From the Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

TRAPC
A NOVEL TRIGGERING RECEPTOR EXPRESSED ON ANTIGEN PRESENTING CELLS

Rutger van der Holst

Karolinska Institutet

Stockholm 2007

Published by Karolinska Institutet. Printed by LarsErics Digital Print, Sundbyberg.

ISBN 978-91-7357-427-3

1 Abstract

The triggering receptor expressed on myeloid cells (TREM) family of receptors plays an important role in innate immunity. They have been described to regulate the course of sepsis, DC maturation, bone modulation and microglial function, and as markers for bacterial infections. We describe a novel activating murine TREM family receptor that is expressed in cells of both myeloid and lymphoid origin, including DCs and monocytes/macrophages and B-cells, but not in T- or NK cells. The molecule was designated triggering receptor expressed in antigen presenting cells (TRAPC). TRAPC belongs to the immunoglobulin superfamily, and associates with the adaptor-signaling molecule DAP12. TRAPC displays the highest identity to human TLT-4 and NKp44. Treating the macrophage cell lines J774.1 and RAW 264.7 with LPS led to increased expression of TRAPC. Cross-linking of TRAPC on macrophages and DCs induced nitric oxide production in both cell types, and up-regulated expression of CD40 on the DCs. Taken together, these results show that TRAPC is an activating receptor that may be involved in regulating immune responses, e.g. during bacterial infections.

In order to investigate if TRAPC indeed plays a regulatory role during bacterial infections we have analyzed the role of TRAPC for its ability to regulate macrophage responses to infections with Gram-negative bacteria and lipopolysaccharide (LPS). Cross-linking of TRAPC on murine macrophage-like J774-A.1 cells resulted in an increase in the expression of iNOS and production of nitric oxide (NO), and in degradation of IκB-α. A more pronounced NO production was achieved when exposing J774-A.1 cells to LPS, or by infecting the cells with *Escherichia coli* or *S. enterica* serovar Typhimurium.

Surprisingly, cross-linking of TRAPC on J774-A.1 cells prior to infection with *E. coli* or *S. enterica* serovar Typhimurium or exposure to LPS reduced the amounts of iNOS and production of NO. For *S. enterica* serovar Typhimurium, the reduced NO levels were accompanied by a substantial increase in intracellular bacterial replication. These results imply a regulatory role for TRAPC during innate immune responses to bacterial infections, acting on the NF-κB signaling pathway.

To gain further insight in the function of TRAPC in immune responses we set out to identify the ligand for TRAPC. Using a soluble TRAPC Fc-fusion protein and flow cytometry, a potential ligand was detected on B cells, macrophages and DCs, i.e. on cells that also express TRAPC. In a first step to identify the ligand, cells positive for the potential ligand were lysed and immunoprecipitated with the TRAPC-Fc fusion protein. After separation in a denaturing polyacrylamide gel, a band potentially containing the ligand was detected. The identification of the ligand for TRAPC is crucial in understanding the function of TRAPC in innate immune responses.

Given the pronounced effect of TRAPC on the response of macrophages to bacterial infections, it is tempting to speculate that bacteria have evolved to use TRAPC and other DAP12 associated receptors to their advantage. In this scenario, by inducing DAP12 mediated inhibition of antibacterial macrophage responses, bacteria would promote their own survival. It remains to be investigated what mechanism(s) underlies the TRAPC-mediated regulation of innate immune effector functions.

2 Contents

3 List of abbreviations

APC	antigen-presenting cell
ATCC	American type cell culture
bp	base pair (only with numbers)
CCL	CC chemokine ligand
CCR	CC chemokine receptor
cDNA	complementary DNA
CD	cluster of differentiation
CDR	complementarity determining region
CFU	colony-forming unit
CNS	central nervous system
CpG	cytosine guanine dinucleotide
CSF	colony-stimulating factor
CTLA	cytolytic T lymphocyte-associated antigen
DAP12	DNAX activation protein of 12 kDa
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorter
Fc	fragment crystallizable
FcR	Fc receptors (e.g. FcεRIγ)
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram (only with numbers)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage CSF
GRB2	growth factor receptor bound protein 2
gp	glycoprotein (e.g. gp100)
h	hour (only with numbers)
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
IFN	interferon (e.g. IFNγ)
Ig	immunoglobulin
IκB	inhibitory NF-κB
IL	interleukin (e.g. IL-2)
iNOS	inducible nitric oxide synthase
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JAK or Jak	Janus kinase
JNK	c-Jun N-terminal kinase
kb	kilobase (only with numbers)

kbp	kilobase pair (only with numbers)
kDa	kilodalton (only with numbers)
LFA	leukocyte (lymphocyte) function-associated Ag
LPS	lipopolysaccharide
LTA	lipoteichoic acid
2-ME	2-mercaptoethanol
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage CSF
mg	milligram (only with numbers)
MHC	major histocompatibility complex
min	minute (only with numbers)
MIP	macrophage-inflammatory protein
ml	milliliter (only with numbers)
mRNA	messenger RNA
μg	microgram (only with numbers)
μl	microliter (only with numbers)
MyD88	myeloid differentiating factor 88
NCR	natural cytotoxicity receptor
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK cell	natural killer cell
NO	nitric oxide
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI3K	phosphatidylinositol 3-kinase
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SHIP	src homology 2-containing inositol 5' phosphatase
STAT	signal transducer and activator of transcription
Th cell	T helper cell
TLR	Toll-like receptor
TLT	TREM like transcript
TNF	tumor necrosis factor
TRAPC	triggering receptor expressed on antigen presenting cells
TREM	triggering receptor expressed on myeloid cells
Tris	tris(hydroxymethyl)aminomethane
VCAM	vascular cell adhesion molecule
Zap70	ζ-associated protein 70 (or ζ-chain-associated protein 70)

4 Preface

When I started my PhD back in 2002 I did not know what kind of a rollercoaster ride this adventure would become. Just like a rollercoaster, research has its ups and downs. Some experiments are going like a perfect free fall and nothing can stop them, but then for some reason, experiments go slow and not the way you want them to. The ever-present process of problem solving is at times highly exhilarating, but at other times very frustrating. Research in biology almost never turns out the way you expect and just like a rollercoaster it swings you from one direction to the other. But just like this process can be very frustrating, this process is the beauty of conducting research. The anticipation of your latest results, the ideas you get when you read the literature and the decisions you take to advance in your research are always very exciting and will lead you somewhere in the end. And, to come back to the rollercoaster, when you get out of your carriage at the end of the line, you look back, reflect and write down your final results in the form of a thesis. You walk away from this attraction and let new people get in, that, just like you, are in for a bumpy ride.

Like this, back in 2002 we started off in a Natural Killer cell laboratory with the following knowledge and ideas, which were soon to be changed resulting in a thesis where NK cells are mentioned only a few times…

5 Introduction

5.1 The Immune System

The immune system is an intricately evolved surveillance system that protects the body from invading pathogens and from cells of the body that undergo a dangerous transformation resulting in tumor formation. The overall immune system can be divided into two major protective arms, the innate and adaptive immunity, that provide us with a vast array of mechanisms to clear infections and tumor cells.

5.1.1 Innate immunity

The first line of defence is the innate immune system. This system first of all consists of skin, mucosal membranes, pH and temperature. These anatomical and physiological barriers make it harder for bacteria, viruses, fungi and parasites to enter the body and many die trying to get through these barriers. If these pathogens do manage to cross these barriers they have to face various molecules and effector cells of the innate immune system. Cytokines, chemokines, defensins, lysozymes and the complement system are circulating the body to attack pathogens and recruit the effector cells of the innate immune system to the site of infection. Neutrophils, monocytes, macrophages and NK cells are the main effector cells of the innate immune system. These cells react fast, i.e. within hours, upon entry of the pathogens.

The innate immune system has to discriminate between the large number of pathogens and self-structures, in order not to damage the body itself. The effector cells of the innate immune system are therefore equipped with a wide variety of receptors that can directly recognize pathogens by means of shared pathogen associated molecular patterns (PAMPs), which are not found in higher eukaryotes. The recognition of these PAMPs is independent of previous antigen encounter and is therefore immediate. Upon recognition, neutrophils, monocytes and macrophages phagocytose and kill pathogens by several complex processes that include the production of highly cytotoxic reactive oxygen species and intermediates at the site of microbial persistence in the phagosomal membranes. In addition, they start secreting many different cytokines and chemokines, which activate and attract more effector cells, initiating an inflammatory response.

This inflammatory response activates two specialized cell types of the immune system, namely macrophages and DCs. These two cells therefore act as the main “sensors” of the innate immune system, with the capacity to activate other parts of the innate system as well as the adaptive system. Even though these cells are capable of phagocytosing and killing pathogens, their second and potentially main specialized job is to process the engulfed pathogens and present their antigens to the second arm of the immune system, the adaptive immune system.

DCs are the major antigen presenting cells (APCs) throughout the entire body. They are strategically located close to the anatomical and physiological barriers of the innate immune system to catch invading pathogens. Upon engulfing pathogens and receiving sufficient inflammatory signals from other innate effector cells they migrate towards the draining lymph node and present antigens of the engulfed pathogen to naïve T cells.

5.1.2 Adaptive immunity

T cells and B cells are the effector cells of the adaptive immune system. The naïve T cells that circulate through the draining lymph nodes are activated upon recognition of a specific antigen by the highly specific T cell receptor (TCR). CD8 positive T cells recognize foreign antigens (mostly from intracellular pathogens) that are presented on major histocompatibility complex (MHC) class I molecules. Upon recognition of the antigen the specific T cell starts to clonally expand and its progeny becomes cytotoxic CD8 positive T cells specialized in killing infected cells that present the same antigen as the one the naïve T cell was activated with.

CD4 positive T cells recognize foreign antigens (mostly from extracellular pathogens) that are presented on MHC class II molecules. Depending on the cytokines present in the environment these CD4 positive T cells will expand into either T helper 1 (Th1) cells or Th2 cells. These two subpopulations differ in the types of cytokines they secrete and which kind of immune response they subsequently induce. The principal function of Th1 cells is in phagocyte-mediated defence against infections, especially with intracellular microbes. The effector function of Th2 cells is in IgE- and eosinophil/mast cell-mediated immune reactions. Both subsets are able to activate B cells to clonally expand and to differentiate into antibody secreting plasma cells.

B cells encompass the humoral adaptive immune system and have the ability to express antigen specific antibodies in a soluble and membrane bound form. Antibodies recognize antigens in their native three-dimensional structure, thus every chemical structure can serve as an antigenic epitope. In addition to their antigen-binding sites, antibodies contain constant regions (Fc) responsible for activation of immune cells through binding to Fc receptors (FcR) on their cell surface. Overall, the adaptive immune response takes more time to fully develop than the innate immune response. Furthermore, the adaptive immune response has the capacity to generate immunological memory. Some cells of the adaptive immune system will develop into memory cells in the inflammatory response. This ensures a more rapid clearance of the pathogen in a recurrent infection.

A characteristic of the adaptive immunity is that it has the ability to recognize a vast array of distinct antigenic specificities and has tolerance towards self-antigens. This is ensured during the development of T and B lymphocytes where they rearrange their receptors to generate the maximum antigenic specificity and undergo a heavy positive and negative selection process so that only lymphocytes that do not recognize self survive and start circulating the body.

In summary, to emerge in good health from an infection, a person needs an effective innate immune response (non clonally distributed leukocytes that react rapidly to pathogens without regard to antigenic specificity), as well as an adaptive immune response (antigen specific cells that are expanded clonally) that has been properly charged by the innate immune response.

5.2 Receptors of the innate immune system

As described above, the innate immune system uses various types of receptors that recognize pathogen associated molecular patterns (PAMPs) [1]. These receptors fall under the common demoninator of pattern recognition receptors (PRRs). G-linked 7 TM domain receptors are such PRRs that trigger inflammatory responses upon recognition of bacterial peptides, lipid mediators, complement factors or pro-inflammatory cytokines [2]. Another group of PRRs are the mannose and scavenger receptors that bind microbes and foreign particles either directly or through the mannose binding lectin that ultimately leads to phagocytosis [2, 3]. A rather different kind of pattern recognition is carried out by Fc receptors. These receptors recognize the constant part of antibodies that have attached specifically to microbes or foreign particles [4]. This coating of microbes and foreign particles is called opsonization. This opsonization is not only performed by antibodies but also by components of the complement system. C3b, for example, can dock to the surface of microbes and mediates the pagocytosis of the microbe via subsequent binding to complement receptors [4, 5].

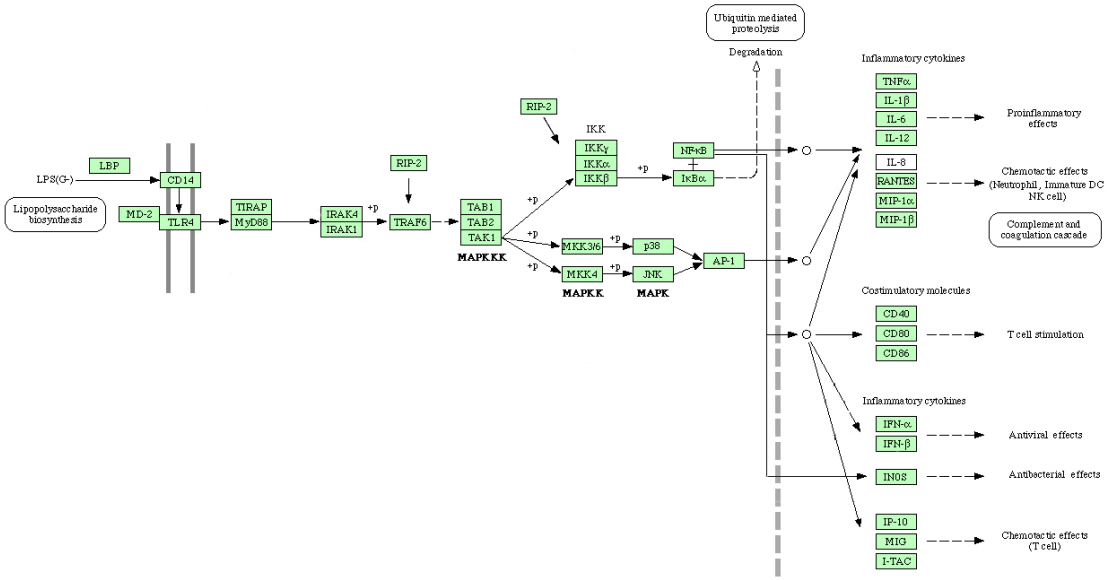


Figure 1. **TLR 4 signaling pathway and functional outcome in macrophages.** Upon binding of LPS together with LPS binding protein (LBP) to the TLR4-CD14-MD2 complex, MyD88 and TIRAP become activated. This protein complex recruits IRAK1 and IRAK4, which upon phosphorylation activate TRAF6. Rip2 is a crucial protein in this step for full activation of the macrophage. TRAF6 activates the MAP kinase pathway going from MAPKKK to MAPKK to MAPK, ultimately leading to p38 and JNK activation that drive the transcription factor AP-1. In parallel TRAF6 activates the degradation of IκBα, causing the activation of NF-κB and its subsequent translocation into the nucleus of the cell where both AP-1 and NF-κB start the transcription of various genes. These genes include genes for pro-inflammatory cytokines and chemotactic cytokines. They also induces the up-regulation of co-stimulatory molecules and the expression of iNOS. (figure modified from www.kegg.com)

5.2.1 Toll like receptors

The family of TLRs (TLR) plays a major role in recognizing bacterial and viral patterns [6]. Toll was originally identified as an essential protein during the embryonic development of Drosophila and was shown to be a key mediator during anti fungal immunity in flies [7-9]. 11 different TLRs have been identified in human, and 13 in mice. TLRs interact with different combinations of adaptor proteins and activate various transcription factors such as NF-κB, activating protein-1 (AP-1) and interferon regulating factors (IRFs). TLR signaling mainly results in transcription of inflammatory cytokines and type I interferons [10, 11].

TLR 4 is the best characterized family member [12]. TLR4 recognizes endotoxin (lipopolysaccharide, LPS), which is an integral component of the outer membranes of Gram-negative bacteria and a prototypic inducer of the bacterial inflammatory response. LPS is recognized by TLR-4 in a complex with the LPS binding protein and CD14. In addition, an accessory molecule, MD2, is required for LPS binding [13].

The intracellular domain of TLR4, and many other TLRs, is termed the TIR domain because it is homologous to the intracellular domain of the IL-1 receptor. The TIR domain supports interaction with the adaptor protein MyD88 [14]. This adaptor protein recruits the IL-1R associated protein kinase 1 (IRAK-1) to the receptor upon TLR4 stimulation with LPS.

5.2.2 TLR signaling

The subsequent phosphorylation of IRAK-1 results in dissociation from the corresponding receptor and association with TNF-R activated factor 6 (TRAF-6), which ultimately leads to activation of $IKK^{\alpha/\beta/\gamma}$ and the mitogen activated protein kinases p38/SAPK and JNK [15, 16]. In addition the adaptor protein RICK/Rip2/CARDIAK is required for optimal activation of phagocytes by TLR2, TLR3, and TLR4 [17]. Once the full activation signaling pathway is completed transcription of inflammatory cytokines and type I interferons is induced, resulting in an inflammatory response (Fig. 1).

In addition to the secretion of cytokines, macrophages expressing TLR4 also produce reactive oxygen intermediates such as nitric oxide. This is a consequence of the induction of transcription of inducible nitric oxide synthase (iNOS) through TLR4 activation signaling pathways. In a MyD88-dependent manner, via several activation and phosphorylation events, IKKs become activated and lead to phosphorylation and subsequently ubiquitinylation of IκBs that targets them for proteasomal degradation. This allows NF-κB, previously bound by IκBs, to translocate into the nucleus and induce iNOS gene transcription [18, 19]

iNOS induction can also be triggered via the Jak-STAT pathway that is activated via IFNγ. Jak-1 and Jak-2 that are associated with the IFNγ receptor become activated and phosphorylate STAT1. Two phosphorylated STAT1 molecules dimerize and transfer to the nucleus where they increase iNOS induction [19].

iNOS catalyzes the conversion of L-arginine to citrulline, releasing freely diffusible nitric oxide (NO) gas [20-22]. Within phagosomes, nitric oxide can combine with oxygen and generate reactive nitrogen oxide intermediates (RNOIs) such as NO_2, N_2O_3 or N_2O_4. Their microbicidal activity is crucial in killing of intracellular bacteria.

Another more recently identified family of receptors plays an important role in the innate immune system, possibly functioning as PRRs. This interesting family is the main focus of this thesis and will be discussed below according to their genomics, structure, signaling and their function in the cell types they are expressed on and in.

5.3 Triggering receptors expressed on myeloid cells – the TREM family

The majority of the cells in the innate immune system express one or more members of a family of molecules that have been termed "triggering receptors expressed on myeloid cells" (TREM receptors). They are grouped into one family based on similar gene and protein organization, and common mapping to syntenic chromosomes in man and mouse. They have been implicated as markers for bacterial infections, regulating the course of sepsis, DC maturation, bone modulation and microglial function as will be discussed in more detail later, and reviewed in [23-25].

The first receptors cloned within the TREM family molecules were human TREM-1 and TREM-2 [26]. These receptors were identified on the basis of homology to the natural cytotoxicity receptor NKp44, a receptor selectively expressed on activated NK cells [27, 28]. TREM-1 is expressed on neutrophils and monocytes/macrophages [26], while TREM-2 is expressed in monocytes/macrophages and DCs [29]. This pattern of expression led to the term TREM - Triggering Receptors Expressed on Myeloid Cells.

Soon after the discovery of these human TREM receptors, the murine homologues of TREM-1 [30] and TREM-2 [31] were identified including a third TREM receptor; murine TREM-3 that has no human counterpart [32]. In addition, several TREM-like transcripts (TLT) have been identified in human and mouse. TLT-1 and TLT-2 are the best characterized among these genes [33-37], while it is not known if the potential TLT-3 and TLT-4 genes encode functional proteins. TREM family orthologues exist in cows, pigs, chickens, skates, and teleost fish, suggesting that the TREM family of molecules is highly conserved during evolution [38-42]. More distant relatives to the TREMs are CMRF-35s and the IREM receptors in humans and the CLM receptors in mice [43-46].

The TREM family genes are encoded in a cluster on human chromosome 6p21 or mouse chromosome 17C3 [32, 35]. Their products share several features, such as an extracellular immunoglobulin like domain, a stalk, a transmembrane region (TM), and an intracellular domain of variable length. The majority of the TREM family molecules are activating receptors without any known signaling motif in the intracellular domain, but with a charged amino acid (lysine) in the TM, allowing association with the adaptor-signaling molecule DAP12 (also known as KARAP/TYROBP) [47].

DAP12 forms a disulphide-bonded homodimer, and associates with receptor chains through complementary charged transmembrane domains that form a salt bridge in the context of the hydrophobic lipid bilayer. DAP12 contains an intracellular immunoreceptor tyrosine-based activating motif (ITAM) [48, 49].

5.3.1 TREM receptor signaling

Ligation of TREM receptors and other DAP12 associated receptors leads to activation of SRC family kinases and subsequent phosphorylation of paired tyrosine residues in the consensus motif, YxxL/I-$(x)_{6-8}$-YxxL/I (x denotes any amino acid), of the ITAM of DAP12. For full activation of the following signaling cascades both tyrosines need to be phosphorylated [50]. After phosphorylation of the tyrosine residues, spleen tyrosine kinase (SYK) and ζ-chain-associated protein kinase of 70 kDa (ZAP70) are recruited to the plasma membrane, leading to activation of phosphatidylinositol 3-kinase (PI3K) [51, 52] and tyrosine phosphorylation of the scaffolding proteins; linker for activation of T cells (LAT) and non-T cell activation linker (NTAL) [53]. LAT, together with phosphatidylinositol-3,4,5-triphosphate generated by PI3K, recruits phospholipase Cγ (PLCγ) . LAT also recruits the protein complex SH2-domain-containing leukocyte protein of 76 kDa (SLP76) – VAV and growth-factor-receptor-bound protein 2 (GRB2) to the signaling complex. The recruitment of these intermediate signaling molecules leads to the activation of protein kinase B (AKT/PKB), protein kinase θ (PKCθ) and mitogen-activated protein kinases (MAPKs), the release of intracellular calcium and the rearrangement of the cytoskeleton. Ultimately the transcription factors AP-1, NF-κB and NFAT are activated leading to various effector functions, such as the secretion of cytokines, and up- or down regulation of surface molecules and the release of granules, depending on the cell type. (Fig. 2).

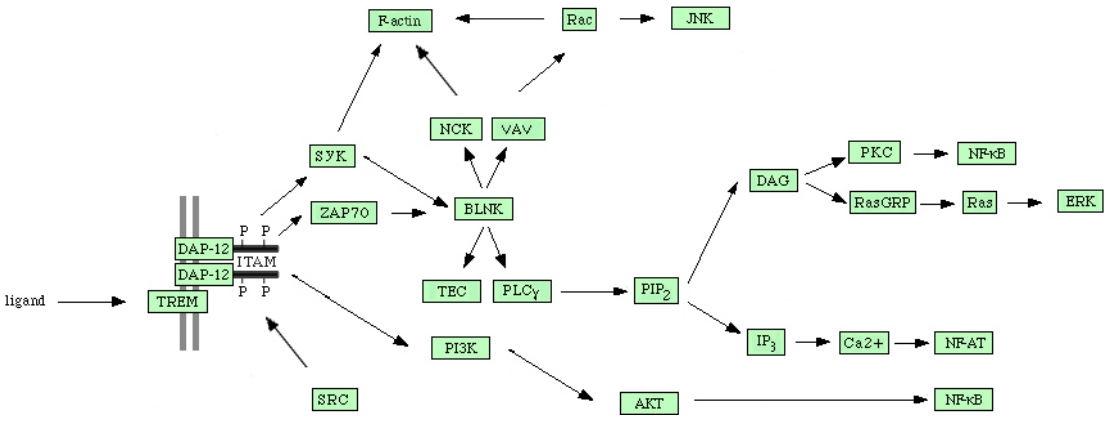


Figure 2. **ITAM signaling pathway.** TREM receptor engagement results in ITAM phosphorylation by Src, allowing recruitment and activation of Syk family protein tyrosine kinases. This event causes the subsequent phosphorylation of the BLNK family of adaptor proteins. This family is able to induce multiple signaling cascades, including activation of PI3K, PLCγ and subsequent induction of Ca^{2+} flux, NFAT translocation to the nucleus, ERK activation and PKC activation resulting in the activation of NF-κB. (figure modified and revised from www.kegg.com)

5.3.2 TREM family member structures

From a structural point of view, all of the TREM family molecules contain the typical Ig-V domain consensus sequence Asp-X-Gly-X-Tyr-X-Cys. Due to this signature the overall folds of both TREM-1 and TLT-1 resemble that of a V-type immunoglobulin domain in crystal structures [54-57]. It is conceivable that TREM-1 and TLT-1 capture their ligand with the CDR equivalent loop regions within the V-type immunoglobulin domain. To date two crystal structures of human TREM-1 exist, but the predictions made about possible dimerization of the receptor differ significantly. The first crystal structure, solved by Radaev et al., predicted that TREM-1 forms a head-to-tail dimer with 4100 $Å^2$ interface area that is partially mediated by a domain swapping between the first strands, and that the antiparallel dimeric TREM-1 most likely contains two distinct ligand-binding sites [57]. While the second crystal structure of human TREM-1, solved by Kelker et al., predicted that the extracellular ectodomain exist as a monomer in solution [56]. This has been demonstrated with the use of analytical ultracentrifugation and ^1H NMR spectroscopy and is backed by the same results for the crystal structure of murine TREM-1 [55]. It is unclear whether TREM-1 forms dimers on the cell surface and if so what implications that could have for its function on neutrophils and monocytes/macrophages.

5.3.3 TREM-like transcripts

The TREM-like transcripts, TLT-1, TLT-2 and TLT-3, differ from the TREM receptors regarding their amino acid sequences in their transmembrane domains and intracellular parts. The transmembrane domain of TLT-1 does not contain a charged amino acid precluding association with the adaptor molecule DAP12. Its intracellular part contains an immunoreceptor tyrosine-based inhibitory motif, suggesting that TLT-1 might be an inhibitory TREM-family member [34, 35]. Indeed, it has been shown that TLT-1 is able to recruit both SHP-1 and SHP-2, potent protein phosphatases that mediate inhibitory signaling [34, 36]. In addition, the intracellular part contains a potential type II polyproline Src homology 3 (SH3) domain binding site. This proline rich domain might be involved in bringing SH3 domain containing phosphoproteins into proximity in order for them to be dephosphorylated by TLT-1-bound SHP-1 [34, 35]. This inhibitory potential of TLT-1 has not been confirmed in functional assays; surprisingly an activating role has been suggested for TLT-1, which will be discussed later [36].

TLT-2, like TLT-1, does not contain a charged amino acid in its transmembrane domain and can therefore most likely not associate with DAP12. In contrast to TLT-1, TLT-2 contains a type I SH3 binding motif. Furthermore, the intracellular part of TLT-2 contains 2 tyrosines, one of which is part of a YxxV endocytosis, STAT-3 recruiting motif and one that is part of a STAT-3 recruiting motif YxxS, that might be involved in the signaling of this receptor [35].

TLT-3 does contain a charged amino acid in its transmembrane domain and might therefore associate with DAP12 in order to signal. However, through hydrophobicity plotting of the amino acid sequence of TLT-3 it is predicted that TLT-3 contains 2 transmembrane domains, a feature that is rather rare in other proteins [35].

In summary, the TREM family of receptors have been evolutionary conserved and their genes are, at least in human and mouse, encoded within a gene cluster. They are all part of the immunoglobulin-like superfamily, a characteristic not only based on the shared Ig-V type consensus motif but also confirmed by crystallography studies. The TREM receptors associate with DAP12 in order to signal while the TREM-like transcripts differ in that respect and have different signaling motifs and additional features in their intracellular amino acid sequence. These receptors are mostly expressed by cell types of myeloid origin, while some studies also report their expression on specific cell types of lymphoid origin.

The function of the TREM family of receptors will now be further discussed in relation to the cell type they are expressed on, as well as their possible role as diagnostic markers in clinical situations (Fig. 3).

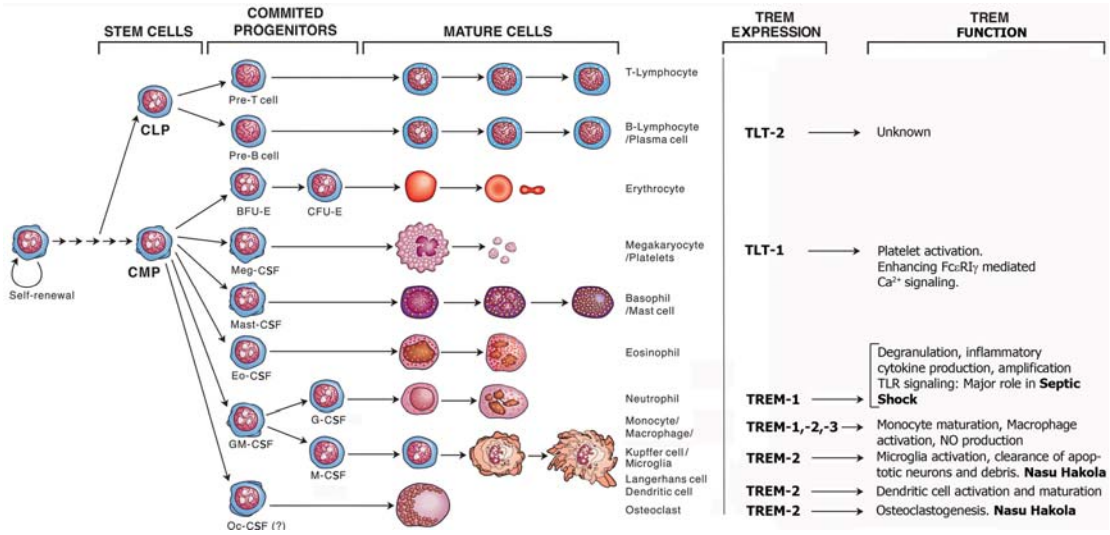


Figure 3. **Overview of the haematopoietic system, the expression of TREMs on different cell types and their function.** A pluripotent stem cell develops into lympoid precursor stem cells and myeloid precursor stem cells. From there the various cell types of the immune system develop. The TREM or TLT expression has been indicated for the cells that have been described to express these receptors. A short functional description has been given for the receptor expressed on a certain cell type, which will be discussed in more detail in the following chapters. (figure modified from www.bloodlines.stemcells.com)

5.4 TREM receptor function and expression on cells of hematopoietic origin

5.4.1 Neutrophils

Neutrophils are the most abundant type of white blood cells and form an integral part of the immune system. Their name derives from staining characteristics on hematoxylin and eosin histological preparations. Whereas basophilic cellular components stain dark blue and eosinophilic components stain bright red, neutrophilic components stain a neutral pink. These phagocytes are normally found in the blood, where they represent the most abundant type of leucocytes. However, during the acute phase of inflammation, particularly as a result of bacterial infection, neutrophils leave the vasculature and migrate toward the site of inflammation in a process called chemotaxis. Neutrophils react within an hour of insult and they are the predominant cells in pus, accounting for its whitish appearance. Neutrophils are active phagocytes, capable of ingesting microorganisms or particles. Upon activation, neutrophils start to produce inflammatory cytokines, chemokines, cytolytic granules and reactive oxygen intermediate and phagocytose infected cells.

Neutrophils express TREM-1 and upon cross-linking of TREM-1, they start to up-regulate the expression of CD26, CD11c, CD49e, CD49d, CD11b, and CD18. This may increase cellular adhesion and especially the adhesion to fibronectin (CD11b/CD18 (Mac-1)), fibrinogen (CD29/CD49d) and VCAM (CD29/CD49e) that play a role in the extravasation of neutrophils and the subsequent migration into the site of infection [26]. TREM-1 ligation causes immediate intracellular calcium mobilization and tyrosine phosphorylation of ERK and PLCγ which are important prerequisites for the activation of neutrophil effector functions like degranulation and the initiation of respiratory burst and phagocytosis in neutrophils [26, 58]. Not only are adhesion molecules up-regulated upon TREM-1 ligation, neutrophils also start to secrete IL-8, a neutrophil recruiting chemokine, MCP-1, a monocyte chemo-attractant protein, and TNFα, a potent pro-inflammatory mediator [26, 58]. TREM-1 activation also induces efficient neutrophil degranulation, measured by the release of myeloperoxidase (MPO), but not phagocytosis [58, 59].

Soon after the cloning and characterization of TREM-1 it became apparent that TREM-1 synergizes with Toll-like receptors. Especially with TLR2, TLR4, and TLR7/8 when stimulated with their respective ligands Pam_3Cys, LPS, and R-848. Functionally this results in an enhanced secretion of IL-6, IL-8, TNFα and IL-1β [30, 58]. Furthermore, the expression of TREM-1 on the surface of neutrophils is up-regulated upon stimulation with LPS, lipoteichoic acid (LTA), and extra cellular bacteria like Pseudomonas Aeruginosa and Staphylococcus Aureus, but not with intracellular bacteria like Bacillus Calmette-Guerin or with mycolic acid. TREM-1 is up-regulated on peritoneal neutrophils of patients with microbial sepsis and mice with experimental LPS induced shock [30, 60]. In contrast with these observations two studies report that TREM-1 protein levels on the cell surface of human neutrophils either stay constant [61] or are rapidly down regulated combined with a gradual increase of protein levels to baseline amounts after LPS administration in healthy human volunteers [62].

The regulation of neutrophil numbers is important to prevent an exacerbation of the immune response that could be detrimental for the host. Therefore neutrophil survival is tightly controlled. In parallel with the activation of neutrophil effector functions, many inflammatory mediators also regulate cell survival by altering apoptosis. Cytokines like GM-CSF, as well as bacterial products like LPS, delay apoptosis, while others, like TNFα, promote programmed cell death [63]. Recently it has been shown that the synergy between TLR-4 and TREM-1 induces neutrophils apoptosis, while TLR4 activation by itself promotes the survival of neutrophils [58]. This suggests a role for TREM-1 in counter-regulating inflammatory responses by promoting neutrophil apoptosis in the presence of TLR mediated activation, as opposed to the synergistic effects of TREM-1 and TLR4 on cytokine secretion.

Another interesting observation is that TREM-1 is recruited to ganglioside M1 lipid rafts in neutrophils upon stimulation with LPS or anti TREM-1. TLR-4 and TREM-1 co-localize upon stimulation [64]. These results could explain why synergy is observed between TREM-1 and TLR4. Upon cross-linking of signaling receptors, lipid rafts become larger and more stable structures often attached to the cytoskeleton, a phenomenon called coalescence. Lipid rafts serve to spatially segregate signaling components in the plasma membrane and as such regulate the initiation and prolongation of signaling resulting in an enhanced end result of the signaling cascade, e.g. cytokine secretion, as seen for TREM-1 and TLR4 [65].

Aging of the host has a clear effect on the function of neutrophils, and especially on the effect of TREM-1 engagement. Neutrophils from the elderly (65-78 years) are not able to modulate the respiratory burst after TREM-1 ligation and TREM-1 ligation cannot reverse neutrophil survival following incubation with LPS or GM-CSF when compared to neutrophils from young individuals (19-25 years). Furthermore, TREM-1 engagement is not able to drive the recruitment of TREM-1 into lipid-rafts of the elderly [66]. This could partly explain the altered response of neutrophils from the elderly. The observed alterations in TREM-1 response are possibly an important contributing factor in the higher incidence of sepsis-related deaths in the elderly population.

5.4.1.1 Sepsis and septic shock

Sepsis and septic shock, which are associated with a mortality rate of 25-80%, are the leading causes of death in most intensive care units in developed countries despite recent advances in critical care medicine. The hallmark of sepsis syndrome is multiple organ dysfunction, frequently accompanied by an uncontrollable decline in blood pressure. Septic shock is characterized by a massive release of pro-inflammatory mediators such as TNFα, IL-1β, macrophage migration inhibitory factor (MIF) and high mobility group box 1 protein (HMGB-1). These pro-inflammatory mediators significantly delay neutrophil apoptosis. However, Inflammatory cytokines, and especially TNFα, are considered to be deleterious, yet they also possess beneficial effects in sepsis as shown by the fatal issue of peritonitis in animals with impaired TNFα responses. In clinical trials the inhibition of TNFα increased mortality.

The finding that sepsis is a frequent complication in rheumatoid arthritis patients treated with TNFα antagonists has highlighted the role of TNFα in the clearance of infection [67]. A disproportionate inflammatory response to microbial products finally leads to tissue damage, haemodynamic changes, multiple organ failure and ultimately death (Fig. 4). Sepsis and septic shock differ from the systemic inflammatory response syndrome (SIRS) simply by the fact that SIRS is not associated with infection. As soon as SIRS is associated with infection, it is called sepsis. The symptoms of sepsis and SIRS are the same, but it is crucial to distinguish between the two phenomena to start with the appropriate treatment.

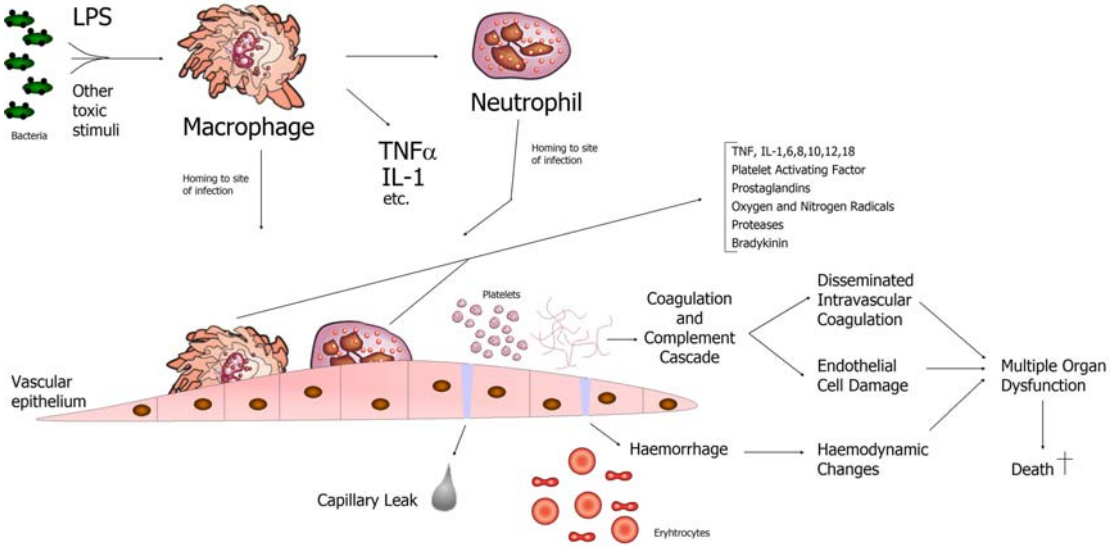


Figure 4. **Overview of septic shock induced by bacteria, LPS or other toxic stimuli.** Bacteria, LPS or other toxic stimuli activate macrophages and neutrophils that will start to secrete many pro-inflammatory cytokines, and start to home to the site of infection. They will also recruit many more inflammatory cells. Eventually these cells have to migrate from the bloodstream into the site of infection. Macrophages and neutrophils attach to the vascular epithelium and start to secrete even more cytokines. These cells can create capillary leaks that will cause the leakage of erythrocytes out of the bloodstream into the underlying tissue inducing a haemorrhage. This leakage will cause severe haemodynamic changes. Due to the damage of the vascular epithelium the coagulation and complement cascades will be activated leading to disseminated intravascular coagulation and even more endothelial cell damage. These effects together with the haemodynamic changes will cause multiple organ dysfunction, that, if left untreated, ultimately will result in death.

Bouchon et al. used their observations of cell surface TREM-1 up-regulation upon LPS stimulation and the enhanced secretion of the pro-inflammatory cytokines, IL-1β and TNFα, as a result of synergy between TREM-1 and TLR4, to investigate the role of TREM-1 in septic shock [30]. Mice that have been induced to get septic shock either by injection with LPS, caecal ligation and puncture or intraperitoneal administration of E.coli, die rapidly within the first 48 hours after the challenge. When these mice were injected with a murine TREM-1-Fc fusion protein, 1 hour before the induction of septic shock, up to 80% of the mice survived, whereas all mice in the control group died. Interestingly, from a therapeutical point of view, 40% of the mice survived septic shock after the injection of the murine TREM-1-Fc up to 4 hours after the induction of septic shock [30].

When blood samples were analysed for typical pro-inflammatory cytokines that cause septic shock, mice that had been injected with the murine TREM-1-Fc had significant lower plasma levels of IL-1β and TNFα. Furthermore the numbers of infiltrating neutrophils and macrophages in the peritoneum were decreased, while the numbers of circulating leukocytes were not affected. These results clearly demonstrate the crucial role of TREM-1 during septic shock and that TREM-1 blockade is sufficient to lower systemic levels of TNFα and Il-1β and to reduce cellular infiltrates at the site of inflammation below levels that are lethal for the host without causing leukopenia [30].

The synergy of TREM-1 with other receptors is not restricted to TLRs. TREM-1 is also capable of amplifying cytokine production (IL-6, IL-1β and TNFα) induced by the second major class of pattern recognition receptors, the NAIP, CIITA, HET-E, TP-1-leucine-rich repeat (NACHT-LRR; NLR) receptors, which recognize intracellular microorganisms through sensing their muropeptide components of peptidoglycan [68]. This makes the role of TREM-1 in bacterial infections even more intricate.

The crucial role of TREM-1 in septic shock and the blockade of TREM-1 with TREM-1 fusion proteins have biological relevance, since a splice variant of TREM-1 (TREM-1sv) has been described. This splice variant lacks a transmembrane domain and might therefore be translated into a soluble receptor with potential as a regulator of myeloid activation [69]. And indeed, both neutrophils and monocytes are capable of secreting soluble TREM-1 (sTREM-1) upon stimulation with LPS or with the cell wall fraction of *Mycobacterium bovis* BCG [61, 70-72]. A synthetic peptide of TREM-1, called LP17, has been generated and tested for its regulatory capacity of myeloid cells. LP17 comprises the CDR3 and the "F" β strand of the extracellular domain of TREM-1, keeping its potential ligand-binding region in tact [71]. LP17 is capable of attenuating cytokine production by human monocytes and protect septic animals from hyper responsiveness and death. LP17 blocks the production of TNFα and IL-1β through abolishing the p65/p50 NF-κB over-activation induced by the engagement of TREM-1, but not the secretion of sTREM-1. The favourable effect of LP17 in septic shock was not related to an enhanced bacterial clearance. The way that LP17 might function is that it could impair TREM-1 dimerization because LP17 still contains the tyrosine residue that potentially mediates dimerization of TREM-1 [57]. LP17 could also compete with the natural ligand for TREM-1 [71]. Gibot et al. elegantly describe the protective effects of LP17 in a rat model of septic shock. P. aeruginosa induces severe pneumonia in rats associated with signs of severe sepsis within the first 24 hours. In septic rats LP17 improved haemodynamic status, attenuated the development of lactic acidosis and hypoxemia, modulated lung and systemic inflammatory responses and coagulation activation, reduced lung histological damage and improved survival [73].

