Identifizierung, Klonierung und Charakterisierung von neuen DAP12-assoziierten Rezeptoren exprimiert von myeloiden Zellen

Identification, Cloning and Characterization of Novel DAP12-associated Receptors Expressed on Myeloid Cells

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1 INTRODUCTION

1.1 The immune system

Vertebrates and, in particular, mammals are continually subjected to attack by pathogens. As a response to parasitic microorganism and viruses (pathogens), they have evolved an elaborate protective array known as **immune system** (Latin: *immunis*: exempt). The immune response to pathogens relies on both innate and adaptive components (Hoffmann et al., 1999). Whereas the innate immune response ensures (i) a broad recognition of shared pathogenic structures and (ii) an immediate response without previous antigen encounter, the adaptive immune response is characterized by (iii) the highly specific recognition of foreign antigens displayed by pathogens coupled to potent mechanisms for their elimination, (iv) the ability to recognize a vast array of distinct antigenic specificities, (v) the capacity of the system to generate an immunological memory, and (vi) the tolerance towards self antigens. The identification and characterization of novel molecular mechanisms responsible for the initiation of innate responses and the efficient elimination of pathogens is the subject of this thesis.

1.1.1 The innate immune system

The innate arm of the immune system is primarily responsible for the early recognition of pathogens that manage to breach the physiological barriers presented by the skin and mucous membranes. Recognition ultimately leads to the initiation of a general immune alert, also known as **inflammation**. The inflammatory response includes a variety of molecular changes ensuring the immediate fight against the invasion, and influences the subsequent, adaptive responses. Thus, the innate immune system links pathogen recognition to efficient destruction.

A key challenge to the innate immune system is the discrimination of a large number of potential pathogens from self, by means of a limited arsenal of receptors. This problem is compounded by the tendency of pathogens to mutate. In the ongoing battle against pathogens hosts have evolved a set of germ line encoded membrane-bound and soluble pathogen-detection receptors that recognize certain invariable pathogen-associated molecular patterns (PAMPs), which are not found in higher eukaryotes. These receptors are therefore termed Pattern Recognition Receptors (PRRs) (Aderem and Ulevitch, 2000; Ezekowitz et al., 1990; Gordon, 1995; Janeway, 1989; Medzhitov and Janeway, 2000), which can be cell-bound or soluble. Cell-bound PRRs are mainly expressed on neutrophils, monocytes and macrophages, which upon pathogen recognition, phagocytose and kill them. This concurrently coordinates additional host responses by synthesizing a wide range of inflammatory mediators (Aderem and Un-

sponses by synthesizing a wide range of inflammatory mediators (Aderem and Underhill, 1999). On top of killing and degradation of pathogens, macrophages have the ability to present components of the pathogen to T cells, resulting in the activation of the adaptive immune response and the establishment of protective immunity (Aderem and Underhill, 1999).

In addition to PRRs, other mechanisms have evolved for the recognition and control of infection or oncogenically transformed cells. Of particular importance are soluble, antibiotic peptides such as defensins (Ganz and Lehrer, 1998) that perforate bacterial membranes, and lytic enzymes, such as lysozyme that digest the cell wall of bacteria (Wilmott et al., 2000). In addition, Natural killer (NK) cells monitor host cells for deregulated expression of certain surface molecules (MHC class I and MHC class I-like molecules), which can occur during viral infection or stress responses due to tumorgenesis, and kill abnormal target cells (Bauer et al., 1999; Colonna et al., 2000; Groh et al., 2001; Groh et al., 1998).

The success of the innate immune system in controlling pathogens is highlighted by the fact that all organisms except for the vertebrates rely exclusively on innate immune responses. Even vertebrates cope with most infections by exclusively using their innate immune system. Under some circumstances, however, the invading pathogen escapes innate immune surveillance. In which case, the adaptive immune response is launched in order to support the innate responses with increased specificity and the ability to generate memory.

1.1.2 The adaptive immune system

Pathogen recognition in the adaptive immune response is based on antigen receptors of the immunoglobulin (Ig) superfamily (SF) that are generated by random specificities by somatic recombination of germ line encoded gene segments (Tonegawa, 1983; Tonegawa, 1988). Cells expressing an antigen-specific receptor are selected upon pathogen binding since receptor triggering induces clonal expansion by proliferation. In addition, the adaptive immune system, in contrast to the innate immune system, displays a receptor diversity that in principal allows for the recognition of every chemical structure. Furthermore, cell clones selected for a certain antigen are retained, thus constituting antigen specific memory.

B and T cells constitute the adaptive immune system. B cells have the ability to express antigen specific antibodies in a soluble and membrane bound form. Secreted antibodies are the main effectors of the humoral response. They recognize antigens in their native three-dimensional structure, thus every chemical structure can serve as an antigenic epitope for B cells. In addition to their antigen-binding sites, antibodies con-

tain constant regions (Fc) responsible for the communication with immune cells via binding to Fc receptors (FcR). T cells utilize a surface T cell receptor (TCR) to recognize cells that are infected by intracellular pathogens persisting in the cytosol, or cells that have internalized pathogens into membrane-enclosed intracellular compartments (Davis et al., 1998). For presentation to TCR, the antigens have to be processed into peptides, stabilized and transported to the cell surface. Presentation is mediated by a family of highly polymorphic molecules encoded in the Major Histocompatibility Complex (MHC). Processing and presentation differs between peptides derived from cytosolic and vesicular pathogens. Cytosol-derived peptides are processed to a length of 8-10 amino acids by the proteasome and delivered to native MHC class I molecules located in the endoplasmic reticulum (ER) by peptide transporters (Rammensee et al., 1993). Peptide-loaded MHC class I molecules are delivered to the cell surface, where they are recognized by $CD8^+$ T cells (Lehner and Cresswell, 1996). In contrast, extracellular antigens internalized in endosomal compartments are processed in the lysosomes, giving rise to peptides of $12 - 24$ amino acid in length (Rudensky et al., 1991), which are loaded onto MHC class II molecules. These are delivered to the cell surface for recognition by $CD4^+$ T cells (Abbas et al., 1996). $CD8^+$ and $CD4^+$ T cells are functionally distinct (Germain, 1994). $CD8^+$ T cells are cytotoxic lymphocytes, which kill target cells upon recognition of viral- and tumor-derived peptides presented by MHC class I. This eliminates the persisting parasite or limits the progression of cellular transformation (Rammensee et al., 1993). In contrast, $CD4^+$ T cells, also known as T helper (T_H) cells, recognize bacteria taken up in the vesicular system and secrete Interferon (IFN)-γ upon stimulation, which in turn helps macrophages to kill the harbored parasites. Furthermore, activated T_H cells induce B cells to differentiate into antibody secreting plasma cells or memory cells (Abbas et al., 1996).

1.1.3 Dendritic cells: the central link between innate and adaptive immunity

The adaptive and innate immune system communicate, interact with, and reley on each other on several levels and various time points during an immune response. Antigen-presenting cells (APCs) are activated during innate immune responses and trigger adaptive immunity, which increases specificity and generates immunological memory. Over the last 25 years, DCs have emerged as the major APC involved in linking the two arms of immunity. DCs are a distinct population of bone marrow-derived leukocytes that initiate primary and secondary immune responses (Banchereau et al., 2000). DCs can be differentiated from monocytes *in vitro* (Bender et al., 1996; Sallusto and Lanzavecchia, 1994) and *in vivo* (Randolph et al., 1999) (Figure 1.1). DCs migrate from the blood to peripheral tissues, where they reside in an

immature state, awaiting antigen encounter. Upon antigen capture, DCs process them into peptides, which are loaded onto MHC molecules for presentation to T cells. As a result of pathogen invasion, inflammation and tissue damage, DCs receive additional activating signals, which induce a profound change in DC phenotype and functions. This process is known as maturation (Figure 1.1) (Banchereau et al., 2000). Mature DCs express the chemokine receptor CCR7, which interacts with the chemokines CCL19 (also known as EBI-1 ligand chemokine (ELC), or Macrophage inflammatory protein 3 β, (MIP-3β)) and CCL21 (also known as secondary lymphoid-tissue chemokine (SLC), or 6-C-Kine) (Cyster, 2000; Zlotnik and Yoshie, 2000). These chemokines are crucial for guiding DCs from peripheral tissues to draining lymph nodes, as demonstrated in natural or targeted genetic deletions of CCL19, CCL21 or CCR7 (Forster et al., 1999; Gunn et al., 1999; Nakano et al., 1998a; Ngo et al., 1999; Saeki et al., 1999). In addition, mature DCs express high levels of stable MHC-peptide complexes on the cell surface, upregulate costimulatory and adhesion molecules and downregulate antigen-capturing molecules. Thus, mature DCs can efficiently present antigens and stimulate naïve T cells located in the T cell-rich areas of lymph nodes (Banchereau et al., 2000; Lanzavecchia, 1998). Here, DCs receive further activating signals from cognate T_H cells, which express CD40 ligand (CD40L) (Armitage et al., 1992), OX40 (Chen et al., 1999; Kopf et al., 1999), and tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE) (Bachmann et al., 1999; Josien et al., 2000; Kong et al., 1999). These stimuli trigger Interleukin (IL)-12 secretion by antigen presenting DCs thus promoting T_H1 type T cell responses (Cella et al., 1996; Heufler et al., 1996; Josien et al., 2000; Koch et al., 1996; Macatonia et al., 1995; Ohshima et al., 1997).

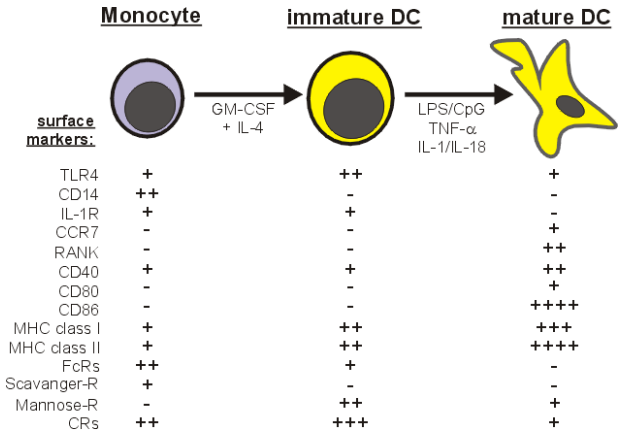

Figure 1.1: Differentiation from monocytes to dendritc cells.

Activating signals induce DC maturation through triggering of several distinct receptors including Toll-like receptors (TLRs), IL-1R, IL-18R, TNF-R1, -R2, CD40, RANK, and FcR (1.3) (Aderem and Ulevitch, 2000; Bachmann et al., 1999; Baeuerle, 1998; Baldwin, 2001; Banchereau et al., 2000; Chen et al., 1999; Josien et al., 2000; Karin and Ben-Neriah, 2000; Kong et al., 1999; Kopf et al., 1999; Medzhitov and Janeway, 2000; Ravetch and Bolland, 2001).

In addition, DC activation and maturation can trigger the production of cytokines such as IL-12, IL-18, or IL-10, which may in turn polarize emerging T cell responses (Reis e Sousa, 2001). Two major subclasses of $CD4^+$ T cells exist according to their secreted cytokine pattern (Mosmann et al., 1986). T_H1 cells secrete cytokines that drastically augment the anti-microbial capacities from phagocytes, thus helping the innate immune system to target and destroy intracellular parasites. T_H2 cells secrete cytokines mainly modulating antibody responses, thus supporting humoral responses in the fight against extracellular pathogens (Asnagli and Murphy, 2001).

T_H1 cells play an essential role in helping phagocytes to destroy persistent intracellular pathogens. Macrophages process phagocytosed microbes and present pathogen-derived peptides in the context of MHC class II to T_H1 cells, which upon stimulation secrete IFN-γ and TNF-α. The combination of these two cytokines leads to macrophage activation, a complex process that includes the production of highly cytotoxic reactive oxygen species (ROS) and intermediates (ROI) at the site of microbial persistence in the phagosomal membranes. These concerted anti-microbial mechanisms are responsible for the elimination of persisting pathogens from macrophages (Carroll and Prodeus, 1998; Le Page et al., 2000).

1.2 Inflammation

1.2.1 The sequence of the inflammatory process

The so-called ‘danger-model’ of an immune response (Matzinger, 1998) predicts that all signals from invading pathogens and damaged or stressed cells initiate an immune response by triggering inflammation. During the inflammatory process, soluble and cellular components work together in the attempt to eliminate the agents causing physical damage or infection. While it is clear that inflammation is crucial to maintain the health of an individual, inflammatory responses can result in massive tissue destruction with a potentially fatal outcome for the host if poorly controlled (Figure 1.2).

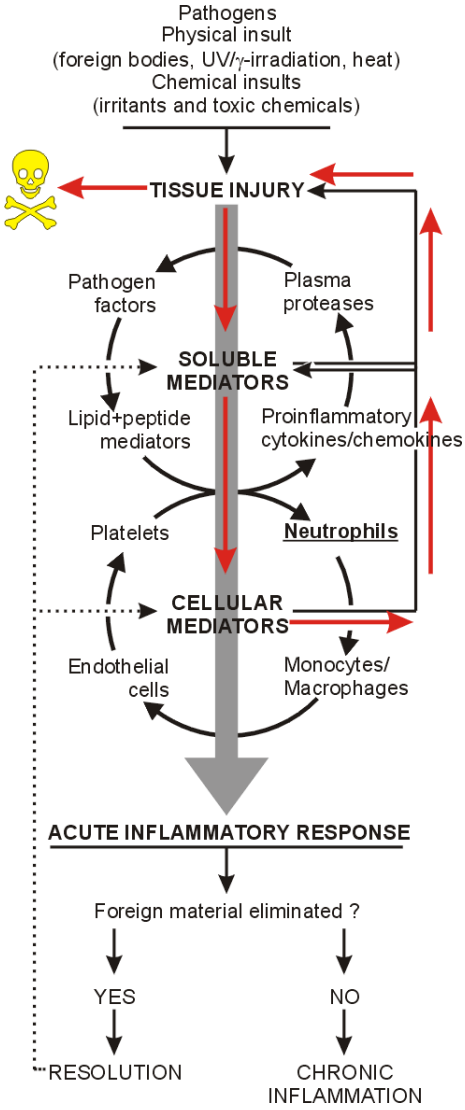

Figure 1.2: The inflammatory process.

The inflammatory process has three potential outcomes: (i) Pathogen elimination and resolution of inflammation; (ii) chronic inflammation or (iii) fatal inflammatory diseases due to an overwhelming and uncontrolled immune response (red arrows).

1.2.1.1 The inflammation causing agents

During invasion, agents, such as pathogens, foreign bodies, or chemicals, harm or destroy epithelial and endothelial cells, which constitute the primary barriers of a multi-cellular organism. Chemical and physical agents are detected indirectly, as injured or dying cells release intracellular degradation products, which in turn activate the plasma protease cascades. In contrast, pathogens can be recognized directly by cell-bound PRRs on innate immune cells (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000).

1.2.1.2 The acute inflammatory response

1.2.1.2.1 Physical responses

Regardless of the initiating agent, inflammation includes four cardinal signs (C Celsus 100 n. Chr.): *rubor* (redness), *dolor* (pain), *calor* (heat), and *tumor* (swelling), which are caused by local vasodilation, increased vascular permeability, recruitment of neutrophils and systemic fever. These physiological reactions ensure proper recruitment of soluble and cellular effectors to the site of infection or injury and a systemic response, which include fever and high serum levels of pentraxins (Paul, 1999).

1.2.1.2.2 Soluble inflammatory mediators and cytotoxic agents

Pro-inflammatory mediators are mainly produced at the site of inflammation and constitute a heterogeneous group of lipid mediators and amines such as leukotriens, prostaglandins, histamine and serotonine, pro-inflammatory cytokines, and chemokines such as TNF-α, IL-1b, IL-6 and IL-8. All these molecules modulate the inflammatory response upon binding to their cognate cell surface receptors (1.3).

In addition, several plasma proteases, in particular the complement proteases are crucially involved in the regulation of inflammation. The *complement system* consists of ~ 25 plasma proteins that interact in two related sets of reactions: the antibody-dependent classical pathway and the antibody-independent alternative pathway. Both pathways largely consist of the sequential activation of a series of serine proteases leading to the elimination of the pathogen in three possible ways: (i) killing of foreign cells or microorganisms by binding to and lysing their cell membrane, a process known as complement fixation, (ii) stimulation of receptor-mediated phagocytosis of foreign particles via FcR or complement receptors (CRs), a process named opsonization, and (iii) triggering a local acute inflammatory reaction that marks the area and attracts neutrophils (Carroll, 1998).

At the site of inflammation, activated macrophages and neutrophils produce highly cytotoxic reactive oxygen species (ROS) and intermediates (ROI) to destroy invading pathogens. Activation-induced NADPH-Oxidase and Myeloperoxidase (MPO) are responsible for the production of a variety of ROS and ROI, such as superoxide-radicals ($O2^{-\bullet}$), hydrogenperoxide (H_2O_2), hydroxylradicals ($HO^{\bullet}$), peroxidradicals ($ROO^{\bullet}$) and hypochlorite (HOCl) (Finkel, 1998; Johnson et al., 1996; Polyak et al., 1998; Segal and Shatwell, 1997). To protect themselves from the self-destructive effects of the produced ROS, macrophages and neutrophils produce radical-scavengers and catabolic enzymes (Finkel, 1998). Interestingly, the production of ROS is also a secondary effect during the progression of necrotic cell death (1.3.4.1), thus leading to

a further increase of ROS production at the site of inflammation (Degli Esposti and McLennan, 1998).

1.2.1.2.3 Cellular components

1.2.1.2.3.1 Neutrophils

Neutrophils are crucial to both immunity and inflammation. It is of note that in the absence of neutrophils (neutropenia) it is exceedingly difficult to clear invading pathogens, thus ultimately leading to inevitable demise as a result of overwhelming infection. Neutrophils represent 40-50 % of the circulating leukocyte population and are quiescent cells, with a short half-life of 6 – 8 hours and a wide range of inflammatory activities upon stimulation (Bicknell et al., 1994; Dransfield et al., 1995; Reveille et al., 1989; Whyte et al., 1993a; Whyte et al., 1993b). The activation leads to the induction of several cell-surface and intracellular proteins required for proper adhesion, phagocytosis, degranulation, oxidative burst and the production of proinflammatory mediators, thus ensuring the ideal and adequate elements for neutrophils to ingest and destroy pathogens.

An intriguing aspect of neutrophil activation is the phenomenon of priming. Neutrophils primed by brief exposure to activating agents exhibit an enhanced response to subsequent stimuli. Both short-term (including changes in cell morphology, oxidative and phagocytic capacity) and long-term (prolonged cell survival) responses to priming agents have been observed. Overall, these observations suggest a two-step process leading first from the non-receptive to a receptive state followed by full neutrophil responsiveness (Downey et al., 1995; Williams and Solomkin, 1999).

1.2.1.2.3.2 Monocytes and macrophages

Monocytes and macrophages complement together with neutrophils the group of professional phagocytes. Like neutrophils, they are attracted to sites of inflammation, invade the tissue, and are capable of secreting antibacterial proteins and proinflammatory mediators (Thepen et al., 1994). In contrast to neutrophils, monocytes and macrophage can act as APCs, which connect the innate to the adaptive immune system (Abbas et al., 1996). Thus, macrophages are of particular importance during chronic inflammation.

1.2.1.2.3.3 Eosinophils

Eosinophils are tissue-localized granulocytes that are recruited to sites of inflammation particularly in response to respiratory, gastrointestinal, dematolgic allergens and helminthic parasites (Martin et al., 1996). In contrast to neutrophils, eosinophils are ineffective phagocytes and release their granules filled with oxidative and cationic

proteins in the extracellular milieu. Whereas the detrimental effects of eosinophils during lung diseases are well characterized, the beneficial effects during inflammations are rather poorly described (Broide, 2001).

1.2.1.3 Resolution of acute inflammatory responses

After the danger to the individual is passed, the inflammatory response must be resolved both to minimize collateral damage to host tissue and to reset the system for further attacks, thus keeping the system sensitive.

1.2.1.3.1 Resolution by regulation of neutrophil apoptosis

It has recently been recognized that extravasated neutrophils undergo apoptosis or programmed cell death if not stimulated by environmental inflammatory mediators (Savill and Haslett, 1995). In particular, bacterial products and proinflammatory cytokines are involved in prolonging neutrophil survival (Colotta et al., 1992; Hachiya et al., 1995; Hiroi et al., 1998; Sheth et al., 2001; Sweeney et al., 1998; Watson et al., 1996; Watson et al., 1998; Watson et al., 1997). An alternative fate for neutrophils during inflammatory responses in tissues is necrosis (1.3.3). In contrast to necrosis, during apoptosis the neutrophils membrane remains intact and potentially damaging granule contents are retained. The intact apoptotic cell is phagocytosed by macrophages. This process actively suppresses the production and secretion of proinflammatory cytokines, thus supporting the resolution of inflammation as well (Fadok et al., 1998; Voll et al., 1997).

1.2.1.3.2 Resolution by immune suppressive cytokines

IL-4 is responsible for the downregulation of IL-6 and NADPH-Oxidase in neutrophils (Abramson and Gallin, 1990).

T-cell-produced IL-10 mainly inhibits cytokine secretion from macrophages and antigen-presentation (Moore et al., 1993; Mosmann, 1994). In addition, Keel and coworkers recently reported (Keel et al., 1997) that IL-10 induces neutrophil apoptosis during the resolution of septic shock.

TGFβ acts as the primary anti-inflammatory mediator promoting several anti-inflammatory effects, such as production of proinflammatory cytokines and inhibition of leukocyte adhesion. The role of TGFβ as a central player during resolution of inflammation is strongly supported by the phenotype observed in TGFβ -deficient mice, which develop severe inflammations in multiple tissues (Kolodziejczyk and Hall, 1996; Lawrence, 1996).

1.2.1.3.3 Hypothalamo-Pituitary-Adrenocortical Axis

One of the more intriguing pathways of investigations is the connection between the central nervous system (CNS), the adrenal cortex, and the resolution of inflammation (Buckingham et al., 1996; Sternberg, 1995; Sternberg and Licinio, 1995). Glucocorticoids, which are produced by the adrenal cortex and mediate immunosuppression and neutrophil apoptosis, are main players in this concept. Numerous studies have suggested that IL-1, IL-6, TNF-α, Macrophage Migration Inhibitory Factor (MIF), but even direct electric stimulation of the hypothalamus, lead to increased pituitary release of ACTH into the serum, which mediates increased corticosterone production in the adrenal cortex, ultimately decreasing inflammatory responses (Borovikova et al., 2000; Buckingham et al., 1996; Petrovsky and Bucala, 2000; Rothwell and Luheshi, 2000).

1.2.1.4 Chronic inflammations

When acute inflammation not resolves, inflammation becomes chronic to prevent the spreading of the infection throughout the body. In contrast to acute inflammation, which is characterized by a primarily neutrophil influx, the histologic hallmarks of chronic inflammations include the accumulation of macrophages, lymphocytes and the growth of fibroblast and vascular tissue. This formation is often referred to as granuloma (Boros, 1994; Wynn and Cheever, 1995). Several unusual cell types are characteristic of granulomata, including epithelioid cells, which are macrophage derivatives, and multinuclear giant cells, which are fusions of epitheloid cells with macrophages. In particular intracellular mycobacteria (e.g. tuberculosis, leprosy) predispose individuals to granuloma formations (Paul, 1999).

1.2.1.5 Fatal inflammatory diseases

In several clinical conditions, including systemic bacterial infection, sepsis, systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS) and reperfusion injuries following ischemic conditions, the inflammatory response is excessive and abnormally prolonged. This culminates not only in tissue damage, but also haemodynamic changes, multiple organ failure and ultimately death. Massive release of cytokines and chemokines (Baggiolini, 2001; Matsumoto et al., 1997; Moldawer, 1994; Morrison and Ryan, 1987; Mukaida et al., 1998; Tracey et al., 1986) and increased resistance of cells to apoptosis (Chitnis et al., 1996; Jimenez et al., 1997) appear to be particularly important during the pathogenesis of these diseases.

Under certain circumstances during bacterial infections the excessive inflammatory response can lead to septic shock (Bone, 1991; Glauser et al., 1991; Morrison and

Ryan, 1987; Tracey et al., 1986). This process is characterized by the massive release of proinflammatory cytokines, in particular TNF-α, IL-1β, macrophage migration inhibitory factor (MIF) and high mobility group-1 (HMG-1) protein (Alexander et al., 1991; Bernhagen et al., 1993; Beutler et al., 1985; Ohlsson et al., 1990; Wang et al., 1999). In general, bacteria do not directly cause lethal shock and tissue damage. Rather, bacterial products, such as lipopolysaccharide (LPS) stimulate the observed overwhelming acute inflammatory response (Moldawer, 1994; Morrison and Ryan, 1987; Tracey et al., 1986).

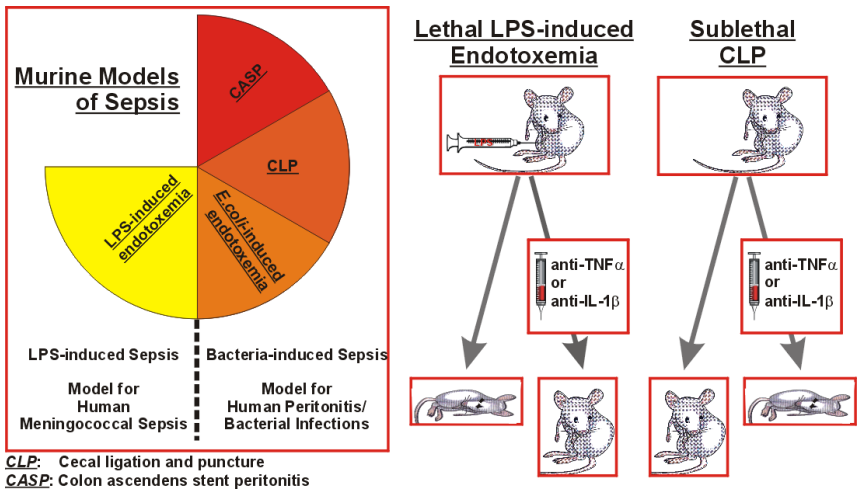

Figure1.3: The TNF-α/IL-1β paradox in mouse models of sepsis

Bolus administration of LPS into mice causes lethal endotoxemia, which can be protected by therapeutic agents that selectively inhibit cytokine action or prevent cytokine release, in particular neutralizing anti-TNF-α Ab (Beutler et al., 1985; Echtenacher et al., 1990) and reduction of functional IL-1β (Alexander et al., 1991; Bianchi et al., 1996; Ohlsson et al., 1990; Wakabayashi et al., 1991). Although endotoxemia reproduces some aspects of septic shock, it is substantially different from human sepsis, since it does not involve the replication and dissemination of bacteria. Thus, three well-characterized mouse models of microbial peritonitis, *Escherichia coli*-induced peritonitis, cecal ligation and puncture (CLP) or colon ascendence stent peritonitis (CASP) are used to mimic the leading cause of septic shock in surgical patients (Figure 1.3) (Cohen and Abraham, 1999; Friedman et al., 1998). Surprisingly, anti-TNF-α treatment is rather deleterious in these models due to imparment of the capacity of the immune system to fight infections (Echtenacher et al., 1990; Echtenacher et al., 1996; Eskandari et al., 1992; Malaviya et al., 1996; Peschon et al., 1998; Pfeffer et al., 1993; Rothe et al., 1993). Thus, the goal of anti-inflammatory therapy is to eliminate the undesirable aspects of a double-edged sword – tissue destruction beyond what is absolutely necessary for eliminating a pathogenic agent.

1.3 The molecular mechanisms responsible for activating and resolving inflammatory responses

Immune cells coordinate their activation and inhibition through complex biochemical signaling systems. Extracellular signals are transmitted via cell-surface bound receptors into the cell. The receptors are linked to intracellular communication pathways, which are maintained by the synthesis or alteration of a great variety of different substances that are often integral components of the process they control. The importance and the complexity of inter- and intracellular signaling is highlighted by the fact that 20 % of the ~32 000 human coding genes encode proteins involved in signal transduction (Blume-Jensen and Hunter, 2001). The following paragraphs will introduce the most important signal-sensing receptors and their connected intracellular signaling pathways that are responsible for activation, inhibition, survival and death of cells involved in innate responses.

1.3.1 Intracellular signaling pathways

1.3.1.1 The activation of Phospholipase C

Upon stimulation of G-protein-coupled receptors (GPCR), such as chemokines recep-

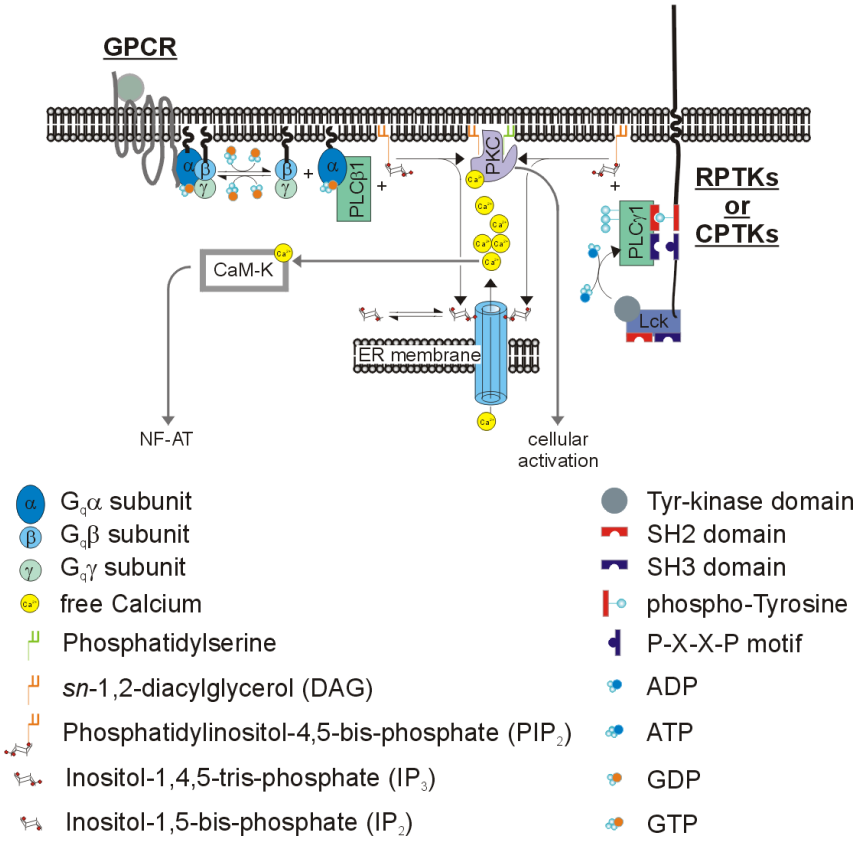

Figure 1.4: Activation of Phospholipase C (PLC) and calcium-dependent pathways

tors (1.3.2.1), GTP induces the dissociation of the receptor-associated G_q- complex to $G_q\alpha$•GTP, and $G_q\gamma/G_q\beta$. Phospholipase C β (PLCβ) is activated and recruited to the plasma membrane by association with $G_q\alpha$•GTP (Rebecchi and Pentyala, 2000) (Figure 1.4). Activated PLCβ hydrolyzes Phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and 1,2-*sn*-Diacylglycerol (DAG). Whereas IP_3 induces calcium-release from the endoplasmic reticulum (ER) to the cytosol by interacting with IP_3 receptors, DAG together with calcium induces the activation of Protein kinase C (PKC). In addition, calcium associates with the calcium-binding protein calmodulin subsequently leading to activation of calcium-calmodulin-dependent proteins such as calcium-calmodulin-dependent kinases (CaM-K). These events activate directly transcription factors responsible for cell differentiation such as NF-AT and induce several downstream signaling pathways (Blume-Jensen and Hunter, 2001; Kurosaki et al., 2000; Latour and Veillette, 2001).

Hydrolysis of PIP_2 can also be mediated by PLCγ. PLCγ is recruited via its SH2-domain and is subsequently phosphorylated and activated by receptor protein tyrosine kinases (RPTK) or cytosolic PTKs (CPTKs) (1.3.2.6.1 and Figure 1.12) (Blume-Jensen and Hunter, 2001). The enzymatic activity of PLCγ is similar to PLCβ resulting inPIP2 hydrolysis connnected downstream pathways.

1.3.1.2 The Mitogen-activated protein kinase pathways

Mitogen-activated protein kinases (MAPKs) are evolutionary conserved enzymes connecting cell surface receptors to critical regulatory targets within the cell. MAPKs respond to biological, chemical or physical stress controlling cell adaptation, survival, and death. MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPK kinase kinase (MAPKKK, MEKK) (Figure 1.5) (English et al., 1999). These modules may be activated by STE20 kinases or GTP-binding proteins (Chang and Karin, 2001).

Mammals express at least four distinct regulated groups of MAPKs, extracellular-signal-regulated kinases (ERK)-1/2, Jun-amino-terminal kinases (JNK)-1/2/3, Stress-activated protein kinases (SAPKs) or p38 proteins (p38α/β/γ/δ) and ERK5. As indicated in Figure 1.5, all these MAPKs are activated by specific MAPKK, which in turn, however, can be activated by several distinct MAPKKK increasing the complexity and diversity of MAPK signaling. Once activated, MAPKs need to find their proper protein targets, which is ensured by anchor amino acid residues (X) flanking the MAPK-target sequence X-X-S/T-P-X-X and an additional docking interaction mediated by an other site on both the kinase and the substrate (Chang and Karin, 2001).

MAPKs are fundamentally involved in transcriptional regulation in response to extracellular stimuli (Treisman, 1996). As shown in Figure 1.4, JNK phosphorylates Jun proteins thereby enhancing their ability to activate transcription without affecting DNA binding (Kallunki et al., 1996). Most MAPKs, in particular ERKs and p38/SAPKs phosphorylate Ets transcription factors (TFs) that are involved in induction of fos genes, whose products heterodimerize with Jun proteins to form activation protein (AP)-1 complexes (Treisman, 1996). In addition, ERK activates Myc (Egan and Weinberg, 1993) and the p38s/SAPKs phosphorylate and enhance the activity of MEF2C and related family members (Han et al., 1997). In all these cases, MAPKs function inside the nucleus and target TF that are pre-bound to DNA.

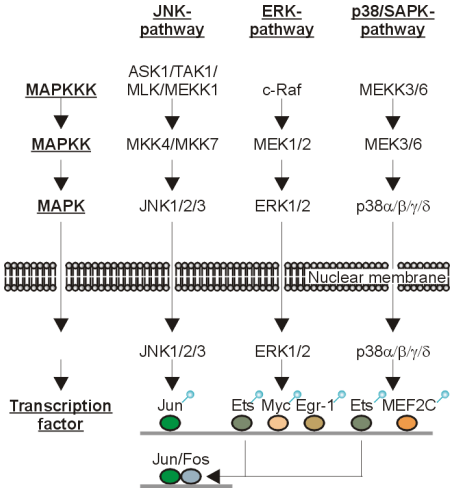

Figure 1.5: The Mitogen-activated protein kinase (MAPK) pathways

Besides acting directly on TFs, MAPKs regulate gene expression post-transcriptionally by mRNA stabilization (Chen et al., 2000). In addition, MAPKs modulate cell proliferation, cell survival and death (Chang and Karin, 2001). Whereas ERK1/2 has been linked to cell survival, JNK and p38 are rather linked to induction of apoptotic cell death (Xia et al., 1995). Interestingly, cells deficient for Jun1 and 2 are only impaired in UV-induced apoptosis, thus suggesting that JNK can directly activate the apoptotic machinery (Tournier et al., 2000). The mechanism by which ERK protects against apoptosis is complex. Erk activation by growth and survival factors prevents apoptosis through ribosomal S6 kinase (RSK), which inactivates the pro-apoptotic protein Bad (Bonni et al., 1999). It was shown recently that ERK may directly induce phosphorylation of Bad, thus leading to cell survival (Klein et al., 2000; Scheid and Duronio, 1998; Scheid et al., 1999).

1.3.1.3 The Iκ kinase (IKK) pathway

On other kinase cascade is responsible for the activation of Nuclear factor (NF)-κB (Malinin et al., 1997) (Figure 1.6). The NF-κB-inducing kinase (NIK) is capable of activating the IκB-kinases IKKα and IKKβ (Nakano et al., 1998b) thus leading to IκB phosphorylation, ubiquitinylation, and degradation by the proteasome (Karin and Ben-

Neriah, 2000). The removal of IκB subsequently activates NF-κB (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1998). An additional possibility for IKK activation is guided by the MAPKKK MEKK1 (Belich et al., 1999; Lin et al., 1999). MEKK1 is activated in part via proteolytic cleavage by a caspase or by a recently identified TRAF6-interacting protein called ECSIT (Kopp et al., 1999) (1.3.2.5.1).

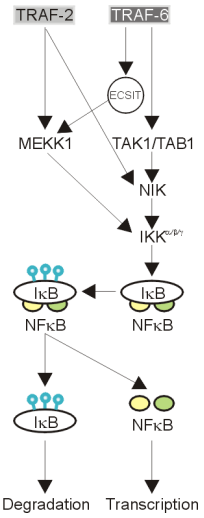

Figure 1.6: The pathways leading to the activation of Nuclear Factor (NF)-κ B

1.3.1.4 The Phosphoinositide 3-OH kinase (PI(3)K) pathways

PI(3)Ks are a family of lipid kinases defined by their ability to phosphorylate the 3'-OH group of the inositol ring in inositol-phospholipids. Class I PI(3)Ks are composed by a catalytic Ser/Thr-kinase domain and a regulatory domain responsible for phosphatidylinositol interaction. PI(3)K can be activated by association to RPTK or CPTK-activated receptors (PI(3)K IA) (Figure 1.7) and by GPCR (PI(3)K IB) (Blume-Jensen and Hunter, 2001). Binding via its SH2 domains allosterically activates the catalytic domain of PI(3)K and leads to the production of Phosphatidylinositol-3,4-bisphosphate ($PI^{(3,4)P}$) or Phosphatidylinositol-3,4,5-trisphosphate ($PI^{(3,4,5)P}$). The cellular effects mediated by Phosphoinositides are mediated through the specific binding of $PI^{(3)P}$ to FYVE domains and $PI^{(3,4)P}$ or $PI^{(3,4,5)P}$ to Pleckstrin-homology (PH) domains, respectively. PH domains are found in numerous proteins but particularly in the protein-serine/threonine kinases, 3'-phosphoinositide-dependent kinase-1 (PDK-1) and Akt, which are central players in the effects induced by phosphoinositides. As shown in Figure 1.7, PI(3)K-mediated production of $PI^{(3,4,5)P}$ and $PI^{(3,4)P}$ leads to the recruitment and co-localization of Akt and PDK-1 via their PH domains to the plasma membrane. The constitutive active PDK-1 phosporylates Akt at Thr^{308}, which stabilizes Akt in an active conformation. Although phosphorylation at Thr^{308} is a prerequisite for kinase activation, a further phosphorylation event targeting the C-terminal located Ser^{467} is essential for full kinase activity. The kinase for this phosphorylation ('PDK-2') remains to be identified.

Akt target proteins are phosphorylated within the same basic motif, R-X-R-X-X-S/T and are all involved in regulating apoptosis, cellular growth and cell-cycle regulation (Blume-Jensen and Hunter, 2001). The substrates involved in apoptosis regulation

include Forkhead TFs, the pro-apoptotic protein Bad, and the cyclic AMP response element-binding protein (CREB) (Datta et al., 1999). In addition, several Akt-substrates are involved in cell-cycle progression finally leading to hyper-phosphorylation and inactivation of the Retinablastoma (Rb) protein (Blume-Jensen and Hunter, 2001; Evan and Vousden, 2001).

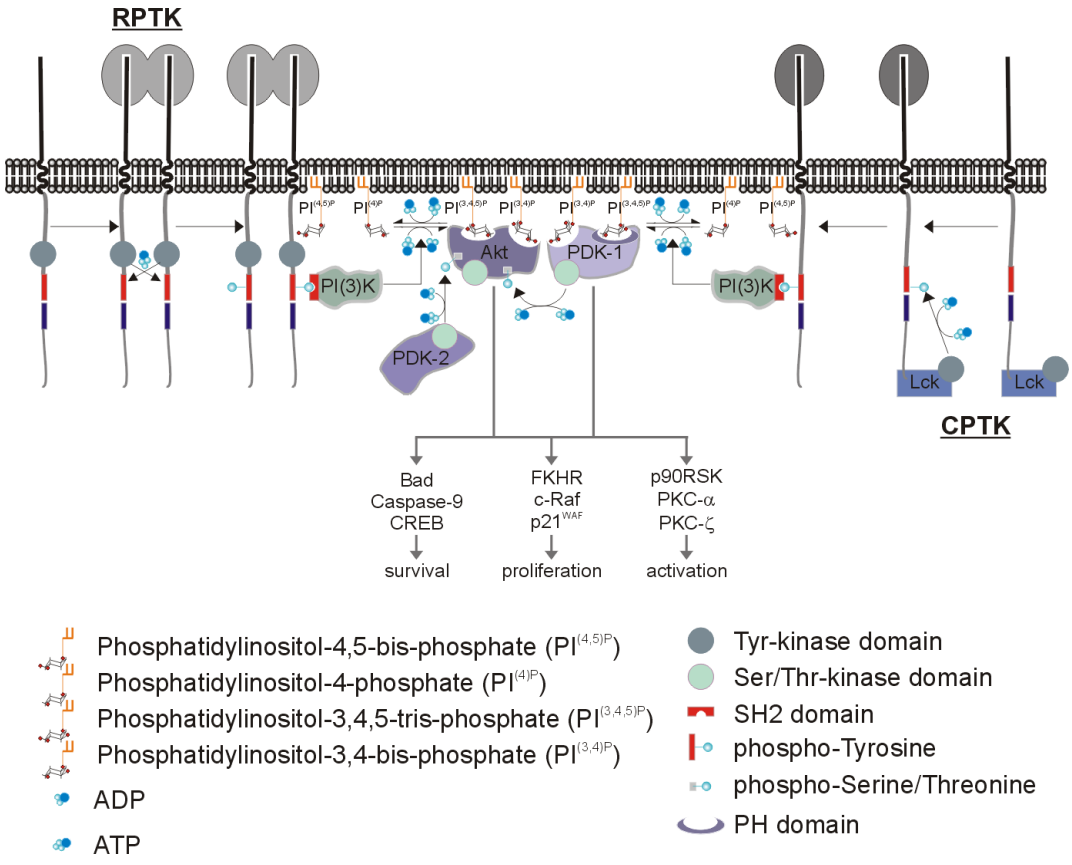

Figure 1.7: The Phosphoinositide 3-OH kinase (PI(3)K)- and PDK-1/Akt-pathway

1.3.2 Activating receptor systems

1.3.2.1 G-protein-coupled receptors (GPCR)

Chemokines such as IL-8, but also other proinflammatory attractants such as Platelet activating factor (PAF), leukotrienes, formyl-Met-Leu-Phe (fMLP) and Complement factor 5a (C5a) interact with a growing family of homologous seven-transmembrane (7T) G protein-coupled receptors (GPCR) (Ben-Baruch et al., 1995; Haribabu et al., 2001; Murphy, 1996). The three intracellular loops of 7T-GPCR contain several serine and threonine residues that are subjected to intensive phosphorylation events modulating receptor sensitivity and signal transduction (Haribabu et al., 2001). Upon ligand binding, GPCRs-mediated intracellular signaling events are transduced by associated G-proteins (Figure 1.4). Depending on the cell type, stimulated GPCR-

induced responses are manifold including chemotaxis, degranulation and induction of NADPH-Oxidase and adhesion molecules (Baggiolini, 1998; Baggiolini, 2001; Baggiolini et al., 1995; Haribabu et al., 2001; Murphy and Tiffany, 1991). Prostaglandins and leukotriens maintain and augment the inflammatory response by influencing pain, vasodilatiation and permeability, and neutrophil-endothelial cell interaction (Ashby, 1994; Cazzola et al., 1995; Ford-Hutchinson, 1994; Goetzl et al., 1995; MacDonald, 1996; Seibert et al., 1995; Vane and Botting, 1995).

1.3.2.2 Receptors of the Tumor necrosis factor-Receptor superfamily

The TNF-R-SF represents a growing family, with over 20 members identified in mammalian cells thus far (Baker and Reddy, 1998; Smith et al., 1994). These proteins share 2 – 6 highly homologues cysteine-rich extracellular domains, and similar intracellular effector domains. Upon ligand binding, these receptors transmit their signals via protein-protein interactions, which convey several activating signals culminating in a broad-spectrum of cellular responses such as apoptosis, survival, proliferation, or differentiation (Baker and Reddy, 1998; Peter et al., 1999). Activation of the receptors is induced upon binding of the cognate ligands that – with the exception of Nerve growth factor (NGF) – constitute the TNF-SF. The members of the families with their interacting ligands are summarized in Figure 1.8. In the following sections, the most important members with their immunological and biochemical properties will be introduced.

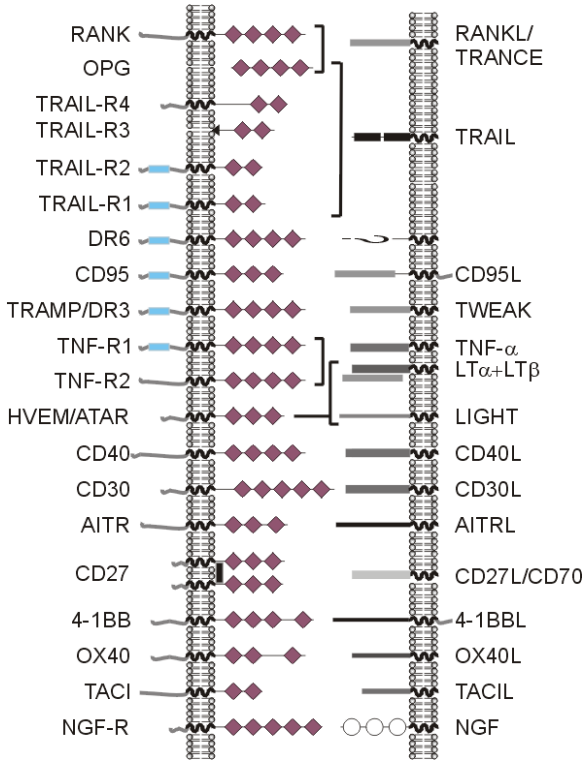

Figure 1.8: Interaction between TNF-R-SF (left) and TNF-SF (right) members

Blue boxes indicate death domains (DDs), diamonds indicate cysteine-rich extracellular domains.

1.3.2.2.1 The Tumor necrosis factor (TNF) system

TNF-α was originally described as a factor responsible for LPS-induced tumor necrosis and is related to Lymphotoxin (LTα/TNF-β). Although produced from different

cell types, TNF-α (from activated macrophages) and LTα (from activated T cells) can interact with the same receptors, TNF-R1 and –R2. Thus TNF-α and LTα produce similar biological activities such as the induction of several proinflammatory responses, fever and the killing of susceptible cells (Lynch, 1996; Peter et al., 1999). Both TNF-Rs are widely expressed. TNF-R1 is mainly responsible for the induction of proinflammatory responses via NF-κB but can also induce apoptosis (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1993; Tartaglia et al., 1991; Wong et al., 1992). In contrast, TNF-R2 is less well defined and seems to be involved in supporting TNF-R1-mediated functions (Baker and Reddy, 1998; Peter et al., 1999). Interestingly, TNF-R2 has an essential role during activation-induced cell death of $CD8^+$ T cells (Zheng et al., 1995).

1.3.2.2.2 The CD40/CD40L system

CD40 was identified in immunochemical studies employing an mAb specific for a 50 kDa protein (Koho et al., 1984; Paulie et al., 1985; Paulie et al., 1984; Stamenkovic et al., 1989a; Stamenkovic et al., 1989b) expressed during all stages of B cell development and differentiation (van Kooten and Banchereau, 2000), whereas its ligand, CD40L, was mainly expressed on activated $CD4^+$ T cells (Armitage et al., 1992; Graf et al., 1992; Hollenbaugh et al., 1992; Lederman et al., 1992; Noelle et al., 1992). Patients suffering from X-linked hyper-IgM syndrome (HIGM) display a mutation in the CD40L gene, thus highlighting the pivotal role of the CD40/CD40L-system in T-cell dependent B cell responses (Callard et al., 1993). Similar deficiencies in mounting immune responses were observed in genetically modified mice with inactivation of either the CD40 or the CD40L gene (Kawabe et al., 1994; Renshaw et al., 1994; Xu et al., 1994). Following these findings, it was observed that CD40 and CD40L expression was much broader than initially thought. One major observation has been the expression of CD40 on monocytes and dendritic cells (Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000). On DCs, CD40 expression seems to be a critical step in the final maturation to fully competent APCs. As a consequence HIGM patients and CD40- and CD40L-deficient mice show abnormalities in priming of $CD4^+$ T cells. Recently, CD40-CD40L interactions was also shown to be necessary for cross-priming of CTL responses by DCs. Moreover, CD40 is widely expressed on non-hematopoietic cells, including endothelial cells, fibroblasts, and epithelial cells (Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000). On these cells, CD40 is involved in the amplification and regulation of inflammatory responses. In line with these results, using either knock-out animals or neutralizing anti-CD40 or -CD40L mAb, interference with CD40-CD40L-interaction was shown to be beneficial

in several disease models, including transplantation, autoimmunity, allergy, and infectious diseases (Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000).

