more time. The cells were resuspended in 100 µl of the ready made staining solutions. Incubation lasted for 20 min in the dark at 4°C and was followed by two washing steps (150 µl/sample, centrifugation at 2000 rpm, 4°C, 1 min). In the case of Biotin-conjugated antibodies, a second staining step with Streptavidin-PE conjugates was attached. Briefly, after the two final washing steps, the cells were resuspended in 100 µl of the staining solutions containing the Streptavidin-PE conjugates for 30 min at 4°C in darkness. Finally, the supernatant was discarded, the cells were washed twice and resuspended in 200 µl FACS buffer. Stained samples were analyzed by flow cytometry using a FACSCalibur (BD Bioscience) and the data were analysed using the software FlowJo.
If peripheral blood samples were analysed, they were treated with Erythrocyte lysis buffer prior to starting general staining procedures.

3.2.6.4 Magnetic activated cell sorting (MACS)

This purification method is based on binding of the cells to antibodies that are attached to magnetic beads. The labelled cells can then be separated from unlabeled cells by means of a magnetic field.

Cell separation was carried out using the Midi-MACS Separator (Miltenyi) according to the manufacturer's protocol for LS columns. Briefly, $1x10^8$ cells were resuspended in 900 µl MACS buffer (1x PBS + 0.5 % BSA). For the positive selection of T cells, 100 µl of an antibody specific for the T cell surface marker CD90 (Thy1.2) (Miltenyi) was added to the cells, mixed and the solution was incubated for 15 min at 4°C. LS columns were equilibrated and used as described in the protocol. Unlabelled cells of the flow through and the positively selected cells were examined for T cells content by flow cytometry.

4 Results

4.1 Expression and regulation of DUSP1

4.1.1 Mining of microarray datasets for regulation of phosphatases in macrophages

To obtain a global view of the expression and regulation of phosphatases in macrophages, I took advantage of two microarray datasets that have been described previously (Lang et al., 2002; Schmitz et al., 2004). In the first experiment, the shaping of the transcriptional response to LPS by IL-10 was investigated after 45 min and 3 h. IL-10-deficient bone marrow-derived macrophages (BMM) were used to eliminate effects of endogenous IL-10. Of the more than 300 probesets of the MG-U74Av2 GeneChip carrying a functional annotation of "phosphatase" 182 were expressed and used for a hierarchical cluster analysis (Figure 5A). Most of these genes did not change expression after stimulation with LPS and IL-10 alone or in combination, but a small group clustering at the bottom of the graph showed induction. A closer look at this group of phosphatases (Figure 5A, right panel) shows that some genes are represented by two probesets, reducing the number of induced phosphatases to ten. Out of these, three are different MAPK phosphatases, namely DUSP1, DUSP2 and DUSP16. The oligonucleotide array MG-U74Av2 contains probesets representing nine different DUSPs, whose expression values under the influence of IL-10 and LPS are depicted in Figure 5B. While some DUSPs were already expressed under baseline conditions, stimulation with LPS led to strongly increased levels of DUSP1, DUSP2 and DUSP16. Expression of DUSP2 was unaffected by IL-10, whereas expression of DUSP16 was increased by the addition of IL-10 to LPS at both time points. DUSP1 mRNA was transiently induced by LPS alone at 45 min and reached baseline levels again after 3h, but addition of IL-10 caused sustained expression at the 3-h time point.

The second microarray dataset consisted of RAW 264.7 macrophages stimulated with different TLR ligands for 6 h to identify common and specific transcriptional responses (Schmitz et al., 2004), and was analysed here for changes in the expression of DUSP family members (Figure 5C). Although the time point analysed was different from the first dataset, LPS induced mainly DUSP1, DUSP2 and DUSP16 expression also in RAW 264.7 macrophages. All ligands signalling through MyD88 induced expression of these three DUSPs, with some minor differences. CpG oligodeoxynucleotides (ODN) were a better stimulus for expression of DUSP1 and DUSP2 compared to the ligands of TLR2 (Pam3Cys,

MALP) and TLR7 (R848), which in turn more strongly induced DUSP16. In contrast, polyIC, which signals *via* TRIF, induced some DUSP16 but had no effect on DUSP1 and DUSP2.

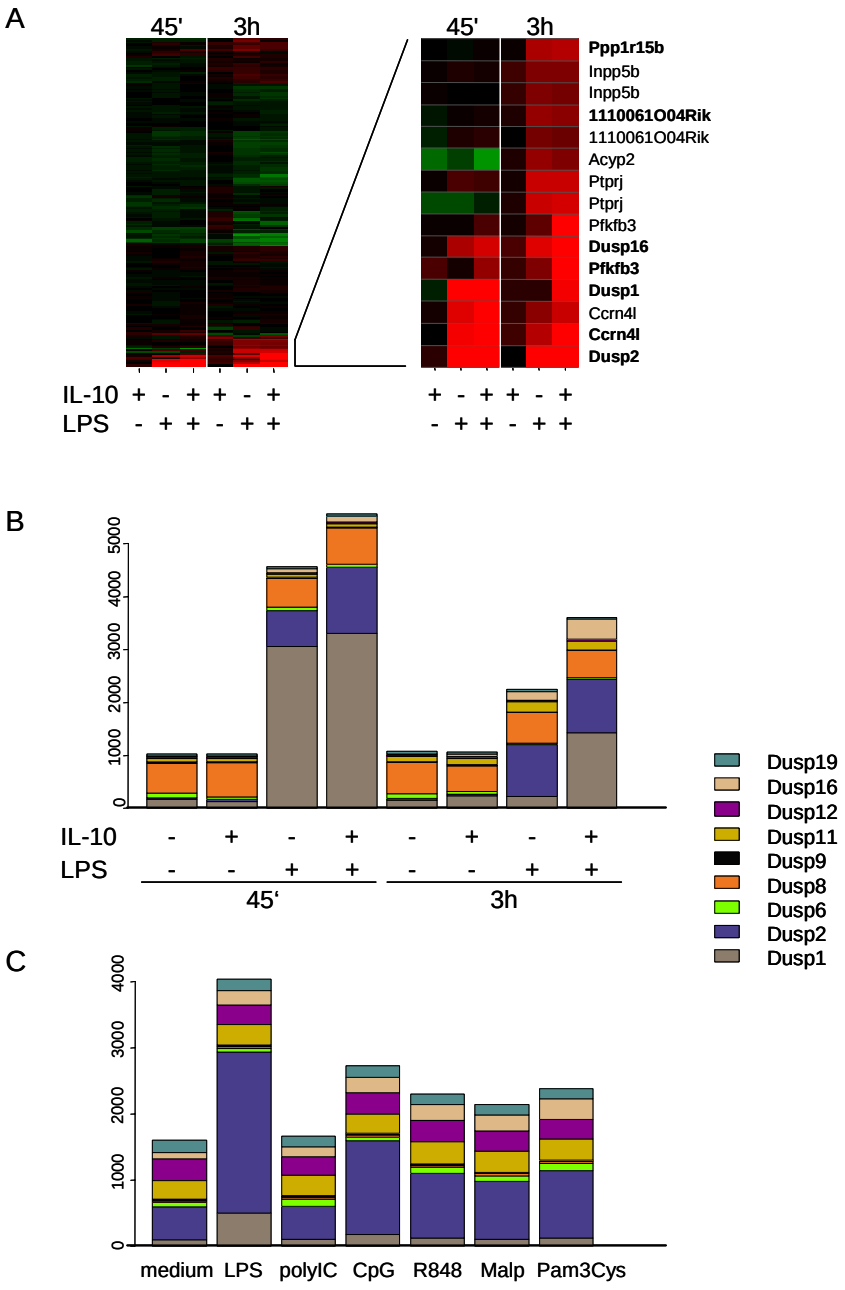


Figure 5. Global changes in the expression levels of phosphatases in activated macrophages under the control of IL-10.

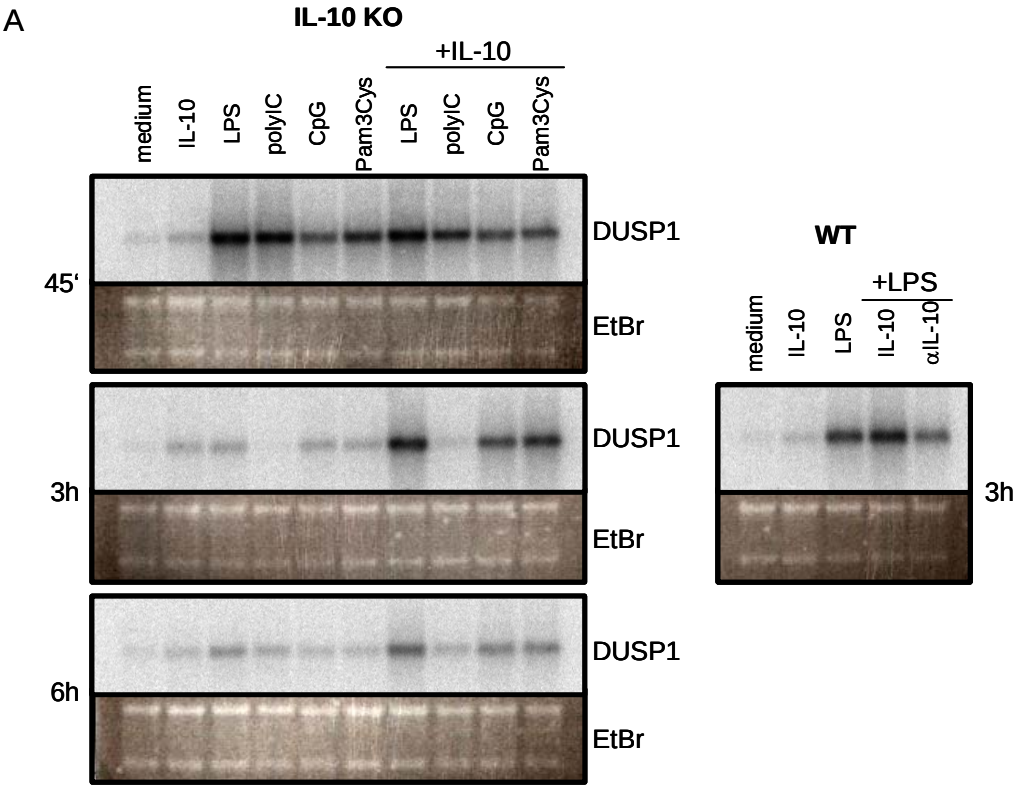
(A) Microarray data from IL-10-deficient macrophages stimulated with IL-10 (10 ng/ml) or LPS (100 ng/ml) were filtered for genes annotated as "phosphatase" and showing an Affymetrix present call in more than one sample. Fold changes relative to the medium control at 45 min and 3 h were calculated from the average expression values as signed ratios. Hierarchical clustering was performed using Spotfire software. Up-regulated genes are shown in red (signed ratio ≥ +5), down-regulated genes in green (signed ratio ≤ -5). The right panel is labelled with gene symbols; bold print indicates an ANOVA p value < 0.01. (B, C) Stacked bar graph representation of expression values for DUSP family members in IL-10-deficient macrophages treated with LPS and IL-10 (B) and in RAW 264.7 macrophages treated with various TLR stimuli (C). Data are presented as normalized average expression values from duplicate (B) and triplicate (C) experiments.

4.1.2 Validation of the microarray results for DUSP1

Further experiments focused on the validation of the analysed microarray experiments and the regulation of DUSP1 by IL-10 in LPS-activated macrophages. Using Northern blot analysis, I observed that the TLR ligands LPS, CpG, Pam3Cys and polyIC induced strong but transient DUSP1 expression in IL-10-deficient macrophages (Figure 6A). Confirming and extending the microarray results, addition of exogenous IL-10 had little effect after 45 min, but synergized with LPS, CpG and Pam3Cys to increase expression of DUSP1 after 3 h, and to a lesser extend after 6 h (Figure 6A). In contrast, polyIC-induced DUSP1 expression was rapidly down-regulated after 3 h and not influenced by IL-10. LPS-induced DUSP1 expression after 3 h was stronger in wild-type macrophages than in IL-10-deficient cells and the addition of IL-10 had only a limited effect. Blocking of endogenous IL-10 in LPS-stimulated cells by addition of an anti-IL-10 antibody partially suppressed DUSP1 mRNA levels (Figure 6A).

The data obtained by Northern blot analysis for DUSP1 mRNA levels could be confirmed and further extended on the protein level by Western blot. At 30 min DUSP1 protein was induced by LPS in wild-type macrophages, whereas addition of IL-10 rather decreased expression at this time point (Figure 6B). DUSP1 levels peaked at 90 min before they decreased again. IL-10 further augmented DUSP1 expression after 90 min and 3 h, confirming the observation on mRNA levels.

To further investigate if the induction of DUSP1 expression in activated macrophages was specific for IL-10, cells were stimulated with exogenous IFN-γ and IL-6 and transcript levels of DUSP1, TNF-α and IL-6 were analysed by Northern blot. The results show that neither IFN-γ nor IL-6 had any influence on DUSP1 expression in activated macrophages (Figure 6C, D). As expected, IFN-γ had a synergistic effect on TNF-α production, whereas IL-6 levels were unaffected by the additional stimuli. Furthermore, in concordance with the known inhibitory effect of IL-10 on macrophage cytokine production, IL-10-induced increases in DUSP1 expression were accompanied by suppression of TNF-α and IL-6 (Figure 6C, D).


A
IL-10 KO
+IL-10
medium
IL-10
LPS
polyIC
CpG
Pam3Cys
LPS
polyIC
CpG
Pam3Cys
45'
DUSP1
EtBr
3h
DUSP1
EtBr
6h
DUSP1
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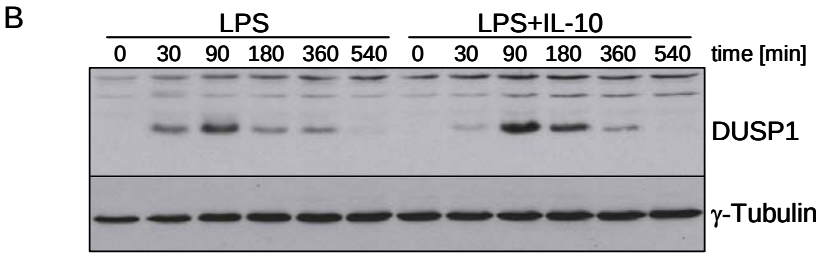
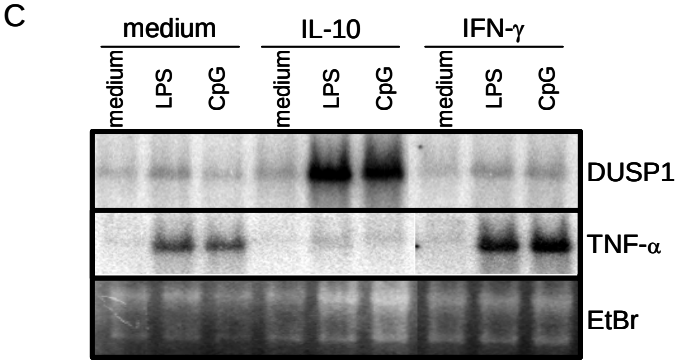
LPS
IL-10
αIL-10
3h


B
LPS
LPS+IL-10
0 30 90 180 360 540 0 30 90 180 360 540
time [min]
DUSP1
γ-Tubulin


C
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LPS
CpG
medium
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medium
LPS
CpG
DUSP1
TNF-α
EtBr

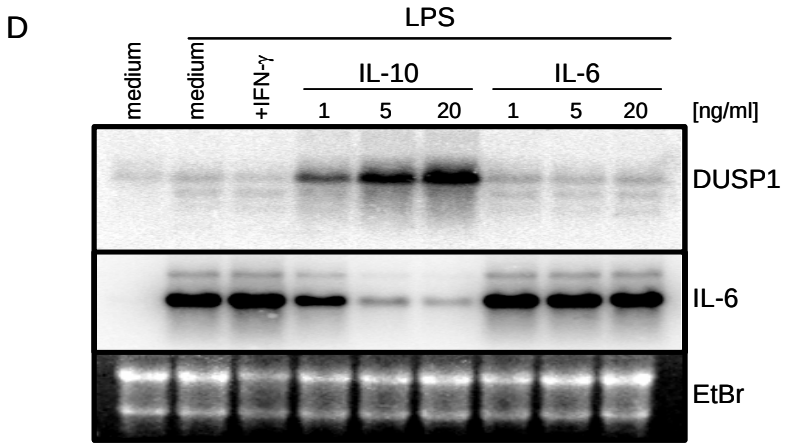


Figure 6. Induction of DUSP1 in activated macrophages.
RNA and protein lysates were prepared from macrophages as described. Total RNA (10 µg) was separated on 1% formaldehyde agarose gels. Ethidium bromide staining was used as a loading control. Total cell lysates (10 µg) were separated by SDS-PAGE and immunoblotted as described. (A) BMM were stimulated with LPS (100 ng/ml), CpG ODN 1668 (1 µM), polyIC (50 µg/ml), Pam3Cys (1 µg/ml) in the presence or absence of IL-10 (10 ng/ml) for the indicated times. Neutralizing mAb against murine IL-10 was added at 10 µg/ml 10 min prior to LPS. (B) Wild-type BMM were stimulated for the indicated times with LPS (100 ng/ml) alone or in combination with IL-10 (10 ng/ml). (C) IL-10 KO BMM were stimulated for 3 h with LPS (100 ng/ml) or CpG ODN 1668 (1 µM) alone or in combination with IL-10 or IFN-γ (both at 10 ng/ml). (D) IL-10 KO BMM were treated for 3 h with LPS alone and in combination with IFN-γ (50 ng/ml) or increasing amounts of IL-10 or IL-6.