The protective effect of LP17 is not only restricted to bacterial infections. Recently it has been shown that Marburg virus and Ebola virus are capable of activating TREM-1, by direct binding of their glycoproteins to TREM-1 on human neutrophils. This results in DAP12 phosphorylation, sTREM-1 secretion, mobilization of intracellular calcium, secretion of pro-inflammatory cytokines and phenotypic changes. LP17 diminished the release of TNFα by filovirus activated human neutrophils in vitro and inhibited the loss of cell surface TREM-1 that otherwise occurred on neutrophils exposed to filovirusses [74].

The source of soluble TREM-1 is debatable. Two different theories have been postulated. One option is that soluble TREM-1 is secreted by human neutrophils in response to LPS challenge in a process involving de novo protein synthesis. Incubation of monocytes or neutrophils with protease inhibitors does not alter the sTREM-1 release [71]. The same phenomenon was observed with the use of cyclohexamide, an inhibitor of protein biosynthesis through interference with peptidyl transferase activity of the 60S ribosome that blocks translational elongation, which completely abrogated the production of soluble TREM-1 [61]. However, immunoblot analysis of monocyte supernatants after treatment with LPS showed that sTREM-1 has a molecular weight of 27 kDa while the predicted molecular weight of the soluble TREM-1 splice variant is 17.5 kDa [71]. In addition, the mRNA levels of TREM-1sv are not elevated upon stimulation with LPS. According to these data the second option, that suggests shedding of TREM-1 from the cell surface rather than de novo protein synthesis, is more likely.

Despite intensive research over the past 20 years, only five interventions have been shown to be effective in large controlled trials considering the reduction of mortality in sepsis, severe sepsis and septic shock [75]. These five interventions include: **1**) Lung protective mechanical ventilation that lowers the systemic levels of IL-6 [76]. **2**) Intensive insulin therapy that prevents hyperglycaemia, which could induce oxidative stress, a hypercoagulable state, phagocyte disfunction, and the formation of advanced glycation end-products that can trigger the receptor for advanced glycation end-products (RAGE) and consequently leads to inflammation [75, 77]. **3**) Early goal directed therapy (EGDT) that balances the systemic oxygen delivery and oxygen demand that may restore and maintain adequate cellular perfusion [78]. **4**) Treatment with low doses of hydrocortisone that increases the mean arterial pressure, systemic vascular resistance, a decline in cardiac index and norepinephrine requirement, possibly by the inhibition of nitric oxide formation [79, 80]. **5**) The treatment with recombinant, human, activated protein C (Xigris) that leads to a faster resolution of cardiovascular and respiratory dysfunction and a slower onset of haematological dysfunction as well as a reduction of IL-6 levels [81, 82]. All these five therapies work considerably well, but not optimal. This is most likely due to the late diagnosis of sepsis and the uncertainty if the underlying cause is an infection or not. This causes a potential delay for the start of treatment with antibiotics in the infected individual or the potentially dangerous unnecessary treatment with antibiotics in patients that do not suffer from infection.

The fact that neutrophils, and monocytes, up-regulate TREM-1 expression on their cell surfaces upon stimulation with bacteria or microbial products and that they induce secretion of soluble TREM-1, together with the interesting observations that a murine TREM-1-Fc fusion protein and a synthetic TREM-1 peptide, LP17, are able to increase the survival of mice that have been induced to get septic shock, led to the investigation of a possible role for soluble TREM-1 and its diagnostic and prognostic properties in human sepsis. Gibot et al. were the first to show that the plasma levels of soluble TREM-1 were correlated to the severity of sepsis and bacterial infections [83]. In this study plasma levels of soluble TREM-1 were measured in patients suffering from sepsis, severe sepsis, septic shock and systemic inflammatory response syndrome, not caused by infection. A plasma soluble TREM-1 level higher than 60ng/ml was more accurate (96%) than any other clinical or laboratory finding, e.g. C reactive protein of procalcitonin, for indicating infection [83, 84]. These results indicate that a rapid measurement of plasma soluble TREM-1 levels in critically ill patients may improve the clinicians' ability to differentiate patients with sepsis from those with systemic inflammation of non-infectious origin.

The measurement of cell surface TREM-1 expression on monocytes throughout the course of sepsis showed a progressive decline of expression in survivors but remained high in nonsurvivors. Cell surface TREM-1 might therefore be an adjunctive diagnostic tool and prove useful in the follow up of septic patients and their antibiotic treatment [85]. In addition, it has been shown that not only surface TREM-1 expression is higher in patients with sepsis as compared to healthy individual or patients that were scheduled to have major abdominal surgery, but also that the levels of TREM-1sv mRNA are elevated and are correlated to the severity of sepsis [86].

The secretion of soluble TREM-1 follows the kinetics of the immunosuppressive cytokine IL-10, indicating that soluble TREM-1 has an immunomodulatory role in sepsis. Furthermore, decreased ratios of soluble TREM-1/TNFα might determine the transition of sepsis into severe sepsis and severe sepsis into septic shock [87, 88]. Early severe sepsis is characterized by CD4 T cell lymphopenia and an increase in NK cell numbers providing a survival benefit of over 20% for the septic patient. The increase of NK cell numbers might be connected to the increase of soluble TREM-1 levels in patients with severe sepsis. If these two observations are functionally connected is unclear [89].

5.4.1.2 TREM-1 and pneumonia

Ventilator associated pneumonia (VAP) is the most frequent nosocomially acquired infection in patients on mechanical ventilation and is associated with prolonged hospital stay and an increased mortality [90]. Pneumonia is an acute infection of the lung parenchyma that is caused by pathogens and is characterized by recruitment of phagocytic cells, in particular alveolar macrophages and neutrophils. These cells act synergistically to generate an acute inflammatory response and thereby eliminate the pathogens by phagocytosis leading to a complete resolution of the infection.

Surface expression of TREM-1 on neutrophils derived from bronchial lavage fluid is increased in patients with pneumonia caused by extracellular bacteria but not in patients with tuberculosis, suggesting surface TREM-1 as a potential biomarker for differential diagnosis [91]. Measuring soluble TREM-1 concentrations in the bronchial lavage fluid of patients on mechanical ventilation revealed that the levels of soluble TREM-1 are increased towards the diagnosis of VAP and that the levels of soluble TREM-1 are increased in patients with bacterial pneumonia but not in patients with fungal pneumonia [92-94]. Interestingly, after the start of antibiotic treatment of the patients with VAP, soluble TREM-1 levels decreased in all patients [92]. These results suggest that soluble TREM-1 is a potential biomarker of VAP and that soluble TREM-1 measurement in the plasma of these patients may provide an opportunity to change the treatment early in the course of patients with VAP, either to intensify treatment when levels stay high or to avoid unnecessary prolonged courses of antibiotics when their levels rapidly decrease [95, 96]. The same results have been obtained for community-acquired pneumonia [97]. Soluble TREM-1 levels are increased in patients with community-acquired pneumonia as compared to healthy controls. However, in this study the level of TREM-1 was only moderately accurate as a marker for the need of antibiotics in lower respiratory tract infections.

The pleurae are dynamic, metabolically active membranes responsible both for maintaining homeostasis and responding to insults resulting in inflammation. Two membranes, the pleurae, surround the lungs. The outer pleura is attached to the chest wall and is known as the parietal pleura; the inner one is attached to the lung and other visceral tissues and is known as the visceral pleura. In between the two is a thin space known as the pleural cavity or pleural space. It is filled with pleural fluid, a serous fluid produced by the pleura. Acute inflammatory processes are associated with the development of an inflammatory cell-rich pleural effusion as well as production of cytokines and chemokines. Such effusions fulfill the criteria of exudates, while non-inflammatory processes usually result in transudative pleural effusions. Concentrations of soluble TREM-1 are higher in infectious and neoplastic pleural effusions than in transudates. In addition, the level of soluble TREM-1 is higher in parapneumonic effusions than in tuberculous effusions, suggesting that the measurement of high soluble TREM-1 levels may exclude tuberculous pleurisy and is indicative of bacterial infection [98].

5.4.1.3 Soluble TREM-1 in infectious diseases

Soluble TREM-1 has also been suggested as a diagnostic and prognostic biomarker for bacterial meningitis. Patients with bacterial meningitis have higher levels of soluble TREM-1 in their cerebro spinal fluid than patients with viral meningitis. Furthermore, patients with bacterial meningitis with lethal outcome had higher levels of soluble TREM-1 than patients who survived the meningitis [99].

Peptic ulcer disease is an ulcer of an area of the gastrointestinal tract that is usually acidic and thus extremely painful. Most ulcers are associated with *Helicobacter pylori*, a spiral-shaped bacterium that lives in the acidic environment of the stomach. The presence of soluble TREM-1 in gastric juice could be a sign of an

inflammatory reaction taking place in the gastric mucosa. Soluble TREM-1 might be an independent factor involving with the peptic ulcerative inflammatory process that is positively correlated with histopathological abnormalities of gastritis. However, the level of soluble TREM-1 was not correlated to the density of *Helicobacter pylori* [100, 101].

Zymosan-A is a TLR2 ligand and a murine CR3 (CD11b/CD18, αMβ2 integrin) receptor ligand, known to strongly induce the stimulation of macrophages, neutrophils and NK cells [102, 103]. In a mouse model of zymosan-A induced hepatic granuloma formation, adenoviral mediated DAP12 gene transfer did not enhance hepatic granuloma formation by day 7, however the overexpression of DAP12 sustained and enhanced granuloma formation beyond day 7. Antibody triggering of DAP12 enhanced granuloma formation by day 7. When soluble TREM-1 was transgenically expressed in the liver as an antagonist of DAP12 signaling via TREM-1, the presence of soluble TREM-1 inhibited zymosan-A induced granuloma formation at all time points examined [104]. These results suggest that DAP12 and TREM-1 are involved in the development of granulomatous responses in the liver.

In a similar study with *Aspargillus fumigatus*, a fungal allergens that can aggrevate asthmatic responses, overexpression of DAP12 resulted in significantly less airway inflammation, airway hyperrsponsiveness and fungal material as compared to mice that received the control adenoviral construct. However, TREM-1 blockade via the overexpression of soluble TREM-1 enhanced allergic airway disease and the presence of fungal material [105, 106]. This study suggests that TREM-1 might have a protective role in the allergic airway disease present in these mice and that TREM-1 could have an impact on the clearance of *Aspargillus fumigatus* from the lungs of allergic mice.

Soluble TREM-1 has also been suggested as a novel mediator in inflammatory bowel disease in correlation with the degree of the inflammatory reaction of the intestinal mucosa [107]. Interestingly, soluble TREM-1 is not a sufficient diagnostic marker for urinary tract infections, where the levels of soluble TREM-1 in urine were undetectable or very low [108].

5.4.1.4 TREM-1 in non-infectious disease

In a study by Wang et al. it was shown that the expression of TREM-1 mRNA is increased in blood leukocytes of patients with acute pancreatitis as compared to healthy controls and these expression levels correlate to the degree of acute pancreatitis [109]. Acute pancreatitis is a self-destructive chemical inflammation of the pancreas that if not treated well could have a lethal outcome. This is the first study that shows up-regulation op TREM-1 in a disease not caused by bacteria, suggesting that TREM-1 may play a role in the occurrence and development of acute pancreatitis.

TREM-1 seems to play a role in another neutrophil related disease. Acute gouty arthritis is an acute inflammatory reaction that develops in response to articular tophacous deposits of monosodium monohydrate (MSU) crystals. Full-blown acute gouty arthritis is dependent on the influx of neutrophils into the joint space and their

subsequent activation that eventually leads to tissue damage. Neutrophil migration into the gouty joint appears to be driven synergistically by MSU crystal induction of IL-1β and TNFα induced activation of the endothelium and E selectin expression, and by critical chemotactic activities of CXCL8 and closely related chemokine ligands of CXCR2 [110]. The MSU crystals are able to up-regulate TREM-1 on phagocytes in vitro and costimulation of phagocytes with MSU crystal and TREM-1 engagement synergistically increased the production of MCP-1 and IL-1β. MSU crystals also induced TREM-1 expression on infiltrating leukocytes, especially neutrophils, in a murine air pouch model of crystal induced acute inflammation [111]. It will be of interest to investigate the inflammatory responses mediated by TLR2 and TLR4, which mediate the recognition of MSU crystals, and TREM-1 in both acute and chronic phases of gouty arthritis for potential prophylaxis and therapy of gouty arthritis.

5.4.1.5 TREM-1 in the clinic: future prospects

As has been described above, soluble TREM-1 seems to be a good diagnostic and even prognostic marker for bacterial infections in humans [88, 112]. In addition, these studies show that soluble TREM-1 has properties of an anti-inflammatory mediator. In a recent study the diagnostic values of various markers for bacterial infections were assessed individually and in combinations of 3 or 6. Plasma C reactive protein and procalcitonin showed a higher diagnostic value than macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor, soluble TREM-1 and neutrophil counts when measured alone. When soluble TREM-1 was combined with C reactive protein and procalcitonin the diagnostic value of bacterial infection was greatly enhanced. The combination of all 6 markers showed the highest diagnostic value [113]. Combining information from several sepsis markers may improve differentiation between patients with bacterial infections and those with systemic inflammation of nonbacterial origin. This would be of great importance in patients in whom diagnosis is not clinically clear-cut. Rapid and adequate treatment of patients suspected of having bacterial sepsis requires accurate and early diagnosis in order to treat the patients in time to increase survival.

From a therapeutical point of view, TREM-1 might be an interesting candidate to block in septic shock, community acquired pneumonia, ventilator associated pneumonia, bacterial meningitis and maybe even acute pancreatitis.

5.4.2 Monocytes and macrophages

Monocytes constitute 3-8% of all leukocytes in blood, and are part of the immune system that protects against blood-borne pathogens. They move quickly, in about 8-12 hours, to sites of infection in tissues. They are usually identified in stained smears by their large bilobed nucleus. Monocytes are produced by the bone marrow from haematopoietic stem cell precursors called monoblasts. Monocytes circulate in the bloodstream for about one to three days and then typically move into tissues throughout the body. Monocytes are responsible for phagocytosis (ingestion) of foreign substances in the body. Monocytes can perform phagocytosis using

intermediary (opsonising) proteins such as antibodies or complement that coat the pathogen, as well as by binding to the microbe directly via pattern-recognition receptors that recognize pathogens. Monocytes are also capable of killing infected host cells via antibody-mediated cellular cytotoxicity (ADCC). When a monocyte enters damaged tissue through the endothelium of a blood vessel it undergoes a series of changes to mature into different types of macrophages at different anatomical locations. Monocytes are attracted to a damaged site by chemical substances through chemotaxis, triggered by a range of stimuli including damaged cells, pathogens, histamine released by mast cells and basophils, and cytokines released by macrophages already at the site.

Macrophages (Greek: "big eaters", *makros = large, phagein = eat*) are cells that phagocytose (engulf and then digest) cellular debris and pathogens either as stationary or mobile cells. On top of the microbicidal functions of macrophages, they are capable of presenting antigens of the pathogens that they have engulfed to the adaptive immune system.

5.4.2.1 TREM-1 expression on monocytes and macrophages

Human and murine monocytes and macrophages express both TREM-1 and TREM-2, while murine macrophages also express TREM-3. Ligation of TREM-1 on the cell surface of monocytes and macrophages results, just like neutrophils, in the up-regulation of adhesion molecules, CD26, CD11c, CD49e, CD49d, CD11b and CD18 [26] and also the up-regulation of costimulatory molecules CD40, CD86, CD54, CD83 and CD32, possibly needed to steer an adequate adaptive immune response. TREM-1 ligation also triggers the release of IL-8, MIP-1α and in synergy with microbial ligands, such as LPS, 19kD, Poly I:C, heat inactivated Gram positive bacteria, gram negative bacteria and fungi, the secretion of TNFα and GM-CSF [26, 59]. These microbial ligands were also able to induce the up-regulation of TREM-1 on monocytes and macrophages, a response that was enhanced by the addition of TNFα or GM-CSF but was inhibited by IL-10. Furthermore, TREM-1 ligation inhibited the production of IL-10 by monocytes [59]. The up-regulation of TREM-1 in monocytes is believed to be caused by post-translational mechanisms, since the level of TREM-1 mRNA remained unchanged whilst protein levels increased upon LPS stimulation [114].

When monocytes are cultured in the presence of GM-CSF and IL-4, or when TREM-1 is triggered, they start to develop into immature DCs. When this happens, they rapidly start to down-regulate the expression of TREM-1 on their cell surface and at the same time up-regulate costimulatory molecules [26, 59]. And indeed, these cells have a higher potential to elicit T cell proliferation and production of IFNγ [59].

The TREM-1 expression on murine peritoneal macrophages is up-regulated upon stimulation with prostaglandin E_2 [115]. Prostaglandins are produced by metabolism of arachidonic acid through activation of cyclooxygenase (COX). The release of prostaglandins is increased in animal models of endotoxemia or sepsis. Prostaglandin E_2 has been shown to function as a mediator of sepsis-induced immunosupression, an inhibitor pro-inflammatory cytokine production by

macrophages and an inducer of IL-10 production [116]. In contrast, prostaglandin E_2 has several detrimental effects in sepsis, including vasodilation and increase in vascular permeability [117]. Prostaglandin E_2 can suppress the production of various cytokines, including TNFα, IL-8, MCP-1, IFNγ-inducible protein-10 and MIP-1β, by LPS stimulated macrophages. Prostaglandin E_2 also induces the expression of soluble TREM-1 that acts as a decoy receptor and inhibits inflammation. However, activation of TREM-1 on prostaglandin E_2 pretreated peripheral blood mononuclear cells enhanced the production of pro-inflammatory cytokines, TNFα and IL-8, indicating that prostaglandin E_2 may modulate the expression of TREM-1 and secretion of soluble TREM-1 by monocytes and macrophages thereby altering the inflammatory response [115].

An interesting observation comes from a study by Schenk et al., where they show that the resident macrophage population in normal human small and large intestine contains only a few TREM-1 expressing macrophages (<10%), whereas the overwhelming majority of monocytes (>90%) and macrophages from lymph nodes or tonsils (>80%) express TREM-1 on the cell surface [118]. This is interesting from a mucosal immunity point of view. The intestinal mucosa normally displays minimal signs of inflammation, even though enourmous amounts of bacteria are present in the colonic mucosa, and mucosal macrophages and luminal bacteria are in close proximity. Adaptations in order to prevent excessive inflammation are, the preferential secretion of IgA and its transport across the epithelial layer, and the presence of several T cell subsets with immunoregulatory properties such as regulatory T cells. Although monocytes and macrophages from spleen, lymph nodes, or tonsils show an increase in oxidative burst after TREM-1 ligation, no effect is seen in intestinal macrophages. In addition, intestinal macrophages fail to up-regulate TREM-1 in response to TNF, LPS or PMA, normally observed in monocytes [118]. Interestingly, intestinal macrophages lack CD14 and CD89 expression and have reduced levels of TLR-4 and TLR-2 expression [119]. The lack of these receptors and the absence of TREM-1 on lamina propria macrophages is likely to prevent excessive inflammatory reactions, and thus, excessive tissue damage in the intestine.

A signaling molecule that keeps the cell activation and cytokine secretion induced by TREM-1 in a myelomonocytic cell line in check is the non T cell activation linker (NTAL, or linker of activation in B cells, LAB). By analyzing receptor-induced tyrosine phosphorylation patterns it was discovered that ligation of TREM-1 leads to tyrosine phosphorylation of NTAL. Knockdown of NTAL with RNAi enhanced ERK1/2 phosphorylation upon TREM-1 ligation. Low levels of NTAL correlated with decreased Ca^{2+} mobilization after TREM-1 triggering. This study suggests that NTAL acts as a negative regulator of TNFα and IL-8 production after stimulation with TREM-1. However, decreased levels of NTAL did not affect cellular differentiation triggered via TREM-1/DAP12. This might indicate that NTAL differentially controls cytokine production and cell differentiation [120].

5.4.2.2 TREM-2 on monocytes and macrophages

TREM-2 is expressed on thioglycollate recruited murine resident peritoneal macrophages, and is almost impossible to detect on any other primary murine cell from the blood, spleen, lung, liver or bone marrow [121]. Culturing bone marrow derived cells with M-CSF drives these cells to become macrophages that express TREM-2. Ligation of TREM-2 on murine monocytes and macrophages leads to the production of nitric oxide (NO) [31]. Even though TREM-2 is an activating receptor [29, 31], recent data suggest that TREM-2 modulates the TLR responses of macrophages. These findings have evolved from the interesting observation that macrophages from DAP12 deficient mice respond to a higher extent to TLR ligands such as LPS, CPG and zymosan [122].

5.4.2.3 DAP12 signaling

DAP12 knock out mice, in which the DAP12 gene is disrupted and DAP12 expression is absent [123], and DAP12 loss of function mice in which one tyrosine of the ITAM sequence of DAP12 is mutated, resulting in non-functional DAP12 expression [124], have been generated. These mice have shed light on the function of DAP12 and its associated receptors. The DAP12 deficient mice showed an enhanced innate immune response to infection with *L. monocytogenis* and are more susceptible to septic shock [122]. In contrast, DAP12 transgenic mice that over express DAP12 are hyperresponsive to septic shock induced by bacterial endotoxin [125]. Moreover, DAP12 also mediates activating signals in macrophages and absence of DAP12 has been demonstrated to decrease mortality and inflammation during septic peritonitis [126]. The role of DAP12 in septic shock and the amplification of TLR responses is thus debated. The fact that transgenic overexpression of DAP12 generates the mice more susceptible to septic shock is not surprising, but the contrasting results in DAP12 deficient mice are in fact surprising. The differences most probably lie in the models used for septic shock. In the study by Hamerman et al. they used mice that were sensitized for septic shock by D-galactosamine, which sensitizes mice to TNFα by blocking hepatic protein synthesis. In this way mice will greatly respond to low levels of endotoxin and the mice die rapid, within hours after endotoxin challenge [122]. Thus DAP12 has a dampening effect on the TLR responses that elicit septic shock in the presence of low doses of endotoxin. In the study by Turnbull et al. they used higher doses of endotoxin without TNFα sensitization. Even in caecal ligation and puncture, the DAP12 deficient mice have a lower response to endotoxin and are protected from septic shock. The LPS levels that circulate in plasma in both mouse [127] and human sepsis [128] are in the range of endotoxin levels tested by Hamerman et al. At the site of infection LPS levels can be much higher, possibly past the range for the inhibitory function of DAP12. A situation can be envisioned where distal to the site of infection, the LPS levels are low and thus prevent unnecessary DAP12-mediated inflammation, whereas at the site of infection, LPS levels are much higher, which fully activates DAP12 so that DAP12 can synergize with TLRs inducing an inflammatory response.

5.4.2.4 TREM-2-mediated regulation of activating signaling

Regarding the above described results it has been hypothesized that low avidity triggering of DAP12 dependent receptors leads to inhibition of TLR mediated responses, while a strong trigger via DAP12 may instead lead to a synergistic effect with TLR signaling [129, 130]. This hypothesis is strengthened by the findings that low avidity ligands for the FcαRI receptor induce inhibition of phagocytosis and IgE mediated exocytosis, while high avidity ligands induced activating signals. In the scenario of low avidity the ITAM of FcαRI is able to recruit the SHP-1 phospatase, a well-known inhibitory signaling molecule [131].

TREM-2 is the first DAP12 associated receptor accounting for the inhibitory effect seen in TLR responses. TREM-2 deficient mice display the same phenotype, i.e. enhanced cytokine secretion, as DAP12 deficient mice in response to TLR ligands such as CpG, LPS, and zymosan, suggesting that TREM-2 accounts for the skewed cytokine production seen in DAP12 deficient mice [121]. Introduction of a TREM-2-DAP12 chimeric receptor, but not a TREM-1-DAP12 chimera, into DAP12 deficient macrophages restored the TLR responses compared to those observed in wild type mice [132]. In addition, TREM-2 knock down with siRNA in wild type macrophages resulted in enhanced cytokine secretion upon CpG and zymosan stimulation [132].

Interestingly, the inhibitory function of DAP12 is not limited to TREM2 or macrophages. Both natural cytoxicity receptor NKp44 and SIGLEC-H (sialic-acid-binding immunoglobulin-like lectin H) are DAP12 associated receptors capable of inhibiting cytokine secretion by plasmacytoid DCs upon antibody-mediated cross-linking [133-136]. Furthermore, DAP12-deficient plasmacytoid DCs produce larger amounts of type I interferon than wild-type cells upon stimulation with CpG and infection with MCMV [134, 137]. These studies suggest that inhibition of cellular activation might be a general function of DAP12, and that DAP12 associated receptors can both activate and inhibit cellular responses, thereby modulating the immune response [130].

5.4.2.5 TREM-3 on murine macrophages

TREM-3 is only expressed on murine macrophages. Functionally TREM-3 is not well characterized. The only data existing on TREM-3 so far is that upon antibody cross-linking it mediates the production of nitric oxide [31]. In humans TREM-3 is most likely a pseudogene.

5.4.2.6 TREM ligands

So far the ligands for the TREMs are unknown, however *Escherichia coli* and *Staphylococcus aureus* bind specifically to TREM-2. Anionic molecules, like LPS or dextran sulphate, can inhibit this binding [138]. This suggests that TREM-2 recognizes pathogens via charged carbohydrates expressed on the pathogen surface. TREM-2-Fc fusion proteins show binding to astrocytoma cell lines [138] and bone marrow derived macrophages [132] suggesting that these cells express a ligand for TREM-2. The inhibitory effect observed for TREM-2 on TLR responses could be due to the presence of the TREM-2 ligand on the same cells, providing a low level of TREM-2 signaling that results in DAP12 mediated inhibition.

In summary, monocytes and macrophages express both TREM-1 and TREM-2 that signal via DAP12. The triggering of the two receptors results in different outcomes. Ligation of TREM-1 induces the secretion of cytokines and the production of soluble TREM-1 that can act as a decoy receptor that modulates the responses of the macrophages themselves. Ligation of TREM-2 results in the production of nitric oxide. Both TREM-1 and TREM-2 act together with TLRs. TREM-1 synergizes with TLRs in the secretion of cytokines and soluble TREM-1, thereby enhancing the inflammatory response, but at the same time trying to get the inflammation under control through the modulatory effects of soluble TREM-1. TREM-2 acts as an attenuator of TLR responses, keeping the inflammatory response in check when TLR ligands are present in low concentrations, but when the levels of these ligands rise, enhancing the inflammatory response. Since these receptors are expressed on the same cell, one could imagine an intricate interplay between TREM-1 and TREM-2, which, depending on their ligands and subsequent effector functions, could modulate each others response. It would be of great interest to study these receptors along side each other and to identify their ligands. This would provide us with information how TREM-1 and TREM-2 work together in an inflammatory response.

5.4.3 Dendritic cells

DCs reside in peripheral tissues in an immature state, where they await the encounter of antigen. Upon antigen capture, DCs process the antigens into peptides, which are loaded on to MHC molecules for presentation to T cells. Through the invasion by pathogens, inflammation and tissue damage, DCs receive additional danger signals. These signals induce changes in DC phenotype and function, and DCs start to migrate from peripheral tissues to the draining lymph nodes. In addition, the activation and maturation of DCs can trigger the production of cytokines such as IL-12, IL-18 and or IL-10. Specific subsets of DCs exert an innate immune defence against bacterial infection by producing TNF/inducible nitic oxide synthase [139].

5.4.3.1 TREM-2 expression on DCs

When monocytes are stimulated with GM-CSF and IL-4, which drives their development into immature DCs, TREM-2 expression is strongly up-regulated. However when these immature DCs start to mature TREM-2 expression is rapidly lost [29]. The first functional data on TREM-2 comes from a study on monocyte derived DCs. These DCs express TREM-2 and ligation of TREM-2 on these cells induces partial DC maturation, as measured by the up regulation of costimulatory molecules, DC survival and the up-regulation of the chemokine receptor CCR7 [29]. CCR7 interacts with CCL19, or MIP-3β and CCL21. These are all chemokines that are crucial for guiding the DCs from the peripheral tissue (i.e. the site of infection) to the draining lymph node, where they are able to elicit a T cell response. It is of interest to note here that DAP12 loss of function mice have an accumulation of DCs in the skin [124]. This could possibly be due to the lack of TREM-2 signaling. DC are not able to up-regulate CCR7 in this scenario, the DCs cannot migrate to the draining lymph node and are therefore getting stuck in the skin. In DAP12 loss of function

mice this is associated with an impaired hapten-specific contact sensitivity, due to inadequte priming of T cells in the draining lymph node [124].

5.4.3.2 DC and NK cell cross-talk via TREM-2

Another interesting study potentially reveals a TREM-2 mediated cross talk between DCs and NK cells [140]. As described above, IL-4 and GM-CSF up-regulate TREM-2 expression on immature DCs. It is also known that IL-4 is mandatory for mouse bone marrow derived DCs grown in GM-CSF to promote NK cell activation in the draining lymph nodes. Gene targeting of DAP12 suppresses the GM-CSF/IL-4 DC capacity to activate NK cells. Interestingly, IL-4 mediated DC-activated NK activity is blocked by soluble TREM-2-Fc molecules [140]. From these data can be hypothesized that by selectively up-regulating TREM-2 molecules, IL-4 facilitates engagement of DAP12 signaling pathways in DCs following encountering with NK cells, ultimately leading to NK cell activation. These studies on TREM-2 in DCs link the TREM family of receptors to the interplay between the innate and the adaptive immune system. Via this cross talk and the TREM-2 induced DC migration to the draining lymph node, the innate immune system might be able to better fight the infection that is at large and steer an adequate adaptive immune response to the infection. However, in this context one should note that the expression of TREM-2 on primary DCs has never been detected [121], making it difficult to extrapolate these in vitro results to an in vivo situation.

5.4.4 Microglia

The tissue macrophages of the brain are the microglia [141, 142]. They are found in all brain regions, often in close apposition with neurons, and compromise between 5 and 15% of cells in the central nervous system (CNS). Microglia, the smallest of the glial cells, can act as phagocytes, cleaning up CNS debris. Microglia are close cousins of other phagocytic cells including macrophages and DCs. Microglia are derived from myeloid progenitor cells that come from the bone marrow. During embryonic development they migrate to the CNS to differentiate into microglia.

5.4.4.1 TREM-2 and Nasu Hakola disease

The fact that research on TREM family molecules has been conducted in the field of microglia comes from the identification of mutations in the gene of TREM-2 that lead to the development of a rare recessive hereditary disease called Nasu Hakola disease [143]. Nasu Hakola disease is also called Polycystic Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy (PLOSL) and was first discovered independently by two different research groups in Finland and Japan [144, 145]. The disease is characterized by multiple bone cyst-like lesions and progressive encephalopathy leading to severe neurodegeneration, which eventually presents itself in the form of pre-senile dementia [146, 147].

The clinical course of PLOSL can be divided into several phases: a latent period up to the age of 20 years, followed by first osseous symptoms and early neurological changes, then leading at the age of approximately 40 to severe neurological abnormalities and massive neurodegeneration [148, 149]. At later stages of the disease patients develop tremor, gait disturbances and finally evolve to a state of profound dementia, being unable to walk due to severe neurological alterations. They often die following a respiratory of urinary tract infection in the fourth of fifth decade of life [148]. Neuropathological analysis demonstrates a generalized cortical atrophy and substantial white matter atrophy, especially in the corpus callosum and basal ganglia. A common feature of all patients is marked loss of axons and myelin, accompanied by axonal spheroids [147].

The underlying defect in this disease has been pin pointed to mutations in the DAP12 gene. The Finnish population caries a homozygous deletion of exons 1-4 of the gene encoding DAP12 [150], and in the Japanese population a loss-of-function mutation of DAP12 was causing the disease [151]. Even though these mutations in DAP12 are responsible for the disease in most of the patients, some patients displaying the same symptoms carried an intact DAP12 gene. A linkage study performed by Paloneva et al. showed that patients with an intact DAP12 gene all carried mutations in the TREM-2 gene [143]. More recently, an Italian family having Nasu Hakola has been described that carries a mutation in the TREM-2 gene that generates a premature stop codon [152]. These findings led to the investigation of TREM-2 in the brain and in bone, especially in microglia and osteoclasts, which will be discussed below. It is of interest to note that DAP12 deficient mice also suffer from bone remodelling problems and hypomyelinosis with synaptic degeneration [153].

5.4.4.2 TREM-2 function on microglial cells

The first report that showed expression of TREM-2 on microglia was a study performed by Schmid et al. [154]. They showed that TREM-2 is expressed by unactivated microglia, and that LPS and IFNγ down-regulate TREM-2 expression. In the healthy CNS, not all microglia express TREM-2. Brain regions with an incomplete blood-brain barrier showed the lowest percentages of TREM-2 expressing microglia, whereas the lateral entorhinal and cingulated cortex had the highest percentages. These regional variations in TREM-2 expression may contribute to the varying sensitivities of different brain regions to similar pathological signals. In the above-described study TREM-2 was only expressed by unactivated microglia. In a later study it became apparent that TREM-2/DAP12 is strongly expressed by microglia both irregularly shaped resting and globular active [155]. TREM-2 is also present in a fraction of neurons, but not in astrocytes and oligodendrocytes. Both in microglia and neurons the receptor appears to be located mostly intracellularly in a discrete compartment(s) partially coinciding with or adjacent to the Golgi complex/trans-Golgi network [155]. This observation was confirmed by a study by Prada et al. where they also showed that TREM-2 was mostly located intracellularly. They extended this finding with the fact that TREM-2 is mostly distributed

intracellularly in two pools: a deposit in the Golgi complex and a population of exocytic vesicles, distinct from endosomes and lysosomes. TREM-2 within these compartments is continuously translocated to and recycled from the cell surface and the exocytosis of these vesicles seems to be regulated by the activation status of the microglia [156]. Through this mechanism microglia might be able to quickly respond to an inflammatory stimulus by quickly shuttling the "pre-packaged" TREM-2 to their cell surfaces.

Functionally TREM-2 engagement on microglia, like on DCs, induced up-regulation of CCR7. The up-regulation of CCR7 was functional because the TREM-2 stimulated microglia showed increased chemotaxis towards CCL19 and CCL21, the ligands for CCR7 [157].

Microglial activation is characteristic to most neurodegenerative diseases such as Parkinson, Alzheimer, MS and AIDS associated dementia as well as ischemia and posttraumatic brain injury. Release of pro-inflammatory cytokines and cytotoxic factors, such as NO and IL-1, by microglia has been suggested to contribute to neurodegeneration. However, microglia also have progenerative and neuroprotective functions as injured neurons recover in the presence of microglia. Microglial cells also produce neuroprotective agents and scavange for cellular debris resulting from injury or disease [142, 149]. It has also been suggested that neurodegeneration might not primarily result from microglial aggression but rather from neglect by these cells [158]. Thus elimination of apoptotic neurons without inflammation is crucial for brain tissue homeostasis. In the light of these findings it has been shown that knock down of TREM-2 in microglia inhibited phagocytosis of apoptotic neurons and it increased gene transcription of TNFα and nitric oxide synthase-2. In the case of overexpression of TREM-2, phagocytosis was increased and microglial pro-inflammatory responses were decreased [157]. This suggests that TREM-2 is important in the homeostasis of the brain by stimulating phagocytosis without inflammation. The TREM-2-DAP12 receptor-signaling complex is required in microglial cells for the removal of apoptotic cells, organic matrix components and macromolecules. A defect of the TREM-2/DAP12 complex would lead to accumulation of toxic products that might directly cause brain damage or over-stimulate the microglial cells to release cytotoxic mediators, which could explain the brain pathology observed in patients with Nasu Hakola disease.

There is some debate about if TREM-2 is expressed in oligodendrocytes [153, 159] or not [155]. These are the myelin producing cells of the CNS and patients with Nasu Hakola disease lose myelin over time. Loss of TREM-2/DAP12 signaling could lead to their apoptotic death or a defect in their differentiation and consequent loss of myelin production [159], thereby contributing to the brain pathology of Nasu Hakola disease. It is of interest to note that no TREM-2 mutations have been observed in Alzheimer's disease and frontotemporal lobar degeneration, both diseases in which microglia are highly active [160].

A splice variant of TREM-2 (TREM-2sv) has also been described. Like TREM-1sv, TREM-2sv has no transmembrane region and could therefore be a soluble form of the receptor [154, 161]. Whether TREM-2sv also functions as a

decoy receptor is not known, but it is tempting to speculate that TREM-2sv has immunomodulatory effects, as well as an impact on brain homeostasis and bone remodelling.

5.4.4.3 DAP12 deficiency and autoimmune disease

DAP12 deficient mice fail to develop autoimmunity due to impaired antigen priming. In an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), where an autoimmune reaction exists against myelin proteins, DAP12 deficient mice, compared to wild type mice, are less susceptible to develop this condition upon immunization with myelin proteins [123]. Intravenous injection of TREM-2-transduced bone marrow-derived myeloid precursor cells in wild type mice at the peak of EAE diminished the clinical symptoms, reduced axonal damage, and prevented further demyelination. The TREM-2-transduced myeloid cells migrated into the inflammatory spinal cord lesions of EAE-diseased mice and displayed an increased lysosomal and phagocytic activity. They cleared degenerated myelin, and created an anti-inflammatory cytokine milieu within the CNS, thereby preventing further progression of EAE [162]. In concordance with the results that TREM-2 is important for brain homeostasis and clearance of apoptotic neurons and degenerated myelin, blockade of TREM-2 on microglia with antibodies has been shown to exacerbate EAE [163]. This makes TREM-2 on microglia an interesting therapeutic target for promotion of repair and resolution of inflammation in multiple sclerosis and other neuroinflammatory diseases.

5.4.5 Osteoclasts

Osteoclasts are large cells that are characterized by multiple nuclei and a cytoplasm with a homogenous, “foamy” appearance. This appearance is due to a high concentration of vesicles and vacuoles. At a site of active bone resorption, the osteoclast forms a specialized cell membrane, the “ruffled border”, which touches the surface of the bone tissue. The ruffled border, which facilitates removal of the bony matrix, is a morphologic characteristic of an osteoclast that is actively resorbing bone [164].

5.4.5.1 TREM-2 on osteoclasts and in Nasu Hakola disease

Nasu Hakola patients suffer from bone cysts, mostly located in their extremities, that are filled with triglycerides [148]. Osteoclasts express both DAP12 and TREM-2 [165]. The first indication that TREM-2 and DAP12 might be involved in the osteoporotic features of Nasu Hakola patients comes from a study by Paloneva et al. [166]. In this study they show that DAP12 deficient and TREM-2 deficient peripheral blood mononuclear cells have an impaired and delayed differentiation into osteoclasts. These osteoclasts also displayed a reduced bone resorption capacity. These results were confirmed by Cella et al. [165]. Normally osteoclast precursor cells develop into multinucleated osteoclasts when they are treated with M-CSF and RANKL. The triggering of TREM-2 with antibodies in combination with M-CSF and RANKL enhances the development of osteoclasts. Knock down of TREM-2 by siRNA in osteoclasts precursor cells resulted in a loss of multinucleated osteoclast

formation in response to M-CSF and RANKL and antibody blockade of TREM-2 decreased bone resorption capacity [167].

5.4.5.2 RANKL-induced osteoclast differentiation

Bone marrow derived monocyte/macrophage precursor cells of hematopoietic origin develop into osteoclasts through cell-cell signaling with mesenchymal cells including osteoblasts. RANKL is expressed mainly as a membrane bound form on osteoblasts and induces the signaling essential for precursor cells to differentiate into osteoclasts, whereas M-CSF, secreted by osteoblasts, provides the survival signal to these cells [168, 169]. Osteoclasts express PIR-A and OSCAR that associate with FcRγ, and TREM-2 and SIRP-β1 that associate with DAP12. Triggering of either receptor by cross-linking with an antibody accelerated RANKL-induced osteoclast differentiation. In absence of RANKL the stimulation of these receptors alone could not induce osteoclast differentiation, suggesting that these receptor-mediated signals act cooperatively with RANKL but cannot substitute the signal [169, 170].

DAP12 deficient osteoclast precursor cells display a severe loss of multinucleated osteoclasts formation upon RANKL stimulation. They show decreased responses in their migration towards M-CSF and have an impaired bone resorption capacity [171]. It is being hypothesized that osteoclast precursor cells themselves express the ligands for DAP12 associated receptors, such as TREM-2 and SIRPβ1. These receptors are continuously activated without the addition of ligands. When DAP12 is absent, this mutual activation is abrogated and osteoclasts formation is impaired [169]. This hypothesis is strengthened by the finding that the osteoclasts precursor cell line RAW264.7 is dependent on cell surface expression of TREM-2 for osteoclastogenesis [167]. It is of interest to note that when DAP12 deficient osteoclasts precursor cells are co-cultured with osteoblasts, they are indeed able to form multinucleated osteoclasts [169]. Thus osteoblasts are needed to compensate for the loss of DAP12. This effect is not seen in DAP12/FcεRIγ double deficient osteoclast precursors. In this case both ITAM signaling pathways are not activated, causing an inefficient activation of the master transcription factor for osteoclastogenesis, NFAT-1c. This blocks the precursor cells to form multinucleated osteoclasts [169, 170, 172].

5.4.5.3 DAP12 deficiency and osteoclasts

Both DAP12 deficient and DAP12/FcεRIγ double deficient mice exhibit severe osteopetrosis, a sclerotic bone disease caused by impaired osteoclastic bone resorption. [153, 169, 170, 173]. This is in sharp contrast with DAP12 deficient humans having Nasu Hakola disease. These patients suffer more from an osteoporotic-like syndrome, resulting in bone cysts. The discrepancy between Nasu Hakola patients and DAP12 deficient mice in regard to bone cysts/osteoporosis in humans and osteopetrosis in mice is difficult to explain. However, this is not the only example where genetically engineered mice exhibit a different phenotype compared with the clinical manifestations that patients present. Homozygous deletion of the TNFRSF11B gene, encoding for osteoprotegerin (OPG, a decoy receptor of

RANKL), leads to juvenile Paget's disease in humans [174]. Paget's disease, otherwise known as osteitis deformans, is a chronic disorder that typically results in enlarged and deformed bones caused by excessive breakdown and formation of bone tissue causing bone to weaken. In contrast to this, OPG deficient mice exhibit a decrease in total bone density resulting in osteoporosis [175].

The picture gets even more complicated with unpublished results describing that TREM-2 deficient mice have an accelerated osteoclastogenesis with more rapid fusion of the cells to form multinucleated functional osteoclasts capable of resorbing bone ex vivo [25]. In addition, these mice do not have osteopetrosis like DAP12 deficient mice. The authors suggest that TREM-2 might rather have an inhibitory effect on osteoclastogenesis and that other DAP12 associated receptors might also be involved in osteoclastogenesis.

5.4.5.4 TREM-2 and plexin-A1 in development of osteoclasts

Another interesting observation comes from a study where they generated plexin-A1 deficient mice [176]. Plexin-A1 deficient mice develop osteopetrosis and have defects in DC function. Plexin-A1 deficient mice have a reduced number of osteoclasts, a lower ratio of osteoclast surface to bone surface and a reduced bone turnover by osteoclasts. These results show the importance of plexin-A1 and its ligand, semaphorin 6D, in osteoclastogenesis. Osteoblasts, however, develop and function normally in plexin-A1 deficient mice [176]. In the same study it was shown that Plexin-A1 associates with TREM-2, linking semaphoring signaling to the ITAM containing adaptor molecule DAP12. In this way TREM-2, Plexin-A1 and DAP12 have been implicated as a receptor complex. Knock down of TREM-2 by siRNA blocked the activation of DCs via Plexin-A1 and semaphoring 6D, showing that the receptor complex is functional and needs all its components to induce signaling [176].

