The role of guanine nucleotide exchange factors in the regulation of immune cell signaling

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Erklärung

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Eidesstattliche Erklärung

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CONTENTS

1. INTRODUCTION

1.1 IMMUNE CELL SIGNALING

The immune system comprises a complex network of cell types which need to communicate effectively among each other to identify and eliminate invading pathogens, e.g. bacteria, viruses, parasites and fungi. This highly elaborated and dynamic exchange of information either occurs through direct cell-to-cell contacts or through a variety of intercellular mediators that include cytokines, chemokines, growth factors and hormones. Immune cells express a wide range of surface receptors to sense these signals and to transmit them from the outside to the inside of the cell. Each receptor type is composed of at least one ligand binding subunit and a variable intracellular transduction machinery. Ligand binding to the receptor stimulates cascades of intracellular reactions, inducing a ligand-specific cellular response, e.g. growth, differentiation, proliferation, survival, death, adhesion or migration. Intracellular signal transduction is mediated by specific signaling molecules, involving GTP-binding proteins, second messenger molecules, protein kinases, ion channels and many other effector proteins. Nearly every intracellular signaling process is regulated at least to some degree by protein phosphorylation. Nonetheless, each receptor class employs specific molecules to transmit signals to the cytoplasm.

Cytokine receptors are connected to the Janus kinase (JAK) family of tyrosine kinases which phosphorylate several signaling proteins which are recruited to the receptor complex. Ligand engagement by these receptors results in the activation of signal transducers and activators of transcription (STATs), a family of transcription factors (O`Shea and Murray, 2008). Chemokines signal through G-protein coupled seven transmembrane receptors to activate specific cellular mechanisms involved in chemotaxis and integrin activation (Zlotnik et al., 2006). The Toll-like receptor family (TLR), a type of pattern recognition receptors of the innate immune system, uses the myeloid differentiation primary response gene 88 (MyD88) as an important universal

adapter protein to initiate the production of cytokines and several other effector molecules via activation of the transcription factor NF-κB (Arancibia et al., 2007). The T-cell and B-cell receptors are responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules, presented on the surface of professional antigen-presenting cells (APC) such as dendritic cells. They generate signals through associated ITAMs, which couple the receptors to the downstream signaling machinery. The intracellular signaling cascades, leading to T-cell or B-cell activation, are initiated by tyrosine kinases which in turn phosphorylate and thus activate several downstream adaptor proteins (Chan and Shaw, 1996). Finally, members of the TNF receptor family largely signal through their cytoplasmic interactions with so-called "death" domain proteins RIP, TRADD, FADD and TRAF (TNF receptor associated factor). Intracellular signaling by these factors either leads to the induction of apoptosis via caspase activation or to the activation of the transcription factors c-Jun and NF-κB (Liu, 2005).

1.2 T-CELL RECEPTOR SIGNAL TRANSDUCTION

The activation of T-cells in response to foreign antigen induces cytokine production (e.g. interleukin-2, IL-2), antigen specific T-cell clonal expansion and differentiation. These responses are initiated by T-cell antigen receptors and co-stimulatory molecules. The second signal is provided by accessory membrane proteins of the B7 family, which are expressed on antigen presenting cells, and which bind CD28, PD-1 or CTLA-4 on T-cells. The mechanism of T-cell activation is tightly controlled by a complex network of intracellular signaling pathways, the precise regulation of which is still not fully understood.

1.2.1 T-CELL RECEPTOR COMPLEX

The majority of mature T-cells expresses the conventional T-cell receptor (TCR), consisting of a disulfide-bonded heterodimer of a TCR-α- and a TCR-ß-subunit. It recognizes foreign peptide bound to major histocompability complex class I or class II (MHC I or MHC II) molecules presented on the surface of professional antigen-presenting cells (APC), e.g. dendritic cells. However, a small percentage of thymocytes and peripheral lymphocytes express a TCR-γ chain associated with another type of polypeptide termed TCR-δ, instead of the classical TCR-α-/ß-heterodimer, which recognize a more limited number of antigens. Although a variety of effector functions have been ascribed to them, the exact biological function of the T-cell population which bears the γ-/δ-TCR is still unknown (Aralcon et al., 1987, O'Brien et al., 2007). Each chain of the TCR is a member of the immunoglobulin superfamily and possesses one N-terminal, extracellular immunoglobulin variable (V) domain which forms the binding surface for the MHC-bound peptide, one constant (C) domain, a transmembrane-spanning region and a short cytoplasmic tail at the C-terminal end. The highly diverse repertoire of α-/ß-T-cell receptors is accountable for the enormous diversity in specificity of the T-cell receptor for processed antigen. The TCR is constitutively and non-covalently associated with a homodimer of two ζ-chains and the CD3-complex, both are essential components for signal transduction. CD3 is a protein complex composed of four distinct chains: a γ-CD3 chain, a δ -CD3 chain, and two ε-CD3 chains (figure 1 and 2).

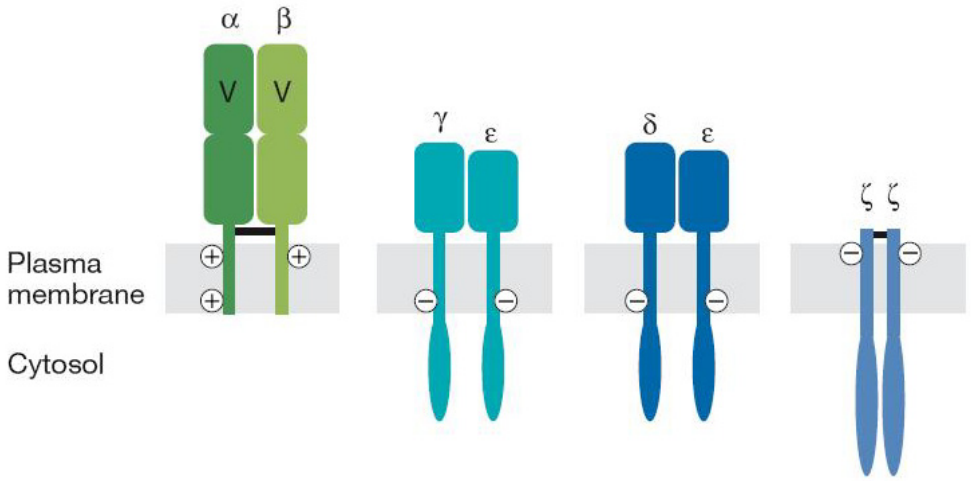

Figure 1: The T-cell antigen receptor complex. The TCR consists of a heterodimer of a TCR-α- and a TCR-ß-subunit (or of a γ-TCR/ δ-TCR-heterodimer, not shown), which is non-covalently associated with a homodimer of two ζ-chains and a CD3-complex (γ-/ε-heterodimer and one δ-/ε-heterodimer). Schematic pictures of the individual components of the TCR complex are shown. The variable immunoglobulin domains of the TCR α-and β-subunit (V) bind to the antigen, whereas the cytoplasmic tails of the CD3-complex (γε and δε) and the ζ-chain homodimer interact with cytosolic-signaling proteins (figure taken from Alarcon et al., 2006).

The CD3-complex, the ζ-chain homodimer and the TCR form together the T-cell receptor complex. Whereas the TCR itself lacks a significant intracellular domain, the associated CD3-complex and the ζ-chain homodimer contain intracellular signaling domains, the so-called immunoreceptor tyrosine-based activation motifs (ITAMs) that couple the TCR-complex to the downstream signaling machinery. The cytoplasmic tail of the CD3-complex contains a single ITAM per chain, whereas the ζ-chain homodimer contains three ITAMs per chain (Malissen, 2008).

It was believed for a long time that the TCR-complex of an unstimulated T-cell exists as a monovalent protein with one ligand-binding site per receptor (figure 3, old model), which is distributed evenly on the plasma membrane (Punt et al., 1994). Recent studies revealed the existence of multivalent (pre-clustered) TCR-complexes, which were detected by fluorescence resonance-energy transfer and electron microscopy (Fernandes Miguel et al., 1999, Schamel et al., 2005). According to the new model,

monovalent and multivalent receptors co-exist in the membrane of naïve T-cells (figure 3, new model). The pre-clustered TCR-complexes respond to ligands with higher sensitivity than monovalent TCRs, and can thus be activated even at low peptide/MHC concentrations (Schamel et al., 2005).

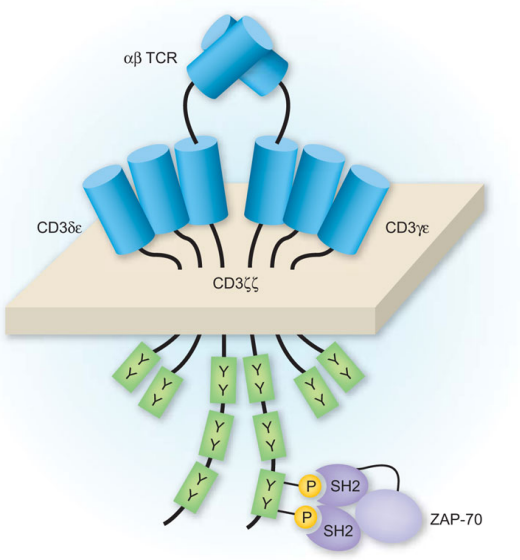

Figure 2: ITAMs of the TCR-complex. The TCR itself lacks a significant intracellular signaling domain. The associated CD3-complex and the ζ-chain homodimer contain intracellular domains, so-called immunoreceptor tyrosine-based activation motifs (ITAMs), that couple the TCR-complex to the downstream signaling machinery. The cytoplasmic tail of the CD3-complex contains a single ITAM per chain, whereas the ζ-chain homodimer contains three ITAMs per chain (figure taken from Malissen, 2008).

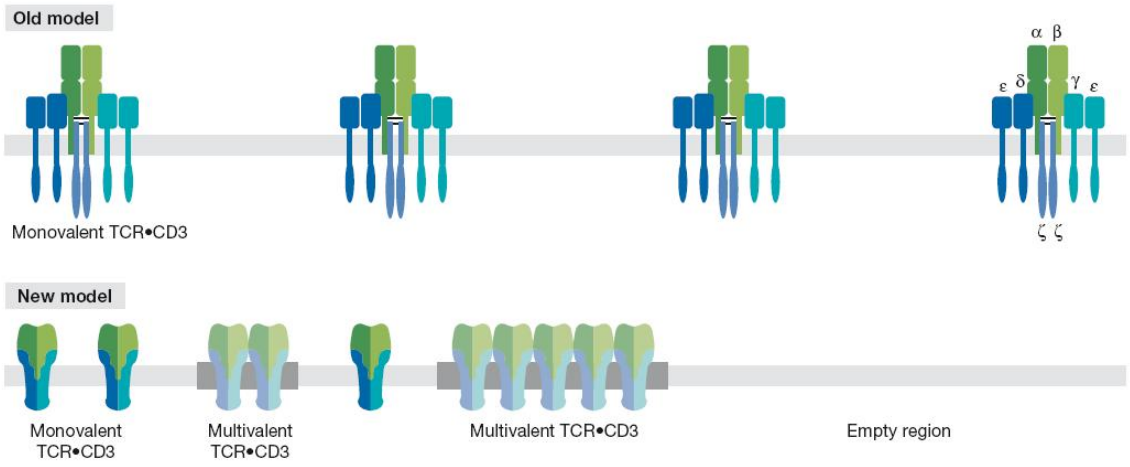

Figure 3: Arrangement of the T-cell antigen receptor complex in the cell membrane of a naïve T-cell. The old model shows only monovalent T-cell receptors which are distributed evenly on the plasma membrane. According to a new model monovalent and multivalent TCR complexes co-exist on the surface of resting T-cells. These multivalent TCR-complexes respond to antigens with higher sensitivity than a monovalent TCR, and can thus be activated even at low peptide/MHC concentrations. The cell surface also contains „empty regions" that do not contain any TCR-complex (figure taken from Alarcon et al., 2006).

1.2.2 SIGNAL TRANSDUCTION THROUGH THE T-CELL RECEPTOR

Binding of a naïve T-cell to a MHC/peptide complex, together with the activation of co-stimulatory molecules, initiates the phosphorylation of ITAMs of the CD3-complex and the ζ-chains, creating binding sites for signaling components that propagate an intracellular signaling cascade leading to T-cell activation (Chan and Shaw, 1996). In a resting T-cell the phosphorylation of these ITAMs is permanently suppressed by tyrosine phosphatases, such as CD45. Binding of the TCR to MHC-bound antigen induces the formation of the immunological synapse, a signaling platform at the interface of T-cells and APCs, where receptors and intracellular proteins cluster into spatially segregated domains (Dustin, 1999). The distance between the T-cell and the APC in the proximity of the MHC/TCR-complex is only ~ 150nm. However, CD45 is a large, highly glycosylated molecule, bearing a large extracellular domain, and will therefore be excluded from this T-cell/APC contact site. The exclusion of CD45 from the APC/T-cell contact site enables the phosphorylation of the ITAMs and thus activates intracellular signaling pathways leading to T-cell activation (Leupin et al., 2000).

TCR-stimulation first leads to the recruitment and activation of tyrosine kinases of Src, Syk and Tec protein families. The association of the Src-family kinase Lck with the CD4 and CD8 T-cell co-receptors brings this kinase into proximity with the ζ-chain homodimer and the CD3-complex. ITAMs of the CD3-complex and of the ζ-chain homodimer are subsequently phosphorylated by the tyrosine Lck, and this creates docking sites for the Syk family protein ZAP-70 (figure 2). ITAM-bound ZAP-70 will furthermore be phosphorylated -and thus activated- by Lck. Activated ZAP-70 phosphorylates several downstream molecules, among them the linker of activated T-cells (LAT) and the SH2 domain containing leukocyte protein of 76kDa (SLP-76) (Myung et al., 2000). Tyrosine phosphorylated SLP-76 and LAT act as central adapter proteins and recruit signaling components that promote e.g. activation of the MAP kinase cascade, calcium-dependent activation of NFAT, activation of NF-κB as well as a reorganization of the actin cytoskeleton (figure 4 and 7).

Calcium-dependent NFAT activation

One central signaling protein recruited to phosphorylated LAT is the enzyme phospholipase C-γ (PLC-γ). PLC-γ activation strictly depends on Vav-1, a guanine nucleotide exchange factor with bears catalytic GEF activity for Rho/Rac GTPases. Vav-1 is required for the formation and stabilization of the LAT/ SLP-76/ PLC-γ complex and for PLC-γ activation via activation of Tec family kinases which directly phosphorylate PLC-γ (Tybulewicz, 2005). The activation of PLC-γ yields production of the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3), which results from the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) at the plasma membrane. IP3 binds to a receptor in the membrane of the endoplasmic reticulum (ER), which initiates an elevation of intracellular calcium (Ca^{2+}). The increased Ca^{2+}-level results in the activation of the protein phosphatase calcineurin which in turn leads to the dephosphorylation, and thus to the activation of the transcription factor NFAT. Dephosphorylated NFAT subsequently translocates to the nucleus, where it cooperates with other factors to induce gene transcription (figure 4).

It has been shown that the rapid Ca^{2+}-release from ER stores is followed by slower Ca^{2+} entry from outside the cell, essential for refilling the intracellular stores. The underlying molecular mechanism of the Ca^{2+}-influx in response to ER-depletion remained unknown for a long time. The stromal interaction molecule 1 (STIM1) was recently identified as a ER calcium-sensor that trans-locates to the plasma membrane following store-depletion (Liou et al., 2005, Roos et al., 2005, Spassova et al., 2006). At the plasma membrane, STIM1 was shown to activate Orai, a tetra-spanning membrane protein which functions as a highly Ca^{2+}-selective channel (Feske et al., 2006, Zhang et al., 2006, Liuk et al., 2008).

Protein kinase C signaling

The second messenger DAG, which is produced by hydrolysis of PIP2 by PLC-γ, activates several isoforms of the protein kinases C (PKC) family. PKC isoforms can be divided into three distinct groups: the conventional calcium- and DAG-dependent PKCs (α, ß1, ß2 and γ), the calcium-independent/DAG-dependent novel PKCs (δ, ε, η and θ) and the atypical PKCs (ξ and λ), which lack calcium or DAG binding domains (Spitaler et al.,

2004). Little is known about the biological function and the proximal targets of these PKC isoforms. It has been shown that PKC-θ is specifically recruited to the immunological synapse following TCR-engagement, where it gets activated by DAG (Monks et al., 1997). Active PKC-θ induces a signaling cascade which leads to activation of the transcription factor NF-κB. PKCs are furthermore involved in the activation of the GTPase Ras. Activation of Ras also requires the guanine nucleotide exchange factors Sos (homologue of the Drosophila "son of sevenless" protein) or RasGRP. RasGRP contains a C1 domain which requires binding to DAG for its function. GTP-bound activated Ras catalyzes the phosphorylation of the protein kinase Raf which leads to the activation of the mitogen activated protein (MAP) kinases Erk1/2. ERK kinases directly activate the heterodimeric transcription factor complex AP-1 (see below).

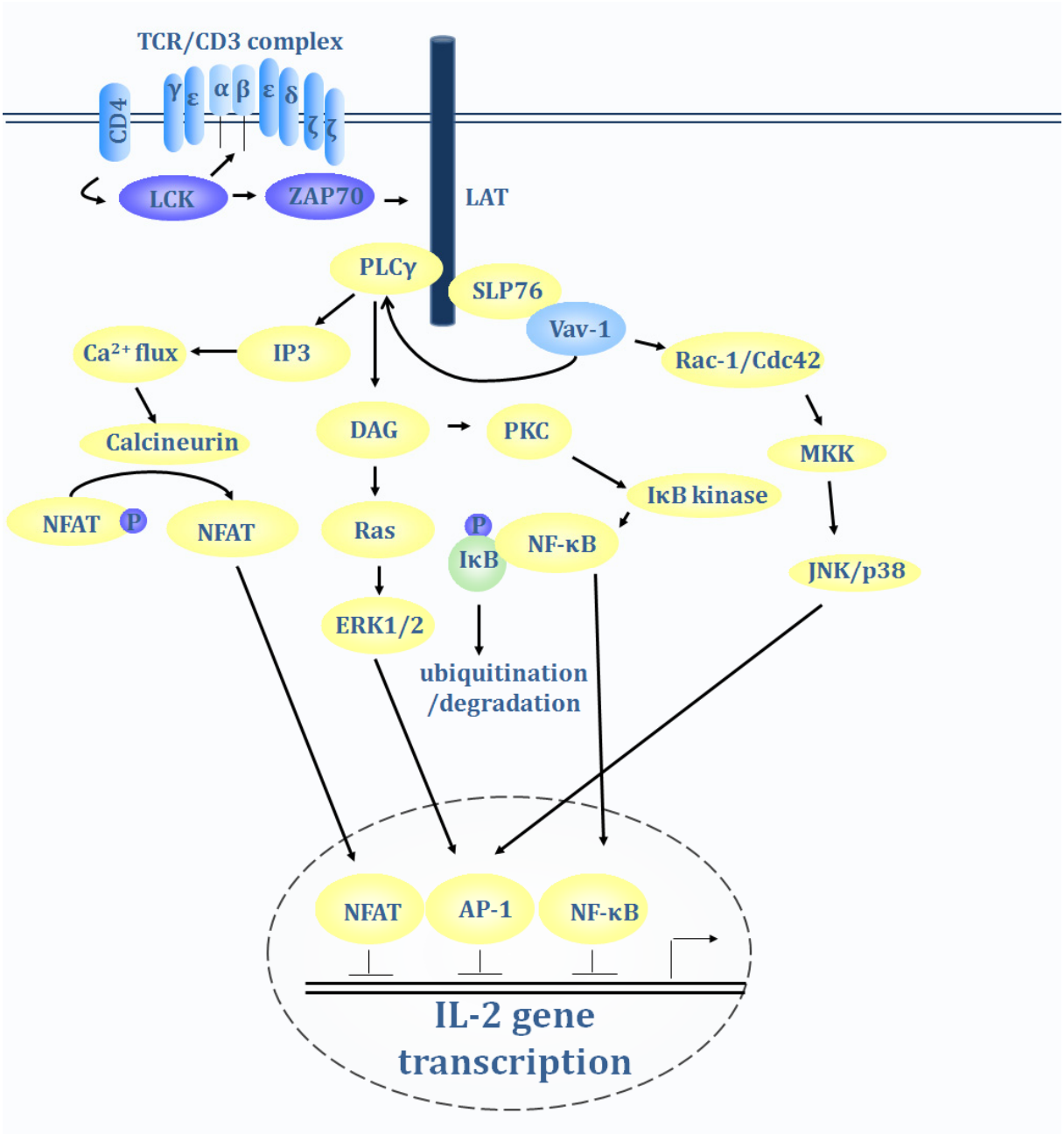

Figure 4: Simplified scheme of the TCR signaling pathway. Stimulation of the TCR leads to the activation of the MAP-kinase cascade, calcium-dependent activation of NFAT and PCK-dependent activation of NF-κB. The specific intracellular signaling pathways are described in the text.

MAP kinase signaling

Ligation of the antigen-receptor on T-cells results in activation of a mitogen-activated protein (MAP) kinase cascade which triggers signaling events ultimately leading to the activation of AP-1, a transcription factor complex composed of Fos and Jun family proteins. Activated AP-1 has been shown to bind to response elements of the IL-2 promotor and therefore takes a central role in the regulation of IL-2 gene transcription. Furthermore, AP-1 participates in the formation of a transcriptionally active NFAT (Jain et al., 1993).

Three major groups of MAP kinases are expressed in T-cells: the extracellular signal-regulated protein kinases (ERK-1 and ERK-2) (Schaefer and Weber, 1999), the p38 MAP kinases (Han and Ulevitch, 1999) and the the c-Jun NH2-terminal kinases (JNK) (Davis, 2000). All MAP kinases exhibit a Thr-X-Tyr motif and the phosphorylation of both the threonine and the tyrosine residues is essential and sufficient for their activation.

Erk pathway

As described above, the ERK pathway can be activated by Ras via the protein kinase Raf. Raf in turn activates the MAP kinase kinases MKK1 and MKK2, which subsequently activate ERK-1 and ERK-2. One target of activated ERK is Elk-1, a protein which is involved in the up-regulation of c-Fos, a member of the AP-1 complex.

p38/JNK pathway

The activation of p38 and JNK requires Rho family GTPases, e.g. Rac and Cdc42. Upon T-cell stimulation the guanine nucleotide exchange factor for Rho/Rac GTPases Vav-1 is recruited to the TCR by binding via its SH2 domain to phosphorylated tyrosines of SLP-76, where it activates Rac-1 and Cdc-42. This initiates the activation of different MAP kinase kinases. Activation of MKK3 and MKK6 induces phosphorylation of p38, whereas JNK pathway is activated by MKK4 and MKK7. The downstream target of JNK is the transcription factor c-Jun. p38 has been shown to activate the transcription factor Elk-1, as well as the activation transcription factor-2 (ATF-2) (Raingeaud et al., 1996). As stated before, Elk-1 has been implicated in the up-regulation of the AP-1 complex

member c-Fos. ATF-2 was shown to form heterodimers with Jun subunits and therefore plays an important role in the regulation of AP-1 activity (Liu et al., 2006).

PI3kinase signaling

Ligation of the TCR and/or co-stimulatory receptor CD28 also leads to activation of phosphoinositide-3 kinase (PI-3-kinase). Activated PI-3-kinase phosphorylates PIP2 within the plasma membrane, creating the signaling phospholipid PIP3. PIP3 is believed to act by recruiting proteins which bear a specialized lipid-binding motif, the so-called pleckstrin-homology (PH) domain. PI-3-kinase regulated proteins include the Rho/Rac exchange factor Vav-1 and the Akt serine/threonine kinases.

Akt (also referred as PKB, protein kinase B) has been shown to regulate cell survival and cell cycle progression by phosphorylating -and thus inhibiting- e.g. the forkhead transcription factor FoxO1 (Appleman et al., 2002). The phosphorylation of FoxO1 promotes its export from the nucleus to the cytoplasm, where it is unable to induce the transcription of the cell cycle inhibitors p27/kip. The resulting reduction of the p27/kip protein expression in an activated T-cell is indispensable for cell cycle progression and clonal expansion (Appleman et al., 2000).

PI3-kinase mediated recruitment of Vav-1 to the plasma membrane leads to activation of Rac/Rho GTPases, which are involved in the dynamic TCR-induced reorganization of the actin cytoskeleton.

1.2.3 THE GUANINE NUCLEOTIDE EXCHANGE FACTOR VAV-1

Vav-1 is a cytoplasmic signal transducer protein, which was initially identified as an oncogene (Katzav et al., 1989). Subsequently, two additional Vav family proteins were isolated in mammals: Vav-2 (Henske et al., 1995) and Vav-3 (Movilla and Bustelo, 1999). Vav homologues are also expressed in *Drosophila* (Dekel et al., 2000) and in *C. elegans* (Yoo and Greenwald, 2005). Vav-1 protein expression was first detected in the fetal liver of E11.5 embryos, and is down-regulated during diversification of hematopoietic activity

in the embryo and up-regulated in thymus and spleen. In newborn and adult mammals, Vav-1 expression was shown to be restricted to the hematopoietic system (Katzav et al., 1989), whereas Vav-2 and Vav-3 proteins are more widely expressed (Henske et al., 1995, Movilla and Bustelo, 1999).

Vav proteins function as GDP/GTP nucleotide exchange factors for different members of Rho/Rac GTPases. While Vav-1 is primary an exchange factor for Rac-1, it also functions less efficiently as GEF for RhoA and Cdc42 (Rapley et al., 2008).

Structure and regulation of Vav-1

Vav-1 contains characteristic domains that are involved in multiple functions. The most important domain is the DBL homology domain (DH), bearing catalytic GEF activity towards Rho/Rac family GTPases. This domain is flanked by several motifs which regulate the GEF activity and mediate protein/protein interactions. These domains include: a calponin-homology domain (CH) which is involved in Ca^{2+} mobilization, an acidic motif (AC) that contains three sites of tyrosine phosphorylation (Y142, Y160 and Y174) and a pleckstrin homology domain (PH) which interacts with poly-phosphoinositides. Furthermore, Vav-1 contains a proline-rich region (Pro), two SRC-homology 3 domains (SH3) and one SRC-homology 2 domain (SH2), which function as protein interaction sites (figure 5). Surprisingly, Vav-1 encodes two nuclear localization signals (NLS). Although primary localized in the cytoplasm, under certain conditions Vav-1 was also detected in the nucleus, where it is thought to regulate gene transcription (Bustelo, 2000).

Vav-1 activity is strictly regulated by phosphorylation of three regulatory tyrosines (Y142, Y160 respectively Y174), which were localized in the acidic motif of the protein. The phosphorylation of Y174 was shown to induce the release of an autoinhibitory loop, which enables Vav-1 to function as a GEF towards Rho/Rac GTPases (Aghazadeh et al., 2000).

All Vav family proteins play a critical role in T- and B-lymphocyte development and function and were shown to have overlapping functions in antigen-receptor mediated

signaling in T- and particularly in B-lymphocytes (Fujikawa et al., 2003, Bustelo, 2000). Among these diverse functions of Vav family proteins, the best known is the role of Vav-1 in T-cell signaling and TCR-induced cytoskeletal rearrangements, described in the following section.

Figure 5: Domain structure of Vav proteins. Vav-proteins consist of a DBL homology domain (DH), a calponin-homology domain (CH), an acidic motif (AC) and a pleckstrin homology domain (PH). Furthermore, Vav-1 contains a proline-rich region (Pro), two SRC-homology 3 domains (SH3) and one SRC-homology 2 domain (SH2), which function as protein interaction sites and surprisingly two nuclear localization signals (NLS). The biological functions of these domains are described in the text.

Vav-1 and the regulation of the cytoskeleton

Engagement of TCR by antigen presented on MHC molecules rapidly results in reorganization of the actin and the microtubular cytoskeleton which strongly depends on the expression on Vav-1.

APC binding induces polymerization of actin filaments beneath the area of cell-cell contact, where it is thought to stabilize integrin-mediated adhesion between APC and T-cells and the formation of the immunological synapse (IS). Many proteins have been identified that regulate local actin polymerization. One of them is the Arp2/3 complex, a major regulator of actin in a wide range of cell-types, which is activated by the Wiskott-Aldrich syndrome protein (WASP). TCR-induced phosphorylation of the adaptor protein SLP-76 creates binding sites for Vav-1 (also see above) and for the non-catalytic region of tyrosine kinase (Nck). The adaptor protein Nck is essentially composed of one SH2 domain and three SH3 domains and functions to recruit WASP to the APC/T-cell contact site, by binding to phosphorylated SLP-76 and to WASP via its SH2 and SH3 domains

respectively. The subsequent activation of WASP requires Vav-1 activity. Vav-1 gets rapidly phosphorylated upon TCR-ligation and promotes activation of Cdc42. WASP activation via Cdc42-GTP consequently induces Arp2/3-dependent actin polymerization at the T-cell-APC contact site (Zeng et al., 2003).

Furthermore, Vav-1 is a preferential exchange factor for the Rho family GTPases Rac-1, which mediates several cytoskeletal-associated processes, including membrane ruffling, cell spreading and formation of lamellipodia. Rac-1 might also control TCR-induced alterations of the actin cytoskeleton by activating the phosphatidylinositol-4 phosphate 5-kinase (PIP5K), which is involved in the activation of talin and vinculin via PLC-γ. Talin and vinculin anchor the cytoskeleton to the plasma membrane and interact constitutively with Vav-1 in a GEF-independent manner (Hornstein et al., 2004).

In addition, TCR-stimulation regulates the microtubular cytoskeleton, too. It was shown that the microtubule organizing center (MTOC) orientates towards the APC/T-cell contact site upon TCR engagement in a Vav-1 dependent manner. It is still uncertain how Vav-1 mediates TCR-induced MTOC polarization, possibly due to its GEF activity towards Rho-/Rac GTPases. A number of studies have implicated these GTPases in the regulation of microtubules, e.g. Cdc42, which is involved in the TCR-induced polarization of the microtubular cytoskeleton (Stowers et al., 1995).

Finally, Vav-1 was found to be required for TCR-induced activation and clustering of the integrin LFA-1, which mediates the adhesion between T-cells and APCs and is therefore indispensable for IS-formation (Ardouin et al., 2003).

Function of Vav-1 in T-cell development and signaling

Besides its role in regulating the cytoskeleton, Vav-1 is a key factor for T-cell development (figure 6) and activation (figure 7). In Vav-1 knock-out mice the thymus size is significantly reduced. This is due to a reduction of $CD4^+CD8^+$ double-positive (DP) as well as single-positive (SP) mature thymocytes, whereas the number of $CD4^-CD8^-$ double-negative (DN) thymocytes remains unchanged in these mice. Thus, Vav-1 is apparently involved in transducing pre-TCR signals, controlling the development of DP

from DN thymocytes. Interestingly, the development of thymocytes, which express a TCR-γ chain associated with TCR-δ, instead of the classical TCR-α-/ß-heterodimer, is Vav-1 independent (Tybulewicz, 2005).

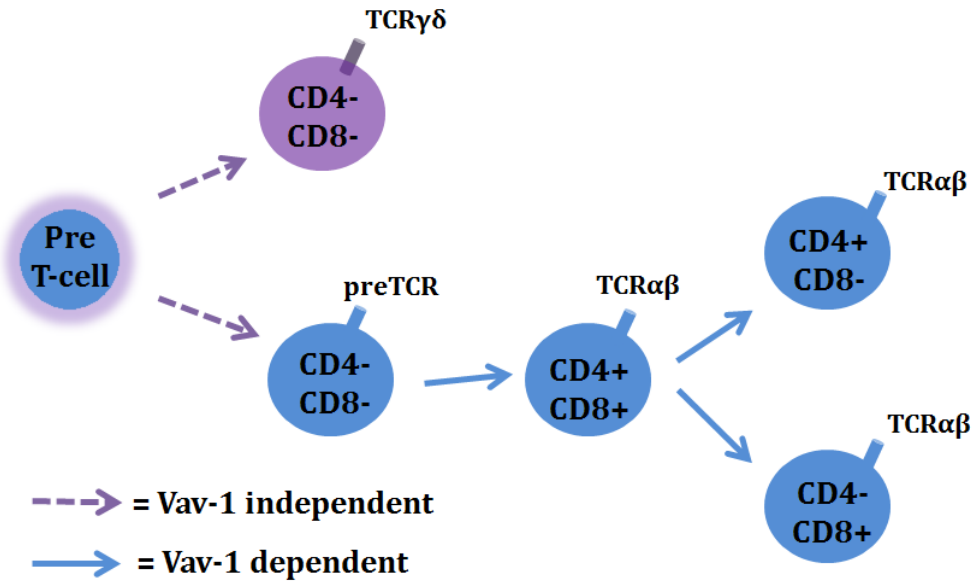

Figure 6: Vav-1 expression is crucial for T-cell development. Vav-1 is involved in pre-TCR signaling, leading to development of DP thymocytes. Furthermore Vav-1 is required for positive selection of SP $CD4^+$ or $CD8^+$ T-cells. However, the development of thymocytes bearing the γδ-TCR is independent of Vav-1 expression. Blue arrows = Vav-1 dependent pathways, purple arrows = Vav-1 independent pathways.

In addition to an impaired T-cell development, the activation of mature T-cells is also defective in mice lacking Vav-1. In Vav-1 knock-out α/β- and γ/δ-T-cells TCR-induced proliferation, up-regulation of activation markers and the production of cytokines is strongly reduced. As already illustrated above (see chapter 1.2.2 and figure 4), Vav-1 is involved in several signaling pathways downstream of the TCR. Hence, Vav-1 deficient T-cells have defects in TCR-induced Ca^{2+}-flux, activation of the transcription factors NFAT and NF-κB and in the activation of the MAP kinase signaling cascade, summarized in figure 7.

Due to a functional redundancy of Vav-1 and the related proteins Vav-2 and Vav-3, the functional lesions in T-cells of Vav-1 knockout mice are incomplete. Consistently, knock-out of all family members had been shown to result in a complete block of TCR-induced signaling (Fujikawa et al., 2003).

