CEACAM1 and ICAM-3 from human granulocytes bind DC-SIGN of dendritic cells through Lewis x residues

DOCTORAL THESIS

by

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Contains

1. ABBREVIATIONS

APS	**Ammoniumpersulfat**
Asn	**Asparagine**
bp	**Base pair**
BSA	**Bovines Serum Albumin**
°C	**Degree Celsius**
cDNA	**complementary DNA**
CAMs	**Cell-adhesion molecules**
CEA	**Carcinoembryonic Antigen**
CEACAM1	**CEA- related cell adhesion molecule-1**
cm	**Centimetre**
Da	**Dalton**
DC-SIGN	**Dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin**
DMEM	**Dulbecco's Modified Minimal Eagle Medium**
DMSO	**Dimethylsulfoxide**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxyribonucleoside triphosphate**
DTT	**Dithiothreitol**
ECM	**Extracellular matrix**
ECL	**Enhanced chemiluminescence**
E. coli	**Escherichia coli**
EDTA	**Ethylenediaminetetraacetic acid**
EGF	**Epidermal growth factor**
EVTs	**Extravillous trophoblasts**
FCS	**Fetal calf serum**
FITC	**Fluorescein isothiocyanate**
fMLP	**N-formyl-methionyl-leucyl-phenylalanine**
Fuc	**Fucose**
FUT(s)	**Fucosyltransferase (s)**

FUTIII	**Fucosyltransferase III**
FUTIV	**Fucosyltransferase IV**
FUTVII	**Fucosyltransferase VII**
FUTIX	**Fucosyltransferase IX**
g	**Gram**
Gal	**Galactose**
GalNAc	**N-Acetylgalactosamine**
GDP-fucose	**Guanosine diphosphate-fucose**
Glc	**Glucose**
GlcNAc	**N-Acetylglucosamine**
GPI	**Glycosylphosphatidylinositol**
h	**Hour (s)**
ICAM	**Intercellular adhesion molecule**
ICAM-3	**Intercellular adhesion molecule-3**
Ig	**Immunoglobulin**
IL	**Interleukin**
ITAM	**Immunoreceptor tyrosine-based activation motif**
ITIM	**Immunoreceptor tyrosine-based inhibition motif**
kb	**Kilo base pair**
kDa	**Kilodalton**
L	**Litre**
LAMP1	**Lysosome-associated membrane protein 1**
LB-Medium	**Luria-Bertani-medium**
Lea	**Lewis a**
Leb	**Lewis b**
Lex	**Lewis x**
Ley	**Lewis y**
LN	**Lactosamine**
M	**Molarity (molar concentration), mol/L** **Molar solution**
MALDI-TOF MS	**Matrix-assisted laser desorption/ionization time-offlight mass spectroscopy**

Man	**Mannose**
mA	**Milliampere**
mg	**Milligram**
min	**Minutes**
mL	**Millilitre**
mM	**Millimolar**
mRNA	**Messenger ribonucleic acid**
NANA	**N-Acetylneuraminic acid (sialic acid)**
NCAMs	**Neural-Cell Adhesion Molecules**
ng	**Nanogram**
nm	**Nanometre**
µg	**Microgram**
µL	**Microlitre**
MIP-1	**Macrophage inflammatory protein-1**
NaAc	**Sodium acetate**
NK-cells	**Natural killer-cells**
OD	**Optical density**
p.a.	**pro analysis (for analytical purposes)**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PDGF	**Platelet-derived growth factor**
PECAMs	**Platelet-Endothelial-Cell Adhesion Molecules**
PE	**Phycoerythrin**
PI	**Propidium iodide**
PMSF	**Polymethylsulfonylfluoride**
PSGs	**Pregnancy-specific glycoproteins**
rpm	**Rounds per minute**
SDS	**Sodiumdodecylsulfate**
sec	**Second (s)**
SSEA-1	**Stage-specific embryonal antigen-1**
TAE	**Tris-Acetate-EDTA**
TBS	**Tris buffered saline**

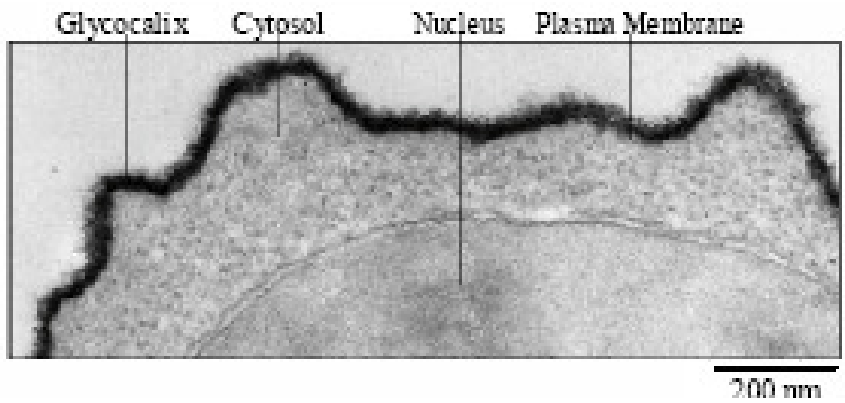
TBS-T	**Tris buffered saline with Tween 20**
TEMED	**N,N,N‘,N‘-Tetramethylethylene diamine**
Tris	**Tris (hydroxymethyl)-aminomethane**
U	**Unit**
UV	**Ultraviolet**
V	**Volt**
VCAM-1	**Vascular cell adhesion molecule-1**
VEGF	**Vascular endothelial growth factor**
v/v	**Volume per volume**
w/v	**Weight per volume**
X-Gal	**5-Bromo-4-chloro-3-indoxyl-ß-D-galactopyranoside**

2. INTRODUCTION

2.1. Glycoproteins

Heterogeneous glycans decorate the extracellular regions of the glycoproteins bound to cell membranes building the so-called glycocalyx. Membrane-associated carbohydrates are in the form of polysaccharides covalently attached to proteins forming glycoproteins, and covalently attached to lipid forming the glycolipids.

a)



b)

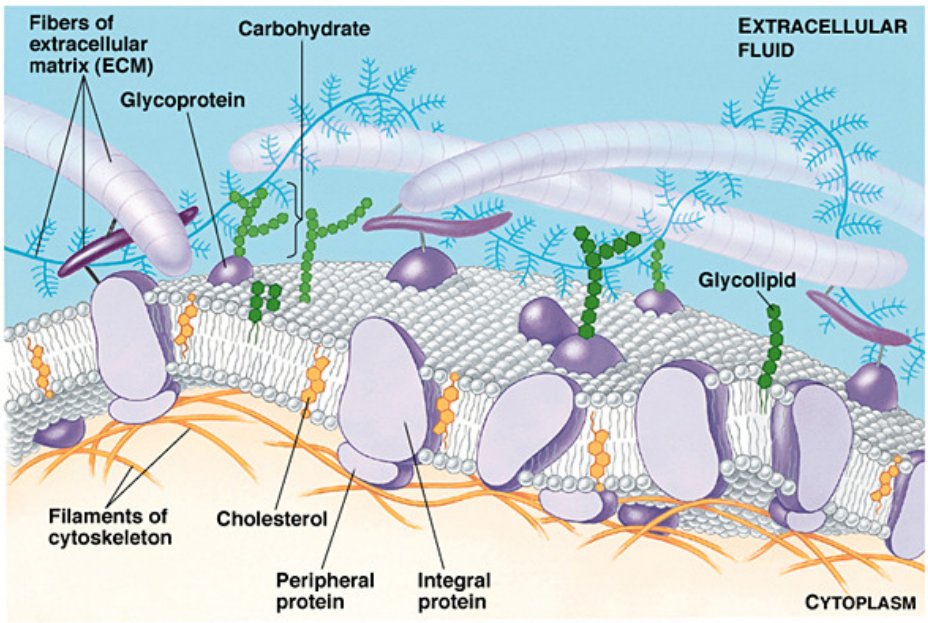


Figure 1. a) Electron micrograph of a lymphocyte showing the carbohydrates layer (Alberts and Bray 1994)**; b) 3D image of protein membrane**

(*fig.cox.miami.edu/Faculty/Dana/104F01_3.html,*) (1999 Addition Wesley Longman Inc)

There are many different types of polysaccharides plus some polyalcohols and amino sugars in the glycocalyx, and the exact makeup is species specific.

The predominant sugars found in glycoproteins are glucose, galactose, mannose, fucose, N-Acetylgalactosamine (GalNAc), N-Acetylglucosamine (GlcNAc) and N-Acetylneuraminic acid (NANA). The distinction between proteoglycans and glycoproteins resides in the level and types of carbohydrate modification. The carbohydrate modifications found in glycoproteins are rarely complex: carbohydrates are linked to the protein component through either O-glycosidic or N-glycosidic bonds. The N-glycosidic linkage is through the amide group of asparagine. The O-glycosidic linkage is to the hydroxyl group of serine, threonine or hydroxylysine. The linkage of carbohydrate to hydroxylysine is generally found only in the collagens. Carbohydrates linked to 4-hydroxylysine are either the single sugar galactose or the disaccharide glucosylgalactose (Glcα1,2Galβ). In Ser- and Thr-type O-linked glycoproteins, the carbohydrate directly attached to the protein is GalNAc. In N-linked glycoproteins, it is GlcNAc.

The predominant carbohydrate attachment in glycoproteins of mammalian cells is via N-glycosidic linkage. The site of carbohydrate attachment to N-linked glycoproteins is found within a consensus sequence of amino acids, Asn-X-S(T), where X is any amino acid except proline. N-linked glycoproteins all contain a common core of carbohydrate attached to the polypeptide. This core consists of three mannose residues and two GlcNAc. Varieties of other sugars are attached to this core and comprise three major N-linked groups: high-mannose type, complex type and hybrid type (Fig. 2).

Cell and tissue-specific glycosylation of glycoproteins (post-translation modification) is mediated by the expression of different glycosyltransferases and glycosidases, which add or remove specific carbohydrates residues.

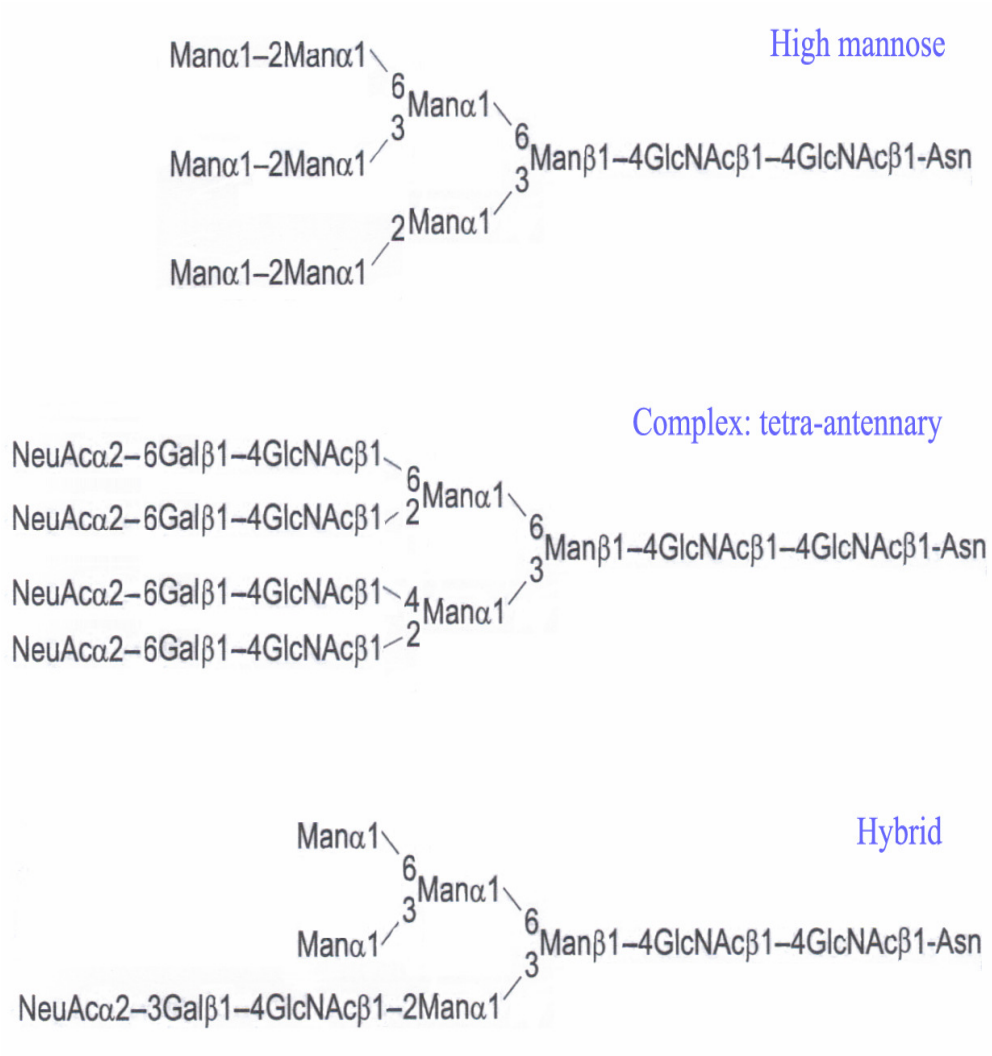


Figure 2. Structures of N-linked glycans (Taylor and Drickamer 2006)

Modified or incomplete structures of lactosaminoglycans have been reported for a number of diseases; both acquired (e.g. cancer diseases, rheumatoid arthritis) and inherited (some congenital dyserythropoietic anemias). In addition, many discoveries have been made showing that oligo/polylactosamines are good binders of various pathogenic microbes and toxic proteins. Lactosaminoglycans may serve as mediators of infections or may contribute to a barrier for the entry of pathogens and toxic proteins.

2.2. Fucosyltransferases

A fucosyltransferase is an enzyme that transfers an L-fucose sugar from a GDP-fucose (Guanosine diphosphate-fucose) donor substrate to an acceptor substrate. The acceptor substrate can be another sugar such as the transfer of a fucose to a core GlcNAc (N-acetylglucosamine) sugar as in the case of N-linked glycans. Fucosylated glycoconjugates play important roles in lymphocyte trafficking, embryogenesis, inflammation, cancer metastasis and the synthesis of blood group antigens.

Fucosyltransferases (FUTs) catalyze the transfer of fucose in α(1,2), α(1,3), α(1,4), and α(1,6) linkages to various oligosaccharide acceptors.

Thirteen fucosyltransferase genes have been identified so far in the human genome, which each generates a unique range of fucosylated products. FUTI and FUTII are α(1,2)-fucosyltransferases responsible for synthesis of the H blood group antigen and related structures (Kelly, et al. 1995). FUT VIII is an α(1,6)-fucosyltransferase that directs addition of fucose to asparagine-linked GlcNAc moieties, a common feature of N-linked glycan core structures (Miyoshi, et al. 1999). Alpha 1,3-fucosyltransferases (α1,3-FUTs) catalyze the final step in the synthesis of a range of important glycoconjugates like those that function in cell adhesion and lymphocyte recirculation (de Vries, et al. 2001)

Six human α1,3 FUT genes have been cloned, FUTIII, FUTIV, FUTV, FUTVI, FUTVII and FUTIX. FUTIV and FUTIX are the most important α1,3-FUTs accountable for synthesis of the Lewis x (Lex) structure.

FUTIX is expressed in human leukocytes, glandular compartments of the stomach and brain, whiles FUTIV is expressed in a variety of tissues. Expression of FUTIV in a number of tissues does not correlate with the expression of Lex (Kaneko, et al. 1999). FUTIX is expressed in human granulocytes and is the main enzyme responsible for the expression of Lex residues on granulocytes. Distal lactosamine units (Galβ1,4GlcNAc; LN) of the polylactosamine chain is

fucosylated through α(1,3)-FUTIX activity to form the Lewis x (Lex; CD15) epitope (Nakayama, et al. 2001).

FUTIX preferentially transfers a fucose to the GlcNAc residues at the distal LN unit of the polylactosamine chain whereas FUTIV to the inner LN units (Nishihara, et al. 1999). FUTVII expressed in leukocytes determines the expression of sialyl Lewis x (sLex) (Clarke and Watkins 1996).

2.3. Lewis x (Lex)

The Lex (CD15) residue has been identified as stage-specific embryonic antigen-1 (SSEA-1) at the morula stage of the mouse embryo and is defined as terminal epitope of oligosaccharide chains with the common structure [Galβ1,4 (Fucα1,3)GlcNAc-R] (Kobata and Ginsburg 1969, Hakomori, et al. 1981).

The Lewis x epitopes are expressed in some tissues, such as epithelial cell of intestinal tissues (Hakomori 1992), certain neurons, glial cells in the central nervous system (Ashwell and Mai 1997), mature granulocytes and all myeloid cells from the promyelocytes stage onwards (Nakayama, et al. 2001). In human leukocytes, CD15 is expressed preferentially in mature neutrophils and monocytes. Lewis x is a member of Lewis blood group antigens that are biosynthetically and structurally related carbohydrate structures.

Oligosaccharides with the Lex determinant are accumulated in large quantities in various adenocarcinomas (Singhal, et al. 1990). Several gram-negative bacteria can express human blood group antigens. *Schistosoma mansonii*, a microbe that causes persistent infections (the majority of infected persons remain without clinical signs), expresses Lex antigen (Nutten, et al. 1999). The Gram-negative bacterium *Helicobacter pylori*, the major cause of chronic gastritis and duodenal ulcers produces LPS O-antigen units that can be posttranslationally fucosylated to generate Lewis x antigens (Appelmelk, et al. 1996).

2.4. Cell-adhesion molecules of the CEA family

Cell-adhesion molecules (CAMs) enable cells to associate with each other and segregate into distinct tissues. Some cell-cell interactions are transient, such as the interactions between cells of the immune system and the interactions that direct white blood cells to sites of tissue inflammation. In other cases, stable cell-cell junctions play a key role in the organization of cells in tissues.

There are five principal classes of CAMs: cadherins, the Immunoglobulin (Ig) superfamily, selectins, mucins and integrins.

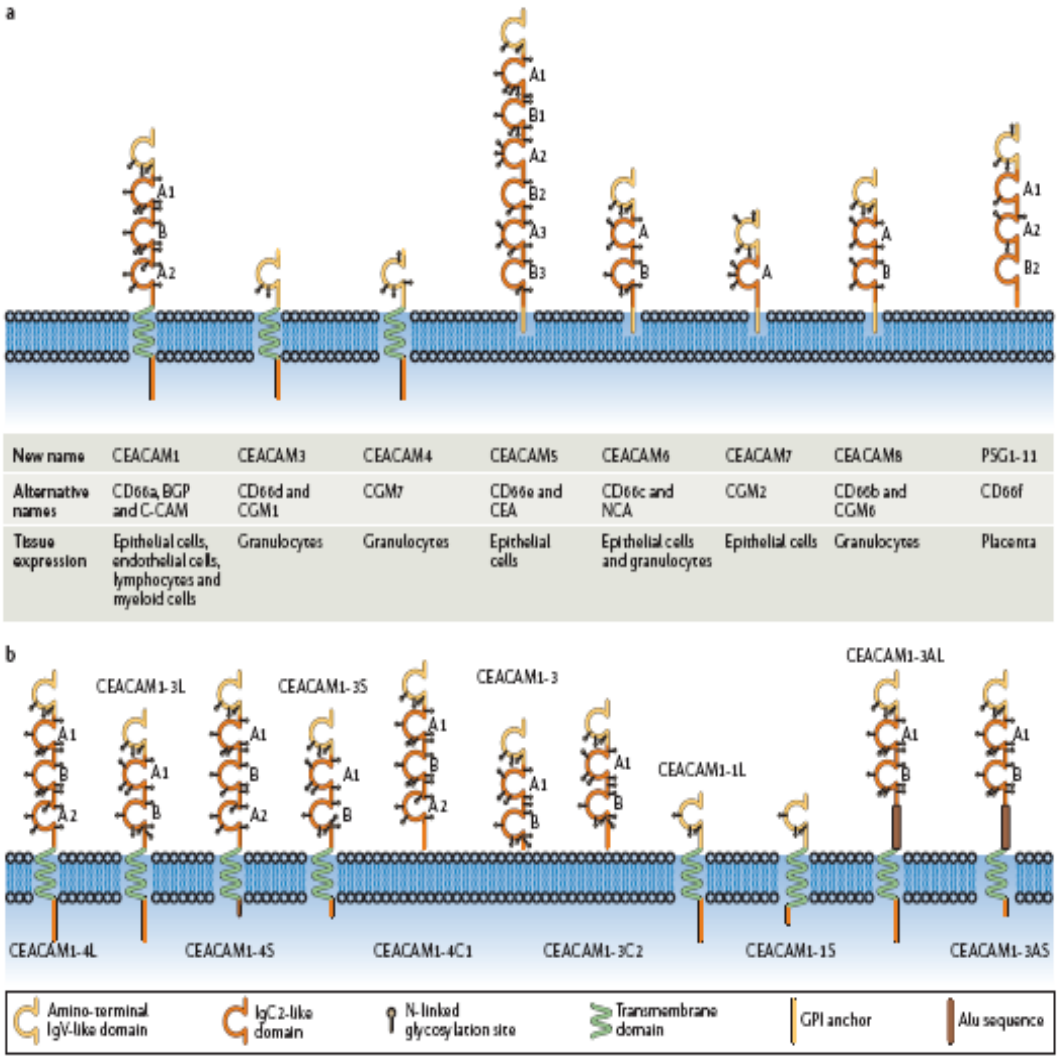
Many proteins involved in cell-cell interaction and antigen recognition in the immune system and other biological systems are members of a protein family called the immunoglobulin superfamily, since they share similar structural features. All members of the immunoglobulin superfamily have at least one immunoglobulin domain or immunoglobulin-like domain. The immunoglobulin domain consists of a sandwich of two β sheets mostly held together by a disulfide bond, and are named "the immunoglobulin fold". There are two main types of immunoglobulin domains: constant-like (C) and variable (V) -like domains.

There are more than 100 members of the Ig superfamily, which mediate a variety of cell-cell interactions, including the carcinoembryonic antigen related cell adhesion molecules (CEACAMs), intercellular cell adhesion molecules (ICAMs), vascular-cell adhesion molecules (VCAMs), platelet-endothelial-cell adhesion molecules (PECAMs), and neural-cell adhesion molecules (NCAMs).

In this work, we have focused on two members of Ig superfamily, the carcinoembryonic antigen related cell adhesion molecule-1 (CEACAM1) and intercellular cell adhesion molecule-3 (ICAM-3).

2.4.1. CEA family

The carcinoembryonic antigen (CEA) family comprises a number of human glycoproteins belonging to the immunoglobulin superfamily.
CEA was discovered as tumor-associated antigen in human colorectal carcinoma (Gold and Freedman 1965).
CEA levels in serum are elevated during the progression of various malignant diseases, such as colon, breast or lung cancers. CEA serves as a clinical tumor marker and is of important prognostic relevance in the evaluation of progression in colonic carcinoma (Thompson, et al. 1991).
After the cDNA of CEA has been cloned and characterized independently by various groups, CEA was assigned to the Ig superfamily (Beauchemin, et al. 1987, Zimmermann, et al. 1987).
The carcinoembryonic antigen (CEA) gene family is a large gene family mapping in the q13.1-2 region of human chromosome 19.
To date, 29 CEA-like genes are known in the human genome and according to their structural similarities, they have been classified into three subgroups: 12 CEA-like-genes, 11 PSG-subgroup (pregnancy-specific glycoproteins), 9 of which are expressed and a group of 6 pseudogens (CEACAM13 through 18). CEACAM1 is also a member of the carcinoembryonic antigen (CEA) gene family that was originally described in the bile ducts of liver as biliary glycoprotein (BGP) and shown to cross-react with anti-CEA antibodies (Svenberg 1976). Later, it was shown to be apically expressed in intestinal epithelia (Svenberg, et al. 1979).



New name	CEACAM1	CEACAM3	CEACAM4	CEACAM5	CEACAM6	CEACAM7	CEACAM8	PSG1-11
Alternative names	CD66a, BGP and C-CAM	CD66d and CGM1	CGM7	CD66e and CEA	CD66c and NCA	CGM2	CD66b and CGM6	CD66f
Tissue expression	Epithelial cells, endothelial cells, lymphocytes and myeloid cells	Granulocytes	Granulocytes	Epithelial cells	Epithelial cells and granulocytes	Epithelial cells	Granulocytes	Placenta

Figure 3. The carcinoembryonic antigen family and naturally occurring isoforms of human CEACAM1 (Gray-Owen and Blumberg 2006)

a) Domain structure and predicted glycosylation pattern of the main isoforms encoded by the CEA gene family. Alternative names are indicated, as is the range of cells expressing each receptor. Each receptor has an amino-terminal immunoglobulin variable-region-like (IgV-like) domain that, owing to its presence at the N terminus of the protein, is often known as the N domain. Most of the rest of the extracellular part of each receptor is comprised of a variable number of immunoglobulin constant-region-type-2-like (IgC2-like) domains. **b)** The gene encoding CEACAM1 consists of nine exons that can be alternatively spliced to generate 11 different isoforms. The domain structure and predicted glycosylation pattern of each protein isoform is illustrated. Letters next to the domains indicate the relationship to A or B subsets of IgC-like domains. Standardized nomenclature for this receptor dictates that the number after CEACAM1 indicates the number of extracellular Ig-like domains, whereas the letter that follows indicates the presence of either a long

(L) or a short (S) cytoplasm tail, a unique terminius (C), or an Alu family repeat sequence present within the open reading frame. GPI, glycosylphosphatidylinositol; PSG, pregnancy-specific glycoprotein.

CEACAM1 has been also referred to as NCA-160, BGP, C-CAM (cell-cell adhesion molecule), C-CAM105, H4A or pp120. In the course of the discovery of new genes and proteins that belong to the CEA gene or protein family, their nomenclature became quite inconsistent. An overview of the CEA-family subgroup is shown in Fig.3.

The common characteristics of all members of the CEA-subgroups are as follows: members of Ig superfamily, heavily glycosylated molecules, homophilic and heterophilic adhesion, regulation of cell growth and differentiation, binding to a variety of different pathogens as well as binding to a variety of intracellular adaptors in central signal transduction cascades (Horst and Wagener 2004).

2.4.2. Structure

All members of CEA subgroup share a common characteristic structure, the so called Ig fold (Boehm, et al. 1996, Tan, et al. 2002).

Structurally, each of the human CEACAMs contains one N-domain of 108-110 amino acid residues, homologous to Ig variable (IgV)-like domains, followed by a different number (zero to six) of Ig constant (IgC)-like domains. The structure of the N-domain is predicted to be of a stacked pair of β-sheets with nine β-strands (Bates, et al. 1992). Following the transmembrane domain, CEACEAMs are linked to the membrane via long or short cytoplasmic tails, or a glycosylphosphatidylinositol (GPI)-anchor. PSGs are soluble. CEACAM1, CEACAM3, and CEACAM4 contain a hydrophobic transmembrane domain followed by a long or short cytoplasmic domain, whereas CEACAM2, CEA (CEACAM5), CEACAM6, and CEACAM7 are attached to the membrane via a glycosylphosphatidyl inositol (GPI) anchor.

Most of the CEA family members are heavily glycosylated molecules, containing zero to six potential N-linked glycostructures per Ig domain, generally more than

half the receptor's molecular weight consists of carbohydrates (Lucka, et al. 2005). The N-linked glycans contain high mannose and complex type oligosaccharides like lactosaminoglycans (glycoconjugates containing Galβ1,4GlcNAc motif/motifs) type I and type II, terminated by fucosyl- and sialyl-residues (Odin, et al. 1986, Kannicht, et al. 1999). A non-glycosylated β-pleated sheet in the amino-terminal domain mediates homophilic intercellular binding (Watt, et al. 2001) and is the target for various bacteria.

The CEACAM1 gene codes for an N-terminal V-like domain, three C2 domains (A1, B1, A2), a transmembrane domain, and a cytoplasmic domain.

Depending of the length of the cytoplasmic domain there are two isoforms of CEACAM1, long form (CEACAM1-L) composed of 73 amino acids and short form (CEACAM1-S) composed of 10-12 amino acids. The cytoplasmic domain of CEACAM1-L contains two tyrosine residues (Tyr 488 and 516). Tyr 516 is embedded into an imperfect ITAM (immunoreceptor tyrosine-based activation motif).Tyr 488 is part of a perfect immunoreceptor tyrosine-based inhibition motif (ITIM)(Obrink 1997).

So far, there are at least 11 known splice variants of CEACAM1 (Barnett, et al. 1993), out of which the most examined are CEACAM1-4L and CEACAM1-4S, both containing four extracellular Ig-like domains (Fig.3).

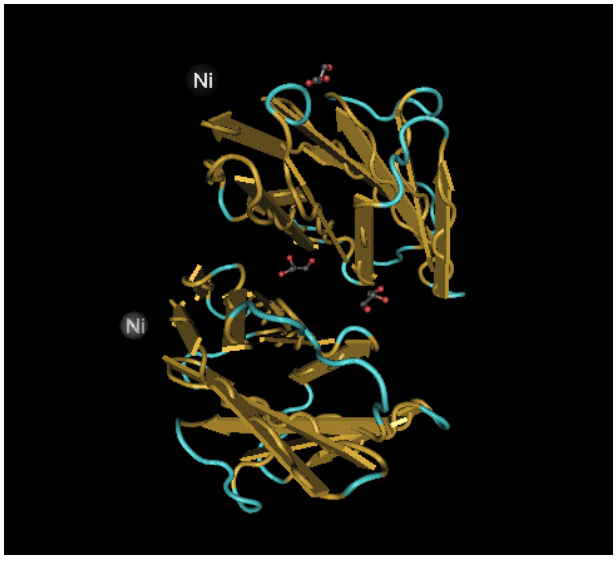


Figure 4. Crystal structure of the N-terminal domain of human CEACAM1 (http://www.ncbi.nlm.nih.gov/)

Both variants, CEACAM1-4L and CEACAM1-4S undergo dimerization, the dimer consist either of one or both isoforms. The equilibrium between monomeric and dimeric forms affects the cellular responses to CEACAM1 binding. As a cis-homodimer, CEACAM1 is found mostly on epithelial cells (Hunter, et al. 1996). In addition, CEACAM1 can occur in trans-homophilic interaction (CEACAM1-CEACAM1) involving the N-terminal domain, and trans-heterophilic (CEACAM1-CEACAM5) cellular interactions (Watt, et al. 2001).

2.4.3. Expression

CEACAMs display a very heterogeneous expression pattern which can be categorized into following major groups (Horst and Wagener 2004):

- selectively epithelial expression (CEA, CEACAM7)
- expression on granulocytes (CEACAM3, CEACAM8)
- broad expression (CEACAM1, CEACAM6)
- predominant expression pattern in the syncytiotrophoblast (PSGs)

CEA, the founding member of this family, is a normal constituent of the glycocalyx of the colonic mucosa (Baranov, et al. 1994, Hammarstrom and Baranov 2001). In addition to CEA, CEACAM1, CEACAM6 and CEACAM7 are expressed in healthy colon. Specifically, all four members are expressed at the apical surface of epithelial cells but not at the basolateral side of epithelial cells. CEA is expressed on many tumour cells but not on leukocytes. CEACAM1, CEACAM8, CEACAM6 and CEACAM3 are expressed on human neutrophils and can transmit activation signals in neutrophils (Skubitz, et al. 2001).

CEA family members can be detected during gestation and embryonic development. CEA and other CEA-gene family members are expressed in early stages of human pregnancy during the first trimester (Wagener, et al. 1983).

PSGs are mainly expressed in the placenta with restriction to the syncytiotrophoblast (Zhou, et al. 1997).

Human CEACAM1, which is a subject of investigation of this study, shows broad expression pattern.

It is expressed on various epithelia, such as oesophagus (glandular epithelial cells), stomach (pyloric mucous cells, Brunner' gland cells), in epithelial cells of the duodenum, jejunum, and ileum; in colon (columnar epithelial cells), pancreas (ductal epithelial cells), liver (bile canaliculi, bile duct epithelial cells), gall bladder (epithelia), in kidney epithelial cells in the proximal tubuli, the urinary bladder (transitional epithelial cells), prostate epithelial cells, cervix, squamous epithelial cells, endometrium, glandular epithelial cells, and in sweat and sebaceous glands (Prall, et al. 1996).

CEACAM1 is expressed in the invasive trophoblasts in the placenta showing high expression during the first trimester of pregnancy. Furthermore, CEACAM1 is present on epithelial cells of endometrium, but the decidua is devoid of CEACAM1. Reactivity of specific antibodies against CEACAM1 is also found in maternal blood vessels and embryonic capillaries (Daniels, et al. 1996, Bamberger, et al. 2000).

In addition to the expression on a wide variety of epithelial and endothelial cells, CEACAM1 is expressed by a variety of hematopoetic cell types including granulocytes, NK cells, B cells, T cells, monocytes, and dendritic cells. On several of these cell types, CEACAM1 expression requires the cell to be activated by various stimuli. CEACAM1 is rapidly up-regulated on all T cells by activation with cytokines (IL-2, IL-7 and IL-15) (Kammerer, et al. 1998, Boulton and Gray-Owen 2002).

In addition, CEACAM1 is expressed in various tumours, either up-or down-regulated. It is up-regulated in gastric carcinomas, squamous lung cell carcinomas (Ohwada, et al. 1994) and in malignant melanomas (Thies, et al. 2002). CEACAM1 has been found to be down regulated in premalignant colorectal adenomas (Nollau, et al. 1997b), prostate and hepatocellular carcinoma, ~30% of breast carcinomas (Riethdorf, et al. 1997) (Huang, et al. 1998) and 90% of colon tumours (Neumaier, et al. 1993).

Tissue	Detection
Oesophagus	Apical membranous staining of epithelia of oesophageal glands; no staining of the squamous epithelium
Stomach	-
Small intestine	Apical membranous staining of enterocytes; in the duodenum apical membranous staining of epithelia of Brunner's glands
Colon	Strong apical membrane staining of superficial absorptive cells; weaker staining of goblet cells
Pancreas	Apical membranous staining of epithelia of large and small ducts; no staining reaction in exocrine acini; endothelia of capilliaries in islets
Liver	Membranous staining reaction of hepatocytes along bile canaliculi; apical membranous staining of bile duct epithelia
Gallbladder	Apical membranous staining of epithelia
Kidney	Strong apical membranous staining of epithelia of proximal tubules; endothelia of glomerular capillaries and vasa recta
Renal pelvis and urinary bladder	Weak to moderate membranous staining of urinary bladder, the transitional epithelium, accentuated in superficial layers
Prostate	Apical membranous staining of epithelia, less pronounced in atrophy: endothelia of small arteries and venules
Uterino portio and cervix	Apical membranous staining of cervical epithelia; membranous staining of squamous epithelia in the metaplastic region
Endometrium	Apical membranous staining of uterine glands; endothelium of small endometrial vessels
Female breast	Apical membranous staining of duct epithelia
Ovaries	-
Testes	-
Lung and bronchi	-
Thyroid gland	Focal staining of the endothelium in active regions
Adrenal gland	Endothelium of sinusoids
Placenta	Endothelium of small arteries, capillaries and venules
Bone marrow	Cytoplasmic staining of granulocytes and their precursors, including myelocytes

Table 1. Immunohistochemistry of normal human tissues with specific CEACAM1 mAb 4D1/C2 (Prall, et al. 1996)

2.4.4. Biological functions of CEACAM1

Although the biological function of CEACAM1 remains elusive, CEACAMs are largely determined by there ability to function as cellular and intercellular adhesion molecules. Adhesion of CEACAMs to each other or with other extracellular ligands induces specific signal transduction though CEACAMs. CEACAM1, CEACAM6 and CEA are capable of homophilic and heterophilic

adhesion, whereas CEACAM8 undergoes heterophilic adhesion with CEACAM6 (Oikawa, et al. 1991).

Homophilic interactions that have been confirmed for the human CEACAM1-4L, -4S, -3L isoforms (Teixeira, et al. 1994), are thought to be important in the embryonic organization of the intestinal epithelium and of hepatocytes in the liver. Homophilic CEACAM1 interactions are involved in neutrophil activation and adhesion during inflammatory response (Skubitz, et al. 1996), in lymphoregulation and immunosurveillance (Morales, et al. 1999), and in placental trophoblasts.

CEACAM1is associated with early stages of angiogenesis (Wagener and Ergun 2000) and negative regulation of cell proliferation (Obrink 1997). Heterophilic adhesion of CEACAM1 to other CEACAM family members, E-selectin (Stocks, et al. 1995), to fimbral proteins of *Escherichia coli* and *Salmonella*, to Opa proteins of *Neisseria meningitidis* and *gonorrhoeae* (Bos, et al. 1997) (Virji, et al. 1996), to *Haemophilus influenzae* (Virji, et al. 2000) and to murine coronaviruses (Holmes, et al. 1993), has also been reported. This heterophilic adhesion mediates the uptake of *Neisseria, Haemophilus* or coronaviruses; facilitates bacterial colonization of the gut and bacterial phagocytosis by neutrophils, and may be involved in the initial tethering of granulocytes to the endothelium prior to their transendothelial migration during the inflammatory response.

Although the mechanism of action is largely unresolved, several reports suggest that CEACAM1 participated in signal transduction by interacting with other membrane or cytoplasmic proteins via its cytoplasmic domain. The human CEACAM1-4L cytoplasmic domain contains an ITIM motif (immunoreceptor tyrosine-based inhibition motif) that have been shown to be phosphorylated by protein kinases of the src family. Subsequent to phosphorylation of these residues, CEACAM1 interacts with components of the cytoskeleton, such as paxillin and actin as well as β_3 integrin (Ebrahimnejad, et al. 2000, Brummer, et al. 2001).

2.5. ICAM family

The members of the intercellular adhesion molecules (ICAMs) subfamily are heavily glycosylated cell surface receptors of the immunoglobulin superfamily (IgSF). They share functional and structural homologies and mediate cell-cell adhesive interactions relevant for the function of the immune system.
Five members of the ICAM subfamily have been described so far (ICAM-1,-2, -3 -4 and -5), all binding to the integrin $\alpha_L\beta_2$ (LFA-1), but showing great variation in tissue distribution (Gahmberg, et al. 1997).
Most thoroughly investigated members are ICAM-1, ICAM-2 and ICAM-3.

2.5.1. Structure

All ICAM proteins are type I transmembrane glycoproteins, containing immunoglobulin-like C2-type domains.
There are large differences in the number of extracellular domains among the members of the ICAM family, ranging from two in ICAM-2 and ICAM-4 to nine in ICAM-5. ICAM-1 exists predominantly as a dimer on the cell surface, whereas ICAM-2 does not (Reilly, et al. 1995). ICAM-3 (CD50) contains five Ig-like domains. Upon activation, ICAM-3 becomes phosphorylated on some of the tyrosine and five serine residues in its cytoplasmic domain by p56 ^{lck} and p59 ^{fyn} tyrosine kinases and protein kinase C, respectively. The LFA-1 integrin (I domain) binds to the first N-terminal domain (D1) of the receptors (Shimaoka, et al. 2003).
ICAMs are N-glycosylated, but the role of those glycans in receptor folding and ligand recognition is poorly understood.
Structural studies revealed that N-linked oligosaccharides of ICAM-3 from human granulocytes are mainly of tri- and tetra-antennary complex-type, about 60% of which contain two to three poly-N-acetyllactosamine chains. In addition a small

amount of high mannose-type oligosaccharides was detected (Funatsu, et al. 2001).

In this study, we have investigated the expression and function of Lex residues on ICAM-3 from human leukocytes.

