The role of triggering receptor expressed on myeloid cells-2 in inflammation

Doctoral Thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy (PhD)

submitted by

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Table of contents

Declaration

The doctoral candidate, Riem Gawish, conducted her PhD studies under the supervision of 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.

For the publication "Triggering receptor expressed on myeloid cells-2 fine-tunes inflammatory responses in murine Gram-negative sepsis" included in chapter 2 of this thesis, Riem Gawish performed the majority of experiments, analyzed the data and wrote the manuscript. Sylvia Knapp funded and conceived the project, supervised the work and wrote the manuscript. Rui Martins and Omar Sharif assisted with experimental work and data analysis. Benedikta Böhm and Terije Antari Wimberger assisted with experiments regarding the expression of TREM-2 on macrophages upon LPS challenge for their master thesis under the co-supervision by Riem Gawish and Sylvia Knapp. Karin Stich provided technical help and Mariane Schmidt generated GFP-expressing *Escherichia coli*.

Omar Sharif performed the crucial experiments leading to the publication "The triggering receptor expressed on myeloid cells 2 inhibits complement component C1q effector mechanisms and exerts detrimental effect during pneumococcal pneumonia", which is also included in this thesis, and wrote the manuscript together with Sylvia Knapp. Riem Gawish substantially contributed to the experimental work. She performed the confocal-based studies that investigated phagocytosis by AMs, generated the TREM-2 overexpressing RAW 267.4 cell-line and helped with the analysis of the microarray study. All other co-authors assisted with experiments or provided experimental tools.

List of Figures

Introduction

Published Figures

Triggering receptor expressed on myeloid cells-2 fine-tunes inflammatory responses in murine Gram-negative sepsis

The triggering receptor expressed on myeloid cells 2 inhibits complement component 1q effector mechanisms and exerts detrimental effects during pneumococcal pneumonia

List of tables

Abstract

Toll-like receptor (TLR) mediated responses have to be tightly controlled in order to first promote the induction of inflammation and efficient clearance of bacteria, while at the same time limiting collateral damage. The recruitment of immune cells and subsequent phagocytosis of bacteria are crucial effector mechanisms during this process. The triggering receptor expressed on myeloid cells (TREM)-2 was shown to negatively regulate the induction of inflammatory cytokines upon TLR activation and has been identified as a phagocytic receptor for bacteria. Considering TREM-2's impact on these important host defense mechanisms, the hypothesis of this thesis was that TREM-2 would play an important role during bacterial infection, as well as during sterile inflammation. Using wild-type (WT) and TREM-2 knockout (TREM-$2^{-/-}$) mice the role of TREM-2 was investigated in primary macrophage populations, and in vivo during Gram-negative sepsis, endotoxemia and pneumococcal pneumonia.

This work shows that the contribution of TREM-2 to the regulation of inflammatory responses and the phagocytic capacity of macrophages strongly depends on the cell-type investigated. While in vitro TREM-2 suppressed TLR-induced cytokine responses and promoted phagocytosis by peritoneal macrophages (PM) and bone marrow-derived macrophages (BMM), it enhanced TLR responses and suppressed phagocytosis by alveolar macrophages (AM). This difference was attributed to the suppression of complement component 1q (C1q) by TREM-2 in AM. In vivo, TREM-2 consistently dampened early inflammation, whereas the effects on outcome strongly depended on the type of injury. During endotoxemia TREM-2 impaired survival by delaying the induction of the otherwise protective negative regulator A20. However, upon infections with viable bacteria, TREM-2's impact on bacterial phagocytosis overshadowed its effects on early inflammation. As such, TREM-2 improved the uptake of *E. coli*, which antagonized the otherwise harmful effect of a delayed early inflammatory response. Conversely, TREM-2 exhibited detrimental effects during pneumococcal pneumonia, by affecting local C1q production and phagocytosis by AM.

In summary, TREM-2 exerts cell-type specific effects, illustrating the importance of studying the role of TREM-2 in a context and disease specific manner.

Kurzfassung

Die Feinabstimmung von TLR-mediierten Immunreaktionen ist ausschlaggebend für die Induktion von Entzündung und die Beseitigung von Bakterien unter gleichzeitiger Vermeidung von entzündungsinduzierten Kollateralschäden. Die Rekrutierung von Immunzellen und darauf folgende Phagozytose ist dabei von höchster Bedeutung für die effiziente Elimination von Pathogenen.

Der „Triggering receptor expressed on myeloid cells (TREM)-2“ unterdrückt einerseits die TLR-mediierte Zytokin Produktion und wurde andererseits als Phagozytoserezeptor für Bakterien beschrieben. In Anbetracht des Einflusses von TREM-2 auf diese essentiellen Mechanismen der Immunantwort wurde die Grundhypothese für diese Arbeit formuliert, nämlich dass TREM-2 eine wichtige Rolle während bakterieller Infektionen sowie während steriler Entzündung spielen würde. Unter Verwendung von Wildtyp (WT) - und TREM-2 defizienten (TREM-$2^{-/-}$) Mäusen, wurde die Funktion von TREM-2 in verschiedenen Makrophagenpopulationen untersucht, sowie in vivo, während Gram-negativer Sepsis, Endotoxemie und Pneumokokken Pneumonie.

Diese Arbeit zeigt, dass der Einfluss von TREM-2 auf die TLR- induzierte Entzündung sowie auf die Phagozytose von Makrophagen stark zelltyp-spezifisch ist. Während TREM-2 in vitro in Peritonealmakrophagen (PM) und „bone marrow-derived“ Makrophagen (BMM) die Produktion von inflammatorischen Faktoren inhibiert und die Phagozytose verstärkt, zeigt er in Alveolarmakrophagen (AM) genau den gegenteiligen Effekt, nämlich eine Verstärkung der Zytokinantwort und eine Verringerung der Phagozytosekapazität. Die unerwarteten Effekte von TREM-2 in AMs konnten auf eine zelltyp-spezifische Unterdrückung der Expression von Komplement Komponente 1q (C1q) zurückgeführt werden.

In vivo, war die Rolle von TREM-2 komplex, da TREM-2 die Immunantwort auf mehreren Ebenen beeinflusst. Während TREM-2 in allen drei Fällen die frühe Entzündungsantwort unterdrückte, war sein Einfluss auf den späteren Verlauf stark abhängig vom jeweiligen Modell. Im Fall der sterilen Entzündung durch Lipopolysaccharid (LPS) war die TREM-2 vermittelte Verzögerung der frühen Entzündung von Nachteil für das Überleben von WT Mäusen, da damit auch eine verspätete Expression des protektiven Negativregulators A20 einherging. Im Vergleich zur Endotoxemie, war während Bakteriellen Infektionen der Einfluss von TREM-2 auf die Phagozytose von größerer Bedeutung und überschattete die Wirkung von TREM-2 auf die frühe Entzündung. Demnach verbesserte TREM-2 die

Phagozytose von *E. coli*, was der Verzögerung der frühen Entzündungsantwort entgegenwirkte. Im Gegensatz dazu, war die Phagozytose von Pneumokokken, durch die TREM-2-mediierte Unterdrückung der C1q Expression verschlechtert.

Zusammengefasst zeigen diese Daten, dass TREM-2 ein wichtiger Modulator der Immunantwort ist und für ein besseres Verständnis seiner Funktionen in einem Krankheits- und Zelltyp-spezifischen Kontext untersucht werden muss.

Publications arising from this thesis

Triggering receptor expressed on myeloid cells-2 fine-tunes inflammatory responses in murine Gram-negative sepsis

Riem Gawish, Rui Martins, Benedikta Böhm, Terje Wimberger, Omar Sharif, Karin Lakovits, Mariane Schmidt, Sylvia Knapp

The FASEB Journal, published online: December 2014, doi: 10.1096/fj.14-260067 fj.14-260067, Print: Vol 29, 2015

The triggering receptor expressed on myeloid cells 2 inhibits complement component 1q effector mechanisms and exerts detrimental effects during pneumococcal pneumonia

Omar Sharif, Riem Gawish, Joanna M. Warszawska, Rui Martins, Karin Lakovits, Anastasiya Hladik, Bianca Doninger, Julia Brunner, Ana Korosec, Roland E. Schwarzenbacher, Tiina Berg, Robert Kralovics, Jacques Colinge, Ildiko Mesteri, Susan Gilfillan, Andrea Salmaggi, Admar Verschoor, Marco Colonna, Sylvia Knapp

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Abbreviations (alphabetically ordered)

AM	alveolar macrophage
AnxA	annexin A1
ATF3	cyclic AMP-dependent transcription factor 3
BAL	bronchoalveolar lavage fluid
BMDC	bone marrow-derived dendritic cell
BMM	bone marrow-derived macrophage
C1q	complement component 1q
cAMP	cyclic Adenosine monophosphate
C. albicans	*Candida albicans*
CHO	chinese hamster ovary
CLP	cecal ligation and puncture
CNS	central nervous system
CRP	C-reactive protein
DAMP	danger-associated molecular pattern
DAP10	DNAX adaptor protein 10
DAP12	DNAX adaptor protein 12
DC	dendritic cell
DNA	deoxyribonucleic acid
DSS	dextran sodium sulfate
EAE	experimental autoimmune encephalitis
E. coli	*Escherichia coli*
ERK	Extracellular-signal regulated kinase
FACS	Fluorescence-activated cell sorting
GM-CSF	granulocyte/macrophage colony stimulating factor
GOLD	golgi dynamics domain
HDAC	histone deacetylase
IBD	inflammatory bowel disease
IFN	interferon

Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IKK	IκB kinase
IκB	inhibitor of kappa B protein
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KC	keratinocyte-derived chemokine (CXCL1)
KIR	killer-cell immunoglobulin like receptor
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTB4	leukotriene B4
MAC	membrane attack complex
MAIR-2	myeloid-associated immunoglobulin-like receptor-2
MAL	MyD88-adapter like
MBL	mannose binding lectin
MCP-1	monocyte chemoattractant protein-1
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
MyD88	myeloid differentiation primary response gene 88
NET	neutrophil extracellular trap
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cells	natural killer cells
NLR	NOD-like receptor
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
P. aeruginosa	*Pseudomonas aeruginosa*

PAMP	pathogen-associated molecular pattern
PDC	plasmacytoid dendritic cell
PDLIM2	PDZ and LIM domain protein 2
PGE2	prostaglandin E2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLF	peritoneal lavage fluid
PLOSL	polycystic lipomembranous osteodysplasia with sklerosing leukoencephalopathy
PM	peritoneal macrophage
PMN	polymorphonuclear leukocytes
PPR-δ	Peroxisome proliferator-activated receptor δ
PTP	phosphotyrosine phosphatase
RIG-I	retinoic acid-inducible gene 1
PRR	pattern recognition receptor
SHP-1/2	Src homology region 2 domain-containing phosphatase-1/2
RANK	Receptor Activator of NF-κB
RANKL	RANK ligand
RLR	RIG-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SH2/SH3	Src-homology domain 2/3
SHIP	SH2 domain-containing inositol 5'-phosphatase
SIRS	systemic inflammatory response syndrome
SOCS	suppressor of cytokine signaling
S. aureus	*Staphylococcus aureus*
S. pneumonia	*Streptococcus pneumonia*
Syk	spleen tyrosine kinase
TAG	TRAM adaptor with GOLD domain
TAK1	TGF-β activated kinase 1
TGF-β	transforming growth factor-β

TIR domain	Toll/interleukin-1 receptor homology domain
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TLT	TREM-like transcript
TNF-α	tumor necrosis factor-α
TRAM	TRIF-related adaptor molecule
TREM	triggering receptor expressed on myeloid cells
TREM-2$^{-/-}$	TREM-2 knockout
TRIF	TIR-domain-containing adaptor inducing IFN-β
WT	wild-type

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1. Introduction

In this chapter, basic mechanisms of innate immunity are summarized in order to provide the basic background information that is required to understand the context of this thesis. This will cover an overview about inflammatory reactions, important mediators and cell-types, as well as a short outline of the general mechanisms that are involved in regulating Toll-like receptor (TLR) signaling. This is followed by a review about the triggering receptor expressed on myeloid cells (TREM)-2 with a focus on the immunologic aspects of TREM-2 and on what is known about its role in inflammatory processes and infectious diseases.

1.1. Innate immunity and Inflammation

1.1.1. Definition and general concepts

The mammalian immune system is organized in different layers, one part being innate and the other part being adaptive immunity (Fig. 1). One part of innate immunity comprises of physical and anatomical barriers that prevent microorganisms, viruses or potentially dangerous substances to enter the body. Once a pathogen or a noxious stimulus has entered, innate immune cells are the first sensors of the injury and are crucial for triggering an inflammatory response that will in turn lead to clearance of the invader, activation of adaptive immunity and restoration of homeostasis (Fig. 1).

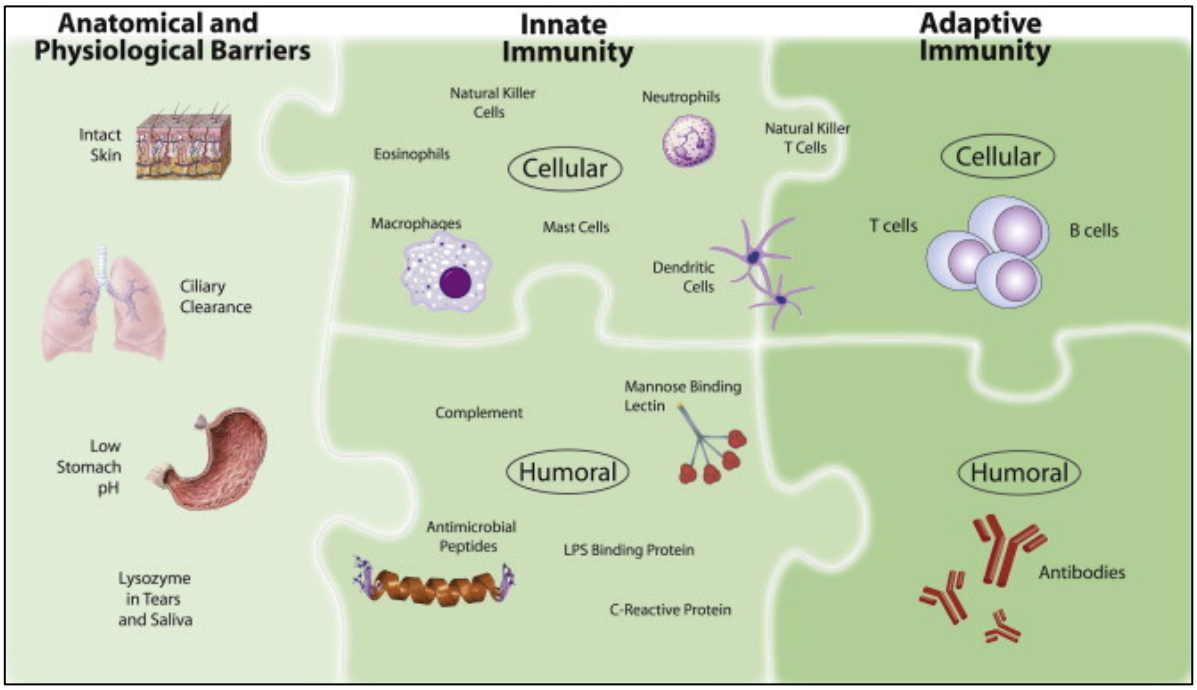

Figure 1: Integrated human immune system (1)

Innate immune responses are quick, and target evolutionary conserved structures. (1) Adaptive responses, in contrast, take days and are highly specific, as they require clonal expansion of T-cells and B-cells that target a specific epitope. In addition, only adaptive immune responses lead to the development of an immunologic memory (2).

Key players of innate immunity are, amongst others, macrophages, dendritic cells (DC), polymorphonuclear leukocytes (PMN, neutrophils), eosinophils, natural killer (NK)-cells and mastcells, all of them being critical in the first phase of an insult and involved in the induction of an inflammatory response (1, 3). Inflammation is known as part of a complex biological response by a cell or a multicellular organism to a potentially dangerous, infectious or sterile trigger, with the purpose to restore homeostasis. As such, inflammation is considered to be a protective response and an essential part of the healing process. Triggers can be pathogens, damaged cells or irritating substances (4). When any body surface is inflamed, the cardinal signs are *dolor* (pain), *calor* (heat), *rubor* (redness), *tumor* (swelling) and were described by Celsus (~ 25 BC – 50 BC). Later *function laesa* (loss of function) was added as a hallmark. While redness, heat and swelling are attributed to vasodilation and increased permeability of the endothelium and pain results from the release of inflammatory mediators that in turn stimulate nerve endings, the loss of function is more complex and can have multiple reasons. In contrast inflammation of internal organs may not cause the full set of the mentioned cardinal signs, for example, pain could only be perceived in the presence of sensory nerves in the inflamed area. (4, 5)

In the context of infections, inflammation is considered as an innate immune response, as it is generic, in contrast to adaptive responses that are specific for each pathogen. In an ideal situation inflammation is a tightly regulated, vital process, with the purpose to eliminate the trigger and restore homeostasis as fast and efficient as possible. However unresolved and/or uncontrolled inflammation can have a variety of pathological consequences including autoimmunity, sepsis, fibrosis and others (Fig. 2). (4, 6, 7)

In line with this, inflammation can be divided into three phases: initiation, acute phase and resolution phase. The initiation phase is mediated by innate immune cells that are residing in the involved tissue, recognize the stimulus via pattern recognition receptors (PRRs) and thereby are triggered to release inflammatory mediators, like cytokines and chemokines, resulting in activation of the endothelium (8). In the acute phase endothelial activation, involving vasodilation and increased vascular permeability, facilitates leukocytes to migrate and extravasate to the site of inflammation, which in turn further contributes to the production

of inflammatory mediators. On a systemic level acute inflammation induces high levels of acute-phase proteins, can cause fever, increases the blood pressure and often changes the number of leukocytes. (9) The acute phase is then followed by resolution, the phase when the system tries to restore homeostasis. Resolution involves the production of anti-inflammatory mediators, the removal of apoptotic material and tissue remodeling. Importantly, resolution is often not successful, resulting in ongoing acute inflammation or transition into chronic inflammation, both being pathologic conditions that can have fatal consequences (Fig. 2). (4, 10)

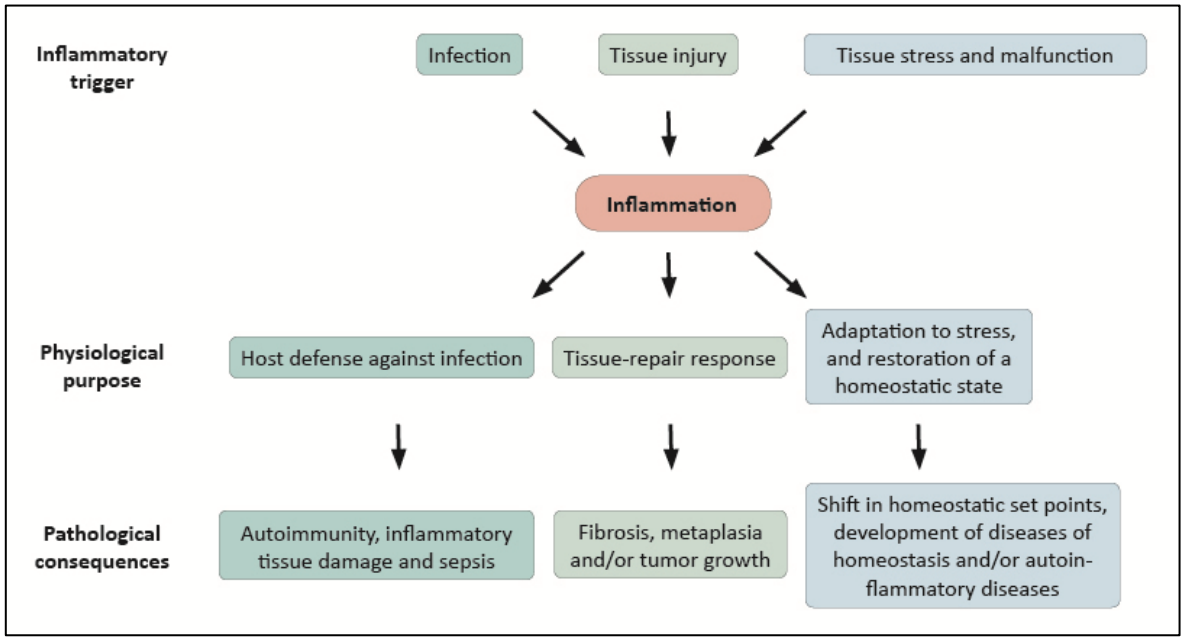

Figure 2: Causes, and physiological and pathological outcomes, of inflammation, modified from (4)

1.1.2. Phases of inflammation

Initiation – Activation of Pattern Recognitions Receptors:

Initially inflammation is elicited by tissue resident innate immune cells, like macrophages that are able to recognize a variety of pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) via germline-encoded PRRs that are expressed on their surface. PRRs bind evolutionary conserved patterns that are universal for certain groups of pathogens or are universal stress signals. Typical PAMPs are for example are lipopolysaccharide (LPS), the highly immunogenic cell wall component of gram-negative bacteria, or viral nucleic acids such as DNA or RNA. Today many different PRRs are known to be crucial for the innate immune system and they are classified according to their function, ligand specificity, localization and/or evolutionary relationship. Membrane bound PRRs

include the Toll-like receptor (TLR) family and the group of C-type lectin receptors. (8, 11-14) Cytoplasmic PRRs are the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the family of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (15, 16). Complement receptors, collectins or pentraxin proteins belong to the secreted PRRs (13, 14, 17). Of high importance for this manuscript are the TLRs, of which at least 13 members are known to exist in mammals, TLR1 to TLR10 in human and TLR1 to TLR9 and TLR11 to TLR13 in mice. (18, 19) TLRs belong to the type I integral membrane proteins are glycosylated and form homodimers, like TLR4-TLR4, or heterodimers like TLR2-TLR1 or TLR2-TLR6. Upon ligand binding they signal via their cytosolic Toll/IL-1 receptor-like (TIR) domain inducing the activation of nuclear factor κB (NFκB), the interferon (IFN) inducible factors (IRF) and other transcription factors, resulting in the expression of genes that mediate immunologic responses like inflammation, cell maturation and proliferation (Fig. 3). (18, 19)

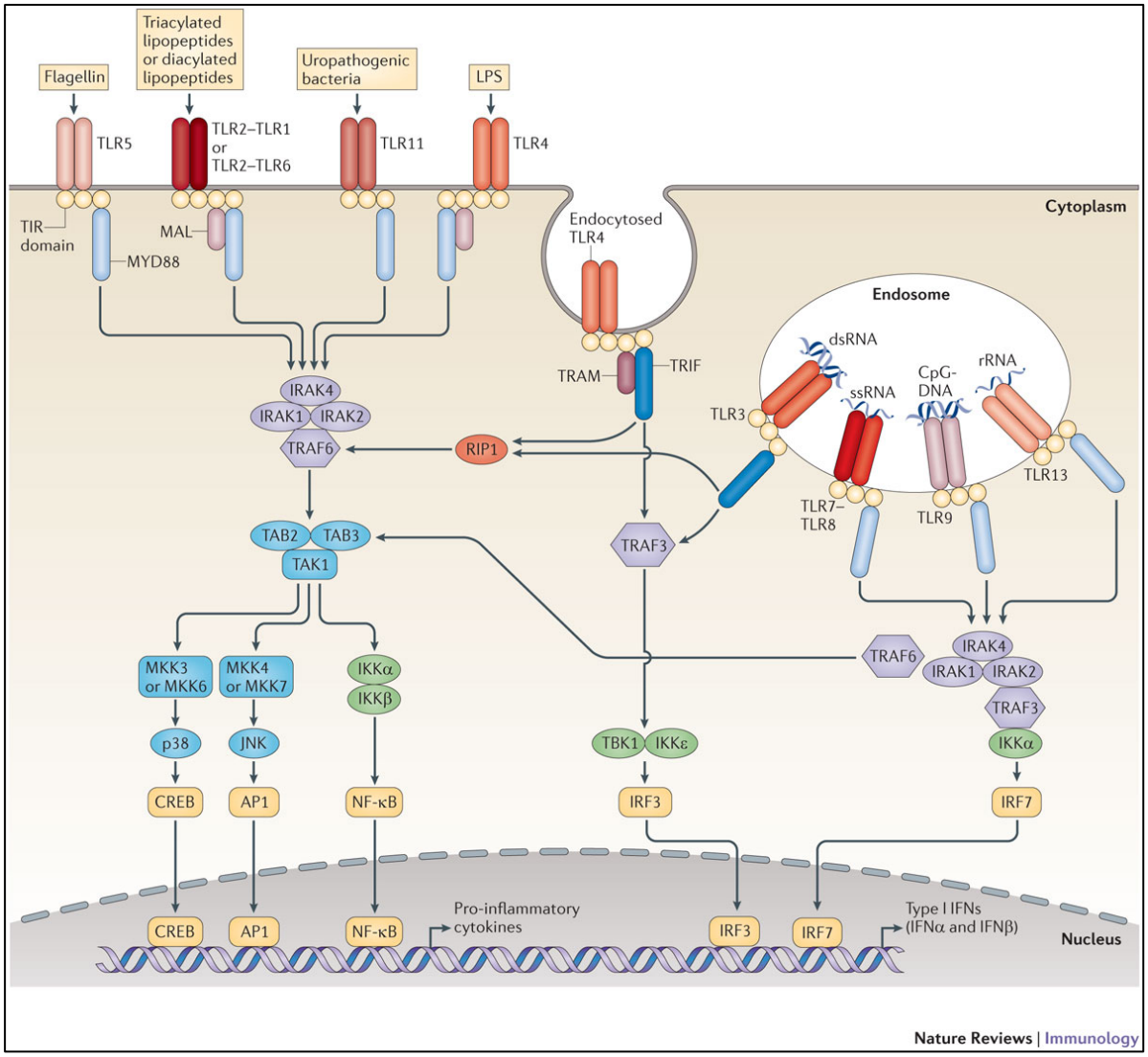

Figure 3: Mammalian TLR signaling pathways (18)

Nuclear factor 'κ-light-chain-enhancer' of activated B-cells (NFκB) proteins (or Rel transcription factors) are crucial for regulating innate and adaptive immune responses. In quiescent cells they are localized in the cytoplasm, associated to the so called inhibitor of NFκB (IκB) proteins. Upon activation of the TLR signaling cascade, IκB proteins get phosphorylated, ubiquitinated and degraded by the proteasome. As a consequence NFκB is released, translocates to the nucleus and induces the transcription of various genes. (20)

Prominent triggers for TLR signaling are LPS that is a potent activator of TLR4, allowing for the recognition of gram-negative bacteria, or lipoteichoic acid (LTA), component of the gram-positive cellwall, that binds TLR2 (19, 21, 22). Table 1 depicts more detailed information about TLRs, their ligands, signaling and associated diseases (Table 1) (23-25).

Receptor	**PAMPs**	**DAMPs**	**Adaptor**	**Associated disease**
TLR1/TLR2	Lipoproteins, Triacyl lipoproteins (Pam_3CSK_4)	n. d.	Myd88/MAL	-
TLR2/TLR6	Diacyl lipoproteins, LTA, zymosan	Heat shock proteins, ECM fragments (versican, hyaluronic acid)	Myd88/MAL	-
TLR3	dsRNA (poly I:C)	mRNA	TRIF	Herpes virus infection
TLR4	LPS,viral envelope proteins	Heat shock proteins, ECM fragments	Myd88/MAL TRIF/TRAM	Sepsis, COPD, Asthma, Artherosclerosis
TLR5	flagellin	n. d.	MyD88	Legionella infection, recurrent cystitis
TLR6/TLR4	n. d.	Amyloid-β, oxidized LDL	MyD88 TRIF	-
TLR7	ssRNA, Imiquimod, R848	ssRNA (immune complexes)	MyD88	Celiac disease
TLR8	ssRNA	ssRNA (immune complexes)	MyD88	Celiac disease
TLR9	DNA, hemozoin	DNA (immune complexes)	MyD88	Malaria, SLE
TLR10	n. d.	n. d.	MyD88	-
TLR11	Profilin-like molecule, uro-pathogenic bacteria	n. d.	MyD88	-
TLR12	Profilin	n. d.	MyD88	-
TLR13	Bacterial rRNA	n. d.	MyD88	-

Table 1: Mammalian TLRs and their respective ligands, signaling pathways and associated diseases (23-25)

Acute phase response and complement activation:

The acute phase response is a result of the initiation phase and occurs via the secretion of acute-phase cytokines, like interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF) α. It involves an upregulation of cytokine receptors in different parts of the brain leading to the classical symptoms of sickness, like alterations in thermoregulation (fever) and loss of

appetite. At the same time the liver starts to produce high amounts of acute-phase proteins that mediate the activation of leukocytes, opsonization of pathogens and thereby promote clearance. The most famous acute-phase protein is the pentraxin, C-reactive protein (CRP), an important clinical parameter for inflammation. (9, 26) It was discovered in the serum of patients with acute inflammation, while being absent in healthy serum. CRP belongs to the opsonins due to its ability to bind phosphocholine on the surface of dying cells and some bacteria. Doing so, it activates the complement system via the complement component C1q. (27)

The complement system is a complex network of small plasma proteins and is an essential part of the humoral innate immune response. It can be activated by three major ways, all resulting in the generation of the key component of the complement pathway, the C3 convertase. Activation of the C3 convertase then induces a sequential and self-amplifying proteolytic cascade, first inducing cleavage of the complement component C3, leading to the generation of the C5 convertase that cleaves C5 and finally results in the assembly of the membrane attack complex (MAC) on the surface of microbes. (28)

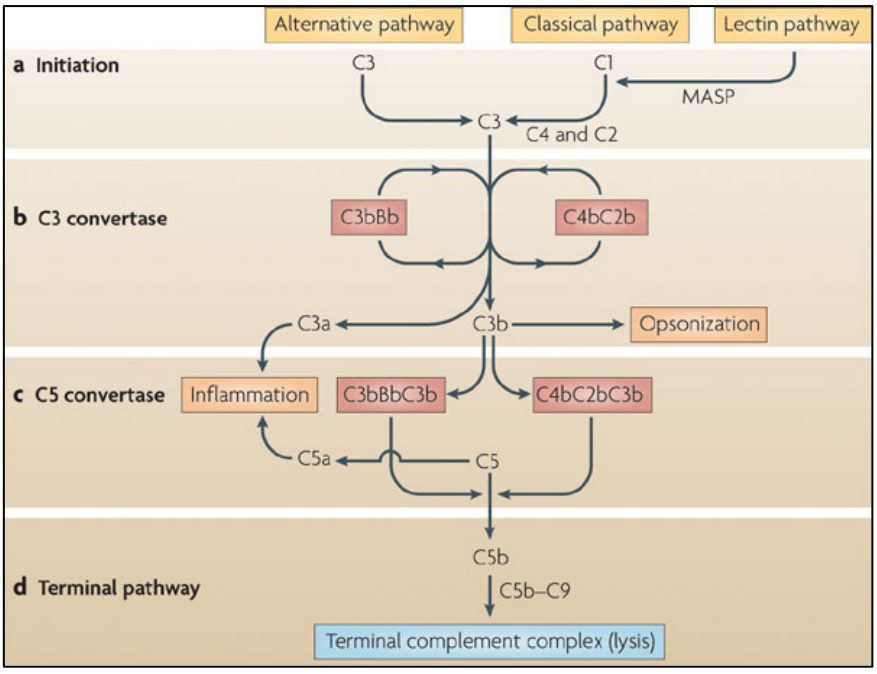

Figure 4: Complement regulators and inhibitory proteins (28)

The classical pathway is initiated by the complement component C1q and its interaction with antibodies bound to the target (29) or by C1q alone by binding to pathogens in an antibody-independent manner (30-32). Spontaneous and constant C3 cleavage happens via the alternative pathway on most biological surfaces within the plasma and other fluids (33). The

third option is the activation of complement via the lectin pathway, which is initiated by recognition of carbohydrates by PRRs, such as the mannose-binding lectin (MBL) (34). Physiological functions of complement activation include defense against pathogens, by enhancing leukocyte recruitment, opsonization of pathogens and pathogen lysis, as well as clearance of apoptotic cells, which is important for the maintenance of homeostasis. As uncontrolled complement activation results in autoimmunity and immunopathology, the complement cascade is tightly regulated at many levels. (28)

Next to a strong humoral response, the acute phase is associated with dramatic changes in the vascular component, which is critical for the aforementioned leukocyte recruitment. Inflammatory mediators released by resident immune cells are sensed by endothelial cells and change them from a homeostatic to an inflammatory state. Important molecules are vasoactive amines, like serotonin and histamine, eicosanoids like prostaglandin E2 (PGE2) or leukotriene B4 (LTB4), cytokines like TNFα and IL-1 or small signaling molecules like nitric oxide (NO). (35, 36) One important consequence is vasodilation, allowing an increased blood flow at the site of inflammation, together with an increase in vascular permeability, which is required for the transport of plasma fluid and the extravasation of cells into the inflamed tissue.