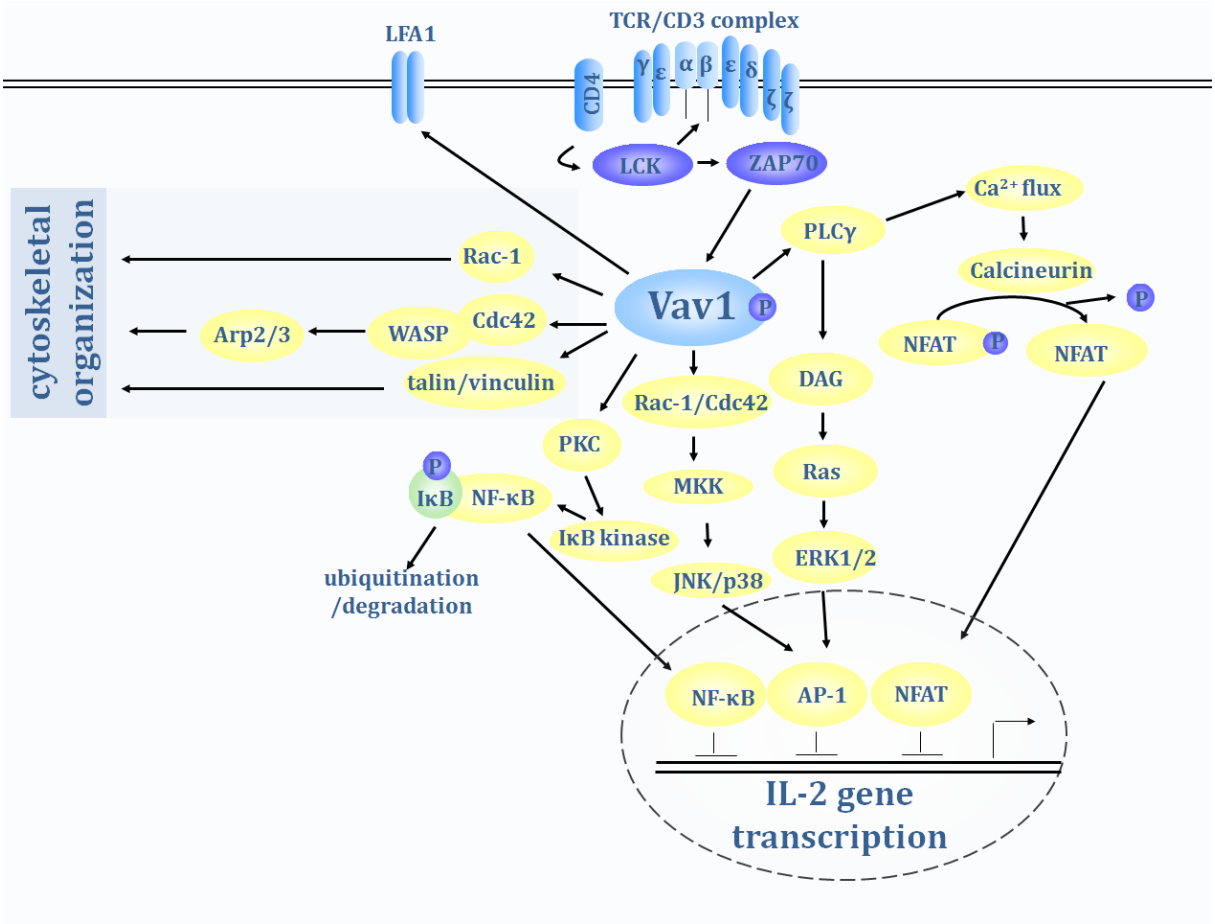

Figure 7: Vav-1 is a key signal transducer downstream of the TCR. Vav-1 is rapidly tyrosine phosphorylated following T-cell receptor stimulation. Active Vav-1 is required to transduce signals from the TCR, leading to TCR-induced Ca^{2+}-flux, activation of the transcription factors NFAT and NF-κB and to the activation of the MAP kinase signaling cascade. Furthermore Vav-1 is involved in the reorganization of the actin and the microtubular cytoskeleton upon TCR engagement. Vav-1 dependent signaling pathways are described precisely in the text.

A novel, pathological function of Vav-1: Role in human cancer

As illustrated here, Vav-1 plays an important, extensively studied role in T-cell development and activation. However, recent studies revealed a so far unknown pathological function of Vav-1 in human cancer. Although a truncated isoform of Vav-1 was already isolated as an oncogene (Katzav et al., 1989), it was only recently shown that the wild-type form of Vav-1 may actually be involved in the development of human cancers (Katzav, 2007). Surprisingly, Vav-1 is expressed in melanoma cell lines, in pancreatic tumors as well as in the majority of tested neuroblastoma samples. The precise function of Vav-1 in the pathogenesis of cancer is still not clear, but ectopic expression of the signaling protein is thought to activate intracellular pathways, which may stimulate or enhance proliferation, cell survival and invasion (Katzav 2007).

1.2.4 CYTOHESIN-1 FUNCTIONS AS A NOVEL SIGNALING FACTOR IN T-CELL ACTIVATION

Cytohesins are a subfamily of guanine nucleotide exchange factors for ADP-ribosylation factor (ARF) GTPases. ARF proteins belong to the Ras family of small GTPases and are critical components of numerous vesicular trafficking pathways and are furthermore implicated in the remodeling of the actin cytoskeleton. They may be grouped into three classes: class I ARFs (ARF1, ARF2, ARF3), class II ARFs (ARF4 and ARF5) and class III ARFs (ARF6). Whereas the precise biological function of class II ARFs is largely unknown, class I ARFs were identified as Golgi-associated GTPases, regulating vesicle formation and the class III ARF ARF6 was shown to be important for membrane traffic between the endosomal compartment and the plasma membrane (D`Souza and Chavrier, 2006).

Like other GTP-binding proteins, ARF proteins cycle between an active GTP-bound and an inactive GDP-bound conformation. Replacement of bound GDP by GTP, which is promoted by guanine nucleotide exchange factors (GEFs), leads to ARF protein activation. Several ARF GEFs have been described and can be divided into two subfamilies: large (~200kDa) ARF GEFs, which can be inhibited by the small molecule

GEF inhibitor brefeldin A (BFA) and small (~47kDa), BFA-insensitive ARF GEFs, which include the cytohesins family proteins.

Structure and regulation of cytohesin family proteins

Thus far, four members of the cytohesin family have been identified: cytohesin-1, cytohesin-2 (also ARNO), cytohesin-3 (also Grp-1 in humans, *Steppke* in *Drosophila*) and cytohesin-4, which share a highly similar domain organization. Cytohesin proteins contain a central sec-7 domain, which bears catalytic GEF activity towards ARF-GTPases (Meacci et al., 1997) and mediates interaction with the integrin ß2-chain CD18 (Kolanus et al., 1996). Furthermore, cytohesins contain a N-terminal coiled-coil domain (CC) and a PH domain (PH), which is followed by a short C-terminal polybasic region (C) (figure 8). The coiled-coil domain at the N-terminus of cytohesin is a known protein/protein interaction motif that is probably involved in dimerization and/or interaction with other proteins.

The PH domain is required for the recruitment of cytohesin to the plasma membrane by binding to membrane phosphoinositides (Nagel et al., 1998). The membrane localization of cytohesin can be regulated by PI3-kinase, which phosphorylates PIP2 within the plasma membrane, creating the signaling phospholipid PIP3. PIP3 is thought to recruit cytohesin proteins by binding to their specialized lipid-binding PH domain.

All cytohesin proteins may be expressed in two different splice variants: a diglycine isoform or a triglycine isoform which differ in their respective binding affinity for PIP3 (Klarlund et al., 2000). The diglycine isoform has two glycines residues located in the inositol-binding site of the PH domain and was shown to have a high affinity for PIP3. In response to several growth factors or insulin, the diglycine splice variant is recruited to the plasma membrane by binding to PIP3. The triglycine isoform has a very low affinity for PIP3 and it remains unclear, how this splice variant is regulated. It was recently proposed that ARF- and ARF-like proteins bind to the PH domain of cytohesin to mediate plasma membrane association, irrespective of whether they are diglycine or triglycine isoforms (Hofmann et al., 2007, Cohen et al., 2007). The PH domain of cytohesin is followed by a short C-terminal polybasic region, which is rich in positively

charged amino acids and mediates membrane association cooperatively with the PH domain. The C-terminal polybasic region markedly stabilizes the interaction of the PH domain with PIP3 (Nagel et al., 1998).

Cytohesin family proteins differ most in structure near the C-terminus. It has been shown that cytohesin-1 and cytohesin-2 bear phosphorylation sites at the C-terminal polybasic region, which are phosphorylated by the protein kinase C upon recruitment to the plasma membrane. Cytohesin-1 contains one threonine- (threonine395) and two serine- (serine 393 and serine 394) phosphorylation sites, whereas cytohesin-2 contains only one serine (serine391) in the polybasic region which is phoshorylated after membrane recruitment. However, the homologous protein cytohesin-3 lacks the carboxy-terminal serine phosphorylation sites present in cytohesin-1 and cytohesin-2 (Kolanus, 2007). This different phosphorylation pattern might explain distinct functions of the otherwise structurally highly similar cytohesin family proteins.

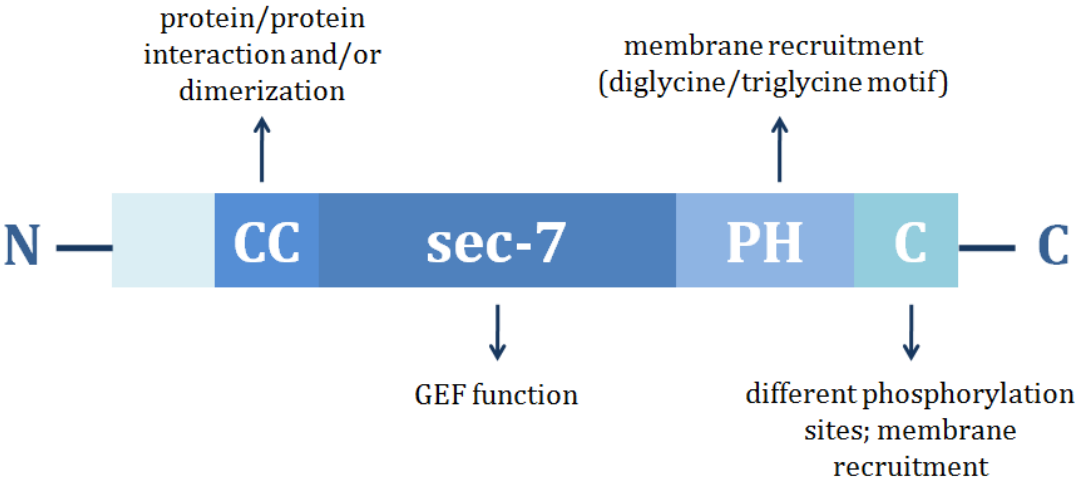

Figure 8: Domain structure of cytohesin proteins. Cytohesin family proteins contain a N-terminal coiled-coil domain (CC), a central sec-7 domain, which bears the GEF activity towards Arf-GTPases and PH domain (PH), which is followed by a short C-terminal polybasic region (C). The biological function of these domains is described in the text.

The role of cytohesin-1 in the regulation of immune cell adhesion and migration

Cytohesin-1 is predominantly expressed in hematopoetic cells and was identified in 1996 as an LFA-1 interacting protein. It specifically binds to the cytoplasmic domain of the integrin ß2-subunit CD18, which was shown by the use of the yeast two-hybrid system (Kolanus et al., 1996). It was subsequently demonstrated that cytohesin-1 acts as an important regulator of ß2-integrin-mediated adhesion, since RNAi of cytohesin-1 markedly reduces static adhesion of human monocyte derived dendritic cells to ICAM-1 (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). Over-expression of cytohesin-1 in Jurkat T-cells, results in an enhanced binding to the LFA-1-ligand ICAM-1 (Kolanus et al., 1996). LFA-1 activation depends on its direct interaction with cytohesin-1 which induces the expression of an extracellular activation epitope. Surprisingly, a point mutant of cytohesin-1 (E157K), in which the ARF-GEF function is disrupted, is also able to induce this extracellular activation epitope by direct binding to LFA-1. These data reveal that GEF activity of cytohesin-1 is not required for the induction of conformational switches leading to integrin activation. However, the GEF activity of cytohesin-1 is required to induce LFA-1-dependent cell spreading, through the activation ARF GTPases (Geiger et al., 2000).

It has recently been demonstrated that integrin-dependent dendritic cell migration in a 3D environment strongly depends on cytohesin-1 function *in vitro* and *in vivo* (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). Cytohesin-1 was shown to mediate its functions by activating the GTPase RhoA, which is required for integrin-dependent functions, such as regulation of cell adhesion and migration (Quast et al., 2009).

The balance between T cell activation and anergy is established by two cytohesin family proteins

Besides its role in signaling pathways, which affect the regulation of immune cell adhesion and migration, cytohesin-1 acts as a novel intracellular signal transducer in T-cell activation. It was demonstrated that the GEF activity of cytohesin-1 is crucial for activation of the IL-2 promotor upon TCR-ligation. The IL-2 promoter contains binding sites for several transcription factors, regulating IL-2 gene transcription. These

transcription factors include NF-κB, NFAT, AP-1, the octamer binding protein (Oct) and the CD28 response element (CD28RE) (Serfling et al., 1995). Recent over-expression analysis indicated that cytohesin-1 is specifically important for TCR-induced activation of the transcription factor AP-1 (Paul, PhD thesis 2007), a transcription factor complex consisting of homo- or heterodimers of Fos/Jun family proteins.

Interestingly, over-expression of the homologous protein cytohesin-3, lacking a carboxy-terminal serine phosphorylation site present in cytohesin-1, represses AP-1-and thus IL-2 promoter activation, suggesting that this highly related protein is a direct antagonist of cytohesin-1 (Paul, PhD thesis 2007). Consistently, the inhibition of T-effector cell proliferation by $CD25^+$ $FoxP3^+$ regulatory T-cells is specifically abrogated in cytohesin-3 knock-down effector cells (Grell et al., in preparation). Fully in line with all the findings, it was demonstrated that cytohesin-3 expression is strongly up-regulated in various types of anergic T-cells (Korthäuer et al., 2000, Paul, PhD thesis 2007, Grell et al., in preparation). Cytohesin-3 thus acts an endogenous regulator of tolerance induction/maintenance through functional inhibition of the cytohesin-1/AP-1 signaling axis (Paul, PhD thesis 2007).

Several other functions have been ascribed to cytohesin proteins, which could not be all depicted here. For a more detailed overview of the cytohesin family and their precise roles in several signal transduction pathways, see the recently published review by W. Kolanus, in which the known cellular functions of cytohesins are discussed in great detail (Kolanus 2007).

1.3 CENTRAL SIGNALING PATHWAYS IN INNATE IMMUNITY

Although the innate immune system lacks the specificity of adaptive immunity, it is able to differentiate between self and nonself. This is achieved by receptors that recognize highly conserved microbial structures, so-called pathogen-associated molecular patterns (PAMPs). The recognized microbe-specific molecules include bacterial carbohydrates

(e.g. lipopolysaccharide (LPS), mannose), nucleic acids (e.g. bacterial or viral DNA or RNA), peptidoglycans, N-formylmethionine, lipoproteins and fungal glucans.

The main family of pattern recognition receptors are Toll-like receptors (TLR) which are able to sense a wide range of microbial motifs (figure 9). They play a major role in the induction of innate immunity and also contribute to the activation of the adaptive immune system. All TLRs contain leucine-rich repeats which mediate ligand binding, and a Toll/interleukin-1 receptor-like domain (TIR domain), which interacts with several intracellular TIR domain-containing adaptor molecules. Signaling through TLRs leads to the activation of the transcription factor NF-κB, finally initiating the production of cytokines (e.g. IL-1 and TNFalpha) and several other effector molecules. In addition to TLRs, many other surface receptors have been proposed to participate in innate immunity and microbial recognition, such as scavenger receptors, intracellular NOD-like receptors (NODs and NALPs), RNA helicases (e.g. RIG-I) and C-type lectin receptors, e.g. dectin-1, a receptor for fungal ß-glucans (see chapter 1.3.2).

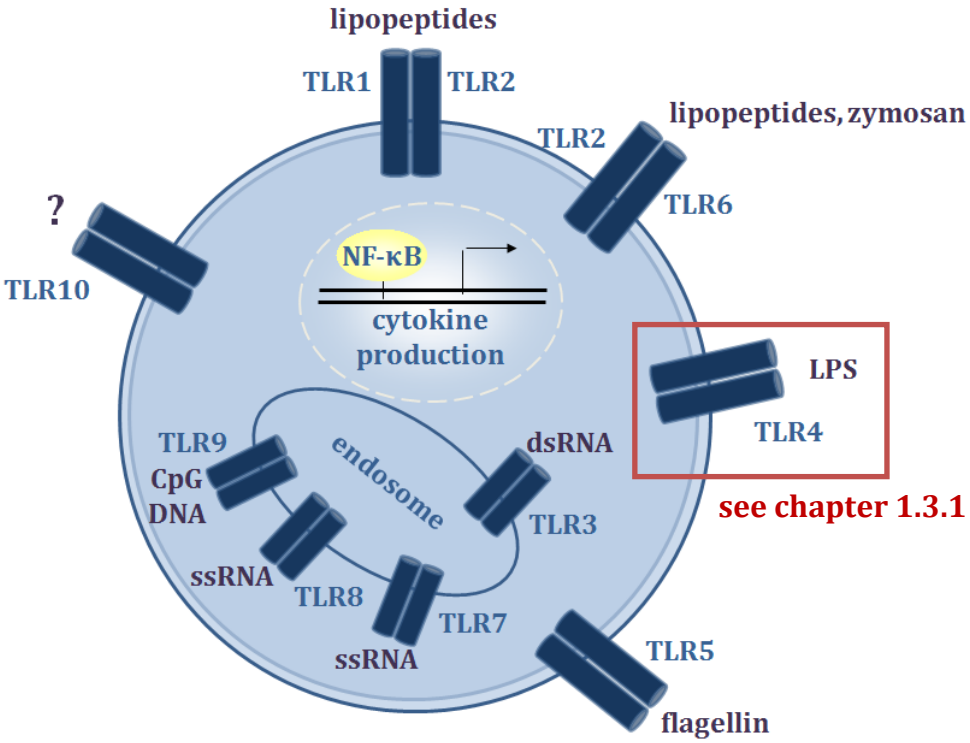

Figure 9: Toll like receptors and their ligands.

1.3.1 THE LPS SIGNALING PATHWAY

Lipopolysaccharide (LPS) is a cell-wall component of gram-negative bacteria and acts as potent activator of cells of the immune system, including macrophages, monocytes and dendritic cells. It is able to induce a dramatic systemic reaction, known as septic shock, by triggering an uncontrolled production of pro-inflammatory cytokines, e.g. tumor necrosis factor alpha (TNFalpha)(see chapter 1.3.3). LPS mediates its effects by signaling through the TLR4/CD14/MD2 receptor complex. The LPS binding protein (LBP) is necessary to deliver LPS to the plasma membrane, where it binds to the cell surface protein CD14 (cluster of differentiation-14)(Schumann et al., 1990). Subsequently, the LPS/CD14 complex interacts with TLR4 and its accessory protein MD2 (da Silvia Correia, et al., 2001). Activation of TLR4 leads to recruitment of TIR domain-containing adaptor molecules (TIRAPs), including the myeloid differentiation factor 88 (MyD88), the MyD88 adaptor-like protein (Mal or TIRAP), the TIR-containing adaptor inducing IFN-ß (TRIF) and the TRIF-related adaptor molecule (TRAM). These adaptor proteins propagate intracellular signaling cascades, leading to activation of the transcription factors IRF3, AP-1 and NF-κB (Akira, et al., 2003).

As illustrated in figure 10, TLR4 ligation activates a MyD88-dependent and a MyD88-independent signaling pathway. The MyD88-dependent pathway activates NF-κB and AP-1 via IL-1R–associated kinases 1 and 4 (IRAK1 and IRAK4) and tumor-necrosis-factor-receptor-associated factor 6 (TRAF6). The MyD88-independent pathway signals through TRIF and TRAM and induces the phosphorylation of the interferon-regulatory factor 3 (IRF3). Phosphorylated IRF3 subsequently translocates from the cytoplasm to the nucleus, where it promotes IFN-ß production which finally results in the transcriptional activation of IFN-inducible genes. Furthermore, it has been demonstrated that the MyD88-independent pathway is involved in late-phase activation of NF-kB via interaction of TRIF with TRAF6 (Dauphinee and Karsan, 2006, Arancibia et al., 2007).

TLR-4 is highly expressed on dendritic cells, which are the most potent antigen-presenting cells of the immune system. In dendritic cells, TLR4 signaling induces their terminal differentiation ("maturation"), which is characterized by robust cell surface expression of MHC-peptide complexes and of so-called co-stimulatory molecules, e.g. CD80, CD86 and CD40. The activation of either the MyD88-dependent or the MyD88-independent pathway is sufficient for the induction of LPS-dependent dendritic cell maturation (Akira et al., 2003).

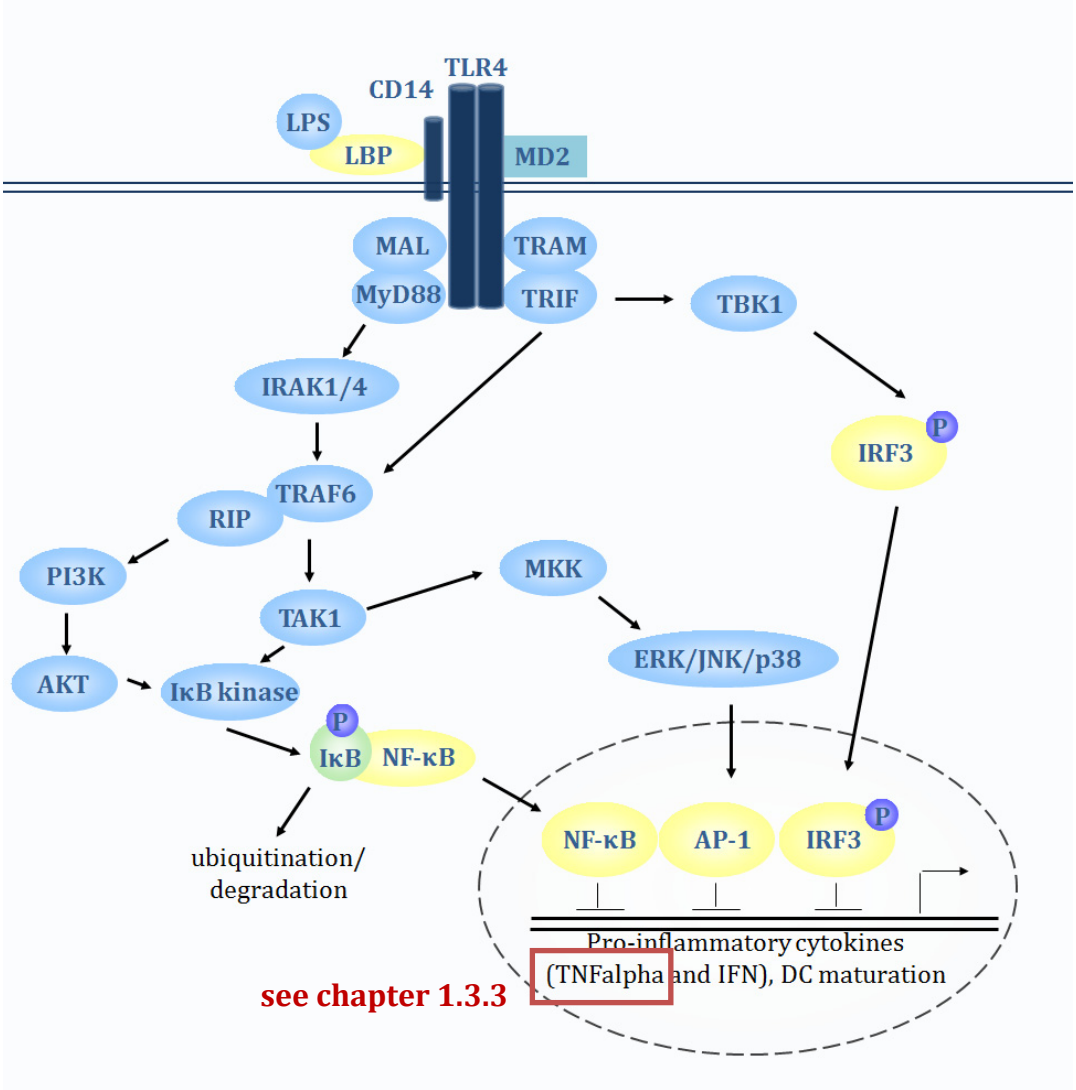

Figure 10: The LPS signaling pathway. Binding of LPS to TLR-4 induces MyD88-dependent activation of NF-κB and AP-1. Furthermore, LPS stimulation leads to the induction of a MyD88-independent pathway, ending in activation of the transcription factor IRF3, IFN-ß production and finally in transcription of IFN-inducible genes.

1.3.2 THE PATTERN RECOGNITION RECEPTOR DECTIN-1

The C-type lectin receptor dectin-1 plays a key role in coordinating the responses of macrophages and dendritic cells to fungal pathogens. It acts as a major receptor for zymosan, curdlan and other 1,3-linked ß-glucans, found in the cell membrane of fungi and also in some bacteria. Engagement of dectin-1 by ß-glucans triggers a variety of cellular responses, e.g. ligand uptake by phagocytosis, respiratory burst and the production of pro-inflammatory cytokines (e.g. TNFalpha, IL-6 and IL-10) as well as chemokines (e.g. CXCL2) (Reid et al., 2009).

Dectin-1 is remarkably similar to other immune receptors, since its cytoplasmic domain contains an immunoreceptor tyrosine-based activation motif (ITAM) related to those of antigen- and Fc-receptors. However, the dectin-1 ITAM is atypical, for only one of its two tyrosine residues participates in signaling (Underhill et al., 2005, Rogers et al., 2005).

Engagement of dectin-1 triggers the phosphorylation of the ITAM by Src kinases and this creates docking sites for the tyrosine kinase Syk (figure 11). Recruitment and subsequent activation of Syk triggers the activation of NF-κB via the caspase recruitment domain 9 (CARD9), which assembles with BCL10 and MALT1 (Gross et al., 2006). Furthermore, dectin-1 collaborates with TLRs through an yet undefined mechanism, in inducing optimal cytokine and chemokine production in response to fungal pathogens. This has been shown in macrophages, where dectin-1 as well as TLR2 are required for TNFalpha production (Dennehy et al., 2008).

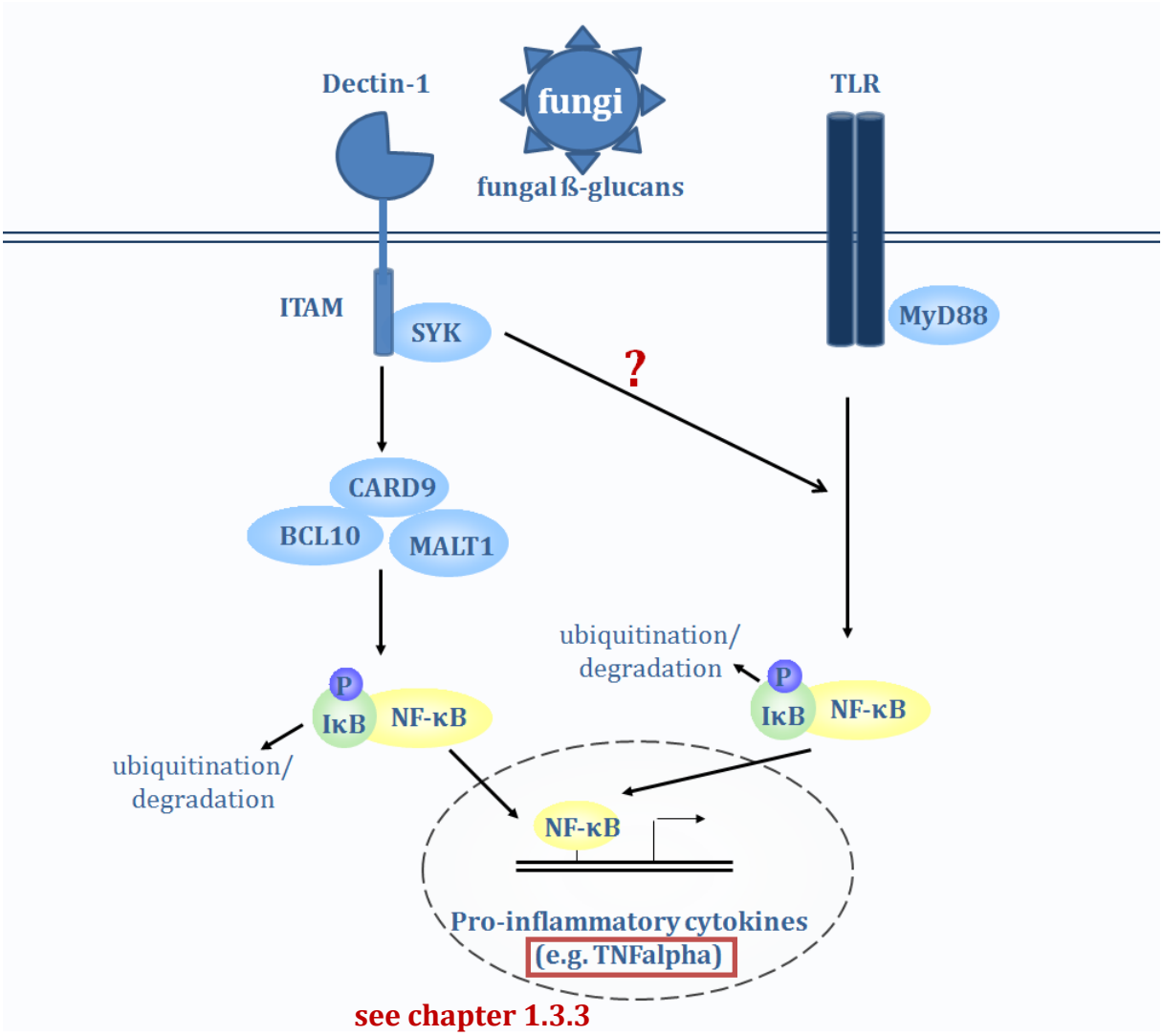

Figure 11: Dectin-1-mediated activation of NF-κB. The C-type lectin receptor dectin-1 recognizes fungal 1,3-linked ß-glucans and triggers an intracellular signaling cascade leading to the activation of NF-κB. Dectin-1 was shown to collaborate with TLRs through an yet undefined mechanism, in inducing optimal cytokine and chemokine production in response to fungal pathogens.

1.3.3 THE TNFALPHA SIGNALING PATHWAY

Tumor necrosis factor alpha (TNFalpha) was first described in 1975 as an endotoxin-induced serum factor, which causes necrosis of tumors in certain mouse model systems (Carswell et al., 1975). It is now known that TNFalpha is a major mediator of apoptosis, inflammation and immunity in a wide range of cell-types. Dysregulated TNFalpha production has been implicated in the pathogenesis of several human diseases, e.g. sepsis, diabetes, rheumatoid arthritis and inflammatory bowel diseases. Furthermore, it has become clear that the described necrotizing ability of TNFalpha does not hold true for all tumor types, and that TNFalpha may even promote cancer progression (Mocellin et al., 2005).

TNFalpha is a homotrimeric molecule primarily produced by activated macrophages, but also by a broad variety of other cell-types, including neutrophils, dendritic cells and mast cells. Large amounts of TNFalpha are released in response to lipopolysaccharide and other bacterial products (see chapter 1.3.1 and 1.3.2). It exerts its cellular effects through two distinct surface receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), both members of the TNFR superfamily. Receptors within this group share a similar extracellular domain, containing multiple cysteine-rich repeats and were thought to signal through ligand-induced receptor trimerization. However, it has been recently demonstrated that TNFR1 and TNFR2 can exist as pre-assembled oligomers, and that this homophilic interaction is mediated by the distal cystein-rich domain, the so-called pre-ligand assembly domain (PLAD). As demonstrated for the TCR (see above and Schamel et al., 2005), these pre-clustered TNFR were thought to respond to ligands with higher sensitivity than monovalent receptors and this may facilitate rapid cellular responses to cytokine stimulation (Chan, 2007).

In the majority of cells, TNFR1 appears to be the key mediator of TNFalpha signaling, therefore this introduction focuses on TNFR1-induced signaling pathways. The ligand-binding induced trimerization of TNFR1 leads to the release of the silencer of death domain (SODD), an inhibitory protein which binds to the intracellular death domain of

the unstimulated receptor (Jiang et al., 1999). This release permits the recruitment of the TNF-receptor associated death domain protein (TRADD) (Hsu et al., 1995). TRADD acts as an adaptor protein which subsequently recruits the Fas-associated death domain (FADD), the TNF-receptor-associated factor 2 (TRAF2) (Hsu et al., 1996) and the receptor-interacting protein kinase (RIP) (Ting et al., 1996). These signaling proteins propagate intracellular signaling cascades leading either to the induction of apoptosis via caspase activation or to activation of the transcription factors c-Jun and NF-κB (figure 12).

TNFalpha-induced activation of NF-κB

TNFalpha-induced activation of NF-κB is mediated via activation of the IKK complex, which is composed of two catalytic subunits, IKKα and IKKβ, and the non-catalytic subunit NEMO (Zandi et al., 1997). The activation of the IKK complex (see below) subsequently leads to the phosphorylation of IκB. Phosphorylated IκB is then targeted for Lys-48-linked poly-ubiquitination which leads to its recognition and degradation by the proteasome. The degradation of IκB allows the trans-location of NF-κB to the nucleus, where it activates the expression of e.g. anti-apoptotic and inflammatory genes.

The activation of the IKK complex is not completely understood, but it has been shown that TNFalpha-induced signaling to NF-κB is regulated by two forms of poly-ubiquitination. As mentioned above, Lys48-linked poly-ubiquitination triggers proteasome-dependent degradation of proteins, e.g of the NF-κB inhibitor IκB, TNFalpha-induced degradation of which enables the activation of NF-κB. In contrast, Lys63-linked poly-ubiquitination does not lead to protein degradation, but is associated with intracellular signaling, e.g. TNFalpha-induced NF-κB activation. Upon recruitment to the TNFR1 complex, Lys63-linked poly-ubiquitin chains are attached to TRAF2 and RIP. The E3 ligase required for RIP ubiquitination is still unknown. There is some evidence that the RING domain containing protein TRAF2 might induce the ubiquitination of RIP (Lee et al., 2004), whereas others have shown that RIP is ubiquitinated by the cellular inhibitor of apoptosis c-IAP1 or c-IAP2 (Park et al., 2004).

The NF-κB essential modulator (NEMO) subsequently binds to these Lys63-linked poly-ubiquitin chains of RIP and gets afterwards also modified by Lys63-linked poly-ubiquitination. The role of the ubiquitination of NEMO is still unclear but is required for activating the IKK complex (Tang et al., 2003).

Together, RIP-dependent phosphorylation of IKKα and IKKβ and the ubiquitination of NEMO are required to fully activate the IKK complex (Tang et al., 2003).

The duration of TNFalpha-induced activation of NF-κB is regulated by multiple inhibitor proteins. One mechanism to terminate signaling to NF-κB is the inducible expression of IκB, which is one of the target genes of NF-κB. Newly expressed IκB bind to NF-κB and masks its nuclear location sequence in order to terminate NF-κB activation. Furthermore, TNFalpha stimulation dramatically increases the expression of A20, a ubiquitin ligase which mediates Lys48-linked poly-ubiquitination as well as Lys63-linked de-ubiquitination of RIP (Lin et al., 2008). Attached Lys48-linked ubiquitin chains mark RIP for proteasomal-degradation and this leads finally to the down-regulation of NF-κB activity.

TNFalpha-induced activation of c-Jun

TNFalpha can activate the JNK pathway in a TRAF2-dependent manner. TRAF2 is thought to activate several MAP kinase kinase kinases (MAPKKK), e.g. MEKK1 and ASK1, which in turn activate a kinase cascade leading to the activation of JNK. The downstream target of JNK is the transcription factor c-Jun, a member of the AP-1 transcription factor complex which plays important roles in a variety of cellular processes, including proliferation, differentiation and prevention of apoptosis (Wajant et al., 2003).

Molecular mechanisms of TNF-induced cell death

Under conditions in which protein synthesis is blocked, or in which NF-κB activation is inhibited, TNFR1 stimulation leads to a strong induction of apoptosis. Apoptosis induced by TNFR1 is mediated via recruitment of the adaptor protein FADD (Hsu et al., 1996). Pro-caspase-8 is then recruited by binding to the death effector domain of FADD, where it becomes activated by cleavage at specific aspartic acid residues, and this initiates an

apoptotic signaling cascade. The induced caspase cascade leads to the activation of caspase-activated DNase (CAD), which results in DNA fragmentation. Furthermore, the mitochondrial apoptotic pathway gets activated, resulting in the release of cytochrome-C which further amplifies the caspase cascade (Mocellin et al., 2005).

