Regulation of anti-inflammatory cytokine IL-10 by the Polycomb Group Protein Bmi1

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Arnold R. Sienerth

aus Hermannstadt

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Gutachter der schriftlichen Arbeit

1. Gutachter: Prof. Dr. Utz Fischer

2. Gutachter: Prof. Dr. Alexander Buchberger

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1. Prüfer: Prof. Dr. Utz Fischer

2. Prüfer: Prof. Dr. Alexander Buchberger

3. Prüfer: PD Dr. Jörg Wischhusen

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To my parents

Summary

Macrophages are important effector cells of the innate and adaptive immune response and exert a wide variety of immunological functions which necessitates a high level of plasticity on the chromatin level. In response to pathogen-associated molecular patterns (PAMPs) or inflammatory signals macrophages undergo a process of cellular activation which is associated with morphologic, functional and biochemical changes. Toll-like receptors (TLR) are able to sense many different PAMPs. TLR4 is an important sensor for lipopolysaccharide (LPS) which elicits a major portion of the host's inflammatory response through the activation of many different signaling pathways such as the NF-κB and the MAPK protein kinase pathways RAS-RAF-MEK-ERK, p38 and JNK.

Polycomb group (PcG) proteins are well known chromatin modifiers which function in large complexes and are required to maintain chromatin structure in a transcriptionally repressed state. It has previously been shown that the PcG protein Bmi1 is phosphorylated by 3pK, a downstream effector kinase of the MAPK protein kinase pathways RAS-RAF-MEK-ERK, p38 and JNK.

In this work I analyzed the role of Bmi1 as a downstream effector of MAPK signaling during macrophage activation. Unexpectedly a rapid up-regulation on the Bmi1 protein level was observed in bone marrow derived macrophages (BMDMs) after LPS treatment. The Bmi1 induction was associated with transient protein phosphorylation that occured downstream of MAPK signaling. LPS treatment of BMDMs in the absence of Bmi1 resulted in a pronounced increase of IL-10 secretion. This secretion of the anti-inflammatory cytokine IL-10 was associated with increased IL-10 mRNA levels. Furthermore, siRNA mediated knock down of Bmi1 in J774A.1 macrophages also resulted in elevated IL-10 mRNA levels in response to LPS. ChIP analysis revealed that Bmi1 binds to throughout the *il-10* locus. Alternative activation of wild type BMDMs via concomitant TLR4 and FcγR activation which triggers high IL-10 expression is paralleled by an attenuated Bmi1 protein expression.

These results identify Bmi1 as a repressor of IL-10 expression during activation of macrophages.

Zusammenfassung

Makrophagen sind wichtige Effektorzellen der angeborenen und adaptiven Immunantwort und üben eine große Fülle von immunologischen Funktionen aus. Deshalb benötigen sie eine hohe Plastizität auf Chromatinebene. Als Antwort auf pathogen-assoziierte molekulare Muster (PAMPs) oder andere inflammatorische Signale machen Makrophagen einen zellulären Aktivierungsprozess durch, der mit morphologischen, funktionellen und biochemischen Veränderungen assoziiert ist. Toll-like Rezeptoren (TLR) sind fähig, solche PAMPs zu erkennen. Der TLR4 ist ein wichtiger Sensor für Lipopolysaccharid (LPS). LPS löst eine inflammatorische Antwort des Wirtes durch die Aktivierung vieler verschiedener Signalwege wie zum Beispiel des NF-κB Signalwegs und der MAPK Proteinkinasensignalwege RAS-RAF-MEK-ERK, p38 und JNK aus.

Polycomb Gruppen (PcG) Proteine arbeiten in großen Proteinkomplexen zusammen und werden benötigt, um das Chromatin in einem transkriptionell reprimierten Zustand beizubehalten. Es konnte gezeigt werden, dass das PcG Protein Bmi1 von 3pK, einer Effektorkinase der MAPK Proteinkinasensignalwege RAS-RAF-MEK-ERK, p38 und JNK, phosphoryliert wird.

In meiner Doktorabeit analysierte ich die Rolle von Bmi1 als downstream-Effektor der MAPK-Signaltransduktion während der Makrophagenaktivierung. Es wurde unerwarteterweise eine schnelle Induktion von Bmi1 auf Proteinebene in Knochenmarks-Makrophagen (BMDMs) beobachtet. Diese Induktion war MAPK-abhängig und mit einer transienten Proteinphosphorylierung assoziiert. Die LPS-Behandlung der BMDMs in Abwesenheit von Bmi1 hatte erhöhte IL-10 Sekretion zur Folge. Diese erhöhte Sekretion des anti-inflammatorischen Zytokines IL-10 war mit erhöhten IL-10 mRNA Expression verbunden. Des Weiteren hatte ein Bmi1 siRNA-vermittelter Gen-Knockdown in J774A.1 Makrophagen eine erhöhte IL-10 mRNA-Expression zur Folge. ChIP Analysen haben gezeigt, dass Bmi1 am ganzen *il-10*-Locus verteilt binden kann. Eine TLR4 und FcγR vermittelte alternative Aktivierung von BMDMs, die mit hoher IL-10 Expression verbunden ist, führte zu einer attenuierten Bmi1 Proteinexpression.

Diese Ergebnisse zusammengenommen weisen Bmi1 als Repressor von IL-10 während der Makrophagenaktivierung aus.

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1 Introduction

Organisms are constantly threatened by invaders and therefore need to build up efficient defence mechanisms. Effective protection requires recognition, activation and mobilization, regulation and resolution. Especially vertebrates have therefore evolved throughout evolution a sophisticated, intermeshed network namely the immune system.

1.1 The immune system

The immune system is able to defend the organism against infections caused by microorganisms such as bacteria, viruses, fungi and parasites such as worms. The vertebrate organism has developed series of tightly coordinated lines of defences.

1.1.1 The adaptive immune system

Once activated through the innate immune system the adaptive immune system is able to learn, to adapt, and remember specific pathogens. The ability to develop a long lasting immunological memory for a great number of pathogenic germs is the most important advantage of the adaptive immune system in comparison to the innate immune system. The effector cells of the adaptive immune system are a type of leukocyte, called a lymphocyte. B cells and T cells are the major types of lymphocytes.

T cells maturation occurs in the thymus and the abbreviation T stands for thymus. In the thymus they learn how to distinguish self from nonself. Mature T cells are stored in secondary lymphoid organs such as the lymph nodes, spleen, tonsils, appendix, and Peyer's patches in the small intestine. Several different subsets of T cells have been discovered, each with a distinct function. Although the specific mechanisms of activation vary slightly between different types of T cells, the activation occurs through the engagement of both the T cell receptor and the CD28 and the T cell and the major histocompatibility complex (MHC) peptide on the antigen-presenting cells (APC).

Helper T cells or helper T cells (Th) divide rapidly and secrete small proteins called cytokines that regulate or assist in the immune response.

Cytotoxic T cells destroy infected cells and tumor cells, and are also implicated in transplant rejection.

Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immunological memory against past infections.

Regulatory T cells also known as suppressor T cells are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells.

B cells are the other essential effector cells of the adaptive immune system. Immature B cells are produced in the bone marrow and B cell development and B-lineage commitment is controlled by a complex interplay between specific cytokine receptors and a range of transcription factors (Monson, 2008). B cells are involved in the production of antibodies that circulate in blood plasma and lymph, known as humoral immunity. In most cases B-cell activation is dependent on a second type of lymphocyte namely helper T cell. Once a helper T cell has been activated by an antigen, it becomes capable of activating a B cell that has already encountered the same antigen (Parker, 1993). Upon activation, B cells produce antibodies, each of which recognizes a unique antigen, and neutralize specific pathogens. Once a B cell encounters its specific antigen it further differentiates into an effector cell, also known as a plasma cell. Plasma cells are short lived cells with a life expectation of 2-3 days which secrete antibodies. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement system. About 10% of plasma cells will survive to become long-lived antigen specific memory B cells. Already primed to produce specific antibodies, these cells can respond quickly if the same germ infects the host again.

1.1.2 The innate immune system

The first line of defence against invaders represents mechanical or physical barriers provided by the epithelial tissues e.g. from the skin and membranes lining the respiratory, digestive, urinary, and reproductive tracts which are positioned at the interface between the host and external environment. The cells of the epithelium are closely packed through cell-cell connections called tight junctions and as long as these barriers remain undamaged, many invaders cannot enter the body. But in addition some epithelial cells are able to secrete fluids like mucus, sweat or tears which are viscous and thereby prevent microorganism invasion or contain enzymes like lysozymes or antimicrobial peptides like defensins and cryptidins and thereby eliminate the microbial menace. Besides this some epithelial tissues are associated with non pathogenic, commensal microorganisms which compete with pathogenic germs for nutrients and adhesion

sites and themselves produce antimicrobial substances such as colicins produced by *Escherichia coli* (*E. coli*) and thereby protect from pathogen infections (Gallo & Nizet, 2008; Krisanaprakornkit et al, 2000).

The next line of defence is the complement system. The complement system is an important immune surveillance system which consists of a number of small proteins in the blood plasma, which normally circulate as inactive enzyme precursors so called zymogens and gets activated through a triggered-enzyme-cascade. The complement activity is important for the clearance of foreign pathogenic and altered host cells and has mainly following functions: Opsonization of microorganisms with activated complement proteins, which promotes phagocytosis by phagocytic cells which display receptors against the proteins of the complement proteins, chemoataxis of other phagocytic cells and lysis of microorganisms mediated through for example pore building in membranes of pathogens (Gros et al, 2008; Tomlinson, 1993).

Yet another line of defence involves multiple cell types derived from the bone marrow which originate from a common, pluripotent hematopoietic stem cell. The cells involved are macrophages, dendritic cells, neutrophils, eosinophils, basophils and natural killer cells which are called white blood cells or leukocytes. Leukocytes belong to an immune response that is immediate, present at birth and has no memory, the innate immunity. The previously mentioned epithelial barriers and the complement system are also considered to participate in innate immune responses.

Neutrophils are the most common type of white blood cell circulating in the bloodstream. They ingest bacteria and other foreign cells. Neutrophils contain pre-packed granules that release enzymes to help kill and digest these cells. Neutrophils must be signalled to leave the bloodstream and enter tissues. The signal often comes from the bacteria themselves, from complement proteins, or from damaged tissue, all of which produce substances that attract neutrophils to a trouble spot. But neutrophils themselves generate chemotactic signals that attract monocytes and dendritic cells (Nathan, 2006).

In response to diverse stimuli, including nonspecific tissue injury, infections, allografts, allergens, and tumors, eosinophils are recruited from the bloodstream circulation into inflammatory foci, where they release their granules that contain cytotoxic, cationic proteins for example eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Rothenberg & Hogan, 2006).

Basophils make up less than 1% of circulating blood leukocytes and provide unique functions particularly during allergic and antihelminth responses. Basophils also produce substances that attract neutrophils and eosinophils to a trouble spot. They contain granules filled with histamine, a substance involved in allergic reactions and released when basophils encounter allergens. Histamine release causes several systemic effects, such as vasodilation, mucous secretion, nerve stimulation and smooth muscle contraction. This results in rhinorrhea, itchiness, dyspnea, and anaphylaxis (Sullivan & Locksley, 2009).

Natural killer cells are important in the initial defense against viral infections because they are able to eliminate pathogen-infected cells and even tumors cells by virtue of their cytolytic capacity. Attached to pathogen-infected cells and tumors cells, they release enzymes and other substances that damage the membranes of these cells (Colucci et al, 2003).

Dendritic cells (DCs) reside mainly in the skin, but also in lymph nodes and tissues throughout the body and their main function is to process macromolecules, that elicit an immune response termed antigens, enabling induction of T cell response (Degli-Esposti & Smyth, 2005). Thus they serve as a link between the innate immunity and a further line of defence developed only by vertebrates and cartilaginous fish the adaptive or acquired immune system (Dempsey et al, 2003). That means that the innate immune system does not only function as the first line of host defense it also functions as an activator and controller of the adaptive immune response.

1.2 Macrophages

As mentioned macrophages play a crucial role in the innate immunity and were initially discovered as phagocytic cells as big eaters implied by their name (Metchnikoff, 1905; Nathan, 2008) representing an evolutionary conserved cell population responsible for pathogen elimination and housekeeping functions in a wide range of organisms, from invertebrates to vertebrates (Martinez et al, 2009). But macrophages are much more than that they play an important role in innate as well as in adaptive immunity and as important members of the immune response they are widely distributed and are found in almost every tissue of the body, and constitute a heterogeneous cell family. Even in a single organ, resident tissue macrophages can be very heterogeneous. The rodent spleen is a particularly good example for this (Table 1.1) (Hume, 2008). The environment of macrophages significantly influences the function of macrophages such that macrophages resident in different tissues display varying patterns of function including wound healing, tissue repair and vascularization (Stout & Suttles, 2004).

Additionally macrophages have important roles in tissue development including functions in branching morphogenesis, neuronal patterning, angiogenesis, bone morphogenesis and the generation of adipose tissue (Pollard, 2009)

Macrophages are derived from pluripotent self-renewing hematopoietic stem cells in bone marrow which further differentiate into myeloid progenitor cells. Myeloid progenitor cells give rise to monoblasts, pro-monocytes and finally monocytes (Figure 1.1). Then monocytes are released from the bone marrow into the bloodstream where they can circulate for several days before entering tissues and replenishing long-lived resident tissue macrophage populations (Gordon & Taylor, 2005).

Macrophages are large, motile, terminally differentiated cells of the mononuclear phagocytic lineage. After exerting their functions, the differentiated cells die via apoptosis (Metcalf, 1991).

Name	Appearance
Monocyte	blood
Macrophages	
Alveolar macrophages	lung
Histiocytes	connective tissue
Kupffer cells	liver
Microglia cells	neural tissue
Osteoclasts	bone
Langerhans cells	epidermis/stratum spinosum
Hofbauer cells	placenta
Osteoclast	bone
White-pulp macrophage	spleen
Red-pulp macrophage	spleen
Marginal-zone macrophage	spleen
Metallophilic macrophage	spleen
Sinusoidal lining cells	spleen

Table 1.1 Macrophage heterogeneity

Overview about the macrophage heterogeneity (Mosser & Edwards, 2008).

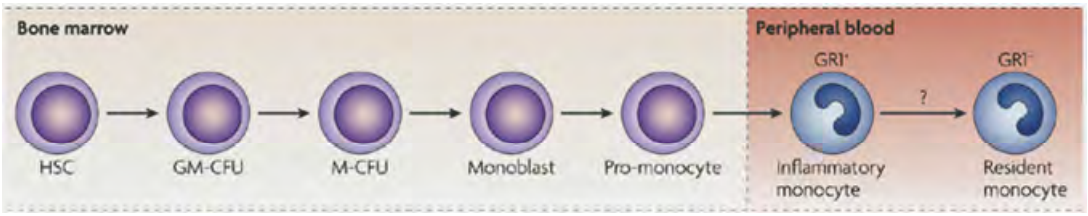

Figure 1.1 Monocyte differentiation

Monocytes originate from a common haematopoietic stem cell (HSC) in the bone marrow. They differentiate to myeloid progenitor cells then to a monoblasts and before they finally differentiate to monocytes which exit the bone marrow and enter the bloodstream. Reproduced from (Mosser & Edwards, 2008) with permission from Nature Publishing Group.

1.2.1 Macrophage functions

Macrophages are considered as professional phagocytes and are uniquely qualified to engulf large (≥ 0.5 mm) particles, including microorganisms (Flannagan et al, 2009). Phagocytosis as a mechanism of innate immune defence represents one of the first line of immunologic defences. By crosslinking of innate receptors on the macrophage surface upon binding to pathogens waves of signal transduction events initiate the rearrangement of the actin cytoskeleton as well as extension of pseudopodia, leading to the internalization of the bacteria into the phagosome. Phagosome formation is not only mediated by pattern recognition receptors it can also be indirectly mediated through opsonins such as immunoglobulin G (IgG) and is subdivided in different developmental steps such as phagosome formation, phagosome maturation and finally microbicidal activity of the phagosome. One of the antimicrobial features of the phagosome is its V-ATPases mediated acidification (Beyenbach & Wieczorek, 2006). Another feature to effectively kill intraphagosomal microorganisms is the production of the toxic reactive oxygen (ROS) and nitrogen species (NOS). A further tool of phagosomes to antagonize microorganisms is the production of antimicrobial proteins and peptides such as defensins, lysozymes and exopeptidases like NRAMP1 (Flannagan et al, 2009). Macrophages phagocyte not only foreign material they are also involved in the removal of cells that undergo apoptosis (Erwig & Henson, 2008).

As antigen-presenting cells (APCs) macrophages function as crucial mediators of host defense serving as a link between innate and adaptive immune responses during infection. Antigens derived from phagocytosed particles or pathogens can be loaded onto major histocompatibility

complex (MHC) class I (Lehner & Cresswell, 2004) or MHC class II molecules (Bryant & Ploegh, 2004) and thereby initiating a T-lymphocyte response.

Macrophages are able to undergo a complex process termed "activation" which is associated with morphologic, functional and biochemical changes in the cells. Activation is mediated by stimuli from endogenous and exogenous sources (Adams & Hamilton, 1984). Macrophages can efficiently respond to many different signals that trigger activation due to their remarkable plasticity to change their phenotype and to change their physiology (Mosser & Edwards, 2008). In response to conserved pathogen-associated molecular patterns (PAMPs) or endogenous danger signals such as interferonγ (INFγ) and tumor-necrosis factor (TNF) recognized by pattern recognition receptors macrophages alter their physiology which can provide them with enhanced microbibial capacity and secretion of high levels of pro-inflammatory cytokines and mediators. This type of macrophage activation is reffered to as “classical activation” and is important for host defence. Classical activation must be tightly controlled because the produced cytokines and mediators can lead to host-tissue damage.

In addition similarly to classically activated macrophages, macrophages change their physiology to promote wound healing in response to an endogenous stimulus like IL-4. These cells are called wound-healing macrophages and were originally termed alternatively activated macrophages. Wound-healing macrophages are able to secrete components of extracellular matrix and molecules important for matrix reorganisation (Mosser & Edwards, 2008). Macrophages treated *in vitro* with IL-4, in contrast to classically activated macrophages, fail to present antigens to T cells, produce minimal amounts of pro-inflammatory cytokines and are less efficient than at killing pathogens (Martinez et al, 2009).

However, environmental signals are not the sole inducers of changes in macrophages which increase immune response or wound-healing function. In response to stress adrenal cells release glucocorticoids which inhibit macrophage-mediated host defence and inflammatory functions by inhibiting the transcription of pro-inflammatory cytokine genes and decreasing mRNA stability giving rise to a population of regulatory macrophages (Mosser & Edwards, 2008). However, not all previously mentioned macrophage functions are affected in glucocorticoid treated regulatory macrophages. Phagocytosis of apoptotic cells seems to remain largely unaffected if not increased in the presence of glucocorticoids (Liu et al, 1999). Glucocorticoid-treated macrophages either fail to present antigen to T cells or induce the development of regulatory T cells that can also inhibit immune responses (Franchimont, 2004). There are many other factors that can provide a signal for the differentiation of regulatory macrophages including prostaglandins, apoptotic cells

and the immunosuppressive cytokine IL-10. Some of these signals like prostaglandins and apoptotic cells generally have little or no stimulatory function on their own. However, when combined with a second stimulus, such as a Toll like receptor (TLR) agonist, the two signals re-programme macrophages to secrete high levels of IL-10. High IL-10 production is the most important and reliable characteristic of regulatory macrophages. Another way to generate IL-10 producing regulatory macrophages is the stimulation with TLR ligands in the presence of immune complexes recognized by the Fc receptor family (Sutterwala et al, 1998). Therefore regulatory macrophages are potent inhibitors of inflammation and their primary role seems to be the attenuation of the immune response and the limitation of inflammation to avoid toxicity and tissue damage (Mosser, 2003).

1.3 Pattern-recognition receptors (PPRs)

Macrophages and other professional phagocytic cells of the immune system recognize ligands indirectly after opsonization with antibodies or activated complement proteins by Fc receptor family or complement receptors. Alternativly direct recognition of conserved pathogens associated molecular patterns (PAMPs) occurs through PRRs. Despite their name, PAMPs are common to all microorganisms regardless of their pathogenicity. PRRs are germline-encoded, nonclonal, expressed on all cells of a specific type and independent of immunological memory in contrast to lymphocyte receptors of the adaptive immune system (Akira et al, 2006). First it was thought that PRRs only recognize microbial nonself structures like PAMPs (Janeway & Medzhitov, 2002) but it has become clear that PPRs are also able to recognize endogenous ligands as well (Rakoff-Nahoum & Medzhitov, 2009). The molecular range of ligands that can be recognized by PRRs is very wide, including proteins, lipids, carbohydrates and nucleic acids of both exogenous and endogenous origin. A single PRR is often able to sense multiple ligands and some ligands can bind to several distinct PRRs. According to their functional properties, PRRs can be subdivided into endocytic PRRs and signalling PRRs. Endocytic PRRs, such as scavenger receptors, mannose receptors, and β-glucan receptors promote above introduced phagocytosis of microorganisms. Signaling PRRs such as RIG-like receptors (retinoid induced gene-I-like receptors, RLR), NOD-LRR proteins (nucleotide binding oligomerization domain-like receptors, NLR) and Toll-like receptors (TLRs), act as sensors by triggering intracellular signaling and orchestrating inflammatory responses (Creagh & O'Neill, 2006).

1.3.1 Toll-like receptors (TLRs)

TLRs constitute a family of proteins structurally related to *Drosophila* Toll which was originally identified as a transmembrane receptor required for the establishment of dorso-ventral polarity in the developing embryo (Hashimoto et al, 1988). Later it was shown that *Drosophila* Toll receptor has a crucial function against fungal infection (Lemaitre et al, 1996). Shortly after the discovery of the role of the *Drosophila* Toll in the host defense, a mammalian homologue of the *Drosophila* Toll was identified (Medzhitov et al, 1997). TLRs are evolutionarily conserved from *Caenorhabditis elegans* to mammals. To date, 12 TLR family members have been identified in mammals (Kumar et al, 2009). The members of the mammalian TLR family are able to sense different microbial PAMPs whereas the nine members of the *Drosophila* Toll family are mostly not involved in anti-microbial host defense (Akira et al, 2006). Based on their primary sequences, TLRs can be further divided into several subfamilies, each of which recognizes related PAMPs. The subfamily of TLR1, TLR2, TLR4 and TLR6 recognizes lipids, whereas the highly related TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (Figure 1.2). TLRs are expressed on various immune cells DCs, B cells, specific types of T cells, including macrophages and even on fibroblasts and epithelial cells (Akira et al, 2006). While TLRs (TLRs 1, 2, 4, 5, and 6) which sense lipid and protein ligands are expressed on the plasma membrane, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes, and their ligands, mainly nucleic acids, require internalization to the endosome before signaling is possible (Rakoff-Nahoum & Medzhitov, 2009).

Despite their ability to recognize a multitude of PAMPs TLRs cannot sense PAMPs in the cytosol. These PAMPs are detected by cytoplasmic NOD-LRR proteins which then trigger signaling pathways. Proteins in this family also possess LRRs that mediate ligand sensing. In addition members of the NOD-LRR protein family have a nucleotide binding oligomerization domain (NOD); and a domain for the initiation of signaling, such as CARD, PYRIN, or baculovirus inhibitor of apoptosis repeat (BIR) domain. (Inohara et al, 2005).

In addition to their role in mammalian host defence from microbial infection, TLRs are involved in various aspects of tissue homeostasis, including tissue repair and regeneration (Rakoff-Nahoum & Medzhitov, 2009).