2.5.2. Expression

ICAM-1 and ICAM-2 are expressed on vascular endothelia (Nortamo, et al. 1991a). ICAM-2 is expressed constitutively on lymphocytes, monocytes, platelets, and most endothelial cells (Nortamo, et al. 1991b) and may be important for leukocyte recirculation in normal uninflamed tissues (Springer 1994). ICAM-1 and ICAM-2 are expressed at a very low level on resting T cells. ICAM-4 is found on red blood cells (Bailly, et al. 1995) and ICAM-5 on neuronal cells of the telencephalon of the brain (Tian, et al. 1997). In contrast to ICAM-1 and ICAM-2, ICAM-3 is absent from endothelia.

ICAM-3 is expressed on resting lymphocytes, monocytes and neutrophils, representing the major LFA-1 ligand on these cells (de Fougerolles and Springer 1992, Fawcett, et al. 1992). Approximately 95% of human B and T lymphocytes, 60% of thymocytes, 70-85% of monocytes/macrophages, 98 % of neutrophils, and many transformed leukocytes (chronic lymphocytic leukaemia, hair cell leukaemia, acute and chronic myleloid leukaemia and multiple myeloma) constitutively express ICAM-3 (Berney, et al. 1999).

ICAM-3 is expressed on human bone marrow endothelial cells and controls endothelial integrity via reactive oxygen species-dependent signalling (van Buul, et al. 2004). ICAM-3 is highly present on the surface of human eosinophils and has a role in the down-regulation of GM-CSF production (Kessel, et al. 2003).

ICAM-3 is constitutively and widely expressed by cells participating in inflammatory dermatoses (including Langerhans cells and T and B lymphocytes), and can be, albeit rarely, induced on endothelial cells and dermal dendrocytes (Montazeri, et al. 1995).

2.5.3. Function

ICAM-1, -2, and -3 play significant roles in the function of the immune system, mediating cell adhesion through interaction with the leukocyte integrin $\alpha_L\beta_2$ in different cellular settings. Like other integrins, $\alpha_L\beta_2$ is a large heterodimeric transmembrane protein that functions as a bidirectional and allosteric signalling molecule. The interaction of $\alpha_L\beta_2$ with ICAMs is critical in immune surveillance and responsiveness of leukocytes. Monoclonal antibodies to all three ICAMs are required to inhibit peripheral blood lymphocyte proliferation in immune responses at the same level as monoclonal antibodies to $\alpha_L\beta_2$, suggesting that multiple αLβ2 ligands are required for different aspects of $\alpha_L\beta_2$-dependent leukocyte functions (de Fougerolles and Springer 1992). ICAM-3 is the major $\alpha_L\beta_2$ ligand on resting T cells, on which ICAM-1 and -2 are expressed either at a very low level or not at all. Moreover, ICAM-3 is a co-stimulatory molecule for both, resting and activated T cells. The finding that adhesion of resting T lymphocytes to LFA-1 occurs primarily via ICAM-3 combined with the fact that ICAM-3 is much higher expressed than other LFA-1 ligands on monocytes and resting lymphocytes implies an important role for ICAM-3 in the initiation of immune responses (Hernandez-Caselles, et al. 1993)

It functions not only as an adhesion molecule, but also as a potent signalling molecule. Recent work has provided direct evidence that ICAM-3 is the primary adhesion molecule involved in antigen-independent conjugate formation between T cells and antigen-presenting cells (APCs). This initial contact of a T cell with the APC creates an opportunity for the T cells to exert exploratory scanning of the APC surface for antigen recognition (Montoya, et al. 2002).

ICAM-3 augments signalling through CD3, functioning as a co-stimulatory molecule for resting T cells in the initial activation step (Berney, et al. 1999).

The recently reported binding of ICAM-2 and ICAM-3 to the C-type lectin dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) has been reported to be relevant for the function of dendritic cells.

Whereas the integrin binding surface in ICAMs is glycan-free (Casasnovas, et al. 1999, Shimaoka, et al. 2003), the recognition of DC-SIGN is dependent on the carbohydrates (Guo, et al. 2004)

2.6. Dendritic cells (DCs)

Dendritic cells (DCs), originally identified by Steinman and his colleagues (1972) represent the pacemakers of the immune response (Steinman and Cohn 1973). During inflammation, immature dendritic cells undergo maturation, which enables them to migrate to the lymph nodes, and to present peptides derived from the acquired antigens to lymph node T-cells. They are crucial to the presentation of peptides and proteins to T and B-lymphocytes and are widely recognized as the key antigen presenting cells (APCs). The T cell receptors (TCRs) on T lymphocytes recognize fragments of antigens (Ags) bound to molecules of the major histocompatibility complex (MHC) on the surfaces of APCs. The peptide binding proteins are of two types, MHC classes I and II, which interact with and stimulate cytotoxic T lymphocytes (CTLs) and T helper cells (Ths), respectively. On entry into APCs, Ags are processed, cleaved into peptides in the cytosol and then reexpressed on the cell surface linked to MHC proteins. When bound to MHC class I molecules, CTLs are generated and activated, and cells in tissues expressing the Ags (e.g. virus infected cells, cancer cells) are recognised and destroyed. Antigens presented on the cell surface linked to MHC class II molecules interact with T helper cells, which, when activated, have profound immune-regulatory effects (Banchereau and Steinman 1998). Thus, DCs play a key role in host defences and a crucial role in putative anti-cancer immune responses.

2.6.1. Receptors on dendritic cells

Dendritic cells express a repertoire of pathogen-recognition receptors, including Toll-like receptors (TLRs) and C-type lectins. TLRs recognize specific pathogen components, such as lipoprotein, lipopolysaccharide (LPS) or bacterial DNA (Akira and Hemmi 2003). C-type type lectins recognize specific carbohydrate structures that are present on cell-wall components of pathogens, and internalize

pathogens for degradation in lysosomal compartments to enhance antigen processing and presentation by DCs (Drickamer 1999). Many of the C-type lectins expressed on dendritic cells have been described, including mannose receptor (CD206), DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN), blood DC antigen 2 (BDCA2), DEC205 (CD205), C-type lectin receptor 1 (CLECA), and DC-associated lectin 1 (DCAL1). Most of C-type lectins are type II transmembrane protein, except mannose receptor and DC205 that are type I.

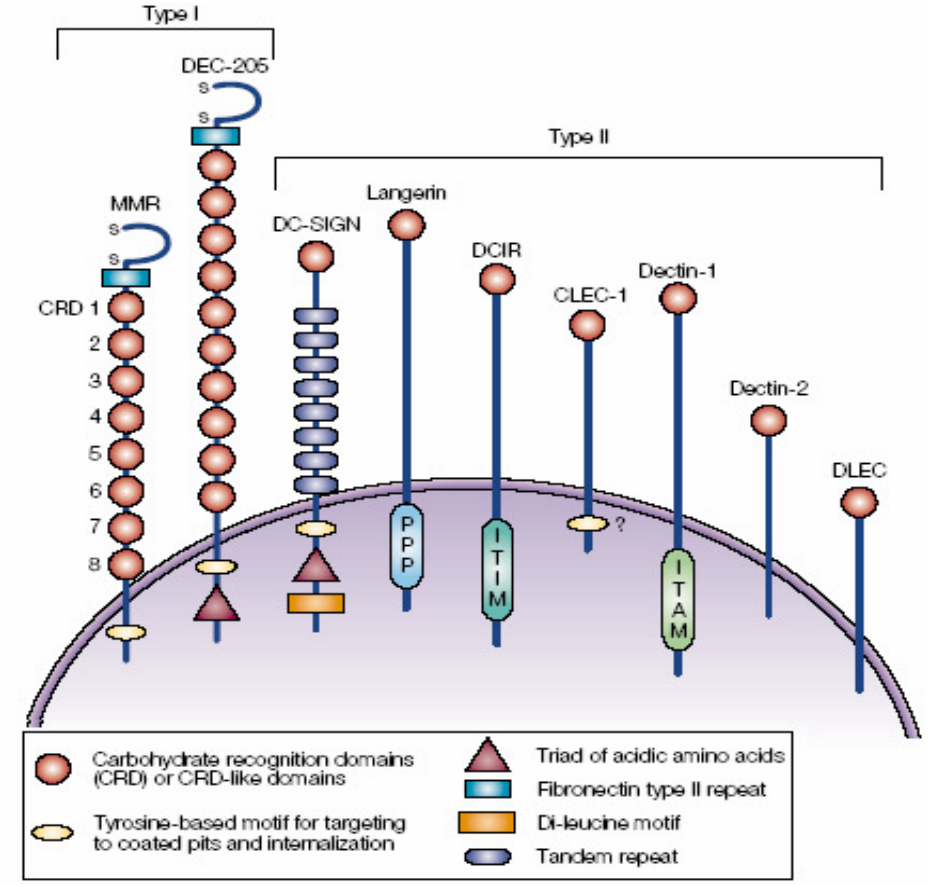


Figure 5. C-type lectins or lectin-like molecules produced by dendritic cells (Figdor, et al. 2002)

CLEC-1, C-type lectin receptor 1; DCIR, dendritic cell immunoreceptor; DC-SIGN, dendritic-cell specificICAM-3 grabbing non-integrin; DLEC, dendritic cell lectin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MMR, macrophage mannose receptor.

2.7. DC-SIGN (CD209)

Dendritic cell-specific intracellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) is a type II transmembrane C-type lectin that has been reported to be a cell-adhesion receptor that mediates the cell interaction between DCs and resting T cells upon binding ICAM-3.

2.7.1. Structure of DC-SIGN

DC-SIGN consists of a carbohydrate recognition domain (CRD), a neck domain which contains seven complete and one incomplete tandem repeat, transmembrane domain (TM), and cytoplasmic domain that includes internalization motifs such as the di-leucine (LL) motif and the tri-acidic (EEE) clusters, and an incomplete immunoreceptor tyrosine-based activation motif (ITAM) (Fig.6). The CRD of DC-SIGN is a globular protein structure consisting of 12β-strands, two α-helices and three disulphide bridges. A loop protrudes form the protein surface and forms part of two Ca^{2+}-bindig sites. Carbohydrate ligands interact directly with Ca^{2+} through hydroxyl groups and hydrogen bonds with amino-acid chains (Glu-Asn) that also act as Ca^{2+} coordination ligands (van Kooyk and Geijtenbeek 2003).

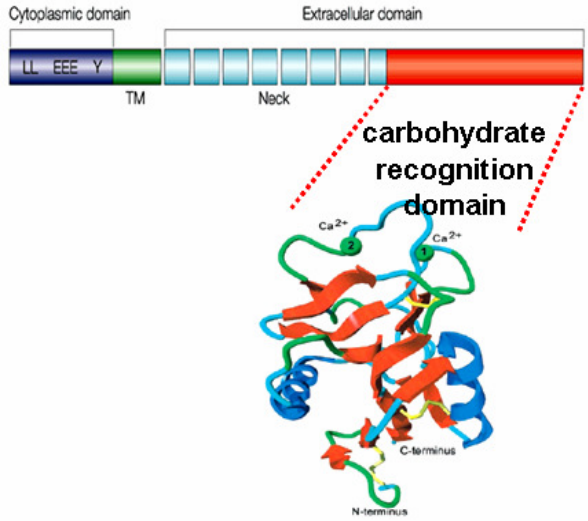


Figure 6. Structure of the CRD of DC-SIGN (Geijtenbeek, et al. 2002)

2.7.2. Function of DC-SIGN

DC-SIGN, as a member of the C-type family, functions as cell-adhesion receptor that recognizes specific carbohydrates structures, such as Lewis blood groups (Lex, Lea, Leb, Ley) and high mannose structures in a Ca^{2+}-dependent manner, through a conserved CRD (Appelmelk, et al. 2003) .

It binds the glycostructure of ICAM-2 and ICAM-3, and functions as receptor that regulates DC migration (Geijtenbeek, et al. 2000a) and DC-T cell interaction (Geijtenbeek, et al. 2000c), respectively. Lex residues present on glycostructures of ICAM-3 are involved in binding to DC-SIGN (Bogoevska, et al. 2007).

DC-SIGN mediates cellular interaction with Mac-1 of neutrophils (van Gisbergen, et al. 2005b).

CEACAM1 has recently been described to contain Lewis x residues on granulocytes (Lucka, et al. 2005), and interaction between DC-SIGN and granulocyte CEACAM1 is mediated by these Lewis x groups (Bogoevska, et al. 2006).

DC-SIGN binds many pathogens (Table 2). DC-SIGN functions as a HIV-1 receptor that captures HIVgp120 and facilitates DC-induced HIV transmission of T cells (Geijtenbeek, et al. 2000b). Internalization motifs in the cytoplasmic tail of DC-SIGN hint to a function of DC-SIGN as endocytic receptor, but the underlying mechanism for being such an efficient phagocytic pathogen-recognition receptor is poorly understood. During development of human monocyte-derived DCs, DC-SIGN becomes organized in well-defined micro-domains (with an average diameter of 200 nm) that co-localize with lipid rafts (Cambi, et al. 2004). These micro-domains containing DC-SIGN act as a docking site for HIV-1 particles, facilitating entry of the virus into DCs.

Recently, DC-SIGN was also shown to bind other viruses like CMV (Halary, et al. 2002), Ebola (Simmons, et al. 2003), Dengue (Navarro-Sanchez, et al. 2003, Tassaneetrithep, et al. 2003), hepatitis C (Pohlmann, et al. 2003), as well as micro-organisms such as *Leishmania* (Colmenares, et al. 2002*)*, *Candida albicans*

(Cambi, et al. 2003), *Mycobacterium* (Tailleux, et al. 2003, van Kooyk, et al. 2003) and *Schistosoma* (van Die, et al. 2003*).*

Pathogen	Antigen	Carbohydrate structure	Other c-type lectin receptors
Viruses			
HIV-1	**gp 120**	**High mannose**	**Mannose receptor and Langerin**
HIV-2	**gp 120**	**?**	
SIV-1	**gp 120**	**?**	
Ebola virus	**GP**	**High mannose**	
Cytomegalo virus	**gB**	**?**	
Hepatitis C virus	**E1/E2**	**?**	
Dengue virus	**gE**	**?**	
Bacteria			
Helicobacter pylori	**LPS**	**Lewis-x**	
Klebsiella pneumoniae	**LPS**	**Mannose**	
Mycobacteria tuberculosis	**ManLAM**	**Di-mannose, tri-mannose**	**Mannose receptor**
Yeast			
Candida albicans	**?**	**?**	**Mannose receptor**
Parasites			
Laishmania pifanoi	**LPG**	**High mannose**	
Schistosoma mansoni	**SEA**	**Lewis-x**	

Table 2. DC-SIGN recognized by pathogens (van Kooyk and Geijtenbeek 2003)

gB,glycoprotein B; gE, glycoprotein E; GP,glycoprotein; Langerin, Langerhans-cell-specific C-type lectin; LPG, Lipophosphoglycan; LPS, lipopolysaccharide; ManLAM, mannose-capped lipoarabinomannan; SEA, soluble egg antigen; SIV, simian immunodeficiency virus

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments

Analytical balance	**Mettler, Giessen**
Autoclave	**Tecnomara, Fernwald**
Blotting instrument	**Biometra, Göttingen**
Flow cytometer	**FACSCalibur, Becton Dickinson**
ELISA-plate-photometer	**SLT, Crailsheim**
Film cassettes and x-ray films	**Amersham/Pharmacia LKB, München**
Fluorescence microscope Leitz DMRB	**Leitz, Wetzlar**
Gel chambers and accessories (SDS-gels)	**Biometra, Göttingen**
Gel chambers and accessories (agarose-gels)	**Hoefer, Scientific Instruments, San Francisco**
Heating blocks	**Flow Laboratories, Meckenheim**
Confocal laser-scanning microscope	**Leica, Bensheim**
Light microscope	**Zeiss Invers410, Jena**
Mini-shaker	**B. Braun, Melsungen**
Polaroid chamber, films	**Polaroid, Hertfordshire**
PCR-instruments	**Minicycler Biozym, Hamburg**
pH-meter	**InoLab,WTW, Weilheim**
Pipettes	**Eppendorf, Hamburg**
PD10 columns	**Amersham Bioscience, Freiburg**

Rotational shaker	**Greiner, Frickenhausen**
Sterile bench	**Flow Laboratories, Meckenheim**
UV-spectrophotometer	**Shimadzu, Kyoto**
Water bath	**GFL, Burgwedel**
Centrifuges	
Sorvall RC5C, rotor SS-34	**Sorvall/DuPont, Bad Homburg**
Eppendorf centrifuge	**Eppendorf, Hamburg**
Hermle ZK 380	**Eppendorf, Hamburg**
Other used materials	
Glass devices (bottles, beakers, cuvettes, graduated cylinders)	**Schott, Mainz**
PVDF membranes	**Immobilon P, Millipore, Schwalbach**
Steril plastic tubes	**Greiner, Frickenhausen** **Nunc, Wiesbaden** **Eppendorf, Hamburg**
Whatman-paper	**Whatman, Kent (UK)**
Ultra filtration devices	**Amicon, Millipore Cooperation, Bedford**

3.1.2. Chemicals

All reagents were obtained at p.a. quality from Merck (Darmstadt), Serva (Heidelberg) and Sigma (Deisenhofen). All solutions were prepared with double distillated water.

Antibodies (Abs)

Primary antibodies	**target of the antibodies**
4D1/C2, murine IgG_1 monoclonal antibody (mAb), Prof. Dr. C. Wagener	human CEACAM1
T84.1, murine IgG_1mAb Prof. Dr. C. Wagener	human CEACAM1, CEACAM5, CEACAM6
L5 mAb, rat IgM, Prof. Dr. M. Schachner	human Lewis x (CD15)
L3 mAb, rat IgM, Prof. Dr. M. Schachner	high mannose structure
L4 mAb, rat IgM, Prof. Dr. M. Schachner	high mannose structure
Anti-Fc, mouse IgG_1, Dianova, Hamburg	human IgG_1
DC28 mAb, mouse IgG_{2a}, R&D Systems	human DC-SIGN and DC-SIGNR
DC-SIGN mAb, 120507, mouse IgG_{2b}	human DC-SIGN
Anti-HA-rabbit, polyclonal hemagglutinin, Santa Cruz	HA-tag (YPYDVPDYA), which corresponding to the partial peptide of Influenza hemagglutinin (HA) protein
Anti-ICAM3-140.11 mAb, mouse IgG_1 Dr. R. Villella, Barcelona University Hospital, Spain	human ICAM-3
ICAM-3 mAb 3.1, mouse IgG_1, Santa Cruz	human ICAM-3
Goat polyclonal mAb N-19, Santa Cruz	human ICAM-3
Anti c-myc (clone 9E10) mAb, mouse IgG_1, Santa Cruz	myc-tag (EQKLISEEDL)
CD15,-CBL144 mAb, mouse IgM, Chemicon	human CD15

Primary labeled monoclonal antibodies

CD83-phycoerythrin (PE), IgG_1, BD Biosciences	human CD83
CD86-PE, mouse $IgG_{1,}$ BD Biosciences	human DC86
CD3-PE mouse IgG_{2a}, Miltenyi Biotec	human CD3
CD14-FITC, mouse IgG_{2a}, Miltenyi Biotec	human CD14
CD19-PE, mouse $IgG_{1,}$ Miltenyi Biotec	human CD19
CD80-FITC, mouse $IgG_{1,}$ BD Biosciences	human CD80
H5G11-PE-labeled, Santa Cruz	human LAMP1
120507-PE, mouse IgG_{2b}, R&D Systems	human DC-SIGN
Goat anti-human IgG, Fc-PE, (Fab_2), Dianova	human IgG_1, Fc
Anti-digoxigenin-HRP, Roshe	digoxigenin

Secondary labeled antibodies

Rabbit anti-mouse IgG-HRP mAb, Dako	mouse IgG
Rabbit anti-human IgG-Fc-HRP mAb, Dako	human Fc
Rabbit anti-mouse IgG-FITC mAb, Dako	mouse IgG
Goat-anti-rabbit-IgG mAb, Santa Cruz	rabbit IgG
Mouse-anti-rat IgG-PE mAb, BD Biosciences	rat IgM
Goat-anti-mouse-PE mAb, BD Biosciences	mouse IgM

Isotype controls for FACS analysis

Rat IgM-PE, BD Biosciences
Mouse IgM-PE, BD Biosciences
Mouse IgG_{2b}-PE, DAKO
Mouse IgG_{2a} -PE, DAKO
Mouse IgG_1-FITC, DAKO
Mouse IgG_1-PE, DAKO

Blocking agents

Purified human IgG,	Sigma, Deisenhofen
Purified mouse IgG,	Sigma, Deisenhofen

Lectin

Galanthus nivalis agglutinin (GNA) - digoxigenin-labeled	Roche, Mannheim

Other reagents

Acrylamid/Bisacrylamid (40%), 29:1	Roth, Karlsruhe
Annexin-V-FLUOS staining kit	Roche, Mannheim
Anti-CD14, anti-CD3, and anti-CD19 magnetic microbeads	Miltenyi Biotec, Bergisch-Gladbach
Blocking reagent	Roche, Mannheim
BSA (10 µg/mL)	New England Biolabs, Frankfurt
Bench mark pre-stained protein ladder	Invitrogen, Karlsruhe
Colloidal Coomassie staining-Roti-Blue	Roth, Karlsruhe
ECL western blotting kit	Amersham Biosciences, Freiburg
Lewis x-BSA	Chemicon, Hampshire,
Protein G-Plus agarose	Santa Cruz, USA
Protein assay	Biorad, Munich
NuPage gradient gel	Invitrogen, Karlsruhe
TEMED	Biorad, Munich
Ficoll-Paque (d = 1.077 g/cm3)	Pharmacia, Freiburg
FITC labelling kit	Calbiochem, Darmstadt

Cell culture reagents

Cell lines

Chinese hamster ovary (CHO) cells	American Type Culture Collection, Manassas
Human embryonal kidney 293 (HEK293)	American Type Culture Collection, Manassas
Human blood cells	Blood transfusion department - UKE, Hamburg

Cell culture medium

Dulbecco's Modified Eagle Medium (DMEM)	PAA, Laboratories, Cölbe
DMSO	Merck, Darmstadt
Feta calf serum (FCS)	PAA Laboratories, Cölbe
Glutamax	Invitrogen, Karlsruhe
G418	Invitrogen, Karlsruhe
Lipofectamin2000®	Invitrogen, Karlsruhe
Penicillin/Streptomycin	Invitrogen, Karlsruhe
RPMI1640	PAA Laboratories, Cölbe,
Serum free medium II (SFM II)	Invitrogen, Karlsruhe
Zeocine	Invitrogen, Karlsruhe
FM® 1-43 membrane staining kit	Molecular Probes, Eugene

Reagents for biomolecular methods

Bacteria

One Shot®INVαF'chemical competent *E. coli*	Invitrogen, Karlsruhe

Expression vectors

pCR 2.1 Vector	Invitrogen, Karlsruhe
pEF-BOS Vector, Dr. B.J. Mayer	Children's Hospital, Harvard Medical School, Boston
pcDNA3.1/G418 Vector	Invitrogen, Karlsruhe
pcDNA3.1/Zeo Vector	Invitrogen, Karlsruhe

Kits

DNA-Sequencing Kit	Applied Biosystems, Warrington
Qiagen Plasmid Maxi Kit	Qiagen, Hilden
QIAEX II Gel Extraction Kit (150)	Qiagen, Hilden
TA-Cloning-Kit Version R	Invitrogen, Karlsruhe

Other reagents

Bacto Yeast Extract	Difco Laboratories, Hamburg
Bacto Trypton	Difco Laboratories, Hamburg
Agarose	Biozym, Hamburg
1 kb DNA-Marker	Invitrogen, Karlsruhe
250 bp DNA-Marker	Invitrogen, Karlsruhe
1kb plus DNA-Marker	Invitrogen, Karlsruhe

Enzymes

Taq-Polymerase, 1 U/µL	Roche, Mannheim
T4-DNA-Ligase, 4 U/µL	Invitrogen, Karlsruhe
*Asp*718-Restriction Endonucleases, 10 U/µL	Roche, Mannheim
*Sal*I-Restriction Endonucleases, 10 U/ µL	Roche, Mannheim
*Not*I-Restriction Endonucleases, 10 U/ µL	Roche, Mannheim
α(1-3,4) Fucosidase	Calbiochem, Darmstadt

3.1.3 Buffers and solutions

DNA-loading buffer, 10x

30% Glycin (v/v), 0.25% Bromphenol blue (w/v), 50 mM EDTA, 0.25 % Xylen Cyanol (w/v), 25% Ficoll (Typ 400) (v/v) in dH_2O

LB (Luria-Bertani) medium, 2x

20 g Bacto Trypton, 10 g Bacto Yeast Extract, 20 g NaCl per1 L dH_2O, adjusted to pH 7.0

FACS-buffer

1% FCS in PBS

FACS-buffer for binding assay

1% FCS in PBS, 1mM $CaCl_2$, 2 mM $MgCl_2$

Final fixing FACS- buffer

1% FCS in PBS, 1% paraformaldehyde

Final fixing FACS- buffer for binding assay

1% FCS in PBS, 1mM $CaCl_2$, 2 mM $MgCl_2$, 1% paraformaldehyde

KLB-lyses buffer

25 mM Tris/HCl (pH 7.4),150 mM NaCl, 5 mM EDTA (pH 8.0), 10% Glycerol (v/v), 1% Triton X-100 (v/v), 10 mM Na-Pyrophosphate, 1 mM Na-Orthovanadate, 10 mM β-Glycerolphosphate

PBS, 10x, pH 7.4

80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 per 1L of dH_2O

SOC medium

2% Trypton (w/v), 0.5% Yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose

TBST (Tris-buffer saline with Tween)

150 mM NaCl, 10 mM Tris (pH 8.0), 0.05% Tween 20

TSM buffer

20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM $CaCl_2$, 2 mM $MgCl_2$

Tris-acetate-EDTA (TAE) -buffer, 50x

242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0) per 1 L of dH_2O

Buffers for plasmid mini-preparation:

Buffer 1

25 mM Tris/HCl (pH 8.0), 5 mM EDTA, RNase A 100 µg/mL

Buffer 2

0.2 M NaOH, 1% SDS (w/v)

Buffer 3:

3 M NaAc, pH 4.8

Buffers for plasmid maxi-preparation:

Buffer P1

50 mM Tris/HCl (pH 8.0), 10 mM EDTA, 100 µg/mL RNase A

Buffer P2

200 mM NaOH, 1% SDS (w/v)

Buffer P3

3.0 M Potassium acetate, pH 5.5

Buffer QBT

750 mM NaCl, 50 mM MOPS, pH 7.0, 15 % Isopropanol (v/v), 0.15% Triton X-100 (v/v)

Buffer QC

1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% Isopropanol (v/v)

Buffer QF

1.25 M NaCl, 50 mM Tris/HCl, pH 8.5, 15% Isopropanol (v/v)

Buffers for SDS-Polyacrylamid gel electrophoresis:

Electrophoresis-running buffer, 10x

30 g Tris, 10 g SDS, 144 g Glycin per 2 L dH_2O

Separating buffer, 4x

1.5 M Tris/HCl, pH 8

Stacking buffer, 4x

0.5 M Tris/HCl, pH 6.8

Stripping buffer

62.5 mM Tris/HCl (pH 6.7), 2 % SDS (w/v)

Protein-loading buffer, (Laemmli) 5x

0.3 M Tris/HCl (pH 6.8), 10% SDS (w/v), 25% β-Mercaptoethanol,
0.1 mg/mL Bromphenol Blue, Glycerol

Western blot transfer buffer - CAPS buffer

10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, pH 11

The CAPS buffer was prepared as 10x stock solutions and mixed with methanol prior to use.

3.2. Methods

3.2.1. Extraction and generation of proteins

3.2.1.1. Isolation of granulocytes

Granulocytes were isolated from fresh buffy coats of normal human blood donors (Blood transfusion department, University Hospital Hamburg-Eppendorf). After diluting 1:4 (v/v) with phosphate-buffered saline (PBS) solution, the blood was layered on Ficoll-Paque (d = 1.077 g/cm3; Pharmacia, Freiburg). Following centrifugation for 20 min (2000 rpm) at 4°C, the upper phase was carefully removed, and granulocytes were harvested. To remove the contaminating erythrocytes, the cells were resuspended in erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 8.0), and incubated for 20 min at room temperature (RT). After centrifugation by 1500 rpm for 5 min, the supernatant was removed, granulocytes were washed three times with PBS, and immediately analyzed. The purity of granulocyte preparations was confirmed by FACS analysis applying anti-human CD15 mAb CBL144 (Chemicon, Hampshire).

Reference:
(Lucka, et al. 2005)

3.2.1.2 Protein extraction (Cell lysates preparation)

To extract the proteins from granulocytes or other cell lines, “cell lysates” were prepared. A crude lysate is the solution produced when cells are destroyed by disrupting their cell membranes in a process known as cytolysis. Here, we prepared lysates from a variety of cell lines like CHO, HEK293 and leukocytes. The cells were lysed in fresh prepared KLB-buffer. Additionally for this procedure, Na-pervanadate solution (100 µL of 50 mM Na-orthovanadate + 16 µL H_2O_2 (30%) incubated for 30 min) was prepared.

Before use, in 10 mL of the KLB-buffer protease inhibitors (Table 3) were added.

Components	Volume/µL
0.1 M PMSF	100
Aprotinin	100
50 mM Na-Pervanadate solution	20
1 M NaF	100

Table 3. The components added to the KLB-buffer

The cells were harvested using a method appropriate to the properties of the specific cell line and the growth vessel. After the growth medium was removed, the cells were carefully washed twice in cold PBS, and placed on ice. 0.5-1 mL of the freshly prepared KLB buffer was added to the cells and the cells were incubated for 30 min on ice. The adherent cells were scraped from the plate with plastic scraper. After the cells suspension was centrifuge by 14000 rpm for 10 min at 4°C, the supernatant was pipetted in new tube without disturbing the pellet, and stored at -20°C.

References:

(Mayer, et al. 1991)
(Nollau and Mayer 2001)

3.2.1.3. Protein extraction from tissue

After the tissue was washed with sterile PBS, it was cut into small pieces and immediately freezed in liquid nitrogen. Since tissues required a more vigorous disruption, we used a bead mill for efficient disruption and homogenization. For total protein sample solubilisation, 1 mL of chilled freshly prepared KLB lysis buffer, as described above, was added for 30 min. Subsequent to centrifugation (14 000 rpm, 10 min, 4 °C), the supernatant was pipetted in new tubes, and the total protein concentration was determined by Bradford assay. The tissue lysates were kept at -20°C.

The lysates from melanoma tissue were obtained from Biocut, Heidelberg.

The placenta tissue as well as normal colon and colorectal cancer tissues were obtained from Institute of Pathology, University Hospital - Eppendorf, Hamburg.

Reference:
(Nollau, et al. 1997b)

3.2.1.4. Purification of native CEACAM1 from human granulocytes

CEACAM1 was purified from crude membrane extracts of human granulocytes by immunoaffinity chromatography using the monoclonal antibody (mAb) T84.1, which binds to a common epitope on CEACAM1, CEACAM5 and CEACAM6, respectively. To attain a higher purity, this protein fraction was further treated by size-exclusion chromatography.
The purification was performed by Christa Frenz (Department of Clinical Chemistry, University Hospital-Eppendorf, Hamburg) and the method in details is described by Lucka, et al. 2005.

Reference:
(Lucka, et al. 2005)

3.2.1.5. Generation of soluble Fc-DC-SIGN

For the generation of soluble recombinant Fc-DC-SIGN protein, culture plates of HEK293 cells were transiently transfected with 5 µg of Fc-DC-SIGN cDNA using Lipofectamin 2000 (Invitrogen) and cultivated for 48h in serum free medium (SFMII, Invitrogen). Cell culture supernatants were harvested, centrifuged by 3000 rpm for 10 min, concentrated by ultrafiltration (Amicon 10; Millipore Cooperation, Bedford, MA) at 3000 rpm, exchanged against PBS by PD10 columns (Amersham Bioscience, Freiburg, Germany), and the protein concentration was determined by Bradford assay (BioRad, München, Germany). The purity of the preparation was

analyzed by Coomassie staining and immunoblotting using the specific anti-DC-SIGN mAb.

Reference:

(Sambrook and Russel 2001)

3.2.1.6. Purification of ICAM-3 from human leukocytes

ICAM-3 was purified from human blood buffy coat cell lysates by affinity chromatography. Human blood buffy coats were acquired from Finnish Red Cross Blood Transfusion Service, Helsinki, Finland.

The purification procedure of ICAM-3 from human leukocytes was performed by Prof. Dr. Carl G.Gahmberg's research group, Department of Bioscience, Division of Biochemistry, University of Helsinki, Finland. The purity of the ICAM-3 preparation was analyzed by Coomassie staining after separation of the proteins by 8 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Reference:

(Funatsu, et al. 2001)

3.2.2. Protein characterisation

3.2.2.1. Determination of the protein concentration by Bradford method

The Bradford assay is a colorimetric assay for measuring total protein concentration in a given solution. It is based on an absorbance shift in the Coomassie brilliant blue G-250 (CBBG) dye when bound to arginine and hydrophobic amino acid residues present in protein. CBBG binds to these residues in the anionic form, which has an absorbance maximum at 595 nm (blue). The free dye in solution is in the cationic form, which has an absorbance maximum at 470 nm (red). The assay is monitored at

595 nm using a spectrophotometer. Increase of the absorbance is proportional to the amount of bound dye and thus to concentration of the proteins present in the sample. A standard curve was made using dilutions of BSA/1x PBS in a range of 30 µg/mL, 15 µg/mL, 7.5 µg/mL, 3.75 µg/mL and 1.875 µg/mL. As recommended by supplier, 200 µL of Bradford-reagent (Biorad, Munich) were pipetted to 800 µL of each dilution, mixed, and incubated for 5 min at RT. From the unknown protein sample, it was pipette 2 µL and mixed with 798 µL 1x PBS and 200 µL Bradford reagent. When it was necessary, the unknown sample was diluted, and the result was corrected. The absorbance was measured at 595 nm.

References:

(Bradford 1976)
(Stoscheck 1990)

3.2.2.2. SDS-polyacrylamide gel electrophoresis (Laemmli method)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their molecular weight and electrical properties as they migrate through a polyacrylamide gel matrix.

Before separation in an electric field based on the molecular weight, the proteins were first denatured and made soluble with detergent, sodium dodecyl sulfate (SDS), and a disulfide bond reducing compound, such as 2-mercaptoehanol or dithiothreitol (DTT). SDS binds to polypeptide chains, destroying most of their complex structure and giving them an overall negative charge.

Polyacrylamide gels are generated by the polymerization of acrylamide monomer and the crosslinking co-monomer N,N'-methylene-bis-acrylamide (referred to as bis). Variations in the concentrations of monomer and crosslinker, lead to polyacrylamide gels with different pore sizes. The polymerization reaction is initiated by a catalyst N,N,N',N'-Tetramethylethylene diamine (TEMED), and an initiator, ammonium persulfate (APS). For separating proteins, usually concentrations of 8-12% polyacrylamide are used with ratios of 19:1 or 29:1 of

acrylamide to bis. Regardless of the system, preparation requires casting of two different layers of acrylamide between glass plates of gel cassettes. The lower layer (separating or resolving gel) is responsible for actually separating polypeptides by their molecular weight. The upper layer (stacking gel) includes the sample wells.

The protein samples and the 8 % SDS-PAGE were prepared as follows:

Protein samples preparation:

6 µL of 5x loading (Laemmli) buffer was pipetted in 24 µL of the protein sample and heated at 95°C for 3 min.

Gel preparation:

The components used for preparing of the separating gel are shown in Table 4.

Components	**8 % separating gel**
Acrylamide/Bisacrylamide (40%), 29:1	1 mL
Separating gel buffer (1.5 M Tris/HCl, pH 8.8)	1.25 mL
SDS (10%)	50 µL
H2O	2.65 µL
APS (10%)	50 µL
TEMED (10%)	1.7 µL
Total volume	5mL

Table 4. Protocol for separating gel

Immediately after pouring the separating gel mix into already prepared cassette, the gel was overlaid with water-saturated butanol for obtaining a smooth, completely level surface on top of the separating gel, in order to obtain straight and uniform bands. Then the gel was left to polymerise.

The components used for preparing of 4 %stacking gel are shown in Table 5.

Components	**4% Stacking gel**
Acrylamide/Bisacrylamide (40%), 29:1	250 µL
0.5 M Tris/HCl, pH 6.8	625 µL
SDS (10%)	25 µL
H_20	1.58 mL
APS (10%)	19 µL
TEMED	2.5 µL
Total volume	2.5 mL

Table 5. Protocol for stacking gel

After the polymerization, the butanol was poured off from the surface of the separating gel, and the stacking gel mix was immediately added into the cassette. A comb was inserted in the stacking gel in order to form sample wells, taking care not to entrap air bubbles under the teeth of the comb. When the stacking gel was polymerised, the comb was removed and the samples were loaded in the sample wells. The gel was run at constant current of 10 mA for stacking gel, increasing to 20 mA for separating gel until the bromophenol blue dye in the samples reached the bottom of the gel. The molecular weight of the unknown proteins was determinated by comparing their relative electrophoretic mobility to standard proteins of known molecular weights (Benchmark pre-stained protein ladder, Invitrogene).

Reference:

(Laemmli 1970)

3.2.2.3. Protein transfer -western blotting

Western blotting, also known as "protein blotting" or "immunoblotting" is a well-established method used to detect a target protein from a mixture of proteins.

Proteins in a mixture (e.g. cell culture lysate, tissue homogenate, etc.) are separated by molecular weight using SDS-polyacrylamide gel electrophoresis as described above. The proteins were transferred (blotted) from the gel onto a membrane for easier handling and manipulation.

We have used 0.45 µm pore size, polyvinylidene difluoride (PVDF) high quality membrane, cut to a same size as the gel (9 x 10 cm). PVDF membrane is particularly suitable for high sensitivity and low background immunoblotting. The membrane was first soaked in 100% methanol for 15 sec, incubated in distillate water for 2-5 min followed by incubation in the transfer buffer (CAPS buffer) until ready for use. A western transfer instrument was prepared by sandwiching one pieces of Whatman paper (10 x 10 cm), the PVDF membrane, the SDS-PAGE gel, and one more piece of Whatman paper, in that order, between two wet porous pads. This sandwich formation was pressed between the two plastic panes of the western transfer

instrument with the PVDF membrane near to the positive electrode of the instrument. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and proteins. The instrument was run for 3 hours with about 400 mA electricity under stirring in a cold room (4°C). The transfer of the pre-stained protein ladder allowed easy evaluation of the transfer efficiency, and afterwards, easy determination of the molecular weight of the proteins.

References:

(Barnett, et al. 1993)
(Towbin, et al. 1979)
Instruction Manual, Millipore, USA

3.2.2.4. Coomassie staining

Coomassie staining was used for visualisation of protein bands on polyacrylamide gel. The method is based on the fact that Coomassie (Brilliant Blue G-250) dye binds the proteins. We have used Roti-Blue colloidal CBBG-250 staining solution, which colloidal properties allow the dye to bind the proteins, and only minimal the gel matrix. For these purpose, polyacrylamide gel was incubated in mix of 60 mL H_20, 20 mL Methanol and 20 mL Roti-Blue (5x concentrate) for 1h under gently shaking. Colloidal dye complexes from gel surface was removed by incubating the gel in washing solution (25 mL Methanol + 75 mL H_20) for 30 min. The gel was stored in 20% Ethanol, sealed in a plastic bag. The molecular weight of the proteins in the samples was determinated by comparing it to the molecular weight of a pre-stained protein marker.