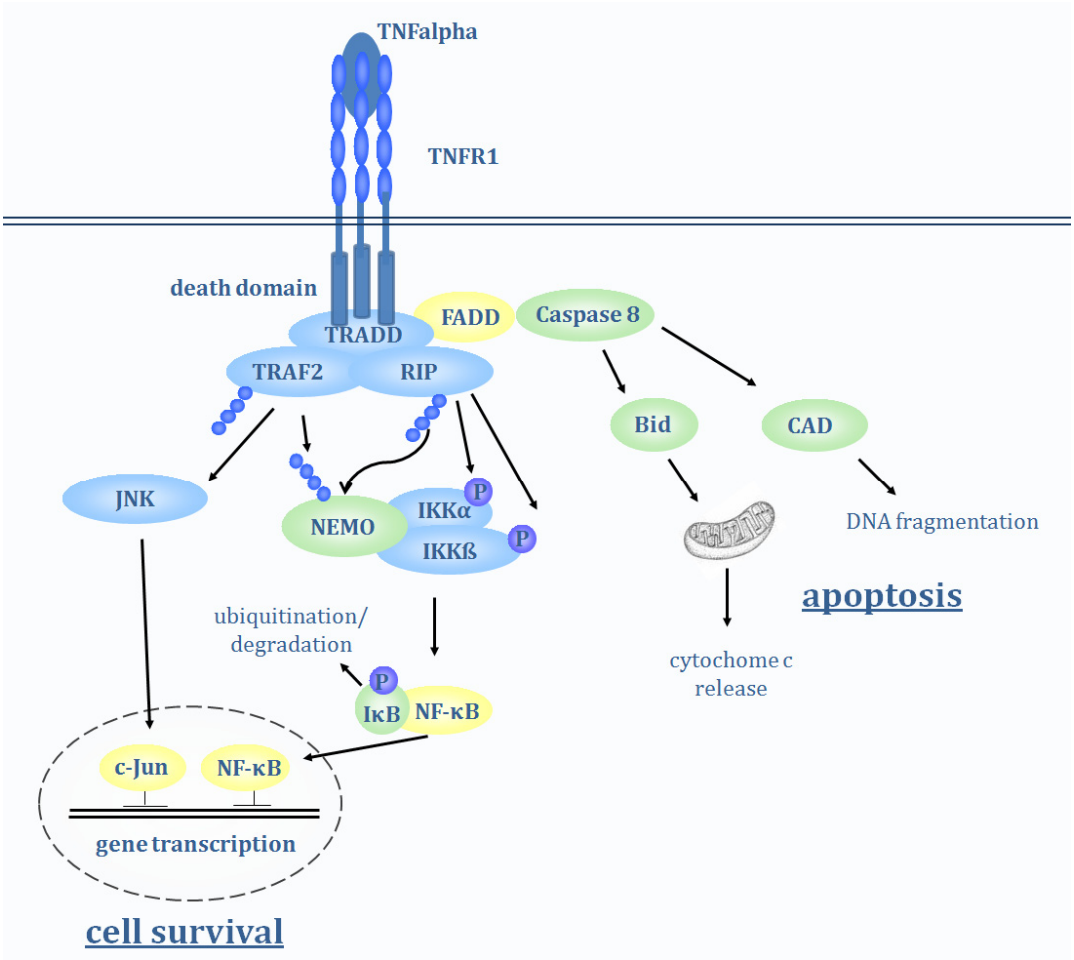

Figure 12: The TNFalpha signaling pathway. Binding of TNFalpha to TNFR1 propagates intracellular signaling cascades leading either to the induction of apoptosis via caspase activation or to the activation of the transcription factors c-Jun and NF-κB.

Cell-specific modulation of TNFR functions

Signaling through the TNRF has been studied in great detail, and was shown to be essentially similar in most cell-types. However, some studies report of cell-specific modulation of TNFR functions, e.g. in controlling homeostatic versus inflammation induced-apoptosis functions of neutrophil granulocytes.

Neutrophils are one of the first group of immune cells which migrate towards the site of inflammation, where they are responsible for eliminating invading pathogens by phagocytosis. The internalization of the pathogen is followed by a rapid release of reactive oxygen species into the phagosome in order to degrade internalized particles and bacteria. However, neutrophil anti-microbial products may also damage surrounding host tissue. To limit the destructive potential of neutrophils, their lifespan is tightly regulated. Neutrophils are cleared from tissues by apoptosis and subsequent phagocytosis by macrophages to inhibit the release of their cytotoxic content into the extracellular milieu. Several cytokines accelerate neutrophil apoptosis, e.g. high doses of TNFalpha. Recently, ß2-integrins where shown to modulate TNFalpha-induced apoptosis of neutrophils. In the absence of apoptosis-inducing cytokines, clustering and activation of ß2-integrins promote cell survival via activation of the survival proteins ERK and AKT. However, activation of β2-integrins in the presence of TNF-α potentiates TNFalpha triggered apoptosis induction. This is due to a an inhibition of AKT activity via SHIP (SH2-containing inositol 5-phosphatase) recruitment to the integrin (Whitlock et al., 2000, Salamone et al., 2004, Gardai et al., 2002).

Besides their function in modulating TNFalpha-induced apoptosis induction, ß2-integrins play a major role in regulating TNFalpha-induced respiratory burst of human neutrophils. TNFalpha triggers the release of large amounts of reactive oxygen intermediates, but this signaling pathway strongly depends on activated ß2- integrins. TNFalpha stimulation failed to induce ROS production of neutrophils in suspension, and the respiratory burst was furthermore shown to be completely inhibited by CD18 or CD11b blocking antibodies (Dapino et al., 1993, Nathan et al., 1989, Decleva et al., 2002). However, the molecular basis of this integrin mediated modulation of TNFalpha signaling still remains largely unknown. Taken together, ß2-integrins are thought to act as signaling partners of TNFR, by cross-talking with TNFalpha-induced signal transduction in a cell-type specific manner.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 EQUIPMENT

Autoclave	Model 135T, H+P (Oberschleißheim)
Centrifuges	Multifuge 4KR, Heraeus instrument GmbH (München)
	Biofuge pico, Heraeus instrument GmbH (München)
	Biofuge fresco, Heraeus instrument GmbH (München)
	Avanti J-20XP, Beckman Coulter (München)
	Optima LE-80K Ultracentrifuge, Beckman Coulter (München)
CO_2-Incubator	Model 381, ThermoForma (Karlsruhe)
Electronic balance	College, MettlerToledo (Greifensee, Switzerland)
Electrophoresis chambers	Perfect blue gel system, Peqlab (Erlangen)
	Protein-Minigel-Apparature, BioRad (München)

Electroporation device	GenePulser Xcell + CE module, Biorad (München)
	Nucleofector, Amaxa (Köln)
Flow cytometer	Epics XL, Beckman Coulter (München)
	FACS Canto II, BD (Heidelberg)
Fluorescence- and absorption analyzer	Synergy HT, MWG (Ebersberg)
Gel dryer	Model 583, BioRad (München)
Heat block	Thermomixer compact, Eppendorf (Hamburg)
Horizontal shaker	Rocky, Fröbel Labortechnik GmbH (Lindau)
	WS 5, Edmund Bühler (Hechingen)
Laminar flow hood type 2 for cell culture	Euroflow, Thermolife (Woerden, Netherlands)
Luminometer	Microlumat plus LB 96V, Berthold (Bad Wildbad)
Magnetic stirrer	ARE, VELP scientifica (Milan, Italy)
MicroPorator, MP-100 system	Peqlab (Erlangen)

Microscopes	Light microscope DMIL, Leica (Wetzlar)
	Fluorescence microscope TE2000, NIKON (Tokyo, Japan)
	confocal Laser-Scanning-Microscope Fluoview 1000, Olympus (Tokyo, Japan)
Oligonucleotide purification columns	Quant 96 G-50 micro columns, GE Healthcare (München)
pH-meter	MP220, Mettler Toledo (Greifensee, Switzerland)
Photometer	Biophotometer, Eppendorf (Hamburg)
Pipettes	P2, P10, P100, P200, P1000, Eppendorf (Hamburg)
Pipette controller	Pipetus-Akku, Hirschmann Laborgeräte (Eberstadt)
Powersupplies for electrophoresis	Elite300Plus, Schütt Labortechnik (Göttingen)
	PS 202, Apelex (Massy Cedex, France)
Precision balance	Mettler Toledo (Greifensee, Switzerland)
Protein-Transfer-Apparature	BioRad (München)
Vortex	Zx3, VELP scientifica (Mailand, Italy)
Water bath	Typ 1004, GFL (Burgwedel)

2.1.2 CONSUMABLES

Cell culture dishes (10cm)	Greiner Bio-one (Frickenhausen)
Cell culture flasks (175/75/25 cm^2)	Greiner Bio-one (Frickenhausen)
Cell culture plates (6-well, 12-well, 24-well)	Greiner Bio-one (Frickenhausen)
Cell scraper	Sarstedt (Nümbrecht)
Cellophane membrane	BioRad (München)
Disposable hypodermic needle, (0.4x20mm)	Sterican, Braun (Melsungen)
Elektroporation cuvettes (4mm)	BioRad (München)
FACS tubes	BD Falcon (Heidelberg)
Filter paper	Whatman Nr. 4, Schleicher & Schuell (Dassel)
	Filter paper used for EMSA (gel drying), BioRad (München)
Filter tips (10µl, 200µl, 1000µl)	MultiGuard, Roth (Karlsruhe)
Microtiter plates (96-Well)	Nunc (Roskilde, Denmark)
Nitrocellulose membrane	PROTRAN, Schleicher & Schuell (Dassel)
Nylon cell strainer (40µm pore)	BD Biosciences (Heidelberg)
Pipette tips (10µl, 200µl, 1000µl)	Roth (Karlsruhe)
Polypropylene-Reaction tubes (0.5/1.5/2.0ml)	Eppendorf Starlab (Helsinki, Finland)

Radiographic film	Hyperfilm™ MP, Amersham Biosciences (Buckinghamshire, UK)
Sterile filters (0.2µm/0.45µm)	Schleicher & Schuell (Dassel)
Syringe, 10ml	Braun (Melsungen)

2.1.3 REAGENTS

Acrylamide, 40%	Roth (Karlsruhe)
Acrylamide/Bisacrylamid-Mix, 30%	Roth (Karlsruhe)
Adenosine 5`-triphosphate, [γ-^{32}P], 10mCi/ml	Perkin Elmer (Massachusetts, USA)
Ammoniumperoxodisulfate (APS)	Roth (Karlsruhe)
BCA-Reagent solutions	Pierce (Rockford, USA)
Bovine serum albumin (BSA)	Roth (Karlsruhe)
Bromphenolblue	Roth (Karlsruhe)
Curdlan	WAKO Chemicals (Neuss)
Dimethylsulfoxide (DMSO)	Roth (Karlsruhe)
Dithiothreitol (DTT)	Roth (Karlsruhe)
DNA loading buffer with ficoll, 6x	Roth (Karlsruhe)
EDTA (ethylene diamine tetraacetic acid)	Roth (Karlsruhe)
EGTA (ethylene glycol tetraacetic acid)	Roth (Karlsruhe)
Ethanol	Roth (Karlsruhe)

Ethidium bromide	Roth (Karlsruhe)
Ficoll, human (Pancoll)	PAN Biotech GmbH (Aidenbach)
G418 sulfate	PAA (Pasching)
Gentamicin	Gibco (Eggenstein)
Glucose	Roth (Karlsruhe)
Glycine	VWR (Darmstadt)
Glycerin	Roth (Karlsruhe)
Glycerolphosphate	Sigma (Taufkirchen)
GM-CSF, human (recombinant)	R&D Systems (Wiesbaden)
GM-CSF, murine	supernatant derived from the murine Ag-cell line stably transfected to express GM-CSF
Guanosine 5′-triphosphate sodium salt solution (GTP)	Sigma (Taufkirchen)
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Roth (Karlsruhe)
HRP-detection system	ECL Western Blotting Analysis System RPN2109, Amersham Biosciences (Buckinghamshire, UK)
Hydrocloric acid (HCL)	Roth (Karlsruhe)
ICAM-1-Fc	supernatant from CV-1 cells over expressing ICAM-1-Fc fusion protein
Igepal	Sigma (Taufkirchen)

Interleukin-4 (IL-4), human (rekombinant)	R&D Systems (Wiesbaden)
Lipopolysaccharide (LPS)	Sigma (Taufkirchen)
L-Glutamine	Gibco (Eggenstein)
Luciferase substrate	Promega (Mannheim)
Magnesium chloride ($MgCl_2$)	Roth (Karlsruhe)
2-Mercaptoethanol	Roth (Karlsruhe)
Methanol	Roth (Karlsruhe)
Milk powder	Roth (Karlsruhe)
Non essential aminoacids	Sigma (Taufkirchen)
Penicillin/Streptomycin	GIBCO (Eggenstein)
Phenylmethanesulphonylfluoride (PMSF)	Sigma (Taufkirchen)
Piceatannol	Sigma (Taufkirchen)
PMA (12-O-Tetradecanoylphorbol-13-acetate)	Sigma (Taufkirchen)
Poly(2′-deoxyinosinic-2′-deoxycytidylic acid) sodium salt (poly-dI-dC)	Sigma (Taufkirchen)
Potassium chloride (KCL)	Roth (Karlsruhe)
2-Propanol	Roth (Karlsruhe)
Protease inhibitors	Antipain, Aprotinin, Benzamidine, Leupeptin, PMSF, Sigma (Taufkirchen)
Protein A-Sepharose™ 6MB	Amersham Biosciences (Buckinghamshire, UK)

Protein standard	Precision Plus Protein All Blue standard, BioRad (München)
Reporter lysisbuffer	Promega (Mannheim)
SecinH3 (20H3)	Calbiochem (Darmstadt)
Sodium dodecyl sulfate (SDS)	Roth (Karlsruhe)
Sodium fluoride (NaF)	Sigma (Taufkirchen)
Sodium hydroxide (NaOH)	Roth (Karlsruhe)
Sodium orthovanadat (Na_3VO_4)	Sigma (Taufkirchen)
Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma (Taufkirchen)
Sucrose	Roth (Karlsruhe)
TBE-buffer, 10x	Roth (Karlsruhe)
6-Thio GTP	Jena Bioscience (Jena)
TEMED	Roth (Karlsruhe)
Tetra-sodium-diphosphate decahydrate	Roth (Karlsruhe)
TNFalpha, human (recombinant)	Peprotech (Hamburg)
TNFalpha, murine (recombinant)	Peprotech (Hamburg)
Tris-[hydroxymethyl]aminomethan (Tris)	Roth (Karlsruhe)
Triton X-100	Roth (Karlsruhe)
Trypan blue solution, 0.4%	Sigma (Taufkirchen)
Zymosan A, from *Saccharomyces cerevisiae*	Sigma (Taufkirchen)

2.1.4 KITS

Human T-cell Nucleofector Kit	AMAXA (Köln)
MidiMACS Separator Kit	Miltenyi Biotec GmbH (Bergisch Gladbach)
Pan T-cell isolation Kit, mouse	Miltenyi Biotec GmbH (Bergisch Gladbach)

2.1.5 MEDIA, SERA AND BUFFER

DMEM, high glucose	PAA (Pasching)
HBSS	Gibco (Eggenstein)
IMDM	PAA (Pasching)
OptiMem, phenolfree	Gibco (Eggenstein)
PBS	Gibco (Eggenstein)
RPMI 1640	Gibco (Eggenstein)
VLE-RPMI 1640	Biochrom (Berlin)

2.1.6 ENZYMES

T4-DNA-ligase	MBI Fermentas (St. Leon-Rot)
T4-polynucleotide-kinase	Roche (Mannheim)
Shrimp Alkaline Phosphatase (SAP)	Roche (Mannheim)
Restrictionenzymes	MBI Fermentas (St. Leon-Rot), NEB (Schwalbach)
RNase	Roche (Mannheim)
Vent-DNA-polymerase	NEB (Schwalbach)

2.1.7 ANTIBODIES

The following antibodies were used against mouse antigens:

Primary Antibody	**Dilution**	**Supplier**
FITC anti-mouse CD54	1:200 (FACS)	BD Biosciences PharMingen (Heidelberg)
FITC anti-mouse CD80	1:200 (FACS)	eBioscience (San Diego, USA)
FITC anti-mouse MHC class II (I-A/I-E)	1:200 (FACS)	eBioscience (San Diego, USA)
PE anti-mouse CD86	1:200 (FACS)	eBioscience (San Diego, USA)

The following primary antibodies were used against human antigens:

Primary Antibody	Dilution	Supplier
Goat anti-human IgG	1:100 (coating)	Dianova (Hamburg)
Rabbit anti-human actin	1:1000 (WB)	Sigma (Taufkirchen)
Rabbit anti-human phosho ERK1/2	1:1000 (WB)	Cell Signalling (Danvers, USA)
Rabbit anti-human phosho JNK	1:1000 (WB)	Cell Signalling (Danvers, USA)
Rabbit anti-human phosho p38	1:1000 (WB)	Cell Signalling (Danvers, USA)
Rabbit anti-human Vav-1 (C-14)	1:1000 (WB), 2µg/500µg protein (IP)	Santa Cruz (Heidelberg)
Rabbit anti-human WASP (H250)	1:1000 (WB), 2µg/500µg protein (IP)	Santa Cruz (Heidelberg)
Rat anti-human cytohesin-1 (clone 7H2)	1:20 (WB)	E. Kremmer (München)
Mouse anti-human CD3 (clone Okt3)	2µg/ml (stimulation)	LGC Promochem, ATCC
Mouse anti-human CD28	1µg/ml (stimulation)	BD Biosciences PharMingen (Heidelberg)
Mouse anti-human CD40	1:200 (FACS)	BD Biosciences PharMingen (Heidelberg)
Mouse anti-human CD80	1:200 (FACS)	BD Biosciences PharMingen (Heidelberg)
Mouse anti-human CD86	1:200 (FACS)	BD Biosciences PharMingen (Heidelberg)
Mouse anti-human Phosphotyrosin (clone 4G10)	1:10000 (WB)	Upstate (Lake Placid, USA)
Mouse anti-human SLP-76	1:250 (WB)	BD Biosciences PharMingen (San Jose, USA)
Mouse anti-human WASP (clone 67B4)	1:1000 (WB) 2µg/500µg protein (IP)	gift from J. Wehland
Normal rabbit IgG	2µg/500µg protein (IP)	Santa Cruz (Heidelberg)

The following secondary antibodies were used for flow cytometry analysis of human cells and for western blot analysis:

Secondary Antibody	Dilution	Supplier
HRP-Donkey-anti-Rat-IgG	1:5000 (WB)	Dianova (Hamburg)
HRP-Goat-anti-Rabbit-IgG	1:5000 (WB)	Rockland (Gilbertsville, USA)
HRP-Goat-anti-Mouse-IgG	1:5000 (WB)	Dianova (Hamburg)
FITC-Sheep-anti-Mouse	1:200 (FACS)	Dianova (Hamburg)

2.1.8 Oligonucleotides and Plasmids

siRNAs

RNAi was performed using oligonucleotides against the following target sequences:

Cytohesin-1 (human) :

5'-AATGACCTCACTCACACTTTCTT-3'

Vav-1 (human):

5'-TGC CAT CAG CAT TAA ATA TAA -3' and 5'-CAG GTG GAG TCA GCC AGC AAA-3'

Vav-2 (human):

5'-CCCGTTATATATCTGTGAATA-3' and 5'-ATCACTGGGTATGCTATT GTA-3'

Vav-3 (human):

5'-CACGACTTTCTCGAACACCTA-3'

Renilla luciferase (used as control siRNA): 5-AAAAACATGCAGAAAATGCTGTT-3’

All siRNAs were purchased from Qiagen (Hilden) or Dharmacon (Chicago, USA), respectively.

DNA oligonucleotides

Cyh1 human Mlu for: 5’-GCGGGGACGCGTGCCACCATGGAGGAGGACGACAGC-3‘

Cyh1 human Not rev: 5’- GGCGGGGCGGCCGCTCAGTGTCGCTTCGTGGAGGAG-3‘

NF-κB sense: 5’-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3‘

NF-κB anti-sense: 5’-CGGAAAGTCCCCAGCGGAAAGTCCCTGAT-3‘

Vav-1 human Mlu for: 5’-GCGGGGACGCGTGCCACCATGGAGCTGTG-3‘

Vav-1 human Not rev: 5’- GGCGGGGCGGCCGCTCAGCAGTATTCAGAA-3‘

Vav-1 L213A for: 5’-ACTGACACGGCGGGCTCCATCCAGCAG-3‘

Vav-1 L213A rev: 5’-GATGGAGCCCGCCGTGTCAGTGTACTT-3‘

Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg).

Plasmids

Over-expression constructs were cloned into the pRK5 vector and commonly bear an amino-terminal flag-tag (see below). They contain the wild-type human cytohesin-1, human cytohesin-1 E157K, wild-type human Vav-1, human Vav-1 L213A, wild-type human RhoA or human RhoA T19N cDNA sequences, respectively. Expression of these flag-tagged fusion protein can easily be monitored, using a tag-specific antibody. The peptide sequence of the flag-tag is as follows: N-DYKDDDDK-C.

The N1:eGFP vector (Promega) was used as marker for transfection in static adhesion assays with transfected human DC and in reporter gene assays with Jurkat T-cells.

2.1.9 BACTERIA STRAINS

Strain	**Genotype**
MC1061	araD139,Δ(ara-leu)7696, galE15, galK16, Δ(lac)$_{\chi 74}$, rpsL(Str$^{\gamma}$), hsdRR2 ($m_{\kappa}^{-}m_{\kappa}^{-}$), mcrA, mcrB
DH5α	endA1, hsdR17($r_{\kappa}^{-}m_{\kappa}^{+}$), supE44,thi1, recA1, gyrA, (Nal$^{\gamma}$), relA1

2.1.10 PRIMARY CELLS

Human dendritic cells were generated from monocytes, isolated from Buffy coats of healthy blood donors. The Institute of Experimental Hematology and Transfusion Medicine of the University of Bonn kindly provided the Buffy coats.

Murine bone marrow derived dendritic cells (BM-DC) were prepared from bone marrow of six week old C57BL/6 WT and Vav-1 knock-out mice.

Murine embryonic fibroblasts (MEFs), isolated from WT and Vav-1/2/3 triple knock-out mice, were kindly provided by W. Swat (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis).

Isolation of primary cells was approved by the local Ethics Committee.

2.1.11 CELL LINES

Cell type	Description
Ag-cells	cell line stably transfected to express GM-CSF
Jurkat E6	human T-cell-line from patients with acute leukemia
TAg Jurkats	stable transfectant line expressing SV40 large T-antigen

2.1.12 MICE

WT C57BL/6 — inbred mouse strain expressing the MHC class I haplotype $H2K^b$

purchased from Charles river laboratories

Vav-1 -/- — deficient of Vav-1 protein expression (C57BL/6 background)

provided by W. Ellmeier (Institute of Immunology, Medical University of Vienna, Austria), in accordance with J.M Penninger (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria), who generated the Vav-1 deficient mice (Fischer, … and Penninger, 1998)

mice were bred in the central animal facility "Haus für Experimentell Therapie" (HET) at the University Hospital Bonn

Animal care and experiments were done in compliance with institutional guidelines and the German law for Welfare of Laboratory Animals. For all experiments mice between 6-10 weeks of age were used.

2.2 METHODS

2.2.1 MOLECULAR CLONING

2.2.1.1 PCR

The polymerase chain reaction (PCR) is a widely used technique in molecular biology. It is used to amplify a desired fragment of DNA by enzymatic replication. As PCR progresses, the generated DNA is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. A typical PCR reaction mix contained the following compounds:

PCR Mix for 50µl Total Reaction Volume:

DNA Template	200ng
10x Buffer	5µl
2.5mM dNTP Mix	5µl
100pmol Primer Forward	1µl
100pmol Primer Reverse	1µl
Taq Polymerase	0.75µl
ad 50µl H_2O	

2.2.1.2 DNA PRECIPITATION

In order to purify DNA, the PCR reaction mix was precipitated with 3M sodium acetate (pH 4.8), added 1/10 of the total volume, and 100% ethanol up to 2.5x of the PCR reaction mix volume. The mix was then centrifuged for 10min at 4°C in a microcentrifuge with 14000 rpm. The pellet was washed carefully with 70% ethanol and dried. Finally, the DNA was resolved in a proper amount of A. bidest.

2.2.1.3 ANALYSIS OF DNA BY RESTRICTION DIGEST

A restriction digest was performed after DNA precipitation, in order to check the obtained products. 2µg of plasmid DNA were digested with the corresponding enzymes, according to the manufacturers protocol. In general, digestion time was 1h at 37°C.

2.2.1.4 PURIFICATION OF DNA FRAGMENTS

In order to purify DNA fragments from enzymes and remaining PCR reactants, DNA was pipetted on a low melting agarose gel, where separation was performed by electrophoresis. After adequate separation/run time, the gel was documented via UV analysis and the DNA fragment was cut out the gel. The small gel block was then melted at 65°C and used for further working procedures.

2.2.1.5 Removal of 5'-phosphate ends in plasmid Vector

Unspecific self-ligation of digested DNA was avoided by removing the 5'-phosphate ends of the DNA. Therefore, 1U of shrimp alkaline phosphatase was given to the plasmid vector and incubated for 10min at 37°C. The enzyme is finally inactivated by heating the sample up to 70°C for 15min.

2.2.1.6 Ligation and Transformation

The ligation is one of the crucial steps in molecular cloning, where the complementary restriction sites of the vector and the PCR product (insert) were fused to each other. This reaction is catalyzed by the T4-Ligase, which covalently fuses the 3'hydroxyl groups of the vector and the 5'phosphate groups of the PCR product. Normally, a ratio of 1:3 (vector to insert) was used for this reaction. Due to this ratio, equimolar amounts were used in the gel separation and a frequently used reaction mix was:

1µl digested vector

3µl digested PCR product

2µl 10x Ligation buffer

1µl T4-Ligase

ad 20µl A. bidest

Reaction took place at room temperature for at least 4h (or overnight at 16°C).

2.2.1.7 TRANSFORMATION OF CHEMO-COMPETENT E. COLI CELLS

For transformation of competent *E. coli* with a new ligated construct, chemo-competent cells were thawed on ice and 80µl of cell suspension were mixed with 0.5 µg of plasmid DNA or 5µl of ligation reaction. After incubation for 5min on ice, cells were subjected to a heat shock for 5min at 37°C, followed by an incubation for 1min on ice. Subsequently, cells were diluted in 1ml LB medium and incubated at 37°C for 30min. Cells were plated in agar plates, containing the specific antibiotic corresponding the one inserted in the plasmid. The plates were incubated at 37°C overnight and single colonies were picked for further cultivation in 5ml LB-medium with the selective antibiotic.

2.2.1.8 ISOLATION OF PLASMID DNA

Mini-preparation

The Mini preparation is a method for rapid isolation and verification of the transformed bacteria with the DNA construct. 2ml of the overnight culture in LB-medium containing the corresponding selective antibiotic were pelleted at 20000xg and resuspended in 250µl solution I. Then, 250µl of solution II were added in order to lyse the bacteria. A precipitation of the chromosomal DNA occurs when adding 350µl solution III to the sample. Afterwards, the sample was centrifuged at 20000xg for 15 min in order to separate chromosomal DNA from RNA and proteins. Approximately 700µl supernatant – which contained the plasmid DNA- were mixed with phenol and after a centrifugation at 20000xg for 5min remaining proteins were separated from the plasmid DNA. The DNA precipitates when adding 0.8% isopropanol and subsequently pelleted with a further centrifugation step (15min, 208000xg at 4°C). The pellet was washed with 70% ethanol, dried and resuspended in 50µl A. bidest. The still remaining RNA was hydrolyzed when adding 10µg RNase A to the sample.

Solution I	10mM EDTA (pH8.0)
Solution II	2M NaOH 1% (w/v) SDS
Solution III	2.5M KOAc 2.5M HOAc (pH4.7)

Maxi-preparation

In the Maxi preparation much larger volumes of bacterial suspension were grown but essentially, this is a scaled-up Mini prep, followed by additional purification. This results in relatively large amounts (several micrograms) of very pure plasmid DNA. 1l of overnight bacterial culture was incubated at 37°C and pelleted at 5000xg. This pellet was then resuspended in 40ml solution I. Cell lysis was alkaline mediated by 80ml solution II and finally lysis was neutralized with 40ml of solution III. The following centrifugation (5000xg for 10min) separated the DNA from the remaining cell material. The supernatant was filtrated, using gauze and the DNA was precipitated adding 250ml isopropanol and centrifuging for 10min at 5000xg and 4°C. The pellet was then dried and solved in 4ml solution I containing 5.5g CsCl and 4.4g Ethidium bromide (EtBr). The solution was then centrifuged for 5min and 12300xg and the supernatant was then given into an ultracentrifugation tube. A gradient centrifugation took place (Rotor type: NVT90) at 50900xg for 4h and a band within the CsCl was clearly visible. This band was then extracted with a syringe. EtBr was removed by mixing with n-butyl alcohol – saturated with 1M NaCl. The DNA was finally precipitated with aquivalent volume of a 1M ammonium acetate solution and 2x the volume of the sample solution with absolute ethanol. A final centrifugation (6700xg at 4°C) helped to obtain a pellet which was then washed with 70% ice cold ethanol and dried overnight. The pellet was then resolved in approx. 500-1000µl A. bidest (depending on the size of the pellet).

2.2.1.9 QUANTIFICATION OF PLASMID DNA

The concentration of DNA solutions was determined, by measuring the absorbance at 260nm, using the Eppendorf Photometer.

2.2.2 CELL CULTURE

2.2.2.1 CELL COUNTING

10µl cell suspension were applied to a Neubauer counting chamber. Determination of total cell number was performed by counting four large squares.

The total cell number was calculated by the formula:

*counted cell number/4*dilution factor*10^4= cell number/ml*

To determine the number of living cells, the cell suspension was diluted 1:1 with trypan blue solution and incubated 3min at RT. Viable cells (= cells that were not stained by trypan blue) were afterwards counted.

2.2.2.2 CELL CULTIVATION

Jurkat T-cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS and 10µg/ml gentamycin at 37°C in an incubator, containing 5% CO_2 and were splitted three times per week to a titer of 2x10^5 cells per ml.

Murine embryonic fibroblast (MEFs) were cultured in DMEM high glucose, containing 10% heat-inactivated FCS, 100mM non essential amino acids, 100mM sodium pyruvat and 2mM L-Glutamine at 37°C in an incubator, containing 5% CO^2. Confluent MEFs were splitted in a 1:3 ratio.

Ag-cells were cultured in IMDM supplemented with 8% heat-inactivated FCS, 1% penicillin/streptomycin, 2mM L-Glutamine and 1mg/ml G418. Ag-cells are semi-adherent cells; non-adherent cells were discarded when splitting the cells. Cells were usually splitted in a 1:5 ratio and collected with 2mM EDTS/PBS.

2.2.2.3 GENERATION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS (MO-DC)

Mo-DC were prepared from standard buffy coat preparations of healthy blood donors by Ficoll density gradient centrifugation. Therefore, the buffy coat was diluted with pre-warmed 2mM EDTA/PBS in a 1:1 ratio and 30ml of diluted blood were then stratified on 15ml Ficoll in a 50ml tube. Cells were then centrifuged at 800xg for 30min at RT in a swinging-bucket rotor without brake. After centrifugation a ring of PBMCs (peripheral blood mononuclear cells) got visible in the interphase between Ficoll and serum. The PBMC fraction, containing monocytes and lymphocytes, was carefully transferred into a new 50ml tube by using a 10ml pipette. The tube was filled up with 2mM EDTA/PBS and centrifuged at 640xg for 7min at RT. The cells were washed until the pellet became white, decreasing the centrifugation speed successively from 640xg to 200xg, in order to get rid of erythrocytes and platelets. The pellet was then resuspended in 5ml VLE-RPMI supplemented with 10% heat-inactivated FCS and 1% Penicillin/Streptomycin for cell counting. A concentration of $5x10^6$ cells per ml was adjusted with supplemented medium. Monocytes were subsequently isolated from the PBMC fraction by adhesion to plastic surfaces. Therefore, isolated PBMCs were seeded in 6-well plates (5ml cell suspension/6-well) and allowed to adhere for 1-2h at 37°C in an incubator, containing 5% CO_2. Afterwards, the supernatant, containing the non-adherent lymphocytes

(peripheral blood lymphocytes, PBL) was taken off. PBLs were usually used immediately for functional assays. The adherent monocytes were washed a least four times with pre-warmed PBS and cultured in VLE-RPMI medium supplemented with 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, 20ng/ml IL-4 and 20ng/ml GM-CSF for 5 to 7 days, to allow differentiation into immature mo-DC. Two days after isolation, medium was half-renewed and fresh cytokines were added (end concentration: 10ng/ml IL-4 and 10ng/ml GM-CSF).

For maturation, mo-DC were stimulated by addition of 1µg/ml LPS and 50ng/ml TNFalpha for 48h. Mo-DC were alternativlely stimulated to mature by adding 100ng/ml TNFalpha or 1µg/ml LPS alone.

2.2.2.4 GENERATION OF MURINE BONE MARROW-DERIVED DENDRITIC CELLS

BM-DC were prepared from bone marrow of six week old C57BL/6 WT or Vav-1-/- mice. Bone marrow was isolated from hind legs of mice. Legs were disinfect with ethanol, before fur and skin were removed. Legs were stored in PBS and muscle tissue was removed. Bone marrow was then flushed out with PBS using a syringe. Total bone marrow was filtered with 40µm pore nylon cell strainers and plated in 10cm non-treated petri-dishes at $5x10^6$ cells in 10ml IMDM supplemented with 10% heat-inactivated FCS, 2mM L-Glutamine, 100 u/ml Penicillin, 0.1mg/ml Streptomycin and 30% GM-CSF (supernatant derived from the murine Ag-cell line stably transfected to express GM-CSF; see below). The culture medium was half-renewed every three days. At day 8-10 of culture, BM-DC were stimulated to mature by adding 100ng/ml TNFalpha for 48h. BM-DC were alternatively stimulated with 100µg/ml zymosan or with 100µg/ml curdlan for 24h to induce dectin-1 mediated maturation.

Production of GM-CSF conditioned medium

For the production of GM-CSF conditioned medium, Ag-cells were harvested and washed three times with PBS to get rid of G418. Afterwards cells were taken up in culture medium (see above) without G418 and seeded in 30cm cell culture dishes ($1.5x10^6$ in 30ml medium cell per culture dish). Cells were incubated for 4 days (or until medium became slightly orange), before collecting the supernatant. The supernatant was centrifuged for 15min at 2000rpm and was then filtered through a sterile 0.2µM pore-size filter. The GM-CSF conditioned medium was stored a -20°C until use.

2.2.2.5 Isolation of murine splenic $CD4^+/CD8^+$ T-cells

Murine splenic $CD4^+/CD8^+$ T-cells were isolated by magnetic activated cell sorting (MACS), using the Pan T-cell isolation kit. The Pan T-cell isolation kit is an indirect magnetic labeling system for the isolation of "untouched" T-cells from single-cell suspensions of mouse spleen or lymph node cells. Highly pure "untouched" T-cells were isolated by depletion of non T-cells. Non T-cells e.g. B-cells, NK cells, dendritic cells, macrophages, granulocytes and erythroid cells were indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119, as well as anti-Biotin MicroBeads. The magnetically labeled non T-cells were depleted by retaining them on a MACS column in the magnetic field of a MACS separator, while the unlabeled T-cells passed through the column. This negative isolation method was used to avoid possible interfering effects, which are due to antibody-mediated cross-linking of surface molecules on the T-cell.

Spleens, taken from 6 week old C57BL/6 WT or Vav-1 -/- mice, were passed through a metal filter, to remove cell clumps and to obtain a single cell suspension. Cells were taken up in suitable amount of MACS-buffer for cell counting. After determination of the cell number, cells were spun down by centrifugation at 300xg for 10min. The cell pellet was resuspended in 40µl of MACS-buffer per 10^7 total cells and 10µl of biotin-antibody

cocktail per 10^7 total cells were added. Cells were incubated for 10min at 4°C. Afterwards 30µl MACS-buffer and 20µl anti-biotin MicroBeads per 10^7 total cells were added and cells were incubated for additional 15min at 4°C. Cells were washed with MACS-buffer by adding 10−20x labeling volume and centrifuged at 300xg for 10min. The cell pellet was resuspended in 500µl MACS-buffer per 10^8 total cells. A LS column was placed in the magnetic field of a suitable MACS separator and rinsed with 3ml MACS-buffer. The cell suspension (1-10ml) was applied onto the column. The effluent fraction with unlabeled cells, representing the enriched T-cell fraction, was collected and the column was washed four times with 3ml MACS-buffer. The entire effluent was collected in the same tube. T-cells were taken up in RPMI medium supplemented with 10% heat-inactivated FCS and used for functional assays 3-4h after isolation.