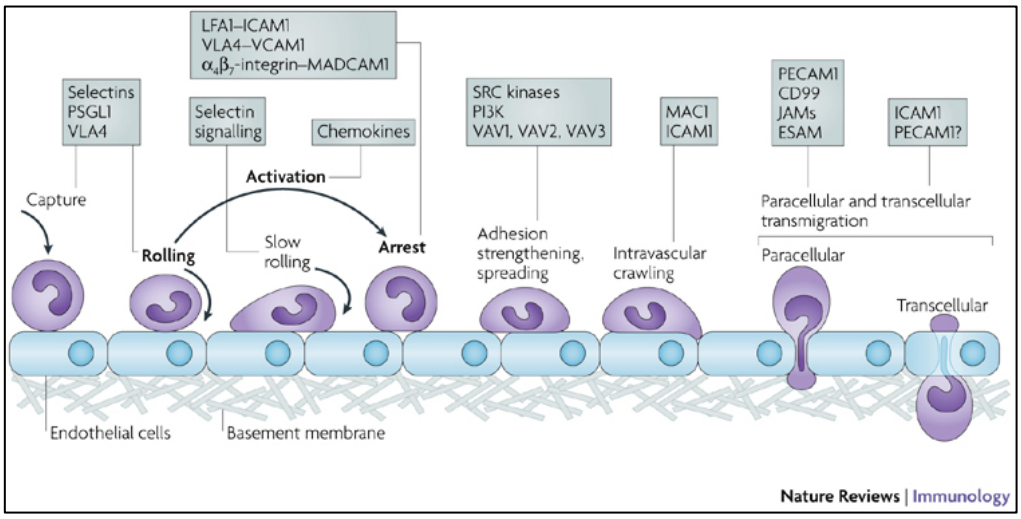

Figure 5: The process of leukocyte extravasation from the lumen of a vessel into the tissue in the course of inflammation, adapted from (37)

Plasma contains important molecules that will further contribute to the process, like Immunoglobulins (Ig), Bradikinin or components of the complement system. At the same time activated endothelial cells upregulate adhesion molecules, like selectins and integrins, on their surface, that will facilitate the recruitment of leukocytes. (37) The recruitment of

immune cells to inflamed tissue is a complex interaction process between leukocyte and endothelial surface molecules as well as extracellular matrix proteins and involves capturing and rolling over the endothelial surface, adhesion and activation, followed by diapedesis and transmigration, illustrated in more detail in figure 5. (37, 38) Typically the acute phase response is strongest in acute bacterial infections (9, 26, 39-41).

Resolution of inflammation:

Resolution is of great importance in order to prevent inflammation caused immunopathology and for restoring tissue homeostasis. It is thought to be an active event, with the purpose to terminate an acute inflammatory response, after the initial trigger of inflammation has been removed, for example a bacterial infection has been cleared successfully. Resolution involves specific pathways, mediators and cells. Failure in resolution pathways can have deleterious consequences, like uncontrolled overwhelming inflammation during sepsis, or result in chronic inflammation. (10, 42) Resolution is a coordinated process and involves a complete removal of the inflammatory trigger, clearance of apoptotic cells and material and the reversion of parenchymal/stromal cells from an inflammatory state to a non-inflammatory state (43).

The early phase of acute inflammation is characterized by the accumulation of neutrophils in the extravascular space and by edema formation. After the removal of the inflammatory trigger, neutrophils undergo apoptosis, a key event in the resolution of inflammation (44), and have to be removed in order to restore homeostasis. Neutrophils in general have a short life span, but during inflammation their survival is enhanced by the inflammatory milieu, for example higher granulocyte/macrophage-colony stimulating factor (GM-CSF) concentrations (10, 44, 45). Slightly later than neutrophils, inflammatory monocytes are also recruited to the site of injury (10). Cell recruitment is then abrogated by mechanisms like chemokine sequestration and proteolysis (46) and dying neutrophils release factors that inhibit further neutrophil infiltration, such as annexin A1 (AnxA1) (47) or lactoferrin (48).

Finally, apoptotic neutrophils promote resolution by changing macrophages from an inflammatory phenotype to a regulatory phenotype. Upon ingestion of apoptotic cells (efferocytosis) macrophages tune down the production of proinflammatory factors and start to secrete anti-inflammatory cytokines like IL-10 and transforming growth factor (TGF)-β (49). IL-10 is an important anti-inflammatory mediator, as it suppresses the production of proinflammatory cytokines and enhances the expression of anti-inflammatory mediators (50).

TGF-β regulates immune responses in multiple ways, having profound effects on adaptive immunity as well as on wound healing. TGF-β deficient mice die early from multi-organ failure due to over-activation of the adaptive immune system. (51, 52) Resolution-phase macrophages have also been shown to have a higher capacity to present antigen and to produce enhanced levels of T- and B-cell chemoattractants, like CCL5 or CXCL13, leading to the recruitment of T- and B-cells in the resolution phase (53).

1.1.3. Innate immune cells – key players of inflammation

Immune cells like macrophages and DC are critical for the initial recognition of potentially dangerous signals. Upon activation they produce chemokines that attract other leukocytes, such as neutrophilic granulocytes that migrate towards high IL-8 concentrations (54), or monocytes, that are attracted by the monocyte chemoattractant protein (MCP)-1 (55, 56).

Neutrophils are of special importance in acute inflammation, for instance during bacterial infections, as they are the main effector cells that kill bacteria (by phagocytosis, degranulation or by forming neutrophil extracellular traps (NET)) thereby preventing bacterial proliferation and dissemination. Neutrophils are usually the first cells that arrive at the site of injury, recruitment happens within minutes. (54)

Monocytes are recruited and can give rise to macrophages or DC, which then in turn fulfill important immunologic functions (57). Macrophages are highly effective in phagocytosing pathogens and apoptotic material, cytokine production and are crucial for restoring homeostasis. (58) DC as professional antigen presenting cells are important for the induction of adaptive responses. They pick up antigen at the site of injury and migrate towards the next lymphnode, were they present the antigen to naïve T-cells. (59)

In chronic inflammatory situations cells of the adaptive immune system participate in the inflammatory response. Changes in total leukocyte numbers are typical for inflammatory conditions. Leukocytosis, an increase of leukocytes, is more frequent and often induced during bacterial infections or allergic reactions. Leukopenia, describing a drop in leukocyte numbers, can for example occur during certain viral infections. (60)

Macrophages and neutrophils are of high importance for this manuscript and therefore will be discussed in detail in this chapter.

Macrophages:

As mentioned before, tissue resident macrophages are crucial during infection for initiating inflammatory responses, phagocytose and kill invading pathogens and perform antigenpresentation (Fig. 6) (58).

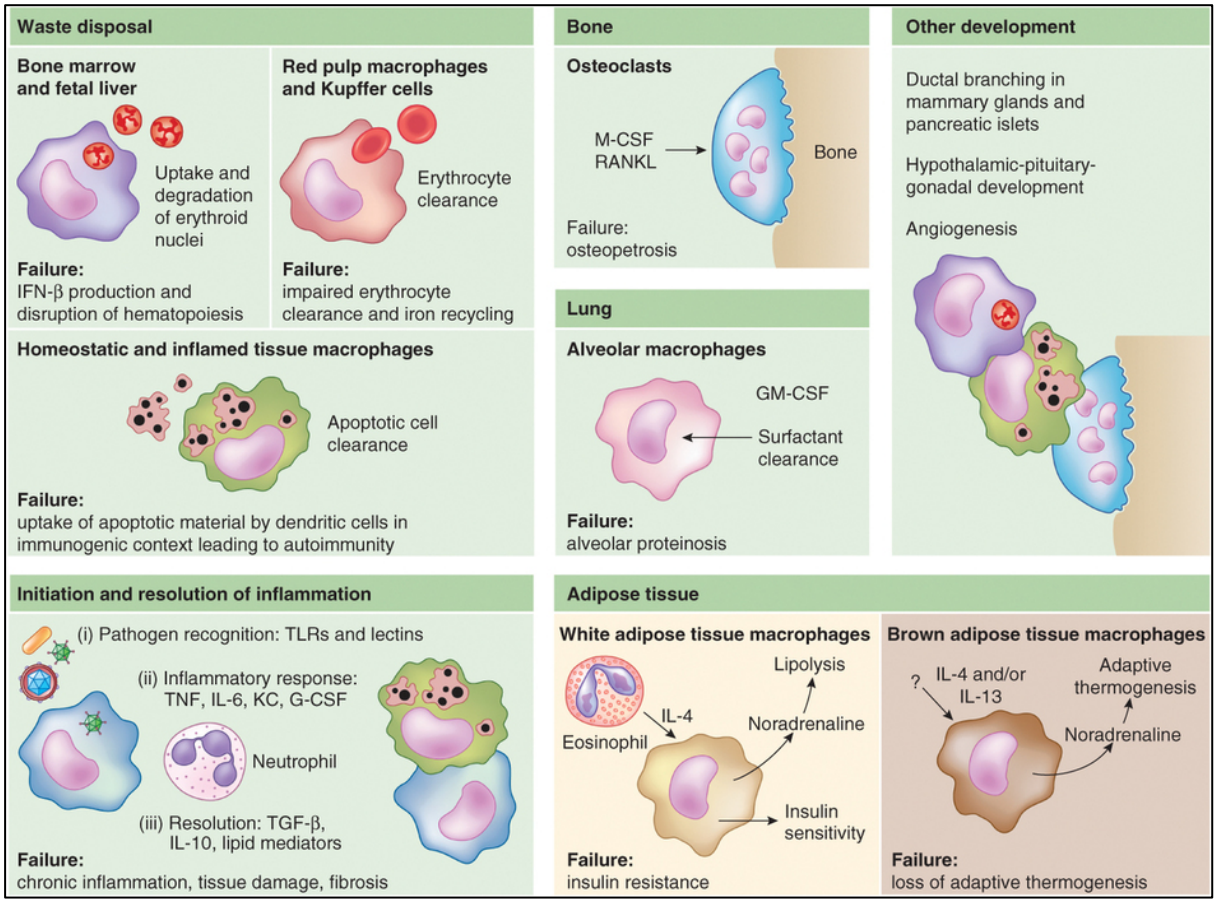

Figure 6: Functions of tissue-resident macrophages (58)

Under steady state conditions they remove cellular debris during tissue remodelling and are crucial for the removal of apoptotic cells, thereby maintaining homeostasis (61). They reside in the majority of tissues in the body and are a very heterogeneous cell population, as they exhibit tissue specific functions and even differ in origin (58). The old paradigm that all macrophages derive from blood monocytes, and therefore are of hematopoietic origin, has been outdated and extended by a model, that includes not just monocytes as the precursors of tissue resident macrophages, but involves self-renewal capacities of some macrophages (62-64), as well as cells that arise from a distinct yolk-sac derived lineage (58, 65). However, especially under inflammatory conditions, inflammatory monocytes are a major source of macrophages at the site of injury (56).

The most important factor for macrophages is M-CSF, regulating their differentiation, proliferation and survival (66). Mice that have a mutation in the *Csf1* gene (encoding for G-CSF) show severe defects in many different macrophage subpopulations. The fact that these mice show severe tissue abnormalities, for example profound osteopetrosis due to a lack of osteoclasts, highlights the importance of macrophages in tissue development and homeostasis (67, 68).

As macrophages already show profound heterogeneity under steady state conditions, it is not surprising that they can also display functional differences upon activation, dependent on the stimulus. This phenomenon is called macrophage polarization and in a simplified way classifies macrophages into 2 different categories, the classic M1 macrophages and the alternatively activated M2 macrophages. (69). Classic activation is caused by stimuli like IFNγ, Type-I IFN or TNFα. Basically all TLR agonists induce this M1 phenotype, characterized by a strong production of proinflammatory cytokines (like IL12), the generation of reactive oxygen species (ROI) and a high capacity to present antigen (61, 70). The so-called M2 phenotype includes a more heterogeneous population of macrophages and involves all phenotypes, other than the classic M1 (61, 70). Of high importance are the wound healing macrophages that develop upon stimulation with IL-4 and/or IL-13. These M2 macrophages are characterized by a different cytokine profile and have a decreased antimicrobial activity compared to the classic M1 macrophages, but enhance the production of extracellular matrix via their high arginase activity (69-71) instead. Regulatory macrophages, another M2 subpopulation, are crucial for the resolution process, as they are important to dampen inflammation by producing high amounts of the anti-inflammatory cytokine IL-10 (72).

Neutrophils:

Under homeostatic conditions neutrophils make up 50-70% of blood leukocytes in human blood and 10-25% in mice. They have a rather short lifespan in the circulation (around 12h in mice) and are constantly produced in the bone marrow from myeloid precursors. During acute inflammation, their production and their lifespan increases. Neutrophils are without any doubt the major effector cells during acute inflammation. They are the first cells responding to the inflammatory response of tissue resident cells and are the first immune cells arriving at the site of infection. (54) The early inflammatory response in many mouse models of infection, i.e. the amount of neutrophils recruited to the site of infection, is a critical factor for the outcome. Their longevity increases dramatically during acute infection (54, 73, 74). This allows them to efficiently perform their effector functions at distinct sites of inflammation,

but might also lead to bystander tissue injury. Neutrophils can kill pathogens by different machanisms namely phagocytosis, degranulation and NET-formation (Fig. 7). (54)

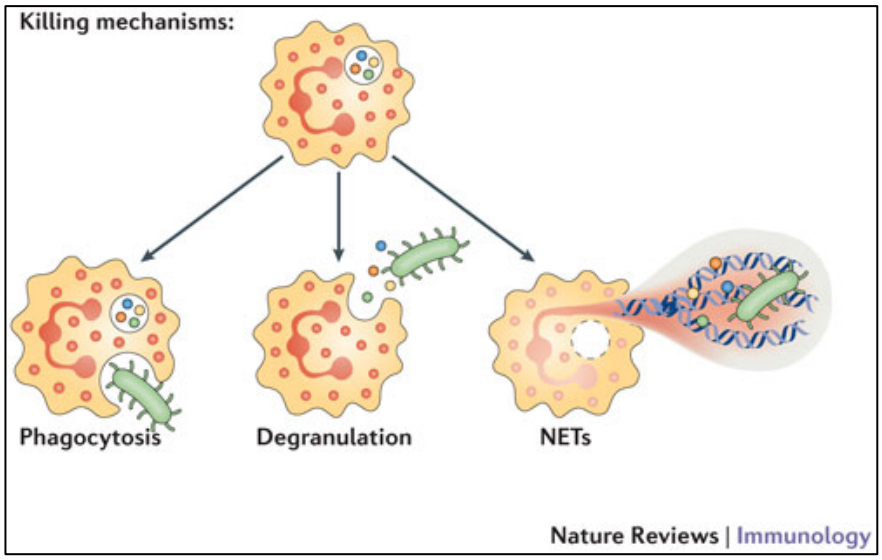

Figure 7: Killing mechanisms of neutrophils (54)

When neutrophils die under physiological conditions they are mainly cleared in the bone marrow, the spleen or the liver. During infection, apoptotic neutrophils are mainly cleared by tissueresident macrophages, most importantly by kupffercells in the liver. While neutrophils were long considered as a homogenous population of innate effector cells that contribute to the inflammatory response, more and more evidence support the existence of different neutrophil subsets. Several reports support the existence of anti-inflammatory IL-10 producing neutrophils (75, 76) and the efferocytosis of neutrophils has been reported to turn macrophages into regulatory IL-10 producing macrophages, an important factor during resolution (49).

1.2. Regulation of TLR-mediated inflammation

It is clear that tight regulation is crucial to protect the system from damage-causing inflammatory responses. Thus, it is not surprising that TLR signaling is controlled by multiple mechanisms, which can be roughly categorized in regulation by i) soluble and decoy factors, ii) disruption of adaptor complexes, iii) degradation of signaling molecules and iv) transcriptional interference.

As negative regulation of TLR signaling is an important topic for this thesis, this chapter will give an overview about the basic mechanisms that are known and try to describe the most prominent molecules that are involved in negatively regulating TLR responses. (23, 24, 77)

1.2.1. Soluble and decoy factors

Soluble forms of TLR2 and TLR4 have been described. For TLR4 several mRNA variants have been detected in mammalian hosts, indicating the presence of different TLR4 isoforms. Furthermore the presence of recombinant sTLR4 significantly reduces LPS-induced TNF production by macrophages in vitro. (78) sTLR2 has been detected in human milk and plasma (REF), is constantly released by human blood monocytes and is able to limit IL-8 and TNF production by monocytes stimulated with lipoprotein (79).

Besides sTLRs a soluble form of CD14 (an important co-receptor for TLRs) has been identified and is negatively regulating TLR4 mediated inflammation (80) and soluble ST2 has been shown to limit the expression of TLR4 and TLR4, thereby reducing cytokine responses (81). (23, 24, 77)

1.2.2. Disruption of adaptor complexes

As illustrated in Figure 3 the complex formation of several adaptor proteins is required for efficient TLR signaling. Of particular importance are MyD88, TIRAP/MAL, TRIF and TRAM, which are therefore targets of regulators that are involved in fine-tuning TLR-induced signal transduction. Mechanisms that lead to complex disruption involve competition, de-phosphorylation by phosphatases and de-ubiquitination. One important negative regulator that acts through competition is for example MyD88s, a short variant of MyD88 that has been detected in the spleen and the brain and is upregulated by THP-1 monocytes upon stimulation with LPS (82, 83). The presence of sMyD88 leads to the formation of sMyD88-MyD88 heterodimers, which still cause the recruitment of IRAK1, but IRAK1 is no longer phosphorylated (82). (77)

Another competitor of TLR signaling is the TRAM adaptor with GOLD domain (TAG), a variant of TRAM. It occupies the TRIF binding site for TRAM and thereby inhibits the TRIF mediated pathway. TAG is also involved in TLR4 degradation after LPS stimulation (84). The src homology 2 domain-containing protein tyrosine phosphatase-1 and -2 (SHP-1, -2) negatively regulate TLR signaling by de-phosphorylation. SHP-1 suppresses IRAK1 and IRAK2 activation, leading to a reduced activation of NF-κB target genes and an enhanced type-I IFN response. SHP-2 inhibits TRIF-mediated induction of type-I IFN. (85-87)

The negative regulator A20 is a prominent example for regulation by deubiquitination and is important for this manuscript. It is the only negative regulator that controls both, MyD88-dependent and -independent signal transduction, as it targets TRAF6, a signaling molecule that is shared by all members of the TLR family. A20 is induced very fast upon LPS and TNF treatment and deficient mice develop inflammation in multiple organs, which can be rescued by antibiotic treatment and by co-deficiency of MyD88. This indicates that A20 is involved in suppressing immune responses against commensal bacteria. (88-90) Additionally A20 can inhibit NF-κB activation independent of deubiquitination, as it was shown to inhibit TAK1 mediated IKK activation without DUB activity (91).

1.2.3. Degradation of signal transducers

Proteasomal degradation, classically regulated by ubiquitination, is an essential process that controls various cellular processes critical for homeostasis, but also immune responses (REF). Suppressor of cytokine signaling (SOCS) proteins are members of the E3 ubiquitin ligase family and are well described negative regulators of TLR-induced cytokine production that induce the degradation of MAL or TRAF proteins (92). Other regulators directly target transcription factors that are activated via TLR signaling. The PDZ and LIM domain containing protein 2 (PDLIM2) promotes degradation of the NF-κB subunit p65, thereby inhibiting TLR responses (93) and also IRF3 and IRF7 are degraded after polyubiquitination by the peptidyl-prolyl isomerase Pin1 (94, 95).

Next to the ubiquitin-proteasome system, autophagy has been shown to negatively regulate the TRIF-dependent pathway. Deficiency in Atg16L1, an important component of the autophagy degradation system, leads to enhanced ROS, IL-1b and IL-18 production in response to LPS. Furthermore Atg16L1 has been associated with the development of Chrons disease, undermining its importance in regulating inflammatory responses. (96, 97)

1.2.4. Transcriptional regulation

It is well established that the transcriptional activity of NF-κB activity is regulated by epigenetic mechanisms. Histone deacetylase 1 (HDAC1) is recruited to promoter regions by the cyclic AMP-dependent transcription factor (ATF3), leading to transcriptional suppression of proinflammatory gene transcription. (98) Other negative regulatory factors inhibit transcription by direct competition with NF-κB binding sites. So does the IκB delta, which selectively blocks IL-6 transcription (99). The regulatory function of the NF-κB subunit p50 is very well understood. p50 can form homodimers with p65 in order to activate transcription.

However p50 homodimers block NF-κB binding sites and blocks transcription of proinflammatory genes, while enhancing IL-10 production, as it has been observed on endotoxin tolerant macrophages. (100, 101)

Another transcriptional mechanism is the simple downregulation of TLR expression, which mainly happens via TLR degradation after ligation (102-104). Transcriptional regulation of TLR regulation has been investigated but the results are inconsistent. TLR4 mRNA has been shown to decrease in LPS stimulated RAW 264.7 cells (105) but to increase in human Leukocytes after LPS stimulation (106), indicating cell-type specific differences.

1.3. Inflammatory diseases

Inflammation comes in different flavors and can be classified as *local* versus *systemic* and *acute* versus *chronic*. While pathologic inflammation was mainly viewed for a long time from the point of acute inflammatory conditions, it becomes more and more evident, that the component of inflammation is playing a role in the pathology of many different diseases. Chronic inflammation is supposed to be responsible for the most common chronic diseases, that industrial countries have to deal with, like metabolic disorders (107) or atherosclerosis (108). (6, 7) All kinds of pathologies caused by inflammation are the result of a failure in controlling and/or resolving inflammation, which becomes most evident during sepsis, described in more detail in the following chapter.

1.3.1. Sepsis – Terms and definitions

Sepsis is a fatal complication of infection and a leading cause of death in hospitalized patients worldwide. In the United States it accounts for 750,000 -1,000,000 cases per year and, despite the successful use of antibiotics, mortality rates range between 20% and 30% (109, 110). This is mainly due to the lack of treatment options that target the collateral damage caused by uncontrolled immune responses and often leads to multiorgan failure.

The current treatment of sepsis is unspecific and includes antibiosis, surgical or radiological approaches with the purpose to eliminate or control the initial trigger. Other treatment approaches try to support organ function like mechanical ventilation and the intravenous administration of fluids (0.9% NaCl, 5% Albumin solution) and ionotropic and/or vasocontricting drugs (vasopressin, norepinephrin). (111)

Sepsis is an imprecise clinical diagnostic term. Per definition "sepsis" was defined for a long time as systemic inflammatory response syndrome (SIRS) together with an infection. "SIRS" is diagnosed when patients show at least two abnormalities in either body temperature (<36 °C or >38°C), heart rate, respiratory rate and/or leukocyte count (112, 113). Sepsis in combination with organ dysfunction is classified as "severe sepsis" and if patients additionally show cardiovascular dysfunction and are not responsive to fluid administration they are classified as having "septic shock". (110, 112, 113) Sepsis is known to be caused by hyperactivation of PRRs, such as TLRs by a potentially dangerous stimulus, which can be either of pathogenic (PAMP) or of endogenous origin (DAMP). The result is a combination of symptoms that do not differ between an infectious situation or a situation of sterile inflammation, caused by tissue injury, making the clear definition of sepsis a matter of discussion. (110, 114) The most recent perception of sepsis by the international sepsis forum was to define sepsis as *"life-threatening condition that arises when the body´s response to an infection injures its own tissues and organs."*(115)

1.3.2. Peritonitis

Under normal conditions, the peritoneum is completely sterile. Peritonitis is defined as inflammation of the peritoneal cavity and in most cases caused by bacterial contamination. Peritoneal infection is a severe condition, as it is a frequent cause of sepsis and still has a high mortality around 30%, despite the efficient use of antibiotics and progress that was made in supporting basic body functions.

Clinical classifications of peritonitis are considering the pathogenesis and distinguish primary, secondary and tertiary peritonitis. (116-118) Primary peritonitis is defined as an infection by an extraperitoneal source. It is usually caused by a single pathogen, in 70% of cases this pathogen is *E. coli*. Secondary peritonitis results from spillover of bacteria from the gastrointestinal flora into the peritoneum, which can occur due to perforation (for example in the course of acute appendicitis), trauma or post-surgery. It is usually caused by a mixture of pathogens, including *E. coli*, *Klebsiella* spp, enterococci, staphylococci and others. (118, 119) Tertiary peritonitis describes persistent and/or recurrent infection, after a primary or secondary peritonitis has been treated already and often happens in patients which are immunesuppressed. (118)

As described above in the chapter about sepsis, also the treatment of peritoneal sepsis is complex. Next to antibiotic therapy and support of organfunctions peritonitis is treated by intra-abdominal lavage (washing). (118)

1.3.3. Innate immunity during peritonitis

One danger of peritoneal infection is the rapid dissemination of bacteria from the peritoneum to the blood stream, immediately resulting in bacteremia and a high risk to develop sepsis (120, 121). The ratio of resident macrophages to lymphocytes in the peritoneum of a healthy mouse is approximately 1:1, with peritoneal B1 cells being the most abundant lymphocyte population (53). Upon infection effective TLR signaling is of critical importance for the host defense. In case of *E. coli* the presence of TLR2 and TLR4 are of special importance, as they recognize bacterial LPS and lipoprotein and trigger the production of inflammatory cytokines and chemokines, which in turn leads to the recruitment of neutrophils to the site of infection. The absence of this protective immune response leads to uncontrolled bacterial replication and dissemination, finally resulting in systemic inflammation and organ failure (122, 123). While inflammation is crucial for the control of bacteria, the downside is the collateral damage, caused by the dramatic inflammatory response, as it occurs during sepsis, a frequent cause of death during peritonitis. This is best illustrated in mouse models of endotoxemia, were the injection of LPS allows studying inflammation uncoupled from bacterial proliferation. TLR4 mice are protected from endotoxemia, again highlighting the importance of TLR4 in regulating inflammation in response to LPS (21) and undermining the requirement for a tight control of inflammation in response to infection, in order to allow for efficient pathogen clearance, while preventing immunopathology.

Mouse models have been developed in order to investigate peritonitis and generally fall in one of the two categories: intoxication or infection. Infection by direct injection of a certain number of bacteria into the peritoneum like for example *E. coli*, allows investigating precise mechanisms to a single pathogen and allows a very good dosage control. Another frequently used model is the cecal ligation and puncture (CLP) model, were the cecum is ligated and punctured with a needle leading to an infection of the peritoneum with intestinal bacteria. This model mimics the situation of patients who suffer from polymicrobial infection, but the disadvantage is a high variation due to a worsened dosage control. (124, 125) Intoxication models, like intraperitoneal (i.p.) injection of LPS or heat killed bacteria are performed to study inflammation irrespective of bacterial replication. (124)

1.3.4. Pneumonia

Despite the development of antibiotics, invasive pneumonia is a major cause of death worldwide and a special threat for children, immunocompromised individuals and adults with an age over 65. (126, 127) The world health organization estimates that around 1.6 million people die from pneumonia annually, including 1 Million children below the age of five and the developing countries being most affected (126). *Streptococcus pneumonia* is the most frequently identified pathogen causing community acquired pneumonia (CAP) (127), in contrast to ventilator-associated pneumonia (affecting hospitalized patients), one of the most common nosocomial infections, caused other microorganisms like *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) or *Candida albicans* (*C. albicans*) (128).

1.3.5. Innate immunity in pneumococcal pneumonia

In contrast to the sterile peritoneum, the lung is constantly exposed to the outside and therefore is protected by multiple unspecific and specific mechanisms and immune responses are under tight control. Innate immunity of the lung involves mucociliary clearance, the production of antimicrobial factors and populations of myeloid cells. (1) Resident alveolar macrophages (AM) are the sentinel cells of the airways, as they are the first line of defense and very important for the early induction of inflammatory responses in case of an infection (129, 130). In many cases small numbers of microbes are cleared by the resident immune system, but a serious infection leads to the recruitment of neutrophils and promote clearance. At the same time resident DC are getting activated, migrate to the lymphnodes and elicit an adaptive immune reaction. Next to their crucial role in adaptive immunity, they are important type I IFN producers during anti-viral responses. (131)

Important TLRs for recognition of *S. pneumoniae* are TLR2, recognizing LTA and peptidoglycan (22, 132), TLR4 by sensing pneumolysin (133) and TLR9, getting activated by pneumococcal DNA, released after autolysis of the bacteria (134). TNFα is one of the first cytokines that is detectable in the bronchoalveolar lavage fluid and tissue of mice during pneumococcal pneumonia. Together with IL-1 it induces the production of KC (the murine IL-8 homologue) that is crucial for the recruitment of neutrophils. (130) Another important factor is IL-6 that prolongs the lifespan of neutrophils and boosts their phagocytic capacity by inducing CRP production in the liver during pneumococcal pneumonia. (135) AMs are also crucial for maintenance of lung homeostasis by producing the anti-inflammatory factors IL-10 and TGFβ (129, 136).

1.4. Triggering receptor expressed on myeloid cells-2

Tiggering receptor expressed on myeloid cells (TREM)-2 belongs to the TREM family of proteins located on chromosome 6p21 in humans and 17C3 in mice. TREMs are a heterogeneous group of surface receptors that are involved in various processes, like inflammation, neurological development and bone homeostasis.

The most prominent and studied members are TREM-1 and TREM-2 which are present in both species, mice and humans. Additionally the TREM family comprises TREM-3, TREM-5 and plasmacytoid dendritic cell (PDC)-TREM, which are only present in mice, as well as the so called TREM-like transcripts (TLTs). (137-139)

Structurally the extracellular domain of all TREM receptors comprises a single immunoglobulin superfamily (IgSF) V-type domain, however intracellular there is more variation. Some of the TLT proteins harbor an immunoreceptor tyrosine-based inhibitory motif (ITIM) for signaling via the phosphatases SHP-1 and/or SHP-2 (140, 141) and the cytoplasmic tail of TLT2 includes a potential SH3 binding motif (139). In contrast to that TREM-1 and TREM-2 do not contain any known motifs for signal transduction and signal transduction occurs via association with the DNAX adaptor protein 12 (DAP12). (137)

1.4.1. Lessons from the Nasu-Hakola disease

First indications for TREM-2 and its functions derive from studying a very rare syndrome, the so called Nasu-Hakola disease, or polycystic lipomembranous osteodysplasia with sklerosing leukoencephalopathy (PLOSL). PLOSL is a severe, recessively inherited, systemic disease caused by mutations in TREM-2 or its adaptor protein DAP12. It was first described simultaneously in the early 1970s by Nasu and Hakola (142, 143).

