The role of Lipocalin 2 in macrophage polarization and the host defense against *Streptococcus pneumoniae*

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Doctor of Philosophy

Submitted by

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DECLARATION

Joanna Maria Warszawska, MD, performed her PhD studies under the supervision of Univ.-Prof. Dr. Sylvia Knapp, PhD at the Department of Medicine 1, Laboratory of Infection Biology, Medical University of Vienna and the Research Center for Molecular Medicine (Ce-M-M-) of the Austrian Academy of Sciences.

Joanna Warszawska designed and conducted all crucial experiments, analyzed data and wrote the manuscript. Sylvia Knapp provided funding, conceived the project, supervised the work and wrote the manuscript.

Riem Gawish Mag., Omar Sharif PhD, and Stefanie Sigel PhD, assisted with experiments and data analysis. Karin Lakovits and Bianca Doninger provided technical assistance.

Prof. Dr. Günter Weiss from the Medical University Innsbruck provided conceptual and experimental advice and Manfred Nairz MD, PhD, performed experiments. LCN2-/- mice that were generated by Alan Aderem (University of Washington, Seattle U.S.) and Shizuo Akira (University of Osaka, Japan) were provided in collaboration with Günter Weiss.

Prof. Dr. Mathias Müller and Birgit Strobl, PhD, from the Veterinary University Vienna provided STAT3 flox/flox mice that were generated by Valeria Poli at the University of Turin and who granted permission to use these mice.

Ildiko Mesteri, MD, from the Clinical Department of Pathology, Medical University of Vienna, did all histological analyses.

Patient samples were collected by Joanna Warszawska during her residency at the intensive care unit (ICU) of the Department of Medicine 3, before starting her PhD, and she received assistance from Peter Schenk MD, Valentin Fuhrmann MD, and Alexander Spiel MD. Joanna Warszawska performed all assays with patient samples during her PhD. The clinical project was initiated by Sylvia Knapp and Peter Schenk, MD, MSc, (Department of Medicine 2, Medical University of Vienna).

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ABSTRACTS IN ENGLISH AND GERMAN

Despite advances in antimicrobial therapies and vaccination protocols, pneumonia remains the most common cause of death of children worldwide. Among the causative pathogens of bacterial pneumonia in humans, *Streptococcus pneumoniae* is the most relevant one.

Innate immunity is the first line of defense against invading lung pathogens. In the course of pneumonia activated macrophages elicit an inflammatory response, leading to the recruitment of neutrophils and clearance of bacteria. The successful elimination of pneumococci requires the immediate induction of this inflammatory response, which ultimately determines the outcome. Thus, the activation and polarization of lung macrophages is considered a crucial factor for the successful defense against *S. pneumoniae*.

We studied the presence of inflammatory molecules in the bronchoalveolar lavage (BAL) fluid of patients suffering from severe pneumonia requiring mechanical ventilation and discovered substantially elevated concentrations of Lipocalin 2 (LCN2). LCN2 is a glycoprotein expressed mainly by innate immune cells such as macrophages, neutrophils and epithelial cells. It is known for its antimicrobial activity against bacteria such as *Escherichia coli* or *Klebsiella pneumoniae*, which is based on LCN2's ability to scavenge a subset of bacterial siderophores, thereby preventing bacterial iron acquisition. Strikingly, although *Streptococcus pneumoniae* is considered as a siderophore-independent pathogen, infection with this bacterium strongly induced LCN2 in humans – and the biological role of this finding was unclear.

We therefore decided to investigate the potential function of LCN2 in the inflammatory response to this bacterium. Considering reports indicating the involvement of deactivating agents in the regulation of LCN2 expression, we hypothesized that LCN2 might be involved in determining the functional properties of macrophages. Using a variety of molecular techniques, we discovered that LCN2 was released by deactivated macrophages – and that LCN2 itself had the capacity to deactivate macrophages in the presence of bacteria. We further revealed that the anti-inflammatory cytokine IL-10 mediated the anti-inflammatory effects in the presence of LCN2. Using a murine pneumococcal pneumoniae model, we were able to reproduce

the deactivating properties of LCN2 in the lungs upon infection, which led to a delayed immune response and consequently enhanced bacterial outgrowth. Mice deficient in LCN2 therefore exhibited an improved immune response and survival from pneumococcal infections. In agreement with data from mouse studies, we further discovered that higher LCN2 levels in the BAL of patients suffering from pneumonia caused by Gram positive bacteria correlated with a bad outcome of the infection.

Our data reveal for the first time a detrimental role for Lcn 2 during severe infections caused by *Streptococcus pneumoniae* and indicate that LCN2 could potentially serve as a biomarker of severe lung infections.

Trotz des Einsatzes von Antibiotika und Impfprogrammen ist die bakterielle Pneumonie, meist verursacht durch *Streptococcus pneumoniae*, auch heute noch die weltweit häufigste Todesursache von Kindern.

In der Lunge residierende Makrophagen, als Teil des angeborenen Immunsystems, spielen bei der Abwehr von *Streptococcus pneumoniae* eine kritische Rolle. Sie sind die ersten Zellen, die auf die Infektion reagieren und essentiell für die Induktion der Entzündung. Bei Aktivierung produzieren die Makrophagen Zytokine und Chemokine, die zur Rekrutierung von Neutrophilen und im Weiteren zur Eliminierung der Bakterien führen. Je stärker und schneller diese frühe Entzündungsreaktion induziert wird, umso effizienter funktioniert die Beseitigung der Pneumokokken und umso besser ist auch der Verlauf der Erkrankung. Demnach ist die Aktivierung und Polarisierung von Lungenmakrophagen ein kritischer Faktor in der Abwehr von *Streptococcus pneumoniae*.

Im Zuge dieser Studie wurde die bronchoalveolare Lavage (BAL) Flüssigkeit von Patienten mit schwerer, beatmungspflichtiger Pneumonie auf das Vorhandensein von verschiedenen Entzündungsmediatoren untersucht. Besonders auffallend waren deutlich erhöhte Konzentrationen von Lipocalin 2 (LCN2), einem antimikrobiellen Glykoprotein das vorwiegend von Zellen des angeborenen Immunsystems, wie Makrophagen, Neutrophilen und Epithelzellen, produziert wird. LCN2 war bis jetzt hauptsächlich für seine Aktivität gegen *Escherichia coli* und *Klebsiella pneumoniae* bekannt, wobei die antimikrobielle Wirkung auf der Fähigkeit von LCN2 beruht bakterielle Siderophoren zu binden und den Bakterien auf diesem Wege Eisen zu entziehen. Obwohl *Streptococcus pneumoniae* bis dato als Siderophor-unabhängiger Keim gilt, fanden wir sehr hohe LCN2 Konzentrationen in Patienten mit Pneumokokken Pneumonie und beschlossen daher die Funktion von LCN2 in der Abwehr von *Streptococcus pneumoniae* genauer zu untersuchen.

Es ist bekannt, dass die LCN2 Expression von immunsuppressiven Substanzen induziert wird. Basierend darauf, stellten wir die Hypothese auf, dass LCN2 direkt an der Regulation der Makrophagenaktivierung und -funktion beteiligt ist und damit eine wichtige Rolle während der Pneumokokken Pneumonie spielt.

Mit Hilfe diverser zellbiologischer Techniken konnten wir zeigen, dass LCN2 i) vor allem von deaktivierten Makrophagen produziert wird und ii), dass die Präsenz von

LCN2 auch essentiell für den Vorgang der Makrophagendeaktivierung ist. Mechanistisch weisen unsere Daten darauf hin, dass die antiinflammatorischen Effekte von LCN2 von IL-10, einem wichtigen antiinflammatorischen Zytokin, vermittelt werden.

Diese, auf in vitro Versuchen basierenden Erkenntnisse, werden auch in vivo reflektiert, was anhand eines Pneumokokken Mausmodells untersucht wurde. Während der Infektion induziertes LCN2 führte in Mäusen zu einer verzögerten frühen Immunantwort und damit zu einer schnelleren Vermehrung der Bakterien. Im Gegensatz dazu zeigten LCN2 defizienten Mäuse eine verstärkte frühe Zytokinproduktion, vermehrte Einwanderung von Neutrophilen in die Lunge und als Konsequenz einen Überlebensvorteil während der Pneumokokken Pneumonie. Im Einklang damit korreliert eine starke LCN2 Induktion mit einer höheren Sterberate bei Patienten mit durch Gram-positive Keime verursachter Lungenentzündung.

Unsere Daten weisen erstmals auf eine schädliche Rolle von LCN2 bei Infektionen mit *Streptococcus pneumoniae* hin und identifizieren LCN2 als potentiellen Biomarker für die Prognose von Pneumokokken Pneumonien.

PUBLICATIONS ARISING FROM THIS THESIS

Lipocalin 2 deactivates macrophages and worsens pneumococcal pneumonia outcomes.

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ABBREVIATIONS

AM	alveolar macrophage(s)
AMAC-1	alternative macrophage activation-associated CC chemokine-1
AP-1	activator protein-1
APC	antigen presenting cell
APOC2	apolipoprotein C-II
APOE	apolipoprotein E
Asc	apoptosis-associated speck-like protein containing a CARD
ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage
Bcl-2, -10	B-cell lymphoma 2, 10
BCL2L11	Bcl-2-like 11
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
BMDM	bone marrow derived macrophages
CAP	community acquired pneumonia
CARD	caspase recruitment domain
Cardif	CARD adaptor inducing IFN-β
CbpA	choline-binding protein A
CCL	C-C chemokine ligand
CD	cluster of differentation
CDP	common dendritic cell precursor
ChoP	phosphorylcholine

CLR	C-type lectin receptors
CSF	colony stimulating factor
CTLD	C-Type Lectin-Like Domain
CXCL	chemokine (C-X-C motif) ligand
DAI	DNA-dependent activator of IFN-regulatory factors
DC	dendritic cell
DUSP	dual specificity protein phosphatises
DALYs	disability adjusted life years
ETS	E26 transformation-specific transcription factor family
FIZZ1	found in inflammatory zone 1
Foxo3a	Forkhead-box-O3 transcription factor
GAS	gamma activated sequences
GM-CSF	granulocyte-macrophage colony stimulating factor
HAP	hospital acquired pneumonia
H&E	hematoxyllin & eosin
HIV	human immunodeficiency virus
HLA	human leukocyte antigens
HO	hemoxygenase
HSC	hematopoietic stem cells
ICU	intensive care unit
ie-DAP	D-γ-glutamyl-meso-diaminopimelic acid
IFN	interferon
IKK	IκB kinase

IL	interleukin
IM	interstitial macrophage(s)
iNOS	inducible nitric oxide synthase
IPS-1	IFN-β promoter stimulator 1
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosin-based inhibitory motif
LCN2	lipocalin 2
(V)LDL	(very) low density lipoproteins
LPS	lipopolysaccharide
LRR	leucine rich repeats
LTA	lipoteichoic acid
MAC	membrane attack complex
Mal	MyD88 adaptor like
Malt-1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	mitogen activated protein kinases
MARCO	macrophage receptor with collagenous structure
MAVS	mitochondrial antiviral signaling
MCP1	monocyte chemotactic protein 1, CCL2
MDA-5	melanoma differentiation-associated gene 5
MDC	macrophage-derived chemokine, CCL22
MDP	macrophage-dendritic cell progenitor
MDP	muramyl dipeptide

MDP-PG	muramyl dipeptide-peptidoglycan
MHC	major histocompatibility complex
MMP	matrix metalloproteinase(s)
MyD88	myeloid differentiation primary response gene 88
NET	neutrophil extracellular traps
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κappa B
NKT	natural killer T cells
NLR	NOD like receptors
NBD	nucleotide-binding oligomerization domain
NOD	NBD domain containing protein
Nrf1	nuclear respiratory factor 1
Nurr1	nuclear receptor related 1
PAFR	platelet-activating factor receptor
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PG	prostaglandins
PGN	peptidoglycan
pia	pneumococcal iron acquisition operon
PIM	pulmonary intravascular macrophage(s)
pit	pneumococcal iron transport operon
piu	pneumococcal iron uptake operon
PI3K	phosphoinositide 3-kinase

PLCγ	phospholipase C gamma
PM	peritoneal macrophage(s)
PPARγ	peroxisome proliferator-activated receptor gamma
PRR	pattern recognition receptors
PsaA	pneumococcal surface antigen A
PspA	pneumococcal surface protein A
PTX	pentraxin
PYD	pyrin domain
RGS-16	regulator of G protein signalling-16
RIG-I	retinoic acid inducible gene I
RIP2	receptor interacting protein 2
RLR	RIG-I-like receptors
ROI/ROS	reactive oxygen intermediates / species
RSV	respiratory syncytial virus
SARM	sterile α- and armadillo-motif-containing protein
SHIP	Src-homology-2-domain -containing inositol phosphatase
SHP-1/-2	Src-homology-2-domain-containing protein tyrosine phosphatase 1/2
SiglecF	sialic acid-binding immunoglobulin-like lectin F
SIGNR1	dendritic cell specific intercellular adhesion molecule grabbing nonintegrin related 1
SLAM	signalling lymphocyte activation molecule
SLC22A17	solute carrier family 22 member 17
SOCS	suppressor of cytokine signalling

SORL1	sortilin-related receptor, L(DLR class) A repeats-containing
Srnx-1	sufiredoxin-1
STAT	Signal Transducer and Activator of Transcription
Syk	spleen tyrosine kinase
TARC	thymus and activation-regulated chemokine, CCL17
TNF	tumor necrosis factor
TGFβ	transforming growth factor β
Thsp1	thrombospondin
TIR	Toll/interleukin-1 receptor domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll like receptors
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF related adaptor molecule
Treg	regulatory T cells
TRIF	TIR-domain-containing adaptor –inducing interferon β
Txnrd	thioredoxin reductase
UNICEF	United Nations International Children's Emergency Fund
VEGF	vascular endothelial growth factor
VISA	virus-induced signalling adaptor
WHO	World Health Organization

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ventilated ICU patients with suspected pneumonia. This project extended my PhD thesis with important clinical data supporting our findings. I appreciate Peter for his competence, enthusiasm, vigor, curiosity, passion for science and his personality.

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1. INTRODUCTION

From an immunological point of view, the lung comprises a very interesting tissue compartment, as it is constantly exposed to microorganisms (Charlson et al, 2011), which requires the presence of a sophisticated defense system. At the same time, to preserve the lung's major biological function – gas exchange – a tightly controlled immune response is required, to maintain sufficient tissue to allow for proper oxygenation.

1.1 LUNG IMMUNITY

Immune responses to pathogens can be generally divided into innate and acquired, which can both be further divided into cellular and humoral (see Table 1). Innate immunity encompasses inborn and rather unspecific mechanisms which are promptly available (Beutler, 2004). On the other hand, acquired immune responses are very specific and directed against distinct epitopes of pathogens, which require more time to evolve (in the order of days to weeks). Therefore, it is the innate immune response that constitutes the first line defense and plays a superior role during acute bacterial lung infections.

Table 1 Immune responses

Responses	Innate	Acquired
Humoral	Complement factors Antimicrobial peptides Lysozyme Lactoferrin Cyto-/chemo-kines	Immunoglobulins
Cellular	Macrophages Granulocytes (neutrophil, basophil and eosinophil) Dendritic cells Innate lymphoid cells Mast cells Epithelial cells Endothelial cells	T-cells B-cells NKT-cells

The lung is not a sterile site. On the contrary, airways are constantly exposed to microorganisms (Charlson et al, 2011). The outcome of any colonization/infection is dictated by the virulence and the number of bacteria on the one hand and host's defense mechanisms on the other hand. In case low numbers of microorganisms enter the airways, the chances are high that they will be successfully trapped in the mucus and cleared from lungs by a coordinated movement of cilia or killed by bactericidal peptides. If some pathogens manage to enter the lower airways, they are encountered by alveolar macrophages. Alveolar macrophages are essential innate immune cells in the pulmonary compartment. Strategically located at the luminal site of the alveolus, alveolar macrophages are considered the first immune cells to encounter invading pathogens. Representing a crucial element of lung defense and physiology, alveolar macrophages gain phagocytic and bactericidal properties upon pathogen recognition. This step is facilitated by a number of pattern recognition receptors (PPRs), which sense diverse pathogen associated molecular patterns (PAMPs), such as conserved cell wall components, bacterial RNA or DNA. Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like helicases or scavenger receptors are such PRRs (Akira et al, 2006; Beutler, 2009; Creagh & O'Neill, 2006). The engagement of TLRs initiates a downstream signaling cascade resulting in the nuclear translocation of nuclear factor kappa B (NFκB) and the induction of inflammatory genes (Kawai & Akira, 2007). This leads to the production and secretion of proinflammatory cytokines and chemokines. Alveolar macrophages are the major cells involved in the initiation of this response, however, other cells of innate immunity (e.g. epithelial cells) also contribute to the production of pro-inflammatory cytokines and chemokines during infection. If the number of bacteria is low, alveolar macrophages engulf and kill bacteria without inducing a relevant inflammatory response. If the pathogen burden, however, exceeds the clearance capacity of the system, alveolar macrophages initiate a strong inflammatory response. As a result, chemokines secreted at the site of bacterial invasion facilitate the immediate recruitment of neutrophils. Neutrophils reside in pulmonary capillaries in a 50 times higher concentration than in the peripheral blood, ready to pass the alveolo-endothelial barrier when needed (Burns et al, 2003). These professional phagocytes are very effective in pathogen phagocytosis and killing, contributing substantially to bacterial clearance. Further, activated neutrophils release neutrophil extracellular traps (NETs) - "a soup" of antimicrobial peptides embedded in a matrix of deoxyribonucleic acids (Yipp & Kubes, 2013). These structures serve to

entrap and kill extracellular bacteria. After neutrophils fulfilled their purpose they undergo apoptosis and are cleared by macrophages – a critical step for the resolution of inflammation (Knapp et al, 2003).

Another important aspect of the inflammatory response involves the extravasation of plasma contents into alveoli. Humoral innate factors such as complement or natural immunoglobulins are present in plasma and divided from alveoli by the thinnest barrier in the body. Mediators secreted by macrophages and other cells upon infection lead to vascular leakage and increased permeability for plasma contents. Complement factors (mainly C3b) and immunoglobulins opsonize bacteria and thus facilitate phagocytosis (Beutler, 2004). Complement factors form the membrane attack complex (MAC), which lyses bacterial cells (Beutler, 2004). All these defense mechanisms are effective in bacterial killing but also lead to unintended side effects and “collateral damage”. It is therefore of high importance to control inflammation and to restore homeostasis after the pathogen has been successfully eradicated. Alveolar macrophages are importantly involved in this step of the inflammatory response via their ability to clear apoptotic and necrotic cells (Knapp et al, 2003) and via the production of anti-inflammatory mediators such as IL-10, TGFβ or prostaglandins.

1.2 MACROPHAGES

Macrophages owe their name to Elie Metchnikoff, who was working on phagocytosis and termed “large eaters” macrophages in contrast to neutrophils, which he called “small eaters” (microphages). Since these days, macrophages have been investigated extensively (Gordon, 2007).

1.2.1 MONOCYTE AND MACROPHAGE POPULATIONS

Monocytes and macrophages are hematopoietic cells. Monocytes originate from macrophage-dendritic cell progenitors (MDP) in the bone marrow. For many years the dogma was that monocytes are released into the blood to circulate a few days before they give rise to most tissue resident macrophages and a subset of dendritic cells

(CD11b hi, CD103-) (Hashimoto et al, 2011) (see Figure 1.). However, more recent data revealed that macrophages originate to a great extent from the proliferation of embryonically derived local population and only minimally from monocytic precursors (see Figure 2) (Epelman et al, 2014; Guilliams et al; Hashimoto et al; Sieweke & Allen, 2013). However, under inflammatory conditions the population of monocytes differentiating to macrophages increases (Epelman et al, 2014). During inflammation also the number of monocyte-derived dendritic cells raises. The majority of dendritic cells under steady state originate, however, from a common dendritic cell precursor (CDP). Alveolar macrophages are long lived cells that were recently found to originate from fetal monocytes in a GM-CSF dependent manner (Guilliams et al).

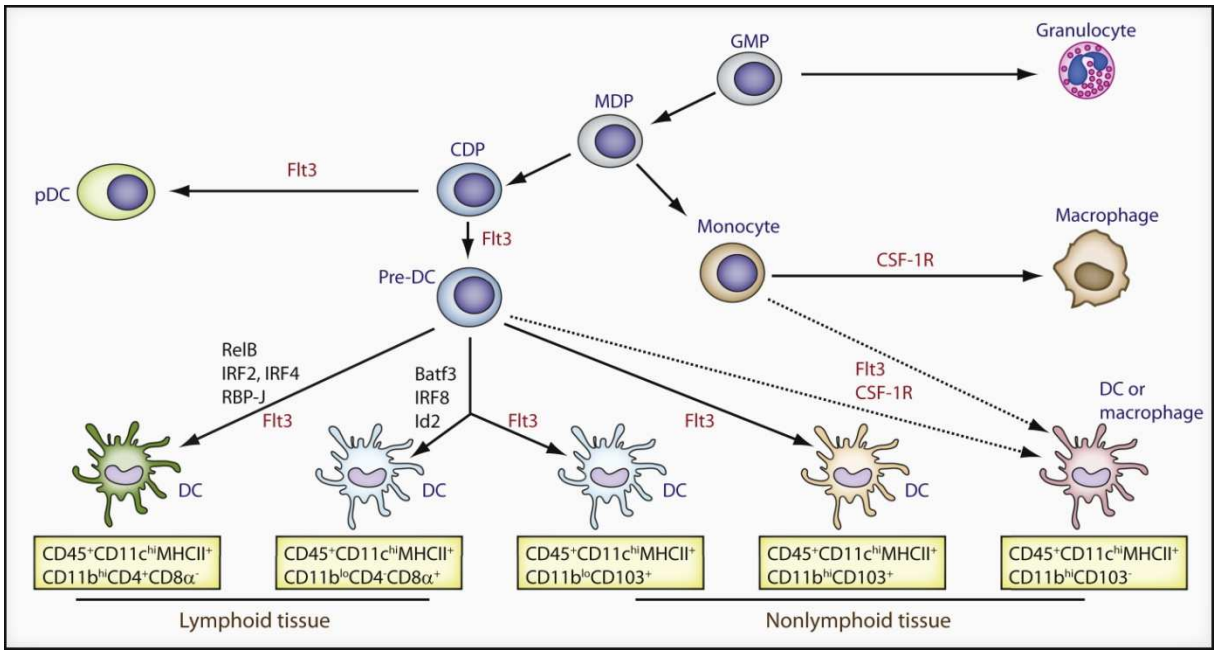

Figure 1 The origin of dendritic cells and macrophages

Adapted from Hashimoto et al. *Immunity* 2011 (Hashimoto et al, 2011).

The differentiation of macrophages requires the colony stimulating factor 1 (Csf-1) and the transcription factor PU.1 (Hashimoto et al, 2011). However, other proteins of the ETS family, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, KIT, proteins of the TNF family and TNF-receptor related proteins are also important determinants of macrophage differentiation (Gordon, 2003). Macrophages are a very heterogeneous group of cells. The phenotype of macrophages depends on the site they reside, and the local micro-environment. In general, lymphoid and non-lymphoid tissue macrophages are distinguished based on their location in lymphoid (spleen,

lymph nodes, bone marrow, thymus) or non-lymphoid organs (lung, liver, gut, skin, kidney). In the spleen, red pulp, marginal zone, marginal zone metalophillic and tingibile body macrophages are differentiated. In the lymph nodes there are subcapsular sinus, medullar, $CD11c^{hi}CD169^{+}$ and tangible body macrophages. The bone marrow is populated by bone marrow $CD169^{+}$ macrophages (Hashimoto et al, 2011). In the thymus, subcapsular, cortex, cortico-medullary and medulla macrophages are found. Non-lymphoid macrophages encompass Kupffer cells in the liver, lamina propria and serosal macrophages in the gut, dermal and deep dermis macrophages in the skin, and kidney and lung macrophages (see below). Microglia in the central nervous system and osteoclasts in the bone represent very specialized macrophage populations.

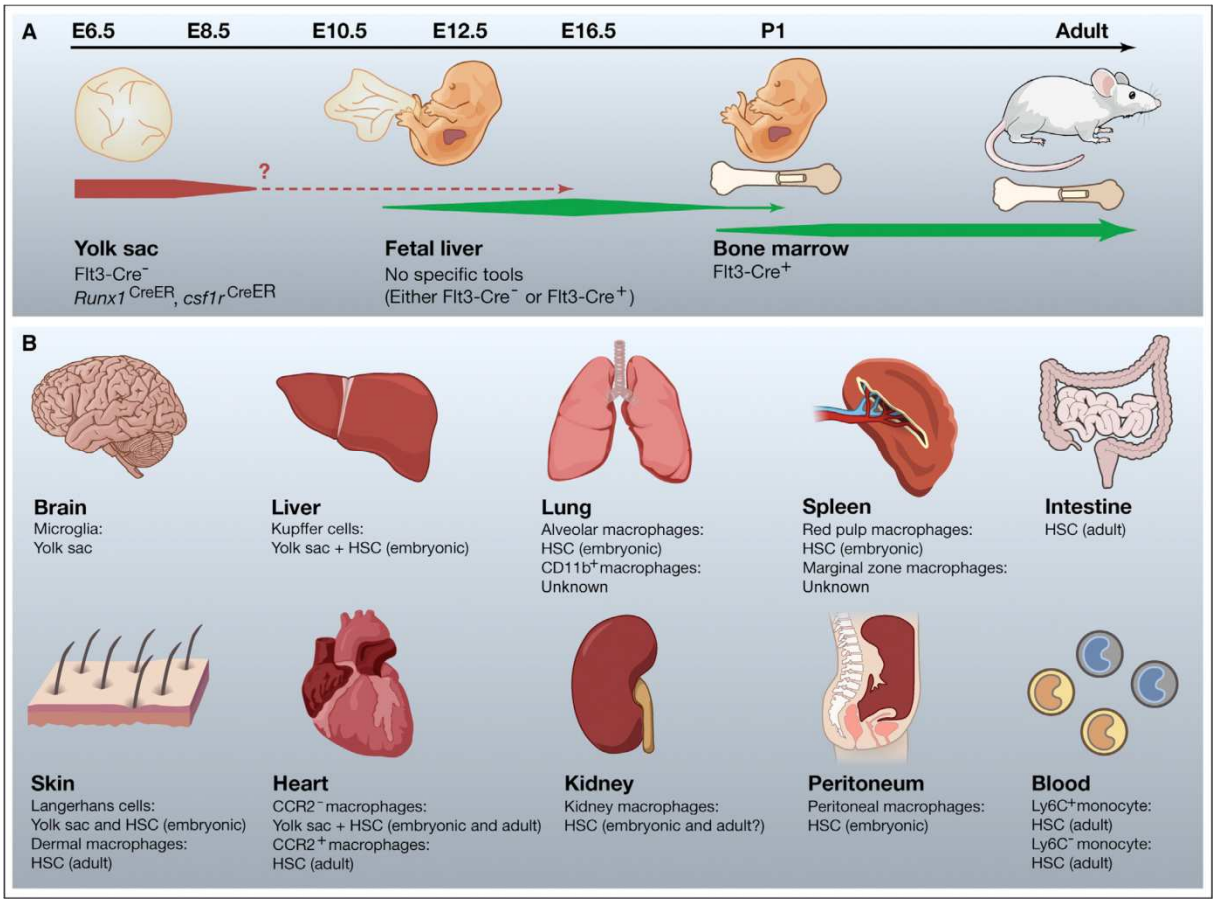

Figure 2 Resident macrophage populations at different anatomical locations and their origins

Yolk sac progenitors, embryonic and adult hematopoietic stem cells (HSC). Macrophages which originate from adult HSC are continually repopulated by monocytes. Adapted from Epelman et al., *Immunity* 2014 (Epelman et al, 2014).