1.3.2.2.3 The RANK/RANKL-system

CD40-deficient mice challenged with viruses are able to mount protective $CD4^+$ T cell responses that produce normal levels of IFNγ (Oxenius et al., 1996). This suggested the existence of CD40/CD40L independent mechanisms for T cell priming. Indeed, another receptor of the TNF-R-SF expressed on DCs that are critically involved in APC-T cell interactions, was recently identified.

The DC-bound Receptor activator for NF-κB (RANK) (Anderson et al., 1997), also called TRANCE-R, interacts with TRANCE/RANKL/OPGL/ODF on T cells leading to the upregulation of costimulatory signals and DC cell survival (Anderson et al., 1997; Bachmann et al., 1999; Josien et al., 2000; Kong et al., 1999; Wong et al., 1997). In addition, RANKL has been demonstrated to play an essential role in osteoclast differentiation and activation (Lacey et al., 1998; Yasuda et al., 1998).

1.3.2.3 The signal transduction of TNF-R family members

1.3.2.3.1 TNF-R associated factors and their downstream signals

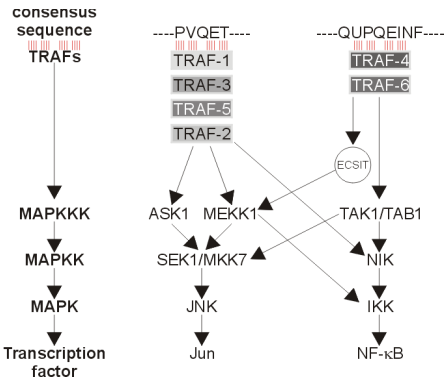

Figure 1.9: TRAF-mediated connection from surface receptors to downstream signaling pathways

The consensus sequence PVQET recruits TRAF-1, -2, -3, and –5, whereas the sequence QUPQEINF is responsible for the

TNF-R-associated factors (TRAFs), in particular TRAF-1 and –2, were first identified as signal transducers from TNF-R2 (Rothe et al., 1994). Since then, four additional members were identified in human and mouse (Inoue et al., 2000). TRAF1, TRAF2, TRAF3 and TRAF5 are highly homologues and bind to the consensus sequence PXQXT in several receptors, whereas TRAF4 and TRAF6 recognize QEPQEINF in the cytoplasmic tail of IL-1R, IL-18R, the TLRs and in IL-1R-associated protein kinase (IRAK)-1 and IRAK-2 (Inoue et al., 2000).

As shown in Figure 1.9, signaling pathways triggered by TRAF2, TRAF5 and TRAF6 link the associated receptors to transcription factors of the NF-κB- and the AP-1-family by activating MAPKKKs and MAPKKs. Whereas the cascade leading to JNK activation is clearly defined by the activation of MMK7, the direct connection to the observed activation of p38/SAPKs remains to be identified (Inoue et al., 2000; Nishi-

toh et al., 1998; Sanchez et al., 1994; Tournier et al., 1997; van Kooten and Banchereau, 2000; Yan et al., 1994).

The role of the TRAFs during signal transduction in the immune system was highlighted by the generation of TRAF-deficient mice. Surprisingly, TRAF2$^{-/-}$ mice and transgenic mice bearing a dominant-negative TRAF2 (DN-TRAF2) showed a dramatic decrease of JNK activity whereas NF-κB activation was almost normal (Lee et al., 1997; Yeh et al., 1997). TRAF5 may compensate for the loss of TRAF2 in NF-κB activation. However, TRAF5$^{-/-}$ mice showed no impairment in signal transduction from receptors of the TNF-R family (Nakano et al., 1999). Although neither TRAF1 nor TRAF3 activates any kinases tested *in vitro* so far, TRAF1 transgenic mice (Speiser et al., 1997) and TRAF3$^{-/-}$ mice (Xu et al., 1996) exhibit marked impairment in their immune systems. TRAF6$^{-/-}$ mice exhibit severe osteoporosis and are impaired in NF-κB activation upon stimulation with LPS, IL-1 and CD40 thus reflecting the involvement of TRAF-6 in the signal transduction from RANK, TLRs, IL-1R and CD40 (Inoue et al., 2000; Lomaga et al., 1999). In conclusion, the gathering of TRAFs at certain intracellular motifs of receptors of the TNF-R and TLR/IL-1R family determines the pathways switched on upon receptor triggering thus leading to cellular responses such as NF-κB-mediated survival or Jun-mediated differentiation (Inoue et al., 2000).

1.3.2.3.2 Protein complexes recruited to receptors of the TNF-R-SF

Stimulation of receptors of the TNF-R family leads to the recruitment of several proteins to intracellular binding domains. The complexes formed upon stimulation of TNF-R1, -R2, CD40 and RANK are depicted in Figure 1.10. Whereas the signal transduction of TNF-R2 and RANK are completely dominated by TRAF-mediated signals (1.3.2.3.1), the intracellular motifs present in TNF-R1 and CD40 allow for the induction of additional pathways.

TNF-R1 contains an intracellular ‘death domain’ (DD), which upon stimulation allows for the formation of a death-inducing signaling complex (DISC), which contains FADD, Caspase-8, TRADD, RIP and TRAF-2 (Kischkel et al., 2000). It was previously shown that FADD$^{-/-}$ and Caspase-8$^{-/-}$ cells are resistant to TNF-induced apoptosis (Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998), thus providing evidence that FADD and Caspase-8 are responsible for TNF-R1-mediated apoptosis. In contrast, TRADD, RIP and TRAF-2 mediate survival signals via JNK- and NF-κB activation (Inoue et al., 2000). As described above, TRAF2$^{-/-}$ mice have a defect in JNK activation, whereas NF-κB activation is normal (Lee et al., 1997). In contrast, RIP$^{-/-}$ mice can activate JNK but not NF-κB (Kelliher et al., 1998), thus indicating that RIP is responsible for NF-κB activation, whereas TRAF-2 mediates JNK

phosphorylation during TNF-R1-induced signaling. The regulation between survival and death signals induced at the TNF-R1 remains unclear. However, it is known that induction of apoptosis via TNF-R1 requires the inhibition of transcription or translation, thus suggesting the presence of apoptosis inhibitors under normal conditions (Leist et al., 1994; Polunovsky et al., 1994).

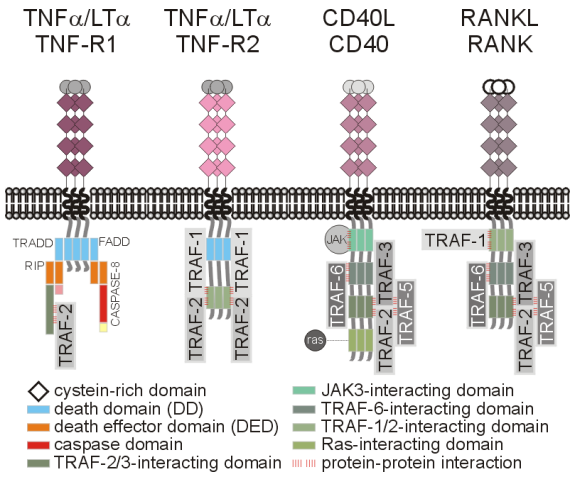

Figure 1.10: Stimulation-induced protein clusters of TNF-R1, R2, CD40 and RANK

Although CD40 does not contain a DD, it displays binding sites not only for TRAF2, TRAF3, TRAF5 and TRAF6 but also additional platforms for the binding of JAK3 and Ras. Furthermore, one motif has been shown to be essential for the activation of the transcription factor NF-AT. Thus, in contrast to other receptors of the TNF-R family, which allow for the activation of JNK and NIK/IKK, CD40 can activate ERK1/2 in a Ras-dependent and independent fashion (Kashiwada et al., 1998), the Janus Kinase (JAK)-3 leading to STAT3 and STAT6 activation (Hanissian and Geha, 1997), and the CamK II resulting in the activation of the transcription factor NF-AT (Genestier et al., 1994; O'Garra et al., 1986; Valentine et al., 1995; Venkataraman et al., 1994).

1.3.2.4 IL1R- and IL18R system

IL-1 is a major inflammatory mediator produced primarily by monocytes and activated macrophages. IL-1 activity is mediated by two proteins, known as IL-1α and IL-1β. A high affinity activating receptor (IL-1RI) for IL-1 is expressed by lymphocytes and fibroblasts whereas a low affinity decoy receptor (IL-1RII) is expressed on several other cell types (Fitzgerald and O'Neill, 2000). An unique feature of IL-1 is the presence of a naturally occurring antagonist, IL-1RA, which is expressed in neutrophils and monocytes (Lennard, 1995). The recently identified cytokine IL-18 is structurally and functionally highly related to IL-1 and induces the same type of responses upon IL-18R stimulation (Dinarello, 2000). The intracellular domain of IL-1R and IL-18R are structural homologues to the Toll-like receptors and therefore share identical signal transduction molecules and pathways (1.3.2.5.1).

1.3.2.5 Pattern recognition receptors (PRRs)

In contrast to the adaptive immune response, requiring induction and selection over a period of days, the innate immune systems insures immediate recognition of invading pathogens. As already described, PRRs recognize a broad spectrum of evolutionary conserved motifs (pathogen associated molecular patterns = PAMPs), which are absent in higher eukaryontes and are pertinent to microbial function (Janeway, 1989). Apparently, PRRs have evolved to recognize patterns in all biological polymers (polypeptides, polysaccharides, polynucleotides; see also Table) generating a detection system with a very broad specificity. The complement receptors, scavenger receptors, the mannose receptor, CD14 and the Toll-like receptors (TLRs) belong to the family of PRRs. In principle, there are two classes of PRRs: those that mediate phagocytosis and those that lead to activation of proinflammatory pathways.

1.3.2.5.1 Toll-like receptors

Toll was identified as an essential protein during embryonic development of *Drosophila* and was subsequently shown to be a key mediator during anti-fungal immunity in flies (Lemaitre et al., 1996). Subsequently, an evolutionary conserved receptor family was identified in mammals (Medzhitov et al., 1997), which display homologies in their intracellular domains to IL-1 receptor (IL-1R) and IL-18 receptor (IL-18R), but have unique extracellular regions (Akira et al., 2001). Ten members of the TLR-family have been reported so far and additional TLR sequences may be retained from several databases (Akira et al., 2001). The surface expression of TLRs seems to be very low, varies among immune cells and depends on certain stimuli (Visintin et al., 2001).

1.3.2.5.1.1 Specificity of TLRs

LPS is an integral component of the outer membranes of Gram-negative bacteria and can provoke septic shock (Ulevitch and Tobias, 1995). A complex of LPS and the serum protein LPS-binding protein (LBP) binds to CD14 on myeloid cells and induces signals through TLR4. TLR4 was identified by analysis of the LPS-hyporesponsive mouse stain C3H/HeJ, which contains a miss-sense point mutation in the cytoplasmic tail of the TLR4 gene leading to a non-functional TLR4 (Poltorak et al., 1998). The generation of $TLR4^{-/-}$ mice and the identification of individuals harboring a mutant form of TLR4 confirmed the central role for TLR4 in LPS-binding and LPS-mediated inflammatory responses (Arbour et al., 2000; Hoshino et al., 1999). However, LPS requires an accessory molecule, MD-2, for the binding of LPS (Shimazu et al., 1999).

The ligand specificity of TLR2 is modulated by heterodimerization with TLR1, TLR6 or another TLR. (Ozinsky et al., 2000). Whereas TLR2 is responsible for the recogni-

tion of the triacetylated NH_2-terminini of lipoproteins (Akira et al., 2001), TLR6 recognizes diacetylated NH_2-termini, as expressed in macrophage-activating lipoprotein-2 (MALP-2) (Akira et al., 2001; Takeuchi et al., 2001). Nevertheless, TLR2 seems to be essential for the signal transduction properties in all heterodimers containing TLR2. Thus, $TLR2^{-/-}$ cells are unresponsive to all lipoproteins whereas $TLR6^{-/-}$ mice are only resistant to MALP-2-induced responses (Akira et al., 2001; Takeuchi et al., 2001).

Table 3.1: Pathogen-associated molecular patterns (PAMPs) and their receptors (PRRs)

PRRs	PAMPs or ligand	Pathogens	Locus	Phenotype of k.o. mice
TLR1	TLR2/TLR1 heterodimers: Ligands unkown		4q14	Not identified
TLR2	TLR2/TLRX heterodimers:		4q31.3-q35	Resistant to LPs
	Lipotechoic acid (LTA)	Gram-positive bacteria		
	Lipoproteins (LP)	Eubacteria		
	Lipoarabinomannan (LAM)	Mycobacteria		
	Peptidoglycan (PGN)	Most bacteria		
	Zymosan	Yeast		
	GPI anchor	Trypanosoma cruzi		
TLR3	Not identified	Not identified	4q31.3-q35	Not identified
TLR4	TLR4/MD2 heterodimers:		9q32-q33	Resistant to LPS
	LPS	Gram-negative bacteria		
	Taxol	Taxus brevifolia		
	F protein	RSV		
	Hsp60	Host		
	Fibronectin	Host		
TLR5	Flagellin	Gram-negative bacteria Gram-positive bacteria	1q33.3-q42	Not identified
TLR6	TLR2/TLR6 heterodimers: MALP-2	Mycobacteria	4q14	Resistant to MALP-2
TLR7	Not identified	Not identified	Xp22	Not identified
TLR8	Not identified	Not identified	Xp22	Not identified
TLR9	CpG DNA	All bacteria	3p21.3	Resistant to CpG DNA
CD14/ LBP	LPS	Gram-negative bacteria		Not identified
	Peptidoglycan (PGN)	Most bacteria		
Mannose Receptor	Zymosan	Yeast		Not identified
	Mannans and Mannoproteins	Yeast		
N-formyl-Met receptor 1 + 2	N-formyl Methionine	Prokaryotes		Not identified

TLR5 recognizes bacterial flagellin from both Gram-negative and –positive bacteria (Hayashi et al., 2001). Flagellin constitutes bacterial flagella, a polymeric rod-like appendages, which extend from the outer membrane and was known for a long time to be a strong adjuvant readily inducing NF-κB and promoting inflammatory responses (Eaves-Pyles et al., 2001; Steiner et al., 2000).

Bacterial DNA contains unmethylated CpG oligonucleotide motifs, which can stimulate immune responses in mammals whereas eukaryotic methylated CpG motifs cannot (Krieg, 2000). Both $TLR2^{-/-}$ and $TLR4^{-/-}$ cells respond normally to CpG DNA (Hacker et al., 2000) whereas $MyD88^{-/-}$ cells do not (Kawai et al., 1999), indicating that CpG recognizes a distinct MyD88-associated receptor. Indeed, it was shown re-

cently that TLR9-deficient mice and isolated TLR9$^{-/-}$ DCs and macrophages are completely unresponsive to CpG DNA (Hemmi et al., 2000).

Interestingly, TLR1, TLR2, TLR6, and TLR9 are expressed in endosomes (Hemmi et al., 2000; Ozinsky et al., 2000) and it was previously observed that at least CpG-mediated responses require phagosomal acidification prior to signal transduction. Thus, it is likely that specific TLRs, according to the nature of the phagocytosed pathogen, are recruited to the phagosomes where they are assembled to trigger proper inflammatory responses (Hacker et al., 2000; Hemmi et al., 2000).

1.3.2.5.1.2 Signaltransduction of TLRs, IL-1R and IL-18R

Sequences homologues to IL-1R type I in their cytoplasmic domains allow TLRs to use the same signaling molecules. This includes the adaptor protein MyD88, which upon TLR, IL-1R or IL-18R stimulation recruits the IL-1R-associated protein kinase 1 (IRAK-1) to the receptor (Medzhitov et al., 1998; Muzio et al., 1997). As shown in Figure 1.11, phosphorylation of IRAK-1 results in dissociation from the receptor and association with TNF-R activated factor 6 (TRAF-6), which ultimately leads to the activation of IKK$^{\alpha/\beta/\gamma}$ and the MAPKs p38/SAPK and JNK. Thus, triggering of TLRs by PAMPs guide the induction of proteins, which in turn arbitrate inflammatory and immune responses.

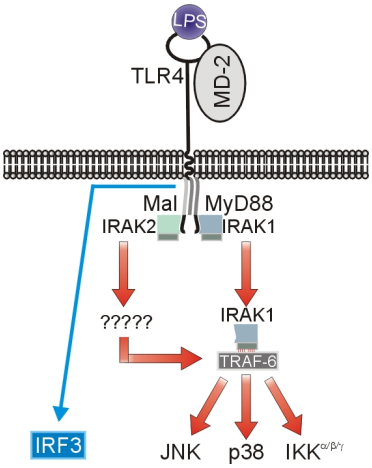

Figure 1.10: TLR4/MD-2 as an example for the signal transduction of the Toll-like receptors (TLRs)

Interestingly, TLR2- but not TLR4-mediated NF-κB activation is completely abolished in MyD88$^{-/-}$ mice, thus proposing MyD88-independent TLR4-pathways to NF-κB (Kawai et al., 1999; Takeuchi et al., 2000). Indeed, the recent discovery of the MyD88-adapter-like (Mal) protein (Fitzgerald et al., 2001) and Toll-IL-1R (TIR) domain-containing adapter protein (TIRAP) (Horng et al., 2001) defines an additional pathway from TLR4 to NF-κB via the recruitment of IRAK-2. In addition, LPS induces several IFNγ-inducible genes most probably via IFN regulatory factor 3 (IRF3) (Akira et al., 2001). Surprisingly, it was shown, that TLR4 can also induce apoptosis using a MyD88-independent pathway (Aliprantis et al., 1999) in THP-1 tumor cells and recent studies have indicated that the anti-cancer drug TAXOL binds and stimulates TLR4 (Kawasaki et al., 2000) thus halting mitosis and inducing cell death (Yamazaki et al., 2000).

1.3.2.5.2 CD14 and LPS-binding protein

The TLR4-mediated response to LPS is enhanced by LPS-binding protein (LBP). LBP is a serum factor that upon binding to LPS allows for binding of the LPS/LBP complex to the GPI-linked surface receptor CD14. CD14 cannot signal by itself, thus it is believed that CD14 increases the local concentration of LPS at the cell surface for proper stimulation of TLR4 (Yang et al., 1998).

1.3.2.5.3 Scavenger receptor

The PRRs on macrophages and neutrophils with the broadest specificity are the scavenger receptors (SRs) (Gough and Gordon, 2000), which bind a broad range of polyanionic ligands including oxidized or modified low density lipoprotein (Brown and Goldstein, 1990). More recently, SRs have been implicated in the recognition of microbes and their products particularly in the binding and phagocytosis of LTA, LPS, Gram-positive and -negative as well as mycobacteria (Gough and Gordon, 2000). However, the signaling pathways induced by SF are not yet defined.

1.3.2.5.4 Mannose receptor

Macrophages, Neutrophils and DCs express a multitude of membrane-bound lectins for the binding and phagocytosis of endogenous and exogenous ligands. The Mannose receptor (MR) is critically involved in microbial recognition and uptake (Aderem and Underhill, 1999; Linehan et al., 2000). The MR binds and internalizes glycoproteins displaying α-linked high-mannose containing saccharide structures terminating in mannose, fucose or N-acetylglucosamine residues (Kery et al., 1992) thus leading to the uptake of all kinds of pathogens as well as endogenous proteins (Linehan et al., 2000). Whether MR is transducing additional activating signals is yet unclear.

1.3.2.5.5 Complement receptor 3 (CR3)

CR3 is a heterodimer constituted by Integrin α_M and Integrin β_2 (also called CD11b/CD18 or Mac-1), which is expressed by macrophages, neutrophils and certain B cells. Apart from binding to the complement component iC3b, CR3 displays multiple binding sites for several endogenous and pathogenic ligands and stimulates cell adhesion, transmigration, and phagocytosis (Linehan et al., 2000). It is of note, that CR3 can also bind to certain pathogen surfaces and to LPS even in the absence of complement factors (Ehlers and Daffe, 1998), thus it shares functions of proinflammatory and phagocytic PRRs. Although CR3 seems to have the potential for signal transduction, full activation is dependent on additional signals by FcR, chemokine receptors or TLRs (Jones et al., 1998).

1.3.2.6 Fc receptors (FcR)

The FcR, with the exception of FcεRII, comprise a still growing family of Ig-SF receptors, which interact with soluble Ig thus connecting the humoral immune response to cellular innate and adaptive responses. The binding of Ig to cells can target them for phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) or mediate activating (1.3.2.6.1)or inhibitory signals (1.3.3).

1.3.2.6.1 Activation via the ITAM-containing FcR gamma chain (FcRγ)

Similar to the BCR and the TCR, the FcR has a short cytoplasmic domain devoid of any signaling motifs, thus requiring accessory proteins for signal transduction. FcγRI, FcγRIIA, FcγRIIIA, FcεRI and FcαR are associated with FcRγ, which contains one immunoreceptor tyrosine-based activation motifs (ITAMs) (Ravetch and Bolland, 2001; Reth, 1989). In general, upon receptor stimulation ITAMs are phosphorylated by PTKs of the Src family in particular Lck (Ravetch and Bolland, 2001; Sicheri and Kuriyan, 1997; Weil and Veillette, 1996). This leads to the recruitment and association of Lck and Syk to the receptor paralleled by a strong increase of their kinase activity. A central substrate of these kinases in T cells (Lin and Weiss, 2001) but also in myeloid cells (Bottino et al., 2000; Tridandapani et al., 2000) is the linker of activated

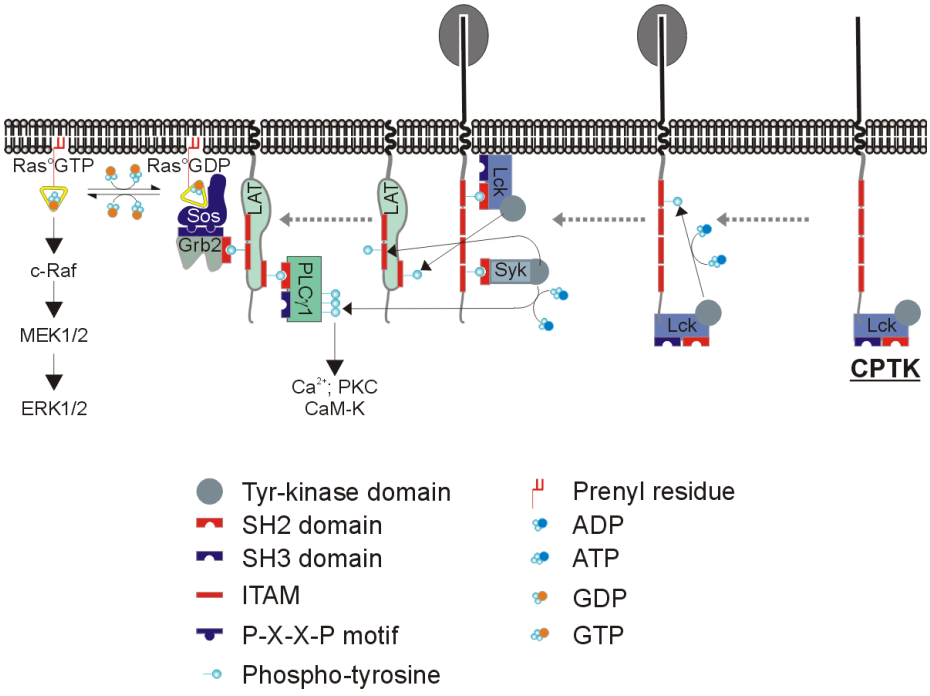

Figure 1.12: Activation of Protein tyrosine kinases recruited to ITAM-containing receptors

T cells (LAT), which upon phorphorylation becomes a platform for PLCγ and Grb-2 (Lin and Weiss, 2001). Grb-2 recruits Sos via two SH3 domains and leads to the acti-

vation of the Ras/c-Raf/MAPKK pathway (Figure 1.12) (Lin and Weiss, 2001). Interestingly, genetic disruption of FcRγ caused animals to become resistant to anaphylaxis (due to impaired mast cell activation via FcεRI), concurrent with defects in ADCC and phagocytosis consistent with the participation of FcRγ in other receptor complexes (Clynes and Ravetch, 1995; Takai et al., 1994).

1.3.2.7 Other activating receptors associated with FcRγ or DAP10

1.3.2.7.1 ILT1/FcRγ

Recently, a novel FcRγ-interacting receptor expressed on monocytes and macrophages called ILT1 was identified (Nakajima et al., 1999). Signal transduction of this receptor closely resembles FcγRI-mediated signaling, suggesting that FcRγ is exclusively responsible for the signal transduction whereas the associated receptor mediates ligand recognition and binding.

1.3.2.7.2 NKG2D/DAP10

A novel signal transducing subunit called DAP10 was reported in association with a cell surface receptor termed NKG2D. The NKG2D/DAP10 complex is a receptor for the stress-inducible and tumor-associated MHC molecule MICA (Wu et al., 1999). In addition, DAP10 is expressed in T cells and monocytes (Chang et al., 1999). DAP10 binds PI(3)K following phosphorylation of a cytoplasmic YINM motif, which results in activation of Akt (Chang et al., 1999; Wu et al., 1999). In addition, DAP10 binds adapter protein Grb2 subsequently leading to activation of the Ras/c-Raf/ERK pathway (Chang et al., 1999; Egan et al., 1993). The role of NKG2D/DAP10 in innate responses is presently unknown.

1.3.3 Inhibiting receptor systems

ITAM-mediated activation signals can be counter-regulated by receptors containing a 13 amino acid consensus sequence termed immunoreceptor tyrosine-based inhibitory motif (ITIM) (Amigorena et al., 1992; Muta et al., 1994; Scharenberg and Kinet, 1996). ITIM-containing receptors are members of the FcR, Killer cell Ig-like receptor (KIRs) and Ig-like transcripts (ILTs) family of surface receptors (Colonna et al., 2000; Ravetch and Bolland, 2001). Clustering of ITIM-bearing inhibitory receptors with activating receptors results in the phosphorylation of ITIMs by PRKs and subsequent recruitment of SH2-containing phosphatases, such as SHP-1, -2 or SHIP (Scharenberg and Kinet, 1996). These phosphatases can act on phosphorylated tyrosine residues thus terminating the required signals for the activation of downstream pathways (Figure 1.12). The important role for counter regulating phosphatases is underlined by the

phenotype of SHP-1-deficient motheaten mice displaying acute inflammations throughout the body as a consequence of uncontrolled immune activation (Zhang et al., 2000).

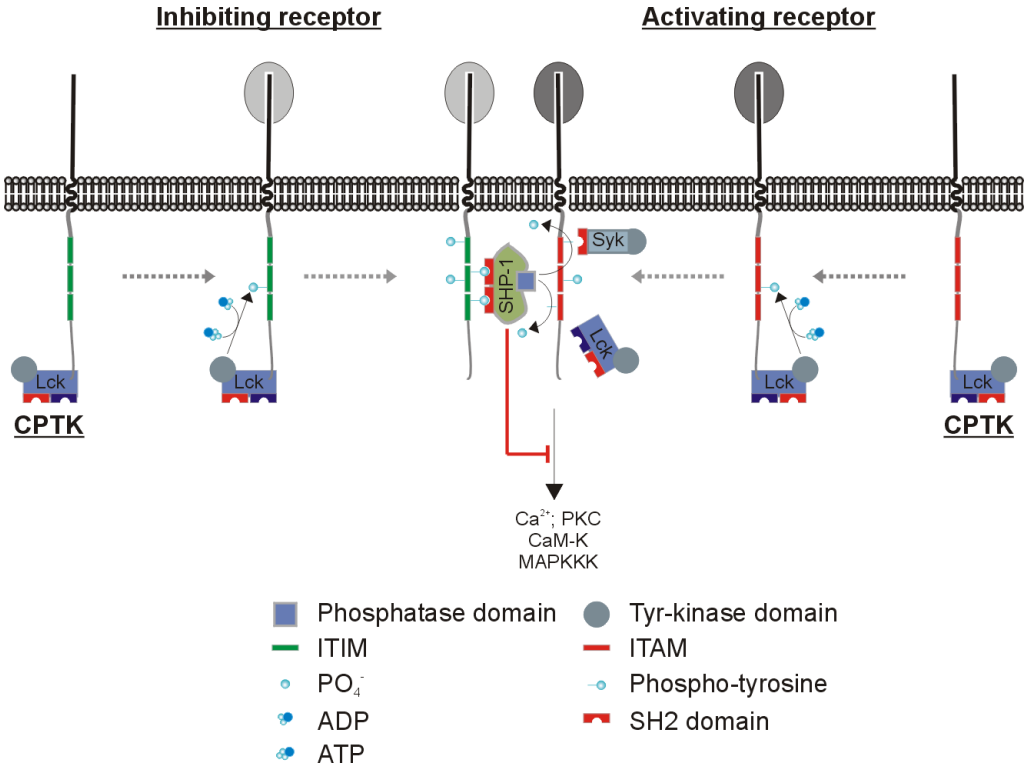

Figure 1.13: Counter-regulation of ITAM-containing receptors by inhibitory receptors

1.3.4 The molecular basis for cell death and survival

1.3.4.1 Necrosis and Apoptosis

During development cell death and proliferation are equally important. In the early 1970s Kerr, Wyllie and Currie recognized that there are in principle to types of cell death: necrosis and apoptosis (Kerr et al., 1972; Wyllie et al., 1981).

Necrosis is mostly due to cell damage caused by toxic agents or injury. Due to the high cellular osmolarity, damage to the plasma membrane ultimately leads to strong water influx culminating in cell swelling (oncose) and finally cell burst with the release of lysosomal and cytosolic proteases into the intercellular space. These proteins induce massive tissue damage thus necrosis is often paralleled or followed by inflammatory responses (Trump et al., 1981).

Apoptosis, or programmed cell death is the physiological form of cell death mediated by a tightly regulated set of receptors, intracellular proteins and proteases. Apoptosis is not only responsible for the removal of excessive cell populations and senescent cells, but also for the elimination of virus-infected and malignant cells (Thompson,

1995). In contrast to necrosis, the highly specific proteins involved during apoptosis induce defined molecular modifications followed by characteristic morphological changes such as chromatin condensation, nucleus fragmentation, and the formation of membrane-encapsulated cell fragments, called apoptotic bodies (Kerr et al., 1972). The most significant biochemical changes include the fragmentation of nuclear DNA by activated endonucleases (Arends et al., 1990; Cohen and Duke, 1984; Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1997; Sakahira et al., 1998; Wyllie et al., 1981), the loss of plasma membrane polarity and integrity (Duvall et al., 1985; Fadok et al., 1992a; Fadok et al., 1992b; Savill et al., 1990), and the disruption of the mitochondrial membrane potential ($\Delta\Psi m$) (Kroemer and Reed, 2000).

1.3.4.2 Death receptor-induced apoptosis

The discovery of CD95 (Fas/APO-1) revealed for the first time a surface receptor capable of inducing apoptosis (Itoh et al., 1991; Oehm et al., 1992; Trauth et al., 1989; Yonehara et al., 1989). CD95 together with TNF-R1, DR3, TRAIL-R1, TRAIL-R2 and DR6 constitute the group of death receptors (DRs) (Peter et al., 1999; Schmitz et al., 2000), a subfamily of the TNF/NGF-SF (Baker and Reddy, 1998; Smith et al., 1994). DRs are characterized by an 80 amino acid intracellular death domain (DD) essential for the induction of apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). Upon DR-oligomerization by specific death-inducing ligands, a so-called ”death inducing signaling complex” (DISC) is assembled (Kischkel et al., 1995) (Figure 1.14). The DISC contains the adapter protein FADD/Mort1 and the pro-caspase-8 or a 10 (Kischkel et al., 2001; Scaffidi et al., 1999). Caspase-8 activation occurs via autoproteolysis and induces the cleavage and activation other caspases in-

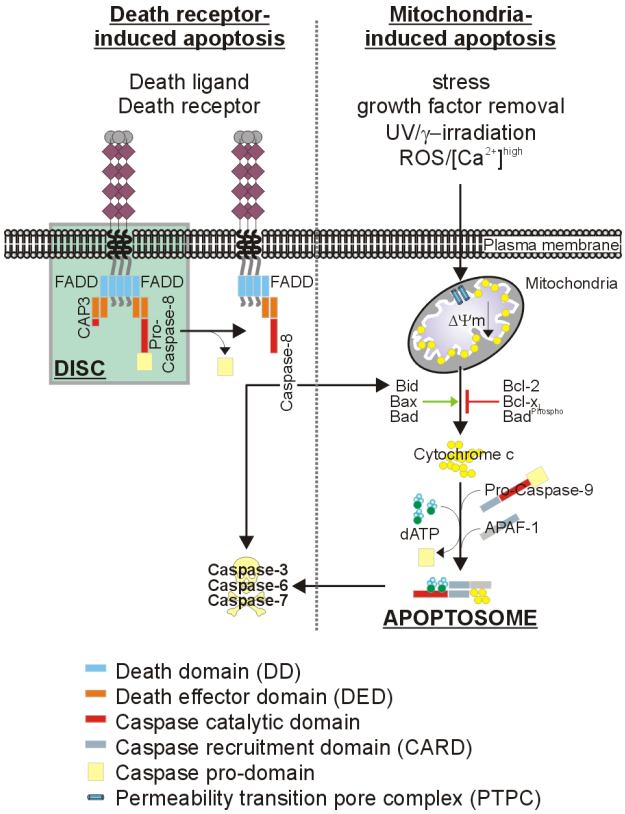

Figure 1.14: Death receptor- and mitochondria-mediated pathways to apoptosis

cluding Caspasen-3, -6 and -7. This cascade ultimately leads to the cleavage of target proteins and nuclear DNA essential for apoptosis and also responsible for the morphological changes observed (Scaffidi et al., 1999) (Figure 1.14). It is of note that Caspase-$8^{-/-}$-cells are resistant to CD95-, TNF-RI-, DR3-, and TRAIL-R2-mediated apoptosis (Varfolomeev et al., 1998) indicating caspase-8 as the central player during DR-induced apoptosis.

1.3.4.3 The mitochondrial pathway to apoptosis

Mitochondria play a crucial role during apoptosis (Kroemer and Reed, 2000). The loss of $\Delta\Psi m$ is one of the earliest hallmarks of apoptosis and can be detected even before DNA-fragmentation (Petit et al., 1995; Zamzami et al., 1995). As shown in Figure 1.14, early after induction of apoptosis, mitochondria permeability transition (PT) leads to the loss of mitochondrial membrane potential ($\Delta\Psi m$). PT is induced by the permeability transition pore complex (PTPC), a channel in the inner mitochondrial membrane (Bernardi et al., 1994; Petit et al., 1995; Petronilli et al., 1994; Zamzami et al., 1995). The opening of the PTPC may be responsible for a massive water influx to the mitochondrial matrix culminating in the burst of the outer membrane and the release of mitochondrial proteins (Green, 2000), such as cytochrome c (Kluck et al., 1997; Liu et al., 1996), pro-Caspase-2, -3, -9 (Mancini et al., 1998; Susin et al., 1999a), apoptosis-inducing factor (Susin et al., 1999b) and SMAC/Diabolo (Du et al., 2000; Verhagen et al., 2000) from the mitochondria to the cytosol can be observed. Together with the cytosolic factors Apaf-1 and dATP, cytochrome c and pro-Caspase-9 form a protein complex called apoptosome (Green, 2000; Hengartner, 2000), which facilitates the autoproteolytic cleavage of pro-caspase-9 leading to subsequent activation of effector caspases and execution of apoptosis (Li et al., 1997; Srinivasula et al., 1998; Yang et al., 1998; Zou et al., 1997). The integrity of mitochondria is regulated by members of the Bcl-2 family. Whereas anti-apoptotic familiy members including Bcl-2 and Bcl-x_L are able to protect mitochondria against various apoptotic and cytotoxic stimuli, some pro-apoptotic members such as Bax, Bad, Bim or Bid are able to induce cytochrome c release, dissipation of $\Delta\Psi m$, and subsequent apoptosis (Adams and Cory, 1998; Green, 2000; Gross et al., 1999; Hengartner, 2000; Huang and Strasser, 2000; Kroemer and Reed, 2000). Bad, in particular, was demonstrated to be important for the balance between cell death and survival (Yang et al., 1995). Induction of Bad phosphorylation on multiple serine residues influences its subcellular distribution, from an association with Bcl-x_L at the mitochondria, to a cytosolic location, associated with 14-3-3 (Zha et al., 1996). The association of Bad with Bcl-x_L is mediated through dimerization of conserved Bcl-2 homology domains (BH) (Kelekar et al., 1997; Zha et al., 1997). Phosphorylation of residues in proximity to the BH3 domain

of Bad may alter the affinity of Bad for Bcl-x_L, promoting dissociation. This relieves Bcl-x_L, thus protecting cells from apoptosis by keeping mitochondrial integrity (Hengartner, 2000). Bad phosphorylation at two specific residues, serine 112 (Ser^{112}) and serine 136 (Ser^{136}) (Zha et al., 1996) and subsequent induction of cell survival can be induced by various cytokines and survival factors (Datta et al., 1997; del Peso et al., 1997; Scheid and Duronio, 1998; Zha et al., 1996). Recent work has illustrated the importance of PI3K-dependent activation of Akt (Burgering and Coffer, 1995; Franke et al., 1995), ERK (Scheid and Duronio, 1998; Scheid et al., 1999; Xia et al., 1995), Protein kinase A (PKA) and p90RSK (Bonni et al., 1999; Harada et al., 1999; Tan et al., 1999) in Bad phosphorylation and cell survival. Thus, the regulation of mitochondrial integrity is central in the mechanism to modulate cell death and survival.

1.3.4.4 Regulation of neutrophil survival during resolution of inflammation

How neutrophil survival and apoptosis during inflammation is regulated is poorly understood. Interestingly, mice carrying spontaneous mutations in the genes for fas ligand (B6/gld) or fas (B6/lpr) show no dysregulation of the lifespan of neutrophils during acute inflammatory responses. This indicates that Fas/FasL system is not involved in the control of neutrophil apoptosis (Fecho et al., 1998; Fecho and Cohen, 1998). The role of TNF-α during neutrophil homeostasis is controversial: some reports suggest TNF-induced neutrophil survival (Dunican et al., 2000; Grey et al., 1996), whereas others imply TNF-mediated apoptosis (Avdi et al., 2001). In an elegant study Dippert and coworkers (Dibbert et al., 1999) show that neutrophils deficient for Bax, a pro-apoptotic member of the Bcl-2 family (Adams and Cory, 1998), are more resistant to apoptosis during inflammation. Thus, it is more likely that the mitochondrial apoptosis pathway regulates neutrophil apoptosis during inflammation, rather than death receptor engagement. In conclusion, the balance between neutrophil survival, apoptosis and necrosis in inflamed tissues may be an important determinant of the degree of tissue injury and the resolution of inflammation. The molecular basis for this process has yet to be defined.

1.4 DAP12, a novel ITAM-bearing protein associated with activating NK cell receptors

1.4.1 Identification and characterization of DAP12

1.4.1.1 Characterization of DAP12 and its signal transduction properties

MHC class I molecules are expressed on the surface of almost all cells in the body. Only upon viral infection or in tumor cells MHC class I can be downregulated to escape the $CD8^+$-mediated cellular cytotoxicity. However, the absence of MHC class I is detected by NK cells in a process known as recognition of "missing self" (Ljunggren and Karre, 1990). This is mediated by cell-surface receptors, which, on binding MHC class I molecules transduce inhibitory signals that block NK-cell-mediated target cell lysis. When MHC class I expression is lost or reduced, NK cells are released from inhibition and thus, can rapidly identify and kill virally infected or transformed cells.

NK cells monitor the expression of MHC class I molecules using various receptors. Cloning of the receptors revealed remarkable diversity (Lanier, 1997; Yokoyama, 1995). Two receptor families have been identified so far: the C-type lectin-like molecules and receptors belonging to the Ig-SF known as human killer-cell immunoglobuline receptors (KIRs). Intriguingly, some MHC class I-binding NK cell receptors promote rather than inhibit target cell lysis. This difference is paralleled by heterogeneity in the cytoplasmic domains of the receptors. Inhibitory receptors have a long cytoplasmic tail that contains ITIMs. In contrast, activating NK cell receptors have short cytoplasmic domains that lack either ITIMs or other signaling motifs. These truncated receptors transmit stimulatory signals to NK cells by activating PTKs and PLCγ, thus leading to NK-mediated cytotoxicity (Biassoni et al., 1996; Houchins et al., 1997; Mason et al., 1996; Moretta et al., 1995). The molecular basis for the signal transduction was unclear for a long time. However early biochemical studies revealed that the activating NK cell receptor KIR2DS receptor is noncovalently associated with a 12 kDa protein, that exists as a disulfide-linked dimer (Olcese et al., 1997). Finally, Lanier and coworkers identified DAP12, an ITAM-bearing transmembrane protein with a predicted molecular mass of 12 kDa that strikingly resembles the FcR-associated FcRγ chain and the TCR-associated CD3ζ polypeptide (Lanier et al., 1998b). The cytoplasmic tail of DAP12 contains a single ITAM, which couples the engagement of associated surface receptors to PTK-dependent pathways. Upon ITAM phosphorylation, DAP12 recruits the SH2-tandem PTKs Syk and/or ZAP70 (Lanier et

al., 1998b; McVicar et al., 1998) (Figure 1.15). *In vitro* studies have shown that phosphorylation of both tyrosine residues present in the ITAM motif are mandatory for the recruitment and activation of Syk and ZAP-70. These results are supported by the low affinity of each SH2 domain for single tyrosine-phosphorylated hemi-ITAM, as well as by the spatial arrangement of the N and C terminus SH-2 domain of ZAP70 that precisely fits the length of the spacer between each of the tyrosine residues in the ITAMs (Chu et al., 1998). Along this line it was shown that a single mutated tyrosine residue in DAP12's ITAM prevents DAP12-dependent cell activation (Tomasello et al., 1998). Syk appears to be the central kinase responsible for DAP12-mediated activation of NK cells (Brumbaugh et al., 1997; McVicar et al., 1998; Tomasello et al., 1998). However, NK cells derived from $Syk^{-/-}$ or $ZAP^{-/-}$ mice as well as from ZAP-deficient patients have been reported to be fully competent for natural cytotoxicity (Colucci et al., 1999; Elder et al., 1994; Negishi et al., 1995). Thus, *in vivo* Syk and ZAP-70 sub-serve redundant functions in NK cells. Syk and/or ZAP-70 further lead to the activation of several downstream signals such as intracellular calcium mobilization, the activation of PLCγ and the MAPK ERK1 and 2 (Campbell et al., 1998; Lanier et al., 1998a; Lanier et al., 1998b; McVicar et al., 1998).

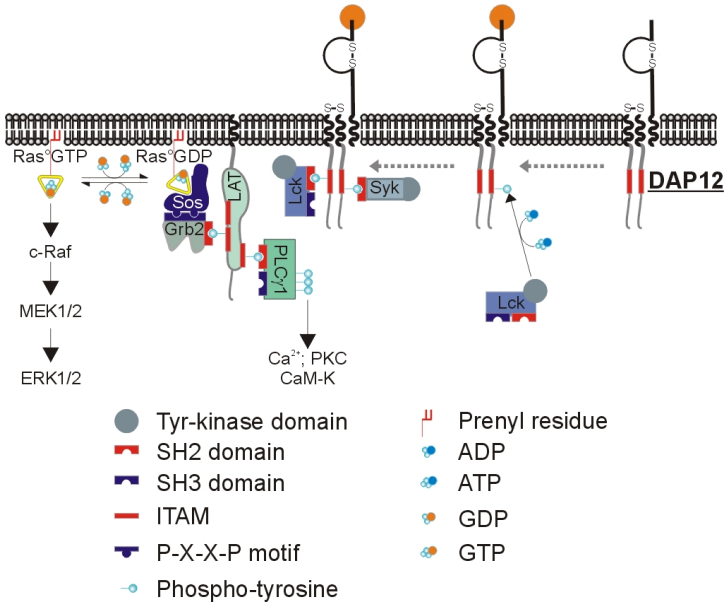

Figure 1.15: DAP12-mediated signaling pathways in NK cells

1.4.1.2 DAP12-associated receptors in NK cells and myeloid cells

Very soon after the discovery of DAP12, it was shown that many NK cell receptors associate with DAP12. These include KIR2DS and NKp44 in humans, as well as Ly49D, Ly49H, and Ly49P in the mouse (Campbell et al., 1998; Makrigiannis et al., 1999; Mason et al., 1996; Mason et al., 2000; Tomasello et al., 1998). In both humans and mice, DAP12 also associates with the CD94/NKG2C receptors for the HLA-E and Qa-1 MHC class Ib molecules, respectively (Lanier et al., 1998a; Vance et al., 1998).

Interestingly, DAP12 was not only detected in NK cells but also in myeloid cells, such as monoytes, macrophages, dendritic cells, and granulocytes (Lanier et al., 1998b; Tomasello et al., 1998). This suggests that not yet identified DAP12-associated receptors may also be involved in activation of these cell types.

1.4.2 Phenotype of Knock-in mice bearing a nonfunctional DAP12 and DAP12-deficient mice

1.4.2.1 Defects in NK cells

In mice, DAP12 was shown to be essential for the surface expression of Ly49D and Ly49H (Smith et al., 1998). Interestingly, surface expression of these receptors was not impaired in Knock-in mice bearing a nonfunctional mutation within the ITAM of DAP12 ($DAP12^{loss\text{-}of\text{-}function\ (lof)}$ knock-in mice) (Tomasello et al., 2000) but was clearly reduced in $DAP^{-/-}$ mice (Bakker et al., 2000), However, Ly49D- and Ly49H-mediated NK cell cytotoxicity was eradicated from both genetically engineered mice (Bakker et al., 2000; Tomasello et al., 2000). These results indicate that DAP12 is indeed required for the surface expression of associated activating receptors (Smith et al., 1998) and that the ITAM is essential for NK cell mediated cytotoxicity.

The mechanisms that govern the shaping of the NK cell repertoire of Ly49 molecules are unclear. It has been proposed that activating NK receptors may contribute to the regulation of inhibitory NK receptor expression on NK cells. Interestingly, no difference in total NK cell numbers and in the cell surface expression of the inhibitory receptors Ly49A, Ly49C, Ly49I, Ly49G2 and NKG2A/NKG2C could be observed in $DAP12^{-/-}$ and $DAP12^{lof}$ knock-in mice. Therefore, the DAP12-associated activating Ly49 receptors are not required for normal expression of inhibitory Ly49 isoforms (Bakker et al., 2000; Tomasello et al., 2000).

1.4.2.2 Defects in myeloid cells

Surprisingly, $DAP12^{-/-}$ and $DAP12^{lof}$ knock-in mice showed several defects in the myeloid cell compartment. $DAP12^{lof}$ knock-in mice showed a dramatic accumulation of DCs in muco-cutaneous epithelia and the skin. In addition, they were resistant to hapten-specific contact sensitivity (CS) (Tomasello et al., 2000) CS is a T cell mediated inflammatory skin reaction in response to topical application of haptens, which are captured by dermal DCs and Langerhans cells. These cells migrate to the paracortical area of the draining LN and prime hapten-specific T cells. Challenge of hapten-sensitized mice with the same hapten at a remote skin site (e.g. ear) induces recruitment of hapten-specific $CD8^+$ T cells, which initiate skin inflammation (Kehren et al.,

1999). In addition, DAP12-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE) induced by immunization with myelin oligodendrocyte glycoprotein (MOG) peptide (Bakker et al., 2000). These phenotypes suggested a role of DAP12 in either modulating inflammatory responses or regulating migration and antigen presentation capacity of DCs or other APCs.