4.1.3 Sustained activation of p38 MAPK in IL-10 knockout macrophages

Given the preference of DUSP1 for the inactivation of p38 MAPK (Franklin and Kraft, 1997), I hypothesized that stunted expression of DUSP1 in LPS-stimulated IL-10-deficient macrophages may cause prolonged phosphorylation of p38 MAPK. To test this, wild-type and IL-10-deficient macrophages were stimulated with LPS and phosphorylation of p38 was analysed by Western blot at different time points. The kinetic analysis indeed showed stronger and prolonged phosphorylation of p38 in IL-10-deficient compared to wild-type cells (Figure 7). Addition of exogenous IL-10 did not affect early p38 activation, but caused faster down-regulation of p38 phosphorylation in IL-10-deficient macrophages, whereas it had little effect in wild-type cells (Figure 7). To show that this IL-10-mediated effect is specific for p38, the same protein lysates were analysed for phosphorylation levels of another MAPK, namely ERK1/2. In contrast to p38, phosphorylation of the ERK1/2 MAPK was induced to similar levels in IL-10-deficient and wild-type macrophages early after LPS stimulation and was rapidly down-regulated, with little obvious influence of endogenous or added IL-10 (Figure 7).

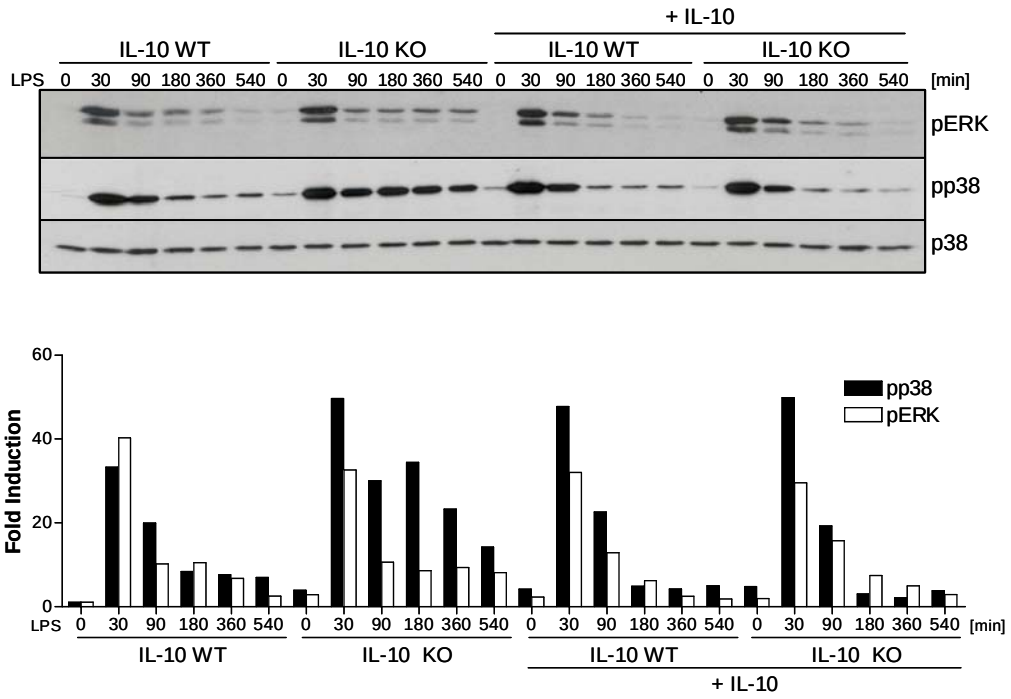


Figure 7. Effect of IL-10 on the time course of p38 and ERK MAPK activation.
Wild-type and IL-10-deficient BMM were pre-treated with IL-10 (10 ng/ml) for 15 min or not, and stimulated with LPS (100 ng/ml) for the indicated times. Total cell lysates (15 µg) were separated by SDS-PAGE and immunoblotted as described. For semiquantitation, densitometry was performed using total p38 and γ-tubulin (not shown) for normalization of pp38 and pERK1/2, respectively. Data presented are representative of three independent experiments.

4.1.4 Stability of DUSP1 mRNA

In an attempt to determine whether DUSP1 is a direct transcriptional target of IL-10, macrophages were stimulated in the presence of the inhibitor of protein synthesis cycloheximide (CHX) (Figure 8A, B). Strikingly, CHX by itself up-regulated DUSP1 mRNA levels without any further enhancement by LPS or IL-10. This effect can be solely attributed to CHX, since its solvent DMSO had no influence on DUSP1 expression. Further, it was specific for DUSP1, since expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as loading control, was unaltered under all conditions (Figure 8A). The mRNA accumulation could be due to abrogated translation of a repressor that inhibits DUSP1 mRNA expression, or to an increase in mRNA stability. To further examine this, the inhibitor of transcription Actinomycin D was used to determine the decay rate of DUSP1 mRNA under basal and stimulated conditions in bone marrow-derived macrophages. Figure 8B shows that the DUSP1 mRNA was highly unstable, regardless of whether LPS alone was the stimulus or applied in combination with IL-10. For comparison, membranes

were also probed for TNF-α, a prototypically unstable mRNA harbouring a classical AU-rich element in the 3'UTR, or GAPDH as controls. The half life of the mRNA for DUSP1 and TNF-α was similar, ranging from 30 to 45 min, with little effect of the addition of IL-10. In contrast, addition of CHX 2 h before Actinomycin D treatment caused substantial stabilization of the DUSP1 mRNA (Figure 8B).

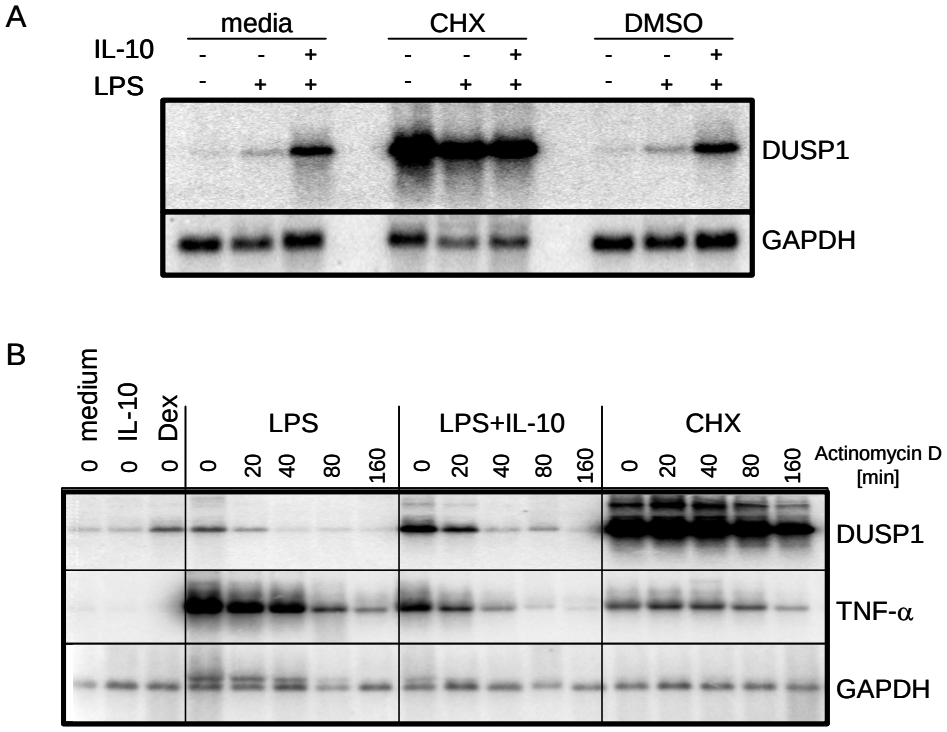


Figure 8. DUSP1 mRNA is unstable and accumulates in the presence of CHX.
(A) IL-10-deficient BMM were pre-treated for 15 min with CHX (20 µg/ml), DMSO or medium, followed by addition of LPS (100 ng/ml) or IL-10 (10 ng/ml) as indicated. After 2 h, RNA was prepared. Total RNA (10 µg) was used for Northern blot analysis as described. (B) IL-10-deficient BMM were treated for 2 h with IL-10 (20 ng/ml), DEX (300 nM), LPS (100 ng/ml) or CHX (20 µg/ml). After addition of Actinomycin D (10 µg/ml), cells were harvested for RNA preparation at the indicated time points. Decay of DUSP1 and TNF-α mRNA was analyzed by Northern blot analysis. The TNF-α blot was reprobed with a GAPDH cDNA and shows some residual signal from the TNF-α probe.

4.1.5 Activation of DUSP1 reporter by LPS but not by IL-10

To assess whether DUSP1 gene expression is regulated by LPS and IL-10 at the promotor level, several fragments of the mouse DUSP1 promotor ranging in size from 0.7 kb to 2 kb were PCR-amplified and cloned upstream of a Luciferase reporter gene (Figure 9A). Inducibility of the reporter constructs by LPS and IL-10 was determined using transient and stable RAW 264.7 transfectants. Since the results were comparable for the various fragments used, and between transient and stable transfection, only the data obtained with a stable transfectant of the 1-kb promotor fragment are presented. DUSP1 promotor fragments

conferred stronger basal expression compared to the pGL3-basic parent reporter construct. I observed inducibility of the DUSP1 reporter after stimulation with LPS (Figure 9B). However, I did not observe induction of reporter gene activity by stimulation of transfected RAW 264.7 macrophages with IL-10, or synergistic induction in combination with LPS (Figure 9B; upper panel), although the stably transfected lines responded to the addition of IL-10 by increased expression of endogenous DUSP1 mRNA, as expected (Figure 9C). In addition to DUSP1, I also probed the membrane for the luciferase (luc) mRNA. Confirming the observation for the protein levels, luc mRNA was induced by LPS, but unaffected when IL-10 was used in combination (Figure 9C). The short half life of the DUSP1 mRNA observed before suggested a similar role for the 3'UTR in the regulation of the DUSP1 mRNA levels as reported for TNF-α mRNA. I therefore created another reporter construct, in which the SV40 polyadenylation site of the pGL3-based reporter construct was replaced with the 3'UTR of the DUSP1 mRNA, and observed a reduced basal luciferase activity compared to the parent construct after stable transfection in RAW 264.7 macrophages (Figure 9B; upper panel). Inducibility by LPS was slightly higher than with the DUSP1 promotor construct containing the SV40-based 3'UTR. However, IL-10 again did not affect reporter gene activity. To exclude confounding effects of LPS-induced IL-10 produced by RAW 264.7 macrophages, I repeated the experiment in the presence of a neutralizing anti-IL-10 antibody, but did not observe any difference in the stimulation of LPS-induced reporter gene activity (not shown). *In silico* analysis of the DUSP1 promotor using MatInspector (Genomatix, Germany) revealed that no STAT3 binding sites were present in the examined promotor sequences, however, three STAT3 binding sites were identified 7 kb, 12 kb and 21 kb upstream of the transcriptional start site (Figure 9A). To test whether these sites might render the reporter responsive to IL-10, I PCR-amplified fragments surrounding these sequences and inserted them into the reporter construct containing the 1-kb DUSP1 promotor sequence and the DUSP1 3'UTR, and into the pGL3-promotor reporter construct, in which the reporter gene is under the control of the SV40 promotor. Since the data obtained from both constructs were comparable, only the results for the construct containing the STAT3 sites in combination with 1-kb DUSP1 promotor and 3'UTR are depicted. Transcription of the reporter gene was induced by LPS in all cases with a slightly higher induction when the third STAT3 site was included (Figure 9B; bottom panel). Furthermore, neither the sole stimulation with IL-10 did induce any reporter gene activity, nor did the combination of LPS and IL-10 further affect reporter gene levels. Finally, an effect of endogenously produced IL-10 could be excluded by

the addition of the neutralizing anti-IL-10 antibody, which did not influence reporter gene activity (Figure 9B).

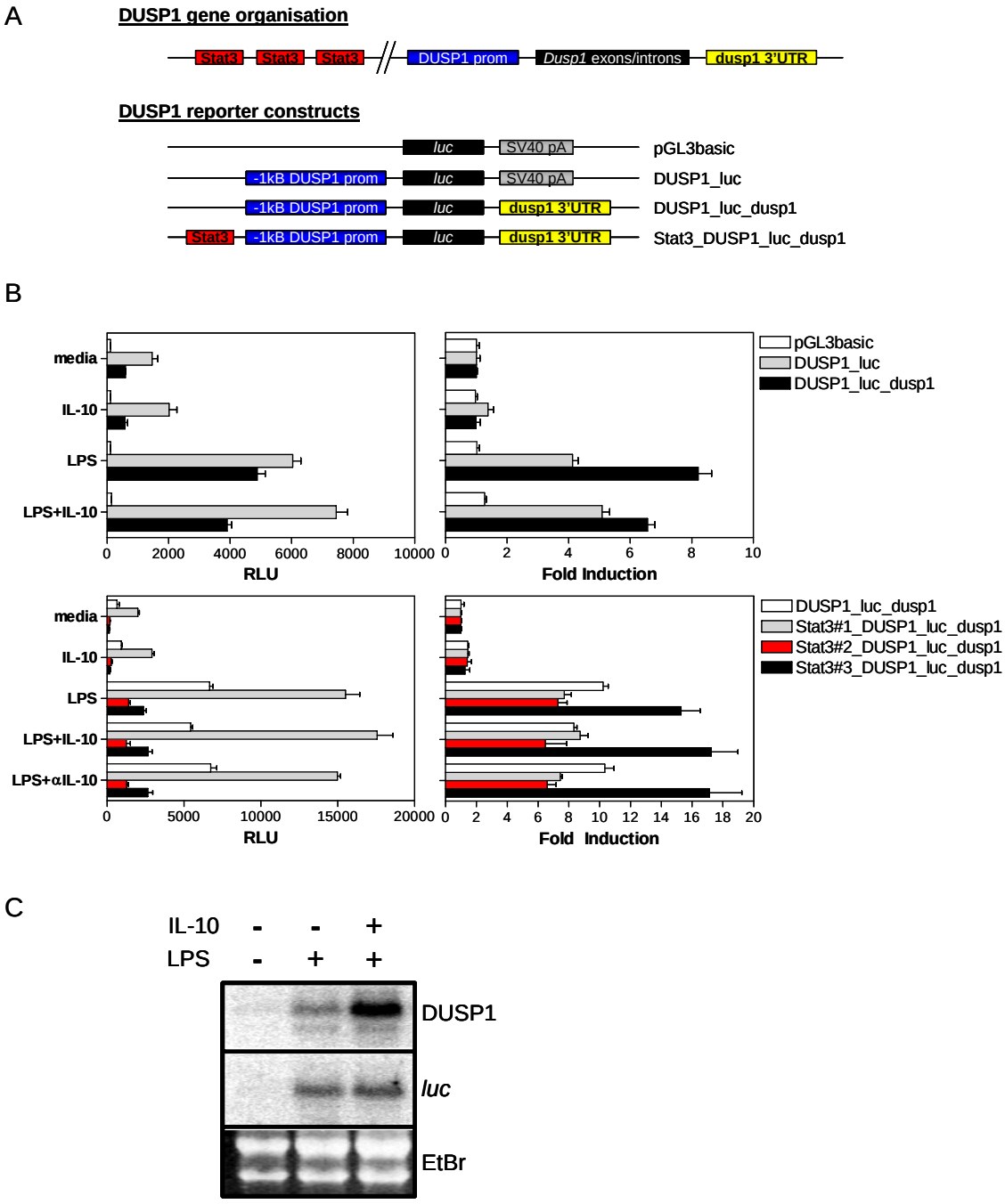


Figure 9. Activation of DUSP1 reporter constructs.
(A) Schematic representation of the DUSP1 gene and the luciferase reporter constructs; 3' untranslated region (UTR); STAT3 binding sites; DUSP1 promotor (prom); luciferase gene (luc), and the simian virus 40 (SV40) late polyadenylation signal (pA) are indicated. (B) RAW 264.7 macrophages stably transfected with the reporter constructs were stimulated with LPS (100 ng/ml) and IL-10 (10 ng/ml) as indicated. Neutralizing mAb against murine IL-10 (αIL-10) was added at 10 µg/ml 10 min prior to LPS. After 8 h, cells were lysed in Reporter lysis buffer and assayed for Luciferase activity as described. Fold inductions refer to unstimulated controls. Data shown are means ± SD of triplicate measurements and representative of three independent experiments. (C) RAW 267.4 macrophages harbouring DUSP1_luc were assayed by Northern blot for expression of DUSP1 mRNA and of the Luciferase reporter gene 2 h after stimulation with LPS alone or in combination with IL-10.

4.1.6 LPS-induced DUSP1 expression is independent of IKKβ

As mentioned in the introduction, previous studies could show that DUSP1 can be induced by LPS *via* activation of ERK or JNK, which in the case of the DUSP1-reporter constructs might also account for their induction. However, the role of the NF-κB pathway in the control of DUSP1 expression after LPS stimulation has not been investigated yet. I addressed this question by using bone marrow-derived macrophages from IKKβ$^{flox/flox}$/LysMcre conditional knockout mice, in which IKKβ is disrupted only in macrophages and neutrophils. This was achieved by breeding of mice carrying a loxP-flanked IKKβ allele (Greten et al., 2004) with LysMcre mice, in which the Cre-recombinase is expressed under the control of the murine lysozyme M gene regulatory region (Clausen et al., 1999). IKKβ is a kinase upstream of the NF-κB transcription factor. Its deletion prevents NF-κB translocation to the nucleus and therefore abrogates NF-κB-mediated signalling. As shown in Figure 10A deletion of IKKβ was not complete, but severely reduced in BMM of IKKβ$^{flox/flox}$/LysMcre mice. Expression of DUSP1 was induced within 30 min after stimulation with LPS, but did not significantly differ between BMM from IKKβ$^{flox/flox}$ and IKKβ$^{flox/flox}$/LysMcre mice. The findings on protein level could further be confirmed on mRNA level by real-time quantitative PCR (Figure 10B). As for the protein, DUSP1 mRNA expression was not significantly altered by IKKβ deficiency 90 min and 3 h after LPS stimulation. In contrast, expression of PTX3, a known target gene of NF-κB and therefore a good control for IKKβ deletion efficiency, showed highly diminished induction in the IKKβ-deleted macrophages (Figure 10B).

