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DISSERTATION

Titel der Dissertation

CLEC-1 and CLEC-2

C-type lectin-like receptors encoded within the myeloid cluster of the NK gene complex

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

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Wien, März 2009

The most exciting phrase to hear in science,
the one that heralds new discoveries, is not Eureka!
but rather, "hmm.... that's funny...."

(Isaac Asimov)

Content

Summary

CLEC-1 and CLEC-2 belong to a family of C-type lectin-like receptors with important functions in the immune system, e.g. as pattern recognition receptors on monocytic and dendritic cells.

The first part of this work was devoted to evaluate the expression of CLEC-2 and CLEC-1 in subsets of peripheral blood cells. In monocytes and granulocytes low to moderate levels of mRNA for both receptors were detected, platelets contained mRNA for CLEC-2. T- and B-lymphocytes as well as NK cells did not display significant transcript levels for any of the two receptors. Using flow cytometry, CLEC-2 protein was detected on platelets and on $CD14^+$ monocytic cells. CLEC-2 protein levels on monocytes showed a clear donor-dependent variation, which could potentially indicate different infection states. Whereas platelets readily adhered in adhesion assays to cells expressing the CLEC-2 ligand podoplanin, the level of CLEC-2 expression on monocytes apparently was not sufficient to reveal binding in this cellular assay.

In contrast to CLEC-2 and despite it contains all characteristics of typical lectin-like receptors, CLEC-1 could never be detected on the cell surface although significant mRNA and intracellular protein levels were detected. Neither pro-inflammatory stimuli nor infection with different pathogens induced its transport to the cell surface. Since immunofluorescence staining and cell fractionation experiments revealed a membrane bound intracellular pattern resembling that of the endoplasmic reticulum resident protein Calnexin, it appears that CLEC-1 may have an intracellular localization and function comparable to some of the Toll-like receptors (TLR). Following treatment of dendritic cells with known ligands of intracellular TLR, a general pattern of downregulation of CLEC-1 mRNA could be detected. As these stimuli trigger maturation of dendritic cells, this might indicate a role for CLEC-1 in immature dendritic cells.

In the fourth part of this work, we conducted an extensive comparison of the human and mouse genomic regions encoding the receptors of the myeloid subfamily of the NK receptor complexes. This revealed a large inversion of part of the region during the evolution of the primate lineage and identified two additional lectin-like genes, which we found to be also expressed in cells of the myeloid lineage.

Zusammenfassung

CLEC-1 und CLEC-2 gehören zu einer Familie von C-Typ lektinähnlichen Rezeptoren mit wichtigen Funktionen im natürlichen Immunsystem wie etwa als Muster-erkennungsrezeptoren auf monozytischen oder dendritischen Zellen.

Der erste Teil dieser Arbeit bestand aus einer Analyse der Expression von CLEC-2 und CLEC-1 in Blutzellfraktionen. In Monozyten und Granulozyten konnten für beide Rezeptoren niedrige bis moderate mRNA Mengen nachgewiesen werden, in Blutplättchen wurde CLEC-2 mRNA detektiert. T- und B-Lymphozyten sowie NK Zellen zeigten für beide Rezeptoren keine signifikanten Transkriptmengen. Durch Durchflusszytometrie konnte CLEC-2 Proteinexpression auf Blutplättchen und auf $CD14^+$ monozytischen Zellen nachgewiesen werden. CLEC-2 Proteinmengen auf Monozyten zeigten klare Donorvarianz, was auf verschiedene Infektionszustände hindeuten könnte. Während Blutplättchen in Adhäsions-Assays klar an Zellen adherierten, die den CLEC-2 Liganden Podoplanin exprimierten, war die Höhe der CLEC-2 Expression auf Monozyten nicht ausreichend, um in diesem Versuch Bindung nachweisen zu können.

Im Gegensatz zu CLEC-2 konnte CLEC-1 trotz nachweisbarer mRNA- und Protein-expression nie an der Zelloberfläche detektiert werden, obwohl CLEC-1 alle Merkmale von typischen lektinähnlichen Rezeptoren aufweist. Weder proinflammatorische Stimulation noch Infektion mit Pathogenen induzierten CLEC-1 Transport an die Zelloberfläche. Da Immunofluoreszenzfärbungen und Zellfraktionierungsexperimente ein membrangebundenes, intrazelluläres Expressionsmuster ähnlich Calnexin, einem Protein aus dem Endoplasmatischen Reticulum, zeigten, könnte CLEC-1 intrazellulär lokalisiert sein und eine Funktion ähnlich den Toll-like Rezeptoren (TLR) aufweisen. Stimualtion von dendritischen Zellen mit bekannten Liganden von intrazellulären TLR führte zu einer generellen Herunterregulierung von CLEC-1 mRNA Mengen. Da diese Stimuli die Reifung von dendritischen Zellen induzieren, könnte das auf eine Rolle von CLEC-1 in unreifen dendritischen Zellen hindeuten.

Im vierten Teil dieser Arbeit, führten wir einen eingehenden Vergleich der humanen und murinen genomischen Regionen der myeloiden Familie des NK Rezeptor Komplexes durch. Dieser Vergleich zeigte eine Inversion eines Teiles der Region im Laufe der Evolution der Primatenlinie und identifizierte zwei zusätzliche in myeloiden Zellen exprimierte lektinähnliche Gene.

1) Introduction

1.1) The innate immune system

The immune system has been divided traditionally into innate and adaptive immunity. Compared to the complexity of the adaptive system, innate immunity seemed to be primitive and unsophisticated and therefore received little attention for a very long time. Although it had been discovered already in 1883 by the Russian immunologist Ilya Mechnikov, who detected mobile cells surrounding, engulfing and destroying newly introduced bacteria in starfish, until about 10 years ago scientists concentrated to a large extent on the adaptive or acquired part of the immunity.

However since the discovery of the Toll like receptors (TLR) in the mid 1990s (Nomura, Miyajima et al. 1994; Poltorak, He et al. 1998) it became evident that the innate immune system is of utmost importance providing not only the very first line of defence against a wide variety of challenges but also activating and especially channelling the adaptive immune system towards the appropriate way of defence against the detected pathogen (Medzhitov and Janeway 2000).

As diverse as its functions are the different players of the innate immune system. The very first line of defense is formed by mechanical barriers like the skin or mucus secreted by the respiratory and gastrointestinal tract, second there are chemical compounds and proteins such as β-defensins, lysozyme and the complement system and at last there exist several different types of innate immune cells.

The most important cell types responsible for implementing the functions of the innate immune system are natural killer (NK) cells, mast cells, eosinophils, basophils and the phagocytic cells including monocytes, macrophages, dendritic cells (DC) and neutrophils. These cell types are capable of identifying and eliminating pathogens due to the expression of various pattern recognition receptors, such as the Toll like family, which recognize a wide range of pathogen-associated molecular patterns (PAMP).

1.2) Monocytic and dendritic cells

Both monocytes and dendritic cells belong to the myeloid lineage of blood cells and play crucial roles in the innate immune system as well as in the activation of appropriate adaptive immune functions.

1.2.1) Monoytes

Monocytes have been known for some time to be able to ingest material aiming on the one hand to simply eliminate waste and debris and on the other hand to destroy invading pathogens. Killing of pathogens is triggered by their binding to pattern recognition receptors expressed by the monocyte, causing the engulfment of the pathogen and the generation of the so called respiratory burst, resulting in release of reactive oxygen species (ROS) which destroy the engulfed pathogenic cell (El-Benna, Dang et al. 2005).

However, in recent years it became obvious that the role of monocytes goes far beyond being a simple degradation machinery and that they also play important regulatory and effector roles in both the innate and the adaptive arm of the immune system (Dale, Boxer et al. 2008). Blood monocytes represent the significant amount of approximately 10% of circulating blood leukocytes in humans, they can be recruited to sites of injury or infection within minutes (Auffray, Fogg et al. 2007; Geissmann, Jung et al. 2003), they can take up antigen in peripheral tissues and transport it to the draining lymph nodes, produce cytokines and inflammatory or anti-inflammatory mediators (Sunderkotter, Nikolic et al. 2004) and they can give rise to macrophages and dendritic cells. Circulating monocytes also have been shown to be recruited directly from the blood to the T-cell areas of the lymph nodes during inflammation in the skin. To reach the peripheral lymph nodes (PLN), monocytes cross the membranes of high endothelial venules (HEV), which is the same route as taken by naive lymphocytes and subsets of memory cells. (Palframan, Jung et al. 2001; Geissmann, Auffray et al. 2008).

Functions of monocytes in PLN have been shown to be neutralization of microorganisms (Delemarre, Kors et al. 1990), maintenance of the phenotype of

HEV (Hendriks, Eestermans et al. 1980), production of angiogenic factors (Sunderkotter, Steinbrink et al. 1994) and generation of lymphotropic cytokines and chemokines (Peters, Dupuis et al. 2000). Depending on the cytokine milieu monocytes may also differentiate into macrophages or DC. Exposure to IL-4 and GM-CSF leads to DC differentiation (Sallusto and Lanzavecchia 1994), whereas stimulation with IL-6 and M-CSF causes generation of macrophages (Mitani, Katayama et al. 2000; Geissmann, Auffray et al. 2008).

Considering these various functions it seems to be obvious, that there have to be distinct subsets of mononuclear phagocytes rather than one homogeneous population. In fact there are two main monocytic subtypes in humans, defined by the expression of CD14 and CD16, being the 'classical' $CD14^+CD16^-$ and the 'pro-inflammatory' $CD14^{+/-}CD16^+$ cells (Passlick, Flieger et al. 1989) (CD nomenclature see Appendix C).

$CD16^+$ monocytes are found in larger numbers in the blood of patients with acute inflammation and infectious diseases (Mizuno, Toma et al. 2005; Fingerle, Pforte et al. 1993; Skrzeczynska, Kobylarz et al. 2002). They were reported to produce pro-inflammatory TNF-α in response to LPS stimulation, whereas $CD14^+CD16^-$ monocytes produce anti-inflammatory IL10 (Frankenberger, Sternsdorf et al. 1996; Belge, Dayyani et al. 2002).

Thus instead of being mere precursors for dendritic cells or macrophages, recent data suggest that monocytes themselves are novel candidates even for shaping T-cell responses. LPS-activated monocytes have been shown to lead to differentiation of naive $CD4^+$ T cells into Th17 helper cells, which have been characterized recently as a distinct lineage of pro-inflammatory T helper cells (Evans, Suddason et al. 2007; Wilson, Boniface et al. 2007). Th17 lymphocytes release the potent inflammatory cytokine IL-17 and are therefore involved in various autoimmune diseases including multiple sclerosis and asthma (Matusevicius, Kivisakk et al. 1999; Linden, Hoshino et al. 2000).

1.2.1) Dendritic cells

Dendritic cells (DC) have been first described in 1972 (Steinman and Cohn 1973) and are by now recognized to be the most important antigen presenting cells (APC), which are in their mature form capable of presenting processed antigenic peptides to T- and B-lymphocytes and thereby initiate potent immune responses to clear invading pathogens. Immature dendritic cells on the other hand, induce tolerance when they take up antigens from their environment in the absence of additional inflammatory stimulation (figure 1).

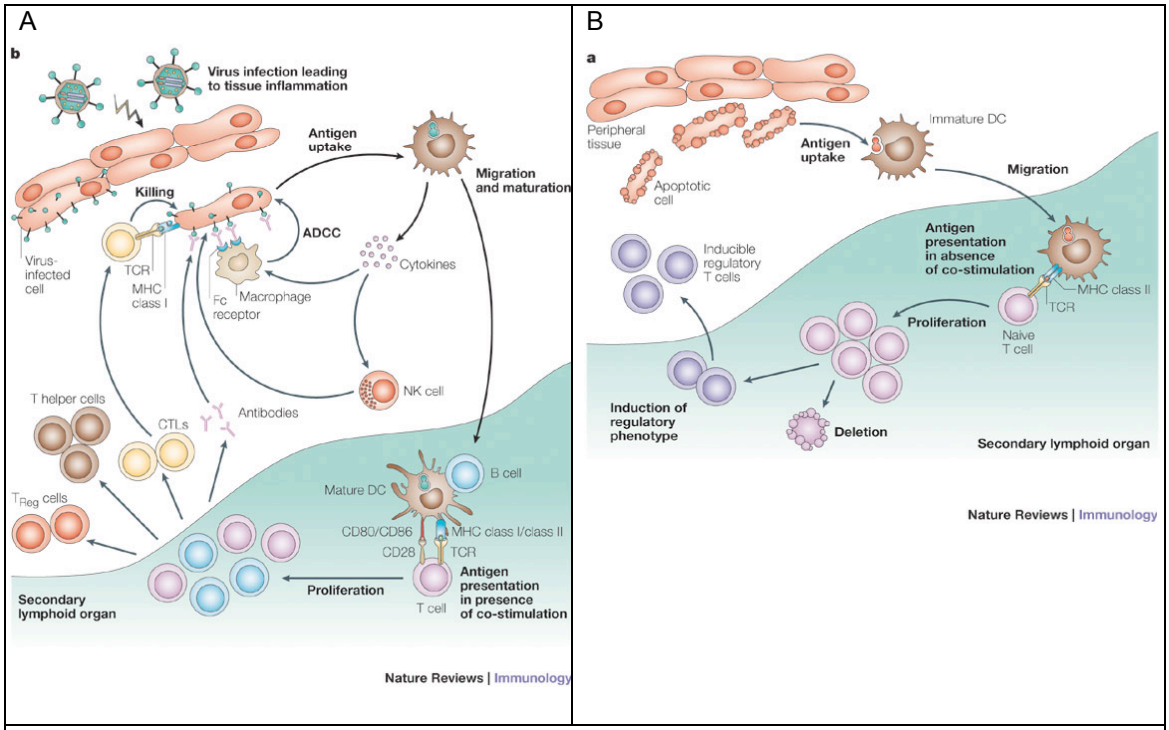


Figure 1: Functions of immature and mature dendritic cells. A. Mature DC induce immunity. Inflammation induces maturation of DC and migration of large numbers of mature DC to draining lymph nodes. Mature DC express co-stimulatory molecules for the appropriate activation of $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-lymphocytes (CTL), B-lymphocytes and thereby initiate an adaptive immune response. $CD4^+CD25^+$ regulatory T-lymphocytes (T_{Reg}) are also expanded. B. Immature tissue DC induce tolerance. In the absence of inflammatory stimuli DC remain immature and antigens are presented to T- lymphocytes in the lymph node without co-stimulation, leading to the deletion of T cells or the generation of regulatory T cells. ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer; TCR, T-cell receptor. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Banchereau and Palucka 2005), copyright (2005)

Dendritic cells can arise from both common myeloid and lymphocyte progenitors derived from $CD34^+$ hematopoietic stem cells (Manz, Traver et al. 2001) and there exist several subsets of dendritic cells defined by marker

expression and function. To date dendritic cells are devided into myeloid/conventional DC, plasmacytoid DC and epidermal Langerhans cells.

In mice myeloid or conventional DC are further divided into two subsets, $CD8^+$- and $CD8^-$-DC, which activate T cells toward Th1 and Th2 differentiation, respectively (Maldonado-Lopez, De Smedt et al. 1999; Vremec, Pooley et al. 2000). They can be found only within lymph nodes, spleen and thymus (Steinman and Cohn 1973). In contrast, human myeloid DC seem to be less heterogeneous and are defined to be $CD11c^+CD14^-CD1a^+$ (Ito, Liu et al. 2005).

Plasmacytoid DC (pDC), which express CD123 as surface marker but are CD11c negative (Ito, Liu et al. 2005), are smaller and have shorter dendrites than conventional DC and resemble lymphocytes when immature. Whereas myeloid DC are very potent in macropinocytosis of antigens from their environment, immature pDC rather produce type 1 interferon (IFN) in response to viral infection (Panoskaltsis, Reid et al. 2004). They reside within lymph nodes, spleen, thymus, and bone marrow (Takeuchi and Furue 2007).

Epidermal resident Langerhans cells (LC) are grouped together with dermal and intestinal dendritic cells. Upon encountering and uptake of invading pathogens, LC migrate into regional lymph nodes to present processed antigens to T lymphocytes (Romani, Holzmann et al. 2003). LC appear to be self-renewing and hardly need to be repopulated by circulating precursors unless their environment is disturbed like in skin damage or inflammation (Merad, Manz et al. 2002). If necessary repopulation is achieved by a subset of blood monocytes ($Gr\text{-}1^{hi}$) which have been identified to be the circulating precursors of LC in vivo. $Gr\text{-}1^{hi}$ monocytes have been shown to migrate to inflamed areas of the epidermis and dermis, where they actively proliferate and subsequently differentiate into either dermal macrophages or epidermal LC (Ginhoux, Tacke et al. 2006). Therefore, apart $CD34^+$ hematopoietic stem cells, monocytes circulating in the peripheral blood provide a further source of DC (Palucka, Taquet et al. 1998; Randolph, Beaulieu et al. 1998) especially the subset of $CD16^+$ monocytes, which seems to be precommitted to differentiate into DC (Randolph, Sanchez-Schmitz et al. 2002). Despite of precommittment, different inflammatory and infectious stimuli such as *ex vivo* applied GM-CSF and IL-4 also drive monocytes to develop into DC (Sallusto and Lanzavecchia 1994).

Considering their function, human mDC and pDC used to be termed DC1 and DC2, because they could be shown to preferentially induce Th1 and Th2 lymphocytes, respectively (Rissoan, Soumelis et al. 1999). However, this view turned out to be too simplified when several studies showed that although there seems to be a tendency of DC subset-specific T-lymphocyte lineage induction, this process is in general highly dependent on the development, maturation or activation conditions applied (Takeuchi and Furue 2007).

1.3) Pattern recognition receptors of the innate immune system

Pattern recognition receptors (PRR) are expressed on innate immune cells, which they render capable to detect and to react to pathogen-associated and danger-associated molecular patterns (PAMP and DAMP). PAMP are invariant pathogen specific structures shared by a class of pathogens and include bacterial carbohydrates such as lipopolysaccharide (LPS) of gram negative bacteria, peptidoglycans and lipotechoic acids from the cell wall of gram positive bacteria, bacterial carbohydrates containing mannose residues, the bacterial amino acid N-formylmethionine, fungal glucans, bacterial flagellin and bacterial and viral nucleic acids. DAMP on the other hand are endogenous molecules named endokines or alarmins which are either secreted actively by immune cells or released passively by dying cells and have activating effects on cells engaged in host defense and tissue repair expressing the corresponding pattern recognition receptor. Innate immune mediators with alarmin function include defensins, eosinophil-derived neurotoxin, cathelicidins and HMGB1 (Oppenheim and Yang 2005).

Pattern recognition receptors can be secreted like the complement activating mannose-binding protein (MBP) (Fraser, Koziel et al. 1998), localized to the cellular cytoplasm like retinoic acid-inducible gene-1 (RIG-1) (Kato, Sato et al. 2005) or membrane associated like the families of Toll like receptors and C-type lectin-like receptors.

1.3.1) C-type lectin-like receptors

C-type lectin-like receptors are a family of type II transmembrane pattern recognition receptors characterized by their extracellular C-type lectin-like domains (CTLD). C-type lectins had been identified as proteins containing Ca^{2+}-dependent carbohydrate-recognition domains (CRD) (Zelensky and Gready 2005; Weis, Taylor et al. 1998). C-type lectin-like receptors on the other hand are not only capable of recognizing carbohydrates but can in fact bind to a wide variety of different ligands and are also involved in other processes like cell-cell contact often even in a Ca^{2+}-independent manner (Weis, Taylor et al. 1998). As shown in figure 2, C-type lectins expressed on dendritic cells contribute to

processes like adhesion to and migration through the endothelial cell layer and T-Lymphocyte activation, and they can function as both soluble and cell surface-bound antigen receptors (Figdor, van Kooyk et al. 2002).

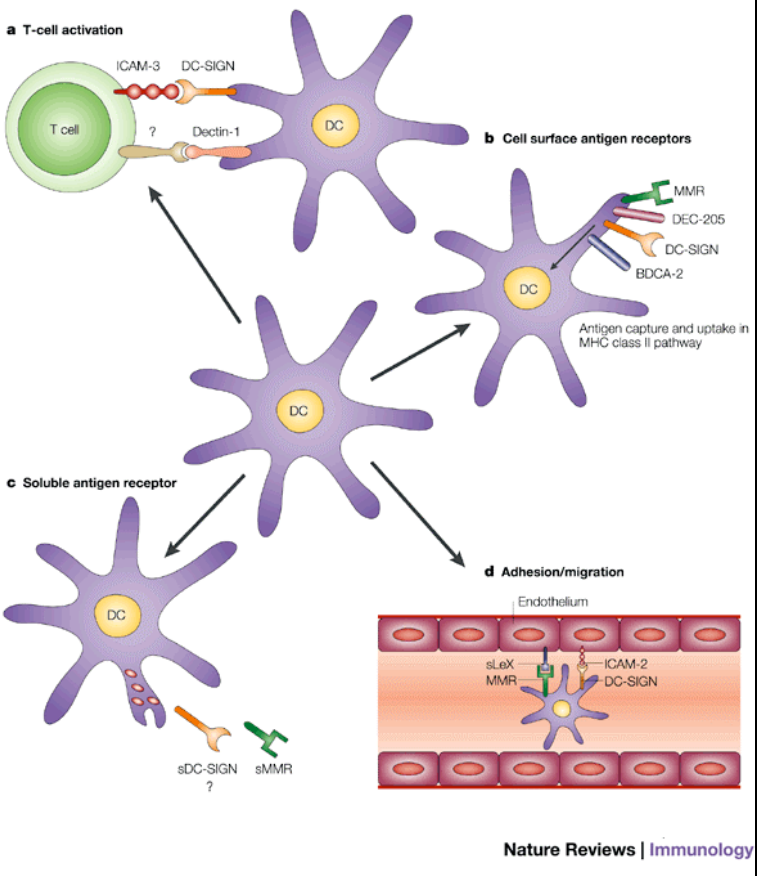


Figure 2: Functions of C-type lectin and C-type lectin-like receptors in dendritic cells. a. DC-SIGN and potentially DECTIN-1 might mediate DC-T-cell interactions. b. Membrane-bound lectins act as endocytic receptors for antigen capture. c. MMR and DC-SIGN may also be secreted as soluble antigen receptors and function as agglutinins and opsonins. d. MMR and DC-SIGN function in DC cell trafficking. BDCA-2 (blood DC antigen 2); DC-SIGN, (dendritic-cell specific ICAM-3 grabbing non-integrin); ICAM, (intercellular adhesion molecule); MHC (major histocompatibility complex); MMR, (macrophage mannose receptor). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Figdor, van Kooyk et al. 2002), copyright (2002)

The common structure shared by all C-type lectins comprises a short cytoplasmic domain involved in downstream signaling, a transmembrane region, a flexible neck domain and the C-type lectin-like domains which contain 6 highly conserved cystein residues and are involved in ligand binding (Zelensky and Gready 2005).

1.3.2) Toll-like receptors

Toll-like receptors (TLR) are a family of type I transmembrane glycoproteins characterized by the extracellular leucine-rich-repeat domain and the cytoplasmatic Toll/interleukin-1 receptor-like (TIR) domain involved in downstream signaling. They are expressed on various immune cells like

dendritic cells, macrophages, B- and some types of T-lymphocytes and comprise one of the most important pattern recognition receptors family of the innate immune system (Miyake 2007). They rapidly sense pathogen invasion and thereby play a major role as the initiator of the innate as well as adaptive immune responses.

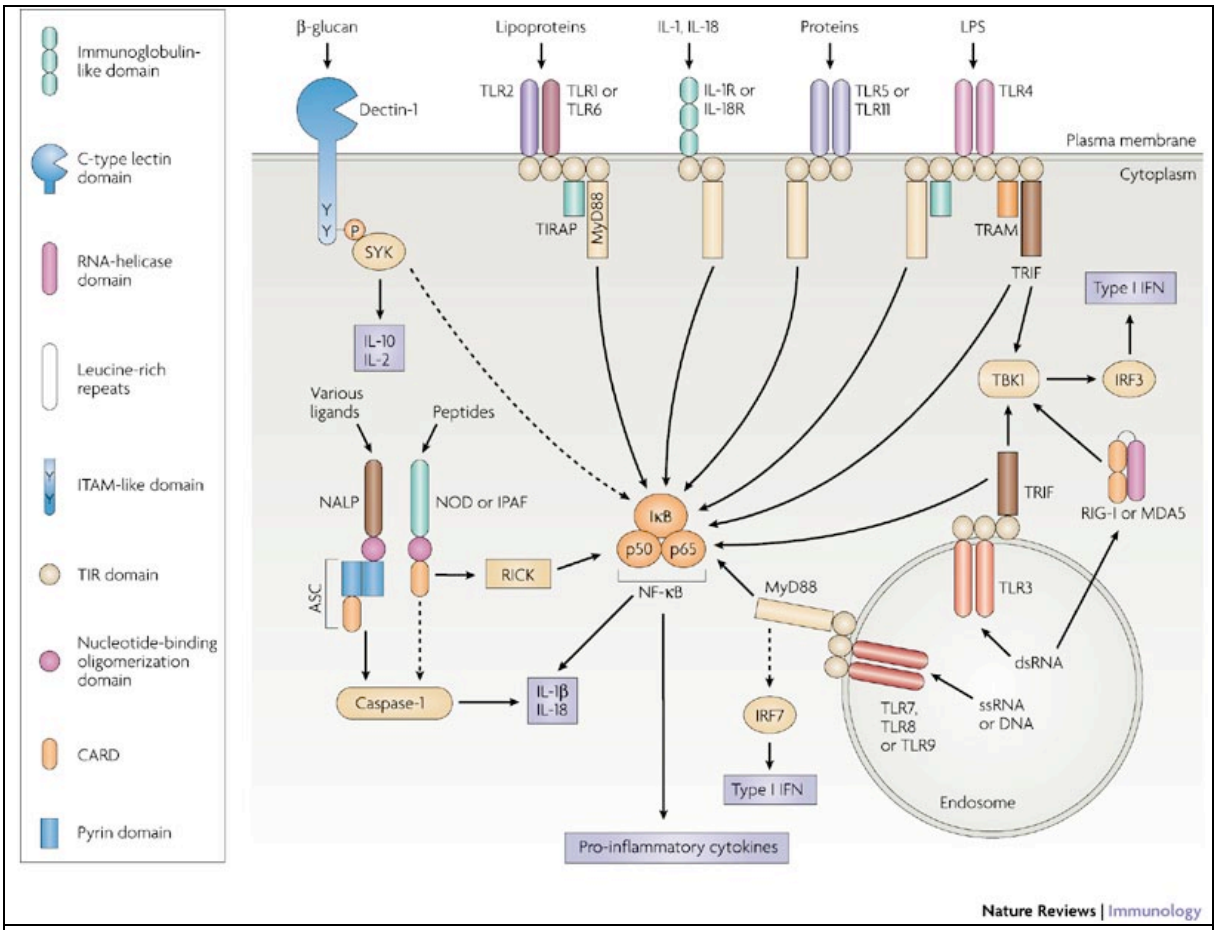


Figure 3: The Toll like receptor signaling pathways and cooperations with other PRR. Overview showing known Toll like receptors as well as other important pattern recognition receptors with their corresponding ligands as well as utilized downstream signaling pathways and outcome of signaling. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, (Trinchieri and Sher 2007; Boothroyd and Dubremetz 2008), copyright (2007)

As shown in figure 3, each TLR specifically binds pathogen associated molecular patterns and thereby activates one of several possible TLR signaling pathways. TLR1, 2, 4, 5 and 6 are expressed on the cell surface and recognize bacterial and fungal cell wall components such as lipoproteins (TLR1, 2 and 6), lipopolysaccharides (TLR4) and flagellin (TLR5). TLR3, 7, 8 and 9 are found mostly within endosomes and recognize microbial nucleic acids such as double-stranded RNA (TLR3), single-stranded RNA (TLR7 and 8) and non-methylated

CpG-motif containing DNA (TLR8). The ligands for TLR10, 12 and 13 still remain unidentified. TLR10 can be found in humans but not in mice, TLR8 is not functional in mice and TLR11, 12 and 13 are expressed in mice but not in humans (Poltorak, He et al. 1998; Aliprantis, Yang et al. 1999; Hemmi, Takeuchi et al. 2000; Alexopoulou, Holt et al. 2001; Hayashi, Smith et al. 2001; Heil, Hemmi et al. 2004).

TLR utilize NFκB-, MAPK-, Myeloid differentiation primary response gene 88 (MyD88)- and TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling pathways, resulting in different cellular responses. For example the intracellular TLR3 couples to the adaptor protein TRIF leading to the activation of TNF Receptor Associated Factor family of proteins-3 (TRAF-3) and Interferon response factor-3 (IRF3) and the secretion of IFN-β, which is required for an effective antiviral response. Other TLR couple to the adapter protein MyD88 (Takeda and Akira 2004; O'Neill 2006) which recruits interleukin-1 receptor associated kinases (IRAK) and TRAF6 causing activation of NF-kB, c-Jun-NH2-kinases (JNK) and p38. The MyD88-coupled TLR signaling results in the synthesis of cytokines such as TNF-α, IL-6 and IL-1 (Kawai and Akira 2005; Akira, Uematsu et al. 2006).

1.4) The human NK receptor complex

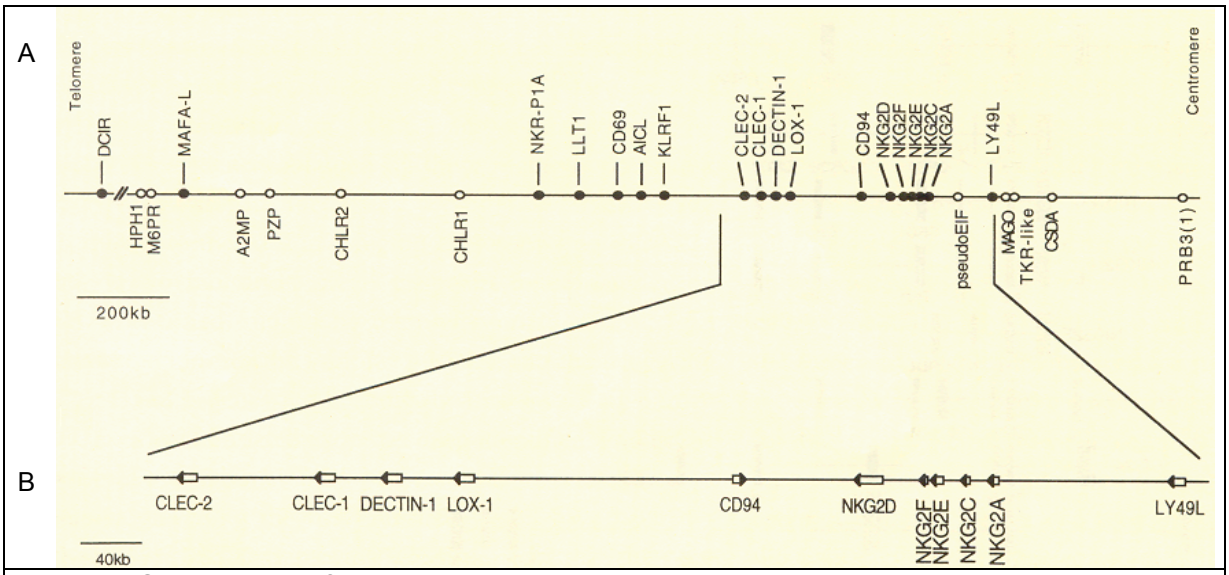


Figure 4: Organization of the human NK receptor complex on chromosome 12. A. Physical map of the 2.5 Mb region extending from the DCIR gene on the telomeric side to the PRB gene cluster on the centromeric side. B. Linkage and orientation of the C-type lectin-like genes in the centromeric part of the NK receptor gene complex (Hofer, Sobanov et al. 2001).

The NK receptor family encoded within the NK gene complex located on the short arm of human chromosome 12 is one highly important family of C-type lectin-like receptors (figure 4). This genomic region has become the focus of intense investigations in recent years because of accumulating evidence supporting an important role of many of the encoded receptors in the immune system (Hofer, Sobanov et al. 2001; Ryan, Naper et al. 2001). Almost all of the proteins encoded in the NK receptor complex belong to the C-type lectin-like receptor family (Schnittger, Hamann et al. 1993; Yabe, McSherry et al. 1993; Lanier, Chang et al. 1994; Chang, Rodriguez et al. 1995), and have been reported to bind to carbohydrates such as β-glucans (Brown and Gordon 2001; Grunebach, Weck et al. 2002) and to various other ligands e.g. oxidised low density lipoprotein (Sawamura, Kume et al. 1997; Sakurai and Sawamura 2003). Although the CTLD of most NK cell receptor proteins do not contain the sequence motifs associated with calcium and sugar binding, carbohydrate binding has been reported for some members of the group. For example, Osteoclast Inhibitory Lectin (OCIL) binds high molecular weight sulphated glycosaminoglycans (Gange, Quinn et al. 2004) and Mast cell Function-

associated Antigen (MAFA) exhibits calcium-dependent binding to terminal mannose (Binsack and Pecht 1997).

According to the expression patterns of the encoded proteins, the centromeric part of the NK receptor complex can be subdivided roughly into two regions. The genes at the centromeric side contain CD94 and the natural killer group 2 (NKG2) gene family expressed mainly on NK and NKT cells (Hofer, Sobanov et al. 2001). Members of this part of the NK cell receptor family have undergone differential expansion in human and mouse. The Ly49 group is present only in mice, whereas the NK receptor protein (NKRP) 1 group, NKG2 proteins and CD94 are present in both species. Inhibitory NK receptors, for instance the NKG2A/CD94 heterodimer inhibit cytolytic activity of NK cell upon recognition of markers of healthy self cells, predominantly major histocompatibility (MHC)-related molecules. Activating receptors like the NKG2C/CD94 heterodimer on the other hand stimulate cytolytic activity upon recognition of infected or transformed cells (Johansson and Hoglund 2006).

