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„Das einzig Beständige ist die Veränderung“

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1. Abstract

Balancing of innate immune responses is critical to all eukaryotic organisms, to ensure effective defense against invading pathogens as well as tissue remodeling and homeostasis, while preventing host damage due to uncontrolled inflammation. Macrophages are at the core of both mediation and implementation of such processes. Triggering receptor expressed on myeloid cells (TREM)-2 is a surface receptor expressed on macrophages and increasing evidence points toward its regulatory function in processes such as bone formation, brain homeostasis and immune mediated pathogen response. To this end, studies suggest an inhibitory role of TREM-2 in modulating inflammation, exerting its influence on Toll-like receptor (TLR) signaling via the ITAM containing mediator DNAX activating protein of 12kDa (DAP12).

This thesis provides evidence that TREM-2 over-expression in murine macrophages has an inhibitory effect on the activation of protein kinase B (Akt, formerly PKB) and extracellular signal regulated kinase (ERK) upon stimulation with lipopolysaccharide (LPS). Further, we prove the existence of several murine TREM-2 variants whose different masses are a result of N-glycosylation in either the endoplasmatic reticulum (ER) or the Golgi apparatus. This is confirmed by confocal experiments, which conclusively show that TREM-2 is localized in the Golgi of macrophages and found in other unidentified cytosolic structures. Investigating the fate of TREM-2 upon LPS stimulation, we observe a rapid decline independent of proteasomal degradation, indicating that TREM-2 has to be removed in order to allow for an appropriate inflammatory response.

2. Zusammenfassung

Neben Umbau und Selbstregulation von Gewebe ist für eine effektive Verteidigung gegen pathogene Eindringlinge und Verminderung schädigender Entzündungsreaktionen eine ausbalancierte Aktivität des angeborenen Immunsystems von zentraler Bedeutung.

Makrophagen sind maßgeblich an der Vermittlung und Umsetzung dieser Prozesse beteiligt. Triggering receptor expressed on myeloid cells (TREM)-2 ist ein auf Makrophagen exprimierter Oberflächenrezeptor, dessen regulatorische Funktionen im Zusammenhang mit Knochenbildung, Nervenzell-Homöostase und immunologischen Abwehrreaktionen zunehmende Aufmerksamkeit erhalten. Vergangene Studien zeigen, dass TREM-2 seine inhibitorischen Effekte auf Entzündungsreaktionen durch das ITAM enthaltende DNAX activating protein of 12kDa (DAP12) vermittelt und damit toll-like receptor (TLR) Signalwege beeinflusst.

Diese Arbeit zeigt, dass eine Überexpression von TREM-2 in Maus Makrophagen die Aktivierung von Proteinkinase B (Akt) und extracellular signal regulated kinase (ERK) als Antwort auf Lipopolysaccharide (LPS) reduziert. Weitere Daten belegen die Existenz von mehreren TREM-2 Varianten in der Maus, dessen unterschiedliche Massen von Glycosylierungen im Endoplasmatischen Retikulum (ER) und dem Golgi Apparat stammen. Weiterführende Experimente in Makrophagen lokalisieren TREM-2 im Golgi und anderen nicht identifizierten, intrazellulären Strukturen. Untersuchungen der TREM-2 Dynamik als Antwort auf LPS Stimulation zeigen ein rasches Verschwinden des Rezeptorproteins auch bei Unterbindung proteasomaler Abbauprozesse.

3. Experiments and thesis

Triggering receptor expressed on myeloid cells (TREM)-2 is an important mediator in a wide array of cellular processes such as neural homeostasis [1], bone formation [2] and inflammation [3]. TREM-2 is an established negative regulator of cytokine production in macrophages [4, 5] and dendritic cells [6, 7] upon TLR activation in vitro [6], but its mode of action remains poorly understood. The current model proposes that TREM-2 acts upon established signaling pathways involved in the induction of cytokine production and exhibits its counter-inflammatory phenotype primarily through the signaling capacity of its intracellular adapter DAP12 [3, 8, 9].

In the context of innate immunity, observed knockout phenotypes are typically adjudicated to effects impacting on TLR4 signaling [10]. Depending on the specific setting, prior investigations of this involvement showed alterations in MAP-kinase or Akt signaling upon inhibition of TREM-2 or its mediators [4, 5]. We hypothesize that TREM-2 responds to LPS stimulation by inhibiting the activation of a central inflammatory mediator of TLR4 signaling, nuclear factor kappa B (NF- κ B), exhibiting its influence via one of these pathways or both.

Research question 1: Which intracellular signaling pathways are affected by TREM-2 activation upon TLR4-mediated stimulation of macrophages?

One of the aims of the outlined thesis is to examine signaling pathways in a macrophage lineage over-expressing TREM-2.

Research question 2: What happens to TREM-2 protein in response to TLR4 stimulation?

Tied with the previous question there is the unresolved issue of TREM-2 expression upon stimulation, to which there are contradicting reports depending on the specific setting. Macrophages abrogate TREM-2 expression upon LPS stimulation on RNA level [9] while it is increased during sepsis [11] and early and chronic inflammation of the spinal cord [12].

Since RNA expression of TREM-2 in response to LPS has been studied in different situations before, we aim to examine the dynamics of TREM-2 on protein expression by western blotting and FACS after TLR4 stimulation in macrophages.

Research question 3: Where is TREM-2 localized and is it subject to post-translational modification?

Considering TREM-2's influence on LPS responses, knowing its intracellular localization is of high importance for the understanding of its mode of action. The likely post-translational modification of TREM-2 [13] will be another field of exploration. While the characteristics of different covalent modifications can give critical clues on the route of transportation on its way to the outer cell membrane, assessing post-translational modifications often hint towards functional dynamics and possibly even ligand affinities.

4. Introduction

4.1. Innate Immunity and inflammation

In mammals, three lines of defense against pathogens are generally distinguished: Physiological barriers, innate immunity and adaptive immune responses. Their order of mentioning addresses both their rising degree of specificity and evolutionary development. Unlike the other two, physiological barriers are not reactive in the sense that they respond to any invading threat specifically. Pathogens are prevented from entering the organism by obstacles like the mechanical barrier of the skin, the low pH of the stomach or degrading enzymes secreted through salivary, lacrimal or similar glands [14]. Physiological barriers are often considered an additional functionality of innate immunity because this second layer of defense works in a similarly general, yet more reactive fashion. Upon activation, the innate immune system mounts an immediate inflammatory response. The limited number of signals recognized by cells of the innate immune system via so-called pattern recognition receptors (PRRs), are either of physiological origin as a result of tissue damage or highly conserved structures associated with a major group of microorganisms, namely pathogen-associated molecular patterns (PAMPs) [15]. As a consequence, the limited number of PRRs is germ line encoded and equally present in all cells of the same cell type [16]. This is contrasted by the antigen-specific set of receptors that are part of the adaptive immune system. Mediated by B- and T-lymphocytes, which have their individual receptors randomly generated to provide affinity against a broad range of very specific epitopes not innate to the host itself, an adaptive immune response is delayed because of the requirement for clonal expansion of the relevant cell type [16, 17].

As the following thesis will examine a receptor presumed to impact on innate immune responses, the involved components will now be summarized. The major process of interest

here is inflammation, encompassing the range of responses initiated by cells of the innate immune system upon activation through a physical, chemical or biological trigger. The symptoms that go along with loss of tissue function in the affected region are classically defined as dolor (pain), calor (heat), rubor (redness) and tumor (swelling) [18]. A cause of these is vasodilatation, where higher permeability of blood vessels allows for increased blood circulation, along with influx and adhesion of further immune cells [15, 16]. The major acting cell types mediating acute inflammatory responses are granulocytes (neutrophils, basophils and eosinophils), mast cells and mononuclear cells (monocytes and macrophages). Their effector functions can be simplified as a combination of direct participation in pathogen removal by phagocytosis and killing on one hand [19], and the production of soluble mediators like cytokines and chemokines on the other. Among many, the most notable of these are considered to be tumor necrosis factor (TNF) α and Interleukins (IL) 1, 6, 8 and 12 [16]. From the vast number of PRRs that have been identified over time, TLRs are both the best studied and the most relevant to our setting, though several other classes of cytosolic PRRs have since been described. Among these, RIG-I-like receptors (RLRs), which recognize RNA viruses and the larger Nod-like receptor (NLR) family composed of over 20 members are important to mention. In contrast to the above mentioned TLRs, these sensors are cytosolic and responsible for intracellular pathogen sensing [20]. TLRs being the most important sensors of microbial infection in particular, elicit responses to a wide range of conserved substances of bacterial and viral origin [21]. They will be described in more detail in the following chapters.

Any inflammatory reaction causes a series of effects that are not solely harmful to the invading microbe, but also to the host itself. While a powerful inflammatory response is crucial in order to fight an infection, overwhelming or uncontrolled inflammation can have serious detrimental effects. Consequently, tight regulation is essential to achieve proper

pathogen clearance while preventing host immunopathology. As a result, the large amount of structures involved in mediating an immune response from detection towards an effective action is highly complex. Key molecules tend to converge otherwise differing pathways in order to adapt a specific response to the changing situational needs. Failure to both amplify and diminish a certain immune response can result in dire consequences for the host, which is why there is a substantial network of regulators involved in fine-tuning any given inflammatory process [22]. The following subchapter outlines a setting where these mechanisms reach a dilemma in achieving host survivability.

4.1.1. Gram negative sepsis

Sepsis is a state of severe system wide inflammation during the course of an infection and continues to pose a life-threatening condition despite the availability of antibiotics. It typically leads to organ dysfunction and hypoperfusion [23]. Further advancement causes lactic acidosis, oliguria and, ultimately, multiple organ dysfunction syndrome. Notably, it is rarely the pathogen which causes the high fatality of this condition, but rather the host immune response to microbial PAMPs or endogenous danger associated molecular patterns (DAMPs) [24]. Views on the major causes of sepsis have been adapted over the past decade. There is consent that the major trigger is bacterial infection, though sepsis is also promoted by fungal or viral infection. The primary sources of pathologies that lead to sepsis are infections of the lungs, abdomen and the urinary tract [25]. In general Gram-positive and Gram-negative sepsis are distinguished. Compared to Gram-negative bacteria, it is increasingly emerging that Gram-positive bacteria are an equal, if not greater cause of sepsis in humans. They have been identified as a causality of sepsis in over 50% of incidences in the past years, most of these attributed to staphylococcal infection [26]. In the case of Gram-negative sepsis, there is a general consent that the endotoxin LPS is the primary trigger of this response [24, 27]. Signaling implications are clarified by reports that LPS shock is abrogated in the absence of

MyD88 [28]. Yet, MyD88 is one of the core upstream mediators of TLR signaling, and the kinase cascade resulting from activation is regulated on so many levels that a collective model is still out of reach. TLR4 is of special importance to this work because it signals the presence of LPS and has been identified as a key mediator in sepsis. TLR4 is required for the protective immune response during Gram-negative peritonitis [29] and has been shown to mediate LPS induced shock in murine models [30]. For a better understanding of this connection, the next chapter will outline the importance of TLR4 mediated signaling in pathogen induced immune responses.

4.1.2. Toll-like receptor (TLR) 4

TLRs are crucial for the induction of innate immune responses and have been shown to recognize a broad range of PAMPs, such as illustrated in Fig. 1. Upon ligand binding, the induced pathways result in the transcription and secretion of several pro-inflammatory cyto-

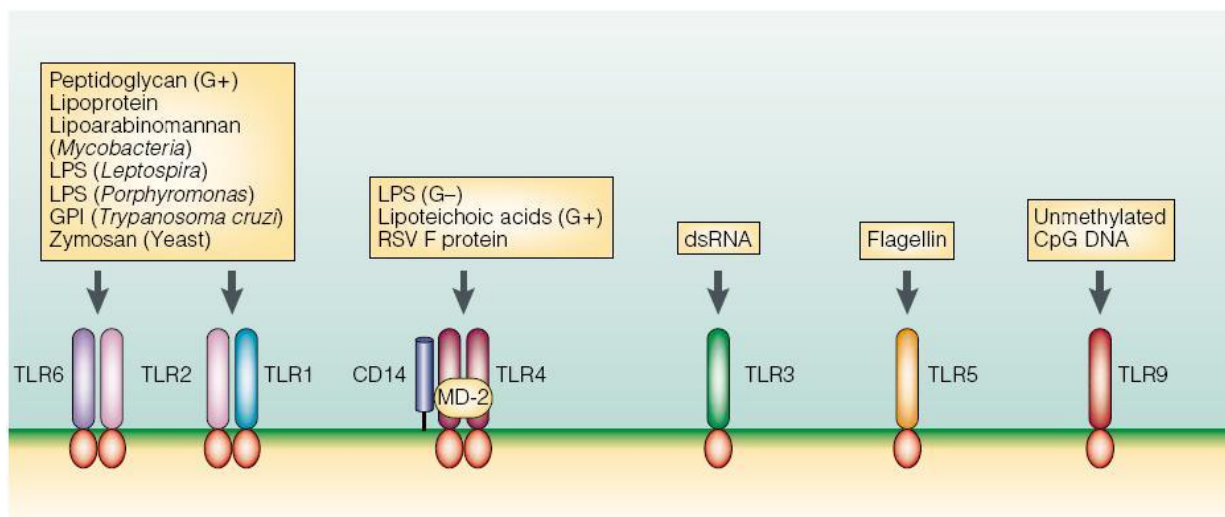


Figure 1 TLR receptors and their ligands. Modified from Medzhitov 2001 [31]

and chemokines. This can be mediated via the activation of either the classical MyD88 dependent or the MyD88 independent TRIF pathway [22], illustrated in Fig. 2. MyD88 mediates signaling of all TLR receptors except TLR3 and is shown to induce activation of the

transcription factor NF- κ B and several MAP kinases which in turn promote to the production of inflammatory cytokines [31]. As mentioned previously, a knockout of MyD88 renders mice resistant to endotoxic shock. It additionally abolishes the production of cytokines such as IL-6 and IL-12 upon DC and macrophage stimulation with a range of PAMPs. Further investigation showed that this does not encompass stimulants of TLR3 and TLR4, which continue to activate transcription factors NF- κ B and interferon regulatory factor (IRF) 3 via

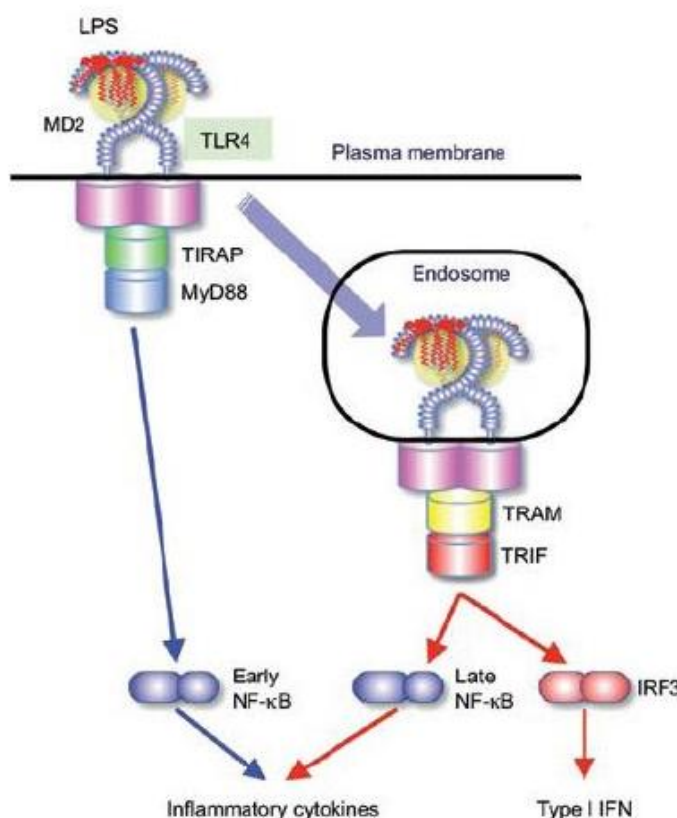


Figure 2 TLR4 signaling. Modified from Kawai and Akira 2010 [20]

an alternative route of TRIF mediated signaling [20, 31]. This became known as the MyD88 independent pathway which induces the production of type I interferon (IFN) and inflammatory cytokines [20]. TLR4 is the only known TLR which is able to signal via both pathways [20, 28]. Activation of IRF 3 via TRIF is of lesser relevance to our bacterial model. Remembering that TLR4 reacts to a range of stimuli, activation of IRF3 most prominently initiates a cellular

response against viral infection [32]. TRIF activation of NF- κ B and MAPK has been reported, but is considered relevant in later stage inflammation [33]. Looking at the MyD88 dependent pathway, we are confronted with signal transducers IL-1 receptor associated kinase (IRAK) 1 and 4, TNF associated factor (TRAF) 6 and transforming growth factor- β -activated kinase (TAK) 1. All of these are known to be critically involved in NF- κ B and MAPK dependent cytokine secretion and represent additional layers of regulation [22, 34, 35].

As mentioned, the most common result of TLR activation is mediated by the transcription factor NF- κ B, which leads to transcriptional reprogramming and the production of cytokines. NF- κ B is inherently present in the cytosol, coupled with I κ B α which prevents it from actively promoting transcription in the nucleus. The nuclear translocation of NF- κ B is generally preceded by a degradation of cytosolic I κ B α [28, 36]. Specific to innate immunity, NF- κ B is critical to the expression of chemokines (IL-8), cytokines (IL-1, IL-1, IL-12, TNF- α) and a range of adhesion molecules [37]. While all of these are vital to an inflammatory process, other NF- κ B functions impact on the fields of apoptosis, development, adaptive immunity and lymphoid architecture [37].

With such a spectrum of different functions, it is obvious that NF- κ B is subject to tight regulation. Signal alteration can impact on any of the mediators involved in NF- κ B activation, only some of which have been mentioned. Since our protein of interest is a proposed negative regulator of inflammation, some of the general strategies observed to inhibit TLR signaling will be presented. One of these leads to the dissociation of adaptor complexes. For instance, IRAK signaling is achieved by the assembly of an adapter complex which can be suppressed by phosphorylation and deubiquitination enzymes such as SHP 1 or 2 [38]. This type of signal suppression will be further elaborated in later sections, since these phosphatases have also been identified as mediators of TREM-2 mediated responses. Other ways to reduce the extent of TLR signaling are achieved by the degradation of signal proteins, expression of soluble decoy factors or transcriptional regulation [22, 38]. An important mechanism to mention is the self regulation of NF- κ B by negative feedback inhibition. I κ B α , mentioned to inhibit NF- κ B activation, is one of its primary target genes [36]. This implies that NF- κ B requires permanent induction to remain constitutively active. Other examples of transcriptional regulation will also be found in subsequent chapters.