Note: Retained cells can be eluted outside of the of the magnetic field, as magnetically labeled (non T-cell) fraction.

MACS buffer
PBS
+2mM EDTA
+0.5% BSA

2.2.3 CELL TRANSFECTION

2.2.3.1 TRANSFECTION OF HUMAN AND MURINE DENDRITIC CELLS WITH SIRNA

Immature human or murine dendritic cells were harvested in 50ml tubes and centrifuged at 200xg for 7min. The pellet was washed with 50ml pre-warmed PBS. After another centrifugation step, cells were resuspended in OptiMem ($4x10^6$ cells/electroporation sample/100µl OptiMem)

An appropriate amount of siRNA (see below) was transferred to a 4mm cuvette and incubated for 3min with $4x10^6$ immature DC in 100µl OptiMem before electroporation in a Gene Pulser X cell, equipped with a CE module. Pulse conditions were square wave, 1000V, 2 pulses and 0.5ms pulse length. Electroporated human mo-DC were quickly placed in 5ml VLE-RPMI 1640 medium, containing 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, 5ng/ml IL-4 and 5ng/ml GM-CSF. Electroporated murine BM-DC were placed in 10ml IMDM supplemented with 10% heat-inactivated FCS, 2mM L-Glutamine, 100 u/ml Penicillin, 0.1mg/ml Streptomycin and 30% GM-CSF supernatant.

48h after electroporation immature DC were stimulated to mature (see above). 96h after electroporation RNAi efficiency was tested, using a standard western blot protocol (see below) and silenced mature DC were used for functional assays.

Used amounts of siRNA:

Vav-1 siRNA	Vav-2 siRNA	Vav-3 siRNA	Cytohesin-1 siRNA	Renilla siRNA
10µg Vav-1-1 10µg Vav-1-2	10µg Vav-2-1 10µg Vav-2-2	20µg	5µg	5µg or 20µg

Note: RNAse-free filter tips were used, when working with siRNA.

2.2.3.2 TRANSFECTION OF MO-DC WITH PLASMID DNA

Immature human mo-DC were transfected with plasmid DNA, using the newly developed MicroPorator MP-100 system, which uses a pipette gold-tip as electroporation space instead of the cuvette. For microporation of mo-DC the MP solution Kit 100µl was used. The cells were transfected according to manufacturing instructions with following modifications. $5x10^5$ immature mo-DC were transfected with 10µg plasmid (prK5:flag:Cyhtohesin1 WT, prK5:flag:Cyhtohesin1 E157K, prK5:flag:RhoA WT or prK5:flag:RhoA T19N). All samples were co-transfected with 10µg N1:eGFP as a marker for transfection. Pulse conditions were 1500V, 30ms, 1 pulse. Electroporated cells were quickly placed in 5ml VLE-RPMI 1640 medium, containing 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, 5ng/ml IL-4 and 5ng/ml GM-CSF. 2h after microporation respective cells were stimulated with 1µg/ml LPS and 50ng/ml TNFalpha to induce maturation. The cells were then cultured overnight at 37°C and were used for functional assays 24h after electroporation. The transfection efficiency was determined, by counting GFP-positive and GFP-negative mo-DC in five fields of view at 10x magnification, using the TE Eclipse fluorescent microscope. The obtained transfection efficiency was usually 1-5%. Due to this low transfection efficiency it was not possible to detect the over-expressed flag-tagged fusion proteins by western blot analysis.

2.2.3.3 TRANSFECTION OF JURKAT T-CELLS WITH SIRNA

Jurkat T-cells were splitted one day before transfection, to achieve log phase growth at time of transfection. Optimal cell titer at the day of transfection: $5x10^5$ cells/ml. Appropriate amount of siRNA (see below) was transferred to a 4mm cuvette and overlaid with $1x10^7$ Jurkat T-cells in 500µl OptiMem. Cells were subsequently electroporated in a Gene Pulser X Cell Electroporator which was equipped with a CE module. Pulse conditions were square wave, 300V, 1 pulse and 20ms pulse length.

Electroporated T-cells were quickly placed in 10ml RPMI 1640 medium containing 10% heat-inactivated FCS and 10µg/ml gentamycin. 48h after electroporation RNAi efficiency was tested using a standard western blot protocol (see below) and silenced T-cells were used for functional assays.

Used amounts of siRNA:

Vav-1 siRNA	Cytohesin-1 siRNA	Renilla siRNA
5µg Vav-1-1 5µg Vav-1-2	5µg	5µg or 10µg

Note: RNAse-free filter tips were used, when working with siRNA.

2.2.3.4 TRANSFECTION OF JURKAT T-CELLS WITH PLASMID DNA

Jurkat T-cells were splitted one day before transfection, to achieve log phase growth at time of transfection. The optimal cell density at the day of transfection is $5x10^5$ cells/ml. Jurkat T-cells were harvested and washed with 50ml PBS. 10-30µg plasmid DNA was transferred to a 4mm cuvette and overlaid with $1x10^7$ Jurkat T-cells in 500µl RPMI/50% FCS. Cells were subsequently electroporated in a Gene Pulser X cell electroporator, using the following settings: exponential protocol, 240V,1500µF.

Cells were incubated overnight (at least 16h) in 10ml culture medium at 37°C in an incubator containing 5% CO_2, before they were used for functional assays. Over-expression of the respective protein was proved using a standard western blot protocol (see below).

2.2.3.5 TRANSFECTION OF PBLS WITH SIRNA

1×10^7 freshly prepared PBLs were suspended in 100µl human T-cell Nucleofector solution, mixed with 10µg siRNA and transferred into an Amaxa certified cuvette. After inserting the cuvette into the cuvette holder of the Nucleofector, cells were electroporated using program U-14. Electroporated PBL were quickly placed in 5ml VLE-RPMI 1640 medium, containing 10% FCS and 1% penicillin/streptomycin and seeded in a 6-well. 48h after electroporation RNAi efficiency was analyzed using a standard western blot protocol (see below) and silenced PBLs were used for functional assays.

Note: RNAse-free filter tips were used, when working with siRNA.

2.2.4 PROTEIN BIOCHEMISTRY

2.2.4.1 PREPARATION OF CELL LYSATES

For the preparation of cell lysates, $5x10^5$ – $2x10^6$ cells were harvested in 1.5ml reaction-tubes and washed with cold PBS. The cell pellet was subsequently resuspended in 60-100µl Igepal-lysisbuffer with freshly added protease-inhibitors (see below) and incubated for 20min on ice. Afterwards, cellular debris was pelleted by centrifugation at 11300xg at 4°C for 10min. The supernatant (=cell lysate) was transferred to a new tube and the pellet was discarded. The protein amount was quantified by a standard BCA-assay (see below).

For experiments in which p38, JNK or ERK phosphorylation levels were analyzed, unstimulated or stimulated cells were lysed in Triton-lysisbuffer, containing phosphatase-inhibitors and freshly added protease-inhibitors (see below). To this end,

the cell pellet was resuspended in 60-100µl Triton-lysisbuffer and immediately frozen in liquid nitrogen. Cell lysate was stored at -80°C until use. When cell lysates was needed, it was thawed on ice, before cellular debris was pelleted by centrifugation at 11300xg at 4°C for 10min. The supernatant was transferred to a new tube and protein content was determined by a standard BCA-assay (see below).

Igepal-lysispuffer
10mM Hepes, pH 7.5
10mM KCl
10mM MgCl
150mM NaCl
1%Igepal

Protease-Inhibitor	**dilution**
Antipain (2mg/ml)	1:1000
Aprotinin (20mg/ml)	1:2000
Benzamidine (1M)	1:1000
Leupeptin (20mg/ml)	1:2000
PMSF (1M)	1:1000

Triton-lysispuffer
10mM Tris HCL, pH 7.5
1mM EGTA
1mM EDTA
10mM Glycerolphosphate
50mM Sodium Fluoride
5mM Sodium Pyrophosphate
1mM Sodium Vanadate
0.27M Sucrose
1% Triton X-100

2.2.4.2 IMMUNOPRECIPITATION

Immunoprecipitation (IP) is a technique for precipitating a protein out of solution, using an antibody that specifically binds to that particular protein. The antibody/protein complex will then be pulled out of the sample using protein A/G-coupled sepharose beads and this physically isolates the protein of interest from the rest of the sample. The sample can then be separated by SDS-PAGE for Western blot analysis.

500µl cell lysate (containing 500µg protein) were incubated with the recommended amount of primary antibody (see above) for 1h at 4°C under rotary agitation. Afterwards, 20µl of appropriate sepharose conjugate suspension (protein-A-sepharose or protein-G-sepharose) was added and the lysate/beads mixture was incubated at 4°C under rotary agitation for 1-2h. When the incubation time was over, tubes were centrifuged for 3min at 200xg and 4°C, the supernatant was removed and the beads were washed three to five times with 1ml ice-cold lysisbuffer. Finally, the supernatant was removed and the sample was boiled with 3x loading buffer at 95°C for 5min, to denature the protein and separate it from the sepharose beads. The samples were either stored at -20°C or directly run on a SDS-PAGE.

2.2.4.3 DETERMINATION OF THE PROTEIN CONCENTRATION BY THE BCA ASSAY

The protein amount of prepared cell lysates was quantified, using the BCA-assay (Pierce). The BCA assay (bicinchoninic acid assay) is a biochemical assay for determining the total level of protein in a solution, similar to Lowry protein assay, Bradford protein assay or biuret reagent. It combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid. The BCA assay primarily relies on two reactions. Firstly, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+} in a temperature dependent reaction. The amount of Cu^{2+}

reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with one Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562nm.

First, a serial dilution for standard curve was performed using bovine serum albumin (BSA):

standard	**dilution**	**final BSA concentration (µg/ml)**
A	5OOµl stock solution (2mg/ml BSA)	2000
B	250µl standard A + 250µl lysisbuffer	1000
C	250µl standard B + 250µl lysisbuffer	500
D	250µl standard C + 250µl lysisbuffer	250
E	250µl standard D + 250µl lysisbuffer	125
F	lysisbuffer	0

3µl of each standard (A-F) and each sample were added to a 96-well microplate. Samples were added in replicates of two. The working reagent was prepared using a ratio of 1:50 (reagent A:reagent B). A green solution formed. 200µl of working reagent was added to each well using a multichannel pipette. This was done rapidly, because the reaction is very time sensitive. The microplate was placed in an incubator for 10min at 65°C or for 30min at 37°C. The protein in each well appeared purple. Absorbance of each well was read at 562nm using an ELISA microplate reader. Standard F was used as the blank on the reader. Amount of protein in each well was calculated afterwards.

2.2.4.4 SDS-PAGE

SDS-PAGE stands for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and is a technique to separate proteins according to their size. SDS is a detergent which denatures proteins and applies a negative charge to every protein. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in charge and folding. Differences in folding patterns e.g. would cause some proteins to better fit through the gel matrix than others. Adding SDS solves this problem, as it denatures the proteins so that they may be separated strictly by length (number of amino acids). SDS binds to the protein in a constant ratio (1.4g SDS per 1g protein), giving a uniform mass:charge ratio to all proteins, so that the distance of migration through the gel is directly related to only the size of the protein.

In this study discontinuous SDS polyacrylamide gel electrophoresis was employed, in which gels are constructed of two different acrylamide gels, one on top of the other. The upper stacking gel contained 5% acrylamide (a very loose gel) and the lower resolving gel (often called running gel), contained a higher acrylamide concentration. The large pore stacking gel concentrated the SDS-coated proteins, whereas the small pore resolving gel afterwards effectively separated proteins.

Polyacrylamide gels were prepared according to standard protocol. The prepared gels were wrapped in wet tissue paper to prevent drying out and stored at 4°C until use. Equal amounts of protein (usually 10-20µg) were mixed with 3x loading buffer and boiled for 5min at 99°C. The gel was clamped in the electrophoresis chamber and buffer chambers were filled with gel running buffer. The boiled sample was pipetted into the gel, including one lane with 15µl of molecular weight protein standard. The gel was run at 80V (stacking gel) and at 120V (resolving gel), until the blue dye front reaches the bottom. The gel was afterwards removed from the power supply and proteins were visualized using Coomassie Brilliant Blue stain or blotted on a nitrocellulose membrane (see below).

1x stacking gel solution (percentage of gel:5%)	
A. bidest	2.05ml
1 M Tris HCl, pH 6.8	375µl
10% SDS	30µl
10% APS	30µl
TEMED	3µl
30% Acrylamid/ Bisacrylamid-Mix	0.5ml

1x resolving gel solution (percentage of gel:10%)	
A. bidest	2ml
1 M Tris/HCl (pH 8.8)	1.25ml
10% SDS	50µl
10% APS	50µl
TEMED	2µl
30% Acrylamid/ Bisacrylamid-Mix	1.515ml

3x loading buffer
150mM Tris HCl, pH 6.8
6% SDS (w/v)
30% Glycerin (v/v)
300mM DTT
0.3% Bromphenolblue (w/v)

1x Laemmli running buffer
25mM Tris
192mM Glycin
0.1% SDS
ad 1l A. bidest

2.2.4.5 WESTERN BLOT ANALYSIS

Sponges, whatman papers and the nitrocellulose membrane were pre-wet in 1x Transfer buffer. Placed on the black site of the western blot cassette were: a sponge, three whatman filter papers, the gel, the nitrocellulose membrane, another three whatman filter papers and a second sponge. The cassette was then closed and placed in the western transfer chamber, filled with cold 1x Transfer buffer. The gel was then transferred at 80V for 2h at 4°C. After transferring proteins onto the nitrocellulose membrane, membrane was blocked overnight with 5% milk powder in TBST at 4°C under gently shaking. Protein was detected next day, by a specific primary antibody (see above) in 10ml TBST, containing 5% BSA. The membrane was incubated for 1h with primary antibody solution at RT (with shaking), and then washed 5 times for 5min with TBST. The primary antibody was then detected with a secondary antibody (see above), conjugated to horseradish peroxidase (HRP). Membrane was washed again 5 times for 5min with TBST, before incubating it for 1min with ECL solution (1ml of solution A and 1ml of solution B). Chemiluminescence was detected by autoradiography. To demonstrate loading of equal amounts of proteins, membranes were "stripped" of antibody, using a "stripping" buffer (20min, 55°C) and were stained for a ubiquitous protein, e.g. actin or GAPDH or for other proteins of interest.

1x Transfer buffer
192mM Glycin
25mM Tris
20% Methanol
0.002% SDS

TBS buffer
50mM Tris, pH 7.5
140mM NaCl

TBST buffer
0.05% Tween-20 (v/v) in TBS buffer

„Stripping“ buffer
2% SDS (w/v)
62.5mM Tris/HCl, pH 6.7
100mM 2-Mercaptoethanol

2.2.5 CELL STIMULATION AND FUNCTIONAL ASSAYS

2.2.5.1 TREATMENT OF CELLS WITH INHIBITORS

Tyrosine kinase SYK was blocked by piceatannol

To inhibit Syk kinase activity during dendritic cell maturation, immature BM-DC were pre-incubated with 25µM piceatannol for 2h. DMSO was used as a control. Afterwards, respective cells were stimulated with 100µg/ml zymosan or 100µg/ml curdlan to induce maturation. 24h later, maturation of the respective cells was assessed by flow-cytometry.

GEF activity of Vav proteins was blocked by 6-Thio-GTP

6-Thio-GTP is a metabolite of Azathioprine, a drug which is widely used in organ transplantation and in the therapy of auto-immune or chronic inflammatory diseases. 6-Thio-GTP binds with high affinity to the nucleotide-free (“empty”) Rac-1 protein and will subsequently get hydrolyzed to 6-Thio-GDP. It is thus capable of displacing its

physiological counterpart GTP in a concentration dependent fashion. Importantly, in the next round of the cycle, Vav proteins will not be able to catalyze the removal of 6-Thio-GDP. Although there are several other GEFs expressed in eukaryotic cells which bear exchange activity towards Rho/Rac GTPases, 6-Thio-GTP will specifically block Vav-catalyzed nucleotide replacement reactions. In consequence, this small molecular nucleotide analog is an indirect, but highly selective inhibitor of the Vav GEF function (Poppe et al., 2006). To inhibit the GEF activity of Vav protein during dendritic maturation, immature cells were pre-incubated with 10µM 6-Thio-GTP. Two days later, 10µM 6-Thio GTP was added once again together with 100ng/ml TNFalpha to induce maturation. Unmodified GTP was used as a control. 48h later, maturation of respective cells was assessed by flow-cytometry.

GEF activity of cytohesin proteins was blocked by two different inhibitors

The small molecule SecinH3 (sec7 inhibitor H3 or 20H3), is a sec7 inhibitor which has been recently identified in an aptamer displacement screen (Hafner et al., 2006). SecinH3 preferentially inhibits the GEF activity of cytohesins 1-3 and the *Drosophila* cytohesin-3 homolog *steppke*, whereas the GEF function of other sec-7 domain containing proteins (e.g. the yeast protein Gea2 or the mammalian ARF6 GEF EFA6) is only weakly affected by SecinH3 (Hafner et al., 2006). It has been demonstrated that SecinH3 potently inhibits cytohesin-catalyzed GDP/GTP exchange on ARF1 and ARF6, as well as cytohesin-3-dependent insulin signal transduction of *Drosophila*, mouse and human cells *in vitro* and *in vivo* (Hafner et al., 2006, Fuss et al., 2006).

Compound 16 is novel potent cytohesin family GEF inhibitor identified by chemo-informatic methods to improve target affinity and selectivity of SecinH3; the details will be published elsewhere (Bajorath et al., in preparation).

Both inhibitors were dissolved in DMSO. Jurkat T-cells were incubated with 25µM SecinH3 or 25µM compound 16 for 1h at 37°C. Equal amounts of DMSO were used as a control.

Inhibited cells were afterwards stimulated with anti-CD3 and anti-CD28 and analyzed for p38 phosphorlyation levels (see below).

2.2.5.2 CELL STIMULATION

Maturation of dendritic cells

see 2.2.2.3 and 2.2.2.4

Stimulation of dendritic cells for adhesion assays

see 2.2.5.5

Stimulation of dendritic cells for EMSA

To induce the activation of NF-κB, immature human dendritic cells were stimulated at 37°C for 1h or 2h with 100ng/ml human TNFalpha.

Stimulation of murine splenic T-cells for EMSA

Murine splenic T-cells were stimulated 3-4h after cell isolation with 100ng/ml murine TNFalpha for 5min or 10min at 37°C.

Stimulation of MEFs for EMSA

WT and Vav-1/2/3 triple knock-out MEFs were seeded in 6-well plates one day before stimulation. The next day, cells were stimulated with 100ng/ml murine TNFalpha for 5min, 10min or 15min at 37°C to induce the activation of NF-κB.

Stimulation of Jurkat T-cells

To induce the phosphorylation of p38, ERK or JNK, Jurkat T-cells were stimulated with 1µg/ml anti-CD28 and 2µg/ml anti-CD3 (OKT3) for 5min, 10min, 30min or 60min at 37°C. For AP-1 and IL-2 reporter gene assays, Jurkat T-cells were stimulated 16-24h

after transfection with 1µg/ml anti-CD28 and 2µg/ml anti-CD3 (OKT3) or with 50ng/ml PMA and 2µg/ml anti-CD3 for 6-7h at 37°C.

Stimulation of PBLs

To induce the phosphorylation of p38, ERK or JNK, PBLs were stimulated with 10µg/ml plate-bound anti-CD28 and 10µg/ml plate-bound anti-CD3 (OKT3) for 5min or 10min, at 37°C. To couple the antibodies to the plate, the bottom a 24-well plate was covered with 200µl antibody solution and incubated for 2-3h at RT or overnight at 4°C under gently shaking. After the incubation time, the plate was washed twice with PBS and the cells were then placed on the antibody-coated wells for 5min or 10min.

Note: In all experiments, cells were stimulated in 200-500µl RPMI, containing 0.5%FCS. 1-2ml cold PBS was added in order to terminate stimulation.

2.2.5.3 EMSA

The activation of endogenous NF-κB was measured with the help of the so-called electrophoretic mobility gel shift assay (EMSA). EMSA is a technique for studying gene regulation and determining protein/DNA interactions, which is based on the observation that protein/DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide gel electrophoresis. If NF-κB subunits are present in isolated nuclear extracts, their interaction with a DNA probe, containing the specific recognition sequence for NF-κB, shifts the band of the ^{32}p-labeled probe up.

Preparation of nuclear extracts

For the preparation of nuclear extracts unstimulated and TNFalpha stimulated cells (1-5x10^6) were washed twice with cold PBS. The cell pellet was afterwards incubated in 400µl of buffer A for 15min on ice. After addition of 25µl of a 10% Igepal solution (buffer B), cells were gently shaked for 1min at 4°C. The nuclei were then pelleted by

centrifugation at 11300xg at 4°C for 5min, resuspended in 50-70µl of high salt buffer C and incubated for 20min at 4°C under constant shaking. Incubation with the high salt buffer led to the lysis of nuclei: the nucleoplasm was extracted into the buffer while the nulear envelop stayed intact and retained the genomic DNA. After centrifugation at 4°C and 11300g for 5min, supernatants (=nuclear extracts) were collected and protein concentration was determined by a standard BCA-assay (see above). Extracts were stored at -80°C until use.

Buffer A
10mM HEPES, pH 7.9
10mM KCL
0.1mM EDTA
0.1mM EGTA
1mM DTT (freshly added)
1mM PMSF (freshly added)

Buffer B
10% Igepal

Buffer C
20mM HEPES, pH 7.9
0.4M NaCl
1mM EDTA
1mM EGTA
1mM DTT (freshly added)

Annealing and labeling of NF-κB oligonucleotides

The lyophilized NF-κB oligonucleotides were resuspended in water, adjusting a concentration of 100pM/µl. 20µg NF-κB sense and 20µg NF-κB antisense strand were then mixed in a 1.5ml eppendorf reaction tube. 10µl 10x Annealing buffer were added and the reaction volume was filled up to 100µl with A. bidest. The tube was incubated in a water bath at 95°C for 5min. The heat block was then turned off, leaving sample in, until water bath reached RT. Slow cooling to room temperature should take 45–60min. The annealed oligonucleotide was diluted with A. bidest to a concentration of 25ng/ml. It was either stored at -20° C or directly labeled.

Annealing buffer
20mM Tris/HCl, pH 6.7
10mM $MgCl_2$
50mM NaCl
1mM DTT

Annealed oligonucleotides were labeled, according to following protocol in a total volume of 50µl.

Annealing reaction:

1µl annealed NF-κB oligonucleotide (=25ng)

5µl 10x PNK reaction buffer

2µl T4-polynucleotide-kinase

12µl gamma-^{32}P-ATP = 120µCi = 4.44 MBq

30µl A. bidest

The tube was incubated for 30min at 37°C under gently shaking. After labeling, radioactive labeled oligonucleotides were purified using Quant 96 G-50 micro columns (GE Healthcare), to remove unincorporated labeled nucleotides from the DNA labeling reaction. The probe was afterwards counted by LSC (liquid scintillation counting) and then put in a radioactive storage box at 4°C until use.

EMSA

For the EMSA, nuclear extracts were incubated with the radioactive labeled oligonucleotide probe, containing the specific recognition sequence for NF-kB. The binding reaction occurs under specific salt/pH conditions in a Binding buffer. Poly-dIdC was added to prevent unspecific binding of proteins to the NF-kB oligonucleotide probe and by this to reduce background.

Binding Mix:

2µl poly dIdC (1µg/µl)

4µl 5x Binding buffer

10µg protein (nuclear extract)

ad. 20µl A.bidest

The binding mix was incubated for 20min at RT, before adding 2µl of the radioactive labeled NF-κB oligonucleotide probe. After 7min incubation time, 6x loading buffer was added, and the samples were separated on a non-denaturating gel at 200V, for 3-4h in a water-cooled electrophoresis chamber (Peqlab). Gel was subsequently dried in a gel dryer (80°C, 2h, under vacuum) and bands were detected by autoradiography, e.g. by exposing the dried gel 24-96h to an autoradiography film at -80°C.

Running buffer
0.25x TBE buffer

5x Binding buffer
25mM HEPES, pH 7.8
25mM $MgCl_2$
250mM KCL
1mM EDTA
50% Glycerol
25mM DTT

Non denaturating gel
40ml A. bidest
1.25ml 10x TBE buffer
7.5ml Acrylamide 40%
312.5µl APS 10%
187.5µl TEMED

Note: The dimensions of the gels were 20x20 cm. 1.5mm spacers and a comb with 15 wells were used.

2.2.5.4 T-CELL ACTIVATION ASSAYS

The secretion of IL-2 by activated T-cells is an important step in the initiation of an immune response. The IL-2 promoter contains binding sites for several transcription factors regulating IL-2 gene transcription. These transcription factors include NF-κB, NFAT, AP-1, the octamer binding protein (Oct) and the CD28 response element (CD28RE) (Serfling et al., 1995).

To monitor the activity of these transcription factors, minimal (m)CMV-driven luciferase-reporter constructs were used which encode the firefly luciferase reporter gene under the control of AP-1 response elements, NF-κB response elements or the full length IL-2 promotor.

Jurkat T-cells were transfected with 15µg luciferase-reporter constructs together with either 20µg expression plasmid or with 5-10µg siRNA. The cells were co-transfected with 5µg N1:eGFP, a plasmid which directs expression of the green fluorescent protein (GFP), as a marker for transfection.

16-24h after transfection T-cells were stimulated as described above (see 2.2.5.2) for 6-7h. T-cells were afterwards centrifuged and the pellet was lysed in 200µl 1x Reporter lysisbuffer. Lysates were stored at -80°C until use. Samples were analyzed, using the Luciferase Assay System (Promega), an extremely sensitive and rapid reagent for the quantitation of firefly luciferase. Lysates were thawed on ice and centrifuged at 11300xg at 4°C for 10min. 100µl of supernatant were added to a opaque 96-well multiwell plate. Samples were added in replicates of three. The plate was then placed into the luminometer, equipped with an injector. The injector added 100µl of Luciferase Assay Reagent per well. The well was read immediately, before injecting reagent in the next well. The photons produced (=relative light units, RLU) were collected for a period of 10sec.

The transfection efficiency was analyzed by flow cytometry, by measuring the number of GFP-positive cells. The total protein content of samples was determined by BCA assay. The measured relative light units were afterwards related to the protein content and to the transfection efficiency: RLU/µg protein/GFP-positive cell.

2.2.5.5 Static adhesion assay with GFP-transfected Mo-DC

Static cell adhesion assays on ICAM-1-Fc were carried out as described previously (Boehm et al., 2003) with following modifications. $2x10^5$ dendritic cells/well in HBSS were placed on ICAM-1-Fc coated 96-well plates and stimulated with 50ng/ml PMA for 60min at 37°C/5% CO_2 to adhere. After 1h unbound cells were washed off carefully with HBSS and the adherent GFP-positive cells which represent the transfected population were counted in five fields of view at 10x magnification using fluorescent microscopy

using the TE Eclipse microsope (Nikon). The number of GFP-positive cells in the unwashed sample (50-100 cells/field of view) was set to 100%.

2.2.5.6 STAINING CELLS FOR FLOW CYTOMETRIC ANALYSIS

$2x10^5$-$5x10^5$ dendritic were stained with saturating concentrations of primary antibodies (see above) for 30min in 1% BSA/PBS on ice. Cells were washed twice with cold in 1% BSA/PBS, before incubating them with the secondary fluorochrome labeled antibody for 30min in 1% BSA/PBS on ice. Afterwards, the cells were again washed twice with cold in 1% BSA/PBS. Measurements were conducted either with a Epics XL coulter equipped with a 488nm laser or with a FACS Canto II equipped with a 488nm laser and a 635nm laser. Data were analyzed using the Expo32 ADC software (Beckman coulter).

3. RESULTS

3.1 THE ROLE OF CYTOHESIN-1 IN THE REGULATION OF T-CELL RECEPTOR SIGNALING

3.1.1 SIRNA-MEDIATED SILENCING OF CYTOHESIN-1 PROTEIN EXPRESSION ABROGATES T-CELL RECEPTOR-INDUCED ACTIVATION OF THE TRANSCRIPTION FACTOR AP-1

The secretion of IL-2 by activated T-cells is an important step in the initiation of an immune response. The IL-2 promoter contains binding sites for several transcription factors regulating IL-2 gene transcription. These transcription factors include NF-κB, NFAT, AP-1, the octamer binding protein (Oct) and the CD28 response element (CD28RE) (Serfling et al., 1995).

Recent over-expression analysis indicated that cytohesin-1 is important for TCR-induced activation of the transcription factor AP-1 (Paul, PhD thesis 2007), a transcription factor complex consisting of homo- or heterodimers of Fos/Jun family proteins (Kaminska et al., 2000). To verify and corroborate this finding, TCR-stimulated activation of AP-1 was analyzed in cytohesin-1 knock-down T-cells. To monitor the activity of AP-1-dependent signal transduction, a minimal (m)CMV-driven AP-1-luciferase-reporter construct was used, which encodes the firefly luciferase reporter gene under the control of AP-1 response elements. RNAi in Jurkat T-cells was employed to specifically inhibit cytohesin-1 protein expression in these cells. For this assay, the T-cell line Jurkat E6 was transiently transfected with the AP-1-luciferase-reporter construct, along with either control siRNA or with cytohesin-1 siRNA. As shown in figure 13a, stimulation of the control cells with 50ng/ml PMA and 2µg/ml anti-CD3 (OKT3) results in a robust activation of AP-1 transcriptional activity, which is strongly inhibited when cytohesin-1 expression was decreased by RNAi (figure 13a), confirming and substantiating that cytohesin-1 expression is important for the activation of AP-1.

Knock-down efficiency of cytohesin-1 protein expression in these cells was confirmed by analyzing cell lysates by immunoblot. RNAi of cytohesin-1 dramatically reduces specific protein expression (figure 13b).

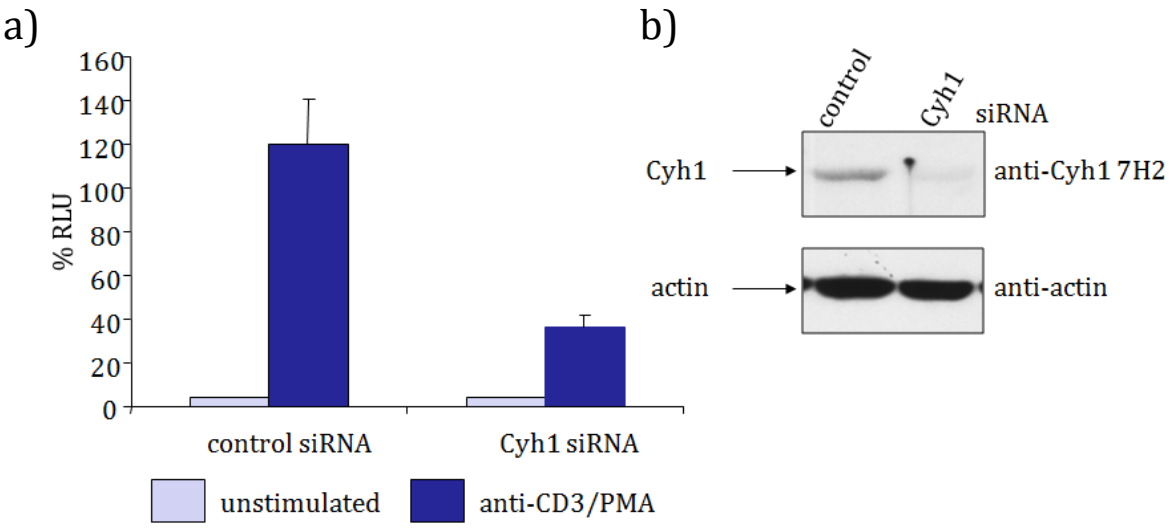

Figure 13: SiRNA-mediated silencing of cytohesin-1 protein expression abrogates T-cell receptor-stimulated activation of AP-1. Jurkat E6 T-cells were transfected with 10µg AP-1-luciferase-reporter construct, together with either 5µg control siRNA (targeting the renilla luciferase but not the firely luciferase encoded by the AP-1-luciferase-reporter construct) or with 5µg cytohesin-1 (Cyh1) siRNA. 48h after transfection, cells were stimulated with 50ng/ml PMA and 2µg/ml anti-CD3 (OKT3) for 6h. TCR-stimulation of control cells leads to robust activation of AP-1 transcriptional activity, which is strongly reduced following transfection with a cytohesin-1 specific siRNA (a). RLU= relative light units. Knock-down efficiency of cytohesin-1 protein expression was analyzed by immunoblot (b).

3.1.2 CYTOHESIN-1 KNOCK-DOWN RESULTS IN IMPAIRED TCR-INDUCED PHOSPHORYLATION OF P38 AND JNK, BUT NOT OF ERK1/2 MAP KINASES

AP-1 is a transcription factor complex consisting of homo- or heterodimers of members of the Fos or Jun families (Kaminska et al., 2000). The TCR-induced formation of the AP-1 complex, as well as the synthesis of Fos and Jun proteins, are controlled by at least two known pathways. One pathway is regulated by the extracellular-signal regulated kinases 1 and 2 (ERK1 and ERK2), whereas the second pathway involves the p38 mitogen-activated protein kinase (p38) and the c-Jun N-terminal kinase (JNK) (Whitmarsh et al., 1996). The data shown above suggest that cytohesin-1 is important for TCR-induced activation of the AP-1-complex, therefore it was investigated in which of these two pathways cytohesin-1 is involved and whether cytohesin-1 expression is required for the activation of one of these kinases.

First, it was confirmed that RNAi of Vav-1, a known signaling component of the pathway leading to activation of AP-1, reduces TCR-induced activation of the MAP kinase p38. Vav-1 is a guanine nucleotide exchange factor for the small GTPase Rac-1. It had been demonstrated that Vav-1-mediated Rac-1 activation leads to TCR-induced phosphorylation (= activation) of JNK and p38 and finally to activation of AP-1 (see introduction for details). As depicted in figure 14, siRNA-mediated knock-down of Vav-1 protein expression abrogates anti-CD3-/anti-CD28-induced phosphorylation of p38. Knock-down of Vav-1 expression in these cells was confirmed by analyzing cell lysates by immunoblot (figure 14).