1.4.3 Phenotype of humans bearing a loss-of-function mutation in DAP12

Recently, Paloneva and coworkers have reported a cohort of patients in Finland and Japan that lacks functional DAP12 genes (Paloneva et al., 2000). These individuals suffer of Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), also known as Nasu-Hakola disease (Verloes et al., 1997). PLOSL is a recessively inherited disease characterized by a combination of psychotic symptoms rapidly progressing to presenile frontal-lobe dementia resulting in death prior to 50 years of age. In addition, these patients suffer from bone cysts restricted to wrists and ankles. PLOSL has a global distribution, although most of the patients have been diagnosed in Finland and Japan, with an estimated population prevalence of 2×10^{-6} (Verloes et al., 1997) in the Finns. A shared 153-kb ancestor haplotype in all Finnish disease alleles between markers *D19S1175* and *D19S608* on chromosome 19q13.1 has been previously identified and the molecular defect of PLOSL was identified as one large deletion in all Finnish PLOSL alleles and another mutation in a Japanese patient, both representing loss-of-function mutations, in the DAP12 gene (Paloneva et al., 2000). In the Finnish patients, the absence of DAP12 was confirmed by Northern and Western blot analysis (Paloneva et al., 2000). Consistent with results obtained in DAP12-deficient mice and $DAP12^{lof}$ knock-in mice, these patients have normal NK cells numbers and show no impairment in their lytic activity against certain NK sensitive target cell lines such as K562 (Bakker et al., 2000; Paloneva et al., 2000; Tomasello et al., 2000). So far, no severe immunodeficiencies have been reported in these patients and the predominant pathologies that arise in the bones and CNS but are of late onset. It is likely that these defects result from the deficiency of DAP12 in osteoclasts and microglial cells, respectively, or in other myeloid cells patrolling these tissues and controlling tissue homeostasis. This might explain the late onset of disease in these patients. It is not currently known, whether PLOSL patients are more resistant to acute onset of inflammatory, allergic or autoimmune pathology, as in the case of EAE mice.

1.5 Aim of the thesis

DAP12 is not only expressed on NK cells but also on myeloid cells (Lanier et al., 1998b; Tomasello et al., 1998). Recent literature demonstrates that mice and humans functional-deficient for DAP12, show several defects in myeloid cell function in several tissues (Bakker et al., 2000; Paloneva et al., 2000; Tomasello et al., 2000). These findings indicate that yet unknown DAP12-associated receptors exist in myeloid cells and most probably regulate their activation.

Thus, the aim of this thesis is to identify, clone, and characterize novel DAP12-associated receptors expressed on myeloid cells and to study their role in immune responses.

2 MATERIAL AND METHODS

2.1 Biochemistry

2.1.1 Basic biochemical methods

2.1.1.1 Determination of Protein concentration

2.1.1.1.1 Bradford assay

Different volumes (1 – 10 µl) of the protein sample were placed in a 96-well plate (Costar) and 200 µl Bradford reagent (100 mg Coomassie G-250, 50 ml 95 % Ethanol, 100 ml conc. H_3PO_4, filled up to 200 ml with ddH_2O, filter solution (0.22 µm filter, Millipore) was added. For the generation of the standard curves, aliquots of 1, 2, 4, 8, and 16 µg of γ–Globulin and BSA were used. Spectrometric analysis was performed with a Spectramax 250 (Molecular Devices) at 595 nm wavelength employing the Softmax software.

2.1.1.1.2 Bicinchioninic acid protein assay

In the presence of detergents like Nonidet P (NP)-40, Triton-x (Tx)-100 or Digitonin (DIG), the Bradford assay was substituted by the Bicinchioninic acid (BCA) protein assay (Pierce) to circumvent high background reactivity. 200 µl of BCA reagent was added to sample and standard samples and the reaction allowed to develop for 30 min at 37°C. Spectrometric analysis was performed at 562 nm.

2.1.1.1.3 Spectrometric analysis at 280 nm wavelength

For fast assessment of protein concentrations during protein purifications, protein samples were analyzed for absorbance at 280 nm compared to a blank containing the cognate buffer. For mAb and soluble IgG fusion proteins an OD_{280} of 1.4 corresponds to 1 mg/ml of protein.

2.1.1.2 SDS-PAGE

For SDS-PAGE the Protean III mini-gel system (Biorad) was used. Running gel components were mixed at the percentage of acryl amide requested according to Table 2.1, adding the TEMED last. The poured gels were overlaid with water-saturated Isobutanol, which was removed after gel polymerization by washing with ddH_2O. Stacking gel was poured and the comb inserted immediately without bubbles. Before loading the sample, slots were rinsed with syringe and needle. Samples containing 1 x Re-

ducing Sample Buffer (1 x RSB: 50 mM Tris-HCl pH 6.8, 2 % SDS, 0.01 % (w/v) Bromphenolblue, 30 % Glycerol) were boiled (10 min, 95°C), centrifuged briefly and applied to the slots. Empty slots were loaded with 1 x RSB. In case of reducing SDS-PAGE, 10 µl 1 x RSB was added onto the molecular weight markers (Amersham). Running conditions: constant voltage (70 V: stacking gel and 80-90 V: running Gel).

Table 2.1: Gel composition for 1 SDS-PAGE Mini gel.

All volumes in ml	15 %	12 %	10 %	8 %
ddH_2O	1.1	1.6	1.9	2.3
30% Acrylamid mix (Biorad)	2.5	2.0	1.7	1.3
1.5 M Tris-HCl pH 8.8	1.3	1.3	1.3	1.3
10 % SDS (in water)	0.05	0.05	0.05	0.05
10 % Ammoniumpersulfate (in water)	0.05	0.05	0.05	0.05
TEMED	0.002	0.02	0.02	0.02

2.1.1.3 Coomassie staining

After SDS-PAGE the gel was placed for 24 h in 50 ml NOVEX staining solution (Invitrogen/NOVEX) under agitation at RT, and then destained with ddH_2O, that was exchanged several times. Gels were then dried by vacuum at 60°C.

2.1.1.4 Silver staining

After SDS-PAGE, the gel was fixed in MeOH/CH_3COOH/H_2O (v/v/v: 45:10:45) for 20 min. After rinsing the gel with H_2O for 60 min to remove acid, the gel was incubated for 90 s in 0.02% (w/v) $Na_2S_2O_3$ (sensitizing solution). The sensitizing solution was discarded and the gel rinsed with two changes of H_2O (1 min each). After incubating the gel for 20 min in 0.1 % (w/v) AgNO3 at 4°C the Gel was rinsed again as described above. For development, the gel was placed in 0.04% (v/v) Formalin, 2 % (w/v) Na_2CO_3. Development was quenched with 1 % CH_3COOH when sufficient staining was obtained. Silver stained gel is stored in the fridge

2.1.1.5 Autoradiography

After SDS-PAGE, the gel was placed on a 3MM paper (Whatman), quickly covered with plastic foil (Alio), dried at 60°C under vacuum for 2 h and subjected to autoradiography using exposure of KODAK MR films for 1 up to 21 days.

2.1.1.6 Semi-dry Protein transfer

Before transfer Hybond PVDF membrane (Amersham) (dimension 6 x 9 cm) was first washed 1 min in Methanol and 1 min in water, 5 min with fresh water and 15 min in 1 x Transfer buffer (TB) (10 x TB: 390mM Glycine, 480mM Tris base (pH_{final}: 8.5), 0.375 % (w/v) SDS; 1 x TB: 10 % (v/v) 10x TB, 20 % (v/v) Methanol, 70% (v/v) wa-

ter). After SDS-PAGE the gel was washed in TB for 15 min. For transfer a sandwich was built up on the anode containing 3 x 3MM paper (soaked in TB), PVDF membrane, gel, 3 x 3MM paper (soaked in TB). The cathode was fitted and the transfer performed for 45 min at constant current (17 A/gel).

2.1.1.7 Western Blot analysis

After the transfer, the membrane was placed for ≥ 1 h in blocking solution (BS) (PBS/0.1 % (v/v) Tween-20/5 % (w/v) Skim milk (Biorad) and subsequently incubated with the first mAb at a concentration of 10 µg/ml in BS for 2 h at RT. The membrane was washed 6 times with washing buffer (WB) (50 ml PBS/0.1 % (v/v) Tween-20) before a second horseradish peroxidase (HRP)-labeled Ab (dilution 1:500 – 1:100000) in BS was added for 30 min at RT. The membrane was washed again before incubating it in Enhanced Chemoluminescence Solution (ECL, Amersham) for 1 min. Excess fluid was removed from the membrane with a paper towel, the membrane placed in transparent foil (Kaliak) and exposed to ECL-film (Kodak), for different time periods. To detect, protein phosphorylation, phosphatases were blocked by adding $Na_2V_2O_5$ at a concentration of 10 µg/ml to all solutions.

2.1.2 Protein production, purification and modification

2.1.2.1 Production, purification and modification of BirA-tagged proteins

For expression of *in vivo* biotinylated TREM-1-BirA^{tag} fusion protein, competent *E. coli* strain AVB101 (Avidity) was transformed with the respective plasmid TREM-1/pAC-4, and grown in 5 x 400 ml of LB-Medium containing Ampicillin (100 µM) and Chloramphenicol (10 µg/ml) in a 2-litre flask at 37°C for 4h. AVB101 contain an isopropyl β-thiogalactoside (IPTG) inducible *birA* gene in a pACYC184 plasmid encoding a biotin ligase capable of biotinylating a particular Lysine residue in the BirA consensus sequence. After induction with 0.5 mM IPTG and addition of 50 µM D-Biotin cells were incubated for additional 8 h, harvested and pelleted. The pellet was suspended in 40 ml of 10 mM HEPES, pH 7.4, 150 mM NaCl containing protease inhibitors (complete, Roche) and lysed with an EmulsiFlex high-pressure homogenizer (G. Heinemann Ultraschall- und Labortechnic). After centrifugation surplus of D-Biotin was removed by dialyzing against 10 mM HEPES, pH 7.4, 150 mM NaCl. Purification of the biotinylated TREM-1-BirA fusion protein was attempted using Sepharose-4B-immobilized Avidine (Pierce). Lysates were pre-cleared using 1 ml Sepharose-4B (Pharmacia) for 2 h at 4°C, followed by 4 h incubation with 0.5 ml Sepharose-4B-immobilized Avidine. The resin was washed two times with 50 ml

10 mM HEPES, pH 7.4, 150 mM NaCl, two times with 50 ml 10mM HEPES, pH 7.4, 2 M NaCl, two times with 10 mM HEPES, pH 7.4, 5 M NaCl, and finally once with 10 mM HEPES, pH 7.4, 150 mM NaCl. The amount and purity of biotinylated TREM-1-BirA coupled to Sepharose-4B-immobilized Avidine was determined by elution of 10 µl of loaded beads with reducing sample buffer, subsequent SDS-PAGE and Coomassie Staining. The average loading was ~ 1mg TREM-1-BirA/1ml Sepharose-4B-immobilized Avidine. Proper biotinylation of the purified TREM-1-BirA was measured by probing Western Blots with HRP-conjugated Streptavidine. For Immunization 200 µl beads (≅ 200µg TREM-1-BirA) were mixed with 200 µl Freund complete adjutants (FCA). To generate biotinylated TREM-1-BirA monomers, the resin was eluted with 500µM D-Biotin solution and recovered biotinylated TREM-1-BirA was dialyses against PBS.

2.1.2.2 Production, purification and modification of huIgG fusion proteins

cDNAs encoding TREM-1-huIgG1, TREM-2-huIgG1, TREM-4-huIgG1, TREM-5-huIgG1, mTREM-1-huIgG1, mTREM-2-huIgG1, or control fusion protein (ILT3-huIgG1 (Cella et al., 1997)), were transfected in mouse myeloma cell line J558L by electroporation at 240mV, 1050µF, and plated in Selectionmedium I (2.2.1.5) in 96-well plates. Supernatants from transfectants were screened by ELISA using anti-huIgG1 as capturing Ab and mouse-adsorbed HRP-labeled goat-anti-human IgG1 (Southern Biotechnologies Associates (SBA)) as detecting antibody. Producing transfectants were sub cloned and expanded. Purification of huIgG1 fusion proteins from culture supernatants (SNs) was performed by ultrafiltration over a (MWCO: 50 kDa) followed by affinity chromatography on Sepharose-coupled Protein A (Sigma) according to manufacturer's protocols. Purified fusion proteins were dialyzed against PBS and sterilized by 0.22 µm filtration (Millipore). Aliquots of purified fusion proteins were either biotinylated (Roche) or labeled with Cy5 (Pharmacia) according to manufacturer's protocols.

2.1.2.3 Production, purification and modification of huIgM fusion proteins

cDNAs encoding TREM-1-huIgM, TREM-2-huIgM, mTREM-1-huIgM, or mTREM-2-huIgM were transfected in mouse myeloma cell line J558L by electroporation at 250 mV, 960 µF, and plated in Selectionmedium I in 96-well plates. SNs from transfectants were screened by ELISA using anti-huIgM as capturing Ab and mouse-adsorbed HRP-labeled goat-anti-human IgM (SBA) as detecting antibody. Producing transfectants were subcloned and expanded. Purification of huIgM fusion proteins from culture supernatants was performed by ultrafiltration over a 300 kDa-MWCO

membrane (Skan AG) followed by affinity chromatography on Sepharose-coupled mouse anti-human IgM mAb (Sigma) according to manufacturer's protocols. Purified fusion proteins were dialyzed against PBS and sterilized by 0.22 µm filtration (Millipore). Aliquots of purified fusion protein were labeled with Cy5 (Pharmacia) according to manufacturer's protocols.

2.1.2.4 Production and modification of mAb

2.1.2.4.1 Purification of control and anti-TREM mAbs

SN from hybridomas 21C7, 9E2, 37F8 (anti-TREM-1, IgG1, κ), 50D1, 100E1, 1A1 (anti-mTREM-1), 29E3, 21E10, 10B11, 20G2 (anti-TREM-2, IgG1,κ), 64F5, 64A9 (anti-TREM-4, IgG1, κ), 8H6, 11F6 (anti-TREM-5, IgG1, κ), 1B7.11 (control IgG1, κ, anti-2,4,6 TNP, American Type Culture Collection (ATCC), Manassas, VA), and (control IgG1, κ, anti-β2 Integrin, Pharmingen) were purified by affinity chromatography using GammaBind-Sepharose (Pharmacia).

2.1.2.4.2 Biotinylation or Cy5-labeling of mAbs

Purified mAbs were either biotinylated (Roche) or labeled with Cy5 (Pharmacia) according to manufacturer's protocols. In brief, affinity purified mAb were concentrated to 2 mg/ml using Biomax concentrators (MWCO 10000; Millipore) and dialyzed against 100 mM Na_3BO_3 pH 8.8. For biotinylation 1 mg of protein was mixed with 200µg (Roche) (10 mg/ml in DMSO) and the solution was incubated for 30 min at room temperature (RT). The reaction was stopped by adding 20 µl 1 M NH_4Cl. For Cy5-labeling, 1 mg of Protein was mixed with Cy5 labeling reagent and incubated for 30 min at RT. For buffer exchange and removal of free biotin or Cy5, a G50 Gelfiltration column (Pharmacia) was equilibrated with PBS. The protein solution was applied onto the column, eluted with PBS and the first two fractions collected. The amount, specificity and purity of biotinylated mAbs were determined by ELISA and SDS-PAGE/Coomassie Staining. Finally, the protein solutions were sterilized by 0.22 µm ultrafiltration (Millipore) or preserved by adding NaN_3 to a final concentration of 0.05 % (w/v).

2.1.2.4.3 Ficin digest for Production of F(ab') and F(ab')$_2$ mAb fragments

F(ab') or F(ab')$_2$ fragments of mAb 29E3 and mAb 21C7 were prepared using the Fab'/F(ab')$_2$ Kit (Pierce). F(ab') and F(ab')$_2$ were separated from the Fc portion by affinity chromatography on protein G-sepharose, followed by gel filtration on a Superdex 75 HR10/30 (Pharmacia). F(ab') and F(ab')$_2$ preparations were tested for the absence of Fc fragments by ELISA. F(ab') and F(ab')$_2$ fragments were biotinylated

allowing for cross-linking by ExtrAvidine (Sigma) or flow cytometry by Streptavidine-allophycocyanin (APC) or -phycoerythrin (PE) (Pharmingen). Alternatively, F(ab')$_2$ fragments were crosslinked using a F(ab')$_2$ goat anti-mouse IgG F(ab')$_2$ specific antibody (Jackson).

2.1.2.5 Selection and Coupling of Peptides for the production of antisera in rabbits

2.1.2.5.1 Peptides for the production of DAP12 antisera

Table 2.2: Summary of peptides used for immunization of rabbits.

Peptide #/rabbit #	Sequence (NH_2→COOH)	Source of sequence	Application
#DAP12-A/#1370	NH_2-RLVPRGRGAAEAATRKQRITETE-COOH	DAP12 AA66-AA89	DAP12 antisera
#DAP12-B/#1376	NH_2-TRKQRITETESPYQELQGQRSD-COOH	DAP12 AA79-AA101	DAP12 antisera
#DAP12-C/#1378	NH_2-ETESPYQELQGQRSDVYSDLNTQRPYYK-COOH	DAP12 AA77-AA113	DAP12 antisera

2.1.2.5.2 Production and conjugation of immunogenic peptides

Peptides were produced by David Avila using an ABM 430A synthesizer (Applied Biosystems), lyophilized and coupled to Keyhole limped hemocyanin (KLH; Pierce) by Glutaraldehyde (Sigma): 200 µl KLH (6 mg/ml in PBS) were mixed with 80 µl Peptide (10 mg/ml in water). Then Glutaraldehyde was added to a final concentration of 10 mM in 5 steps of 1.2 µl (5% solution in water). After each addition the peptide/KLH mix was incubated at RT for 5 min and finally incubated on ice for additional 30 min. To stop the reaction 8 µl 1 M Glycine pH 8.5 was added. 7 aliquots of 500 µg coupled peptides/1 ml PBS were used for immunization and further aliquots stored at –20°C.

2.1.2.6 Purification and modification of DAP12 antisera from rabbits

CNBr-Sepharose-4B with a capacity of 40 mg peptide/2 ml resin (Pharmacia) was washed 6 x with 50 ml 10 mM HCl and 3 x with 50 ml 500 mM Na_3BO_3 pH 7.8. 2 ml of the CNBr-Sepharose-4B was resuspended in 50 ml 100 mM Na_3BO_3 pH 7.8 and 40 mg of peptide was added. After rotating for 2 h at RT, free CNBr was blocked by adding 3 ml of 1 M Tris-HCl pH 9 for additional 2 h. To remove unspecific binding, the resin was washed 5 x with 1 M Glycine pH 1.8 and 1 M Tris-HCl pH 9. Finally, the peptide/CNBr-Sepharose-4B was washed in RPMI/HEPES until neutralized. 50 ml antiserum was applied on the peptide/CNBr-Sepharose-4B over night at 4°C. After washing the resin with 50 ml PBS and 50 ml 3 M NaCl, bound antibodies were eluted using 100 mM Glycine pH 1.8. Amount, specificity and purity of the antiserum was tested by Bradford assay, ELISA and SDS-PAGE/Coomassie staining. Biotinylation was performed as described in 2.1.2.4.2.

2.1.3 Determination of protein phosphorylation and activation

2.1.3.1 Protein tyrosine phosphorylation, ERK-, JNK, and p38/SAPK activation

$2x10^6$ cells (Monocytes, Neutrophils, or MDCs)/0.5ml were incubated at 37°C with $F(ab')_2$ 21C7 (anti-TREM-1), $F(ab')_2$ 29E3 (anti-TREM-2) or control $F(ab')_2$ (1F11 (anti-β2-Integrin) or 1B7.11 anti-2,4,6 TNP) in the presence of $F(ab')_2$ goat anti-mouse $F(ab')_2$-specific mAb (Jackson) for different time periods. After stimulation, cells were lysed in reducing sample buffer. Specific induction of tyrosine phosphorylation and phosphorylation of ERK, p38/SAPK and JNK was determined by reducing Western Blot analysis using HRP-labeled PY20 anti-phosphotyrosine mAb (Transduction Laboratories), anti-Phospho-ERK$^{Thr202/Tyr204}$, anti-ERK, anti-Phospho-p38/SAPK, anti-p38/SAPK, anti-Phospho-JNK and anti-JNK antibodies (all from New England Biolabs (NEB)).

2.1.3.2 PLCγ phosphorylation and activation

For the determination of PLCγ phosphorylation, $2x10^7$ cells were stimulated as described above and lysed in 1% Tx-100, 100 mM Tris-HCl pH 7.4, 150 mM NaCl, protease inhibitors (Complete, Roche). Lysates were subjected to immunoprecipitation with PY20 mAb at 4°C for 3h. Immunocomplexes were precipitated by addition of protein-G-Sepharose 4B for 1h at 4°C. Precipitates were washed 4 times with lysis buffer, followed by a final wash with 0.5% Tx-100, 100 mM Tris-HCl p H7.4, 150 mM NaCl, and resuspended in reducing sample buffer. Lysates were subjected to SDS-PAGE and Western blot analysis with an anti-PLCγ or anti-Hck mAbs (Transduction Laboratories).

2.1.3.3 IκB-phosphorylation and -degradation

2×10^6 cells/0.5ml were incubated at 37°C with $F(ab')_2$ 29E3 (anti-TREM-2) $F(ab')_2$ 21C7 (anti-TREM-1), or control $F(ab')_2$ mAbs (1F11 anti-β2-Integrin or 1B7.11 anti-2,4,6 TNP) in the presence of $F(ab')_2$ goat anti-mouse $F(ab')_2$-specific mAb (Jackson) as a cross-linker for different time periods. After stimulation, cells were lysed in reducing sample buffer. Specific induction of IκB-degradation and -phosphorylation was determined by reducing Western Blot analysis using anti-IκB (NEB) and anti-phospho-IκB antibodies (NEB). To confirm equal loading blots were incubated for 30 min in a buffer containing 62.5 mM Tris/HCl pH 6.8, 2% SDS and 100 mM β-mercaptoethanol at 60°C. Then blots were washed six times for 10 min in PBS/Tween, blocked with PBS/Tween/ 5% Skim milk and analyzed with HRP-labeled anti-Actin mAb (Sigma).

2.1.3.4 Phosphorylation of Bad, Akt and ERK

2 x 10^6 neutrophils/0.5ml were incubated at 37°C with biotinylated F(ab')$_2$ mAb 21C7 (anti-TREM-1) or control F(ab')$_2$ (29E3 anti-TREM-2, 1F11 anti-β2-Integrin or 1B7.11 anti-2,4,6 TNP) in the presence of ExtrAvidine (Sigma) as a cross-linker for different time periods. After stimulation, cells were lysed in reducing sample buffer and subjected to Western Blot analysis using anti-Phospho-ERK$^{Thr202/Tyr204}$, anti-ERK, anti-Akt^{Thr208}, anti-Akt, anti-Bad^{Ser112}, anti-Bad^{Ser136}, anti-Bad^{Ser155}, and anti-Bad Abs (NEB).

2.1.3.5 Detection and Monitoring of Bad-Bcl-x_L heterodimers

For the determination of Bcl-x_L-Bad heterodimers, 10 x 10^7 neutrophils were stimulated as described in 2.1.3.4 and lysed in 1.2% DIG, 100mM Tris-HCl pH 7.4, 150mM NaCl, protease inhibitors (Complete, Roche). Lysates were subjected to immunoprecipitation with anti-Bcl-x_L mAb (Pharmingen) at 4°C for 3h. Immunocomplexes were precipitated by addition of Protein G-Sepharose 4B (Pharmacia) for 1h at 4°C. Precipitates were washed 4 times with lysis buffer, followed by a final wash with 0.5% DIG, 100 mM Tris-HCl pH 7.4, 150 mM NaCl, and resuspended in reducing sample buffer. Lysates were subjected to SDS-PAGE and Western blot analysis with an anti-Bad Ab and anti-Bcl-x Ab (NEB).

2.1.3.6 Detection of Cytochrome c release from mitochondria

100 x 10^6 neutrophils/time point were incubated at 37°C on plastic-bound F(ab')$_2$ mAb 21C7 (anti-TREM-1) or control F(ab')$_2$ (anti-TREM-2 mAb 29E3). After stimulation for different time periods, cells were harvested by centrifugation at 600 x g for 10 min at 4°C. The cell pellets were washed twice in PBS, resuspended in 160 µl isotonic buffer (250 mM sucrose, 20 mM HEPES, 20 mM KCl, 1,5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) supplemented with protease inhibitors (17 µg/ml PMSF, 8 µg/ml Aprotinin, 2 µg/ml Leupeptin), and allowed to swell during a 20 min incubation period on ice. The cells were gently broken up using a Dounce homogenizer (Kontes Glass company). Small aliquots of the lysate were taken and stained with trypan blue to determine the progression of cell lysis. Homogenization was continued until ≤ 80% of the cells were broken. The homogenate was centrifuged once at 750 x g at 4°C to remove unbroken cells and nuclei. The supernatant was centrifuged at 10.000 x g for 25 min at 4°C to collect the mitochondria-enriched heavy membrane pellet (HM: marked as **Mit** in figure legends). This supernatant was centrifuged at 100.000 x g for 60 min at 4°C to yield the light membrane pellet (LM) and the soluble cytosolic fraction (S100: marked as **Cyt** in figure legends). Protein concentrations of

S100 and HM fractions were determined and 5 µg total protein examined by Western Blot analysis for Cytochrome c using anti-Cytochrome c mAb (7H8.2C12: 1:500; Pharmingen). To confirm equal loading and purity of the cytosolic fraction, western blots were also developed either with an antibody directed against against mitochondrial cytochrome c oxidase (12C4-F12: 1:1000; Molecular Probes, Inc.).

2.1.4 Determination of receptor size and receptor-associated molecules

2.1.4.1 [^{35}S] metabolic labeling, biotinylation and pervanadate treatment

Purified monocytes were incubated in cysteine- and methionine-free DMEM (Sigma) containing 2% dialyzed FCS for 1h. [^{35}S]-labeled cysteine and methionine (Promix, Amersham) were then added at 1 µCi/ml and incubation was continued for 6 h. For surface biotinylation, monocyte-derived DCs were washed three times in PBS followed by incubation with Sulfo-NHS-Biotin according to the manufacturer's protocol (Pierce). For pervanadate treatment, cells were incubated with 200 µM pervanadate and 200 µM H_2O_2 at 37°C for 5 min. Biotinylation or pervanadate stimulation was stopped by washing the cells 3 x in PBS/10% FCS/200 µM pervanadate and 1 x with ice cold PBS/200 µM pervanadate.

2.1.4.2 Immunoprecipitations and Coimmunopreciptiations

Surface-biotinylated, metabolically labeled or pervanadate-treated cells were lysed in 1% DIG, 100 mM Tris-HCl pH 7.4, 150 mM NaCl, protease inhibitors (Complete, Roche). After overnight preclearing with normal mouse serum coupled to GammaBind-Sepharose 4B (Pharmacia), lysates were subjected to Immunoprecipitations (Ips) with 5 µg/ml of 21C7 (anti-TREM-1), 29E3 (anti-TREM-2), 1F11 (anti-MHC class I) or 1B7.11 (anti-2,4,6 TNP) at 4°C for 3 h. Immunocomplexes were precipitated by addition of GammaBind-Sepharose 4B for 3h at 4°C. Precipitates were washed 4 times with lysis buffer, followed by a final wash with 0.5% DIG, 100 mM Tris-HCl pH7.4, 150 mM NaCl.

Proteins bound to GammaBind-Sepharose were eluted using RSB and separated by SDS-PAGE. Co-Immunoprecipitates (Co-Ips) were analyzed by Western blot with anti-phosphotyrosine PY20-HRP (Transduction Laboratories) anti-DAP12, anti-DAP10, or anti-FcRγ rabbit antiserum (kindly provided by Dr. H. Nakajima, National Institute for Longevity Science, Japan) for receptor-associated molecules.

2.1.4.3 Deglycosylation and Determination of receptor size

In deglycosylation experiments Proteins bound to GammaBind-Sepharose were eluted with Deglycosylation buffer (20µl buffer for 20µl GammaBind-Sepharose: 8.5 M Urea, 0.7 % (w/v) SDS, 0.7 % (v/v) β-ME, 600 mM Tris-HCl pH 7.6) and boiled for 5 min. After addition of 20 µl 7.5 % (v/v) NP-40 and 77.5 µl water IPs were incubated with or without N-Glycanase F (3 U), O-Glycanase (0.5 U), Galactosaminidase (3 U) or Sialylase (1 U) (Roche) for 18 h at 37°C. For separation by SDS-PAGE, RSB was added (final concentration 1xRSB) and precipitates were analyzed by autoradiography (lysats from metabolically labeled cells), or by Western Blot with HRP-conjugated streptavidine (lysates from surface biotinylated cells).

2.2 Cellular Biology

2.2.1 Basic cell biological methods

2.2.1.1 Determination of cell number

An aliquot of cell suspension was diluted 1:1 with trypan blue solution (0.05 % w/v) and cells were counted with a Neubauer counting chamber (0.1 mm depth). The number of live cells in 16 small squares multiplied by 2 x 10^4 corresponds to the density in cell/ml.

2.2.1.2 Freezing and thawing of cells

For freezing, cell suspensions were prepared, centrifuged (200 x g, 5 min, 25°C) and the pellet resuspended in Freezing medium (2.2.1.5) at a minimal concentration of 5 x 10^6 cells/ml. 1 ml aliquots in freezing tubes (Costar) were placed in ice-cooled freezing containers and placed for 48 h at –80°C, Tubes were then transferred to liquid N_2 tank.

Cell thawing was performed quickly in a 60°C water bath until a ~ 10% of suspension remained frozen. The suspension was immediately diluted into 10 ml of pre-warmed appropriate medium and centrifuged. Pelletted cells were resuspended in medium and cultured at 37°C, 5 % CO_2.

2.2.1.3 Splitting of adherent cells

The weakly adherent cell lines COS and 293 were resuspended for splitting or freezing merely by pipetting.

Monocyte-derived Dendritic cells (MDCs) or Macrophages (MMϕs) were washed once with PBS, the cell layer covered with Trypsin/0.5 mM EDTA in HBBS (GibcoBRL) and incubated at 37°C for 5-10 min checking the progress of detachment with the microscope- After the appropriate time, trypsin was quenched with 2 volumes of R-Medium, either splitted into new cell culture plates, centrifuged (200 x g, 5 min, 4°C) for freezing or analysed.

In case, MDCs or MMϕs were detached for flow cytometric analysis of surface markers or stimulation experiments, cells were washed once with PBS, the cell layer covered with PBS/0.5 mM EDTA and incubated at 37°C for up to 15 min checking the progress of detachment with the microscope. After appropriate time, cells were

washed 3 times to remove EDTA, centrifuged (200 x g, 5 min, 4°C) and used for analysis.

2.2.1.4 Splitting of cell suspensions

Cells in suspension reaching a density of 1 x 10^6 cell/ml were splitted in a ratio of 1:2 up to 1:10 by adding the appropriate volume of Medium.

2.2.1.5 Media for cell culture of eukaryotic cells

Table 2.3: Summary of cell culture media.

Name	Basic composition	Supplements	Application
R-Medium	RPMI 1640, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS		Culture of human cell lines, neutrophils and monocytes
I-Medium	IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS		Culture of SP2/0 for fusion
I-Medium for mouse cells	IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS	2-Mercaptoethanol (50µM)	Culture of mouse cell lines
Selection medium I	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS	HAT supplement, Mouse IL-6 (2 % SN ≅ 50 ng/ml)	Selection of Hybridomas up to 1 ml cell suspension
Selection medium II	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS	HT supplement, Mouse IL-6 (2 % SN ≅ 50 ng/ml)	Selection of Hybridomas up to 5 ml cell suspension
Selection medium III	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 5 % ultra-low IgG FCS	HT supplement, Mouse IL-6 (2 % SN ≅ 50 ng/ml)	Selection of Hybridomas up to 30 ml cell suspension
Selection medium IV	RPMI 1640, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS	G418 (1 mg/ml)	Culture of cell lines carrying a neomycin-resistance gene
Selection medium V	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 5 % ultra-low IgG FCS	Xanthine (125 µg/ml); Myco-phenolic acid (4 µg/ml)	Culture of cell lines carrying a gpt resistance gene
Production medium I	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 5 % ultra-low IgG FCS		Culture of Hybridomas and IgG fusionprotein producing J558L
Production medium II	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 2 % ultra-low IgG FCS		Culture of Hybridomas and IgG fusionprotein producing J558L
Production medium III	SF-IMDM, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 2 % low-endotoxin FCS		Culture of IgM fusionprotein producing J558L
MDC-Medium	RPMI 1640, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS	IL-4 (2 % SN ≅ 50 ng/ml), GM-CSF (50 ng/ml)	Differentiation of MDCs from human Monocytes
MDM-Medium	RPMI 1640, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % low-endotoxin FCS	M-CSF (50 ng/ml)	Differentiation of MMϕs from human Monocytes
T-Medium	RPMI 1640, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 10 % human serum	IL-2 (2 % SN ≅ 50 ng/ml),	Culture of T cell clones
Labeling medium	RPMI 1640 w/o L-cystein and L-methionin, L-Glutamin (2 mM), Kanamycin (50 µg/ml), 2 % FCS (dialyzed against PBS)	Promix (1 µCi/ml)	Labeling of cells with ^{35}[S]
Freezing medium	90 % low-endotoxin FCS	10 %DMSO	Storage of cells in liquid N_2

All media and supplements were obtained from GibcoBRL, with the exception of low-endotoxin FCS (Hyclone), GM-CSF and M-CSF (R&D Systems), Xanthine (Sigma), Mycophenolic acid (Calbiochem) and IL-2-, IL-4-, IL-6-containing SNs (produced in our laboratory).

2.2.2 Production of monoclonal antibodies (mAb) and antisera

2.2.2.1 Production of mouse mAb

2.2.2.1.1 Production of anti-human TREM-1 mAbs

6-wk-old BALB/c mice (Iffa-Credo) were immunized with Streptavidine-coupled TREM-1-BirA-Biotin (1^{st} immunization: day 0: 200µl TREM-1-BirA-Biotin/Streptavidine-Sepharose (1:1 in FCA) (≅ 100 µg TREM-1-BirA-Biotin)) and TREM-1-huIgG1 (2^{nd} immunization: day 7: 50 µg TREM-1-huIgG1 s.c. (in PBS); 3^{rd} immunization: day 14: 5 µg TREM-1-huIgG1 i.v. (in PBS, sterile)). Spleen cells were fused 3 days after the last immunization with SP2/0 myeloma cells and hybridomas plated in Selectionmedium I in 96-well plates. Hybridoma SNs were screened by ELISA using TREM-2-huIgG1 as capturing protein and human-adsorbed HRP-labeled goat-anti-mouse IgG (PharMingen) as detecting antibody. ELISA-positive SNs were then tested by flow cytometry for staining 293 cells expressing FLAG-tagged TREM-1. Hybridomas producing ELISA- and transfection-positive SNs were expanded up to 1 ml in Selectionmedium II. 500 µl cell suspension were frozen in Freezing medium (2.2.1.5), 10 µl cell suspension was used for subcloning in Selectionmedium III and the remaining cells were adapted to either Selectionmedium III, R-Medium or Productionmedium I (2.2.1.5).

2.2.2.1.2 Production of anti-human TREM-2 mAbs.

6-wk-old BALB/c mice (Iffa-Credo) were immunized with purified TREM-2-huIgM (1^{st} immunization: day 0: 50 µg TREM-2-huIgM s.c. (1:1 in FCA); 2^{nd} immunization: day 10: 50 µg TREM-2-huIgM s.c. (1:1 in Freund incomplete adjutants (FIA)); 3^{rd} immunization: day 17: 20 µg TREM-2-huIgM s.c. (in PBS); 4^{th} immunization: day 23: 5 µg TREM-2-huIgM i.v. (in PBS, sterile)). Spleen cells were fused 3 days after the last immunization with the SP2/0 myeloma cells and selected as described in 2.2.2.1.1. Hybridoma SNs were screened by ELISA using TREM-2-HuIgG1 as capturing protein and human-adsorbed HRP-labeled goat-anti-mouse IgG (PharMingen) as detecting antibody. ELISA-positive hybridoma supernatants were then tested by flow cytometry for staining 293 cells expressing FLAG-tagged TREM-2. Hybridomas producing ELISA- and transfection-positive SNs were treated as described in 2.2.2.1.1.

2.2.2.1.3 Production of anti-human TREM-4 and TREM-5 mAb

6-wk-old BALB/c mice (Iffa-Credo) were immunized with purified TREM-4-huIgG1 or TREM-5-huIgG1 (immunization schema: 2.2.2.1.2). Spleen cells were fused 3 days

after the last immunization with the SP2/0 myeloma cells and selected as described in 2.2.2.1.1. Hybridoma SNs were screened by ELISA using TREM-2-HuIgG1 as capturing protein and human-adsorbed HRP-labeled goat-anti-mouse IgG (PharMingen) as detecting antibody. ELISA-positive hybridoma SNs were then tested by flow cytometry for staining 293 cells expressing FLAG-tagged TREM-5 or HA-tagged TREM-4. Hybridomas producing ELISA- and transfection-positive SNs were treated as described in 2.2.2.1.1.

2.2.2.2 Production of rat anti-mouse TREM-1 mAb

Rat anti-mouse mAb were produced using the Kearney's subtractive immunization method (Kearney et al., 1986). 6-wk-old Wistar rats (Iffa-Credo) were immunized as follows:

Day -3: 1st immunization right footpad: 50 µg huIgG1 s.c. (1:1 in FCA)
Day 0: 2nd immunization right footpad: 50 µg huIgG1 s.c. (1:1 in FIA)
1st immunization left footpad: 50 µg mTREM-1-huIgG1 s.c. (1:1 in FCA)
Day 5: 3rd immunization right footpad: 50 µg huIgG1 s.c. (in PBS)
2nd immunization left footpad: 50 µg mTREM-1-huIgG1 s.c. (1:1 in FIA)
Day 10: 4th immunization right footpad: 50 µg huIgG1 s.c. (in PBS)
3rd immunization left footpad: 50 µg mTREM-1-huIgG1 s.c. (in PBS)
Day 15: 5th immunization right footpad: 50 µg huIgG1 s.c. (1:1 in PBS)
4th immunization left footpad: 50 µg mTREM-1-huIgG1 s.c. (in PBS)
Day 18: 6th immunization right footpad: 30 µg huIgG1 s.c. (in PBS)
5th immunization left footpad: 30 µg mTREM-1-huIgG1 s.c. (in PBS)
Day 21: 7th immunization right footpad: 10 µg huIgG1 s.c. (1:1 in PBS)
6th immunization left footpad: 10 µg mTREM-1-huIgG1 s.c. (in PBS)

Poplitary lymph node cells from the left leg were fused 2 days after the last immunization with the SP2/0 myeloma cells, hybridomas were selected as described under 2.2.2.1.1 and hybridoma SNs were screened by ELISA using mTREM-1-HuIgG1 as capturing protein and multiple-adsorbed HRP-labeled goat-anti-rat Ig (PharMingen) as detecting antibody. ELISA-positive hybridoma supernatants were then tested by flow cytometry for staining 293 cells expressing FLAG-tagged mTREM-1. Hybridomas producing ELISA- and transfection-positive SN were treated as described in 2.2.2.1.1.

2.2.2.3 Production of DAP12 rabbit antisera

Prior to first immunization with 500 µg peptide/KLH-conjugate (s.c. 1:1 in FCA) rabbits were bled to obtain pre-immune serum. After two weeks the animals were given 6 booster immunizations of 300 µg conjugate/KLH-conjugate (s.c. 1:1 in FIA) in weekly intervals. One weeks after the last immunization rabbits were bleed and antisera were tested in Western Blot analysis on lysates from 293 cells expressing FLAG-tagged DAP12 (Dietrich et al., 2000).

2.2.2.4 Summary: monoclonal antibodies and antisera

Table 2.4: Summary of all produced mAb and antisera.

Clone	Specificity	Host	Isotype	Application	Produced by
Monoclonal Abs					
Anti-huTREM-1					
21C7	Human TREM-1	Mouse	IgG1, κ	FACS, WBA, IP, IHC, ELISA, Stimulation	A. Bouchon
9E2	Human TREM-1	Mouse	IgG1, κ	FACS, WBA, IP, IHC, ELISA	A. Bouchon
37F8	Human TREM-1	Mouse	IgG1, κ	FACS, ELISA (recognizes different epitope than 21C7 and 9E2)	A. Bouchon
Anti-huTREM-2					
29E3	Human TREM-2	Mouse	IgG1, κ	FACS, WBA, IP, ELISA, Stimulation	A. Bouchon
10B11	Human TREM-2	Mouse	IgG1, κ	FACS, WBA, IP, ELISA	A. Bouchon
25C10	Human TREM-2	Mouse	IgG1, κ	FACS, WBA, IP, ELISA	A. Bouchon
20G3	Human TREM-2	Mouse	IgG1, κ	FACS, WBA, IP, ELISA, IHC	A. Bouchon
21E10	Human TREM-2 Mouse TREM-2	Mouse	IgG1, κ	FACS, WBA, IP, ELISA	A. Bouchon
Anti-huTREM-4					
64A9	Human TREM-4	Mouse	IgG1, κ	FACS, WBA, IP, ELISA	A. Bouchon
64F5	Human TREM-4	Mouse	IgG1, κ	FACS, WBA, IP, ELISA, IHC	A. Bouchon
	Human TREM-4	Mouse	IgG1, κ	FACS, WBA, IP, ELISA	A. Bouchon
	Human TREM-4	Mouse	IgG1, κ	FACS, WBA, IP, ELISA	A. Bouchon
	Human TREM-4	Mouse	IgM	FACS, WBA, IP, ELISA	A. Bouchon
Anti-huTREM-5					
8H6	Human TREM-5	Mouse	IgG1, κ	FACS, WBA, ELISA, IHC	A. Bouchon
11F6	Human TREM-5	Mouse	IgG1, κ	FACS, WBA, ELISA	A. Bouchon
11A12	Human TREM-5	Mouse	IgM, κ	FACS, WBA, ELISA	A. Bouchon
Anti-mTREM-1					
50D1	Mouse TREM-1	Rat	IgG2b, κ	FACS, ELISA	A. Bouchon
1A1	Mouse TREM-1	Rat	IgG2b, κ	FACS, ELISA	A. Bouchon
100E1	Mouse TREM-1	Rat	IgG2b, κ	FACS, ELISA	A. Bouchon
Antisera					
DAP10 antiserum					
1178	Human DAP10	Rabbit	IgG	WBA, IP	Dietrich/Bouchon
DAP12 antiserum					
1370	Human DAP12	Rabbit	IgG	WBA, IP	Dietrich/Bouchon
1378	Human DAP12 Mouse DAP12	Rabbit	IgG	WBA, IP	Dietrich/Bouchon

Table 2.4 does not display all clones selected from every fusion. It summarizes all mAb and antisera frequently used in this thesis together with their Isotype and capability to work in flow cytometry (FACS), Western blot analysis (WBA), IPs, immunohistochemistry (IHC), enzyme-linked immunosorbant assay (ELISA) and stimulation assays without using a cross-linking antibody.

2.2.3 Transfection of cell lines

Jurkat cells engineered to express SV40 large T antigen (Jurkat^{SV40}) or J558L where washed in R-Medium, resuspended in 400 µl R-Medium at a concentration of 1 x 10^7 cells/ml and mixed with 30 µg plasmid DNA. Electroporation occurred at 260 mV and 960/1050µF (Jurkat^{SV40}/J558L). After electroporation, cells were collected, placed in 30 ml R-Medium and analyzed after 10-12 h by flow cytometry or lysed for Western Blot analysis. For the generation of stable transfectants, cells where incubated 24 h in R-Medium before they where plated in Selectionmedium IV or V, according to the resistance gene introduced in the expression plasmid.

293 or COS cells were transiently transfected with 3-5 µg of plasmid DNA using Lipofectene (Stratagene) or Cytofectene (Biorad), according to the manufacturer's protocols.

2.2.4 Isolation and maintenance of different cell populations from blood

2.2.4.1 Human neutrophils

2.2.4.1.1 Isolation by Ficoll gradient

Human blood was mixed with one volume of 3% Dextran T-500 (Pharmacia) in 0.9% NaCl to sediment red blood cells (RBC). Leukocytes in the supernatant were further separated by gradient density centrifugation on lymphocyte separation medium (LSM) (ICN Biomedicals) into Peripheral blood mononuclear cells (PBMC) and neutrophils. Any remaining RBC were removed by hypotonic lysis with 0.2% NaCl solution for 30 s. Neutrophil preparations routinely contained > 91% neutrophils as determined by FACS analysis, and were > 99% viable.

2.2.4.1.2 Isolation by Percoll gradient

Neutrophils were isolated as previously described (Klein et al., 2000). Briefly, human blood was mixed with an equal volume of Dextran solution (3% Dextran T-500 (Pharmacia) in 0.9% NaCl) and left for sedimentation (30 min) to remove RBC. Blood leucocytes in the supernatant were separated by density centrifugation on a two-level Percoll (Pharmacia) gradient (50.5% and 42.0%). Neutrophils were aspirated from the 42 to 50.5 interface and washed in RPMI/HEPES. Any remaining RBC were removed by hypotonic lysis with 0.2% NaCl solution for 30 s. Neutrophil preparations routinely contained > 97% neutrophils as determined by FACS analysis, and were > 99% viable.

2.2.4.2 Human monocytes

2.2.4.2.1 Isolation of human monocytes by magnetic cell sorting

PBMC were purified from human blood by gradient density centrifugation on LSM (ICN Biomedicals). After three washes in RPMI/HEPES, PBMC were resuspended in MACS-buffer (PBS/10%FCS/500 µM EDTA) and incubated with CD14 Micro Beads (Miltenyi) for 30 min. After three washes with magnetic cell sorting (MACS)-buffer, $CD14^+$ monocytes were purified from PBMCs by MACS (Miltenyi). After MACS, cells were washed 3 times in RPMI/HEPES and finally resuspended in R-Medium

(experiments with monocytes), MDC-Medium (Differentiation to MDCs), or MMϕ-Medium (Differentiation to MMϕs) (2.2.1.5).

2.2.4.2.2 Isolation of human monocytes by Percoll gradient

In stimulation experiments with anti-TREM-1 antibodies, monocytes were prepared from PBMCs by Percoll gradient density centrifugation as previously described (Nakajima et al., 1999), to avoid potential interferences of cell surface-bound anti-CD14 antibodies with TREM1-mediated signaling.

2.2.4.3 Human monocyte-derived DCs and macrophages

Monocyte-derived DCs (MDCs) and macrophages (MMϕs) were prepared from $CD14^+$-monocytes as described (Bender et al., 1996; Sallusto and Lanzavecchia, 1994; Vincent et al., 1992). In brief, $CD14^+$-monocytes were washed three times in RPMI/HEPES, counted and adjusted to a concentration of 300 000 cells/ml in MDC- or MMϕ-Medium. After 4-6 days the cells cultured in MDC-medium display morphology and phenotype of immature DCs. Cells kept in MMϕ-medium appear as Macrophages after 10-14 days.

2.2.4.4 Culturing of the human T-cell clone VIP13

VIP13 was maintained in T-Medium (2.2.1.5) as previously described (Lanzavecchia et al., 1988). For induction of proliferation, PBMC were isolated, irradiated (3000 rad) and mixed with VIP13 cells at a relation of 1:10 (PBMCs:VIP13).

2.2.5 Stimulation of cells and flow cytometry

2.2.5.1 Stimulation of cells

2.2.5.1.1 Stimulation of cells with mAb coated on plastic

$F(ab')_2$ (21C7 anti-TREM-1 mAb), $F(ab')_2$ 29E3 (anti-TREM-2 mAb), or control $F(ab')_2$ mAb (see) were coated for 6 h at 37°C on MAXISorp 96-well flat-bottom plates (Costar) (sterilized for 10 min by UV-irradiation) with a final concentration of 20 µg/ml in PBS. Neutrophils, monocytes, or MDCs were plated at concentration of 0.5 - 5 x 10^5 cells/well and simultaneous contact to the plate was induced by short centrifugation (100 g, 1 min, 25°C). Supernatants and cells were separated after 6, 12, 24, 36, 48 or 72 h and tested by ELISA or flow cytometry, respectively.

2.2.5.1.2 Stimulation of cells with mAb in solution

0.5 - 5 x 10^5 neutrophils, monocytes, or MDCs were stimulated with F(ab')$_2$ (21C7 anti-TREM-1 mAb), F(ab')$_2$ 29E3 (anti-TREM-2 mAb), or control F(ab')$_2$ mAb at a final concentration of 10 µg/ml. Cross-linking was induced by adding F(ab')$_2$ goat-anti-mouse F(ab')$_2$-specific Ab (Jackson) at a concentration of 5 µg/ml. Supernatants and cells were separated after 6, 12, 24, 36, 48 or 72 h and tested by ELISA, Western Blot or flow cytometry.

2.2.5.1.3 Stimulation of neutrophils and monocytes with bacterial products

Purified monocytes and neutrophils were cultured in the absence or presence of heat-inactivated *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Bacillus of Calmette-Guerin* (ratios of monocytes/neutrophils : bacteria were ~1:10 – 1:100), LPS (100ng/ml), LTA (100ng/ml), mycolic acid (10µg/ml) for different time points. Cells and SN were separated and analyzed by flow cytometry and ELISA.