Affected individuals initially develop normally, being in a latent disease-free state until the onset of the disease that is usually at an age around 20 +/- 10 years. First symptoms are bone fractures, mild pain and swelling of ankles, characteristics of the so called osseous state. This is followed by an early neurological state, when patients develop the first neurological symptoms like personality changes and behavioral abnormalities, which then progresses to a late neurological stage, characterized by fully developed dementia and a loss of motoric abilities. Patients at this stage get usually bedridden and finally die in the 4^{th} to 5^{th} decade of their life. (144)

Most individuals suffering from PLOSL carry a homozygous mutation in the DAP12 gene, as shown by genetic analyses of Japanese, Finnish and Brazilian patients. These mutations usually are loss of function mutations, causing the complete absence of DAP12 or the production of non-functional DAP12 protein. The minority of patients is homozygous for loss of function mutations of TREM-2. In both cases, the result is a complete or at least substantial loss of function of DAP12/TREM-2 signaling. (144)

Several interesting conclusions can be drawn from studying the Nasu Hakola disease. At first, it has been shown that individuals with DAP12 deficiency also show decreased expression of TREM-2. In contrast, DAP12 expression levels seem to be unaffected by TREM-2 deficiency. (145) This fits with the fact that DAP12 can associate with many other receptors and does not need TREM-2 to be stably expressed. TREM-2 on the contrary seems to be dependent on DAP12. However this raises the question about what happens with other DAP12 associated receptors and their signaling, if DAP12 is absent. Secondly, despite the fact, that TREM-2/DAP12 signaling seems to impact immunologic functions, there are no reports about higher incidences of infections or neoplasias in individuals suffering from PLOSL. Interestingly, in vitro studies could not show any dysfunctions of receptors other than TREM-2, in situations of DAP12 deficiency, (146) indicating that i) PLOSL is caused by the dysfunction of TREM-2, ii) DAP12 associated receptors other than TREM-2, might be able to compensate for the loss of DAP12 in its absence and iii) compensation for the disrupted TREM-2/DAP12 axis might fail in the brain and in bones, the organs were PLOSL is manifested. (144, 146)

1.4.2. TREM-2/DAP12 signaling

As TREM-2 signals via DAP12, understanding TREM-2 signaling requires an understanding of DAP12 and ITAM/ITIM signaling, respectively. DAP12 is a very small single pass transmembrane domain protein, consisting of only 113-114 amino acids. Like many other ITAM receptors, it is a promiscuous protein and known to associate with many different receptors such as the killer-cell immunoglobulin like receptor (KIR) or the Ly49 family on NK-cells, as well as the myeloid-associated immunoglobulin-like receptor (MAIR)-2 or Siglec expressed on macrophages (147, 148).

As shown in Figure 8 the presence of positively charged aminoacids, like Lysine, within the transmembrane domain of a receptor, is critical for the association with DAP12 (148, 149).

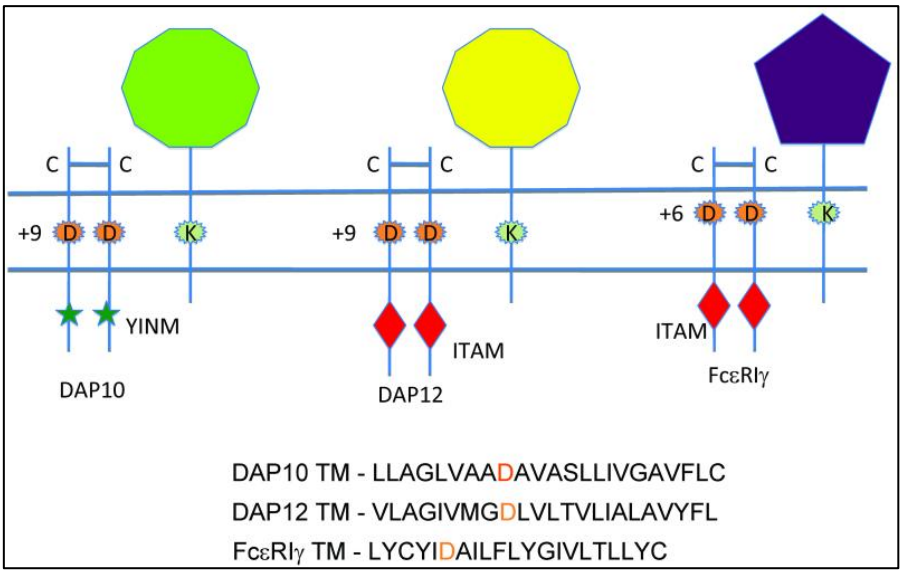

Figure 8: Interaction of charged redidues within the transmembrane domains of FcRγ, DAP10 and DAP12 and their associated receptors (adapted from Lanier 2009) (148)

Signal transduction of DAP12, as well as of other ITAM-bearing receptors has been a mystery since many years. For a long time it was believed that, based on their names, ITAMs, defined by the sequence D/ExxYxxL/Ix_{6-8}YxxL/I, would always mediate cellular activation, while ITIMs, defined by the consensus sequence S/I/V/LxYxxI/V/L would exert inhibitory signaling. However this concept turned out to be incomplete, as recent publications identified ITAMs to be involved in cellular inhibition (141, 150, 151), while ITIMs were shown to mediate activating functions and interfere with ITAM signaling (152, 153). Another layer of complexity is added by the fact that DAP12 has been shown to harbor an ITIM within its ITAM motif (141).

The activating pathway is pretty well understood. Upon activation of its associated receptor, tyrosin residues within the ITAM of DAP12 get phosphorylated by members of the Src kinase family. Fully phosphorylated ITAMs promote the activation of SH2 domain containing Syk kinases (Syk and/or Zap70), which then induce the phosphorylation of multiple substrates, like the phosphatidyl inositol 3-kinase (PI3K), the extracellular-signal regulated kinase (ERK) or phospholipase Cγ1 (PLCγ1) and subsequently mediate cellular activation. (154) In contrast, only little is known about inhibitory DAP12 signaling. Phosphorylation of ITIM containing receptors classically lead to the recruitment of phosphatases, mediating dephosphorylation and thereby inhibit otherwise activated effector molecules. Involved are phosphotyrosine phosphatases (PTPs), like SHP-1 or SHP-2 or the inositolphosphatase SHIP. (141)

In an attempt to explain the ambiguity of DAP12/ITAM signaling, Barrow et al. developed a working model in which high affinity/avidity ligation of a DAP12 associated receptor would induce full (dual) ITAM phosphorylation and thereby lead to activation, while low affinity/avidity binding would only result in partial phosphorylation of the closet ITIM and subsequent inhibitory signaling. (141, 147)

Given the limited knowledge about DAP12 signaling, it is clear that even less is known about the mechanisms of TREM-2 signal transduction. However, a role for Syk in TREM-2 signaling is well accepted and supported by multiple studies (155-157). ERK has been shown to be involved in TREM-2 mediated signaling in human osteoclasts as well as in mouse DC (155, 158). In Osteoclasts, TREM-2 activates a co-stimulatory pathway that involves Syk and triggers to ERK phosphorylation and NFATc1 induction. This pathway crucially supports RANK signaling, is therefore critical for proper osteoclastogenesis and has been shown to be inhibited by IL-10 (155). In mouse DC ERK activation is induced upon crosslinking of TREM-2 with $F(ab')_2$ 29E3 fragments, thereby promoting DC survival. In addition TREM-2 ligation results in upregulation of CCR7, MHC class II, CD86 and CD40 indicating a role for TREM-2 in the maturation process of DC. (158) A link between TREM-2 and PI3K/Akt signaling has been reported several times. Firstly, PI3K activation is critical for TREM-2 mediated immunity against *P. aeruginosa* (159, 160). Furthermore, experiments with bone marrow derived macrophages (BMM) and osteoclasts, showed TREM-2 to activate ERK, PI3K and the guanine nucleotide exchange factor Vav3, thereby triggering Calcium (Ca^{2+}) mobilization, cytoskeletal rearrangements and survival. This study also showed DAP10 to participate in TREM-2 complex formation and SHIP1 to compete with PI3K for binding to DAP12. (161)

1.4.3. TREM-2 and the brain

TREM-2 and DAP12 are already abundant in the embryonic mousebrain on day17 of development. Colocalization studies showed that mainly microglia are positive for their expression and also in humans TREM-2 is expressed on microglia. (145) Together with the fact that impaired TREM-2 signaling is causing the Nasu Hakola disease, it was always widely accepted that there is an important role for TREM-2 in the maintenance of CNS immune homeostasis. (145, 162) Recent studies have identified heterozygous mutations in TREM-2, to be associated with early onset dementia and point towards TREM-2 being important in the pathogenesis of alzheimers disease (163).

Mechanisms how TREM-2 impacts brain homeostasis still need to be elucidated, but there is evidence that TREM-2 might be crucial for the clearance of apoptotic neurons (162, 164) and blockade of TREM-2 worsens brain inflammation in a mouse model of autoimmune encephalitis (165). A very recent publication by Wang et al. shows that TREM-2 seems to be critical for the microglial sensing of various anionic lipids, associated with the death of neurons and the formation of β-Amyloid plaques. Interestingly, microglia that express a TREM-2 variant carrying a mutation that had been identified as a risk factor for early onset of Alzheimers disease, were impaired in their ability to detect these damage associated lipids. (166) This study is in line with another report showing TREM-2 to bind a wide array of anionic ligands (167).

1.4.4. TREM-2 expression

Indicated by its name, TREM-2 has been detected on a variety of myeloid cells. Amongst macrophages, AM, BMM, hepatic macrophages, peritoneal macrophages, microglia and osteoclasts have been shown to express TREM-2 using various techniques (146, 155, 168-173). In addition, TREM-2 is found on immature monocyte derived DC (158) as well as on many different cell lines like J774.2, RAW 264.7, IC21 and MT2 (macrophages), THP-1 and U937 (monocytes/premonocytes), N9 and CHME-5 (microglia) and the neuroblastoma cell lines N2A and T98G (139, 165, 173, 174). There are no reports about TREM-2 being expressed by cells of the lymphocyte lineage but there is evidence about its presence on hepatic endothelial cells (168).

As mentioned above, TREM-2 expression seems to be dependent on the presence of DAP12 (145) and is suppressed by IL-10 (155). Furthermore it seems to get downregulated on RNA and protein level upon stimulation of the TLR4 pathway (168, 169) as well as during treatment with IL-1β and TNFα (168) and one study showed that TREM-2 undergoes ectodomain shedding and is cleaved within the transmembrane domain by γ-secretase when overexpressed in COS7 cells (175), indicating that there are multiple mechanisms involved in regulating TREM-2 levels. This data support the fact that a soluble form of TREM-2 has been detected in human serum and cerebrospinal fluid and is increased in individuals suffering from inflammatory neurologic diseases, like multiple sclerosis (176).

TREM-2 is also strongly induced in human and mouse lungs upon cigarette smoke exposure (177), upon chronic 2nd hand smoke exposure in mouse AM (178) as well as during alternative macrophage activation with IL-4 and IL-13 (169).

Regarding its cellular localization, TREM-2 has been found to be glycosylated and shuttle between surface and golgi apparatus in microglia (179) and also other studies performed with microglia and neurons found the majority of TREM-2 to be localized intracellular (173).

1.4.5. TREM-2 and inflammation

Many studies have examined the impact of TREM-2 signaling in different immunological settings. In contrast to the closely related TREM-1 (180, 181), which is involved in the amplification of immune responses, TREM-2 seems to rather inhibit inflammatory responses. As such, TREM-2 has been shown to negatively regulate the production of TNFα by BMM challenged with LPS, CpG or Zymosan (169). Interestingly, DAP12 deficiency phenocopies the lack of TREM-2 in this respect, as DAP12 knockout BMM also show an enhanced TNFα response upon TLR activation (172). In this study normal cytokine production was restored by transfection of DAP12 deficient BMM with a TREM-2/DAP12 fusionprotein, consisting of the extracellular domain of TREM-2 and the intracellular part of DAP12, further strengthening the model of an inhibitory TREM-2/DAP12 signaling axis in macrophages. Similar results were obtained with peritoneal macrophages, as newly (thioglycollate) recruited PM release more TNFα and IL-6 in response to LPS (169). In line with the fact that TREM-2 is upregulated by alternative stimuli, like IL-4 (169), the TREM-2/DAP12/Syk axis seems to be important for the IL-4 induced formation of multinucleated giant cells by macrophage fusion (182).

Next to macrophages, also DC are affected by TREM-2 signaling. Bone marrow derived DC (BMDC) that lack TREM-2 produce more IL-6, IL-12 and TNF upon stimulation with LPS, CpG or Zymosan and also the production of type I IFN seems to be suppressed by TREM-2, according to this study (183). Furthermore TREM-2 deficient BMDCs display increased maturation, as indicated by higher MHC class II levels, upon TLR ligation and were more capable in inducing T-cell proliferation, as compared to WT BMDC. (183) However, these data are in conflict with publications showing a role for TREM-2 in antigen uptake and maturation of DC (158, 184) and another report showing TREM-2 to boost T-cell activation during DSS colitis (185).

However, the role of TREM-2 in inflammation has been only partially studied in vivo. To this end, TREM-2 has been investigated in mouse models of experimental autoimmune encephalitis (EAE), in DSS colitis and during infections with *P. aeruginosa*, *S. pneumonia* as well as during *E. coli* induced and polymicrobial sepsis.

During EAE TREM-2 signaling has been shown to be detrimental, as blockade of TREM-2 worsens clinical symptoms of mice and CNS inflammation (165). However, it is important to mention that this study made use of a TREM-2 blocking antibody, which was thus far not used in any other study in vivo and without showing data that would confirm that TREM-2 signaling is effectively inhibited.

A more reliable report, focused on TREM-2 role during DSS colitis, a mouse model for inflammatory bowel disease (185). This study was more straightforward, as it shows TREM-2 to be upregulated in the inflamed mucosa of IBD patients and in addition made use of TREM-2 deficient mice and compared their response to WT mice upon repeated exposure of DSS. Interestingly the absence of TREM-2 is beneficial for mice during DSS colitis, which is at first counterintuitive in the context of its supposed negative regulatory role in inflammation. However, DSS colitis has been shown to be dependent on the gut microbiome and the authors of this paper claim, that TREM-2 deficiency would impair the capability of DCs in the lamina propria to activate T-cells, thereby reducing mucosal inflammation. (185)

It seems to be clear that TREM-2 is crucial for proper host defense during infection with the gram-negative pathogen *P. aeruginosa*. In one study induction of keratitis by *P. aeruginosa* elevates TREM-2 expression and silencing of TREM-2 leads to bacterial outgrowth and more inflammation, whereas activation of TREM-2 was beneficial for the host resistance (159). This study is supported by in vitro data showing TREM-2 to be crucial for successful intracellular killing of *P. aeruginosa* by macrophages (160).

One study showed TREM-2 to promote bacterial clearance in a CLP model of Gram-negative sepsis, further confirming TREM-2´s function as a phagocytic receptor (186). This study shows that the administration of TREM-2 overexpressing bone marrow derived cells is able to reduce bacterial burden during CLP. However, no effect on inflammation was observed.

This thesis aims to further investigate the role of TREM-2 during infection, in particular during Gram-negative sepsis, as well as during pneumococcal pneumonia. The two publications arising from this work are part of this manuscript and will be discussed in the context with the current literature in the discussion section.

1.4.6. TREM-2 as a phagocytic receptor

TREM-2 has been manifold shown to crucially regulate phagocytosis. The first and most convincing report regarding this issue shows that co-overexpression of TREM-2 and DAP12

enables Chinese hamster ovary (CHO) cells to uptake gram-negative bacteria, like *E. coli*, *Francisella tularensis* and *P. aeruginosa*, as well as the gram positive pathogen, *Staphylococcus aureus* (157). This process showed to be dependent on actin rearrangements and requires the activity of Rac and Cdc42 as well as Syk signaling.

Further evidence for TREM-2´s role in phagocytosis come from the already mentioned study in which TREM-2 overexpressing cells enhance bacterial clearance in a CLP model (186).

1.4.7. TREM-2 and bone formation

Despite the fact that there are some discrepancies between mice and humans, it is evident that TREM-2 importantly regulates osteoclastogenesis. PLOSL patients suffer from osteoporosis. This is in line with the fact that DAP12 and TREM-2 deficient monocytes derived from individuals suffering from PLOSL are hampered in differentiating to osteoclasts in vitro (187) and show an osteoporotic phenotype (146). Confocal studies reveal that these cells show an abberant morphology. They are smaller and display alterations in their actin cytoskeletal organization, as compared to healthy control cells. (146, 187) Confusingly, these data are in conflict with studies that were performed in mice. While DAP12 deficiency leads to impaired osteoclastogenesis in vitro and mild osteopetrosis in vivo, experiments performed with TREM-2 deficient cells surprisingly show accelerated osteoclastogenesis in the absence of TREM-2 without resulting in an in vivo phenotype (188).

Later, TREM-2 was found to be involved in an important co-stimulatory pathway that regulates RANK signaling and therefore is critical for osteoclast development (155).

1.5. Aim of the thesis

Efficient TLR signaling and pathogen clearance are the key determinants that influence the outcome from infections. Given that i) TREM-2 has been shown to negatively regulate TLR-mediated cytokine production and ii) has been identified to mediate phagocytosis of bacteria, we hypothesized that TREM-2 exerts important functions in the course of bacterial infection in vivo.

Therefore the aim of this thesis was to better characterize the role of TREM-2 during sterile inflammatory conditions and medically relevant infectious diseases. In more detail, this manuscript aims to elucidate i) the role of TREM-2 in Gram-negative sepsis, ii) during sterile injury induced by administration of lipopolysaccharide as well as iii) in pneumonia caused by the Gram-positive pathogen *Streptococcus pneumoniae*.

We accomplished this by a combination of in vivo studies together with cell-biologic approaches in vitro, comparing the effects of TREM-2 on different cell-types.

2. Results

2.1. The role of TREM-2 in Gram-negative sepsis

Triggering receptor expressed on myeloid cells-2 fine-tunes inflammatory responses in murine Gram-negative sepsis

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List of abbreviations

BMM, bone marrow-derived macrophages

CHO, Chinese hamster ovary

CLP, cecal ligation and puncture

DAP12, DNAX-activation protein of 12kDa

DOK3, downstream of kinase 3

E. coli, *Escherichia coli*

ITAM, immunoreceptor tyrosine-based activating motif

KC, keratinocyte-derived chemokine

KIR, killer cell immunoglobulin-like receptors

LPS, lipopolysaccharide

LTA, lipoteichoic acid

MAIR-II, myeloid-associated immunoglobulin-like receptor-II

MOI, multiplicity of infection

NFκB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

PLF, peritoneal lavage fluid

PM, peritoneal macrophage

S. aureus, *Staphylococcus aureus*

SHIP-1, Phosphatidylinositol-3,4,5-trisphosphate 5 phosphatase 1

Siglec, Sialic acid-binding immunoglobulin-type lectins

TG-PM, thioglycolate-recruited peritoneal macrophages

TLR, Toll-like receptor

TNFα, tumor necrosis factor α

TREM-2, Triggering receptor expressed on myeloid cells -2

Abstract

During infections, Toll-like receptor (TLR)-mediated responses require tight regulation to allow for pathogen removal, while preventing overwhelming inflammation and immunopathology. The triggering receptor-expressed on myeloid cells (TREM)-2 negatively regulates inflammation by macrophages and impacts on phagocytosis, but the function of endogenous TREM-2 during infections is poorly understood.

We investigated TREM-2's role in regulating TLR4-mediated inflammation by studying WT and TREM-$2^{-/-}$ mice challenged with LPS, and found TREM-2 to dampen early inflammation. Augmented early inflammation in TREM-$2^{-/-}$ animals was followed by an accelerated resolution and ultimately improved survival, associated with the induction of the negative regulator A20. Upon infection with E. coli, the otherwise beneficial effect of an exaggerated early immune response in TREM-$2^{-/-}$ animals was counteracted by a 50% reduction in bacterial phagocytosis. In line, TREM-$2^{-/-}$ PM exhibited augmented inflammation following TLR4 stimulation, demonstrating the presence and negative regulatory functionality of TREM-2 on primary PM. Significantly, we identified a high turnover rate, as TREM-2 RNA is 25-fold down-regulated and the protein proteasomally degraded upon LPS encounter, thus ensuring a tightly regulated and versatile system that regulates inflammation.

Our results illustrate TREM-2's effects on infection-triggered inflammation and identify TREM-2 as a potential target to prevent overwhelming inflammation while preserving anti-bacterial-effector functions.

Key words:

Peritonitis, Inflammation, macrophages, negative regulator

Introduction

Peritonitis is an infection of the otherwise sterile peritoneal cavity by intestinal bacteria like *Escherichia coli* (*E. coli*) and a frequent source of sepsis (1). Despite the availability of antibiotics, mortality rates remain high, mostly due to uncontrolled systemic inflammation and organ failure that cannot be treated as of today (2).

Pattern recognition receptors expressed on immune cells are the first sensors of invading pathogens and crucial in inducing protective inflammatory responses (3, 4). TLR2 and TLR4 are of particular importance as they detect bacterial lipoproteins and lipopolysaccharide (LPS) from *E. coli* to then elicit immune responses including the production of cytokines and chemokines that in turn attract neutrophils to the site of infection (5, 6). Thus, impaired TLR signaling substantially compromises these protective immune responses against *E. coli*, leading to uncontrolled bacterial spread, systemic inflammation and subsequent organ failure (5, 6). Systemic inflammation and subsequent organ failure are the hallmarks of sepsis (7, 8), and the most frequent cause of death from peritonitis in humans. The unfettered and overwhelming inflammatory response during sepsis either develops as a consequence of inefficient elimination of bacteria and persistent triggering of immune responses or due to an impaired resolution of inflammatory responses in the absence of pathogens. The fatal effects of persistent inflammation are best illustrated in models of endotoxemia, in which high doses of LPS induce a deadly systemic inflammatory response from which TLR4 deficient mice are protected (9). The outcome from severe infections and sepsis are therefore determined by a tightly regulated inflammatory response that ensures efficient bacterial clearance without causing inflammation-induced immunopathology. In concurrence with this notion, a number of regulatory molecules that modulate and fine-tune TLR-mediated inflammation have been identified over the last years (8, 10).

The triggering receptor expressed on myeloid cells (TREM)-2 is as such a prototypic regulator of TLR-mediated inflammation (11). TREM-2 is expressed on a variety of innate immune cells, including macrophages, dendritic cells and microglia (11-13). To this end, several in vitro studies showed that TREM-2 negatively regulates TLR-induced responses, by a yet unknown mechanism. Bone marrow-derived macrophages (BMM), thioglycolate-recruited peritoneal macrophages (TG-PM) and dendritic cells of TREM-2 knockout (TREM-$2^{-/-}$) mice produce more inflammatory cytokines compared to wild-type (WT) macrophages in response to LPS or CpG (12-16). While, to this end, an endogenous ligand remains unknown,

one report demonstrated that TREM-2 might bind bacterial cell wall components like LPS and lipoteichoic acid (LTA) (17). Significantly, TREM-2 is involved in bacterial phagocytosis. As such, TREM-2 deficient BMM were shown to less efficiently phagocytose *E. coli*, while TREM-2 transfection enabled non-phagocytic Chinese hamster ovary (CHO) cells to internalize *E. coli and Staphylococcus aureus* (*S. aureus*) (18) and i.v. injection of TREM-2 overexpressing myeloid cells improved bacterial clearance and outcome in a model of cecal ligation and puncture (CLP) (19).

TREM-2 is a single pass transmembrane protein of the immunoglobulin superfamily and contains a very small intracellular domain with no known motifs for signal transduction (11, 12, 20). TREM-2 signaling occurs via pairing with the immunoreceptor tyrosine-based activating motif (ITAM)-bearing adaptor DNAX-activation protein of 12kDa (DAP12) (11, 12), which is a promiscuous protein that interacts with different members of the TREM family and other receptors such as KIR, Ly49, MAIR-II or Siglec (14, 21). Upon ligation of its associated receptor, downstream signaling events are shaped by Src-family kinase mediated phosphorylation of DAP12's ITAM domain, followed by recruitment of the Syk protein tyrosine kinase. Syk is importantly involved in downstream engagement of diverse signaling pathways, including NFκB, PI3K and PLCγ (14, 21, 22). Considering TREM-2's inhibitory effects on macrophage activation, the phosphatase SHIP was found to negatively regulate PI3K recruitment to the TREM-2/DAP-12 signaling complex (23). Moreover, the requirement for DAP12 dependent phosphorylation of downstream of kinase (DOK)3 was shown recently to be involved in the TREM-2 associated prevention of inflammatory responses to low-dose LPS (24).

Based on the finding that mutations in the TREM-2 or DAP12 gene in humans lead to a condition called Nasu Hakola disease, which is characterized by bone cysts and presenile dementia (25-27), most previous studies focused on TREM-2's role in bone formation and the maintenance of brain homeostasis. As such, TREM-2 was found to be important in the differentiation of osteoclasts (28-31) and as a receptor for apoptotic neurons (32). Very recent data illustrate that rare heterozygous variants of TREM-2 are associated with an increased risk of Alzheimer's disease (33).

Despite TREM-2's reported function in inflammation and phagocytosis in vitro and in contrast to the closely related receptor TREM-1, which augments TLR-mediated inflammation and whose involvement was found associated with progressive inflammation

during human and murine sepsis (8, 34-37), the biological role of TREM-2 during infections in vivo is poorly understood. To this end, one report found TREM-2 involved in the bacterial clearance and control of inflammation during *Pseudomonas aeruginosa* keratitis (38) and one publication showed TREM-2 overexpressing bone-marrow macrophages to enhance phagocytosis of bacteria in vivo (19).

We recently investigated the function of TREM-2 during pneumococcal pneumonia and discovered a detrimental, and cell-type specific role for TREM-2. We found TREM-2 on alveolar macrophages to selectively suppress the production of the complement component C1q, which in turn impaired bacterial phagocytosis and outcome form pneumonia (39). However, the role of endogenous TREM-2 during Gram-negative infection and sepsis and the individual contribution of TREM-2 to the function of different immune cells in the course of inflammation therein are unclear. Using TREM-2 deficient mice, we studied the biological function of TREM-2 in regulating TLR4-induced responses in vivo during endotoxemia and Gram-negative sepsis, as well as on a cellular level by investigating TREM-2´s expression and function on different macrophage subsets, including resident peritoneal macrophages.

Materials and Methods

Animals:

Age matched 8-12 week old female TREM-2$^{-/-}$ mice and WT C57BL/6 controls were used. TREM-2$^{-/-}$ mice were kindly provided by Marco Colonna, Washington University, and back-crossed onto a >98% B6 C57BL/6 background facilitated by genome-wide SSLP typing at 10cM intervals (Speed Congenics Facility of the Rheumatic Diseases Core Center) (13). All in vivo experiments were approved by the Institutional Review Board of the Medical University of Vienna and the Austrian Ministry of Sciences (BMWF-66.009/065-II/3b/2011).

Mouse models of endotoxemia and E. coli peritonitis:

Mice were i.p. injected with 37mg/kg LPS from *E. coli* or 1 x 10^4 CFU *E. coli* (O18.K1) as described previously (40-42) and sacrificed at indicated times to collect peritoneal lavage fluid (PLF), blood and organs (41). Body temperature was measured rectally. PLF supernatants were collected for ELISA measurements; pelleted cells were stained with Türk's solution and counted using a hemocytometer. Differential cell counts were performed on cytospin preparations stained with Giemsa. Liver samples were homogenized using Precellys 24^{TM} (Peqlab) and prepared for PCR and ELISA as described earlier (43). Cytokines and chemokines were quantified using commercially available ELISAs (RnD Systems). Transaminases were measured as described earlier (40). Bacterial loads were determined by plating serial dilutions of PLF, blood or organ homogenates on blood agar plates. Plates were incubated overnight at 37°C and colonies were counted the next day. For survival studies mice were treated with either 37mg/kg or 43mg/kg LPS or infected with 1x10^4 CFU *E. coli* and their health state was checked every 2 hours.

Cell isolation, culture and stimulation:

Peritoneal macrophages (PM) were isolated by peritoneal lavage and BMM were isolated and differentiated as described earlier (44). Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS (Gibco) and 1% penicillin/streptomycin. For in vitro stimulations cells were plated at 2.5 x 10^5/ml in 96-well plates and let adhere overnight. Subsequently, cells were washed with PBS and stimulated with LPS (100ng/ml) from *E. coli* or heat-killed *E. coli* (4 x 10^7 CFU/ml) for indicated time points. The translation inhibitor cyclohexamide and the proteasome inhibitor MG132 were used at 100µg/ml.

Generation of TREM-2 overexpressing RAW264.7 cells:

Expression plasmids were generated by Gateway cloning using pDONR™201 as a shuttle vector and pCMV-StrepIII-HA-GW as expression vector. The following primers were used to obtain Gateway compatible fragments (containing *att* sites) by PCR: sense attB1 GFP 5´*ggggacaagtttgtacaaaaaagcaggctagactgccatggtgagcaagggc*3´, antisense-attB1 GFP 5´*ggggaccactttgtacaagaaagctgggttcttgtacagctcgtccat*3´, sense-attB1 TREM-2 5´*ggggacaagtttgtacaaaaaagcaggctagactgccatgggaccctctccac*3´, antisense-attB1 TREM-2 5´*ggggaccactttgtacaagaaagctgggttcgtacctccgggtcc*3´. Constructs were stably overexpressed in RAW264.7 cells by retroviral transfection as previously described (45).

Western blotting

For preparation of whole cell extracts, cells (1-5 x 10^6) were stimulated with LPS (100ng/ml) and lysed in RIPA-buffer (50mM Tris-HCl, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% DOC, complete protease inhibitor cocktail tablets (Roche), 10mM NaF, 1mM orthovanadate). After centrifugation, supernatants were frozen at -80°C. Protein concentrations were determined using the Pierce BCA assay (Thermo scientific) and western blotting was conducted as described earlier (46) using a biotinylated sheep anti-mouse TREM-2 Ab (RnD Systems) and Streptavidin-HRP (RnD Systems).

Phagocytosis assays

In vitro FACS based phagocytosis-assays using FITC-labeled *E. coli* were performed as previously described (43). In vivo phagocytosis was assessed by injecting FITC-labelled *E. coli* (1x10^7 CFU/mouse) intraperitoneally. Peritoneal exudate cells were harvested 1h later and analyzed by FACS using antibodies against CD3 (eBioscience), F4/80 and Ly6G (Biolegend).

RT-PCR

mRNA was isolated from cells using TRIzol® Reagent (Invitrogen) and cDNA was generated using the iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using iTaq SYBR Green Supermix (Bio-Rad), the StepOnePlus™ cycler (Applied Biosystems) and specific primers for TREM-2, A20, IL-6 (TREM-2: 200bp, forward *ttgctggaaccgtcaccatc*, reverse *cacttgggcaccctcgaaac*; A20, 296bp, forward *catccacaaagcacttattgaca*, reverse *aacaggactttgctacgacactc*; IL-6: 187bp, forward *ccacggccttccctacttca*, reverse

tgcaagtgcatcgttgttc; TLR4: 365bp, forward *ctcctgcctgacaccaggaagcttga*, reverse *aggccaattttgtctccacagccacca*).