TREM-2 is clearly involved in osteoclastogenesis, but the discrepancies between mice and humans in regard to TREM-2 deficiency remains puzzling. However, targeting TREM-2 in osteoporosis or osteopetrosis seems to be an interesting option for optimizing treatment of the two conditions.

5.4.6 B cells

B cells are lymphocytes that play a large role in the humoral immune response as opposed to the cell-mediated immune response that is governed by T cells. The abbreviation "B" comes from bursa of Fabricius that is an organ in birds in which avian B cells mature. The principal function of B cells is to make antibodies against soluble antigens. B cells are an essential component of the adaptive immune system.

5.4.6.1 TREM receptor family members expressed by B cells

B cells do not express TREM-1, TREM-2 and TREM-3 but they do express the TREM-like transcript TLT-2 [37]. B cells subpopulations express different levels of TLT-2. B1 cells express more TLT-2 than marginal zone/transitional 2 B cells, that in turn express more TLT-2 than transitional 1 B cells. The lowest TLT-2 levels were observed in follicular B cells [37]. TLT-2 does not associate with DAP12 and has no

clear signaling motif in its cytoplasmic domain [35]. Functionally it is still unclear what TLT-2 might trigger in B cells. Stimulation of splenic B cells with anti-TLT-2 mAb in soluble or plate-bound form did not potentiate survival, induce proliferation, or up-regulate expression of activation markers, including CD69 and CD86. But what TLT-2 really does is currently unknown. Its potential intracellular SH3 binding domain has not been demonstrated to bind SH3 yet. Its putative endocytosis sequence lacks functional data as well. The only clear result is that TLT-2 expression is up-regulated upon stimulation of neutrophils and macrophages with LPS, while TLT-2 expression is unaltered on B cells [37]. This TREM family member needs to be characterized further in order to identify its function on B cells, macrophages and neutrophils.

5.4.7 Platelets and megakaryocytes

Platelets or thrombocytes are the cell fragments circulating in the blood that are involved in the cellular mechanisms of primary haemostasis leading to the formation of blood clots. Platelets are produced in the bone marrow; the progenitor cell for platelets is the megakaryocyte. It is about twelve times larger than an erythrocyte, possesses a lobed nucleus and sheds platelets into the circulation. Platelets are highly reactive cells that carry large quantities of both soluble and cell-bound cargo in 2 principal types of secretory granules, α-granules and dense granules. Upon vascular damage, platelets become activated and release their granules containing highly reactive compounds and/or receptor. Activated platelets express high levels of CD62P that facilitate the rolling required for additional platelet receptors to further interrogate the vascular wall when damage is present.

5.4.7.1 TLT-1 expression in platelets and megakaryocytes

Platelets and megakaryocytes express the TREM-like transcript TLT-1 where it is located within the α-granules together with CD62P [33, 36]. Upon platelet activation TLT-1 is up-regulated, due to the translocation of the α-granules to the platelet surface. TLT-1 has been suggested to be an inhibitory TREM family member because it contains two ITIMs at Y245 and Y281. It also exists as a splice variant lacking these ITIMs [34, 54]. Upon cross-linking of TLT-1 the phosphatase SHP-2 is recruited to the classical ITIM sequence at Y281. However, cross-linking of TLT-1 enhances FcεRIγ-mediated calcium signaling, which is dependent on the recruitment of SHP-2 [36]. Platelet activation is one of the first steps in a cascade of thrombocytic events, including clot formation and retraction. Platelet activation induces the up-regulation of TLT-1 and the release of soluble TLT-1 [54]. Therefore, TLT-1 may play a role in maintaining vascular homeostasis and regulating coagulation and inflammation at sites of injury.

Platelets appear to have another role in the biology of TREM family receptors. A recent study by Haselmayer et al. suggest that platelets express a TREM-1 ligand, shown by the binding of TREM-1-Fc fusion proteins to the platelet surface [177]. The binding of the TREM-1 ligand on platelets to TREM-1 on neutrophils enhanced the effector functions of neutrophils induced by LPS. These findings shed new light on

TREM-1 and its role during innate inflammatory response to infections, and may contribute to the development of future concepts to treat sepsis.

5.5 Bacterial infections

Phagocytes, i.e. mainly neutrophils and macrophages, are important effector cells in the immune defense against bacterial infections. Phagocytosis and subsequent killing of the bacteria are their main function in eliminating the infection. Bacteria can be classified by a number of criteria, one being if they are extracellular or intracellular pathogens. Two pathogens that differ in this respect commonly used in infection models are *Escherichia coli* and *Samonella enterica*.

5.5.1 Escherichia coli

Escherichia coli is one of the main species of Gram-negative bacteria living in the lower intestines of mammals. When located in the large intestine, it actually assists with waste processing, vitamin K production, and food absorption. Besides these beneficial properties of the bacteria in the intestine, *E. coli* can also cause several intestinal and extra-intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, sepsis and pneumonia. Even though *E. coli* can cause inflammation, it is not able to replicate within macrophages. One of the main bacterial components that induces an inflammatory response is LPS. LPS is a highly conserved glycolipid and is a major component of the outer membrane of Gram-negative bacteria and is important for membrane stability. LPS is detected by immune cells through TLR4, a receptor that can induce strong inflammatory responses. The response of this receptor can be modulated in various ways, including activation of TREM family molecules.

5.5.2 Samonella enterica

In contrast with *E. coli*, *Samonella enterica* sv Typhimurium is a genetically tractable, facultative intracellular pathogen, whose capacity to cause systemic disease in humans and mice depends upon its ability to survive and replicate within macrophages and DCs [178, 179]. *S. enterica* can cause a variety of diseases ranging from mild gastroenteritis to severe systemic infections, including typhoid fever. Fever, headache, diarrhea, muscle pain, and enlarged liver and spleen due to bacterial replication in these organs characterize typhoid fever [180].

In mice and humans, *S. enterica* spreads via the faecal oral route. After the bacteria reach the distal ileum and caecum they start to invade M cells and endothelial cells of the intestinal epithelium and work their way through this epithelial barrier. In the underlying Peyer's patches they encounter resident phagocytic cells such as macrophages and DCs [181]. These cells actively phagocytose the bacteria and induce an inflammatory reaction, starting with the production of IL-8 and antimicrobial mechanisms such as the release of reactive oxygen intermediates. Even though macrophages and DCs play a crucial role in controlling a *S. enterica* infection, they also represent an important site for bacterial replication [182, 183].

S. enterica actively maintains itself within an intracellular vacuole, thereby shielding itself from an antibacterial activity of host macrophage cytosol. *S. enterica* controls the maturation of its vacuole, segregating itself from the macrophage degradative pathway. Like several other pathogens, *S. enterica* reduces the effectiveness of bacteriocidal and bacteriostatic free radicals generated by macrophages, by synthesizing enzymes and products that counteract them [184-188].

Reactive oxygen intermediates, produced during the oxidative burst, and NO production are the most important macrophage defence mechanisms during an infection with *Samonella enterica* sv Typhimurium [189-192]. These superoxides can pass through the outer membranes of Gram-negative bacteria and may damage periplasmic proteins, but are unable to penetrate through the cytoplasmic membrane. In the periplasm, however, they undergo spontaneous or enzyme-mediated dismutations to generate more reactive hydrogen peroxide, which is able to penetrate the cytoplasmic domain and enter the bacterial cytoplasm in order to damage vital bacterial proteins and induce bacterial death [193].

These two Gram-negative bacteria were used in this thesis to study the function of TRAPC during infection of macrophages.

6 Aims of the thesis

Our initial aim was to clone and characterize the murine homologue of human NKp44. At that time it was not known NKp44 existed in mice and the genome of the mouse was nearly completely sequenced. This provided the basis for the initial identification of a putative cDNA for murine NKp44. Soon after the cloning of the potential murine homologue of NKp44 we discovered that the identified cDNA was not expressed by NK cells, but was expressed on antigen presenting cells. We named this novel receptor Triggering Receptor expressed on Antigen Presenting Cells (TRAPC).

The specific aims of this thesis were to:

I. Clone and characterize the TRAPC receptor

II. Define the adaptor-signaling molecule associated with TRAPC

III. Analyze the expression profile and properties of TRAPC

IV. Determine the function of TRAPC in macrophages and DCs

V. Investigate the role of TRAPC during bacterial infection of macrophages

VI. Identify the ligand for TRAPC

The studies in this thesis were performed in collaboration with:

Jonas Sundbäck
Klas Kärre
Mikael Rhen
Eva Bjur
Daniel Ågren
Ingrid Helander
Caroline Helmstetter

7 Results and discussion

7.1 Cloning and genomic localization of TRAPC

7.1.1 Cloning of TRAPC

In the last 2 decades, a family of NK cell specific activating receptors has been identified in human, which mediate killing of human and mouse tumor cells in a MHC independent manner [194]. These natural cytotoxicity receptors (NCR) NKp46, NKp44 and NKp30 belong to the immunoglobulin superfamily. NCRs are selectively expressed on all NK cells and are associated with ITAM-containing signal transducing adaptor molecules CD3ζ, DAP12 or FcεRIγ [195]. NKp46 and NKp44 have been shown to bind the hemagglutinin of influenza virus and hemagglutinin-neuraminidase of parainfluenza virus, but due to tumor cell sensitivity also other ligands must exist [196, 197].

NKp44 is encoded within the TREM gene cluster and differs from the other TREM family molecules in that it is expressed on NK cells that are of lymphoid origin [27, 28]. NKp44 has recently shown to be expressed on IL3 stimulated plasmacytoid DCs [136]. The NCRs were defined as a group by function rather than genetic linkage; NKp46 and NKp30 are not encoded within the TREM gene cluster [195]. NKp44 and NKp30 have not been cloned in mouse, but it has been shown that human probes of these receptors hybridize to mouse genomic DNA in northern blots [28, 198], The only NCR identified in mouse is NKp46, (MAR-1 or Ncr1) [199], while murine NKp30 has been described as a pseudogene in *mus musculus* [200].

Murine expressed sequence tags (EST, NCBI database) were searched with the BLASTN algorithm program for sequences bearing homology with the human NKp44 cDNA sequence (AJ225109). One EST (BG148075) was selected that shared a high degree of sequence identity with the NKp44 sequence, particularly in exon 2. B searching the Celera Discovery System mouse genome database with a short stretch (71bp) of this EST that showed the highest homology to NKp44, a genomic sequence on chromosome 17 close to the recently identified TREM cluster was identified. Using the GENSCAN server (http://genes.mit.edu/GENSCAN.html) we found a putative 756 bp open reading frame coding for a protein homologous to NKp44. Based on this predicted gene sequence, RT-PCR was performed using mRNA extracted from organs of a C57BL/6 mouse. We amplified and cloned a cDNA containing a complete open reading frame of 780 bp coding for a 259 amino acid protein, henceforth called TRAPC (Triggering Receptor expressed in Antigen Presenting Cells) (Fig. 5).

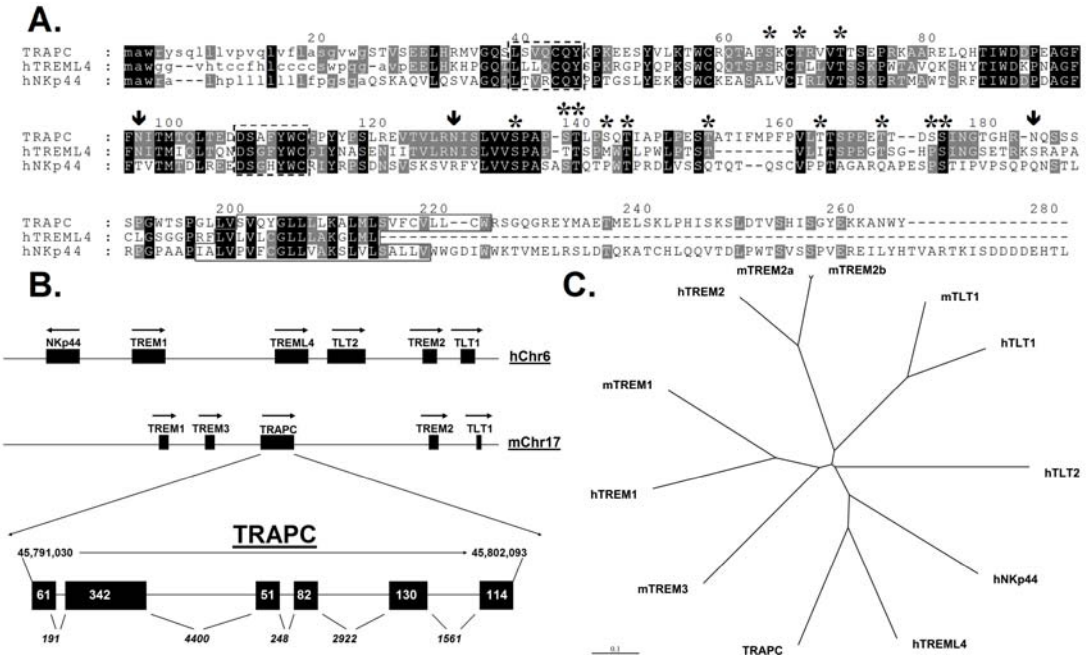


Figure 5. **Characterization of TRAPC.** (**A**) Alignment of predicted amino acid sequences of TRAPC (DQ186654) with NKp44 (HSA225109) and TREML4 (NM198153). Leader sequences are depicted in lowercase. Transmembrane domains are boxed. V-type immunoglobulindomain consensus motifs are boxed with arced lines. Potential O-glycosylation sites are marked with asterixes (*). Potential N-glycosylation sites are marked with arrows (↓). (**B**) Genomic localization and exon usage of TRAPC. Numbers are taken from the ENSEMBL database. (**C**) Phylogenetic analysis of human and murine proteins encoded within the TREM-cluster. The extent of the sequence relatedness between two proteins is shown by the total distance of the lines that connect those proteins.

7.1.2 Sequence analysis of TRAPC

The TRAPC protein is characterized by 24-residue signal peptide, an ectodomain of 171 amino acids with one putative V-type immunoglobulin like (Ig-V) domain formed by the cysteine residues in position 43 and 112, a 23-residue long transmembrane domain, and an intracellular part of 41 amino acids lacking any known signaling motifs. The transmembrane domain contains the positively charged amino acid Lysine (position 205) suggesting that TRAPC could signal via interaction with an adaptor molecule such as DAP12 that contains the negatively charged residue aspartic acid in its transmembrane domain. Two additional cysteine residues on position 58 and 66 are present within the predicted Ig-V domain of TRAPC. These may form an extra disulfide bridge as observed in the crystal structure of NKp44 [201, 202]. The TRAPC protein contains 3 potential N-glycosylation sites and 14 potential O-glycosylation sites.

Figure 5A shows the alignment of the full-length amino acid sequence of TRAPC with the full-length amino acid sequences of NKp44 and the human TREM-like transcript 4 (TREML4) [35]. TRAPC shows 40% sequence identity with TREML4 and 29% with NKp44, however, taking chemical characteristics of the amino acids in account, sequence homology is much higher, 51% and 45% respectively.

Sequence alignment of the predicted Ig-V domains alone of TRAPC, TREML4 and NKp44 reveals an even higher sequence identity (59% with TREML4 and 51% with NKp44) and homology (78% with TREML4 and 67% with NKp44). NKp44 has been shown to associate with DAP12 through interaction of the positively charged lysine in its transmembrane domain. This lysine is conserved in both TRAPC and TREML4.

The TRAPC gene consists of 6 exons spanning a total genomic region of 10102 bp. (Fig. 5B). A comparative phylogenetic analysis with the known TREM and TLT family members in human and mouse, showed the closest homology of TRAPC to TREML4 and NKp44 (Fig. 5C). Figure 5B also shows the genomic localization of the TRAPC gene based on the ENSEMBL database. The gene maps within the family of single Ig-domain receptors consisting of the TREM molecules and the TREM-like transcripts. The position of the gene corresponds to the position of the TREML4 gene on the syntenic human chromosome 6, the TREM family gene that is most homologous to TRAPC. The generic name of TRAPC in the common databases is also TLT4 and TREML4, in concordance with the predicted human TREML4 gene.

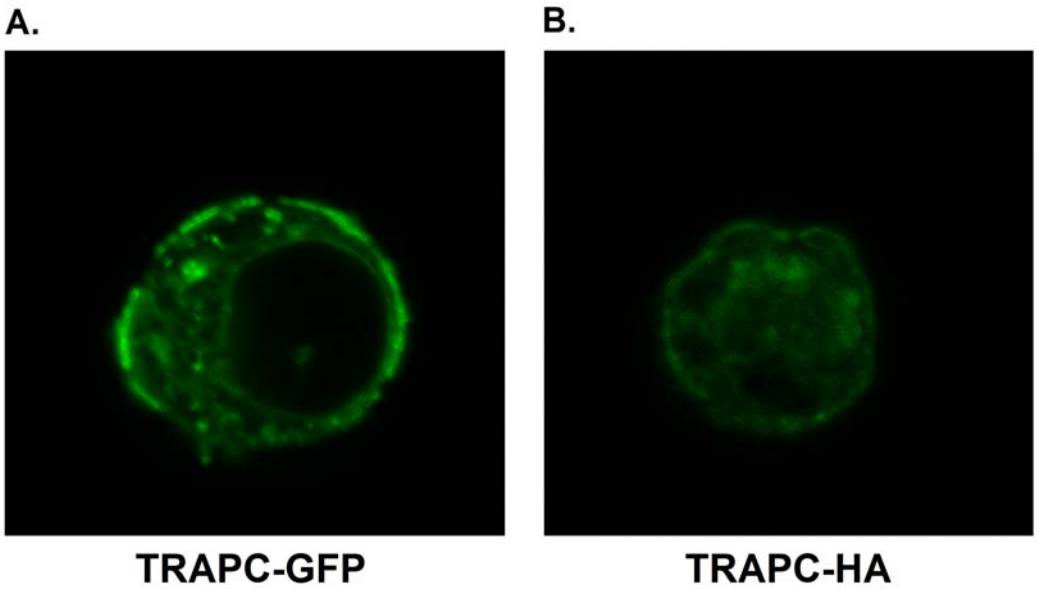


Figure 6. **Cell surface expression of TRAPC.** (**A**) 293T cells transiently transfected with C-terminal GFP tagged TRAPC. (**B**) 293T cells transiently transfected with N-terminal HA tagged TRAPC. Cells were stained with mouse anti-HA antibodies and secondary FITC anti-mouse antibodies. These figures are representatives from 3 independent experiments.

7.1.3 TRAPC is expressed as a transmembrane protein in transfectants

To verify that TRAPC was a cell surface expressed receptor, we subcloned the cDNA of TRAPC into the expression vector pEGFP-N3 as a fusion construct in frame with EGFP (EGFP linked C-terminal to TRAPC, i.e. intracellular EGFP) (details described in material and methods section). Transfection of 293T and CHO cells with the TRAPC-GFP fusion vector showed expression of GFP lining the cell membrane in confocal fluorescence microscopy, indicating that TRAPC was expressed on the cell surface (Fig. 6).

When GFP was transfected alone into 293T cells or CHO cell it is expressed throughout the whole cytoplasm, but not in the nucleus (data not shown). The cell surface expression was confirmed by transfecting 293T and CHO cells with a N-terminal HA tagged TRAPC vector, i.e. extracellular HA, and detecting cell surface expression of TRAPC-HA by flow cytometry and fluorescence microscopy with anti HA antibodies (Fig. 6). However, ín figure 6, there is also TRAPC expression inside the cytoplasm. This can be explained by the fact that we overexpressed TRAPC in these experiments and that the TRAPC observed in the cytoplasm is TRAPC still trapped in the Golgi apparatus, where it needs to be processed and glycosylated in order for it to appear at the cell surface.

Overall, based on the cloning, sequence analysis, genomic localization and the fact that TRAPC is expressed as a cell surface molecule we conclude that TRAPC is a new TREM family member.

Nomenclature within the existing genome databases can be rather confusing. Predicted genes are present under different names than the eventual cloned versions of the same gene and sometimes the same gene has been cloned by several groups and has therefore different names. This is the case for TRAPC, which has many different aliases in the mouse genome; MGI:1923239, 5031403H21Rik, IDCP1, Treml4 and TLT3, within the common accessible databases (Genbank, Ensembl, etc.). TRAPC is named TREML4 by homology to the human predicted TREML4 gene in the Ensembl database (Entrez gene GeneID: 224840), and is published in genbank under the name of IG-like domain containing protein c (IDCP1c), cloned from spleen RNA with different splice variants (genbank Acc No: AY 557629, AY 465530, AY 465531, AY 465532), however still unpublished in any journal. Recently TRAPC has also been termed TREM6 in a work by Hollyoake *et al*. [200].

The original aim of the study was to clone and characterize the murine NKp44 gene and protein. However, based on our findings regarding the cloning of TRAPC and on database searches, we conclude that an orthologue to hNKp44 does not exist in *mus musculus*. In a study by Hollyoake et al., where they investigate the presence of NKp30 in 12 different mouse strains, they also concluded that NKp44 does not exist in mouse [200].

Given that this thesis also describes the expression pattern of this TREM family receptor, we suggest that the name triggering receptor expresses in antigen presenting cells, TRAPC, should be used.

7.2 Association of TRAPC with adaptor molecules

7.2.1 Cloning of DAP12-FLAG, CD3ζ-FLAG and FcεRIγ-FLAG

TRAPC contains three tyrosines in the intracellular domain but it lacks any known signaling motif. TRAPC contains a positively charged amino acid in its transmembrane domain that was predicted to be used to associate with a signaling adaptor molecule, in similarity with other TREM family molecules.

To test this, we cloned and transiently transfected 293T cells with cDNA constructs containing HA-tagged TRAPC together with FLAG tagged cDNAs of the adaptor molecules DAP12, CD3ζ and FcεRIγ.

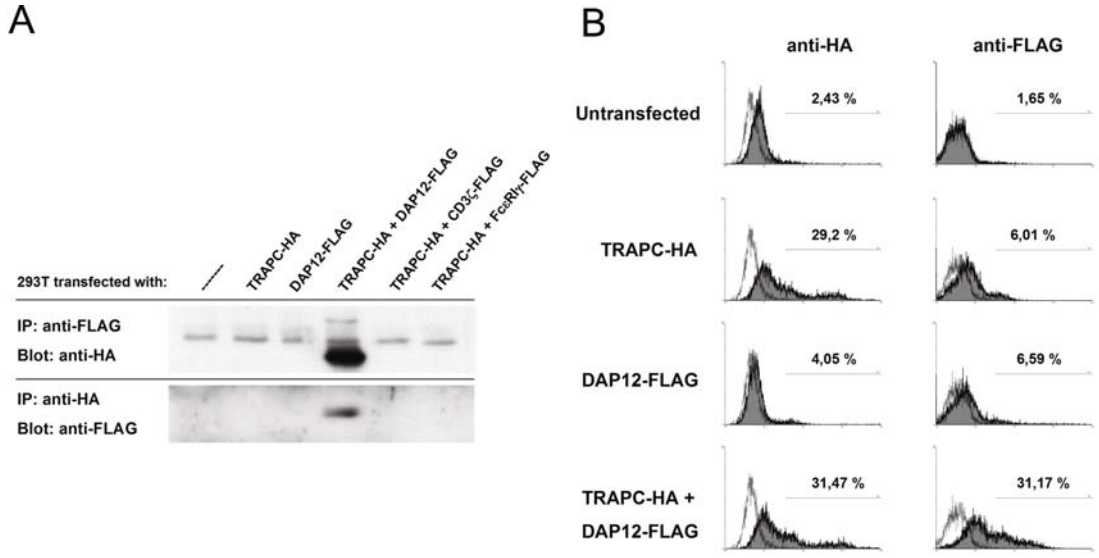


Figure 7. **TRAPC association with DAP12.** (**A**) 293T cells were transfected with the indicated plasmids. Immunoprecipitations were carried out either with anti FLAG or anti HA, and blotted with anti HA or anti FLAG as indicated. This is a representative of 3 independent experiments. (**B**) 293T cells were transfected with the indicated plasmids and stained with anti-HA and anti-FLAG antibodies and analyzed by flow cytometry. This is a representative of 6 independent experiments.

7.2.2 TRAPC associates with DAP12

Immunoprecipitation with an anti-HA-antibody and subsequent western blots revealed that TRAPC associated with DAP12, as shown by the presence of a FLAG specific ~14 kDa band. We did not detect any FLAG specific bands when TRAPC-HA was cotransfected with FLAG-tagged CD3ζ or FcεRIγ (Fig. 7A). In the reversed experiment we immunoprecipitated with anti-FLAG antibodies. A specific HA band (~37 kDa) was detected in the cell lysate from cells co-transfected with FLAG-DAP12 and HA-TRAPC, whilst the cell lysates from cells co-transfected with HA-TRAPC and FLAG-CD3ζ or FLAG-FcεRIγ showed no HA-specific band. As a control for the antibody specificity, non-immunoprecipitated lysates of the transfected cells were blotted and stained with both anti-HA and anti-FLAG antibodies. These blots revealed the appropriate bands for HA-TRAPC, FLAG-DAP12, FLAG-CD3ζ and FLAG-FcεRIγ (data not shown). We concluded that TRAPC associated with DAP12, similar to other activating TREM family molecules.

7.2.3 FACS analysis of TRAPC association with DAP12

The results obtained from the immunoprecipitation experiments were confirmed by flow cytometry analysis of transiently transfected 293T cells (Fig. 7B). The cell surface expression of DAP12 is dependent on the association with an appropriate receptor [31]. Co-transfection of 293T cells with HA-TRAPC and FLAG-tagged DAP12 showed a marked increased expression of cell surface FLAG-DAP12 compared to expression of FLAG-tagged DAP12 alone. These results showed that co-expression of TRAPC was needed to get cell surface expression of DAP12, and confirmed the association of TRAPC with DAP12 as shown with western blot.

Co-transfection of 293T cells with TRAPC-HA and FLAG-CD3ζ or FLAG-FcεRIγ did not increase the expression of FLAG-CD3ζ or FLAG-FcεRIγ compared to the levels of expression of FLAG-CD3ζ or FLAG-FcεRIγ when they were transfected into 293T cells alone (data not shown). The requirement for physical association between adaptor and receptor molecule is often mutual, i.e. receptors generally do not come to the cell surface in the absence of DAP12. However, the level of expression of HA-TRAPC was similar in the absence and presence of FLAG-DAP12, indicating that TRAPC does not need to physically associate with DAP12 in order to be expressed on the cell surface similar to e.g. Ly-49D [123], at least when it is over-expressed as in our *in vitro* experimental setting.

7.3 Properties of TRAPC and analysis of expression profile.

7.3.1 TRAPC is expressed on antigen presenting cells

To investigate the tissue distribution of TRAPC, nested RT-PCR was performed on RNA extracted from multiple organs of female C57BL/6 mice. As shown in figure 8 TRAPC could be detected in tissue from lymph node, liver, kidney, lung, uterus and spleen but not in brain.

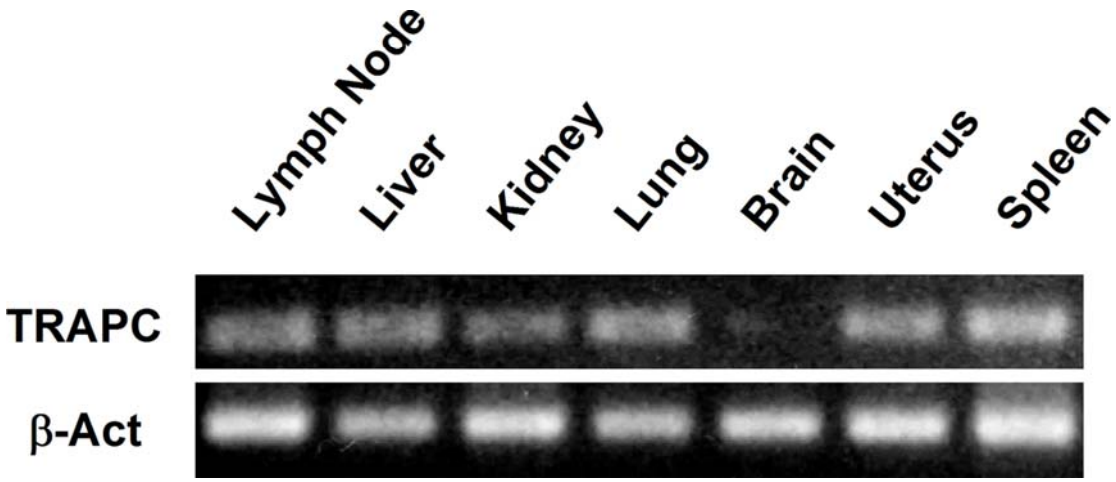


Figure 8. **Organ distribution of TRAPC.** Expression pattern of TRAPC measured by nested RT-PCR on RNA extracted from various tissues. This is a representative from 3 independent experiments.

Table I. Cellular expression of TRAPC[a]			
Origin	**Mouse strain**	**Culture conditions / name**	**TRAPC**
Cultured/Sorted primary cells:			
Dendritic Cell			
	BALB/C	**7d GMCSF bonemarrow DC**	**+**
	C57BL/6	**7d GMCSF bonemarrow DC**	**+**
	C57BL/6	**7d GMCSF CD11c+ (MACS)**	**+**
B cell			
	C57BL/6	**B220+ (FACS)**	**+**
	C57BL/6	**CD19+ (FACS)**	**+**
	C57BL/6	**CD19+ (MACS)**	**+**
Monocyte			
	C57BL/6	**CD14+ (MACS)**	**+**
Macrophage			
	C57BL/6	**F4/80+ (MACS)**	**+**
NK cell			
	C57BL/6	**5d IL-2 LAK**	**-**
	C57BL/6	**NK1.1+ CD3- (FACS)**	**-**
	NOD	**TMb-1+ CD3- (FACS)**	**-**
T cell			
	C57BL/6	**CD3+ NK1.1- (FACS)**	**-**
	NOD	**CD3+ TMb-1- (FACS)**	**-**
Cell lines:			
Dendritic Cell			
	BDF	**FSDC**	**+**
	DBA/2	**CB-1**	**-**
	BDF	**D2SC/1**	**+**
B cell			
	BALB/C	**WEHI 231**	**+**
	BDF	**70Z/3**	**-**
	BALB/C	**J558**	**-**
	BALB/C	**S194**	**+**
Macrophage			
	BALB/C	**RAW 264.7**	**+**
	BALB/C	**J774.1**	**+**
	C57BL/6	**SV40 MES-13**	**-**
Microglial			
	C57BL/6	**BV-2b**	**-**
Mast/Mast-Basophil			
	BALB/C	**MCP5/L**	**-**
	DBA/2	**IC-2**	**-**
	DBA/2	**P815**	**-**
T cell			
	C57BL/6	**RMA**	**-**
	C57BL/6	**ALC**	**-**
	C57BL/6	**CTLL-2**	**-**

[a] Detection of TRAPC expression by nested RT-PCR on RNA of sorted primary cells, cultured primary cells and cell lines of different origin. Sorting was performed either by FACS or MACS as indicated in the table. Purity of the different sorted cell populations were ~98% in all cases. The table represents overall data from 5 independent experiments

Expression of TRAPC mRNA was also analyzed in sorted primary cells by nested RT-PCR (Table I). When primary cells were sorted from murine spleens, either by magnetic beads (MACS) or by flow cytometry (FACS), TRAPC mRNA could be detected in B cells ($CD19^+$, $B220^+$), monocytes ($CD14^+$) and macrophages ($F4/80^+$), but not in T cells ($CD3^+$, $NK1.1^-$) or NK cells ($NK1.1^+$, $CD3^-$ or $TM\beta1^+$, $CD3^-$).To analyze the expression of TRAPC more extensively in NK cells we used splenocytes cultured for 5 days in IL-2 (lymphokine activated killer cells, LAK), a condition that mainly induces activated NK cells. These cultures were negative for TRAPC mRNA expression (Table I).

TRAPC mRNA could be detected in DCs derived from bone marrow cultured for 7 days in presence of GM-CSF. Since these cultures essentially but not exclusively consist of DCs, we isolated DCs with CD11c magnetic beads from these cultures. Also the isolated $CD11c^+$ DCs showed expression of TRAPC mRNA (Table I). We detected expression of TRAPC in B220 sorted flt3-ligand derived plasmacytoid DCs by RT-PCR and western blot, and in isolated primary microglia but not astrocytes by RT-PCR (data not shown).

Since sorted cells might not be 100% pure and contaminated with other cells, we verified the expression pattern of TRAPC in different cell lines by RT-PCR (Table I and Fig. 9). We detected TRAPC mRNA in 2 out of 3 DC lines, 2 out of 3 macrophage cell lines and in 2 out of 4 B cell lines. Cell lines of T-cell, mast cell, basophil and microglial origin that we tested were all negative for TRAPC mRNA using RT-PCR. From these results we concluded that TRAPC was expressed by antigen presenting cells.

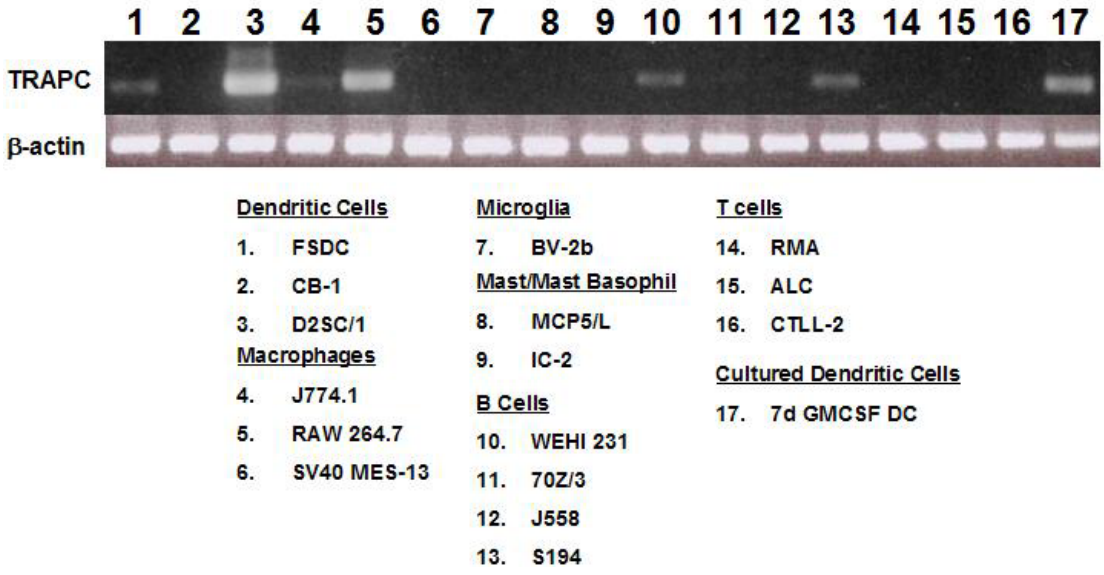


Figure 9. **Expression of TRAPC in cell lines.** Expression pattern of TRAPC measured by nested RT-PCR on RNA extracted from various cell lines. This is a representative from 5 independent experiments.

7.3.2 *Anti-TRAPC polyclonal antibodies*

In order to predict accessible epitopes of TRAPC to be used for producing antibodies, the 3D structure of TRAPC was modeled based on homology with the crystal structure of NKp44 (RCSB protein data bank (pdb) accession number 1HKF) using the software Deep View Swiss-PdbViewer [203] (Fig. 10).

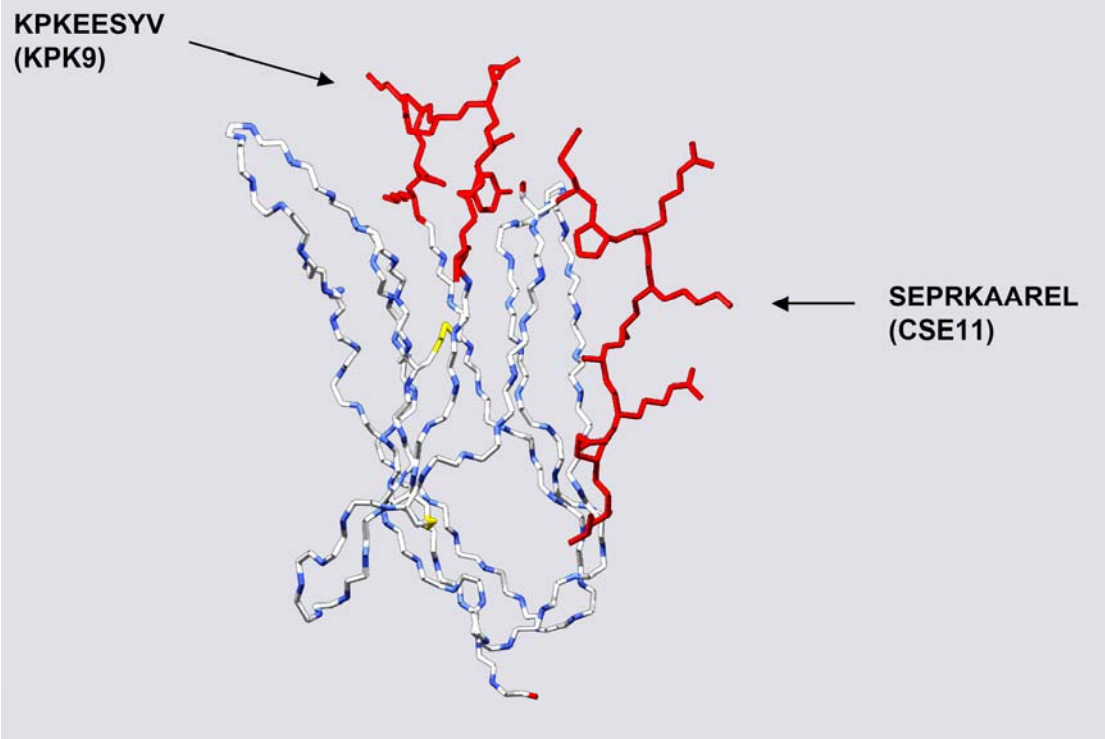


Figure 10. **3D model of TRAPC.** TRAPC was modeled according to the crystal structure of human NKp44 using Deep View Swiss-Pdb Viewer software. There are 3 loops in the structure of which 2 are depicted in red. These 2 loops have been selected to generate peptides from, i.e. KPK9 and CSE11, which were used to immunize rabbits and hens, in order to obtain polyclonal antibodies. In yellow, the 2 cysteines are depicted that form the crucial S-S bridges for the formation of the Ig-V domain.

Two predicted loops in the model were selected as peptide antigens for producing polyclonal antibodies. These peptides, KPKEESYVC (hereafter named KPK9) and CSEPRKAAREL (CSE11), were synthesized and used to immunize 1 rabbit (IgG) and 1 hen (IgY) respectively, in order to obtain antibodies that would work in both western blot and in flow cytometry with the benefit of avoiding FcR binding by using hen antibodies (IgY). High titers in the serum and the egg yolk were confirmed by ELISA. The polyclonal antibodies were affinity purified using the respective peptide used for immunization. Peptides and antibodies were made by/purchased from Agrisera (Umeå, Sweden).

7.3.3 FACS analysis of TRAPC expression profile

To investigate the cellular distribution of TRAPC as well as its characteristics we transfected 293T cells with TRAPC-GFP and compared the staining of these cells with the polyclonal antibodies compared to untransfected 293T cells. Figure 11A shows the specific staining of 293T cells transfected with TRAPC-GFP by KPK9 and CSE11 (both IgY). Both the rabbit IgG polyclonal antibodies also specifically stained the TRAPC transfected 293T cells (data not shown).

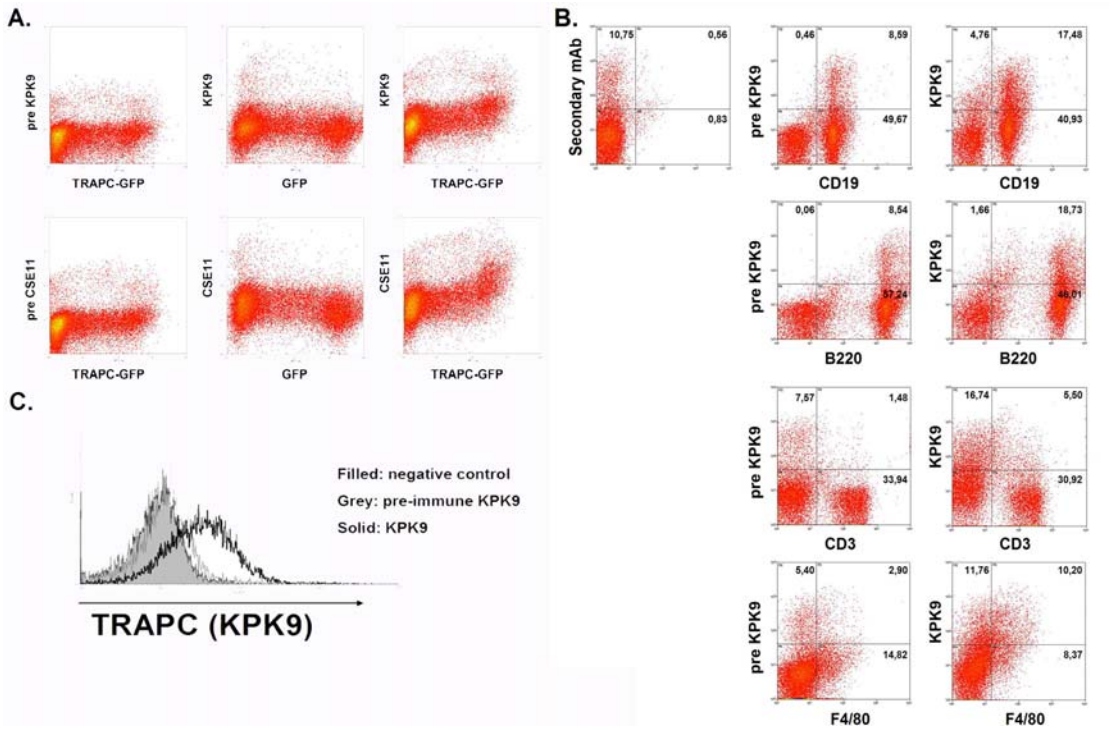


Figure 11. **Cell surface expression of TRAPC measured with polyclonal anti TRAPC antibodies.** (**A**) Analysis of anti-TRAPC polyclonal antibodies KPK9 and CSE11 (both IgY) and their respective pre-immune IgY on 293T cells 24 hours after transfection with TRAPC-GFP or GFP only. (**B**) Splenocytes stained with the polyclonal antibody KPK9 (IgY) together with anti CD3-FITC, anti-B220-PE, anti-CD19-FITC or anti-F4/80-FITC. (**C**) Bone marrow derived myeloid DCs (7 day GM-CSF culture) were stained with the polyclonal antibody KPK9 (IgY) and the respective pre-immune IgY. These figures are representatives from 6 independent experiments.

The anti-TRAPC polyclonal antibodies were used to analyze TRAPC expression on C57BL/6 splenocytes. Figure 11B shows that TRAPC was expressed on subsets of B-cells (both $CD19^+$ and $B220^+$), and macrophages ($F4/80^+$) using the polyclonal antibodies KPK9 (IgY), which is consistent with our RT-PCR data. A small subset of T-cells ($CD3^+$) stained positive for KPK9, but we could not detect TRAPC mRNA from isolated $CD3^+$ cell by RT-PCR (Table I). Staining with pre-serum was similar as staining with the secondary antibody control alone. Therefore we conclude that increase in staining (2 fold and more) represents specific TRAPC staining on the various celltypes.