2.2.5.1.4 Differentiation of mature DCs from MDCs

Maturation of MDC was induced by LPS (100 ng/ml), LTA (100 ng/ml), CpG oligonucleotides (1 µg/ml), TNF-α (10 ng/ml), TNF-β (20 ng/ml) or plate-bound IgG (coated at a concentration of 20 µg/ml) for different time periods. Full maturation was observed after 24-48 h. Induction of maturation via CD40 was induced by co-culture MDCs with irradiated CD40L-transfected J558L cells (3000 rad) at a relation of 1 : 10 (MDCs : J558L).

2.2.5.1.5 Activation of MMϕs

MMϕ were stimulated with LPS (100 ng/ml), LTA (100 ng/ml), CpG oligonucleotides (1 µg/ml), TNF-α (10 ng/ml), TNF-β (20 ng/ml) or plate-bound IgG (coating with 20 µg/ml) for different time periods. Full activation could be observed after 12-24 h. Induction of maturation via CD40 was induced by co-culture MMϕs with irradiated CD40L-transfected J558L cells (3000 rad) at a concentration of 1 : 10 (MMϕs : J558L).

2.2.5.1.6 Pharmacological inhibition

In blocking experiments, inhibitors (PD98059 [20 µM], LY294002 [10 µM], SB203580 [2 µM], PP2 [1 µM] (all from Calbiochem), TPCK [20 µM] (Sigma), SN50 [20 µM] (Biomol) were added 60 min before stimulation.

2.2.5.2 Flow cytometry of human cells

Before staining, all cells were incubated with PBS/20% human serum for 1h on ice to block FcRs. Whole blood leukocytes were incubated with mAbs 21C7 (anti-TREM-1, IgG1), 3C10 (anti-CD14, IgG2b) and L243 (anti-HLA-DR, IgG2a) followed by isotype-specific FITC/PE/biotin-conjugated secondary antibodies. After a further incubation step with APC-labeled streptavidine, cells were analyzed on a FACSCalibur using CELLquest software (Beckton Dickinson). Dead cells were excluded by gating on PI-negative cells. In single-color-staining, cells stimulated as described in 2.4, were stained with mAb 21C7, 29E3, or control mAbs followed by human-adsorbed PE-conjugated goat anti-mouse IgG (SBA).

2.2.6 Detection of cellular activation markers

2.2.6.1 Measurement of intracellular calcium mobilization

Monocyte-derived DCs were loaded with Indo-1^{AM} (Sigma) for 30 min at 37°C, washed 3 times and resuspended in RPMI 1640/10 mM HEPES/5% FCS. Cytoplasmic Ca^{2+} levels were monitored in individual cells by measuring 405/525 spectral emission ratio of loaded Indo-1^{AM} dye by flow cytometry. After a baseline was acquired for at least 30s, 29E3, 21C7, F(ab') 29E3, F(ab') 21C7, $F(ab')_2$ 29E3, $F(ab')_2$ 21C7, control F(ab'), $F(ab')_2$,or mAb were added to a final concentration of 1µg/ml and analysis was continued up to 512 s. In some experiments, $F(ab')_2$ goat-anti-mouse $F(ab')_2$-specific Ab (Jackson) was used as a cross-linking antibody. In case antibodies and antibody fragments were biotinylated, ExtraAvidine (Sigma) was added as cross-linker.

2.2.6.2 Measurement of surface activation markers

To measure stimulation-dependent changes in the expression of cell surface markers cells were stimulated as described in 2.4. Cells were harvested at different time points and stained with anti-TREM-1 (21C7) -TREM-2 (29E3, 21E10, 10B11, 25C10 or 20G2), -TREM-4 (64A9 or 64F5), -TREM-5 (8H6 or 11F6), -MHC class I, -MHC class II, -CD1a, -CD11a, -CD11b, -CD11c, -CD14, -CD16, -CD18, -CD29, -CD32, -CD35, -CD38, -CD40-, -CD41, -CD49a, -CD49b, -CD49c, -CD49d, -CD49e, -CD49f, -CD54, -CD61, -CD64, -CD80, -CD83, -CD86, -CD89, -CD103, -CD115, -CD116, -CCR5, -CCR6, -CXCR4, or -Mannose receptor conjugated with Cy5-, PE- or FITC (all from Immunotech, Pharmingen or self labeled). Anti-CCR7 mAb (PharMingen) was followed by $F(ab')_2$ PE-labeled goat anti-mouse IgM Ab (SBA).

Cells were analyzed on a FACSCalibur using CELLquest software (Beckton Dickinson). Dead cells were excluded by gating on PI-negative cells.

2.2.6.3 Measurement of cell secreted cytokines and chemokines

2.2.6.3.1 ELISA

To measure stimulation-dependent changes in cytokine secretion, cells were stimulated as described in 2.4. Supernatants were collected and tested for production of IL-6, IL-8, IL-10, TGF-β, IL-12p40, IL-12p75, IL-13, IL-15, IL-18, IL-1α, IL-1β, TNF-α, and MCP-1 by ELISA (PharMingen or R&D systems).

2.2.6.3.2 Intracellular flow cytometry

To measure stimulation-dependent changes in cytokine production on single-cell level, cells were stimulated as described in 2.4 in the presence of Brefeldine A (10 mg/ml; Sigma). Cells were harvested at different time points, fixed (3% Paraformaldehyde (PFA) in PBS) and permeabilized (PBS, 10 % low-endotoxin FCS, 0.5 % Saponin, 10 mM HEPES). Detection of cytokines was performed using PE-, FITC- or APC-labeled anti-IL-6, -IL-8, -IL-10, -TGF-β, –IL-12p40, -IL-12p75, -IL-13, -IL-15, IL-18, -IL-1α, -IL-1β, -TNF-α, and -MCP-1 (PharMingen) followed by flow cytometry.

2.2.6.4 Neutrophil degranulation assay

Purified neutrophils were stimulated in 96-well flat-bottom plates coated with 21C7 (anti-TREM-1) or 1F11 (anti-MHC class I) mAbs at a concentration of $1x10^5$ cells/well. After 16 h or 36 h, the cell suspensions were centrifuged, and SNs analyzed by ELISA for myeloperoxidase (MPO) (R&D Systems).

2.2.6.5 Detection of leukotriene production

Purified neutrophils and monocytes were stimulated as described in 2.5.4. After different time points SNs were analyzed by ELISA for Leukotriene B4 (LTB4) (R&D Systems) according to the manufacturer´s instructions.

2.2.6.6 Chemotaxis assay

MDCs (1×10^7) were treated for 24 h with F(ab)'$_2$ 21C7, F(ab)'$_2$ 29E3 coated on plastic (20 µg/ml), or LPS (1 µg/ml). Cells (5×10^5 in 100 µl IMDM/0.5% BSA) were incubated for 1h at 37°C. Cell were subsequently loaded into collagen-coated transwells (Costar, 3 µm pore filter), which were placed onto 24-well plates containing

450 µl medium supplement with 100 ng/ml CCL19 (ELC/MIP-3β) or CCL20 (6-C-Kine/SLC) (both from Peprotec). After incubation period of 4 h at 37°C, cells that had migrated to the lower chamber were collected and counted on a cytofluorimeter (FACSCalibur, constant time acquisition). In blocking experiments cells were pre-incubated with anti-CCR7 mAb (10 µg/ml) and added to the transwell.

2.2.6.7 Antigen-uptake and presentation

2.2.6.7.1 Receptor-internalization assay

To determine internalization of TREM-1 and -2, cells were incubated in R-Medium at a cell density of 2 $x10^5$ cells/ml at 37°C or 4°C with Cy5-conjugated anti-TREM-1 mAb 21C7, anti-TREM-2 29E3 or control mAb. At different time points after stimulation, aliquots of cell suspension were washed in ice-cold R-Medium and immediately treated with 300 µl 0.5 M NaCl, 0.5 M acetic acid, pH 2.2 for 10 s. The acid-resistant fluorescence of the cells (representing internalized anti-TREM-1, anti-TREM-2 or control mAb) was measured by flow cytometry. The percentage of internalized mAb to cell surface-bound mAb was subsequently calculated using the equation: *HAR - CA*R/*CT*. In this equation, *HAR* is the mean fluorescence intensity (MFI) of acid-treated cells incubated at 37C; *CAR* is the MFI of acid-treated cells incubated at 4°C, and *CT* is the MFI of untreated cells incubated at 4°C. For each construct at least three different clones were analyzed.

2.2.6.7.2 Antigen-presentation assay

2.5 x 10^4 irradiate DCs (3000 rad) were co-cultured with 5 x 10^4 cells of the VIP13 T cell clone in 96-well flat-bottom microplates (Costar) in the presence of serial dilutions of IgG1 mAbs or F(ab')$_2$ mAb fragments (Lanzavecchia et al., 1988). mAbs used in the assay were the following: ZM3.8 (anti-ILT3, IgG1,κ) (Cella et al., 1997), 9E2 (anti-TREM-1, IgG1, κ), 29E3 (anti-TREM-1, IgG1, κ), and ICRF44 (anti-CD11b/Mac-1, IgG1, κ, Pharmingen). F(ab')$_2$ mAb fragments used in the assay were F(ab')$_2$ 9E2 and F(ab')$_2$ 29E3. After 72 h, the cultures were pulsed with [^{3}H]-Thymidine (1µCi/well, specific activity 5 Ci/mmol), and the radioactivity incorporated was measured after additional 16 h. The data plotted against the concentration of mAbs determined by ELISA using a purified mouse IgG1, κ or F(ab')$_2$ IgG1, κ as a standard.

2.2.7 Methods for the detection of cell death and survival

2.2.7.1 Detection of apoptosis and necrosis *in vitro*

2.2.7.1.1 Determination of mitochondrial membrane potential

Measurement of mitochondrial membrane potential ($\Delta\Psi m$) was performed as described previously (Bouchon et al., 2000; Metivier et al., 1998). Briefly, 5 x 10^4 cells were incubated in PBS containing 40 nM 3,3-Dihexyloxocarbo-cyanine iodide ($DiOC_6(3)$ or 10 nM JC-1 (Molecular Probes Inc.) for 15 min at 37°C in the dark followed by analysis on a FACScalibur (Becton Dickinson) for reduced fluorescence 525 nm. For simultaneous detection of dead cells and loss of ($\Delta\Psi m$) 7-Aminoactinomycin D (7-AAD, Molecular Probes) or PI (Sigma) were added as described in 2.2.7.1.3. $DiOC_6(3)^+$/7-AAD^- cells correspond to live cells, $DiOC_6(3)^{low}$/7-AAD^- cells to early apoptotic cells, $DiOC_6(3)^+$/7-AAD^+ cells to necrotic cells, and $DiOC_6(3)^{low}$/7-AAD^+ cells to late apoptotic cells.

2.2.7.1.2 Determination of Reactive Oxygen Species (ROS)

Measurement of generation of ROS was performed by incubating 5 x 10^4 cells in PBS containing 2 µM dihydroethidin (DHE, Molecular Probes Inc.) for 15 min at 37°C in the dark followed by analysis on a FACScalibur cytometer (Becton Dickinson) for increased fluorescence at 575 nm by ROS-mediated oxidation of DHE to Ethidine. DHE can be also used for the detection of oxidative burst in neutrophils, monocytes and macrophages.

2.2.7.1.3 Measurement of cell viability

To determine the integrity of the cell membrane, cells were incubated for 5 min in 4 µg/ml 7- 7-AAD (Molecular Probes) or 2,5 µg/ml PI (Sigma) at 4°C in the dark followed by analysis on a FACScan (Becton Dickinson) for increased fluorescence at 625 nm and 575 nm after 7-AAD and PI-uptake, respectively.

2.2.7.1.4 Determination of DNA fragmentation

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described (Nicoletti et al., 1991). Briefly, 2.5 x 10^5 cells were incubated in 24-well plates (Costar) with or without apoptotic stimuli in 0.5 ml medium at 37°C. Cells were collected by centrifugation at 600 x g for 10 min at 4°C, washed twice with PBS and then resuspended in 100 µl lysis solution containing 0,1% (v/v) Tx100, 0,1% (w/v) sodium citrate and 50 µg/ml PI. Apoptosis was quantitatively determined by flow cytometry after incubation at 4°C in the dark for at least 24 h as cells containing nuclei with subdiploid DNA content.

2.2.7.1.5 Determination of Phosphatidyl-serine

$1x10^5$ cells were incubated in 96-well plates (Costar) with or without apoptotic stimuli in 200 µl medium at 37°C. Cells were collected by centrifugation at 600 x g for 10 min at 25°C, washed twice in binding buffer (2,5 mM $CaCl_2$, 140 mM NaCl, 10 mM HEPES pH 7,4) and then resuspended in 50 µl staining solution (0,2 µg/ml Annexin-V-FITC (R&D Systems), 4 µg/ml 7-AAD (Molecular Probes), 2,5 mM $CaCl_2$, 140 mM NaCl, 10 mM HEPES pH 7,4). After incubation for 15 min in the dark at 37°C, 150 µl cold binding buffer was added and the degree of single positive ($FITC^{bright}$ / $7\text{-}AAD^{dull}$: early apoptotic cells) and double-positive cells ($FITC^{bright}$ / $7\text{-}AAD^{bright}$: late apoptotic and necrotic cells) determined by flow cytometry.

2.2.7.2 Detection of cell proliferation by ^{3}[H]-Thymidine incorporation

To determine the proliferation capacities of cells after stimulation or co-culture, cells were plated at a concentration of 50 000/well in round-bottom 96-well plates and stimulated as described in 2.4 for 48 h. Cells were pulsed with 1 µCi ^{3}[H]-Thymidine (Amersham) for 16 h before harvesting onto glass fiber filters (Wallac). Filters were dried and added to 5 ml scintillation fluid (FSA Laboratory Supplies). The number of counts per minute (cpm) was determined using a beta scintillation counter (Wallac)

2.2.7.3 Detection of apoptosis and necrosis *in vivo* and *ex vivo*

2.2.7.3.1 Detection of neutrophil apoptosis during endotoxemia *in vivo*.

To detect apoptotic neutrophils during LPS-induced shock we modified and extended a previous report (Zamzami et al., 1995). C57BL/6 mice (female, 8-10 weeks, 19-22 g) were randomly grouped (20 mice per group) and injected intravenously (i.v.) with 10µg $DiOC_6(3)$ (Molecular Probes) in sterile saline. 1 hour later, mice were injected intraperioneally (i.p.) with 500 µg/mouse of purified huIgG1,κ (Sigma), mTREM-1-IgG1, or mTREM-1-IgG1 with a mutated Fc part to reduce FcR binding and complement fixation ($mTREM\text{-}1\text{-}IgG1^{mutant}$). LPS (LPS from E. coli 055:B5 (25 mg/kg; Sigma) was administred one hour later i.p. 10 of 20 mice were kept and viability of treated mice was monitored 4-6 times a day for at least 10 days whereas 10 of 20 mice were sacrificed at different time points after LPS administration and peritoneal lavage cells (PLCs) were carefully isolated. To minimize adherence of activated live phagocytes and progression of apoptosis ex vivo isolated PLCs were surface stained with Cy5-labeled anti mTREM-1 mAb and PE-labeled anti-LY-6G mAb (Pharmingen) for 5 minutes on ice and immediately analysis on a FACSCalibur. Cells destroyed during the harvest were excluded by 7-AAD staining. $mTREM\text{-}1^+$/Ly-

$6G^+/DiOC_6(3)$ cells correspond to early apoptotic neutrophils and were measured as indicated.

2.2.7.3.2 Monitoring of neutrophil apoptosis *ex vivo*.

C57BL/6 mice (female, 8-10 weeks, 19-22 g; 30 animals per group) were treated as described in 2.2.7.3.1. 10 of 30 mice were kept and viability of treated mice was monitored 4-6 times a day for at least 10 days whereas 20 of 30 mice were sacrificed 6 hours after LPS administration and PLCs were carefully isolated. LY-$6G^+$ were sorted on a MoFlo (Cytomation) and subsequently cultured at a concentration of $5x10^5$/ml in R-Medium. Determination of $\Delta\Psi m$ the integrity of the cell membrane was performed 0, 2, 4, 6, 8, 12, and 24 hours after sorting by staining with $Dioc_6(3)$ and PI as described in 2.2.7.1.1.

2.3 Molecular Biology

2.3.1 Primers

Table 2.5: Summary: human TREM-1 primers.

Number	Sequence (5' → 3')	Application; restriction sites
Cloning of human TREM-1/pCR2.1-TOPO		
21107	GCTGGTGCACAGGAAGGATG	forward oligo for cloning of full length TREM-1 cDNA into pCR2.1
21108	GGCTGGAAGTCAGAGGACATT	reverse oligo for cloning of full length TREM-1 cDNA into pCR2.1
21134	AAGGGACGGAGAGATGCCC	Sequencing
21135	TGTTCGATCGCATCCGCTT	Sequencing
21332	CCTCCCCACTTGGACTGGA	Sequencing
Cloning of human TREM-1/pAC-4		
21313	ACCAAA*CATGG*AACTCCGAGCTGCAACTAAAT	Cloning of TREM-1 extracellular region in pAC-4; 5';*Nco*I
21314	ACCAAA*GGATCC*CCTGATGATATCTGTCACATTTG	Cloning of TREM-1 extracellular region in pAC-4;3';*Bam*HI
Cloning of human TREM-1/pCMV1FLAG		
21142	TAGTAG*GCGGCCGC*GGAACTCCGAGCTGCAACTAAA	Cloning of TREM-1 in pCMV-1FLAG;5' *Not*I
21143	TAGTAG*TCTAGA*CTAGGGTACAAATGACCTCAG	Cloning of TREM-1 in pCMV-1FLAG;3' *Xba*I
Cloning of human TREM-1/pCD4huIgG1		
21281	TAGTAG*GAGCTC*ACAGGAAGGATGAGGAAGACCAGG-CTC	Cloning of TREM-1 extracellular region in pCD4huIgG1;5' *Sst*I
21282	AAGCTTATACTTACCCCTGATGATATCTGTCACATTTGT	Cloning of TREM-1 extracellular region in pCD4huIgG1;3' blunt

Table 2.6: Summary: human TREM-2 primers.

Number	Sequence (5' → 3')	Application
Cloning of human TREM-2/pCR2.1-TOPO		
22067	TGATCCTCTCTTTTCTGCAG	forward oligo for cloning of full length TREM-2 cDNA into pCR2.1
22068	TCAAGGGAAAGACGAGATC	Nested forward oligo for cloning of full length TREM-2 cDNA into pCR2.1
22070	GTGTTTAAAATGTCCAATATT	reverse oligo for cloning of full length TREM-2 cDNA into pCR2.1
22071	CAGAAGTTGTCAGGTGTTCTT	Nested reverse oligo for cloning of full length TREM-2 cDNA into pCR2.1
22060	GGCAGTGAGGCTGACACCC	Sequencing
22061	GGGTGTCAGCCTCACTGCC	Sequencing
22075	GAGAAGGGCCCATGCCAGCGTGT	Sequencing
22076	GCAGAGTTTGGAGCTGATACCC	Sequencing
22505	CACTGGGGGAGGCGCAA	Sequencing
22629	GAGCCTCTTGGAAGGAGAAA	Sequencing
22630	GATCCAGGGGGTCTGCCA	Sequencing
Cloning of human TREM-2/pCMV1FLAG		
22084	TAGTAG*AAGCTT*CACAACACCACAGTGTTCCAGGG	Cloning of TREM-2 in pCMV-1FLAG;5' *Hin*-dIII
22085	TAGTAG*TCTAGA*TCACGTGTCTCTCAGCCCTGGCAG	Cloning of TREM-2 in pCMV-1FLAG;3' *Xba*I
Cloning of human TREM-2/pCD4huIgG1		
22072	TAGTAG*AAGCTT*ATACTTACCGGGTGGGAAAGGGATT-TCTCCTTCCAA	Cloning of TREM-1 extracellular region in pCD4huIgG1;3' *Hin*DIII
22069	TAGTAG*GAATTC*ACTCTGCTTCTGCCCTTGGCTGGG	Cloning of TREM-1 extracellular region in pCD4huIgG1;5' *Eco*RI

Table 2.7: Summary: human TREM-4 primers.

Number	Sequence (5' → 3')	Application
Cloning of TREM-4/pCR2.1		
22625	AGACGCTGGGGAGTACTGGT	Trem-4 3'-end race forward 1
22626	GTGGGGTCGAGAAACGGG	Trem-4 3'-end race forward 2
22627	CCCCCGATGAGTCTTTACTGAT	Trem-4 3'-end race forward 3
22751	GGTCCGCATACTGGCCCCA	Trem-4 3'-end race forward 4
22752	CTGCTGAGCCTTCTGTCAGCC	Trem-4 3'-end race forward 5
22719	AATGCGGCTTCTGGTCCTGCT	forward oligo for cloning of partial TREM-4 cDNA into pCR2.1
22720	ATCAGTAAAGACTCATCGGGG	reverse oligo for cloning of partial TREM-4 cDNA into pCR2.1
Cloning of TREM-4/pCD4huIgG1		
22769	TAGTAG*GAATTC*ATGCGGCTTCTGGTCCTGCTATGG-GGTT	Cloning of TREM-4 extracellular region in pCD4huIgG1;5' *Eco*RI
22770	TAGATG*AAGCTT*ATACTTACCGGACACCCTGGGCT-TAGAGCTG	Cloning of TREM-4 extracellular region in pCD4huIgG1;3' *Hin*dIII
Cloning of TREM-4/pDISPLAY		
23624	TAGTAG*AGATCT*TATGAAGCCCTGGAGGGGCCAGA	Cloning of TREM-4 extracellular region in pDISPLAY;5' *Bgl*II
23625	TAGTAG*GTCGAC*GGACACCCTGGGCTTAGAGCT	Cloning of TREM-4 extracellular region in pDISPLAY;3' *Sal*I

Table 2.8: Summary: human TREM-5 primers.

Number	Sequence (5' → 3')	Application
Cloning of TREM-5/pCR2.1		
23922	ACGAGGAGCCGGGAAGGCAGA	forward oligo for cloning of full length TREM-5 cDNA into pCR2.1
23923	AGGCTCTGCAGATCCATCTC	reverse oligo for cloning of full length TREM-5 cDNA into pCR2.1
Cloning of TREM-5/pCD4huIgG1		
23924	TAGATG*GAATTC*ATGTGGCTGCCCCCTGCTCTGCT	Cloning of TREM-5 extracellular region in pCD4huIgG1;5' *Eco*RI
23925	TAGTAG*AAGCTT*ATACTTACCGTAGTGGTTCCTCTTG-TGGGAG	Cloning of TREM-5 extracellular region in pCD4huIgG1;3' *Hin*DIII
Cloning of TREM-5/pCMV-1FLAG		
24256	TAGTAG*AAGCTT*GAGTCTGTGAGAGCCCCAGAGCAG-GGG	Cloning of TREM-5 extracellular region in pCMV-1FLAG;5' *Hin*DIII
24257	TAGTAG*TCTAGA*CTCTGCAGATCCATCTCTCTAAGT	Cloning of TREM-5 extracellular region in pCMV-1FLAG;3' *Xba*I

Table 2.9: Summary: mouse TREM-1 primers.

Number	Sequence (5' → 3')	Application
Cloning of mTREM-1/pCR2.1 TOPO		
22005	ACTCTGGATTGTATCGTTGTGTGATT	mTrem-1 3'-end race forward 1
22006	ATCCTCCGAATGACCCTGTTGTG	mTrem-1 3'-end race forward 2
22007	CTTCGATCCTGTCCGCCTGGTGG	mTrem-1 3'-end race forward 3
22079	GAGCTTGAAGGATGAGGAAG	forward oligo for cloning of full length TREM-5 cDNA into pCR2.1
22080	AAGCTCTGGAGTTTCTCTTTATTTC	reverse oligo for cloning of full length TREM-5 cDNA into pCR2.1
Cloning of mTREM-1/pCD4huIgG1		
22209	TAGTAG*AAGCTT*ATACTTACCGTCAGCATCTGTCC-CATTTAT	Cloning of mTREM-1 extracellular region into pCD4huIgG1;3'*Hin*dIII
22208	TAGTAG*GAATTC*AGGATGAGGAAGGCTGGG	Cloning of mTREM-1 extracellular region into pCD4huIgG1;5' *Eco*RI
Cloning of mTREM-1/pCMV-1FLAG		
23135	TAGTAGAAGCTTGAAGTCAAAGCTGCCATTGTTCTA	Cloning of mTREM-1 extracellular region in pCMV-1FLAG;5' *Hin*dIII
23136	TAGTAGGAATTCTCATCCAAATGTCCTCTTTGTGACAA	Cloning of mTREM-1 extracellular region in pCMV-1FLAG;3' *Xba*I

Table 2.10: Summary: mouse TREM-2 primers.

Number	Sequence (5' → 3')	Application
Cloning mTREM-2/pCR2.1		
22017	CTTGCACAAGGTCCCCTCC	forward oligo for cloning of full length mTREM-2 cDNA into pCR2.1 #1
22018	GGCTGGCTGCTGGCAAAGG	Nested forward oligo for cloning of full length mTREM-2 cDNA into pCR2.1 #2
22019	AAAAGTAGCAGAAACAGAAGT	reverse oligo for cloning of full length mTREM-2 cDNA into pCR2.1 #1
22020	CATGCAGGCTGGATTGACTCC	Nested reverse oligo for cloning of full length mTREM-2 cDNA into pCR2.1 #2
22129	CCATGCCAGCGTGTGGTGAGCA	Sequencing
22130	AAACTTGCTCAGGAGAACGCA	Sequencing
Cloning mTREM-2/pCD4huIgG1		
22880	TAGTAG*GAATTC*GCCATGGGACCTCTCCACAGTTTC-TCCTGC	Cloning of mTREM-2 extracellular region in pCD4huIgG1;5' *EcoR*I
22881	TAGTAG*AAGCTT*ATACTTACCGGAGGTGGGTGG-GAAGGAGGT	Cloning of mTREM-2 extracellular region in pCD4huIgG1;3' blunt
Cloning mTREM-2/pCD4huIgM		
22889	TAGTAG*GTCGAC*ATACTTACCGGAGGTGGGTGG-GAAGGAGGT	Cloning of mTREM-2 extracellular region in pCD4huIgM; 5' *Sal* I
22881	TAGTAG*AAGCTT*ATACTTACCGGAGGTGGGTGG-GAAGGAGGT	Cloning of mTREM-2 extracellular region in pCD4huIgM1;3' *Hin*dIII
Cloning mTREM-2/pCMV-1FLAG		
M88	TAGTAG*AAGCTT*CTCAACACCACGGTGCTG	Cloning of mTREM-2 extracellular region in pCMV-1FLAG;5' *Hin*dIII
M89	TAGTAG*GGATCC*TCACGTACCTCCGGGTCCAG	Cloning of mTREM-2 extracellular region in pCMV-1FLAG;3' *Bam*HI

Table 2.11: Summary: mouse TREM-3 primers.

Number	Sequence (5' → 3')	Application	Designed by
Cloning of mTREM-3			
22123	CTCCAGAGACTCTGGTGCTC	mTrem-3 3'-end race forward 1	Colonna/Bouchon
22124	ACAAACACCAGAAAGGCAGA	mTrem-3 3'-end race forward 2	Colonna/Bouchon
22125	GCCAGGGCTGGGAAGTACTT	mTrem-3 3'-end race forward 3	Colonna/Bouchon
22203	TTATCAGAAGAGAGATAGGT	reverse oligo for cloning of full length mTREM-3 cDNA into pCR2.1 #1	Colonna/Bouchon
22204	TACATCATATAGGTTTTACC	Nested reverse oligo for cloning of full length mTREM-3 cDNA into pCR2.1 #2	Colonna/Bouchon
22205	CACCAGGAAGGAGCTTCATA	forward oligo for cloning of full length mTREM-3 cDNA into pCR2.1 #1	Colonna/Bouchon
22206	CAGAGGAGG-CAGGGACCTGGGGGATG	Nested forward oligo for cloning of full length mTREM-3 cDNA into pCR2.1 #2	Colonna/Bouchon
22207	CACAGCTCTCCTCAGAGCTG	Sequencing	Colonna/Bouchon

2.3.2 Vectors and constructs

Table 2.12: Summary of used vectors

Name	Reference	Application
pCR2.1 TOPO	Invitrogen	Cloning vector, direct cloning of PCR products using the TOPO cloning kit (Invitrogen); Template cDNA for subcloning
pCMV-1^{FLAG}	Sigma	Eukaryotic expression vector, containing a N-terminal FLAG-tag for detection of expression, CMV promoter for high expression
pDISPLAY^{HA}	Invitrogen.	Eukaryotic expression vector. The extracellular domain of the target receptor is fused to transmembrane region of platelet derived growth factor receptor to ensure surface expression. In addition, it contains a N-terminal Hemaglutinin (HA)-tag for detection of expression
pAC-4	Avidity	Prokaryotic expression vector for the production of monomeric, biotinylated proteins, extracellular receptor domain(s) are fused to a BirA-tag that is in vivo biotinylated by AVB101 bacteria expressing BirA enzyme
pCD4huIgG1	K. Karijalainen, BII	Eukaryotic expression vector for the production of dimeric receptor-huIgG1 fusion proteins secreted from the myeloma cell line J558L
pCD4huIgM	K. Karijalainen, BII	Eukaryotic expression vector for the production of decameric receptor-huIgM fusion proteins secreted from the myeloma cell line J558L
pCD4huIgG1^{mutant}	K. Karijalainen, BII	Insect expression vector for the production of dimeric receptor-huIgG1 fusion proteins secreted from *Drosophila* Schneider S2 cells, the Fc part is mutated to diminish FcR binding

Table 2.13: Summary of cloned constructs

Name	Vector	Insert (cloning sites)
huTREM-1/pCR2.1	PCR2.1-TOPO	TREM-1 (-)
huTREM-1/pAC-4	pAC-4	TREM-1 (*Nco*I, *Bam*HI)
huTREM-1/pCD4huIgG1	pCD4huIgG1	TREM-1 (*Sst*I, blunt)
huTREM-1/pCD4huIgM	pCD4huIgM	TREM-1 (*Sal*I, *Hin*dIII)
huTREM-1/pCMV-1^{FLAG}	pCMV-1^{FLAG}	TREM-1 (*Hin*dIII and *Xba*I)
huTREM-2/pCR2.1	PCR2.1-TOPO	TREM-2 (-)
huTREM-2/pCD4huIgG1	pCD4huIgG1	TREM-2 (*Eco*RI and *Hin*dIII)
huTREM-2/pCD4huIgM	pCD4huIgM	TREM-2 (*Sal*I, *Hin*dIII)
huTREM-2/pCMV-1^{FLAG}	pCMV-1^{FLAG}	TREM-2 (*Hin*dIII and *Xba*I)
huTREM-4/pCR2.1	PCR2.1-TOPO	TREM-4 (-)
huTREM-4/pCD4huIgG1	pCD4huIgG1	TREM-4 (*Eco*RI and *Hin*dIII)
huTREM-4/pDISPLAY^{HA}	pDISPLAY^{HA}	TREM-4 (*Bgl*I and *Sal*I)
huTREM-5/pCR2.1	PCR2.1-TOPO	TREM-5 (-)
huTREM-5/pCD4huIgG1	pCD4huIgG1	TREM-5 (*Eco*RI and *Hin*dIII)
huTREM-5/pCMV-1^{FLAG}	pCMV-1^{FLAG}	TREM-5 (*Hin*dIII and *Xba*I)
mTREM-1/pCR2.1	PCR2.1-TOPO	mTREM-1 (-)
mTREM-1/pCD4huIgG1^{mutant}	pCD4huIgG1^{mutant}	mTREM-1
mTREM-1/pCD4huIgG1	pCD4huIgG1	mTREM-1 (*Eco*RI and *Hin*dIII)
mTREM-1/pCMV-1^{FLAG}	pCMV-1^{FLAG}	mTREM-1 (*Hin*dIII and *Xba*I)
mTREM-2/pCR2.1	PCR2.1-TOPO	mTREM-2 (-)
mTREM-2/pCD4huIgG1	pCD4huIgG1	mTREM-2 (*Eco*RI and *Hin*dIII)
mTREM-2/pCD4huIgM	pCD4huIgM	mTREM-2 (*Sal*I, *Hin*dIII)
mTREM-2/pCMV-1^{FLAG}	pCMV-1^{FLAG}	mTREM-2 (*Hin*dIII and *Xba*I)
mTREM-3pCR2.1	PCR2.1-TOPO	mTREM-3 (-)

2.3.3 Basic molecular biological methods

2.3.3.1 Determination of nucleic acid concentrations

The concentration of dsDNA, ssDNA and RNA was determined at 260 nm wavelength (Spectramax 250 (Molecular Devices)). Concentrations were calculated as follows

OD_{260} (dsDNA) x 50 x dilution = c(dsDNA) [µg/ml]

OD_{260} (ssDNA) x 33 x dilution = c(ssDNA) [µg/ml]

OD_{260} (RNA) x 40 x dilution = c(RNA) [µg/ml]

2.3.3.2 Agarose gel electrophoresis

Gel electrophoretic separation of DNA for analytical or preparative purposes was performed if not otherwise stated, using 1 % gels of low-endotoxin agarose (Roche) in TAE buffer (2M Tris-HCl pH 8.0, 1 mM EDTA) already containing 10 µg/ml ethidium bromide (EtBr) (Sigma). 6 x sample buffer (Promega) was added to the sample. Electrophoresis was performed at 3 – 5 V/cm corresponding to 50 - 85 V using Horizon 58 gel electrophoresis chambers (BRL).

2.3.3.3 Preparation of transformation competent bacteria

2.3.3.3.1 Electrocompetent cells

2 x 5 ml Luria broth (LB) medium (Tryptone (Difco) 10 g/l, Yeast extract (Difco) 5 g/l, NaCl (Sigma) 10 g/l) were inoculated with a single colony and grown until turbidity was just visible (3-4 h). 4 x 2.5 ml were transferred to 4 x 250 ml LB in 2 l Erlenmeyer flasks and shaken at 37°C until OD_{600} reached 0.4 (3-4 h). The bacterial suspension was chilled for 10 min on ice, and cells were harvested by centrifugation (8000 g, 10 min, 4°C). The following manipulations were performed on ice and with pre-cold materials. The bacterial pellet was resuspended in 2 x 10 ml 1 mM HEPES pH 7.0 (sterile), diluted to 2 x 500 ml with 1 mM HEPES pH 7.0 (sterile), and centrifuged as before. After resuspension in 2 x 200 ml 1 mM HEPES pH 7.0 (50 ml BLUEMAX tubes (Falcon)) and centrifugation (1800 g, 15 min, 4°C), the pellet was resuspended once more in 2 x 1 ml 10 % Glycerol in H_2O (sterile), cells were aliquoted in 40 µl portions and frozen in liquid nitrogen. Storage occurred at – 80°C.

2.3.3.3.2 Chemical competent cells (*E. coli* AVB101)

A single colony of AVB101 was grown over night (ON) in 100 ml LB/0.4% Maltose. 100 ml LB/0.4% Maltose was inoculated with 1.4 ml ON culture and grown until OD600 reached 0.8. The bacterial suspension was chilled for 10 min on ice, and cells were harvested by centrifugation in 50 ml BLUEMAX tubes (Falcon) (4000 g, 10 min, 4°C). The following manipulations were performed on ice and with pre-cold materials. The bacterial pellet was resuspended in 40 ml 50 mM $CaCl_2$ and kept on ice for 20 min. After centrifugation (4000 g, 10 min, 4°C) cells were resuspended in 1.5-2.5 ml 50 mM $CaCl_2$. After adding an equal amount of 15 % (v/v) ice-cold Glycerol,

cells were aliquoted in 40 µl portions and frozen in liquid nitrogen. Storage occurred at – 80°C.

2.3.3.4 EST-search and full length cDNA assembly

GenBank expressed sequence tagged database (dbEST) was initally searched with the amino acid sequences of NKp44 (Cantoni et al., 1999) and CMRF35 (Jackson et al., 1992) using the tblastn algorithm. One sequence (accession n. D78812) with no matches in the GenBank non-redundant database (nr) was selected and re-analyzed against the dbEST database to detect overlapping cDNAs. Seventeen distinct cDNAs were assembled in a single contig using the gel-assemble function of Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI. This contig contained an open reading frame encoding TREM-1 putative polypeptide. This open reading frame was used to search the databases for TREM-1 homologues, leading to the identification of contigs encoding TREM-2, -3, -4 and –5.

2.3.3.5 Reverse Transcriptase-Polymerase chain reaction (RT-PCR)

2.3.3.5.1 Isolation of total RNA

The method used is modified from the single-step RNA isolation method developed by Chomcynski and Sacchi (Chomczynski and Sacchi, 1987) using 1 ml TRIzol Reagent (Gibco, BRL)/1 x 10^6 cells. Total RNA was prepared according to the manufacturer's protocol.

2.3.3.5.2 Reverse Transcription (RT)

The reverse transcription (RT) of eukaryotic mRNA was performed using the "SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis" (GibcoBRL) according to the manufacturer's protocol.

2.3.3.5.3 Polymerase chain reaction (PCR)

Table 2.14: Set up and programs for PCR

	Normal PCR	Long-range PCR	High fidelity PCR
MIX I:	5 µl 10 x buffer 19 µl H_2O 1 µl Taq (2.5 U)	5 µl 10 x buffer 3 17 µl H_2O 3 µl Pfu/Taq mix (10 U; Roche)	5 µl 10 x *Pfu* buffer 19 µl H_2O 1 µl Pfu (5 U; Roche)
MIX II:	10 µl dNTPs (stock: 4 x 2.5 mM) 5 µl Primer 1 (c_{Final}: 250 nM) 5 µl Primer 2 (c_{Final}: 250 nM) 1 µl Template DNA (50-100 ng) 4 µl H_2O	10 µl dNTPs (stock: 4 x 2.5 mM) 5 µl Primer 1 (c_{Final}: 500 nM) 5 µl Primer 2 (c_{Final}: 500 nM) 1 µl Template DNA (500 ng) 4 µl H_2O	10 µl dNTPs (stock: 4 x 2.5 mM) 5 µl Primer 1 (c_{Final}: 250 nM) 5 µl Primer 2 (c_{Final}: 250 nM) 1 µl Template DNA (50-100 ng) 4 µl H_2O
Program	1) Hot start 94°C for 5 min 2) 94°C for 45 s 50-62°C for 15°s 72°C for 60 s 25-35 cycles 3) 72°C 10 min 4) hold at 4°C	1) Hot start 95°C for 5 min 2) 95°C for 60 s 50-62°C for 60°s 72°C for up to 13 min 35 cycles 3) 72°C 10 min 4) hold at 4°C	1) Hot start 95°C for 5 min 2) 95°C for 45 s 50-62°C for 15°s 72°C for 60 s 25-35 cycles 3) 72°C 10 min 4) hold at 4°C

The annealing temperature of primers was determined considering template-matching nucleotides (Σ {[(A+T) x 2°C]+ [(G+C) x 4°C]}). When RT-PCR yielded low or undecteable amount of DNA, the amplified product was subjected to a further amplification using nested oligonucleotides. For long-range PCR (product size > 2 kb) the "Expand long PCR template System™" (Roche) was used according to manufacturer's protocol. For normal-range PCR (product size < 2 kb) 10 x PCR buffer was used (500 mM KCl, 100 mM Tris-HCl pH 8.4, 15 mM $MgCl_2$, 1 mg/ml gelatin). High-fidelity PCR (constructs for fusion proteins) was performed using *Pfu* polymerase with the appropriate buffers (Promega) according to the manufacturer's protocol. PCR reaction solutions and programs are summarized in Table 2.15.

For TREM-2, TREM-4, mTREM-1, and mTREM-3, blast search of NCBI databases yielded cDNA sequences lacking a 3' end. In these cases, 3'end was cloned by 3' Rapid Amplification of cDNA Ends (RACE) using the marathon kit (Clonetech) according to the manufacturer's protocol.

2.3.3.6 General procedure for cloning PCR products

2.3.3.6.1 Preparation of plasmid DNA from *E.coli* cultures

All plasmid DNAs were prepared with QIAGEN Maxi-prep and Mini-prep Kits according to the manufacturer's protocols and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8).

2.3.3.6.2 Restriction enzyme digest of plasmid DNA

To prepare the DNA fragments for ligation or to analyze the products of ligation after transformation, restriction digests at 37°C were performed. 0.1 – 5 µg of fragment or plasmid DNA were digested in the appropriate restriction enzyme buffer in a volume of 5 – 20 µl per µg DNA. For analytic purposes, 0.5 µg were digested for 60 min with an enzyme activity of 5 U/µg DNA. Preparative digests were performed for at least 2 h using 10-20 U/µg DNA. All restriction enzymes were obtained from NEB or Promega together with the provided buffers. After digestion the enzymes were inactivated at 60°C or 80°C according to manufacturer's protocols.

2.3.3.6.3 Phosphatase treatment of DNA

For removal of 5'-phosphate groups from digested vectors to prevent religation, 1 U of calf intestine alkaline phosphatase (AP, Roche) was added after the digest to the reaction mixture and incubated for another 30 min at 37°C. AP was inactivated by adding EDTA to a final concentration of 5 mM and heating for 10 min at 75°C. To remove the AP, agarose gel electrophoresis was performed and the dephosphorylated DNA fragments were purified from the gel.

2.3.3.6.4 Ethanol precipitation of DNA

1/10 volume of 3 M $Na^+CH_3COO^-$ pH 5.3 and 2 volumes of ethanol were added to the DNA containing solution and thoroughly mixed. After incubation at – 80°C for 15 min, the DNA was pelleted by centrifugation (20 000 x g, 30 min, 4°C), washed 2 x with 70 % Ethanol and air-dried. DNA was dissolved in water or TE buffer, incubated for 10 min at 65°C and then resuspended.

2.3.3.6.5 Purification of DNA from agarose gels

Agarose gels were examined under UV light at 366 nm wavelength and the bands of interest excised. Purification of DNA fragments was performed using the QIAquick gel extraction kit (QIAGEN) according to the manufacturer's protocol.

2.3.3.6.6 Ligation of DNA fragments into vectors

Direct cloning of PCR products into pCR2.1-TOPO was performed using the TOPO TA cloning system (Invitrogen) according to the manufacturer's protocol. Ligation of fragments with cohesive ends were performed at a molar ratio between vector (up to 0.5 µg) and insert of 1 : 4 in a volume of 10 µl using the T4 ligase and ligase buffer from NEB.

2.3.3.6.7 Transformation of E.coli

Ligations performed with the TOPO TA cloning system (Invitrogen) were directly transformed in One Shot TOP10 chemically competent *E. coli* (Invitrogen) according to the manufacturer's protocol.

Electro competent E.coli XL-1 blue were transformed as follows: 50 µl aliquots were thaw on ice, 2 µl ligation mix added and the suspension transferred to a pre-cooled electroporation cuvette (0.1 or 0.2 cm electrode distance, Biorad); the bacteria were pulsed with 2.5 kV, 3 µF (0.2 cm cuvettes) or 1.8kV, 3 µM (0.1 cm cuvettes) and immediately taken up in 1 ^ml of pre-warmed SOC-medium (Tryptone (Difco) 20 g/l, Yeast-extract (Difco) 5 g/l, NaCl (Sigma) 10 mM, KCl (Sigma) 4 mM, $MgCl_2$ (Sigma) 20 mM, Glucose (Fluka) 20 mM); after shaking (250 rpm, 60 min, 37°C) bacteria were centrifuged (500 x g, 10 min, RT), resuspended in 100 µl SOC-Medium and plated onto selecting agar plates; after incubation ON at 37°C, colonies were expanded in selectionmedium, mini-preps performed and isolated DNA analyzed for correct ligation by restriction digest.

2.3.3.7 Sequencing of Plasmid, λ-phage and BAC-DNA

DNA sequencing was performed by capillary electrophoresis (ABI PRISM, Genetic Analyzer 310) using fluorescent terminator di-desoxynucleotides supplied in a ready to go solution (Big Dye, Perkin Elmer). The sequencing reaction was performed on a GeneAmp PCR System 9600 (Perkin Elemer) (Table 2.15). DNA products were precipitated adding 3 volumes of isopropanol and incubated at RT for 15 min. After centrifugation, the pellet was washed in Ethanol and 70 % Ethanol. The SN was removed completely and the sample dried in a vacuum centrifuge for 10 – 15 min. The pellet was resuspended in 30 µl Template Suppression reagent (Perkin Elmer), vortexed, boiled for 2 min and chilled on ice. Sequence analysis was performed using the ABI PRISM Autoassembler software (version 1.4.0).

Table 2.15: Set up and programs for Cycle sequencing

	Plasmid DNA	λ-Phage DNA	BAC DNA
Mix: DNA	11.8 µl (1 µg DNA)	23.6 µl (2 µg DNA)	59 µl (2 µg DNA)
Primer	0.2 µl (40 ng)	0.4 µl (40 ng)	1 µl (60 ng)
Big Dye	8 µl	16 µl	40 µl
Program:	Hot start	Hot start	Hot start
	96°C for 30 s	96°C for 30 s	96°C for 30 s
	50°C for 15°s	50°C for 20°s	50°C for 20°s
	60°C for 60 s	60°C for 4 min	60°C for 4 min
	25 cycles	50 cycles	75 cycles
	hold at 4°C	hold at 4°C	hold at 4°C

2.3.4 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Schreiber et al. (Schreiber et al., 1989) with some modifications. Stimulation of monocyte-derived human DCs (10^7) with control or anti-TREM-2 antibody or with LPS was carried out for 0.5 or 4 h at 37°C as described above (2.2.5). Cells were washed in PBS, resuspended in 10 ml of ice-cold buffer A (10 mM Tris-HCl pH 7.9, 60 mM KCl, 1 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin), and incubated for 15 min on ice. NP-40 was added from a 10% stock solution to a final concentration of 0.6%, and samples were vortexed for 10 s. After incubation for 3 min on ice, samples were centrifuged at 3000 rpm for 10 min at 4°C. Nuclei were washed in 10 ml of ice-cold buffer A and resuspended in 30 ml of ice-cold buffer C (20 mM Tris-HCl pH 8, 0.4 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 25% glycerol). Nuclei were incubated for 30 min at 4°C, and nuclear extracts were separated from debris by centrifugation at 15.000 x g for 15 min at 4°C. Protein concentrations were determined by Bradford assay. NF-κB consensus and mutant binding sites were 5'-AGTTGAGGGGACTTTCCCAGGC and 5'-AGTTGAGGCGACTT-TCCCAGGC, respectively. Annealed binding sites were radiolabeled using polynucleotide T4 kinase and $\gamma[^{32}P]$-ATP. Radiolabeled oligonucleotides were purified by electrophoresis through an 8% polyacrylamide gel containing 22.5 mM Tris-borate and 0.5 mM EDTA, overnight elution from gel slices at 37°C, concentration using Elutip-d columns (Schleider & Schuell), and ethanol precipitation. EMSAs were performed as described previously (Hernandez-Munain et al., 1994) with some modifications. Nuclear extracts (2µg) were incubated with 1µg of poly(dI-dC) carrier and 1 µg of BSA in a 25 µl of reaction mix containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol for 10 min at 4°C in the presence or absence of 25-fold excess of unlabeled oligonucleotide competitors. Labeled binding-site probes (15 fmols, ~ 5 x 10^4 cpm) were then added for an additional 20 min of incubation at 4°C. Samples were electrophoresed through a 4% polyacrylamide gel containing 22.5 mM Tris-borate and 0.5 mM EDTA at 4°C.

2.4 Animal models

2.4.1 Disease models

2.4.1.1 Models for Septic Shock

2.4.1.1.1 High dose LPS-induced Endotoxemia.

C57BL/6 mice (female, 8-10 weeks, 19-22 g) were randomly grouped (5-10 mice per group) and injected intraperitoneally (i.p.) with different concentrations of LPS from E. coli 055:B5 (Sigma), in a blinded fashion. 500 µg/mouse of purified huIgG1,κ (Sigma), mTREM-1-IgG1, huILT3-IgG1 (Cella et al., 1997), or heat-inactivated mTREM-1-IgG1 (30 min, 95°C) was administrated i.p. at 1, 2, 4, 6 h after or 1 h prior to LPS administration. Viability of treated mice was monitored 4-6 times a day for at least 10 days. To monitor the inflammatory process blood and peritoneal lavage was isolated and tested as described in 2.4.2.

2.4.1.1.2 *E. coli* peritonitis model.

E coli peritonitis was induced in mice as described previously (Appelmelk et al., 1986). Briefly, C57BL/6 mice (female, 8-10 weeks, 19-22 g) were weighed and randomly distributed into groups of 5-15 animals of equal body weight. Mice were injected i.p. with 500 µg of mTREM-1-IgG1 or control huIgG1 prior to i.p. administration of 500 µl of a suspension of *E. coli* O111:B4 (1.6-2.1 x 10^6 CFU per mouse).