Statistical analysis

Statistical evaluation was performed using GraphPad Prism software. Data are represented as mean ± SEM and were analyzed using either Student´s t-test, comparing two groups, or one-way ANOVA analysis, followed by Tukey multiple comparison test, for more groups. Survival studies were analyzed using a Log rank (Mantel-Cox) test. Differences with a p-value ≤ 0.05 were considered significant.

Results

TREM-2 delays LPS-induced inflammation and increases mortality from endotoxemia

TREM-2 has been shown to regulate TLR4 mediated responses (15) and to bind LPS (17). To investigate the impact of TREM-2 on the in vivo inflammatory response to LPS, we challenged WT and TREM-2$^{-/-}$ mice i.p. with LPS from *E. coli* and harvested organs 6h later, at a time point before mice show any clinical signs of disease. In line with published data showing that TREM-2 negatively impacts TLR4 mediated inflammation in vitro, the absence of endogenous TREM-2 resulted in a more pronounced early (6h) inflammatory response. This was indicated by a substantially increased influx of cells (Fig. 1A), consisting of macrophages and neutrophils, and enhanced IL-6 and KC levels (Fig. 1B) in the peritoneal cavity of TREM-2$^{-/-}$ as compared to WT animals. TNF levels and body temperature were similar between WT and knockout animals at this early time point (Fig. 1C, Fig. 1D).

Interestingly, this augmented early inflammatory response in TREM-2$^{-/-}$ mice was followed by an accelerated resolution of inflammation, as indicated by a rapid decline of peritoneal cells (Fig. 1A) and IL-6 (Fig. 1B) 20h after LPS administration. Lower plasma TNFα levels (Fig. 1C) at this late time point correlated with less pronounced hypothermia (Fig. 1D) in TREM-2 deficient animals, whereas WT mice still displayed signs of ongoing inflammation (Fig. 1B, 1D). As a result of the rapidly resolving inflammatory response, TREM-2$^{-/-}$ mice showed a significantly improved survival from LPS-induced shock (Fig. 1E). The dynamic of an enhanced early inflammatory response, followed by an accelerated resolution in the absence of TREM-2, led us to hypothesize that TREM-2 might not only tune down inflammatory genes, but might also affect the expression of negative regulators of TLR signaling that promote the resolution of inflammation. We therefore tested WT and TREM-2$^{-/-}$ livers for the induction of classical negative regulators of TLR4-signaling such as IRAK-M, Tollip and A20 (47). In line with this idea, we discovered robustly increased expression levels of the early NFκB target gene A20 in TREM-2 deficient mice early (6h) after administration of LPS compared to their WT counterparts (Fig. 1F), while TREM-2 did not impact on the other genes tested (data not shown). Together, TREM-2 deficiency was associated with an augmented immediate inflammatory response to LPS, an earlier induction of the negative regulator A20 and an accelerated resolution of inflammation that ultimately resulted in an improved survival.

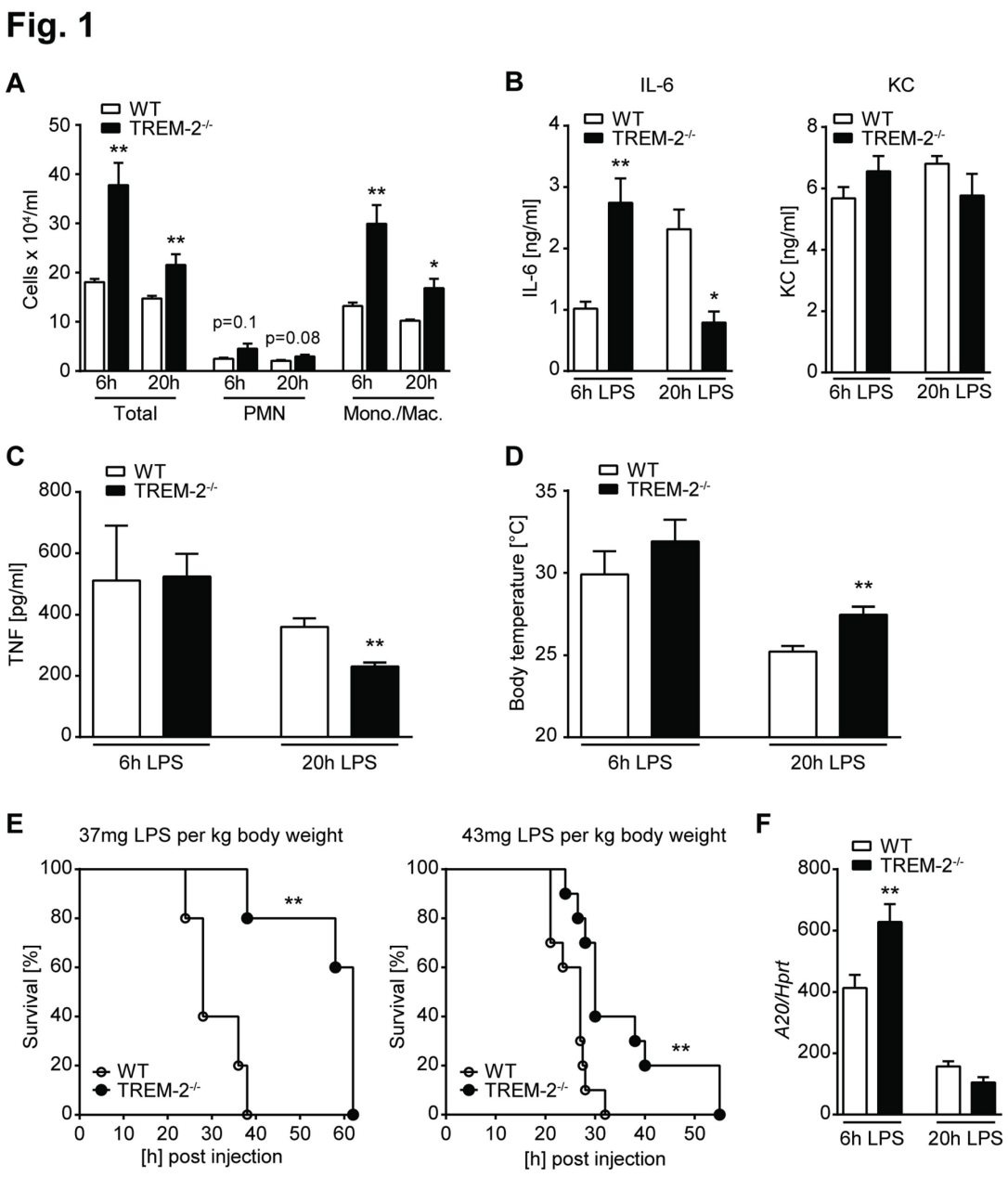

Figure 1. TREM-2 exerts detrimental effects during endotoxemia. WT and TREM-2$^{-/-}$ mice were i.p. injected with 37mg/kg LPS. Peritoneal cell numbers (A), cytokines in PLF (B) and plasma (C) were assessed at 6h and 20h post injection. Body temperature was measured at 6h and 20h post injection (D). Survival (n=5 per group) was monitored following i.p. LPS administration (37mg/kg or 43mg/kg) over a period of 72h (E). A20 was detected in liver samples 20h after LPS administration by RT-PCR (F). Data presented in A-D and F are mean+/-SEM of n=6/group and time, all data are representative of 2 independent experiments. * indicates $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Unaltered bacterial counts despite an enhanced inflammation in TREM-2 deficient mice

Based on these data, we wanted to study the potential role of endogenous TREM-2 in a clinically relevant model of Gram-negative peritonitis. WT and TREM-2$^{-/-}$ mice were infected i.p. with *E. coli* and sacrificed either 6h post-infection, to evaluate the early inflammatory response, or 16h post-infection, at a time when differences in bacterial clearance and organ damage can be assessed. Concurrent with our observations during endotoxemia, we discovered significantly higher numbers of macrophages and neutrophils in the peritoneal exudate of TREM-2 deficient mice 6h post-infection, indicating a stronger early innate immune response to the bacteria (Fig. 2A).

Based on the enhanced recruitment of phagocytes to the site of infection in TREM-2 deficient animals, we anticipated this response to positively contribute to bacterial clearance and containment of infection at later stages of the disease. However, the enhanced cell influx was not associated with an improved bacterial clearance at later stages, as WT and TREM-2$^{-/-}$ animals had comparable bacterial numbers in the peritoneal cavity, blood and distant organs (Fig. 2B) and TREM-2 deficient mice continued to exhibit more inflammatory cells in the peritoneal lavage fluid (Fig. 2C), indicating ongoing inflammation. This was accompanied by elevated peritoneal concentrations of MCP-1 and KC (Fig. 2D), more pronounced signs of systemic inflammation and sepsis-associated liver injury, as illustrated by elevated plasma levels of IL-6 (Fig. 2E) and of the transaminases ASAT and ALAT (Fig. 2F) in TREM-2$^{-/-}$ mice. Despite these differences in the inflammatory response, but in line with our finding of an unaltered bacterial clearance, the absence of TREM-2 did not impact on survival during *E. coli* peritonitis (Fig. 2G).

The fact that TREM-2 deficiency neither improved bacterial clearance nor survival despite an enhanced early influx of inflammatory cells suggested that TREM-2 might in parallel affect crucial antibacterial effector mechanisms of phagocytes. Since TREM-2 was proposed to be a phagocytic receptor on BMM in vitro (18), we next assayed the ability of WT and TREM-2$^{-/-}$ PM to phagocytose *E. coli* and found TREM-2$^{-/-}$ primary PM to be strongly impaired in their capability to ingest *E. coli* (Fig. 2H). In order to validate our in vitro finding, we then performed an in vivo phagocytosis assay and injected FITC-labelled bacteria intraperitoneally into WT and TREM-2 deficient mice to then analyze the bacterial uptake by peritoneal exudate cells.

Fig. 2

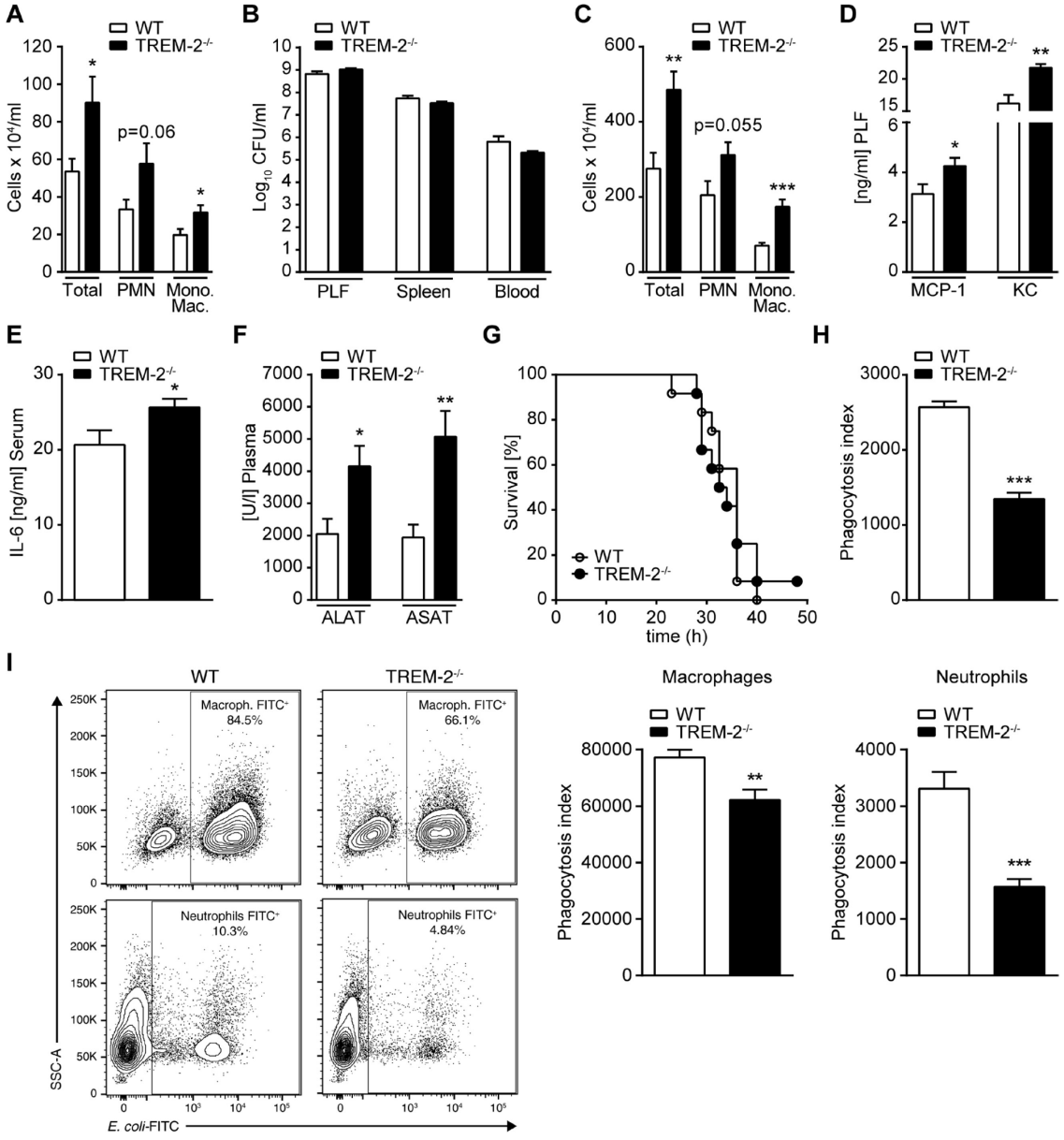

Figure 2. TREM-2 dampens inflammation and promotes bacterial clearance in *E. coli* peritonitis. WT and TREM-2$^{-/-}$ mice (n=8 per group) were i.p. infected with 1x10^4 CFU *E. coli* and sacrificed either 6h or 16h later. Peritoneal cell influx was accessed at 6h post infection (A). At 16h post infection, bacterial counts in PLF, spleen and blood (B) and peritoneal cell numbers (C) were determined. MCP-1 and KC was measured in PLF (D) and IL-6 in plasma samples (E) by ELISA. Transaminase levels were detected in plasma (F). WT and TREM-2$^{-/-}$ mice (n=12 per group) were infected i.p. with 1 x 10^4 CFU *E. coli* and survival was monitored (G). Primary PM of WT and TREM-2$^{-/-}$ mice were incubated for 1h with FITC-labeled *E. coli* at 37°C and the phagocytic uptake of bacteria was quantified by FACS (H). WT and TREM-2-/- mice were injected i.p. with FITC-labelled bacteria and the bacterial uptake by peritoneal exudate cells was analyzed 1h later by FACS (I). Data are representative

of at least two independent experiments and depicted as mean+/-SEM. * indicates $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Supporting and extending our in vitro data, we found TREM-2 deficient PMs and neutrophils to exhibit an impaired ability to phagocytose bacteria (Fig. 2I). Collectively, these results illustrate an ambiguous role of TREM-2 during bacterial infection, as it negatively regulates early inflammation while at the same time being critical for the uptake of *E. coli*.

Glycosylated TREM-2 is present on resident peritoneal macrophages

TREM-2$^{-/-}$ mice consistently exhibited an augmented early inflammatory response in the peritoneal cavity upon LPS or bacterial infection. PMs are strategically positioned immune cells in the peritoneal cavity and are crucial in eliciting early inflammation upon invasion of microbes (48, 49). Our data therefore suggest an important role for TREM-2 in regulating PM responses, although earlier reports pronounced TREM-2 to not be expressed on resident PM (13). We therefore decided to challenge this notion and investigated the potential presence and regulation of TREM-2 on PMs. We first compared TREM-2 mRNA expression by primary PM, BMM and RAW 264.7 cells, a peritoneal macrophage-like cell line. We revealed substantial amounts of TREM-2 transcript in primary PM (Fig. 3A), though slightly lower than transcript levels of BMM, which were reported earlier to express TREM-2. Significantly, using western blotting, we further confirmed the presence of TREM-2 protein in PM, RAW 264.7 cells and BMM (Fig. 3B). TREM-2$^{-/-}$ PM were used to verify the specificity of the antibody (Fig. 3C).

Interestingly, we discovered at least four TREM-2 specific bands, a double band slightly above 26 kDa and another double band around 35 kDa (Fig. 3B). TREM-2 was earlier proposed to be posttranslationally modified, with two predicted sites of N-linked glycosylation (50). To test this experimentally, we digested BMM lysate with the specific glycosidases endoglycosidase H (Endo H) and peptide-*N*-glycosidase F (PGNase F) and found the smaller size fragments to be Endo H sensitive, indicating core-glycosylation, and the larger fragments around 35 kDa to be Endo H resistant, resembling the fully glycosylated forms of TREM-2 (Fig. 3D). These data suggest that TREM-2 is core-glycosylated in the ER of macrophages and then further shuttled through the Golgi apparatus, where it gets further glycosylated.

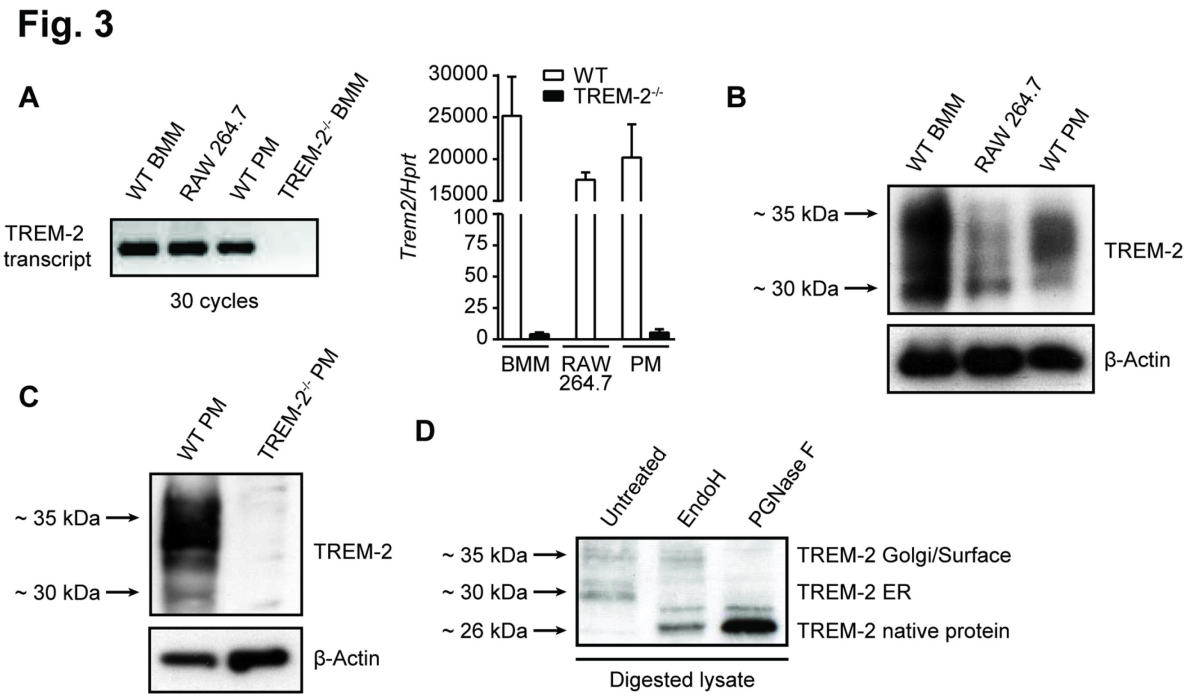

Figure 3. TREM-2 is expressed in resident PM and post-translationally modified. TREM-2 expression was assessed in BMM, RAW 264.7 cells and resident PM on transcript level after 30 PCR cycles as well as by RT-PCR (A) or on a protein level by western blotting (B, C). BMM lysates were digested with either Endo H or PGNAse F and TREM-2 was detected by westernblotting (D). Data are representative of two independent experiments.

TREM-2 has a short half-life and is rapidly down-regulated in response to LPS

To finally understand why TREM-2's effects on inflammation in vivo seemed restricted to early time points after microbial challenge we decided to examine the regulation and expression dynamic of TREM-2 in more detail. We thus assessed TREM-2 protein expression in selected macrophage populations upon LPS challenge and observed a rapid decline of TREM-2 protein levels upon LPS encounter, irrespective of the macrophage subset tested (Fig. 4A). To investigate how LPS induced the prompt decline of TREM-2 protein, we first focused on TREM-2's transcriptional regulation. Following 1h of LPS treatment, TREM-2 transcript levels were strongly reduced in primary PM (Fig. 4B). This supported a role for transcriptional regulation, but also suggested that TREM-2 has a very short half-life and has to be continuously resynthesized, in order to maintain baseline expression. We tested this hypothesis using cyclohexamide, an established inhibitor of translation, and observed a rapid decline of TREM-2 expression (Fig. 4C), comparable to the decline we observed upon LPS treatment (Fig. 4A).

To then assess the type of protein degradation, we studied the impact of proteasomal degradation of TREM-2 and added the proteasomal inhibitor MG132 to cells to discover a solid and immediate accumulation of TREM-2 protein (Fig. 4D).

Fig. 4

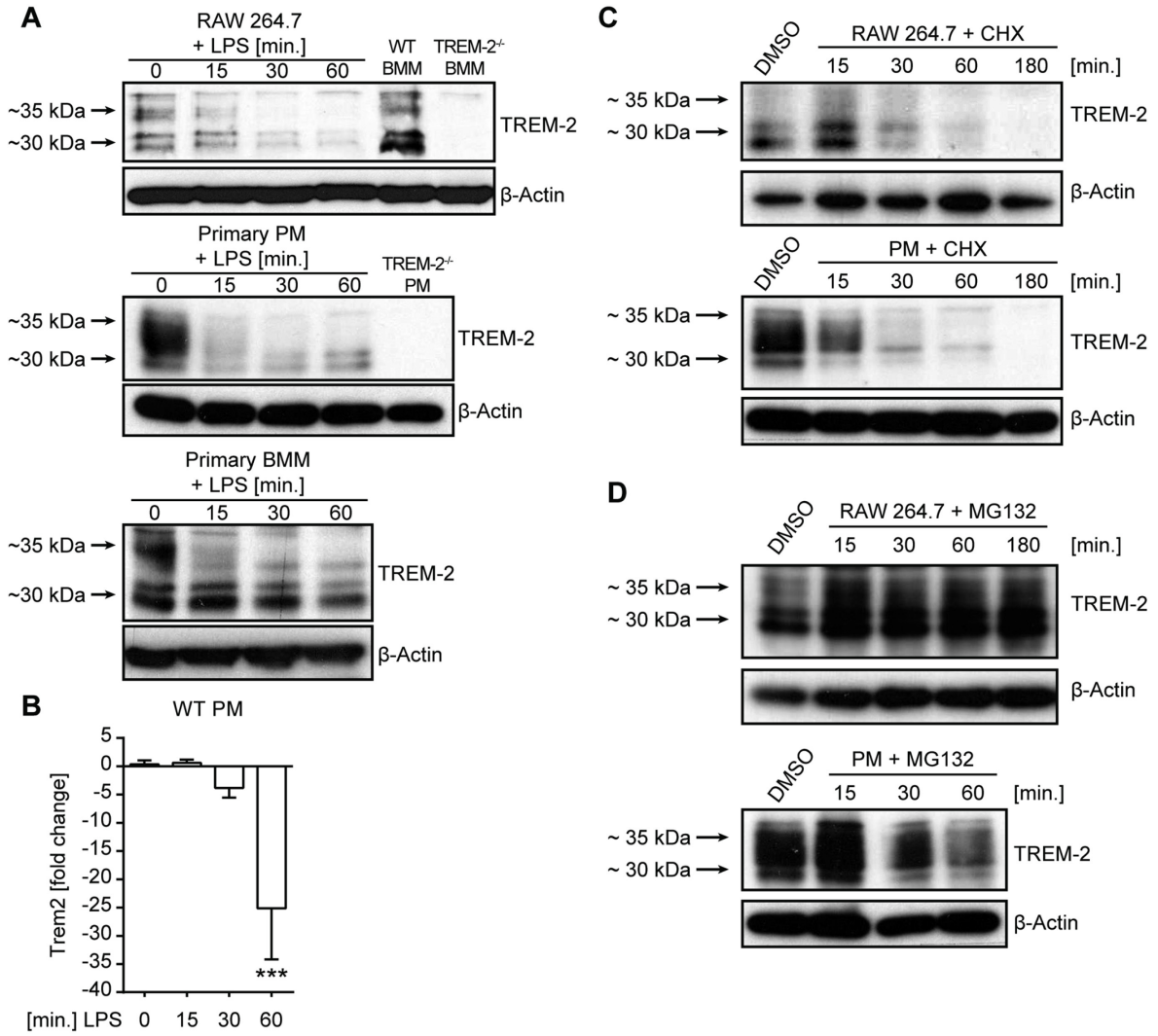

Figure 4. TREM-2 is rapidly downregulated in response to LPS. TREM-2 protein levels in RAW 264.7 cells, primary PM and BMM were detected upon LPS (100ng/ml) stimulation over time (A). TREM-$2^{-/-}$ PM were stimulated with LPS (100ng/ml) and TREM-2 RNA levels, expressed as fold change compared to untreated WT, were determined at indicated timepoints (B). RAW 264.7 cells and primary PM were cultured in the presence of either 100 μg/ml cyclohexamide (C) or 100 μg/ml MG132 (D) and TREM-2 protein levels were assessed. RNA measurements were performed in quadruplicates. Westernblots are representative of at least two independent experiments. * indicates $p \leq 0.05$, and *** $p < 0.001$

Collectively, our data suggest that TREM-2 has a very high turnover rate in macrophages and that TREM-2 expression is tightly controlled by continuous proteasomal degradation and re-synthesis, thus ensuring a tightly regulated and very versatile system that regulates the inflammatory response.

TREM-2 negatively regulates TLR-4 mediated cytokine responses in primary PM

Having established that TREM-2 is expressed and tightly regulated on primary PM, we wanted to finally address the potential effect of endogenous TREM-2 in regulating the inflammatory responses by PM. We stimulated resident PM from WT and TREM-$2^{-/-}$ mice with LPS or *E. coli* and discovered substantially higher IL-6 induction by TREM-2 deficient resident PM as compared to WT PM on mRNA (Fig. 5A) as well as on protein level (Fig. 5B). This was not only true for IL-6, but also for other genes such as KC (Cxcl1), TNFα, COX-2, TREM-1 and IκBα (Fig. 5C), which are typically induced by LPS, clearly suggesting TREM-2 to be present and functional as a negative regulator on these primary cells. As a control we tested TREM-2 overexpressing RAW264.7 macrophages upon stimulation with LPS or *E. coli* and discovered significantly less IL-6 in response to both stimuli (Fig. 5D).

Based on a publication showing enhanced TLR4 expression in LPS treated TREM-2 deficient alveolar macrophages (51), we compared TLR4 mRNA levels in quiescent and LPS challenged primary PM of WT and TREM-$2^{-/-}$ mice. While we did not find any differences in baseline expression, TREM-2 deficient cells failed to downregulate TLR4 mRNA in response to LPS (Fig. 5E), which might contribute to the augmented inflammatory response we observed in TREM-2 deficient PMs. Together with the boosted early inflammation we have observed in vivo, our data show that endogenous TREM-2 expression is strictly controlled on primary PM and importantly contributes to the negative regulation of TLR4-triggered inflammation in vitro and in vivo.

Fig. 5

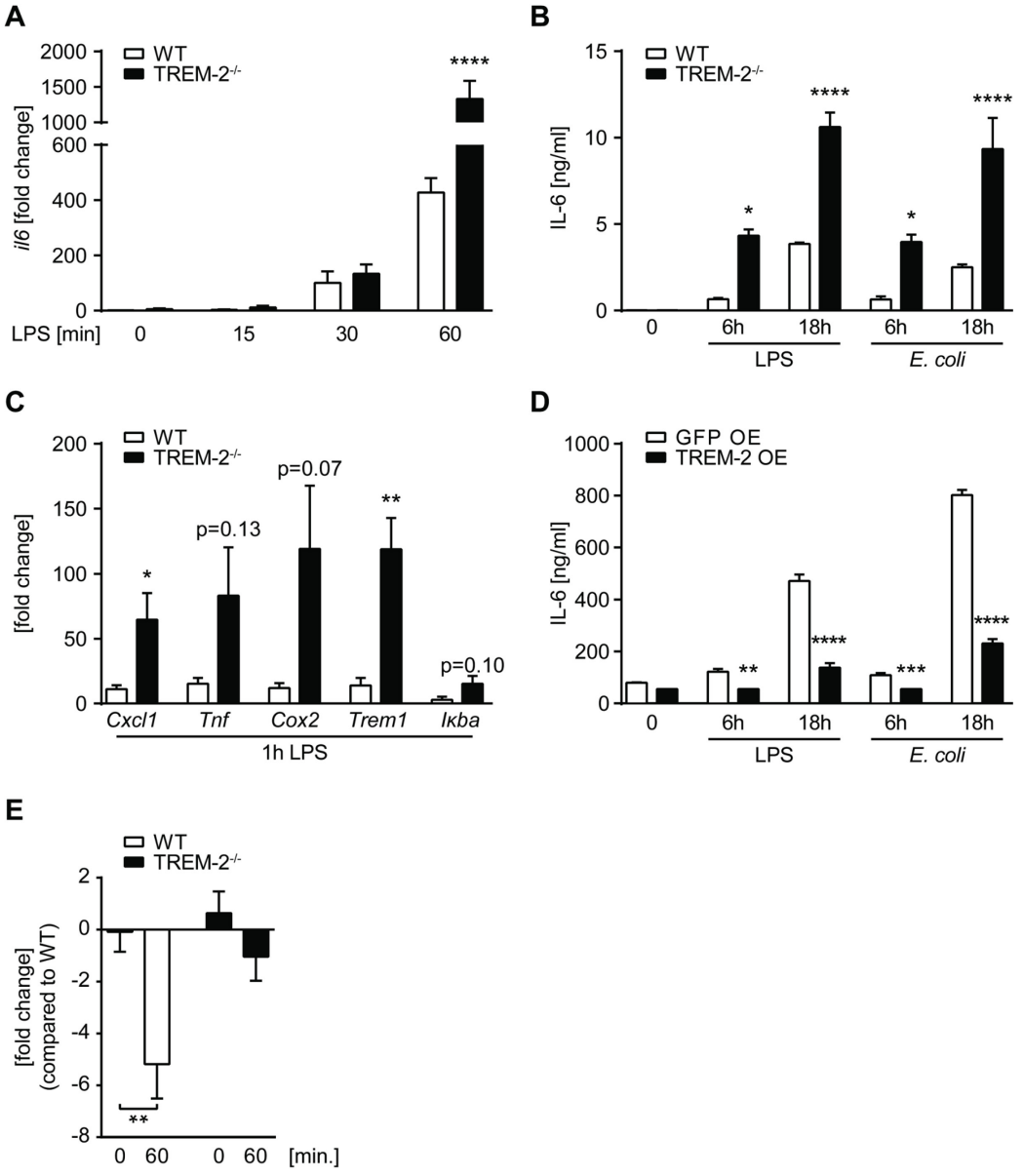

Figure 5. TREM-2 negatively regulates TLR4-mediated cytokine responses by PM. Primary PM from WT and TREM-2$^{-/-}$ mice were stimulated with LPS (100ng/ml) or 4x10^7 CFU/ml *E. coli* for indicated times and RNA levels, expressed as fold change compared to untreated WT, of different inflammatory genes (A, C) or protein levels of IL-6 (B) were quantified. RAW264.7 cells overexpressing TREM-2 and GFP-control cells were stimulated with 100ng/ml LPS or 4x10^7 CFU/ml *E. coli* and IL-6 protein levels were assessed after 6h and 18h in supernatants by ELISA (D). TREM-2$^{-/-}$ PM were stimulated with LPS (100ng/ml) and TLR4 mRNA levels, expressed as fold change compared to untreated WT, were assessed on resting and stimulated cells (E). Data are mean+/-SEM of quadruplicates and representative of two independent experiments. * indicates $p \leq 0.05$, ** $p \leq 0.01$, and *** $p < 0.001$.