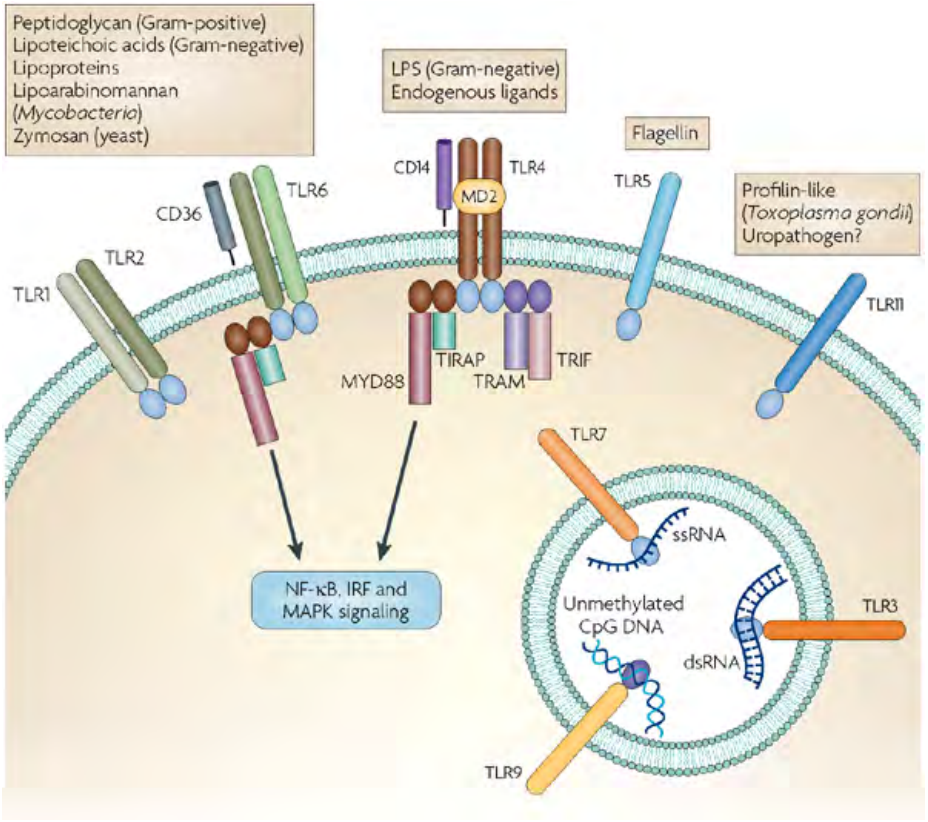

Figure 1.2 Toll-like receptors recognize a range of PAMPs

Bacteria are sensed by five TLRs in humans: lipopolysaccharide (LPS) is the main bacterial ligand for TLR4; lipotechoic acid and diacylated lipopeptides are sensed by TLR2–TLR6 dimers; triacylated lipopeptides are sensed by TLR2–TLR1 dimers and flagellin is sensed by TLR5. For anti-fungal responses a TLR2–TLR6 dimer senses zymosan. Double-stranded RNA (polyI:C) is sensed by TLR3 and TLR7 (human only) and TLR8 (human and mouse) sense single-stranded viral RNA (ssRNA). Viral CpG motifs are sensed by TLR9. Protozoal products such as glycosylphosphatidylinositol (GPI)-anchor proteins are also sensed by TLR2. Reproduced from (Rakoff-Nahoum & Medzhitov, 2009) with permission from Nature Publishing Group.

1.3.2 TLR structure

TLR family members are type I transmembrane glycoproteins structurally characterized by the presence of an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a intracellular Toll/IL-1 1R homologue (TIR) domain (Figure 1.3) (Bowie & O'Neill, 2000).TLRs contain 16–28 LRRs which form multiple LRR modules. The individual LRR modules are 20–30 amino acids long and are responsible for binding PAMPs (Medzhitov, 2001). The intracellular TIR domains consist of about 150 amino acid residues and have a common fold containing a five-stranded β-sheet surrounded by five α-helices which play an important role in TLR signal transduction through TIR dimerization and/or adaptor recruitment (Jin & Lee, 2008).

The key signaling TIR domain, which is unique to the TLR system, interacts with TIR-domain containing adaptors. Similar to the TLRs, the TIR-containing adaptors are conserved across many species. These five adaptors are MyD88 (myeloid differentiation primary-response protein 88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFNγ (TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM; also known as TICAM2) and sterile α- and armadillo-motif-containing protein (SARM) (O'Neill & Bowie, 2007). TLR signal transduction requires recruitment of further downstream signaling molecules IL-1R-associated kinases (IRAKs), transforming growth factor-β (TGF- β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6). Activation of TLRs leads to activation of NF-κB, mitogen-activated protein kinases (MAPKs), JuN N-terminal kinases (JNKs), p38 and ERKs, and interferon regulatory factor (IRF3, IRF5 and IRF7) signaling pathways. These signals are essential for the orchestration of host innate and adaptive immune responses (Rakoff-Nahoum & Medzhitov, 2009).

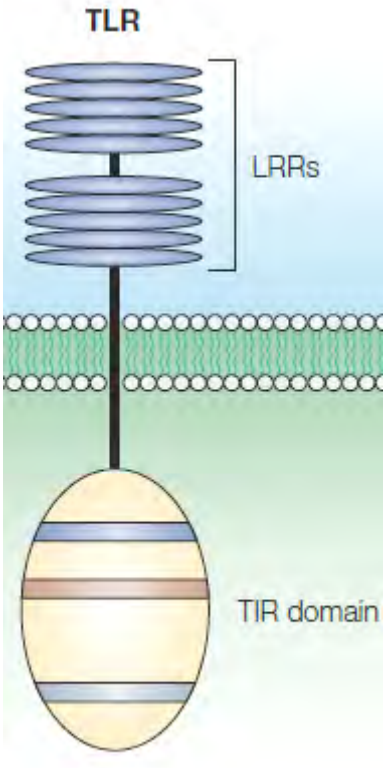

Figure 1.1 TLR structure

TLRs have a conserved cytoplasmic domain that is known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions (known as boxes 1, 2). In adddition TLRs have tandem repeats of leucine-rich regions. Reproduced from (Akira & Takeda, 2004) with permission from Nature Publishing Group.

1.3.3 TLR4 signaling

TLR4, the first mammalian TLR identified, induces several inflammatory cytokines and costimulatory molecules (Medzhitov et al, 1997). It was shown that TLR4 is an important sensor for lipopolysaccharide (LPS) (Poltorak et al, 1998). LPS is one of the best studied immunostimulatory components and it is the major phosphoglycolipid component of the bacterial Gram-negative outer membrane and consists of three parts: a core oligosaccharide, an O side chain and lipid A. Lipid A or endotoxin, is the only region of LPS recognized by the innate immune system (Miller et al, 2005). It is reported that several other PAMPs can activate TLR4 such as fusion (F) protein from respiratory syncytial virus (RSV) and the envelope protein from mouse mammary tumor virus (MMTV). In addition, endogenous molecules can also interact directly or indirectly with TLR4, such as heat-shock proteins, hyaluronic acid and β-defensin 2 (Lu et al, 2008).

LPS mediated activation requires different interactions of several proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4. LBP is a soluble shuttle protein which directly converts oligomeric micelles of LPS to a monomer for delivery to CD14. The glycosylphosphatidylinositol-anchored protein CD14 concentrates LPS for binding to the TLR4–MD2 complex (Lu et al, 2008).

Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR domains (O'Neill & Bowie, 2007).

TLR4 signaling feeds into two pathways the MyD88-dependent and MyD88-independent (TRIF-dependent) pathway. Several studies have shown that the MyD88-dependent pathway is responsible for the induction of pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the induction of Type I interferons and interferon-inducible genes important for antiviral and antibacterial responses (Akira & Takeda, 2004).

1.3.3.1 The MyD88 dependent pathway

Upon ligand binding, dimerization the TLR4 is able to recruit the adaptor molecule MyD88, which consists of a C-terminal TIR and an N-terminal death domain (DD). The latter interacts with the DD of a serin/threonin protein kinase, IRAK4. Binding to MyD88 enables IRAK4 to phosphorylate the subsequently recruited IRAK1, thereby inducing the activation of its kinase activity. Autophosphorylated IRAK1 subsequently dissociates from the receptor complex and associates with TRAF6. Then the IRAK1-TRAF6 complex interacts at the plasma membrane

with another preformed complex consisting of TAK1, TAB1, and TAB2 or TAB3. This interaction induces phosphorylation of TAB2/TAB3 and TAK1, leading to their translocation to the cytoplasm together with TRAF6 and TAB1, whereas IRAK1 is degraded at the plasma membrane. In the cytoplasm, TAK1 is then activated, resulting in the activation of IKKs (IkB kinases), which then phosphorylate the IκBs (inhibitor of NF-κB). This phosphorylation allows ubiquitylation and subsequent degradation of IκB, thereby releasing NF-κB (nuclear factor-κB). NF-κB is consequently free to translocate into the nucleus and induce the expression of its target genes (Doyle & O'Neill, 2006).

Activation of TAK1 also results in the activation of the MAPK (mitogen-associated protein kinases) ERK (extracellular-regulated kinase) and p38 as well as the SAP (stress-associated protein)-kinase JNK/SAPK (JNK for JUN N-terminal kinase), finally leading to the phosphorylation and activation of Jun and Fos, which together form the AP-1 (activator protein 1) transcription factor. AP-1 has also a role in the expression of pro-inflammatory cytokines (Lu et al, 2008).

1.3.3.2 The MyD88 independent (TRIF dependent) pathway

MyD88-independent response is entirely dependent on TRIF. TRIF is an important TIR-containing adaptor protein that activates IRF3 through the recruitment of TRAF3. TRAF3 themselves can associate with TANK (TRAF family member-associated NF-κB activator), TBK1 (TANK binding kinase 1) and IKKi to mediate downstream signalling. TBK1 and IKKi are important for the dimerization and translocation of IRF3. IRF3, together with NF-κB, activates the transcription of target genes, such as Type I interferons (Lu et al, 2008).

TRIF can also activate NF-κB through an alternative pathway. The C-terminal end of TRIF possesses a RIP homotypic interaction motif (RHIM), and it associates with receptor interacting protein 1 (RIP1) through homophilic interaction of RHIM domains and mediates NF-κB activation (Cusson-Hermance et al, 2005).

Furthermore, phosphoinositide 3-kinase (PI3K) exerts multiple effects on both MyD88-dependent and -independent TLR pathways (Hazeki et al, 2007).

The complexity of adaptor use and differences in the following signaling transduction pathway enables a cell to regulate TLR action and to react in a coordinated way on pathogenic threat. One of the main functions of TLR4 activation is to trigger cytokine secretion.

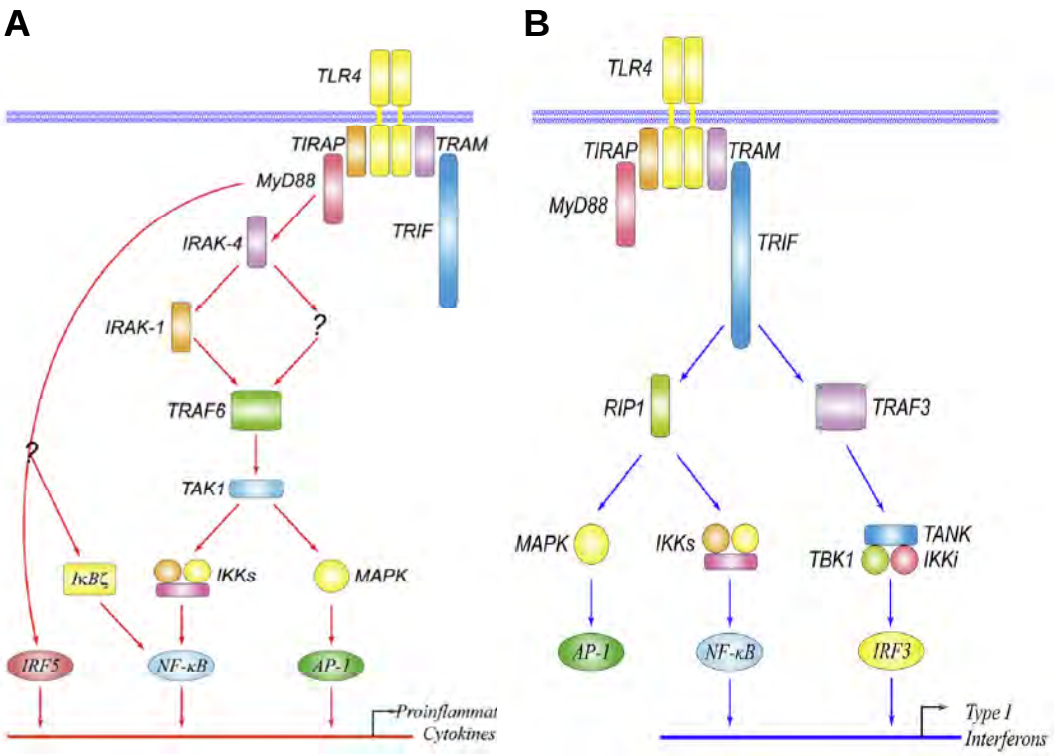

Figure 1.3 (A) The MyD88-dependent pathway and (B) the MyD88-independent pathway

(**A**) MyD88 activates IRAKs/TRAF6 as well as the transcription factors NF-κB, AP-1 and IRF5 further downstream. These transcription factors induce of pro-inflammatory cytokine expression.

(**B**) TRIF signals the induction of Type I interferons by recruiting TRAF3 and RIP1 to activate transcription factor IRF3, as well as NF-κB and AP-1. Reproduced from (Lu et al, 2008) with permission from Elsevier.

1.4 Cytokines

The characteristic response triggered by pathogen recognition and/or tissue damage is called inflammation, within which various cytokines communicate with the environment to regulate this process (Parkin & Cohen, 2001). Cytokines are major regulators of macrophage activation that regulate the qualitative nature of macrophage activation to achieve effective clearance of pathogens, while limiting the amount of inflammation to avoid toxicity and tissue damage (Hu et al, 2008).

Cytokine is a general name of a diverse group of small soluble proteins and glycoproteins that act as humoral regulators at nano- to picomolar concentrations. Cytokines are secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced *de novo* in response to an immune stimulus. They generally act over short distances on the cells that secrete them (autocrine action), on nearby cells (paracrine action) but in some instances on distant cells (endocrine action). Cytokines act by binding to specific membrane receptors, which

then signal the cell via second messengers, often tyrosine kinases, to alter its gene expression. Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. Cytokines include interferons chemokines and interleukins.

1.4.1 Interferons, chemokines and interleukins

Interferons (IFNs) were the first discovered cytokines which act as agents interfering with viral replication (Isaacs & Lindenmann, 1957). IFNs consist of a heterogeneous group of proteins which are classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs are comprised of multiple IFNα subtypes (14–20, depending on species), IFNβ, IFNω, and IFNτ, all of them are structurally related and bind to a common heterodimeric receptor (IFNAR, comprised of IFNAR1 and IFNAR2 chains). IFNγ is the only type II interferon. It is structurally unrelated to type I IFNs and binds to a different receptor (Schroder et al, 2004). The IFNs possess a broad spectrum of activity and are involved in complex interactions. They display antiviral activity, impact cellular metabolism and differentiation, and possess antitumor activity (Jonasch & Haluska, 2001). Nevertheless, IFNs are mainly known for their antiviral activities against a wide spectrum of viruses. IFNs are synthesized by virus-infected cells to protect other non-infected but virus-sensitive cells against infection. In addition IFN are also known to have protective effects against some non-viral pathogens (Schroder et al, 2004).

Chemokines are a group of small (8–14 kDa), mostly basic, structurally related molecules that regulate cell trafficking and homing of various types of leukocytes at sites of inflammation through interactions with a subset of seven-transmembrane, G protein–coupled receptors. About 40-50 chemokines have now been identified in humans which additionally play fundamental roles in the development and homeostasis (Mantovani, 1999; Zlotnik & Yoshie, 2000). According to the structural organization of their NH_2-terminal Cysteine (C) residues, chemokines are structurally divided into the CC, CXC, CX_3C, and C chemokines (Schutyser et al, 2005).

Interleukins were first described as cytokines that act specifically as mediators between leucocytes. Currently, it is well-known that in addition to leucocytes many other cells of the body produce and use these molecules as means of signaling. Interleukins are the primary messengers and directors of the immune system which can cause cellular proliferation, cell activation, inflammation, physiological changes such as fever and pain, allergies and growth.

There are currently 35 described interleukins. Usually interleukins are synthesized after cell activation as a consequence of a physiological or non-physiological stimulus.

1.4.2 Interleukin-10 (IL-10)

IL-10 was first identified as factor produced by mouse Th2 cells which inhibited activation and production of cytokines by Th1 cells and was therefore called cytokine synthesis inhibitory factor (CSIF) (Fiorentino et al, 1989). It is now known that IL-10 can be expressed by a variety of cells, usually in response to an activation stimulus, including macrophages, DC, B cells, and various subsets of T cells (Couper et al, 2008). It is also reported that IL-10 is produced by non-immune cells such as keratinocytes, epithelial cells and tumor cells (Mosser & Zhang, 2008). IL-10 is a Type II cytokine and the first cytokine described of a family of cytokines conprising IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29 (Commins et al, 2008).

IL-10 inhibits a broad spectrum of activated macrophage functions, including the major inflammatory cytokines, IL-1α and β, IL-6, IL-12, IL-18 and TNFα. The NO production as well as the MHC class II expression were also inhibited by IL-10. Additionally inflammatory chemokines of both the CC and CXC type and the production of macrophage matrix metalloproteases are also suppressed by IL-10. Further IL-10 can inhibit inflammation by increasing the production of the release of IL-1 receptor antagonists by macrophages (Akdis & Blaser, 2001). This suppression of immune responses by IL-10 is reinforced by the phenotype of IL-10-deficient mice, which develop colitis in the presence of normal gut flora (Kuhn et al, 1993). IL-10-deficient mice are able to clear certain intracellular pathogens more efficiently; this is however often accompanied by immunopathology, which can be lethal to the host (O'Garra et al, 2008). Through its suppressive effect on macrophges IL-10 also limits Th1 and Th2 effector responses. Thus IL-10 has emerged as a key immunoregulator which confines excessive immune activation after infection which is accompanied by impaired tissue damage and increased host mortality (Couper et al, 2008).

As a key immunoregulator IL-10 and in particular its regulation is a potent target for various pathogens. Several viruses and bacteria like human immunodeficiency virus (HIV), cytomegaloviruses (CMV), rhinovirus or *Mycobacterium* spp can induce production of IL-10 during the course of infection to dampen the host immune response and to suppress their elimination from the host. Certain viruses including human Epstein-Barr virus, equine herpesvirus-2, poxvirus Orf and cytomegaloviruses encode their own viral homologs of IL-10 to

overcome the host immune response (Imlach et al, 2002; Kotenko et al, 2000; Spencer et al, 2002).

IL-10 signals through a two-receptor heterotetramer complex consisting of each two copies of IL-10 receptor 1 (IL-10R1) and IL-10R2 .The binding of IL-10 to the receptor complex activates the Janus tyrosine kinases, JAK1 and Tyk2, associated with IL-10R1 and IL-10R2, respectively, to phosphorylate the cytoplasmic tails of the receptors and this results in the recruitment of the signal transducer and activator of transcription 3 (STAT3) to the IL-10R1. The site of STAT3 recruitment is conserved between the human and murine receptor. The homodimerization of STAT3 results in its release from the receptor and translocation of the STAT homodimer into the nucleus, where it binds to STAT-binding elements at the promoters of various genes (Donnelly et al, 1999; O'Shea & Murray, 2008).

The human and mouse *il-10* gene are similarly organized. The human *il-10* gene spans about 4.7 kb on chromosome 1q21–32 and contains five exons that are separated by four introns. The murine *il-10* gene is located on chromosome 1E4 and also contains five exons. The open reading frames (ORF) encode secreted proteins of ~178 amino acids with rather well conserved sequences. The mouse and human Il-10 show a ~73% amino acid sequence homology and have an α-helical bundle structure similar to interferons and other cytokines (Moore et al, 2001).

The *il-10* gene forms with the adjacent genes *il-24*, *il-20* and *il-19* an approximately 200 kb gene cluster. This *il-10* gene cluster is found in both human and mouse and is flanked by two noncytokine genes: PIGR upstream of IL-24; and MAPKAPK2 downstream of IL-10 (Mosser & Zhang, 2008).

1.5 *Il-10* gene regulation

The timing as well as the relative amounts of pro-inflammatory and anti-inflammatory cytokine production are critical for safe resolution of infection. Therefore *il-10* gene expression must be tightly controlled and regulated.

1.5.1 IL-10 transcriptional regulation

Many transcription factors were found to positively regulate IL-10 transcription. The promoters for IL-10 in all the cells producing IL-10 are essentially the same and therefore the transcription factors that initiate transcription are conserved. But the activated signaling pathways that induce IL-10 are stimuli and cell type specific. The murine and human promoter are organised in a

similar fashion. The promoter in the murine *il-10* gene contains a TATA box between 98 and 95 bp upstream of the first methionine codon, while in the human *il-10* gene locus, its location is between 91 and 88 bp. A CCAAT box is located between −233 and −237 bp in human *il-10* gene and between −244 and −240 bp in the murine *il-10* gene.

As mentioned many transcription factors have an influence on IL-10 expression (Mosser & Zhang, 2008). For example Sp1 plays a prominent role in LPS mediated induction of the IL-10 promoter (Brightbill et al, 2000). Furthermore a proximal putative site has been identified as a c-Maf-binding element for LPS-induced IL-10 production in macrophages (Cao et al, 2005b). c-Maf is the cellular homolog of the avian viral oncogene v-mal. In contrast NF-κB proteins had only minor effects on LPS-mediated IL-10 production (Bondeson et al, 1999).

1.5.2 Regulation of the *il-10* locus on the chromatin level

The influences of epigenetic mechanisms, including covalent and noncovalent modifications of DNA and histones on *il-10* gene expression have been studied in a variety of different cells and appear to play significant roles in *il-10* gene regulation.

In macrophages, for the control of *il-10* gene expression histone, phosphorylation rather appears to be more important than histone acetylation. Transient phosphorylation of serine 10 of histone H3 (H3S10) correlates temporally with the initiation of *il-10* gene transcription in macrophages and seems to be sufficient because the IL-10 mRNA levels are not dramatically affected by treatment with deacetylases inhibitors (Lucas et al, 2005; Zhang et al, 2006).

Various DNase I hypersensitive sites (HSS) were found in the neighbourhood of the *il-10* gene in different cell types. Nucleosome-poor regions are generally HSS, which are often located at the regions close to protein-bound regulatory elements. These nucleosome-poor regions can be enhancers, locus control regions, matrix attachment regions, or insulator/boundary elements. In macrophages, HSS +1.65, HSS −0.12, HSS −2.0 and as well as HSS −4.5 are found. HSS −4.5 is specifically present in LPS-stimulated macrophages and contains an NF-κB-binding motif (Saraiva et al, 2005). All of this HSS are characterized by constitutive hyperacetylation at histones H3 and H4.

GATA3 has been described as a chromatin remodeling molecule and not as a transcriptional activator. In primary $CD4^+$ T cells GATA3 can bind to DNase I HSS residing on both the 5'-proximal region as well as intron 4, and then induce remodeling of chromatin structure by

increasing histone acetylation and thereby stabilizing the *il-10* locus into a transcriptionally competent status (Shoemaker et al, 2006).

DNA methylation status at CpG sites is also associated with regulation of IL-10 cytokine expression. Different types of T cells produce different amounts of IL-10 when the IL-2-STAT5 signaling axis is activated to the same extent. A cluster of CpGs around intron 4 of the *il-10* gene is suggested to suppress the *il-10* gene expression through CpG methylation (Tsuji-Takayama et al, 2008).

1.6 Polycomb group proteins (PcG)

Epigenetic regulators change the activities and abilities of a cell without affecting and mutating the sequence of DNA through covalent and noncovalent modifications of DNA and histone proteins that alter the overall chromatin structure. PcG proteins are well known chromatin modifier discovered and best studied in the fruit fly *Drosophila melanogaster*. The first Polycomb group (PcG) mutations, *extra sex combs* (*esc*) and *Polycomb* (*Pc*), were identified in the 1940s. These mutations cause additional sex combs on the second and third legs of males, instead of only one the first leg, where they normally belong. This phenotype is caused by loss of correct *Hox* gene repression, leading to the transformation of segments along the anteriorposterior axis (Ringrose & Paro, 2004). *Hox* genes are transcription factors with a homeo-box domain which are encoded in gene clusters and play a crucial role in the proper development and subsequent maintenance of the body plan, in both vertebrates and invertebrates (Krumlauf, 1994). Together with antagonistic trithorax group (trxG) proteins which activate *Hox* gene expression PcG proteins form a binary epigenetic switch providing a cellular memory mechanism. PcG proteins are evolutionary conserved. Beside flies and mammals, PcG-mediated gene regulation plays a crucial role in the development even of flowering plants and *Caenorhabditis elegans* and underlined the importance of this ancient family of chromatin modifiers (Lund & van Lohuizen, 2004). Recent studies revealed that PcG proteins are involved in the control of hundreds of other genes in mammals and insects (Schwartz & Pirrotta, 2007).

1.6.1 PcG complexes

PcG proteins assemble as biochemically and functionally distinct high molecular weight complexes. They were initially purified from *Drosophila*, but homologous complexes were later isolated in other organisms too. *Drosophila* has three different PcG complexes – Polycomb repressive complex 1 (PRC1), Polycomb repressive complex 2 (PRC2) and Pleiohomeotic

repressive complex (PhoRC). In mammals at least two different PRC were identified the PRC1 and the PRC2 (Table 1.2).