2.4.1.2 Experimental autoimmune Encephalomyelitis

2.4.1.2.1 Myelin oligodendrocyte glycoprotein (MOG) peptids

MOG^{35-55} (MEVGWYRSPFSRVVHLYRNGK) and MOG^{92-106} (DEGGYTCFFRDHSYQ) were synthesized by David Avila at a BII peptide facility on a ABM 430A synthesizer (Applied Biosystems) using fluorenylmethoxycarbonyl (F-MOC) chemistry. The peptides were >92 % pure, as determined by HPLC.

2.4.1.2.2 Induction and Evaluation of EAE

C57BL/6 mice (female, 10 weeks, 19-21 g) were randomly grouped (5-10 mice per group) and injected with an emulsion of 100 µg MOG25-55 in FCA H37Ra (Difco # 231131) s.c. in all four flanks (50 µl emulsion/25 µg peptide per flank) together with an i.p. injection of 200 ng pertussis toxin in 200 µl PBS (Fluka # 77339). 400 µg/mouse of purified huIgG1,κ (Sigma), purified huIgM (Sigma), mTREM-1-huIgG1, or mTREM-2-huIgM was administrated in 400 µl PBS i.p. one day before, 3

and 6 days after EAE induction. Animals were monitored daily for onset of disease, clinical symptoms and weight for up to 50 days after EAE induction.

For the clinical evaluation of EAE, the following scale was used: 0, no clinical disease; 0.5, partial tail weakness; 1, tail weakness; 1.5, paraparesis type I (incomplete paralysis of the hip); 2, paraparesis type II (incomplete paralysis of one ore two hind limbs); 3, paraplegia (complete paralysis of one or two hind limbs); 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead animals.

2.4.2 Investigations of Inflammatory parameters

2.4.2.1 Isolation of organs and lavage fluids

2.4.2.1.1 Blood

Blood (250 µl) was collected from the tail vein of treated and control mice. In some experiments, blood was obtained from the vena cava. In these cases, 20 µl Heparin solution was injected i.p. before opening the peritoneal cavity. Blood was withdrawn from the peritoneal cavity after cutting the vena cava. For flow cytometry total blood cells were used. For detection of cytokines in the serum, blood was collected into a Serum Separator Tube (Becton Dickinson) and the obtained serum was analyzed by ELISA.

2.4.2.1.2 Peritoneal lavage cells (PLCs)

The peritoneal cavity of individual mice were cannulated with a 22-G needle, 3 – 5 ml ice-cold PBS was injected and withdrawn repeatedly with a syringe. This procedure was repeated three times until a final volume of 10 ml PL was collected. PLCs were harvested and used for determination of total cell numbers per PLCs within a Coulter Counter (IG Instrumenten Gesellschaft) for preparation of differential cell counts and flow cytometry analysis. PL Fluid (PLF) was concentrated over a Biomax 5K membrane (MWCO 5000Da, Millipore) and used to determine TNF-α and IL-1β levels by ELISA.

2.4.2.1.3 Bronchoalveolar lavage (BAL) cells

The tracheae of individual mice were cannulated with a 22-G needle surrounded by a plastic tubing (Polyethylene [PE] Intramedic Tubing; Becton Dickinson). 0.3 ml PBS was injected and withdrawn repeatedly with a syringe. This procedure was repeated three times until a final volume of 1.2 ml BAL was collected. BAL cells were harvested and used for determination of total cell numbers per BAL within a Coulter Counter (IG Instrumenten Gesellschaft) for preparation of differential cell counts and

flow cytometry analysis. Bronchoalveolar lavage fluid (BALF) was concentrated over a Biomax 5K membrane (MWCO 5000Da, Millipore) and used to determine TNF-α and IL-1β levels by ELISA.

2.4.2.1.4 Bone marrow (BM) cells

Animals were sacrificed using CO_2; hind legs were isolated and freed from all feer and muscle tissue using sterile forceps and scissors. The most upper part of the femur (1 mm) was cut off and the leg placed in a P-200 yellow pipette Tipp with femur pointing downwards. The loaded pipette Tipp was put in a 15 ml Bluemax tube (Falcon) containing 500 µl I-Medium and centrifuged at 4°C, 800 x g, for 8 min. the cell pellet was resuspended and kept on ice until use

2.4.2.1.5 Spleen and lymph node (LN) cells

Animals were sacrificed; LN or Spleen was excised using sterile forceps and scissors and kept in ice-cold I-Medium. Organs were teased to single-cell suspension using 70 µm cell strainers (Falcon, New Jersey, USA) and a 2 ml syringe plunger (Falcon). Cells were sedimented (10 min, 180 x g, 4°C), resuspended in I-Medium and kept on ice until use

2.4.2.1.6 Mononuclear cells from Brain

Brain was isolated using steril forzeps and sicors and kept in a 6 well plate containing I-Medium. The brain was further cut in to smaller pieces using a scalpel. Brain parts were teased to single-cell suspension using 70 µm cell strainers (Falcon, New Jersey, USA) and a 2 ml syringe plunger (Falcon). Cells were sedimented (10 min, 180 x g, 4°C) and RBC were lysed by adding 3 ml ddH_2O for 15 s followed by 3 ml 2 x PBS. Cells were washed 2 x in I-Medium and resuspended in 7 ml 70 % Percoll solution (100%: 90% Percoll + 10% 10 x PBS) (7 ml/brain). 7 ml of 40% Percoll solution and 7 ml PBS were added on top of the 70% Percoll solution layer and the resulting gradient was centrifuged for 40 min, 400 x g, w/o brakes. After removal of the top layers (fat and debris), mononuclear cells were isolated from the 40%-70% interface of the gradient. Cells were washed 3 times with PBS counted (brain from naïve or endotoxemia mice: 0.3×10^6/mouse; brain from EAE mice: 0.8×10^6/mouse) and used for flow cytometry.

2.4.2.2 Cell counting

For cell counting using a Coulter Counter (IG Instrumenten Gesellschaft AG), cells were diluted into a coulter isoton II (10 ml, Coultronics) containing 20 µl ZAP-OGLOBIN (Coulter Electonics) to lyse RBC. The number of particles in 0.5 ml was

determined with the appropriate lower and upper threshold settings, excluding aggregates, dead cells and debris.

2.4.2.3 Morphological differentiation

5 x 10^4 isolated cells from individual mice were fixed onto glass slides in PBS supplemented with 20% BSA by cytospins (600 x g, 10 minutes). Cells were differentiated by May-Grünwald/Giemsa staining: After fixation with methanol (2.30 minutes) cells were stained with undiluted May-Grünwald solution (Fluka) for 3 minutes. Thereafter the staining was performed in a 50% May-Grünwald solution for further 3 minutes. In a last step cells were stained in 7% Giemsa solution (Fluka) for 12 minutes. Slides were rinsed with tap water and air-dried over night. The following day cells were embedded within Optiprep solution. The number of morphological differentiated cells in 200 total cells was counted (x%) and the total cell number of each individual cell type in the isolated fluid or organ was calculated.

2.4.2.4 Flow cytometry of mouse cells

2.4.2.4.1 Flow cytometry of PLCs

Four-color analysis of peritoneal leukocytes was performed after blocking FcR (FcR blocking agent, Pharmingen) for 30 min, using anti-mTREM-1, anti-Ly-6G (Pharmingen), anti-Mac-1 (Pharmingen) mAbs conjugated with APC, PE and FITC, respectively, and biotinylated anti-F4/80 followed by streptavidine-CyChrome (Pharmingen).

2.4.2.4.2 Flow cytometry of BAL cells

BAL cells were incubated with anti-CD32/CD16 mAb in PBS/0.1%BSA for 30 minutes at 4°C to block unspecific binding to FcR. After blocking cells were washed with PBS/0.1%BSA followed by surface staining with Cy5-labeled anti-mTREM-1 mAb 50D1, FITC-labeled anti-Mac-1 (BD PharMingen) and Pe-labeled anti-Ly-6G (BD PharMingen). Subsequently, cells were washed with PBS/2% FCS and resuspended in PBS/ 2% FCS to analyze cells by flow cytometry. To analyze intracellular cytokine expression in mTREM-1-positive cells, BAL cells were incubated four hours prior harvesting with Brefeldin A (10 mg/ml; Sigma) to retain cytokines in the cytoplasm. Thereafter cells were washed with PBS/2% FCS and incubated with anti-CD32/CD16 mAb for 30 minutes at 4°C to block Fc-binding. After staining of surface mTREM-1 with Cy5-labeled anti-TREM-1 mAb, cells were washed with PBS/ 0.1%BSA, fixed with 2% PFA for further 30 minutes at room temperature followed by intracellular staining in permeabilization buffer containing 0.5% saponin, PBS/ 1% BSA, Biotin-

labeled anti-IL-1β and PE-labeled anti-TNF-α (BD PharMingen). After a final staining step using Streptavidine-FITC, cells were resuspended in PBS/1%BSA for analysis by three-color flow cytometry. Data were analyzed using CellQUEST^{TM} software (Becton Dickinson).

2.4.2.4.3 Flow cytometry of brain, LN, BM and spleen cells

Four-color analysis of leukocytes was performed after blocking FcR (FcR blocking agent, Pharmingen) for 30 min, using anti-mTREM-1, anti-Ly-6G (Pharmingen), anti-Mac-1 (Pharmingen) mAbs conjugated with Cy5, PE and FITC, respectively. In some experiments cells were stained using anti-TREM-1, anti-Ly-6C (Pharmingen), and anti-PECAM mAb (Pharmingen) conjugated with Cy5, PE and FITC, respectively. Dead cells were excluded by gating on PI-negative cells.

3 RESULTS

3.1 Cloning of a novel group of receptors: Triggering receptors-expressed on myeloid cells (TREMs)

3.1.1 Molecular characterization of the TREM family

3.1.1.1 Identification and cloning of a novel family of transmembrane proteins of the Ig SF by EST database search

The GenBank EST database was searched with NKp44 (Cantoni et al., 1999) and CMRF35 polypeptides (Jackson et al., 1992) and several overlapping cDNAs were found (Table 3.1). These were assembled in a contig which contained an open reading frame encoding a protein of 234 amino acids with a predicted molecular mass of ~26 kDa (Figure 3.1). A cDNA containing the entire open reading frame was amplified by RT-PCR from monocytes and granulocytes, exclusively (data not shown).

Table 3.1 : EST cloning of the Triggering receptors expressed on myeloid cells (TREMs)

Name	Bait for search	ESTs Accesion no.	Full length (AA/bp)	Cloning source	Accession no. of cDNA
TREM-1	NKp44, CMRF35	D78812, AI337247, AW139572, AW274906,AW139573,AI394041, AI621023, AI186456, AI968134, AI394092, AI681036, AI962750, AA494171,AA099288,AW139363, AW135801, AA101983	234/702	Human Monocytes	NM018643
TREM-2	TREM-1	N41388	230/690	Human MDCs	NM018965
TREM-3	TREM-2	AL391903 (genomic sequence)		No cDNA cloned	Not released
TREM-4a	CMRF-35	U70073	233/699	Human MDCs	Not released
TREM-4b	CMRF-35	U70073	222/666	Human MDCs	Not released
TREM-5	CMRF-35	BI908345	201/603	Human BM	Not released
mTREM-1	TREM-1	AA895986	230/690	Mouse neutrophils	NM021406
mTREM-2b	TREM-2	AA030138	227/681	Mouse BM	NM021410
mTREM-3	TREM-1	AA896129, AA052136	183/549	Mouse Liver	NM021407
mTREM-4	TREM-4	AK009375	222/666	Mouse tongue	Not released
mTREM-5	TREM-5	BG245629	196/588	Mouse mammary tumor	Not released

Therefore, this molecule was designated "**T**riggering **R**eceptor **E**xpressed on **M**yeloid cells**-1**" (TREM-1). The GenBank EST database was then searched with TREM-1 polypeptide and several novel cDNAs encoding TREM-1-homologs in mouse (m) and man were identified. Accordingly, these molecules were named TREM–2, –3, –4a, –4b, –5 and mTREM–1, –2, –3, –4 and –5, respectively (Figure 3.1). Full-length cDNA clones from TREM–1, –2, –5, mTREM–1, –2, and –3 were obtained from different sources of myeloid cells but not from others cell types such as lymphocytes (Table 3.1). Interestingly, TREM-3 seems to be a pseudo-gene in humans, since the cDNA displays a nucleotide substitution, which results in a premature in-frame STOP codon (Figure 3.1). With the exception of the intracellular domain of TREM-4, everything

could be cloned from monocytes-derived DCs. Database searches revealed two potential intracellular regions for TREM-4, thus the according sequences were termed TREM-4a and –4b. Recently, Daws and coworkers identified an additional gene highly homologues to mTREM-2, called mTREM-2a. mTREM-2 was henceforth referred to as mTREM-2b (Daws et al., 2001).

3.1.1.2 Sequence comparison between TREMs

As shown in Figures 3.1, all TREM amino acid sequences start with a hydrophobic signal peptide followed by an extracellular region composed of a single Ig-SF domain containing three (TREM-1), one (TREM-2, TREM-4a, TREM-4b, mTREM-1, mTREM-3, mTREM-4, mTREM-5,) or no (TREM-5, mTREM-2a, -2b, mTREM-5) potential N-glycosylation sites. The length of the Ig-fold and the characteristic motifs

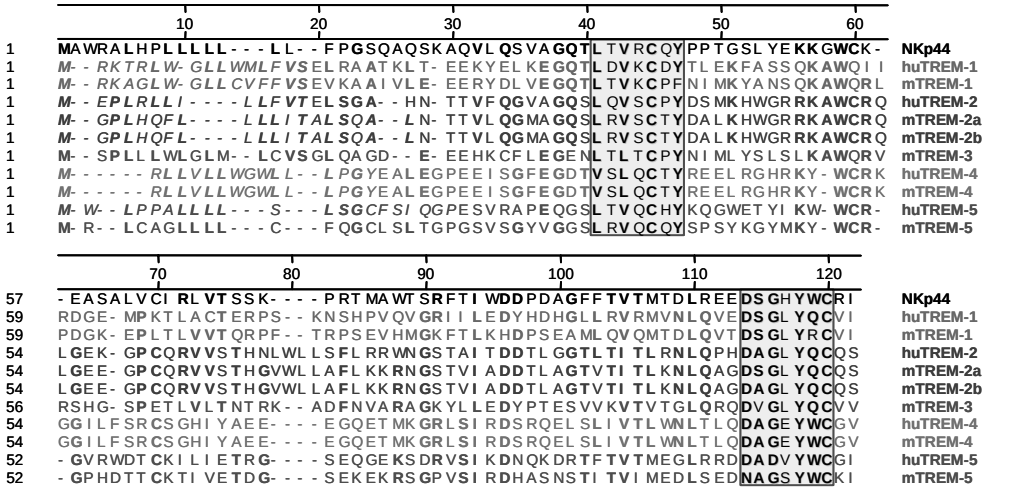

Figure 3.1: Signal sequences and V-type domain of human and mouse TREMs aligned with NKp44.

Alignment of the V-type domain from human and mouse TREMs (TREM-1 (red), TREM-2 (blue), TREM-3 (grey), TREM-4 (light blue), TREM-5 (green) and NKp44 (black). The signal peptide is indicated in italic letters. The cysteines potentially involved in generating the intra-chain disulfide bridge of the Ig-SF V-type fold are boxed in yellow and their flanking consensus sequences are shown in bold. TREM-1, -2, and –3 sequences have been submitted to GenBank database under accession no. indicated in Table 3.1. Amino acids identical in at least four out of 11 sequences are bold. The alignment was generated by Clustal method. Gaps (dashes) were introduced to maximize homologies.

flanking the cysteins (**L/VxL/VxCxY** and **D/NxGxYxC**) indicate that the Ig-fold is of the V-type (Barclay Leukocyte Facts book, second edition, Academic Press). It is of note that TREM-1, mTREM-1 and mTREM-3 contain a longer connective peptide than the other TREMs (Figure 3.1). In contrast to the extracellular domain, the cytoplasmic tails are variable in length (2 amino acids (mTREM-3) to 44 amino acids (TREM-4a)), however they do not contain any evident signaling motifs. The homologies and phylogenetic connections between the human TREMs and mouse TREMs are indicated in Figure 3.2 and 3.3.

The transmembrane domain of all TREMs contains a charged amino acid residue, either a lysine (human and mouse TREM-1, -2, -3 and -5) or an arginine residue (human and mouse TREM-4). Similar transmembrane and cytoplasmic domains are present in activating NK cell receptors, which pair with the transmembrane adapter protein DAP12 (Lanier and Bakker, 2000; Moretta et al., 2001), DAP10 (Chang et al., 1999; Wu et al., 1999) or FcRγ (Ravetch and Bolland, 2001). Alignment of the transmembrane regions revealed that TREM-1, -2, mTREM-1, -2a, -2b, and –3 and the known DAP12-associated receptors KIR2DS4 (Olcese et al., 1997), NKp44 (Cantoni et al., 1999), NKp44 like gene 1 and 2 (Accesion no. AJ010099 and AJ010100) have a highly homologues transmembrane domain containing the consensus sequence **CxxLxKxLxxSxL** (Figure 3.4). Surprisingly, human and mouse TREM-5, although containing a lysine residue in the transmembrane domain, do not have this motif (Figure 3.4). Similar to NKG2D, CD64, CD16 and ILT1, mouse and human TREM-4 display an Arginine residue instead of a lysine. DAP10 or FcRγ were previously described to pair with such receptors (Chang et al., 1999; Wu et al., 1999) (Ravetch and Bolland, 2001). Alignment revealed that TREM-4 transmembrane domains have little in common with NKG2D, CD64 and CD16, but are highly identical to the transmembrane domain of ILT1 (Figure 3.4). Interestingly, these receptors share a **RxxAxxLVLxxL** motif. Biochemical methods will be required to determine whether any of these motifs are indeed responsible for the specific association with DAP12/KARAP (Lanier and Bakker, 2000; Moretta et al., 2001), DAP10 (Chang et al., 1999; Wu et al., 1999) or FcRγ (Ravetch and Bolland, 2001).

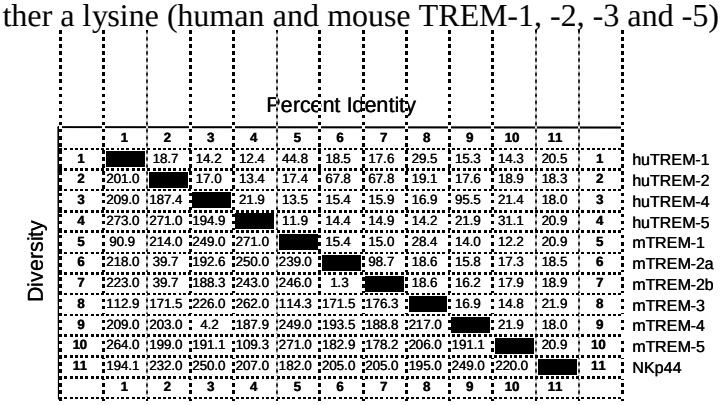

Figure 3.2: Identity (%) and diversity of the complete amino acid sequences of TREM family members and NKp44.

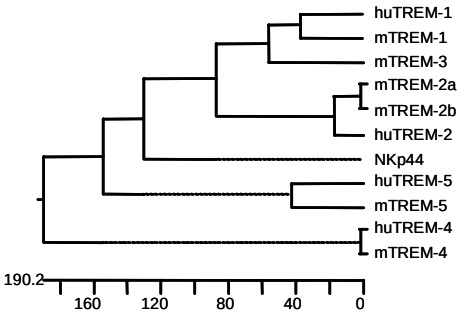

Figure 3.3: Phylogenetic tree of TREM family members.
Three groups can be clearly identified. One includes NK44, TREM-1, -2, and –3, whereas TREM-4 and –5 constitutes seperate groups. The scale represents million of years.

The analysis of somatic cell hybrids containing different human chromosomes demonstrated that the genes encoding TREM-1, -2, and 3 map on human chromosome 6, like the NKp44 gene (data not shown), whereas TREM-4 and –5 map to chromosome 17, respectively. This location was confirmed by the recent release of human chromosome 6 (NT_010672.6 | Hs17_10829) and 17 (NT_010755.6 | Hs17_10912) genomic sequences.

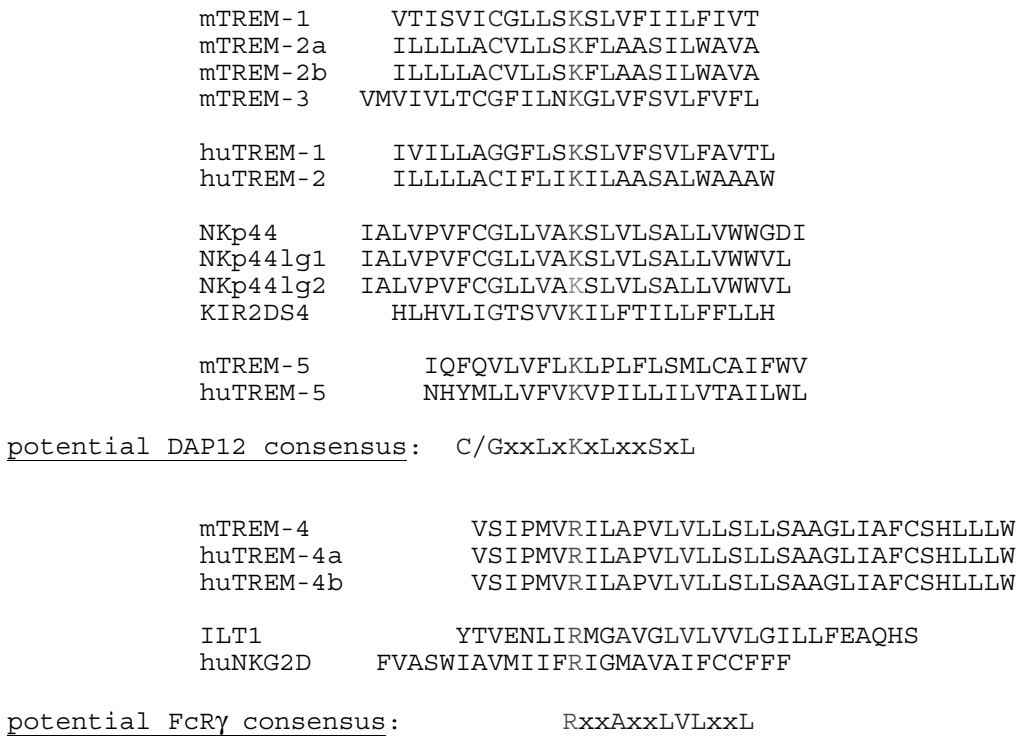

Figure 3.4: Sequence comparison of transmembrane domains from TREMs and other activating receptors

embrane regions compared to NKp44, NKp44lg1, NKp44lg2, KIR2DS4, ILT1, NKG2D. Charged residues are shown in red, identical anchor residues are shown in blue. Consensus sequences predicted to be responsible for interaction with DAP12 and FcRγ are indicated.

3.2 Characterization of human and mouse TREM-1

3.2.1 Characterization of human TREM-1 *in vitro*

3.2.1.1 TREM-1 is selectively expressed on blood neutrophils, monocytes and alveolar macrophages

To investigate the cellular distribution of TREM-1, anti-TREM-1 mAbs were produced. As shown in Figure 3.5A, the mAb 21C7 stained TREM-1-transfected COS7 cells, as compared to control transfectants. In addition, expression of TREM-1 was partially increased by co-transfection of DAP12 cDNA, suggesting that cell surface expression of TREM-1 may require association with either DAP12 or a related signaling molecule. In peripheral blood of different donors, 21C7 stained neutrophils and, to a lesser extent, $CD14^{high}$ monocytes. $CD14^{dim}$ monocytes, DCs, or lymphocytes were TREM-1 negative (Figure 3.5B). To investigate the expression of TREM-1 during

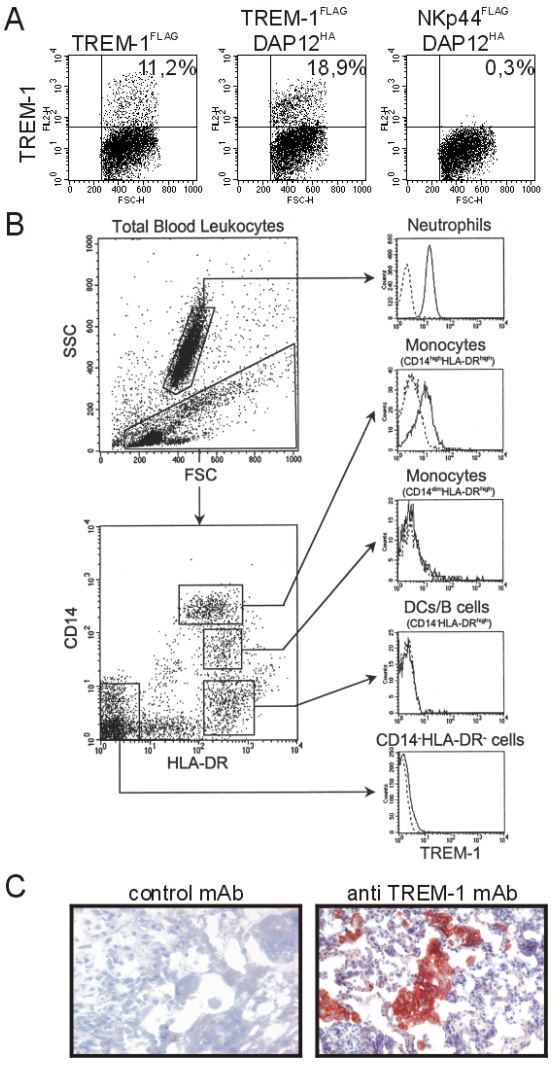

Figure 3.5: TREM-1 is selectively expressed on neutrophils, $CD14^{high}$ monocytes and alveolar macrophages

A. mAb 21C7 recognizes selectively TREM-1. FLAG-tagged TREM-1 ($TREM\text{-}1^{FLAG}$) was expressed in COS7 cells without (left panel) or with HA-tagged DAP12 ($DAP12^{HA}$) (middle panel). Cells were analyzed by FACS with mAb 21C7, as compared to $NKp44^{FLAG}/DAP12^{HA}$ co-transfected cells (right panel). The percentage of TREM-1-positive cells (upper right quadrant) is indicated. Expression of $TREM\text{-}1^{FLAG}$, $NKp44^{FLAG}$ and $DAP12^{HA}$ was confirmed using anti-FLAG and anti-HA mAbs (data not shown). Cells stained with a control antibody were contained within the lower right quadrant.
B. Three color FACS analysis of whole blood leukocytes. High side scatter cells correspond to $TREM\text{-}1^{+}$ neutrophils. Low side scatter cells include $CD14^{high}/HLA\text{-}DR^{+}$ cells (monocytes), $CD14^{high}/HLA\text{-}DR^{+}$ (monocytes), $CD14^{-}/HLA\text{-}DR^{+}$ cells (which include B cells and DCs), and $CD14^{-}/HLA\text{-}DR^{-}$ cells (mostly lymphocytes).
C. TREM-1 is strongly expressed on alveolar macrophages in situ. TREM-1 was detected in lung sections from normal individuals using mAb 21C7 (right panel). A staining with an isotype-matched control mAb are shown in the left panel.

differentiation of $CD14^+$ monocytes into either MDCs or MMϕ, surface TREM-1 levels were monitored over time in the presence of GM-CSF/IL-4 or M-CSF, respectively. TREM-1 was completely downregulated on these cells after three days of culture (data not shown). Stimulation of DCs with LPS, heat-inactivated gram-positive bacteria, gram-negative bacteria, or fungi did not induce TREM-1 expression (data not shown). Interestingly, immunohistochemical analysis of tissue sections revealed that alveolar macrophages were expressing TREM-1 at very high levels (Figure 3.5C) while macrophages in other tissues did not (data not shown). This selective expression of TREM-1 on some of the major initiators of innate responses suggested that it plays a role in acute inflammatory responses.

3.2.1.2 TREM-1 triggers the release of pro-inflammatory chemokines and cytokines, and increases surface expression of activation markers.

To examine whether TREM-1 can trigger acute inflammatory responses, neutrophils and monocytes were plated on a plastic surface coated with F(ab')$_2$ goat anti-mouse IgG and the mAb 21C7 and tested for secretion of chemokines, cytokines and for release of granule components. In neutrophils, cross-linking of TREM-1 induced the secretion of IL-8 and the release of myeloperoxidase (MPO) (Figure 3.6A, B). In monocytes, cross-linking of TREM-1 triggered the release of large amounts of IL-8 as well as MCP-1 and TNF-α (Figure 3.6C-E). In control experiments, neutrophils and monocytes were stimulated with isotype-matched antibodies which either bind (such as anti-MHC class I mAbs) or do not bind (such as an anti-2,4,6 TNP mAb) cells. In both cases, secretion of cytokines, chemokines and MPO was 5- to 50-fold lower than that induced via TREM-1 (Figure 3.6 and data not shown). Thus, the activation of neutrophils and monocytes induced by anti-TREM-1 mAb is not due to engagement of Fc receptors. Similar results were obtained in further experiments using F(ab')$_2$ fragments of the mAb 21C7, confirming the independence of TREM-1-induced effects from FcR binding of stimu-

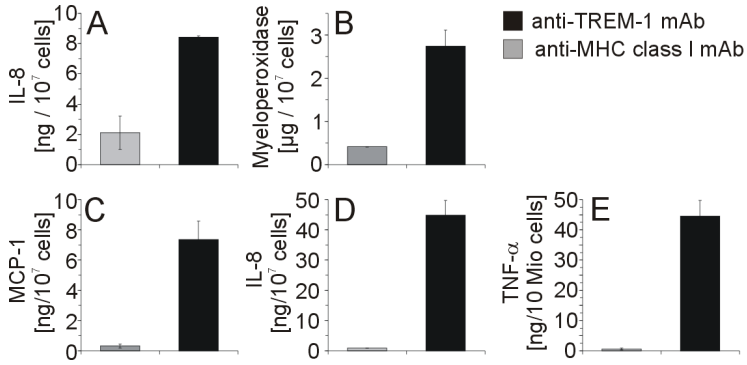

Figure 3.6: TREM-1-mediated cytokine production and degranulation.

TREM-1 triggers release of IL-8 (**A**) and MPO (**B**) in neutrophils and secretion of MCP-1 (**C**), IL-8 (**D**), and TNF-α (**E**) in monocytes. Monocytes or neutrophils were stimulated for 24 h with either anti-TREM-1 or control IgG_1 mAbs (anti-MHC class I mAb) coated on plastic and supernatants were analyzed by ELISA. All data points correspond to the mean and the standard deviation of four independent experiments.

lating mAb (data not shown). Secretion of IL-6, IL-10, IL-12 or type I IFN, were not increased by the engagement of TREM-1 (data not shown).

Table 3.3: TREM-1-dependent regulation of surface markers.

Monocytes or neutrophils were stimulated as in Figure 3.6 and analyzed by flow cytometry. Numerical values indicate specific Mean fluorescence intensity (MFI) after subtraction of the fluorescence detected with an isotype-matched control. The shown data are representative for seven experiments.

	Stimulation for 24h			
	Neutrophils		Monocytes	
	anti-MHC class I	Anti-TREM-1	anti-MHC class I	anti-TREM-1
CD40	-	-	23.9	254.1
CD80/B7.1	-	-	0.6	0.1
CD86/B7.2	-	-	32.6	521.5
CD54/ICAM1	10.9	35.6	27.0	97.5
CD11b	0.4	27.9	234.9	256.8
CD11c	75.3	85.7	175.6	385.5
CD18	54.8	76.9	198.8	211.9
CD49d	21.8	30.2	0.1	4.8
CD49e	76.1	91.9	14.9	46.5
CD29	2.7	14.7	23.2	76.9
CD32/FcRII	86.2	100.2	72.1	114.0
CD83	-	-	0.9	44.6

The rapid migration of neutrophils and monocytes from the blood to the inflammatory site requires their adhesion to the endothelium and to extracellular matrix proteins (Springer, 1994). Therefore it was tested whether engagement of TREM-1 stimulated up-regulation of adhesion molecules. As shown in Table 3.3, cell surface expression of CD29, CD11c, CD49e, and to a lesser extent CD11b, CD49d and CD18, were increased on both neutrophils and monocytes. Thus, TREM-1 may increase cellular adhesion to fibronectin, fibrinogen and VCAM by upregulating CD11b/CD18 (Mac-1), CD29/CD49d and CD29/CD49e heterodimers, respectively. In addition, TREM-1 stimulation led to a strong upregulation of the co-stimulatory molecules CD40, CD86 (B7.2) and CD54 (ICAM-1), as well as of CD83 and CD32 (FcRII) on monocytes. Interestingly, CD83 upregulation could not be observed using $F(ab')_2$ 21C7 for stimulation (data not shown). Thus, TREM-1 is not only capable of increasing adhesion of myeloid cells to endothelium and extracellular matrix molecules but can also prepare monocytes for co-stimulation of other cells recruited to the inflammatory lesions.

3.2.1.3 Stimulation of TREM-1 induces calcium mobilization and tyrosine phosphorylation

Activation of neutrophils and monocytes is often accompanied by a number of intracellular changes. Indeed, ligation of TREM-1 with the mAb 21C7 elicited a rapid rise in intracellular Ca^{2+} concentration (Figure 3.7A). In addition, cross-linking of TREM-1 stimulated tyrosine phosphorylation of several proteins with apparent molecular masses of ~40, ~60, ~70, and ~100 kDa (Figure 3.7B). The observed ~40 kDa tyrosine phosphorylated proteins correspond to mitogen activated protein (MAP) kinases, as demonstrated by anti-phospho-ERK1/2 immunoblotting (Figure 3.7B). Precipita-

tion of tyrosine phosphorylated proteins and immunoblotting with an anti-PLCγ mAb, revealed that the observed ~100 kDa phosphoprotein corresponds to PLCγ (Figure 3.7D), thus explaining the observed Ca^{2+} influx.

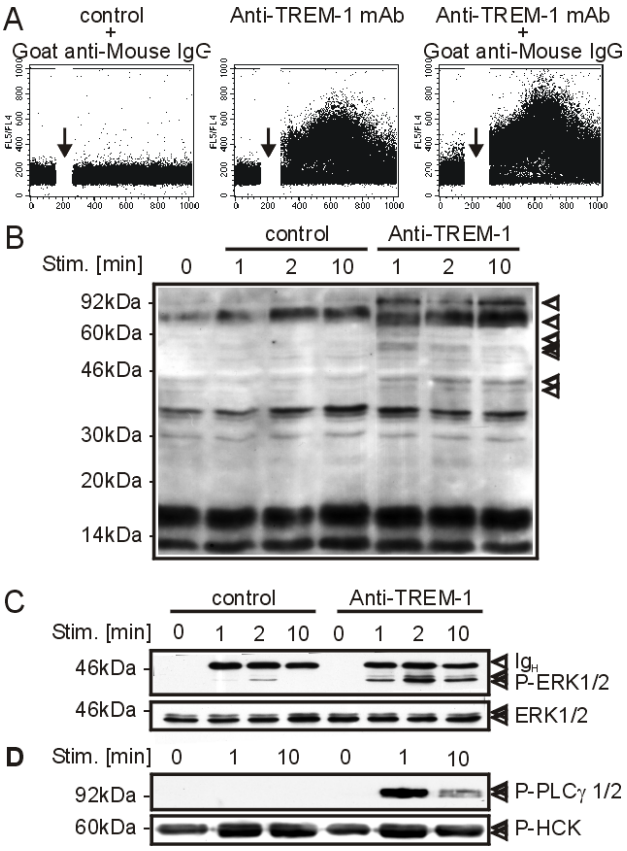

Figure 3.7: TREM-1 triggers intracellular Ca^{2+} mobilization and tyrosine phosphorylation of ERK1, ERK2 and, PLC-γ.

A. anti-TREM-1 mAb induces intracellular Ca^{2+} mobilization in monocytes as compared to a control IgG_1 mAb (anti-MHC class I). Ca^{2+} mobilization occurred even in the absence of a cross-linking Ab. Addition of antibodies is indicated by an arrow.
B. Anti-phosphotyrosine blot of cell lysates from monocytes stimulated with anti-TREM-1 or control IgG_1 mAbs in the presence of a cross-linking Ab for the indicated time periods.
C. Monocytes were stimulated as indicated in (B) and examined by western blot analysis using anti-phospho-ERK 1/2 (upper panel) and anti-ERK 1/2 (lower panel) mAbs.
D. Tyrosine phosphorylated proteins were precipitated from monocytes stimulated as indicated in (B) and immunoblotted with anti-PLC-γ (upper panel) or anti-Hck (lower panel) antibodies. Anti-Hck blotting was performed as a loading control, since phosphorylation of Hck is similar in both stimulated and unstimulated monocytes. Phosphorylated proteins are indicated by arrows in all panels. Molecular weight markers are shown.

3.2.1.4 TREM-1 is a ~30 kDa glycoprotein associated with DAP12.

Biochemical analysis of TREM-1 immunoprecipitated from surface-biotinylated monocytes revealed that TREM-1 is a glycoprotein of ~30 kDa, that is reduced to 26 kDa after N-deglycosylation, in agreement with the predicted molecular mass of TREM-1 (Figure 3.8A). Since TREM-1 lacks known signaling motifs in the cytoplasmic domain, it should associate with a separate signal transduction subunit to mediate activating signals. Adapter molecules, such as DAP12, DAP10 or FcRγ, are tyrosine phosphorylated upon cell treatment with the phosphatase-inhibitor pervanadate (Chang et al., 1999; Lanier et al., 1998b; Nakajima et al., 1999; Wu et al., 1999). Indeed, anti-phosphotyrosine blotting of TREM-1 immunoprecipitates from pervanadate-stimulated monocytes revealed a phosphorylated protein of ~12 kDa and ~24 kDa under reducing and non-reducing conditions, respectively (Figure 3.8B). An identical pattern was observed following the immunoprecipitation of SIRPβ1, which is associated with DAP12 (Dietrich et al., 2000). Indeed, immunoblotting of TREM-1

immunoprecipitates with DAP12, DAP10 or FcRγ antiserum demonstrated that TREM-1 associates with DAP12 (Figure 3.8C and data not shown).

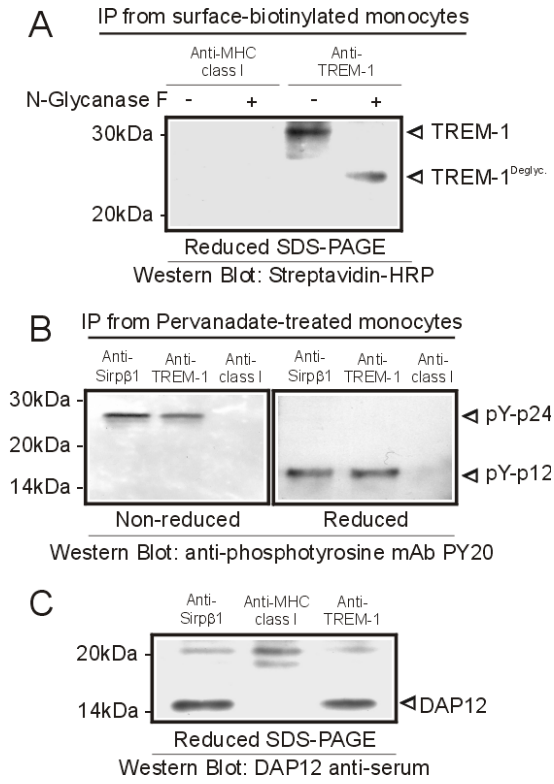

Figure 3.8: TREM-1 is a ~30kDa glycoprotein associated with the adaptor protein DAP12.

A. anti-TREM-1 mAb or control IgG_1 (anti-MHC class I mAb) immunoprecipitates from surface biotinylated monocytes were left untreated or treated with N-Glycanase F, and analyzed by Western Blot analysis with Streptavidine-HRP.
B. Pervanadate-treated monocytes were subjected to immunoprecipitation with anti-TREM-1 mAb, anti-SIRP mAb as a positive control, or control IgG_1. The precipitates were analyzed by anti-phosphotyrosine blot under reducing and non-reducing conditions.
C. Anti-DAP12 blot analysis of a TREM-1 immunoprecipitate from monocytes (reducing conditions). Control IgG_1 (anti-MHC class I mAb) and anti-SIRP mAb immunoprecipitates were included as negative and positive control, respectively. TREM-1 and DAP12 are indicated by arrows. Molecular weight markers are shown.

3.2.1.5 TREM-1 expression and function is potentiated by bacterial stimuli.

The ability of TREM-1 to trigger secretion of pro-inflammatory mediators, prompted the investigation of its role in inflammations caused by bacteria. TREM-1 expression was determined by flow cytometry on neutrophils and monocytes incubated *in vitro* with heat-inactivated gram-positive bacteria, gram-negative bacteria, mycobacteria, or with bacterial cell wall components. As shown in Figure 3.9, TREM-1 expression was strongly upregulated by extracellular bacteria, including *Pseudomonas aeruginosa* and *Staphylococcus aureus,* as well as lipoteichoic acid (LTA) and LPS. In contrast, intracellular bacteria, such as *Bacillus of Calmette-Guerin (BCG),* and mycolic acid had no effect. These results suggested that TREM-1 is upregulated under inflammatory conditions caused by extracellular bacteria. Importantly, LPS-mediated TREM-1 upregulation was paralleled by an increased ability of TREM-1 to trigger the secretion of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 beta (IL-1β) (Figure 3.9B). In contrast, TREM-1 was rapidly downregulated upon stimulation with immune suppressive cytokines such as IL-10 or TGF-β whereas other proinflammatory cytokines including TNF-α, IL-1β, and IL-18 but not others (TNF-β and IL-1α) induced TREM-1 upregulation (Figure 3.9C and data not shown).

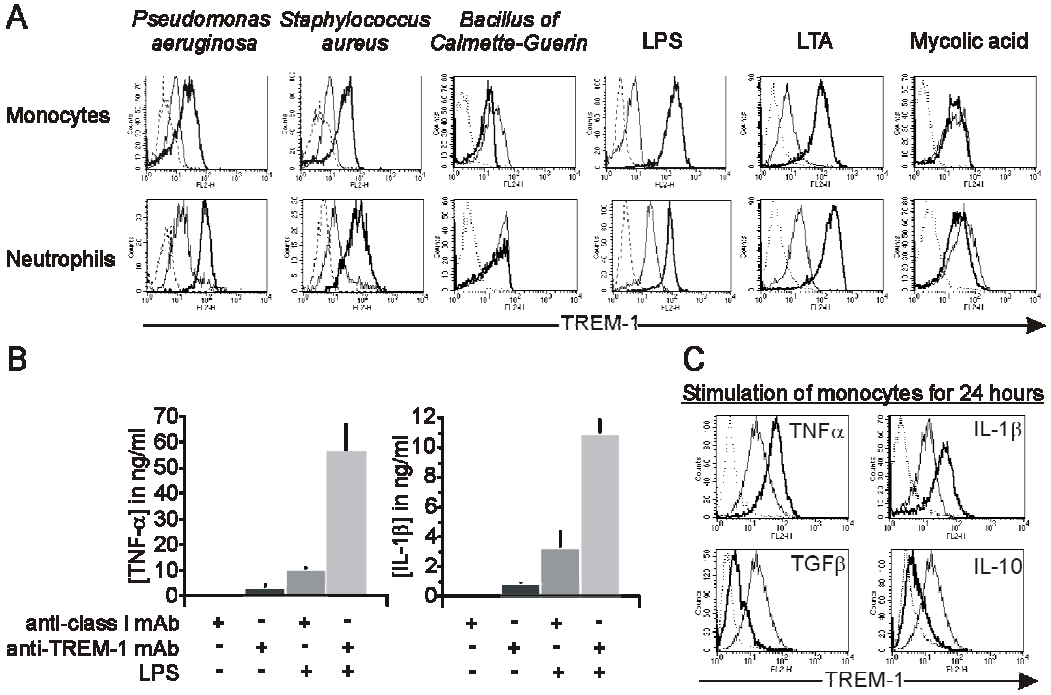

Figure 3.9: Regulation of human TREM-1 surface expression and function *in vitro*.

A. TREM-1 is strongly upregulated after incubation of neutrophils and monocytes with heat-inactivated *Staphylococcus aureus*, *Pseudomonas aeruginosa,* LTA and LPS (left and middle panels) but not by *Bacillus of Calmette-Guerin* and mycolic acid (right panels). Stimulated cells (solid bold line) were analysed for cell surface expression of TREM-1, as compared to non-stimulated cells (solid line). Dashed profiles indicate background staining of stimulated cells with a control IgG1 mAb.
B. Ligation of TREM-1 potentiates LPS-mediated cytokine release. Monocytes were challenged for 16 hours as indicated. Supernatants were analysed for TNF-α (left panel) and IL-1β (right panel). All data points correspond to the mean and the standard deviation of four independent experiments.
C. TREM-1 is differentially regulated by pro- and anti-inflammatory cytokines. Monocytes were incubated with TNF-α, IL-1β (top panels) or IL-10 and TGFβ (bottom panels) Stimulated cells (solid bold line) were analysed for cell surface expression of TREM-1, as compared to non-stimulated cells (solid line). Dashed profiles indicate background staining of stimulated cells with a control IgG1 mAb.

3.2.2 The role of TREM-1 in microbial infections

3.2.2.1 Human TREM-1 is strongly expressed in acute inflammatory lesions caused by bacteria and fungi but not in chronic inflammations.

In vivo TREM-1 expression was determined in tissue specimens derived from acute or granulomatous inflammatory lesions caused by bacterial, fungal or non-microbial agents. TREM-1 was highly expressed in neutrophils associated with skin lesions caused by *Staphylococcus aureus*, such as folliculitis and impetigo (Figure 3.10A-D). In addition, increased TREM-1 expression was observed in neutrophils associated with granulomatous lymphadenitides caused by *Bartonella henselae* and *Aspergillus fumigatus* (Figure 3.10E-H). In the latter, TREM-1 was also expressed in epithelioid and multinucleated giant cells surrounding the granulomas (Figure 3.10D). In contrast, TREM-1 expression was either weak or absent in granulomatous lymphadenitides caused by *Mycobacterium tuberculosis* as well as in sarcoid and foreign body

granulomas (data not shown). In addition, TREM-1 was hardly detectable in non-microbial inflammations, such as psoriasis, ulcerative colitis and vasculitis caused by immune complexes, despite a considerable infiltration of neutrophils and monocytes (Figure 3.11A-F and data not shown). Together, these results are consistent with a predominant role of TREM-1 in acute and granulomatous inflammations caused by microbial products.

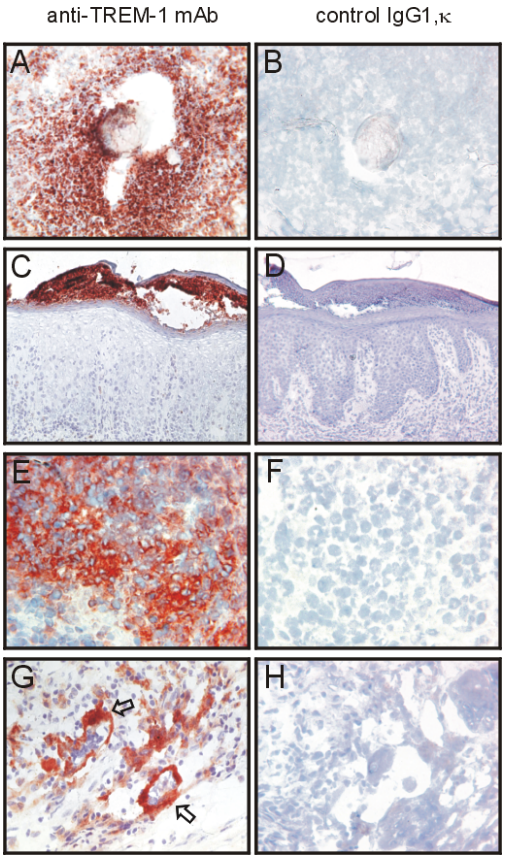

Figure 3.10: Human TREM-1 is strongly expressed in acute inflammatory lesions caused by bacteria and fungi.

TREM-1 expression was detected using mAb 21C7 in acute cutaneous folliculitis (**A**) and impetigo (**C**) due to *Staphylococcus aureus*, in cat scratch granuloma induced by *Bartonella henselae* (**E**) and granuloma due to *Aspergillus fumigatus* (**G**). It is of note that within the latter, TREM-1 is expressed not only on infiltrating neutrophils but also on the multinucleated giant cells (arrow) surrounding the granuloma. Staining with control mAb is shown in **B, D, F, H**, respectively.