A second part of the centromeric region of the NK complex directly telomeric of the CD94 gene codes for proteins predominantly expressed by cells of the myeloid lineage implying common regulatory mechanisms for each cluster. Therefore these clusters will subsequently be referred to as the NK and the myeloid cluster, respectively.

The myeloid cluster spanning from the myeloid inhibitory C-type lectin-like receptor (MICL) to the CD94 gene, has been shown in recent years to code for several lectin-like receptors, namely C-type lectin-like receptor (CLEC)-1, CLEC-2, DECTIN-1 and oxidized low-density lipoprotein receptor-1 (LOX-1) (Sobanov, Bernreiter et al. 2001).

1.4) The myeloid subfamily of the NK receptor complex

LOX-1, DECTIN-1, CLEC-1 and CLEC-2 encoded in the telomeric part of the human NK gene complex form the myeloid subfamily among the NK gene receptors. In contrast to the members of the NK subfamily they are not expressed on NK or NKT cells but show a more diverse expression pattern having been found among others in monocytic, dendritic and endothelial cells (Hofer, Sobanov et al. 2001; Sobanov, Bernreiter et al. 2001). All four receptors are type II transmembrane glycoproteins with a short N-terminal cytoplasmic region, a single transmembrane domain, a stalk and extracellular C-type lectin-like domains. These C-type lectin-like domains are highly conserved at six cysteine residues, which are necessary for forming the lectin-like fold (Shi, Niimi et al. 2001). Furthermore, all four receptors are likely to form homodimers via disulfide bonds within the stalk region (Xie, Matsunaga et al. 2004; Ishigaki, Ohki et al. 2007). Concerning ligands and functions little is known about CLEC-1 whereas in part CLEC-2, but especially LOX-1 and DECTIN-1 have already been studied intensively (Kanazawa 2007).

1.4.1) Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)

LOX-1 was first identified in endothelial cells (Sawamura, Kume et al. 1997) but subsequently it could be shown to be also expressed on macrophages (Yoshida, Kondratenko et al. 1998), smooth muscle cells, monocytes (Draude, Hrboticky et al. 1999) and platelets (Chen, Kakutani et al. 2001). Its structure shows the common C-type lectin-like features and was revealed by crystallization of the CTLD and modeling of the neck domain based on the homology to the myosin heavy chain coiled-coil structure (figure 5A, 5B) (Ohki, Ishigaki et al. 2005). Its expression is regulated by pro-inflammatory stimuli such as tumour necrosis factor-α (TNF-α), phorbol ester (Kume, Murase et al. 1998), transforming growth factor-β (TGF-β) (Minami, Kume et al. 2000), angiotensin II (Li, Zhang et al. 1999) and fluid shear stress (Murase, Kume et al. 1998).

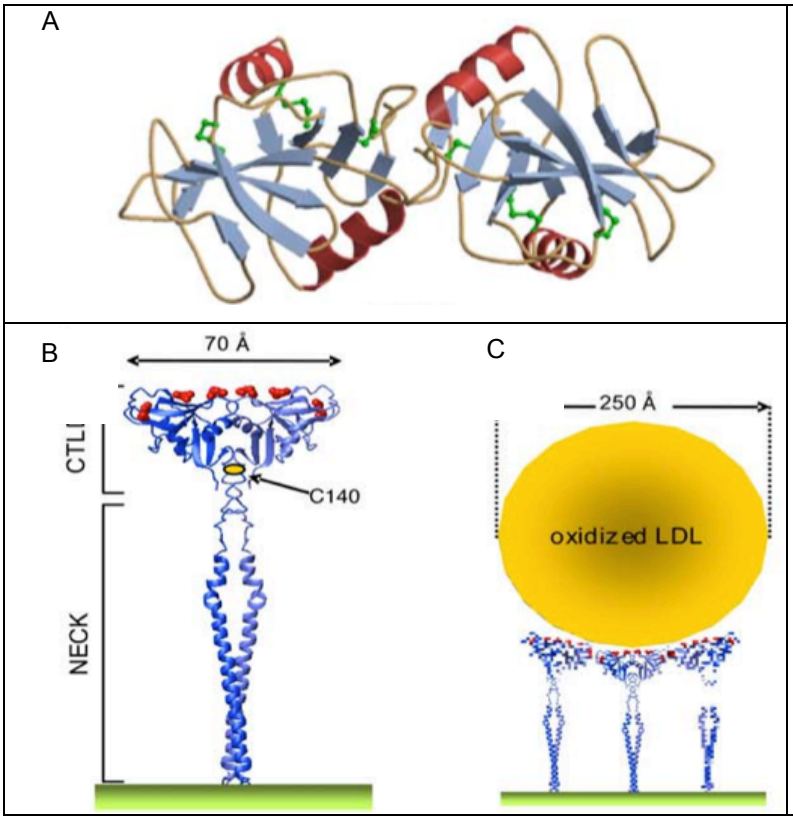


Figure 5: Crystal structure of LOX-1.
A: Ribbon representation of the CTLD dimer structures of LOX-1
B: Whole structure of LOX-1, modeled based on the crystal structure for the CTLD and the predicted model structure for the NECK domain.
C: Comparison between the size of the bound OxLDL particle and three LOX-1 dimers. Reprinted from Structure 13, Ohki, I., T. Ishigaki, et al., "Crystal structure of human lectin-like, oxidized low-density lipoprotein receptor 1 ligand binding domain and its ligand recognition mode to OxLDL.", (6): 905-17, Copyright (2005), with permission from Elsevier.

LOX-1 levels are elevated in early atherosclerotic lesions, suggesting that LOX-1 binding its ligand oxidized low density lipoprotein (oxLDL) is involved in the formation of atherosclerotic plaques (Chen, Kakutani et al. 2000; Mehta, Sanada et al. 2007). Taking into account the diameter of oxLDL (Segrest, Jones et al. 2001) and the fact that LOX-1 had previously been reported to exist as a hexamer on the cell surface (Xie, Matsunaga et al. 2004) the assembled structure of LOX-1 binding to oxLDL might look as depicted in figure 5C. Because of binding to oxLDL, LOX-1 has been postulated to act as scavenger receptor, which are defined by their ability to bind negatively charged molecules (Vohra, Murphy et al. 2006).

The precise epitope on oxLDL recognized by LOX-1 is not known but it is thought to be peptide-based (Moriwaki, Kume et al. 1998). LOX-1 also recognizes other modified lipoprotein particles including hypochlorite-modified high-density lipoprotein (Marsche, Levak-Frank et al. 2001) but not native LDL (Moriwaki, Kume et al. 1998). Furthermore, LOX-1 binds a high diversity of different ligands, being anionic polymers such as polyinosinic acid and carrageenan (Moriwaki, Kume et al. 1998), anionic phospholipids including phosphatidylserine (Murphy, Tacon et al. 2006), apoptotic bodies,

aged cells (Oka, Sawamura et al. 1998), activated platelets (Kakutani, Masaki et al. 2000), AGE (advanced glycation end-products) (Jono, Miyazaki et al. 2002) and both gram-positive and gram-negative bacteria (Shimaoka, Kume et al. 2001).

Ligand binding to LOX-1 elevates reactive oxygen species (ROS) levels rapidly via activation of a membrane-bound nicotinamide adenine dinucleotide phosphate- (NADPH) oxidase (Chen, Xun et al. 2007). ROS then can activate two signal transduction pathways involving either p38 mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K), both causing activation of nuclear factor κB (NF-κB) and subsequent regulation of pro-inflammatory gene expression (Li, Saldeen et al. 2000; Nishimura, Akagi et al. 2004; Chen, Xun et al. 2007; Cominacini, Pasini et al. 2000). LOX-1 activation can also elevate CD40 and CD40L (CD40 ligand) levels. CD40-regulated intracellular signaling is an early immune response event and stimulates cytokine and chemokine secretion (Li, Liu et al. 2003; Dunn, Vohra et al. 2008).

1.4.2) Dendritic cell associated C-type lectin-1 (DECTIN-1)

DECTIN-1 also shares the common C-type lectin-like tertiary structure in its extracellular CTLD (figure 6A) and has been found to be expressed on different myeloid cells such as macrophages and dendritic cells (figure 6B). DECTIN-1 mRNA can be alternatively spliced generating two predominant functional isoforms, one of which lacks the stalk region. Whereas the absence of the stalk does not seem to have significant effects on the ability of DECTIN-1 to recognize its ligand, the two isoforms show slightly different expression patterns (Willment, Gordon et al. 2001; Brown 2006; Heinsbroek, Taylor et al. 2006). Both isoforms, like most other C-type lectins, possess an extracellular carbohydrate-recognition domain (CRD) consisting of three CTLD and a cytoplasmic tail containing an immunoreceptor tyrosine-based activation (ITAM)-like motif (Brown 2006).

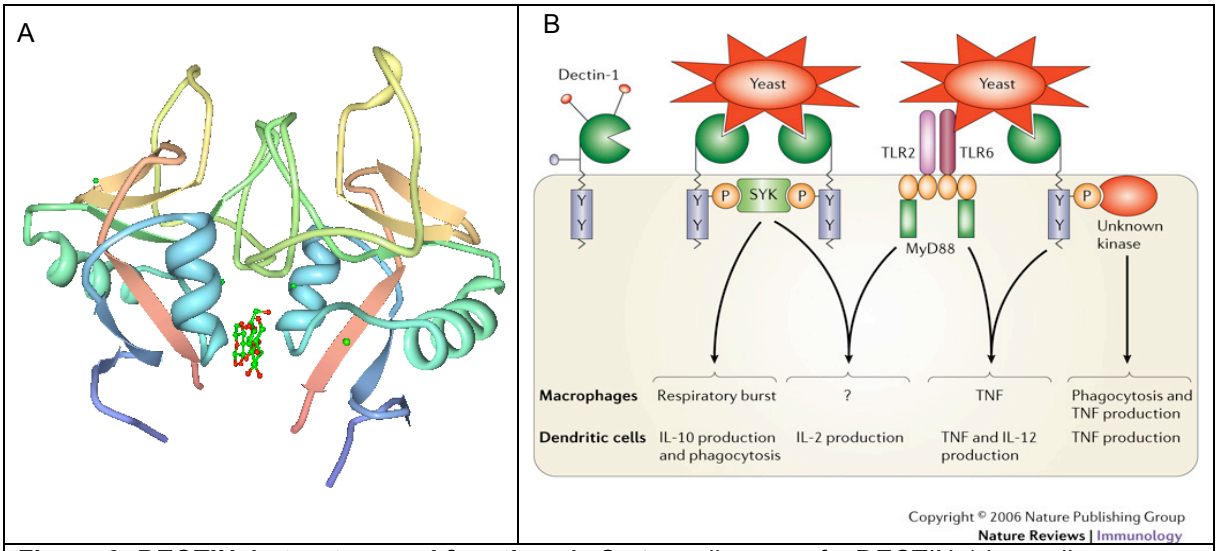


Figure 6: DECTIN-1 structure and function. A: Cartoon diagram of a DECTIN-1 homodimer binding a β-glucan molecule. Monomers are colored from blue at the N-terminus to red at the C-terminus. The β-glucan is shown as green and red balls-and-sticks. Obtained from RCSB Protein Data Bank using entry ID: 2cl8, (Berman, Westbrook et al. 2000), (Brown, O'Callaghan et al. 2007). B: DECTIN-1-mediated signal transduction. On ligand binding, DECTIN-1 becomes tyrosine phosphorylated and induces an intracellular signaling cascade that results in various cell-specific responses. Due to the bridging of two DECTIN-1 molecules, their cytoplasmic tails can interact with spleen tyrosine kinase (SYK). Interactions with SYK can directly induce cellular responses, such as respiratory burst and interleukin-10 (IL-10) production. For the production of IL-2 additional signals from the TLR pathway are required. SYK contributes to phagocytosis in dendritic cells, but it is not required for this process in macrophages. Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Immunol 6(1): 33-43 (Brown 2006), copyright (2006)

DECTIN-1 recognizes β-1,3-glucans on a wide variety of fungal species, including *Saccharomyces*, *Candida*, *Coccidoides*, *Pneumocystis* and *Aspergillus* in a calcium-independent fashion (Brown 2006; Palma, Feizi et al. 2006) inducing a variety of cellular responses. These responses include respiratory burst, activation and regulation of phospholipase A_2 (PLA_2) and cyclooxygenase 2 (COX2), endocytosis and phagocytosis of ligands and the production of cytokines and chemokines, such as TNF-α, macrophage inflammatory protein 2 (MIP-2), IL-2, IL-10, IL-6 and IL-23 (figure 6B) (Brown 2006), (LeibundGut-Landmann, Gross et al. 2007).

DECTIN-1 signaling involves the spleen tyrosine kinase (SYK) leading to downstream signaling through caspase-recruitment domain 9 (CARD9) (Brown 2006). The CARD9 pathway has been reported recently to induce DC maturation and direct T helper 17 (Th17)-cell responses (LeibundGut-Landmann, Gross et al. 2007; Gross, Gewies et al. 2006). This response is independent of Toll like receptors, but there are also other DECTIN-1-mediated

responses like the production of pro-inflammatory cytokines and chemokines which require collaborative signaling from TLR (figure 6B) (Brown 2006).

1.4.3) C-type lectin-like receptor-2 (CLEC-2)

Human CLEC-2 has been identified to be a novel activating receptor that is expressed on the surface of platelets and megakaryocytes (Suzuki-Inoue, Fuller et al. 2006) and responsible for platelet activation by the snake toxin rhodocytin (Suzuki-Inoue, Fuller et al. 2006).

As for other members of the C-type lectin-like family of receptors, its putative carbohydrate-recognition domain lacks the key structural features that confer binding to carbohydrates, suggesting that its endogenous ligand may be a protein. Recently, crystallized CLEC-2 was shown to have a compact C-type lectin-like domain with a flexible loop on its surface which has been suggested to play an important role in ligand binding (Watson, Brown et al. 2007).

Additionally, CLEC-2 has been reported to facilitate the capture of HIV-1 by CLEC-2-transfected HEK293 cells and platelets. However, CLEC-2 does not bind to a viral envelope protein but to a cell surface protein derived from the host HEK293 cell (later identified to be podoplanin) integrated into the viral envelope upon budding (Chaipan, Soilleux et al. 2006). In line with these findings, several studies have identified podoplanin on tumour cells, as an endogenous ligand for CLEC-2 (Suzuki-Inoue, Kato et al. 2007; Christou, Pearce et al. 2008). The interaction between CLEC-2 on platelets and podoplanin on tumour cells has been shown to be essential for the platelet aggregation-inducing activity of tumour cells. Therefore it was proposed that the interaction between podoplanin and CLEC-2 may regulate tumour metastasis and nestling, the former by covering tumour cells with platelets and thereby hiding it from detection by the immune system and the latter by activating platelets to release growth factors important in angiogenesis and tumour growth (Kato, Fujita et al. 2003; Kato, Kaneko et al. 2008).

The CLEC-2 signaling cascade is initiated by a single YXXL (Tyr-Xaa-Xaa-Leu) motif in its cytoplasmic domain (Fuller, Williams et al. 2007). As previously reported for DECTIN-1 (Brown 2006), cross-linking of CLEC-2 induces Src kinase-dependent tyrosine phosphorylation of the YXXL sequence, resulting in

the activation of the tyrosine kinase SYK and initiation of a signaling pathway that culminates in activation of phospholipase Cγ2 (PLCγ2) (Fuller, Williams et al. 2007).

1.4.4) C-type lectin-like receptor-1 (CLEC-1)

CLEC-1 transcripts could be detected in endothelial, dendritic and monocytic cells (Colonna, Samaridis et al. 2000; Sobanov, Bernreiter et al. 2001). Although it shows high similarity in structure and sequence to the other myeloid subfamily members of the NK gene complex, neither ligands nor functions could yet be assigned to CLEC-1. Interestingly, as described in this work CLEC-1 does not show surface expression on any cell type tested, therefore it might be possible that the endogenous location of CLEC-1 is to be found in intracellular membranes rather that on the cell surface, like it is the case for intracellular TLR.

Endothelial cells are known to contain CLEC-1 mRNA, although at first sight it seems gratuitous for this cell type to express a member of a receptor family having primarily immune functions. Nevertheless endothelial cells also express LOX-1, a highly homologous family member of CLEC-1, which is known to be of high importance in the formation of atherosclerositic plaques upon binding to its most prominent ligand oxLDL (Chen, Kakutani et al. 2000; Mehta, Sanada et al. 2007).

Endothelial cells form a thin cell layer at the interior surface of blood vessels, providing an interface between circulating blood and surrounding vessel. Nevertheless, they are much more than mere lining cells having a diverse array of functions in vascular biology and immunology. Endothelial cells are essential in the control of blood pressure, blood clotting and angiogenesis, as well as in immunology e.g. in controlling inflammation in response to histamines or in diapedesis of immune cells (Dejana 2004). Immune cell diapedesis through blood vessel endothelium is an essential process underlying most innate and adaptive immune responses and is defined by leukocyte adhesion to the endothelial cells, squeezing through the space between between two adjacent endothelial cells and infiltration into the underlying tissue (Muller 2003).

Furthermore, endothelial cells have been shown to express Toll like receptors and therefore are able to directly bind and react to pathogenic patterns (Maaser, Heidemann et al. 2004) (Hijiya, Miyake et al. 2002). All these facts clearly show the importance of endothelial cells in immunity and therefore it might indeed be possible that CLEC-1 serves an important function in this cell type.

1.5) Eukaryotic and prokaryotic Pathogens

The main task for the immune system is to detect and destroy invading pathogens of any kind. Cells of the innate immune system such as dendritic cells, monocytes and macrophages sense pathogens via their various pattern recognition receptors leading to a cascade of immune reactions to finally clear the infection.

Pathogens on the other hand have developed a wide range of immune evasion strategies to avoid detection by the immune system. Obligate intracellular pathogens are either able to resist killing inside phagocytic cells or even actively invade non-phagocytic cells. Once having avoided destruction by lysosomal enzymes, the intracellular environment protects the pathogen not only against defence mechanisms of the immune system but also against drugs administered as therapy.

1.5.1) *Toxoplasma gondii*

Toxoplasma gondii is an ubiquitous protozoan parasite that requires an intracellular site for growth and replication and causes toxoplasmosis in animals and humans. Although the definite host of *T. gondii*, where it can conclude the sexual part of its life cycle, are felines, the asexual part of the cycle can take place in any warm-blooded animal as depicted in figure 7A.

The invasive form of the parasite shown in figure 7B is the motile haploid tachyzoite, which is rapidly dividing and can infect a wide range of mammalian host cells including immune and non-immune cells (Kim and Weiss 2004). Inside the cell the parasite propagates by a series of binary fissions, finally causing the infected cell to burst and to release the tachyzoites into the surrounding environment (Dubey 2002). There the haploid tachyzoites reversibly transform into latent bradyzoites to produce tissue cysts mainly in brain and muscle tissue (McCabe and Remington 1988). If tissue cysts are taken up by felines the parasites infect epithelial cells of the small intestine where they undergo sexual reproduction and oocyst formation. Oocysts are then shed with the feces and can be taken up by intermediate hosts again.

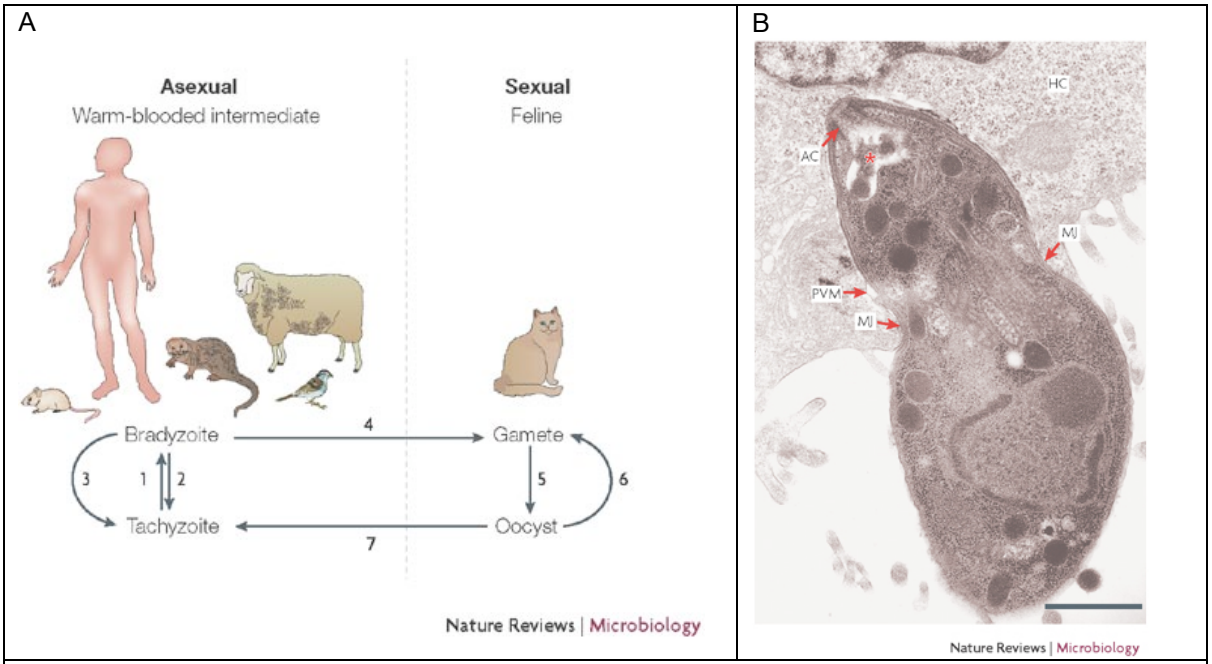


Figure 7: The *Toxoplasma gondii* life cycle. A: Schematic overview showing the asexual and sexual part of the *Toxoplasma gondii* life cycle. *Toxoplasma gondii* is able to infect any warm-blooded animal, were it forms tissue cysts taken up by further hosts with food. If taken up by felines, *Toxoplasma gondii* can complete the sexual part of its life cycle and form oocysts. B: *T. gondii* tachyzoite invading a HeLa cell (HC). AC: apical cytoskeleton, PVM: parasitophorous vacuole membrane, MJ: moving junction, where the invasion is thought to be driven by parasite motors. scale bar represents 0.5 μm. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Boothroyd and Dubremetz 2008), copyright (2008) and from Trends in Microbiology, 6, Jean François Dubremetz, "Host cell invasion by Toxoplasma gondii", 4, Copyright (1998) (Dubremetz 1998), with permission from Elsevier.

The invasive process of the parasite into the host cell involves cellular recognition and adhesion, secretion of penetrating enhancing factors (PEF), conoid penetration and finally the induction of a parasitophorous vacuole, a protective and exchange site. This invasion process is an active, oriented and specific process (Bonhomme, Pingret et al. 1992). The central role of the parasitophorous vacuole is to act as the interface between the parasite and its immediate environment the host cytoplasm, functioning in structural organization, nutrient acquisitions and signaling (Sinai 2008).

1.5.2) *Listeria monocytogenes*

Listeria monocytogenes are gram-positive rod-shaped bacilli and the causative agent of listeriosis, a rare but lethal food-borne infection characterized by meningitis, meningo-encephalitis, materno-fetal and perinatal infections.

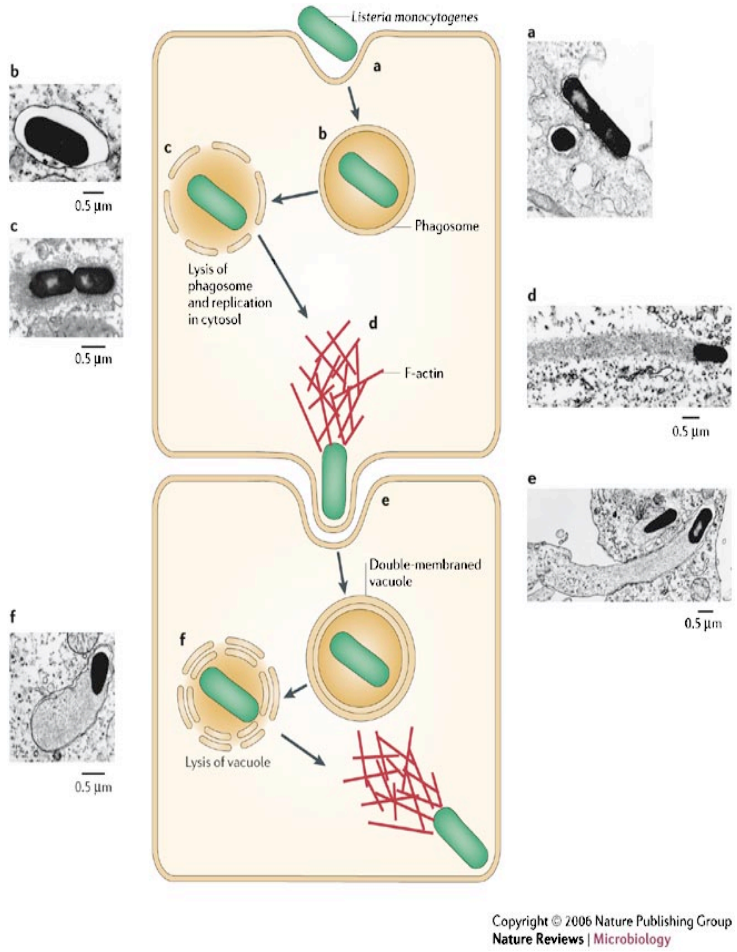


Figure 8: Listeria monocytogenes cell infection process. Electron-micrographs and schematic representation of the successive steps of cell infection by L.monocytogenes. a. L.monocytogenes induces its entry into a cell using InlA, B (InternalinA,B). b. Bacteria are internalized in a phagosome. c, d. membrane of the vacuole is disrupted by secretion of PlcA and PlcB (phosphatidylinositol-specific phospholipase A/B) and the pore-forming toxin LLO (listeriolysin O). Bacteria are released into the cytoplasm, where they multiply and polymerize actin. e. Bacteria enter neighbouring cells by forming protrusions in the plasma membrane. f. On entry into the neighbouring cell, bacteria are present in a double-membraned vacuole. F-actin, filamentous actin. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Boothroyd and Dubremetz 2008) copyright (2006) and (Cossart and Lecuit 1998) copyright (1998) and from Cell, 68/3, C. Kocks et. al., "L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein", 11, Copyright (1998), with permission from Elsevier (Kocks, Gouin et al. 1992).

After crossing the intestinal barrier the bacteria reach the liver via lymph and blood, where they replicate in hepatocytes. They can also cross the blood-brain barrier and the materno-fetal barrier and reach the brain and the placenta, respectively. *Listeria monocytogenes* is able to resist intracellular

killing when phagocytosed by macrophages and to invade many types of cells, which are normally non-phagocytic.

L.monocytogenes enters non-phagocytic cells and can spread from cell to cell (Pizarro-Cerda and Cossart 2006; Cossart and Sansonetti 2004; Cossart, Pizarro-Cerda et al. 2003) by the “zipper mechanism” which involves the progressive interaction of bacterial surface ligands with their respective cellular receptors to form a tight apposition of the plasma membrane around the incoming microbe (figure 8).

After entry into cells, the bacteria are entrapped into a vacuole from which they escape into the cytosol where they replicate and recruit actin. They polymerize actin at one pole of the bacteria and the resulting network of branched filaments is used to propel bacteria to move through the cytoplasm. At the plasma membrane, bacteria push the membrane and induce the formation of protrusions which invade neighboring cells and generate a two membrane vacuole from which bacteria escape, allowing a new cycle of replication to take place in a second infected cell.

The major bacterial genes and factors critical for the cell infection process include internalin A (InlA) and InlB for the entry process (Bierne, Sabet et al. 2007) and the pore-forming toxin LLO for the escape from the vacuole (Hamon, Batsche et al. 2007; Lecuit, Sonnenburg et al. 2007), whereas the actin-based motility is mediated by the surface protein ActA (Gouin, Welch et al. 2005).

1.6) Aims

CLEC-1 and CLEC-2 are C-type lectin-like receptors encoded in the myeloid subfamily region of the human NK gene complex. Like other receptors of this subfamily, they are expressed on cells of the myeloid lineage rather than on NK cells. Considering their homology to two other closely related members of the subfamily, LOX-1 and DECTIN-1, which function as innate pattern recognition receptors, a similarly important function can be predicted for CLEC-1 and CLEC-2.

A general aim of this study was to investigate the distribution of CLEC-1 and CLEC-2 expression in different subtypes of human peripheral blood leukocytes and to find indications about potential functions of both receptors. Furthermore, the genomic regions of the murine and human myeloid subfamilies were investigated in regard to their evolutinary relationship and to identify additional related genes.

Specific aims of this study were:

- to analyse the expression of CLEC-1 and CLEC-2 in subsets of human peripheral blood cells.
- to study possible functional implications of CLEC-1 and CLEC-2 expression.
- to perform a comparative analysis of the murine and human genomic regions containing the myeloid subfamily of the NK gene complex.

2) Materials and methods

2.1) Electrophoresis

Agarose gel electrophoresis

The required volume of the DNA sample was mixed with 6x loading buffer (Fermentas International, Canada) and loaded onto a 1% agarose gel. The DNA was directly stained during separation by adding ethidium bromide to the gel. The separation was done by applying 70V for 30min. As a molecular weight standard marker a GeneRuler 123bp DNA ladder (Fermentas, Germany). Tris-acetate-EDTA (TAE)-buffer (Invitrogen Life Technologies, Austria) was used for the preparation of the gel and as electrophoreses buffer.

SDS polyacrylamide gel electrophoresis

$1x10^5$ Cells were lysed in 10µl 4x sodium dodecyl sulphate (SDS) sample buffer and three times frozen in liquid nitrogen and heated to 95°C.

The samples were then loaded onto the polyacrylamide gel and electrophoresis was carried out in the presence of 1xSDS electrophoresis buffer (Appendix A) at the appropriate current and voltage. As marker a Low Molecular Weight Marker (14,4 – 97kDa) (GE Healthcare Europe, Germany) was used.

2.2) Western blotting

After SDS polyacrylamid gel electrophoresis proteins were transferred to Immobilon polyvinylidene fluoride (PVDF) membranes (Millipore / Biomedica Medizinprodukte, Austria) by semi-dry blotting (PeqLab Biotechnology, Germany) at 350 mA for 1,5 h. The transferred proteins could be visualized by staining the membrane with PonceauS staining solution (SERVA Electrophoreses, Germany).

Membranes were incubated in blocking buffer (Appendix A) for 30min at room temperature. Primary antibodies were diluted in blocking buffer as recommended by the company (see table below) and incubated for 1h at room temperature or over night at 4°C on a shaker. Membranes were then washed

three times for 5 min with 1% Tween in PBS+ (PBS+T). Secondary antibodies labeled with horse radish peroxidase (HRP) were diluted in PBS+T as recommended by the company (see table below) and added to the membranes for at least 1 hour at room temperature followed by washing steps as described above. Afterwards the membranes were incubated with LumiGlo Reagents (Cell Signaling Technology, USA) as recommended in the company's protocol. Signals were detected by exposing a film (Fujifilm, Germany) to the membranes and thereafter the films were developed using the film developer Curix60 (Agfa HealthCare, Austria).

If membranes needed to be stained with another antibody they were incubated for 20min at 50°C in prewarmed stripping buffer (Appendix A). Then the membranes were washed three times for 5 min with PBS+T. Afterwards the antibody staining procedure was carried out as described above.

<table>
<tr><td colspan="3">Primary antibodies used for Western blotting</td></tr>
<tr><td>antibody</td><td>company</td><td>working concentration / dilution</td></tr>
<tr><td>mouse anti-human CLEC-1</td><td rowspan="2">R&D Systems, USA</td><td rowspan="2">0,3µg/ml</td></tr>
<tr><td>mouse anti-human CLEC-2</td></tr>
<tr><td>mouse anti-human COX-1</td><td rowspan="2">kindly provided by Dr. Wolfgang Gregor (Veterinary University Vienna)</td><td rowspan="2">1:3000</td></tr>
<tr><td>mouse anti-human Nucleoporin</td></tr>
<tr><td>rabbit anti-human LAMP-1</td><td>Santa Cruz Biotechnology, USA</td><td>1:500</td></tr>
<tr><td>mouse anti-human Calnexin</td><td>Abcam, USA</td><td>1:4000</td></tr>
<tr><td colspan="3">Secondary antibodies used for Western blotting</td></tr>
<tr><td>sheep anti-mouse Ig, HRP-linked</td><td rowspan="2">GE Healthcare Europe, Germany</td><td rowspan="2">1:6000</td></tr>
<tr><td>sheep anti-rabbit Ig, HRP-linked</td></tr>
</table>

2.3) Cell culture techniques

Maintenance of cells

Human fibroblasts GM5520 used for *Toxoplasma gondii* amplification were grown in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific, USA) with 10% foetal calf serum (FCS, Lonza, Germany), 10 mM L-Glutamine (Sigma-Aldrich, UK) and 5ml/500ml Penicillin/Streptomycin mixture (10.000U/ml Penicillin / 10.000U/ml Streptomycin, Lonza, Germany) in a 5% CO_2 atmosphere at 37°C.

The human breast cancer cell line MCF-7 stably transfected to express podoplanin (kindly provided by Prof. Michael Detmar, ETH Zürich) was grown in DMEM with 10% FCS, 10mM L-Glutamine and 5ml/500ml Penicillin/Streptomycin mixture, 60 μg/ml Hygromycin B (Invitrogen Life Technologies, Austria) and 150 μg/ml G418 (Gibco Invitrogen Corporation, Austria) in a 5% CO_2 atmosphere at 37°C.

Human embryonic kidney (HEK) 293 cells used for adenovirus amplifications were grown in Minimum Essential Medium Alpha (MEM alpha, Gibco Invitrogen Corporation, Austria) supplemented with 10% newborn calf serum (NCS, Fisher Scientific, Austria), 10mM L-Glutamine and 5ml/500ml penicillin/streptomycin mixture in a 5% CO_2 atmosphere at 37°C.