Among the several TLR receptors in existence, TLR4 is of special importance to this work due to its unique affinity to LPS [39]. LPS is critical to the structural integrity of the outer cell wall of Gram-negative bacteria and consequently presents an ideal structure for the sensory mechanisms required for their detection [40]. Disruption of TLR4 leads to complete

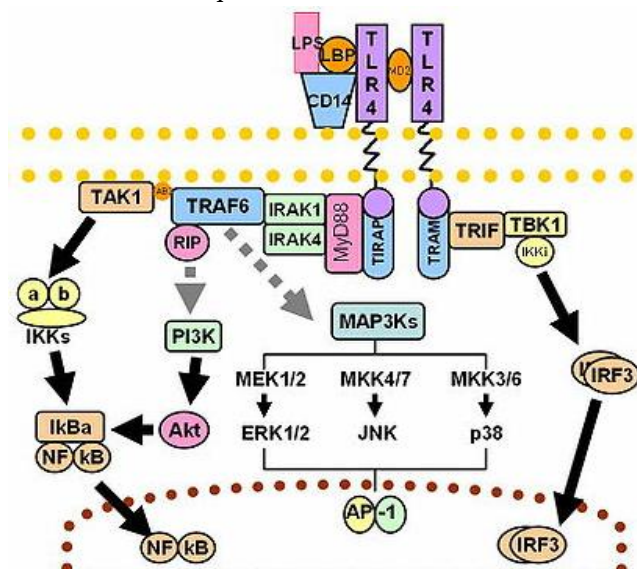


Figure 3 Adapted from Georgia State University, Neuroscience institute [143]

irresponsiveness to most types of LPS, emphasizing its importance in a respective infection scenario [41]. TLR4 is known to be expressed in antigen presenting cells (such as DCs, macrophages and certain B cells), endothelia, epithelial cells, myocytes, adipocytes as well as endometrial, thyroid and mesangial cells

[42]. Fig. 3 is a sketch of selected TLR4

interactions during Gram-negative infection or LPS stimulation. An important note here, is that LPS is directed to TLR4 via LPS binding protein (LBP) which has a high affinity to the associated receptor CD14 and MD-2. It is the assembly of this complex that ultimately initiates an inflammatory reaction to the stimulus [14, 43, 44]. All TLRs act as either hetero- or homodimers [14] and the illustration correctly points out the homodimeric assembly of TLR4 upon activation.

4.2. Glycosylation and protein maturation

Glycosylation is a common trait of immune receptors in general and is additionally known to play a vital role in TLR recognition [45], TLR4 specifically among these [46].

Oligosaccharides bound to cell surface receptors can directly modulate protein function and signaling, but also stabilize them against denaturation or proteolysis, change stability and

turnover or their solubility and charge [47]. Others may act as ligands that allow for cell adhesion, macromolecule interaction or pathogen invasion [48]. The process of protein glycosylation begins with the co-translational transfer of a high-mannose group to the emerging protein at the ER membrane. This is important for proper folding and vital to quality control in the ER [49].

The two primary forms of vertebrate glycan addition can be divided into N- and O-glycosylation, of which N-glycosylation describes the addition of sugar residues to an amide

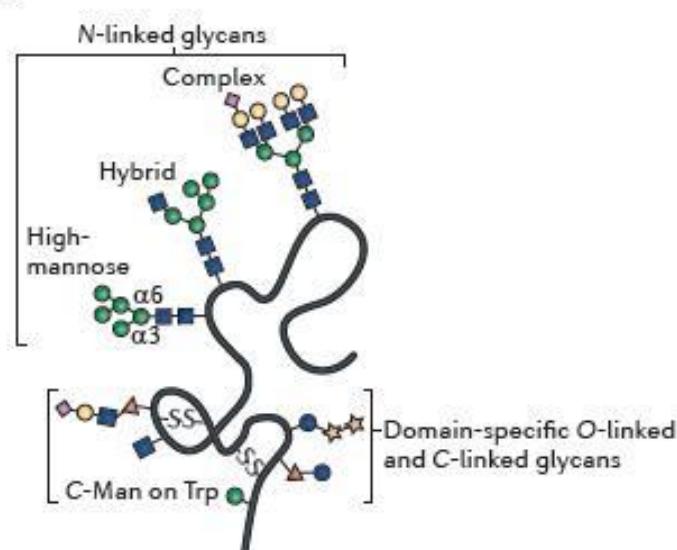


Figure 4 Major types of glycosylation. Modified from Nairn 2012 [48]

group of asparagine, while O-glycosylation constitutes any addition to the hydroxyl groups of serine or threonine [50]. About 90% of glycoproteins carry N-glycosylations that commonly raise their mass by 3kDa [48].

Most N-glycan additions and all O-glycosylations occur in the Golgi apparatus [50]. The Golgi consists

of several layers called cisterns which are organized from cis near the ER to trans toward the outer membrane. The main process of glyco- and thus protein maturation takes place in the medial and trans-cisterns [51]. It is still not entirely clear how this increasing complexity is reliably reproduced in a system of constant vesicular rearrangement [50].

Increasing reports on protein glycosylation result, at large, from the usage of glycosidase assays. Endoglycosylase H (EndoH) is an enzyme that cleaves all sugar chains that are added co-translationally to any nascent ER protein before they are further processed in the Golgi [52], where the addition of fucose to the sugar base make them unsusceptible to EndoH cleavage. Peptide-N4-(N-acetyl-beta-glucosaminy)asparagine amidase (PNGase) on the other

hand, is able to cleave the overwhelming portion of all existing N-glycosylations [53]. As a consequence, if a protein is cleaved by PNGase but not EndoH, this protein has undergone further modification in the Golgi apparatus.

It has been established that the complexity of the glycome increases with the evolutionary complexity of organisms. This has led to the suggestion that PRRs like the phyloglycomic recognition system subset of TLRs have developed the ability to detect the distinct glycans of lower organisms as a mean of non-self discrimination [45].

4.3. Macrophages in innate immunity

4.3.1. Macrophage development, function and polarization

Being the first line of defense and equipped with a broad range of PRRs in order to sense invading pathogens, macrophages will be described in the following chapter. Macrophages are cells derived from the hematopoietic lineage and a major player in innate immunity. The term itself describes a greatly heterogeneous cell type with the ability to specialize on a number of tasks depending on the tissue of residency. They differentiate from a circulating population of monocytes which is itself heterogeneous before assuming their terminal tasks when reaching a destination [54, 55].

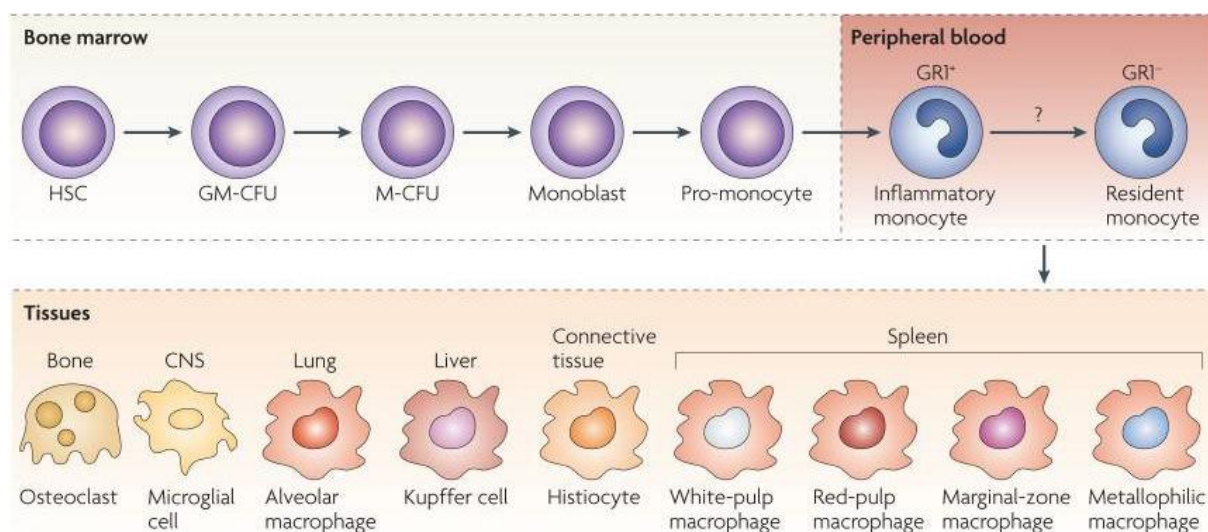


Figure 5 Monocyte heterogeneity. Modified from Mosser, 2008 [58]

The great variance of macrophages appears to be both prerequisite and consequence of their presence in nearly all tissues and the overwhelming range of tasks they are involved in. Aside the central role in innate immunity, their functions are equally critical to the fields of tissue repair, homeostasis and embryonic development [56, 57]. As visualized in Fig. 5, macrophages are derived from hematopoietic stem cells (HSCs), proceed to differentiate into monocytes via specific, increasingly defined colony forming units (CFU) and, latterly, the monoblast stage [58]. These continue to give rise to the greater part of macrophages, of which only the most notable are shown, including a notion of their differing morphologies. Aside these, it has also been shown that a portion of resident macrophages are derived from colony forming cells in resident tissue [55]. An important note on inflammatory monocytes is that their influx constitutes the main population of macrophages at an infection site eliciting an inflammatory response, quickly surpassing the number of resident ones [55, 57].

Macrophages are one of the primary sensors of both cellular debris, necrotic cells and the before mentioned PAMPs through TLR signaling pathways. Their plasticity is remarkable. Depending on the incoming signals, macrophages can be conveyed to an immunologically active state in which they play a key role in phagocytosis, cytokine production, bacterial killing and antigen presentation. With a different set of stimuli, such as received during tissue homeostasis, they assume other vital tasks like tissue remodeling or clearing their vicinity of apoptotic cells and cellular debris [58].

This implies that, rather than switching between active and inactive forms, macrophages constantly reassign their tasks according to the signals they receive. Initial hypothesis distinguished the pro-inflammatory or classically activated M1 state and the alternatively activated M2 state serving non-pathogen associated tasks [57]. However, more detailed investigations concluded that the simplicity of a singular M2 polarization could not be reconciled with the spectrum of tasks during non-inflammatory situations [59].

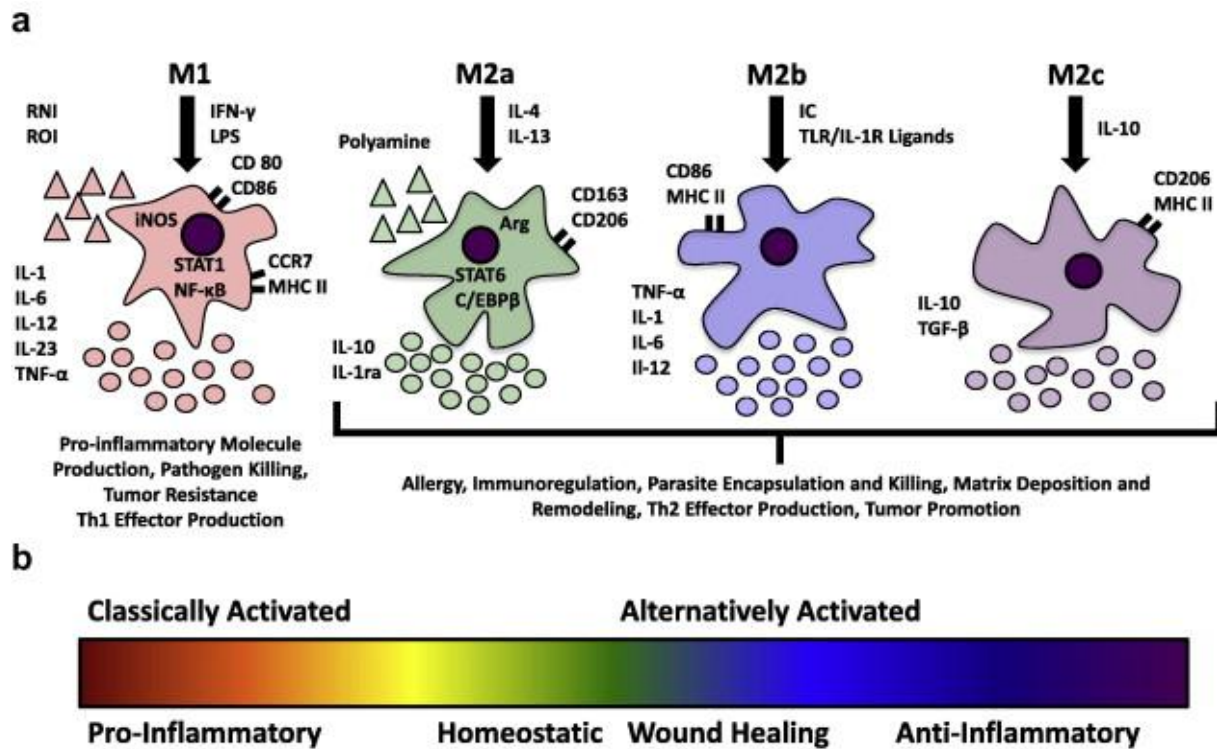


Figure 6 modified from Brown and Badylak, 2013 [59]

Fig. 6 sums up the current view on macrophage plasticity, addressing the incoming signals that lead to a certain polarization along with the according cytokine production. The two extremes are composed of the pro-inflammatory and the equally important anti-inflammatory state, latterly renamed M2c. Additionally, the alternatively activated conditions M2a and M2b were introduced as waypoints in the ever changing expression patterns. These polarizations are most common due to their involvement in homeostasis, repair and other largely housekeeping associated functions. Anti-inflammatory macrophages are characterized by the down regulation of pro-inflammatory cytokines and upregulation of IL-10 [60]. The specific function of these remains poorly understood, but might be important in returning to a non-inflammatory state after an infection has been cleared.

4.3.2. Peritoneal and bone marrow derived macrophages

There are two specific types of macrophages that will be subject of investigation in this thesis. One is the immortalized cell line of RAW 264.7 macrophages, isolated from a tumor induced in the peritoneum of a male mouse by injection of Abelson Leukemia Virus (A-MuLV) [61]. Relevant to our setting, it has been shown that these cells show responsiveness to LPS stimulation by activation of NF- κ B [62] that results in cytokine secretion similar to that of primary peritoneal macrophages [44]. The other is composed of macrophage precursors from murine bone marrow, isolated and in vitro differentiated into macrophages by a protocol outlined in the method section.

4.3.3. Pattern recognition in macrophages and effector functions

This chapter will provide more specific insights to the signaling and effector mechanisms of macrophages upon pathogen recognition, with emphasis on cytokine responses.

Macrophages express a broad range of PRRs, including the described TLRs which are crucial for the induction of quick and appropriate cytokine responses upon pathogen encounter. Activation of TLR4 by LPS in macrophages leads to classical NF- κ B activation, which has been shown to enhance or repress the expression of at least 70 target genes in several macrophage lineages [63]. Among these, we find IL-1 β , TNF- α , MIP1-3, KC and IL-6 [63-65], all of which are associated with induced sepsis models. As a consequence, the regulation of NF- κ B activation plays a central role in LPS induced inflammation [65] as mediated by the resulting production of these pro-inflammatory cytokines.

The signal mediators named phosphatidylinositol 3-kinases (PI3Ks) are at the center of a cascade, namely the PI3K/Akt pathway, which is crucial to all eukaryotic cells. It is classically known to regulate cell proliferation and survival and therefore mainly associated

with its role in different kinds of cancer [66]. In macrophages, PI3K has been shown to critically influence development, homeostasis and activation [67]. It is also involved in arthritis and lymphoma, where interaction is known to promote anti-inflammatory effects or B-cell survival, respectively [66, 68]. Crosstalk between the PI3K- and the NF- κ B pathway has been observed in many different settings, but remains poorly understood. Oncogenic mutations in PI3K lead to aberrant NF- κ B dependent cytokine responses upon growth factor deprivation in human breast cancer [69]. While it is known as a downstream mediator of several receptors such as IGF-1R, Fc ϵ R1 and c-kit, we will continue to focus on its important implications for TLR4 signaling. Knockdown or inhibition of the PI3K pathway in BMDMs and PMs was shown to reduce the production of NF- κ B dependent pro-inflammatory cytokines, whereas co-stimulation of both TLR4 and PI3K specifically enhances the expression of IL-6 and TNF- α [70, 71]. Interestingly, the reverse effect was observed for the production of IL-1 β [70], an equally established target of NF- κ B. Although the background of this differential effect on cytokine production is not entirely clear, it adds to the notion of how the PI3K pathway impacts on the cytokine response of macrophages.

While being well known for their involvement in a wide range of cellular processes, several MAPK pathways are also crucially involved in shaping the inflammatory responses of innate immune cells. For instance, anthrax induces cleavage of both extracellular signal-regulated kinase (ERK) and p38, resulting in a significant reduction of pro-inflammatory cytokines associated with NF- κ B activation, such as TNF- α , IL-6 and IL-1 [72]. Further, p38 is the main target of the anti-inflammatory drug SB203580, a pyridinyl imidazole inhibitor which decreases TNF- α and IL-1 secretion upon action [73, 74]. Other studies suggest a rather indirect role of p38 in inflammation, such as the removal of otherwise repressive chromatin modifications [75]. Overall, the influence of p38 on macrophage inflammation is unquestioned, as associations with chemotaxis, mRNA stability and cytokine expression have

all been established [74]. The MAP kinase ERK has a suffocating amount of signaling implications and is involved in a wide range of cellular processes [74, 76]. Studies on its role in inflammation report involvement in LPS-induced macrophage migration [77] and cytokine production in response to influenza infection [78]. There is evidence to suggest that many of the attributed actions arise from its influence on macrophage polarization [79]. Specifically, it has been shown that stimulation of the ERK pathway promotes the interaction between CREB-binding protein (CBP) and one of its modulators, RSK1 [74]. Though the nature of this modulation is not entirely clear, CBP is known to influence the binding of other co-factors to NF- κ B [80].