3.2.2.2 Human TREM-1 is strongly upregulated in infiltrating neutrophils of septic patients

Under certain circumstances the excessive inflammatory response to infectious agents can lead to septic shock (Beutler et al., 1985; Bone, 1991; Glauser et al., 1991; Morrison and Ryan, 1987; Tracey et al., 1986). This process is characterized by the massive release of proinflammatory cytokines, such as TNF-α, IL-1β, macrophage migration inhibitory factor (MIF) and high mobility group-1 (HMG-1) protein, which mediate not only tissue damage, but also haemodynamic changes, multiple organ failure and ultimately death (Alexander et al., 1991; Bernhagen et al., 1993; Beutler et al., 1985; Ohlsson et al., 1990; Wang et al., 1999). Consistent with the role of TREM-1 in bacterial infections, TREM-1 surface expression was increased considerably on neutrophils infiltrating the peritoneal cavity of patients with septic shock due to bacterial peritonitis (Figure 3.12B). In contrast, peritoneal lavage cells of patients with a systemic inflammatory response syndrome (SIRS) caused by non-microbial peritoneal inflammation showed normal levels of TREM-1 (Figure 3.12A).

3.2.2.3 Mouse TREM-1 is expressed on mouse neutrophils, alveolar macrophages and is strongly upregulated during experimental LPS-induced shock

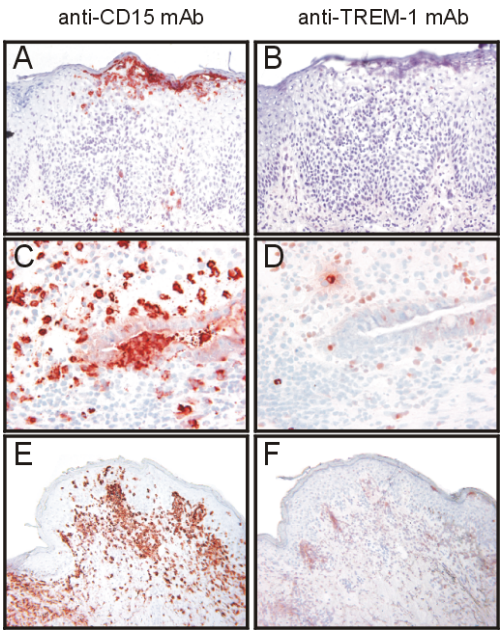

Figure 3.11: Human TREM-1 is only weakly expressed in neutrophils and monocytes accumulating in non-pathogenic inflammations.

Psoriasis (**A**, **B**), Ulcerative Colitis (**C, D**), and Vasculitis caused by immune complexes (E, F) are characterised by inflammatory infiltrates of neutrophils, as detected by anti-CD15 mAb (left panels). However, TREM-1 expression is weak or absent (right panels) in consecutive/serial sections.

If TREM-1 were involved in inflammatory responses to microbial products, inhibition of TREM-1 using soluble TREM-1 as a receptor decoy would be expected to reduce inflammation, eventually preventing lethal shock. To test this hypothesis in murine models of sepsis, we cloned the murine homologue of human TREM-1 (mTREM-1) and generated the mTREM-1-specific mAb 50D1. Similar to human TREM-1, mTREM-1 is weakly expressed on blood neutrophils, alveolar macrophages and neutrophil precursers in the BM (data not shown). Surprisingly, Mac-1^{high}/Ly-$6G^{-}$ blood monocytes did not express mTREM-1. Using 50D1, we observed that mTREM-1 expression was upregulated in peritoneal neutrophils during experimental LPS-induced shock (Figure 3.12C,D) similar to microbial septic shock in humans (Figure 3.12A,B).

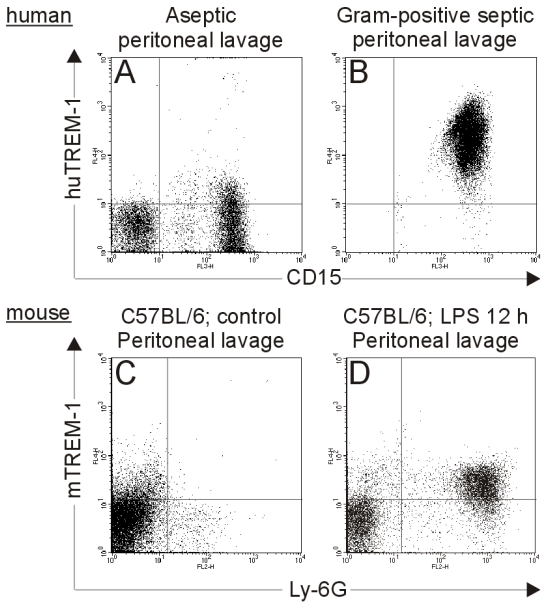

Figure 3.12: TREM-1 is strongly upregulated on peritoneal neutrophils during septic shock in humans and mice.

Flow cytometric analysis of peritoneal lavage cells from patients with aseptic SIRS due to aseptic cholecystitis (A) or polymicrobial gram-positive sepsis caused by bowel perforation (B). $CD15^{high}$ cells correspond to neutrophils. Four-colour analysis of peritoneal leucocytes from LPS-treated C57BL/6 mice (D) compared to control animals (C). Ly-$6G^{high}$ /TREM-1^{high} cells correspond to murine neutrophils. The Ly-$6G^{low-negative}$/TREM-1^{high} cells are CD11b/Mac-1^{+}(data not shown) and therefore correspond to peritoneal macrophages. Staining with isotype-matched control mAbs were set to the indicated lower quadrants.

3.2.2.4 Inhibition of mTREM-1 signaling blocks endotoxic shock and inflammatory responses *in vivo*.

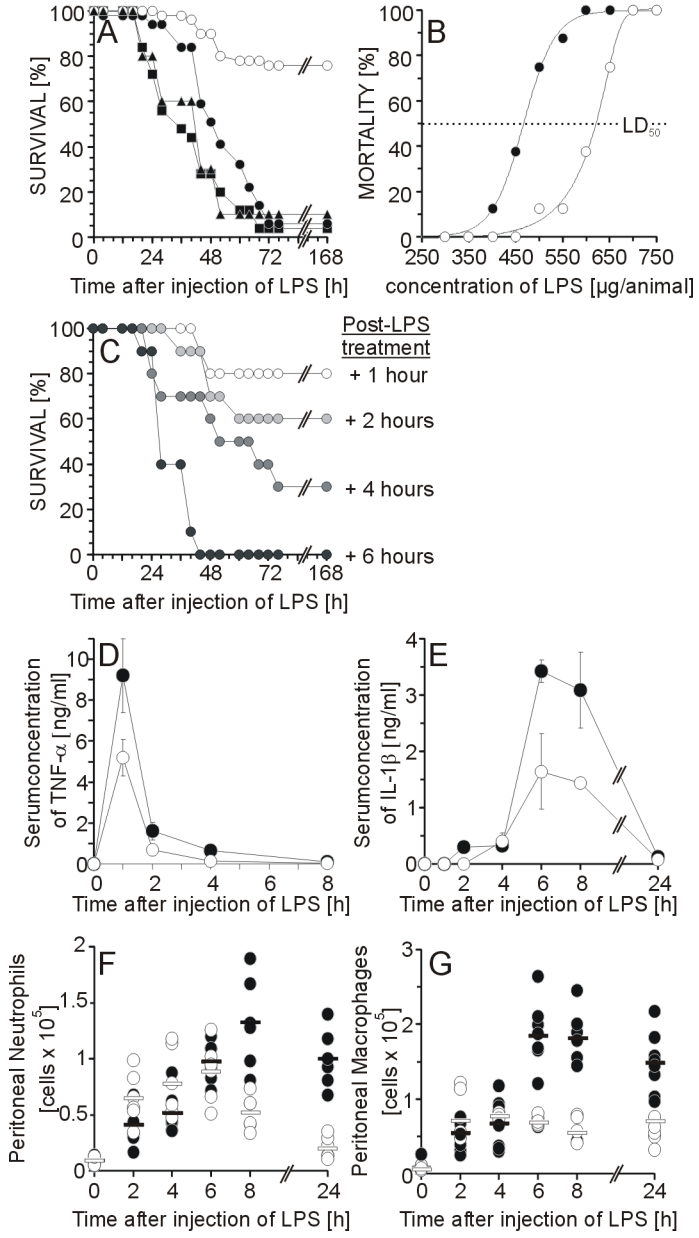

Figure 3.13: Inhibition of mTREM-1 signaling blocks endotoxic shock and inflammatory responses *in vivo*.

A. C57BL/6 mice were treated with control huIgG1 (closed circles) or mTREM-1-IgG1 (open circles) 1 hour prior to administration of LPS. Data points are from seven independent experiments, each of which included 5-10 animals per group. Survival was 76% (37 of 49) in mice treated with mTREM-1-IgG1 and 6% (3 of 49) in mice treated with huIgG1 ($P = 0.0002$, two-tailed Fisher's exact test). In additional controls, mice received injections with purified human ILT3-IgG1 (closed squares, $n = 25$) or heat-inactivated mTREM-1-IgG1 (closed triangles; $n = 10$) before induction of endotoxemia.

B. Estimation of the LPS LD_{50} in mice treated with mTREM-1-IgG1 or huIgG1. Mice were randomly assigned to 20 groups each containing 10 animals. Ten groups received intraperitoneal injections of mTREM-1-IgG1, whereas 10 groups were injected with huIgG1. One hour later, endotoxemia was induced by application of various quantities of LPS as indicated. Calculation of LD_{50} was accomplished as previously described ($LD_{50}^{mTREM\text{-}1\text{-}IgG1}$ = 621 µg, LD_{50}^{IgG1} = 467 µg; $P < 0.0001$)[6].

C. mTREM-1-IgG1 protects against LPS-induced lethal peritonitis when given after challenge. Mice were injected with LPS one (white circles), two (light grey circles), four (dark grey circles) and six hours (black circles) prior to administration of mTREM-1-IgG1. Data points are from two independent experiments, which included 3-7 animals per group. Survival was 80% ($P = 0.0007$, two-tailed Fisher's exact test), 60% ($P = 0.0108$, two-tailed Fisher's exact test), 40% and 0%, respectively.

D.-G. Analysis of inflammatory parameters during fatal endotoxemia. Mice were treated as described in (**A**). Serum levels of TNF-α (**D**) and IL-1β (**E**) and numbers of peritoneal neutrophils (**F**) and macrophages (**G**) were determined at the indicated time points. Data points correspond to the mean and the standard deviation of two independent experiments, each of which included 4-6 mice per treatment group.

A chimeric protein containing mTREM-1 extracellular domain and human-IgG1 Fc portion (mTREM-1-IgG1) was produced and injected into the peritoneal cavity 1 hour before the induction of endotoxemia. Lethality was monitored over time as compared to animals, which had received control injections of human IgG1 (huIgG1), control-IgG1 fusion protein (ILT3-IgG1) (Cella et al., 1997) or heat-inactivated mTREM-1-

IgG1 prior to LPS administration. As shown in Figure 3.13A, 76% of the mice treated with mTREM-IgG1 survived endotoxemia as compared to 7% of control mice. To precisely quantify the protection provided by mTREM-1-IgG1, groups of mice pre-treated with mTREM-1-IgG1 or huIgG1 were challenged with various doses of LPS. The LD_{50} of LPS in animals treated with mTREM-1-IgG1 (LD_{50} = 621 µg) was significantly higher than the LD_{50} in control animals (LD_{50} = 467 µg) (Figure 3.13B). Furthermore, it was monitored whether mTREM-1-IgG1 is still protective when administered 1 hour, 2 hours, 4 hours and 6 hours after LPS injection. Remarkably, mTREM-1-IgG1 conferred 80% protection against endotoxic shock when applied 1 hour after LPS injection. Partial protection was also observed two and four hours later (Figure 3.13C). Thus, soluble TREM-1 is effective even when injected subsequent to the outbreak of endotoxemia.

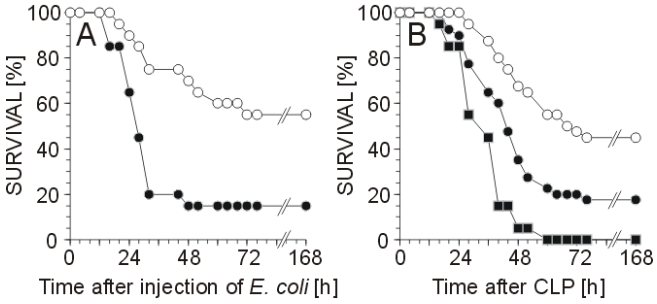

Figure 3.14: mTREM-1 is protective in bacterial peritonitis.

A. C57BL/6 mice were injected intraperitoneally with mTREM-1-IgG1 (open circles) or huIgG1 (closed circles) one hour before intraperitoneal administration of *E. coli*. Data points are from two independent experiments, which included 5-15 animals per group. Survival was 55% (11 of 20) in mice treated with mTREM-1-IgG1 and 15% (3 of 20) in mice treated with control huIgG1 (P = 0.0187, two-tailed Fisher's exact test).
B. mTREM-1-IgG1 protects against fatal septic shock induced by CLP. Mice were injected intraperitoneally with mTREM-1-IgG1 (open circles), huIgG1 (closed circles) or TNF-R1-IgG1 (closed squares) immediately after CLP. Data points are from four independent experiments, which included 5-10 animals per group. Survival was 45% (18 of 40) in mice treated with mTREM-1-IgG1, 17.5% (7 of 40) in mice treated with control huIgG1 (P = 0.015, two-tailed Fisher's exact test) and 0% (0 of 20) in mice treated with TNF-R1-IgG1.

Analysis of blood samples taken from mice pre-treated with TREM-1-IgG1 and control animals at different time points following LPS administration revealed a significant reduction of the plasma concentrations of both TNF-α and IL-1β (Figure 3.13D,E). Furthermore, differential count analysis revealed a significant reduction in the total cell number of neutrophils and monocytes/macrophages infiltrating the peritoneum 6-8 hours after LPS injection in mTREM-1-IgG1-pre-treated animals as compared to controls (Figure 3.13F,G). The reduced accumulation of leukocytes at the inflammatory site was not caused by a putative capacity of mTREM-1-IgG1 to target leukocytes for sequestration or destruction. In fact, injection of TREM-1-IgG in normal mice did not affect the levels of circulating leukocytes (data not shown). Thus, inhibition of TREM-1-mediated responses is sufficient to lower serum concentrations of TNF-α and IL-1β and cellular infiltrates below levels that are lethal for the host under conditions of LPS-mediated shock, without causing leukopenia.

3.2.2.5 mTREM-1-IgG1 is protective in bacterial peritonitis

Endotoxic shock imitates human sepsis only in part, as it does not involve the replication and dissemination of bacteria. In these conditions a complete block of TREM-1 signalling could be deleterious by impairing the capacity of the immune system to fight infections, as previously observed for anti-TNF-α treatments (Echtenacher et al., 1990; Echtenacher et al., 1996; Eskandari et al., 1992; Malaviya et al., 1996; Peschon et al., 1998; Pfeffer et al., 1993; Rothe et al., 1993). It was therefore investigated whether mTREM-1-IgG1 protects against septic shock in two models of microbial peritonitis and sepsis caused by intraperitoneal administration of *Escherichia coli* or by cecal ligation and puncture (CLP) (Appelmelk et al., 1986; Bone, 1991; Calandra et al., 2000; Echtenacher et al., 1990; Glauser et al., 1991). As shown in Figure 3.14, injection of mTREM-1-IgG1 conferred significant protection against lethal *E. coli* peritonitis and CLP-induced septic shock (the CLP model was performed by Dr. M. Weigand, University of Heidelberg, Germany) compared to control huIgG1, whereas treatment with TNF-R1-IgG1 caused accelerated and complete death of all animals (Figure 3.14B). Thus, mTREM-1-IgG1 reduces inflammatory responses but still allows sufficient control of the bacterial infection.

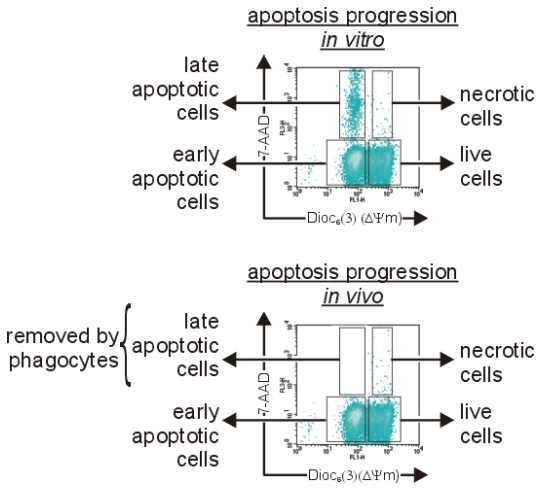

Figure 3.15: Schematic view of apoptotic cells *in vitro* and *in vivo*.

Predicted flow cytometric analysis of apoptotic cells kept in vitro (top panel) or isolated from mice (bottom panel) using Propidium iodide (detection of late apoptotic cells) and $Dioc_6(3)$ (detection of early apoptotic cells).

3.2.2.6 TREM-1 mediates neutrophil survival *in vivo*

3.2.2.6.1 Detection of apoptosis during endotoxemia *in vivo*

mTREM-1-IgG1-pretreated animals showed a significant reduction in the number of peritoneum infiltrating neutrophils compared to controls. Interestingly, this reduction was paralleled by a much higher degree of dead neutrophils observed during differential count analysis. In particular, morphological changes such as chromatin condensation, nucleus fragmentation and cell fragmentation into apoptotic bodies (Kerr et al., 1972) indicated neutrophil apoptosis and suggested that mTREM-1-IgG1 may influence neutrophil homeostasis. Therefore, it was assessed whether blockade of TREM-1

signaling inhibits neutrophil survival during LPS-induced shock. Unfortunately, identification and quantification of apoptotic cell death *in vivo* is limited by the action of macrophages, which recognize and subsequently phagocytose cells displaying cell surface signals of onset and progression of apoptosis (Savill and Fadok, 2000). Thus, methods detecting late morphological changes during apoptosis (such as PI-uptake detecting the loss of plasma membrane integrity) cannot be used *in vivo* (for a schematic view see Figure 3.15). However, it was previously shown that, even *in vivo*,

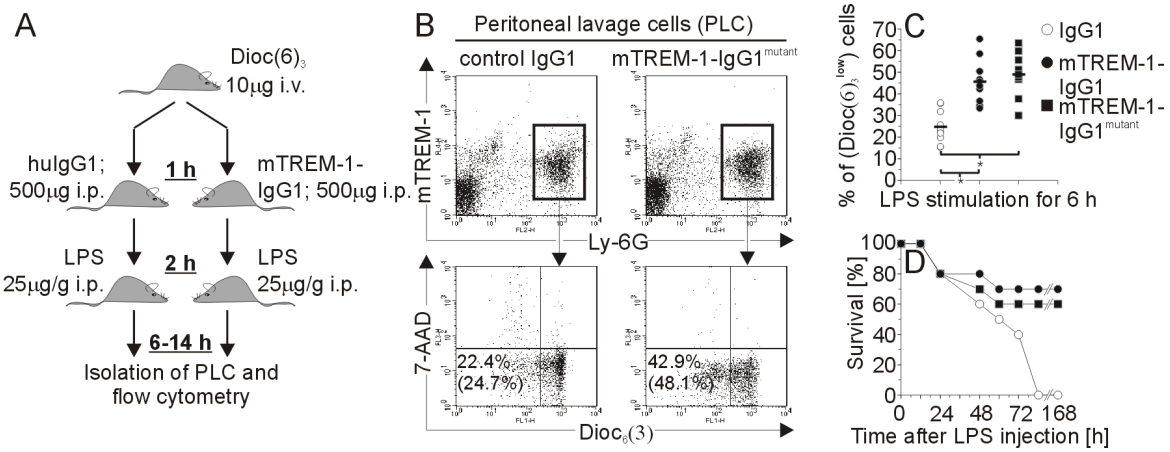

Figure 3.16: Inhibition of TREM-1 signaling accelerates onset of neutrophil apoptosis *in vivo*.

A. Experimental set up: female C56BL/6 mice were randomly grouped (20 mice per group) and injected with 10 µg DiOC6(3) i.v. One hour later, 500 µg of mTREM-1-IgG1, mTREM-1-IgG^{mutant} or control huIgG1 were administered i.p. in a blinded fashion. Endotoxemia was induced by injecting 25 g/kg LPS (LD_{100}) i.p. one hour later. Mice of each treatment group were randomly separated in two subgroups of 10 mice each. 10 mice were kept for 10 days and viability was monitored 4-6 times a day. The residual 10 mice/group were sacrificed after 6 hours, peritoneal lavage cells (PLC) were isolated and placed on ice immediately.

B. Detection of early apoptotic neutrophils by FACS analysis. Freshly isolated PLC were kept on ice during staining for 10 min with anti-mTREM-1-Cy5, anti-Ly-6G-PE and 7-AAD and subsequently analyzed on a FACScalibur. mTREM-1$^+$/Ly-6G$^+$ neutrophils were gated and the percentages of $DiOC_6(3)^{low}$/7-AAD$^-$ neutrophil cells, corresponding to early apoptotic neutrophils, was determined and indicated in the corresponding quadrant. 7-AAD$^+$ cells were excluded.

C. mTREM-1-IgG1 reduces neutrophil survival *in vivo*. Mice were treated as indicated in (A) and (B) and percentages of mTREM-1$^+$/Ly-6G$^+$/DiOC6(3)^{low}/7-AAD$^-$ were determined. Data points are representative for three independent experiments (10 animals per treatment group). The amount of early apoptotic cells was 48.1% in neutrophils from mice treated with mTREM-1-IgG1, 45.7% in neutrophils from mice treated with mTREM-1-IgG1^{mutant}, and 24.7% in neutrophils from mice treated with huIgG1 (*P < 0.05, Student t test).

D. mTREM-1-IgG1 and mTREM-1-IgG1^{mutant} protect mice against endotoxemia. Mice were treated as indicated in (A) and (B) and survival kinetics was determined over a 10 day-period. Data points are representative for three independent experiments (10 animals per treatment group). Survival was 70% in mice treated with mTREM-1-IgG1, 60% in mice treated with mTREM-1-IgG1^{mutant}, 10% in mice treated with huIgG1 (P < 0.02, Student t test).

early changes in mitochondrial membrane potential can be detected (Metivier et al., 1998) (for a schematic view see Figure 15). Despite the biological limitations, a system was established that allows the identification of apoptotic neutrophils during LPS-induced peritonitis (Figure 3.16A,B). Before induction of endotoxemia, total blood leucocytes were labeled by injecting the fluorescent dye $DiOC_6(3)$ intravenously. $DiOC_6(3)$ is known as an indicator for intact mitochondrial membrane potential, thus allowing the detection of early mitochondrial defects as reduction of fluorescence at 525 nm (Metivier et al., 1998). One hour after $DiOC_6(3)$ administration, mice were treated with control huIgG1, soluble decoy TREM-1 receptor (mTREM-1-IgG1) or

TREM-1-IgG1 fusion protein with a mutated Fc portion (TREM-1-IgG1^{mutant}). Endotoxemia was induced one hour later, peritoneal lavage cells were isolated at different time points after LPS administration, surface stained with anti Ly-6G and anti-mTREM-1 and analyzed by FACS for mTREM-1^{+}/Ly-6G^{+}/DiOC$_6$(3)^{low}/7-AAD^{-} early apoptotic neutrophils (Figure 3.16B).

3.2.2.6.2 mTREM-IgG1 reduces neutrophil survival during endotoxemia *in vivo*

Using the described system the progression of neutrophil apoptosis during LPS-induced shock was investigated. To confirm protection against septic death, half of the treated mice were kept and monitored for mortality over time (Figure 3.16D) whereas the other half of the mice was investigated for neutrophil apoptosis (Figure 3.16C). Strikingly, in mice protected against septic death by mTREM-1-IgG1 or

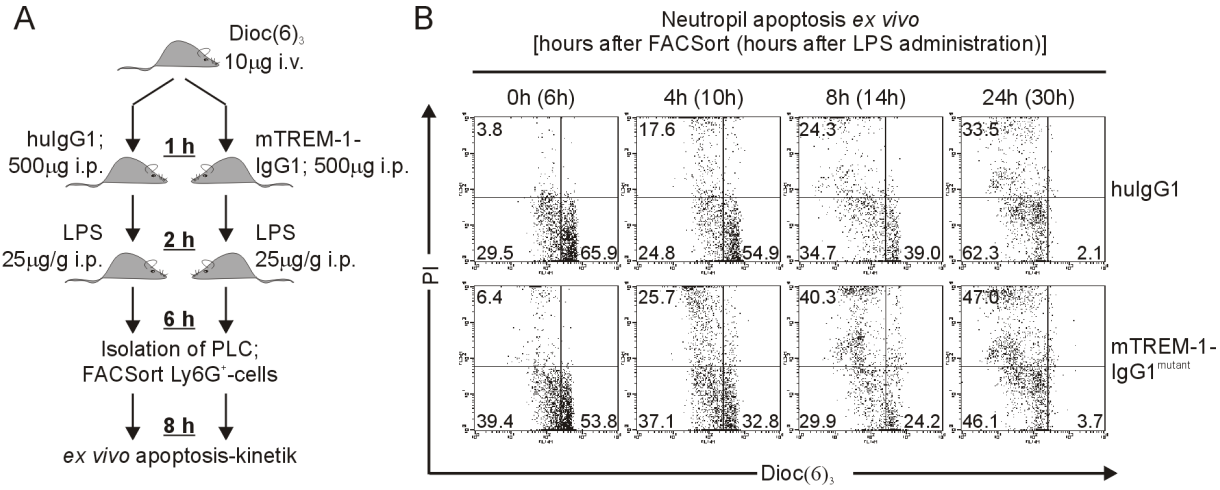

Figure 3.17: Inhibition of TREM-1 blocks initiation but not progression of apoptosis.

A. Experimental set up: mice (30 mice per group) were treated as indicated in Fig. 3A. 6 hours after LPS administration PLC were isolated, Ly-6G^{+} cells were purified by flow cytometry and cultured at 37°C.
B. Loss of $\Delta\Psi$m [reduced fluorescence with DiOC$_6$(3)] and cell death (increased fluorescence at 575 nm/PI uptake) was determined after LPS administration and FACSorting at the indicated time points (in brackets). Percentages displayed in the quadrants correspond to late apoptotic cells (PI^{+}/Dioc$_6$(3)^{low}), early apoptotic cells (PI^{-}/Dioc$_6$(3)^{low}) and live cells (PI^{-}/Dioc$_6$(3)^{high}). Data points are representative for two independent experiments (30 animals per treatment group).

mTREM-1-IgG1^{mutant}, the amount of early apoptotic neutrophils 6 hours after LPS administration were significantly increased (48.1 and 45.7%, respectively) compared to control animals (24.7%) (Figure 3.16C). Interestingly, a difference in apoptotic cell death in mice treated with mTREM-1-IgG1 could already be observed 2 hours after LPS administration (data not shown), thus indicating that TREM-1-mediated survival signals seem to be essential from early on during LPS-induced shock.

We also isolated Ly-6G^{+} neutrophils from the peritoneal cavity 6 hours after LPS injection and we monitored *in vitro* loss of $\Delta\Psi$m and integrity of the plasma membrane over time (Figure 3.17A). Neutrophils derived from mTREM-1-IgG1 mutant-treated

animals revealed an accelerated apoptosis as compared to neutrophils isolated from control animals (Figure 3.17B). However, the rate of transition from early ($Dioc_6(3)^{low}$/Propidium iodine $(PI)^-$) to late ($Dioc_6(3)^{low}/PI^+$) apoptosis was not significantly altered in the presence of mTREM-1-IgG1 mutant, thus indicating that TREM-1-signaling prevents initiation but not progression of neutrophil apoptosis.

3.2.2.7 The molecular mechanism of TREM-1-mediated neutrophil survival

3.2.2.7.1 TREM-1 promotes neutrophil survival through cytokine secretion and direct intracellular signaling

To investigate the molecular mechanisms of TREM-1-anti-apoptotic effect, we analyzed *in vitro* survival of human neutrophils stimulated with plastic bound $F(ab')_2$ anti-TREM-1 mAb. As shown in Figure 3.18A, the vast majority of TREM-1-stimulated neutrophils remain viable up to 48 h as compared to neutrophils stimulated by control $F(ab')_2$ mAb, which underwent apoptosis just after 8 h in culture. Similar effects were obtained with isolated mouse neutrophils stimulated with anti-mTREM-1 mAb 50D1 (data not shown). These data confirm that triggering of TREM-1 leads to resistance to apoptosis in neutrophils and show that this effect can be achieved in the

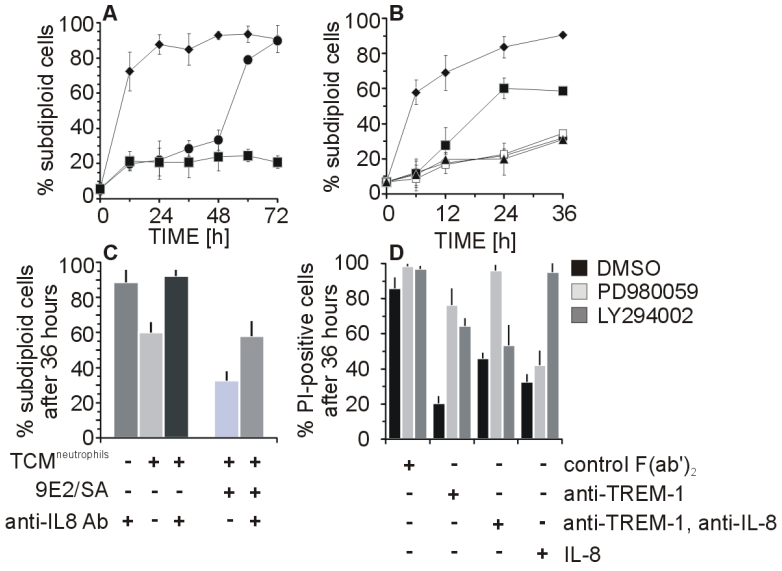

Figure 3.18: Stimulation of TREM-1 mediates survival of neutrophils *in vitro*

A. Freshly isolated neutrophils were stimulated with plastic-bound control $F(ab')_2$ (black diamonds), $F(ab')_2$ anti-TREM-1 mAb 9E2 (black circles). DNA fragmentation was determined at the indicated time points. All data are the mean and standard deviation of three independent experiments.

B. Freshly isolated neutrophils were stimulated with plastic-bound control $F(ab')_2$ (black diamonds) or $F(ab')_2$ anti-TREM-1 mAb 9E2 (black circles) in the presence of neutralizing anti-TNF-α (white squares), anti-IL-8 (black squares), anti-IL-1β (black triangles squares). DNA fragmentation was determined at the indicated time points. All data are the mean and standard deviation of three independent experiments.

C. TREM-1-conditioned Medium (TCM) was produced by stimulating or neutrophils (TCM) for 24 hours with plastic bound $F(ab')_2$ anti-TREM-1 mAb 9E2. TCM were then transfered to unstimulated neutrophils in the presence or absence of biotinylated $F(ab')_2$ anti-TREM-1 mAb 9E2/Streptavidine (9E2/SA), neutralizing Ab against IL-8 as indicated. DNA fragmentation was determined 36 hours after stimulation. All data are the mean and standard deviation of four independent experiments.

D. (C) Freshly isolated neutrophils were stimulated with plastic-bound control $F(ab')_2$, $F(ab')_2$ anti-TREM-1 mAb 9E2, $F(ab')_2$ anti-TREM-1 mAb 9E2 together with neutralizing anti-IL-8 mAbs, or IL-8 alone, in the absence (black bars) or presence of PD98059 (Erk-inhibitor, light grey bars) and LY94002 (PI(3)K-inhibitor, dark grey bars). Cell death (increased fluorescence at 575 nm after PI uptake) was determined after 36 hours.

absence of LPS stimulation. Since TREM-1 triggers the release of cytokines, such as IL-8, TNF-α and IL-1β *in vitro*, it was unclear whether the observed survival effect of TREM-1 was mediated by these cytokines in an autocrine fashion or rather by TREM-1-induced intracellular signaling pathways. Therefore, neutrophils were stimulated through TREM-1 in the presence of neutralizing antibodies against TNF-α, IL-8, and IL-1β. As shown in Figure 3.18B, no effect of anti-TNF-α or IL-1β could be documented in this assay. In the presence of anti-IL-8 mAb, a reduction of neutrophil survival was detected, but a considerable number of cells were still protected from apoptosis. IL-8-neutralized supernatant from TREM-1 stimulated neutrophils (TREM-1 conditioned medium: TCM) did not induce any survival on unstimulated neutrophils thus ruling out other soluble survival factors secreted after TREM-1 stimulation (Figure 3.18C). Thus, TREM-1 protects against spontaneous apoptosis by inducing the secretion of IL-8 as well as by direct activation of intracellular pathways.

TREM-1 stimulation induces activation and phosphorylation of the ERK1/2 in monocytes (Figure 3.7) and neutrophils (Figure 3.19A). ERK, together with other kinases, Akt, protein kinase A (PKA) and 90-kDa ribosomal S6 kinases (p90RSK) are implicated in transmitting survival signals (Bonni et al., 1999; Burgering and Coffer, 1995; Franke et al., 1995; Scheid and Duronio, 1998; Scheid et al., 1999; Tan et al., 1999; Xia et al., 1995). In particular, it has been shown that IL-8 inhibits apoptosis by inducing PI(3)K-mediated activation of Akt (Klein et al., 2000). To assess the role of these kinases, we tested the effect of the ERK inhibitor PD98059 and the PI(3)K inhibitor LY294002 on TREM-1-mediated survival. As shown in Figure 3.18D, both PD98059 and LY294002 reduced TREM-1-mediated neutrophil survival. In the presence of neutralizing anti-IL-8 mAb, only PD98059 retained its inhibitory ability. In contrast, IL-8-mediated survival was highly sensitive to the PI(3)K inhibitor LY294002. Thus, TREM-1 stimulation induces neutrophil survival through an IL-8/PI(3)K/Akt-dependent pathway and through a receptor-proximal ERK-dependent survival signal.

3.2.2.7.2 TREM-1 triggers the phosphorylation of Bad and the release of Bcl-x_L in neutrophils

Neutrophils are characterized by a high rate of constitutive apoptosis that can be explained by the constitutive expression of pro-apoptotic members of the Bcl-2 family (Bad, Bax, Bid and Bak), as well as by low levels of anti-apoptotic Bcl-2 and Bcl-x_L (Adams and Cory, 1998; Akgul et al., 2001). Despite the limited number of mitochondria in neutrophils, several studies suggest that integrity of mitochondria controlled by Bcl-2 family members play an important role in the apoptotic pathways in these cells (Adams and Cory, 1998; Akgul et al., 2001; Green, 2000; Gross et al., 1999; Hengartner, 2000; Huang and Strasser, 2000; Kroemer and Reed, 2000). Recent

work showed that phosphorylation of Bad by Akt at Ser^{136} (Blume-Jensen et al., 1998; Brunet et al., 1999; Datta et al., 1997; del Peso et al., 1997; Klein et al., 2000; Lizcano et al., 2000; Scheid and Duronio, 1998; Scheid et al., 1999; Zhou et al., 2000) or ERK at Ser^{112} (Klein et al., 2000; Scheid and Duronio, 1998; Scheid et al., 1999) is crucial to cell survival in particular for neutrophils (Klein et al., 2000). Therefore it was investigated whether TREM-1 stimulation induces ERK- and/or Akt -dependent phosphorylation of Bad. As shown in Figure 3.19A, TREM-1 stimulation led to the phosphorylation of ERK but not of Akt/PKB, at least during early time points. Consistent with this observation, TREM-1 induced phosphorylation of Bad at Ser^{112} (Figure 3.19A). Treatment of neutrophils with the ERK inhibitor PD98059 blocked TREM-1-induced Bad phosphorylation entirely, while a PI(3)K inhibitor had no effect (Figure 3.19B). Stimulation of cells with IL-8 induced activation of both ERK and Akt (Figure 3.19A). Although both IL-8 and TREM-1 induced similar ERK activation, IL-8 induced a predominant phosphorylation of Bad at Ser^{136}, whereas phosphorylation of Bad at Ser^{112} was hardly detectable (Figure 3.19A). PKA has been shown to induce survival by phosphorylating Bad at Serine 155 (Ser^{155}) (Harada et al., 1999; Lizcano et al., 2000). However, neither IL-8 nor anti-TREM-1 mAb stimulation led to the phosphorylation of Bad at Ser^{155} (Figure 3.19A), excluding involvement of PKA during TREM-1-induced neutrophil survival.

The presence of Bcl-x_L proteins in neutrophils is debated (Akgul et al., 2001; Chuang et al., 1998; Moulding et al., 1998; Ohta et al., 1995; Santos-Beneit and Mollinedo, 2000; Weinmann et al., 1999). Here, however, low levels of the 28 kDa Bcl-x_L protein could be detected in neutrophil lysates by immunoblotting of anti-Bcl-x immunoprecipitates using an anti-Bcl-x antibody (Figure 3.19B). To demonstrate that TREM-1-mediated phosphorylation of Bad at Ser^{112} influences Bad-Bcl-x_L-heterodimerization and Bcl-x_L-mediated protection of mitochondrial integrity, we quantified Bcl-x_L-Bad heterocomplexes in anti-Bcl-x immunoprecipitates. As shown in Figure 3.19C, Bad is rapidly released from Bcl-x_L upon stimulation with TREM-1 mAb, indicating that phosphorylation of Bad at Ser^{112} is sufficient to the release of Bcl-x_L from Bad-Bcl-x_L-heterodimers. Interestingly, IL-8 also induces dissociation of Bad from Bcl-x_L (Figure 3.19C), however this effect could be reversed entirely with the addition of PI(3)K inhibitors suggesting that the IL-8-mediated activation of Bad is dependent on the PI(3)K/Akt pathway (data not shown).

To demonstrate that the disruption of Bad-Bcl-x_L heterodimers protects mitochondrial integrity, we monitored the distribution of cytochrome c between mitochondria and cytosol during TREM-1 stimulation. Cytochrome c is normally found within mitochondria. When cytochrome c is released in the cytosol, it cooperates with Apaf-1 and dATP cofactors in inducing autocatalytic cleavage of caspase-9 and subsequent apop-

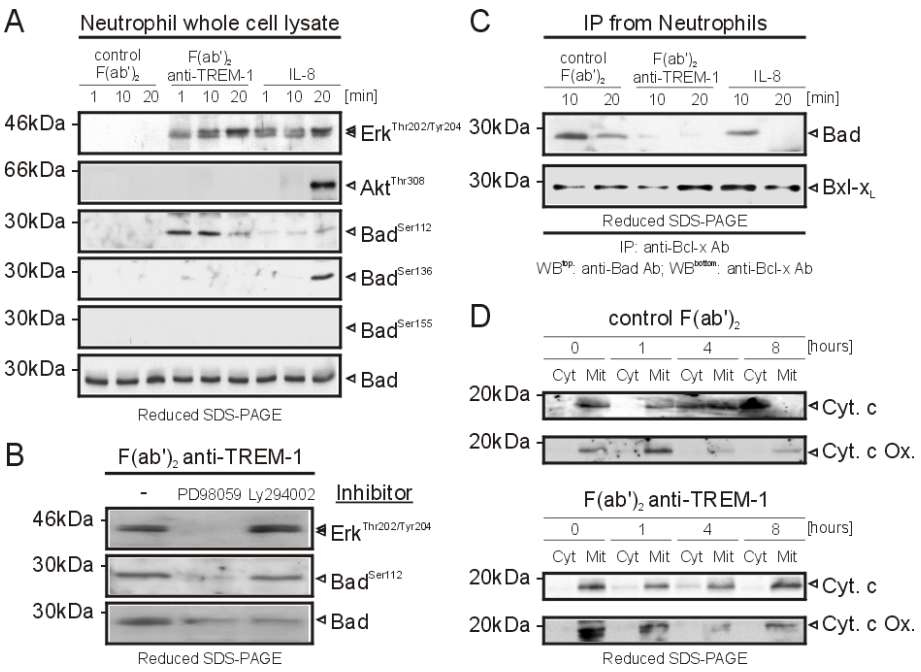

Figure 3.19: Stimulation of TREM-1 leads to Erk-induced phosphorylation of Bad at Ser^{112} and subsequent disruption of Bad-Bcl-x_L interaction and protection of mitochondrial integrity

A. Neutrophils were stimulated with F(ab')$_2$ control, F(ab')$_2$ anti-TREM-1 mAb 9E2 or IL-8 for the indicated time periods, lysed, subjected to reduced SDS-PAGE on 12% polyacrylamide gels and analyzed by immunoblot for phosphorylation of Erk, Akt, and Bad at Thr^{202}/Tyr^{204}, Thr^{308}, Ser^{112}, Ser^{135}, and Ser^{155}, respectively. The blots were stripped and subsequently reprobed with anti-Bad Ab.

B. Neutrophils were stimulated as indicated in (A) and subjected to immunoprecipitation with anti-Bcl-x Ab. The precipitates were analyzed by anti-Bad (upper panel) and anti-Bcl-x (lower panel) immunoblot under reducing conditions.

C. Neutrophils were stimulated with F(ab')$_2$ anti-TREM-1 mAb 9E2 in the absence or presence of PD98059 and LY294002 for 10 min. Whole cell lysates were prepared and subjected to SDS-PAGE and subsequent immunoblot analysis for phospho-$ERK^{Thr202/Tyr204}$ and phospho-Bad^{Ser112}. The blots were stripped and subsequently reprobed with anti-Bad Ab.

D. Freshly isolated human neutrophils were treated with F(ab')$_2$ anti-TREM-1 mAb (lower panels) or control F(ab')$_2$ mAb (top panels) for the indicated time periods. Subcellular fractions were prepared and analyzed by immunoblot. Cytosolic (Cyt) and mitochondrial fractions (Mit) were first analyzed with 7H8.2C12 anti-cytochrome c mAb (1st and 3rd panel), subsequently stripped and developed with 12C4-F12 anti-cytochrome c oxidase/subunit II mAb to control equal loading and purity of mitochondrial fractions (2nd and 4th panel).

tosis (Kluck et al., 1997; Li et al., 1997; Liu et al., 1996; Srinivasula et al., 1998; Yang et al., 1998; Zhou et al., 2000). In neutrophils stimulated through TREM-1 cytochome c was primarily found inside the mitochondria (Figure 3.19D). In contrast, unstimulated cells displayed rapid release of cytochrome c from the mitochondria to the cytosol, allowing for caspase-9 cleavage and induction of apoptosis (Figure

3.19E). Thus, upon TREM-1 stimulation, Bcl-x_L is released from the inhibitory function of Bad and can protect the integrity of mitochondria.

3.2.3 The hunt for TREM-1 ligand (TREM-1L)

TREM-1 is engaged on neutrophils during LPS-induced shock. The observation that mTREM-1-IgG1 is actively blocking endotoxemia (Figure 3.13C) suggests that the unknown TREM-1L is induced early after LPS stimulation (0 – 4 hours after LPS administration). TREM-1L can be a cell surface molecule expressed on immune cell infiltrating the peritoneum or on endothelial cell. In addition, it would be possible that TREM-1L is secreted thus acting as a proinflammatory cytokine. To address this first possibility, soluble TREM-1-huIgG1 was modified by biotinylation or with the fluorescent dye Cy5 and used for the staining of different cell types, including cells extracted from the peritoneum of LPS-treated mice after 2 – 12 hours. Flow cytometry analysis revealed that Ly-6G$^+$/Mac-1$^+$ neutrophils infiltrating the peritoneum already 2 – 4 hours after LPS administration are TREM-1L$^+$ (Figure 3.20). Interestingly Mac-1$^+$/IgM$^+$/CD19$^+$ B cells and F4/80$^+$/Mac-1$^+$ macrophages are TREM-1L$^-$ (Figure 3.20). Neutrophils isolated from blood, spleen or PL from normal mice did not bind to TREM-1-IgG1 (data not shown). In addition, isolated blood neutrophils from human and mouse stimulated *in vitro* with LPS did not bind to TREM-1-IgG1 (data not shown). This set of data clearly indicates that TREM-1L is, in particular, induced during endotoxemia but not with LPS alone. Further experiments using either expression cloning or a biochemical approach will be attempted to clone TREM-1L from neutrophils.

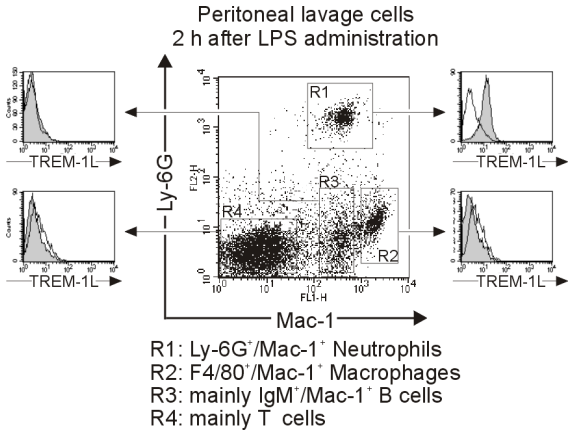

Figure 3.20: TREM-1L is expressed on neutrophils infiltrating the peritoneum during LPS-induced shock
Four-colour flow cytometric analysis of peritoneal leucocytes from LPS-treated C57BL/6 mice. Gating on Ly-6G^{high}/Mac-1^{high} murine neutrophils, Ly-6G$^{low\text{-}negative}$/Mac-1^{high}/F4/80$^+$ activated, peritoneal macrophages, and Ly-6G-/Mac-1+/IgM$^+$ B cells shows strong binding of mTREM-1-IgG1-Cy5 (solid, grey profiles) exclusively to neutrophils compared to control huIgG1-Cy5 (solid, white profiles). Staining with isotype-matched control mAbs were set to the

3.3 Characterization of human and mouse TREM-2

3.3.1 Characterization of human TREM-2 *in vitro* and *in situ*

3.3.1.1 Human immature monocyte-derived DCs express TREM-2, a ~40 kDa glycoprotein which is associated with DAP12.

In initial studies, TREM-2 transcript was selectively detected in monocyte-derived DCs by reverse-transcriptase polymerase chain reaction (data not shown). To pre-

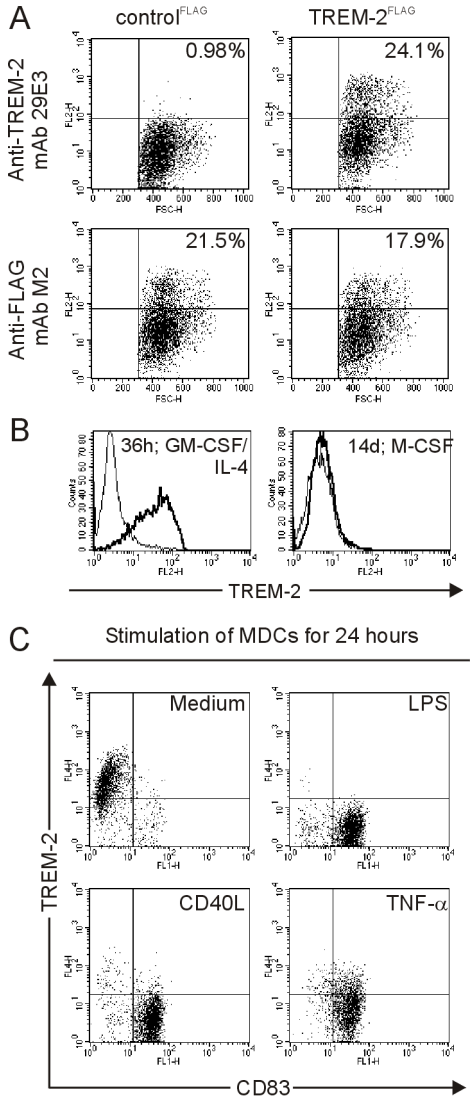

Figure 3.21: TREM-2 is selectively expressed on immature DCs

A. mAb 29E3 recognizes selectively TREM-2. 293 cells transfected with a cDNA encoding FLAG-tagged TREM-2 (TREM-2^{FLAG}) (right panels) were stained with mAb 29E3 (upper panel), as compared to cells transfected with a control cDNA (control^{FLAG}) (left panels). The percentages of positive cells (upper right quadrants) are indicated. Expression of TREM-2^{FLAG} and control^{FLAG} was confirmed using an anti-FLAG mAb (lower panels). Cells stained with an isotype-matched control mAbs were comprised within the indicated lower quadrant.

B. TREM-2 is strongly unregulated after stimulation of monocytes with GM-CSF and IL-4. Monocytes treated with GM-CSF/IL-4 (left panel) or M-CSF (right panel) were analyzed by flow cytometry for cell surface expression of TREM-2 (solid bold line) after 36 h or up to 14 days, respectively. Dashed profiles indicate background staining with a control IgG_1 mAb.

