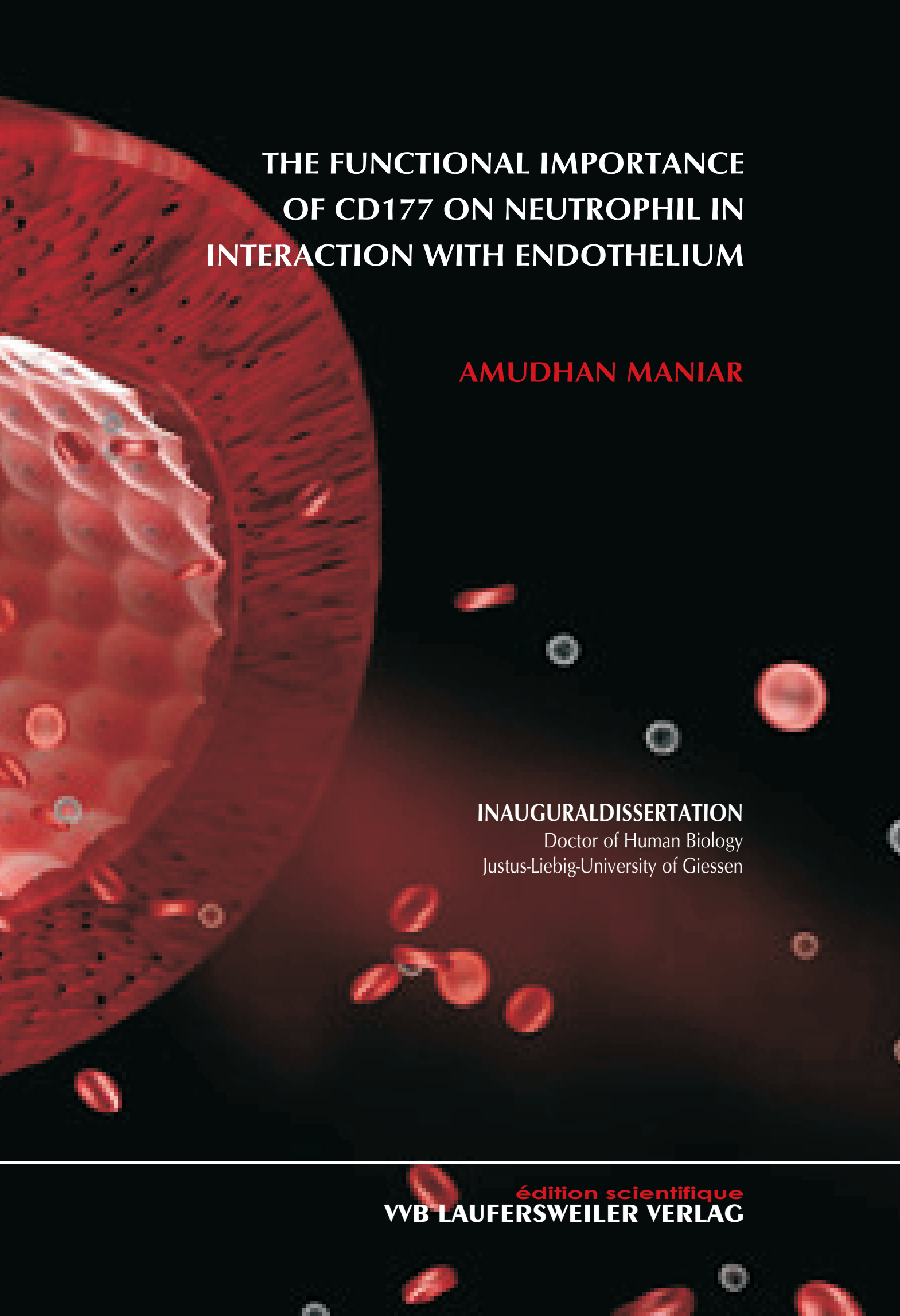

THE FUNCTIONAL IMPORTANCE OF CD177 ON NEUTROPHIL IN INTERACTION WITH ENDOTHELIUM

AMUDHAN MANIAR

INAUGURALDISSERTATION
Doctor of Human Biology
Justus-Liebig-University of Giessen