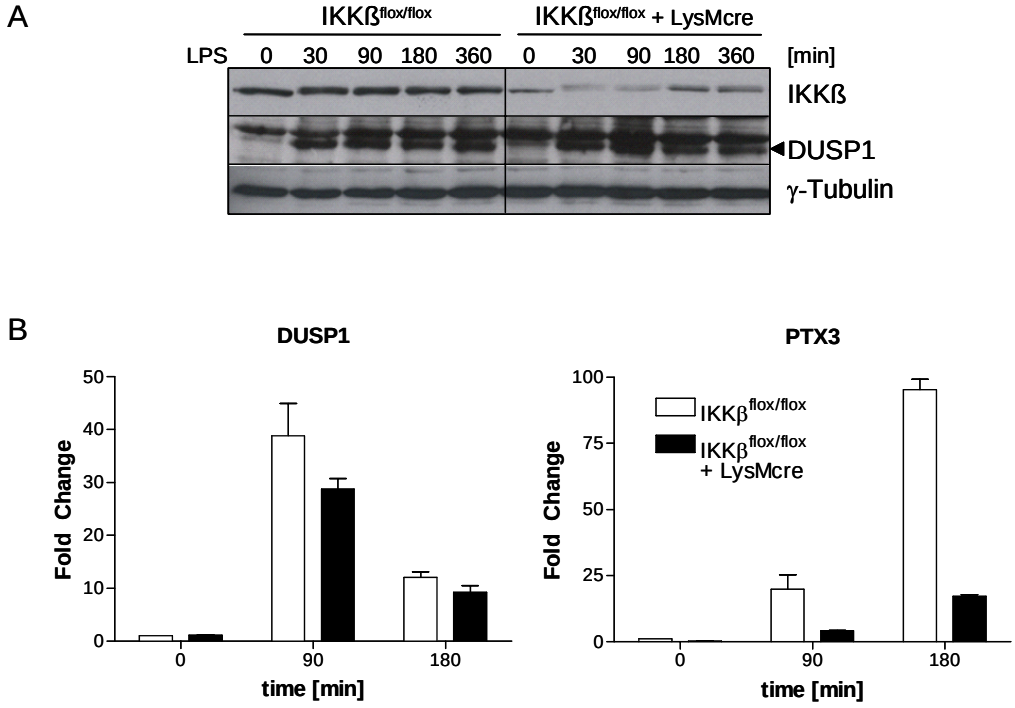


Figure 10. DUSP1 expression is independent of IKKβ in activated macrophages.
(A) BMM from IKKβ$^{flox/flox}$ and IKKβ$^{flox/flox}$/LysMcre mice were stimulated with LPS (100 ng/ml) for the indicated time. Total cell lysates (15 µg) were analyzed by immunoblotting for IKKβ, DUSP1 and γ-tubulin levels. The DUSP1 antibody detects a double band of which the lower represents DUSP1. (B) IKKβ$^{flox/flox}$ (white bars) and IKKβ$^{flox/flox}$/LysMcre (black bars) BMM were stimulated with LPS (100 ng/ml) for 90 and 180 min. RNAs were extracted as described. DUSP1 and PTX3 mRNA levels were assessed by quantitative real-time PCR and normalized to β-actin. Data are expressed as the fold change in mRNA levels in stimulated relative to unstimulated IKKβ$^{flox/flox}$ cells. Values are means ± SD of two independent experiments.

4.1.7 Synergistic effect of IL-10 and dexamethasone on DUSP1 expression in activated macrophages

As described in the introduction, the synthetic glucocorticoid dexamethasone (DEX) shares immunosuppressive effects with IL-10 and is a known inducer of DUSP1 expression in macrophages and mast cells. I asked whether both agents use the same pathway for induction of DUSP1 expression and performed combination experiments (Figure 11). In contrast to IL-10, DEX dose-dependently increased DUSP1 mRNA already in resting macrophages. Addition of IL-10 did not significantly increase the levels of DEX-induced DUSP1 mRNA in resting macrophages, but IL-10 and DEX synergistically enhanced DUSP1 expression in the presence of LPS (Figure 11A). The high level of DUSP1 expression with the combination of IL-10 and DEX in activated macrophages was paralleled by a more pronounced inhibition of p38 phosphorylation in LPS-activated IL-10-deficient macrophages at the 6-h time point than observed by IL-10, and particularly DEX, alone (Figure 11B). Finally, it was examined if this

synergistic effect could also be observed on the level of cytokine production by activated macrophages. Production of the pro-inflammatory cytokines IL-12p40 and IL-6 was dose-dependently inhibited by IL-10 and DEX alone, but in combination stronger inhibition was observed at lower concentrations of both reagents (Figure 11C).

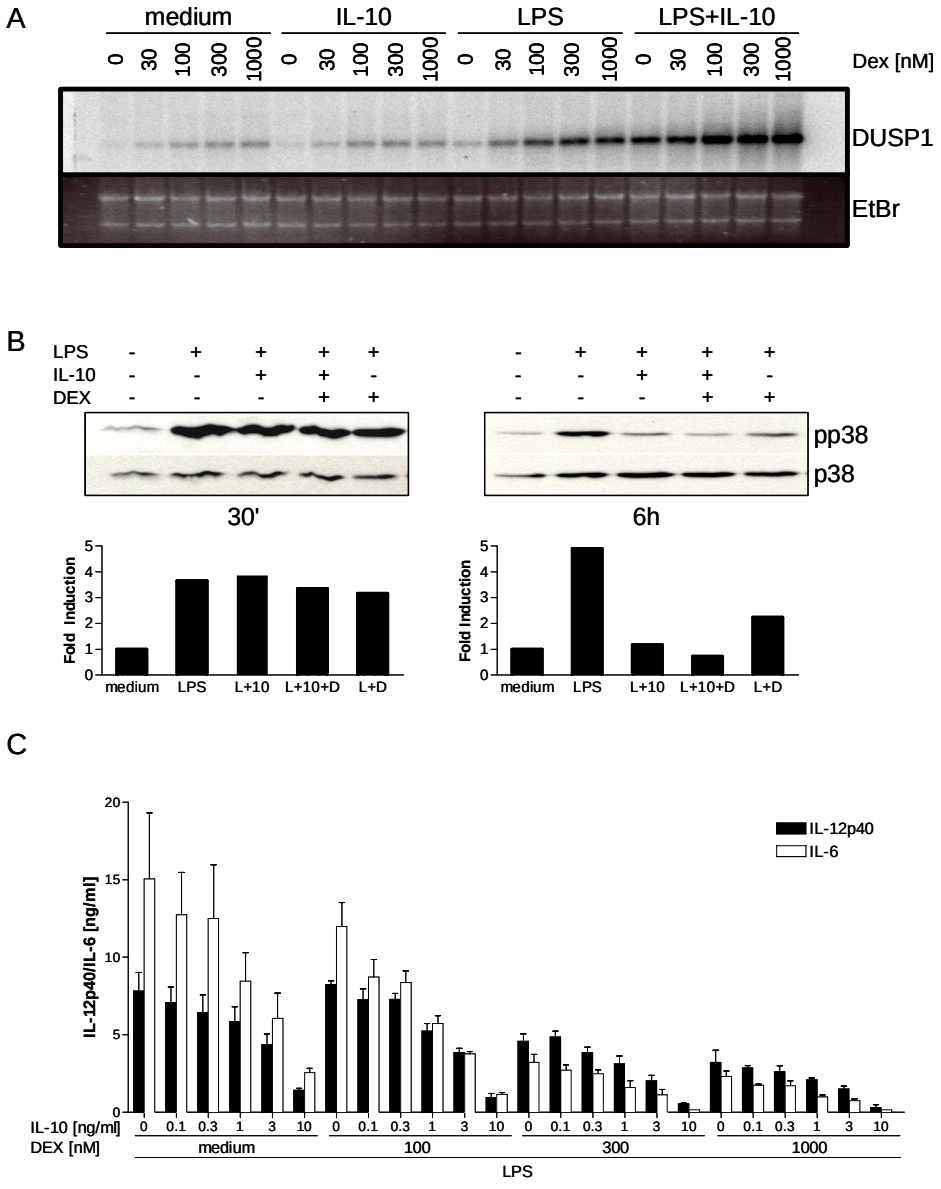


Figure 11. IL-10 and DEX synergize to increase DUSP1 expression and inhibit cytokine production.
(A) IL-10 KO BMM were stimulated for 3 h or not with IL-10 (10 ng/ml), LPS (100 ng/ml) and the indicated concentrations of DEX. Expression of DUSP1 mRNA was determined by Northern blot analysis. Ethidium bromide staining was used as a loading control. (B) IL-10 KO BMM were pre-treated for 15 min with medium, IL-10 (20 ng/ml) or DEX (1000 nM), followed by stimulation with LPS (100 ng/ml) for 30 min or 6 h. Total cell lysates (10 µg) were analyzed by immunoblotting for phosphorylated and total p38 MAPK levels. For semiquantitation, densitometry was performed using total p38 for normalization of pp38. (C) BMM were pre-treated for 15 min with titrated amounts of IL-10 and DEX, and then stimulated with LPS for 20 h. Supernatents were assayed by ELISA for production of IL-6 (white bars) and IL-12p40 (black bars). Shown are means ± SD of quadruplicate measurements from a representative experiment.

4.2 The role of DUSP1 in inflammation and sepsis

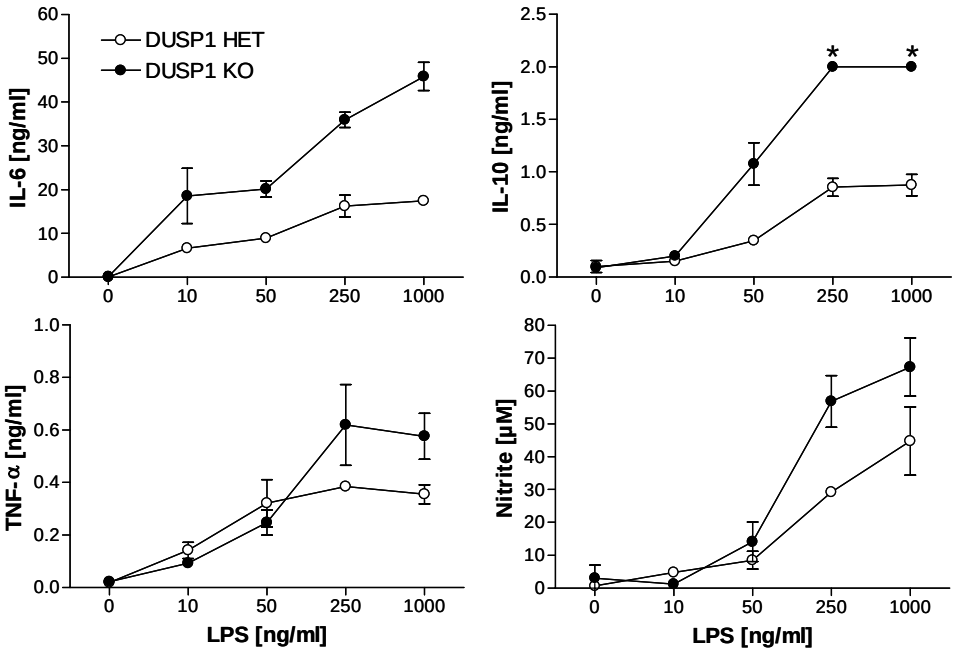
To further investigate the requirement of DUSP1 for the regulation of macrophage activity and the innate immune response in general, I made use of the previously described DUSP1-deficient mice (Dorfman et al., 1996).

4.2.1 LPS-induced cytokine production and MAPK activation in DUSP1-deficient macrophages

Using primary BMM from heterozygous (HET) and DUSP1-deficient mice, I observed significantly higher production of IL-6 and IL-10 in response to titrated amounts of LPS in DUSP1-deficient cells (Figure 12A). The secretion of TNF-α and NO by LPS-stimulated macrophages was less affected by DUSP1 deficiency but higher than in heterozygous control cells (Figure 12A). To further elucidate the mechanisms that led to the up-regulation of these cytokines, a kinetic analysis of MAPK phosphorylation was performed. I found primarily an effect of DUSP1 deficiency on the down-regulation of p38 activation that led to markedly increased phospho-p38 levels at later time points (Figure 12B). In contrast, the kinetics of ERK1/2 activation were similar in control and DUSP1-deficient macrophages, whereas JNK showed a transiently increased phosphorylation status in the absence of DUSP1 (Figure 12B). Further confirmation of prolonged p38 activation was brought about by the investigation of typical downstream targets of p38. The transcription factor ATF-2 and the protein kinase MK2 are well described physiological targets of p38 and both play critical roles in the innate immune response and LPS-induced cytokine production (Kotlyarov et al., 1999; Tsai et al., 2000). Consistent with the sustained p38 activation in DUSP1-deficient BMM, LPS stimulation also resulted in prolonged ATF-2 and MK2 activation and in the case of ATF-2 also in stronger phosphorylation even at earlier time points (Figure 12C). To prove that the increased activity of p38 and its downstream targets is responsible for the enhanced levels of cytokines, macrophages from heterozygous and DUSP1-deficient mice were treated with titrated amounts of the p38-specific inhibitor SB203580 before they were challenged with LPS (Figure 12D). The results revealed that cytokine levels of heterozygous and DUSP1-deficient cells approximate with increasing concentrations of the inhibitor. At concentrations above 5 μM no significant differences in cytokine production were observed anymore. The diminished differences in cytokine production observed at higher concentrations of the inhibitor were accompanied by reduced MK2 phosphorylation in LPS-stimulated macrophages (Figure 12D). Although phospho-MK2 levels were again higher in the DUSP1-

deficient cells compared to heterozygous cells, there was no difference detectable when the cells were pre-treated with an inhibitor concentration of 10 µM.

A



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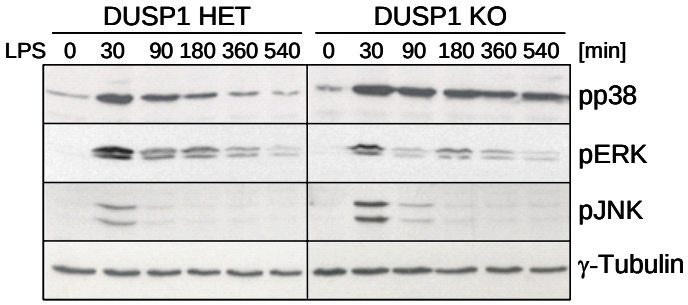

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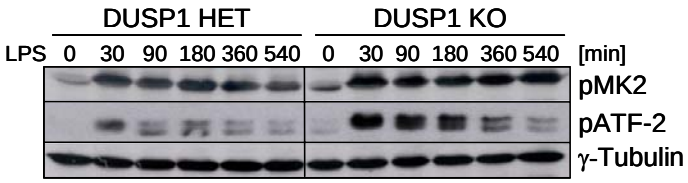


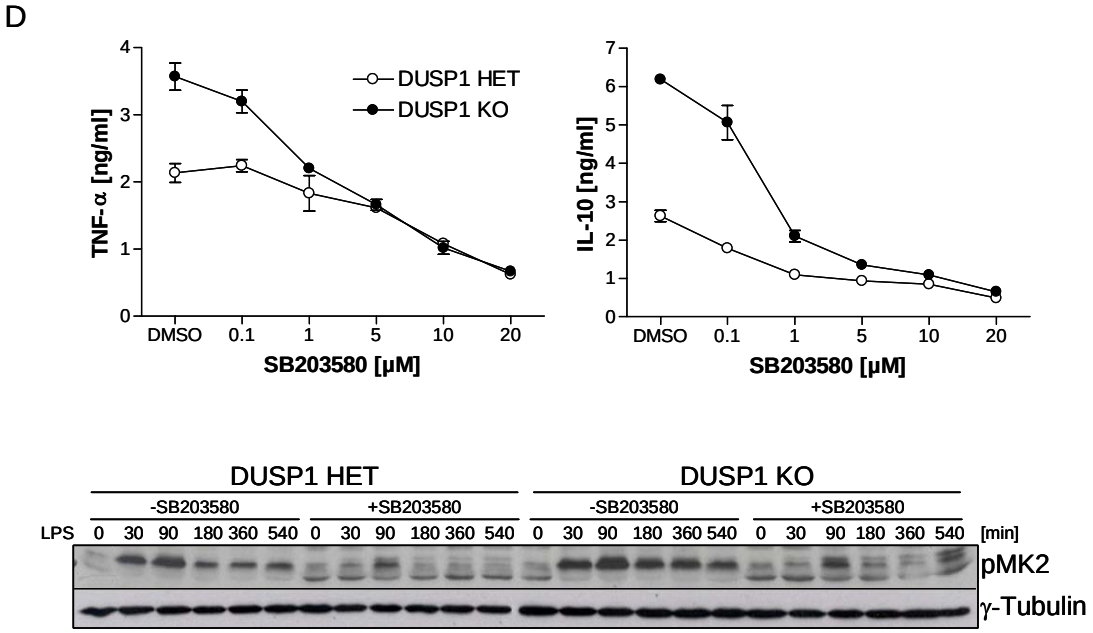


Figure 12. *In vitro* analysis of macrophages from DUSP1-deficient mice.
(A) BMM from DUSP1 HET or DUSP1 KO mice were stimulated with LPS (100 ng/ml) and supernatants were harvested after 5 h (TNF-α), 8 h (IL-6 and IL-10) and 48 h (nitrite). The supernatants were analyzed by ELISA or NO-assay. Data shown are means ± SD of triplicate measurements from a representative experiment. Two data points marked by asterisks (*) were beyond the maximum of the standard curve. (B and C) Kinetics of MAPK activation and downstream targets induced by LPS (100 ng/ml) in BMM from DUSP1 HET or DUSP1 KO mice. Cells were stimulated for the indicated times, and whole cell lysates were analyzed by Western blot using antibodies to phosphorylated p38, ERK1/2, JNK, MK2, ATF2 and to γ-tubulin. Data shown are from one representative experiment out of three performed. (D) BMM from DUSP1 HET or DUSP1 KO mice were pre-treated with titrated amounts of SB203580 or DMSO as control for 30 min prior to stimulation with LPS (100 ng/ml). TNF-α and IL-10 were measured by ELISA after 5 h and 8 h, respectively. Data shown are means ± SD of triplicate measurements from a representative experiment. For detection of phospho-MK2 and γ-tubulin in whole cell lysates by Western blot, BMM were pre-treated with SB203580 (10 µM) prior stimulation with LPS (100 ng/ml) for the indicated times. Data shown are from one representative experiment out of three performed.