To verify that TRAPC was expressed on the cell surface of myeloid DCs, we stained a 7 days GM-CSF culture of CD11c purified DCs with the polyclonal antibodies KPK9 (IgY) (Fig. 11C). All DCs stained positive for TRAPC, while the pre-serum staining was comparable to the secondary antibody only negative control.

7.3.4 TRAPC protein expression analyzed by western blot

To test the specificity of the polyclonal antibodies in western blots, we compared CSE11 (IgY) staining of cell lysates from 293T cells transiently transfected with TRAPC-HA with untransfected 293T cells. CSE11 stained only TRAPC-HA transfected cells (Fig. 12A). When the blot was stripped and re-probed with a monoclonal antibody against HA, identical bands of 37 kDa and 60 kDa were detected as in the CSE11 staining. In addition, CSE11 (IgG) specifically stained highly purified recombinant extra cellular domain of TRAPC, 12 kDa, produced in *E. coli* (Fig. 12B).

In agreement with the RT-PCR data we detected protein of TRAPC in lysates of the murine macrophage cell lines J774.1 and RAW 264.7 and in lysates of bone marrow derived DCs using the CSE11 (IgG) antibody (Fig. 12B), but not in lysates from the murine T cell line 2B4. All four polyclonal antibodies, raised against two different peptides of TRAPC, showed similar staining of cell lysates from the macrophage cell line J774.1 (Fig. 12B), where CSE11 (IgG) gave the clearest staining and was chosen for subsequent experiments.

When using the polyclonal antisera against TRAPC in reducing western blots, we detected bands of 37 and 60 kDa that is heavier than the predicted molecular weight of 26 kDa of TRAPC, and we could not detect any 26 kDa band in our blots. TRAPC contains several potential N- and O-glycosylation sites (Fig. 5A). To determine the influence of these glycans on the migration of TRAPC, cell lysates from LPS stimulated macrophage cell line J774.1 were enzymatically deglycosylated. Blotting with CSE11 (IgG) demonstrated a shift in the bands when compared to non-treated cell lysates (Fig. 12C), showing that TRAPC was indeed glycosylated. The in the kit enclosed fetuin was used to ensure the function of the deglycosylation kit.

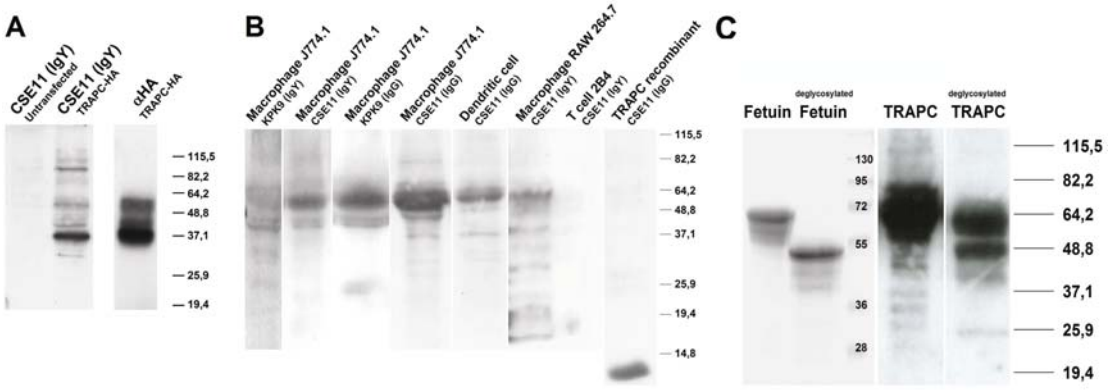


Figure 12. **Westernblot analysis of TRAPC expression in cell lines and primary cells.** (**A**) Polyclonal antibody CSE11 (IgY) specifically recognizes TRAPC-HA. Equal amounts of total cell lysates of TRAPC-HA transfected 293T cells were used in western blot. This is a representative from 3 independent experiments. (**B**) Detection of TRAPC by polyclonal antibodies in total cell lysates of the macrophage cell line J774.1 and RAW 264.7, bone marrow derived DCs cultured for 10 days with Flt3L, T cell line 2B4, and purified recombinant TRAPC. This is a representative from 5 independent experiments. (**C**) Total cell lysates of the cell line J774.1 stimulated with 10 ng/ml LPS were deglycosylated using a kit that removes N- and O-linked sugars. Membranes were stained with CSE11 (IgG). The fetuin control of the deglycosylation kit has been included. This is a representative from 4 independent experiments.

Cell lysates from HA-TRAPC transfected cells showed similar patterns when blotted with the polyclonal anti-TRAPC antibodies as when blotted with anti-HA antibodies, which showed that the polyclonal antisera recognized TRAPC specifically. This combined with the similar TRAPC detection of all four polyclonal antisera in cell lysates from J774.1 and RAW264.7, the fact that the 60 kDa band is also present with the anti-HA antibody (see Fig. 12A) and that all four polyclonal antisera specifically recognized recombinant TRAPC, convinced us that the polyclonal antisera were indeed specific for TRAPC and that the size differences are due to post-translational mechanisms.

TRAPC contains three potential N-glycosylation sites and fourteen potential O-glycosylation sites that may account for the 37 kDa band but are unlikely to explain the 60 kDa band. After deglycosylation of a full J774.1 cell lysate and subsequent reducing western blotting using the polyclonal antisera a weak 26 kDa band could be detected, corresponding to the predicted molecular weight of TRAPC. However an additional 50 kDa band appeared and part of the 60 kDa band remained. The deglycosylation assay was repeated 3 times and the same band pattern (26, 50 and 60 kDa bands) was detected in all cases and for all 4 different polyclonal antibodies, while the control protein was fully deglycosylated in all instances.

We cannot explain why the 60 kDa TRAPC band remained after deglycosylation since the control fetuin protein was fully deglycosylated, but we believe it is unlikely that the detected 60 kDa band represents unspecific staining by all 4 anti-TRAPC polyclonal antibodies. Proteins may behave different in these experiments when using complex cell lysates, while the control included in the kit is a pure protein optimally deglycosylated by the use of the kit. The 60 kDa band may represent a fully glycosylated TRAPC that, due to the presence of other proteins in a full cell lysate and potential different chemical properties of the lysate and/or the TRAPC molecule itself, could not be deglycosylated by the use of the deglycosylation kit in question.

Another possibility would be that TRAPC form homo- or hetero-dimers. However, it remains questionable if TREM family molecules can multimerize. The majority of TREM receptors are described as monomers, except TLT-1 that has been suggested to form a homodimer [34, 54]. There is debate if TREM-1 forms a dimer in solution. One crystal structure of human TREM-1 predicts a head to tail dimer, achieved through domain swapping [57], while different crystal structures of both human and murine TREM-1 contradict this finding and predict a monomeric state for TREM-1 in solution [55, 56].

Based on the presence of the 60 kDa band and the 50 kDa band after deglycosylation in our western blots it might be possible that TRAPC forms a dimer on the cell surface, may it be homo- or heterodimers, under certain circumstances. Interestingly, TRAPC contains two cysteines in the transmembrane domain that could be involved in dimerization. A helical wheel prediction of the transmembrane region of TRAPC predicts these two cysteines to be positioned right above each other (Fig. 13). It could be envisioned that these two cysteines interact with cysteines in another TRAPC protein or in a different protein, forming strong disulphide bridges, which aid in the dimerization of TRAPC.

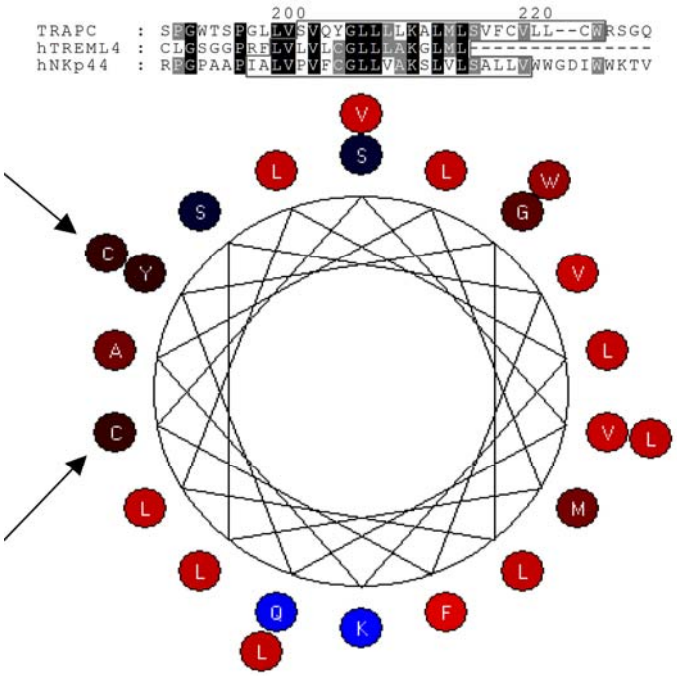


Figure 13. **Helical wheel prediction of TRAPC transmembrane domain.** Helical wheel prediction of the transmembrane region of TRAPC was performed using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Transmembrane regions of TRAPC, hTREML4 and hNKp44 are depicted and boxed. The cysteines residues are indicated with arrows.

Intra-membrane disulphide bridge dependent dimerization has been described before [204]. However, disulphide bridges can be dissociated under reducing conditions. Nevertheless, under our reducing conditions the predicted 60 kDa dimer was not completely dissociated, even when the samples were boiled for five hours and in the presence of iodoacetamide (data not shown). This indicates that if a dimer is formed it is extremely difficult to dissociate the proteins, which has been described for other disulphide linked proteins [205].

It remains to be elucidated if TRAPC may form a homo- or heterodimer. This is an intriguing question to solve, since it may have implications for its function and could potentially give clues to with which ligand(s) TRAPC may interact.

7.4 Functional analysis of TRAPC in macrophages and DCs

7.4.1 LPS up-regulates TRAPC expression on macrophages

Already in the report of the cloning of the first TREM family member, TREM-1, it was shown that its expression could be increased by treating cells with the bacterial endotoxin LPS. To investigate if it was possible to regulate the expression of TRAPC, we examined the effect of LPS treatment of the murine macrophage cell line J774.1. Using the CSE11 (IgG) antibody in western blot, TRAPC expression was up-regulated in a dose dependent manner in J774.1 cells (Fig. 14A). As a control that equal amounts of protein per sample were loaded on the western blot, the membranes were stripped and subsequently incubated with anti-actin antibodies.

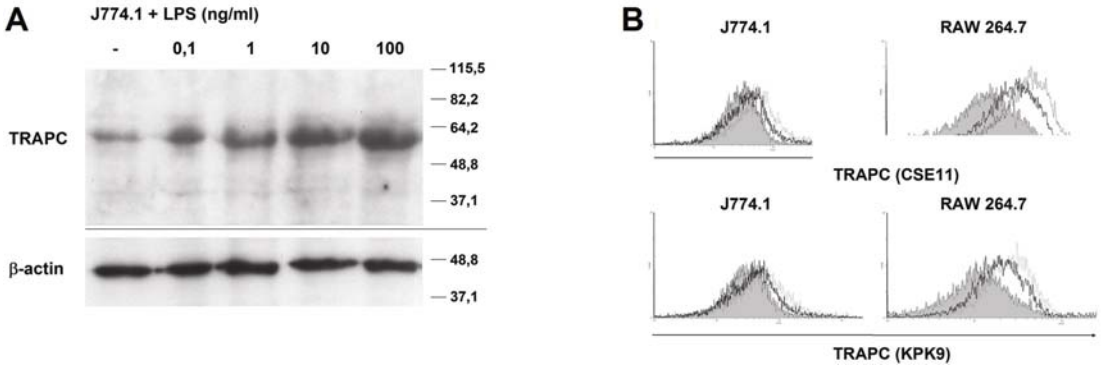


Figure 14. **TRAPC is up-regulated in macrophages upon LPS stimulation.** (**A**) J774.1 cells were stimulated for 16 hours with 0.1-100 ng/ml LPS. Equal protein amounts of cell lysates were loaded on reducing 12% SDS-PAGE gels and the membranes were incubated with CSE11 (IgG). This figure is a representative of 3 independent experiments. (**B**) J774.1 and RAW 264.7 cells were stimulated with either 100 ng/ml LPS (grey line), 10 ng/ml LPS (black line) or unstimulated (filled histogram) and cell surface expression levels of TRAPC were measured by flow cytometry using the polyclonal antibodies KPK9 (IgY) and CSE11 (IgY). When treated with 1, 0.1 and 0.01 ng/ml LPS the levels of TRAPC were slightly increased in a dose dependent manner as compared to the untreated control (data not shown). This figure is a representative of 4 independent experiments.

In addition, we showed by flow cytometry that TRAPC was expressed on the cell surface of the macrophage cell lines J774.1 and RAW 264.7, and that LPS treatment up-regulated the expression levels in a dose dependent manner (Fig. 14B). The LPS-induced up-regulation of TRAPC on RAW 264.7 was consistently higher than on J774.1, which correlated with the activation status as measured by NO production (data not shown). These results showed that TRAPC expression could be regulated by a bacterial product, suggesting that TRAPC may play a role during infections.

7.4.2 TRAPC cross-linking activates macrophages and DCs

To analyze the function of TRAPC on macrophages and DCs we used the polyclonal antibodies KPK9 and CSE11 (both IgY to avoid FcR binding) coated on plastic surface to crosslink TRAPC. After 16 hours of stimulation, nitric oxide levels were measured in the supernatant of the macrophage cell line J774.1 through the indirect Griess reagent method that measures nitrite. Cross-linking of TRAPC by both antibodies induced production of NO as compared to the macrophages cultured on the plate bound pre-immune serum or uncoated wells (Fig. 15A). However, the production of NO via TRAPC cross-linking was not as potent as stimulating the macrophages with LPS, which was used as a positive control.

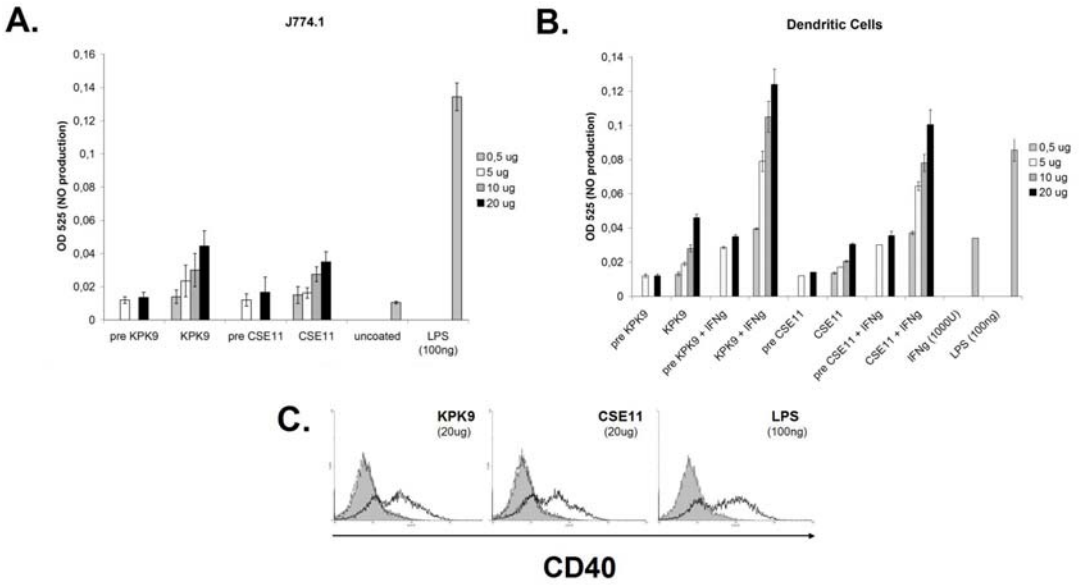


Figure 15. **TRAPC cross-linking induces NO production in macrophages and DCs.** (**A**) NO production after cross-linking of TRAPC on J774.1 cells by increasing amounts of the polyclonal antibodies KPK9 (IgY) and CSE11 (IgY). As a positive control J774.1 cells were stimulated with 100 ng/ml LPS. (**B**) NO production after cross-linking of TRAPC on primary bone marrow derived myeloid DCs (7 day GM-CSF culture) by increasing amounts of the polyclonal antibodies KPK9 (IgY) and CSE11 (IgY). For A and B mean values of 3 independent experiments using triplicates are displayed. (**C**) CD40 expression by DCs upon cross-linking of TRAPC with 20 µg/ml of the polyclonal antibodies KPK9 (IgY) and CSE11 (IgY) or 100 ng/ml LPS analyzed by flow cytometry. This is a representative from 3 independent experiments.

Cross-linking of TRAPC on sorted DCs triggered production of NO to levels comparable with the macrophages (Fig. 15B). The respective pre-immune sera did not stimulate NO production. Moreover, cross-linking of TRAPC in the presence of 1000 units IFNγ greatly enhanced the production of NO compared to IFNγ as a single stimulus. This suggests that IFNγ stimulation synergizes with the triggering of TRAPC, as seen also for other stimuli [206]. The expression of the activation markers CD40, CD80, CD86 and MHC class II were also analyzed on the DCs. The expression level of CD40 was increased upon cross-linking of TRAPC by both KPK9 and CSE11 polyclonal antibodies, while stimulation with the respective pre-immune sera in the same concentrations did not show this increase (Fig. 15C). LPS treatment resulted in similar CD40 expression levels as cross-linking of TRAPC.

The already high expression levels of CD80, CD86 and MHC class II remained constant (data not shown).

To summarize the results described so far, we have presented a novel activating murine TREM like receptor that is expressed on leukocytes of both myeloid and lymphoid origin, which we have named triggering receptor expressed in antigen presenting cells (TRAPC).

We showed that TRAPC is associated with DAP12, but not with CD3ζ or FcεRIγ. DAP12 is expressed in a wide variety of haematopoietic cells and several different cell surface receptors are associated with DAP12 to form a signaling complex. Monocytes and macrophages express many DAP12 associated receptors. The majority of these receptors, i.e. TREM-1, TREM-3, IREM-2, CD200R3, MAIR-II, MDL-1 and SIRP-β1, have been shown to induce production of NO and TNFα upon cross-linking [31, 44, 207-210].

Cross-linking of TRAPC on macrophages induced a NO response that correlated with the amount of triggering stimuli. We showed that TRAPC is up-regulated upon LPS stimulation on macrophage cell lines.

The regulation of TRAPC expression by LPS, and the ability of TRAPC to induce NO production, is common to other TREM receptors and other DAP12 associated molecules, e.g. TREM-1 expression is up-regulated by LPS stimulation and it is involved in regulating immune response during bacterial infections [24]. It will be of importance to identify all receptors involved in DAP12 signaling and to identify their ligands in order to fully understand how an inflammatory response is regulated.

DCs accumulate in mucosal tissue and skin in DAP12 loss of function mice and DAP12 knock out mice. These mice have an impaired contact sensitivity [124] and fail to develop experimental autoimmune encephalomyelitis [123] while DAP12 transgenic mice have an increased susceptibility to endotoxic shock and an enhanced resistance to infection with certain intracellular bacterial infections [125].

DCs express the DAP12 associated receptors TREM-2, CD200RLa, CD200RLb and PIRL-β [29, 207, 211] TRAPC is expressed on bone marrow derived DCs, and cross-linking of TRAPC on these cells up-regulated the costimulatory molecule CD40, a sign of DC activation. Similar to the macrophages, we observed a dose dependent increase of NO production upon cross-linking of TRAPC on the DCs. Moreover, NO production by the DCs was markedly increased when adding IFNγ during the cross-linking of TRAPC, showing that TRAPC engagement could be important during an inflammatory response. We suggest that a contributory factor in the phenotype of DAP12 deficient mice may be due to lack of a functional TRAPC receptor.

B cells express the DAP12 associated receptors MAIRII and Ly49D, but to our knowledge TRAPC is the first DAP12 associated TREM receptor that is expressed in B cells. Recently it was shown that the TREM family molecule TLT-2 was also is expressed in B cells, but this receptor is not associated with DAP12 [37]. Ly-49D is expressed on immature B cells and binding to its ligand MHC class I induces the secretion of low levels of IFNγ [212]. MAIRII is expressed on marginal zone B cells and the expression is up-regulated upon LPS stimulation [209].

Staining of C56BL/6 splenocytes with the polyclonal antibody KPK9 (anti-TRAPC) and the B cell marker CD19 showed that a subpopulation of B cells express TRAPC on the cell surface (Fig. 11B). It will be important to further characterize the function of TRAPC in B cells.

In conclusion, we show that TRAPC is a DAP12 associated receptor that is expressed in antigen presenting cells, and that LPS regulates the expression of TRAPC. Cross-linking of TRAPC on macrophages and DCs induces NO production, and up-regulates expression of CD40 on DCs. TREM receptors have an important role in amplifying the immune response during infections, and in regulating bone formation and housekeeping in the brain. The expression pattern, the ability to be regulated by LPS, and the activating capabilities of TRAPC suggest that it is involved in innate immune responses. We were therefore interested in the function of TRAPC during the onset of an immune response, in the defence against microorganisms. That is why we continued to study the function of TRAPC in an *in vitro* model of bacterial infections of macrophages.

7.5 Fine tuning of macrophage anti-bacterial responses by TRAPC

7.5.1 Triggering of TRAPC on J774-A.1 cells decreased NO production during E. coli infection

We showed that cross-linking of TRAPC on murine macrophage-like cells and primary murine DCs resulted in production of NO. Additionally, cross-linking of TRAPC on DCs in the presence of IFNγ was shown to greatly enhance the production of NO compared to when cells were treated with IFNγ alone. These results, in combination with the fact that macrophage production of NO is an important antibacterial host defense mechanism, intrigued us to study whether cross-linking of TRAPC would have any biological relevance during bacterial infection of macrophages.

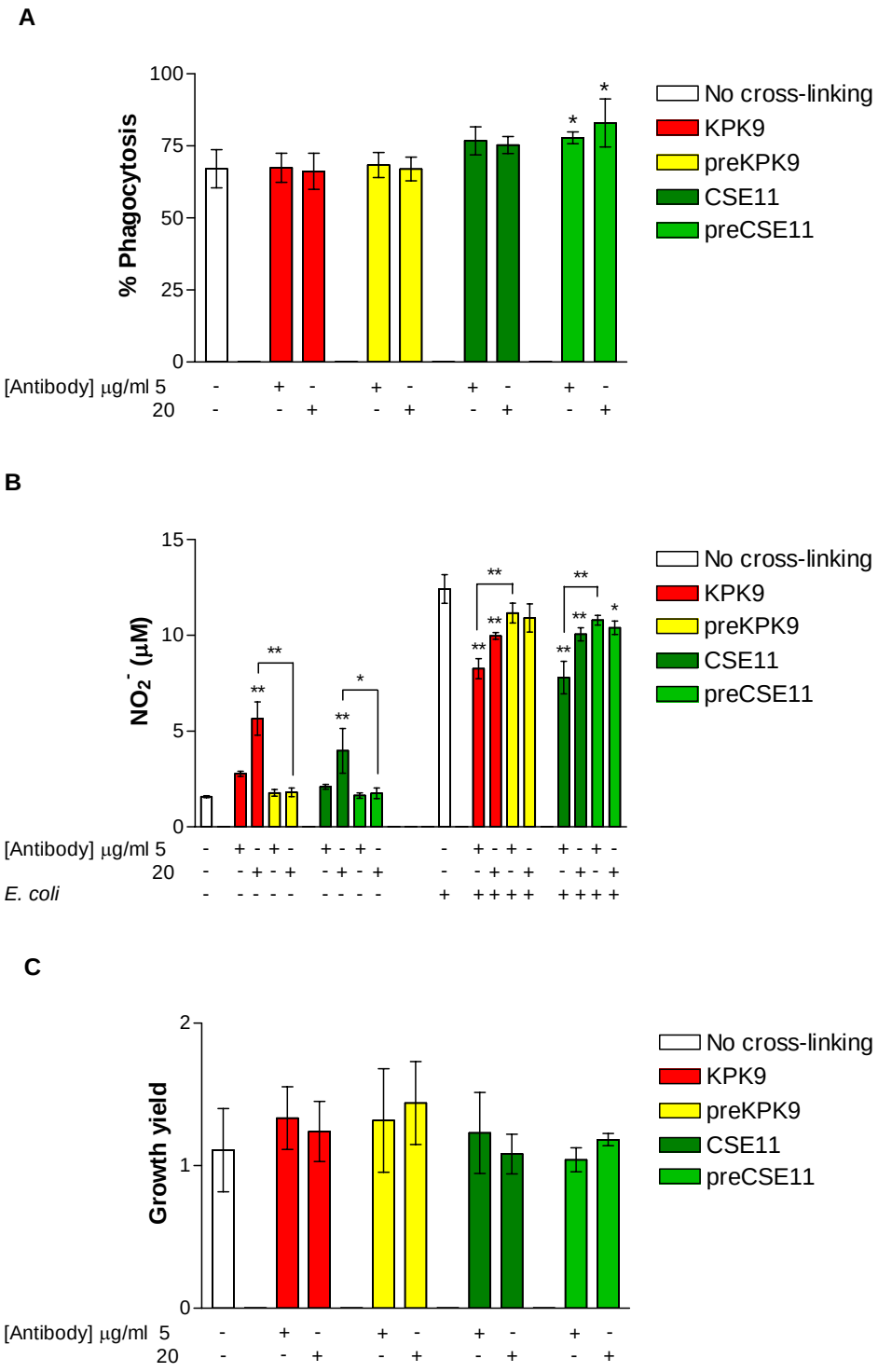


Figure 16. **Cross-linking of TRAPC inhibits host cell NO production during infection of J774-A.1 with *E. coli*.** Cells were infected with *E.coli* TG1 and the effect of TRAPC cross-linking on (**A**) phagocytosis, (**B**) NO induction and (**C**) bacterial replication were studied. 1h post infection cells were treated with gentamicin (50 µg/ml) for 45 minutes to kill extracellular bacteria. At 2h and 16h post infection, cells were lysed for measurement of phagocytosis efficacy (cfu counts at 2h/cfu counts seeded on the cells) and growth yields (cfu counts at 16h/cfu counts at 2h) respectively. NO production was measured 16h post infection. * $p < 0.05$ ** $p < 0.001$ in a two-sided T-test with Bonferroni corrections n = 3. In **B**, unless otherwise indicated, all non-infected samples were compared to the non-cross-linked non-infected control and all infected samples were compared to the non-cross-linked infected control. Additionally, in **B** all infected samples differed significantly from the respective uninfected control with a p-value < 0.001 and for all samples cross-linked with KPK9 or CSE11 the difference between the high and the low antibody concentration was significant with a p-value < 0.001 for uninfected KPK9 and a p-value < 0.05 for all other samples.

The macrophage-like cell line J774-A.1 was grown in 24-well cell culture plates coated with two different concentrations (5 or 20 µg/ml) of affinity-purified hen antibodies (IgY), KPK9 or CSE11, directed against two separate peptide epitopes of TRAPC. Isolated pre-immune IgY served as the negative control. The cells were cross-linked for 4h and subsequently infected with the non-pathogenic *E. coli* strain TG1.

Cells were lysed 2 and 16h post infection to determine phagocytosis efficacy (cfu at 2h/cfu seeded on the cells) and bacterial growth yield (cfu at 16h/cfu at 2h) respectively. Macrophage production of NO was measured as accumulation of nitrite in the cell culture medium 16h post infection.

The bacterial uptake was highly comparable for cells grown in coated or non-coated wells (Fig. 16A). A statistically significant minor increase in phagocytosis efficacy, compared to non-coated controls, was observed for cells grown in preCSE11-coated wells (Fig. 16A). However, considering the low magnitude of the increase it is unlikely to be biologically relevant.

In accordance with our previous observations, an increase in NO levels was observed for cells cross-linked with KPK9 or CSE11 compared to non-cross-linked controls (Fig. 15). Infection of non-cross-linked J774-A.1 cells with *E. coli* TG1 resulted in a substantial production of NO (Fig. 16B), and similar levels of NO were produced in infected cells grown in pre-immune IgY-coated plates.

Surprisingly, cross-linking with anti-TRAPC antibodies during infection resulted in a significantly decreased NO production, as compared to the infected non-cross-linked control, and to infected cells coated with pre-immune IgY (Fig. 16B). Furthermore, the decrease in NO production was more pronounced when cross-linking TRAPC with the lower concentration of antibodies. As *E.coli* is not adapted to grow inside macrophages it was not surprising that *E. coli* TG1 was unable to replicate in J774-A.1 cells both in presence and absence of cross-linking (Fig. 16C).

7.5.2 Cross-linking of TRAPC allows increased replication of a facultative intracellular pathogen

Expression of NO by phagocytic cells constitutes a growth-suppressing antimicrobial effector mechanism against many intracellular pathogens. Since the laboratory-adapted *E. coli* TG1 does not replicate in murine macrophage-like J774-A.1 cells (Fig. 16C), it was not possible to determine whether the differences in NO production had any significant impact on bacterial intracellular replication. *S. enterica* serovar Typhimurium is a facultative intracellular pathogen that replicates in phagocytic cells [182, 213]. The intracellular replication of *S. enterica* serovar Typhimurium is controlled by host cell production of NO in J774-A.1 cells [189, 192]. Therefore *S. enterica* serovar Typhimurium was used as a model bacterium to study whether TRAPC cross-linking could affect intracellular bacterial replication.

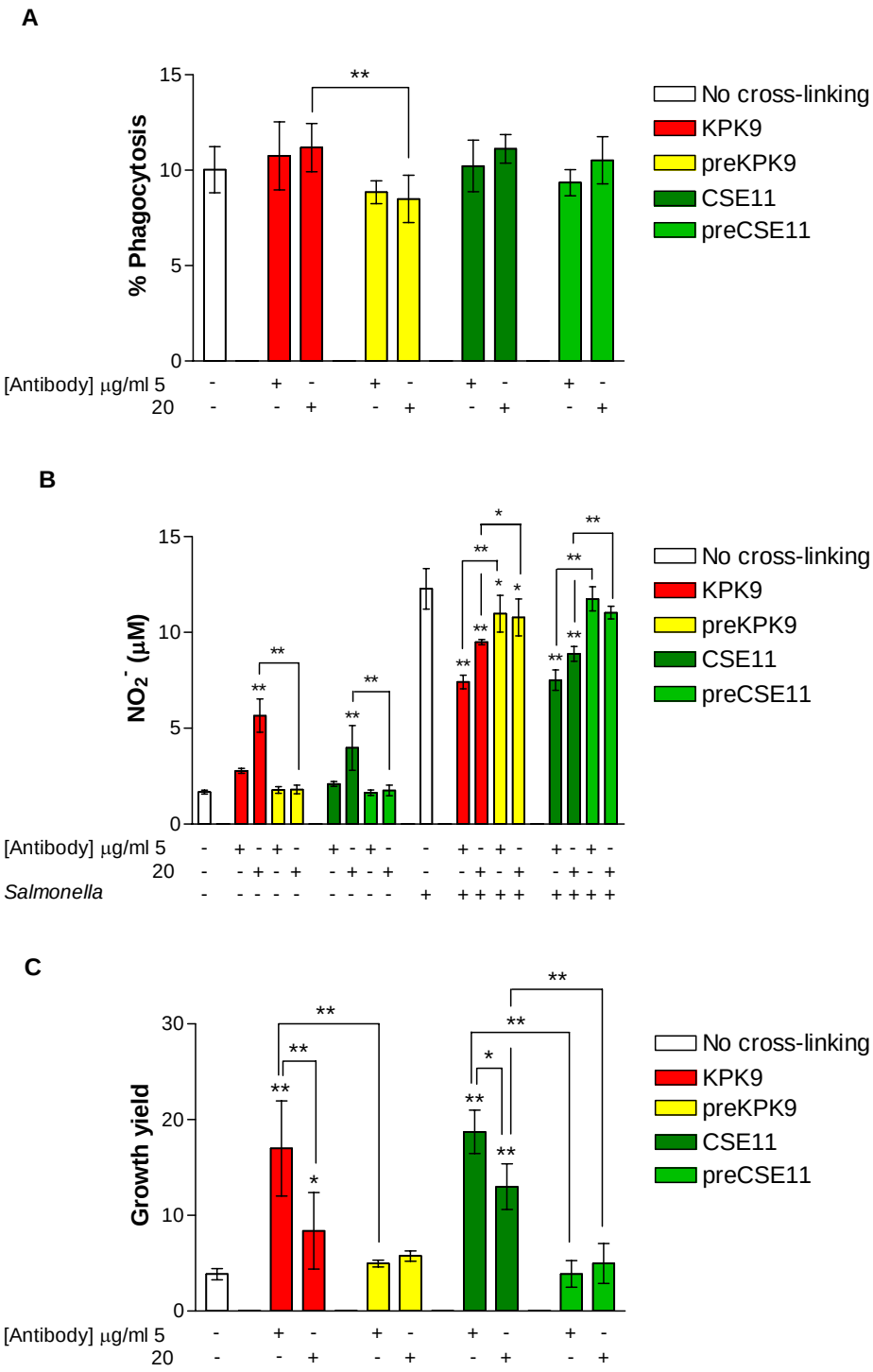


Figure 17. **Cross-linking of TRAPC inhibits host cell NO production and promotes bacterial replication during infection of J774-A.1 with *S. enterica* serovar Typhimurium.** Cells were infected with *S. enterica* serovar Typhimurium. The effects of TRAPC cross-linking on phagocytosis (**A**), NO induction (**B**) and bacterial replication (**C**) were studied. Cells were treated as described in Fig. 1 and lysed for measurement of phagocytosis efficacy (cfu counts at 2h/cfu counts seeded on the cells) and growth yields (cfu counts at 16h/cfu counts at 2h) at 2h and 16h post infection respectively. NO production was measured 16h post infection. In **B**, unless otherwise indicated, all non-infected samples were compared to the non-cross-linked non-infected control and all infected samples were compared to the non-cross-linked infected control. * $p < 0.05$ ** $p < 0.001$ in a two-sided T-test with Bonferroni corrections $n \geq 3$. Additionally, in **B** all infected samples differed significantly from the respective uninfected control with a p-value < 0.001 and for all samples cross-linked with KPK9 or CSE11 the difference between the high and the low antibody concentration was significant with a p-value < 0.05 for infected CSE11 and a p-value < 0.001 for all other samples.

As in the experiment using *E. coli* TG1, only a minor effect on *S. enterica* phagocytosis efficacy was observed (Fig. 17A). Cross-linking with preKPK9 resulted in a statistically significant minor decrease in phagocytosis compared to cross-linking with KPK9. However, the decrease was small and not statistically significant compared to non-cross-linked control. Furthermore it did not parallel the phagocytosis of *E. coli* TG1 in KPK9-coated wells (Fig. 17A).

Cross-linking of TRAPC with either KPK9 or CSE11 caused suppression of NO production by infected J774-A.1 cells at 16 hours post infection. As in the *E. coli* experiment, a slight decrease in NO levels was observed during infection of cells treated with pre-immune IgY compared to the non-cross-linked infected control. However, as in the experiment with *E. coli*, it was not as pronounced as the decrease obtained by cross-linking with KPK9 or CSE11. Again the NO levels obtained with KPK9 and CSE11 during infection were significantly lower, particularly at the lower concentration of cross-linking antibodies, compared to those obtained with infected cells treated with the corresponding pre-immune IgY (Fig. 17B). Although treatment with pre-immune IgY caused a slight decrease in NO production it did not affect the bacterial growth yields (Fig. 17C). In contrast, cross-linking of TRAPC with KPK9 or CSE11 caused a substantial and significant increase in bacterial growth yield 16h post infection compared to the non-cross-linked controls and cells treated with the pre-immune IgY (Fig. 17B and C). Thus, cross-linking of TRAPC resulted in an increased bacterial proliferation that correlated inversely with the NO production by the infected cells.

7.5.3 Cross-linking of TRAPC caused suppression in the NO response of J774-A.1 cells during stimulation with LPS

LPS is one of the major activating molecules involved in Gram-negative bacterial infections of macrophages. We therefore tested whether triggering of TRAPC on J774-A.1 cells could affect LPS mediated responses. The J774-A.1 cells were cross-linked for 4h as described above and subsequently stimulated with LPS at different concentrations. The effect on NO production was analyzed after 16h of stimulation. In concordance with the infection experiments, cross-linking with anti-TRAPC antibodies resulted in a decreased NO production during LPS treatment as compared to the non-cross-linked control, and to cells treated with pre-immune IgY for all but the lowest LPS concentration (Fig. 18). This implies that TRAPC triggering may affect the TLR4 signaling pathway.

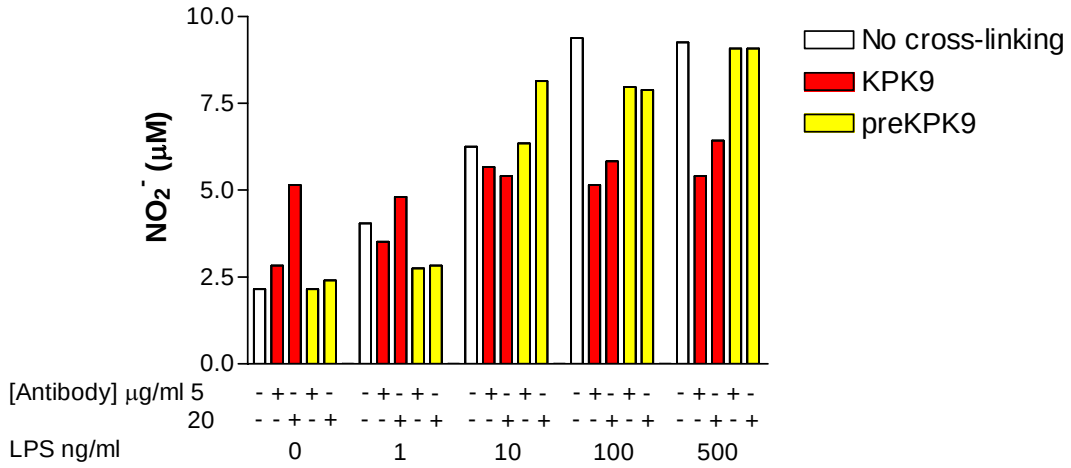


Figure 18. **Cross-linking of TRAPC inhibits host cell NO production during LPS stimulation of J774-A.1 cells.** J774-A.1 cells were cultured in non-coated wells or wells coated with the anti-TRAPC antibodies KPK9 or CSE11, or the corresponding pre-immune IgY at two different concentrations (5 and 20 μg/ml). After 4h of cross-linking cells were stimulated with LPS at different concentrations and the effect on NO production was measured after16h. Bars represent the mean of triplicates from 1 experiment.

7.5.4 Cross-linking of TRAPC during bacterial infection reduced the protein level of iNOS

Both during bacterial infection and LPS stimulation, we observed a reduction in NO production during TRAPC cross-linking. This interference with NO production could be due either to inhibition of iNOS expression or interference with the enzymatic activity of iNOS. Immunoblotting of *S. enterica*-infected and uninfected cells 16h post infection with anti-iNOS antibodies indicated a decrease in iNOS expression during cross-linking TRAPC with the lower concentration of the anti-TRAPC antibodies, compared to the pre-immune IgY (Fig. 19). The decrease was verified by densitometry, determining the iNOS/actin ratios (KPK9=1.36 vs. preKPK9=1.61 and CSE11=1.52 vs. preCSE11=2.57). Thus, at least for the lower concentration of anti-TRAPC, the reduction in NO levels, observed during infection with *S. enterica* serovar Typhimurium, is accompanied by a decrease in iNOS levels.

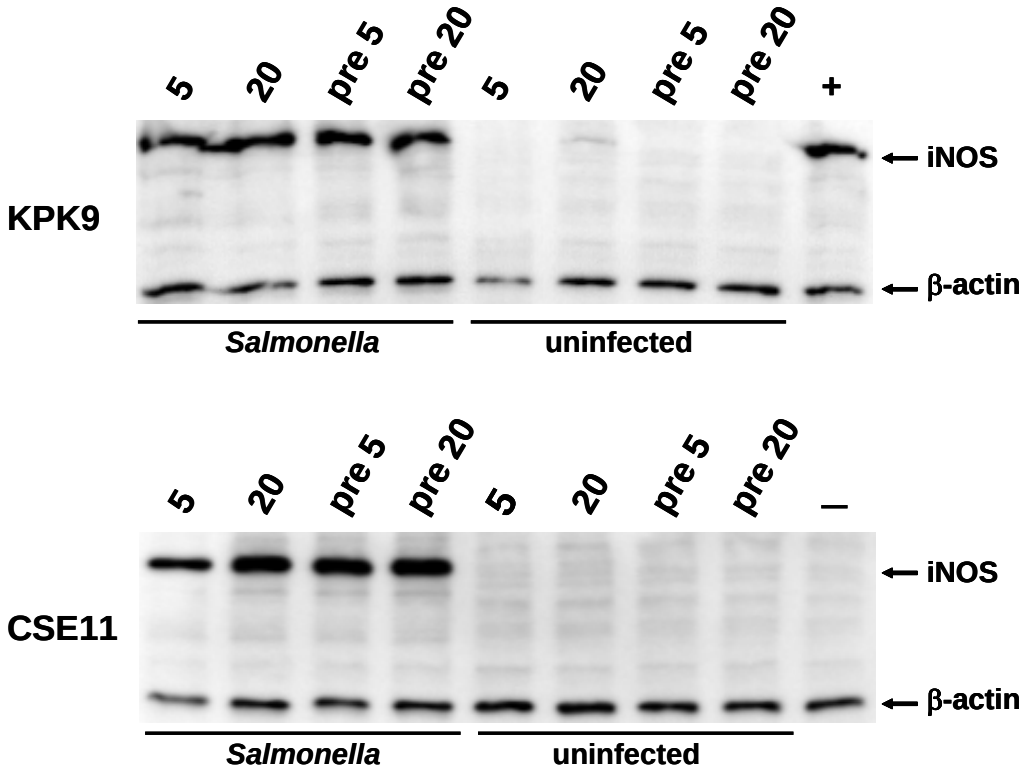


Figure 19. **Cross-linking of TRAPC inhibits host cell iNOS induction during infection of J774-A.1 with *S. enterica* serovar Typhimurium.** Cells were treated as described in Fig. 1 and subsequently infected with *S. enterica* serovar Typhimurium. Induction of iNOS was measured by immunoblotting 16 hours post infection. As a positive control (+) non cross-linked J774-A.1 cells infected with *S. enterica* serovar Typhimurium were used. β-actin levels were measured on the same blot to enable comparison of the band intensities.

7.5.5 Cross-linking of TRAPC affects the NF-κB signaling pathway

Many antimicrobial defense mechanisms expressed by macrophages during infection, e.g. induction of iNOS, depend on the NF-κB signaling pathway for their activation. Activation of this pathway requires degradation of IκB. Therefore we next studied the effect of TRAPC cross-linking on the NF-κB signaling pathway by following the degradation of IκB-α. J774-A.1 cells were cross-linked with KPK9 for 20, 40, 60 minutes or 4h. In this experiment, cross-linking of TRAPC triggered degradation of IκB-α (Fig. 20), implying a connection between TRAPC cross-linking and the NF-κB signaling pathway.