Reference:

Instruction for use, Roth, Karlsruhe, Germany

3.2.2.5. Protein immunodetection (Standard immunodetection method)

After separation by SDS-PAGE, the proteins were transferred to PVDF membrane, and the non-specific binding sites were blocked by incubating the membrane in a buffer-salt solution, such as TBST (tris-buffered saline with tween), containing BSA (3 %) or 1% blocking reagent (Roche) for overnight.

Subsequent to blocking, the membrane was placed in a plastic bag and treated with the primary antibody (1 mg/mL) in TBST by incubation for 1h at RT on rotary platform shaker. The first antibody was specific for the protein of interest. Afterwards, the membrane was washed three times with TBST for 5 min, and incubated at a dilution of 1:25000 to 1:50000 with the appropriate secondary antibody, horseradish peroxidase (HRP)-labeled, for 1h at RT. During the incubation with the second antibody and the washing steps, the protein membrane was agitated on shaker.

Subsequent to the washing step (3h at RT in TBST, the washing buffer was changed every 15 min), the signals were detected by Enhanced Chemiluminescence (ECL) reagents purchased from Amersham Company. Following the supplier recommended protocol, the substrate working solution was prepared by mixing equal parts of the detection reagents 1 and 2. After the washing buffer was removed, the membrane was incubated in the working solution for 1 min at RT. The excess liquid was removed, and the membrane was placed in a plastic sheet protector in a film cassette. The membrane was exposed to X-ray film from 30 sec to several hours in order to be obtained optimal results.

References:

(Batteiger, et al. 1982)

(Harlow 1988)

Manuel protocol, ECL Western Blotting Kit, Amersham Biosciences

3.2.2.6. Membrane stripping (by heat and detergent)

Membrane stripping by heat and detergent is applicable to any chemiluminescent substrate system and uses a combination of detergent and heat to release the antibodies bound to the membrane. After stripping, it is still possible to analyze the blot with another antibody specific to a different target protein. The blot should not get dry between rounds of immunodetection. If allowed to dry, any residual antibody molecule will bind permanently to the membrane.

The membranes were incubated in 10 mL stripping buffer containing 63µL β-mercaptoethanol (100 mM) at 50°C in water bath for 30 min sealed in plastic bag.

Subsequent to washing in TBST washing buffer, twice for 10 min, blocking step in TBST/3% BSA buffer or in 1% blocking reagent (Roche) was proceed before the next round of detection.

Reference:

(Sambrook and Russel 2001)

3.2.2.7. DC-SIGN overlay assay

Whole cellular extracts, immunoprecipitates or purified proteins were separated by SDS-PAGE and transferred to PVDF-membranes as described above. After blocking overnight at 4°C in 1% blocking reagent (Roche), membranes were incubated with recombinant soluble Fc-DC-SIGN (5 µg/mL) in TSM-buffer for 2h at 4°C under shaking. The membranes were washed three times for 5 min in cold TSM-buffer, and the bound Fc-DC-SIGN was detected by HRP-labeled rabbit anti-human IgG at dilution of 1:25000 in cold TSM buffer. Subsequent to incubation for 1 h, the membranes were washed three times for 15 min in TSM buffer, and the bands were visualized using chemiluminescence.

Reference:

(Appelmelk, et al. 2003)

3.2.2.8. Immunoprecipitation (IP)

Immunoprecipitation (IP) involves the interaction between a protein and its specific antibody, the separation of these immune complexes with Protein G or Protein A, and the subsequent analysis by SDS-PAGE. This technique provides a rapid and simple means to separate a specific protein from whole cell lysates or culture supernatants. In our experiments, we have used the Protein G-plus coupled to insoluble matrix, such as agarose beads.

The whole cellular lysates were prepared as describe above (section cell lysates preparation) and precleared with 20 µL Protein G-plus agarose beads (pre-washed two times with 500 µL KLB-buffer) by incubation for 3h on ice, and the beads were pellet at 2500 rpm for 1 min. The pre-clearing step was incorporated to decrease the amount of non-specific contamination and to remove proteins with high affinity for Protein G-plus agarose. 250-500 µg of the precleared whole cellular lysates were incubated with 1-5 µg of the primary specific antibody for 3h on ice. For each antibody used for precipitation, the concentration was titrated and the amount of cell lysates in preliminary experiments was determinated in other to achieve optimal conditions. 20 µL of the pre-washed (with KLB-buffer) protein G-plus agarose beads were added, and incubated overnight at 4°C on rotary shaker. The beads were pellet at 2500 rpm for 1 min and carefully washed three times with 500 µL KLB-buffer, freshly prepared. After the third washing step, 24 µL of the supernatant were kept in which 6µL of 5x Laemmli sample buffer were added, and boiled at 95 °C for 3 min. Subsequent to centrifugation (2500 rpm, 30 sec), the supernatant was loaded on 8% SDS-PAGE and the proteins were transferred to PVDF membrane. The detection of the targeted protein was performed with specific antibody as previously described in the section immunodetection.

References:

(Sambrook and Russel 2001)

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Manufacturer's instructions for Protein G-plus agarose, Santa Cruz

3.2.2.9. Defucosylation of the glycoproteins

The glycoproteins were defucosylated by treatment with α(1-3,4) fucosidase, an enzyme that catalyzes the hydrolysis of α1-3 and α1-4-linked branched, non-reducing terminal fucose from complex carbohydrates. Lex residues present on glycostructure of the glycoproteins were completely destroyed by treatment of the glycoproteins with α(1-3,4) fucosidase. The whole cellular lysates were prepared and precleared as described above. 200-500 µg of the precleared whole cellular lysates were incubated with 1-5 µg of the specific mAb at 4°C for 3h, subsequently 20 µL of Protein G Plus agarose were added and the reaction was incubated overnight at 4°C on rotary shaker. The beads were washed three times with 500µL of 50 mM sodium phosphate buffer (pH 5.0). After the third washing step, 35 µL of the supernatant was kept and 25 mU/mL of α(1-3,4) fucosidase was added. The digestion with α(1-3,4) fucosidase was carried out overnight at 37°C. The beads were boiled for 3 min at 95°C in 1 x Laemmli-buffer and subsequent to centrifugation (2500 rpm, 15 sec), the supernatant containing the fucosidase treated proteins was separated by SDS-PAGE.

References:

Product information of α (1-3,4) fucosidase, Calbiochem
(Sambrook and Russel 2001)

3.2.3. Cell biological methods

3.2.3.1. Freezing cultured cells

2 x 10^6 cells (in late log phase growth) were centrifuged at 900 rpm. The cells were re-suspend in 1 mL freezing medium (FCS, 10% DMSO), and placed in a sterile tube. The tubes were placed in styrofoam container with lid and freezed at -80°C. Once frozen, the cells were stored in liquid nitrogen.

Reference:
(Sambrook and Russel 2001)

3.2.3.2. Thawing cultured cells

After the cells were retrieved from liquid nitrogen storage, they were thawed in water bath at 37°C. 10 mL warm corresponding media cell culture was added, mixed and spin down at 900 rpm for 5 min. The cells were re-suspended in fresh complete growth medium into a sterile flask, diluted to the appropriate concentration. They were incubated at 37°C in a presence of 5% CO_2 until confluent, and subsequently split into desired flask size. The culture reagents were purchased from PAA Laboratories (Cölbe, Germany).

Reference:
(Sambrook and Russel 2001)

3.2.3.3. Cell lines and tissue culture

For transfection studies, we have used two cell lines: Human Embryonic Kidney-293 (HEK293) and Chinese Hamster Ovary (CHO). HEK293 and CHO cells were

obtained from the American Type Culture Collection and cultured in Dulbeco's modified Eagle's medium and RPMI1640, respectively, supplemented by 10% fetal calf serum, 100 U/mL penicillin and 10 mg/mL streptomycine. The culture reagents were purchased from PAA Laboratories (Cölbe, Germany).
Atmosphere conditions required to support the growth include 5 % CO_2 in humidified incubator at 37°C.

References:

Manuel protocol, American Type Culture Collection, Manassas
Manuel protocol, PAA Laboratories, Cölbe

3.2.3.4. Transient transfection

The transient transfections of CHO and HEK293 cells were performed with 5 µg of plasmid DNA applying Lipofectamine 2000™ as recommended by the supplier (Invitrogene, Karlsruhe, Germany). A day before the transfection, the cells (1 x 10^6) were cultivated in culture plates (diameter: 10 cm) in corresponding growth medium so that they were 70% confluent at the time of transfection.
The plasmid DNA was incubated with 500µL culture medium (without FCS and antibiotics) for 5 min. At the same time, 25 µL of the Lipofectamine 2000™ with 500µL of the culture media (without FCS and antibiotics) were incubated for 5 min. After the incubation, both solutions, diluted DNA and diluted Lipofectamin 2000™ were combined, mixed gently and incubated for 20 min at room temperature in order to allow the DNA-Lipofectamin 2000™ complexes to be formed. In meantime, the cells were washed 3 times with cell culture medium (without FCS and antibiotics), and covered with 3 mL of the same medium. The DNA-Lipofectamin 2000™ complexes were added to the plate, mixed gently by rocking the plate back and forth, and the cells were incubated at 37°C in a CO_2 incubator. After 3 hours of incubation, the medium was replaced with 10 mL fresh medium (containing FCS and antibiotics), and the cells were incubated for 48 hours. The expression of the recombinant proteins was confirmed by FACS and western blot analyses.

Reference:

Manuel protocol, Invitrogene, Karlsruhe, Germany.

3.2.3.5. Stable transfection

CHO cells were transfected with 5µg CEACAM1 cDNA (vector: pcDNA3.1/G418) or in combination with 5µg FUTIX cDNA (vector: pcDNA3.1/Zeo) as described above and the stable transfectants were selected in present of G418 or G418 in combination with zeocine, respectively. After selection, the stable CHO transfectants expressing CEACAM1 were cultivated in RPMI1640/FCS medium in the presence of G418 while the CHO transfectants co-expressing CEACAM1 and FUTIX in presence of G418 and Zeocine, at a final concentration of 0.5 mg/mL. The expression of CEACAM1 and FUTIX was confirmed applying western blot analyses. Additional, the expression level of CEACAM1 and Lex structure on the surface of the transfected cells was demonstrated by FACS analyses.

Reference:

(Ebrahimnejad, et al. 2004)

3.2.3.6. Generation of dendritic cells (DCs)

For the generation of immature dendritic cells, mononuclear cells from normal human blood donors were isolated from the interphase after density centrifugation as described previously (section “isolation of granulocytes”). Monocytes were isolated by anti-CD14 magnetic microbeads following the protocol of the supplier (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany).

After isolation, the monocytes were cultivated for 5 to 7 days in RPMI1640/10% fetal calf serum (FCS) in the presence of interleukin-4 (IL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF) in concentration of 500 U/mL and

800 U/mL, respectively. IL-4 and GM-CSF were purchased from PromoKine (Heidelberg, Germany). Differentiation of the monocytes to immature dendritic cells was confirmed by FACS-analysis using the DC-SIGN specific mAb 120507 and antibodies directed against the surface markers CD14, CD83 and CD86, respectively. Lack of CD14 expression was demonstrated, as well as high levels of DC-SIGN and low to moderate levels of expression of CD83 and CD86, which is typical for immature dendritic cells.

Reference:

(Romani, et al. 1994)

3.2.3.7. Cell binding assay

Plasma membranes of CHO cells were fluorescently labelled by FM, 1-43FX as recommended by the supplier (Molecular Probes, Eugene, OR). Cells were washed in PBS, resuspended in cell-culture medium containing 1 mM $CaCl_2$ and 2mM $MgCl_2$, respectively, and incubated at a ratio of 1:1 with 1 x 10^5 immature dendritic cells cultivated on Lab Tek glass chamber slides (surface area: 1.8 cm^2; Nunc, Wiesbaden, Germany). After incubation for 30 min at 37°C, slides were washed fives times in pre-warmed PBS, cells were fixed in 4% paraformaldehyde/PBS and binding of CHO cells was visualized by fluorescence microscopy (Leitz DMRB fluorescence microscope, Leitz, Wetzlar, Germany) and photographically documented by a digital camera. Cell numbers per microscopic field were determined from digital images applying the cell-counting tool of the NIH image software package (version 1.63; NIH, Bethesda, MR). For blocking studies, immature DCs were pre-incubated by DC-SIGN mAb DC28 (20 mg/mL) for 30 min at 37°C. Levels of statistical significance were calculated by the Student's t-test.

Reference:

(Sambrook and Russel 2001)

Manuel protocol, Molecular Probes, Eugene, OR

3.2.4. Flow cytometry analysis

Flow cytometry is a technology that simultaneously measures and analyzes multiple physical characteristic of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity and relative fluorescence intensity. This technology is frequently used to determine the types of markers and receptors on the surface of a cell. For these experiments, a fluorescent dye is attached to antibodies or receptor ligands and then the cells containing labeled antibodies can be subjected to flow cytometry. The amount of receptor on cell surface is detected as a level of fluorescence. Flow cytometry can be also used to sort out cells of a particular type from a mixer. Fluorescence-activated cell sorter (FACS) is an instrument based on flow cytometry that can select one cell from thousands of other cells.

In this study, flow cytometry analyses were performed for following investigations: determination of the purity of the granulocytes or other populations isolated from peripheral human blood leukocytes, protein expression level on cell surface by transient and stable transfectomas, differentiation of monocytes to dendritic cells, Fc-DC-SIGN binding assay and granulocyte apoptosis.

After the cells were harvested or purified from peripheral blood in corresponding buffer solution, they were washed with cold PBS and counted in Neubauer's-chamber. The cells isolated from peripheral blood were pre-incubated by mouse or human polyclonal IgG (Sigma, Taufkirchen, Germany) at a final concentration of 200 µg/mL for 30 min at 4°C in order to block the potential binding of Fc-DC-SIGN or antibodies by Fc-receptors. Cells (10^5) were placed in 5 ml FACS-tube in 100 µl working volume of FACS-buffer and incubated with the appropriate antibody (0.5 -2 µg) or Fc-DC-SIGN (2.5 µg) for 30 min at 4°C. Subsequent to washing (twice with 4 ml FACS-buffer), cells were pelleted at 1200 rpm at 4°C and fixed in 500 µL 1% paraformaldehyde/FACS-buffer.

For blocking experiments, cells were pre-incubated by the appropriate blocking antibodies at a final concentration of 20 µg/mL for monoclonal and at 100 µg/mL for polyclonal antibodies for 30 min at 4°C. Isotypes of the same subclass, species and

fluorochrome, as the specific antibodies were used in the experiments. The isotype controls were used at the same concentration as the specific antibodies usually as recommended by the supplier.

References:

(Battye and Shortman 1991)

A learning guide, BD Biosciences, San Jose, CA

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3.2.4.1. Interpretation of the flow cytometry data

The FACS analyses were carried out on FACS Calibur flow cytometer and the data were analysed by the programme CELLQUEST™ (Becton Dickinson Immunocytometry System, Manisfield, MA). The data were processed with 10^5 cells pro sample and 10^4 counts (events). Isotype control of the same subclass, species and fluorochrome as the specific antibody were included in order to provide some evidence of non-specific binding from the specific antibody.

Once a data file has been saved, cell population can be displayed in several formats like histogram or dot plot format. Single parameters such as fluorescence (FITC-F1, PE-F2) are represented in histograms format, whereas the horizontal axis represent the parameter's signal value in channel numbers and the vertical axis represents the number of events per channel number.

Two parameters are displayed simultaneously in a two-dimensional dot plot, where one parameter is displayed on the x-axis (FITC) and the other parameter on the y-axis (PE). A quadrant marker divides two-parameter plots into four sections to distinguish population considered negative, single positive or double positive. The lower-left quadrant displays events that are negative for both parameters. The upper-left quadrant display events that are positive for the y-axis parameter, but negative for x-axis parameter. The lower-right quadrant display events positive for x-axis parameter, but negative for y-axis parameter. The upper-right quadrant contains events that are positive for both parameters.

Reference:

A learning guide, BD Biosciences, San Jose, CA

3.2.5. Biomolecular methods

3.2.5.1. Polymerase chain reaction-PCR

The polymerase chain reaction (PCR) has revolutionized molecular genetics by permitting rapid cloning and analysis of DNA. PCR is a rapid and versatile in vitro method for amplifying defined target DNA sequences present within a source of DNA. To permit such selective amplification two synthetic oligonucleotide primers (often about 15-20 nucleotides long) complementary to the 3' ends of the target DNA are required. The PCR technique uses DNA Polymerase like, *Taq* polymerase from *Thermus aquaticus*, *Pfu* polymerase from *Pyrococcus furiosus*, or *Vent* polymerase from *Thermococcus litoralis*, which in general have maximal catalytic activity at 75 to 80°C. The reaction involves sequential cycles composed of three steps: denaturing the double-stranded DNA (has to be heated to 94-96°C in order to separate the strands); annealing (60°C), the primers hybridized to their complementary sequences in the target DNA; and elongation step at 72°C, where the DNA-polymerase extends each primer from its 3'end generating newly synthesised strands. Repeated cycles (around 30 times), synthesis (cooling) and melting (heating) quickly amplify the sequences of interest. At the end, follows one elongation step of 10 min, at 72 °C in order the incomplete PCR-fragment to become completed DNA-strand. Critical parameters to optimize include incubation times and temperatures, concentration of DNA Polymerase, primers, $MgCl_2$, and template DNA.

References:

(Mullis and Faloona 1987)

(Saiki, et al. 1988)

3.2.5.1.1. Generation of CEACAM1 cDNA by PCR

The entire coding regions of CEACAM1 was amplified from human cDNA (provided from Dr. Peter Nollau, Institute for Clinical Chemistry, University Hospital Hamburg-Eppendorf) by polymerase chain reaction and cloned into a pEF-BOS expression vector (Dr. B.J. Mayer, Children's Hospital, Harvard Medical School, Boston, USA). For precipitation and detection, a myc-tag was attached to C-terminus of CEACAM1 cDNA. The template DNA was 1:100 diluted with sterile deionized water and from this dilution, 1 µL was pipetted. *Pfu*-polymerase was used as well as its corresponding reaction buffer. In the upper primer *Sal*I and in the lower primer *Asp*718, endonucleases restriction sites were incorporated. The primers were obtained form MWG-Biotech AG, Martinsried, Germany. A control reaction, omitting template DNA, was performed to confirm the absence of contamination. The reaction probe was set as shown in Table 6.

Components	**Volume/µL**
10x *Pfu*-Reaction buffer	5
dNTPs (50 x)	1
Upper -*Primer* (20 pmol/µL)	1
Lower - *Primer* (20 pmol/µl)	1
DNA-template	1
Pfu-Polymerase	1
H_2O	40
Total volume	50

Table 6. Reagents for PCR amplification of CEACAM1 cDNA

The reaction mixture was overlaid with 1 drop of mineral oil. The amplification was performed in Biorad Minicycler using the program as shown in Table 7.

Reaction step	**Temperature /°C**	**Time**
1. initial denaturing	95	5 min
2. denaturing	95	30 sec
3. annealing	60	40 sec
4. elongation	72	2.5 min
5. final elongation	72	10 min

Table 7. Program for PCR amplification of CEACAM1 cDNA

Thirty cycles (from step 2 to 4 step) were performed.

Primers (restriction sites are underlined):

Upper: 5'-ACGCGTCGACACCATGGGGCACCTCTCAGCCCCAC-3'

Lower: 5'CCGGTACCCTGCTTTTTTACTTCTGAATAAATTATTTCTGTGGC-3'

3.2.5.1.2 Generation of ICAM-3 cDNA by PCR

DNA encoding regions of full-length ICAM-3 was amplified from human cDNA (provided by Dr. Peter Nollau, Institute for Clinical Chemistry, University Hospital Hamburg-Eppendorf) and cloned into a mammalian pEF-BOS expression vector. For precipitation and detection, a myc-tag was attached to C-terminus of ICAM-3 cDNA. The template DNA was 1:100 diluted with sterile deionized water and from this dilution, 1 µL was pipetted. It was used *Pfu*-polymerase as well as its corresponding reaction buffer. In the upper primer *Sal*I and in the lower primer *Not*I, endonucleases restriction sites were incorporated. A control reaction, omitting template DNA, was performed to confirm the absence of contamination. The reaction probe was set as shown in Table 8.

Components	**Volume/µL**
10x *Pfu*-Reaction buffer	5
dNTPs (50 x)	1
Upper -*Primer* (20 pmol/µL)	1
Lower - *Primer* (20 pmol/µL)	1
DNA-template	1
Pfu-Polymerase	1
DMSO	5
H_2O	35
Total volume	50

Table 8. Reagents for PCR amplification of ICAM-3 cDNA

The reaction mixture was overlaid with 1 drop of mineral oil. The amplification was performed in Biorad Minicycler using the program as shown in Table 9.

Reaction step	Temperature /°C	Time
1. initial denaturing	95	5 min
2. denaturing	95	30 sec
3. annealing	62	30 sec
4. elongation	72	2.5 min
5. final elongation	72	10 min

Table 9. Program for PCR amplification of ICAM-3 cDNA

Thirty cycles (from step 2 to 4 step) were performed.

Primers (restriction sites are underlined):

Upper: 5'-GATCTGTCGACACCATGGCCACCATGGTACCATC-3'

Lower: 5'-TTGCGGCCGCCAAGTCCTCTTCAGAAATGAGCTTTTGCTCCTCAGCT CTGGACGGTTCTTC-3'

3.2.5.1.3. Generation of Fc- DC-SIGN cDNA construct by overlap PCR

For the Fc-DC-SIGN overlay assay, the extracellular part of DC-SIGN was amplified by PCR. A full-length of DC-SIGN cDNA was used as template for amplification of the extracellular part of DC-SIGN. As a detection region, a human Fc DNA-fragment was attached to the N-terminus of the DC-SIGN DNA fragment, and the construct was cloned into a pEF-BOS based expression vector. This construct was cloned by Charsten Fisher, Institute of Clinical Chemistry, University Hospital Hamburg-Eppendorf. Briefly, two fragments of the DNA template were generated and than anneal-extend to reconstitute the whole DNA construct. The two fragments were overlapping on a decent length. The first part is a standard PCR followed by overlap PCR. The reaction was set up into a sterile 0.5-mL microcentrifuge tube as shown in Table 10.

Components	Volume/µL
10x *Pfu*-Reaction buffer	5
dNTPs (50 x)	1
Upper -*Primer* (20pmol/µL)	1
Lower - *Primer* (20 pmol/µL)	1
DNA-template	1
Pfu-Polymerase	1
DMSO	5
H_2O	35
Total volume	50

Table 10. Setting of the standard PCR1 and PCR2 for Fc-DC-SIGN cDNA amplification

Reaction step	Temperature /°C	Time
1. initial denaturing	95	5 min
2. denaturing	95	30 sec
3. annealing	60	40 sec
4. elongation	72	2.5 min
5. final elongation	72	10 min

Table 11. Standard PCR program for amplification of Fc-DC-SIGN cDNA

The setting of the reaction probe for the overlap PCR was performed as shown in Table 12.

Components	Volume/µL
10x *Pfu*-Reaction buffer	5
dNTPs (50 x)	1
DMSO	5
H_2O	34
DNA from PCR1	1
DNA from PCR 2	1
Upper-*Primer* (20 pmol/µL)	1
Lower-*Primer* (20 pmol/µL)	1
Pfu-Polymerase	1
Total volume	50

Table 12. Reagents for overlap PCR amplification of Fc-DC-SIGN cDNA

The reaction mixture was overlaid with 1 drop of mineral oil. The amplification was performed in Biorad Minicycler using the PCR program in Table 13.

Reaction step	Temperature /°C	Time
1. initial denaturing	95	5 min
2. hybridization of DNA fragment form PCR1 and PCR2	60	5 min
3. elongation	72	8 min
4. denaturing	95	30 sec
5. Hybridisation of primers	60	30 sec
6. elongation	72	2.5 min
7. final elongation	72	10 min

Table 13. Overlap PCR program for amplification of Fc-DC-SIGN cDNA construct

In total 30 cycles (from step 4 to step 6) were performed.

Primers (the restriction sites for *Sal*I and *Asp*718 are underlined):

DC-SIGN upper I: 5'-CATTGTGCTGGATCTGTCGACG-3'

DC-SIGN lower I:

5'ACCACCCAAGTCCTCTTCAGAAATGAGCTTTTGCTCACCAGTGGAACCTGGAACCCAGAGC-3'

DC-SIGN upper II:

5'GAGCAAAAGCTCATTTCTGAAGAGGACTTGGGTGGTAGCTCCATAAGTCAGGAACAATC-3'

DC-SIGN lower II: 5'-AGCTTTTGCTCGGTACCCTACG-3'

3.2.5.1.4. Generation of fucosyltransferases (FUTs) cDNA

The entire coding regions of the FUTs FUTIII, IV, VII and FUTIX were amplified from human cDNA by polymerase chain reaction and cloned into a pEF-BOS expression vector by Heike Sander and Kevin Dierck, Institute for Clinical Chemistry, University Hospital Hamburg-Eppendorf. For detection, a HA-tag was attached to the fucosyltransferases cDNA.

3.2.5.2. Agarose gel electrophoresis

Agarose gel electrophoresis is an easy way to separate DNA fragments by their size and to visualize them.

The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbones. For this reason, when an electrical potential is placed on, the DNA will move toward the positive pole. The molecular weight of the DNA fragment is a logarithmic function from the distance passed within a certain time limit (i.e. small fragments travel much faster than large fragments). Adequate amount of agarose (a typical gel was 1% agarose) was dissolved and melted in high temperature (microwave) in a buffer such as 1x TAE (Tris-Acetate/EDTA). After short cooling of the agarose gel (approximately to 60°C), ethidium bromide was added (5 µL/100 mL gel) and the mix was pored out in a gel-casting chamber containing the well-forming comb. When the gel had received the needed hardness, the samples with appropriate amount of 10x loading buffer were loaded into the pre-formed sample wells. The separation was achieved after 60 min running at 80V in a horizontal position. The detection of the DNA bands was done using the UV light and Polaroid camera.

Reference:

(Sambrook and Russel 2001)

3.2.5.3. Purification of the PCR products from agarose gel

For purification of the PCR products from agarose gels, QIAEX II Gel Extraction Kit (Qiagen, Hilden) was used following the protocol of the supplier. Briefly, the DNA band was excised from the agarose gel with a clean, sharp scalpel and placed in a 1.5-mL microcentrifuge tube. Three volumes of buffer QX1 (Qiagen) to 1 volume of gel for DNA fragments in the range 100 bp – 4 kb, were added. QIAEX II was resuspended by vortexing for 30 sec and then 20 µL QIAEX II to the samples were

added. To solubilize the agarose, the solution (gel with buffer) was incubated at 50°C for 10 min. Every 2 min the tube was mixed by inverting to keep the QIAEX II beads in suspension. The colour of the mixture had to be yellow. The sample was centrifuged for 30 sec and the supernatant was carefully removed with a pipette. The pellet was washed with 500 µL of buffer QX1, twice with 500 µL of buffer PE (Qiagen) and air-dried for 10-15 min or until the pellet became white.

To elute DNA, 20 µL of H_2O were pipetted and the pellet was resuspended by vortexing, followed by incubation at room temperature for 5 min. After centrifugation for 30 sec, the supernatant was carefully pipetted into a clean tube. The supernatant contained the purified DNA.

Reference:

Manuel QIAEX II gel extraction kit, Qiagen, Hilden

3.2.5.4. Ligation of PCR products in the expression vector

TOPO TA Cloning® (Invitrogen) kit provided a highly efficient, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. The *Taq* polymerase has a non template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in the kit had single, overhanging 3´ deoxythymidine (T) residues, which allows the PCR inserts to be ligated efficiently with the vector. For ligation of the PCR products in the pCR 2.1 vector T4-DNA Ligase and corresponding ligase buffer were used (Table 14).

Components	Volume /µL
Purified PCR-Product-insert	6
Ligation puffer 10x	1
PCR 2.1 vector	2
Ligase T4 DNA	1
Total volume	10

Table 14. Protocol for ligation of PCR-products in pCR2.1 vector

The desired DNA constructs (inserts) were cleaved from PCR 2.1 vector, purified from agarose gel and legated in pEF-BOS expression vector as shown in Table 15.

Components	Volume /µL
DNA construct (inserts)	7
Ligation puffer 10x	1
pEF-BOS vector	1
Ligase T4 DNA	1
Total volume	10

Table 15. Protocol for ligation of the DNA fragments in pEF-BOS vector

The reaction was incubating over night at 16°C in Biozym Minicycler. The transformation of the legated product was proceed in One Shot INVαF'-Z-cells.

Reference:

User manual, TOPO TA cloning kit, Invitrogen

3.2.5.5. DNA sequencing (Sanger method)

For sequencing the DNA fragments incorporated in the expression vector, the Sanger sequencing method was carried out. The Sanger method is also called *dideoxy* sequencing because it involves the use of 2', 3'- dideoxynucleosides triphosphates (ddNTPs), which lacks a 3'-hydroxyl group. These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides. This occurs because a phosphodiester bond cannot be formed between the dideoxynucleotide and the next incoming nucleotide, and thus the DNA chain is terminated. In this method, the single strand DNA serves as the template strand for in vitro DNA synthesis whereas, as a primer a synthetic 5'-end-labeled oligodeoxynucleotide is used. With the automated procedures, the reactions were carried out in a single tube containing all four ddNTP's, each labeled with a different colour dye. The DNA was separated on a polyacrylamide gel since the four dyes fluoresce at different wavelengths, and a laser then reads the gel to determine the identity of each band according to the wavelengths at which it fluoresces.

The DNA-sequencing kit was purchased form the company Applied Biosystems. According to the supplier, the reaction was set as shown in table 16.

Components	Volume /µL
Ready reaction mix	4
Buffer	4
primer	1 (10 pmol/µL)
Template DNA	1 (1µg)
H_20	10
Total volume	20

Table 16. Sequencing protocol

T7 primer (sequence 5'-3' direction in the insert) and M13 primer (3'-5' direction in the insert) were used for sequencing of the desired insert in the pCR 2.1 vector. Complementary priming sites were already incorporated in the pCR 2.1 vector. In addition, internal primers were used for sequencing in pEF-BOS vector.
The amplification was performed as shown in Table 17.

Reaction step	Temperature /°C	Time
denaturing	96	30 sec
annealing	50	15 sec
elongation	60	4 min

Table 17. Sequencing amplification program

In total, 25 cycles were performed. The oil was carefully removed from the samples and the amplified products were pipetted into new tubes containing 50 µL Ethanol (100%) and 2 µL NaAc (3M, pH 5.2). The samples were incubate for 30 min on ice and then centrifuged for another 30 min at 13000 rpm. The supernatant was removed and the pellet subsequent to washing with 250 µL Ethanol (70%) was air-dried at 37°C. The sequencing analyses were performed at ABI PrismTM377 DNA Sequencer (Applied Biosystems) at the Institute of Cell biology and Clinical Neurobiology, University Hospital- Eppendorf, Hamburg.

References:

(Sanger, et al. 1977)
Manuel protocol, DNA-Sequencing Kit, Applied Biosystems

3.2.5.6. Restriction digestion

The desired DNA fragments were cleaved from pCR 2.1 vector and than re-cloned in pEF-BOS expression vector by performing restriction digestion (Table 18).

Components	Volume /µL
Plasmid DNA, pCR2.1 (mini preparation)	5
*Sal*I	1
*Asp*718 (or *Not*I for ICAM-3)	1
Buffer H (10x), (Roche)	1
H_2O	1
Total volume	10

Table 18. Protocol for restriction digestion of the desired DNA fragment cloned in pCR2.1 vector

The pEF-BOS vector was pre-cut with enzymes *Sal*I and *Asp*718 or with *Sal*I and *Not*I, by using the protocol in Table 19.

Components	Volume /µL
pEF-BOS vector (maxi preparation)	1
*Sal*I	1
*Asp*718 (or *Not*I for ICAM-3)	1
Buffer H (10x), (Roche)	1
H_2O	15
Total volume	20

Table 19. Protocol for restriction digestion of pEF-BOS expression vector

In order to prove that the expression vector contains the desired DNA fragment, additional digestion was done (Table 20).

Components	Volume /µL
DNA-maxi preparation	1
*Sal*I	1
*Asp*718 (or *Not*I for ICAM-3)	1
Buffer H (10x), (Roche)	1
H_2O	15
Total volume	20

Table 20. Protocol for restriction digestion of the desired DNA fragment cloned in pEF-BOS expression vector

The restriction digestions were carried out for 2 hours at 37°C.

Reference:
(Sambrook and Russel 2001)

3.2.5.7. Bacteria plate preparation

In 250 mL water, 7.5 g agar was dissolved, and the solution was autoclaved for 1 hour at 120 °C at 1 bar. Under the same conditions, the 2x LB medium was autoclaved. Both mediums where mixed in ratio 1:1. In addition, antibiotic ampicilin (100 µL/mL) was added, and poured into plates (diameter 10 cm). The plates were left to harden, inverted, and stored at 4°C.

3.2.5.8. Transformation

The transformation of One Shot INVαF'-Z-cells were performed following the procedure of TA-Cloning-Kit Version R (Invitrogene, Karlsruhe). Briefly, 50 µL vial of frozen One Shot INVαF'competent cells for each transformation was thawed on ice. 1 µL of the ligation reaction was pipetted in the cells and incubated for 30 min on ice. The heat shock for exactly 30 sec was done in a water bath at 42°C. After removing them from the water bath, the vials were placed on ice and 250 µL of SOC medium (at room temperature) was added to each vial. The vials were incubated at 37°C for 1 hour at 50 rpm in a rotary shaking incubator. 50 to 150 µL from the transformation reaction were spread on LB-agar plates and incubated over nigh at 37°C.
When a pCR 2.1 vector was used, 40 µL 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-Gal, 40 mg/mL in dimethylformamide dissolved) were added before spreading the samples on the plates for blue-white screening. The pCR2.1 vector contains lac promoter for expression of lacZα fragments, which encodes the

first 146 amino acids of β-galactosidase. X-Gal, a histochemical substrate was used to detect the β-galactosidase enzyme. When the plasmid contained the insert, the coding sequences for the enzyme were broken and the bacterial colonies were white while the other bacterial colonies were blue.

Reference:

TA-cloning kit Version R, Invitrogene, Karlsruhe

3.2.5.9. Isolation of plasmid DNA (mini-preparation)

The white single bacterial colony was picked from the plates with sterile tip, cultured in 3 mL 1xLB medium containing 3µL ampicilin (100 mg/mL), and incubated overnight at 37°C in shacking incubator (155 rpm). The bacteria were harvested by centrifugation of 2 mL bacterial culture in Eppendorf-centrifuge tubes at 14 000 rpm. The bacterial pellet was resuspended in 200 µL of buffer 1. By adding the 200 µL of buffer 2 and incubation for 5 min at room temperature, the bacteria were lysed. During this procedure, the use of a vortex must be avoided to minimize shearing of the contaminating chromosomal DNA. The reaction was neutralized by adding 200 µL of buffer 3. After 5 min of incubation, 600 µL phenol-chloroform-isoamylalcohol (25:24:1) were added and centrifuged for 10 min by 14000 rpm to separate the phases. 500 µL from the aqueous (top) phase was removed in new Eppendorf-tubes and 1 mL of 100% ethanol was added. The reaction was incubated for 10 min at room temperature. After centrifugation for 15 min at 14000 rpm, the DNA pellet was washed with 70% ethanol and air-dried. DNA pellet was resuspended in 50 µL double distillate water.

Reference:

Manuel protocol, Clinical Chemistry, University Hospital-Eppendorf, Hamburg

3.2.5.10. Isolation of plasmid DNA (maxi-preparation)

For maxi-preparation, Qiagen Plasmid Maxi Kit was applied. Briefly, approximately 500 µL of over night incubated bacterial culture from mini-preparation was inoculated in 100 mL 1xLB medium containing 100 µL selective antibiotic ampicilin (100 mg/mL), and incubated overnight at 37°C under vigorous shaking (155 rpm). The bacterial culture was transferred in sorvall tubes and bacterial cells were harvested by centrifugation at 6000xg for 15 min at 4°C in Sorvall RC5C centrifuge (rotor GSA). Bacterial pellet was completely resuspended in 10 mL buffer P1. 10 mL of buffer P2 was added, gently mixed by inverting 4-6 times, and incubated at RT for 5 min. Another 10 mL of chilled Buffer P3 were pipetted, immediately but gently mixed by inverting 4-6 times, and incubate on ice for 20 min. The precipitated material contained genomic DNA, proteins, cell debris, and SDS. The next step was centrifugation at 13000 rpm for 30 min at 4°C. The supernatant containing plasmid DNA was removed and loaded onto the QIAGEN-tip 500 (previously equilibrates by applying 10 mL buffer QBT) allowing entering in the column by gravity flow. After the QIAGEN-Tip 500 column was washed twice with 30 mL buffer QC, the DNA was eluted with 15 mL buffer QF and collected in sorvall tubes, precipitated with 10.5 mL isopropanol, mixed, and at the end immediately centrifuged at 11 000 rpm (Sorvall centrifuge-rotor SS-34) for 30 min at 4°C. Carefully the supernatant was decanted, the pellet with 4ml of 70% ethanol was washed, and divided in 2 mL Eppendorf tubes. After centrifugation for 10 min at 14 000 rpm, the pellet was air-dried and dissolved in each tube with 100 µL water. The DNA preparation was stored at -20°C.

Reference:

Protocol, Qiagen Plasmid Maxi Kit, Qiagen

3.2.5.11. Photometric determination of DNA concentration and quality

The concentration of DNA was determined by measuring the absorbance at 260 nm (A_{260}, where DNA absorbs the light most strongly) in a spectrophotometer. Correct measurements are obtained when the DNA is free of RNA and readings are at values between 0.1 and 1 absorption units.

The most common purity calculation is the ratio of the absorbance at 260 nm divided by the absorbance at 280 nm, where aromatic amino acids, if present as contamination in the DNA solution, absorb the light most strongly. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0.

DNA preparations were shortly vortexed, diluted 1:50 and 1:100 with water and measured at 260 nm and 280 nm. Water was used as a blank probe.

$$\text{DNA concentration } (\mu g/mL) = (A_{260nm}) \text{ x dilution factor x F}$$

F= (DNA extinction coefficient, 50 for double strand DNA)

Reference:

(Sambrook and Russel 2001)

4. RESULTS

4.1. CEACAM1 from human granulocytes is a major carrier of Lex residues

4.1.1. Detection of CEACAM1 from human granulocytes by western blot analysis

In order to identify CEACAM1 in whole cellular extracts of human granulocytes, western blot analyses were performed (Fig. 7). For the detection of CEACAM1, three monoclonal antibodies (mAbs) were applied. The monoclonal antibody T84.1 binds CEACAM6 in addition to CEACAM1. mAbs 12140-4 and 4D1C2 recognize only CEACAM1 among the CEACAMs expressed in human granulocytes. Applying mAb T84.1, CEACAM1 and CEACAM6 were detected as broad bands typical for glycoproteins with an apparent molecular weight of ~ 160 and 90 kDa, respectively. mAb 12140-4 and mAb 4D1C2 specifically recognize CEACAM1 confirming the identity and expression of CEACAM1 in human granulocytes.