4.2.2 Enhanced IFN-β production in DUSP1-deficient mice

The induction of type I interferons by viruses and other pathogens is a crucial event for innate immunity. It is known that particularly IFN-β plays a critical role in LPS-induced shock (Karaghiosoff et al., 2003). Analysis of LPS-induced IFN-β production in macrophages by ELISA revealed elevated levels in DUSP1-deficient cells compared to wild-type cells (Figure 13A). Ligation of IFN-β to its receptor induces signal transduction *via* phosphorylation and activation of the transcription factor STAT1, which forms homodimers but also heterodimers with STAT2. I therefore assessed whether STAT1 phosphorylation is altered in DUSP1-deficient macrophages. As depicted in Figure 13B serine phosphorylation of STAT1 could be detected already 30 min after LPS stimulation, whereas tyrosine phosphorylation was first

detectable after 90 min. However, phosphorylation of both amino acids was much stronger in the DUSP1-deficient macrophages, whereas the total amount of STAT1 protein remained unchanged. The interferon regulatory factors (IRFs) comprise a family of DNA-binding proteins that have been implicated in the transcriptional regulation of IFN and certain IFN-inducible genes. IRF-1 was the first member that was discovered to activate promoters in type I IFN genes (Miyamoto et al., 1988). Further studies revealed that IRF-1 is also involved in the response to LPS (Barber et al., 1995). To examine whether IRF-1 plays a role in the enhanced IFN-β response in DUSP1-deficient macrophages, I checked for IRF-1 expression after LPS stimulation (Figure 13B). Confirming previous reports, I found IRF-1 to be induced by LPS with peak levels 3 h after stimulation. Additionally, IRF-1 was much stronger induced in the macrophages deficient for DUSP1 compared to wild-type, especially at the late time points.

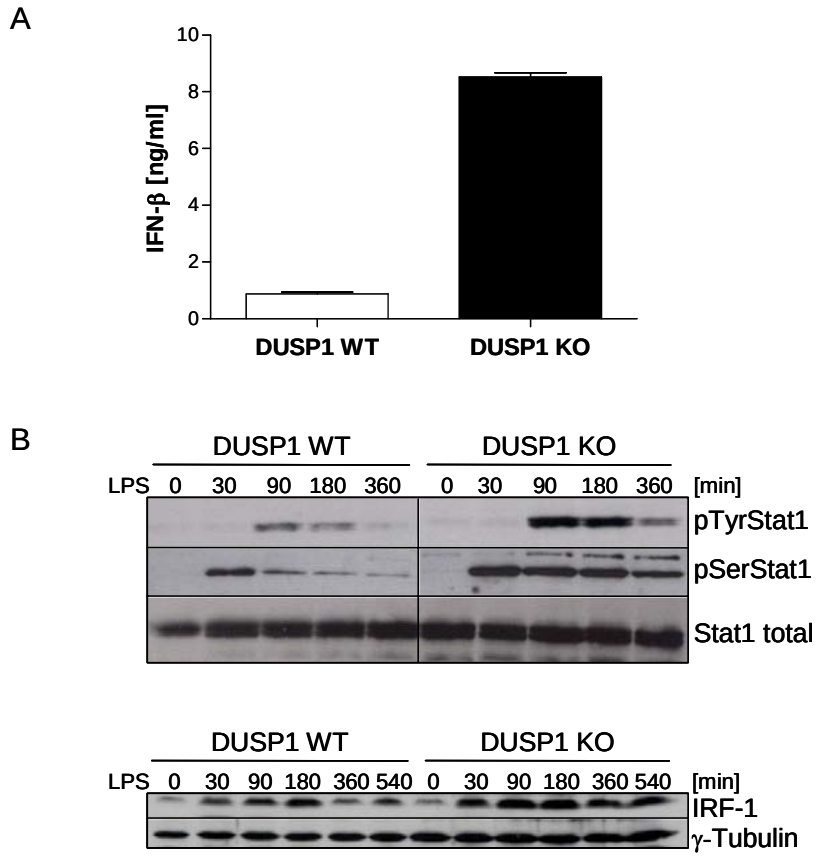


Figure 13. DUSP1-deficient macrophages exhibit enhanced IFN-β production.
(A) BMM from DUSP1 WT or DUSP1 KO mice were stimulated with LPS (100 ng/ml). Supernatents were harvested after 8 h and production of IFN-β was analyzed by ELISA. The means ± SD of triplicate measurements from one representative experiment out of four are shown. (B) BMM from DUSP1 WT or DUSP1 KO mice were stimulated with LPS (100 ng/ml) for the indicated times. Whole cell lysates were analyzed by Western blot using antibodies to phosphorylated STAT1, total STAT1, IRF-1 and γ-tubulin. Data shown are from one representative experiment out of five performed.

4.2.3 Increased susceptibility of DUSP1-deficient mice to lethal LPS shock

Based on these *in vitro* data, I asked whether DUSP1 determines the response to LPS *in vivo*. In a first approach, wild-type mice were injected intraperitoneally (i.p.) with 10 µg/g body weight of LPS and sacrificed at different time points to obtain spleens, livers and lungs for mRNA preparation. As shown in Figure 14A, all three organs showed rapidly induced DUSP1 mRNA expression with peak levels 6 h after LPS stimulation.

In order to test whether this induction of DUSP1 expression is required to limit the inflammatory response by controlling p38 activity in a negative feedback loop, I used DUSP1-deficient mice in a high-dose LPS shock model. For this study, wild-type and DUSP1-deficient mice were injected i.p. with 25 µg/g body weight of LPS. All of the DUSP1-deficient mice died between 20 and 48 h after injection, whereas more than 90 % of the wild-type littermates survived (Figure 14B).

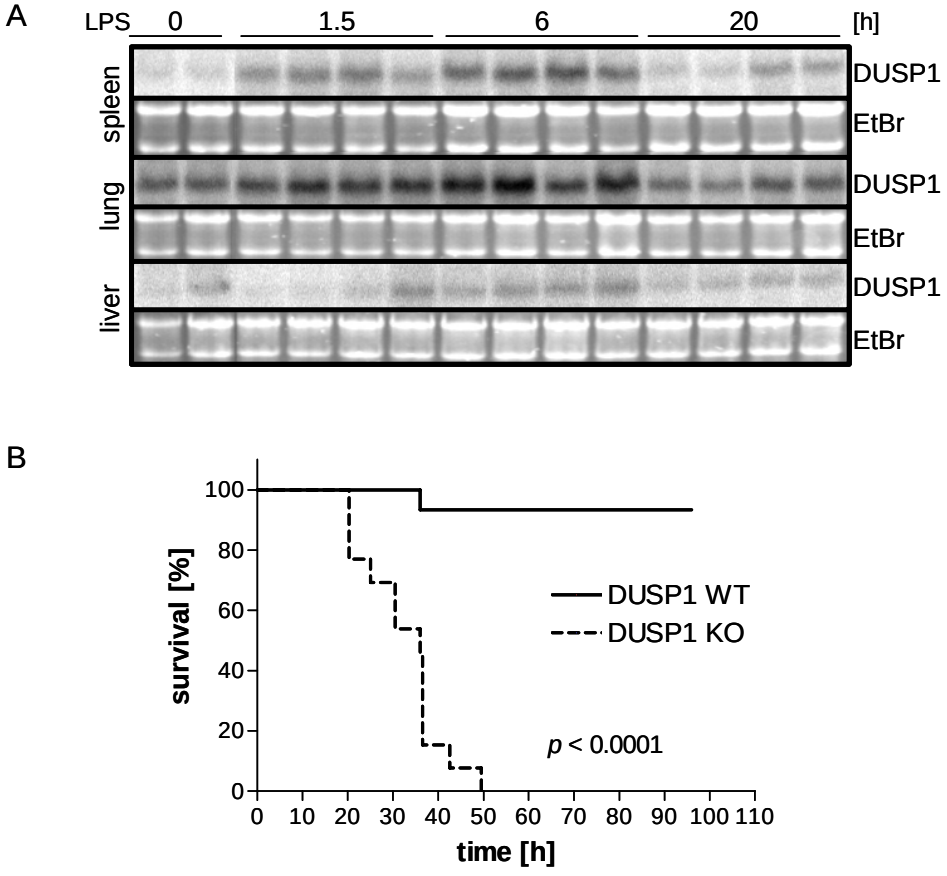


Figure 14. DUSP1 is induced after injection of LPS and controls survival.
(A) Expression levels of DUSP1 in the spleens, lungs and livers of wild-type mice are determined by Northern blot analysis of 10 µg total RNA at the indicated times after i.p. injection of 10 µg/g LPS. Data shown are from individual mice (n = 3-4) from one representative experiment. (B) Survival of DUSP1 WT (n = 15) and DUSP1 KO (n = 13) mice after i.p. injection of 25 µg/g LPS. Data are pooled from two experiments. *P* was determined using Student's *t* test.

4.2.4 Cytokine production *in vivo*

Serum levels of cytokines with a known role in the pathogenesis of endotoxin shock were analyzed to investigate the mechanisms underlying the high susceptibility to LPS in the absence of DUSP1 (Figure 15A). For this purpose, wild-type and DUSP1-deficient mice were injected i.p. with 10 µg/g body weight of LPS. Already 1.5 h after injection, the levels of TNF-α and IL-6 were significantly elevated in the mice lacking DUSP1. At the later time points, differences were even more pronounced for IL-6, whereas TNF-α was down-regulated in the DUSP1-deficient mice, although still higher than in wild-type mice (Figure 15A; left). Because DUSP1-deficient and wild-type mice were on a mixed genetic background, I confirmed the differences in IL-6 and TNF-α levels 6 h after LPS injection in mice back-crossed on pure backgrounds to exclude confounding by modifier genes (Figure 15A; right). I also analyzed IFN-γ and IL-12p40, both known to contribute to lethal outcome of endotoxin shock (Kohler et al., 1993; Wysocka et al., 1995; Zisman et al., 1997), and found no significant effect of DUSP1 deficiency 6 h after injection (Figure 15B; not shown for IFN-γ). IL-10-deficient mice are known to be also highly susceptible to LPS-induced shock (Berg et al., 1995). I therefore compared cytokine production of mice deficient for DUSP1 and IL-10, respectively. In contrast to the DUSP1-deficient mice, IL-10-deficient mice exhibited uncontrolled release not only of IL-6 and TNF-α, but also of IFN-γ and IL-12p40 upon stimulation with LPS (Figure 15B and Berg et al., 1995).

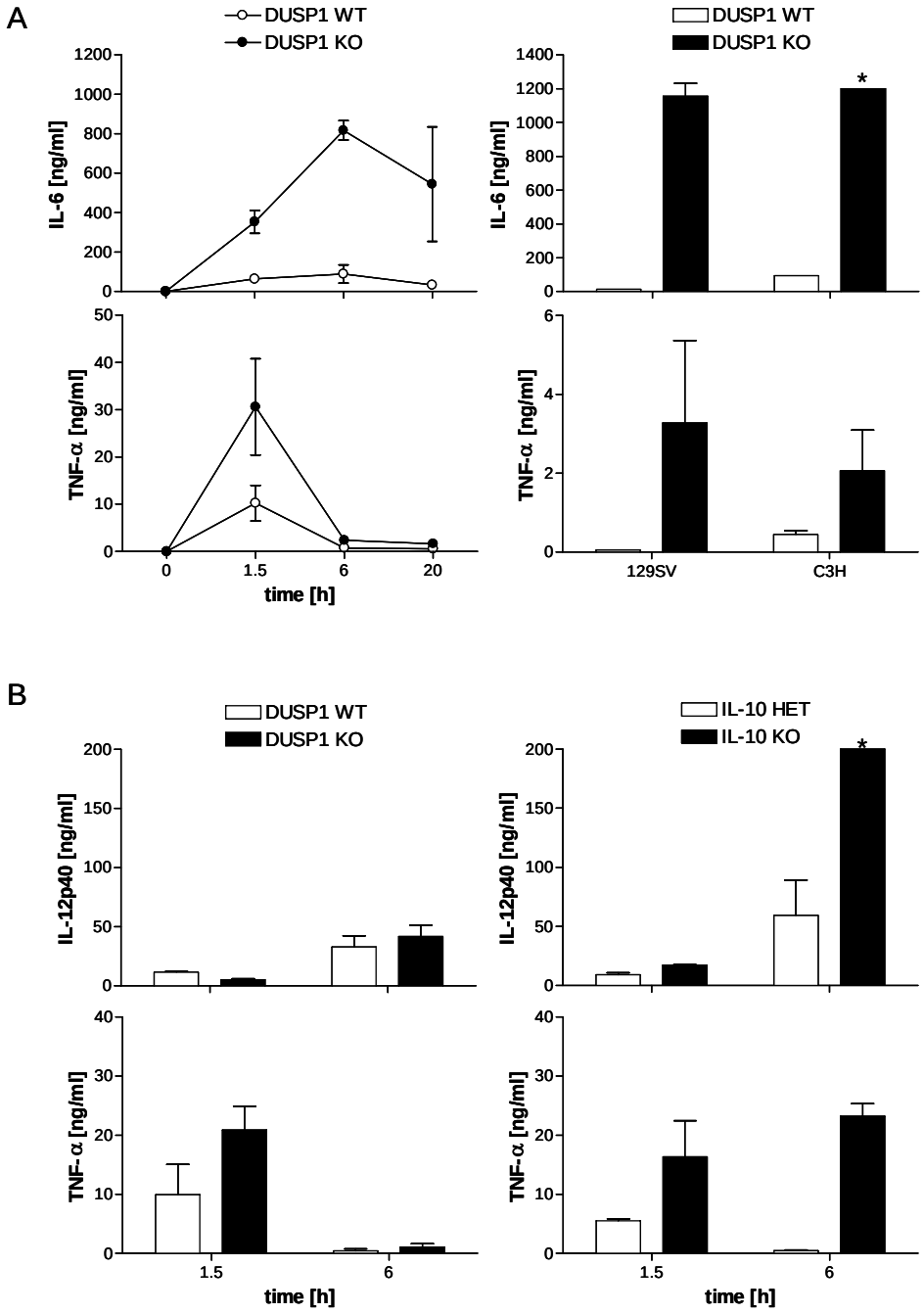


Figure 15. DUSP1 controls cytokine production *in vivo*.
(A and B) DUSP1 KO, IL-10 KO and control mice were injected with 10 µg/g LPS i.p. and serum cytokine levels were determined by ELISA at the indicated time points. Data shown are means ± SD (n = 3-4 mice per data point) from representative experiments. Two data points marked by asterisks (*) were beyond the maximum of the standard curve. (A) TNF-α and IL-6 in DUSP1 WT and DUSP1 KO mice on a mixed 129Sv x Bl/6 (left) or pure 129Sv and C3H (right) background (6 h after LPS). (B) Comparison of the effect of deficiency in DUSP1 or IL-10 on serum levels of TNF-α and IL-12p40 at the indicated times.

4.2.5 Haematopoietic cells are responsible for the elevated cytokine levels in DUSP1-deficient mice

The higher cytokine production observed in DUSP1-deficient compared to wild-type mice after LPS injection (Figure 15) corresponds to the observations in macrophages (Figure 12), however, the differences were much higher *in vivo* than *in vitro*. In an attempt to clarify the cellular source of the elevated cytokine levels observed in the DUSP1-deficient mice after injection of LPS, I adoptively transferred bone marrow from DUSP1-deficient mice and heterozygous littermates into irradiated C57BL/6 wild-type mice. Since the donor mice were on a different genetic background than the recipient mice, and therefore possess a different MHC haplotype, the bone marrow of the donor mice was depleted of T cells using an antibody against the T cell specific marker Thy1.2 and the MACS-technique described in Material and Methods, in order to prevent graft-versus-host disease. After T cell depletion, the bone marrow was intravenously (i.v.) injected in C57BL/6 recipient mice that had been irradiated 24 h before to destroy bone marrow stem cells. The efficiency of the bone marrow reconstitution was analyzed 4 weeks after injection. Mice were bled and the peripheral blood mononuclear cells (PBMCs) were analyzed by FACS (Figure 16A). To distinguish between residual cells of the recipient mice and progenitors of the injected bone marrow cells of the donor mice, two surface markers, namely CD45.1 and CD45.2, were used. The cells of the recipient mice were heterozygous and therefore positive for both markers. In contrast, the donor cells only expressed CD45.2. Whereas in the non-irradiated C57BL/6 control mice all cells were double-positive, the irradiated mice that received bone marrow contained 92-95 % CD45.2 single-positive cells (Figure 16A, depicted only for one reconstituted mouse per group), confirming successful reconstitution of the bone marrow with cells from DUSP1-deficient and control mice, respectively. The next step was to analyze cytokine production of these mice 6 h after i.p. injection of 10 µg/g body weight of LPS. Comparable to the experiments with DUSP1-deficient mice, the mice that were reconstituted with bone marrow from the mice lacking DUSP1 produced significantly more IL-6, IL-10, CCL3 and CCL4 than the heterozygous reconstituted control mice (Figure 16B), indicating that mainly the haematopoietic cells are responsible for the higher susceptibility of DUSP1-deficient mice to LPS-induced shock.