The heterogeneity among monocytes is less well understood. However, at least 2 distinct phenotypes can be found in mice and humans. In mice, based on the expression of Ly6C, CD43 and CX_3CR1, classical also known as inflammatory ($Ly6C^{hi}$, CD43-, $CX3CR1^{low}$) and non-classical monocytes ($CX3CR1^{hi}$, CD43+, $Ly6C^{low}$) are distinguished (Epelman et al, 2014; Gordon & Taylor, 2005). Inflammatory monocytes migrate towards inflammatory sites in response to chemotactic agents such as C-chemokine ligand 2 (CCL2; also known as MCP1) as they express the CCL2 receptor CCR2, and CD62L (L-selectin) (see Table 2). However, recent reports suggest that this monocyte subset can exit the vasculature also under resting conditions (Epelman et al, 2014). The major task of classical monocytes is patrolling of the extravascular space for any foreign material, which is then conveyed to lymph nodes. Under inflammatory conditions classical monocytes differentiate to tissue macrophages after they have left the vessels. Non-classical monocytes are believed to patrol and clear the intravascular space from damaged endothelial cells. These monocytes differentiate from classical monocytes through a NR4A1 dependent mechanism. Murine monocyte subsets are depicted in Figure 3. Interestingly, analogous populations among human monocytes are found. As such, human CD14hi CD16- (classic) monocytes express CCR2 and low levels of CX3CR1 and correspond to the murine classical monocyte subset (Gordon & Taylor, 2005). Human CD14+CD16+ monocytes express high levels of CCR5, MHC class II and similar to murine non-classical monocytes high amounts of CX3CR1. Recently another subset was identified within human CD14+CD16+ monocytes, characterized by high expression of CD64 (FcγRI), CD86 and HLA-DR (Grage-Griebenow et al, 2001). This group showed characteristics of monocytes and dendritic cells with high phagocytic activity and cytokine production, and high stimulatory activity on T cells, respectively.

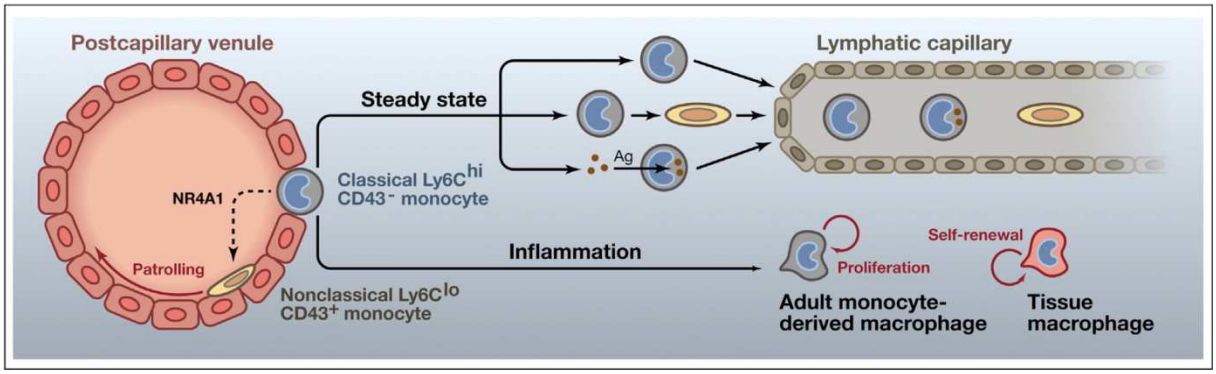

Figure 3 Murine monocyte subsets

Adapted from Epelman et al, *Immunity* 2014 (Epelman et al, 2014).

Table 2 Two major monocyte subsets in human and mice (receptor comparison)

Adapted from Gordon and Taylor, *Nat Rev Immunol* 2005 (Gordon & Taylor, 2005).

Antigen	Human $CD14^{hi}CD16^{-}$ 'inflammatory' monocytes	Human $CD14^{+}CD16^{+}$ 'resident' monocytes	Mouse $CCR2^{+}$ CX_3CR1^{low} 'inflammatory' monocytes	Mouse $CCR2^{-}$ CX_3CR1^{hi} 'resident' monocytes
Chemokine receptors				
CCR1	+	–	ND	ND
CCR2‡	+	–	+	–
CCR4	+	–	ND	ND
CCR5	–	+	ND	ND
CCR7	+	–	ND	ND
CXCR1	+	–	ND	ND
CXCR2	+	–	ND	ND
CXCR4	+	++	ND	ND
CX_3CR1‡	+	++	+	++

Other receptors				
CD4	+	+	ND	ND
CD11a	ND	ND	+	++
CD11b	++	++	++	++
CD11c[‡]	++	+++	–	+
CD14	+++	+	ND	ND
CD31	+++	+++	++	+
CD32	+++	+	ND	ND
CD33	+++	+	ND	ND
CD43	ND	ND	–	+
CD49b	ND	ND	+	–
CD62L[‡]	++	–	+	–
CD80	ND	ND	ND	ND
CD86	+	++	ND	ND
CD115	++	++	++	++
CD116	++	++	++[§]	++[§]
CD200R	ND	ND	ND	ND
F4/80	ND	ND	+	+
Ly6C	ND	ND	+	–
7/4	ND	ND	+	–
MHC class II	+	++	–	–

1.2.2 PULMONARY MACROPHAGES

In the lower respiratory tract, the organ of interest of this work, three tissue compartments, e.g. alveolar, interstitial and intravascular, are distinguished. Alveoli are air containing vesicles lined by pulmonary epithelial cells (pneumocytes) and a thin surfactant layer. This compartment is constantly exposed to microorganisms. The interstitial compartment is a space located between alveoli and vessels. It consists mainly of extracellular matrix. The intravascular compartment encompasses lung capillaries. Interstitial and intravascular compartments are usually sterile. Each compartment harbors a specific macrophage population. Thus, alveolar (AM), interstitial (IM) and in some species (cattle, horse, goat or pig) also pulmonary intravascular macrophages (PIM) are known (Schneberger et al). Alveolar macrophages are large cells that reside in the alveolar space. They are characterized by the expression of CD11c, mannose receptor, CD200R, CD11a, CD18, CD169,

CD68, SiglecF, MARCO, Mac-2 and low F4/80, CD14 and MHC class II expression (Bedoret et al, 2009; Hashimoto et al, 2011; Hussell & Bell, 2014; Lohmann-Matthes et al, 1994; Maus et al, 2001). Alveolar macrophages express multiple PRRs and can bind a variety of molecules (see Table 3 and 4). There are conflicting data whether alveolar macrophages are pro- or anti-inflammatory (Schneberger et al). So far, the pro-inflammatory actions of alveolar macrophages were shown in vitro (Schneberger et al). In vivo, epithelial cells affect the function of alveolar macrophages both directly and indirectly (Hussell & Bell, 2014). Thus, bronchial epithelial cells secrete IL-10 in a constitutive manner (Bonfield et al, 1995). Further, surfactant lipids and proteins are important factors of epithelial origin shaping the immune responses of alveolar macrophages by modulation of TLR function (Henning et al, 2008). Another model emphasizes the role of αvβ6 integrin and TGFβ at the epithelial-macrophage interface, which keeps alveolar macrophages in a quiescent state under steady-state conditions (low proinflammatory cytokine production, poor phagocytosis, suppression of adaptive immune responses) (Lambrecht, 2006; Munger et al, 1999). In this model, the switch to pro-inflammatory functions (activation) is facilitated by TLR ligation. Activated alveolar macrophages loose contact to epithelial cells and tonic αvβ6-TGFβ inhibition and become excellent phagocytes and potent in killing of ingested pathogens. (Lambrecht, 2006). Finally, epithelial cells limit the inflammatory response of alveolar macrophages via CD200-CD200R interaction (Snelgrove et al, 2008).

Table 3 Ligands bound by alveolar macrophages

Adapted from Lohmann-Matthes et al., *Eur Respir J* 1994 (Lohmann-Matthes et al, 1994).

	Mouse	Human
Immunoglobulins	IgG1, IgG2a, IgG2b, IgG3	IgG1, IgG3 monomers, IgG complexes
Proteins	fibronectin, fibrin, lactoferrin, transferrin, GM-CSF, CSF-1, INF-γ, IL-4, IL-1Ra, IL-2, IL-10, TGFb, insulin	
Surface markers	Class II molecules, CD11a, CD11b, CD11c, CD14, CD18, CD54	
Complement	C3b, iC3b, C4b, C3d, C3dg, C5a	
Lipoproteins	LDL, β-VLDL	
Lectins	α-linked galactose, N-acetylgalactosamine, N-acetylglucosamine, α-linked fucose, mannose, N-acetylneuramine residues	

Table 4 Differences in Toll like receptor (TLR) expression between murine and human monocytes and lung macrophages

Adopted from Schneberger et al., *Cell Tissue Res* 2011 (Schneberger et al).

		Mouse		**Human**			
		+	**-**	**+**	**-**	**+/-**	**?**
Blood	*Monocytes*	TLR-4,9	TLR-10	TLR-1,2,4, 5,6,7,8		TLR-3	TLR-7,9
Lung	*alveolar macrophages*	TLR-2,3,4, 5,7,9	TLR-10	TLR-2,4,9	TLR-3		
	interstitial macrophages	TLR-2,4	TLR-10	?	TLR-3		
References		2, 4-6, 8, 11-13, 16		1-3, 7, 9, 10, 14, 15			

(+) expressed, (-) not expressed, (+/-) expressed at low level, (?) uncertain/disputed expression

(1) Visintin et al. 2001, (2) Hornung et al. 2002, (3) O'Mahony et al. 2008, (4) Kadowaki et al. 2001, (5) Klinman 2004, (6) Hawn et al. 2007, (7) Maris et al. 2006, (8) Cabanski et al. 2008, (9) Droemann et al. 2005, (10) Zarember and Godowski 2002, (11) Bedoret at al. 2009, (12) Bessa et al. 2009, (13) Punturieri et al. 2004, (14) Chang et al. 2007, (15) Juarez et al. 2010, (16) Leon et al. 2004

Interstitial macrophages are located in the lung interstitium. They are smaller than alveolar macrophages and express a distinct receptor pattern: $CD11c^{-}$ $F4/80^{+}CD68^{+}MHCII^{+}$ (Hashimoto et al, 2011). This macrophage population is less well studied as it is difficult to isolate. Compared to alveolar macrophages they are equally effective regarding Fcγ receptor mediated pahagocytosis, less effective regarding Fcγ receptor independent phagocytosis, produce less reactive oxygen and nitrogen species, TNFα and type I interferons, but are better in antigen presentation due to higher MHC Class II receptor expression and higher accessory function (Lohmann-Matthes et al, 1994) (see Figures 4 and 5). Recent studies indicate that interstitial macrophages are rather anti-inflammatory, as they secrete spontaneously high amounts of IL-10 and are able to prevent airway allergy (Bedoret et al, 2009).

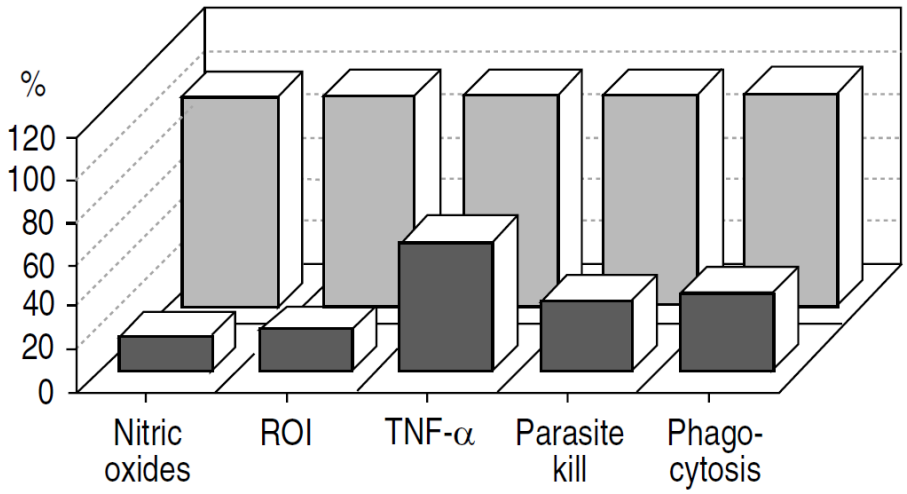

Figure 4 Comparison of alveolar (light grey bars) and interstitial (dark grey bars) macrophages

Columns 1-3: treatment with LPS; columns 4 and 5 at baseline; ROI reactive oxygen intermediates. Adopted from Lohmann-Matthes et al., *Eur Respir J* 1994 (Lohmann-Matthes et al, 1994).

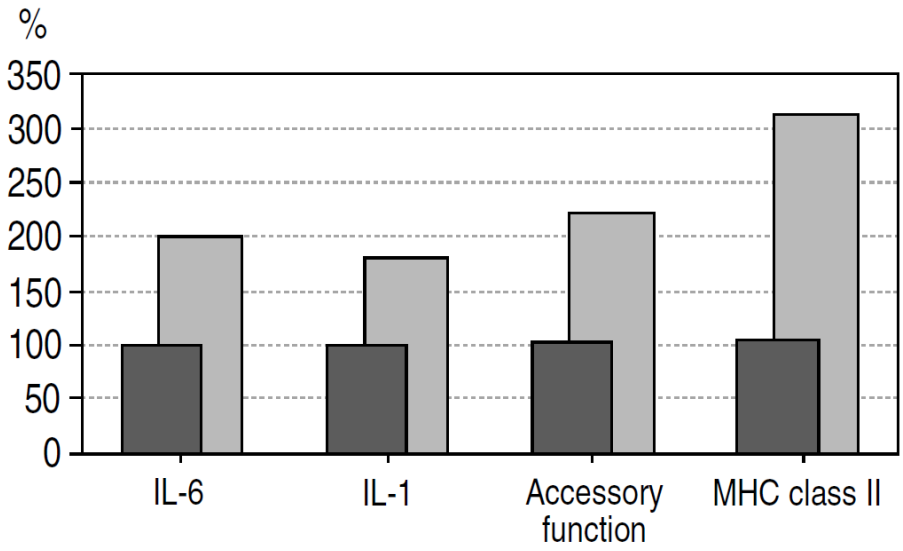

Figure 5 Comparison of alveolar (dark grey bars) and interstitial (light grey bars) macrophages

Columns 1 and 2: after stimulation with LPS; columns 3 and 4 at baseline. Adapted from Lohmann-Matthes et al., *Eur Respir J* 1994 (Lohmann-Matthes et al, 1994).

Pulmonary intravascular macrophages are a group of lung macrophages identified last (Staub, 1994). These macrophages adhere to pulmonary vessels in some domestic animals (see above). They are responsible for the elimination of foreign material,

which entered the bloodstream. They seem to be pro-inflammatory as they render animals more susceptible to lung inflammation (Gill et al, 2008).

Lung dendritic cells are localized mostly in the lung interstitium and resemble DCs found in the spleen. They are positive for CD11c, highly positive for MHC Class II and negative for F4/80 and FcγR (Lohmann-Matthes et al, 1994). They are poor phagocytes but specialized in antigen presentation and accessory function.

1.2.3 MONOCYTES AND MACROPHAGES IN VITRO

Monocytes can be isolated from murine blood, i.e. from peripheral blood mononuclear cells (PBMC), which contain besides monocytes also lymphocytes. Peritoneal (PM), bone-marrow derived (BMDM) and alveolar macrophages (AM) are the most popular murine primary macrophages in cell culture. Resident peritoneal macrophages or e.g. thioglycollate elicited peritoneal macrophages are retrieved from the peritoneal cavity by peritoneal lavage (50 and 70% of retrieved cells respectively). Bone-marrow-derived macrophages are differentiated from bone marrow progenitors in vitro in the presence of M-CSF. Alveolar macrophages represent approximately 95% of cells obtained by bronchoalveolar lavage (Snelgrove et al, 2008). Alveolar macrophages cannot be replaced by peritoneal or bone marrow derived macrophages in in vitro tests as they differ substantially from each other in many aspects. The environmental factors affect e.g. the way of energy production. Alveolar macrophages are located in the airways where they are in direct contact with air. Thus, they carry out predominantly aerobic phosphorylation (Zhang et al, 2008b). In contrast peritoneal and bone marrow derived macrophages deploy mainly anaerobic glycolysis. While peritoneal and bone marrow derived macrophages express F4/80 high, CD11b high and CD68+, alveolar macrophages are F4/80 low, CD11b low and CD68+ (Zhang et al, 2008b) (and also Figure 6). Peritoneal macrophages express higher levels of type I and type II IL-4 receptor and therefore respond to IL-4 and IL-13 at much lower concentrations than bone marrow derived macrophages.

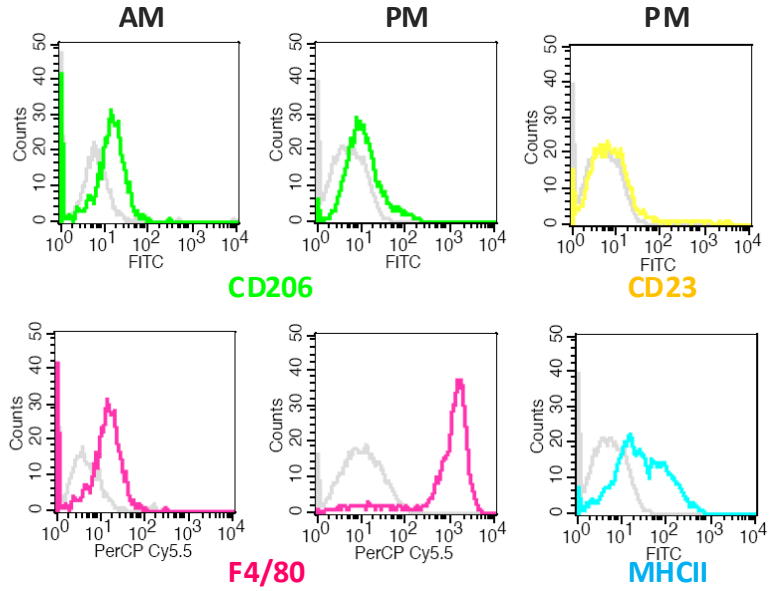

Figure 6 Flow-cytometric analysis of the cell surface expression of macrophage receptors

CD206 (mannose receptor), and F4/80 on alveolar and peritoneal macrophages and CD23 (low affinity receptor for immunoglobulin (Ig)E - FcεRII) and MHCII (major histocompatibility complex Class II) on peritoneal macrophages (unpublished data).

1.2.4 MACROPHAGE POLARIZATION

Beside the heterogeneity based on the anatomic location, the inflammatory state of the environment dictates the phenotype and polarization of macrophages. The ability of terminally differentiated macrophages to adjust their phenotype (surface receptor expression) and effector functions to local requirements in response to pathogens/cytokines etc. is referred to as macrophage plasticity (see Figures 7 and 8). It has been first described in vitro (Gordon, 2003; Mantovani et al, 2004; Mosser & Edwards, 2008; Mosser & Zhang, 2008; Stein et al, 1992) but there is mounting evidence that this phenomenon exists in vivo in different pathological conditions (Benoit et al, 2008; Ghassabeh et al, 2006).

Classically activated macrophages (M1) arise after two-step activation. The first step – also referred to as priming – includes IFNγ mediated STAT1/2 transcription factor binding to gamma-activated sequences (GAS) (Gordon, 2003; Mosser & Edwards, 2008; Mosser & Zhang, 2008). The second step is usually the engagement of one of the TLRs or TNFR (TNF secretion results from TLR stimulation) and NFκB

activation. Macrophages polarized with TLR ligands (e.g. bacterial PAMPs) develop a M1-like phenotype (innate activation). TLR signaling can induce type I interferon production which makes the stimulation with IFNγ redundant (Mosser & Edwards, 2008).

M1 phenotypes are characterized by the secretion of pro-inflammatory mediators (cytokines e.g. TNF, IL-6, IL1β, IL-12, and chemokines e.g. CCL5, CXCL9, CXCL10 and CXCL11) and high bactericidal and tumoricidal properties due to the high activity of iNOS and enhanced oxidative burst (Mantovani et al, 2004; Martinez et al, 2009; Mosser & Edwards, 2008; Murray et al, 2014). M1 macrophages also up-regulate MHC class II and co-stimulatory molecules (CD80, CD86) and opsonic receptors (e.g. FcγRIII (CD16)). In vivo they are thought to play a protective role during an acute phase of infections with intracellular pathogens such as mycobacteria, Listeria monocytogenes, Salmonella spp. or Chlamydia (Benoit et al, 2008). However, unlimited M1 activation (magnitude or duration) can be also detrimen1tal. This is e.g. the case during *E. coli* induced peritonitis and sepsis (Mehta et al, 2004). The M1 phenotype was also shown to negatively influence the outcome in secondary pneumococcal pneumonia after influenza infection (Smith et al, 2007). M1 macrophages play further a controversial role in tumor biology (Mosser & Edwards, 2008). On the one hand they could contribute to tumor surveillance owing to their tumoricidal properties shown in vitro (Klimp et al, 2002; Romieu-Mourez et al, 2006). On the other hand M1 macrophages could contribute to inflammation-induced tumorigenesis through the production of free radicals (Swann et al, 2008).

Alternatively activated (M2) macrophages are a heterogeneous group. This group comprises wound-healing macrophages (first described as alternatively activated) M2a, regulatory macrophages M2b, and deactivated macrophages M2c (Gordon, 2003; Mantovani et al, 2004; Mosser & Edwards, 2008). Wound-healing M2a macrophages are the best studied subset of alternatively activated macrophages. They up-regulate non-opsonic receptors e.g. mannose receptor, CD23 and CD163 receptor, MHC II and costimulatory molecules and secrete high amounts of chemokines (CCL17/TARC, CCL18/AMAC1, CCL22/MDC), cytokines (IL-10, TGFβ, IL-1ra/IL-1 decoy receptor) and other products (FIZZ1, Ym1/2) (Mantovani et al, 2004; Mosser & Edwards, 2008). M2a macrophages further up-regulate arginase1, an enzyme which competes for arginine (the substrate) with iNOS. The products of

arginase enzymatic activity are L-ornithine and polyamines which are the substrate for proline and collagen production and enhance cell proliferation, respectively (Gordon, 2003). The polarization of M2a macrophages critically depends on the transcription factor STAT6 (Junttila et al, 2008). Wound-healing M2a macrophages play a role in the pathogenesis of allergy, parasitic infections and fibrosis (Mosser & Edwards, 2008). Tumor associated macrophages are macrophages recruited to tumor sites and polarized to a M2 like phenotype within tumor environment (Allavena & Mantovani, 2012). They are believed to promote tumor growth and metastasis through multiple mechanisms: the secretion of pro-proliferative and anti-apoptotic factors, the suppression of T-cell activation, the induction of angiogenesis and the extracellular matrix deposition and remodeling (Allavena & Mantovani, 2012).

Alternatively activated M2b macropages (Mantovani et al, 2004), also known as regulatory macrophages (Mosser & Edwards, 2008), emerge in the presence of immune-complexes and TLR ligands or IL1R agonists (Mosser & Zhang, 2008). The addition of immune-complexes potently induces the expression of IL-10 and suppresses the expression of IL-12 while other pro-inflammatory cytokines remain high (Mantovani et al, 2004; Mosser, 2003; Mosser & Edwards, 2008). Further, regulatory macrophages secrete high amounts of the chemokine CCL1, promoting the recruitment of cells expressing CCR8 (Th2 and Treg lymphocytes and eosinophils), involved in tissue repair and immunoregulation (Mantovani et al, 2004). Since the development of immune-complexes requires the involvement of adaptive immunity, immune-complexes and regulatory macrophages appear in vivo at later stages of the inflammatory response (Mosser, 2003; Mosser & Edwards, 2008). Regulatory macrophages were shown to prevent lethality from endotoxemia in mice due to their ability to secrete vast amounts of IL-10 (Mosser, 2003). Thus M2b macrophages protect from overwhelming inflammation.