Discussion

The fine-tuning of TLR-mediated signaling events is required to elicit a profound and powerful immune response while simultaneously triggering regulatory pathways that prevent overwhelming inflammation and ultimately restore tissue homeostasis (52). This beneficial balance is achieved through the production of inflammatory mediators upon infection and the simultaneous induction of genes that negatively feedback on inflammation (52, 53).

In the present study we investigated the biological function of TREM-2, which is considered a negative regulator of inflammation. We discovered that TREM-2 is essential in negatively regulating early inflammation during endotoxemia and Gram-negative sepsis in vivo and propose these effects to be attributed to TREM-2´s effects on resident peritoneal macrophages. The TREM-2 mediated delay of early inflammation proved detrimental during LPS induced shock and was associated with a diminished induction of the negative regulator A20, ultimately resulting in prolonged inflammation and impaired survival of WT animals (Fig. 1). Upon infection with *E. coli*, TREM-2's inhibitory impact on early inflammation was counteracted by its crucial role as a phagocytic receptor, which eventually resulted in an unaffected outcome when comparing WT and TREM-2$^{-/-}$ mice (Fig. 2). Given that the presence of TREM-2 consistently dampened early peritoneal inflammation in response to both, LPS and live bacteria, and taking into account the principal importance of resident PM in sensing the presence of pathogens in the otherwise sterile peritoneal cavity (48, 49), we investigated expression and function of TREM-2 on resident PM in more detail. We here show that primary, resting PM express substantial amounts of TREM-2 (Fig. 3) and that this is functionally relevant both in vitro (Fig. 5) as well as during endotoxemia (Fig. 1) and bacterial infection (Fig. 2) in vivo.

While it seems established that TREM-2 is expressed on inflammatory macrophages, this receptor was thus far considered absent on resident PM, based on surface FACS staining (13). The current unavailability of commercial antibodies that reliably detect TREM-2 on cells together with the rapid downregulation upon activation makes it challenging to conclusively demonstrate the presence of TREM-2 on primary PM. However, functional data derived from our in vivo studies that exhibit changes in phagocytosis already one hour after injection of bacteria (Fig. 2) as well as the altered inflammatory response early during peritonitis (Fig. 1 and 2), which was shown earlier to be induced by PM (48, 49), strongly suggest the presence of TREM-2 on resident peritoneal phagocytes.

Our data illustrate that a large proportion of TREM-2 is stored intracellular and that especially the surface band is highly sensitive to degradation (Fig. 4) and probably also shedding, as was shown by other groups (50). Based on the rapid downregulation of (surface) TREM-2 upon activation of cells, we believe that westernblotting might be a more sensitive method to detect the presence of TREM-2, as this method does not require lengthy handling of cells and furthermore allows the simultaneous detection of intracellular and surface protein. In line with this, we believe that differences in TREM-2 protein levels between PM and BMM might result from PM activation and protein degradation as a result of time consuming isolation procedures, while BMM represent truly resting cells. TREM-2 deficient primary PM exhibited an enhanced inflammatory reaction upon TLR4 activation in vitro. Based on publications showing TREM-2 to affect ERK signaling in human osteoclasts (30) and murine dendritic cells (54), we attempted to study ERK phosphorylation in primary PM but failed to obtain reproducible results. However, and in contrast to WT cells, we found TREM-2 deficient PM to maintain steady levels of TLR4 mRNA following LPS stimulation, which was in line with previously published data in alveolar macrophages, where TREM-2 levels were reduced using siRNA (51). Downregulation of TLR4 in response to LPS is considered a negative regulatory mechanism that contributes to controlling the inflammatory response (47) and we suggest that the preservation of TLR4 expression might in part account for the hyperinflammatory phenotype of TREM-2 deficient PMs.

On a cellular level we could show that processing of TREM-2 in macrophages involves glycosylation at two distinct sites and that all tested macrophage-subsets, namely BMM, RAW 276.4 cells and resident PM, store substantial amounts of TREM-2 in the ER (Fig. 3). We further observed that TREM-2 glycosylation seems to be cell type- and site-specific, as we discovered different glycosylation patterns between BMM and PM. This is not unusual, as cell type- and site-specific glycosylation patterns have been described for several proteins (55-57) and might regulate cell type-specific binding affinities to respective ligands. A recent publication showed that TREM-2, when overexpressed in COS-7 or HEK293 cells, undergoes ectodomain shedding, and that the remaining C-terminal fragment is further degraded by γ-secretase (50). However, we could not detect endogenous soluble TREM-2 in the serum of WT mice nor in the supernatant of peritoneal macrophages after TCA-precipitation in vitro (data not shown). This could either indicate cell-type-specific processing of TREM-2 or a lack of assay sensitivity. Looking closer into the regulation of TREM-2 expression in peritoneal

macrophages, we found that TREM-2 protein levels are tightly regulated by continuous proteasomal degradation and resynthesis. LPS rapidly turns off TREM-2 transcription and this immediately impacts on TREM-2 protein levels, due to its high turnover rate (Fig. 4). TREM-2 downregulation was demonstrated earlier on both mRNA level in liver macrophages during endotoxemia (58) as well as on protein level upon overnight LPS challenge in vitro (13), but the celerity of TREM-2 downregulation we discovered here upon LPS treatment was not known before.

Considering the fact that mortality from endotoxemia is attributed to overwhelming inflammation and that the absence of classical negative regulators leads to a higher susceptibility to septic shock, it seems counterintuitive at first that TREM-2 would exert detrimental effects in this model. TREM-2´s presence on resting cells and its rapid decline upon stimulation is in contrast to what is known about classical negative regulators of TLR signaling (47). A20 and IRAK-M are prototypic negative regulators, which are barely expressed or even absent in resting cells, but strongly induced upon TLR ligation to then negatively feedback on the TLR-signaling cascade (47, 59, 60). We therefore believe that tonic TREM-2 signaling fine-tunes the sensitivity of quiescent cells to a certain stimulus and we propose that the mere presence of TREM-2 at the time of LPS administration was sufficient to delay the onset of inflammation. Thus, TREM-2 not only postponed the induction of the early immune response in WT mice but at the same time delayed the induction of the TLR-induced, early NFκB target gene A20 (Fig. 1), a ubiquitin-editing protein that attenuates NFκB signaling by promoting the degradation of RIPK1 and TRAF6, crucial mediators of TNF and TLR-induced NFκB activation (61). As such, A20 negatively regulates cytokine responses and contributes to the termination of inflammation in mice and humans (52, 59, 62) and it was shown earlier that hepatic overexpression of A20 was sufficient to protect mice from LPS-induced shock (63).

We encountered a different situation when we challenged mice with live *E. coli*, i.e. a persistent stimulus, which is not as rapidly removed as LPS, and continuously replicates in vivo. Despite the fact that TREM-2 knockout mice displayed no difference in survival during *E. coli* induced peritonitis, their immune response was severely altered (Fig. 2). Just like upon LPS administration, the absence of endogenous TREM-2 during *E. coli* infection led to accelerated phagocyte recruitment to the site of infection, but did not affect the outcome from this disease. Since both a strong inflammatory response and an efficient elimination of

bacteria via phagocytosis are required to clear bacterial peritonitis, we propose that the augmented inflammatory response was ultimately counteracted by impairment in bacterial phagocytosis, which, in line with previous studies (18, 19, 64), emphasizes the importance of TREM-2 as a phagocytic receptor. Our data demonstrate that in vivo, not only macrophages, but also neutrophils were hampered in their ability to ingest E. coli, thus supporting our hypothesis that TREM-2's impact on inflammation was counteracted by its effects on phagocytosis. We did not assess later timepoints after infection but expect also newly recruited macrophages, which were earlier shown to express TREM-2 (13), to phagocytose less bacteria in the absence of TREM-2. Regarding TREM-2's effects on neutrophils, we can only speculate about TREM-2 presence on these cells, as this has not been shown before. Alternatively, neutrophils might be affected by the altered inflammatory milieu or soluble TREM-2, which has been shown to bind various bacteria (17) might be released in vivo to impact on the phagocytic capacity of neutrophils. Of interest, we recently observed that TREM-2 deficiency was beneficial during murine pneumococcal pneumonia, which was explained by an increased bacterial uptake by alveolar macrophages in the absence of TREM-2 (39). Mechanistically we discovered a cell type-specific effect, as TREM-$2^{-/-}$ alveolar macrophages exhibited significantly higher levels of the complement component C1q that was sufficient to enhance the phagocytosis of bacteria. However, primary PM from either wt or TREM-$2^{-/-}$ mice did not differ in C1q production (data not shown) and we therefore believe that the differences in the uptake of bacteria by PM are directly caused by the absence of TREM-2 and its function as a phagocytic receptor.

Taken together, we propose that TREM-2 plays a dual role during infection. TREM-2 crucially fine-tunes immediate TLR4-induced inflammation on resident PM, resulting in delayed immediate inflammatory responses in vivo and in vitro, while at the same time acting as a phagocytic receptor for *E. coli*.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

2.2. TREM-2 and pneumococcal pneumonia

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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Running Title:

The role of TREM-2 in pneumococcal pneumonia.

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Abstract

Phagocytosis and inflammation within the lungs is crucial for host defense during bacterial pneumonia. Triggering receptor expressed on myeloid cells (TREM)-2 was proposed to negatively regulate TLR-mediated responses and enhance phagocytosis by macrophages, but the role of TREM-2 in respiratory tract infections is unknown. Here, we established the presence of TREM-2 on alveolar macrophages (AM) and explored the function of TREM-2 in the innate immune response to pneumococcal infection in vivo. Unexpectedly, we found *Trem-2*$^{-/-}$ AM to display augmented bacterial phagocytosis in vitro and in vivo compared to WT AM. Mechanistically, we detected that in the absence of TREM-2, pulmonary macrophages selectively produced elevated complement component 1q (*C1q*) levels. We found that these increased C1q levels depended on peroxisome proliferator-activated receptor-δ (PPAR-δ) activity and were responsible for the enhanced phagocytosis of bacteria. Upon infection with *S. pneumoniae*, *Trem-2*$^{-/-}$ mice exhibited an augmented bacterial clearance from lungs, decreased bacteremia and improved survival compared to their WT counterparts. This work is the first to disclose a role for TREM-2 in clinically relevant respiratory tract infections and demonstrates a previously unknown link between TREM-2 and opsonin production within the lungs.

Author Summary

Bacterial respiratory tract infections are a major cause of morbidity and mortality and *Streptococcus pneumoniae* (*S. pneumoniae*) remains the main cause of community acquired pneumonia worldwide. The continued rise in antibiotic resistance stresses the need for better insights into the host defense mechanisms associated with pneumococcal pneumonia. The early innate immune response that constitutes bacterial phagocytosis, complement activation and inflammation is critical for the outcome during pneumonia. The triggering receptor expressed on myeloid cells 2 (TREM-2) has recently been shown to be both a negative regulator of the inflammatory response and a promoter of phagocytosis, but its contribution to pneumonia remains unknown. In our study, we unexpectedly found that alveolar macrophage expressed TREM-2 is detrimental in bacterial phagocytosis and clearance during pneumococcal pneumonia. This occured via the suppressive effects of TREM-2 on complement component 1q (C1q), an important regulator of bacterial phagocytosis that is crucial for the host response during pneumonia. Thus, targeting the TREM-2 pathway could be used as a novel strategy for modulating C1q production and pulmonary innate immune responses, which could be of clinical relevance during pneumonia and other respiratory tract infections.

Introduction

Phagocytosis is the process by which cells ingest particles and is a major effector mechanism of the innate immune system. Professional phagocytes such as macrophages use a variety of surface receptors including scavenger-, Fc- and opsonin-receptors to internalize microbes. In addition, innate immune receptors such as Toll like receptors (TLRs) recognize conserved microbial structures and trigger the production of pro-inflammatory cytokines and chemokines, thereby shaping both the innate and adaptive immune response.

TLR activation and phagocytosis are intimately linked. Upon phagocytosis of Gram positive and negative bacteria, TLR2 and 4 initially located at the plasma membrane accumulate into phagosomes, sample their contents and elicit immune responses to products which only become accessible after digestion of the cell wall of bacteria (1-3). TLR activation also enhances the expression of phagocytic receptors such as scavenger receptor A or MARCO and elevates opsonin levels (4-7). The prime aim of such inflammatory phagocytosis is to urgently remove invading pathogens before they multiply, invade other tissues and spread systemically. The rapid elimination of pathogens thus prevents excessive inflammation that can otherwise result in immunopathology and organ failure. Understanding the mechanisms that control phagocytosis and limit inflammation is important as this has implications in the survival from infectious diseases. The triggering receptor expressed on myeloid cells 2 (TREM-2) has been proposed to regulate both processes.

TREM-2 belongs to a conserved but functionally distinct gene family of proteins, with the best studied family members including TREM-1 and 2 (8, 9). TREM-2 is a receptor with an unknown ligand and is expressed by several cell types including bone marrow derived macrophages (BMDM), microglia and osteoclasts (10-12). Humans with mutations in TREM-2 develop Nasu-Hakola disease, which is characterized by progressive dementia and bone cysts (12, 13). Furthermore, recent evidence shows individuals who possess heterozygous rare variants of TREM-2 are at increased risk of Alzheimer's disease (14, 15). TREM-2 signals via the immunoreceptor tyrosine-based activation motif (ITAM) of the adaptor protein DNAX activation protein of 12kDa (DAP-12) to mediate its downstream effects. DAP-12 is rather promiscuous and is used by many other receptors having both activating and inhibitory functions (16). When TREM-1 is engaged, it potentiates the immune response to bacteria and TLR ligands (17-19). Conversely, TREM-2 was reported to function as a negative regulator of TLR mediated inflammation (10, 11, 20). Therefore, TREM family members act as fine

tuners of TLR mediated innate immune responses. Significantly, TREM-2 also plays an important role in phagocytosis. Over-expression of TREM-2 in Chinese hamster ovary (CHO) cells confers binding of both *Staphylococcus aureus* and *Escherichia coli* and TREM-2 mediates phagocytosis of *E. coli* in BMDM (21). The in vivo relevance of the phagocytic capacity of TREM-2 in relation to *E. coli* peritonitis has been corroborated by a recent study showing that administration of bone marrow cells that over-express TREM-2 enhances bacterial clearance and improves survival in a cecal ligation and puncture (CLP) model (22).

In this report we examine the function of TREM-2 in the context of *Streptococcus pneumoniae* infection, the most frequent cause of community acquired pneumonia (23). We chose to examine TREM-2 in this context for several reasons. Firstly, TREM-2 is expressed on human AM as well as mouse bronchial epithelial cells, suggesting a role for TREM-2 within the pulmonary compartment (24, 25). Secondly, pulmonary expression of TREM-2 and DAP-12 increases in mice following *Mycobacterium bovis* infection (26), but the functional importance of TREM-2 in lung host defense is unknown. Lastly, both the early innate immune response and phagocytosis are critical for the outcome during pneumoccal pneumonia (27-30). Thus, we hypothesized that studying TREM-2 in the context of pneumococcal pneumonia would provide an ideal model system for examining the cross-talk between TLR signaling and phagocytosis within the lungs.

Results

Pulmonary TREM-2 expression and function during early pneumococcal pneumonia

To investigate the role of pulmonary TREM-2 in the context of bacterial pneumonia, we first established which cell types expressed TREM-2 within the lungs. We determined TREM transcript levels in primary AM and respiratory epithelial lung cells (pEC) as well as epithelial cell lines such as alveolar MLE-12 and bronchial MLE-15 cells, respectively (31), and included RAW 264.7 macrophages as a positive control (32, 33). While TREM-1 expression was predominantly restricted to macrophages, we discovered TREM-2 to be strongly expressed in both AM and respiratory epithelial cells (Fig. 1A). We confirmed these results by demonstrating expression of TREM-2 on primary AM using western blot (Fig. 1B). Interestingly, we detected two bands in AM, with the upper band probably corresponding to a glycosylated form of TREM-2 as previously observed (34). Specificity for the antibody was provided as minimal detection of TREM-2 was observed in TREM-2 deficient AM. We then sought to determine pulmonary TREM-2 expression upon *S. pneumoniae* infection and found a time dependent upregulation in whole lung transcript levels, with highest expression 48h post infection (Fig. 1C). This increase in pulmonary TREM-2 expression during infection most likely reflected the influx of TREM-2 expressing cells, since TREM-2 transcript levels on primary AM declined following *S. pneumoniae* treatment (Fig. 1D). Together, TREM-2 was abundantly expressed within healthy lungs and further induced upon infection with *S. pneumoniae*.

To exploit the functional role of this constitutive pulmonary TREM-2 expression in relation to early pulmonary inflammatory responses following bacterial infection, we next infected WT and *Trem-2*$^{-/-}$ mice with *S. pneumoniae* for 6h. Much to our surprise, since TREM-2 was earlier considered a negative regulator of inflammation (10, 20), we did not identify any differences in levels of several inflammatory mediators tested, such as TNF-α, MCP-1, IL-1β and IL-6 (Fig. 1E and F). In fact *Trem-2*$^{-/-}$ mice only displayed elevated levels of KC, a chemokine required for neutrophil influx following bacterial respiratory tract infections (35) (Fig. 1E and F). This was accompanied by a modest increase in recruited neutrophils (Fig. 1G).

Altogether, these data demonstrate that lung TREM-2 only partially dampened inflammation following *S. pneumoniae* infection in vivo.

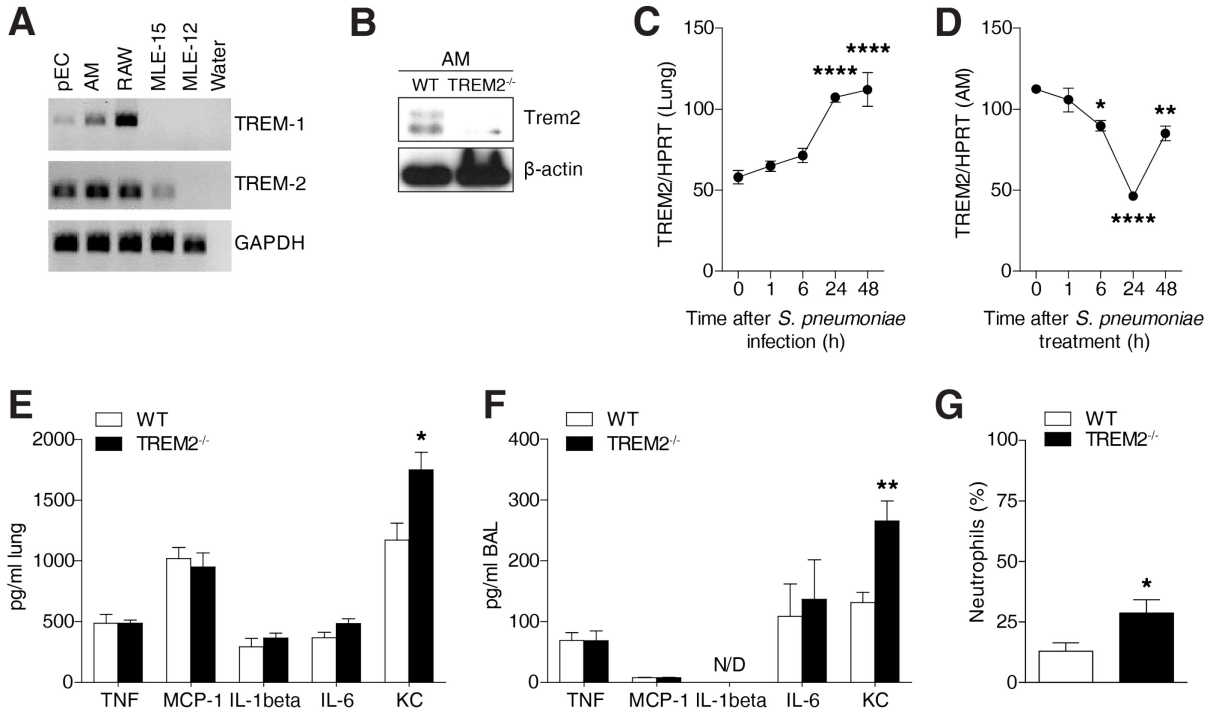

Figure 1: Pulmonary TREM-2 expression and function during *S. pneumoniae* induced inflammation

(A) TREM-1 and TREM-2 expression was evaluated in the indicated cell types using RT-PCR. (B) Western blot was used to evaluate TREM-2 expression on AM. (C) WT mice (n=6 per time point) were intranasally inoculated with 10^5 CFU *S. pneumoniae* and after indicated time points TREM-2 lung transcript levels were evaluated. (D) WT AM were treated with 2 x 10^7CFU/ml *S. pneumoniae* for indicated time points and TREM-2 RT-PCR was conducted. (E-G) WT and *Trem-2*$^{-/-}$ mice (n = 7 per genotype) were intranasally infected with 10^5 CFU *S. pneumoniae* for 6h and levels of indicated cytokines were evaluated in the lung (E) or BALF (F) and neutrophil counts were determined in the BALF (G). Data represent mean ± SEM and are (A-G) representative of two independent experiments. Differences were calculated versus time point 0 (C/D) or versus WT (E-G) and are indicated as * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$.

Decreased TLR2 mediated cytokine production in TREM-2 deficient AM

Given that TREM-2 is considered a negative regulator of inflammation, our observation of unaltered cytokine release in lungs of *Trem-2*$^{-/-}$ mice following *S. pneumoniae* infection was surprising. We therefore first re-examined the regulatory function of TREM-2 and concentrated on macrophage responses to bacteria. We found *S. pneumoniae* or LPS induced TNF-α and KC synthesis by *Trem2*$^{-/-}$ BMDM augmented (Fig. 2A and B), supporting previous observations of TREM-2 negatively regulating TLR mediated cytokine synthesis (10).

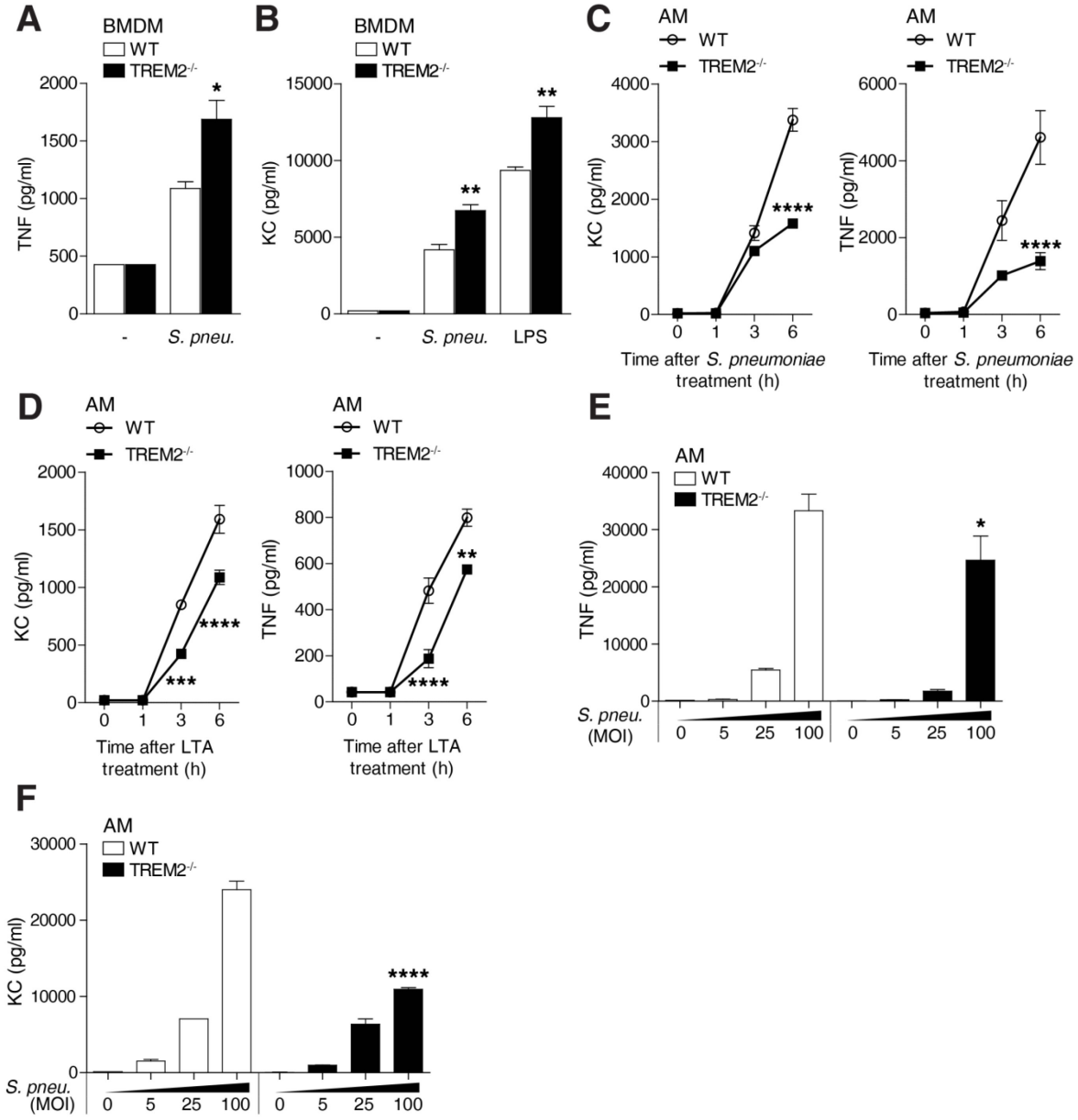

Figure 2: Cell type specific effects of TREM-2 on *S. pneumoniae* and TLR2 mediated cytokine production

(A and B) WT and *Trem-2*$^{-/-}$ BMDM (n = 4 per genotype) were treated with 2 x 10^7 CFU/ml *S. pneumoniae* or 100ng/ml LPS for 6h and TNF-α (A) or KC (B) levels were measured in the supernatant. (C and D) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype/time point) were treated with 2 x 10^7 CFU/ml *S. pneumoniae* (C) or 10μg/ml *S. pneumoniae* LTA (D) for the indicated times and KC and TNF-α levels were measured in the supernatant. (E and F) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype/MOI) were treated with the indicated doses of *S. pneumoniae* and TNF-α (E) and KC (F) levels were determined in the supernatant. Statistical comparisons are done versus WT cells (A/B) at a given timepoint (C/D), or at a particular MOI of *S. pneumoniae* (E/F), and indicated as: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p = < 0.0001$. All data represent mean ± SEM and are representative of two independent experiments.

Getting back to the specific role of TREM-2 within the lungs, we then determined whether *Trem-2*$^{-/-}$ AM would behave similarly to BMDM in response to *S. pneumoniae* or its lipotechoic acid (LTA), a known TLR2 ligand (36). Strikingly, *Trem-2*$^{-/-}$ AM displayed opposite effects to *Trem-2*$^{-/-}$ BMDM. We discovered that TREM-2 deficient AM exhibited decreased inflammation in response to either stimuli (Fig. 2C and D). The mechanism whereby ITAM coupled receptors generate inhibitory or activating signals is not well understood. However, the avidity of receptor ligation and thus ligand density has been described as a potential reason for pro- versus anti-inflammatory responses by ITAM associated receptors (16, 37). To rule out that the dose of bacteria could alter the effect of TREM-2 on TLR signaling, we stimulated primary AM from WT and *Trem-2*$^{-/-}$ mice with increasing doses of *S. pneumoniae*. While TNF-α and KC release was not different between WT and *Trem-2*$^{-/-}$ AM at an MOI of 25, synthesis was significantly lower in *Trem-2*$^{-/-}$ AM stimulated with an MOI of 100 *S. pneumoniae* (Fig. 2E and F).

These data suggest that TREM-2 regulated TLR2 mediated cytokine synthesis in a cell type specific manner, i.e. it diminished inflammation in BMDM and human monocyte-derived macrophages, but partially enhanced it in AM. Furthermore, given that BMDM and AM were stimulated with an identical dose of bacteria, these data indicate that the cell type specific effects of TREM-2 cannot be explained by differences in receptor ligation and avidity.

Increased phagocytic activity in TREM-2 deficient alveolar macrophages in vitro and in vivo

Considering that the elimination of pathogens is the crucial step in host defense during pneumonia, we then more closely examined the anti-bacterial properties of macrophages and the role of TREM-2 herein. In agreement with published reports that indicated TREM-2 to be a phagocytic receptor for *E. coli* (21) we first identified that *Trem-2*$^{-/-}$ BMDM exhibited a decreased uptake of *S. pneumoniae* compared to WT cells (Fig. 3A). Although we anticipated that *Trem-2*$^{-/-}$ AM would behave similarly, this postulate proved to be incorrect as *Trem-2*$^{-/-}$ AM surprisingly exhibited an enhanced uptake of *S. pneumoniae* compared to WT AM (Fig. 3B). We could rule out differences in phagocytosis between the cell-types due to the kind of bacterium used, since uptake of *E. coli* was equally enhanced in *Trem-2*$^{-/-}$ AM compared to WT AM (Fig. 3C). We further confirmed the enhanced uptake of *S. pneumoniae* in *Trem-2*$^{-/-}$ AM using confocal microscopy (Fig. 3D and Fig. S1) and corroborated previously published observations showing that *Trem-2*$^{-/-}$ BMDM exhibit decreased uptake of *E. coli* compared to

WT BMDM (21) (Fig. S2). Increased phagocytosis of *S. pneumoniae* by TREM-2 deficient AM was also found following opsonisation of bacteria with either pneumococcal serotype 3 capsular antibodies or 10% WT serum, indicating that TREM-2 can inhibit both FcR-dependent and independent phagocytosis of *S. pneumoniae* (Fig. 3E and F). Uptake of FITC labeled BSA was unchanged between the genotypes of AM, while in the same experiment uptake of *S. pneumoniae* was significantly higher, thus illustrating that TREM-2 affects phagocytosis of bacteria but not endocytosis of BSA (Fig. 3G). Further, to extend these studies to other serotypes of *S. pneumoniae*, we tested uptake of serotype 19A *S. pneumoniae*, a common serotype that causes invasive pneumococcal disease in children (38). By doing so, we could observe enhanced uptake of 19A *S. pneumoniae* in *Trem-2*$^{-/-}$ AM compared to their WT counterparts (Fig. 3H).

We next set out to investigate if the enhanced phagocytosis of *S. pneumoniae* by TREM-2 deficient AM would also be observed in vivo. To this end, we inoculated WT and *Trem-2*$^{-/-}$ mice with FITC-labeled *S. pneumoniae* and assessed the uptake of *S. pneumoniae* by phagocytes in vivo using flow cytometry. By doing so, we could verify that *Trem-2*$^{-/-}$ F4/80^{+} CD11c^{+} AM did indeed display augmented bacterial phagocytosis in vivo compared to their WT counterparts (Fig. 3I and J). Considering the importance of infiltrating neutrophils in phagocytosing bacteria during pneumonia, we then examined the potential contribution of neutrophils to bacterial clearance in vivo. Following intranasal infection with FITC labeled *S. pneumoniae,* we found a tendency for decreased phagocytosis by Ly6G^{+}CD11b^{+} lung neutrophils from *Trem-2*$^{-/-}$ mice compared to their WT counterparts, suggesting that the modest increase in neutrophil numbers early during infection (Fig. 1G) was not responsible for the improved bacterial clearance of *Trem-2*$^{-/-}$ mice (Fig. 3K and L). We finally asked if the enhanced phagocytosis by *Trem-2*$^{-/-}$ AM would result in an improved bacterial clearance during pneumococcal pneumonia in vivo. We infected mice with *S. pneumoniae* for 24h and recovered significantly fewer bacteria from lungs and bronchoalveolar lavage fluid (BALF) from *Trem-2*$^{-/-}$ mice (Fig. 3M).