Another crossing point between MAPK and NF- κ B signaling appears to be the transcription factor AP-1. Both are similarly inhibited as a consequence of suppressing the phosphorylations of ERK, JNK and p38 MAPK [81]. Among the many involvements of transcription factor AP-1, its most vital contribution to macrophage inflammatory responses is the transcriptional activation of TNF- α , IL-6, cyclooxygenase 2 (Cox-2) and monocyte chemoattractant protein-1 (MCP-1) [82, 83]. PAMPs tend to activate both AP-1 and NF- κ B in synchrony, and it has been shown that they sometimes augment each other in the promotion of a specific transcript [84]. The best established connection between MAPK and AP-1 is probably that of c-Jun N-terminal kinases (JNK). It is shown to directly influence the expression of all the above mentioned cytokines [82, 84].

Besides cytokine production, phagocytosis is another key feature of the macrophage immune response [85]. Phagocytosis in general, is the cellular process of engulfing large particles and also applies to the clearance of apoptotic cells and cellular debris, which is vital to tissue remodeling and homeostasis [86]. However, this brief overview will focus on its immunological role. Phagocytosis is also mediated by PRRs, which induce rearrangements of the actin cytoskeleton that lead to internalization of the recognized pathogen [87].

The PI3K/Akt pathway is established as a key component of both phagocytosis and phagosome formation in macrophages [88, 89]. Aside another attributed function in macropinocytosis [90], its central role in actin remodeling upon pathogen encounter has been extensively studied [91]. Signaling of the MAPK ERK has also been reported to influence certain types of phagocytosis in neutrophils and macrophages, such as mediated by FcγR [86]. Yet the investigation of specific instances has shown that phagocytosis can occur without mediation through either pathways as well [92], such as the phagocytosis of IgG coated erythrocytes by phagocytic monocytes.

The importance of regulating TLR responses having been discussed, we now turn to TREM-2. It is as the center of this thesis and a proposed negative regulator of TLR signaling [3]. The following sections will elaborate on the TREM protein family, TREM-2 signaling and its proposed functions.

4.4. TREM-2

4.4.1. The TREM receptor family

TREM-2 belongs to the proteins family known as triggering receptors expressed on myeloid cells (TREM), which consists of surface receptors that impact on a range of signaling pathways. From the vital processes that TREM-2 specifically participates in, neurological development, bone modeling, inflammation and, more recently, Alzheimer's disease have received the most attention [93, 94]. It remains an orphan receptor as the extracellular ligand has not been identified [3].

The TREM genes are located in a cluster on chromosome 6p21 of the human genome which includes the genes translating into TREM-1 and -2. On the 17c3 chromosome of mice, additional expression of TREM-3 and -5, TREM like transcripts (TLT) -1 and -2, and a

TREM version found on plasmacytoid dendritic cells (PDC-TREM) have all been shown [3, 95-97]. TREM-1, -2 and -3 share the feature of an extracellular single immunoglobulin variable superfamily (IgSF-V) domain, a charged transmembrane domain and a short cytoplasmic tail [97]. As the tail does not contain any known signaling motifs, the question of how downstream signaling is facilitated was clarified by showing that, representatively, TREM-1 activated by antibody mediated receptor crosslinking associates with the adapter molecule DNAX activation protein of 12kDa (DAP12) [98]. DAP12 contains an immunoreceptor tyrosine-based activating motif (ITAM) and will be discussed separately as it is imperative to TREM signaling [3]. Contrastingly, genetic analysis has shown that TLTs contain a potential immunoreceptor tyrosine-based inhibiting motif (ITIM) which recruits SHP phosphatases [99]. Studies on TREM receptors in inflammatory settings report soluble forms of TREM-1, TREM-2 and TLT-1 both *in-* and *ex-vivo*, the origin of which has yet to be resolved. While there are indications of alternative splice variants resulting in a soluble form, other experiments have shown that proteolytic cleavage of the surface variants can sufficiently explain these observations [3, 95, 100].

Of all the TREM family members, TREM-1 has been studied most extensively. It is widely accepted as an amplifier of innate immune responses. For instance, a knockout of TREM-1 results in a reduced production of cytokines such as IL-1 β and TNF in murine Kupffer cells and diminishes the activation of NF- κ B and several MAPKs [101]. A soluble form, abbreviated sTREM-1, is reported to be released into various bodily fluids and has since been recognized as an important biomarker for a range of infectious diseases such as pneumonia and sepsis [102, 103]. The dynamics of sTREM-1 levels assessed in septic patients allow for accurate predictions of survival and mortality when compared to other classic biomarkers [103].

In accordance to their given name, TREM family members have been found expressed on a large and growing number of myeloid cell types, meriting the extrapolation that they are ubiquitous in this lineage [3, 95]. Beyond it, TREM 1-3 expression has been found in hepatic endothelial cells [104] and TLT-2 expression has been confirmed in cells of the lymphoid lineage [95].

4.4.2. Activating and inhibitory functions of DAP12

The signal transduction adaptor molecule DAP12 is equally expressed in cells of the myeloid, but also the lymphoid lineage and known to exhibit activating influence on pathways involving ERK, PI3K or phospholipase Cy1 [100, 105]. DAP12 also goes by the name of killer cell activating receptor associated protein (KARAP), which points towards its initial discovery in NK cells, and tyrosine binding protein (TYROBP), addressing its interaction with the protein-tyrosine kinases ZAP-70 and Syk [106, 107]. It is a small single pass transmembrane protein of 114 amino acids, the major part of which makes up the intracellular ITAM [108]. Of the known ITAM containing transcripts sharing the “YxxL/I–x6-8–YxxL/I” motif, DAP12 is classified as one of many transmembrane signaling adaptors. Its detection in humans, pigs, rodents and bony fishes exemplifies the conserved nature of its entangled role in vertebrate signaling [107].

In the past years, the classical notion of an ITAM conveying an activating response and ITIM containing proteins mediating a contrasting inhibitory response has been challenged by an increasing number of contradicting reports [109]. Updated models account for the ambiguous character of these motifs and thus also DAP12. One of these is based on the finding that DAP12 contains an ITIM consensus sequence within its ITAM [109]. The derived ambiguity of signaling suggests that a double phosphorylation of the larger ITAM and subsequent binding of Syk kinases continues to activate other pathways, while single phosphorylation of

the smaller ITIM instead leads to binding of the phosphatase SHP-1, which elicits inhibitory effects [109, 110]. Latter investigations, especially of the ITAM containing human immunoglobulin A Fc receptor (Fc α RI, or CD89), introduce the term inhibisome for the lipid raft structures formed as a consequence of SHP-1 recruitment. The inhibitory action is determined by the recruitment of activating signaling kinases to these structures upon which their phosphorylation is impaired and does not result in further signaling [110-112].

DAP12 acts as a signaling adaptor for well over 40 different immune receptors in myeloid and lymphoid immune cells. Besides the TREM family, the main groups of these consist of CD15 transcripts important to chemotaxis and phagocytosis, Ly-49 receptors involved in self recognition of NK cells and sialic acid binding immunoglobulin-type lectins (Siglecs) which play a part in cell adhesion and phagocytosis [113]. Though DAP12 mediation of TREM signaling has shown to be imperative, our specific interest goes beyond its ability to bind this receptor family among many others. From the sum of these interactions, TREM-2 and DAP12 deficiency in human results in a strikingly similar phenotype partially characterized by loss of myelin and basal ganglial calcification, often accompanied bone cysts. This form of presenile dementia is also referred to as the Nasu-Hakola disease [114]. Because of mediation via either of these two associated proteins, we continue to look more closely at the details of this condition.

4.4.3. Nasu-Hakola disease

Nasu-Hakola disease (NHD) is also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS), was first described in the 1970s and is directly associated to recessively inherited mutations of the TREM-2, the DAP12 gene or both [115, 116]. In spite of individual deviances in progression, it can be generalized that homozygous mutations result in increased bone fracture incidences, bone cysts primarily in wrists and ankles and onset of neurological symptoms in the 2nd and 3rd decade of life. As the disease

progresses, severe dementia and premature death increasingly occur toward the 4th and 5th decades of life [93, 117]. Of the recorded incidences, time between onset of symptoms and decease of a subject spans between 3 and 35 years [117]. Depending on the specific mutation of TREM-2, a number of incidences have been reported where the characteristics of NHD presenile dementia is not accompanied by the formation of bone cysts [94].

Having examined some of the many binding partners of DAP12 in prior, the fact that loss of its function appears to be compensated in the case of all receptors except TREM-2 is still puzzling. In vitro studies conducted with DAP12 deficient cells did not show any disruption in signaling of the other associated receptors, proposing that its absence is somehow compensated for [8]. If we consider this eliminatory information to be complete, it implies that NHD is in essence caused by TREM-2 loss of function. How this causes the described symptoms has yet to be clarified in detail. Recent reports show a significant increase of phosphorylated Syk (pSyk) expression in the cerebral cortex and hippocampus of NHD brains [118]. Remembering the ITAM binding property of Syk kinases, this offers a possible route of action to be further investigated.

Somewhat intriguingly, recessive heterozygous TREM-2 mutations in carrier individuals have recently been identified as a significant genetic risk factor of Alzheimer's disease [93, 94, 119], which will be addressed in a separate chapter.

4.4.4. TREM-2 expression and localization

The following chapter is of special importance to this work, as the cellular localization of TREM-2 stands one of the major points addressed in the presented experiments. As previously touched upon when introducing the TREM receptor family, TREM-2 is found in a wide range of cells. Expression in dendritic cells and in vitro derived macrophages has long been established [9]. Since then, it has been found in bone-marrow derived macrophages

(BMDMs), microglia, endothelial cells, resident peritoneal and alveolar macrophages and hepatic macrophages. Specifically, its presence has been shown by various means in the following cell types: THP-1 (monocytes), U937 (pre-monocytes), CHME-5 and N9 (microglia), T98G and N2A (neuroblastoma), J774.2, IC21 and MT2 (macrophages) and RAW 264.7 (monocyte/macrophage) [3]. The close connection between TREM-2 and DAP12 is also reflected in their expression patterns. Bone marrow derived dendritic cells (BMDCs) have been shown to express TREM-2 in a DAP12 dependent manner. A knockout of DAP12 leads to a near total depletion of TREM-2 expression in these cells [6]. The same observation was made in DCs isolated from patients suffering from NHD as a consequence of either DAP12 or TREM-2 loss of function mutations. It was additionally identified that a lack of TREM-2 does not in turn down-regulate DAP12 expression, possibly as a consequence of its several other binding partners [7]. In terms of magnitude, the expression of both proteins in the CNS is reported to be highest in microglia and oligodendrocytes, with higher DAP12 levels as compared to TREM2 [120]. Both are expressed from an early embryonic stage (day 17) through adulthood. Co-localization with a microglia/macrophage marker, substantiated by lack of TREM-2/DAP12 expression in PU.1KO specimen missing these cell types, is another indication of the signaling importance in neural immunology and/or debris clearance [121]. Expression is also reported in “foamy” macrophages [122]. These are considered anti-inflammatory type M2 macrophages and originate from infiltrating macrophages and resident microglia [122, 123].

The existence of soluble TREM-1 forms has long been established [124]. A soluble form of TREM-2 (sTREM-2) has also been reported in the cerebral spinal fluid (CSF) of mice studied in the context of multiple sclerosis and CNS inflammation. Compared to the control group, it was significantly elevated in the CSF of all the groups with inflammatory neurological disorders presented [122]. Genetic studies preceding this biochemical one, had identified a

cDNA sequence very similar to the TREM-2 gene but with only four of its five total exons [97]. The missing exon 4 was known to constitute the transmembrane region, predicting a soluble form to be expressed. This alternatively spliced form shifts the reading frame of the remaining gene, and would lengthen the TREM-2 tail region of this version by 54 amino acids [97]. Since the origin of sTREM-2 has not been established and it is now known whether it arises from alternative splicing or receptor cleavage, the existence of a soluble form remains a matter of debate.

Very little is known about the cellular localization of TREM-2. Studies on human microglia and glioblastoma cells suggest that TREM-2 is primarily found in the intracellular compartment of unactivated cells [124]. The localization of TREM-2 within murine N9 microglial cells has been examined more closely, showing it is mainly distributed in two pools of intracellular compartments. One was identified as a deposit in the Golgi by its co-localization with trans-Golgi network (TGN) markers [125]. The second consists of exocytic vesicles not defined further than as standing apart from endo- and lysosomes, showing very low co-localization with markers of these distinct vesicles. The TREM-2 containing vesicles appeared to be continuously shuttled towards and then recycled back from the cell surface [125]. Stimulation experiments showed that the addition of ionomycin, which increases the intracellular Ca^{2+} concentration, leads to a rapid increase in TREM-2 surface density [124, 125], apparently shifting the recycling dynamics of vesicles towards the surface. Though at a lesser rate, the same has observed upon LPS stimulation in the specific case of N9 cells [125].

4.4.5. Immunological aspects of TREM-2 signaling

The paradigm of TREM-1 generally augmenting innate immune responses and TREM-2 inhibiting their extent [3], still continues to coincide with more recent studies on the topic, although arguments will only be presented for the role of TREM-2. While the introduction to

the signaling adapter of both receptors, DAP12, has touched upon the ambiguous character of ITAM signaling [108, 109], the fact that two opposing effects can be mediated by the same molecule remains an exciting feature of these systems. Because of the established, close knit connection between the two proteins, most of the cited studies use transgenic variants of DAP12 in attributing the observed effects to TREM-2. The idea of TREM-2 being a negative regulator of cytokine responses stems from two early in vitro studies using macrophages [9, 126]. The observation of DAP12 knockout macrophages showing an increase in cytokine secretion as opposed to similar wild-type macrophages upon TLR and FcR stimulation [126] was followed by an attempt to link this phenotype to a specific binding partner of DAP12. Using TNF secretion as a readout, the introduction of a TREM-2/DAP12 fusion construct to otherwise DAP12 deficient cells was able to reverse the effect [126]. A separate study on endogenously activated microglia shows that TREM-2 over-expression reduces the amounts of TNF produced upon stimulation with apoptotic neurons, whereas a TREM-2 knockout lead to a two-fold increase in measured amounts [1]. It was further shown that TREM-2 positively correlated with efferocytosis in the same system [1]. As a consequence of the broad involvement of macrophages, the latter case does not clarify whether the reproduced inhibitory function of TREM-2 is immunologically relevant, or an example of its role in neural homeostasis. The same goes for another report showing the requirement for TREM-2 signaling in the formation of multinucleated giant cells [127]. While the involvement may be of primary relevance to the formation of multinuclear osteoclasts, it is postulated that NHD patients might display a detrimental phenotype when challenged with granulomatous infection [127], which has yet to be confirmed.

A study using pathogen derived TLR ligands directly links TREM-2 deficiency to an increase in pro-inflammatory cytokines produced by BMDCs after 16 hours [6]. Various concentrations of LPS, CpG DNA and Zymosan all increased the production of IL-12 p70

and, though less significantly, TNF when compared to wild-type BMDCs. Additionally, CpG DNA stimulation leads to increased type I IFN mRNA levels in TREM-2 deficient BMDCs [6]. The same study showed TREM-2 to limit DC maturation and negatively regulate their ability to induce antigen specific T-cell proliferation [6]. Furthermore, TREM-2 was recently reported to reduce cytokine production of TNF- α , MIP-2, and IL-1 β in thioglycollate recruited peritoneal macrophages challenged with LPS specifically via the PI3K/Akt pathway. Inhibition of this pathway restores cytokine production in this setting [4]. Another study conducted in BMDMs also observes that inflammatory cytokine production is increased when knocking out DOK3, a mediator of TREM-2/DAP12 signaling. Though in this case, investigators attributed the effect to an increase in ERK phosphorylation [5]. This demonstrates the many layers on which TREM-2 inhibits or delays both innate and adaptive pathogen responses. Notably, a recent study observing the effects of a TREM-2 knockout on a model of DSS and TNBS induced colitis in mice reports a contradictory phenotype [128]. Absence of TREM-2 had a protective effect combined with a reduction of mucosal TNF- α , IL-1 β and IL-10 production. Similarly, TLR stimulation of isolated TREM-2 knockout DCs lead to a reduction of cytokine secretion in this model [128].

TREM-2 was also shown to be involved in the formation of higher order complexes [129], based on the finding that it associates with plexin-A1 and DAP12 in dendritic cells and fibroblasts transfected to express these proteins [129].

TREM-2 has further been recognized as a phagocytic receptor of bacteria [10, 11]. It enables otherwise incompetent Chinese hamster ovary cells to internalize *P. Aeruginosa*, *F. Tularensis* and *S. Aureus*. In addition, BMDMs deficient of TREM-2 and DAP12 show a decrease in their phagocytotic action [10]. In a separate study, the same group provided further clues on the possible ligand interactions of TREM-2 [130]. The experimental setup included a TREM-2/Ig fusion protein, which was shown to have a high affinity toward

bacterial species such as *E. Coli*, *P. Mirabilis*, *S. Pyogenes* and *S. Aureus*, which could be reversed by the addition of excess soluble TREM-2. Lesser affinity binding was observed with *P. Aeruginosa* and *S. Xylosus* while the fusion protein did not at all associate with *C. Albicans*. The suggestion of TREM-2 affinity toward anionic ligands was put forward by showing that interactions could be blocked by anionic carbohydrates and bacterial components [130]. The notion of TREM-2 involvement in phagocytosis is substantiated by a report that TREM-2/DAP12 signaling promotes ERK phosphorylation in microglia and DCs, thus inducing F-Actin polarization without influencing the PI3K/Akt pathway [1].

Although this was reported in a non infectious setting, the combined findings presented in this section have resulted in speculation that TREM-2 promotes phagocytosis at the same time as reducing inflammatory action of the same cells.

4.4.6. TREM-2 and the brain

Both TREM-2 and DAP12 are expressed in the central nervous tissue of mammals and the importance of their contribution is obviated by an increasing range of pathologies arising from their malfunction. Other than the previously discussed role of TREM-2 in NHD [131], brain homeostasis and efferocytosis of neurons [1], the number of reports on its involvement in Alzheimer's disease and multiple sclerosis is increasing.

Experimental autoimmune encephalomyelitis (EAE) is an animal model for multiple sclerosis. It has been shown that TREM-2 expression is up-regulated in microglia of the CNS and spinal cord during both early and chronic stages [12]. A blocking of TREM-2 results in higher inflammatory infiltration of the CNS and increased parenchymal demyelination, overall enhancing the detrimental phenotype of this model [12]. A complementing study shows that

the intravenous administration of TREM-2 transduced myeloid precursors lead to an amelioration of these enhanced clinical symptoms [132].

