Novel Small Molecule Inhibitors Dissecting GEF–dependent and –independent Cytohesin Functions in Immune Cell Signaling

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Contents

1 Introduction

1.1 The immune system of vertebrates

Vertebrates have developed a highly complex system to combat pathogens that constantly aim to destroy the integrity of organisms, the immune system.

Immunity against pathogenic attacks is generated by two different branches of the immune system, innate and adaptive immunity. Innate immunity serves as the first defense during the course of an infection and fights pathogens with unspecialized, but effective clearance of infections via the complement system and phagocytes. The complement system consists of soluble plasma proteins that target bacterial, viral and fungal structures and thereby tags these pathogens for phagocytosis. Furthermore, the assembly of certain complement factors can lead to a pore formation in the bacterial membrane for instance followed by destruction of the pathogen (Janeway and Medzhitov 2002). Phagocytes are another major component of the innate immune system. As a reaction to viral or bacterial infection a specialized group of cells from myeloid origin, e.g. macrophages, granulocytes, natural killer cells (NK cells) and dendritic cells (DCs) serve as sentinels and incorporate soluble antigens as well as virus, bacteria and already infected cells to clear the infection. Therefore, they express a set of surface receptors, the toll like receptors (TLR) as a member of PRR (pattern recognition receptors) which recognize conserved antigens of the microbial metabolism, e.g. LPS (lipopolysaccharides), proteogylcans, microbial DNA and double-stranded RNA (Takeda 2004; Beutler 2004).

In addition, dendritic cells serve as a link between the innate and the adaptive immune system and therefore adopt the role of inducing an immune response that is able to specifically target the intruder and develop a memory for this pathogen that in turn leads to a stronger and faster defense the next time this pathogen threatens the organisms. Dendritic cells do therefore not only incorporate the pathogen but process it and present the antigenic peptides on specialized molecule receptors on the cell surface. The so called MHC II (major histocompatibility complex II) molecules (Snell 1948) also play a crucial role in the discrimination between self and foreign antigens, an important feature of the immune system in order to save the organism from self-destruction by autoimmunity. MHC class I molecules are ubiquitously expressed in almost every cell of the organism. MHC class I receptors are loaded with antigens from the cytoplasm which include host peptides as well as particles from virus and other infectious antigens. Once loaded on the MHC receptor, the receptor is transported to the cell surface and is recognized by $CD8^+$ T cells (CD, cluster of differentiation). If these cytotoxic T cells recognize the MHC molecule together

with the presented antigen, they degranulate and thereby destroy the infected cells. In contrast to that, antigen-loaded MHC class II molecules are recognized by $CD4^+$ T cells, the so called helper cells. These $CD4^+$ T cells are able to stimulate B lymphocytes that in turn develop to plasma cells. Plasma cells then produce antibodies that are specific for the presented antigen.

During antigen uptake and presentation of antigen on MHC class II molecules, dendritic cells undergo certain changes in their morphology and receptor expression patterns on their cell surface, a process that is termed maturation. This in turn leads to the ability to efficiently activate the adaptive part of the immune system. Activated mature dendritic cells migrate towards the draining lymph nodes, where they enter the paracortex and present the processed antigens to T cells that are part of the adaptive immune system. This long lasting and more pathogen-specific branch of the immune system is mainly developed and maintained by lymphocytes. Two subclasses of lymphocytes can be separated from each other. B cells and T cells develop from a common progenitor in the bone marrow. Whereas B cells fully develop in the bone marrow, T cells leave the bone marrow in earlier steps of their development and undergo certain positive and negative selection steps in the thymus that avoid autoimmunity. The positive selection regulates the ability of TCR subsets to be able to interact with MHC class I and II molecules, respectively. T cells that fail to recognize MHC molecules presented by the thymus epithelial are deleted during T cell development. The negative selection leads to elimination of thymocytes that interact with MHC molecules that present self-antigens. Only if both selection mechanisms are successfully overcome, the T cells are leaving the thymus as naïve T cells, circulate through the blood stream and subsequently enter the lymph nodes to be activated by antigen presenting cells (APCs).

After recognition of the foreign antigen that is presented by APCs in the lymph node, T cells become activated, proliferate and differentiate into effector or helper T cells. Effector $CD8^+$ T cells migrate to infected tissues and clear the infection by release of cytotoxic granules which destroy the pathogen and infected apoptotic cells. $CD4^+$ T helper cells are in turn able to activate B cells that differentiate into plasma cells which produce antigen specific antibodies and into memory cells that stay in the organism for an extended period and are able to secrete specific antibodies in the course of a new infection with the once recognized pathogen. The development of an immunological memory enables the organisms to clear a new infection with the same pathogen much faster and with higher efficacy.

1.1.1 Dendritic cells as cross-linkers of innate and adaptive immunity

Dendritic cells (DCs) were first discovered by Steinman and Cohn in peripheral tissues in mice (Steinman and Cohn 1973). They are a particularly heterogeneous group of antigen presenting phagocytes that develop out of myeloid or lymphoid progenitors, respectively, specialized in the uptake of antigens, the processing and consecutive presentation of antigen to T cells in the lymph node. Both, myeloid and lymphoid progenitors reside in the bone marrow. Myeloid progenitors can develop into monocytes that are characterized by a defined subset of surface markers ($CD14^+$and $CD11c^+$) and are circulating in the blood. After development of monocytic precursors in the bone marrow, they migrate mainly via the blood stream to non-lymphatic tissues. M-CSF (macrophage colony-stimulating factor) alone induces the differentiation into macrophages. In contrast, monocytes can develop into immature DCs after encountering the cytokines GM-CSF (granulocyte-macrophage-colony stimulating factor) and IL-4 (interleukin-4) (Inaba 1992; Sallusto and Lanzavecchia 1994). In this immature state, dendritic cells have the ability to roam the tissue and search for pathogens, which they take up by phagocytosis (Sallusto 1995; Sallusto and Lanzavecchia 1994; Fanger et al. 1996; Slepnev and De 2000). The surface expression of MHC II and costimulatory B7 molecules is low and they have a characteristic cell shape with dendritic protrusions.

Langerhans cells (LC) or Langerhans macrophages develop out of $CD14^+$, $CD11c^+$ and $CD1^+$ progenitor cells by stimulation with the cytokines GM-CSF, IL-4 and TGF-β or M-CSF alone, respectively. Langerhans cells and Langerhans macrophages reside in peripheral, mostly epidermal tissue and mature upon encountering CD40L, the CD40 ligand on T cells, or pathogenic DNA.

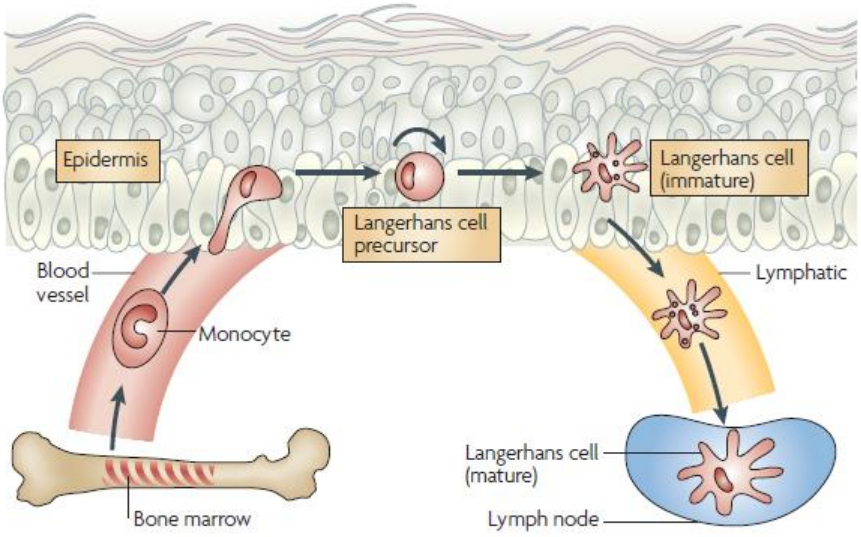

Figure 1: Life cycle of a dendritic cell.
Progenitors of the Langerhans cell leave the bone marrow and enter the tissue via the blood stream. There, they develop into precursor cells and into immature dendritic cells. Upon antigen-uptake, DCs leave the tissue and enter the lymphatics, where they mature. Fully matured DCs reside in the lymph nodes and present the processed antigens to T cells (Shortman and Naik 2007).

After encountering an antigen or sense inflammatory factors such as LPS, TNF-α or CD40L, respectively, immature dendritic cells are able to produce proinflammatory factors, e.g. IL-12 and type I and II interferons that in turn stimulate the innate immunity (Mellman and Steinman 2001). Moreover, they undergo a dramatic change not only in their morphology but also in the expression pattern of surface receptors and actin rearrangement, a developmental program that is called maturation (cf. figure 1). Morphologically, this change is accompanied by rounder cell shape and the formation of migration-relevant lamellipodia. The maturation process enables dendritic cells to migrate through afferent vessels towards the lymph node and serve as effective antigen presenting cells. Therefore, reciprocal downregulation of the chemokine receptors CCR1 and CCR3, respectively and upregulation of CCR7 occurs, a chemokine receptor that signals through stimulation via CCL19 and CCL21 (Dieu et al. 1998; Sozzani et al. 1998). These chemokines are expressed and secreted by lymphatic endothelial cells and stromal lymphoid tissue in the draining lymph nodes and serve as chemoattractants for the directed migration of DCs to the lymph node a process that is named chemotaxis (Martín-Fontecha et al. 2003; Saeki et al. 1999). Furthermore, adhesion molecules, e.g. ICAM-1 (intercellular adhesion molecule 1, CD54) and co-stimulatory molecules such as CD80 and CD86 are upregulated in the course of maturation. This upregulation is a key feature of mature dendritic cells that enables them to successfully activate naïve T cells for clonal expansion and differentiation into T cell subsets. In addition, maturation of the professional APC is accompanied by the downregulation of endocytosis. After migration into

the paracortical T cell zone of the lymph node dendritic cells are attracted to T cells and form initial, however transient contacts with them. After recognition of the T cell-specific antigen presented by the MHC complex, a more stable interaction can occur, that leads to full activation of T cells and is accompanied by clonal expansion and differentiation followed by a phase of detachment (Mempel et al. 2004).

1.1.2 T cell receptor signaling and T cell activation

Naïve T cells that successfully passed positive and negative selection rounds in the thymus are released in the blood stream and enter lymph nodes, where they migrate along fibroblastic reticular cell (FC) and DC-networks, respectively (Bajenoff et al. 2006). In the paracortical T cell zone they encounter APCs. This contact serves as the starting point in T cell activation. If the T cell receptor (TCR) recognizes its specific antigen peptide presented by MHC molecules, a signaling cascade is initiated that finally leads to fully activated $CD4^+$ or $CD8^+$T cells, respectively. $CD4^+$ T cells differentiate afterwards into T helper cells of the type 1 or type 2, respectively, dependent on cytokine stimulation. For TH_1 helper cells the activated T cell is dependent on the presence of IL-12 that is mainly produced by macrophages and B cells. Activated inflammatory TH_1 cells in turn secrete interferon-γ (IFN γ) and tumor necrosis factor- α (TNF-α) for the activation of macrophages. Differentiation into TH_2 helper cells is dependent on IL-4, secreted by T cells and mast cells. After stimulation, these cells secrete IL-4, IL-5, IL-10 and IL-13 and activate B cells for the effective production of antibodies for long-term immunity. $CD8^+$ T cells differentiate into cytotoxic T cells that clear viral infections by destruction of infected cells.

In order to establish this activation not only the TCR needs to be involved but furthermore the costimulatory molecule B7/CD28. Only if these two receptors are synchronously stimulated, T cells can be activated. Sole engagement of the TCR leads to anergy of T cells which is characterized by the unresponsiveness of T cells to further stimulation.

The T cell receptor (TCR) consists of a heterodimer of an α- and a β-chain of the immunoglobulin superfamily linked by a disulfide-bridge. α- and β-chains exhibit a variable immunoglobulin region that is located at the extracellular N-terminus followed by a constant transmembrane region and a short cytoplasmic tail at the C-terminus. The variable region is responsible for the binding of the peptide/MHC complex. Moreover the TCR receptor complex harbors a homodimer of ζ-chains and the CD3 complex. The CD3 complex consists of a γ-CD3 chain, a δ-CD3 chain and two ε-CD3 chains. The latter components of the TCR complex are indispensable for the signal transduction

upon peptide/MHC complex recognition, since they possess intracellular signaling domains that bear ITAM-motifs (immunoreceptor tyrosine-based activation motifs) and serve as phosphorylation sites for kinases that transfer the signal from the outside into the cell. In the ε- , γ-, and δ-CD3 chain there is only one ITAM, whereas in the ζ-chain, three ITAMs can be found. These motifs are phosphorylated upon TCR ligation and serve as interaction sites for phosphotyrosine kinases (PTK) (Chan et al. 1992). This initial phosphorylation of ITAMs is thought to occur after a conformational change in the CD3-ε chain upon TCR ligation which leads to an easier accessibility for the lymphocyte-specific protein tyrosine kinase Lck and Fyn (cf. figure 2). This in turn leads to the recruitment of ZAP-70 to the signaling complex. ZAP-70 has two major target proteins, LAT (linker for the activation of T cells) and SLP-76 (Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa). The nine tyrosines in LAT are phosphorylated after TCR engagement and bind PLCγ1 (phospholipase C) and PI3K (phosphatidylinositol 3-kinase) as well as GRB2 (growth factor receptor-bound protein 2) and Gads (GRB2-related adapter downstream of Shc). The latter recruits SLP-76 to phosphorylated LAT. A signaling network is formed by the attachment of Vav1, Nck and Itk to the complex.

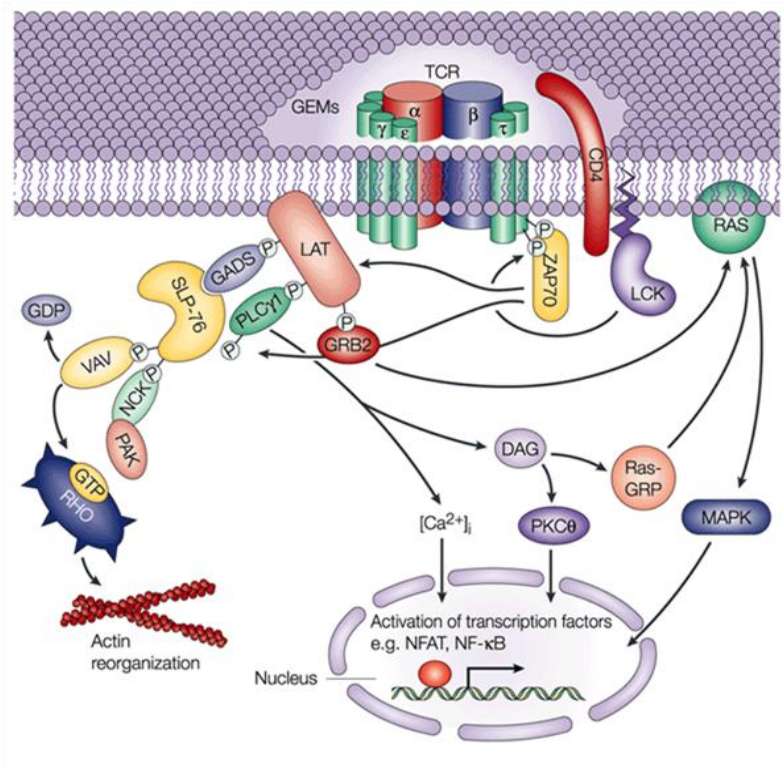

Figure 2: Signal transduction after TCR engagement.
Clustering of the T cell receptor leads to intracellular phosphorylation events that initiate a signaling cascade ultimately leading to actin rearrangements and the activation of transcription factors (figure taken from Koretzky and Myung 2001)

The formation of this signaling complex leads to the activation of PLCγ1 by Itk. Phospholipase C is a crucial factor in T cell activation. It catalyzes the hydrolysis of $PI(4,5)P_2$ to the second messengers DAG (diacylglycerol) and IP_3 (inositol triphosphate). DAG activates two pathways in the course of T cell activation. On the one hand DAG activates protein kinase C theta (PKCθ) which regulates NF-κB activation. In the course of activation NF-κB translocates to the nucleus and activates several genes that are involved in homeostasis and survival of T cells. On the other hand, DAG activates Ras. Ras is a GTPase that activates Raf1, a MAPKKK (mitogen-activated protein kinase kinase kinase) that activates MAPK kinases (MAPKK) that ultimately activate the MAPK extracellular signal-regulated kinases ERK1 and ERK2. ERK1/2 activate the AP-1 complex, a transcription factor that positively regulates the *IL-2* gene. IL-2 is the most important cytokine for the activation of T cells. IL-2 secretion leads in an autocrine feedback loop to sustained T cell activation and serves as a proliferation signal for the clonal expansion of T cells (Nelson et al. 1994).

IP_3 signaling occurs mainly via Ca^{2+} ions. IP_3 receptors on the membrane of the endoplasmic reticulum (ER) are activated by the PLCγ1 product and release Ca^{2+} ions from the sarcoplasm into the cytosol. Increased calcium concentrations in the cytoplasm activate CRAC (calcium release activated calcium) channels which open for Ca^{2+} ion influx into the T cell. Elevated cytosolic calcium-ion levels activate calcineurin that dephosphorylates nuclear factor of activated T cells (NFAT), a transcription factor that subsequently translocates into the nucleus. Together with AP-1 it is responsible for the transcription of the *IL-2* gene and other genes important for T cell activation.

1.2 Adhesion and migration of immune cells

1.2.1 Integrins in adhesion and migration of immune cells

Integrins are a family of proteins that serve as adhesion molecules and signal transmitters. They are heterodimeric transmembrane receptors that consist of an α-chain and a β-chain. Thus far, 8 different α- and 18 different β-chains are known that can in combination build up to 24 different heterodimers. Integrins play a crucial role in the mediation of cell-cell and cell-matrix contacts and are therefore important proteins in cellular processes such as wound healing, embryogenesis and the extravasation of leukocytes and formation of the immunological synapse (Lauffenburger et al. 1996; Dustin and Springer 1991; Springer and Dustin 2012).

The α-chain consists of an I-domain at the N-terminal extracellular site (only nine of the 18 α-chains bear this motif), followed by a seven-bladed β-propeller and additional three β-sandwich domains (named Thigh, Calf1 and Calf2). Furthermore, they consist of a transmembrane region and a short cytoplasmic C-terminal tail.

The β-chains contain an N-terminal I-like domain, a β-sandwich hybrid domain followed by a cystein-rich PSI (Plexin-semaphorin-integrin) domain, four integrin EGF-like domains and a β-tail domain. Also β-chain integrins are inserted in the plasma membrane and bear a short intracellular domain which serves as binding site for talin and α-actinin (Stanley et al. 2007).

The integrin signaling is bidirectional and consists of inside-out and outside-in signaling. Inside-out signaling refers to the conformational change in the integrin extracellular domain that leads to higher binding affinities often accompanied by clustering of the integrin receptors for higher binding avidity. Outside-in signaling means the signals that are transduced after binding of integrins in their high affinity conformation to its ligands (Abram and Lowell 2009).

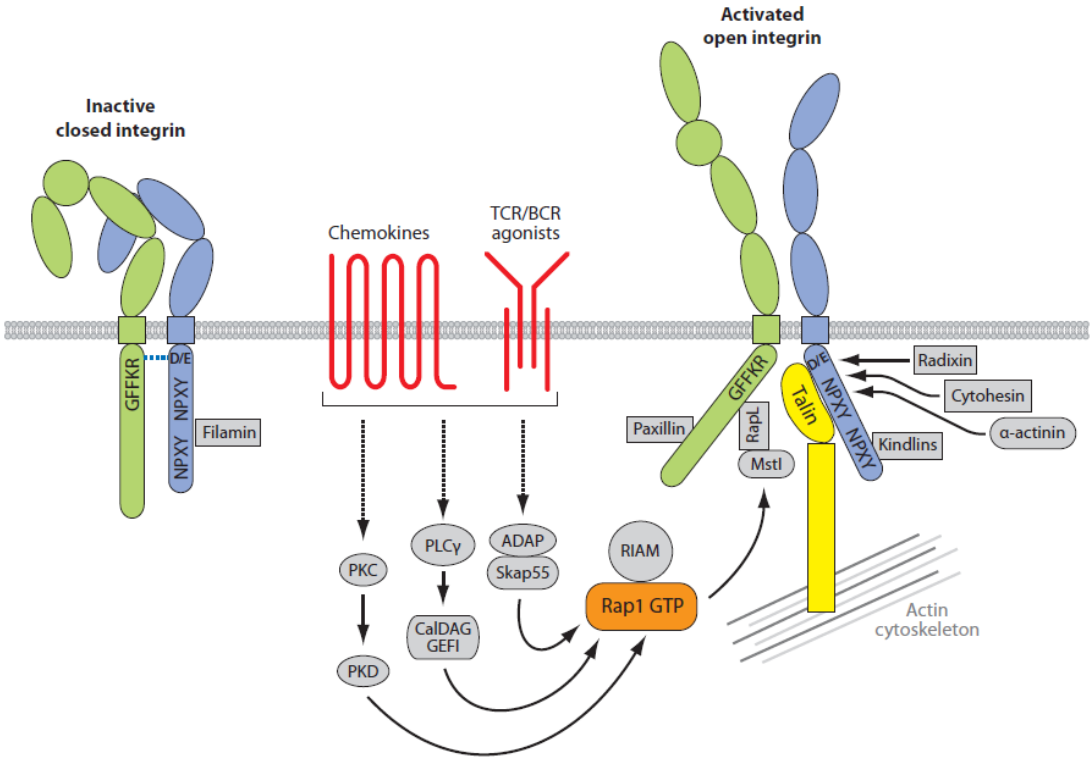

Figure 3: Integrin-inside-out signaling in immune cells.
Before stimulation the integrins are bent in an inactive form with a closed conformation of the α- and β-cytoplasmic integrin chains. Upon stimulation of immune cells with chemokine or TCR engagement, several kinases are activated that recruit Rap1-GTP and talin to the cytoplasmic tail of integrins, leading to a separation of the α- and β-chain. This in turn, changes the arrangement of the extracellular domains which are then in an active and opened conformation. (Abram and Lowell 2009)

Inside-out signaling occurs mainly after stimulation of the cell by chemokines or the cross-linking of extracellular receptors, e.g. the TCR (cf. figure 3). Engagement of the TCR leads to a signaling cascade that ultimately results in the activation of β_2-integrins in T cells. Substrate-binding to chemokine receptors or TCR activates several signaling cascades that result in the recruitment of GTP-loaded Rap1 to the α-chain of integrins and talin to the β-chain. Talin connects the integrin with the actin cytoskeleton and therefore enables physical coupling for forward movement. Several other proteins bind to the cytoplasmic tails of integrins upon stimulation which leads to the separation of the integrin α- and β-chain. This cytoplasmic tail separation results in conformational changes in the integrin extracellular domain structure converting them into an opened and active conformation, that enables binding of adhesion molecules (Abram and Lowell 2009).

1.2.2 Cell Migration

Cell migration serves an indispensable basis for many biological processes. In embryogenesis, migration of cells is required for gastrulation and neurulation. In adult organisms migratory processes are necessary for wound healing and for the proper functionality of the immune system. Furthermore, migration plays a major role in tumor development and cancer progression via metastasis (Lauffenburger et al. 1996).

During cell migration, a tight regulation of spatial and temporal development of cell polarity and signal transduction is of crucial importance. Migration can be divided into several fundamental steps. Forward locomotion of cells is dependent on the formation of membrane protrusions at the leading edge (figure 4). Afterwards the cell prolaps attaches to the substrate, e.g. ECM (extracellular matrix) or adhesion molecules on cells. Firm adhesion to the substrate in turn serves as anchorage for the subsequent contraction of the cell body and nucleus and drives the delocalization. The last step in this concerted process is the detachment of the cell at the trailing edge (Sheetz 1994).

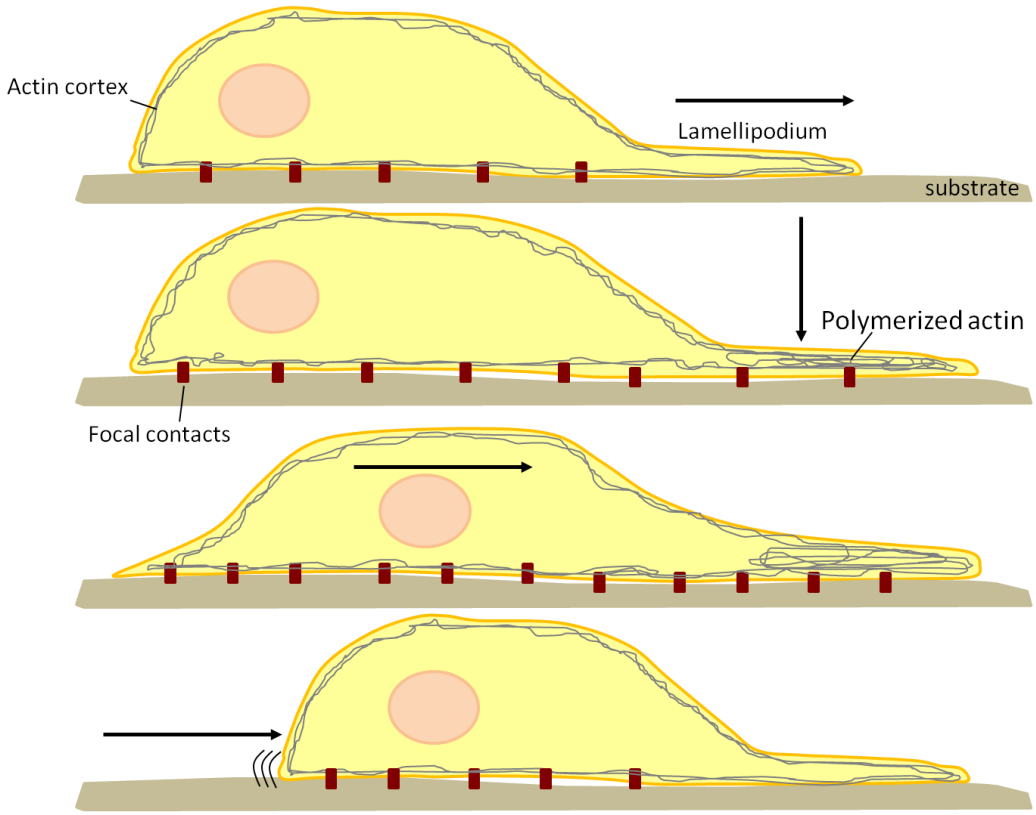

Figure 4: The fundamental steps of cell migration
Step 1: formation of membrane protrusions and polymerization of actin at the cell front. Step 2: adhesion of the protrusion to the substrate by development of focal contacts. Step 3: tension of the cell cortex and movement of the cell body. Step 4: Detachment of the cell rear by mechanical force or proteolytic cleavage of the focal contacts

Migration, especially in immune cells is not a random process, but often led by a gradient of chemokines which serve as guidance for the directionality of cell movement, a process known as chemotaxis. Chemokine sensing leads to the directed formation of membrane protrusions and to development of cell polarity (Van and Devreotes 2004).

Development of cell polarity is mediated by heterotrimeric G protein coupled receptors (GPCR) that recognize chemokines and signaling of ECM receptors such as integrins subsequently lead to the activation of signaling cascades. These signaling pathways mainly depend on small GTPases of the Rho-family, e.g. Rho A, Rac1 and Cdc42. Their function is supported by two classes of proteins. GEF proteins (Guanine nucleotide exchange factors) activate Rho-family GTPases by the exchange of GDP for GTP. In contrast, GAPs (GTPase activating proteins) inactivate GTPases by facilitating the hydrolysis of GTP to GDP. RhoA acts on the leading and the trailing edge of migrating immune cells. In transmigrating T cells downregulation of RhoA by RNAi leads to unpolarized cells and defects in the retraction of the uropod (Heasman et al. 2010). Rac1 and Cdc42 can be found at the

leading edge of migrating cells. They activate the Arp2/3 complex which is necessary for actin polymerization and leads to the formation of membrane protrusions. Furthermore, Rac1 activates IRSp53 which leads to the formation of a branched actin network (Nakagawa et al. 2003). Cdc42 and Rac1 positively regulate microtubule orientation and stabilization. Therefore, they interact with PAK that phosphorylates Stathmin, a negative regulator of microtubule formation which is inactivated upon phosphorylation (Wittmann et al. 2004). By targeting IQGAP1 that translocates the microtubule interacting protein CLIP-170 to actin filaments (Fukata et al. 2002), Rac1 and Cdc42 serve as providers of an anchorage site at the leading edge of migrating cells. Another important factor in the organization of cell polarity are the downstream target of Rho-GTPases of the ROCK family and Dia1. ROCK leads to the activation of the LIM-kinase that inhibits cofilin and thereby inhibiting actin depolymerization. Dia1 induces stress fibers mainly via interaction with src kinases and leads to activation of the Arp2/3 complex by interactions with IRSp53 (Ridley 2006).

Leukocytes are able to use integrin–dependent and –independent mechanisms for migration according to the surrounding environment (Friedl et al. 1998; Lämmermann et al. 2008). They move in an amoeboid manner which enables them to move very fast along the ECM by squeezing through it rather than by degrading ECM structures (Mandeville et al. 1997; Friedl and Wolf 2003). A further characteristic of amoeboid migration in leukocytes is the lack of strong adhesive contacts between the cells and the substrate (Wolf et al. 2003). At the leading edge, a network of filamentous actin is rapidly formed and membrane protrusions are probing the surrounding. This scanning is accompanied by a local concentration of diverse receptors involved in the establishment of signal transduction, e.g. TCR, β_2-integrins, Fc- and chemokine receptors. The mid region of the migrating cell contains the nucleus and signs responsible for the maintenance of the front-rear axis. The trailing edge of the cells contains the uropod and shows a high concentration of adhesion receptors, e.g. ICAM-1, β_1-integrins and ERM (ezrin radixin moesin)-adaptor proteins, which are responsible for the mediation of cell-cell and cell-matrix interaction and is postulated to have an anchoring function (Friedl and Weigelin 2008; Wolf et al. 2003).

In contrast to migration on two-dimensional surfaces, the migration in a three-dimensional surrounding is independent of the engagement of integrins (Friedl et al. 1998; Friedl and Weigelin 2008; Friedl and Wolf 2003; Lämmermann et al. 2008). Locomotion is provided by a dynamic actin-flow squeezing the cells through the ECM. The confinement of the ECM itself provides the anchorage for the cells to generate forward flow (Lämmermann et al. 2008).

1.3 Cytohesin and ARF proteins and their role in immune cell signaling

In humans and mice there are four known members of the cytohesin-family, namely cytohesin-1, cytohesin-2 (ARNO), cytohesin-3 (Grp1) and cytohesin-4. For *Drosophila* only one homologue of this family is known, *steppke*. Cytohesins serve as guanine nucleotide exchange factors for ADP-ribosylation factors (ARFs) (Chardin et al. 1996). *In vitro*, cytohesin-1 interacts with ARF1 and ARF6, respectively.

Initially, ARF proteins were discovered as cofactors in the cholera-toxin mediated ADP-ribosylation of heterotrimeric G-proteins (Kahn and Gilman 1986). Later on it became clear that they are important proteins in vesicular membrane trafficking (Moss and Vaughan 1995). Due to similarities in their sequence, ARF-proteins can be grouped in three classes. ARF1, ARF2 and ARF3 belong to class I ARFs and regulate COPI-coat assembly and the budding of vesicles that are transported from the ER to compartments of the Golgi-network. Class II ARFs (ARF4 and ARF5), in particular ARF5 might be involved in recruitment of coat-particles to trans-Golgi membranes, however, the precise function of class II ARFs remains unclear. ARF6 is the only member of the class III ARFs. The protein is localized at the plasma membrane (Peters et al. 1995) and regulates clathrin–dependent and –independent endocytosis e.g. of MHC I molecules and integrin β1 (Brown et al. 2001). ARF6 is capable of activating phosphatidyl 4-phosphate 5-kinase (PIP5K) and phospholipase D (PLD) and therefore has an impact on the phospholipid metabolism. It thereby leads to actin remodeling via Rac1 (D'Souza-Schorey et al. 1998; Radhakrishna and Donaldson 1997).

1.3.1 Structure of cytohesin proteins

In the main, cytohesin proteins show a highly conserved modular domain structure (cf. figure 5). All members possess a coiled-coil domain at the N-terminus, that is responsible for protein interactions e.g. with CYTIP (Boehm et al. 2003) and possibly for dimerization of cytohesin proteins. The central Sec7 domain bears the catalytic activity responsible for GDP-/GTP-exchange on ARF-GTPases (Chardin et al. 1996; Meacci et al. 1997) and furthermore interacts with LFA-1 (Chardin et al. 1996; Geiger et al. 2000; Kolanus et al. 1996). This interaction leads to an enhancement of TCR-mediated adhesion of T cells to the LFA-1 specific ligand ICAM-1 (intercellular adhesion molecule-1) as part of the inside-out signaling. At the C-terminus, a PH-domain is located, followed by a polybasic c-domain. The PH-domain is a regulatory element

important for the integration of the cytohesin proteins into the plasma membrane (Nagel et al. 1998). All cytohesin family members have the possibility to be expressed in a diglycine and a triglycine variant (Cronin et al. 2004; Klarlund et al. 2000). The diglycine variant shows a high affinity to PIP_3, which is a phosphorylation product of the PI3-kinase, and is responsible for the insertion of cytohesin proteins into the plasma membrane. The triglycine variant is not correlated with a high affinity to PIP_3, however proteins of the ARF family might serve as regulators of membrane recruitment as it is proposed by Hofmann and Cohen. These authors reported, that ARL4 and ARF6 are able to translocate proteins of the cytohesin family to the plasma membrane independently from the expression of the diglycine or the triglycine variant (Cohen et al. 2007; Hofmann et al. 2007).

Figure 5: Modular domain structure of cytohesin proteins.

Furthermore, the polybasic C-terminal region in cytohesin-1 and cytohesin-2 contains phosphorylation sites. Cytohesin-2 phosphorylation leads to the removal from the plasma membrane (Santy et al. 1999). Cytohesin-1 bears phosphorylation sites at position Ser 393, Ser 394 and Thr 395 in contrast to cytohesin-3 that is lacking the amino acid sequence leading to phosphorylation events (Dierks et al. 2001; Kolanus 2007). Thus, phosphorylation events might play a crucial role in the regulation of cytohesin proteins especially regarding the localization and differential roles in cell signaling.

1.3.2 Cytohesin proteins in immune cell signaling

Cytohesin-1 is ubiquitously expressed in many tissues but very highly expressed in NK cells and activated T cells. It was first discovered as an interactor of LFA-1 ($\alpha_L\beta_2$- integrin) in a yeast-two-hybrid screen with LFA-1 serving as bait (Kolanus et al. 1996). Further investigation in the same study revealed that the interaction between LFA-1 and cytohesin-1 was provided by direct interaction of the Sec7 domain of cytohesin-1 and the β_2-cytoplasmic tail of LFA-1. Cytohesin-1 is a positive regulator for the activation of LFA-1 mediated adhesion in activated T cells and in mature MoDCs (Kolanus et al. 1996; Quast et al. 2009). Mainly responsible for this activation is

the Sec7 domain of cytohesin-1 and its related GEF-activity since the dominant-negative point mutant cytohesin-1 E157K failed in the activation of T cell adhesion to ICAM-1 (Geiger et al. 2000). However, overexpression of the C-terminal PH domain of cytohesin-1 only, had a dominant-negative effect on the activation of LFA-1 mediated adhesion, leading to the conclusion that the PH domain could serve as a regulatory element in this context (Kolanus 2007).

LFA-1 activation is mediated via conformational changes in the integrin's extracellular domain structure. Transition into the high-affinity conformation allowing leukocyte arrest on endothelial tissues is a very fast process in which cytohesin-1 is involved in T cells and mature dendritic cells (Quast et al. 2009; Weber et al. 2001).

Correct localization of cytohesin-1 is important for the function of the protein and might be guided by the small GTPase ARF6 (Weber et al. 2001). In activated T cells and stimulated mature dendritic cells cytohesin-1 is recruited to the plasma membrane. In migrating T cells cytohesin-1 and ARF6 show differential functions leading to the conclusion that they do not act in the same pathway (Weber et al. 2001).

Another important regulator of cytohesin-1 function in immune cells is CYTIP (cytohesin-interacting protein, cybr) (Boehm et al. 2003). CYTIP interacts with cytohesin-1 via its coiled-coil domain and leads to the removal of cytohesin-1 from the plasma membrane. In this respect, CYTIP serves as negative regulator of T cell adhesion by binding cytohesin-1 and therefore translocating the protein from the plasma membrane which ultimately results in the abrogation of LFA-1 activation (Boehm et al. 2003).