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1. Auflage 2007

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Inaugural Dissertation

Doctor of Human Biology

Justus-Liebig-University of Giessen

Amudhan Maniar

Vaniyambadi, India

Giessen, 2006

Institute for Clinical Immunology and Transfusion Medicine

Director: Prof. med. Gregor Bein

Medical School

Justus Liebig University of Giessen

Examiner 1: **Prof. Gregor Bein**

Examiner 2: **PD. Dr. Ralf Shermuly**

Date of disputation: **10.10.2007**

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M. AMUDHAN.

CONTENTS

ABSTRACT

Human neutrophil specific antigen CD177 plays a role in various clinical and immunological syndrome such as immune mediated neutropenia, transfusion related acute lung injury (TRALI) and polycythemia rubra vera (PRV). Recently, strong upregulation of CD177 was observed during bacterial infection and sepsis. Although the clinical importance of CD177 has been studied for nearly three decades, not much is known about the function of CD177 on neutrophils. Strong upregulation of CD177 noted during bacterial infection and sepsis.

To study the possible role of CD177 in the first step of the inflammation process, we sought to determine the counter receptor of CD177 on endothelium. For this purpose, we first produced CD177-Fc fusion protein in insect cells to capture endothelial CD177 counter receptor. Immunofluorescence analysis showed specific binding of CD177-Fc to the human umbilical vein endothelial cell (HUVEC). Immunoprecipitation of biotin surface labeled HUVEC with CD177-Fc protein yielded a specific band of 130 kDa. By use of a panel of monoclonal antibodies in immunoblotting experiment, we could identify this band as platelet endothelial cell adhesion molecule-1 (PECAM-1). This result could be confirmed by real-time protein-protein analysis using surface plasmon resonance technology. Purified PECAM-1 bound to CD177 with a Kd of $8.5*10^6$ in cation-dependent manner. Analysis of CD177 transfected cells on PECAM-1 protein showed a specific adhesion, which is inhibited by mab against CD177 (clone MEM166) as well as mabs specific for PECAM-1 (clones PECAM 1.1 and 1.2). Similar findings were observed when the adhesion of human neutrophil expressing CD177 on HUVEC was analyzed.

In summary, we identified endothelial PECAM-1 as the counter receptor of neutrophil CD177. This heterophilic binding opens the new concept of multistep interaction between neutrophil and endothelium, as an important process in the inflammation.

1. INTRODUCTION

CD177 is belongs to the Ly-6 gene super family, which is also known as the snake toxin family, Ly-6 genes were first described in mice encodes family of leukocyte surface glycoproteins expressed by a subpopulation of lymphoid and myeloid cells during haematopoiesis from multipotential stem cells to lineage committed precursor cells. Proteins encoded by the Ly-6 genes are divided in to two subfamilies as one subfamily encodes GPI anchored proteins and the second subfamily encodes secretory proteins. The Ly-6 family is characterized by the presence of 70-100 conserved amino acids and 8 to 10 cysteine rich domains. Human Ly-6 genes encodes the GPI anchored proteins with a one or three cysteine rich domains, urokinase plasminogen activator receptor (uPAR) and CD59, respectively, are described. uPAR is expressed by neutrophils, monocytes and their precursors and serves as a high affinity receptor for urokinase plasminogen activator (uPA). uPAR also plays a role in cell migration, adhesion and cellular invasion. CD59 or membrane inhibitor of reactive lysis is expressed by erythrocytes as well on leukocytes is an important erythrocyte membrane molecule that inhibits complement mediated lyses and protects cells from membrane attack complex induced lysis.