Primary human umbilical vascular endothelial cells (HUVEC) were grown in M199 medium (Lonza, Germany) supplemented with 20% foetal calf serum, 2ml/500ml endothelial cell growth supplement (ECGS, PromoCell/ Biomedica Medizinprodukte, Austria), 2U/ml Heparin (Roche Diagnostics, Germany) und 10ml/500ml PSFG (Penicillin 10.000U/ml, Streptomycin 10mg/ml, Fungizon, 200mmol Glutamin, Lonza, Germany) in a 5% CO_2 atmosphere at 37°C. Human cords were obtained from the Lainzer Hospital (Vienna) and HUVEC were isolated and cultured as described (Sobanov, Bernreiter et al. 2001).

All adherent cells were passaged by treatment with Trypsin (Gibco Invitrogen Corporation, Austria) after washing two times with 1xPBS.

Suspension cell lines used: 721.221, Mono-Mac-6, K-562, Jurkat, U-937, CCRF-CEM, P815, NK-92, RPMI-8866 were all grown in RPMI1640 medium containing 10% FCS, 10mM L-Glutamine and 5ml/500ml penicillin/streptomycin

mixture in a 5% CO_2 atmosphere at 37°C. NK-92 cultures were supplemented in addition with 1mM sodium pyruvate, 50mM β-mercaptoethanol (Sigma-Aldrich, UK) and human rIL-2 (R&D Systems, USA) at a final concentration of 20 IU/ml.

Toxoplasma gondii RH (kindly provided by Prof. John Trowsdale, University of Cambridge) was amplified by infection of the human GM5520 fibroblast cell line. The parasites were isolated from 100% infected GM5520 by scraping the cell monolayer and centrifugation at 500xg for 10min. The resuspended cell pellet was aspirated three times through a 20 g needle and another three times trough an 18 g needle. The suspension was filtered through a 3μm filter (Nuclepore Whatman, UK) and centrifuged again for 10min at 500xg. The pellet containing the isolated parasites was resuspended and used further for infection of cells at a multiplicity of infection (MOI) of 10 and for flow cytometry analysis.

Listeria monocytogenes LLO- (kindly provided by Prof. Thomas Decker, University of Vienna) were grown on BHI (brain heart infusion) agar plates and one colony was used to prepare a 5ml over night culture. Density of bacteria was determined by measuring OD600 and used immediately for FACS analysis or to infect HUVEC at a MOI of 100 for 2h at 37°C.

Isolation of PBMC and CBMC

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque™ PLUS (GE Healthcare Europe, Germany) gradient centrifugation according to the manufacturer's instructions. Venous peripheral blood was obtained from healthy volunteers or purchased from the Red Cross, cord blood was kindly provided by Anita Jandrositz/Prof. Karl-Heinz Preisegger, EccoCell, Austria.
For subsequent assays either whole PBMC or positively isolated subsets ($CD14^+$, $CD3^+$, $CD19^+$) were used as described in 2.3.5.

Isolation of platelets

Whole blood was centrifuged for 15min at 800rpm to separate blood cells from serum. Serum was collected and centrifuged at 3000rpm for 10min to pellet platelets. Platelets were washed two times and processed immediately for flow cytometry staining, RNA isolation or Western blotting.

Isolation of granulocytes

Granulocytes were isolated using the pellet resulting from Ficoll-Paque™ PLUS (GE Healthcare Europe, Germany) gradient centrifugation as described in 2.3.2. After removal of Ficollsupernatant and three times washing of the granulocyte-erythrocyte mixture at 1200rpm for 5min, the pellet was incubated with NH_4Cl buffer (Appendix A) for 10 min at 4°C to lyse erythrocytes. After washing the lyses step was repeated a second time. Granulocytes were processed immediately for RNA isolation, flow cytometry staining or Western blotting or maintained in RPMI1640 medium for a maximum of one day.

Positive isolation of PBMC and CBMC subsets

$CD14^+$, $CD3^+$ and $CD19^+$ subsets of PBMC were isolated using the respective magnetic cell sorting (MACS) positive isolation kits (Milteny Biotech, Germany) according to the manufacturer's instructions and processed immediately for flow cytometry staining, RNA isolation or Western blotting or kept in RPMI1640 medium over night.

$CD34^+$ progenitor cells were isolated from cord blood mononuclear cells using the MACS $CD34^+$ cell isolation kit (Milteny Biotech, Germany) and expanded in RPMI1640 medium supplemented with stem cell factor (SCF, 50ng/ml; ImmunoTools, Germany), FMS-like tyrosine kinase 3-ligand (Flt3-L, 50ng/ml; ImmunoTools, Germany) and Thrombopoietin (TPO, 50ng/ml; Peprotech, UK) for three days.

Differentiation of $CD34^+$ cells to cord blood derived dendritic cells

Cord blood derived dendritic cells were prepared as described (Sobanov, Bernreiter et al. 2001). In short; after expansion for three days as described in 2.3.5, differentiation was induced in the presence of 100ng/ml granulocyte-

macrophage colony-stimulating factor (GM-SCF, Peprotech, UK), 50ng/ml Flt3-L (Peprotech, UK), 2,5ng/ml TNF-α (R&D Systems, USA), 20ng/ml stem cell factor (SCF, Peprotech, UK), 25ng/ml Interleukin-4 (IL-4, Peprotech, UK) for 6 to 9 days. Medium was changed every third day.

Infection of cells with adenoviruses and pathogens

Adherent cells were infected at a MOI of 100 by adding the virus to the usual growth medium and incubating the cells for 48h. For infection of suspension cells virus was pre-incubated in serum free medium with 0,2μM $LnCl_3$ (Palmer, Stoddart et al. 2008) and added to the cells at a MOI of 10.000.

For infection with *Listeria monocytogenes* and *Toxoplasma gondii* HUVEC were seeded the day before infection and incubated with isolated *Listeria monocytogenes* at a MOI of 100 and with isolated *Toxoplasma gondii* at a MOI of 10 for two days.

Stimulation of cells

Cordblood derived dendritic cells (CBDC) and HUVEC were incubated with 1μM CpG-ODN M362 (sequence: 5'-tcg tcg tcg ttc gaa cga cgt tga t-3', from InvivoGen, France), 1μM control-ODN M362 (sequence: 5'- tgc tgc tgc ttg caa gca gct tga t-3', from InvivoGen, France), 25μg/ml poly(I:C) (InvivoGen, France), 10μg/ml Imiquimod (InvivoGen, France), 100ng/ml LPS (Sigma-Aldrich, UK), 10ng/ml TNFα (R&D Systems, USA), 1 μg/ml phorbol myristate acetate (PMA, Sigma-Aldrich, UK) at the respective usual growth conditions for the indicated durations.

CBDC kindly provided by Dr. Frank Kalthoff (Novartis, Austria) were stimulated for maturation with 100 ng/ml LPS (Sigma-Aldrich, UK), 4 μg/ml of anti-CD40 mAb (mAb clone 626.2) cross-linked in solution by the addition of 2 μg/ml of F(ab')2-fragments of goat-anti mouse IgG (Pierce Chemical, USA), 25 μg/ml Zymosan A (Sigma-Aldrich, UK) or 10 ng/ml IFNγ for 6 hours at the usual growth conditions.

Stimulation of the cells was verified by Real-Time RT-PCR showing the upregulation of E-Selectin mRNA in HUVEC and CCL22 (chemokine (C-C motif) ligand 22) mRNA in dendritic cells by Real-Time RT-PCR. After

stimulation and infection the cells were washed thoroughly and processed for flow cytometry analysis, immunofluorescence staining or RNA isolation and subsequent cDNA synthesis for Real-Time RT-PCR.

Adhesion assay

Adhesion assays were performed to show binding of CLEC-2 expressing cells to podoplanin expressing cells. Adherent cells were incubated with DiO-labeled (Molecular Probes, USA) suspension cells on a shaker for 30min (15min horizontal, 15min vertical shaking) at 37°C in a 5% CO_2 atmosphere using the growth medium appropriate for the adherent cells. Cells were washed thoroughly, detached by trypsinization and analysed by flow cytometry. Adherent cells used were MCF-7 with or without podoplanin (kindly provided by Michael Detmar, ETH Zürich), HUVEC and LEC-TERT (TERT (Telomerase Reverse Transcriptase)-immortalized Lymphatic endothelial cells, kindly provided by Heide Niederleithner/Peter Petzelbauer, Medical University Vienna, Austria). Suspension cells used were PBMC, $CD14^+$ monocytes, $CD3^+$ lymphocytes, $CD19^+$ lymphocytes, granulocytes, platelets and K-562 as well as several other suspension cell lines. K-562 infected with AdVCLEC-2 were used as positive control for the adhesion of CLEC-2 expressing cells to podoplanin expressing cells.

Immunofluorescence staining

Endothelial cells were seeded in chamber slides (Lab-Tek, Nunc, Germany) and infected with the respective adenovirus or pathogen as described in 2.5.4 the next day. If applicable cells were stimulated as described in 2.6, washed, fixed for 15 min with 4% paraformaldehyd (Sigma-Aldrich, UK) and optionally permeabilized for 15 min with 0,5% TritonX (Sigma-Aldrich, UK).

Cord blood derived dendritic cells were infected with the respective adenovirus as described in 2.5.4 and stimulated as described in 2.6. After washing cells were put on ice and incubated with 2 µl Fc-blocking solution (Miltenyi Biotech, Germany) per $1x10^6$ cells for 20min. Afterwards cells were attached to adhesion slides (Marienfeld, laboratory glassware, Germany) according to the manufacturer's instructions. Fixation and permeabilization was achieved by incubation with ice cold methanol for 30min. Following fixation and

permeabilization endothelial and dendritic cells were incubated with 5% FCS in PBS for 15min on ice. Primary antibodies and, after two washing steps, secondary antibodies were added to the cells at the concentrations recommended by the manufacturer (see table below) for 30 min at 4°C. To visualize the nuclei, cells were stained in addition with the fluorescent DNA dye 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, UK) for 15 min before mounting the slide.

Results were analyzed in a Nikon Diaphot TMD fluorescence microscope (Nikon, UK).

Primary antibodies used for immunofluorescence staining		
antibody	**company**	**working concentration / dilution**
mouse anti-human CLEC-1	R&D Systems, USA	1µg/ml
mouse anti-human CLEC-2		
mouse anti-human DECTIN-1		
mouse IgG2b isotype control		
mouse anti-human Golgin-97	Molecular probes, USA	1:200
mouse anti-human Calnexin	Abcam, USA	1:100
Secondary antibodies used for immunofluorescence staining		
goat anti-mouse IgG-AlexaFluor 488	Molecular probes, USA	1:500
rabbit anti-mouse IgG-FITC F(ab)2	Dako, Denmark	1:100

2.4) Adenovirus techniques

Generation of primary adenoviruses

cDNA of CLEC-1, CLEC-2 and DECTIN-1 was subcloned into the pAC CMVplpASR(+), shuttle vector (Gomez-Foix, Coats et al. 1992) and the resulting vectors and the adenoviral backbone vector pJM17 (McGrory, Bautista et al. 1988) were co-transfected into HEK293 cells by $Ca_3(PO_4)_2$-precipitation using the Mammalian Transfection Kit (Biomedica, Austria) according to the manufacturer's instructions. After the formation of plaques, adenovirus clones were isolated by a three times freeze-thaw lysing cycle and centrifuging the HEK293 cells as described before (Gomez-Foix, Coats et al. 1992).

pAC-DR1: DECTIN-1 with C-terminal FLAG-Tag between EcoR I and Xba I cutting sites in expression vector pAC CMVpLpA SR(+).

pAC-CR1: CLEC-1 with C-terminal FLAG-Tag between *EcoR I* and *Xba I* cutting sites in expression vector pAC CMVpLpA SR(+).

pAC-CLEC2: CLEC-2 between EcoR I and Xho I cutting sites in expression vector pAC CMVpLpA SR(+).

Selection and amplification of monoclonal adenoviruses

Monoclonal adenoviruses were isolated by infecting HEK293 cells in a 96 well plate with serial dilutions of the primary adenovirus isolate. Wells containing one single plaque after 5-7 days of incubation were selected and the adenovirus clones were isolated by harvesting the cells and lysing them by a three times freeze-thaw cycle in HE-buffer (Appendix A). Monoclonal adenoviruses were amplified by infection and lyses of increasing amounts of cells.

Purification of adenoviruses

A highly concentrated adenovirus suspension, resulting from harvesting the adenoviruses from 40 T175 cell culture flasks of infected cells, was centrifuged two times over a CsCl-gradient. The first centrifugation step was done at 20°C with 48.000 rpm for 1 hour and the second step with 55.000 rpm over night.

The purified virus was collected and dialysed for 4 hours at 4°C to remove the remaining CsCl. After 4 hours the dialysis buffer (Appendix A) was changed and the dialysis was continued over night at 4°C.

2.5) Flow cytometry

Cells were washed and adjusted to $3x10^5$ cells per 50 µl sample and incubated with 2 µl FcR-blocking solution (Miltenyi Biotech, Germany) or 5% FCS for 15 min. Primary antibody was added using the concentration recommended by the manufacturer (see table below) and incubated for 30 min on ice. Samples were washed and incubated for another 30 min with the appropriate secondary antibody at the recommended concentrations (see table below). After a final washing step, cells were fixed and FACS analysis was performed with a BD FACSCalibur.

Binding of flag-tagged soluble CLEC-1 and DECTIN-1 (kindly provided by Mag. Irene Michl, Medical University Vienna, Austria) was tested after blocking by incubation of 2 µg / 50 µl soluble protein for 1,5 hour at 4°C with the cells in question and detection of the bound protein by FITC-labeled anti-FLAG antibody.

Primary antibodies used for flow cytometry		
antibody	**company**	**working concentration / dilution**
mouse anti-human CLEC-1	R&D Systems; USA	1µg/50µl
mouse anti-human CLEC-2		
mouse anti-human DECTIN-1		
mouse IgG2b isotype control		
mouse anti-human CD15 PE-conjugate	Miltenyi Biotech, Germany	0,25µg/50µl
mouse anti-human CD3 APC-conjugate		
mouse anti-human CD14 FITC-conjugate		
mouse anti-human CD11b PE-conjugate		
mouse anti-human CD1a APC-conjugate		
mouse anti-human CD19 PE-conjugate		
mouse IgG isotype control PE-conjugate		
mouse IgG isotype control APC-conjugate		
mouse IgG isotype control FITC-conjugate		
mouse anti-FLAG M2 FITC conjugate	Sigma-Aldrich, UK	0,25µg/50µl
Secondary antibodies used for flow cytometry		
rabbit anti-mouse IgG-FITC F(ab)2	Dako, Denmark	1:100

2.6) Subcellular fractionation

Cells were harvested in Et_3N buffer (Appendix A) with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Germany) and lysed in a Potter homogenizer until cells were broken. The raw homogenate was centrifuged at 2900 rpm for 10 min. Supernatant 1 was centrifuged again at 3500 rpm at 10 min and pellet 2 was added to pellet 1. Both pellets were resuspended in 5 ml 2,2 M sucrose and centrifuged at 24000 rpm for 80 min to purify nuclei from mitochondria and to obtain the final nuclear fraction. Supernatant 2 was centrifuged at 9000 rpm for 15 min. Pellet 3 was washed and centrifuged a second time like before to obtain the final mitochondrial fraction. Supernatant 3 was centrifuged at 13000 rpm for 15 min. The resulting pellet 4 was

resuspended and centrifuged a second time to obtain the final lysosomal fraction. Supernatant 4 was centrifuged at 39000 rpm for 40 min. Supernatant 5 contained the cytosolic fraction and pellet 5 was resuspended and repelletet to obtain the final microsomal fraction.

2.7) RNA techniques and Real-Time RT-PCR

After incubation with the indicated stimulating agents, adherent cells were treated with RNAlater (Ambion, USA), washed once with DEPC-treated water, lysed in 1ml Trizol (Invitrogen, Austria) per $1x10^6$ cells and processed for RNA isolation.

Suspension cells were pelleted and directly lysed in 1ml Trizol per $1x10^6$ cells. RNA was isolated by chloroform extraction and subsequent precipitation using Isopropanol. 2 µg of total RNA were reverse transcribed into cDNA using SuperscriptTM II RT (Invitrogen, Austria) and oligo-dT primers (Applied Biosystems, USA) according to the manufacturer's protocol.

To monitor gene expression Real-Time RT-PCR was performed using a Light Cycler instrument and the Fast Start DNA-Mastermix SybrGreen1 (Roche Diagnostics, Germany) according to established procedures (Brostjan, Bellon et al. 2002). As internal standard for normalization β2-microglobulin or β-actin mRNA was used. Quantification and standardization was performed as described (Brostjan, Bellon et al. 2002). Briefly, linearized plasmids containing the genes of interest were used as standards. A standard dilution series of the plasmids was performed for every gene of interest. Since standard samples were based on pure preparations of plasmid DNA fragments rather than total cDNA mixtures, test dilution series of plasmids in water as well as in PBMC cDNA were performed to verify that the sensitivity of the Real-Time RT-PCR assay was not affected by the presence of unrelated cDNA molecules. The oligonucleotide primers used (obtained from Invitrogen GmbH, Austria) were as follows.

oligonucleotide primer	sequence from 5' end
ß2microglobulin-forward	GATGAGTATGCCTGCCGTGTG
ß2microglobulin-reverse	CAATCCAAATGCGGCATCT
DECTIN-1-forward	ACCATGGGGGTTCTTTCC
DECTIN1-reverse	CCATGGTACCTCAGTCTG
CLEC-1-forward	GGGGGCTTTTGTTTTTTC
CLEC-1-reverse	GCTTTGTTATACAGCTCACG
CLEC-2-forward	GGATTTGGTCTGTCATGC
CLEC-2-reverse	GCAGTACTGCTTACTCTC
Actin-forward	GTGATGGTGGGCATGGGTCA
Actin-reverse	TTAATGTCACGCACGATTTCCC
LOX-1-forward	GCATGCAATTATCCCAGG
LOX-1-reverse	GCTACTCTCTTCAGTGTTT
CLEC9a-forward	TGGAGCATTTGGCACACCAG
CLEC9a-reverse	CAACCCCACCCAGTAATCATAGC
GABARAPL1-forward	TGTCAACAACACCATCCCTCC
GABARAPL1-reverse	CTTCCAACCACTCATTTCCCATAG
CLEC12b-CTLD1-forward	TGAGGAGAAAACCTGGGCTA
CLEC12b-CTLD2-reverse	GCCAGAGGAGTCCCATGATA
CLEC12b-deletion-stalk-forward	TGGGGATGATGTTTTTGCAG
CLEC12b-isertion-CTLD2-reverse	TCCATGGAAAGCTTGTGTTT
CLEC-2-complete-forward	GCAAAGTCATTGAACTCTGAGC
CLEC2-complete-reverse	TCCTGTCCACCTCTTTGCAT
CLEC9a-complete-forward	ATGCACGAGGAAGAAATATACAC
CLEC9a-complete-reverse	TCAGACAGAGGATCTCAACGC
CCL22 s	CGCGTGGTGAAACACTTCTA
CCL22 as	ATAATGGCAGGGAGGTAGGG
E-Selectin LP	CGCTGTAAAATCTTGGCACA
E-Selectin RP	CTGTGGGCATTCAACATCTG
Plasmids used for standardization	**source**
pZErO-DECTIN-1	described previously (Sobanov 2001)
pZErO-CLEC-1	
pZErO-CLEC12b	clone IRAKp961A2448Q2 (RZPD, Germany)
pZErO-FLJ31166	clone HU3_p983D11229D2 (RZPD, Germany)
pZErO-GABARAPL1	clone IRATp970E1244D6 (RZPD, Germany)
pZErO-CLEC-2	PBMC cDNA amplified by RT-PCR using CLEC-2complete-forward, CLEC2-complete-reverse and CLEC9a-complete-forward, CLEC9a-complete-reverse, respectively and cloned into pZErO™-2 (Invitrogen, The Netherlands)
pZErO-CLEC-9a	

2.8) Bioinformatics

Search for additional genes and homologs of known genes

Novel genes were searched for by comparing sequences available from the UCSC Genome Browser (available at: http://genome.ucsc.edu/) and the NCBI (National Center for Biotechnology Information) Map Viewer (available at: www.ncbi.nlm.nih.gov/mapview). The human reference sequence (human assembly may 2004, hg17) is based on NCBI Build 35 and was produced by the International Human Genome Sequencing Consortium. Mouse genome data (mouse assembly march 2005, mm6) was obtained from the build 34 assembly by NCBI. Sequences of other species (Pan troglodytes: panTro2, Rhesus macaques: rheMac2, Bos taurus bosTau4, Canis familiaris: canFam2) were also obtained from UCSC Genome Browser and NCBI Map Viewer. For the search of genes already known in one species (e.g. NKG2i in mice) the NCBI BLAST (blastn) algorithm was used (available at: http://www.ncbi.nlm.nih.gov/BLAST/) to find possibly existing additional mRNA or expressed sequence tag (EST) of a potential homolog of the already known gene.

Accession numbers of the sequences used:

HUMAN: MICL NM_138337 / AY547296, CLEC2 NM_016509 / AY358599, CLEC12B UNQ5782 / AK128243, CLEC9A NM_207345 / AY358265, CLEC1 NM_016511 / BC067746, DECTIN-1 NM_022570 / AF313469, LOX-1 NM_002543 / AB010710, FLJ31166 NM_153022 / AY358845, GABARAPL1 NM_031412 / AF287012, CD94 NM_002262 / AY227806

MOUSE: MICL NM_177686 / AK084335, CLEC12b AK016908, CLEC2 NM_019985 / BC064054, CLEC9a NM_172732 / AK036399, CLEC1 NM_175526 / AK031121, DECTIN-1 NM_020008 / AF262985, LOX-1 NM_138648 / AK154687, “mouse FLJ31166” XM_284236, GABARAPL1 NM_020590 / AK168921, NKG2i NM_153590 / AY100458, CD94 AK136548

CHIMP: MICL XM_520734, CLEC2 XM_520735, CLEC12B XM_520736, (CLEC9A sequence – no accession number), CLEC1 XM_520737, DECTIN-1 XM_528732, LOX-1 XM_528733, “FLJ31166” (incl. GABARAPL1 sequence) XM_520738, CD94 NM_001009062

DOG: MICL XM_534891, CLEC12b XM_849067, CLEC2 XM_543823, CLEC9a XM_849058, CLEC1 XM_543822, DECTIN-1 XM_849050, LOX-1 XM_543821, "FLJ31166" M_849040, GABARAPL1 XM_848051 / XM_861857, CD94 XM_849017

Sequence alignments and detection of homologies

For alignments of shorter DNA and protein sequences the MacVector7.0 software was used. For bigger alignments and alignments that should make genomic rearrangements detectable the Shuffle LAGAN tool (available at: http://lagan.stanford.edu/lagan_web/index.shtml) was used.

Homologies of large genomic sequences were detected and plotted by the mVista Browser (available at: http://genome.lbl.gov/vista/index.shtml) using the AVID algorithm. For the detection of homologies between multiple short DNA and protein sequences the ClustalW algorithm of the MacVector7.0 software or the BLAST 2 SEQUENCES Version of the NCBI BLAST algorithm was used.

For interspecies detection of conserved regions the ECR-Browser was used (available at: http://ecrbrowser.dcode.org/).

Investigation of exon-intron structure, protein domains

The exon-intron structure of the additional proteins was determined by aligning their mRNA sequences to the corresponding genomic region. When available the aligned sequences were obtained from the UCSC Genome Browser, otherwise alignments were produced using the MacVector7.0 software. All exon-intron boundaries were checked for the proper GT-AG boundary consensus sequence.

To predict the protein domains of the additional proteins the EMBL SMART algorithm set to standard mode (available at: http://smart.embl-heidelberg.de/ (Schultz, Milpetz et al. 1998; Letunic, Copley et al. 2006)) was used. The predicted domains were then matched to the already determined exons of the gene.

The SMART server provides the integrated output of several programs; the TMHMM2 (available at: http://www.cbs.dtu.dk/services/TMHMM/) program for prediction of transmembrane segments, the Coils2 program (available at: http://www.ch.embnet.org/software/COILS_form.html) for coiled coil regions, the

SignalP program (available at: http://www.cbs.dtu.dk/services/SignalP/) for Signal peptides and DisEMBL (available at: http://dis.embl.de/) for disordered regions (Linding, Jensen et al. 2003), (Bendtsen, Nielsen et al. 2004), (Nielsen, Engelbrecht et al. 1997). These programs were used for more detailed protein domain analysis.

Construction of phylogenetic trees

Because the C-terminal part of the lectin-like genes is highly variable and could bias the resulting phylogenetic tree only the CTLD sequences were used. The phylogenetic tree was constructed using default parameters of the ClustalW tool of the EBI (available at http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Wilbur and Lipman 1983), (Myers and Miller 1988).

Scanning of UTR sequences

The investigation of the human CLEC9A UTR was performed using UTRScan, UTRdb and UTRblast (all available at: http://bighost.area.ba.cnr.it/BIG/UTRHome/).

Structure Modeling

Structure of not crystallized proteins was predicted based on the homology to a template sequence using SWISS MODEL WorkSPACE (available at: http:// swissmodel.expasy.org/workspace). Crystalized structure of dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, 1sl6A) was used as a template for the CLEC-1 structure model.

3) Results

3.1) Expression of myeloid subfamily members in peripheral blood cells and endothelial cells

To provide a comprehensive overview of the expression of CLEC-1, CLEC-2 and DECTIN-1 in different subsets of human peripheral blood cells and endothelial cells, we investigated the corresponding mRNA expression and the surface localization of these receptors.

3.1.1) Expression of CLEC-1, CLEC-2 and DECTIN-1 mRNA in peripheral blood cells

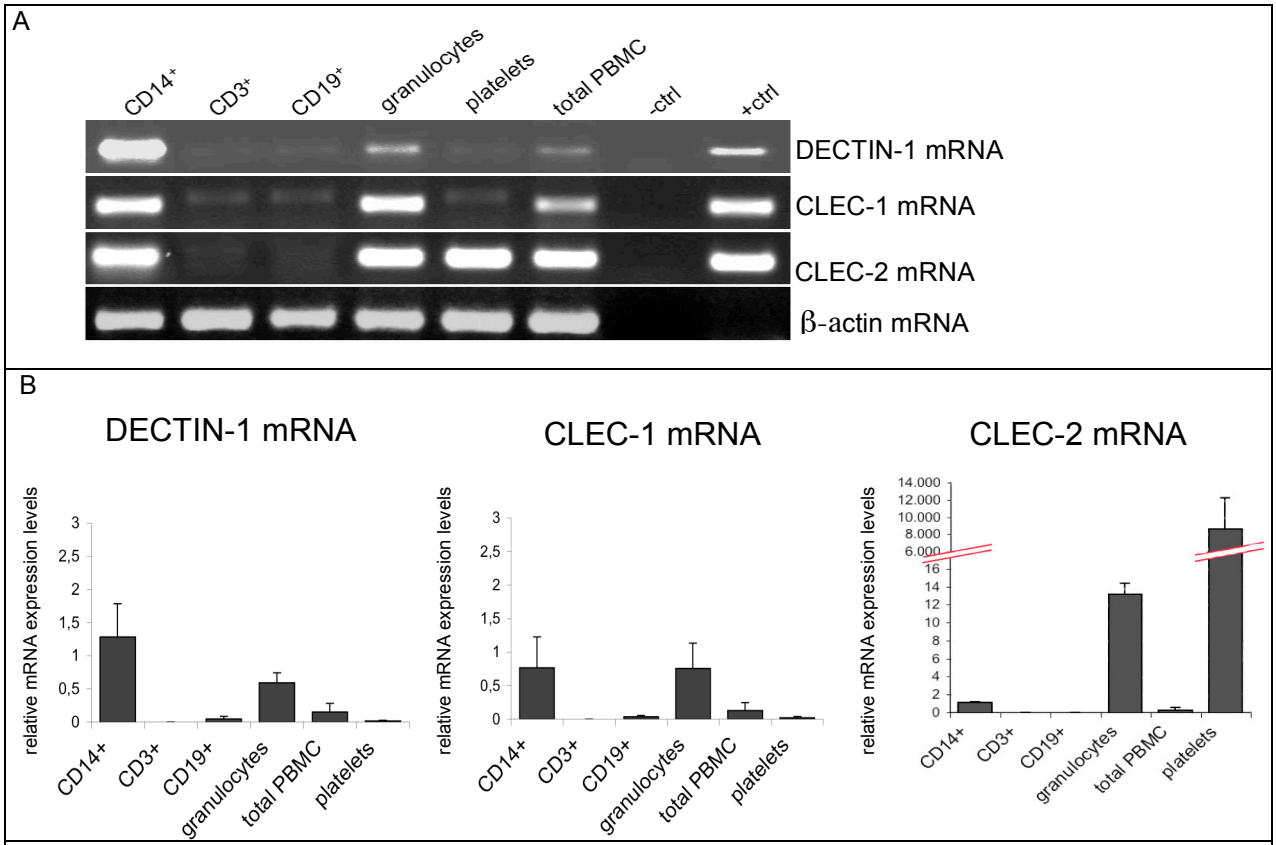


Figure 9: CLEC-1, CLEC-2 and DECTIN-1 mRNA expression in blood cell subsets. Real-Time RT-PCR was performed using cDNAs obtained from peripheral blood cell subsets as templates. β-actin was used as internal control for normalization. Plasmids containing the corresponding cDNA were used as positive control (+ctrl), plasmids with unrelated cDNA served as negative control (-ctrl). Purity of cell fractions was controlled by FACS analysis using antibodies against appropriate cell marker molecules (see 3.1.2). A: 1% agarose gel showing the end products of a representative Real-Time RT-PCR experiment. B: Relative expression levels of CLEC-1, CLEC-2 and DECTIN-1 mRNA in blood cell subsets of one representative individual. Measurements were done in triplicates. The relative strength of expression was determined by normalizing the CLEC-1, CLEC-2 and DECTIN-1 mRNA levels to the corresponding β-actin mRNA levels.

To examine CLEC-1, CLEC-2 and DECTIN-1 mRNA levels, granulocytes, platelets, $CD14^+$ monocytes, $CD19^+$ B-lymphocytes and $CD3^+$ T-lymphocytes were isolated from PBMC of healthy volunteers by MACS and processed for RNA isolation and cDNA synthesis. cDNA was then used for Real-Time RT-PCR and the reaction end products were loaded on an agarose gel. Normalization of Real-Time RT-PCR data was carried out against β-actin as reference transcript, assuming that β-actin mRNA is present in similar amounts in the different cell types including platelets (Akbiyik, Ray et al. 2004).

The data displayed the presence of mRNA for all three receptors in the whole PBMC population, in $CD14^+$ monocytic cells as well as in granulocytes (figure 9A). CLEC-2 mRNA was further present in platelets, whereas no transcripts for any of the receptors were detected in the $CD3^+$ and $CD19^+$ lymphocytic fractions.

As shown in figure 9B, CLEC-2 mRNA levels as determined by quantitative Real-Time RT-PCR normalized to β-actin are significantly higher in granulocytes than in monocytes, and in platelets CLEC-2 mRNA even showed an about 1.000-fold higher level than in granulocytes.

For DECTIN-1 mRNA levels were highest in monocytes, whereas CLEC-1 mRNA levels seem to be similar for monocytes and granulocytes. As expected, due to the fact that PBMC consist of different cell populations, mRNA levels for all three receptors are lower in the total PBMC population as compared to some of the the isolated cell types. Significant expression in lymphocytic populations could not be detected (figure 9B).

3.1.2) Surface expression of CLEC-1, CLEC-2 and DECTIN-1 in peripheral blood cells

To investigate whether all cell types expressing mRNA for CLEC-1, CLEC-2 and DECTIN-1 would produce the protein and display the receptors on the cell surface, cells were stained with the respective antibodies and analysed by flow cytometry. The purity of the individual cell populations was confirmed using antibodies against the surface marker CD151 (platelets), CD15 (granulocytes), CD14 (monocytes) or CD3 (lymphocytes). As C-type lectin-like receptors of the NK gene complex are type II transmembrane proteins and are usually transported to the cell surface, non-permeabilized cells were stained. As in the case for the mRNA, surface staining for CLEC-1 and CLEC-2 was studied in comparison to DECTIN-1, for which coherent data were already available (Kanazawa 2007).

As shown in figure 10, CLEC-2 could be detected on the surface of platelets and monocytes, but not on granulocytes, whereas DECTIN-1 was strongly expressed on monocytes and at lower levels on granulocytes. CLEC-1 was not detectable on the cell surface of any of the cell fractions tested. None of the receptors could be shown in the lymphocytic fraction.

In regard of DECTIN-1 this data are consistent with the detected mRNA expression in monocytes and granulocytes. However, CLEC-1 could not be detected on the cell surface of any of the cell types, despite CLEC-1 mRNA was clearly identified at low but significant levels in monocytes as well as in granulocytes. Further, CLEC-2 transcripts can be found in platelets, monocytes and granulocytes, whereas flow cytometry detected CLEC-2 surface localization only for platelets and monocytes, but not for granulocytes. Compared to the high level of CLEC-2 mRNA in platelets, the surface staining of platelets appears to be only moderate (figure 10).

Therefore, it seems that despite the presence of CLEC-1 and CLEC-2 mRNA in certain cell types the corresponding receptor is not expressed on the cell surface.