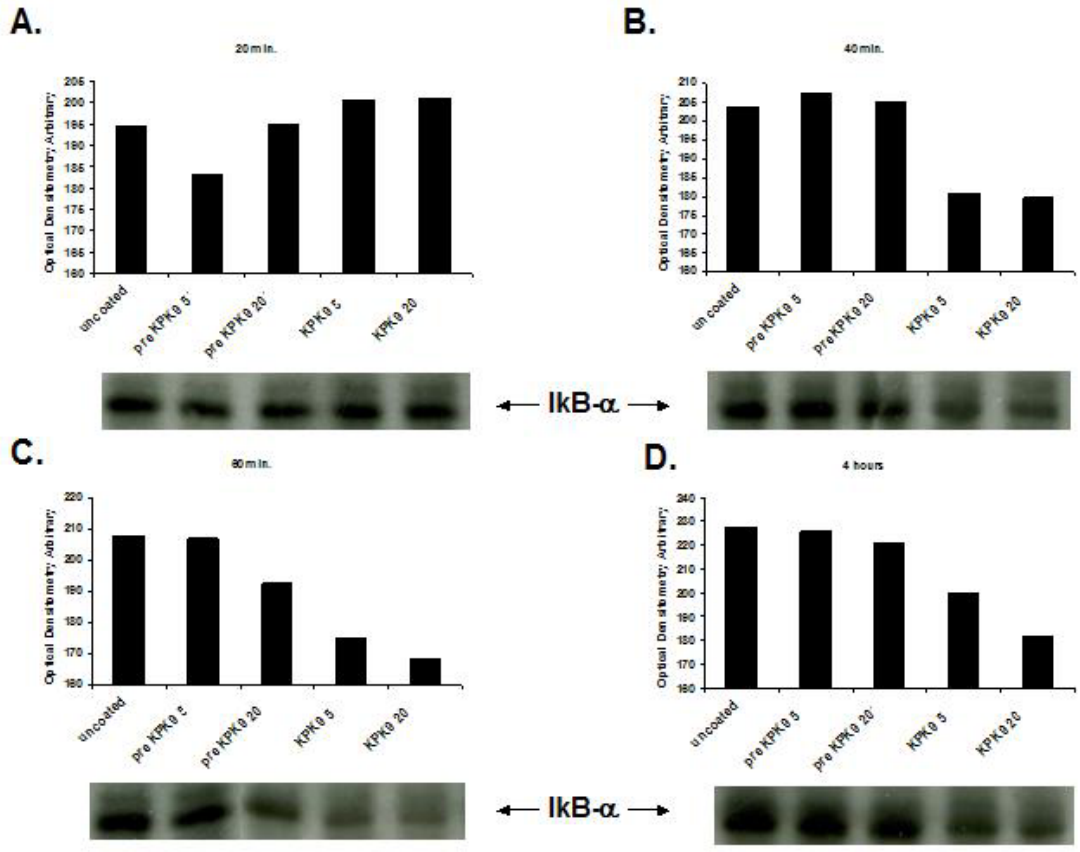


Figure 20. **TRAPC cross-linking affects IκB-α degradation.** J774-A.1 cells were cross linked with KPK9, or the corresponding pre-immune IgY at two different concentrations (5 and 20 μg/ml), for 20, 40 or 60 minutes or 4 hours. 4.10^6 cells were lysed and analysed by SDS-PAGE followed by immunoblotting with anti- IκB-α. Bars represent arbitrary optical densitometry values. This is a representative from 2 independent experiments.

In the first part of this thesis we presented the cloning and characterization of a new member of the mouse TREM receptor family, TRAPC. Cross-linking of TRAPC on phagocytic cells triggered NO production in these cells; hence TRAPC was likely to play a role in innate responses to infection. Here we have followed up the initial characterization of TRAPC by studying the role of TRAPC cross-linking during bacterial infection of the murine macrophage-like cell line J774-A.1.

As expected cross-linking of TRAPC alone triggered NO production in J774-A.1 cells (Fig. 16B and 17B). Surprisingly, combined with infection with *E. coli* TG1 or *S. enterica* serovar Typhimurium, TRAPC cross-linking on J774-A.1 cells inhibited host cell NO production and reduced the protein levels of iNOS. *E. coli* TG1 is unable to grow in macrophage-like cells it was therefore not surprising that the reduced NO levels did not affect the bacterial growth yields (Fig. 16C). However, for *S. enterica* serovar Typhimurium the reduction of host cell NO and iNOS production correlated with an increased growth yield (Fig. 17C). *S. enterica* serovar Typhimurium relies on several specific virulence factors to enable growth in phagocytic cells. Some of these virulence functions are directed at inhibiting the activities or subcellular localization of phagocyte NADPH oxidase and iNOS [214, 215].

However, as *E. coli* TG1 lacks the genetic determinants for such interference, the inhibition of NO production upon cross-linking of TRAPC is more likely caused by the cross-linking itself rather than by an indirect effect mediated by virulence factors of *S. enterica* serovar Typhimurium.

Based on unpublished data (E. Bjur), the increase in bacterial growth yield was notably larger than would be expected from the decrease in NO production and it therefore seems likely that cross-linking of TRAPC mediates inhibition not only of NO production but also of other antimicrobial macrophage functions.

Recognition of LPS by macrophages, and hence of Gram-negative bacteria, is mediated via TLR4 which triggers MyD88 signaling, eventually leading to nuclear translocation of NF-κB and pro-inflammatory responses such as up-regulation of iNOS expression. Interestingly, cross-linking of TRAPC on uninfected J774-A.1 cells leads to degradation of IκB-α, a sign of activation and nuclear translocation of NF-κB. This suggests that signaling mediated via TRAPC and signaling that occur during bacterial recognition converge in the NF-κB pathway. Thereby it seems likely that the reduction in NO production, observed when cross-linking of TRAPC is combined with bacterial infection or LPS stimulation, is mediated through inhibition of the NF-κB pathway. If that is proven to be the case, it remains to be elucidated at what stage the inhibition of the NF-κB pathway is taking place, and which signal transducers that are affected by cross-linking of TRAPC during bacterial infections. However, we cannot rule out yet that other signaling pathways are activated by bacterial infection, e.g. the MAPK pathway, on which TRAPC cross-linking could have an inhibitory effect.

Cross-linking with pre-immune IgY was also observed to give a slight reduction in the expression of NO during infection. However, which of the two pre-immune IgYs that had a statistically significant effect varied between the *E. coli* and *S. enterica* experiments, and the NO levels induced during infection of cells cross-linked with KPK9 or CSE11 was significantly lower than the NO levels obtained with the corresponding pre-immune IgY (Fig. 17B). More important, the reduction in NO production triggered during infection of cells treated with the pre-immune IgY did not affect the bacterial growth yields (Fig. 17C). Furthermore, no evidence for the degradation of IκB-α was observed when applying pre-immune IgY (Fig. 20). This implies that the minor effects observed with the pre-immune sera are unspecific.

If pathways activated by two single ligands regulate the transcription of a gene independent of each other, stimulation with both ligands at the same time would result in an additive response equal to the sum of the responses induced by the two ligands individually. In contrast, if the pathways modulate each other, application of the ligand pair would lead to non-additive responses, which can be either greater or less than the expected additive response. In an eloquent and thorough study by Zhu et al. it was shown that different stimuli used in combination with LPS to trigger macrophage responses in the RAW264.7 cell line could have additive and inhibitory effects [216].

LPS enhanced the expression IFNγ-response genes in the early phase and IFNγ attenuated the late LPS transcriptional response, primarily through inducing SOCS-1 expression. 2-methyl-thio-ATP mainly enhanced the LPS response through induction of IFNβ that suppresses IL-10 production, while prostaglandin-E_2 and isoproterenol inhibit the LPS response through inducing the anti-inflammatory cytokine IL-10.

In this light it was expected that TRAPC triggering, that induced the production of NO in a DAP12 dependent manner, would act synergistically with the stimulation of the macrophages through bacterial infection. The signaling pathways induced by bacterial infection, mainly activated via TLR4, differ from the downstream signaling cascade of DAP12. Therefore it was surprising that we observed an inhibitory effect of TRAPC cross-linking on the macrophage response to bacterial infection.

Recent publications however, have demonstrated that DAP12 can have a modulating effect on TLR signaling. Macrophages from DAP12 knockout mice respond to a higher extent to TLR ligands such as LPS, CpG and zymosan [122]. Re-introduction of DAP12 into these cells restored TLR signaling to wild type levels. This DAP12 mediated inhibition of TLR signaling was confirmed and it was furthermore shown that TREM-2 specifically is involved in this inhibition [121, 132].

It has been hypothesized that low avidity triggering of DAP12 dependent receptors leads to inhibition of TLR mediated responses, while a strong triggering via DAP12 may instead lead to a synergistic effect with TLR signaling [129]. This hypothesis is strengthened by the findings that low avidity ligands for the FcαRI receptor induce inhibition of phagocytosis and IgE mediated exocytosis, while high avidity ligands induced activating signals. In the scenario of low avidity ligands, the ITAM of FcαRI is able to recruit the SHP-1 phosphatase, a well-known inhibitory signaling molecule [131]. We show that triggering of TRAPC inhibits macrophage responses to bacterial infection, allowing bacteria to thrive better inside these cells. This inhibition was more pronounced when a weaker cross-linking stimulus in terms of lower antibody concentrations was used which is in line with the above-described hypothesis.

Furthermore, DAP12 knockout mice show an enhanced innate immune response to infection with *Listeria monocytogenis* and are more susceptible to septic shock [122]. However, DAP12 also mediate activating signals in e.g. macrophages and absence of DAP12 has been demonstrated to decrease mortality and inflammation during septic peritonitis [126]. The host depends on induction of innate immune responses to control an infection [192, 217, 218]. However, uncontrolled activation of these responses would be harmful and a dampening of the response may be necessary for survival [219-221]. DAP12 seems to be involved in balancing the response through its ability to mediate both activating and inhibitory signals.

SOCS-1 has been shown to be a crucial regulator of LPS responses in macrophages. SOCS-1 deficient mice are highly sensitive to LPS-induced shock and produce increased levels of inflammatory cytokines. In particular SOCS-1 inhibited LPS-induced NF-κB and STAT-1 activation in macrophages [221].

TLR responses also induce the production of IRAK-M that prevents dissociation of IRAK and IRAK-4 from MyD88, the crucial TLR adaptor-signaling molecule, and the formation of IRAK-TRAF6 complexes, thereby inhibiting the TLR response. IRAK-M deficient showed increased inflammatory responses to bacterial infection [220].

Whereas DAP12 associated receptors can induce the expression of SOCS-1 and IRAK-M by themselves or are able to enhance the expression through synergy with TLR signaling in order to regulate the TLR responses remains to be investigated.

Likewise, PI3K has been shown to play a modulatory role in TLR signaling [222]. PI3K inhibits TLR signaling in the early phase and modulates the magnitude of primary activation. Mice lacking the p85α regulatory subunit of class I_A PI3K show impaired Th2 responses and have enhanced Th1 responses [219, 223]. This is most likely due to the imbalance of Th1 and Th2 responses through the altered production of IL-12. PI3K deficient DCs produce much more IL-12 than wild type mice [219]. This can also be achieved through the use of specific PI3K inhibitors. In these DCs PI3K seems to block the p38 activation pathway. PI3K has also been shown to block the MAPK and NF-κB pathway in monocytes through the activation of Akt [224].

In contrast to two other studies showing that PI3K inhibition decreased LPS-induced transcriptional activity of NF-κB [225, 226], inhibition of PI3K seems to augment the phosphorylation and degradation of IκB-α, resulting in nuclear translocation of NF-κB in monocytes [224]. These latest results are strengthened by studies showing that PI3K inhibition reduces endotoxin tolerance [227], which is a state of macrophage tolerance to LPS acquired through continuous exposure to small amounts of LPS [228]. This is of special importance in intestinal mucosal immunity where bacteria and macrophage are located in constant close proximity. The existence of endotoxin tolerance reflects and strengthens the hypothesis that low avidity triggering of a DAP12 associated receptor induces inhibition.

Manipulation of the endogenous PI3K/Akt signaling pathway may even represent a new and novel therapeutic approach to management of important diseases, especially septic shock [229].

Even though DAP10 is normally seen as the major PI3K dependent adaptor molecule, DAP12 is also able to activate PI3K in a Syk dependent manner [230]. Through this mechanism it is conceivable that DAP12 can trigger the above-described inhibitory effects on TLR signaling. In the same way triggering of TRAPC prior to infection with *E.coli* or *S. enterica* could induce the recruitment of a pool of PI3K. At the time when bacteria infect the macrophage and trigger TLR signaling, in our experimental system, this pool of PI3K is still present and could potentially immediately start to inhibit the TLR signaling pathway. This hypothesis is further strengthened by the fact that TRAPC triggering induces IκB-α degradation and NF-κB translocation to the nucleus and PI3K has been shown to have profound effects on these events.

Another mechanism through which TLR activation can be modulated is via PLCγ that was suggested to decrease the recruitment of MyD88, thereby inhibiting TLR activation. It has been shown that the recruitment of the TLR signaling adaptor MyD88 requires the interaction of the scaffolding protein TIRAP (Toll-/IL-1-receptor-containing adaptor protein) with phosphatidylinositol-4,5-bisphosphate (PIP_2). Notably, PIP_2 is also the preferred substrate of active PLCγ.

PLCγ could inhibit the recruitment of TIRAP and MyD88 to the developing TLR-signaling complexes by cleaving PtdIns(4,5)P2 into the second messengers diacylglycerol and inositol-1,4,5-trisphosphate, reducing the amount of PtdIns(4,5)P2 available to facilitate the recruitment of TIRAP and MyD88 to the TLR-signaling complex. DAP12 is able to activate PLCγ [52] and therefore DAP12 signaling induced via its associated receptors, including TRAPC, could proximally inhibit TLR activation by decreasing the recruitment of MyD88 to the TLRs through PLCγ.

In contrast to the studies published on the inhibitory role of DAP12 [122] and, more recently, TREM-2 [121, 132], our study shows a direct physiological role for the inhibition mediated by TRAPC. To our knowledge this is the first study that directly triggers a DAP12 associated receptor in a direct system where an inhibitory effect is observed. In the case of TREM-2, the studies were conducted using either TREM-2 deficient macrophages or chimeric TREM-2 receptors, where TREM-2 was coupled to the intracellular part of DAP12. These two studies indirectly show that TREM-2 is able to mediate an inhibitory signal. We show that direct triggering of TRAPC with antibodies has an inhibitory effect on the macrophage response to bacterial infection and that through this inhibitory effect bacterial survival is enhanced.

An interesting observation is that TREM-2 is able to bind specifically to Gram-negative and Gram-positive bacteria and to yeast and this binding can be inhibited by bacterial products such as LPS, lipoteichoic acid, and peptidoglycan but also anionic carbohydrate molecules, including dextran sulfate, suggesting that ligand recognition is based partly on charge [138]. It is tempting to speculate that bacteria have evolved to use DAP12 associated receptors to their advantage. In this scenario, by inducing DAP12 mediated inhibition of antibacterial macrophage responses, bacteria would promote their own survival. It remains to be investigated if bacteria are able to bind these DAP12 associated receptors, including TRAPC, directly, and what mechanism(s) underlies the TRAPC-mediated regulation of innate immune effector functions. In order to answer this question, we have initiated a project to identify the ligand(s) for TRAPC.

7.6 Towards the identification of the TRAPC ligand

7.6.1 TRAPC fusion protein production and analysis.

By fusing the extracellular domain of a receptor with the constant part from an antibody, the resulting fusion protein can be used as an antibody to screen for binding to potential ligands. This was the strategy chosen also by us to search for ligands for TRAPC. The TRAPC-Fc fusion protein was constructed to contain the V-type Ig-domain and the complete stalk region from TRAPC, put together with the Fc part of a human IgG1 immunoglobulin and the leader sequence of CD5 to achieve high secretion when expressed in eukaryotic cells (Fig. 21).

Figure 21. **Domains of the TRAPC-Fc fusion protein.** The TRAPC-Fc construct is inserted in pCDM8 plasmid vector. It consists of the leader sequence of CD5, the extracellular part of TRAPC containing a single V-type Ig-domain and a short stalk region connected to the Fc part of human IgG1.

The TRAPC-Fc fusion protein was produced in 293T cells as a soluble protein and was purified from the supernatant using a protein G column. The quality of the purified TRAPC-Fc protein was assessed on an SDS-gel in three different dilutions where Coomassie blue staining showed an approximately 70 kDa band (Fig. 22). A second gel was loaded identically and was probed with anti-human IgG – HRP, which showed a band of 70 kDa confirming that it consisted of Fc-fusion protein. As a control, CTLA-4-Fc was loaded on both gels and this fusion protein showed a band at 60 kDa, corresponding to the predicted size of CTLA-4-Fc and a weaker band at approximately 25 kDa, possibly a degradation product.

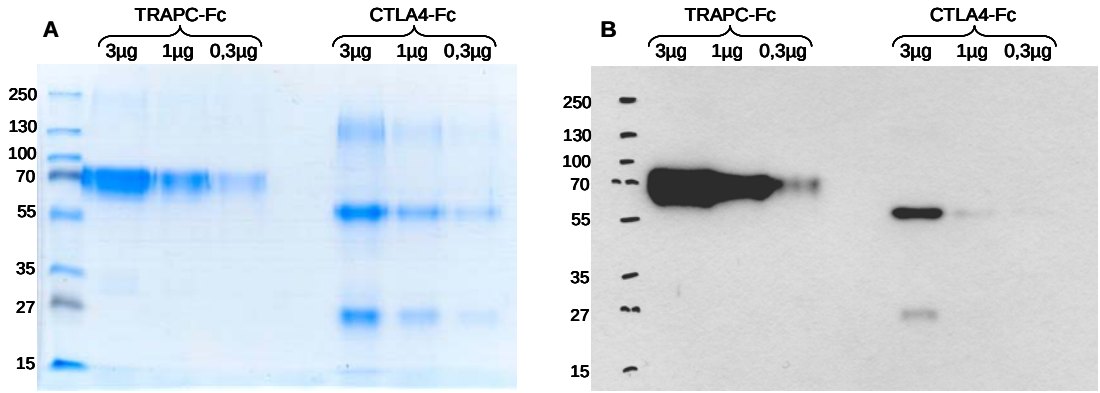


Figure 22. **Purity of TRAPC-Fc and CTLA-4-Fc fusion proteins.** Fusion proteins were separated by SDS-PAGE (12%). Different amounts of protein have been applied as indicated. (A) Coomassie Blue G250 staining for 1h. (B) The proteins were transferred to a PDVF membrane and blotted using an anti-human IgG-HRP antibody. This is a representative from 2 independent experiments.

7.6.2 TRAPC-Fc staining of eukaryotic cells

To study if eukaryotic cells expressed a ligand for TRAPC, the TRAPC-Fc fusion protein was used in flow cytometry to stain cell lines of different origin including T cells, mast cells or macrophages. TRAPC-Fc fusion protein binding was detected using a secondary anti-human IgG-FITC antibody. As a control, cells were incubation with only the secondary antibody, or labeled with CTLA-4-Fc fusion protein that is known to bind to CD80 and CD86. Cell lines of T – and mast cell origin, i.e. RMA-S, P815 and YAC-1, stained negative for TRAPC-Fc binding, while the macrophage-like cell line J774.1 bound the TRAPC-Fc fusion protein specifically (Fig. 23).

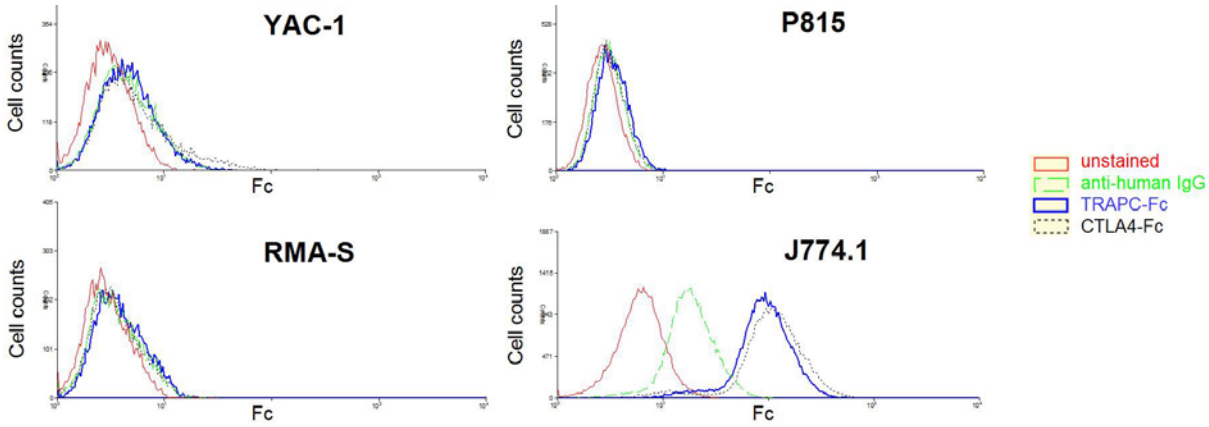


Figure 23. **TRAPC-Fc fusion protein staining of mouse cell lines.** Cells were cultured in RPMI, around $1*10^6$ cells were harvested for labeling per sample. Labeling with TRAPC-Fc (blue line) or CTLA-4-Fc (black line), each at $1\mu g/1*10^6$ cells, was followed by detection with anti-human IgG–FITC. Unstained cells are shown in red and the negative control staining using the secondary antibody only is indicated in green. These figures are representatives from 3 independent experiments.

The cells were also analyzed for TRAPC receptor expression using the polyclonal chicken antibody KPK9. The pre-immunized antibodies preKPK9 was used as control. As we previously shown, J774.1 cells stained positive for TRAPC as compared to the controls (Fig. 24B). J774.1 cells also bound the CTLA-4-Fc fusion protein, indicating that the J774.1 cells expressed CD80 and CD86, which was confirmed by flow cytometry (Fig. 24A).

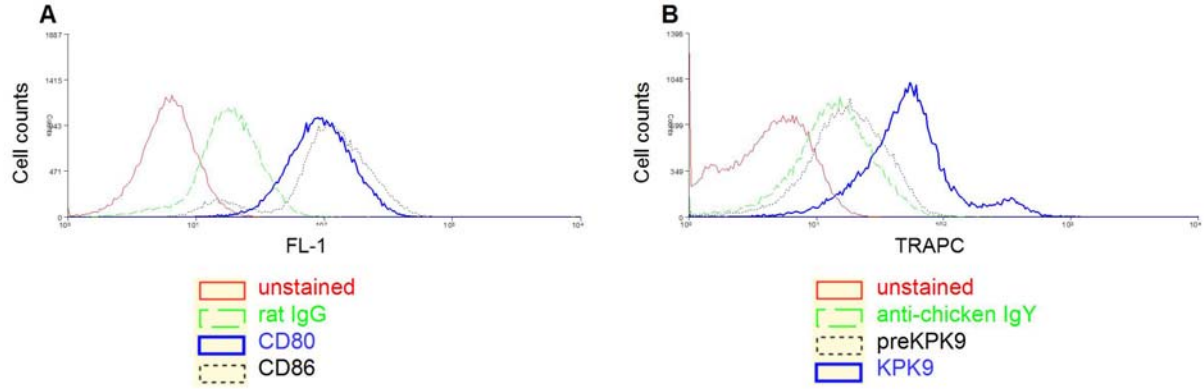


Figure 24. **CD80, CD86 and TRAPC expression on J774.1 cells.** (A) J774.1 cells were labeled with CD80, CD86 and a rat IgG-isotype control. (B) J774.1 cells labeled with anti-TRAPC antibody KPK9 (1:1000) as well as the pre-serum (preKPK9, corresponding concentration) and the anti-chicken IgG only as a control. These figures are representatives from 3 independent experiments.

In summary, we show that the J774.1 cells bound both the TRAPC-Fc fusion protein, and expressed the TRAPC receptor.

7.6.3 No alteration in TRAPC-Fc binding upon bacterial infection of J774.1 cells

Since the J774.1 stained positive for the TRAPC-Fc fusion protein, one obvious question was if the potential ligand expression was regulated by bacterial infection. Therefore J774.1 macrophages were infected with invasive, *S. enterica* serovar Typhimurium, or non-invasive bacteria *E. coli*, laboratory strain DH5α. The cells were infected with 10^6 bacteria/ml for one hour, harvested at 2h and 16h post infection, labeled with TRAPC-Fc fusion protein and analyzed by flow cytometry.

No difference in TRAPC ligand expression in uninfected, *E.coli*- or *S. enterica*-infected cells could be detected 2h post infection (Fig 25). No change in expression of CD80 or CD86 could either be detected by CTLA-4-Fc fusion protein binding within this timeframe.

16h post infection (Fig. 25), a moderate increase in TRAPC-Fc fusion protein binding was observed, but this was also seen for the negative control, i.e. secondary antibody alone. CTLA-4-Fc binding was considerably increased as compared to the 2h sample, which confirmed successful infection and activation of the macrophages that lead to an up-regulation of the co-stimulatory molecules CD80 and CD86.

Bacterial infection did not influence the binding of the TRAPC-Fc fusion protein to J774.1 cells.

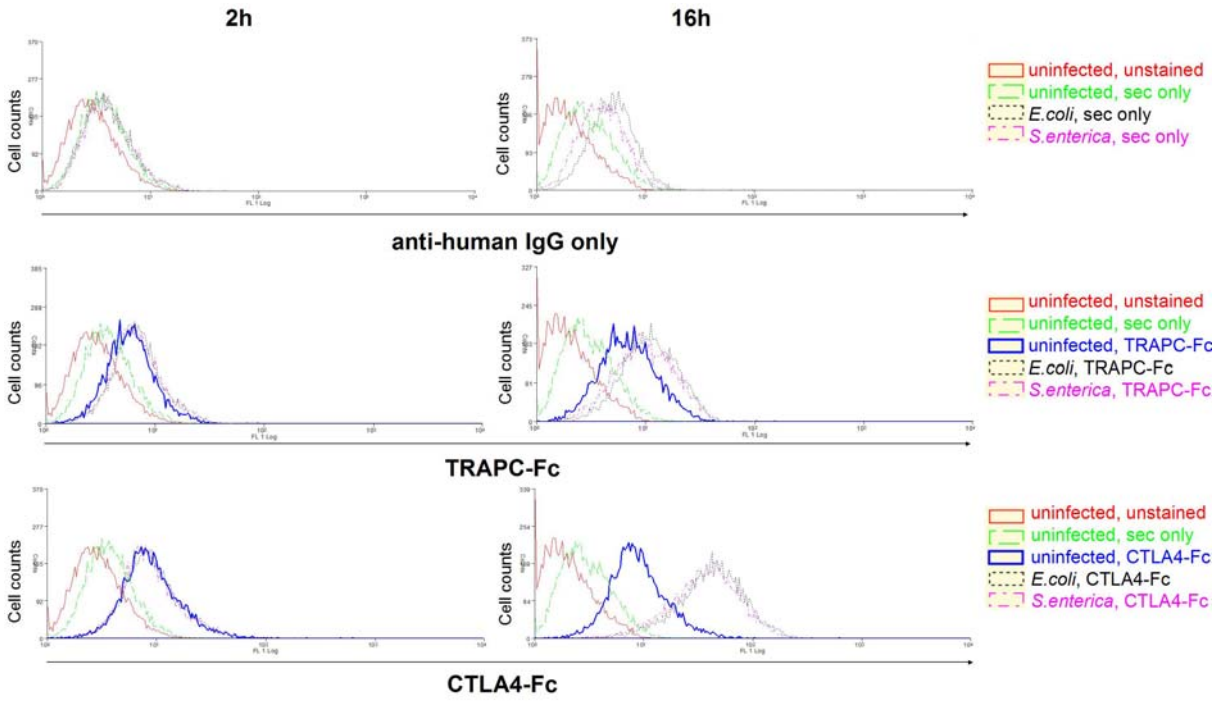


***Figure 25.* TRAPC ligand staining on infected J774.1 cells.** J774.1 cells were infected with *E.coli* or *S. enterica*, respectively. Cells were harvested 2hrs and 16hrs after infection and labeled with TRAPC-Fc and CTLA-4-Fc fusion proteins. Staining with anti-human IgG-FITC antibody alone is shown in the upper panel. The middle panel represents TRAPC ligand staining while the lower panel shows binding of CTLA-4-Fc. Data are representative for three independent experiments.

7.6.4 TRAPC-Fc binding is not affected by TLR ligands or IFNγ

Since we did not observe any difference in TRAPC-Fc fusion protein binding during bacterial infection, we were interested in if other stimuli could regulate the cell surface expression of the potential TRAPC ligand. Three cell activating reagents that utilize different receptors and signaling pathways were chosen; LPS, bacterial polysaccharide that stimulate cells via TLR4 and CD14 and signals via the MyD88 - IRAK pathway; CpG DNA, which is found in bacteria and protozoa and is recognized by TLR9 in endosomal compartments of innate immune cells and leads to regulation of inflammatory responses by activating the MyD88 - IRAK pathway as well; and IFNγ which binds to the IFNγ receptor on the macrophage and signal via STAT1. The cells were also infected with *S. enterica*. The J774.1 cells were treated with the different reagents for 2h or 16h, and ligand expression was analyzed by flow cytometry.

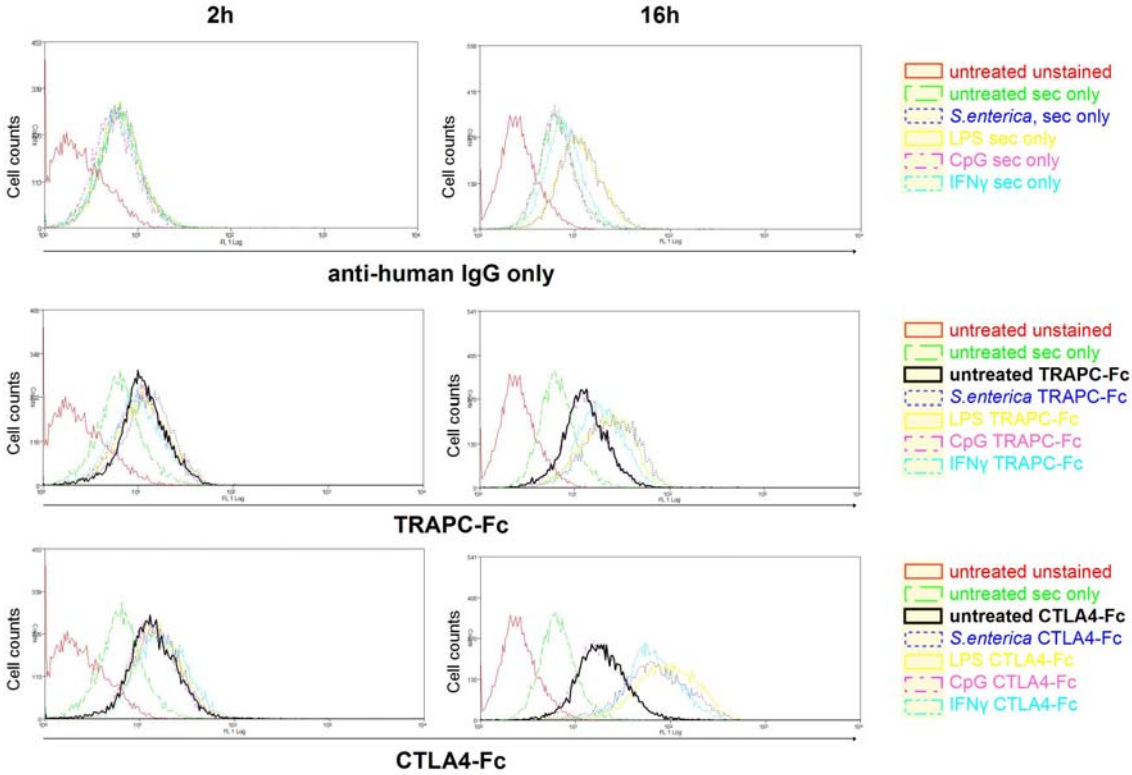


Figure 26. **Stimulation of J774.1 cells with TLR ligands and IFNγ.** Cells were stimulated with IFNγ, CpG or LPS, respectively. Additionally, infection with *S. enterica* was performed. Cells were harvested and labeled with TRAPC-Fc and CTLA-4-Fc 2hrs and 16hrs post stimulation/infection. Staining with anti-human IgG-FITC antibody alone is shown in the upper panel. The middle panel represents TRAPC ligand staining while the lower panel shows binding of CTLA-4-Fc. Data are representative for two independent experiments.

CTLA-4-Fc was included to follow the regulation of CD80 and CD86 as a marker of stimulation, and as a Fc fusion control. As shown in Figure 26, 2h after stimulation, none of the treatments had affected the TRAPC-Fc fusion protein binding. CTLA-4-Fc binding was not affected either.

After 16h, CpG DNA had no effect on the TRAPC-Fc binding, while IFNγ treated cells showed a small increase in binding. LPS as well as *S. enterica*-infected cells were even more positive when labeled with the TRAPC-Fc fusion protein (middle panel). However, the background staining with the anti-human IgG – FITC secondary antibody only on LPS treated and infected cells was higher as well (Fig. 26, upper panel), showing that the increase in TRAPC-Fc binding by LPS treatment or *S. enterica* infection was due to increase in secondary antibody binding. The CTLA-4-Fc binding was considerably increased after 16 hrs of infection and stimulation with IFNγ or LPS, but not with CpG (Fig. 26, lower panel).

We concluded that the potential ligand for TRAPC expressed on J774.1 cells is not regulated by any of the stimuli used in our experiments.

7.6.5 TRAPC receptor and ligand expression on DCs

Since the TRAPC receptor is expressed on B cells, macrophages and DCs, and we have shown presence of a TRAPC ligand on a macrophage cell line using TRAPC-Fc fusion proteins, we were interested in if also DCs and B cells express a TRAPC ligand. To test this, we cultured bone marrow derived DCs from C57BL/6 mice and analyzed them for expression of TRAPC and its ligand. As we previously described, bone marrow derived DCs have high expression of the TRAPC receptor (Fig. 27B). The bone marrow derived DCs did also express a potential ligand for TRAPC on their cell surface, by labeling with the TRAPC-Fc fusion protein, like the macrophage cell line J774.1 (Fig. 27A). DCs express CD80 and CD86 and as shown in figure 27A, the DCs bound the CTLA-4-Fc fusion proteins.

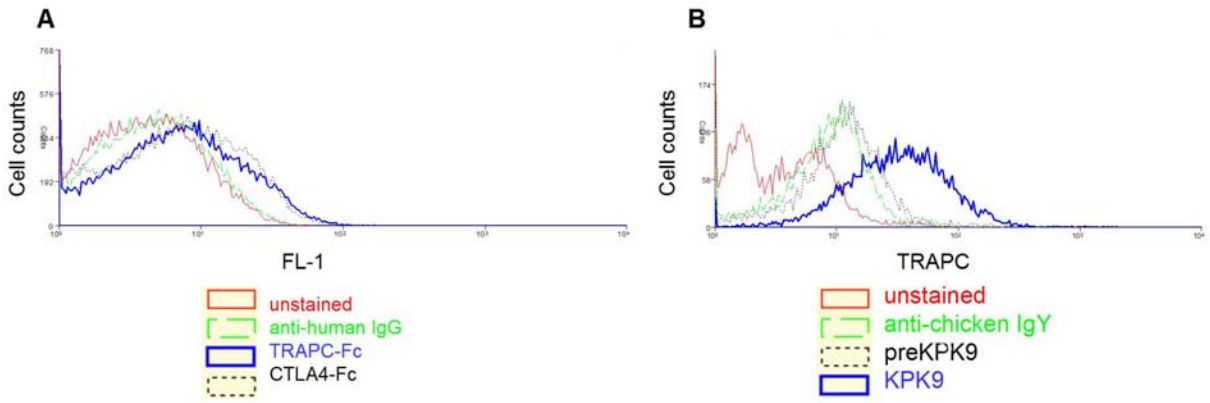


Figure 27. **TRAPC ligand and TRAPC expression on DCs.** (**A**) TRAPC ligand staining on DCs. Bone-marrow derived DCs were cultured in presence of GM-CSF for 6 days, re-plated in medium without GM-CSF and harvested for analysis the following day. Labeling with TRAPC-Fc (blue line) or CTLA-4-Fc (black line), each at 1µg/1*10^6 bone marrow derived DCs, was followed by detection with anti-human IgG–FITC. Unstained cells are shown in red and the negative control staining using the secondary antibody only is indicated in green. (**B**) TRAPC staining on DCs. Bone marrow derived cells were labeled with anti-TRAPC antibody KPK9 (1:1000) as well as the pre-serum (preKPK9, corresponding concentration) and the anti-chicken IgG only as a control.

7.6.6 Both the TRAPC receptor and its ligand was expressed also on B cells

To study if the TRAPC-Fc fusion protein could also bind to B cells, we used freshly isolated splenocytes form C57BL/6 mice. The splenocytes were labeled with TRAPC-Fc to detect TRAPC ligand expression (Fig. 28). All B cell, as defined with an anti-CD19 antibody, stained positive for the TRAPC-Fc fusion protein, while the $CD3^+$ T cells were negative. NK cells and neutrophils did not bind the TRAPC-Fc fusion protein (data not shown).

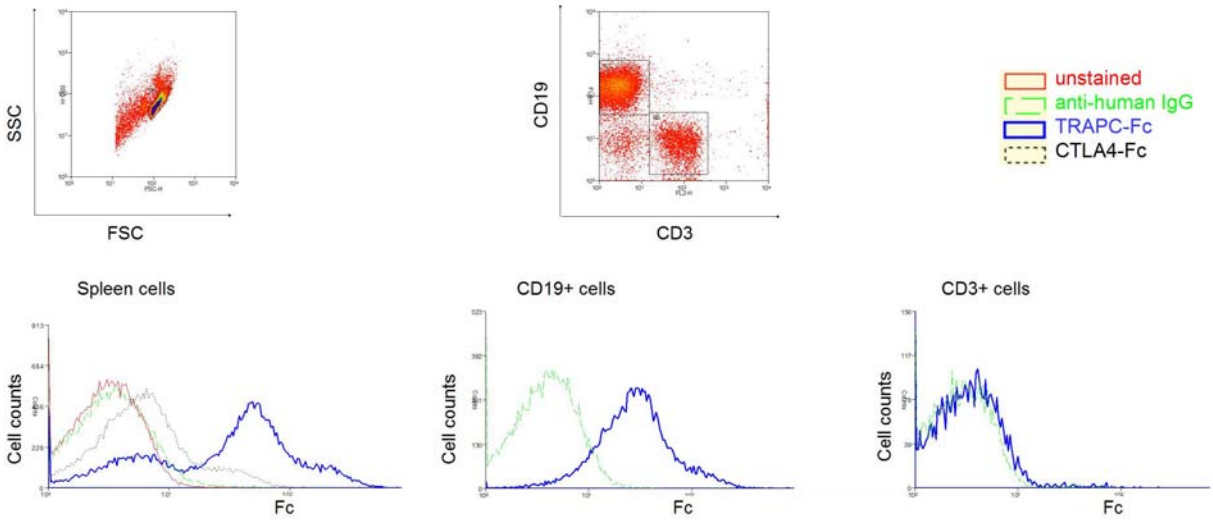


Figure 28. **B cells express a potential TRAPC ligand.** Freshly isolated spleen cells from B6 mice were labeled with CD3 and CD19 antibodies as well as the TRAPC-Fc fusion protein. The upper right dot plot shows the $CD3^+$ and $CD19^+$ cell populations. The expression of TRAPC ligand is shown in the histograms below. Data shown is one representative of three independent experiments.

The splenocytes were also stained with anti TRAPC antibodies (KPK9), and as we previously had shown, B cells ($B220^+$) expressed the TRAPC receptor while T cells ($CD3^+$) did not (Fig. 29).

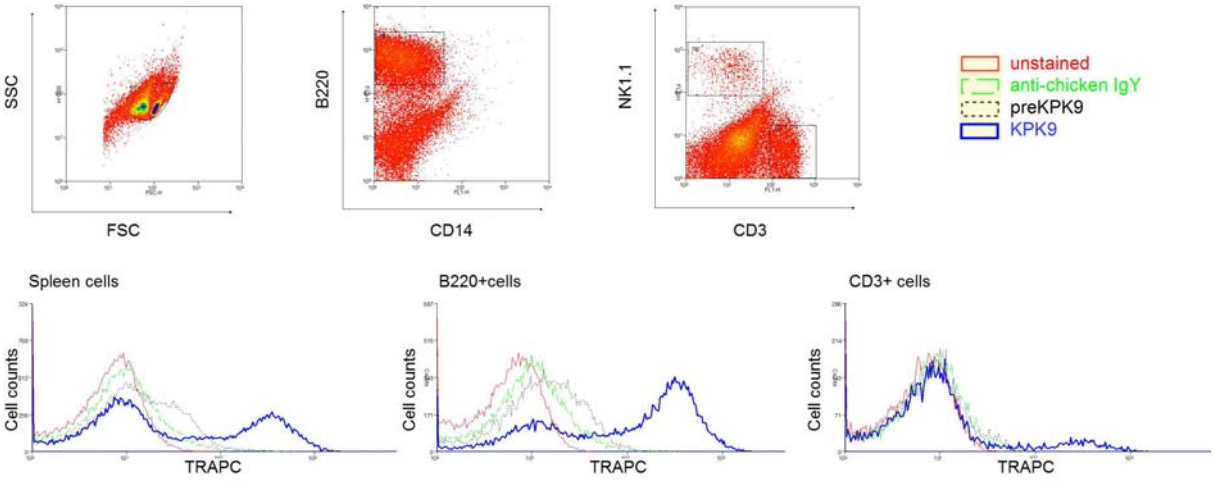


Figure 29. **TRAPC expression on spleen cells.** Spleen cells from C57Bl/6 mice were isolated and labeled with anti- B220 and CD14 or CD3 and NK1.1 antibodies. In addition, anti-TRAPC antibody KPK9 or the corresponding controls (anti-chicken IgY or KPK9 pre-serum) were added. FSC - SSC gates and on the subpopulations is shown in the upper panel. The histograms below show TRAPC expression on all spleen cells, $B220^+$ cells (B cells) and $CD3^+$ cells (T cells). Data shown is one representative of three independent experiments.

Freshly isolated bone marrow cells were stained for TRAPC-Fc binding and TRAPC receptor expression. In accordance with the result of the spleen cell, B cells ($CD19^{+}$) showed high expression of the TRAPC ligand, whereas T cells ($CD3^{+)}$, NK cells ($NK1.1^{+)}$ and neutrophils ($Gr1^{+}$) were negative for TRAPC-Fc staining (data not shown). These findings suggest that the cells that express the TRAPC receptor, i.e. macrophages, DCs and B cells, did also express a potential ligand for TRAPC.

7.6.7 Visualization of TRAPC and its potential ligand

In order to visualize the expression of TRAPC and its ligand on the surface of macrophages and B cells, and to get an insight in the expression pattern and localization of TRAPC and its ligand in relation to each other, we utilized confocal imaging. (These experiments have not been conducted by the author, but by Caroline Helmstetter under the supervision of the author. These figures have been take up in this thesis because they confirm expression of TRAPC on B cells and macrophages and give clues to where the ligand of TRAPC may be expressed.)

A majority of the J774.1 macrophage-like cells did stain positive with the TRAPC-Fc fusion protein in the confocal microscopy. Interestingly, in a minority of J774.1 macrophages, a polarization in TRAPC ligand expression could be observed (Fig. 30). The TRAPC-Fc fusion protein stained the cell surface of J774.1 cells in a patchy pattern.