Drosophila melanogaster	**Mouse**	**Human**	**Protein domains**	**Functions**
PhorRC				
dSfmbt	L3MBTL2/mbTD1	SFMBT1	MBT repeats, SAM	Binding methylated lysine residues
Pho	YY1/YY2	YY1/YY2	Zink-finger domain	Sequence-specific DNA binding
PRC2				
Esc	Eed	EED	WD40 repeats[1]	
E(z)	Ezh1/Enx2	EZH1	SET domain[4]	Histone methyltransferase
	Ezh2	EZH2		
Su(z)12	Suz12	SUZ12	Zinc-finger domain	
PCL	PHF19/MTF2	PHF1	PHD, Tudor	
PRC1				
Pc	Cbx2/Hpc1	CBX2/M33	Chromodomain	Methyl-lysine binding
	Cbx4/Hpc2	CBX4/MPC2		
	Cbx8/Hpc3	CBX8/PC3		
Ph	Edr1/Hph1	EDR1/MPH1/RAE28	Zinc-finger SPM domain[3]	
	Edr2/Hph2	EDR2/MPH2		
	Edr3/Hph3	EDR3		
dRing	Ring1/Rnf1/Ring1A	RING1/RING1A	RING-finger[1] domain	Ubiquitin ligase
	Rnf2/Ring1B	RNF2/RING1B		
Psc	Bmi1	BMI1	RING-finger[1] domain	
	Rnf110/Zfp144/Pcgf2	RNF110/ZFP144/MEL18		
	Znf134	ZNF134		
Scm	Scml1	SCMH1	Zinc-finger, SPM domain[3]	
TBP-assciated factors				

Table 1.2 PcG complexes and PcG nomenclature

1WD repeats are a conserved domain that usually ends with tryptophan and aspartic acid (WD). 2A RING finger is a conserved cysteine-rich domain named after the gene RING in which it was first identified. 3SPM refers to the

presence of this domain in SCM, PHO and MBT proteins. 4 The SET domain is named after the first letter of the three proteins in which the domain was found: SU(VAR)3-9, E(Z) and TRX.
ESC, extra sex combs; EED, embryonic ectoderm development; E(Z), Enhancer of zeste; PC, Polycomb; PCL, Polycomb-like; PH, polyhomeotic; PHD, plant homeodomain; Pho, pleiohomeotic; PSC, Posterior sex combs; SU(Z), Suppressor of zeste; SCM, Sex combs on midleg; MTF, metal response element-binding transcription factor; CBX, chromobox; homologue PHF19, PHD-finger protein 19.

Biochemical purified PRC2 from *Drosophila melanogaster* contains four core components: E(z) (Enhancer of zeste), Esc (Extra sex combs), Su(z)12 (Suppressor of zeste 12) and Nurf-55 (in humans, EZH2, EED, SUZ12 and RbAp46/48). The SET domain-containing E(z) and the mammalian homologue EZH2 subunit trimethylates lysine 27 of histone H3 (H3K27me3) and histone H1 lysine 26 (H1K26me3). In mammals, PRC2, PRC3 and PRC4 complexes have been biochemically characterized. They only differ by the presence of different isoforms of EED, the homologue of the fly ESC. In humans the PRC2 core components are EZH2, EED, SUZ12 and RbAp46/48, respectively (Schuettengruber et al, 2007) (Table 1.2).

Drosophila PRC1 contains Pc, Polyhomeotic (Ph), Posterior sex combs (Psc) and dRing, in addition to several other components, including TBP-associated factors. The mammalian PRC1 complex has been isolated from HeLa cells using exogenously expressed tagged protein components and affinity purification. The purified complex(es) include HPC1, 2 and 3, HPH1, 2 and 3, RING1A and RING1B, BMI1 and, potentially, its homologue MEL18 (Schwartz & Pirrotta, 2007). RING in flies and RING1A and B in mammals which all contain RING domains have been shown to function as E3 ubiquitin ligases that mono-ubiquitylate lysine 119 of histone H2A (H2AK119) (Table 1.2).

PhoRC only found in *Drosophila* includes the sequence specific DNA binding protein Pleiohomeotic (Pho) as well as the dSfmbt protein [*Scm*-related gene containing four malignant brain tumor (MBT) domains], which binds specifically to mono- and dimethylated H3K9 and H4K20 via its MBT repeats. PhoRC is also involved in homeotic gene silencing (Schuettengruber et al, 2007) (Table 1.2).

1.6.2 PcG mode of action

Several specific DNA regions of PcG target genes have been identified which are necessary and sufficient for PcG-mediated repression in *D. melanogaster*. All three PcG complexes bind to such sequences, called Polycomb response elements (PREs). Nevertheless neither PRC2 nor PRC1 core complexes contain sequence specific DNA binding proteins and thus the recruiting

mechanism is still unknown. Much of the information about their structure and function comes from work on a few specific PREs: the engrailed PRE, the Fab7 PRE and the bxd PRE. PREs are complex structures, which include many conserved short motifs which are recognized by known DNA-binding proteins and comprise several kilobases. *Drosophila melanogaster* PREs often contain clusters of GAGAG motifs, which bind GAGA factor (GAF) and Pipsqueak (PSQ), both BTP/POZ proteins that are reported to associate with PcG complexes. Over decades PREs have been functionally validated only in the fly (Schwartz & Pirrotta, 2007) but recently functional PREs have also been identified in mammels (Sing et al, 2009).

In mammals, members of the PRC1 display discrete nuclear foci called PcG bodies preferentially located to pericentric chromosome territories (Saurin et al, 1998). This unique localization of PcG bodies suggests that the complex might be specifically targeted to these repetitive DNA sequences. The functional significance of these nuclear domains remains unknown, but pericentric heterochromatin integrity in general is essential for proper chromosome segregation, genomic stability, and transcriptional silencing (Peters et al, 2001; Taddei et al, 2001).

The histone methyltransferase EZH2 methylates lysine 27 of the histone H3 tail (H3K27me3). This histone modification can be recognized through the chromodomain of protein from the PRC1 which then propagate the silenced state. The chromodomain is a highly conserved motif found in the PRC1 proteins Pc and its mammalian homologue CBX (Sparmann & van Lohuizen, 2006). Consequently H3K27me3 has a crucial role in the stable binding of PcG complexes.

Mouse and human PRC2 components bind throughout the H3K27me3 regions (Bracken et al, 2006), whereas *Drosophila* PRC2 members bind to restricted regions, presumably PREs, even though H3K27me3 covers large domains (Schwartz et al, 2006).

The precise molecular mechanism of transcriptional repression by PcG is still poorly understood It is thought that PRC-mediated repression is mediated directly by blocking the transcription initiation machinery or indirectly by preventing ATP-dependent nucleosome remodeling by the SWI/SNF complex and thereby preventing the transcription initiation machinery (Sparmann & van Lohuizen, 2006). As mentioned above PRC1 possesses an ubiqutin E3 ligase, which targets H2AK119 and this modification is associated with gene repression (Cao et al, 2005a; Wang et al, 2004). It has also been shown that PRC2 also exhibits methyltransferase activity towards lysine 26 of the linker histone H1. Methylated H1K26 can recruit heterochromatin binding protein 1 (HP1) to chromatin, and could thereby influence higher order chromatin structure (Sparmann & van Lohuizen, 2006). In addition, a report revealed a role for the RNA interference (RNAi)

machinery in the nuclear clustering of PcG target sites in *Drosophila*, suggesting that RNAi factors might increase PcG-mediated silencing through an effect on nuclear organization (Grimaud et al, 2006). Furthermore it has been proposed that EZH2 was able to recruit DNA methyltransferases (DNMTs) to select target genes. DNA hypermethylation of gene promoter regions induces transcriptional repression (Vire et al, 2006).

1.6.3 Bmi1

Bmi1 (B cell-specific Moloney murine leukaemia virus integration site 1) is one of the best studied PRC1 members and was originally identified as a collaborating proto-oncogene in c-Myc induced murine lymphomagenesis (Haupt et al, 1991; van Lohuizen et al, 1991b). The murine Bmi1 protein shares domains of high homology with the Drosophila PcG proteins Psc (Posterior sex combs) and Su(z)2 (Suppressor-2 of Zeste) (Brunk et al, 1991; van Lohuizen et al, 1991a). Deletion of the Bmi1 gene in mice leads to transformations of the anteriorposterior axis of the skeleton, severe haematopoietic defects and progressive loss of cerebellar neurons resulting in neurological abnormalities (van der Lugt et al, 1994).

Identification of Bmi1 as a negative regulator of the *ink4a/arf* locus encoding the cell cycle regulators and tumor suppressors $p16^{Ink4a}$ and $p19^{Arf}$ ($p14^{ARF}$ in humans) (Jacobs et al, 1999), points to a key role for Bmi1 as a determining factor in cell cycle control. $p16^{Ink4a}$ and $p19^{Arf}$ are two structurally distinct proteins. Whereas $p16^{Ink4a}$ is a cyclin–dependent kinase inhibitor (CDKI) that activates the retinoblastoma (Rb) pathway, $p19^{Arf}$ is a critical regulator of the p53 pathway by inhibiting the MDM2 function. Both pathways are known tumor suppressor pathways and because both pathways are frequently deregulated in human cancer, altered expression of Bmi1 might be involved in tumor formation as well. In humans, overexpression of BMI1 was found in high grade B-cell non-Hodgkin lymphomas (NHLs) (Bea et al, 2001), breast carcinoma (Dimri et al, 2002) and non-small cell lung cancer (NSCLC) (Vonlanthen et al, 2001).

Bmi1 is implicated in self-renewal of multiple stem/progenitor cells for example haematopoietic stem cells and early cerebellar progenitors (Lessard & Sauvageau, 2003; Molofsky et al, 2003; Park et al, 2003) and it is highly expressed in differentiating primitive haematopoietic cells (Ohta et al, 2002).

It was shown that Bmi1 activates telomerase in human mammary epithelial cells but not fibroblasts (Dimri et al, 2002). A new key role was attributed to Bmi1 and its PcG partner RING1A. Bmi1 represents an essential cofactor of the RING1/2 monoubiquitin E3 ligase implicated in the ubiquitination of histone H2AK119. Ubiquitin E3 ligase activity is associated

with the repression of *HOX* genes (Buchwald et al, 2006; Cao et al, 2005a) and monoubiquitinated histones H2A are enriched on the inactive female X-chromosome and are thought to be involved in its stable silencing. A recent report has shown that Bmi1 has an unexpected role in maintaining mitochondrial function and redox homeostasis (Liu et al, 2009).

Bmi1 has been shown to be a downstream target of a major developmental signaling pathway, the Sonic hedgehog pathway in mouse cerebellum (Leung et al, 2004). There is additional evidence supporting implicating MAPK signaling in the function of Polycomb group proteins, for example MAPK signal transduction cascades target PcG protein complex/chromatin interaction through phosphorylation. MAPK-activated protein kinase 3 (3pK) acts as a Bmi1 kinase *in vitro* and *in vivo* and causes PcG protein dissociation from chromatin. Phosphorylation of Bmi1 resulted in de-repression of the *ink4/arf* locus. Bmi1 phosphorylation is driven through 3pk via serum/TPA or arsenite stimulation in human cancer cells. This suggests that Bmi1 is a target of both, stress stimuli as well as mitogenic signals through MAPK pathways (Voncken et al, 2005).

1.7 Aim of the work

Macrophages play a crucial role in the innate as well as in the adaptive immune system. Activated macrophages exert a great number of inflammatory mediators which are important for a functional host defense. These mediators need to be mobilized rapidly after infectious challenge to achieve effectiveness and must be well regulated to prevent toxicity and tissue damage. During macrophage activation a multitude of signaling pathways, among them MAPK pathway, are activated which control the cellular response.

This work aims at beginning to understand the role of the PRC1 member Bmi1 as a putative downstream effector of TLR4 signaling during the process of macrophage activation.

2 Materials and Methods

2.1 Materials

2.1.1 Instruments

Hardware	Manufacturer
Bacterial incubator	Heraeus Instruments
Bacterial shaker	New Brunswick Scientific
Cell culture hood	Heraeus Instruments
Cell culture incubator	Heraeus Instruments
Cell culture microscope	Leica
Combi spin	PEQLAB Biotechnologie
Developing machine	AGFA
Digital pH meter	WTW
Electrophoresis power supply	Bio-Rad, Pharmacia Biotech
Electrophoresis unit	Bio-Rad
FACSCalibur	Becton Dickinson
Fine scale	Scaltec
Heat block	Liebisch, Eppendorf
Horizontal electrophoresis gel system	Scientific workshop of the MSZ
Inverted DMIRBE microscope	Leica
Mega centrifuge	Sorval, Heraeus Instruments
Mini centrifuge	Eppendorf
Pipettes	Eppendorf
Protein blotting cells	Bio-Rad
Radiographic cassette	Dr. GOOS SUPREMA
Real-time thermocycler Rotorgene2000	Corbet
Scale	Sartorius Mechatronics,
Shakers	PEQLAB, Edmund Bühler, Heidolph
Sonicator sonoplus HD 70	BANDELIN electronic
Spectrophotometer	Pharmacia Biotech, Eppendorf
Thermocycler Primus 96 advanced	PEQLAB Biotechnologie
Vortex	Scientific Industries
Water bath	Memmert

2.1.2 General chemicals

Reagents	Manufacturer
5-bromo-4-chloro-3-indolyl	Roth
Acetone	Roth
Acrylamide (30%)/Bisacrylamide (0.8%)	AppliChem
Agarose, ultra pure	Roth, PEQLAB Biotechnologie
Ammonium Acetate	Roth
Ammonium peroxydisulfate (APS)	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Aprotinin	Sigma-Aldrich
Bacto-Agar	Roth
Bovine serum albumin (BSA)	AppliChem
Bromphenolblue	Merck
Calciumchloride (CaCl2)	Roth
Chloralhydrate	Roth
Chloroform	AppliChem
Citric acid	Roth
DAPI (4',6-Diamidino-2-phenylindol)	Roth
Deoxycholate (DOC)	Roth
dNTPs	Promega, PEQLAB Biotechnologie
EGTA	Sigma-Aldrich
EGTA	Roth
Ethanol	AppliChem, Roth
Ethanol	AppliChem
Ethidiumbromide	Roth
Ethidiumbromide	Roth
Ethylenediaminetetraacetic acid-disodium salt (EDTA)	Roth
Ethylenediaminetetraacetic acid-disodium salt (EDTA)	Roth
Formaldehyde	Merck
Formaldehyde	AppliChem
Formamide	Roth
Gel blotting paper	Whatman
Gelatine	AppliChem
Glutathion-sepharose	Roth
Glycerol	Roth
Glycine	Sigma-Aldrich
Hydrochloride (HCl)	Merck
IGEPAL (NP-40)	Roth
Isopropanol	Roth, AppliChem
Leupeptin	Sigma-Aldrich

Loading dye	Fermentas
Magnesiumchloride (MgCl)	AppliChem
Methanol	AppliChem
MOWIOL	Calbiochem
Natrium chloride (NaCl)	AppliChem
Nitrocellulose transfer membrane Protean pore size 0,45 µm	Whatman
P3-D-galactoside	Roth
Paraformaldehyde (PFA)	Merck
Phenol	AppliChem
Phenol/Chloroform (TE saturated)	AppliChem
Phenol:Chloroform:Isoamylacohol	AppliChem
Phosphate buffered saline (PBS) tablets	Fluka
PIPES (Piperazin-1,4-bis(2-ethansulfonacid))	Roth
Ponceau S	Sigma-Aldrich
Potassium acetate (KAc)	Roth
Potassium ferricyanid	Roth
Potassium ferrocyanide	Roth
Potassiumchloride (KCl)	Merck
Potassiumdihydrophosphate (KH_2PO_4)	Roth
Propidium iodide (PI)	Sigma-Aldrich
Skimmed milk powder	AppliChem
Sodium citrate	Roth
Sodium dodecyl sulfate (SDS) ultra pure	Sigma-Aldrich
Sodium orthovanadate	Roth
Sodium phosphate,	Roth
Sodiumdihydrophosphate (NaH2PO4)	Merck
Sodiumhydrophosphate (NaHPO4)	Merck
Sodiumhydroxide (NaOH)	Sigma-Aldrich
TEMED	AppliChem
Tris-(hydroxymethyl)-aminomethane (Tris)	Merck, Roth
Triton-X100	Sigma-Aldrich
Tween 20	Roth
X ray films	AGFA, Fuji
X-gal	Sigma-Aldrich
β-Mercaptoethanol	Roth

2.1.3 Cell culture materials and additives

Reagents and materials	Manufacturer
Actinomycin D	Sigma-Aldrich
Anthrax lethal toxin	Emmanuel Lemiche, Université de Nice-Sophia-Antipolis
Bürker hemacytometer	Marienfeld-Superior
Cell scaper	SARSTEDT
Cell strainer 40, 70, 100 m pore size	BD Falcon™
DMEM	GibcoBRL
DMSO	AppliChem
Fetal bovine serum (FBS)	PAN-Biotech, Sigma-Aldrich
HBSS	GibcoBRL
Lipopolysaccharide (LPS) *E. coli* 0111:B4	Sigma-Aldrich
Phosphate buffered saline (Dulbecco) (PBS) solution without Ca/Mg	Biochrom
Puromycin, Dihydrochloride	Calbiochem
Recombinant Mouse Interleukin-3 (IL-3)	Calbiochem
Recombinant Mouse Macrophage colony-stimulating factor (M-CSF)	Calbiochem
HBSS	GibcoBRL
RPMI 1640	GibcoBRL
SB203580	Calbiochem
SP600125	Calbiochem
Tissue culture dishes, different sizes	SARSTEDT
Tissue culture dishes, Permanox^{TM} coated	NUNC
Tissue culture flasks, vented	SARSTEDT
Tissue culture plates, 6 well	Greiner Bio-One
Trypsine-EDTA	GibcoBRL
UO126	Promega
Water cell culture tested	Sigma-Aldrich

2.1.4 Antibodies

Antibodies	Manufacturer
Anti-mouse IgG conjugated peroxidase	Millipore, GE healthcare
Anti-rabbit IgG conjugated peroxidase	Millipore
Bmi1 (clone F6)	Millipore
CD11b Fluorescein isothiocynate (FITC)	Miltenyi Biotec GmbH
C-RAF (C-20)	Santa Cruz
Dendritic cell (DC) marker (PE)	eBioscience
ERK (#9102)	Cell Signaling
F4/80 Phycoerythrin (PE)	Acris
F4/80-Like receptor	dianova
GAPDH (clone 6C5)	Millipore
Gr-1 Phycoerythrin (PE)	Miltenyi Biotec GmbH
H3K27me3 (ab6002)	Abcam
HA High Affinity (clone 3F10)	Roche
Histone H3 (#06-775)	Millipore
Histone H4 pan (clone 62-14113)	Millipore
I B (FL-359)	Santa Cruz
JNK (clone 56G8)	Cell Signaling
M2PK (Clone DF4)	ScheBo Biotech
MHC-class II Phycoerythrin (PE)	Miltenyi Biotec GmbH
MKK3 (H-70)	Santa Cruz
p38 (#9212)	Cell Signaling
pAkt (Ser473)	Cell Signaling
pERK (clone 12D4)	Santa Cruz
pJNK (Thr183/Tyr185)	Cell Signaling
pp38 (anti-ACTIVE ®)	Promega
Sheep red blood cells stroma IgG	Sigma-Aldrich
TLR4 (H-80)	Santa Cruz

2.1.5 Enzymes

Enzymes	Manufacturer
DNase I	Fermentas
Proteinase K	PEQLAB Biotechnologie
RNase A (DNase-free)	Sigma-Aldrich
Shrimp alkaline phosphatase (SAP)	Fermentas
Taq DNA Polymerase	New England Biolabs

2.1.6 Kits

Kits	Manufacturer
DyNAmo™ HS SYBR® Green qPCR Kit	Finnzymes
First Strand cDNA Synthesis Kit	Fermentas
SensiMix™ SYBR & ROX Kit	Quantace Ltd
peqGOLD TriFast™ Kit	PEQLAB
SuperSignal West Femto Maximum Sensitivity Substrate	Millipore
QIAfilter Plasmid Maxi Kit	Quiagen
Chromatin Immunoprecipitation (ChIP) Assay Kit	Millipore

2.1.7 Oligonucleotides

2.1.7.1 Primers genotyping

Primers		Primer sequence
Bmi1 knock out allele	NL11	CGTCTGTCGAGAAGTTTCTG
	NL12	AGAAGAAGATGTTGGCGACC
Bmi1 wild type allele	NL2	CAGCAATGACTGTGATGC
	NL5	TCACTCCCAGAGTCACTTTC

2.1.7.2 RT-qPCR primers

Primers	Primer sequence
AcRP0 for	TTATCAGCTGCACATCACTCAG
AcRP0 rev	CGAGAAGACCTCCTTCTTCCA
IL-10 for	CAGGGATCTTAGCTAACGGAAA
IL-10 rev	GCTCAGTGAATAAATAGAATGGGAAC
Bmi1 s	CTGATGCTGCCAATGGCTCC
Bmi1 as	AGTCATTGCTGCTGGGCATC
HPRT s	CACAGGACTAGAACACCT
HPRT as	GCTGGTGAAAAGGACCTCT
p16 for	CGTGAGGGCACTGCTGGAAG
p16 rev	ACCAGCGTGTCCAGGAAGCC
p19 for	GCGCTCTGGCTTTCGTGAAC
p19 rev	CGTGTCCAGGAAGCCTTCCC

2.1.8 Chromatin immunepreciptation (ChIP) primers

Primer		Primer coordinates	Primer sequence
Primer 1	for	12605 - 12624	AGGTTGTCCTGGAAACAGCTCT
	rev	12775 - 12755	CATCTACCAACTGCCCACCCT
Primer 2	for	15611 - 15630	GTGCTCGCTCTGCTACCAAC
	rev	15812 - 15792	TCTGGAGCCTCCACTGAAGGG
Primer 3	for	27390 - 27409	CCTCTTGAATCCGCAGCTCC
	rev	27575 - 27556	GGCGCACACTGGGAAAAGCC
Primer 4	for	29937 - 29957	AACCTTTGCCAGGAAGGCCCC
	rev	30144 - 30125	TCCCGGCTGTACTGGCCCCT
Primer 5	for	32712 - 32732	TAAGTGGCAAAGGGGGCGAGT
	rev	32887 - 32868	TGCCAGAGACGCTTTGCAGA
GAPDH	for	CCCACTTGCCTCTGTATTGG	
	rev	CTGTGGGGAGTCCTTTTC	

2.1.9 DNA Constructs

pcDNA3HA-Bmi1 Christian Scheuermann, MSZ

2.1.10 Cell lines and mouse lines

2.1.10.1 Cell line

J774A.1 mouse BALB/c monocytes-macrophages (Ralph et al, 1976; Ralph & Nakoinz, 1975; Ralph et al, 1975)

2.1.10.2 Mouse lines

FVB/N strain (Taketo et al, 1991)

All animal studies were approved by the Bavarian State authorities for animal experimentation. The animals were housed at the Institut für Medizinische Strahlenkunde und Zellforschung (MSZ) animal care facility.

2.1.10.3 Sheep erythrocytes

Sheep red blood cells were purchased from Dunn Labortechnik GmbH, Asbach, Germany

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Preparation of competent bacteria ($CaCl_2$-method)

A single bacterial colony was picked of an overnight incubated LB-Agar plate and 250 ml SOB-medium were inoculated. Bacteria were grown at 18°C (165rpm) for about 2 days, until OD_{600} = 0,6. The 10 min chilled bacteria were pelleted in a cooled Beckman centrifuge (J-6B) at 3000 rpm for 10 min and the pellet was gently resuspended in 20 ml chilled TB-buffer. After 10 min on ice bacteria were pelleted again and resuspended in 20 ml TB-buffer. 1,6 ml DMSO were added and bacteria were incubated for another 10 min on ice. The suspension was aliquoted, frozen in liquid nitrogen and stored at -80°C.

- SOB

  2 % Trypton/Pepton
  0,5% Yeast extract
  10 mM NaCl
  2,5 mM KCl
  autoclave media and let it cool down
  10 mM $MgCl_2$ (0,2 µm steril filtered)
  10 mM $MgSO_4$ (0,2 µm steril filtered)

- TB-Puffer

  10 mM PIPES (Piperazin-1,4-bis(2-ethansulfonacid))
  55 mM $MnCl_2$
  15 mM $CaCl_2$
  250 mM KCl

2.2.1.2 Transformation of competent bacteria

Competent bacteria were thawed on ice and desired plasmid-DNA (~0,5-1 µg) was added to 100 µl of bacteria and mixed carefully. The tube was incubated on ice for 30 min subsequently heatshocked at 42°C for 45 s. The bacterial suspension was immediately put back on ice for 2 min and 900 µl of antibiotic-free SOC-medium was added. The bacteria were incubated on 37°C,

600 rpm on a thermomixer for 60 min. Thereafter, 100 µl of the suspension were plated on an antibiotic-containing LB-plate. Only bacteria that have taken up the plasmid-DNA, which contains the antibiotic-resistence casette can grow on later the agar plates. A single colony can be expanded in LB-medium and used for DNA-preparation.