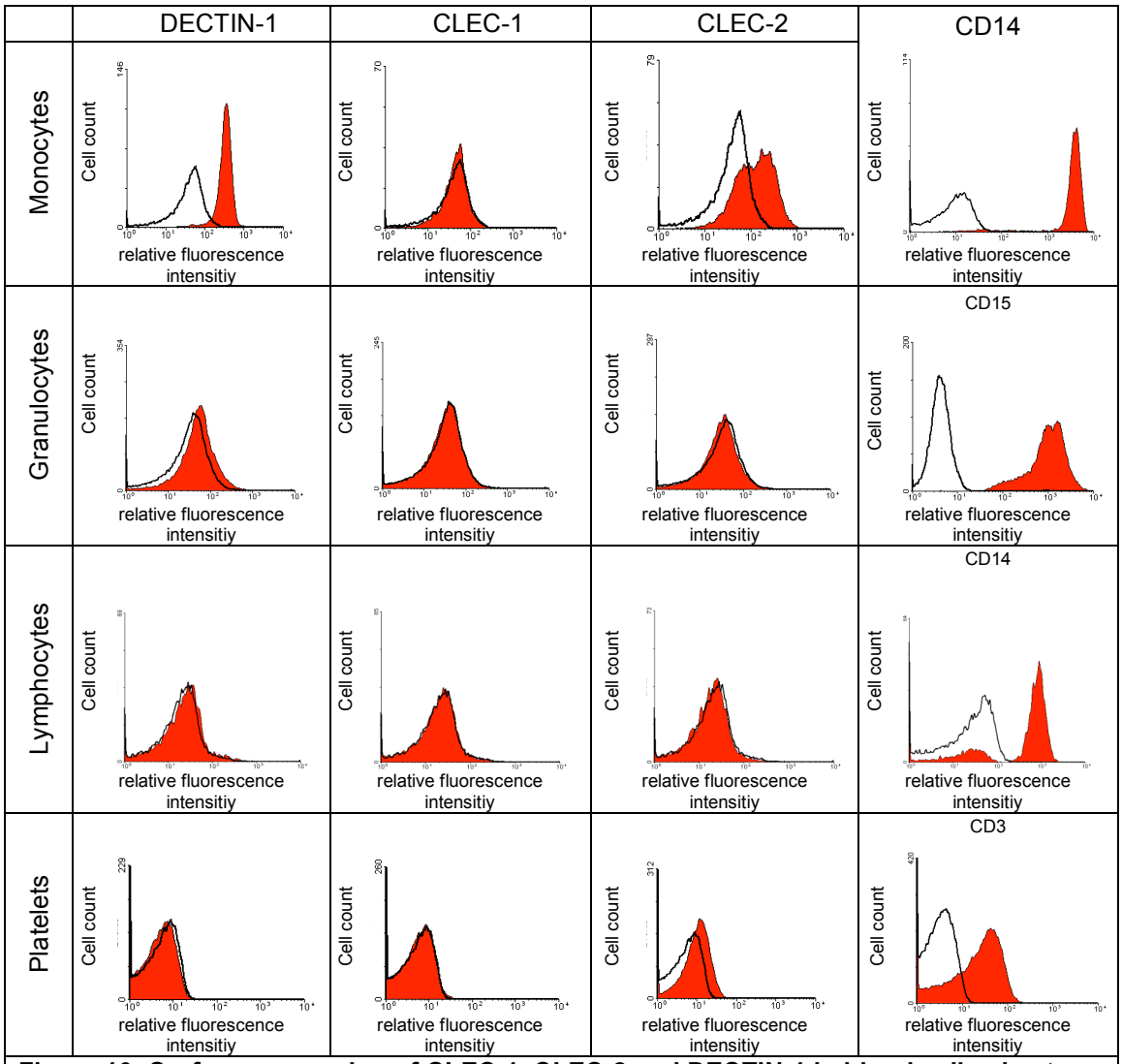


Figure 10: Surface expression of CLEC-1, CLEC-2 and DECTIN-1 in blood cell subsets. Surface expression of endogenous CLEC-1, CLEC-2 and DECTIN-1 in platelets, granulocytes, monocytes and lymphocytes was tested by flow cytometry after staining with the appropriate primary (mouse anti-human CLEC-1, CLEC-2 or DECTIN-1) antibody and a secondary FITC-labeled anti-mouse antibody. Platelets and granulocytes were isolated from whole peripheral blood, monocytes and lymphocytes are populations gated from stained total PBMC samples as depicted in figure 13. Unfilled, black peak corresponds to isotype control staining. Stainings of cells of one individual representative for high CLEC-2 expressing donors are shown.

In addition to the primary cell types isolated from blood, the lymphocytic cell lines Jurkat (acute T-lymphocyte leukemia cell line), 721.221 (EBV transformed B-lymphoblastoid cell line), NK-92 (NK lymphoma cell line) and the myeloid cell lines Mono-Mac-6 (acute monocytic leukemia cell line) and K-562 (human chronic myeloid leukemia cell line) were tested for surface expression of CLEC-1, CLEC-2 and DECTIN-1. None of the cell types tested showed expression of CLEC-2 or CLEC-1, only K-562 stained positive for DECTIN-1 surface expression (figure 11).

-

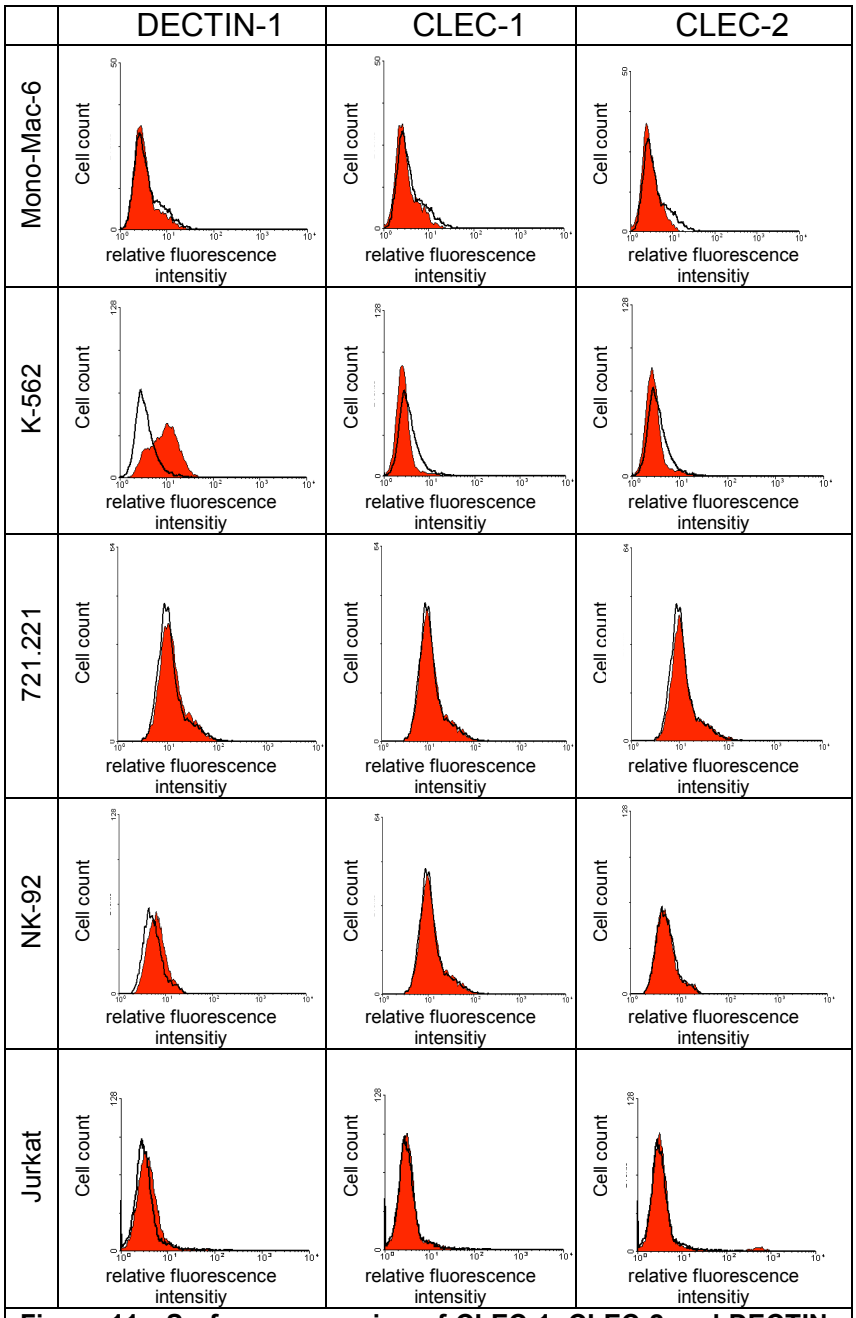


Figure 11: Surface expression of CLEC-1, CLEC-2 and DECTIN-1 on leukemia cell lines. Expression of endogenous CLEC-1, CLEC-2 and DECTIN-1 was investigated by flow cytometry after staining with the appropriate primary (mouse anti-human CLEC-1/CLEC-2/DECTIN-1) antibody and a secondary FITC-labeled anti-mouse antibody. Unfilled, black peak corresponds to isotype control staining. One exemplary experiment out of three performed is shown.

3.1.3) Expression of the receptors in endothelial cells

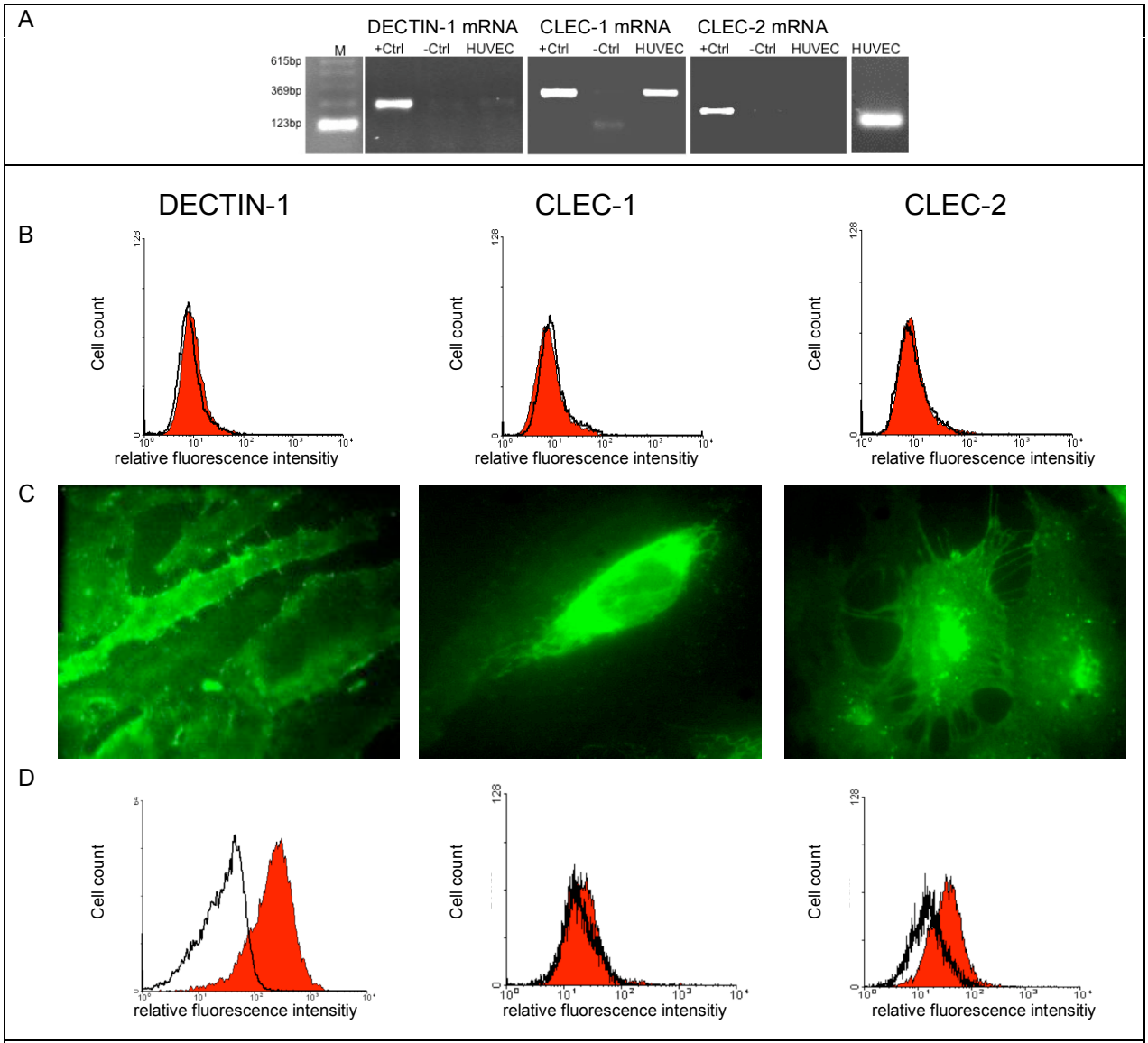


Figure 12: Expression of endogenous and recombinant CLEC-1, CLEC-2 and DECTIN-1 receptors in HUVEC. The presence of endogenous CLEC-1, CLEC-2 and DECTIN-1 mRNA was tested by RT-PCR and a potential surface expression for CLEC-1 evaluated by flow cytometry. The intracellular localization of the receptors following recombinant expression of CLEC-1, CLEC-2 and DECTIN-1 in HUVEC was investigated by immunofluorescence staining and flow cytometry. One representative experiment is shown. A: 1% agarose gel showing PCR products corresponding to CLEC-1, CLEC-2 and DECTIN-1 mRNA in cDNA preparations from HUVEC. Plasmids containing the appropriate or unrelated cDNA were used as positive or negative control, respectively. B: FACS analysis of non-permeabilized HUVEC stained with mouse anti-human CLEC-1, CLEC-2 or DECTIN-1 antibodies and secondary anti-mouse FITC labeled antibodies. (red peak: mouse anti-human CLEC-1, CLEC-2 or DECTIN-1 antibodies, black peak: antibody isotype control) C: Immunofluorescence staining of permeabilized HUVEC infected with the recombinant adenoviruses AdVCLEC-1, AdVCLEC-2 or AdVDECTIN-1. Cells were stained with the corresponding mouse anti-human CLEC-1, CLEC-2 or DECTIN-1 antibodies and secondary anti-mouse FITC labeled antibodies. Magnification used: 1000x. D: FACS analysis of non-permeabilized, adenovirus-infected HUVEC. HUVEC infected with AdVCLEC-2 were stained with mouse anti-human CLEC-2 and secondary anti-mouse FITC labeled antibody. HUVEC infected with AdVCLEC-1 or AdVDECTIN-1 were stained with anti-FLAG FITC labeled antibody (red peak: AdVCLEC-1, DECTIN-1, black peak: AdVCtrl).

Human umbilical vein endothelial cells (HUVEC) had been shown previously to contain detectable amounts of CLEC-1 mRNA (Sobanov, Bernreiter et al. 2001), but not CLEC-2 and DECTIN-1 mRNA. Consistently, mRNA for CLEC-1 could be detected in HUVEC (figure 12A). However, when HUVEC were tested for surface expression of CLEC-1, no staining on the cell surface was detectable (figure 12B).

As the expression levels of endogenous CLEC-1 and CLEC-2 in HUVEC and monocytes are quite low, the detection of the endogenous receptors with the available antibodies by flow cytometry and immunofluorescence staining frequently proved difficult. Therefore recombinant adenoviruses for the expression of the receptors were generated to facilitate studies of intracellular localization and of ligand binding properties. Following infection with the respective recombinant adenoviruses, cells were either permeabilized and processed for immunofluorescence to test for detectable protein inside the cells, or not permeabilized and analysed by flow cytometry to investigate surface localisation of the receptors. Whereas DECTIN-1 and CLEC-2 showed clear surface expression in immunofluorescence as well as in flow cytometry, recombinant CLEC-1 was not exposed at detectable levels at the cellular surface although there was strong intracellular expression ((figure 12C, 12D). It seems therefore that not only endogenous CLEC-1, but also overexpressed recombinant CLEC-1 in HUVEC is not transported to the cell surface and CLEC-1 may have a normal intracellular localization (see below in chapter 3.3) Intracellular localization of CLEC-1).

3.2) Expression of CLEC-2 on monocytes

While this work was in progress, CLEC-2 expressed on the surface of platelets has been demonstrated to be the physiological ligand of podoplanin, a highly glycosylated cell surface protein (Suzuki-Inoue, Kato et al. 2007). As we found, in addition to expression on platelets, significant CLEC-2 receptor expression also on primary blood $CD14^+$ monocytic cells, we investigated the potential significance of CLEC-2 for monocytes. This appeared to be of interest, as CLEC-2 might mediate binding of monocytes to cell types with high podoplanin expression such as lymphatic endothelial cells or tumor cells.

3.2.1) $CD14^+$ monocytes express CLEC-2 in varying amounts

To investigate CLEC-2 expression on monocytes in more detail, whole PBMC isolated from peripheral blood of healthy adult volunteers and CBMC from cord blood (kindly provided by Anita Jandrositz/Karl-Heinz Preisegger, EccoCell Biotechnology, Austria) were tested for CLEC-2 staining in comparison to CLEC-1 and DECTIN-1 staining by flow cytometry. Both CLEC-2 and DECTIN-1 could be shown to be expressed on the gated monocytic subpopulation of PBMC as well as of CBMC. Positive CLEC-1 staining could never be detected despite the presence of the corresponding mRNA (see figure 12A).

DECTIN-1 staining defined a homogeneous DECTIN-1 positive cell population, which corresponds clearly to the $CD14^+$ monocytic subpoplation in PBMC and CBMC. Further, DECTIN-1 seems to be equally strong expressed in PBMC and CBMC (figure 13) and always showed similar strength of expression in samples from different donors (data not shown).

CLEC-2 antibodies on the other hand only stained a subpopulation of monocytic cells, suggesting that there might be two subpopulations of monocytes, one expressing CLEC-2 and the other lacking CLEC-2 expression (figure 13).

It is intriguing, that CLEC-2 staining of monocytes seemed to be generally weaker on CBMC samples and showed clear variation in the level of expression when different donors are compared (figure 13, 14).

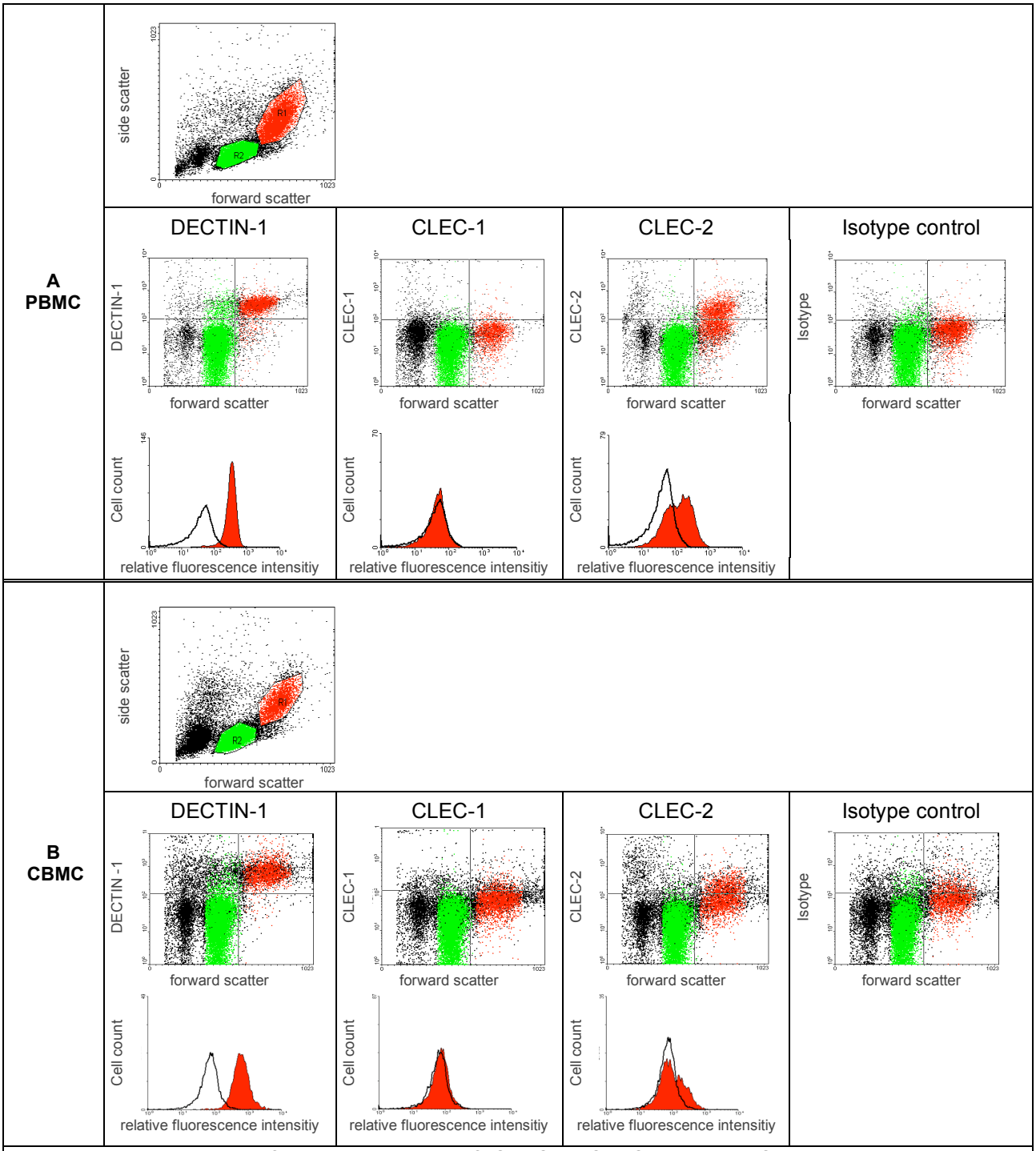


Figure 13: Evaluation of the expression of CLEC-1, CLEC-2 and DECTIN-1 receptors on the monocytic subpopulation of PBMC and CBMC. PBMC were isolated from peripheral blood, CBMC from cord blood and stained using primary mouse anti-human CLEC-1, CLEC-2 or DECTIN-1 antibodies and a secondary FITC-labeled anti-mouse antibody. FITC-labeled cells in the total PBMC or CBMC population are shown. The lymphocytic subpopulation is displayed in green (gate R1 in forward versus side scatter dot plot), the monocytic subpopulation in red (gate R2 in forward versus side scatter dot plot). Quadrants are drawn to delineate the staining of the isotype control antibody. Histograms show the expression of CLEC-1, CLEC-2 and DECTIN-1 on the gated monocytic subpopulation. Red peak: anti- CLEC-1, CLEC-2 or DECTIN-1 staining, black peak: isotype control staining. One PBMC sample representative for donors with strong CLEC-2 expression (out of 10 samples) and one representative CBMC sample (out of 6 samples) is shown. A: PBMC B: CBMC

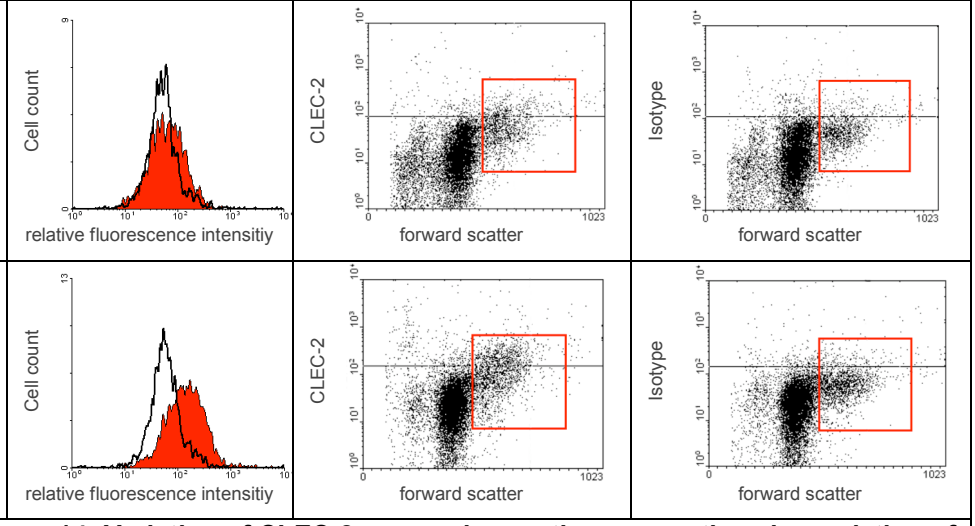


Figure 14: Variation of CLEC-2 expression on the monocytic subpopulation of PBMC. PBMC were isolated from peripheral blood and stained with a primary mouse anti-human CLEC-2 antibody and a secondary FITC-labeled anti-mouse antibody. Expression of CLEC-2 on the monocytic subpopulation of two different PBMC samples, one representative for strong (10 samples) and one representative for weak (6 samples) CLEC-2 expressing donors. Histograms show the gated monocytic fractions corresponding to the populations boxed in red squares in the dot plots. Red peak: anti-CLEC-2 staining, black peak: isotype control staining. Dot plots show FITC-labeled cells in total PBMC, the monocytic subpopulation displayed in in red boxes. The horizontal lines are drawn to delineate the area of cells incubated with the isotype control antibody.

In total PBMC from 18 and CBMC from 11 donors were isolated and investigated for CLEC-2 expression on monocytes. In PBMC more than half of the donors showed high (>20% CLEC-2 positive cells) and about one third low (<20% CLEC-2 positive cells) expression of CLEC-2, about 10% of all samples tested were negative (table 1). For CBMC, none of the donors expressed CLEC-2 strongly and only about 50% of all samples were weakly positive for CLEC-2 expression.

PBMC			CBMC		
> 20% CLEC-2$^+$	**< 20% CLEC-2$^+$**	**negative**	**> 20% CLEC-2$^+$**	**< 20% CLEC-2$^+$**	**negative**
10 (**56%**)	6 (**34%**)	2 (**11%**)	0 (**0%**)	6 (**55%**)	5 (**45%**)

Table1: Statistics of CLEC-2 expression on PBMC and CBMC. All PBMC and CBMC samples tested were counted and classified according to the level of CLEC-2 expression in three groups. High: >20% CLEC-2$^+$, low: <20% CLEC-2$^+$ and negative samples.

We found varying CLEC-2 levels not only when samplesobtained from different donors where tested, but also when we collected blood from one single donor at different times. PBMC of one donor were investigated for expression of CLEC-2 and DECTIN-1 at four different times (about 2 months apart) showing that CLEC-2 expression levels varied significantly. The highest expression levels were found the second time and third time we tested this donor (figure 15). The strength of DECTIN-1 expression on the other hand was equally strong at every time point tested.

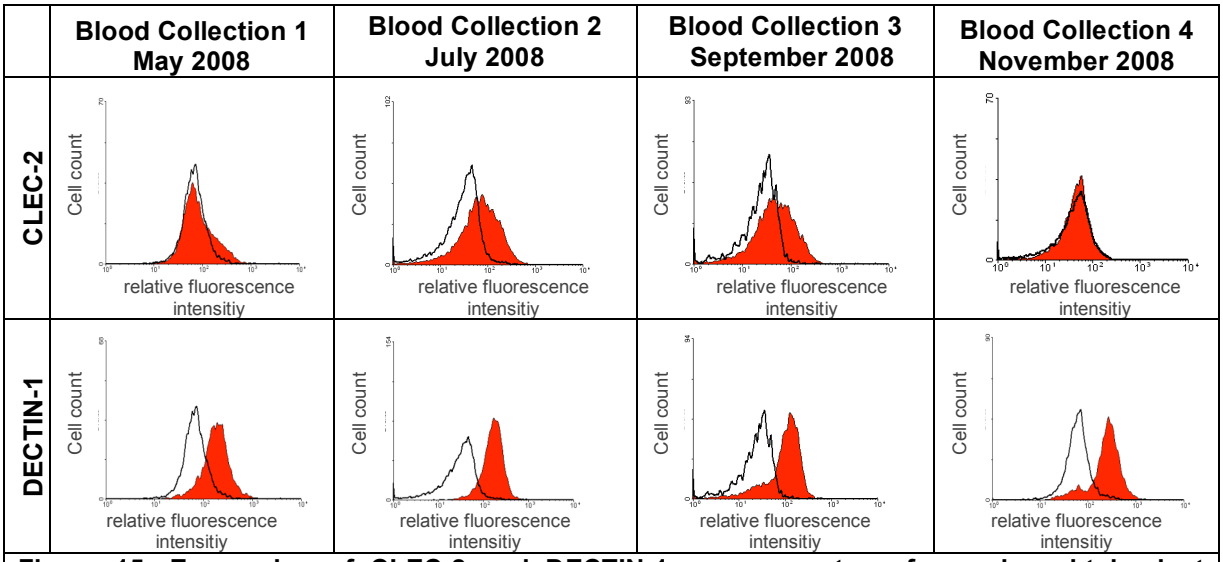


Figure 15: Expression of CLEC-2 and DECTIN-1 on monocytes of samples obtained at different time points from one donor. PBMC were isolated from peripheral blood and stained with primary antibodies against CLEC-2 and DECTIN-1 (mouse anti-human CLEC-2 or DECTIN-1) and a secondary FITC-labeled anti-mouse antibody. CLEC-2 and DECTIN-1 staining on the gated monocytic fraction (gating see figure 13) of PBMC obtained at four different times are shown. Red peaks correspond to anti-CLEC-2 or anti-DECTIN-1 staining, black peaks correspond to staining with iisotype control.

3.2.2) CLEC-2 protein expression in peripheral blood cell subsets

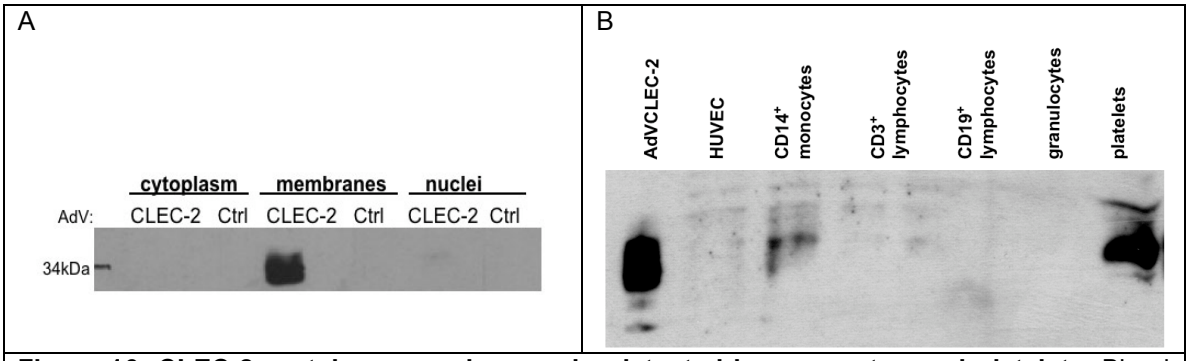


Figure 16: CLEC-2 protein expression can be detected in monocytes and platelets. Blood cell subsets were isolated and fractionated into membrane, cytoplasmic and nucleic fractions. Fractions were processed for Western Blotting. CLEC-2 protein was detected using a primary mouse anti-human CLEC-2 and a secondary HRP-labeled anti-mouse antibody. A: cytoplasmic, membrane and nucleic fraction of AdVCLEC-2 infected HUVEC used as positive control. B: membrane fractions of blood cell subtypes.

To further confirm data about CLEC-2 expression on subsets of peripheral blood cells obtained by flow cytometry, we performed Western Blot analysis. Taking into account previous findings by FACS and RT-PCR, we could expect only low protein levels in monocytic cells. Therefore we isolated the membrane fractions of the various cell types to obtain samples enriched for CLEC-2 expression. Western blot analysis of endothelial cells overexpressing CLEC-2 following infection with recombiant adenoviruses confirmed that CLEC-2 can be detected exclusively in the membrane fraction (figure 16A).

In accordance with the FACS data, the Western blot clearly displays the presence of CLEC-2 in the membrane fraction of $CD14^+$ monocytes (figure 16B). As expected, high amounts of CLEC-2 protein was also detected in membranes of platelets (Suzuki-Inoue, Fuller et al. 2006), whereas endothelial cells as well as $CD3^+$ and $CD19^+$ lymphocytic cells did not show detectable CLEC-2 protein expression, which is in line with the absence of corresponding mRNAs in these cell types. However, membrane fractions of granulocytes did not contain detectable amounts of CLEC-2 protein, despite CLEC-2 mRNA was clearly detectable in granulocytes (see figure 9A), suggesting a translational control level for CLEC-2 protein production.