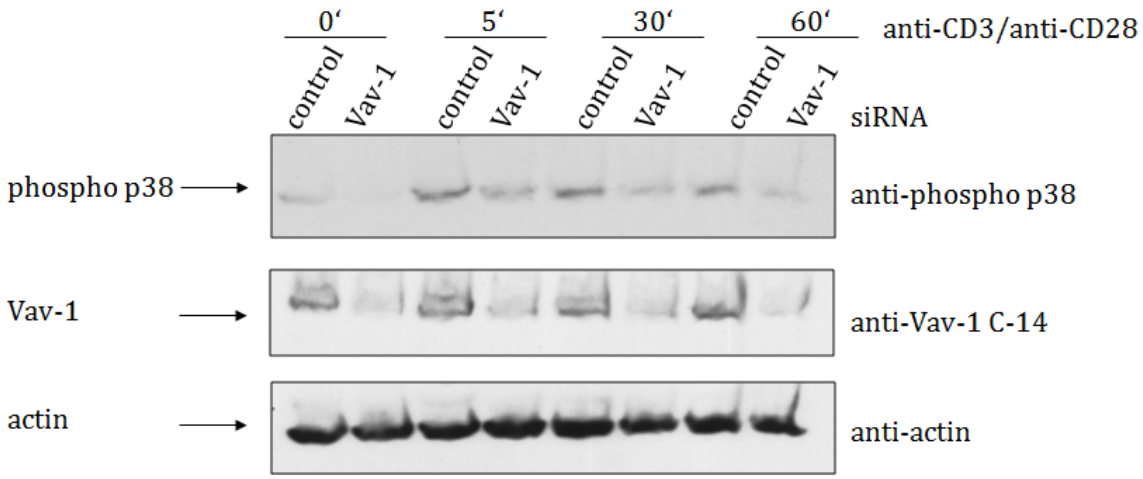

Figure 14: siRNA-mediated knock-down of Vav-1 strongly inhibits TCR-induced phosphorylation of p38. Jurkat E6 T-cells were transfected with either 5µg control siRNA or with 5µg Vav-1 siRNA to reduce the Vav-1 protein expression in these cells. 48h after transfection, cells were stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 for 5min, 30min or 60min and analyzed for p38 phosphorylation levels. Stimulation of control cells with anti-CD3 and anti-CD28 results in phosphorylation of p38. Knock-down of Vav-1 abrogates TCR-induced phosphorylation and thus activation of p38. Knock-down of Vav-1 protein

Since RNAi of the employed positive control protein Vav-1 blocks TCR-induced phosphorylation of p38 in our system, it was next analyzed whether siRNA-mediated knock-down of cytohesin-1 also leads to an impaired activation of the MAP kinase p38. For these experiments, primary T-cells (peripheral blood lymphocytes, PBL) were transfected using the AMAXA nucleoporation system with either control siRNA or with cytohesin-1 siRNA. 48h after transfection, cells were stimulated with 10µg/ml plate-bound anti-CD3 (OKT3) and 10µg/ml plate-bound anti-CD28 for 5min and analyzed for p38 phosphorylation levels. As shown in figure 15, stimulation of control cells with anti-CD3 and anti-CD28 yields a strong phosphorylation, and thus activation of p38. However, RNAi of cytohesin-1 results in an abrogation of TCR-induced phosphorylation of p38 (figure 15).

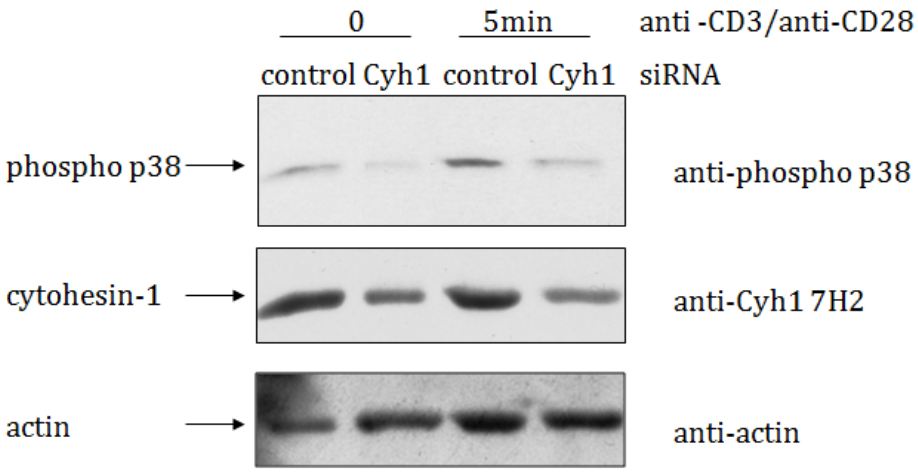

Figure 15: Silencing of cytohesin-1 results in impaired TCR-induced phosphorylation of the MAP kinase p38. Primary T-cells (peripheral blood lymphocytes, PBL) isolated from human buffy-coats were transfected with either 10µg control siRNA or with 10µg cytohesin-1 siRNA using the AMAXA nucleoporation system to reduce the cytohesin-1 protein expression. 48h after transfection, T-cells were stimulated with 10µg/ml plate-bound anti-CD3 and 10µg/ml plate-bound anti-CD28 for 5min and analyzed for p38 phosphorylation levels. Stimulation of control cells with anti-CD3 and anti-CD28 leads to a strong phosphorylation of p38, which is completely blocked after siRNA-mediated knock-down of cytohesin-1 expression. Knock-down of cythesin-1 protein expression was confirmed by immunoblot stained for cytohesin-1. Actin expression was used as protein loading control.

These results clearly indicate, that the expression of cytohesin-1 is required for the phosphorylation of p38, and thus for the activation of the transcription factor AP-1 via this route. Furthermore it has been recently shown in our lab, that silencing of cytohesin-1 leads to a reduced TCR-induced phosphorylation of JNK, but not to a decrease of ERK1/ERK2 MAP kinase activation (figure 16, Windheim, unpublished). Thus, cytohesin-1 is involved in the activation of the p38/JNK-, but not in the ERK1/ERK2-branch of the MAP kinase signaling cascade. Respective data of B. Paul (Paul, PhD thesis 2007), concerning an involvement of cytohesin-1 in TCR-induced ERK-activation, could not be confirmed.

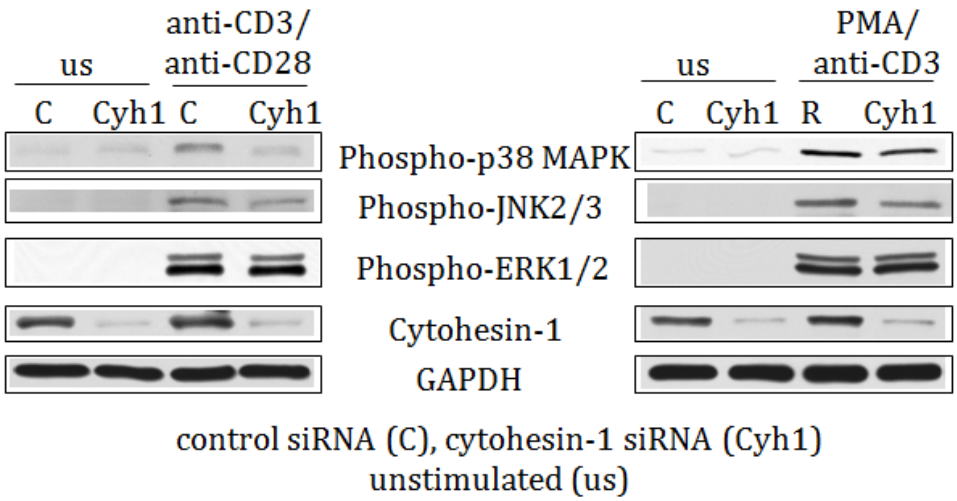

Figure 16: RNAi of cytohesin-1 reduces TCR-induced phosphorylation of p38 and JNK, but not of ERK1/2 MAP kinases. Jurkat T-cells were transfected with either 5µg control siRNA or with 5µg cytohesin-1 siRNA to reduce the cytohesin-1 protein expression. 48h after transfection, cells were stimulated either with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 (left blot) or with 50ng/ml PMA and 2µg/ml anti-CD3 (right blot) for 5min and analyzed for phosphorylation levels of p38, JNK2/3 and ERK1/2. Stimulation of control cells leads to a strong phosphorylation of p38, JNK2/3 and ERK1/2. Silencing of cytohesin-1 abrogates TCR-induced phosphorylation of p38 and JNK, but not of ERK1/2. Knock-down of cytohesin-1 protein expression was confirmed by immunoblots stained for cytohesin-1. GAPDH expression served as loading control (Windheim, unpublished).

Next it was analyzed, whether the cytohesin-1 GEF function is required for TCR-induced signaling to p38. To this end, cytohesin-1 WT or the dominant negative GEF mutant cytohesin-1 E157K was over-expressed in Jurkat T-cells. The cells were subsequently stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 for 5min to induce the phosphorylation of p38. In control cells, the stimulation leads to a robust phosphorylation of the MAP kinase p38, which is augmented by over-expression of cytohesin-1 WT (figure 17). However, over-expression of cytohesin-1 E157K completely blocks TCR-induced phosphorylation of p38 (figure 17), which indeed indicates that the GEF activity of cytohesin-1 is required for induced phosphorylation, and thus the activation of p38 during T-cell activation.

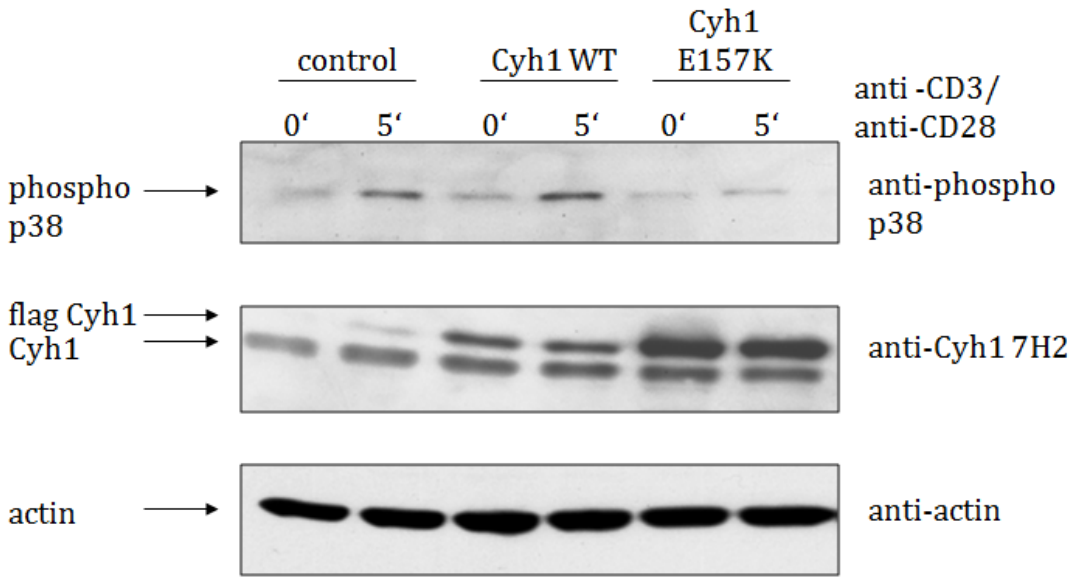

Figure 17: TCR-induced phosphorylation of p38 requires an intact GEF activity of cytohesin-1. Cytohesin-1 WT or the dominant negative GEF mutant cytohesin-1 E157K was over-expressed in Jurkat E6 T-cells. Cells were afterwards stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 to induce the phosphorylation of p38. In control cells TCR-stimulation leads to a strong phosphorylation of p38. Over-expression of cytohesin-1 WT leads to an increased phosphorylation of p38, which is completely blocked by the over-expression of the dominant-negative GEF mutant cytohesin-1 E157K. Over-expression was confirmed by immunoblot stained for cytohesin-1.

The GEF activity of cytohesin proteins can alternatively be blocked by the small molecule SecinH3 (sec-7 inhibitor H3 or 20H3), a novel sec-7 inhibitor, which has been recently identified in an aptamer displacement screen (Hafner et al., 2006). SecinH3 preferentially inhibits the GEF activity of cytohesins 1-3 and the *Drosophila* cytohesin homologue *steppke*, whereas the GEF function of other sec-7 domain containing proteins (e.g. the yeast protein Gea2 or the mammalian ARF6 GEF EFA6) is only weakly affected by SecinH3 (Hafner et al., 2006). It has been demonstrated that SecinH3 potently inhibits cytohesin-catalyzed GDP/GTP exchange on ARF1 and ARF6, as well as cytohesin-3-dependent insulin signal transduction of *Drosophila*, mouse and human cells *in vitro* and *in vivo* (Hafner et al., 2006, Fuss et al., 2006).

To analyze whether SecinH3 inhibits TCR-induced phosphorylation of p38, Jurkat T-cells were pre-incubated with 25µM SecinH3 for 1h and stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 for 5min, 10min, 30min or 60min. In DMSO treated control cells, the stimulation induces a robust phosphorylation of p38, which is decreased in SecinH3 treated cells (figure 18a). However, these effects are not as strong as expected, when using a potent chemical GEF inhibitor. These data are fully in line with the observation, that SecinH3 only partially inhibits cytohesin-mediated migration and adhesion (Novak and Quast, unpublished).

Compound 16 is a novel potent cytohesin family GEF inhibitor, which was recently identified with the help of chemo-informatic methods (Bajorath et al., in preparation). To analyze whether this compound also inhibits TCR-induced phosphorylation of p38, Jurkat T-cells were incubated with either 25µM SecinH3 or with 25µM compound 16 for 1h and stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 for 10min. In DMSO treated control cells, the stimulation induces a robust phosphorylation of p38, which is again only slightly decreased in SecinH3 treated cells (figure 18b). However, inhibition of the GEF function of cytohesin by compound 16 completely abrogates TCR-induced phosphorylation of p38 (figure 18b). These results substantiate the finding that compound 16 is a novel potent cytohesin GEF inhibitor, enormously stronger than SecinH3. Furthermore, these experiments confirm and corroborate the assumption, that an intact GEF function of cytohesin-1 is essential for the activation of p38, and thus of AP-1.

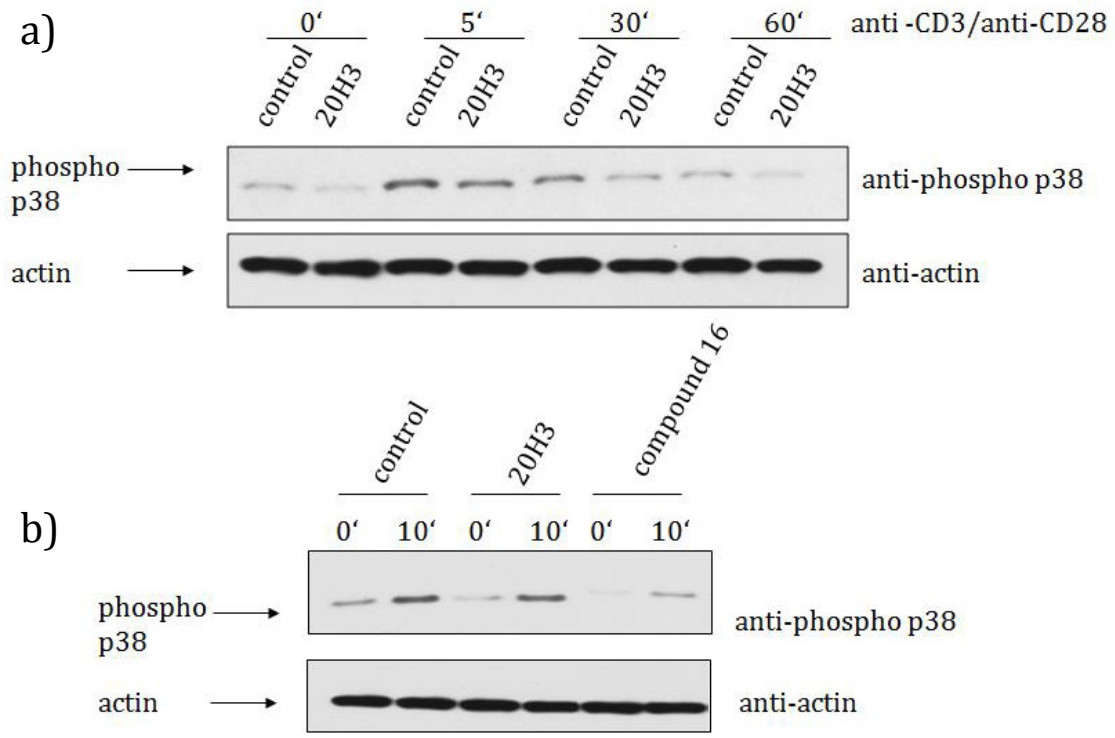

Figure 18: Inhibition of cytohesin by specific GEF inhibitors leads to decreased TCR-induced phosphorylation of p38. Jurkat T-cells were pre-treated with 25µM cytohesin GEF inhibitor SecinH3 (a and b) or with 25µM compound 16 (b) for 1h to inhibit the GEF activity of cytohesin proteins. Cells were afterwards stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 to induce the phosphorylation of p38. In DMSO treated control cells anti-CD3-/anti-CD28-stimulation leads to a strong phosphorylation of p38 (a and b). In SecinH3 treated cells this phosphorylation is slightly decreased (a and b). However, inhibition of the GEF function of cytohesin by compound 16 completely abrogates TCR-induced phosphorylation of p38 (b), substantiating that compound 16 is a stronger cytohesin GEF inhibitor than SecinH3. Furthermore, these result confirm the assumption, that an intact GEF function of cytohesin-1 is essential for TCR-induced signaling to p38.

3.1.3 CYTOHESIN-1 IS NOT LOCATED UPSTREAM OF VAV-1

Similar to what has been described for cytohesin-1, the Rac GEF Vav-1 enhances TCR – mediated signaling by increasing the phosphorylation of p38 and JNK, which finally leads to AP-1 transcriptional activity. Although a direct interaction between cytohesin-1 and Vav-1 had not been detected yet (data not shown), it was analyzed whether cytohesin-1 influences Vav-1 activation or whether cytohesin-1 acts downstream of Vav-1. The activity of Vav-1 is regulated by phosphorylation of the three regulatory tyrosines Y142, Y160 and Y174. A large body of evidence suggests that TCR engagement results in a strong phosphorylation of Vav-1, which in turn activates the GEF function of the protein towards Rho/Rac GTPases (see introduction for details). It was therefore analyzed, whether cytohesin-1 expression modulates TCR-induced Vav-1 phosphorylation. To this end, Jurkat T-cells were either transfected with control siRNA or with cytohesin-1 siRNA. 48h later, respective T-cells were stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 for 5min or 10min to induce the phosphorylation of Vav-1. Immunoprecipitates from unstimulated and TCR-stimulated Jurkat T-cell lysates were afterwards prepared, using a polyclonal rabbit antibody against Vav-1. Anti-phosphotyrosine immunoblots of these Vav-1 immunoprecipitates show that Vav-1 is tyrosine phosphorylated upon TCR cross-linking in control cells (figure 19a). In cells in which the protein level of cytohesin-1 was reduced by RNAi by nearly 100%, TCR-induced Vav-1 phosphorylation is not altered (figure 19). These data clearly show that the activation of Vav-1 does not require cytohesin-1 function and indicate that cytohesin-1 acts either downstream of Vav-1 or acts in a pathway which alternatively regulates the phosphorylation of p38 and JNK, and thus the activation of the transcription factor AP-1.

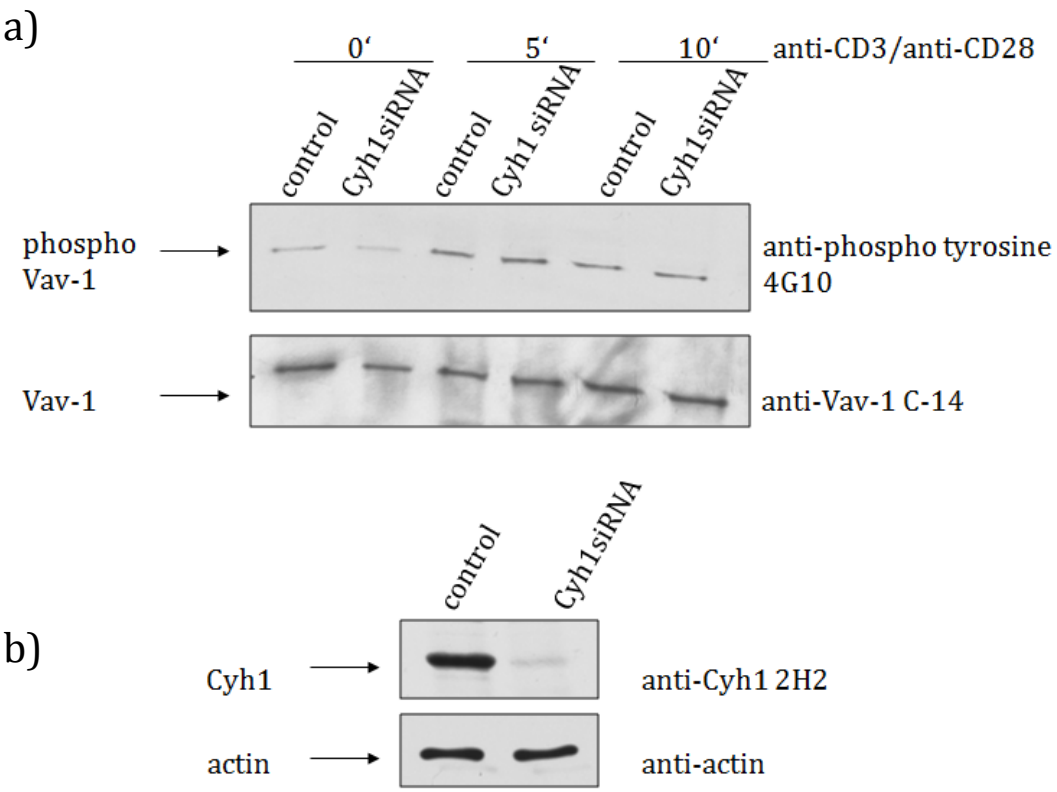

Figure 19: Cytohesin-1 is not involved in TCR-induced phosphorylation of Vav-1. Jurkat T-cells were either transfected with 5µg control siRNA or with 5µg cytohesin-1 siRNA. 48h later, T-cells were stimulated with 2µg/ml anti-CD3 (OKT3) and 1µg/ml anti-CD28 for 5min and 10min to induce Vav-1-phosphorylation. Immunoprecipitates of cell lysates were afterwards prepared, using a polyclonal rabbit antibody against Vav-1. Anti-phosphotyrosine immunoblots of these Vav-1 immunoprecipitates show that Vav-1 is tyrosine phosphorylated upon TCR cross-linking in control cells (a). In cells in which the protein level of cytohesin-1 was reduced, TCR-induced Vav-1 phosphorylation is not altered (a). These data clearly show that the activation of Vav-1 does not require cytohesin-1 function. Knock-down of cytohesin-1 protein expression in these cells was confirmed by analyzing cell lysates by immunoblot. RNAi of cytohesin-1 dramatically reduces specific protein expression (b).

3.1.4 CYTOHESIN-1 DOES NOT INTERACT WITH THE WASP-COMPLEX

T-cell receptor stimulation by foreign antigen induces the recruitment and activation of the protein tyrosine kinases Fyn, Lck and ZAP-70, leading to the phosphorylation and activation of several signaling components of the T-cell signal transduction pathway. Among these substrates are the hematopoietic cell-specific molecule SLP-76 (SH2 domain containing leukocyte phosphoprotein of 76 kDa) and Vav-1, a guanine nucleotide exchange factor which acts on members of the Rac/Rho family of small GTP-binding proteins (Crespo et al., 1997). Both proteins play critical roles in the regulation of TCR signals and associate with each other via the SH2 domain of Vav-1 and phosphorylated tyrosines of SLP-76 (Tuosto et al., 1996). The phosphorylation of SLP-76 also creates binding sites for Nck. Nck then functions to recruit the Wiskott-Aldrich syndrome protein (WASP), which is activated by binding to Cdc42-GTP and induces Arp2/3-dependent actin polymerization at the T-cell-APC contact site (Zeng et al., 2003). WASP is linked to the actin cytoskeleton through direct binding to the Arp2/3 complex (Machesky and Insall, 1998). It therefore connects the T-cell receptor signaling machinery with the actin cytoskeleton and enables cytoskeletal reorganization during T-cell activation, needed for the induction of a specific immune response. Biochemical analysis of T-cell lysates has shown that WASP, SPL-76 and Nck are present in large complexes in the T-cell (Krause et al., 2000). As shown, cytohesin-1 expression also influences both T-cell receptor signaling (shown here and Paul, PhD thesis 2007) as well as cytoskeletal reorganization during integrin-dependent adhesion and migration (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). For this reason, it was analyzed, whether cytohesin-1 is a member of this WASP/SLP-76/Nck protein complex, which links the T-cell receptor signaling machinery to the actin cytoskeleton. Immunoprecipitates from unstimulated and anti CD3-/anti CD28-stimulated Jurkat T-cell lysates were prepared, using a polyclonal rabbit antibody against WASP. However, cytohesin-1 protein was not detectable in WASP immunoprecipitates neither of unstimulated nor of stimulated Jurkat T-cells (figure 20a). As positive control, WASP immunoprecipitates were analyzed using a SLP-76 antibody. Since the control protein

SLP-76 was also not detectable in these WASP immunoprecipitates, I used a different WASP antibody to pull-down the WASP/SLP-76/Nck complex. To this end, immunoprecipitates of unstimulated or of stimulated Jurkat T-cells extracts were prepared, using a monoclonal mouse WASP antibody. With the help of this monoclonal antibody, it was possible to co-precipitate WASP and SLP-76, but again cytohesin-1 was not present in this complex (figure 20b) (Novak, unpublished).

Hence, a direct interaction between cytohesin-1 and WASP/SLP-76/Nck complex had not been detected, yet, suggesting that cytohesin-1 is located in an alternative protein complex, where it regulates T-cell activation as well as integrin-dependent migration and adhesion.

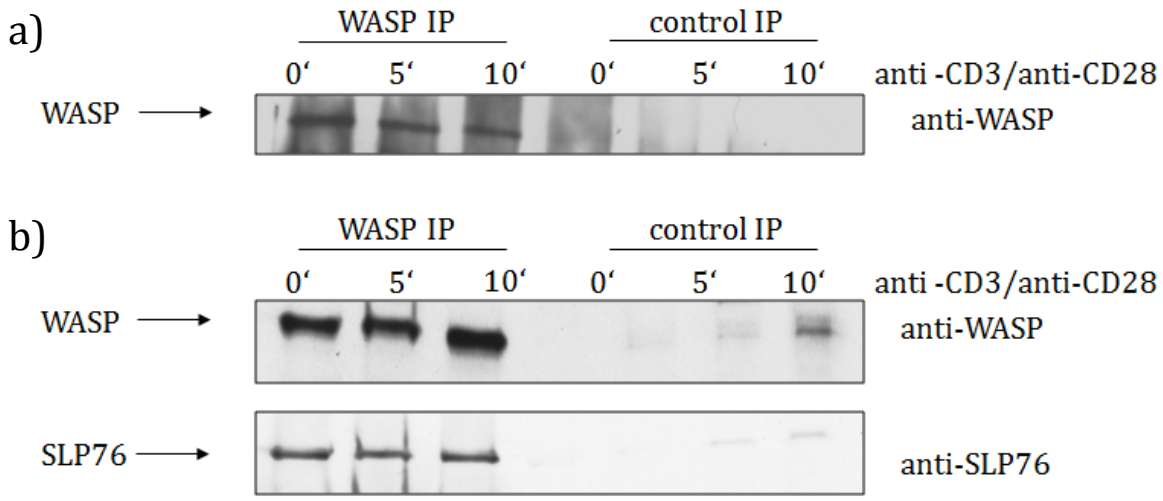

Figure 20: Cytohesin-1 does not interact with the WASP complex. WASP immuno-precipitation with Jurkat T-cells. Jurkat T-cells were stimulated with 2µg/ml anti-CD3 and 1µg/ml anti-CD28 for 5min or 10min. Immunoprecipitates from either unstimulated or TCR-stimulated Jurkat T-cell lysates were prepared using either a polyclonal rabbit antibody (a) or a monoclonal mouse antibody against WASP (b). Only the monoclonal WASP antibody is able to pull-down the WASP/SLP-76 complex (b). Cytohesin-1 protein does not interact with WASP, neither in unstimulated nor in stimulated Jurkat T-cells (a and b). For negative control IPs either normal rabbit IgG (a) or only protein-A-sepharose beads (b) were used. (figure b: Novak, unpublished)

3.2 ROLE OF GUANINE NUCLEOTIDE EXCHANGE FACTORS IN DC MIGRATION AND MATURATION

3.2.1 BETA 2-INTEGRIN MEDIATED CELL ADHESION REQUIRES AN INTACT CYTOHESIN-1 GEF ACTIVITY

Besides its role in T-cell activation, cytohesin-1 plays an important role in signaling pathways, which affect the regulation of immune cell adhesion and migration. It has recently been demonstrated that integrin-dependent dendritic cell 3D migration strongly depends on cytohesin-1 function *in vitro* and *in vivo* (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). Furthermore, cytohesin-1 acts as an important regulator of ß2-integrin mediated adhesion, since RNAi of cytohesin-1 markedly reduces static adhesion of human mo-DC to ICAM-1 (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). Until now, there is no clear evidence suggesting that the GEF activity of cytohesin-1 is required for regulating adhesive events of dendritic cells. To address this issue, a novel protocol was established, which enables over-expression analysis of cell adhesion and migration event in human monocyte derived dendritic cells (mo-DC). Human mo-DC were transfected with control vector or with expression constructs encoding the wild type cytohesin-1 protein (Cyh1 WT) or the GEF deficient, dominant-negative cytohesin-1 mutant E157K (Cyh1 E157k), using the newly developed MicroPorator MP-100 system (Peqlab), which uses a pipette gold-tip as electroporation space instead of the cuvette. The cells were co-transfected with a marker plasmid, which directs expression of the green fluorescent protein (GFP). It was shown that static cell adhesion to ICAM-1 is markedly enhanced in GFP-positive human mo-DC over-expressing cytohesin-1 WT, but not in DC over-expressing the GEF-deficient cytohesin-1 E157K mutant (figure 21). This proves for the first time that the GEF function of cytohesin-1 is required for ß2-integrin mediated dendritic cell adhesion.

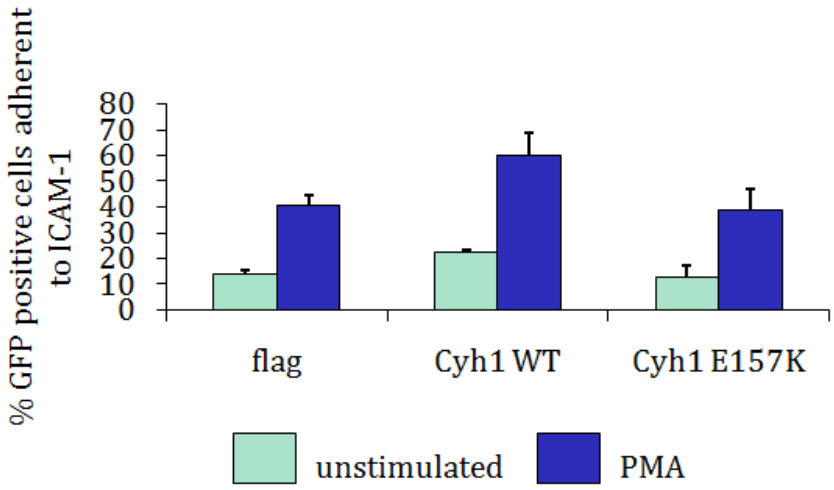

Figure 21: Beta2-integrin mediated cell adhesion requires an intact cytohesin-1 GEF activity. Static cell adhesion assay on ICAM-1 with human monocyte-derived dendritic cells. Immature human mo-DC were transfected with 10µg control vector, or with 10µg expression constructs encoding the wild type cytohesin-1 protein (Cyh1 WT) or the GEF deficient, dominant-negative cytohesin-1 mutant E157K (Cyh1 E157k), using the newly developed MicroPorator MP-100 system (Peqlab). All samples were co-transfected with 10µg N1:eGFP as a marker for transfection. 2h after microporation, respective cells were stimulated with 1µg/ml LPS and 50ng/ml TNFalpha to induce maturation. The cells were then cultured overnight in VLE-RPMI at 37°C and used for static adhesion assays 24h later. The obtained transfection efficiency was 1%-5%. The cells were then placed on ICAM-1-Fc coated 96-well plates and stimulated with 50ng/ml PMA for 1h to adhere. Unbound cells were washed off carefully with HBSS and the adherent GFP-positive cells which represent the transfected population were counted in five fields of view at 10x magnification using fluorescent microscopy. The number of GFP-positive cells in the unwashed sample was set to 100%. Static cell adhesion to ICAM-1 is strongly enhanced in GFP-positive dendritic cells over-expressing cytohesin-1 WT, but not in dendritic cells over-expressing the GEF-deficient cytohesin-1 E157K mutant. This analysis shows that the GEF function of cytohesin-1 is crucial for ß2-integrin mediated dendritic cell adhesion.

Furthermore, it has recently been shown that cytohesin-1 controls the activation of the GTPase RhoA in several cell-types (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). RhoA is an important regulator of chemokine-induced cell adhesion and cell migration, and cytohesin-1 acts upstream of RhoA in directing integrin-dependent adhesion and

migration (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). In line with this data, it is shown that over-expression of RhoA WT enhances static adhesion of mo-DC to ICAM1, whereas over-expression of the dominant negative GTP-loading deficient mutant RhoA T19N abrogates this effect, indicating that cytohesin-1 and RhoA are acting in the same pathway (figure 22).

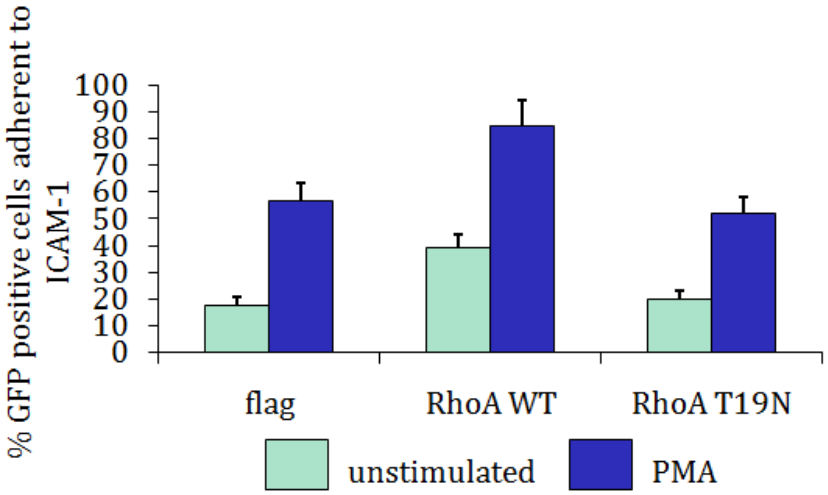

Figure 22: Over-expression of RhoA WT enhances static adhesion of human mo-DC to ICAM1, whereas over-expression of the dominant negative mutant RhoA T19N abrogates this effect. Static cell adhesion assay on ICAM-1 with human mo-DC. Immature human mo-DC were transfected with 10µg control vector, or with 10µg expression constructs encoding the wild type RhoA protein (RhoA WT), respectively the dominant negative GTP-loading deficient mutant RhoA T19N, using the MicroPorator MP-100 system (Peqlab). All samples were co-transfected with 10µg N1:eGFP as a marker for transfection. 2h after microporation, the cells were stimulated with 1µg/ml LPS and 50ng/ml TNFalpha to induce maturation. The cells were then cultured overnight in VLE-RPMI at 37°C and were used for static adhesion assays 24h later. The cells were then placed on ICAM-1-Fc coated 96-well plates and stimulated with 50ng/ml PMA for 1h to adhere. Unbound cells were washed off carefully with HBSS and the adherent GFP-positive cells were counted in five fields of view at 10x magnification using fluorescent microscopy. The number of GFP-positive cells in the unwashed sample was set to 100%. Static cell adhesion to ICAM-1 is strongly enhanced in GFP-positive dendritic cells over-expressing RhoA WT, but not in dendritic cells over-expressing the dominant negative GTP-loading deficient mutant RhoA T19N.

3.2.2 EXPRESSION OF THE RHO/RAC GEF VAV-1 IS CRUCIAL FOR TNFALPHA-INDUCED MATURATION OF HUMAN MO-DC

Dendritic cells are the most potent antigen-presenting cells of the immune system. Their binding to pathogen-borne structures (pathogen-associated molecular patterns or PAMPs) induces the terminal differentiation ("maturation") of the cells, which is characterized by robust cell surface expression of MHC-peptide complexes and of so-called co-stimulatory molecules. DC maturation is further accompanied by a strong stimulation of the migratory capacity of these cells. To stimulate this maturation process *in vitro,* mo-DC were treated with LPS and TNFalpha, as the combination of both stimuli induces higher DC maturation than LPS or TNFalpha alone.