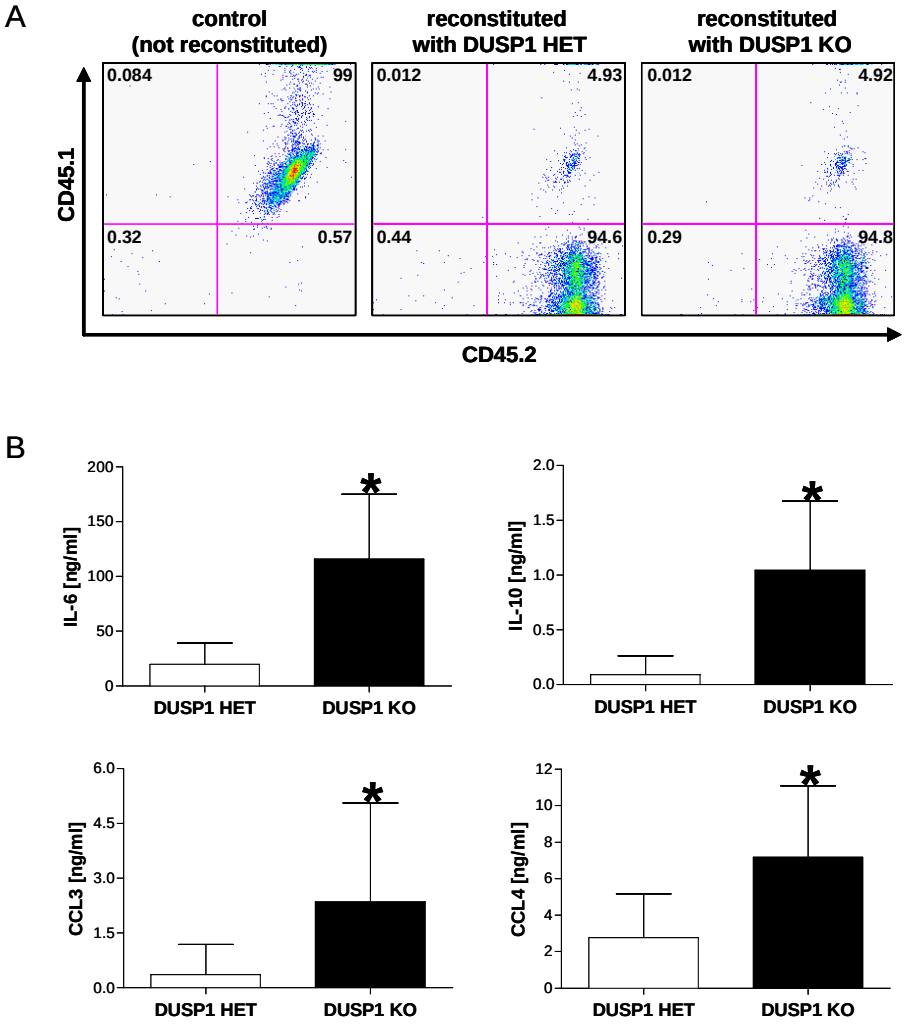


Figure 16. DUSP1 bone marrow-chimeric mice are more susceptible to LPS than heterozygous reconstituted control mice.

(A and B) Bone marrow cells (5 x 10^6) from DUSP1 HET and DUSP1 KO mice were depleted of T cells and then adoptively transferred into irradiated CD45.1/CD45.2 recipient C57Bl/6 mice. (A) After 4 weeks PBMCs of the recipient mice were stained for CD45.1 and CD45.2 and analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on life cells. Data shown are from one representative mouse per group (n = 10 per group). (B) After 6 weeks, the reconstituted mice were injected i.p. with 10 µg/g LPS. Serum cytokine levels after 6 h were measured by ELISA. Data shown are the means ± SD from five mice per group (IL-6 and CCL3) or ten mice per group (IL-10 and CCL4) from representative experiments. *, DUSP1 KO different from DUSP1 HET, $p < 0.05$ determined using Student's *t* test.

4.2.6 Genome-wide analysis of DUSP1-regulated gene expression in LPS-challenged mice

To obtain a more global view of the impact DUSP1 has on LPS-induced gene expression, transcriptional profiling was performed using spleen RNA prepared 6 h after LPS challenge. The RNA was labelled and hybridized to Affymetrix GeneChips® MOE430A 2.0 as described in Material and Methods. The list of significantly regulated genes was achieved by applying the SAM multiclass algorithm (Tusher et al., 2001) of the samr package for R (FDR < 1 %: 1465 probe sets). Further filtering included a minimum fold-change criterion between all four experimental conditions of ± 2 (1372 probe sets) and a max (all mean expression values) – min (all mean expression values) filter of > 50 (1215 probe sets). Of these genes, a total of 608 were up-regulated. In both groups of mice, LPS induced substantial changes in gene expression, with considerable overlap in the genes induced in wild-type or DUSP1-deficient mice (Figure 17A, Venn diagram and Table 8 for selected genes). However, in DUSP1-deficient mice, nearly threefold more genes were uniquely up-regulated by LPS compared to wild-type, which is also evident from the hierarchical clustering analysis, with cluster C containing 229 genes induced more strongly in the absence of DUSP1 (Figure 17A, B). The microarray data corroborate most of the cytokine data obtained by ELISA from serum samples (Figure 15). IL-6 was up-regulated in DUSP1-deficient mice, whereas IL-12p40 and IFN-γ showed no difference in expression. A notable exception is TNF-α, whose expression was not increased in DUSP1-deficient mice at the RNA level, a result that was confirmed by Northern blot analysis. Additionally to TNF-α, I validated the microarray data by Northern blot analysis using probes for IL-6, SOCS3 and N-MYC downstream regulated gene 1 (NDR1) (Figure 17C). In accordance with the microarray results all three genes showed higher mRNA expression in the spleens of DUSP1-deficient mice.

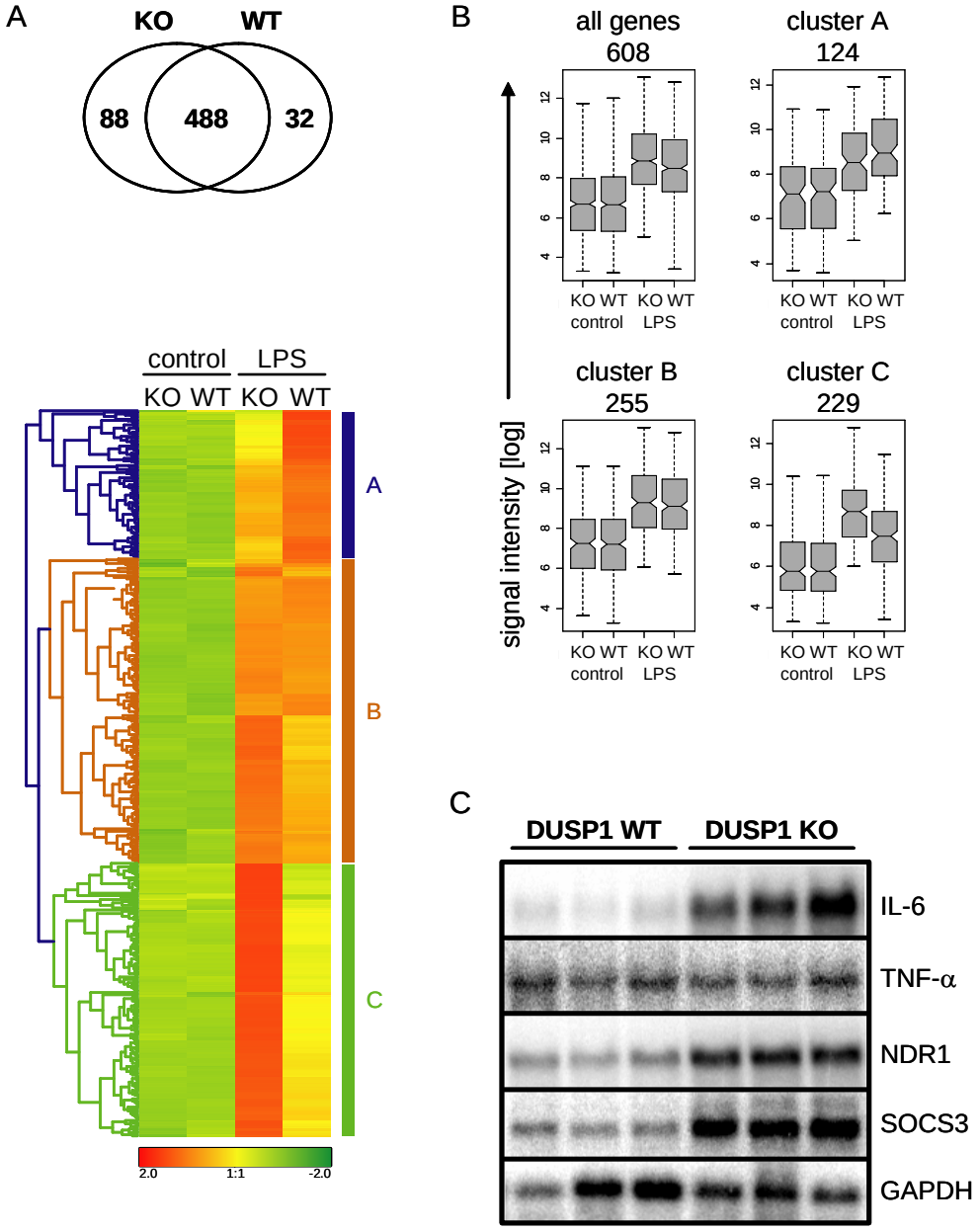


Figure 17. Genome-wide analysis of LPS-induced gene expression in the spleens of DUSP1-deficient and wild-type mice.

(A) Total splenic RNA was prepared from DUSP1 WT and DUSP1 KO mice 6 h after i.p. injection of 10 µg/g LPS (n = 3 per group) and was processed for Affymetrix GeneChip analysis as described. Venn diagram comparing the numbers of genes significantly up-regulated in DUSP1 WT and DUSP1 KO mice by LPS (for criteria see Material and Methods). Z-score-normalized expression values of LPS-induced genes were subjected to hierarchical clustering analysis. Cluster A contains a group of genes induced more strongly in the spleen of DUSP1 WT mice. In contrast, cluster C represents a larger subset of LPS-induced genes up-regulated in the absence of DUSP1. Expression data of these 608 genes are published (Hammer et al., 2005). (B) Box plots for all genes and clusters A, B and C. (C) Confirmation of the microarray expression data for selected genes. Total splenic RNA (10 µg) of DUSP1 WT and DUSP1 KO mice 6 h after i.p. injection of LPS (10 µg/g) was analyzed by Northern blot for the expression of IL-6, TNF-α, NDR1 and SOCS3. GAPDH expression was used as loading control. Data shown are from individual mice (n = 3) from one representative experiment.

Affymetrix Probeset ID	Gene Symbol	KO Control (Average)	KO LPS (Average)	WT Control (Average)	WT LPS (Average)	KO LPS Fold Change as signed ratio	WT LPS Fold Change as signed ratio	p-value *t* test WT LPS/KO LPS
1421473_at	Il1a	25	160	19	155	8,32	8,07	0,9488
1450297_at	**Il6**	**28**	**2717**	**13**	**457**	**217,16**	**36,51**	**0,0030**
1450330_at	**Il10**	**15**	**245**	**14**	**74**	**16,99**	**5,14**	**0,0058**
1425454_a_at	Il12a	76	171	52	145	3,26	2,76	0,5097
1419530_at	Il12b	17	107	14	51	7,46	3,57	0,2395
1417932_at	Il18	895	1445	811	1312	1,78	1,62	0,6966
1419607_at	Tnf	83	129	67	340	1,92	5,07	0,0103
1419561_at	**Ccl3**	**31**	**2633**	**21**	**617**	**123,85**	**29,02**	**0,0003**
1421578_at	**Ccl4**	**50**	**3689**	**46**	**683**	**79,94**	**14,79**	**0,0004**
1449984_at	**Cxcl2**	**27**	**2539**	**17**	**707**	**147,62**	**41,08**	**0,0004**
1418930_at	Cxcl10	400	6921	279	6039	24,82	21,65	0,2119
1422305_at	Ifnb1	15	16	14	17	1,14	1,23	0,6746
1422332_at	Ifna11	9	9	8	9	1,02	1,01	0,8015
1422408_at	Ifna4	13	11	12	13	-1,07	1,07	0,1031
1425947_at	Ifng	16	189	13	276	14,66	21,36	0,4069
1416576_at	**SOCS3**	**192**	**2923**	**138**	**1311**	**15,21**	**9,47**	**0,0016**
1418932_at	**NFIL3**	**40**	**508**	**40**	**180**	**12,83**	**4,46**	**0,0025**
1420760_s_at	**Ndrl**	**402**	**1849**	**528**	**901**	**4,60**	**1,71**	**0,0047**
1418666_at	**Ptx3**	**11**	**187**	**11**	**39**	**16,27**	**3,53**	**0,0001**
1417408_at	**F3**	**23**	**85**	**23**	**25**	**3,68**	**1,10**	**0,0035**
1460283_at	Mefv	141	183	133	276	1,30	2,07	0,2150
1453851_a_at	**Gadd45g**	**76**	**662**	**55**	**297**	**8,68**	**5,38**	**0,0001**

Table 8. Expression levels of LPS-induced genes in the spleens of DUSP1-deficient and wild-type mice.
Genes induced after 6 h by LPS in spleens of DUSP1 WT and DUSP1 KO mice. Depicted are the expression levels of the most important inflammatory mediators. Marked in bold are the probe sets showing significant differences in expression between DUSP1 WT and DUSP1 KO mice after LPS challenge with a p-value of < 0.01. Data shown are averages from individual mice (n = 3) from one representative experiment. A complete list is available online (Hammer et al., 2005).

4.2.7 Control of selected inflammatory molecules by DUSP1

I mined the microarray data with regard to the question of how DUSP1 deficiency causes excess lethality in LPS challenge. Although some downstream effectors of LPS-induced lethality may not yet be expressed at the relatively early time point analyzed in the microarray experiment, DUSP1-deficient mice already appeared sicker clinically. Therefore, it seems reasonable to assume a contribution to the severe inflammatory response for at least some of the genes overexpressed in the absence of DUSP1. Among the cytokines that have been implicated as contributors to the pathogenesis of lethal endotoxin shock, interferons type I and II as well as IL-12, TNF-α, IL-1α and IL-18 were expressed at similar levels in spleens from wild-type and DUSP1-deficient mice (Table 8). On the other hand, the chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), and CXCL2 (MIP-2) were among the LPS targets overexpressed most strongly in DUSP1-deficient spleens (Table 8). To confirm and extend this finding, I analysed the levels of these chemokines in the serum of wild-type and DUSP1-deficient mice 6 and 20 h after i.p. injection of LPS (Figure 18A). All three chemokines showed elevated protein levels in DUSP1-deficient mice, corresponding to the mRNA levels measured in the spleen. In contrast, CXCL10 (IP-10), which was not affected on the mRNA level in the spleen, was also not increased in the serum of DUSP1-deficient compared to wild-type mice (Figure 18A).

Pentraxin-related gene (PTX3) and coagulation factor III (F3) are other genes that are more strongly up-regulated in DUSP1-deficient spleens, whereas MEFV, the gene encoding for pyrin, is induced in wild-type but not in DUSP1-deficient spleens (Table 8). PTX3 belongs to the group of long pentraxins and is supposed to act as a component of the humoral arm of innate immunity with non-redundant roles in matrix-remodelling and initiation of innate defence mechanisms against various pathogens (Bottazzi et al., 2006). The type I membrane protein F3 plays an important role in blood coagulation and in matrix-remodelling upon tissue injury and in cancer (Milsom and Rak, 2005). Mutations in the pyrin protein cause the inherited disorder familial mediterranean fever (FMF). In a study using mice with targeted disruption of the pyrin encoding gene MEFV, it could be shown that these mice exhibit heightened sensitivity to endotoxin and a defect in macrophage apoptosis (Chae et al., 2003). The confirmation of the expression levels of these genes observed in the microarray experiment was brought about by quantitative real-time PCR. The results in Figure 18B reveal up-regulation of PTX3 in the spleen of DUSP1-deficient mice compared to wild-type mice 6 and 20 h after LPS injection, which is consistent with the microarray data. F3 expression was unaltered at the 6-h time-point, but significantly up-regulated at the 20-h time point. MEFV

on the other hand, was induced 2.5-fold in wild-type, but unaltered in DUSP1-deficient spleens after 6 h, further confirming the microarray data. Comparable differences in expression of these genes between wild-type and DUSP1-deficient mice could also be detected in the livers of the respective mice (data not shown).

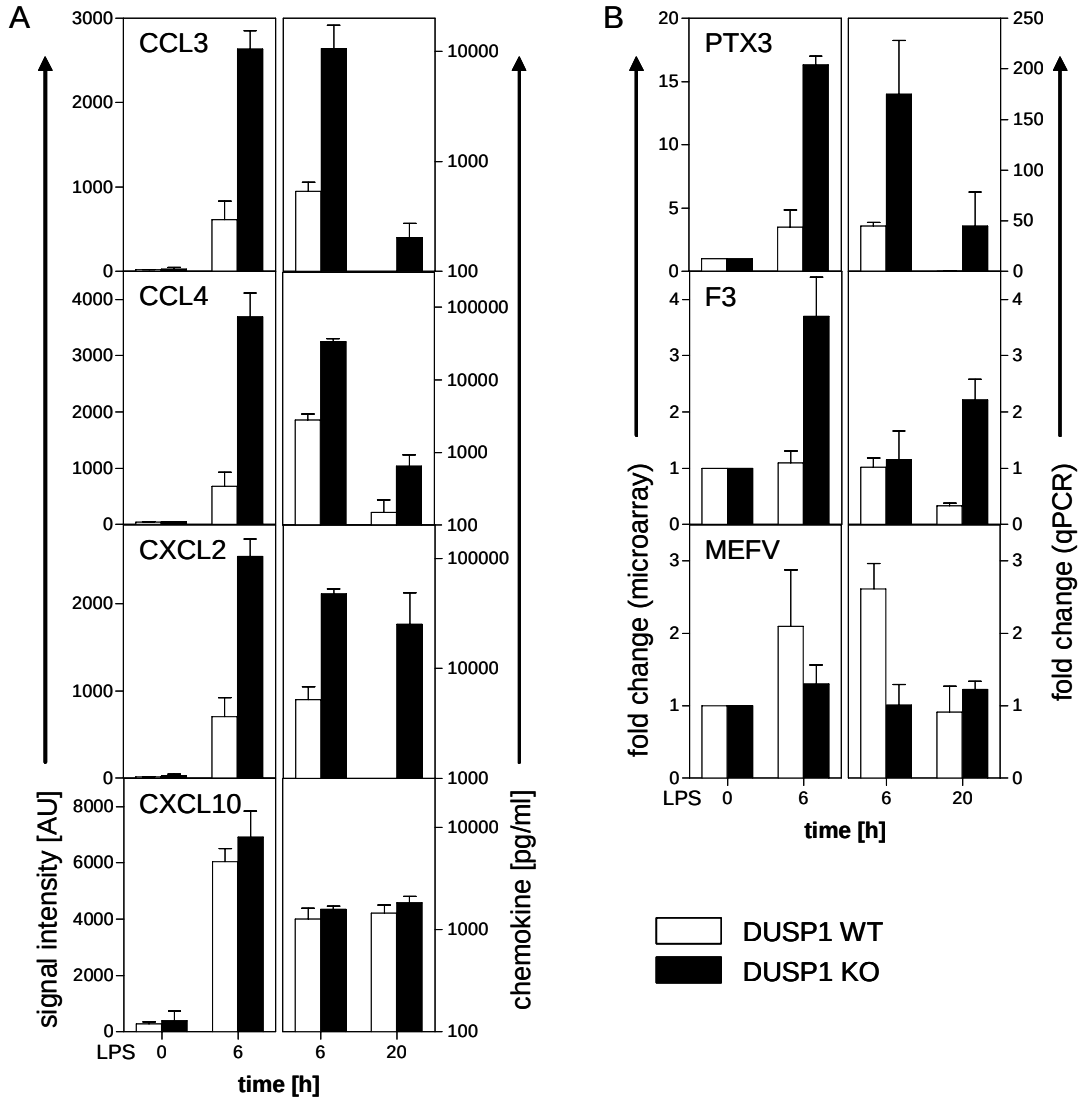


Figure 18. Control of selected inflammatory molecules by DUSP1.
(A) Differential control of the chemokines CCL3, CCL4, CXCL2 and CXCL10 by DUSP1 at the indicated times after i.p. injection of LPS (10 µg/g). Confirmation of the microarray expression data (left: average after 6 h and ± SD of signal intensity values) by ELISA determination of serum levels (right: means ± SD, n = 3-4). (B) PTX3, F3 and MEFV are differentially expressed in DUSP1 KO mice at the indicated times after i.p. injection of LPS (10 µg/g). Microarray expression data (left: average fold change in stimulated relative to unstimulated mice; values are means ± SD of three mice) were confirmed by quantitative real-time PCR of splenic mRNA of DUSP1 WT and DUSP1 KO mice after i.p. injection of LPS (10 µg/g) for the indicated times and normalized to β-actin (right). Data are expressed as fold change in mRNA levels in stimulated relative to unstimulated mice. Values are means ± SD of two independent experiments.