C. TREM-2 is rapidly downregulated upon maturation of DCs. LPS- (top right panel), CD40L- (lower left panel), TNF-α-stimulated (lower right panel), or unstimulated monocyte-derived DCs (top left panel) were analyzed by flow cytometry for cell surface expression of TREM-2 and CD83 after 36h. Cells stained with an isotype-matched control mAbs were comprised within the indicated lower quadrants.

cisely investigate the cellular distribution of TREM-2 as well as its biochemical characteristics and functions, anti-TREM-2 mAb were produced. The mAb 29E3 stained TREM-2-transfected 293 cells specifically, as compared to control transfectants (Figure 3.21A). In agreement with RT-PCR data, TREM-2 was highly expressed on DCs

derived from peripheral blood monocytes upon *in vitro* culture with GM-CSF and IL-4 (Figure 3.21B). DC maturation induced by lipopolysaccaride (LPS), TNF-α, CD40L-expressing cells (Figure 3.21C), IL-1β, CpG oligonucleotides, or aggregated IgG (data not shown) led to complete downregulation of TREM-2. TREM-2 was undetectable on macrophages obtained by culturing monocytes up to 14 days with M-CSF (Figure 3.21B), on primary DCs of peripheral blood, and on immature DCs in tissues or Langerhans cells in the skin (data not shown). Thus, TREM-2 is preferentially expressed on immature monocyte-derived DCs.

Immunoprecipitation of TREM-2 from surface-biotinylated monocyte-derived DCs revealed that TREM-2 is a glycoprotein of ~40 kDa, that is reduced to 26 kDa after N-deglycosylation (Figure 3.22A). This result is in agreement with the predicted molecular mass of TREM-2. Since TREM-2 lacks known signaling motifs in the cytoplasmic domain and displays a charged lysine residue in the transmembrane domain, it was likely to be associated with a separate adapter molecule to transduce signals. Adapter molecules, such as DAP12, DAP10 and FcRγ are tyrosine phosphorylated upon cell treatment with the phosphatase-inhibitor pervanadate (Lanier et al., 1998b; Nakajima et al., 1999; Wu et al., 1999). Indeed, anti-

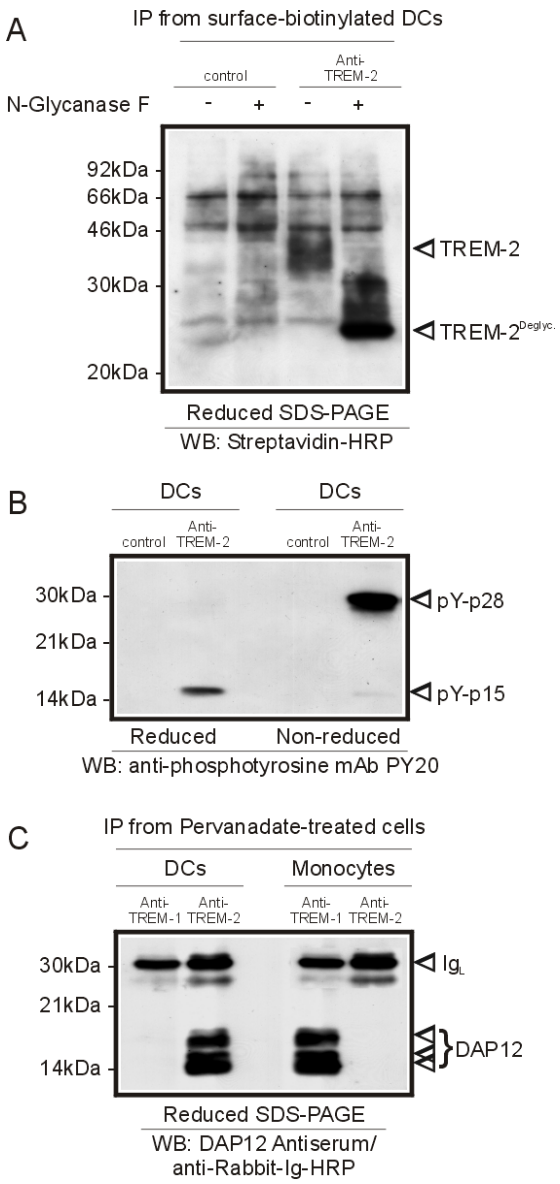

Figure 3.22: TREM-1 is a 40 kDa glycoprotein associated with the adaptor protein DAP12

A. Surface-biotinylated monocyte-derived DCs were lysed and subjected to immunoprecipitation with 29E3 anti-TREM-2 mAb (right lanes) or control IgG_1 mAbs (21C7 anti-TREM-1 mAb). Immunoprecipitates were left untreated or treated with N-Glycanase F and analyzed by Western Blot analysis with Streptavidine-HRP. Molecular weight markers and specific protein bands are indicated.
B. Pervanadate-treated monocyte-derived DCs were subjected to immunoprecipitation with 29E3 anti-TREM-2 mAb or control IgG_1 mAb (21C7 anti-TREM-1 mAb). The precipitates were analyzed by anti-phosphotyrosine blot under reducing (left lanes) and non-reducing (right lanes) conditions. Tyrosine phosphorylated proteins are marked by arrows. Molecular weight markers are indicated.
C. Anti-DAP12 blot analysis of TREM-2 immunoprecipitate from monocyte-derived DCs (left lanes) and monocytes (right lanes) after pervanadate-stimulation (reducing conditions). TREM-1 immunoprecipitates from monocytes and monocyte-derived DCs were included as positive and negative controls, respectively. Molecular weight markers and specific protein bands are indicated.

phosphotyrosine blotting of TREM-2 immunoprecipitates from pervanadate-stimulated monocyte-derived DCs revealed a phosphorylated protein of ~14 kDa and ~28 kDa under reducing and non-reducing conditions, respectively (Figure 3.22B). This pattern was consistent with the association of TREM-2 with a tyrosine-phosphorylated protein that forms a disulfide-linked homodimer. Immunoblotting of TREM-2 immunoprecipitates with anti-DAP12, -DAP10 and -FcRγ antiserum demonstrated that TREM-2, like TREM-1, associates with only DAP12 (Figure 3.22C and data not shown). Thus, TREM-2 is capable of stimulating DAP12-linked signaling pathways in DCs.

3.3.1.2 TREM-2 induces ERK activation and survival of dendritic cells.

To see whether the TREM-2/DAP12 complex transduces activating signals in DCs as other DAP12-associated receptors do in NK cells, monocytes and neutrophils (Lanier and Bakker, 2000), TREM-2 was stimulated with 29E3 mAb or with its $F(ab')_2$ fragment. In both cases, ligation of TREM-2 elicited a rapid rise in intracellular Ca^{2+} concentration of DCs (Figure 23B). However, monovalent engagement of TREM-2 using F(ab') 29E3 did not induce calcium mobilization, indicating that TREM-2-mediated activation requires at least two cross-linked receptors (data not shown). Cross-linking of TREM-2 with $F(ab')_2$ 29E3 stimulated tyrosine phosphorylation of several proteins with approximate molecular masses of ~110, ~90, ~60-70, and ~30-40 kDa (Figure 3.23C). The observed ~40 kDa tyrosine phosphorylated proteins corresponded to the ERK1/2, as demonstrated by anti-phospho-ERK1/2 immunoblotting (Figure 3.23D). It was previously shown that survival of LPS-stimulated DCs is dependent on ERK (Rescigno et al., 1998) and PI(3)K (Ardeshna et al., 2000), while maturation is mainly mediated through NF-κB. Therefore, it was tested whether stimulation of TREM-2 leads to prolonged survival of DCs kept in culture in the absence of GM-CSF or IL-4. As shown in Figure 3.23E, cross-linking of TREM-2 with $F(ab')_2$ 29E3 prolonged DC survival for almost 8 days. Treatment of TREM-2-stimulated DC with the ERK inhibitor PD98059 blocked this survival effect. Inhibitors of PI-3K, IκB-phosphorylation, or IκB-degradation had no effect (Figure 3.23F and data not shown). These observations indicate the TREM-2 induces survival of DCs through activation of the ERK pathway.

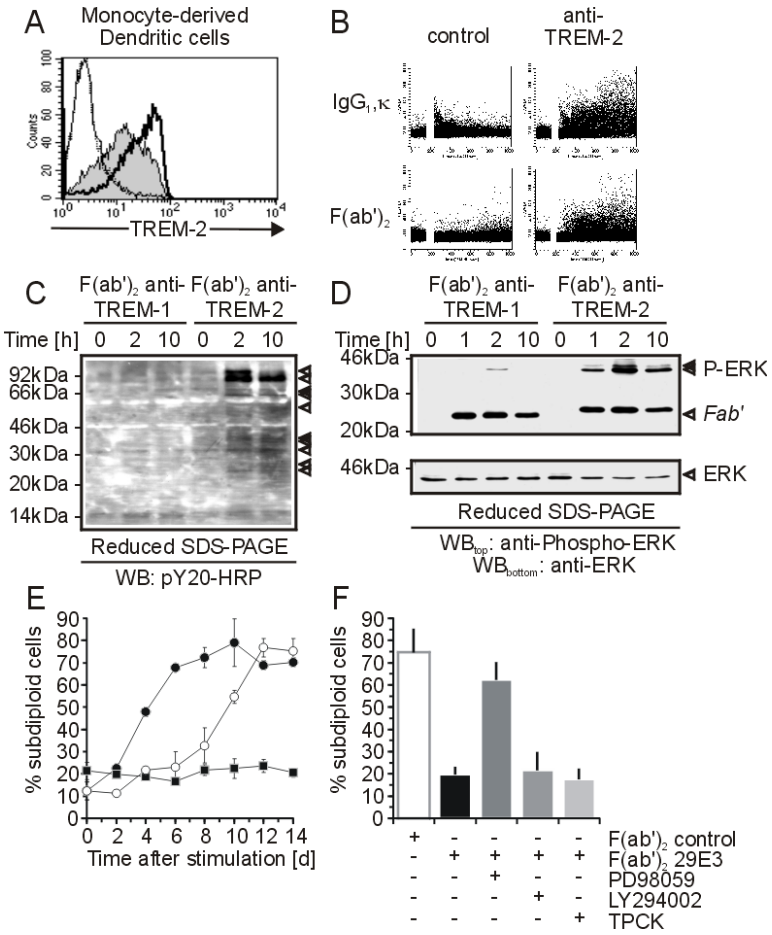

Figure 3.23: Stimulation of monocyte-derived DCs via TREM-2 induces Calcium mobilization, tyrosine phosphorylation and prolongs DC survival by an Erk-dependent pathway.

A. Functional characterization of F(ab') and F(ab')$_2$ 29E3. Monocyte-derived DCs were analyzed by flow cytometry for cell surface expression of TREM-2 using either biotinylated F(ab')$_2$ 29E3 (solid bold profile), F(ab') 29E3 (grey profile), control F(ab') (dashed profile) or F(ab')$_2$ (solid profile) followed by Streptavidine-PE.

B. Stimulation of TREM-2 induces intracellular Ca^{2+} mobilization. Bivalent crosslinking of TREM-2 using IgG1,κ 29E3, or Fc-free F(ab')$_2$ 29E3 induces intracellular Ca^{2+}-mobilization in contrast to control mAbs (21C7 anti-TREM-1 mAb; left panels; 1A11 anti-MHC class I mAb; data not shown).

C. Anti-phosphotyrosine blot of cell lysats from monocyte-derived DCs stimulated with F(ab')$_2$ 29E3 (anti-TREM-2) or control F(ab')$_2$ (anti-TREM-1 mAb) for the indicated time periods.

D. Monocyte-derived DCs were stimulated as indicated in (C) and examined by Western blot analysis for anti-phospho-Erk1/2 (upper panel) compared to anti-Erk 1/2 (lower panel). Arrows indicate phosphorylated proteins in all panels. Molecular weight markers are shown.

E. Monocyte-derived DCs were washed five times to remove GM-CSF / IL-4 before stimulation with plastic-bound F(ab')$_2$ 29E3, control F(ab')$_2$ (21C7 anti-TREM-1 mAb) or GM-CSF for the indicated time periods. Apoptotic cell death was determined by measurement of DNA fragmentation.

F. Monocyte-derived DCs were stimulated plastic-bound F(ab')$_2$ 29E3 in the presence or absence of PD98059 Erk-Inhibition (PD98059 [20µM]), PI3K-Inhibition (LY294002 [10µM]) or Inhibition of IκB-degradation (TPCK [20µM]). Apoptotic cell death was determined after 8 days as compared to DCs stimulated with control F(ab')$_2$.by measuring subdiploid DNA content.

3.3.1.3 TREM-2 triggers rapid upregulation of CCR7 and increased expression of MHC class II, CD86 and CD40.

To examine whether TREM-2 can trigger migration of DCs and/or their maturation into efficient antigen-presenting cells, immature DCs were stimulated with F(ab')$_2$ 29E3 mAb coated on a plastic surface. These cells were tested for the expression of cell surface molecules involved in migration, antigen-presentation, costimulation and

Table 3.4: TREM-2-dependent regulation of cell surface activation markers.

DCs were cultured for 48 h in plates coated with control $F(ab')_2$, $F(ab')_2$ anti-TREM-2 mAb or LPS as indicated in Figure 3.26. Cells were subsequently analyzed by flow cytometry for the indicated cell surface molecules. Numerical values indicate specific mean fluorescence intensity after subtraction of the fluorescence detected with an isotype-matched control. The data shown are representative for 4 independent experiments.

	$F(ab')_2$ anti-TREM-1	$F(ab')_2$ anti-TREM-2	LPS
TREM-2	112.23	3.45	7.1
MHC class I	67.8	65.3	107.1
MHC class II	119.12	278.65	454.67
CD40	171.35	598.6	635.89
CD80	32.1	39.4	104.6
CD86/B7.2	14.04	387.91	683.56
CCR5	12.95	13.56	3.12
CCR6	3.68	3.45	4.01
CCR7	6.82	21.98	12.45
CXCR4	5.13	4.56	17.8
CD11a	10.92	6.78	13.72
CD11b	53.9	65.7	23.1
CD11c	91.1	65.7	123.5
CD29	38.22	37.56	37.5
CD41	4.54	4.67	4.39
CD54/ICAM-1	56.87	54.78	271.45
CD61	4.95	5.03	4.21
CD103	3.63	3.96	3.26
Mannose-R	81.8	82.9	30.9
CD64/FcγR I	9.8	10.1	2.3
CD32/FcγR II	17.21	16.78	2.34
CD89/FcγR	4.54	4.75	4.96
CD35/CR 1	3.94	4.23	3.67
M-CSF-R	14.6	4.23	5.21
GM-CSF-R	15.6	13.7	13.5
CD38	2.5	2.2	43.5
CD83	3.34	3.23	26.7
CD1a	106.76	134.9	87.54

adhesion, as well as for the production of cytokines. Upon TREM-2 ligation, CCR7 surface expression was rapidly increased (Figure 3.24A). CCR7 was functionally competent, as TREM-2-stimulated DCs showed a specific chemotactic response towards the CCL19 and CCL21, which could be inhibited by anti-CCR7 mAb (Figure 3.24B). The amplitude and kinetics of CCR7 upregulation induced via TREM-2 were different as compared to those induced by LPS stimulation (Figure 3.24A). While expression of CCR7 was detectable by 6 hours after TREM-2 stimulation, LPS-induced upregulation of CCR7 was weaker and occurred only after 24 hours of cell stimulation. Regardless of CCR7 surface levels, however, LPS-stimulated DCs displayed a stronger chemotactic response towards CCL19 and CCL21 than TREM-2-stimulated DCs.

Therefore, DC mobility and chemotaxis to CCL19 and CCL21 is not solely related to CCR7 expression levels. Indeed, it has been shown that other receptors, such as the Multidrug Resistance-associated Protein 1 (MRP-1), can contribute to optimal mobilization of DCs from skin to secondary lymphoid organs (Robbiani et al., 2000).

Ligation of TREM-2 also induced increased cell surface expression of several molecules involved in T cell stimulation, such as MHC-class II, CD40 and CD86 (Table 3.4). In contrast to LPS-activated DCs, CD83 and ICAM-1 were not upregulated. Furthermore, antigen-capturing molecules, such as CD32, CD64, CD89, and the mannose receptor, were not downregulated (Table 3.4). DCs activated through TREM-2 did not secrete either IL-12, or other cytokines (data not shown). Thus, TREM-2 mediates a unique pattern of DC activation, characterized by strong upregulation of CCR7, expression of some T cell stimulatory molecules and lack of cytokine secretion.

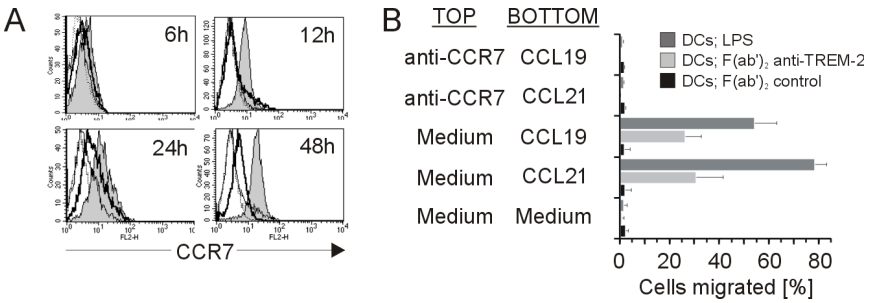

Figure 3.24: Stimulation of TREM-2 induces CCR7 expression and chemotactic response towards CCL19 and CCL21.

A. CCR7 is rapidly upregulated after stimulation of TREM-2 on DCs. DCs were stimulated with F(ab')$_2$ anti-TREM-2 mAb (grey profils), control F(ab')$_2$ (21C7; anti-TREM-1; solid line profils), or LPS (solid bold profils). After the indicated time periods cells were harvested and analyzed by flow cytometry for cell surface expression of CCR7 by anti-CCR7 mAb (mouse IgM) followed by PE-labeled goat anti-mouse IgM. Dashed profiles indicate background staining with a control IgM mAb.
B. TREM-2 stimulation directs migration of DCs towards CCL19 and CCL21 by a CCR7-dependent pathway. DCs stimulated for 24 h with plastic-coated control F(ab')$_2$ (21C7, anti-TREM-1; black bars), F(ab')$_2$ anti-TREM-2 mAb (light-grey bars), or LPS (dark-grey bars) were tested in transwell chemotaxis assays for the ability to migrate towards medium alone, medium supplemented with 100 ng/ml CCL19 or CCL21 (BOTTOM well). In control experiments, DCs were preincubated for 15 min with anti-CCR7 mAb before placing them in the TOP well for assessment of chemotaxis towards medium alone, medium supplemented with CCL19 or CCL21 placed in the BOTTOM well.

3.3.1.4 TREM-2 does not activate IκBα/NF-κB or p38/SAPK signaling pathways.

LPS-induced maturation of DCs requires the activity of NF-κB transcription factor (Doi et al., 1997; Kontgen et al., 1995; Sha et al., 1995). Prior to activation, NF-κB is retained in the cytoplasm through binding to the inhibitory IκB protein. Stimulation of cells with LPS leads to IκB kinase (IKK)-mediated phosphorylation of IκB. This is followed by rapid ubiquitination and proteolytic degradation of IκB, allowing nuclear translocation of NF-κB and binding to κB-promoter elements (Baeuerle, 1998; Baldwin, 2001; Karin and Ben-Neriah, 2000). To study whether TREM-2 activates the IκBα/NF-κB pathway, DCs were stimulated through TREM-2 followed by the analysis of phosphorylation of IκBα and the assessment of nuclear translocation of NF-κB by searching for NF-κB-containing complexes in electrophoretic mobility shift assays (EMSA). In striking contrast to LPS (Ardeshna et al., 2000), antibody-mediated ligation of TREM-2 did not lead to phosphorylation and degradation of IκBα (Figure 3.25A) or nuclear translocation of NF-κB (Figure 3.25B). It was previously shown that LPS-induced maturation of DCs is also mediated by activation of p38/SAPK (Ardeshna et al., 2000; Arrighi et al., 2001). To see whether TREM-2 activates p38/SAPK, TREM-2 was crosslinked on DCs with F(ab')$_2$ 29E3 and lysats were analyzed for tyrosine phosphorylation of p38/SAPK by western blot analysis. In contrast to LPS, TREM-2 did not induce p38/SAPK tyrosine phosphorylation (Figure 3.25C). Thus, TREM-2-induced activation pathway is NF-κB- and p38/SAPK-independent.

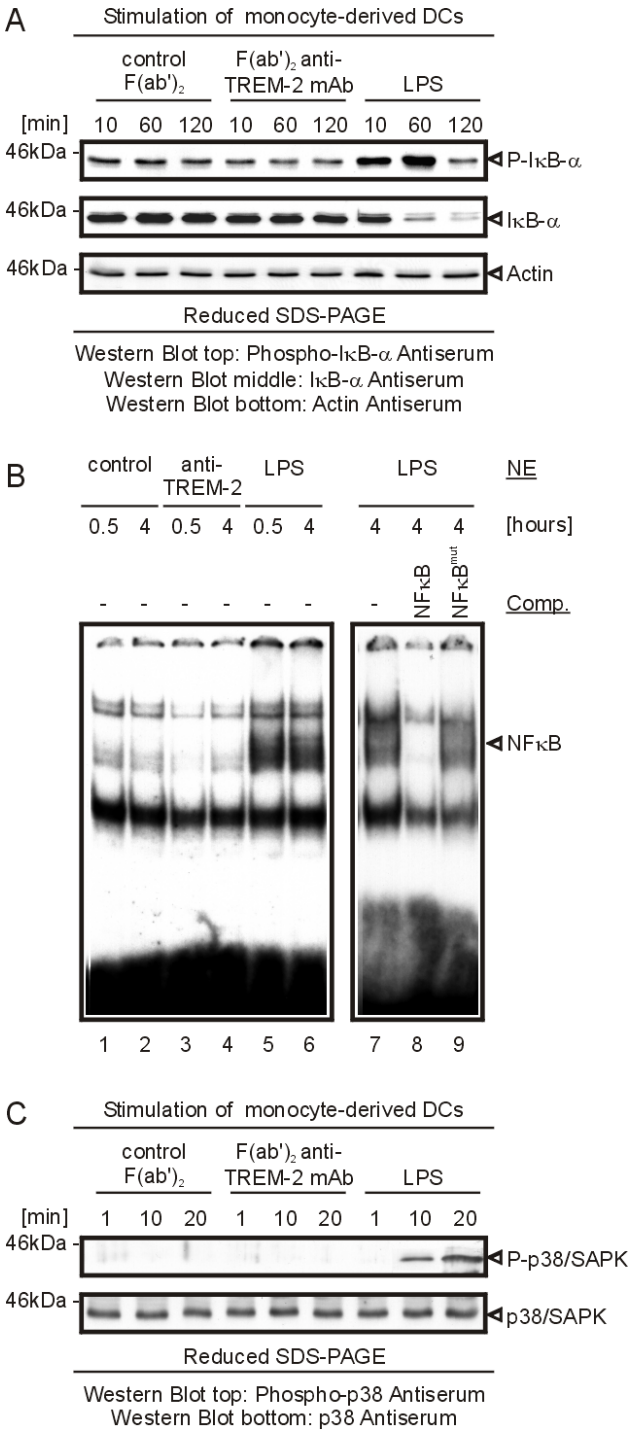

Figure 3.25: TREM-2 does not initiate IκBα/NFκB- and p38/SAPK-dependent pathways.

A. Lack of IκBα phosphorylation and degradation upon TREM-2 stimulation of monocyte-derived DCs. Monocyte-derived DCs were stimulated by LPS or plastic-bound $F(ab')_2$ anti-TREM-2 mAb or control $F(ab')_2$ for the indicated times. Protein lysates were tested for IκBα phosphorylation and degradation by Western Blot analysis. The same blot was sequentially stripped and reprobed with anti-phospho-IκBα, anti IκBα, and anti-Actin (loading control) antibodies.

B. Lack of NFκB translocation upon TREM-2 triggering. Monocyte-derived DCs were stimulated for the indicated time points as described in (A) and nuclear extracts were obtained. Radiolabeled NFκB consensus double-stranded oligonucleotides were incubated with the indicated nuclear extracts in the absence of competing oligonucleotides or in the presence of a 25-fold molar excess of wild-type (NFκB) or mutant (NFκBmut) competing oligonucleotides. DNA-protein complexes were resolved by electrophoresis. The NFκB-containing complex is marked.

C. Absence of p38/SAPK phosphorylation upon TREM-2 stimulation of monocyte-derived DCs. Monocyte-derived DCs were stimulated for the indicated times as described in (A) Protein lysates were tested for p38/SAPK phosphorylation (upper panel) by Western Blot analysis. The same blot was sequentially stripped and reprobed with anti p38/SAPK antibodies (lower panel). Arrows indicate proteins in all panels. Molecular weight markers are shown.

To confirm proper stimulation of TREM-2/DAP12 in all experimental settings an aliquot of stimulated DCs was kept and tested after 48 hours for upregulation of MHC class II and CD86 (data not shown).

3.3.1.5 TREM-2 induces DC maturation through an ERK- and PTK-dependent, NF-κB and p38/SAPK-independent pathway.

To further characterize the signaling molecules involved in TREM-2-mediated DCs maturation, DCs were incubated with inhibitors of ERK (PD98049), NF-κB (TPCK), p38/SAPK (SB203580) and PTKs (PP2). Treated cells were stimulated with either $F(ab')_2$ anti-TREM-2 mAb, LPS or immobilized IgG and subsequently analyzed for cell surface expression of maturation markers, such as CCR7, MHC class II, ICAM-1

CD83, CD40 and CD86. TREM-2 induced upregulation of CCR7, CD86, class II and CD40, while cell surface expression of ICAM-1 and CD83 was not increased (Figure 3.26, red bars). Remarkably, TREM-2-induced expression of CCR7, CD86, class II and CD40 was completely blocked by a PTK inhibitor and partially blocked by an ERK inhibitor. This differential inhibitory capacity suggests that PTK may activate downstream signaling molecules other than ERK, which concur to TREM-2-mediated DC maturation. Incubation of DCs with NF-κB and p38/SAPK inhibitors had virtually no effect (Figure 3.26, red bars).

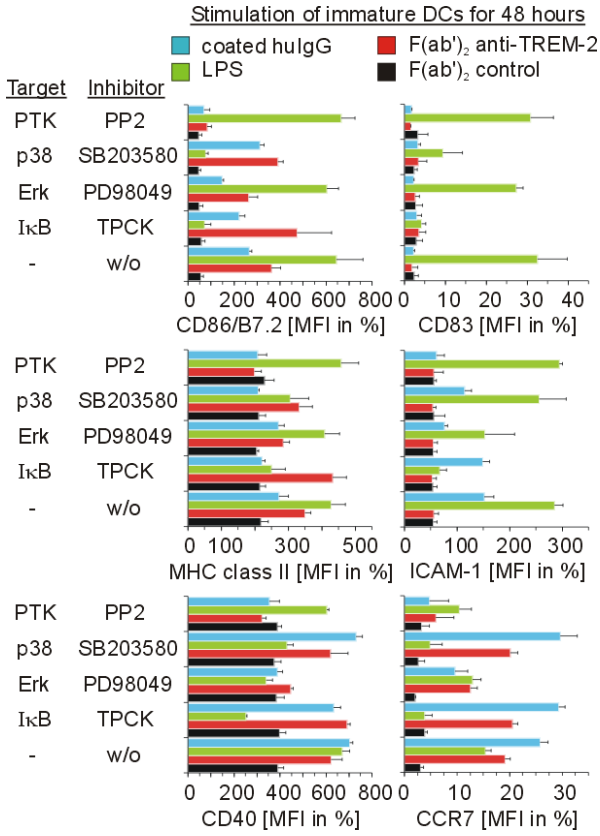

Figure 3.26: Comparison of TREM-2-, TLR- and FcR-mediated maturation pathways.

Monocyte-derived DCs were stimulated with plastic-bound control $F(ab')_2$ (black bars), $F(ab')_2$ anti-TREM-2 (red bars), human IgG (blue bars), or LPS (green bars) in the presence of inhibitors for Erk (PD98059), p38/SAPK (SB203580), PTK (PP2), inhibition of IκBα degradation (TPCK) or an equal volume of DMSO as a control. After 48 hours, cell surface expression of CD86 (top left panel), MHC class II (middle left panel), CD40 (bottom left panel), CD83 (top right panel), ICAM-1 (middle right panel) or CCR7 (bottom right panel) was determined by flow cytometry. Data shown are representative for four independent experiments and display the mean and standard deviation of 3 independent samples.

LPS-induced maturation pathway was totally distinct from that mediated by TREM-2. LPS induced upregulation of CCR7, MHC class II, ICAM-1 CD83, CD40 and CD86 (Figure 3.26, green bars) (Banchereau et al., 1998). Incubation of DCs with NF-κB and p38/SAPK inhibitors prevented LPS-induced maturation, whereas ERK inhibitor had a modest effect, as previously described (Figure 3.26, green bars) (Ardeshna et al., 2000; Arrighi et al., 2001). Finally, engagement of FcR by immobilized IgG induced a maturation pattern, which was similar to that induced by TREM-2, with the exception of an upregulation of ICAM-1 (Figure 3.26, blue bars). Incubation of DCs with PTK and ERK inhibitors resulted in total and partial inhibition of FcR-induced maturation, respectively. These observations provide evidence that TREM-2 mediates DC maturation by PTK/ERK-dependent pathways. These pathways overlap with

those initiated by FcRs, but are distinct from the IκBα/NF-κB and p38/SAPK-dependent pathways triggered by LPS.

3.3.1.6 Crosslinking of TREM-2 on MDCs results in receptor internalization and delivery into the antigen-processing compartment

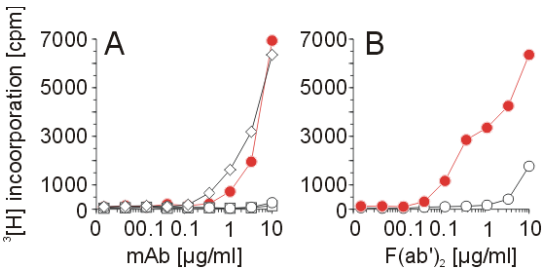

Figure 3.27: TREM-2 can act as a antigen-capturing surface receptor

Presentation of anti-TREM-2 mAb to a T cell clone specific for mouse IgG1 (VIP13) by irradiated DCs. (A) DCs were stimulated with the indicated concentrations of anti-ILT3 mAb (white diamonds), anti-TREM-2 mAb (red circles), anti-TREM-1 mAb (white squares), anti-CD11b mAb (white circles), (B) F(ab') $_2$ anti-TREM-2 (red circles)or F(ab')$_2$ anti-TREM-1 mAb (white circles). F(ab') $_2$ anti-TREM-2 is presented ~100-fold more efficiently than F(ab')$_2$ anti-TREM-1. The shown data are representative for 3 independent experiments

TREM-2 is selectively expressed on professional antigen capturing and processing cells. In addition, the signaling subunit of TREM-2, DAP12 displays cytoplasmic Y-x-x-V/L motifs, which have been reported to mediate internalization . Therefore, the ability of TREM-2 to endocytose and deliver its ligand(s) to an antigen-processing and loading compartment was analyzed. Strikingly, TREM-2 was internalized almost completely from the cell surface of monocyte-derived DCs already after 10-30 min compared to control stimulation (data not shown). Rapid internalization of surface receptors upon binding to their ligands is a common feature of antigen-capturing receptors, such as FcR, Mannose-Receptor or Scavanger-Receptor. Upon internalization, all of these receptors with their bound ligands are delivered to the antigen-processing machinery, which subsequently leads to presentation of ligand-peptides in the context of MHC-class II molecules. To further investigate a similar role for TREM-2 in antigen capture, we used the 29E3 anti-TREM-2 mAb as a ligand for TREM-2 and evaluated the ability of DCs to present peptides derived from 29E3 to a CD4$^+$-class II-restricted T cell clone specific for a mouse IgG1 peptide epitope (Lanzavecchia et al., 1988). The presentation of the 29E3 mAb was compared to that of mAbs that bind either to a known antigen-capturing receptor (anti ILT-3 mAb

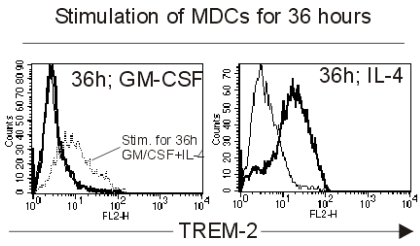

Figure 3.28: TREM-2 is strongly upregulated after stimulation of monocytes with IL-4.

IL-4- (right panel), or GM-CSF- (left panel) treated monocytes were analyzed by FACS for cell surface expression of TREM-2 (solid bold line) after 36 h. Dashed profiles correspond to background staining with a control IgG_1 mAb. The dotted inset in the left panel indicates the upregulation using GM-CSF/IL-4 for a stimulation of 36 hours.

ZM3.8; (Cella et al., 1997)), to cell surface-expressed CD11b, or do not bind to surface molecules and thus are taken up in the fluid phase (anti-TREM-1 mAb 9E2). As shown in Figure 3.27, DCs presented 29E3 mAb to T cells 50-100 fold more efficiently than anti-CD11b or control mAb. The presentation is comparable to the engagement of ILT3 with mAb ZM3.8 (Cella et al., 1997). To reduce the unspecific contribution of FcR to the observed degree of T cell stimulation, we further investigated the potential of DCs to present peptides from $F(ab')_2$ anti-TREM-2 mAb. Similar to results observed with IgG1,κ mAb, the $F(ab)_2$ anti-TREM-2 mAb is presented with 100fold higher efficiency presented than control $F(ab')_2$ mAb. Together, these experiments indicate that TREM-2 can efficiently deliver its ligand(s) to an intracellular compartment where MHC class II loading can occur. This is in line with the observed strong upregulation of MHC class II. Since TREM-2-stimulation leads in parallel to increased expression of CD40 or CD86, the sum of these events generate the optimal environment for the stimulation of antigen-specific T cells in the lymph node.

3.3.1.7 Regulation of human TREM-2 and characterization of human TREM-2 *in situ*

As shown in Figure 3.28, the observed TREM-2 upregulation in monocyte-derived DCs after 1 – 2 days was due to IL-4 rather than GM-CSF, since IL-4 alone induced a strong TREM-2 upregulation just after 24 – 36 hours in isolated monocytes and human blood monocytes (data not shown). This observation opens the possibility that TREM-2 may play its particular role in conditions such as parasitic infections or allergic diseases, which are predominantly controlled by IL-4. In addition, it suggests that TREM-2 might not only be expressed by DCs but also by other myeloid cells responsive to IL-4, such as mast cells (Hassuneh et al., 1997). This conclusion is further supported by the characterization of human TREM-2 *in situ*. Indeed, TREM-2 expression was detected not only on tonsillar DCs (data not shown) but also on tissue mast cells

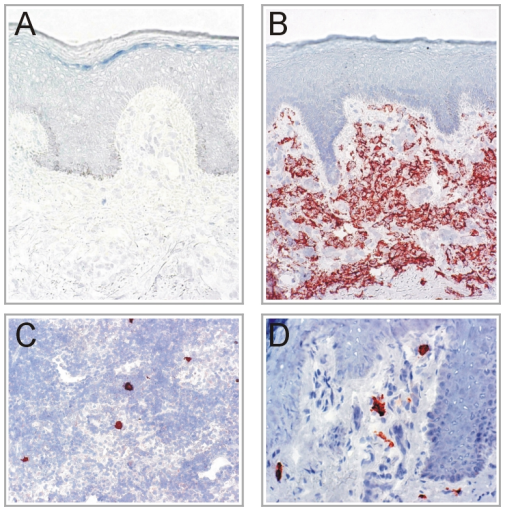

Figure 3.29: Human TREM-2 is expressed on mast cells *in situ*

TREM-2 expression was detected using mAb 20G2 in mastocytoma (**A, B**), spleen (**C**) and lymph node (**D**) from normal individuals. Staining with control mAb is shown in **B**.

from normal individuals in the lymphnode and spleen (Figure 3.29C,D) and in mastocytomas (Figure 3.29B) using the TREM-2 mAb 20G2.

3.3.2 The role of mTREM-2 in experimental autoimmune encephalomyelitis

In preliminary experiments, anti-human TREM-2 mAb 21E10 stained mouse BM-derived DCs cultured in IL-3/GM-CSF/IL-4, suggesting a strong structural and functional homology between mouse and human TREM-2. Thus, mTREM-2 appears to be expressed on DCs, just like human TREM-2Whether the mAb 21E10 is capable of recognizing mouse mast cells is currently under investigation.

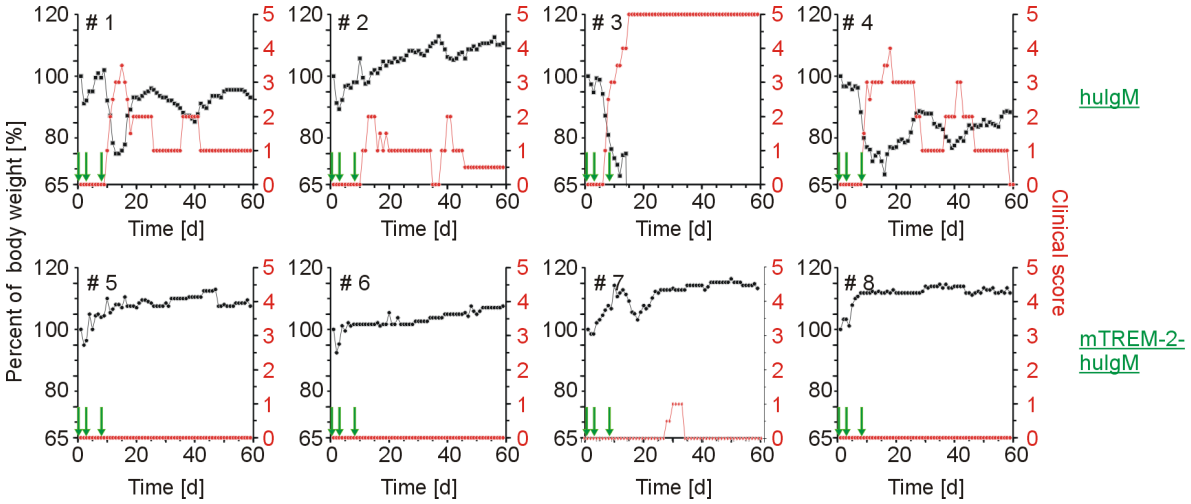

Figure 3.30: MOG-induced EAE in C57BL/6 treated with mTREM-2-IgM or huIgM.

Development of clinical EAE in four mice treated with mTREM-2-IgM (bottom) and four mice injected with control huIgM after immunization with peptide MOG^{35-55} in CFA. The clinical score (red curves) and weight (black curves) were monitored daily over a period of 60 days (x-axis) after injection. Day of injections with 400 µg of protein/animal are depicted as green arows. The shown data are representative of a larger group of immunized animals summarized in Table 3.5.

$DAP12^{-/-}$ mice are resistant to experimental autoimmune encephalomyelitis (EAE) and it was shown that this effect was paralleled by improper T cell stimulation by APCs. TREM-2 was shown to play a central role in APC maturation, migration and T cell priming in human MDCs. It was therefore tested, whether blocking of mTREM-2 signaling by mTREM-2-IgM influences EAE compared to animals injected with control human IgM. 400 µg mTREM-2-IgM fusion protein or control huIgG1 was injected intraperitoneally in C57BL/6 mice 6 h before,and 3 and 9 days after EAE induction. Animal weight and clinical symptoms were monitored every day for 60 days. As shown in Figure 3.30 and Table 3.6, control animals were highly responsive to immunization with MOG^{35-55} peptide showing an early onset of disease (day 6 - 15), a high mean maximal disease score (3.1 ± 1.4) and a milder relapsing course of disease after 33 – 39 days. In striking contrast to the high susceptibility of control mice, ani-

mals treated with mTREM-2-IgM are resistant to immunization with MOG^{35-55}. In treated animals the incidence of MOG^{35-55}-induced disease was 30 %, whereas in control mice, the incidence was 90 %. In addition, the maximal disease score was only 0.2 ± 0.4 and disease onset was delayed up to 25 days as compared to control animals. Whether TREM-2 plays a role in the direct co-stimulation beween T cells and APCs or is rather involved in the recruitment of APC to the T cell area remains unknown.

Table 3.5 : MOG-induced EAE in C57BL/6 treated with mTREM-2-IgM or huIgM.

C57BL/6 (two independent experiments containing 5 mice per group) were challenged with MOG^{35-55} peptide under treatment with mTREM-2-IgM or control IgM. The disease incident, the mean maximal disease score, mean day of onset and relapse of clinical EAE were calculated for each group of immunized mice.

Antigen	Treatment (3 x 400 µg/animal)	Disease incident (%)	Mean maximal disease score ±SE	Mean day of onset (range)	Mean day of relapse (range)
MOG^{35-55}	huIgM	9/10 (90)	3.1 ± 1.4	9.2 (6 – 15)	37.4 (33 – 39)
MOG^{35-55}	mTREM-2-IgM	3/10 (30)	0.2 ± 0.3	27.1 (25 – 31)	No relapse

3.4 Characterization of TREM-3, -4, -5

3.4.1 Characterization of TREM-3

3.4.1.1 First characterization of mTREM-3 by Northern blot analysis

As human TREM-3 is a pseudogene, efforts concentrated on the characterizations of mouse TREM-3. Northern Blot analysis revealed that mTREM-3 mRNA is mainly expressed in the liver, and not in other tissues or organs of the immune system. The production of anti-mTREM-3 mAb is in progress.

3.4.2 Characterization of TREM-4

3.4.2.1 Northern blot analysis

In initial studies, TREM-4 transcript was selectively detected in MDCs by reverse-transcriptase polymerase chain reaction (data not shown) suggesting the expression of TREM-4 in immature DCs. Using Northern blot analysis TREM-4 mRNA could be detected in the testis, lung and heart (data not shown).

3.4.2.2 Production and characterization of anti-TREM-4 mAb *in vitro* and *in situ*

To determine the cellular distribution of TREM-4, anti-TREM-4 mAb were produced. The mAb 64F5 stained TREM-4-transfected Jurkat cells specifically, as compared to control transfectants (Figure 3.31A). In disagreement with RT-PCR data, TREM-4 could be detected neither on immature MDCs nor on mature DCs (Figure 3.31B). Interestingly, immunohistochemistry revealed that TREM-4 is strongly expressed on sertoli cells in testis and on endothelial cells in particular in the heart. Since TREM-4 contains an Arginine residue in the transmembrane region it might interact with FcRγ or DAP10 rather than with DAP12. Identification of cell lines expressing TREM-4 will pave the way to further biochemical insights about the signal transduction properties of TREM-4.

3.4.3 Characterization of TREM-5

3.4.3.1 Northern blot analysis

TREM-5 transcript was initally detected in PBL by Northern Blot analysis (data not shown) and cloned from human BM indicating expression of TREM-5 in immune cells.

3.4.3.2 Production and chracterization of anti-TREM-5 mAb *in vitro* and *in situ*

To accurately investigate the cellular distribution of TREM-5 as well as its biochemical characteristics and functions, anti-TREM-5 mAb were produced. In transfected

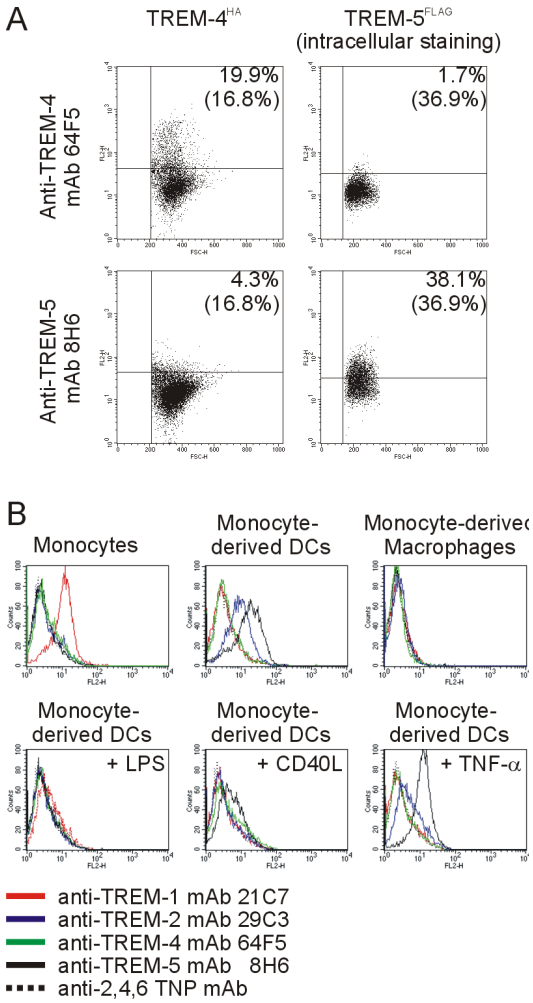

Figure 3.31: Characterization of anti-TREM-4 and anti-TREM-5 mAbs.

A. mAb 64F5 and 8H6 recognize TREM-4 and TREM-5, respectively. Jurkat cells transfected with a cDNA encoding FLAG-tagged TREM-5 (TREM-5^{FLAG}) (right panels) or HA-tagged TREM-4 (TREM-4^{HA}) were stained with anti-TREM-4 mAb 64F5 (upper panel) and anti-TREM-5 mAb 8H6 (lower panels). TREM-5^{FLAG} transfectants were stained for intracellular expression. The percentages of positive cells (upper right quadrants) are indicated. Expression of TREM-4^{HA} and TREM-5^{FLAG} was confirmed using an anti-HA and an anti-FLAG mAb, respectively (Percentages obtained with the tag-specific mAb are shown in brakets (upper right quadrants)). Cells stained with an isotype-matched control mAbs were comprised within the indicated lower quadrant.

B. Top panels: TREM-5 is strongly unregulated after stimulation of monocytes with GM-CSF and IL-4. Monocytes untreated (left panel) or treated with GM-CSF/IL-4 (middle panel) or M-CSF (right panel) were analyzed by flow cytometry for cell surface expression of TREM-1 (red profiles), -2 (blue profiles), -4 (green profiles), and -5 (black bold profiles) after 3 d or up to 14 days, respectively. Dashed profiles indicate background staining with a control IgG_1 mAb.

Bottom panels: TREM-5 is downregulated upon maturation of DCs. LPS- (right panel), CD40L- (middle panel), or TNF-α-stimulated MDCs (right panel) were analyzed by flow cytometry as described in (B).

Jurkat cells TREM-5 was expressed intracellularly and was not delivered to the cell surface. Nevertheless, the mAb 8H6 specifically stained TREM-5-transfected Jurkat cells intracellularly, as compared to control transfectants (Figure 3.31A). Flow cy-

tometry analysis from PBL revealed a yet undefined population of TREM-5$^+$ cells, which has the cell size of monocytes and blood DCs according to FSC-analysis (data not shown). Interestingly, TREM-5 was not expressed on isolated CD14$^+$ monocytes, thus strongly suggesting that blood DCs are TREM-5$^+$. Indeed, TREM-5 was expressed even stronger on MDCs than TREM-2, whereas it could not be detected on MMϕs (Figure 3.31B). In contrast to the regulation of TREM-2, TREM-5 is induced by GM-CSF + IL-4, but not by culturing monocytes in either GM-CSF or IL-4 alone (data not shown). DC maturation induced by lipopolysaccaride (LPS), CD40L-expressing cells, but not by TNF-α, IL-1β, or aggregated IgG led to complete downregulation of TREM-5 (Figure 3.31B data not shown).

Interestingly, the downregulation of TREM-5 seems to occur with different kinetics and upon different stimuli compared to TREM-2 (Figure 3.31B) thus suggesting that these molecules are differentially regulated. In addition, the transmembrane region important for interaction with a signaling subunit is different between the two proteins (Figure 3.4). It is therefore possible, that in contrast to TREM-2, TREM-5 might not interact with DAP12. Whether TREM-5 interacts with DAP12 and whether stimulation of TREM-2 and –5 leads to identical biochemical and cellular changes remains to be determined.

4 DISCUSSION

4.1 Amplification of neutrophil and monocyte responses

4.1.1 The TREM-1 amplification system

Biological amplification systems require a tightly controlled initiation, a rapid and – if possible - exponential amplification of the desired responses, and a reliable mechanism to terminate the responses once the biological effect is achieved (Voet and Voet, 1998).

In the immune system, amplification systems are switched on during potentially dangerous situations such as pathogen invasion (Matzinger, 1998). Under conditions of microbial or viral infection the immune system faces a toxin-producing, rapidly dividing intruder that can potentially overwhelm immune surveillance. A control is therefore only possible if the immune response acts with the highest possible efficiency right from the beginning. The results presented here strongly suggest a role of TREM-1 as an amplifier of inflammatory responses triggered by bacterial and fungal infections.