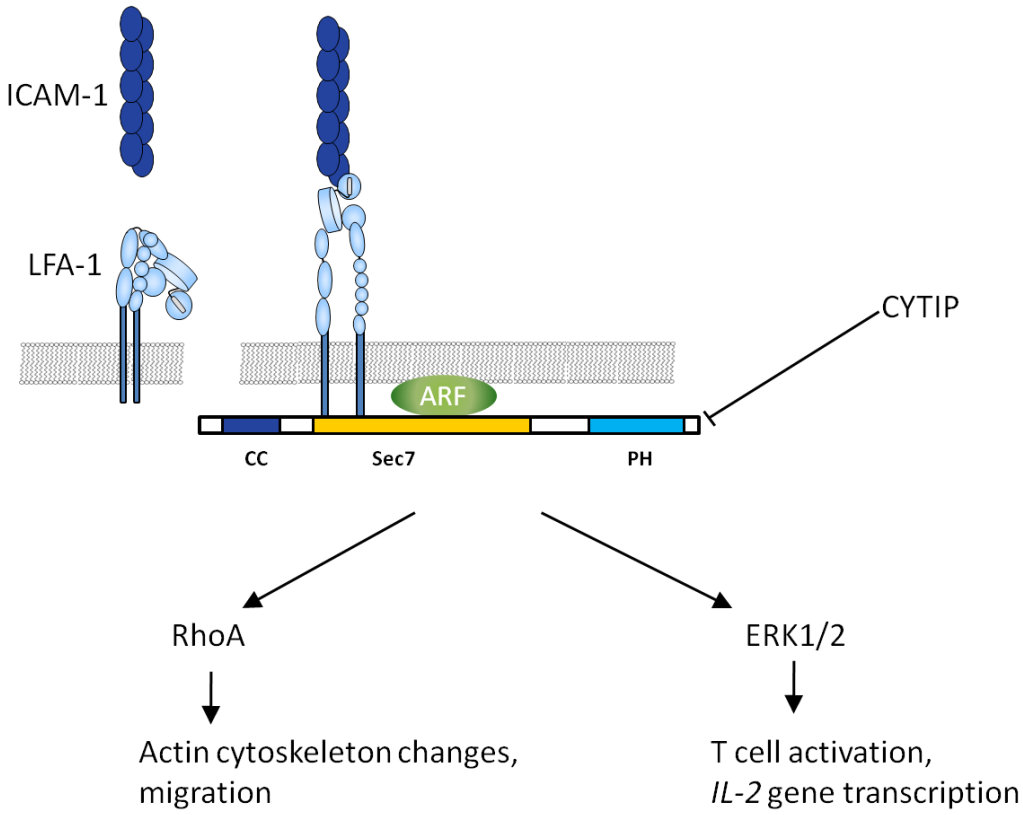

Figure 6: Cytohesin-1 in immune cell signaling.
Cytohesin-1 interacts with the cytoplasmic tail of CD18 and leads to LFA-1 activation. Membrane recruitment of cytohesin-1 is mediated by ARF proteins which in turn are activated by the catalytic GTP-exchange activity of the cytohesin Sec7 domain. Membrane recruitment is negatively regulated by CYTIP. It interacts with the coiled-coil domain of cytohesin and leads to its retraction from the plasma membrane. Cytohesin-1 activates RhoA and the MAPK cascade resulting in actin structural rearrangements and IL-2 production, respectively.

Cytohesin-1 is not only involved in inside-out signaling in T cells by activating LFA-1 after TCR engagement, but also important for outside-in mediated signals leading to T cell activation and proliferation. This can be concluded from experiments in which cell spreading was investigated (Geiger et al. 2000). Jurkat T cells that overexpress cytohesin-1 induced cell spreading when plated on ICAM-1 coated surfaces. In contrast to cells, that overexpressed the dominant-negative cytohesin-1 E157K mutant, which were unable to spread. This finding clearly underlines the role of the Sec7 domain as a central component of the outside-in signaling cascade. However, the cytohesin-1 E157K mutant does not influence the expression of the mAb24 activation epitope in LFA-1. Furthermore Perez could show that activation of LFA-1 resulted in the phosphorylation of cytohesin-1 and the activation of the c-Jun and ERK1/2 pathway (Perez et al. 2003). The cytohesin-1 dependent activation of the MAPK signaling cascade was also described by Kliche who further investigated in the involvement of the GEF-activity of cytohesin-1 in ERK1/2 activation

(Kliche et al. 2001). Overexpression of Kaposin A, a component of human Herpesvirus 8 (HHV8), was able to activate ERK1/2 signaling leading to activation of the AP-1 response element in 293 cells. Activation of AP-1 only occurred when cytohesin-1 wildtype was overexpressed but not when the GEF-inactive E157K mutant was overexpressed. Activation of the MAPK signaling cascade is ultimately linked to genetic programs as cell survival and proliferation.

AP-1 is part of the promoter region of the *IL-2* gene and IL-2 production is a hallmark of T cell activation. Cytohesin-1 is a positive regulator of *IL-2* gene transcription and therefore of T cell activation, most likely via the ERK1/2 signaling route. It could be demonstrated that cytohesin-1 positively regulates ERK1/2 phosphorylation and activates AP-1 (Paul 2007). Furthermore, cytohesin-3, which is upregulated in anergic T cells (Korthauer et al. 2000), inhibits AP-1 and therefore, *IL-2* gene transcription ultimately enhancing the unresponsive state of anergic T cells (Paul 2007).

Another involvement of cytohesin-1 in immune cell signaling was recently demonstrated by Quast et al. (cf. figure 6). Cytohesin-1 is a positive regulator of RhoA activity in dendritic cells as well as in HeLa cells (Quast et al. 2009). This activation is PI3-kinase-dependent and can be blocked by the employment of the PI3-kinase inhibitor LY-294002 (Quast et al. 2009). PI3-kinase is activated in the course of events after TCR-engagement in T cell activation. PI3-kinase phosphorylates its target PIP_2 and increases thereby the PIP_3-content of the plasma membrane leading to plasma membrane recruitment of proteins via their PH domains. Especially the diglycine variant of cytohesin proteins has a high affinity for PIP_3 and can be regulated by PI3-kinase. Furthermore, PI3-kinase negatively regulates the expression of cytohesin-3 in peripheral blood lymphocytes (PBL) (Paul 2007). Interestingly, in insulin signaling cytohesin-3 and its homologue *steppke* is located upstream of PI3-kinase in *Drosophila* as well as in murine and human insulin signaling, respectively (Hafner et al. 2006; Fuss et al. 2006)

1.4 Specific inhibition of protein function

Proteomics is the science concerned with the identification of protein functions in biological processes. This field emerged and gained further importance as a consequence of unraveling the human genome. Proteins are the key players in almost every cellular process, including signal transduction, transport of ions and second messengers and building structures in the cell.

Investigating protein function and their interplay in a physiological background is challenging, and different approaches that aim to target inhibition of protein expression or function are available at the present time.

A complete exclusion of the protein of interest can be achieved by knockout technology. In this approach the genetic information of a protein is eliminated from the genome by deleting an exon of the gene of interest or by creating a frame shift in the gene locus that leads to a stop signal and therefore the genetic information is not or only partially translated. Gene knockout is created by recombining a reporter gene into the gene locus of interest in embryonic stem cells. After successful recombination the embryonic stem cells can be transferred into a murine blastocyst of pseudopregnant surrogate mothers. This leads to the development of a chimera with a homozygous manipulated gene locus. For full gene knockout, the chimeric animals have to be crossed to produce a homozygous animal. The phenotype of the desired knockout or knock-in is then investigated in the organism in whole or in some tissues or cell types. Creating a knockout or knock-in organism, respectively, can be very time consuming and costly, especially in higher developed organisms since breeding periods are long and the genetic manipulation has to be germline transmissive. Furthermore, the outcome is uncertain since there is the possibility that the deletion of a gene could lead to lethality in early steps in development. This can be overcome by creating conditional knockout animals by tissue- or cell-type specific recombination into CRE-mice. These mice express CRE-recombinase in a tissue-specific manner. Injection of tamoxifen then leads to recombination in the selected tissue and creates a partial knockout. A further drawback in this approach is, that multifunctional proteins which possess binding sides for more than one interacting protein, are completely removed so that there is no facile way to investigate a separable function of the protein without leaving other functions untouched.

Another technology used for the analysis of protein function is the employment of short interfering RNA (siRNA technology). This method takes advantage of a common mechanism within the cell: After the transcription of the gene into mRNA, this single stranded ribonucleic acid can be degraded when bound within the RISC (RNA-induced silencing complex) to its complementary

sequence. Thereby a downregulation of protein expression is achieved. Introduction of RNA complementary to the mRNA of the protein of interest can therefore lead to a downregulation of the desired protein content in the organism.

RNAi technology provides a very useful tool in the investigation of protein function, because the gene of interest can be specifically targeted and this method is applicable to almost every transfectable cell type *in vitro*. Furthermore, in comparison to knockout strategies, this technique is faster and less cost intensive. Nevertheless, there are some disadvantages in this method. The use of RNAi is not suitable for proteins that have a long-time turnover, because the protein is targeted indirectly and in order to gain sufficient knockdown efficiencies, the proteins that are already produced, have to be degraded first. Furthermore, siRNA transfections are only transient and knockdown efficiency is only of short duration especially in fast dividing cells. Sometimes the downregulation of the protein is incomplete and therefore the loss of function cannot be observed, because even small amounts of the protein are sufficient to remain the functionality in the investigated context. Furthermore, particularly in immune cells, transfection can lead to cell activation as a side effect by the activation of TLRs (Toll-like receptors) (Hornung et al. 2005; Heil 2004; Diebold 2004), mainly TLR 3, 7 and 8. This hurdle can be overcome by substitution of the 2'-hydroxyl-group with a 2'-O-methyl-group (Judge et al. 2006). The modification has an immunosuppressive activity leading to both, downregulation of the desired gene product and avoidance of undesired upregulation of inflammatory responses. Another obstacle in the employment of siRNA technology is the absence of a reliable delivery system *in vivo*. To date there is no possibility to introduce small interfering RNAs into whole mammalian organisms or deliver it for long-term application.

Another opportunity to effectively inhibit protein function *in vitro* and *in vivo* is provided by the use of small molecules as inhibitors of cellular processes. Small molecules play a major role as second messenger molecules, e.g. cAMP.

The approach to selectively target a protein of interest by means of the addition of organic molecular probes is termed "chemical genetics" (O' Connor et al. 2011). This method is applicable in whole organisms, cell types and single cells. Advantages of the use of small molecules as inhibitors of protein function are versatile. During the last decade a plethora of small organic molecules has been developed and applied to investigate protein functions *in vivo* and *in vitro*. Small compounds are easy to apply to organisms by injection or to cultured cells and enter the cells quickly due to their hydrophobicity. Small molecule compounds can also easily be removed from cell culture by washing it out. This can provide the opportunity to investigate kinetics of

protein turnover. Furthermore, specific sites of protein function can be targeted while other functions of the protein are left untouched. This offers the advantage of focusing on separate pathways the protein might be involved in. Another advantage of the system is that different concentrations can be used. In comparison, a knock-out of a complete gene or even gene family can result in impacts on several pathways. If the concentration of the inhibitor is chosen carefully, it can result in phenotypes comparable to a partial gene knockout and might provide information about dose response relations.

The biggest benefit from using small molecules as tools for inhibition of protein function however might arise from the opportunity to use it as drugs in the treatment of diseases. Since it is possible to design small molecules for almost all kinds of proteins, specific disease resulting from overproduction or overfunctionality of proteins might be targeted. In contrast to therapeutic antibodies that are also very specific for the abrogation of protein-protein interaction and hence provide a versatile tool for the cure of specific diseases, small molecules are cell-permeable and therefore not only restricted to targeting cell surface receptors. However, also in the use of small molecules some disadvantages have to be taken into account. On one hand there is always the risk of toxicity of the compound itself or by products that develop during metabolism of these compounds. On the other hand the solubility of compounds can cause problems in the bioavailability, because organic molecules tend to build aggregates in hydrophilic surrounding. These major disadvantages might be overcome in parts by adding functional groups to the molecule that lead to higher solubility of the compound without affecting target specificity and functionality. Adding reporter groups to the molecule can be also very useful for the *in vitro* and *in vivo* investigation of protein function. For example, using compounds with fluorophores could provide information about spatio-temporal distribution of the target protein in the living cell if the protein of interest cannot be targeted directly by overexpression of a fusion protein or if overexpression in this context leads to undesired side effects.

In summary, all these techniques have their merits and demerits notwithstanding small molecules have some key advantages especially regarding their potential as starting points for the development of therapeutics.

Secin H3, a novel small molecule inhibitor for cytohesin function

Inhibition of the ARF-GEFs of the cytohesin family was impossible for a long time. The ARF-inhibitor Brefeldin A was known and its mode of action was investigated and unraveled (Mossessova et al. 2003; Nebenfuhr et al. 2002), however, it was ineffective in the inhibition of cytohesins. Recently Hafner et al. identified a small organic molecule effective in the inhibition of cytohesin GEF function towards ARF-GTPases (Hafner et al. 2006). In a very elegant approach, an aptamer, namely M69, that binds to cytohesins was fluorescently labeled and several compound libraries were screened for their ability to displace this aptamer, revealing a better and specific binding to cytohesin proteins, resulting in an increase of fluorescence by the released aptamer. With the help of this aptamer-displacement assay, the novel small molecule inhibitor Secin H3 (Sec7 inhibitor H3, inhibitor of cytohesin function) could be identified. Secin H3 was able to inhibit GTP-exchange towards ARF1 and ARF6 for cytohesin-1, -2 and -3 without affecting Golgi-network structures (Hafner et al. 2006). Furthermore, with the help of this small molecule inhibitor, cytohesin proteins could be linked to non-immune insulin signaling in humans and in *Drosophila* (Fuss et al. 2006; Hafner et al. 2006), and to Erb receptor signaling (Bill et al. 2010).

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Autoclave Model 135T	H+P (Oberschleißheim)
Avanti J-20XP	Beckman Coulter (München)
Biofuge fresco	Heraeus instrument GmbH (München)
Biofuge pico	Heraeus instrument GmbH (München)
Centrifuges Multifuge 4KR	Heraeus instrument GmbH (München)
CO_2-Incubator	Binder (Great River, USA)
Dual channel SPR 7600DC	Reichert Technologies (Depew, USA)
Electronic balance College	MettlerToledo (Greifensee, Switzerland)
Electrophoresis chambers perfect blue gel system	Peqlab (Erlangen)
Electroporation device GenePulser Xcell + CE module	Biorad (München)
FACS Canto II	BD (Heidelberg)
Fluorescence- and absorption analyzer Synergy HT	MWG (Ebersberg)
Fluorescence microscope Eclipse TE2000-ε	NIKON (Tokyo, Japan)
Gel dryer Model 583	BioRad (München)
Heat block Thermo mixer compact	Eppendorf (Hamburg)
Horizontal shaker Rocky	Fröbel Labortechnik GmbH (Lindau)
Laminar flow hood type 2 for cell culture Euroflow	Thermolife (Woerden, Netherlands)
Luminometer Microlumat plus LB 96V	Berthold (Bad Wildbad)
Magnetic stirrer ARE	VELP scientific (Milan, Italy)
MicroPorator, MP-100 system	Peqlab (Erlangen)
Light microscope DMIL	Leica (Wetzlar)
Nucleofector	Amaxa (Köln)
Oligonucleotide purification columns Quant 96 G-50 micro columns	GE Healthcare (München)

Optima LE-80K Ultracentrifuge	Beckman Coulter (München)
pH meter MP220	Mettler Toledo (Greifensee, Switzerland)
Photometer Biophotometer	Eppendorf (Hamburg)
Pipette controller Pipetus-Akku	Hirschmann Laborgeräte (Eberstadt)
Pipettes Pipetman P2, P20, P100, P200, P100	Gilson (Middleton, WI, USA)
Power supplies for electrophoresis Elite300Plus	Schütt Labortechnik (Göttingen)
Precision balance	Mettler Toledo (Greifensee, Switzerland)
Protein-Minigel-Apparature	BioRad(München)
Protein-Transfer-Apparature	BioRad (München)
Sonifier GM300	Bandelin (Berlin)
Vortex Zx3	VELP scientifica (Mailand, Italy)
Water bath Type 1004	GFL (Burgwedel)
WS 5 rocker	Edmund Bühler (Hechingen)

2.1.2 Consumables

Cell culture dishes (10 cm)	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)
Chip SPR HC1500m	Xantec bioanalytics GmbH (Düsseldorf)
Disposable hypodermic needle, (0.4x20 mm) Sterican	Braun (Melsungen)
Electroporation cuvettes (4 mm)	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 (10 µL, 200 µL, 1000 µL)	Starlab (Ahrensburg)
Microtiter plates (96-well)	Nunc (Roskilde, Denmark)
Nitrocellulose membrane PROTRAN	Schleicher & Schuell (Dassel)
Nylon cell strainer (40 µm pore)	BD Biosciences (Heidelberg)
Polypropylene reaction tubes (0.5/1.5/2.0 mL)	Starlab (Ahrensburg)
Radiographic film Hyperfilm™ MP,	GE Healthcare (München)

Sterile filters (0.2 µm/0.45 µm)	Schleicher & Schuell (Dassel)
Syringe, 10 mL	Braun (Melsungen)

2.1.3 Reagents

Acrylamide, 40%	Roth (Karlsruhe)
Acrylamide/Bisacrylamide-Mix, 30%	Roth (Karlsruhe)
Adenosine 5`-triphosphate, [γ-^{32}P], 10 mCi/mL	Perkin Elmer (Massachusetts, USA)
Ammonium peroxodisulfate (APS)	Roth (Karlsruhe)
Anhydrotetracyline	IBA-technologies (Göttingen)
BCA-Reagent solutions	Pierce (Rockford, USA)
Bovine serum albumin (BSA)	Roth (Karlsruhe)
Bromophenol blue	Roth (Karlsruhe)
Dimethyl sulfoxide (DMSO)	Roth (Karlsruhe)
Dithiothreitol (DTT)	Roth (Karlsruhe)
DNA loading buffer with ficoll, 6x	Roth (Karlsruhe)
ethylene diamine tetraacetic acid (EDTA)	Roth (Karlsruhe)
ethylene glycol tetraacetic acid (EGTA)	Roth (Karlsruhe)
Ethanol	Roth (Karlsruhe)
Ethidium bromide	Roth (Karlsruhe)
Ficoll, human (Pancoll)	PAN Biotech GmbH (Aidenbach)
Fibrinogen	Sigma (Taufkirchen)
G418 sulfate	PAA (Pasching)
Gentamicin	Gibco (Eggenstein)
Glucose	Roth (Karlsruhe)
Glycine	VWR (Darmstadt)
Glycerin	Roth (Karlsruhe)
Glycerolphosphate	Sigma (Taufkirchen)
GM-CSF, murine (recombinant)	R&D Systems (Wiesbaden), Immunotools (Friesoythe)
Guanosine 5'-triphosphate sodium salt (GTP)	Sigma (Taufkirchen)
4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)	Roth (Karlsruhe)

HRP detection system ECL Western Blotting Analysis	Thermo Scientific (Rockford, IL, USA)
Hydrochloric acid (HCl)	Roth (Karlsruhe)
ICAM-1-Fc supernatant from CV-1 cells over-expressing ICAM-1-Fc fusion protein	
Igepal	Sigma (Taufkirchen)
Imidazole	Roth (Karlsruhe)
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 amino acids	Sigma (Taufkirchen)
Ni-NTA	QIAGEN, Hilden
Penicillin/Streptomycin	GIBCO (Eggenstein)
Phenylmethanesulphonylfluoride (PMSF)	Sigma (Taufkirchen)
12-O-Tetradecanoylphorbol-13-acetate (PMA)	Sigma (Taufkirchen)
Potassium chloride (KCl)	Roth (Karlsruhe)
2-Propanol	Roth (Karlsruhe)
Protease inhibitors Antipain, Aprotinin, Benzamidine, Leupeptin, PMSF	Sigma (Taufkirchen)
Protein A-Sepharose™ 6MB	GE Healthcare (München)
Precision Plus Protein All Blue Standard	BioRad (München)
Secin 16, ZINC ID 00843734	ASINEX (Moscow, Russia)
Secin 144, ZINC ID 08188318	Vitas-M Laboratory (Moscow, Russia)
Secin H3	Calbiochem (Darmstadt)
Sodium dodecyl sulfate (SDS)	Roth (Karlsruhe)
Sodium fluoride (NaF)	Sigma (Taufkirchen)
Sodium hydroxide (NaOH)	Roth (Karlsruhe)
Sodium orthovanadate (Na_3VO_4)	Sigma (Taufkirchen)
Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma (Taufkirchen)

Sucrose	Roth (Karlsruhe)
TBE-buffer, 10x	Roth (Karlsruhe)
TEMED	Roth (Karlsruhe)
Tetra-sodium-diphosphate decahydrate	Roth (Karlsruhe)
Tris-[hydroxymethyl]aminomethane (Tris)	Roth (Karlsruhe)
Triton X-100	Roth (Karlsruhe)
Trypan blue solution, 0.4%	Sigma (Taufkirchen)
VCAM-1-Fc supernatant from HEK293T cells over-expressing VCAM-1-Fc fusion protein	

2.1.4 Kits

MidiMACS Separator Kit	Miltenyi Biotec GmbH (Bergisch Gladbach)
$CD4^+$ T Cell Isolation Kit II, human	Miltenyi Biotec GmbH (Bergisch Gladbach)
$CD8a^+$ T Cell Isolation Kit II, mouse	Miltenyi Biotec GmbH (Bergisch Gladbach)
CD11c MicroBeads, mouse	Miltenyi Biotec GmbH (Bergisch Gladbach)

2.1.5 Media, sera and buffers

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

2.1.6 Enzymes

T4 DNA ligase	MBI Fermentas (St. Leon-Rot)
Shrimp Alkaline Phosphatase (SAP)	Roche (Mannheim)
Restriction enzymes	MBI Fermentas (St. Leon-Rot),
RNase A	Roche (Mannheim)
Phusion Polymerase	Thermo Scientific (Rockford, IL, USA)

2.1.7 Antibodies

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 Signaling (Danvers, USA)
Rabbit anti-human ERK1/2	1:1000 (WB)	Cell Signaling (Danvers, USA)
Mouse anti-human CD3 (clone OKT3)	5 µg/mL	LGC Promochem, ATCC (Wesel)
Mouse anti-human CD28	1 µg/mL (stimulation)	BD Biosciences PharMingen (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)	Santa Cruz (St. Cruz, USA)
HRP-Goat-anti-Rabbit-IgG	1:5000 (WB)	Santa Cruz (St. Cruz, USA)
HRP-Goat-anti-Mouse-IgG	1:5000 (WB)	Santa Cruz (St. Cruz, USA)
FITC-Sheep-anti-Mouse	1:200 (FACS)	Dianova (Hamburg)

2.1.8 Oligonucleotides and plasmids

2.1.8.1 DNA oligonucleotides

All oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg).

primer	sequence 5' – 3'
pASK_C1Sec7_for	GCGGGGCTCGAGATGGAAAACCTGGGATCCACA
pASK_C1Sec7_rev	GCGGGGAAGCTTTTTAAGTGTGAGTGAGGTCATT
pASK_C2Sec7_Mlu_f	GCGGGGACGCGTATGGTGGAGGGGCTGGAGGCCAATGAGGGC
pASK_C2Sec7_Not_r	GCGGGGGCGGCCGCTTTAGGTGTGGGTCAGGTCATTCCCGTC
pASK_C3Sec7_Mlu_f	GCGGGGACGCGTATGATCGACAATCTAACTTCCGTAGAGGAG
pASK_C3Sec7_not_r	GCGGGGGCGGCCGCTTTAGGTGTGGGTCAGGTCGTTCCCGTC
pASK_C4Sec7_Mlu_f	GCGGGGACGCGTATGATCGACTGCTTCGAGAGTGCGGAGGAG
pASK_C3Sec7_not_r	GCGGGGGCGGCCGCTTTAGGTGTGAGTGAGGTCATTGCCGTC

Arf1(Δ17N)_Nde_f	GCGGGGCATATGCGCATCCTCATGGTGGGCCTG
Arf1_XhoI_r	GCGGGGCTCGAGTTTACTTCTGGTTCCGGAGCTGATT

2.1.8.2 Plasmids

Expression constructs were cloned into the pASK IBA43 plus vector and commonly bear an amino-terminal hexahistidine-tag. The Sec7 domain (amino acids 49-249) of human cytohesin-1 and Δ17N ARF1 (amino acids 18 - 181) were inserted into the MCS using the restriction enzymes XhoI and HindIII which leads to the deletion of the C-terminal strep-tag.

2.1.9 Bacteria strains

Strain	Genotype	Application	Reference
BL21	B F^- ompT $hsdS_B$ (r^-_B m^-_B) gal dcm (DE3) (Cam^r)	Expression of 6xHIS fusion proteins	Studier and Moffat, 1986
MC1061	araD139, Δ(ara-leu)7696, galE15, galK16, $\Delta(lac)_{\chi 74}$, rpsL(Str^l), hsdR2 ($m_\kappa^- m_\kappa^-$), mcrA, mcrB (P3)additional with yeast plasmid p3: kann, bla(amp)-am, tet-am	Amplification of plasmids (p3): CDM constructs	Casadaban and Cohen, 1980
DH5α	endA1, hsdR17($r_\kappa^- m_\kappa^+$), supE44,thi1, recA1, gyrA, (Nal^y), relA1	Amplification of plasmids	Hanahan, 1983

2.1.10 Primary cells

Human peripheral blood lymphocytes (PBL) were 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 (BmDC) were prepared from bone marrow of six to eight weeks old WT C57BL/6 mice.

HUVECs were a kind gift from George Whitesides.

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

2.1.11 Cell lines

Cell type	Description
Jurkat E6.1	human T cell-line from patients with acute leukemia (ATCC)
TAg Jurkats	stable transfectant line expressing SV40 large T-antigen
CV-1	monkey kidney cell line
HEK293T	Human embryonic kidney cell line expressing the SV40 large T-antigen

2.1.12 Mice

WT C57BL/6 inbred mouse strain expressing the MHC class I haplotype H2Kb were purchased from Charles river laboratories. 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 six to eight weeks of age were used.

2.2 Methods

2.2.1 Molecular Cloning

2.2.1.1 PCR

In order to amplify DNA fragments, polymerase chain reaction (PCR) was used. This is the standard method for the enzymatic amplification of DNA. The chain reaction is divided into three parts characterized by different temperatures. First, the desired DNA fragment is reversely denatured by applying temperatures of 95°C. Oligonucleotides (primers) subsequently anneal to the sequence specific complement region of single-stranded DNA. The temperature for this step is dependent on the nature of the oligonucleotide and usually is between 55 and 65°C. In a final step the polymerase elongates the complement strand by adding free nucleotides to the 3’ end of the primer at a temperature of 72°C. In each cycle the DNA template is theoretically duplicated.

A 50 µL reaction mix for PCR contained the following ingredients:

DNA template	200 ng
5 x Buffer	10 µl
dNTP Mix (2.5 mM)	5 µL
10 pmol forward primer	1 µl
10 pmol reverse primer	1 µl
Taq Polymerase	0.5 µl
Ad 50 µL H_20 bidest.	

2.2.1.2 DNA precipitation

In order to clear the DNA from enzymes and unbound nucleotides, the PCR reaction mix was phenol-chloroform extracted by adjusting the volume to 400 µL with A. bidest. 300 µL 1:1 phenol-chloroform mix was added. The solution was vortexed and spun down to separate the aqueous phase from the organic phase. DNA was precipitated with 40 µL 4 M lithium chloride and 1 mL 100% ethanol at -20°C for 20 minutes. The mix was then centrifuged for 12 minutes at 4°C in a micro centrifuge at 13000 rpm. The pellet was washed carefully with 70% ethanol and dried. Finally, the DNA was dissolved in a 40 µL of A. bidest.

2.2.1.3 Analysis of DNA by restriction digest

In order to obtain cut products ready for the ligation in the desired vector and to prepare the vector backbone for ligation the DNA has to be digested by restriction endonucleases. Therefore 40 µL of cleaned PCR product or 3 µg plasmid DNA, respectively were mixed with 5 µL of the adequate 10x restriction buffer suitable for the chosen enzyme and 1 µL of the enzyme. The final volume was adjusted to 50 µL and the digest mix was incubated at 37°C for one hour.

2.2.1.4 Removal of 5'phosphate ends in plasmid vectors

To avoid self-ligation of the plasmid backbone the 5'phosphate was removed by incubating the digestion mix with 1 U of shrimp alkaline phosphatase (SAP) and 5.7 µL of 10x SAP-buffer for additional 10 minutes at 37°C.

2.2.1.5 Purification of DNA fragments

For the removal of proteins and undesired DNA fragments after the restriction digest, the DNA was loaded onto a low melting agarose gel and separated by electrophoresis. After separation the

desired fragments were excised under UV-light and the gel block was melted and diluted with 40 µL H_2O bidest to set the DNA free from the agarose.

2.2.1.6 Ligation and transformation

The ligation of the vector backbone and the insert generates a new plasmid which contains the information for the expression of proteins. The complementary ends of plasmid and DNA-insert are fused to each other with the help of T4 DNA ligase which links the 3'OH group of the vector to the 5'phosphate group of the insert. Vector and insert were used at a ratio of 1:3. The reaction contained additionally 1 µL T4 DNA ligase and 2 µL 10x ligation buffer the final volume was adjusted to 20 µL with H_20 bidest. The reaction was performed at room temperature for 2 hours.

2.2.1.7 Transformation of chemo-competent **E. coli** *cells*

E. coli are used to amplify the plasmid DNA or to recombinantly express protein encoded by the transformed plasmid. For the transformation chemo-competent bacteria were carefully thawed on ice and 70 µL of the cell suspension was mixed with either 1 µL of plasmid DNA or 10 µL of ligation mix and incubated for 10 minutes on ice. Cells were then heat-shocked for 3 minutes at 37°C and subsequently incubated on ice for 5 minutes. The transformed bacteria were incubated with 1 mL LB-medium without antibiotics for 30 minutes at 37°C and plated on LB-agar containing the appropriate antibiotic for the selection of the plasmid.

2.2.1.8 Isolation of plasmid DNA

Minipreparation

The mini preparation is a method for isolation of plasmid DNA from bacteria from a small volume of bacterial culture. 2 mL of an overnight culture in LB-medium containing the corresponding selective antibiotic were pelleted at 20000 x g and resuspended in 250 µL solution I. Then, 250 µL of solution II were added in order to lyse the bacteria. Precipitation of the chromosomal DNA occurs when 350 µL solution III were added to the sample. Afterwards, the sample was centrifuged at 20000 x g for 15 minutes in order to separate chromosomal DNA, RNA and proteins from plasmid DNA. 700 µl of supernatant were mixed with phenol and aqueous and organic phase were separated by centrifugation at 20000 x g for 5 minutes for extraction of remaining proteins. By adding 0.7 volumes of isopropanol and incubating the mix at – 20°C for 20 minutes, plasmid DNA was precipitated. The DNA was pelleted by centrifugation at 20000 x g for 10 minutes. The pellet was subsequently washed with 70% ethanol and 100% ethanol, respectively dried and

resuspended in 50µl A. bidest. The remaining RNA was hydrolyzed by adding 10 µg RNase A to the sample.

Solution I	Solution II	Solution III
10 mM EDTA	2 M NaOH	2.5 M KOAc
25 mM Tris-Cl (pH 8.0)	1% (w/v) SDS	2.5 M HOAc (pH 4.7)

Maxipreparation

To gain high amounts of very pure plasmid DNA, maxipreparations were performed. A maxipreparation follows initially the same procedure as a minipreparation, followed by an additional purification of the DNA by a CsCl density gradient centrifugation. Bacteria from 1 liter overnight culture in LB-medium with the respective selective antibiotics were pelleted at 5000 x g for 20 minutes. The pellet was resuspended in 40 mL solution I. Alkaline lysis was achieved by adding 80 mL solution II. 40 mL solution III was added subsequently to neutralize the mix and the precipitate was pelleted at 5000 x g for 10 minutes. The supernatant was filtered and DNA was precipitated by adding 100 mL isopropanol and centrifuging for 10min at 5000 x g and 4°C. The pellet was then dried and solved in 4 mL solution I containing 5.5 g CsCl and 500 µL ethidium bromide. The solution was centrifuged for 5min and 5000 x g and the supernatant was then loaded into an ultracentrifugation tube. The mix was centrifuged for 4 hours at 50000 x g in an ultracentrifuge. The DNA band was isolated and contaminating ethidium bromide was removed by for consecutive extracting steps with n-butyl-alcohol. DNA was precipitated by adding an equal volume of 1 M ammonium acetate and two volumes of ethanol. Precipitated DNA was pelleted by a centrifugation at 5000 x g at 4°C. The pellet was washed with 70% ethanol and 100% ethanol and dried overnight. It was resuspended in 500 – 1000 µL 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 260 nm, using an Eppendorf photometer.

2.2.2 Cell culture

2.2.2.1 Cell counting

10 µL cell suspension were mixed with an adequate amount of trypan blue staining solution to detect dead cells and 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:

$$\textit{No. of cells counted / 4} \cdot \textit{dilution factor} \cdot 10^4 = \textit{cell number/mL}$$

2.2.2.2 Cell cultivation

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

2.2.2.3 Generation of human PBL

PBL were prepared from standard buffy coat preparations of healthy blood donors by Ficoll density gradient centrifugation. Hence, the buffy coat was diluted with prewarmed 2 mM EDTA/PBS in a 1:1 ratio and 30 mL of diluted blood were then overlaid on 15 mL Ficoll in a 50 mL-tube. Cells were then centrifuged at 800 x g for 30 minutes at RT in a swinging-bucket rotor without brake. After centrifugation a ring of PBMCs (peripheral blood mononuclear cells) became visible in the interphase between Ficoll and serum. The PBMC fraction, containing monocytes and lymphocytes, was carefully transferred into a fresh 50 mL tube. The cells were diluted with 2 mM EDTA/PBS to a volume of 50 mL and centrifuged at 640 x g for 7 minutes at room temperature. The cells were washed until the pellet became white, decreasing the centrifugation speed successively from 640 x g to 200 x g, in order to clear the cells from contaminating erythrocytes and platelets. The pellet was then resuspended in 5 mL 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. Isolated PBMCs were seeded in 6-well plates (5 mL cell suspension /6-well) and allowed to adhere for 1-2 hours at 37°C in an incubator with 5% CO_2-atmosphere. Afterwards, the supernatant, containing the non-adherent lymphocytes (peripheral blood lymphocytes, PBL) was collected and further cultivated. PBLs were transferred into a fresh flask the next day in order to further remove adherent monocytes.

2.2.2.4 Generation of murine bone marrow-derived dendritic cells

BM-DC were prepared from bone marrow of six to eight week old C57BL/6 WT mice. Bone marrow was isolated from hind legs of mice. Therefore legs were disinfected with ethanol, before tibia and femur were isolated. Bone marrow was then flushed out of the bone with PBS using a syringe. Total bone marrow was filtered with 40 µm pore nylon cell strainers and plated in 10 cm non-treated petri-dishes at 5 x 10^6 cells in 10 mL VLE-RPMI supplemented with 10% heat-inactivated FCS, 2 mM L-Glutamine, 100 U/mL Penicillin, 0.1 mg/mL Streptomycin and 10 ng/mL murine recombinant GM-CSF. The culture medium was half-renewed every three days. At day 8-10 of culture, BM-DC were stimulated to mature by adding 200 ng/mL LPS for 48 hours.

2.2.2.5 Isolation of murine splenic CD8+ T cells and dendritic cells

Murine splenic $CD8^+$ T-cells were derived from NK-depleted OT-I mice and wild type DC were obtained from WT C57BL/6 mice by magnetic activated cell sorting (MACS), using CD11c micro beads or $CD8a^+$ T Cell Isolation Kit (Miltenyi), respectively. The latter kit is an indirect magnetic labeling system for the isolation of "untouched" T cells from single-cell suspensions of mouse spleen. Highly pure "untouched" T cells were obtained by depletion of non T cells. Non T cells e.g. B-cells, NK cells, dendritic cells, macrophages, granulocytes and erythrocytes 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. For DC and T cell isolation spleens were passed through a metal filter with a pore size of 250 µm, to remove cell aggregates and connective tissue and to obtain a single cell suspension. T cells were additionally loaded onto a column filled with medium equilibrated nylon wool and incubated for 1 hour at 37°C. Column was washed with twice the column volume of medium and flow-through, containing the T cells was collected. T cells and DC were counted, pelleted and taken up in an appropriate volume of MACS-buffer and micro beads. Cells were incubated for 15 minutes at 4°C. Afterwards two consecutive washing steps were performed to remove unbound antibodies. The cell pellet was then resuspended in 500 µL MACS-buffer per 10^8 total cells and applied to an AutoMACS automate which performed the magnetic separation. T cells and DCs were taken up in RPMI-medium supplemented with 10% heat inactivated FCS,100 U/mL Penicillin and 0.1 mg/mL Streptomycin and used for

functional assays 3-4 hours after isolation. DCs were pulsed with OVA-peptide at a concentration of 1 mg/mL for one hour prior to cocultivation with OT-1 T cells.

MACS buffer

PBS

2 mM EDTA

0.5% BSA

2.2.2.6 Generation of ICAM-1-Fc and VCAM-1-Fc supernatants

Production of supernatant containing ICAM-1-Fc

CV-1 cell were grown to confluency in a 15 cm petri-dish in DMEM supplemented with 2 mM L-Glutamine, 10% heat-inactivated FCS and 10 µg/mL gentamicin. Cells were rinsed with PBS and 5 mL of DMEM without supplements was added. 50 µL of Vaccinia virus stock bearing the ICAM-1-Fc DNA (for details see Kolanus et al. 1996) was added to the medium for one hour and 25 mL DMEM medium was added afterwards. Cells were grown for two more days or until the cell shape was rounded. The supernatant was collected and sterile-filtered (pore size 0.2 µm) to clean the supernatant from contaminating cells and virus. Supernatant was used in cell adhesion assays.

Production of supernatant containing VCAM-1-Fc

HEK293 T cells were grown to 50 – 60 % confluency and transfected with the CDM8 VCAM-1 plasmid via standard Calcium-phosphate method (Graham and van der Eb 1973). Cells were further grown in DMEM medium supplemented with 2 mM L-Glutamine, 10% heat-inactivated FCS and 10 µg/mL gentamicin for four to five days and supernatant containing VCAM-1-Fc fusion protein was collected and sterile filtered.

