The complex role of the immunoproteasome in *E. coli*-induced immune responses in macrophages

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II List of Abbreviations

AKT / PKB	RAC-alpha serine/threonine-protein kinase / Protein kinase B
APC	Antigen-presenting cell
BMC	Bone marrow cells
BMDM	Bone marrow derived macrophages
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CFU	Colony forming units
DMEM	Dulbecco's Modified Eagle's medium
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
E. coli	*Escherichia coli*
F4/80 / EMR-1	EGF-like module-containing mucin-like hormone receptor-like 1
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
gMFI	geometric mean of fluorescence intensity
GTP / GDP	Guanosine triphosphate / Guanosine diphosphate
IFNγ	Interferon gamma
IκB	Inhibitor of NfκB
IL	Interleukine
iNOS	Inducible nitric oxide synthase
IP	Immunoproteasome
ITAM	Immunoreceptor tyrosine-based activation motif
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LCCM	conditioned media produced by CCL-1 mouse fibroblast cell line
LDH	Lactate dehydrogenase

LMP2	Low-molecular-mass polypeptide 2
LMP7	Low-molecular-mass polypeptide 7
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MECL-1	Multicatalytic endopeptidase complex subunit
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
MYD88	Myeloid differentiation primary response gene 88
M-CSF	Macrophage colony-stimulating factor
NfκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PA28	Proteasome activator
PBS	Phosphate buffered saline
PD-L1	Programmed cell-death ligand 1
POMP	Proteasome maturation protein
ROS	Reactive oxygen species
RT	Room temperature
S	Svedberg unit
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SSC	Sideward scatter
STAT	Signal transducer and activator of transcription
TAP1	Transporter associated with antigen processing 1
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNFα	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β

III Summary

The immunoproteasome (IP) is a multimeric protein complex with proteolytic activity which is upregulated under inflammatory conditions to maintain cellular protein homeostasis. Apart from its role in protein homeostasis a number of studies indicate that the IP is involved in the regulation of anti-viral immune responses. However, very little is known about the role of the IP in innate host immune responses to bacterial infections. To close this gap of knowledge the present thesis aimed to investigate the impact of IP activity on antibacterial macrophage effector functions using primary murine IP-deficient macrophages and relevant bacterial triggers, including LPS and *E. coli*.

The results of the present thesis reveal that the immunoproteasome is involved in a number of distinct macrophage effector functions. The secretion pattern of crucial macrophage cytokines / chemokines including IFNγ, IL-1β, IL-6 and CCL4 in response to bacterial triggers is altered in IP-deficient macrophages. Importantly, the findings of unchanged gene induction as well as early proximal TLR4 signaling clearly indicate that the immunoproteasome modulates cytokine / chemokine production on post-transcriptional levels, rather than on transcriptional level. Moreover, the study shows that phagocytosis of *E. coli* is not regulated by immunoproteasome activity. However, the extracellular bacterial killing of macrophages might be impaired since the thesis reveals that the release of nitric oxide upon exposure to bacterial triggers is severely impaired in an IP-deficient background. By studying stress-induced cell death as well as intracellular accumulation of reactive oxygen species, the present thesis further shows that IP-deficient macrophages are more susceptible to pathogen-induced cellular stress.

In addition to its role in innate macrophage immune responses, the data of this thesis indicate that the immunoproteasome modulates the ability of macrophages to regulate adaptive T-cell immunity. Surface upregulation of MHC class I and the T-cell co-stimulatory molecule CD86 is impaired in IP-deficient macrophages with potential implications to macrophage-mediated adaptive T-cell activation during bacterial infections.

In conclusion, the data presented here convincingly show that the immunoproteasome plays a pivotal role in innate macrophage effector functions in bacterial infections and further provides evidence that the immunoproteasome is involved in the induction of adaptive T-cell immunity.

IV Zusammenfassung

Das Immunoproteasom (IP) ist ein multiproteolytischer Proteinkomplex, der während einer Infektion gebildet wird um die Proteinhomöostase der Zelle aufrechtzuerhalten. Bisherige Studien haben die Bedeutung des IPs in viralen Infektionen erforscht. Hingegen ist bisher wenig bekannt über die Rolle des IPs in der antibakteriellen Immunantwort. Die vorliegende Dissertation untersuchte erstmals die Rolle des Immunoproteasoms in Makrophagen bei einer bakteriellen Infektion. Um ein umfassendes Verständnis zu gewinnen, wie das IP wichtige Makrophagen-Effektorfunktionen moduliert, wurden primäre murine IP-K.O. Makrophagen sowie relevante bakterielle Stimuli, wie LPS und *E. coli*, verwendet. Die Ergebnisse der Arbeit zeigen, dass das IP an zahlreichen fundamentalen Makrophagen-Effektorfunktionen beteiligt ist. Als Antwort auf bakterielle Reize weisen IP-K.O. Makrophagen ein verändertes Sekretionsmuster wichtiger Zytokine / Chemokine, wie z. B. IFNγ, IL-1β, IL-6 und CCL4 auf. In diesem Zusammenhang zeigen gleiche Zytokin- / Chemokin-Genexpressionsmuster sowie eine unveränderte proximale TLR4-Signaltransduktion, dass das IP die Zytokin- / Chemokin-Antwort ausschließlich auf post-transkriptionaler Ebene reguliert. Darüber hinaus belegen die Daten der Studie, dass die Phagozytose von Bakterien nicht vom IP reguliert wird. Hingegen könnte das extrazelluläre Abtöten von Pathogenen gestört sein, da IP-defiziente Makrophagen eine reduzierte Sekretion von Stickstoffmonoxid nach Stimulation mit LPS und *E. coli* aufweisen. Aufgrund erhöhter Zelltodraten sowie intrazellulärer Akkumulation von reaktiven Sauerstoffspezies in IP-K.O. Makrophagen, als Antwort auf bakterielle Reize, kommt die Dissertation weiterhin zu dem Schluss, dass das Fehlen der IP-Aktivität zu einer verminderten Stresstoleranz von Makrophagen bei Bakterieninfektionen führt. Zusätzlich zu der gezeigten Bedeutung bei der angeborenen antibakteriellen Immunantwort weisen die Daten darauf hin, dass das IP die Fähigkeit von Makrophagen reguliert, die adaptive T-Zell-Antwort zu aktivieren. Die Oberflächenexpression von MHC-I und die des T-Zell kostimulatorischen Moleküls CD86 ist in IP-K.O. Makrophagen reduziert, was zu einer gestörten Makrophagen-vermittelnden T-Zell-Aktivierung bei bakteriellen Infektionen führen könnte. Zusammenfassend zeigt die vorliegende Dissertation, dass das IP eine wichtige Rolle bei der Regulation von angeborenen und adaptiven Makrophagen-Effektorfunktionen während bakterieller Infektionen spielt.

1. Introduction

1.1 Innate and adaptive immune responses

The immune system of higher organisms consists of a complex network of tissues, cells and molecules that fight potentially hazardous foreign pathogens as well as eliminate transformed endogenous cells, such as cancer cells or virus-infected cells. A proper immune system of an organism is a prerequisite to prevent disease and thus, ensure survival. The immune system of an organism can be sub-classified into the adaptive and the innate immune system although this classification is not mutually exclusive (Kennedy 2010).

1.1.1 Innate immune system

The innate immune system recognizes virtually all foreign pathogen antigens and is activated immediately or within hours after infection by specific and non-specific defense mechanisms. The non-specific defense mechanisms include physical barriers, such as the skin and mucous membranes. In addition, the specific defense mechanisms involve immune cells, such as phagocytes and mast cells. With the help of distinct receptors they recognize antigen structures of invading pathogens ultimately inducing the host response against the invading pathogens including pathogen clearance as well as recruitment and activation of other immune cells of the innate and adaptive immune system. (Kennedy 2010)

1.1.2 Adaptive immune system

Similar to the innate immune system, the adaptive immune system consists of highly specialized immune cells, such as B- and T-lymphocytes, that recognize foreign pathogen antigens by specific receptor complexes. In contrast to innate immune responses, the antigen recognition by T- and B-cells requires antigen presentation by antigen-presenting-cells (APCs), including dendritic cells or macrophages. Importantly, by genetic recombination of antigen receptor gene segments the adaptive immune system is highly

diverse generating lymphocytes that recognize one particular antigen. Moreover, by generation of long-living memory T- and B-cells in infections, a long-lasting immunity is acquired by the organism against the particular pathogen. (Kennedy 2010)

1.2 Macrophages

1.2.1 Macrophages – cellular components of the immune system

Macrophages represent a group of immune cells which are found in virtually all tissues within the organism including blood, lymph nodes and peritoneal tissues. Depending on the location and tissue function, macrophages exhibit a wide functional range and heterogeneity. Macrophages originate from bone marrow hematopoietic stem cells which continuously differentiate into monocyte progenitor cells in presence of macrophage colony-stimulating factor (M-CSF). These generated monocytes are released from the bone marrow into the bloodstream. Upon tissue damage and / or infection circulating monocytes are rapidly recruited to the affected tissue where they finally differentiate into macrophages that exert a broad repertoire of effector functions in response to the respective immunological trigger (Mantovani et al. 2004, Yang et al. 2014).

1.2.2 Macrophages – bridge between the adaptive and innate immune system

Macrophages are crucial players of the innate immune system since they are part of the first line of defense against invading pathogens. Recognition of pathogens by certain receptors on the macrophage surface leads to activation of signaling pathways ultimately resulting in the production of immunomodulatory mediators, such as cytokines and chemokines. Through the production of immunomodulatory mediators, macrophages are capable to orchestrate effector immune responses and recruit other immune cells of the innate and adaptive immune system to the site of infection (Arango Duque and Descoteaux 2014). In addition, macrophages are able to distinguish cellular antigens between self and non-self thereby preventing auto-immune activity. Upon phagocytosis of invading pathogens or foreign particles, non-self-antigens and pathogen proteins are processed into small peptides that are subsequently presented on the cell surface of macrophages to

activate cells of the adaptive immune system, e. g. T-cells and B-cells (Flannagan et al. 2012). Activated T-cells fight invading pathogens by cytotoxic activity or activation of B-cells to produce antibodies. In addition, both T- and B-cells contribute to long time immunity against the respective invading pathogen by generation of persistent memory cells that are rapidly activated by reencounter with the infectious trigger (Hwang and Actor 2001).

1.2.3 Pathogen recognition by macrophages

Macrophages recognize invading pathogens by their *pathogen-associated molecular patterns* (PAMPS), e. g. microbial proteins, lipids and nucleic acids as well as cell wall components, such as *lipopolysaccharide* (LPS). PAMPS are recognized by *pattern recognition receptors* (PRRs) on the surface of macrophages and other cells of the innate immune system (Takeuchi and Akira 2010). The most prominent PRRs are *Toll-like receptors* (TLRs), surface receptors that recognize molecules derived from pathogens. In particular, the TLR4 receptor complex possesses a key function in innate immunity since it recognizes LPS, a ubiquitous cell wall component of many gram-negative bacteria (Akira et al. 2001). Additionally, macrophages harbor the *mannose receptor*, the *dectin-1 receptor*, the *scavenger receptor A* and *opsonin receptors* on their surface (Flannagan et al. 2012). After receptor-mediated recognition of PAMPS, several distinct signaling pathways are induced in macrophages to exert a set of various effector functions to fight invading pathogens.

1.3 Macrophage effector functions

1.3.1 Overview of macrophage effector functions

The present thesis focuses on the role of the immunoproteasome in the modulation of innate and adaptive macrophage effector functions during bacterial infections.

To effectively fight infections and orchestrate innate and adaptive immune responses macrophages possess a wide range of effector functions (Fig. 1).

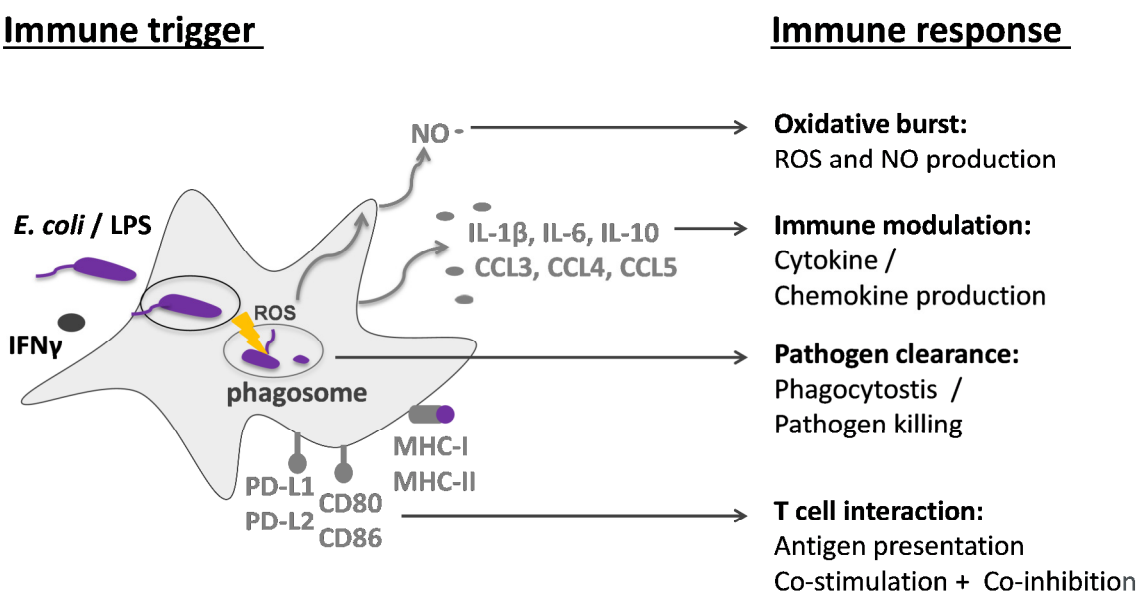

Figure 1. Schematic overview of macrophage effector functions
Upon immunological triggers, i. e. *E. coli* or IFNγ macrophages respond with production of an broad spectrum of immune modulatory molecules including reactive oxygen species (ROS), nitric oxide (NO) and cytokines / chemokines. Furthermore, macrophages engulf invading pathogens and present processed pathogen proteins on the cell surface to activate T-cells. In addition to antigen presentation, macrophages express co-stimulatory i. e. CD80 and CD86 as well as co-inhibitory surface molecules, such as PD-L1 to modulate T-cell responses.

It is important to note that all macrophage effector functions are not isolated processes but rather are interconnected and exerted simultaneously. The nature of the used repertoire of macrophage effector functions is based on the type of infection, e. g. viral or bacterial, site of infection and other conditions. In addition to the effector functions described here, macrophages possess other effector functions which are not further discussed. The introduction focuses on the effector functions which were investigated in the context of this study.

1.3.2 Cytokine and chemokine production

Cytokines and chemokines are immunomodulatory agents produced by macrophages and other immune cells. By orchestrating immune processes and linking innate with adaptive immunity, cytokines and chemokines play an important role in the regulation of effective immune responses.

Cytokines

Cytokines are group of small soluble signaling glycoproteins. Upon exposure to immunological triggers macrophages produce a broad spectrum of pro- and anti-inflammatory cytokines leading to enhancement or suppression of the immune response by activation or inhibition of other immune cells.

The most prominent pro-inflammatory cytokines which are produced during the early immune response by macrophages are *tumor necrosis factor alpha* (TNFα) and *interleukin-1beta* (IL-1β). TNFα is secreted by macrophages in order to regulate the release of neutrophil-attracting chemokines, such as CXCL1, CXCL2 and CXCL5 (Griffin et al. 2012). Furthermore, TNFα is involved in macrophage-mediated phagocytosis by expanding the membrane facilitate the engulfment of pathogens (Murray et al. 2005).

Similar to TNFα, the main function of IL-1β is the recruitment of granulocytes (Arango Duque and Descoteaux 2014). Furthermore, IL-1β directly enhances the expansion and differentiation of T-cells (Ben-Sasson et al. 2009).

Another macrophage-produced cytokine is *interleukin-6* (IL-6), that acts as a pleiotropic cytokine with pro- and anti-inflammatory functions. In the context of macrophage immune responses IL-6 promotes differentiation of B-cells into plasma cells and activates cytotoxic T-cells (Arango Duque and Descoteaux 2014).

A prominent example for an anti-inflammatory cytokine produced by macrophages and other immune cells is *interleukin-10* (IL-10). IL-10 suppresses macrophage activation and production of TNFα, IL-1β, IL-6, IL-8, IL-12 and M-CSF (Fiorentino et al. 1991). Furthermore, IL-10 acts in an autocrine fashion suppressing the anti-microbial activity and diminishing the capacity of macrophages to respond to IFNγ (Oswald et al. 1992, Cunha et al. 1992). Additionally, IL-10 suppresses *major histocompatibility complex* (MHC) *class II* expression in activated macrophages thereby preventing an overwhelming immune response of the organism (Chadban et al. 1998).

Chemokines

Chemokines are a specific group of cytokines that act as chemoattractants guiding cellular migration to inflammatory sites during infection in a process known as chemotaxis. Inflammatory chemokines are released upon exposure to immunological triggers, such as pathogens or pro-inflammatory cytokines (Arango Duque and Descoteaux 2014).

The main chemokines which are released by macrophages are CCL3, CCL4 and CCL5 (CCL: *chemokine (C-C motif) ligand*) (Mantovani et al. 2004). The chemokines CCL3 and CCL4 also known as *macrophage inflammatory protein-1α* and *β* (MIP-1α and MIP-1β) act together to attract natural killer cells, monocytes as well as macrophages (Menten et al. 2002). In addition to CCL3 and CCL4, CCL5 (RANTES) represent a chemotactic chemokine which recruits T-cells and leukocytes to the site of infection / inflammation (Arango Duque and Descoteaux 2014).

1.3.3 Production of reactive oxygen species and nitric oxide

The clearance of invading pathogens is a key effector function of macrophages. Bacterial killing by macrophages is exerted primarily by production of *reactive oxygen species* (ROS) and *nitric oxide* (NO) via the induction of the *NADPH oxidase system* (NOX) and the *nitric oxide synthases* (NOSs; NOS1-3), respectively. Both processes are collectively referred to as *oxidative burst*. Macrophages use ROS to kill intracellular pathogens during phagocytosis and NO to kill intra- and extracellular pathogens as well as tumor cells (Lorsbach et al. 1993).

Nitric oxide

Nitric oxide is a free radical with a half-life of only a few seconds in blood. It is produced by the NADPH-dependent conversion of L-arginine into L-citrulline catalyzed by the enzyme family of nitric oxide synthases. The *inducible* isoform of the *nitric oxide synthase* (iNOS) is involved in macrophage immune responses. Maximal induction of the iNOS requires stimulation with pro-inflammatory cytokines, e. g. IL-1β and TNF-α followed by subsequent stimulation with a microbial stimulus (Fang, 2004).

Reactive oxygen species

Intracellular reactive oxygen species are produced by NADPH oxidase complexes in various cell organelles, including cell membranes, mitochondria, peroxisomes and the endoplasmic reticulum. It is important to note that ROS products, such as superoxide anions (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO) are also formed as by-products in physiological cellular metabolic processes as well as under inflammatory stress conditions. ROS products are induced rapidly after exposure to pathogens or pathogen

products, whereas nitric oxide production requires a *de novo* protein synthesis and a longer timeframe of up to 24 hours (Fang 2004).

ROS- and NO-mediated killing of pathogens relies on (i) protein oxidation destroying protein functionality and (ii) DNA damage leading to strand breaks and erroneous DNA replication (Fang, 2004). In addition to its cytotoxic role, low intracellular doses of NO have been shown to act as important cell signaling molecules involved in many physiological and pathophysiological processes, e. g. vasodilation and post-translational regulation of proteins (Hou et al. 1999).

Long-term environmental stress of macrophages can lead to increased production of reactive oxygen species resulting in damage of certain cell structures. In detail, elevated intracellular ROS levels can lead to (i) oxidation of amino acids in proteins and poly-unsaturated fatty acids in lipids, (ii) oxidative deactivation of specific enzymes by oxidation of co-factors and (iii) damage of DNA (Schieber and Chandel 2014).

1.3.4 Phagocytosis

One of the key functions of macrophages is the engulfment and killing of invading pathogens by a process that is referred to as *phagocytosis*. Phagocytosis is a receptor-mediated mechanism which includes the recognition, binding, internalizing and degradation of pathogens. The receptor-mediated recognition of pathogens induces signaling pathways ultimately leading to rearrangement of the actin cytoskeleton thereby enabling macrophages to engulf the invading pathogens (Aderem and Underhill 1999). Within the macrophage pathogens are trapped in a membrane-bound vacuole referred to as *phagosome*. After maturation of the phagosome, the fusion of the phagosome with a *lysosome* ultimately leads to the formation of the *phagolysosome*. The precise mechanisms of phagosomal maturation and phagosomal killing of bacteria are not fully elucidated yet. However, it is generally accepted that fusion with the lysosome results in establishing an acidic (pH = 4,5) and oxidative environment that is enriched with hydrolytic enzymes leading to killing of the internalized pathogens by degradation (Flannagan et al. 2012). After degradation of pathogen proteins, pathogen peptide fragments are released into the cytoplasm and coupled with the *major histocompatibility complex class II* (MHC class II) for presentation on the macrophage surface (Neefjes et al. 2011).

1.3.5 Antigen presentation

In addition to its anti-microbial functions, macrophages are also crucial for the activation and regulation of the adaptive immune response. Antigen-dependent activation of B- and T-cells by *professional antigen-presenting cells* (APC) is one crucial step in the activation of the adaptive immune response. Along with dendritic cells, macrophages are the most important antigen-presenting cells.

By presenting processed peptides via major histocompatibility complex (MHC) class I and class II on the surface, macrophages are capable to activate T-cells (Fig. 2). MHC class I and class II molecules are similar in function, since both present processed peptides at the cell surface to *cytotoxic T-cells* (CD8(+) T-cells) and to *helper T-cells* (CD4(+) T-cells), respectively. However, the processed peptides originate from different sources: intracellular sources for MHC class I presentation and exogenous sources for MHC class II presentation (Neefjes et al. 2011) (Fig.2).

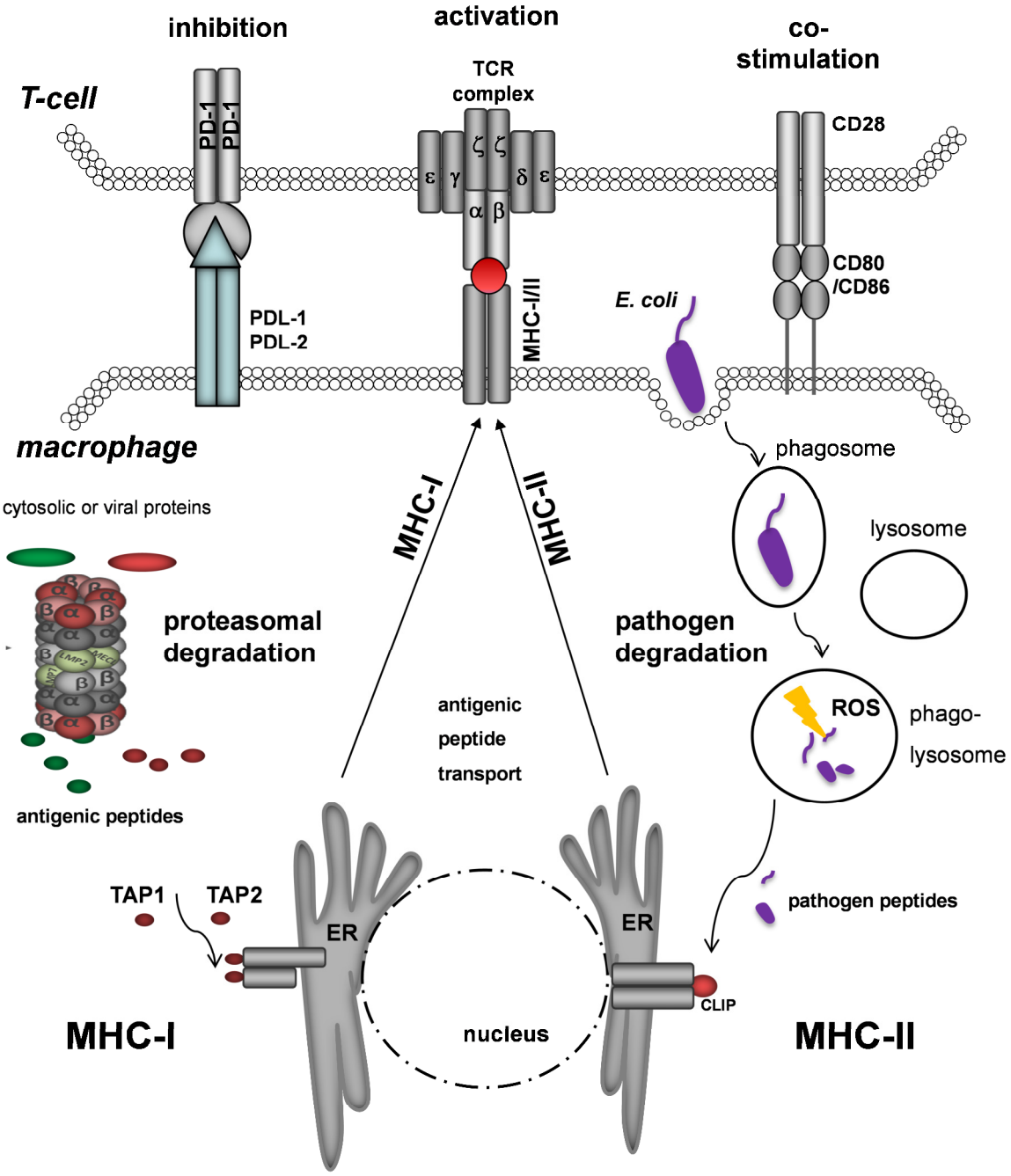

Figure 2. Schematic overview of antigen presentation and expression of T-cell co-stimulatory and co-inhibitory molecules by macrophages
Processed peptides of cytosolic proteins, viral proteins and engulfed pathogens are presented by major histocompatibility complex (MHC) class I and MHC class II molecules on the macrophage surface. In addition to antigen presentation, macrophages express the co-stimulatory ligands CD80 (B7.1) and CD86 (B7.2) which stimulate the CD28 receptor expressed on T-cells resulting in T-cell co-stimulation including proliferation and survival. PD-L1 (B7-H1, programmed death-ligand 1) is expressed on macrophages upon LPS and IFNγ stimulation leading to T-cell inhibition including cell cycle arrest and apoptosis.

MHC class I antigen presentation

MHC class I molecules are found on the surface of all nucleated cells and predominantly present cytosolic and processed intracellular pathogen peptides. The standard proteasome as well as the immunoproteasome are crucial components in MHC class I peptide presentation since they process cellular proteins and engulfed pathogen proteins into small peptides of 8–9 amino acids. The processed antigens are translocated via the *transporter associated with antigen presentation* (TAP1 and TAP2) from the cytosol into the lumen of the *endoplasmic reticulum* (ER). Within the lumen of the ER, the processed peptides are loaded on MHC I complexes. The loading process involves several ER *chaperons* and molecules including *tapasin*, *calreticulin*, *calnexin* and *Erp57* which mediate the assembly of the antigen-MHC class I complexes. The formed complexes are released from the ER followed by Golgi-mediated transport to the plasma membrane where they finally are presented to cytotoxic CD8(+) T-cells (Neefjes et al. 2011). Activated cytotoxic CD8(+) T-cells kill the infected antigen-presenting cell by release of cytotoxic agents, such as *perforin, granzymes, granulysin* and others as well as triggering the infected antigen-presenting cell to undergo apoptosis. Additionally, CD8(+) T-cells secrete cytokines, including TNFα and IFNγ, to activate phagocytes to remove apoptotic particles and to further enhance the immune response against infected cells (Zhang and Bevan 2011).

MHC class II antigen presentation

MHC class II molecules are exclusively found on the surface of professional antigen-presenting cells, including macrophages and dendritic cells. The antigens presented by MHC class II molecules are derived from extracellular proteins independently of degradation by the proteasomal system. Peptides for the MHC class II pathway are generated during endocytosis and phagocytosis of engulfed particles or pathogens within the phagolysosome (see detailed description of phagocytosis). In the context of T-cell immunity, antigen-presenting cells present antigen peptides to CD4(+) T-cells via MHC class II (Neefjes et al. 2011). Antigen-activated CD4(+) T-cells proliferate and differentiate into effector cells exerting cell-mediated immune responses by activation of macrophages, neutrophils and dendritic cells and humoral immune responses by activation of B-cells to produce antibodies (Zhou et al. 2009).

1.3.6 Expression of T-cell co-stimulatory and co-inhibitory signaling molecules

In addition to MHC antigen presentation, macrophages are capable to modulate T-cell responses by surface expression of CD80, CD86 and PD-L1 (Fig. 2). In this manner, macrophages can regulate activation as well as inhibition of T-cell immunity in infections underlining the importance of macrophages in linking innate and adaptive immune responses. T-cell activation, expansion and effector T-cell differentiation is regulated by activated macrophages through expression of co-stimulatory proteins along with MHC-dependent antigen presentation (Fig.2).

The most important and best investigated co-stimulatory molecules are CD80 (B7.1) and CD86 (B7.2) which are physiological ligands for the T-cell co-receptor CD28 (Allison 1994). Interaction of CD80 and 86 with CD28 stimulates the PI_3-kinase / AKT-pathway in T-cells (PI_3-kinase: *phosphatidylinositol-4,5-bisphosphate 3-kinase;* AKT: *protein kinase B*) promoting survival, differentiation and proliferation of T-cells (Lenschow and Bluestone 1993, Lanier et al. 1995).