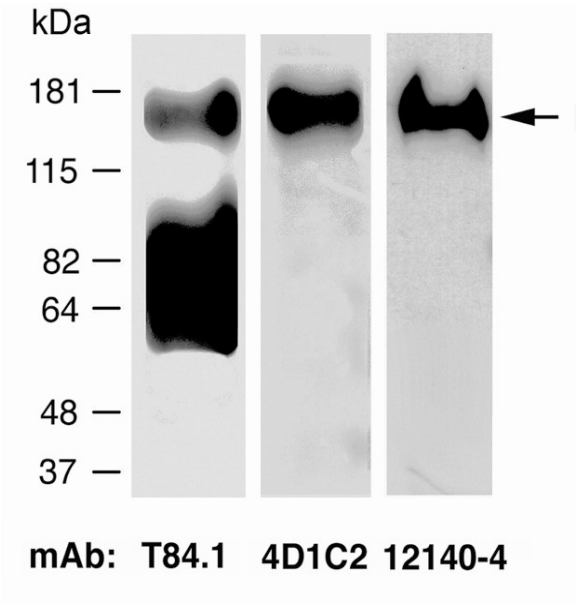


Figure 7. Detection of CEACAM1 by western blot analyses of whole cellular lysates of human granulocytes. CEACAM1 (marked by arrow) is specifically recognized by mAb 4D1C2 and mAb 12140-4, whereas CEACAM1 and CEACAM6 are detected by mAb T84.1 The Horseradish peroxidase (HRP)-labeled anti-mouse IgG mAb was applied as secondary antibody.

4.1.2. CEACAM1 expressed on human granulocytes is a major carrier of Lex groups

Western blot analysis revealed that CEACAM1 is a major carrier of Lex groups among the glycoproteins of human granulocytes.

The presence of Lex residues on CEACAM1 from human granulocytes was detected by Lex specific mAb L5, using whole cellular lysates of granulocytes and precipitates of CEACAM1 by T84.1 mAb (Fig. 8). In addition, the precipitated CEACAM1 was incubated with α (1-3, 4) fucosidase, an enzyme that specifically removes the fucose from Lex residues. In whole cellular extracts of human granulocytes, strong binding of mAb L5 was observed to a band of ~160 kDa well comparable with CEACAM1.

In contrast, only weak binding of mAb L5 occurred to proteins of ~50, 80, and 90 kDa. The identity of the 80 and 90 kDa proteins is presently unknown. The 50-kDa band was also detectable by the secondary Ab indicating unspecific binding.

Strong binding of mAb L5 to the precipitated CEACAM1 was observed which confirmed that the Lex-carrying 160 kDa protein observed in whole cellular lysates of granulocytes represents CEACAM1. The binding to the precipitated CEACAM1 was completely abrogated after the Lex residues were removed by treatment with α (1-3, 4) fucosidase.

Interestingly, Lex epitopes were undetectable on CEACAM6 indicating that the attachment of Lex residues is specific to CEACAM1. Taken together, these data demonstrate that CEACAM1 is the major carrier of Lex epitopes on human granulocytes.

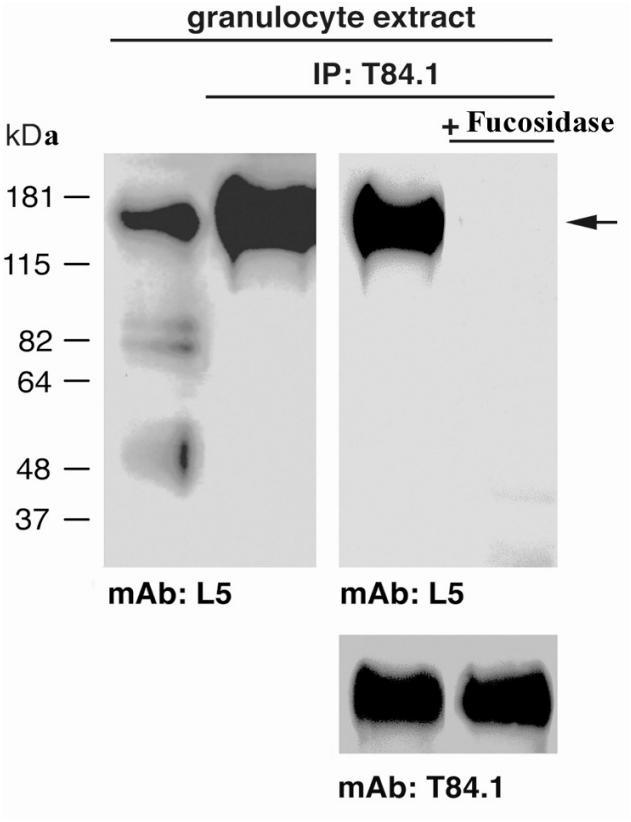


Figure 8. CEACAM1 from human granulocytes is a major carrier of Lex groups

Lex groups on CEACAM1 are recognized by Lex-specific mAb L5 in whole cellular extracts and after immunoprecipitation (IP) of CEACAM1 by mAb T84.1 identifying CEACAM1 as a major carrier of Lex groups in human granulocytes. The presence of Lex residues on CEACAM1 was confirmed by treatment of precipitated CEACAM1 by α (1-3, 4) Fucosidase. After stripping of the membrane, reprobing by mAb T84.1 demonstrated that untreated and treated CEACAM1 were present at comparable amounts (lower panel).

4.2. CEACAM1 carrying Lex groups is the major ligand of DC-SIGN

DC-SIGN has been identified as a C-type lectin that displays affinity for mannose-containing glycoconjugates and fucose-containing structures like Lex or other Lewis glycans. Since CEACAM1 carries Lex-groups, we asked if the Lex residues on CEACAM1 and on other members of the CEA family are ligands of DC-SIGN. To investigate binding of DC-SIGN to CEACAM1, the extracellular domain of human

DC-SIGN carrying a Fc-tag (human IgG_1) at the N-terminus for detection was expressed in human embryonic kidney 293 (HEK293) cells. Subsequent to purification from the cell-culture supernatants, recombinant soluble Fc-DC-SIGN was applied as a probe in blot overlay assays.

4.2.1. Determination of Fc-DC-SIGN concentration by Bradford method

The total protein concentration of the Fc-DC-SIGN preparation was measured by Bradford method (Fig. 9). For creating the standard curve, BSA in a range from 1,875 to 30 µg/mL in PBS was diluted and the Bradford reagent was added. Fc-DC-SIGN samples were prepared in the same way as the standard samples. The optical density was measured at 595 nm.

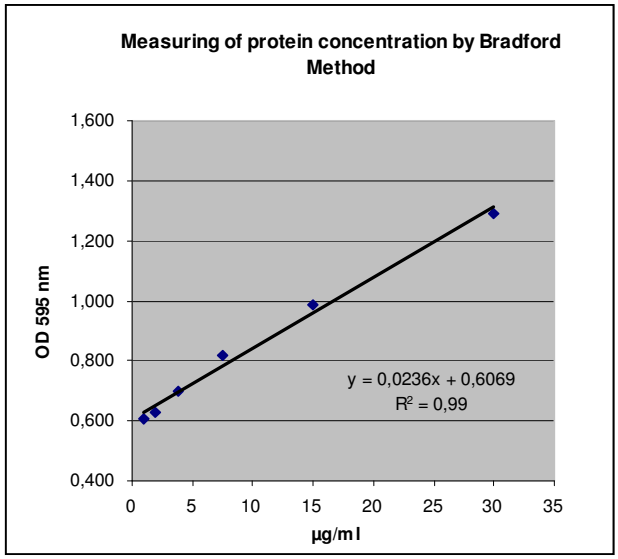


Figure 9. Standard curve was created for measuring the protein concentration of Fc-DC-SIGN by Bradford method

For creation of the standard curve 1.875, 3.75, 7.5, 15 and 30 µg/mL BSA standard solutions were prepared. The optical density was measured at 595 nm and the blank sample was adjusted against the Bradford reagent. The concentration of the proteins was calculated by measuring optical density (OD) using the following equation: X= (OD-0.6069)/0.0236.

4.2.2. Detection of soluble Fc-DC-SIGN in the culture supernatant preparation

To prove that Fc-DC-SIGN was successfully expressed in HEK293 cells and properly purified from cell-culture supernatants, western blot analysis and Coomassie staining were performed (Fig 10). By the western blot analysis after the Fc-DC-SIGN preparation was separated on SDS-PAGE, the protein membrane was incubated with anti-DC-SIGN antibody followed by rabbit anti mouse IgG-HRP labeled antibody. In addition, the detection of Fc-DC-SIGN with a molecular weight of 64 kDa was confirmed by Coomassie staining.

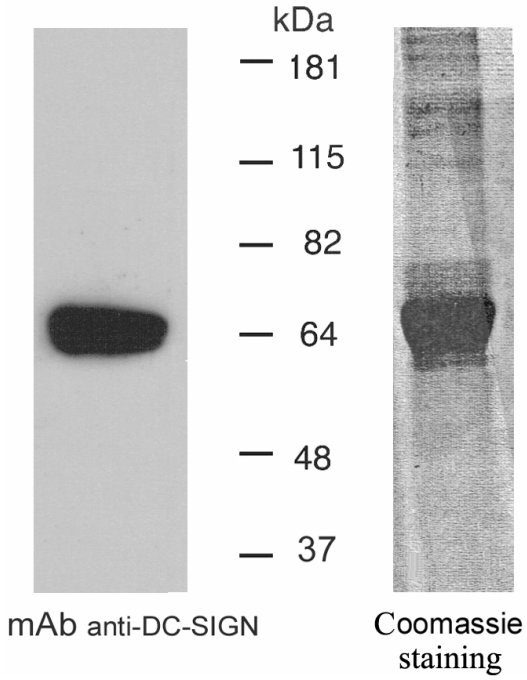


Figure 10. Purity of the Fc-DC-SIGN preparation demonstrated by western blot analysis and Coomassie staining

Culture supernatants from Fc-DC-SIGN transfectomas were collected and concentrated. After the medium was exchanged by the PD10 column to PBS, the preparation was analysed for DC-SIGN expression. Performing western blot analysis, DC-SIGN (64 kDa) was detected by anti-DC-SIGN mAb. In addition, the purity of the Fc-DC-SIGN preparation was confirmed by Coomassie staining.

4.2.3. CEACAM1 from human granulocytes carrying Lex is the major ligand of DC-SIGN

Having identified CEACAM1 as the major carrier of Lex epitopes on human granulocytes we have investigated the interaction of CEACAM1 with DC-SIGN by overlay assay of whole cellular extracts of human granulocytes (Fig. 11).
The proteins were separated by SDS-PAGE and transferred to PVDF membrane.
In whole cellular extracts of human granulocytes strong and selective binding of Fc-DC-SIGN was observed to a protein of approximately 160 kDa well comparable to CEACAM1. The additional band of approximately 55 kDa corresponds to human IgG present in the granulocyte preparation that was also detectable by the anti-human Fc-antiserum applied for detection.
Immunoprecipitation of whole cellular extracts of granulocytes by mAb T84.1 confirmed that the 160 kDa protein represents CEACAM1, as strong binding of Fc-DC-SIGN to CEACAM1 occurred after precipitation. Treatment of precipitates by α(1-3,4) fucosidase further confirmed that the binding of DC-SIGN was solely dependent on Lex residues on CEACAM1.
Most importantly, no binding of Fc-DC-SIGN to whole cellular extracts of granulocytes was observed in the supernatant subsequent to precipitation, clearly demonstrating that Lex-carrying CEACAM1 is the major binding partner of DC-SIGN in extracts of human granulocytes.

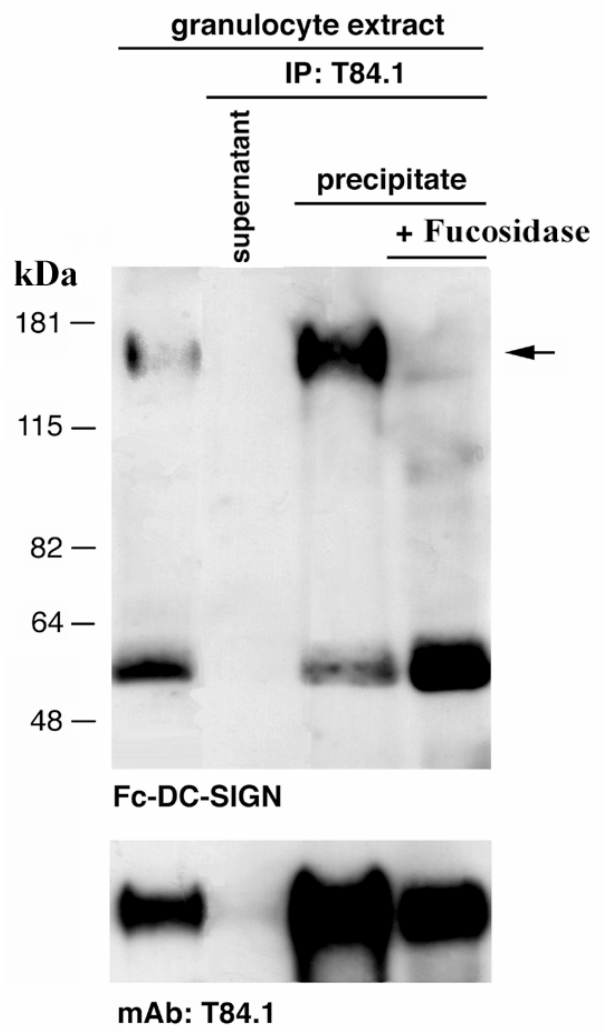


Figure 11. Lex residues on CEACAM1 from human granulocytes are specifically recognized by DC-SIGN

Binding of Fc-DC-SIGN to CEACAM1 (marked by arrow) was demonstrated by blot overlay assay of whole cellular extracts of human granulocytes. Whole cellular extract of human granulocytes, precipitated CEACAM1 by mAb T84.1, supernatant (subsequent to precipitation) and α(1-3,4) fucosidase treated precipitates were separated by SDS-PAGE. HRP-labeled anti-human Fc-antiserum was used for detection of bound Fc-DC-SIGN. IP of CEACAM1 confirmed specific binding of Fc-DC-SIGN to CEACAM1. Treatment of precipitates by α(1-3,4) fucosidase prior to western blot analysis demonstrates that binding of DC-SIGN to CEACAM1 is Lex-dependent. Identity of CEACAM1 and equal loading was confirmed by reprobing of the membrane by mAb T84.1 after stripping (lower panel).

4.3. Binding of Fc-DC-SIGN to Lex-residues on CEACAM1 from human granulocytes studied by FACS analyses

In addition to western blot analysis, the interaction of Fc-DC-SIGN to Lex residues on CEACAM1 from human granulocytes was studied by flow cytometry. Here, we have demonstrated strong binding of DC-SIGN to the surface of human granulocytes involving CEACAM1. The binding of Fc-DC-SIGN to CEACAM1 was significantly inhibited applying anti-DC-SIGN blocking mAb and polyclonal anti-CEACAM1 antiserum.

4.3.1. Determination of the purity of the granulocyte preparation by FACS analysis

To investigate the surface binding of Fc-DC-SIGN to human granulocytes, fresh granulocytes were isolated from buffy coats of human blood donors applying gradient centrifugation. Prior to binding studies, the purity of the granulocytes preparation was proved by flow cytometry. The granulocytes were stained with mouse anti-human IgM-CD15-PE labeled antibody (Fig. 12).

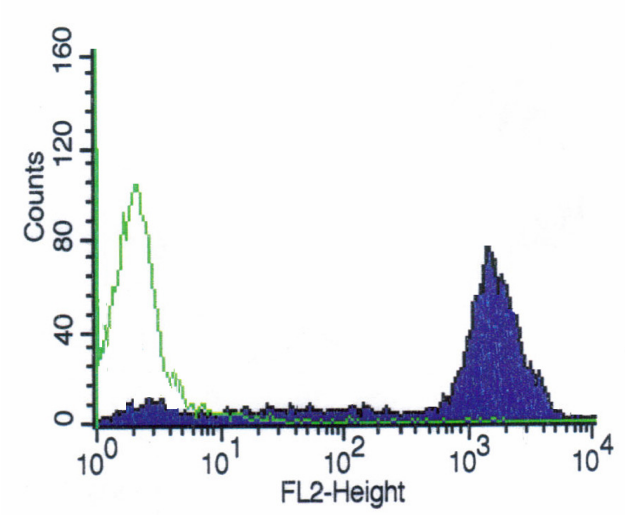


Figure 12. Determination of the purity of the granulocyte preparation by FACS analysis Subsequent to purification, the granulocytes were incubated with anti human CD15 CBl144-PE

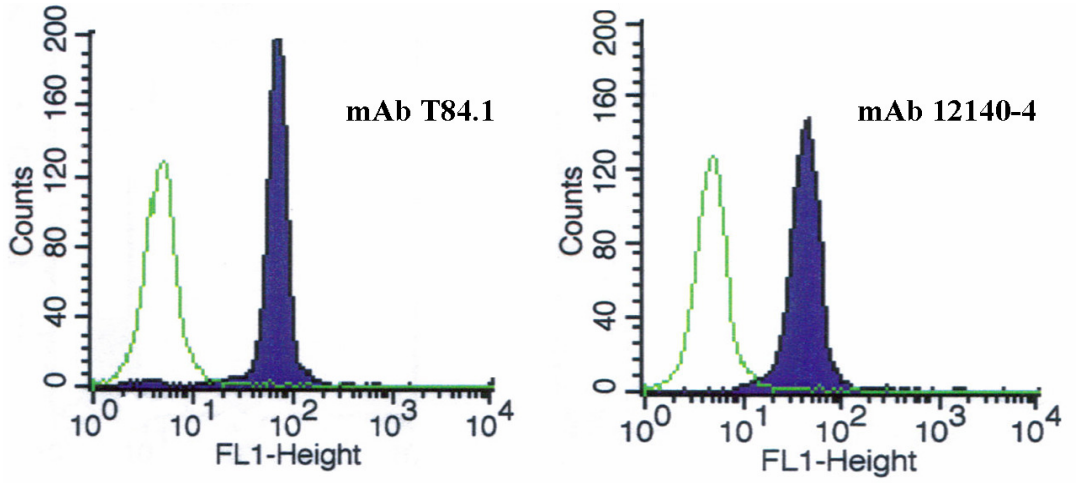
labeled mAb. The flow cytometry data are demonstrated as histogram (fluorescence intensity log 10 vs. counts). The high purity of the granulocyte preparation is represented by filled histogram in blue. Corresponding isotype control is shown by hollow histogram in green.

4.3.2. Surface expression of CEACAM1 on human granulocytes

In order to investigate the binding of Fc-DC-SIGN to Lex residues on CEACAM1, the surface expression of CEACAM1 on human granulocytes was analysed by FACS analyses (Fig 13). For this experiment, human granulocytes were isolated from buffy coats of normal human blood donors and CEACAM1 specific antibodies were directly labeled by FITC.

Binding of mAb T84.1 to granulocytes indicates that CEACAM1 and/or CEACAM6 are expressed on the cell surface of human granulocytes. CEACAM1 was specifically detected by applying the mAbs 12140-4 and 4D1C2. The strong and specific binding of mAb 12140–4 demonstrates that CEACAM1 is expressed at high levels on the cell surface of human granulocytes. The lower intensity achieved by mAb 4D1C2 most probably is explained by its lower affinity for CEACAM1.

To the analysed granulocytes, no stimulative substances were added. The up-regulation of CEACAM1 was increased when granulocytes were stimulated for 10 min with fMLP.



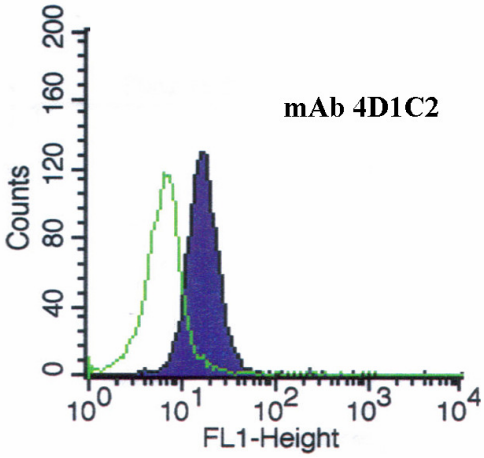


Figure 13. Surface expression of CEACAM1 on human granulocytes by FACS analysis
After the granulocytes were isolated, they were analysed for surface expression of CEACAM1. Detection was performed with T84.1, 12140-4 and 4D1C2 mAbs, all labeled by FITC. The expression of CEACAM1 is shown by filled histograms in blue while the hollow green histograms represent the isotype controls for the CEACAM1 specific antibodies.

4.3.3. Surface binding of Fc-DC-SIGN to human granulocytes involving CEACAM1 demonstrated by FACS-analyses

In addition to western blot analysis, binding of Fc-DC-SIGN to Lex residues on CEACAM1 was studied by FACS analyses (Fig. 14).
Prior to Fc-DC-SIGN incubation, potential unspecific binding sites were blocked by preincubation of the granulocytes with human polyclonal IgG.
Surface binding of DC-SIGN to the granulocytes involving CEACAM1 was demonstrated by double staining of the granulocytes with the FITC-labeled CEACAM1 mAb 12140-4 and the PE-labeled mAb 120507 specific for DC-SIGN. The dot plot data revealed that most granulocytes (>95%) were positive for CEACAM1 and DC-SIGN suggesting strong binding of DC-SIGN to the surface of human granulocytes involving CEACAM1.
According to FACS measurements with two labeled antibodies (two colour measurements), single staining with FITC-labeled mAb 12140-4 and PE-labeled mAb 120507 were included.

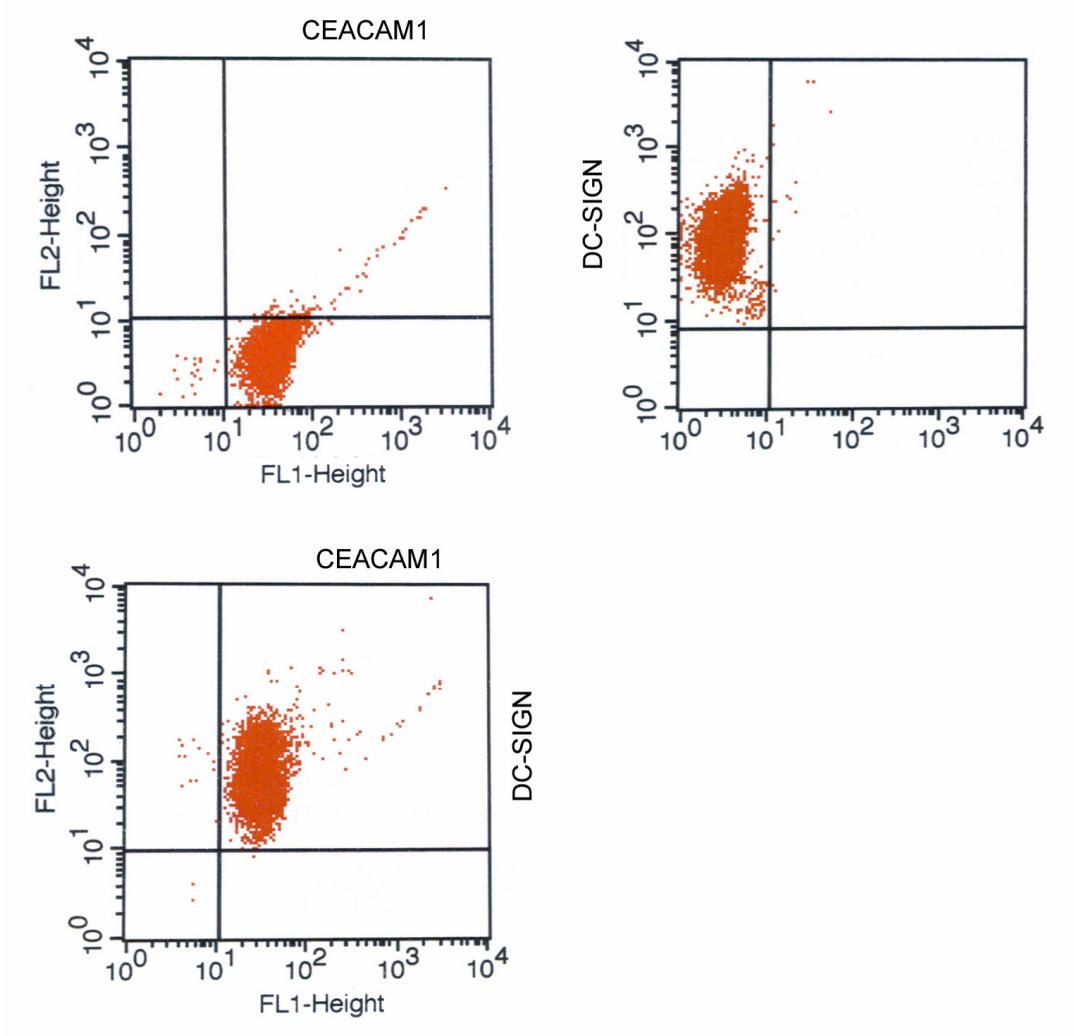


Figure 14. Surface binding of Fc-DC-SIGN to human granulocytes involving CEACAM1 by FACS analyses

Freshly prepared granulocytes were incubated with Fc-DC-SIGN. For detection of the bound DC-SIGN, the granulocytes were either single stained by FITC-labeled mAb 12140-4 (single staining upper left) and by PE-labeled 120507 DC-SIGN specific human mAb (single staining upper right) or double stained with both mAbs (lower left). The results are represented as dot plot analyses suggesting strong binding of Fc-DC-SIGN to the surface of human granulocytes involving CEACAM1. Isotype controls like human $IgG2_b$-PE-labeled (for DC-SIGN mAb) and human IgG_1-FITC labeled (for CEACAM1 mAb) were included.

4.3.4. Inhibition of the binding between DC-SIGN and CEACAM1 on the surface of granulocytes

To proof the specificity of the binding, the granulocytes were preincubated by Lex-specific mAb L5, a polyclonal antiserum directed against CEACAM1 and mannose specific mAb L3.

Prior to the inhibition, the potential binding of Fc-DC-SIGN or antibodies by Fc-receptors was blocked by human polyclonal IgG.

Following the incubation by Fc-DC-SIGN, the bound Fc-DC-SIGN was detected by staining of the granulocytes with DC-SIGN 120507-PE labeled mAb.

Binding of Fc-DC-SIGN to CEACAM1was inhibited after blocking with mAb L5 demonstrating that Lex residues of CEACAM1 on the surface of human granulocytes are specifically recognized by DC-SIGN.

With respect to mAb L5, incomplete inhibition of DC-SIGN binding may be explained by the differences in binding affinities or concentration of inhibiting antibodies.

Regarding the CEA antiserum, incomplete coverage of Lex residues on CEACAM1 or other ligands should also be considered. In both instances, DC-SIGN may interact with membrane structures, which are not recognized by the Abs. Lex residues present on other glycoproteins or on glycolipids may bind DC-SIGN, but not mAb L5. Alternatively, DC-SIGN may bind to high-mannose residues.

It should be noted, however, that the binding of DC-SIGN was not inhibited by the high-mannose-specific mAb L3.

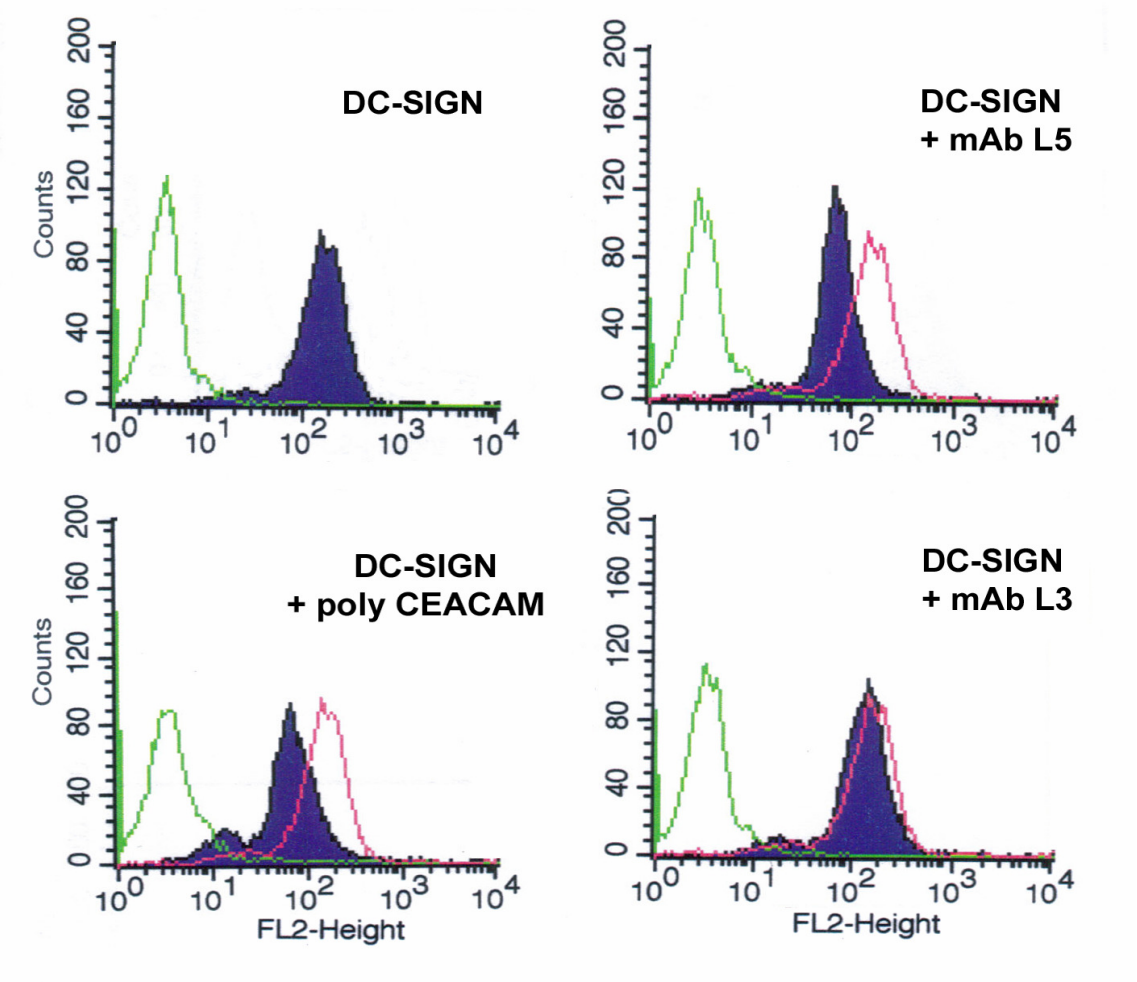


Figure 15. Inhibition of the binding between DC-SIGN and CEACAM1 on the surface of the human granulocytes by FACS analyses

Inhibition of binding between DC-SIGN and CEACAM1 was investigated by preincubation of the granulocytes by Lex-specific mAb L5, polyclonal anti-CEACAM antiserum and high-mannose specific mAb followed by FACS-analysis. Compared to untreated granulocytes (upper left), DC-SIGN binding was decreased in the presence of Lex-specific mAb L5 and polyclonal anti-CEACAM antiserum (filled versus hollow histograms in pink). No shift in binding was observed in the presence of high-mannose specific mAb L3. The hollow histograms in green represent the isotype control for PE-labeled DC-SIGN 120507 mAb.

The data shown in histograms: fluorescence intensity (FL2-height) vs. the number of events (counts).

4.4. α(1,3) Fucosyltransferase IX (FUTIX) specifically transfers fucose residues to recombinant CEACAM1

4.4.1. FUTIX is the major fucosyltransferase that transfers fucose residues to CEACAM1

In order to investigate which fucosyltransferase is involved in catalysing the synthesis of Lex groups in CEACAM1, recombinant CEACAM1 was transfected in the HEK293 cell line in combination with human fucosyltransferases FUTIII, IV, VII and IX. 48h post-transfection, whole cellular lysates were prepared and separated by 8% SDS-PAGE (Fig. 16).

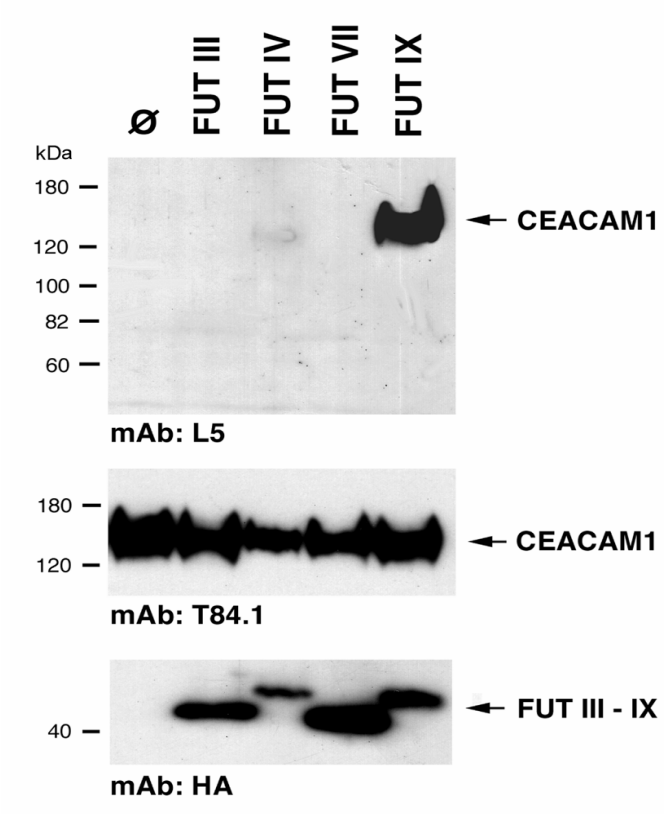
Lex residues were detectable on CEACAM1 by western blot analysis applying the Lex-specific mAb L5, when CEACAM1 was co-expressed with fucosyltransferase IX (FUTIX) and only weakly detectable in the presence of FUTIV. No presence of Lex residues was detected on CEACAM1 co-expressed with FUTIII and FUTVII.

The T84.1 mAb confirmed equal expression level of CEACAM1 in each transient transfections. Since all recombinant fucosyltransferases FUTIII, IV, VII and IX had human HA-tag, an anti-human HA antibody was applied to confirm the co-expression of the fucosyltransferases.

In addition to western blot analysis, the preferential fucosylation of CEACAM1 by FUTIX is further supported by FACS analyses of HEK293 cells transfected with CEACAM1 in combination with FUTIV and FUTIX, respectively, applying the L5 mAb.

As FUTIX is expressed at high levels in mature granulocytes transferring fucose to GlcNAc residues at distal lactosamine units (Nakayama, et al. 2001), our findings indicate that FUTIX plays a central role in the formation of Lex epitopes on CEACAM1 in human granulocytes.

a)



b)

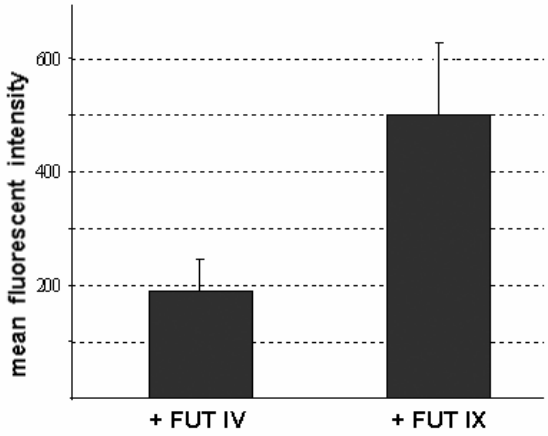


Figure 16. Lex-residues are specifically transferred to CEACAM1 by FUTIX as demonstrated by co-expression of CEACAM1 and different fucosyltransferases.

a) HEK293 cells were transiently co-transfected with CEACAM1 in combination with human fucosyltransferases FUTIII, FUTIV, FUTVII and FUTIX, respectively, (Ø, transfected by CEACAM1 only). Whole cellular lysates were prepared, separated by 7% SDS-PAGE and transferred to PVDF-membrane. Western blot analysis was performed applying the Lex-specific mAb L5. The membrane was subsequently stripped and the expression of CEACAM1 was confirmed by mAb T84.1, showing

that equal amount of CEACAM1 is loaded on the gel. The expression of HA-tagged fucosyltransferases was confirmed by applying polyclonal anti-HA antiserum.

b) FACS analysis of HEK293 cells co-transfected by CEACAM1 in combination with FUTIV and FUTIX, applying the mAb L5. Mean fluorescent intensity was determinate from three independent measurements, and levels of intensity were corrected for binding of mAb L5 to HEK 293 cells transfected by CEACAM1 only.

4.4.2. Different terminal glycostructures of recombinant CEACAM1 are recognized by DC-SIGN

Having identified FUTIX as the major enzyme transferring Lex residues to CEACAM1, we have tested the binding of DC-SIGN to CEACAM1 from HEK293 and CHO cells transfected by FUTIX.

HEK293 and CHO cells were transiently transfected with CEACAM1 alone or co-transfected with FUTIX. CEACAM1 was precipitated from whole cell lysates by T84.1 mAb. Subsequent to separation of the precipitates by SDS-PAGE, the PVDF membrane was incubated with soluble Fc-DC-SIGN (Fig. 17).

In both cell lines, strong binding of Fc-DC-SIGN to CEACAM1 transfected in combination with FUTIX (CEACAM1/FUTIX) was demonstrated. Surprisingly, CEACAM1 expressed alone in HEK293 cells showed strong binding to Fc-DC-SIGN, unlike CEACAM1 from CHO cells. Thus, strong binding of Fc-DC-SIGN to CEACAM1 was observed in HEK293 cells independent of the expression of FUTIX. The Lex epitopes on CEACAM1 transfected in CHO cells were only present when CEACAM1 was co-transfected with FUTIX. Recombinant CEACAM1 expressed in CHO cells did not bind DC-SIGN or a monoclonal Lex antibody. BSA was used as a control for the binding of Fc-DC-SIGN to Lex groups. Identity of CEACAM1 and equal loading was confirmed by reprobing of the membrane by mAb T84.1, after stripping.

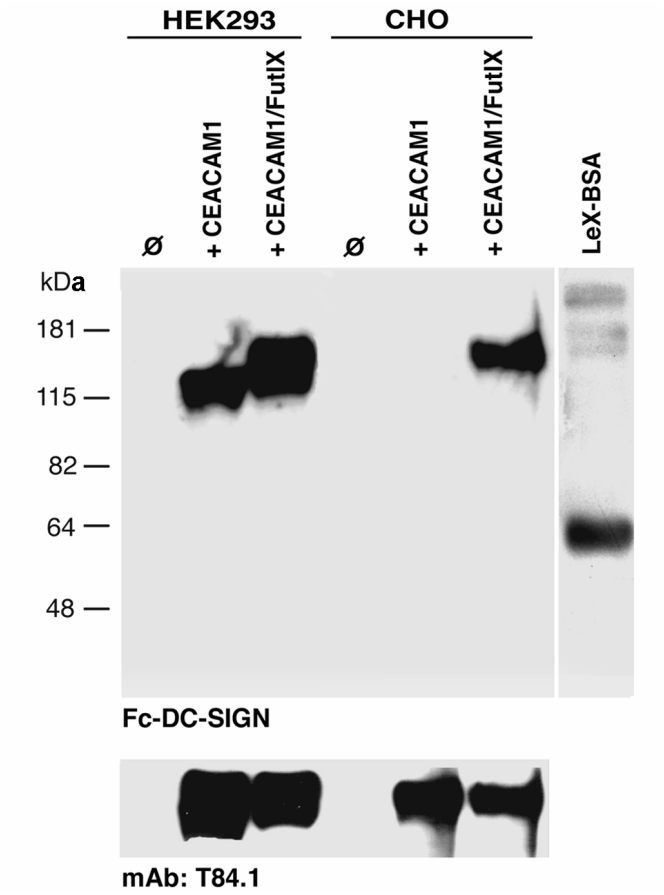


Figure 17. Terminal glycostructures on recombinant CEACAM1 recognized by DC-SIGN are different depending on the cell type analyzed

HEK293 and CHO cells were transiently transfected with CEACAM1 alone or in combination with FUTIX, (Ø, untransfected cells). CEACAM1 was precipitated from whole cellular lysates by mAb T84.1. Blot overlay was performed applying soluble Fc-DC-SIGN and HRP labeled anti-human Fc antiserum for detection. Lex-BSA was used as a control for the binding of DC-SIGN to Lex residues. The membrane was stripped and probed with mAb T84.1 showing that CEACAM1 was present at comparable amounts (lower panel).