Earlier this year, two separate study groups convincingly presented a significant correlation between specific TREM-2 heterozygous mutations and the prevalence of Alzheimer's disease [6, 119]. Substitutions in the TREM-2 amino acid sequence such as R47H or Q33X are believed to increase the risk of late onset Alzheimer's, proposedly by failing to inhibit CNS inflammation, as no amyloid-plaque formation has been reported in NHD patients [9]. Because certainty of the TREM-2 – Alzheimer's correlation was established very recently, specific details remain and are likely to follow. One current suggestion was put forward by a study examining the effects of aluminum, an established cause of Alzheimer's disease, on murine microglia [133]. It shows that aluminum induces the up-regulation of a specific micro RNA which in turn significantly decreases TREM-2 expression in the brain. The proposed model implies that reducing TREM-2 expression in the brain impairs phagocytic responses and thus augments the developmental causes of the disease [133].

5. Material and Methods

5.1. Material

5.1.1. Media and supplements

Media: RPMI 1640 (GIBCO 21875-034 and GE healthcare E15-840)
 DMEM Dulbeccos modified eagle medium (GIBCO 31885-023 and Sigma-aldrich D5796)
 RPMI 1640 without phenol red for immunofluorescence (GIBCO 11835-063)

Antibiotics: Pen/strep (GIBCO 15140, 10000U/ml penicillin, 10000µg/ml Streptomycin)

Additives: FCS (fetal bovine serum, GIBCO 10082-147)
 L929 supernatant

RAW 264.7 Media: DMEM + 10%FCS + 1% pen/strep

L929 Media: RPMI + 10% FCS + 1% pen/strep

BMDM Media: RPMI + 10%FCS + 10% L929 supernatant, +1% pen/strep

5.1.2. Chemicals

5.1.2.1. Buffers and solutions

Buffer	Ingredients/supplier	Application
10x PBS	Morphisto (1123700500)	Cell culture, WB, IF
5x RAW detachment buffer RdB	15mM KAc, 150mM KCl in ddH ₂ O	Cell culture
RIPA buffer	150mM NaCl, 0.5% Na-Deoxycholate, 0.1% SDS, 1% Triton-X, 50mM, Tris pH 7.5; 1mM Na ₃ VO ₄ , 10mM NaF, Benzoase (1:1000), Complete protease inhibitor (Roche, 1:50)	Preparation of WB samples
BenchMark™ Pre-Stained Protein Ladder	Invitrogen, 10748-010	Western blotting
10x running buffer	per [l]: 30.3g Trizma base, 150g Glycine, 10g SDS	
10x Semi-dry blotting buffer	48mM (=58g) Trizma base (Sigma; T-1258), 39mM (=29g) Glycine (Merck; 104201), 0.01% (=1g) SDS (Sigma; L-3771) in 1 ddH ₂ O;	
Blocking solution	5% milk powder in PBS	
Washing solution	PBS with 0.1% Tween-20	
Stripping buffer	200mM Glycine, 150mM NaCl, 0.01% Tween-20 in ddH ₂ O; pH 2.5	
Fix&Perm fixation solution	ADG Bioresearch, GAS-001S100	FACS
Fix&Perm permeabilization solution	ADG Bioresearch, GAS-002S100	FACS / Immunofluorescence

Paraformaldehyde (PFA)	3.7% in PBS	Immunofluorescence
Blocking solution	0.5% cold water fish gelatine in PBS	

5.1.2.2. Reagents, stimuli, inhibitors and kits

Substance	Ingredients	Application
Ibidi mounting medium	Ibidi 50001	IF
DAPI	Sigma 32670	IF
Instant milk powder	Maresi	Blocking
Bovine serum albumine	Fraction V, Roche, 10735094001	
Cold water fish gelatin	Sigma	
LPS	Sigma	Stimulus
Lidocain	GebroPharma, Xylanest 2% with Epinephrin(1:200000)	Cell culture
MG132	Biomol PI 102-0005	Inhibitor
BCA Protein Assay Reagent (bicinchoninic acid)	Thermo scientific, 23225	WB, Protein concentration
ECL protein detection reagent	Amersham, RPN 2106OL/AF	WB, Protein detection
EndOH	New England Biolabs, P0702S	Endoglycosidase
PNGase F	New England Biolabs, P0704S	Endoglycosidase

5.1.3. Antibodies

Primary Antibodies (all anti-mouse)				
Antibody	Company	Host Species	Application	Dilution
TREM-2 - biotin	R&D Systems, BAF1729	Sheep	WB, IF	1:200, 1:40
TREM-2	R&D Systems, AF1729	Sheep	WB, IF	1:200, 1:40
TREM-2 (G-16)	Santa Cruz, sc-22634	Goat	IF	1:200
TREM-2 - APC	R&D Systems, FAB17291A	Rat	FACS	1:20
total I κ B α (C-21)	Santa Cruz, sc-371	Rabbit	WB	1:1000
Phosphor I κ B α (Ser 32/36)	Cell Signaling, 9246S	Mouse	WB	1:1000
total Erk (p44/42)	Cell Signaling, 9107	Mouse	WB	1:1000
phospho Erk	Cell Signaling, 9106S	Mouse	WB	1:1000
pan Akt (total)	Cell Signaling, 4691	Rabbit	WB	1:1000
phospho Akt (Ser473)	Cell Signaling, 4060	Rabbit	WB	1:1000
β -Actin	Sigma-Aldrich, A5441	Mouse	WB	1:1000
Golgin 97	Abcam Ltd., AB84340	Rabbit	IF	1:200
Secondary Antibodies				
Antibody	Company	Host Species	Application	Dilution
Anti-streptavidin HRP conjugate	Sigma-Aldrich, S 2438	n/a	WB	1:200
Anti-streptavidin HRP conjugate	R&D systems, 890903	n/a	WB	1:200
Anti-mouse-IgG HRP conjugate	BIO-RAD, 170-6516	Goat	WB	1:25 000
Anti-rat-Ig HRP conjugate	Invitrogen, A21210	Rabbit	WB	1:10 000
Anti-rabbit IgG HRP conjugate	Cell Signaling, 7074S	n/a	WB	1:2000

Anti-Sheep IgG (H+L), F(ab') ₂ fragment Texas Red conjugate	Sigma-Aldrich, SAB3700742	Donkey	IF	1:1000
Anti-rabbit IgG (H+L) DyLight 405 conjugate	Jackson ImmunoResearch, 711-475-152	Donkey	IF	1:1000

5.2. Methods

5.2.1. Cell culture routine

5.2.1.1. L929 Fibroblasts

Fibroblasts were cultured in RPMI medium with 10% FCS and 1% Pen/strep and split 1:10 every other day. Cells were detached by incubation with trypsin for 5 minutes after washing with PBS. New media containing FCS was added to inactivate trypsin. Cells were then centrifuged and re-suspended in an appropriate volume of fresh growth medium for re-plating.

5.2.1.2. RAW 264.7 cells

RAW 264.7 cells were cultured in designated RAW medium and split every 2-3 days or when confluent. Cells were washed with PBS and detached by the use of RAW detachment buffer. An appropriate volume was added and cells were detached by repeated flushing after 5 minutes of incubation. They were then centrifuged at room temperature, re-suspended and split 1:10 for further growth.

5.2.1.3. BMDMs

Isolation from mouse bone marrow

Removed fur of sacrificed mice and cut legs which were disinfected in 70% Ethanol and stored in cold saline until transfer to a sterile working space. Hinges were cut to reveal bone marrow which was flushed out with 5ml RPMI media per bone using a 27G needle. Lumps

were re-suspended and transferred to a falcon tube for gentle centrifugation (1250rpm, 5min, RT, 0 breaks). Supernatant was discarded and all cells derived from the same mouse were re-suspended in 1ml of BMDM freezing media before transfer to Mr. Frosty freezing container (Thermo Scientific) in individual cryotubes. Subsequent freezing of the bone marrow to -80°C is considered to kill of erythrocytes while leaving the desired macrophage population intact. If storage for several days was required, cells were transferred to liquid nitrogen after 24 hours.

Seeding

Previously harvested BMDM aliquots stored in liquid nitrogen were transferred to 50ml RAW media for thawing, then centrifuged and re-suspended in an appropriate volume.

Cells were counted and plated with a specified density, receive additional BMDM medium on day 5 in order to allow full differentiation on day 7. The following table specifies plating densities and volumes for designated plates.

	Area (cm ²)	Number of cells	Volume of Medium	Cells/mL	Volume to add (5 th day)
Petri dish (10cm)	78,5	10.000.000	10 mL	1.000.000	2 mL
6 well plate	9,6	1.250.000	2,5 mL	500.000	500 µL
12 well plate	3,9	500.000	1 mL	500.000	250 µL
24 well plate	1,9	250.000	500 µL	500.000	100 µL
48 well plate	1	130.000	250 µL	520.000	75 µL
96 well plate	0,3	40.000~50.000	150 µL	267.000	50 µL

Freezing and thawing cells

Freezing:

Designated cells were suspended in 1ml appropriate freezing media (containing 20% FCS and 10% DMSO), transferred to labeled cryotubes and put into Mr. Frosty (Thermo Scientific) pre-cooled to 4°C. Mr. Frosty is further cooled to -80°C and tubes transferred to liquid nitrogen the next day for storage.

Thawing:

Cells taken from liquid nitrogen were pre-thawed to enable transfer to 50ml to cell specific medium. Once defrosted, they were centrifuged at 1250rpm (RT). Discarded supernatant was replaced by an appropriate amount of medium and re-suspended cells were plated for further use.

5.2.1.4. *Cell counting*

Cell suspensions were diluted 1:1 with trypan blue to exclude dead cells and debris. 25 squares in counting chamber (= 0.1µl) were manually counted in order to calculate the concentration. $\text{Cell count} \times 2 \times 10^4 = \text{cells/ml of cell suspension}$

5.2.2. **Western blotting**

5.2.2.1. *Preparation of protein samples for western blotting*

Cells were washed twice with cold PBS and RIPA-buffer (For 10cm plate: 100µl) was added directly to the plate. Cells were scraped off, transferred to a 1.5ml tube and left on ice for 30min. After centrifugation (14000x rpm, 10min, 4°C), supernatant was transferred to a new safe-lock tube and the pellet discarded.

Samples were then fried in liquid N₂ and stored at -80°C

5.2.2.2. *Determination of Protein Concentration*

Protein concentrations of cell lysates were determined using a commercial BCA protein detection kit by Pierce in accordance with the manufacturer's instructions. Please refer to supplementary method 12.3.1 for details.

5.2.2.3. *Western blotting*

After preparing appropriate SDS gels (10% or 12%), equal amounts of all protein samples and protein ladder were loaded to respective chambers. Gels were run in a wet chamber for about

2h with 120 Volts applied before being transferred to a PVDF membrane (Immobilon-FL transfer membrane by millipore, Cat.no. IPFL00010, Pore size: 0.45µm), which was activated with methanol and equilibrated in 1x western blot transfer buffer.

Page gels and extra thick filter paper (BioRad, Cat. No. 1703960) were equally equilibrated in 1x transfer buffer and prepared in the order paper-membrane-gel-paper. After rolling out bubbles and closing the "Transfer-blot SD Semi-dry transfer cell by BioRad", 12 Volts were applied for 60minutes.

After disassembly, the membrane was washed with PBS and blocked in PBS +5% milk powder for 1h. Primary antibodies were added and incubated at 4°C over night (or 2h at RT) Washed 3x with PBS + 0.01% Tween 20 for about 20min at RT, applied secondary, HRP-conjugated antibody solution and incubated 2hours at room temperature. After 3 washing steps with PBS + 0.01% tween-20 for about 20min each, blots were left in PBS for 10min to remove additives. ECL western blot detection system was applied according to manufacturer's instructions. Blots illuminate HP Hyperfilms (Amersham, 28906846) for differing amounts of time. These are then developed in a dark chamber for visualization of protein bands.

5.2.2.4. *Proteasome inhibition*

All cells designated for proteasome inhibition received 10ml fresh 3%FCS Medium with 10µM proteasome inhibitor MG-132 one hour prior to LPS-stimulation in 10cm plates. All other cultures used as controls received fresh Medium with 3%FCS only.

5.2.2.5. *Glycosidase assay*

Reaction mixtures were prepared and combined according to manufacturers instructions. For details, see supplementary protocols 2 and 3 for EndoH and PNGase, respectively. Refer to supplementary method 12.3.1 for details.

Reaction mixtures for BMDM wt sample were adapted according to its concentration of 1602 µg protein per ml RIPA buffer (as determined by Pierce-BCA kit).

3times: 6,5 sample + 1ul 10x GDB buffer + 2,5ul ddH₂O

➔ Boil 100°C 10min

Control: Add 2ul 10x G5 Reaction buffer + 8ul ddH₂O

EndoH: Add 2ul 10x G5 Reaction buffer + 7ul ddH₂O + 1ul EndoH

PNGase: Add 2ul 10x G7 Reaction buffer + 2ul NP-40 + 5ul ddH₂O + 1ul PNGase

5.2.3. Flow cytometry

Stimulation:

Cells were pre-treated with mock, 100ng/ml or 1mg/ml LPS in 3% FCS – Medium for 1h in a 12-well chamber and then washed off stimulant with 3x ice cold PBS. Cells were then distributed to corresponding FACS tubes (one well/tube)

While keeping any non-permeabilized tubes on ice, the BMDMs group to be permeabilized underwent additional incubation steps. They were centrifuges at 1250rpm for 5min at 4°C, supernatant was discarded and cells were fixed by applying 100µl ADG fixation medium for 15min. Again, cells were spinned at 1250rpm for 5min at 4°C, supernatant was removed and 100µl ADG permeabilization medium applied for 15min.

After spinning and removing media of all cells in the same way, the protocol continued equally for all samples.

Added 95µl of PBS (1%BSA) + 5µl of TREM-2 APC (R&D Systems FAB17291A, diluted 1:20) to corresponding tubes except negative and isotype controls and incubated for 30min RT.

Cells are spinned, supernatant removed and washed with 3ml PBS (1%BSA), then spinned again and resuspended in 260µl of PBS (1%BSA) for subsequent flow cytometry.

5.2.4. Immunofluorescence staining and sample preparation

The immunofluorescence staining protocol has been adapted several times as the specific needs and requirements for a meaningful readout became clear. The following shows the final protocol from which data in the results section is derived. For details on the development of this protocol, refer to supplementary section 11.2.

Cells were plated at a density of 50 000 cells per well in 8-well Ibidi µ-chambers (product no. 80826) as outlined in subchapter 5.2.1.3, 7 days before staining. On the last day, cells were washed twice with 200µl PBS and fixed with 3.7% PFA for 10min at room temperature. After another 2 washing steps with 200µl PBS, autofluorescence was quenched by incubation with 0.1M Glycine in PBS for 5 min. Cells were then washed again, permeabilized for 10 min at room temperature using 200µl Perm Solution (ADG Fix&Perm). Blocking was carried out for 20-60min at room temperature using cold water fish scale (cwfs-) gelatin 0,5%in PBS. Then cells were incubated for 1.5h at room temperature with 100µl primary antibody mix diluted in PBS +0,2% cwfs-gelatin.

Primary Antibody 1: Anti-mouse TREM 2 (R&D Systems, AF1729,
Stock 200 µg/ml in Dilution Buffer, work conc. 5µg/ml)

Primary Antibody 2: Anti-mouse Golgin-97 (Abcam Ltd., AB84340, Stock 1000
µg/ml in Dilution Buffer, work conc. 5µg/ml)

After incubation with the primary antibodies, cells were washed with 200µl/well PBS containing 0,5M NaCl and then washed once with PBS only, before treating them with 50µl Perm solution and 150µl (0,5%) cwfs gelatin. Subsequently 100µl secondary antibody diluted in PBS containing 0,2% cwfs-gelatin was added for 1h at room temperature.

Secondary Antibody 1: Donkey anti-sheep Texas Red (Sigma-Aldrich SAB3700742, 1:1000)

Secondary Antibody 2: Goat anti-rabbit DyLight 405 (Jackson ImmunoResearch, 711-475-152, 1:200)

After washing away the secondary antibody with PBS, cells were flushed with ddH₂O to remove Salt. After letting cells dry they were embedded with Ibidi mounting medium (glycerin based) and stored at room temperature.

5.2.5. Imaging and deconvolution

Immunofluorescence images were scanned using a LSM 780 confocal laser microscope and its 63x oil immersion objective. Blue channel was excited by a 405nm UV laser and recorded wavelengths span 410-450nm. Green signal was excited with a 488nm laser with recorded wavelengths of 491-580nm. Red channel was excited by a 594nm laser and shows signals from 600-670nm. All single layer images used 1.0AU pinholes in all channels for better visualization. Stacks had pinholes of 0.85, 0.63 and 0.44 for the blue, green and red channel, respectively. This led to an equal section thickness of 0,52 μ m in all channels and resulted in an interval of 260nm. This was required for subsequent deconvolution according to a theoretical point-spread function (PSF) by the Huygens Essentials software. Theoretical PSF calculations were merited by the minor practical drift of the objective and determined lack of chromatic aberration (not shown). Deconvolution was performed at 20 iterations with a signal/noise ratio of 1:10. Background intensity was adapted from software as calculated by the lowest noise algorithm setting.

6. Results

6.1. TREM-2 affects MAPK- and PI3K signaling

MAPK- as well as PI3K signaling has been previously shown to impact on NF- κ B activation in different settings [71, 80]. To understand which signaling pathways are influenced by TREM-2, we examined the phosphorylation states of selected MAPK, Akt and I κ B α upon LPS stimulation in transgenic RAW 264.7 cells. RAW TREM-2-OE cells, modified to over-express TREM-2 were compared to RAW GFP cells, which only express GFP to exclude the transfection process as a source of the studied effects. RAW GFP cells show the presence of an unspecific protein band around 50kDa in all samples and blots, which is not shown for better comparison of the relevant bands.