3.2.3) Evaluation of a potential role of monocytic CLEC-2 in cell-cell adhesion

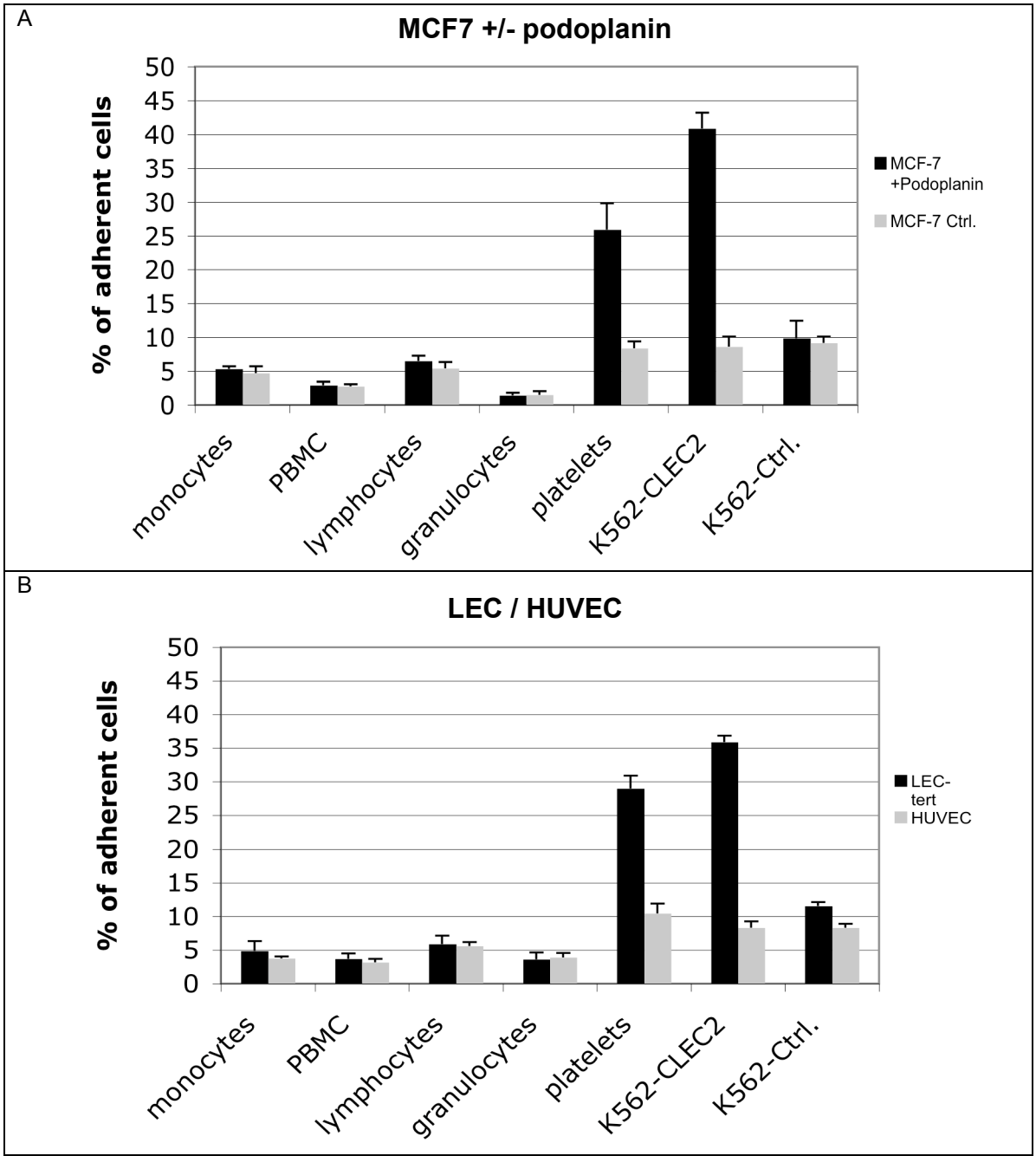


Figure 17: Adhesion of blood cell subpopulations to podoplanin expressing cells. Blood cell subtypes were isolated from peripheral blood, DiO-labeled and incubated with a monolayer of podoplanin expressing cells on a shaker. MCF-7 stably transfected with podoplanin or lymphatic endothelial cells expressing podoplanin in combination with AdVCLEC-2-infected K-562 were used as positive control, MCF-7 or HUVEC without podoplanin expression in combination with AdVCtrl-infected K-562 were used as negative controls. The percentages of labeled cells retained on the cellular monolayers were calculated from flow cytometry data obtained after washing and trypsinization of the cells. Graphs correspond to one of two experiments perfomed in triplicates. Incubation was done with various DiO-labeled blood cell subpopulations as indicated. A: monolayer cells are MCF-7 with or without podoplanin. B: monolayer cells are immortalized lymphatic endothelial cells (LEC-tert) or HUVEC.

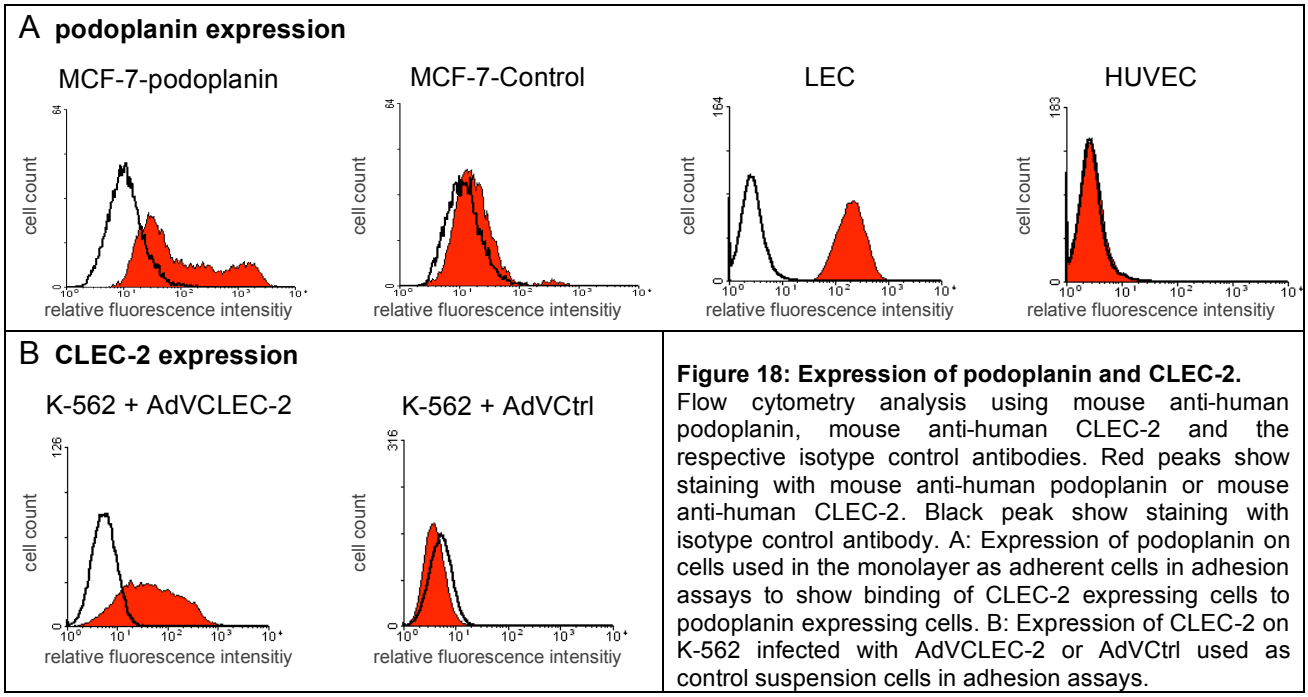


Figure 18: Expression of podoplanin and CLEC-2. Flow cytometry analysis using mouse anti-human podoplanin, mouse anti-human CLEC-2 and the respective isotype control antibodies. Red peaks show staining with mouse anti-human podoplanin or mouse anti-human CLEC-2. Black peak show staining with isotype control antibody. A: Expression of podoplanin on cells used in the monolayer as adherent cells in adhesion assays to show binding of CLEC-2 expressing cells to podoplanin expressing cells. B: Expression of CLEC-2 on K-562 infected with AdVCLEC-2 or AdVCtrl used as control suspension cells in adhesion assays.

Podoplanin has been reported to be the physiological ligand of CLEC-2 (Kato, Kaneko et al. 2008), therefore it was of interest to investigate, whether CLEC-2 expression on monocytes would mediate monocyte binding to podoplanin expressing cells. Adhesion assays were performed by incubating labeled suspension cells with CLEC-2 expression with adherent monolayer cells expressing podoplanin (figure 18) for 30min on a shaking platform. Afterwards cells in suspension were washed away and the bound suspension cells were harvested together with the monolayer cells by trypsinization and counted by flow cytometry.

K-562 infected with AdVCLEC-2 used as positive control adhered strongly to podoplanin-transfected MCF-7 cells as well as to lymphatic endothelial cells expressing podoplanin, but did not adhere to control MCF-7 and HUVEC. As expected platelets also adhered strongly to both podoplanin$^+$ MCF-7 and LEC, but not to the podoplanin$^-$ cells.

Unfortunately, despite expression of moderate levels of CLEC-2 on monocytes there was no significant adherence of monocytes to podoplanin expressing cells as compared to podoplanin negative cells (figure 17) suggesting that the CLEC-2 expression levels on monocytes do not suffice to reveal binding in the employed assay.

Further, we could not detect any specific cell adhesion by granulocytes and lymphocytes, which is in line with the findings that lymphocytes and granulocytes do not express CLEC-2 on their surface (figure 17).

3.3) Intracellular localization of CLEC-1

CLEC-1 is the least investigated receptor of the myeloid subfamily of the human NK gene complex and only little information is available about this protein. It has been reported by our group that CLEC-1 mRNA is found in endothelial and dendritic cells, but information about its ligands and functions are not available so far, although the gene encoding CLEC-1 has been identified some years ago (Sobanov et al., 2001, Colonna, Samaridis et al. 2000). Considering the importance of the other family members in the natural immune system, it is likely that CLEC-1 has a similar important function.

3.3.1) CLEC-1 shows all characteristics of C-type lectin-like receptors

CLEC-1 is encoded in the NK gene complex together with several other C-type lectin-like receptors. It is flanked by its closest homologs CLEC-2 (telomeric) and DECTIN-1 (centromeric). Despite high homology is observed between CLEC-1 and these genes (Sobanov, Bernreiter et al. 2001), it does not seem to share the important attribute of surface expression with its other family members (see firgure 21). To investigate whether this striking difference could be explained by variations of the DNA and protein sequence, an analysis of the CLEC-1 sequence by bioinformatical approaches was conducted.

By comparing the genomic region encoding human CLEC-1 with sequences available from chimpanzee, rhesus monkey, cow, mouse, rat and dog, we found the human and the two primate species tested (chimpanzee, rhesus monkey) to be almost identical. This high homology spreads over the whole genomic CLEC-1 sequence including exons as well as intronic areas. Further, there are still regions with the notable homology of about 50 to 70% when the human sequence is compared to the sequences of the non-primate species (cow, mouse, rat, dog). However, in non-primate species, significant homology is basically limited to protein coding exon regions. There are also some short but highly conserved areas in non-coding regions, which might show the existence of important regulatory elements (figure 19A).

An alignment of the human CLEC-1 and CLEC-2 protein sequences shows a high degree of sequence identity for 73 out of 280 amino acids (26%) and in addition 21% similar amino acids (figure 19A). The transmembrane regions display even 48% identity.

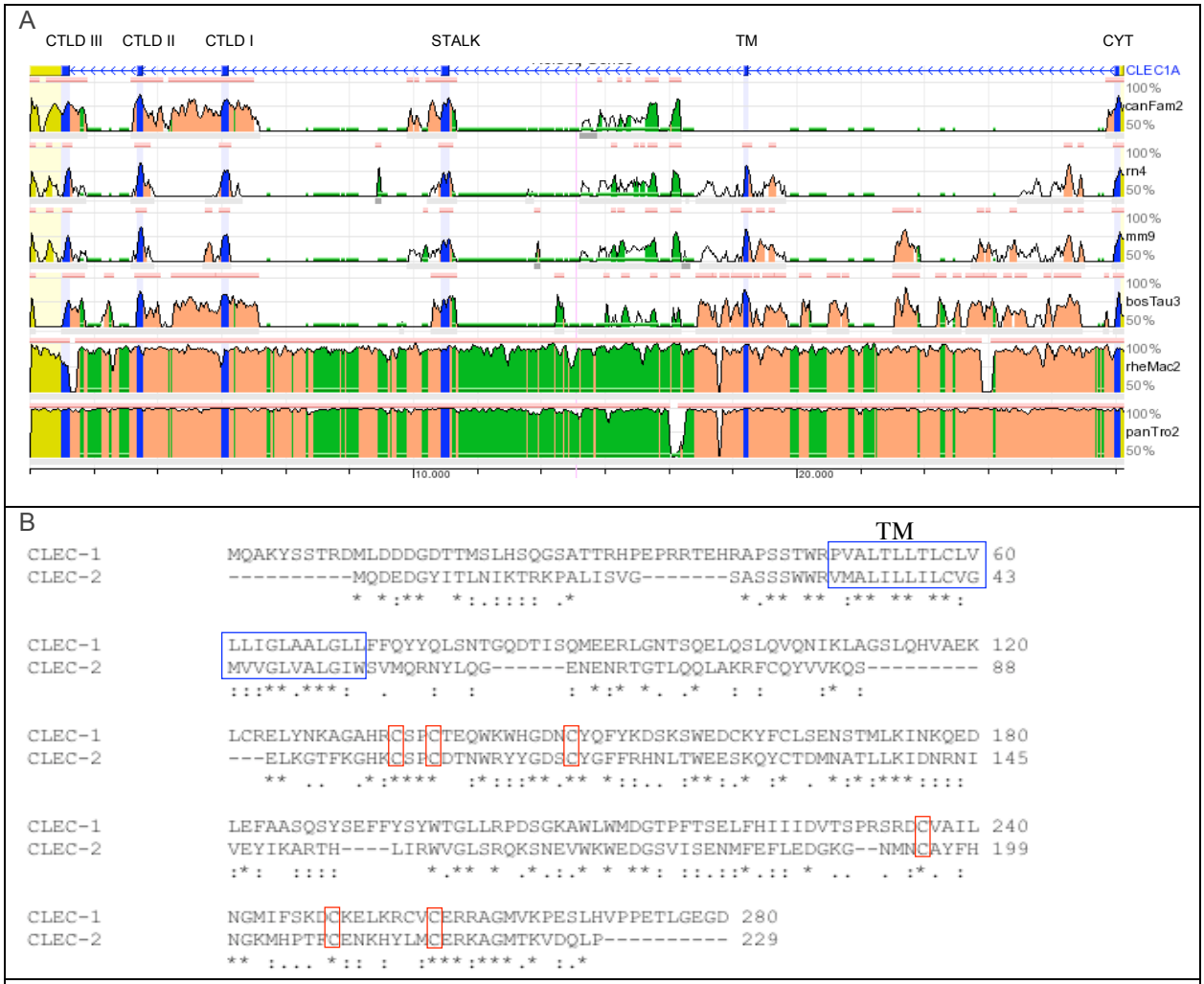
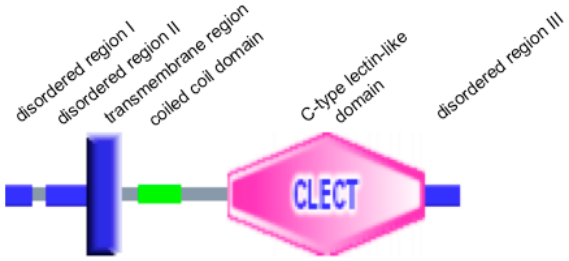
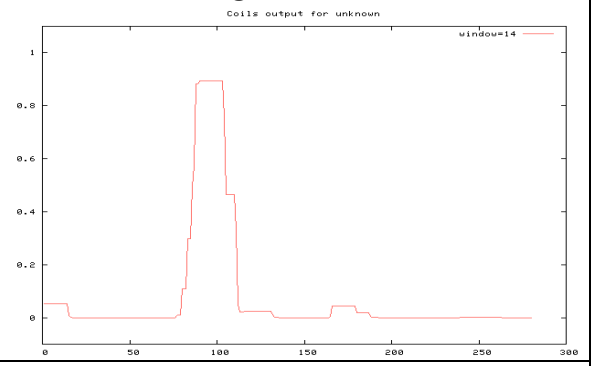


Figure 19: CLEC-1 DNA and protein sequence analysis. A: Genomic sequence alignment showing the degree of similarity of the CLEC-1 genes of chimpanzee (panTro2), rhesus monkey (rheMac2), cow (bosTau3), mouse (mm9), rat (rn4) and dog (canFam2) with the human CLEC-1 gene (hg18). The height of the peaks indicate per cent similarity. Colours define different genomic regions. yellow: UTR, blue: coding exons, salmon: intronic regions, green: transposons and simple repeats. Alignment obtained by using the ECR Browser available at http://ecrbrowser.dcode.org
B: Alignment of the human CLEC-1 and CLEC-2 protein sequences using the ClustalW2 algorithm set to default parameters (available at http://www.ebi.ac.uk/Tools/clustalw2/index.html?). asterisk: identity, colon: conserved substitutions, period: semi-conserved substitutions, TM: transmembrane region boxed in blue, conserved cysteins boxed in red.

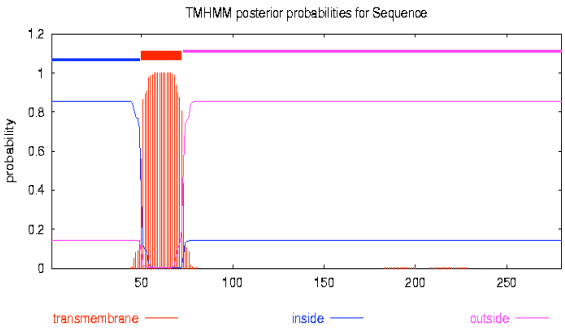
A CLEC-1 domain overview



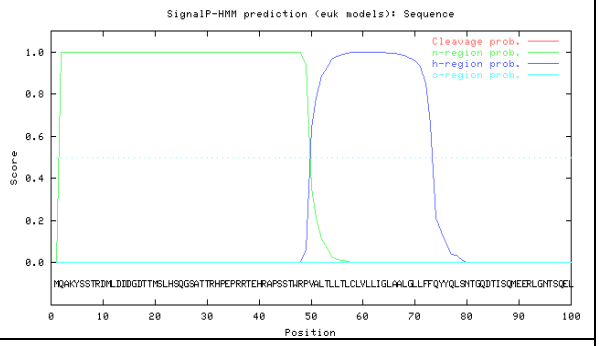
B Coiled Coil region



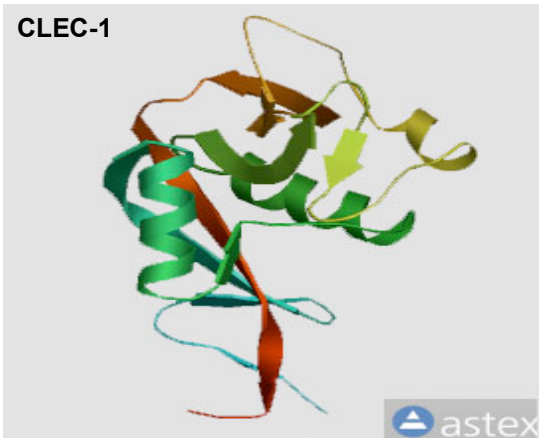
C Transmembrane region



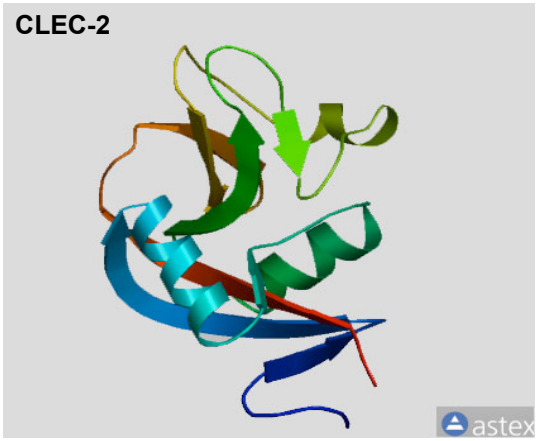
D Signal Anchor



E CLEC-1 and CLEC-2 Protein tertiary structure



template: DC-SIGN 1sl6A (2.25 Å)
sequence identity: 19,774%
E-value: 1.50e-26



template: CLEC-2 2c6uA (1.60 Å)
sequence Identity: 100%
E-value: 3.11e-70

Figure 20: Bioinformatic analysis of CLEC-1 protein sequence. A: Protein domain overview obtained using SMART (available at http://smart.embl-heidelberg.de/smart), horizontal blue box: region of intrinsic disorder, vertical blue box: transmembrane region, horizontal green box: coiled coil domain, pink CLECT box: **C**-type **lect**in region. B: Prediction of coiled coil regions using COILS Server (available at http://www.ch.embnet.org/software/COILS_form.html). C: Prediction of transmembrane region using TMHMM Server v. 2.0 (available at http://www.cbs.dtu.dk/services/TMHMM/). D: Prediction of signal peptides/anchors using SignalP 3.0 Server (available at http://www.cbs.dtu.dk/services/SignalP/) h-region: hydrophobic core region, n-region: positively charged NH2-terminal segment, c-region: COOH-terminal cleavage region. E: B: Structure model of CLEC-1 compared to CLEC-2 obtained using Swiss-Model Algorithm (available at http://swissmodel.expasy.org/workspace/index.php). DC-SIGN (1sl6A) was used as template sequence for CLEC-1. Template for the CLEC-2 model was the crystallized structure of CLEC-2 (2c6uA) itself.

Further investigation of the CLEC-1 protein sequence using bioinfomatics tools revealed a domain structure typical for all C-type lectin-like proteins. Like the other C-type lectins, CLEC-1 is predicted to contain a transmembrane region and C-type lectin-like domains, and in addition three regions of intrinsic disorder and a coiled coil domain could be identified (figure 20A).

To characterize the predicted domains in more detail we used the programs generating the SMART predictions. Emphasis was given to the transmembrane region, which was predicted by the TMHMM Server calculating the total probability of a residue being located within a transmembrane helix, inside or outside of the membrane. In the CLEC-1 protein sequence the predicted transmembrane region spans 22 amino acids from residue 50 to residue 72, leaving 49 amino acids on the N-terminus as short cytoplasmic part of the protein and 208 amino acids at the outer side of the membrane harbouring the C-type lectin-like domains (figure 20C). Close to the membrane domain one of the programs also used by SMART (COILS) predicted a coiled coil region of 27 amino acids length, which nicely correlates with exon three encoding the stalk of the protein (figure 20B). As expected for a type II transmembrane protein the prediction program used (SignalP) did exclude the existence of an N-terminal signal peptide, but it could identify a signal anchor with a probability of 100% (figure 20D) corresponding nicely to the predicted transmembrane region. Therefore the bioinformatics analysis strongly supports a type II transmembrane localization for CLEC-1 similar to the other members of the family. Finally SMART also detected regions of intrinsic disorder on the N-terminal as well as on the C-terminal end of the protein. Finally, a model of the CLEC-1 tertiary structure was produced using the Swiss Model Workspace. The program automatically chose the already crystallized DC-SIGN (Guo, Feinberg et al. 2004) as the most suitable template and generated a model based on the homology to DC-SIGN. For comparison we also modeled the CLEC-2 structure. In this case the crystallized CLEC-2 itself was used as template. As depivted in figure 20E, a striking similarity between the CLEC-1 and the CLEC-2 structures is apparent. Like CLEC-2, CLEC-1 is predicted to consist of three α-helices, one of which is very short, associated with several β-sheets, causing a very similar general conformation.

3.3.2) The intracellular localization of CLEC-1 is not changed by cell activation

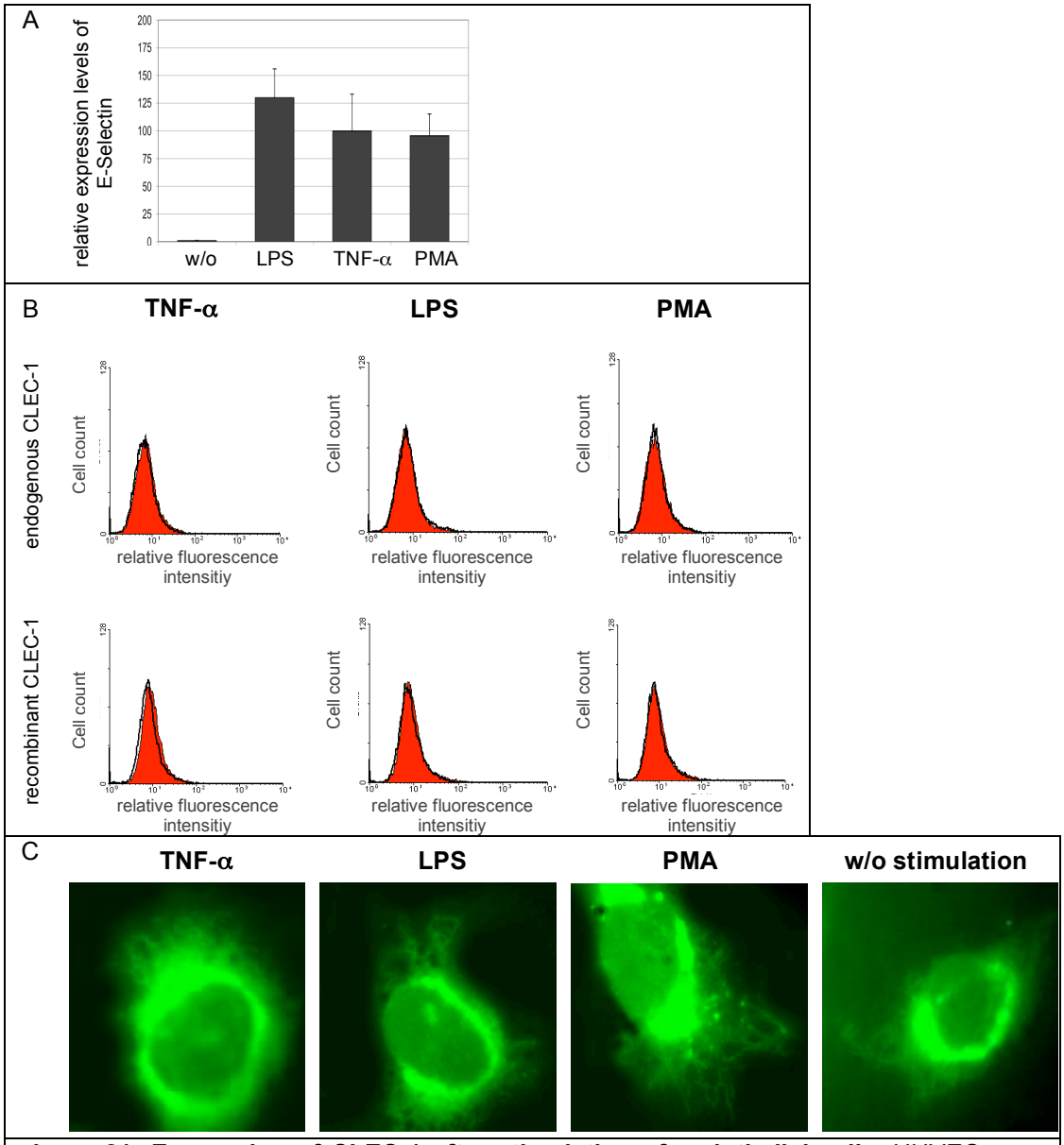


Figure 21: Expression of CLEC-1 after stimulation of endothelial cells. HUVEC were infected with AdVCLEC-1, stimulated for 5h with 10ng/ml TNF-α, 100ng/ml LPS or 1 µg/ml PMA and processed for Real-Time RT-PCR, flow cytometry and immunofluorescence staining using a primary mouse anti-human CLEC-1 and a secondary FITC-labeled anti-mouse antibody, respectively. A: Relative levels of E-Selectin mRNA in HUVEC analysed by Real-Time RT-PCR to confirm the activation of HUVEC used for immunofluorescence and flow cytometry. Measurements were done in triplicates for each experiment. B: Flow cytometry analysis of endogenous CLEC-1 and recombinant CLEC-1 overexpressed by adenoviral infection on non permeabilized HUVEC. Red peaks show staining of stimulated cells. Black peak show staining of non-stimulated cells. Experiments were done twice in duplicates, one representative sample is shown. C: Immunofluorescence staining of recombinant CLEC-1 in permeabilized HUVEC. Experiments were done twice, one representative picture is shown. Magnification used: 1000x

As bioinformatics did not display any unusual feature in the CLEC-1 sequence and strongly supported a transmembrane localization similar to the other members of the family, the question arose why CLEC-1 could not be detected on the surface of cells with CLEC-1 mRNA. One possible explanation might be that CLEC-1 is retained intracellularly until the cell is challenged with a specific stimulus. As mRNA of CLEC-1 was found in endothelial cells, we used the inflammatory stimuli LPS and TNFα as well as PMA to stimulate HUVEC for 5h and tested for detectable surface expression of endogenous CLEC-1 by flow cytometry. To exclude that a failure to detect CLEC-1 would be caused by the low expression level of the endogenous CLEC-1 we also used recombinant overexpressio of CLEC-1 with a corresponding adenoviral construct. Although all three stimuli used strongly activated endothelial cells as shown by the upregulation of E-Selectin (figure 21A), we could not detect any surface expression of endogenous or overexpressed CLEC-1 (figure 21B). Permabilized endothelial cells overexpressing CLEC-1 showed an intracellular pattern of CLEC-1 staining without any detectable changes after stimulation (figure 21C) supporting a normal intracellular localization not influenced by the activation state of the cell.

3.3.3) The intracellular staining of CLEC-1 shows ER-like pattern

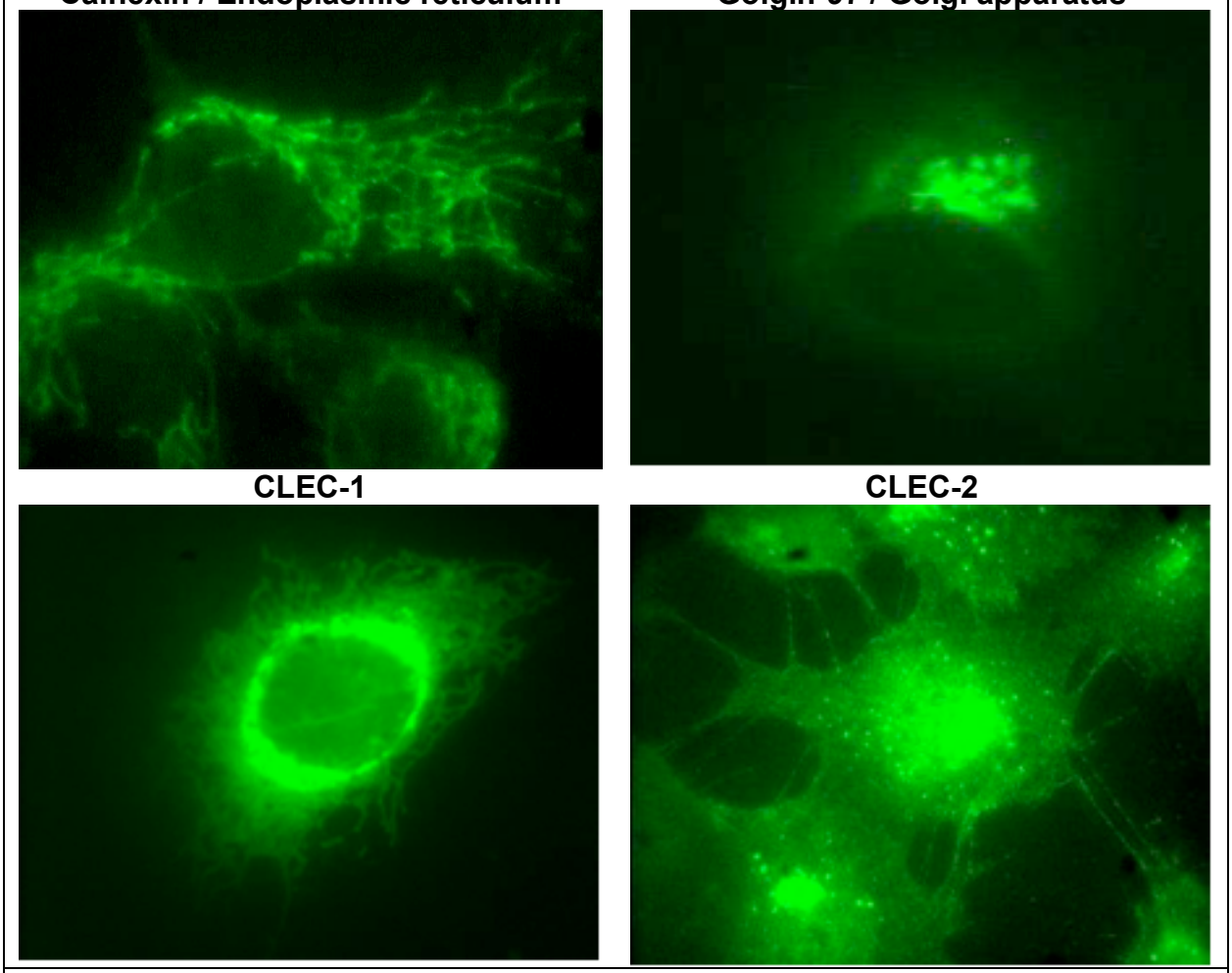


Figure 22: Intracellular localization of recombinant CLEC-1 in HUVEC. HUVEC infected with AdVCLEC-1 were permeabilized and stained for CLEC-1 using a primary mouse anti-human CLEC-1 and a secondary FITC-labeled anti-mouse antibody. Calnexin, Golgin-97 and CLEC-2 staining with corresponding primary mouse anti-human Calnexin, Golgin-97, or CLEC-2 antibodies and a secondary FITC-labeled anti-mouse antibody were used to compare the staining pattern of CLEC-1 to proteins with different localization within the cell. Stainings were done three times, one representative picture is shown. Magnification used: 1000x

Intracellular staining of recombinant CLEC-1 in endothelial cells always showed perinuclear accumulation and thin structures with a net-like appearance reaching out towards the borders of the cell. Considering the fact that CLEC-1 does not seem to be present on the cellular surface as indicated by flow cytometry data as well as immunoflourescence staining, but still possesses all characteristics of a transmembrane protein, it is plausible to propose that the normal physiological localisation of CLEC-1 is at an intracellular membrane. To test this hypothesis we performed immunofluorescence staining of endothelial cells infected with AdVCLEC-1 and AdVCLEC-2 as a control for surface

staining. We further stained non-infected cells with antibodies against Calnexin, a specific marker for the endoplasmic reticulum, and Golgin-97, used as marker for the Golgi apparatus. Comparing the staining patterns of the differently stained cells it became apparent that CLEC-1 staining did not resemble surface staining or Golgi-97 staining (figure 22). The observed CLEC-1 patterns most closely resembled calnexin staining. Therefore it appears that CLEC-1 is indeed preferentially localized to the endoplasmic reticulum membranes.