4.4.3. Identification of high-mannose structures on CEACAM1 expressed in HEK293 cells by *Galanthus nivalis* agglutinin (GNA)

To characterize the glycostructures of CEACAM1 in HEK293 cells recognized by DC-SIGN but lacking Lex residues, blot overlay assays with GNA, a high-mannose specific lectin, were performed (Fig. 18). Strong binding of GNA to CEACAM1 expressed in HEK293 cells demonstrates the presence of high-mannose groups on CEACAM1 independent of the expression of FUTIX. High-mannose structures were not detectable on CEACAM1 expressed in CHO cells. This finding indicates that the high-mannose structures on CEACAM1 from HEK293 cells bind to DC-SIGN.

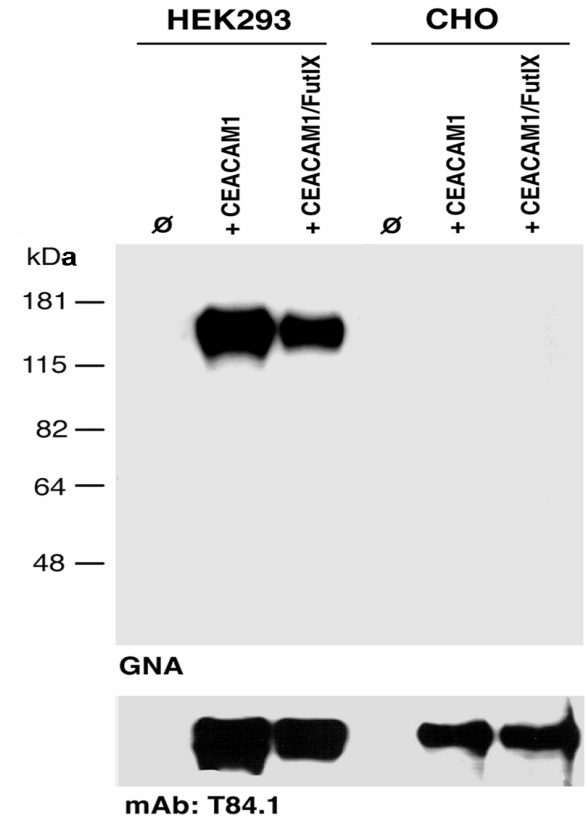


Figure 18. Identification of high-mannose structure on CEACAM1 expressed in HEK293 cells by *Galanthus nivalis* agglutinin (GNA) using blot overlay assay

HEK293 and CHO cells were transfected with CEACAM1 alone or in combination with FUTIX (Ø, untransfected cells). CEACAM1 was precipitated by T84.1 mAb and precipitates were probed by digoxigenin-labeled GNA, followed by anti-digoxigenin-HRP labeled Ab. The membrane was stripped and probed with mAb T84.1 showing that CEACAM1 was present at comparable amounts.

4.4.4. α(1-3,4) fucosidase treatment of recombinant CEACAM1 confirms that the binding of DC-SIGN to CEACAM1 is Lex dependent

CEACAM1 was transiently co-transfected with FUTIX in CHO cells. Using the mAb T84.1, CEACAM1 was precipitated from whole cellular lysates of the transfected cells. The untreated and the α(1-3,4) fucosidase treated precipitates were submitted to SDS-PAGE and western blot (Fig. 19). Treatment of CEACAM1 by α(1-3,4) fucosidase completely abolished the binding of mAb L5 and soluble Fc-DC-SIGN, respectively, confirming that the binding of DC-SIGN to CEACAM1 is dependent on the presence of the Lex residues.

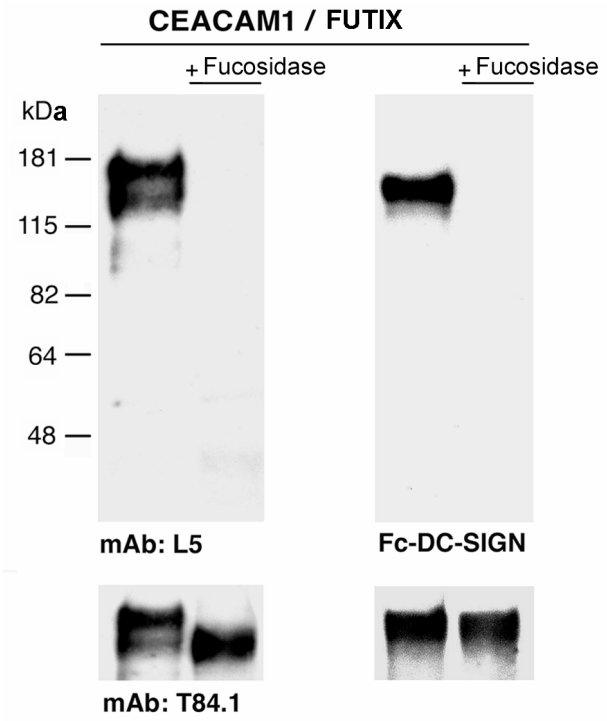


Figure 19. The binding of DC-SIGN to CEACAM1 co-expressed with FUTIX (CEACAM1/FUTIX) in CHO cells is Lex dependent

Lysates of CHO cells transiently co-transfected by CEACAM1 and FUTIX were precipitated by mAb T84.1. α(1-3,4) fucosidase treated and untreated precipitates of CEACAM1 were analysed for binding of Lex specific mAb L5 and Fc-DC-SIGN, respectively. No binding was observed by α(1-3,4) fucosidase treated precipitates. Subsequent to stripping of the membrane, reprobing by mAb T84.1 demonstrated that untreated and treated CEACAM1 were present at comparable amounts.

4.5. Interaction between CEACAM1 and DC-SIGN at the cellular level

Having identified CEACAM1 as a specific ligand of DC-SIGN and the major carrier of Lex epitopes on human granulocytes, we have investigated the interaction between CEACAM1 and DC-SIGN at the cellular level. For this purpose, human dendritic cells were generated from monocytes, CHO cells stably expressing CEACAM1 or coexpressing FUTIX were fluorescently labeled and native CEACAM1 purified from human granulocytes was attached to fluorescence microbeads.

4.5.1. Purity of monocyte preparations determined by FACS analysis

Monocytes were isolated from peripheral human blood using anti-CD14 magnetic microbeads following the protocol of the supplier. Anti-CD14 mAb-FITC labeled and anti-DC-SIGN-PE labeled mAb were used for detection of CD14 and DC-SIGN, respectively, applying flow cytometry analysis (Fig. 20). As expected, monocytes were expressing high levels of CD14 but were lacking DC-SIGN expression.

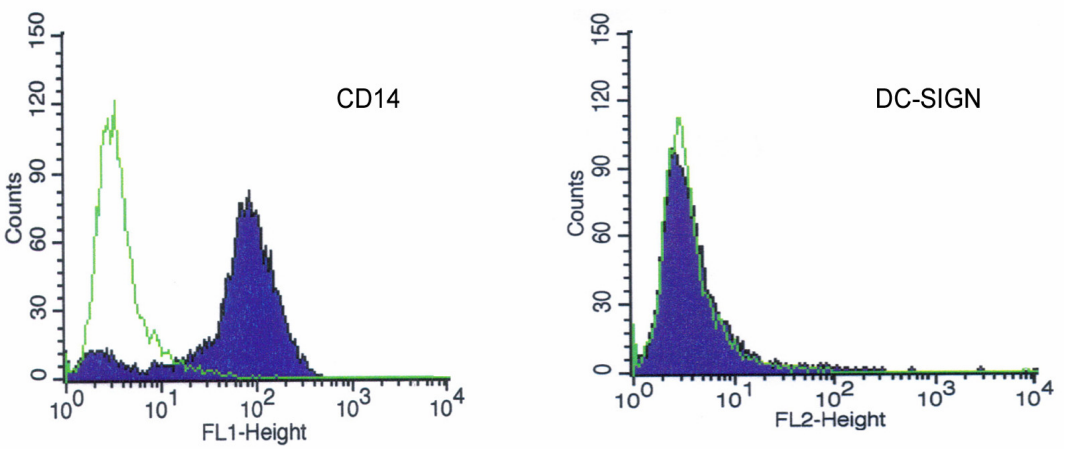


Figure 20. Purity of a monocyte preparation confirmed by FACS analysis

The data are shown by histogram, fluorescence intensity log 10 (FL1-channel-FITC; FL2-PE) vs. events (counts). Surface expression of CD14 on isolated human monocytes was detected by anti-CD14 mAb-FITC labeled (filled histogram in blue, left). No detection of DC-SIGN on the monocytes was observed by staining with anti-DC-SIGN mAb-PE labeled (filled histogram in blue, right). The hollow histograms in green represent the isotype controls.

4.5.2. Differentiation of dendritic cells (DCs) demonstrated by FACS analysis

Monocytes were isolated from peripheral human blood and differentiated by IL-4 and GM-CSF to immature dendritic cells marked by high levels of DC-SIGN expression. Differentiation of monocytes to immature dendritic cells was confirmed by FACS-analysis using DC-SIGN specific mAb 120507 and antibodies directed against the surface markers CD14, CD83 and CD86, respectively, demonstrating high levels of DC-SIGN expression, low to moderate levels of expression of CD83 and CD86, respectively, and lack of CD14 expression typical for immature dendritic cells.

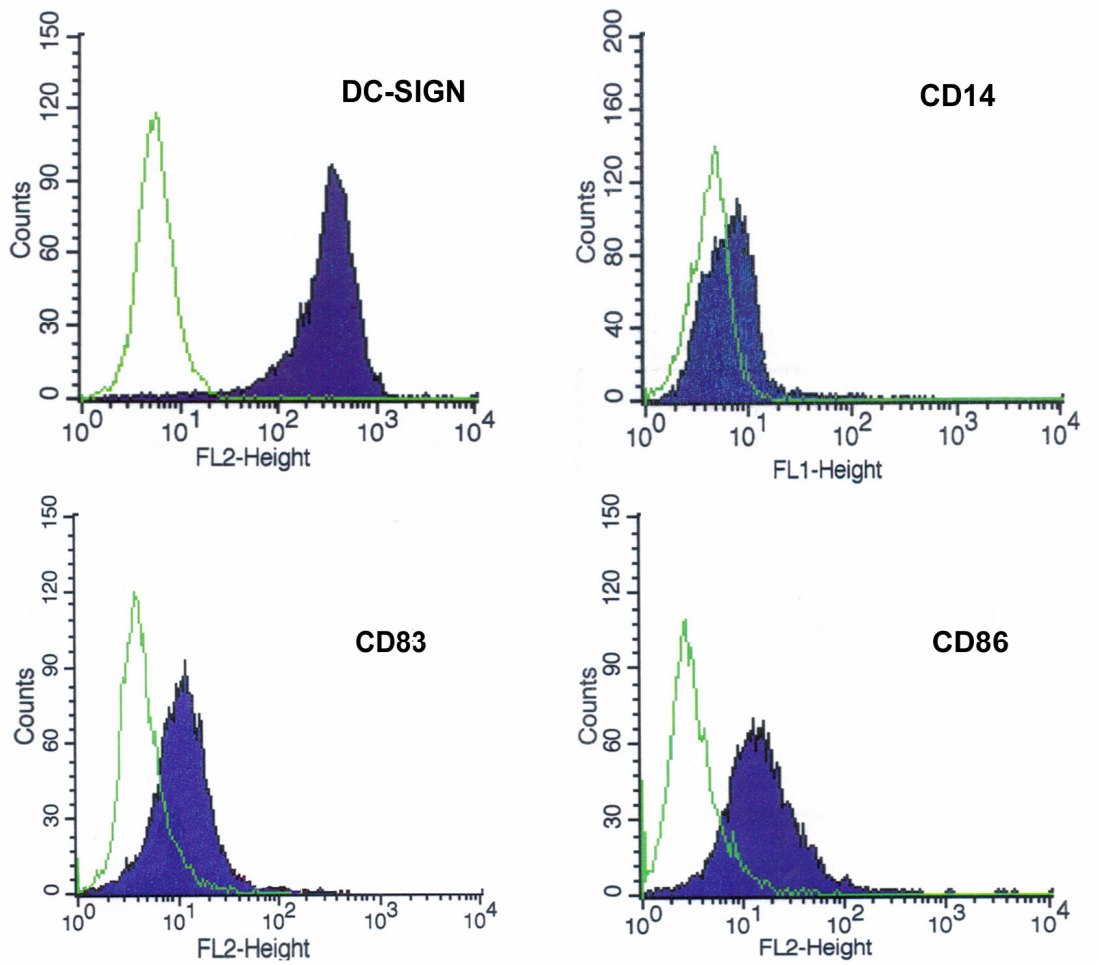


Figure 21. Differentiation of dendritic cells demonstrated by FACS analysis

The data are shown by histograms, fluorescence intensity log 10 (FL1-channel-FITC; FL2-PE) vs. events (counts). Detection of DC-SIGN, CD14, CD83 and CD86 expression on surface of DC was

performed by anti-DC-SIGN-PE labeled mAb 120507, anti-CD14-FITC labeled mAb, anti-CD83-PE labeled mAb and anti-D86-PE labeled mAb, respectively (field histograms in blue).
The hollow histograms in green represent the isotype controls.

4.5.3. Determination of CEACAM1 expression in CHO cells stably transfected with CEACAM1 alone or in combination with FUTIX by flow cytometry

In order to investigate the binding of CEACAM1 to DC-SIGN at the cellular level, CEACAM1 was stably expressed in CHO cells in the presence or absence of FUTIX (Fig. 22 & Fig. 23). CHO transfectomas were analysed for surface expression of CEACAM1 and Lex residues by FACS-analysis. CEACAM1 was detected by T84.1-FITC labeled mAb while the determination of Lex groups was done by L5 mAb followed by anti-rat IgM-PE labeled mAb.

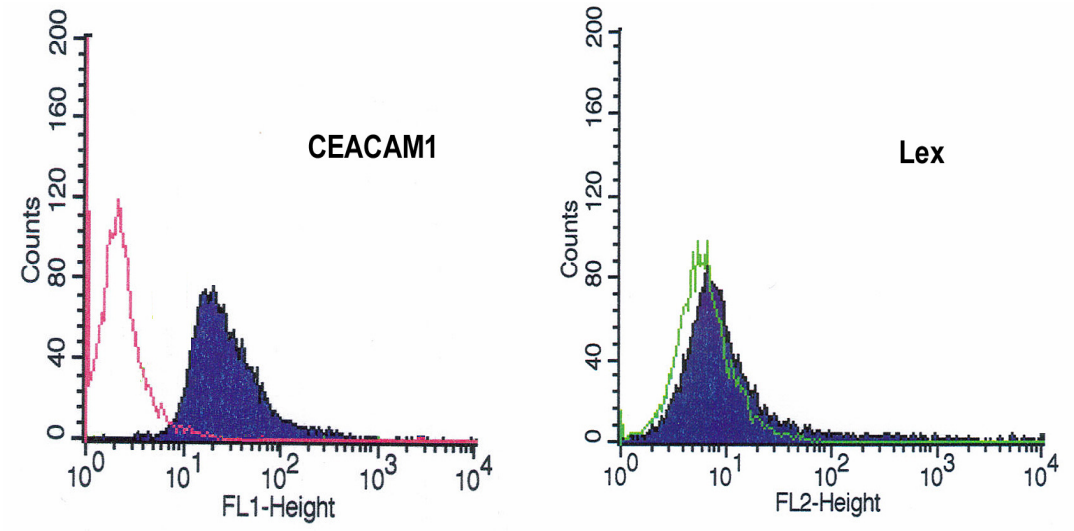


Figure 22. CEACAM1 expression on the surface of CHO cells stably transfected with CEACAM1 cDNA analysed by FACS analyses

CHO cells were stably transfected with CEACAM1 cDNA. Detection of CEACAM1 on the surface of transfectomas was confirmed by T84.1-FITC labeled mAb (field histogram in blue). Lex residues were not detected on the surface of the transfectomas as demonstrated by applying the Lex-specific mAb followed by mouse-anti rat IgM PE-labeled mAb. Isotype controls were also included presented by the hollow histograms.

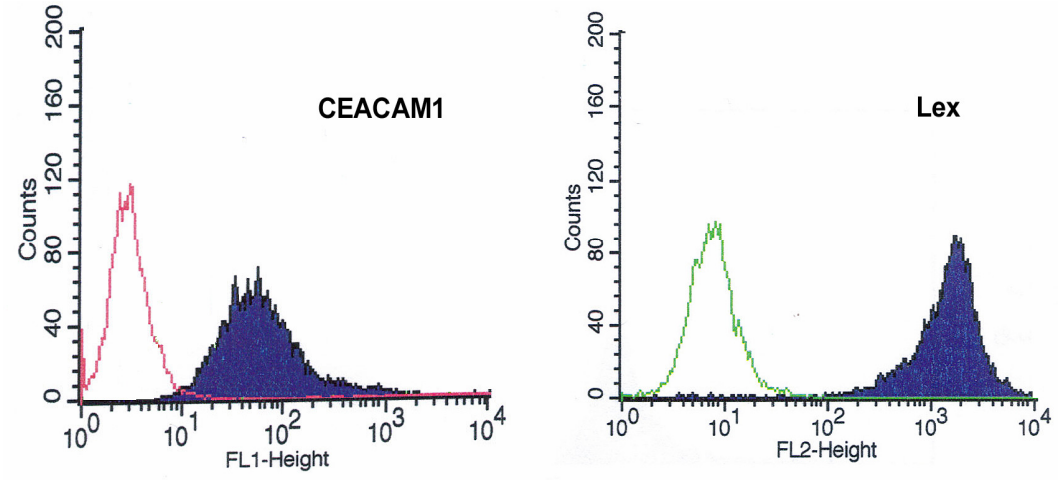


Figure 23. CEACAM1 expression on the surface of CHO cells stably transfected with CEACAM1 in combination with FUTIX detected by FACS analyses

CHO cells were stably co-transfected by CEACAM1 and FUTIX. CEACAM1 was detected on the cell surface of the transfectomas by T84.1-FITC labeled mAb.

Strong surface expression of Lex residues on CHO transfectomas applying Lex specific mAb L5 followed by mouse-anti rat IgM-PE labeled mAb was observed. The hollow histograms represent the isotype controls.

4.5.4 Binding studies between CHO cells stably expressing CEACAM1 alone or in combination with FUTIX and dendritic cells

The binding studies performed with CHO cells stably expressing CEACAM1 in presence or absence of expression of FUTIX, and DCs, revealed that Lex residues on CEACAM1 specifically mediate the binding to DC-SIGN on DCs. CEACAM1 and CEACAM1/FUTIX transfectomas were fluorescently labeled, incubated with human immature DCs, and the bound cells were visualized and photographically documented by fluorescence microscopy. Compared with transfectomas expressing CEACAM1 only, the number of CEACAM1/FUTIX transfectomas bound to immature DCs was ~3-fold increased (Fig. 24).

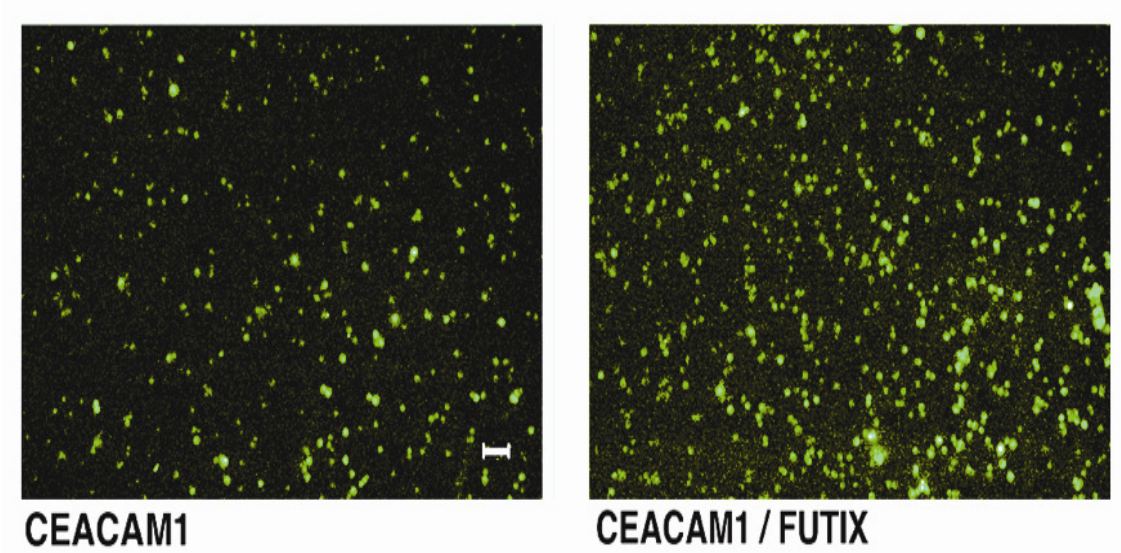


Figure 24. Attachment of CHO cells stably expressing either CEACAM1 or CEACAM1 in combination with FUTIX to DCs demonstrated by fluorescence microscopy

CHO cells were stably transfected with CEACAM1 alone, or CEACAM1 was co-transfected with FUTIX (CEACAM1/FUTIX). Fluorescently labeled CHO transfectomas were incubated with human immature DCs and the bound cells were quantified and documented by fluorescence microscopy (scale bar represents 200 µm). The number of CEACAM1/FUTIX transfected cells (right image) bound to immature dendritic cell was approximately 3-fold increased compared to the cells transfected with CEACAM1 only.

4.5.5. Inhibition studies of binding between CHO cells stably expressing CEACAM1 in combination with FUTIX and immature DCs

Binding of CEACAM1/FUTIX-transfectomas to immature DCs was substantially decreased to background levels when Lex epitopes on CEACAM1 were blocked by Lex-specific mAb L5. In addition, the attachment of transfectomas to DCs was inhibited by incubation of the transfectomas with polyclonal anti-CEACAM antiserum or incubation of the dendritic cells with DC-SIGN specific mAb DC28. The irrelevant polyclonal antiserum raised against HA was applied as a control (Fig. 25 & Fig. 26).

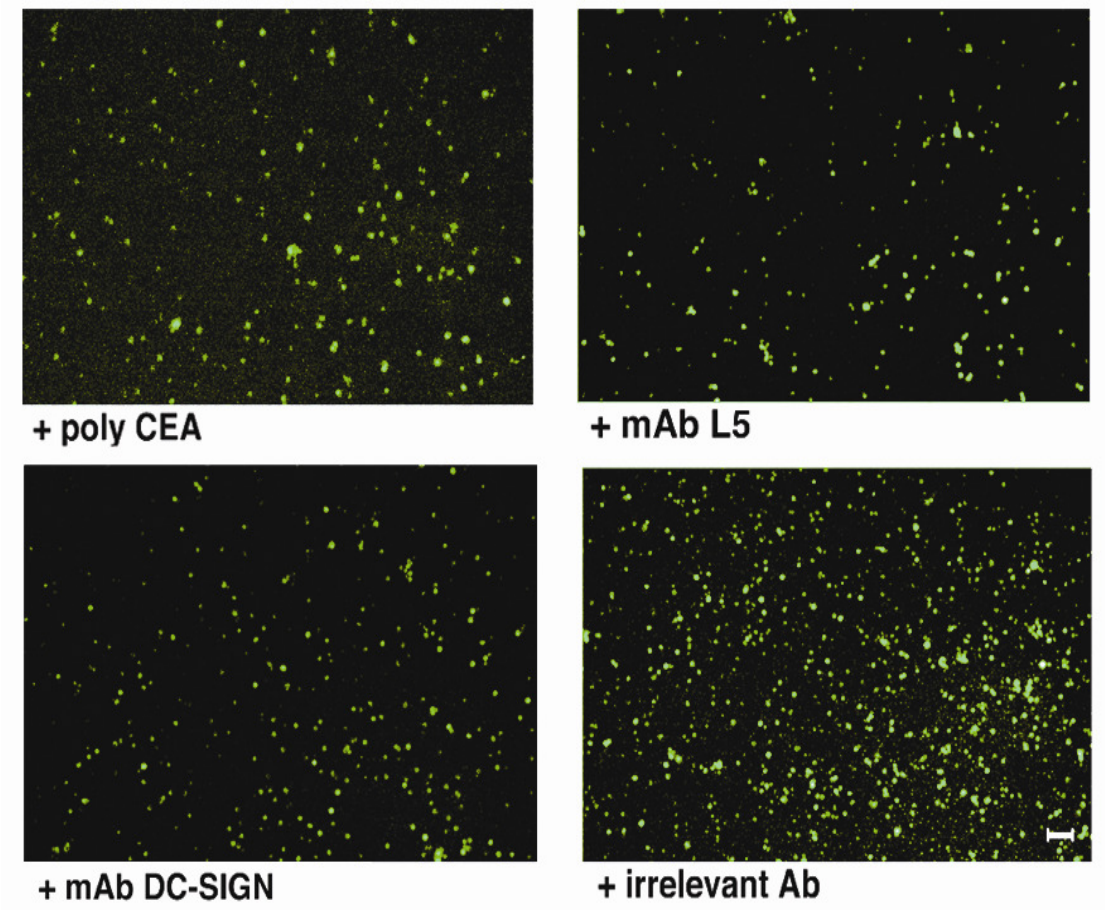


Figure 25. Inhibition of the interaction of CEACAM1/FUTIX transfectomas with immature DCs

The attachment of CHO cells stably co-transfected with CEACAM1 and FUTIX to human immature DCs was inhibited by mAb L5 or the polyclonal anti-CEACAM antiserum, respectively. Binding of

CHO cells to DCs was inhibited by DC-SIGN mAb DC28 blocking the interaction of DC-SIGN and carbohydrates. As a control, the irrelevant polyclonal antiserum raised against HA was included. Scale bar represents 200 µm.

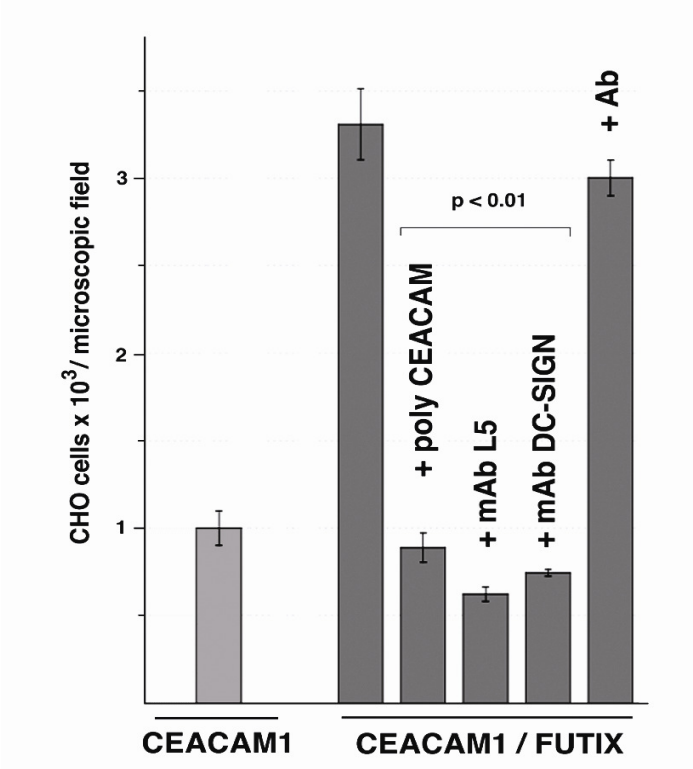


Figure 26. Statistical analysis of the inhibition of the interaction of CEACAM1 and CEACAM1/FUTIX CHO-transfectomas with immature DCs

The absolute cell numbers of the bound cells per microscopic field (Fig. 24 and Fig. 25) were quantified from the digital images applying the cell-counting tool of the NIH image software package (version 1.63; NIH, Bethesda, MR). The levels of statistical significance were calculated by the Student's t-test. Binding studies were performed in three independent experiments.

4.6. Lex groups mediate the binding and internalization of CEACAM1 in DCs

4.6.1. Strong binding of native CEACAM1 to immature DCs

Native CEACAM1 isolated by affinity chromatography from extracts of human granulocytes was covalently coupled to fluorescent microbeads. BSA and Lex-BSA were also covalently attached to fluorescent microbeads to determine the background binding and as a positive control for DC-SIGN binding, respectively. After the immature DCs were incubated with the coated beads, the DCs were fixed and analysed by fluorescence microscopy. Compared to the background binding of BSA-coated beads, strong binding of native CEACAM1-coated beads to DCs was observed (Fig. 27).

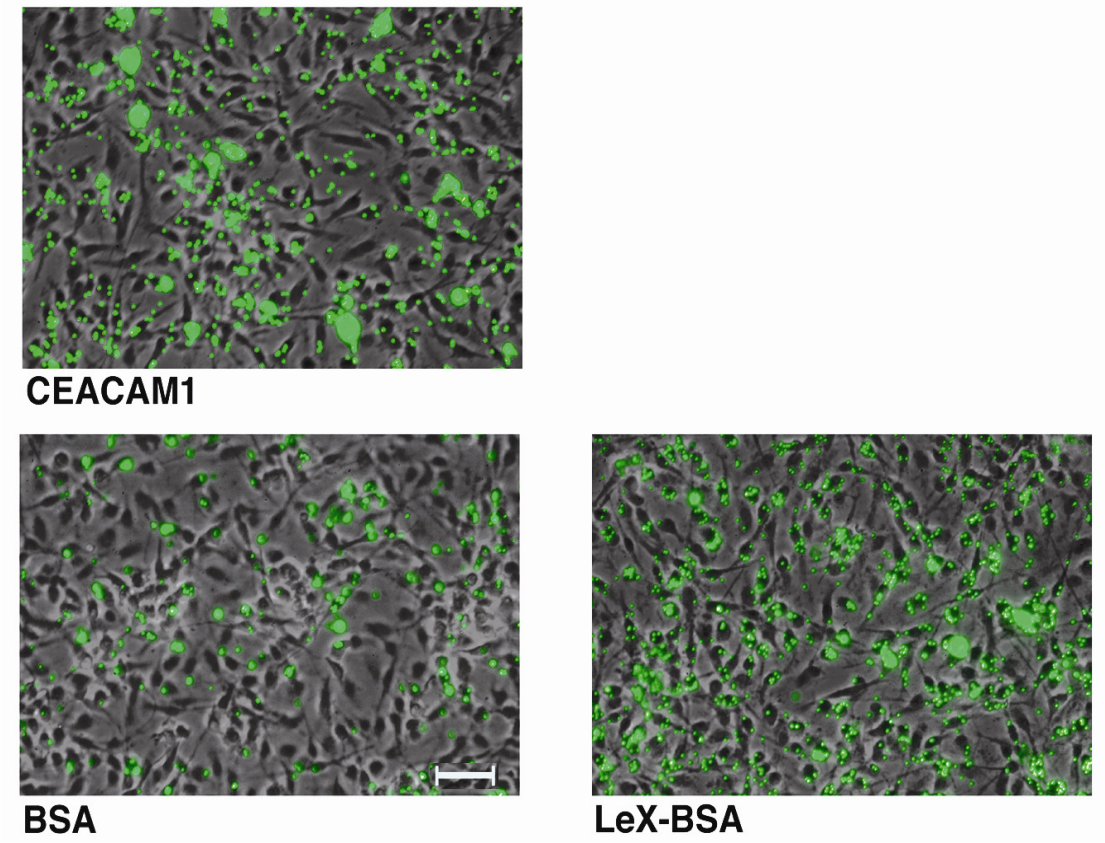


Figure 27. Binding of native CEACAM1 from human granulocytes to dendritic cells

Native CEACAM1, BSA and Lex-BSA coated fluorescent microbeads (green colour) were incubated with immature DCs, and the bound beads were visualized by fluorescence microscopy. In contrast, to the BSA-coupled beads, strong binding of the beads coated by native CEACAM1 was demonstrated.

Lex-BSA coupled beads were used as a positive control. Dendritic cells are shown by phase contrast microscopy (scale bar represents 80 µm).

4.6.2. Inhibition studies revealed that Lex groups on native CEACAM1 mediate the binding to immature DCs

To prove the specificity of the binding between the fluorescent CEACAM1-coated beads and DCs, inhibition studies were performed. The DCs were preincubated with DC-SIGN mAb DC28 or CEACAM1-coated beads were pretreated by α(1-3,4) fucosidase. Lex-BSA-coated fluorescent beads were used as positive control. Binding of CEACAM1 and Lex-BSA-coupled beads was significantly decreased when DCs were preincubated with DC-SIGN-blocking Ab DC28. The interaction of native CEACAM1 to DCs was inhibited by over 50% when CEACAM1-coated beads were pretreated by α(1-3,4) fucosidase, confirming that Lex residues on CEACAM1 mediate the interaction to DC-SIGN on DCs (Fig. 28).

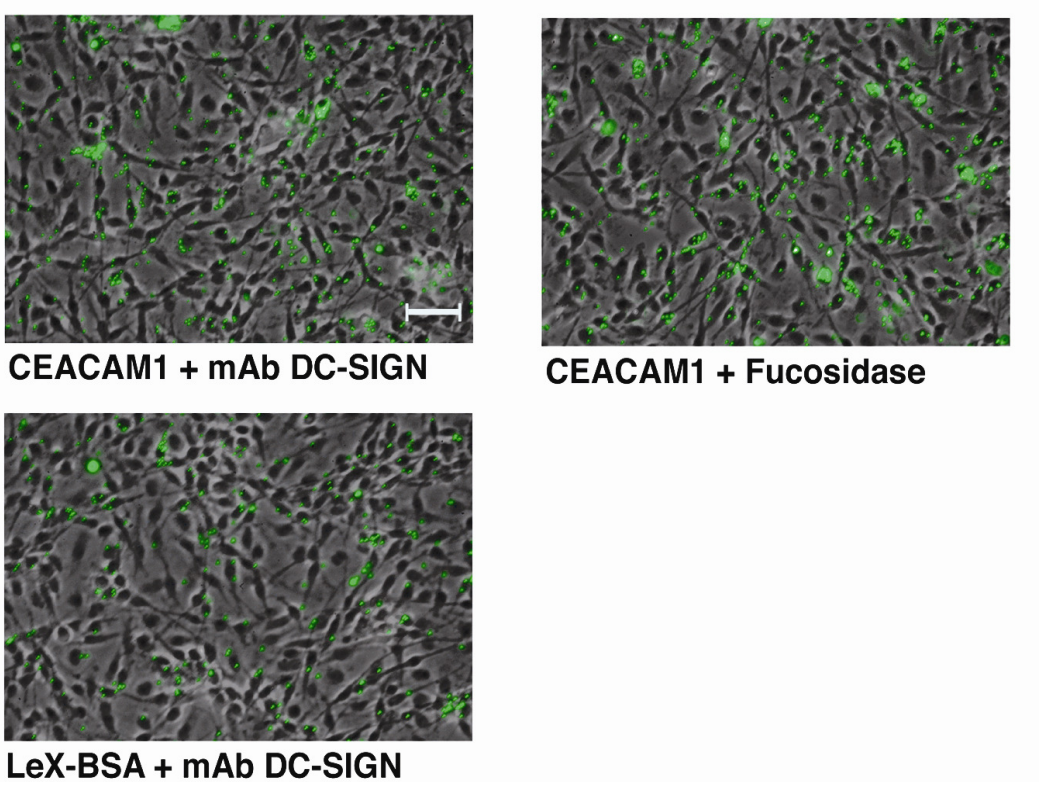


Figure 28. Microbeads coated by native CEACAM1 (green colour) are specifically bound via Lex-groups to DC-SIGN on immature dendritic cells

The binding of CEACAM1-coated beads and Lex-BSA-coated beads to DCs was significantly decreased when DCs were pretreated by DC-SIGN blocking mAb DC28. Binding of CEACAM1 coated microbeads was inhibited when CEACAM1-coated beads were pretreated by α(1-3,4) fucosidase. DCs are shown by phase contrast microscopy (scale bar represents 80 µm).

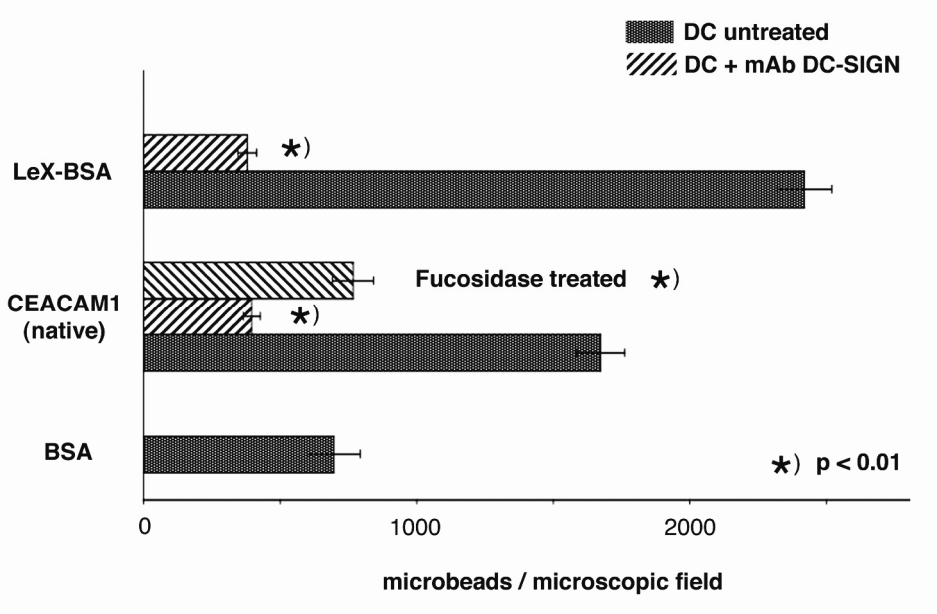


Figure 29. Statistical analysis of inhibition of the binding of native CEACAM1 to immature DCs
Absolute numbers of CEACAM1, Lex-BSA or BSA coated fluorescent microbeads bound to DCs per microscopic field were quantified from digital images (Fig. 27 and Fig. 28). Binding experiments were performed in triplicate. Statistical significance was calculated using the Student's t-test.

4.6.3. Internalization of native CEACAM1 and co-localization with LAMP1 detected by confocal microscopy

Confocal microscopy studies were performed to investigate the internalization of native CEACAM1 by DCs and co-localization with lysosome-associated membrane protein 1 (LAMP1) (Fig.30). Native CEACAM1 was isolated by affinity chromatography from extracts of human granulocytes and covalently attached to fluorescent microbeads. Fluorescent CEACAM1-coated microbeads were incubated with immature dendritic cells cultivated on glass chamber slides, and after washing, the bound beads were visualized and photographically documented by confocal microscopy. Large numbers of beads were clustered in the vicinity of the cell nucleus

suggesting the internalization of CEACAM1 coated microbeads into the cytoplasm of immature DCs. The uptake was drastically reduced when DC-SIGN on DCs was blocked by the DC-SIGN blocking Ab DC28 indicating that binding and uptake of CEACAM1 is specifically mediated by DC-SIGN. Parallel staining of the lysosome-associated membrane protein 1 (LAMP1) showed that the fluorescent beads coated by native CEACAM1 are co-localized with LAMP1 demonstrating that the majority of beads are internalized and subsequently transferred to lysosomal compartments.