4.2.8 The production of IL-10 is regulated by DUSP1

As described above, DUSP1 can be induced by the immunoregulatory cytokine IL-10. The microarray analysis now identified IL-10 itself to be induced 3.3-fold stronger in the DUSP1-deficient spleen, which was confirmed by Northern blot analysis revealing peak levels of IL-10 6 h after LPS injection (Figure 19A). This difference was strikingly more pronounced in the liver, in which IL-10 expression was elevated even 20 h after LPS injection. To investigate, whether this increased IL-10 mRNA levels also affects protein levels, I analyzed serum from wild-type and DUSP1-deficient mice 6 and 20 h after i.p. injection of LPS. As depicted in Figure 19B, the increased mRNA levels were indeed accompanied by 10-fold higher serum IL-10 levels in DUSP1-deficient mice.

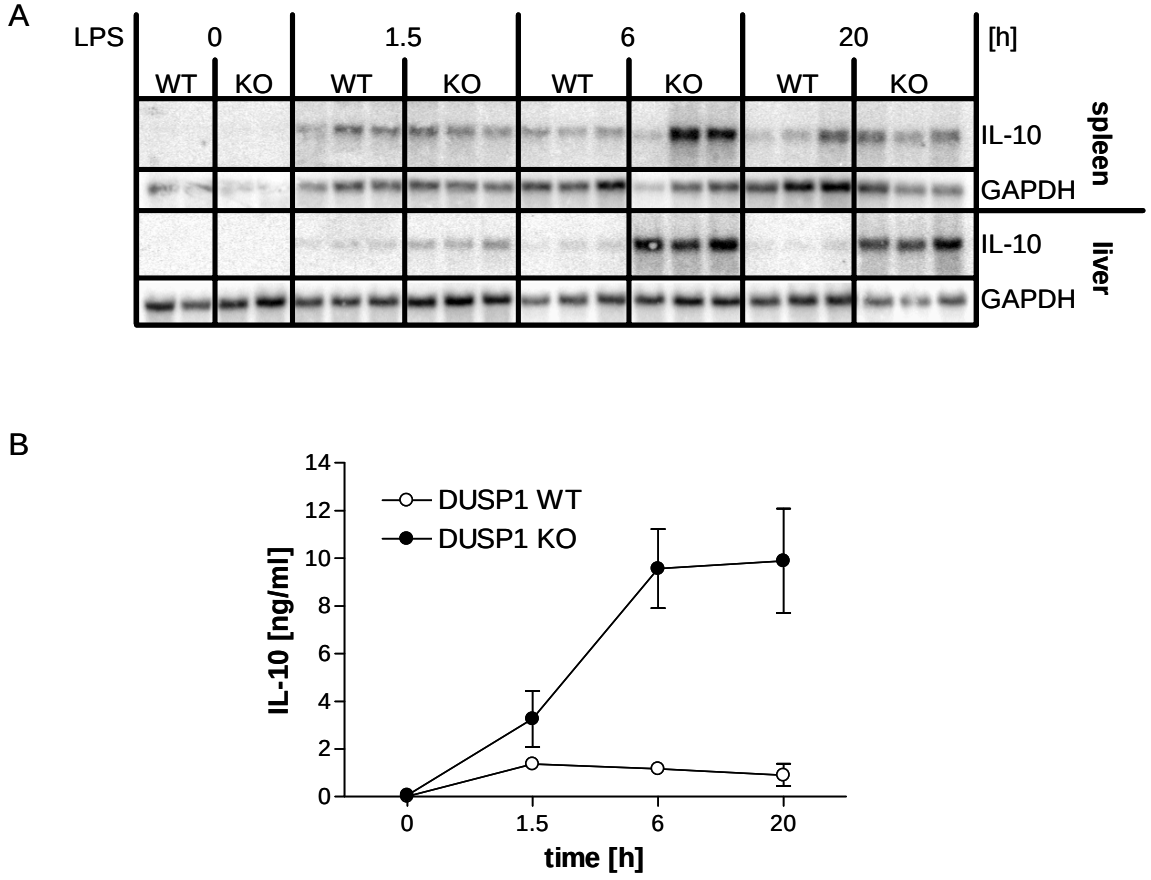
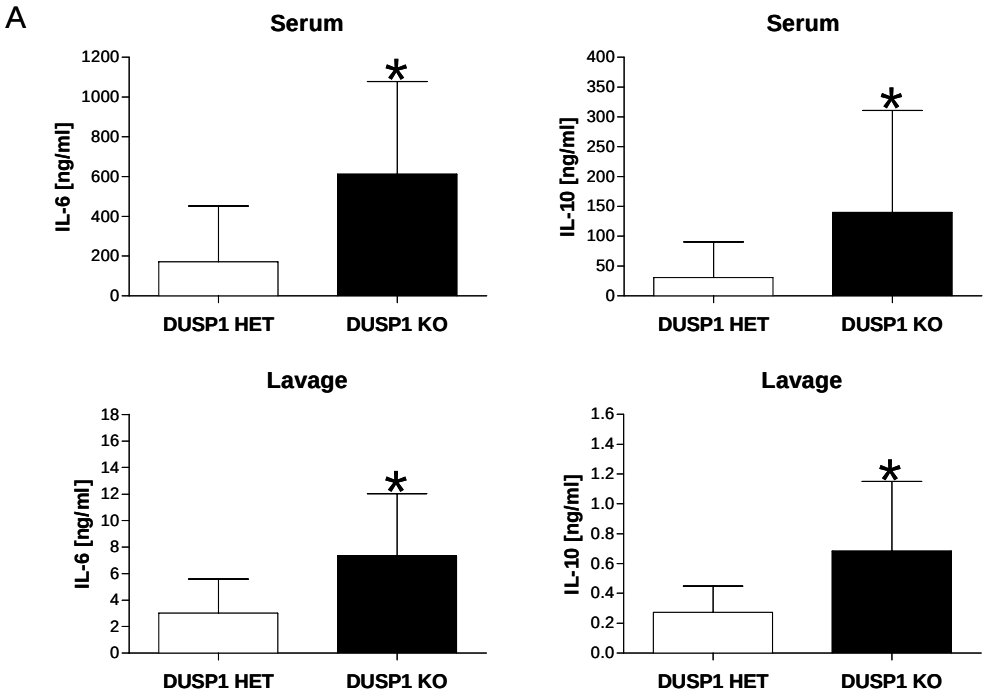


Figure 19. DUSP1 controls LPS-induced IL-10 production.
(A and B) DUSP1 WT and DUSP1 KO mice were i.p. injected with LPS (10 µg/g) and analysed at the indicated time points. (A) Total RNA from spleens and livers were prepared and 10 µg were analyzed by Northern blot for the expression of IL-10. GAPDH expression was used as loading control. Data are shown from individual mice (n = 2-3) from one representative experiment. (B) Serum levels of IL-10 after the indicated times were determined by ELISA. Data are the means ± SD (n = 3-4) from one representative experiment.

4.2.9 Polymicrobial sepsis in DUSP1-deficient mice

To elucidate the potential role of DUSP1 for the immune defence against polymicrobial infection, we analyzed DUSP1-deficient mice in a well established murine model of abdominal sepsis termed colon ascendens stent peritonitis (CASP). In CASP, a stent is inserted into the ascending colon, which generates a septic focus and leads to a rapid invasion of bacteria into the peritoneal cavity and the blood. As a consequence, endotoxemia occurs, inflammatory cells are recruited, and a systemic inflammatory response syndrome develops (Zantl et al., 1998). DUSP1-deficient mice and heterozygous littermates were sacrificed 12 h after they underwent surgery. As demonstrated in Figure 20A, IL-6 and IL-10 levels were significantly higher in the serum and the peritoneal lavage of DUSP1-deficient mice. However, the elevated cytokine levels did not affect the bacterial burden in the liver, lung or peritoneal cavity, as there was no significant difference detected between DUSP1-deficient and heterozygous control mice (Figure 20B). This observation was accompanied by an unaltered influx of granulocytes and macrophages into the peritoneal cavity of mice deficient for DUSP1 compared to heterozygous littermates (data not shown). Genetic deficiency for DUSP1 did not significantly alter the survival of polymicrobial septic peritonitis after insertion of an 18-gauge venous catheter. The lethality of the heterozygous mice was already very high and comparable to DUSP1-deficient mice (Figure 20C). However, the usage of a smaller catheter (20-gauge) resulted in a survival of 70 % of heterozygous mice, whereas only 20 % of the DUSP1-deficient mice were alive at 7 days after sepsis induction (Figure 20C).



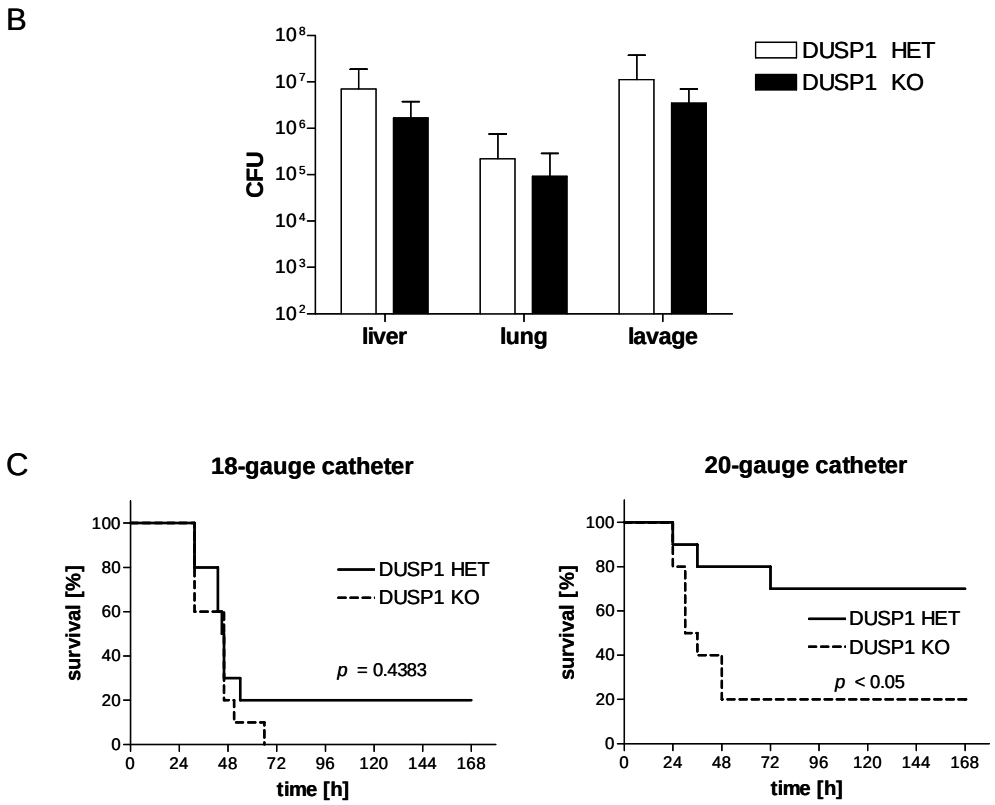


Figure 20. The role of DUSP1 in polymicrobial sepsis.
(A-C) CASP was induced in DUSP1 HET and DUSP1 KO mice with a venous catheter (20 gauge) as described in Material and Methods. (A) Mice were sacrificed 12 h after surgery and cytokine levels in the serum and the peritoneal lavage were measured by ELISA. Data are pooled from three experiments and presented as means ± SD (n = 11-12 per group). *, DUSP1 KO different from DUSP1 HET, $p < 0.05$ determined using Student's *t* test. (B) Normal bacterial clearance in DUSP1-deficient mice. Peritoneal lavage fluids, livers and lungs were obtained 12 h after CASP from DUSP1 HET or DUSP1 KO and total bacterial counts were determined. Results are derived from 8 mice per group. There was no significant difference between DUSP1 HET and DUSP1 KO mice as determined using Student's *t* test. (C) Survival of DUSP1 HET and DUSP1 KO (n = 10 per group) after CASP with a venous catheter (20 or 18 gauge). Data are pooled from two experiments. *P* determined using Student's *t* test.

5 Discussion

5.1 IL-10 controls the expression of DUSP1 in activated macrophages

In the 17 years since the discovery of IL-10, major advances have been made in understanding the function of this important cytokine. Genetic studies using IL-10-deficient and IL-10 transgenic mice established the unequivocal importance of IL-10 in controlling inflammation initiated and perpetuated by pro-inflammatory signals in acute and chronic diseases (Moore et al., 2001). The major physiological function of IL-10 is to regulate macrophages activated by pathogens and their products. At the molecular level, however, major gaps remain in understanding how IL-10 deactivates macrophages. It is known that STAT3-dependent induction of target genes is required for the inhibitory effect of IL-10 (Bogdan et al., 1992; Takeda et al., 1999), but the identity of the molecular players mediating specific or general inhibition, as well as the level at which they interfere with inflammatory gene expression are only beginning to be understood.

5.1.1 TLR ligands and IL-10 synergize to induce DUSP1 expression

As mentioned above, interference with activating signalling pathways seems to be a major mechanism by which IL-10 is able to deactivate macrophages. MAPKs are consistently activated by pro-inflammatory agonists. They contribute to subsequent inflammatory or innate immune responses by regulating the expression of numerous effector genes at transcriptional or post-transcriptional levels. The largest group of phosphatases dedicated to the regulation of MAPK signalling are the dual-specificity phosphatases (DUSP). Analysis of our microarray data revealed three DUSP family members, namely DUSP1, DUSP2 and DUSP16, to be induced by LPS and with some minor differences by other TLR ligands as well, confirming and extending previous reports (Chen et al., 2002; Shepherd et al., 2004; Zhang et al., 2004; Hammer et al., 2005; Jeffrey et al., 2006). In the case of DUSP1, Chi and colleagues demonstrated that its expression can be induced by the MyD88- as well as the TRIF-dependent pathway and that both pathways were required for the full induction of DUSP1 in LPS-treated macrophages (Chi et al., 2006). Additional stimulation with IL-10 had different effects on the three DUSPs. Whereas DUSP2 expression was not influenced by IL-10, DUSP16 expression was enhanced at the early and the later time point. However, the greatest effect of IL-10 was observed on DUSP1, where it led to sustained expression in LPS-activated

macrophages after 3 h, while the DUSP1 induction was transient when only LPS was used. This observation was new and suggested DUSP1 as a potential mediator of the anti-inflammatory IL-10 effect. The fact that LPS alone induced DUSP1 much stronger in wild-type than in IL-10-deficient macrophages after 3 h can be explained by endogenous IL-10 production, since addition of an anti-IL-10 antibody partially suppressed DUSP1 levels.

5.1.2 Concerted induction of DUSP1 expression by the anti-inflammatory glucocorticoid dexamethasone and IL-10

In several studies it has been shown that DEX and other glucocorticoids induce expression of DUSP1 in HeLa cells, macrophages or mast cells. This was accompanied by attenuated activation of one or more MAPK and resulted in down-regulated expression of several inflammatory mediators (Kassel et al., 2001; Chen et al., 2002; Lasa et al., 2002; Hermoso et al., 2004; Zhao et al., 2005). Thus, in addition to transrepression of NF-κB and AP-1 by ligand-bound, dimerized glucocorticoid receptor, down-regulation of MAPK by DUSP1 likely contributes to the anti-inflammatory effects of glucocorticoids (Clark, 2003; Abraham et al., 2006). These findings could recently be extended by Abraham and colleagues who showed in a murine model of acute inflammation, that DEX exerted significant anti-inflammatory effects in DUSP1 wild-type but not in DUSP1-deficient mice, implicating DUSP1 as an important mediator of anti-inflammatory actions of glucocorticoids *in vivo* (Abraham et al., 2006). In the present study, I observed synergistic induction of DUSP1 expression in activated macrophages by the combination of IL-10 and DEX. This correlated with a more pronounced p38 inhibition and a greatly enhanced inhibitory effect on cytokine release (Hammer et al., 2005). A similarly enhanced suppression of inflammatory cytokines by the combination of DEX and IL-10 has previously been described in human PBMC and THP-1 cells (Joyce et al., 1996). Since several side effects of glucocorticoids often limit their therapeutic use (Saklatvala, 2002), the combination with IL-10, which acts primarily on haematopoietic cells, may be a favourable strategy to reduce the dose required for an anti-inflammatory effect (Herfarth et al., 1998). In contrast to DEX, IL-10 increases DUSP1 expression only in activated but not in resting macrophages, suggesting that different mechanisms are operative. This interpretation is supported by the fact that IL-10 and DEX are synergistic for increasing DUSP1 expression.