2.2.3 Protein Biochemistry

2.2.3.1 Isolation and purification of recombinantly expressed proteins

BL21 (DE3) bacteria transformed with the plasmid pASK-IBA-43plus cytohesin-1 Sec7 were seeded in 1 liter LB-medium at an optical density at 600 nm (OD_{600}) of 0.1 and shaken at 150 rpm at 37°C. At an OD_{600} between 0.6 and 0.8 protein expression was induced by adding 200 µg/L anhydrotetracyline and bacteria were grown at 37°C for 6 more hours. Anhydrotetracycline binds

to the tetracycline promoter/operator and leads to the transcription and bacterial recombinant expression of the desired plasmid information.

Bacteria were pelleted by centrifugation at 5000 x g for 20 minutes and pellet was immediately used for purification or stored at -80°C.

For purification affinity chromatography technique was applied. The expressed hexahistidine-tag of the protein binds to a Ni-NTA (nickel nitrilotriacetic acid) sepharose. Ni-NTA is a chelating agent that binds Ni^{2+} ions. The histamine tag of the protein coordinates Ni-ions by the nucleophilic imidazole in the histidine side chain and hence binds to the immobilized Ni-NTA. Untagged proteins and proteins with lower histidine content do not bind and can be removed by intensive washing. Afterwards the protein can be eluted from the sepharose by adding high concentrations of imidazole.

The bacterial pellet from 1 liter of culture was lysed in 20 mL lysis buffer and the bacteria were sonicated (Sonifier GM300) for 15 minutes at an intensity of 70% for complete disrupture of bacterial membranes. Cell debris and insoluble protein was removed by centrifugation at 6000 x g at 4°C. Supernatant was incubated with 1 mL Ni-NTA sepharose slurry and incubated for one hour at 4°C under gentle agitation. Unbound protein was removed by four consecutive washing steps with washing buffer and protein was eluted by two elution steps adding 1 mL of elution buffer and incubating for 5 minutes on ice. Imidazole was removed by gel filtration, loading the eluate on a PD-10 column and the protein was eluted by 8 consecutive elution steps with 500 µL dialysis buffer, each.

Buffers for the purification of cytohesin proteins:

Lysis buffer:	**Wash buffer:**	**Dialysis buffer:**	**Elution buffer:**
100 mM Tris/HCL, pH 7.8	50 mM Tris/HCl, pH 7.8	PBS	50 mM Tris/HCl, pH 7.8
300 mM NaCl	300 mM NaCl		300 mM NaCl
3 mM $MgCl_2$	3 mM $MgCl_2$		3 mM $MgCl_2$
proteinase inhibitors:	25 mM imidazole		500 mM imidazol
Aprotinin (20 mg/mL) 1:2000			
Leupeptin (20 mg/mL) 1:2000			
PMSF (1 M) 1:1000			

Buffers for the purification of (Δ17N)ARF1:

Lysis buffer/Wash buffer:	**Elution buffer:**	**Dialysis buffer:**
50 mM Tris/HCl (pH 8.0)	50 mM Tris/HCl (pH 7.4)	50 mM Tris/HCl (pH 7.4)
150 mM KCl	150 mM KCl	150 mM KCl
10 mM imidazole	100 µM GDP	3 mM $MgCl_2$
100 µM GDP	1 M imidazole	100 µM GDP
proteinase inhibitors (only in lysisbuffer):		
Aprotinin (20 mg/mL) 1:2000		
Leupeptin (20 mg/mL) 1:2000		
PMSF (1 M) 1:1000		

2.2.3.2 GTP-exchange assay

In order to investigate the cytohesin-mediated replacement of GDP with GTP on ARF1 a special characteristic of the ARF1 protein was exploited. Under normal circumstances ARF1 is expressed with a myristoylation site which is responsible for plasma membrane insertion *in vivo*. However expression lacking the first 17 N-terminal amino acids of the protein leads to better solubility coupled with higher activity of the protein (Ahmadian et al. 2002; Antonny et al. 1997; Northup et al. 1982). Secondly tryptophans within ARF1 display autofluorescence that can be detected at a wavelength of 280 nm and 340 nm for excitation and emission, respectively. Upon binding of GTP and replacement of GDP this fluorescence increases dramatically and can be measured in a plate reader. The increase in fluorescence is proportional to the amount of GTP loaded on the ARF1 protein and thus represents therefore for the activity of the used cytohesin protein.

To this end cytohesin Sec7 domain was diluted in PBS buffer to a final concentration of 160 – 200 nM supplemented with 3 mM $MgCl_2$ and various concentrations of Secin inhibitors and incubated at 37°C for 10 minutes. ARF1(Δ17N) protein (final concentration 500 nM) was preincubated with 3 mM $MgCl_2$, 500 µM EDTA and 20 µM GDP in PBS at 37°C for 15 minutes. The proteins were mixed and incubated in a 96-well plate at 37°C. Exchange reaction was started by adding GTP at a final concentration of 50 µM to the protein mix. Changes in fluorescence were followed by measuring at wavelength of 280 nm and 340 nm for excitation and emission, respectively every 5 seconds until saturation was reached. The velocity of the reaction was calculated from the linear fluorescence increase area by determining the slope printed against the logarithm of the used concentrations of inhibitor.

2.2.3.3 Interaction studies of cytohesin protein with small organic molecules

SPR (surface plasmon resonance) is a versatile tool for studying molecular interactions. From whole cells down to small molecules interactions can be followed with this technique. The mechanism underlying this optometric method is based on changes in the angle of total reflection on a gold surface. In this study protein was loaded on one half of a gold-chip surface, the other half was left blank. Previously, the gold-chip was coated with a hydroxycarboxylate-hydrogel. This gel improves the resolution of interaction measurement since it increases the thickness of the layer in which interactions influence the angle of total reflection. Change of this angle occurs after mass increase on top of the gold surface and displays dependent on the curve of association and dissociation of the analyte interaction. Small molecules were loaded on the chip surface. If interaction occurred, an increase in the response was observable. The blank half of the chip served as a reference for unspecific interactions of the analyte with the chip itself.

SPR experiments were performed using a dual-channel SR7000DC system (Reichert Inc., USA). Recombinant Cytohesin-1 Sec7 domain was covalently immobilized on an HC1500m chip. The surface was activated with activation buffer (0.1 M NHS, 0.7% EDC, 0.05 M MES, pH 5.0) and the protein was applied at a concentration of 50 µg/mL in 5 mM acetic acid, pH 4.5 to sample channel, only. Unreacted residues on the chip surface were quenched with 1 M ethanolamine, pH 8.5 (see detailed protocol below). Binding and dissociation were performed in 1% DMSO containing PBS at a flow rate of 50 µL/min. Regeneration of the chip surface was achieved by injection of 10 mM glycine-HCl, pH 3.0. The netto sample channel response (which was calculated by subtracting the response from the reference channel from that of the sample channel) was corrected for blank buffer injection and DMSO injection. Data processing and curve fitting was done using SPR V4.0.17 (Reichert Inc., USA) and Scrubber2 software (BioLogic Software, Australia).

<u>Detailed immobilization protocol</u>

Sensorchip: NHS activated HC1500m

- System flushed with H_2O
- Elution buffer (1 M NaCl/0.01 M NaOH, pH 9.0) for 3 minutes, flow rate 50 µL/min
- H_20 2-5 min
- Injection of activation buffer (freshly prepared: 0.1 M NHS, 0.05M MES, pH 5.0 and 0.7% EDC) for 10 minutes (flow rate 10 µL/min)
- 2 minutes H_20 50 µL/min
- Unhook reference channel

- Purge line with H_2O 100 µL/min
- Injection of ligand (50 µg/mL) for 15 minutes (3 µL/min)
- H_2O 15 min 3 µL/min + 120 s 50 µL/min
- Put reference channel in line again (flow rate 50 µL/min)
- 1 M Ethanolamine 10 µL/minute for 15 minutes
- Flush system with H_2O flow rate 50 µL/min
- Switch system to PBS/ 1% DMSO

2.2.3.4 Preparation of cell lysates

For the preparation of cell lysates, 2 x 10^6 cells were harvested and washed with cold PBS. The cell pellet was subsequently resuspended in 60 - 100 µL Igepal lysis buffer with freshly added protease inhibitors and incubated for 20 minutes on ice. Cell debris was pelleted by centrifugation at 13000 x g at 4°C for 10 minutes. The cell lysate was transferred to a new tube and the pellet was discarded. The protein amount was quantified by using a standard BCA assay. For experiments in which ERK phosphorylation levels were analyzed, unstimulated or stimulated cells were lysed in Triton lysisbuffer, containing phosphatase inhibitors and freshly added protease inhibitors. To this end, the cell pellet was resuspended in 60-100 µL Triton lysisbuffer and immediately frozen in liquid nitrogen. Cell lysate was stored at -80°C until use. When cell lysates were needed, it was thawed on ice, before cellular debris was pelleted by centrifugation at 13000 x g at 4°C for 10 minutes. The supernatant was transferred to a new tube and protein content was determined by a standard BCA assay

Igepal lysis buffer	**Triton lysis buffer**	**Protease inhibitor dilution**
10 mM HEPES, pH 7.5	10 mM Tris HCl, pH 7.5	Antipain (2 mg/mL) 1:1000
10 mM KCl	1 mM EGTA	Aprotinin (20 mg/mL) 1:2000
10 mM MgCl	1 mM EDTA	Benzamidine (1 M) 1:1000
150 mM NaCl	10 mM Glycerol phosphate	Leupeptin (20 mg/mL) 1:2000
1% Igepal	50 mM Sodium Fluoride	PMSF (1 M) 1:1000
	5 mM Sodium Pyrophosphate	
	1 mM Sodium Vanadate	
	0.27 M Sucrose	
	1% Triton X-100	

2.2.3.5 Determination of protein concentration by BCA assay

Protein amount of cell lysates were 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. First, a serial dilution for standard curve was performed using bovine serum albumin ranging from 2 mg/mL to

0 mg/mL. 3 µL of each standard and each sample were added to a 96-well microplate. Samples were added in two replicates. The detection reagent was prepared using a ratio of one part reagent A : 50 parts reagent B). 200 µL of working reagent was added to each well. The 96-well plate was placed in an incubator for 10 minutes at 65°C. Absorbance of each well was read at 562 nm using a microplate reader. Standard 0 mg/mL was used as the blank control. Amount of protein in each well was calculated afterwards from linear regression of the standard's absorption.

2.2.3.6 SDS-PAGE

SDS-PAGE is the standard method for separating proteins according to their size. SDS binds to the protein in a constant ratio (1.4 g SDS/g protein), giving a uniform mass-to-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 and the lower resolving 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 due to a shift in pH. Polyacrylamide gels were prepared according to standard protocol. 10 µg of protein were mixed with 3x loading buffer and boiled for 5 minutes at 99°C. The gel was clamped in the electrophoresis chamber and buffer chambers were filled with gel running buffer. The boiled sample was loaded onto the gel, including one lane with 6 µL of molecular weight protein standard. The gel was run at 80 V (stacking gel) and at 120 V (resolving gel), until the blue dye front reaches the bottom. Afterwards the aperture was removed from the power supply and proteins were visualized using Coomassie Brilliant Blue stain or blotted on a nitrocellulose membrane.

1x stacking gel solution (5%)
2.05 mL A. bidest.
375 µL 1 M Tris/HCl (pH 6.8)
30 µL 10% SDS
30 µL 10% APS
3 µL TEMED
0.5 mL 30% acrylamide/bisacrylamide-Mix

1 x resolving gel solution (10%)
2 mL A. bidest.
1.25 mL 1 M Tris/HCl (pH 8.8)
50 µL 10% SDS
50 µL 10% APS
2 µL TEMED
1.515 mL 30% acrylamide/bisacrylamide-Mix

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

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

2.2.3.7 Western Blot analysis

Western Blot was performed to immobilize proteins separated by SDS-PAGE on a nitrocellulose membrane for immunostaining. 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. This was done in fresh 1x transfer buffer. The cassette was then closed and placed in the western transfer chamber, filled with cold 1x transfer buffer. The proteins were then transferred from the gel to the nitrocellulose membrane at 300 mA per chamber for one hour at 4°C. Unoccupied spaces on the nitrocellulose membrane were blocked overnight with 5% milk powder in TBS-T at 4 °C under gentle agitation. Protein detection was achieved by a specific primary antibody in 10 mL TBS-T, containing 5% BSA. The membrane was incubated for one hour with primary antibody solution at room temperature (with gentle agitation), and then washed 4 times for 5 minutes with TBS-T. The primary antibody was then detected with a secondary antibody, conjugated to horseradish peroxidase (HRP). Membrane was washed again 4 times for 5min with TBS-T, before incubating it for 1 minute with ECL solution (1 mL of solution A and 1 mL of solution B, Pierce). Chemiluminescence was detected by autoradiography. To demonstrate loading of equal amounts of proteins, membranes were "stripped" of antibody, using a "stripping" buffer (20 minutes, 55°C) and were stained for a ubiquitous protein, e.g. actin.

1x Transfer buffer
192 mM glycine
25 mM Tris
20% methanol
0.002% SDS

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

TBS buffer
50 mM Tris, pH 7.5
140 mM NaCl

"Stripping" buffer
2% (w/v) SDS
62.5 mM Tris/HCl, pH 6.7
100 mM 2-mercaptoethanol

2.2.4 Cell stimulation and functional assays

2.2.4.1 Inhibitor treatment

The small molecule SecinH3 (Sec7 inhibitor H3), 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 Sec7 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, as well as cytohesin-3-dependent insulin signal transduction of *Drosophila*, mouse and human cells *in vitro* and *in vivo* (Fuss et al. 2006; Hafner et al. 2006).

Secin 16 and Secin 144 are novel potent cytohesin family GEF inhibitors identified by chemoinformatic methods to improve target affinity and selectivity of SecinH3 (Stumpfe et al. 2010). Both inhibitors were dissolved in 0.5% DMSO. Immune cells were incubated with SecinH3, Secin 16 or Secin 144 for 1h at 37°C. Equal amounts of DMSO were used as a control. Inhibited cells were afterwards used in assays as indicated.

2.2.4.2 Cell stimulation

Stimulation of murine splenic T cells

Murine splenic T-cells were stimulated 3-4h after cell isolation by cocultivation with OVA-pulsed murine DC.

Stimulation of Jurkat T cells

For adhesion assays Jurkat T cells were stimulated with 5 µg anti-CD3 (OKT3) or 50 ng/mL PMA for 15 – 20 minutes at 37°C.

Stimulation of PBLs

To induce the phosphorylation of ERK PBLs were stimulated with aAPC at a ratio of 1 cell : 1 bead. The aAPCs were coated with anti-CD3 (OKT3), anti-CD28 and MHC I in a ratio of 5% : 1 : 7. In all experiments, cells were stimulated in 200-500 µL VLE-RPMI, containing 0.5% FCS. 1 - 2 mL cold PBS was added in order to terminate stimulation.

Maturation of dendritic cells

see 2.2.2.4

Stimulation of Jurkat T cells and PBL for cell adhesion assays

see 2.2.4.6

2.2.4.3 CBA assay and ELISA

The production of cytokines is a hallmark for the activation of T cells and the development of different T cell subsets. After engagement of the TCR and costimulation of CD28 T cells undergo changes that finally lead to differentiation into T cell subsets and clonal expansion. In order to investigate the activation and differentiation status of T cells, they were isolated and stimulated in the presence of either aAPCs (human system) or OVA-pulsed DC (murine system) and incubated for several days. The supernatant, containing cytokines released from the cells was collected and frozen at -80°C. Cytokine production was measured by CBA or IL-2- and Interferon-γ-ELISA according to the manufacturer's instruction.

2.2.4.4 Proliferation assay

The measurement of proliferation is an important tool to investigate the influence of inhibitors on T cell activation. To this end, cells are stained with a viability dye (in this case CFSE) which is resides in the cytosol of the cells and is diluted by every cell division. Therefore a decrease in fluorescence with every cell division can easily be measured by flow cytometry.

$CD4^+$ untouched T cells were stained with 0.5 µM CFSE dye in PBS for eight minutes. Reaction was stopped by adding FCS to the reaction mix which quenches unincorporated CFSE. Excessive dye was removed by two consecutive washing steps and cells were resuspended in VLE-RPMI supplemented with 10% heat-inactivated FCS and 100 U/mL Penicillin, 0.1 mg/mL Streptomycin at a concentration of 1 x 10^6 cells/mL. T cells were preincubated with the respective inhibitor (at concentrations indicated) for one hour at 37°C in medium. Stimulation was achieved by mixing cells and aAPCs at a final ratio of 1:1 and the cells were incubated at 37°C and 5% CO_2. After three – four days CFSE fluorescence was measured by flowcytometry. Therefore cells were washed in PBS and the mean fluorescence intensity of the samples was measured in the FITC-channel.

2.2.4.5 Viability assay

In order to find out if the application of inhibitors for cytohesin functions have an impact on the viability of T cells, Annexin V staining in combination with propidium iodide staining was performed. Annexin V is a protein that binds to phosphatidylserin (PS). PS is located in the inner plasma membrane. Upon induction of apoptosis, PS translocates to the outer leaflet of the plasma membrane and can be detected by fluorescently labeled Annexin V. In contrast, propidium iodide stains dead cells by entering the cell, because of missing plasma membrane integrity. Hence, a combination of both dyes gives information about necrosis and apoptosis induction.

In the viability assays 1 x 10^6 cells were incubated with cytohesin-inhibitors at different concentrations in 0.5% DMSO or DMSO alone as control and cultivated in media at 37°C and 5% CO_2. After three days the cells were collected and washed with PBS. The staining with Annexin V and propidium iodide was performed according to the manufacturer's protocol. Annexin V positive and propidium iodide positive cells were quantified by flow cytometry.

2.2.4.6 Static cell adhesion assays

Static cell adhesion assays on ICAM-1-Fc or VCAM-1-Fc were carried out as described previously (Boehm et al. 2003; Kolanus et al. 1996) with following modifications. 2x10^5 Jurkat/well in HBSS were preincubated with inhibitors diluted in 0.5% DMSO/HBSS for one hour at 37°C and 5% CO_2 and stained with H33342 bisbenzimide (6 µg/mL) 30 minutes prior to stimulation. Cells were then placed on ICAM-1-Fc/VCAM-1-Fc coated 96-well plates and stimulated with either 5 µg/mL anti-CD3 (OKT3) antibody or 50 ng/mL PMA for 15 minutes at 37°C and 5% CO_2 to adhere. Unbound cells were washed off carefully with HBSS and the adherent cells were quantified by measuring bisbenzimide fluorescence at a wavelength of 360 nm and 485 nm for excitation and emission, respectively. The number of adherent cells was calculated by comparison with an unwashed control. All samples were measured in triplicates.

Adhesion assays with PBL were carried out in the same fashion, except that a concentration of 1 x 10^6 cells/mL were used and the stimulation time was increased to 30 minutes.

2.2.4.7 Adhesion under flow conditions

In order to investigate early events in the activation of integrins, adhesion assays were performed in which PBLs were drawn over a confluent layer of endothelial cells. After a short period of rolling some PBLs attach firmly to the endothelial cells and can be counted (Weber et al. 2001).

HUVEC cells were grown to confluency in 35 mm Petri-dishes and stimulated with 10 ng/mL TNF-alpha over night. Two hours prior to experiment cells were loaded with human CXCL12 (500 ng/mL). HUVECs were assembled in a parallel flow-chamber. PBL ($1x10^6$ cells/mL in HH-buffer) were incubated with 10 µM Secin 16 in 0.5% DMSO or DMSO alone as control prior to experiment. PBL were drawn through the chamber with a laminar flow at a shear rate of 1.5 dyn/cm^2. The cells that were attached to HUVEC after 6 minutes of wash with HH-buffer were counted.

HH-buffer:

HBSS w/o Mg^{2+} and Ca^{2+}
0.5% BSA
10 mM HEPES pH 7.4
1 mM $MgCl_2$
1 mM $CaCl_2$

2.2.4.8 Transwell migration assays

One method for the quantification of chemotaxis is a transwell migration assay. To this end polycarbonate filters of a pore size of 5 µm in a modified Boyden chamber were used. Cells are loaded on top of the filter and are attracted by a chemokine gradient that is applied to the lower chamber and squeeze through the mesh of the polycarbonate filter. Migrated cells can then be easily quantified by counting the cells in the lower compartment of the chamber.

2 x 10^5 cells (Jurkat or BmDCs) were preincubated with inhibitors in 0.5% DMSO or DMSO alone as control in VLE-RPMI containing additionally 10% heat-inactivated FCS and 100 U/mL penicillin, 0.1 mg/mL streptomycin. Cells were loaded on top of the filter and incubated for one hour at 37°C and 5% CO_2 to adhere. Afterwards, cells were stimulated with 200 ng/mL human CXCL12 or murine CCL19, respectively. After three hours (Jurkat T cells) or 24 hours (BmDC) cells in the lower compartment of the chamber were counted and compared to the total number of cells in the assay to determine the migration rate.

2.2.4.9 2D migration assays

Ibidi slides (15 µ-slideVI) were coated with either 5 µg/cm^2 fibronectin, 50 µg/mL fibrinogen or 12.5 µg/mL murine ICAM-1-Fc for 1 hour at room temperature and washed with PBS. For human ICAM-1-Fc coating, slides were preincubated with goat anti-human antibody (16 µg/mL) at pH 9.5 for 90 minutes followed by two washing steps in 50 mM Tris pH 9.5. To avoid unspecific binding to the plastic surface slides were subsequently coated with 1% BSA/PBS for 60 minutes at room

temperature or overnight at 4°C. ICAM-1-Fc supernatants from vaccinia infected CV-1 cells were then applied for 90 minutes at room temperature. Unbound ICAM-1 was removed by two washing steps with HBSS.

1.5 x 10^5 cells were preincubated with the respective inhibitor or DMSO as control in 30 µL growth medium supplemented with 0.5% FCS and seeded on the slides. After one hour 10 µL chemokine (200 µg/mL) was applied to one side of the slide. Cell migration was recorded with a fully automated inverted phase-contrast TE Eclipse microscope with a 10x objective, motorized xyz-stage (Marzhäuser, Wetzlar) and a CCD-1300 Vosskühler camera at 37°C. In every sample 30 cells were tracked and their motility, y- forward migration index and velocity was analyzed with ImageJ software and the manual tracking and chemotaxis (ibidi) tool. The y-forward migration index is determined by dividing the y-value of a given track by its respective accumulated track length. It is a factor for the directionality of movement towards a chemokine.

2.2.4.10 3D collagen gel chemotaxis assay

2 x 10^5 BmDCs or PBL per assay in 50 µL VLE-RPMI with 0.5% FCS were mixed with 100 µL of collagen mix (kept strictly on ice to avoid polymerization of the collagen) and the respective inhibitor at a final concentration of 10 µM in 0.5% DMSO or DMSO alone as control. The mixture was loaded into a custom made chemotaxis chamber and incubated for 1 hour at 37°C and 5% CO_2. After the collagen was polymerized, 600 ng/mL murine CCL19 or human CXCL12, respectively were applied on top of the gel which creates a chemokine gradient throughout the collagen. The chambers were sealed with liquid wax and the chambers were adjusted on a microscope in a climate chamber with 37°C. Time-lapse phase-contrast series were recorded using a fully automated microscope. For every experiment pictures were taken at a rate of 2 min/ frame for four hours. Chemotactic mobility was determined by manual tracking of cells over the complete period of the assay using ImageJ's manual tracking plug-in tool. FMI and velocity were calculated in ImageJ using the ibidi chemotaxis plug-in.

Collagen mix:
50 µL $NaHCO_3$ (7.5%)
100 µL 10x MEM
750 µL Collagen I

3 Results

Cytohesins are a family of guanine nucleotide exchange factors (GEFs) for small ARF-GTPases. The family consists of four known members in mammals, termed cytohesin-1 (Kolanus et al. 1996), cytohesin-2 (ARNO) (Chardin et al. 1996), cytohesin-3 (also Grp1 in humans and *steppke* in *Drosophila*, respectively) (Fuss et al. 2006; Klarlund et al. 1997) and cytohesin-4 (Ogasawara et al. 2000). Cytohesins are reported to serve as important regulators in cellular processes, e.g. cell adhesion and migration of immune cells, in non-immune insulin signaling and EGF receptor signaling (Kolanus et al. 1996; Quast et al. 2009; Fuss et al. 2006; Bill et al. 2010). The GEF-activity however seems to be dispensable for at least some of these signal transduction pathways (Geiger et al. 2000; Bill et al. 2010).

In contrast to other ARF-GEF proteins, e.g. BIG1/2 and GBF, cytohesins are insensitive to inhibition with Brefeldin A (Mossessova et al. 2003; Renault et al. 2003). However, recently the small molecule inhibitor Secin H3 (Sec7 inhibitor H3, inhibitor of cytohesin function, cf. figure 7), 1-,2-,4-substituted triazole, was discovered. In the course of a small molecule screening for GEF activity inhibitors, Secin H3 was found to be able to replace the cytohesin specific aptamer M69 (Mayer et al. 2001) bound to the Sec7 domain of cytohesin-2. It inhibits the GEF-activity of cytohesin-proteins towards ARF1 selectively, and binds to their respective Sec7 domains. In contrast, only minor effects on other Sec7 domain bearing GEF proteins (e.g. EFA6) can be observed (Hafner et al. 2006).

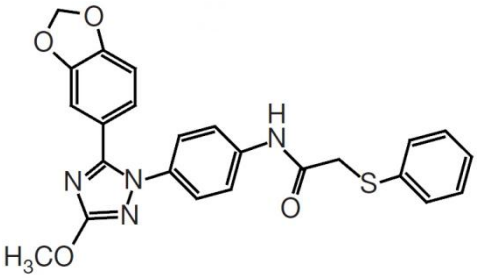

Figure 7: Molecular structure of Secin H3 (Hafner et al. 2006).

With the help of Secin H3, it was possible to identify cytohesin-2 and -3 in humans and the *Drosophila* homolog *steppke* as regulators of insulin signaling upstream of PI3-kinase (Fuss et al. 2006; Hafner et al. 2006). Furthermore, very recently cytohesin-2 was reported to act in Erb signaling (Bill et al. 2010).

Despite the efficacy of Secin H3 in inhibiting cytohesin activity in insulin and EGFR signaling, cytohesin functions in immune cells were only poorly inhibited as compared to cytohesin knockdown experiments. Hence, there was a need for more potent inhibitors for this class of enzymes. To this end, Secin H3 was used to serve as the lead structure for a similarity based virtual compound screen (Hafner et al. 2006; Stumpfe et al. 2010). This bioinformatic approach combined two virtual screening (VS) methods. Both methodologies were target-structure based, that means, Secin H3 was used as reference compound in the search of novel compounds. Both approaches used two-dimensional representations of Secin H3, so called 2D fingerprints as the basis for virtual search. Yet, one approach, termed “similarity search”, focused on similar structures to the lead compound Secin H3. Starting from a reference compound, virtual molecule libraries are screened for divergent but similar molecular structures (Stumpfe et al. 2010). The other approach, termed “diversity search” was focusing on finding structures with different structural scaffolds compared to the lead-structure Secin H3 (Stumpfe et al. 2010).

Using these criteria, 145 compounds were selected as potential interactors and further investigated in various *in vitro* and *in vivo* assays regarding their efficacy in inhibiting reported cytohesin functions.

As depicted in figure 8, these assays included a gene expression assay for d4EB-P1 in S2 cells after insulin stimulation, an *in vitro* GTP-exchange assay with isolated ARF1 and cytohesin-2 (ARNO) Sec7, and a cell adhesion assay using Jurkat T cells on ICAM-1 coated surfaces.

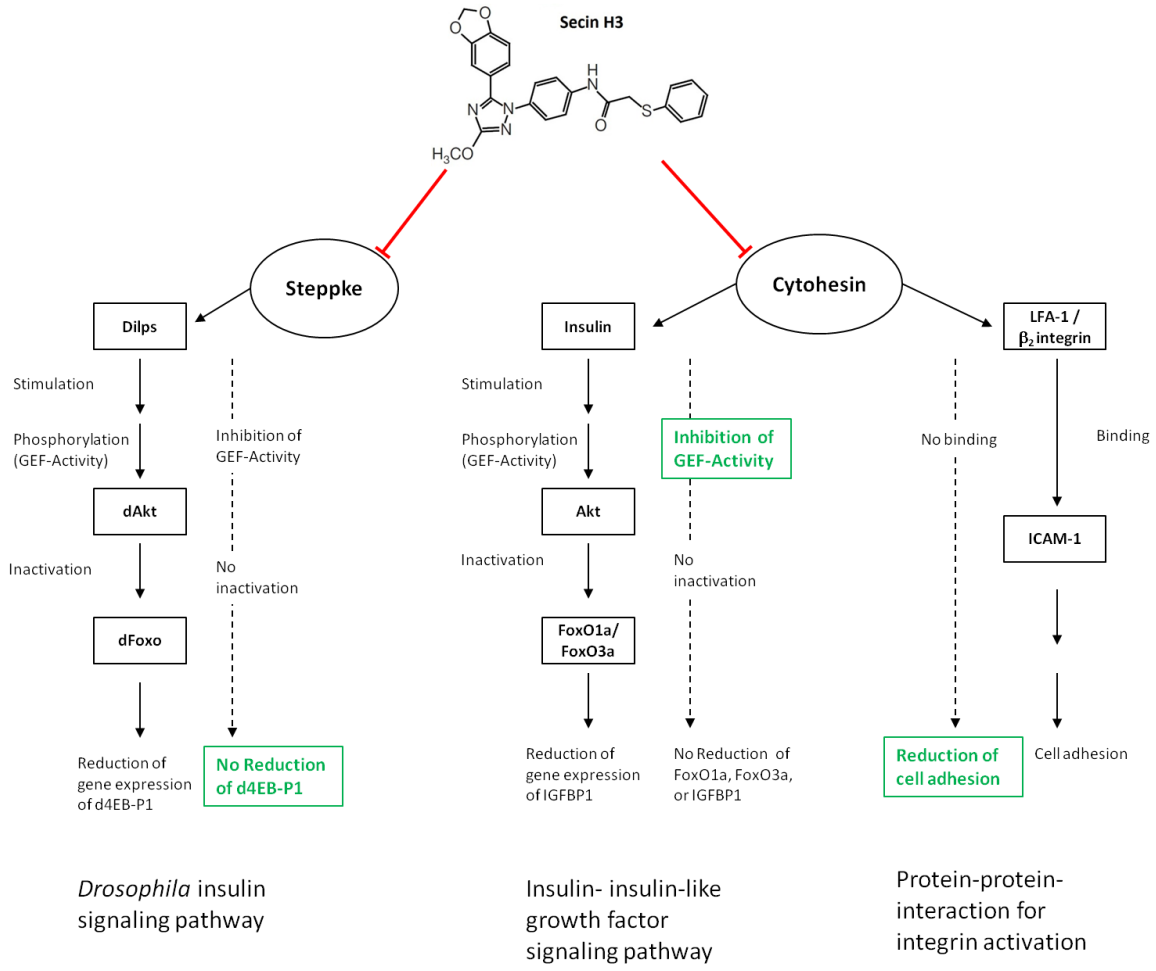

Figure 8: Pathways influenced through Secin H3-mediated inhibition of *steppke* and cytohesin, respectively.
Full arrows indicate the signaling pathways without inhibition by SecinH3, dashed arrows indicate the consequences of inhibiting *steppke* or cytohesin, respectively. The effects of inhibition displayed in the green boxes served as read out for the three assays performed in the screening (modified from Stumpfe et al. 2010)

The compounds were screened for their ability to interfere with cytohesin-dependent insulin-signaling in *Drosophila* S2 tissue cells. Inhibition of the *Drosophila* cytohesin *steppke* leads to elevated levels of dFOXO target genes, e.g. *d*4EB-P1, since their transcription is reduced after phosphorylation of dFOXO under normal conditions. The mRNA levels of *d*4EB-P1 served as read out for the functionality of the compounds (Stumpfe et al. 2010).

In the second screening assay the compounds were tested for their inhibitory capacity of the catalytic GEF-activity of ARNO Sec7 protein towards ARF1 GTPase. Cytohesin GEF-activity was therefore measured *in vitro* with isolated proteins (Stumpfe et al. 2010).

In the third screening, the compounds were assayed for their ability to inhibit cytohesin-1 mediated adhesion of Jurkat T cells to ICAM-1 after TCR engagement or PMA-stimulation (Stumpfe et al. 2010).

As a result, two compounds showed interesting inhibition profiles which were further investigated. These compounds were Secin 16 (cf. figure 9), a small molecule comprising a phenyl diamide core and Secin 144 (cf. figure 12), a 5-oxa-1,2-diazole with aromatic appendices. The aim of this study was to investigate the inhibitory profile of these compounds and consequences for cytohesin-related processes in immune cells.

3.1 The cytohesin inhibitor Secin 16 is a potent pan-cytohesin inhibitor

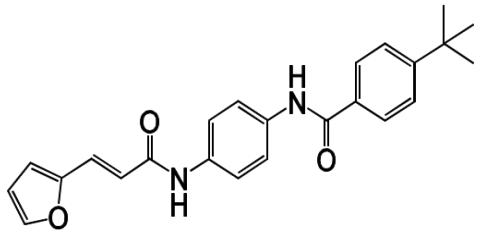

Figure 9: Molecular structure of Secin 16 (molecular structure from ASINEX).

3.1.1 Secin 16 inhibits cytohesin-dependent GTP-exchange, insulin signaling in *Drosophila* and adhesion of Jurkat T cells to ICAM-1

The *in silico* identified small molecule compounds had to be investigated in cytohesin-regulated processes in order to determine their inhibitory efficacy. These assays included the guanine-nucleotide exchange of ARNO-Sec7 towards the ADP ribosylation factor 1 (ARF1) (Chardin et al. 1996), for their ability to increase mRNA levels of *d*4E-BP1, a downstream target of the transcription factor *d*FOXO (Fuss et al. 2006; Southgate et al. 2007), and for inhibiting adhesion of Jurkat T cells to ICAM-1 coated surfaces (Kolanus et al. 1996).

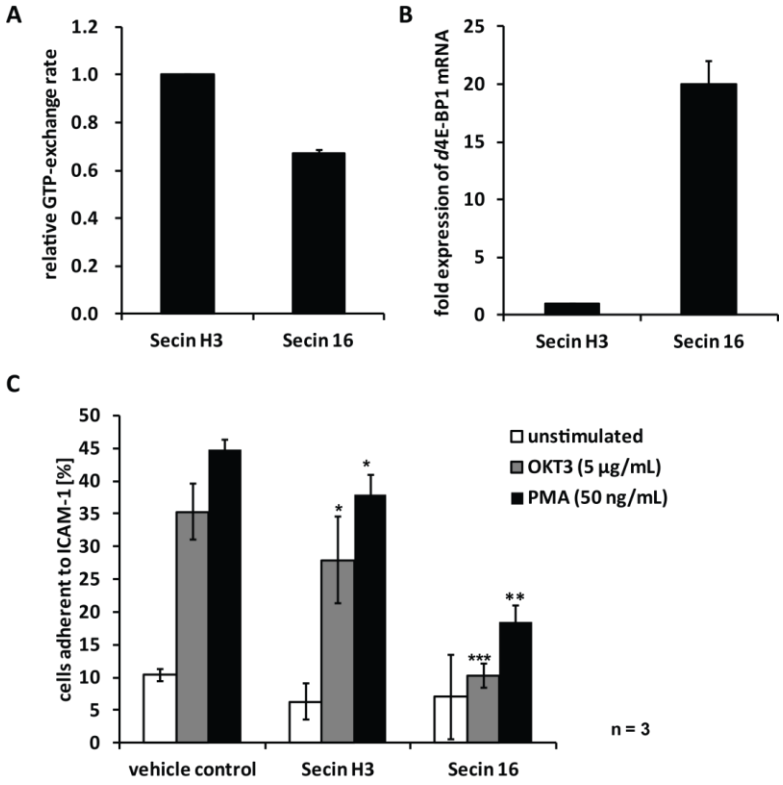

Figure 10: Secin 16 is a more potent inhibitor of cytohesin-2-mediated guanine nucleotide exchange towards ARF1, *steppke*-mediated insulin-signaling and cytohesin-1 regulated T cell adhesion to ICAM-1 as compared to Secin H3.

A: Shown is the GTP-exchange rate of ARNO towards ARF1 normalized to the activity of Secin H3. GTP-exchange rate was determined by measuring the tryptophan fluorescence of 250 nM ARF1 (Δ17N) (amino acids 18 - 181) after addition of 50 µM GTP in the presence of 10 nM ARNO-Sec7 (amino acids 50 - 255) and 3 mM magnesium ions. The concentration of the compounds was 5 µM. The average of five independent experiments is displayed. **B**: Shown is the expression of d4E-BP1 mRNA in *Drosophila* S2 cells after 18 hour preincubation with the respective inhibitor at a concentration of 10 µM and insulin stimulation. mRNA levels were quantified via qRT-PCR. The average of three independent experiments normalized to Secin H3 is shown. (A and B modified from Stumpfe et al. 2010). **C**: Adhesion of Jurkat T cells to ICAM-1 coated surfaces. Cells were preincubated with 25 µM Secin H3, Secin 16 or DMSO as vehicle control, respectively, stained with H33342 and placed on ICAM-1. Jurkat T cells were stimulated with anti-CD3 (OKT3) or phorbol ester (PMA), respectively for 15 minutes. Unattached cells were removed by 5 consecutive washing steps and adherent cells were determined by measuring H33342 fluorescence at 485 nm. Shown is the average of three independent experiments as percentage of adherent cells compared to an unwashed control (100%). Asterisks indicate significant deviation from the vehicle control with respective stimulation calculated by Student's t-test (* = $P < 0.05$, ** = $P < 0.01$; *** = $P < 0.001$).