In order to attenuate T-cell activation, e. g. after an infection has been cleared, co-inhibitory signaling molecules are expressed on the macrophage surface leading to decreased T-cell activation, inhibition of growth-factor production, inhibition of cell cycle progression and in some cases to T-cell death (Collins et al. 2005). The most prominent co-inhibitory signaling molecule expressed by macrophages is PD-L1 (B7-H1) (*programmed death-ligand 1*). It is recognized by the PD-1 receptor on antigen-specific CD8(+) T-cells (Yamazaki et al. 2002, Loke and Allison 2003). Although it is constitutively expressed on the macrophage surface, PD-L1 expression is increased upon exposure to LPS under inflammatory conditions (Loke and Allison 2003).

1.4 IFNγ and TLR4 signaling in macrophages

1.4.1 IFNγ signaling

IFNγ is a cytokine which is involved in innate and adaptive immune responses of macrophages. In the early phase of the immune response IFNγ is produced by *natural killer cells* (NK cells) and professional antigen-presenting cells (monocytes, macrophages,

dendritic cells) for *autocrine* activation and *paracrine* induction of neighboring cells (Gessani and Belardelli 1998, Frucht et al. 2001). At later stages of the immune response, antigen-activated T-cells are the main source of IFNγ to regulate macrophage activation (Frucht et al. 2001). IFNγ is a central regulator of macrophage immune responses since it is involved in the regulation of a variety of effector functions. For example, by inducing the expression of MHC class I and II complexes, IFNy modulates the antigen presentation of macrophages thereby activating adaptive immune responses. In addition, IFNγ has a pivotal role in macrophage-mediated orchestration of other immune processes by regulating the secretion of cytokines and chemokines. Importantly, via autocrine activation, IFNγ also directly regulates antibacterial functions of macrophages in inflammation, including induction of nitric oxide synthesis. (Schroder et al. 2004)

On the macrophage surface IFNγ is recognized by the *IFNγ receptor* (IFNGR) which consists of two subunits IFNG1 and IFNG2 that transmit signals via the JAK-STAT pathway (Rauch et al. 2013). This pathway involves activation of *Janus kinases* (JAKs) and STAT proteins (*Signal Transducer and Activator of Transcription*) by phosphorylation leading to activation of IFNγ regulated genes, e. g. IRF-1, iNOS or IFNβ (Schroder et al. 2004) (Fig. 3).

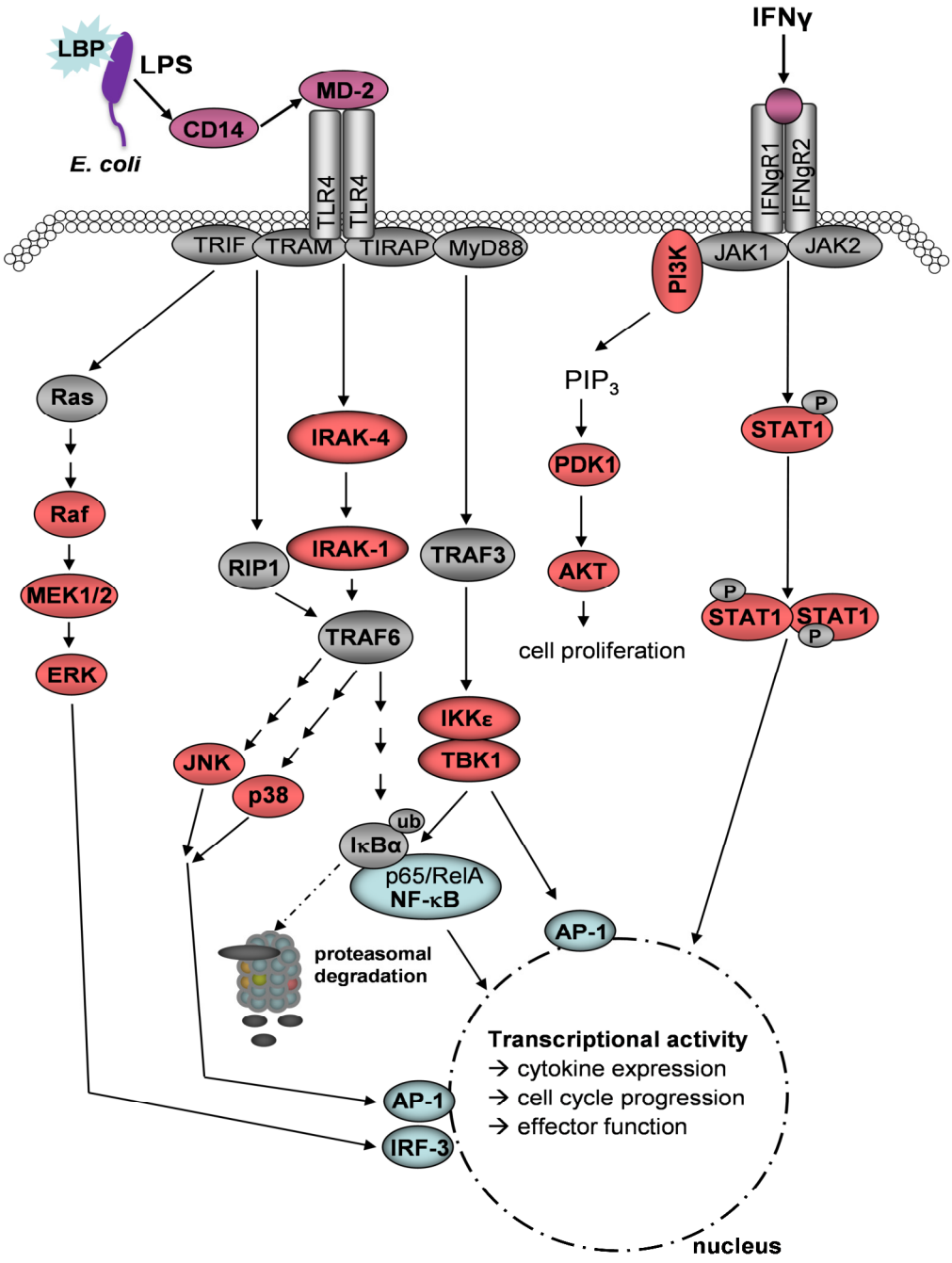

Figure 3. Schematic overview of TLR4 and IFNγ signaling in macrophages
Lipopolysaccharide (LPS) of gram(-) bacterial cell walls is bound by the LPS-binding protein (LBP) and CD14. The LPS/LBP/CD14 complex is recognized and bound by the TLR4-MD2 receptor complex leading to activation of the adapter molecules TRIF/TRAM and MyD88 followed by downstream activation / phosphorylation of ERK-1/2, JNK-1/2 and p38 MAPK. Induction of the TRIF-and MyD88-dependent pathways results in the activation of the transcription factors AP-1, IRF-3 and NFκB.
IFNγ is recognized by the IFNγ receptor (IFNGR) followed by intracellular signal transduction by the JAK-STAT pathway. This pathway involves the activation of JAK and STAT proteins by phosphorylation and results in induction of IFNγ-regulated genes, such as iNOS and IRF-1. Signaling via IFNγ and LPS induces transcriptional modulation of numerous effector genes, mediating cytokine expression, cell cycle progression and immune effector functions.

1.4.2 TLR4 signaling

Bacterial cell wall compounds are known to be potent activators of innate immune responses, since they are recognized by specialized pattern recognition receptors on the surface of macrophages and other immune cells. Lipopolysaccharide (LPS) is the prototype and best studied cell wall compound of the outer membrane of gram-negative bacteria which activates a broad spectrum of macrophage effector functions. High concentrations of LPS can lead to excessive production of pro-inflammatory cytokines resulting in hyper inflammation and the progression of a life threating septic shock (Qureshi et al. 2012).

Several molecules are involved in the recognition of LPS by macrophages. First, LPS is extracted from bacterial cell walls by the *LPS-binding protein* (LBP). Second, the LPS-LPB complex is recognized and bound by the macrophage membrane protein CD14 which in turn transfers LPS to the TLR4-MD-2 receptor complex on the macrophage surface (Maeshima and Fernandez 2013). Upon binding of LPS, the TLR4-MD-2 complex dimerizes and forms an intracellular scaffold for the recruitment of cellular adaptor molecules. TLR4 activation by LPS results in the induction of two distinct intracellular pathways, the MyD88-dependent pathway and the TRIF-dependent pathway (MyD88: *myeloid differentiation primary-response gene 88*; TRIF: *TIR-domain-containing adaptor protein*) (Maeshima and Fernandez 2013) (Fig. 3).

In the TRIF-dependent pathway, TLR4 is internalized into endosomes where it recruits the adaptor molecules TRAM and TRIF (TRAM: *TRIF-related adaptor molecule*). These adaptor molecules activate the transcription factor *interferon regulatory factor-3* (IRF3) mediating the production of type I interferons as well as a delayed activation of the transcription factor NFκB (*nuclear factor kappa-light-chain-enhancer of activated B*-cells) (Kawai and Akira 2010).

In the MyD88-dependent pathway, dimerization of TLR4-MD-2 at the macrophage surface recruits the intracellular adaptor molecules TIRAP (*toll-interleukin 1 receptor domain containing adaptor protein*) and MyD88. Recruitment of TIRAP and MyD88 induces a signaling cascade which results in the activation of NFκB promoting the gene expression and secretion of pro- and anti-inflammatory cytokines (Kagan and Medzhitov 2006). Activation of the transcription factor NFκB is initiated by the degradation of *NFκB inhibitors* (IκBs) that bind, and thus inhibit, NFκB molecules. Proteasomal degradation of the NFκB inhibitors requires phosphorylation by the *IκB kinase* (IKK) followed by

ubiquitination with K48-linked ubiquitin. As a result, the released NFκB complex is capable to translocate into the cell nucleus where it acts as a transcription factor. In addition to NF-κB, the transcription factors *activated protein 1* (AP-1) and *interferon regulatory factor 3* (IRF-3) are activated by the described pathways involving the three main *mitogen activated protein kinases* (MAPKs) JNK (c-*Jun N-terminal kinase*), p38 MAPK and ERK (*extracellular-regulated kinase*). AP-1-regulated genes are involved in crucial cellular processes including proliferation, differentiation and apoptosis. The MyD88 dependent pathway leads to rapid activation of MAPK, AP-1 and NFκB and production of cytokines whereas the TRIF-dependent pathway is known for a delayed activation of these transcription factors. (Schroder et al. 2004)

1.4.3 IFNγ enhancement of the LPS response

Numerous studies observed an enhancement of the macrophage immune response upon LPS stimulation by pre-treating cells with IFNγ (Schroder et al. 2004, Held et al. 1999, Bosisio et al. 2002). This effect is often described as *IFNγ priming* and relies on the fact that LPS and IFNγ cross-regulate signaling molecules of each other pathways (Schroder et al. 2004). IFNγ promotes the LPS recognition and signaling process by enhancing the LPS binding capacity of macrophages by upregulation of the TLR4 / MD-2 complex, MyD88 adaptor molecules and IRAK signaling molecules (Bosisio et al. 2002). Furthermore, IFNγ pre-treatment supports the LPS-induced NFκB activation by faster degradation of NFκB inhibitors and a higher DNA binding capacity (Held et al. 1999). Additionally, LPS triggers macrophages to produce type I interferons, such as IFNα and IFNβ which act in an autocrine and paracrine manner ultimately enhancing the IFNγ response (Gao et al. 1998). LPS- / IFNγ-activated signaling molecules often bind to the same promoter regions of target genes to synergistically regulate transcription. For example the promoter of the inducible nitrite synthase harbors two binding sites for STAT1 and NFκ-B and maximal expression requires both signals (Gao et al. 1998, Gao et al. 1997).

1.4.4 Macrophage activation states

A classical commonly applied classification of macrophage activity / differentiation is the M1 / M2 paradigm. In this classification system macrophages are referred to as either

classical activated macrophages (M1 macrophages) or *alternatively activated macrophages* (M2 macrophages).

M1 "killer" macrophages are described to exhibit strong anti-microbial properties with high production of the pro-inflammatory cytokines TNFα, IL-1β, IL-12 and antimicrobial effectors, such as nitric oxide and defensins upon stimulation with IFNγ and LPS. In contrast, M2 "repair" macrophages produce high levels of IL-10, TGF-β and low levels of IL-12 upon stimulation with IL-4. This type of macrophages is predominantly involved in processes like wound healing and tissue repair. Furthermore, M2 macrophages are described as the phenotype of resident tissue macrophages. (Mosser and Edwards 2008) However, recent research studies question this rather simple dichotomy of macrophage classification in favor of more complex models (Martinez and Gordon 2014).

1.5 The proteasome system

The *proteasome* is a multimeric protein complex with protease activity which is found in the cytoplasm of pro- and eukaryotes. The central function of the proteasome is the non-lysosomal degradation of proteins, e. g. damaged and misfolded proteins as well as not-required proteins. Hence, the proteasome is a pivotal cell structure for regulation of protein homeostasis within the cell. Due to the broad substrate spectrum of the proteasome, it is involved in many cellular processes including protein quality control, cell growth, cell differentiation, regulation of gene expression and supply of antigenic peptides for the MHC class I complex. In order to be recognized for degradation by the proteasome, proteins need to be poly-ubiquitinated during a process called *ubiquitination (Bedford et al. 2010)*. Ubiquitin is a highly conserved polypeptide with 76 amino acids (8.5 kDa) that is ubiquitously found in all eukaryotic cells. It serves as a substrate for ubiquitination to label proteins for proteasomal degradation. Ubiquitination is a reversible process of post-translational protein modification in which ubiquitin is bound to a target protein (Komander 2009).

1.5.1 Proteasome structure

The general structure of all mammalian proteasomes is a cylindrical core complex with one regulator complex at each end. The core complex consists of four rings, each composed of seven different proteins, whereby the two inner rings harbor the three proteolytic active subunits. The two outer rings are essential to bind the regulator complexes. The regulator complex consists of 19 different proteins and facilitates the recognition, binding and unfolding of ubiquitinated proteins in an ATP-dependent fashion. Based on distinct structural features, different proteasome types have been described, such as the *standard proteasome* and the *immunoproteasome* (Fig. 4).

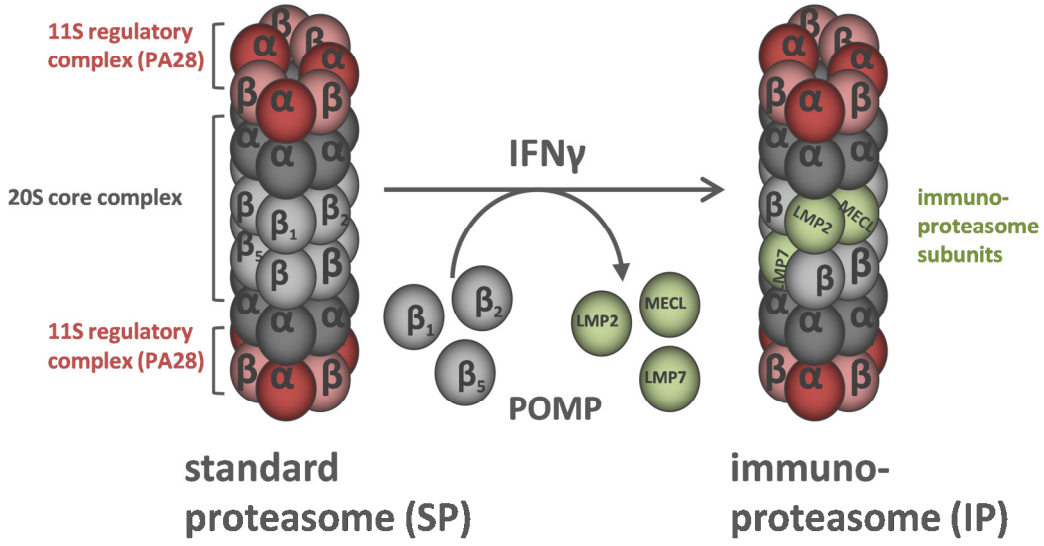

Figure 4. Structure of the standard proteasome and the immunoproteasome
The standard proteasome is formed by the *20S core complex* and a regulatory complex, which can be either the *19S regulator* (not shown) or the *11S regulator* (PA28). The 20S core complex is built by a heptameric ring structure with two outer rings and two inner rings formed by the α-subunits (α1 - α7) and β-subunits (β1−β7), respectively. The central β1, β2 and β5 subunits represent the active site of the standard proteasome. The *11S regulator (PA28-proteasome activator 28)* contains three PA28α and four P28β-subunits which form heptameric rings that can bind to the two outer rings of the 20S complex of the standard proteasome as well as the *20S immunoproteasome*. The immunoproteasome is preferentially formed upon IFNγ stimulation by incorporation of the subunits LMP7, LMP2 and MECL-1 into the 20S complex substituting the standard proteasome subunits β1, β2 and β5. In addition, IFNγ induces synthesis of PA28 as well as the synthesis of the proteasome maturation protein (POMP).

Structure of the standard 26S proteasome

The majority of ubiquitinated proteins in eukaryotic cells is degraded by the 26S proteasome, also referred to as the standard or constitutive proteasome (Bedford et al. 2010). The standard 26S proteasome consists of a *20S core complex* which interacts with the *19S regulator complex* and / or *PA28 activator complex*.

The 20S core complex contains an outer ring and an inner ring, each with seven subunits (α1-α7 and β1-β7). The two inner rings harbor the three proteolytic active subunits: beta1 (β1), beta2 (β2) and beta5 (β5) with N-terminal threonine residues, which exhibit a nucleophile hydrolase activity for peptide bound cleavage (Fig. 4). Each of these subunits has a distinct proteolytic activity: beta1 has a *caspase-like activity* (cleavage after acidic amino acids), beta2 has a *trypsin-like activity* (cleavage after basic amino acids) and beta5 has *chymotrypsin-like activity* (cleavage after hydrophobic amino acids). The multi-specificity of the proteolytic β-subunits facilitates the formation of a broad range of processed peptides for MHC-presentation or protein-metabolism. (Bedford et al. 2010)

The *19S regulator complex* (PA700) facilitates the recognition and binding of poly-ubiquitinated proteins and serves as gate to the proteolytic active core of the 20S core complex. The 19S regulator complex contains 18 distinct subunits which are divided into a lid and a base part. The base-complex includes proteins (Rpn1, Rpn2, Rpn10) and a ring composed of six ATPases (Rpt1-Rpt6). The binding of ATP to the 19S ATPase subunits mediates the assembly of the 19S regulator complex with the 20S core complex. ATP hydrolysis is further required for the assembled complex to degrade folded and ubiquitinated proteins. The 19S lid contains up to ten non-ATPase subunits which are responsible for the recognition and binding of poly-ubiquitinated proteins. (Kloetzel 2001)

1.5.2 Proteasome activator PA28

In place of the 19S regulator complex, the *proteasome activator 28* (PA28) can bind at one end to the 20S core complex, thereby forming the *19S-20S-PA28 proteasome (Tanahashi et al. 2000)*. In addition, the PA28 regulator complex can bind at both ends of the 20S core complex forming the *PA28-20S-PA28 proteasome (Cascio et al. 2002)* (Fig. 4). Due to the gene deficient mouse model lacking PA28αβ utilized in the present study, the 20S core complex coupled with PA28 is described in more detail. The PA28 complex has a heptameric ring structure composed of two related IFNγ-inducible proteins, PA28α and

PA28β which do not contain any ATPases (Ahn et al. 1996) (Fig. 4). As a result, the PA28 complex cannot unfold larger ubiquitinated proteins containing native or denatured structures. It is speculated that PA28 promotes the degradation of polypeptides of intermediate size that were generated by the standard 26S proteasome (Tanahashi et al. 2000). Furthermore, it was shown that PA28 leads to an increased capacity to selectively degrade oxidant-damaged proteins as well as promoting a more efficient antigen presentation (Pickering et al. 2010). An IFNγ-dependent upregulation of the PA28 expression was shown suggesting a role in regulation of immunoproteasome functions (Ferrington and Gregerson 2012).

1.6 The immunoproteasome

The discovery of an alternate proteasome complex in immune cells treated with IFNγ made it necessary to discriminate the newly described proteasome from the standard proteasome. Due to its formation under inflammatory conditions the alternative proteasome was named immunoproteasome (IP) (Aki et al. 1994, Ferrington and Gregerson 2012).

1.6.1 Structure of the immunoproteasome

The immunoproteasome is structurally similar to the 26S standard proteasome except of three catalytic β-subunits (β1, β2, β5) in the 20S core complex. Within the 20S core complex of the immunoproteasome the β-subunits are substituted by the β1 homolog LMP2/β1i (*low molecular-weight polypeptide 2*), the β5 homolog LMP7/β5i (*low molecular-weight polypeptide 7*) and the β2 homolog MECL-1/β2i (*multicatalytic endopeptidase complex 1*) (Fig. 4). Rather than exhibiting the pronounced caspase activity of the β1 subunit, LMP2/β1i possess a chymotrypsin-like activity, which promotes the generation of MHC class I-compatible peptides containing hydrophobic C-terminal anchors. In contrast, the subunits MECL-1/β2i and LMP7/β5i show the same proteolytic activity as the corresponding standard proteasome subunits (Ferrington and Gregerson 2012).

1.6.2 Assembly of the immunoproteasome

The main steps of the assembly process are similar for the standard 26S proteasome as well as for the immunoproteasome, despite of the integration of the proteolytic subunits. The incorporation of the IP-specific subunits is mediated by the *proteasome maturation protein* (POMP) and is executed by two sequential steps. First, the pro-peptide of LMP2 is integrated in the newly processed 20S core complex followed by the pro-peptide MECL-1 forming the pre-immunoproteasome. Second, the LMP7 subunit is incorporated leading to successful maturation of the functional immunoproteasome by assisting the catalytic lysis of the pro-peptides of LMP2 and MECL-1 (Ferrington and Gregerson 2012, Griffin et al. 1998).

In cells which co-express standard and IP-subunits upon IFNγ stimulation the formation of immunoproteasomes is preferred. This favored incorporation of the IP-subunits is known as *cooperative assembly* and is based on the following mechanism. First, LMP2 integration occurs earlier in the proteasome formation process than the standard proteasome subunit β1. Second, POMP which is concomitantly expressed under IFNy stimulation, binds the pro-peptide of LMP7/β5i with greater affinity than the pro-peptide of β5 (De et al. 2003, Ferrington and Gregerson 2012, Johnston-Carey et al. 2016). Therefore, the assembly and maturation process of the immunoproteasome upon IFNγ stimulation is supposed to be four times faster than the standard proteasome (Johnston-Carey et al. 2016). In addition, the IP has a shorter half-life (27h) than the standard proteasome (133h), potentially enabling faster adaption to environmental changes, e. g. invasion of pathogens (Ferrington and Gregerson 2012).

1.6.3 The expression of the immunoproteasome in immune cells and non-immune cells

High expression of the immunoproteasome was primarily found in immune cells including professional antigen-presenting cells (macrophages, dendritic cells, B-cells) (Ebstein et al. 2012). For a long period of time it was believed that non-immune cells and tissues express immunoproteasomes only upon an environmental stimulation with IFNγ. This was confounded by studies showing that non-immune cells and organs constitutively express the subunit LMP7 (e. g. epithelia cells, colon, liver, vein cells) as well as the complete set

of IP-subunits (liver, kidney) (Ebstein et al. 2012). However, the immunoproteasome expression in these cells is generally lower under non-stimulating conditions whereby immune cells show a high in vivo and in vitro IP expression independent of environmental stimulation (Ebstein et al. 2012).

1.6.4 Activation of the immunoproteasome subunit gene expression

The promoter regions of the immunoproteasome genes (LMP7 and LMP2) harbor multiple binding sites for transcription factors, including STAT-1 and IRF-1 (Ferrington and Gregerson 2012). The promoter also includes additional binding sites for transcription factors, such as NFκB, AP-1 and CREB (*cAMP responsive element binding protein*) (Ferrington and Gregerson 2012). Therefore, several inflammatory immune triggers, including TNFα, type I interferons (IFNα/β), nitric oxide and certain pathogens are able to induce immunoproteasome expression (Johnston-Carey et al. 2016). IFNγ is the best studied immune trigger that induces the expression of immunoproteasome subunits. The IFNγ-dependent upregulation of the IP-subunits results from binding of the transcription factors IRF-1 and STAT-1 to multiple IFN-γ consensus / activation sequences in the promoter region of the genes LMP7, LMP2 and MECL-1 (Ferrington and Gregerson 2012).

1.6.5 Immune- and non-immune functions of the immunoproteasome

Since the IP was discovered, numerous studies with diverse experimental approaches have sought to elucidate the functional differences between the immunoproteasome and the standard proteasome. However, findings of these studies are often very contradictory, potentially explained by different experimental settings, utilized IP-deficient mouse and cell model as well as different chemical approaches which selectively inhibit the proteolytic subunits of the standard and immunoproteasome. In general, both the immunoproteasome and the standard proteasome share similar cellular functions.

1.6.6 The immunoproteasome in MHC class I antigen presentation

Antigen presentation via MHC class I and class II molecules is one major macrophage effector function linking innate and adaptive immune responses. Originally, it was believed

that the main function of the immunoproteasome is the generation of peptides for MHC class I-mediated antigen presentation to activate adaptive immune responses. This assumption was based on the finding that the genes for LMP7 and LMP2 were found in the region of the MHC locus on chromosome 17 in mice upstream of the TAP1 and TAP2 genes (Ebstein et al. 2012, Ferrington and Gregerson 2012). Degradation of proteins for peptide supply for the MHC class I complex is one of the major functions of the immunoproteasome and the standard proteasome. Both proteasome types generate peptides with a size of eight to nine amino acids which are loaded on MHC class I complexes. However, due to the enhanced chymotrypsin-activity by the proteolytic subunits LMP7 and LMP2, the immunoproteasome generates more peptides with a hydrophobic C-terminal anchor which have a higher affinity to the MHC I peptide binding site (Ferrington and Gregerson 2012). Furthermore, a study using a mass spectrometry approach revealed that immunoproteasomes have a specific cleavage preference for unstructured protein regions (i. e. regions lacking secondary and tertiary structures) increasing the abundance and diversity of MHC I-associated peptides compared to standard proteasomes (de Verteuil et al. 2010). The recently elucidated crystal structure of the mouse immunoproteasome provides an explanation for the enhanced antigen processing by the IP. The LMP2 substrate-binding channel has an increased hydrophobicity in contrast to the β1 substrate-binding channel resulting in the formation of epitopes with small, nonpolar residues which are more eligible for presentation on MHC-I molecules (Huber et al. 2012). However, although published data have shown the association between immunoproteasome activity and MHC-I-mediated peptide presentation, the regulation of the MHC I expression in IP-deficient macrophages has not been investigated yet.

1.6.7 The immunoproteasome in the regulation of signaling pathways and cytokine production

Studies using different cell and infection models have investigated the role of the immunoproteasome in cytokine expression and secretion. These findings suggest that the immunoproteasome has a pleiotropic role in modulation of the cytokine response. Pro- and anti-inflammatory cytokines have been shown to be positively or negatively regulated by the immunoproteasome. Cytokines which have been linked to be modulated by immunoproteasome activity are interferons (e. g. IFNβ and IFNγ) and several interleukins

(e. g. IL-1β, IL-10 and IL-23) (Ferrington and Gregerson 2012, Reis et al. 2011b). The reduced ability to produce certain cytokines in IP-deficient mice and cell models has often been associated with reduced NFκB activation. For none-immune cells one study revealed that LMP2-deficient mice show an impaired proteolytic processing of NFκB precursors and decreased degradation of the NFκB inhibitor IκBα. (Maldonado et al. 2013). In line with this, two studies observed that the immunoproteasome has a higher degradation turnover for IκBα than standard proteasomes (Seifert et al. 2010, Visekruna et al. 2006). In contrast, another study reported that the immunoproteasome subunits LMP2 and LMP7 are not required for NFκB activation (Jang et al. 2012). These contradictory findings point out that more studies are required to find out how the immunoproteasome is involved in the NFκB pathway. However, despite of the NFκB pathway, the cytokine expression is regulated by many other signaling pathways. The potential involvement of the immunoproteasome in these pathways needs to be investigated to fully understand its role in the inflammatory cytokine response in inflammation.

1.6.8 The immunoproteasome during protein homeostasis

Protein degradation of oxidant-damaged, misfolded or not required proteins by the proteasome system including immuno- and standard proteasomes is an important mechanism in maintaining intracellular protein homeostasis. Microbial stimuli and various types of cell stress can lead to temporal accumulation of protein aggregates in various cells and tissues. Contradictory findings have been reported on the role of the immunoproteasome and standard proteasome in eliminating non-functional protein aggregates upon oxidative stress. One study reported that in contrast to the standard proteasome, the immunoproteasome has higher capacity to degrade these protein aggregates upon IFNγ stimulation. This notion might be explained by a higher ability of the immunoproteasome to degrade K48-linked poly-ubiquitinated substrates. The higher ability is described with a significantly increased poly-ubiquitin-substrate turnover capacity that is two- to three-fold higher than measured for standard proteasomes (Seifert et al. 2010). However, the underlining mechanism leading to a higher-proteolytic activity remains unknown. In contrast, another study using the same experimental settings as Seifert et al. observed no differences in the degradation capacity of poly-ubiquitinated proteins between the standard proteasome and the immunoproteasome (Nathan et al.

2013). In conclusion, further investigations are required to precisely decipher the importance of immunoproteasome activity for maintaining protein homeostasis under cellular stress conditions.

2. Aims and Objectives

The immunoproteasome (IP) is a multimeric protein complex with proteolytic activity which is formed under inflammatory and cellular stress conditions to maintain protein homeostasis within the cell. Besides its classical function in protein homeostasis, a number of studies using viral infection models in different IP-K.O. mice indicate that the immunoproteasome is involved in the regulation of anti-viral immune responses (Vankaer et al. 1994, Chen et al. 2001, Basler et al. 2011). Only a few recently published studies have investigated the role of the IP in bacterial and fungal infections (Kirschner et al. 2016, Mundt et al. 2016). However, the role of the immunoproteasome in bacterial infections is not fully understood yet. In particular, the impact of the lack of immunoproteasome activity on macrophage effector functions in bacterial infections is poorly understood. In vivo experiments by our group showed that mice lacking immunoproteasome activity by genetic K.O. of major immunoproteasome subunits exhibit an increased mortality in *E. coli*-induced infections associated with a higher bacterial burden in several organs. The biological background of the observed immune-compromised phenotype of IP-deficient mice remains unclear. The increased mortality accompanied by impaired bacterial clearance early after infection, suggests that the IP is involved in early innate immune responses against bacteria with particular focus on macrophage effector functions.