- SOC

  SOB-Media

  20 mM Glucose (0,2 µm steril filtered)

- LB-Medium

  10 g Trypton/Pepton
  5 g Hefeextrakt
  10 g NaCl
  ad 1000 ml H_2O and adjust to pH 7,5 with NaOH
  For ager plates, add 1,5% of Bacto-agar

2.2.1.3 Glycerol stocks

E. coli strains can be stored for many years at –70°C in 15% glycerol. Add 0.85 ml of a logarithmic-phase *E. coli* culture to a vial with 0.15 ml (100%) pre-sterilized glycerol and vortex the vial vigorously to ensure even mixing of the bacterial culture and the glycerol. Freeze in liquid nitrogen and store at –70°C.

2.2.1.4 DNA-isolation from competent bacteria

To isolate DNA from transformed bacteria QIAfilter Plasmid Maxi Kit were used according to the manufacturer's protocols.

2.2.1.5 Electrophoresis on agarose gel

Double stranded DNA fragments and plasmids can be separated according to their size on an agarose gel. Agarose is added to 1xTAE buffer to obtain a 0,8-2% final concentration. The suspension is boiled in the microwave until the agarose is completely solubilised. Add ethidiumbromide up to a concentration of 0,5 µg/ml and pour the solution into the gel apparatus. DNA gel loading buffer was added to the DNA sample and applied on the gel. We electrophoresed in 1xTAE buffer at 100-140 Volts. The DNA can be visualised under UV-light through the incorporation of ethidiumbromide into the DNA.

- 50 x TAE

  242 g Tris 100 ml

  0,5 M EDTA pH 8,0

  57,1 ml acetic acid 100%

  ad 1000 ml H_2O

2.2.2 Mammalian tissue culture

2.2.2.1 J774A.1 culture conditions

J774A.1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. The cells were cultured in tissue culture flasks in an incubator at 37°C, 7% CO_2 and 100% humidity. Tissue culture work was done under sterile conditions. J774A.1 cells were cultured till confluency, then the cells were detached by cell scraper from the flask and diluted with fresh pre warmed medium.

2.2.2.2 Freezing and thawing of cells

For freezing, J774 cells were resuspended in cold freezing medium and aliquoted in 1 ml cryo-tubes. The cells were frozen for 24 - 72 h at -80°C, before they were stored in liquid nitrogen.

- Freezing medium:

  DMEM

  20% FCS

  10% DMSO

The frozen cells were quickly thawed and resuspended in 5 ml tissue culture medium. The resuspended cells were centrifuged at 1100 x rpm for 5 min. The supernatant was discarded and the cells were subsequently resuspendet in pre warmed tissue culture media and further cultured in tissue culture flasks.

2.2.2.3 Transfection

Protocol was adjusted for one well of a six-well plate (5-6x10^5 cells). 2 µg DNA and 6 µl Fugene HD were added to 100 µl DEMEM media without any supplements. The solution was mixed and vortexed. After 15 min of incubation the transfection mix was applied on cells which were covered with fresh normal growth media.

2.2.3 Bone marrow derived macrophages (BMDMs)

2.2.3.1 Genotyping of mice

Small piece of mouse tail (0.5-1 cm) were digested in 200 µl of the tail lysis buffer with 0.4 mg/ml Proteinase K overnight at 56°C. The obtained lysates were centrifuged for 10 min at 14000 rpm. 5 µl supernatant was transferred to a new tube and diluted in 45 µl H_2O. DNA dilutions were used for genotyping by PCR using the reaction mix and thermo cycler conditions listed below.

- Tail Lysis Buffer

  50 mM EDTA

  50 mM Tris pH 8

  0,5% (w/v) SDS

- Mastermix for 1 x reaction (25µl):

  1 µl template DNA

  0,25 µl Primer sense (50 pmol/µl)

  0,25 µl Primer antisense (50 pmol/µl)

  2,5 µl 10 x Polymerase buffer

  1 µl dNTPs (10 mM)

  0,25 Taq DNA Polymerase

  19,75 µl H_2O

- Programme:

	Bmi1 WT allele			Bmi1 knock out allele	
	5 min	94°C	Activation	5 min	94°C
35 cycles	30 s	94°C	Denaturation	30 s	94°C
	60 s	53°C	Annealing	90 s	56°C
	60 s	72°C	Extension	60 s	72°C
	10 min	72°C		10 min	72°C

2.2.3.2 Generation of BMDMs

To generate BMDMs4-6 week old mice were sacrificed. Usually sex matched litters were used for BMDMs cultivation. The femurs were cut out and the muscles were removed. The ends of the bones were cut as far towards the end as possible and afterwards the bone marrow was flushed into a 50 ml conical tube with RPMI 1640 medium using a 25 G needle and a 1 ml syringe. The bone marrow suspension was poured through a 70 µm cell strainer and subsequently centrifuged at 1100 rpm for 10 min. The supernatant was discarded and the cells were resuspended in erythrocyte lysis buffer and again centrifuged at 1100 rpm for 10 min. The supernatant was again discarded and the cells were resuspended in RPMI 1640 medium and counted with a Bürker hemacytometer. To obtain BMDMs the bone marrow derived cells were propagated in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine in the presence of 10 ng/ml recombinant M-CSF and 1 ng/ml recombinant IL-3 in an incubator at 37°C, 7% CO_2 and 100% humidity for eight days.

- Erythrocyte lysis buffer

  155 mM NH_4Cl

  50 mM Tris pH 7,2

2.2.4 Experimental assays and treatments

2.2.4.1 Bacterial uptake and phagocytosis assay

BMDMs were cultivated as described above. For *in vitro* macrophage infection the cells were rinsed twice and resuspended in complete medium without antibiotics. The OD of *E. coli* (DH5α) grown to logarithmic growth phase was determined immediately before infection. 20 min cytochalasine D, a cell permeable and potent inhibitor of actin polymerization, pretreatment

of BMDMs. was served as control for active bacterial uptake. For infection at a MOI 1:50, the cells were centrifuged at 300 x g for 5 min and incubated for 25 min (t=0; bacterial uptake) or 180 min (t=165 min; phagocytosis) at normal cell culture conditions followed by a 30 min treatment with 100 µg/ml gentamycine. The cells were subsequently harvested to determine CFU. For the determination of intracellular CFU the infected cells were rinsed in PBS and subsequently lysed with 200 µl 1% Triton-X-100 in PBS to release intracellular bacilli. 800 µl LB media was added to bacterial suspensions, serially diluted and plated on agar plates. The number of viable bacilli was evaluated by counting individual colonies.

2.2.4.2 Erythrocyte binding to macrophages

Sheep red blood cells (SRBC) were opsonized with rabbit sheep red blood cells stroma IgG (E-IgG) at nonagglutinating titers at a final dilution of 1:150 for 40 min at room temperature. Opsonized SRBC were washed and resuspended in HBSS before their addition to macrophages for different time periods. Erythrocytes were added to BMDMs at a ratio of 20:1.

2.2.4.3 Anthrax lethal toxin (LT) assay

Protective antigen (PA) and lethal factor (LF) are the two components of LT the bacterial toxin from *Bacillus anthracis*. PA is the pore forming component and LF is the highly specific protease that exclusively cleaves MKKs (Turk, 2007). BMDMs were preteated 30 min before LPS stimulation with simultaneously and as control separately with PA and LF at final concentrations of 3µg/ml respectively in normal culture medium.

2.2.4.4 Senescence-associated β-galactosidase staining

Cells were rinsed in PBS and subsequently fixed at room temperature for 3-5 min in 3% formaldehydę. Senescence associated (SA) -β-galactosidase staining was performed as previously described (Dimri et al, 1995).

2.2.4.5 siRNA interference

siRNA has become a widely used tool for down-regulating gene expression of a specific gene. For siRNA experiments 4-6 x 10^5 J774A.1 cells were seeded in 6 well tissue culture plates and transfected with siRNA directed against Bmi1 (Santa Cruz) and nonsilencing/scrambled siRNAs (Santa Cruz) as controls. Briefly, 15 µl of 10 µM siRNA were diluted in 200 µl culture medium without serum per well. 20 µl HiPerFect Transfection Reagent per well were added to the diluted siRNA and mixed by vortexing and incubated for 5-15 min at room temperature to allow the

formation of transfection complexes. The complexes were added drop-wise onto the cells and the plate was gently swirled to ensure uniform the distribution of the transfection complexes. The cells with the transfection complexes were incubated under their normal growth conditions for 6 h. After the incubation time 1500 µl culture medium containing serum and antibiotics was added to the cells and incubated at normal growth conditions. The murine macrophage cell line J774A.1 was transfected twice at intervals of 24 h and analysed 12 h later.

2.2.5 RNA methods

The method of choice for the quantification of different mRNAs is real-time reverse transcription polymerase chain reaction (RT-qPCR). RT-qPCR is a combination of several steps: (i) the RNA extraction (ii) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA and (iii) the amplification of the cDNA using the PCR followed by detection and quantification of amplification products in real time.

2.2.5.1 RNA extraction

The RNAs from primary or cell line cells were extracted using a commercial RNA tri-reagent based extraction peqGOLD TriFast™ Kit from PEQLAB, following the manufacturer's instructions precisely. The obtained RNA was dissolved in DEPC-H_2O and could be stored at -80°C. The yield and quality of thus obtained RNA was determined by analysis of the A260/A280 ratio with a spectrophotometer. Additionally an equal amount of RNA was subsequently treated with DNase I according to the manufacturer's instructions.

2.2.5.2 Reverse transcription PCR (RT-PCR)

Equal amounts (1 µg) of DNase I treated RNA were used for reverse transcription. Reverse transcription was carried out in 20 µl final volume using the First Strand cDNA Synthesis Kit from Fermentas according to the manufacturer's instructions. The cDNA was synthesized using random hexamer primers qRT-PCR provided by the kit.

2.2.6 Quantitative real time PCR (qPCR)

The qPCR was performed monitoring the fluorescent nucleic acid dye SYBR green incorporation in the presence of the reference dye ROX using the DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes).

- Mastermix for 1 x reaction (20 µl):

  1 µl cDNA

  1µl Primer sense (10 µM)

  1µl Primer antisense (10 µM)

  0,4 µl ROX

  10 µl Mastermix

  6,6 µl H_2O

The cDNA was amplified and detected on a centrifugal rotary real-time thermal cycler Corbett Rotorgene 2000 using the following conditions.

- Programme:

	15 min	94°C	Activation
45 cycles	30 s	94°C	Denaturation
	30 s	var.	Annealing
	60 s	72°C	Extension
	5 min	72°C	
	5 min	25°C	
		Melting curve analysis	

Data was analyzed by Rotor-Gene Real-Time Analysis Software.

2.2.7 Protein methodologies

2.2.7.1 Cytokine detection assay

After BMDM generation the old media was discarded and the cells were once rinsed with PBS. The macrophages were covered with 1,5 ml fresh pre warmed RPMI media and 12 hours later the cells were treated with 100 ng/ml LPS and collected at different time points. As controls two supernatants of untreated BMDMs were collected, one immediately with the start of the experiment and the second one at the end of the experiment. Supernatants of WT and Bmi1-/- BMDMs were analyzed by enzyme-linked immunoabsorbent assay (ELISA) for cytokine production performed by IMMUMED GmbH.

2.2.7.2 Immunocytochemistry (ICC)

ICC uses antibodies that target specific protein antigens and allows evaluating whether the cells express the protein in question and in which sub-cellular compartments it is expressed. To perform ICC the cells were grown on glass coverslips. Then the cells were washed twice with PBS and subsequently fixed for 10 min with fresh 3,7% paraformaldehyd (PFA) in PBS at room temperature. If needed the cells were additionally quenched in 50 mM NH_4Cl in PBS for at least 5 min. To permeabilize the cells they were incubated in 4°C chilled acetone for 10 min at 4°C. The cells were rinsed immediately with PBS. To proceed with the staining the cells were then incubated for 1 h in the primary antibody solution (antibody dilution 1:200 in PBS + 0,2% gelatine) at room temperature and then rinsed for 3 times in PBS. Further the cells were incubated for 1 h in corresponding fluorochrome-conjugated secondary antibody solution (antibody dilution 1:200 in PBS + 0,2% gelatine) at room temperature followed by 3 times rinsing in PBS. After two further rinsing steps the cells were additionally counterstained with 4',6-Diamidino-2-phenylindol (DAPI) for 10 min at room temperature. The coverslips were mounted using MOWIOL. Immunofluorescence pictures were acquired on inverted DMIRBE microscope under oil immersion. Images were captured and stored as Openlab LIF files.

2.2.7.3 Shrimp alkaline phosphatase (SAP) dephosphorylation assay

4-5 x 10^5 J774A.1 macrophages were rinsed twice with 4°C cold PBS. Subsequently the cells were collected in 200 ml 1 x SAP buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL aprotinin and 1 mg/mL pepstatin A) and lysed by shearing through a 22G injection needle. 10 U SAP were added to the lysates and incubated for 1 hour at 37°C. After incubation the lysates were resuspended in 2 x denaturing sample buffer and heated at 95°C for 5 min. The lysates were stored at -20°C.

2.2.7.4 Chromatin fractionation assay

4 x 107 J774A.1 cells for each condition were collected for preparation of subcellular fractions as previously described (Wysocka et al, 2001). To obtain equal protein ratios the fractions were resuspended in adequate amount of denaturing sample buffer heated at 95°C for 5 min and stored at -20°C.

2.2.7.5 Preparation of whole cell lysat (WCL) from mammalian cells

To obtain WCL the cells were washed in cold PBS. After that the cells were resuspended in 2 x denaturing sample buffer. The extracts were sheared by syringing through a 22G needle and then heated at 95°C for 5 min. The WCLs were stored at -20°C.

- 2 x denaturing sample buffer

  50 mM Tris-HCL pH 6,8

  5% (v/v) 2-mercaptoethanol

  0,005% (w/v) bromphenol blue

  4% (w/v) SDS

  20% (v/v) glycerol

2.2.7.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE is the method used to separate proteins by mass under denaturing conditions. Sodium dodecyl sulfate (SDS) is an anionic detergent that disrupts nearly all noncovalent interactions in native proteins and applies a negative charge to each protein. WCLs were loaded on polyacrylamide gels that are composed of two layers: a large pore polyacrylamide gel (5%) stacking gel (pH 6.8) which insures a simultaneous entry of the proteins into the resolving gel and a (8 - 14% acrylamide monomer) resolving gel (pH 8.8) which separates the proteins according to their size.

- Stacking gel buffer

  0,5 M Tris-HCL pH 6,8

- Resolving gel buffer

  1,5 M Tris-HCL pH 8,8

- Resolving gel (14% Acryamide)

Acrylamide (30%)/Bisacrylamide (0,8%)	11,67 ml
Resolving gel buffer	9,5 ml
10 % (w/v) SDS	250 µl
H_2O	3,35 ml
Amonium persulfate (APS)	250 µl
TEMED	12,5 µl

- Stacking gel

Acrylamide (30%)/Bisacrylamide (0,8%)	1,5 ml
Stacking gel buffer	2,5 ml
10 % (w/v) SDS	100 µl
H_2O	5,7 ml
Amonium persulfate (APS)	100 µl
TEMED	10 µl

- 1 x SDS-PAGE running buffer: (1 l) 10 x SDS-PAGE running buffer (1 l):

25 mM Tris-HCL pH 6,8	3,38 g	38,28 g
192 mM Glycine	14,41 g	144,13
0,1% (w/v) SDS	1,00 g	10,00 g

2.2.7.7 Immunoblotting

The Western blot (alternatively, immunoblot) is a method used to detect specific proteins in a given sample of tissue homogenate or extract using antibodies specific to the target protein. For this purpose SDS-PAGE gels were electroblotted at 300 mA to nitrocellulose transfer membrane using the BioRad “Wet Blot system”. Therefore, the acrylamid gel was assembled in direct contact with the nitrocellulose membrane flanked by gel blotting papers. This “sandwich” was placed in a blotting chamber filled with blotting buffer. To check if the transfer has occurred Ponceau S fixative dye solution was used to stain the membranes, which were washed afterwards with deionised water. For Western blot analysis the membranes were incubated in blocking buffer for 1 h at RT or overnight at 4°C on a shaker. Subsequently the membranes were sealed in a plastic bag and incubated for 2 h at RT or overnight at 4°C on a shaker with an appropriate dilution of the primary antibody in blocking buffer directed against the protein of interest. The membranes were washed three times with PBS, each time for 10 min. The appropriate peroxidase-conjugated (HRP) secondary antibody was diluted in blocking buffer, and added to the membrane. The nitrocellulose membranes were subsequently incubated for one additional hour at RT on a shaker. Afterwards the membranes were washed three times with PBS, each time for 10 min. Finally the antigen-antibody complexes were detected using the SuperSignal West Femto Maximum Sensitivity Substrate ECL kit and autoradiography according to the manufacturer’s instructions.

- 1 x blotting buffer: (1 l) 10 x SDS-PAGE running buffer (1 l):

25 mM Tris-HCL pH 6,8	3,38 g	38,28 g
192 mM Glycine	14,41 g	144,13g
20% (v/v) Methanol	200 ml	

- Blocking buffer

  1 x PBS

  0,05% (v/v) Tween 20

  5% (w/v) skimmed milk powder

2.2.7.8 Immunoblot stripping

The removal of primary and secondary antibodies from a membrane is possible, so that the membrane can be re-probed with alternative antibodies. First the nitrocellulose membrane was incubated in distilled H_2O for 10 min. Next the membrane was incubated in 0,2 M NaOH for 10 min followed by an additional 10 min incubation in distilled H_2O. The membrane should be incubated in blocking buffer for 1 h at RT or overnight at 4°C on a shaker before it can be re-probed as described above.

2.2.7.9 Chromatin immune precipitation (ChIP)

ChIP assays are used to evaluate the association of proteins with specific regions of chromatin. The technique involves cross linking of proteins with DNA, fragmentation and preparation of soluble chromatin followed by immunoprecipitation with antibodies recognizing the proteins of interest. The cross linking of the DNA and proteins was then reversed and then identified by PCR amplification. For ChIP analysis 1 x 10^6 cells per condition was used. ChIP analyses were performed with ChIP Assay Kit (Millipore) according to manufacture's instructions. The DNA fragmentation was obtained by sonification. The parameters for sonification were: pulse for 30 seconds with 60 pulses per minute and 60% of output, each sample was pulsed 4 times. Shearing efficiency was checked by agarose gel electrophoresis after reversion of cross-links. DNA pulled down was analysed by qPCR using the SensiMix™ SYBR & ROX Kit (Quantace Ltd). Data are represented as the fold of enrichment of DNA pulled down with the specific antibody over the input.

2.2.8 Fluorescence activated cell sorter (FACS)

FACS is a powerful tool to measure and analyze of characteristics of single cells suspended in a flowing saline stream through a beam of light. The properties measured include relative cell size, relative cell granularity, surface molecules or intracellular constituents.

2.2.8.1 Quantification of surface antigens by FACS

BMDMs were cultivated as described above with a little change the bone marrow cells were seeded on Permanox^{TM} coated tissue culture dishes. Floating cells and old culture media were removed and the adherent macrophages were washed twice with ice cold 1 x PBS. To remove the BMDM the cells were incubated with Trypsine-EDTA for 1 min at 37°C and then gently scraped from the tissue culture dish. The BMDMs were poured through a 40 µm cell strainer. In order to avoid FcγII/III receptor mediated non-specific antibodies binding which could contribute to background staining the cells were incubated with blocking antibodies (FcR, CD16/CD32) for 15 min at room temperature and subsequently stained with fluorochrome-conjugated antibodies according to the manufactures instructions'. Stainings with appropriate isotype controls were served as controls. FACS analysis was performed on a FACSCalibur flow cytometer and the data were analyzed by CellQuest Pro 4 (Becton Dickinson) software.

2.2.8.2 Cell-cycle analysis

The most commonly used DNA dye is propidium iodide (PI). Its advantage is the excited light can be used on most common flow cytometers. Bone marrow cells were seeded on Permanox^{TM} coated tissue culture dishes. Floating cells and old culture media were removed and the adherent macrophages were washed twice with ice cold 1 x PBS. To remove the BMDM the cells were incubated with Trypsine-EDTA for 1 min at 37°C and then gently scraped from the tissue culture dish. The BMDMs were poured through a 40 µm cell strainer and after harvesting and washing, the cells were resuspended in 1 ml 1x PBS and fixed with 4 ml -20°C cold absolute ethanol while vortexing. Fixed samples could be stored for up to 14 days at -20°C. The fixed cells were resuspended in 1 ml PBS and accessorily 50 µl PI (1 mg/ml)and 20 µl DNase-free RNase A (10 mg/ml) were added. The samples were incubated for 30 min in the dark and then analyzed by FACS. The obtained data were evaluated by CellQuest Pro 4 (Becton Dickinson) software.

2.2.9 Statistical analysis

Unless otherwise stated, all statistical tests were done using Microsoft Office Excel 2003 and STATGRAPHICS centurion XV software.

3 Results

3.1 Bmi1 is rapidly up-regulated on the protein level in bone marrow derived macrophages (BMDMs) after LPS treatment

To investigate whether Bmi1 is a downstream target of LPS-mediated TLR signaling, we analysed BMDMs for their expression of Bmi1 in response to the endotoxin LPS, a major constituent of the outer membrane of Gram negative bacteria and a potent macrophage activator. Analysis by Western blot revealed that BMDMs express Bmi1 protein levels close to detection limit in the basal state. After LPS treatment, however, Bmi1 was very rapidly up-regulated. The increase in protein level was readily visible after 10 min of LPS treatment. This up-regulation went along with a transient change in SDS gel migrating properties which have previously been shown to be associated with the phosphorylation status of Bmi1 (Voncken et al, 1999). The first time points of LPS treatment showed an upper band which represents the hyperphosphorylated form of Bmi1. After 3 hours of LPS treatment a lower band appeared which corresponds to the hypophosphorylated form of the Bmi1 protein. This band remained visible for subsequent time points (Figure 3.1A).

The rapid Bmi1 protein up-regulation was not paralleled by rapid elevation of Bmi1 RNA levels, which remained unchanged within the first hours of LPS treatment. Only after 5 hours of LPS stimulation an increase in Bmi1 mRNA expression was measured (Figure 3.1B) suggesting that the rapid Bmi1 induction is not regulated on the transcriptional level in response to LPS treatment.

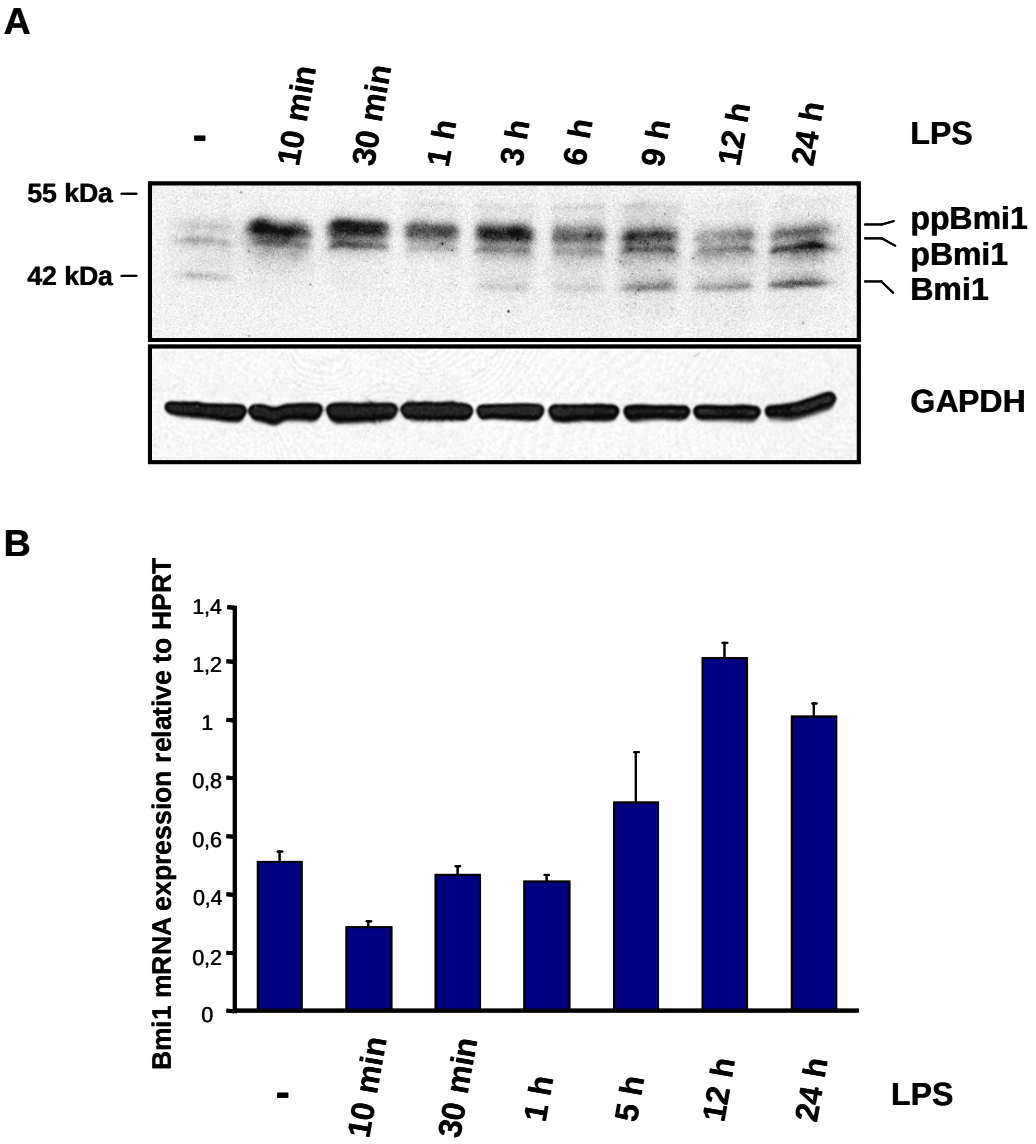

Figure 3.1 Transient Bmi1 up-regulation and phosphorylation in macrophages after LPS treatment

(**A**-**B**) BMDMs from WT mice were treated with 100 ng/ml LPS for the indicated time periods. (**A**) WCLs of BMDMs were immunoblotted with antibodies against Bmi1and GAPDH as loading control. (**B**) Total RNA was isolated from BMDMs and subjected to RT-qPCR for the analysis of Bmi1 mRNA expression. Expression levels of Bmi1 mRNA were related to the HPRT mRNA expression levels.