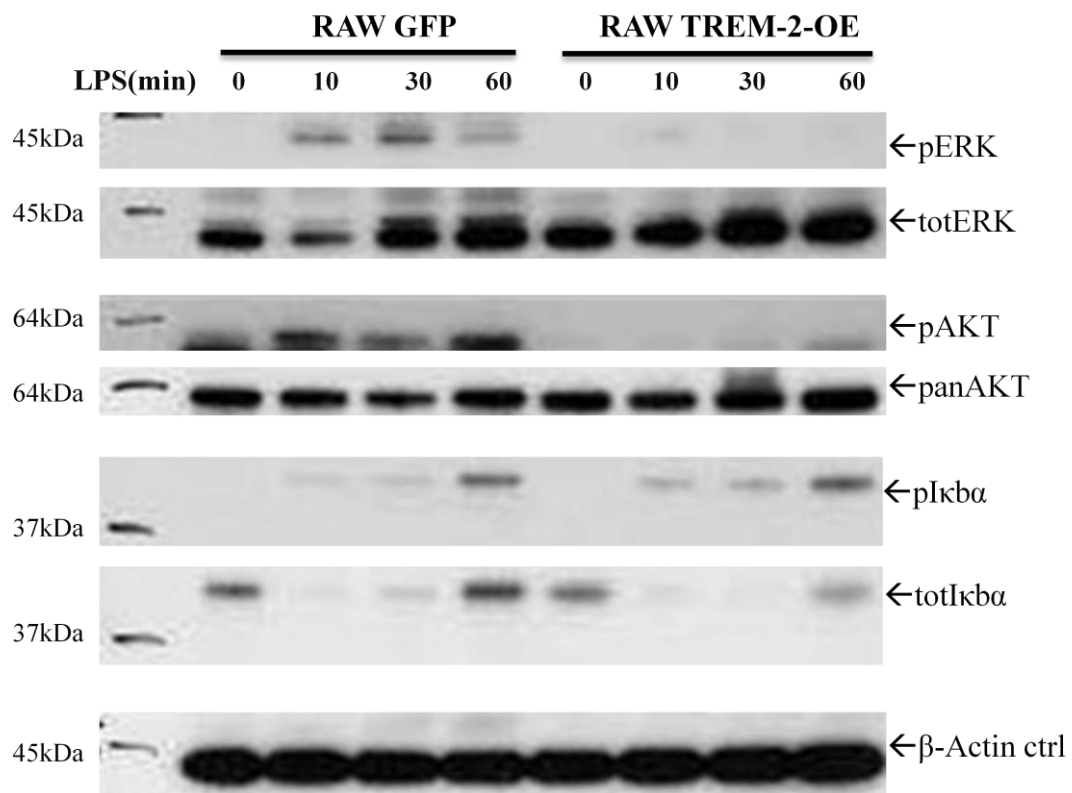


Figure 7 Bands depict protein levels of respective cell isolates after a given time of stimulation with 100ng/ml LPS as determined by western blot. Top lanes for each pair represent the phosphorylated form, bottom lanes represent total amounts. Sizes for all kinases: ERK = 42/44kDa, AKT = 60kDa, I κ B α = 40kDa, β -actin = 43kDa. β -Actin loading control accounts for all blots as all were loaded with equal amounts of the same sample.

TREM-2 OE cells as well as control cells show equal and substantial phosphorylation of I κ B α upon LPS treatment, indicating that the stimulation worked for both cell types. Phosphorylation of I κ B α goes along with a rapid decrease in total I κ B α (due to proteasomal degradation), which starts to get resynthesized at 60min. Total I κ B α in overexpressing cells shows similar dynamics as the control, though it appears to reconstitute less rapidly.

As expected upon LPS challenge, control cells show an increase in phosphorylated ERK and Akt. ERK phosphorylation peaks at 30 min and seems to go along with a decrease in total protein. Akt was tricky to separate from the previously mentioned GFP band, but reaches a peak at 60 min in control cells. Interestingly ERK as well as Akt phosphorylation seems to be abrogated by the overexpression of TREM-2, indicating TREM-2 signaling to impact on these pathways.

6.2. TREM-2 protein levels decrease within minutes of LPS treatment

Going further into the mechanics of TREM-2 upon TLR4 stimulation, we proceeded to have a look at what happens to TREM-2 itself on protein level upon LPS treatment.

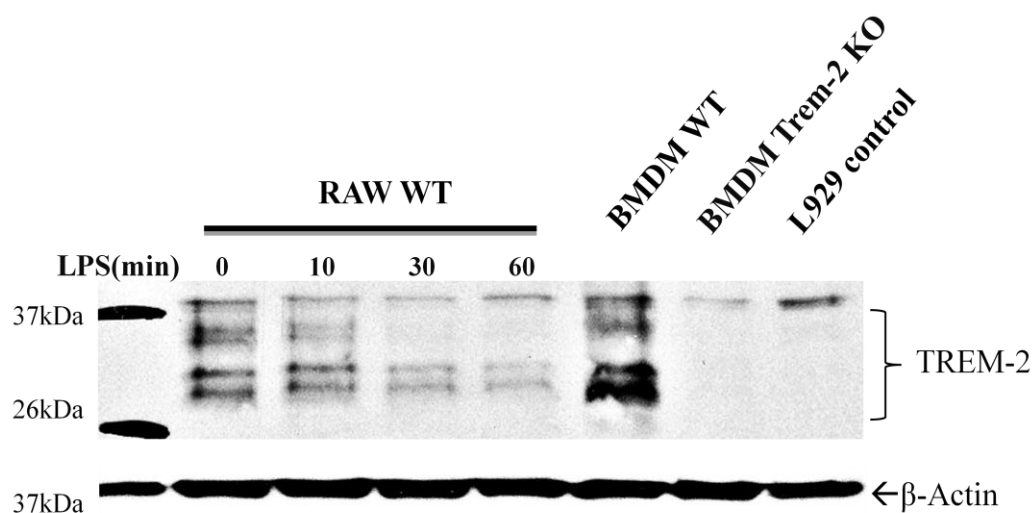


Figure 8 shows TREM-2 levels in resting wild-type- and knockout BMDMs and L929 fibroblasts. RAW 264.7 cells were stimulated with 100ng LPS for given amount of time. Note that the expected size for TREM-2 is around 27kDa.

Western blots show both RAW 264.7 and BMDM wild-type cells to be positive for TREM-2, which appeared in multiple bands between 26 and 37 kDa. Proving specificity of the antibody, the same bands were not detected in either BMDMs, isolated from TREM-2 knockout animals or L929 fibroblast controls. The band around 38kDa was found in all cell types blotted for TREM-2 with this antibody, including negative controls. It was therefore considered a result of unspecific binding and will not be shown or elaborated in latter results.

Note that the predicted size of the native TREM-2 protein lies near 25kDa (Uniprot, accession number Q99NH8). However, all TREM-2 specific bands were of bigger size than the predicted native protein, pointing toward its further processing and posttranslational modification.

Notably, TREM-2 expression successively decreased upon LPS stimulation of the treated RAW 264.7 cells, while levels of the larger product appear to decline more rapidly than the smaller product.

Increasingly interested in this rapid reduction of TREM-2 protein expression upon LPS challenge, we tested this effect in different other cell types, namely RAW cells overexpressing TREM-2 or a chimeric TREM-2/DAP12 protein. Again, the TREM-2 antibody in use detects several bands of differing sizes in both cell types. Importantly, we continue to observe the same rapid decrease of the bigger TREM-2 product upon LPS challenge while the levels of the smaller product change slightly or remained unaffected (Fig. 9).

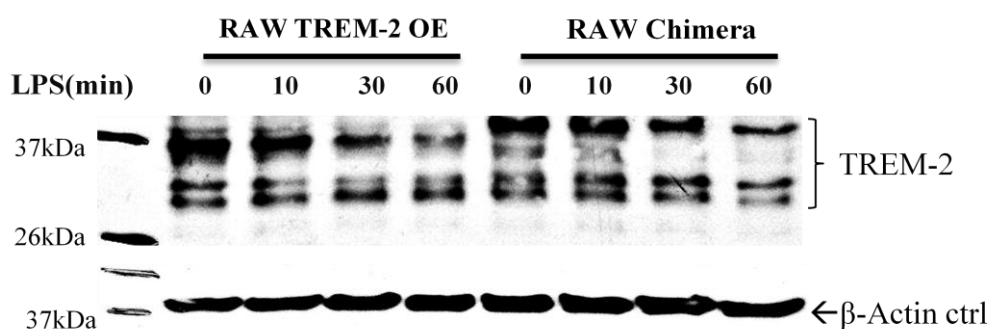


Figure 9 depicts TREM-2 levels in RAW TREM-2 OE and RAW TREM-2/DAP12 Chimera cells upon stimulation with 100ng LPS for given amount of time.

To back up this so far consistent data on the decrease of TREM-2 protein upon LPS stimulation, we designed a preliminary FACS experiment to reproduce this effect using both a different method and a different, APC-conjugated antibody.

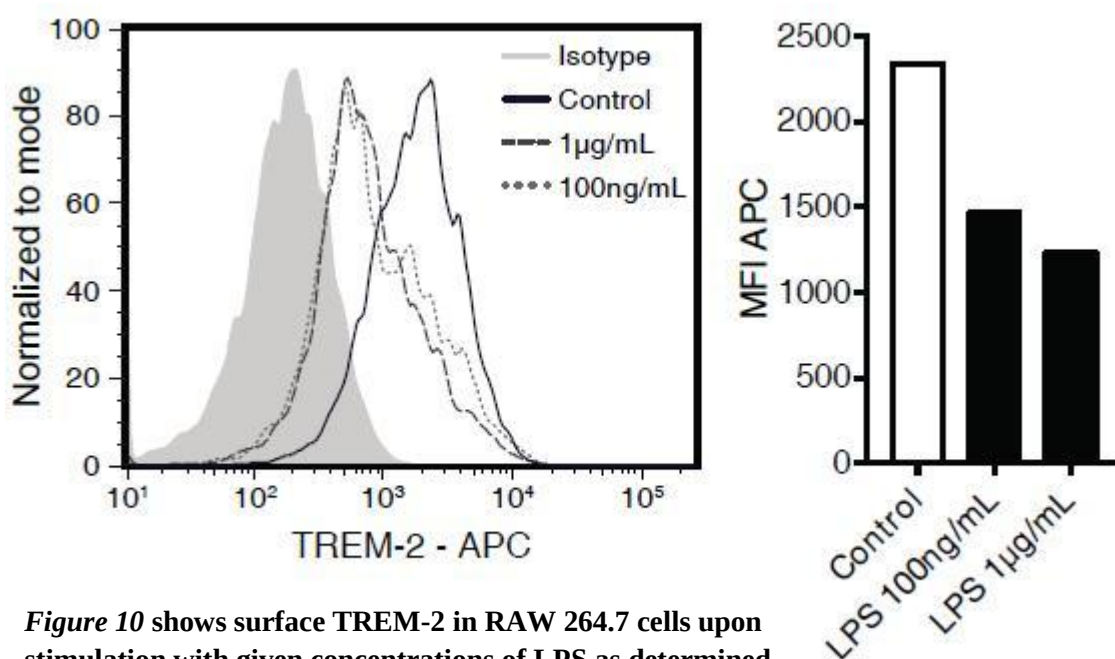


Figure 10 shows surface TREM-2 in RAW 264.7 cells upon stimulation with given concentrations of LPS as determined by flow cytometry. Stimulation: 60 minutes.

We observe that the mean fluorescence intensity of surface-bound APC decreases upon LPS stimulation of RAW 264.7 cells as compared to unstimulated control cells, confirming the results obtained by western blotting. The effect also appears to be dose dependent as the TREM-2 signal decreased in correlation to the amount of LPS added during stimulation.

Examination of the same effect in BMDMs failed to reproduce the result obtained for RAW cells. A decrease in surface expression could not be observed using FACS (Fig. 11). Given that, in contrast to RAW 264.7 cells, BMDMs show a much stronger expression of the smaller TREM-2 product (double band slightly above 26kDa) compared to the bigger product (double band around 37kDa) and that this was the band which only slightly decreased upon LPS treatment (Fig. 8 and 9), we decided to permeabilize the cells to obtain additional information on the cellular localization of TREM-2.

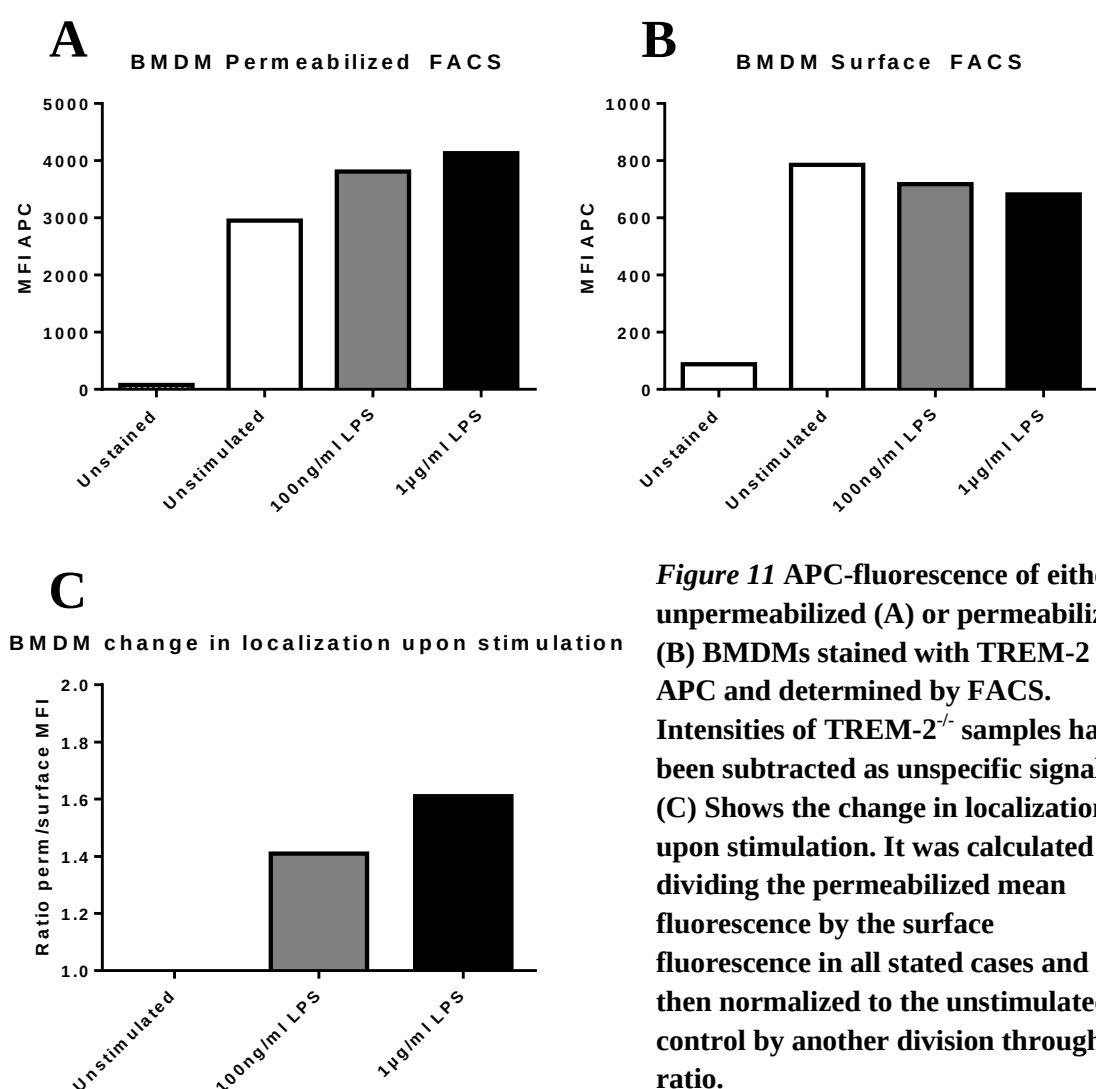


Figure 11 APC-fluorescence of either unpermeabilized (A) or permeabilized (B) BMDMs stained with TREM-2 APC and determined by FACS. Intensities of TREM-2^{-/-} samples has been subtracted as unspecific signal. (C) Shows the change in localization upon stimulation. It was calculated by dividing the permeabilized mean fluorescence by the surface fluorescence in all stated cases and then normalized to the unstimulated control by another division through its ratio.

Absolute values of mean fluorescence measured show that TREM-2 is approximately three times more abundant in the intracellular domains of BMDMs as compared to their surface,

confirming the results of previous western blots. The observed reduction of surface TREM-2 upon LPS stimulation is moderate but consistent when compared to previous experiments. Permeabilized BMDMs show an unexpected increase on fluorescence upon stimulation and both effects are more pronounced with rising LPS concentrations. Calculating the ratio of permeabilized to surface signal gives an indication of the changing location of TREM-2 upon stimulation. As compared to the distribution in unstimulated BMDMs, LPS leads to an increasing shift towards the intracellular.

6.3. Proteasome inhibition leads to accumulation of TREM-2 at baseline, but does not prevent LPS dependent decline

With respect to the observed LPS dependent disappearance of TREM-2, we hypothesized that this could be due to proteasomal degradation and proceeded to examine whether proteasomal inhibition by MG132 can abolish this effect in RAW 264.7 cells.

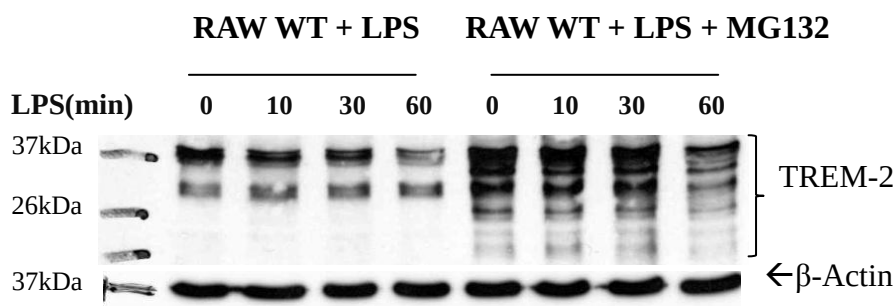


Figure 12 compares TREM-2 in RAW 264.7 cells stimulated with 100ng/ml LPS for given amount of time with equally stimulated RAW cells after pre-incubation with 10µg/ml MG132 1 hour prior to LPS addition.

Though not as pronounced as in previous instances, subsequent decrease of TREM-2 protein levels upon LPS stimulation occur equally in both groups. While proteasomal inhibition did not diminish this effect, it led to a significant accumulation in protein levels at baseline and consequently throughout the time course. The number of differently sized TREM-2 variants is also substantially increased. Interestingly, we observe a protein band at the predicted size of

native TREM-2 protein around 26kDa upon proteasomal inhibition. These results point towards proteasomal regulation of TREM-2 under resting conditions, while the decrease of TREM-2 protein expression upon LPS challenge seems to be independent of the proteasome.