The aim of the study is to investigate the role of the IP in antibacterial macrophage effector functions. For this purpose, primary macrophages from an immunoproteasome K.O. mouse model lacking the two IP-subunits LMP7, MECL-1 and the proteasome-activator complex PA28, were used as an in vitro cell model. To account for the complex nature of macrophage responses, the present thesis addresses a set of various macrophage effector functions, including

- TLR4- and IFNγ-induced proximal intracellular signal transduction
- induction and secretion of crucial macrophage cytokines and chemokines
- phagocytosis and intracellular killing of *E. coli*
- production of reactive oxygen and nitrogen species (oxidative burst)
- cellular resistance to pathogen-induced stress
- capacity of macrophages to modulate adaptive T-cell responses, including macrophage MHC class I upregulation and surface expression of T-cell co-stimulatory and co-inhibitory molecules

3. Material and Methods

3.1 Material

Bacteria culture

Yeast extract	(Carl Roth)
Trypto / pepton	(Carl Roth)
Sodiumchlorid	(Carl Roth)
Agar-agar	(Sigma-Aldrich)
Mitomycin C	(Sigma-Aldrich)

Cell culture

Dulbecco's modified Eagle's medium DMEM	(Gibco)
Dulbeccos PBS 1x	(Gibco)
Fetal calf serum (FCS)	(Biochrom)
Trypan blue 0.4 %	(Invitrogen)
Penicillin / Streptomycin	(Invitrogen)
Gentamicin	(Life Technologies)
ACK-Lysing Buffer	(Invitrogen)
Lipopolysaccharide from *E. coli* 0111:B4	(InvivoGen)
E. coli BioParticles® Alexa Fluor® 488	(Life Technologies)
E. coli BioParticles® Opsonizing Reagent	(Life Technologies)
Nuclease free water	(Ambion)
0.5 x Tris-EDTA-Puffer	(FLUKA)
Recombinant murine IFNγ	(Peprotech)

Flow cytometry

Cytofix / Cytoperm Solution	(BD Pharmingen™)
10x PermWash buffer	(BD Pharmingen™)
Golgi Plug™ Protein Transport Inhibitor	(BD Pharmingen™)
Paraformaldehyde	(Carl Roth GmbH)
Attune Focusing Fluid	(Life Technologies)
Sodium hypochlorite solution	(Carl Roth)
Attune Performance Tracking Beads	(Life Technologies)
Attune Wash Solution	(Life Technologies)
Attune 10X Shutdown Solution	(Life Technologies)
Paraformaldehyde	(Carl Roth)

Nitrogen monoxide (NO) assay

N-(1-naphtyl) ethylenendiamine dihydrochloride	(Sigma-Aldrich)
Phosphoric acid	(Sigma-Aldrich)
Sulfanilamide	(Sigma-Aldrich)
Nitrite standard	(Sigma-Aldrich)

Reactive oxygen species (ROS) assay

2',7'-dichlorodihydrofluorescein diacetate	(Invitrogen
Phorbol myristate acetate (PMA)	(InvivoGen)
Hydrogen peroxide solution (H_2O_2)	(Sigma-Aldrich)

Western Blot

Enhanced Luminol Reagent Plus	(PerkinElmer Inc.)
Ethanol, 96 %	(Nordbrand)
SuperSignal®West Pico Chemieluminescent substrate	(Thermo Scientific)
Milkpowder	(Carl Roth)
Tris	(Sigma-Aldrich)
Glycin	(Sigma-Aldrich)
Tween	(Sigma-Aldrich)
BSA (Albumin Fraktion V)	(Sigma-Aldrich)
Acrylamide (Rotiphorese® 37.5 %)	(Carl Roth)
Sodiumchloride	(Carl Roth)
ProSieveTM 50 Gel Solution	(Lonza Group Ltd)
DTT	(Sigma-Aldrich)
NuPAGE® LDS Puffer (4x)	(Life Technologies)
Protease inhibitor cocktail (PIC)	(Merck)
cOmplete™, EDTA-free mini	(Roche)
MG132 (cell-permeable proteasome inhibitor)	(Callbiochem)
N-Ethylmaleimide (NEM)	(Sigma-Aldrich)
Sodiumchloride (NaCl)	(Carl Roth)
Magnesiumchloride ($MgCl_2$)	(Sigma-Aldrich)
HEPES	(Sigma-Aldrich)
EDTA	(Sigma-Aldrich)
PhosSTOP (phosphatase inhibitor)	(Roche)
TEMED	(Sigma-Aldrich)
Triton X-100	(Sigma-Aldrich)
Mini-Protean TGX Gels 4-15 %	(Biorad)

Kits

Pierce™ BCA Protein Assay Kit	(Thermo Scientific)
RNeasy Mini Kit	(QIAGEN)
High Capacity cDNA Reverse Transkription Kit	(Applied Biosystems)
Rnase Inhibitor 20 U/µl	(Applied Biosystems)
2x SensiMixTM SYBR® No-ROX Kit	(Bioline)
InnuPREP RNA Mini Kit	(Analtik Jena AG)

Mouse IL-6 ELISA Ready-SET-Go	(eBioscience)
Mouse IL-1 beta ELISA Ready-SET-Go	(eBioscience)
Mouse IL-12 p70 ELISA Ready-SET-Go	(eBioscience)
14 Plex Kit - ProcartaPlex®	(eBioscience)

Primer

Table 1: List of primer for mouse genotyping

Gene	Forward Primer (Fw) Reverse Primer (Rv)	Forward Primer Neomycin cassette
PA-Neo	Fw: CCGACGGCGAGGATCTCGTCGTGA Rv: AGCGAGCACGTACTCGGATG	
PA28a	Fw: CAGGCAGTGTCCTCAATGGT Rv: CTCAGGCTGGTTGCACAGTA	
PA28b	Fw: TCAGAGATGCAGGTCTTCAA Rv: TCACAGAAAGCTGTGAGCGT	
LMP7	Fw: GGACCAGGACTTTACTACGTAGATG Rv: CTTGTACAGCAGGTCACTGACATCG	CCGACGGCGAGGATCTCGTCGTGA
MECL-1	Fw: AGAGAGAAACACGTGACAGACTGG Rv: CAGGACAGGTGTGGTTCCAGGAGC	CCGACGGCGAGGATCTCGTCGTGA

Table 2: List of primer for quantitative and semiquantitative PCR

Gene		Forwad Primer (Fw) Reverse Primer (Rv)
Beta1	proteasome subunit, beta type	Fw: CGTTTTCGCCTTATGCCTT Rv: TGGCCTTGTTATTGGAATGCT
Beta2	proteasome subunit, beta type 2	Fw: GCTATGGTGCCTTCCTGACT Rv:CTCCAGCCTCTCCAACACAT
Beta5	proteasome subunit, beta type 5	Fw: AGCTTCGCAATAAGGAACGC Rv: TAGCCTCGATCCATAACGCC
Ccl2	chemokine (C-C motif) ligand 2	Fw: CACTCACCTGCTGCTACTCA Rv: ACCCATTCCTTCTTGGGGTC
Ccl4	chemokine (C-C motif) ligand 4	Fw: TTCTGTGCTCCAGGGTTCTC Rv: AGCAAAGACTGCTGGTCTCA
Ccl7	chemokine (C-C motif) ligand 7	Fw: GAAGCCAGCTCTCTCACTCT Rv: CACCGACTACTGGTGATCCTT
Ccl12	chemokine (C-C motif) ligand 12	Fw: CCACCATCAGTCCTCAGGTA Rv: GGACACTGGCTGCTTGTGAT
Hprt	Hypoxanthin-Phosphoribosyl-Transferase	Fw: TGTAATCCAGCAGGTCAGCA Rv: GGCGCGAACGACAAGAAA
Cd14	CD14 antigen	Fw: TGCGAGCTAGACGAGGAAAG Rv: CCGCCCCCAAACAATTGAAA
Cxcl1	chemokine (C-X-C motif) ligand 1	Fw: TGGCTGGGATTCACCTCAAG Rv: TCTCCGTTACTTGGGGACAC
IFN-β	interferon beta	Fw: CCAGCTCCAAGAAAGGACGA Rv: GCCCTGTAGGTGAGGTTGAT
IFNγR1	interferon gamma receptor R1	Fw: TGCCTGTACCGACGAATGTT Rv: TTGGTGCAGGAATCAGTCCA
IFNγR2	interferon gamma receptor R2	Fw: TCCTCGCCAGACTCGTTTTC Rv: CAGCAACCTATGCCAAGAGC
IL-1α	interleukin 1alpha	Fw: CGCTTGAGTCGGCAAAGAAA Rv: GATACTGTCACCCGGCTCTC

IL-1β	interleukin 1beta	Fw: TGGCAGCTACCTGTGTCTTT Rv: CAGCTCATATGGGTCCGACA
IL-1R	interleukin 1 receptor	Fw: ACGAGAAACAACCAGCTCAT Rv: AGGTCAATAGGCACCATGTCT
IL-6	interleukin 6	Fw: ACCACTTCACAAGTCGGAGG Rv: TCTGCAAGTGCATCATCGTT
Kc	interleukin 8	Fw: ACCTCAAGAACATCCAGAGCTT Rv: CGACCATTCTTGAGTGTGGC
IL-10	interleukin 10	Fw: TGGGTGAGAAGCTGAAGACC Rv: GCTCCACTGCCTTGCTCTTA
iNOS, NOS2	inducible nitric oxide synthase 2	Fw: GGTGAAGGGACTGAGCTGTT Rv: CTGAGAACAGCACAAGGGGT
Ly96/Md2	Ly96 lymphocyte antigen 96	Fw: AGCAACAGTGGTTCTGCAAC Rv: TCCATTGGTTCCCCTCAGTC
Psmb8/ LMP7	proteasome subunit beta type-8	Fw: TTCCTGAGGTCCTTTGGTGG Rv: TACAACCTGCACTCCTTGGC
Psmb9/ LMP2	proteasome subunit beta type-9	Fw: TTCTGTGCCCTCTCAGGTTC Rv: TTCTTCACCACGTTTGCAGC
Psmb1/ MECL1	proteasome subunit beta type-10	Fw: CGGGGTTGATTTGAACGGAC Rv: GTGATGGCTTCCACCAACAG
Psme1/ PA28α	proteasome activator complex subunit 1	Fw: AGGTTTCGAGCTGTGCTTTC Rv: CCCAAGCAGGTTCTCTGTCT
Psme2/ PA28β	proteasome activator complex subunit 2	Fw: CAGCACCTGATCCCCAAGAT Rv: CTGCTTCATCTCGCTCATGC
Rpl13a	ribosomal protein L13A	Fw: TACGCTGTGAAGGCATCAAC Rv: CTCGGGAGGGGTTGGTATTC
Tap1	transporter 1, ATP-binding cassette, sub-family B	Fw: CGGCAACCTTGTCTCATTCG Rv: CTGGACTTTGGGCTGGTTTG
TNFα	tumor-necrose factor alpha	Fw: GGCCTCCCTCTCATCAGTTC Rv: TTTGCTACGACGTGGGCTAC
TLR4	toll-like receptor 4	Fw: CCTGACACCAGGAAGCTTGA Rv: TCAAGGGGTTGAAGCTCAGA

Antibodies

Table 3: List of antibodies for western blot analyses

antibody	target protein size (kDa)	species	dilution	diluted in	company
Beta-actin(15E5)	45	rabbit	1:1000	TBS-T + 1% milk	Cell Signaling
Caspase1	42	rat	1:500	TBS-T + 1% milk	ebioscience
Caspase3	39, 19, 17	rabbit	1:1000	TBS-T + 1% milk	Cell Signaling
IkBalpha	39	rabbit	1:200	TBS-T + 1% milk	Santa Cruz
LMP2	20	rabbit	1:500	TBS-T + 1% milk	abcam
LMP7	20	rabbit	1:1000	TBS-T + 1% BSA	laboratory stock
MECL-1	25	rabbit	1:5000	TBS-T + 1% BSA	laboratory stock
PA28alpha	28	rabbit	1:1000	TBS-T + 1% milk	Cell Signaling
PA28beta	28	rabbit	1:500	TBS-T + 1% milk	Cell Signaling
P44/42 MAPK (Erk1/2)	44, 42	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	44, 42	rabbit	1:2000	TBS-T + 1% BSA	Cell Signaling
PanErk p44/p42 MAPK	44,42	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling

(ERK1/2)					
p38 MAPK	38-43	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling
Phospho-p38 MAPK (Thry180/Tyr182)	38-43	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling
Pan-14-3-3	20-30	mouse	1:5000	TBS-T + 1% milk	Santa Cruz
Phospho-Akt (Ser473)	60	rabbit	1:2000	TBS-T + 1% BSA	Cell Signaling
PanAkt	60	rabbit	1:2000	TBS-T + 1% BSA	Cell Signaling
Vinculin	124	rabbit	1:1000	TBS-T + 1% milk	Cell Signaling

Table 4: List of antibodies for flow cytometry analyses

antibody	isotype	fluorphore	diluted in	company
CD11b	rat IgG2a	FITC	1xPBS +2 % FCS	ImmounoTools
CD11b	rat IgG2b	PE	1xPBS +2 % FCS	Becton, Dickinson
F4/80	rat IgG2a K	eflour 660	1xPBS +2 % FCS	ebioscience
CD86	rat IgG2a K	FITC	1xPBS +2 % FCS	ebioscience
CD86 (B7-2)	rat IgG2aK	PE	1xPBS +2 % FCS	ebioscience
CD80	hamster IgG	APC	1xPBS +2 % FCS	ebioscience
MHC-I (H-2Kb)	mouse IgG2a K	PE	1xPBS +2 % FCS	ebioscience
MHC-I (H-2Db)	mouse IgG2a K	APC	1xPBS +2 % FCS	ebioscience
Fixable Viability Dye	-	eFluor®780	1xPBS	ebioscience
Propidium iodide	-	PE	1xPBS +2 % FCS	Life technologies
Annexin V	-	APC	Annexin V binding buffer	ebioscience
CD16/CD32	-	unlabeled	1xPBS +2 % FCS	ebioscience
CD274 (B7-H1) PD-L1	rat IgG2a K	PE	1xPBS +2 % FCS	ebioscience

Buffers and media

DMEM: DMEM with Glutamax, 4500 mg/L D-Glucose,
10 % FCS
1 % penicillin / streptomycin
10 mM sodium Pyruvate

DMEM w/o phenol red: DMEM
10 % FCS

FACS buffer: 1x PBS pH 7.4
2 % FCS

LB media:
1 % yeast extract
0.5 % trypton / pepton
1 % NaCl
up to 1 liter with Aqua dest

LB agar:
1 % yeast extract
0.5 % trypton / pepton
1 % NaCl
10 g/l agar-agar
up to 1 liter with Aqua dest

5% stacking gel:
for 10 mL
6.3 mL dist. water
2.6 mL 0.5M Tris pH 6.8
1 mL acrylamide
50 µL 20 % SDS
50 µL 20 % ammonium persulfate
10 µL TEMED

10 % separation gel:
for 30 mL
18.1 mL dist. water
5.6 mL 2 M Tris pH 8.6
6 mL ProSieve^{TM} 50 Gel Solution
150 µL 20 % SDS
150 µL ammonium persulfate
12 µL TEMED

10 x PAGE buffer:
250 mM Tris
2 M glycine
35 mM SDS
Adjust to pH 8.3; dilute 1:10 with dist. water prior use

10 x transfer buffer:
250 M Tris
2 M glycine
10 % methanol, add prior use
adjust to pH 10; dilute 1:10 with dist. water prior use

10 x TBS-Tween:
100 mM Tris
1 M NaCl
1 % Tween 20
adjust to pH 7.6; dilute 1:10 with dist. water prior use

Stripping buffer:
100 mM 2-mercapto-ethanol
62.5 mM Tris
2 % SDS
adjust to pH 6.7

HBSM buffer:	150 mM NaCl 5 mM $MgCl_2$ 20 mM HEPES Adjust to pH 7.2
4 x Protein sample buffer:	1 mM DTT NuPAGE® LDS Puffer (4x)
lysis buffer 1:	50 mM Hepes pH 7.5 140 mM NaCl 5 mM $MgCl_2$ 1 mM EGTA pH 7.5 1 % NP-40 0.1 % lauryl maltoside 1:200 protease inhibitor cocktail
lysis buffer 2:	HBSM buffer 1:200 protease inhibitor cocktail 1 % Triton X-100 1 tablet / 10 mL PhosSTOP

3.2 Methods

3.2.1 Laboratory mice

β5i/LMP7 + β2i/MECL-1 and PA28αβ gene-deficient mice were provided by AJ. Sijts (University of Utrecht) and backcrossed to C57BL/6J background for 10 generations by in-house breeding (de Graaf et al. 2011). β5i/LMP7 + β2i/MECL-1 and PA28αβ gene-deficient mice are referred to as immunoproteasome knock-out mice (IP-K.O.) and the corresponding control mice C57BL/6J are referred to as wild-type mice (WT). The β5i/LMP7+β2i/MECL-1 and PA28αβ gene-deficient mice do not exhibit any phenotypic characteristics or abnormalities in growth or fertility (de Graaf et al. 2011). In order to confirm the absence of the genes β5i/LMP7 + β2i/MECL-1 and PA28αβ, the knock-out mice were regularly genotyped via PCR using the primer pairs listed in table 1. All animal experiments were performed with 8 to 12 weeks old mice which were kept in a specific-pathogen-free (SPF) facility at the University Hospital of Jena. The experiments were performed in accordance with the German legislation on protection of animals and under permission of the regional animal welfare committee of Thuringia.

3.2.2 Bacteria cultivation

The *Escherichia coli (E. coli)* serotype O18:K1:H7 (DSMZ 10724) was purchased from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ). *Escherichia coli* O18:K1:H7 is an extra intestinal pathogen (Johnson et al. 2001) and was originally isolated from an appendicitis patient (Achtman et al. 1983).

Bacteria were cultivated in Luria-Bertani (LB)-media at 37 °C, 5 % CO_2 under continuous shaking with 200 rpm until they reached exponential growth. Afterwards, a stimulation suspension of *E. coli* was prepared in *Dulbecco's phosphate-buffered saline* (PBS) with a defined amount of bacteria quantified by *Colony Forming Units* (CFUs). To ensure a stable bacteria number over long time stimulations (e. g. 24 hours) further growth of *E. coli* was stopped by mitomycin C. Mitomycin C is an antibiotic which at low concentrations inhibits DNA synthesis and thus, inhibits bacterial cell growth but does not kill the bacteria. In detail, 2 x 10^9 CFU_S of *E. coli* were incubated with 50 µg/mL mitomycin C for 2 hours at 37 °C, 5 % CO_2 and 200 rpm. Afterwards, growth arrested *E. coli* O18:K1:H7 were used for cell stimulation experiments.

3.2.3 Cultivation of L-929 cell line and preparation of differentiation media

The adherent murine fibroblast like cell line L-929 was purchased from ATCC. L929 cells constitutively express the macrophage-colony stimulating factor (M-CSF). The supernatant of this cell line was used to differentiate murine bone marrow cells into macrophages (see below). L929 cells were seeded on cell culture dishes and were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin / streptomycin at 37 °C and 5 % CO_2. After the cells reached confluence, cells were cultivated for additional 5 days. The cell supernatant was harvested daily and replaced with fresh media. The collected cell supernatant was sterile filtered (pore size 0.2 µM) followed aliquotation and storage at -20 °C.

3.2.4 Isolation and differentiation of bone marrow derived macrophages

In order to isolate bone marrow cells and differentiate them to *bone marrow derived macrophages*, wild-type and IP-K.O. mice were sacrificed by rapid cervical dislocation. Tibias and femurs were carefully dissected without cutting the bone ends followed by cleaning the bones from attached tissues. Unopened bones were sterilized with 70 % ethanol and stored in 1 x PBS on ice until further procedure. Bones were cut off on both ends and flushed out with 0.9 x sodium chloride using a syringe with a needle size of 25 gauge. Bone marrow cells were passed through a cell strainer (40 µm). The collected bone marrow cells were centrifuged at 500 g for 5 minutes. Afterwards, the supernatant was removed and the cells were resuspended with Ammonium-Chloride-Potassium (ACK)-lysis buffer to lyse erythrocytes. The erythrocyte lysis was stopped with excess amounts of PBS and cells were centrifuged by 500 g for 5 minutes. The supernatant was removed and cells were resuspended in DMEM supplemented with FCS, penicillin / streptomycin, sodium pyruvate and 20 % L-929 cell supernatant followed by subsequent incubation over night at 37 °C and 5 % CO_2 in cell culture dishes. On the following day, non-adherent cells, compromising hematopoietic progenitor cells, were transferred into new cell culture dishes and fresh DMEM supplemented with FCS, penicillin / streptomycin, sodium pyruvate and 20 % L-929 cell supernatant was added. On day 5 post-isolation cells have been grown confluent and were gently harvested using a cell scraper and transferred into new culture plates. The successful differentiation and maturation of bone marrow derived macrophages from bone marrow cells was confirmed by the detection of expression of the mature macrophage marker F4/80 and CD11b via flow cytometry as well as morphological analyses via light microscopy.

3.2.5 Macrophage stimulation experiments

For all cell stimulation experiments 100 Units of recombinant murine IFNγ and various concentrations of lipopolysaccharide from *E. coli* 0111:B4 (LPS-EB) were used. The precise amount of LPS used for in vitro macrophage stimulation is stated in the figure legend of the respective experiments. The used *multiplicity of infection* (MOI) i. e. the ratio

of *E. coli* per macrophage utilized in the experiments is indicated in the respective figure legends.

3.2.6 RNA isolation and reverse transcription

To quantify the relative gene expression of target genes upon stimulation, total RNA was isolated from 3×10^6 macrophages using the *Qiagen RNeasy mini kit* following the manufacturer's instructions. An additional DNA digestion was included to remove the residual genomic DNA using *DNaseI*. The total RNA was eluted in 20 µl RNase free water. The amount and quality of the RNA was analyzed using a *NanoDrop D-1000* Spectrophotometer (Thermo-Fisher Scientific). For quantitative Real- Time PCR analysis, 1 µg RNA was reverse transcribed into complementary DNA (cDNA) using the *High Capacity cDNA Reverse Transcription Kit*. PCR of the cDNA was carried out on a *S1000™ Thermal Cycler* in a 20 µl reaction volume containing: 2 µl RT-buffer (10x), 2 µl random primers (10x), 1 µl *MultiScribe® Reverse Transcriptase* (50 U/µL), 0.8 µl dNTPs (100 mM) and 1 µl RNAse inhibitor. The thermal conditions of the PCR protocol included an initial 10 minutes annealing step of the random primers at 25 °C, followed by extension of the complementary strand for 120 minutes at 37 °C and completed by 5 minutes at 85 °C to inactivate the reverse transcriptase. To protect the newly synthesized cDNA from DNases, the cDNA was resuspended in 180 µl 0.5 % Tris-EDTA Buffer pH 7.4 (TE-buffer) and stored at -20 °C until use for quantitative Real-Time PCR.

3.2.7 Primer design

Specific primer pairs for each target gene were self-designed with the help of three free online software tools. First, the *eEnsembl* (http://www.ensembl.org/Mus_musculus/Info/Index) tool was used to search for sequence annotation. Second, the *Primer Blast* tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) provided by the National Center for Biotechnology Information (NCBI) was used for primer design. Third, the *Mfold Web Server* (http://unafold.rna.albany.edu/?q=mfold/dna-folding-form) was used for analysing possible secondary structures.

The efficiency of the primers was evaluated by a standard curve based on a serial dilution of cDNA containing the target gene. The measured cycle threshold (Ct) values were plotted versus the logarithm of the sample concentrations. The data were subsequently fitted to the equation (1) with the help of the *Rotor Gene 6000* Software (Qiagen). The intercept of the standard curve corresponds to the Ct(1) of a diluted standard containing only a single target molecule (Kubista et al. 2006). The primer efficiency (E) was calculated from the slope (see equation (2)). Primer specificity of each target gene was examined by a melt curve analysis and the size of the PCR product was verified by gel electrophoresis. The sequences of all primers used for semiquantitative and quantitative Real-Time PCR are listed in table 2.

$$Ct = k \times \log(no) + Ct(1) \quad (1)$$

$$\text{Equation } E = \left(10 \times \frac{-1}{slope}\right) - 1 \quad (2)$$

3.2.8 Quantitative Real-Time PCR

The quantitative Real-Time PCR (qPCR) was conducted using a *Corbett Rotor-Gene 6000* (Qiagen). Each sample was analyzed in duplicates in a total reaction volume of 20 µl containing 10 µl of 2 × *SensiMix SYBR Master Mix* (Bioline) and 0.2 µM of each primer (see table 2). All qPCRs were set up using a *CAS-1200 pipetting robot* (Qiagen). The cycling conditions were 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. For each experiment a RT-negative sample was included as control containing sample RNA and nuclease free water. The relative expression of target genes was analyzed using a modified *Pfaffl* method (Pfaffl 2001, Rieu and Powers 2009). To determine significant differences in the mRNA expression between different experimental conditions, the relative quantity (RQ) for each sample was calculated using the equation (3), where E is the efficiency of the primer and Ct the threshold cycle (Klassert et al. 2014). The expression levels were normalized to the geometric mean of the housekeeping gene 60S ribosomal protein L13a B (Rrpl13a) and to the unstimulated control of the wild-type and IP-knock-out according to equation (4). The stability of the housekeeping genes was assessed using the *BestKeeper algorithm* (Pfaffl et al. 2004). The

normalized RQ (NRQ) values were log2-transformed for further statistical analysis with *GraphPad PRISM v5.0*.

$$RQ = \frac{1}{E^{\Delta CT}} \quad (3)$$

$$NRQ = \frac{E(target\ gene)^{\Delta CT(control-stimulation)}}{E(housekeeping\ gene)^{\Delta CT(control-stimulation)}} \quad (4)$$

3.2.9 Western blot analysis

In order to analyze TLR-4 signaling of wild-type and immunoproteasome-deficient macrophages, short time stimulation (0 min, 15 min, 30 min, 60 min) with (i) 1 µg/mL LPS, (ii) 100 Units/mL IFNγ or (iii) combination of both were performed in 6 well plates with 2 x 10^6 macrophages. After stimulation cells were washed with ice-cooled PBS followed by cell lysis using lysis buffer 1 or lysis buffer 2. Cells were scraped and the cell lysates were transferred into vials followed by 15 minutes incubation on ice. Afterwards, samples were centrifuged for 15 minutes at maximum speed at 4 °C and cleared lysates were transferred into vials containing 5x sample buffer followed by boiling at 95 °C for 5 minutes and storage at -20 °C. The total protein concentrations of the cell lysates were calculated based on a bovine serum albumin (BSA) standard curve using Pierce™ BCA Protein Assay Kit and a TECAN plate reader. Equal volumes of protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10 % gradient separation gel and a 5 % stacking gel. Gels were run in 1x PAGE buffer at 45 mA and max. 400 Volt for approximately 100 minutes using the *PROTEAN® II xi Cell* (Bio-Rad) electrophoresis apparatus. A wet/tank blotting system (*Trans-Blot®* Cell, Bio-Rad) was used to transfer the proteins from the gel matrix to a polyvinylidene fluoride (PVDF) membrane. The protein transfer was performed at 0.75 mA per gel for 100 minutes. Subsequently, the membranes were blocked with 1x TBS-T + 1 % BSA for 30 minutes and were cut into pieces in order to detect several proteins with different protein sizes at the same time. Membranes were incubated with primary antibody dilutions over night at 4 °C under continuous shaking. The antibodies used for analysis of signaling proteins are listed in table 3. After incubation with the primary antibody, membranes were washed with 1x TBS-T followed by incubation with the respective secondary antibodies for 2 hours at room temperature or overnight at 4 °C under continuous shaking.

Membranes were washed with 1x TBS-T and 1 x TBS. For protein detection, membranes were soaked with *Oxidizing Reagent Plus* and *enhanced Luminol Reagent Plus* (PerkinElmer Inc.) for 1 minute. The horseradish peroxidase-catalysed chemiluminescence reaction was detected with the *FujiFilm LAS-3000* (FujiFilm) imaging system. In order to remove primary and secondary antibodies after protein imaging, membranes were incubated with stripping buffer for 30 minutes at 50 °C. After membrane stripping, membranes were rinsed with distilled water and washed with 1x TBS-T followed by a new protein detection cycle.

3.2.10 Cytokine detection

To characterize the cytokine protein expression / secretion profile upon stimulation with IFNγ and *E. coli*, 0.1 x 10^6 macrophages were stimulated for 24 hours at 37 °C and 5 % CO_2. After stimulation the cell supernatants were stored at -20 °C until further analyses using *ProcartaPlexTM Immunoassay Kit* (eBioscience) according to the manufacturer's instructions.