With the observation of a very rapid increase of Bmi1 protein levels in BMDMs after LPS treatment and due to the function of Bmi1 as a repressor of the *ink4a/arf* locus which encodes the cell cycle regulators $p16^{Ink4a}$ and $p19^{Arf}$ (Jacobs et al, 1999), we analyzed the $p16^{Ink4a}$ and $p19^{Arf}$ mRNA expression after LPS stimulation. Both $p16^{Ink4a}$ as well as $p19^{Arf}$ showed only minor changes on mRNA expression level during a 24 hours time course (Figure 3.2A+B)

indicating that LPS induced Bmi1 protein levels and changes in the phosphorylation status have no effect on $p16^{Ink4a}$ and $p19^{Arf}$ mRNA expression.

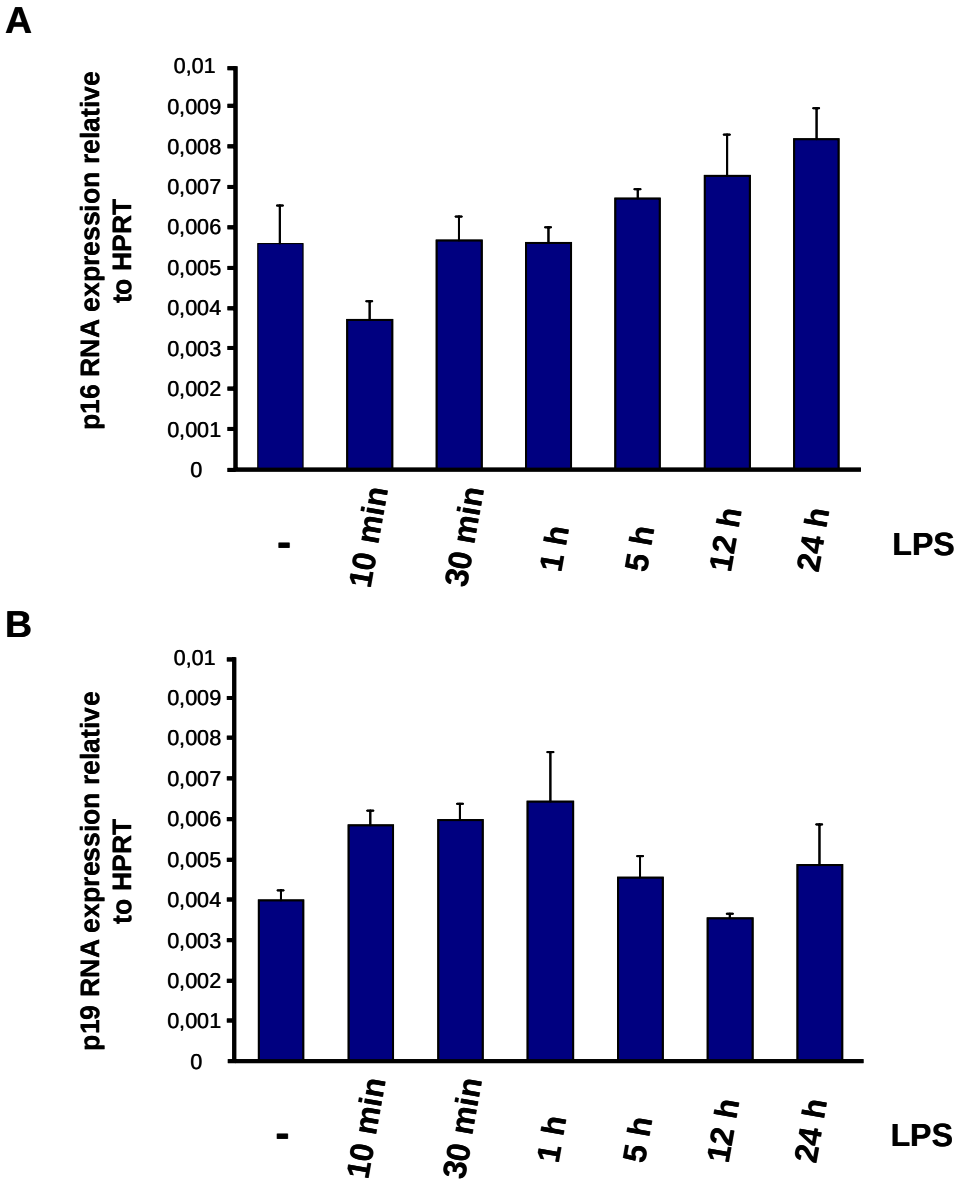

Figure 3.2 Low levels of $p16^{Ink4a}$ and $p19^{Arf}$ mRNA expression in BMDMs after LPS treatment

(**A-B**) BMDMs from WT mice were treated with 100 ng/ml LPS for the indicated time periods. Afterwards total RNA was isolated and subjected to RT-qPCR. (**A**) $p16^{Ink4a}$ mRNA expression was related to HPRT mRNA expression. (**B**) Relative $p19^{Arf}$ mRNA expression levels were related to HPRT mRNA expression.

To begin to understand which signaling cascade(s) mediate Bmi1 phsphorylation inhibitor studies were performed. Previous analysis identified Bmi1 as a target of MAPK signaling including the mitogen-activated (Ras-RAF-MEK-ERK) as well as stress-induced phosphorylation cascades p38/JNK (Voncken et al, 2005). Therefore BMDMs were pretreated

with anthrax lethal toxin (LT) from *Bacillus anthracis*, a protease which exclusively inhibits MAPK signaling via the cleavage of MKKs. Anthrax LT, the active form of the toxin, is composed of the protective antigen (PA) and the lethal factor (LF) (Turk, 2007). LT totally blocked LPS induced Bmi1 expression. While BMDMs without LT pretreatment or only pretreated with either the protective antigen (PA), the pore forming component, or lethal factor (LF) showed no effect on LPS induced Bmi1 protein expression. The combined application of LF and PA completely blocked LPS induced Bmi1 expression (Figure 3.3). Control immunoblots probed with phospho specific antibodies against ERK, p38 JNK and antibodies against MKK3 showed that the LPS activated MAPK signaling pathways were interrupted by LT (Figure 3.3). In summary LPS induced Bmi1 expression depends on MAPK signaling.

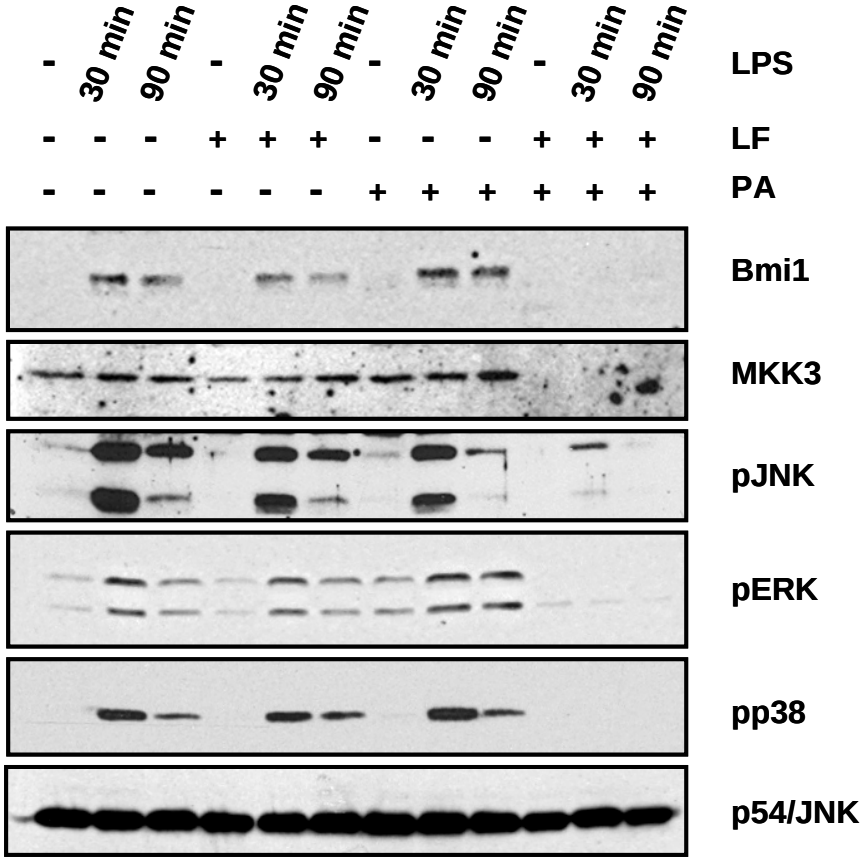

Figure 3.3 Anthrax LT toxin mediated blocking of LPS induced Bmi1 expression

BMDMs were pretreated for 30 min with either lethal factor (LF) or protective antigen (PA) alone or LF and PA in combination followed by 100 ng/ml LPS treatment at indicated time points. The obtained WCLs were immunoblotted with indicated antibodies. p54/JNK specific antibody was used as loading control.

3.2 Characterization of WT and Bmi1-/- BMDMs

The rapid Bmi1 protein induction and phosphorylation after LPS treatment argued for a function of Bmi1 during acute activation of BMDMs. To address the role of Bmi1 during macrophage activation BMDMs from WT and Bmi1-/- mice were analysed. First bone marrow derived cells obtained from WT and Bmi1-/- mice were differentiated to macrophages. The differentiation status of Bmi1-/- and WT BMDMs was analysed by FACS. Expression analysis of macrophage surface marker F4/80 and CD11b (McKnight & Gordon, 1998) revealed that WT as well as Bmi1-/- macrophages showed the same percentage (approximatly 80%) of F4/80 and CD11b double positive cells (Figure 3.4A).

To gain more insight into possible variability in differentiation between bone marrow cells from WT and Bmi1-/- mice in our selected BMDM culture conditions, macrophages were stained with CD11b and additional with other surface markers of specific for the myeloid lineage. No differences in either expression of Gr1, a common granulocyte marker, or in expression of DC-marker, a dendritic cell marker, could be determined (Figure 3.4B+C). These results indicate that differentiation along the monocyte/macrophage lineage is imperturbed in Bmi1-/- bone marrow progenitors under the chosen cultivation conditions.

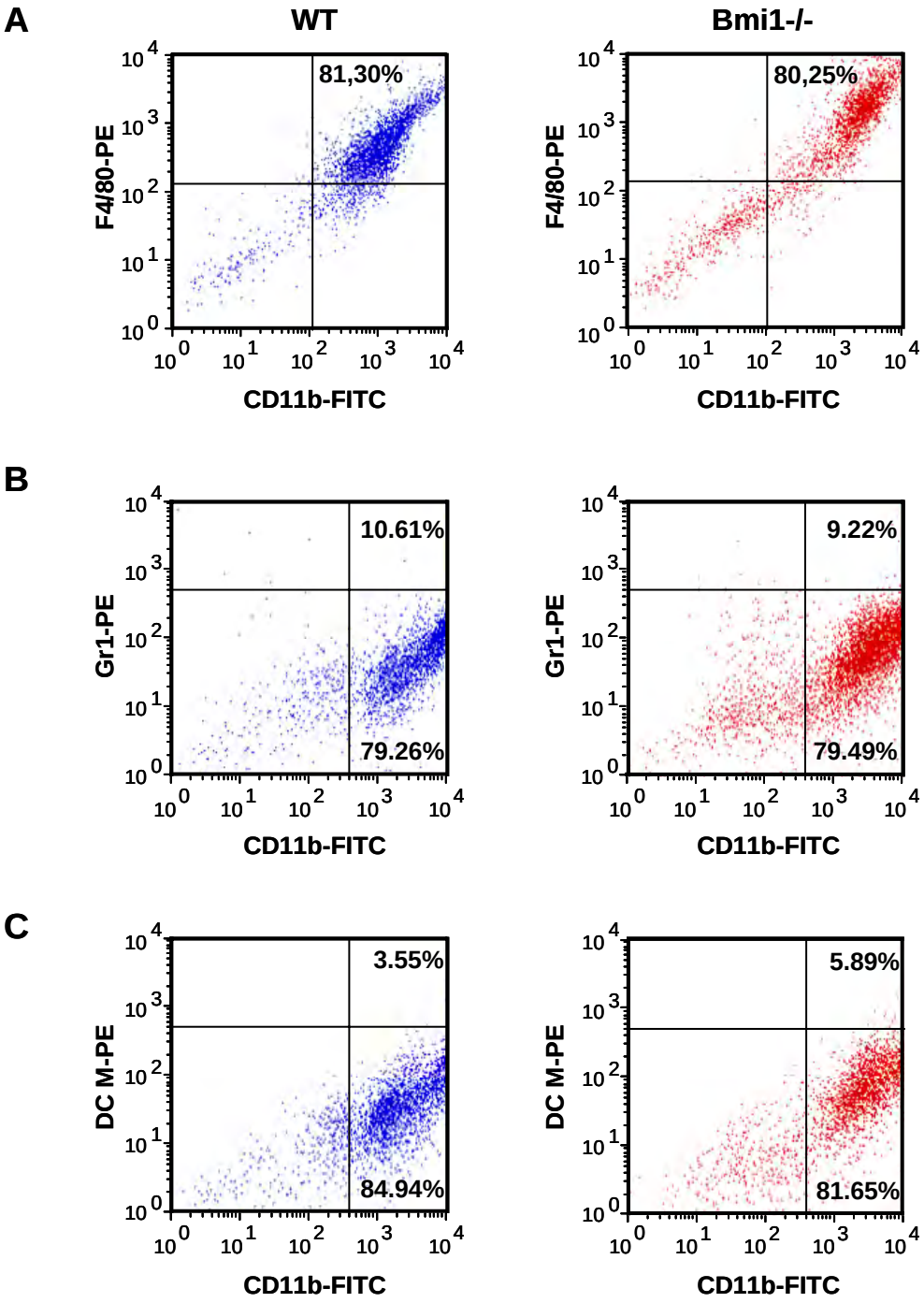

Figure 3.4 No differences in differentiation between WT and Bmi1-/- bone marrow cells

WT and Bmi1-/- BMDMs were analysed by FACS. (**A**-**C**) The cells were stained with the indicated fluorochrome-conjugated antibodies.

To test the relevance of Bmi1 abrogation for macrophage function we analysed Bmi1-/- and WT BMDMs in a functional bacterial uptake assay. This experiment was perfomed under the guidance of Antoine Galmiche. In this assay Bmi1-/- macrophages showed the same capacity to internalize bacteria and the same rate of killing of internalized bacteria as BMDMs from WT mice (Figure 3.5) indicating that BMDMs derived from Bmi1-/- were functional at this level.

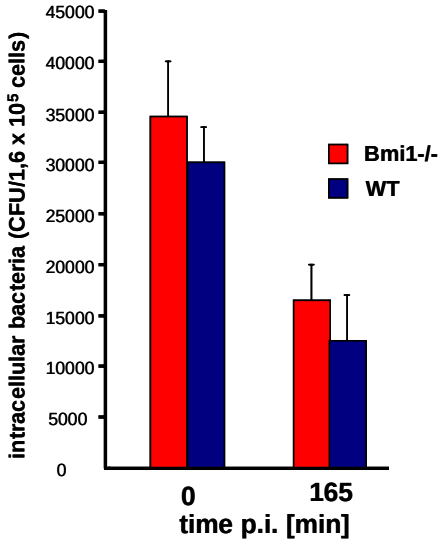

Figure 3.5 Indiscriminative difference in bacterial uptake and phagocytosis between WT and Bmi1-/- macrophages

WT and Bmi1-/- BMDMs were exposed to *E.coli* (DH5α) as described in the Material and Methods part at a MOI of 1:50. The CFU was determined by plating serial dilutions of cell lysates on LB agar plates.

3.3 Bmi1 abrogation affects LPS induced cytokine expression

Macrophages respond to LPS with the rapid expression of cytokines. As Bmi1 is rapidly regulated in response to LPS we next analysed LPS induced cytokine expression in Bmi1-/- and WT BMDMs. First cell culture supernatants from WT and Bmi1-/- BMDMs were analysed by ELISA by IMMUMED GmbH for their cytokine content after LPS treatment (Figure 3.6). The secretion of TGFβ was unaffected by LPS treatment in WT and Bmi1-/- BMDMs. Pro-inflammatory cytokine secretion was strongly induced in both WT as well as Bmi1-/- macrophages after LPS treatment and no differences were detectable in secretion levels of the pro-inflammatory cytokines IL-1β, INFγ and IL-12(p70). A lower secretion of the pro-inflammatory cytokines IL-12(p40), IL-6 and TNFα was measured in supernatant derived from Bmi1-/- BMDMs after LPS treatment especially after 12 hours of LPS treatment. The anti-

inflammatory cytokine IL-10 secretion was also induced in WT and Bmi1-/- BMDMs after LPS treatment but BMDMs from Bmi1-/- mice secreted significant more IL-10 in comparison to their WT counterparts (Figure 3.6).

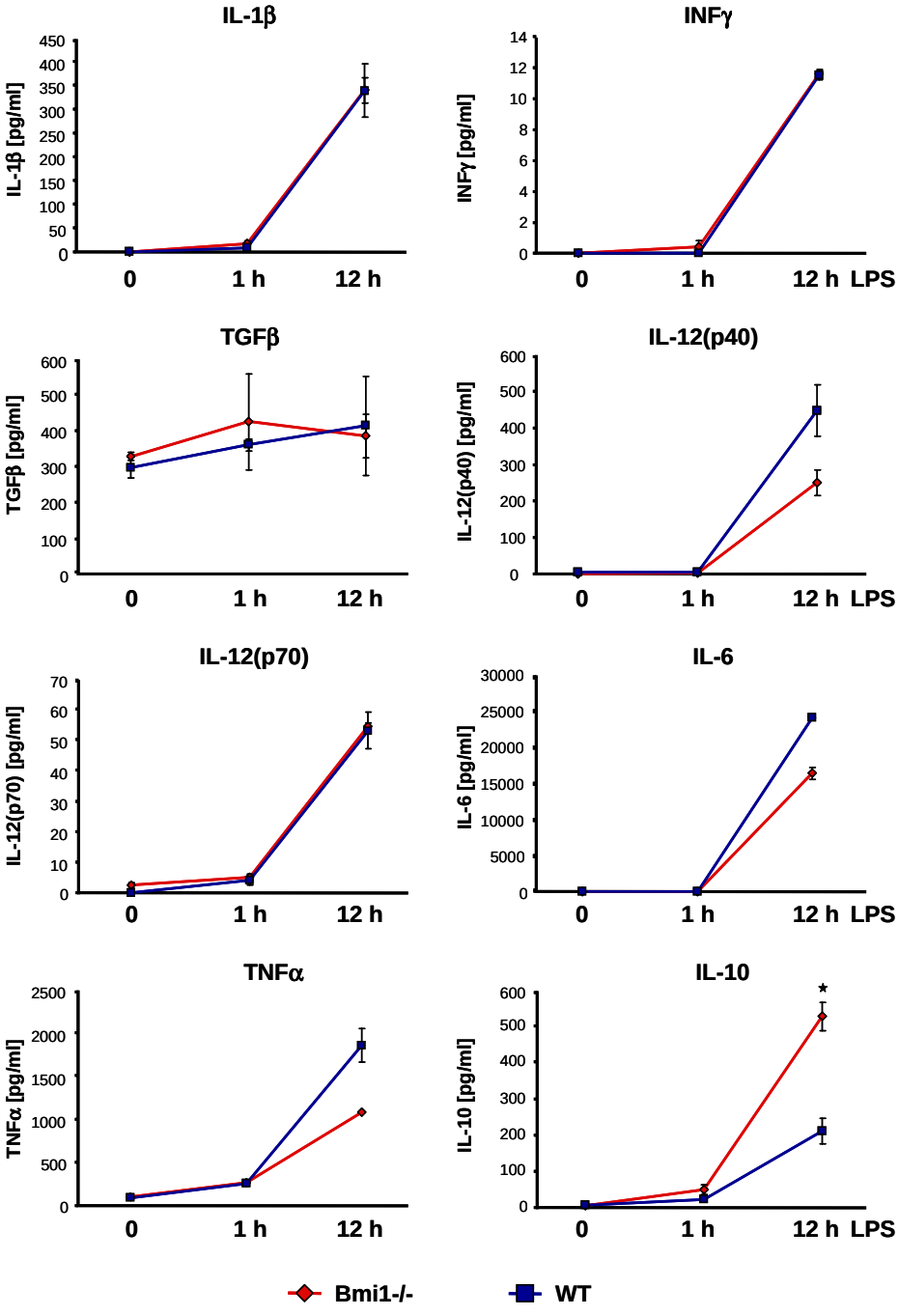

Figure 3.6 Reduced pro-inflammatory cytokine levels and elevated anti-inflammatory cytokine IL-10 level in Bmi1-/- macrophages

WT and Bmi1-/- BMDMs were treated with 100 ng/ml LPS for indicated periods. Supernatants were analyzed by ELISA for cytokine production. Data are presented as mean ± SD from two individual donors. Asterisks indicate statistically significant differences ($p<0,05$ paired Student`s t-test).

3.4 Increased IL-10 mRNA expression in Bmi1-/- BMDMs

IL- 10 expression is tightly transcriptional and post-transcriptional regulated during macrophage activation (Mosser & Zhang, 2008). We therefore asked whether the increase of IL-10 secretion in Bmi1-/- BMDMs correlates with higher IL-10 mRNA expression after LPS-mediated activation. RT-qPCR analysis revealed that Bmi1-/- BMDMs show similar kinetics of IL-10 mRNA expression. IL-10 mRNA levels were 4-5-fold higher in Bmi1-/- BMDMs in comparison to WT BMDMs after LPS treatment (Figure 3.7A). The basal IL-10 mRNA level in Bmi1-/- BMDMs was only slightly elevated in the uninduced state (Figure 3.7A).

To ask whether the differences in IL-10 mRNA levels are the result of higher transcription rate or a product of higher RNA stability WT and Bmi1-/- BMDMs were pretreated with actinomycin D (ActD), a RNA synthesis inhibitor (Reich & Goldberg, 1964). ActD fully abrogated LPS-mediated IL-10 mRNA induction in both WT as well as Bmi1-/-BMDMs (Figure 3.7B). Therefore the higher LPS induced IL-10 secretion in Bmi1-/- BMDMs resulted from the increased *il-10* gene transcription. This result was consistent with a function of Bmi1 as a negative regulator of IL-10 expression in response to LPS-mediated macrophage activation.