6.4. TREM-2 is glycosylated and shuttles through the Golgi

Based on the assumption that multiple TREM-2 bands observed in western blots are a consequence of post translational modification, we continued to define the possible nature of these. Literature suggests that human TREM-2 is N-glycosylated, based on a study showing that a 40kDa variant (we observed a 37kDa variant in our setting) was reduced to 26kDa as a result of N-glycosylation cleavage [13]. Either N- or O-glycosylation of a proposed soluble TREM-2 variant has also been suggested by a similar experimental setup using a mix of endoglycosidases [122]. The most prominent online predictors of these modifications, NetNGlyc 1.0 [134] and NetOGlyc 4.0 [135] originally designed for human proteins, predict one high probability N-glycosylation site at amino acid 23 [134] and one O-glycosylation site with a lesser probability of 0.5 [135].

To further this given information, the following experiment was set up not only to show whether the multiple TREM-2 bands observed in our murine extracts are a result of N-glycosylation, but also to identify their source. A mixture of different endoglycosidases, which were described in the introduction (section 4.2) was used to define whether the size of a specific band is a consequence of glycosylation on the ER or the Golgi apparatus.

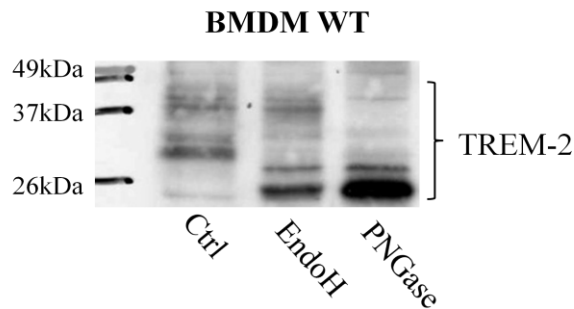


Figure 13 shows TREM-2 in wild-type BMDMs upon treatment with EndoH or PNGase as compared to the control without enzyme.

Results show the usual multitude of TREM-2 bands previously seen in untreated control groups. Samples treated with EndoH show a complete disappearance of the lower TREM-2 double band, indicating that these are TREM-2 products which have not undergone Golgi processing. In contrast, the higher molecular weight bands were resistant to EndoH treatment. The appearance of native TREM-2 (26kDa) upon glycosidase treatment underlines this result. PNGase treatment diminished TREM-2 bands of all sizes. This is mirrored by the production of more native TREM-2 product than in the case of EndOH. Interestingly, there are two cleavage products in both of the treated samples, indicative of additional, unidentified type of post translational modification. All protein bands that disappear upon glycosidase treatment are a result of N-glycosylation.

6.5. TREM-2 is localized in the Golgi of BMDMs

One implication of the previous result is the post-translational modification of TREM-2 in the Golgi apparatus. The following experiment substantiates this notion by a second method and show the cellular localization of TREM-2 relative to the Golgi within unstimulated BMDMs.

Initial 2D imaging of TREM-2 and Golgin 97 shows several instances of significant signal

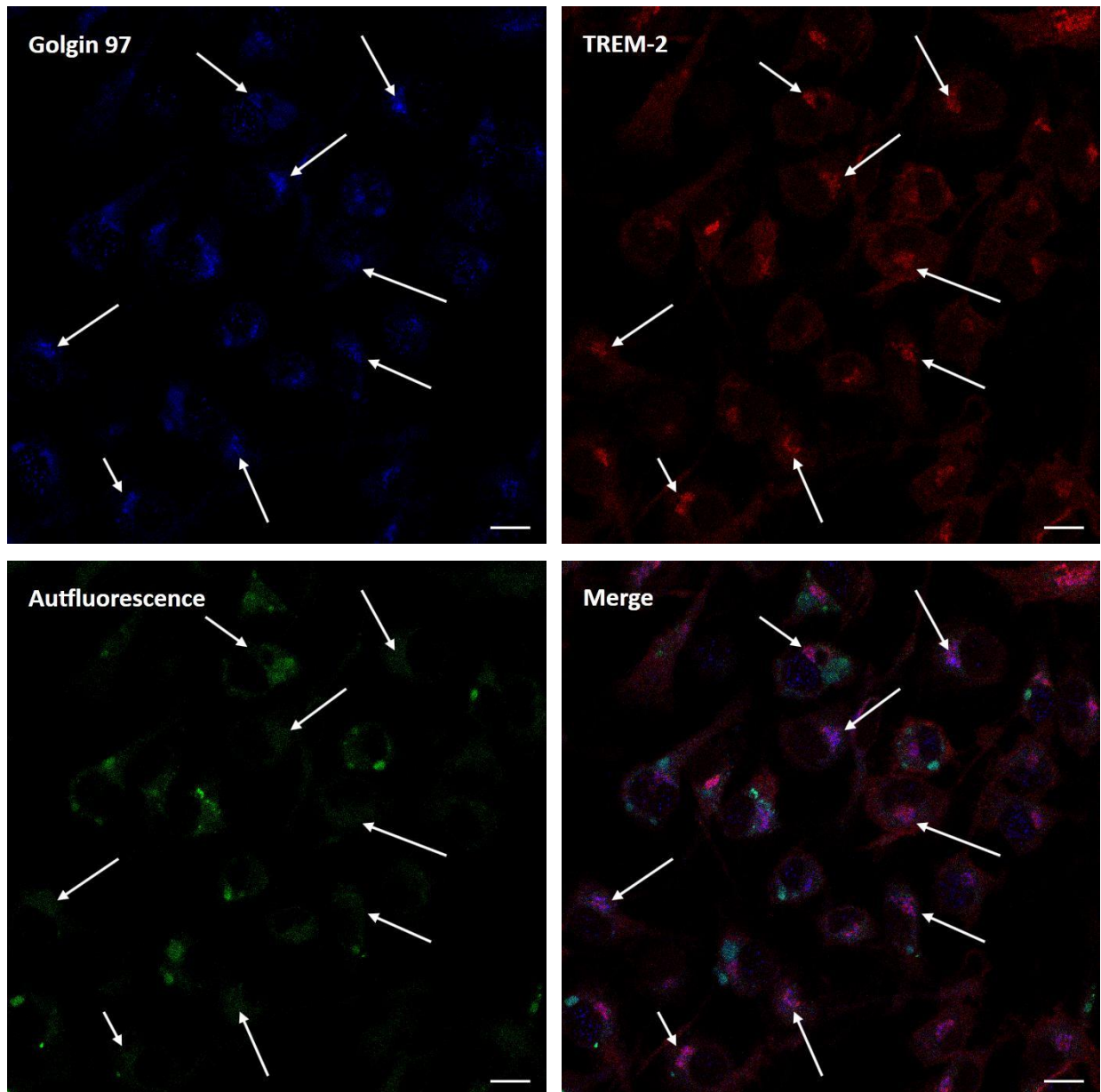


Figure 14 shows recorded channels of the same 2D image as labeled. White arrows show direct high-intensity signal co-localization between TREM-2 and Golgin 97 that does not have a relevant corresponding intensity in the green, autofluorescent channel. Bar: 10 μ m

overlap as represented by arrows (Fig. 14). In addition, TREM-2 localization at the surface and to other intracellular structures can be made out in certain cells. The implications of high intensity autofluorescence in BMDMs and the reasons for showing this channel are discussed in supplementary section 12.2. Controls are shown in supplementary Fig. 1, where staining with either Golgin 97 or TREM-2 leads to significant loss of signal in the respective channel.

To get a closer look at the signal overlap between TREM-2 and Golgin 97, the following images have been deconvolved as outlined in the method section. In achieving accurate 3D reconstruction, deconvolution is not only superior to common filtering techniques [136] but also essential in this experimental setup to exclude otherwise common false-positive results in colocalization analysis [137].

The first set of images shows the co-localization of Golgin-97 and TREM-2 along a vector through the Golgi apparatus in a representative z-stack. Its purpose is to detail the proposed overlap along a Golgi mid-section and to discriminate from green autofluorescence.

Fig. 14 shows a significant overlap of signal peaks in the TREM-2 and Golgin 97 channel for the plotted locations. Importantly, an insignificant amount of observed signal in these channels corresponds to an intensity peak in the green channel after deconvolution, instances of which have been highlighted.

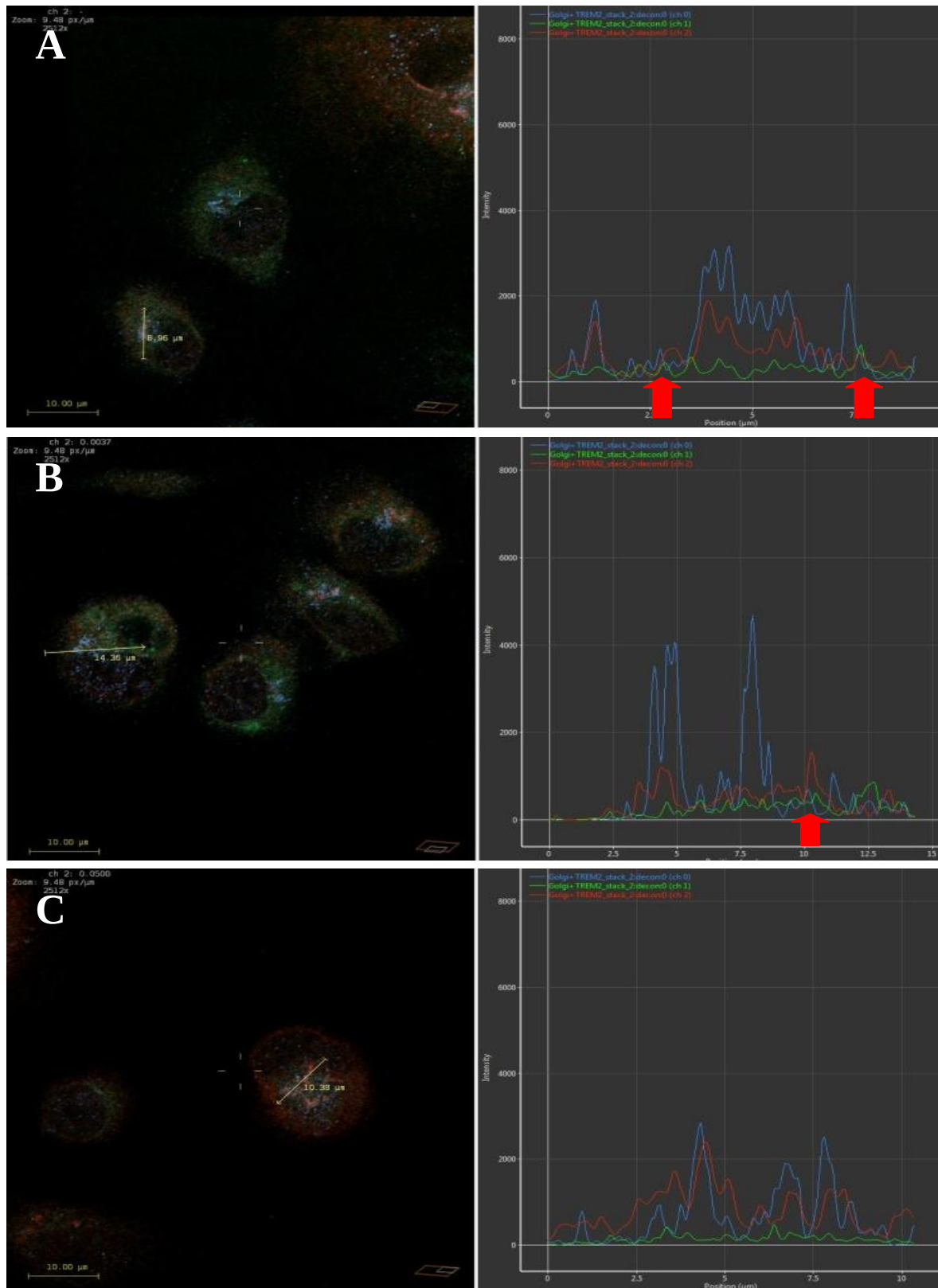


Figure 15 BMDMs imaged for TREM-2 (Red), Golgin 97 (Blue) and autofluorescence (green). A, B and C represent different instances of a mid-section (left) in the same deconvolved confocal Z-stack, with an orthogonal representation (right) of pixel intensities along an introduced vector. The x-axis of the intensity projection shows the pixel positions along this line, whereas the y-axis reflects their respective intensity in the equally colored channel. Red arrows highlight peaks that must be neglected in all channels due to a possible autofluorescent source.

We continue to substantiate the proposed co-localization with another Z-stack that has been equally deconvolved. Fig 15A is a presentation of this cell in the midsection of a deconvolved Z-stack, with an orthogonal view of pixel intensity along the introduced line. Again, we observe a general high-intensity signal overlap between the TREM-2 (red) and Golgi (blue) channel. The minor intensity peak in the green channel highlighted by the red arrow, exemplifies that the amount of peaks we discarded in our assessment due to autofluorescence poses a vast minority. Fig. 15B shows 3D object reconstruction of the highlighted cell, accounting for pixels emitting the top 30% of all recorded intensities. The blue structure shows the Golgi apparatus with individual cisternae not distinguishable by the given resolution. The red structures represent TREM-2 signal which clearly colocalizes with the Golgi and is otherwise distributed around the cytoplasm and cell surface. Affiliations of the smaller objects remain to be determined.

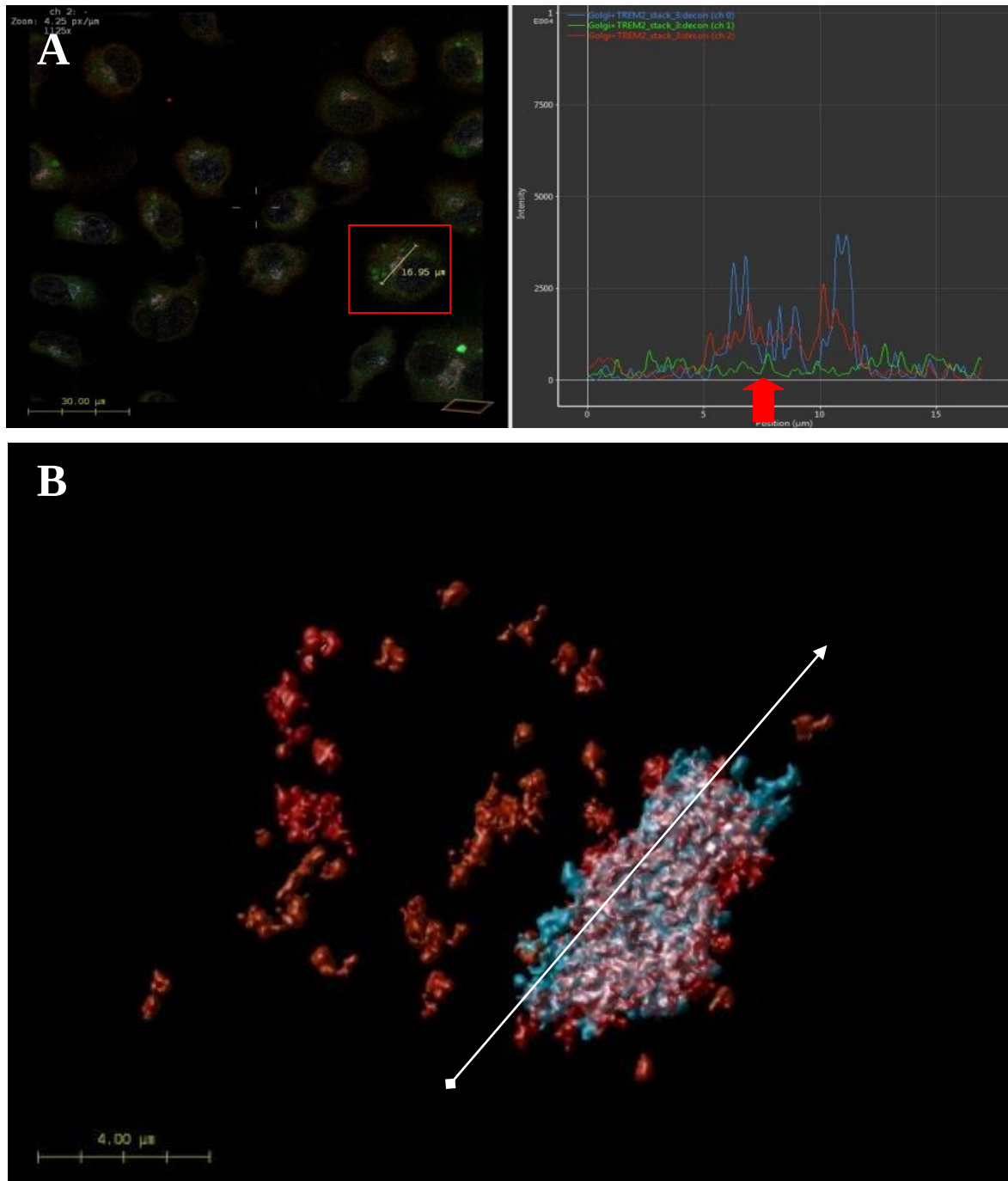


Figure 16 (A) Z-stack midsection with an orthogonal representation of intensities along the drawn vector. Red box highlights the cell used for 3D object reconstruction and the red arrow exemplifies a peak that would have to be neglected due to an autofluorescent signal source. (B) 3D object reconstruction of pixels with the top 30% intensity in the TREM-2 (red) and Golgin 97 (blue) channel. Relative to its presentation in A, the cell of interest is viewed from below. The vector in B is an approximation of the one introduced to A and merely serves to assist orientation.

7. Discussion

Special interest in TREM-2 is evoked by its involvement in human pathologies such as NHD [115, 116] and the emerging connection to Alzheimer's disease [6, 119], but also by its impact on various inflammatory conditions, which were studied using mouse models. In such, TREM-2 has been shown to be important during EAE, a mouse model of multiple sclerosis [12], to regulate inflammation during *Pseudomonas aeruginosa* infection [4] and DSS induced colitis [128]. Furthermore, its important role as a phagocytic receptor in vivo has been shown in a cecal ligation and puncture (CLP) model of gram-negative sepsis [11]. While an increasing number of disease studies support the idea of TREM-2 being a crucial regulator of immune responses, its mechanism and mode of action remain poorly understood. Whether in brain homeostasis or pathogen induced inflammatory settings, TREM-2/DAP12 signaling is consistently reported to reduce the production of pro-inflammatory cytokines [1, 4, 126] while increasing phagocytic action [1, 127]. Yet the details of how this combination is mediated have not been clarified. This work, aimed to investigate the reactive dynamics of TREM-2 in macrophages using two different approaches, focuses primarily on its regulatory functions during cellular responses to LPS on one hand, and its cellular localization on the other.