5.1.3 Mechanisms of DUSP1 induction by LPS and IL-10

In an attempt to clarify which mechanisms underlie the regulation of DUSP1 by IL-10 and LPS on the promotor level, I used luciferase reporter gene assays and found inducibility of the

DUSP1 promoter by LPS in RAW macrophages (Hammer et al., 2005). The relatively high basal activity of the promoter constructs is similar to what was observed previously in a pituitary cell line. In these experiments thyrotropin release hormone increased expression dependent on GC boxes, Cre sites and E-boxes in the proximal promoter (Ryser et al., 2004). Although not tested here, these sites are also likely to play a role in the activation by LPS because TLR signalling induces transcription factors that bind to Cre sites as well as E-boxes. I did not observe any effect of IL-10 on the activity of a reporter gene in resting or LPS-activated RAW macrophages. The absence of STAT3 binding sites can explain that no enhancement of luciferase expression was brought about by IL-10 in the constructs carrying the first 2 kb of the sequence upstream of the transcriptional start site. Even the constructs that additionally carry fragments containing one of the three STAT3 binding sites from the upstream region of the proximal promotor were not rendered responsive to IL-10. However, it might be possible that a concerted binding of STAT3 proteins to all three binding sites can induce DUSP1 transcription. The usage of STAT3-deficient macrophages would be necessary to completely elucidate the requirement of STAT3 for IL-10-induced DUSP1 expression. Another approach made to address the question whether the increased induction of DUSP1 in the presence of IL-10 was a direct transcriptional effect, e.g. *via* STAT3 activation, used the inhibitor of protein synthesis CHX. Unfortunately, DUSP1 mRNA was massively up-regulated by CHX itself, making a clear statement impossible. An alternative method detects and compares the levels of the unspliced primary transcript of DUSP1 before and after stimulation with IL-10. A PCR protocol is currently established and will hopefully provide the answer to this question. Although the data presented here do not exclude a direct activation of the DUSP1 promotor by STAT3 in primary macrophages, it has so far proven elusive.

The DUSP1 promotor region also contains several NF-κB sites. If DUSP1 is a NF-κB target of LPS signalling has not been addressed so far. Using IKKβ-deficient macrophages I found DUSP1 induction to be IKKβ-independent. Although activity of IKKβ is required in the classical pathway, it does not rule out that alternative NF-κB pathways might still be active to transmit the LPS signal. In one report for example, it was demonstrated that the LPS-inducible MAPKKK TPL-2 binds to the C-terminal half of NF-κB1 p105 and that overexpressed TPL-2 stimulates p105 proteolysis, thereby activating NF-κB (Belich et al., 1999; Hayden and Ghosh, 2004).

Generally, regulation of DUSP1 gene expression remains puzzling. The p38 MAPK, ERK, JNK and PKC pathways have all been implicated in LPS-dependent DUSP1 induction,

although transcription factors that transduce these signals have not been identified (Li et al., 2001; Chen et al., 2002; Lasa et al., 2002; Xiao et al., 2002; Abraham and Clark, 2006). A study using fibroblasts for instance could show that activation of the JNK pathway, but not ERK, resulted in increased DUSP1 mRNA (Bokemeyer et al., 1996). Similarly, inhibition of MEK-1 by PD98059 did not interfere with LPS-induced expression of DUSP1 in macrophages (Valledor et al., 1999; Valledor et al., 2000). However, the role of ERK signalling in DUSP1 mRNA expression is not entirely clear, because the MEK-1 inhibitor U0126 was reported to block LPS-induced increases of DUSP1 at the mRNA and the protein level (Chen et al., 2002). Only recently published work by the group of Celeda could now shed some light on this controversial question. They demonstrated that at least in primary macrophages the transcriptional induction of DUSP1 by M-CSF and LPS solely depends on JNK, and specifically on the JNK1 isoform, but not on the other MAPKs ERK or p38 (Sanchez-Tillo et al., 2007). Although TLR signalling activates the DUSP1 promotor, mRNA levels may also be controlled. The finding of a very short half-life of DUSP1 mRNA was an unexpected result of this study since the mRNA does not contain a canonical AU-rich element (ARE) and is not in the ARED database (Bakheet et al., 2003). Nevertheless, LPS-induced DUSP1 mRNA was as unstable as that for TNF-α (Hammer et al., 2005). A role for the 3'UTR of DUSP1 in regulation of mRNA steady state levels is also suggested by the reduced basal luciferase activity and the conserved responsiveness to LPS when the endogenous 3'UTR was inserted into the DUSP1 reporter construct. However, the stability of DUSP1 mRNA was only slightly increased by addition of IL-10 to primary or RAW macrophages, and this difference does not appear to explain the robust increase of steady state mRNA levels. Thus, mechanisms other than control of mRNA stability and control of the proximal promoter are operating in the IL-10-induced increase of DUSP1 mRNA expression in activated macrophages.

5.2 DUSP1 negatively regulates innate immune responses

5.2.1 Inhibition of p38 MAPK by IL-10

The MAPK p38 is the preferred substrate of DUSP1 in human and mouse macrophages (Franklin and Kraft, 1997; Chen et al., 2002), leading me to hypothesize that increased expression of DUSP1 in IL-10-treated macrophages results in attenuated p38 phosphorylation, which I confirmed experimentally. However, the effect of IL-10 on the levels of phosphorylated p38 MAPK was incomplete and modest, especially when compared to the

strong inhibition of IL-6 and IL-12p40 release (Hammer et al., 2005). Inhibitory effects of IL-10 on p38 have been implicated by earlier studies in human monocytes (Geng et al., 1994; Niiro et al., 1998). Further, Kontoyiannis et al. have proposed that the main effect of IL-10 signalling in murine macrophages is the inhibition of p38, which prevents activation of its downstream target MK2, thereby inhibiting TNF-α protein translation (Kontoyiannis et al., 2001). However, the question whether, and if so how strongly, IL-10 inhibits p38 MAPK activation is still controversial, since other investigators have failed to show any effect of IL-10 on p38 activity (Donnelly et al., 1999; Berlato et al., 2002; Denys et al., 2002; Biswas et al., 2003; Driessler et al., 2004). They rather suggest p38-independent effects at the level of NF-κB activity or post-transcriptional control. The exact reasons for these differences are not obvious. In my hands, the use of IL-10-deficient macrophages was required to demonstrate an effect of exogenous IL-10. Also, it should be stressed that IL-10 did not inhibit the early peak in the activation of p38, but induced a faster down-regulation of the response. Another important parameter that might be responsible for the conflicting observations is the timing of IL-10 treatment. For example, pre-treatment with IL-10 inhibits the promotor region of a reporter construct, whereas a simultaneous incubation of IL-10 with LPS rather initiates mRNA degradation *via* the 3'UTR (Denys et al., 2002). Moreover, the use of different cellular targets, such as mouse primary macrophages, human monocytes, human DCs and several cell lines (J774, THP-1, RAW 267.4) makes a direct comparison of the different studies rather difficult. Nevertheless, given the kinetics of DUSP1 mRNA expression in IL-10-treated activated macrophages observed in this study, control of p38 activation provides an operational link between IL-10-induced DUSP1 expression and inhibition of cytokine production.

5.2.2 DUSP1 is an essential negative regulator of p38 MAPK and cytokine production *in vitro*

DUSP1-deficient mice were used to validate the relevance of DUSP1 as potential mediator of the inhibitory effect of IL-10 on p38 activity and cytokine production by activated macrophages and its requirement for regulation of the innate immune response to LPS. In the absence of DUSP1 expression, the LPS-induced activation of p38, and to a lesser extend JNK, was prolonged in bone marrow-derived macrophages, whereas the time course of ERK activation was not affected (Hammer et al., 2005).

The sustained p38 activation had direct effects on downstream signalling, since the activation of the physiological targets MK2 and ATF-2 was also prolonged after LPS stimulation. Thus, DUSP1 is essential for the control of p38 activity, which is consistent with earlier data

showing selectivity of this MAPK phosphatase for p38 and JNK over ERK (Franklin and Kraft, 1997; Chen et al., 2002). It also corroborates previous reports that demonstrated prolonged p38 activation in DUSP1-deficient embryonic fibroblasts and alveolar macrophages, respectively (Wu and Bennett, 2005; Zhao et al., 2005). The fact that early peaks of p38 activity did not markedly differ between heterozygous control and DUSP1-deficient macrophages, suggests that induction of DUSP1 expression is predominantly a negative feedback mechanism to regulate the switch-off of MAPK signalling. Albeit slowly, even in the cells lacking DUSP1 the levels of phosphorylated p38 did decline. This might be due to the turnover of phosphorylated MAPKs or to dephosphorylation by other phosphatases. As shown here and by others, LPS not only induces DUSP1 in macrophages, but also DUSP2, DUSP10, and DUSP16 (Matsuguchi et al., 2001; Zhang et al., 2004; Hammer et al., 2005). Apparently, however, these cannot fully compensate for a lack of DUSP1 at least in terms of p38 regulation, but may be more effective in regulating JNK and ERK activity. It is interesting to mention that a series of three independent studies, each one investigating DUSP1-deficient mice, was published simultaneously to the work presented here (Zhao et al., 2005; Chi et al., 2006; Salojin et al., 2006). In accordance with my own results, these studies suggested p38 as the primary target of DUSP1 in activated macrophages, followed by JNK with no or very little effect on ERK activation.

It is obvious that altered p38 activity should influence the production of pro-inflammatory mediators by macrophages. Indeed, the extended p38 activation led to augmented production of the pro-inflammatory cytokines TNF-α and IL-6, as well as elevated NO levels. This confirms and extends the data by Zhao et al. who observed elevated TNF-α levels in conjunction with the prolonged p38 activity. Interestingly, the production of IL-10 in macrophages was also dramatically increased in DUSP1-deficient cells. Possible explanations for this counterintuitive finding will be discussed in more detail below. The increased production of TNF-α and IL-10, as well as the concurrent prolonged MK2 phosphorylation in DUSP1-deficient macrophages were completely abrogated when the p38-specific pharmacological inhibitor SB203580 was used. Two recent publications confirmed and extended these results, showing that no such abrogation was obtained when JNK or ERK were inhibited (Chi et al., 2006; Salojin et al., 2006). Compared to TNF-α and IL-10, the production of IL-6 remained unaffected by p38 inhibition, indicating that alternative mechanisms are operative. One possibility is that IL-6 is mainly induced by JNK, which was transiently increased in the absence of DUSP1. The p38/MK2 axis is very well known for its ability to mediate TNF-α production by enhancing the stability of TNF-α mRNA and

accelerating its translation (Kotlyarov et al., 1999; Lehner et al., 2002; Neininger et al., 2002). Taking into account my own observations, I therefore suggest that DUSP1 negatively regulates LPS-induced TNF-α production by inhibiting p38, which in turn leads to reduced MK2 activity and a subsequent slowdown of TNF-α protein synthesis. Notably, TNF-α and IL-6 are also overexpressed by DUSP10-deficient macrophages, suggesting that this phosphatase may at least partially overlap in function with DUSP1 (Zhang et al., 2004). LPS-activated DUSP2-deficient macrophages in contrast, express less TNF-α and IL-6 than wild-type controls, therefore DUSP2 may serve a distinct, positive regulatory role in innate immunity (Jeffrey et al., 2006).

5.2.3 DUSP1 controls IFN-β production in activated macrophages

Ligation of TLRs by viral products leads to the induction of type I interferons, providing a crucial mechanism of anti-viral defence. Moreover, LPS-induced signalling through TLR4 can also trigger the induction of type I responses, particularly IFN-β, in a MyD88-independent manner (Takeda and Akira, 2005). Examination of IFN-β levels in the supernatants of LPS-stimulated wild-type and DUSP1-deficient macrophages revealed significantly higher production of IFN-β by the cells lacking DUSP1. Induction of IFN-β transcription does not require *de novo* protein synthesis; rather, it occurs through posttranslational modification of transcription factors, which bind to specific positive regulatory domains within the IFN-β promoter (Falvo et al., 2000). It is well established that TLR4 stimulation leads to the activation of the cellular transcription factors NF-κB, interferon regulatory factor 3 (IRF-3), and ATF-2/c-JUN, which in conjunction with the transcriptional coactivator p300/CREB binding protein (CBP) activate transcription from the IFN-β promoter (Merika et al., 1998; Wathelet et al., 1998; Falvo et al., 2000; Kawai et al., 2001; Colonna, 2007). Whereas NF-κB and ATF-2/c-JUN are activated by various stimuli, IRF-3 is mainly activated in response to viral infection. However, several studies could show that IRF-3 can be activated by LPS as well (Kawai et al., 1999; Navarro and David, 1999; Shinobu et al., 2002). The higher IFN-β production by the DUSP1-deficient cells may be due to a direct effect on the activity of one or more of these transcription factors by p38. As already shown here, prolonged p38 activity led to enhanced phosphorylation and activation of ATF-2. Whether this enhanced ATF-2 activity alone caused the higher IFN-β production has to be determined. An additional mechanism is suggested by Navarro and David who observed that LPS-induced nuclear translocation of IRF-3 and subsequent induction of IFN-β requires the kinase activity of p38. Altered p38 activity would therefore positively affect IRF-3 activity

(Navarro and David, 1999). Finally, Saccani and colleagues propose a model of p38-dependent control of NF-κB activity by enhancing the accessibility of the NF-κB binding sites found in a subset of NF-κB-dependent promotors. However, if the IFN-β promotor belongs to this class of promotors has not been tested yet (Saccani et al., 2002).

5.2.4 DUSP1 deficiency affects STAT1 activity

Secreted IFN-β acts in an autocrine or paracrine fashion to amplify the response by binding to the type I interferon receptor, which activates STAT1 and ultimately results in the production of several IFN-inducible genes with immunomodulatory and antiviral properties (Toshchakov et al., 2002). I found that higher production of IFN-β by DUSP1-deficient compared to wild-type macrophages correlated with stronger phosphorylation of STAT1 on both, the tyrosine and the serine residue, albeit with different kinetics. The increased phosphorylation of the tyrosine residue is an indirect effect of DUSP1-deficiency due to the enhanced IFN-β production. It is very well known that binding of IFN-β to its receptor induces phosphorylation of STAT1 at its tyrosine residue (Darnell, 1997; Platanias, 2005). In contrast, serine phosphorylation of STAT1 occurs earlier after stimulation with LPS suggesting a direct effect. A possible explanation is brought about by the observation that p38 can directly phosphorylate the serine residue of STAT1 in macrophages upon LPS treatment (Kovarik et al., 1999). Curiously, there are reports from two different groups suggesting that DUSP1 dephosphorylates STAT1 at tyrosine residues in hepatocytes and vascular smooth muscle cells, which would imply a more direct interaction with the JAK-STAT pathway (Venema et al., 1998; Liu et al., 2002). However, these claims were contested by a yeast two-hybrid investigation of DUSP1 binding determinants that concluded that direct interactions are limited to the MAPK family (Slack et al., 2001). As mentioned above, the activation of STAT1 results in the production of several IFN-inducible genes. The stronger activation of the IFN-β pathway in DUSP1-deficient cells was in fact accompanied by higher IRF-1 levels. IRF-1 is mostly known as critical mediator in IFN-γ-mediated signalling, driving IL-12p35 and iNOS (Martin et al., 1994; Liu et al., 2003). However, Schmitz et al. recently implicated IRF-1 in the control of TLR9-dependent IFN-β induction in myeloid DCs (Schmitz et al., 2007).

5.2.5 DUSP1 plays a critical role in suppressing endotoxin shock

The pro-inflammatory cytokines TNF-α and IL-6 play a pivotal role in the pathogenesis of septic shock (Pfeffer et al., 1993; Ulloa and Tracey, 2005). Given the finding of their

augmented production by DUSP1-deficient macrophages after LPS stimulation, it was obvious to ask for the consequences of DUSP1 deficiency for the response to LPS *in vivo*. DUSP1 mRNA was rapidly induced in spleens, livers and lungs after injection of LPS. The absence of DUSP1 in a high-dose LPS shock model rendered these mice highly susceptible to the lethal effects of endotoxin compared to their wild-type counterparts, which again indicates a requirement of DUSP1 in a negative feedback loop that limits the inflammatory response. DUSP1-deficient mice produced dramatically more TNF-α and IL-6 after LPS challenge, which further validates the critical role of DUSP1 in the control of these two pro-inflammatory cytokines *in vivo*. Three additional groups independently reported very similar phenotypes of DUSP1-deficient mice in the endotoxin model (Zhao et al., 2005; Chi et al., 2006; Salojin et al., 2006). Consistent with the lethal outcome, mice lacking DUSP1 displayed the hallmarks of septic shock, such as depressed circulation, kidney failure, and inflammatory infiltrates in the lung and other tissues, much more pronounced than the surviving wild-type mice (Zhao et al., 2005). Salojin et al. further demonstrated in a model of autoimmune disease that collagen-induced arthritis is markedly more severe in DUSP1-deficient mice, with increased joint swelling and production of IL-6 and TNF-α (Salojin et al., 2006). DUSP1 is therefore an essential endogenous negative regulator of the systemic and local innate inflammatory response, phenotypically similar in effect to the cytokine IL-10 or the phosphatase SHIP. In the light of the proposed role for DUSP1 as a mediator of the deactivating IL-10 effects, it is interesting that DUSP1-deficiency had no significant effect on production of IL-12p40 and IFN-γ, whereas IL-10-deficient mice produced significantly more of these cytokines in response to LPS. Unaltered IFN-γ levels between DUSP1-deficient and wild-type mice were also observed by Chi et al. In contrast, Salojin et al. reported elevated IFN-γ levels in the sera of DUSP1-deficient mice after LPS challenge (Chi et al., 2006; Salojin et al., 2006). These conflicting data remain to be resolved, but might be due to the different time points of the analysis or the amount of injected LPS.