3.3.4) Endogenous CLEC-1 is localized in the ER of endothelial cells

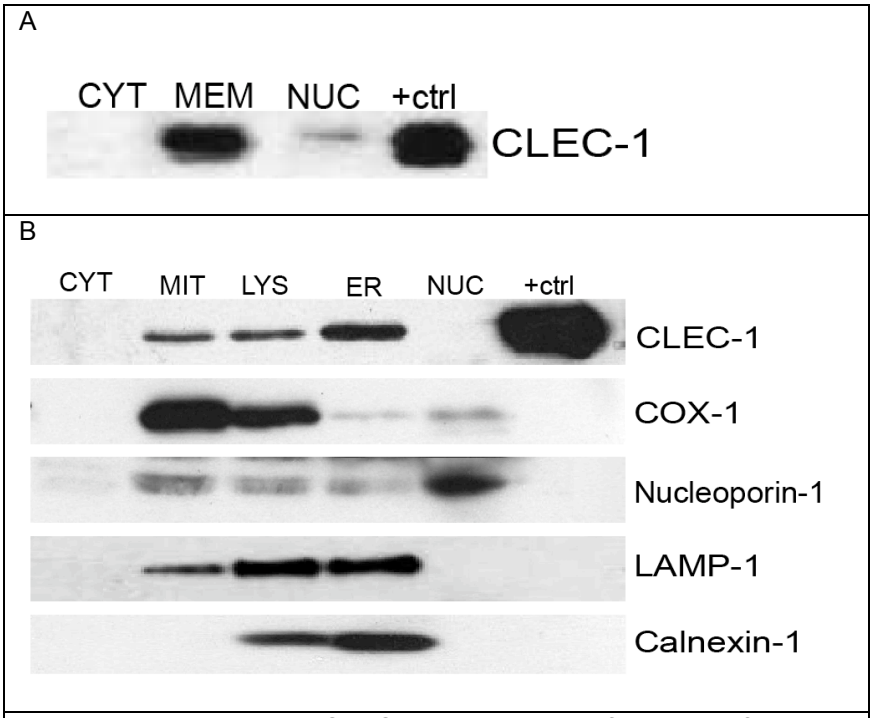


Figure 23: Endogenous CLEC-1 in subcellular fractions of HUVEC. Subcellular fractions of endothelial cells were isolated and tested in Western Blots using primary mouse or rabbit antibodies against human CLEC-1, COX-1, nucleoporin-1, LAMP-1 and Calnexin-1. Proteins were detected using secondary HRP-labeled anti-mouse or anti-rabbit antibodies. Cells infected with AdVCLEC-1 were used as positive control (+ctrl) for CLEC-1 expression. A: Cytoplasmic, membrane and nucleic fractions of HUVEC tested for CLEC-1. B: Cytoplasmic, mitochondrial, lysosomal, microsomal and nucleic fractions of HUVEC tested for CLEC-1. As organelle marker proteins COX-1 (mitochondria), Nucleoporin (nuclei), LAMP-1 (lysosomes) and Calnexin (Endoplasmic reticulum) were used.

To further confirm that CLEC-1 is localized in intracellular membranes we produced subcellular fractions of endothelial cells and tested them for the presence of endogenous CLEC-1 by Western Blotting. We could clearly detect a preferential presence of CLEC-1 in the membrane fraction, whereas as expected CLEC-1 was absent from the cytoplasmic and nuclear fractions (figure 23A). The hypothesis that endogenous CLEC-1 might be localized to the ER membrane could be further supported by another cell fractionation experiment. Subcellular fractions of endothelial cells enriched in cytoplasmic, mitochondrial, lysosomal, microsomal and nucleic proteins were prepared by differential centrifugation. As differential centrifugation can not achieve totally pure fractions, it was important to stain for marker proteins in each fraction to define

the degree of purity. Marker proteins used were cytochrome c oxidase subunit 1 (COX-1) for the mitochondrial fraction, nucleoporin for the nucleic fraction, lysosomal-associated membrane protein 1 (LAMP-1) for the lysosomal fraction and calnexin for the endoplasmic reticulum fraction. This experiment showed that CLEC-1 staining was clearly strongest in the ER fraction (figure 23B, although smaller amounts of CLEC-1 could also be detected in the mitochondrial and lysosomal fractions, which is probably due to contamination of these fractions with ER proteins. As expected, no staining of CLEC-1 in the cytoplasmic and the nuclear fraction could be observed.

3.3.5) CLEC-1 and intracellular TLR ligands

The fact that CLEC-1 is found only intracellularly but is a member of a family of mainly surface located receptors is reminiscent of the situation in the TLR receptor family. Furthermore, similar to TLRs some of the C-type lectin-like receptors show characteristics of pattern recognition receptors. In the case of the intracellular TLR9 it has been described that upon binding to its ligand TLR9 translocates between different intracellular compartments (Latz, Schoenemeyer et al. 2004). Since it was possible that CLEC-1 would have similar ligands as TLRs or would translocate between different compartments together with TLRs, possible effects of ligands for the intracellular TLR3, 7 and 9 on CLEC-1 mRNA expression level and intracellular localization were investigated. As endogenous CLEC-1 is expressed in dendritic cells, we differentiated cord blood derived DC, stimulated them with the TLR ligands poly(I:C), Imiquimod and CpG-ODN and analysed CLEC-1 expression by Real-Time RT-PCR and immunofluorescence staining.

3.3.5.1) Marker expression on cord blood derived dendritic cells

Dendritic cells were differentiated from $CD34^+$ progenitor cells isolated from cord blood and tested for expression of CD11b and CD1a as markers for DC. Cells were confirmed to express both CD11b and CD1a, but not CD14, a monocytic marker (figure 24A, 24B). mRNA transcripts for DECTIN-1, CLEC-1 and CLEC-2 were clearly detected by PCR (figure 24C).

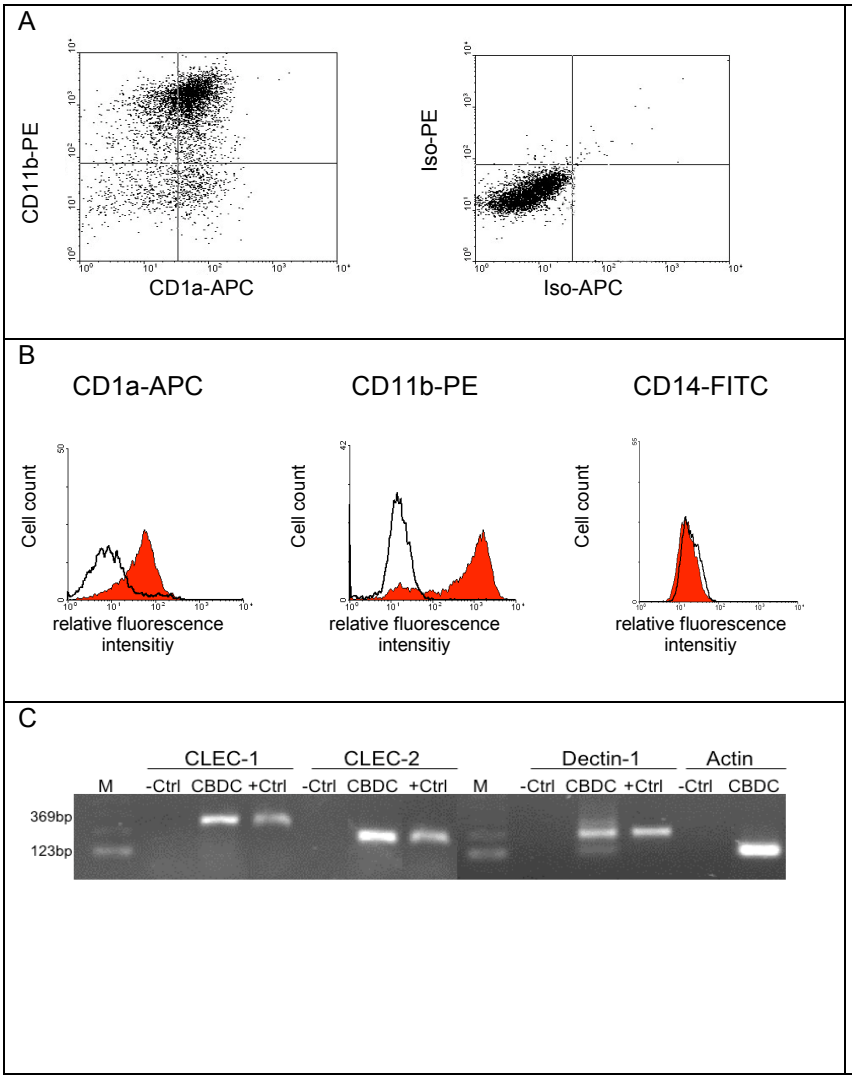


Figure 24: Analysis of surface markers and DECTIN-1, CLEC-1 and CLEC-2 mRNA expression in CBDC. CBDC were differentiated from $CD34^+$ cord blood progenitor cells and tested for the expression of the dendritic cell markers CD11b and CD1a and the monocytic marker CD14 by flow cytometry using mouse anti-human CD11b-PE, CD1a-APC and CD14-FITC antibodies and the corresponding isotype controls. Analysis of marker expression was done after each differentiation, one representative sample is shown. PCR was used to detect CLEC-1, CLEC-2 and DECTIN-1 transcripts in CBDC cDNA. A: Dot plot confirming double staining of both CD11b and CD1a on CBDC. B: Histograms showing expression of CD11b, CD1a and CD14. Red peaks show staining with mouse anti-human CD11b-PE, CD1a-APC or CD14-FITC. Black peaks show staining with the corresponding isotype control antibody. C: 1% agarose gel showing PCR products corresponding to CLEC-1, CLEC-2 and DECTIN-1 mRNA in CBDC cDNA preparations. Plasmids containing the appropriate or unrelated cDNA were used as positive or negative control, resp.

3.3.5.2) CLEC-1 mRNA is downregulated upon activation of dendritic cells

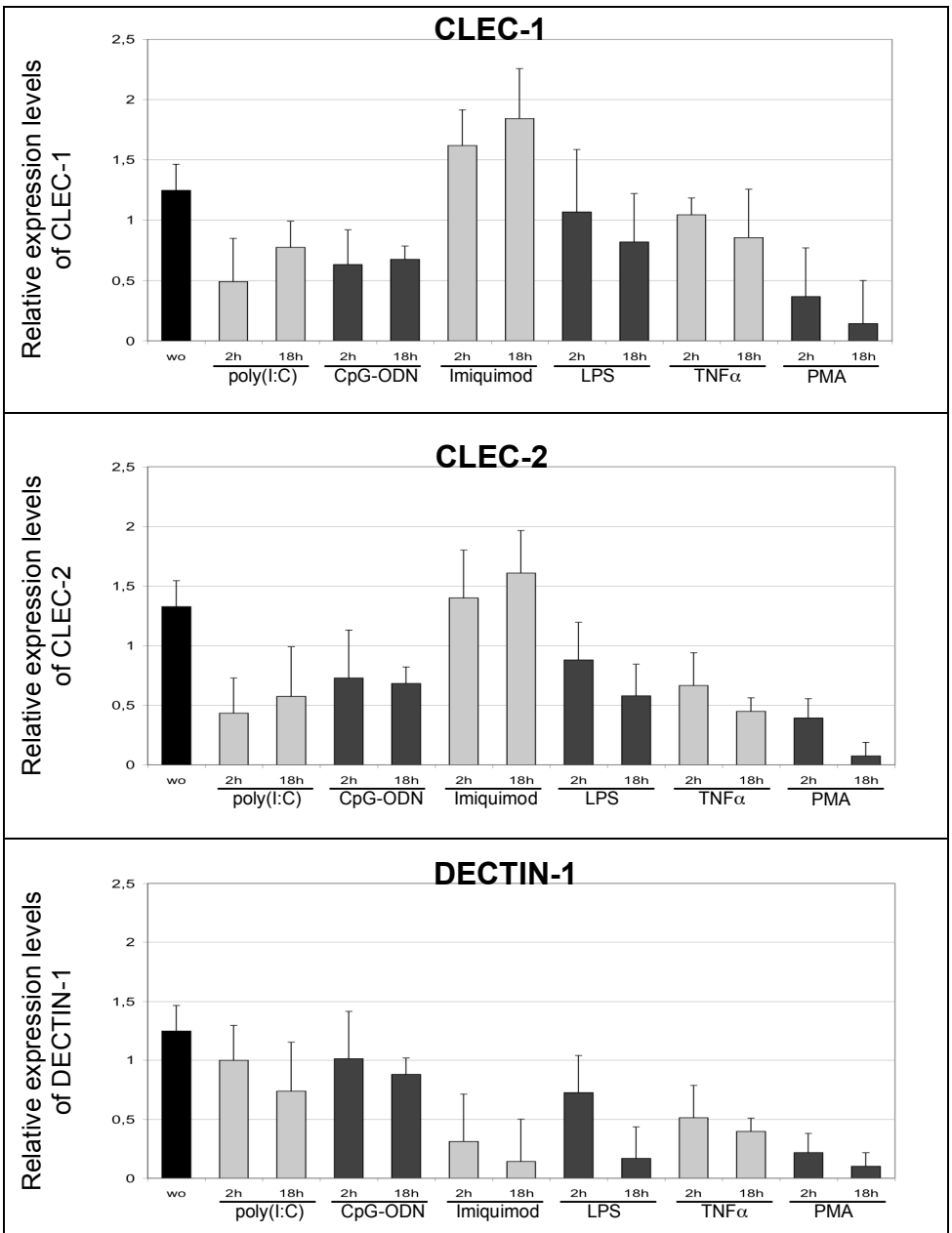


Figure 25: Regulation of CLEC-1, CLEC-2 and DECTIN-1 in CBDC. Regulation of mRNA levels of CLEC-1, CLEC-2 and DECTIN-1 in CBDC upon simulation with 25μg/ml poly(I:C), 1μM CpG-ODN, 10μg/ml Imoquimod, 100ng/ml LPS, 10ng/ml TNF-α and 1 μg/ml PMA. CBDC were stimulated for 2h and over night with various stimuli and processed for RNA isolation and cDNA synthesis. Real-Time RT-PCR was performed using β-actin as internal control for normalization. mRNA levels in stimulated cells relative to unstimulated cells are shown for CLEC-1, CLEC-2 and DECTIN-1. Measurements were done in triplicates.

Considering the possibility that CLEC-1 might be an intracellular pattern recognition receptor, it was of interest to investigate whether TLR ligands or other inflammatory stimuli would show any regulatory effect on CLEC-1

expression in dendritic and endothelial cells. To this end dendritic cells were differentiated from $CD34^+$ positive progenitor cells from cord blood. The obtained cells were then stimulated with 25μg/ml poly(I:C), 1μM CpG-ODN (CpG oligo-desoxy-nucleotide), 10μg/ml Imiquimod (R484), 100ng/ml LPS, 10ng/ml TNF-α and 1 μg/ml PMA for different time points and processed for RNA isolation, cDNA synthesis and Real-Time RT-PCR detection of CLEC-1 transcript levels. CLEC-2 and DECTIN-1 mRNA levels were included for comparison. Poly(I:C), CpG-ODN and Imiquimod mimic bacterial and viral nucleic acids and are used as synthetic ligands for TLR-3 (binds viral dsRNA in the endosome), TLR-9 (binds unmethylated bacterial DNA) and TLR-7 (binds viral ssRNA) respectively. LPS, TNF-α and PMA were included as additional known stimuli of DC maturation.

As shown in figure 25, the substances used generally seemed to cause downregulation of all three receptors in cord blood derived dendritic cells, presumably indicative of a stimulus for maturation of the cells following triggering of the respective receptors. When compared to DECTIN-1, CLEC-1 downregulation was most pronounced upon treatment of CBDC with poly(I:C) and CpG-ODN, whereas imiquimod did not seem to affect CLEC-1 mRNA levels at all. A similar pattern of downregulation was observed for CLEC-2, whereas DECTIN-1 was affected most strongly by Imiquimod. CpG-ODN showed no significant effect on DECTIN-1 mRNA levels. Control non-CpG-ODN (GpC instead of CpG dinucleotides) did not have any effect on any of the receptors (data not shown). LPS, TNF-α and PMA caused downregulation of all three receptors, the effect being most pronounced for mRNA levels of DECTIN-1.

3.3.6) CLEC-1 and intracellular pathogens

It is generally accepted that receptors of the myeloid receptor family, for which the ligands are known, function as pattern recognition receptors (Kanazawa 2007). As our data suggest that CLEC-1 is expressed intracellularly and may have an intracellular "pattern recognition" function, we investigated whether there might be any effect on CLEC-1 upon infection of the cells with intracellular pathogens indicative of a role of the receptor in the response towards these pathogens. As examples for common intracellular pathogens the eukaryotic parasite *Toxoplasma gondii* and the gram-positive bacteria *Listeria monocytogenes* were investigated.

Inside the host cell *T.gondii* is surrounded by a cell membrane creating the parasitophorous vacuole in which the parasite is protected from being killed by cellular defence mechanisms. The infection process of *Listeria monocytogenes* on the other hand results in the bacteria being surrounded by a two-membrane vacuole from which they need to escape into the cytosol where they replicate.

It might be possible that the host cell senses *Toxoplasma gondii* or *Listera monocytogenes* infection via a receptor in these vacuole membranes, and given the already known characteristics of CLEC-1 it could be a possible candidate for this function. Therefore we tested different possibilities of interactions between CLEC-1 and the two chosen pathogens, *Toxoplasma gondii* and *Listeria monocytogenes*.

3.3.6.1) Toxoplasma gondii and Listeria monocytogenes

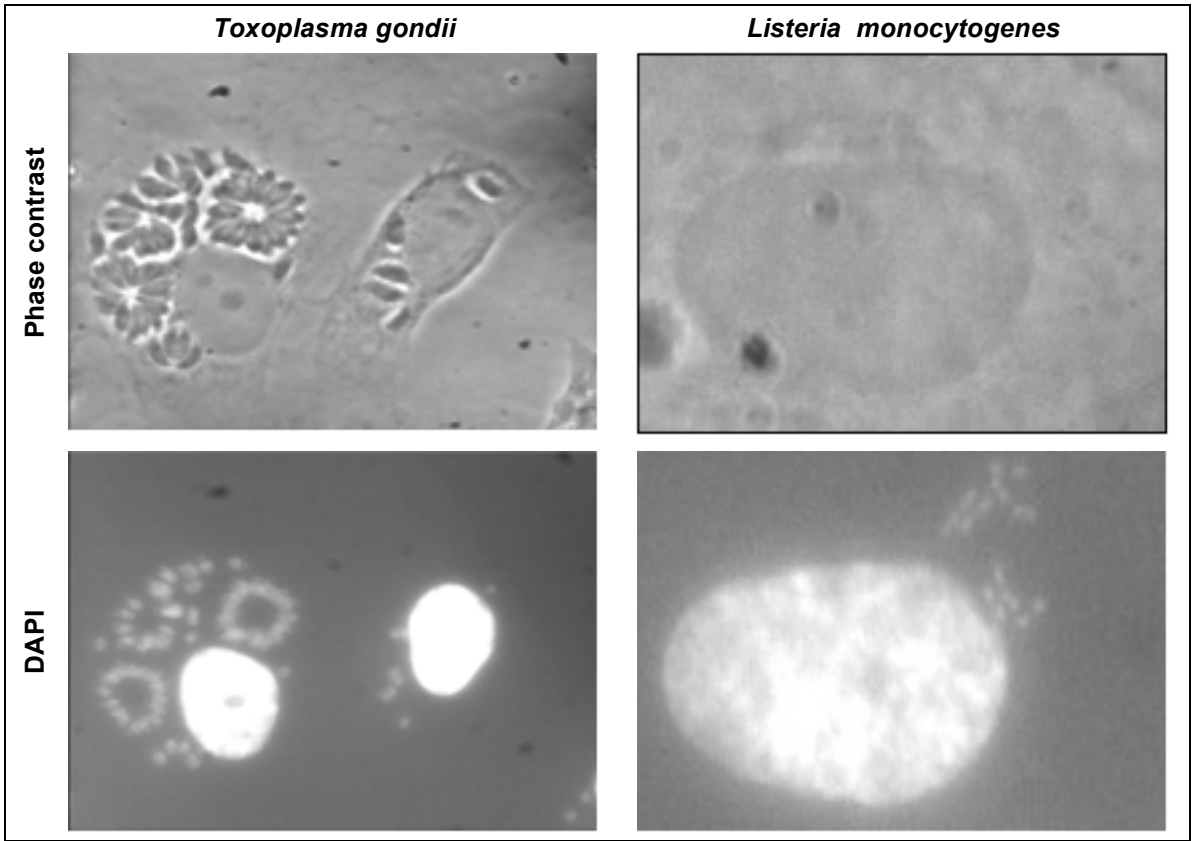


Figure 26: HUVEC infected with *Listeria monocytogenes* and *Toxoplasma gondii*. HUVEC were seeded and infected for two days with *Listeria monocytogenes* and *Toxoplasma gondii* at a MOI of 100 and 10, respectively. Pictures were taken under phase contrast and following staining with DAPI using UV light. Magnification used: *Toxoplasma gondii*: 500x, *Listeria monocytogenes 1000x.*

To investigate possible effects of *Toxoplasma gondii* and *Listeria monocytogenes* infection on CLEC-1, *Toxoplasma gondii* and *Listeria monocytogenes* were amplified and either used for infection of cells or purified and directly used for binding studies. Infection of cells was done at a MOI of 100 for *Listeria monocytogenes* and a MOI of 10 for *Toxoplasma gondii*. Infected endothelial cells were analysed by microscopy, *Toxoplasma gondii* having a size of approximately 6x2 µm are visible in phase contrast at a 500-fold magnification and can be stained with the DNA dye DAPI and detected in the UV channel. *Listeria monocytogenes* can only be detected by DAPI staining (figure 26).

3.3.6.2) CLEC-1 is not affected by T. gondii and L. monocytogenes

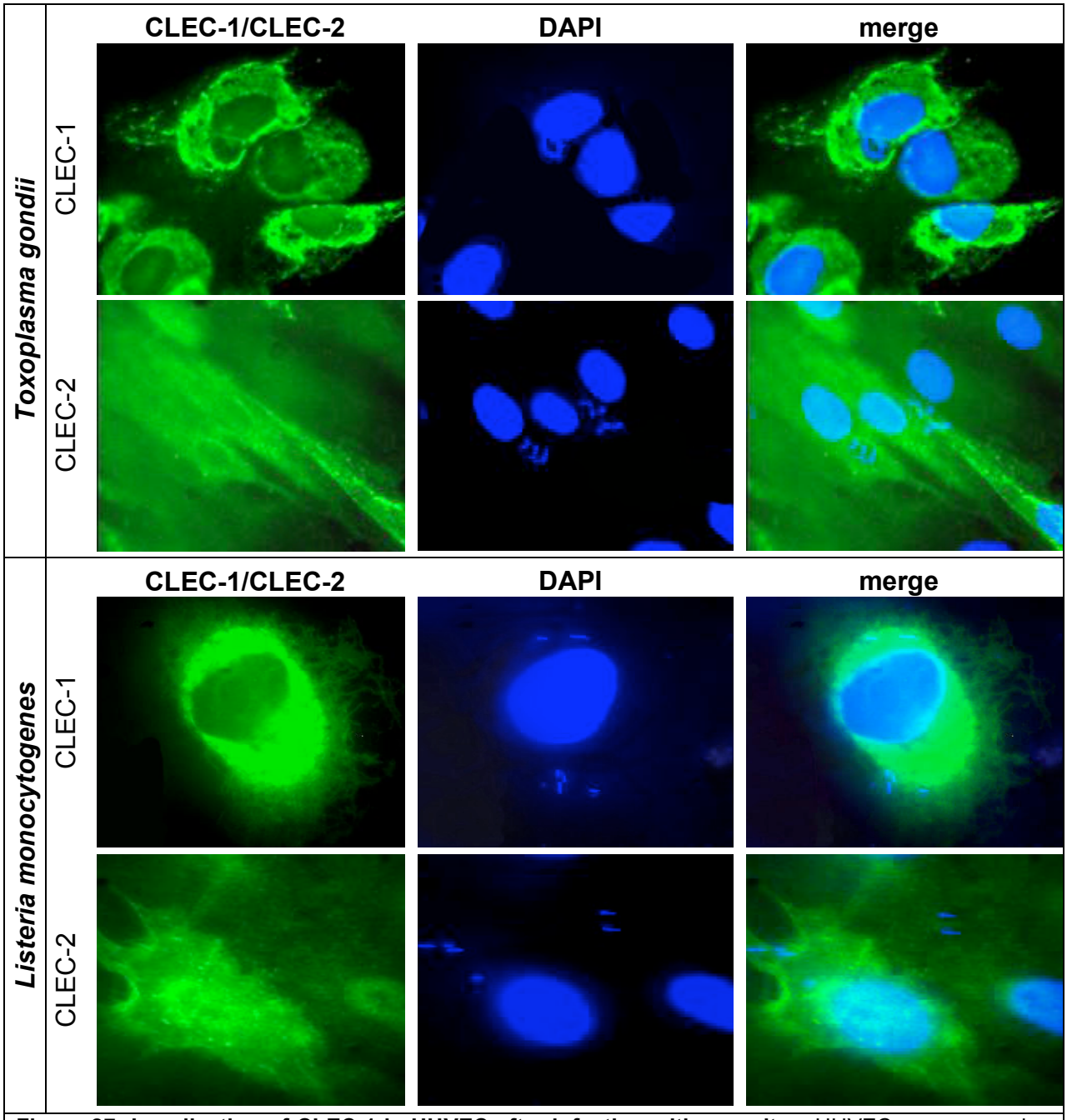


Figure 27: Localisation of CLEC-1 in HUVEC after infection with parasites. HUVEC overexpressing CLEC-1 were infected with *Toxoplasma gondii* and *Listeria monocytogenes* and tested for intracellular CLEC-1 relocalisation by immunofluorescence staining using a primary mouse anti-human CLEC-1 and a secondary FITC-labeled anti-mouse antibody. Cell nuclei were visualized using DAPI (blue). Magnification used: 1000x

It was conceivable, that if CLEC-1 would interact with *Toxoplasma gondii* or *Listeria monocytogenes* within the cell, it would be possible to detect CLEC-1 in close proximity to the pathogens, as a membrane receptor potentially accumulating in the vacuole membrane. To test this hypothesis we performed

immunofluorescence staining of cells overexpressing CLEC-1 in comparison to cells overexpressing CLEC-2 and infected with *Toxoplasma gondii* or *Listeria monocytogenes*. CLEC-1 and CLEC-2 staining showed the usual pattern, the first being expressed intracellularly and the second on the cell surface, but no changes in localization of either CLEC-1 or CLEC-2 was apparent when the cells were infected with *Toxoplasma gondii* or *Listeria monocytogenes* (figure 27). It could be shown that the lumen of the parasitophorous vacuole clearly lacks CLEC-1 expression and that there is no re-localisation to the vacuole membrane causing accumulation of CLEC-1. The same holds true for CLEC-2.

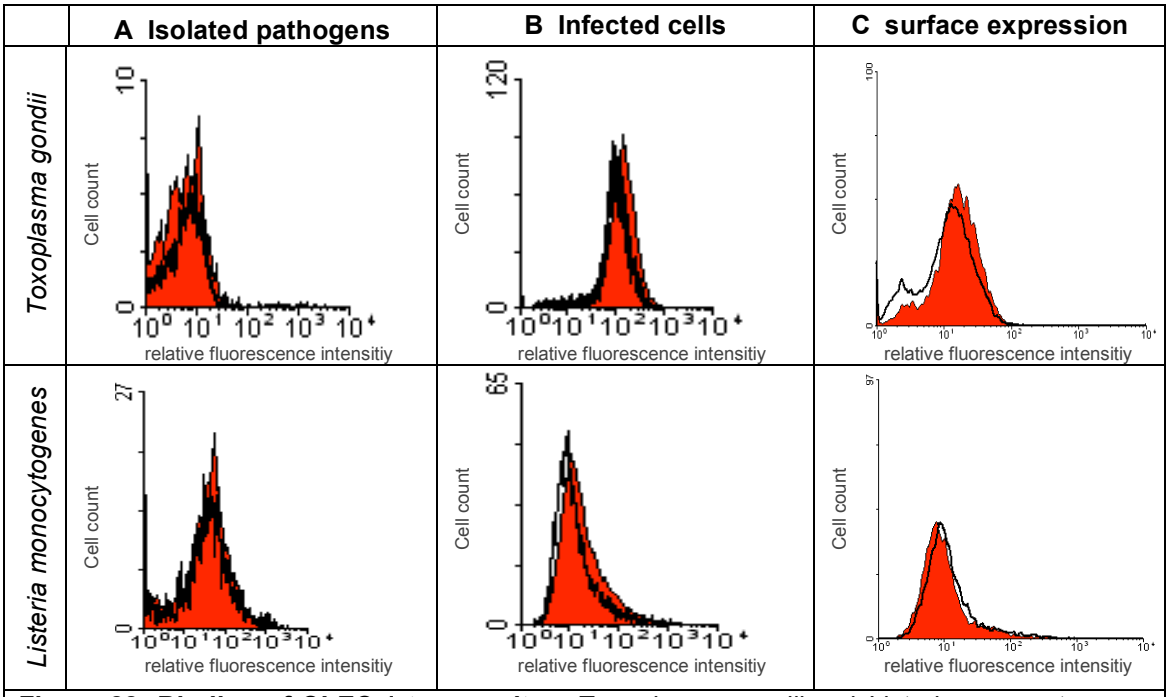


Figure 28: Binding of CLEC-1 to parasites. *Toxoplasma gondii* and *Listeria monocytogenes* were isolated and directly tested for binding to soluble CLEC-1 or used for infection of HUVEC. A: Isolated parasites were incubated with soluble CLEC-1, washed thoroughly, stained using a FITC-labeled anti-FLAG antibody and analysed by flow cytometry. The black peak shows background staining of parasites incubated without soluble CLEC-1 protein. B: Infected HUVEC were incubated with soluble CLEC-1, washed thoroughly, stained using a FITC-labeled anti-FLAG antibody and analysed by flow cytometry. The black peak shows background staining of non-infected HUVEC. C: Infected HUVEC expressing recombinant CLEC-1 were tested for CLEC-1 surface expression, using a primary mouse anti-human CLEC-1 and a secondary FITC-labeled anti-mouse antibody. The black peak shows background staining of non-infected HUVEC.

To test whether CLEC-1 is capable of binding directly to isolated *Toxoplasma gondii* or *Listeria monocytogens*, we amplified and isolated the pathogens and incubated them with soluble CLEC-1. We further tested, whether CLEC-1 would bind a ligand upregulated on the cell surface after infection of the cell with either

Toxoplasma gondii or *Listeria monocytogenes*, by infecting HUVEC with both pathogens and incubating the infected cells with soluble CLEC-1.

However, we could not detect any specific binding of soluble CLEC-1, neither directly to the isolated parasites nor to the surface of infected cells (figure 28A, 28B). The same is true for soluble DECTIN-1, which we used in addition as a control (data not shown). Soluble proteins containing the CLEC-1 or DECTIN-1 ectodomaim, as well as a FLAG-tag, were kindly provided by Mag. Irene Karas.

Although we never found surface expression of CLEC-1 on any cell type and propose that CLEC-1 might function intracellularly, it still can not be excluded completely that CLEC-1 is only expressed on the cell surface upon the appropriate stimulation of the cell. Therefore we further tested whether *Toxoplasma gondii* or *Listeria monocytogenes* infection would cause CLEC-1 to be transported to the surface. To this end HUVEC overexpressing CLEC-1 were infected with *Toxoplasma gondii* and *Listeria monocytogenes*, stained for CLEC-1 surface expression and analysed by flow cytometry. As shown in figure 28C CLEC-1 could not be shown to be localized on the cell surface after *Toxoplasma gondii* or *Listeria monocytogenes* infection.

3.4) Genomic analysis of the myeloid cluster of the murine and human NK gene complex

The genes encoded in the myeloid receptor part of the human NK receptor complex have become the focus of intense investigations in recent years, in particular since several of them, such as LOX-1 and DECTIN-1, were shown to play a pivotal role in the initiation and regulation of immune responses. When this work was initiated, there were still large unknown genomic regions between these genes, which had not been analysed before for protein encoding regions, therefore we decided to study these regions to potentially identify additional lectin-like genes.

To this end an extensive comparison of the sequences of the myeloid receptor part of the human, chimpanzee, mouse and rat NK complexes was conducted, based on sequences which have recently become available by whole genome sequencing.

3.4.1) Identification of additional C-type lectin-like genes

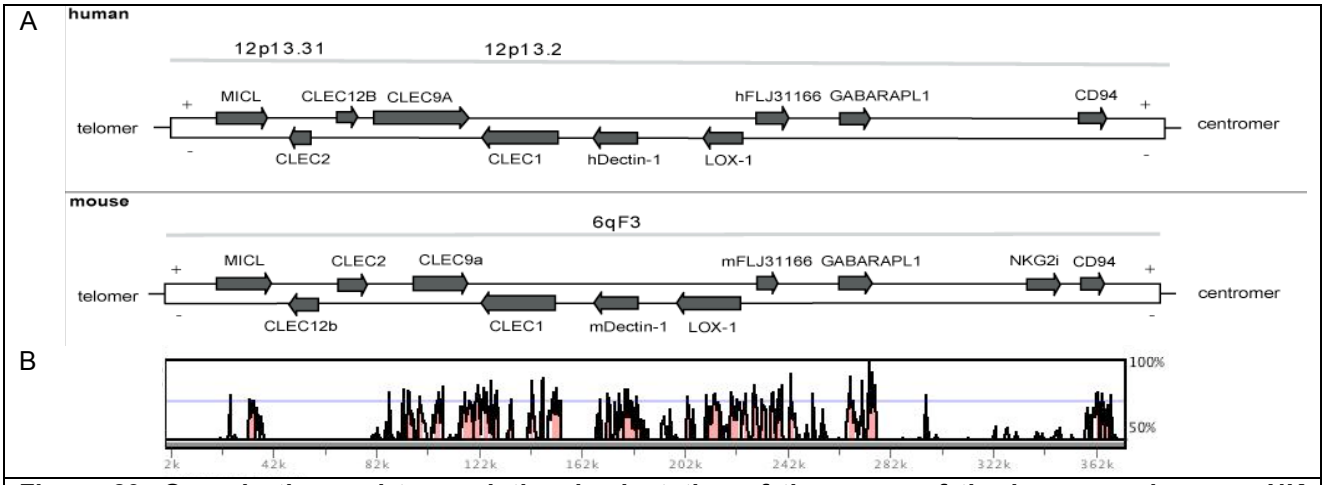


Figure 29: Organization and transcriptional orientation of the genes of the human and mouse NK receptor complex between MICL and CD94. Physical map of the approx. 380 kb region extending from the MICL gene on the telomeric side to the CD94 gene on the centromeric side of the NK receptor complex. By searching for mRNA and EST sequences four additional genes were identified in the human as well as in the murine NK receptor complex. Linkage, orientation and distance between the genes in the centromeric part were delineated from sequence information available from the human genome project. A: Linkage and orientation of the lectin-like genes in the centromeric part of the human NK receptor gene complex on the short arm of chromosome 12. B: Linkage and orientation of the lectin-like genes in the syntenic region of the murine NK receptor gene complex on chromosome 6. C: Homology blot showing the homology between the corresponding human and the murine sequences of the NK gene complex. The Blot was produced using mVista (VistaBrowser, http://genome.lbl.gov/vista/mvista/submit.shtml) with the human genome as base genome.