Finally, alternatively activated macrophages also comprise deactivated (M2c) macrophages. This is a heterogeneous group, which encompasses macrophages polarized with TLR ligands together with diverse stimuli such as IL-10, glucocorticoids, prostaglandins or apoptotic cells (Gordon, 2003; Mantovani et al, 2004; Mosser & Edwards, 2008). Although macrophages deactivated with different agents show distinct expression patterns of surface receptors and secreted molecules, they share some functional characteristics. Although M2c macrophages suppress M1-type

proinflammatory cytokines and chemokines through the inhibition of NFκB and STAT1, this phenotype is more sophisticated and not restricted to simple deactivation. Thus, macrophages deactivated with IL-10 upregulate long pentraxin (PTX3) (Mantovani et al, 2004), which has antimicrobial properties (Moalli et al, 2011), versican, a proteoglycan of the extracellular matrix, IL-7 and CXCL14 affecting function of B-cells and lymphogenesis and a panel of anti-inflammatory mediators such as the signaling lymphocytic activation molecule (SLAM, CD150), suppressor of cytokine signaling 3 (SOCS3), regulator of G protein signalling-16 (RGS-16) and phosphatases (Mantovani et al, 2004). Glucocorticoids deactivate macrophages through multiple mechanisms with the transrepression of the major proinflammatory transcription factors NFκB and AP-1 being the best established (Clark et al, 2008). Other mechanisms involve the downregulation of STAT1 expression (Hu et al, 2003) or DUSP mediated inactivation of MAP kinases (Clark et al, 2008). Consequently, the deactivation with glucocorticoids results in the suppression of proinflammatory cytokines and chemokines while other responses such as phagocytosis of apoptotic cells or upregulation of CCR2 and CCR5 are enhanced (Mantovani et al, 2004) (Liu et al, 1999). The impact of glucocorticoid mediated deactivation of macrophages becomes apparent in mice lacking glucocorticoid receptors in the myeloid compartment (Bhattacharyya et al, 2007). These animals are highly susceptible to lethal endotoxemia, indicating that glucocorticoid deactivated macrophages are crucial in limiting overwhelming immune responses.

Oxidized phospholipids generated in atherosclerotic lesions promote the development of a distinct macrophage phenotype, termed Mox (Kadl et al). This subset of macrophages is characterized by the up-regulation of Nrf2 dependent redox-regulatory genes such as HO-1, sufiredoxin-1 (Srnx1) or Txnrd, and Nrf2 independent genes such as VEGF, Dusp-1 and -5, Nurr-1, or Thsp (Kadl et al). Functionally, Mox macrophages show impaired phagocytosis and chemotaxis compared to M1 and M2 phenotypes. The high abundance of Mox macrophages in advanced atherosclerotic lesions indicates a role for this subset in the pathophysiology of atherosclerosis and possibly other chronic inflammatory conditions.

CXC chemokine ligand 4 induces another distinct macrophage phenotype, termed M4 polarization (Gleissner et al). These macrophages display some features of M1 and M2 macrophages but also show some unique characteristics. They

upregulate genes involved in the immune response (CCL18, TRAIL), the APC function (MHC Class II and costimulatory molecule CD86) and lipid metabolism (APOC2, APOE, SORL1). The biological role of this subset is unclear.

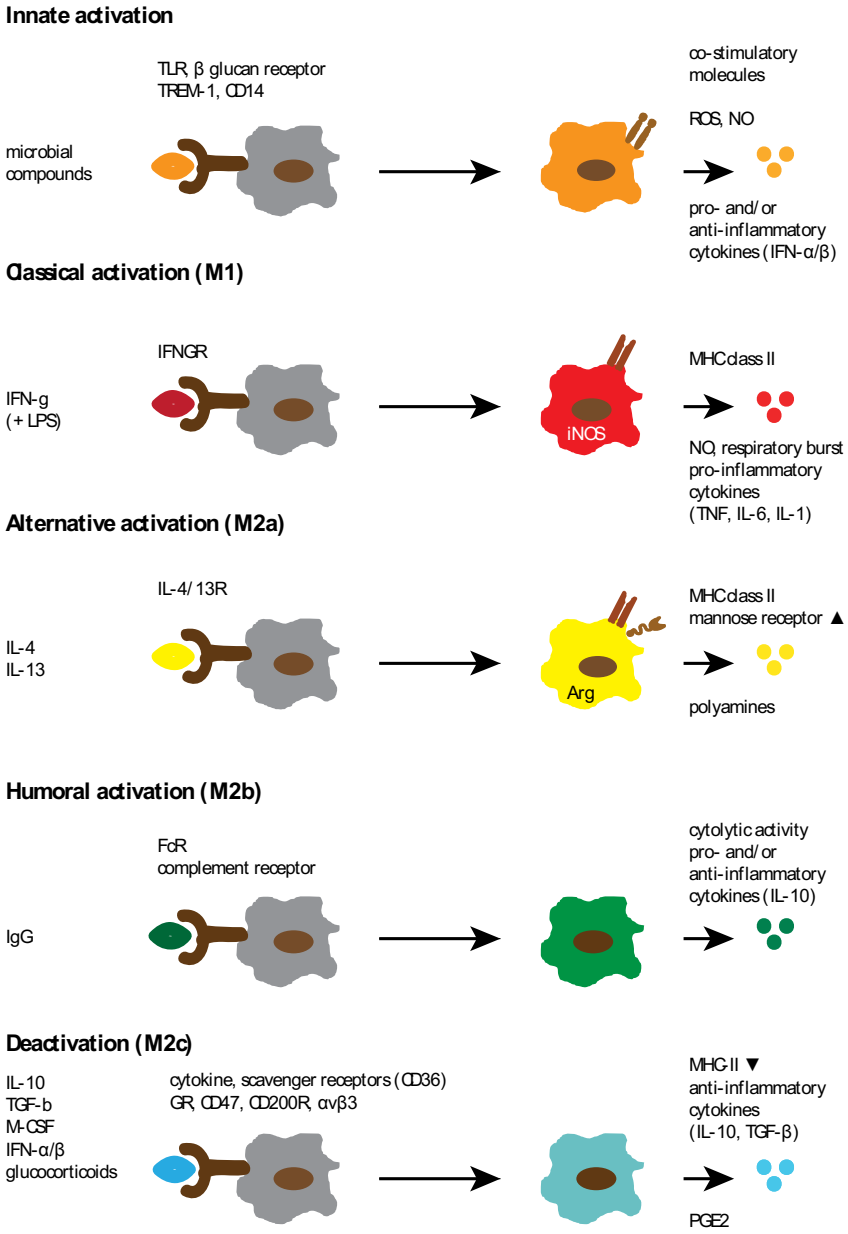

Figure 7 Activation of macrophages

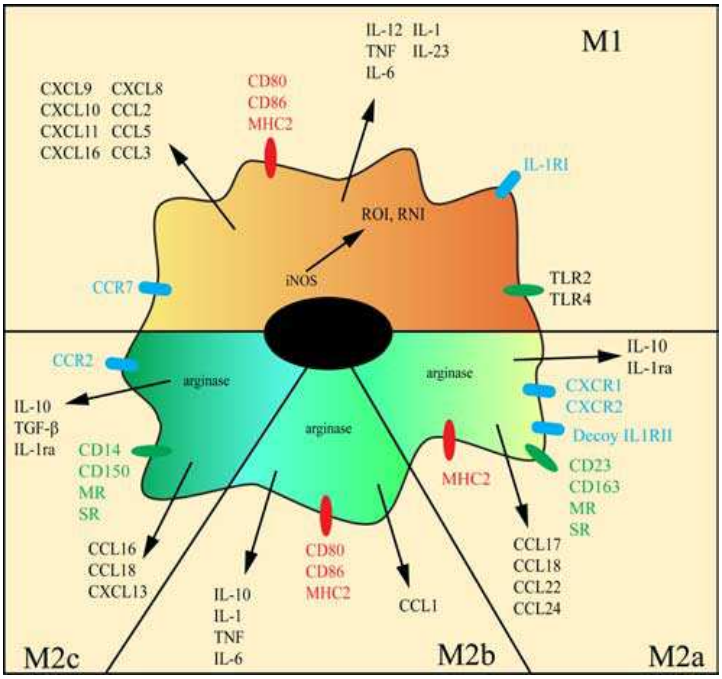

Figure 8 Markers of different macrophage polarization states

Adapted from Benoit et al, *J Immunol* 2008 (Benoit et al, 2008).

1.3 LIPOCALIN 2

Lipocalin 2 (LCN2), also known as neutrophil gelatinase associated lipocalin, siderocalin, uterocalin or 24p3 (mouse), is a mammalian protein of 25 kDa, which belongs to the lipocalin family of proteins that bind small lipophilic ligands. LCN2 was first discovered by Kjeldsen et al. in the specific granules of human neutrophilic granulocytes, where it associates with human matrix metalloproteinase 9 (MMP9, gelatinase B) (Kjeldsen et al, 1993). LCN2 was later shown to be highly expressed at sites which stay in close contact with the environment, such as the skin or mucosal surfaces by both myeloid (macrophages, neutrophils) and non-myeloid cells (epithelial cells) (Chan et al, 2009).

1.3.1 ANTIBACTERIAL HOST DEFENSE

The expression of LCN2 by immune cells at the host-pathogen interface strongly suggested a role for LCN2 in the immune responses. Goetz and colleagues showed

for the first time that LCN2 interferes with siderophore mediated bacterial iron acquisition (Goetz et al, 2002). Siderophores are low molecular weight iron chelators, produced by certain microbes to facilitate iron uptake (Saha et al, 2013). In general three types of siderophores can be distinguished based on the structure of the iron-coordinating moiety: the catecholate type, the hydroxymate type and the mixed type (Saha et al, 2013). The catecholate-type siderophores are for example enterobactin from *Streptomyces*, vibriobactin from *Vibrio cholerae* and pyochelin produced by *Pseudomonas aeruginosa*. Staphylopherrin (*Staphylococcus* spp.) and alcaligin (*Alcaligenes denitrificans*) represent siderophores of the hydroxymate type. Petrobactin from *Bacillus anthracis* and mycobactin produced by *Mycobacterium tuberculosis* are examples of mixed type siderophores (Saha et al, 2013). LCN2 is known to sequester siderophores of certain bacteria such as *E. coli* (Goetz et al, 2002), *Klebsiella pneumoniae* (Chan et al, 2009), *Salmonella thyphimurium* (Nairz et al, 2008) or *Mycobacterium tuberculosis* (Holmes et al, 2005). Several groups showed subsequently the relevance of this finding for the host defense against these pathogens. Thus, Flo and colleagues showed that LCN2 is essential for the host defense against *E.coli* as almost all LCN2 deficient mice succumbed after an i.p. injection of *E.coli* at a sub-lethal dose (Flo et al, 2004). Another group found that LCN2 protected from *Salmonella typhimurium* infection as Hfe-deficiency led to increased LCN2 expression and consecutively improved survival from *Salmonella thyphimurium* sepsis *(Nairz et al, 2009).*The role of LCN2 in the pulmonary host defense is discussed below.

1.3.2 APOPTOSIS

Apoptosis is another important cellular process involving LCN2. LCN2 was shown to mediate apoptosis of several primary cells such as murine primary thymocytes, splenocytes and bone marrow cells, human primary neutrophils and peripheral blood lymphocytes or leukocytic cell lines such as the IL-3 dependent murine cell line FL5.12 or the IL-3 independent human cell line MT-4 (Devireddy et al, 2001). LCN2 was highly induced and secreted upon cytokine withdrawal and promoted apoptosis in an autocrine manner. Another group delineated the mechanism of LCN2 induction upon IL-3 withdrawal (Park et al, 2009). According to this group Foxo3a is one of the

transcription factors responsible for LCN2 expression. IL-3 deprivation abrogates PI3K/Akt mediated phosphorylation of Foxo3a, which under normal conditions prevents Foxo3a binding to the LCN2 promoter and transcriptional activity (Park et al, 2009).

Secreted LCN2 forms complexes with bacterial siderophores (Goetz et al, 2002), which can be internalized via two putative receptors, the 24p3 receptor (Slc22a17, BOCT) and megalin (Chakraborty et al, 2012; Devireddy et al, 2005) (see Figure 9). Recently, Devireddy and colleagues reported the existence of mammalian siderophores (Devireddy et al, 2010). Depending on the iron status of the LCN2-siderophore complexes (iron loaded (holo-LCN2) or iron-lacking (apo-LCN2)) it was proposed that the internalization process leads to either iron transport or apoptosis, respectively. Uptake of apo-LCN2 was shown to lead to the induction of the proapoptotic protein BCL2L11, a mitochondrial protein of the Bcl2 family (Devireddy et al, 2005). Associated with this concept, Liu et al. found increased cell numbers in the bone marrow, the peripheral blood and the spleen in LCN2-deficient mice when compared to wild-type littermates (Liu et al, 2011). The authors argued that these differences resulted from the defective apoptosis of neutrophils, thymocytes, erythroid cells and cytokine-dependent mast cells (Liu et al, 2011). Another group assessed the effects of LCN2 on human hematopoiesis and found that LCN2 induced apoptosis in mature cells of the erythroid and monocyte/macrophage lineage (Miharada et al, 2008). However, in another report, LCN2 was found to protect immune cells from apoptosis during endotoxic shock in mice (Srinivasan et al, 2012a).

1.3.3 IMMUNMODULATION

Beside its role in host defense and apoptosis, LCN2 was also shown to modulate the inflammatory response. Zhang et al. studied the effects of exogenously administered LCN2 on inflammation in adipocytes and macrophages (Zhang et al, 2008a). In this study the addition of LCN2 to media of 3T3-L1 adipocytes stimulated with TNF induced the expression of the key anti-inflammatory transcription factor and the master regulator of adipogenesis PPARγ and its downstream target adiponectin. This resulted in improved insulin sensitivity. Pretreatment of RAW264.7 macrophages with LCN2

decreased proinflammatory cytokine secretions in response to a subsequent stimulation with LPS (Zhang et al, 2008a). Another group investigated the effects of LCN2 on the immune response of the human alveolar epithelial cell line A549 (Bachman et al, 2009; Nelson et al, 2007). In this context LCN2 exerted proinflammatory effects, as it increased IL-8 secretion in response to the bacterial siderophore enterobactin. Whether this discrepancy is cell or stimulus specific is not clear.

Recently, LCN2 was shown to exert anti-inflammatory effects in primary peritoneal and bone marrow derived macrophages, as LCN2 deficient cells secreted higher levels of proinflammatory cytokines and chemokines than wild-type cells in response to LPS (Srinivasan et al, 2012a). Consequently, LCN2 deficient mice displayed significantly higher mortality than wild-type littermates in an endotoxin shock model (Srinivasan et al, 2012a).

1.3.4 LCN2 AND PULMONARY HOST DEFENSE

LCN2 was also shown to play an essential role in the host defense against relevant pulmonary pathogens.s such, Saiga and colleagues found that LCN2 inhibited mycobacterial growth in vitro, which could be reversed by the supplementation of iron (Saiga et al, 2008). As a consequence, LCN2 deficient mice were highly susceptible to pulmonary infection with *Mycobacterium tuberculosis*. Alveolar macrophages and epithelial cells both secreted LCN2 during mycobacterial infection but only epithelia cell-derived LCN2 was found required for the successful clearance of mycobacteria (Saiga et al, 2008). The infection with another pulmonary pathogen, *Klebsiella pneumoniae,* led to TLR4 dependent induction of LCN2 (Chan et al, 2009). Further, LCN2 dose-dependently inhibited the growth of non-aerobactin producing strains of *Klebsiella pneumoniae* - an effect which was reversed by iron addition to the media. As expected, LCN2 deficient mice displayed an impaired clearance of this strain of *Klebsiella pneumoniae* (Chan et al, 2009). Another group investigated clinical isolates of *Klebsiella pneumoniae* and found that LCN2 protected only against strains producing the siderophore enterobactin, while isolates producing salmochelin (glycosylated enterobactin) or yersiniabactin evaded LCN2 mediated growth inhibition

(Bachman et al, 2011). *Escherichia coli*, another pathogen depending on enterobactin for iron acquisition, can also, under some circumstances, cause pneumonia (Cheng et al, 2014). Wu and co-workers showed that LCN2 was highly induced in the lung during *E.coli* infection (Wu et al, 2010). LCN2 deficient mice displayed an impaired bacterial clearance and survival compared to wild-type animals and LCN2 deficient survivors showed higher bacterial burdens in the spleens (Wu et al, 2010). Together, these data points towards a protective, siderophore-dependent role of LCN2 within the pulmonary compartment.

However, LCN2 is not only induced by siderophore-dependent microbes. Nelson and colleagues showed that LCN2 was upregulated in the upper airways upon colonization with bacteria, which do not produce siderophores like *Streptococcus pneumoniae* or *Haemophilus influenzae* (Nelson et al, 2005) and the role of this induction was not known.

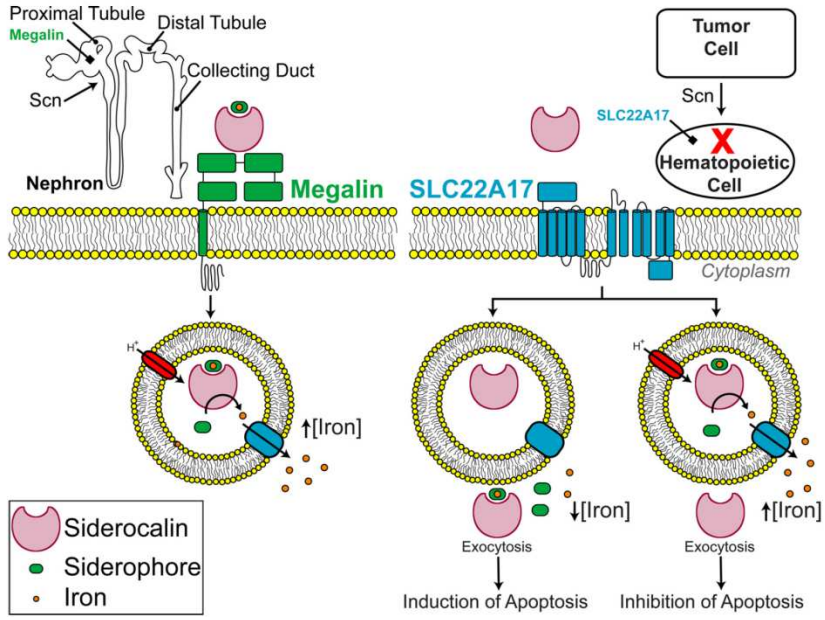

Figure 9 LCN2 (siderocalin) mediated iron trafficking in different cells

Two putative LCN2 receptors: megalin in the renal proximal tubule and Slc22a17 in hematopoietic cells. Adapted from Correnti et al, *J Biol Chem* 2012 (Correnti & Strong, 2012).

1.4 SIGNALING

1.4.1 PATTERN RECOGNITION RECEPTORS

Pattern recognition receptors (PRRs) can be classified in five major groups: Toll like receptors (TLRs), Nod like receptors (NLRs), RIG- like receptors (RLRs) (Creagh & O'Neill, 2006), C-type lectin receptors (CLRs) (Osorio & Reis e Sousa, 2011) and scavenger receptors (Mukhopadhyay & Gordon, 2004).

TLRs are type I transmembrane proteins located either in the cytoplasmic (TLR-1,-2,-4,-5, and -6) or endosomal membrane (TLR-3,-7,-8,-9) (Akira et al, 2006). These receptors recognize a wide range of microbial ligands such as lipopolysaccharide (LPS) of Gram negative bacteria (TLR-4), lipoteichoic acid (LTA), peptidoglycan and lipoproteins of Gram-positive bacteria (TLR-2/TLR1 or TLR-2/TLR-6), bacterial flagellin (TLR-5), viral double-stranded RNA (TLR-3) and single-stranded RNA (TLR-7 and TLR-8), or bacterial CpG DNA and viral DNA (TLR-9)(Akira et al, 2006). Upon ligand binding TLRs build homo- or heterodimers, a step initiating downstream signal transduction (see Figure 10). All TLRs have leucine-rich-repeat (LRR) motifs facing the extracellular (cytoplasmic TLRs) or the endosomal (endosomal TLRs) space, responsible for ligand binding. The TLR-domain facing the cytoplasm contains a Toll/Interleukin 1 receptor (TIR) domain, responsible for binding of adaptor proteins. Currently there are five TIR domain containing proteins involved in TLR signaling known (O'Neill & Bowie, 2007) (see Figure 11): myeloid differentiation primary response protein 88 (MyD88) (Kawai et al, 1999), MyD88 adaptor like (Mal) (Fitzgerald et al, 2001) alias TIR domain containing adaptor protein (TIRAP) (Horng et al, 2001), TIR domain containing adaptor inducing IFN-β (TRIF) (Yamamoto et al, 2003), the TRIF related adaptor molecule (TRAM) (Fitzgerald et al, 2003).and sterile α- and armadillo-motif-containing protein (SARM), which negatively regulates TRIF (Carty et al, 2006). The downstream signaling pathways involve many proteins but, in general, MyD88 dependent signaling activates mitogen activated protein kinases (MAPKs) and IκB kinases (IKKs) resulting in activator protein 1 (AP-1) and NFκB activation, while TRIF-dependent signaling leads to type I interferon (IFN) production and antiviral immunity (see Figure 10) (Creagh & O'Neill, 2006).

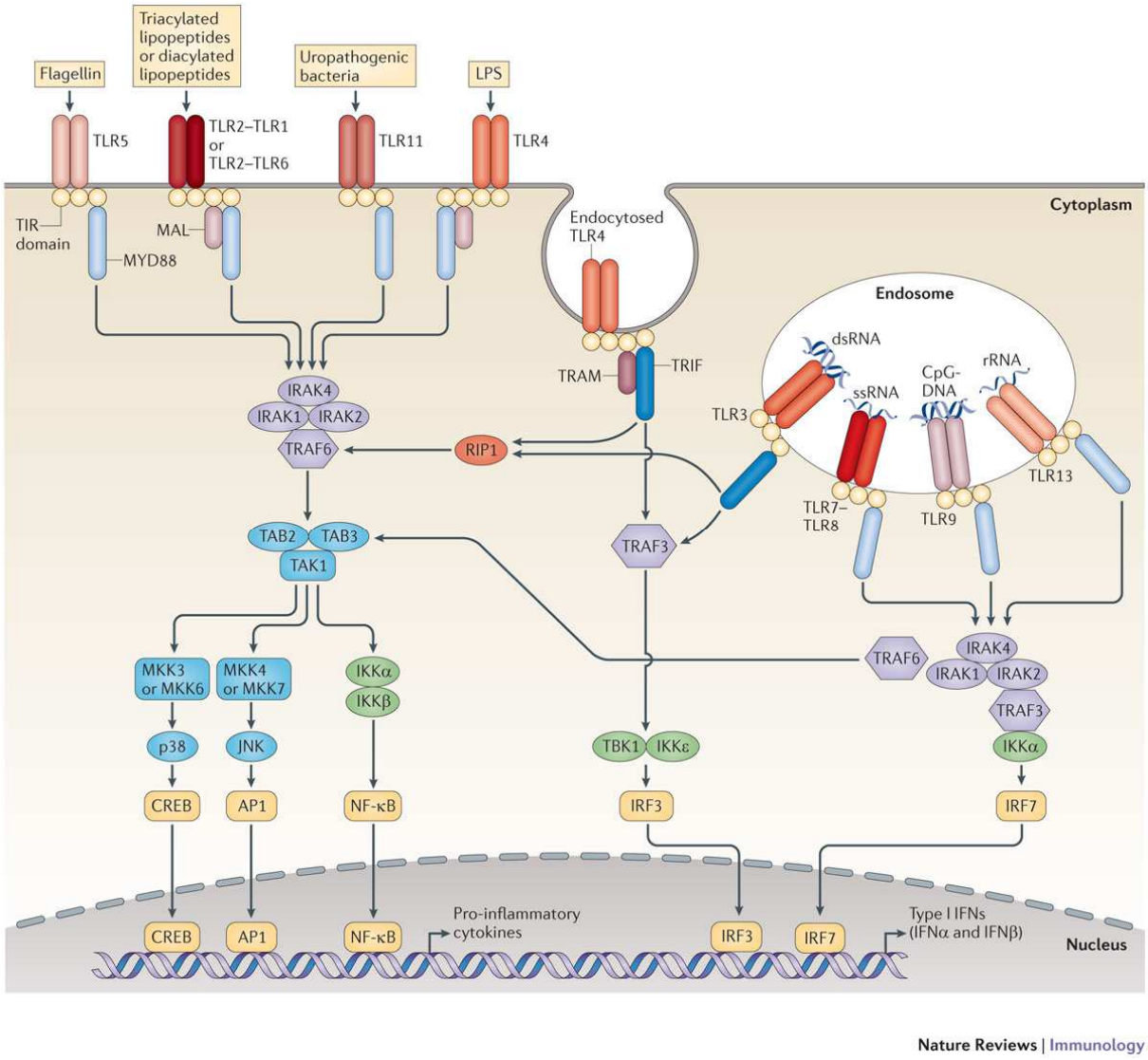

Figure 10 TLR signaling

Adapted from O'Neill et al., *Nat Rev Immunol* 2013 (O'Neill et al, 2013).