3.2.11 Detection of nitric oxide

Nitric oxide (NO) is a free radical and important cellular signaling molecule which is produced by macrophages in response to inflammatory stimulation. Nitric oxide has a short half-life is rapidly oxidized to nitrite. To characterize the NO production of wild-type and immunoproteasome-deficient cells, 0.1 x 10^6/mL cells were seeded and stimulated with (i) IFNγ, (ii), LPS, (iii) growth arrested *E. coli*, (iv) LPS + IFNγ and (v) *E. coli* + IFNγ for 24 hours at 37 °C and 5 % CO_2. Afterwards, the cell-free supernatant was transferred in a 96 well plate and stored at -20 °C until further use. Nitric oxide was indirectly measured by detection of nitrite in the cell supernatant using Griess Reagent containing *naphthylethylenediamine dihydrochloride* and *sulphanilamide* in phosphoric acid. A standard curve of nitrite ranging from 160 µM to 2,5 µM was prepared. 50 µl of either nitrite standards or samples were transferred to a flat bottom 96 well plate and 50 µl of Griess Reagent was added. This Griess Reagent converts nitrite to a deep purple azo compound and the absorption intensity was measured at 550 nm using an *Infinite M200 reader* (Tecan). The amount of the azochromophore reflects the amount of nitrite and thus,

the concentration of NO within the samples. The concentration of nitric oxide was calculated from the nitrite standard curve.

3.2.12 Detection of intracellular reactive oxygen species

To monitor *reactive oxygen species* (ROS) upon stimulation with IFNγ, *E. coli* and *E. coli* + IFNγ macrophages were incubated for 30 minutes with the non-flourescent cell permeable 2',7'-*dichlorodihydrofluorescein diacetate* (H2DCFDA) at 37 °C and 5 % CO_2. Afterwards, macrophages were washed and incubated with IFNγ, *E. coli* or a combination of both for 6 hours. The production of reactive oxygen species within the macrophages leads to deacetylation of H2DCFDA which is thereby converted to the fluorescent 2′,7′-dichlorofluorescein (DCF). The DCF was subsequently detected after 6 hours stimulation with an *Infinite M200 reader* (Tecan) measuring the fluorescence intensity at 532 nm emission upon excitation at 488 nm.

3.2.13 Phagocytosis assay

To analyze the capacity of the macrophages to engulf bacteria, 1 x 10^6 macrophages were incubated for 30, 60 and 90 minutes with 1 x 10^7 opsonized *fluorescein isothiocyanate* (FITC)-conjugated *E. coli BioParticels*. Phagocytosis was stopped by ice-cooled PBS. Macrophages were washed, mechanical detached and resuspended in PBS supplemented with 2 % FCS followed by flow cytometry analysis. Preparation of macrophages for flow cytometry included the removal of any bacterial particles from the surface of the macrophages in order to exclusively measure engulfed intracellular bacteria. Discrimination of live and dead macrophages was performed using flow cytometry analysis with propidium iodide staining.

3.2.14 Intracellular killing assay

To characterize the ability of macrophages to kill engulfed *E. coli* intracellularly, 1 x 10^6 macrophages were incubated with 1 x 10^7 CFU/mL growths arrested *E. coli* representing a multiplicity of infection (MOI) of 10 at 37 °C and 5 % CO_2 for 90 minutes. After stimulation several dilutions of the cell supernatant were prepared and plated on LB agar to quantify the following day the colony forming Units (CFUs) of the extracellular

(non-phagocytosed) bacteria. In order to remove remaining extracellular bacteria, macrophages were washed with PBS and incubated 2 hours with 100 µg/mL gentamycin. Afterwards cells were washed with PBS and lysed by adding 0.02 % Triton X using *lysing matrix* tubes and a *FastPrep-24* homogenisator (MP Biomedicals). In order to determine the number of intracellular (engulfed) bacteria, serial dilutions of the cell lysates were plated on LB agar plates and next day CFU's were determined. The number of killed bacteria was calculated by subtracting the sum of the number of extracellular and intracellular determined *E. coli* from the total number of initially used *E. coli*.

3.2.15 Flow cytometry

To analyze the expression of target cell surface proteins in the respective experiments, macrophages were washed with PBS, mechanical detached and resuspended in PBS containing 2 % FCS. In order to prevent unspecific antibody binding, Fc-receptors of macrophages were blocked by incubation with mouse CD16/CD32 antibodies. Afterwards, cells were washed with PBS and stained with indicated antibodies or corresponding isotype controls (see table 4). Due to safety requirements cells were fixed with *paraformaldehyde* prior to flow cytometry analyses. Flow cytometry data were acquired using an *Attune® Acoustic Focusing Cytometer* (Life Technologies) and analyzed with *FlowJo software* (TreeStar Inc., Ashland, USA). The general gating strategy for flow cytometry analyses consisted of three principle steps. First, duplet cell aggregates were excluded from further analyses by FSC-H vs. FSC-A gating. Second, singlet cells were differentiated according their respective viability determined by staining with propidium iodide or fixability viability dye. Third, viable singlet cells were included for the analysis of the surface expression of the target protein.

3.2.16 Statistical analyses

Statistical analyses were performed using *GraphPad PRISM v5.0 software* (San Diego, USA). Unless stated otherwise, the following statistical tests were used in order to evaluate statistical significant differences:

- TWO-way ANOVA and Bonferroni post-test with a 95 % confidence interval was used for gene expression data
- Wilcoxon Mann-Whitney U-test with a 95 % confidence interval and a two-tailed p value was used for protein expression data
- Student t-test with a 95 % confidence interval

4. Results

4.1 Characterization of the primary macrophage cell model

4.1.1 In vitro differentiation of bone marrow derived macrophages

The present study aimed to investigate the role of the *immunoproteasome* (IP) in the regulation of innate and adaptive immune responses of macrophages with particular focus on antibacterial macrophage effector functions. For this purpose, *bone marrow derived macrophages* (BMDMs) were selected as a primary cell model since BMDMs share biological and physiological features with peripheral macrophages.

In the present study BMDMs were generated by in vitro differentiation of freshly isolated *bone marrow cells* (BMCs) in the presence of the *murine macrophage colony stimulating factor* (M-CSF). M-CSF is a lineage-specific growth factor which is involved in proliferation, survival and differentiation of bone marrow cells into cells of the macrophage lineage (Hamilton 2008). The differentiation of bone marrow cells into macrophages is associated with an increased expression of distinct surface marker proteins and a morphological change of the cell.

To confirm the differentiation of macrophages from bone marrow cells under the used experimental setting, the expression patterns of the mature macrophage-specific surface proteins F4/80 and CD11b were analyzed via flow cytometry. Isolated BMCs were analyzed immediately after isolation (day 0) and over the course of six days cultivation (day 1 to day 6) in the presence of M-CSF (Fig. 5). The percentage of CD11b and F4/80 double-positive cells increased from 14 % on the day of isolation to 92 % on day six post-isolation (Fig. 5A). In addition to flow cytometry analyses, the morphological appearance of bone marrow cells was monitored by light microscopy (Fig. 5C). The morphology of the isolated cells changed from small round shaped non-adherent bone marrow cells (day 0 to day 2) to long branched adherent macrophages (day 6). These results show that the used experimental setting is appropriate to generate a sufficient pure population of mature BMDMs in vitro.

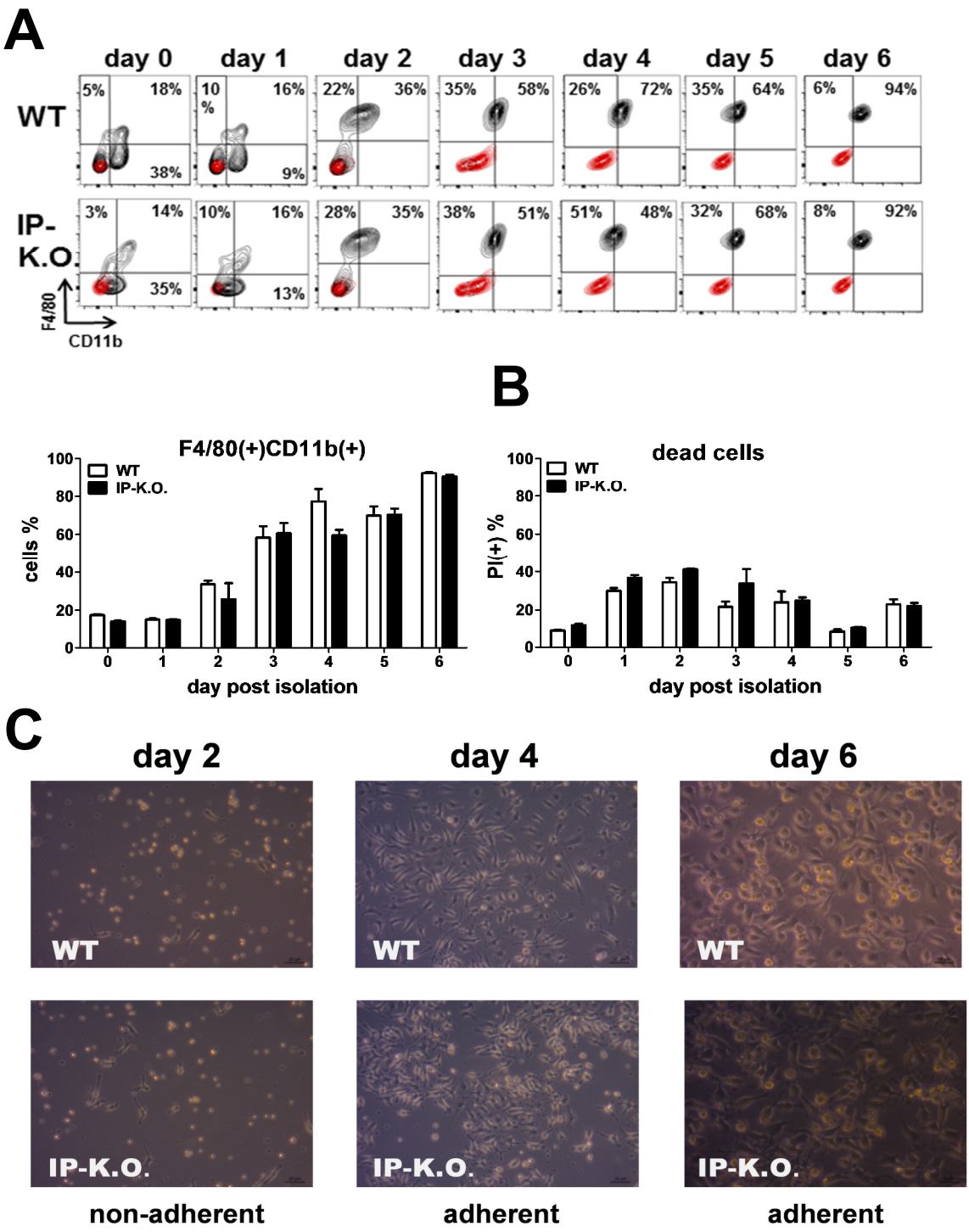

Figure 5. In vitro differentiation of bone marrow derived macrophages
Flow cytometry analysis of bone marrow derived macrophages from wild-type and immunoproteasome-knockout mice over the course of six days post isolation of bone marrow cells using anti-F4/80 and anti-CD11b surface markers. **(A)** Representative flow cytometry contour plots are depicted with F4/80 and CD11b double staining (black) and corresponding isotype controls (red). Percentage of F4/80 and CD11b double positive cells immediately after isolation (0 day) until day six of in vitro differentiation. **(B)** Percentage of dead cells within the BMDM population of WT and IP-K.O. macrophages determined with propidium iodide (PI) staining followed by flow cytometry analysis. All data are presented as means + standard error of mean (SEM) with at least four mice per group. **(C)** Light microscopic images of bone

marrow cells on day 2, day 4 and day 6 post-isolation. 20 x magnification on day 2 and day 4, 40 x magnification on day 6.

By comparing the differentiation of IP-K.O. and wild-type macrophages, both experimental groups showed a similar expression pattern of the macrophage surface markers CD11b and F4/80 as well as the same morphological appearance. Furthermore, over the course of the in vitro differentiation, the number of dead cells was determined using propidium iodide staining followed by flow cytometry analysis (Fig. 5B). The number of non-viable cells did not differ between IP-K.O. macrophages and their wild-type counterparts.

In summary, both WT and IP-K.O. macrophages show the same differentiation capacity in terms of morphological appearance and expression of mature macrophage surface marker proteins. For all experiments described this in vitro macrophage differentiation protocol was used ensuring comparable inter-experimental results.

4.1.2 Immunoproteasome-subunit expression of wild-type and IP-K.O. macrophages upon IFNγ and LPS stimulation

Until now, no data exist for IP-subunit expression patterns on protein level for peripheral macrophages. In this context it has been shown that the IP-subunits are constitutively expressed in vivo in immune cells including professional antigen-presenting cells (APCs), e. g. human dendritic cells (Ebstein et al. 2012) (see introduction).

To investigate the IP-subunit expression on protein level in macrophages, protein lysates of non-stimulated and LPS-stimulated macrophages from wild-type and LMP7/β5i+MECL-1/β2i and PA28αβ gene-deficient mice were prepared followed by western blot analyses using specific antibodies against the IP-subunits and the subunits of the activator complex PA28 (Fig. 6A). As expected, in protein lysates of the wild-type macrophages the immunoproteasome-subunits LMP7 and MECL-1 and the activator ring subunits PA28α and PA28β were detected, while LMP7/β5i+MECL-1/β2i and PA28αβ gene-deficient macrophages did not show an expression of these subunits. Exposure of the wild-type macrophages with LPS for one hour did not further enhance the protein expression of the studied IP-subunits. These results indicate that wild-type macrophages constitutively express the immunoproteasome-subunits LMP7 and MECL-1 and the activator ring subunits PA28α and PA28β independently of inflammatory conditions. Moreover, these

experiments confirm the IP-deficient phenotype of the utilized genetically modified IP-K.O. mouse strain.

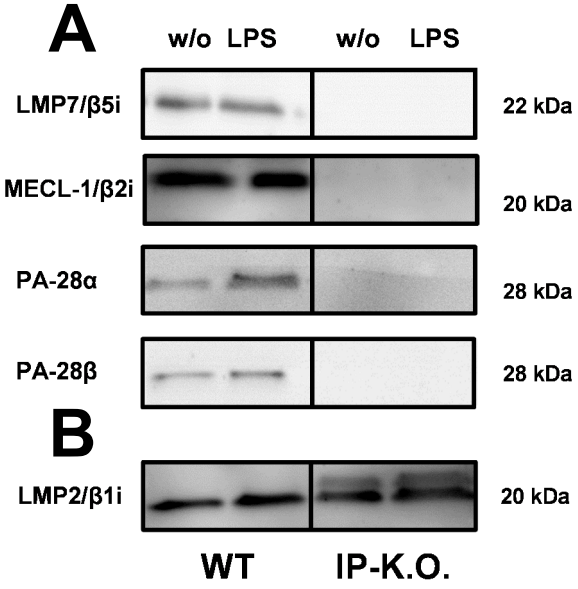

Figure 6. IP-subunit protein expression of WT and IP-K.O. macrophages
Macrophages were stimulated with LPS (1 µg/mL) or non-stimulated (w/o) for 1 hour followed by cell lysis. Cell lysates were prepared and subjected to standard western blot analysis. **(A)** Total protein levels of the immunoproteasome subunits LMP7/β5i and MECL-1/β2i as well as the activator complex PA28α and PA28β. **(B)** Total protein levels of the immunoproteasome subunit LMP2/β1i. Protein marker sizes in kilo Dalton (kDa) are shown on the right side of the panel.

Furthermore, the protein expression of the third immunoproteasome-subunit LMP2 was investigated in both experimental groups (Fig. 6B). In the protein lysate of the wild-type macrophages one distinct protein band was detected for LMP2, while LMP7/β5i+MECL-1/β2i and PA28αβ gene-deficient macrophages showed two distinct protein bands. In addition to the 20 kDa LMP2 protein band, a second band was observed with a molecular weight of approx. 25-30 kDa. This additional band might present the pre-form of unprocessed LMP2 as described for other LMP7 deficient cells (De et al. 2003). Since 20S immunoproteasomes containing LMP2/β1i inefficiently assemble in the absence of LMP7/β5i, cells of the used LMP7/β5i+MECL-1/β2i and PA28αβ K.O. mice contain predominantly standard proteasomes and are referred to as IP-K.O. macrophages in the present study(de Graaf et al. 2011, De et al. 2003).

4.1.3 Transcriptional expression of genes involved in recognition of *E. coli*, LPS and IFNγ

To study the role of the immunoproteasome in macrophage immune responses to bacterial infections, IFNγ, viable *E. coli* (± IFNγ) and LPS (± IFNγ) were employed as bacterial immune triggers in the present study. Appropriate recognition of *E. coli* and LPS by macrophages requires the expression of the macrophage surface proteins CD14 and the Toll-like receptor 4 / MD-2 complex (see introduction). To confirm the expression of these genes in the in vitro differentiated wild-type and IP-deficient primary macrophages, a semi-quantitative PCR was conducted using appropriate primer pairs. Transcriptional expression of CD14, TLR4 and MD-2 was verified for IP-deficient and wild-type macrophages by a clear PCR product (Fig. 7A). Furthermore, the transcriptional expression of the IFNγ receptor subunits IFNGR1 and IFNGR was confirmed in both experimental groups by semi-quantitative PCR (Fig. 7B).

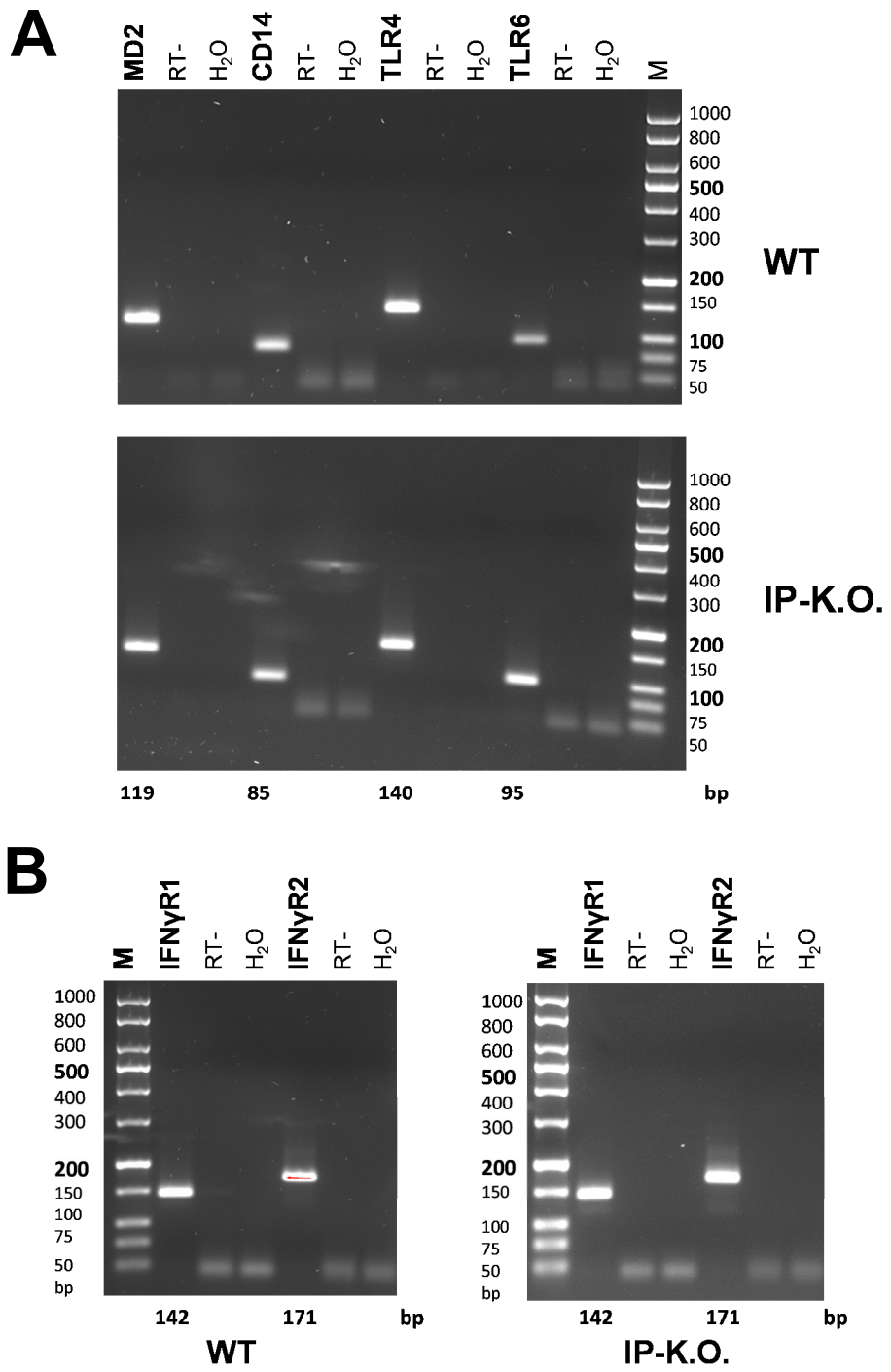

Figure 7. Gene expression of CD14, MD-2, TLR 4, 6 and IFNγ receptor
The gene expression was analyzed by semi-quantitative PCR and gel electrophoresis of PCR products using a 2 % agarose gel. **(A)** Semi-quantitative PCR products of depicted genes in wild-type and IP-K.O. macrophages. **(B)** Semi-quantitative PCR products of the IFNγ receptor subunits IFNγR1 and IFNγR2 in wild-type and IP-K.O. macrophages. DNA fragment sizes are listed in base pairs (bp). RT- = reverse transcription negative control, M = DNA low range marker.

4.1.4 Immunoproteasome gene expression upon stimulation in wild-type macrophages

Studies using different immunoproteasome cell models have revealed that IFNγ and LPS significantly alter the subunit composition of the immunoproteasome on transcriptional level (Reis et al. 2011a). To determine whether these immune triggers induce IP-subunit expression in the macrophage cell model used in this study, macrophages of wild-type mice were stimulated with IFNγ and LPS derived from *E. coli* (Fig. 8). Additionally, a combination of both immune triggers (IFNγ + LPS) was included in all experiments. IFNγ is able to enhance the LPS response in macrophages, a process that is often referred to as cell priming (see introduction section) (Schroder et al. 2004). After stimulation of macrophages with the described immune triggers for 2 and 8 hours, RNA was extracted followed by analysis of the gene expression of the IP-subunits LMP2, LMP7 and MECL-1 as well as the PA28 subunits using quantitative PCR. After 2 hours macrophage stimulation no or only a weak upregulation of the analyzed genes was observed (Fig. 8). In contrast, 8 hours after stimulation all IP-subunit genes (LMP2, LMP7 and MECL-1) were markedly upregulated under IFNγ or LPS stimulation (Fig. 8A). Under LPS stimulation macrophages exhibit a two-fold higher IP-subunit expression compared to IFNγ treatment. The combination of both triggers (IFNγ + LPS) did not lead to an enhancement of the LPS-induced subunit gene expression in macrophages. Hence, IFNγ has no amplifying effect on the gene expression of IP-subunits as described for other genes, e. g. iNOS (Schroder et al. 2004). With respect to the activator complex PA28, a different gene expression pattern was observed (Fig. 8B). IFNγ treatment of macrophages resulted in upregulation of the PA28β subunit, while PA28α expression was not altered. In contrast, LPS stimulation induced the upregulation of both activator complex subunits PA28α and PA28β. Furthermore, the macrophage gene expression of the PA28β subunit was eight-fold higher than gene expression of the PA28α subunit possibly owing to a constitutive PA28α gene expression in macrophages.

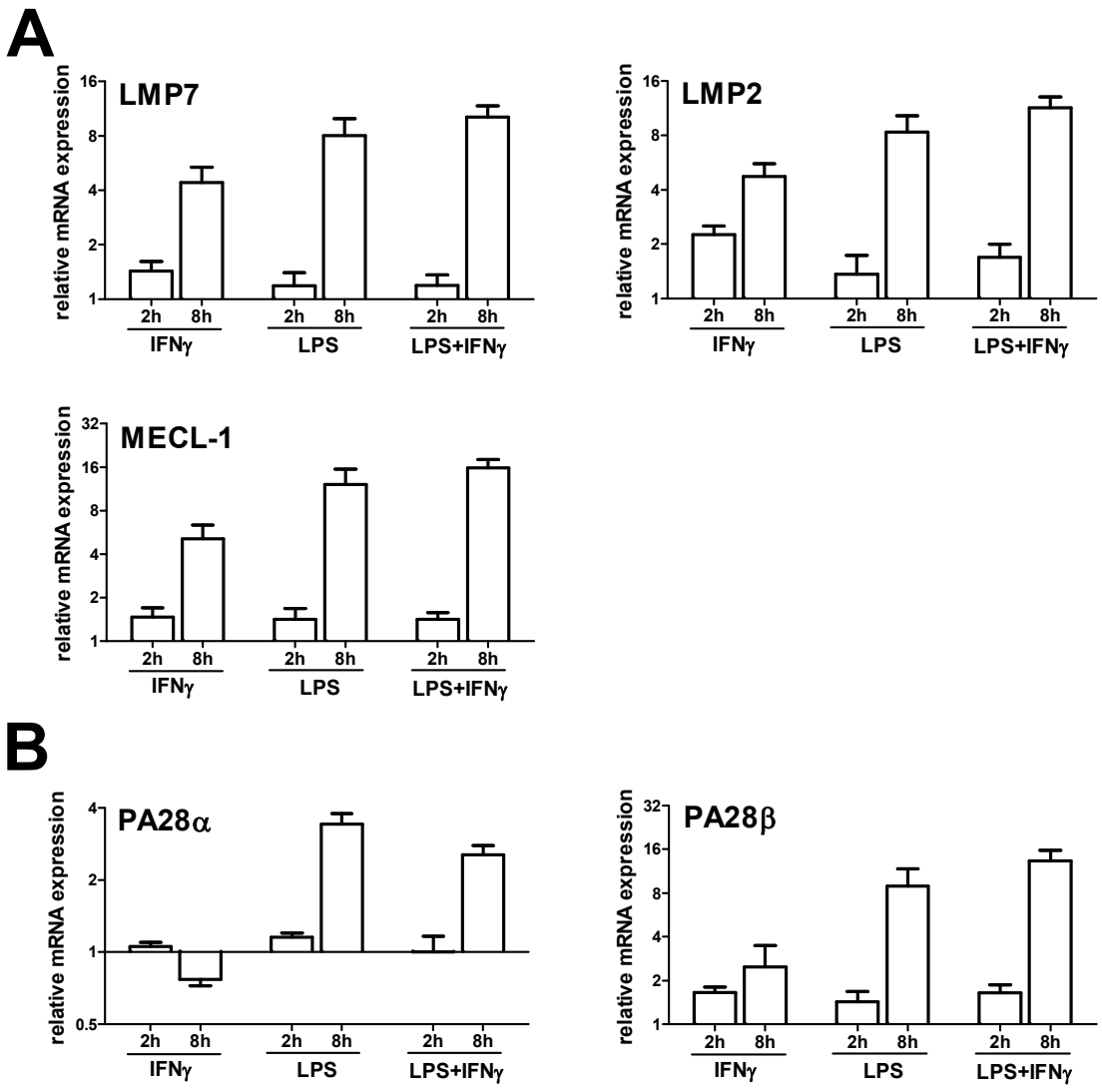

Figure 8. Gene expression of IP-subunits and PA28α/β in WT macrophages
Relative mRNA expression levels of the **(A)** immunoproteasome-subunits LMP7, LMP2 and MECL-1 and **(B)** the proteasome activator 28 subunits (PA28α and β) were measured by quantitative PCR. Macrophages were stimulated with IFNγ (100 Units/mL), LPS (1 μg/mL) or a combination of LPS and IFNγ for 2 and 8 hours. Data are presented as means (n=3) + standard error of mean (SEM) of the fold-change relative to non-stimulated macrophages.

In summary, the two hour macrophages stimulation with IFNγ and LPS did not result in a pronounced induction of the gene expression of immunoproteasome subunits. In contrast, prolonged macrophage stimulation (8 hours) strongly induced gene expression of the IP-subunits studied. Hence, these results convincingly show that IFNγ and LPS are bacterial immune triggers that are capable to induce IP-subunit expression in the utilized primary macrophage cell model.

To investigate a potential compensation of the absence of the immunoproteasome subunits LMP7 and MECL-1 by upregulation of the standard proteasome subunits beta1, beta2 and beta5 in IP-deficient macrophages, the gene expression of these subunits was studied by quantitative PCR (Fig. 9). No differences in the expression levels of the standard proteasome subunits were observed between wild-type and IP-K.O. macrophages after 2 and 8 hours stimulation. Thus, no compensatory effect of the K.O. macrophages on the transcriptional level was found.