The inflammatory phenotype observed in this study and by others is dependent on haematopoietic DUSP1 because I was able to transfer the phenotype by reconstitution of wild-type mice with bone marrow from DUSP1-deficient mice. Notably, the absolute serum levels of the examined cytokines after LPS challenge were lower in the reconstituted mice compared to the non-irradiated wild-type and DUSP1-deficient mice. This is probably due to an incomplete reconstitution of the haematopoietic system and a resulting lower cell number in the reconstituted mice. It cannot be completely ruled out, however, that cells of non-haematopoietic origin might also contribute to the excessive cytokine production. Kano et al.,

for example, reported that conditional knockout of STAT3 in endothelial cells renders mice more susceptible to LPS-induced shock and implicated non-haematopoietic cells as an important source for pro-inflammatory cytokine production in this model (Kano et al., 2003). One possibility to test this is the reconstitution of DUSP1-deficient mice with bone-marrow from wild-type mice. If the phenotype solely depends on cells of the haematopoietic system, they should produce equal amounts of cytokines compared to wild-type mice reconstituted with wild-type bone-marrow after LPS injection. Experiments testing this hypothesis are currently underway.

5.2.6 DUSP1 controls a subset of LPS-induced genes

To further dissect the impact of DUSP1 on the control of inflammatory mediators a microarray analysis of splenic RNA was performed. I found about three times more genes up-regulated in DUSP1-deficient mice compared to wild-type after stimulation with LPS. Confirming the findings on the protein level, IL-6 was much stronger induced in the DUSP1-deficient mice, whereas IL-12p40 and IFN-γ displayed no differences. TNF-α expression was not increased on the mRNA level in DUSP1-deficient mice, which is in contrast to the observation at the protein level. A possible explanation could be posttranscriptional effects that influence TNF-α mRNA stability or translation. Closer examination of the genes that have been implicated in endotoxin shock revealed that not only IL-12 and IFN-γ were expressed at similar levels in DUSP1-deficient and wild-type mice, but also IL-18 and type I interferons. Why the augmented IFN-β levels in macrophages cannot be monitored *in vivo* has so far proven elusive. Beside typical cytokines, the chemokines CCL3, CCL4 and CXCL2 were also found to be overexpressed in DUSP1-deficient mice. CCL3 is a known promotor of leukocyte recruitment to the lungs and increases lethality, an effect that may be enhanced by the closely related CCL4 and CXCL2 thereby contributing to the development of shock (Standiford et al., 1995). However, as observed for the cytokines, expression of some but not all chemokines was DUSP1-dependent. CXCL10 levels, for example, did not differ between DUSP1-deficient and wild-type mice, suggesting that only a subset of the LPS-induced genes is under the control of DUSP1. Beside typical mediators of endotoxic shock, several other genes were also differentially expressed in DUSP1-deficient animals upon LPS treatment. The long pentraxins PTX3 and coagulation factor III (F3) were both up-regulated in DUSP1-deficient spleens. In contrast, MEFV, the gene for familial mediterranean fever, was more strongly induced in wild-type mice. Although all three genes have been implicated in innate immunity and inflammation their contribution to the lethal outcome of LPS-induced shock in

DUSP1-deficient mice still needs to be clarified (Chae et al., 2003; Bottazzi et al., 2006; Ahamed et al., 2007).

5.2.7 Stronger inflammation is not equivalent to better protection in DUSP1-deficient mice

The higher cytokine production by DUSP1-deficient mice after LPS challenge prompted me to ask whether DUSP1 also affects the protection from infection with live bacteria. To answer this question, I investigated its role in a model of septic peritonitis. Similar to the observations in the LPS-induced endotoxic shock model, this severe mixed bacterial infection resulted in augmented levels of IL-6 and IL-10 in the serum and the peritoneal lavage of DUSP1-deficient compared to heterozygous control mice. Furthermore, DUSP1 deficiency renders mice more susceptible to the lethal effects of sepsis after polymicrobial peritonitis. However, it has to be mentioned that a difference in mortality could only be detected when a smaller venous catheter was used. Immediate recruitment of neutrophils to the site of infection is considered as an essential mechanism to eliminate certain classes of pathogens. Also, neutrophils were shown to be of critical importance for the immune defence against intraabdominal bacterial infections in mice (Haziot et al., 1996; Weighardt et al., 2000). In the present study, increased accumulation of neutrophils and macrophages in the peritoneal cavity was observed, but did not differ between control and DUSP1-deficent mice. Further, although bacterial loads in spleens, livers and the peritoneal cavities varied by hundred- even thousand-fold between individual mice, they were similar in the two groups. This indicates that the higher cytokine levels measured in DUSP1-deficient mice did not correlate with improved clearance of the bacteria. These observations suggest that DUSP1 plays a crucial role in controlling exaggerated cytokine production, and that DUSP1-deficient mice die rather due to an overwhelming cytokine storm than to their inability to control the infection.

5.2.8 Mutual regulation of expression between DUSP1 and IL-10

An intriguing finding from this study is the counterintuitive, overwhelming release of the anti-inflammatory cytokine IL-10 in the absence of DUSP1 both in isolated macrophages and *in vivo*, which might be expected to bring about down-regulation of the inflammatory reaction but obviously fails to do so. Similarly, in the myeloid-specific STAT3-deficient mouse, abrogated IL-10 signalling leads to overshooting, lethal cytokine production that includes IL-10 (Takeda et al., 1999). It has been shown that p38 is crucial for the production of IL-10 during LPS stimulation (Ma et al., 2001; Guo et al., 2003; Hu et al., 2006). Therefore the increase of IL-10 production in DUSP1-deficient cells reflects the increased p38 activity.

However, the increased levels of IL-10 in DUSP1-deficient mice may not be produced early enough, or in sufficient amounts to counteract the detrimental effects of pro-inflammatory cytokines in this model. Among the genes overexpressed in DUSP1-deficient spleens, I observed a considerable number of transcripts previously identified as IL-10-induced genes, including SOCS3, NFIL3, NDR1, BCL-3 and GADD45γ. This probably reflects secondary effects of the overshooting production of IL-10 and IL-6 that both activate transcription *via* STAT3 signalling (Lang et al., 2002). Although IL-10 signalling seems to be intact in the absence of DUSP1, the high levels were not effective in down-regulating synthesis of IL-6 or the chemokines CCL3, CCL4, or CXCL2. Thus, DUSP1 may be required for the inhibition of at least some IL-10-regulated genes. This hypothesis would also explain the fact that the production of other classical inflammatory mediators like IL-12p40 and IFN-γ is unaltered in DUSP1-deficient mice compared to wild-type, due to a DUSP1-independent inhibitory action of IL-10 (Figure 21). Similarly, TNF-α overproduction is only seen at early time points after exposure to LPS. The reduction at later time points probably reflects the then elevated IL-10 levels that suppress TNF-α production. In support of this mechanism Chi and colleagues showed that TNF-α mRNA levels could be enhanced by the exposure of DUSP1-deficient macrophages to a neutralizing IL-10-specific antibody prior to LPS challenge (Chi et al., 2006). At any rate, the link between IL-10, p38 and DUSP1 now appears rather complex (Figure 21). The anti-inflammatory cytokine lies both upstream of DUSP1, modulating its expression in response to LPS, and downstream of DUSP1, where it acts as a target of the p38 MAPK signalling pathway.

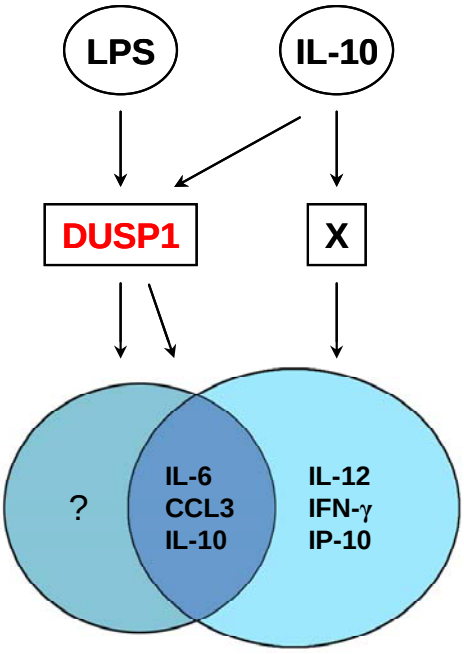


Figure 21. Proposed model for the negative control of inflammatory mediators by IL-10 and DUSP1.
IL-10 lies both upstream of DUSP1, where it modulates its expression, and downstream of DUSP1 as target of the p38 MAPK signalling pathway. Only a part of the IL-10 controlled genes depend on DUSP1, whereas the other portion is regulated by different IL-10 mediators. In addition, there must be genes that are influenced by DUSP1, but do not underlie the control of IL-10. However, the existence of these genes needs to be confirmed.

5.2.9 DUSP1 and other candidate mediators of the IL-10 effect

The question, whether DUSP1 really mediates anti-inflammatory effects of IL-10 and especially to which degree, turned out to be difficult to test directly because of the strong overexpression of IL-10 by DUSP1-deficient macrophages. To examine the nature of this complex interaction I generated IL-10/DUSP1 double-deficient mice. If DUSP1 is the essential mediator of IL-10, one would expect that the double-deficient mice produce equal amounts of pro-inflammatory mediators like IL-6, CCL3 and CCL4 compared to the mice solely deficient for DUSP1 or IL-10. First *in vitro* experiments with bone marrow-derived macrophages showed that this is probably not the case, since the DUSP1-deficient as well as the double-deficient cells still responded to IL-10 in a dose dependent manner. This finding is further supported by preliminary *in vivo* data. The IL-10-deficient mice produced more pro-inflammatory mediators after injection of LPS than their DUSP1-deficient counterparts, whereas the double-deficient mice produced even higher amounts than the mice lacking IL-10. Interestingly, half of the double-deficient mice died at doses that are sublethal for the single-deficient mice, which implies that DUSP1 controls a subset of anti-inflammatory genes that are not induced by IL-10 (Figure 21). Although additional experiments are necessary to

confirm and extend these observations, they so far favour the model that DUSP1 is required for the inhibition of some, but not all IL-10-regulated genes. In fact, DUSP1 is not the only potential mediator of the anti-inflammatory IL-10 effect in macrophages that we identified from our previously described microarray experiments (Lang et al., 2002). SOCS3 was one of the genes induced most strongly by IL-10 in resting macrophages. However, it was subsequently shown not to be required for inhibition of the production of the pro-inflammatory cytokines TNF-α and IL-12p40 by LPS-activated macrophages (Lang et al., 2003). Potential mediators of the IL-10-induced suppression of cytokine production are genes that negatively influence activating signalling pathways or transcription. I therefore focused on the analysis of genes with repressing properties. NFIL3 and B-ATF are both members of the basic leucine zipper family (bZIP) of transcription factors with the ability to act as transcriptional repressors (Cowell et al., 1992; Williams et al., 2001). Therefore they represent candidate mediators of the IL-10 effect. Another transcription factor found to be induced by IL-10 is BCL-3, a member of the IκB protein family. Although it is known that BCL-3 influences NF-κB activity it is controversial whether it acts as positive or negative regulator (Bours et al., 1993; Lenardo and Siebenlist, 1994; Brasier et al., 2001). Wessells and colleagues proposed that BCL-3 and p50 function co-ordinately to limit the pro-inflammatory response of activated macrophages by attenuating transcription of pro-inflammatory cytokines on the one hand and activating expression of IL-10 on the other (Wessells et al., 2004). In a first attempt to examine their influence on LPS-induced cytokine expression, overexpression studies of these transcription factors as well as of the IL-10-induced kinases TPL-2 and NDR1 were performed in RAW 264.7 macrophages. However, overexpression and subsequent detection of the proteins was difficult. During the course of these experiments another gene array analysis in murine macrophages also revealed up-regulation of BCL-3 by IL-10 or LPS, thus confirming our own results (Kuwata et al., 2003). In this study, overexpression of BCL-3 led to the inhibition of LPS-induced TNF-α and mice lacking BCL-3 showed reduced inhibition of TNF-α production by IL-10. In contrast, IL-6 production was not affected at all. Furthermore, Cao et al. could show that c-MAF, another member of the bZIP family of transcription factors, is induced by IL-10 in human monocytes (Cao et al., 2002). When overexpressed in primary murine macrophages c-MAF selectively inhibits IL-12 production, but potently activates IL-10 production. According to that, mice deficient for c-MAF showed a reduced IL-10 production. However, IL-12 production was normal after stimulation with LPS and was still inhibited by IL-10 in c-MAF-deficient mice. Thus, Bcl-3 and c-MAF, respectively, are able to mediate some but not all of the anti-inflammatory activities of IL-10.

Since the overexpression of potential IL-10 mediators in the macrophage cell line RAW 264.7 was not a very suitable approach, I decided to create a transgenic mouse model, which conditionally overexpresses NFIL3. So far, positively transfected embryonic stem cells (ESC) were injected into foster mice. The chimeric offspring is currently screened for founder mice, which will hopefully help to clarify whether NFIL3 is another important component of the anti-inflammatory response to IL-10.

5.3 Outlook

The unexpected finding of a non-redundant role for DUSP1 in balancing the inflammatory response suggests that various members of the DUSP family fulfil specific regulatory roles in immune cells. Two recent reports describing the function of DUSP2 and DUSP10 further substantiate this hypothesis (Zhang et al., 2004; Jeffrey et al., 2006). Deletion of either of these phosphatases leads to elevated JNK signalling. However, in the case of DUSP10 it results in enhanced cytokine production, whereas mice lacking DUSP2 display a deficit in cytokine gene expression. In the case of DUSP1, the p38 MAPK pathway likely is the major target, and DUSP1 deficiency unleashes overwhelming production of a rather selective set of pro-inflammatory mediators. An important challenge in the field is to understand this apparent division of labor between different MAPK phosphatases in the control of immune responses in relation to various MAPKs. In terms of infections with pathogens it is an important question whether changes in the production of inflammatory cytokines and mediators depending on the functionality of DUSP confer a state of more efficient pathogen control to the innate immune system, or may in contrast lead to increased immunopathology. Future work will therefore focus on the contribution of DUSP1 to the host response to viral and bacterial infections like *Chlamydia pneumoniae* and *Listeria monocytogenes*. By comparing the expression profiles of the double- and single-deficient mice after LPS challenge, we further hope to shed some more light on the question, which subsets of inflammatory mediators are controlled by DUSP1 and IL-10, respectively. Besides that, we are currently designing a phosphoproteomic approach to investigate whether MAPKs are the only physiological targets of DUSP1. Using chromatin-immunoprecipitation we want to dissect how DUSP1 expression is controlled, since understanding its regulation might help to specifically interfere with uncontrolled DUSP1 activity as observed in various human cancers (Liao et al., 2003; Vicent et al., 2004).

6 Summary

The detection of and the response to microbial infections by macrophages and dendritic cells depend largely on the family of Toll-like receptors (TLR) that recognize specific molecular patterns present in the pathogens. One of the key pathways activated by TLR signalling in innate immune cells is the MAPK cascade. Active p38, JNK and ERK MAPK phosphorylate a plethora of downstream transcription factors and other target proteins, which contribute to the transcriptional regulation of cytokines and inflammatory mediators. However, TLR activation is a double-edged sword. It is essential for provoking the innate response and enhancing adaptive immunity against pathogens. On the other hand, members of the TLR family are also involved in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases. The anti-inflammatory cytokine IL-10 plays an irreplaceable role in the negative regulation of macrophage activity and inflammation, primarily by blocking the expression of the pro-inflammatory mediators. To date, it is largely unclear which IL-10-induced genes are operative in macrophage deactivation and which level of cytokine gene expression they may target. An obvious possibility to negatively regulate the response to infectious non-self is the control of MAPK activity by specific phosphatases.

In this study, I have mined microarray datasets for changes in the expression of MAPK phosphatases in resting and TLR-activated macrophages. LPS transiently induced expression of the dual-specificity phosphatase 1 (DUSP1), while the addition of IL-10 resulted in a continued and more robust expression of DUSP1. LPS-induced phosphorylation of p38 MAPK was prolonged in IL-10-deficient macrophages, whereas addition of exogenous IL-10 partially corrected this effect. Interestingly, IL-10 also functioned synergistically with the immunosuppressive glucocorticoid dexamethasone in inducing DUSP1 expression and in suppressing production of IL-12-p40 and IL-6. Investigating the regulation of DUSP1 expression, I found that DUSP1 mRNA is unstable, suggesting that at least part of its regulation is post-transcriptional. However, the stability of DUSP1 mRNA was not substantially affected by IL-10. In reporter gene assays using DUSP1 promoter constructs, I could show that LPS activated the promoter, but IL-10 had no effect on the reporter activity. Furthermore, LPS induces DUSP1 expression *via* pathways that are IKKβ-independent.

A non-redundant role for DUSP1 in balancing the inflammatory response was revealed using DUSP1-deficient mice. DUSP1 knockout prolonged the activation of p38 and its downstream targets in LPS-stimulated macrophages. This was associated with substantially elevated production of TNF-α, IL-6, IFN-β and NO. Transcriptional profiling revealed that DUSP1

controls a significant fraction of LPS-induced genes *in vivo*, including IL-6 and IL-10 as well as the chemokines CCL3, CCL4, and CXCL2. The source of these elevated levels is of haematopoietic origin as shown by adoptive transfer experiments. Although the expression of the important mediators of endotoxin lethality, IFN-γ and IL-12, was not altered by the absence of DUSP1, significantly higher lethality was observed in these mice upon LPS injection. Another interesting finding was that the lethal outcome of LPS challenge in DUSP1-deficient mice was correlated with excessive production of the anti-inflammatory cytokine IL-10, indicating that an essential fraction of IL-10-induced macrophage deactivation may therefore depend on functional DUSP1. Mice lacking DUSP1 were also more susceptible in a model of polymicrobial sepsis, indicated by increased levels of IL-6 and IL-10 and a higher mortality of the mice lacking DUSP1. In contrast, bacterial cell numbers showed no differences between DUSP1-deficient and control mice.

In conclusion, this study provides evidence for a link connecting the macrophage-deactivating cytokine IL-10 with a member of the DUSP family that controls macrophage function at the level of MAPK activity. In addition, it suggests a critical and non-redundant role for DUSP1 in the innate immune response to LPS and in septic peritonitis. Taking into account recent reports from other labs on the effects of genetic deletion of DUSP2 and DUSP10, there appears an unexpected specificity in the regulation of the MAPK proteins by individual DUSPs, which makes them an attractive new pharmacological target for immunomodulation. Small molecule inhibitors have recently been identified and hold promise for selective therapeutic intervention

7 References

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