ARNO-Sec7 and ARF1(Δ17N) were overexpressed in *E. coli* and purified. ARF1(Δ17N) lacks the first 17 amino-terminal amino acids which contain a myristoylation site, hence deletion of this site leads to better activity and solubility of the protein (Franco et al. 1993). The GTP-exchange was measured by tryptophan fluorescence of ARF1(Δ17N) which changes due to significant conformational shifts in the protein after loading of ARF1 with GTP (Antonny et al. 1997; Northup et al. 1982). ARF1 was pre-incubated with GDP. ARNO-Sec7 and the respective inhibitor were then

mixed with the ARF1 protein and the reaction was started by addition of GTP. The fluorescence was measured. Figure 10A shows the relative GTP-exchange rate of ARNO Sec7 towards ARF1 after inhibition with Secin 16 compared to Secin H3. Secin 16 was able to inhibit the GTP-exchange 33% stronger as compared to Secin H3 at a concentration of 5 µM (Stumpfe et al. 2010).

Inhibition of *steppke* in insulin-mediated signaling reduces Akt-dependent *d*FOXO1 phosphorylation (Fuss et al. 2006). This in turn, leads to the exclusion from the nucleus, where *d*FOXO1 can no longer act as a repressor of *d*4EB-P1 gene expression (Brownawell et al. 2001). Elevated levels of *d*4EB-P1 mRNA hence serve as positive read out for inhibition of *steppke.* For the quantification of *d*4E-BP1 mRNA levels *Drosophila* S2 cells were preincubated with the inhibitors in a concentration of 10 µM for 18 hours. mRNA was isolated after insulin stimulation (5 µg/mL) and *d*4E-BP1 levels were quantified via quantitative RT-PCR after cDNA synthesis. The mRNA expression of *d*4E-BP1 (cf. figure 10B) following incubation with Secin 16 is 20-fold increased compared to the one for Secin H3 (Stumpfe et al. 2010).

It is known that cytohesin-1 is an important regulator for adhesion of immune cells to ICAM-1 (Geiger et al. 2000; Kolanus et al. 1996; Quast et al. 2009). However, Secin H3, a pan-cytohesin inhibitor did not lead to an adhesion phenotype to the same extend than knock-down of cytohesin proteins by RNAi did. In order to investigate, if Secin 16 is able to inhibit this cytohesin function more efficiently than Secin H3, adhesion assays with Jurkat T E6.1 cells were performed. Hence, Jurkat T cells were preincubated with 25 µM of the respective inhibitor for one hour, stained with Hoechst H33342 and subsequently placed onto ICAM-1 coated surfaces. After 15 minutes of stimulation with 5 µg/mL OKT3 or 50 ng/mL PMA, respectively, unattached cells were carefully removed and the remaining cells were quantified by measuring the H33342-fluorescence.

Adhesion of Jurkat E6.1 T cells to ICAM-1 coated surfaces is strongly reduced following inhibition with Secin 16. Cells preincubated with Secin 16 show a reduction in adhesion of 59% after PMA stimulation and of 71% after TCR stimulation with anti-CD3 (OKT3) antibody, respectively, compared to the vehicle-treated control. In contrast, Secin H3 reduces the amount of adherent cells only by 15% after PMA stimulation and of 21% after TCR stimulation compared to the DMSO control. This effect is much weaker than that of inhibition by Secin 16. These results indicate that Secin 16 inhibits cytohesin-1 regulated cell adhesion more efficiently as compared to Secin H3.

Taken together, these data suggest that compared to Secin H3, Secin 16 was much more effective in inhibiting multiple cytohesin-functions, e.g. ARNO-dependent guanine nucleotide exchange

towards ARF-GTPase 1, *steppke*-dependent insulin-signaling and cytohesin-1-dependent regulation of Jurkat T cell adhesion (Stumpfe et al. 2010). Therefore, the cytohesin-inhibitor Secin 16 was chosen for further investigation.

3.1.2 Secin 16 binds to the Sec7 domain of cytohesin-1

The diversity of the subsequently employed screening assay was a first attempt to address the question of specificity of the novel small molecules for cytohesin proteins. Therefore, known cytohesin functions are exploited for the Grp-1 homologue *steppke* in *Drosophila*, for guanine-nucleotide exchange in ARNO and for cytohesin-1-mediated cell adhesion of Jurkat T cells.

Since all these cytohesin-mediated processes were inhibited by the use of Secin 16, it can be assumed that Secin 16 is a specific compound for the inhibition of all cytohesin proteins. Furthermore, the observed inhibition of the *in vitro* GTP-exchange only involved the isolated Sec7 domain of ARNO and the ARF1(Δ17N) proteins therefore, interaction with the cytohesin Sec7 domain can be concluded.

Nevertheless, it was necessary to show that Secin 16 is able to bind its target protein. Hence, SPR (surface plasmon resonance) was used to ascertain that Secin 16 specifically binds to the Sec7 domain of cytohesin-1.

In a dual-channel SPR setup the Sec7 domain of cytohesin-1 purified from *E. coli* was covalently linked to the sample channel of an HC1500m chip. Binding of Secin 16 was measured as the net channel response (displayed as difference between sample channel response and reference channel response) corrected for blank buffer injections (1% DMSO, PBS) and for DMSO injections in different concentrations in 1% DMSO/PBS.

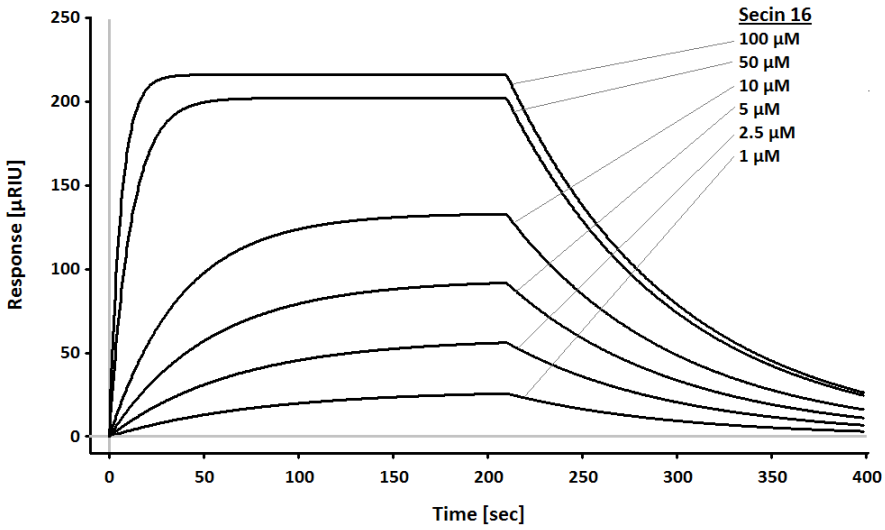

Figure 11: Secin 16 interacts with cytohesin-1 Sec7 domain.
Secin 16 shows dose-response dependence in the binding to Sec7-domain of cytohesin-1. Shown are the fitted binding and dissociation curves of Secin 16 interaction with the Sec7 domain of cytohesin-1 immobilized to an HC1500m polycarboxylate hydrogel chip at the indicated concentrations in 1% DMSO/PBS. The data are corrected for blank buffer and solvent injections. Each line is calculated from at least three independent injections.

Secin 16 binds in a concentration-dependent manner to cytohesin-1 Sec7 domain as displayed in figure 11. At concentrations of 10 µM the binding of the protein already reaches saturation after 200 seconds of injection. Coating of the chip surface with ARF1(Δ17N) or BSA, respectively did not lead to binding of the compound even after elongating the incubation time with the proteins. This clearly indicates that Secin 16 binds specifically to the Sec7 domain of cytohesin proteins and can be used in concentrations of 10 µM for further experiments.

3.2 The cytohesin inhibitor Secin 144 is a partial inhibitor of cytohesin function

The virtual screen performed to find novel inhibitors for cytohesin function, led to the discovery of 145 potential novel molecules that could serve for this purpose. In the consecutive functional screen these compounds were evaluated for their potential to inhibit cytohesin-related functions, e.g. guanine nucleotide exchange of cytohesin-2 towards ARF1, steppke-dependent regulation of insulin signaling and cytohesin-1-regulated adhesion of Jurkat T cells to ICAM-1. Amongst others, Secin 144 showed an interesting inhibition profile (Stumpfe et al. 2010).

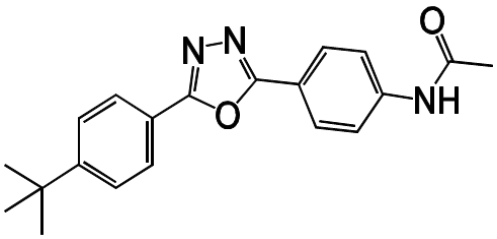

Figure 12: Molecular structure of Secin 144 (molecule structure from VitasM laboratories).

3.2.1 Secin 144 does not influence cytohesin-2-mediated GTP-exchange towards ARF1 and *steppke*-dependent Foxo signaling, but abrogates adhesion of Jurkat T cells to ICAM-1

In order to verify the inhibitory potential of compounds that were found in a virtual screen, based on Secin H3 as lead compound, the novel Secin molecules were subjected to functional screening assays (Stumpfe et al. 2010).
Cytohesin proteins are known GEFs for the ADP-ribosylation factors, e.g. ARF1 and ARF6 (Chardin et al. 1996; Meacci et al. 1997). Inhibition of this primary cytohesin function leads to a decreased exchange of GTP towards ARF1 as it can be measured by tryptophan autofluorescence (Ahmadian et al. 2002).

As depicted in figure 13A, Secin 144 was tested for the ability to inhibit GTP-exchange. In order to compare the inhibitory capacity with the small molecule Secin H3, *in vitro* GTP-exchange assays with ARNO Sec7 towards ARF1 were performed. To this end, cytohesin-2 was preincubated with 5 µM Secin 144 or Secin H3 in DMSO, respectively, and ARF1(Δ17N) was added to the reaction. GTP-exchange was measured after injection of GTP by following the increase of the intrinsic ARF1 tryptophan fluorescence (Antonny et al. 1997; Northup et al. 1982).

The addition of Secin 144 did not lead to an improved inhibition of GTP-exchange activity of cytohesin-2 towards ARF1. As depicted in figure 13A, the relative exchange rate was even increased about 35% compared to Secin H3 treatment.

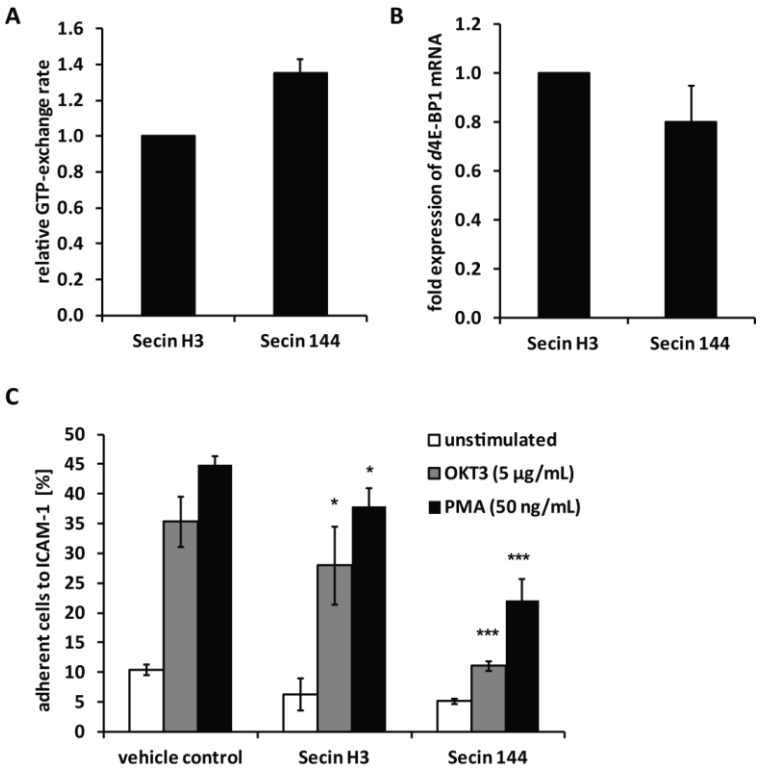

Figure 13: Secin 144 shows no significant inhibition of *Drosophila* insulin signaling and cytohesin-2 GTP-exchange towards ARF1 as compared to Secin H3, however it is a potent inhibitor of cytohesin-1-mediated adhesion of Jurkat T cells to ICAM-1.

A: Shown is the GTP-exchange rate normalized to the activity of Secin H3. Secin 144 leads to elevated exchange rates compared to Secin H3 thus cannot be considered as an improved inhibitor of GTP-exchange towards ARF1. Secin 144 exchange rate was determined by measuring the tryptophan fluorescence at 250 nM ARF1(Δ17N) (amino acids 18 - 181) after addition of 50 µM GTP in the presence of 10 nM ARNO-Sec7 (amino acids 50 - 255) and 3 mM magnesium ions. The concentration of the compounds was 5 µM. Secin 16 shows a relative increase of exchange activity of 1.35. The average of five independent experiments is shown. (modified from Stumpfe et al. 2010) **B**: Shown is the expression of d4E-BP1 mRNA in *Drosophila* S2 cells after 18 hour preincubation with the respective inhibitor at a concentration of 10 µM and insulin stimulation. mRNA levels were quantified via RT-PCR. The average of three independent experiments is shown. The level of d4E-BP1 mRNA is decreased compared to the mRNA level that is expressed after incubation with Secin H3. (modified from Stumpfe et al. 2010) **C**: Adhesion of Jurkat E6.1 T cells to ICAM-1. Cells were preincubated with 0.5% DMSO (vehicle control) or 25 µM of the indicated compound in 0.5% DMSO for one hour and placed in 96-well plates coated with ICAM-1-Fc. Cells were then stimulated for 15 minutes and unattached cells were carefully removed by 5 consecutive washing steps. Shown is the average of three independent experiments as percentage of adherent cells compared to an unwashed control (100%). Asterisks indicate the significance to the comparably stimulated vehicle control determined via Student's t-test (* $P < 0.05$, *** $P < 0.001$).

The *Drosophila* cytohesin-3 homologue *steppke* was reported to act in insulin signaling leading to the phosphorylation of *d*FOXO1. After phosphorylation FOXO1 is retained in the cytosol and hence, fails to act as a transcription factor for *d*4EB-P1 (Fuss et al. 2006). Thus, elevated *d*4EB-P1 mRNA levels served as readout for cytohesin inhibition. In order to test the effects of the novel small molecules in cytohesin function they were applied to well-established and robust functional

assays. For the determination of *d*4EB-P1 mRNA levels, S2 cells were starved and preincubated with the inhibitors 18 hours prior to stimulation with insulin. Cells were lysed and cDNA was produced out of isolated mRNA via reverse transcription. cDNA levels were determined by qRT-PCR.
Secin 144 leads to a decreased level of *d*4EB-P1 mRNA of about 20% compared to Secin H3, equivalent to ineffective inhibition of *steppke* function (cf. figure 13B).

Interestingly, Secin 144 was found to be a strong inhibitor of T cell adhesion to ICAM-1 as depicted in figure 13C. Jurkat T cells were preincubated with 25 µM Secin 144 and placed onto ICAM-1-Fc-coated surfaces. For stimulation 5 µg/mL anti-CD3 (OKT3) or 50 ng/mL PMA were used, respectively.
Application of Secin 144 in Jurkat T cells results in a strong reduction of T cell adhesion. Adhesion to ICAM-1 was abrogated irrespective of the kind of stimulation. This inhibitory effect on T cell adhesion was much stronger as compared to T cells that were treated with Secin H3.

3.2.2 Secin 144 binds to the Sec7 domain of cytohesin-1

Secin 144 proved to be only a partial inhibitor of cytohesin function as shown by the functional screening assays (cf. figure 13). In GTP-exchange assays and Foxo1-activation assays, Secin 144 had no effect on the inhibition of cytohesin functions. In stark contrast to that, Secin 144 was one of the most potent inhibitors of T cell adhesion to ICAM-1 *in vitro*. Since adhesion is a cellular process which involves a plethora of signaling cascades and therefore protein components, it was also possible that Secin 144 did not affect cytohesin-1 but other proteins responsible for proper adhesion in T cells. To this end, it was tested, whether Secin 144 interacts with the Sec7 domain of cytohesin-1 responsible for the mediation of T cell adhesion (Kolanus et al. 1996; Geiger et al. 2000).

A dual-channel SPR setup was used to investigate the interaction of Secin 144 with the Sec7 domain of cytohesin-1. Sec7 domain of cytohesin-1 purified from *E. coli* was covalently linked to the sample channel of an HC1500m chip. The reference channel was left unloaded. Binding of Secin 144 was measured at various concentrations as the net channel response (difference between sample channel and unloaded reference channel). Therefore, Secin 144 was freshly dissolved in water-free DMSO and subjected to ultrasound treatment for 15 minutes. Undissolved particles were pelleted by centrifugation at 13,000 x g for 15 minutes. The stock solution was then diluted in 1% DMSO/PBS at suitable concentrations immediately before injection. The resulting

response was corrected for blank buffer injections and DMSO as vehicle control and association and dissociation was depicted as a function of response versus time.

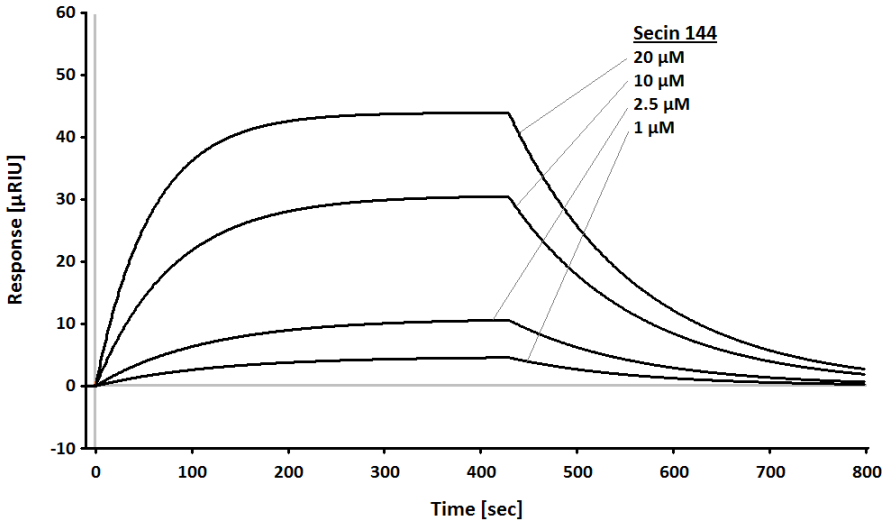

Figure 14: SPR sensorgram of Secin 144 interaction with cytohesin-1 Sec7 domain.
Shown are the fitted binding and dissociation curves of Secin 144 interaction with the Sec7 domain of cytohesin-1 immobilized to an HC 1500m polycarboxylate hydrogel chip at the indicated concentrations in 1% DMSO/PBS. The data are corrected for blank buffer and solvent injections. Each line is calculated from at least three independent injections.

As depicted in figure 14 the interaction of Secin 144 with the Sec7 domain of cytohesin-1 can be clearly proven with this method. Association of Secin 144 is concentration-dependent and its saturation occurs already at a concentration of 10 µM after 400 seconds. Hence, it can be concluded that Secin 144 binds specifically to cytohesin-1. This implies that the inhibitory effects observed upon use of Secin 144 in Jurkat T cell adhesion on ICAM-1, are due to the fact that Secin 144 is able to inhibit specifically cytohesin-1 function.

3.3 Dissecting GEF-dependent and GEF-independent functions of cytohesin-1

3.3.1 GTP-exchange of cytohesin-1 Sec7 domain towards ARF1 GTPase is only inhibited by Secin 16 but not by Secin 144

Secin 16 was shown to be a potent small molecule compound for the inhibition of GTP exchange towards ARF1 mediated by cytohesin-2 (figure 10A). To investigate a possible inhibition of Secin 16 in the cytohesin-1 mediated GTP-exchange, assays were performed in the same manner for the isolated Sec7 domain of cytohesin-1 and ARF1(Δ17N).

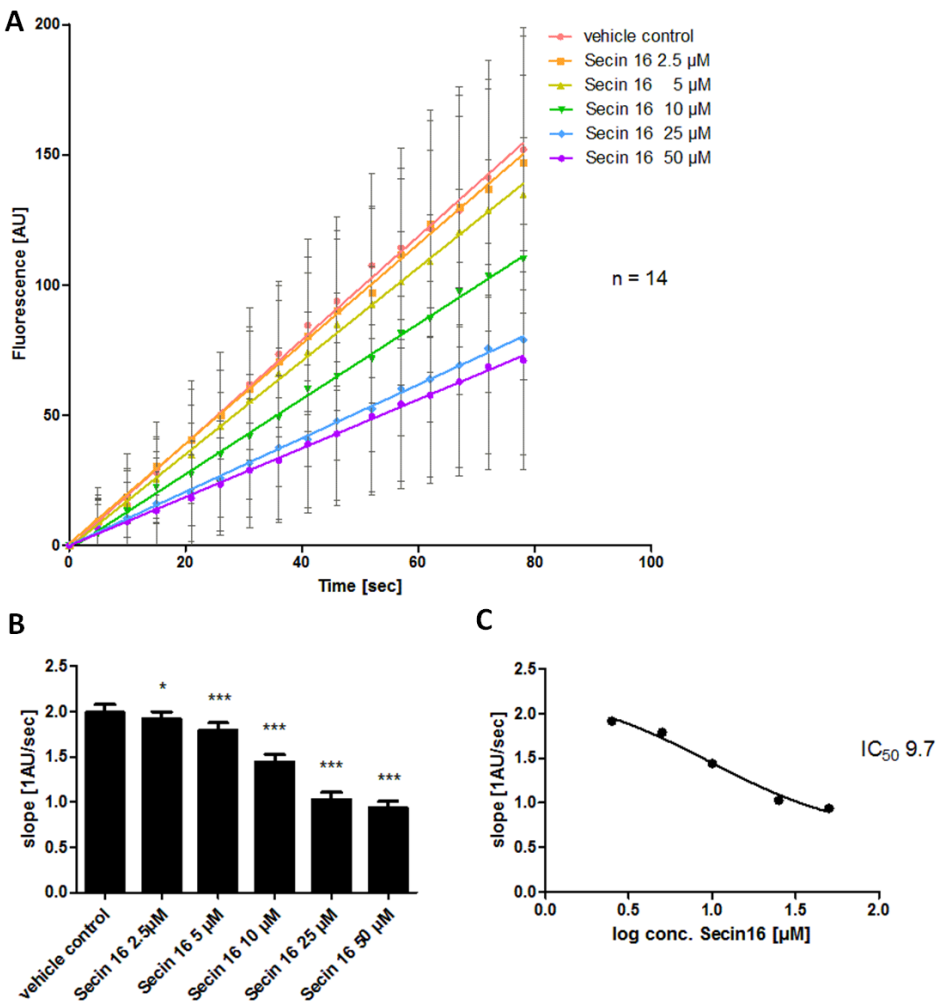

Figure 15: Secin 16 inhibits cytohesin-1 guanine nucleotide exchange towards ARF1 dose-dependent.

ARF1(Δ17N) (final concentration 500 nM) was loaded with GDP in the presence of 2 mM EDTA. Stabilizing magnesium was added at a final concentration of 3 mM. Cytohesin-1 Sec7 domain (amino acids 49 – 249) at a final concentration of 80 nM preincubated with DMSO as vehicle control or the compound at the indicated concentrations was added and the reaction was started by injection of GTP at a final concentration of 50 µM. The reaction was followed by measuring the tryptophan fluorescence of ARF1 protein at excitation and emission wavelengths of 280 nm and 340 nm, respectively. **A**: The linear region of fluorescence increase is displayed versus the time. Lines show the fitted linear regression. **B**: Calculated slope of the fluorescence increase is displayed versus Secin 16 concentration. Only the linear region of the fluorescence increase was used to determine the slope. Asterisks indicate significance compared to the vehicle control as calculated via Student's t-test (* P< 0.05, *** P< 0.001). **C**: IC_{50} of Secin 16 in the GTP-exchange assay. Shown is the slope versus the log of Secin 16 concentration. IC_{50} was calculated as 9.7 µM via GraphPad software. The graphs are the averaged result of 14 independent experiments.

Secin 16 was used in concentrations from 2.5 µM to 50 µM in the GTP-exchange reaction of cytohesin-1 Sec7 domain towards the ARF1 protein. ARF1(Δ17N) and cytohesin-1 Sec7 domain were overexpressed in *E. coli* and purified by Ni-NTA affinity chromatography. ARF1 was loaded with GDP in the presence of magnesium ions prior to the assay and the Sec7 domain of

cytohesin-1 was preincubated with DMSO as control or Secin 16 at concentrations indicated. Both proteins were then mixed and the reaction was initiated by injection of GTP at a final concentration of 50 µM. GTP-exchange was determined by measuring the tryptophan fluorescence of ARF1 protein (Antonny et al. 1997; Northup et al. 1982). As displayed in figure 15A, significant inhibition of the reaction can be observed already at concentrations of 2.5 µM. The calculated IC_{50} from the *in vitro* GTP-exchange assay is 9.7 µM and thus corresponds to the concentration used in the other assays to inhibit cytohesin function. These results show that not only the GEF-function of ARNO, but also of cytohesin-1 Sec7 domain is effectively inhibited by Secin 16 *in vitro*.

Secin 144 failed to inhibit GTP-exchange functions of cytohesin-2 towards ARF1 GTPase. However, in adhesion experiments Secin 144 proved to be a very potent inhibitor of adhesion to ICAM-1 mediated by cytohesin-1 in Jurkat T cells. Furthermore, SPR experiments showed a specific interaction of Secin 144 with its target cytohesin-1.

The virtual ligand-based screen for the identification of novel inhibitors for cytohesin proteins was based on the fact that Secin H3 specifically interacts with the Sec7 domain of cytohesin proteins. The family of cytohesin proteins shows a highly conserved domain structure. It was therefore unlikely that the failure of Secin 144 to inhibit GTP-exchange of cytohesin-2 Sec7 domain towards ARF1 was due to the fact that it is a specific inhibitor for cytohesin-1 functions. However, to exclude this possibility, GTP-exchange experiments with the Sec7 domain of cytohesin-1 have been performed.

Hence, both ARF1(Δ17N) or cytohesin-1 Sec7 domain were overexpressed in *E. coli* and purified by Ni-NTA affinity chromatography. Cytohesin-1 was preincubated with Secin 144 at concentrations between 1 µM and 20 µM. ARF1 was preincubated with GDP, EDTA and $MgCl_2$ and mixed with cytohesin-1. Reaction was monitored following addition of GTP by measuring the tryptophan fluorescence of ARF1 protein (Antonny et al. 1997; Northup et al. 1982).

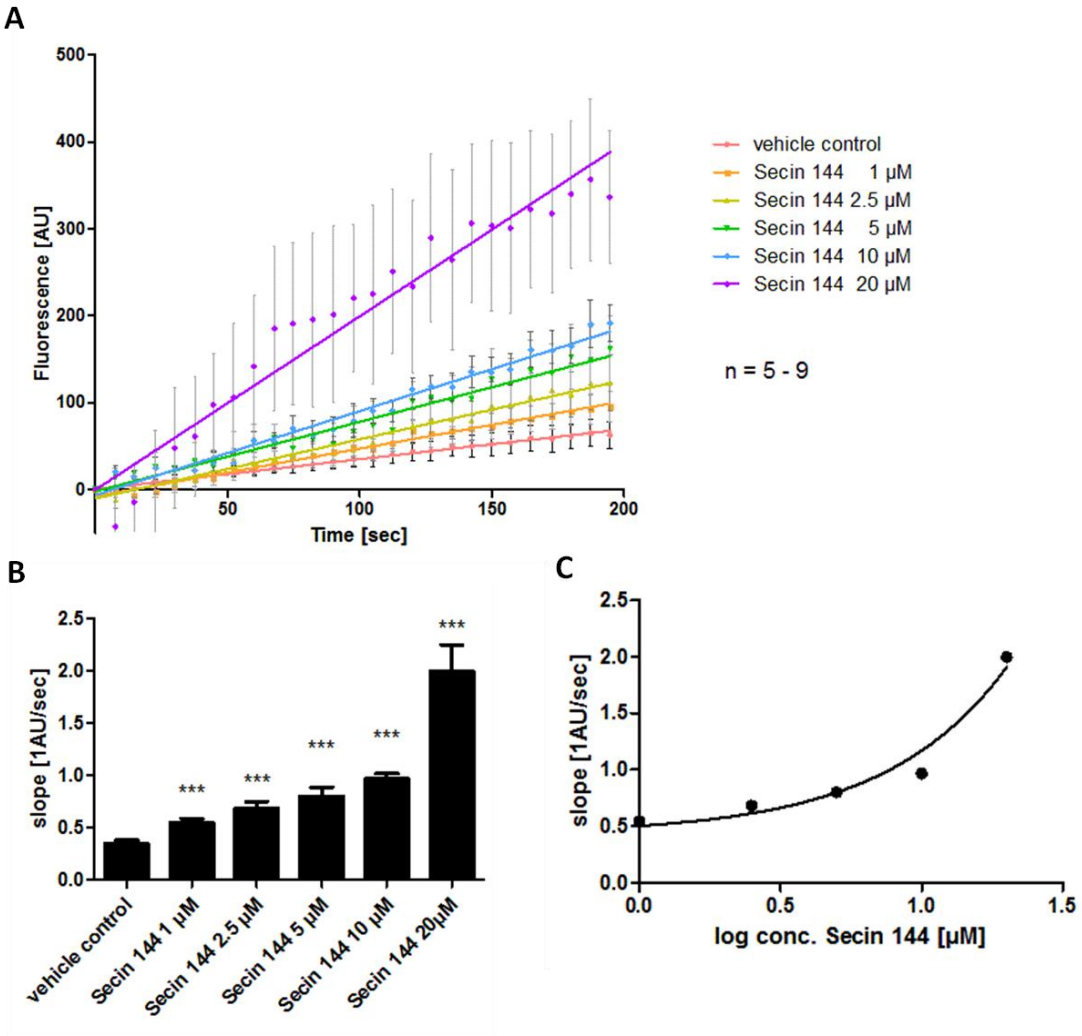

Figure 16: Secin 144 fails in inhibiting cytohesin-1-mediated GTP-exchange towards ARF1 *in vitro.*

ARF1(Δ17N) (final concentration 500 nM) was loaded with GDP in the presence of 2 mM EDTA. Stabilizing magnesium was added at a final concentration of 3 mM. Cytohesin-1 Sec7 domain (amino acids 49 – 249) at a final concentration of 80 nM preincubated with DMSO as control or the compound at the indicated concentrations was added and the reaction was started by injection of GTP at a final concentration of 50 µM. The reaction was followed by measuring the tryptophan fluorescence of ARF1 protein at excitation and emission wavelengths of 280 nm and 340 nm, respectively. **A**: The linear region of fluorescence increase is displayed versus the time. Lines show the baseline corrected linear regression, error bars depict ±SD. **B**: Calculated slope of the fluorescence increase is displayed versus Secin 144 concentration. Only the linear region of the fluorescence increase was used to determine the slope. Asterisks indicate significance compared to the DMSO control as calculated via Student's t-test (*** P< 0.001). **C**: Concentration-activity plot of Secin 144 in GTP-exchange assay. Shown is the slope versus the log of Secin 144 concentration. The graphs are the averaged result of up to nine independent experiments.

As displayed in figure 16, no inhibition of the cytohesin-1-mediated GTP-exchange reaction towards ARF1 can be observed after addition of Secin 144. In contrast, an increase in the GTP-exchange activity of cytohesin-1 towards ARF1 is visible. However, the half-maximal effective

concentration, EC_{50}, for the GTP-exchange was calculated to be more than 310 mM (GraphPad Prism). Especially since the GTP-exchange was observed in an *in vitro* system in the sole presence of the two interacting proteins, cytohesin-1 and ARF1, the effect is likely even smaller in a cytosolic surrounding. Therefore, it can be concluded, that the applied concentrations of Secin 144 have no enhancing effect on the GTP-exchange activity of cytohesin-1 proteins *in vivo*.
Furthermore, it can be concluded that the observed inhibition effects of Secin 144 in Jurkat T cell adhesion are not due to a specific inhibition of only cytohesin-1 in contrast to other cytohesins, since there is also no inhibition of GTP-exchange activity of cytohesin-1 towards ARF1.
This result indicates that the inhibitory function of Secin 144 on cytohesin is limited to the adhesion-related branch of cytohesin-functions. Since the GEF function of cytohesin is not inhibited, it can be postulated that Secin 144 only inhibits the regulation of β_2-integrin activation. This qualifies Secin 144 as a potent tool for dissecting GEF-dependent and -independent functions of cytohesin for the first time.

3.3.2 The influence of cytohesin-1 on the activation of β_2-integrins

The activation of LFA-1 is a key function of cytohesin-1 (Kolanus et al. 1996; Geiger et al. 2000). Cytohesin-1 is recruited to the plasma membrane after T cell activation and interacts with the cytoplasmic tail of β_2-integrin thus, leading to conformational changes that result in higher affinity of LFA-1 for its corresponding ligand ICAM-1.

It was reported that cytohesin-1 directly interacts with the β_2-integrin CD18 (Geiger et al. 2000; Kolanus et al. 1996). The novel small molecule inhibitor Secin 144 specifically inhibits the activation of cytohesin mediated β_2-integrin activation which could be due to loss of interaction between cytohesin-1 and LFA-1. Unfortunately, several attempts to co-immunoprecipitate cytohesin-1 with CD18 and *in vitro* interaction studies with purified cytohesin Sec7 domain and cell lysates were unsuccessful regarding the detection of direct interaction between these two proteins in the course of this study.

3.3.2.1 Novel inhibitors for cytohesin function abrogate primary T cell adhesion to ICAM-1 and VCAM-1

Secin 16 and Secin 144 proved to be potent inhibitors of Jurkat T cell adhesion to ICAM-1. The small molecule inhibitor Secin 16 does not only inhibit the cytohesin-mediated activation of LFA-1, but is also able to abrogate GEF-activity of cytohesin proteins towards ARF-GTPases. Moreover, cytohesin-mediated regulation of GEF-activity was reported to be important for T cell adhesion

(Geiger et al. 2000; Kolanus et al. 1996). Thus, it was of great interest to test, whether sole inhibition of LFA-1 activation could be sufficient to abrogate adhesion in PBL. Furthermore, the influence of the inhibitors on activation of other integrins was tested by adhesion assays on VCAM-1, a ligand for β_1-integrins. To this end, PBL were preincubated with 10 µM Secin 16 or Secin 144, stained with H33342 (bisbenzimide) and placed on ICAM-1-Fc or VCAM-1-Fc coated 96-well plates, respectively. After one hour cells were stimulated with anti-CD3 (OKT3, 5 µg/mL) or PMA (50 ng/mL) to induce adhesion for 30 minutes. Unattached cells were carefully removed by several washing steps and adherent cells were quantified by measuring incorporated bisbenzimide fluorescence at 485 nm.

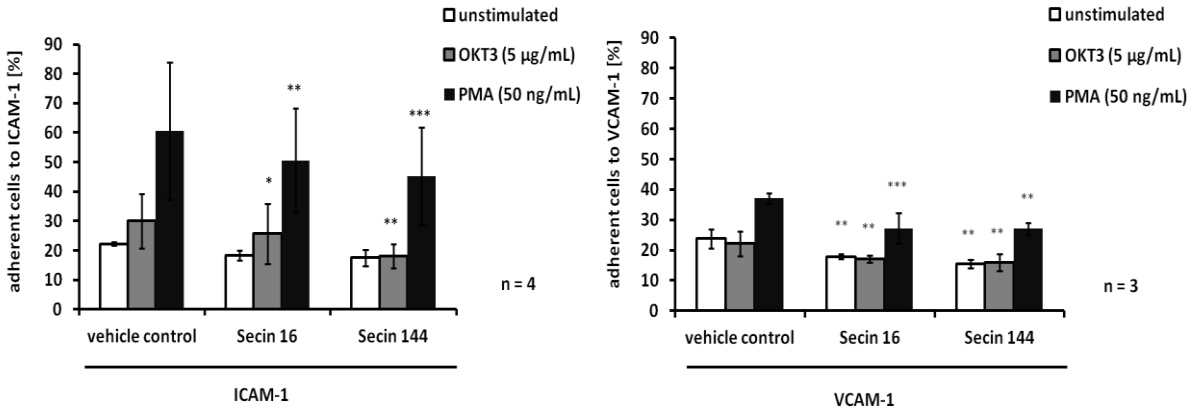

Figure 17: Adhesion of PBL to ICAM-1 and VCAM-1 is strongly reduced after inhibition of cytohesin proteins with Secin 144 and Secin 16.

PBL were preincubated with 10 µM Secin 144 in 0.5% DMSO or DMSO alone, respectively, and 6 µg/mL H33342 for one hour in HBSS. Cells were seeded onto ICAM-1 or VCAM-1 coated 96-well plates in triplicates and stimulated with OKT3 or PMA, respectively for 15 minutes. 5 consecutive washing steps removed not attached cells. Adherent cells were quantified by measuring the fluorescence of incorporated H33342. Depicted is the percentage of adherent cells compared to an unwashed control (100%). The average of 4 and 3 independent experiments is shown. Error bars represent ± SD. Asterisks indicate significant deviation from the corresponding DMSO control as calculated by Student's t-test (** = P< 0.01; *** = P< 0.001)

As depicted in figure 17, Secin 16 is able to reduce adhesion of PBL to ICAM-1. This effect is milder than in Jurkat T cells, however a significant reduction can be observed. Furthermore, the adhesion to VCAM-1 is also impaired after Secin 16 inhibited cytohesin-function. PBL seeded on VCAM-1 are not as responsive to stimulation by anti-CD3 (OKT3) and phorbol ester as compared to cells seeded on ICAM-1. However a reduction in the number of adherent cells can still be observed indicating that other integrins are also influenced by cytohesin function.