This thesis shows evidence for TREM-2 impacting on TLR4 mediated responses by suppressing the PI3K as well as the ERK pathway. In addition, it adds important insights toward TREM-2 localization and modification in BMDM, showing that TREM-2 is glycosylated and shuttled through the Golgi apparatus while mainly stored in intracellular compartments in resting cells.

To improve understanding of how TREM-2 influences TLR4 mediated responses, we studied the activation of NF- κ B, the PI3K- and the ERK pathway in TREM-2 overexpressing macrophages upon LPS challenge. NF- κ B is classically activated by TLR4 signaling which leads to the phosphorylation and subsequent degradation of I κ B α [138]. Importantly, TREM-2 overexpression did not alter LPS induced I κ B α phosphorylation (Fig. 7), suggesting that TREM-2 does not directly interfere with TLR4 mediated I κ B phosphorylation and subsequent NF- κ B activation. However, we observe a reduced reconstitution of I κ B α at 60min in TREM-2 overexpressing cells (Fig. 7). This initially seems counter-intuitive, because it would suggest a lack of negative feedback inhibition of NF- κ B in TREM-2 overexpressing cells, which would not fit with a suppression of cytokine responses. However, I κ B α is one of the first NF- κ B target genes [36] along with several proinflammatory mediators and a lack of reconstitution might also reflect a suppressed transcription of NF- κ B target genes by a different mechanism.

In contrast, TREM-2 over-expression abolished both ERK and Akt phosphorylation at all time points, while total baseline protein levels remain equal for both samples. This confirms previous reports on the influence of TREM-2 on MAPK and PI3K dynamics [1, 4], though simultaneous signal reduction upon LPS stimulation was not assessed in these cases. The data presented in this thesis extend observations to the cell type in question and additionally suggest that TREM-2 exerts its effects by impacting on PI3K and ERK signaling in different cell types. More directly, the inhibition of Akt phosphorylation, shown to play a role in inhibiting inflammatory cytokine production [139, 140], suggests that the counter-inflammatory effects of TREM-2 are mediated via this pathway. This is supported by an in vitro study that showed a recruitment of the regulatory PI3K subunit P100 to the TREM-2/DAP12 complex [84] and an in vivo study, where the impact of TREM-2 on cytokine responses could be blocked by the use of wortmannin, a known inhibitor of PI3K [4].

The suppression of ERK phosphorylation by TREM-2 overexpression in RAW cells is equally in line with previous reports [1, 5]. ERK has been linked to TREM-2 mediated increase in phagocytic activity of microglia in neural homeostasis [1] and to negative regulation of inflammatory cytokines in an LPS induced BMDMs [5]. Our supportive data is the first to report a similar connection in RAW 264.7 macrophages.

Leaving these implications standing, we continued to assess TREM-2 protein patterns in macrophages and the impact of LPS stimulation on TREM-2 levels. Using L929 fibroblasts and TREM-2 deficient BMDMs as negative controls, we clearly distinguish four different TREM-2 products in RAW cells and BMDMs, all of which exceed the expected size of 26kDa predicted for the native protein (Fig. 8).

Upon LPS challenge, we observe a rapid decline of TREM-2 levels in RAW cells (Fig. 8), as well as in TREM-2 overexpressing cells and a cell line overexpressing a TREM-2/DAP12 chimeric protein (Fig.9). The larger TREM-2 products completely disappeared within 30 minutes, while the lower bands either decreased at a lesser pace through all time points in RAW cells or seem unaffected in overexpressing cells. The fact that the rapid decrease in TREM-2 protein levels is ubiquitous for all of these cells, suggests that this is a general behavior of TREM-2 upon LPS encounter. We could confirm this effect in RAW 264.7 macrophages using flow cytometry, showing a clear and dose dependent decrease in TREM-2 surface expression upon LPS encounter (Fig. 10). Since we only measured protein localized on the surface of these cells, and assuming that internalization would retain fluorescence, we could not assess whether the signal reduction was a result of protein degradation or receptor cleavage.

The effect was further examined in BMDMs (Fig. 11), introducing a second group that was stained after permeabilization to quantify intracellular TREM-2 in comparison to its surface levels. Though marginal, we observe a trend of a dose dependent decrease in surface TREM-

2. Importantly and much more pronounced, an equally dose dependent increase in intracellular TREM-2 fluorescence was detected, allowing us to quantify the ratio of surface vs. intracellular signal. This ratio increases with rising amounts of LPS when normalized to that of the unstimulated control, indicating a shift of TREM-2 towards the intracellular. Though this result is preliminary and cannot prove receptor internalization, it dissents the possibility of receptor cleavage in which both signals would have to decrease similarly in this case and favors a model were TREM-2 gets internalized upon LPS encounter. Only a few studies have so far investigated the faith of the TREM-2 protein upon cellular stimulation. Previous observations showed a shift toward the surface [124, 125], thereby contradicting our results. However, these results were obtained in other celltypes, namely N9 microglia [125] and used ionomycin for cell activation in the major part of their experiments. The increase of intracellular Calcium caused by ionomycin has been attributed to perforation of the membrane and is shown to induce apoptosis or necrosis in a range of cell types [141, 142]. Thus, only taking into account the contrasting observation for microglial cells, we propose the differential view that TREM-2 receptor dynamics upon LPS stimulation causes internalization in BMDMs, as opposed to a surface shift in microglia [125]. Our results are also in line with the similar deviation reported for TREM-2 RNA expression levels, showing LPS dependent abrogation in macrophages [9] and up-regulation in microglia [12]. A possible explanation might be that TREM-2 inhibition needs to be stopped to effectively counter a starting bacterial infection, i.e. when an acute inflammatory response is needed, while its continuing expression is required for containment of chronic inflammation.

Overall, the extent of LPS induced TREM-2 disappearance observed in RAW 264.7 cells is unequaled in BMDMs, which suggests cellular differences as well. Interestingly this ties in with western blot results (Fig. 8), showing that the ratio between the bigger and the smaller TREM-2 product substantially differs between RAW cells and BMDMs. BMDMs contain

much more of the smaller product, which does not react to LPS as pronouncedly as the bigger kDa Product (Fig. 8 and 9).

We further examined whether LPS induced degradation of TREM-2 was mediated via the proteasome. The results of this experiment shown in Fig. 12 imply that this is not the case, as subsequent reduction of TREM-2 protein levels still occurs upon proteasomal inhibition. However, inhibition of the proteasome for one hour prior to stimulation leads to accumulation of TREM-2 at baseline, implying that TREM-2 expression on protein level is regulated by the proteasome under resting conditions and suggests a rapid protein turnover. Proteasomal inhibition also allowed the detection of a band around 25kDa that accounts for the native protein, indicating that native TREM-2 is either immediately modified in the ER or rapidly degraded. In line with previously mentioned RNA expression data [9, 12], results from our lab also propose that the LPS induced reduction of TREM-2 is a consequence of regulation on RNA level (unpublished data). Tied with these clues of rapid protein turnover in general, it could explain how RNA level expression control can lead to such a quick reduction of mature protein upon LPS stimulation as observed in previous results.

The appearance of TREM-2 in multiple bands suggesting post-translational modification, became the subject of further investigation. Currently, there are few concise reports on the post-translational modification of TREM-2 [13, 122], nor are there any studies showing where it gets modified on the way to its designated location. Applying a common glycosidase digest, we show that the larger TREM-2 bands around 37kDa account for a pool of TREM-2 which has been processed in the Golgi, as these products were cleaved only by PNGase but resistant to EndoH (Fig. 13). Interestingly, the smaller kDa product around roughly 30kDa was cleaved by both enzymes, which is a clear indication that the additional mass stems from glycosylations innate to the endoplasmatic reticulum and shows that a substantial pool of TREM-2 is not fully processed through the Golgi. Given that the ER and the further

glycosylated TREM-2 protein pool always appears in two sets of double bands, we are left with the proposition that each set of these is derived from a different type of glycosylation descending from either ER in the case of lower mass bands, or Golgi-processed in the case of the high mass bands. Importantly, two cleavage products remain upon full removal of sugars, both with sizes in the vicinity of the proposed endogenous mass of TREM-2 around 25kDa. There are faint but visible correspondences to these in the control group, each of which is enriched by the enzymatic reactions, indicating that there might be TREM-2 pools which are subject to alternative splicing or another type of posttranslational modification other than glycosylation. Comparing TREM-2 expression patterns of RAW cells and BMDMs from fig. 5 in the light of this new knowledge, we can conclude that BMDMs express a lot more of the ER processed TREM-2 variant while Golgi-processed levels remain largely equal in both cell types.

Having established that, next to a not further glycosylated ER pool, a substantial proportion of TREM-2 is processed through the Golgi, we continued to examine whether it is also stored in the Golgi under unstimulated conditions in macrophages using confocal microscopy. In doing so, we could show that TREM-2 clearly co-localizes with the Golgi (Fig. 14) in resting macrophages. The initial determination of these instances is the result of an explicitly conservative assessment, made for reasons outlined in supplementary section 12.2. Briefly described, certain structures contained in BMDMs emit substantial autofluorescence in all channels. To pinpoint co-localization of signal derived from two specific antibody stainings, we had to discriminate against autofluorescent structures. This was achieved by deconvolution of 3D images (Fig.15), thereby excluding false-positive colocalization signals often produced in 2D pictures, due to chromatic and spherical aberration innate to microscopic systems [137]. Furthermore, deconvolution allows additional discrimination against autofluorescent structures. While we observe complete colocalization in some cases

(Fig. 15A and 15C), we find TREM-2 localized to some parts of the Golgi while others were free of signal (15B). Given the design of the Golgi apparatus, examples of this must be expected considering the cisternal process of protein maturation. Importantly, we exemplify that an irrelevant amount of colocalized signal is a consequence of autofluorescence, as only a small number of peripheral peaks show additional alignment with the green channel (red arrows). This confirms the establishment of a robust and precise method for the localization of TREM-2 in BMDMs.

To provide a more demonstrative account of TREM-2 distribution and colocalization with the Golgi, we proceeded with 3D object reconstruction of all high intensity structures in a randomly chosen cell.

Fig. 16B obviates colocalization between TREM-2 and the Golgi as both signals make up the largest overlapping structure. At this point, we can only speculate toward the nature of the remaining, cytoplasmic TREM-2 objects. Though the expected localization at the surface of BMDMs is exemplified by some cells in fig. 14, it is possible that their emittance was discarded during reconstruction of high intensity signal. For a meaningful readout, low fluorescence sourcing from a few molecules must be discarded, pointing toward a non-centralized surface distribution of TREM-2. As a consequence, we are looking at objects containing significant concentrations of TREM-2 protein. Previous observations on the matter report that intracellular TREM-2 does not colocalize with markers of endo- and lysosomes [125] and suggest its presence in otherwise unidentified exocytic vesicles. Our data confirms the existence of further intracellular structures containing TREM-2 without allowing claims toward their specific functional nature.

8. Conclusion and outlook

Our data shows that TREM-2 over expression leads to a significant reduction in Akt and ERK phosphorylation upon LPS stimulation of RAW 264.7 macrophages, without directly influencing the phosphorylation of I κ B α . Experiments using pharmacological inhibitors of these pathways will be required to prove that this is the way in which TREM-2 mediates its impact on cytokine secretion.

It is further shown that TREM-2 protein level is quickly down regulated upon LPS encounter. Although these data are preliminary, we show evidence that this does not happen via proteasomal degradation and further studies will be required to test for other mechanisms, like transcriptional regulation or receptor cleavage. Further experiments revealed that decrease of surface TREM-2 is consistent in RAW 264.7 cells and BMDMs, while latter results additionally point toward receptor internalization. We are also able to conclude that murine TREM-2 appears in two groups of post-translationally modified variants, determining that one group is the result of N-glycosylation in the ER, while the second group results from further glyco-processing in the Golgi. Two variants still remain after cleavage of sugar groups and the nature of this additional difference has yet to be determined. Latter confocal experiments detailed that TREM-2 is localized in the Golgi apparatus of resting macrophages and present in additional cytosolic structures. The establishment of a solid method for TREM-2 localization in murine BMDMs provides an opportunity to further study the nature of these yet unidentified compartments and could be used to study changes in TREM-2 localization upon stimulation in the future.

9. Abbreviations

AP-1	Activator protein 1
BMDM	Bone marrow derived macrophage
CNS	Central nervous system
CWFS-Gelatin	Cold water fish scale gelatin
CSF	Cerebral spinal fluid
DAMP	Danger associated molecular pattern
DAP12	DNAX activation protein of 12kDa
DC	Dendritic cell
E. Coli	Escherichia Coli
EndOH	Endoglycosidase H
ER	Endoplasmatic reticulum
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
GFP	Green fluorescent protein
HSC	Hematopoetic stem cell
I κ B α	inhibitor of kappa light chain gene enhancer in B cells
IL	Interleukin
IRF	Interferon regulatory factor
ITAM	Tyrosine-based activating motif
ITIM	Tyrosine-based inhibiting motif
JNK	c-Jun N-terminal kinases
KO	Knockout
LBP	LPS-binding protein
LPS	lipopolysaccharide
MAPK	Mitogen activated protein kinases
MG132	Proteasome inhibitor
NF- κ B	Nuclear factor kabba B
NHD	Nasu-Hakola disease
NLR	NOD-like receptor
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PNGase	Peptide -N-Glycosidase
PRR	Pathogen recognition receptor
PSF	Point spread function
TLR	Toll-like receptor
TLT	TREM-like transcript
TNF	Tumor necrosis facto
TREM	Triggering receptor expressed on myeloid cells
WT	Wild type

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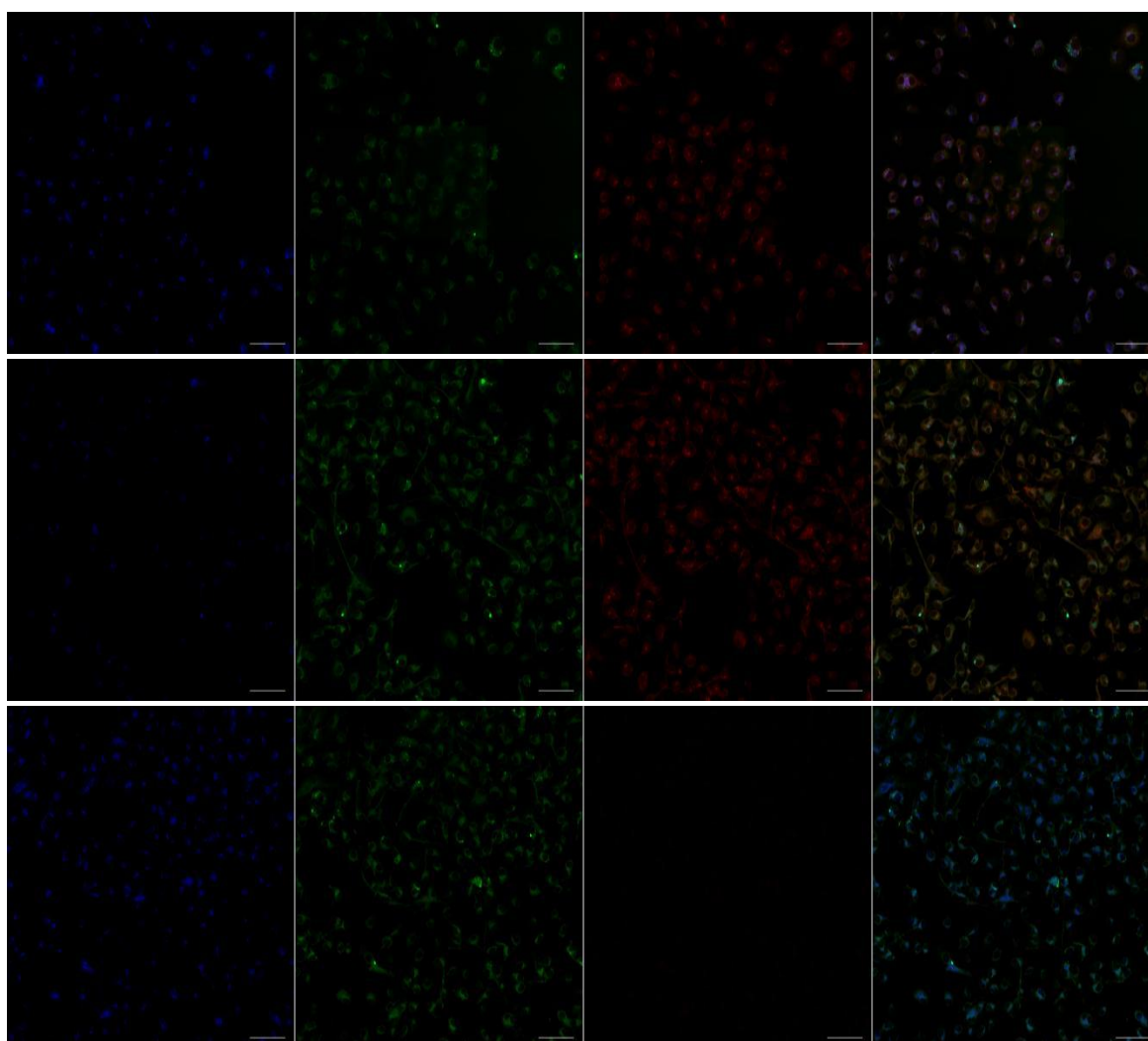
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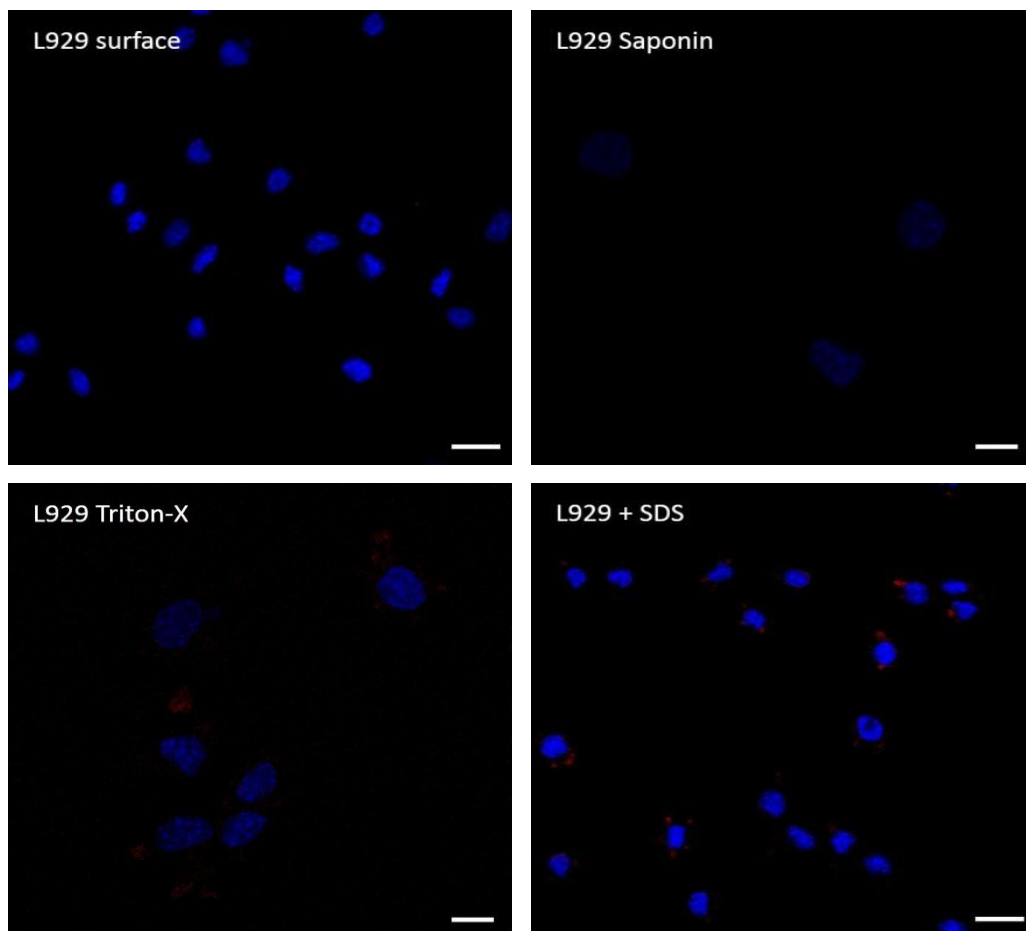
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11. Appendix