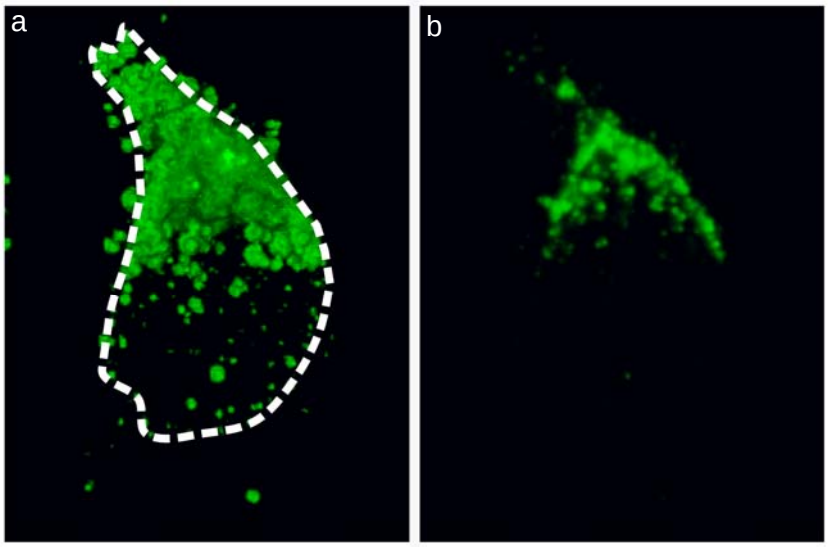


Figure 30. **Macrophage labeled with TRAPC-Fc.** Visualization of TRAPC-Fc staining on a J774.1 cell by confocal microscopy. (**A**) The brightness of the picture was increased in order to outline the whole cell. (**B**) Unmodified image. These figures are representatives from 2 independent experiments.

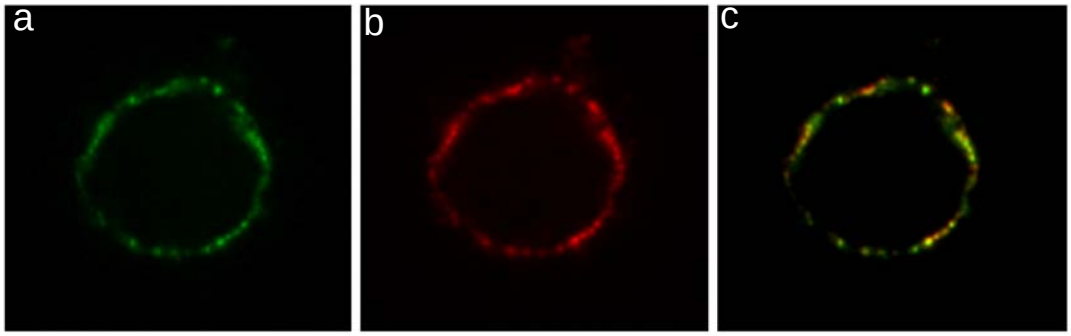


Figure 31. **J774.1 cell labeled with TRAPC-Fc and anti-TRAPC.** (**A**) Visualization of the potential TRAPC ligand with the TRAPC-Fc fusion protein detected with anti-human IgG-FITC (green). (**B**) TRAPC staining with anti-TRAPC (KPK9) antibody detected by anti-chicken IgY-Alexa647 (red). (**C**) Overlay illustrates the co-localization of TRAPC and its ligand on the cell surface (yellow). These figures are representatives from 2 independent experiments.

J774.1 macrophage-like cells were labeled with anti-TRAPC (KPK9) antibody (red) and the TRAPC-Fc fusion protein (green). Figure 31 shows the expression of the TRAPC receptor and its potential ligand a J774.1 cell. Both the ligand and the receptor were expressed on the cell surface in a patchy pattern, which was perfectly overlapping (Fig. 31).

Not all of J774.1 cells stained positive with the TRAPC-Fc fusion protein (Fig. 32A), but those that were positive did also express the TRAPC receptor (Fig. 32B + C). In addition, some cells that did express the TRAPC receptor, did not express the potential TRAPC ligand (Fig. 32C). As a control for the TRAPC receptor labeling, cells were labeled with KPK9-pre-serum. No background staining was observed (Fig. 32D). Moreover, no detectable unspecific binding of the secondary anti-human IgG-FITC antibody was observed (data not shown).

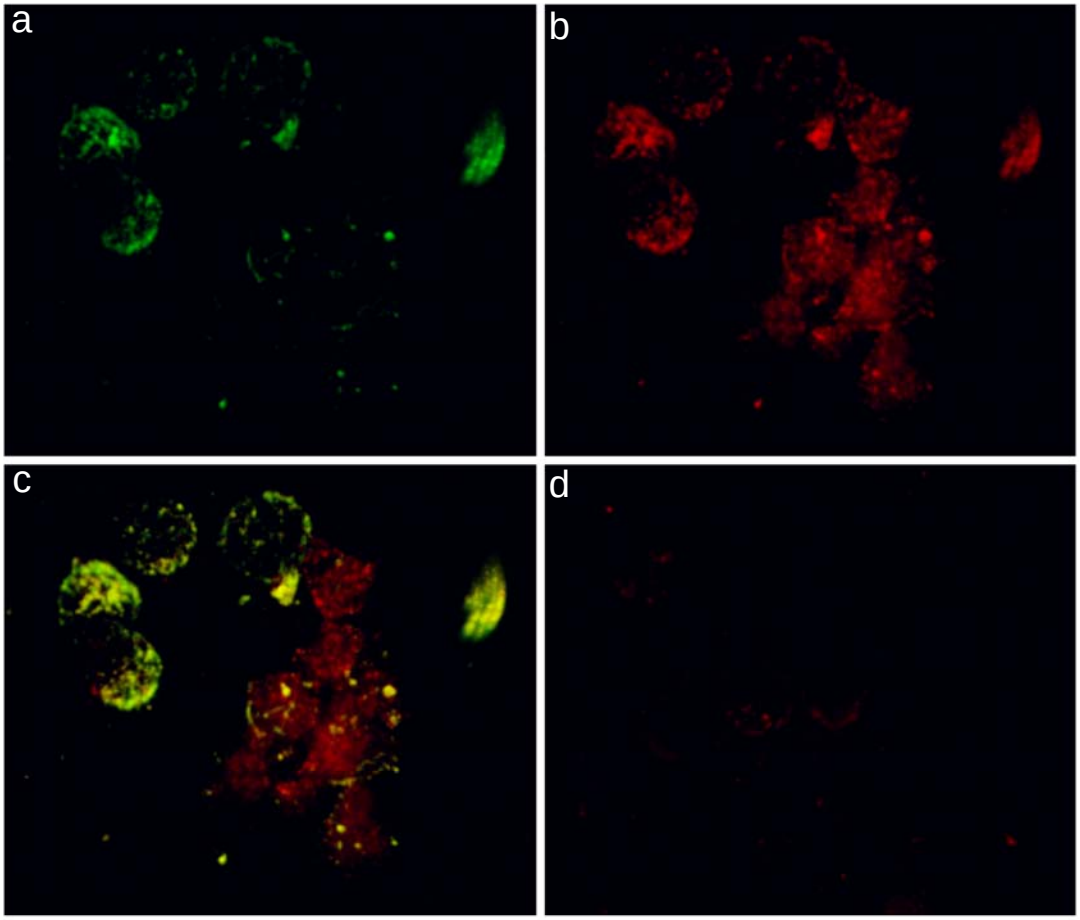


Figure 32. **J774.1 macrophages labeled with TRAPC-Fc and anti-TRAPC.** Visualization of (**A**) TRAPC ligand, and (**B**) TRAPC, expression on J774.1 macrophages by confocal microscopy. (**C**) An overlay illustrates the co-localization on the cell surface and shows cells expressing TRAPC but not its ligand. (**D**) Cells labeled with KPK9-pre-serum as negative control for the detection of TRAPC. These figures are representatives from 2 independent experiments.

7.6.8 TRAPC and TRAPC ligand expression on splenocytes

Since we previously showed that it was mainly the B cells in spleen that bound the TRAPC-Fc fusion protein, splenocytes were labeled with anti-CD19-APC to be able to distinguish B cells from other spleen cells, and with TRAPC-Fc fusion protein to detect ligand expression. Some spleen cells clearly bound the TRAPC-Fc fusion protein (Fig. 32A). Overlaying the $CD19^+$ cells (Fig. 32B, shown in red) with the ligand expressing splenocytes showed that B cells did express a potential TRAPC ligand (Fig. 32C), confirming our results obtained by flow cytometry (Fig. 28). We could also detect $CD19^+$ splenocytes that did not express the potential TRAPC ligand, and $CD19^-$ splenocytes that did express the ligand (Fig. 32C).

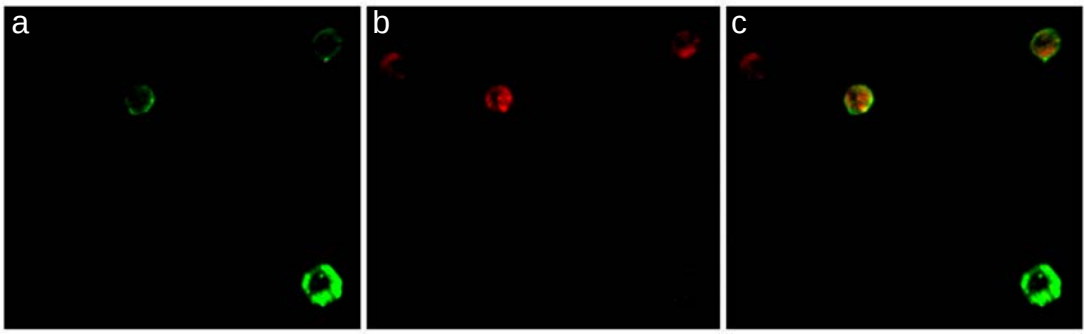


Figure 33. **Spleen cells labeled with TRAPC-Fc and CD19 antibodies.** (**A**) Splenocytes expressing TRAPC ligand. (**B**) Splenocytes positive for the B cell marker CD19. (**C**) Overlay showing that most B cells were positive for a potential TRAPC ligand. These figures are representatives from 2 independent experiments.

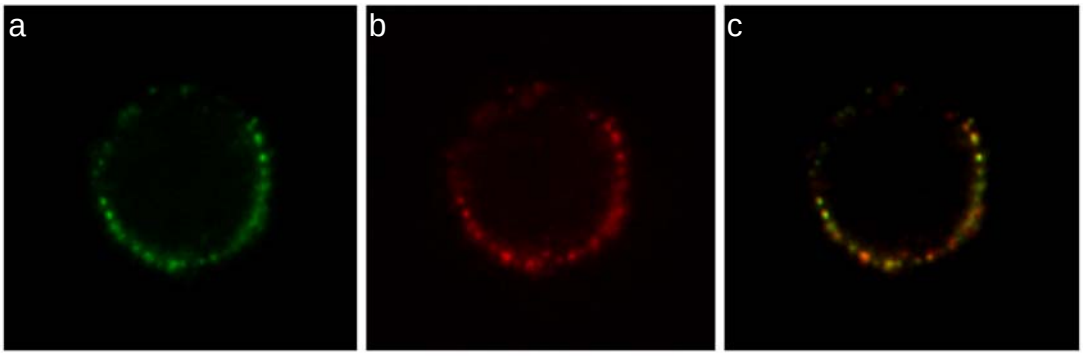


Figure 34. **Splenocyte expressing both TRAPC and its ligand.** Visualization of a splenocyte labeled with (**A**) anti-TRAPC and (**B**) TRAPC-Fc. (**C**) Co-localization of TRAPC and its potential ligand. These figures are representatives from 2 independent experiments.

Staining of splenocytes with both anti-TRAPC antibodies (KPK9) and TRAPC-Fc fusion protein showed that TRAPC and its ligand also co-localize on splenocytes in a patchy expression pattern (Fig. 34).

7.6.9 Immunoprecipitation of a potential ligand on B cells

Since we detected bright staining on especially B cells with the TRAPC-Fc fusion protein, we reasoned that this was due to either high affinity or abundant expression of a potential ligand on these cells, and therefore that it might be possible to immunoprecipitate the ligand with the TRAPC-Fc fusion protein form those cells.

Therefore we enriched for splenic B cells by negatively sorting using magnetic cell sorting (MACS), where T and NK cells were sorted away from the rest of the splenocytes, leaving mainly untouched B cells. The purity of the negatively sorted B cell population was 73.2 %, while the positively sorted mixed T and NK cell population, used as negative control in the immunoprecipitation, consisted of 78.2 % T cells and 4,8 % B cells (Fig. 35).

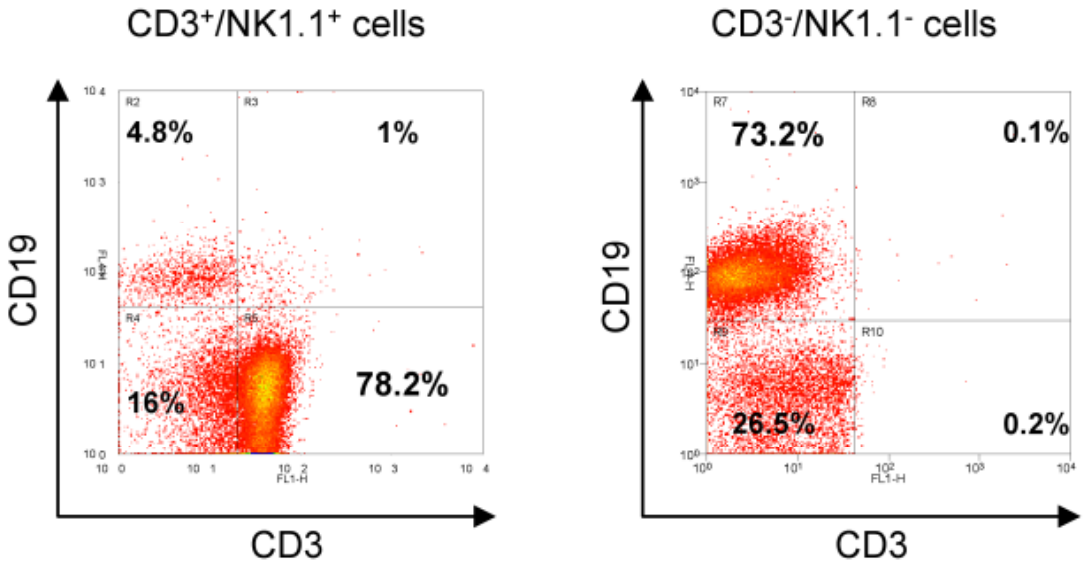
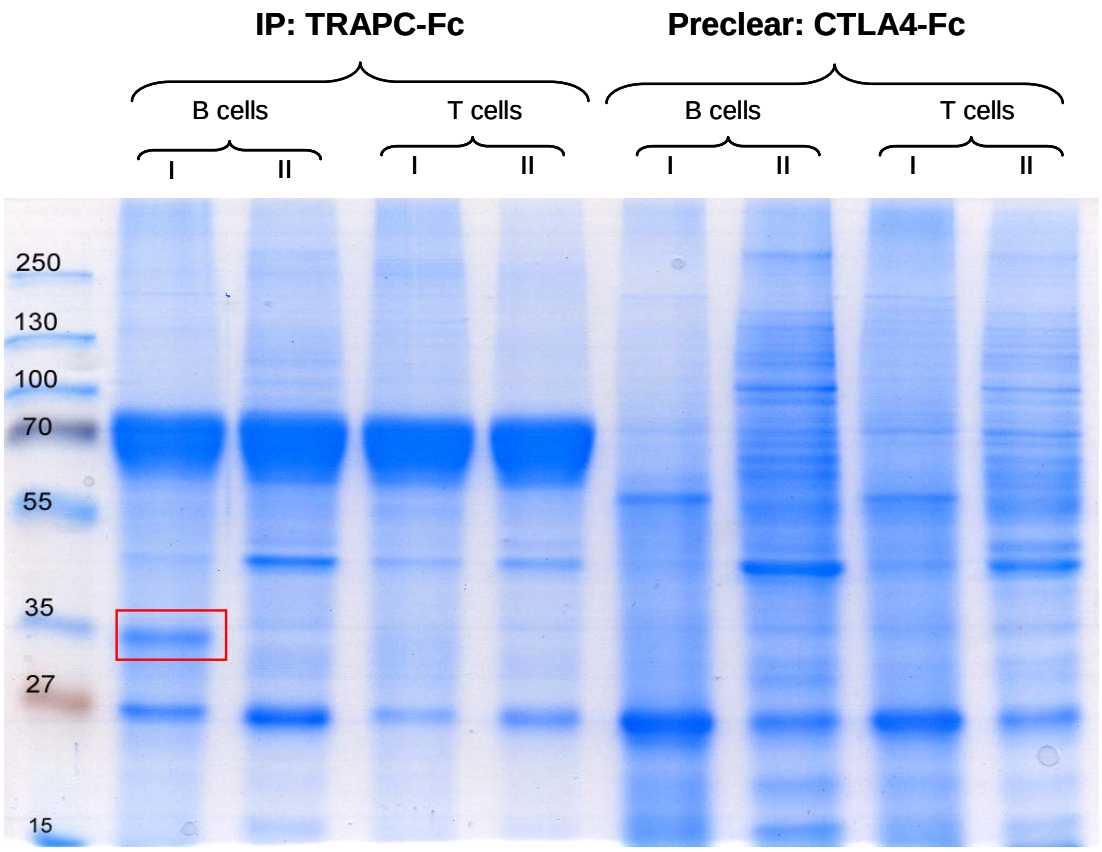


Figure 35. **B cell and T cell population purity after MACSort.** B cells were negatively sorted by MACS using anti-CD3 and anti-NK1.1 labeling and separated with anti-FITC microbeads. B cell population purity was 73.2%. The remaining B cells in the $CD3^+$ / $NK1.1^+$ cells added up to 4.8%. These figures are representatives from 2 independent experiments.

The enriched B cells, and the T and NK cell pool, were lysed with buffers containing either NP40 or octylglucoside as detergents, pre-cleared with the CTLA-4-Fc fusion protein and subsequently immunoprecipitated using the TRAPC-Fc fusion protein. The immunoprecipitates were separated on a SDS-PAGE gel, were it was shown that the immunoprecipitate from the B cells contained a additional 35 kDa band compared to the T and NK cell lysate, that could possibly represent the TRAPC ligand (Fig. 36). This 35 kDa band was completely absent in the pre-clear control of both B cells and T cells, indicating that it is not an unspecific band. Further, the 35 kDa band was immunoprecipitated using lysis buffer I (NP40), showing that this buffer represented the best condition for optimal immunoprecipitation of the potential ligand.



I : Lysis buffer I (NP40)
II : Lysis buffer II (Octylglucoside)

Figure 36. **A 35 kDa protein from B cell possibly represent the ligand for TRAPC.** $30*10^6$ B cells and T cells were lysed in two different lysis buffers. After pre-clearing with the CTLA-4-Fc fusion protein, the lysates were applied to the TRAPC-Fc coupled beads. Over night incubation was followed by extensive washing of the beads, before they were boiled in sample buffer. Protein-containing supernatants were separated by SDS-PAGE and Coomassie staining was performed. The 70 kDa band represents the TRAPC-Fc fusion protein. The 35 kDa band in the B cell lysates has been highlighted and could possibly represent the ligand for TRAPC. These figures are representatives from 2 independent experiments.

The immunoprecipitations has to be repeated using B cell lysates and preferably also lysates from DC and macrophages to confirm the potential ligand for TRAPC of 35 kDa. When confirmed, it will be sequenced by mass spectrometry.

Screening of murine cell lines of different origin revealed that a potential ligand for TRAPC was expressed on the macrophage cell line J774.1. We could not detect binding of the TRAPC-Fc fusion protein to cell lines of T and mast cell origins. Additionally, TRAPC-Fc fusion protein binding was detected on bone marrow-derived DCs that were cultured for 6 days in presence of GM-CSF. On splenocytes, TRAPC-Fc binding was detected on $CD19^+$ B cells, but not on $CD3^+$ T cells. A similar expression pattern was observed in bone marrow cells from C57BL/6 mice. The B cell population stained brightly for TRAPC-Fc, while T cells, NK cells and neutrophils were negative.

Taken together, these results demonstrated that those cells we previously shown to express the TRAPC receptor also bound the TRAPC-Fc fusion protein, indicating that the cells expressed both the ligand and the receptor.

In the case of other TREM family molecules, co-expression of the receptor and its ligand has been observed as well. Even though the ligand for TREM-1 still remains to be identified, stainings with a TREM-1/IgG1 tetramer revealed ligand expression on 80% of the neutrophils infiltrating the peritoneum during septic shock in a mouse model. In contrast, TREM-1 ligand (TREM-1L) was not expressed on neutrophils from non-septic animals [231]. This suggests that ligand up-regulation is important during endotoxemia. Neutrophils that were recruited to extra vascular sites in septic shock carried both TREM-1 and TREM-1L allowing an increased activation of the cells resulting in higher production of pro-inflammatory cytokines.

Like TREM-1, other receptor-ligand pairs expressed on the same cell type are regulated by the same stimuli. Fas and Fas Ligand (FasL) are expressed on several cell types, including T cells, and the interaction between Fas and FasL is crucial in T cell development and tolerance. Upon repeated stimulation of the T cells, Fas and FasL are co-expressed on T cells and lead to activation-induced cell death [232-235]. Based on these observations we investigated if the ligand for TRAPC could be regulated by the same stimuli that also induced increased TRAPC receptor expression, i.e. TLR ligands and bacterial infections.

The stimuli used to investigate a possible regulation of the expression of the ligand for TRAPC included different TLR ligands, IFNγ and bacterial infection with *E. coli* and *S. enterica*. Ligand expression was analyzed by flow cytometry with TRAPC-Fc fusion proteins at 5 different time points between 1h and 16hrs after infection or stimulation. Surprisingly, no regulation of ligand expression could be observed through the stimuli used in this study. In comparison, staining with the CTLA-4-Fc fusion protein showed that CD80 and CD86 are up-regulated on macrophages as a result of IFNγ or LPS-stimulation as well as bacterial infection. This effect could not be observed as early as 2h, but was clearly present 16hrs after infection or stimulation. In conclusion, either the ligand of TRAPC is expressed at a constant rate independently of the activation state of the cells, or it is regulated by stimuli different from the ones we used in our system.

TREM-2, which is expressed on bone marrow-derived DCs and on macrophages, including osteoclasts and microglia, has been shown to bind to Gram-positive and Gram-negative bacteria and yeast, suggesting TREM-2 may act like a pattern recognition receptor. Staining cell lines with a TREM-2A-Fc fusion protein revealed TREM-2 ligand expression on astrocytoma cell lines. In both cases binding could be disrupted by anionic carbohydrates, leading to the conclusion that ligand recognition by TREM-2 is dependent on positively charged carbohydrates on the receptor surface [138].

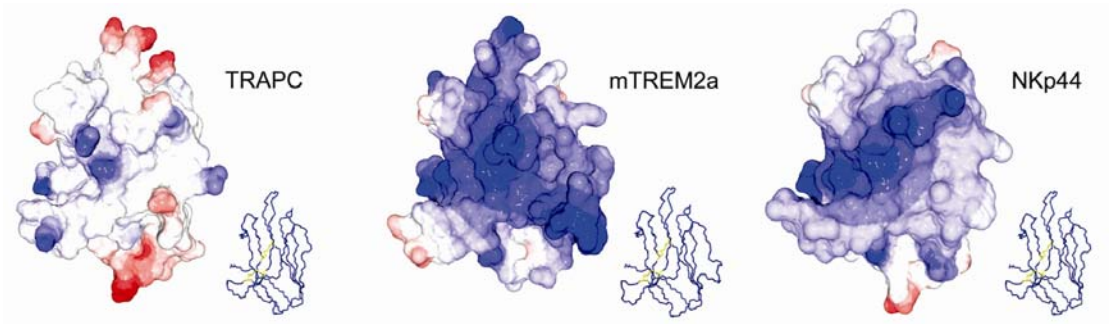


Figure 37. **Model of TRAPC, mTREM2a and hNKp44 structure.** The molecular surfaces of TRAPC and mTREM2a are shown modelled according to the crystal structure of NKp44 [201]. Surface colors indicate electrostatic potential spanning between -5 (red) and 5 (blue).

NKp44 binds to viral heamagglutinin (49). The 3D structure of NKp44 has been solved by X-ray crystallography [201, 202]. The surface of NKp44 shows a positively charged groove, possibly involved in ligand binding. Modeling the 3D structure of TREM-2A based on the structure of NKp44 shows a similar positively charged area as NKp44, which is in agreement with TREM-2A binding anionic ligands.

Modeling the 3D structure of TRAPC according to the crystal structure of NKp44 showed that it did not contain a similar positively charged surface area as NKp44, which suggests that TRAPC binds to ligands of different nature than TREM-2A (Fig. 37).

Recently, bone marrow-derived macrophages were shown to express a TREM-2 ligand [132]. It has been speculated that the presence of TREM-2 and its ligand on the same cell would have functional consequences. TREM-2 is able to, via DAP12, inhibit macrophage responses upon TLR4 and FcR stimulation. This could be due to a continuous basal TREM-2 triggering via ligands expressed on the macrophages themselves that give rise to an inhibitory signal. Similarly to TREM-2, this could also be valid for TRAPC.

The co-expression and co-localization of both TRAPC receptor and ligand on the cell surface of macrophages and B cells, as shown by FACS and confocal microscopy, may imply a cis-interaction between the TRAPC receptor and its ligand, i.e. interaction between the two molecules on the same cell, which would induce a continuous basal signaling in order to keep the cells in a steady-state condition. In case of a bacterial infection or LPS stimulation, the TRAPC receptor is up-regulated, possibly engaging more ligands on the same cell or on other cells leading to increased cellular activation. One could also envision that potentially higher-affinity ligands expressed by eukaryotic cells or pathogens binds to TRAPC during infections, which would induce activation of the TRAPC expressing cells leading to an enhanced immune response.

8 Concluding remarks

In this thesis we describe a novel activating murine TREM family receptor that is expressed on cells of both myeloid and lymphoid origin, including dendritic cells and monocytes/macrophages and B-cells, but not in T- or NK cells. The molecule was designated triggering receptor expressed in antigen presenting cells (TRAPC). TRAPC belongs to the immunoglobulin superfamily, and associates with the adaptor-signaling molecule DAP12. The human homologue to TRAPC is TREML-4. Treating the macrophage cell lines J774.1 and RAW 264.7 with LPS led to increased expression of TRAPC. Cross-linking of TRAPC on macrophages and dendritic cells induced nitric oxide production in both cell types, and up-regulated expression of CD40 on the dendritic cells.

The work in this thesis has been focused on the expression pattern and function of TRAPC in macrophages and dendritic cells. We observed that cross-linking of TRAPC on murine macrophage-like J774-A.1 cells resulted in an increase in the expression of iNOS and nitric oxide (NO), and in degradation of IκB-α. A more pronounced NO production was achieved when exposing J774-A.1 cells to LPS, or by infecting the cells with *Escherichia coli* or *S. enterica* serovar Typhimurium. These results showed that triggering macrophages via the TRAPC receptor induced activation of NFκB. We plan to investigate which signal transducing molecules are involved in the signaling pathway triggered by TRAPC. It would also be of interest to elucidate which genes are transcribed upon NFκB, or other signaling molecules activation via TRAPC.

The most surprising results of our study was that cross-linking of TRAPC on J774-A.1 cells prior to infection with *E. coli* or *S. enterica* serovar Typhimurium or exposure to LPS *reduced* the amounts of iNOS and production of NO. For *S. enterica* serovar Typhimurium, the reduced NO levels were accompanied by a substantial increase in intracellular bacterial replication. It would be highly interesting to elucidate at which point in the signaling cascade TRAPC would interfere with the macrophage response to bacteria. Our system may allow us to study the mechanism of DAP12 inhibition under conditions of direct infection of macrophages, as compared to what so far has been published using DAP12 knock out mice or chimeric receptors.

In an attempt to identify the ligand for TRAPC using a soluble TRAPC Fc-fusion protein we have detected a potential ligand on B cells, macrophages and dendritic cells, i.e. on cells that also express TRAPC. Furthermore, confocal microscopy of cells labeled with anti-TRAPC polyclonal antibodies and the TRAPC-Fc fusion protein indicated that the receptor and ligand co-localized on the cell surface. In a first step to identify the ligand, cells positive for the potential ligand were lysed and immunoprecipitated with the TRAPC-Fc fusion protein. After separation in a denaturing polyacrylamide gel, a band potentially containing the ligand was detected. This band remains to be sequenced by mass spectrometry. The identification of the ligand for TRAPC is crucial in understanding the function of TRAPC in innate immune responses.

It would be of importance to elucidate the function of TRAPC on B cells as well as its expression pattern during different B cell development stages. Since TRAPC is expressed on antigen presenting cells it will be fundamental to investigate the role of TRAPC in the process of antigen presentation and the subsequent stimulation of T cell responses. Does triggering of TRAPC influence antigen presentation? Does triggering of TRAPC induce cytokine production that could skew the subsequent T cell response into a Th1 or a Th2 type response? And if it does, could this response also be regulated dependent on ligand density or avidity as for the anti-bacterial response by macrophages?

Overall we have presented a novel TREM family member, TRAPC, which associated with the adaptor molecule DAP12. TRAPC is an activating receptor on macrophages and dendritic cells that was able to trigger nitric oxide production and up-regulated activation markers. TRAPC expression is up-regulated on macrophages upon stimulation with LPS and the cellular activation triggered by TRAPC is most likely a result of NFκB activation. Cells that expressed the TRAPC receptor did also express a potential ligand for TRAPC, suggesting that this receptor – ligand pair could be involved in auto-regulating these cells, which remains to be tested. Most importantly, we showed that macrophage response towards bacterial infections could be modulated depending on the extent of stimulation of the TRAPC receptor.

9 Material and methods

9.1 Materials

9.1.1 Chemicals and biochemicals

40% acrylamide-bisacryamide (37.5:1)	Bio-Rad
APS (Ammoniumpersulfate)	Sigma
β-Mercaptoethanol	Sigma
BenchMark pre-stained protein ladder	Invitrogen
Bromphenol blue	Merck
BSA	Sigma
CpG DNA (355pmol/µl)	Cybergene
DMSO(Dimethylsulfoxide)	Sigma
EDTA	Sigma
Filter paper	Whatman
Glycerol	Sigma
Glycine	Sigma
Griess reagent	Merck
Halt™ protease inhibitor cocktail	Pierce
HCl	Merck
IFNγ	Pepro Tech
Isopropanol	Merck
Lipofectamine™ 2000 Reagent	Invitrogen
LPS	Sigma
Methanol	Sigma
Milk powder	Semper
Murine GM-CSF	Pepro Tech
NaCl	Merck
NP-40	Sigma
Octylglucoside (OG)	Roche Applied Science
Polyinosinic-Polycytidylic acid (Poly:IC)	Sigma
SDS (Sodium dodecyl sulfate)	Sigma
Sucrose	Merck
TEMED (N,N,N',N''-Tetramethylethylendiamin)	Sigma
Tris	Sigma
Trypan blue	Fluka
Tween20	

9.1.2 Enzymes

Platinum Taq polymerase	Invitrogen
BglII	Invitrogen
EcoRI	Invitrogen
PstI	Invitrogen
BamHI	Invitrogen
NotI	Invitrogen
XhoI	Invitrogen

9.1.3 Buffers

Mild washing buffer	50 mM Tris pH 7.5-8 150 mM NaCl 0.1% NP-40 1 mM EDTA pH 8 1% protease inhibitors
Incubation Buffer	50 mM Tris pH 7.5 150 mM NaCl 5 mM EDTA 0.25% NP-40 2% BSA
5x reducing sample buffer	50% Sucrose 0.625M Tris 1% SDS 10% β-Mercaptoethanol → pH 6.8 Bromphenol blue
10x running buffer	0.25M Tris 1.92M Glycine
1x running buffer	10x running buffer 0.1% SDS ddH_2O
1x transfer buffer	10x running buffer 10% (v/v) MetOH 0.1% SDS ddH_2O
10x TTBS	0.2 M Tris pH 7.5 1.5 M NaCl
1x TTBS	10x TTBS 0.05% Tween20 ddH_2O
Stripping buffer	25mM glycine 1% SDS → pH 2,0
FACS buffer	PBS 2% FCS
MACS buffer	PBS 0.5% FCS 2mM EDTA

Cell lysis buffer I	50mM Tris 150mM NaCl 1mM EDTA 10% Glycerol; → pH 7.54 1% NP-40 1% protease inhibitors
Cell lysis buffer II	50mM Tris 150mM NaCl; → pH 7.54 1% Octylglucoside 1% protease inhibitors

9.2 Cell culture media

9.2.1 Antibiotics

Penicillin/streptomycin (10 000 units penicillin and 10mg streptamycin/ml in 0.9% NaCl)	Sigma
Gentamicin (50mg/ml)	Sigma

9.2.2 Media supplements

Foetal calf serum	Gibco
L-Glutamin (200mM)	Sigma
Sodium pyruvate (100mM)	Sigma
MEM non-essential amino acid solution (100x)	Sigma
β-Mercaptoethanol solution (50mM)	Sigma

Cell culture media

Complete RPMI:

RPMI – 1640	Sigma
10% fetal calf serum	
2mM L-Glutamin	
1% (v/v) Penicillin/streptamycin	
1mM Sodium pyruvate	
1x MEM	

Macrophage medium:

RPMI – 1640 (endotoxin tested) + L-Glut	Gibco
10% fetal calf serum	
10 mM L-Glutamin (final concentration)	
10µg/ml Gentamicine	
20mM HEPES	

Complete DMEM:

DMEM	Gibco
10% fetal calf serum	
2mM L-Glutamin	
1% (v/v) Penicillin/streptamycin	
10mM HEPES	

9.3 Antibodies and beads

CD3 – FITC	BD Pharmingen
CD3 – PE	BD Pharmingen
CD14 – FITC	BD Pharmingen
NK1.1 – FITC	BD Pharmingen
NK1.1 – APC	BD Pharmingen
NK1.1 – PE	BD Pharmingen
TMβ-1– FITC	
F4/80 – FITC	Caltag Laboratories
B220 – FITC	Caltag Laboratories
B220 – PE	BD Pharmingen
CD11c – PE	BD Pharmingen
Gr1 – PE	BD Pharmingen
CD19 – APC	BD Pharmingen
CD19 – FITC	BD Pharmingen
CD80– FITC	BD Pharmingen
CD86– FITC	BD Pharmingen
anti-IκBa	CellSignaling Technology
anti-TRAPC: KPK9, chicken polyclonal (IgY)	Agrisera
CSE11, chicken polyclonal (IgY)	Agrisera
KPK9, rabbit polyclonal (IgG)	Agrisera
CSE11, rabbit polyclonal (IgG)	Agrisera
anti-FLAG (polyclonal)	Sigma
anti-Flag (M2)	Sigma
anti-HA.11	Nordic Biosite
anti-human IgG – FITC	Jackson
anti-chicken – Alexa Fluor 647	Molecular Probes
anti-rabbit IgG – Alexa Fluor 647	Molecular Probes
anti-rat IgG – FITC	Caltag Laboratories
anti-human IgG – HRP	Caltag Laboratories
anti-rabbit IgG – HRP	Sigma
anti-chicken IgG – HRP	Sigma
anti-mouse IgG – HRP	Sigma
anti-FITC-MicroBeads	Miltenyi Biotech
anti-CD11c-Microbeads	Miltenyi Biotech
PROSEP-A High Capacity	Millipore
Protein G Sepharose 4 Fast Flow	GE Healthcare

9.4 Cell lines

RMA-S, a TAP deficient murine T cell lymphoma	Karolinska Institutet
P815, murine mastocytoma	ATCC
YAC-1, murine lymphoma	ATCC
J774.1, murine macrophage like cell line	ATCC
RAW 264.7, murine macrophage like cell line	ATCC
HEK 293T, human embryonic kidney cell line	ATCC
CHO, Chinese hamster ovary cell line	ATCC

9.5 Kits

RNeasy kit	Qiagen
TOPO-TA Cloning kit	Invitrogen
d-rhodamine terminator cycle sequencing kit	Perkin Elmer
ECL chemoluminescence detection kit	Amersham
Enzymatic deglycosylation kit	Prozyme
Coomassie Plus Protein Assay Reagent Kit	Pierce
HiTrap Protein A HP columns	Amersham Biosciences

9.6 Instruments

Centrifuge 5810R (rotor: A-4-62)	Eppendorf
Electrophoresis apparatus	Bio-Rad
ELISA reader	Molecular devices
FACScan	Becton Dickinson
FACSort	Becton Dickinson
Heating block	Grant instruments
MidiMACS separator	Miltenyi Biotech
Microscope, confocal	Leica
Spectrophotometer	Pharmacia Biotech
Table top centrifuge (rotor: 220.59 V07)	Hermle
377 Applied Biosystems automatic sequencer	Perkin Elmer

9.7 Software

Flow cytometry acquisition	CellQuestPro
Flow cytometry analysis	DAKO Summit
Confocal image analysis	Volocity
	Adobe Photoshop
Statistics	Graph Pad Prism

Molecular Biology:

BioEdit	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
ClustalX	http://bips.u-strasbg.fr/fr/Documentation/ClustalX/
Staden Package	http://staden.sourceforge.net/
Genedoc	http://www.nrbsc.org/gfx/genedoc/index.html
pDRAW	http://www.geocities.com/acaclone/
Treeview	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
Oligo6	Molecular Biology Insights, Inc.

9.8 Methods

9.8.1 RT-PCR

Total RNA from C57BL/6 spleen and liver cells was purified using the RNeasy kit. The cDNA (TRAPC) containing the complete open reading frame (756 bp) was obtained using the primers: 5' CCT TTT CTC CTC TCC TCT AC (TRAPC-for) and 5' TTT GGC CTG TTC TGC CTT AG (TRAPC-rev). Amplification was performed with 20 pmoles of each primer for 35 cycles (1 min at 95^{o}C, 30 s at 52^{o}C, 1 min at 72^{o}C), followed by 7 min incubation at 72^{o}C, utilizing Platinum Taq polymerase. The amplification products were subcloned in pCR2.1 vector by TOPO-TA Cloning kit and were sequenced in both directions. All animal experiments were approved by the Committee for Animal Ethics in Stockholm, Sweden.

9.8.2 Analysis of TRAPC tissue and cell line expression by nested RT-PCR

Total RNA from unstimulated and tilorone stimulated C57/B6 spleen, liver, lymph node, lung, kidney, uterus, and brain cells were purified using the RNeasy kit. RNA of the following cell lines was used: fetal skin dendritic cells (FSDC), CB-1, D2SC/1), RAW 264.7, J774.1, SV40 MES-13, BV-2b, MCP5/L, IC-2, P815, WEHI 231, 70Z/3, J558, S194, RMA, ALC and CTLL-2 (kindly provided by Dr. S. Applequist, Karolinska Institute, Stockholm, Sweden [236]).

In the first round of the nested PCR the TRAPC-for and TRAPC-rev primers were used (see above). Amplification was performed with 20 pmoles of each primer for 20 cycles (1 min at 95^{o}C, 30 s at 54^{o}C, 40 s at 72^{o}C), followed by 7 min incubation at 72^{o}C, utilizing Platinum Taq polymerase.

In the second round of the nested PCR the following primers were used to amplify a 373 bp fragment of the TRAPC product: 5' AAC TGC TCC TGG TCC CTG TG (TRAPC-nested-for) and 5' GTT GAT GGG GCT GGC GAC AC (TRAPC-nested-rev). Amplification was performed with 20 pmoles of each primer for 25 cycles (1 min at 95^{o}C, 30 s at 54^{o}C, 40 s at 72^{o}C), followed by 7 min incubation at 72^{o}C, utilizing Platinum Taq polymerase.

As a control of the quality of the starting material a set of primers specific for β-actin was used: 5' CAT CCA TCA TGA AGT GTG ACG (Act-for) and 5' CAT ACT CCT GCT TGC TGA TCC (Act-rev). The PCRs were performed in a 35-cycle reaction (1 min at 95^{o}C, 30 s at 54^{o}C, 40 s at 72^{o}C).

9.8.3 Preparation of monocyte, macrophage, T-, NK- and B-cell populations

Dendritic cells from 7-day GMCSF bone marrow dendritic cell cultures from C57BL/6 mice were isolated with magnetic cell sorting (MACS) using MiniMACS or MidiMACS. Cells were labeled with anti-CD11c microbeads for 15 minutes at 4^{o}C. The cells were washed and separation was performed according to the manufacturer's instructions. The purity of the sorted CD11c+ dendritic cells was usually ~ 98%.

To purify monocyte and B-cell populations erythrocyte-depleted splenocytes from a tilorone stimulated female C57BL/6 mouse were stained with CD14-FITC, B220-FITC, CD19-FITC, F4/80-FITC antibodies for 20 minutes at 4^{o}C. Subsequently these cells were incubated with anti-FITC microbeads for 15 minutes at 4^{o}C.

The cells were washed and separation was performed according to the manufacturer's instructions. The purity of these cell populations was usually ~ 98%.

In order to purify NK-cells and T cells erythrocyte-depleted splenocytes from a tilorone stimulated female C57BL/6 or NOD mouse were stained with CD3-PE and NK1.1-FITC, CD3-PE and TMβ-1-FITC respectively. $CD3^+NK1.1^-$, $CD3^+TM\beta\text{-}1^-$ and $CD3^-NK1.1^+$, $CD3^-TM\beta\text{-}1^+$ cell populations were sorted in a FACSDiva. B-cell populations were also sorted in this way by using B220-FITC, CD19-FITC.

RNA was extracted using the RNeasy kit and nested RT-PCR as described above was performed.

9.8.4 Constructing TRAPC-HA, TRAPC-GFP, DAP12-FLAG, CD3ζ-FLAG and FcεRIγ-FLAG plasmids

For constructing the TRAPC-HA construct the following primer set was used: 5' AGA TCT TCC ACA GTA TCT GAA (TRAPC-BglII-for containing the BglII restriction site) and 5' CTG CAG TTA GTA CCA GTT AGC (TRAPC-PstI-rev containing the PstI restriction site). Amplification on the pCR2.1 vector, containing the full length TRAPC cDNA, was performed with 20 pmoles of each primer in 35 cycles (1 min at 95°C, 30 s at 52°C, 1 min at 72°C), followed by a 7 min incubation at 72°C, utilizing Platinum Taq polymerase.

The amplification product was subcloned in pCR2.1 vector by TOPO-TA Cloning kit and was sequenced in both directions. The plasmid was digested with BglII and PstI restriction enzymes and subsequently cloned in frame with the Ig κ-chain signal sequence into the pDisplay vector.

Primers used for the constructing TRAPC-GFP construct were the following: 5' CCT TTT CTC CTC TCC TCT AC (TRAPC-for) and 5' GGA TCC GTA CCA GTT AGC CTT C (TRAPC-BamHI-rev, containing the BamHI restriction site). Amplification was performed with 20 pmoles of each primer in 35 cycles (1 min at 95°C, 30 s at 52°C, 1 min at 72°C), followed by 7 min incubation at 72°C, utilizing Platinum Taq polymerase. The amplification product was subcloned in pCR2.1 vector by TOPO-TA Cloning kit and was sequenced in both directions. The product was then cloned into the pEGFP-N3 expression vector (Clontech) using the EcoRI restriction site of the pCR2.1 vector and the BamHI restriction site.