Adhesion to both, ICAM-1 and VCAM-1 coated surfaces, is abrogated after incubating PBL with Secin 144. Adhesion of PBL to VCAM-1 is not as strong as to ICAM-1 and stimulation does not increase the adherence of PBL to the same extent than in PBL seeded on ICAM-1. Secin 144 treatment leads to inhibition of PBL attachment below unstimulated baseline levels after TCR stimulation in both experimental setups. The inhibitory effect of Secin 144 on the adhesion of PBL to VCAM-1 might be due to the fact that Secin 144 does not only inhibit cytohesin-1. Cytohesin-2 and -3 are reported to be important regulators of β_1-integrin recycling (Oh and Santy 2010). It is therefore possible that the inhibition of other proteins of the cytohesin family might have an influence on the adhesion to β_1-integrin ligands as VCAM-1.

3.3.2.2 Adhesion under flow is reduced in Secin 144 treated PBL

T cells need to firmly attach to endothelial vessels under shear flow of the blood stream for their extravasation into inflamed tissues. Therefore, a rapid conformational change of integrins is necessary to respond to chemokines, released from the center of infection (Shamri et al. 2005). Quast et al. reported that BmDCs were diminished in their ability to firmly attach to endothelial cells under flow conditions when cytohesin expression was downregulated by RNAi (Quast et al. 2009).

In order to find out the role of cytohesin-1 in the development of activated LFA-1 conformations in human PBL, the small molecule inhibitors Secin 16 and Secin 144 were used in adhesion assays under flow conditions.

PBL were preincubated with Secin 16 or Secin 144 in 0.5% DMSO or DMSO alone as vehicle control, respectively, for one hour and subsequently perfused in a chamber with a confluent layer of HUVECs stimulated with TNF-α to induce ICAM-1 surface expression and loaded with human CXCL12 for the induction of LFA-1 high affinity conformation. Adherent cells were quantified.

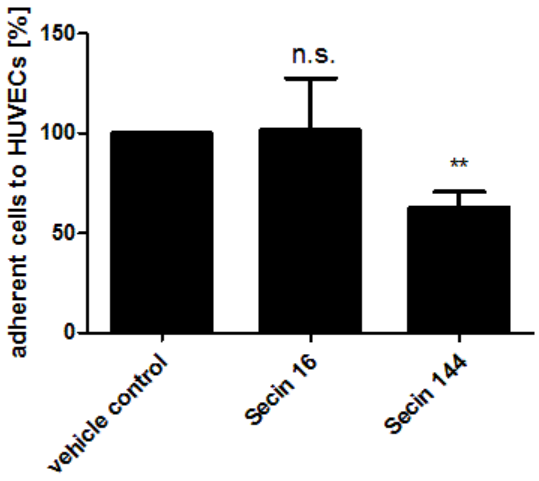

Figure 18: Adhesion of PBL to HUVECs is unaffected under flow-conditions by the inhibition with Secin 16 but reduced after inhibition with Secin 144.
HUVECs were grown to confluency and stimulated with 10 ng/mL TNF-α over night. Two hours prior to experiment, cells were stimulated with human CXCL12 (500 ng/mL). The HUVECs containing petri dish was assembled in a parallel flow-chamber. PBL (1 x 10^6 cells/mL in HH-buffer) were incubated with 10 µM Secin 16 in 0.5% DMSO or DMSO alone (control) prior to experiment. PBL were drawn through the chamber with a laminar flow at a shear rate of 1.5 dyn/cm^2. The cells that were attached to HUVECs after 6 minutes of wash were counted. Shown is the average of the adhesion rate from four independent experiments. (n.s. = not significant; ** = $P < 0.01$).

As it can be seen in figure 18 the inhibition of cytohesin-1 function with Secin 144 reduces the ability of PBL to develop LFA-1 high-affinity conformation. In contrast, Secin 16 treatment did not lead to alterations in the development of the high affinity state of LFA-1 as depicted by unaltered numbers of attached cells to HUVECs.

A possible explanation for this is, that the compound uses a different site for the inhibition of cytohesin-1 therefore inhibition of LFA-1 activation only occurs when Secin 144 is used, because Secin 144 is able to fully inhibit the interaction of cytohesin-1 with LFA-1.

3.3.3 Cytohesin-mediated activation of T cells is not exclusively GEF-dependent

Cytohesins, especially cytohesin-2 are reported to play an important role in cell signaling, e.g. insulin signaling in mice, humans and *Drosophila* via the PI3K/Akt pathway (Fuss et al. 2006; Hafner et al. 2006) and Erb signaling (Bill et al. 2010), connecting cytohesin proteins to very important metabolic cell functions and the development of cancer. Since cytohesin-1 is the most abundantly expressed cytohesin protein in leukocytes (Moss and Vaughan 2002) it is likely, that this protein also plays an important role in immune cell signaling since common signaling cascades are involved. Cytohesin-1 was shown to have an important role in T cell activation after TCR

engagement (Paul 2007; Grell 2009). The complete role of cytohesin-1 in T cell activation remains elusive, however. Especially the dual role of cytohesin proteins as GEFs for ARF-GTPases and LFA-1 activators was inseparable in the investigation of cytohesin-1 function. The comparison of inhibition profiles of Secin 16 in combination with Secin 144 could help to give further insights into this question. Therefore, these compounds were used to investigate the role of cytohesins in T cell activation and cell signaling.

3.3.3.1 In murine T cells, IL-2 and interferon-γ cytokine production is abrogated after inhibition with Secin 16

A major read out for the activation of T cells is the production of the interleukin IL-2. This cytokine is produced after engagement of the TCR complex and CD28 and regulates via a positive feedback loop the proliferation and clonal expansion of T cells (Serfling et al. 1995; Malek 2008). To measure, whether the inhibition of cytohesins has an impact on the production of IL-2, murine $CD8^+$ T cells were isolated from spleens of OT-1 mice and preincubated with Secin 16 for one hour. Stimulation of the T cells was performed by co-cultivation with OVA-pulsed spleen dendritic cells for up to 4 days. IL-2 and interferon-γ production were quantified by ELISA.

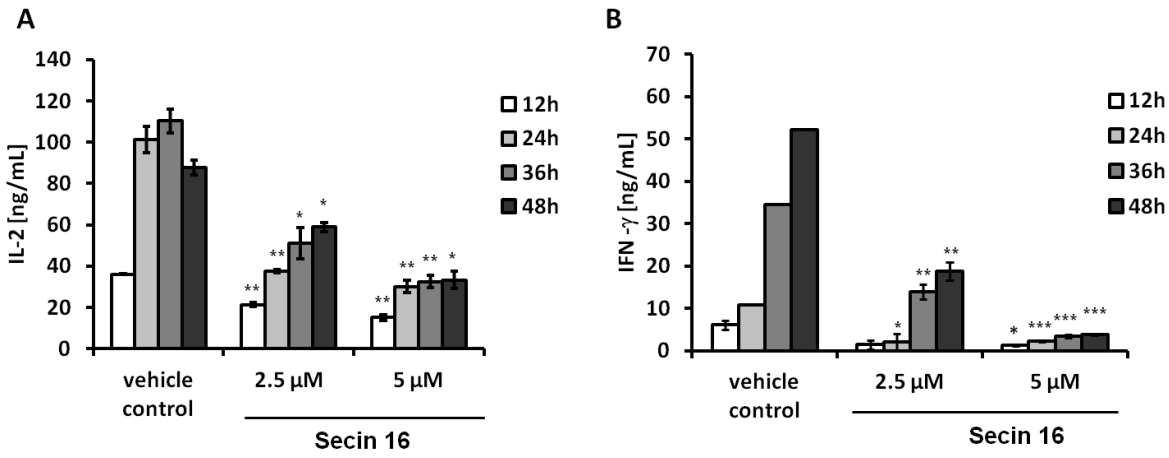

Figure 19: IL-2 and interferon-γ production in murine T cells is abrogated after incubation with Secin 16

Murine CD8+ T cells were isolated from OT-1 mice spleens by MACS separation and pre-incubated with the indicated concentrations of Secin 16 in 0.5% DMSO or DMSO alone (control) in RPMI-medium for one hour. DC from b/6 wild-type mice were isolated from spleen via MACS-separation and pulsed with 1 mg/mL OVA-peptide. After one hour cells were mixed and the compound concentration was adjusted to the increased volume. After indicated time points, supernatant was collected and cytokine concentration was determined by ELISA. Shown results are the average of four experiments. Asterisks indicate significant deviation from the corresponding control at the indicated time points as determined by Student's t-test (* = $P < 0.05$; ** = $P< 0.01$; *** = $P< 0.001$)(results in collaboration with Dr. Rike Schulte, IMMEI, Bonn).

As shown in figure 19, IL-2 and Interferon-γ production in murine T cells is abrogated after inhibition of cytohesin by Secin 16. 5 µM Secin 16 inhibit the production of IL-2 by more than 70% as compared to the corresponding vehicle control, the use of 2.5 µM Secin 16 inhibits the Interleukin-2 production by 30 - 64%, depending on the time point. Interferon-γ production is even more inhibited by the use of Secin 16 as compared to DMSO control. At a concentration of 2.5 µM Secin 16 the interferon-γ production is reduced by 60 – 80% whereas at a concentration of 5 µM Secin 16 a reduction of 80-90% can be observed. Thus the inhibitor shows a dose-response dependency and significantly inhibits the production of activating cytokines. This corroborates findings of Grell and Paul that cytohesin plays an important role in T cell signaling upon activation, who could show that cytohesin-1 is an upstream-regulator of IL-2 gene transcription (Grell 2009; Paul 2007).

3.3.3.2 Secin 144 treatment of $CD4^+$ T cells leads to reduced cytokine production

Cytokine production is an important readout for the activation status of T cells. Cytokines determine differentiation of T cell subsets and lead to autocrine activation and clonal expansion of T cells. In order to investigate the role of cytohesin-1 for the activation of human T cells, cytokine production was measured in human $CD4^+$ T cells. Therefore, $CD4^+$ T cells were isolated by magnetic-activated cell sorting and incubated with Secin 16 or Secin 144, respectively, for one hour prior to stimulation. T cells were stimulated by the use of artificial antigen presenting cells (aAPC, a bead that is coated with anti-CD3 and anti-CD28, for full T cell activation) for two days. Supernatants were collected and the cytokines were quantified by a cytometric bead array via flow cytometry.

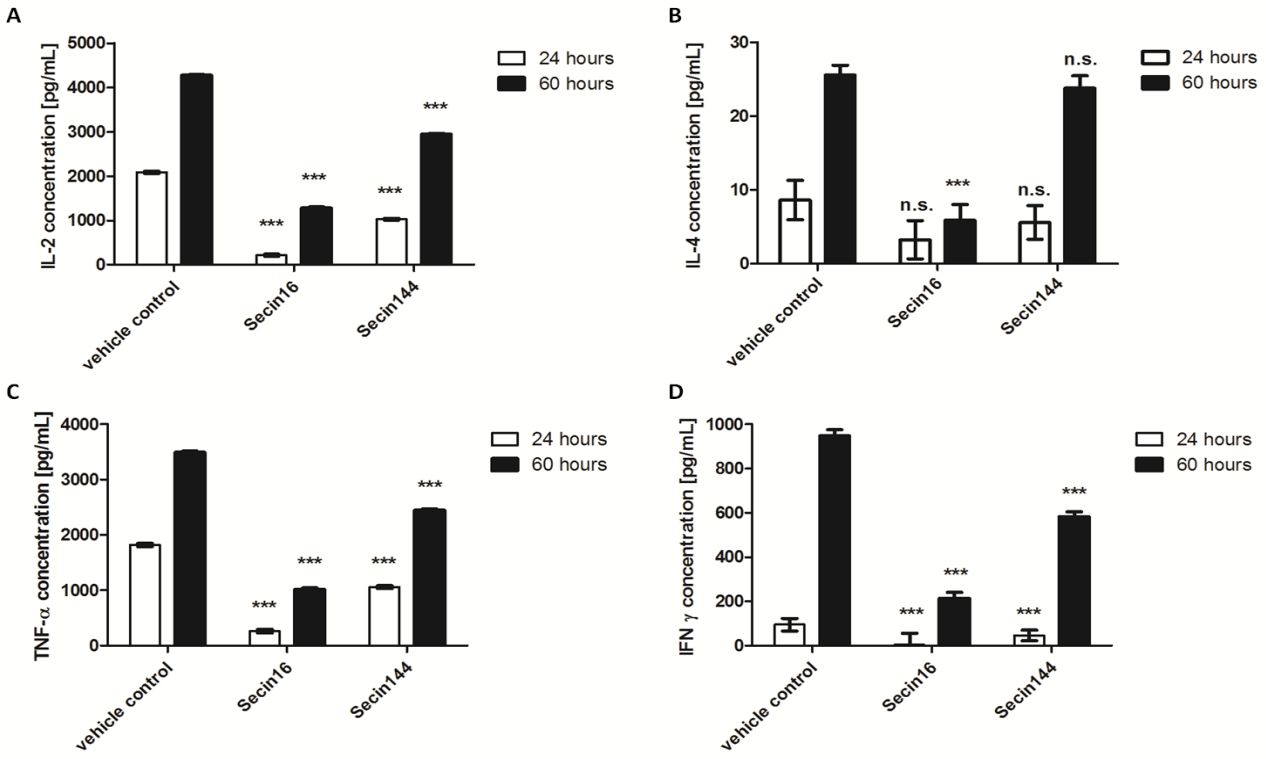

Figure 20: CD4+ T cell cytokine production is inhibited by Secin 16 and Secin 144.
Human CD4+ T cells were isolated by magnetic-activated cellsorting, preincubated with 10 µM Secin 16 or Secin144, respectively, in 0.5% DMSO or DMSO alone (vehicle control) and stimulated with anti-CD3/anti-CD28 beads for 24 and 60 hours. Supernatants were collected at the indicated time points and the cytokine concentration was quantified by CBA (cytometric bead array). Shown is a representative out of three independent experiments.

IL-2 is the most important cytokine for the activation of T cells. After TCR engagement together with its costimulatory factor CD28, IL-2 is produced in large amounts to stimulate proliferation and differentiation of naïve T cells into effector cells. As shown in figure 20A the inhibition of cytohesin-1 by Secin 16 and Secin 144 leads to a strong reduction of IL-2 synthesis. The amount of IL-2 is 3 – 6 times lower than in the stimulated control. Treatment of $CD4^+$ T cells with Secin 144 prior to aAPC-stimulation also reduces IL-2 levels after 24 and 60 hours, however, to a lower extent as compared to Secin 16 treated cells indicating that both, cytohesin GEF-function as well as regulation of LFA-1 activation are important factors in the development of activated T cells.

Interleukin-4 is an important cytokine for the differentiation of naïve T cells into TH2 cells. It is secreted at the site of T cell-APC contact and leads to differentiation and antibody synthesis in B cells (Leavy 2010). Inhibition of cytohesin-1 with Secin 16 leads to a reduction of IL-4 synthesis down to 30% compared to the stimulated vehicle control. Preincubation of $CD4^+$ T cells with Secin144 does not significantly alter the produced amounts of IL-4.

Furthermore, incubation of T cells with Secin 16 leads to a strong reduction of TNF-α production (about 30% of the stimulated vehicle control). TNF-α is a cytokine that is produced as acute phase response of infectious processes and leads to apoptosis of infected cells. Secin 144 treatment also reduces TNF-α production, however to a lower extent than inhibition with Secin 16.

Interferon-γ is an important cytokine for the differentiation of naïve T cells into TH_1 cells, that finally leads to an upregulation of cell based immunity. The interferon-γ level after Secin 16 treatment is decreased to 75% of the level for the stimulated control. In this context, inhibition of cytohesin function by Secin 144 also leads to a decreased level of cytokine production. However, the reduction is milder as compared to Secin 16 treatment and therefore indicates that not only cytohesin-regulated activation of LFA-1 is of importance in T cell activation but also GEF-dependent processes.

These results suggest, that cytohesin-1 is a crucial factor in the activation and differentiation of T cells. Furthermore, since the synthesis of major cytokines is relevant in the differentiation of T cells to various T cell subsets is reduced after the inhibition of cytohesin-function, cytohesin-1 appears to be involved in early signaling events after TCR engagement.

Cytohesin-1 plays an important role in the activation of T cells (Grell 2009; Paul 2007) and the novel cytohesin inhibitors Secin 16 and Secin 144 corroborate these findings by the abrogation of cytokine production of IL-2, IL-4, TNF-α and interferon-γ.

Production of several cytokines is reduced after treatment of $CD4^+$ T cells with Secin 144. This reduction however, is lower than in Secin 16-treated T cells (cf. figure 20) indicating that on one hand LFA-1 activation plays a role in cytokine production and on the other hand it also shows that the GEF activity of cytohesin-1 is the main factor for full T cell activation and therefore cytokine production.

3.3.3.3 Proliferation of $CD4^+$ T cells is impaired after inhibition of cytohesin function

Cytokine production, especially secretion of IL-2, is a hallmark of activated T cells. Furthermore, activated T cells expand clonally and divide. Clonal expansion of T cells is a process that involves cytokine signaling, TCR- and LFA-1-engagement (Varga et al. 2010; Li et al. 2009; Smith-Garvin et al. 2009). Cytohesin proteins are reported to play an important role in signaling events, that are initiated by T cell receptor clustering or LFA-1 activation (Geiger et al. 2000; Kolanus et al. 1996). It is likely, that the signaling events, mediated by cytohesin proteins, are not solely dependent on GEF-activity, but that other functions of the protein are of importance. To further address the

question, if proliferation of T cells is regulated by cytohesin-1 and if this regulation is caused by the GEF-activity of cytohesins, Secin 16 and Secin 144 were used as differential inhibitors of cytohesin function in proliferation assays. $CD4^+$ T cells were stained with CFSE and were preincubated with inhibitor or DMSO as vehicle control for one hour. Subsequently, cells were cultivated in the presence of aAPCs and the compound for several days. Cell proliferation was followed by measuring the average fluorescence of every cell generation by flow cytometry, since the CSFE-dye dilutes with every cell division, leading to populations with distinct lower fluorescence intensity as compared to undivided cells.

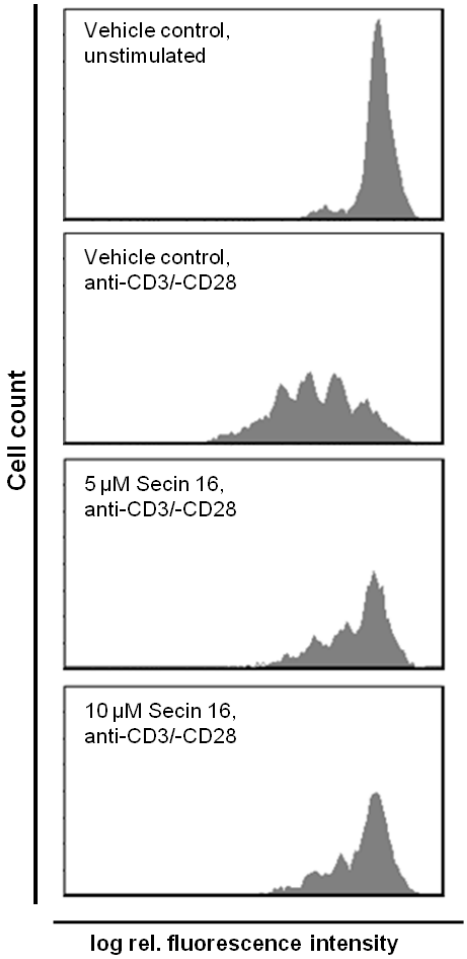

Figure 21: Proliferation of PBL is inhibited by Secin 16.
$CD4^+$ T cells were isolated from buffy coats, stained with CFSE and incubated with 5 µM or 10 µM Secin 16, respectively in 0.5% DMSO or DMSO alone for one hour prior to stimulation with aAPC for 5 days. Cells were analyzed by flow cytometry. The shown histograms are a representative of five independent experiments. Secin 16 strongly inhibits the proliferation of primary T cells.

As shown in figure 21, proliferation of $CD4^+$ T cells is strongly reduced after inhibiting the cytohesin protein with Secin 16. In contrast to the stimulated DMSO control, T cells, preincubated

with Secin 16, show almost no proliferation and the main fraction of the population remains in an undivided state. This result shows that not only the production of cytokines but also the clonal expansion of activated T cells is disturbed after inhibiting cytohesin proteins.

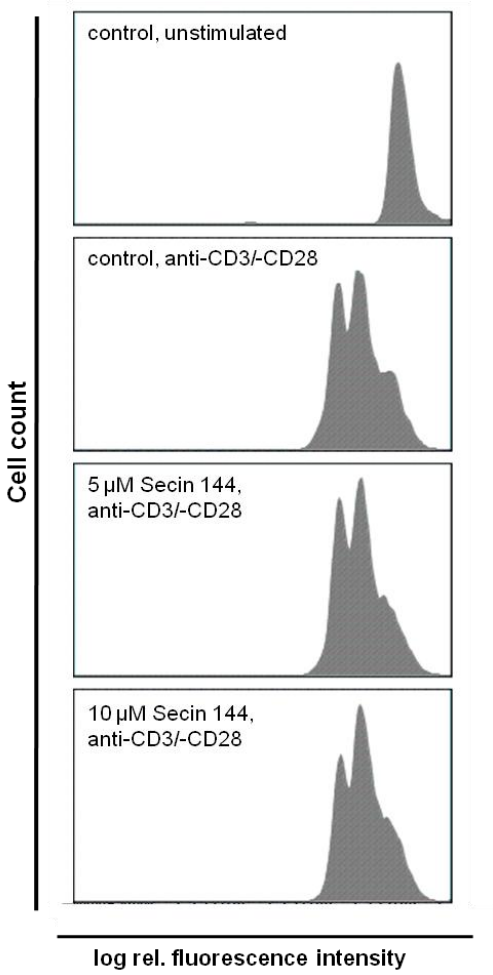

Figure 22: Proliferation is marginally influenced by inhibition of cytohesin-1 with Secin 144.
T cell proliferation is reduced after treatment of $CD4^+$ T cells with Secin 144 in a concentration-dependent manner. Proliferation inhibition is low compared to the effects induced by use of Secin 16. $CD4^+$ T cells were isolated, stained with CFSE and incubated with 5 µM or 10 µM Secin 144, respectively in 0.5% DMSO or DMSO alone for one hour prior to stimulation with aAPC for 3 days. Cells were analyzed by flow cytometry. The shown histograms are a representative of five independent experiments.

As it is depicted in figure 22, inhibition of cytohesin function with Secin 144 leads to a marginal decrease in proliferation of T cells after TCR engagement. These mild effects in T cell proliferation are indicating that mainly the GEF-function of cytohesin is of importance in the clonal expansion of T cells, especially in comparison with Secin 16 treated cells (cf. figure 21). These findings are in good accordance to earlier results in which it was observed, that IL-2 production is reduced in

Secin 144-treated cells, however to a smaller extend than cells in which cytohesin function was inhibited by the use of Secin 16. The reduction of proliferation is likely to be caused by inhibition of LFA-1 activation by Secin 144.

3.3.3.4 Novel inhibitors for cytohesin function do not cause cell death in primary T cells

Secin 16 proved to be a potent inhibitor of T cell proliferation and cytokine production. To ascertain that these effects are not caused by increased cell death due to toxic side effects of the compound, cells were incubated with increasing concentrations of Secin 16 and cell death was quantified by Annexin V and propidium iodide staining.

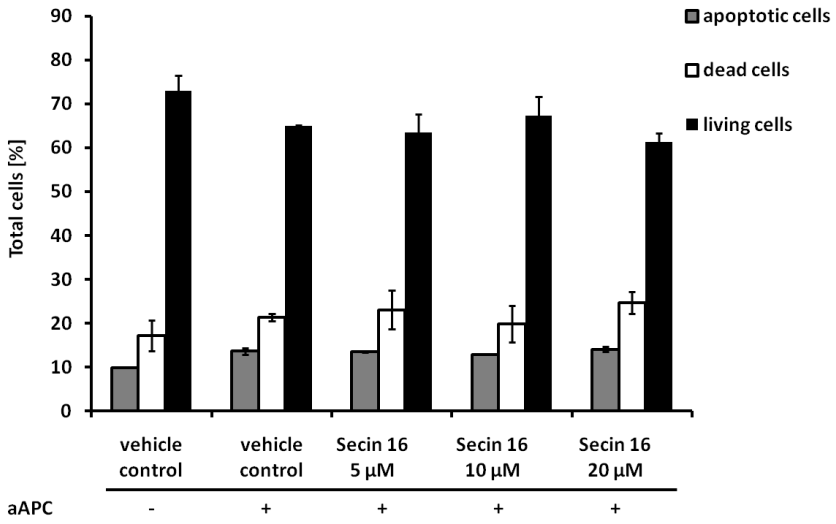

Figure 23: Secin 16 does not alter the viability of PBL.
Cells were incubated with Secin 16 at concentrations indicated and stimulated with aAPC for three days. Cells were subsequently stained with anti-Annexin V-FITC for the detection of apoptosis and propidium iodide for the detection of dead cells. The amount of living, apoptotic and dead cells was quantified by flow cytometry. Shown is the average of two independent experiments. Error bars indicate ±SD.

As depicted in figure 23 Secin 16 has no significant impact on the viability of human PBL. The amount of living cells in the aAPC-stimulated DMSO control is 71 %. Comparable results are derived for the Secin 16-incubated samples. The changes in the number of living, apoptotic and dead cells proved to be not significant and a dose-response dependency cannot be observed. This indicates that the reduction in cytokine production and proliferation after incubating T cells with Secin 16 is not caused by toxicity of the small molecule inhibitor but dependent on the inhibition of cytohesin function.

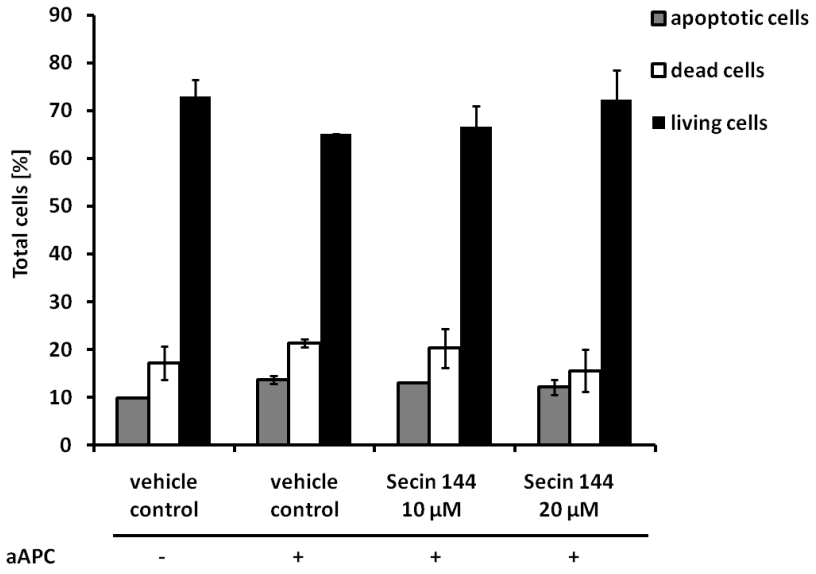

Figure 24: Viability of human PBL is not affected by the use of Secin 144 as inhibitor for cytohesin-function.
Cells were incubated with Secin 144 at concentrations indicated and stimulated with aAPC for three days. Cells were subsequently stained with anti-Annexin V-FITC for the detection of apoptosis and propidium iodide for the detection of dead cells. The amount of living, apoptotic and dead cells was quantified by flow cytometry. Shown is the average of two independent experiments. Error bars indicate ±SD.

In order to show that effects observed in T cell activation, cytokine production and T cell proliferation are not caused by enhanced cell death due to the application of the compound, viability stains were also performed for Secin 144 treated cells. Figure 24 displays the results of the viability stain. Treatment of primary T cells with Secin 144 does not lead to increased apoptosis or necrosis as displayed by unaltered levels of dead and apoptotic cells, respectively compared to vehicle control treated cells.

3.3.3.5 Ca^{2+}-flux in Jurkat T cells is not affected by Secin 16 treatment

T cell signaling consists of a complex network of signaling cascades influencing and signaling back to each other. Since it is reported, that cytohesin plays no role in the mobilization of intracellular calcium (Paul 2007; El Azreq et al. 2010) and to further examine the specificity of the inhibitory compounds for cytohesin-mediated signaling events, Ca^{2+}-flux experiments were performed. To this end, Jurkat T cells were preincubated with Secin 16 for one hour and stained with Fluo-3, a calcium-sensitive dye. Fluorescence of Fluo-3 was quantified by flow cytometry and stimulation of Ca^{2+}-flux was induced by adding 2 µg of anti-CD3 (OKT3) antibody.

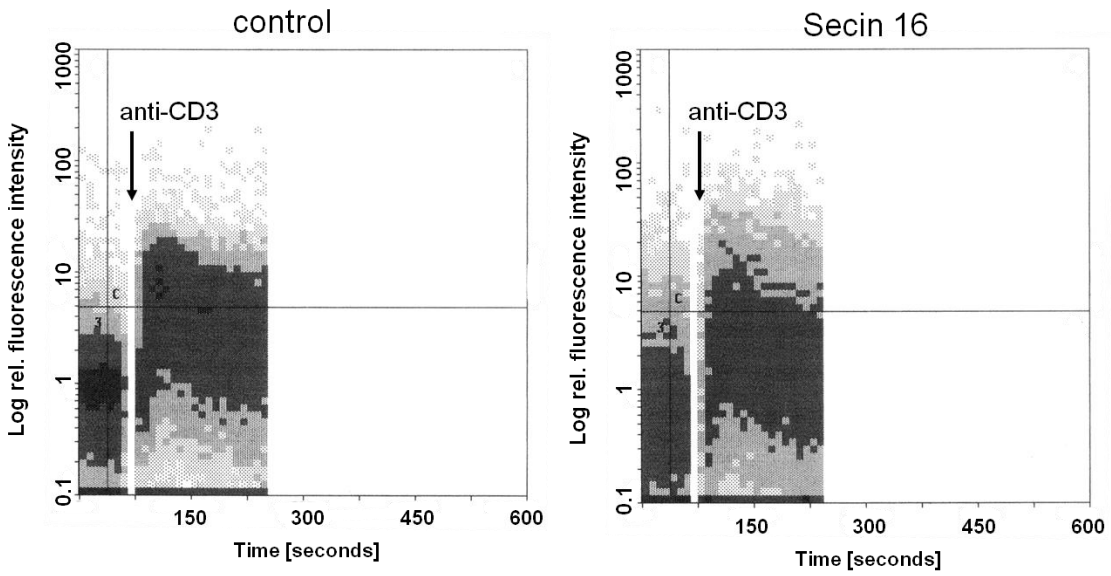

Figure 25: Ca^{2+}-flux is unaltered in Jurkat T cells after inhibition with Secin 16.
Jurkat T cells were incubated with 10 µM Secin 16 in 0.5% DMSO or DMSO alone for one hour prior to experiment and stained with the Ca^{2+}-ion sensitive dye Fluo-3. Ca^{2+}-flux was measured with a flow cytometer. The baseline was recorded and Ca^{2+}-flux was induced by addition of 2 µg anti-CD3 antibody OKT3. The increase of fluorescence indicates an increase in free Ca^{2+}-ions released into the cytosol. Left site depicts Ca^{2+}-flux histogram for the DMSO control, right site shows histogram for 10 µM Secin 16.

As depicted in figure 25, no differences in the calcium-release profile between Secin 16-treated Jurkat T cells and DMSO-treated cells can be observed. The fluorescence increase in DMSO vehicle control and Secin 16 treated Jurkat T cells is about 10-fold higher after TCR-stimulation. Thus, the use of Secin 16 for inhibition of cytohesin has no impact on the signaling cascade responsible for calcium release. This observation is in full agreement with earlier results of Paul, who showed that transfection of Jurkat T cells with siRNA targeting cytohesin-1 did not alter calcium-levels within the cell as compared to control cells (Paul 2007).

3.3.3.6 Secin 144 does not inhibit the phosphorylation of MAPK ERK1/2

MAP kinases play an important role in signal transduction during T cell proliferation, eventually leading to the production of IL-2 (Serfling et al. 1995). The phosphorylation of the MAPK ERK1/2 is reported to be positively regulated by cytohesin function in T cells after LFA-1 engagement (Perez et al. 2003). To investigate, how cytohesin-1 mediates the activation and phosphorylation of ERK1/2 and, if LFA-1 engagement is a prerequisite for this phosphorylation, $CD4^+$ T cells were stimulated with aAPCs after preincubation with Secin 16 or Secin 144, respectively. The phosphorylation of ERK1/2 was investigated by the use of western blot analysis.

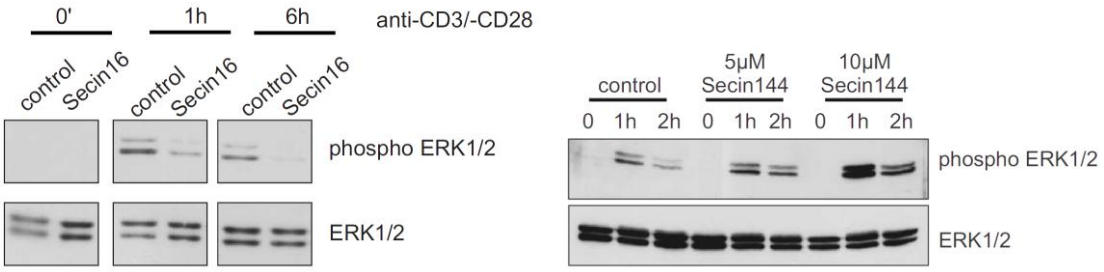

Figure 26: ERK1/2 phosphorylation is diminished by Secin 16 and enhanced by Secin 144
$CD4^+$ T cells were incubated with 10 µM Secin 16 or Secin 144 in 0.5% DMSO, respectively or 0.5% DMSO alone (control) for one hour prior to stimulation. Cells were stimulated by aAPC for the indicated times. After stimulation cells were lysed and 10 µg of total protein were separated by SDS-PAGE and analyzed for phospho-ERK1/2 content via western blot. (unpublished data from Dr. Jessica Grell)

As depicted in figure 26, phosphorylation of ERK1/2 is abrogated in Secin 16 pretreated $CD4^+$ T cells. Total ERK1/2 expression is unaltered. This underlines the importance of cytohesin-1 in the activation of T cells. Cytohesin-1 overexpression leads to a strong upregulation of AP-1 expression in AP-1 promoter experiments (Paul 2007). The result indicates that this activation is mediated by the MAP kinase ERK1/2 underlining the importance of cytohesin-1 mediated signal transduction in T cells.

Secin 144 inhibits the cytohesin-regulated activation of LFA-1 in adhesion however, GTP-exchange activity of cytohesin-1 is not inhibited. Figure 26 shows that the phosphorylation of ERK1/2 is not inhibited in $CD4^+$ T cells after inhibition of LFA-1 activation via cytohesin-1 with Secin 144. In contrast, Secin 144 treatment leads to an increased level of ERK1/2 phosphorylation. This activation occurs only after stimulation with aAPCs and not in unstimulated T cells. This indicates that not only the catalytic GEF-activity of cytohesin proteins, but also that signaling after TCR engagement is necessary for proper signal propagation during T cell activation.

3.3.4 Influence of cytohesin-1 on the chemotaxis of immune cells

Cytohesin-1 is reported to be an important regulator of dendritic cell migration (Quast et al. 2009). Secin H3 failed to inhibit these complex processes at reasonable concentrations. Due to the fact that Secin 16 and Secin 144 were able to inhibit cytohesin-1 dependent adhesion more efficiently as compared to Secin H3, it was tested, whether these compounds are also able to inhibit cytohesin-dependent functions in immune cell migration.

Furthermore, Secin 16 and Secin 144 show a differential inhibitory profile dissecting GEF-dependent and -independent cytohesin functions. It was therefore interesting to investigate into the influence of these cytohesin functions in immune cell chemotaxis.

3.3.4.1 Secin 16 but not Secin 144 inhibits migration of Jurkat T cells and mature BmDCs

Cytohesin-1 is reported to be important for the migration of mature dendritic cells (Quast et al. 2009). To further characterize Secin 16 and Secin 144, the inhibitors were utilized to inhibit cytohesin-1 function in transwell migration assays. Therefore, mature BmDCs were preincubated with Secin 16 or Secin 144, respectively at a concentration of 10 µM for one hour and placed on top of a modified Boyden chamber. Cells were stimulated with 200 ng/mL murine CCL19 or human CXCL12, respectively for 3 hours. Migrated cells were quantified by counting the cells in the lower compartment of the chamber and displayed as percent of migrated cells compared to the vehicle control (DMSO).