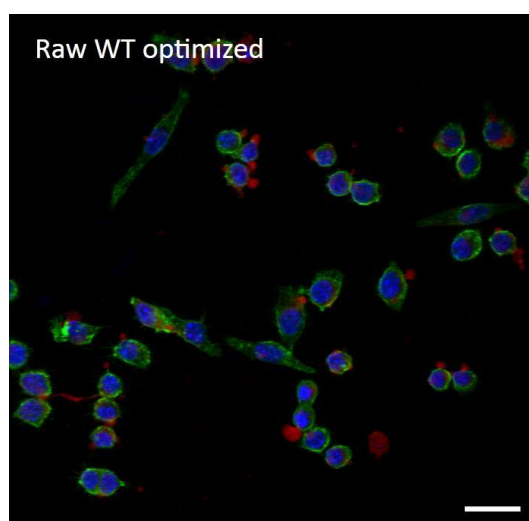
11.1. Supplementary figures



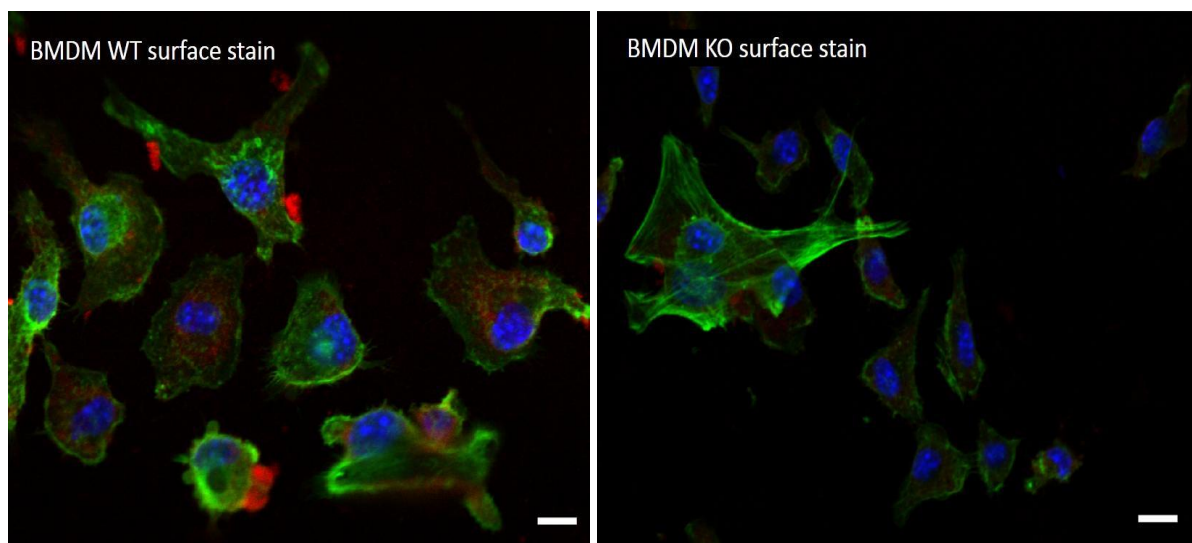
Supplementary Figure 1 shows 3x3 tile scans of TREM-2 and golgin-97 stained sample slides (A) along with controls for TREM-2 only (B) and golgin-97 only (C). Intensities in the blue channel are digitally amplified for better vision, all other channels are unchanged. Notice that in (B), blue signal is perfectly co-localized with high intensity green autofluorescence. Bar: 50 μ m



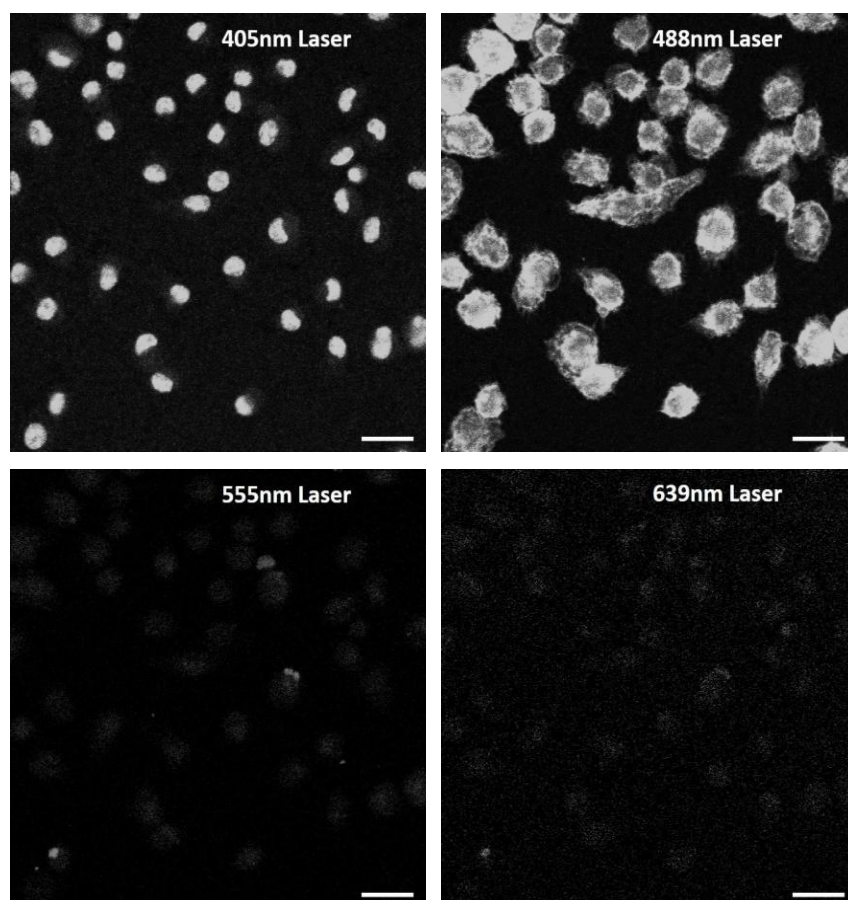
Supplementary Figure 2 shows confocal images of L929 fibroblasts permeabilized with respective agents. Bar: 10 μ m for surface/SDS, others 20 μ m. Stained for DNA (blue), TREM-2 (red) and actin (green, removed for better vision of other channels). Used as a negative control, L929 cells should not show signal in the red channel.



Supplementary Figure 3 shows permeabilized RAW 264.7 cells stained for DNA (blue), TREM-2 (red) and actin (green). Intensity of red channel digitally enhanced. Bar: 10 μ m



Supplementary Figure 4 shows a stain of both wild-type (left) and knockout (right) BMDMs without permeabilization. Channels stained for DNA (blue), TREM-2 (red) and actin (green). Bar: 10 μ m



Supplementary Figure 6 shows images of BMDMs stained with DAPI and excited with the given laser at 2% power and recorded without filter settings. Bar: 10 μ m

11.2. Developing a novel approach for object colocalization in BMDMs

There were barely a handful of commercial antibodies against murine TREM-2 available for purchase, none of which are sold specifically for application in immunofluorescence microscopy (IF) at the time experiments were conducted. While this is probably the reason why there have been equally few publications with confocal work on TREM-2, the basic consequence is that we had to start from scratch with no reliable sources on optimal conditions.

Testing out several antibody concentrations ranging from 1-50µg/ml (data not shown), we found that a final working dilution of 5µg/ml was optimal for the R&D Systems antibody. It was applied to all images depicting TREM-2 in this chapter.

Initial testing of classical reagents found PFA (3,7%) to present the only rational choice for a fixative, surpassing methanol in all properties relevant to this experiment. Blocking with a combination of normal mouse IgG (1:30) in 5% goat serum + 1%BSA in PBS was found suitable at this stage, though it was adapted later to suit the optimization of secondary antibodies, used for subsequent colocalization studies. The final protocols use 0,5% cold water fish scale gelatin since it contains no potentially cross-reactive sugar groups and is prepared from species completely unrelated to the ones used for production of the various antibodies applied.

Several reagents were tested for permeabilization. While very early results with RAW 264.7 cells show suspiciously intense intracellular signal with the use of stronger permeabilization agents, the finding that L929 are suitable as a TREM-2 negative control soon attributed this signal increase to the detergic properties of the applied substance. Supplementary Fig. 2 shows how the signal in L929 fibroblasts increases from none in saponin-permeabilized samples to a rising intensity when triton-X and SDS were applied, respectively. Thus the signal obtained with these membrane-permeabilizing substances is likely based on their

otherwise denaturing effects on protein (and was considered as unspecific, which can be also seen in westernblots, showing unspecific bands in L929 fibroblasts and TREM-2 deficient BMDMs under denaturing conditions). The binding affinity to this cross-reactive protein seems minimal in the natural state, since we did not observe any signal using unpermeabilized cells or cells treated with saponin. Having thus identified saponin as the only practicable permeabilization agent for this antibody, the sum of gathered experimental input resulted in an optimization of our TREM-2 stain in RAW 264.7 cells. An example of the outcome is shown in Supp. Fig. 3.

This also included several minor modifications to the staining protocol. To abolish the reactive potential of the remaining oxygen residues which might crosslink to NH_3 - groups of an applied antibody, glycine was added to samples after PFA fixation. Instead of using solutions prepared from pulverized saponin, which is a heterogeneous powder prepared from biological sources containing several saponin derivatives, we decided to apply a commercial saponin-based permeabilization solution originally designed for FACS to obtain cleaner results. Furthermore, some of the washing steps were adapted for the final protocol. After each antibody incubation, the samples were washed with PBS containing 0.7M instead of the regular 0.2M NaCl to wash out more unspecific interactions, while specific antibody binding is uninfluenced by salt molarities below 1M. For final colocalization studies, we also exchanged the initially used biotinylated TREM-2 antibody by an unconjugated, goat derived version that enables signal amplification. Finally, the samples were rinsed with milliq- H_2O following the last washing step. This is to ensure that the contents of PBS do not crystallize during the drying process. Crystals scatter the microscopes lasers and skew the resulting images. This sum of adaptations resulted in a notable reduction of unspecific binding and led to an increase in overall image quality.

However, to sufficiently differentiate intracellular objects to an extent necessary for colocalization studies, we decided to use BMDMs instead of the smaller RAW cells. The combination of a small cytoplasm and the maximum resolution of 200nm achieved by a light microscope was not optimal for a meaningful readout on colocalization. Initial experiments were performed on RAW 264.7 cells because they are easy and quick to culture and do not require the scarification of mice. Subsequent experiments are performed with BMDMs obtained from both WT and TREM-2 deficient mice as described in methods. Initial results obtained with BMDMs following the thus far optimized staining show the quite promising results depicted in Supp. Fig. 4.

After scanning innate BMDM autofluorescence as shown in Supp. Fig. 6, it became clear that this was going to become an obstacle in further colocalization studies. To rule out any reagents used in the staining procedure as a source, autofluorescence of unstained cells was compared after each incubation step showing no difference in any of the channels (not shown).

Considering the strong innate autofluorescence of macrophages and the solid but comparably weak TREM-2 signal, we had to change the imaging approach entirely. Auto-fluorescence showed to be lowest in the range of 400-450nm, which is why we switched to a goat derived anti-rabbit DyLight 405 conjugated secondary antibody for organelle detection.

Since our microscope is limited in the low-fluorescent far red, with a maximum high-pass filter at 700nm, the choice for the TREM-2 secondary antibody with its 615nm emission maximum was still adequate for this experiment. An important information gathered from studying BMDM autofluorescence is that high intensity emitting structures yield signal in all images wavelengths, peaking somewhere in the green light range.

Exclusion of any unspecific signal in the stained channels by overlaying it with an additional image of the unstained green channel, allows to exclude false positive results caused by the

fact that self emitting signal is highly co-localized. What we expect to image from these samples is a largely background free organelle signal in the UV range, a green channel containing exclusively autofluorescent signal and a red channel showing a combination of both TREM-2 and autofluorescence. By then negating any UV or red signal that has a clear correspondance in the green channel, we are left with the only information relevant to our readout: Positioning of respective organelles and signal emitted from our antibodies. The results of these experiments are detailed in section 5.5.

11.3. Supplementary Methods

11.3.1. Pierce-BCA protein assay

Equipment:

Pierce BCA Kit, Thermo Scientific #23225

RIPA buffer

96 well plate flat bottom

1,5ml Eppendorf tubes

Pipettes and tips

ELISA Reader

Samples in RIPA buffer

Procedure:

Defrost samples at room temperature, prepare standards as follows:

Standard	RIPA [μ l]	BSA [μ l]	Final BSA conc. [μ g/ml]
Blank	100	-	0
S1		100 of Stock*	2000
S2	100	100 of Stock*	1000
S3	100	60 of Stock*	750
S4	100	100 of S2	500
S5	100	100 of S4	250
S6	100	100 of S5	125
S7	400	100 of S6	25

* prepare fresh

Prepare 200 μ l working reagent (WR) per sample: Mix BCA Reagent A and B in the ratio 50:1

Pipet 200 μ l WR/well and add 25 μ l Standard or sample

Cover plate and incubate at 37°C for 20min

Cool plate to room temperature before measuring on ELISA Reader (A562)

11.3.2. Endoglycosidase reactions

EndoH

- Combine 1-20 µg of glycoprotein, 1 µg of 10X Glycoprotein Denaturing Buffer and H₂O (if necessary) to make a 10 µl total reaction volume.
- Denature glycoprotein by heating reaction at 100°C for 10 minutes.
- Make a total reaction volume of 20 µl by adding 2 µl of 10X G5 Reaction Buffer, H₂O and 1-5 µl Endo H.
- Incubate reaction at 37°C for 1 hour.

PNGase

- Combine 1-20 µg of glycoprotein, 1 µl of 10X Glycoprotein Denaturing Buffer and H₂O (if necessary) to make a 10 µl total reaction volume.
- Denature glycoprotein by heating reaction at 100°C for 10 minutes.
- Make a total reaction volume of 20 µl by adding 2 µl of 10X G7 Reaction Buffer, 2 µl of 10% NP-40, H₂O and 1-2 µl PNGase F.
- Incubate reaction at 37°C for 1 hour.

12. Curriculum Vitae

Demographic Data

Date of birth: 04.01.1986

Nationality: Austria, Norway

Home address: Rechte Wienzeile 71/1/15, 1050 Wien

Education

1992 – 1995	Primary school Volksschule Wienerwald-Sittendorf, Austria
1995 – 1996	Primary school Ridabu Barneskole Hamar, Norway
1996 – 1999	Gymnasium Anton-Kriegergasse, Austria
1999 – 2001	Ringshaugh Ungdomskole Tønsberg, Norway
2001 – 2004	Skagerak International School, Norway
2004	Final exam and graduation with International Baccalaureate degree
2004 – 2005	Academic study of chemistry at the University of Vienna
2005 – date	Academic study of molecular biology at the University of Vienna
Nov. 2013	Expected graduation date, awarding of academic title Mag. rer. nat.

Practice

July 2010	Internship at the Laboratory for Immunohistochemistry, Department of Pathology, Rikshospitalet, Norway
Oct – Dec 2010	Advanced techniques in Biochemistry. Teige group, Department of Biochemistry, Max F. Perutz Laboratories, University of Vienna
April – Aug 2011	Advanced techniques in cell biology and molecular medicine. Weitzer group, Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna
2012 – date	Diploma student, Knapp group: Laboratory of Infection Biology at the Medical University of Vienna and , CeMM, Center for Molecular Medicine of the Austrian Academy of Sciences

Publications

- | | |
|-----------|--|
| July 2010 | Simon Stael, Agostinho G. Rocha, Terje Wimberger , Dorothea Anrather, Ute C. Vothknecht and Markus Teige. <i>Cross-talk between calcium signalling and protein phosphorylation at the thylakoid</i> . J Exp Bot, 2012. 63(4): p. 1725-33. |
| Sept 2013 | Riem Gawish, Rui Martins, Terje Wimberger , Omar Sharif, Karin Lakovits, Bianca Doninger, Mariane Schmidt and Sylvia Knapp. <i>TREM-2 fine tunes inflammatory responses and enhances in vivo phagocytosis of bacteria during Gram-negative sepsis</i> . J Leuk Biol submitted |