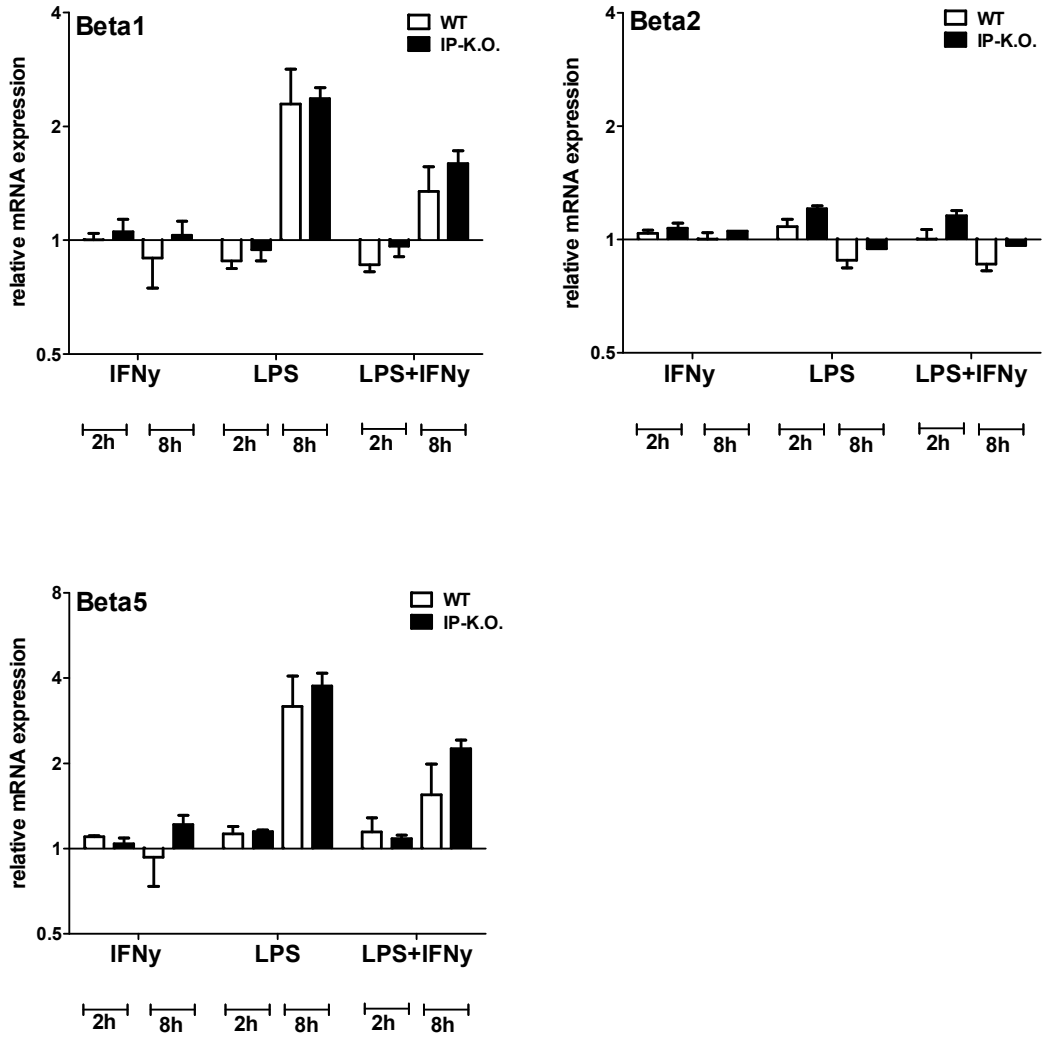

Figure 9. Gene expression profile of standard proteasome subunits in WT and IP-K.O. macrophages
Relative mRNA expression of the standard proteasome subunits beta1, beta2 and beta5 were measured by quantitative PCR. Macrophages were stimulated with IFNγ (100 Units/mL), LPS (1 µg/mL) or a combination of LPS and IFNγ for 2 and 8 hours. Data are presented as means (n=3) + standard error of mean (SEM) of the fold change relative to unstimulated macrophages. Significant differences were evaluated using a TWO-way ANOVA and Bonferroni post-test.

4.2 The role of the immunoproteasome in innate macrophage effector functions

4.2.1 IP-deficient macrophages exhibit an altered cytokine secretion profile

The expression and secretion of immunomodulatory cytokines and chemokines upon exposure to microbial triggers are major effector functions of macrophages. Cytokines and chemokines are central mediators of the adaptive and innate immune system.

The present study investigated the role of the immunoproteasome in the macrophage cytokine / chemokine response by in vitro stimulation of IP-deficient and wild-type macrophages with IFNγ, LPS and *E. coli* (Fig. 10 – 12). To study the immunomodulatory cytokine / chemokine response in its entire complexity, the expression of (i) pro-inflammatory, (ii) anti-inflammatory as well as (iii) chemotactic mediators were examined. IFNγ, IFNβ, IL-6, IL-1β, TNFα and G-CSF were investigated, since they represent key cytokines of the pro-inflammatory immune response, whereas IL-10 production was studied as a major anti-inflammatory immune mediator. The chemotactic response of macrophages was assessed via measurement of the expression of CCL2 (MCP-1), CCL4 (MIP-1β), CCL5 (RANTES), CXCL-1 (GRO-α) and CXCL10 (IP-10) which are involved in immune cell recruitment. To account for the multifaceted regulation of protein expression, the expression profile was studied on the transcriptional level by quantitative PCR (Fig. 10) as well as on the level of secreted proteins using ELISA (Fig. 11 and 12). To meet the complex temporal kinetics of the mRNA induction of the studied cytokines, two time points (2h and 8h) were chosen reflecting an early immune response (2h) and a late immune response (8h).

Neither after 2 hours (data not shown), nor after 8 hours stimulation, differences in the transcriptional activation of IL-1β, IL-6, KC, IL-10, TNFα and CCL2 were observed between wild-type and IP-K.O. macrophages suggesting that the IP is not involved in the gene regulation of these effector cytokines / chemokines (Fig. 10).

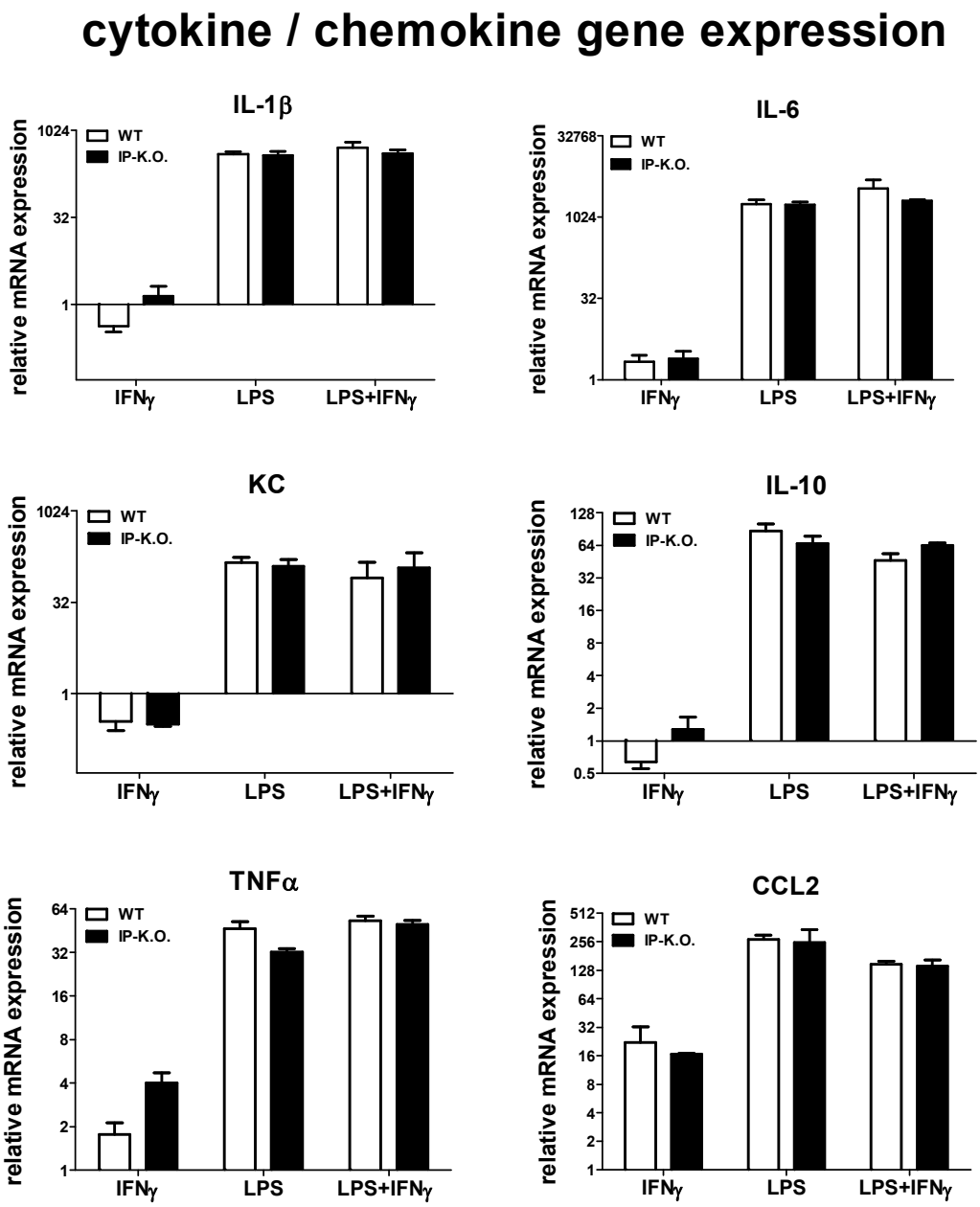

Figure 10. IP-deficient macrophages do not show an altered cytokine / chemokine expression on transcriptional level
Relative mRNA expression of the depicted cytokine genes and the chemokine CCL2 were measured by quantitative PCR. Macrophages were stimulated with IFNγ (100 Units/mL), LPS (1 µg/mL) or a combination of LPS and IFNγ for 8 hours. Data are presented as means (n=3) + standard error of mean (SEM) of the fold change relative to non-stimulated macrophages. Significant differences were evaluated using a TWO-way ANOVA and Bonferroni post-test.

In contrast, on protein level IP-deficient macrophages showed an altered cytokine / chemokine response compared to wild-type macrophages upon stimulation with IFNγ, LPS and *E. coli* for 24 hours (Fig. 11 and 12). A compromised cytokine secretion

was detected for the pro-inflammatory cytokines IL-6, IFNβ and IFNγ in IP-K.O. macrophages compared to wild-type macrophages (Fig. 11B, D, G). In contrast, IL-10, IL-1β and the chemokine CLL4 were elevated in IP-K.O. macrophages (Fig. 11A, F and Fig. 12B). TNFα secretion levels as well as the secretion patterns of the chemokines CCL2, CCL5, CXCL-1 and CXCL-10 were similar between IP-K.O. and wild-type macrophages under all studied inflammatory conditions (Fig. 11E and Fig. 12A, D, E, C). Interestingly, a bivalent effect of the lack of immunoproteasome activity was observed for the secretion pattern of G-CSF (Fig. 11C). Upon LPS and *E. coli* stimulation a compromised secretion of G-CSF was observed in IP-K.O. macrophages while under *E. coli* stimulation in presence of IFNγ an increased secretion was detected.

In summary, the results described above indicate that the immunoproteasome is involved in the cytokine / chemokine response in macrophages acting on post-transcriptional level rather than on transcriptional level. However, the role of the IP in cytokine / chemokine regulation during inflammation appears to be complex since some mediators are upregulated while others are reduced in the absence of the immunoproteasome.

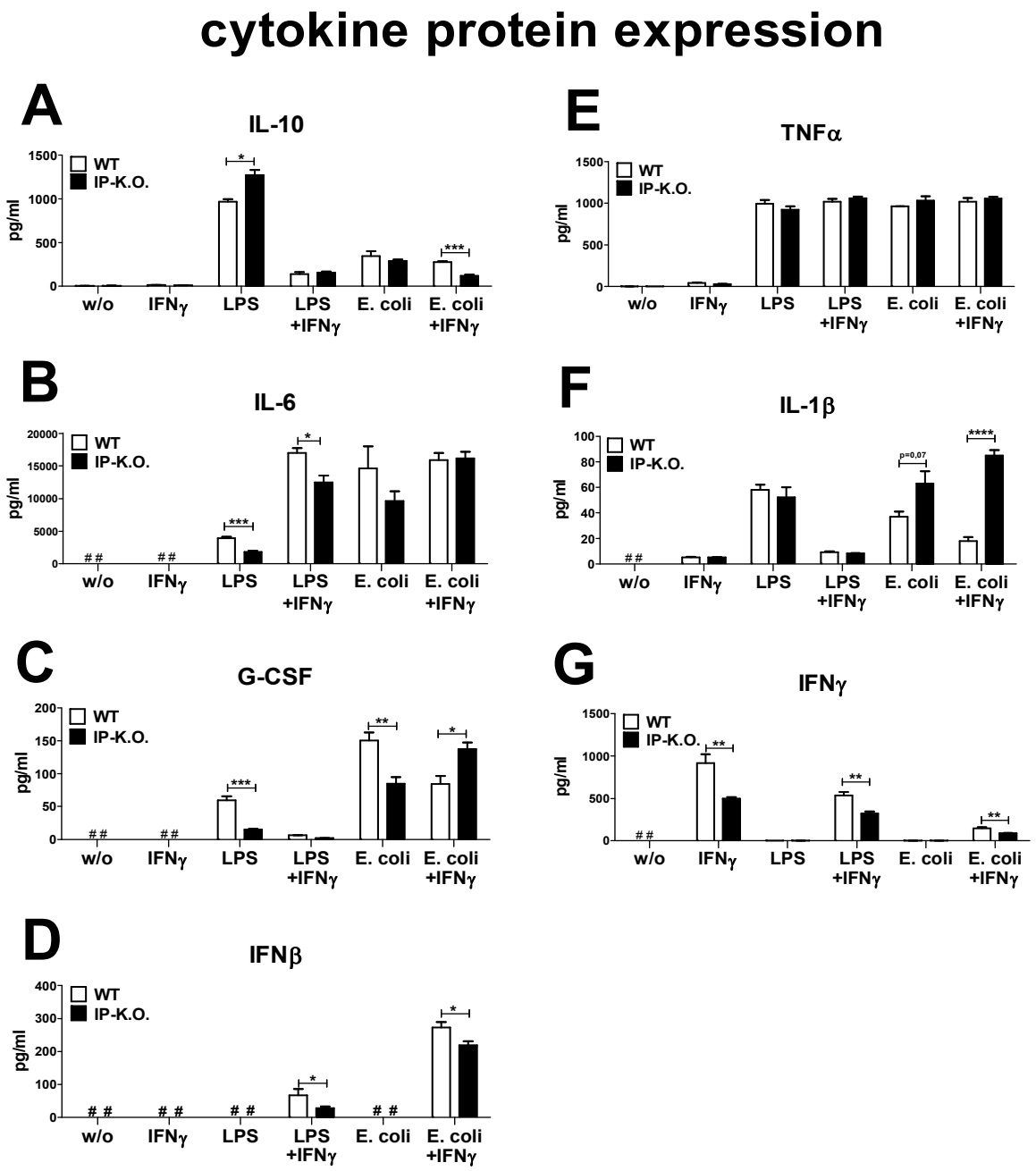

Figure 11. IP-deficient macrophages show an altered cytokine protein expression
Macrophages were stimulated with IFNγ (100 Units/mL), LPS (10 ng/mL), *E. coli* (multiplicity of infection [MOI] 0.1) and LPS in combination with IFNγ for 24 hours. Cell supernatants were analyzed for the indicated cytokines **(A - G)** by multiplex ELISA. Data are presented as means + standard error of mean (SEM) with at least three animals per group. Significant differences were evaluated using student t-test with a 95% confidence interval (* $0.01 \leq p \leq 0.05$; ** $0.001 \leq p < 0.01$; *** $p < 0.001$. # below limit of detection, w/o without stimulation).

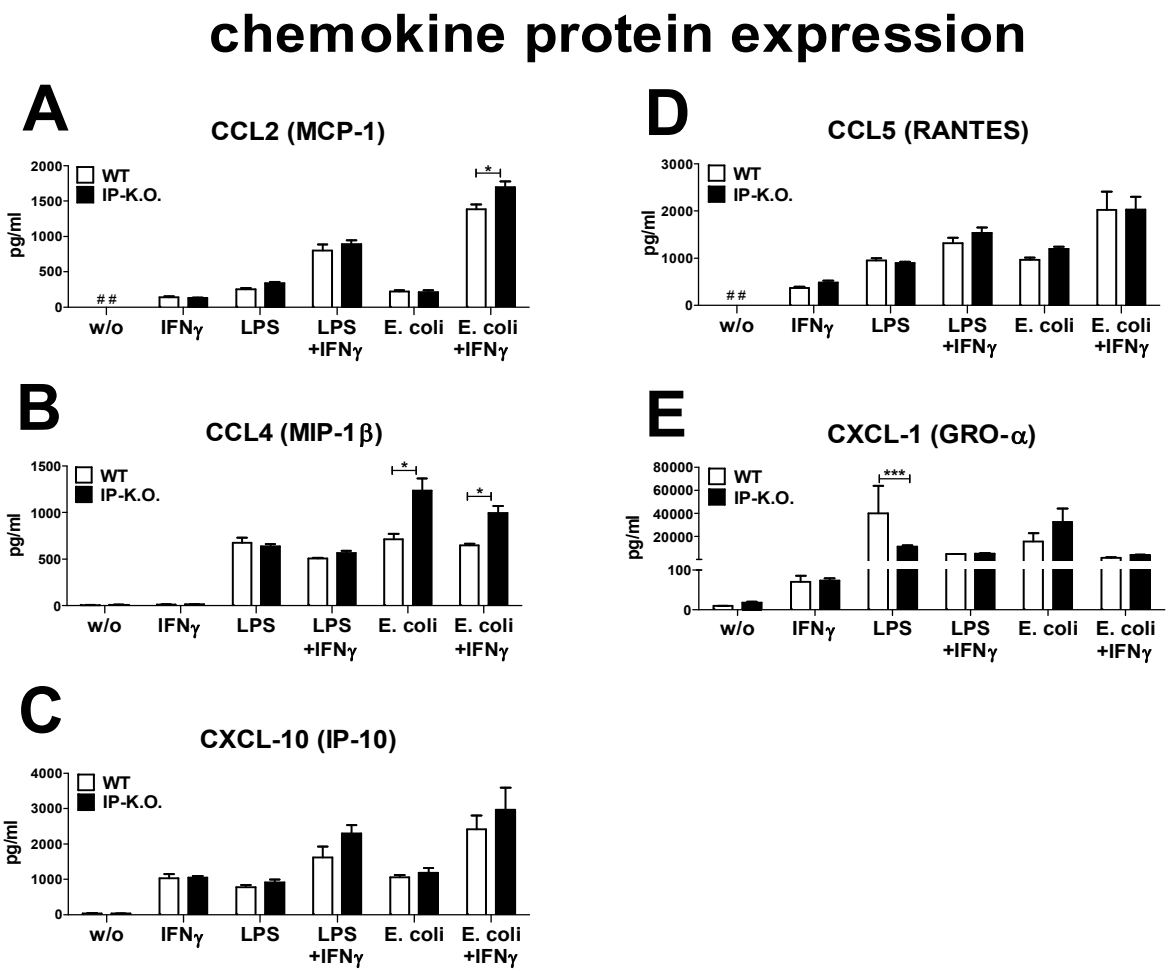

Figure 12. IP-deficient macrophages show an altered chemokine protein expression
Macrophages were stimulated with IFNγ (100 Units/mL), LPS (10 ng/mL), *E. coli* (multiplicity of infection [MOI] of 0.1) and LPS in combination with IFNγ for 24 hours. Cell supernatants were analyzed for the indicated chemokines **(A - E)** by multiplex ELISA. Data are presented as means + standard error of mean (SEM) with at least three animals per group. Significant differences were evaluated using student t-test with a 95% confidence interval (* $0.01 \leq p \leq 0.05$; ** $0.001 \leq p < 0.01$; *** $p < 0.001$. w/o without stimulation, # below limit of detection, w/o without stimulation).

4.2.2 The lack of immunoproteasome activity does not impact intracellular TLR4 signaling of macrophages

The experiments presented in figure 10 indicate that the cytokine and chemokine gene expression under LPS exposure is not altered in IP-deficient macrophages on transcriptional level suggesting that TLR4 -mediated induction of cytokine and chemokine genes is not modulated by immunoproteasome activity. In order to in investigate this notion in more detail, the activation pattern of crucial TLR4 and IFNγ signaling molecules upon stimulation with LPS (± IFNγ) and IFNγ was studied in IP-K.O. and wild-type macrophages. Activation of TLR4 / IFNγ signaling by bacterial trigger leads to the

induction of various interconnected signaling cascades followed by the activation of transcription factors that regulate the induction of cytokine / chemokine expression as well as other cellular processes (for detailed description of TLR4 / IFNγ signaling see introduction). To examine whether the immunoproteasome is involved in the activation of crucial TLR4 signal molecules, the activation / phosphorylation kinetics of AKT, ERK and p38 MAPK and the degradation of IκBα were assessed via western blot analyses (Fig. 13).
To account for the different temporal kinetics of activation and degradation of the studied signaling proteins, macrophages of both experimental groups were stimulated for 0, 15, 30 and 60 minutes with IFNγ, LPS and a combination of both triggers (Fig. 13). IFNγ treatment led to a very weak phosphorylation / activation of ERK, p38 and AKT in both experimental groups exhibiting no differences. In contrast, upon LPS and LPS + IFN*γ* treatment AKT, ERK and p38 were markedly phosphorylated / activated as indicated by strong distinct protein bands in both experimental groups. However, the activation pattern of the three studied signaling molecules was indistinguishable between IP-K.O. and wild-type macrophages suggesting no involvement of the immunoproteasome in the activation of TLR4 signaling molecules. Furthermore, upon LPS and LPS + IFNγ treatment no altered IκBα-degradation kinetics were observed in IP-K.O. macrophages suggesting that the IP does not alter NFκB activation in the investigated period of time.
In summary, the results of the signal transduction analyses of mayor signaling molecules as well as the degradation of IκBα indicate that the immunoproteasome is not involved in early induction of the proximal TLR4 signaling cascade.

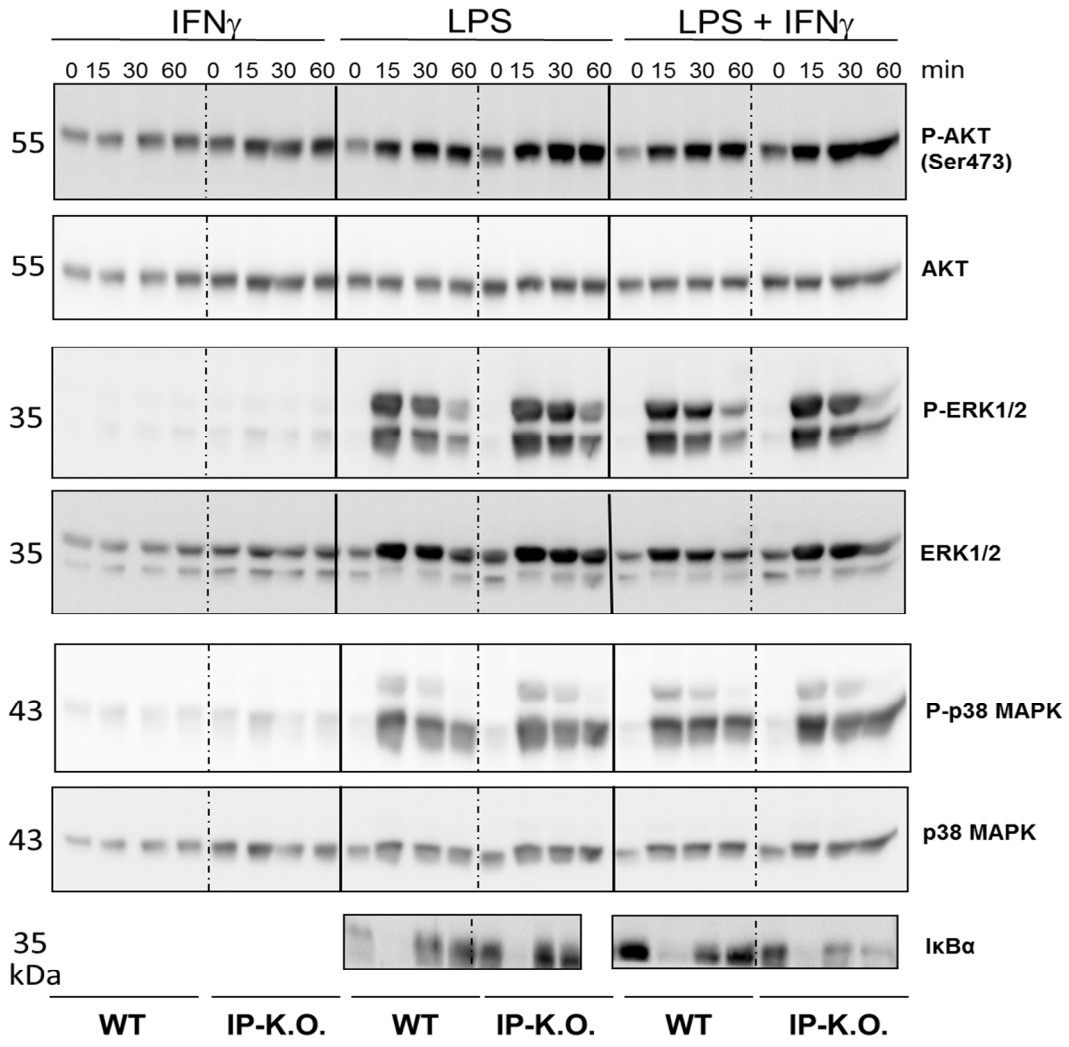

Figure 13. TLR4 signaling in IP-deficient macrophages is not altered after LPS stimulation Macrophages of wild-type and IP-K.O. mice were challenged with IFNγ (100 Units/mL), LPS (1µg/mL) or a combination of LPS and IFNγ for the indicated periods of time followed by cell lysis. Cell lysates were subjected to western blot analysis of phosphorylated and total protein levels of the indicated signaling proteins. Representative blots are shown from three independent experiments. Protein marker sizes in kilo Dalton (kDa) are shown on the left side of the panel.

4.2.3 IP-deficiency in macrophages does not impact phagocytosis and intracellular killing

Recent data of our group showed that *E. coli* infection of IP-deficient mice resulted in an increased mortality associated with a higher bacterial burden in several organs and tissues, i. e. blood, liver and peritoneal fluid (data not shown). The increased number of *E. coli* in IP-deficient mice over the course of the infection was detected 12 hours post-infection. These observations suggest that the early innate antibacterial immune response in IP-deficient mice is compromised. Since phagocytosis of invading pathogens and subsequent

intracellular killing are key features of antibacterial macrophage responses, it can be assumed that the lack of immunoproteasome activity might lead to a compromised exertion of both effector functions resulting in the observed immune-suppressed phenotype of the *E. coli*-infected IP-K.O. mice.

To study the phagocytosis capacity of IP-deficient and wild-type macrophages in vitro, macrophages were incubated with fluorophore-conjugated *E. coli*-particles for 30, 60 and 90 minutes. In addition to *E. coli* stimulation alone, pre-stimulation with IFNγ and LPS for 16 hours was added to the experimental setup to enhance the macrophage phagocytosis capacity. The engulfment of the fluorophore-labelled *E. coli* particles leads to an accumulation of fluorophore within the macrophages which was analyzed via flow cytometry. In flow cytometry analyses, fluorophore-positive macrophages indicate the engulfment of *E. coli* particles and thus, these macrophages are defined as 'phagocytosis positive'.

The data presented in figure 14A show that over the course of the experiment the frequency of macrophages that engulfed *E. coli* particles increased from 20 % after 30 minutes to 40 % after 90 minutes in both experimental groups. However, no differences were observed between IP-K.O. and wild-type macrophages. In addition to the frequency of macrophages that engulfed bacteria, the total amount of engulfed *E. coli* was quantified on *per cell* base using the *geometric mean of fluorescence intensity* (gMFI) method (Fig. 14B). IP-K.O. macrophages did not exhibit differences in the total cellular amount of engulfed bacteria compared to wild-type macrophages.

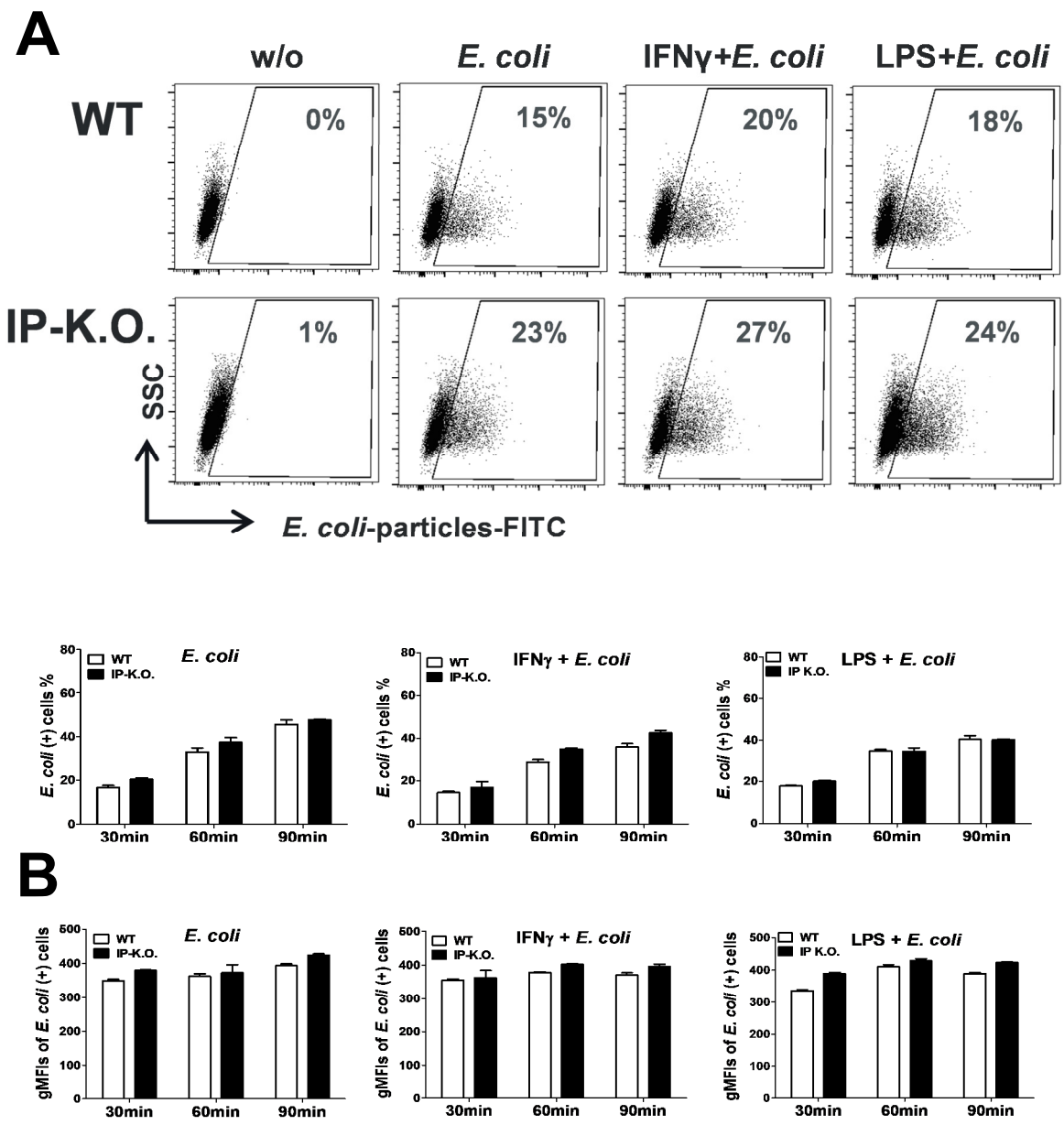

Figure 14. Phagocytosis capacity of *E.* coli is not altered in IP-deficient macrophages
To measure the early phase of phagocytosis, macrophages were challenged with FITC-conjugated *E. coli*-particles (multiplicity of infection [MOI] 10) for the indicated periods of time. The percentage of *E. coli*-positive macrophages was determined via flow cytometry. Dead cells were excluded in the flow cytometry analysis using propidium iodide staining. Representative dot plots of cells without *E. coli*-particles (w/o), with *E. coli*-particles and stimulation with IFNγ (100 Units/mL) or LPS (100 ng/mL) for 16 hours prior to incubation with *E. coli*-particles for 30 minutes. **(A)** Quantification of *E. coli* positive cells. **(B)** Geometric mean of the fluorescence intensity (gMFIs) of the *E. coli* positive-cells. Data are presented as mean + standard error of mean (SEM) of four mice per group and are representative of three experiments.