It has been recently shown that Vav-1 is involved in signaling pathways leading to functional and morphological differentiation of human mo-DC, since RNAi-mediated knock-down of Vav-1 strongly impairs LPS/TNFalpha-induced maturation (Nickel, diploma thesis 2005). Vav-1 silenced mo-DC exhibit an immature phenotype in morphology and, furthermore, LPS/TNFalpha-dependent up-regulation of characteristic maturation markers as CD40, the MHC class II protein HLA-DR and the co-stimulatory molecules CD80 and CD86 is decreased in these cells (Nickel, diploma thesis 2005).

In mammals, the Vav family of proteins consists of the three known members Vav-1, Vav-2 and Vav-3. Importantly, Vav proteins were shown to have strongly overlapping functions in antigen-receptor mediated signaling in T- and particularly in B-lymphocytes. It was therefore analyzed here, whether the other Vav family proteins Vav-2 and Vav-3 might also contribute to LPS/TNFalpha-induced maturation of human mo-DC. Using a special square wave electroporation protocol, immature mo-DC were transfected with either control siRNA, with Vav-1 siRNA or with Vav-1, Vav-2 and Vav-3 siRNA. 48h later, the cells were stimulated with 1µg/ml LPS and 50ng/ml TNFalpha for 48h to stimulate their maturation. Mo-DC maturation was determined by analyzing the expression of the characteristic maturation markers CD80 and CD40.

As depicted in figure 23, maturation of mo-DC is decreased following transfection with a Vav-1 specific siRNA, as compared to control cells. However, knock-down of all three Vav family proteins does not lead to stronger inhibition of LPS/TNFalpha-induced up-regulation of maturation marker expression, in comparison to Vav-1 knock-down mo-DC (figure 23), indicating that Vav-2 and Vav-3 are not involved in maturation signaling of dendritic cells.

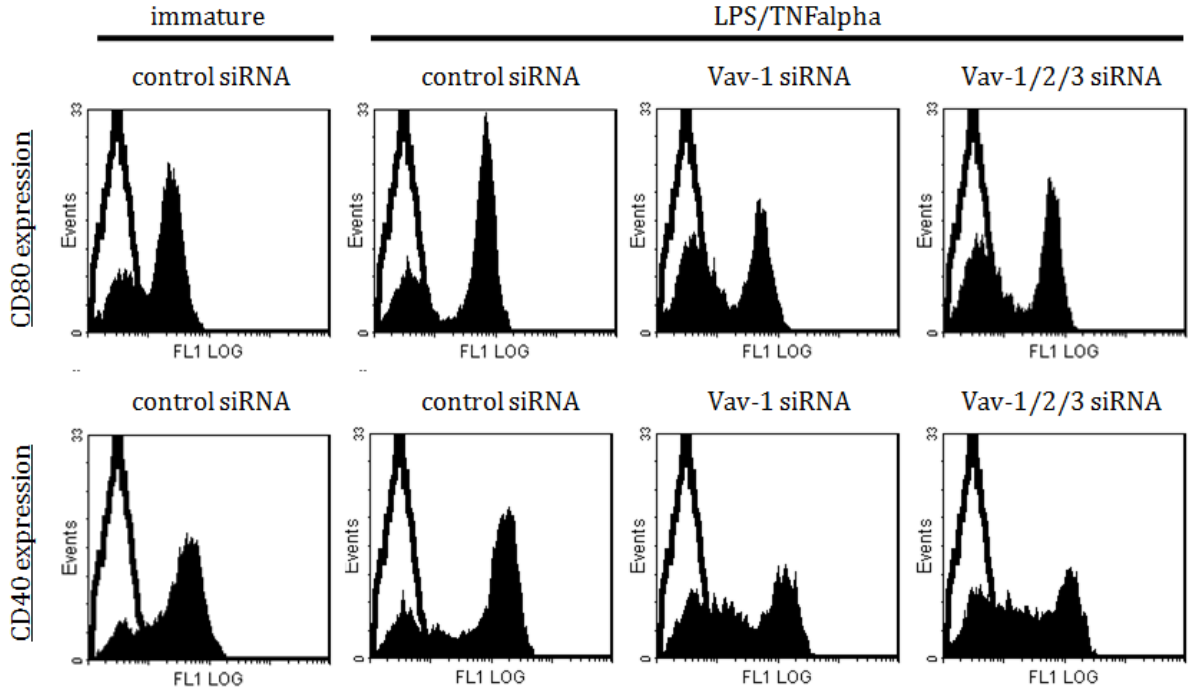

Figure 23: Knock-down of Vav-2 and Vav-3 does not affect DC maturation. Using a special square wave electroporation protocol, human immature mo-DC were transfected with control siRNA, Vav-1 siRNA or with Vav-1, Vav-2 and Vav-3 siRNA. 48h later, cells were stimulated with 1µg/ml LPS and 50ng/ml TNFalpha to induce maturation. Mo-DC maturation was determined two days after stimulation by analyzing the expression of the characteristic maturation markers CD80 and CD40, using flow-cytometry. LPS/TNFalpha stimulation of control cells results in robust up-regulation of the co-stimulatory molecules, which is markedly decreased in Vav-1 knock-down mo-DC, indicating that Vav-1 expression is crucial for maturation of human mo-DC. However, knock-down of all three Vav family proteins does not lead to stronger inhibition of LPS/TNFalpha-induced up-regulation of maturation marker expression, in comparison to Vav-1 knock-down mo-DC.

Although binding of LPS to Toll-like receptor 4 (TLR4) and binding of TNFalpha to TNF receptors (TNFR1 and TNFR2) eventually result in NF-κB activation, intracellular signaling from Toll- and TNFalpha-receptors are quite distinct (see introduction). To test whether RNAi of Vav-1 interferes with LPS- or with TNFalpha-induced maturation of dendritic cells or whether Vav-1 is important for both signaling pathways, Vav-1 protein expression was silenced during LPS- or TNFalpha-induced maturation of mo-DC, respectively. Immature mo-DC were transfected with control siRNA or with Vav-1 siRNA. 48h later, the cells were either stimulated with 1µg/ml LPS or with 100ng/ml TNFalpha for 48h to induce maturation. Mo-DC maturation was determined by analyzing the expression of characteristic maturation markers. Stimulation of control cells with either LPS (figure 25) or with TNFalpha (figure 24) leads to a robust up-regulation of the co-stimulatory molecules CD80 and CD86. Figure 25 shows that LPS-induced maturation of mo-DC is not altered following transfection with a Vav-1 specific siRNA, as compared to control cells. However, TNFalpha-induced up-regulation of the co-stimulatory molecules CD80 and CD86 is markedly decreased in Vav-1 knock-down mo-DC (figure 24). These data clearly show that Vav-1 is crucial for TNFalpha-, but not for LPS-induced maturation of human mo-DC.

RNAi of Vav-1 strongly reduces specific protein expression, as shown by immunoblot (figure 26).

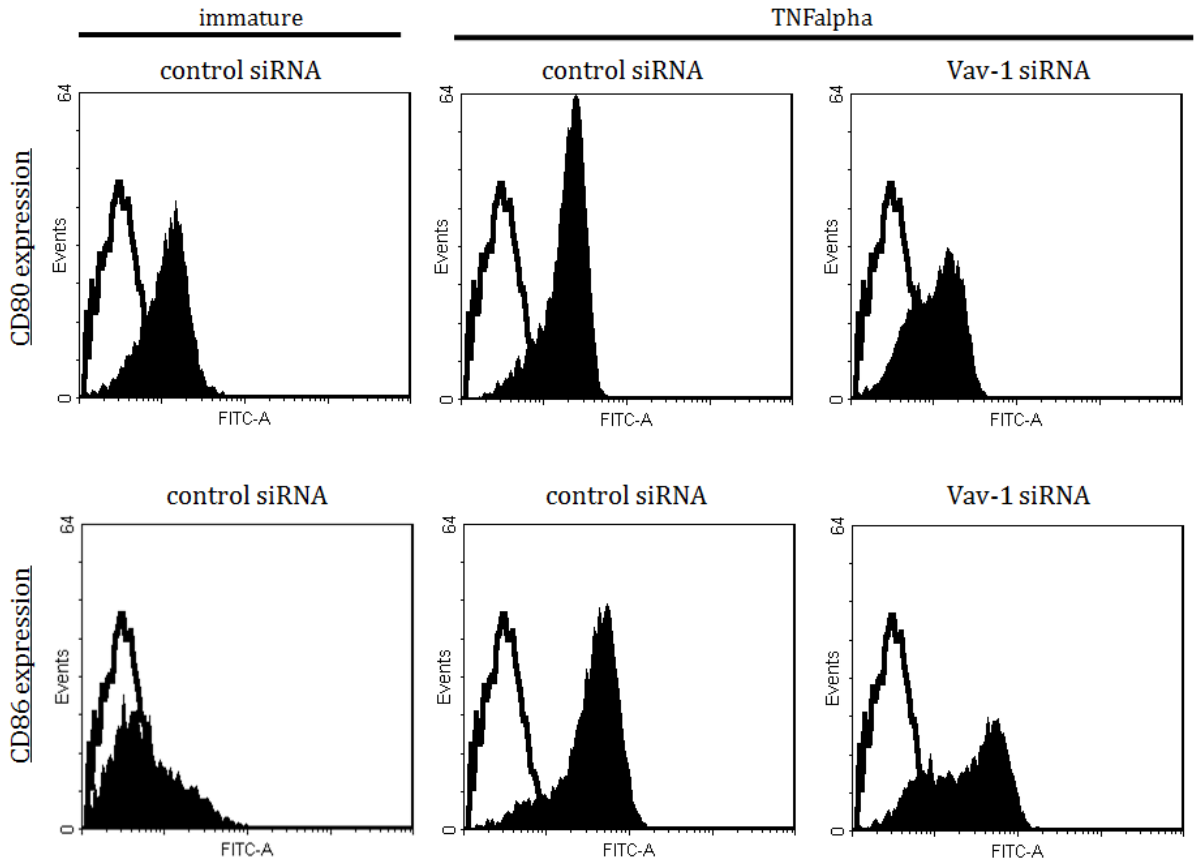

Figure 24: RNAi of Vav-1 reduces TNFalpha-induced up-regulation of the co-stimulatory molecules CD80 and CD86. Vav-1 protein expression was silenced during TNFalpha-induced maturation of human mo-DC. Using a special square wave electroporation protocol, human immature mo-DC were transfected with either 20µg control siRNA or with 20µg Vav-1 siRNA. 48h later, the cells were stimulated with 100ng/ml TNFalpha to induce maturation. Mo-DC maturation was determined two days after stimulation by controlling the expression of the characteristic maturation markers CD80 and CD86, using flow-cytometry. TNFalpha stimulation of control cells results in robust up-regulation of the co-stimulatory molecules CD80 and CD86, which is markedly decreased in Vav-1 knock-down mo-DC, indicating that Vav-1 expression is important for TNFalpha-induced maturation of human mo-DC.

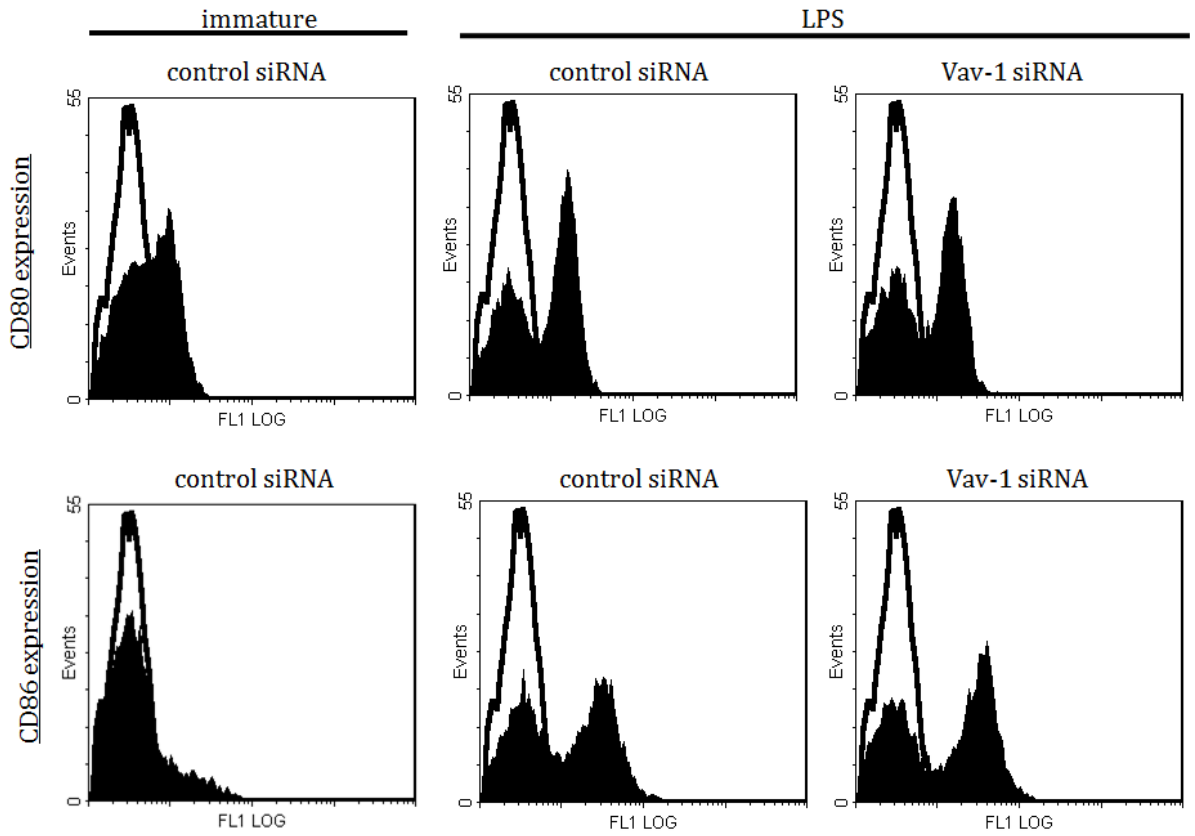

Figure 25: RNAi of Vav-1 does not interfere with LPS-induced up-regulation of maturation markers. Vav-1 protein expression was silenced during LPS-induced maturation of mo-DC. Using a special square wave electroporation protocol, human immature mo-DC were transfected with either 20µg control siRNA or with 20µg Vav-1 siRNA. 48h later, the cells were either stimulated with 1µg/ml LPS for 48h to induce maturation. Mo-DC maturation was determined by controlling the expression of the maturation markers CD80 and CD86 with the help of flow-cytometry. Stimulation of the control cells with LPS leads to robust up-regulation of the co-stimulatory molecules CD80 and CD86. LPS-induced maturation of mo-DC is not altered following transfection with a Vav-1 specific siRNA, as compared to control cells, indicating that Vav-1 is important for TNF-alpha-, but not for LPS-induced maturation of human mo-DC.

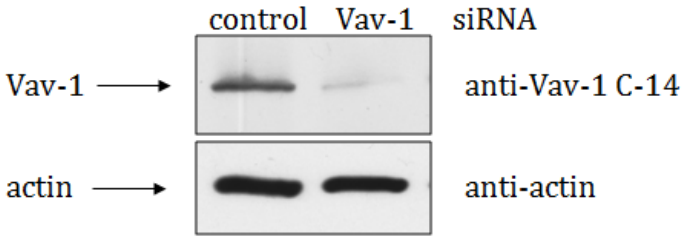

Figure 26: Employing square wave electroporation, Vav-1 expression was efficiently silenced in mo-DC. Using a special square wave electroporation protocol, immature mo-DC were transfected with either 20µg control siRNA or with 20µg Vav-1 siRNA. 48h later cells were stimulated with 100ng/ml TNFalpha to induce maturation. Knock-down of Vav-1 protein expression in these cells was confirmed 96h after transfection by analyzing cell lysates by immunoblot. Actin protein expression was used as protein loading control.

Vav proteins are guanine nucleotide exchange factors which bear catalytic GEF activity towards a broad range of Rho/Rac-family GTPases (Crespo et al., 1997). However, there is specificity in this system, too, because Rac-1 is primarily targeted by Vav-1 (Crespo et al., 1997). To analyze, whether the TNFalpha-induced maturation of mo-DC depends on an intact GEF function of Vav-1, the Vav GEF inhibitor 6-Thio-GTP was employed.

6-Thio-GTP is a metabolite of Azathioprine, a drug which is widely used in organ transplantation and in the therapy of auto-immune or chronic inflammatory diseases. 6-Thio-GTP binds with high affinity to the nucleotide-free ("empty") Rac-1 protein and will subsequently get hydrolyzed to 6-Thio-GDP. It is thus capable of displacing its physiological counterpart GTP in a concentration dependent fashion. Importantly, in the next round of the cycle, Vav proteins will not be able to catalyze the removal of 6-Thio-GDP. Although there are several other GEFs expressed in eukaryotic cells which bear exchange activity towards Rho/Rac GTPases, 6-Thio-GTP will specifically block Vav-catalyzed nucleotide replacement reactions. In consequence, this small molecular nucleotide analog is an indirect, but highly selective inhibitor of the Vav GEF function (Poppe et al., 2006).

Immature mo-DC were thus pre-incubated for 2 days with 10µM 6-Thio-GTP before they were stimulated with 100ng/ml TNFalpha to induce maturation. Unmodified GTP was used as a control. 48h later, maturation of the respective cells was assessed by flow-cytometry. As can be seen in figure 27, stimulation of control cells with TNFalpha leads to strong up-regulation of the co-stimulatory molecules CD80, CD86 and CD40. However, TNFalpha-induced maturation marker expression is markedly decreased in cells that had been per-incubated with 6-Thio-GTP. Thus, inhibition of the Vav-1 GEF function by 6-Thio-GTP results in an impairment of TNFalpha-dependent up-regulation of important co-stimulatory molecules (figure 27). These data strongly suggest that TNFalpha-induced maturation of mo-DC requires an intact Vav-1 GEF domain.

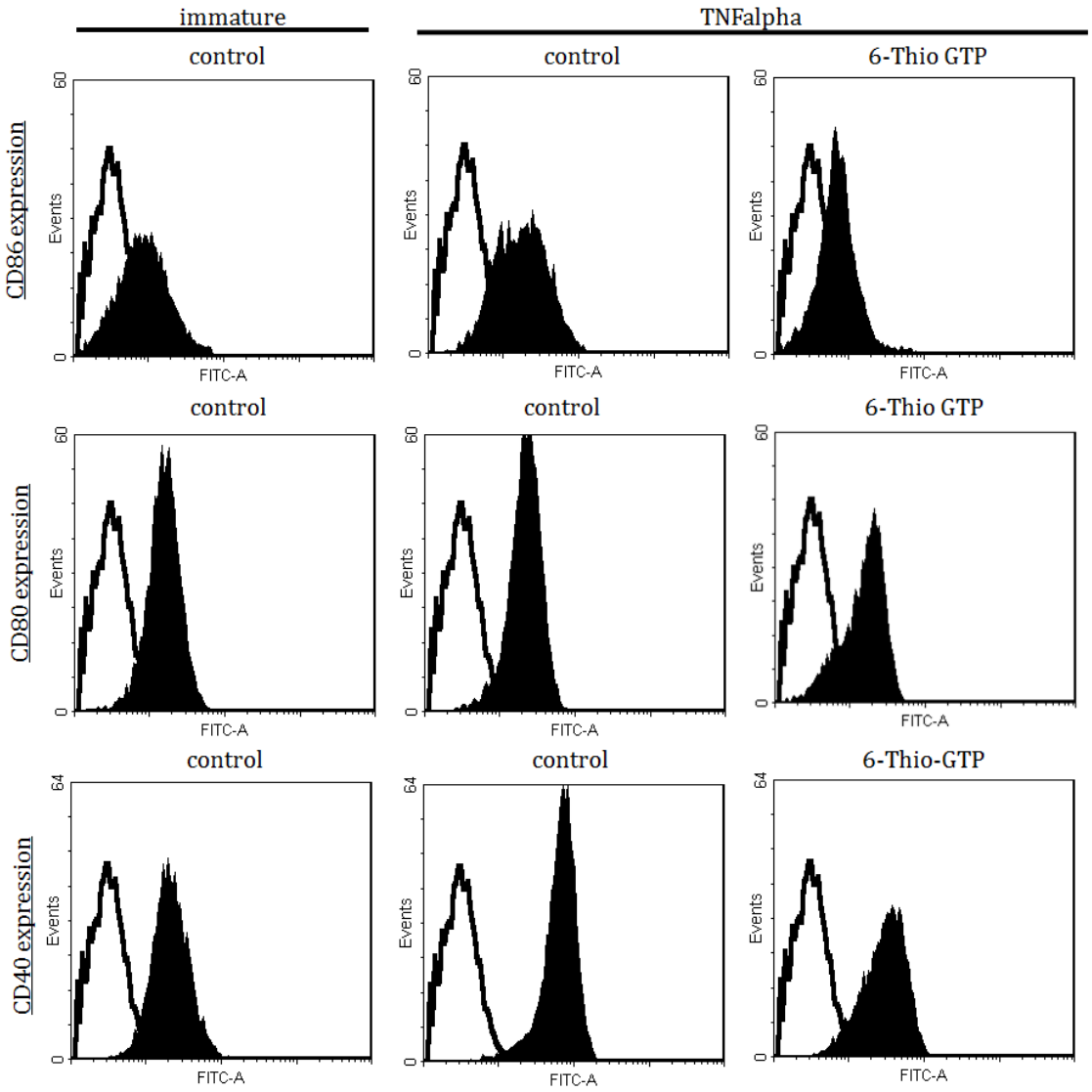

Figure 27: Vav-1 GEF function is essential for TNFalpha-induced up-regulation of maturation markers. To analyze whether the Vav GEF function is required for TNFalpha-induced mo-DC maturation, immature mo-DC were pre-incubated for 2 days with 10µM of the Vav GEF inhibitor 6-Thio-GTP, before they were stimulated with 100ng/ml TNFalpha to induce maturation. Unmodified GTP was used as a control. After 48h maturation stage of the cells was assessed by detecting the surface markers CD40, CD80 and CD86, using flow-cytometry. Stimulation of control cells with TNFalpha leads to an up-regulation of co-stimulatory molecules, which is markedly decreased in cells where the Vav-1 GEF function is inhibited by 6-Thio-GTP. These data suggest that an intact GEF activity of Vav-1 is essential for TNF-alpha induced maturation of mo-DC.

3.2.3 siRNA-MEDIATED KNOCK-DOWN OF VAV-1 LEADS TO REDUCED EXPRESSION OF TNFALPHA TARGET GENES

Employing microarray-based transcriptome analysis, it has previously been shown that the expression of 120 genes is reduced in LPS/TNFalpha-stimulated Vav-1 knock-down mo-DC, as compared to cells which had been transfected with control siRNA (Nickel, diploma thesis 2005, in collaboration with J. Schultze, Tumor Immunology, Cologne Medical School). Since Vav-1 appears to be specifically involved in TNFalpha receptor signaling in mo-DC, these data now have been re-evaluated. Comparing these data with microarray analyses of TNFalpha stimulated WT mo-DC, it could be shown that among these 120 regulated genes, 31 of them are known TNFalpha target genes, as for example the intercellular adhesion molecule 1 (CD54, ICAM1), the chemokine receptor CCR1, the maturation marker CD83 and the co-stimulatory molecule CD80 (figure 28). These results are consistent with the finding that Vav-1 regulates TNFalpha-dependent signaling pathways in human mo-DC.

TNFalpha regulated genes
Homo sapiens intercellular adhesion molecule 1 (CD54, ICAM1)
Homo sapiens CD209 antigen (CD209)
Homo sapiens colony stimulating factor 1 receptor
Homo sapiens CD33 antigen (CD33)
Homo sapiens prion protein (p27-30)
Homo sapiens Fc fragment of IgG, (FCGR2A)
Homo sapiens interleukin 1 receptor, type I (IL1R1)
Homo sapiens CD84 antigen (leukocyte antigen) (CD84)
Homo sapiens glycoprotein Ib (platelet), alpha polypeptide
Homo sapiens CD36 antigen (CD36)
Homo sapiens platelet/endothelial cell adhesion molecule (PECAM1)
Homo sapiens CD69 antigen (CD69)

Homo sapiens TNF receptor superfamily, member 9 (TNFRSF9)
Homo sapiens interleukin 13 receptor, alpha 1 (IL13RA1)
Homo sapiens lysosomal-associated membrane protein 3 (LAMP3)
Homo sapiens plexin C1 (PLXNC1)
Homo sapiens programmed cell death 1 ligand 2
Homo sapiens chemokine (C-C motif) receptor 1 (CCR1)
Homo sapiens CD83 antigen (CD83)
Homo sapiens signaling lymphocytic activation molecule family member 1 (SLAMF1)
Homo sapiens TNF receptor superfamily, member 14
Homo sapiens TNF receptor superfamily, member 1B (TNFRSF1B)
Homo sapiens F11 receptor (F11R)
Homo sapiens fms-related tyrosine kinase 3 (FLT3)
Homo sapiens lymphocyte antigen 75 (LY75)
Homo sapiens poliovirus receptor (PVR)
Homo sapiens chemokine (C-C motif) receptor 7 (CCR7)
Homo sapiens indoleamine-pyrrole 2,3 dioxygenase (INDO)
Homo sapiens CD80 antigen (CD80)

Figure 28: SiRNA-mediated knock-down of Vav-1 leads to reduced expression of TNFalpha target genes. Using a special square wave electroporation protocol, immature mo-DC were transfected with either 20µg control or with 20µg Vav-1 siRNA. 48h later, the cells were stimulated with 1µg/ml LPS and 50ng/ml TNFalpha for 48h to induce maturation. Isolated RNA was then analyzed by microarray analysis in the group of J. Schultze. In LPS/TNFalpha-stimulated Vav-1 knock-down mo-DC the expression of 120 genes is reduced (Nickel, diploma thesis 2005). Comparing these data with microarray analysis of TNFalpha stimulated WT mo-DC, it could be shown, that among these 120 Vav-1-regulated genes, 31 are known TNFalpha target genes. These analysis are consistent with claim that Vav-1 is involved the TNFalpha mediated gene transcription in human mo-DC.

3.2.4 VAV-1 IS REQUIRED FOR TNFALPHA-INDUCED ACTIVATION OF NF-κB IN HUMAN MO-DC

The interaction of TNFalpha with its cognate receptors leads to the activation of survival factors or of pro-inflammatory genes through the transcription factor NF-κB, a heterodimeric transcription factor complex, consisting of Rel family proteins. Five mammalian NF-κB/Rel family members have been identified so far: NF-κB1 (also called p50), NF-κB2 (also named p52), RelA (also known as p65), RelB and c-Rel. They all share a highly conserved Rel homology domain, containing DNA-binding and dimerization domains and the nuclear localization signal of the Rel proteins. Upon stimulation the NF-κB complex translocates to the nucleus and directly induces target gene expression by binding to NF- κB binding sites, which contain a specific decameric DNA sequence (GGG ACT TTC C).

In dendritic cells, TNFalpha-induced activation of NF-κB leads to e.g. the expression of maturation markers, such as the co-stimulatory molecules CD40, CD80 and CD86. To analyze, whether Vav-1 is involved in TNFalpha-mediated signaling to the NF-κB complex in mo-DC, the activation of endogenous NF-κB was measured with the help of the so-called electrophoretic mobility gel shift assay (EMSA). EMSA is a technique for studying gene regulation and determining protein/DNA interactions, which is based on the observation that protein/DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide gel electrophoresis. If NF-κB subunits are present in the isolated nuclear extracts, their interaction with a DNA probe, containing the specific recognition sequence for NF-κB, shifts the band of the ^{32}p-labeled probe up.

Using square wave electroporation, immature mo-DC were transfected with either control siRNA or with siRNA specific for Vav-1. Four days after transfection, the cells were treated with 100ng/ml TNFalpha and nuclear extracts were prepared 1h or 2h after stimulation. The nuclear extracts were incubated with a ^{32}p-labeled, double-stranded oligonucleotide probe, which contains the specific recognition sequence for

NF-κB (figure 29) and were then assayed for NF-κB-mediated electrophoretic retardation of the labeled probe by EMSA. As shown in figure 30, stimulation with TNFalpha results in robust activation of NF-κB in control cells. However, TNFalpha-induced activation of NF-κB is strongly reduced in Vav-1 knock-down mo-DC, indicating that Vav-1 protein expression is crucial for TNFalpha-dependent mobilization of NF-κB in this cell-type.

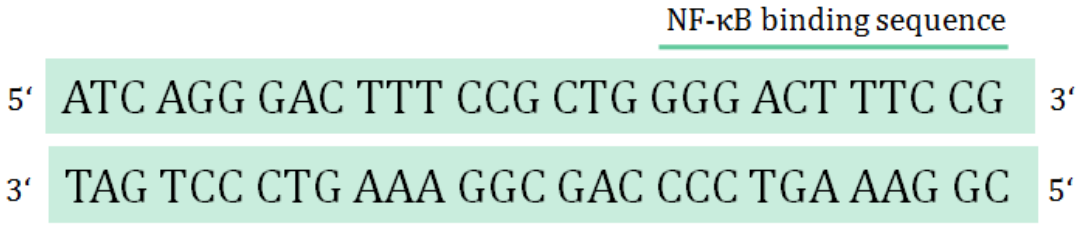

Figure 29: Double-stranded oligonucleotide probe, containing the specific recognition sequence for NF-κB.

Under conditions in which protein synthesis is blocked, or NF-κB activation is inhibited, TNFR1 stimulation leads to a strong induction of apoptosis. Apoptosis induced by TNFR1 is mediated via recruitment of the adaptor protein FADD (Hsu et al., 1996). Pro-caspase-8 is then recruited by binding to the death effector domain of FADD, where it becomes activated by cleavage at specific aspartic acid residues, and this initiates a apoptotic signaling cascade. Vav-1 expression was shown here to be crucial for TNFalpha-induced signaling to NF-κB, and this finding implies that TNFR stimulation leads to a strong induction of apoptosis in Vav-1 knock-down mo-DC. It was indeed shown here that siRNA-mediated knock-down of Vav-1 induces cell death in response to TNFalpha treatment (figure 31).

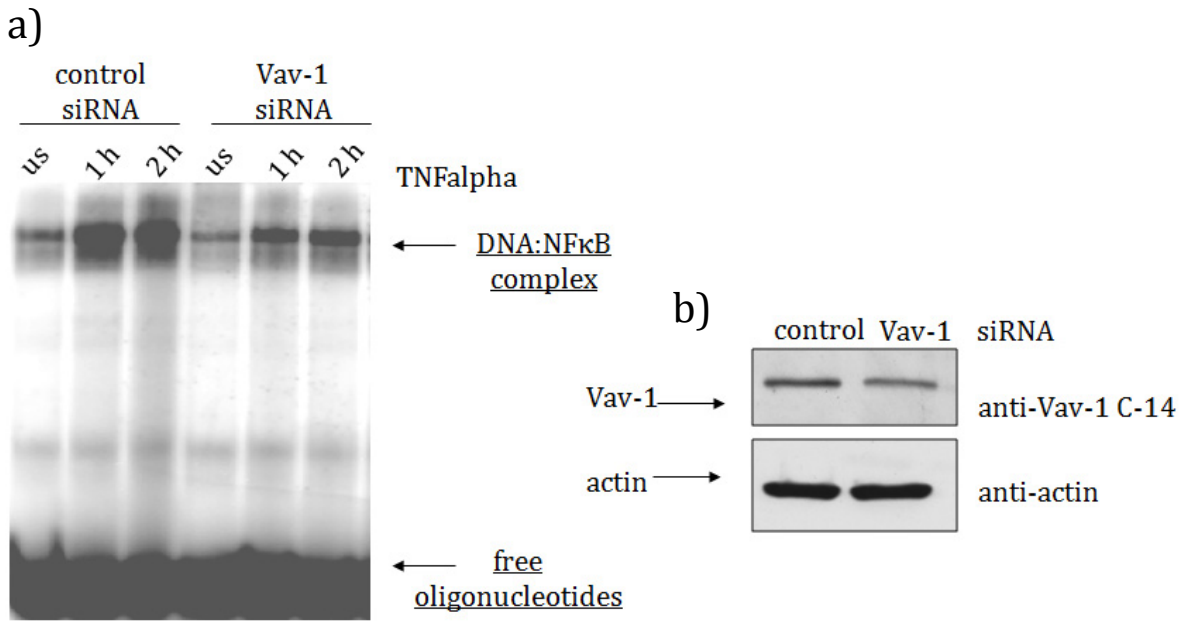

Figure 30: Employment of RNAi in mo-DC reveals, that Vav-1 expression is crucial for TNFalpha-induced signaling to NF-κB. RNAi was used to specifically inhibit Vav-1 protein expression in mo-DC. Using a special square wave electroporation protocol, human immature mo-DC were transfected with either 20µg control siRNA or with 20µg Vav-1 siRNA. 96h after transfection the cells were treated with 100ng/ml TNFalpha and nuclear extracts were prepared 1h or 2h after treatment. The nuclear extracts were incubated with a ^{32}p-labeled double-stranded oligonucleotide probe, containing the specific recognition sequence for NF-κB and were then assayed for NF-κB activation by EMSA. In control cells stimulation with TNFalpha results in robust activation of NF-κB, which is strongly reduced in Vav-1 knock-down mo-DC (a). According to this data, Vav-1 protein expression is involved in TNFalpha-induced signaling to NF-κB in human mo-DC. Specific knock-down of Vav-1 is shown in immunoblots (b).

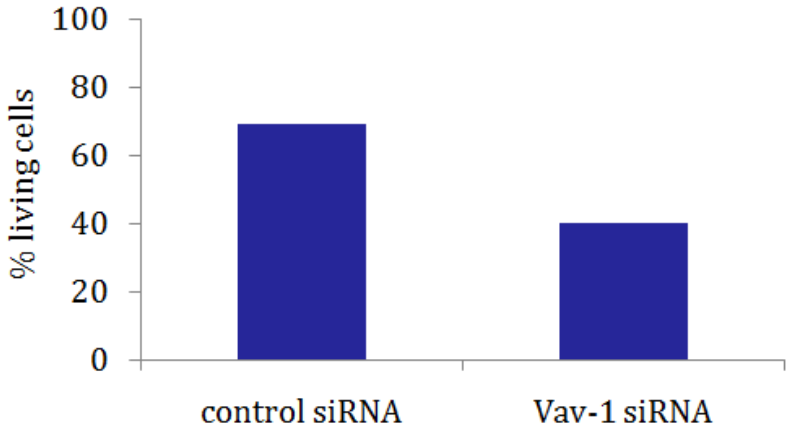

Figure 31: siRNA-mediated knock-down of Vav-1 induces cell death in response to TNFalpha treatment. Using a square wave electroporation protocol, immature mo-DC were transfected with either 20µg control or with 20µg Vav-1 siRNA. 48h later, respective cells were either stimulated with 100ng/ml TNFalpha for 48h to induce maturation. 96h after transfection, the number of viable cells was determined by a trypan blue exclusion assay. Since Vav-1 expression was shown to be crucial for TNFalpha-induced signaling to NF-κB, TNFR stimulation induces cell death of Vav-1 knock-down mo-DC.