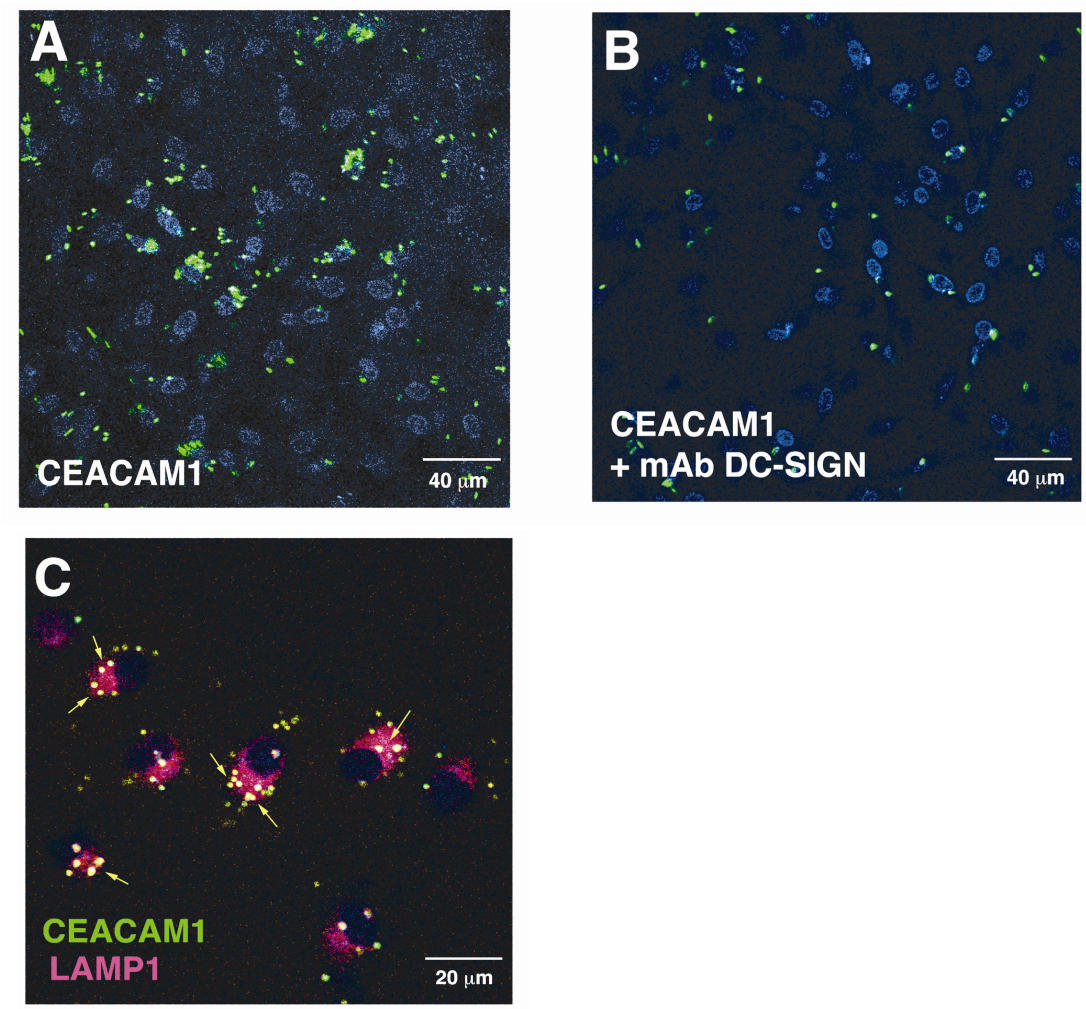


Figure 30. Native CEACAM1 coupled to microbeads is internalized by immature dendritic cells and co-localizes with LAMP1

Fluorescent microbeads coated by native CEACAM1 were incubated with immature DCs. A) As shown by confocal microscopy of representative areas, beads (green colour) accumulated in clusters in the cytoplasm of dendritic cells close to the nuclei stained in blue by TOTO3. Scale bar represents 40 µm. B) When DC-SIGN was blocked by incubation of DCs with DC-SIGN mAb DC28 the internalization of the microbeads was strongly decreased. Scale bar represents 40 µm.

C) Staining of immature dendritic cells by PE-labeled anti-LAMP1 antibody demonstrates that CEACAM1 coated beads are co-localized with LAMP1 (marked by yellow arrows) expressed in the cytoplasm. Scale bar represents 20 µm.

4.7. The interaction between granulocytes and Fc-DC-SIGN alters the spontaneous apoptosis of the human granulocytes

To evaluate the biological function of the interaction of granulocytes with DC-SIGN, we have investigated if this interaction has effect on apoptosis of granulocytes using flow cytometry analyses.

In the early stage of apoptosis, changes occur at the cell surface. An early marker for apoptosis is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to outer layer, by which the PS becomes exposed at the extracellular surface of the apoptotic cells. PS can be detected with FITC-labeled annexin V.

In order to make a distinction between early apoptotic and late apoptotic cells, the granulocytes were labeled with propidium iodide (PI), a fluorescent nucleic dye.

The granulocytes were isolated from human peripheral blood and cultured for 18h with PBS, soluble Fc-DC-SIGN (10µg/mL, T84.1 mAb (30 µg/mL), mAb 4D1C2 (30 µg/mL), human IgG containing serum (30 µg/mL), mouse IgG containing serum (30 µg/mL), anti-HA mAb (25 µg/mL), mAb L5 (30 µg/mL) and fMLP (10^{-6}M), respectively.

Double staining with FITC-annexin V and PI revealed higher PS expression and PI intercalation when granulocytes were incubated with soluble Fc-DC-SIGN compared to the antibodies or fMLP. Actually, by double staining three populations of the stained granulocytes were observed: annexin V-positive/PI positive cells (late stage of apoptosis), annexin V-positive/PI-negative (early apoptotic granulocytes) and annexin V-negative/PI-negative cells (living granulocytes).

Significant increase of the granulocytes in the late stage of apoptosis in the presence of Fc-DC-SIGN indicates that the DC-SIGN-granulocyte interaction has influence on the survival of human granulocytes.

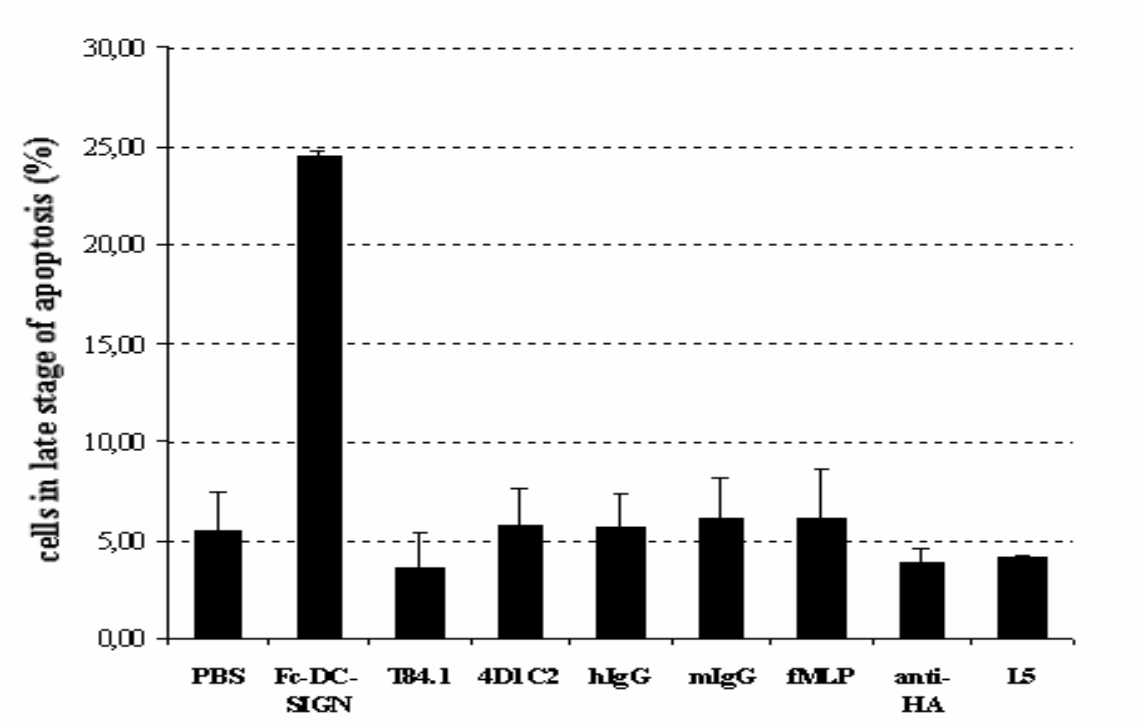


Figure 31. Fc-DC-SIGN triggers spontaneous apoptosis in human granulocytes as demonstrated by FACS analysis

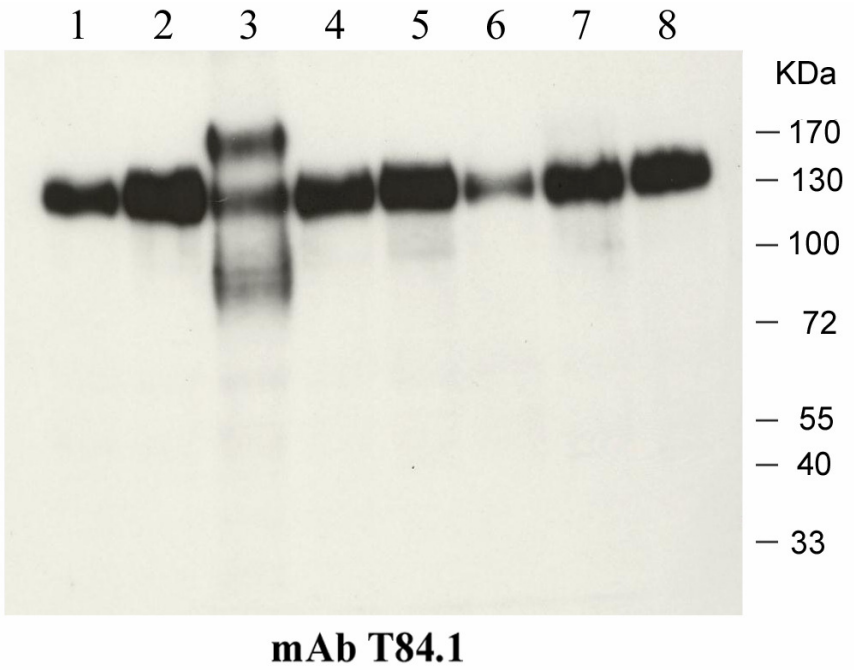
Granulocytes were incubated with PBS, Fc-DC-SIGN, mAb T84.1, mAb 4D1C2, human IgG containing serum, mouse IgG containing serum, anti-HA mAb, mAb L5 and fMLP, respectively, for 18h and double stained with FITC-labeled annexin V and PI. Incubation with Fc-DC-SIGN resulted in significant increase in annexin V-positive/PI-positive granulocytes (late stage of apoptosis) compared with PBS and antibody treated granulocytes. The data are representative of three independent experiments ($p<0.01$).

4.8. CEACAM1 from human placenta caries Lex residues and binds DC-SIGN

Since our data provide evidence for the presence of Lex residues on CEACAM1 from human granulocytes as well as binding to DC-SIGN we have tested if the Lex epitopes are expressed on CEACAM1 from human placenta, CEACAM1 from human malignant melanoma and to other members of CEA family in the colonic mucosa and colorectal carcinoma.

4.8.1. Detection of CEACAM1 expression in human placenta

To identify CEACAM1 in whole cellular extracts of human placenta tissue western blot analyses were performed (Fig. 32). First trimester human placenta tissue was cut into small pieces. Eight specimens were lysed and screened for CEACAM1 expression. Equal amount of each lysate was separated on 8% SDS-PAGE and transferred to PVDF membrane. Applying mAb T84.1, CEACAM1 was detected as a band that corresponds to the molecular weight (130 kDa) of CEACAM1 expressed in human placenta. For specific detection of CEACAM1, the mAb 4D1C2 was applied.



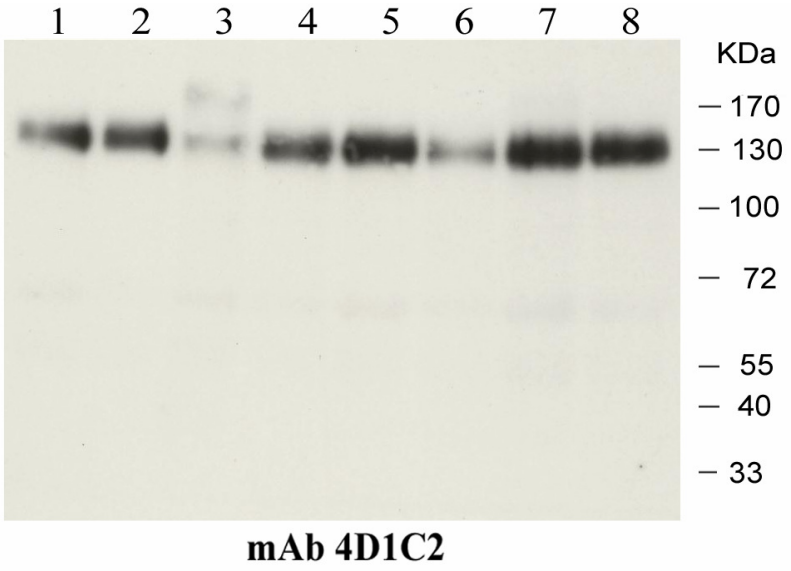


Figure 32. Western blot analyses of whole cellular extracts of human placenta tissue revealed expression of CEACAM1

Eight whole cellular extracts were prepared from human placenta tissue and screened for CEACAM1 expression by mAbs T84.1 and 4D1C2, respectively. CEACAM1 was detected in whole cellular lysates as a band with molecular weight of 130 kDa. In the third column of the blot detected by T84.1 mAb additional bands of CEACAMs were detected, most probably from granulocytes present in the tissue. The molecular weight of the detected CEACAM1 protein was estimated by comparison with a molecular weight standard (marked right).

4.8.2. CEACAM1 expressed in human placenta is a carrier of Lex residues

We have investigated the expression of Lex groups on CEACAM1 from human first trimester placenta. Western blot analysis by Lex specific antibody L5 demonstrates that CEACAM1 from first trimester placenta carries Lex residues (Fig. 33). In whole cellular extracts of human placenta tissue, strong binding of mAb L5 was observed to a band of 130 kDa corresponding to a molecular weight of CEACAM1. To prove that this band represents CEACAM1, immunoprecipitation of CEACAM1 by mAb T84.1 was performed. Strong binding of mAb L5 to the precipitated CEACAM1 was detected, demonstrating that CEACAM1 from human placenta is a major carrier of Lex residues.

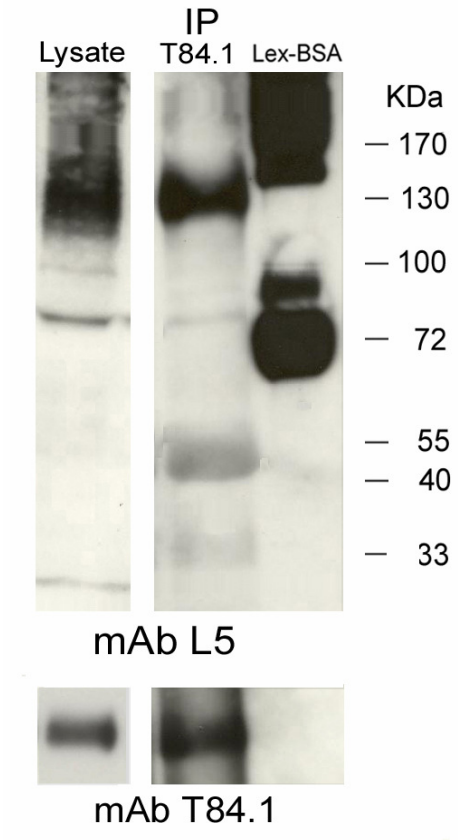


Figure 33. Detection of Lex residues on CEACAM1 from human placenta by western blot analysis

Whole cellular extracts were prepared from human placenta tissue, CEACAM1 was precipitated by mAb T84.1, the proteins were separated by 8% SDS-PAGE and transferred to PVDF membrane. Western blot analysis was performed applying the Lex-specific mAb L5.

Lex groups on CEACAM1 are recognized by mAb L5 in whole cellular extracts and after immunoprecipitation of CEACAM1, identifying CEACAM1 as a major carrier of Lex groups in human first trimester placenta. Lex-BSA was used as a control for the binding of the mAb L5 to Lex residues. Identity of CEACAM1 was confirmed by reprobing the membrane with mAb T84.1, after stripping (lower panel).

4.8.3. CEACAM1 from human placenta carrying Lex residues binds DC-SIGN

Since CEACAM1 is expressed in normal first trimester human placenta and decorated with Lex groups, we have investigated the binding of CEACAM1 to Fc-DC-SIGN by blot overlay assay (Fig. 34). Immunoprecipitation of CEACAM1 was performed by incubating the whole cellular extracts with T84.1 mAb. Subsequent to separation of the proteins by 8% SDS-PAGE, the PVDF membrane was probed with Fc-DC-SIGN and the detection was performed by HRP-labeled anti-human Fc-antiserum. Strong binding of Fc-DC-SIGN was observed in a range of 130 kDa, corresponding to the molecular weight of CEACAM1. These data demonstrate that CEACAM1 from human placenta is a ligand of DC-SIGN.

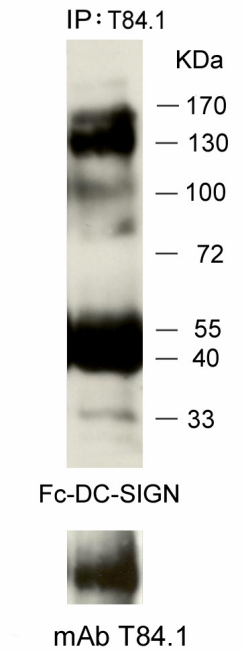


Figure 34. CEACAM1 from human placenta binds DC-SIGN in a blot overlay assay

CEACAM1 (130 kDa) from whole cellular extracts of human placenta tissue is recognised by Fc-DC-SIGN after precipitation of CEACAM1 by T84.1 mAb. HRP-labeled anti-human Fc-antiserum was used for detection of bound Fc-DC-SIGN. In addition, a band of approximately 50 kDa was detectable by the anti-human Fc-antiserum indicating unspecific binding. Subsequent to stripping, the membrane was reprobed by mAb T84.1 demonstrating the presence of CEACAM1 (lower panel).

4.9. Lex residues were not detected on CEACAM1 from human malignant melanoma

4.9.1. Detection of CEACAM1 in human malignant melanoma

So far, there is no evidence that glycan structures of CEACAM1 from malignant melanoma are containing Lex residues. Therefore, we have examined whole cellular lysates of malignant melanoma from 15 patients. Expression of CEACAM1 in whole cellular lysates of malignant melanoma tissue was analyzed by western blot using the mAb T84.1. CEACAM1 (~130 kDa) was successfully detected in 11 out of 15 whole cellular lysates of malignant melanoma (Fig. 35).

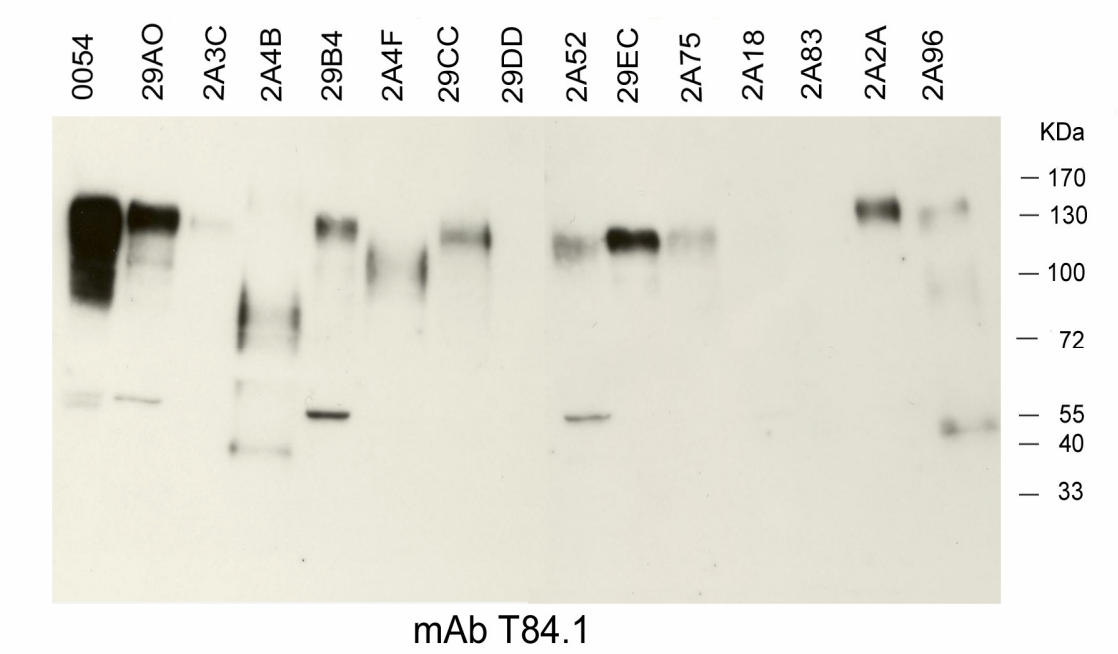


Figure 35. Detection of CEACAM1 in lysates of malignant melanoma by western blot analysis
To identify CEACAM1 in western blots of whole cellular lysates of malignant melanoma tissue the mAb T84.1 was applied. The numbers represent the ID of each patient. The bands corresponding to ~130 kDa represent CEACAM1.

4.9.2. CEACAM1 from malignant melanoma is not a carrier of Lex residues

To investigate whether CEACAM1 from human malignant melanoma contains Lex groups, we have examined the binding of Lex specific mAb L5 to immunoprecipitated CEACAM1 from lysates of malignant melanoma. CEACAM1 was precipitate by mAb T84.1 from equal amount of each lysate sample. Lex-BSA was used as a control for binding of L5 mAb. By western blot analysis, no binding of mAb L5 to CEACAM1 from human malignant melanoma was observed (Fig. 36). The band in the column 4 corresponds to the Mr of CEACAM1 isolated from human granulocytes.

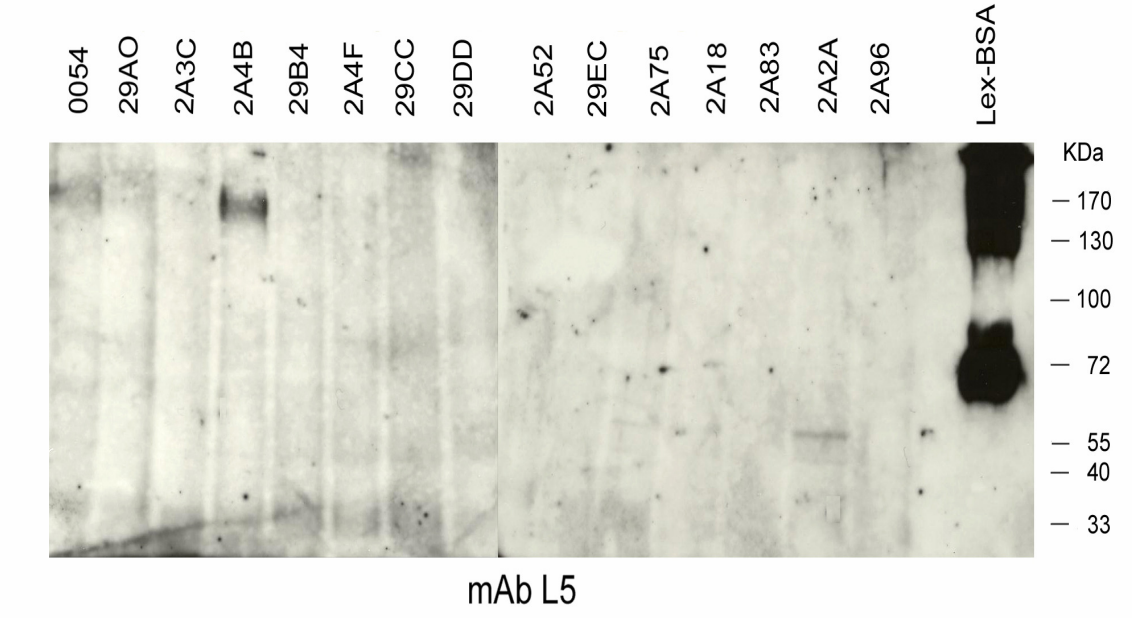


Figure 36. CEACAM1 from malignant melanoma is not a carrier of Lex residues

CEACAM1 was immunoprecipitated (mAb T84.1) from whole cellular lysates of 15 patients with malignant melanoma. Lex-BSA was used as a positive control of binding of L5 mAb. The band that is detected in column 4 (~160 kDa) corresponds to the molecular weight of CEACAM1 from human granulocytes that can be a result of contamination of the tissues.

4.10. CEA from colorectal carcinoma contains Lex residues and binds DC-SIGN

4.10.1. Detection of CEA and other members of the CEA family in human normal colon mucosa and colorectal carcinoma

Whole cellular extracts from normal colon mucosa and colorectal carcinoma were submitted to SDS-PAGE and western blot. The blot was incubated with mAb T84.1, which binds CEACAM1, CEA (CEACAM5) and other members of the CEA family. In whole cellular extracts of normal colon mucosa, binding of mAb T84.1 was observed to the proteins of >180 kDa and ~160 kDa well comparable to CEA and CEACAM1, respectively. In contrast to normal colon mucosa, additional members of CEA family were detected in colorectal carcinoma (Fig. 37).

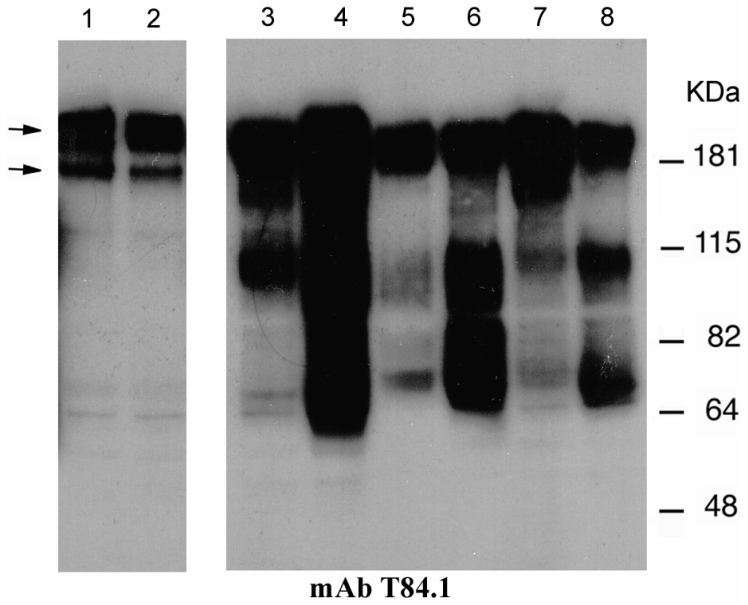


Figure 37. Detection of members of the CEA family in whole cellular extract of normal colon mucosa and colorectal carcinoma

Detection of the members of the CEA family in whole cellular extract of normal colon mucosa and colorectal carcinoma was performed by mAb T84.1. Columns 1 and 2 represent whole cellular extracts of normal colon mucosa; columns 3 to 8 represent whole cellular extracts of colorectal carcinoma. Upper arrow: CEA; lower arrow: CEACAM1. In colorectal carcinoma tissue, additional family members were recognized.

4.10.2. High expression of Lex residues on CEA from colorectal carcinoma

To investigate whether CEACAM1, CEA (CEACAM5) or other member of the CEA family expressed in normal colon mucosa or colorectal carcinoma recognized by the mAb T84.1 contain Lex epitopes, western blot analyses were performed.

T84.1 precipitates from whole cellular extracts of normal colon mucosa (2 patients) and colorectal carcinoma (6 patients) were separated on SDS-PAGE and the protein membrane was probed with Lex specific mAb L5. Strong binding of mAb L5 in a range of the molecular weight of CEA from colorectal carcinoma extracts demonstrates that CEA contains high levels of Lex groups (Fig. 38). Notably, the fucosylation of CEA from whole cellular extracts of normal colon mucosa is significantly lower as demonstrated by weak binding of mAb L5. This finding indicates that increased fucosylation of CEA occurs when colon cells are transformed to malignation.

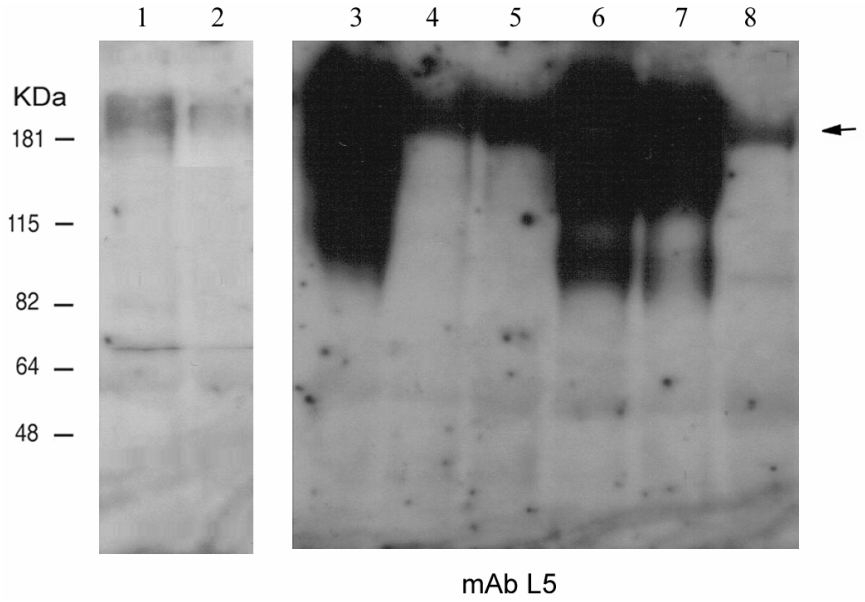


Figure 38. Increased expression of Lex residues on CEA from colorectal carcinoma

Precipitates by mAb T84.1 from whole cellular extracts of normal colon mucosa (column 1 and 2) and colorectal carcinoma (column 3-8) were separated on SDS-PAGE and immunoblotted with mAb L5. Compared with CEA from normal colon mucosa increased expression of Lex residues on CEA (marked by arrow) in colorectal carcinoma was observed.

4.10.3. CEA from colorectal carcinoma binds DC-SIGN

Carcinoembryonic antigen (CEA) is a tumour-associated antigen that is highly expressed in the majority of human colorectal carcinomas. Since CEA (CEACAM5) from colorectal carcinoma tissue contains Lex residues we have investigated the interaction with Fc-DC-SIGN. Whole cellular extracts of human normal colon mucosa and malignant colorectal carcinoma were immunoprecipitated using T84.1 mAb. The precipitates were separated by 8% SDS-PAGE and immunoblotted with Fc-DC-SIGN (Fig. 39). Strong binding of Fc-DC-SIGN to CEA from human colorectal carcinoma was observed indicating that Lex residues are recognized by DC-SIGN.

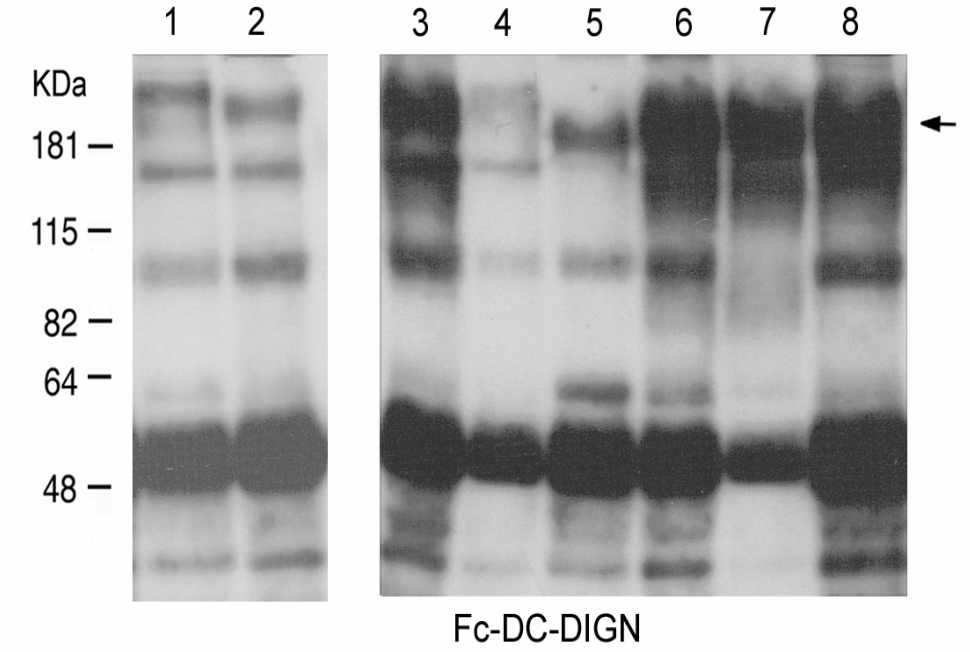


Figure 39. CEA from human colorectal carcinoma binds Fc-DC-SIGN

Column 1 & 2 represent precipitates by T84.1 mAb from whole cellular extracts of healthy colon mucosa (2 patients), while the columns 3-8 represent precipitates from lysates of colorectal carcinoma (6 patients). Blot overlay was performed applying soluble Fc-DC-SIGN and HRP-labeled anti-human Fc-antiserum for detection. Strong binding of Fc-DC-SIGN to CEA was observed (marked with arrow) in patients with colorectal carcinomas, compared to only weakly binding in healthy colon mucosa. The other bands (55 & 95 kDa) are unspecific binding of the anti-human Fc-antiserum.

4.11. DC-SIGN binds ICAM-3 isolated from peripheral human leukocytes through Lewis x residues

Since so far, the glycan structure mediating the interaction of native ICAM-3 with DC-SIGN is undefined, we have investigated which glycan structures may be responsible for the interaction of ICAM-3 with DC-SIGN.
For this purpose, ICAM-3 was purified from human blood buffy coats by affinity chromatography, and binding studies with DC-SIGN on DCs were performed.
Our results indicate that ICAM-3 from peripheral granulocytes, but not from peripheral lymphocytes and monocytes, contains Lewis x residues and binds DC-SIGN.

4.11.1 Determination of the purity of ICAM-3 preparation

The purity of the ICAM-3 preparation was analysed by Coomassie staining and western blot analysis, after separation of the proteins by SDS-PAGE (Fig. 40).
The ICAM-3 preparation displayed a diffuse band with an apparent molecular weight of 120-170 kDa, reflecting heterogeneous glycosylation. The broad band was also apparent in western blots by ICAM-3 mAb 140.11. A minor sharper band corresponding to a molecular weight of >180 kDa may represent aggregates.

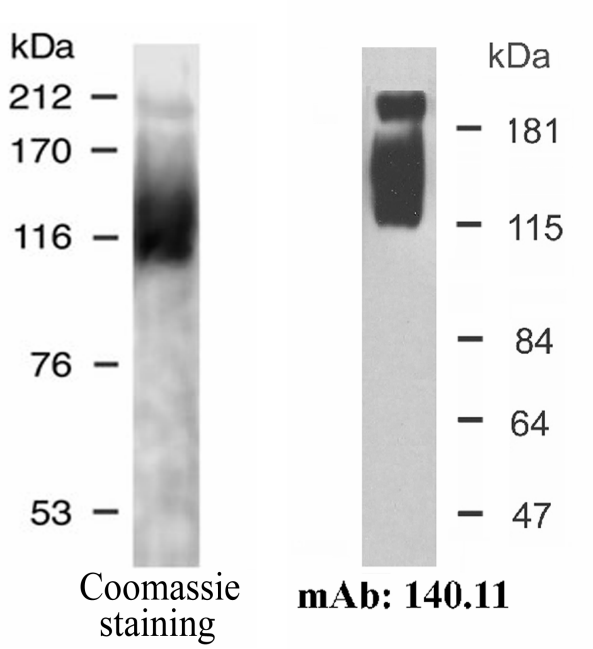


Figure 40. Determination of the purity of the ICAM-3 preparation by Coomassie staining and western blot analysis

Purity of immunaffinity purified human ICAM-3 demonstrated by Coomassie staining and western blot analysis by the ICAM-3 specific mAb 140.11.

The strong diffuse band with molecular weight of 130-170 kDa represents ICAM-3.

4.11.2. ICAM-3 from human leukocytes is a carrier of Lex residues

Expression of Lex residues on ICAM-3 was studied by western blot analysis.

The purified ICAM-3 preparation was separated by 8 % SDS-PAGE and the protein transfer was performed to a PVDF membrane.

Applying the already well described Lex-specific mAb L5, we demonstrated that ICAM-3 from human leukocytes is a carrier of Lex residues. The molecular weight of the band detected by the mAb L5 corresponds to the band detected with anti-ICAM-3 mAb 140.11. Lex-Bovine Serum Albumin (Lex-BSA) was used as a positive control for binding of mAb L5 (Fig. 41).

When, the ICAM-3 preparation was treated with α(1-3,4) fucosidase the binding of mAb L5 was completely abolished. The α(1-3,4) fucosidase treatment confirms the presence of Lex epitopes on ICAM-3.

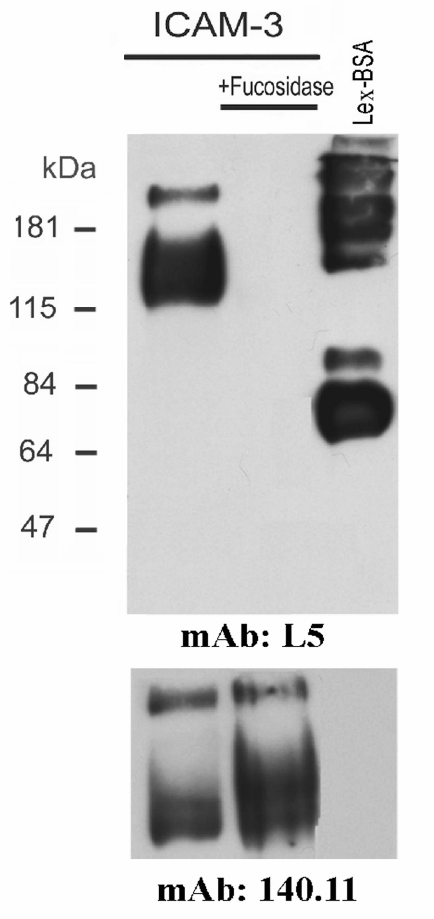


Figure 41. Lex residues on ICAM-3 from human leukocytes detected by western blot analysis
Western blot analysis of the ICAM-3 preparation by mAb L5 followed by anti-rat IgM mAb-HRP labeled was performed. When the ICAM-3 preparation was preincubated with α(1-3,4) fucosidase, no binding of mAb L5 was observed. Lex-BSA conjugate served as a positive control. Subsequent to stripping, the membrane was probed with ICAM-3 specific mAb 140.11 demonstrating equal loading of ICAM-3 (lower panel).