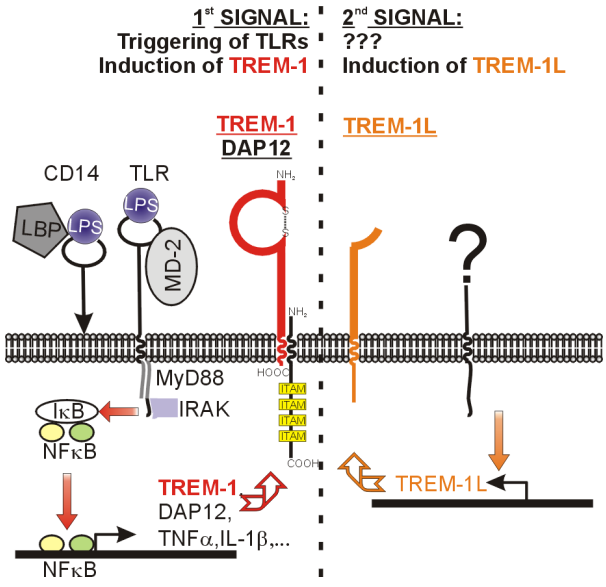

Figure 4.1: Induction of the TREM-1 system

As schematically shown in Figure 4.1, during the early phase of infection, neutrophils and monocytes initiate the inflammatory response following the engagement of PRRs such as the TLRs by microbial products (Akira et al., 2001; Medzhitov and Janeway, 2000). Bacterial products induce TREM-1 and investigations of the TREM-1 promoter revealed several NF-κB responsive elements (data not shown). This indicates TREM-1 as a target gene for NF-κB, which can be induced by TLRs, TNF-R1 or CD40 (Inoue et al., 2000). The cross-talk between the signaling pathways induced by microbial challenge and DAP12-dependent pathways is also exemplified by the upregulation of DAP12 expression

upon LPS treatment (Aoki et al., 2000). It is of particular interest, however, that receptor and ligand of the TREM-1 system appear to be differentially regulated. Whereas TREM-1 upregulation is clearly NF-κB-dependent, TREM-1L is not induced solely by LPS stimulation of neutrophils *in vitro* (data not shown), thus indicating that TLR-triggering is not sufficient for the upregulation of TREM-1L. Since TREM-1L expression could be detected on neutrophils during endotoxemia *in vivo*, it is likely that distinct or additional signals to TLRs are need for the induction of TREM-1L. Thus, the activation of the TREM-1 amplification system requires the concerted induction of different switches and proofreading mechanisms further ensuring a tight control for the induction.

TREM-1 induces amplification of inflammatory responses in two ways: prolonged survival of effector cells (4.1.2) and cooperation with other signaling pathways (Figure 4.2). It has been shown that TLRs initiate host responses to pathogens through NF-κB as well as by activating JNK- and p38/SAPK-dependent transcription factors (TFs) such as Jun and MEF2C (Aderem and Ulevitch, 2000; Akira et al., 2001; Medzhitov and Janeway, 2000; Medzhitov et al., 1997). In contrast, TREM-1/DAP12 initiate PLCγ-, PTK-, calcium- and ERK1/2-dependent pathways finally culminating in the activation of distinct TFs such NF-AT, Myc, Ets-1, and Egr-1. TFs of the Ets-1 family are responsible for the transcription of Fos, which together with Jun form the TF AP-1 (Blume-Jensen and Hunter, 2001). Therefore, by complementation TREM-1/DAP12- and TLR-dependent pathways act synergistically by activating a broad set of MAPK and TFs such as NF-κB, AP-1 (Fos/Jun heterodimers), Myc, and NF-AT that cooperate for the induction of an optimal pro-inflammatory responses (Figure 4.2). Taken

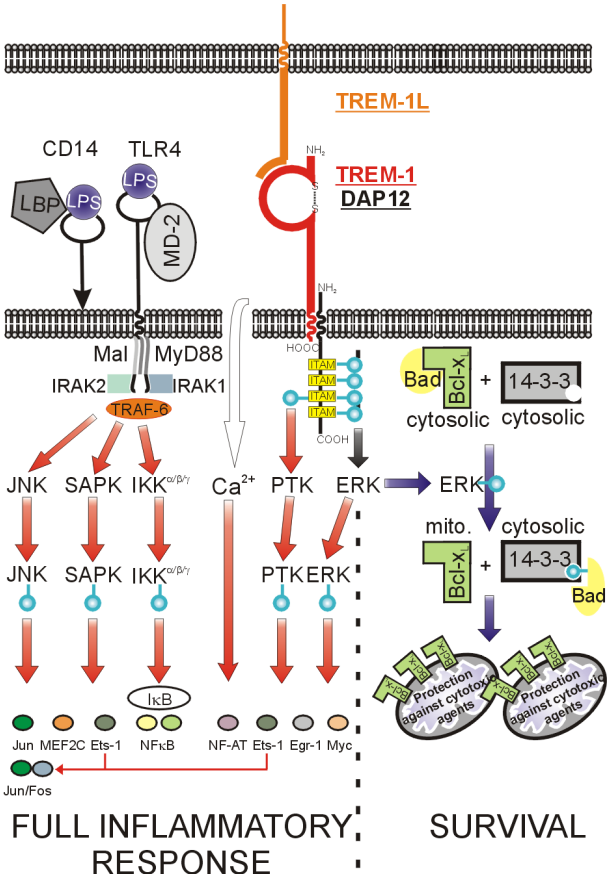

Figure 4.2: Signal amplification by the TREM-1 system

together, the data presented here indicate that TREM-1 amplifies inflammation by intensifying the quality and prolonging the duration of the response (Figure 4.2).

After the invading pathogens are cleared, all systems including TREM-1 must be reset to ensure the resolution of inflammation and the sensitivity of the system. Potential mechanisms can either act intracellularly on DAP12-induced signals (Figure 1.13) or directly interfere with the TREM-1/TREM-1L interaction on the cell surface (Figure 4.3). In NK cells, stimulation via DAP12-associated receptors is counterregulated by inhibitory receptors containing ITIM-motifs capable of binding phosphatases such as SHP-1, SHP-2 or SHIP (Scharenberg and Kinet, 1996). Receptor-proximal activation of phosphatases results in dephosphorylation of ITAMs, thus interfering with activating signals (Amigorena et al., 1992; Muta et al., 1994). So far only few inhibitory receptors expressed on myeloid cells are known. ILT3, an inhibitory receptor expressed on monocytes and DCs, is indeed capable of recruiting SHP-1 and subsequently counteracting activating receptor signals (Cella et al., 1997). Thus, it is likely that phosphatases are responsible for the down regulation of TREM/DAP12-mediated activation signals (Figure 1.13). Similar to TREM-2, TREM-1 is rapidly internalized after triggering with specific mAb (data not shown). This is most probably due to the putative Y-x-x-V/L motif of TREM-1-associated DAP12, which resembles an internalization sequence. Thus, the duration of TREM-1 signaling may be regulated by stimulation-induced receptor internalization (Figure 4.3). Further investigations using the cognate TREM-1L are necessary to determine whether receptor internalization is indeed a mechanism to terminate TREM-1-mediated signaling. The recent identification of a splice variant of TREM-1 that lacks the transmembrane region offers an additional possibility for the modulation of TREM-1 signals (sequence and

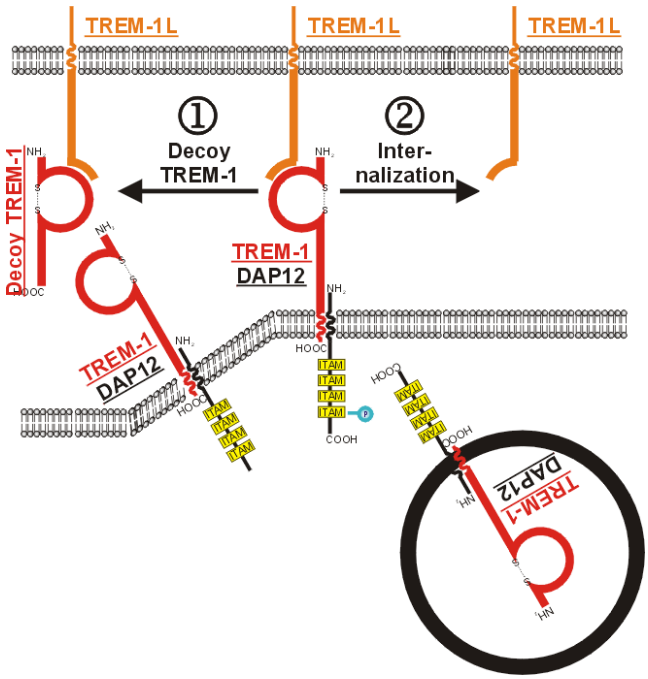

Figure 4.3: Downregulation of the TREM-1 system

TREM-1 signaling can be counterregulated by inhibitory receptors (not shown), receptor internalization (2) or the secretion of a TREM-1 DcR (1).

data not shown). This transcript would lead to a soluble TREM-1 (sTREM-1) that could be secreted and might act as a receptor decoy (DcR). Using an ELISA system with two TREM-1 specific mAbs, it was in fact possible to detect sTREM-1 in the serum of normal individuals at concentrations of 50 - 400 pg/ml (data not shown). Most strikingly, sTREM-1 could not be detected in the serum of several septic patients (data not shown). It would be important to assess whether TREM-1, in particular, is absent in patients with abnormally prolonged inflammatory responses. Further investigations focusing on the regulation of sTREM-1 *in vitro* and *in vivo* will provide evidence to whether sTREM-1 is a DcR and can act as an endogenous brake for the immune system (Figure 4.3).

Induction of TREM-1 and TREM-1L by microbial stimuli initially leads to the protection of neutrophil against apoptosis and amplification of inflammatory responses (Figure 4.2). Under normal conditions this leads to the clearance of the pathogen, followed by the disruption of the TREM-1 amplification loop by either receptor internalization, secretion of soluble TREM-1 or intracellular activation of phosphatases (Figure 1.13 and 4.3). Under certain circumstances the immune system is not capable of clearing the invading microorganisms. Further pathogen spreading is then terminated by granuloma formation (Figure 3.10 and 3.11). An effective control of the encapsulated pathogens requires immune cells highly resistant to cytotoxic effects of microbial toxins and self-secreted ROS, which are produced to destroy the pathogen. Indeed, the highest TREM-1 expression of all systems tested was observed on neutrophils and giant cells forming granulomas (Figure 3.10 and 3.11). This strongly supports the idea that TREM-1 and TREM-1L are expressed on the same cell type thus self-amplifying their own survival and anti-microbial responses. In addition, it is of note that signaling through DAP12 *in vitro* induces a marked morphological change of mouse myeloblastic leukemic cell transfectants into multinucleated giant cells (Aoki et al., 2000) suggesting that TREM-1 may be also involved in the formation of giant cells from monocytic precursors.

Finally, the observation that TREM-1 and –2 are located on chromosome 6, which was implicated in several inflammatory and autoimmune disorders, suggests that indeed these genes may be centrally involved during regulation of inflammation. It will therefore be important to see if TREM-1 is polymorphic and if the polymorphism is linked to disease.

4.1.2 The function of TREM-1 in disease and neutrophil homeostasis

Recently, mice overexpressing a DAP12 transgene (Tg-DAP12) were generated (Bouchon et al., submitted for publication, data not shown). Interestingly, these mice

show several defects in the homeostasis of neutrophils, such as the development of neutrophilia, which is associated with a fatal inflammatory syndrome in the lungs and an increased sensitivity to LPS-induced shock.

The hyperresponsiveness of Tg-DAP12 mice to LPS injection further supports the model presented here, in which DAP12-mediated pathways, particulary TREM-1/DAP12, act as amplification signals for innate responses to microbial challenge. Interestingly, the regulation of TREM-1 during septic shock in humans parallels the situation in mice (Figure 3.12). Remarkably, inhibition of TREM-1 in LPS-induced shock in mice is sufficient to reduce serum TNF-α and IL-1β to sublethal levels, preventing shock and death. However, TNF-α and IL-1β are not removed entirely by mTREM-1-IgG1, allowing for clearance of bacterial infections (Echtenacher et al., 1990; Echtenacher et al., 1996; Eskandari et al., 1992; Malaviya et al., 1996; Peschon et al., 1998; Pfeffer et al., 1993; Rothe et al., 1993). Therefore mTREM-1-IgG1 also protects against bacterial peritonitis, in contrast to prophylactic treatment with anti-TNF-α antibodies (Beutler et al., 1985) or IL-1R antagonists, which increase lethality (Alexander et al., 1991; McNamara et al., 1993; Ohlsson et al., 1990) (Figure 1.14). mTREM-1-IgG1 provided protection even after the injection of LPS, an effect that was previously only reported for inhibition of MIF and HMG-1 (Calandra et al., 2000; Wang et al., 1999). Thus, post-infection administration of soluble TREM-1 might be a suitable therapeutic tool to treat septic shock as well as other microbial-mediated diseases. In addition, the identification of sTREM-1 in human serum suggests the possibility that humans may use an endogenous TREM-1 to shut off the TREM-1 system that is misbalanced only under certain conditions such as septic shock. Thus, administration of sTREM-1 to septic patients should rerely reset the system thus being highly beneficial and having few side effects.

It is interesting to note that TREM-1/DAP12 oligomeric complexes may initiate inflammatory reactions in the absence of microbial challenge. This proinflammatory role of TREM-1/DAP12-dependent pathways is suggested by the progressive development of a fatal inflammatory syndrome in Tg-DAP12 mice, which spontaneously occurs after 4 weeks of age and causes premature death within 1-2 months. Cells and DAP12-associated cell surface receptors, which cause the onset of this syndrome, need to be elucidated. However, neutrophilia and lung infiltration of macrophage-like cells strongly suggest that TREM-1-expressing neutrophils and alveolar macrophages concur to pathogenesis of the wasting syndrome. Interestingly, neutrophilia is also observed in G-CSF-transgenic mice, but is relatively benign (Serizawa et al., 2000). Thus, the development of wasting syndrome requires not only the expansion of but also activation of myeloid cells. Two additional observations are along this line: (i) cross-linking of TREM-1 triggers secretion of inflammatory cytokines (TNF-α, IL-

1β) and chemokines (IL-8, MCP-1), and up-regulation of costimulatory and adhesion molecules, and (ii) TREM-1 can be up regulated by TNF-α and IL-1β, indicating that TREM-1 might act as a self-amplifying system. In conclusion, all these results demonstrate that DAP12-associated receptors, in particular TREM-1, are involved in all kinds of inflammatory process and that an abnormal function of DAP12-associated receptors may be sufficient in itself to cause inflammation.

Finally, the presence of neutrophilia in Tg-DAP-12 mice indicates a role of DAP12 pathways in neutrophil homeostasis. Regulation of neutrophil homeostasis is essential for effective host responses against pathogens (Haslett et al., 1994). Under normal conditions, circulating neutrophils have a short lifespan, as they undergo apoptosis. During bacterial infections, apoptosis is delayed, preserving the inflammatory function of neutrophils at the sites of infection. However, excessive neutrophil survival may block the resolution of inflammation, leading to tissue damage and systemic inflammatory syndromes, such as sepsis. LPS and bacterial products delay neutrophil apoptosis by activating the TLRs/NF-κB pathway and by promoting the release of anti-apoptotic inflammatory mediators and cytokines, such as LTB4, IL-8 and GM-CSF (Brach et al., 1992; Chitnis et al., 1996; Colotta et al., 1992; Jimenez et al., 1997; Matsumoto et al., 1997). In Tg-DAP12 mice neutrophilia may partly be the consequence of high serum levels of G-CSF and TNF-α (data not shown). Yet, the activation of DAP12 signaling pathways influences neutrophil survival by a more direct mechanism, as exemplified by TREM-1 engagement. At early time points, TREM-1 promotes survival of neutrophils by ERK-dependent Bad-phosphorylation at Ser^{112}. This leads to subsequent disruption of Bad-Bcl-x_L-heterodimers followed by Bcl-x_L-mediated protection of mitochondrial integrity. Only at later time points, TREM-1 mediates secretion of IL-8, which promotes Bad-phosphorylation at Ser^{136} by a PI(3)K/Akt-dependent pathway. The expression of Bcl-2-family members in human neutrophils has been controversial (Klein et al., 2000; Moulding et al., 1998; Ohta et al., 1995; Santos-Beneit and Mollinedo, 2000). Although Bcl-x_L was hardly detectable in neutrophils by western blot analysis, we were able to precipitate Bad-Bcl-x_L heterodimers with an anti-Bcl-x antibody. These results support previous reports describing a very low expression of Bcl-x_L (Moulding et al., 1998; Ohta et al., 1995). However, it is possible that, in addition to Bcl-x_L, other anti-apoptotic Bcl-2 family members, such as A1 or Mcl-1, are expressed in neutrophils and interact with Bad (Santos-Beneit and Mollinedo, 2000; Weinmann et al., 1999). Interestingly, TREM-1 stimulated neutrophils are also more resistant to apoptosis induced via death receptors such as CD95 and TRAIL-Rs (data not shown). Thus, TREM-1 may not only induce protection of mitochondria against cytopathic agents but also other mechanisms that provide resistance against death inducing ligands. Apparently,

TREM-1-mediated signals ensure protection against mitochondria-induced and death-receptor-induced apoptosis. Thus, TREM-1 may have the ability to promote neutrophil survival, not only during acute inflammation but also during normal homeostasis, which explains the observed neutrophilia and spontaneous onset of wasting syndrome in DAP12 transgenic mice. Most likely, the protective effect of mTREM-1-IgG1 during septic shock is due not only to reduction of TREM-1-induced secretion of inflammatory cytokines but also to accelerated apoptosis of neutrophils. In several clinical conditions, including burns, acute respiratory distress syndrome and reperfusion injuries following ischemic conditions, the inflammatory response to the insulting agent is excessive and abnormally prolonged, culminating in tissue damage. Massive release of chemokines, specifically IL-8, and increased resistance of neutrophils to apoptosis appear to be especially important in the pathogenesis of these diseases (Baggiolini, 1998; Chitnis et al., 1996; Jimenez et al., 1997; Matsumoto et al., 1997). Inhibition of KARAP/DAP12 and of TREM-1 signaling *in vivo* may thus contribute to the resolution of inflammation and to an improved outcome of these pathological conditions.

4.1.3 The ligand(s) for TREM-1 (TREM-1L)

The production of a functional TREM-1 fusion protein provided insight into the regulatory mechanisms steering the TREM-1 system *in vivo*. In particular the identification of TREM-1L on neutrophils infiltrating the site of inflammation completed the model of TREM-1/TREM-1L as a system to ensure neutrophil survival and full responsiveness during inflammation.

Several cytokines such as TNF-α, IL-1β in monocytes (Mangan et al., 1991), IL-2 in T cells (Van Parijs et al., 1999) and GM-CSF in neutrophils (Klein et al., 2000), are known to induce survival in an autocrine fashion. In contrast, TREM-1 and TREM-1L are both membrane bound proteins. The observations made in granulomas strongly support the concept that TREM-1L stimulates TREM-1 function on neighboring cells. It is not yet known whether TREM-1L can stimulate TREM-1-mediated responses on the same cell as it was reported for other receptor-ligand pairs (Dhein et al., 1995). In addition, it would be possible that TREM-1L is produced in a membrane-bound and a soluble isoform as reported for several other ligands (Kapsogeorgou et al., 2001; Orlinick and Chao, 1998; Peter et al., 1999).

Expression cloning using a cDNA library prepared from peritoneal neutrophils of endotoxemic mice did not lead to the identification of TREM-1L. Perhaps, this technique is limited by intracellular modifications or the association of cofactors to TREM-1L thus altering the affinity for TREM-1. It will be necessary to establish bio-

chemical approaches, which are currently limited by the small cell numbers available from peritoneal lavages of endotoxemic mice. Further investigation on the regulation and expression pattern of TREM-1L is necessary to identify the potential stimuli that are responsible for TREM-1L induction. By means of this crucial information it will be possible to induce TREM-1L on cell lines in amounts sufficient to set up a biochemical system to identify TREM-1L.

4.2 Amplification of DC responses

4.2.1 Homeostasis or amplification? Two potential roles for TREM-2

We have shown that TREM-2 is an activating receptor expressed on monocyte-derived DCs, which activates PTK and ERK signaling through the association with DAP12, an ITAM-containing adapter molecule (Lanier and Bakker, 2000). TREM-2/DAP12-mediated signaling promotes survival of DCs and up regulation of CCR7, MHC class II, CD86 and CD40. As compared to the classical DC activation, triggered by LPS, the TREM2/DAP12 pathway does not lead to the up-regulation of ICAM-1 and CD83 or secretion of IL-12, and is entirely independent of NF-κB and p38/SAPK signaling. TREM2/DAP12-induced DC maturation is more similar to that initiated by the FcRs, through the association FcRγ, another ITAM-containing adapter molecule [Amigorena, 1999 #67]. Indeed, it is shown here that FcR-mediated maturation is dependent on PTK and ERK signaling.

This study is the first to show that a DAP12-mediated pathway can activate human DCs. What could be the physiological significance of this maturation pathway? On one hand, DAP12-mediated DC activation could be important in the normal homeostasis of DCs (Figure 4.4). After the triggering of TREM-2, by a yet unknown ligand, it induces up-regulation of CCR7, which plays a pivotal role in directing DCs from the periphery to the T cell rich areas of draining lymph nodes (Forster et al., 1999; Gunn et al., 1999; Ngo et al., 1999; Saeki et al., 1999). Thus, in the absence of pathogens, DAP12-associated receptors could regulate the homeostatic circulation of DCs from the periphery to the lymph nodes, allowing for the renewal of lymph node DCs. In addition, TREM-2 induces upregulation of some T cell stimulatory molecules, such as MHC class II, CD40 and CD86. Thus, DAP12-mediated maturation of DCs may promote partial activation of T cells in the absence of exogenous antigens. Presumably, this activation is critical for the survival of T cells

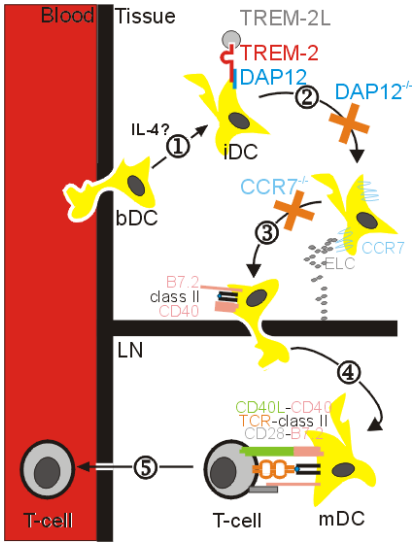

Figure 4.4: The role of TREM-2 during DC homeostasis

Under normal conditions immature DC patrol from the blood into the tissue where they become TREM-2$^+$ (1). In the tissue triggering of TREM-2 induces CCR7 (2) thus redirecting the DC to the lymph node (3). During their travel, DCs partially mature, particularly upregulate costimulatory molecules, which are necessary to trigger T cells (4). In the absence of cytokines, TREM-2 signaling transmits survival signals to the T cells thus ensuring T cell homeostasis and tolerance.

and the homeostasis of T cell populations (Tanchot et al., 1997). The physiological functions of DAP12-mediated DC activation are consistent with the reported phenotype of knock-in mice bearing a nonfunctional DAP12, which showed an accumulation of DCs in muco-cutaneous epithelia, associated with an impaired hapten-specific contact sensitivity [Tomasello, 2000 #44]. Our data suggest that this phenotype may be explained in part by a reduced ability of DCs to up regulate CCR7 expression and to respond to CCL19 and CCL21, affecting the migration of DCs to the T-cell zone of draining lymph nodes. In addition, cross-linking of TREM-2 promotes DC survival essential for the migration from the periphery to the draining LN. This observation is consistent with the previous demonstration that ERK signaling prevents apoptosis of LPS-stimulated DCs (Rescigno et al., 1998) and the results obtained from TREM-1/DAP12. Therefore, it is likely that DAP12-mediated ERK-activation in DCs induces phosphorylation of Bad or other Bcl-2 inhibitors (Klein et al., 2000; Scheid and Duronio, 1998; Scheid et al., 1999). Once released from inhibition, Bcl-2 could translocate into the mitochondria and inhibit DC apoptosis (Adams and Cory, 1998; Green, 2000; Yang et al., 1995). In conclusion, TREM-2 may act on DC homeostasis by providing fuel and protection (survival signals), and the proper navigation systems (CCRs) so that DCs can travel between blood, tissue and LN.

On the other hand, TREM-2 or other DAP12-associated receptors could synergize with cell surface receptors, which activate DCs through NF-κB (Figure 4.5). DAP12-deficient mice were resistant to EAE and resistance was associated with a severely diminished production of IFN-γ by myelin-reactive $CD4^+$ T cells [Bakker, 2000 #45]. Since T cells from normal $DAP12^{+/+}$ mice do not express DAP12, the resistance to EAE probably results from improper APC function at different stages during induction of the immune response. This ultimately results in the failure to induce autoreactive T cells in $DAP12^{-/-}$ mice. The results obtained on TREM-2 function *in vitro*, strongly support the idea that DAP12-associated DC receptors could amplify maturation signals transduced by other receptors allowing for optimal antigen-presentation and subsequent T cell function. Most interestingly, administration of mTREM-2-IgM leads to complete resistance against EAE in mice. Whether this resistance is indeed associated with diminished APC function and subsequent T cell priming is yet unclear. Protection against EAE was retained in these mice even after mTREM-2-IgM was cleared from the blood (data not shown), thus indicating that TREM-2/DAP12 acts during the initiation of EAE and is most likely required for complete APC maturation or proper migration of APCs to the areas of T cell priming in the LN. The role of TREM-2/DAP12-dependent pathways during APC maturation is further emphasized by the observation that mTREM-2-IgM reduces T cell priming and differentiation to IL-4-secreting effector $CD4^+$ T cells during Ovalbumine (OVA)-induced

asthma (pers. communication Nicole Schmitz, BII). Consistent with previous publications, the reduced levels of IL-4 lead to the improper induction of IL-5 followed by reduced lung eosinophilia, and impaired B cell functions such as inhibition of isotype switch resulting in decreased serum levels of IgE (Kopf et al., 1995). Thus, complete DC maturation during both T_H1 and T_H2-mediated responses is mediated by a synergistic involvement of several DC receptors including DAP12-associated receptors.

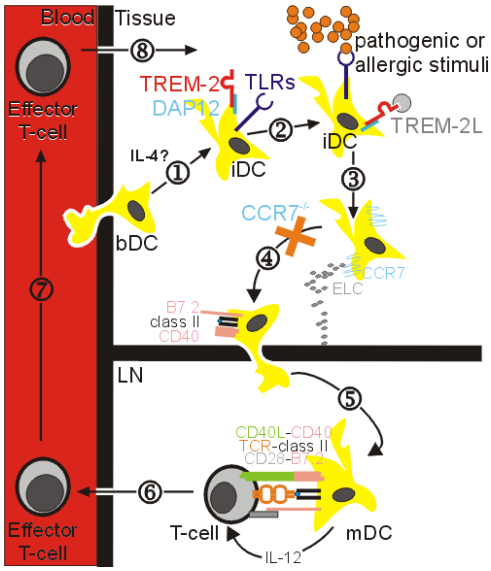

Figure 4.5: The role of TREM-2 during inflammation

Infections lead to the recruitment of DCs into the tissue (1,2). Triggering of PRRs together with TREM-2 are responsible for the induction of full DC maturation (3) and migration to the draining lymph node (LN) (4). In the LN, stimulation of T cells by costimulatory molecules and DC-secreted cytokines (5) induces effector T cells (6), which travel via the blood stream (/) to the site of infection (8).

Up to now, resistance to EAE has also been observed in CD40 ligand (CD40L)-deficient mice that express a transgenic T cell receptor specific for myelin basic protein (MBP) due to impaired CD80 and CD86 expression on the APC (Grewal et al., 1996). Immunization of these mice with MBP peptide, along with the simultaneous co-administration of CD80-overexpressing APC, primed the myelin-reactive T cells for IFNγ production and induced EAE. Their results demonstrated that restoration of costimulatory activity on APC *in vivo* could restore EAE induction. The cross-linking of CD40 on DCs up regulates MHC class II, the costimulatory molecules CD80 and CD86 and OX40L, and the chemokine receptor CCR7, and induces IL-12 secretion (Grewal and Flavell, 1998; van Kooten and Banchereau, 2000; Yanagihara et al., 1998). Therefore the CD40/CD40L interaction has been implicated as a key mediator of DC maturation. Although $CD40^{-/-}$ mice fail to mount proper T_H1 responses to a number of pathogens, CD40-deficient mice challenged with viruses are able to mount protective $CD4^+$ T cell responses that produce normal levels of IFN-γ (Oxenius et al., 1996). This suggests the existence of CD40/CD40L independent mechanisms for T cell priming. In addition to the CD40/CD40L pathways, other pathways, such as TRANCE-R/TRANCE and OX40/OX40L have been implicated in APC-T-cell interaction. Stimulation of TLR4 or TLR9 by bacterial products induces IL-12 production and enhances surface expression of costimulatory molecules on DCs. MyD88 seems to be essential for the cytokine production, however it was shown that $MyD88^{-/-}$ DCs can still mount allogeneic T cell activation paralleled with

up-regulation of co-stimulatory molecules (Akira et al., 2001; Kaisho et al., 2001). This implies that stimulation by LPS or CpG DNA can engage other pathways that are either started form TLRs in a MyD88-independent way or that further receptors cooperate with TLRs to mediate the completion of DC maturation. Our results show that DAP12-mediated pathways constitute an additional pathway leading to APC maturation. Remarkably, these pathways are independent of $IKK^{\alpha,\beta,\gamma}$/NFκB and p38/SAPK in contrast to TLRs and receptors of the TNF-R family. Thus, DAP12-associated DC complement signals from TLRs and TNF-R family members ultimately leading to full DC maturation.

4.2.2 The regulation and cellular distribution of TREM-2 and TREM-2L

Human TREM-2 was the first DAP12-associated receptor identified on DCs. TREM-2 is rapidly up regulated by IL-4 on human monocytes, on MDCs and even on MMϕs but is not expressed on mature DCs. In addition, mTREM-2 mRNA was detected in mouse macrophage cell lines [Daws, 2001 #116]. Thus, it is likely that TREM-2 can be induced also on distinct myeloid cell types by stimulation with IL-4 or additional unknown stimuli. Interestingly, TREM-2 could be detected on tonsillar DCs but also on normal and transformed mast cells in malignant mastocytomas. Mast cells are a main source of IL-4 under these (Hassuneh et al., 1997) and other conditions (Broide, 2001), thus supporting the idea that TREM-2 can be upregulated by IL-4 on distinct types of myeloid cells.

Obviously, the cellular response induced by TREM-2/DAP12 is dependent on the properties of the cell type stimulated. Biochemical characterization of TREM-2 on DCs revealed its main function in inducing cell survival and generating the phenotype of mature APC. mTREM-2a and 2b were found to be expressed on a macrophage cell line. While antibody-mediated ligation of TREM-2a in this cell line leads to oxidative burst, measured by the release of nitric oxide (Daws et al., 2001) it is not yet clear whether it also induces cell survival or CCR7 upregulation. Whether stimulation of TREM-2/DAP12 on mast cells leads to degranulation, induction of costimulatory molecules and MHC class II or cell survival is currently under investigation. It is of note, that mast-cell deficient WBB6/F1-Kit^{W}/Kit^{Wv}(W/W^{v}) mice exhibit a significantly reduced EAE disease incidence, delayed EAE disease onset, and decreased mean clinical scores when compared with their wild-type congenic littermates (WBB6/F1-Kit^{+}/Kit^{+}) (Secor et al., 2000). The idea that mast cells contribute to the pathogenesis of EAE in mice and multiple sclerosis (MS) in humans is not new. Over 100 years ago, mast cells were observed in the CNS plaques of MS patients (Neuman

et al., 1890). Subsequent studies reported a correlation between the number and/or distribution of mast cells in MS or EAE pathology (Lafaille et al., 1997; Olsson, 1974; Toms et al., 1990). Sites of inflammatory demyelination are also sites of mast cell accumulation in the brain and spinal cord, and the percentage of degranulated mast cells in the CNS correlates with the clinical onset of disease symptoms in acute EAE (Brenner et al., 1994). Mast cells also produce cytokines, particularly IL-4, that have been implicated in either EAE disease pathology or protection from diseases (Begolka et al., 1998; Gordon and Galli, 1990; Khoury et al., 1992; Renno et al., 1995; Selmaj et al., 1991). Furthermore, levels of tryptase, a mast cell specific proteolytic enzyme, are elevated in the cerebrospinal fluid in MS patients (Rozniecki et al., 1995) and mast cell-derived proteases are capable of degrading myelin (Dietsch and Hinrichs, 1989; Johnson et al., 1988; Watson et al., 1994). It is therefore likely that impaired mast cell function contributes to the observed phenotype during EAE in mice treated with mTREM-2-IgM and DAP12$^{-/-}$ mice. Thus, to fully understand TREM-2 function *in vivo*, it is essential to determine all cell types expressing TREM-2 and its regulation.

Taking the traveling route of APCs from the periphery to the LN into consideration, the putative TREM-2L has to be strategically placed in the periphery to redirect the APCs to the draining LN. Thus the ligand can be an extracellular matrix protein, sugar, or a secreted or membrane-bound protein expressed on tissues or pathogens. In addition, it is possible TREM-2 is directly involved in APC-T-cell contact, in which case it would be expressed on T cells. Using TREM-2-IgM or mTREM-2-IgM fusion protein, binding was tested on several distinct cell lines and primary T cells stimulated in different ways, however so far no binding has been observed. The results obtained from mice with EAE and OVA-induced asthma strongly suggests that TREM-2L could be an endogenous ligand. Thus future investigations will concentrate on isolated cell populations from these mice.

4.3 Conclusions

TREMs constitute a novel family of receptors that are involved in the control of inflammation and the connection between innate and adaptive immune responses. While PRRs are mainly involved in the recognition of pathogens and the initiation of innate responses, TREMs build a second front line, ready to be activated by endogenous factors, which are produced during the initial phase of inflammation. Therefore, TREMs construct an amplification loop that is tightly controlled and supports innate responses by prolonging effector cell survival and increasing the quality of the required response. In addition, TREMs are able to initiate innate responses autonomously, thus providing an additional receptor network for the activation of innate responses, which complement TLRs, receptors of the TNF-R-SF and GPCR. Observations obtained from neutrophils and DCs suggest further that, in the absence of pathogenic stimuli TREMs are used for the regulation of myeloid cell homeostasis. Thus, while invertebrates, use the Toll-system for initiation and amplification of innate responses (Dushay and Eldon, 1998; Meister et al., 1997), in vertebrates a separation of initiation of inflammation (TLRs) from amplification (TREMs) has evolved. Such a separation allows for the control of a higher maximum power to use against pathogens combined with a mechanism for the fine tuning of immune responses according to the microorganism encountered. Immunological amplification systems such as the TREMs are appealing targets for therapeutic intervention in situations of excessive systemic inflammatory responses. As demonstrated here, exogenous modulations of TREM-1 signaling is sufficient to block septic shock in mice and may be sufficient to restore correct innate responses during certain pathological conditions in humans.

5 SUMMARY

In the work presented here, a new group of activating receptors expressed on myeloid cells was identified, cloned and characterized. Due to selective expression on myeloid cells, the receptors were designated **T**riggering **R**eceptors **E**xpressed on **M**yeloid cells (**TREM**s). Five TREMs were identified. All of them are characterized by an extracellular Immunoglobulin (Ig) domain of the V-type, a charged lysine or arginine residue in the transmembrane domain, and no intracellular signaling motifs. At least three members of this family associate with a transmembrane adapter molecule called DAP12. DAP12 recruits protein tyrosine kinases, which initiate a cascade of phosphorylation events leading to cell activation.

Functional studies focused on TREM-1 and TREM-2. TREM-1 is selectively expressed on blood neutrophils, monocytes and alveolar macrophages and associates with DAP12. Engagement of TREM-1 *in vitro* triggers secretion of proinflammatory cytokines and chemokines and promotes survival of neutrophils. Resistance to apoptosis is mediated by phosphorylation and inactivation of a proapoptotic member of the Bcl family, called Bad, which regulates mitochondrial integrity. *In vivo*, TREM-1 is expressed at very high levels on neutrophils and monocytes that accumulate in human tissues infected with bacteria. In addition, it is upregulated on peritoneal neutrophils of patients with microbial sepsis and mice with experimental sepsis. Strikingly, blockade of TREM-1 during experimental sepsis reduces inflammation, increases neutrophil apoptosis and protects mice against sepsis. Thus, TREM-1-driven signals play a central role in neutrophil function and in acute inflammatory responses to bacteria.

TREM-2 is a cell surface receptor on dendritic cells (DCs), which is associated with DAP12, like TREM-1. In vitro, TREM-2/DAP12 induces upregulation of CC chemokine receptor 7, which promotes homing of DCs to lymph nodes, where they encounter naïve T cells. In addition, TREM-2 promotes DC survival and induces partial DC maturation, increasing the T cell stimulatory activity of DCs. In vivo, blockade of TREM-2 signaling in mice reduces the ability of DCs to stimulate autoimmune T cells, which cause experimental autoimmune encephalomyelitis (EAE). Thus, TREM-2/DAP12 enhances the antigen presenting capability of DCs.

In conclusion, TREMs constitute a novel group of receptors involved in the control of neutrophil inflammatory responses and DC antigen presenting function. They provide and effective pathway to amplify innate as well as adaptive responses to pathogenic invasion and represent a new therapeutic target for the control of diseases with immunological pathogenesis.

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7 ABBREVIATIONS

β-ME	beta-Mercaptoethanol
$\Delta\Psi_m$	Mitochondrial membrane potential
λ	Wavelength
µg	microgram
µl	microliter
µM	micromolar
2n	diploid
[^{3}H]	Tritium
A	Alanine
7-AAD	7-Aminoactinomycin
Ab	Antibody
ADCC	Ab-dependent cell cytotoxicity
AP	Alkaline phosphatases
APC	allophycocyanin
APCs	Antigen-presenting cells
APS	Ammonium persulfate
ARDS	Acute respiratory distress syndrome
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavages fluid
BCA	Bicinchioninic acid
BCG	Bacillus of Calmette-Guerin
bcl-2	B cell lymphoma gene 2
BCR	B-cell receptor
BH	Bcl-2 homology domain
BM	Bone marrow
Bp	Base pair
BS	Blocking solution
BSA	Bovine serum albumin
C	Cysteine
°C	Degrees centigrade
C5a	Complement factor 5a
CaM-K	Calcium-calmodulin-dependent kinases
CASP	Colon ascendens stent peritonitis
CD	Cluster of differentiation
cDNA	Complementary DNA
CCR	CC Chemokine receptor
CCL	CC Chemokine ligand
CLP	Cecal ligation and puncture
CNS	Central nervous system
Co-IP	Co-immunoprecipitations
cpm	counts per minute
CR	Complement-receptor
CREB	cyclic AMP response element-binding protein
CS	Contact sensitivity
CXCR	CXC chemokines receptor
D	Aspartic acid
DAG	1,2-*sn*-Diacylglycerol
DAP10	DNAX activating protein 10
DAP12	DNAX activating protein 12
DC	Dendritic cell
DD	Death domain
ddH_2O	double-distilled water
DcR	Decoy receptor
DED	Death effector domain
DHE	Dihydroethidine
DIG	Digitonin
$DiOC_6$(3)	3,3´-Dihexyloxacarbozy-aniniodide
DISC	Death-inducing-signaling complex
DMSO	Dimethylsulfoxide
DN	dominant negative
DNA	Desoxyribonucleic acid
DR	Death receptor
ds	double stranded
E	Glutamic acid
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemoluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetate
ELC	EBI-1 ligand chemokine
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmatic reticulum
ERK	Extracellular-signal regulated kinase
EST	Expressed sequence tagged
et al.	*et alii*
Eth	Ethidin
EthBr	Ethidium bromide
F	Phenylalanine
$F(ab´)_2$	Antigen binding fragment
Fc	Crystallizing fragment
FACS	Fluorescence-assisted cell sorting
FADD	Fas-associated death domain protein
FCA	Freund complete adjuvant
FCS	Fetal calf serum
FcR	Fc receptor
FcRγ	Fc receptor γ chain
FIA	Freund incomplete adjuvans
FITC	Fluorescein-isothiocyanate
FMLP	formyl-Met-Leu-Phe
f-MOC	fluorenylmethoxycarbonyl
FSC	Forward scatter
G	Glycine
GC	Germinal center
GPCR	G-protein-coupled receptor
H	Histidine
h	*hora* (hour)
HMG-1	High mobility group-1
HRP	Horseradish peroxidase
hu	human
I	Isoleucine
Ig	Immunoglobulin
IHC	Immunohistochemistry
IKK	Iκ kinase
IL	Interleukin
ILT	Ig-like transcript
IFN	Interferon
IP	Immunoprecipitations
i.p.	intraperitoneally

IP_3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl β-thiogalactoside
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
IRAK	IL-1R-associated protein kinase
IRF	IFN regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibitory motif
i.v.	intravenously
K	Lysine
kDa	kilo Dalton
KIR	Killer cell Ig-like receptor
KLH	Keyhole limped hemocyanin
L	Leucine
LAT	linker of activated T cells
LB	Luria broth
LN	Lymph node
LPS	lipopolysaccharide
LSM	Lymphocyte separation medium
LT	Lymphotoxin
LT	Leukotriene
LTA	Lipotechoic acid
M	Methionine
M	molar (mol/l)
m	monoclonal
mA	milliampere
MACS	Magnetic cell sorting
Mal	MyD88-adapter-like
MALP2	Macrophage-activating lipoprotein-2
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MDC	Monocyte-derived DC
MMϕ	Monocyte-derived macrophage
MFI	Mean fluorescence intensity
mg	milligram
MHC	Major Histocompatibility Complex
MIF	Macrophage migration inhibitory factor
min	minutes
ml	milliliter
mM	millimolar
MOG	Myelin oligodendrocyte glycoprotein
MPO	Myeloperoxidase
MR	Mannose receptor
mRNA	Messenger RNA
MRP-1	Multidrug Resistance-associated Protein-1
MS	Multiple sclerosis
mu	murine
MW	Molecular weight
MWCO	Molecular weight cut off
N	Asparagine
N_2	Nitrogen
NC	Nitrocellulose
NF-AT	Nuclear Factor of activated T-cells
NF-κB	Nuclear Factor κ B
ng	nanogram
NGF	Nerve growth factor
NIK	NF-κB-inducing kinase
NK	Natural killer
nM	nanomolar
NO	Nitric oxide
NP	Nonidet
ON	over night
OPG	Osteoprotegerin
OVA	Ovalbumine
Q	Glutamine
P	Proline
p	polyclonal
p90RSK	90-kDa ribosomal S6 kinases
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDK-1	3'-phosphoinositide-dependent kinase-1
PE	β-Phycoerythrin
PFA	Paraformaldehyde
PH	Pleckstrin-homology
pH	potentia hydrogeni
PI	Propidium iodide
PI(3)K	Phosphoinositide 3-OH kinase
$PI^{(4)P}$	Phosphatidylinositol-4-phosphate
$PI^{(4,5)P}$	Phosphatidylinositol-4,5-bisphosphate
$PI^{(3,4)P}$	Phosphatidylinositol-3,4-bisphosphate
$PI^{(3,4,5)P}$	Phosphatidylinositol-3,4,5-triphosphate
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PL	Peritoneal lavage
PLOSL	Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PLCs	Peritoneal lavage cells
PLF	Peritoneal lavage fluid
PRR	Pattern recognition receptor
PS	Phosphatidyl-serine
PTB	Protein-tyrosine binding
PTK	Protein tyrosine kinase
PTPC	Permeability transition pore complex
R	Arginine
RA	Receptor antagonist
RACE	3' Rapid amplification of cDNA ends
RANK	Receptor activator for NF-□B
Rb	Retinablastoma
RBC	Red blood cells
RIP	„Receptor interacting protein"
RNA	Ribonucleic acid
rpm	Rotations per minute
RSB	Reducing sample buffer
ROI	Reactive oxygen intermediates

ROS	Reactive oxygen species
RT	Reverse transcription
RT	Room temperature (20-25°C)
< 2n	subdiploid
S	Serine
SAPK	Stress-activated protein kinase
s.c.	subcutaneously
SDS	Sodiumdodecylsulfate
SF	Superfamily
SH2	Src-homology 2
SHP	SH2-containing phosphatase
SIRS	Systemic inflammatory response syndrome
SLC	secondary lymphoid-tissue chemokine
SN	supernatant
Sos	Son of sevenless
SR	Scavanger receptor
ss	single stranded
SSC	Side scatter
t	*tempus* (time)
T	Threonine
TB	Transfer buffer
TCR	T cell receptor
TEMED	Tetraetylmethylendiamine
TF	Transcription factor
Tg	transgenic
T_H	T helper
TIR	Toll/IL-1R
TIRAP	Toll-IL-1R (TIR) domain-containing adapter protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNP	Tri-nitrophenol
TRADD	TNF-receptor associated death domain
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation-induced cytokine
TREM	Triggering receptor expressed on myeloid cells
Tris	Tris(hydroxymethyl)-methylamine
Tx	Triton x
UV	ultraviolet
V	Valine
v/v	Volume per volume
v/w	Volume per weight
WB	Washing buffer
WBA	Western blot analysis

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Prof. Dr. P. H. Krammer, Dr. H. Walczak

10 PUBLICATIONS

1. **Bouchon A.**, Krammer P.H., and Walczak H. (2000) Critical role for mitochondria in B cell receptor-mediated apoptosis. *Eur. J. Immunol*. 30: 69-77.

2. **Bouchon A.**, Dietrich J., and Colonna M. (2000) Cutting edge: Inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164: 4991-4995.

3. Walczak H.*****, **Bouchon A.***, Stahl H., and Krammer P.H. (2000) Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2- or Bcl-x_L-overexpressing chemotherapy-resistant tumor cells. *Cancer. Res.* 60: 3051-3057.

4. Nakajima H., Cella M., **Bouchon A.**, Grierson H.L., Lewis J., Duckett C.S., Cohen J.I., and Colonna M. (2000) Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur. J. Immunol*. 2000 30: 3309-3318.

5. **Bouchon A.**, Facchetti F., Weigand M.A., and Colonna M. (2001) TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature*. 2001 410: 1103-1107.

6. **Bouchon A.**, Hernández-Munain C., Cella M., and Colonna M. (2001) A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. *J. Exp. Med.* 2001 194: 1111-1122.

7. **Bouchon A.**, Cella M., Grierson H.L., Cohen J.I., and Colonna M. (2001) Activation of NK cell-mediated cytotoxicity by CD2-like receptor activating cytotoxic cells (CRACC), a SAP-independent receptor of the CD2 family. *J. Immunol.* 167: 5494-5499.

***** equally contributing authors

11 WORKSHOP PRESENTATIONS

21th-23th of March 2001: Oral Presentation at the **Wolfsberg Symposium**, Wolfsberg, Switzerland: *"TREM-1 amplifies inflammation and is a crucial mediator of septic shock."*

29th-31th of March 2001: Oral Presentation at the **III. Roche Milano Ricerche Symposium**, Milano, Italy: *"TREM-1 amplifies inflammation and is a crucial mediator of septic shock."*

20th-22th of April 2001: Oral Presentation at the **I. Thymus and Immunity Symposium**, Dresden, Germany: *"TREM-1, a novel activating receptor expressed on neutrophils, which amplifies inflammation."*

12 CURRICULUM VITAE

I. PERSÖNLICHE DATEN

Name:	Axel Bouchon
Geburtstag:	28. März 1973
Geburtsort:	Reutlingen, Deutschland
Familienstand:	ledig

II. AUSBILDUNG

1979 – 1992 Abitur am Dietrich-Bonhoeffer-Gymnasium Metzingen

1992 – 1994 Julius-Maximilian Universität in Würzburg (Chemie Vordiplom)
1995 – 1996 Eberhard-Karl Universität in Tübingen (Biochemie Vordiplom)
1996 – 1997 Ludwig-Maximilian Universität in München, Fakultät für Biochemie (offizieller Austausch zwischen Tübingen und München)
1997 – 1998 Eberhard-Karl Universität in Tübingen (30.11.1998: Diplom in Biochemie: Gesamtnote: sehr gut)

4/1998-12/1998 Diplomarbeit in der Abteilung von Prof. P. H. Krammer, Tumor Immunologie Programm, Deutsches Krebsforschungszentrum, Heidelberg:
Characterization of activation-induced cell death in the human B cell line BL60.

2/1999-12/2001 Promotion in der Gruppe von Prof. Marco Colonna am Basel Institut für Immunologie, Basel, Schweiz:
Identification and Characterization of novel DAP12-associated receptors expressed on myeloid cells.

Tübingen, den 29.10.2001