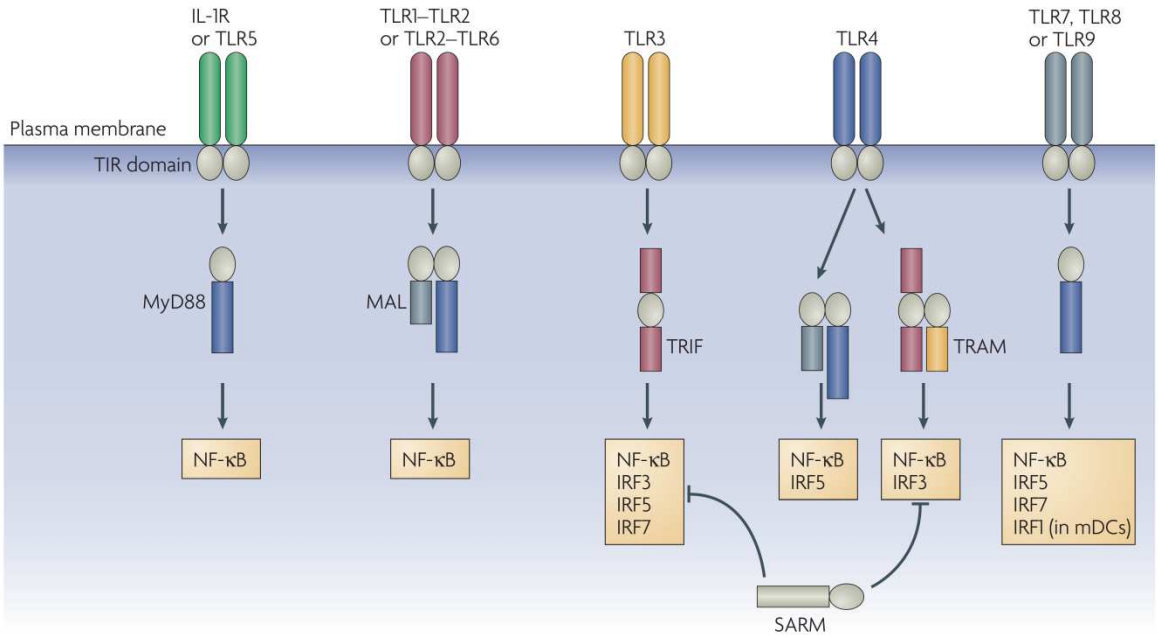

Figure 11 TLR signaling – adaptor proteins

Adapted from O'Neill and Bowie, *Nat Rev Immunol* 2007 (O'Neill & Bowie, 2007).

NLRs are a family of intracellular proteins with a typical structure: similar to TLRs, most NLRs possess LRR motifs for ligand binding, a nucleotide-binding oligomerization domain (NBD) domain for ATP-dependent oligomerization, a caspase recruitment domain (CARD), and a pyrin domain (PYD) or baculovirus inhibitor of apoptosis repeat (BIR) domain for signaling (see Figure 12)(Akira et al, 2006; Creagh & O'Neill, 2006). NLR family members include, among others, NBD - containing protein 1 and 2 (NODs 1 and 2) and proteins building up the multimeric protein complexes called inflammasomes, such as NLRP1, NLRP3 or NLRC4. The proteins of the NOD family recognize the D-γ-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptides (MDP), respectively, the constituents of bacterial peptidoglycan (PGN). Ligand binding results in the receptor interacting protein 2 (RIP2) dependent activation of NFκB (Akira et al, 2006). The inflammasome associated NLRs recognize anthrax toxin (NLRP1), bacterial flagellin (NLRC4) or bacterial RNA, toxins, ATP, uric acid and many more (NLRP3). The ligand binding results in the formation of a multiprotein complex, which ultimately activates caspase 1 that in turn cleaves and activates pro-IL1-β and IL-18 (see Figure 12)(Akira et al, 2006).

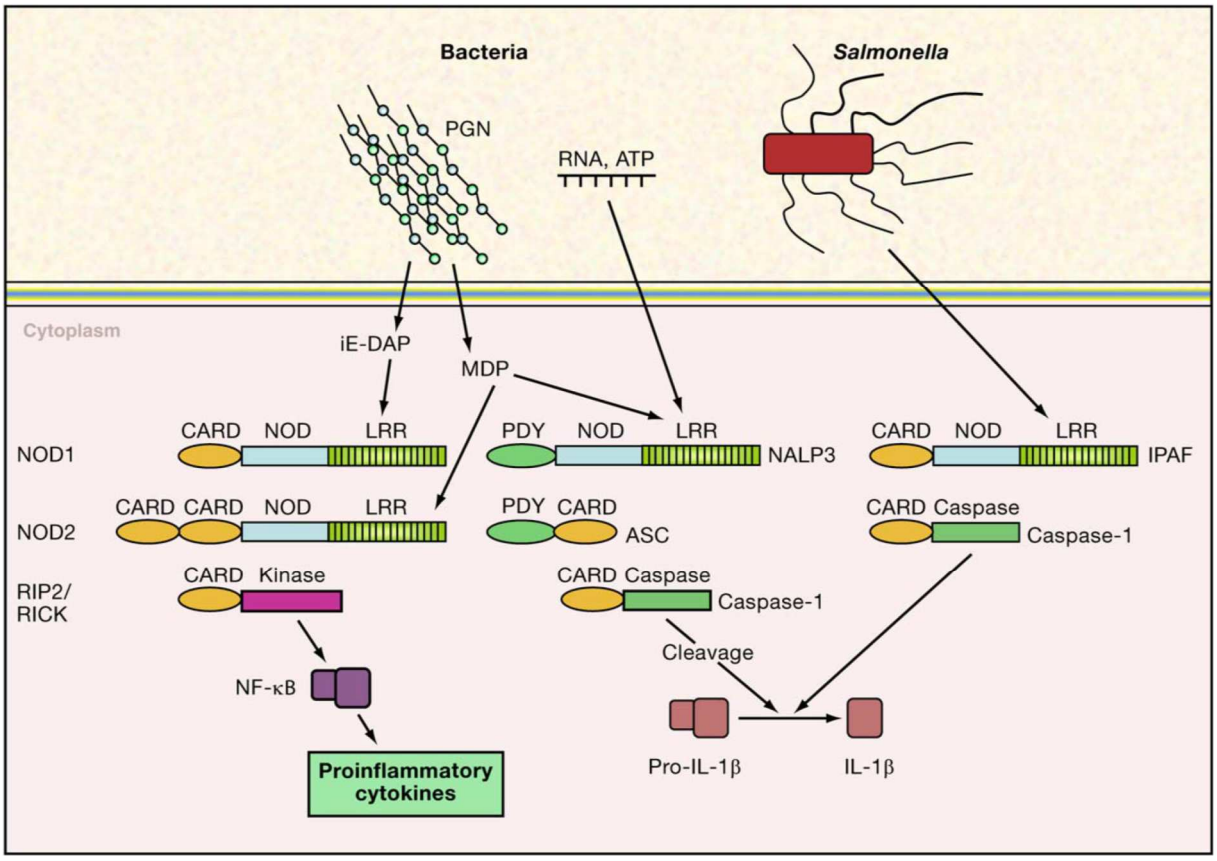

Figure 12 NLRs – structure and signaling

Adapted from Akira et al., *Cell* 2006 (Akira et al, 2006).

Retinoic acid inducible gene-1 (RIG-I)-like receptors include two members: RIG-I and melanoma differentiation-associated gene 5 (MDA 5) (Creagh & O'Neill, 2006). RLRs are located intracellularly and recognize viral double stranded RNA from picornavirus (MDA5) and influenza virus, paramyxoviruses and others (RIG-I) (Creagh & O'Neill, 2006). RLRs share some features with antiviral TLRs and others with NLRs. On the one hand, analogue to TLR-signaling, RLRs activate NFκB and IRF3 transcription factors, resulting in type I IFN production. On the other hand, RLRs contain, similar to NLRs, CARD domains and activate through CARD-CARD interaction the IFN-β promoter stimulator 1 (IPS-1; other names: mitochondrial antiviral signalling protein – MAVS, virus-induced signalling adaptor – VISA, CARD adaptor inducing IFN-β – Cardif) (Akira et al, 2006; Creagh & O'Neill, 2006).

CLRs constitute a big family of transmembrane proteins with the characteristic C-type lectin like domain (CTLD) (Osorio & Reis e Sousa, 2011). Many CLRs bind carbohydrates in a Ca^{2+}-dependent manner and are involved in the recognition of

sugar moieties of fungi, bacteria, and viruses (Osorio & Reis e Sousa, 2011). Therefore it is not surprising that CLRs or their adapter proteins were published to play an important role in anti-fungal immunity (Gross et al, 2006; LeibundGut-Landmann et al, 2007; Saijo et al, 2010; Taylor et al, 2007). Some CLRs, such as mannose receptor or langerin, act as endocytic receptors, that internalize their ligands but, according to the current knowledge, do not initiate signaling (Osorio & Reis e Sousa, 2011). Other CLRs recruit and activate spleen tyrosin kinase (Syk) through the immunoreceptor tyrosin-based activation motif (ITAM). Thus, the ligand binding leads to the phosphorylation of the two tyrosin residues within the ITAM motif and the subsequent binding of the SH2 domains of the Syk. Syk activation has multiple effects: it leads to CARD9/Malt-1/Bcl-10 dependent activation of NFκB, MAPK-signaling, PLCγ-mediated Ca^{2+} increase and calcineurin-dependent NFAT activation and finally generation of reactive oxygen species (ROS) with subsequent NLRP3 inflammasome activation and IL1β production (see Figure 13) (Osorio & Reis e Sousa, 2011). Some receptors (e.g. dectin 1) contain an ITAM motif in the cytoplasmic region and thus can activate Syk directly (the so called hemITAM signaling). Others (e.g. dectin 2) activate Syk indirectly through ITAM containing adapters (the so called ITAM signaling) (Osorio & Reis e Sousa, 2011). Finally, there is a group of CLRs containing immunoreceptor tyrosin-based inhibitory motif (ITIM). These CLRs bind phosphatases (SHP-1, SHP-2 and SHIP), thus counteracting Syk and TLR signaling (Osorio & Reis e Sousa, 2011).

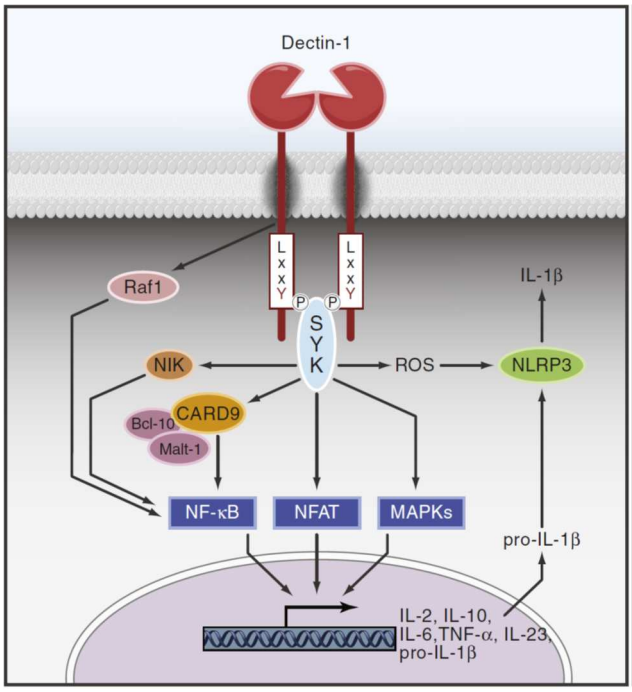

Figure 13 Signaling via the CLR - dectin 1

Adapted from Osario and Reis e Sousa, *Immunity* 2011 (Osorio & Reis e Sousa, 2011).

1.4.2 IL-10 SIGNALING

IL-10 is a major anti-inflammatory cytokine that importantly regulates inflammatory processes (see Figure 14). IL-10 affects cytokine and chemokine levels indirectly through STAT3-SOCS3-dependent signaling (inhibition of NFκB) (Shouval et al, 2014). Additionally, IL-10 inhibits STAT1 phosphorylation, thereby suppressing e.g. CXCL9 and CXCL10 expression. IL-10 is further known to decrease KC expression through decreased mRNA stability (Biswas et al, 2003). Finally, the transcription factor and proto-oncogene c-Maf was also reported to mediate some of IL-10's antiinflammatory effects (Cao et al, 2002).

IL-10 was also shown to activate phosphatidylinositol 3-kinase and p70 S6 kinase, which mediates the proliferative and not anti-inflammatory IL-10 actions (Crawley et al, 1996).

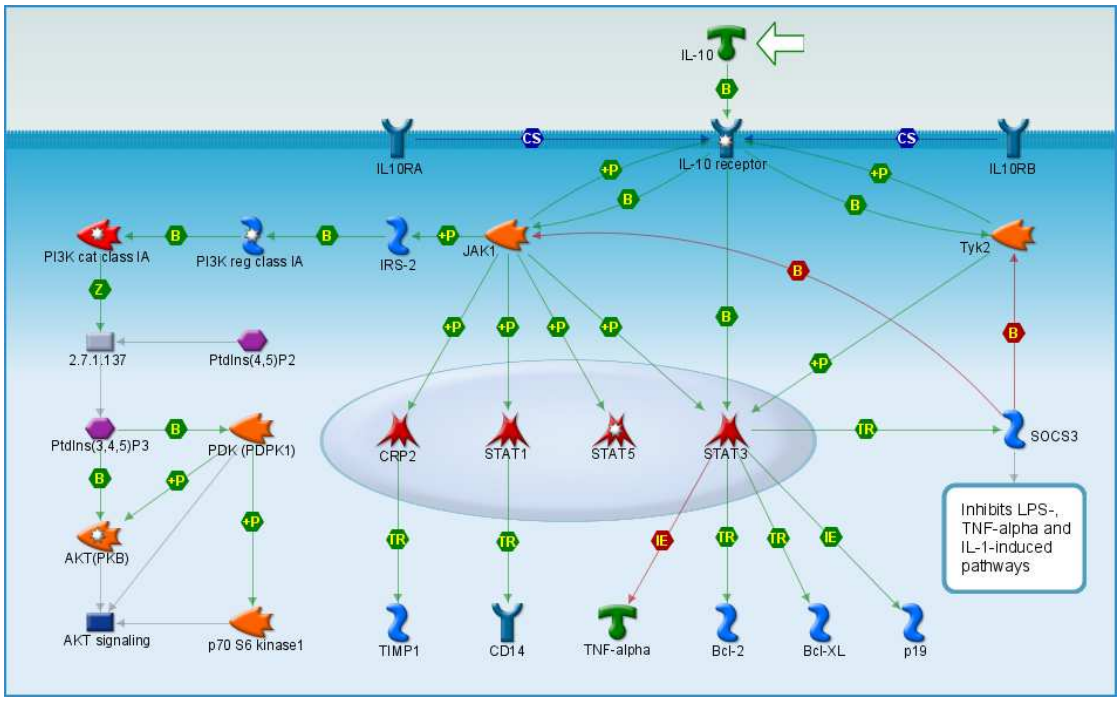

Figure 14 IL-10 signaling

Source: http://pathwaymaps.com/maps/531/

1.5 PNEUMONIA

Pneumonia is an infection of the lower respiratory tract. Despite the considerable progress in prophylaxis and treatment options over the past decades, pneumonia remains a global concern. According to a WHO report in 2006, pneumonia is the major killer of children under 5 years (2 million deaths, almost 20% of the overall mortality due to pneumonia) (UNICEF, 2006)(REF). Respiratory tract infections are moreover important from the socio-economical point of view since they cause the greatest 'global burden of disease' worldwide measured by DALYs (disability adjusted life years) (Mizgerd, 2006).

Lower respiratory tract infections can be generally divided into community-acquired (CAP) and hospital-acquired (nosocomial, HAP) pneumonia. Ventilator-associated pneumonia is an important sub-type of HAP.

1.5.1 COMMUNITY-ACQUIRED PNEUMONIA (CAP)

Community acquired pneumonia is the infection of lower airways in patients without prior hospitalization. Up to 15% of all cases are caused by viruses, the other cases are of bacterial origin. Among bacterial pathogens, *Streptococcus pneumoniae* is the single most frequent causative pathogen, while influenza viruses are the most frequent cause of viral lung infections (Bartlett & Mundy, 1995). In children the percentage of viral pneumonia is 50 or higher. The most common pathogens are respiratory syncytial virus (RSV), influenza, parainfluenza and rhinovirus (Dodman et al, 1999; Juven et al, 2000). Bacteria are responsible for around 50% of pediatric pneumonia cases with *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* being the major causative pathogens (Dodman et al, 1999; Juven et al, 2000). Secondary pneumococcal or staphylococcal pneumonia is often complicating the course of influenza infection (van der Sluijs et al, 2004).

1.5.2 STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae is a Gram positive, aerotolerant, anaerobic, extracellular bacterium. It is a pathogen which colonizes the upper respiratory tract without causing an infection in most cases. The colonization rate is more than 60% in infants and declining with increasing age to approximately 10% in adults (Austrian, 1986; van der Poll & Opal, 2009). However, in some cases an invasive pneumococcal disease such as pneumonia, sepsis or meningitis develops. This depends on bacterial virulence factors (invasive vs. persistent colonization phenotype) and host factors (e.g. immunocompromised host)(van der Poll & Opal, 2009). Furthermore, the clinical presentation of invasive pneumococcal diseases depends on the biofilm formation capability. Biofilm-forming pneumococci lead more often to meningitis or pneumonia while untethered bacteria are more prone to induce bacteremia. Children (< 2 a) and elderly people (> 65 a), splenectomized patients, alcoholics, diabetics, patients with abnormal humoral immunity (HIV infected, complement or immunoglobulin deficiency) and patients with a chronic lung disease e.g. chronic obstructive pulmonary disease, or suffering from recent influenza infections are groups at risk of developing an invasive pneumococcal disease (van der Poll & Opal, 2009). Pneumococcal pneumonia presents usually as a lobar pneumonia affecting one or more lobes. Patients suffer from purulent tracheobronchial secretions, cough, fever or hypothermia, leukocytosis or leukopenia, and in severe cases show serious respiratory symptoms (hypoxemia, hyperventilation). The course of pneumococcal pneumonia can be complicated by sepsis and/or respiratory failure, requiring mechanical ventilation.

Since the sequencing of the pneumococcal genome in 1997, multiple virulence factors have been identified (Kadioglu et al, 2008; Mitchell, 2000). The most prominent and well established virulence factor long before the era of sequencing is the polysaccharide capsule. In general, two forms can be observed: a thin capsule during the initial colonization phase and a thick one, which protects pneumococci from opsonisation and phagocytosis by phagocytes in the invasive phase (Kadioglu et al, 2008). Capsules further protect bacteria from being removed by the mucociliary escalator and they form a barrier for some antibiotics (van der Poll & Opal, 2009). Another important virulence factor expressed by virtually all invasive clones is pneumolysin. It has very pleiotropic, mainly pro-inflammatory effects: cytolytic activity,

TLR4 stimulation, induction of cytokines and chemokines, complement activation, activation of CD4+ T cells, inhibition of ciliary movement and alleviation of the respiratory burst by phagocytic cells (van der Poll & Opal, 2009). Other virulence factors facilitate adhesion, invasion, acquisition of iron and other essential heavy metals, protection from oxidative stress, generation of biofilms, quorum sensing, bacterial competition or escape from the host immune system. Important examples include bacteriocin (acting against competing bacteria), choline-binding protein A (CbpA) – responsible for adherence, IgA binding, inhibition of complement activation; pneumococcal surface protein A (PspA) – known for lactoferrin binding and inhibition of complement activation, pneumococcal surface antigen A (PsaA) – responsible, among others, for adhesion, protection against oxidative stress and zinc and manganese transport or enzymes (e.g. neuraminidases, hyaluronidase, superoxide dismutase, autolysin) (Mitchell, 2000; van der Poll & Opal, 2009) (see Figure 15).

In the context of the role of LCN2 during pneumococcal infection it is important to mention that *Streptococcus pneumoniae* does not depend on siderophores for iron uptake (Tettelin et al, 2001). Instead, at least three ABC transport systems exist in *S. pneumoniae*, encoded by three operons: pneumococcal iron transport, pneumococcal iron acquisition and pneumococcal iron uptake (pit, pia and piu, respectively) (Brown et al, 2002; Kadioglu et al, 2008; van der Poll & Opal, 2009). Each operon encodes for an iron binding protein, an ATPase and a membrane permease. PiuA and PiuA are lipoproteins which bind hemin and hemoglobin and facilitate their uptake by *S. pneumoniae,* while PitA seems to bind Fe3+ ions (Brown et al, 2002; Kadioglu et al, 2008; Tai et al, 2003). It is consistent with the reports that *S. pneumoniae* grows in the iron-depleted media if Fe2+, Fe3+ or hem-containing proteins are repleted (Brown et al, 2002).

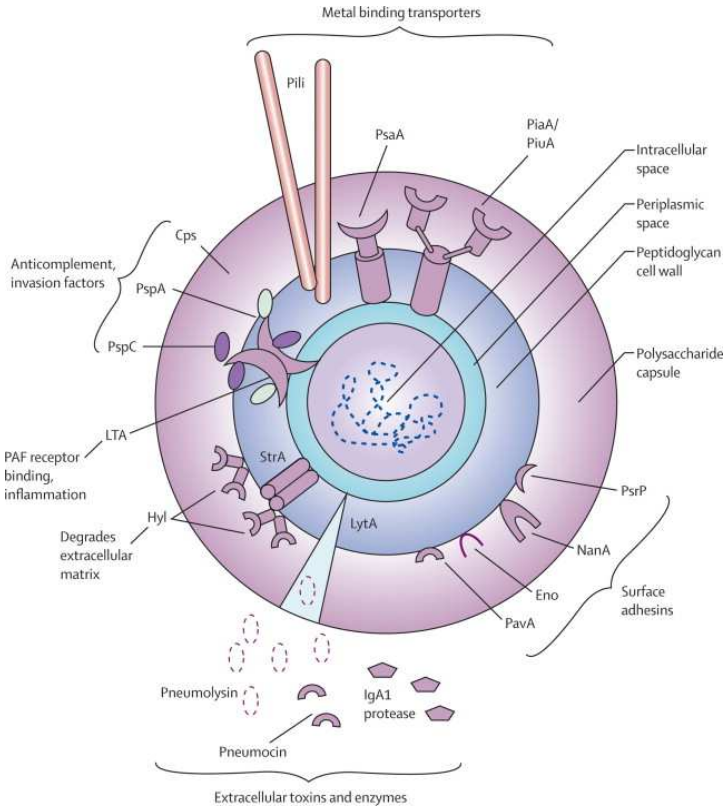

Figure 15 Virulence factors of *S. pneumoniae*

Adapted from van der Poll and Opal, *Lancet* 2009 (van der Poll & Opal, 2009).

The first step in the recognition of *S. pneumoniae* is the binding of host receptors to intact bacteria or bacterial structures either directly or indirectly (see Figure 15) (van der Poll & Opal, 2009). Thus, macrophage receptor with collagenous structure (MARCO) was shown to be a phagocytic receptor for *S. pneumoniae* in vitro (Arredouani et al, 2004). Another receptor, the dendritic cell specific intercellular adhesion molecule grabbing nonintegrin related 1 (SIGNR1), is a C type lectin expressed on marginal-zone macrophages, probably involved in the presentation of pneumococcal capsular polysaccharides to B cells (Kang et al, 2004; van der Poll & Opal, 2009).The phagocytic complement receptors and Fcγ receptors bind pneumococci indirectly after opsonisation with iC3b or IgG, respectively (van der Poll & Opal, 2009). The acute phase protein C-reactive protein binds C1q and promotes complement activation after binding of pneumococcal cell wall phosphorylcholine (Suresh et al, 2006). Some receptors such as CD14 (Dessing et al, 2007) or the receptor for platelet activating factor (PAF), which also recognizes phosporylcholine in the cell wall of bacteria, are utilized by pneumococci to spread systemically (Cundell

et al, 1995; van der Poll & Opal, 2009) There is furthermore considerable data on the receptors involved in the recognition of *S. pneumoniae* proteins and DNA (see Figure 16). Thus, pneumococcal lipoproteins and lipoteichoic acid are recognized by TLR2, while pneumolysin activates TLR4 and the NLRP3 inflammasome. Bacterial DNA is recognized by DAI and CpG DNA activates the endosomal TLR-9. Finally peptidoglycan (muramyl dipeptide) is a Nod2 ligand (Dockrell et al, 2012).

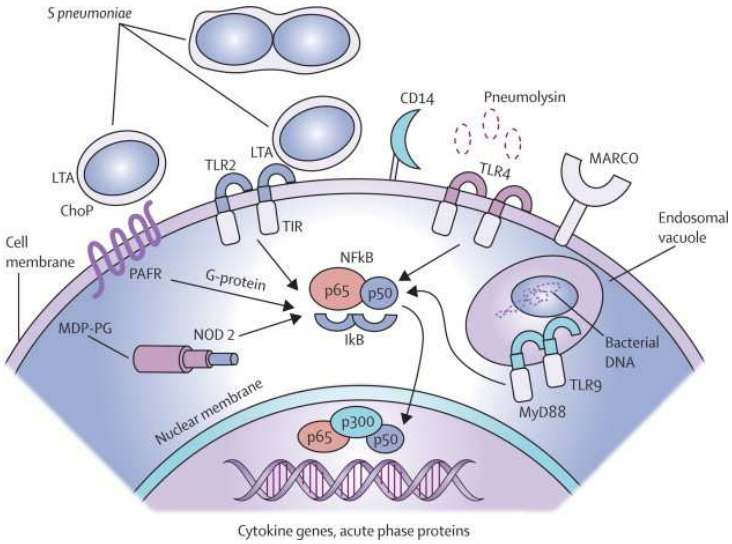

Figure 16 Recognition of *S. pneumoniae*

Adapted from van der Poll and Opal, *Lancet* 2009 (van der Poll & Opal, 2009).

LTA – lipoteichoic acid, ChoP - phosphorylcholine, CD14 – cluster of differentiation 14, MARCO – ma, PAFR – platelet activating factor receptor, MyD88 – myeloid differentiation 88, iKB - , NFκB - TLR – Toll like receptor; MDP-PG – muramyl dipeptide - peptidoglycan; NOD2 – nucleotide binding oligomerization domain containing protein 2;

Animal models showed that an initial strong pro-inflammatory response is a prerequisite for the successful control of infection (van der Poll & Opal, 2009). Thus, TNFα and, to lesser extent, IL1β were shown to be crucial for the clearance of pneumococci (Rijneveld et al, 2001; van der Poll et al, 1997a). Other pro-inflammatory cytokines, such as IL-6 and IL-18, are beneficial but the role of these cytokines is less important (Lauw et al, 2002; van der Poll et al, 1997b). Consistent with the idea of the protective role of inflammation in the host defense against *S. pneumoniae*, the anti-inflammatory cytokine IL-10 renders animals more susceptible to pneumococcal disease (van der Poll et al, 1996). Interestingly, IFNγ has negative effects on the clearance of pneumococci possibly by inhibiting the bactericidal properties of macrophages (Rijneveld et al, 2002). The data on the role of IL-12 are inconclusive (van der Poll & Opal, 2009).