These data were surprising and important because they challenged the general view of TREM-2 acting as a phagocytic receptor and negative regulator of inflammation but suggested that TREM-2 can modulate phagocytosis and inflammation in a very cell-type specific manner. With uptake of bacteria being higher in resident AM from TREM-2 deficient mice, this macrophage type obviously displays opposite effects from BMDM.

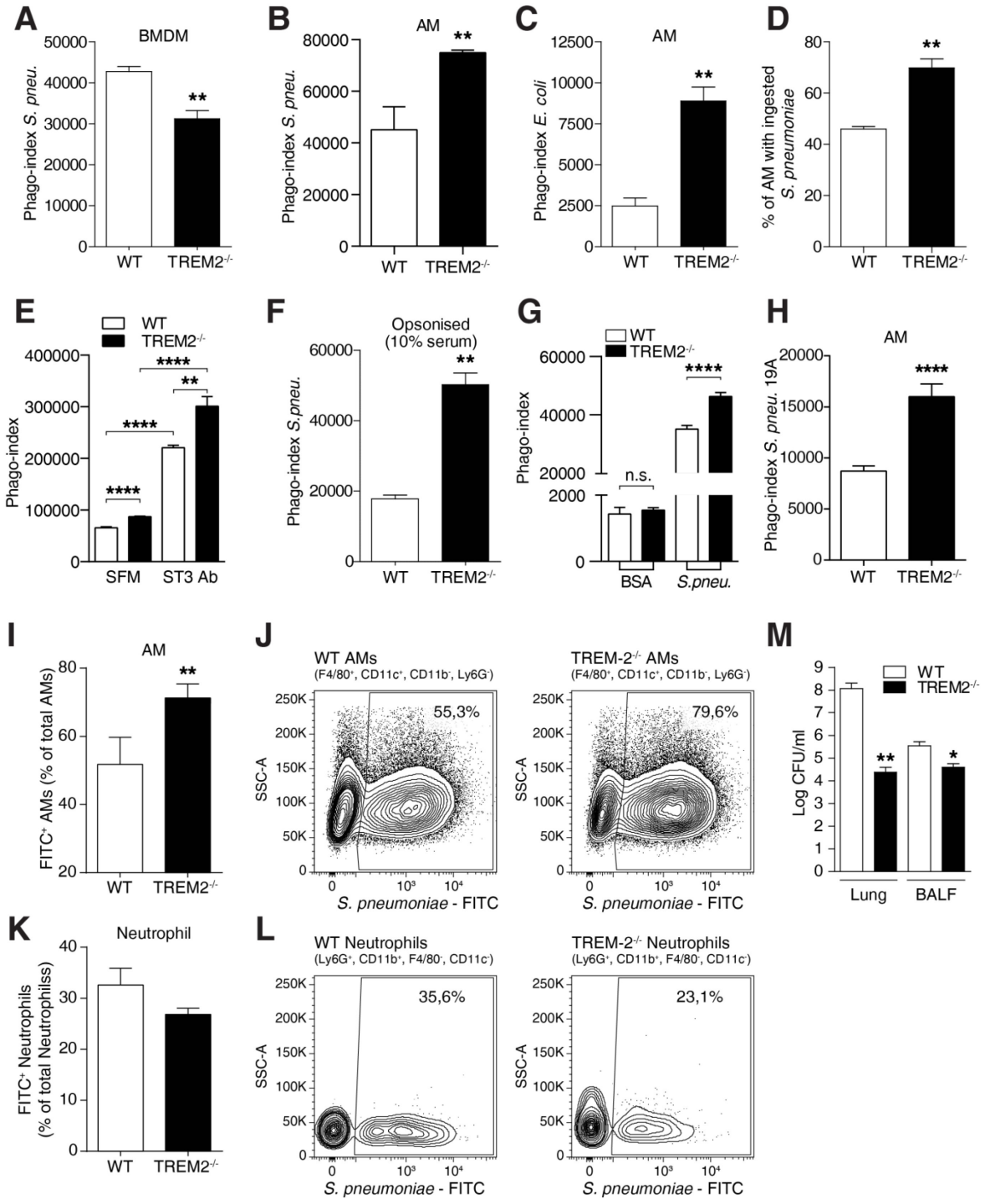

Figure 3: Elevated phagocytosis of bacteria by TREM-2 deficient AM

(A) WT and *Trem-2*$^{-/-}$ BMDM (n = 4-5 per genotype) were incubated with FITC labeled *S. pneumoniae* (MOI 100) and after 1h phagocytosis was assessed using FACS. (B and C) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype) were incubated with FITC labeled *S. pneumoniae* (B) or *E. coli* (C) (MOI of 100) and phagocytosis was assessed using FACS 1h later. (D) Elevated phagocytosis of *S. pneumoniae* by *Trem-2*$^{-/-}$ AM as determined using confocal microscopy as described in the M&M section. The percentage of cells that contain bacteria is depicted (n= 4-5 per genotype). (E-F) WT and *Trem-2*$^{-/-}$ AM (n= 4-5 per genotype) were incubated with FITC labeled *S.*

pneumoniae (MOI 100) under either serum free conditions (SFM) or the bacteria were pre-opsonised with 10% anti-pneumococcal serotype III capsular antibody (ST3-Ab) (E) or 10% pooled WT mouse serum (F) for 30 min before addition to the cells. Phagocytosis was assessed 1h later. (G) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype) were incubated with 1μg/ml FITC labeled BSA or FITC labeled *S. pneumoniae* (MOI 100) and phagocytosis was assessed 1h later by FACS. (H) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype) were incubated with FITC labeled *S. pneumoniae* strain 19A (MOI 100) and phagocytosis was assessed 1h later by FACS. (I-L) WT and *Trem-2*$^{-/-}$ mice (n = 7 mice per genotype) were intranasally infected with 10^6 CFU FITC labeled *S. pneumoniae* for 4h and in vivo phagocytosis by AM (I-J) and neutrophils (K-L) was determined. J and L show representative FACS plots of data in I and K. (M) WT and *Trem-2*$^{-/-}$ mice (n = 6 mice per genotype) were intranasally infected with 10^5 CFU *S. pneumoniae* and bacterial CFUs were enumerated 24h post infection in the lung and BALF. All data represent mean ± SEM versus WT unless otherwise indicated. Data in (A-C, F and H) are representative of three independent experiments and all other data are representative of two independent experiments. * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$.

Increased C1q production via PPAR-δ in TREM-2-deficient alveolar macrophages

Given the importance of lung macrophages in pneumococcal clearance (29, 39), we next focused on determining the molecular targets by which TREM-2 deficiency in AM was able to influence pneumococcal uptake and we conducted a genome wide transcriptome analysis of both genotypes of AM. While we discovered no difference in the expression levels of important phagocytic receptors such as the scavenger receptors *Cd36, Marco*, *Sra-1* and *Lox-1* and the complement receptors *Cr1 (Cd35)* and *Cr3 (itgb2/itgam; Cd11b/Cd18)* (Fig. 4A and Fig. S3), we importantly found that *Trem-2*$^{-/-}$ AM expressed higher baseline levels of opsonins such as *C1qa*, *C1qb*, *C1qc* and *Thbs1* (encoding thrombospodin; Fig. 4A). We confirmed higher basal *C1qb* and *Thbs1* levels in TREM-2 deficient AMs by RT-PCR (Fig. 4B) and verified enhanced intracellular C1q protein levels in *Trem-2*$^{-/-}$ AM by flow cytometry (Fig. 4C). In search for specific factors that would explain differences in TREM-2 mediated responses between AM and BMDM, we found that *C1qb* levels were exclusively increased in *Trem2*$^{-/-}$ AM, whereas *Thbs1* levels were elevated in both TREM-2 deficient AM and BMDM (Fig. 4D). This led us to hypothesize that altered C1q expression could account for differential phagocytic effects between AM and BMDM. As microarray data suggested that TREM-2 had the ability to influence basal C1q production in AM, we decided to test this idea and to determine *C1qb* and *C1qc* transcript levels in RAW264.7 macrophages over-expressing TREM-2. Over-expression of TREM-2 was able to lower basal *C1qb* and *C1qc* transcript levels, proving that TREM-2 had the ability to regulate C1q transcription (Fig. 4E and Fig. S4).

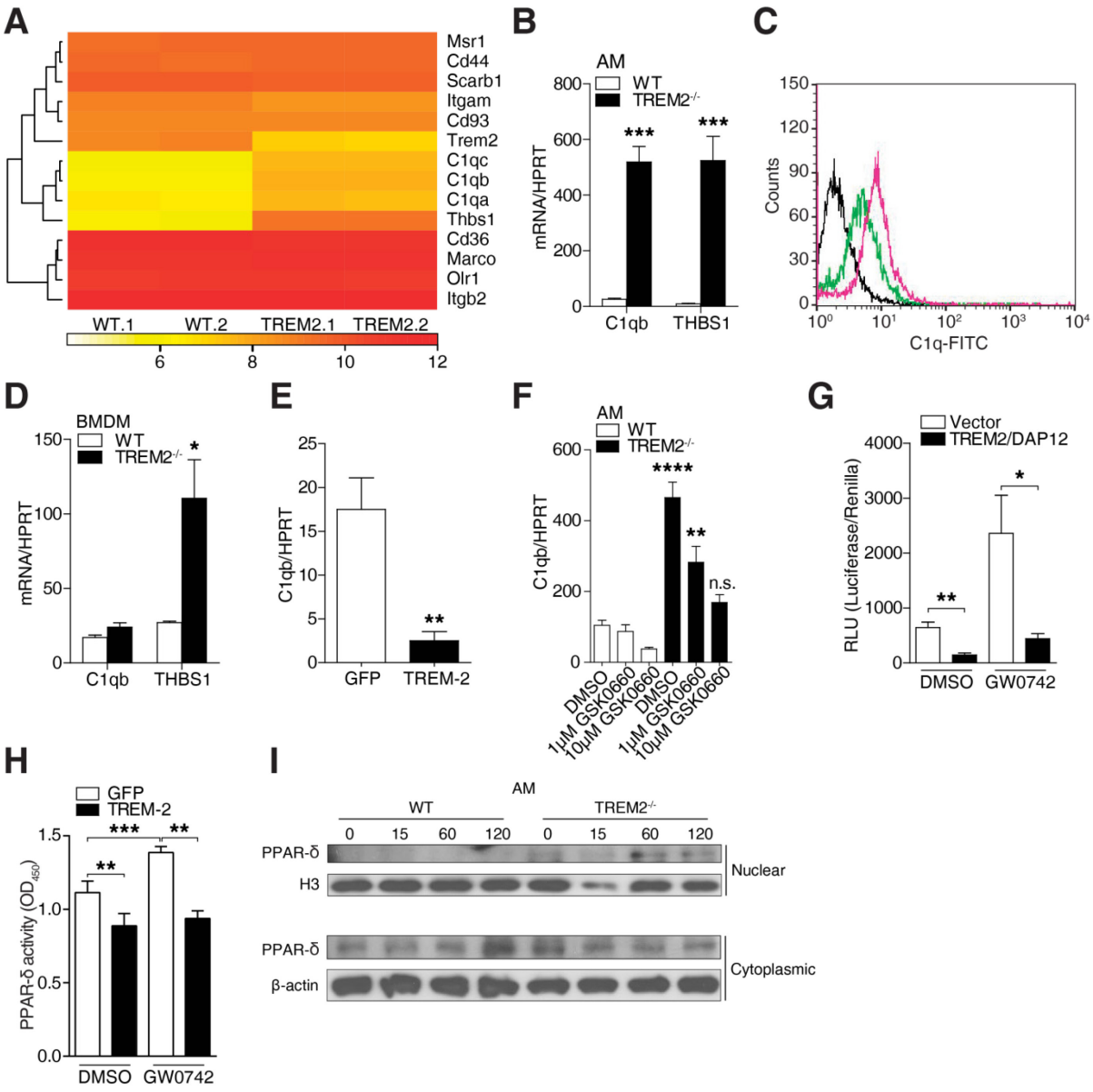

Figure 4: Elevated C1q production via PPAR-δ in TREM-2 deficient AM

(A) Heat map from microarray data depicting baseline expression of selected genes in WT and *Trem-2*$^{-/-}$ AMs. (B) Verification of enhanced basal expression of the opsonins *C1qb* and *Thbs1* in AM using RT-PCR (n = 4 per genotype). (C) Basal expression of *C1qb* in WT versus *Trem-2*$^{-/-}$ AMs as determined by intracellular FACS. Green line depicts WT macrophages, pink *Trem-2*$^{-/-}$ macrophages and black represents isotype control antibody. (D) *C1qb* and *Thbs1* basal expression was determined in WT and *Trem-2*$^{-/-}$ BMDM (n = 4 per genotype) using RT-PCR. (E) *C1qb* expression was quantified in RAW264.7 cells over-expressing TREM-2 or GFP control (n = 4 per condition). (F) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype/condition) were pre-treated for 24h with the indicated doses of the PPAR-δ inhibitor GSK0660 or DMSO control after which RT-PCR of *C1qb* was performed. (G) HEK cells were transfected with a PPRE reporter plasmid together with TREM-2 and DAP-12 or a vector control, stimulated with 1μM of the PPAR-δ activator GW0742 or DMSO 24h post transfection, and luciferase activity was assayed 48h post transfection (n = 4 per condition). (H) RAW264.7 cells over-expressing

TREM-2 or GFP control (n = 4 per condition), were treated with 1μM of the PPAR-δ activator GW0742 or DMSO control for 24h, nuclear extracts were prepared and PPAR-δ activity levels were monitored as described in the methods. (I) WT and *Trem-2*$^{-/-}$ AM were treated with 1μM of the PPAR-δ activator GW0742 or DMSO control for the indicated time points, nuclear and cytoplasmic extracts were prepared and blotted for PPAR-δ. All data are representative of two independent experiments except for data in (E), which is representative of three independent experiments, and represent mean ± SEM versus WT (B/D), GFP control cells (E), DMSO (F) or vector (G). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

The nuclear receptor peroxisome proliferator-activated receptor-δ (PPAR-δ) has previously been shown to regulate C1q and the C1q promoter contains binding sites for this transcription factor (40). To understand the potential role of PPAR-δ in our cell system, we treated *Trem-2*$^{-/-}$ AM with the PPAR-δ inhibitor GSK0660 (41) and could revert *C1qb* transcript levels to those of WT AM in a dose dependent manner (Fig. 4F). We observed similar results using another PPAR-δ inhibitor, arguing against inhibitor-specific effects in down regulating C1q expression (Fig. S5). Specificity of these compounds for PPAR-δ and not other PPARs such as PPAR-γ was evaluated, since activation of PPAR-δ with the PPAR-δ activator GW0742 (40) increased transcript levels of *C1qb* and *C1qc* in WT AM in a dose dependent manner and co-incubation with PPAR-δ inhibitors abrogated this increase (Fig. S5). These data confirmed that PPAR-δ activity determines C1q transcription in AM. There was no striking difference in transcript and total protein levels of PPAR-δ between WT and *Trem-2*$^{-/-}$ AM, suggesting that the ability of TREM-2 to regulate C1q via PPAR-δ was not due to enhanced PPAR-δ levels (Fig. S6).

To demonstrate that TREM-2 itself downregulates PPAR-δ activity, we made use of TREM-2 over-expressing HEK cells and quantified PPAR-δ activation using a reporter system. Over-expression of TREM-2 was able to lower both basal and ligand-induced PPAR-δ reporter activity (Fig. 4G). To rule out differences between HEK cells and macrophages, and to further confirm the reporter gene experiments, we next stimulated TREM-2 or GFP control over-expressing RAW264.7 macrophages with GW0742 or DMSO control and monitored nuclear PPAR-δ levels. These data corroborated the experiments in Fig. 4G and demonstrated that TREM-2 could suppress PPAR-δ activity in RAW264.7 macrophages (Fig. 4H). To understand the specific effect of TREM-2 on PPAR-δ activation in AM, we next monitored nuclear and cytoplasmic levels of PPAR-δ in WT and *Trem-2*$^{-/-}$ AM following GW0742

treatment. *Trem-2*$^{-/-}$ AM exhibited an early translocation of ligand-induced PPAR-δ from the cytoplasm to the nucleus, compared to their WT counterparts (Fig 4I). Altogether, these data demonstrate that TREM-2 can suppress basal levels of C1q and suggest that the elevated C1q production observed in *Trem-2*$^{-/-}$ AM occurs via effects of TREM-2 on the nuclear receptor PPAR-δ, a known activator of C1q transcription (40).

Elevated S. pneumoniae phagocytosis and bacterial clearance in TREM-2 deficient AM and mice is dependent on C1q

C1q is unique among the complement factors, as it is exclusively produced by macrophages and not hepatocytes (42-46). While C1q is well-known for its role in initiating the classical complement pathway, it also exerts complement-independent functions such as clearance of immune-complexes or apoptotic cells (47-49). The importance of complement in general, including C1q, in protecting against pneumococcal infections is well established (30, 50) and has been based on the importance of C3b-mediated opsonization of bacteria. Since we observed that TREM-2 deficient AM produced more C1q (Fig. 4A, B, C and F) and that *Trem-2*$^{-/-}$ mice showed a modestly elevated neutrophil influx early during pneumococcal pneumonia (Fig. 1G), we speculated that classical complement activation could be increased in the pulmonary compartment of *Trem-2*$^{-/-}$ mice following *S. pneumoniae* infection and monitored levels of C3a and C5a, anaphylatoxins, which promote neutrophil influx (51). However, we did not observe any differences in C3a and C5a in the BALF of *Trem-2*$^{-/-}$ mice both 6h and 24h post *S. pneumoniae* infection, suggesting that classical complement activation was not generally elevated in the pulmonary compartment of *Trem-2*$^{-/-}$ mice following *S. pneumoniae* infection (Fig. S7).

We therefore studied the potential direct contribution of C1q to bacterial phagocytosis, and pre-incubated WT AM with C1q or control BSA and quantified the uptake of *S. pneumoniae*. C1q significantly increased bacterial phagocytosis by AM (Fig. 5A), thus confirming a direct role for C1q in phagocytosis, independent of complement activation. Further, these data are consistent with data, showing that C1q can bind to *S. pneumoniae*, independent of IgG and increase internalization (52). As our data indicated that PPAR-δ activation enhanced C1q levels in WT AM (Fig. S5) and that PPAR-δ inhibition could lower C1q levels (Fig. 4F), we next decided to examine the role of PPAR-δ mediated C1q production in the context of *S. pneumoniae* phagocytosis within WT AMs.

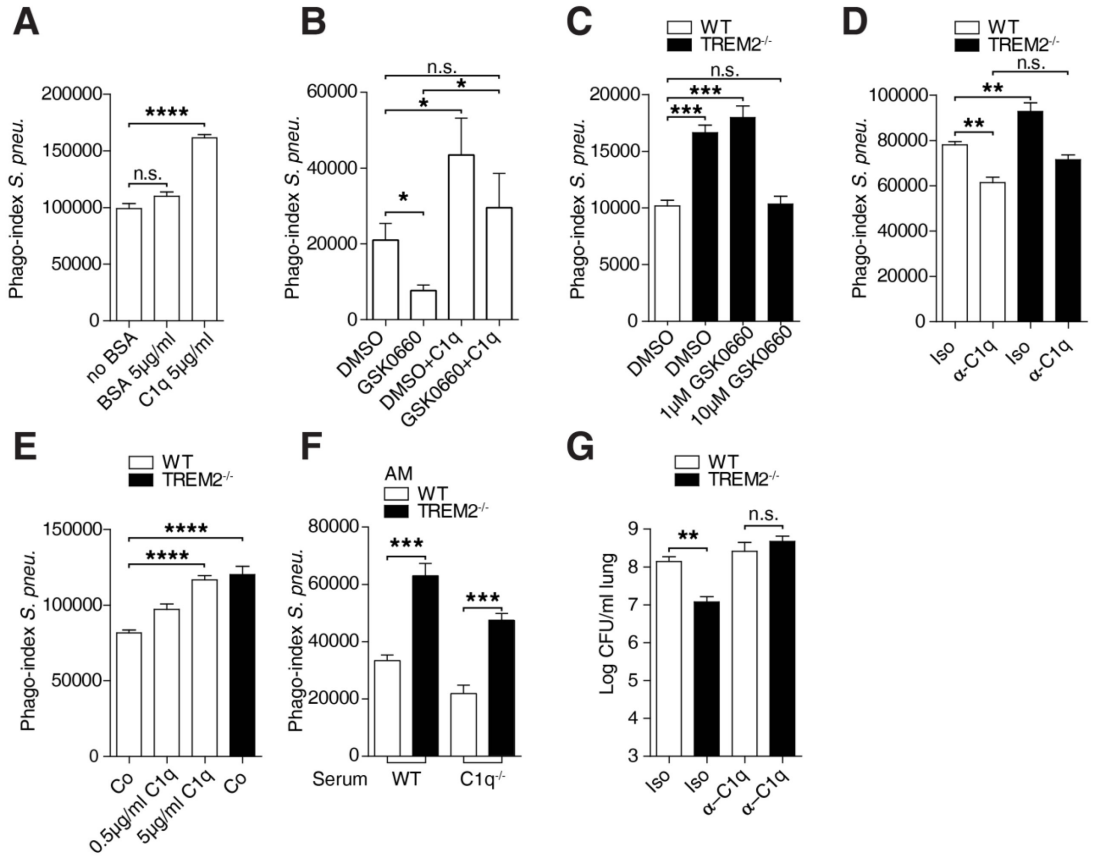

Figure 5: Enhanced phagocytosis by *Trem-2*$^{-/-}$ AM depends on C1q.

(A) WT AM were adhered to C1q, control BSA or uncoated plates for 3h prior to incubation with FITC labeled *S. pneumoniae* (MOI 100) and phagocytosis was assessed using FACS 1h later (n = 3-4 per condition). (B) WT AM (n = 6-7 per condition) were pre-treated for 24h with 10µM PPAR-δ inhibitor GSK0660 or DMSO control after which, the cells were adhered to C1q or control plates for 3h and phagocytosis of FITC labeled *S. pneumoniae* (MOI 100) was assessed 1h later using FACS. (C) WT and *Trem-2*$^{-/-}$ AM (n = 4 per genotype/condition) were pre-treated for 24h with the indicated doses of the PPAR-δ inhibitor GSK0660 or DMSO control after which phagocytosis of FITC labeled *S. pneumoniae* (MOI 100) was assessed 1h later using FACS. (D) WT and *Trem-2*$^{-/-}$ AM were pre-treated for 1h with 10µg/ml C1q blocking antibody or isotype control and phagocytosis of FITC labeled *S. pneumoniae* (MOI 100) was assessed 1h later using FACS (n = 4 per genotype/condition). (E) WT and *Trem-2*$^{-/-}$ AM (n = 4-7 per condition) were adhered for 3h to plates coated with the indicated conditions prior to incubation with FITC labeled *S. pneumoniae* (MOI 100) and phagocytosis was assessed using FACS. (F) WT and *Trem-2*$^{-/-}$ AM (n= 4 per genotype) were incubated with FITC labeled *S. pneumoniae* (MOI 100) that were pre-opsonised with 10% WT or C1qa$^{-/-}$ serum for 30 min after which phagocytosis was assessed 1h later using FACS (G) WT and *Trem-2*$^{-/-}$ mice (n = 17 per condition) were intranasally treated with 125µg C1q blocking antibody or isotype control prior to infection with 6 x 10^4 CFU *S. pneumoniae*. 48h post infection lung bacterial CFUs were enumerated. Data represent mean ± SEM; ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$. Data in (A, C-F) are representative of two independent experiments, (B and G) are pooled data from two independent experiments.

AMs, where PPAR-δ had been inhibited exhibited lower *S. pneumoniae* uptake compared to DMSO control cells. Importantly, the attenuated *S. pneumoniae* phagocytosis upon PPAR-δ inhibition was increased to levels of DMSO control cells upon addition of C1q (Fig. 5B). These data strongly suggest that in AM the principal target for PPAR-δ is C1q for the regulation of *S. pneumoniae* phagocytosis. Given that PPAR-δ inhibitors can lower basal C1q production in *Trem-2*$^{-/-}$ AM (Fig. 4F and Fig. S5), we next postulated that inhibiting C1q using PPAR-δ inhibitors would revert the enhanced phagocytosis by *Trem-2*$^{-/-}$ AM to WT levels. Significantly, at the dose of inhibitor where *C1qb* transcript levels in TREM-2 deficient AM were lowered to that of WT AM (i.e. 10μM GSK0660 (Fig. 4F)), phagocytosis was no longer different between WT and *Trem-2*$^{-/-}$ AM (Fig. 5C).

These data linked elevated C1qb levels, produced in a PPAR-δ dependent manner, with enhanced phagocytosis. To confirm the dependence of enhanced phagocytosis by *Trem-2*$^{-/-}$ AM on higher C1q levels, we blocked C1q using a blocking antibody and discovered that C1q blockage abolished any differences in phagocytosis between WT and *Trem-2*$^{-/-}$ AM (Fig. 5D). To then test if exogenous administration of C1q would enhance phagocytosis of WT AMs to *Trem-2*$^{-/-}$ levels, we adhered WT AM to C1q coated plates and quantified uptake of *S. pneumoniae* compared to *Trem-2*$^{-/-}$ AM. Consistent with results in Fig. 5A, C1q enhanced phagocytosis of *S. pneumoniae* by WT AM in a dose dependent manner to reach *Trem-2*$^{-/-}$ levels (Fig. 5E). Thus, using two independent approaches of inhibiting C1q in *Trem-2*$^{-/-}$ AM, as well as exogenously supplementing C1q in WT AM, we demonstrated that the enhanced phagocytosis of *S. pneumoniae* by *Trem-2*$^{-/-}$ AM depended on macrophage derived C1q.

Bone marrow transplantation experiments indicate that transplantation of WT bone marrow into *C1qa*$^{-/-}$ mice restores levels of serum C1q to WT, clearly demonstrating that C1q is produced by myeloid cells [46]. To further ratify the importance of macrophage derived C1q versus serum C1q, we next opsonised *S. pneumoniae* with serum from WT or *C1qa*$^{-/-}$ mice and examined phagocytosis. Our hypothesis was that the difference in phagocytosis between WT and *Trem-2*$^{-/-}$ AM would still be visible in the presence of C1q-deficient serum, although overall phagocytosis levels would be reduced in both genotypes. Indeed, this was the case, strongly suggesting that AM are the source of C1q and that elevated C1q levels produced by *Trem-2*$^{-/-}$ AM mediate the enhanced phagocytosis of *S. pneumoniae* (Fig. 5F).

To finally study the importance of elevated C1q production by *Trem-2*$^{-/-}$ AM in vivo, we blocked C1q in lungs of mice before infection with *S. pneumoniae* and quantified bacterial clearance. Indeed, blocking pulmonary C1q could reverse the difference in bacterial counts between WT and *Trem-2*$^{-/-}$ animals (Fig. 5G). We conclude that the improved bacterial clearance we observed in TREM-2 deficient animals was intimately linked to enhanced C1q production by TREM-2 deficient AM.

TREM-2 is deleterious during pneumococcal pneumonia

The enhanced bacterial phagocytosis of *S. pneumoniae* in *Trem-2*$^{-/-}$ mice finally led us to monitor the survival of WT and *Trem-2*$^{-/-}$ mice during pneumonia. Ninety five hours post infection, before the first *Trem-2*$^{-/-}$ mouse succumbed, 60% of WT mice were dead, demonstrating conclusively that WT mice displayed enhanced mortality compared to their TREM-2 deficient counterparts during pneumococcal pneumonia (Fig. 6A). To determine the reasons for this effect, we evaluated pulmonary bacterial loads and bacteremia shortly before the first mouse succumbed. TREM-2 deficient mice exhibited a thousand fold decrease in *S. pneumoniae* burden in lungs 48h post infection (Fig. 6B). These data corroborated our earlier time points of infection (Fig. 3M). However, although, we had observed that *Trem-2*$^{-/-}$ mice reproducibly exhibit enhanced bacterial clearance compared to WT mice (Fig. 3M, 5G and 6B), there were some differences in the degree of bacterial clearance. Natural variation in animal experiments or differences in experimental setup could explain this. Strikingly, while seven out of nine WT mice exhibited bacteremia, *S. pneumoniae* could only be detected in the blood of one *Trem-2*$^{-/-}$ mouse (Fig. 6C). Elevated bacteremia in WT mice resulted in enhanced systemic inflammation as determined by plasma IL-6 measurements, compared to TREM-2 deficient mice (Fig. 6D).

We next evaluated lung pathology and inflammation. Lung histology revealed that the levels of interstitial inflammation, pleuritis and edema formation were greatly decreased in mice deficient for TREM-2 compared to WT mice (Fig. 6E and 6F). In agreement with this, we detected diminished pulmonary cytokine levels such as TNF-α, IL-1β, MCP-1 and IL-6 (Fig. 6G).