4.11.3. ICAM-3 from human leukocytes binds DC-SIGN via Lex residues

Binding of ICAM-3 from human leukocytes to Fc-DC-SIGN was studied by overlay assay. Equal amount of the untreated and α(1-3,4) fucosidase treated ICAM-3 preparation was loaded on SDS-PAGE and transferred to a PVDF membrane. Applying the soluble Fc-DC-SIGN, strong binding comparable to the molecular weight of ICAM-3 was observed. No residual binding of Fc-DC-SIGN to α(1-3,4) fucosidase treated ICAM-3 was observed (Fig. 42). These findings indicate that Lex residues on native ICAM-3 from peripheral leukocytes mediate the binding of ICAM-3 to DC-SIGN.

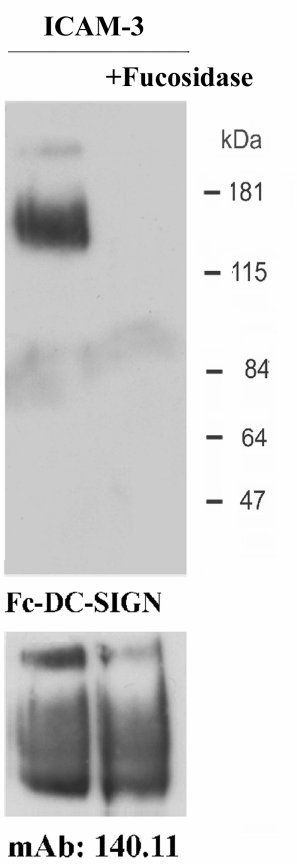


Figure 42. ICAM-3 from human leukocytes binds DC-SIGN

The band corresponding to ICAM-3 demonstrates the binding of Fc-DC-SIGN to ICAM-3 from human leukocytes. On contrary, no binding of Fc-DC-SIGN was observed when the preparation was incubated with α(1-3,4) fucosidase demonstrating that the binding of DC-SIGN to ICAM-3 is Lex dependent. The bound Fc-DC-SIGN was detected by HRP labeled anti-human Fc mAb. After stripping, equal loading of ICAM-3 was confirmed by mAb 140.11 (lower panel).

4.11.4. Lewis x groups mediate the binding of ICAM-3 to dendritic cells

4.11.4.1. Binding of ICAM-3 coated fluorescent microbeads to immature dendritic cells

In order to investigate the interaction of Lex-carrying ICAM-3 and DC-SIGN of DCs, isolated ICAM-3 was covalently attached to fluorescent microbeads and incubated with immature dendritic cells. Monocytes were isolated from peripheral human blood and differentiated by interleukin-4 (IL4) and granulocyte macrophage colony-stimulating factor (GM-CSF) to immature DCs showing high levels of DC-SIGN expression. Bound beads were visualized by fluorescence microscopy and photographically documented with a digital camera. Strong binding of ICAM-3-coated beads to DCs was observed (Fig. 43).

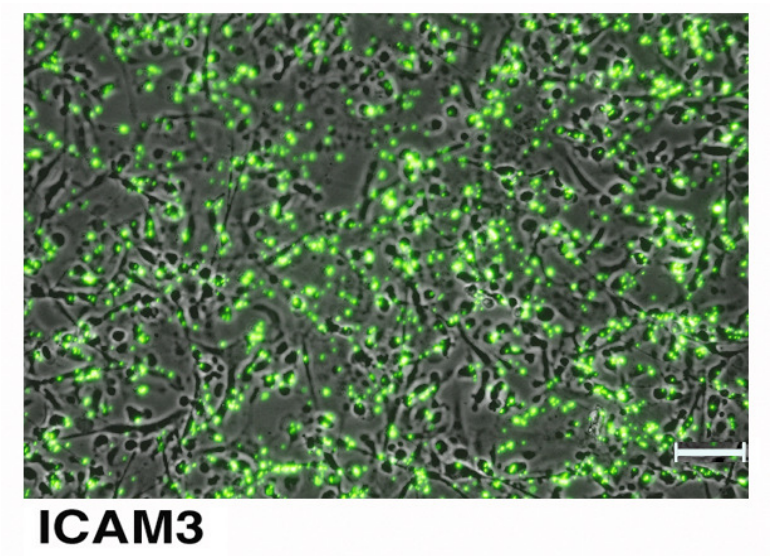


Figure 43. Binding of ICAM-3 coated fluorescent microbeads to immature dendritic cells
Immature dendritic cells were incubated with ICAM-3 coupled fluorescent microbeads (green colour). A fluorescence microscope was used for visualization of the bound ICAM-3 coated beads. The dendritic cells are shown by phase contrast microscopy (scale bar represents 80 µm).

4.11.4.2. Inhibition studies revealed that the Lex groups on ICAM-3 mediate the binding to immature DC

To prove the specificity of ICAM-3-DC-SIGN interaction blocking studies were performed. The binding of the ICAM-3 coated fluorescent beads to the DCs was significantly reduced when defucosylation of ICAM-3 was done by α(1-3,4) fucosidase, when Lex residues on ICAM-3 were blocked by mAb L5 or immature DCs were preincubated by DC-SIGN blocking mAb DC28 (Fig. 44 & Fig. 45).

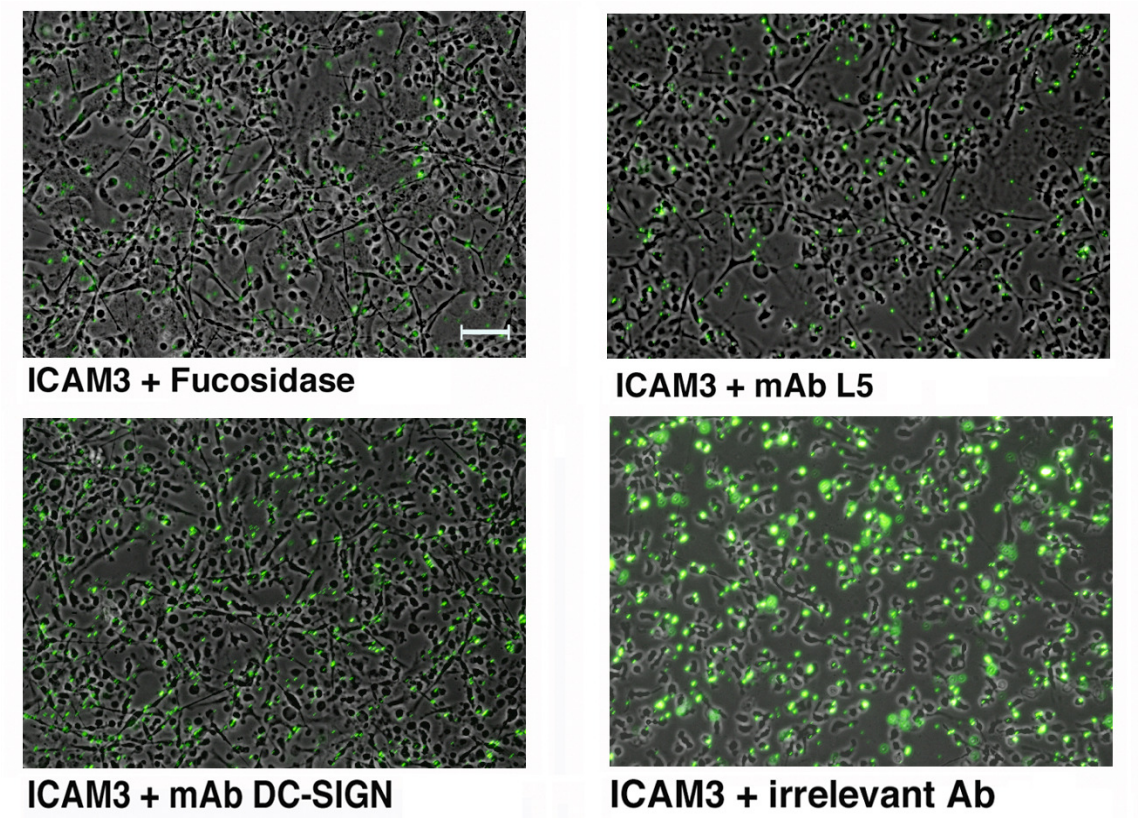


Figure 44. Inhibition studies of binding of ICAM-3-coated beads to DC-SIGN on DCs

Binding of the fluorescent beads coated by ICAM-3 was strongly reduced after removal of Lex residues from ICAM-3 by α(1-3,4) fucosidase, when DC-SIGN was blocked by preincubation of DCs by blocking DC-SIGN antibody DC28 or after Lex residues on ICAM-3 were blocked by Lex-specific mAb L5. No inhibition was observed by preincubation of DCs with an irrelevant Ab. A fluorescence microscope was used for visualization of the bound ICAM-3 coated beads. Dendritic cells are shown by phase contrast microscopy (scale bar represents 80 μm).

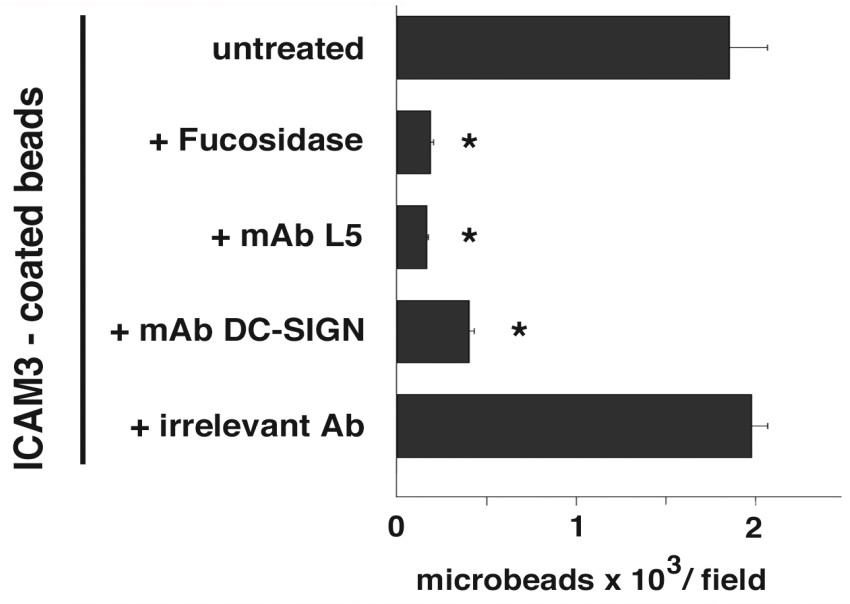


Figure 45. Quantitative data of binding and inhibition studies of ICAM-3 coated fluorescent microbeads to DCs

Absolute numbers of microbeads bound to DCs (Fig. 43 and Fig. 44) were quantified from digital images. For statistical significance, the Student's t-test was performed. Binding experiments were performed in triplicate. Compared to untreated beads, the highly significant decrease in binding is marked by asterisks ($P < 0.01$).

4.11.5. Analyses of ICAM-3 from different peripheral blood cells populations revealed that only ICAM-3 from granulocytes binds DC-SIGN

The ICAM-3 preparation used in this study was isolated from buffy coats of healthy donors. In order to verify which of the populations of human peripheral blood leukocytes contained ICAM-3 and bound the Lewis x antibody and soluble Fc- DC-SIGN, respectively, monocytes, T lymphocytes, B lymphocytes, and granulocytes were isolated by gradient centrifugation followed by cell-type specific magnetic cell sorting. The purity of the isolated cell populations was confirmed by flow cytometry.

4.11.5.1. Purity of the peripheral blood cell populations confirmed by FACS-analysis

In order to verify the purity and homogeneity of the isolated cell fractions FACS analyses were performed. Monocytes, granulocytes, T and B lymphocytes were incubated with anti-CD14-FITC, anti-CD15-PE, anti-CD3-PE, and anti-CD19-PE mAbs, respectively. Isotype controls for each antibody were included. The FACS analysis confirmed the homogeneity and high purity of the isolated cell populations of human peripheral blood leukocytes (Fig. 46).

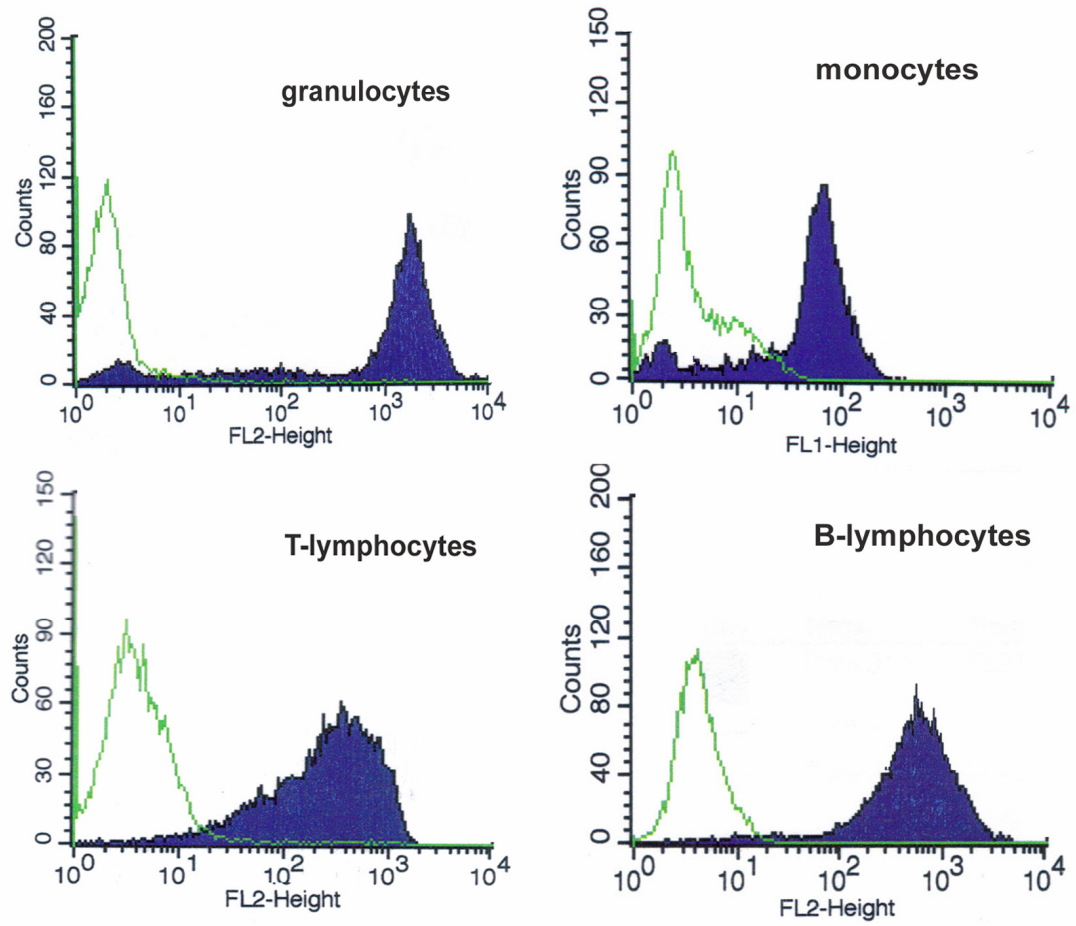


Figure 46. Purity of the peripheral blood cell populations confirmed by FACS-analysis

Isolated granulocytes, monocytes, T lymphocytes and B lymphocytes were incubated with anti-CD15-PE, anti-CD14-FITC, anti-CD3-PE and anti-CD19-PE, respectively. The data are shown by histograms, fluorescence intensity log 10 (FL1-channel-FITC; FL2-PE) vs. events (counts). Isotype controls were included (hollow histograms in green).

4.11.5.2. Detection of ICAM-3 in purified populations of peripheral blood leukocytes

Subsequent to isolation, the peripheral blood cell populations (monocytes, granulocytes, T and B lymphocytes) were lysed and pre-cleared by protein G Plus agarose. ICAM-3 was precipitated from whole cellular lysates of each isolated population of the peripheral blood leukocytes by ICAM-3 specific mAb 3.1. The precipitates were loaded on NuPage gradient gel and transferred to PVDF membrane. ICAM-3 was identified in each of the populations using the goat polyclonal ICAM-3 Ab N-19.

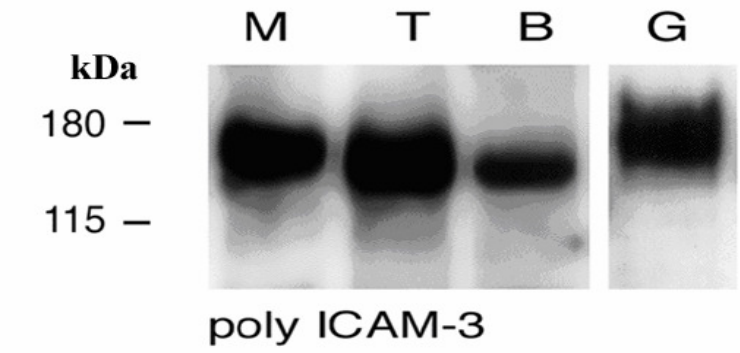


Figure 47. Detection of ICAM-3 in purified populations of peripheral blood leukocytes by western blot analysis

Western blot analysis was performed for detection of ICAM-3 immunoprecipitated by ICAM-3-specific mAb 3.1 from whole cellular extracts of purified populations of peripheral blood leukocytes. M, monocytes, T, T lymphocytes, B, B lymphocytes, G, granulocytes. ICAM-3 was detected in each of the isolated populations applying the polyclonal ICAM-3 antibody N-19.

4.11.5.3. ICAM-3 from human granulocytes carries Lex residues

After ICAM-3 was identified in whole cellular lysates of the isolated peripheral blood cell populations, the same lysates were submitted for detection of Lex residues. ICAM-3 was immunoprecipitated from whole cellular lysates of human monocytes, granulocytes, T and B lymphocytes, respectively, using ICAM-3 specific mAb 3.1. The precipitates were separated on Nu Page gradient gel and the protein membrane was incubated with Lex specific mAb L5 (Fig. 48). Strong binding of the mAb L5 to ICAM-3 precipitated from human granulocytes was observed. In contrast, no binding of the L5 mAb was detected on ICAM-3 from monocytes and T or B lymphocytes, respectively, indicating that among human peripheral blood leukocytes, only granulocyte ICAM-3 carries Lewis x residues.

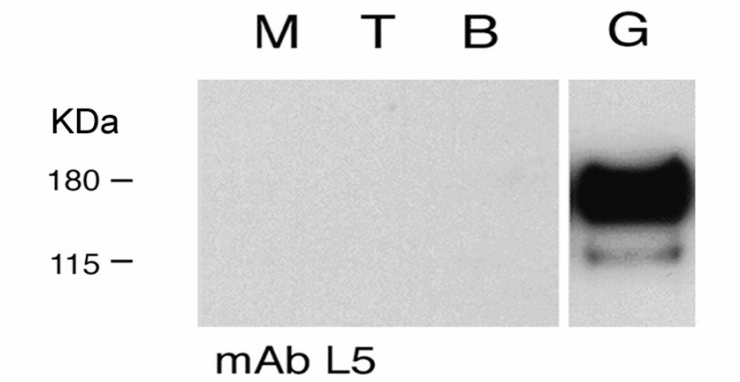


Figure 48. Among peripheral blood leukocytes, only ICAM-3 from granulocytes carries Lex residues

Western blot analysis was performed for detection of Lex residues on ICAM-3 immunoprecipitated from whole cellular lysates of purified populations of peripheral blood leukocytes (M, monocytes, T, T lymphocytes, B, B lymphocytes, G, granulocytes). Lex residues were detected only on ICAM-3 from human granulocytes by Lex-specific mAb L5.

4.11.5.4. ICAM-3 from human granulocytes binds Fc-DC-SIGN

Precipitates of ICAM-3 from whole cellular lysates of purified peripheral blood cell populations were investigated for binding to Fc-DC-SIGN. By overlay assay, we demonstrated that ICAM-3 from human granulocytes was recognized by Fc-DC-SIGN (Fig. 49). The bound Fc-DC-SIGN was detected by anti-human Fc-HRP labeled antiserum. In contrast, ICAM-3 precipitated from monocytes and T or B lymphocytes, respectively, was not recognized by soluble Fc-DC-SIGN.

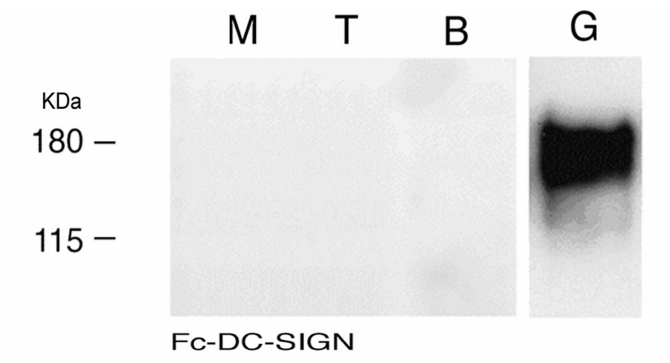


Figure 49. ICAM-3 from human granulocytes binds Fc-DC-SIGN

M, monocytes, T, T lymphocytes, B, B lymphocytes, G, granulocytes.

Precipitates of ICAM-3 by polyclonal ICAM-3 specific mAb 3.1 from lysates of human peripheral blood leukocytes were analyzed for binding to Fc-DC-SIGN. Strong binding of Fc-DC-SIGN only to ICAM-3 from human granulocytes was observed. The bound Fc-DC-SIGN was detected by HRP-labeled anti-human Fc-antiserum followed by chemiluminescence.

4.11.5.5. High-mannose structures were not identified on ICAM-3 from human leukocytes populations applying GNA

For further characterization of the glycostructure of ICAM-3 from isolated populations of human leukocytes, blot overlay assay was performed with *Galanthus nivalis* agglutinin (GNA), a lectin binding to high-mannose structures.
Monocytes, granulocytes, T and B lymphocytes, were isolated from peripheral blood cells of human leukocytes, lysed, and ICAM-3 was immunoprecipitated with ICAM-3 specific mAb 3.1 (Fig. 50). No significant binding of GNA to the precipitated ICAM-3 was observed.

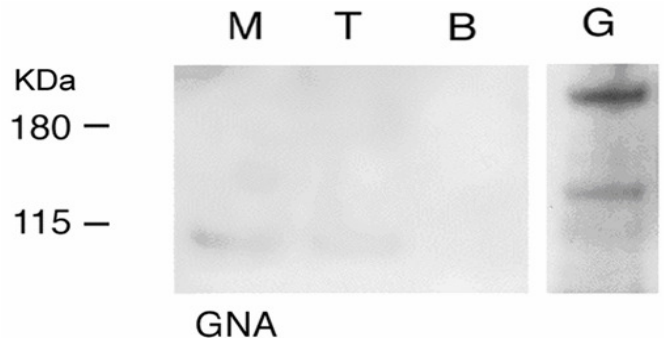


Figure 50. High-mannose structures were not identified on ICAM-3 from peripheral blood populations by blot overlay using GNA

M, monocytes, T, T lymphocytes, B, B lymphocytes, G, granulocytes.

The precipitates of ICAM-3 (mAb 3.1) were probed by digoxigenin-labeled GNA followed by incubation with a HRP-labeled anti-digoxigenin antibody, for detection. No significant binding of the mannose-specific GNA was observed.

4.11.5.6. Recombinant ICAM-3 binds Lewis x antibody L5 and DC-SIGN after coexpression with fucosyltransferase IX

Human fucosyltransferases FUTIII, IV, VII and IX, respectively, involved in terminal modifications of glycoproteins were coexpressed with CEACAM1 in HEK293 cells (Fig. 51).

Binding of the Lewis x-specific mAb L5 to ICAM-3 was observed in the presence of FUT IX. A band with a corresponding electrophoretic mobility was detected by Fc-DC-SIGN. With FUT IV, some binding of the monoclonal Lewis x antibody and a faint binding of Fc-DC-SIGN were present. No Lewis x residues were detectable in ICAM-3 coexpressed with FUT III or FUT VII.

In all of the HEK293 transfectants, binding of DC-SIGN to a band of higher electrophoretic mobility was observed in each of the cotransfectants. In the loading control using an antibody against the myc-tagged ICAM-3, it is apparent that this is the main form of expressed recombinant ICAM-3. Since a band of identical electrophoretic mobility binds the high-mannose-specific GNA, the lower molecular weight form of ICAM-3 expressed in HEK293 cells probably binds DC-SIGN via high-mannose residues.

These results indicate that FUT IX and, to a lesser extent, FUT IV mediate the synthesis of Lewis x residues in ICAM-3, and that these residues bind DC-SIGN. In addition, a recombinant form of ICAM-3 devoid of Lewis x residues interacts with Fc-DC-SIGN, probably via high-mannose residues.

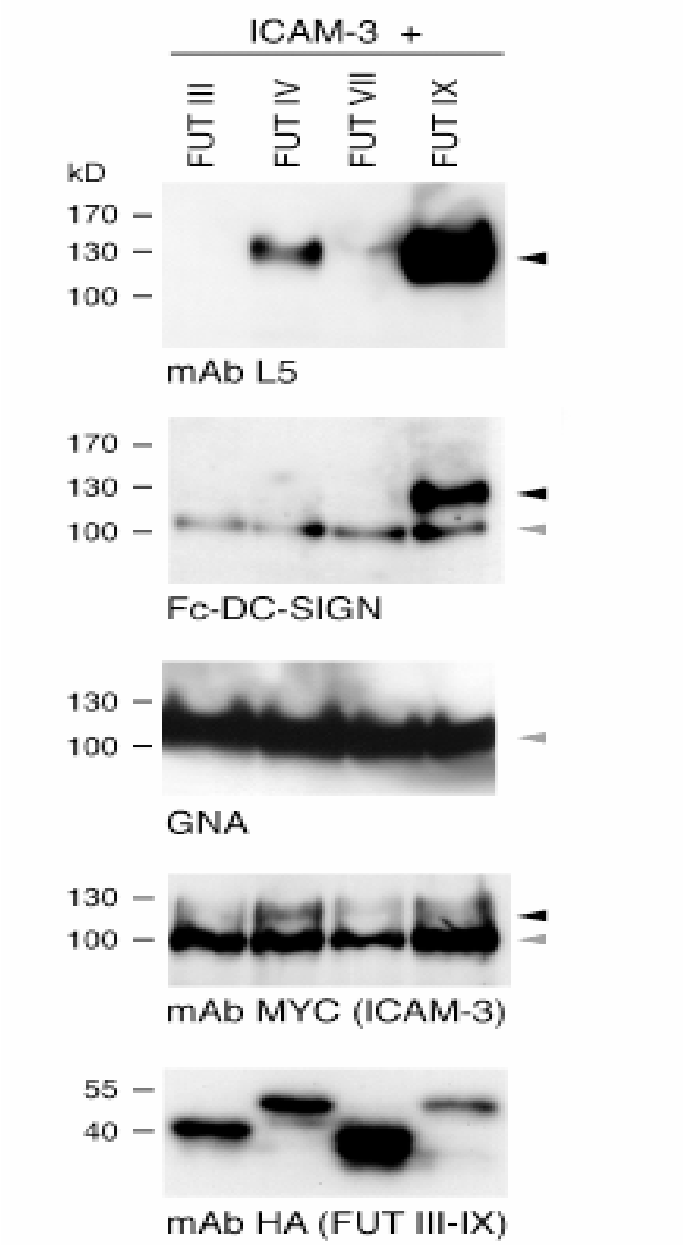


Figure 51. Western blots of recombinant ICAM-3 preparations produced by HEK293 cells coexpressed with different FUTs

HEK293 cells were cotransfected by ICAM-3 cDNA in combination with different FUT cDNAs. mAb L5, monoclonal Lewis x antibody; Fc-DC-SIGN, recombinant fusion protein. GNA, *Galanthus nivalis* agglutinin; mAb MYC, monoclonal antibody directed against the MYC tag of recombinant ICAM-3; mAb HA, monoclonal antibody directed against the hemagglutinin tag of the FUTs. Lewis x-carrying ICAM-3 is marked by black arrowheads and the fraction of ICAM-3 positive for GNA- and Fc-DC-SIGN-binding is marked by gray arrowheads.

4.12. Results from the biomolecular experiments

For the experimental work DNA-encoding human CEACAM1, ICAM-3 and Fc-DC-SIGN, were amplified by PCR using human cDNA molecules and specific primer combinations. The PCR products were inserted into a pCR 2.1 plasmid vector that provides a quick, efficient and one-step cloning strategy. Subsequent to sequencing, the desired DNA constructs (inserts) were cleaved from pCR 2.1 vector, purified from agarose gel and ligated into the pEF-BOS expression vector.

4.12.1. PCR characterisation of ICAM-3 cDNA

The DNA fragment amplified by PCR that contained the sequence of ICAM-3 cDNA (1600 bp), was separated by electrophoresis on 1% agarose gel containing ethidium bromide. QIAEX II Gel Extraction Kit (Qiagen, Hilden) was used for purification of the PCR product from the agarose gel.

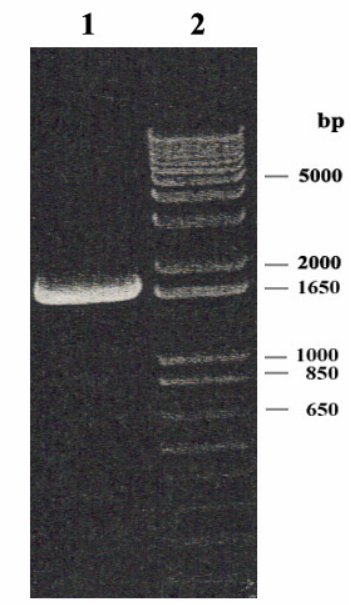


Figure 52. The amplified product of ICAM-3 cDNA by PCR

10 µL of the PCR product was separated on 1% agarose gel. The DNA fragment of ICAM-3 (column 1; 1600bp) was compared with standard DNA marker (column 2; 1kb plus).

4.12.2. Restriction digestion of ICAM-3 cDNA in pCR 2.1 vector

Following the purification of the amplified full length ICAM-3 cDNA fragment from the agarose gel, it was ligated in pCR 2.1 vector at 16°C, overnight. The transformation was performed in One Shot INVαF'competent cells. In order to verify the presence of the desired insert (ICAM-3, 1600 bp), six clones were selected, and following the plasmid DNA isolation from bacterial culture, restriction digestion with enzymes *Sal*I and *Not*I was performed (Fig. 53).

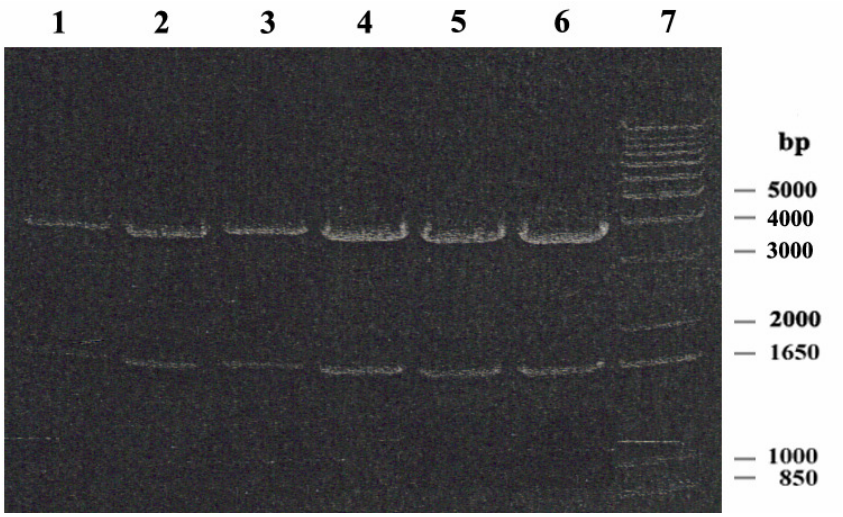


Figure 53. Restriction digestion of plasmid DNA (ICAM-3 cDNA cloned in pCR2.1 vector)
In order to verify the presence of the required ICAM-3 cDNA fragment inserted (1600 bp) in pCR2.1 vector, restriction digestions of six DNA-mini preparations (5µL) with the enzymes SalI and *Not*I were performed (column 1-6). Column 7: 1kb plus DNA marker.

4.12.3. Cloning of the ICAM-3 cDNA in pEF-BOS vector

In other to cleave the ICAM-3 cDNA fragment from pCR2.1 vector, 5 µL of the plasmid-DNA mini-preparation were digested with restriction enzymes *Sal*I and *Not*I. 1µL of DNA maxi-preparation (vector pEF-BOS) was precleaved with the same restriction enzymes (Fig. 54). Subsequent to purification from the agarose gel (1%), both, the pre-cut pEF-BOS vector and the DNA-insert (ICAM-3, 1600 bp) were ligated at 16°C overnight, and the ligated product was transformed in One Shot

INVαF'competent cells. After the plasmid DNA was isolated from the bacterial culture, restriction digestion with the enzymes *Sal*I and *Not*I was performed for verification of the presence of the desired insert (Fig. 55). For further investigation, DNA-maxi preparation containing the recombinant ICAM-3 in pEF-BOS vector (Fig. 55, clone 8) was performed and used for transfection studies.

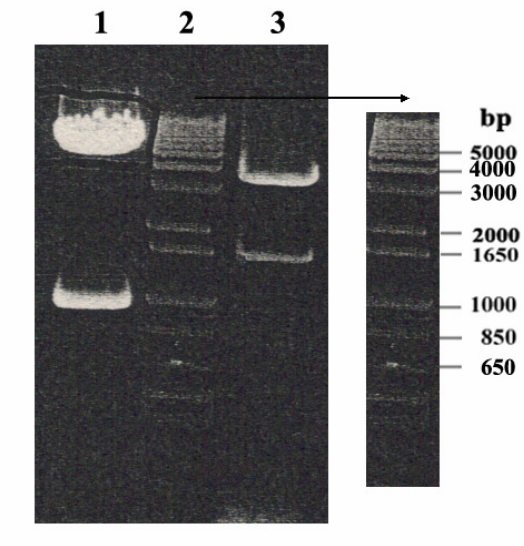


Figure 54. Restriction digestion of pEF-BOS vector and ICAM-3 cDNA, cloned in pCR2.1 vector Column 1: the pEF-BOS vector pre-cut with the enzymes *Sal*I and *Not*I; column 2: DNA ladder-1kb plus; column 3: mini-preparation of pcR2.1 vector containing ICAM-3cDNA digested with the enzymes *Sal*I and *Not*I.

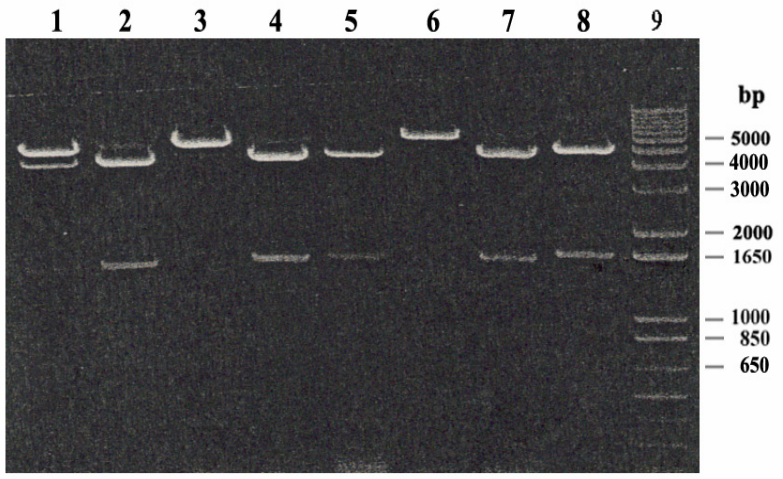


Figure 55. Restriction digestion of plasmid DNA of ICAM-3 cDNA cloned in pEF-BOS vector DNA-mini preparation was prepared from eight clones and digested with restriction endonucleases *Sal*I and *Not*I (column 1-8). The cleaved DNA fragment (1600 bp) represents ICAM-3 DNA. Column 9: 1kb plus ladder.

4.12.4. Restriction digestion of Fc-DC-SIGN cDNA

The PCR product of Fc-DC-SIGN cDNA was extracted from 1% agarose gel, ligated in pEF-BOS vector, and the ligated product was transformed in One Shot INVαF'competent cells. After the DNA was isolated from the bacterial culture (two clones were selected), restriction digestion with enzymes *Sal*I and *Asp*718 was performed to verify the presence of the Fc-DC-SIGN cDNA fragment (1800 bp). Agarose gel electrophoresis was employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and the purity of DNA isolation and to size fractionate DNA (Fig.56). After the presences of the desired DNA insert (Fc-DC-SIGN) in the pEF-BOS vector was confirmed, DNA-maxi preparation was carried out. For restriction digestion, 1 µL of plasmid DNA (maxi preparation) was digested by the enzymes *Asp*718 and *Sal*I for 2 hours at 37°C. The restriction digestion was monitored by agarose gel electrophoresis (Fig 57).

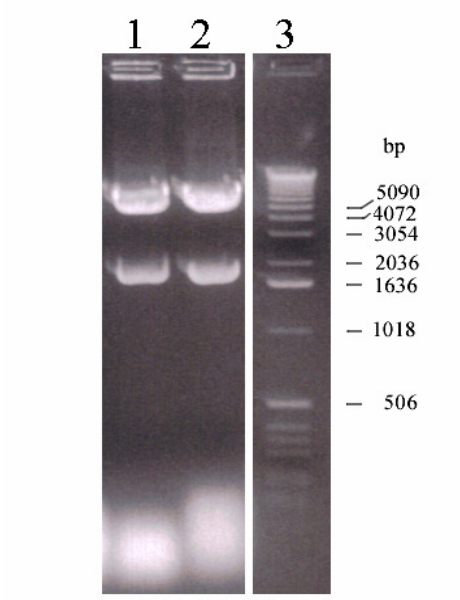


Figure 56. Restriction digestion of DNA mini preparation of Fc-DC-SIGN cDNA cloned in pEF-BOS vector

5 µL of DNA mini preparation were digested with restriction endonucleases *Asp*718 and *Sal*I (2h, 37°C). The digested products were separated on 1% agarose gel and the expected insert of Fc-DC-SIGN fragment in length of 1800 bp was visualized (column 1 and 2). The marker of 1 Kb was co-electrophoresed with DNA samples for fragment size determination (column 3).

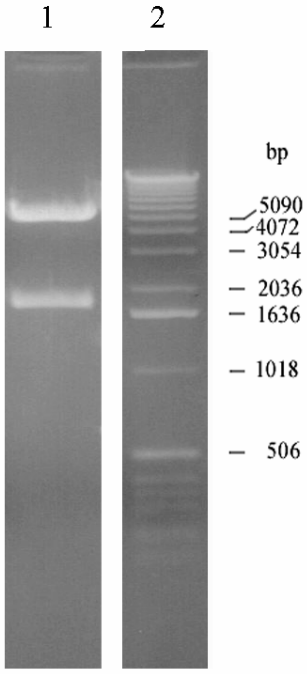


Figure 57. Restriction digestion of DNA maxi preparation of Fc-DC-SIGN cDNA in pEF-BOS vector

1 µL of plasmid DNA maxi preparation with restriction endonucleases *Asp*718 and *Sal*I was digested and the digested products were separated on 1% agarose gel. Column 1: Fc-DC-SIGN cDNA fragment (1800 bp) in pEF-BOS vector. Column 2: 1 kb DNA-ladder.

4.12.5. Restriction digestion of CEACAM1 cDNA

In order to confirm, that CEACAM1 cDNA is properly inserted in the pEF-BOS vector, restriction digestion of the plasmid DNA mini-preparation was performed. 5µL of plasmid DNA preparation was digested with *Sal*I and *Asp*718 restriction endonucleases. 1% agarose gel stained with ethidium bromide was used for separation of the DNA fragments (Fig. 58). Restriction digestion of the plasmid DNA maxi-preparation was performed at the same conditions (Fig. 59).