Many cell types are involved in the host defense against *S. pneumoniae*. Macrophages are the first cells to encounter the bacteria. They are crucial for the clearance as they

phagocytose and kill the microbes. Macrophage apoptosis is another step helping to kill ingested pneumococci (Dockrell et al, 2003). Finally, macrophages exert protective anti-inflammatory actions through the removal of apoptotic neutrophils in the resolution phase of pneumococcal pneumonia (Knapp et al, 2003). If the bacterial inoculum is too high, macrophages recruit neutrophils. The role of neutrophils depends on the strain of *S. pneumoniae*. While neutrophils are very important for the successful clearance of serotype 3 and high doses of serotype 4 pneumococci, mice infected with serotype 8 *S. pneumoniae* profit from neutrophil depletion (van der Poll & Opal, 2009). Host-derived Galectin-3, α chemokines, the pneumococcus-derived N-formyl-methionyl-leucyl-phenylalanine and pneumolysin are the major neutrophil chemoattracting agents during pneumococcal pneumonia (van der Poll & Opal, 2009).

Further, epithelial cells contribute to the host defense against *S. pneumoniae* by secretion of cytokines, chemokines, antimicrobial peptides and the pneumococcal binding protein (van der Poll & Opal, 2009).

Other cell types shown to play a role in the host defense against *S. pneumoniae* are natural killer T cells, CD4+ T cells and γδ T cells (van der Poll & Opal, 2009). This explains why HIV infected patients are more susceptible to invasive pneumococcal disease.

2. AIMS OF THE THESIS

- To investigate the presence of inflammatory mediators in patients suffering from severe bacterial pneumonia requiring mechanical ventilation.
- To determine the role of Lipocalin 2 in the pulmonary host response to *S. pneumoniae*.
- To determine polarization status of pulmonary macrophages and the role of LCN2 herein.

3. RESULTS

LIPOCALIN-2 DEACTIVATES MACROPHAGES AND WORSENS PNEUMOCOCCAL PNEUMONIA OUTCOMES

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Conflict of interest:

The authors declare no financial or commercial conflict of interest.

Abstract:

Macrophages play a key role in responding to pathogens, and initiate an inflammatory response to combat microbe multiplication. Deactivation of macrophages facilitates resolution of the inflammatory response. Deactivated macrophages are characterized by an immunosuppressive phenotype, but the lack of unique markers that can reliably identify these cells explains the poorly defined biological role of this macrophage subset. We identified Lipocalin 2 (LCN2) as both a marker of deactivated macrophages and a macrophage deactivator. We show that LCN2 attenuated the early inflammatory response and impaired bacterial clearance, leading to impaired survival of mice suffering from pneumococcal pneumonia. LCN2 induced IL-10 formation by macrophages, skewing macrophage polarization in a STAT3-dependent manner. Pulmonary LCN2 levels were tremendously elevated during bacterial pneumonia in humans and high LCN2 levels were indicative of a detrimental outcome from pneumonia with Gram-positive bacteria. Our data emphasize the importance of macrophage deactivation for the outcome of pneumococcal infections and highlight the role of LCN2 and IL-10 as determinants of macrophage performance in the respiratory tract.

Introduction:

Macrophages are innate immune cells involved in the maintenance of tissue homeostasis and in the pathogenesis of diverse conditions such as metabolic, allergic, fibrotic, autoimmune, and neoplastic diseases (Murray & Wynn, 2011). Macrophages are well known to play decisive roles during infections, as they express a number of pattern recognition receptors that enable them to sense pathogens (Gordon, 2003). In doing so, macrophages initiate an inflammatory response to combat microbe multiplication, which is followed by a deactivation process that facilitates the resolution of inflammation after microbial elimination. The basis for these pleiotropic functions of macrophages is their enormous phenotypic and functional plasticity, also referred to as 'macrophage polarization'.

Bacterial pneumonia is an excellent model to study the poorly understood plasticity of macrophages in vivo, where macrophages must rapidly adjust to an environment of constantly changing stimuli (Mizgerd, 2008). In the setting of the clinically important model of pneumococcal pneumonia, it is well established that an initial pro-inflammatory response facilitates the clearance of pathogens and that the subsequent anti-inflammatory response limits tissue damage by phagocytosis of apoptotic neutrophils and cell debris (Knapp et al, 2003; Lagler et al, 2009; Mitchell, 2000; van der Poll & Opal, 2009). Pulmonary macrophages are importantly involved in shaping the inflammatory response upon infection, albeit the precise pathways that govern these steps in vivo are incompletely understood.

In vitro, macrophages polarize to pro-inflammatory, classically activated M1 macrophages in the presence of IFNγ and LPS, and to alternatively activated, anti-inflammatory M2 macrophages in the presence of IL-4 and/or IL-13 (Gordon, 2003; Mosser & Edwards, 2008; Stein et al, 1992). The field of macrophage polarization expanded substantially over the past years, further reflecting the importance and relevance of different macrophage populations. As such, further characterizations of alternative phenotypes resulted in the discovery of novel subsets, such as regulatory, deactivated and tumor associated macrophages (Gordon, 2003; Mantovani et al, 2004; Mantovani et al, 2002; Mosser & Edwards, 2008). Deactivated macrophages constitute a remarkably heterogeneous group that arises upon stimulation with Toll-like receptor (TLR) ligands in combination with glucocorticoids, IL-10, apoptotic cells

(ACs), or prostaglandins (Gordon, 2003; Mosser & Edwards, 2008). Deactivated macrophages are characterized by the down-regulation of pro-inflammatory mediators and the up-regulation of anti-inflammatory cytokines such as IL-10 (Mantovani et al, 2004). While macrophage activation has been studied extensively over the past years, the process of macrophage deactivation is less well understood.

Lipocalin 2 (LCN2) (Kjeldsen et al, 1994; Kjeldsen et al, 1993), also known as neutrophil gelatinase-associated lipocalin, is a mammalian protein expressed by myeloid and epithelial cells in response to TLR-activation during infections (Chan et al, 2009; Flo et al, 2004). LCN2 is mainly known as an anti-microbial defense mediator that scavenges a subset of bacterial siderophores, thereby restricting iron acquisition by bacteria such as Escherichia coli (Berger et al, 2006; Flo et al, 2004), Salmonella typhimurium (Nairz et al, 2009), Klebsiella pneumoniae (Chan et al, 2009) or mycobacteria (Saiga et al, 2008). However, Streptococcus pneumoniae, the most prevalent respiratory pathogen, does not depend on siderophores for iron acquisition (Tettelin et al, 2001). We and others (Nelson et al, 2005) found that S. pneumoniae induced remarkably high LCN2 levels in the respiratory tract. This finding alerted us to the possibility of a siderophore-independent role of LCN2 within the pulmonary compartment. Upon analysis of the published LCN2 promoter (Park et al, 2009) we noticed NFκB binding sites, but also glucocorticoid response elements and several binding sites for transcription factors which are critically involved in the polarization of M2 macrophages (Lawrence & Natoli, 2011), such as STAT3, CREB and C/EBPβ. Considering that LCN2 may modulate inflammation (Nelson et al, 2007; Zhang et al, 2008a), we hypothesized that LCN2 can play a role in macrophage polarization and thereby impact on the host defense against pathogens such as S. pneumoniae.

Results

LCN2 is highly expressed in deactivated macrophages

To first determine whether LCN2 expression depends on the macrophage polarization status, we treated primary alveolar macrophages (AM) with classically activating M1 (LPS and IFNγ), alternatively activating M2 (IL-4 and IL-13) or deactivating (LPS and dexamethasone) stimuli and quantified LCN2 secretion. These stimuli were specific, as the stable endproduct of nitric oxide, nitrite, was only detectable in supernatants of M1 stimulated cells (Ehrt et al, 2001), and mannose receptor (MRC1) (Stein et al, 1992) expression was exclusively induced in M2 polarized macrophages (Figure 1A). Interestingly, LCN2 was tremendously up-regulated upon deactivation with LPS and dexamethasone (Figure 1A). We confirmed these observations using the AM cell line MH-S (Supplemental Figure 1A). To test if LCN2 induction was restricted to glucocorticoid-induced responses or a general phenomenon of deactivated macrophages, we treated primary AM with LPS or S. pneumoniae together with the deactivating stimuli dexamethasone, IL-10 or apoptotic cells (ACs). The addition of either dexamethasone or IL-10 markedly enhanced the LCN2 release by AM (Figure 1B) while at the same time decreasing the release of the pro-inflammatory mediator keratinocyte-derived chemokine (KC) (Figure 1C). ACs solely inhibited the pro-inflammatory KC response upon S. pneumoniae stimulation (Figure 1C) but did not affect LCN2 secretion (Figure 1B). We next sought to determine whether LCN2 up-regulation was an exclusive feature of deactivated AM or if this induction can be extended to other macrophage populations. We therefore repeated the above described experiments using bone marrow-derived macrophages (BMDM). Dexamethasone enhanced LCN2 release by BMDM similar to what we found in AM, whereas IL-10 exerted no effects on LCN2 secretion by BMDM (Supplemental Figure 1B and 1C). To investigate the deactivation of macrophages over time, we polarized BMDM for 1, 6 and 16h and assessed the induction of LCN2 and IL-10, the latter being an established marker for deactivated macrophages (Mantovani et al, 2004). This time-course disclosed that IL-10 induction preceded LCN2 releases upon dexamethasone-mediated deactivation of macrophages (Figure 1D and 1E). Together, high LCN2 induction is a feature of dexamethasone deactivated macrophages and a robust marker of IL-10 polarized AM.

LCN2 skews macrophages towards a deactivated phenotype

Having established that LCN2 was highly induced in deactivated macrophages, we next wondered whether LCN2, in analogy to IL-10, itself exerted immunomodulatory effects on these cells. Comparing TLR-induced responses of wild-type (WT) and LCN2-/- BMDM or AM we observed that the absence of LCN2 resulted in a sustained pro-inflammatory immune response. LCN2-/- macrophages produced significantly more KC, TNFα, and IL-6 in response to S. pneumoniae (Figure 2A and B; Supplemental Figure 2A and B) or the TLR2 ligand lipoteichoic acid (LTA) (Supplemental Figure 2C). At the same time, we found the anti-inflammatory response impaired, as illustrated by a diminished IL-10 response by LCN2-/- macrophages (Figure 2C). Since LCN2 modulates intracellular iron homeostasis (Nairz et al, 2009) and because iron negatively affects pro-inflammatory immune effector pathways in macrophages (Oexle et al, 2003; Weiss et al, 1994), we excluded the possibility that differences in the iron status mediated these effects. In fact, neither the addition of deferoxamine nor of Fe(III) salts changed the KC response to S. pneumoniae by WT and LCN2-/- BMDM (Supplemental Figure 2D). We furthermore studied whether the presence of bacterial siderophores is critical for the LCN2-mediated effects on inflammation, and repeated the above described experiments in the presence or absence of enterobactin. The addition of enterobactin did not change the deactivating effects of endogenous LCN2 on macrophages following S. pneumoniae stimulation (Supplemental Figure 2E).

The prominent pro-inflammatory immune response of LCN2-/- macrophages suggested that LCN2 exerted anti-inflammatory functions and may itself contribute to the deactivation of macrophages exposed to S. pneumoniae. To next assess the direct effect of LCN2, we treated AM with S. pneumoniae in the presence or absence of recombinant LCN2. To exclude the possibility of modifying effects of siderophores on cellular iron homeostasis, we used LCN2 expressed in a mouse myeloma cell line, i.e. lacking enterobactin (apo-LCN2). Addition of exogenous apo-LCN2 indeed dampened the pro-inflammatory response while enhancing the expression of IL-10 (Figure 2D; Supplemental Figure 2F). We verified the dose-dependent suppressive effects of apo-

LCN2 using the alveolar macrophage cell line MH-S, stimulated with LTA from S. pneumoniae or S. aureus, or LPS, in the presence of exogenous apo-LCN2 (Supplemental Figure 2G and H). Consistent with the idea of LCN2 dampening inflammation, LCN2 overexpression in RAW264.7 macrophages prevented IL-6 induction upon S. pneumoniae challenge (Figure 2E).

To ultimately investigate the potential impact of endogenous LCN2 on pulmonary macrophages in vivo, we infected WT and LCN2-/- mice with S. pneumoniae for 6h, isolated alveolar cells (consisting of 88% alveolar macrophages and 12% neutrophils) by lavage and quantified KC and IL-10 transcript levels. We found endogenous LCN2 capable of keeping alveolar cells in a deactivated state, as illustrated by increased IL-10 and suppressed KC mRNA expression upon S. pneumoniae infection in WT but not LCN2-deficient mice (Figure 2F). Collectively, these overexpression, substitution and deletion experiments illustrate that LCN2 deactivates macrophages in vitro and in vivo.

LCN2 is induced during infection in humans and mice

The finding that endogenous LCN2 deactivated AM during pneumococcal infection (Figure 2F) encouraged us to examine the potential function of LCN2 during pneumonia in more detail. To start with, we tested the possible induction of pulmonary LCN2, which has never been studied in patients with bacterial lung infections. Because we wanted to investigate the systemic and local, pulmonary, induction of LCN2, we concentrated on mechanically ventilated intensive care unit (ICU) patients, where alveolar lavage samples were made available during routinely performed bronchoscopies. We found LCN2 concentrations in bronchoalveolar lavage (BAL)-fluid and plasma significantly enhanced in patients with confirmed bacterial pneumonia (pulmonary infiltrates in chest X-rays and bacterial CFU counts > 104 /ml BALF; Figure 3A). LCN2 concentrations in BAL-fluid were approximately 10-fold higher than in plasma, thus indicating the local production of LCN2 at the site of infection (Figure 3A). Notably, inhaled glucocorticoid therapy (as part of the acute treatment) increased pulmonary LCN2 levels in patients with confirmed bacterial pneumonia (Fig. 3B), suggesting that these potent macrophage deactivating drugs also induced LCN2 in

vivo in humans. Intriguingly, we found increased LCN2 levels not only in patients with pneumonia caused by pathogens producing siderophores that are recognized by LCN2 such as Klebsiella or E. coli, but also in patients infected with bacteria whose siderophores are not recognized by LCN2, like S. aureus, or bacteria that do not utilize siderophores at all, such as S. pneumoniae (Figure 3C). These findings hinted towards a siderophore-independent role of LCN2 during pulmonary infections.

To examine this potential siderophore-independent role of LCN2, we made use of a murine pneumococcal pneumonia model and at first quantified pulmonary LCN2 concentrations upon infection. We observed a steady increase in LCN2 levels starting 6h after infection with S. pneumoniae (Figure 3D). We next searched for the cellular origin of LCN2 during pneumococcal infection and immunohistochemical stainings of lungs revealed LCN2 expression in both hematopoietic and epithelial cells 48h after S. pneumoniae infection (Figure 3E). We therefore tested LCN2 levels in primary AM and primary respiratory epithelial cells treated with S. pneumoniae and found a more pronounced mRNA (Figure 3, F and G) and protein (Figure 3H) expression in AM than in epithelial cells. Since apart from macrophages also neutrophils (PMN) were reported as cellular sources of LCN2 (Kjeldsen et al, 1994), we stained for LCN2 in neutrophils of whole lung cell suspensions obtained from mice infected with S. pneumoniae. These assays showed that all neutrophils contained preformed LCN2 in their granules at baseline while upon progression of pneumonia the proportion of LCN2-positive PMN gradually decreased (Supplemental Figure 3). These data demonstrate the importance of neutrophil-derived LCN2 and suggest secretion of preformed LCN2 from neutrophilic granules. Considering the rapid recruitment of PMNs during pneumonia, early increments in lung LCN2 most likely originated from neutrophils. Together, pulmonary LCN2 is highly induced in the course of bacterial pneumonia in both mice and humans.

LCN2 impairs bacterial clearance in vivo

Having found LCN2 to exert anti-inflammatory effects and LCN2 to be strongly induced during pneumonia, we next explored the biological function of LCN2 during pneumococcal pneumonia and analyzed the antibacterial response in WT and LCN2-/- mice. First, we studied a late time-point, where the efficacy of antibacterial effector mechanisms can be appreciated by the decline in bacterial counts. By doing so, we

revealed a significantly improved bacterial clearance in LCN2-/- mice when compared to WT animals after 48h of infection, indicating an improved anti-bacterial response in the absence of LCN2 (Figure 4A). As a consequence of the reduced bacterial burden in LCN2-/- mice, we found an accelerated resolution of inflammation as evidenced by lower levels of inflammatory cytokines in the lung (Figure 4B) and by less severe signs of lung inflammation (Figures 4, C and D). We then sought to determine the respective contribution of myeloid and epithelial cells to the observed phenotype and generated bone marrow chimeric mice in order to subject them to S. pneumoniae for 48h. Although we observed a tendency towards improved bacterial clearance in chimeric LCN2-/- mice that have been reconstituted with WT bone-marrow (KO-WT), only mice completely lacking LCN2 in both the hematopoietic and radioresistant epithelial compartment (KO-KO) displayed significantly lower bacterial counts in their lungs after 48h (Figure 4E). As a consequence, bacterial dissemination (Figure 4F), as well as pulmonary (Figure 4G) and systemic cytokine formation (Figure 4H) were most robustly reduced in KO-KO animals. Together, LCN2 produced by both epithelial and myeloid cells was required to impair the host defense against S. pneumoniae in vivo.

LCN2 dampens the early immune response

In order to elucidate the mechanism underlying this improved host defense in the absence of LCN2 we evaluated the early (6h) immune response to S. pneumoniae in mice, as this early time-point resembles the induction phase of inflammation that in turn determines the efficacy of the antibacterial response later on. After infecting WT and LCN2-/- animals with S. pneumoniae for 6h we detected a significantly increased release of proinflammatory cytokines and chemokines in LCN2-/- animals (Figure 5A). The neutrophil chemoattractant KC was most impressively increased in LCN2-/- mice, which resulted in a consecutively enhanced influx of neutrophils (Figure 5B). Since LCN2 has been reported to affect apoptosis (Devireddy et al, 2001), we excluded the possibility that increased neutrophil numbers in the BAL-fluid of LCN2-/- mice resulted from altered rates of apoptosis (Supplemental Figure 4, A to C) or from a-priori differences in white blood cell counts between WT and gene-deficient animals (Supplemental Figure 4D). Interestingly, the anti-inflammatory mediator IL-10, but not TGFβ, was significantly decreased in LCN2-/- as compared to WT mice (Figure 5C). Hence, these in vivo data support our hypothesis of LCN2 being a deactivator of

macrophages (Figure 2) as we found LCN2 to dampen the early immune response upon S. pneumoniae infection in mice. The resulting delay in the attraction of neutrophils to the site of infection in turn can explain the subsequently impaired bacterial clearance in WT mice (Fig. 4A).

LCN2 impairs bacterial clearance in an IL-10-dependent manner

Having observed increased IL-10 levels upon S. pneumoniae stimulation in the presence of LCN2 in vitro and in vivo (Figure 2, C, D and F; Figure 5C), we hypothesized that the anti-inflammatory properties of LCN2 might depend on the induction of IL-10. To test this hypothesis, we inhibited IL-10 activity using a blocking antibody before treating BMDMs with S. pneumoniae and LCN2. Indeed, IL-10 neutralization completely abolished the inhibitory effects of LCN2, confirming that LCN2 required IL-10 to suppress KC releases (Figure 6A). In agreement with this statement, macrophages deficient in STAT3, the major downstream transcription factor of IL-10, did not display reduced KC releases in the presence of exogenous LCN2 (Figure 6B). Having established that LCN2 dampens inflammation in a largely IL-10 dependent manner, we next wondered if LCN2 directly affected transcription of IL-10 mRNA. Using RAW264.7 macrophages stably overexpressing LCN2 we discovered an enhanced baseline IL-10 promoter activity, which was significantly augmented following stimulation with S. pneumoniae (Figure 6C), indicating that LCN2 enhanced IL-10 transcription, especially in the presence of bacteria. In accordance, BMDM treated with exogenous LCN2 in the absence of bacteria did not induce IL-10 release; whereas LCN2 dose-dependently enhanced the production of IL-10 when concomitantly stimulated with S. pneumoniae (Figure 6D). To summarize, we revealed that LCN2 deactivates macrophages through an IL-10-STAT3 dependent mechanism via the induction of IL-10 in the presence of bacteria.

Given the in vitro anti-inflammatory properties of LCN2 were mediated via IL-10 (Figure 6A and 6B) and considering the detrimental effects of IL-10 during pneumococcal pneumonia (van der Poll et al, 1996; van der Sluijs et al, 2004), we hypothesized that LCN2 might dampen inflammation and thus impair the host defense to S. pneumoniae via induction of IL-10 in vivo. To examine this idea, we blocked IL-

10 in WT and LCN2-/- animals that were infected with S. pneumoniae for 48h. Consistent with previous data (Figure 4A), LCN2-/- mice treated with isotype Ab cleared S. pneumoniae from their lungs more efficiently than respective WT animals (Figure 6E). In agreement with earlier reports (van der Poll et al, 1996), blocking of IL-10 decreased bacterial counts in WT animals, while no significant change was observed in LCN2-/- mice. Of note, application of the anti-IL-10 antibody completely abolished the differences in pulmonary and systemic bacterial clearance between WT and LCN2-/- animals (Figure 6E and 6F) and accelerated the resolution of inflammation (Figure 6G). In conclusion, our results confirm that LCN2 impairs bacterial clearance in a largely IL-10 dependent manner in vivo.

LCN2 exerts detrimental effects during pneumococcal pneumonia in mice and humans

The improved bacterial clearance in the absence of LCN2 suggested that LCN2 might negatively affect the outcome from pneumococcal pneumonia. To test this hypothesis we infected WT and LCN2-/- mice with S. pneumoniae and monitored survival over 7 days. While 70% of LCN2-/- mice recovered, more than 90% of WT mice succumbed to the infection (Figure 7A). In accordance with these murine data, high LCN2 levels in the BAL-fluid of ICU patients suffering from pneumonia caused by Gram-positive bacteria (that were either siderophore-independent or produced siderophores not recognized by LCN2; Supplemental Table 1) but not by Gram-negative bacteria (which, to a high degree depended on LCN2 compatible siderophores) were indicative for an adverse clinical outcome (Figure 7B). Of note, none of the non-survivors suffering from pneumonia with Gram-positive bacteria received inhaled glucocorticoids. Together these data strengthen the hypothesis that LCN2 exerts detrimental effects during pneumococcal pneumonia in mice and indicate that enhanced pulmonary LCN2 levels in humans suffering from pneumonia with Gram-positive bacteria were predictive for a poor outcome.

Discussion

Deactivated macrophages are characterized by down-regulating pro-inflammatory cytokines while up-regulating IL-10 (Mantovani et al, 2004). Compared to M1 or M2 polarized cells, the precise functions of deactivated macrophages are still poorly understood, and even less is known about their biological role during infections. Here, we identified LCN2 as a novel marker and mediator of deactivated macrophages in mice. Our studies illustrate that the presence of LCN2 was associated with macrophage deactivation in the lungs, which resulted in an impaired immune response to the clinically important pathogen S. pneumoniae. Mechanistically, we found IL-10 to mediate the anti-inflammatory effects of LCN2 upon infection.

Among the macrophage subsets we studied, highest LCN2 levels were released by AM, and AM were the only cells where IL-10 further enhanced the synthesis of LCN2. Since AM is the only macrophage subset that is constantly exposed to environmental microbes, a tight control of its activation status is required. Our data suggest an essential role for LCN2 herein, which is consistent with the homeostatic and anti-inflammatory role of AM (Knapp et al, 2003) and the important regulatory function of IL-10 in the lungs (Sun et al, 2009). Although our findings indicate that LCN2 preferentially impacts the function of AM, it is tempting to speculate that other anti-inflammatory macrophage subsets might also respond to IL-10-LCN2 induction. Potential candidates are intestinal myeloid regulatory cells (Mreg), given the importance of IL-10 in intestinal homeostasis (Kayama et al, 2012), but also tumor-associated macrophages since LCN2 was found significantly elevated in plasma of tumor bearing mice (Whiteaker et al, 2011). In fact, while this manuscript was in preparation, Jung et al. reported that IL-10 induced LCN2 in human macrophages (Jung et al, 2012c). By keeping breast cancer cells in either conditioned medium from IL-10 stimulated macrophages or medium containing exogenous LCN2, an enhanced growth rate of tumor cells was observed. This led the authors to argue that IL-10-induced LCN2 might play a role in tumor-associated macrophage biology (Jung et al, 2012c). These data strengthen our observation of LCN2 as a mediator of deactivated macrophages and extend these findings to human cells. In contrast to this publication, we only identified LCN2 induction following stimulation of macrophages with IL-10 in the presence of bacterial TLR ligands, while IL-10 alone did not induce any LCN2. In addition to IL-10, we established dexamethasone to be a major inducer of LCN2 associated macrophage deactivation. In support of our data, an expression profiling

study done with murine splenic dendritic cells revealed a strong synergistic effect of dexamethasone and LPS on LCN2 expression (Vizzardelli et al, 2006). The interesting fact that inhalative glucocorticoids enhanced LCN2 levels in BAL-fluid from patients with pneumonia extends these findings to humans as it supports the idea that pulmonary macrophages also express LCN2 in humans. Based on our data and published reports, we postulate that LCN2 secretion is specifically restricted to IL-10 or dexamethasone associated macrophage deactivation. In support of this notion, published reports illustrate that LCN2 is not induced in M1 or M2-polarized macrophages (Recalcati et al, 2010), and even down-regulated in regulatory macrophages that were generated in the presence of immune complexes (Edwards et al, 2006).