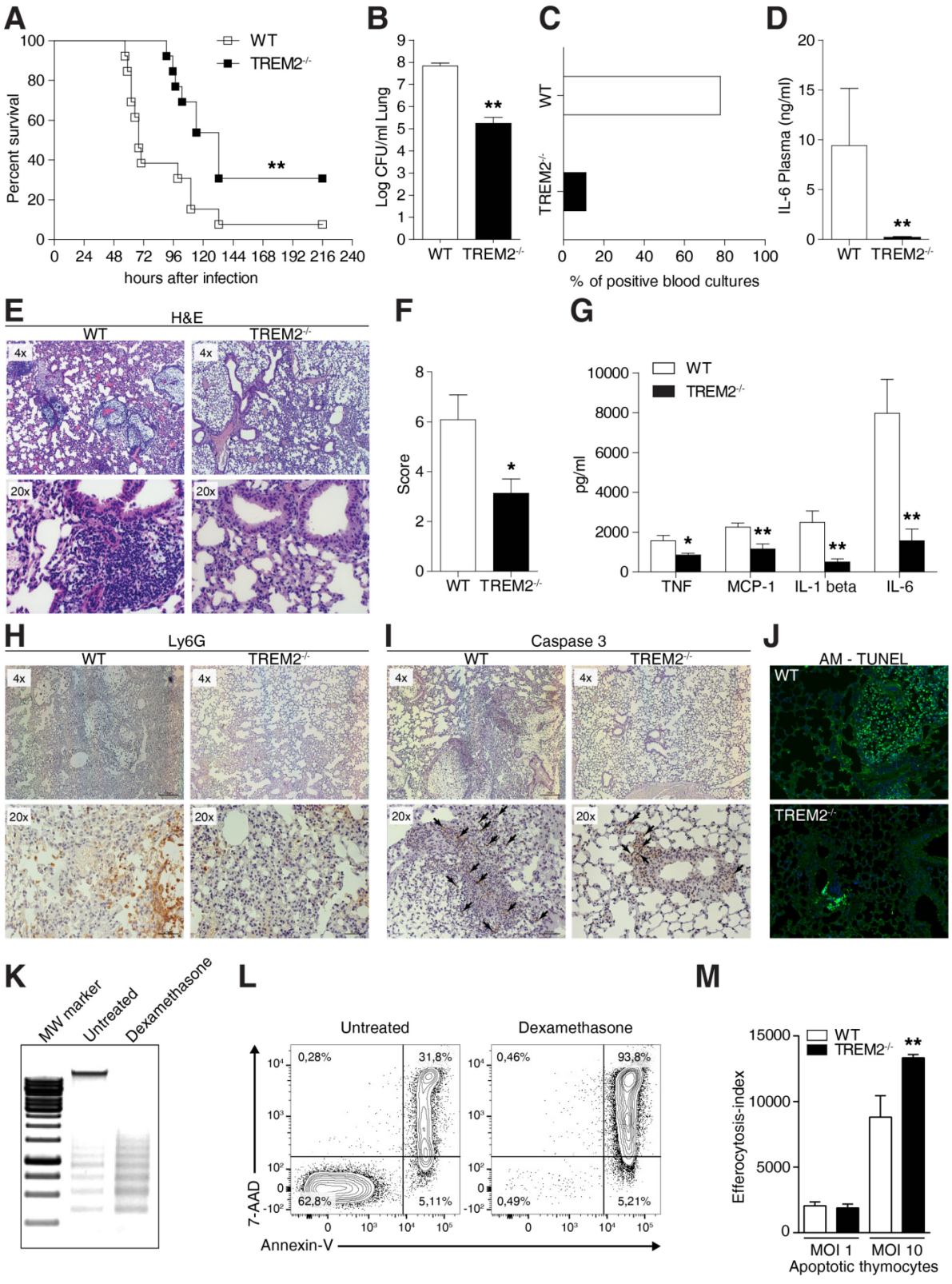

Figure 6: TREM-2 deficiency improves outcome during pneumococcal pneumonia

(A) WT and *Trem-2*$^{-/-}$ mice were intranasally infected with 10^5 CFU *S. pneumoniae* and survival was monitored for 10 days (n=13/genotype). **(B-G)** WT and *Trem-2*$^{-/-}$ mice (n=9 mice per genotype) were intranasally infected with 1 x 10^5 CFU *S. pneumoniae* and **(B)** lung bacterial CFUs were enumerated 48h post infection. (**C**) Blood

cultures were monitored for *S. pneumoniae* **(D)**, IL-6 levels were evaluated in the plasma using ELISA. **(E)** Representative H/E staining of lungs 48h post infection. **(F)** Lung inflammation score, as described in the Methods section. **(G)** Levels of lung cytokines were evaluated using ELISA. **(H-J)** Representative Ly6G **(H)**, active caspase 3 **(I)** and TUNEL **(J)** staining of lungs 48h post infection. Magnification depicted for TUNEL stains is 20 x and arrows indicate caspase 3 positive cells. **(K-L)** Thymocytes (n=4) were treated with 1μM dexamethasone and apoptosis was evaluated using DNA laddering **(K)** or Annexin-V/7-AAD positivity **(L)**. **(M)** WT and *Trem-2*$^{-/-}$ AM (n = 8 per genotype and condition), were fed CFSE labeled apoptotic cells and efferocytosis was assessed 1h later using FACS. Data in **B**, **D**, **F**, **G** and **M** are presented as mean ± SEM, WT versus TREM-2$^{-/-}$. Data in **A-G** are representative of two independent experiments. Data in **M** is pooled data from 2 independent experiments; * p = < 0.05, ** p = 0.005.

Attenuated pulmonary inflammation is not only associated with decreased bacterial burden within the lungs but can also be attributed to improved clearance of apoptotic neutrophils by AM, which promotes the resolution of inflammation (53-56). To examine the possibility that the higher inflammation in WT mice might be associated with more apoptotic cells, we monitored pulmonary neutrophil infiltration and active caspase 3 levels in WT and *Trem-2*$^{-/-}$ mice using Ly6G and active caspase 3 staining. We could observe that *Trem-2*$^{-/-}$ mice displayed attenuated neutrophil and active caspase 3 levels, particularly in the interstitial space, 48h post *S. pneumoniae* infection (Fig. 6H, 6I). Elevated pulmonary cell apoptosis of WT mice late during pneumococcal pneumonia was confirmed using TUNEL staining (Fig. 6J). Specificity for all stainings was verified as no positive signal was detected in the respective isotype controls (Fig. S8).

We next tested the hypothesis that aside from improved *S. pneumoniae* phagocytosis (Fig. 3 and 5), *Trem-2*$^{-/-}$ AM may exhibit elevated apoptotic cell uptake, also known as efferocytosis. To model this we measured the uptake of CFSE labeled apoptotic thymocytes, a well-established and widely used method for determining efferocytosis. Thymocyte apoptosis was confirmed using both DNA laddering and Annexin V/7-AAD positivity (Fig. 6K and L). While there was no difference in the clearance of CFSE labeled apoptotic bodies at low doses between the genotypes of AM, importantly, at a MOI of 10, *Trem-2*$^{-/-}$ AM exhibited significantly elevated efferocytosis compared to WT AM (Fig. 6M).

We conclude that TREM-2 deficiency improved bacterial and apoptotic cell clearance, lung pathology and prevented systemic inflammation during pneumococcal pneumonia, all of which ultimately led to improved survival.

Discussion

In this study we examined the effects of TREM-2 on bacterial phagocytosis and pulmonary inflammation within the context of bacterial pneumonia. We unexpectedly discovered a cell-type specific role for TREM-2, as TREM-2 suppresses bacterial phagocytosis via repression of C1q in AM. These findings demonstrate a previously unknown link between ITAM associated receptor expression and opsonin production in resident AM and explains the detrimental function of TREM-2 during pneumococcal pneumonia.

C1q is a member of the defense collagen family that is important for initiating the classical complement pathway and thereby crucial for host defense against pneumococci (30, 50, 57). C1q consists of 18 polypeptide chains that associate together in a "bouquet of tulips" like configuration, with each C1q chain containing a C-terminal globular region that recognizes PAMPs, and a N-terminal collagenous region that associates with phagocytic receptors on macrophages to enhance bacterial phagocytosis (45, 51). Within the pulmonary compartment, C1q is produced locally by AM (45, 46), i.e. by those cells that provide the first phagocytic line of defense against *S. pneumoniae* (29, 39). C1q has been shown to act as a molecular bridge between *S. pneumoniae* and host cells, independently of IgG and serotype, facilitating increased adherence and bacterial uptake (52). Our data reveal a previously unknown link between C1q and TREM-2 and suggest that TREM-2 suppresses C1q production by AM via a mechanism that involves PPAR-δ associated pathways (Fig. 4). The consequence of this suppressive effect by TREM-2 in AM is important as it explains the detrimental impact of TREM-2 during pneumococcal pneumonia. Our findings that blocking the locally enhanced production of pulmonary C1q was sufficient to reverse the improved bacterial clearance by *Trem-2*$^{-/-}$ AM in vivo and in vitro support this argument (Fig. 5).

Beside AM, neutrophils are considered important in phagocytosing bacteria upon lung infections and since we observed a modest increase in neutrophil influx early during pneumococcal pneumonia in *Trem-2*$^{-/-}$ mice, these cells could contribute to the enhanced bacterial clearance in these mice. Although we cannot exclusively rule out this possibility, we importantly discovered that neutrophils from TREM-2 deficient mice exhibit a tendency towards lower uptake of *S. pneumoniae* in vivo, while *Trem-2*$^{-/-}$ AM exhibit increased phagocytosis in vitro and in vivo (Fig. 3). Underscoring the importance of AM in bacterial clearance during pneumonia over neutrophils, previous studies by our group show that selective changes in KC secretion and neutrophil influx occurring in a TLR2 dependent

manner are not sufficient to induce altered bacterial clearance or differences in outcome during pneumococcal pneumonia in vivo (58).

But how does TREM-2 influence C1q production? PPARs are nuclear receptors that are ligand inducible transcription factors and activate target genes through binding to PPAR-response elements (PPREs) as heterodimers with the retinoid X receptor family (59). PPARs shuttle between the cytoplasm and nucleus in response to ligand activation (60). Three lines of evidence link PPAR-δ to the enhanced C1q production in AM: 1) PPAR-δ activation enhances production of C1q by AM; 2) PPAR-δ inhibition lowers levels of C1q in *Trem-2*$^{-/-}$ AM to WT AM; 3) Over-expression of TREM-2 lowers levels of C1q and PPAR-δ activation. Interestingly the early ligand induced activation kinetics and nuclear shuttling of PPAR-δ are elevated in *Trem-2*$^{-/-}$ AM, while PPAR-δ appears to be primarily localized in the cytoplasm of WT AM (Fig. 4I). These data suggest that the manner by which TREM-2 influences C1q transcription is mediated by effects on PPAR-δ activation, possibly via interference with PPAR-δ ligand binding, as already suggested by our PPRE reporter experiments and activity assays (Fig. 4G and H). Regardless of the exact mechanism whereby TREM-2 inhibits PPAR-δ activity, our data suggests that C1q is the principal target for PPAR-δ in the regulation of *S. pneumoniae* phagocytosis within AM (Fig. 5B-C).

Our observations of enhanced phagocytosis in TREM-2 deficient AM are important as they raise awareness of conceptually labeling "TREM-2 as a phagocytic receptor for bacteria." We do not dispute previous studies showing that TREM-2 deficiency in BMDM leads to lower bacterial phagocytosis (21). In fact, we were able to reproduce these findings but believe that the effects of TREM-2 on phagocytosis are cell-type specific. While AM and BMDM are related cell types, they present substantial differences with the former cell type being a resident macrophage isolated using lavage and the latter isolated from bone marrow and in vitro differentiated with M-CSF. Although differential C1q expression upon TREM-2 deficiency may account for the phagocytic differences between the cell types, it is highly likely that in BMDM, additional factors play a role.

The cell-type specific effects of TREM-2 on phagocytosis are reminiscent of its effects on antigen presentation and osteoclastogenesis, which clearly differ between different cell types. TREM-2 stimulation of immature dendritic cells induces expression of MHC class II and co-stimulatory molecules that are required for antigen presentation (61), but this effect is not

observed in microglia (11). TREM-2 deficiency in RAW 264.7 macrophages and human monocytes leads to a reduced capacity to generate osteoclast precursors but bone marrow cells from *Trem-2*$^{-/-}$ mice exhibit accelerated osteoclastogenesis (12, 32, 62). Furthermore, although TREM-2 has been shown to be a phagocytic receptor for apoptotic neurons (11), uptake of microspheres is unchanged following knockdown of TREM-2, indicating TREM-2 is not essential for all types of phagocytosis (63).

Interestingly, our data show that AM expressed TREM-2 not only suppresses bacterial phagocytosis but also efferocytosis (Fig. 6M). These data are in contrast to previous studies suggesting that TREM-2 promotes the uptake of apoptotic neurons by microglia (11, 63). C1q is important for the uptake of apoptotic cells (47-49). Indeed, as C1qa$^{-/-}$ mice age they develop multiple apoptotic bodies and autoimmunity compared to WT controls (47). Within the lung, improved clearance of apoptotic neutrophils by AM promotes the resolution of inflammation (53-56). Our data raise the intriguing possibility, that AM expressed TREM-2 might influence efferocytosis through C1q. This hypothesis is consistent with our observations of TREM-2 specifically modulating bacterial phagocytosis and efferocytosis but not the uptake of BSA by AM. These findings make perfect sense in the context of TREM-2 modulating these processes via C1q, as C1q acts as a bacterial opsonin and is also critical for the uptake of apoptotic cells.

The ability of TREM-2 to regulate cellular responses in a cell-type specific manner is not limited to phagocytosis and efferocytosis. In this study we show that TREM-2 also modulates *S. pneumoniae* and TLR2 induced inflammation in a cell-type specific manner, with TREM-2 deficient AMs displaying less inflammation in response to either stimulus. Importantly, these observations are consistent with very recent studies by Correale *et al.*, who demonstrated that dendritic cells from *Trem-2*$^{-/-}$ mice display attenuated inflammation in response to TLR ligands (64). Further, we find in vivo that, other than KC, no other inflammatory mediator tested was higher in *Trem-2*$^{-/-}$ mice following early pneumococcal infection compared to their WT counterparts. In this regard it is important to note that recent observations indicate that the anti-inflammatory activity of TREM-2 in vivo may differ depending on the disease context. For example, *Trem-2*$^{-/-}$ mice exhibit attenuated inflammation following DSS induced colitis and stroke, rather than augmented inflammation as expected (64, 65). In summary, our study, together with others present in the literature, provides credence for the claim that TREM-2 impacts cellular responses in a cell-type, stimulus- and disease context-specific manner. Since we found TREM-2 to suppress C1q secretion, it is interesting that C1q has

been shown to dampen TLR mediated cytokine synthesis, although the exact mechanism behind this is unknown (66, 67). It is tempting to speculate that the suppressive effects of TREM-2 on C1q production in AM not only modulate bacterial phagocytosis but also dampen TLR mediated inflammation.

This is the first report demonstrating a function for TREM-2 in the pulmonary compartment. Importantly, we clearly show that the ability of TREM-2 to confer bacterial phagocytosis is cell-type specific and that TREM-2 modulates C1q production by AM. It is tempting to speculate that targeting the TREM-2 pathway could be used as a novel strategy for modulating C1q production and pulmonary innate immune responses, which might be of relevance to other respiratory tract infections and possibly autoimmune diseases.

Materials and Methods

ELISA and Reagents

ELISA kits for mouse TNF, MCP-1, IL-1β, IL-6, KC and C5a were from R & D Systems, for human IL-6 from eBioscience and the mouse C3a ELISA was from Uscn Life Science Inc. All ELISAs were performed according to the manufacturer's instructions. GSK0660 was from Tocris Biochemicals. GW0742 and GSK3787 and recombinant C1q were purchased from Sigma. *S. pneum*oniae LTA was a kind gift from Sonja von Aulock (University of Konstanz, Germany). C1q antibodies used for FACS and blocking experiments were clones 7H-8 (in vitro and in vivo) and JL-1 (in vitro) respectively, both purchased from Hycult Biotech as was the MARCO (clone ED-31) antibody. Isotype control antibody IgG2bκ (559530) used for C1q blocking experiments was from BD Bioscience. CD36 (clone 63) antibody was from Millipore. CD45-V500 (clone 30-F11) was from BD Bioscience. Ly6G-PE (clone 1A8), CD11c-APC (clone N418), F4/80 (clone BM8) antibodies were from Biolegend. CD11b-Alexa Fluor 700 (clone M1/70) was from eBioscience. TREM-2 (BAF1729), PPAR-δ (ab8937) and β-actin (clone AC15) antibodies for western blotting were from R/D systems, AbCaM and Sigma respectively. Recombinant PPAR-δ was supplied in the PPAR-δ activity assay (Abcam ab133106). Ly6G (clone 1A8) and active caspase 3 (Asp175 – clone 5A1E) antibodies used in IHC were from BD Bioscience and Cell Signaling respectively. As secondary reagents we used PE conjugated rat anti-mouse (eBioscience), FITC conjugated goat anti-rat $F(ab')_2$ (Jackson Immunoresearch), anti-rabbit HRP (Cell Signaling), streptavidin HRP (R/D systems), anti-mouse HRP (BioRad), biotinylated anti-rat IgG (Vector Laboratories) and biotinylated, swine anti-rabbit IgG (Dako) .

Animals

Trem-2$^{-/-}$ mice were generated as previously described (10) and the TREM-2 mutation was backcrossed onto a >98% B6 C57BL/6 background facilitated by genome-wide SSLP typing at 10cM intervals (done by the Speed Congenics Facility of the Rheumatic Diseases Core Center). Wild type mice were purchased from Charles River and all mice were bred at the Medical University of Vienna Animal facility under pathogen free conditions. Age (8-10 week) and sex matched mice were used in all experiments.

Ethics Statement

All animal experiments were discussed and approved through the Animal Care and Use Committee of the Medical University of Vienna and the Austrian Ministry of Sciences and were carried out in strict accordance with Austrian law (Tierversuchsgesetz; BMWF-66.009/0321-II/10b/2008).

Bacteria

S. pneumoniae serotype 3 was obtained from American Type Culture Collection (ATCC 6303). Serotype 19A *S. pneumoniae* was a clinical isolate from a patient suffering from severe invasive pneumococcal disease and confirmation of the serotype was provided by antibodies specific to the capsule (Statens Serum Institute). Both strains were grown for 6h to mid-logarithmic phase at 37°C in Todd-Hewitt broth (Difco), harvested by centrifugation at 4000rpm for 15 min at 4°C, and washed twice in sterile saline. *S. pneumoniae* serotype 3, was used for all in vivo experiments and most in-vitro experiments (except Fig. 3H). Bacteria were diluted in sterile saline to obtain an estimated concentration of 10^5 CFU per 50µl for intranasal inoculation of mice. The true concentration was determined by growing serial 10-fold dilutions on sheep blood agar plates overnight.

Mouse Model of Pneumococcal Pneumonia

Pneumonia was induced by intranasal administration of a bacterial suspension containing 10^5 CFU *S. pneumoniae* (ATCC 6303) as described earlier (19, 58, 68). Six, 24, or 48 h after infection, mice were anesthetized with ketamine (Pfizer) and sacrificed. Blood was collected in EDTA-containing tubes and plated on blood agar plates to determine bacterial counts, plasma was stored at −20°C. Whole lungs were homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products), after which serial 10-fold dilutions in sterile saline were plated on blood agar plates, left at 37°C and CFU were counted 16h later. Remaining lung homogenates were incubated for 30min in lysis buffer (containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and pepstatin A, leupeptin, and aprotinin (all 20 ng/ml; pH 7.4; Sigma-Aldrich)) at 4°C, centrifuged at 1500 *g* at 4°C, and supernatants were stored at −20°C until cytokine measurements were performed. Lungs for histology were harvested 48h post infection, fixed in 10% formalin, and embedded in

paraffin. Four-µm sections were stained with H&E and analyzed by a pathologist blinded for groups, who scored lung inflammation and damage as previously described (19, 58).
In separate experiments, bronchoalveolar lavage (BAL) was performed 6h and 24h after infection *by exposing the trachea of mice* through a midline incision, canulating it with a sterile 18-gauge venflon (BD Biosciences) and instilling two 500µl aliquots of sterile saline. Approximately, 0.9ml was retrieved per mouse. Total cell numbers were counted using a hemocytometer (Türck chamber); differential cell counts were done on cytospin preparations stained with Giemsa. BALF supernatant was stored at –20°C for cytokine measurements. In some experiments, to assess survival, mice were intranasally infected with *S. pneumoniae* and survival was monitored regularly for 10 days. In experiments involving C1q blocking, 125µg of C1q blocking antibody (kindly provided by Admar Verschoor, Technical University of Munich) or isotype control were intranasally instilled prior *to S. pneumoniae* infection.

Immunohistochemistry and TUNEL staining

Paraffin embedded lungs from WT and *Trem-2*$^{-/-}$ mice were deparaffinized in xylene and ethanol and subjected to antigen-retrieval using citrate buffer pH6.0 (Vector laboratories). Thereafter endogenous peroxidase activity was blocked with 1.6% H_2O_2 in PBS. Following washing and blocking steps using 10% swine or rabbit serum (Vector laboratories) in PBS for the active caspase 3 (Cell signaling) and Ly6G (BD Biosciences) stains respectively, sections were incubated either overnight at 4ºC or 1h room temperature with the active caspase 3 diluted to 1:25 in swine serum overnight and the Ly6G antibody diluted 1 in 50 in rabbit serum. After washing, endogenous biotin and avidin sites were blocked using the avidin biotin blocking kit (Vector laboratories). Sections were washed, and incubated with either biotinylated anti-rat IgG (Vector Laboratories) or biotinylated, swine anti-rabbit IgG (Dako) for the Ly6G and active caspase 3 stains respectively. Binding was visualized using the Vectastain ABC kit (Vector laboratories) followed by either a step where the sections were incubated with DAB conjugated to HRP (active caspase 3) or Nova Red stain (Vector laboratories, Ly6G stain). Sections were countered with hematoxylin, dehydrated and subjected to light microscopy.
Tunnel stain was performed using the in situ cell death detection kit, according to the manufacturer's instructions (Roche 1684809). Briefly, paraffin embedded lungs from WT and *Trem-2*$^{-/-}$ mice were deparaffinized in xylene and ethanol and subjected to antigen-retrieval

using Pronase E (Sigma). Following washing and incubation with Tunnel reaction mix, nuclei were stained using DAPI (Sigma). Slides were visualized under fluorescence illumination (Zeiss, AxioImager.M2).

Cell isolation, culture and stimulations

AM were obtained by BAL from healthy WT or *Trem-2*$^{-/-}$ mice. Cells were resuspended in RPMI 1640 containing 1% penicillin/streptomycin (pen/strep) and 10% FCS and plated for either phagocytosis assays or stimulations at the appropriate density. In some cases this was on C1q (Sigma-Aldrich) coated or BSA coated plates and cells were left to adhere for 3h or overnight before stimulations. BMDMs were retrieved from the tibia and the femur of mice and differentiated in RPMI 1640 supplemented with 1% pen/strep, 10% FCS and 10% L929-conditioned medium for 7 days. Primary lung epithelial cells were isolated as previously described (69) and were cultured in in HITES media as were MLE-12 and 15 cells as previously described (31). RAW 264.7 cells cultured in RPMI 1640 containing 1% pen/strep and 10% FCS. In all experiments related to cytokine production cells were stimulated with 2 x 10^7 CFU/ml *S. pneumoniae* (MOI 100) or 10μg/ml LTA for 6h unless otherwise indicated.

Generation of TREM-2 over expressing RAW 264.7 cells

Retroviral transfection was used to generate RAW264.7 cells that were stably transfected with GFP or TREM-2. Briefly, the packaging cell line GP-293 HEK (Clontech) was transfected with TREM-2 expression plasmid (pORF-hTREM-2 originally purchased from InvivoGen) 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.

Luciferase assays

For reporter gene assays, HEK cells seeded at 1.5×10^5cells/ml, were transfected with PPRE luciferase promoter constructs (provided by Nikolina Papac, Medical University of Vienna), expression vectors encoding PPAR-δ, RXR-α (provided by Ajay Chawla, University of California, San Francisco), vector control (pIRES, Stratagene) or a combination of PPAR-δ, RXR-α together with TREM-2 and DAP-12 (subcloned into the pIRES backbone) using

calcium phosphate. 24h post transfection, cells were stimulated with 1μM GW0742 and 48h post transfection luciferase activity was determined using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). All transfectants contained the pRenilla luciferase gene vector (Promega) as an internal control for transfection efficiency and luciferase values were normalized to renilla.

Preparation of FITC labeled bacteria and CFSE labeled apoptotic cells

S. pneumoniae (ATCC 6303 and serotype 19A) or *E.coli* (018:K1) were grown in Todd-Hewitt broth (Difco) or Luria Bertani medium respectively, washed twice and resuspended in saline at a concentration in the range of 10^9 CFU/ml as determined by OD_{600}. Bacteria were heat killed at 65°C, for 30 min, washed once with 10ml 0.1M $NaHCO_3$, resuspended in a solution containing 0.2 mg/ml FITC dissolved in 0.1M $NaHCO_3$ and incubated under constant stirring in the dark at 37°C for 1h. Bacteria were washed twice with PBS and the concentration was set to obtain 2 x 10^9 CFU/ml.

Thymocytes were isolated from 4 week old C57BL/6 female mice and apoptosis was induced by culturing 6 x 10^6 freshly isolated thymocytes in RPMI/10% FCS with 1μM dexamethasone for 16h as previously described (70). Apoptotic laddering was assayed using a commercially available kit (Roche Diagnostics). 7-AAD/Annexin V staining was performed by resuspending the thymocytes in staining buffer (0.1M Hepes, 1.4M NaCl, 25mM $CaCl_2$, pH 7.4), after which Annexin V (BD Biosciences) was added at 1:20 for 15 min, following which 7-AAD (eBioscience) was added at 1:50 for 15 min. Cells were analyzed by flow cytometry (BD LSR Fortessa). Following apoptosis, thymocytes were labeled with CFSE, according to the manufacturer's instructions (Molecular Probes).

Phagocytosis and efferocytosis assays

AM or BMDM from WT and *Trem-2*$^{-/-}$ mice were plated at 0.5x10^6/ml in 12-well microtiter plates (Greiner) and allowed to adhere overnight. After washing steps, FITC-labeled heat-killed *S. pneumoniae* or *E. coli* (O18:K1) was added in the presence of RPMI for 1h (MOI 100) at 37°C or 4°C (as a negative control), respectively. Cells were treated with proteinase K at 50μg/ml for 15 min at room temperature to remove adherent but not internalized bacteria and subsequently placed on ice and washed. In some experiments, bacteria were pre-opsonised with either 10% pooled WT mouse serum, C1qa$^{-/-}$ serum (47)or 10% Type III

capsular antibody (Statens Serum Institute, Denmark) in RPMI for 30 min before addition to the cells. Uptake was analyzed using a flow cytometer (Beckton Dickinson FACScalibur). The phagocytosis index of each sample was calculated: (mean fluorescence x % positive cells at 37°C) minus (mean fluorescence x % positive cells at 4°C).

Verification of phagocytosis results obtained via FACS was conducted using confocal microscopy as previously described (68). Briefly, AM plated at 3 x 10^5 in 8 well chamberslides (Lab-Tek Chamberslide system) were incubated with FITC-labeled heat-killed *S. pneumoniae* at a MOI 100 for 1h at 37°C. After washing steps, lysosomes were stained with Lysotracker red and nuclei with DAPI (Invitrogen), followed by visualization using confocal laser scanning microscopy (LSM 510, Zeiss). The ratio of engulfed bacteria (as determined by overlay of green bacteria and red lysosomes) was quantified by an independent researcher from 300 counted cells per well and is expressed as percentage of cells that contain bacteria.

For the in vivo phagocytosis assays WT and *Trem-2*$^{-/-}$ mice were inoculated intranasally with 5x10^6 CFU (MOI 10, assuming 5 x 10^5 cells in a naïve mouse) FITC-labeled *S. pneumoniae*. BALF was collected 4h later, cells were resuspended in a PBS supplemented with 1% FCS and antibodies against Ly6G, CD11b, F4/80, CD11c and CD45 and incubated for 30min. After a washing step, cells were resuspended in PBS and analyzed by flow cytometry (BD LSR Fortessa). AMs were identified as F4/80$^+$, CD11c$^+$, Ly6G$^-$, CD11b$^-$ cells. Neutrophils were identified as the Ly6G$^+$, CD11b$^+$, F4/80$^-$ and CD11c$^-$ population. To control for background FITC signals and non-specific binding of bacteria, the % of FITC-positive CD45$^-$ cells (i.e. non-phagocytosing cells) was subtracted from the % of FITC-positive CD45$^+$ cells (i.e. containing phagocytosing cells).

For efferocytosis assays AM from WT and *Trem-2*$^{-/-}$ mice were incubated with CFSE labelled apoptotic thymocytes at the indicated MOI for 1h at 37°C or 4°C (as a negative control), respectively. AM were subsequently removed and stained using APC conjugated CD11c antibody (clone N418, eBioscience). Uptake was analyzed using a flow cytometer (Beckton Dickinson FACScalibur). The efferocytosis index of each sample was calculated: (mean fluorescence x % CD11c$^+$ CFSE$^+$ cells at 37°C) minus (mean fluorescence x % CD11c$^+$ CFSE$^+$ at 4°C).

RT-PCR

Trizol was used for RNA extraction from primary cells and cDNA was converted using the Superscript III first strand synthesis system as recommended by the supplier (Invitrogen). RT-PCR was conducted according to the LightCycler FastStart DNA MasterPLUS SYBR Green I system using the Roche Light cycler II sequence detector (Roche Applied Science). Mouse gene-specific primer sequences used are shown in supporting information table 1. All transcript levels studied were normalized to HPRT.

Western blotting

5 x 10^6 cells were treated as indicated in the figure legends and whole cell extracts were prepared. Cells were washed once with cold PBS, after which the cell pellet was solubilized and lysed in ice cold whole cell extract buffer (20 mM Hepes pH 7.6, 400 mM NaCl, 1 mM EDTA, 5 mM NaF, 500 μM Na3VO4, 25% glycerol, 0.1% NP-40, 1 mM PMSF, 1 mM DTT, 0.1 mg/ml aprotonin). Lysates were centrifuged at 14,000 rpm for 15 minutes and stored at −80°C. Equal amounts of protein were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. Antibodies specific for TREM-2 and PPAR-δ were used at 1:1000 and β-actin at 1:500. Immunoreactive proteins were detected by enhanced chemiluminescent protocol (GE Healthcare).

PPAR-δ activity assay

5 x 10^6 cells were treated as indicated in the figure legends and nuclear extracts were prepared by washing the cells with ice cold PBS, followed by three washes in 1 ml of ice cold hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl) to induce swelling. Thereafter, the pellet was resuspended in hypotonic buffer supplemented with 0.1% Nonidet P-40 and incubated on ice for 5 minutes to release nuclei. Subsequently, samples were centrifuged at 4°C to pellet nuclei, the cytoplasmic fraction was removed and the nuclear pellett resuspended in 50 μl of ice cold high salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 25% glycerol) and incubated for 15 minutes at 4°C. Disrupted nuclei were centrifuged at full speed in a table top centrifuge for 15 minutes at 4°C, after which the supernatant (nuclear fraction) removed. PPAR-δ DNA binding activity in nuclear extracts was

determined using the PPAR-δ transcription factor assay kit according to the manufacturer's instructions (Abcam). In brief, nuclear extracts were incubated on a 96 well plate to which the PPRE consensus sequence was immobilized. After binding and washing, PPAR-δ activity within nuclear extracts was detected using a PPAR-δ specific antibody followed by incubation with a secondary HRP conjugated antibody and quantification using spectrophotometry. The specific OD was calculated by subtracting the non-specific binding wells (i.e. wells where nuclear extract was absent), and normalized to protein content.

Microarray

Isolated total RNA was purified using the RNeasy kit per manufacturer's instructions (Qiagen). Total RNA (200 ng) was then used for GeneChip analysis. Preparation of terminal-labeled cDNA, hybridization to genome-wide murine GeneLevel 1.0 ST GeneChips (Affymetrix), and scanning of the arrays were carried out according to manufacturer's protocols. Affymetrix microarray cell intensity files were combined, and expression was normalized using the robust multi-array average algorithm (71), generating an expression matrix. Identification of differentially regulated genes was performed with significance analysis of microarrays as described previously (72); a false-discovery rate of 5% was imposed. All data are deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and the accession ID is GSE51378.

Statistical analysis

Data are presented as the mean ± SEM. Comparisons between groups was assessed using either T-test or ANOVA followed by Tukey's multiple comparisons analysis, where appropriate. Survival data was analyzed by Log rank (Mantel-Cox) test using GraphPad Prism Software.