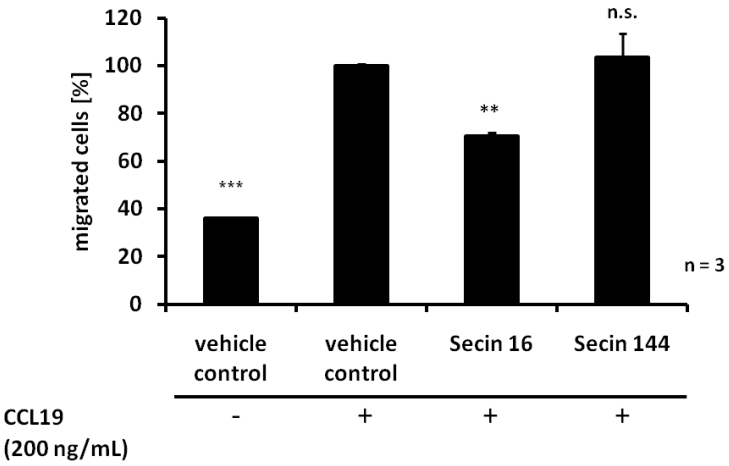

Figure 27: Transwell migration of mature BmDCs is inhibited by Secin 16 but not by Secin 144.
Mature BmDCs were preincubated with 0.5% DMSO (vehicle control), 10 µM Secin 16 or 10 µM Secin144 in 0.5% DMSO, respectively, for one hour and seeded in a modified Boyden chamber. Cells were stimulated with 200 ng/mL murine CCL19. Migrated cells were quantified after three hours of incubation at 37°C by counting the cells in the lower compartment of the chamber. The average of three independent experiments is shown normalized to the migration rate of the stimulated vehicle control. Asterisks indicate significant deviation from the stimulated control as determined by Student's t-test (**= P < 0.01; *** = P < 0.001)

It is depicted in figure 27 that migration of LPS-stimulated mature BmDCs after the inhibition of cytohesin function with the inhibitor Secin 16 is reduced by more than 25% as compared to the CCL19-stimulated vehicle control. This inhibition is in concordance with the findings, that RNAi of cytohesin-1 strongly reduced CCL19-induced chemotaxis of LPS-stimulated mature DCs (Quast et al. 2009).

Interestingly, transwell migration of mature BmDCs is unaffected by Secin 144 employment. Secin 144 does not abrogate chemokine-induced migration as compared to cells that are treated with vehicle control.

To ascertain if the involvement of cytohesin-1 in migration is also important for T cells, transwell migration assays were employed and compound-treated Jurkat T cells were examined.

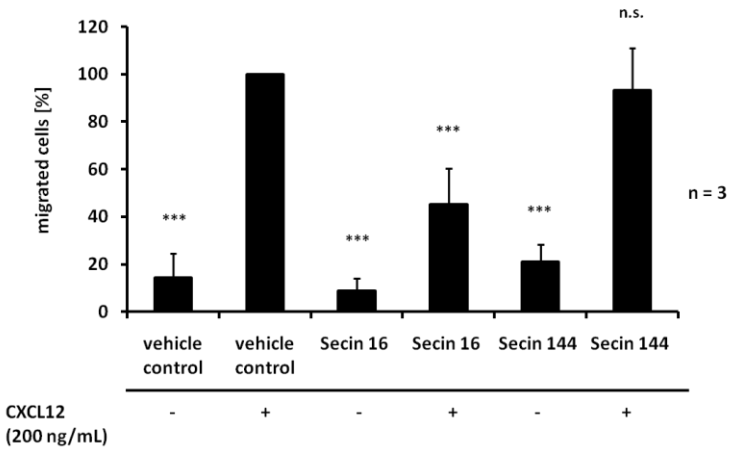

Figure 28: Jurkat T cells migration is abrogated by inhibition of cytohesin-1 function with Secin16, but not with Secin 144.

Jurkat T cells were preincubated with 0.5% DMSO (vehicle control), 10 µM Secin 16 or Secin 144 in 0.5% DMSO, respectively, for one hour and seeded in a modified Boyden chamber. Cells were stimulated with 200 ng/mL human CXCL12 for three hours. Migrated cells were quantified after three hours of incubation at 37°C by counting the cells in the lower compartment of the chamber. The average of three independent experiments is shown normalized to the migration rate of the stimulated DMSO control. Asterisks indicate significant deviation from the stimulated control as determined by Student's t-test (***=P< 0.001, n.s. = not significant)

In human T cells, Secin 16 inhibits chemokine-induced transwell migration even more efficiently than compared to murine, mature BmDCs. As depicted in figure 28, transwell migration ability of Jurkat T cells is reduced about 50% in the presence of Secin 16. Thus, Secin 16 is also a potent inhibitor of human immune cell migration mediated by cytohesin-1.

However, transwell migration of mature BmDCs and Jurkat T cells is unaffected by Secin 144 employment (cf. figure 27 and figure 28). The compound does not alter chemokine-induced migration as compared to cells that are incubated with DMSO as control. Since Secin 144 mainly inhibits the cytohesin-mediated activation of LFA-1 this result is explainable, because in this experimental setup no specific ligand for LFA-1 was present.

Transwell migration seems to be mainly influenced by GEF-activity of cytohesin proteins as it can be concluded from the results comparing effects of Secin 16 and Secin 144 if no specific ligand is present.

3.3.4.2 The role of cytohesin-1 in immune cell migration on two-dimensional substrates

On two-dimensional surfaces dendritic cells use integrins as a force generator for forward locomotion (Johnson et al. 2006; Alon and Dustin 2007). It is known, that cytohesin interacts specifically with LFA-1 and therefore leads to an activation of this integrin (Geiger et al. 2000; Kolanus et al. 1996). Furthermore, cytohesin-2 and cytohesin-3 are reported to act in a complementary fashion in the recycling of β_1-integrins and, as a consequence, influence their activity as well (Oh and Santy 2010). To further characterize Secin 16 and Secin 144, respectively, and therefore the role of cytohesin proteins in immune cell migration, the chemotaxis of immune cells on two-dimensional substrates was investigated.

Cell migration on two-dimensional surfaces is strongly integrin-dependent (Alon and Dustin 2007; Johnson et al. 2006) and inhibition of the cytohesin-1-mediated activation of β_2-integrins should lead to an alteration of chemotaxis in immune cells. Since Secin 144 does not have an influence on the GEF-function of cytohesin-1 it is interesting to investigate, which impact the sole inhibition of cytohesin-regulated LFA-1 activation has on the chemotaxis of immune cells.
To this end, two-dimensional migration assays were performed. Cells were seeded on fibronectin, fibrinogen or ICAM-1 coated surfaces, respectively, and stimulated with 600 ng/mL chemokine (for immature BmDCs CCL3, for PBL CXCL12, respectively). Motility of the cells was monitored by live cell imaging.

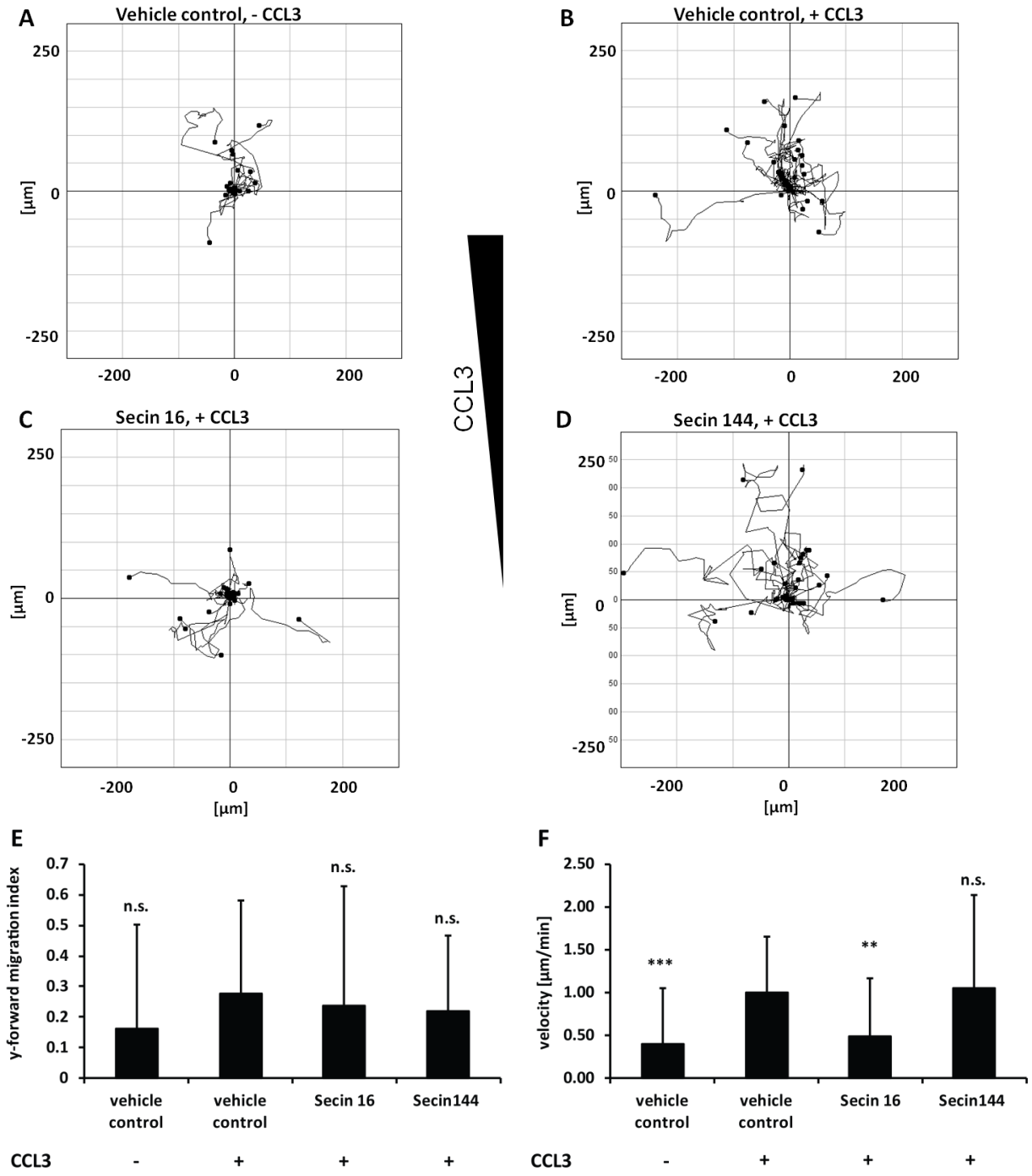

Figure 29: Inhibition of cytohesin leads to diminished migration of immature BmDCs on fibronectin mediated by cytohesin-dependent GEF-activity.

Cells were preincubated with 10 µM Secin16 or Secin 144 in 0.5% DMSO or DMSO alone as control, respectively, in minimal medium and seeded on ibidi slides coated with 50 µg/cm^2 fibronectin for one hour. A chemokine gradient was applied from the upper side of the slide (CCL3 200 ng/mL as indicated by the arrow) and cells were followed via live cell imaging. For each sample 30 cells were tracked using ImageJ manual tracking tool and chemotaxis plots, y-forward migration and velocity were determined using ibidi's chemotaxis plugin tool in Image J. Error bars depict ± SD, asterisks indicate significant deviation compared to the stimulated DMSO control (n.s. = not significant; ** = P < 0.01; *** = P< 0.001 determined by student's t-test)

Immature BmDCs show an impairment in chemotaxis, when they migrate towards a CCL3 gradient on the extracellular matrix protein fibronectin (cf. figure 29) only after Secin 16 treatment. The velocity is strongly reduced when cytohesin-1 is inhibited by Secin 16. Furthermore, the distance covered by immature BmDCs, when migrating towards CCL3 is reduced after Secin 16 treatment. This supports findings of Quast et al. who could show that chemotaxis is impaired when mature BmDCs migrate through fibronectin coated transwell filters after RNAi of cytohesin-1. Treatment of immature BmDCs with Secin 144 does not lead to alterations in chemokine-induced migration on fibronectin.

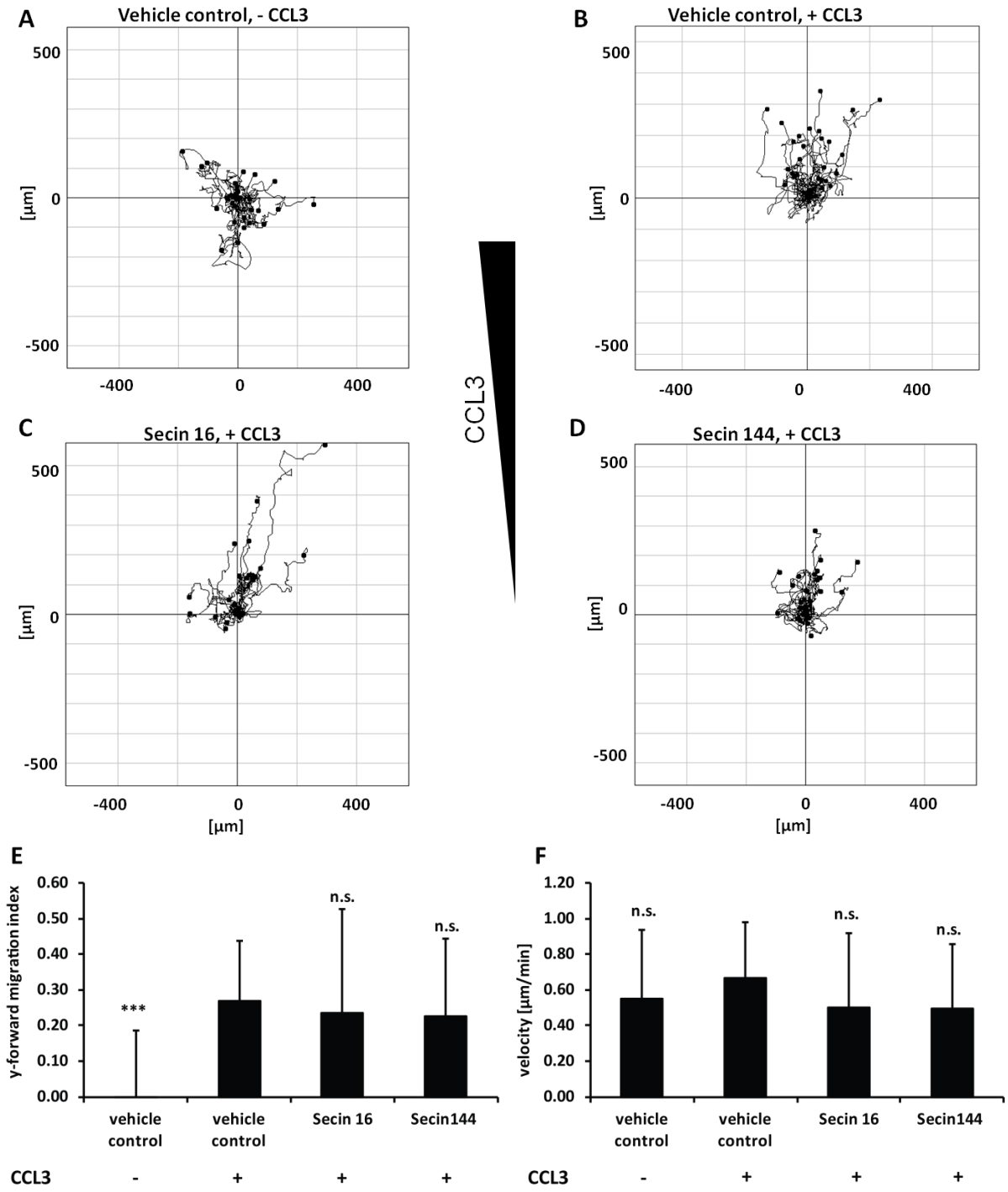

Figure 30: Immature BmDCs show no significant reduction in chemotaxis on fibrinogen after inhibition of cytohesin functions.

Cells were preincubated with 10 µM Secin16 or Secin 144 in 0.5% DMSO or DMSO alone as vehicle control, respectively in minimal medium and seeded on ibidi slides coated with 50 µg/mL fibrinogen for one hour. A chemokine gradient was applied from the upper side of the slide (CCL3 200 ng/mL as indicated by the arrow) and cells were followed via live cell imaging. For each sample 30 cells were tracked using ImageJ manual tracking tool and chemotaxis plots (A-D), y-forward migration (E) and velocity (F) were determined using ibidi's chemotaxis plugin tool in Image J. Error bars depict ± SD, asterisks indicate significant deviation compared to the stimulated DMSO control (n.s. = not significant; *** = P< 0.001 determined by student's t-test)

In immature, murine BmDCs, two-dimensional migration on fibrinogen is hardly affected, when cytohesin-1 function is inhibited by Secin 16 or Secin 144, respectively. Neither the track lengths nor y-forward migration index or velocity-values are significantly changed by treatment of the cells with the small molecule inhibitors Secin 16 and Secin 144. Interestingly, unstimulated cells also show a high motility as depicted by the velocity of the cells (cf. figure 30F). Stimulation in this context leads to directing the cells towards the chemokine gradient as displayed by the y-forward migration index. This index is calculated as the ratio of movement into chemokine-direction (y-movement) and the length that is covered, and gives information about the degree of directionality of the forward locomotion.

Figure 31 displays the two-dimensional migration of immature BmDCs when they migrate towards CCL3 on ICAM-1. CCL3-induced chemotaxis of immature BmDCs on ICAM-1 is abrogated, if cells are preincubated with Secin 16 as compared to the CCL3-stimulated vehicle control. The velocity of Secin 16 treated cells is strongly reduced, but also the distances that are covered by these cells are much lower than in the stimulated control. In contrast, Secin 144 treatment only slightly alters path lengths and velocities of immature BmDCs when they migrate towards CCL3 on ICAM-1.

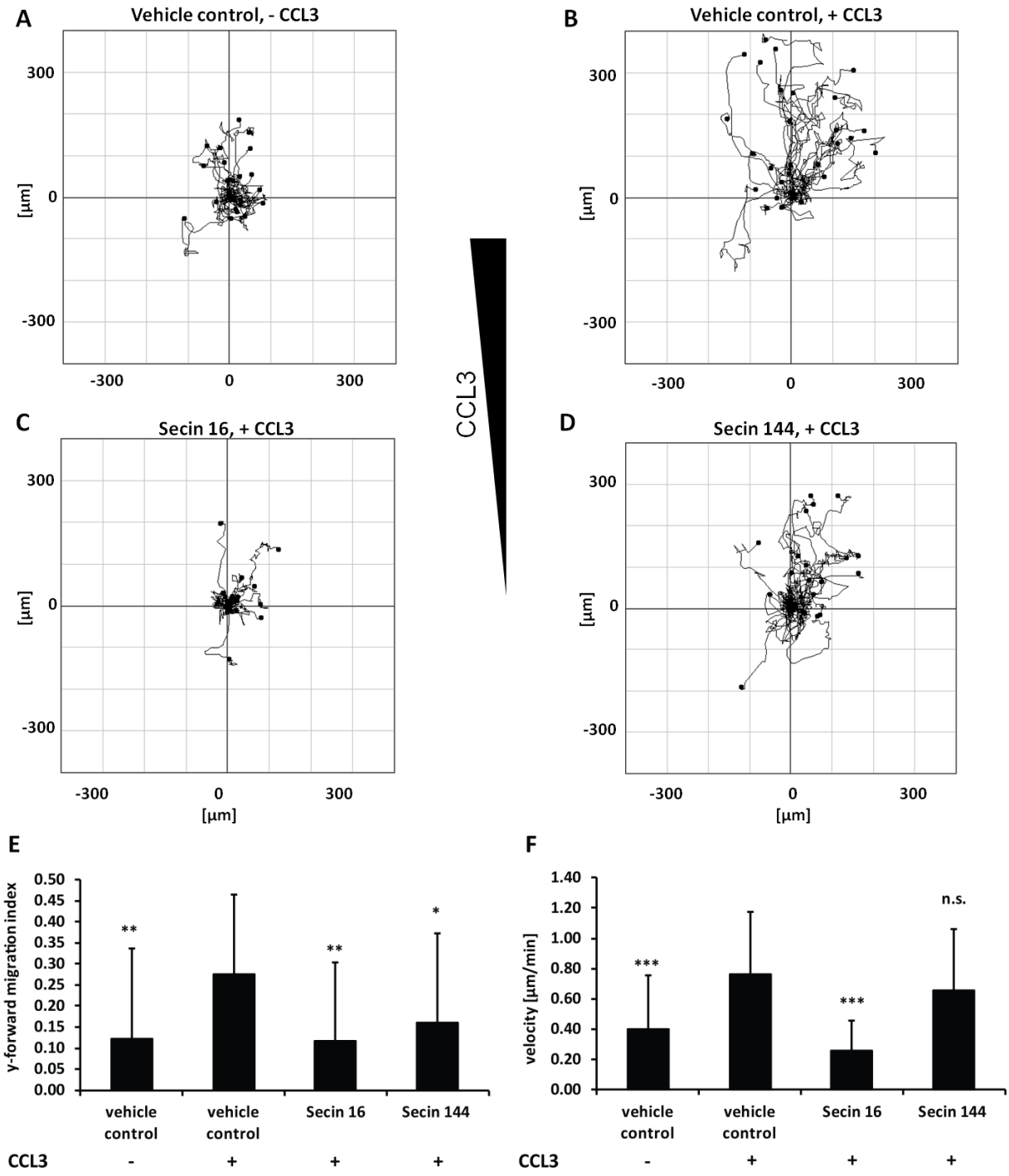

Figure 31: Migration on a two-dimensional surface of immature BmDCs on ICAM-1 is abrogated if cytohesin is inhibited by Secin 16 treatment, and to lower extend by Secin 144.

Immature BmDCs were preincubated with 10 µM Secin16 or Secin 144 in 0.5% DMSO or DMSO alone as vehicle control, respectively, in minimal medium and seeded on ibidi slides coated with ICAM-1-Fc supernatants for one hour. CCL3 (200 ng/mL) was applied to one side of the slide for the induction of a chemokine gradient (as indicated by the arrow) and cells were followed via live cell imaging. For each sample, 30 cells were tracked using ImageJ manual tracking tool and chemotaxis plots (A-D), y-forward migration (E) and velocity (F) were determined using ibidi's chemotaxis plugin tool in Image J. Error bars depict ± SD, asterisks indicate significant deviation compared to the stimulated DMSO control (n.s. = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ determined by student's t-test)

Taken together, these findings indicate that if a specific ligand for LFA-1 is present, e.g. fibronectin or ICAM-1, respectively, inhibition of cytohesin-1 and therefore inhibition of LFA-1 activation leads to a reduction in chemokine-induced migration of murine immature dendritic cells. This reduction however seems to be mainly dependent on the guanine nucleotide exchange function of cytohesin-1, since inhibition with Secin 144, which is shown to only influence the regulation of LFA-1 activation, only effects chemotaxis to a lower extend.

This shows on one hand the importance of cytohesin-1 in the migration of immune cells on the other hand, it also indicates that not only integrin-dependent processes are responsible for the forward locomotion of immune cells in a chemokine gradient, but also intracellular processes mediated and regulated by cytohesin-1 seem to be of great importance, which are controlled and supported by the GEF-activity of this protein.

It was furthermore interesting to investigate the role of cytohesin proteins in the two-dimensional migration of T cells. To this end, PBL were preincubated with Secin 16 in 0.5% DMSO or DMSO alone as control and seeded on fibrinogen and ICAM-1 coated surfaces.

The influence of cytohesin-1 on the migration of PBL on a two-dimensional fibrinogen substrate is displayed in figure 32. Chemokine-induced migration is strongly impaired after application of Secin 16 to PBL. Lymphocytes migrate slower and forward locomotion is abrogated. Secin 144 does not significantly alter CXCL12-induced chemotaxis of PBL

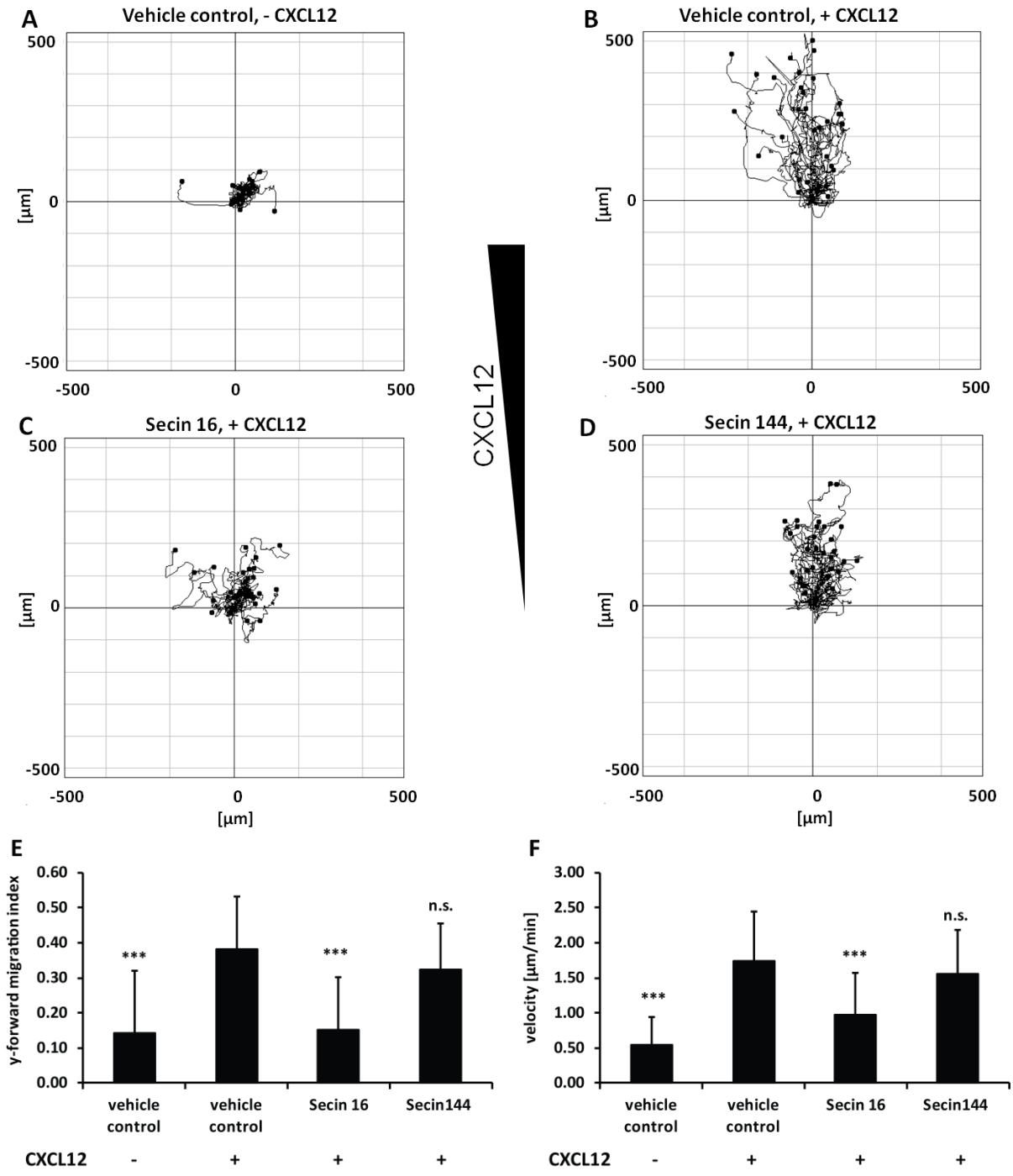

Figure 32: Chemotaxis of PBL on two-dimensional fibrinogen is abrogated after inhibiting cytohesin-1 functions with Secin 16, but not by Secin 144 treatment.

Cells were preincubated with 10 µM Secin16 or Secin 144 in 0.5% DMSO or DMSO alone as control, respectively, in minimal medium and seeded on ibidi slides coated with 50 µg/mL fibrinogen for one hour. A chemokine gradient (arrow) was applied from the upper side of the slide (CXCL12 200 ng/mL) and chemotaxis was followed via live cell imaging at 37°C. For each sample 30 cells were tracked using ImageJ manual tracking tool and chemotaxis plots. Error bars depict ± SD, asterisks indicate significant deviation compared to the stimulated DMSO control (n.s. = not significant; *** = P< 0.001 determined by student's t-test)

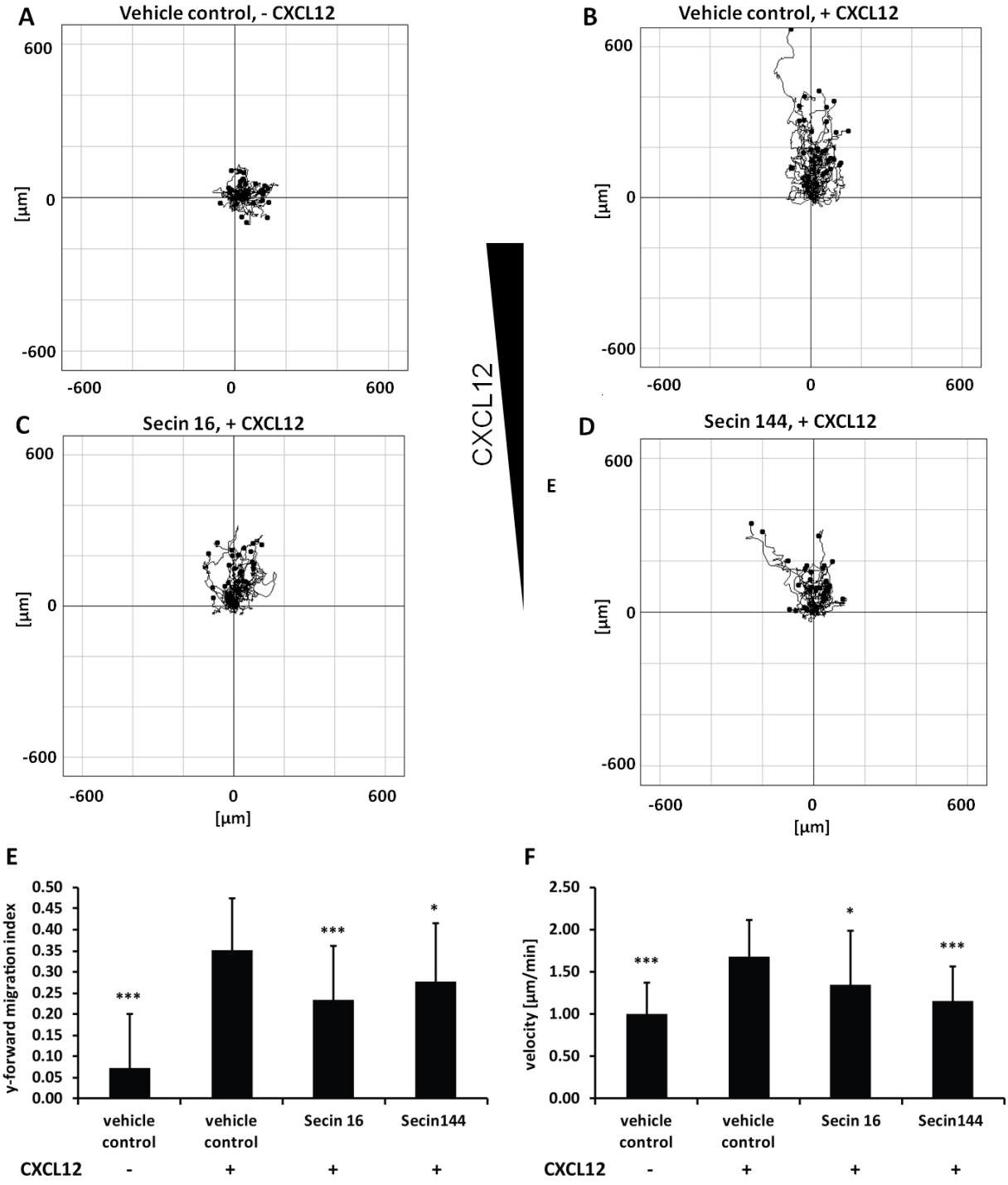

Figure 33: Chemotaxis is reduced after inhibition of cytohesin function in PBL migrating on two-dimensional ICAM-1.

Cells were preincubated with 10 µM Secin 16 or Secin 144 in 0.5% DMSO or DMSO alone as vehicle control, respectively in minimal medium and seeded on ibidi slides coated with ICAM-1-Fc for one hour. A chemokine gradient was applied from the upper side of the slide (CXCL12 200 ng/mL) and cells were monitored via live cell imaging. For each sample 30 cells were tracked using ImageJ manual tracking tool and chemotaxis plots (A-C), y-forward migration (D) and velocity (E) were determined using ibidi's chemotaxis plugin tool in Image J. Error bars depict ± SD, asterisks indicate significant deviation compared

to the stimulated DMSO control (n.s. = not significant; ** = P < 0.01; *** = P< 0.001 determined by student's t-test)

Two-dimensional migration of PBL is also significantly reduced on ICAM-1 coated surfaces after inhibition of cytohesin function by the use of Secin 16. Additionally to decreased track lengths also the forward migration ability and velocity are significantly reduced as delineated in figure 33. Interestingly, in this setup also the inhibition of cytohesin-1 function by Secin 144, that is only limited to regulation of LFA-1 activation, leads to a decrease in chemotaxis of PBL.

3.3.4.3 Immune cell chemotaxis in three-dimensional environment is dependent on cytohesin-GEF activity

Since immune cells mainly migrate in a three-dimensional tissue environment under natural conditions, transwell migration and 2D migration are not completely representative for the *in vivo* situation. Taking that into account, 3D migration experiments were performed in which cells migrate through a three-dimensional collagen gel matrix *in vitro*. Mature BmDCs were mixed with collagen and the small molecule inhibitors Secin 16 and Secin 144, respectively, and incubated for one hour at 37°C prior to stimulation. 600 ng/mL murine CCL19 were then applied on top of the collagen to create a chemokine gradient allowing cells to migrate directionally. The cells were observed via live cell microscopy in a climate chamber at 37°C and tracks were monitored by live cell imaging over a period of four hours at a frame rate of 2 minutes/frame.

As it can be seen in figure 34, Secin 16 strongly inhibits the chemotactic migration of murine mBmDCs in a 3D environment as compared to the DMSO-treated vehicle control. Not only the distance that is covered by the migrating cells is strongly reduced compared to the control but also the directionality as depicted by the y-forward migration index and the velocity are significantly reduced by at least 50% of the vehicle control. In contrast, treatment of cells with Secin 144 does not significantly alter chemotactic migration of mature BmDCs in collagen matrices.

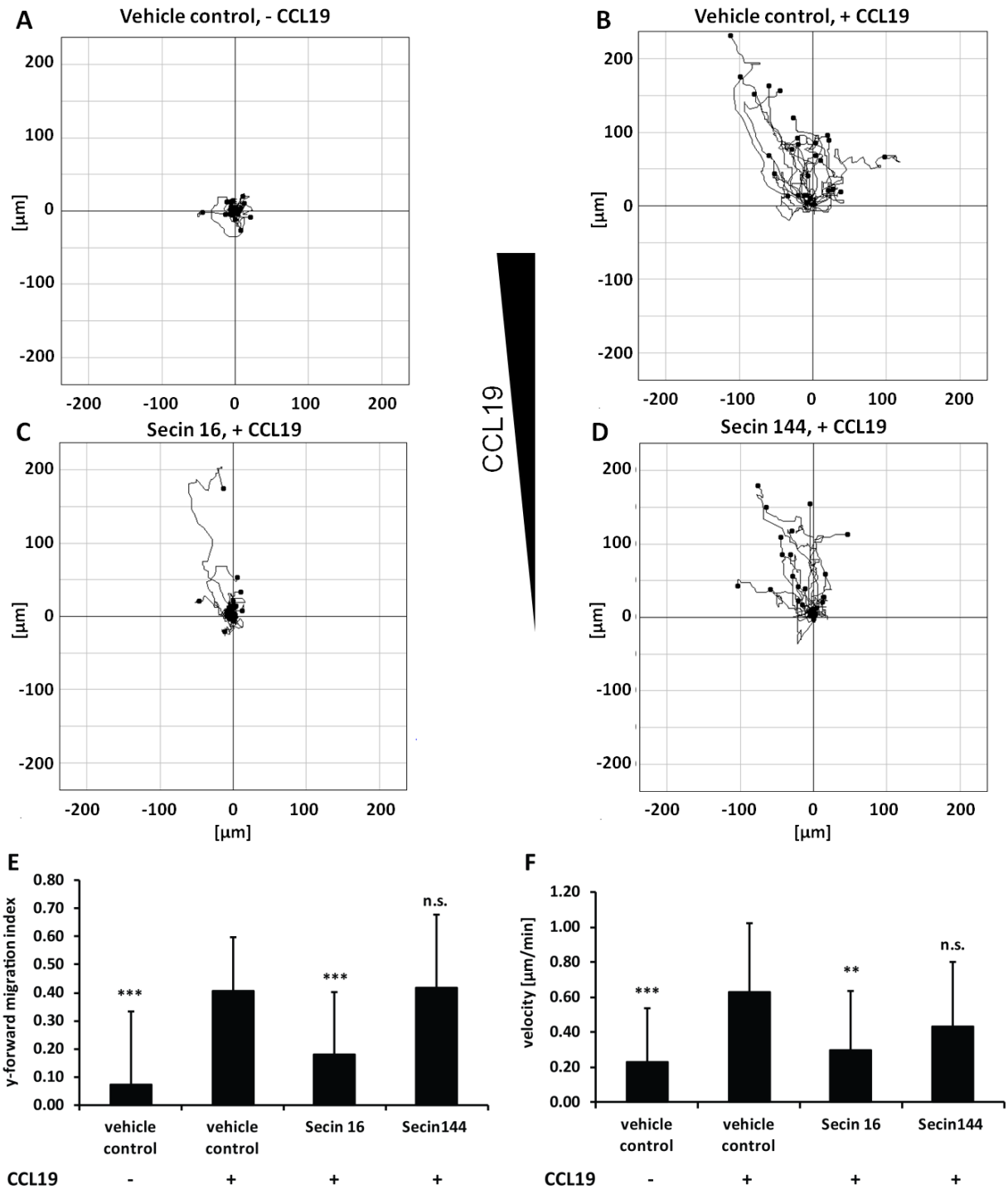

Figure 34: Migration of mBmDCs in three-dimensional collagen matrices is reduced after Secin 16 treatment but unaffected by inhibition of cytohesin function with Secin144.