To construct FLAG tagged DAP12, CD3ζ and FcεRIγ we amplified CD8-leader sequence and the FLAG tag from the BSRα-TREM-1-FLAG vector (kindly provided by dr. M. Daws, University of California, San Fransisco) using the following primer set: GCG GCC GCA TGG CCT TAC CAG (CD8-leader-FLAG-NotI-for, containing the NotI restriction site) and CTC GAG GCT TGT CGT CAT CGT (CD8-leader-FLAG-XhoI-rev, containing the XhoI restriction site). Amplification was performed with 20 pmoles of each primer in 35 cycles (1 min at 95°C, 30 s at 52°C, 1 min at 72°C), followed by a 7 min incubation at 72°C, utilizing Platinum Taq polymerase. The amplification product was subcloned in pCR2.1 vector by TOPO-TA Cloning kit and was sequenced in both directions. The PCR product was subsequently cloned into the pCDNA3.1 (zeo+) vector with the NotI and XhoI restriction enzymes and sequenced to check for correct insertion and ORF.

Into this pCDNA3.1 (zeo+) containing the CD8-leader-FLAG DNA we cloned in the DAP12, CD3ζ and FcεRIγ cDNA lacking the leader sequence using the restriction enzyme XhoI in order to create the FLAG-tagged adaptor molecules. For this reason the adaptor molecules were amplified with the following primer sets: DAP12, CGC TCG AGC CCA GAG TGA CAC TT (DAP12-XhoI-noleader-for, containing the XhoI restriction site) and GGC ATA GAG TGG GCT CAT CTG (DAP12-rev), CD3ζ, CGC TCG AGC ACA GAG CTT TGG TC (CD3ζ-XhoI-noleader-for, containing the XhoI restriction site) and TGG GAA ATG AAT AAA CTG AG (CD3ζ-rev) and FcεRIγ, CGC TCG AGC CCT GGG AGA GCC GC (FcεRIγ-XhoI-noleader-for, containing the XhoI restriction site) and AGC TAC TGG GGT GGT TTT TC (FcεRIγ-rev). Amplification was performed with 20 pmoles of each primer in 35 cycles (1 min at 95^{o}C, 30 s at 52^{o}C, 1 min at 72^{o}C), followed by 7 min incubation at 72^{o}C, utilizing Platinum Taq polymerase. The amplification products were subcloned in pCR2.1 vector by TOPO-TA Cloning kit and were sequenced in both directions.

9.8.5 DNA sequencing

All clones were sequenced in both directions by a d-rhodamine terminator cycle sequencing kit and a 377 Applied Biosystems automatic sequencer.

9.8.6 Passaging cells

All cell culture works were performed under sterile conditions (laminar flow) using 25cm^2 or 75cm^2 cell culture flasks. Incubations were all done at 37°C with 7.5% CO_2 if not otherwise indicated. Suspension cells were grown in complete RPMI and split every other day on a 1:10 ratio. Slightly adherent cells were cultured in the same way except the fact that they had to be detached from the bottom of the flask by rigorous pipetting.

The murine macrophage like cell lines J774.1 and RAW 264.7 that grow adherently to a confluent monolayer were cultured in a special batch of RPMI medium which had been tested for endotoxin and supplemented with endotoxin-free FCS to ensure that the cells did not become activated before functional assays were performed. Cells split every other day in a 1:6 ratio.

9.8.7 Freezing and thawing of cells

In order to keep cells stored for longer periods they were frozen at -80°C. To prepare the cells for freezing, they were centrifuged (5min, 1500rpm) in a falcon tube and the pellet was resuspended in FCS/10% DMSO (1ml/1*10^6 cells). Then the cells were divided in aliquots of 1 million cells each in 1ml cryotubes. The tubes were placed in the -80°C freezer in a freezing box filled with isopropanol to ensure slow and even cooling.

When taking up frozen cells again, they were thawed quickly and transferred directly to 10ml of pre-warmed medium. After incubating at 37°C for 5 minutes to allow the cells to recover, they were pelleted and seeded out in fresh medium.

9.8.8 Transfections

293T cells were transiently transfected with either TRAPC-HA or DAP12-FLAG using Lipofectamine 2000 according to the manufacturers protocol.

For co-transfection of 293Ts with TRAPC-HA + DAP12-FLAG, TRAPC-HA + CD3ζ-FLAG, or TRAPC-HA + FcεRIγ-FLAG a modified lipofectamine 2000 protocol was used; 18 µl of Lipofectamine 2000 reagent was added to 250 µl Optimem medium and left for 5 min at room temperature (RT). For each co-transfection, 3 µg of DNA of both plasmids was added to 250 µl Optimem. The 2 mixes were added together and incubated for 20 min at RT. Subsequently, the mix was added drop-wise to the cells in 6 well plates containing RPMI and 10% FCS and incubated at 37^{o}C for 16 hours.

9.8.9 Polyclonal antibodies

The predicted amino acid sequences of TRAPC (genbank accession number DQ186654) and mouse TREM-2a (genbank AY024348) were submitted to Swiss-Model (http://swissmodel.expasy.org/), an automated protein-modeling server [237]. The acquired 3D models were analyzed with the program Deep View Swiss-PdbViewer (http://www.expasy.org/spdbv/) were the molecular surface was computed showing the electrostatic potential spanning –5 (red) to 5 (blue) [203]

Two predicted loops in the model were selected as peptide antigens for producing polyclonal antibodies. Each of these peptides, KPKEESYVC (hereafter named KPK9) and CSEPRKAAREL (CSE11), were synthesized and used to immunize 1 rabbit and 1 hen respectively. High titers in serum/egg yolk were confirmed by ELISA, and the polyclonal antibodies were purified using the respective peptide antigen. Peptides and antibodies were made by/purchased from Agrisera.

9.8.10 Flow cytometry

Harvested or isolated cells were washed in PBS and counted. 3-10*10^{5} cells were taken for a staining. All following incubations were carried out on ice; FACS buffer (PBS supplemented with 2% FCS) was used for washing and diluting antibodies and all centrifugations were done at 1500rpm for 5 minutes. Cells were pelleted in a 5ml FACS tubes or a 96 well v-bottom plate, depending on the number of samples. In order to avoid unspecific antibody binding, especially to Fc receptors, cells were blocked for 15 minutes in 50µl FACS buffer containing mouse serum in a 1:50 dilution. The diluted antibody was added directly into the blocking solution and incubated for 30 minutes. Final antibody concentration was approximately 1µg/10^{6} cells. Two washing steps followed before the cells were re-suspended in 300µl FACS buffer and measured by flow cytometry.

9.8.11 Immunoprecipitation

Cells were harvested or isolated and washed in PBS according to previous descriptions. The cell pellet was loosened and kept on ice for the complete lysis procedure. Lysis buffer I or II was added at a volume of 500µl/30*10^6 cells. The lysed cells were mixed by pipetting and vortexing and incubated on ice for 20 min. Lysates were centrifuged (15 min, 13000rpm) in a tabletop centrifuge to get rid of nuclei and debris.

The beads were washed three times in mild washing buffer. For each sample, 75µl of Protein G beads were incubated with 5µg antibody or fusion protein as indicated in the figure legends and 500µl incubation buffer on a rotating wheel at 4°C over night.

The beads coupled to the irrelevant fusion proteins or antibodies were washed for three times in mild washing buffer before 500µl of cell lysate was added. The mixtures were incubated for 2h at 4°C on a rotating wheel. Supernatants cleared from unspecific binding proteins were transferred to the beads coupled to the fusion protein or antibody of interest (that had been washed before (3x)).

The immunoprecipitation took place over night at 4°C on a rotating wheel. Thereafter, the beads were washed extensively (10x) in mild washing buffer and finally boiled (95°C) in 15µl 1x sample buffer for 5 minutes. The tubes were centrifuged again and the supernatant was loaded on a 12% SDS-PAGE gel and subsequently stained with coomassie or used for western blotting.

9.8.12 Western blotting

Proteins were transferred to Hybond P PVDF membranes and blocked for 1h in a 5% solution of non-fat dried milk dissolved in TTBS with gentle agitation on an orbital shaker. The membranes were probed with antibodies as indicated in the respective figure legends. All antibody incubations were done for one hour and after each step, the membrane was washed extensively (3x 15min) with TTBS. Membranes were developed using ECL according to the manufacturer's protocol.

In some instances membranes were stripped for 45 minutes using stripping buffer. The membranes were washed in TTBS and blocked and probed again with other antibodies. ECL detection was performed before re-probing.

9.8.13 LPS stimulation of macrophages and detection of TRAPC

Murine macrophage cell line (J774.1) was plated out in 6 well plates, 500.000 cells/well, and stimulated for 16 hours with 0,1 – 100 ng/ml LPS (Sigma, from E.coli 026:B6). Cells were harvested and lysed with RIPA buffer (1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Protein concentration was measured by Coomassie Plus Protein Assay Reagent and spectophotometry. Samples were diluted to equal protein concentrations and reducing sample buffer (50% sucrose, 0.625M Tris, 1%SDS, 10% β-mercapto ethanol) was added. The samples were boiled for 5 minutes prior to resolution by SDS-PAGE using 12% polyacrylamide gels.

Membranes were probed with CSE11 (IgY and IgG) to detect TRAPC. Subsequently the membranes were incubated with a secondary HRP-conjugated goat anti-rabbit IgG antibody or a secondary HRP-conjugated rabbit anti-chicken IgG antibody and specific signals were detected with ECL reagents. Subsequently the membranes were stripped for 30 minutes in low pH stripping buffer (1% SDS, 25mM glycine, pH 2,0) and incubated with a mouse anti actin antibody followed by a secondary HRP-conjugated sheep anti-mouse IgG antibody.

9.8.14 In vitro *cell culture infection model.*

E. coli TG1, *E. coli* DH5a and *S. enterica* serovar Typhimurium TT16729 [238] were used in this study.

J774-A.1 cells, were cultivated in RPMI medium supplemented with 10% FBS, L-glutamine (10 mM final concentration), HEPES (10 mM final concentration) and gentamicin (10 μg/ml final concentration).

24-well cell culture plates were coated with the polyclonal hen antibodies KPK9 or CSE11, or the corresponding pre-immune IgY, for 48 hours at 4°C. Thereafter the plates were washed and seeded with $4x10^5$ J774-A.1 cells per well. One set of wells that were left non-coated served as control. The cells were left to cross-link and adhere for 4h before infection. Bacteria used for infection were cultured on LB agar plates over night at 37˚C to ensure a non-invasive phenotype [239]. Prior to infection bacteria were suspended in phosphate-buffered saline and opsonized *in vitro* for 30 minutes at 37˚C using 10% fresh pre-immune serum from BALB/c mice. Thereafter bacteria were diluted in cell culture medium supplemented only with HEPES (10 mM final concentration) and subsequently seeded on the J774-A.1 cells. The cell-culture plates were centrifuged 5 minutes at 1500 rpm to facilitate infection. One hour post infection, medium containing gentamicin at a concentration of 50 μg/ml was applied and cells were incubated for 40 minutes to kill extracellular bacteria. For continued incubations, the killing medium was replaced by maintenance medium containing 10 μg/ml of gentamicin. At indicated time points cells were lysed by hypotonic lysis and the amount of intracellular bacteria was determined by cfu counts of viable bacteria [189].

9.8.15 Statistical analysis

Data from cell culture experiments were analysed by a one-way ANOVA. This indicated differences between the groups with a p-value < 0.0001 in all experiments except the intracellular growth yields for *E. coli* and the phagocytosis assay for *S. enterica* serovar Typhimurium which gave p-values > 0.05 and < 0.001 respectively. The ANOVA analysis was followed by a two-sided T-test with Bonferroni corrections to determine significant differences between the different groups. All p-values from the T-test analysis are specified in the figure legends.

9.8.16 Preparation of dendritic cells from mouse bone marrow

Hind legs from B6 mice were used to isolate bone marrow cells. The femoral bone was cut on both ends and flushed with DMEM into a petri dish. The cells were passed via a cell strainer into a 50ml falcon tube and centrifuged at 1000rpm for 10 minutes. The cells were taken up in DMEM supplemented with murine GM-CSF at a final concentration of 10ng/ml in a volume of 18ml per leg and plated in a 12 well cell culture dish adding 3ml per well and incubated for 6 days. Subsequently, loose and loosely adherent cells were harvested and re-suspended in DMEM without GM-CSF and incubated for 1 day.

9.8.17 Isolation of murine spleen cells

C57BL/6 spleens were mashed and harvested. Red blood cells were lysed by hypotonic shock, i.e. 4.5ml of sterile ddH2O was added followed very quickly by 0.5ml of 10x PBS. After centrifuging and re-suspending in fresh medium, cell were counted.

9.8.18 Stimulation of cells

The macrophage cell line J774.1 was used to stimulate with IFNγ as well as different TLR stimuli, i.e. LPS recognized by TLR 4 and CpG recognized by TLR 9. $5*10^5$ cells/well in 1ml of macrophages medium were plated out in 24 well plates the day before and incubated over night. The stimulating agents were diluted in medium and added to the cells in the following concentrations: IFNγ at 1000U/ml, LPS 1µg/ml and CpG 507pmol/ml. Half of the cells were harvested after two hours for flow cytometry staining, the other cells were incubated until 16 hours post stimulation and then harvested, stained and analyzed.

9.8.19 Bacterial infection of cells

J774.1 macrophages were grown in $75cm^2$ cell culture flasks in macrophage medium and plated out in 24 well plates at a ratio of $5*10^5$ cells per well in 1 ml one day prior to infection. Bacteria were freshly streaked on LB-agar plates and incubated over night at 37°C. On the day of infection, several bacterial colonies were harvested and re-suspended in 3ml PBS. The OD at 600nm was acquired with a spectrophotometer and the bacteria were diluted with PBS until an OD^{600} between 0.2 and 0.5 was obtained. Subsequently, bacteria were opsonized for 15 minutes at 37°C by adding 10% mouse serum to 100µl of the bacteria-PBS solution.

Thereafter, bacteria were diluted in macrophage medium according to the result of the following equation which allows to estimate the number of bacteria to infect with: $(0.488/OD^{600}) * 2.1 = x$ µl. X determines the volume of bacterial solution to be added per ml of macrophage medium to reach an amount of approximately 10^6 bacteria/ml. The cells which had been plated out the previous day were washed twice in RPMI / 20mM Hepes to get rid off any remaining gentamicine that would kill bacteria and prevent from a successful infection. 1 ml of medium containing bacteria was added to each well; plates were centrifuged (5min, 1500rpm, without acceleration and brake) and incubated for 1 hour at 37°C.

Subsequently, supernatants were discarded and bacteria that had not been phagocytosed were killed by incubation with 1ml of macrophage medium containing 250µg/ml gentamicine for 45 minutes at 37°C. Cells were washed twice in PBS and 2h samples were harvested at that time point. In the residual wells, medium was changed to the usual macrophage medium and the cells were incubated until harvesting.

9.8.20 Measurement of NO

Host cell NO production was analyzed by following the accumulation of nitrite in the cell culture medium using Griess reagent (nitrite test, Merck, Darmstadt, Germany). A standard curve, using sodium nitrite, was used to convert values from absorbance to µM.

9.8.21 TRAPC-Fc fusion protein

The extracellular domain of TRAPC, lacking the leader sequence and its transmembrane domain, was amplified with the primers GCTAGCTCCACAGTATCTGAAGA (TRAPC-noleader-NheI) and GCGGATCCCCATATTGCACAGAG (TRAPC-noTM-BamHI), and cloned in frame into the pCDM8 vector containing the CD5 leader sequence and the human IgG1 Fc construct using the restriction enzymes NheI and BamHI.

The TRAPC-Fc fusion plasmid was used to transfect 293T cells using lipofectamine 2000 according to the manufacturer's protocol. Plates were incubated over night. Subsequently, cells were harvested and washed twice in PBS before re-suspension in RPMI supplemented with 5% of FCS depleted of immunoglobulins.

After 5-6 days, the cell culture supernatants were collected, pooled and centrifuged (2x 15min, 3600rpm) to remove cells and cell debris. The supernatant was run over HiTrap Protein A HP columns according to manufacturer's protocol. The buffer of the purified TRAPC-Fc fusion protein was subsequently exchanged to PBS using either dialysis or PD-10 columns containing SephadexTM G-25 according to manufacturer's instructions. Briefly, they were equilibrated with 25ml of PBS and the flowthrough was discarded. Then a sample volume of 2.5ml was added and the flowthrough was again discarded. This was followed by elution with 3.5ml PBS and collecting the flowthrough containing the sample proteins in PBS now.

Protein concentration was determined using the Coomassie Plus protein assay, measured by absorption maximum from 465nm to 595nm with a concomitant color change from brown to blue. Protein concentrations were estimated by reference to absorbencies obtained from a standard curve ranging from 62.5µg/ml to 2000µg/ml.

9.8.22 Preparation of cells for confocal microscopy

Both, J774.1 cells and freshly isolated spleen cells were used in confocal microscopy. After washing and counting the cells, cell were stained similar as for flow cytometry. The antibodies and Fc fusion proteins were used in the same concentrations and incubated with the cells. Primary antibody or fusion proteins were incubated for 40 min, and secondary fluorescently labelled antibody for 10 min. Subsequently the cell suspension was placed on an object slide and sealed with nail polish. Slides were kept on ice in the dark and analyzed in a confocal microscope using the 63-times magnification.

10 Acknowledgements

This is the part that is read the first and written the last in any thesis I have come across so far. Therefore I have not written it in the last minute, in order read and rewrite this part as many times as I saw fit and to have more time to reflect back and think of ways to thank people in the best and most sincere way.

First of all I would like to thank my main supervisor **Jonas Sundbäck**. When I came to Sweden the first time back in 1999, to do my practical training, you came to pick me up from Arlanda. You wrote to me in an email: Look for the bold guy with the beard! I have to say: It was easy to find you! From that moment on we had a click and performed research on the highest level in the lab as well with yeast outside the lab! You taught me all there is to know on Molecular Biology and Freeware Molecular Biology programs. You shared your enthousiasm for science with me and I was hooked. That's why I came back after finishing my studies in Holland to do my full PhD under your supervision. We rolled into the TRAPC business together and you made me the researcher I am now. Through you I became independent and conducted my own experiments, in the end merely showing you the results to discuss the next experiments to be done. You truly were a good supervisor but also a friend to whom I could always talk about everything, including computer programs, jazz music, movies and of course exquisite food and whisky. Jonas, thank you for everything and most of all for believing in me!

Then my Professor and Co-supervisor **Klas Kärre**. Klas, thank you for having me in your lab and for asking me after my practical training period if I wanted to come back to Sweden to do my full PhD in your lab. The decision I made for myself to accept your invitation was one of the best decisions in my life. Thank you for all the advise you have given me on my research throughout the years, all scientific discussions, help with writing papers and my thesis and for backing me up when I made the decision to write my thesis in a monograph format and doing that in Holland. In the thesis of Håkan you have been mentioned as the Gandalf of the lab and Hanna rather called you Professor Dumbledore. I feel I cannot stay behind in comparing you to someone. So, to maybe start a tradition in your lab, I would like to compare you to the mighty First Wizard: **Zeddicus Zu'l Zorander**. (Try to figure this one out!)

My collaborators:

Mikael Rhen: Thank you for your infectious enthusiasm about our macrophage research, your ever present help by any means and your incredible quick way of designing experiments to test hypotheses in such a way that any result is a succes. You truly are a walking molecular biochemistry encyclopaedia.

Eva Bjur: You called me you macrophage friend, and that is very true. It was a joy to work together with you. The way you worked, the pace you worked in and your planning of experiments completely matched with my ways. By this we were a real 2-man team achieving perfectly reproducible results, which is every researchers dream! Thank you for getting my project to an intriguing higher level. I wish you all the luck in Canada!

Gunther Schneider and **Daniel Ågren**: Even though we never reached a breakthrough it was a pleasure to have worked with you. I know that somewhere out there is a condition in which TRAPC will form perfect crystals!

My laboratory colleagues:

Bruno Vanherberghen (a.k.a Bruney, Bruuuuuunnnnnnniiiieeeeeeee and Da Brunonizer): Thank you for being an awesome friend and writing room buddy. Thanks for being there for me to annoy you almost every moment of the day! For all the fun in the lab, our exquisite dancing moves, practical jokes and endless discussions over many a good beer and BAM! In the end I finally tricked you into listening to POWER METAL!!!! Oh, what a suffering it was for all our other colleagues working in the lab! It has been an honour to know you. When will you come visit in Amsterdam?

Michael Uhlin (a.k.a The Uhlin and The Loser): Even though we never hung out in your Masucci period, I was truly happy that you came and worked with us the last 2 years! You are a great friend and BAM buddy! I loved all our gossip stories during our running sessions in Haga Parken or at our intensive tredmill running at Friskis and Svettis. It was awesome to share views with you on the life of Hirad Coldheart and The Unknown Warrior. Raven with me!!!! Yet again, It has been an honour to know you! Poopelipoo!!!

Gustaf Vahlne (a.k.a Moustache Man, Da Vahlnenizer, Prince of Göteborg and The Hulk): You are so good looking! If there was a club only for beautiful people we would be number 1 and 2 on the guestlist! Thanks for being an excellent friend and sharing your view on how to apply for a job in the industry. For the third time: It has been an honour to know you.

To all 3: You guys were the best lunch buddies ever! Always in for a good laugh and never shied a way from harsh views on various things in life! Thanks for all the great nights out and an incredible amount of fun in the lab. Without you guys, my stay at MTC would not have been so nice! Thanks again!

Lunch brings me to **Hjulet (a.k.a The Wheel)**: My favourite lunch place with excellent service! Everyday I felt a warm welcome there. Always a smile on the faces of the people serving the food! Over the years colleagues of mine called you guys my Hjulet buddies, and somehow that's just how it felt. Thanks so much for all the free Thursday pancakes! You guys rock!

Hanna Sjölin (a.k.a The Yolin): It has been a pleasure to have you as a friend and colleague. Your eagerness to discuss my latest results on DAP12 and other science-related matters was contagious. The fact that you loved Richard Rahl, Kahlan Amnell, Nathan Rahl and Zedd was something I highly valued! Even Ender was a beloved guest of our conversations. Remember: Never trust a person that does not eat cheese!

Håkan Hall: Thanks for being there as a friend since all the way back in 1999. The endless Americas Army sessions were wonderfull. Too bad you moved away to Canada, so we could not see each other so often anymore. Good luck with your MBA!

The rest of my colleagues **Petter Höglund, Björn Örnfeldt, Katja Andersson, Danika Schepis, Louise Berg, Maria Johansson, Petter Brodin, Hanna Brauner, Eleftheria Rosmaraki, Alexander Rölle, Cristina Matos, Marjet Elemans, Mantas Okas, Stina Wickström, Andre Ortlieb, Elena Sandalova, Maxim Pavlenko**: Thank you all for being in the lab, for scientific discussions, for all the help I needed and for being the persons who you all are. Good luck in everything that you are doing now and are going to do in the future.
The CIM invasion:

Adnane Achour: Thanks for always being who you are! Enthusiastic, knowledgeable, smart, funny and uplifting! Thanks for believing in me as a scientist, even though I will not take your advice to stay in academics.

Daniel Badia, Melissa Norström and Elias Nehme: Thanks for being in the lab and making the corridor a lot more fun! Practical jokes, student-supervisor humor and gloves filled with dry ice were ever present with you guys around!

Benedict Chambers: Thanks for all the help and scientific discussions. You're a funny guy, but maybe you should take singing lessons! He he. Thanks for having loads of fun in the making of the famous MTC Christmas movies and the MTC calendar of 2000.

The students I supervised during the years:

Caroline Helmstetter: My favourite german student! You weren't actually my student, but we were more of a team trying to identify the TRAPC ligand. You were excellent! You could even handle my jokes about the germans. All the luck at the Max Planck Institute in Berlin!

Zijang Yang (a.k.a Jay): Thanks for doing a thousand minpreps and trying to clone TREML4. Also for showing me how to drink tea with double flavours and of course the excellent tie I got from you when you left for Switzerland. All the luck there with your PhD.

Thanusha Paramanathan: Thank you for your determination to work with our retroviral vectors. Sad to say but I never got them to work nicely. You were also an excellent student and I wish you all the luck in Sweden, while your "dude" is still in Sydney.

Ingrid Helander: My first student that helped me on my way towards my PhD. Your immunoprecipitations steered me into intriguing directions! Thanks a million for that. Oh, and not to forget that time when you played me and Gustaf Iron Maiden on your pirate radio station for which you worked as a dj!

My first supervisor ever **Thorbald van Hall**: Thank you for your supervision back in 1998 and for arranging my practical training in Sweden. Without you I would have never been in Sweden to do my PhD. Surprisingly enough, you even worked a while in Stockholm where we met again! Thanks!

Then I would like to express my thanks to people who made my stay in Sweden unforgettable and who have unknowingly contributed to my successful research and the writing of my thesis.

Marieke Verschuren: The girl I met on the net with a wish to move to Sweden. I helped her out with some arrangements and gave tips on how to go through all the Swedish burocracy. Who later turned out to be my lovely neighbour in Svedmyra! Marieke, thank you for all the fun we had, the late night talks about life over many a beer in Kellys before you moved here and later as a very good friend and neighbour during our barbecues. You truly are a good friend and I hope we will see each other often, now that I am back in Holland. Now we have to sing "De Vlieger" and the Dutch anthem somewhere in a bar in Amsterdam, or when I am there at Kellys!!!

Kristian Niemann: My guitar teacher, Therion buddy, neighbour and friend. Even though I wasn't the perfect student, we had fun anyways! Metal evenings, beers, whisky and barbecues were all a pleasure with you! You're awesome! Hope to see you often, be it either on stage or visiting me in Amsterdam!

Johan Niemann: The ever-present brother of Kristian and Therion buddy. I laughed my ass off with you! You're a cool dude who always knows every song played in the bar where we were drinking beer! Remember that KTH graduation party we crashed in our metal outfits?

Stijn Heessen: You are great! Thanks for bringing me into your group of friends. It was loads of fun with the Red Babs (Stijn, Kristian, Richard and me!). The many times we played "kick the can" and we went dancing in Göta Källare. All the MTC pubs we organized were excellent because of us. Hope to see you more often in Amsterdam!

Sebastiaan Janssen and **Judith Goes**: My Hyves friends! The first blind date I ever went on was a good one. You guys are great friends. Thanks for inviting us to the POW WOW. That was excellent. May we meet each other often!

Michelle Smid: Thank you for taking care of Cyntha! Because of you we had a great time in Stockholm together.

Gunnar Frykfors and **Pennicha Sabandar**: Thank you for letting me cook for Rent a Chef! That was truly excellent. I learned a lot from this cooking business! I'm still thinking of doing this myself in Holland! I'll keep in touch!

There are of course many more people I would like to thank, but to keep the list within a certain limit I cannot mention them all. So in this way I want to thank them all for their contribution to my excellent stay in Sweden and the succesfull conduction of my research during the last 6 years.

I also would like to express my thanks to my family.

My brother **Menno**: It has been fantastic to have you visit at least once a year in Stockholm! We had so much fun. It did not matter if it was hiking in Norway, camping on Sandhamn or drinking beer with my friends in Stockholm it was always fun. Lets not spend 160 euros again on beer in 1 night ok? On top of that you're an incredible brother! Thanks for everything! Stay Metal! (Too bad I cannot put a grunt in words here, cause that would have sufficed!)

My sister **Marije**: You are the best sister one could wish for. We had a lot of fun when you visited me at the student corridor Jägargatan. Keep the good things going and never let the caring side of you go away! You're the sweetest!

Opa en Oma van Prooijen en **Opa en Oma van der Holst**: Ik wil jullie graag bedanken voor alle goede zorgen die jullie hebben laten zien, al die jaren dat ik in Zweden heb gewoond en gestudeerd. Er werd iedere keer vol interesse gevraagd hoe het met mijn muizen ging! Sommigen hebben het niet overleefd, maar de dierenverzorgers op ons laboratorium dragen perfect zorg voor deze lieve beestjes!

Nico en Tine van Heerwaarden: I couldn't wish for nicer parents in law to be! Thank you for always being interested in what I was doing and that you let your daughter move to Sweden. The times you visited were awesome. Elitloppet, Skansen and Der Untergang were truly amazing with you! Thank you.

Papa and Mama: It is hard to put what I feel for you in just a few words here in my thesis. I'll try anyway. Thank you for being there for me throughout my whole life and supporting me wherever you could. For believing in me and letting me make my own choices. It must have been hard at times to have a son that lives abroad. For that I want to thank you with all my heart and soul, I love you!

Jonatan Pinkse: Here's the odd one out in my family, cause he isn't family! But he is my best friend for the last 16 years. Even though contact has sometimes not been as frequent as it should, every time I see you and talk to you its like we met each other yesterday for the last time. Unfortunately I cannot return the favour of you being my "Paranymf" at my defence, simply because the Swedish defence tradition is different. Being your "Paranymf" truly was an honour for me! But don't worry, I'll return the favour in another form very soon! Thank you for being you and being my friend! You're the best!

I would also like to thank **Me**: Because without Me this thesis would not have been possible. I thank Me for excellent scientific discussions, outstanding experimental skills and for always believing in Me.
(Some people will understand why I put this here! If you do not understand, you can either ask Daniel Davis, or me, to explain this for you!)

Last and most important I would like to thank

My wife to be, **Cyntha**: Thank you for being so courageous to move to Sweden and live with me. From the moment you moved in with me I couldn't imagine life without you anymore. Everything fell into place. Thank you for being there during the last stage of my PhD, for listening to me about what was going on in the lab, not understanding a word about the science I performed. You inspired me to go on when research sometimes wasn't easy. Thank you for pushing me to make clear choices and acting upon these choices.
And most of all, thank you for saying **Yes** to the most important question I ever asked in my whole life! My love for you is so overwhelming that it cannot be described in words. Every time I say to you these three words: "I Love You", they mean so much more than just that. Thank you for being my Cyntha, I love you with all my heart!

11 References

1 **Janeway, C. A., Jr.,** Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 1989. **54 Pt 1**: 1-13.

2 **Baggiolini, M., Dewald, B. and Moser, B.,** Human chemokines: an update. *Annu Rev Immunol* 1997. **15**: 675-705.

3 **Linehan, S. A., Martinez-Pomares, L. and Gordon, S.,** Mannose receptor and scavenger receptor: two macrophage pattern recognition receptors with diverse functions in tissue homeostasis and host defense. *Adv Exp Med Biol* 2000. **479**: 1-14.

4 **Barrington, R., Zhang, M., Fischer, M. and Carroll, M. C.,** The role of complement in inflammation and adaptive immunity. *Immunol Rev* 2001. **180**: 5-15.

5 **Aderem, A. and Underhill, D. M.,** Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 1999. **17**: 593-623.

6 **Hoebe, K., Jiang, Z., Tabeta, K., Du, X., Georgel, P., Crozat, K. and Beutler, B.,** Genetic analysis of innate immunity. *Adv Immunol* 2006. **91**: 175-226.

7 **Stein, D., Roth, S., Vogelsang, E. and Nusslein-Volhard, C.,** The polarity of the dorsoventral axis in the Drosophila embryo is defined by an extracellular signal. *Cell* 1991. **65**: 725-735.

8 **Hashimoto, C., Hudson, K. L. and Anderson, K. V.,** The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 1988. **52**: 269-279.

9 **Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. and Hoffmann, J. A.,** The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 1996. **86**: 973-983.

10 **Akira, S. and Takeda, K.,** Functions of toll-like receptors: lessons from KO mice. *C R Biol* 2004. **327**: 581-589.

11 **Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A., Jr.,** A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997. **388**: 394-397.

12 **Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B.,** Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998. **282**: 2085-2088.

13 **Aderem, A. and Ulevitch, R. J.,** Toll-like receptors in the induction of the innate immune response. *Nature* 2000. **406**: 782-787.

14 **Beutler, B., Hoebe, K., Georgel, P., Tabeta, K. and Du, X.,** Genetic analysis of innate immunity: identification and function of the TIR adapter proteins. *Adv Exp Med Biol* 2005. **560**: 29-39.

15 **Gan, L. and Li, L.,** Regulations and roles of the interleukin-1 receptor associated kinases (IRAKs) in innate and adaptive immunity. *Immunol Res* 2006. **35**: 295-302.

16 **O'Neill, L. A.,** How Toll-like receptors signal: what we know and what we don't know. *Curr Opin Immunol* 2006. **18**: 3-9.

17 **Kobayashi, K., Inohara, N., Hernandez, L. D., Galan, J. E., Nunez, G., Janeway, C. A., Medzhitov, R. and Flavell, R. A.,** RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 2002. **416**: 194-199.

18 **Hayden, M. S. and Ghosh, S.,** Signaling to NF-kappaB. *Genes Dev* 2004. **18**: 2195-2224.

19 **Rao, K. M.,** Molecular mechanisms regulating iNOS expression in various cell types. *J Toxicol Environ Health B Crit Rev* 2000. **3**: 27-58.

20 **Alderton, W. K., Cooper, C. E. and Knowles, R. G.,** Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001. **357**: 593-615.

21 **Mayer, B. and Hemmens, B.,** Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem Sci* 1997. **22**: 477-481.

22 **Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L. and Wiseman, J.,** N omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J Biol Chem* 1991. **266**: 6259-6263.

23 **Colonna, M.,** TREMs in the immune system and beyond. *Nat Rev Immunol* 2003. **3**: 445-453.

24 **Colonna, M. and Facchetti, F.,** TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. *J Infect Dis* 2003. **187 Suppl 2**: S397-401.

25 **Klesney-Tait, J., Turnbull, I. R. and Colonna, M.,** The TREM receptor family and signal integration. *Nat Immunol* 2006. **7**: 1266-1273.
26 **Bouchon, A., Dietrich, J. and Colonna, M.,** Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 2000. **164**: 4991-4995.
27 **Cantoni, C., Bottino, C., Vitale, M., Pessino, A., Augugliaro, R., Malaspina, A., Parolini, S., Moretta, L., Moretta, A. and Biassoni, R.,** NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med* 1999. **189**: 787-796.
28 **Vitale, M., Bottino, C., Sivori, S., Sanseverino, L., Castriconi, R., Marcenaro, E., Augugliaro, R., Moretta, L. and Moretta, A.,** NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med* 1998. **187**: 2065-2072.
29 **Bouchon, A., Hernandez-Munain, C., Cella, M. and Colonna, M.,** A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. *J Exp Med* 2001. **194**: 1111-1122.
30 **Bouchon, A., Facchetti, F., Weigand, M. A. and Colonna, M.,** TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 2001. **410**: 1103-1107.
31 **Daws, M. R., Lanier, L. L., Seaman, W. E. and Ryan, J. C.,** Cloning and characterization of a novel mouse myeloid DAP12-associated receptor family. *Eur J Immunol* 2001. **31**: 783-791.
32 **Chung, D. H., Seaman, W. E. and Daws, M. R.,** Characterization of TREM-3, an activating receptor on mouse macrophages: definition of a family of single Ig domain receptors on mouse chromosome 17. *Eur J Immunol* 2002. **32**: 59-66.
33 **Washington, A. V., Schubert, R. L., Quigley, L., Disipio, T., Feltz, R., Cho, E. H. and McVicar, D. W.,** A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 2004. **104**: 1042-1047.
34 **Washington, A. V., Quigley, L. and McVicar, D. W.,** Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood* 2002. **100**: 3822-3824.
35 **Allcock, R. J., Barrow, A. D., Forbes, S., Beck, S. and Trowsdale, J.,** The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur J Immunol* 2003. **33**: 567-577.
36 **Barrow, A. D., Astoul, E., Floto, A., Brooke, G., Relou, I. A., Jennings, N. S., Smith, K. G., Ouwehand, W., Farndale, R. W., Alexander, D. R. and Trowsdale, J.,** Cutting edge: TREM-like transcript-1, a platelet immunoreceptor tyrosine-based inhibition motif encoding costimulatory immunoreceptor that enhances, rather than inhibits, calcium signaling via SHP-2. *J Immunol* 2004. **172**: 5838-5842.
37 **King, R. G., Herrin, B. R. and Justement, L. B.,** Trem-like transcript 2 is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. *J Immunol* 2006. **176**: 6012-6021.
38 **Cannon, J. P., Haire, R. N., Mueller, M. G., Litman, R. T., Eason, D. D., Tinnemore, D., Amemiya, C. T., Ota, T. and Litman, G. W.,** Ancient divergence of a complex family of immune-type receptor genes. *Immunogenetics* 2006. **58**: 362-373.
39 **Ramanathan, B., Minton, J. E., Ross, C. R. and Blecha, F.,** Characterization of bovine cDNA encoding triggering receptor expressed on myeloid cells 1 (TREM-1). *Vet Immunol Immunopathol* 2004. **102**: 85-89.
40 **Ramanathan, B., Minton, J. E., Ross, C. R. and Blecha, F.,** Cloning of porcine triggering receptor expressed on myeloid cells-1 (TREM-1) and its induction by lipopolysaccharide, peptidoglycan, and Salmonella enterica serovar Typhimurium infection. *Dev Comp Immunol* 2005. **29**: 1-7.
41 **Stet, R. J., Hermsen, T., Westphal, A. H., Jukes, J., Engelsma, M., Lidy Verburg-van Kemenade, B. M., Dortmans, J., Aveiro, J. and Savelkoul, H. F.,** Novel immunoglobulin-like transcripts in teleost fish encode polymorphic receptors with cytoplasmic ITAM or ITIM and a new structural Ig domain similar to the natural cytotoxicity receptor NKp44. *Immunogenetics* 2005. **57**: 77-89.
42 **Viertlboeck, B. C., Schmitt, R. and Gobel, T. W.,** The chicken immunoregulatory receptor families SIRP, TREM, and CMRF35/CD300L. *Immunogenetics* 2006. **58**: 180-190.

43 **Alvarez-Errico, D., Aguilar, H., Kitzig, F., Brckalo, T., Sayos, J. and Lopez-Botet, M.,** IREM-1 is a novel inhibitory receptor expressed by myeloid cells. *Eur J Immunol* 2004. **34**: 3690-3701.

44 **Aguilar, H., Alvarez-Errico, D., Garcia-Montero, A. C., Orfao, A., Sayos, J. and Lopez-Botet, M.,** Molecular characterization of a novel immune receptor restricted to the monocytic lineage. *J Immunol* 2004. **173**: 6703-6711.

45 **Chung, D. H., Humphrey, M. B., Nakamura, M. C., Ginzinger, D. G., Seaman, W. E. and Daws, M. R.,** CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. *J Immunol* 2003. **171**: 6541-6548.

46 **Daish, A., Starling, G. C., McKenzie, J. L., Nimmo, J. C., Jackson, D. G. and Hart, D. N.,** Expression of the CMRF-35 antigen, a new member of the immunoglobulin gene superfamily, is differentially regulated on leucocytes. *Immunology* 1993. **79**: 55-63.

47 **Tomasello, E. and Vivier, E.,** KARAP/DAP12/TYROBP: three names and a multiplicity of biological functions. *Eur J Immunol* 2005. **35**: 1670-1677.

48 **Tomasello, E., Olcese, L., Vely, F., Geourgeon, C., Blery, M., Moqrich, A., Gautheret, D., Djabali, M., Mattei, M. G. and Vivier, E.,** Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12. *J Biol Chem* 1998. **273**: 34115-34119.

49 **Lanier, L. L., Corliss, B. C., Wu, J., Leong, C. and Phillips, J. H.,** Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* 1998. **391**: 703-707.

50 **Lanier, L. L.,** NK cell recognition. *Annu Rev Immunol* 2005. **23**: 225-274.

51 **Wu, J., Cherwinski, H., Spies, T., Phillips, J. H. and Lanier, L. L.,** DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. *J Exp Med* 2000. **192**: 1059-1068.

52 **McVicar, D. W., Taylor, L. S., Gosselin, P., Willette-Brown, J., Mikhael, A. I., Geahlen, R. L., Nakamura, M. C., Linnemeyer, P., Seaman, W. E., Anderson, S. K., Ortaldo, J. R. and Mason, L. H.,** DAP12-mediated signal transduction in natural killer cells. A dominant role for the Syk protein-tyrosine kinase. *J Biol Chem* 1998. **273**: 32934-32942.

53 **Chiesa, S., Mingueneau, M., Fuseri, N., Malissen, B., Raulet, D. H., Malissen, M., Vivier, E. and Tomasello, E.,** Multiplicity and plasticity of natural killer cell signaling pathways. *Blood* 2006. **107**: 2364-2372.

54 **Gattis, J. L., Washington, A. V., Chisholm, M. M., Quigley, L., Szyk, A., McVicar, D. W. and Lubkowski, J.,** The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *J Biol Chem* 2006. **281**: 13396-13403.

55 **Kelker, M. S., Debler, E. W. and Wilson, I. A.,** Crystal structure of mouse triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.76 A. *J Mol Biol* 2004. **344**: 1175-1181.

56 **Kelker, M. S., Foss, T. R., Peti, W., Teyton, L., Kelly, J. W., Wuthrich, K. and Wilson, I. A.,** Crystal structure of human triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.47 A. *J Mol Biol* 2004. **342**: 1237-1248.

57 **Radaev, S., Kattah, M., Rostro, B., Colonna, M. and Sun, P. D.,** Crystal structure of the human myeloid cell activating receptor TREM-1. *Structure* 2003. **11**: 1527-1535.

58 **Radsak, M. P., Salih, H. R., Rammensee, H. G. and Schild, H.,** Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. *J Immunol* 2004. **172**: 4956-4963.

59 **Bleharski, J. R., Kiessler, V., Buonsanti, C., Sieling, P. A., Stenger, S., Colonna, M. and Modlin, R. L.,** A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol* 2003. **170**: 3812-3818.

60 **Gibot, S., Massin, F., Le Renard, P., Bene, M. C., Faure, G. C., Bollaert, P. E. and Levy, B.,** Surface and soluble triggering receptor expressed on myeloid cells-1: expression patterns in murine sepsis. *Crit Care Med* 2005. **33**: 1787-1793.

61 **Mahdy, A. M., Lowes, D. A., Galley, H. F., Bruce, J. E. and Webster, N. R.,** Production of soluble triggering receptor expressed on myeloid cells by lipopolysaccharide-stimulated human neutrophils involves de novo protein synthesis. *Clin Vaccine Immunol* 2006. **13**: 492-495.

62 **Knapp, S., Gibot, S., de Vos, A., Versteeg, H. H., Colonna, M. and van der Poll, T.,** Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia. *J Immunol* 2004. **173**: 7131-7134.

63 **Simon, H. U.,** Neutrophil apoptosis pathways and their modifications in inflammation. *Immunol Rev* 2003. **193**: 101-110.

64 **Fortin, C. F., Lesur, O. and Fulop, T., Jr.,** Effects of TREM-1 activation in human neutrophils: activation of signaling pathways, recruitment into lipid rafts and association with TLR4. *Int Immunol* 2006.

65 **Dykstra, M., Cherukuri, A., Sohn, H. W., Tzeng, S. J. and Pierce, S. K.,** Location is everything: lipid rafts and immune cell signaling. *Annu Rev Immunol* 2003. **21**: 457-481.

66 **Fortin, C. F., Lesur, O. and Fulop, T., Jr.,** Effects of aging on triggering receptor expressed on myeloid cells (TREM)-1-induced PMN functions. *FEBS Lett* 2007.

67 **Hotchkiss, R. S. and Karl, I. E.,** The pathophysiology and treatment of sepsis. *N Engl J Med* 2003. **348**: 138-150.