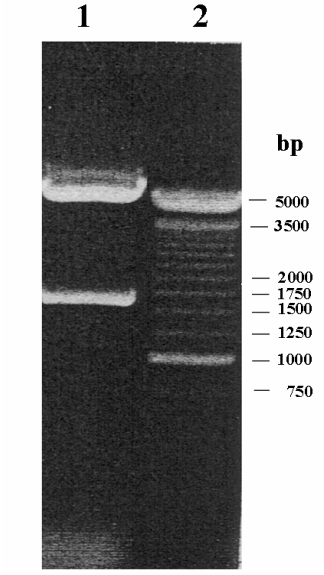


Figure 58. Restriction digestion of DNA mini-preparation of CEACAM1 cDNA cloned in pEF-BOS expression vector

Restriction digestion of 5 µL plasmid-DNA (pEF-BOS vector containing CEACAM1 cDNA), mini-preparation, with *Sal*I and *Asp*718 restriction endonucleases. The cleaved DNA fragment in length of 1600 bp represents CEACAM1 cDNA (column 1). 250 bp DNA ladder was used as marker (column 2).

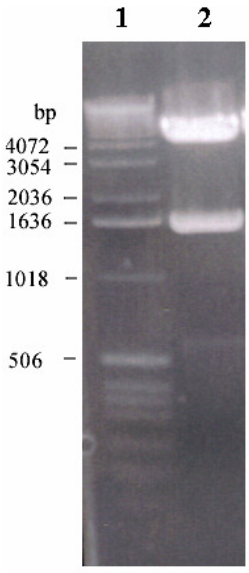


Figure 59. Restriction digestion of DNA maxi-preparation of CEACAM1 cDNA cloned in pEF-BOS vector

Plasmid-DNA (CEACAM1 cDNA cloned in pEF-BOS expression vector) was incubated with restriction endonucleases *Sal*I and *Asp*718. Column 1: 1kb DNA marker; column 2: plasmid-DNA, CEACAM1 cDNA fragment (1600 bp).

5. DISCUSSION

In the present studies, evidence is provided that, under physiological conditions, CEACAM1 and ICAM-3 bind DC-SIGN via Lex residues.

We have identified CEACAM1 on granulocytes as a new ligand of DC-SIGN. The structural analysis of CEACAM1 supported by mass spectrometry revealed that this interaction is mediated through Lex epitopes present of CEACAM1.

DC-SIGN expressed on dendritic cells has been described as a cellular ligand of ICAM-3 on resting T cells.

Since so far it is not clear which glycan epitopes of native ICAM-3 are responsible for binding of DC-SIGN, we have investigated the glycostructure of ICAM-3 from human leukocytes. Here, we provide evidence that native ICAM-3 from human granulocytes but not from T-cell binds DC-SIGN via Lex residues.

For these studies, native CEACAM1 was isolated form human granulocytes and native ICAM-3 was purified from human leukocytes by affinity chromatography.

5.1. CEACAM1 expressed on human granulocytes is a major carrier of Lewis x residues

Cellular adhesion molecules (CAMs) are important participants in cell-cell interactions and interactions between cells and components of the extracellular matrix. These molecules have been implicated in a wide variety of cellular functions including signal transduction, cellular communication and recognition, embryogenesis, inflammatory and immune responses, and apoptosis.

Among the members of the CEA family, the CEA-related cell adhesion molecule 1 (CEACAM1) has attracted particular interest since it is involved in several important cellular activities, such as tumor progression, angiogenesis, and immune modulation.

Like other CEA family members, CEACAM1 is highly glycosylated molecule. Studies of the glycostructures of CEACAM1 contribute to understand their biological function.

Biological functions of CEACAM1 glycans have been shown for high-mannose structures through the specific binding to type 1 fimbriae (Leusch, et al. 1990) (Sauter, et al. 1993) and for lactosamine glycan structures through the binding to galectin-1 and galectin-3 (Feuk-Lagerstedt, et al. 1999), (Ohannesian, et al. 1995). It has been postulated that the glycan structure of CEACAM1 mediates the binding of granulocytes to endothelial cells (Stocks, et al. 1995).

Previously, it has been suggested that CEACAM1 may also carry sLex groups (Stocks and Kerr 1993). However, in recent investigations, sLex antibodies such as KM93, HECA452, 2H5, or CSLEX1 did not bind to native granulocyte CEACAM1 (Lucka, et al. 2005).

Lex structures, also known as SSEA-1 or CD15, are defined as terminal epitopes of oligosaccharide chains that are expressed on glycoproteins and glycolipids (Spooncer, et al. 1984). Recently, several Lex glycan epitopes on native CEACAM1 from human granulocytes were identified by MALDI-TOF mass spectrometry (Lucka, et al. 2005).

In the present study, detection of Lex groups on CEACAM1 isolated from whole cellular extracts of human granulocytes is demonstrated by western blot analysis applying the well-established mAb L5. The antibody has been shown to recognize Lex structures contained by neural N-glycans (Streit, et al. 1996).

Furthermore, treatment of CEACAM1 purified from human granulocytes with α(1-3,4) fucosidase, an enzyme that releases the fucose residues bound to the antennae but not to the core structure, completely abolished the binding of mAb L5. This supports the finding that CEACAM1 expressed on human granulocytes is a major carrier of Lewis x residues.

Moreover, Lex residues were not detectable on CEACAM6, a related member of the CEACAM family expressed on granulocytes, indicting that CEACAM6 is lacking some specific structural characteristics that are essential for fucosylation.

5.2. CEACAM1 expressed on human granulocytes is a major ligand of DC-SIGN

DC-SIGN is a C-type lectin present on both, dendritic cells and macrophages. DC-SIGN contains a carbohydrate recognition domain with specificity for high-mannose moieties and non-sialylated Lewis antigens. This enables DC-SIGN to function as a pathogen receptor with broad specificity that binds viruses, such as human immunodeficiency virus-1 (HIV-1) (Geijtenbeek, et al. 2000b) and hepatitis C virus (HCV) (Pohlmann, et al. 2003), bacteria such as *Mycobacterium tuberculosis* (Tailleux, et al. 2003) and parasites, such as *Schistosoma mansonii* (van Die, et al. 2003). In addition, DC-SIGN has been postulated to interact with cellular ligands and to mediate binding of DCs to intercellular adhesion molecule (ICAM-2) on endothelium (Geijtenbeek, et al. 2000a), and ICAM-3 on T cells (Geijtenbeek, et al. 2000c).

Having identified CEACAM1 from human granulocytes as a carrier of Lex residues, we have raised a hypothesis that Lex residues of CEACAM1 or other members of CEA family can be recognized by DC-SIGN.

To investigate the binding of DC-SIGN to CEACAM1, recombinant soluble Fc-DC-SIGN containing the extracellular domain caring a human Fc-tag (IgG_1) was generated, and blot overlay assays were performed.

In western blots of crude extracts of human granulocytes, strong binding of Fc-DC-SIGN to a band was detected that corresponds to the molecular weight of CEACAM1 (~160 kDa).

To proof that this band represents CEACAM1, immunoprecipitation of CEACAM1 by CEACAM1-reactive mAb was carried out. Strong binding of Fc-DC-SIGN to precipitated CEACAM1 was observed. After the precipitation of CEACAM1, no binding of Fc-DC-SIGN was detected in the supernatant, thus confirming that CEACAM1 is the major ligand of DC-SIGN in whole cellular extracts of human granulocytes.

Treatment of the precipitated CEACAM1 by α(1-3,4) fucosidase completely abrogated the binding of Fc-DC-SIGN. α(1-3,4) fucosidase is an enzyme that specifically targets fucose bound to GlcNAc in α1-3 or α1-4 linkage and removes the fucose moiety essential for binding to DC-SIGN. This enzyme completely removes the Lex epitopes from CEACAM1, since CEACAM1 treated with α(1-3,4) fucosidase did not react with Lex specific monoclonal Ab L5.

In addition to blot overlay assays, surface binding of Fc-DC-SIGN to human granulocytes was investigated by flow cytometry. Granulocytes were isolated from fresh buffy coats, and before Fc-DC-SIGN incubation, unspecific binding of Abs or Fc-Dc-SIGN was blocked by preincubation of the granulocytes with human IgG.
Double staining with anti-DC-SIGN mAb and CEACAM1-specific mAb suggests surface binding of Fc-DC-SIGN to the granulocytes involving CEACAM1.
The specificity of the interaction between DC-SIGN and CEACAM1 was proved by inhibition studies. Preincubation of granulocytes by a Lex-specific mAb and a polyclonal antiserum directed against CEACAM significantly decreased the binding of DC-SIGN. In contrast, no inhibition was observed when granulocytes were pre-incubated with Ab against high-mannose residues.

5.3. α(1-3) fucosyltransferase IX specifically transfers fucose residues to CEACAM1

Granulocytes have a unique glycosylation pattern, and in contrast to other leukocytes, express Lewis x carbohydrates on the cell surface (Gooi, et al. 1983). Although granulocytes express high levels of Lex, so far only several proteins on granulocytes are found as carriers of Lex groups, including CEACAM1, ICAM -3 and Mac1(Lucka, et al. 2005),(Bogoevska, et al. 2007), (Skubitz and Snook 1987), (van Gisbergen, et al. 2005b).
Little is known on the regulation of Lex expression of these proteins, but the differential expression of fucosyltransferases appears to play a role. Since granulocytes express the fucosyltransferase IV (FUTIV) and IX (FUTIX), we posed

the question if they might be involved in the formation of Lex epitopes on CEACAM1.

In our work, we have investigated which fucosyltransferase particularly determines the expression of Lex residues on CEACAM1 applying western blot and FACS analysis.

Recombinant CEACAM1 cDNA was co-transfected in HEK293 cells in combination with DNAs encoding fucosyltransferases FUTIII, FUTIV, FUTVII and FUTIX. Lex residues were present on CEACAM1 when CEACAM1 was co-expressed with FUTIX. A weak signal was observed when CEACAM1 and FUTIV were co-expressed. This implied that FUTIX exhibits stronger activity than other FUTs for forming the Lex groups on CEACAM1 that are recognized by anti-Lex Ab.

Although FUTIX preferentially fucosylates CEACAM1, fucosylation of other ligands of DC-SIGN cannot be excluded. In our investigations, we have found that CEACAM1 and ICAM-3 are fucosylated by FUTIX, but there is no evidence what determines Lex synthesis on other potential ligands of DC-SIGN in granulocytes. Further efforts are required to determine whether FUTIX or other fucosyltransferases are involved in Lex formation on other ligands of DC-SIGN expressed on granulocytes.

5.4. Cell-specific glycosylation of CEACAM1 regulates binding to DC-SIGN

After FUTIX was identified as the major fucosyltransferase involved in the fucose transfer to CEACAM1, co-transfection studies in CHO and HEK293 cells were conducted with the intention to investigate the binding of DC-SIGN to recombinant CEACAM1.

Recombinant CEACAM1 expressed in CHO cells did not bind to Fc-DC-SIGN.

When CEACAM1 was co-expressed with FUTIX strong binding of Fc-DC-SIGN to CEACAM1 was observed. Treatment of CEACAM1 co-transfected with FUTIX by α(1-3,4) fucosidase completely abrogated the binding of Fc-DC-SIGN.

Strong binding of Fc-DC-SIGN was also detected when recombinant CEACAM1 was expressed in combination with FUTIX in HEK293 cells.

Surprisingly, CEACAM1 expressed in HEK293 in the absence of FUTIX showed also distinct binding to Fc-DC-SIGN. Lacking of Lex residues on CEACAM1 from HEK293 cells was confirmed applying a Lex specific antibody.

Since DC-SIGN also recognizes high-mannose, binding of GNA (*Galanthus nivalis* agglutinin), a high-mannose specific lectin, was tested.

Strong binding of GNA to recombinant CEACAM1 expressed in HEK293 cells in the presence or absence of FUTIX was detected. In contrast, no binding of GNA to CEACAM1 expressed in CHO cells was observed. These data support the assumption that high-mannose groups present on CEACAM1 from HEK293 cells mediate the binding to DC-SIGN.

Here we have demonstrated that the glycosylation of CEACAM1 is cell specific. N-linked carbohydrate content and composition determines the CEACAM1 binding efficiency to DC-SIGN.

Taken together, these results suggest that glycans expressed in in-vitro systems may display different binding properties and may not reflect the in vivo situation.

5.5. CEACAM1 specifically mediates the interaction to cellular DC-SIGN on dendritic cells via Lex residues

Monocyte-derived immature dendritic cells express DC-SIGN at high levels (Sallusto and Lanzavecchia 1994). On these cells, DC-SIGN is the major receptor that interacts with cellular Lewis x groups and Lewis x-expressing pathogens such as *Schistosoma mansonii* and *Helicobacter pylori* (Appelmelk, et al. 2003), (van Die, et al. 2003).

Since Lex residues on CEACAM1 mediate the binding to DC-SIGN, the adhesion of CEACAM1 and DC-SIGN was investigated at the cellular level. Binding studies with immature dendritic cells expressing DC-SIGN and CHO cells stably expressing CEACAM1 or CEACAM1 co-expressed with FUTIX were conducted. In comparison to the CHO-transfectomas, expressing only CEACAM1, strong

attachment of CHO-transfectomas co-expressing CEACAM1, and FUTIX (CEACAM1/FUTIX) was shown.

The attachment was significantly inhibited by the Lex mAb, by the polyclonal CEACAM antiserum and anti-DC-SIGN antibody, showing that immature DCs bind CEACAM1/FUTIX transfectomas through DC-SIGN-CEACAM1 interaction. Blocking with Lex-specific mAb indicates that Lex residues on CEACAM1 are involved in binding of CEACAM1/FUTIX transfectants to the adhesion receptor DC-SIGN on dendritic cells.

In addition, the cellular adhesion between freshly prepared granulocytes and immature DCs was tested. No significant blocking of the adhesion by DC-SIGN or CEACAM1 Abs was observed indicating that Lex residues of CEACAM1 may contribute to cell-cell attachment depending on the cell system investigated and the absence or presence of other adhesion molecules involved in cell adhesion.

Taken together, these data imply that Lex residues on CEACAM1 are involved in the cellular interaction with DC-SIGN expressed by immature DCs.

5.6. Lex residues of native CEACAM1 mediate the binding and internalization of CEACAM1 through DC-SIGN

To gain insight into the functional role of the interaction of Lex epitopes present on CEACAM1, native CEACAM1 isolated form human granulocytes by affinity chromatography was attached to fluorescent microbeads. Strong binding of native CEACAM1 to immature dendritic cells was observed. To prove that the Lex groups on granulocyte CEACAM1 mediate the binding to cellular DC-SIGN, inhibition studies were performed by preincubation of native CEACAM1-coated beads with α(1-3,4) fucosidase. After the Lex residues were destroyed by α(1-3,4) fucosidase, the binding of native CEACAM1 was significantly decreased. In addition, a blocking DC-SIGN antibody inhibited the binding of dendritic cells to CEACAM1 from granulocytes, confirming that CEACAM1 recognized specifically DC-SIGN on dendritic cells.

Native CEACAM1-coated beads were internalized by immature DCs and clustered near the cell nucleus. Internalized CEACAM1 coated beads were co-localized with lysosomal-associated membrane protein 1 (LAMP1) showing that CEACAM1 is targeted into lysosomal compartments of dendritic cells.
This finding suggests that CEACAM1 could also mediate the uptake of damaged granulocytes or cell debris in inflammatory situations such as bacterial infections**.**

5.7. CEA from colorectal carcinoma binds DC-SIGN via Lex residues

CEA (CEACAM5) is present on most normal epithelia and shows retained or enhanced expression after tumour development. It is highly expressed on almost all human colorectal and pancreatic adenocarcinomas. The glycosylation pattern of CEA changes upon malignant transformation (Garcia, et al. 1991).
CEACAM1 is also present on normal colon epithelia (Prall, et al. 1996) but down-regulated in colorectal cancer (Nollau, et al. 1997a).
Having identified native CEA purified from colon cancer metastases as a carrier of Lex residues (Lucka, et al. 2005), we have investigated the binding of CEA from lysates of normal colon mucosa and colorectal carcinoma to DC-SIGN. We hypothesized that CEA and DC-SIGN may be involved in the interaction of colorectal cancer cells with dendritic cells, since DC-SIGN displays high affinity for high-mannose and nonsialylated Lewis glycans.
Here, we shown that colorectal cancer cells express higher levels of Lex residues compared with normal colon mucosa. Strong expression of Lex residues was observed on CEA precipitated from lysates of colorectal carcinoma in contrast to CEA from normal colon mucosa. When the precipitates were submitted to overlay assays with Fc-DC-SIGN, strong binding of Fc-DC-SIGN to CEA from colorectal carcinoma was detected.
Further mass spectrometry analyses of CEA and CEACAM1 from carcinoma and normal colon epithelia, as well as additional binding studies are required to analyze the carbohydrates on these glycoproteins. While our studies were in progress,

similar results were published (van Gisbergen, et al. 2005a) that support our findings.

Our results suggest that the glycosylation-dependent interaction of CEACAM5 and DC-SIGN my enable a cross-talk between colorectal cancer cells and immature dendritic cells. The interaction between CEA and DC-SIGN may also play a role in tolerance induction to tumour cells.

5.8. CEACAM1 from first trimester human placenta binds DC-SIGN

CEACAM1 is expressed in normal human placenta. On the fetal side of the maternal-fetal interface, CEACAM1 is strongly expressed by the extravillous (intermediate) trophoblast at the implantation site. On the maternal side, CEACAM1 is present of the apical pole of endometrial epithelial cells of first trimester endometrium, and in small endometrial vessels, whereas endometrial stromal (decidua) cells are not expressing CEACAM1 (Bamberger, et al. 2000).

Human pregnancy can be regarded as an immunological paradox in that the maternal immune system accepts the allogeneic embryo without immune response. There is evidence that dendritic cell subsets are present in maternal and fetal side of early pregnancy placenta which may be involved in peripheral tolerance (Kammerer, et al. 2000). Recently, uterine immature DC populations expressing DC-SIGN that appear only in pregnancy-associated tissue was described (Soilleux, et al. 2001). So far, the function of DC-SIGN in human placenta is poorly understood.

Since, so far there is no evidence of glycostructures of CEACAM1 expressed in human first trimester placenta, we have assumed that CEACAM1 from human placenta may contain Lex residues. Indeed, Lex residues were detected on CEACAM1 precipitated from whole cellular extracts of first trimester placenta by Lex specific Ab. Performing binding assays with Fc-DC-SIGN, strong binding of DC-SIGN to precipitated CEACAM1was observed.

Further studies are required to analyze the CEACAM1-DC-SIGN interaction that might be functionally implicated in mediating immune tolerance in the human placenta.

Our results contribute to the structural and functional characterization of the N-glycostructure of CEACAM1 expressed in human placenta.

5.9. Lex residues were not detected on CEACAM1 from human malignant melanoma

CEACAM1 is expressed in human malignant melanoma tissue, often at the invading tumour front (Thies, et al. 2002), and the presence of CEACAM1 markedly enhances cell invasion and migration (Ebrahimnejad, et al. 2004).

We have assumed that CEACAM1 from malignant melanoma may also be decorated with Lex epitopes. However, we could not detect the presence of Lex epitopes on CEACAM1 precipitated form whole cellular lysates of human malignant melanoma.

In addition, the CEACAM1 from melanoma cell line G361 was tested for Lex expression but no binding of the Lex specific Ab was observed.

Thus, CEACAM1 on melanoma cells is differently glycosylated compared to CEACAM1 expressed on other cells. Our data contribute to the characterization of the glycostructure of CEACAM1 expressed in malignant melanoma.

5.10. Lex residues on ICAM-3 from human leukocytes bind to DC-SIGN

Another focus of our experimental studies was set on ICAM-3 from human leukocytes and its interaction with DC-SIGN.

ICAM-3 is a glycoprotein constitutively expressed on human leukocytes, such as monocytes, neutrophils, T and B lymphocytes and recognizes the $\alpha_L\beta_2$- leukocyte integrin, as its receptor.

DC-SIGN was described as an adhesion receptor on DCs that establishes cellular interactions with T cells through ICAM-3 (Geijtenbeek, et al. 2000c). It was postulated that this interaction is mediated through glycostructures that are present on ICAM-3. Several studies have shown that high-mannose structures present on ICAM-3 are responsible for DC-SIGN-ICAM-3 interaction (Geijtenbeek, et al. 2000c), (Jimenez, et al. 2005). Unfortunately, in all previous studies the investigation concerning the glycostructure involved in binding to DC-SIGN are based on recombinant ICAM-3 preparation generated from different transfected cell lines. We showed that the glycostructure of recombinant proteins in transfected cell culture may not reflect the in vivo situation. However, so far there are no clear data describing which glycan structures on native ICAM-3 from human leukocytes are responsible for binding to DC-SIGN. Therefore, native ICAM-3 from human peripheral leukocytes was purified. Here, we demonstrated that ICAM-3 derived from human leukocytes contains Lex residues, and that these residues meditate the interaction with DC-SIGN. The presence of Lex residues was detected by a Lex specific antibody, and the binding was completely abolished by preincubation of the ICAM-3 preparation with α(1-3,4) fucosidase. In addition, strong binding of DC-SIGN to native ICAM-3 was found. To proof that Lex epitopes present on ICAM-3 are recognized by DC-SIGN, the Lex residues were removed by α(1-3,4) fucosidase. After α(1-3,4) fucosidase treatment no binding of DC-SIGN was observed.

The presence of Lex residues in ICAM-3 was confirmed by MALDI-TOF analysis, combined with the enzymatic digestion of the N-glycostructure of ICAM-3 (Bogoevska, et al. 2007). In addition to Lex groups, high-mannose structures were also detected by mass spectrometry.

Since the binding of DC-SIGN to the ICAM-3 preparation was completely abrogated by α(1-3,4) fucosidase treatment, the assumption that high-mannose present on ICAM-3 may be involved in the binding with DC-SIGN is not supported. This suggests that high-mannose residues present of ICAM-3 may not have the required structure needed for DC-SIGN binding.

5.11. Lewis x residues on ICAM-3 mediate the binding to cellular DC-SIGN on DCs

DC-SIGN is present in clusters on immature dendritic cells (Cambi, et al. 2004) and forms tetramers within the cell membrane (Feinberg, et al. 2001), which may influence its function compared with recombinant DC-SIGN. Therefore, we explored the binding of ICAM-3 from human leukocytes to cellular DC-SIGN. Purified ICAM-3 was covalently attached to fluorescent microbeads and incubated with immature DCs. Strong binding of native ICAM-3 coupled beads to immature DCs was demonstrated. To investigate the involvement of Lex residues on ICAM-3, the beads were pretreated with α(1-3,4) fucosidase. After the treatment, the binding of ICAM-3 lacking Lex residues decreased significantly. In addition, binding inhibition was demonstrated by blocking of Lex residues by L5 mAb or by preincubation of DCs with anti DC-SIGN mAb.
Taken together, these data prove that Lex residues present on ICAM-3 mediate the interaction of ICAM-3 with cellular DC-SIGN.

5.12. DC-SIGN binds only to ICAM-3 from granulocytes

ICAM-3 is expressed in almost all populations of leukocytes.
In order to prove which of the populations of peripheral blood leukocytes contains ICAM-3 and bind the Lex-specific antibody and Fc-DC-SIGN, respectively, monocytes, granulocytes, T and B lymphocytes were isolated from peripheral blood cells. Immunoprecipitated ICAM-3 was detected in each isolated population by an ICAM-3 mAb. Western blot analyses showed that only ICAM-3 from human granulocytes contains Lex residues, and that these residues are recognised by Fc-DC-SIGN. Surprisingly, no binding of Lex specific Ab or recombinant Fc-DC-SIGN was detected to ICAM-3 from monocytes or T and B lymphocytes, respectively. The high mannose-specific lectin *Galanthus nivalis* agglutinin (GNA) did not bind to any of the ICAM-3 preparations.

Collectively, our findings indicate that only granulocyte ICAM-3 from human peripheral blood cells contains Lex residues recognized by DC-SIGN.

These results are in contrast to previous studies showing that ICAM-3 from human T lymphocytes binds DC-SIGN most probably via high mannose structure (Geijtenbeek, et al. 2000c). However, as pointed out above, this interpretation was based on recombinant ICAM-3 preparations generated in cell cultures. In these preparations, the glycostructure may differ from native preparations. It was proposed that ICAM-3 is a major ligand of DC-SIGN in resting T lymphocytes since the interaction of DCs with resting T cells could be inhibited with DC-SIGN antibodies. Most probably, other molecules on resting T cells are involved in the binding to DC-SIGN on dendritic cells.

Our results show that ICAM-3 from human granulocytes contains Lex residues, and that these residues mediate the interaction to DC-SIGN of dendritic cells.

5.13. Fucosyltransferase IX mediates the synthesis of Lex residues in recombinant ICAM-3, which interact with DC-SIGN

α(1,3) fucosyltransferases are involved in the formation of Lex residues. FUTIV and FUTIX are the most important α(1,3)-FUTs in the synthesis of the Lewis x structure. Since ICAM-3 from human granulocytes contains Lex residues we assumed that FUTIX may transfer fucose to ICAM-3. Therefore, ICAM-3 was expressed in HEK293 cells in combination with FUTIII, FUTIV, FUTVII and FUTIX. Strong binding of the Lex specific antibody was observed when ICAM-3 was co-expressed with FUTIX, and weak binding was present when ICAM-3 was co-expressed with FUTIV. Lex residues were not detected by co-expression of ICAM-3 with FUTIII and FUTVII, respectively.

Lex-carrying ICAM-3 was recognized by Fc-DC-SIGN. An additional Fc-DC-SIGN band of lower molecular weight in all HEK293 transfectomas was detected in western blots. This was the main form of expressed recombinant ICAM-3. Appling the high mannose specific GNA (*Galanthus nivalis* agglutinin) lectin the same band was recognized in each HEK293 transfectants.

We demonstrated that FUTIX is involved in the synthesis of Lex residues on ICAM-3, and that these residues interact with DC-SIGN, while recombinant ICAM-3 in the absence of Lex residues binds DC-SIGN via high-mannose residues.

5.14. Interactions between granulocytes and DC-SIGN mediate spontaneous apoptosis

During the acute phase of inflammation, polymorphonuclear granulocytes (PMN) play a crucial role in innate immunity by destroying the microorganisms such as bacteria, fungi and parasites. This role in immune defence requires granulocytes to arrive at the site of infection at early time points, and makes them ideally suited to control the recruitment of other cell types to the site of infection (Simon 2003). Through the release of MIP-1α and MIP-1β , neutrophils attract T-cells, monocytes, macrophages and immature DCs, while by release of IL-8 other neutrophils are attracted (Scapini, et al. 2000). Moreover, immature DCs also produce IL-8 early upon stimulation, thereby attracting neutrophils and contributing to co-localization of neutrophils and immature DC (Sallusto, et al. 1999). This ensures that both, granulocytes and DCs, are present simultaneously at the site of infection. However, so far, little is know about the crosstalk between granulocytes and immature dendritic cells.

Although activated neutrophils are able to induce DC maturation (van Gisbergen, et al. 2005c), their role in the regulation of DC-maturation is not clear, because pathogens are inducing DCs directly through interaction with TLRs (Gasperini, et al. 1995).

Here, we shown that formation of the cellular contact between granulocytes and DCs is regulated via CEACAM1-DC-SIGN and ICAM-3-DC-SIGN interactions. Currently, it is unknown whether these interactions induce signal transduction pathways in either granulocytes or DCs. The interaction between DC-SIGN and Mac-1 does not induce signals that result in DC maturation (van Gisbergen, et al. 2005c).

Since granulocytes arrive earlier at the side of infection then DCs and on the other hand, the granulocytes are short-lived cells (6-20 h in the circulation) the activity of which must be firmly controlled, we speculated that DC-granulocyte interactions might be involved in later events of inflammatory reactions such as granulocyte apoptosis. Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development.

We incubated freshly prepared granulocytes with soluble Fc-DC-SIGN, different antibodies as well as fMLP, which upregulates CEACAM1. The apoptosis rate was examined by FACS analysis with double-staining with annexin V and PI. Surprisingly, when granulocytes were incubated with Fc-DC-SIGN but not with antibodies or other compounds, significant increase in the spontaneous late stage of granulocyte apoptosis was observed.

These findings support our assumption that the interactions between granulocytes and DCs might be involved in the regulation of granulocyte apoptosis.

SUMMARY

In the present study, the structure and function of the glycans of native glycoproteins, CEACAM1 (CEA related cell adhesion molecule 1) and ICAM-3 (Intercellular adhesion molecule-3) was investigated.

CEACAM1, a member of the carcinoembryonic antigen family, is a highly glycosylated protein expressed on the surface of human granulocytes and lymphocytes, endothelia, and many epithelia. It is involved in the regulation of important biological processes, such as tumor growth, angiogenesis, and modulation of the immune response.

ICAM-3 is a transmembrane glycoprotein mainly expressed on leukocytes. Its function in the immune system is through the interaction with the $\alpha_L\beta_2$ leukocyte integrin. Defined glycostructures on cell surface proteins are important for mediating the recognition, activation and regulation of inflammatory and immunological responses. Characterizing the CEACAM1 and ICAM-3 glycan structure is of pivotal importance in revealing their biological function.

Here, we have demonstrated that native CEACAM1 from human granulocytes is a major carrier of Lewis x (Lex) epitopes.

Since Lex residues of pathogens bind to the C-type lectin dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN), expressed on human dendritic cells (DCs), we hypothesized that Lex glycans of CEACAM1 are recognized by DC-SIGN. Here, we provide evidence that Lex residues on CEACAM1 from human granulocytes are specifically recognized by DC-SIGN mediating the internalization of CEACAM1 into immature DCs.

In addition, we have demonstrated by immunological methods that CEACAM1 from whole cellular lysates of first trimester human placenta and CEA (CEACAM5) from whole cellular lysates of colorectal carcinomas are also decorated with Lex residues recognized by DC-SIGN. Notably, no expression of

Lex groups on CEACAM1 from whole cellular lysates of human malignant melanoma was observed.

DC-SIGN was proposed as additional ligand of ICAM-3 mediating the contact between T cells and dendritic cells. So far, however, the carbohydrate structure of native ICAM-3 involved in the interaction with DC-SIGN has not been identified.
Here, we provide conclusive evidence that ICAM-3 from human leukocytes contains Lewis x residues, and that these residues interact with DC-SIGN.
Investigations on different peripheral blood cell populations revealed that only ICAM-3 from granulocytes bound DC-SIGN.
α(1,3)-fucosyltransferases (FUTs) are responsible for the cellular synthesis of Lex residues. Fucosyltransferase IV (FUTIV) and fucosyltransferase IX (FUTIX) are the most important α(1,3)-FUTs accountable for the formation of the Lewis x structures. In order to explore which fucosyltransferase mediates the terminal fucosylation of CEACAM1 and ICAM-3, co-expression studies with different FUTs were performed. Our findings indicate that FUTIX is the main fucosyltransferase that mediates the synthesis of Lex residues on CEACAM1 and ICAM-3.
Taken together, the results indicate that CEACAM1 and ICAM-3 are involved in the interaction between granulocytes and dendritic cells. Furthermore, the granulocyte-DC-SIGN interactions may affect apoptosis in granulocytes.
In addition, our findings suggest that ICAM-3, CEACAM1 and other members of the CEA family are participating in immune regulation in physiological as well as in pathological conditions such as inflammation, autoimmune diseases and cancer.

ZUSAMMENFASSUNG

In der hier vorgestellten Studie haben wurde die Struktur und die Funktion der Glycane von nativen Glycoproteinen, CEACAM-1 (CEA related cell adhesion molecule 1) und ICAM-3 (Intercellular adhesion molecule-3) untersucht.

CEACAM1, ein Mitglied des Familie des carcinoembryonalen Antigens, ist ein stark glykolysiertes Protein, das auf der Oberfläche von humanen Granulocyten und Lymphocyten, Endothelzellen und vielen Epithelien exprimiert wird. Es ist an der Regulation von wichtigen biologischen Prozessen, wie Tumorwachstum, Angiogenese und der Modulation der Immun-Antwort beteiligt.
ICAM-3 ist ein transmembranöses Glycoprotein, welches vor allem auf Leukocyten exprimiert wird und dessen Funktion im Immunsystem über die Interaktion mit dem $\alpha_L\beta_2$ Leukocyten Integrin erfolgt. Glycostrukturen von Zellmembranproteinen sind an Erkennungs- und Aktivirunsmechanismen und an des Regulation der Immunantwort beteiligt.
Vorherige massenspektrometrische Analysen und die hier dargestellten Befunde belegen, dass natives CEACAM1, das aus Granulozyten isoliert wurde, Lex-Gruppen trägt. Da bekannt war, dass Lex-Reste von Pathogenen das dendritische Lektin DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) binden, wurde untersucht, ob CEACAM1 mit DC-SIGN interagiert. Unsere Untersuchungen belegen, dass rekombinantes DC-SIGN in der Tat CEACAM1 bindet. Mikropartikel, die mit nativem CEACAM1 beladen waren, wurden von dendritischen Zellen über membranständiges DC-SIGN gebunden und internalisiert. Da CEACAM1 auch in der Plazenta und in Melanomen vorkommt, könnte die Interaktion der Lex-Gruppen von CEACAM1 mit DC-SIGN dendritischer Zellen die Immunreaktion modulieren. Der Befund, dass Lex-Gruppen auf CEACAM1 aus humaner Plazenta DC-SIGN binden, unterstützt diese Hypothese. CEACAM1 aus Melanomen trug jedoch keine Lex-Gruppen. Es wurde weiterhin gezeigt, dass CEA aus kolorektalen Karzinomen über Lex-Gruppen mit DC-SIGN interagiert.

Die Bezeichnung ‚DC-SIGN' basiert auf dem Befund, dass rekombinantes ICAM-3 die Interaktion von T-Lymphozyten mit dendritischen Zellen hemmte. Da die Struktur der Glykostrukturen von ICAM-3, die mit DC-SIGN interagierten, nicht bekannt war, wurde eine native ICAM-3-Präparation aus humanen Leukozyten hinsichtlich der Bindung von DC-SIGN und der Struktur der Glykane untersucht. Die hier dargestellten Befunde belegen, dass, vergleichbar mit CEACAM1, Lex-Gruppen die Bindung von ICAM-3 an DC-SIGN vermitteln. Allerdings wurde DC-SIGN nur durch ICAM-3 aus Granulozyten, nicht jedoch aus Monozyten, B-Lymphozyten und insbesondere T-Lymphozyten gebunden. Der Befund, dass ICAM-3 die Interaktion von T-Lymphozyten mit dendritischen Zellen vermittelt, konnte nicht bestätigt werden.

Die Lewis x-Struktur ist durch einen Fucose-Rest gekennzeichnet, der in α(1,3)-glykosidischer Bindung mit einem terminalen GlucNAc-Rest verknüpft ist. Die terminale Fucose spielt somit in der Struktur der Lex-Gruppe eine entscheidende Rolle. In Ko-Expressionsversuchen wurde untersucht, welche Fucosyltransferase(n) für die Anheftung der terminalen Fucose verantwortlich ist. Sowohl für CEACAM1 als auch für ICAM-3 gilt, dass in erster Linie Fucosyltransferase IX (FUTIX) die Synthese von Lex-Gruppen vermittelt. Eine gewisse Aktivität wurde für FUTIV nachgewiesen, FUTIII und FUTVII waren hingegen nicht an der Synthese von Lex-Gruppen beteiligt.

Aus den hier dargestellten Ergebnisse kann geschlossen werden, dass Lex-Gruppen von CEACAM1 und ICAM-3 an der Interaktion von Granulozyten mit dendritischen Zellen beteiligt sind. Möglicherweise spielt diese Interaktion bei der Regulation der Apoptose von Granulozyten im Verlauf der Abwehrreaktion eine Rolle.

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Risk and Safety Phrases

The Risk and Safety Phrases for labeling dangerous chemicals were taken from the catalog and from the safety databank of the supplier (Merck).

Chemicals/hazard symbols	**Risk (R-) phrases**	**Safety (S-) phrases**
Acrylamide/Bisacrylamide solution **(T)**	45-46-E20/21-E25-36/38-43-E48/23/24/25-62	53-36/37-45
Ammonium persulfate **(Xn)**	8-22-36/37/38-42/43	-
β-Mercapthoethanol **(T, Xn)**	24-20/22-41-37/38	53-45-26-36/37/39
Dithiothreitol **(Xn)**	22-36/38	-
Dimethylformamide **(T)**	61-E20/21-36	53-45
Ethanol **(F)**	11	7-16
Ethidium Bromide **(T+)**	22-26-36/37/38-68	26-28. 2-36/37-45
Hydrogen Peroxide **(Xn)**	22-41	26-39
Isopropanol **(F, Xi)**	11-36-67	7-16-24/25-26
Methanol **(F,T)**	11-23/24/25-39/23/24/25	7-16-36/37-45
Paraformaldehyde **(Xn)**	20/22-36/37/38-40-43	22-26-36/37
Phenol **(T)**	25/25-34	28.6-45
Phenol/Chloroform/Isoamyl Alcohol (25:24:1v/v) **(T, Xn)**	23/24/25-34-40	26-27-36/37/39-45
Phenylmethylsulfonylfluorid (PMSF), **(T)**	23/24/25-34-40	26-36/37/39-45
Sodium-Orthovanadate **(Xn)**	20/21/22	22-36
Sodium Aside **(T+, N)**	28-32-50/53	28.1-45-60-61
Sodium Dodecyl Sulfate **(F, Xn)**	11-21/22-36/37/38	26-36/37
Xylene Cyanol **(Xi)**	36	24
Triton X-100 **(Xi)**	22-41	24-26-39

Publications:

Bogoevska, V., Horst, A., Klampe, B., Lucka, L., Wagener, C. and Nollau, P. (2006) CEACAM1, an adhesion molecule of human granulocytes, is fucosylated by fucosyltransferase IX and interacts with DC-SIGN of dendritic cells via Lewis x residues. Glycobiology, 16, 197-209.

Bogoevska, V., Nollau, P., Lucka, L., Grunow, D., Klampe, B., Uotila, L.M., Samsen, A., Gahmberg, C.G. and Wagener, C. (2007) DC-SIGN binds ICAM-3 isolated from peripheral human leukocytes through Lewis x residues. Glycobiology, 17, 324-333.

Posters:

V. Bogoevska, A. Horst, P. Nollau and C. Wagener. Fucosyltransferase IX mediates the synthesis of Lewis x (Lex)-groups on CEACAM1: Interaction of CEACAM1 and DC-SIGN on dendritic cells. Symposium of the Program Project Grant (SFB 470), University of Hamburg, Department of Chemistry, January 2006.

V. Bogoevska, A. Horst, L. Lucka, P. Nollau and C. Wagener. Structural and functional characterization of Lewis x (Lex) - residues on CEACAM1. International CEA-Workshop, Charité campus Benjamin Franklin, Berlin, Germany. July 2005

V. Bogoevska, A. Horst, P. Nollau and C. Wagener. Structural and functional characterization of Lewis x (Lex) residues on CEACAM1. Symposium of the Program Project Grant (SFB 470), University of Hamburg, Department of Chemistry, December 2004.

Curriculum vitae

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E r k l ä r u n g

Hiermit versichere ich ausdrücklich, daß ich die vorliegende Arbeit selbstständig verfaßt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Ich erkläre hiermit, daß ich diese Dissertation nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Hamburg, 2008 Valentina Bogoevska