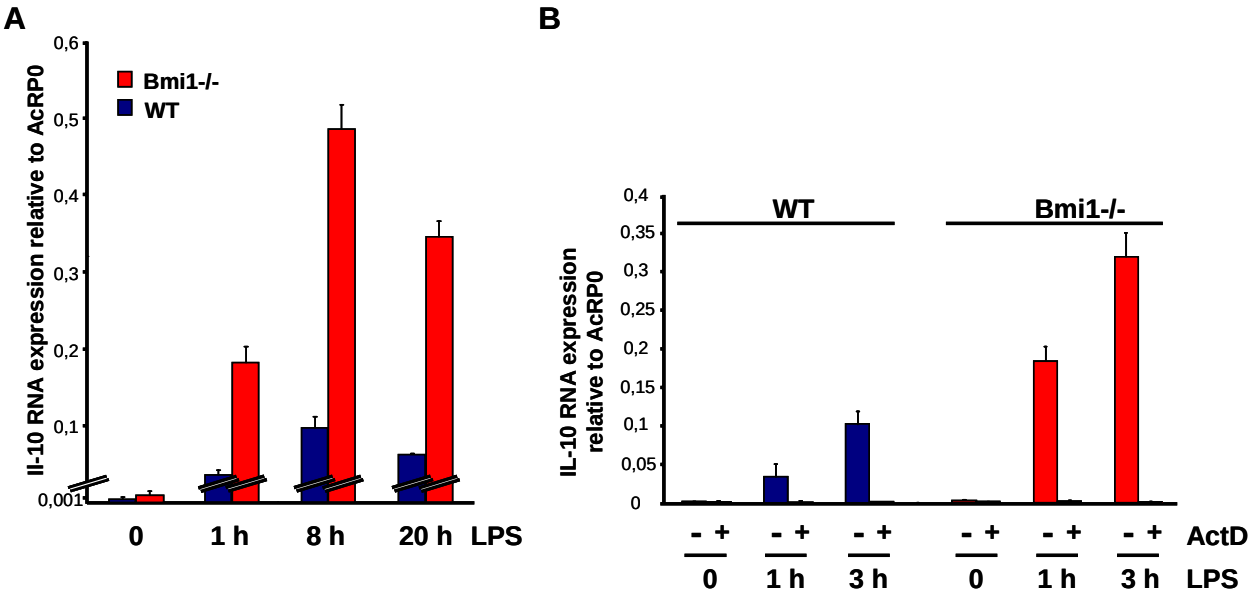

Figure 3.7 Increased transciptionally regulated IL-10 mRNA levels in Bmi1-/- BMDMs after LPS treatment
(**A**) IL-10 mRNA levels in WT and Bmi1-/- BMDMs were analysed by RT-qPCR. The cells were treated with 100 ng/ml LPS at indicated time points. Total RNA was isolated and RT-qPCR analysis was carried out with primers specific for IL-10. IL-10 RNA levels were presented relative to AcRP0 mRNA levels. (**B**) BMDMs from WT and Bmi1-/- mice were 30 min pretreated with 2,5 µg/ml actinomycin D (ActD) before the LPS treatment at indicated

time points. Total RNA was isolated and RT-qPCR analysis was carried out with primers specific for IL-10. IL-10 RNA levels were presented relative to AcRP0 mRNA levels.

3.5 TLR4 signaling is unaffected in Bmi1-/- BMDMs

IL-10 expression in macrophages has previously been shown to be critically dependent on signaling cascades downstream of TLR4 (Mosser & Zhang, 2008). We therefore analysed protein levels of known downstream targets of TLR4 signaling by Western blot analysis (Figure 3.8). As expected only WT BMDMs displayed a rapid increase of Bmi1 in response to LPS. TLR4 expression levels were comparable in WT and Bmi1-/- BMDMs. LPS activation of the MAPK signaling pathways such as the mitogenic (Ras-RAF-MEK-ERK) and stress-induced phosphorylation cascades p38/JNK revealed the same phosphorylation kinetics in WT and Bmi1-/- BMDMs. The function of the NF-κB signaling pathway was unaffected in Bmi1-/- and WT macrophages as shown by similar IκBβ protein degradation kinetics in Bmi1-/- and WT BMDMs after LPS treatment. The AKT pathway was also unaltered and showed the same phosphorylation kinetics of AKT in WT and Bmi1-/- BMDMs after LPS treatment. In summary all known TLR4 signaling pathway demonstrated the identical activity (Figure 3.8) and revealed no differences in LPS-mediated TLR4 signal transduction in WT and Bmi1-/- BMDMs.

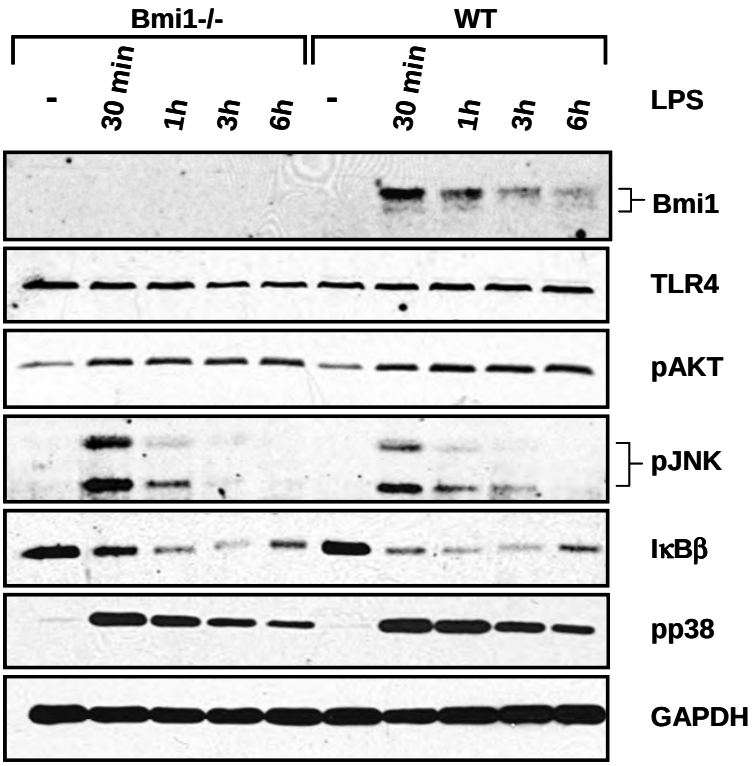

Figure 3.8 TLR4 signal transduction in WT and Bmi1-/- BMDMs

BMDMs from WT and Bmi1-/ mice were treated with 100 ng/ml LPS for the indicated time periods. The obtained WCLs of WT and Bmi1 -/- BMDMs were analyzed by Western Blot which was probed with indicated antibodies. GAPDH was served as loading control.

3.6 Bmi1-/- BMDMs show a senescent phenotype

In the course of the experiments we noticed that the Bmi1-/- BMDMs were bigger in size than their WT counterparts (Figure 3.9A).

Forward scatter (FSC) and side scatter (SSC) analysis performed by flow cytometer confirmed that Bmi1-/- BMDMs were bigger in comparison to WT BMDMs and additionally revealed that Bmi1-/- BMDMs were more granulated than WT BMDMs (Figure 3.9B).

To determine the identity and differentiation status of the bigger and more granulated Bmi1-/- macrophages FACS analysis were performed. The subset of Bmi1-/- macrophages which represented the bigger and more granulated cells (red gate) was analysed for the expression of the F4/80 and CD11b macrophage surface antigens. 97,53% of bigger and more granulated Bmi1-/- BMDMs were double positive for F4/80 and CD11b expression (Figure 3.9C) indicating that these cells with a changed morphlogy were macrophages and did not present an altered cellular identity.

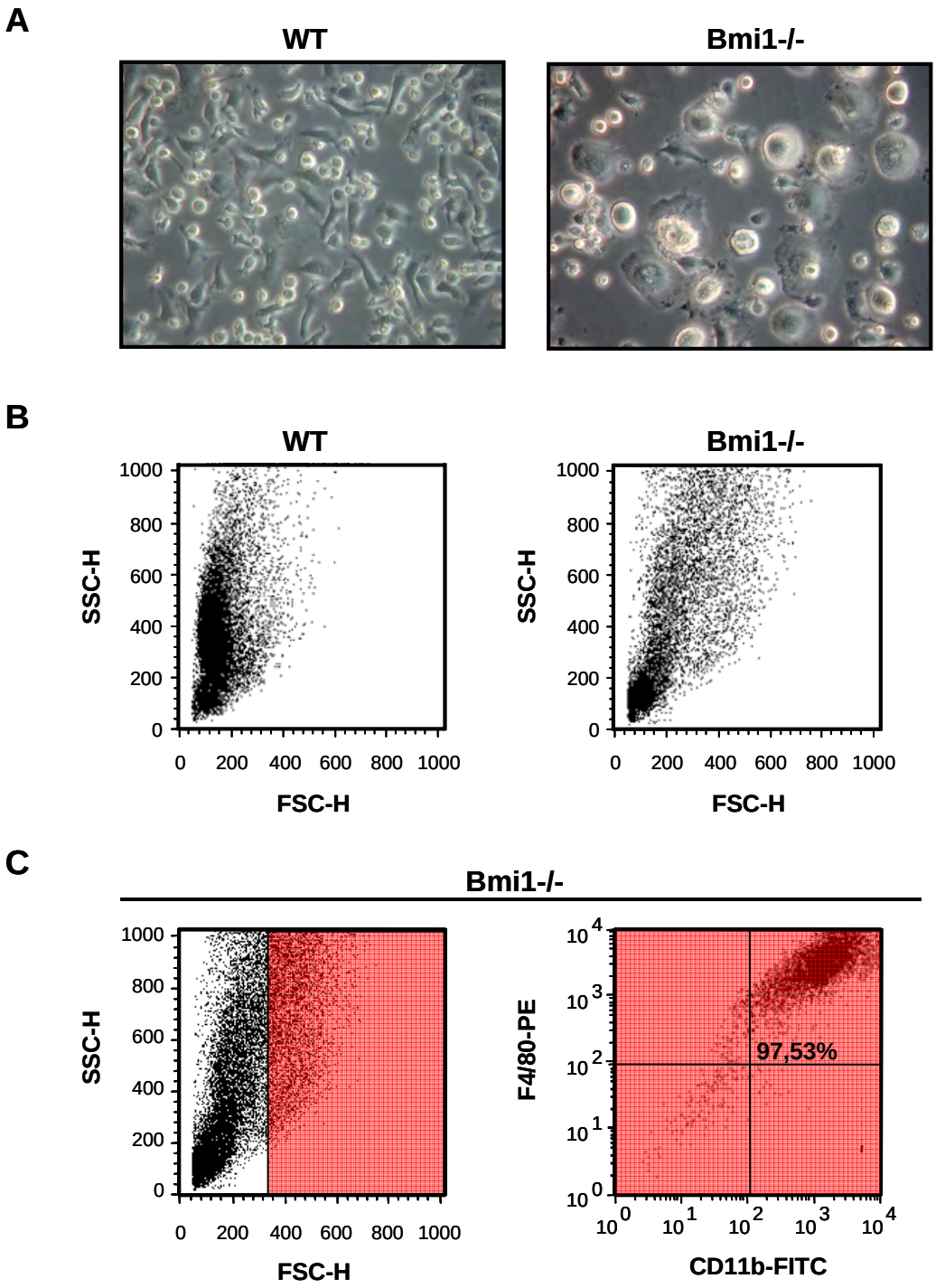

Figure 3.9 Bmi1-/- BMDMs are bigger in size but still display macrophage surface markers

(**A-B**) BMDMs were cultivated from WT and Bmi1-/- mice. (**A**) Representative inverted light microscope pictures are shown for WT and Bmi1-/- BMDMs. (**B**) Representative FSC and SSC dot plot of WT and Bmi1-/- BMDMs. (**C**) For further analysis the Bmi1-/- BMDMs were stained with CD11b-FITC and F4/80-PE conjugated antibodies and the red gate of the FSC and SSC dot plot representing the bigger and more granulated cells was taken to indentify the percentage of CD11b and F4/80 double positive cells.

FACS analysis further revealed that Bmi1-/- BMDMs expressed lower levels of MHC class II molecules (Figure 3.10). Lower expression of MHC class II molecules by macrophages is associated with aging (Herrero et al, 2002). This finding and the differences in size indicate that Bmi1-/- BMDMs have differences in their aging status (Adams, 2007).

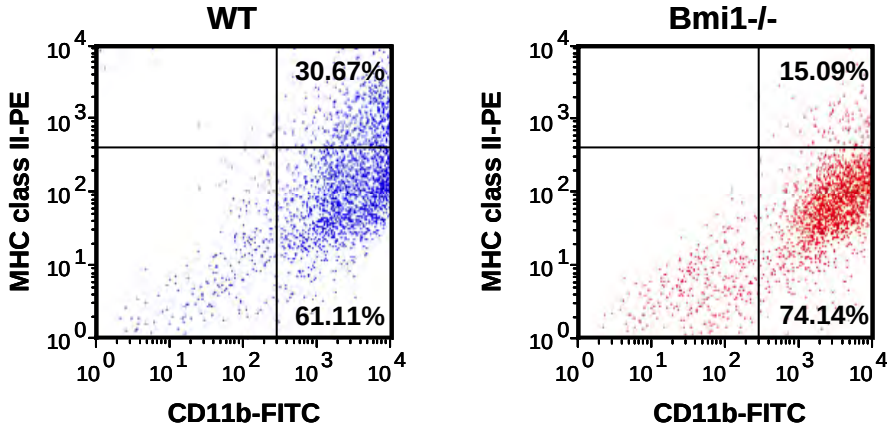

Figure 3.10 Bmi1-/- BMDMs express less MHC class II molecules

BMDMs were cultivated from WT and Bmi1-/- mice. FACS analysis of WT and Bmi1-/- BMDMs was performed with MHC class II and CD11b fluorochrome-conjugated antibody.

Large cell morphology is a cytological marker for cellular senescence (Adams, 2007). Senescence-associated β-galactosidase is widely used as a biomarker of senescence. Therefore β-galactosidase stainings were performed with WT and Bmi1-/- BMDMs. Bmi1-/- macrophages were positive for β-galactosidase staining (Figure 3.11A). Especially the bigger Bmi1-/- macrophages were β-galactosidase positive. But by far not all bigger Bmi1-/- BMDMs showed senescence-associated β-galactosidase staining. In contrast β-galactosidase positive WT BMDMs were not detectable (Figure 3.12A).

Bmi1 is known to repress the *ink4a/arf* locus which encodes the $p16^{Ink4a}$ and the $p19^{Arf}$ proteins. $p16^{Ink4a}$ is involved cell cycle arrest and cellular senescence (Bringold & Serrano, 2000; Jacobs et al, 1999). Western blot analysis revealed that Bmi1-/- BMDMs express higher $p16^{Ink4a}$ protein levels than WT BMDMs (Figure 3.11B).

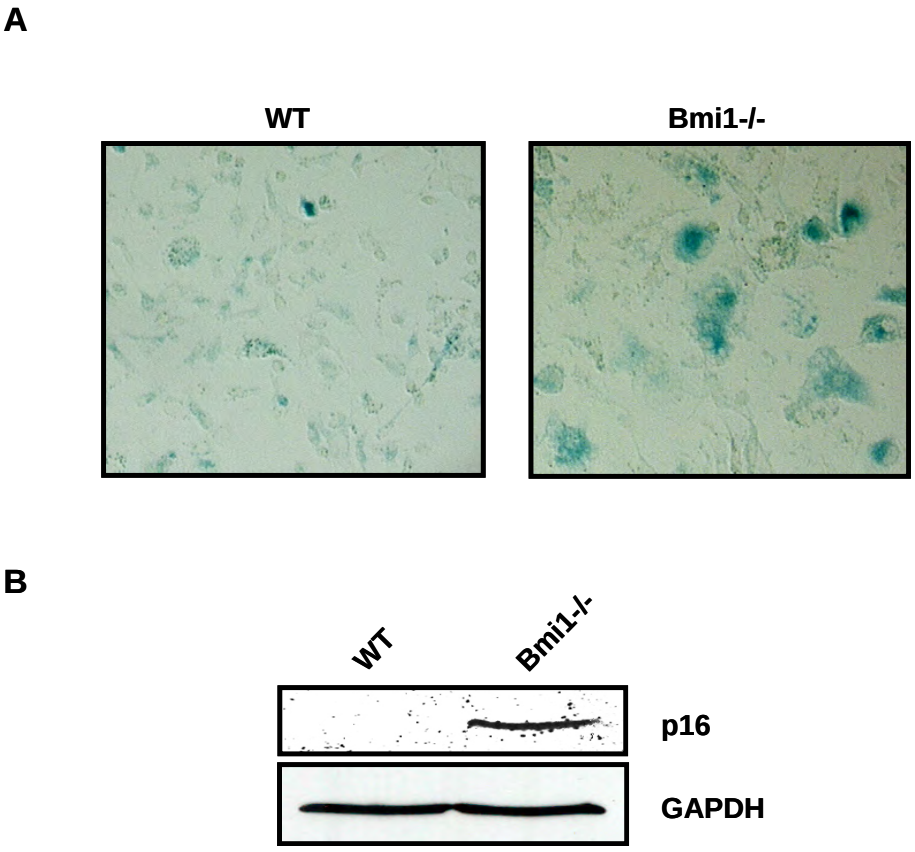

Figure 3.11 Bmi1-/- BMDMs display signs of senescence

(**A**) Senescence-associated β-galactosidase staining of WT and Bmi1-/- BMDMs was performed according the Material and Methods part. Senescent cells were identified as blue stained cells by inverted light microscopy. (**B**) WCLs of WT and Bmi1-/- BMDMs were immunoblotted with antibodies against $p16^{Ink4a}$ and GAPDH as loading control.

In conclusion Bmi1-/- BMDMs secreted more IL-10 in comparison to WT BMDMs in response to LPS. This increase in secretion resulted from a higher IL-10 mRNA expression. Addditionally Bmi1-/- BMDMs showed features of senescence.

3.7 Senescence independent regulation of IL-10 by Bmi1

Previous reports have shown an effect of aging/senescence on the cytokine expression profile of macrophages (Panda et al, 2009; Weiskopf et al, 2009). To analyze if the observed effect on IL-10 expression after Bmi1 abrogation is independent of senescence we decided to employ the murine J774A.1 macrophage cell line (Ralph et al, 1976; Ralph & Nakoinz, 1975; Ralph et al, 1975) as a further model system for Bmi1 function during macrophage activation. siRNA-mediated Bmi1 knock down led to a pronounced reduction of Bmi1 expression on protein level (Figure 3.12A). Previous results revealed an interdependence of Bmi1 expression and the PRC2 protein Ezh2 (Raaphorst et al, 2001). In contrast to these results no changes in Ezh2 protein levels were detectable after siRNA-mediated Bmi1 knock down (Figure 3.12A).

The transient knock down of Bmi1 in J774A.1 cells did not result in a in a senescent phenotype as evident by the lack of significant $p16^{Ink4a}$ mRNA expression (Figure 3.13C) and no altered morphology as revealed by FACS analysis (Figure 3.12D).

Although senescence was not observed after Bmi1 knock down J774A.1 macrophages showed significantly enhanced IL-10 mRNA expression after 1 hour and after 20 hours of LPS treatment (Figure 3.12B).These data confirm the involvement of Bmi1 in the acute repression of IL-10 during LPS mediated macrophage activation and is not a result of aging/senescence linked alterations in the cytokine expression profile of macrophages.

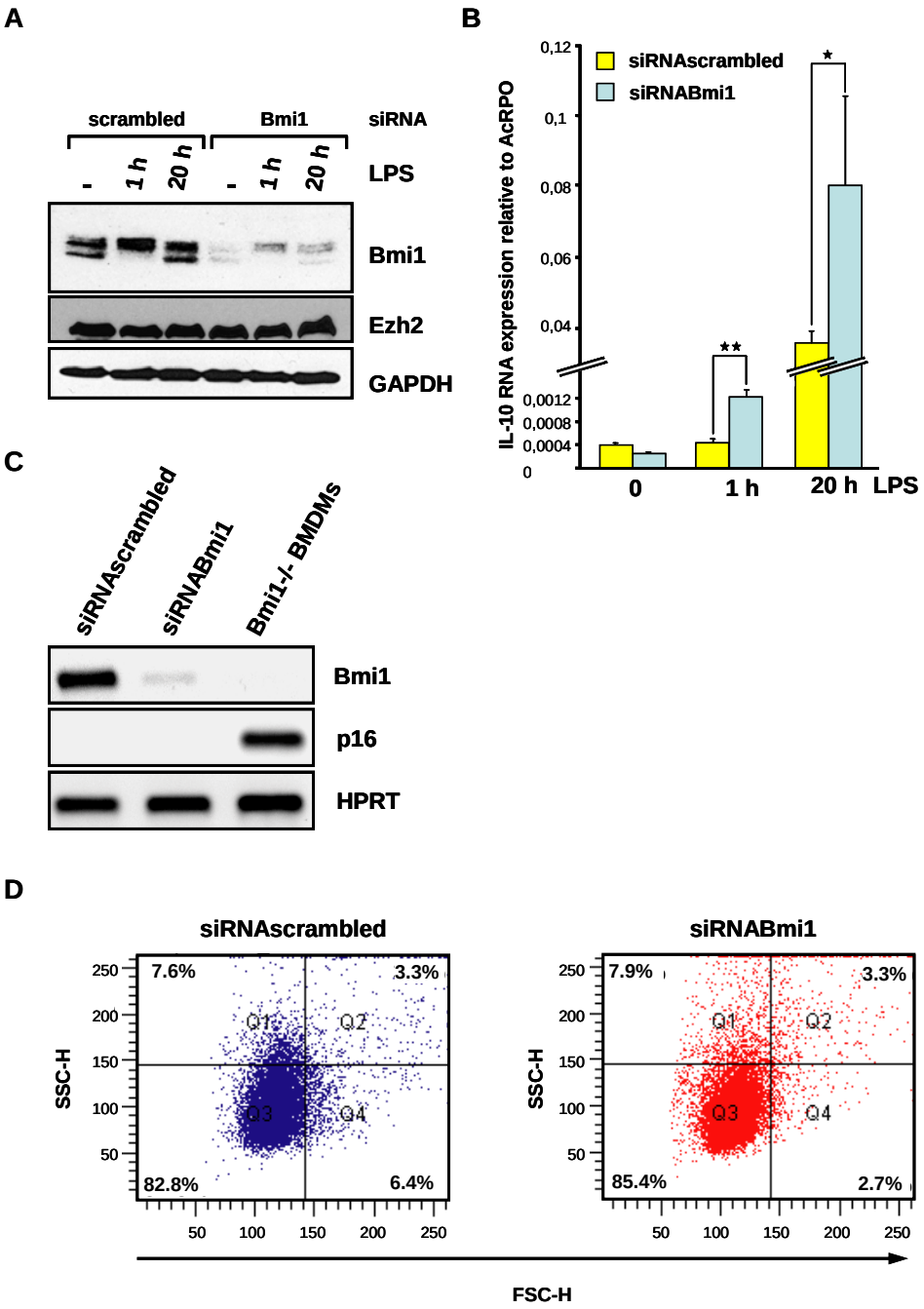

Figure 3.12 Bmi1 regulates the IL-10 mRNA expression in J774A.1 cells

(**A-D**) J774A.1 macrophages were transfected with scrambled as control or Bmi1 siRNAs respectively (**A**) The WCLs were obtained from cells treated with LPS for the indicated time periods. WCLs were immunoblotted with antibodies against Bmi1, Ezh2 and GAPDH. GAPDH was served as loading control. (**B**) Total RNA was isolated and the IL-10 mRNA levels were carried out by RT-qPCR analysis with primers specific for IL-10 and AcRP0. Expression levels of IL-10 mRNA were related to AcRPO mRNA levels. Asterisks indicate statistically significant differences (one $p<0,05$ and two $p<0,01$ paired Student`s t-test).(**C**) Total RNA was isolated from siRNA transfected J774 cells and from Bmi1-/- BMDMs. Semi quantitative RT-qPCR analysis was carried out with primers specific for Bmi1, $p16^{Ink4a}$ and HPRT as control. (**D**) FACS analysis of either scrambled or Bmi1 siRNA transfected J774 cells are presented as FSC and SSC dot plot.

3.8 Reduced IL-10 expression is correlated with increased Bmi1 protein levels in J774A.1 macrophages

In the course of our experiments and in agreement with previous reports (Jones & Flavell, 2005) J774A.1 macrophages express low IL-10 mRNA levels in response to LPS measured by RT-qPCR (Figure 3.13A).

Western blot analysis with WCLs obtained from equal cell numbers revealed that J774A.1 cells express higher Bmi1 protein levels than WT BMDMs irrespective of LPS treatment (Figure 3.13B). These data in combination with the above described Bmi1 abrogation and knock down studies are consistent with an inverse correlation of Bmi1 protein levels with *il-10* gene expression.

Our studies so far revealed that MAPK signaling is important for Bmi1 protein up-regulation. A possible explanation for the elevated Bmi1 protein levels in J774A.1 cells could be activated MAPK signaling transduction pathways in this cell line. To research this Western blot analysis of WCLs obtained from equal cell numbers of WT BMDMs and J774A.1 macrophages were performed. The analysis revealed that the mitogen-activated Ras-RAF-MEK-ERK signaling pathway and the JNK stress-induced phosphorylation cascades were indeed activated in J774A.1 cells (Figure 3.13C).