The human NK receptor complex spans a region of approximately 2 Mb on the short arm of the human chromosome 12 (12p12.3-p13.2) (Renedo, Arce et al. 1997; Sobanov, Glienke et al. 1999) whereas the syntenic region in mice is located on chromosome 6 (6qF3) (Brown, Fulmek et al. 1997), in rats on chromosome 4 (4q42) (Dissen, Ryan et al. 1996) and in cow sequences of the genes encoded in the human complex can be aligned to chromosome 5.

In order to identify additional genes, sequence databases for human and murine mRNA and EST sequences aligning to the NK receptor complex were searched. We focused on the myeloid receptor cluster of the NK complex extending from the MICL (CLEC12a) gene on the telomeric side to the CD94 gene on the centromeric side. This region is known to encode lectin-like receptors predominantly expressed in cells of the myeloid lineages and spans about 343 kb in humans, whereas it is about 100 kb shorter in mice (248 kb). This difference in size can be explained on the one hand by two insertions into the human genome comprising 30 and 50 kb, respectively, and on the other hand by a higher percentage of repetitive elements in the human cluster (51,38%) compared to the murine cluster (32,88%), which is mainly due to a higher amount of Alu elements (human: 15,12%, mice: 2,12% of whole sequence).

Sequence comparisons identified mRNA and EST sequences for four additional genes (figure 29A). Two of these, CLEC12b (corresponding to Refseq.UNQ5782) and CLEC9A (corresponding to Refseq.NM_207345), are encoded between CLEC-2 and CLEC-1 whereas the other two genes, FLJ31166 and GABA(A) receptor-associated protein like 1 (GABARAPL1), are located centromeric of LOX-1. Interestingly, CLEC-2 and CLEC12b occur in exchanged order and transcriptional orientation in mice when compared to men whereas the other genes show identical order and orientation (figure 29A).

Human GABARAPL1 shares 87% amino acid sequence identity with GABA(A) receptor-associated protein (GABARAP) and is known to be expressed at high levels in the central nervous system (Nemos, Mansuy et al. 2003) and in various other organs (Xin, Yu et al. 2001), but has not yet been described in the context of the human NK gene complex. GABARAPL1 is a member of a group of three human paralogues and two mouse orthologues of human GABARAP, which is encoded on human chromosome 17, and belongs to a microtubule-

associated protein family. It was the first protein identified to interact with the γ2 subunit of GABA(A) receptor (Xin, Yu et al. 2001)(Wang, Bedford et al. 1999).
One gene which could be identified in the murine but not in the human complex is located telomeric of CD94, described as murine NKG2i, also called KLRE1. KLRE1 was shown recently to function as a heterodimer with the ITIM-bearing KLRI1 or KLRI2, thereby generating an inhibitory receptor complex on NK cells and a subpopulation of $CD3^+$ cells (Westgaard, Dissen et al. 2003; Saether, Westgaard et al. 2008). It was not possible to find a human homolog to murine NKG2i in the corresponding region of the human NK gene complex.
As depicted in figure 29B, a clear homology is visible between the genomic regions of the human and murine myeloid clusters, which displays the difference in conservation between coding and non-coding regions. In the region encoding CLEC-2 and CLEC12b no detectable homology between the human and mouse sequences is detected in the analysis, which is explained by the finding that this region is inverted in the murine compared to the human genome as will be discussed below.
Homology analysis further detects areas of low homology in the alignment of the genomic sequence of the human and murine region between GABARAPL1 and CD94. Although this region in mice contains the NKG2i gene, the areas in this region displaying some homology do not correspond to the murine NKG2i exons. In the human genome the low homology areas are actually within an insertion of about 60 kb present only in the human cluster.
In addition, centromeric of GABARAPL1 a short stretch of approximately 140 bp seems to be highly conserved between human and mouse. By searching for regulatory elements a putative binding site for the OCT1 transcription factor could be detected there, assuming that this region might play a role in regulation of one of the surrounding genes (figure 29B).

3.4.2) Gene arrangement in primate and non-primate species

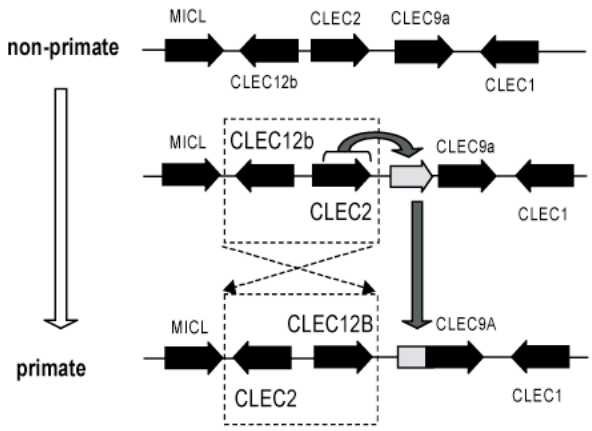


Figure 30: Conformation of the NK gene complex between MICL and CLEC1. Possible events leading from the non-primate to the primate gene arrangement in the part of the NK complex between the genes encoding MICL and CLEC-1. In the non-primate arrangement CLEC-2 and CLEC9a show the same transcriptional orientation and CLEC9a contains the 6 common C-type lectin-like exons. Duplication of 5 CLEC-2 exons followed by the inversion of the region encoding CLEC-2 and CLEC12b could have resulted in the opposite orientation of CLEC-2 and CLEC9A and the addition of three upstream exons to CLEC9A in primates.

The myeloid cluster of the human NK complex seems to be a genomic region showing a high evolutionary activity in more recent times indicated by the AluS/AluJ ratio of 5,25 (147 AluS and 28 AluJ), which is relatively high as compared to the whole genome ratio of 3 (Trowsdale, Barten et al. 2001). AluJ repeats being the evolutionary oldest subfamily diverged 60 million years ago from a common source element, whereas the AluS subfamily was active about 30 million years ago in the ancestral human genome after its divergence from rodents (Price, Eskin et al. 2004; Kapitonov and Jurka 1996; Kumar and Hedges 1998). It is further of interest to note that this region harbours as many AluY, which are the most recently active (24 million years ago) Alu repeats (Carroll, Roy-Engel et al. 2001), as AluJ elements.

Despite the movement of the Alu sequences, the order and orientation of most genes in the myeloid cluster seem to be preserved between mice and men except in the relatively small region of about 40kb containing the CLEC-2 and CLEC12B genes. It was thus of interest to determine the order of the genes of the corresponding myeloid clusters of the syntenic regions in other species such as chimp, rhesus monkey, dog, cow and rat. Interestingly, as shown in figure 30 the gene order and orientation was found to correspond to the human complex only in primate genomes, while those of the other species were similar to the murine NK receptor complex.

Additionally, in the primate complexes, sequences highly homologous to five exons of CLEC-2 were found in the genomic region directly upstream of the coding region of CLEC9A (CLEC-2 exon 2: 96%, CLEC-2 exon 3: 91%, CLEC-2 exon 4: 90%, CLEC-2 exon 5: 87%, CLEC-2 exon 6: 88%). This suggests that a duplication of exons 2-6 of the CLEC-2 gene followed by an inversion of the region containing the complete CLEC-2 and CLEC12B genes has taken place in a common primate ancestor (figure 30).

Interestingly, sequences highly homologous to parts of the CLEC2 gene were also found in the 5'-UTR of CLEC9A mRNA (AY358265) indicating that upstream non-coding exons 1 and 3 of CLEC9A are derived from intronic regions while exon 2 is derived from the second CTLD exon of an ancestral CLEC2 gene. These three exons upstream of the coding region of CLEC9A form a 5'-UTR of about 640bp which contains an open reading frame (ORF) of 273bp starting at position -362bp and ending at position -87bp relative to the CLEC9A translation initiation site. Since mini ORFs in the untranslated region of several genes have been shown to interfere with the translation of the corresponding proteins (Geballe and Morris 1994; Parola and Kobilka 1994; Garnier, Circolo et al. 1995) it is of interest to note that a putative internal ribosomal entry site (IRES) could be identified directly 5' of the start codon (position –93 to –1). This putative IRES could mediate 5`-end-independent ribosomal attachment to an internal position in the mRNA and could thereby facilitate CLEC9A translation as was shown for other genes (Hellen and Sarnow 2001).

3.4.3) Exon-intron structure of the additional genes

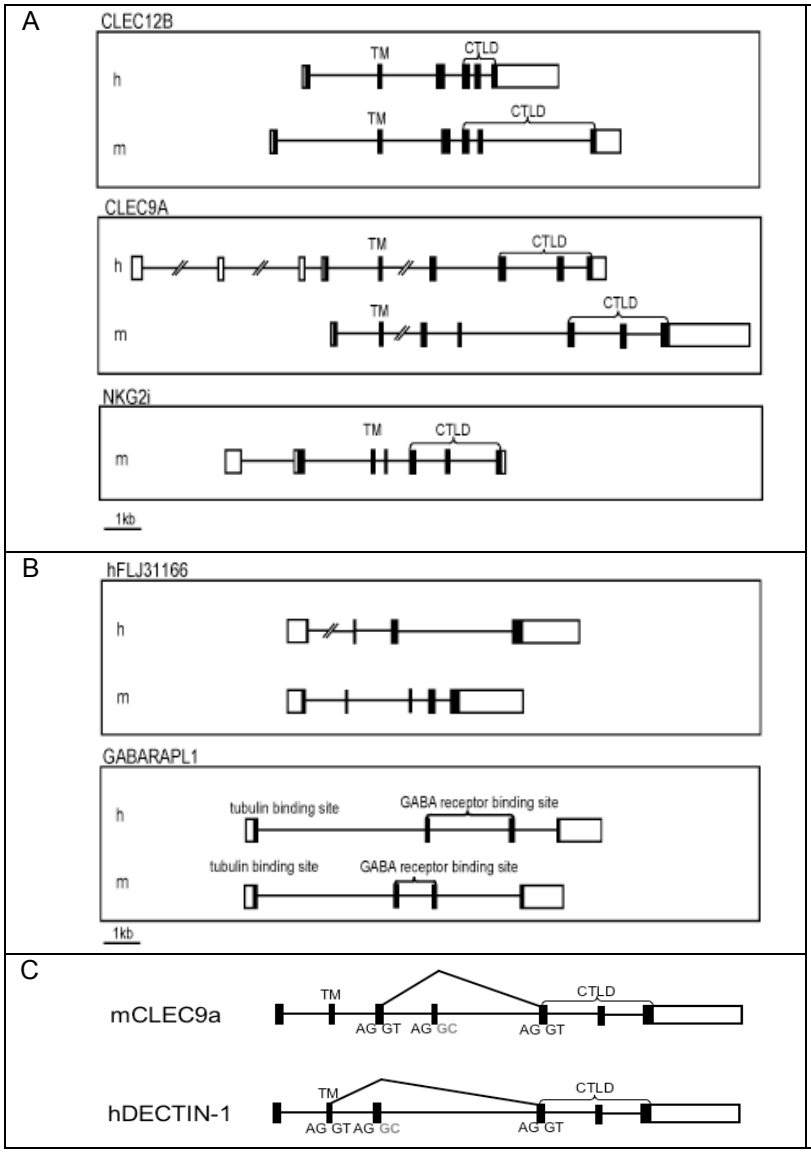


Figure 31: Exon-intron structures of the additional genes of the NK gene complex. The exon-intron structures for the additional genes of the NK gene complex were analyzed by aligning the mRNA sequences of the genes to the corresponding genomic region. The protein domains were identified and assigned to the exons by SMART-analysis. A: Exon-intron structures of the additional genes encoding the lectin-like proteins CLEC12B, CLEC9A and NKG2i. B: Exon-intron structures of the additional genes encoding non-lectin-like proteins FLJ31166 and GABARAPL1. C: Alternative splicing of murine CLEC9a in comparison to human DECTIN-1.

Most of the proteins encoded in the myeloid cluster of the NK gene complex are lectin-like receptors characterized by a type II transmembrane orientation and three C-type lectin-like domains encoded by three exons (Drickamer 1999). Based on an analysis of the exon-intron structure and of the potential protein domains and motifs, the four additional genes and proteins can be classified into two distinct subgroups (figure 31A, 31B).

The first group of genes indeed encodes lectin-like receptors that show the typical exon-intron structure consisting of six exons coding for a N-terminal cytoplasmic region, a transmembrane region, a neck and three C-type lectin-like domains (Drickamer 1999). The intron-exon structure as well as the

predicted protein structure of CLEC12B fits the canonical lectin-like structure, although at least four human CLEC12B mRNA and EST sequences have been found in sequence databases (NCBI) indicating that CLEC12B is differentially spliced.

The mRNA sequence of human CLEC9A contains three untranslated upstream exons in addition to the six exons common to all C-type lectins. As noted before the second UTR exon shows a striking homology of 90% to the first CTLD exon of CLEC-2. Sequences homologous to the other two CTLD exons, the transmembrane and the neck domain of CLEC-2 as well as the corresponding intron and 3'UTR regions were detected in the genomic sequence between UTR exon 1 and UTR exon 3 of the CLEC9A upstream region.

In contrast to human CLEC9A, no additional upstream exons could be identified in murine CLEC9a. However, murine CLEC9a contains two neck exons and two alternatively spliced mRNAs have been reported. The first splice variant shows the common C-type lectin-like exon-intron structure with a single neck domain, whereas the second splice variant contains both exons leading to an extended neck domain in the corresponding protein. As shown in figure 31C this alternative splicing event causing an incorporation of the second neck exon or not, is probably due to a mutation from GT to GC at the 5'-splice site of intron 4, which renders this splice site less efficient as compared to a AG/GT site. A similar mutation at the 5'-splice site of exon 3 probably leads to an alternatively spliced mRNA lacking the stalk exon in DECTIN-1 (Sobanov, Bernreiter et al. 2001).

Importantly, the other two additional genes found in the myeloid cluster, FLJ31166 and GABARAPL1, do not code for lectin-like receptors. For human and murine FLJ31166 transmembrane regions, but no other protein domains or homologies to other proteins could be detected. The exon-intron structure of human und murine GABARAPL1 is made up of four coding exons and the protein does not contain a transmembrane region. The first exon has been reported to encode a tubulin binding site, whereas the sequence of exon three and four codes for a GABA receptor binding site (Xin, Yu et al. 2001).

3.4.4) Amino acid sequences of the additional lectin-like proteins

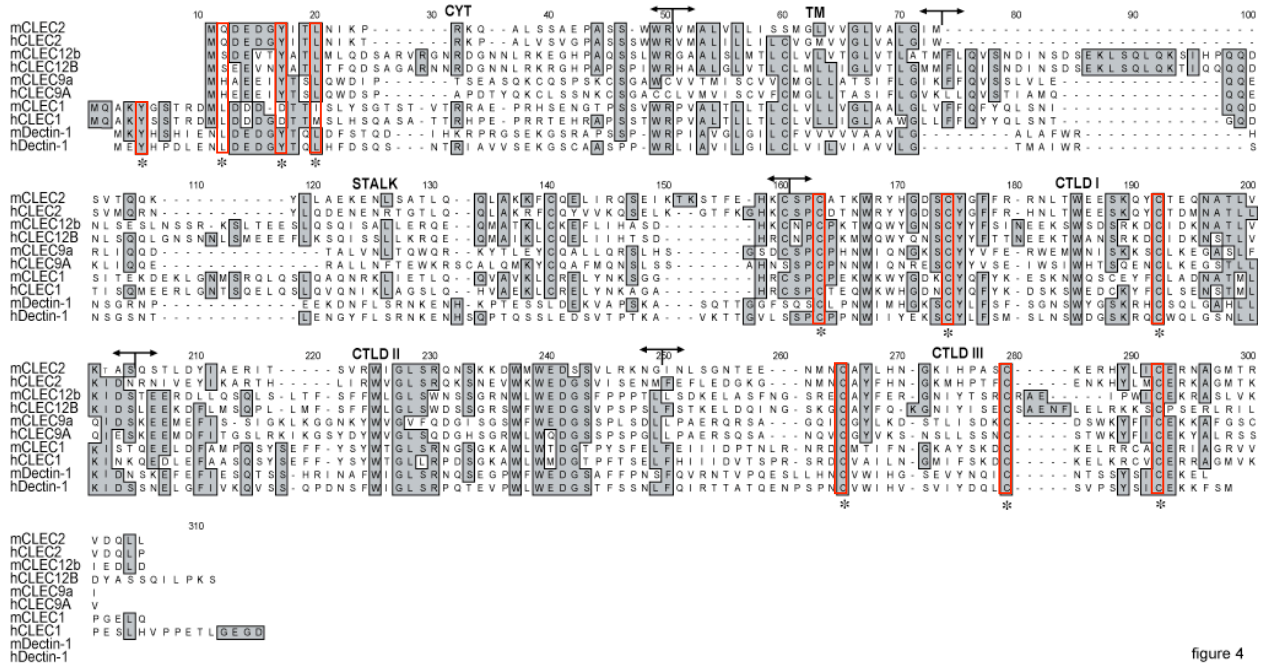


Figure 32: Amino acid sequence of the additional proteins in comparison to known lectin-like receptors. The amino acid sequences of the human and murine homologs of the additional lectin-like proteins CLEC12A and CLEC9A in comparison to the known lectin-like receptors CLEC-1, CLEC-2 and DECTIN-1 are shown. The proteins were aligned using the ClustalW algorithm of the MacVector software. Identical and similar amino acids are marked by boxes. Filled grey boxes indicate identical amino acids. Red boxes highlight ITAM-like motifs in the cytoplasmic part and the highly conserved cysteins in the CTLD, respectively.

The amino acid sequences of lectin-like receptors share common characteristics, such as six highly conserved cystein residues in the extracellular part of the protein and some also contain motifs involved in Ca^{2+}- and ligand binding, namely EPN (mannose binding) / QPD (galactose binding) and WND (Drickamer 1999; Zelensky and Gready 2005). The human and murine homologs of the additional lectin-like proteins CLEC12B and CLEC9A show most typical features of lectin-like receptors. Both proteins in human and mice contain the 6 conserved cystein residues in the first and in the third CTLD, as well as an ITAM-like YXXL-motif in the cytoplasmic tail. This cytoplasmic motif is highly similar to motifs found in the cytoplasmic region of DECTIN-1 and CLEC-2 which have been shown to be essential in DECTIN-1-mediated phagocytosis of Zymosan (Herre, Marshall et al. 2004) and in CLEC-2 mediated platelet activation (Suzuki-Inoue, Fuller et al.) (figure 32).

Similar to most of the other C-type lectin-like proteins of the myeloid cluster, which do not contain known motifs involved in Ca^{2+}- or sugar binding, no such pattern could be identified in CLEC12B and CLEC9A. The only exception in the myeloid cluster is DECTIN-1, which contains one QPD motif. Although these motifs are characteristic for sugar binding and even indicate whether mannose or galactose is bound, their absence does not rule out that the corresponding receptor binds sugars (Zelensky and Gready 2005).

No significant sequence similarities were detected between the lectin-like receptors and FLJ31166 or GABARAPL1 (data not shown). Moreover, these two genes do not share any common characteristics and appear evolutionary not related.

3.4.5) Evolutionary relationship between the lectin-like receptors

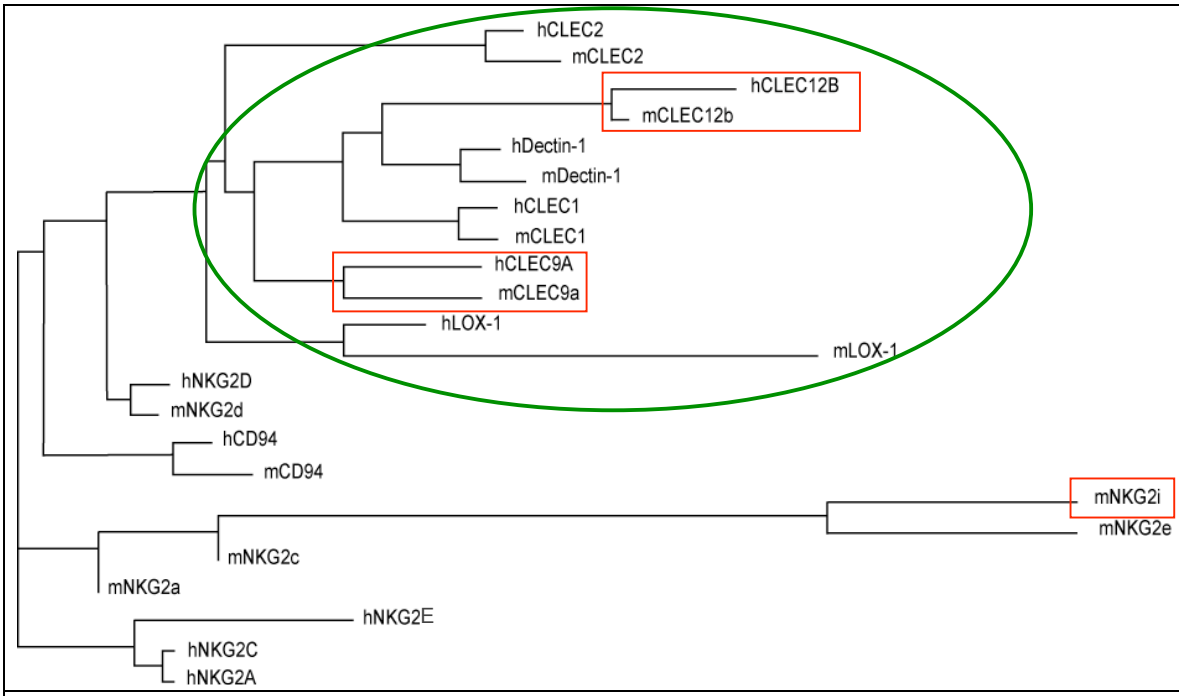


Figure 33: Phylogenetic tree of the genes of the human and mouse NK gene complex based on the CTLD sequences. The phylogenetic tree showing the evolutionary relationship between CLEC12B, CLEC9a and other lectin-like proteins of the NK gene complex. The CTLD parts of the amino acid sequences displayed in figure 3 as well as additional sequences of the human and murine NKG2 gene family were used to construct this phylogenetic tree using the ClustalW algorithm provided by EMBL/EBI (www.ebi.ac.uk/clustalw/). Additional genes boxed in red, myeloid subfamily encircled in green.

To reveal the evolutionary relationship of the additional lectin-like receptors CLEC12B, CLEC9A (and murine NKG2i) to the other C-type lectin-like proteins encoded in the centromeric part of the NK gene complex, a phylogenetic tree based on the amino acid sequences of the CTLD was constructed (figure 33). These C-type lectin-like receptors clearly fall into two separate groups, namely the myeloid and NK receptor groups, and CLEC9A and CLEC12B clearly belong to the myeloid subfamily. The tree furthermore shows that CLEC12B is most closely related to DECTIN-1. CLEC9A is similarly high related to CLEC-1, DECTIN-1 and CLEC12B. mNKG2i on the other hand is most highly related to mNKG2e and is clearly a member of the NK-receptor subfamily. Thus the relationship displayed by the phylogenetic tree corresponds to the arrangement of the receptors in the NK gene complex (see figure 29). It is of interest to note, that in the myeloid subgroup the sequences of the human receptors show highest homology to their murine homologues whereas the human NKG2A, C

and E receptors appear to show higher homology with each other than with the murine homologues, providing an example for convergent evolution of these three receptor chains.

3.4.6) Expression of the additional lectin-like genes CLE12B and CLE9A

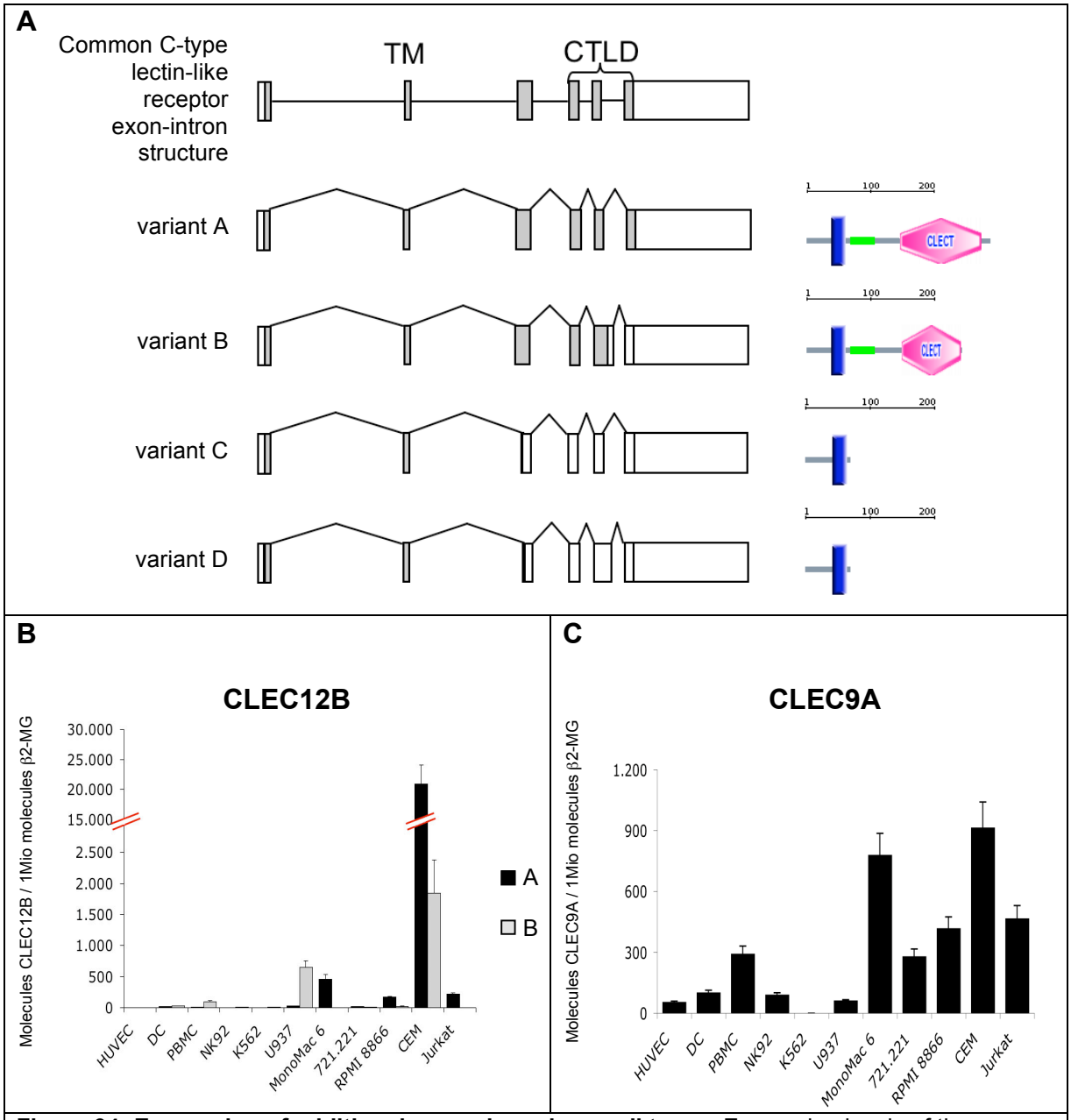


Figure 34: Expression of additional genes in various cell types. Expression levels of the additional lectin-like genes were measured using quantitative Real-Time RT-PCR. Absolute amounts of copies of cDNA were calculated by using standardized amounts of cloned templates. Values obtained for the individual mRNAs were normalized to the corresponding values obtained for β2-microglobulin as internal control and are expressed as molecules / 10^6 molecules β2-microglobulin. Shown are the mean values (± SD) of at least two independent experiments performed in triplicate. A: Schematic representation of splicing events resulting in the four splice variants A to D of CLEC12B (detected by PCR) and the domain structure of the corresponding protein obtained using SMART (available at http://smart.embl-heidelberg.de/smart), vertical blue box: transmembrane region, horizontal green box: coiled coil domain, pink CLECT box: C-type lectin-like domain. B: Expression levels of CLEC12B splice variants A and B in various cell types. C: Expression levels of CLEC9A in in various cell types.

The genes encoded in the human NK receptor complex can be subdivided into two groups according to their expression pattern. The genes of the NKG2/CD94 subfamily are expressed predominantly on NK cells and some on NKT and subpopulations of T cells. In contrast the genes of the myeloid subfamily are expressed on myeloid cells and some on endothelial cells. It was therefore of interest to investigate the expression of the genes newly annotated to the NK receptor complex in cells of various lineages of haematopoietic origin using quantitative Real-Time RT-PCR. GABARAPL1 was found in all cell types tested, whereas expression of FLJ31166 could not be detected in any of the cells (data not shown).

Expression of the C-type lectin-like gene CLEC9A was very low (<100 molecules / one million molecules of β2-microblobulin) in DC, HUVEC, the NK cell line NK-92, the monocytic cell line U-937 and the myeloid-erythroid line K-562. CLEC9A expression was higher (>300 molecules / one million molecules of β2-microglobulin) in the B lymphoid line RPMI 8866, the B-lymphoblastoid line 721.221 and the T cell line Jurkat. The highest levels of expression were detected in the monocytic line Mono-Mac-6 and the T cell line CCRF-CEM (figure 34C)

Using PCR to isolate the complete cDNA of CLEC12B from PBMC we found the mRNA of this molecule to be differentially spliced (figure 34A). Four different splice variants of CLEC12B were detected resulting from two independent differential splicing events. Splice variant A codes for a protein which shows the canonical lectin-like structure of an intracellular domain, a transmembrane domain and a stalk domain encoded by one exon each followed by three exons coding for three CTLD. A differential splicing event at the 3'-end of the second CTLD exon leads to an extension of this exon which contains a stop codon giving rise to a protein lacking the last of the three CTLD. A second differential splicing event does not join the transmembrane coding exon to the 5'-end of the stalk exon but instead uses a potential splice site 8 bp further downstream in the stalk exon. This causes the deletion of 8 bp of the mRNA resulting in a frame shift and the immediate stop of translation. The putative resulting protein contains only the cytoplasmatic and transmembrane domains. Since these differential splicing events also give rise to truncated, potentially non-functional

proteins it was of interest not only to determine the overall expression levels of CLEC12b but also to discriminate especially between putative functional and non-functional isoforms using different sets of primers. Isoforms A and B of CLEC12B are not expressed by HUVEC, the myeloid-erythroid line K-562, the B cell lines 721.221 and RPMI 8866, and the NK cell line NK-92. Low expression could be detected in DC, the monocytic lines U-937 and Mono-Mac-6 and the T-lymphocyte line Jurkat. The T-lymphocyte line CCRF-CEM expressed the highest levels of mRNA. In general, the majority of the transcripts detected in these cells contain the 8 bp deletion in the stalk exon probably rendering the translated product non-functional (data not shown). Only CCRF-CEM cells express substantial levels of CLEC12B mRNA that probably codes for a functional protein (figure 34B). Thus it seems that CLEC12B and CLEC9A do not display the myeloid-specific expression observed for CLEC-1, CLEC-2 and DECTIN-1, but rather are more broadly expressed in the myeloid as well as the lymphocyte lineage.

3.4.7) Regulation of the additional lectin-like genes in dendritic cells.

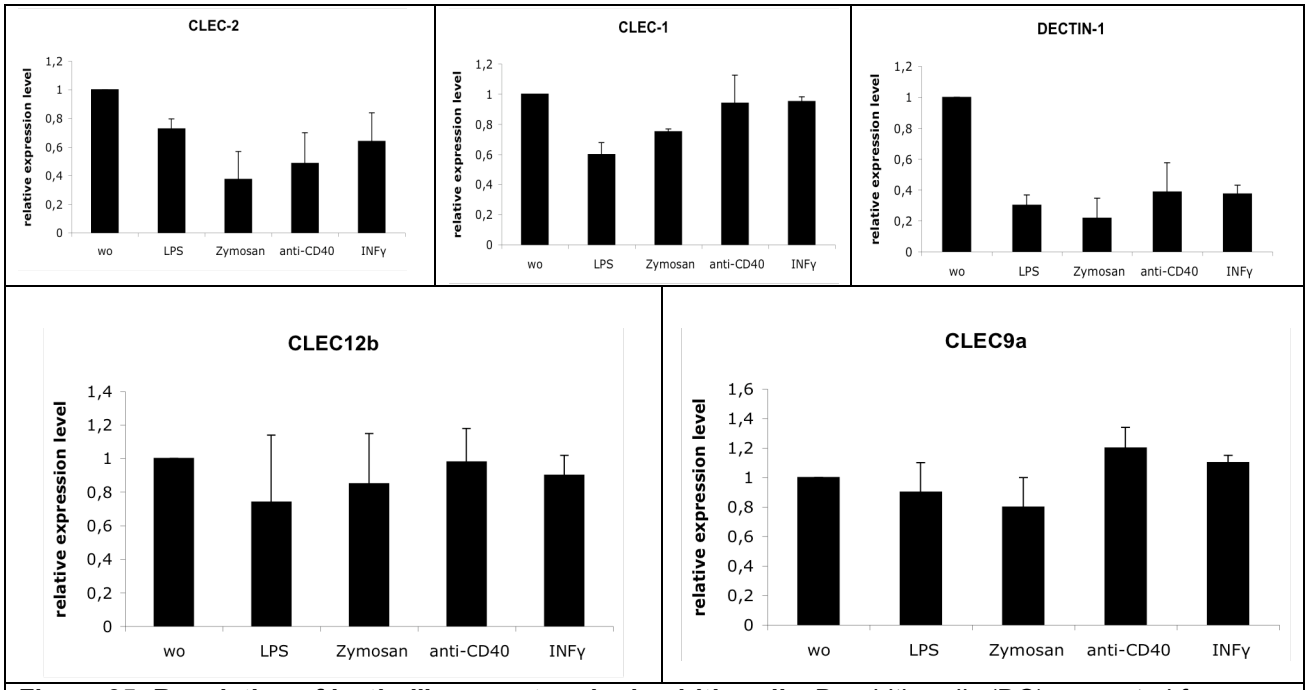


Figure 35: Regulation of lectin-like receptors in dendritic cells. Dendritic cells (DC) generated from $CD34^+$ cells isolated from cord blood were stimulated using LPS, Zymosan, anti-CD40 and IFNγ. Expression levels of the additionals genes were measured using quantitative Real-Time RT-PCR. Absolute amounts of copies of cDNA were calculated by using standardized amounts of cloned template. Values obtained for the individual mRNAs were normalized to the corresponding values obtained for β2-microglobulin as internal control and are expressed as molecules / 10^6 molecules β2-microglobulin. Shown are the mean values (± SD) of at least two independent experiments performed in triplicate.