Recently, a new member of the Ly-6 gene family, human CD177, has been described as a gene located on chromosome 19q13.2, comprised of 9 exons and an open reading frame of 1311 bp which encodes 437 amino acids with an N-terminal signal peptide of 21 amino acids. CD177 consists of two highly homologous cysteine rich domains of 188 amino acids each containing 6 cysteine residues, three potential N-glycosylation sites, and a hydrophobic C-terminus with a GPI (Glycosyl Phosphatidyl Inositol) anchor. CD177 of 58-64 kDa are expressed by neutrophils and their precursors. The CD177 expression is unusual as only expressed on subpopulations of neutrophils with the mean size of 45-65%. The reason for this phenomenon is unclear. CD177 is polymorphic

and comprises of two alleles PRV-1 (polycytemia rubra vera-1) and NB1 (neutrophil antigen B-1 or termed as Human Neutrophil Antigen-2).

In the last decade, studies in our group and in others were focused on the structure of CD177, polymorphisms, antigen expression and their relevance in immune mediated neutropenia and bacterial infections. However, little is known about the function of CD177. Preliminary studies indicated that CD177 on neutrophils may interact with endothelial cells. In this study, we characterized the functional role of CD177 in respect to neutrophil-endothelial cell-cell interaction (see Figure 1). The primary objective of this study is to identify CD177 ligand on endothelium to gain a hint about the cellular function of CD177.

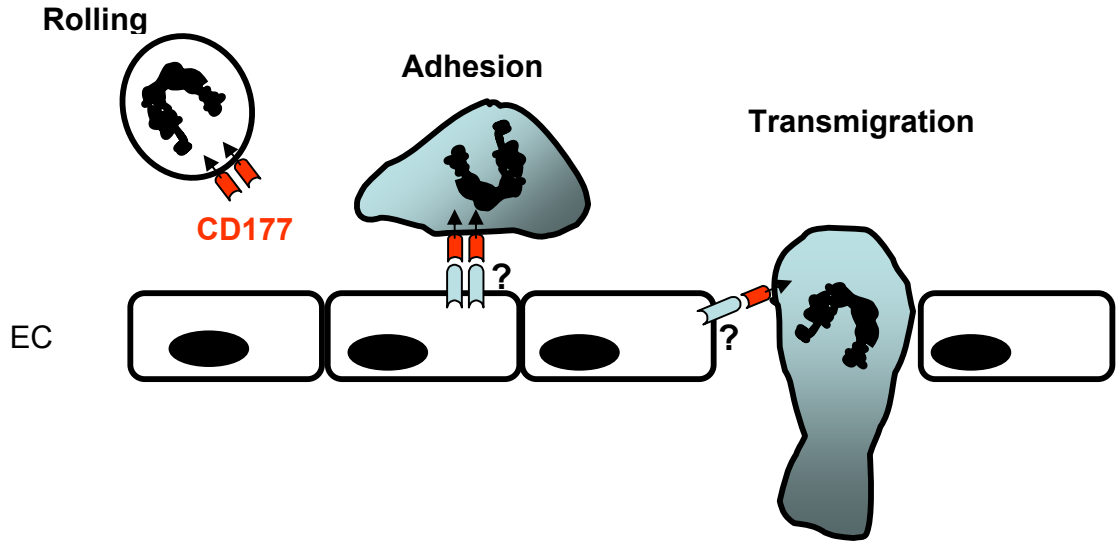

Figure 1: Hypothesis of the functional role of CD177 on the adhesion and transmigration process of neutrophils through endothelial cells (EC). Rolling,adhesion and transmigration process of neutrophils described schematically. The putative CD177 counter-receptor is indicated by question mark (?).

1.1. REVIEW OF LITERATURE

1.1.1. Neutrophils

Neutrophils are white blood cells also known as polymorpho nuclear leukocytes represent 50 to 60% of the total circulating leukocytes which functions as the first line of defense against infectious agents or non self substances that penetrate the body's physical barriers such as bacteria, fungi, protozoa, viruses, infected cells and tumors. Neutrophils develop in bone marrow, where they undergo proliferation and differentiation which takes about two weeks. Neutrophils and macrophages eliminate pathogens via opsonization and kill them by proteolytic enzymes and reactive oxygen species (Kobayashi et al 2005; Boos et al 2006). Under normal conditions neutrophils roll along vascular walls, but during inflammatory conditions neutrophils can adhere, penetrate the endothelial monolayer and migrate through connective tissues to sites of infection along the chemotactic gradient.