To additionally study whether the intracellular killing of the engulfed *E. coli* is altered in IP-deficient macrophages, cells of both experimental groups were incubated with viable *E. coli* for 1 hour. The same *E. coli* strain was used for this in vitro cell experiments as for

the in vivo mice *E. coli* infection experiment described above. After 1 hour *E. coli* stimulation several dilutions of the macrophage cell supernatants including non-phagocytosed *E. coli* bacteria were plated on agar plates. Additionally, several dilutions of the lysed macrophages were plated on agar plates for quantification of the engulfed bacteria. After overnight cultivation the amounts of colony forming units per milliliter (CFUs / mL) of extracellular (non-phagocytosed) and intracellular (engulfed) bacteria were determined. Based on these numbers and the total number of initially used *E. coli* for the 1 hour infection, the number of killed bacteria was calculated (Fig. 15).

The results presented in figure 15 show that the numbers of extracellular and intracellular *E. coli* were not significantly different in IP-K.O. macrophages compared to the wild-type counterparts under all experimental settings (*E. coli*, IFNγ + *E. coli* and LPS + *E. coli*). Therefore, the calculated numbers of killed bacteria in both experimental groups did not differ between the IP-deficient and wild-type macrophages.

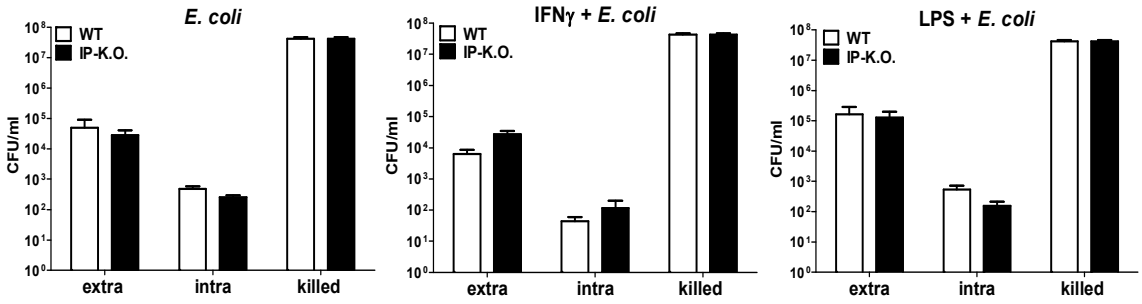

Figure 15. The intracellular killing of *E. coli* is not altered in IP-deficient macrophages
Macrophages without pre-stimulation or with pre-stimulation with IFNγ (100 Units/mL) or LPS (100 ng/mL) for 16 hours were cultured with viable growth arrested *E. coli* (multiplicity of infection [MOI] 10). After 1 hour incubation, cell supernatants and lysed cells were plated on LB agar plates. After overnight culture colony forming units per mL (CFU/mL) of extracellular (extra) and intracellular (intra) bacteria were determined. The number of killed bacteria was calculated as described in the methods section. Data are presented as means + standard error of mean (SEM) with at least four animals per group and are representative of two independent experiments.

Collectively, the findings presented in figure 14 and 15 suggest that the immunoproteasome is not involved in the phagocytosis as well as the intracellular killing capacity of macrophages. Therefore, the higher bacterial burden observed in *E. coli*-infected IP-K.O. mice (data not shown) cannot be attributed to an impaired macrophage phagocytosis and bacterial killing capacity.

4.2.4 IP-K.O. macrophages exhibit a decreased secretion of nitric oxide

In addition to phagocytosis and intracellular degradation of pathogens, the production and secretion of the free radical *nitric oxide* (NO) is another major mechanism of macrophages to fight invading bacteria. Upon autocrine and paracrine IFNγ stimulation during innate immune responses, macrophages activate the *inducible nitric oxide synthase* (iNOS) that produces NO, which in turn is released to kill extracellular pathogens. To study the potential influence of the immunoproteasome in NO production under various inflammatory conditions, IP-K.O. and wild-type macrophages were stimulated with (i) IFNγ, (ii) LPS, (iii) LPS + IFNγ, (iv) viable *E. coli* and (v) viable *E. coli* + IFNγ for 24 hours. The formation and release of NO was indirectly determined by measuring nitrite in the supernatant (Fig. 16).

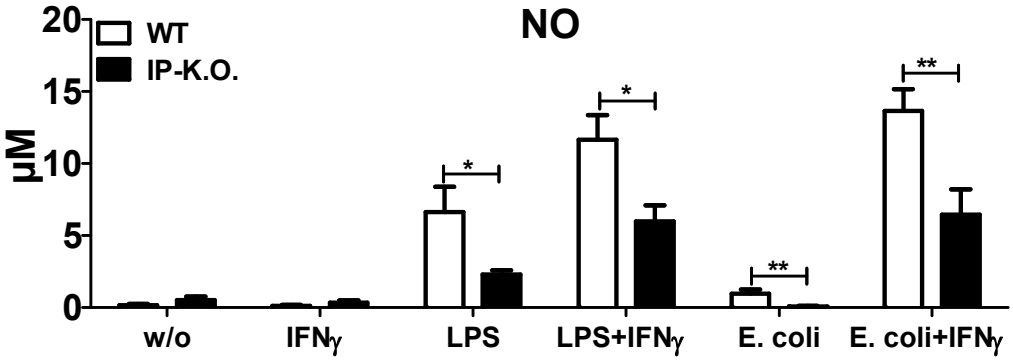

Figure 16. IP-deficiency in macrophages leads to diminished levels of nitric oxide
Primary macrophages from wild-type and IP-knock-out mice were challenged with IFNγ (100 Units/mL), LPS (1 µg/mL), growth arrested viable *E. coli* (multiplicity of infection [MOI] 1) and LPS and *E. coli* combined with IFNγ for 24 h. Nitric oxide (NO) levels were determined indirectly via measurement of nitrite concentrations in the cell supernatants using Griess Reagent. Data are presented as means + standard error of mean (SEM) of at least six mice per group. Statistical significant differences were evaluated using student t-test with a 95 % confidence interval (* $0.01 \leq p \leq 0.05$; ** $0.001 \leq p < 0.01$, w/o without stimulation).

Wild-type macrophages and IP-K.O. macrophages exhibited only a weak NO production after 24 hours at basal level as well as under IFNγ and *E. coli* treatment. In contrast, under LPS treatment the NO production was notably induced in both experimental groups. Furthermore, adding IFNγ to LPS and *E. coli* treatment strongly enhanced the NO production of both experimental groups. Importantly, the results of figure 16 show that the absence of the immunoproteasome resulted in a markedly reduced production of NO upon stimulation with LPS and *E. coli* as well as in combination of both with IFNγ. These

findings strongly suggest that the immunoproteasome is involved in the regulation of NO production during inflammatory processes.

4.2.5 Immunoproteasome-deficient macrophages are more sensitive to cell death and exhibit elevated levels of reactive oxygen species

By maintaining protein homeostasis the immunoproteasome mediates cellular stress resistance under inflammatory conditions. We hypothesized that the absence of the immunoproteasome in macrophages during inflammation leads to increased cellular stress ultimately resulting in cell death. To induce pathogen-induced stress in vitro *E. coli* alone as well as in combination with IFNγ and LPS was used. The frequency of dead cells was studied via flow cytometry using a viability fluorescence dye (Fig. 17A).

A similar rate of dead cells was observed in IP-deficient macrophages compared to wild-type cells in the naïve state without any inflammatory stimulus. Inflammatory stress induced by *E. coli* alone did not result in increased cell death rates in wild-type macrophages whereas in the population of IP-deficient macrophages a significantly higher rate of dead cells was observed. Exposure to *E. coli* in combination with IFNγ or LPS moderately induced cell death in the wild-type macrophage population. Importantly, also under these inflammatory stress conditions IP-deficient macrophages exhibited noticeable higher dead cells rates than their wild-type counterparts. In conclusion, the findings described in figure 17A indicate that the absence of the immunoproteasome results in decreased stress tolerance under pathogen-induced inflammatory conditions.

One potential stress mechanism which is associated with increased cell death is the accumulation of intracellular reactive oxygen species (ROS) leading to oxidative stress. The excessive formation of intracellular ROS leads to damage of cell components, such as signaling proteins, transcription factors and DNA (see introduction).

To study whether IP-deficient macrophages exhibit elevated levels of intracellular reactive oxygen species under inflammatory conditions, a fluorometric assay was conducted. H2DCFDA was used as an intracellular ROS detection reagent, since it is oxidized by ROS forming a fluorescence active reagent (see material and methods) (Fig. 17B).

The results presented in figure 17B show that wild-type cells did not exhibit an intracellular accumulation of ROS upon exposure to *E. coli* (± IFNγ) compared to naïve

conditions. In contrast, increased levels of intracellular ROS upon *E. coli* stimulation were observed in IP-deficient macrophages. From these results one can conclude that the lack of immunoproteasome activity results in increased levels of reactive oxygen species under inflammatory stress conditions.

In summary, the results presented in figure 17 show that the lack of the immunoproteasome activity during pathogen-induced stress is associated with elevated cell death rates and increased intracellular ROS level suggesting that the immunoproteasome mediates cellular stress tolerance in macrophages during bacterial infections.

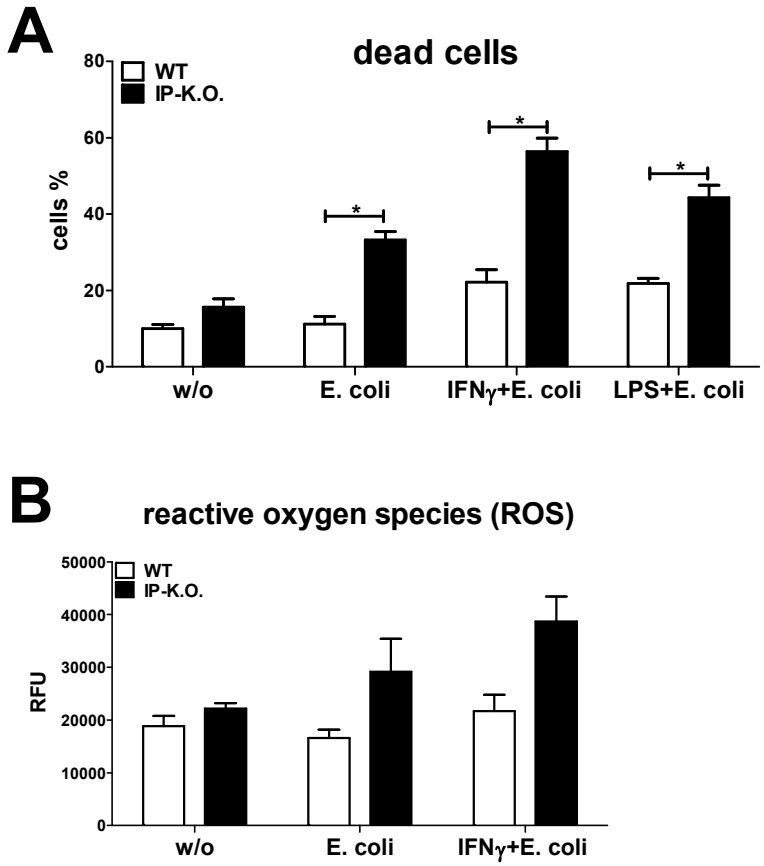

Figure 17. IP-deficient macrophages are more sensitive to cell death and exhibit elevated levels of intracellular reactive oxygen species

Macrophages were pre-stimulated with IFNy (100 Units/mL) or LPS for 16 hours followed by stimulation with *E. coli* (multiplicity of infection [MOI] of 10) for 1 hour or *E. coli* alone **(A)** The frequency of dead cells was detected by flow cytometry using propidium iodide. **(B)** Intracellular levels of reactive oxygen species (ROS) in response to *E. coli* (multiplicity of infection [MOI] of 10) and in combination with IFNγ (100 Units/mL) after 6 hours stimulation. ROS levels were measured by H2DCFDA staining and fluorescence analysis. Data are displayed as mean + standard error of mean (SEM) of four animals per group and are representative of two independent experiments. Statistical significant differences were evaluated using Wilcoxon Mann-Whitney U-test (* $0.01 \leq p < 0.05$; RFU = relative fluorescence units).

4.3 The role of the immunoproteasome in adaptive immune responses of macrophages

4.3.1 IP-deficient macrophages exhibit a reduced expression of MHC class I epitopes

Macrophages are crucial for the activation and regulation of T-cells by presenting processed peptides via *major histocompatibility complex class I* (MHC class I) together with the expression of T-cell co-stimulatory signal proteins. Several studies using different IP-deficient mice lacking one or more immunoproteasome subunits, observed reduced expression of MHC class I molecules on splenic T- and B-lymphocytes as well as on dendritic cells (Kincaid et al. 2012). However, there is a lack of data on surface MHC class I expression in macrophages in an IP-deficient background. To study the role of immunoproteasome activity in MHC class I regulation, basal expression and bacterial-induced upregulation of the two major MHC class I epitopes H-2Kb and H-2Db on IP-deficient macrophages was investigated using flow cytometry analyses (Fig. 18). IP-deficient macrophages and wild-type macrophages were stimulated with IFNy, LPS and *E. coli* + IFNγ for 16 hours. The flow cytometry data were analyzed with two distinct strategies. First, the frequency of H-2Kb- and H-2Db-positive macrophages was quantified. Second, the total surface amount of H-2Kb and H-2Db on per cell base was quantified by the geometric mean of fluorescence intensity (gMFI) within the H-2Kb- and H-2Db-positive cell population (Fig. 18).

The results presented in figure 18A show that under naïve conditions approximately 10 % of wild-type macrophages exhibited H-2Kb expression, while only 2 % of the IP-K.O. macrophages showed an H-2Kb expression. Stimulation with the immunological triggers IFNγ, LPS and *E. coli* + IFNγ resulted in an increased frequency of H-2Kb-positive cells in both experimental groups. Importantly, in the IP-K.O. group less macrophages exhibited an induction of H-2Kb expression compared to the wild-type counterparts in all studied stimulations. A similar pattern was observed for H-2Db-positive cells, albeit in the naïve state the difference between wild-type and IP-K.O. macrophages was more pronounced (Fig. 18B). In contrast, upon stimulation with IFNy, LPS and *E. coli* + IFNγ the difference in the number of H-2Db-positive cells between both experimental groups was less

noticeable but statistically significant. Moreover and importantly, the results in figure 18B show that the cellular expression of MHC class I epitopes was diminished in IP-deficient macrophages that are positive for H-2Kb or H-2Db. Upon stimulation with IFNy, LPS and *E. coli* + IFNγ the cellular expression of H-2Kb and H-2Db was 20 % - 40 % reduced in IP-deficient macrophages compared to the wild-type counterparts.

In summary, the results displayed in figure 18 indicate that under naïve and inflammatory conditions IP-deficiency leads (i) to a lower frequency of MHC class I positive macrophages and (ii) to a reduced cellular expression level of MHC class I in macrophages expressing either of both molecules. These findings strongly suggest that the immunoproteasome is involved in the basal regulation as well as in the infection-induced upregulation of MHC class I during the early immune response.

This notion is additionally supported by the finding that gene expression of TAP1 (*transporter associated with antigen processing 1*) was reduced under all studied conditions in IP-deficient macrophages, albeit only under LPS stimulation this effect is statistically significant (Fig 19). TAP1 is a carrier molecule which transports processed antigens to the *endoplasmic reticulum* (ER). At the ER the antigen is coupled to the MHC class I complex. This finding indicates that in addition to impaired MHC class I expression, the macrophage-mediated peptide presentation in an IP-deficient background is also inhibited through reduced induction of TAP1.

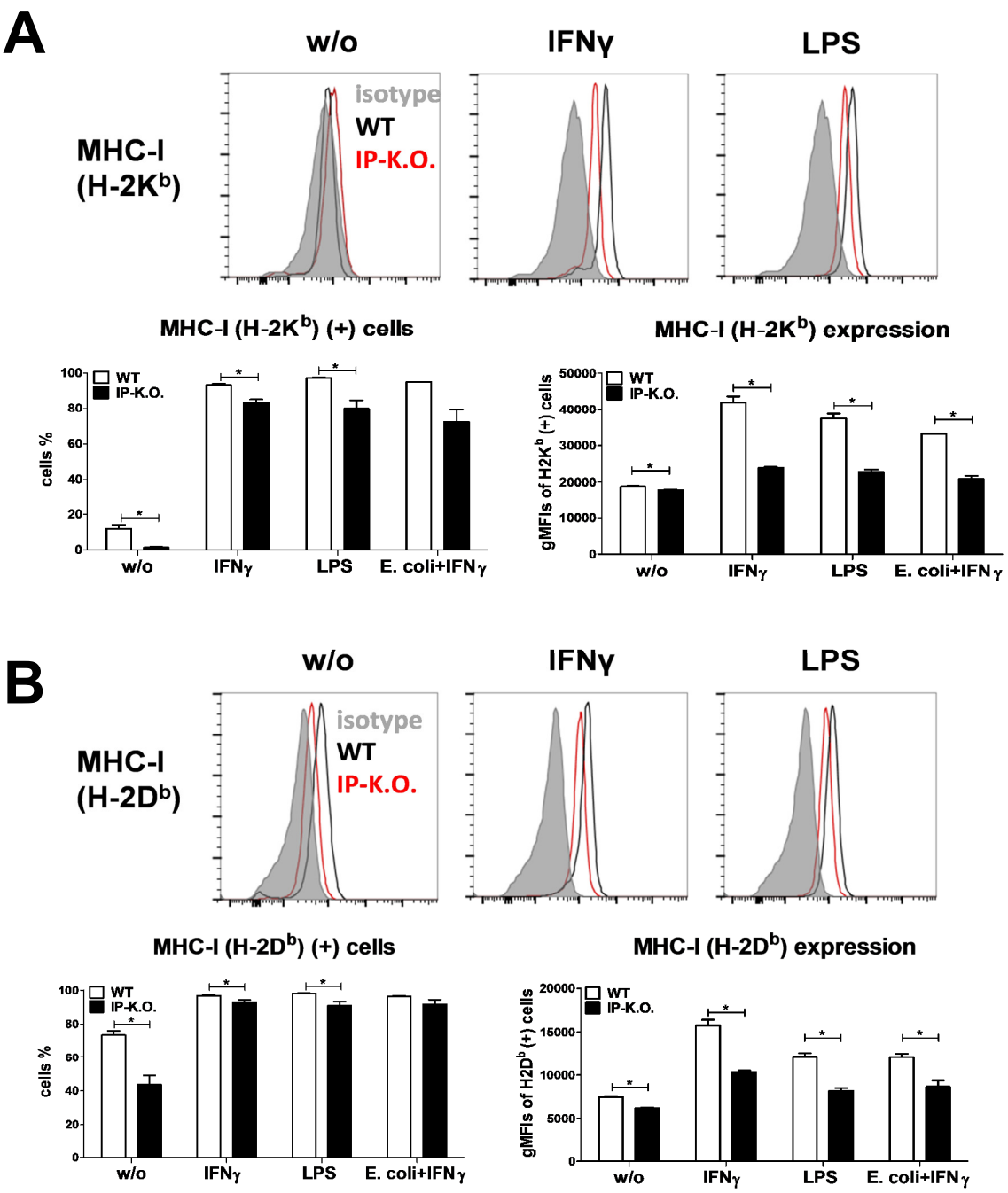

Figure 18. IP-deficient macrophages exhibit reduced surface expression of MHC class I antigens
The expression of the MHC class I epitopes H-2Db and H-2Kb on primary macrophages from wild-type and IP-K.O. mice were determined using flow cytometry. Macrophages were stimulated with IFNγ (100 Units/mL), LPS (100 ng/mL) for 16 hours and E. coli (multiplicity of infection [MOI] 10) + IFNγ (100 Units/mL) for 1 hour. Representative histograms of wild-type macrophages (black) and IP-K.O. macrophages (red) stained with anti-MHC-I (H-2Kb), anti-MHC-I (H-2Db) and corresponding isotype controls (grey shaded) are displayed. **(A)** Quantification of H-2Kb-positive-cells and cellular H-2Kb surface expression. **(B)** Quantification of H-2Db-positive-cells and cellular H-2Db surface expression. The cellular surface expression levels of the MHC-I molecules are determined by the geometric mean of the fluorescence intensity (gMFIs) of the MHC class I positive macrophage population. Data are displayed as mean + standard error of mean (SEM) of four animals per group and are representative of two independent experiments. Statistical significant differences were evaluated using Wilcoxon Mann-Whitney U-test (* $0.01 \leq p < 0.05$, w/o unstimulated).

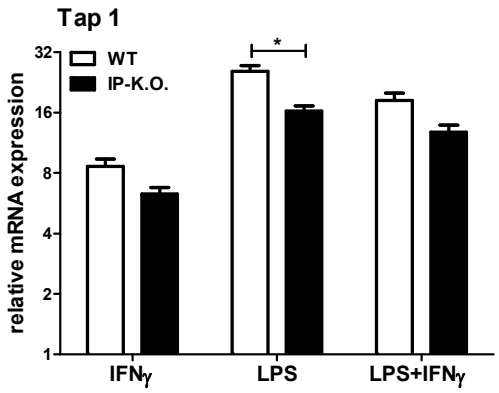

Figure 19. Relative mRNA expression of TAP1
TAP1 gene expression was measured by quantitative PCR. Macrophages were stimulated with IFNγ (100 Units/mL), LPS (1 µg/mL) or a combination of LPS and IFNγ for 8 hours. Data are presented as means (n=3) + standard error of mean (SEM) of the fold change relative to non-stimulated macrophages. Statistical significant differences were evaluated using an TWO-way ANOVA and Bonferroni post-tests (* $0.01 \leq p < 0.05$).

4.3.2 IP-K.O. macrophages show a decreased expression of the T-cell co-stimulatory signal protein CD86

In addition to antigen presentation, macrophages further modulate T-cell responses by surface expression of co-stimulatory molecules including CD86 and CD80. Both molecules are expressed in tandem and provide co-stimulatory signals required for appropriate T-cell activation and survival (Lanier et al. 1995). In order to analyze whether the immunoproteasome is involved in macrophage expression of CD86 and CD80, the surface expression of these molecules was analyzed by flow cytometry in both experimental groups (Fig. 20).

In the naïve state as well as under stimulation with IFNγ and IFNγ + *E. coli* IP-deficient macrophages exhibited significantly reduced surface expression of CD86 (Fig. 20B). LPS stimulation resulted in downregulation of CD86 in both experimental groups. In the case of CD80, all studied inflammatory triggers moderately induced CD80 surface expression exhibiting no differences in both experimental groups (Fig. 20A). Interestingly, CD80 levels of IP-deficient macrophages were elevated in the naïve state. These data suggest that the IP is involved in the regulation of CD86 under inflammatory and non-inflammatory conditions, whereas CD80 expression is only affected in basal states.

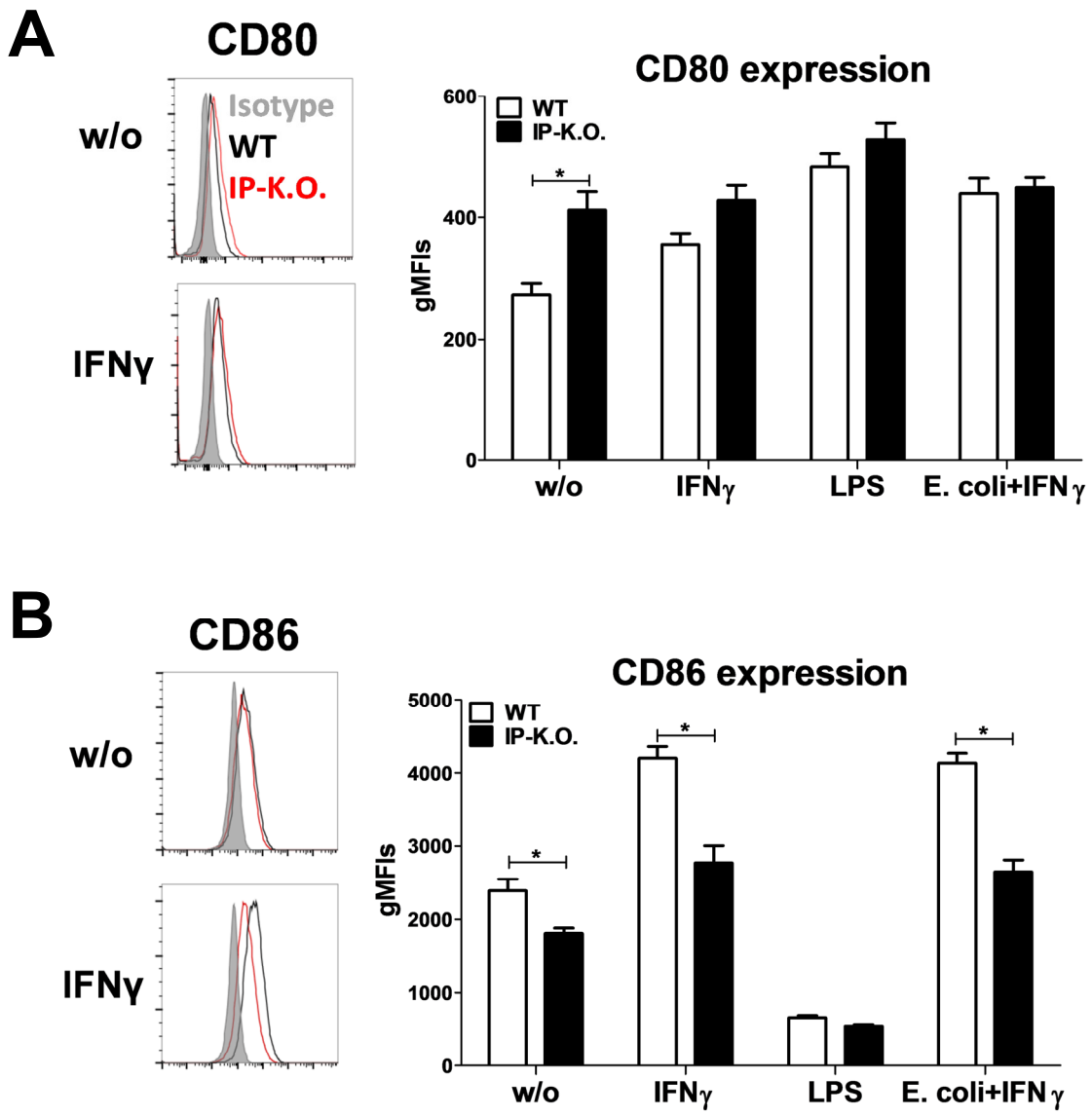

Figure 20. IP-deficient macrophages exhibit altered surface expression patterns of the T-cell costimulatory proteins CD80 and CD86
Surface expression of CD80 and CD86 on wild-type and IP-K.O. macrophages were determined using flow cytometry. Macrophages were stimulated with IFNγ (100 Units/mL) and LPS (100 ng/mL) for 16 hours and *E. coli* (multiplicity of infection [MOI] 10) + IFNγ (100 Units/mL) for 1 hour. Representative histograms of unstimulated (w/o) and stimulated (IFNγ) wild-type macrophages (black) and IP-K.O. macrophages (red) stained with anti-CD80 or anti-CD86 and corresponding isotype controls (grey shaded) are displayed. Cellular surface protein expression of CD86 **(A)** and CD80 **(B)** was quantified by determining the geometric mean of the fluorescence intensity (gMFI) of the total macrophage population. Data are displayed as mean of four animals per group and are representative of two independent experiments. Statistical significant differences were evaluated using Wilcoxon Mann-Whitney U-test (* $0.01 \leq p < 0.05$).

4.3.3 IP-deficient macrophages do not exhibit an altered expression of the T-cell co-inhibitory molecule PD-L1

Besides CD80 and CD86 expression, macrophages regulate T-cell responses by surface expression of *programmed death-ligand 1* (PD-L1). During inflammation, e. g. IFNγ or LPS exposure, macrophages express PD-L1 providing inhibitory signals to effector CD8(+) T-cells expressing PD-1 leading to decreased proliferation, apoptosis-mediated T-cell death as well as other T-cell inhibitory processes (Loke and Allison 2003). Flow cytometry analyses were used to investigate the involvement of the immunoproteasome in the regulation of PD-L1 surface expression (Fig. 21).