Mature BmDCs were incubated with 10 µM Secin 16 or Secin 144 in 0.5% DMSO or DMSO alone (vehicle control), respectively for one hour prior to stimulation. 600 ng/mL murine CCL 19 was applied on top of the collagen to create a chemokine gradient. Results of the quantification of live cell imaging are depicted. For each experiment 30 cells tracks were followed over a period of 4 hours in which every two minutes a picture was taken. Acquired data were analyzed with chemotaxis and migration plug-in tools (ibidi) in Image J and chemotaxis plots, y-forward migration index and velocity were calculated and shown as diagrams. Error bars indicate ± SD, asterisks indicate significant deviation from the stimulated control as determined

in Student's t-test (n.s. = not significant; **=P< 0.01; ***=P< 0.001). The data are representative for five independent experiments.

In another experimental setup chemotaxis of PBL in collagen matrices was investigated after inhibition of cytohesin-function with the cytohesin-inhibitors Secin 16 and Secin 144. PBL were preincubated with 10 µM of the respective small molecule inhibitor in 0.5% DMSO or DMSO alone as vehicle control, respectively and mixed with collagen. After one hour 600 ng/mL CXCL12 were applied on top of the collagen to evoke a chemokine gradient and cell movement was monitored by live cell microscopy in a climate chamber at 37°C.

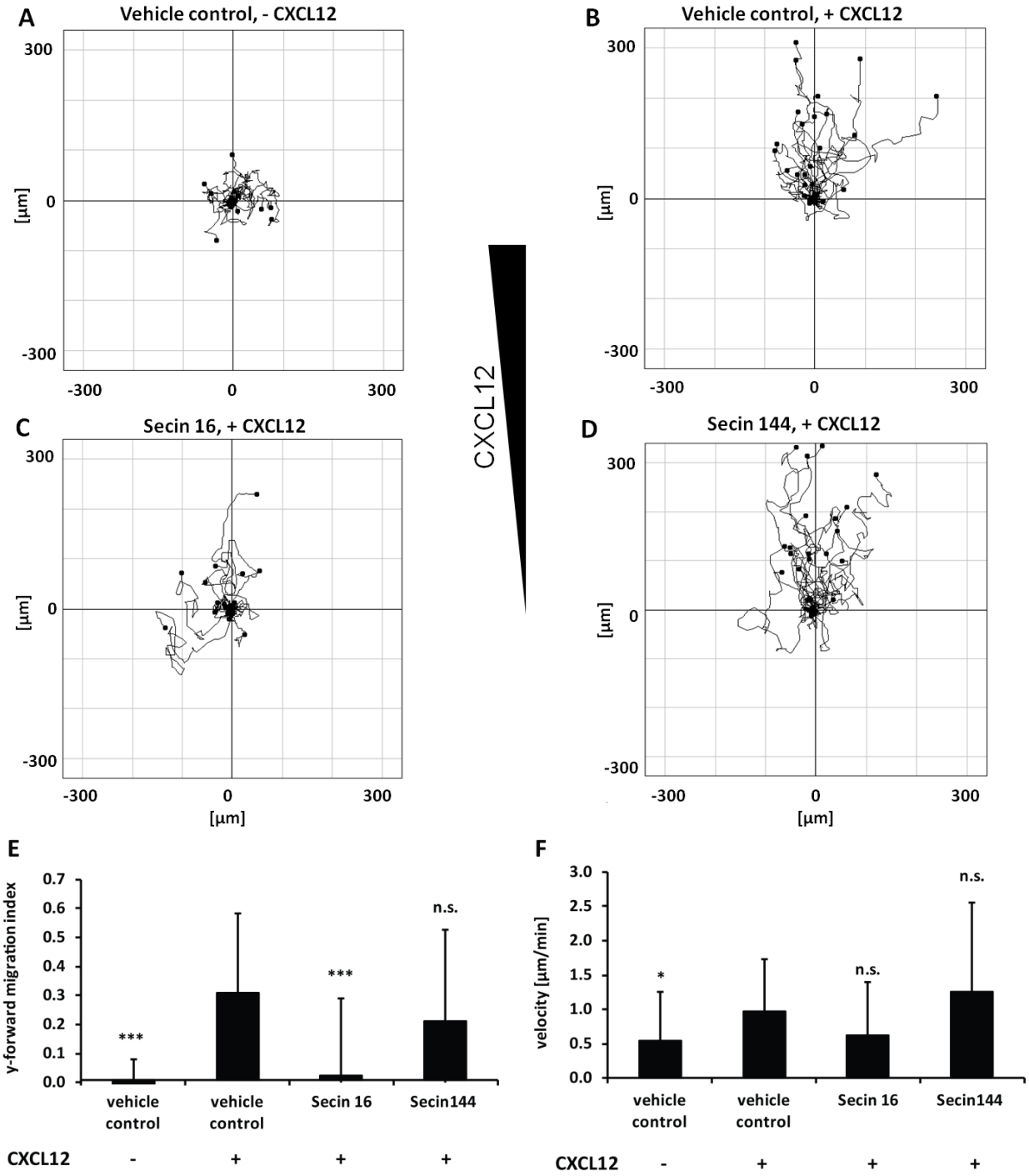

Figure 35: PBL chemotaxis in three-dimensional collagen matrices is only reduced after inhibiting cytohesin-function with Secin 16 but not with Secin 144.

PBL were incubated with 10 µM Secin 16 or Secin 144 in 0.5% DMSO or DMSO alone (vehicle control), respectively for one hour prior to stimulation. 600 ng/mL human CXCL12 was applied on top of the collagen to create a chemokine gradient. Results of the quantification of live cell imaging are depicted. For each experiment 30 cells tracks were followed over a period of 4 hours in which every two minutes a picture was taken. Acquired data were analyzed with chemotaxis and migration plug-in tools (ibidi) in Image J and chemotaxis, y-forward migration index and velocity were calculated and shown as diagrams. Error bars indicate ± SD, asterisks indicate significant deviation from the stimulated control as determined in Student's t-test (n.s. = not significant; *=P< 0.05; ***=P< 0.001). The data are a representative for five independent experiments.

As displayed in figure 35, the migratory capability of PBL is strongly reduced after inhibiting cytohesin-1 function with Secin 16. The overall migration is lower and also directionality as represented by the y-forward migration index is abrogated. The velocity is reduced, however to a lower extent than compared to velocity reduction in mature BmDCs, which might be due to a higher motility of PBL. Thus, even in unstimulated samples the chemotaxis and velocity are higher than in mBmDCs. Secin 144 treatment however, does not reduce chemotactic movement of PBL in a three-dimensional collagen gel. All measured parameters e.g. track length, y-forward migration and velocity do not significantly alter as compared to the CXCL12-stimulated vehicle control.

Taken together, these findings confirm, that in three-dimensional chemotaxis, cytohesin-1 function is an important factor for the migration of immune cells, e.g. mature BmDCs and PBL. Furthermore, for this migration mode, the functions of cytohesin-1 that are related to guanine nucleotide exchange are indispensable, whereas cytohesin-1 mediated activation of LFA-1 is redundant. These findings are augmented by reports that dendritic cells move integrin-independently in three-dimensional surroundings (Lämmermann et al. 2008; Quast et al. 2009) and further imply that integrins are also dispensable for locomotion of T cells in a three-dimensional substrate.

3.3.4.4 Chemokine-induced ex vivo *migration of Langerhans Cells is dependent on the GEF-function of cytohesin-1*

The inhibition of cytohesin-1 with Secin 16 proved to be a useful tool to investigate the function of cytohesin-1 in migration. Furthermore it was shown before that Secin 144 is a partial inhibitor of cytohesin-mediated functions that are involved in integrin activation (see chapter 3.2). Neither in mature BmDCs nor in T cells, transwell migration and chemotaxis in three-dimensional surroundings were altered after inhibition of cytohesin functions by employment of Secin 144. However these experiments are limited in mimicking tissue environment and, since no specific ligand for LFA-1 was present in the experimental setup, an influence on immune cell migration could not be observed. To investigate further impacts of cytohesin-1 on migration in a three-dimensional situation, and the role of cytohesin-mediated activation of LFA-1 function, *ex vivo* migration experiments, in which the chemotaxis of Langerhans cells migrating from ear tissue towards a chemokine, were performed.

Mouse ears of WT C57BL/6 mice were divided in dorsal and ventral halves and floated on medium, containing 10 µM Secin 16 or 10 µM Secin 144 in 0.5% DMSO or DMSO alone as vehicle

control, respectively. After one hour of incubation 100 ng/mL CCL19 was added to the medium in presence of vehicle and compounds. The ears were cultivated for three days and emigrated Langerhans cells (LC) were subsequently quantified.

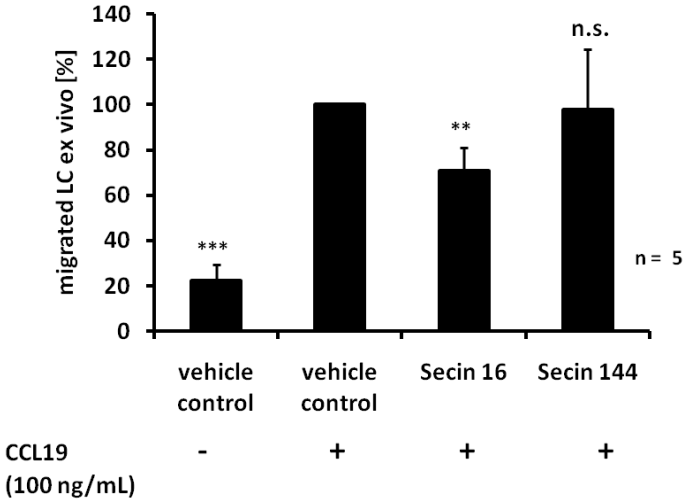

Figure 36: Migration of Langerhans cells out of ear tissue is not disturbed by the application of Secin 144 as inhibitor for cytohesin function however, Secin 16 treatment reduces emigrated cell numbers.

Mouse ears were separated into dorsal and ventral halves and were laid with the dermal site down onto growth medium containing 0.5% DMSO (vehicle control) or 10 µM Secin 16 or Secin 144 in 0.5% DMSO, respectively. After one hour 100 ng/mL murine CCL19 was added as stimulation where indicated. Ears were cultivated for three days and migrated LCs were quantified. Shown is the average migration rate determined from five independent experiments. Asterisks indicate significance as determined via Student's t-test (n.s. = not significant; ** = P< 0.01; *** = P<0.001).

Cytohesin-1 seems to be important for the migration of Langerhans cells. Inhibition of cytohesin-1 functions with Secin 16 leads to a reduction of over 30% in the number of cells that migrate out of the ear in direction towards the chemokine as compared to the stimulated vehicle control. This indicates that also in more complex tissue surroundings cytohesin-1 is needed for proper migration of immune cells towards a chemokine.

However, the results depicted in figure 36 also show clearly that the partial inhibition of cytohesin function that is related to regulation of integrin activation does not cause a decrease in the migration-ability of Langerhans cells towards a chemokine. Thus, chemotaxis in tissue environment does not need activated integrins on dendritic cells for locomotion and therefore activation via cytohesin-1 is dispensable. These findings indicate that the GEF-function of cytohesin-1 is an important factor for the chemokine-induced chemotaxis of many immune cells, e.g. T cells and dendritic cells in mice and men.

4 Discussion

4.1 Novel small molecule inhibitors for cytohesin protein function

This study describes the discovery of novel small molecules that serve as inhibitors for cytohesin function, and its implications in the further investigation of GEF–dependent and –independent cytohesin function in immune cells.

Cytohesin proteins were first discovered as regulators of LFA-1 mediated cell adhesion (Kolanus et al. 1996). Furthermore they serve as guanine-nucleotide exchange factors for ADP-ribosylation factors (ARF) (Chardin et al. 1996). There are four known members of the cytohesin family in mammals, namely cytohesin-1, cytohesin-2 (ARNO), cytohesin-3 (Grp1 and *steppke* in *Drosophila*) and cytohesin-4 (Ogasawara et al. 2000). Except for cytohesin-4, which is only expressed in cells of hematopoietic origin (Ogasawara et al. 2000), all other cytohesins are ubiquitously expressed. All guanine nucleotide exchange factors for ARF proteins share a common domain, the Sec7 domain which harbours the catalytic GEF-activity.
This function can be inhibited by Brefeldin A, however, the small ARF-GEFs of the cytohesin family are insensitive to treatment with this compound. Until very recently, no chemical inhibitor for cytohesin proteins was available and hence the investigation of cytohesin function relied on overexpression and RNAi-knockdown experiments. The small molecule Secin H3 (Sec7 domain inhibitor H3) was discovered in an aptamer displacement assay (Hafner et al. 2006). In this assay, Secin H3 was able to replace the aptamer M69 (Mayer et al. 2001), that specifically binds to cytohesin proteins. Furthermore, the small molecule specifically inhibited cytohesin function and had no influence on other ARF-GEFs, e.g. EFA6. By the use of this inhibitor it was possible to link cytohesin function to signaling pathways including insulin signaling and EGFR signaling (Bill et al. 2010; Hafner et al. 2006).

Notwithstanding the efficacy of Secin H3 to inhibit cytohesin function in signaling pathways, such as insulin signaling and EGFR signaling, in immune cells, this small molecule was only insufficiently capable of inhibiting cytohesin functions. Therefore – and because no knockout model is available at the moment – it was necessary to find novel small molecules with higher efficacy for the inhibition of cytohesin function especially in immune cells.

This task was addressed by a novel approach combining bioinformatics for *in silico* identification of potential novel small molecule candidates with several *in vitro* experiments for functional

screening, regarding their efficacy in inhibiting known cytohesin functions. The bioinformatic strategy included two separate screening methodologies for the identification of small molecules that are similar to the lead compound Secin H3 yet divergent enough to display a different inhibition profile for cytohesin functions (Stumpfe et al. 2010).

In the outcome of the virtual *in silico* screening approach, 145 compounds were selected due to their theoretical potential to serve as cytohesin inhibitors and their commercial availability and were subjected to further investigation. In three different assays, the inhibitory potential of these compounds was tested. These specific assays were chosen, because they are an established representation of the reported cytohesin functions. Furthermore, in these initial screenings the combination of the assays was selected in such a way, that three members of the cytohesin family were involved and a combination of various *in vitro* experiments was performed with cytohesins from different organisms(cf. figure 8).

One screening experiment was the GDP/GTP-exchange assay, in which the Sec7 domain of cytohesin-2 (ARNO) catalyzes the GDP-to-GTP-exchange on ARF1(Δ17N) *in vitro* (Stumpfe et al. 2010). GTP-loading onto ARF1 leads to an increase in the intrinsic tryptophan autofluorescence, caused by a conformational change in the protein. Inhibition of ARNO would lead to diminished incorporation of GTP in ARF1 and therefore less increase in fluorescence.

The second experiment was based on the reported involvement of the *Drosophila* cytohesin *steppke* in FOXO1-dependent insulin signaling (Stumpfe et al. 2010; Fuss et al. 2006). Upon stimulation of starved S2 cells with insulin, phosphorylation of *d*Foxo1 by Akt/PKB can be observed (Fuss et al. 2006). The transcription factor *d*Foxo1 is thereby retained in the cytosol and unable to regulate the transcription of *d*4EB-P1. Inhibition of *steppke* leads to decreased *d*Foxo1 phosphorylation, therefore, *d*Foxo1 can enter the nucleus and can regulate the *d*4EB-P1 transcription which in turn leads to elevated *d*4EB-P1 mRNA levels.

The third experiment in this study setup exploited the reported function of cytohesin-1 to increase adhesive properties of immune cells to ICAM-1 coated surfaces upon TCR engagement or stimulation of T cells with the DAG-analog PMA (Stumpfe et al. 2010). Inhibition of cytohesin-1 as a direct interaction partner of LFA-1 leads to diminished adhesion of T cells to ICAM-1 (Kolanus et al. 1996). Quantification of T cells that are attached to ICAM-1 coated surfaces after treatment with the novel small molecules served as readout in this assay.

In combination, the outcome of these three different assays can provide valuable information for creating holistic inhibition profiles of the putative novel small molecule inhibitors, since all these assays address different reported functions of the cytohesin family and furthermore diverse cytohesins are involved.

In this initial screen two novel molecules, namely Secin 16 and Secin 144, were identified amongst others, which showed interesting inhibition profiles. These small molecules were further selected for the investigation of cytohesin function in the present study.

Secin 16 was able to inhibit the reported cytohesin functions in all of the three initial screening experiments with significantly higher efficacy compared to Secin H3 treatment. Thus, it inhibited the *in vitro* GTP-exchange of ARF1(Δ17N) catalyzed by ARNO, led to elevated *d*4EB-P1 mRNA levels upon stimulation of S2 cells with insulin and abrogated adhesion of Jurkat T cells to ICAM-1 coated surfaces (cf. figure 10).

In contrast, Secin 144 was only able to inhibit cytohesin function in one of the three screening experiments. Secin 144 could efficiently block adhesion of Jurkat T cells to ICAM-1 coated surfaces. However, neither inhibition of ARNO-catalyzed GDP/GTP-exchange nor influence on the increase of *d*4EB-P1 could be observed (cf. figure 13)

This was an interesting finding and raised more questions concerning the different character of these small molecule compounds.

First of all, it was of great importance to ascertain that the observed inhibitory phenotypes are due to a specific interaction of the compounds with its target protein, cytohesin. To address this question, SPR experiments were performed with the Sec7 domain of cytohesin-1. Indeed, an interaction between the small molecule compounds Secin 16 and Secin 144 and the Sec7 domain of cytohesin-1 could be observed (figure 11 and figure 14). The interaction proved to occur in a concentration-dependent fashion. Furthermore, application of LY294002, a small molecule specific for the inhibition of PI3-kinase, served as a negative control and did not alter SPR curves. Unspecific interaction of the small molecules with other proteins was further excluded by measuring interaction of the small molecules to covalently linked BSA or ARF1 via SPR. Also in this experimental setup, no interaction could be observed. This led to the conclusion that the small molecules are able to specifically interact with cytohesin-1 proteins. However, it cannot be fully ruled out that these compounds influence other proteins than the family of cytohesin proteins. Nevertheless, there are some points that can serve as arguments against this, since on one hand

these molecules are structurally derived from the small molecule inhibitor Secin H3 that specifically interacts with the Sec7 domain of cytohesin proteins, and therefore, these novel compounds are likely to interact in a comparable fashion with the target protein. On the other hand, the assays chosen for the functional screening are very divers concerning the signaling pathways that are involved and the only protein that is involved in all these pathways is cytohesin. Furthermore, in the *in vitro* GTP-exchange assay only ARF1 and the Sec7 domain of cytohesin are present, and since the SPR experiments showed that the compounds do not interact with ARF1, cytohesin proteins are the only potential targets in this assay.

In conclusion, this multi-disciplinary approach of bioinformatics, chemistry and biology was very successful in the identification of novel small molecule compounds that can serve as inhibitors for cytohesin function (Stumpfe et al. 2010).

It was then of great interest to characterize the nature of these small molecule compounds, especially, since a differential inhibitory profile was observed regarding the compounds Secin 16 and Secin 144 in the GTP-exchange and *drosophila* FOXO1 assay.

Secin 144 treatment solely affected adhesion of Jurkat T cells to ICAM-1 but did not exhibit significant alterations in the *in vitro* GTP-exchange assay or in the expression levels of *d*4EB-P1 mRNA in starved S2 cells after insulin stimulation (figure 13). Although all proteins of the cytohesin family share a high similarity, which is especially true for their Sec7 domain, it had to be investigated whether this discrepancy in inhibitory ability was due to a sole inhibition of cytohesin-1 proteins with Secin 144. To rule out this possibility, *in vitro* GTP-exchange assays with the Sec7 domain of cytohesins-1 and ARF1(Δ17N) were performed. Also in this experimental setup the use of Secin 144 showed no reduction of cytohesin-catalyzed GTP-exchange towards ARF1 (figure 16). In contrast, the use of Secin 16 efficiently decreased GTP-loading onto ARF1 by cytohesin-1 Sec7 domain (figure 15). It can therefore not only be concluded that Secin 144 targets specifically cytohesin proteins but also that the different inhibition profile compared to Secin 16 is due to a separate inhibitory mechanism by which Secin 144 abrogates cytohesin function in T cell adhesion. A possible explanation is the alteration in the binding site of the small molecule to the Sec7 domain. Binding of the compound to the Sec7 domain seems to exclusively inhibit the interaction between the Sec7 domain and LFA-1, although this interaction site has to be in close proximity to the position of the catalytic GEF-activity of the cytohesin proteins. This can be concluded from the observation that Secin 16 in contrast is able to inhibit both functions, the GEF-activity as well as the adhesion of T cells to ICAM-1. To fully understand the nature of

inhibition of these two small molecule inhibitors it would be of great importance to identify the exact binding sides of Secin 16 and Secin 144, respectively. This could be done by X-ray crystallography or binding studies e.g. SPR experiments with point mutants of the cytohesin Sec7 domain.

Furthermore, these results clearly show that the influence of cytohesin-1 on the regulation of LFA-1 and therefore T cell adhesion is not dependent on its catalytic activity as a GEF for ARF proteins.

In comparison, the E157K mutant affects both, the GTP-exchange ability of cytohesin proteins as well as the LFA-1 mediated adhesion of immune cells (Knorr et al. 2000; Geiger et al. 2000). Thus, it can be concluded that the mutation at this position and the resulting conformational changes affect the interaction with proteins of the ARF family and interaction with other regulatory proteins as those of the integrin family. Secin 144 now separates these functions that were thought to be inseparable and the use of this compound might provide valuable information about the function of this class of proteins.

Combination of the two novel inhibitors in assays that serve as readout for cytohesin function can possibly provide information important to dissect GEF-dependent and -independent regulation of cytohesin proteins for the first time.

4.2 GEF-dependent and independent functions of cytohesin in T cell activation

Cytohesin-1 is reported to be a direct interactor of β_2-integrins (Geiger et al. 2000; Kolanus et al. 1996). In this regard, overexpression of cytohesin-1 regulates the activation of LFA-1 and leads to enhanced adhesion of Jurkat T cells to ICAM-1 coated surfaces after TCR engagement (Geiger et al. 2000; Kolanus et al. 1996).

Inhibition of cytohesin function by application of the two novel small molecule compounds Secin 16 or Secin 144 indeed leads to abrogated adhesion of T cells to ICAM-1 coated surfaces after TCR engagement (cf. figure 5, figure 10 and figure 13). This inhibition of adhesion is most probably due to interference with the interaction between β2-integrin and cytohesin-1. The molecules inhibit this process with higher efficacy than the earlier described Secin H3 compound (Hafner et al. 2006) and can be used in lower concentrations. The exact mode of action was not investigated in this study but it would be interesting to investigate where the differences for

example in the binding sites of the compounds to the Sec7 domain are. Furthermore, inhibition of adhesion is not due to the inhibition of the catalytic GEF-activity of the cytohesin proteins, but can be separated by the use of the small molecule Secin 144, which is an exclusive inhibitor for the reported cytohesin-1 involvement in the establishment of T cell adhesion to ICAM-1.

However, inhibition of T cell adhesion does not only occur on ICAM-1 coated surfaces but can be also observed for lymphocyte adhesion on VCAM-1, a ligand for VLA-4 (very late antigen-4; $\alpha 4\beta 1$) (figure 17). It was reported that cytohesin-1 only interacts with β_2-integrins directly, but not with β_1-integrins (Kolanus et al. 1996). In contrast, cytohesin-2 and -3 are reported to be responsible for the regulation of integrins of the β_1-family and treatment of HeLa cells with Secin H3 or transfection of these cells with siRNA targeting ARNO led to diminished adhesion to fibronectin, another β_1-integrin ligand (Oh and Santy 2010). It can therefore be concluded that the novel small molecule inhibitors for cytohesin function are able to diminish the interaction between proteins of the cytohesin family and proteins of the integrin β_1-and β_2-family. Although cytohesin-3 seems to have an opposing effect on adhesion and migration in comparison to cytohesin-2 in HeLa cells, chemical inhibition of both proteins at the same time seems to have only an influence on cytohesin-2. The situation in PBL is slightly different, since the expression levels of cytohesin-3 are marginal (Korthauer et al. 2000). Moreover, cytohesin-1 and -2 seem to act in the same direction in the adhesion pathway.

Adhesion under static conditions is mainly dependent on the presence of a suitable ligand. In contrast, the formation of adhesive contacts between T cells and the endothelium in the blood stream relies on the fast development of the integrin high-affinity conformation. In earlier studies it was shown that cytohesin-1 and the dominant negative cytohesin-1 E157K mutant directly interact with the cytoplasmic tail of CD18 and that overexpression of cytohesin-1 wildtype and the cytohesin-1 E157K mutant positively regulate the accessibility of the intermediate LFA-1 conformation mAB24-epitope (Geiger et al. 2000). In contrast, cytohesin-1, but not the cytohesin-1 E157K mutant could increase the number of adherent T cells to endothelium under flow conditions (Weber et al. 2001). The employment of the novel small molecule compounds corroborates the findings of Geiger et al., that it is not the GEF-activity of cytohesins that regulates the LFA-1 conformational change (Geiger et al. 2000), since Secin 144 which is unable to inhibit the catalytic GEF-activity of cytohesin-1 decreases adhesion of PBL to endothelium under flow conditions. In contrast, Secin 16 which is an inhibitor for both, LFA-1 mediated adhesion and GEF-activity, fails to inhibit the fast conformational change in LFA-1 in this context (cf. figure 18). This interesting finding indicates that the catalytic GEF-function of cytohesin-1 is dispensable in

this process. Furthermore, it implicates that the dominant-negative cytohesin-1 E157K mutant acts in a yet undefined mechanism that does not only rely on catalytic GEF-activity. Variations in the target interaction sites of the two compounds Secin 16 and Secin 144 with the Sec7 domain of cytohesin-1 seem to be a possible explanation for differences in the inhibition of T cell adhesion to endothelium under flow. For example, the binding of Secin 144 could abrogate the interaction between LFA-1 and cytohesin-1 leading to a reduction in T cell adhesion to endothelium, whereas inhibition of T cells with Secin 16 only insufficiently interferes in this process but nevertheless inhibits long-term establishment of T cell adhesion to ICAM-1.

It is reported for cytohesin-1 to be part of the outside-in signaling in which cytohesin-1 signs responsible for the signal transduction in the MAPK cascade, especially ERK1/2, (Paul 2007; Perez et al. 2003) leading to the production of cytokines and proliferation signals. These proliferation signals are dependent on the production of IL-2, as a hallmark of T cell activation (Serfling et al. 1995). IL-2 reporter activity is positively regulated when cytohesin-1 was overexpressed (Paul 2007). Inhibition of cytohesin-function with the novel small molecule compounds reduces the levels of produced IL-2 in murine and human T cells (figure 19 and figure 20). The reduction of IL-2 production by inhibition of cytohesin-1 corroborates findings of Grell and Paul who could report reduction in the IL-2 reporter gene assay after downregulation of cytohesin-1 and upregulation following overexpression in T cells. Interestingly, the employment of Secin 144 only leads to a partial IL-2 decrease, whereas the use of Secin 16 almost abrogates IL-2 production in murine and human T cells. This observation indicates that the activation of LFA-1 is not the main factor for initiation of IL-2 production. In contrast, the GEF-function of cytohesin-1 appears to be indispensable in this process. The fact, that a slight reduction in the cytokine production after application of Secin 144 can be observed, might be explained by a minor impact of LFA-1. Although the aAPCs are not coated with a specific ligand for LFA-1, T cell – T cell contacts could provide the respective ICAM-1 as ligand in the course of LFA-1 activation and these intercellular interactions are then also inhibited by the small molecule compounds. Taken together, the catalytic GTP-exchange function of cytohesin-1 and its involvement in the positive regulation of integrin activation seem to work synergistically in the process of cytokine production. Comparable results are also achieved when the levels of other cytokines where analyzed. Also for IL-4, TNF-α and Interferon-γ a slight decrease in cytokine production could be noted, when primary T cells were inhibited by the use of Secin 144. In contrast, the employment of Secin 16 led to a strong reduction of effector cytokine production.

As a result of reduced IL-2 production, inhibition of cytohesin function should also lead to reduced proliferation of T cells after TCR engagement. Indeed, the inhibition of cytohesin proteins with Secin 16 almost abrogates T cell proliferation (figure 21). In contrast, Secin 144 employment inhibits T cell proliferation only marginally (figure 22). Although ICAM-1 as specific LFA-1 ligand is not present on the beads, intercellular contacts to other T cells could provide LFA-1 activation and therefore subsequent signaling needed for full cytohesin-mediated T cell proliferation. Secin 144 would also inhibit these activation events, resulting in a minor decrease in proliferation levels. Taken together, diminished proliferation as a result of cytohesin inhibition is fully in line with the previous findings of reduced cytokine production.

It can be concluded, that cytohesin-1 is an important factor for the establishment of T cell activation. Clonal T cell expansion depends on cytokine production and is regulated in parts by signals derived from the MAP kinase cascade. It was shown, that ERK1/2 phosphorylation is positively regulated by cytohesin-1 as part of the outside-in signaling after activation of LFA-1 (Perez et al. 2003). With the help of the novel small molecule inhibitors for cytohesin function it was now not only possible to investigate the role of cytohesin in ERK1/2 phosphorylation. Additionally, by the combination of the two inhibitors Secin 16 and Secin 144 it was furthermore feasible to investigate the influence of LFA-1 in this context. Since Secin 144 seems to solely inhibit the LFA-1 activation of cytohesin-1 proteins, the use of this compound should give further insides into the question, if LFA-1 activation is an indispensable prerequisite for cytohesin-mediated ERK phosphorylation. Inhibition of the cytohesin GTP-exchange activity by the use of Secin 16 abrogates phosphorylation events of the MAP kinase ERK1/2, whereas sole inhibition of cytohesin-mediated LFA-1 activation by Secin 144 employment does not lead to decreased phosphorylation levels (figure 26). In contrast, an increase in MAPK ERK1/2 phosphorylation can be observed. This puzzling finding might be explained by a slightly enhanced GTP-exchange activity of cytohesins after Secin 144 employment that could be observed *in vitro*, but since this effect was very small it is more likely that another, yet unknown factor leads to this enhancement of phosphorylation.

Since no specific ligand for LFA-1 was present in this experimental setup and the stimulation of the T cells was achieved by aAPCs that only provide CD3 and CD28 stimulation, it can be concluded that the role of cytohesin-1 in the phosphorylation of ERK1/2 during T cell activation is not only dependent on LFA-1 induced cytohesin function in primary T cells. This finding strengthens previous results of Paul, who could observe a reduction in the ERK1/2 target protein phosphorylation of Elk1 after the use of siRNA targeting cytohesin-1 in Jurkat T cells.

To further address the question, whether the novel small molecule cytohesin inhibitors show undesired side effects in T cell activation associated signaling pathways, intracellular calcium-flux after TCR ligation was investigated. Intracellular calcium is released as a consequence of PLC activation after TCR engagement. Secin 16 leaves the calcium-levels in T cells unaltered as compared to the vehicle control (figure 25). This finding corroborates findings that downregulation of cytohesins (Paul 2007) and chemical inhibition of cytohesin proteins by Secin H3 (El Azreq et al. 2010) have no influence on the mobilization of intracellular calcium ions.

Toxicity of the inhibitors could cause reduced cytokine production and lower proliferation rates. Therefore, viability studies had to ascertain, that no toxic side effects of the chemical compounds influence the outcome of the *in vitro* experiments. As depicted in figure 23 and figure 24 the use of the chemical inhibitors does not impair T cell viability in the course of the experiment. Thus, it can be concluded, that the observed effects on proliferation and cytokine production in T cells are a result of cytohesin inhibition. Moreover, loss of GTP-exchange activity has the highest impact in this context and can only be observed if Secin 16 is used to inhibit cytohesin function.

4.3 GEF-dependent and -independent cytohesin functions in immune cell migration

Immune cell migration is one fundamental component for establishment and maintenance of immunity. Immature APCs cross the blood vessels and migrate into tissues, where they mature after encountering an antigen. During maturation, dendritic cells leave the residual tissue and enter lymphatic vessels that direct them chemotactically to draining lymph nodes. In the lymph node, antigens are presented to the lymphocytes. They undergo activation and further differentiation and migrate, guided by chemokines, through the blood stream to inflamed tissues, where they fulfill pathogen clearance. Without the chemokinetic information provided by inflamed tissue or persistent secretion of chemokines by the lymph nodes, no directed movement would be possible. This in turn, would lead to prolonged response time, in which pathogenic threats could cause severe damage to the organism. Investigation of migration events in immune cells therefore is an important task in understanding the nature of the pathogen-host homeostasis.

In this study, two novel molecules were characterized that serve as differential inhibitors for cytohesin function, Secin 16 and Secin 144, respectively. Secin 16 application leads to strong

inhibition of all reported cytohesin functions in immune cells at a concentration of 10 µM including GEF-activity towards ARF proteins and LFA-1 activation. In contrast, Secin 144 occurs to be a bonafide inhibitor exclusively affecting cytohesin-mediated integrin activation.

Overexpression of the dominant negative cytohesin-1 E157K mutant in dendritic cells and primary T cells results in low transfection rates and hence, is insufficient for the investigation of the impact of cytohesin GEF-activity on immune cell migration. Furthermore, Secin H3, a recently discovered cytohesin inhibitor, does not allow for discrimination between GEF-dependent and –independent cytohesin functions. The novel small molecule inhibitors described in this study could overcome this hurdle and moreover appear to be more potent in inhibition of cytohesin-mediated events as compared to inhibition of cytohesin function with Secin H3.

It was therefore interesting to inhibit cytohesin function in different immune cells and subject them to a variety of migration experiments. Transwell migration assays were first used to validate the inhibitory potential of Secin 16 and Secin 144. Application of the inhibitors to migrating mature BmDCs resulted in a reduction of migration, when Secin 16 was used to inhibit cytohesin function. However, employment of Secin 144 for this purpose did not alter the number of migrating mature BmDCs after chemokine stimulation compared to vehicle treated cells (Figure 1figure 27). This indicates, that migration of mature BmDCs is cytohesin dependent and furthermore, that the catalytic GEF-function of this protein is indispensable. This finding is in full accordance with earlier results of Quast et al., who reported that dendritic cells transfected with siRNA targeting cytohesin-1 or overexpressing the dominant negative E157K mutant show a reduction in chemotaxis (Quast et al. 2009). Since no specific ligand for LFA-1 was present, the influence of cytohesin-mediated LFA-1 regulation was expected to be marginal. In agreement with the observations for mature BmDCs, also Jurkat T cells migrate in a GEF-dependent fashion and migration could only be inhibited by the use of Secin 16, but not by application of Secin 144 (cf. figure 28).

Transwell assays represent a rather simple model for the investigation of chemotaxis. Furthermore, integrins are employed by the cell to generate forward locomotion for chemotaxis on two-dimensional substrates (Alon and Dustin 2007; Johnson et al. 2006), but are dispensable for immune cells migrating in three-dimensional surroundings (Lämmermann et al. 2008). Hence, more complex experiments had to be performed for the investigation of cytohesin-involvement in immune cell chemotaxis.

Chemotaxis of immature BmDCs on two-dimensional substrates seems to be mainly dependent on cytohesin GEF activity. In the presence of ligands for β_1- or β_2-integrins, inhibition of cytohesin protein function with Secin 16 leads to reduced chemotaxis (cf. figure 29 and figure 31). This is especially true for surfaces that are coated with fibronectin and ICAM-1. In contrast, inhibition of cytohesin-1 mediated LFA-1 activation by Secin 144 has only a minor impact on the migratory capacity of immature BmDCs. These observations hint to a role for cytohesin proteins in the activation of GTPases rather than in the mediation of integrin binding in this process. In fact, it was reported for mature dendritic cells that overexpression of the GEF-inactive cytohesin-1 E157K mutant reduced chemotaxis in transwell assays (Quast et al. 2009)

In comparison, chemotaxis of PBL appears to be stronger dependent on cytohesin-mediated LFA-1 activation during chemotaxis. Chemotaxis on fibrinogen as a substrate is only impaired when the GEF-activity of cytohesin proteins is inhibited by the use of Secin 16 (figure 32). This is an interesting finding since fibrinogen is not a specific ligand for integrins expressed by lymphocytes and chemotaxis of BmDCs is unaffected by cytohesin-1 inhibition in this context. However, the main influence of cytohesin function on migration can be observed when the GTP-exchange ability of the protein is inhibited by Secin 16 inhibition (figure 33). Nevertheless, with ICAM-1 as substrate for two-dimensional chemotaxis and the employment of Secin 144 as an inhibitor of cytohesin-1 mediated LFA-1 activation, migration of T cells is more severely reduced as compared to the same situation in immature BmDCs. ICAM-1 is the specific ligand for the β_2-integrin LFA-1. Presumably this is the reason for the impairment of forward locomotion of PBL, because PBL cannot use attachment sites to generate traction for chemotaxis.

This might indicate that the migration mode of lymphocytes on two-dimensional surfaces slightly differs from the one in dendritic cells hinting to a movement that is stronger dependent on the involvement of LFA-1. This finding is also underlined by work of Alon and coworkers in which they show that the stimulation of lymphocytes with chemokines leads to the clustering of LFA-1 molecules (Woolf et al. 2007), suggesting that the distribution of active LFA-1 molecules on the surface has a stronger impact in PBL.