3.2.5 VAV-1 EXPRESSION IS NOT REQUIRED FOR TNFALPHA-INDUCED ACTIVATION OF NF-KB IN HUMAN AND MURINE T-CELLS

Signaling from the TNF receptor to the NF-kB complex has been studied in great detail, and was shown to be essentially similar in most cell-types (see introduction). However, many studies report of cell-specific modulation of TNF-receptor functions, e.g. in controlling homeostatic versus inflammation induced-apoptosis functions of neutrophil granulocytes (see introduction). Since the apparent requirement of Vav-1 for TNFalpha signaling in mo-DC is a highly surprising finding, and because Vav-1 expression is largely restricted to immune cells, it was next analyzed whether Vav-1 is involved in TNFalpha-induced signaling in other immune cell-types, too.

Vav-1 signaling is of essential importance for the activation of T-cells by cognate antigen, and it was therefore next analyzed, whether the expression of this protein is also limiting for TNFalpha-induced activation of NF-κB in human T-cells. To this end, a

minimal (m)CMV-driven NF-κB-luciferase-reporter construct was used, which encodes the firefly luciferase reporter gene under the control of NF-κB response elements. TAg Jurkat T-cells were transiently transfected with this NF-κB-luciferase-reporter construct along with either control vector, or with expression constructs encoding the wild type Vav-1 protein (Vav-1 WT) respectively the GEF deficient, dominant-negative Vav-1 mutant L213A. As shown in figure 32a, stimulation of the control cells with 100ng/ml TNFalpha for 6h induces robust transcriptional activity of the NF-κB-dependent reporter gene. On the other hand, TNFalpha-induced activation of the NF-κB reporter is not increased in cells over-expressing Vav-1 WT, and, consistently, over-expression of the dominant-negative GEF mutant Vav-1 L213A does not interfere with the respective reporter gene activity either (figure 32a).

As positive control for the experimental approach, T-cells were co-transfected with an IL-2-luciferase-reporter construct along with either Vav-1 WT or Vav-1 L123A expression plasmids, because Vav-1 is an important mediator of TCR-induced IL-2 promoter activation. Thus, over-expression of the Vav-1 constructs is expected to have a marked impact on TCR-dependent signaling in this cell-type. The transfected TAg Jurkat T-cells were stimulated with 50ng/ml PMA and 2µg/ml anti-CD3 (OKT3) for 6h. Stimulation with PMA and anti-CD3 leads to a robust activation of IL-2 promoter activity, which is strongly increased in cells over-expressing Vav-1 WT. As expected, over-expression of the dominant-negative Vav-1 GEF mutant L213A completely blocks PMA-/anti-CD3-induced activation of the IL-2 promoter (figure 32b), showing that the GEF activity of Vav-1 is required for TCR-induced IL-2 promoter activation.

The employed positive control behaves as expected, suggesting that the experimental approach is in principle working and that Vav-1 expression is not required for TNFalpha-induced signaling to NF-κB complexes in human T-cells.

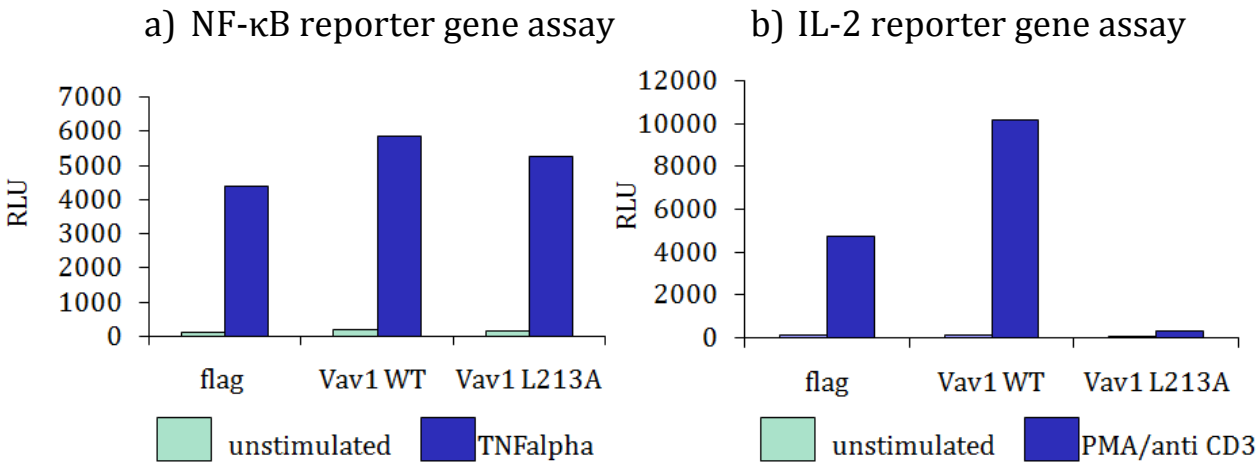

Figure 32: Vav-1 expression is not required for TNFalpha-induced activation of the NF-κB complex in human T-cells. TAg Jurkat T-cells were transfected with 10µg NF-κB-luciferase-reporter construct, together with either 10µg control vector, or with 10µg of expression constructs encoding the wild type Vav-1 protein (Vav-1 WT) respectively the GEF deficient, dominant-negative Vav-1 mutant L213A. 24h after transfection cells were stimulated with 100ng/ml TNFalpha for 6h. Stimulation of control cells induces robust transcriptional activity of the NF-κB-dependent reporter gene. TNFalpha-induced activation of NF-κB is not increased in cells over-expressing Vav-1 WT and over-expression of the dominant-negative GEF mutant Vav-1 L213A does not interfere with the respective reporter gene activity either (a). As positive control TAg Jurkat T-cells were transfected with 10µg IL-2-luciferase-reporter construct, together with 10µg of expression constructs encoding Vav-1 WT or Vav-1 L123A. Respective cells were stimulated with 50ng/ml PMA and 2µg/ml anti-CD3 (OKT3) for 6h. Stimulation with PMA and anti-CD3 leads to a robust activation of IL-2 promoter activity, which is strongly increased in cells over-expressing Vav-1 WT. As already published, over-expression of the dominant-negative mutant Vav-1 L213A completely blocks PMA-/anti-CD3-induced activation of the IL-2 promoter (b).

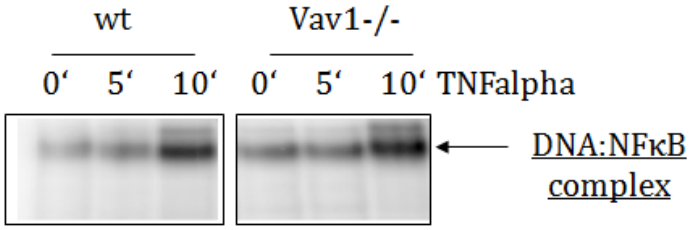

Figure 33: Vav-1 protein expression is not required for TNFalpha-mediated signaling to the NF-κB complex in murine splenic T-cells. "Untouched" CD4+/CD8+ T-cells were negatively isolated from single cell spleen preparations of six week old WT C57/B6 or Vav-1 knock-out C57/B6 mice, by magnetic separation, using the Pan T-cell isolation kit (Miltenyi). Activation of endogenous NF-κB in the isolated CD4+/CD8+ T-cells was measured with the help of electrophoretic gel shift assays. T-cells were stimulated for 5min or 10min with 100ng/ml murine TNFalpha. Nuclear extracts were prepared and incubated with a ^{32}p-labeled double-

TNFalpha-induced NF-κB activation was also analyzed in splenic T-cells derived from Vav-1 knock-out mice. "Untouched" $CD4^+/CD8^+$ T-cells were negatively isolated from single cell spleen preparations of six week old WT C57/B6 or Vav-1 knock-out C57/B6 mice by magnetic separation, using the Pan T-cell isolation kit (Miltenyi). In these isolated T-cells, the activation of endogenous NF-κB was measured, with the help of the EMSA technique. Cells were stimulated for 5min or 10min with 100ng/ml murine TNFalpha and nuclear extracts were prepared as described before. The resulting lysates were subsequently incubated with a ^{32}p-labeled double-stranded, NF-κB specific oligonucleotide probe and finally analyzed by electrophoresis. As shown in figure 33, stimulation with TNFalpha results in robust activation of NF-κB in WT and in Vav-1 knock-out T-cells, indicating that Vav-1 protein expression is not required for TNFalpha-induced activation of NF-κB in murine splenic T-cells.

Taken together, it was shown here that Vav-1 is important for TNFalpha-dependent mobilization of NF-κB in human mo-DC, but neither in human Jurkat T-cells, nor in murine splenic T-cells.

3.2.6 NORMAL TNFALPHA-INDUCED MATURATION OF VAV-1KNOCK-OUT BM-DC

According to the fact that Vav-1 is involved in TNFalpha-induced signaling to the NF-κB complex in human mo-DC, but not in human or murine T-cells, it was considered if Vav-1 is exclusively required for TNFalpha signaling in dendritic cells. For this reason, murine bone marrow derived dendritic cells (BM-DC) were analyzed for a possible involvement of Vav-1 in TNFalpha-induced DC maturation. BM-DC were prepared from bone marrow of six week old WT C57BL/6 mice and Vav-1 knock-out C57BL/6 mice and incubated in GM-CSF-containing culture medium for 10 days. The BM-DC were then stimulated with 100ng/ml TNFalpha for two days. Cell surface expression of maturation markers was measured by flow-cytometry. As shown in figure 34, in WT BM-DC TNFalpha stimulation leads to a robust up-regulation of the intercellular adhesion molecule 1 (ICAM-1,CD54) and to a slight up-regulation of the co-stimulatory molecule CD86, but not of CD80 (data not shown). TNFalpha-induced up-regulation of CD86 and CD54 is not altered in Vav-1 knockout BM-DC, as compared to WT BM-DC (figure 34).

Hence, Vav-1 appears to be specifically involved in TNFalpha-induced signaling pathways of human mo-DC.

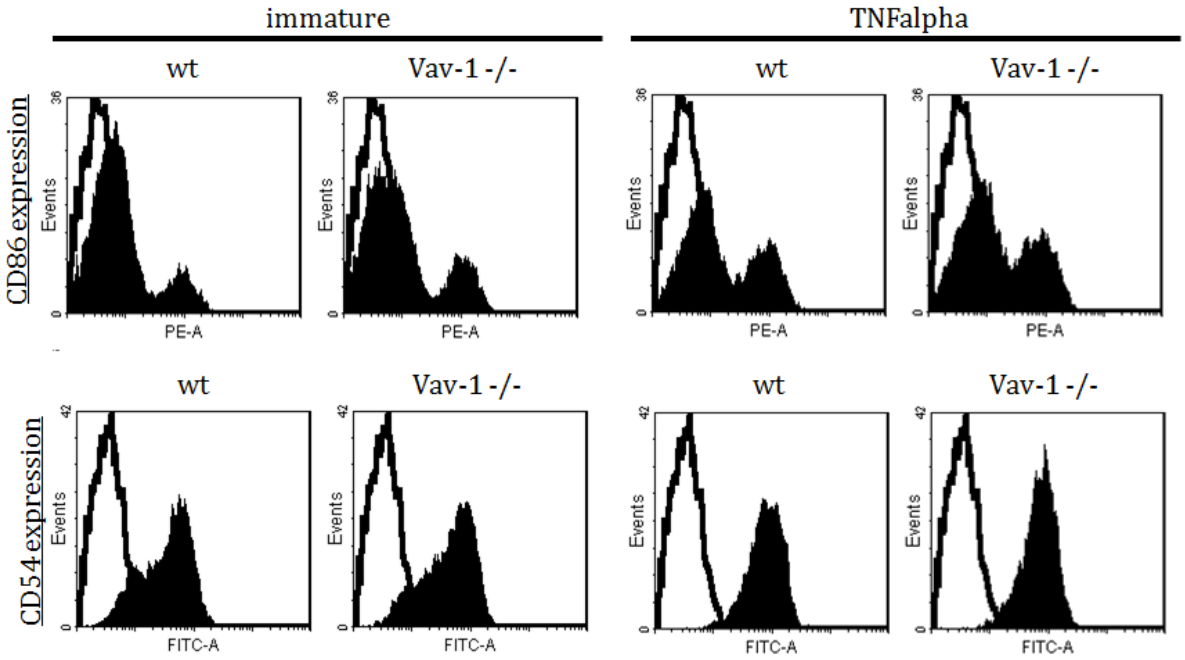

Figure 34: TNFalpha-dependent up-regulation of CD54 and CD86 is not impaired in Vav-1 knock-out BM-DC. BM-DC were prepared from bone marrow of six week old WT C57BL/6 mice and Vav-1 knock-out C57BL/6 mice and incubated in GM-CSF-containing culture medium for 10 days, before stimulating them with 100ng/ml TNFalpha for two days. Cell surface expression of CD54 and CD86 was measured by flow-cytometry. Treatment of control cells with TNFalpha results in strong up-regulation of CD54 and slight up-regulation of CD86. TNFalpha-stimulated up-regulation of both tested markers is not impaired in Vav-1 knock-out BM-DC, compared to WT BM-DC.

3.2.7 Normal TNFalpha-induced activation of NF-κB in Vav-1/2/3 knock-out MEFs

According to the presented data, Vav-1 expression is not important for TNFalpha-mediated signaling, in all tested immune cell-types, excepting human mo-DC. To finally exclude that this is due to a functional redundancy of the Vav family proteins, which masks a possible role of Vav-1 in TNFalpha-dependent signaling pathways, TNFalpha-induced NF-κB activation was analyzed in murine embryonic fibroblasts (MEFs), lacking all three Vav family members. WT and Vav-1/2/3 knock-out MEFs were stimulated for

5min, 10min or 15min with 100ng/ml murine TNFalpha to induce the activation of NF-κB. Nuclear extracts were prepared as described above and then assayed for NF-κB-mediated electrophoretic retardation of the labeled probe by EMSA. As presented in figure 35, stimulation with TNFalpha results in robust activation of NF-κB in WT control cells. A complete knock-out of all Vav family proteins does not impair TNFalpha-induced signaling to the NF-κB complex (figure 35). This experiment clearly eliminates that functional redundancy between Vav-1, Vav-2 and Vav-3 masks a possible role of Vav-1 in TNFalpha signaling in other cell-types, and supports the claim that Vav-1 is exclusively required for TNFalpha-mediated signaling in human mo-DC.

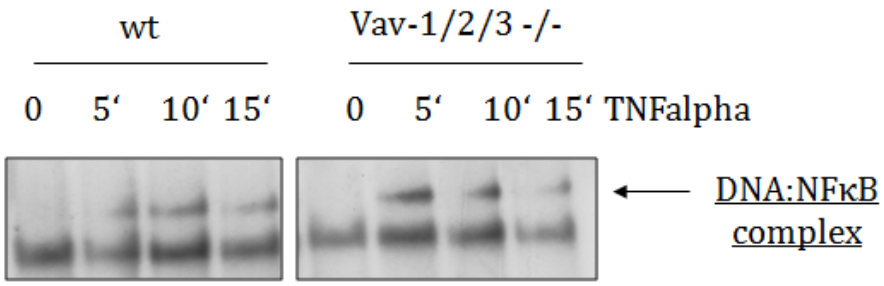

Figure 35: Vav proteins are not required for TNFalpha-induced signaling to NF-κB in murine embryonic fibroblast. The activation of endogenous NF-κB was measured with the help of electrophoretic gel shift assays (EMSA). WT MEFs and Vav-1/2/3 knock-out MEFs were stimulated for 5min, 10min or 15min with 100ng/ml murine TNFalpha. Nuclear extracts were prepared and incubated with a ^{32}p-labeled double-stranded oligonucleotide probe, containing the specific recognition sequence for NF-κB. Nuclear extracts were then assayed for NF-κB-mediated electrophoretic retardation. Stimulation with TNFalpha results in strong activation of NF-κB in WT MEFs, which is not decreased in Vav-1/2/3 knock-out MEFs. This analysis finally eliminates that a functional redundancy of the Vav family members masks a possible role of Vav-1 in TNFalpha-dependent signaling pathways in cell-types other than mo-DC.

3.2.8 DECTIN-1-MEDIATED BM-DC MATURATION STRONGLY DEPENDS ON SYK- BUT IS INDEPENDENT OF VAV-1 EXPRESSION

The C-type lectin receptor dectin-1 has a key role in coordinating the responses of macrophages and dendritic cells to fungal pathogens. Dectin-1 has an immunreceptor tyrosine-based activation motif (ITAM) and is able to trigger the phosphorylation of the Syk tyrosine kinase. It acts as a major receptor for zymosan, curdlan other ß-glucans and signals via the Syk kinase to regulate expression of innate response genes (see introduction). Recognition of zymosan or curdlan by dectin-1 induces the terminal differentiation ("maturation") of dendritic cells, which is characterized by robust cell surface expression of MHC-peptide complexes and of so-called co-stimulatory molecules.

Because of the demonstrated involvement of the Syk tyrosine kinase in dectin-1-mediated signaling pathways, a possible role of the guanine nucleotide exchange factor Vav-1 downstream of dectin-1 and Syk was investigated here. BM-DC were analyzed for a possible involvement of Vav-1 in zymosan- or curdlan-induced maturation. To this end, BM-DC were prepared from bone marrow of six week old WT C57BL/6 mice and Vav-1 knock-out C57BL/6 mice and incubated in GM-CSF-containing culture medium for 10 days, before they were stimulated with 100µg/ml curdlan or 100µg/ml zymosan for 24h. Cell surface expression of maturation markers was subsequently measured by flow-cytometry. In WT BM-DC, treatment with curdlan or zymosan induces an up-regulation MHC class II protein expression and an up-regulation of the co-stimulatory molecules CD80 and CD86 (figure 36). Neither zymosan- nor curdlan-induced up-regulation of MHC II, CD80 and CD86 protein expression is altered in Vav-1 knock-out BM-DC, in comparison to WT BM-DC (figure 36). As positive control for these assays, WT BM-DC were pre-treated with 25µM Syk kinase inhibitor piceatannol 2h before stimulation with curdlan or zymosan. Inhibition of the tyrosine kinase Syk abrogates curdlan- or zymosan induced up-regulation of MHC II, CD80 and CD86 surface expression (figure 37). These findings indeed indicate that dectin-1-mediated signaling pathways, leading to BM-DC maturation, are Syk-dependent but unexpectedly independent of Vav-1 protein expression.

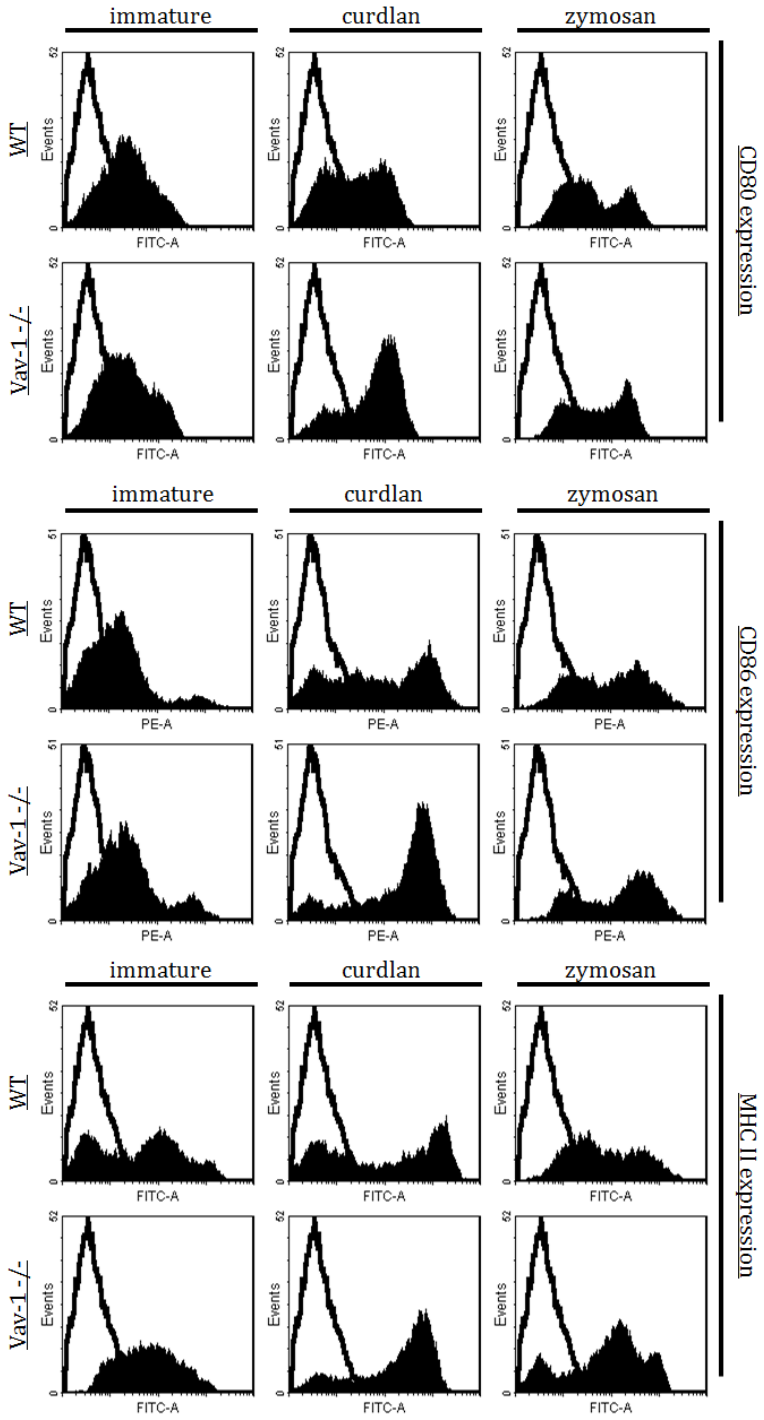

Figure 36: Vav-1 expression is not crucial for dectin-1 mediated BM-DC maturation. BM-DC were prepared from bone marrow of WT C57BL/6 mice and Vav-1 knock-out C57BL/6 mice and incubated in GM-CSF-containing medium for 10 days, before they were stimulated with 100µg/ml zymosan or curdlan. 24h later cell surface expression of maturation markers was measured by flow-cytometry. Treatment with curdlan respectively zymosan results in robust up-regulation of MHC II, CD80 and CD86 surface expression in WT BM-DC and in Vav-1 knock-out BM-DC.

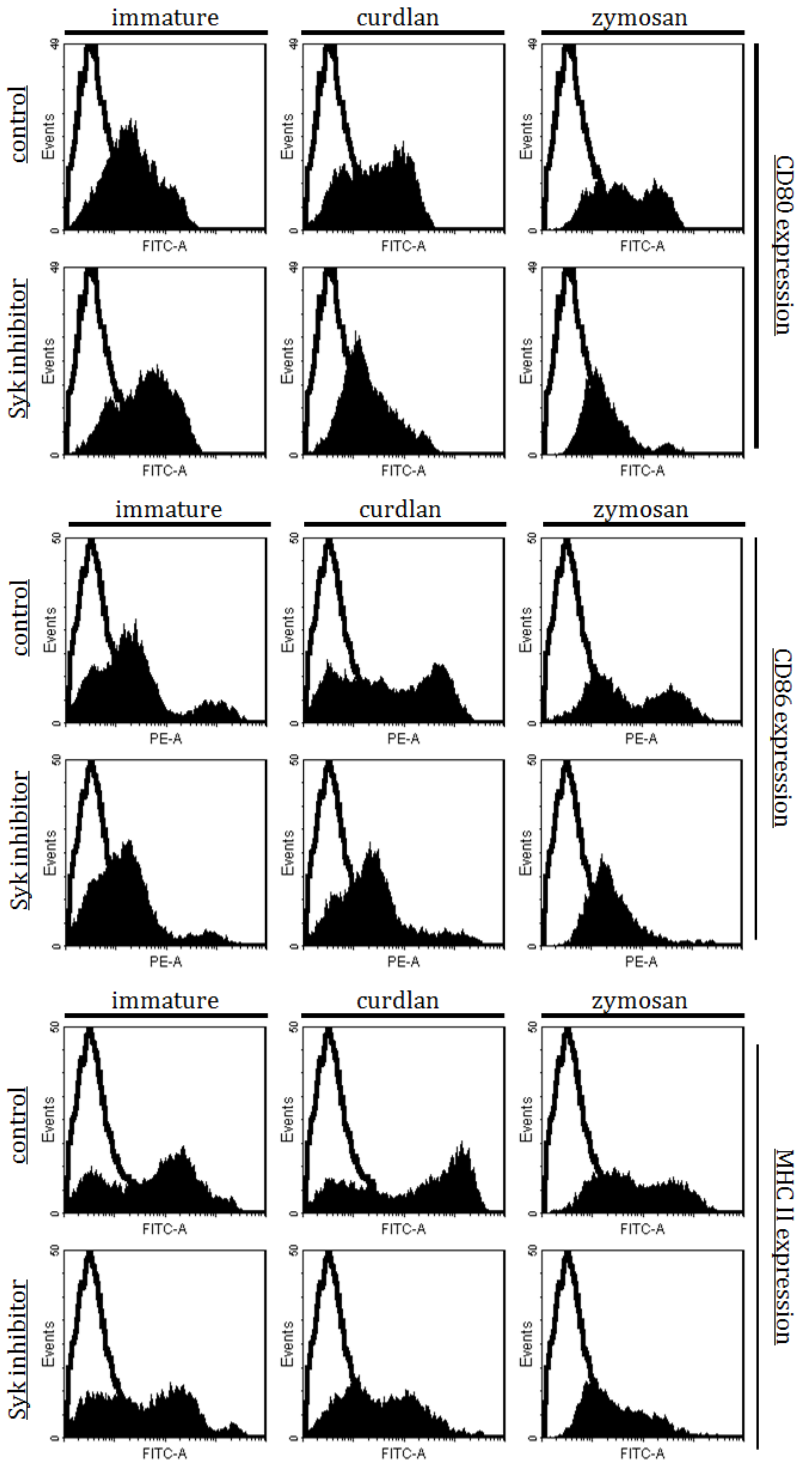

Figure 37: Curdlan- and zymosan-induced BM-DC maturation strongly depends on Syk activity. WT BM-DC were pre-incubated with 25µM Syk kinase inhibitor piceatannol 2h before treatment with curdlan or zymosan for 24h. Cell surface expression of maturation markers was afterwards analyzed, using flow-cytometry. Inhibition of the tyrosine kinase Syk abrogates curdlan- and zymosan induced up-regulation of MHC II, CD80 and CD86 surface expression.

4. DISCUSSION

4.1 THE ROLE OF CYTOHESIN-1 IN REGULATING T-CELL RECEPTOR SIGNALING AND ADHESION AND MIGRATION PROCESSES

4.1.1 CYTOHESIN-1 MEDIATES TCR-INDUCED AP-1 ACTIVATION THROUGH THE REGULATION OF THE P38/JNK PATHWAY

This study describes an important role of the guanine nucleotide exchange factor cytohesin-1 in the regulation of intracellular signaling pathways during T-cell activation. It was recently demonstrated that the GEF activity of cytohesin-1 is crucial for activation of the IL-2 promotor upon TCR ligation (Paul, PhD thesis 2007). The IL-2 promoter contains binding sites for several transcription factors, regulating IL-2 gene transcription. These transcription factors include NF-κB, NFAT, AP-1, the octamer binding protein (Oct) and the CD28 response element (CD28RE) (Serfling et al., 1995). Recent over-expression analysis indicated that cytohesin-1 is specifically important for TCR-induced activation of the transcription factor AP-1 (Paul, PhD thesis 2007), a transcription factor complex consisting of homo- or heterodimers of Fos/Jun family proteins. In this study, these data were confirmed and substantiated by use of RNAi, to specifically inhibit cytohesin-1 protein expression in human T-cells. It could be shown here that AP-1 transcriptional activity is strongly inhibited when cytohesin-1 expression was decreased by RNAi (figure 13a).

Downstream of the TCR, two different MAP kinase pathways control the phosphorylation of Fos/Jun family proteins and were shown to be essential for the formation of an active AP-1 complex. One pathway is regulated by ERK1 and ERK2, whereas the second pathway involves the mitogen-activated protein kinases p38 and JNK (Whitmarsh et al., 1996). As demonstrated in this study, cytohesin-1 mediates positive regulation of AP-1 by activating the p38/JNK- but not the ERK1/ERK2-branch of

the MAP kinase signaling cascade (figure 15 and 16). The ability to activate the p38/JNK signaling branch, strictly depends on its GEF activity, as over-expression of the dominant negative GEF mutant cytohesin-1 E157K completely blocks TCR-induced phosphorylation, and thus activation of p38 (figure 17). To eliminate over-expression artifacts, these data were also confirmed by the use of two different chemical cytohesin GEF inhibitors: the small molecule SecinH3 (Hafner et al., 2006) and the recently identified compound 16 which had been modeled by chemo-informatic methods to improve target affinity and selectivity of SecinH3 (Bajorath et al., in preparation). Whereas TCR-induced signaling to p38 was decreased by SecinH3 treatment, inhibition of the GEF function of cytohesin by compound 16, completely abrogates TCR-induced phosphorylation of p38 (figure 18). These results confirm and corroborate the assumption that an intact GEF function of cytohesin-1 is essential for the activation of p38, and thus of AP-1. Furthermore, it was experimentally confirmed that compound 16 is a novel potent cytohesin family GEF inhibitor.

Taken together, cytohesin-1 expression positively regulates TCR-induced transcriptional activation of the IL-2 gene via signaling to p38, and thus by activating AP-1. Consistently, inhibition of the p38 signaling cascade down-regulates IL-2 promoter activity and IL-2 production in Jurkat T-cells (Smith et al., 2003) as well as IL-2 production in primary murine T-cells (Zhang et al., 1999).

How does cytohesin-1 regulate TCR-induced activation of the p38/JNK MAP kinase cascade? Cytohesin proteins are defined by characteristic protein domains, including a sec-7 homology domain required for GEF activity, a coiled-coil region and a pleckstrin homology domain for membrane localization through binding to phosphatidylinositols. It has previously been shown that the cytokine-inducible protein CYTIP (also known as Cybr or CASP) interacts with cytohesin-1, and that this interaction is mediated by the homotypical coiled-coil domains of the two proteins (Tang et al., 2002). Through this interaction, CYTIP is able to regulate cytohesin-1's GEF activity for ARF GTPases (Tang et al., 2002). CYTIP mRNA and protein levels are increased upon TCR engagement, suggesting a possible role of the protein in intracellular signaling pathways during T-cell activation (Chen et al., 2006). It was indeed demonstrated that over-expression of CYTIP

results in augmented transcriptional activity of AP-1 by activating JNK and p38 MAP kinases (Chen et al., 2006). Therefore, one likely possibility is that upon TCR engagement CYTIP interacts with cytohesin-1, which leads to activation of the GEF activity of cytohesin-1, enabling its signaling to p38/JNK and thus to AP-1.

Activation of the p38/JNK MAPK cascade has been shown to require phosphorylation of the guanine nucleotide exchange factor Vav-1 by Zap-70, and subsequent activation of Rac-1 and Cdc42. CD28 co-stimulation augments the recruitment of Vav-1 to LAT and Zap-70 and increases Zap-70-mediated Vav-1 phosphorylation (Salojin et al., 1999). Rac-1 and Cdc42 elicit the p38 MAPK cascade via the downstream mediator PAK, although the exact mechanism remains unclear, as PAK does not directly activate a MKKK (Zhang et al., 1995). CYTIP was recently implicated in the activation of Vav-1, since over-expression of CYTIP results in increased Vav-1 phosphorylation (Chen et al., 2006). Therefore, it was investigated here, whether cytohesin-1 is also involved in the phosphorylation and thus in the activation of Vav-1. However, conversely to what has been shown for CYTIP, TCR-induced phosphorylation of Vav-1 does not require cytohesin-1 function (figure 19). These results clearly indicate that cytohesin-1 acts either downstream of Vav-1 or acts in a pathway which alternatively regulates the phosphorylation of p38 and JNK, and thus the activation of the transcription factor AP-1.

Additional evidence supports the notion of positioning cytohesin-1 downstream of Vav-1. Cytohesin-1 bears phosphorylation sites at the C-terminal polybasic region, and its phosphorylation was shown to be essential for activation of the IL-2 promotor upon TCR-ligation (Paul, PhD thesis 2007), probably by enhancing the GEF activity of cytohesin and/or by creating docking sites for downstream signaling molecules. Cytohesin-1 is phosphorylated by protein kinase C, which gets activated during T-cell activation by PLC-γ in a Vav-1 dependent manner (see introduction). For this reason, one would expect an impaired cytohesin-1 phosphorylation/activation in cells lacking Vav-1. Cytohesin-1 might therefore considered to be an indirect downstream target of active Vav-1, and it is therefore unlikely, that cytohesin-1 is located upstream of Vav-1, by mediating its phosphorylation.

The assumption that cytohesin-1 is located downstream of Vav-1 is further supported by the finding that cytohesin-1 is specifically involved in the activation of p38, JNK and AP-1. In cytohesin-1 knock-down cells, we see no effect on ERK1/2 activation and also no effect on NF-κB activation, which are both downstream targets of Vav-1. If cytohesin acts as central activator of Vav-1, one would expect a more global T-cell phenotype, similar to that of Vav-1 lacking T-cells.

Additionally, there is evidence against a "co-signaling" of cytohesin-1 and CYTIP downstream of the TCR. Cytohesin-1 dependent phosphorylation of p38 occurs 5min to 30min after TCR cross-linking. However, at this early stage CYTIP mRNA and protein expression are barely detectable in PBMC and undetectable in Jurkat T-cells (Tang et al., 2002, Chen et al., 2006). In freshly isolated PBMC, CYTIP mRNA was shown to be induced 1h after TCR cross-linking and was enhanced again after 24h. In Jurkat T-cells, CYTIP mRNA expression was observed after 2h and peaked after 6h of TCR stimulation. CYTIP protein levels were detected in PBMC not earlier than 8h-16h after stimulation and in Jurkat T-cells 2h-24h after treatment with anti-CD3 (Chen et al., 2006). Taken together, this knowledge leads to the following signaling model: TCR stimulation induces activation of cytohesin-1, mediating the phosphorylation of p38 and JNK, by acting independently from CYTIP and downstream of Vav-1 (figure 38a). At later points in time, TCR stimulation induces CYTIP protein expression. CYTIP then augments TCR-stimulated JNK, p38 and thus AP-1 activation by increasing Vav-1 phosphorylation (figure 38b). Since CYTIP expression was shown to be preferentially elevated in differentiated T helper 1 (Th1) cells (Tang et al., 2002), it is possible that this CYTIP-mediated late phase activation of p38/JNK and AP-1 is responsible for Th1 cell differentiation. Consistent with this, IL-2 production of Th1 cells was shown to be dependent of AP-1 and NFAT, whereas AP-1 is not required for IL-4 production and Th2 cell differentiation (Rooney et al., 1994). It is still unacquainted how CYTIP expression preferentially activates the p38/JNK- and not the ERK1/2 MAP kinase branch.

Recently, two reports described knock-out mice, in which the CYTIP gene had been targeted by homologous recombination. Surprisingly, the phenotypes of these independently generated mutant mice are inconsistent. The CYTIP knock-out mouse

published by Watford et al. has no defect in T-cell functions, including proliferation, cytokine secretion and T helper cell differentiation (Watford et al., 2006). The lack of a T-cell phenotype was assumed to be due to redundancy by functionally similar molecules (e.g. tamalin or the GRP-1-associated protein 1) or to species differences between humans and mice.

However, at the same time Copolla et al. found that their CYTIP knock-out mice showed deficiencies in inflammatory immune cell trafficking (Copolla et al., 2006). One potential explanation for these inconsistent phenotypes is that loss of CYTIP expression affects immune functions only during severe systemic inflammation, as e.g. during aseptic peritonitis (Copolla et al., 2006). Alternatively, differences in gene targeting strategies and/or in the genetic background might be responsible for the observed discrepancies.