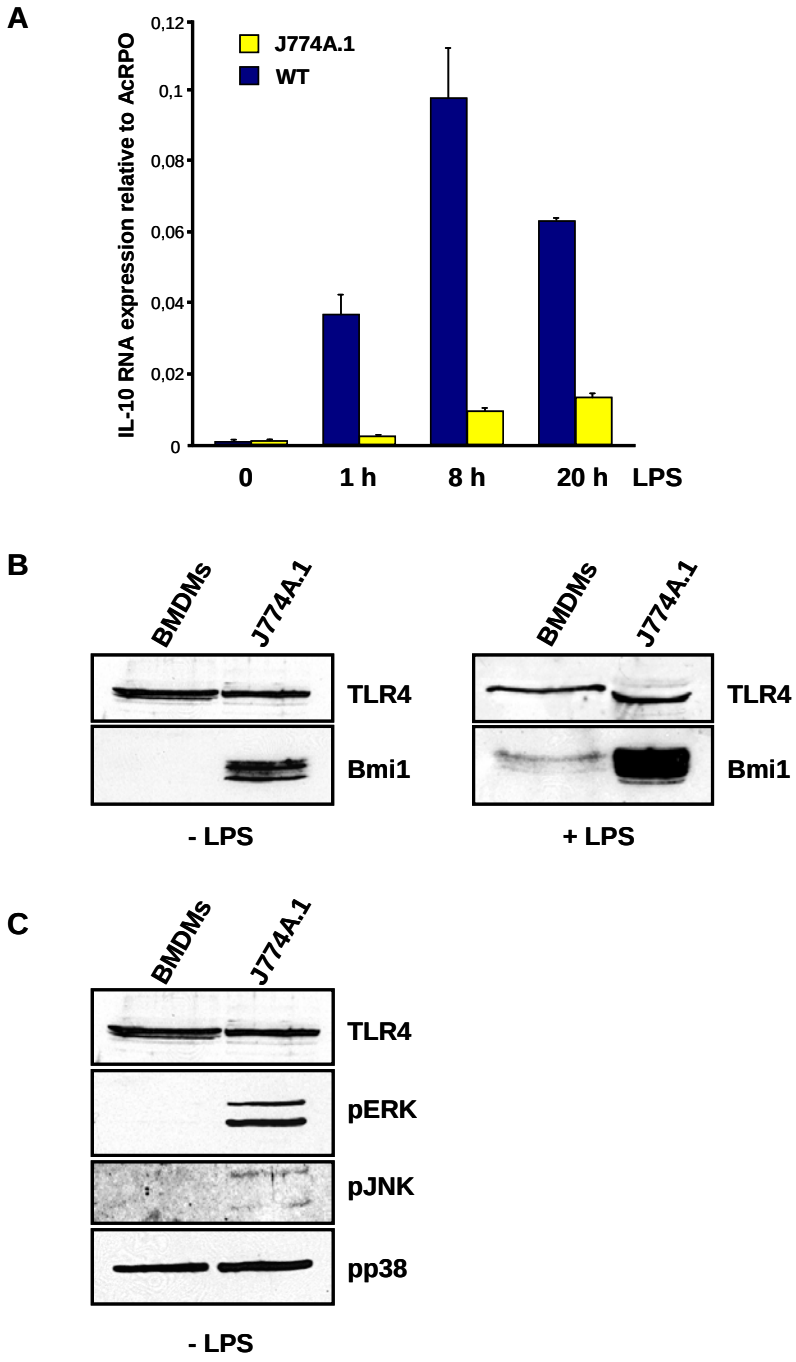

Figure 3.13 Reduced IL-10 expression in response to LPS treatment and elevated Bmi1 protein levels in J774A.1 macrophages

(**A**) IL-10 mRNA levels in WT BMDMs and in the murine macrophage cell line J774A.1 were analysed by RT-qPCR. The cells were treated with 100 ng/ml LPS at indicated time points. Total RNA was isolated and RT-qPCR analysis was carried out with primers specific for IL-10. IL-10 RNA levels were presented relative to AcRP0 mRNA levels. (**B**-**C**) WCLs prepared from equal cell numbers of WT BMDMs and J774A.1 macrophages were immunoblotted and protein levels were quantified with indicated antibodies. (**B**) Left panel: analysis of untreated cells. Right panel: analysis of cells that were treated with 100 ng/ml LPS for 30 min. (**C**) WCLs prepared from equal cell numbers of untreated WT BMDMs and J774A.1 macrophages were immunoblotted and protein levels were quantified with indicated antibodies.

3.9 Bmi1 chromatin interaction is independent of its phosphorylation stratus

Western blot analysis revealed similar Bmi1 phosphorylation kinetics in J774A.1 murine macrophages and WT BMDMs after LPS treatment (Figure. 3.1A and Figure 3.14A).

Previously published data reported that Bmi1 hyperphosphorylation is associated with subsequent chromatin dissociation (Voncken et al, 2005; Voncken et al, 1999). To address this issue J774A.1 macrophages were treated with LPS at different time periods and the obtained cells were lysed and subsequently incubated with shrimp alkaline phosphatase (SAP). SAP is recommended for the dephosphorylation of proteins (Khosravi et al, 1999). The upper LPS induced Bmi1 protein bands disappeared upon treatment with SAP (Figure 3.14B) indicating that phosphorylation is the main post-translational modification in macrophages after LPS activation.

In order to investigate the subcellular localization of Bmi1 in J774A.1 cells after LPS mediated phosphorylation we performed a centrifugation-based fractionation method (Wysocka et al, 2001). This experiment was performed with the kind help of Christian Scheuermann. As shown in Figure 3.14B, Bmi1 could only be detected in the chromatin bound fraction. Both hyperphosphorylated as well as hypophosphorylated forms of the Bmi1 protein were associated with the chromatin fraction (Figure 3.14B). These data suggest that LPS mediated Bmi1 protein phosphorylation does not interfere with the chromatin association in J774A.1 macrophages during activation.

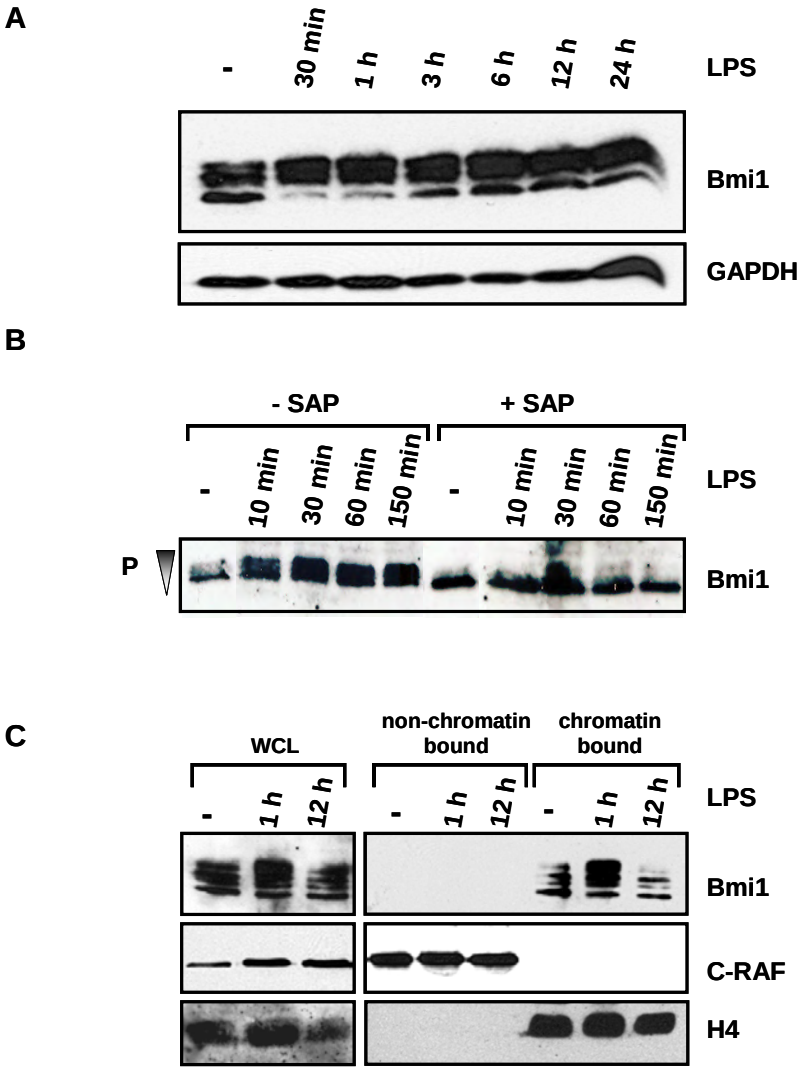

Figure 3.14 LPS mediated Bmi1 phosphorylation in J774A.1 cells and Bmi1 chromatin association independent of its phosphorylation status

(**A**) J774A.1 macrophages were treated with 100 ng/ml LPS for the indicated time periods. The obtained WCLs were immunoblotted with antibodies against Bmi1and GAPDH as loading control. (**B**) J774A.1 macrophages were treated with 100 ng/ml LPS for indicated time periods and were subsequently lysed and incubated with 10 U SAP for 1 hour at 37°C. The SAP treated and untreated control WCLs were immunoblotted with antibodies against Bmi1. (**C**) J774A.1 cells were treated with 100 ng/ml LPS for indicated time periods and were subjected to biochemical fractionation. Equal protein ratios of the fractions were probed by immunoblotting with Bmi1. The quality of the fractionation was assessed by probing with antibodies specific against C-RAF as non-chromatin bound control and H4 as chromatin bound control.

3.10 Bmi1 localizes at the *il-10* locus

As the results so far implicate Bmi1 in the transcriptional regulation of IL-10 we asked whether Bmi1 physically interacted with putative regulatory regions in the *il-10* locus. To this end chromatin immunoprecipitation (ChIP) analysis were performed. The selected primer pairs for DNA amplification are grouped in primers located at previously described hyper sensitive sites (HSS) within the murine *il-10* locus (Jones & Flavell, 2005) and primers that were randomly chosen within the murine *il-10* locus.

First J774A.1 cells were transfected with an expression construct (pcDNA3HA-Bmi1) encoding a HA-Bmi1 fusion protein and an empty expression vector (pcDNA3HA) as control. Western blot analysis of cell extracts obtained from pcDNA3HA-Bmi1 transfected cells revealed expression of the fusion protein (Figure 3.15B).

ChIP analysis of chromatin prepared from pcDNA3HA-Bmi1 and control vector (pcDNA3HA) transfected cells showed that HA-Bmi1 fusion protein localized to all sites within the il-10 locus monitored in this experiment (Figure 3.15C). The enrichment of Bmi1 at the known HSS upstream of the transcribed region (-17 kb) was stronger compared to randomly selected sites in this area. Bmi1 was also found to be enriched at the HSS inside the transcribed region of the *il-10* gene (+0,92 kb and +6,45 kb). Bmi1 did not interact with the *gapdh* gene (Figure 3.15C). Hence, there is a clear correlation between the repression of gene expression and the recruitment of Bmi1 to the *il-10* locus in J774A.1 cells.

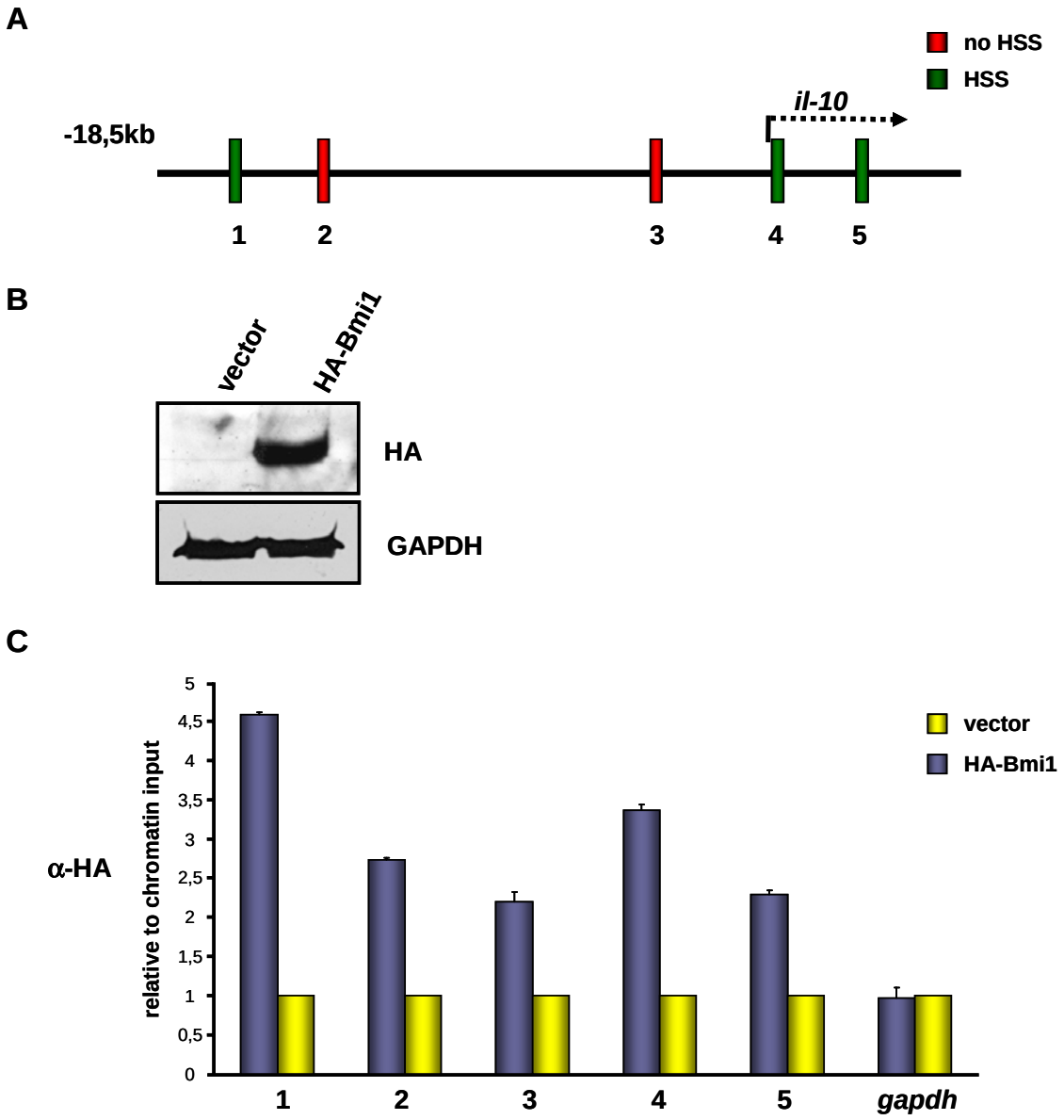

Figure 3.15 Bmi1 is located on the *il-10* locus

(**A**) Schematic representation of the *il-10* locus. Relative positions of the ChIP primer pairs are indicated and numbered 1-5 (green bars represent known hyper sensitive sites (HSS) of the *il-10* locus, red bars represent primer pairs chosen randomly). The exact primer coordinates are listed in the Material and Methods part. (**B**-**C**) J774A.1 cells were transfected either with empty vector (pcDNA3HA) or with pcDNA3HA-Bmi1 construct. (**B**) Bmi1 protein expression was determined by Western blot analysis. The obtained WCLs were immunoblotted with antibodies against HA and GAPDH as loading control. (**C**) RT-qPCR analysis of DNA precipitated with HA-specific antibodies using primers specific for the regions indicated by numbers (1-5) in panel A. DNA enrichment obtained with the corresponding primer pairs from cells transfected with the empty vector (pcDNA3HA) were set one and were served as control (yellow bars). The other enrichments are presented as relative to increase of the control and relative to the total chromatin input. With HA antibodies precipitated DNA for *gapdh* gene served as control.

3.11 Differential expression of Bmi1 in BMDMs after combined TLR4 and FcγR activation

Previous results have shown that LPS is not a potent stimulator of IL-10 in WT BMDMs. However, when this stimulus is combined with immune complexes, the two stimuli synergize to induce high levels of IL-10 (Sutterwala et al, 1998). To further analyse the role of Bmi1 in the regulation of IL-10 secretion, we took advantage of this model and stimulated WT and Bmi1-/- BMDMs with E-IgG (sheep red blood cells opsonized with rabbit sheep red blood cells stroma IgGs) alone or LPS alone or E-IgG in combination with LPS and quantified the IL-10 mRNA expression level by RT-qPCR (Figure 3.16A). WT BMDMs expressed significantly higher IL-10 mRNA levels after combined LPS/E-IgG stimulation as compared to LPS only treated WT BMDMs. The approximately two fold change in IL-10 mRNA expression was comparable to results obtained in studies with murine peritoneal macrophages (Polumuri et al, 2007). Bmi1-/- BMDMs express very high IL-10 mRNA level irrespective of LPS alone or combined LPS/E-IgG treatment. The combined E-IgG and LPS treatment did not induce further elevated IL-10 mRNA expression levels in Bmi1-/- BMDMs. WT as well as Bmi1-/- BMDMs reacted with a slight increase in IL-10 mRNA expression in response to E-IgG alone treatment. However, E-IgG alone treatment induced higher IL-10 mRNA expression in Bmi1-/- BMDMs compared to WT BMDMs (Figure 3.16A).

Next Western blot analysis were performed with protein extracts prepared from WT BMDMs after two hours of either LPS alone, E-IgG alone or combined LPS/E-IgG treatment. Up-regulation of Bmi1 protein levels were observed under all three conditions. However, LPS alone treatment induced higher Bmi1 protein levels then the combined LPS/E-IgG treatment. E-IgG alone treatment also induced low level of Bmi1 protein expression (Figure 3.16B).

As E-IgG alone was sufficient to induce Bmi1 we reasoned that the stimulus may not operate by simply blocking Bmi1 protein expression when applied in combination with LPS. We therefore speculated that E-IgG may alter the kinetics of LPS induced Bmi1 protein expression. To address this issue WT BMDMs were treated with LPS alone or E-IgG alone or LPS/E-IgG in combination for different time periods followed by Western blot analysis of Bmi1 protein levels. The results presented in Figure 3.16C show that 30 minutes of either LPS alone or combined LPS/E-IgG treatment induced comparable Bmi1 protein levels. In contrast 3 hours and 9 hours of combined LPS/E-IgG treatment yielded considerably reduced Bmi1 protein levels compared to LPS only induced Bmi1 protein levels. E-IgG treatment alone induced Bmi1 protein

expression at a constantly low level throughout the monitored time points. No differences in Ezh2 protein levels were detected irrespective of different treatment conditions and periods (Figure 3.17C).

In summary the combined LPS/E-IgG stimulus which induces elevated IL-10 levels led to an attenuation of Bmi1 expression. These results provide further support for a function of Bmi1 as a repressor of IL-10.

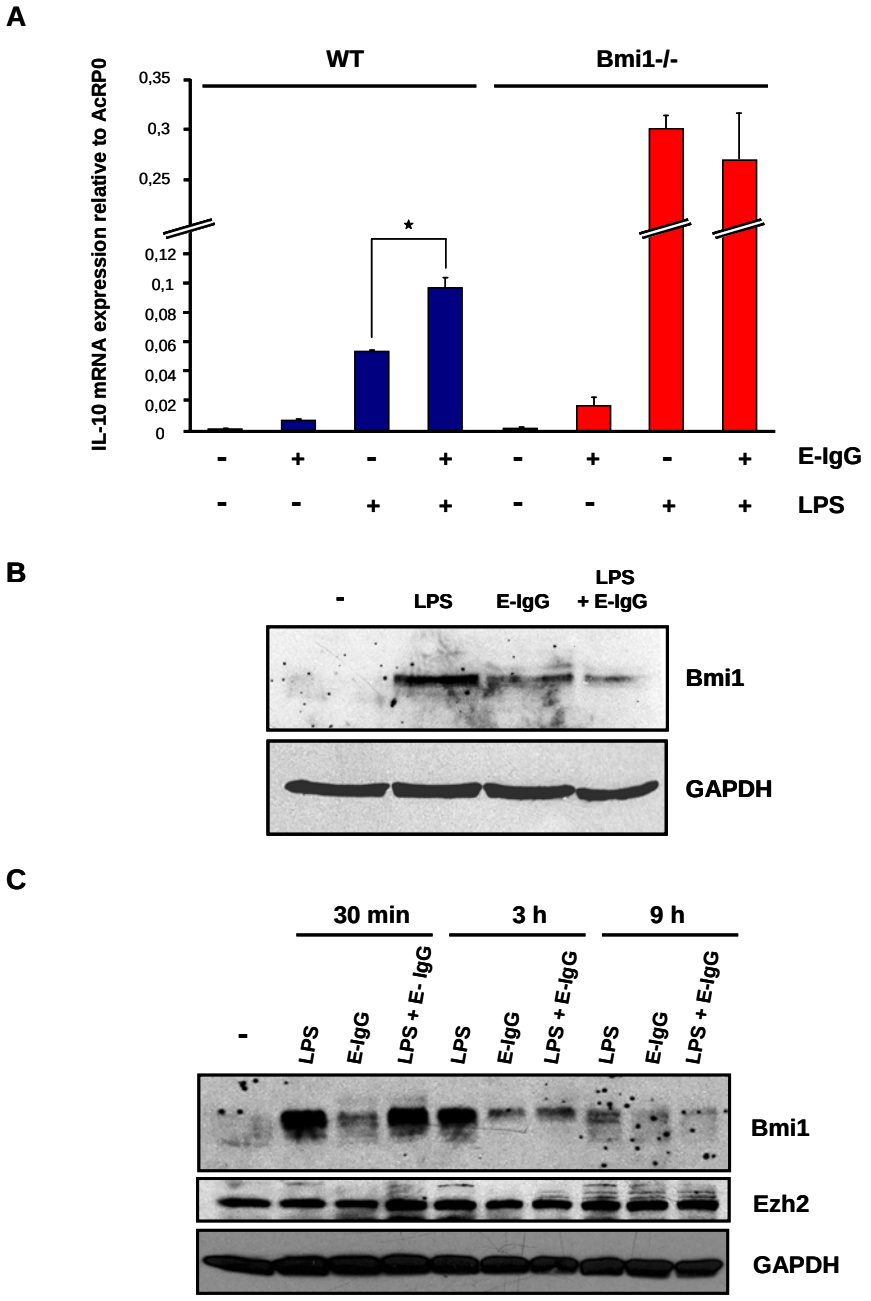

Figure 3.16 Enhanced LPS induced and FcγR ligation mediated IL-10 production is Bmi1 regulated

(**A-B**) BMDMs were exposed to either LPS or E-IgG (sheep red blood cells opsonized with rabbit sheep red blood cells stroma IgGs at subagglutination titers) alone or LPS in combination with E-IgG for 2 hours. (**A**) Total RNA

was isolated and RT-qPCR analysis was carried out with primers specific for IL-10. IL-10 RNA levels were presented relative to AcRP0 mRNA levels. Asterisks indicate statistically significant differences ($p<0,05$ paired Student`s t-test) (**B**) WCLs prepared from WT BMDMs treated with either LPS or E-IgG alone or LPS in combination with E-IgG for 2 hours were immunoblotted with antibodies against Bmi1 and GAPDH as loading control. (**C**) Time course of WT BMDMs treated with either LPS or E-IgG alone or LPS in combination with E-IgG for indicated time points. The obtained WCLs were immunoblotted with antibodies against Bmi1 and GAPDH as loading control.

4 Discussion

The work presented here shows a rapid LPS induced and stable expression of the PcG protein Bmi1 in BMDMs. This LPS mediated protein induction went along with a transient Bmi1 phosphorylation. The regulation of Bmi1 expression was not at the transcriptional level and was MAPK signaling dependent.

This study also shows that Bmi1 levels were crucial for the acute transcriptional repression of the anti-inflammatory cytokine IL-10 in response to LPS. BMDMs in the absence of Bmi1 showed a pronounced increase of IL-10 secretion after stimulation with LPS. This elevated secretion was associated with increased IL-10 mRNA levels. Additionally siRNA mediated knock down of Bmi1 in J774A.1 macrophages also resulted in elevated IL-10 mRNA levels in response to LPS. Conversely, activation conditions that favour IL-10 expression led to a more transient Bmi1 protein expression.

Collectively, these results show that the PcG protein Bmi1 plays a critical role in the regulation of the anti-inflammatory cytokine IL-10 during macrophage activation and provide a link between polycomb-mediated gene regulation and TLR-mediated cell signaling.

4.1 Bmi1 protein and phosphorylation levels in macrophages

PcG proteins are crucial factors for the establishment and maintenance of cellular identity. Up to now only little is known about how PcG activity is regulated in response to signals such as developmental cues. Recent reports revealed that PcG proteins such as Mel18 and Bmi1 are putative targets of various signaling cascades among them those that are constituted by MAPKs (Elderkin et al, 2007; Lee et al, 2005; Voncken et al, 2005). A further report showed the regulation of PcG genes on the transcriptional level in the early response to B-cell antigen receptor cross linking (Hasegawa et al.1998). Though the functional relevance of this observation is so far elusive this and the above mentioned examples point to a rapid function of PcG proteins in response to a broad range of stimuli. This work describes for the first time the rapid regulation of a PcG factor Bmi1 on the protein level in response to an extracellular stimulus. PcG proteins have to adapt polycomb target gene expression to cellular context, lineage commitment and differentiation status, revealing dynamic regulation of polycomb

function (Niessen et al, 2009). LPS, an outer membrane component of Gram-negative bacteria, triggered the rapid induction of Bmi1 on the protein level in bone marrow derived macrophages. LPS is one of the best-characterized agonist of host inflammatory signaling responses and is specifically recognized by the toll like receptor 4 (TLR4). A previous study reported that LPS mediated TLR signaling drove differentiation of myeloid progenitors to macrophages, bypassing some normal growth and differentiation requirements and thereby providing a rapid replenishment of effector cells of the innate immune system during infection (Nagai et al, 2006). The rapid induction of Bmi1, a member of the PcG protein complexes which are known to be involved in setting differentiation programmes (Bantignies & Cavalli, 2006), by LPS could provide a means of setting a stimulus dependent macrophage activation programme. This work focuses on the PcG protein Bmi1. However our studies did not show any differences in protein expression of Ezh2, a histone-lysine N-methyltransferase and a PRC2 protein responsible for setting the PcG specific histone H3K27 methylation mark, after LPS treatment. Further analyses are necessary to clarify whether other PcG proteins are also induced in BMDMs after LPS treatment.