Under naïve conditions only a small fraction of macrophages (< 10 %) expressed PD-L1 exhibiting no statistically significant differences between IP-K.O. and wild-type cells. As expected, upon stimulation with IFNγ, LPS and *E. coli* + IFNγ, surface protein expression of PD-L1 was markedly upregulated in virtually all macrophages. However, the expression pattern of PD-L1 was indistinguishable between wild-type and IP-deficient macrophages. In addition, the expression level of PD-L1 on a cellular base was investigated by determining the geometric mean fluorescence intensity via flow cytometry (Fig. 21). In line with the findings of non-altered frequencies of PD-L1-positive macrophages, the per cell amount of PD-L1 did not differ between IP-K.O. and wild-type macrophages under naïve and inflammatory conditions. In conclusion, the results shown in figure 21 suggest that the immunoproteasome is not involved in the regulation of the T-cell inhibitory molecule PD-L1 in bacterial infections.

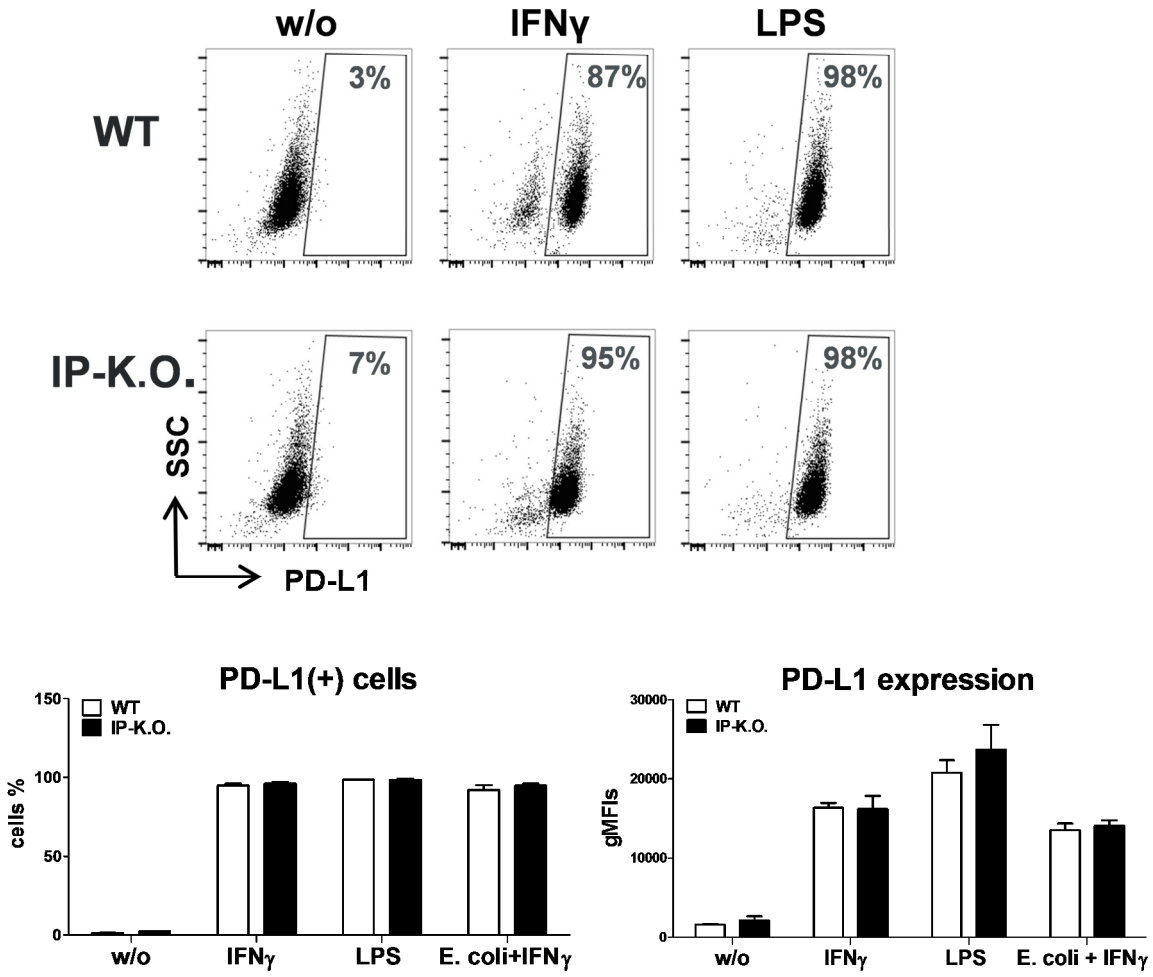

Figure 21. IP-deficient macrophages do not exhibit an altered surface protein expression of PD-L1
Surface expression of PD-L1 on primary macrophages from wild-type and IP-K.O. mice was determined using flow cytometric staining of PD-L1. Macrophages were stimulated with IFNγ (100 Units/mL) and LPS (100 ng/mL) for 16 hours and *E. coli* (multiplicity of infection [MOI] 10) + IFNγ (100 Units/mL) for 1 hour. Representative dot blots are displayed. The frequency of PD-L1-postive cells in (%) and the cellular expression level of PD-L1 (geometric mean of the fluorescence intensity [gMFIs]) of the total macrophage population are displayed. Data are presented as mean + standard error of mean of four animals per group and are representative of two independent experiments. Statistical significant differences were evaluated using Wilcoxon Mann-Whitney U-test

5. Discussion

The immunoproteasome is a multi-subunit protein complex that is involved in protein homeostasis during immunological stress, i. e. viral and bacterial infections. It was long believed that the primary function of the immunoproteasome during inflammation is the processing and supply of viral and cellular peptides for MHC class I antigen presentation. Therefore, many studies have investigated the role of the immunoproteasome in viral infections. However, very little is known about the role of the immunoproteasome in bacterial infections. Since macrophages are crucial cells in the host defense against bacterial infections, it is particularly important to understand the role of the immunoproteasome in the immunological activity of these immune cells. To close this gap of knowledge the present thesis investigated the role of the immunoproteasome in immune effector functions of macrophages in bacterial infections using an appropriate IP-knock-out mouse model together with relevant bacterial triggers.

5.1 The mouse model of immunoproteasome-deficiency

The immunoproteasome differs from the standard proteasome by the preferential incorporation of LMP2, LMP7 and MECL-1 in place of the standard proteasome subunits beta1, beta2 and beta5. The utilized knock-out mouse model genetically lacks two of the major catalytic IP-subunits LMP7/β5i and MECL-1/β2i as well as both subunits of the proteasome activator PA28α and PA28β. Deficiency of these IP-subunits was confirmed by western blot analyses (Fig. 6A).

Furthermore, protein expression of the immunoproteasome subunit LMP2 was investigated in both experimental groups (Fig. 6B). In the protein lysate of the wild-type macrophages one distinct band was detected for LMP2, while β5i/LMP7+β2i/MECL-1 and PA28αβ gene-deficient macrophages showed two distinct bands. This additional band might represent the pre-form of unprocessed LMP2. Griffin et al. made a similar observation, i. e. that absence of LMP7 leads to accumulation of LMP2 precursors. They proposed that the formation of the immunoproteasome is a corporative process and the pre-forms of LMP2 and MECL-1 require LMP7 for maturation and successful incorporation (Griffin et al.

1998). Furthermore, another research group showed that the assembly of immunoproteasomes is greatly inhibited in mice lacking the PA28 activator complex (Preckel et al. 1999). Due to the absence of LMP7, MECL-1 and the PA28 activator in the mouse model used in this study, the β5i/LMP7 + β2i/MECL-1 and PA28αβ gene-deficient macrophages are not capable to form a functional immunoproteasome. Hence, in the present study the β5i/LMP7+β2i/MECL-1 and PA28αβ gene-deficient macrophages are referred to as immunoproteasome-knock-out macrophages (IP-K.O.).

5.1.1 Advantage of genetically modified mouse models

The advantage of the usage of a genetically modified mouse model over a siRNA-mediated downregulation approach is based on the fact that a genetically knock-out leads to a total depletion of the target gene(s) in all cells and tissues of the organism. Moreover, the genetic knock-down of target gene(s) is stable, whereas siRNA-mediated downregulation is transient not allowing long-time in vitro experiments which have been performed in this thesis. In addition, siRNA-downregulation of three proteins is technically complex likely not achieving sufficient transfection efficiencies for all three genes. Another commonly utilized approach in human immunoproteasome research is the usage of pharmacological / chemical compounds that inhibit the proteolytic activity of the IP-subunits. However, compounds selectively targeting all three IP-subunits are not available. Only single subunits can be targeted by inhibitors and currently no inhibitor is available for the IP-subunit MECL-1 (McCarthy and Weinberg 2015).

5.2 The macrophage cell model and bacterial trigger to study the role of the immunoproteasome during immune responses

To draw reasonable conclusions for the role of the immunoproteasome in antibacterial macrophage effector functions, an appropriate *in vitro* macrophage cell model and relevant bacterial triggers have been used.

5.2.1 Macrophage cell model

The utilized primary cell model is based on the isolation of bone marrow cells containing macrophage progenitor cells followed by M-CSF-induced in vitro differentiation into *bone marrow derived macrophages* (BMDM). BMDMs are an ideal in vitro model to study macrophage immune effector functions because they represent a homogenous population of unstimulated macrophages. Moreover, the BMDM cell model is technically simple, reproducible and yields high cell numbers per mice with a long lifespan.

5.2.2 Bacterial triggers

Since LPS is a prominent cell wall compound of gram-negative bacteria and is known to be a potent activator of macrophages, it represents a suitable and established experimental tool to study antibacterial macrophage effector functions. For some experiments *E. coli* was used as a bacterial trigger since it is the prototype of gram-negative bacteria and represents one of the most frequent trigger of numerous common bacterial infections as well as septic syndromes underlining the biological and clinical importance of this immune stimulus (Vincent et al. 2015).

IFNγ was used in all experiments because it has been clearly linked with the formation of immunoproteasome complexes and moreover, plays a crucial role in inflammatory processes. The LPS- and IFNγ-mediated induction of the immunoproteasome in the used macrophage cell model was confirmed by quantitative PCR (Fig. 8) underlining the relevance of the used bacterial triggers.

5.3 The role of the immunoproteasome in innate macrophage effector functions in bacterial infections

5.3.1 In vitro differentiation of bone marrow derived macrophages

No differences in macrophage differentiation, obtained purity, cell numbers and morphology, were observed between wild-type and IP-deficient cells (Fig. 5). This finding clearly suggests that the immunoproteasome complex is not involved in early macrophage differentiation / maturation from bone marrow macrophage progenitor cells. Interestingly,

data from literature indicate that the subsequent polarization of mature macrophages into M1 and M2 macrophages is altered towards M2 polarization in LMP7-deficient alveolar macrophages (Chen et al. 2016) suggesting that later steps of macrophages differentiation require immunoproteasome activity.

5.3.2 The impact of immunoproteasome activity on cytokine / chemokine expression of macrophages

Cytokine and chemokine production upon exposure to immunological triggers is one major effector function of macrophages. Cytokines and chemokines are crucial mediators that modulate immune responses and recruit other immune cells to the infection site. It is not fully understood whether the immunoproteasome impacts the macrophage cytokine and chemokine production / secretion during bacterial infections. In the present study the macrophage cytokine response was investigated by in vitro stimulation of IP-deficient and wild-type macrophages with IFNγ, LPS and *E. coli*. To examine the immunomodulatory cytokine / chemokine response in its entire complexity, the expression of (i) pro-inflammatory, (ii) anti-inflammatory as well as (iii) chemotactic mediators were studied. To account for the complex regulation of cytokine / chemokine production, the expression profile was studied on transcriptional level as well as on the level of secreted proteins. This approach additionally facilitates to determine on which level (transcriptional or protein secretion level) IP-deficiency may impact cytokine / chemokine production, thereby providing first evidence for the underlying mechanisms of IP-mediated cytokine / chemokine profile alterations.

On transcriptional level, neither after 2 hours stimulation (data not shown), reflecting an early immune response, nor after 8 hours stimulation, representing a late immune response, different cytokine or chemokine expression patterns were observed in both experimental groups (Fig. 10). On the other hand, on protein level IP-deficient macrophages show an altered secretion pattern for some cytokines or chemokines compared to WT macrophages upon stimulation with IFNγ, LPS and *E. coli* (Fig. 11 and Fig. 12).

5.3.3 Discrepancy of RNA and protein data

The observed discrepancy between gene expression profiles and protein levels corresponds to other studies. Only about 40 % of cellular protein levels can be predicted from mRNA measurements (Shebl et al. 2010, de Sousa Abreu et al. 2009). The discrepancy between mRNA and protein profiles is based on the fact that mRNA gene expression and subsequent protein synthesis (mRNA translation) rely on distinct mechanisms involving different protein machineries and temporal kinetics. For example, protein production can be dynamically regulated by modulating the ribosomal translation process and post-translational protein modifications (e. g. phosphorylation or ubiquitination). Furthermore, the different time points of the sample collection after stimulation of 2 and 8 hours for RNA samples and 24 hours for protein samples might also account for differences in the observed profiles. In addition, protein profiles were measured on the level of secreted cytokines instead of total intracellular protein levels. That means the observed discrepancies between the mRNA and protein data may also be explained by differential regulation of the secretion machineries in both experimental groups.

In summary, the results show that during bacterial infections the immunoproteasome in macrophages is not involved in the cytokine / chemokine response on the transcriptional level. Therefore it can be concluded that the immunoproteasome does not regulate any transcription factors that modulate mRNA transcription of cytokines, such as NfκB, AP-1 or STAT protein family.

5.3.4 Cytokine / chemokine secretion profiles

As mentioned above the lack of immunoproteasome activity in macrophages under inflammatory conditions results in altered secretion profiles of particular cytokines, while other studied cytokines are not changed. The biological consequences of the findings for each cytokine are discussed in detail in the following sections.

IL-10

The secretion of the anti-inflammatory cytokine IL-10 is significantly increased upon LPS stimulation in IP-deficient macrophages compared to wild-type macrophages (Fig. 11A). IL-10 is known to suppress macrophage activation and the production of various pro-inflammatory cytokines including IL-1β, IL-6 TNFα and G-CSF (see introduction)

(Arango Duque and Descoteaux 2014). This suppressive effect is clearly observed in IP-deficient macrophages which exhibit decreased levels of IL-6 and G-CSF under LPS stimulation compared to the wild-type counterparts (Fig. 11B and C). Based on these findings, one can conclude that the immunoproteasome is involved in the regulation of the anti-inflammatory IL-10 response upon LPS exposure, thereby indirectly influencing the pro-inflammatory macrophage immune responses. However, for cytokines of the early immune response, like TNFα and IL-1β (Fig. 11E and 11F), this anti-inflammatory effect of IL-10 is not observed. It is important to note that IP-deficient and wild-type macrophages do not differ in their IL-10 secretion pattern in response to *E. coli* questioning the observations from LPS-stimulated macrophages. In conclusion, the conflicting data presented and discussed here are not sufficient to reveal the role of the IP in the IL-10 response of macrophages in bacterial infections.

IL-6

The results presented in figure 11B show that IL-6 secretion upon exposure to LPS and *E. coli* is reduced in IP-deficient macrophages indicating that the immunoproteasome is involved in the regulation of this crucial innate immune regulator. However, no different secretion patterns of IL-6 were observed in IP-deficient and wild-type macrophages in the presence of IFNγ during *E. coli* stimulation. Reduced IL-6 production was also shown for peripheral blood mononuclear cells (Muchamuel et al. 2009) and peripheral macrophages (Reis et al. 2011b) under inflammatory conditions in a background of a different IP-K.O. model supporting the conclusion drawn here. IL-6 regulates a spectrum of various processes during innate and adaptive host immune responses, such as inhibition of pro-inflammatory cytokine expression and modulation of T-cell polarization (Jones 2005). Hence, an impaired IL-6 response can ultimately lead to a defective antibacterial immune response.

TNFα

The secretion of TNFα, one of the most important mediator of pro-inflammatory early immune responses, was shown not to be different in wild-type and IP-deficient macrophages on transcriptional (Fig. 10) and protein level (Fig. 11E) under all studied inflammatory conditions. TNFα regulates the chemokine induction of macrophages which is essential for immune cell recruitment to the site of infection and bacterial clearance

(Algood et al. 2004, Roach et al. 2002). This notion is supported by the findings of the present thesis showing that IP-deficient and wild-type macrophages exhibit no different chemokine expression profiles of CCL2, CCL5 and CXCL10 under the same inflammatory conditions (Fig. 12). In conclusion, the findings discussed here convincingly show that the immunoproteasome is not involved in the pro-inflammatory TNFα-induced chemokine response of macrophages in bacterial infections.

IL-1β

In addition to TNFα, the experiments presented in figure 11F show that the secretion pattern of another crucial early pro-inflammatory cytokine, IL-1β, is indistinguishable between IP-deficient and wild-type macrophages upon LPS stimulation. However, upon stimulation with *E. coli* the absence of the immunoproteasome leads to a significantly elevated secretion of IL-1β compared to wild-type macrophages. From this finding one could conclude that the immunoproteasome is a direct or indirect negative regulator of the early IL-1β macrophage response during bacterial infections. Based on findings from human hepatocytes (Zhang et al. 2003) the biological significance of this result might be that IL-1β is a regulator of the secretion of the chemokine CCL4 that is a crucial chemoattractant for phagocytes (Bystry et al. 2001). Experiments studying the secretion of CCL4 clearly support this observation since CCL4 follows the secretion pattern of IL-1β with increased IL-1β levels in IP-deficient macrophages upon *E. coli* stimulation (compare Fig. 11F and Fig. 12B). In summary, these data clearly suggest that the lack of immunoproteasome activity enhances IL-1β pro-inflammatory responses with implications to CCL4-mediated cell recruitment in bacterial infections. In addition to CCL4 regulation, IL-1β secretion is also associated with ROS production as well as cell survival / death which is discussed in section 5.3.12.

IFNγ

IFNγ is a crucial pro-inflammatory cytokine that modulates adaptive as well as innate immune processes in viral and bacterial infections, such as macrophage activation and MHC expression (see introduction). Macrophage IFNγ production is primarily induced by other cytokines (e. g. IL-2, IL-12, IL-17) as well as IFNγ in an autocrine manner (Di Marzio et al. 1994). In contrast, bacterial compounds have not been shown to be potent activators of IFNγ expression in macrophages (Gessani and Belardelli 1998). This fact is

line with the findings from the present thesis showing that LPS and *E. coli* alone do not induce IFNγ production in both experimental groups (Fig. 11G). On the other hand, the presence of IFNγ ultimately leads to IFNγ secretion. Importantly, IP-deficient macrophages exhibit significantly reduced secretion levels of IFNγ compared to the wild-type counterparts upon stimulation with LPS and *E. coli* in combination with IFNγ (Fig. 11G). This finding leads to the conclusion that the immunoproteasome is involved in intracellular IFNγ signaling since IFNγ secretion is regulated by autocrine IFNγ signals (Di Marzio et al. 1994). This conclusion is supported by the fact that in wild-type macrophages IFNγ possesses an inhibitory effect on the macrophage production of IL-1β and G-CSF (Eigenbrod et al. 2013, Ogawa et al. 1994). Interestingly, immunoproteasome-deficient macrophages do not exhibit this inhibitory effect of IFNγ on G-CSF and IL-1β induction upon TLR4 stimulation (Fig. 11C and 11F) further supporting the conclusion that IFNγ signaling is impaired in immunoproteasome-deficient macrophages. An impaired IFNγ signaling due to the lack of proper immunoproteasome activity was also reported by Reis et al. (Reis et al. 2011b) showing that phosphorylation and thus, activation of crucial IFNγ signaling proteins, i. e. STAT1, STAT3 and IRF-3 is impaired in LMP7/MECL-1 knock-out macrophages treated with LPS providing a molecular explanation for the conclusions drawn here.

G-CSF

The results presented in figure 11C show that the lack of immunoproteasome activity in macrophages alters the secretion profile of G-CSF compared to wild-type cells under *E. coli* stimulation. While G-CSF secretion of IP-deficient macrophages is impaired under *E. coli* stimulation alone, an increased production of G-CSF is observed upon *E. coli* exposure in combination with IFNγ. Interestingly, the opposite G-CSF pattern is observed for wild-type macrophages. This effect might be explained by the fact that IFNγ has been shown to be a negative regulator of G-CSF production (Ogawa et al. 1994). Accordingly, the presence of IFNγ reduces the *E. coli*-induced production of G-CSF in wild-type macrophages. In line with the data discussed above which suggest that the lack of immunoproteasome activity impairs IFNγ signaling, this IFNγ-inhibitory effect on G-CSF cannot be observed in IP-deficient macrophages.

IFNβ

The secretion of IFNβ is reduced in IP-deficient macrophages upon exposure to LPS and *E. coli* in the presence of IFNγ (Fig. 11D) suggesting that immunoproteasome activity is involved in the regulation of the macrophage IFNβ response in bacterial infection. IFNβ induction is mainly regulated by TLR4-induced TRIF / TRAM-dependent signaling rather than by the MyD88-dependent pathway (Toshchakov et al. 2002). In this context Reis et al. (Reis et al. 2011b) postulate that this signaling cascade is impaired in an IP-deficient background in peripheral macrophages providing a molecular explanation for the observed impaired IFNβ production in IP-K.O. macrophages. Compromised IFNβ production as well as reduced IFNγ secretion can lead to an impaired NO response of macrophages during bacterial infections as discussed in more detail below (see section 5.3.11).

5.3.5 Chemokines

In addition to the cytokine-mediated immune modulation, the recruitment of other immune cells to the site of infection via secretion of chemokines is another crucial effector function of macrophages during bacterial infections. However, the precise role of immunoproteasome activity in chemokine production of macrophage in inflammation is not understood well. Therefore, the secretion of important chemokines was studied in the present thesis using the same experimental conditions as in the cytokine studies (Fig. 12). The experiments show that the secretion patterns of CCL2, CCL5, CXCL-1 and CXCL-10 between wild-type and IP-deficient macrophages are similar suggesting that immunoproteasome activity does not influence chemokine secretion in bacterial infections. One can conclude that the macrophage-mediated recruitment of other immune cells, such as granulocytes or lymphocytes, is not regulated by the immunoproteasome in inflammation. This conclusion is supported by in vivo experiments from colleagues studying cell recruitment in wild-type and IP-deficient mice that were infected with *E. coli*. These experiments show that the recruitment of leucocytes to the site of infection was not altered in IP-deficient mice under bacterial infections (manuscript in preparation).

In contrast to CCL2, CCL5, CXCL-1 and CXCL-10, levels of CCL4 are elevated upon *E. coli* stimulation in IP-deficient macrophages compared to the wild-type counterparts (Fig. 12B). Since CCL4 (MIP-1β) is implicated with the recruitment of T-cells, monocytes and a variety of other immune cells (Bystry et al. 2001), one could conclude that attraction

of these cell populations is altered in bacterial infections. However, whether elevated CCL4 levels in IP-deficient macrophages indeed result in an enhanced T-cell and monocyte recruitment *in vivo* remains unclear, since the before mentioned *in vivo* experiment as well as the observed secretion profile of the other studied chemokines argue against this conclusion.

5.3.6 Summary - the role of the immunoproteasome in the cytokine / chemokine response of macrophages

The observed and discussed differences in the cytokine / chemokine secretion profile upon bacterial stimulation between IP-deficient and wild-type cells suggest that the immunoproteasome impacts the cytokine response of macrophages on post-transcriptional regulation steps or on the level of secretion. However, the role of the immunoproteasome in cytokine / chemokine regulation during bacterial inflammation appears to be multifaceted since some mediators are unchanged while others are reduced or even upregulated in the absence of the immunoproteasome (see Fig. 11 and Fig. 12). Moreover, the lack of immunoproteasome activity does not exclusively impact the secretion of either pro- or anti-inflammatory cytokines additionally impeding the interpretation of these results regarding the biological consequences for in vivo antibacterial macrophage immune responses.

The cytokine response of macrophages in bacterial infections is very complex forming a network of autocrine acting cytokines that modulate each other's production and secretion (Callard et al. 1999). Moreover, during bacterial infections cytokines from other immune and non-immune cells act on macrophages in a paracrine fashion inducing the production / secretion of macrophage cytokines (Arango Duque and Descoteaux 2014). As a consequence, the interpretation of the role of the immunoproteasome on the observed cytokine profiles is difficult. Any observed alteration in the cytokine secretion of a particular cytokine between wild-type and IP-deficient macrophages can be interpreted either as a direct effect of the immunoproteasome or as an indirect effect. A direct effect means that the immunoproteasome is directly involved in the production of a particular cytokine, for instance directly acting on the production and secretion machinery of the cytokine. On the other hand, an indirect effect describes a process where the

immunoproteasome initially alters the secretion of a '*primary*' cytokine that in turn, regulates the secretion of the 'secondary' cytokine in an autocrine manner.

5.3.7 Differential effects of LPS and *E. coli*

It is important to note that the results presented above show different effects of immunoproteasome-deficiency on the secretion pattern of some cytokines / chemokines with respect to the inflammatory stimulus, i. e. LPS and *E. coli*. Although both triggers represent archetypal bacterial stimuli, the observed differences can be explained by distinct biological features of both triggers. LPS is one virulence factor of *E. coli* that is recognized by macrophages primary via TLR4. In contrast, *E. coli* possesses additional virulence factors, such as peptidoglycan, adhesins and flagellin that provide multifactorial immune signals to macrophages via activation of different TLR signaling pathways (Kawai and Akira 2010) providing an explanation for different outcomes for LPS and *E. coli* stimulation.

5.3.8 The impact of the immunoproteasome on proximal TLR4 signaling

The experiments discussed above reveal that the secretion profile for some cytokines / chemokines is altered in IP-deficient macrophages upon stimulation with LPS and / or *E. coli*. However, these experiments do not provide evidence for the underlying molecular mechanism behind the immunoproteasome-modulated cytokine / chemokine production. Studying proximal TLR4 signaling upon stimulation with LPS and *E. coli* represents one approach to reveal the molecular mechanism how the immunoproteasome impacts cytokine / chemokine production. It is important to note that various signaling pathways have been described that modulate cytokine / chemokine expression under inflammatory conditions (Leonard and Lin 2000). One prominent pathway which is associated with cytokine / chemokine expression is the NfκB pathway (see introduction). Since the activation of the NfκB pathway involves proteasomal degradation steps and hence, potentially depends on immunoproteasome activity, studying this pathway is important to understand possible molecular mechanisms of IP-mediated alteration of cytokine / chemokine expression. The classical induction of the NFκB pathway is initiated

by the signal-induced release of NFκB from *NfκB inhibitor proteins* (IκB's). The release of NFκB from IκB inhibition requires the proteasomal degradation of IκB molecules that is triggered by the phosphorylation of IκB molecules by *IκB kinases* (IKK). In the present study, degradation of IκBα upon TLR4 stimulation was studied over a time course of 0, 15, 30 and 60 minutes (Fig. 13) and even longer periods of 2 and 6 hours (data not shown). No altered degradation kinetics of IκBα were observed between wild-type and IP-deficient macrophages. These results suggest that the lack of the immunoproteasome does not alter the activation of NFκB by degradation of IκBα in proximal TLR4 signaling. Therefore, the altered cytokine secretion profiles of some cytokines above discussed cannot be attributed to an impaired activation of the classical NFκB pathway. This conclusion is supported by the finding that the phosphorylation / activation profile of AKT (Proteinkinase B) upon TLR4 stimulation is also indistinguishable between IP-K.O. and wild-type macrophages (Fig. 13). In the context of TLR4-induced cytokine induction, AKT is an important activator of IKK that in turn phosphorylate IκBα molecules for subsequent ubiquitination and degradation (Kane et al. 1999).

The discussed results provide evidence that the immunoproteasome regulates cytokine expression in an NFκB-independent manner. In this context Basler et al proposed that the immunoproteasome might selectively process an NFκB-independent factor that is required for regulating cytokine production (Basler et al. 2015). However, such a factor has not been identified so far.

In addition to NFκB activation, other signaling pathways, i. e. MAPK pathways, are involved in the cytokine responses of macrophages in inflammation (Carter et al. 1999, Yang et al. 2014). The experiments displayed in figure 13 clearly show that LPS-induced p38 and ERK activation is indistinguishable between wild-type and IP-macrophages. From these data on can conclude that MAPK-dependent cytokine induction, such as TNFα and IL-6, is not regulated by immunoproteasome activity in bacterial infections.

5.3.9 Summary - the role of the immunoproteasome in the cytokine / chemokine signaling

In summary, the findings of unaltered cytokine mRNA expression (Fig. 10) AND unchanged NFκB as well MAPK activation upon TLR4 stimulation indicate that the lack of the immunoproteasome does not lead to altered TLR4-dependent cytokine gene

induction in macrophages during bacterial infections. Both findings clearly suggest that any IP-mediated modulation of cytokine secretion observed in the present study occurs on the level of mRNA translation or post-translational regulation steps (e. g. post-translational modifications or secretion regulation).

It is important to note that the sole lack of the immunoproteasome subunit LMP2 has been shown to result in a reduced NFκB activation upon LPS stimulation in B-cells (Hensley et al. 2010). However, in line with the findings discussed here, another study using LMP7 and MECL-1 K.O. mice does not observe an altered IκBα degradation and thus, NfκB activation, in peritoneal macrophages upon LPS treatment (Reis et al. 2011b). However, this study postulates an involvement of the immunoproteasome in late NfκB-mediated cytokine induction. This might be another explanation for the discrepancy between altered cytokine profiles and unchanged mRNA and IκBα degradation patterns observed in the present study. This explanation is supported by the finding that the LPS-induced secretion of the early immune mediator TNFα is not changed in immunoproteasome deficient macrophages, while IL-6 as a late immune modulator shows an impaired secretion.