Besides the interference with siderophore-dependent bacterial iron acquisition (Flo et al, 2004; Goetz et al, 2002), LCN2 was shown to influence cellular processes such as mammalian iron homeostasis (Nairz et al, 2009; Yang et al, 2002), apoptosis (Devireddy et al, 2005; Devireddy et al, 2001), or inflammatory responses (Bachman et al, 2009; Zhang et al, 2008a). In accordance with our data, Zhang et al. reported that LCN2 reduced the LPS-triggered induction of cytokines by RAW264.7 macrophages (Zhang et al, 2008a). During completion of this manuscript another group reported enhanced levels of LPS-induced proinflammatory cytokines secreted by LCN2-deficient peritoneal macrophages and BMDM as compared to WT cells (Srinivasan et al, 2012b). However, in contrast to our data, this report also showed increased IL-10 releases by LCN2-/- peritoneal macrophages, which might be due to macrophage-subset specific differences or the fact that we focused on S. pneumoniae. In contrast to observations in macrophages, LCN2 was found to augment IL-8 releases by human lung epithelial cells and neutrophil-influx when triggered by the bacterial siderophore (aferric) enterobactin (Bachman et al, 2009; Nelson et al, 2007). We did not observe any pro-inflammatory effect when incubating murine macrophages with aferric enterobactin in the presence of endogenous LCN2 (data not shown). In conjunction with our findings of an anti-inflammatory role of LCN2 described here, these reports suggest that LCN2 exerts pro-inflammatory effects only in the presence of bacteria that release enterobactin, but not in response to bacteria that lack siderophores like S. pneumoniae. The idea that LCN2 modulates inflammation and scavenges ferric siderophores might explain why the upper respiratory tract is

protected from colonization with bacteria releasing enterobactin and is in agreement with the recent observation that the lung microbiome indeed constitutes predominantly of bacteria lacking LCN2-compatible siderophores (Charlson et al, 2011). The presence of pulmonary LCN2 per se could concurrently explain the dominance of LCN2-resistant bacteria such as S. pneumoniae or Haemophilus influenzae as causative pathogens of pneumonia. Klebsiella pneumoniae, another relevant lung pathogen that depends on enterobactin-like siderophores, evolved LCN2-resistant strains to evade LCN2-mediated growth inhibition (Bachman et al, 2011).

The question remains why do host cells induce LCN2 upon infection with siderophore-independent bacteria like S. pneumoniae? Since we discovered LCN2 induction to follow the initial release of IL-10 by macrophages, we postulate that LCN2 might provide a means to prevent overwhelming inflammation and to promote tissue repair after clearance of infectious pathogens. Neutrophils that released substantial amounts of LCN2 late in infection could additionally contribute to the dampening of the immune response and resolution of inflammation through the proposed mechanism, which is in accordance with the recent finding that neutrophils regulate inflammation (Zhang et al, 2009). This pathway might prove safe in uncomplicated infections and even contribute to resolution, which is supported by a report demonstrating a pro-regenerative function for LCN2 in a model of renal ischemia-reperfusion injury (Jung et al, 2012a). However, in ICU patients with severe pneumonia who require mechanical ventilation, elevated LCN2 levels may be a result of an exaggerated immune response to higher bacterial concentrations. In this setting, elevated LCN2 might deactivate macrophages and in turn prevent efficient bacterial clearance and aggravate the disease. In agreement, we report a tendency for a worse outcome during severe pneumonia with Gram positive bacteria in ICU patients with elevated LCN2 levels in the BAL, although the number of patients we examined in this pilot study is certainly too low to draw definitive conclusions. Corticosteroids can further enhance LCN2 formation and macrophage deactivation, which is undesirable for the clearance of viable bacteria. Since we found that inhaled corticosteroids enhanced LCN2 levels in ICU patients with bacterial pneumonia, the use of these drugs might be harmful during severe infections with pathogens that do not produce siderophores recognizable by LCN2. Thus, LCN2 appears to play a significant role in the course of

S. pneumoniae pneumonia and evaluating LCN2 in human BALF may provide significant information on the course of the infection or the necessity to adjust therapy.

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Author contributions

J.M.W. conceived the study, designed and performed experiments, analyzed data and wrote the manuscript. R.G., O.S., S.S., B.D. and K.L. designed and performed experiments. I.M. scored histological sections. M.N., B.S. and M.M. provided mice and important technical advice. L.B. supplied antibodies for the in vivo blocking experiment. J.M.W., A.S., V.F. and P.S. recruited ICU patients and collected clinical samples. G.W. provided important technical advice and contributed conceptionally to the study design. S.K. conceived and supervised the study, designed experiments, analyzed data and wrote the manuscript.

Methods

Human subjects

Sixty-four (21 female, 43 male; age 56 ± 17 years, mean ± SD) intubated and mechanically ventilated ICU patients with suspected pneumonia, defined by the presence of new infiltrates in the chest X-ray were examined (n=26 community-acquired, and n=38 hospital-acquired (out of which n=21 had suspected ventilator-associated pneumonia)). Diagnosis of pneumonia was considered confirmed if microbiological assays revealed >104CFU/ml of potentially pathogenic bacteria in the BAL-fluid (these patients are referred to as having pneumonia in Fig. 3A-3C). Twelve age-matched outpatient subjects (five female, seven male; age 58 ± 15 years) undergoing diagnostic bronchoscopy for non-infectious causes were included as controls. BAL samples were obtained via bronchoscopy and EDTA blood was withdrawn from ICU patients before bronchoscopy. BAL and plasma hLCN2 levels were quantified by ELISA (R&D).

Mice

Pathogen-free 8-10 week old female C57BL/6 wild-type littermate and LCN2-/- mice were used for experiments. LCN2-/- mice (Flo et al, 2004) were kindly provided by Shizuo Akira, University Osaka, and were backcrossed to a C57BL/6 background for ten generations. LysMCre/Cre (Clausen et al, 1999) were bred to STAT3flox/flox (Alonzi et al, 2001) mice, which were kindly provided by Valeria Poli, University of Turin. Cells derived from Stat3+/+,Cre/Cre and Statflox/flox,Cre/Cre littermates were used.

Murine pneumonia model

Pneumococcal pneumonia was induced in mice as described earlier (Knapp et al, 2003; Rijneveld et al, 2001). Briefly, S. pneumoniae serotype 3 (ATCC 6303) was cultured in Todd-Hewitt broth to mid-logarithmic phase, washed twice and inoculated into anesthetized mice intranasally with 104-105 CFU/50µl. Six, 24 or 48 hours after infection mice were sacrificed with ketamin and xylazine and blood samples were collected in EDTA coated tubes. Lungs were harvested and homogenized in sterile saline (Precellys 24TM, Peqlab) and bacterial growth was quantified by plating 10-fold serial dilutions on blood agar plates. The remaining lung homogenates were incubated

in Greenberger lysis buffer as described (Knapp et al, 2004a) and supernatants were stored at -20°C until cytokines were assayed. BAL processing: BAL was performed six hours after infection as described before (Knapp et al, 2004b). Cell-free BAL supernatants were stored at -20°C for cytokine measurement. Cells numbers were determined using a hemocytometer and Giemsa stained cytospin preparations were used for differential cell counts. IL-10 blocking in vivo: mice were anesthetized by i.p. injection of ketamin and xylazine and administered 150µg of either anti-IL-10 (clone JES5.2A5, Bioceros) or isotype Ab (clone GL113, Bioceros), intranasally 15 min following S. pneumoniae infection. Histologic examination: Lungs were fixed in 10% formaldehyde and embedded in paraffin. Lung sections (4µm) were stained with hematoxylin and eosin and the severity of inflammation and pneumonia were analyzed by a trained pathologist blinded for group assignments according to a scoring system. In detail, lungs were examined for the presence of interstitial inflammation, alveolar inflammation, pleuritis, bronchitis and endothelitis. Each parameter was scored in the range of 0 to 3 points with 0 representing absent, 1 mild, 2 moderate, and 3 severe. Additionally, 1 point was scored for the presence of each pneumonia, edema and thrombi formation, and 0.5 point for every infiltrate covering 10% of lung area. The sum of all parameters indicated the total histology score. LCN2 immunostaining was performed on paraffin-embedded lung sections as described previously (Knapp et al, 2004b). Briefly, antigen retrieval was performed using a Tris/EDTA, 0.05% Tween 20 buffer at pH 8.0 (Sigma). Lung slides were incubated with rat anti-mouse LCN2 (clone: 228418; R&D; coupled to N-hydroxysuccinimide biotin (Calbiochem) in 0.1M NaHCO3 buffer, pH 7.8) or a corresponding isotype control Ab, followed by HRP-conjugated secondary antibody (DakoCytomation) and visualized with 3,3-diaminobenzidine tetrahydrochloride (Vector Laboratories). Nuclei were counterstained with hematoxylin.

Generation of bone marrow chimeric mice

Nine week old female WT and LCN2-/- (KO) mice were irradiated by a single administration of γ irradiation (9Gy) and bone-marrow was immediately reconstituted by intravenous injection of 2x106 freshly isolated bone marrow cells of the same (WT-WT, KO-KO mice) or the opposite genotype (chimeric WT-KO, KO-WT mice). One mouse of each genotype was left without reconstitution to control for efficiency of irradiation. Mice were kept under sterile conditions for 12 weeks. The proper

repopulation of bone marrow-derived cells was confirmed by isolating genomic (g)DNA from blood, AM, peritoneal macrophages, and bone marrow followed by genotyping (PCR). The recipient background was confirmed by genotyping gDNA obtained from tails (Supplemental Figure 5).

Cell culture and stimulations

AM and BMDM were isolated as described elsewhere (Lagler et al, 2009; Zhang et al, 2008b). Primary cells were cultured in RPMI 1640 (Gibco) containing 1% penicillin and streptomycin, and 10% fetal calf serum (FCS) at 37°C. MH-S cells (ATCC CRL-2019) were cultured in the same media supplemented with 50 mM 2-mercaptoethanol. For all stimulations RPMI 1640 containing 3% FCS was used. Primary epithelial cells were isolated as described earlier (Raoust et al, 2009) and cultured in RPMI 1640 containing 1% penicillin/streptomycin, 2% FCS, 2mM L-glutamine, 5µg/ml insulin, 10 nM hydrocortisone, 10nM β-estradiol, 100µg/ml transferrin, 30nM sodium selenite (all Sigma). In brief, single cell lung suspensions were obtained by digesting perfused lungs with dispase and DNase I, followed by several steps of filtration via nylon mesh filters. Enrichment for epithelial cells was achieved by negative selection using plates coated with Ab against CD45 and CD16/CD32. IFNγ (PeproTech), LPS from E. coli 055:B5 (Sigma-Aldrich), rmIL-4 (R&D), rmIL-13 (R&D), dexamethasone (ratiopharm), rmIL-10 (eBioscience), LTA from S. pneumoniae (TIGR4) (Draing et al, 2006) and LTA from S. aureus (Morath et al, 2001), enterobactin-free rmLCN2 produced in a mouse myeloma cell-line (R&D; Catalog No. 1857-LC), deferoxamine (Calbiochem), ammonium Fe (III) citrate (Sigma) and enterobactin (Fe-free) (EMC Microcollections) were used at indicated concentrations for cell stimulations.

Analysis of soluble factors

Mouse LCN2, TNF-α, IL-6, KC, IL-1β, macrophage inflammatory protein 2 (MIP-2), IL-10, TGFβ1 and human LCN2 were measured using specific ELISAs (R&D Systems or Bender MedSystems for IL-10) according to the manufacturers' instructions. Nitrite levels were estimated using the Griess Reagent System (Promega).

RT-PCR

Messenger RNA was isolated using TRIzol® Reagent (Invitrogen) and transcribed into cDNA using iScriptTM cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed using iTaq SYBR Green Supermix (Bio-Rad) and the StepOnePlusTM cycler (Applied Biosystems). Primers for LCN2, MRC1, IL-10, KC, IL-6, and hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) were obtained from Sigma (Supplemental Table 2).

Luciferase gene reporter assays

Stable overexpressing RAW264.7 cells were generated by retroviral transfection. Briefly, the packaging cell line GP-293 HEK (Clontech) was transfected with LCN2 expression plasmid (kindly provided by Dr. R. B. Arlinghaus, The University of Texas, Houston) or GFP control plasmids and VSV-G (retroviral vector). RAW 264.7 cells were infected with the virus containing supernatants from HEK cells and successfully transfected, GFP expressing cells were sorted by flow cytometry. For more details see Supplemental Table 1. LCN2 expressing and GFP control cells were then transiently transfected with IL-10 promoter firefly luciferase (Brightbill et al, 2000) (pGL2B, -1538/+64; Addgene) and renilla luciferase (Promega) using the Amaxa Nucleofector Kit (Lonza) according to the manufacturer's instructions. Cells were stimulated 24h later with S. pneumoniae and luminescence was assessed after 24h. Data are presented as relative light units (RLU) after correction for renilla.

Flow cytometry

Lungs were perfused through the heart with PBS, cut into pieces and placed in RPMI 1640 containing 5% FCS, collagenase I (Invitrogen) and DNase I (Sigma) for 1h at 37°C. Single cell suspensions were obtained using a glass homogenizer (KONTES scientific glassware/instruments) followed by passage through 70µm and 40µm cell strainers (BD). Cells were counted and 106 cells/ staining were used. Unspecific binding was blocked using mouse IgG (6µg/staining; Invitrogen). Cell surface markers were stained in PBS containing 2%FCS. Antibodies: HorizonTM V500 conjugated anti-CD45 (30-F11; BD), FITC conjugated anti-Ly6G (1A8; Biolegend), Alexa Fluor 700 conjugated anti-CD11b (M1/70; eBioscience). Viability of cells was assessed using Fixable Viability Dye eFluor® 780 (eBioscience) according to the manufacturers' instructions. Intracellular stainings were performed using the Fix&Perm kit (ADG - An der Grub Bio-Research). Antibodies: anti-LCN2 (228418; R&D; coupled to N-

hydroxysuccinimide biotin (Calbiochem) in 0.1M NaHCO3 buffer, pH 7.8), biotin conjugated rat IgG2a (LO-DNP-16; Caltag), PE conjugated Streptavidin (eBioscience). Assessment of apoptosis: Thymocytes or BAL cells were washed twice with PBS and stained for FITC Annexin V and 7-Amino-Actinomycin D (7-AAD) (both BD) in Annexin V binding buffer according to the manufacturers' instructions.

Generation of apoptotic cells

To generate apoptotic cells, the thymi were isolated from 4-5 week old C57BL/6 mice and passed twice through a cell strainer. The cells were treated with 10µM etoposide (Sigma) for 5h and the percentage of apoptotic cells was assessed by flow-cytometry. The cells were thoroughly washed to remove etoposide before being used for stimulations.

Statistical analysis

Data are expressed as mean ± SEM. Two groups were compared using unpaired t test, more than 2 groups using one-way ANOVA followed by Bonferroni multiple comparison test or Kruskal-Wallis test combined with Dunn´s multiple comparison test, depending on the distribution of the data (parametric or non-parametric, respectively). Survival data were analyzed by Kaplan-Meier and log-rank statistic. A P value < 0.05 was regarded as statistically significant.

Study approval

The human study was approved by the Institutional Review Board of the Medical University of Vienna; written informed consent was obtained from conscious participants prior to enrollment or after regaining consciousness (according to regulations by Austrian law). All animal experiments were approved by the Institutional Review Board of the Medical University of Vienna and the Ministry of Sciences (BMWF-66.009/0318-II/10b/2008).

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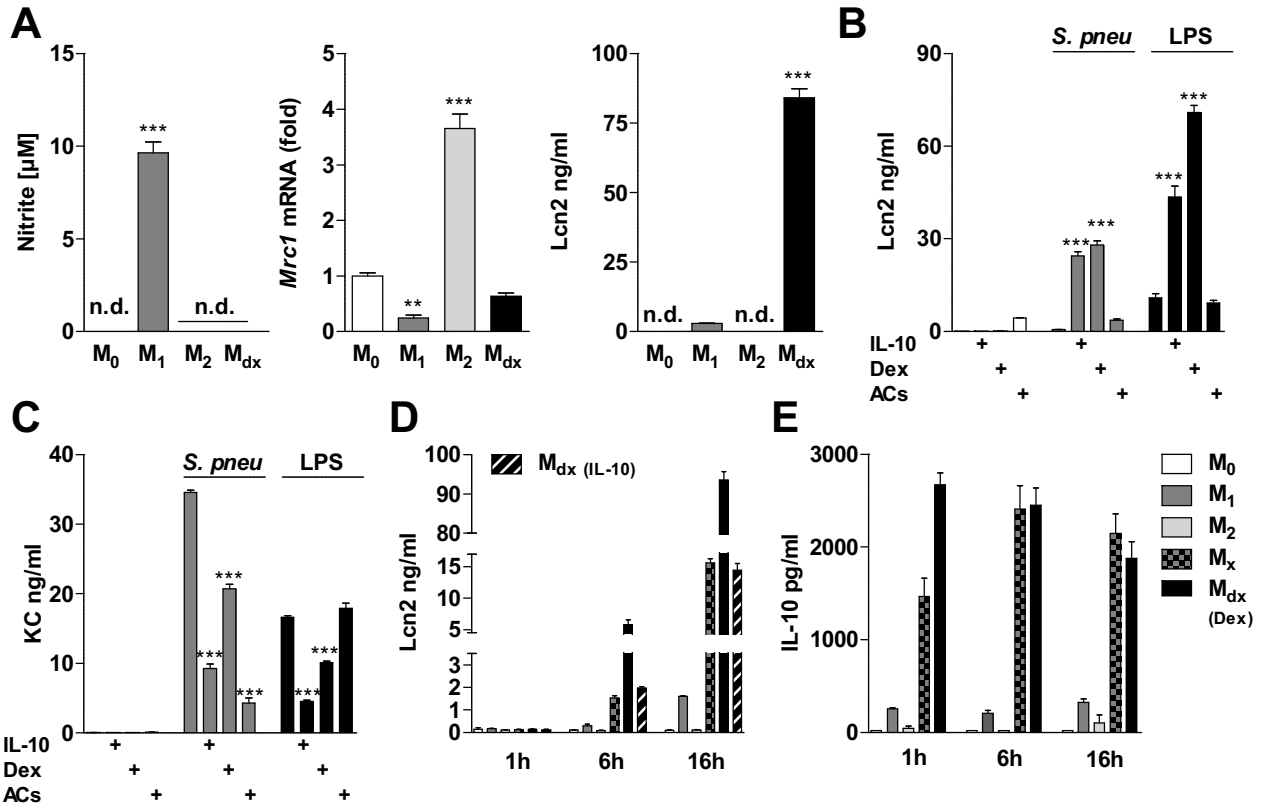

Figure 1: LCN2 is highly expressed in deactivated macrophages

(A) Primary AM were polarized with M1 (100ng/ml LPS, 200U/ml IFNγ), M2 (10ng/ml IL-4 and IL-13) or deactivating (Mdx; 10ng/ml LPS, 100µM dexamethasone) stimuli for 16h. Nitrite and LCN2 were measured in supernatants using the Griess reagent or ELISA. Mrc1 mRNA levels were determined by qRT-PCR, normalized to HPRT and expressed as fold-change compared to control cells. (B,C) Primary AM were treated with 4x107 CFU/ml S. pneumoniae (grey bars) or 10ng/ml LPS (black bars) with or without defined deactivating stimuli (10ng/ml IL-10, 100µM dexamethasone (Dex), or 107/ml apoptotic cells (ACs)) for 16h. LCN2 (B) and KC (C) levels were quantified in supernatants by ELISA. (D,E) BMDM were polarized with M1 (10ng/ml LPS, 200U/ml IFNγ), M2 (10ng/ml IL-4 and IL-13), activated with 10ng/ml LPS (Mx) or deactivated (Mdx) with 10ng/ml LPS and (D,E) 100µM dexamethasone or (D) 10ng/ml IL-10 for 1, 6 and 16h. LCN2 (D) and IL-10 (E) levels were assessed in supernatants by ELISA. Data are presented as mean ± SEM of quadruplicates and are representative of two independent experiments. n.d., not detectable; */ **/ *** indicate $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ versus M0 (A) or versus S. pneumoniae or LPS alone (B,C) (ANOVA).

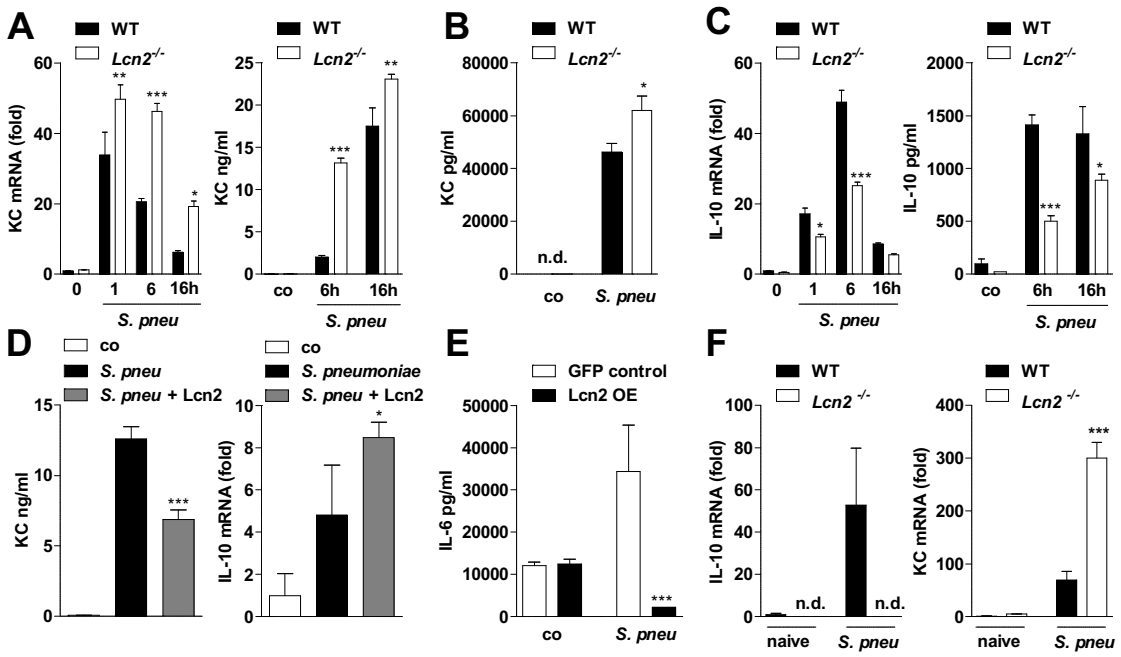

Figure 2: LCN2 skews macrophages towards a deactivated phenotype

(A,C) WT and LCN2-/- BMDM were treated with 4x107 CFU/ml S. pneumoniae for 1, 6 and 16h. KC (A) and IL-10 (C) expression was measured by qRT-PCR, normalized to HPRT and expressed as fold-change compared to untreated WT cells. KC (A) and IL-10 (C) protein levels were assessed in supernatants (ELISA). (B) KC levels in supernatants of WT and LCN2-/- AM stimulated with 4x107 CFU/ml S. pneumoniae for 16h. (D) WT AM were stimulated with 4x107 CFU/ml S. pneumoniae ± 100ng/ml LCN2. KC levels were assessed in supernatants after 16h. IL-10 expression was determined after 1h by qRT-PCR, normalized to HPRT and expressed as fold-change compared to control cells. (E) IL-6 release by RAW264.7 cells overexpressing LCN2 or GFP, treated with 4x107 CFU/ml S. pneumoniae for 16h. (F) WT and LCN2-/- mice were infected with 105 CFU S. pneumoniae. Alveolar cells were isolated after 6h by lavage and KC and IL-10 transcript levels quantified by qRT-PCR, normalized to HPRT and expressed as fold-change compared to cells from uninfected WT animals. Data are presented as mean ± SEM of quadruplicates and representative of two independent experiments. */ **/ *** indicate $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ versus respective WT condition (A-C, F) (ANOVA), S. pneumoniae alone (D) (t test), or GFP control (E) (t test)

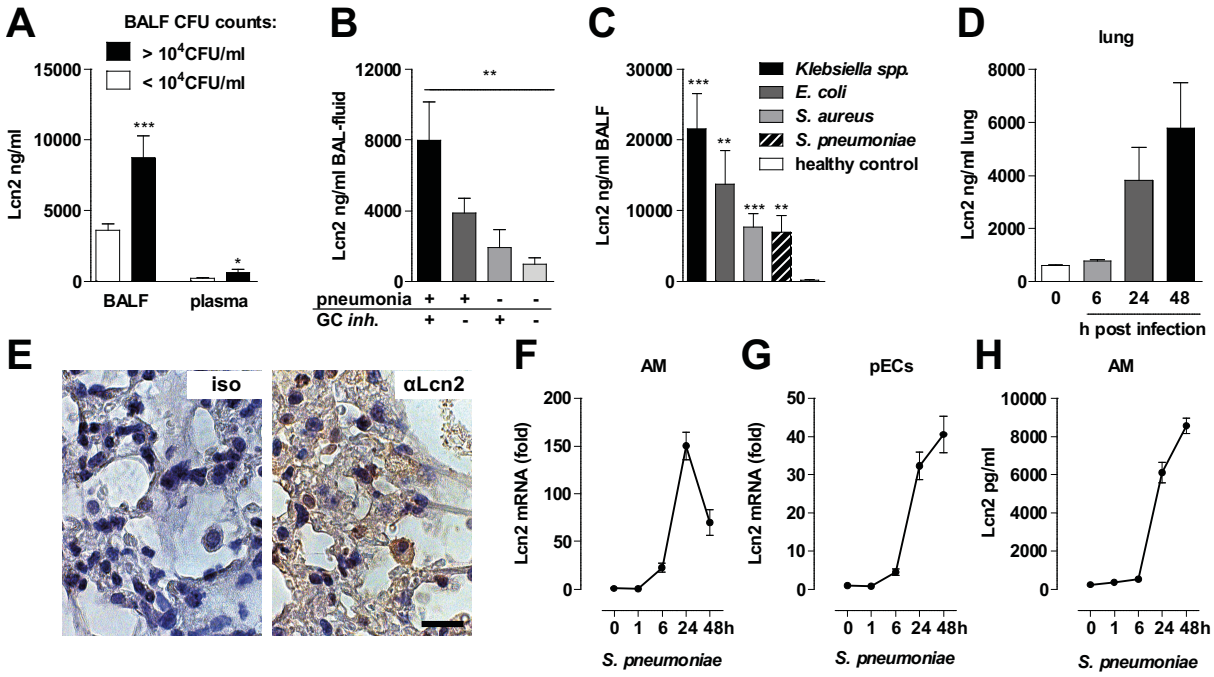

Figure 3: LCN2 is induced during infection in humans and mice

(A-C) LCN2 levels were measured in bronchoalveolar lavage (BAL) fluid and plasma of mechanically ventilated ICU patients with suspected pneumonia (pulmonary infiltrate in the chest x-ray; n=64) (A-C) and healthy controls (n=10) (C). LCN2 concentrations were analyzed according to the presence of bacteria (> 104 CFU/ml) in BAL-fluid (A), according to the presence of pneumonia and history of treatment with inhalative glucocorticoids prior to bronchoscopy (B) and per identified pathogen (Klebsiella spp. n=5 , E. coli n=5; S. aureus n=6, S. pneumoniae n=6, healthy n=12) (C). (D,E) WT mice were infected with 105 CFU S. pneumoniae and (D) pulmonary LCN2 concentrations were quantified over time. (E) Lungs were stained immunochistochemically using rat IgG2a (left) or anti-LCN2 Ab (right panel) 48h after infection; scale bar, 20µm. (F-H) Primary AM (F,H) or primary epithelial cells (G) were treated with 2x107 CFU/ml S. pneumoniae for the indicated time. (F,G) LCN2 transcript levels were assessed by qRT-PCR, normalized to HPRT and expressed as fold-change versus baseline. (H) LCN2 release was evaluated in supernatants by ELISA. Data are expressed as mean ± SEM (n=8 (D,E), n=4 (F-H)) and are representative of two independent experiments. */ **/ *** indicate P < 0.05/ P <0.001/ P < 0.0001 versus ICU patients without pneumonia (A) (t test) or healthy subjects (C) (ANOVA).