Neutrophil extravasation is a multistep process comprising of rolling, adhesion and transendothelial migration (Figure 2). Rolling is mediated by E- and P-selectins (endothelial and platelet selectins) on endothelial cells, which bind to selectin ligand (PSGL) on neutrophils. This process is then followed by adhesion of neutrophils, which involves the additional binding of integrins on neutrophils (α_2-integrins) to the Ig-like molecules on endothelial cells (ICAMs). Finally, bound neutrophils migrate between the endothelial cells to leave the blood vessels and enter the tissue. Neutrophils are then attracted to the injured site by the chemokines.

Specific receptors and counter receptors interactions are involved in the process of inflammation (Figure 2). The first step, rolling is mediated by selectins on endothelial cell and its counter receptor P-selectin glycoprotein ligand (PSGL). This process is then followed by firm adhesion of neutrophils on endothelium by interaction between integrins on neutrophils (VLA-4, LFA-1 and Mac-1) to their counter receptors on endothelium (e.g VCAM-1, ICAM-1) (Davenpeck et al 2000;

Albelda et al, 1994; Bevilacqua, 1993). The next crucial step is transendothelial migration of neutrophils is not well understood. One of the most important molecule is PECAM-1, which is expressed on both neutrophils and endothelial cells (Albelda et al, 1991; Newman et al, 1992). In addition, another cell adhesion molecule, CD99 plays a role in neutrophil transendothelial migration (Allport et al, 1997; Cooper et al, 1995).

Recently, our group demonstrated that interaction between $\alpha M\beta_2$ integrin (Mac-1) on neutrophils and junctional adhesion molecule-C (JAM-C) is important for the first step of neutrophil migration through endothelial cells (Santoso et al, 2002; Chavakis et al, 2004).

Recently, a member of the JAM family is known to interact in both homophilic and heterophilic manner (Keiper et al, 2005). Therefore, it is obvious to presume that endothelial PECAM-1 might also serves as a heterophilic ligand for neutrophil receptor(s). At the last stage of neutrophil transmigration through tight junctions, PECAM-1 dependent transmigration may then follow by VE-cadherin assisted diapedesis.

Several sources of evidence demonstrated that PECAM-1 on endothelial cells play an important role in transendothelial migration of monocytes. However, little is known about its role in neutrophil transmigration (Wakelin et al, 1996; Albelda et al, 1994).

In the next chapter 1.1.6 the role of PECAM-1 in neutrophil transmigration will be discussed in detail.