In three-dimensional surroundings, inhibition of the GEF-activity of cytohesins by Secin 16 application leads to a strong reduction of chemotaxis in mature BmDCs as well as in PBL (cf. figure 34 and figure 35). Utilization of Secin 144 to inhibit cytohesin-mediated LFA-1 activation has no impact on the migratory behavior of the examined immune cells. Although collagen is not a specific ligand for LFA-1, the same influence of cytohesin proteins can be observed in an *in vivo*

situation. Langerhans cells (LC) that are incubated with the respective compounds also show an impairment in chemotaxis only when the cytohesin GEF-activity is inhibited by the use of Secin 16. Preincubation with Secin 144 has no influence on LC chemotaxis (figure 36). Lämmermann et al. reported that immune cells do not rely on integrin-dependent movement in a three-dimensional surrounding (Lämmermann et al. 2008). They migrate mainly by using the environmental traction to generate forward locomotion by squeezing. Changes in chemotaxis by inhibition of integrin-activation is therefore likely not observable.

Taken together, all chemotactic events in the examined cell types and experimental setups lead to the conclusion, that the main function of cytohesin proteins during chemotaxis is the activation of GTPases. Cytohesin-mediated integrin activation plays a minor role in immune cell chemotaxis as it can be concluded by employment of Secin 144, which solely inhibits this cytohesin function and only has a slight influence in chemotactic immune cell movement. The effector molecules that are targeted by cytohesin-regulation remain unclear and need to be further investigated. However, it is very likely that cells use comparable signaling pathways to provide spatio-temporal information for chemotaxis. Therefore it can be assumed, that -as reported for mature DCs and HeLa cells- also in immature DCs and lymphocytes cytohesin-1 mediates signals by regulation of RhoA (Quast et al. 2009). Other targets for cytohesin proteins during chemotaxis are possible, e.g. it was reported that ARL4D is able to interact with the PH-domain of all four members of the cytohesin family and recruits them to the plasma membrane.

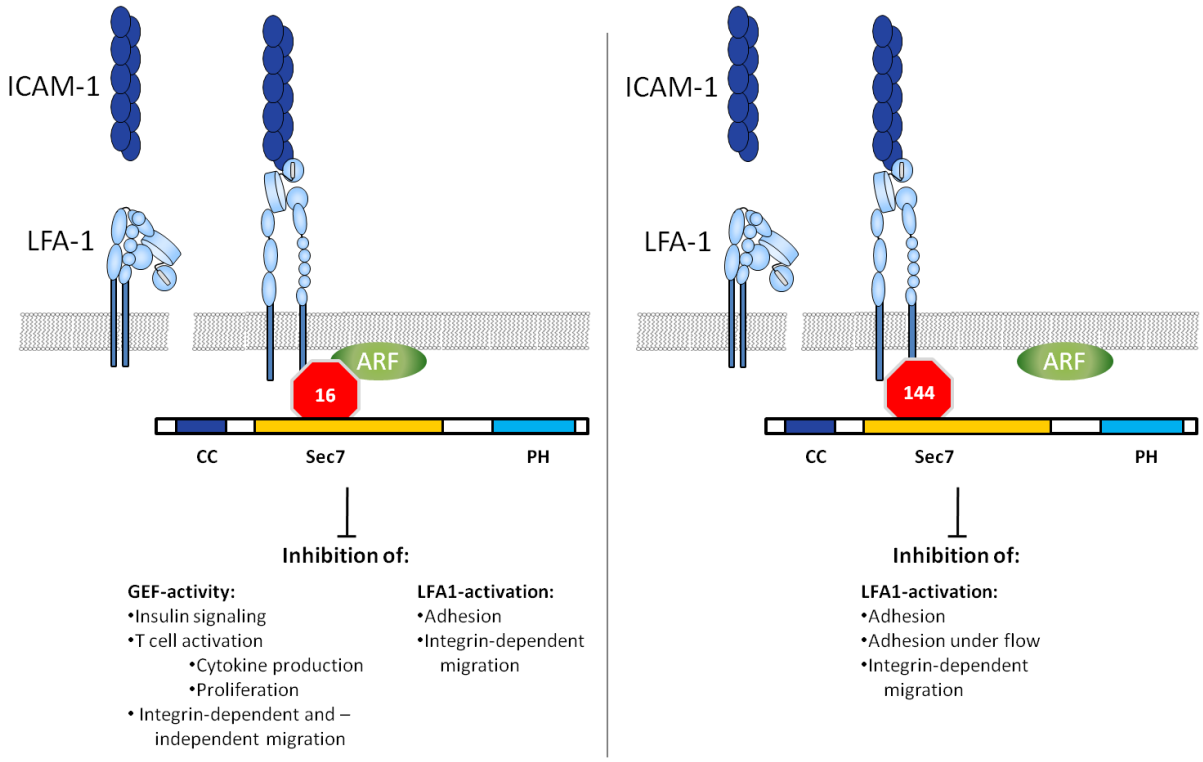

Figure 37: Overview of the effects of Secin 16 and Secin 144 as differential inhibitors for cytohesin function.
Secin 16 inhibits the cytohesin-regulated activation of ARF proteins and LFA-1 in immune cells. Secin 144 exclusively inhibits cytohesin-mediated LFA-1 activation, leading to reduced T cell adhesion and integrin-dependent migration.

Figure 37 summarizes and illustrates the role of cytohesin function as investigated by the inhibition of cytohesin function with Secin 16 or Secin 144, respectively. Inhibition of cytohesin by application of Secin 16 leads to diminished LFA-1 activation. This negatively influences adhesion of T cells to ICAM-1 and VCAM-1, respectively, and the integrin-dependent migration of T cells and mature and immature BmDCs. Furthermore inhibition of cytohesin with Secin 16 strongly reduces the catalytic GEF-activity of cytohesin proteins, resulting in decreased GTP- loading to ARF1 *in vitro* and diminished insulin signaling in *drosophila* S2 cells. Moreover, it inhibits T cell activation through reduced cytokine production and proliferation, T cell signaling via the MAPK pathway and chemotaxis in immune cells during integrin-dependent and -independent migration.

In contrast, Secin 144 only inhibits the cytohesin-mediated activation of LFA-1, resulting in a strong reduction in T cell adhesion to ICAM-1 and VCAM-1 and a diminished development of the active, high affinity LFA-1 conformational state during adhesion under flow conditions.

Chemotaxis in this context only is inhibited, when integrins are indispensable for forward locomotion, e.g. in two-dimensional migration on ICAM-1 as a substrate. The influence of cytohesin-mediated LFA-1 activation in the course of T cell activation is of minor importance, since observed effects in T cell proliferation and cytokine production as well as in T cell signaling are marginal.

5 Summary

Cytohesins are guanine nucleotide exchange factors (GEFs) for the small GTPases of the ARF family. They play important roles in immune cell signaling, immune cell adhesion and migration and furthermore in non-immune insulin signaling and regulation of Erb activation. Cytohesin-1 was first discovered as an interactor with the α_L/β_2-integrin LFA-1 and later as important regulator of T cell signaling in the course of T cell activation.

Investigation of cytohesin function relied on overexpression and downregulation of the protein in a cellular context until recently the small molecule inhibitor Secin H3 was discovered. However, in immune cells Secin H3 was only insufficiently capable of inhibiting cytohesin-mediated processes. In a multidisciplinary approach, bioinformatic *in silico* predictions for novel molecules derived from the structurally similar Secin H3 were examined for their ability to serve as inhibitors for cytohesin functions in various screening assays. These assays were selected, because they involved different cytohesin functions and cytohesin family members, respectively.

In the outcome of these functional screening assays, two novel small molecules, namely Secin 16 and Secin 144, showed interesting inhibition profiles. With the use of Secin 16, it was possible to inhibit GTP-exchange of ARNO towards ARF1, *steppke*-dependent insulin signaling and T cell adhesion to ICAM-1 with significantly more potency compared to Secin H3. In contrast, Secin 144 employment abrogated cytohesin-1 mediated T cell adhesion to ICAM-1, whereas GTP-exchange activity or *drosophila* insulin signaling was left unaffected. Further characterization of these two small molecule inhibitors revealed that they are able to inhibit reported functions of all cytohesin family members without toxic side effects and that they specifically interact with the Sec7 domain of cytohesin-1 *in vitro*. However, Secin 144 was a specific inhibitor for cytohesin-mediated integrin activation and did not interfere with cytohesin functions related to its GEF-activity. In contrast, Secin 16 was able to inhibit both, adhesion- and GEF-related processes that are linked to cytohesin activity.

The combination of these two novel inhibitors was then further exploited for the investigation of cytohesin-mediated processes and their dependency on catalytic cytohesin GEF-activity. It is shown here, that cytohesin-mediated leukocyte adhesion on endothelial cells is mainly achieved by the interaction between cytohesin-1 and LFA-1 and independent of the catalytic GEF-activity of cytohesin proteins. Furthermore, cytohesin-mediated signaling in T cell activation is mainly dependent on the ability of cytohesin GTP-exchange. Hence, cytokine production, T cell

proliferation and ERK1/2 phosphorylation are abrogated, when the pan-cytohesin inhibitor Secin 16 was used. In contrast, the employment of Secin 144 had no significant impact on the inhibition of cytohesin-mediated T cell activation.

Furthermore, immune cell migration is dependent on both, cytohesin GTP-exchange activity and cytohesin-mediated activation of LFA-1. Immune cells are able to switch between integrin-dependent and –independent modes of migration. In integrin-dependent migration the GEF-activity as well as the LFA-1 activation mediated by cytohesin proteins is indispensable for proper forward locomotion in various immune cells. However, in three-dimensional environment integrins are not required for immune cell migration. In this context, only inhibition of the catalytic GEF-activity of cytohesin proteins is impairing migration. In contrast, inhibition of cytohesin-mediated LFA-1 activation does not impair three-dimensional chemotaxis.

In conclusion, the novel small molecule inhibitors for cytohesin function are potent tools for the dissection of GEF-dependent and -independent cytohesin functions.

Zusammenfassung

Cytohesine sind Guaninnukleotid-Austausch Faktoren für die kleinen GTPasen der ARF-Protein-Familie. Sie spielen sowohl in der Signaltransduktion, der Adhäsion und der Migration von Immunzellen als auch in der Insulinsignalkaskade und der ErbB Regulation in Nicht-Immunzellen eine entscheidende Rolle. Cytohesin-1 wurde zunächst als Interaktionspartner des α_L/β_2-Integrins LFA-1 entdeckt und später als wichtiger Regulator der T Zell-Aktivierungskaskade und Aktivator von RhoA in der Migration dendritischer Zellen identifiziert.

Die Untersuchung der Cytohesin Funktion in Immunzellen basierte vornehmlich auf Überexpressionsstudien und Herunterregulierung des Proteins auf mRNA-Ebene mit Hilfe von RNAi bis der organische Molekülinhibitor Secin H3 entdeckt wurde, mit dessen Hilfe eine effektive Inhibition der Cytohesin-Aktivität erreicht werden konnte. Im immunologischen Kontext allerdings zeigte der Inhibitor eine unzureichende Effizienz in der Inhibition von Cytohesin-vermittelten Funktionen. In einem interdisziplinären Ansatz aus Bioinformatik, Chemie und Biologie wurden deshalb neue Moleküle identifiziert, die als Cytohesin Inhibitoren mit verbesserter Wirkeffizienz fungieren sollten. Ausgehend von Secin H3 als Leitstruktur wurden zu diesem Zweck *in silico* Vorhersagen für kleine organische Moleküle getroffen, die im Folgenden auf ihre Fähigkeit zur Inhibition von Cytohesin Proteinen funktionell untersucht wurden. Hierzu wurden Screening-Assays benutzt, in denen sowohl unterschiedliche Cytohesine als auch unterschiedliche Cytohesin Funktionen betroffen waren.

Mit Hilfe der funktionellen Screening-Assays wurden zwei neuartige Moleküle, Secin 16 und Secin 144 identifiziert, die interessante Inhibitionsprofile zeigten. Der Einsatz von Secin 16 in diesen Assays ermöglichte die Inhibition sowohl des GTP-Austauschs an ARF1 von Cytohesin-2, als auch *steppke*-vermittelte Insulin-Signaltransduktion in der Fruchtfliege und T-Zell-Adhäsion an ICAM-1 durch Cytohesin-1. Verglichen mit Secin H3 konnte eine signifikant stärkere Inhibition in allen Assays beobachtet werden. Im Gegensatz dazu konnte durch die Verwendung von Secin 144 ausschließlich die T-Zell Adhäsion auf ICAM-1-Untergrund inhibiert werden, während sowohl die GTP-Austausch-Aktivität von Cytohesin-2 an ARF1 als auch die Insulin-Signaltransduktion in *D. melanogaster* unverändert blieben. Durch die weitere Charakterisierung dieser kleinen organischen Moleküle konnte gezeigt werden, dass diese Inhibitoren spezifisch an die Sec7 Domäne von Cytohesin-1 binden. Des Weiteren zeigten Secin 16 und Secin 144 in kultivierten Zellen keine toxischen Nebeneffekte. Allerdings konnte mit Hilfe von Secin 144 ausschließlich die Cytohesin-vermittelte Integrinaktivierung unterbunden werden, nicht aber Cytohesin-Funktionen,

die durch die GEF-Aktivität der Proteine bedingt waren. Im Gegensatz dazu war es möglich durch den Gebrauch von Secin 16 beide bekannten Cytohesin-1 Funktionen, nämlich LFA-1 Aktivierung und GTP-Austausch an ARF-GTPasen mit großer Effizienz zu inhibieren.

Im Folgenden wurden deshalb beide neuen Inhibitoren in Kombination eingesetzt, um Cytohesin-regulierte Prozesse auf ihre GEF-Abhängigkeit zu untersuchen. Es konnte hier gezeigt werden, dass Cytohesin-vermittelte Leukozyten-Adhäsion an Endothelzellen hauptsächlich, wenn nicht ausschließlich auf der Interaktion zwischen Cytohesin-1 und LFA-1 beruht und unabhängig von der katalytischen GEF-Aktivität von Cytohesin-Proteinen ist. Weiterhin ist die T-Zell Aktivierung in weiten Teilen abhängig von der GEF-Funktion der Cytohesine, weshalb Zytokin-Produktion, T-Zell Proliferation und ERK1/2 Phosphorylierung stark reduziert waren, wenn Secin 16 als Cytohesin-Inhibitor eingesetzt wurde, nicht aber wenn Secin 144 benutzt wurde.

Immunzell-Migration ist hingegen sowohl abhängig von der vollständigen Aktivierung von Integrinen durch Cytohesine als auch von der katalytischen GEF-Aktivität derselben. Immunzellen können zwischen Integrin-abhängiger und –unabhängiger Chemotaxis wechseln, abhängig von der Umgebung. Während der Integrin-abhängigen Migration sind sowohl die Cytohesin-vermittelte Integrinativierung als auch die GEF-Aktivität vonnöten, um gerichtete Vorwärtsbewegungen verschiedener Immunzellen zu gewährleisten. Im Gegensatz dazu werden Integrine in dreidimensionalen Umgebungen nicht für die Immunzellmigration benötigt. In diesem Zusammenhang kann eine Verminderung der Chemotaxis ausschließlich beobachtet werden, wenn die katalytische GEF-Aktivität der Cytohesine durch Secin 16 inhibiert ist. Der Gebrauch von Secin 144 führte zu keiner negativen Beeinflussung der Migrationsfähigkeit in einer dreidimensionalen Collagenmatrix.

Zusammenfassend stellen die neuartigen, kleinen organischen Moleküle zur Inhibition von Cytohesin Funktionen ein wertvolles Werkzeug dar, mit dem sich GEF-abhängige und -unabhängige Cytohesin Funktionen unterscheiden und weiter untersuchen lassen.

Abbreviations

aAPC	artificial antigen presenting cell, bead
ARF1	ADP ribosylation factor 1
ARNO	Cytohesin-2, ARF nucleotide-binding site opener
ATP	Adenosine-5'-triphospate
CD	Cluster of Differentiation
cf.	latin: confer, compare
DC	dendritic cell
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immuno sorbent assay
ERK	extracellular signal-related kinase
et al.	et alii, and others
FCS	fetal calf serum
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	Guanosine-5'-triphosphate
HUVEC	Human Umbilical Vein Endothelial Cells
IFN	Interferon
IL	Interleukin
immBmDC	immature bone marrow derived dendritic cells
LC	Langerhans cell
MAPK	Mitogen-activated protein kinase
mBmDC	mature bone marrow derived dendritic cells
MHC I	major histocompatibility complex I
PBL	peripheral blood lymphocytes
PI3K	Phosphoinositide 3-kinase
SD	standard deviation

SD	Standard deviation
Secin	Sec7-inhibitor
TCR	T cell receptor
TH_1 cells	T Helper cells type 1
TH_2 cells	T Helper cells type 2
TNF	Tumor necrosis factor
VS	virtual similarity

Figure Index

References

Abram, ClareL; Lowell, CliffordA (2009): The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol* 27, S. 339–362.

Ahmadian, Mohammad Reza; Wittinghofer, Alfred; Herrmann, Christian (2002): Fluorescence methods in the study of small GTP-binding proteins. *Methods Mol. Biol* 189, S. 45–63.

Alon, Ronen; Dustin, Michael L. (2007): Force as a Facilitator of Integrin Conformational Changes during Leukocyte Arrest on Blood Vessels and Antigen-Presenting Cells. *Immunity* 26 (1), S. 17–27.

Antonny, B.; Beraud-Dufour, S.; Chardin, P.; Chabre, M. (1997): N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry* 36 (15), S. 4675–4684.

Bajenoff, Marc; Egen, JacksonG; Koo, LilyY; Laugier, JeanPierre; Brau, Frederic; Glaichenhaus, Nicolas; Germain, RonaldN (2006): Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity* 25 (6), S. 989–1001.

Beutler, Bruce (2004): Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 430 (6996), S. 257–263.

Bill, Anke; Schmitz, Anton; Albertoni, Barbara; Song, Jin-Na; Heukamp, LukasC; Walrafen, David et al. (2010): Cytohesins are cytoplasmic ErbB receptor activators. *Cell* 143 (2), S. 201–211.

Boehm, Thomas; Hofer, Susanne; Winklehner, Patricia; Kellersch, Bettina; Geiger, Christiane; Trockenbacher, Alexander et al. (2003): Attenuation of cell adhesion in lymphocytes is regulated by CYTIP, a protein which mediates signal complex sequestration. *EMBO J* 22 (5), S. 1014–1024.

Brown, F. D.; Rozelle, A. L.; Yin, H. L.; Balla, T.; Donaldson, J. G. (2001): Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol* 154 (5), S. 1007–1017.

Brownawell, A. M.; Kops, G. J. P. L.; Macara, I. G.; Burgering, B. M. T. (2001): Inhibition of Nuclear Import by Protein Kinase B (Akt) Regulates the Subcellular Distribution and Activity of the Forkhead Transcription Factor AFX. *Molecular and Cellular Biology* 21 (10), S. 3534–3546.

Chan, A. C.; Iwashima, M.; Turck, C. W.; Weiss, A. (1992): ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71 (4), S. 649–662.

Chardin, P.; Paris, S.; Antonny, B.; Robineau, S.; Béraud-Dufour, S.; Jackson, C. L.; Chabre, M. (1996): A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature* 384 (6608), S. 481–484.

Cohen, Lee Ann; Honda, Akira; Varnai, Peter; Brown, Fraser D.; Balla, Tamas; Donaldson, Julie G. (2007): Active Arf6 recruits ARNO/cytohesin GEFs to the PM by binding their PH domains. *Mol. Biol. Cell* 18 (6), S. 2244–2253.

Cronin, T. C.; Di Nitto, J. P.; Czech, M. P.; Lambright, D.G (2004): Structural determinants of phosphoinositide selectivity in splice variants of Grp1 family PH domains. *EMBO J* 23 (19), S. 3711–3720.Diebold, S. S. (2004): Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA. *Science* 303 (5663), S. 1529–1531.

Dierks, H.; Kolanus, J.; Kolanus, W. (2001): Actin cytoskeletal association of cytohesin-1 is regulated by specific phosphorylation of its carboxyl-terminal polybasic domain. *J. Biol. Chem* 276 (40), S. 37472–37481.

Dieu, M. C.; Vanbervliet, B.; Vicari, A.; Bridon, J. M.; Oldham, E.; Ait-Yahia, S. et al. (1998): Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 188 (2), S. 373–386.

D'Souza-Schorey, C.; Boettner, B.; Van, AelstL (1998): Rac regulates integrin-mediated spreading and increased adhesion of T lymphocytes. *Mol Cell Biol* 18 (7), S. 3936–3946.

Dustin, M. L.; Springer, T. A. (1991): Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu Rev Immunol* 9, S. 27–66.

El, AzreqMohammed-Amine; Garceau, Valerie; Harbour, Danielle; Pivot-Pajot, Christophe; Bourgoin, SylvainG (2010): Cytohesin-1 regulates the Arf6-phospholipase D signaling axis in human neutrophils: impact on superoxide anion production and secretion. *J Immunol* 184 (2), S. 637–649.

Fanger, N. A.; Wardwell, K.; Shen, L.; Tedder, T. F.; Guyre, P. M. (1996): Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. *J Immunol* 157 (2), S. 541–548.

Franco, M.; Chardin, P.; Chabre, M.; Paris, S. (1993): Myristoylation is not required for GTP-dependent binding of ADP-ribosylation factor ARF1 to phospholipids. *J Biol Chem* 268 (33), S. 24531–24534.

Friedl, P.; Entschladen, F.; Conrad, C.; Niggemann, B.; Zanker, K. S. (1998): CD4+ T lymphocytes migrating in three-dimensional collagen lattices lack focal adhesions and utilize beta1 integrin-independent strategies for polarization, interaction with collagen fibers and locomotion. *Eur J Immunol* 28 (8), S. 2331–2343.

Friedl, Peter; Weigelin, Bettina (2008): Interstitial leukocyte migration and immune function. *Nat Immunol* 9 (9), S. 960–969.

Friedl, Peter; Wolf, Katarina (2003): Proteolytic and non-proteolytic migration of tumour cells and leucocytes. *Biochem Soc Symp* (70), S. 277–285.

Fukata, Masaki; Watanabe, Takashi; Noritake, Jun; Nakagawa, Masato; Yamaga, Masaki; Kuroda, Shinya et al. (2002): Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. *Cell* 109 (7), S. 873–885.

Fuss, Bernhard; Becker, Thomas; Zinke, Ingo; Hoch, Michael (2006): The cytohesin Steppke is essential for insulin signalling in Drosophila. *Nature* 444 (7121), S. 945–948.

Geiger, C.; Nagel, W.; Boehm, T.; van Kooyk, Y.; Figdor, C. G.; Kremmer, E. et al. (2000): Cytohesin-1 regulates beta-2 integrin-mediated adhesion through both ARF-GEF function and interaction with LFA-1. *EMBO J* 19 (11), S. 2525–2536.

Graham, F. L.; van der Eb, A. J. (1973): A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52 (2), S. 456–467.

Grell, Jessica (2009): The role of guanine nucleotide exchange factors in the regulation of immune cell signaling. PhD thesis.

Hafner, Markus; Schmitz, Anton; Grüne, Imke; Srivatsan, Seergazhi G.; Paul, Bianca; Kolanus, Waldemar et al. (2006): Inhibition of cytohesins by SecinH3 leads to hepatic insulin resistance. *Nature* 444 (7121), S. 941–944.

Heasman, S. J.; Carlin, L. M.; Cox, S.; Ng, T.; Ridley, A. J. (2010): Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. *The Journal of Cell Biology* 190 (4), S. 553–563.

Heil, F. (2004): Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science* 303 (5663), S. 1526–1529.

Hofmann, Irmgard; Thompson, Amanda; Sanderson, Christopher M.; Munro, Sean (2007): The Arl4 family of small G proteins can recruit the cytohesin Arf6 exchange factors to the plasma membrane. *Curr. Biol* 17 (8), S. 711–716.

Hornung, Veit; Guenthner-Biller, Margit; Bourquin, Carole; Ablasser, Andrea; Schlee, Martin; Uematsu, Satoshi et al. (2005): Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 11 (3), S. 263–270.

Inaba, K. (1992): Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *Journal of Experimental Medicine* 176 (6), S. 1693–1702.

Janeway, Charles A.; Medzhitov, Ruslan (2002): Innate Immune Recognition. *Annu. Rev. Immunol* 20 (1), S. 197–216.

Johnson, LouiseA; Clasper, Steven; Holt, AndrewP; Lalor, PatriciaF; Baban, Dilair; Jackson, DavidG (2006): An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. *J Exp Med* 203 (12), S. 2763–2777.

Judge, A.; Bola, G.; Lee, A.; Machlachlan, I. (2006): Design of Noninflammatory Synthetic siRNA Mediating Potent Gene Silencing in Vivo. *Molecular Therapy* 13 (3), S. 494–505.

Kahn, R. A.; Gilman, A. G. (1986): The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J Biol Chem* 261 (17), S. 7906–7911.

Klarlund, J. K.; Guilherme, A.; Holik, J. J.; Virbasius, J. V.; Chawla, A.; Czech, M. P. (1997): Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science* 275 (5308), S. 1927–1930.

Klarlund, J. K.; Tsiaras, W.; Holik, J. J.; Chawla, A.; Czech, M. P. (2000): Distinct polyphosphoinositide binding selectivities for pleckstrin homology domains of GRP1-like proteins based on diglycine versus triglycine motifs. *J Biol Chem* 275 (42), S. 32816–32821.

Kliche, S.; Nagel, W.; Kremmer, E.; Atzler, C.; Ege, A.; Knorr, T. et al. (2001): Signaling by human herpesvirus 8 kaposin A through direct membrane recruitment of cytohesin-1. *Mol. Cell* 7 (4), S. 833–843.

Knorr, Thomas; Nagel, Wolfgang; Kolanus, Waldemar (2000): Phosphoinositides determine specificity of the guanine-nucleotide exchange activity of cytohesin-1 for ADP-ribosylation factors derived from a mammalian expression system. *European Journal of Biochemistry* 267 (12), S. 3784–3791.

Kolanus, W.; Nagel, W.; Schiller, B.; Zeitlmann, L.; Godar, S.; Stockinger, H.; Seed, B. (1996): Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule. *Cell* 86 (2), S. 233–242.

Kolanus, Waldemar (2007): Guanine nucleotide exchange factors of the cytohesin family and their roles in signal transduction. *Immunol. Rev* 218, S. 102–113.

Koretzky, Gary A.; Myung, Peggy S. (2001): Positive and Negative Regulation of T-Cell Activaiton by Adaptor Proteins. *Nat Rev Immunol* 1 (2), S. 95–107.

Korthauer, U.; Nagel, W.; Davis, E. M.; Le, BeauMM; Menon, R. S.; Mitchell, E. O. et al. (2000): Anergic T lymphocytes selectively express an integrin regulatory protein of the cytohesin family. *J Immunol* 164 (1), S. 308–318.

Lämmermann, Tim; Bader, Bernhard L.; Monkley, Susan J.; Worbs, Tim; Wedlich-Söldner, Roland; Hirsch, Karin et al. (2008): Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature* 453 (7191), S. 51–55.

Lauffenburger, D. A.; Horwitz, A. F. (1996): Cell migration: a physically integrated molecular process. *Cell* 84 (3), S. 359–369.

Leavy, Olive (2010): T cells: Shaping Il4 gene expression. *Nat Rev Immunol* 11 (1), S. 3.

Li, D.; Molldrem, J. J.; Ma, Q. (2009): LFA-1 Regulates CD8+ T Cell Activation via T Cell Receptor-mediated and LFA-1-mediated Erk1/2 Signal Pathways. *Journal of Biological Chemistry* 284 (31), S. 21001–21010.

Malek, ThomasR (2008): The biology of interleukin-2. *Annu Rev Immunol* 26, S. 453–479.

Mandeville, J. T.; Lawson, M. A.; Maxfield, F. R. (1997): Dynamic imaging of neutrophil migration in three dimensions: mechanical interactions between cells and matrix. *J. Leukoc. Biol* 61 (2), S. 188–200.

Martín-Fontecha, Alfonso; Sebastiani, Silvia; Hopken, UtaE; Uguccioni, Mariagrazia; Lipp, Martin; Lanzavecchia, Antonio; Sallusto, Federica (2003): Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* 198 (4), S. 615–621.

Mayer, G.; Blind, M.; Nagel, W.; Bohm, T.; Knorr, T.; Jackson, C. L. et al. (2001): Controlling small guanine-nucleotide-exchange factor function through cytoplasmic RNA intramers. *Proc Natl Acad Sci U S A* 98 (9), S. 4961–4965.

Meacci, E.; Tsai, S. C.; Adamik, R.; Moss, J.; Vaughan, M. (1997): Cytohesin-1, a cytosolic guanine nucleotide-exchange protein for ADP-ribosylation factor. *Proc. Natl. Acad. Sci. U.S.A* 94 (5), S. 1745–1748.

Mellman, I.; Steinman, R. M. (2001): Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106 (3), S. 255–258.

Mempel, Thorsten R.; Henrickson, Sarah E.; Andrian, Ulrich H. von (2004): T-cell priming by dendriticcells in lymph nodes occurs in three distinct phases. *Nature* 427 (6970), S. 154–159.

Moss, J.; Vaughan, M. (1995): Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J Biol Chem* 270 (21), S. 12327–12330.

Moss, Joel; Vaughan, Martha (2002): Cytohesin-1 in 2001. *Arch Biochem Biophys* 397 (2), S. 156–161.

Mossessova, Elena; Corpina, RichardA; Goldberg, Jonathan (2003): Crystal structure of ARF1*Sec7 complexed with Brefeldin A and its implications for the guanine nucleotide exchange mechanism. *Mol Cell* 12 (6), S. 1403–1411.

Nagel, W.; Schilcher, P.; Zeitlmann, L.; Kolanus, W. (1998): The PH domain and the polybasic c domain of cytohesin-1 cooperate specifically in plasma membrane association and cellular function. *Mol Biol Cell* 9 (8), S. 1981–1994.

Nakagawa, Hiroyuki; Miki, Hiroaki; Nozumi, Motohiro; Takenawa, Tadaomi; Miyamoto, Shigeaki; Wehland, Jürgen; Small, J. Victor (2003): IRSp53 is colocalised with WAVE2 at the tips of protruding lamellipodia and filopodia independently of Mena. *J. Cell. Sci* 116 (Pt 12), S. 2577–2583.

Nebenfuhr, Andreas; Ritzenthaler, Christophe; Robinson, DavidG (2002): Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiol* 130 (3), S. 1102–1108.

Nelson, B. H.; Lord, J. D.; Greenberg, P. D. (1994): Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. *Nature* 369 (6478), S. 333–336.

Northup, J. K.; Smigel, M. D.; Gilman, A. G. (1982): The guanine nucleotide activating site of the regulatory component of adenylate cyclase. Identification by ligand binding. *J. Biol. Chem* 257 (19), S. 11416–11423.

O' Connor, Cornelius J.; Laraia, Luca; Spring, David R. (2011): Chemical genetics. *Chem. Soc. Rev* 40 (8), S. 4332.

Ogasawara, M.; Kim, S. C.; Adamik, R.; Togawa, A.; Ferrans, V. J.; Takeda, K. et al. (2000): Similarities in function and gene structure of cytohesin-4 and cytohesin-1, guanine nucleotide-exchange proteins for ADP-ribosylation factors. *J Biol Chem* 275 (5), S. 3221–3230.

Oh, Seung Ja; Santy, Lorraine C. (2010): Differential effects of cytohesins 2 and 3 on beta1 integrin recycling. *J. Biol. Chem* 285 (19), S. 14610–14616.

Paul, Bianca (2007): Bedeutung von Cytohesin-3 in der Signaltransduktions-Kontrolle der T-Zellaktivierung. PhD thesis.

Perez, Omar D.; Mitchell, Dennis; Jager, Gina C.; South, Sharon; Murriel, Chris; McBride, Jacqueline et al. (2003): Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nat. Immunol* 4 (11), S. 1083–1092.

Peters, P. J.; Hsu, V. W.; Ooi, C. E.; Finazzi, D.; Teal, S. B.; Oorschot, V. et al. (1995): Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of nonoverlapping membrane compartments. *J Cell Biol* 128 (6), S. 1003–1017.

Quast, Thomas; Tappertzhofen, Barbara; Schild, Cora; Grell, Jessica; Czeloth, Niklas; Förster, Reinhold et al. (2009): Cytohesin-1 controls the activation of RhoA and modulates integrin-dependent adhesion and migration of dendritic cells. *Blood* 113 (23), S. 5801–5810.

Radhakrishna, H.; Donaldson, J. G. (1997): ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J. Cell Biol* 139 (1), S. 49–61.

Renault, Louis; Guibert, Bernard; Cherfils, Jacqueline (2003): Structural snapshots of the mechanism and inhibition of a guanine nucleotide exchange factor. *Nature* 426 (6966), S. 525–530.

Ridley, Anne J. (2006): Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol* 16 (10), S. 522–529.

Ripphausen, Peter; Nisius, Britta; Bajorath, Jürgen (2011): State-of-the-art in ligand-based virtual screening. *Drug Discovery Today* 16 (9-10), S. 372–376.

Saeki, H.; Moore, A. M.; Brown, M. J.; Hwang, S. T. (1999): Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* 162 (5), S. 2472–2475.

Sallusto, F.; Lanzavecchia, A. (1994): Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179 (4), S. 1109–1118.

Sallusto, F.; Cella, M.; Danieli, C.; Lanzavecchia, A. (1995): Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 182 (2), S. 389–400.

Santy, L. C.; Frank, S. R.; Hatfield, J. C.; Casanova, J. E. (1999): Regulation of ARNO nucleotide exchange by a PH domain electrostatic switch. *Curr Biol* 9 (20), S. 1173–1176.

Serfling, E.; Avots, A.; Neumann, M. (1995): The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. *Biochim. Biophys. Acta* 1263 (3), S. 181–200.

Shamri, Revital; Grabovsky, Valentin; Gauguet, Jean-Marc; Feigelson, Sara; Manevich, Eugenia; Kolanus, Waldemar et al. (2005): Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat Immunol* 6 (5), S. 497–506.

Sheetz, M. P. (1994): Cell migration by graded attachment to substrates and contraction. *Semin Cell Biol* 5 (3), S. 149–155.

Shortman, Ken; Naik, ShalinH (2007): Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7 (1), S. 19–30.

Slepnev, V. I.; De, CamilliP (2000): Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nat Rev Neurosci* 1 (3), S. 161–172.

Smith-Garvin, Jennifer E.; Koretzky, Gary A.; Jordan, Martha S. (2009): T Cell Activation. *Annu. Rev. Immunol* 27 (1), S. 591–619.

Snell, G. D. (1948): Methods for the study of histocompatibility genes. *Journ. of Genetics* 49 (2), S. 87–108.

Southgate, RobertJ; Neill, Bronwyn; Prelovsek, Oja; El-Osta, Assam; Kamei, Yasutomi; Miura, Shinji et al. (2007): FOXO1 regulates the expression of 4E-BP1 and inhibits mTOR signaling in mammalian skeletal muscle. *J Biol Chem* 282 (29), S. 21176–21186.

Sozzani, S.; Allavena, P.; D'Amico, G.; Luini, W.; Bianchi, G.; Kataura, M. et al. (1998): Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. J Immunol 161 (3), S. 1083–1086.

Springer, T. A; Dustin, M. (2012): Integrin inside-out signaling and the immunological synapse. Curr Opin Cell Biol 24 (1), S. 107–115.

Stanley, Paula; Smith, Andrew; McDowall, Alison; Nicol, Alastair; Zicha, Daniel; Hogg, Nancy (2007): Intermediate-affinity LFA-1 binds α-actinin-1 to control migration at the leading edge of the T cell. *EMBO J* 27 (1), S. 62–75.

Steinman, R. M.; Cohn, Z. A. (1973): Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137 (5), S. 1142–1162.

Stumpfe, Dagmar; Bill, Anke; Novak, Nina; Loch, Gerrit; Blockus, Heike; Geppert, Hanna et al. (2010): Targeting multifunctional proteins by virtual screening: structurally diverse cytohesin inhibitors with differentiated biological functions. *ACS Chem. Biol* 5 (9), S. 839–849.

Takeda, K. (2004): Toll-like receptors in innate immunity. *International Immunology* 17 (1), S. 1–14.

Van, HaastertPeterJM; Devreotes, PeterN (2004): Chemotaxis: signalling the way forward. *Nat Rev Mol Cell Biol* 5 (8), S. 626–634.

Varga, Georg; Nippe, Nadine; Balkow, Sandra; Peters, Thorsten; Wild, Martin K.; Seeliger, Stephan et al. (2010): LFA-1 Contributes to Signal I of T-Cell Activation and to the Production of Th1 Cytokines. *J Investig Dermatol* 130 (4), S. 1005–1012.

Weber, K. S.; Weber, C.; Ostermann, G.; Dierks, H.; Nagel, W.; Kolanus, W. (2001): Cytohesin-1 is a dynamic regulator of distinct LFA-1 functions in leukocyte arrest and transmigration triggered by chemokines. *Curr Biol* 11 (24), S. 1969–1974.

Wittmann, Torsten; Bokoch, GaryM; Waterman-Storer, ClareM (2004): Regulation of microtubule destabilizing activity of Op18/stathmin downstream of Rac1. *J Biol Chem* 279 (7), S. 6196–6203.

Wolf, Katarina; Muller, Regina; Borgmann, Stefan; Brocker, Eva-B; Friedl, Peter (2003): Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. *Blood* 102 (9), S. 3262–3269.

Woolf, Eilon; Grigorova, Irina; Sagiv, Adi; Grabovsky, Valentin; Feigelson, Sara W.; Shulman, Ziv et al. (2007): Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat. Immunol* 8 (10), S. 1076–1085