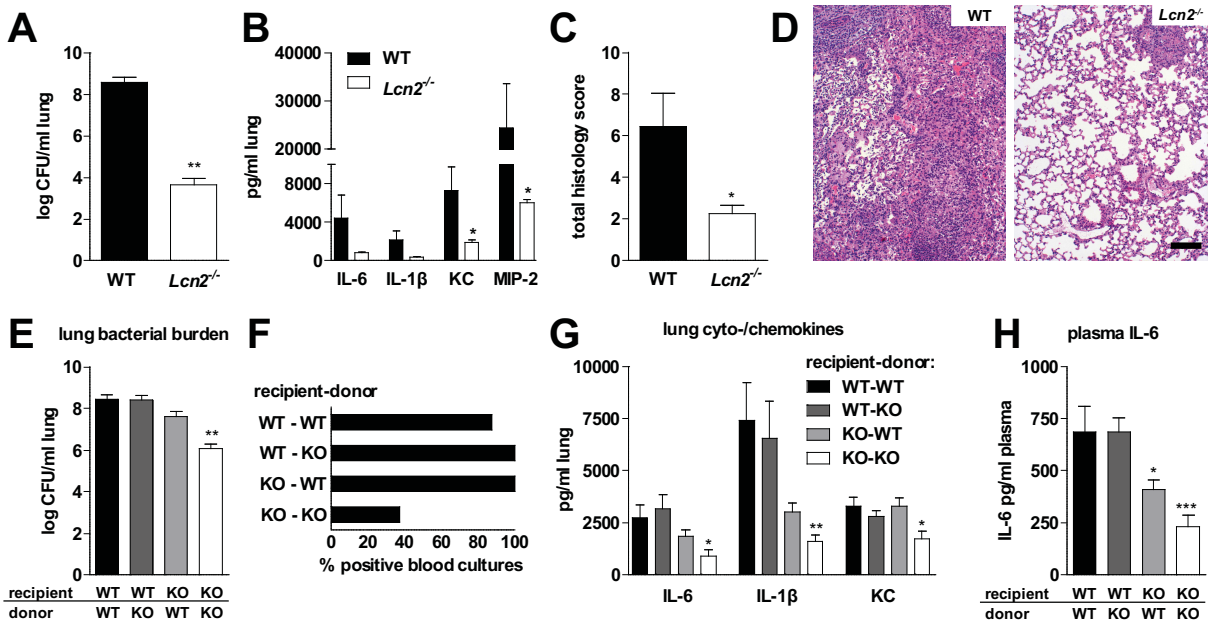

Figure 4: LCN2 impairs bacterial clearance in vivo

(**A-D**) WT and *LCN2*$^{-/-}$ mice were intranasally infected with 2x10^4 CFU *S. pneumoniae* for 48h. (**A**) Bacterial CFU counts were enumerated in lungs and (**B**) cytokine secretion was quantified in lung homogenates by ELISA. (**C**) Lung sections (H&E staining) were scored as described in the Methods, representative images are shown in (**D**); scale bar, 100µm. (**E-H**) WT mice reconstituted with WT bone-marrow (WT-WT), *LCN2*$^{-/-}$ mice reconstituted with *LCN2*$^{-/-}$ bone marrow (KO-KO) and chimeric mice (WT-KO and KO-WT) were infected intranasally with 10^5 CFU *S. pneumoniae*, 48h after infection bacterial growth was quantified in (**E**) lung homogenates and (**F**) blood. IL-6, IL-1β and KC levels in lungs (**G**) and IL-6 levels in plasma (**H**) were quantified by ELISA. Data are presented as mean ± SEM (*n*=8/group) and are representative of two independent experiments. */ **/*** indicate $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ compared to WT using t test (**A**,**B**,**C**) or compared to WT-WT using ANOVA (**E**,**G**,**H**).

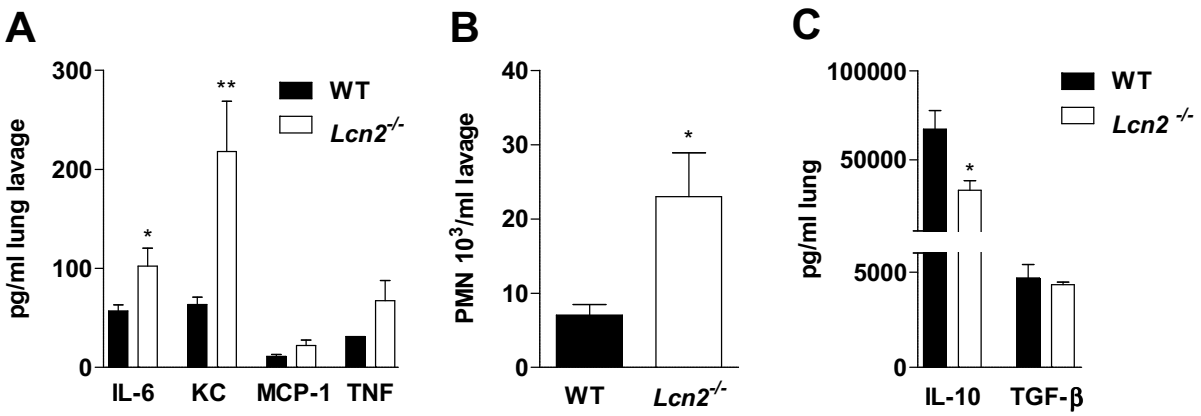

Figure 5: LCN2 dampens the early immune response

(A-C) WT and LCN2-/- mice were intranasally infected with 3x104 CFU S. pneumoniae for 6h. Secretion of IL-6, KC, MCP-1, TNF in BAL-fluid (A) and IL-10 and TGFβ in lungs (C) was quantified by ELISA.

(B) Neutrophil influx was determined by differential cell counts of cytospin preparations. Data are presented as mean ± SEM (n=8/group) and are representative of two independent experiments. */ ** indicate $P < 0.05$/ $p < 0.001$ compared to WT (t test).

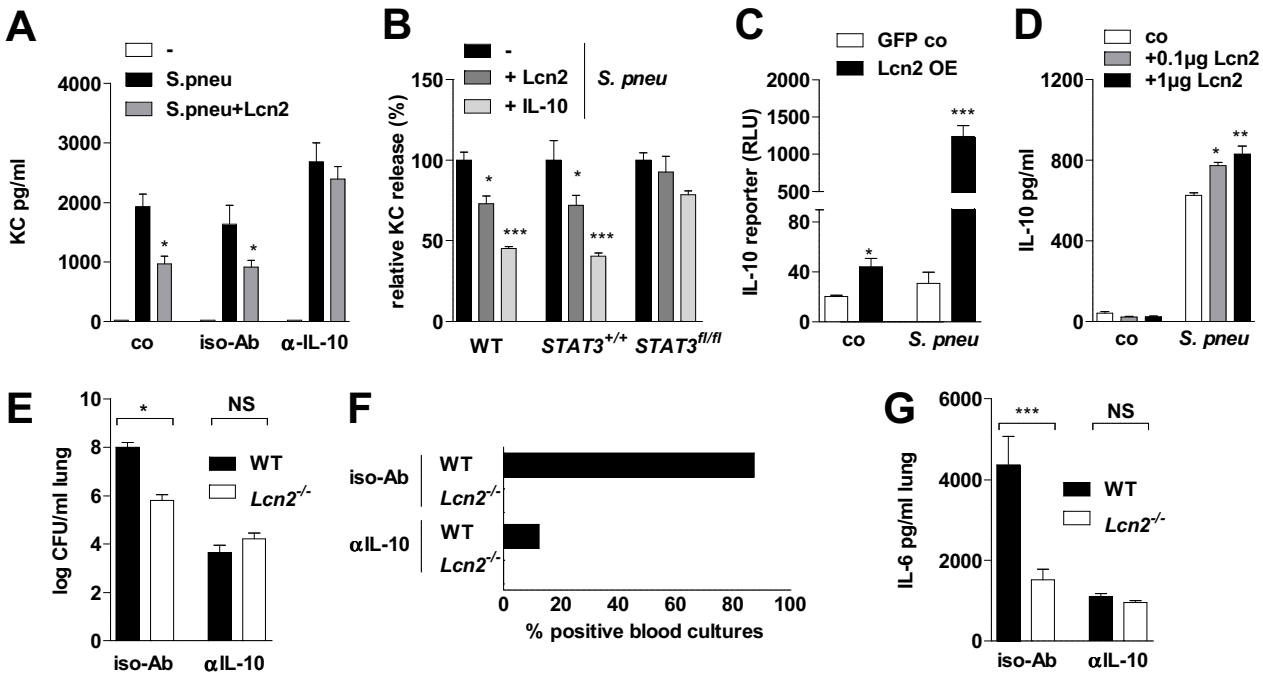

Figure 6: LCN2 impairs bacterial clearance in an IL-10-dependent manner

(A) KC levels in supernatants of WT BMDM stimulated with 4x107 CFU/ml S. pneumoniae ± 100ng/ml LCN2 ± 10µg/ml anti-IL-10 or isotype control antibody for 6h. (B) Normalized KC secretion from WT, STAT3+/+,Cre/Cre, and STAT3-/- (STAT3flox/flox,Cre/Cre) BMDM treated with 4x107 CFU/ml S. pneumoniae plus LCN2 (100ng/ml) or IL-10 (10ng/ml) for 6h. (C) RAW264.7 overexpressing LCN2 or GFP, transfected with an IL-10 reporter and stimulated with 4x107 CFU/ml S. pneumoniae for 24h. Reporter gene activity was measured in cell lysates and normalized to renilla. (D) WT BMDM were treated with 4x107 CFU/ml S. pneumoniae plus LCN2 (0.1µg/ml and 1µg/ml) for 6h. IL-10 release was measured in supernatants by ELISA. (E-G) WT and LCN2-/- mice were intranasally infected with 105 CFU S. pneumoniae, followed by intranasal administration of 150µg anti-IL-10 mAb or isotype control Ab. Bacterial counts in lung (E) and blood (F) were quantified 48h after infection. (G) IL-6 secretions in lung homogenates were determined by ELISA. Data are presented as mean ± SEM (n=4 (A-D); n=8/group (E-G)). NS, not significant; */ **/ *** indicates $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ compared to S. pneumoniae alone (A,B,D) (ANOVA), GFP control (C) (t test) or WT (E, G) ANOVA.

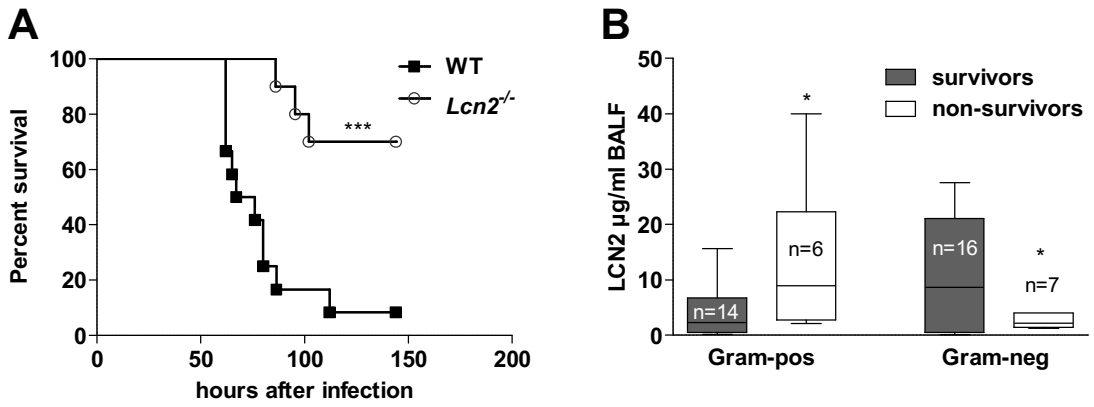

Figure 7: LCN2 is detrimental during pneumococcal pneumonia in mice and humans

(A) WT and LCN2-/- mice were infected with 4x104 CFU S. pneumoniae and monitored for survival (n=12 per group). *** indicates p < 0.0001 versus WT (log-rank). (B) LCN2 levels in BAL-fluid of ICU patients with microbiologically confirmed bacterial pneumonia were analyzed according to the causative pathogen: Gram positive (n=20) or Gram negative pneumonia (n=23). Data are expressed as box (median, 25% and 75% percentile) and whiskers (bar indicates min and max) blot. * indicates P < 0.05 versus survivors (t test).

Supplemental Information

Lipocalin-2 deactivates macrophages and worsens pneumococcal pneumonia outcomes

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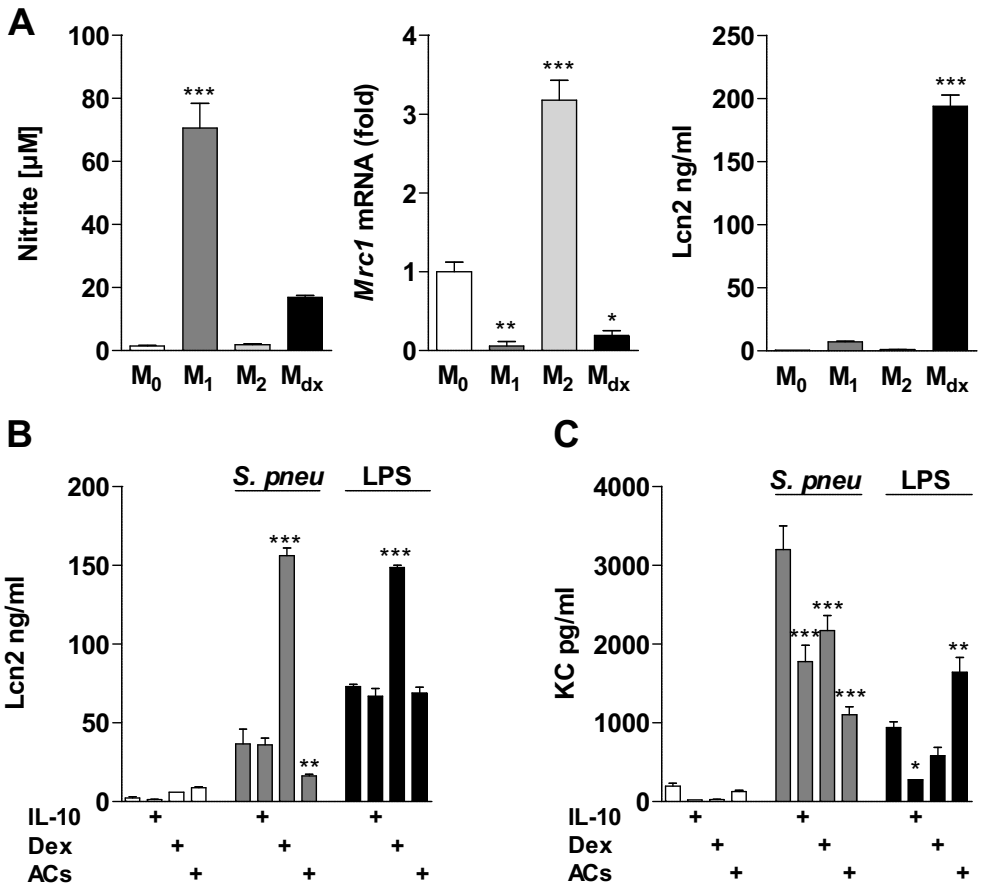
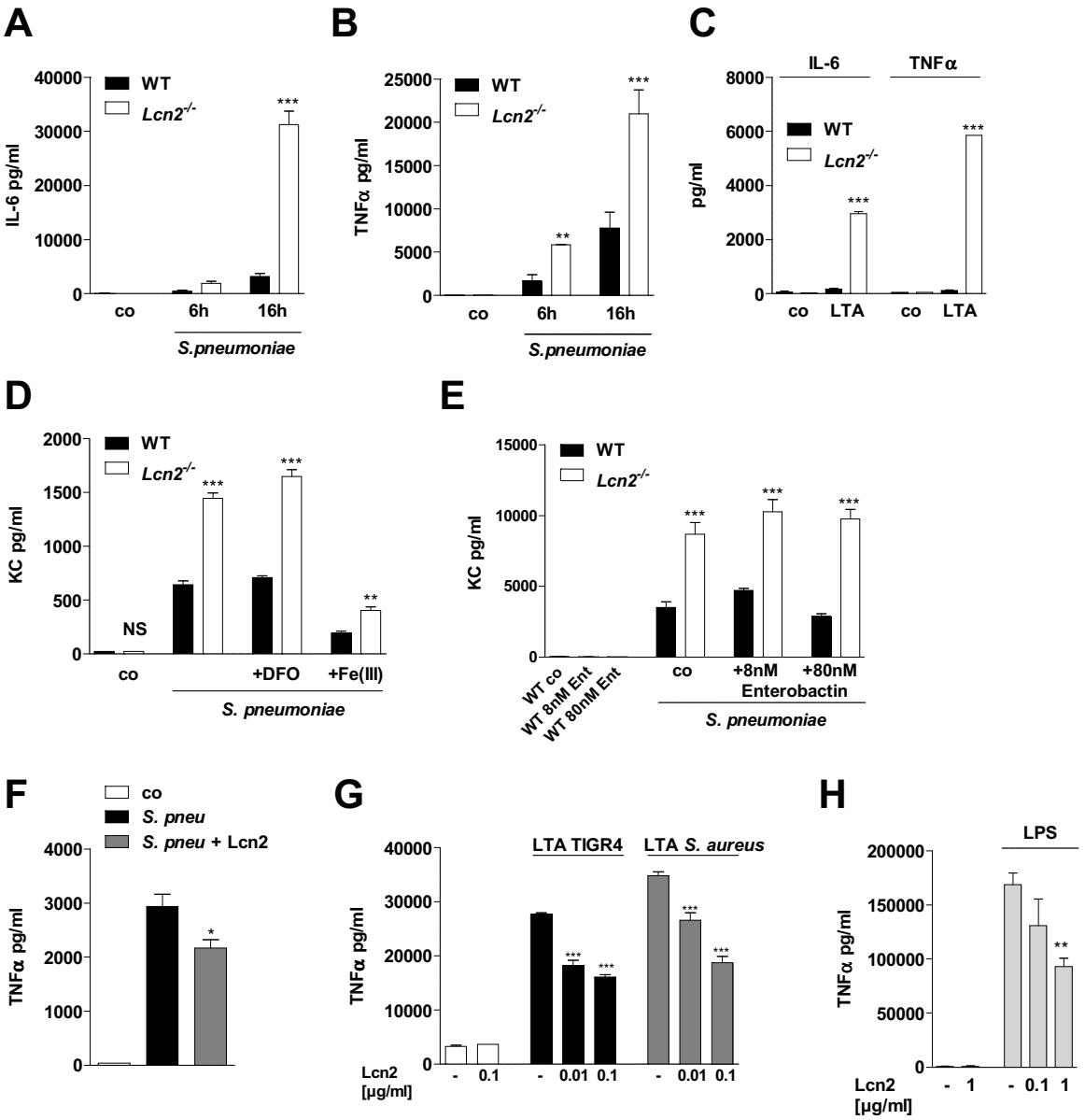


Supplemental Figure 1: LCN2 is highly expressed in deactivated macrophages

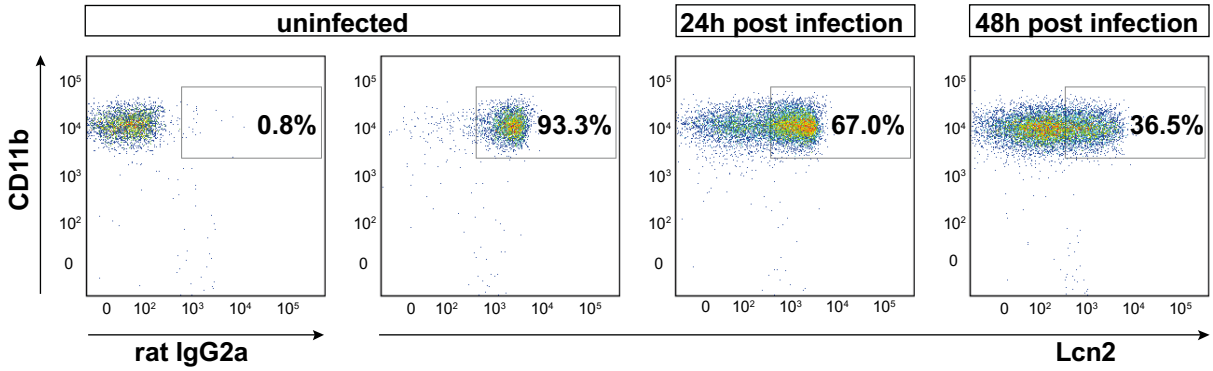
(A) MH-S cells were polarized with M1 (100ng/ml LPS, 200U/ml IFNγ), M2 (10ng/ml IL-13, 10ng/ml IL-4) or deactivating (Mdx; 10ng/ml LPS, 100µM dexamethasone) stimuli for 24h. Nitrite concentration was measured in supernatants and Mrc1 expression was determined by qRT-PCR. LCN2 release was assessed in supernatants by ELISA. The mRNA expression data were normalized to HPRT and expressed as a fold change compared to M0 control cells. (B, C) BMDM were treated with 4x107 CFU/ml S. pneumoniae (grey bars) or 10ng/ml LPS (black bars) alone or in combination with indicated deactivating stimuli (10ng/ml IL-10, 100µM dexamethasone (Dex), or 107/ml apoptotic cells (ACs)) for 16h. Secretions of LCN2 (B) and KC (C) were assessed in supernatants by ELISA. Data are presented as mean ± SEM of triplicate (A) or quadruplicate (B, C) data and representative of at least two independent experiments. */ **/ *** indicate $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ versus M0 (A) or versus respective conditions without deactivating stimuli (B, C) (ANOVA).