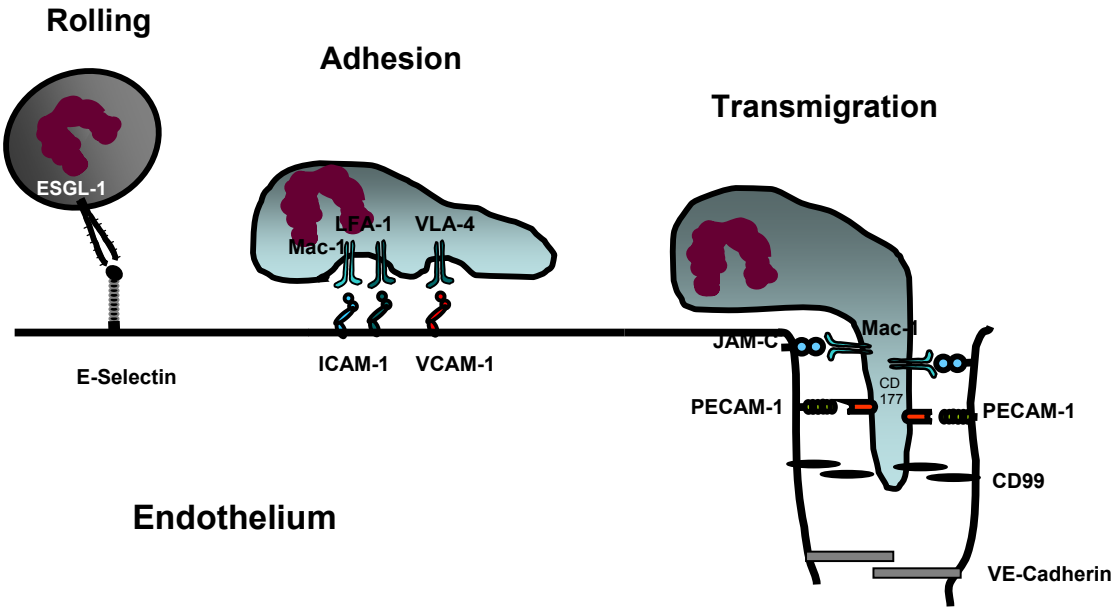

Figure 2: Overview of receptors and counter receptors involved in neutrophil extravasation. ESGL-1-E-selectin glycoprotein ligand-1, ICAM-1: Inter cellular adhesion molecule-1, VCAM-1: Vascular endothelial cell adhesion molecule-1, Mac-1:CD11b/CD18; alpha (M) Beta (2), LFA-1: CD11a/CD18; Lymphocyte function associated antigen-1, VLA-4: CD49d/CD29; very late antigen-4, JAM-C: Junctional adhesion molecule-C, CD99: Glycoprotein expressed on leukocytes. CD177: Human neutrophil antigen 2, PECAM-1; platelet endothelial cell adhesion molecule-1.

1.1.2. CD177

Cellular distribution

CD177 is only found on neutrophils but not on other blood cells (Goldschmeding et al, 1991). The expression of CD177 on the neutrophil surface is unique. In 97% of our population, CD177 are detectable on the cell's surface, but the expression, however, is heterogeneous. In one individual, CD177 negative as well as CD177 positive neutrophil populations could be observed. The reason for this heterogeneity is not yet known. Single nucleotide polymorphisms of CD177 seem to be associated with an increase in the size of the neutrophil population. Our group and others have found that the G42C

dimorphism of CD177 was associated with the size of the neutrophil population expressing CD177 (Wolff et al, 2003; Carruccio et al, 2004).

Recent data from *in vivo* studies showed that stimulation of neutrophils with granulocyte-colony stimulating factor (G-CSF) led to the upregulation of CD177 transcripts (Stroncek et al, 1998). Up regulation in the CD177 gene expression was also observed in granulocyte progenitors upon inactivation by estrogen-dependent form of E2a-Pbx1 oncoprotein (Sykes et al, 2003). Recent analysis on neutrophils collected from women both early and late in pregnancy showed that the expression of CD177 antigen increases during the pregnancy (Carrucio et al, 2003). All these observations indicated that besides gene polymorphisms the variability in neutrophil expression of CD177 among individuals may also cause by differences in gene regulation or in post-translational protein modification.

In rare cases, individuals lacking CD177 were described approximately as 3% of Caucasians, 5% of African American and 11% of Japanese having CD177 deficient neutrophils (Matsuo et al, 2000; Stroncek et al, 2004). One of the causes of this deficiency is mRNA splicing defect (Kiessel et al, 2002). Elevated levels of CD177 transcript in neutrophils were recently found in patients with bacterial infections, but not in patients with viral hepatitis (Gohring et al, 2004)

Molecular nature of CD177

CD177 is a GPI linked membrane protein with a molecular mass of 54-68 KDa. CD177 glycoprotein consists of 437 amino acids with a signal peptide of 21 residues, two cysteine rich domains (residues 133-210 ; 325-399), three potential N-linked glycosylation sites and short transmembrane and cytoplasmic segments including a GPI attachment site (Kiessel et al, 2001).

CD177 is polymorphic in human and comprises of two alleles, NB1 (neutrophil antigen B-1 or termed as Human Neutrophil Antigen-2) and PRV-1 (polycytemia rubra vera-1). The most common allele of CD177 is PRV-1 (Termeniac et al, 2000). The NB1 allele differ only 4 nucleotides (G42C, C390T, G1003A and

T1171C) that result in four amino acid exchanges (Ala3Pro, Leu119Phe, Arg323Gln and Phe379Ser) (Kiessel et al, 2002; Bettinoti et al, 2002). In addition, immediately adjacent to CD177 is a pseudogene that is highly homologous to exons 4 through 9 of CD177 in tail to head orientation (Bettinoti et al, 2002).