5.3.10 The role of the immunoproteasome in phagocytosis and intracellular killing

Phagocytosis, i. e. engulfment of invading pathogens followed by intracellular killing, is one key effector function of macrophages during bacterial infections. It is poorly understood, whether the immunoproteasome influences the phagocytosis capacity of macrophages in bacterial infections. Recent *in vivo* data from colleagues show that a severe *E. coli* infection of IP-deficient mice leads to an increased mortality associated with a higher bacterial burden in several organs and tissues compared to wild-type mice (data not shown). Based on these findings we hypothesized that the phagocytosis capacity of macrophages is altered in IP-deficient mice. To confirm this hypothesis, the phagocytosis capacity of macrophages including bacteria uptake and intracellular killing was studied *in vitro* in IP-deficient and wild-type macrophages.

The data presented in figure 14 show that IP-deficient macrophages display the same capacity to engulf *E. coli* particles compared to the wild-type counterparts indicating that immunoproteasome activity is not involved in macrophage-mediated phagocytosis of pathogens. This result is in line with the finding of unchanged secretion patterns of TNFα

(Fig. 11E), a cytokine that plays a pivotal role in the regulation of phagocytosis by expanding the membrane facilitating the engulfment of pathogens (Arango Duque and Descoteaux 2014). In addition, the TLR4-induced activation of p38-MAPK, an important regulator of phagocytosis (Doyle et al. 2004), is not changed in IP-deficient macrophages (Fig. 13) further supporting the conclusions drawn here.

Moreover, the subsequent intracellular killing of engulfed bacteria is also not disturbed in IP-deficient macrophages (Fig. 15) suggesting that immunoproteasome activity is not required in processes that mediate intracellular killing of engulfed bacteria. These findings demonstrate that phagocytosis as well as the intracellular killing capacity of IP-deficient macrophages is not disturbed in bacterial infections. Based on a recent paper showing similar phagocytosis data of neutrophils in fungal infected LMP7-K.O. mice, this conclusion may be extended to other phagocytic cells (Mundt et al. 2016). In conclusion, the higher mortality accompanied by a higher bacterial burden observed in *E. coli*-infected IP-K.O. mice likely is not associated with an impaired macrophage phagocytosis and intracellular killing capacity. Thus, other explanations are required to link the lack of immunoproteasome activity to the immune-compromised phenotype of *E. coli*-infected mice.

5.3.11 The impact of the immunoproteasome on the secretion of nitric oxide

The clearance of invading bacteria via production and release of nitric oxide (NO) is one pivotal effector function of macrophages. It is poorly understood whether immunoproteasome activity is involved in the antibacterial NO response in macrophages. The experiments depicted in figure 16 convincingly show that IP-deficiency results in an impaired NO production by macrophages in response to LPS and *E. coli*. These data suggest that the immunoproteasome is involved in the regulation of NO release in bacterial infections, thereby modulating a crucial antibacterial innate macrophage response. In the first instance, the experiments shown here do not allow to draw any conclusion about the mechanism behind the interconnection of immunoproteasome activity and NO production. However, the finding of impaired IFNβ and IFNγ secretion in IP-deficient macrophages (Fig. 11D and G) might represent a molecular explanation since both cytokines are crucial regulators of NO production (Gessani and Belardelli 1998, Sheikh et al. 2014). In this

context it has been shown that LPS-dependent NO synthesis in macrophages requires autocrine and paracrine signals of IFNβ (Gao et al. 1998). Based on this notion one can conclude that the immunoproteasome modulates antibacterial NO responses indirectly by regulating IFNβ and IFNγ secretion.

5.3.12 The role of immunoproteasome activity on cellular stress tolerance of macrophages

Mediating cellular stress resistance by maintaining protein homeostasis is one of the major functions of the immunoproteasome during inflammatory processes. However, no studies investigated macrophage stress resistance during bacterial infections in an immunoproteasome-deficient background.

To investigate cellular stress tolerance of IP-deficient macrophages under bacterial stress conditions, cell death rates (Fig. 17A) and the accumulation of intracellular reactive oxygen species (ROS) (Fig. 17B) were studied by the present thesis. The experiments displayed in figure 17A clearly show that IP-deficient macrophages exhibit elevated cell death rates under inflammatory conditions. While wild-type macrophages do not show increased stress-induced cell death upon exposure to *E. coli* alone, the presence of *E. coli* strongly induces cell death in IP-deficient macrophages. Stimulation of macrophages with *E. coli* in combination with IFNγ or LPS represents a strong pathogen stress stimulus resulting in a moderate induction of cell death in the wild-type cell population and, to significantly greater extent, in IP-deficient macrophages. These results clearly indicate that the lack of immunoproteasome activity leads to an increased susceptibility of macrophages to pathogen-induced cellular stress. Defective cellular stress resistance associated with increased cell death can lead to impaired *in vivo* antibacterial macrophage responses with implications to the overall innate host immune response to pathogens.

The experiments discussed above show decreased stress resistance of IP-deficient macrophages but do not provide a molecular explanation of IP-mediated protection to cellular stress. In this context it has been described that IFNγ is an important survival factor for macrophages that prevents pathogen-induced apoptosis under inflammatory stress conditions (Schroder et al. 2004, Xaus et al. 1999). In line with this notion the present thesis shows that the lack of immunoproteasome activity leads to an impaired IFNγ secretion and thus, autocrine IFNγ signals upon bacterial stimulation (Fig. 11G) providing

a molecular mechanism of IP-mediated stress resistance. Importantly, providing IFNγ during *E. coli* stimulation does not prevent stress-induced cell death in IP-deficient macrophages further supporting the conclusion discussed above that the lack of immunoproteasome activity impairs proximal intracellular IFNγ signaling (see section 5.3.4). Moreover, IL-1β has been linked to increased cell death in macrophages during bacterial stress conditions (Brough and Rothwell 2007, Martin-Sanchez et al. 2016). In agreement with this notion, the observed increased IL-1β secretion pattern in IP-deficient macrophages under *E. coli* stimulation (Fig. 11F) represents another possible explanation for elevated cell death rates in an IP-deficient background.

In addition to cytokine-mediated macrophage survival / death, the accumulation of reactive oxygen species is another important indicator of cellular stress that is also associated with cell death. In line with the findings discussed above, IP-deficient macrophages exhibit elevated levels of intracellular ROS under inflammatory conditions (Fig. 17B). Increased concentrations of reactive oxygen species can result in protein and DNA damage, thereby leading to cell death. In this manner elevated ROS levels might provide an additional molecular explanation of stress-induced cell death in IP-deficient macrophages. Interestingly, it has been shown that LPS-induced ROS accumulation in macrophages results in increased IL-1β production (Hsu and Wen 2002) providing another link between elevated ROS levels and increased cell death rates in IP-deficient macrophages. However, it is important to note that the accumulation of ROS was measured after 6 hours stimulation, whereas the cell death rates were determined after 1 hour exposure to inflammatory stress, limiting the evidence for an association between elevated ROS levels and increased cell death rates.

In summary, the experiments discussed here clearly show that IP-deficient macrophages exhibit an increased susceptibility to pathogen-induced stress underlining the importance of immunoproteasome activity in stress resistance under inflammatory conditions.

5.4 The role of the immunoproteasome in the modulation of macrophage-mediated T-cell immunity

In addition to innate antibacterial immunity, macrophages are crucial regulators of the adaptive immunity, including T-cell responses. During infection, macrophages modulate T-cell immunity by (i) MHC-mediated antigen-dependent T-cell activation, (ii) T-cell co-stimulation by expression of co-stimulatory molecules and (iii) inhibition of T-cell responses by the expression of co-inhibitory molecules (see introduction). Furthermore, under physiological conditions macrophages regulate thymic T-cell development by providing crucial signals during T-cell selection. However, the role of immunoproteasome activity in macrophage-mediated modulation of T-cell immunity is not fully understood.

5.4.1 The impact of immunoproteasome activity on surface expression of MHC Class I epitopes

In naïve conditions macrophages as well as other antigen-presenting cells (APCs) regulate thymic T-cell development by presenting self-antigens via MHC complexes during T-cell selection (Anderson et al. 1996). The importance of the immunoproteasome for thymic T-cell development was shown by a study of Nil et al. that found that negative T-cell selection primarily involves immunoproteasome-processed peptides from medulla epithelial cells and thymic dendritic cells (Nil et al. 2004). In the context of thymic T-cell development a number of studies as well as our own observations have shown that immunoproteasome-deficient mice exhibit reduced amounts of CD8(+) T-cells in the thymus and periphery accompanied by increased CD4 / CD8 T-cell ratios (Kincaid et al. 2012, Basler et al. 2011, McCarthy and Weinberg 2015). For an optimal development of CD8(+) T-cells in the thymus, macrophages as well as other APCs present self-antigens in the context of MHC class I. The reduced numbers of CD8(+) T-cells in naïve IP-deficient mice might be explained by lower MHC class I expression on macrophages during thymic CD8(+) T-cell maturation. As a matter of fact, in an IP-deficient background reduced expression levels of MHC class I were reported for splenic T- and B-lymphocytes as well as for dendritic cells (Kincaid et al. 2012). However, no data exist for MHC class I expression on peripheral IP-deficient macrophages. To analyze the macrophage expression

pattern of the important MHC class I surface molecules H2-Kb and H2-Db, flow cytometry analyses were conducted (Fig. 18).

In the naïve state without any inflammatory trigger the IP-K.O. macrophage population exhibit a reduced number of macrophages which are positive for the major MHC class I surface molecules H-2Kb and H-2Db. Furthermore, analyses of the cellular amount of both MHC class I epitopes within the MHC-positive cells revealed that the *per cell* expression of H-2Kb and H-2Db is diminished in IP-deficient macrophages compared to wild-type cells. These results indicate that the immunoproteasome is involved in the regulation of macrophage MHC class I surface expression at the basal state. One can conclude that the lack of immunoproteasome activity in macrophages may lead to an impaired MHC-I-dependent presentation of self-antigens to developing CD8(+) T-cell precursors possibly explaining the described reduced thymic output of mature CD8(+) T-cells.

In addition to reduced basal expression levels of MHC class I molecules in IP-deficient macrophages, the results presented in figure 18 show that the cellular induction of MHC class I is also significantly impaired under inflammatory conditions. These data clearly indicate that immunoproteasome activity is required for proper MHC class I upregulation during infection. Furthermore, one can conclude that standard proteasome activity cannot compensate the lack of immunoproteasome activity. In the context of bacterial infections, the observed defective MHC class I upregulation by macrophages might result in an impaired activation of CD8(+) T-cells and hence, to compromised adaptive T-cell responses.

One possible explanation for the finding of decreased MHC class I induction is that IP-deficiency results in impaired secretion and (autocrine) signaling of IFNγ (see section 5.3.4), a crucial regulator of the induction of MHC class I in inflammation (Schroder et al. 2004). A second potential mechanism is that the lack of immunoproteasome protease activity leads to lower peptide supply for MHC presentation during inflammation. In this context, decreased peptide supply for MHC presentation was shown to be associated with a reduced expression of MHC class I (Serwold et al. 2002). This notion is supported by the finding of the present study that gene expression of the *transporter associated with antigen processing 1* (TAP1) is reduced in IP-deficient macrophages upon stimulation with IFNγ and LPS (Fig. 19). TAP1 is a crucial carrier molecule that transports processed peptides from the immunoproteasome to the endoplasmic reticulum where the peptides are coupled

to assembled MHC class I molecules. The significance of reduced TAP1 levels for MHC-mediated peptide presentation is underlined by the fact that TAP1 is the preferred carrier for peptides processed by the immunoproteasome (Nathan et al. 2013).

5.4.2 The role of the immunoproteasome in the expression of the T-cell co-stimulatory molecules CD80 and CD86

As discussed above, the lack of immunoproteasome activity results in a significantly decreased surface expression of MHC class I molecules under basal and inflammatory conditions with potential implications for thymic T-cell development as well as induction of antigen-dependent T-cell responses. In addition to MHC-mediated T-cell activation, macrophages also co-stimulate T-cells via the expression of the T-cell co-stimulatory molecules CD80 and CD86 which interact with the CD28 co-receptor on the surface of T-cells. Proper T-cell activation by macrophages in inflammation requires both signals, (i) MHC-mediated antigen-stimulation of the TCR-complex as well as (ii) co-stimulation by CD80 and CD86. It is not known whether the immunoproteasome is involved in the expression of CD80 / CD86 in macrophages during bacterial infections. Therefore, the surface expression of both co-stimulatory molecules was studied via flow cytometry in IP-deficient and wild-type macrophage.

The results presented in figure 20 clearly indicate that the immunoproteasome plays a pivotal role in the regulation of CD86 expression in macrophages. CD86 expression levels are reduced in IP-deficient macrophages under naïve and inflammatory conditions compared to the wild-type counterparts (Fig. 20B). It is important to note that the observed expression of CD86 in response to the studied inflammatory stimuli can be attributed solely to the presence of IFNγ rather than the presence of *E. coli*. However, IFNγ is an important immune mediator that plays a crucial role in the host defense against viral as well as bacterial pathogens (Shtrichman and Samuel 2001). It has been shown that the upregulation of CD86 depends on the TRIF / IFNβ signaling axis (Hoebe et al. 2003). The secretion of IFNβ in macrophages associated with autocrine induction of CD86 has been shown to be reduced in IP-deficient macrophages (Fig. 11D) providing a possible mechanism of reduced CD86 induction in IP-deficient macrophages.

In contrast to CD86, the expression profile of CD80 is similar between IP-deficient and wild-type macrophages under inflammatory conditions. Interestingly,

immunoproteasome-deficiency results in elevated CD80 levels under basal conditions. From these findings one can conclude that immunoproteasome activity is involved in the basal regulation of CD80 but is not required for the upregulation of CD80 under inflammatory conditions.

The biological consequences of altered CD80 and CD86 expression on the induction of lymphocyte responses during inflammation are difficult to understand since there are conflicting reports on functional differences between CD80 and CD86 (Sansom 2000). However, a well-balanced CD80 / CD86 co-stimulatory signal provided by macrophages is important for proper T-cell activation in the host response to pathogens. In this context it has been shown that a dysregulated CD80 / CD86 co-stimulation of T-cells can lead to a state of T-cell unresponsiveness (Schwartz 2003) with implications for adaptive antibacterial immune responses. However, the complex nature of CD80 / CD86 T-cell co-stimulation does not allow to draw final conclusions whether the immunoproteasome impacts macrophage-mediated T-cell responses during infection.

In addition to CD80 / CD86 expression patterns under *inflammatory conditions*, it is important to discuss the biological significance of the observed differential expression of both molecules in IP-deficient macrophages under *naïve conditions* (Fig. 20). Under non-stimulatory conditions macrophages as well as other professional antigen-presenting cells are involved in the thymic T-cell maturation by providing crucial stimuli to developing thymocytes during T-cell selection (Wood 1985). It has been shown that a well-balanced co-stimulation of CD28 is crucial for proper T-cell selection during thymic T-cell development (Williams et al. 2005). The observation that the lack of the immunoproteasome leads to an altered basal CD80 and CD86 expression profile in macrophages potentially changes the nature of co-stimulatory signals to developing T-cells. As a consequence, thymic T-cell selection might be altered possibly leading to a dysregulated thymic output of mature T-cells. This conclusion is supported by the fact that under naïve conditions IP-deficient mice exhibit an altered composition of peripheral T-cell populations with an increased CD4(+) / CD8(+) T-cell ratio (data not shown).

5.4.3 The impact of the immunoproteasome on surface expression of the T-cell co-inhibitory molecule PD-L1

In addition, to CD80–and CD86-mediated T-cell co-stimulation, macrophages regulate T-cell responses by providing co-inhibitory signals. One of the most important co-inhibitory molecule expressed by macrophages is the *Programmed death-ligand 1* (PD-L1). PD-L1 on the macrophage surface interacts with its respective receptor PD-1 expressed on activated T-cells providing co-inhibitory signals leading to decreased proliferation and / or apoptosis-mediated T-cell death as well as other T-cell suppressive effects (Loke and Allison 2003). By means of PD-L1 induction during inflammation, macrophages prevent overwhelming host-damaging T-cell responses as well terminate T-cell activity after an infection is cleared. Since PD-L1 belongs to the same protein family (B-7) as CD80 and CD86 and both have been shown to be altered in IP-deficient macrophages, we hypothesized that PD-L1 expression is also affected by the lack of immunoproteasome activity.

Flow cytometry analyses were used to study PD-L1 expression in IP-deficient and wild-type macrophages upon stimulation with IFNγ, LPS and *E. coli*. The results displayed in figure 21 show that the surface upregulation of PD-L1 is not altered in IP-deficient macrophages under all studied inflammatory triggers. These results indicate that in contrast to CD80 and CD86 the immunoproteasome is not involved in the expression of the co-inhibitory molecule PD-L1. From these data one can conclude that IP-deficient macrophages are capable to provide proper co-inhibitory PD-L1 signals to T-cells. Furthermore, this notion suggests that the immunoproteasome is not involved in the macrophage-PD-L1-mediated negative regulation of T-cell responses. The finding of unchanged PD-L1 expression patterns is in line with the observation of unaltered TLR4 signal transduction (Fig. 13), since PD-L1 expression has been shown to be regulated by the TLR4-induced MyD88/TRAF6 and MEK/ERK pathways (Ritprajak and Azuma 2015).

5.4.4 Summary - the role of the immunoproteasome in the modulation of macrophage-mediated T-cell immunity

The experiments shown in figure 18 - 21 investigated the role of the immunoproteasome in macrophage-mediated modulation of T-cell immunity during bacterial inflammation. The findings discussed above allow the conclusion that the lack of immunoproteasome activity

in macrophages prevents optimal induction of T-cell immunity, since two major macrophage-dependent T-cell activation mechanisms are compromised. First, MHC class I upregulation is impaired which can potentially have implications for antigen-dependent CD8(+) T-cell activation. Second, the altered expression profile of the co-stimulatory molecule CD86 might lead to an unbalanced co-stimulation of T-cells. Furthermore, the altered cytokine secretion profile in IP-deficient macrophages observed in this study might also result in disturbed T-cell immunity, since the nature of T-cell responses depends on a well-defined cytokine environment. For example, whether CD4(+) T-cell responses preferentially shift towards cell-mediated immunity (activation of phagocytes) or humoral immunity (activation of B-cells) is tightly orchestrated by a specific cytokine milieu (Zhou et al. 2009).

However, the experiments discussed here, do not finally allow to draw the conclusion that IP-deficiency in macrophages impacts macrophage-mediated T-cells responses in bacterial infections. On the one hand, other T-cell regulatory factors not studied here, are involved in the modulation of T-cell immunity during infection. On the other hand, the present study only investigated T-cell regulatory molecules on macrophage level and not on the level of T-cell effector responses. Further experiments are required to confirm the concept proposed here, describing that the immunoproteasome is involved in the regulation of macrophage-mediated T-cell responses during bacterial infections. In this manner the following conceivable experiment can be conducted: bacterial-stimulated wild-type and IP-deficient macrophages are loaded with a particular antigen peptide (e. g. chicken ovalbumin [Ova]) followed by adding clonal T-cells that are specific for the respective antigen-peptide (i. e. OT-I CD8(+) or OT-II CD4(+) T-cells). As a consequence of antigen-peptide presentation and co-stimulation provided by macrophages, the added T-cells are activated eventually exhibiting a variety of effector functions, such as cytotoxic activity, proliferation or effector cytokine secretion. By studying and comparing the respective T-cell responses induced by wild-type and IP-deficient macrophages, one could convincingly confirm that the lack of immunoproteasome activity in macrophages leads to defective macrophage-mediated adaptive T-cell responses in bacterial infections.

5.5 Conclusion

5.5.1 The role of the immunoproteasome in antibacterial macrophage effector functions

Until now, very little is known about the role of the immunoproteasome in the host defense against bacterial infections. Data from our group revealed that the lack of immunoproteasome activity in mice leads to higher mortality in severe *E. coli* infection associated with an increased bacterial burden in several organs. Since macrophages are crucial cells in the host defense against bacterial infections, the present thesis investigated the role of the immunoproteasome in antibacterial macrophage effector functions. To decipher the link of immunoproteasome activity and the complex nature of antibacterial macrophage responses, the present thesis addressed a set of various crucial macrophage effector functions, including (i) cytokine / chemokine responses, (ii) early TLR4 signaling, (iii) phagocytosis of bacteria, (iv) oxidative burst (NO and ROS production), (v) resistance to pathogen-induced stress and (vi) the capacity of macrophages to modulate adaptive T-cell responses.

Investigating the transcriptional induction of important macrophage cytokine and chemokine genes upon exposure to LPS revealed no differences between IP-deficient and wild-type macrophages. In line with this result, the present thesis showed that the immunoproteasome is not involved in the activation pattern of important proximal TLR4 molecules that are associated with cytokine / chemokine gene induction.

In contrast, the lack of immunoproteasome activity results in an altered protein secretion profile of some studied cytokines and chemokines. From these findings one can conclude that the immunoproteasome impacts the *post-transcriptional* regulation of the cytokine and chemokine response in macrophages rather than gene regulatory processes. Importantly, the role of the immunoproteasome in the cytokine / chemokine response during bacterial inflammation appears to be multifaceted since some mediators are unchanged while others are reduced or even upregulated in the absence of the immunoproteasome. Moreover, the lack of immunoproteasome activity does not exclusively impact the secretion of either pro- or anti-inflammatory cytokines. Since it is known that innate immune responses require a

tightly balanced cytokine / chemokine milieu, the altered cytokine / chemokine profile of IP-deficient macrophages might result in compromised innate immune responses.

Studying the engulfment and subsequent intracellular killing of *E. coli* by macrophages showed that the immunoproteasome is not involved in the phagocytic clearance of bacteria. However, the finding of a significantly reduced release of nitric oxide (NO) in IP-deficient macrophages under inflammatory conditions suggests that the lack of immunoproteasome activity might lead to an impaired extracellular clearance of pathogens by macrophages.

In addition to compromised NO release, the lack of immunoproteasome activity also results in decreased cellular resistance to *E. coli*-induced stress as shown by elevated cell death rates as well as intracellular accumulation of reactive oxygen species.

In conclusion, by showing that the lack of the immunoproteasome leads to altered innate macrophage effector functions the present thesis reveals that immunoproteasome activity plays a pivotal role in the macrophage-mediated host response against bacterial infections (summarized in Fig. 22). Hence, the present study provides possible explanations for the observed immune-comprised phenotype of IP-deficient mice during *E. coli* infection *in-vivo*.

In addition to its role in innate macrophage immune responses, the data of this thesis further indicate that the immunoproteasome modulates the ability of macrophages to regulate adaptive T-cell responses (summarized in Fig. 22). Compromised inflammation-induced upregulation of crucial MHC-class I epitopes and TAP1 suggests that antigen-dependent T-cell activation by macrophages is disturbed in the absence of immunoproteasome activity during bacterial infections. This conclusion is supported by the finding that the immunoproteasome is involved in the induction of the macrophage surface expression of the T-cell co-stimulatory molecule CD86. However, the expression patterns of the T-cell co-stimulatory molecule CD80 and the T-cell inhibitory molecule PD-L1 are not altered in an IP-deficient background.

In summary, the present study provides first evidence that the immunoproteasome plays a role in the modulation of macrophage-mediated adaptive T-cell immunity during bacterial infections. However, additional experiments addressing T-cell responses in more detail are required to confirm this interesting finding.

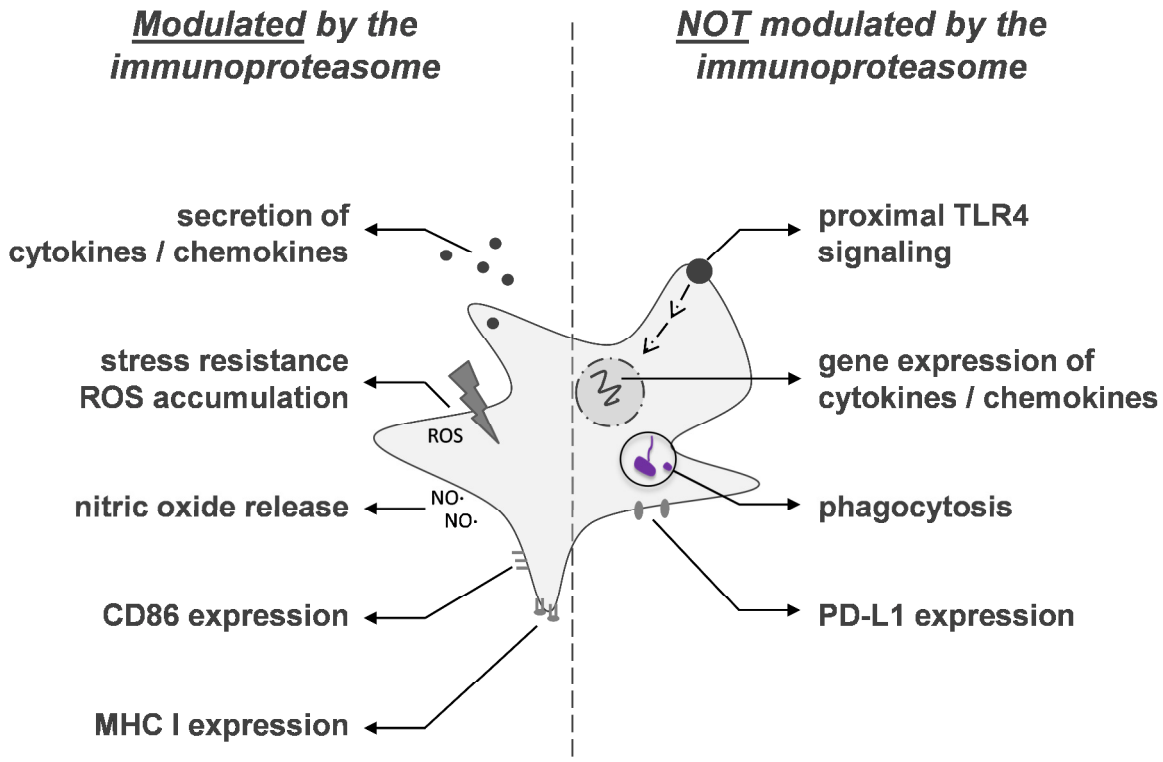

Figure 22. Summary - the role of the immunoproteasome in macrophage effector functions during bacterial infection
The present thesis reveals that the lack of immunoproteasome activity impacts a number of crucial macrophage effector functions during bacterial infections (summarized on the left side of the figure). On the other hand, the data of the present study show that other macrophage effector functions are not modulated by the immunoproteasome (summarized on the right side of the figure).

5.5.2 Compensation of lacking immunoproteasome activity

Since the immunoproteasome and the standard proteasome share similar cellular and proteolytic functions, it is reasonable that the standard proteasome can compensate the lack of immunoproteasome activity in IP-deficient macrophages to some extent. This notion is important for the interpretation of the role of the immunoproteasome in macrophage effector functions during bacterial infections investigated and discussed in the present thesis. It is possible that compensatory mechanisms, e. g. increased standard proteasome expression and / or activity, mask the effects of lacking immunoproteasome activity ultimately leading to wrong conclusions about the involvement of the immunoproteasome in particular macrophage effector functions.

However, the results presented in figure 9 show that under inflammatory conditions the transcriptional expression profile of the standard proteasome subunits beta1, beta2 and beta5 is indistinguishable between wild-type and IP-deficient macrophages. From these

results one can conclude that no compensatory effect exists on the level of transcriptional upregulation of standard proteasome subunits. However, a compensatory effect can occur on protein level that was not studied here. Based on the results that show differences between wild-type and IP-deficient macrophages, compensatory mechanisms for these particular macrophage effector functions can be excluded. In the context of possible compensatory mechanisms there are two ways to interpret all experiments that did not reveal differences between wild-type and IP-deficient macrophages. First, the immunoproteasome is not involved in this particular macrophage function or second, compensatory mechanisms mask the *de facto* involvement of the immunoproteasome in the modulation of this particular macrophage response.

5.5.3 Transferability of the presented results to the human system

Drawing conclusions from murine studies for biological processes raises the question whether experiments with murine cells or organisms reflect the situation in human biological systems. In the context of the present study it is important to discuss the transferability of the presented results to human macrophages on two levels: (i) similarity of the murine and human immunoproteasome system and (ii) biological relevance of murine macrophage cell models and utilized bacterial triggers.

The studied murine IP-subunits share a high amino acid sequence homology with the human homologs. The primary sequence of the proteolytic IP-subunits of human and mice exhibit a 83–96 % sequence identity and a 90–100 % sequence similarity. Moreover, it has been shown that the murine and human IP-genes share the same proteolytic activity (Ferrington and Gregerson 2012). Hence, the functional homology of the murine and human immunoproteasome genes principally allows the transfer of the findings of the present study to the human immunoproteasome system.

Although murine macrophages are widely used to investigate macrophage functions in basic as well as preclinical research, surprisingly little is known about the comparability of mouse and human macrophage biology. In general, macrophages from both species share fundamental biological functions (Reynolds and Haniffa 2015). However, some effector functions, such as NO production, have been reported to be differently regulated in human macrophages (Schneemann and Schoedon 2002).

LPS and *E. coli* were used to mimic (gram-negative) bacterial infections to study macrophage antibacterial effector functions. Both triggers have also been shown to be potent activators of human macrophages (Nau et al. 2002).

In conclusion, although findings from the present murine study might not be entirely transferable to human macrophages, they provide a general understanding of the role of the immunoproteasome in macrophage effector functions. In this context the present thesis is a basis for targeted human studies although the limited availability of appropriate IP-inhibitors impedes human IP studies.

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VI Attachment

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List of Publications

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Detection of vancomycin resistances in enterococci within 3 ½ hours.
*These authors contributed equally to this work.

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Integrating classifiers across datasets improves consistency of biomarker predictions for sepsis

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der

Herstellung des Manuskripts unterstützt haben: Carolin von Lachner, Tilman Klassert und Robby Markwart,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere

wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 18.04.2017