68 **Netea, M. G., Azam, T., Ferwerda, G., Girardin, S. E., Kim, S. H. and Dinarello, C. A.,** Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. *J Leukoc Biol* 2006. **80**: 1454-1461.

69 **Gingras, M. C., Lapillonne, H. and Margolin, J. F.,** TREM-1, MDL-1, and DAP12 expression is associated with a mature stage of myeloid development. *Mol Immunol* 2002. **38**: 817-824.

70 **Begum, N. A., Ishii, K., Kurita-Taniguchi, M., Tanabe, M., Kobayashi, M., Moriwaki, Y., Matsumoto, M., Fukumori, Y., Azuma, I., Toyoshima, K. and Seya, T.,** Mycobacterium bovis BCG cell wall-specific differentially expressed genes identified by differential display and cDNA subtraction in human macrophages. *Infect Immun* 2004. **72**: 937-948.

71 **Gibot, S., Kolopp-Sarda, M. N., Bene, M. C., Bollaert, P. E., Lozniewski, A., Mory, F., Levy, B. and Faure, G. C.,** A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J Exp Med* 2004. **200**: 1419-1426.

72 **Plachouras, D., Routsi, C., Giamarellos-Bourboulis, E. J., Spyridaki, E., Andrianakis, I., Metzelopoulos, S., Tsaganos, T., Floros, I., Douzinas, E. E., Armaganidis, A., Roussos, C. and Giamarellou, H.,** Monocytes as a site of production of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) in the septic host. *Scand J Infect Dis* 2006. **38**: 909-915.

73 **Gibot, S., Alauzet, C., Massin, F., Sennoune, N., Faure, G. C., Bene, M. C., Lozniewski, A., Bollaert, P. E. and Levy, B.,** Modulation of the triggering receptor expressed on myeloid cells-1 pathway during pneumonia in rats. *J Infect Dis* 2006. **194**: 975-983.

74 **Mohamadzadeh, M., Coberley, S. S., Olinger, G. G., Kalina, W. V., Ruthel, G., Fuller, C. L., Swenson, D. L., Pratt, W. D., Kuhns, D. B. and Schmaljohn, A. L.,** Activation of triggering receptor expressed on myeloid cells-1 on human neutrophils by marburg and ebola viruses. *J Virol* 2006. **80**: 7235-7244.

75 **Weigand, M. A., Horner, C., Bardenheuer, H. J. and Bouchon, A.,** The systemic inflammatory response syndrome. *Best Pract Res Clin Anaesthesiol* 2004. **18**: 455-475.

76 Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. *N Engl J Med* 2000. **342**: 1301-1308.

77 **van den Berghe, G., Wouters, P., Weekers, F., Verwaest, C., Bruyninckx, F., Schetz, M., Vlasselaers, D., Ferdinande, P., Lauwers, P. and Bouillon, R.,** Intensive insulin therapy in the critically ill patients. *N Engl J Med* 2001. **345**: 1359-1367.

78 **Rivers, E., Nguyen, B., Havstad, S., Ressler, J., Muzzin, A., Knoblich, B., Peterson, E. and Tomlanovich, M.,** Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N Engl J Med* 2001. **345**: 1368-1377.

79 **Annane, D., Sebille, V., Charpentier, C., Bollaert, P. E., Francois, B., Korach, J. M., Capellier, G., Cohen, Y., Azoulay, E., Troche, G., Chaumet-Riffaut, P. and Bellissant, E.,** Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. *Jama* 2002. **288**: 862-871.

80 **Annane, D., Sebille, V. and Bellissant, E.,** Effect of low doses of corticosteroids in septic shock patients with or without early acute respiratory distress syndrome. *Crit Care Med* 2006. **34**: 22-30.

81 **Vincent, J. L., Bernard, G. R., Beale, R., Doig, C., Putensen, C., Dhainaut, J. F., Artigas, A., Fumagalli, R., Macias, W., Wright, T., Wong, K., Sundin, D. P., Turlo, M. A. and Janes, J.,** Drotrecogin alfa (activated) treatment in severe sepsis from the global open-label trial ENHANCE: further evidence for survival and safety and implications for early treatment. *Crit Care Med* 2005. **33**: 2266-2277.

82 **Bernard, G. R., Vincent, J. L., Laterre, P. F., LaRosa, S. P., Dhainaut, J. F., Lopez-Rodriguez, A., Steingrub, J. S., Garber, G. E., Helterbrand, J. D., Ely, E. W. and Fisher, C. J., Jr.,** Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med* 2001. **344**: 699-709.

83 **Gibot, S., Kolopp-Sarda, M. N., Bene, M. C., Cravoisy, A., Levy, B., Faure, G. C. and Bollaert, P. E.,** Plasma level of a triggering receptor expressed on myeloid cells-1: its diagnostic accuracy in patients with suspected sepsis. *Ann Intern Med* 2004. **141**: 9-15.

84 **Gibot, S., Cravoisy, A., Kolopp-Sarda, M. N., Bene, M. C., Faure, G., Bollaert, P. E. and Levy, B.,** Time-course of sTREM (soluble triggering receptor expressed on myeloid cells)-1, procalcitonin, and C-reactive protein plasma concentrations during sepsis. *Crit Care Med* 2005. **33**: 792-796.

85 **Gibot, S., Le Renard, P. E., Bollaert, P. E., Kolopp-Sarda, M. N., Bene, M. C., Faure, G. C. and Levy, B.,** Surface triggering receptor expressed on myeloid cells 1 expression patterns in septic shock. *Intensive Care Med* 2005. **31**: 594-597.

86 **Gonzalez-Roldan, N., Ferat-Osorio, E., Aduna-Vicente, R., Wong-Baeza, I., Esquivel-Callejas, N., Astudillo-de la Vega, H., Sanchez-Fernandez, P., Arriaga-Pizano, L., Villasis-Keever, M. A., Lopez-Macias, C. and Isibasi, A.,** Expression of triggering receptor on myeloid cell 1 and histocompatibility complex molecules in sepsis and major abdominal surgery. *World J Gastroenterol* 2005. **11**: 7473-7479.

87 **Giamarellos-Bourboulis, E. J., Zakynthinos, S., Baziaka, F., Papadomichelakis, E., Virtzili, S., Koutoukas, P., Armaganidis, A., Giamarellou, H. and Roussos, C.,** Soluble triggering receptor expressed on myeloid cells 1 as an anti-inflammatory mediator in sepsis. *Intensive Care Med* 2006. **32**: 237-243.

88 **Gibot, S. and Massin, F.,** Soluble form of the triggering receptor expressed on myeloid cells 1: An anti-inflammatory mediator? *Intensive Care Med* 2006. **32**: 185-187.

89 **Giamarellos-Bourboulis, E. J., Tsaganos, T., Spyridaki, E., Mouktaroudi, M., Plachouras, D., Vaki, I., Karagianni, V., Antonopoulou, A., Veloni, V. and Giamarellou, H.,** Early changes of CD4-positive lymphocytes and NK cells in patients with severe Gram-negative sepsis. *Crit Care* 2006. **10**: R166.

90 **Torres, A. and Ewig, S.,** Diagnosing ventilator-associated pneumonia. *N Engl J Med* 2004. **350**: 433-435.

91 **Richeldi, L., Mariani, M., Losi, M., Maselli, F., Corbetta, L., Buonsanti, C., Colonna, M., Sinigaglia, F., Panina-Bordignon, P. and Fabbri, L. M.,** Triggering receptor expressed on myeloid cells: role in the diagnosis of lung infections. *Eur Respir J* 2004. **24**: 247-250.

92 **Determann, R. M., Millo, J. L., Gibot, S., Korevaar, J. C., Vroom, M. B., van der Poll, T., Garrard, C. S. and Schultz, M. J.,** Serial changes in soluble triggering receptor expressed on myeloid cells in the lung during development of ventilator-associated pneumonia. *Intensive Care Med* 2005. **31**: 1495-1500.

93 **Gibot, S., Cravoisy, A., Levy, B., Bene, M. C., Faure, G. and Bollaert, P. E.,** Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med* 2004. **350**: 451-458.

94 **Routsi, C., Giamarellos-Bourboulis, E. J., Antonopoulou, A., Kollias, S., Siasiakou, S., Koronaios, A., Zakynthinos, S., Armaganidis, A., Giamarellou, H. and Roussos, C.,** Does soluble triggering receptor expressed on myeloid cells-1 play any role in the pathogenesis of septic shock? *Clin Exp Immunol* 2005. **142**: 62-67.

95 **Chastre, J., Luyt, C. E., Trouillet, J. L. and Combes, A.,** New diagnostic and prognostic markers of ventilator-associated pneumonia. *Curr Opin Crit Care* 2006. **12**: 446-451.

96 **Gibot, S. and Cravoisy, A.,** Soluble form of the triggering receptor expressed on myeloid cells-1 as a marker of microbial infection. *Clin Med Res* 2004. **2**: 181-187.

97 **Phua, J., Koay, E. S., Zhang, D., Tai, L. K., Boo, X. L., Lim, K. C. and Lim, T. K.,** Soluble triggering receptor expressed on myeloid cells-1 in acute respiratory infections. *Eur Respir J* 2006. **28**: 695-702.

98 **Liu, C. L., Hsieh, W. Y., Wu, C. L., Kuo, H. T. and Lu, Y. T.,** Triggering receptor expressed on myeloid cells-1 in pleural effusions: A marker of inflammatory disease. *Respir Med* 2006.

99 **Determann, R. M., Weisfelt, M., de Gans, J., van der Ende, A., Schultz, M. J. and van de Beek, D.,** Soluble triggering receptor expressed on myeloid cells 1: a biomarker for bacterial meningitis. *Intensive Care Med* 2006. **32**: 1243-1247.

100 **Koussoulas, V., Vassiliou, S., Demonakou, M., Tassias, G., Giamarellos-Bourboulis, E. J., Mouktaroudi, M., Giamarellou, H. and Barbatzas, C.,** Soluble triggering receptor expressed on myeloid cells (sTREM-1): a new mediator involved in the pathogenesis of peptic ulcer disease. *Eur J Gastroenterol Hepatol* 2006. **18**: 375-379.

101 **Koussoulas, V., Tzivras, M., Giamarellos-Bourboulis, E. J., Demonakou, M., Vassilliou, S., Pelekanou, A., Papadopoulos, A., Giamarellou, H. and Barbatzas, C.,** Can Soluble Triggering Receptor Expressed on Myeloid Cells (sTREM-1) Be Considered an Anti-Inflammatory Mediator in the Pathogenesis of Peptic Ulcer Disease? *Dig Dis Sci* 2007.

102 **Xia, Y., Vetvicka, V., Yan, J., Hanikyrova, M., Mayadas, T. and Ross, G. D.,** The beta-glucan-binding lectin site of mouse CR3 (CD11b/CD18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to iC3b-opsonized target cells. *J Immunol* 1999. **162**: 2281-2290.

103 **Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L. and Aderem, A.,** The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* 2000. **97**: 13766-13771.

104 **Nochi, H., Aoki, N., Oikawa, K., Yanai, M., Takiyama, Y., Atsuta, Y., Kobayashi, H., Sato, K., Tateno, M., Matsuno, T., Katagiri, M., Xing, Z. and Kimura, S.,** Modulation of hepatic granulomatous responses by transgene expression of DAP12 or TREM-1-Ig molecules. *Am J Pathol* 2003. **162**: 1191-1201.

105 **Carpenter, K. J., Buckland, K. F., Xing, Z. and Hogaboam, C. M.,** Intrapulmonary, adenovirus-mediated overexpression of KARAP/DAP12 enhances fungal clearance during invasive aspergillosis. *Infect Immun* 2005. **73**: 8402-8406.

106 **Hogaboam, C. M., Carpenter, K. J., Schuh, J. M. and Buckland, K. F.,** Aspergillus and asthma--any link? *Med Mycol* 2005. **43 Suppl 1**: S197-202.

107 **Tzivras, M., Koussoulas, V., Giamarellos-Bourboulis, E. J., Tzivras, D., Tsaganos, T., Koutoukas, P., Giamarellou, H. and Archimandritis, A.,** Role of soluble triggering receptor expressed on myeloid cells in inflammatory bowel disease. *World J Gastroenterol* 2006. **12**: 3416-3419.

108 **Determann, R. M., Schultz, M. J. and Geerlings, S. E.,** Soluble triggering receptor expressed on myeloid cells-1 is not a sufficient biological marker for infection of the urinary tract. *J Infect* 2007.

109 **Wang, D. Y., Qin, R. Y., Liu, Z. R., Gupta, M. K. and Chang, Q.,** Expression of TREM-1 mRNA in acute pancreatitis. *World J Gastroenterol* 2004. **10**: 2744-2746.

110 **Liu-Bryan, R. and Terkeltaub, R.,** Evil humors take their toll as innate immunity makes gouty joints TREM-ble. *Arthritis Rheum* 2006. **54**: 383-386.

111 **Murakami, Y., Akahoshi, T., Hayashi, I., Endo, H., Kawai, S., Inoue, M., Kondo, H. and Kitasato, H.,** Induction of triggering receptor expressed on myeloid cells 1 in murine resident peritoneal macrophages by monosodium urate monohydrate crystals. *Arthritis Rheum* 2006. **54**: 455-462.

112 **Gibot, S.,** Clinical review: role of triggering receptor expressed on myeloid cells-1 during sepsis. *Crit Care* 2005. **9**: 485-489.

113 **Kofoed, K., Andersen, O., Kronborg, G., Tvede, M., Petersen, J., Eugen-Olsen, J. and Larsen, K.,** Use of plasma C-reactive protein, procalcitonin, neutrophils, macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor, and soluble triggering receptor expressed on myeloid cells-1 in combination to diagnose infections: a prospective study. *Crit Care* 2007. **11**: R38.

114 **Wong-Baeza, I., Gonzalez-Roldan, N., Ferat-Osorio, E., Esquivel-Callejas, N., Aduna-Vicente, R., Arriaga-Pizano, L., Astudillo-de la Vega, H., Villasis-Keever, M. A., Torres-Gonzalez, R., Estrada-Garcia, I., Lopez-Macias, C. and Isibasi, A.,** Triggering receptor expressed on myeloid cells (TREM-1) is regulated post-transcriptionally and its ligand is present in the sera of some septic patients. *Clin Exp Immunol* 2006. **145**: 448-455.

115 **Murakami, Y., Kohsaka, H., Kitasato, H. and Akahoshi, T.,** Lipopolysaccharide-induced up-regulation of triggering receptor expressed on myeloid cells-1 expression on macrophages is regulated by endogenous prostaglandin E2. *J Immunol* 2007. **178**: 1144-1150.

116 **Strassmann, G., Patil-Koota, V., Finkelman, F., Fong, M. and Kambayashi, T.,** Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med* 1994. **180**: 2365-2370.

117 **Portanova, J. P., Zhang, Y., Anderson, G. D., Hauser, S. D., Masferrer, J. L., Seibert, K., Gregory, S. A. and Isakson, P. C.,** Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J Exp Med* 1996. **184**: 883-891.

118 **Schenk, M., Bouchon, A., Birrer, S., Colonna, M. and Mueller, C.,** Macrophages expressing triggering receptor expressed on myeloid cells-1 are underrepresented in the human intestine. *J Immunol* 2005. **174**: 517-524.

119 **Hausmann, M., Kiessling, S., Mestermann, S., Webb, G., Spottl, T., Andus, T., Scholmerich, J., Herfarth, H., Ray, K., Falk, W. and Rogler, G.,** Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* 2002. **122**: 1987-2000.

120 **Tessarz, A. S., Weiler, S., Zanzinger, K., Angelisova, P., Horejsi, V. and Cerwenka, A.,** Non-T cell activation linker (NTAL) negatively regulates TREM-1/DAP12-induced inflammatory cytokine production in myeloid cells. *J Immunol* 2007. **178**: 1991-1999.

121 **Turnbull, I. R., Gilfillan, S., Cella, M., Aoshi, T., Miller, M., Piccio, L., Hernandez, M. and Colonna, M.,** Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol* 2006. **177**: 3520-3524.

122 **Hamerman, J. A., Tchao, N. K., Lowell, C. A. and Lanier, L. L.,** Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol* 2005. **6**: 579-586.

123 **Bakker, A. B., Hoek, R. M., Cerwenka, A., Blom, B., Lucian, L., McNeil, T., Murray, R., Phillips, L. H., Sedgwick, J. D. and Lanier, L. L.,** DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. *Immunity* 2000. **13**: 345-353.

124 **Tomasello, E., Desmoulins, P. O., Chemin, K., Guia, S., Cremer, H., Ortaldo, J., Love, P., Kaiserlian, D. and Vivier, E.,** Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice. *Immunity* 2000. **13**: 355-364.

125 **Lucas, M., Daniel, L., Tomasello, E., Guia, S., Horschowski, N., Aoki, N., Figarella-Branger, D., Gomez, S. and Vivier, E.,** Massive inflammatory syndrome and lymphocytic immunodeficiency in KARAP/DAP12-transgenic mice. *Eur J Immunol* 2002. **32**: 2653-2663.

126 **Turnbull, I. R., McDunn, J. E., Takai, T., Townsend, R. R., Cobb, J. P. and Colonna, M.,** DAP12 (KARAP) amplifies inflammation and increases mortality from endotoxemia and septic peritonitis. *J Exp Med* 2005. **202**: 363-369.

127 **Opal, S. M., Palardy, J. E., Parejo, N. A. and Creasey, A. A.,** The activity of tissue factor pathway inhibitor in experimental models of superantigen-induced shock and polymicrobial intra-abdominal sepsis. *Crit Care Med* 2001. **29**: 13-17.

128 **Opal, S. M., Scannon, P. J., Vincent, J. L., White, M., Carroll, S. F., Palardy, J. E., Parejo, N. A., Pribble, J. P. and Lemke, J. H.,** Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis* 1999. **180**: 1584-1589.

129 **Hamerman, J. A. and Lanier, L. L.,** Inhibition of immune responses by ITAM-bearing receptors. *Sci STKE* 2006. **2006**: re1.

130 **Turnbull, I. R. and Colonna, M.,** Activating and inhibitory functions of DAP12. *Nat Rev Immunol* 2007. **7**: 155-161.

131 **Pasquier, B., Launay, P., Kanamaru, Y., Moura, I. C., Pfirsch, S., Ruffie, C., Henin, D., Benhamou, M., Pretolani, M., Blank, U. and Monteiro, R. C.,** Identification of FcalphaRI as an inhibitory receptor that controls inflammation: dual role of FcRgamma ITAM. *Immunity* 2005. **22**: 31-42.

132 **Hamerman, J. A., Jarjoura, J. R., Humphrey, M. B., Nakamura, M. C., Seaman, W. E. and Lanier, L. L.,** Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J Immunol* 2006. **177**: 2051-2055.

133 **Blasius, A., Vermi, W., Krug, A., Facchetti, F., Cella, M. and Colonna, M.,** A cell-surface molecule selectively expressed on murine natural interferon-producing cells that blocks secretion of interferon-alpha. *Blood* 2004. **103**: 4201-4206.

134 **Blasius, A. L., Cella, M., Maldonado, J., Takai, T. and Colonna, M.,** Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood* 2006. **107**: 2474-2476.

135 **Blasius, A. L. and Colonna, M.,** Sampling and signaling in plasmacytoid dendritic cells: the potential roles of Siglec-H. *Trends Immunol* 2006. **27**: 255-260.

136 **Fuchs, A., Cella, M., Kondo, T. and Colonna, M.,** Paradoxic inhibition of human natural interferon-producing cells by the activating receptor NKp44. *Blood* 2005. **106**: 2076-2082.

137 **Sjolin, H., Robbins, S. H., Bessou, G., Hidmark, A., Tomasello, E., Johansson, M., Hall, H., Charifi, F., Karlsson Hedestam, G. B., Biron, C. A., Karre, K., Hoglund, P., Vivier, E. and Dalod, M.,** DAP12 signaling regulates plasmacytoid dendritic cell homeostasis and down-modulates their function during viral infection. *J Immunol* 2006. **177**: 2908-2916.

138 **Daws, M. R., Sullam, P. M., Niemi, E. C., Chen, T. T., Tchao, N. K. and Seaman, W. E.,** Pattern recognition by TREM-2: binding of anionic ligands. *J Immunol* 2003. **171**: 594-599.

139 **Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K.,** Immunobiology of dendritic cells. *Annu Rev Immunol* 2000. **18**: 767-811.

140 **Terme, M., Tomasello, E., Maruyama, K., Crepineau, F., Chaput, N., Flament, C., Marolleau, J. P., Angevin, E., Wagner, E. F., Salomon, B., Lemonnier, F. A., Wakasugi, H., Colonna, M., Vivier, E. and Zitvogel, L.,** IL-4 confers NK stimulatory capacity to murine dendritic cells: a signaling pathway involving KARAP/DAP12-triggering receptor expressed on myeloid cell 2 molecules. *J Immunol* 2004. **172**: 5957-5966.

141 **Melchior, B., Puntambekar, S. S. and Carson, M. J.,** Microglia and the control of autoreactive T cell responses. *Neurochem Int* 2006. **49**: 145-153.

142 **Rock, R. B., Gekker, G., Hu, S., Sheng, W. S., Cheeran, M., Lokensgard, J. R. and Peterson, P. K.,** Role of microglia in central nervous system infections. *Clin Microbiol Rev* 2004. **17**: 942-964, table of contents.

143 **Paloneva, J., Manninen, T., Christman, G., Hovanes, K., Mandelin, J., Adolfsson, R., Bianchin, M., Bird, T., Miranda, R., Salmaggi, A., Tranebjaerg, L., Konttinen, Y. and Peltonen, L.,** Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. *Am J Hum Genet* 2002. **71**: 656-662.

144 **Hakola, H. P.,** Neuropsychiatric and genetic aspects of a new hereditary disease characterized by progressive dementia and lipomembranous polycystic osteodysplasia. *Acta Psychiatr Scand Suppl* 1972. **232**: 1-173.

145 **Nasu, T., Tsukahara, Y. and Terayama, K.,** A lipid metabolic disease-"membranous lipodystrophy"-an autopsy case demonstrating numerous peculiar membrane-structures composed of compound lipid in bone and bone marrow and various adipose tissues. *Acta Pathol Jpn* 1973. **23**: 539-558.

146 **Hakola, H. P. and Puranen, M.,** Neuropsychiatric and brain CT findings in polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy. *Acta Neurol Scand* 1993. **88**: 370-375.

147 **Paloneva, J., Autti, T., Raininko, R., Partanen, J., Salonen, O., Puranen, M., Hakola, P. and Haltia, M.,** CNS manifestations of Nasu-Hakola disease: a frontal dementia with bone cysts. *Neurology* 2001. **56**: 1552-1558.

148 **Bianchin, M. M., Capella, H. M., Chaves, D. L., Steindel, M., Grisard, E. C., Ganev, G. G., da Silva Junior, J. P., Neto Evaldo, S., Poffo, M. A., Walz, R., Carlotti Junior, C. G. and Sakamoto, A. C.,** Nasu-Hakola disease (polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy--PLOSL): a dementia associated with bone cystic lesions. From clinical to genetic and molecular aspects. *Cell Mol Neurobiol* 2004. **24**: 1-24.

149 **Neumann, H. and Takahashi, K.,** Essential role of the microglial triggering receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue immune homeostasis. *J Neuroimmunol* 2007.

150 **Paloneva, J., Kestila, M., Wu, J., Salminen, A., Bohling, T., Ruotsalainen, V., Hakola, P., Bakker, A. B., Phillips, J. H., Pekkarinen, P., Lanier, L. L., Timonen, T. and Peltonen, L.,** Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. *Nat Genet* 2000. **25**: 357-361.

151 **Kondo, T., Takahashi, K., Kohara, N., Takahashi, Y., Hayashi, S., Takahashi, H., Matsuo, H., Yamazaki, M., Inoue, K., Miyamoto, K. and Yamamura, T.,** Heterogeneity of presenile dementia with bone cysts (Nasu-Hakola disease): three genetic forms. *Neurology* 2002. **59**: 1105-1107.

152 **Soragna, D., Papi, L., Ratti, M. T., Sestini, R., Tupler, R. and Montalbetti, L.,** An Italian family affected by Nasu-Hakola disease with a novel genetic mutation in the TREM2 gene. *J Neurol Neurosurg Psychiatry* 2003. **74**: 825-826.

153 **Kaifu, T., Nakahara, J., Inui, M., Mishima, K., Momiyama, T., Kaji, M., Sugahara, A., Koito, H., Ujike-Asai, A., Nakamura, A., Kanazawa, K., Tan-Takeuchi, K., Iwasaki, K., Yokoyama, W. M., Kudo, A., Fujiwara, M., Asou, H. and Takai, T.,** Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice. *J Clin Invest* 2003. **111**: 323-332.

154 **Schmid, C. D., Sautkulis, L. N., Danielson, P. E., Cooper, J., Hasel, K. W., Hilbush, B. S., Sutcliffe, J. G. and Carson, M. J.,** Heterogeneous expression of the triggering receptor expressed on myeloid cells-2 on adult murine microglia. *J Neurochem* 2002. **83**: 1309-1320.

155 **Sessa, G., Podini, P., Mariani, M., Meroni, A., Spreafico, R., Sinigaglia, F., Colonna, M., Panina, P. and Meldolesi, J.,** Distribution and signaling of TREM2/DAP12, the receptor system mutated in human polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy dementia. *Eur J Neurosci* 2004. **20**: 2617-2628.

156 **Prada, I., Ongania, G. N., Buonsanti, C., Panina-Bordignon, P. and Meldolesi, J.,** Triggering receptor expressed in myeloid cells 2 (TREM2) trafficking in microglial cells: continuous shuttling to and from the plasma membrane regulated by cell stimulation. *Neuroscience* 2006. **140**: 1139-1148.

157 **Takahashi, K., Rochford, C. D. and Neumann, H.,** Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 2005. **201**: 647-657.

158 **Streit, W. J.,** Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* 2002. **40**: 133-139.

159 **Kiialainen, A., Hovanes, K., Paloneva, J., Kopra, O. and Peltonen, L.,** Dap12 and Trem2, molecules involved in innate immunity and neurodegeneration, are co-expressed in the CNS. *Neurobiol Dis* 2005. **18**: 314-322.

160 **Fenoglio, C., Galimberti, D., Piccio, L., Scalabrini, D., Panina, P., Buonsanti, C., Venturelli, E., Lovati, C., Forloni, G., Mariani, C., Bresolin, N. and Scarpini, E.,** Absence of TREM2 polymorphisms in patients with Alzheimer's disease and Frontotemporal Lobar Degeneration. *Neurosci Lett* 2007. **411**: 133-137.

161 **Carson, M. J., Thrash, J. C. and Lo, D.,** Analysis of microglial gene expression: identifying targets for CNS neurodegenerative and autoimmune disease. *Am J Pharmacogenomics* 2004. **4**: 321-330.

162 **Takahashi, K., Prinz, M., Stagi, M., Chechneva, O. and Neumann, H.,** TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med* 2007. **4**: e124.

163 **Piccio, L., Buonsanti, C., Mariani, M., Cella, M., Gilfillan, S., Cross, A. H., Colonna, M. and Panina-Bordignon, P.,** Blockade of TREM-2 exacerbates experimental autoimmune encephalomyelitis. *Eur J Immunol* 2007. **37**: 1290-1301.

164 **Teitelbaum, S. L.,** Osteoclasts: what do they do and how do they do it? *Am J Pathol* 2007. **170**: 427-435.

165 **Cella, M., Buonsanti, C., Strader, C., Kondo, T., Salmaggi, A. and Colonna, M.,** Impaired differentiation of osteoclasts in TREM-2-deficient individuals. *J Exp Med* 2003. **198**: 645-651.

166 **Paloneva, J., Mandelin, J., Kiialainen, A., Bohling, T., Prudlo, J., Hakola, P., Haltia, M., Konttinen, Y. T. and Peltonen, L.,** DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. *J Exp Med* 2003. **198**: 669-675.

167 **Humphrey, M. B., Daws, M. R., Spusta, S. C., Niemi, E. C., Torchia, J. A., Lanier, L. L., Seaman, W. E. and Nakamura, M. C.,** TREM2, a DAP12-associated receptor, regulates osteoclast differentiation and function. *J Bone Miner Res* 2006. **21**: 237-245.

168 **Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J. and Suda, T.,** Osteoblastic cells are involved in osteoclast formation. *Endocrinology* 1988. **123**: 2600-2602.

169 **Takayanagi, H.,** Mechanistic insight into osteoclast differentiation in osteoimmunology. *J Mol Med* 2005. **83**: 170-179.

170 **Humphrey, M. B., Lanier, L. L. and Nakamura, M. C.,** Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. *Immunol Rev* 2005. **208**: 50-65.

171 **Humphrey, M. B., Ogasawara, K., Yao, W., Spusta, S. C., Daws, M. R., Lane, N. E., Lanier, L. L. and Nakamura, M. C.,** The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. *J Bone Miner Res* 2004. **19**: 224-234.

172 **Koga, T., Inui, M., Inoue, K., Kim, S., Suematsu, A., Kobayashi, E., Iwata, T., Ohnishi, H., Matozaki, T., Kodama, T., Taniguchi, T., Takayanagi, H. and Takai, T.,** Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature* 2004. **428**: 758-763.

173 **Mocsai, A., Humphrey, M. B., Van Ziffle, J. A., Hu, Y., Burghardt, A., Spusta, S. C., Majumdar, S., Lanier, L. L., Lowell, C. A. and Nakamura, M. C.,** The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proc Natl Acad Sci U S A* 2004. **101**: 6158-6163.

174 **Whyte, M. P., Obrecht, S. E., Finnegan, P. M., Jones, J. L., Podgornik, M. N., McAlister, W. H. and Mumm, S.,** Osteoprotegerin deficiency and juvenile Paget's disease. *N Engl J Med* 2002. **347**: 175-184.

175 **Bucay, N., Sarosi, I., Dunstan, C. R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H. L., Xu, W., Lacey, D. L., Boyle, W. J. and Simonet, W. S.,** osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998. **12**: 1260-1268.

176 **Takegahara, N., Takamatsu, H., Toyofuku, T., Tsujimura, T., Okuno, T., Yukawa, K., Mizui, M., Yamamoto, M., Prasad, D. V., Suzuki, K., Ishii, M., Terai, K., Moriya, M., Nakatsuji, Y., Sakoda, S., Sato, S., Akira, S., Takeda, K., Inui, M., Takai, T., Ikawa, M., Okabe, M., Kumanogoh, A. and Kikutani, H.,** Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat Cell Biol* 2006. **8**: 615-622.

177 **Haselmayer, P., Grosse-Hovest, L., Landenberg, P. V., Schild, H. and Radsak, M. P.,** TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood* 2007.

178 **Linehan, S. A. and Holden, D. W.,** The interplay between Salmonella typhimurium and its macrophage host--what can it teach us about innate immunity? *Immunol Lett* 2003. **85**: 183-192.

179 **Biedzka-Sarek, M. and El Skurnik, M.,** How to outwit the enemy: dendritic cells face Salmonella. *Apmis* 2006. **114**: 589-600.

180 **Ohl, M. E. and Miller, S. I.,** Salmonella: a model for bacterial pathogenesis. *Annu Rev Med* 2001. **52**: 259-274.

181 **Vazquez-Torres, A. and Fang, F. C.,** Cellular routes of invasion by enteropathogens. *Curr Opin Microbiol* 2000. **3**: 54-59.

182 **Richter-Dahlfors, A., Buchan, A. M. and Finlay, B. B.,** Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *J Exp Med* 1997. **186**: 569-580.

183 **Salcedo, S. P., Noursadeghi, M., Cohen, J. and Holden, D. W.,** Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. *Cell Microbiol* 2001. **3**: 587-597.
184 **Mastroeni, P. and Sheppard, M.,** Salmonella infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. *Microbes Infect* 2004. **6**: 398-405.
185 **Mastroeni, P.,** Immunity to systemic Salmonella infections. *Curr Mol Med* 2002. **2**: 393-406.
186 **Wick, M. J.,** Living in the danger zone: innate immunity to Salmonella. *Curr Opin Microbiol* 2004. **7**: 51-57.
187 **Hueffer, K. and Galan, J. E.,** Salmonella-induced macrophage death: multiple mechanisms, different outcomes. *Cell Microbiol* 2004. **6**: 1019-1025.
188 **Tierrez, A. and Garcia-del Portillo, F.,** New concepts in Salmonella virulence: the importance of reducing the intracellular growth rate in the host. *Cell Microbiol* 2005. **7**: 901-909.
189 **Eriksson, S., Bjorkman, J., Borg, S., Syk, A., Pettersson, S., Andersson, D. I. and Rhen, M.,** Salmonella typhimurium mutants that downregulate phagocyte nitric oxide production. *Cell Microbiol* 2000. **2**: 239-250.
190 **Mastroeni, P., Vazquez-Torres, A., Fang, F. C., Xu, Y., Khan, S., Hormaeche, C. E. and Dougan, G.,** Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J Exp Med* 2000. **192**: 237-248.
191 **Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinauer, M. and Nathan, C.,** Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* 1999. **10**: 29-38.
192 **Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. and Fang, F. C.,** Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med* 2000. **192**: 227-236.
193 **Janssen, R., van der Straaten, T., van Diepen, A. and van Dissel, J. T.,** Responses to reactive oxygen intermediates and virulence of Salmonella typhimurium. *Microbes Infect* 2003. **5**: 527-534.
194 **Biassoni, R., Cantoni, C., Pende, D., Sivori, S., Parolini, S., Vitale, M., Bottino, C. and Moretta, A.,** Human natural killer cell receptors and co-receptors. *Immunol Rev* 2001. **181**: 203-214.
195 **Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., Biassoni, R. and Moretta, L.,** Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* 2001. **19**: 197-223.
196 **Arnon, T. I., Lev, M., Katz, G., Chernobrov, Y., Porgador, A. and Mandelboim, O.,** Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* 2001. **31**: 2680-2689.
197 **Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T. I., Bushkin, Y., Davis, D. M., Strominger, J. L., Yewdell, J. W. and Porgador, A.,** Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 2001. **409**: 1055-1060.
198 **Pende, D., Parolini, S., Pessino, A., Sivori, S., Augugliaro, R., Morelli, L., Marcenaro, E., Accame, L., Malaspina, A., Biassoni, R., Bottino, C., Moretta, L. and Moretta, A.,** Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med* 1999. **190**: 1505-1516.
199 **Biassoni, R., Pessino, A., Bottino, C., Pende, D., Moretta, L. and Moretta, A.,** The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur J Immunol* 1999. **29**: 1014-1020.
200 **Hollyoake, M., Campbell, R. D. and Aguado, B.,** NKp30 (NCR3) is a pseudogene in 12 inbred and wild mouse strains, but an expressed gene in Mus caroli. *Mol Biol Evol* 2005. **22**: 1661-1672.
201 **Cantoni, C., Ponassi, M., Biassoni, R., Conte, R., Spallarossa, A., Moretta, A., Moretta, L., Bolognesi, M. and Bordo, D.,** The three-dimensional structure of the human NK cell receptor NKp44, a triggering partner in natural cytotoxicity. *Structure* 2003. **11**: 725-734.

202 **Cantoni, C., Ponassi, M., Biassoni, R., Conte, R., Spallarossa, A., Moretta, A., Moretta, L., Bolognesi, M. and Bordo, D.,** Crystallization and preliminary crystallographic characterization of the extracellular Ig-like domain of human natural killer cell activating receptor NKp44. *Acta Crystallogr D Biol Crystallogr* 2002. **58**: 1843-1845.

203 **Guex, N. and Peitsch, M. C.,** SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997. **18**: 2714-2723.

204 **Moriki, T., Maruyama, H. and Maruyama, I. N.,** Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. *J Mol Biol* 2001. **311**: 1011-1026.

205 **Grigorian, A. L., Bustamante, J. J., Hernandez, P., Martinez, A. O. and Haro, L. S.,** Extraordinarily stable disulfide-linked homodimer of human growth hormone. *Protein Sci* 2005. **14**: 902-913.

206 **Dauer, M., Schad, K., Junkmann, J., Bauer, C., Herten, J., Kiefl, R., Schnurr, M., Endres, S. and Eigler, A.,** IFN-alpha promotes definitive maturation of dendritic cells generated by short-term culture of monocytes with GM-CSF and IL-4. *J Leukoc Biol* 2006. **80**: 278-286.

207 **Gorczynski, R. M., Chen, Z., Clark, D. A., Kai, Y., Lee, L., Nachman, J., Wong, S. and Marsden, P.,** Structural and functional heterogeneity in the CD200R family of immunoregulatory molecules and their expression at the feto-maternal interface. *Am J Reprod Immunol* 2004. **52**: 147-163.

208 **Bakker, A. B., Baker, E., Sutherland, G. R., Phillips, J. H. and Lanier, L. L.,** Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. *Proc Natl Acad Sci U S A* 1999. **96**: 9792-9796.

209 **Yotsumoto, K., Okoshi, Y., Shibuya, K., Yamazaki, S., Tahara-Hanaoka, S., Honda, S., Osawa, M., Kuroiwa, A., Matsuda, Y., Tenen, D. G., Iwama, A., Nakauchi, H. and Shibuya, A.,** Paired activating and inhibitory immunoglobulin-like receptors, MAIR-I and MAIR-II, regulate mast cell and macrophage activation. *J Exp Med* 2003. **198**: 223-233.

210 **Barclay, A. N. and Brown, M. H.,** The SIRP family of receptors and immune regulation. *Nat Rev Immunol* 2006. **6**: 457-464.

211 **Shiratori, I., Ogasawara, K., Saito, T., Lanier, L. L. and Arase, H.,** Activation of natural killer cells and dendritic cells upon recognition of a novel CD99-like ligand by paired immunoglobulin-like type 2 receptor. *J Exp Med* 2004. **199**: 525-533.

212 **Hart, G., Flaishon, L., Becker-Herman, S. and Shachar, I.,** Ly49D receptor expressed on immature B cells regulates their IFN-gamma secretion, actin polymerization, and homing. *J Immunol* 2003. **171**: 4630-4638.

213 **Fields, P. I., Swanson, R. V., Haidaris, C. G. and Heffron, F.,** Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A* 1986. **83**: 5189-5193.

214 **Chakravortty, D., Hansen-Wester, I. and Hensel, M.,** Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. *J Exp Med* 2002. **195**: 1155-1166.

215 **Vazquez-Torres, A., Fantuzzi, G., Edwards, C. K., 3rd, Dinarello, C. A. and Fang, F. C.,** Defective localization of the NADPH phagocyte oxidase to Salmonella-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proc Natl Acad Sci U S A* 2001. **98**: 2561-2565.

216 **Zhu, X., Chang, M. S., Hsueh, R. C., Taussig, R., Smith, K. D., Simon, M. I. and Choi, S.,** Dual ligand stimulation of RAW 264.7 cells uncovers feedback mechanisms that regulate TLR-mediated gene expression. *J Immunol* 2006. **177**: 4299-4310.

217 **Pamer, E. G.,** Immune responses to Listeria monocytogenes. *Nat Rev Immunol* 2004. **4**: 812-823.

218 **Chan, E. D., Chan, J. and Schluger, N. W.,** What is the role of nitric oxide in murine and human host defense against tuberculosis?Current knowledge. *Am J Respir Cell Mol Biol* 2001. **25**: 606-612.

219 **Fukao, T., Tanabe, M., Terauchi, Y., Ota, T., Matsuda, S., Asano, T., Kadowaki, T., Takeuchi, T. and Koyasu, S.,** PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat Immunol* 2002. **3**: 875-881.

220 **Kobayashi, K., Hernandez, L. D., Galan, J. E., Janeway, C. A., Jr., Medzhitov, R. and Flavell, R. A.,** IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 2002. **110**: 191-202.

221 **Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., Akira, S., Yamanishi, K., Kawase, I., Nakanishi, K. and Kishimoto, T.,** SOCS-1 participates in negative regulation of LPS responses. *Immunity* 2002. **17**: 677-687.

222 **Fukao, T. and Koyasu, S.,** PI3K and negative regulation of TLR signaling. *Trends Immunol* 2003. **24**: 358-363.

223 **Fukao, T., Yamada, T., Tanabe, M., Terauchi, Y., Ota, T., Takayama, T., Asano, T., Takeuchi, T., Kadowaki, T., Hata Ji, J. and Koyasu, S.,** Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. *Nat Immunol* 2002. **3**: 295-304.

224 **Guha, M. and Mackman, N.,** The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* 2002. **277**: 32124-32132.

225 **Ojaniemi, M., Glumoff, V., Harju, K., Liljeroos, M., Vuori, K. and Hallman, M.,** Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur J Immunol* 2003. **33**: 597-605.

226 **Arbibe, L., Mira, J. P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P. J., Ulevitch, R. J. and Knaus, U. G.,** Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat Immunol* 2000. **1**: 533-540.

227 **Bowling, W. M., Flye, M. W., Qiu, Y. Y. and Callery, M. P.,** Inhibition of phosphatidylinositol-3'-kinase prevents induction of endotoxin tolerance in vitro. *J Surg Res* 1996. **63**: 287-292.

228 **Fan, H. and Cook, J. A.,** Molecular mechanisms of endotoxin tolerance. *J Endotoxin Res* 2004. **10**: 71-84.

229 **Williams, D. L., Ozment-Skelton, T. and Li, C.,** Modulation of the phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. *Shock* 2006. **25**: 432-439.

230 **Jiang, K., Zhong, B., Gilvary, D. L., Corliss, B. C., Vivier, E., Hong-Geller, E., Wei, S. and Djeu, J. Y.,** Syk regulation of phosphoinositide 3-kinase-dependent NK cell function. *J Immunol* 2002. **168**: 3155-3164.

231 **Gibot, S., Buonsanti, C., Massin, F., Romano, M., Kolopp-Sarda, M. N., Benigni, F., Faure, G. C., Bene, M. C., Panina-Bordignon, P., Passini, N. and Levy, B.,** Modulation of the triggering receptor expressed on the myeloid cell type 1 pathway in murine septic shock. *Infect Immun* 2006. **74**: 2823-2830.

232 **Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J., Force, W. R., Lynch, D. H., Ware, C. F. and et al.,** Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* 1995. **373**: 441-444.

233 **Dhein, J., Walczak, H., Baumler, C., Debatin, K. M. and Krammer, P. H.,** Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* 1995. **373**: 438-441.

234 **Ju, S. T., Panka, D. J., Cui, H., Ettinger, R., el-Khatib, M., Sherr, D. H., Stanger, B. Z. and Marshak-Rothstein, A.,** Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* 1995. **373**: 444-448.

235 **Yang, Y., Mercep, M., Ware, C. F. and Ashwell, J. D.,** Fas and activation-induced Fas ligand mediate apoptosis of T cell hybridomas: inhibition of Fas ligand expression by retinoic acid and glucocorticoids. *J Exp Med* 1995. **181**: 1673-1682.

236 **Applequist, S. E., Wallin, R. P. and Ljunggren, H. G.,** Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunol* 2002. **14**: 1065-1074.

237 **Schwede, T., Kopp, J., Guex, N. and Peitsch, M. C.,** SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res* 2003. **31**: 3381-3385.

238 **Bjorkman, J., Rhen, M. and Anderson, D. I.,** Salmonella typhimurium cob mutants are not hyper-virulent. *FEMS Microbiol Lett* 1996. **139**: 121-126.

239 **Bjur, E., Eriksson-Ygberg, S. and Rhen, M.,** The O-antigen affects replication of Salmonella enterica serovar Typhimurium in murine macrophage-like J774-A.1 cells through modulation of host cell nitric oxide production. *Microbes Infect* 2006. **8**: 1826-1838.