Alloimmunization against neutrophil specific antigen, NB1, has been described three decades ago by Lalezari et al (1971) in a case of alloimmune mediated neutropenia. For the systematic naming of neutrophil antigens a new nomenclature for human neutrophil alloantigens (HNA) was introduced. This nomenclature is based on the antigen glycoprotein location (Table 1). To date, five different glycoproteins are known as carriers of antigenic determinants on neutrophils. The first alloantigen comprises three polymorphic isoforms, HNA-1a, HNA-lb and HNA-1c, are localized on FcγIIIb (CD16b). NB1 was renamed as HNA-2a. The HNA-3a, -4a and -5a are located on a 70-95 kDa glycoprotein, CD11b and CD11a integrin, respectively (Bux et al, 2002)

ANTIGEN SYSTEM	ANTIGEN	LOCATION	FORMER NAME	ALLELES
HNA-1	HNA1a HNA-1b HNA-1c	FcγRIIIb	NA1 NA2 SH	FCGR3B*1 FCGR3B*2 FCGR3B*3
HNA-2	HNA-2a	CD177	CD177	CD177*1
HNA-3	HNA-3a	70-95 kDa	5b	Not defined.
HNA-4	HNA-4a	CD11b	Mart	CD11b*1
HNA-5	HNA-5a	CD11a	Ond	CD11a*1

Table 1: Nomenclature of human neutrophil antigens (HNAs) adapted from Stroncek et al, 2004. Illustration of antigen location and alleles depicting glycoprotein polymorphism.

1.1.3. Clinical aspects of CD177

The human neutrophil antigen CD177 plays an important role in several immune-mediated disorders such as autoimmune neutropenia, alloimmune neutropenia,

febrile transfusion reactions, and also transfusion-associated lung injury, TRALI (Stroncek et al, 2002). In the pathogenesis of TRALI, neutrophil-specific antibodies as well as HLA antibodies in the plasma of both blood donors and receipients have been implicated. The final pathway of the pathogenic mechanism of TRALI is increased pulmonary capillary permeability, which results in movement of plasma into the alveolar space causing pulmonary edema. Currently, TRALI is the leading cause of transfusion-associated mortality, (Roberts et al, 2004).
Several approaches were discussed today to prevent TRALI, including leukocyte reduction, shortening of red cells, platelets storage times, use of detergent treated plasma as well as donor selection in respect to neutrophil alloantibodies (Sachs et al, 2006).

Several studies have demonstrated an over expression of neutrophil CD177 transcript in patients with Polycythemia Rubra Vera (PRV), and two other myeloproliferative disorders, essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF) (Stroncek et al, 2004). Myeloproliferative disorders are heterogeneous diseases that occur at the level of a multipotent hematopoietic stem cell. They are characterized by increased blood cell production related to cytokine hypersensitivity and virtually normal cell maturation. Recently, a unique mutation in JAK2 (V617F) was found in patients with PRV, ET and IMF. This mutation is located in the JH2 domain (pseudokinase autoinhibitory domain) resulting in constitutive kinase activity and induces cytokine hypersensitivity or independence of factor-dependent cell lines (Vainchenker & Constantinescu, 2005).

1.1.4. Function of CD177

One study suggests that CD177 may play a role in the adhesion of neutrophils to endothelial cells (Stroncek et al, 1994). Analysis of neutrophil endothelial interaction showed that the adhesion of resting CD177-positive neutrophils is lower in comparison to CD177-negative cells. When the neutrophils were

stimulated with C5a, PMA, or fMLP, no differences were found in the adhesion of CD177-positive and negative cells. Furthermore, no differences were found in chemotaxis to C5a, respiratory burst response to PMA, or opsonized zymogen. These observations indicated that CD177 may inhibit the adhesion of unstimulated neutrophils to endothelial cells. People with CD177 deficient neutrophils are healthy indicating that the function of CD177 may be duplicated by another protein. However, only few CD177 deficient individuals have been studied to determine if the lack of CD177 has a subtle effect on neutrophil counts, neutrophil function, host defense, or host response to inflammation.