Supplemental Figure 2: LCN2 deactivates macrophages

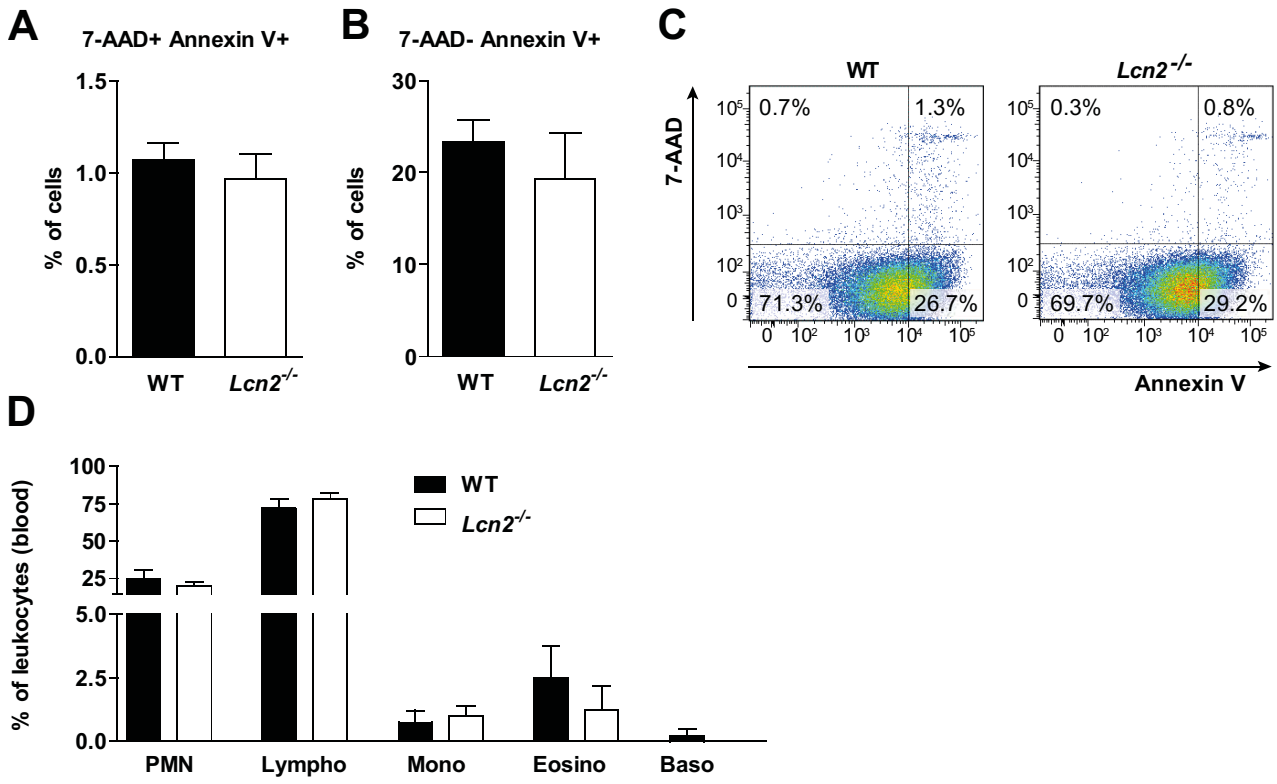
(A-C) WT (black bars) and LCN2-/- (white bars) BMDM were stimulated with 4x107 CFU/ml S. pneumoniae for 6 and 16h (A,B) or 10µg/ml LTA from S. aureus for 16h (C). IL-6 (A,C) and TNFα (B,C) were measured in supernatants by ELISA. (D, E) BMDM from WT (black bars) and LCN2-/- mice (white bars) were exposed to 4x107 CFU/ml S. pneumoniae in the presence or absence of (D) 50µM deferoxamine (DFO) or 50µM Fe (III) ammonium citrate for 6h or (E) 8-80nM enterobactin for 16h. KC was assessed in supernatants by ELISA. (F) Primary WT AM were treated with 4x107 CFU/ml S.pneumoniae with or without 100ng/ml LCN2 for 16h. TNFα levels were quantified in supernatants by ELISA. (G, H) MH-S cells were stimulated with 10µg/ml LTA from S. pneumoniae (TIGR4), 10µg/ml LTA from S. aureus (G) or 1µg/ml LPS (H) with or without increasing doses of LCN2 (0.01µg/ml-0.1µg/ml (G); 0.1-1µg/ml (H)). TNFα

was measured after 16h by ELISA. All data are presented as mean ± SEM of quadruplicates and representative of two independent experiments. NS, not significant; */ **/ *** indicate P < 0.05/ P < 0.001/ P < 0.0001 compared to respective WT condition (A-E) (ANOVA), S. pneumoniae (F) (t test), or respective condition without addition of LCN2 (G, H) (ANOVA).



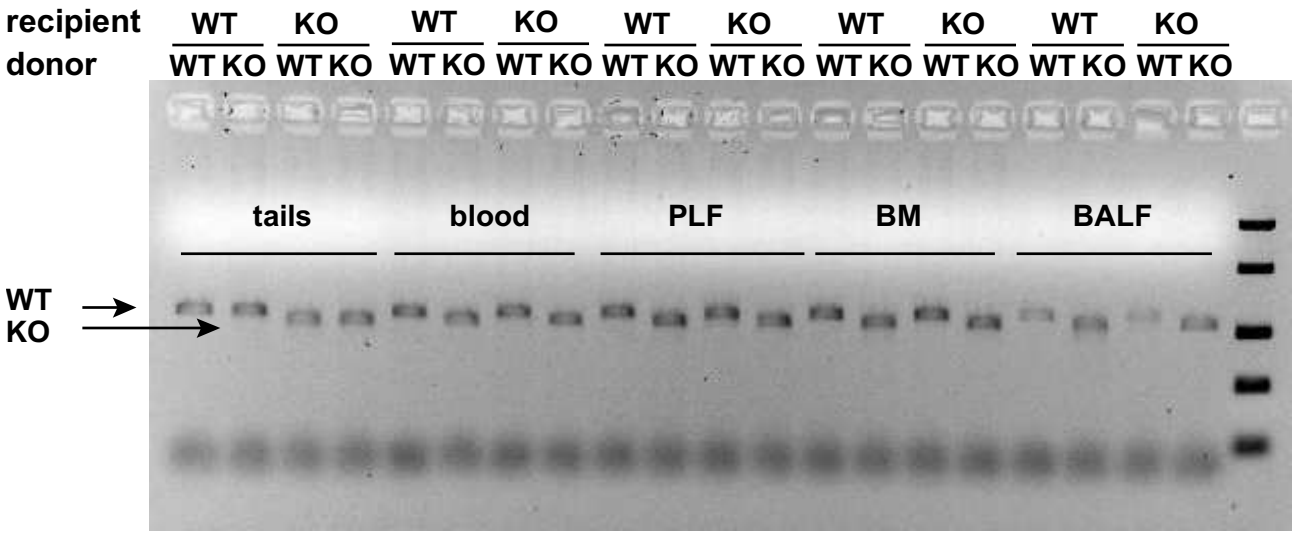
Supplemental Figure 3: Neutrophils release LCN2 during pneumococcal pneumonia

WT mice were infected with 105 CFU S. pneumoniae. CD45+ Ly6G+ CD11b+ cells were analyzed by flow cytometry for intracellular LCN2 expression.



Supplemental Figure 4: Apoptosis and a-priori white blood cell counts are similar in WT and LCN2-/- mice

(A-C) WT and LCN2-/- mice were infected with 105 CFU S. pneumoniae for 12h. Bronchoalveolar lavage was performed and isolated cells were stained with Annexin-V and 7-AAD. The percentage of 7-AAD and Annexin-V double positive or Annexin-V single positive cells is shown in (A) or (B), respectively. Representative dot plots of WT (left) and LCN2-/- (right) lung cells are shown in (C). Data are expressed as mean ± SEM of quadruplicates and representative of two independent experiments. (D) Differential cell counts of blood leukocytes from healthy WT and LCN2-/- mice. Data are expressed as mean ± SEM of quadruplicates. PMN – polymorphonuclear cells; Lympho – lymphocytes; Mono – monocytes; Eosino – eosinophils; Baso – basophils.



Supplemental Figure 5: Reconstitution of bone marrow chimeric mice

WT and KO recipient mice were lethally irradiated and immediately reconstituted with bone marrow from either syngeneic (WT-WT and KO-KO chimeras) or allogeneic donors (WT-KO and KO-WT chimeras). Three months after transplantation, one mouse per group was sacrificed and genomic DNA was isolated from blood cells, peritoneal macrophages (by peritoneal lavage; PLF), bone marrow (BM), alveolar macrophages (by bronchoalveolar lavage (BALF)) and genotyped by PCR. Tail biopsies were tested to ensure proper recipient status. WT-specific PCR products: 471bp, KO specific PCR products: ~450bp.

Supplemental Table 1:

Group	Pathogen	n
Gram-positive; survivors	*Streptococcus pneumoniae*	6
	Staphylococcus aureus	4
	Enterococcus faecium	2
	β-haemolysing *streptoccoci*	2
Gram-positive; non-survivors	*Enterococcus faecalis*	4
	Staphylococcus aureus	2
Gram-negative; survivors	*Escherichia coli*	5
	Klebsiella spp.	5
	Citrobacter freundii	2
	Proteus mirabilis	2
	Haemophilus influenzae	2
Gram-negative; non-survivors	*Acinetobacter baumanni*	2
	Pseudomonas aeruginosa	2
	Legionella pneumophila	2
	Enterobacter cloacae	1

Supplemental Table 2: Primer sequences

Primer name	Full name	Sequence (sense, antisense)
mHPRT	hypoxanthine guanine phosphoribosyl transferase 1	5‘-GTTAAGCAGTACAGCCCCAAAATG-3‘, 5‘-AAATCCAACAAAGTCTGGCCTGTA-3‘
mIL10	interleukin 10	5‘-TGAGGCGCTGTCATCGATTT-3‘, 5‘-CATGGCCTTGTAGACACCTT-3‘
mIL6	interleukin 6	5‘-CCACGGCCTTCCCTACTTCA-3‘, 5‘-TGCAAGTGCATCGTTGTTC-3‘
mKC	chemokine (C-X-C motif) ligand 1	5‘-GACCATGGCTGGGATTCACC-3‘, 5‘-TCAGAAGCCAGCGTTCACCA-3‘
mLCN2	lipocalin 2	5‘-CCTCCATCCTGGTCAGGGAC-3‘, 5‘-TAGTCCGTGGTGGCCACTTG-3‘
mMrc1	mannose receptor, C type 1	5‘-TCTGGGCCATGAGGCTTCTC-3‘, 5‘-CACGCAGCGCTTGTGATCTT-3‘

We generated LCN2 or GFP expression plasmids by Gateway cloning after adding N- and C-terminal att-sites via PCR. We used pDONRTM201 as a shuttle vector. The destination vector was pCMV StrepIIIHA GW.

Primer name	Sequence (sense, antisense)
attB1 GFP	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGACTGCCATGGTGAGCAAGGGC-3’, 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTTGTACAGCTCGTCCAT-3’
attB1 LCN2	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGACTGCCATGGCCCTGAGTGTC-3’, 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTGTCAATGCATTGGTC-3’

4. FINAL DISCUSSION

We found LCN2 expressed in healthy lungs and highly up-regulated in the pulmonary compartment upon infection in mice and humans. This finding raised the fundamental question: what is the role of LCN2 in the lungs in steady-state and during infection? We subsequently performed a number of experiments and discovered that LCN2 up-regulates IL-10 and deactivates macrophages. Thus, it is possible that LCN2 is part of the immunological “brake”, which counteracts the immune response to low number of less virulent pathogens based on the absence of LCN2 sensitive enterobactin-like siderophores. In this hypothetical model, siderophores might alert the immune system to the presence of more virulent bacteria. In the presence of siderophore producing i.e. more virulent bacteria LCN2 might act proinflammatory in order to support the immune response and host defense (Bachman et al, 2009). On the other hand LCN2 was reported to have pro-regenerative and proliferative effects on kidney tubular epithelia (Jung et al, 2012b; Schmidt-Ott et al, 2007), so one might speculate that LCN2 also contributes to tissue repair and regeneration in the lungs at steady state and during the resolution of infection. The fact that LCN2-deficient mice do not show any abnormalities in the resolution process doesn’t preclude this role. However, if LCN2 indeed promotes regeneration in the lung it seems to play a redundant role at least at steady state and during pneumococcal pneumonia. It is possible however that the pro-regenerative effects of LCN2 are important in other models such as influenza pneumonia, where the epithelial damage due to a direct cytopathic effect of the virus or secondary to inflammation occurs (Mauad et al, 2010; Oda et al, 1989; van der Sluijs et al, 2004).

Inhalative glucocorticoids are an integral component of therapy in advanced chronic pulmonary obstructive disease (COPD) and asthma. Interestingly, these particular patient groups are at increased risk of developing pneumococcal pneumonia (Sibila et al, 2013; Talbot et al, 2005). The exact mechanism of this enhanced susceptibility is not known. LCN2 was shown to be increased in induced sputum (Keatings & Barnes, 1997) and plasma (Eagan et al, 2010) of patients with COPD and asthma. Since we observed increased LCN2 secretion in patients receiving inhalative glucocorticoids, we speculate that inhalative glucocorticoids are at least partially responsible for elevated LCN2 levels in COPD and asthma patients. Further, increased LCN2 levels

might contribute to enhanced susceptibility to pneumococcal pneumonia in these patients. An interesting, not yet investigated, aspect is the effect of cigarette smoking on pulmonary LCN2 levels. This would be interesting as smoking was shown to reprogram alveolar macrophages to M2 phenotype (Shaykhiev et al, 2009) and smokers are at increased risk of developing CAP (Torres et al, 2013).

We found LCN2 to promote the deactivation of murine macrophages both in vitro and in vivo. Since inhalative glucocorticoids (potent deactivating agents) increased LCN2 in humans, we speculate that LCN2 also deactivates human macrophages. The LCN2 mediated deactivation is IL-10 and STAT3 dependent, but the exact molecular mechanism remains elusive. Thus, it is unclear how LCN2 leads to IL-10 expression. We found that LCN2 increased IL-10 transcription in macrophages but how this precisely works is unclear. We excluded that LCN2 mediated IL-10 up-regulation through shifts of intracellular iron. One possibility is that LCN2 acts directly as a transcription factor. This is however unlikely as LCN2 is not reported to localize in the nucleus or bind to DNA as it would be expected from a protein with transcription factor activity. Alternatively LCN2 could act indirectly through activation/inhibition of pathways which affect IL-10 transcription. This would imply that LCN2 e.g. activate or inhibit a receptor. To date two receptors have been reported to bind LCN2: solute carrier family 22 member 17 (Slc22a17, also known as brain type organic cation transporter (BOCT)) and low-density lipoprotein receptor-related protein 2 (LRP2, also known as megalin or glycoprotein (gp) 330) (Miharada et al, 2008). Miharada and colleagues showed the expression of both receptors in monocytes and macrophages (Miharada et al, 2008). Slc22a17 is a transmembrane endocytic receptor without any known signaling function. It was shown by Devireddy and colleagues to bind LCN2 and mediate its uptake (Devireddy et al, 2005). LRP2 is a big transmembrane protein with structural homology to LDL family members. It is also an endocytic receptor shown to bind LCN2 with high affinity (Hvidberg et al, 2005). However, megalin is a very pleiotropic receptor with multiple ligands e.g. albumin, lipoprotein lipase, lactoferrin, hormones/ hormone receptors or vitamins/ vitamine receptors (Marzolo & Farfan, 2011). LRP2 is constitutively phosphorylated by GSK3 and has also phosphorylation sites for other kinases such as protein kinase A and C (PKA and PKC) and Ca2+/calmodulin-dependent protein kinase II (CaMKII)(Marzolo & Farfan, 2011) in the cytoplasmic region. The cytoplasmic tail of megalin contains a PDZ-binding motif

and proline-rich sequences reported to interact with signaling proteins, e.g. synectin, megalin-binding protein, SKI-interacting protein (SKIP), Disabled 2 (Dab2) and adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1 (APPL1) (Marzolo & Farfan, 2011). Thus, it is possible that LRP2 regulates signaling pathways and is involved in LCN2 effects on IL-10 expression.

LCN2 (our data) and IL-10 (van der Poll et al, 1996) are both detrimental during primary pneumococcal pneumonia. The question remains if the effects of LCN2 are restricted to pneumococcal pneumonia or can be extended to lung infections caused by other siderophore-independent pathogens such as *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Teixeira and colleagues found that higher bacterial burden in the lungs of mice infected with *S. aureus* correlated with higher IL-10 levels indicating a negative role of IL-10 in this model (Teixeira et al, 2008). Since IL-10 induces LCN2 and vice-versa and the effects of both factors partially depend on one another (Jung et al, 2012b; Warszawska et al), this finding points towards a negative role for LCN2 during staphylococcal infection. Further, LCN2 could potentially affect the course of the secondary pneumonia after initial viral lung infection. Given that IL-10 plays a harmful role during secondary pneumococcal pneumonia after influenza infection (Sun et al, 2009; van der Sluijs et al, 2004), we expect a similar role for LCN2.

Our findings of a detrimental role for LCN2 during severe pneumococcal pneumonia might have implications for clinical practice. One possible implementation of this knowledge could be an attempt to block LCN2 with e.g. antibodies (Nairz et al, 2009) in order to improve patients' outcome. LCN2 is a small protein, which implies that the development of small molecules inhibiting LCN2 function or binding to the receptor, as an alternative to blocking antibodies, might be feasible. Another possibility is the pharmacological modulation of endogenous LCN2 secretion. The LCN2 promoter has binding sites for several transcription factors, among others NFκB, Foxo3a (AKT target), cAMP responsive element binding protein (CREB) or C/EBP β (CCAAT/enhancer binding protein) (Park et al, 2009). Thus, it is highly likely that there are drugs/compounds other than glucocorticoids and IL-10, which modulate LCN2 expression. If we consider our findings, we should use inhalative glucocorticoids in patients with severe pneumonia caused by siderophore independent pathogens with caution. Finally, LCN2 could be used as a biomarker in patients with pneumonia to identify those at risk for progression.

We investigated the effects of LCN2 on the course of pneumococcal pneumonia. In this model the infection is limited to the pulmonary compartment. The successful clearance of pathogens, and in consequence survival, relies on the strong initial immune response. Therefore anti-inflammatory actions of LCN2 are undesirable. However, if we would apply another route of infection e.g. intravenous, leading to a systemic infection with systemic inflammatory response, the anti-inflammatory effects of LCN2 might prove beneficial. A recently published study by Srinivasan et al., showing a protective role for LCN2 during endotoxemia, supports this hypothesis (Srinivasan et al, 2012a).

Concluding, we discovered an important regulator of innate immunity within the pulmonary compartment during bacterial infection. Considering the importance of a tightly regulated immune response within the lungs, LCN2 might serve as an important regulator that assists in the preservation of tissue homeostasis. Its detrimental role during pneumococcal pneumonia calls for its use as a biomarker – and even inspires thinking about its potential role as a therapeutic target to improve immunity during the acute phase of disease.

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Zhang X, Goncalves R, Mosser DM (2008b) The isolation and characterization of murine macrophages. *Curr Protoc Immunol* **Chapter 14:** Unit 14 11

Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R (2009) Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* **31:** 761-771

CURRICULUM VITAE

Personal information

Name	Joanna Maria Warszawska
Date of birth	23.05.1979
Place of birth	Krosno, Poland
Nationality	Polish

Education

10.2008 – 11.2012	PhD studies at the Medical University of Vienna, Ce-M-M- PhD program (Center for Molecular Medicine of the Austrian Academy of Sciences). Supervisor: Prof. Dr. PhD Sylvia Knapp
10.2000 – 06.2005	Medical studies at the Medical University of Vienna, Graduation June 2005 (Dr. med. univ.)
10.1998 – 06.2000	Medical studies at the Jagiellonian University in Cracow (Collegium Medicum UJ), Poland
09.1994 – 05.1998	Secondary School in Krosno, Poland; GCE-A levels 05.06.1998

Professional experience

Since 08.2013	Residency in Anesthesiology and Intensive Medicine at the Dept. of Anesthesia, General Intensive Care and Pain Management, Division of Cardiothoracic and Vascular Anesthesia, Medical University Vienna
02.2013 – 07.2013	Residency in General Medicine, Krankenhaus Hietzing; Vienna
08.2006 – 09.2008	Residency in Internal Medicine at the Dept. of Medicine 3, medical Intensive Care Unit, Medical University Vienna
08.2005 – 07.2006	

	Research assistant at the Dept. of Medicine 3, Division of Gastroenterology and Hepatology, Medical University Vienna

Postgradual trainings

10.2010 – 10.2011	Curriculum “Schrittweise”: Mentoring program for young women in science; Medical University Vienna
2006-2010	Biomedical statistics I,II Design, Analysis and Interpretation of Clinical Studies Publication workshop Medical English I: Scientific Writing Touch typing workshop Giving a good talk/lecture (Microteaching)
2006-2008	Various trainings in medical intensive care such as Center of Excellence of Medical Intensive Care (CEMIC) lecture series, mechanical ventilation & weaning from mechanical ventilation; antibiotic therapy workshops

Grants

2006	Österreichische Nationalbank and Hochschuljubiläumsstiftung grants for the project: „Is depression a risk factor for mortality in medical ICU patients?” Principal Investigator: Wolfgang Miehsler; Contribution: drafted proposal, research associate
2007	Bürgermeisterfonds grant for the project: „ Soluble Haemoglobin Scavenger Receptor (sCD163) in Bronchoalveolar Lavage Fluid of Patients with Suspected Nosocomial Pneumonia.” Project number: 07100; Principal Investigators: Peter Schenk & Sylvia Knapp; Contribution: conception and wrote main parts of proposal. Lead scientist.

Peer-reviewed Publications

1. Köffel R, Meshcheryakova A, **Warszawska J**, Hennig A, Wagner K, Jörgl A, Gubi D, Moser D, Hladik A, Hoffmann U, Fischer MB, van den Berg W, Koenders M, Scheinecker C, Gesslbauer B, Knapp S, Strobl H. Monocytic cell differentiation from band-stage neutrophils under inflammatory conditions via MKK6 activation.
   Blood. 2014 Sep 11. pii: blood-2014-07-588178. [Epub ahead of print]

2. Sharif O, Gawish R, **Warszawska JM**, Martins R, Lakovits K, Hladik A, Doninger B, Brunner J, Korosec A, Schwarzenbacher RE, Berg T, Kralovics R, Colinge J, Mesteri I, Gilfillan S, Salmaggi A, Verschoor A, Colonna M, Knapp S. The Triggering Receptor Expressed on Myeloid Cells 2 Inhibits Complement Component 1q Effector Mechanisms and Exerts Detrimental Effects during Pneumococcal Pneumonia.
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3. Brunner R, Rinner W, Haberler C, Kitzberger R, Sycha T, Herkner H, **Warszawska J**, Madl C, Holzinger U. Early treatment with IgM-enriched intravenous immunoglobulin does not mitigate critical illness polyneuropathy and/or myopathy in patients with multiple organ failure and SIRS/sepsis: a prospective, randomized, placebo-controlled, double-blinded trial.
   Crit Care 17: R213 (2013)

4. **Warszawska J,** Gawish R, Sharif O, Sigel S, Doninger B, Lakovits K, Mesteri I, Nairz M, Boon L, Spiel A, Fuhrmann V, Strobl B, Müller M, Schenk P, Weiss G, Knapp S. Lipocalin-2 deactivates macrophages and worsens pneumococcal pneumonia outcomes
   J Clin Invest. 123: 3363-3372 (2013)

5. Kneidinger N, **Warszawska J**, Schenk P, Fuhrmann V, Bojic A, Hirschl A, Herkner H, Madl C, Makristathis A. Storage of bronchoalveolar lavage fluid and accuracy of microbiological diagnostics in the ICU: a prospective observational study.
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6. Schenk P, **Warszawska J,** Fuhrmann V, König F, Madl C, Ratheiser K. Health-related quality of life of long-term survivors of intensive care: changes after intensive care treatment: Experience of an Austrian intensive care unit.
   Wien Klin Wochenschr. 124:624-632 (2012)

7. Schabbauer G, Matt U, Günzl P, **Warszawska J,** Furtner T, Hainzl E, Elbau I, Mesteri I, Doninger B, Binder BR, Knapp S. Myeloid PTEN promotes inflammation but impairs bactericidal activities during murine pneumococcal pneumonia.
   J Immunol. 185: 468-476 (2010).

8. Holzinger U, **Warszawska J,** Kitzberger R, Wewalka M, Miehsler W, Herkner H, Madl C. Real-time continuous glucose monitoring in critically ill patients: a prospective randomized trial.

Diabetes Care. 33: 467-472 (2010)

9. Matt U, **Warszawska JM,** Bauer M, Dietl W, Mesteri I, Doninger B, Haslinger I, Schabbauer G, Perkmann T, Binder CJ, Reingruber S, Petzelbauer P, Knapp S. Bbeta(15-42) protects against acid-induced acute lung injury and secondary pseudomonas pneumonia in vivo.
Am J Respir Crit Care Med. 180: 1208-1217 (2009)

10. Fuhrmann V, Kneidinger N, Herkner H, Heinz G, Nikfardjam M, Bojic A, Schellongowski P, Angermayr B, Kitzberger R, **Warszawska J**, Holzinger U, Schenk P, Madl C. Hypoxic hepatitis: underlying conditions and risk factors for mortality in critically ill patients.
Intensive Care Med. 35: 1397-1405 (2009)

11. Holzinger U, **Warszawska J,** Kitzberger R, Herkner H, Metnitz PG, Madl C. Impact of shock requiring norepinephrine on the accuracy and reliability of subcutaneous continuous glucose monitoring.
Intensive Care Med. 35: 1383-1389 (2009)

12. Madl C, Holzinger U, Kitzberger R, **Warszawska J**. Stress hyperglycemia--effect on morbidity and mortality in acute patients.
Wien Klin Wochenschr. 120: 387-389 (2008)

13. Fuhrmann V, Schenk P, Jaeger W, Miksits M, Kneidinger N, **Warszawska J,** Holzinger U, Kitzberger R, Thalhammer F. Pharmacokinetics of voriconazole during continuous venovenous haemodiafiltration.
J Antimicrob Chemother. 60: 1085-1090 (2007)

Conference Participation and Presentations:

November 2013	10th Medical Postgraduate Conference, Hradec Kralove, Czech Republic – oral presentation, 2nd prize
October 2013	Annual Meeting of the Austrian Society of Pneumology – oral presentation, award for the best scientific poster
December 2012	Viennese Symposium on Immunology, Infection biology and Inflammation (VI^3) – oral presentation
November 2012	Annual Meeting of the Austrian Society for Allergology and Immunology (ÖGAI), Vienna – oral presentation; best oral presentation award
June 2012	Young Scientist Association of the Medical University of Vienna (YSA) PhD Symposium 2012, Vienna – oral presentation

March 2012	Keystone Symposium: Innate Immunity: Sensing the Microbes and Damage Signals (Q7), Keystone, Colorado – poster presentation
September 2011	The EMBO meeting 2011, Vienna – poster presentation
May 2011	Toll 2011 Conference, Riva del Garda – poster presentation
December 2010	14th Scientific Meeting of the European Society of Chemotherapy / Infectious Diseases (ESCID), Vienna – oral presentation
December 2010	Annual Meeting of the Austrian Society for Allergology and Immunology (ÖGAI), Vienna – oral presentation
October 2010	BioMed Conference „Macrophages and inflammation", Barcelona – poster presentation
September 2010	Joint FEBS-EFIS workshop „Inflammatory Diseases and Immune Response: Basic Aspects, Novel Approaches and Experimental Models", Vienna – poster presentation
June 2010	Young Scientist Association of the Medical University of Vienna (YSA) PhD Symposium 2010, Vienna – oral presentation
May 2010	4. Österreichischer Infektionskongress (ÖIK), Saalfelden – oral presentation
April 2010	20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna – oral presentation