TLR4 signaling involves a broad range of downstream effectors among them also MAPKs (Guha & Mackman, 2001). The experiments presented in this thesis shows that Bmi1 protein level regulation in response to LPS was downstream of MAPK signaling. One of the main functions of MAPK signaling is regulation of gene expression in response to extracellular stimuli mostly by either activating or repressing transcription factor activity (Chang & Karin, 2001). Indeed the MAPK JNK has been shown to affect PcG expression through the transcription factor AP1 during a process termed transdifferentiation in *Drosophila melanogaster* imaginal discs (Lee et al, 2005). The rapid nature of Bmi1 protein up-regulation in the absence of concomitant modulation mRNA levels after LPS treatment, however, suggests regulation at the protein stability or translational level. Therefore a mechanism targeting translational regulation through MAPK activated enhanced polysome assembly (Pyronnet et al, 1999) is a candidate mechanism for the observed effect on Bmi1 protein levels. Alternatively, Bmi1 levels could also be regulated similar to the previously described JNK and p38α mediated stabilization of the $p21^{Waf1/Cip1}$ protein (Kim et al, 2002). It is also known that phosphorylation can prevent ubiquitylation-mediated protein degradation by direct competition for modification of a single amino acid residue in a protein, or indirect by masking the recognition site for a second post-translational modification. This phenomenon is termed negative crosstalk (Hunter, 2007). As we observe extensive phosphorylation of Bmi1 in response to LPS treatment, negative crosstalk

could provide an additional explanation for the rapid increase of Bmi1 protein level in BMDMs after LPS mediated phosphorylation of Bmi1.

LPS induced Bmi1 protein expression was only observed in primary BMDMs but not in the murine macrophage J774A.1 cell line. J774A.1 cells expressed consistently higher Bmi1 protein levels then BMDMs independent of LPS treatment. The observation that some MAPK signaling pathways were constitutively activated in J774A.1 macrophages gives a potential explanation for high basal Bmi1 protein levels that can not be further elevated by extracellular stimuli. In accordance with these results, Voncken and colleagues (Voncken et al, 2005) reported a MAPK dependent phosphorylation of Bmi1 in the human osteosarcoma cell line U2OS without concomitant changes in Bmi1 expression levels. U2OS as well as J774A.1 cells are of tumor origin. A pronounced increase in Bmi1 protein level expression as compared to tissue of origin has previously been reported for various malignancies (Breuer et al, 2004; Dukers et al, 2004; Kang et al, 2007; Kim et al, 2004; Vonlanthen et al, 2001). Bmi1 has originally been identified as a cooperating oncogene (Haupt et al, 1991; van Lohuizen et al, 1991b) and tumor growth has been found to be critically dependent on Bmi1 expression (Becker et al, 2009). The pronounced expression of this protein in tumor cells is not surprising. A recent report has revealed high Bmi1 protein levels in tumors without concomitantly elevated Bmi1 mRNA levels (Hoffmann et al, 2007). High Bmi1 levels may therefore be a direct consequence of high basal MAPK signaling which is frequently observed in tumor cells (Dhillon et al, 2007). The exact molecular mechanism underlying LPS mediated Bmi1 induction is therefore of great interest not only in connection to the Bmi1 function in macrophages but also regarding possible therapeutic intervention strategies for cancer treatment.

Bmi1 has previously been identified as a repressor of the *ink4a/arf* locus (Jacobs et al, 1999). The $p19^{Arf}$ and $p16^{Ink4a}$ proteins encoded in this locus are involved in negative cell cycle regulation. We could not detect significant changes in $p19^{Arf}$ and $p16^{Ink4a}$ mRNA expression after the rapid and massive LPS-mediated Bmi1 protein induction in BMDMs. This observation is consistent with previous reports that identified $p21^{Waf1/Cip1}$ as mediator of cell cycle arrest in activated macrophages (Xaus et al, 1999). This finding argued for a cell cycle regulation independent function of Bmi1 in response to LPS.

We observed that Bmi1 induction went along with Bmi1 phosphorylation in BMDMs. Phosphorylation of Bmi1 has previously been shown to be inversely correlated with its chromatin association (Voncken et al, 1999) and results in the up-regulation of $p19^{Arf}$ in U2OS cells (Voncken et al, 2005). The Bmi1 chromatin disassociation could explain the absence of

changes in *ink4a* and *arf* gene expression in spite of the LPS-mediated Bmi1 induction in BMDMs.

Our study has demonstrated a rapid and transient Bmi1 phosphorylation with similar phosphorylation kinetics upon LPS stimulation in both WT BMDMs as well as in J774A.1 cell line macrophages. Many other different post-translational modifications like ubiquitylation, sumoylation and N-acetylglucosamineylation are described for PcG proteins (Niessen et al, 2009). It is known that phosphorylation regulates the localization of PcG proteins (Niessen et al, 2009). For example unmodified CBX2/M33, another PRC1 member, resides in the cytoplasm in mouse liver cells, whereas post-translational modified isoforms of CBX2/M33 localize to the nucleus (Noguchi et al, 2002). In contrast we detected Bmi1 only on chromatin independent of its phosphorylation status. Similar observations were made with Mel18. Different Mel18 protein phosphorylation levels did not affect chromatin association (Elderkin et al, 2007). Many phosphorylation sites of Bmi1 were found in mouse and human cells (Niessen et al, 2009), suggesting that regulation of Bmi1 chromatin association could depend on phosphorylation of different residues which are cell type and stimulus specific.

4.2 Role of Bmi1 in IL-10 regulation

Based on the following observations our results suggest that Bmi1 protein levels modulate IL-10 expression in activated macrophages. First IL-10 was expressed at high levels in Bmi1-/- BMDMs in response to LPS. Second siRNA mediated knock down of Bmi1 in J774A.1 macrophages resulted in elevated IL-10 levels in response to LPS. Third J774A.1 cells which express high Bmi1 protein levels express IL-10 at very low levels in response to LPS. And finally LPS stimulation in the presence of immune complexes which favours IL-10 expression in BMDMs led to attenuated Bmi1 expression at the protein level as compared to LPS treatment alone.

Previously it has been shown that PcG proteins are involved in the regulation of chemokines and cytokines as well as chemokine receptor expression during Th cell differentiation (Hosokawa et al, 2006; Koyanagi et al, 2005; Miyazaki et al, 2002). A more recent report suggests that PcG proteins, among them the Bmi1 homologue Mel18, differentially interact with the *il-4* and *ifnγ* loci in Th1 and Th2 cells. PcG proteins bind to these loci rapidly upon T cell stimulation (Jacob et al, 2008).

Timing as well as the relative amounts of pro-inflammatory and anti-inflammatory cytokine production are of critical importance for safe resolution of infection. Therefore, their expression must be tightly controlled and regulated. IL-10 is the best studied anti-inflammatory cytokine. Due to its immune regulatory function not only activation of different transcription factors as well as many changes at chromatin level at the *il-10* locus play a crucial role in IL-10 regulation. Several chromatin transitions have previously been identified based on DNase I hypersensitivity assays of the *il-10* locus (Jones & Flavell, 2005). Most DNase I hypersensitive sites (HSS) are conserved among various cell types of the immune system. Only one was identified as macrophage specific and its activation proved to be required for optimal *il-10* gene expression in LPS-stimulated macrophages (Saraiva et al, 2005). Our results clearly demonstrated that Bmi1 binds to throughout the *il-10* locus even in the transcribed region of the *il-10* gene with minor differences between known HSS and randomly chosen sites. Similar results were obtained in a study where Bracken and colleagues could show that Bmi1 binds throughout the *ink4a-arf* locus (Bracken et al, 2007). Changes in Bmi1 interaction with the *il-10* locus upon LPS treatment remain elusive. Nevertheless our results show chromatin association of Bmi1 irrespective of its LPS-mediated phosphorylation status suggesting a Bmi1 protein level depended IL-10 regulation.

Additionally, this work could show that the LPS mediated induction of IL-10 was completely regulated on transcriptional level in WT as well as in Bmi1-/- BMDMs. This may argue for a role of the transcriptional repressor Bmi1 in the direct regulation of IL-10 expression.

The work presented here shows a rapid up-regulation of Bmi1 in BMDMs after a pathogen derived stimulus. To gain deeper insight into the function of Bmi1 in macrophage activation BMDMs from WT and Bmi1-/- mice were compared. The most dramatic difference in cytokine expression between WT and Bmi1-/- BMDMs was observed for IL-10 secretion. IL-10 was expressed at higher levels by Bmi1-/- BMDMs in response to LPS treatment as in WT BMDMs. We also observed a slight reduction in the secretion of the pro-inflammatory cytokines IL-6, IL-12 and TNFα by Bmi1-/- BMDMs in response to LPS treatment. These cytokines are all known to be repressed following exposure of macrophages to IL-10 (Fiorentino et al, 1991; Mosser & Zhang, 2008). Therefore elevated IL-10 secretion by Bmi1-/- BMDMs could explain the observed differences in expression of pro-inflammatory cytokines.

The observed differences in the cytokine secretion spectrum of Bmi1-/- BMDMs may be a consequence of an altered differentiation potential that may arise as a consequence of lacking polycomb function in these cells. However, various assays did not reveal any differences in

cellular identity of analysed terminally differentiated BMDMs with and without abrogated Bmi1 expression: 1) We could not detect any discrepancy with respect to number of cells that express the macrophage surface markers CD11b and F4/80. 2) We could also not detect varieties in granulocyte and DC, both members of the myeloid differentiation lineage to which macrophages belong, surface marker expression. 3) No differences in bacterial uptake and phagocytosis were observed between WT and Bmi1-/- macrophages indicating that Bmi1 abrogation does not affect phagocytosis, a main macrophage function.

Nevertheless Bmi1-/- BMDMs were bigger in size and showed a higher granularity. These bigger and more granulated Bmi1-/-BMDMs expressed the CD11b and F4/80 macrophage markers indicating that they did not represent a population of aberrantly differentiated cells. Senescent cells adopt a large and flat morphology (Collado & Serrano, 2010). It has previously been shown that the polycomb group protein Bmi1 is a crucial factor for senescence through the repression of the *ink4a/arf* locus encoding the proteins $p16^{Ink4a}$ and $p19^{Arf}$ (Jacobs et al, 1999). In agreement with a senescent phenotype our results revealed that Bmi1-/- BMDMs express high levels of $p16^{Ink4a}$ protein and were positive for senescence-associated (SA) β-galactosidase staining. Both $p16^{Ink4a}$ as well as SA-β-galactosidase are accepted and widely used biomarker for senescence (Dimri et al, 1995; Krishnamurthy et al, 2004). Furthermore our results revealed that Bmi1-/- BMDMs showed senescence-associated features like reduced MHC class II molecule expression, G1 arrest and bigger nuclei (data not shown) (Campisi & d'Adda di Fagagna, 2007; Herrero et al, 2002; Mehta et al, 2007). In summary, Bmi1-/- BMDMs show senescent phenotypes. Senescence has been described as state or process of aging at the cellular level, and is thought to relate to age-related diseases (Campisi, 2005).

In agreement with a previous report senescence did not affect the expression of TLR4 on macrophages (Boehmer et al, 2005). Moreover the senescence phenotype of Bmi1-/- BMDMs and the absence of Bmi1 did not affect TLR4 signaling and the expression of downstream TLR4 effectors. In contrast to these results, previous reports have shown that senescence in macrophages is accompanied by a reduction in total levels of the MAPKs p38 and Jun N-terminal kinase (JNK) (Boehmer et al, 2004; Boehmer et al, 2005). The usage of peritoneal macrophages of aged mice could explain the discrepancy between our results and the results presented in the published study.

Cytokine production, as determined using *in vitro* murine models, is altered in senescent macrophages (Chelvarajan et al, 2005; Gomez et al, 2005; Kovacs et al, 2009). Therefore the senescence phenotype is potentially relevant with respect to the observed differences in the

cytokine secretion between Bmi1-/- BMDMs and their WT counterparts. To avoid senescence we employed the murine macrophage cell line J774A.1. siRNA mediated knock down of Bmi1 in J774A.1 macrophages resulted in significant elevated IL-10 mRNA expression in response to LPS, without causing a senescent phenotypes, most likely due to the short termed and incomplete abrogation of Bmi1 expression.

We and others (Jones & Flavell, 2005) could also show that J774A.1 macrophages express low IL-10 mRNA levels upon LPS treatment. Our results further revealed that J774A.1 cells express very high Bmi1 protein levels independent of their activation status. Taking together our results suggest that Bmi1 levels have an influence on IL-10 expression in both J774A.1 macrophages as well as BMDMs.

A previous report described conditions that trigger a regulatory macrophage phenotype which is characterized by secretion of high IL-10 levels (Sutterwala et al, 1998). In agreement with this report we observed high level of IL-10 expression in WT BMDMs after LPS treatment in the presence of immune complexes (E-IgG). Our IL-10 mRNA induction was similar to presented data obtained with peritoneal macrophages (Polumuri et al, 2007). It has been previously shown that this response to immune complexes occurs in macrophages taken from a variety of different species, including mice and human, from various anatomic locations, including the peritoneum, lung and blood (Anderson & Mosser, 2002; Polumuri et al, 2007). We observed that coupling LPS and E-IgG stimulation which induces high IL-10 expression in macrophages altered the expression kinetics of Bmi1 towards a more transient Bmi1 expression at the protein level as compared to LPS only induced Bmi1 protein expression in WT BMDMs. Furthermore LPS treatment in the presence of immune complexes was not able to elevate the IL-10 mRNA expression over the already high IL-10 mRNA expression levels induced by LPS alone in Bmi1-/- BMDMs. Taken together these results suggest that Bmi1 is involved in level dependent negative regulation of IL-10. Many reports have correlated cytokine production with MAPK activation (Akira & Takeda, 2004) and some of them have suggested that differential activation of the MAPKs may lead to differences in cytokine production (Mathur et al, 2004). Potentially faster ERK activation kinetics is linked with increased IL-10 expression (Lucas et al, 2005) and associated with rapid and evanescent phosphorylation of histone H3 at serine 10 (H3S10) along the IL-10 promoter (Zhang et al, 2006). So far it is unclear what was responsible for the altered expression kinetics of Bmi1 when BMDMs were activated in the presence LPS and E-IgG, but differential activation of the MAPKs may be involved in this process.

4.3 LPS induced chromatin remodeling

It was shown that histone H2A lysine 119 ubiquitylation is regulated over Mel18 phosphorylation at multiple serine residues revealing a post-translational modification dependent function of PcG proteins (Elderkin et al, 2007). The Bmi1 homolog Mel18 is another PRC1 member 70% identical to Bmi1 at the amino acid level. The crosstalk between different types of reversal post-translational modifications, like phosphorylation and ubiquitylation, is currently an emerging field (Hunter, 2007).

H2A lysine 119 ubiquitylation is also a target of LPS-induced signaling which triggers gene-specific decrease of H2A lysine 119 ubiquitylation and thereby hence gene induction (Zhou et al, 2008). LPS activation of TLR4 is of great importance for the host to clear a bacterial pathogen. LPS stimulation and associated chromatin structure remodeling has been established leading to modulation of the host transcriptional response (Hamon & Cossart, 2008; Medzhitov & Horng, 2009). Especially histone H3S10 phosphorylation, which is also involved in IL-10 regulation, is a main target of TLR4 activation. The importance of this post-translational histone modification becomes evident through the fact that many different bacteria interfere with components of the signaling pathways to alter the H3S10 phosphorylation status and thereby changing the host's transcriptional immune response programme. During a *Shigella flexneri* infection OspF, a phosphatase, specifically targeted the ERK and p38 MAPKs for dephosphorylation and therby preventing MAPK-dependent phosphorylation of H3S10 at the promoter of a specific subset of genes. (Arbibe et al, 2007). *Listeria monocytogenes* was also found to induce a dramatic decrease in the level of phosphorylated H3S10. The secreted virulence factor listeriolysin O was identified as a main effector and was sufficient for decreasing the level of modified histones (Hamon et al, 2007). These two examples are representative for a reduction of histone H3S10 phosphorylation. But *Helicobacter pylori* mediated TLR4 activation induced NF-κB, ERK and p38 MAPK signaling which correlated with phosphorylation of H3S10 at the IL-6 promoter. IL-6 is one of the cytokines that is responsible for *Helicobacter pylori* induced tissue invasion by macrophages (Pathak et al, 2006).

A recent report has shown that Jmjd3, a demethylase of the PcG specific H3 K27 methylation mark is regulated in a similarly dynamic fashion as Bmi1 in BMDMs in response to LPS. Jmjd3 controlled the H3 K27me3 levels in a variety of target genes during LPS activation (De Santa et al, 2007). Therefore Bmi1 and other PcG proteins could potentially affect a broader range of target genes during macrophage activation.

4.4 Conclusion and future perspectives

Macrophages are able to efficiently respond to stress, tissue damage or other homeostatic processes. Their remarkable plasticity allows them to specifically respond to different stimuli and change their phenotype and their physiology. Thereby sets of genes and functional programmes become active. Epigenetic mechanisms are fundamental for the control this functional programmes. In the presented study here we investigated how extracellular signals control the epigenetic regulator Bmi1 and its influence of gene expression profiles in macrophages via protein kinase cascades. The rapid expression kinetics, the differentially level of induction upon different stimuli and the implication in IL-10 regulation point towards an unique involvement of PcG protein Bmi1 as a regulatory switch which allows macrophages to efficiently respond to various environmental signals either with pro-inflammatory or anti-inflammatory activation programme.

IL-10 is known as a broadly immunosuppressive cytokine and it plays a central role in establishing a balance between pathology and protection. To treat inflammatory diseases such as severe forms of psoriasis, Crohn's disease, colitis or rheumatoid arthritis with the application of recombinant IL-10 were initiated more than a decade ago. But these clinical trails showed only modest clinical effects (Fedorak et al, 2000; Mosser & Zhang, 2008; Schreiber et al, 2000). Understanding IL-10 regulation has the potential to lead to the development of a novel class of anti-inflammatory compounds that work by modulating expression programmes inducing IL-10 production from macrophages.

On the other hand IL-10 intervention has been used in the area of anti-viral responses and vaccinations against viral infections by transiently blocking IL-10 (Brooks et al, 2008; Ejrnaes et al, 2006). Nevertheless, improved knowledge of mechanisms involved in macrophage and other immune cells gene expression programmes could provide therapeutic targets to inhibit the IL-10 production of these cells during viral and various other infections.

Furthermore IL-10 has been associated with tumor progression (Mosser & Zhang, 2008). High levels of IL-10 derive from tumor as well as host cells. Tumor-associated macrophages produce minimal amounts of the immunostimulatory cytokines but they constitutively produce relatively high levels of IL-10 (Martinez et al, 2008). Understanding of *il-10* gene regulation may allow us to manipulate tumor-associated macrophages to program them to preferentially produce pro-inflammatory cytokines rather than the anti-inflammatory cytokine IL-10. Blocking IL-10

secretion and thereby blocking the IL-10 effects can reverse and restore immune responsiveness and could provide a promising anti cancer therapy.

However there can be no doubt, that manipulating IL-10 secretion carries high risk due to the fine balance between immunopathology and immunosuppression. Further work on IL-10 regulation can provide means of therapeutic approaches by making small and transient alterations in the levels of IL-10 production or in the cellular location where it is produced and thereby enhance immune responses or prevente immunopathology.

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6 Appendix

6.1 Abbreviations

3pK	MAPK-activated protein kinase 3
AcRP0	Acidic ribosomal protein 0
ActD	actinomycin D
AP-1	activator protein 1
APC	antigen-presenting cell
ATP	adenosine-5'-triphosphate
BMDMs	bone marrow derived macrophages
Bmi1	B cell-specific Moloney murine leukaemia virus integration site 1
bp	base pairs
C/EBP	CCATT/enhancer-binding protein
cAMP	cyclic adenosine monophosphate
CARD	caspase recruitment domains
CD	cluster of differentiation
c-Maf	musculoaponeurotic fibrosarcoma oncogene homolog (avian)
CRD	cysteine rich domain
CREB	cAMP response element binding
DBD	DNA-binding domain
DC	dendritic cell
DMEM	dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Drosophila	*Drosophila melanogaster*
dsRNA	double-stranded RNA
DTT	dithiothreitol
E(z)	Enhancer of zeste
E. coli	*Escherichia coli*
ECL	enhanced chemoluminiscence
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor

ER	estrogene receptor
ERK	extracellular signal-regulated kinase
et al.	et alii
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDA	food and drug administration
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
Grb2	growth factor receptor-bound protein 2
HOX genes	homeobox containing genes
HRP	horseradish peroxidise
HPRT	hypoxanthine phosphoribosyltransferase 1
HSS	hypersensitive site
HSC	haematopoietic stem cell
IAP	inhibitor of apoptosis protein
Ig	immunoglobulin
IKKs	IκB kinases
IL	interleukin
IL-10	interleukin-10
INF	interferon
IRAK4, IRAK1	IL-1R-associated kinase 4, IL-1R-associated kinase 1
IRF	IFN regulatory factor
IRF	interferon regulatory facto
ISRE	IFN-stimulated response element
IκB	inhibitor of NF-κB
JAK	Janus kinase
JNK	jun N-terminal kinase
kDa	kilo Dalton
LF	lethal factor
LPS	lipopolysaccharide
LRR motif	leucin-rich repeat motif
LT	lethal toxin
MAL	MyD88-adaptor-like protein
MAPK	mitogen-activated protein kinase

M-CSF	macrophage colony stimulating factor
MEF	mouse embryonic fibroblast
MEK	mitogen-activated protein kinase kinase
MEKK	MAPK/ERK activating kinase kinase
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88, myeloid differentiation primary-response protein 88
NOD	nucleotide-binding oligomerization domain
NOD-LRR proteins	nucleotide binding oligomerization domain-like receptors, NLR
NOS	nitrogen species
NSCLC	non-small cell lung cancer
OD	optical density
ORF	open reading frame
PA	protective antigen
PAK	P21 activated kinase
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
Pbx	pre-B-cell leukemia transcription factor
PC12	rat pheochromocytoma
PcG	polycomb group proteins
PCR	polymerase chain reaction
PE	phycoerythrin
pH	potentia hydrogenii
PI3K	phosphatidyl inositide 3-kinase
PKA	protein kinase A
PKB (AKT)	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylflourid
PP1, 2A	protein phosphatase1, 2A
PPRs	pattern-recognition receptors
PRC	polycomb repressor complex

PRE	PcG response elements
Prep-1	Pbx-regulating protein 1
RAF	rapidly growing fibrosarcoma
RBD	Ras binding domain
RHD	Rel homology domain
RHIM	RIP homotypic interaction motif
RIG-like receptors	retinoid induced gene-I-like receptors, RLR
RIP1	receptor interacting protein 1
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
Rsk	Ribosomal S6 kinase
RT	Room temperature
RT-qPCR	reverse transcription quantitative real time PCR
RTK	receptor tyrosine kinase
SAP	shrimp alkaline phosphorylase
SAPK	stress-activated protein kinases
SA-β-galactosidase	senescence associated -β-galactosidase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET domain	Su(var)3-9, Enhancer-of-zeste, Trithorax domain
siRNA	small interfering RNA
SOS	son of sevenless
Sp1	specificity protein 1
ssDNA	single stranded DNA
STAT	signal transducers and activator of transcription
Su(z)2	Suppressor-2 of Zeste
T cell	thymus cell
TAB1/TAB2/TAB3	TAK1-binding proteins
TAK1	transforming growth factor-b(TGF-b)-activated kinase
TCR	T cell receptor
Th	T helper cell

TICAM1	TIR-domain-containing molecule 1, also known as TRIF
TICAM2	TIR-domain-containing molecule 2, also known as TRAM
TIR domain	Toll/IL-1 receptor domain
TIRAP	TIR domain containing adaptor protein, also known as MAL
TLR	Toll-like receptors
TNF	tumor-necrosis factor
TRAF	Tumor-necrosis factor (TNF)-receptor-associated factor
TRAM	TRIF-related adaptor molecule, also known as TICAM2
TRIF	TIR domain-containing adapter inducing IFNβ, also known as TICAM1
trxG	Trithorax group
Tyk2	tyrosine kinase 2
UTR	untranslated region
V	Volt
WT	wild type

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