Recently, Mnjoyan et al (2005) described that CD177 transfected cells grew faster than non transfected cells. Even after 5 days of exposure to serum-free media, cells expressing CD177 continue to proliferate, whereas the control cells ceased.

1.1.5. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1; CD31).

PECAM-1 is a member of the immunoglobulin gene super family (IgSF) and has an important role in a number of endothelial cell functions including inflammation, integrin activation, cell-cell adhesion, angiogenesis and wound healing. PECAM1 is a 130 kDa glycoprotein that is expressed on the surface of circulating platelets, monocytes, neutrophils, T cell subsets and is a major constituent of endothelial cells (>10^6 molecules/cell), but PECAM-1 is not present on fibroblasts, epithelium, muscle or other nonvascular cells (for review see Newman & Newman, 2006).

PECAM-1 is encoded by a 75-kb gene that resides at the end of the long arm of chromosome 17 and is comprised of 16 exons. The 574 amino acids that comprise the extra cellular portion of this molecule are organized into six Ig-like homology domains encoded by exons 2-8, a short transmembrane domain and a long cytoplasmic domain containing 118-amino acids. The cytoplasmic domain contains at least five serine and tyrosine phophorylation sites. Two consensus Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) centered on tyrosine

residues 663 and 686 were found. These ITIMs serve to recruit cytosolic signaling molecules (in particular SHP-2) during a variety of cellular activation events. The extracellular domain of PECAM-1 mediates PECAM-1/PECAM-1 homophilic interactions between cells and is responsible for capturing PECAM-1 at cell-cell junctions. These properties are important in PECAM-1 mediated leukocyte transendothelial migration and in maintaining endothelial cell junctional integrity. Analysis with monoclonal antibodies against PECAM-1 showed that the N-terminal domain (first and second Ig-like domains) is important for the homophilic PECAM-1/PECAM-1 binding (Yan et al, 1995).

In addition to homophilic interactions, several studies have suggested that PECAM-1 may also capable of interacting heterophilically with other components of the cell surface which is dependent upon the presence of divalent cations (Albelda et al, JCB; 1991; Muller et al, J Exp Med, 1992). The binding regions of mabs that blocked PECAM-1 mediated heterophilic interaction were located in either in Ig-like domain 2 or Ig-like domain 6. Further analysis by site-directed mutagenesis pinpointed that a hexapeptide, CAVNEG, within the Ig-like domain 6 is important for the heterophilic PECAM-1 interaction (Yan et al, 1995). Two ligands, cell surface glycosaminoglycans and αvβ3 integrin, were reported as potential counter receptor for heterophilic PECAM-1 interaction (DeLisser et al, 1993; Piali et al, 1995; Buckley et al, 1996). However, recent studies argue whether this interaction of physiological importance (Abelda et al, 1991; Sun et al, 1996).

1.1.6. Role of PECAM-1 on endothelium

Early study from Muller et al (1993) described the important role of endothelial cell PECAM-1 in mediating transendothelial migration of leukocytes. This process involves homophilic interaction between the first extracellular Ig-like domain of leukocyte and endothelial cell PECAM-1 (Liao et al, 1997). Futhermore, PECAM-1 deficient mice exhibit a marked defect in neutrophil and monocyte migration in response to inflammatory stimuli (Schenkel et al, 2004). The reason for this is not clear yet. Recent study suggests that the primary function of endothelial PECAM-

1 in the transmigration process is to serve as a passive ligand for leukocyte PECAM-1. Homophilic interaction between PECAM-1 on leukocyte and PECAM-1 on endothelial cell sends activation signal into the leukocyte that upregulate integrin function and facilitate the latter stages of transendothelial migration (Dangerfield et al, 2002). Treatment with anti-PECAM-1 monoclonal antibodies inhibits leukocyte transendothelial migration by blocking their transit through the basement membrane (collagen- and laminin-rich barrier). This transit requires activated leukocyte integrins (Liao et al, 1995; Thompson et al, 2000). In addition, studies with PECAM-1 deficient mice showed that leukocytes from these mice became transiently delayed while transversing the perivascular basement membrane indicating the involvement of PECAM-1 in modulation of integrin function (Duncan et al, 1999; Thompson et al, 2001).