The C-type lectin-like receptors CLEC-1, CLEC-2 and DECTIN-1 are known to be expressed in dendritic cells (Colonna, Samaridis et al. 2000; Sobanov, Bernreiter et al. 2001; Delneste, Magistrelli et al.) and DECTIN-1 has been shown to be downregulated upon activation of DC (Sobanov, Bernreiter et al. 2001; Delneste, Charbonnier et al.). We therefore investigated the regulation of CLEC12B and CLEC9A in comparison to DECTIN-1, CLEC-1 and CLEC-2 in DC after treatment with various maturation stimuli. To this end DC derived from $CD34^+$ cord blood cells were treated with 100 ng/ml LPS, 25 µg/ml Zymosan A, 4 µg/ml anti-CD40 mAb cross-linked by F(ab')2-fragments of goat anti-mouse IgG and 10 ng/ml INFγ for 6 hours and mRNA levels were measured using Real-Time RT-PCR. Stimulated cells were kindly provided by Dr. Frank Kalthoff (Novartis, Austria).

As expected stimulation of these cells with any of the stimuli used led to a clear downregulation of DECTIN-1. Similar to DECTIN-1, the expression of CLEC-2

was downregulated upon stimulation of DC, however to a lesser extent. CLEC-1 expression on the other hand was only significantly effected in DC stimulated with either LPS or Zymosan but not with anti-CD40 antibody or INFγ.

In contrast, neither expression of CLEC9A nor CLEC12B was significantly altered by treatment of DC with any of the maturation inducing stimuli used (figure 35).

4) Discussion

4.1) CLEC-1 and CLEC-2 are expressed in myeloid cells of PBMC

CLEC-1 and CLEC-2 together with DECTIN-1 and LOX-1 are members of the myeloid subfamily of genes encoded in the NK receptor complex. The first genes identified to be encoded in this complex were found to be specifically expressed in NK and NKT cells, such as the NKG2A, -C and -E genes, which encode inhibitory and activating NK receptor chains. Unlike the NKG2 receptors CLEC-1 and CLEC-2 are not expressed on NK cells, but show a much broader expression pattern. CLEC-2 seems to be restricted to leukocytes, but CLEC-1 mRNA was also found in endothelial cells (Sobanov, Bernreiter et al. 2001; Colonna, Samaridis et al. 2000). As there were no consistent and comparable expression data available for CLEC-1 and CLEC-2 for different blood cell types, the aim of the expression studies was to give a comprehensive overview of the expression of these receptors in the different subtypes of immune cells from peripheral blood.

In our analysis, the presence of CLEC-2 mRNA was detected in total PBMC, granulocytes and platelets. In addition, we found CLEC-2 mRNA in the $CD14^+$ (CD nomenclature see Appendix C) monocytic fraction of PBMC, although the mRNA level was only about one tenth of the level in granulocytes. There was no detectable CLEC-2 mRNA in $CD3^+$ and $CD19^+$ lymphocytic fractions of PBMC. In line with the mRNA data, flow cytometry analysis displayed CLEC-2 on platelets and monocytes. However, despite clear presence of CLEC-2 mRNA in granulocytes, there was no detectable surface expression on this cell type, suggesting additional levels of control either at translation, protein stability or transport to the surface. CLEC-2 mRNA expression in monocytes corresponds with previous reports, which described CLEC-2 mRNA in liver and in blood cells of myeloid origin, including monocytes, granulocytes, and dendritic cells (Colonna, Samaridis et al. 2000). However, protein expression was until now only reported for platelets, where it had been shown to function in platelet activation (Suzuki-Inoue, Fuller et al. 2006).

CLEC-1 mRNA was found at a similar level in granulocytes and $CD14^+$ monocytes and as for CLEC-2 no expression could be detected in $CD3^+$ T-lymphocytes and in $CD19^+$ B-lymphocytes. In contrast to CLEC-2, platelets did not contain significant amounts of CLEC-1 mRNA. Whereas CLEC-2 was expressed on the surface of cells containing CLEC-2 mRNA, with the exception of granulocytes, CLEC-1 could not be detected on the surface of any cell despite clear presence of CLEC-1 mRNA. Similarily, ectopically expressed recombinant CLEC-2 was transported to the cellular surface, whereas CLEC-1 produced from viral vectors in endothelial cells did not lead to detectable surface expression, in spite of a high intracellular accumulation of the protein with an endoplasmic reticulum-like pattern.

The obvious discrepancy between the presence of mRNA on the one hand and the absence of surface protein expression on the other hand could have been caused by translational control, protein instability or the lack of transport to the cell surface. For example, negative translational control of mRNA could take place at any step during protein translation either by regulatory proteins or microRNA recognizing specific motifs present in the 5’ and/or 3’UTR (Gebauer and Hentze 2004). Already translated protein could be degraded immediately or could accumulate in intracellular membranes and be prevented from being transported to the surface. As a case in point, SNAT2 has been demonstrated to move from an endosomal fraction to the surface in myocytes and adipocytes only after stimulation with insulin (Hyde, Christie et al. 2001; Hyde, Peyrollier et al. 2002). Further, PCR analysis can not exclude the possibility, that the detected transcripts are incomplete splice variants or lack any of the essential structural and regulatory features needed for effective translation. In the case of viral-mediated expression, intracellular retention could also be due to an overexpression artifact. The enormous expression of a recombinant protein could overwhelm the protein transport machinery or cause insufficiencies in the availability of a potentially needed surface expression partner.

In the case of the absence of CLEC-2 protein on the surface of granulocytes our experiments suggest either a translational block or a rapid protein degradation, as granulocytes do also not contain detectable amounts of intracellular CLEC-2 protein. Furthermore, ectopic expression of CLEC-2 in

endothelial cells leads to immediate strong surface expression arguing against any problems connected with the surface transport of CLEC-2.

CLEC-1 on the other hand seems to be deficient for surface transport and to be a constituent of intracellular membranes. This is supported by the fact that ectopically expressed CLEC-1 does not reach the cellular surface in endothelial cells. Further, endogenous CLEC-1 protein could be clearly localized to intracellular membranes in endothelial cells as discussed in more detail below.

4.2) Peripheral blood monocytes display CLEC-2 surface expression

During our analysis of blood cell subsets, CLEC-2 could be identified on primary $CD14^+$ monocytes on mRNA as well as on protein level. This might be of relevance as CLEC-2 has been recently reported to bind to podoplanin, a highly O-glycosylated type-1 transmembrane protein, which is expressed on lymphatic but not on blood vessel endothelium. It is therefore widely used as a specific marker for lymphatic endothelial cells (Breiteneder-Geleff, Soleiman et al. 1999). Podoplanin has further been identified in a wide variety of healthy tissues such as kidney, skeletal muscle, placenta, lung and heart (Breiteneder-Geleff, Soleiman et al. 1999; Martin-Villar, Scholl et al. 2005) and shown to be upregulated in several human cancers (Martin-Villar, Scholl et al. 2005; Schacht, Dadras et al. 2005; Shibahara, Kashima et al. 2006; Wicki, Lehembre et al. 2006).

So far, CLEC-2 has been mainly decribed to function as activating receptor on peripheral blood platelets (Suzuki-Inoue, Fuller et al. 2006). In a pathologic situation it is thought that interaction between podoplanin on tumour cells and CLEC-2 on platelets is capable of regulating tumour metastasis, invasion and growth by inducing platelet activation and aggregation on the tumour cell surface (Kato, Kaneko et al. 2008). In normal physiological conditions it is still unknown whether an interaction between podoplanin on lymphatic endothelium and CLEC-2 on platelets plays an important role. Podoplanin is only expressed on lymphatic endothelial cells, but not on blood endothelial cells and it is unclear under which conditions platelets would come in contact with the lymphatics (Suzuki-Inoue, Kato et al. 2007). Podoplanin-deficient mice show defects in lymphatic vessel pattern formation (Schacht, Ramirez et al. 2003), therefore it might be possible that podoplanin CLEC-2 interaction is important during organ development.

Taking all this in consideration, it seems likely that CLEC-2 expressed on monocytes might be an interaction partner for podoplanin on lymphatic endothelial cells, e.g. mediating homing of monocytes to the lymphatics or, in a

pathologic situation, recruitment of monocytes to podoplanin-expressing tumours.

Several PBMC and CBMC samples obtained from healthy volunteers were therefore tested for CLEC-2 expression on their monocytic fraction. In general it was found that CLEC-2 expression was more prominent in PBMC than in CBMC monocytes, although there was a clear donor-dependent variation in the percentage of monocytes with CLEC-2 expression. Whereas most CBMC samples tested were negative or only partly positive for CLEC-2, two thirds of all PBMC samples tested were classified to be strong expressers with more than 20% of monocytes being positive for CLEC-2. Generally, different samples seemed to differ in the percentage of monocytic cells displaying CLEC-2 staining rather than in the strength of the signal. The data are compatible with the possibility that usually a subpopulation of peripheral monocytes expresses CLEC-2 and the size of this subpopulation varies depending on the donor.

Furthermore, not only different donors showed differences in the expression level of CLEC-2, but also single donors tested at different times displayed significant variation. It is conceivable that this variation is explained by the infection status of the donor, causing monocytes to up- or downregulate CLEC-2 surface expression. This hypothesis would also be in line with the fact that CBMC monocytes showed low CLEC-2 expression, as monocytes from cord blood samples are unlikely to have already been exposed to infections.

Western blotting clearly confirmed the presence of CLEC-2 in monocytes, although at a lower level as compared to platelets.

To obtain further clues about the potential importance of CLEC-2 expression on monocytes, we investigated adhesion of CLEC-2 expressing blood cell subsets on different podoplanin expressing cell monolayers. We used MCF-7 cells stably transfected to overexpress podoplanin and lymphatic endothelial cells expressing podoplanin at physiological levels. For adhesion we used DiO-labeled $CD14^+$ monocytes, whole PMBC, lymphocytes, granulocytes, platelets and K-562 overexpressing CLEC-2 as positive control. As expected we found that CLEC-2 expressing platelets showed a clear adhesion to $podoplanin^+$ MCF-7 cells as well as to LEC. In line with the findings that lymphocytes and granulocytes do not express CLEC-2 on their surface, no specific adhesion of lymphocytes and granulocytes to podoplanin expressing cells was detecetd.

Probably due to the low CLEC-2 expression levels, no clear adherence of monocytes to podoplanin$^+$ MCF-7 could be shown, although a tendency of monocytes to adhere slightly stronger to LEC as to HUVEC may be revealed, but would need further evaluation.

As the interaction between CLEC-2 and podoplanin has been reported to have a dissociation constant of 24,5 µM suggesting a weak affinity interaction (Christou, Pearce et al. 2008), the lack of binding could be explained by a too low binding avidity in the *in vitro* assay due to the low CLEC-2 expression on monocytes. Nevertheless this low expression could potentially be sufficient for an interaction of monocytes and lymphatic endothelium *in vivo*.

In summary, this work has supplied clear evidence for CLEC-2 expression on primary CD14$^+$ monocytes, on mRNA level as well as on protein level. However, protein expression levels were found to be variable and the rather low expression will require additional *in vivo* data to reveal the functional implications of CLEC-2 expression by monocytes, e.g. in corresponding CLEC-2 and podoplanin knock-out mice. Nevertheless, the expression of CLEC-2 on monocytes suggests an additional contact and homing pathway for monocytes to lymphatic endothelium.

4.3) CLEC-1 is localized intracellularly in endothelial cells

CLEC-1 shows high homology to CLEC-2 and to the other receptors of the myeloid subfamily of the NK gene complex and in bioinformatic analysis displays all characteristics of a functional type II C-type lectin-like receptor, i.e. a signal anchor, a transmembrane region and three C-type lectin-like domains. It further harbours a predicted coiled coil region adjacent to the transmembrane domain, matching the neck region of C-type lectins. The model of the CLEC-1 tertiary structure based on the homology to the DC-SIGN sequences shows high similarity to the structures of already crystallized family members, such as CLEC-2. Protein sequence analysis further detected regions of intrinsic disorder, which had been reported to be involved in regulation, signaling and control pathways (Uversky, Oldfield et al. 2005; Iakoucheva, Brown et al. 2002; Dunker, Cortese et al. 2005). These regions in CLEC-1 could be detected on the very N-terminus and are probably involved in interacting with partners of the downstream signaling cascade as well as on the very C-terminus possibly involved in ligand binding.

Considering a possible signaling function, the CLEC-1 cytoplasmic region does contain an ITAM-like motif highly similar to DECTIN-1, although DECTIN-1 has been shown to signal exclusively via another ITAM-like motif closer to the transmembrane region (Herre, Marshall et al. 2004), which is shared with CLEC-2 and other members of the family but not present in CLEC-1. A putative signaling function of CLEC-1 remains therefore uncertain. It could be that CLEC-1 has lost its signaling capacity and might be a non-functional protein which has acquired additonal mutations leading to a lack of surface expression. However, the observed 50 to 75% conservation of the CLEC-1 coding sequences between species as far apart as humans and rodents strongly suggests a protein with an important function. In addition, considering that genes of the immune system usually evolve faster than common genes, this degree of conservation is even more striking (Uhrberg 2005; Hughes and Yeager 1997).

Taken together all this evidence supports that CLEC-1 might be a functional protein that should be inserted into cellular membranes with a putative role as

surface receptor. However, although significant levels of mRNA were detected in different blood cells as well as in endothelial cells, CLEC-1 could not be detected on the surface of any cell tested. Furthermore, recombinant CLEC-1 expressed from adenoviral vectors would only accumulate intracellularly and did not reach the cell surface.

It was also conceivable, that CLEC-1 would require a special stimulus to be transported to the cell surface, we therefore triggered endothelial cells with the pro-inflammatory stimuli TNF-α, LPS and PMA and tested for CLEC-1 relocalisation to the cell surface. In addition ligands of TLRs and infection of the cells with selected pathogens was tested to reveal any potential relocalization within or towards the surface of the cell. However, none of the stimuli tested led to surface exposure or any other relocalization of CLEC-1.

Therefore we started to consider the possibility that the cellular surface may not be the physiological location of CLEC-1 within the cell. However, considering the presence of a transmembrane region as well as a type II signal anchor in CLEC-1, the integration into an intracellular membrane was likely.

Indeed, fractionation of endothelial cells known to express endogenous CLEC-1 into cytoplasmic, membrane and nucleic fraction and subsequent analysis of these fractions by Western blotting confirmed the proposed membrane-bound localisation of the protein. Further, immunofluorescence staining of CLEC-1 done in comparison to surface-localized CLEC-2 and markers of the endoplasmic reticulum (ER) and the golgi apparatus, clearly showed that CLEC-1 staining was highly similar to the pattern of ER resident proteins. There was clearly no golgi-like expression and no surface expression comparable to CLEC-2 staining.

To further specify the intracellular localisation of CLEC-1 to the endoplasmic reticulum, we used differential centrifugation of endothelial cell lysates for preparing fractions enriched in cytoplasmic, mitochondrial, lysosomal, microsomal and nucleic proteins. CLEC-1 again was most prominently present in the ER fraction displaying similar staining as the ER protein calnexin supporting an integration of the protein into endoplasmic reticulum membranes. As expected there was clearly no CLEC-1 detected in the cytoplasm as well as in the nucleus, although some CLEC-1 was detectable in the mitochondrial and lysosomal fractions, probably due to unsufficient purity of the fractions. It is

known, that ER resident proteins are localized within the ER because they are actively prevented from entering the secretory pathway by retention and retrieval signals. The classical retention signal for luminal ER proteins such as protein disulfide isomerase (PDI) is a Lys-Asp-Glu-Leu (KDEL) sequence (Luz and Lennarz 1996), whereas most integral membrane proteins such as the chaperon calnexin carry positively charged cytosolic residues like a KKXX (where X is any amino acid) motif (Nilsson and Warren 1994; Andersson, Kappeler et al. 1999). Simple sequence analysis did not reveal a classical motif which could fulfill such a function in CLEC-1, but as it has been proposed for other proteins like LMF1 (Lipase maturation factor 1) its location within the ER may be ensured by interaction with ER proteins possessing a retention signal (Péterfy 2008).

The data thus obtained using recombinant overexpressd CLEC-1 as well as studying endogenous CLEC-1 in endothelial cells strongly support that CLEC-1 may have an intracellular localization and function. It is likely an intracellular member of the myeloid family of pattern recognition receptors of the NK gene complex. In this regard CLEC-1 could be compared to the intracellular Toll like receptors TLR 3, 7 and 9 which recognize microbial nucleic acids such as double-stranded RNA (TLR 3) (Alexopoulou, Holt et al. 2001), single-stranded RNA (TLR 7 and 8) (Diebold, Kaisho et al. 2004) and non-methylated CpG-containing DNA (TLR 9) (Hoene, Peiser et al. 2006). TLR9 for example has been shown to be expressed in the endoplasmic reticulum but to be relocalized to lysosomes upon activation of the cell by CpG-ODN (Latz, Schoenemeyer et al. 2004). Given the similarities in localization we decided to test whether ligands of intracellular Toll like receptors or other pro-inflammatory stimuli would show effects on CLEC-1 expression or in terms of preferential ER or lysosomal localization. However, intracellular relocalisation of CLEC-1 in dendritic cells overexpressing CLEC-1 could not be detected upon stimulation with any of the reagents tested including CpG-ODN, poly(I:C) and Imiquimod. However, Real-Time RT-PCR using cDNA preparations of stimulated dendritic cells revealed a downregulation of CLEC-1 mRNA upon poly(I:C), CpG, TNF-α, LPS and PMA stimulation, whereas incubation with imiquimod did not show any significant effect. The absence of downregulation following imiquimod treatment is in contrast to the downregulation pattern observed for other receptors of the family

such as DECTIN-1, which is efficiently downmodulated by imiquimod as well. It remains to be seen whether this could indicate a role of CLEC-1 in the response to single-stranded RNA viruses. At the moment the general pattern of downregulation in dendritic cells might rather suggest that the effect is not specific for CLEC-1 but is a common effect of maturation of the dendritic cells. Upon encounter of pathogens, dendritic cells start to mature initiating the downregulation of receptors no longer needed for antigen uptake, whereas on the other hand receptors for migration to lymph nodes and antigen presentation are upregulated. Although the downregulation of CLEC-1 upon dendritic cell maturation does not define a specific role in the process of pattern recognition it could be taken as evidence compatible with a function of CLEC-1 in the process of pathogen detection in the immature stages of DC.

Given the intracellular localization of CLEC-1 it appeared possible that CLEC-1 could play a role in the response to intracellular pathogens. We therefore investigated whether CLEC-1 would be able to bind to selected examples of eucaryotic and procaryotic intracellular parasites, namely *Toxoplasma gondii* and *Listeria monocytogenes*. We isolated both *Toxoplasma gondii* and *Listeria monocytogenes* and tested on the one hand the direct binding of soluble CLEC-1 to the isolated pathogens and on the other hand intracellular (co)localization of CLEC-1 with the pathogens in infected cells. However, CLEC-1 neither bound the isolated parasites or bacteria in a way similar to the binding of yeast cell walls by DECTIN-1 nor could we detect any signs of interaction, e.g. accumulation of CLEC-1 around sites of infection within infected cells. Thus, it appears that CLEC-1 is not involved in the response to *Toxoplasma gondii* and *Listeria monocytogenes* and a definition of the function of this protein will likely have to await serial testings of a large number of additional candidates.

4.4) The myeloid subfamily of the NK gene complex contains additional lectin-like genes

The centromeric part of the NK-gene complex contains two different subgroups of genes. One subfamily of C-type lectin-like receptor genes comprises the NKG2 and CD94 genes, which are expressed on NK, NKT and some T cell subpopulations (Hofer, Sobanov et al. 2001). The genes localized more telomeric in the centromeric half of the complex are more broadly expressed, preferentially by cells of the myeloid lineage (Hofer, Sobanov et al. 2001). The members of these two subfamilies do not only show similar expression patterns but also share the highest sequence similarities within each family. Furthermore, the genomic distances between the genes of one subfamily are short, whereas the stretch of non-coding sequences physically separating the myeloid from the NK subfamily, is much longer, suggesting that these families originated from consecutive gene duplications.

In this work we concentrated on the myeloid cluster encoding among others genes identified in our laboratory (Sobanov, Bernreiter et al. 2001).

In the human complex two additional C-type lectin-like genes, CLEC9A and CLEC12B, were shown to be encoded between CLEC-1 and CLEC-2. Because of their location and high sequence homology to the neighbouring receptors of the myeloid cluster, both genes clearly are members of the myeloid subgroup. Like the other receptors of the myeloid cluster of the NK receptor complex, the CLEC9A and CLEC12B genes have orthologues in the murine genome and were therefore probably already present in a common human-murine ancestral genome.

In addition to CLEC12B and CLEC9A, two genes not coding for C-type lectin-like proteins, FLJ31166 and GABARAPL1, were identified between the two subgroups but in close proximity to the centromeric end of the myeloid cluster. The proteins encoded by those genes do not show any homology to the lectin-like receptors of the myeloid cluster or to those of the NK cluster. These genes are also regulated differently from the other genes of the NK complex. FLJ31166 appears not to be expressed in cells of the haematopoietic lineage since we could not detect any mRNA, neither in any of the cell lines tested nor

in PBMC (data not shown). In contrast GABARAPL1 seems to be expressed ubiquitously in a variety of tissues (Nemos, Mansuy et al. 2003), including all haematopoietic cells tested (data not shown). This indicates that these genes stand apart from the lectin-like genes characterized in the NK gene complex. Another gene belonging to the NK receptor subfamily, NKG2i, is encoded telomeric of CD94 in the murine complex. The presence of this gene in the murine complex is a major difference between the human and the murine cluster, because the syntenic human region does not contain an NKG2i homolog. Instead, it displays an additional stretch of non-coding DNA of about 60 kb showing no considerable homology to the murine cluster. As this region is only present in the human genome, this difference could have resulted either from an insertion into the human or a deletion from the murine sequence. The members of the NKG2 subfamily appear to have arosen from gene duplications of one single common ancestral sequence (Barten, Torkar et al. 2001), it might be that the murine NKG2i is the result of a relative recent duplication event, which did not occur in humans.

Another prominent difference between the primate and the rodent cluster is an inversion between CLEC9A and MICL that causes the primate CLEC-2 and CLEC12B to display opposite transcriptional orientation as compared to their homologous in the other species investigated. As in addition a stretch of sequence upstream of the primate CLEC9A coding region shows high homology to CLEC-2 we suggest that this inversion took place after a partial duplication of the gene encoding CLEC-2 in the genome of a common primate ancestor.

The additional genes CLEC9A and CLEC12B show all typical characteristics of C-type lectin-like genes as far as amino acid sequences, exon-intron structure and corresponding protein domains are concerned. CLEC9A is unusual as far as it contains three non-coding upstream exons, probably originating from duplication of part of the CLEC-2 gene.

CLEC12B has been reported shortly after we finished our analysis to function as inhibitory receptor in macrophages by recruiting the phosphatases SHP-1 and SHP-2 through its ITIM (immunoreceptor tyrosine-based inhibition motif) domain (Hoffmann, Schellack et al. 2007). Our analysis found CLEC12B to be differentially spliced. In addition to an mRNA coding for a regular lectin-like

protein three additional splice variants were identified resulting from two independent alternative splicing events. All these differential splicing events lead to truncations and probably non-functional proteins.

Alternatively spliced isoforms have been described for other receptors of this complex. In particular mature mRNAs of DECTIN-1 and CD94 have been demonstrated to be generated by multiple splicing events leading to various isoforms of which some code for truncated and potentially non-functional proteins (Willment, Gordon et al. 2001; Gavino, Chung et al. 2005; Lieto, Maasho et al. 2006). Moreover, functional isoforms lacking the stalk exon of NKG2A, known as NKG2B, DECTIN-1 and CD94 have been shown to exist (Willment, Gordon et al. 2001; Furukawa, Yabe et al. 1998; Lieto, Maasho et al. 2006). Curiously, in the case of CLEC12B these truncated mRNAs encoding probably non-functional proteins, constitute the majority of transcripts in most cell types tested. It is however possible that mRNAs coding for full length CLEC12B are transcribed only in certain cell types or upon certain stimuli not tested in this study.

CLEC9A contains an ITAM-like motif in its cytoplasmic tail similar to that found in DECTIN-1 and CLEC-2. The same motif in DECTIN-1 has been shown to be essential in phagocytosis of Zymosan (Herre, Marshall et al. 2004), which results in the phosphorylation of the tyrosine residue of the YxxL motif. This in turn leads to recruitment of the spleen tyrosine kinase (Syk) which is then activated by phosphorylation (Rogers, Slack et al. 2005; Underhill, Rossnagle et al. 2005). This is similar to the ITAM-like motif in CLEC-2 which is involved in platelet activation and has also been implicated in recruitment and activation of Syk (Suzuki-Inoue, Fuller et al. 2006). It is therefore tempting to speculate that the ITAM-like motifs in CLEC9A may be involved in cellular activation. Indeed, very recent studies by *Huysamen et al.* showed that CLEC9A recruits Syk and mediates endocytosis (Huysamen, Willment et al. 2008).

Because both CLEC12B and CLEC9A share all major characteristics with the other lectin-like receptors encoded by genes of the myeloid cluster it is possible that these proteins fulfil similar functions. However, the pattern of expression of these two genes shows some differences as compared to the other members of the myeloid subfamily. Although CLEC12B and CLEC9A are expressed in cells of the myeloid lineage similar to CLEC-1, CLEC-2 and DECTIN-1, highest

expression was detected in the T cell line CCRF-CEM. Moreover, neither CLEC12B nor CLEC9A expression is significantly downregulated upon stimulation of dendritic cells using different stimuli, a feature common to the other C-type lectin-like receptors of the myeloid subfamily. Thus these two genes may differ to some extent from the others of the subfamily displaying differences in cell type specific expression and regulation. For example, CLEC9A expression was recently described to be present specifically only on $BDCA3^+$ dendritic cells and on a small subset of $CD14^+CD16^-$ monocytes (Huysamen, Willment et al. 2008).

In summary, we have identified additional genes in the human and murine NK receptor complex and have investigated their sequence characteristics, phylogenetic relationship and expression pattern. While two of these genes do not belong to the lectin-like receptor family, the other two clearly are members of the myeloid subfamily of C-type lectin-like receptors based on sequence homology and expression patterns.

5) References

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Acknowledgements

I would like to give my deepest thank and appreciation to all people who helped me to achieve this goal:

Supervisor: Erhard Hofer

Thesis and defence reviewers: John Trowsdale, Pavel Kovarik

Collaborators: John Trowsdale, Thomas Decker, Michael Detmar, Frank Kalthoff, Wolfgang Gregor, Karl-Heinz Preisegger

Colleagues & Fellows: Hormas Ghadially, Dorit Reiche, Irene Karas, Isaak Quast, Manuela Macek, Caterina Sturtzel, Julia Testori, Karoline Lipnik, Tom Bobrzynski

Family and Friends

Thank you very much!

6) Appendix

A: List of buffer ingredients

buffer	ingredients
Blocking buffer (Western Blot)or	5% BSA (w/v) 0,5% skim milk powder (w/v) 0,1% Tween 20 (v/v) in PBS
Blotting buffer (Western Blot)	25mM Tris-HCl 192mM glycine 20% methanol (v/v)
Dialysis buffer (Adenovirus generation)	40% glycerin (v/v) 20mM HEPES 50mM NaCl
Et_3N buffer (Cell Fractionation)	250mM sucrose 10mM Et_3N 1mM EGTA pH7,4
FACS buffer (Flow cytometry)	2 % FCS 2mM EDTA 0.1% NaN_3 in PBS
HE buffer (Adenovirus generation)	10mM HEPES 1mM EDTA
NH_4Cl buffer (Erythrocyte Lyses)	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM EDTA pH 7.4
PBS	140 mM NaCl 10 mM KCl 6,4 mM $Na_2HPO_4x\ 2H_2O$ 2 mM KH_2PO_4
Sample Buffer (SDS-PAGE)	0,0625mM Tris-HCl pH6,8 10% glycerol (v/v) 2% SDS (w/v) 5% mercaptoethanol (v/v) 0,05% bromphenolblue (w/v)
SDS electrophoresis buffer (SDS-PAGE)	25mM Tris 250mM glycine 0,1% SDS (w/v)
Stripping buffer (Western Blot)	50mM Tris 2% SDS (w/v) 100mM mercaptoethanol, pH 6.8

B: Abbreviations

abbreviation	full name
ActA	Actin-assembly inducing protein precursor
AGE	Advanced glycation end product
BHI	Brain heart infusion
BSA	Bovine Serum albumin
CARD9	Caspase-recruitment domain 9
CBDC	Cod blood dendritic cells
CBMC	Cord blood mononuclear cells
CLEC-1/2	C-type lectin-like receptor-1/2
COX-1	Cytochrome c oxidase subunit 1
COX2	Cyclooxygenase 2
CpG-ODN	CpG-oligodesoxynucleotide
CRD	Carbohydrate recognition domain
CTLD	C-type lectin-like domain
DAPI	4',6-diamidino-2-phenylindole
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEC205	Dendritic and epithelial cells, 205 kD
DECTIN-1	Dendritic cell-associated C-type lectin-1
DEPC	Diethylenepyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
EST	Expressed sequence tag
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3-L	FMS-like tyrosine kinase 3-ligand
GABARAPL1	GABA(A) receptor-associated protein like 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEK293	Human embryonic kidney 293
HEV	High endothelial venules
Hpt	Hexose phosphate transporter
HRP	horse radish peroxidase
HSV	Herpes simples virus
HUVEC	Human umbilical vein endothelial cells
IFN-β	Interferon-β
IL-1	Interleukin-1
InlA	Internalin A
IRES	Internal ribosomal entry site
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun-NH2-kinases
LAMP-1	lysosomal-associated membrane protein 1
LEC	Lymphatic endothelial cell
LLO	Listeriolysin
LOX-1	Oxidized low-density lipoprotein receptor-1
LplA1	Lipoate ligase A1
LPS	Lipopolysaccharid
m/pDC	Myeloid/plasmacytoid dendritic cells
MACS	Magnetic cell sorting
MAFA	Mast cell Function-associated Antigen

MAPK	Mitogen-activated protein kinase
MBP	Mannose Binding Protein
MCMV	Murine Cytomegalovirus
M-CSF	macrophage colony-stimulating factor
MEMalpha	Minimum Essential Medium Alpha
MHC	Major histocompatibility complex
MICL	myeloid inhibitory C-type lectin-like receptor
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor κB
NK cells	Natural killer cells
NKG2	natural-killer group 2
NKRP	NK-cell receptor protein
NKT	Natural Killer T cell
OCIL	Osteoclast inhibitory lectin
ORF	Open reading frame
oxLDL	Oxidized low density lipoprotein
PAMP	pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS (+T)	Phosphate buffered saline (+Tween)
PEF	penetrating enhancing factors
PI3K	phosphoinositide 3-kinase
PI-PLC	phosphatidylinositol-specific phospholipase C
PLA_2	phospholipase A_2
PLCγ2	phospholipase Cγ2
PLN	Peripheral lymph node
PMA	phorbol myristate acetate
PMSF	Phenylmethylsulfonylfluorid
PVDF	Polyvinylidene fluoride
RIG-1	Retinoic acid-inducible gene-1
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TGF-β	transforming growth factor-β (
TIR	Toll/interleukin-1 receptor-like domain
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-α
TPO	Thrombopoietin
TRAF	TNF Receptor Associated Factor family of proteins
TRIF	TIR-domain-containing adapter-inducing interferon-β
UTR	Untranslated region

C: CD molecules

CD	Alternative name	Expression pattern
CD107a	LAMP-1	Lysosomal marker, surface of NK upon activation
CD11b	MAC-1	T cells, B cells, NK cells, monocytes, granulocytes
CD14	LPS-R	Monocytes, granulocytes
CD15	LewisX	Monocytes, granulocytes
CD151	SFA-1, PETA-3	platelets, stem cells, endothelium, epithelium
CD16b	FcγR	T cells, NK cells, monocytes, granulocytes, endothelium
CD19	B4	B cells, DC
CD1a	R4T6, HTA-1	DC
CD3	T3	T cells
CD34	gp105-120	Stem cells, endothelium
CD4	L3T4, T4	T cells, monocytes, granulocytes
CD40	Bp50	B cells, DC, stem cells, monocytes, endothelium, epithelium
CD154	CD40L, CD40 Ligand	Activated T cells
CD62L	L-Selectin	T cells, B cells, NK cells
CD83	HB15	B cells, DC
CD94	Kp43	T cells, NK cells

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