Supporting information:

Supporting Table 1

Sequences of primers used for RT-PCR

Gene	**5‘**	**3‘**
C1qb	GCAGCAGGCTCTGGGCTCTGGGA	ATGCCAGGGGGCCCGGTGCA
C1qc	CTGTCTGGGAGAACAGGACGTCTCT	GGCATGCCAGGCTCGCCCTT
THBS1	CCCCGGTGCACACAGGCTCCG	TCGGCGACCGGGGCCCCTTC
TREM-1	ATGACCTAGTGGAGGGCCAG	GCACAACAGGGTCATTCGGAG
TREM-2	TTGCTGGAACCGTCACCATC	CACTTGGGCACCCTCGAAAC
HPRT	GTTAAGCAGTACAGCCCCAAAATG	AAATCCAACAAAGTCTGGCCTGTA

Supporting Figure 1:

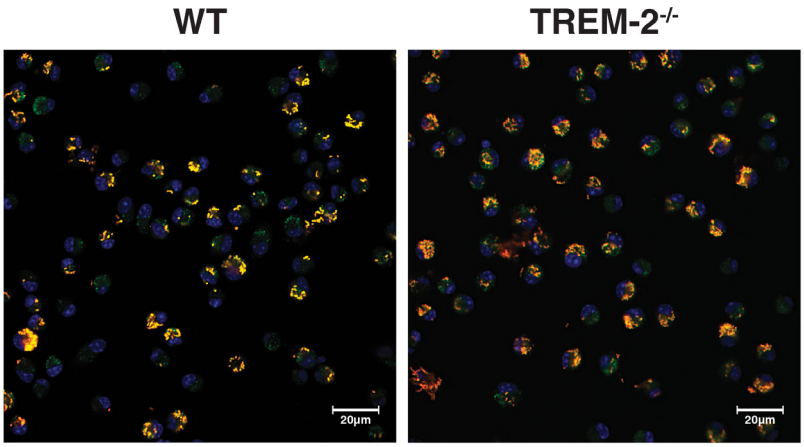

Figure S1: TREM-2 deficient AM exhibit enhanced phagcytosis of *S. pneumoniae* as determined by confocal microscopy

WT and *Trem-2*$^{-/-}$ AM were incubated with FITC labeled *S. pneumoniae* at an MOI of 100 and confocal microscopy was conducted as described in the materials and methods. Depicted are the original pictures. Magnification x 100.

Supporting Figure 2:

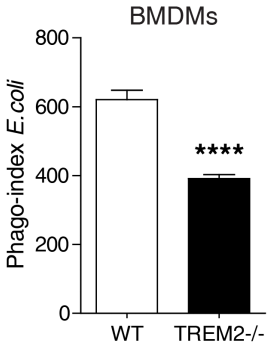

Figure S2: TREM-2 deficient BMDM exhibit lower phagocytosis of *E.coli*

WT and *Trem-2*$^{-/-}$ BMDM (n = 5 per genotype) were incubated with FITC labeled *E. coli* at an MOI of 100 and phagocytosis was assessed 1h later by FACS. Data are presented as mean ± SEM versus WT, **** $p < 0.0001$ and are representative of two independent experiments.

Supporting Figure 3:

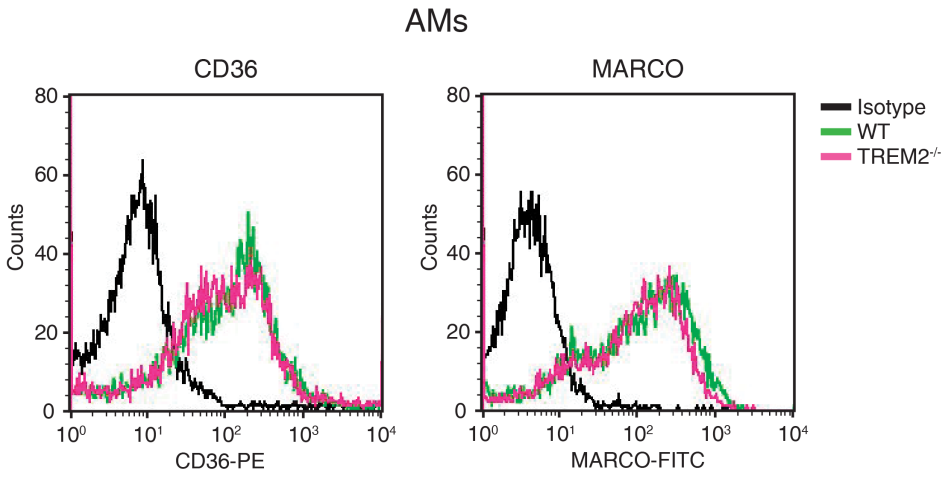

Figure S3: No difference in surface expression of the phagocytic receptors CD36 and MARCO between WT and TREM-2$^{-/-}$ AM

Basal surface expression of CD36 and MARCO in WT versus *Trem-2*$^{-/-}$ AMs as determined by FACS. Green lines depict WT macrophages, pink *Trem-2*$^{-/-}$ macrophages and black represents isotype control antibody.

Supporting Figure 4:

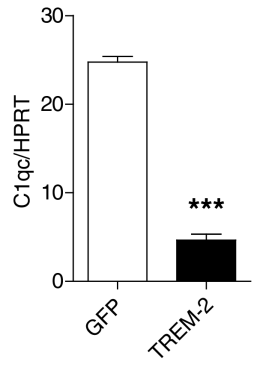

Figure S4: Overexpression of TREM-2 lowers *C1qc* levels

C1qc basal levels were determined in GFP control or TREM-2 overexpressing RAW 264.7 macrophages using RT-PCR (n = 4 per condition). Data are presented as mean ± SEM and are representative of two independent experiments, *** indicates $p < 0.001$.

Supporting Figure 5:

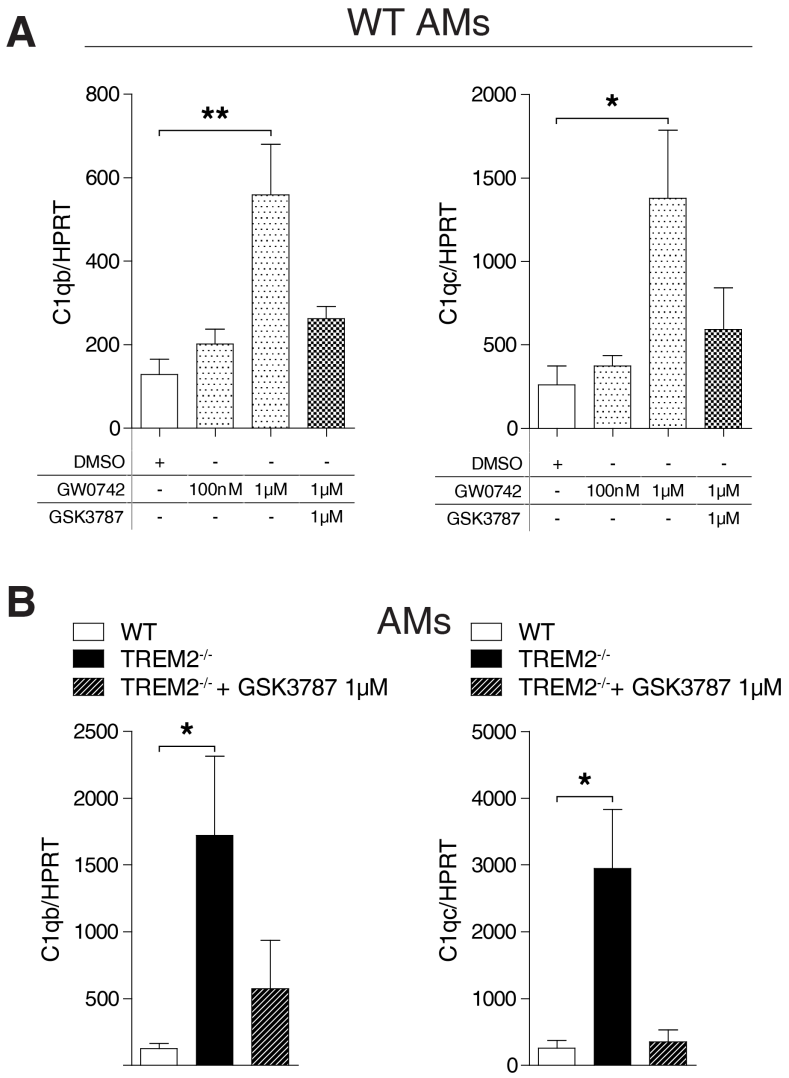

Figure S5: Dependence of C1q expression in AMs on PPAR-δ

(A) WT AM (n = 3-4 per condition) were pre-treated for 24h with the indicated doses of the PPAR-δ activator GW0742 in combination with the PPAR-δ inhibitor GSK3787 along with a DMSO control after which RT-PCR of *C1qb* and *C1qc* was performed. (B) WT and *Trem-2*$^{-/-}$ AM (n = 4 per condition) were treated with DMSO or the PPAR-δ inhibitor GSK3787 after which RT-PCR of *C1qb* and *C1qc* was performed. Data are presented as mean ± SEM and compared versus WT with * $p < 0.05$, ** $p < 0.005$.

Supporting Figure 6:

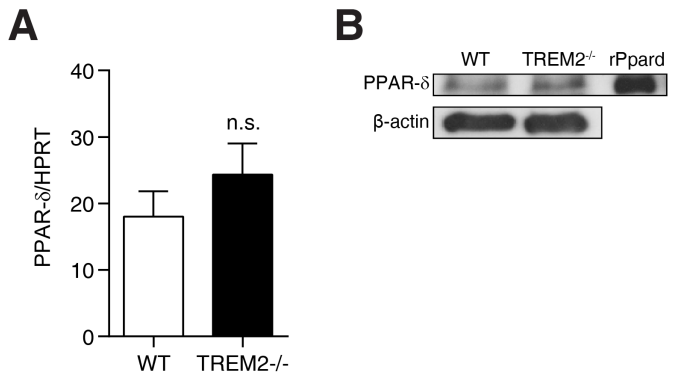

Figure S6: No difference in PPAR-δ levels between WT and *TREM-2*$^{-/-}$ AM.

(A) *PPAR-δ* basal expression was determined in WT and *Trem-2*$^{-/-}$ AM using RT-PCR (n = 3-4 per genotype). (B) PPAR-δ protein levels from whole cell extracts of WT and *Trem-2*$^{-/-}$ AM. The specificity of the antibody was indicated by recombinant PPAR-δ (rPPAR-δ). Data are representative of two independent experiments.

Supporting Figure 7:

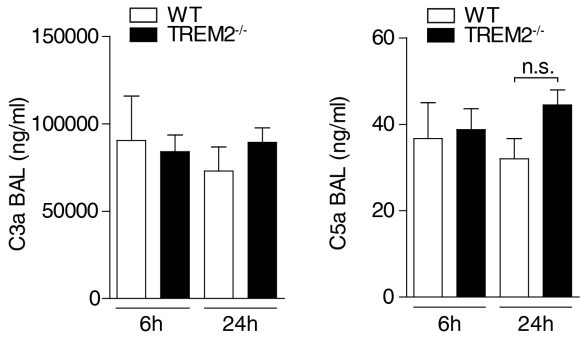

Figure S7: Unaltered C3a and C5a levels in the BALF of *TREM-2*$^{-/-}$ mice following *S. pneumoniae* infection.

WT and *Trem-2*$^{-/-}$ mice (n = 6-7 mice per genotype) were intranasally infected with 10^5 *S. pneumoniae* and C3a and C5a levels were determined in the BALF 6 and 24h post infection. Data represent mean ± SEM versus WT.

Supporting Figure 8:

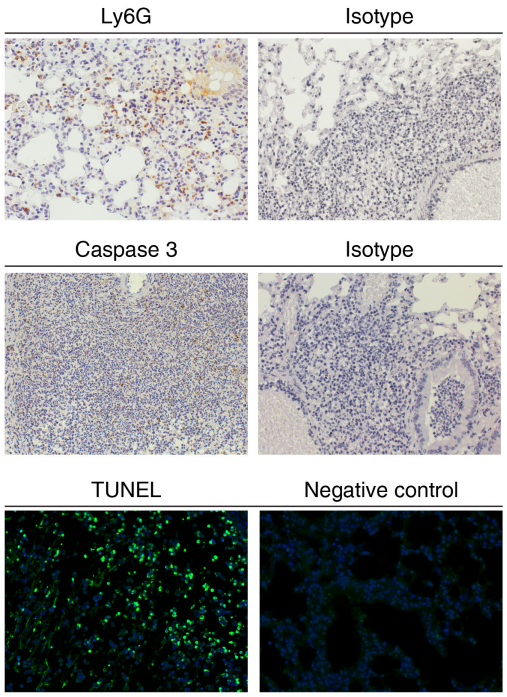

Figure S8: Specificity of antibodies used in Immunohistochemistry and TUNEL

Representative Ly6G**,** active caspase 3, and TUNEL staining of lungs 48h post infection of mice infected with 10^5 *S. pneumoniae* (n= 9) depicting the specificites of the antibodies used.

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3. Discussion

Toll-like receptor mediated inflammation as well as efficient clearance of pathogens by phagocytosis are key determinants for the outcome of infections. Inflammation, as a vital response, is crucial for the initiation of an effective immune response against potentially dangerous stimuli. Tissue resident cells are activated (via PRRs expressed on their surface) to produce proinflammatory cytokines and chemokines, thereby facilitating the recruitment of mononuclear phagocytes to the site of infection that will subsequently promote the elimination of bacteria. (14, 189, 190) However, the downside of inflammation is that it can, if uncontrolled, cause severe tissue damage with dramatic consequences for the host, a phenomenon that is often happening during sepsis (109). Thus, inflammation has to be under tight control in order to allow for pathogen clearance, while preventing immunopathology (77).

Phagocytosis is the process of actin dependent uptake of large particles (> 0.5 μm diameter) carried out by leukocytes in order to remove harmful material from the body. It is thereby critical for the host to defend against invading microorganisms and to maintain and restore homeostasis (including the removal of apoptotic cells). (190)

The triggering receptor expressed on myeloid cells (TREM)-2 has been identified as a negative regulator of TLR-mediated inflammation (169, 172) as well as a phagocytic receptor for bacteria (157, 186) and apoptotic cells (164, 171). Considering its impact on these important features of host defense, the basic assumption for this work was that TREM-2 would play an important role during bacterial infections. Therefore, this manuscript aims to better understand the function of TREM-2 in a context of inflammation and infectious disease in vivo. This was approached by investigating TREM-2 in different in vivo models of inflammation: *E. coli* peritonitis, a model for Gram-negative sepsis, LPS-induced endotoxemia, a model for septic shock and sterile inflammation as well as during pneumococcal pneumonia, an infection of the lung with a Gram-positive pathogen. Complementing in vitro studies were performed for a better understanding of the effects TREM-2 exerts on different macrophage subsets.

Interestingly TREM-2 signaling turned out to be detrimental during endotoxemia, as well as during pneumococcal pneumonia, while it seems to have no impact on the outcome from *E. coli* peritonitis (191, 192). However a more detailed analysis showed that also during *E. coli*

infection, TREM-2 critically shaped immune responses, but due to counterbalancing effects, this did not result in altered survival of TREM-2 deficient mice as compared to wild type controls (191).

As the results of each study are already discussed in detail in the discussion part of the respective paper, this discussion will rather focus on dissecting the two studies next to each other in the context of the current literature and analyze where they show similarities and where they differ. In addition cell-type and model-type specific effects of TREM-2 will be discussed in detail, followed by a short section with general conclusions that can be drawn from this work.

The investigation of TREM-2's function during Gam-negative sepsis and endotoxemia yielded in the following interesting conclusions: i) TREM-2 is expressed on resident peritoneal macrophages (PM), which was not shown before and ii) critically suppresses the TLR4 mediated production of inflammatory molecules by PM in vitro and in vivo, resulting in augmented neutrophil influx in the absence of TREM-2 upon injection of a deadly dose of either live bacteria or LPS. iii) While this accelerated early inflammation was correlated with an augmented early induction of the negative regulator A20 and subsequently improved the survival of TREM-2 knockout mice during endotoxemia, during infection with live *E. coli* the advantage of a strong early response was antagonized by TREM-2's impact on phagocytosis, resulting in unaltered survival. (191)

In line with the literature (169, 172, 183), TREM-2 clearly seems to play a negative regulatory role in TLR4 induced inflammation in vitro and in vivo. The fact that the enhanced early inflammatory response to LPS in the absence of TREM-2 resulted in an improved survival of TREM-2$^{-/-}$ mice, seemed to be controversial at first, as deficiency or malfunction of typical negative regulators is usually detrimental in this model (77, 193, 194). However, upon closer examination, it became clear that while TREM-2 suppressed the production of proinflammatory genes it also delayed the induction of the important negative regulator A20 (191), a NF-κB target gene that is protective during endotoxemia (88, 195). These results are also supported by data showing that also deficiency in DAP12 improves the outcome of LPS induced peritonitis (196). In contrast to sterile inflammation, during infection with live bacteria, the otherwise protective effect of a boosted early inflammatory response was counteracted by TREM-2's important function as a phagocytic receptor. Although in the absence of TREM-2, phagocytes were present in higher numbers, they were impaired in their

most important effector function, as they showed a reduced ability to take up bacteria. This resulted in an unaltered survival phenotype, despite the changes in the initial immune response (191). The importance of TREM-2 as a phagocytic receptor has been described earlier in a study showing that the application of TREM-2 overexpressing bone-marrow derived cells promoted bacterial clearance in a CLP model (186).

Studying TREM-2's role during pneumococcal pneumonia showed that TREM-2 exerts clear detrimental effects, as TREM-2 deficiency improved bacterial clearance and survival. This could be attributed on its effects on alveolar macrophages (AM) that are residing in the lung and are, like PM in the peritoneum, critical in inducing an early inflammatory response to an infection. (192)

However, looking more closely into the mechanism uncovered an unexpected and cell-type specific function for TREM-2 on AMs. Controversially, AMs showed to phagocytose more efficient in the absence of TREM-2 than in its presence and TREM-2 seemed to rather amplify than suppress cytokine production. (192) This was confusing at first, because of TREM-2's established phagocytic function (157, 186) and its proposed negative regulatory role in inflammation (172, 191), but then turned out to be the result of higher levels of the complement component C1q in the absence of TREM-2 (192), which is locally produced by AMs in the lung (197, 198) and can mediate both: enhanced phagocytosis of bacteria (199, 200) and apoptotic cells (201, 202) as well as a suppression of cytokine production (203, 204). Furthermore this study could show that C1q expression is dependent on PPR-δ activity and that TREM-2 suppresses PPR-δ in AM. (192) In addition (and in accordance with the results obtained with *E. coli* and LPS) the absence of TREM-2 led to an accelerated infiltration of neutrophils, which might have partially contributed to the phenotype. (192)

Interestingly, a very recent study showed that also DAP12 is deleterious during pneumococcal pneumonia. Similar to TREM-2, DAP12 deficiency led to a better clearance of bacteria and was associated with an improved capacity of AMs to phagocytose *S. pneumoniae*. (205)

Studying TREM-2 expression and function on different types of macrophages, TREM-2 showed to exert cell-type specific effects. In all macrophage subsets tested (namely AM, PM, BMM and RAW 264.7 cells) TREM-2 appears to be posttranslationally modified, indicated by the detection of multiple TREM-2 specific bands by westernblot analysis. But while in in PMs, BMM and RAW 264.7 cells four different bands were detectable (191), only two appeared to be present in AM (192). Further analysis was carried out in BMM and revealed

that the double band detectable around 28-30kDa was core-glycosylated TREM-2 that was still stored in the endoplasmatic reticulum or at least has not undergone full processing in the golgi apparatus. The doubleband detectable around 37-40 kDa was identified as a fully glycosylated (surface) form of TREM-2 that has been already fully processed. (191) This higher molecular weight bands were absent in AM, indicating that AM seem to store mainly intracellular pools of TREM-2, which might partially account for the cell-type specific differences we have observed functionally. (192) In addition, glycosylation patterns seem to slightly differ also between BMM and PM (191). Cell-type- and site-specific glycosylation is a common phenomenon that has been observed in many proteins (206-208) and might be an important determinant of cell-type specific responses and binding of ligands.

Regarding its impact on inflammation, a very reproducible result from all in vivo studies was a clear suppressive effect of TREM-2 on the early recruitment of neutrophils, as we found higher neutrophil numbers in the PLF of TREM-$2^{-/-}$ mice as compared to WT in response to *E. coli* and LPS (191) and also during pneumococcal pneumonia more neutrophils migrated to the lung in the absence of TREM-2 (192). It is therefore intriguing to propose a role for TREM-2 in leukocyte migration. Interestingly, we could not identify the reason for these changes in neutrophil migration in all cases: While during endotoxemia and pneumonia higher neutrophil numbers correlated with augmented levels of the important neutrophil chemoattractant KC in the PLF (191) or in BAL fluid respectively (192), TREM-2 didn´t seem to impact on early cytokine or chemokine production in response to live *E. coli* (data not shown). Only at later stages, when the mice already show severe organ damage and signs of sepsis, TREM-2 deficient mice displayed higher levels of MCP-1 and KC (191). Given that we only determined levels of IL-6, KC and MCP-1 during *E. coli* peritonitis, we cannot exclude changes in other factors that might influence the migratory behavior of neutrophils. However, together with the fact that TREM-2 deficient neutrophils seem to be impaired in phagocytosis (191, 192) it is intriguing to speculate about cell intrinsic alterations in the migratory behavior of neutrophils that lack TREM-2.

Examining how TREM-2 regulates cytokine induction on a cellular level, we found in vitro that it clearly inhibited responses by PM to LPS and *E. coli* (191) as well as by BMM in response to LPS and *S. pneumoniae*, while it amplifies the production of KC and TNF in AM challenged with LTA or pneumococci (192). Interestingly, in the case of *S. pneumoniae*, in vitro data did not match with what was found in vivo, as there was enhanced KC production

after 6h pneumonia in TREM-2 deficient mice (192). Taking into account that lung epithelial cells express a variety of TLRs and are able to release KC upon activation (209, 210), this points towards a role for epithelial cells in the early production of KC in this model, and indicates that TREM-2 might also negatively regulate their inflammatory potential. In vitro, the results obtained with AM were clearly in conflict with data generated with other macrophage types (172, 191, 192) and the proposed negative regulatory role of TREM-2 (169, 172). Elucidating the mechanism how TREM-2 might impact in such a cell-type-specific manner on basic macrophage functions involved a microarray study of AMs unstimulated and stimulated with *S. pneumoniae* and revealed that, as mentioned earlier, TREM-2 is an important suppressor of C1q expression by inhibiting PPR-δ activity in AM. (192) C1q has been shown to suppress cytokine responses. In LPS challenged monocytes C1q as well as MBL are able to inhibit the expression of proinflammatory cytokines (203, 204) and it was shown that C1q is doing so by promoting the formation of NF-κB p50 homodimers (203). We have also measured C1q in BMM (192) and peritoneal macrophages (data not shown) and could not find any differences between WT and TREM-2 deficient cells, further supporting the idea that the cell-type specific effects TREM-2 exerts in AMs are largely due to its influence on C1q expression.

A closer study of TREM-2's impact on phagocytosis, by performing in vitro as well as in vivo phagocytosis assays, revealed that TREM-2 definitely affects the phagocytic capacity of macrophages and (unexpectedly) of neutrophils. While in PM and neutrophils phagocytosis was hampered in the absence of TREM-2, TREM-2 deficiency on AMs again showed an unexpected phenotype and surprisingly enhanced their capacity to engulf *S. pneumoniae* (191, 192). The effect on PMs can be simply explained by TREM-2's presence on these cells and is in line with other publications that identify TREM-2 as a phagocytic receptor (157, 186). In contrast, the effects that are observed on AMs and neutrophils are more difficult to explain. Like it is the case for the unexpected effects TREM-2 exerts on cytokine release, the phagocytic phenotype of TREM-2 deficient AM could be linked with the augmented local production of C1q in AM. C1q has been shown to enhance phagocytosis of bacteria (199, 200), perfectly explaining the unexpected phenotype of AM, lacking TREM-2. Phagocytosis of TREM-$2^{-/-}$ could be reduced down to WT levels by blocking PPR-δ or C1q and it was possible to boost phagocytosis of WT AM by adding C1q (192). The observation that also neutrophils were affected in their phagocytic behavior in the absence of TREM-2 raised many

questions, as the expression of TREM-2 by neutrophils has never been shown and is a matter of debate. FACS experiments carried out in order to prove TREM-2's presence on neutrophils failed due to the lack of reliable antibodies. However, the functional effect was very solid and reproducible as hampered phagocytosis of TREM-2$^{-/-}$ neutrophils was observed in two different infection models (*E. coli* peritonitis and pneumococcal pneumonia) (191, 192) and could be explained by indirect factors, like the altered inflammatory milieu or changes in complement activation in the absence of TREM-2. In addition to the in vivo studies, in vitro experiments with primary cells revealed that TREM-2 rapidly disappears in PM upon LPS challenge and that its expression levels are very instable, as TREM-2 constantly undergoes proteasomal degradation and resynthesis. (191) Together with another study that showed ectodomain shedding and proteolytic processing of TREM-2 in COS-7 cells (175) and the existence of soluble TREM-1 during sepsis (211, 212), it cannot be ruled out that a soluble form of TREM-2 might be produced during sepsis and affect phagocytosis and possibly also migration of cells that themselves do not express TREM-2.

In summary this work shows that TREM-2 critically shapes the immune response in bacterial infection and during sterile injury. Neutrophils were not the focus of this work, however their migratory behavior as well as their phagocytic capacity seems to be strongly affected by TREM-2. While it is well established that TREM-2 is present on inflammatory, newly recruited macrophages, its presence on neutrophils has never been shown due to a lack of good FACS antibodies. Further studies are required to answer the question how TREM-2 influences the behavior of neutrophils. In macrophages (with the exception of AMs, where direct effects of TREM-2 are overshadowed by its secondary impact on C1q expression) TREM-2 functions as a suppressor of TLR-induced cytokine responses. However, TREM-2 is not a typical negative regulator but rather seems to fine-tune the reactivity of a cell to a certain stimulus. In contrast to classical negative regulators, such as A20 or IRAK-M that are absent under baseline conditions and are induced upon TLR ligation (77), TREM-2 is expressed on quiescent cells and rapidly disappears upon cellular activation (191). This is interesting considering that TREM-2 can bind anionic ligands like LPS and fatty acids (166, 167, 213). One could therefore imagine that TREM-2 acts as a scavenger for anionic ligands like LPS or LTA, thereby competing with TLRs. Upon stimulation TREM-2 would bind, get internalized and degraded and would thereby delay TLR-mediated signal transduction. TREM-2 might further interfere with TLR signaling by activating other mediators that crosstalk with the TLR pathway. As such, TREM-2 has been found in a complex involving PI3K, SHIP1, DAP12 and

DAP10 (161). PI3K has been shown to negatively regulate TLR-signaling, by a yet unknown mechanism (214) and is in addition an important player during the process of phagocytosis (215).

The in vivo data clearly indicate that TREM-2 is critical for the regulation of early inflammation, in different settings of infection as well as during sterile injury. Furthermore TREM-2 substantially promotes cellular effector functions like phagocytosis on macrophages (again AMs represent an exceptional case, due to TREM-2's impact on C1q expression) and surprisingly also on neutrophils. As shown by others, DAP12 deficiency phenocopies TREM-2 deficiency in models of endotoxemia (196) and pneumococcal pneumonia (205), again highlighting the importance of the TREM-2/DAP12 axis. All these effects together result in a rather detrimental role for TREM-2 during infection and inflammation and suggest that blocking of TREM-2 might be a potential therapeutic option in acute inflammatory conditions.

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Curriculum vitae

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Education

2010	Mag[a] rer. nat. in Molecular Biology, University of Vienna, Austria
2003 - 2009	Studies in Molecular Biology and Philosophy, University of Vienna, Austria Studies of Journalism and Content Management, School for higher education, Vienna, Austria
1997 - 2003	Technical High-School for Chemical Industry (Rosensteingasse), Vienna, Austria

Career/Work History

April – Nov. 2013	Internship at Newcastle University, United Kingdom, Prof. Derek Mann (Liver Fibrosis lab)
2010 – ongoing	Start of PhD Thesis in the lab of Sylvia Knapp at CeMM and the Medical University of Vienna, within the Doctoral Program CCHD (Cell Communication in Health and Disease)
2010 – 2013	Deputy Speaker of the Students Council of the Doctoral Program CCHD
2009 – 2010	Master Thesis, Sylvia Knapp Group, MUW (Medical University Vienna) and CeMM (Center for Molecular Medicine of the Austrian Academy of Sciences), Advisor: Dr. Omar Sharif Diploma-thesis: The role of TREM-2 in bacterial lung infections
March – May 2009	Internship, Dept. of Cellbiology, Univ. Vienna – Group Gerhard Wiche, Advisor: Dr. Martin Gregor Quantitative characterization of plectin isoform 1a interaction with integrin β4 and calmodulin
January 2009	Internship, Dept. of Biochemistry, Univ. Vienna, Advisor: Prof. Dr. Franz Koller Investigating the mechanism of SSC-A

November 2008	Internship, Dept. of Immunology, Medical Univ. Vienna - Group Karl Kuchler, Advisor: Dr. Christelle Bourgeois Innate pattern recognition of fungal pathogens and induction of a Type1 IFN Response
Aug./Sept. 2008	Internship, CellMed Research GmbH Optimization of the production protocol of double pulsed dendritic cells with TERT-mRNA and surviving-peptide
Aug. 2005	Internship, Technology review (Hannover)
July 02, July/Aug. 03	Internship, Austrian agency for health-, food security (AGES)
Aug. 2000	Internship, Boehringer Ingelheim Austria (Dept. of Molecular Biology)

Publications

1. **Gawish R**., Wimberger T., Sharif O., Lakovits K., Doninger B., Knapp S.
   Triggering receptor expressed on myeloid cells-2 fine-tunes inflammatory responses in murine Gram-negative sepsis. FASEB 1014

2. Sharif O., **Gawish R.**, Warszawska J. M., Lakovits K., Doninger B., Schwarzenbacher R., Berg T., Kralovics R., Collinge J., Mesteri I., Gilfillan S., Salmaggi A., Colonna M. and Knapp S.
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4. Matt U., Sharif O., Martins R., Furtner T., Langeberg L., **Gawish R.**, Elbau I., Zivkovic A., Lakovits K., Oskolkova O., Doninger B., Vychytil A., Perkmann T., Schabbauer G., Binder C. J., Bochkov V. N., Scott J. D., Knapp S.
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   The lipid-modifying enzyme SMPDL3B negatively regulates innate immunity, revised version submitted to Cell Reports

Awards and Fellowships

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September 2013	Travel grant from PAO Heyendal, to give a talk at the "IL-1 mediated inflammation and diabetes symposium", Radboud University, Nijmegen, Netherlands (Talk: The ambiguous role of TREM-2 in inflammation)
August 2013	Boehringer Ingelheim travel grant, funding 3 month of my stay at Newcastle University

Conference Participation

February 2013	7th bridging the gap (Symposium organized by the PhD students of the doctoral program Cell Communication in Health and Disease)
October 2013	IL-1 mediated inflammation and diabetes symposium, Radboud University, Nijmegen, the Netherlands (Talk: The ambiguous role of TREM-2 in inflammation)
Nov 2012	Annual Meeting of the Austrian Society of Immunology (Poster)
June 2012	8th PhD Symposium of the Medical University of Vienna (Poster)
Mar 2012	Keystone Conference 2012, Innate Immunity: Sensing the Microbes and Damage Signals (Poster)
Mar 2012	5th International Workshop on Cell Communication in Health & Disease (Poster)
Sep 2011	EMDS 2011 - Annual meeting of the European Macrophage and Dendritic Cell Society, Brussels, Belgium (Poster)
Sep 2011	EMBO 2011 meeting, 10th – 13th of September, Vienna, Austria
June 2011	7th PhD Symposium of the Medical University of Vienna (Poster)
Feb 2011	4th International Workshop on Cell Communication in Health & Disease
Feb 2011	1st TranSVIR Workshop, Imaging the Immune System, Vienna, Austria

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