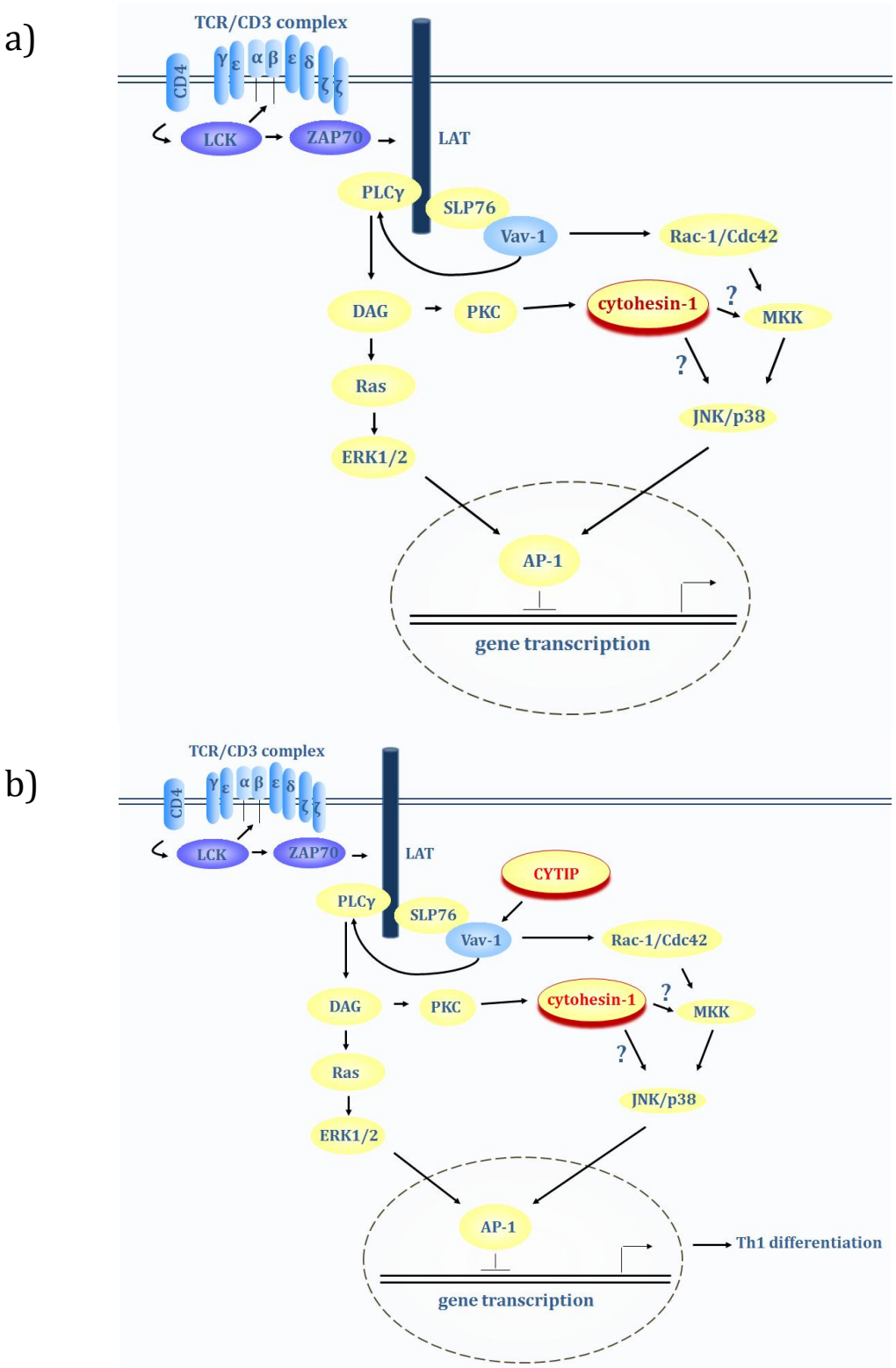

Figure 38: Cytohesin-1 mediates TCR-induced AP-1 activation through regulation of the p38/JNK pathway (a). TCR cross-linking induces the expression of CYTIP, which then augments TCR-stimulated JNK, p38 and thus AP-1 activation, by increasing Vav-1 phosphorylation. CYTIP-mediated late phase activation of p38/JNK and AP-1 is probably responsible for Th1 cell differentiation (b).

4.1.2 THE ROLE OF CYTOHESIN-1 IN REGULATING IMMUNE CELL MIGRATION AND ADHESION

Besides its role in T-cell activation, cytohesin-1 plays an important role in signaling pathways, which affect the regulation of immune cell adhesion and migration. Cytohesin-1 was originally identified as a ß2-integrin binding protein, which when over-expressed induces activation of LFA-1-mediated adhesion in Jurkat T-cells (Kolanus et al., 1996). Recently, it has been demonstrated that integrin-dependent dendritic cell migration in 3D environments, strongly depends on cytohesin-1 function *in vitro* and *in vivo* (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). Furthermore, cytohesin-1 acts as an important regulator of ß2-integrin mediated adhesion, since RNAi of cytohesin-1 markedly reduces static adhesion of human mo-DC to ICAM-1 (Tappertzhofen, PhD thesis 2007, Quast et al., 2009). In the present study, it was demonstrated that the GEF activity of cyohesin-1 is required for regulating adhesive events of dendritic cells. To address this issue, a novel protocol was established, which enables for the first time over-expression analysis with human mo-DC. It could be shown that static cell adhesion to ICAM-1 is markedly enhanced in GFP-positive human mo-DC over-expressing cytohesin-1 WT, but not in mo-DC over-expressing the GEF-deficient cytohesin-1 E157K mutant (figure 21).

4.1.3 CONCLUSION AND OUTLOOK

Besides its known role in T-cell adhesion (Kolanus et al., 1996) cytohesin-1 signaling appears to be critically involved in T-cell activation (shown here and Paul, PhD thesis 2007) and in the control of dendritic cell migration and adhesion (shown here and Tappertzhofen, PhD thesis 2007, Quast et al., 2009). However, there are several unanswered questions, e.g. which molecular signaling complex enables cytohesin-1 to influence T-cell receptor signaling and cytoskeletal reorganization during integrin-dependent adhesion and migration. A possible candidate is the WASP/SLP-76/Nck/Vav-

1 protein complex which links the T-cell receptor signaling machinery to the actin cytoskeleton. Upon T-cell receptor stimulation by foreign antigen, SLP-76 and Vav-1 are recruited to the TCR and subsequently activated by phosphorylation. The phosphorylation of SLP-76 creates binding sites for Nck. Nck functions to recruit WASP, which is activated in a Vav-1 dependent fashion, by binding to Cdc42-GTP. Activated WASP induces Arp2/3-dependent actin polymerization at the T-cell-APC contact site (Zeng et al., 2003). WASP is linked to the actin cytoskeleton through direct binding to the Arp2/3 complex (Machesky and Insall, 1998). It therefore connects the T-cell receptor signaling machinery to the actin cytoskeleton and enables cytoskeletal reorganization in the course of T-cell activation which is required for the induction of specific immune responses. However, in co-precipitation experiments, a direct interaction of cytohesin-1 and WASP (figure 20) or of cytohesin-1 and Vav-1 (not shown) was not observed. A possible association of cytohesin-1 with other intracellular immune signaling components, e.g. SLP-76, Nck or LAT needs to be investigated.

Another likely possibility is that cytohesin-1 does not interact directly with classical components of the TCR machinery, but mediates its effects by binding and activating the ß2-integrin LFA-1, which co-stimulatory activity probably increases activation of the p38/JNK MAP kinase signaling branch. A co-stimulatory contribution of adhesion receptors to different signaling pathways, e.g. to TNFalpha signaling, had often been debated, but the mechanism remained unclear and requests further research (see introduction).

One protein which probably links cytohesin-1-mediated integrin signaling to p38/JNK MAP kinase signaling is the small GTPase RhoA. Recently, a study published by Quast et al. positioned cytohesin-1 upstream of RhoA, in directing ß2-integrin-dependent cell adhesion and migration (Quast et al., 2009). Cytohesin-1 was shown to control the activation of RhoA in primary dendritic cells and in Hela cells (Quast et al., 2009). RhoA is a small GTPase which links integrin signaling to actin cytoskeletal rearrangements in many cell-types. Notably, there is also evidence that activated RhoA can elicit the p38/JNK signaling cascade. Whitmarsh and coworkers have demonstrated that RhoA activates the endogenous JNK and p38 MAP kinase signaling pathways in CHO cells

(Whitmarsh et al., 1997). Furthermore, rhinoviral infections activate p38 MAP kinases via RhoA. Dumitru et al. have showm that inhibition of RhoA, by transfection of transdominant inhibitory T19N RhoA, reduces activation of p38 upon infection of Hela cells with the human rhinovirus strain 14 (RV14) (Dumitru et al., 2006). Additionally, RhoA is able to potently stimulate the expression of c-Jun and the activity of the c-Jun promoter, by selectively activating p38γ in NIH-3T3 cells (Marinissen et al., 2001). Rho GTPases cycle between an active GTP-bound and an inactive GDP-bound state, and the active GTP-loaded enzyme selectively interacts with downstream effector molecules. Recent efforts led to the identification of a number of RhoA effectors, including the RhoA-binding kinase (ROKα), the protein kinase N (PKN) and the PKN-related protein rhophilin (Leung et al., 1995, Watanabe et al., 1996, Amano et al., 1996). Whether these RhoA effector proteins mediate signaling to the JNK and p38 MAP kinases has not been established yet. RhoA also gets activated upon TCR stimulation and plays an important role in T-cell biology, e.g. by controlling thymocyte development and T-cell adhesion and migration (Cantrell, 2003). Although, a function of RhoA in TCR-induced p38/JNK- and thus AP-1 activation remains to be shown, cytohesin-1 might activate RhoA during T-cell activation, and activated RhoA might then elicit the p38/JNK signaling cascade, leading to AP-1 mediated gene transcription.

Thus far, four members of the cytohesin family have been identified: cytohesin-1, cytohesin-2 (also ARNO), cytohesin-3 (also Grp-1 in humans, *Steppke* in *Drosophila*) and cytohesin-4. Although they share a highly similar domain organization, functional differences between individual family members are emerging. Interestingly, over-expression of cytohesin-3, which lacks the C-terminal serine phosphorylation site present in cytohesin-1, represses AP-1- and thus IL-2-promoter activation, suggesting that this highly related protein is a direct antagonist of cytohesin-1 (Paul, PhD thesis 2007). Consistently, the inhibition of T-effector cell proliferation by $CD25^+$ $FoxP3^+$ regulatory T-cells is specifically abrogated in cytohesin-3 knock-down effector cells (Grell et al., in preparation). Fully in line with all the findings it was demonstrated that cytohesin-3 expression is strongly up-regulated in various types of anergic T-cells (Korthäuer et al., 2000, Grell et al., in preparation). Cytohesin-3 thus acts as an

endogenous regulator of tolerance induction/maintenance probably through functional inhibition of the cytohesin-1/AP-1 signaling axis (Paul, PhD thesis 2007, Grell et al., in preparation). Whether cytohesin-3 mediates its antagonistic signaling effects by inhibiting p38 and JNK phosphorylation needs to be examined.

4.2 A NOVEL, UNEXPECTED ROLE OF THE GUANINE NUCLEOTIDE EXCHANGE FACTOR VAV-1 IN INNATE IMMUNITY

4.2.1 EXPRESSION OF VAV-1 IS CRUCIAL FOR TNFALPHA-INDUCED MATURATION OF HUMAN MO-DC

Members of the Vav family of Rho guanine nucleotide exchange factors are thought to control a diverse array of signaling pathways emanating from antigen receptors in lymphocytes, although the exact mechanism by which this GEF exerts its function is only beginning to emerge. Among the diverse functions of Vav family proteins the role of Vav-1 in T-cell signaling is best understood. Vav-1 was shown to regulate cytoskeletal rearrangement during T-cell activation, and mediates diverse other cellular functions, including activation of JNK, ERK, Ras, NF-κB and NFAT pathways (see introduction for details).

Whereas Vav-1 has been known for a long time to be essential for the formation of the adaptive immune system, less information is available about a potential role of the protein in regulating innate immune responses. Innate immunity plays a critical role in first line host defense against invading pathogens. Although the innate immune system lacks the specificity of adaptive immunity, it is also able to differentiate between self and nonself, and this is achieved by receptors that recognize highly conserved microbial structures, so-called pathogen-associated molecular patterns (PAMPs). Recognition of microbe-specific molecules by these pattern recognition receptors activates defense pathways, including the activation of the complement system, recruitment of immune cells to sites of infection, inflammation, and finally activation of the adaptive immune

system. Dendritic cells are the most potent antigen-presenting cells of the immune system. Binding to pathogen-borne structures induces their terminal differentiation ("maturation"), which is characterized by robust cell surface expression of antigen-presenting MHC peptide complexes and of so-called co-stimulatory molecules. Dendritic cell maturation is further accompanied by a strong stimulation of their migratory capacity. Recently, it has been shown that Vav-1 is involved in signaling pathways leading to functional and morphological differentiation of human mo-DC, since RNAi-mediated knock-down of Vav-1 strongly impairs LPS/TNFalpha-induced maturation (Nickel, diploma thesis 2005). Vav-1 silenced mo-DC exhibit an immature phenotype in morphology, and furthermore, LPS/TNFalpha-dependent up-regulation of characteristic maturation markers as CD40, the MHC class II protein HLA-DR and the co-stimulatory molecules CD80 and CD86 is decreased in these cells (Nickel, diploma thesis 2005).

Although binding of LPS to Toll-like receptor 4 (TLR4) and binding of TNFalpha to TNF receptors (TNFR1 and TNFR2) eventually result in NF-κB activation, intracellular signaling pathways from Toll-like and TNFalpha receptors are quite distinct (see introduction). It was therefore investigated in the present study, whether RNAi of Vav-1 interferes with LPS- or with TNFalpha-induced maturation of dendritic cells, or if Vav-1 expression is important for both signaling pathways. The presented data clearly show that Vav-1 expression is crucial for TNFalpha-, but interestingly enough not for LPS-induced maturation of human mo-DC (figure 24 and 25).

The finding that Vav-1 is not required for LPS-induced maturation of human dendritic cells was surprising, because it was recently demonstrated that Vav proteins are required for B-cell responses to LPS (Hebeis et al., 2005). Activation of B-cells by LPS triggers immunoglobulin (Ig) secretion, Ig class switching and promotes the ability of B-cells to function as antigen-presenting cells, by increasing expression of MHC class II and co-stimulatory molecules (Yuan et al., 1983, Hathcock et al., 1994, Lenschow et al., 1996). B-cells express two receptors that function as recognition and signal transducing receptors for LPS: TLR-4 and CD180 (previously called RP105), which both contain a leucine-rich extracellular domain (Nagai et al., 2002). Both receptors work in concert to control B-cell recognition and signaling of LPS. It has been shown that signaling

pathways activated by CD180 require the function of Vav-1, while LPS signaling through TLR-4 can bypass the loss of Vav-1, presumably by utilizing Vav-2 (Hebeis et al., 2005). The redundancy between Vav family proteins might possibly explain, why siRNA-mediated knock-down of Vav-1 did not affect LPS-depended up-regulation of maturation markers in human mo-DC. Hence, the contribution of Vav-2 and Vav-3 to LPS-mediated mo-DC maturation was investigated to exclude that functional redundancy masks a possible role of Vav proteins in LPS signaling of dendritic cells. However, as depicted in figure 23, knock-down of Vav-2 and Vav-3 does not affect mo-DC maturation.

Hebeis and co-workers analyzed the involvement of Vav proteins in LPS-mediated signaling pathways only in murine B-cells (Hebeis et al., 2005). To rule out that species differences are responsible for the observed discrepancies, LPS-induced maturation was also analyzed in murine BM-DC, which were isolated from Vav-1 knock-out mice. Similar to what is shown here for Vav-1 knock-down mo-DC, LPS-induced maturation of Vav-1 deficient murine BM-DC is not impaired, too (data not shown). Notably, Vav proteins seem to be specifically required for B-cells to respond efficiently to LPS.

However, the presented results clearly demonstrate that Vav-1 regulates TNFalpha-dependent signaling pathways in human mo-DC. Employment of RNAi in mo-DC revealed that Vav-1 expression is crucial for TNFalpha-induced surface expression of the co-stimulatory molecules CD40, CD80 and CD86 (figure 24). Consistent with this, microarray-based transcriptome analysis show that siRNA-mediated knock-down of Vav-1 leads to reduced expression of TNFalpha target genes (figure 28).

Vav proteins are guanine nucleotide exchange factors which bear catalytic GEF activity towards a broad range of Rho/Rac family GTPases (Crespo et al., 1997). Although Vav proteins might regulate signaling cascades in a GEF-independent manner, most of the affected cellular responses, including regulation of gene transcription, cell proliferation, survival, migration and differentiation depend on an intact GEF function (Turner and Billadeau, 2002). To analyze, whether Vav-1 regulates TNFalpha signaling in a GEF-independent or in a GEF-dependent manner, Vav-1 GEF activity was inhibited during TNFalpha-induced mo-DC maturation, using the small molecular nucleotide analog 6-

Thio-GTP. Employment of this indirect but highly selective Vav GEF inhibitor reveals that TNFalpha-induced up-regulation of surface markers during human mo-DC maturation strongly depends on an intact Vav-1 GEF domain (figure 27).

The interaction of TNFalpha with its cognate receptors leads to activation of survival factors or of pro-inflammatory genes through the transcription factor NF-κB, a heterodimeric transcription factor complex, consisting of Rel family proteins. In dendritic cells, TNFalpha-induced activation of NF-κB leads to e.g. the expression of maturation markers, such as the co-stimulatory molecules CD40, CD80 and CD86. Since up-regulation of these markers is impaired in TNFalpha treated Vav-1 knock-down mo-DC, one likely possibility is that Vav-1 is involved in the activation of NF-κB. Using electrophoretic mobility gel shift assays, it was indeed shown here that Vav-1 is important for TNFalpha-dependent mobilization of NF-κB in human mo-DC (figure 30).

Under conditions in which protein synthesis is blocked, or in which NF-κB activation is inhibited, TNFalpha stimulation leads to a strong induction of apoptosis via recruitment of the adaptor protein FADD (Hsu et al., 1996). Vav-1 expression is crucial for TNF-induced signaling to NF-κB, and this finding implies that TNFR stimulation leads to strong induction of apoptosis in Vav-1 knock-down mo-DC. Consistently, siRNA-mediated knock-down of Vav-1 was shown to induce cell death in response to TNFalpha treatment (figure 31).

Taken together, this study reveals a novel, so far unexpected role of the guanine nucleotide exchange factor Vav-1 in innate immunity. Vav-1 is essential for TNFalpha-induced terminal differentiation of human mo-DC, since it is involved in the up-regulation of important maturation markers the expression of which is controlled by NF-κB activity.

4.2.2 VAV-1 IS NOT A COMMON COMPONENT OF TNFALPHA SIGNALING

The cytokine TNFalpha elicits an unusually wide range of biological responses, including inflammation, tumor necrosis, cell proliferation, differentiation and apoptosis. TNFalpha receptors are expressed in most normal and transformed cell-types. This broad TNFR expression pattern prompted me to investigate, whether Vav-1 is a global or cell-type specific regulator of TNFalpha-induced responses. To this end, we decided to analyze the importance of Vav-1 in regulating TNFalpha signaling in a variety of immune and also of non-immune cells. The up-regulation of surface markers during TNFalpha-induced maturation of murine Vav-1 knock-out BM-DC is not impaired (figure 34). Furthermore, Vav-1 is not required for TNFalpha-mediated signaling to NF-κB neither in human nor in murine T-cells (figure 32 and 33). To finally eliminate that a functional redundancy between Vav-1, Vav-2 and Vav-3 masks a possible role of Vav-1 in TNFalpha signaling in other cell-types, TNFalpha-induced NF-κB activation was analyzed in murine embryonic fibroblasts (MEFs), lacking all three Vav family members. However, Vav-1/2/3 knock-out MEFs also respond normally to TNFalpha treatment (figure 35). In conclusion, Vav-1 expression appears to influence TNFalpha signaling in a cell-type specific manner, by specifically regulating TNFalpha-induced NF-κB activation and thus differentiation and maturation of human mo-DC.

4.2.3 CONCLUSION AND OUTLOOK

The present study describes an unexpected role of the guanine nucleotide exchange factor Vav-1 in the cell-type specific modulation of TNFalpha signaling pathways. Signaling through the TNRF has been studied in great detail, and seems to be essentially similar in most analyzed cell-types. However, a considerable number of studies report of a cell-specific modulation of TNFR functions. Recently, ß2-integrins have been shown to modulate TNFalpha-induced apoptosis signaling and respiratory burst of neutrophil granulocytes (see introduction for details). Furthermore, a co-stimulatory contribution

of ß2-integrins to human mo-DC maturation has been lately demonstrated (Varughese, diploma thesis 2005). SiRNA-mediated knock-down of the integrin ß2-chain CD18 impairs LPS/TNFalpha-induced DC maturation, since CD18-silenced mo-DC exhibit an immature phenotype in morphology, and furthermore, up-regulation of characteristic surface markers during maturation is decreased in respective cells (Varughese, diploma thesis 2005).

It is well established that Vav-1 acts as crucial regulator of the inside-out activation of ß2-integrins, and the GEF had also been implicated in outside-in signaling pathways downstream of integrins (Hornstein et al., 2004). Therefore, one likely possibility is that Vav-1 does not directly interact with classical components of the TNFR signaling machinery, but mediates its effects by binding and activating ß2-integrins, the co-stimulatory activity of which probably increase TNFalpha-induced activation of NF-κB in a cell-type specific manner (figure 39). However, the mechanism by which ß2-integrins modulate TNFalpha signaling remains unclear and requires further research.

As depicted in figure 24 and 27, neither siRNA-mediated Vav-1 knock-down, nor the inhibition of the Vav GEF activity by 6-Thio-GTP, completely block TNFalpha-induced expression of surface markers during mo-DC maturation. These data indicate that Vav-1 expression might not be crucial for maturation of the entire mo-DC population, but only for the TNFalpha-dependent activation of a special mo-DC subset.

Human monocytes, a generalized source for *in vitro* generation of dendritic cells, have been long time considered as a homogeneous cell population. However, in the last 20 years a number of studies reported of heterogeneity within the human monocyte population. The blood monocytes can be divided into different subsets based on the expression of the lipopolysaccharide receptor (CD14) and FcγIII receptor (CD16) antigens. $CD14^{++}/CD16^{-}$ and $CD14^{+}/CD16+$ are the major types of monocytes (Ziegler-Heitbrock et al., 1988, Passlick et al., 1989, Ziegler-Heitbrock et al., 2007). Additionally, it was reported that these monocyte subsets might be further subdivided into $CD64^{+}$ and $CD64^{-}$ cell populations (Grage-Griebenow et al., 1993). Although heterogeneity within the human monocyte population has been extensively reported, only few studies were

conducted to address the characterization of DC generated from these separate populations of blood monocytes. It has previously been demonstrated that TNFalpha-matured $CD16^-$ and $CD16^+$ human mo-DC differentially stimulate naive $CD4^+$ lymphocytes by inducing Th1- and Th2-like responses, respectively (Sánchez-Torres et al., 2001, Rivas-Carvalho et al., 2004). Recent research led to the identification of mouse monocyte subsets that closely resemble human $CD16^+$ ($Gr\text{-}1^{low}$) and $CD16^-$ ($Gr\text{-}1^{high}$) monocytes (Geissmann et al., 2003). This heterogeneity demands further analysis of an involvement of Vav-1 in TNFalpha-induced maturation of human mo-DC, generated from these separate human and murine monocyte subpopulations.

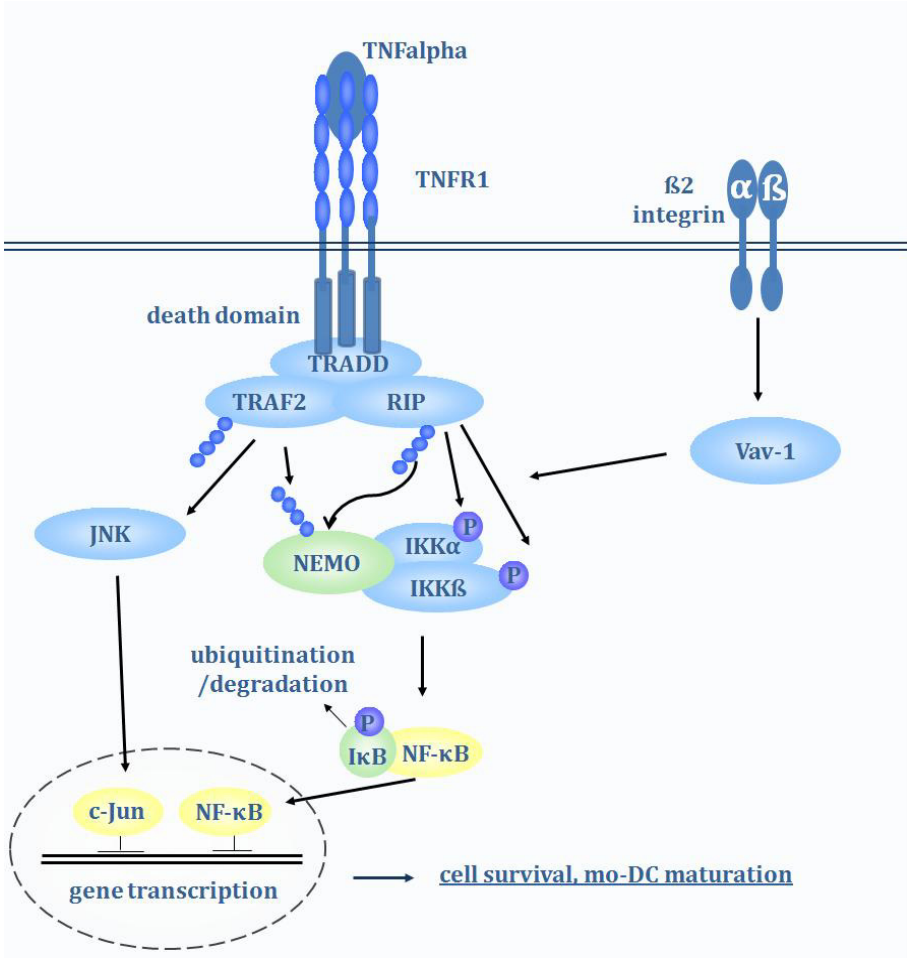

Figure 39: Vav-1 expression influences TNFalpha signaling in a cell-type specific manner, by specifically regulating TNFalpha-induced NF-κB activation and thus final differentiation of human mo-DC. According to our model, Vav-1 does not interact directly with classical components of the TNFR signaling machinery, but mediates its effects by binding and activating ß2-integrins, which co-stimulatory activity probably increase TNFalpha-induced activation of NF-κB.

4.2.4 DECTIN-1-MEDIATED BM-DC MATURATION STRONGLY DEPENDS ON SYK- BUT IS INDEPENDENT OF VAV-1 EXPRESSION

Dectin-1 plays a key role in coordinating responses of macrophages and dendritic cells to fungal pathogens, by acting as major receptor for zymosan, curdlan or other ß-glucans. It is remarkably similar to lymphocyte antigen receptors in that it contains an ITAM, which mediates much of the receptors signaling functions. At least one tyrosine residue within the dectin-1 ITAM is phosphorylated by Src family kinases upon receptor engagement. The phosphorylated ITAM is then thought to serve as a docking site for the tyrosine kinase Syk. Recruitment and activation of Syk is thought to be an important step for various downstream signaling events. As mentioned before, Vav-1 is activated downstream of various ITAM-containing receptors and Syk family kinases were shown to phosphorylate Vav-1 both *in vivo* and *in vitro* (Deckert et al., 1996, Bustelo, 2000, Turner and Billadeau, 2002). This indicates a potential role of Vav-1 in dectin-1-mediated signaling pathways, and it was indeed demonstrated that dectin-1-mediated phagocytosis of ß-glucans by microglia required Vav-1 activity (Shah et al., 2009). Microglia are an important immune component of the brain, which mediate innate and adaptive immune responses (Aloisi, 2001, Kreutzberg, 1996). Like other immune cells, microglia recognize highly conserved microbial structures through pattern recognition receptors (Bsibsi et al., 2002, Kielian, 2006, Laflamme et al., 2001). Upon interaction with PAMPs, microglia undergo an activation process, characterized by phagocytosis of the pathogens and production of pro-inflammatory mediators, including cytokines, chemokines, and ROS (Rock et al., 2004).

All these findings prompted me to analyze a possible role of the guanine nucleotide factor Vav-1 in the regulation of dectin-1-mediated signaling in dendritic cells. Recognition of zymosan or curdlan by dectin-1 induces the terminal differentiation ("maturation") of dendritic cells, which is characterized by robust cell surface expression of MHC peptide complexes and of so-called co-stimulatory molecules. However, neither zymosan- or curdlan-induced up-regulation maturation markers

(figure 36), nor dectin-1 mediated activation of NF-κB (data not shown) is altered in Vav-1 knock-out BM-DC, in comparison to WT BM-DC. In contrast, inhibition of the tyrosine kinase Syk abrogates curdlan- or zymosan induced up-regulation of MHC class II, CD80 and CD86 surface expression (figure 37). These findings indicate that dectin-1-mediated signaling pathways, leading to BM-DC maturation, are Syk-dependent but unexpectedly independent of Vav-1 protein expression. Whether signaling through dectin-1 to NF-κB can bypass the loss of Vav-1, by utilizing Vav-2 and/or Vav-3 needs to be investigated.

Interestingly, in macrophages (Underhill et al., 2005, Brown, 2006) as well as in microglia (Shah et al., 2008), Syk is activated in response to β-glucan stimulation, but pharmacological inhibition of these kinases did not inhibit phagocytosis. In contrast, dectin-1-mediated superoxide generation by microglia (Shah et al., 2008) and BMDC maturation (shown here) required Syk activity. Taken together, these results suggest a complex arrangement of underlying signaling proteins downstream of dectin-1, in which Vav-1 is only partly involved.

5. SUMMARY

The ARF GEF cytohesin-1 was originally identified as ß2-integrin binding protein, and was shown to play an important role in the control of integrin-dependent immune cell migration and adhesion via activating the small GTPase RhoA. To analyze whether the GEF activity of cyohesin-1 is required for regulating adhesive events of dendritic cells, a novel protocol was established, which enables over-expression analysis of cell adhesion and migration events in human mo-DC. It is shown that over-expression of wild type cytohesin-1, but not of the GEF-deficient E157K mutant, markedly enhances adhesion of mo-DC to ICAM-1. These results prove for the first time that the GEF function of cytohesin-1 is required for ß2-integrin-mediated adhesion of dendritic cells. Additionally, there is now strong evidence that cytohesin proteins play major roles in the induction of cellular gene expression, e.g. by regulating insulin signal transduction or MAP kinase activation during tumor cell proliferation. Fully in line with these results, and substantially extending the notion of a broader role of ARF-GEFs in cell signaling, cytohesin-1 is shown here to regulate important intracellular events in the course of T-cell activation. Employment of RNAi reveals that cytohesin-1 expression positively regulates TCR-induced transcriptional activation of the IL-2 gene via activation of the AP-1 transcription factor complex. Cytohesin-1 mediates positive regulation of AP-1 by selectively activating the p38/JNK- but not the ERK1/ERK2-branch of the MAP kinase signaling cascade. The ability of cytohesin-1 to activate p38/JNK signaling depends on the GEF function of the protein, since over-expression of the dominant negative GEF mutant E157K, or treatment with cytohesin-specific chemical GEF inhibitors completely blocks TCR-induced activation of p38. Similar to what has been described here for cytohesin-1, the Vav-1 GEF plays a dual role in the regulation of T-cell signal transduction and cytoskeletal reorganization via activation of Rho GTPases. Therefore, a possible functional interaction between cytohesin-1 and Vav-1 was investigated. However, co-precipitation experiments did not reveal a direct association of Vav-1 with

cytohesin-1. Furthermore, its expression is not required for TCR-induced phosphorylation, and thus for activation of Vav-1. These results indicate that cytohesin-1 might either be located in a specific signaling branch downstream of Vav-1, or acts in a pathway which alternatively regulates activation of p38/JNK and AP-1.

Vav-1 is known to be essential for proper development of the adaptive immune system. However, less information is available about a potential role of the protein in the regulation of innate immune responses. The so-called dendritic cells bridge innate and adaptive immune defense mechanism through a process known as antigen-presentation. Binding to pathogen-borne structures induces the terminal differentiation ("maturation") of this cell type, which is characterized by robust cell surface expression of antigen-presenting MHC peptide complexes and of co-stimulatory molecules, which supports T-cell activation. This study reveals a novel, so far unexpected role of Vav-1 in innate immunity. Vav-1 expression is shown to be essential for TNFalpha-induced terminal differentiation of human mo-DC, since it is involved in the up-regulation of important inflammatory cell surface proteins (maturation markers), the expression of which is controlled by NF-κB activity. It has furthermore been established that Vav-1 expression modulates TNFalpha signaling in a cell-type specific manner, since the up-regulation of surface markers during TNFalpha-induced maturation of murine Vav-1 knock-out BM-DC is not impaired. Consistent with this hypothesis, Vav-1 is not required for TNFalpha-mediated signaling to NF-κB neither in human nor in murine T-cells. In conclusion, the presented data suggest that Vav-1 does not directly interact with classical components of the TNFR signaling machinery, but mediates its effects by binding and activating ß2-integrins, the co-stimulatory activity of which might augment TNFalpha-induced activation of NF-κB in a cell-type specific manner.

6. ABBREVIATIONS

A. bidest	aqua bidestillata, double-distilled water
AP-1	activator protein 1
APS	Ammoniumperoxodisulfate
APC	antigen presenting cell
ATP	Adenosine 5`-triphosphate
BM-DC	bone marrow derived dendritic cell
BCA	bicinchoninic acid assay
BSA	Bovine serum albumin
CD	cluster of differentiation
DC	dendritic cell
dIdC	2′-deoxyinosinic-2′-deoxycytidylic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	ethylene diamine tetraacetic acid
e.g.	lat.: exempli gratia (for example)
EGTA	ethylene glycol tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay

ER	Endoplasmic reticulum
et al.	lat.: et alteres (and others)
EtBr	Ethidium bromide
FACS	fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat
g	gravity, gram
GFP	green fluorescent protein
GTP	guanosine 5′-triphosphate
h	hour
HRP	horseradish peroxidase
HBSS	Hank's buffered salt solution
HCL	Hydrocloric acid
ICAM	intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IFN	Interferon
IP	Immunoprecipitation
KCL	Kaliumchloride
l	litre

LSC	liquid scintillation counting
LPS	Lipopolysaccharide
m	meter, milli
M	Molar
MACS	magnetic activated cell sorting
MEF	Murine embryonic fibroblast
$MgCl_2$	Magnesiumchloride
MHC	Major Histocompability complex
min	minute
mo-DC	monocyte derived dendritic cell
n	nano
NaF	Sodium Fluoride
NaOH	Sodium hydroxide
Na_3VO_4	Sodiumorthovanadat
NFAT	Nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Oct	octamer binding protein
PAMPS	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBL	peripheral blood lymphocytes

PBS	phosphate buffered saline
PE	Phycoerythrin
PKN	Protein kinase N
PMSF	Phenylmethanesulphonylfluoride
PMA	12-O-Tetradecanoylphorbol-13-acetate
RIP	Receptor interacting protein
RLU	relative light unit
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
sec	second
SHIP	SH2-containing inositol 5-phosphatase
siRNA	short interfering RNA
SLP-76	SH2 domain containing leukocyte phosphoprotein of 76 kDa
TBE	Tris/Borate/EDTA
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TNFalpha	Tumor necrosis factor alpha
Th1	T helper cell 1
Th2	T helper cell 2
Tris	Tris-[hydroxymethyl]aminomethan

RNA	Ribonucleic acid
ROK	RhoA-binding kinase
RV14	rhinovirus strain 14
VLE	very low endotoxin
WASP	Wiskott-Aldrich syndrome protein
WB	Western blot
WT	wild-type

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