1.1.7. Role of PECAM-1 on neutrophils

Although neutrophils carry PECAM-1 on the cell surface, little is known about its function. During inflammation, neutrophils migrate from the vascular lumen into the extravascular sites. Both, *in vitro* and *in vivo* experiments have suggested that PECAM-1 is required for the transmigration of neutrophils across endothelial monolayers (Vaporciyan et al, 1993; Bogen et al 1994; Muller et al, 1995). Using an *in vitro* model of leukocyte transendothelial migration, Muller et al (1995) demonstrated that the treatment of either leukocytes or the endothelial monolayer with soluble PECAM-1 or mabs against PECAM-1 blocked leukocyte transmigration without affecting adhesion. These results could be confirmed by *in vivo* experiments in a murine model of peritonitis and in murine model for studying leukocyte-endothelial interactions involving the human vasculature (Christofidou-Solomidou et al, 1997). In addition, the authors demonstrated that extravasated neutrophils displayed decreased surface expression of PECAM-1, possibly as result of engagement of its ligand.

Berman and coworkers (1995, 1996) observed that the ligation of PECAM-1 on monocytes and neutrophils increases the binding capacity of integrin CD11b/CD18 (Mac-1). This integrin activation is critical for the transmigration of neutrophils at sites of inflammation. Homophilic PECAM-1 interaction regulates integrin mediated adhesion (Buckley et al 1998, Tanaka et al 1992) as well as other heterophilic cell adhesion by enhancing the expression of heterophilic receptors as in the case of $\alpha v\beta_3$ (Dangerfield et al 2002).
Recent data showed that endothelial PECAM-1 functions as a passive homophilic ligand for neutrophilic PECAM-1, which after engagement leads to neutrophil signal transduction, integrin activation, and transmigration in a stimulus-specific manner. Whereas IL-1β elicit PECAM-1 dependent signalling, IL-8 mediated transmigration is PECAM-1 independent (O'Brien et al, 2003).
Recently, Dangerfield et al (2002, 2005) reported the role of integrin $\alpha_6\beta_1$ upregulation in neutrophil migration through the perivascular membrane, which appears to be associated with PECAM-1 engagement. In *in vivo* murine model, blockade of $\alpha_6\beta_1$ inhibits neutrophil migration induces by IL-1β, but not TNFα.

Study in PECAM-1 deficient mice showed that PECAM-/- neutrophils are much less polarized and spreading in response to IL-8 gradient, indicating that PECAM-1 on neutrophils has a major role in modulating neutrophil-directed migration (Wu et al 2005). Comparison of eosinophils, neutrophils and monocyte transendothelial migration *in vivo* showed that neutrophil and monocyte tissue recruitment is PECAM-1 dependent, whereas eosinophils tissue recruitment seems to be PECAM-1 independent (Miller et al, 2001).

Furthermore, antibody mediated ligation of PECAM-1 enhances the cell adhesion of neutrophils onto human dermal microvascular endothelial, which supports the fact that PECAM-1 may activate other adhesive protein(s) or complex cascade *in cis* manner. Furthermore, antibody engagement of PECAM-1 on suspended neutrophils triggered a prompt intracellular Ca^{++} signal leads to changes in the neutrophil cytoskeleton activity (Kuwabara et al 1992, Elias et al 1998).

2. MATERIALS AND METHODS

All chemical and reagents are purchased in Germany unless otherwise mentioned.

2.1. Materials

Antibiotics and drugs

DEPC (Di Ethyl Pyro Carbonate)	Sigma
Gentamycin	PAN Biotech
Penicillin / Streptomycin	PAN Biotech
Protease Inhibitor-Cocktail	Roche
Trypsin/EDTA	PAN Biotech

Chemicals and reagents

Ammonium persulfate	Sigma
Acrylamide/bisacrylamide	Roth
Agarose ultra pure	Roth
Ammonium persulfate (APS)	Serva
Ampicillin	Roche
Boric acid	Sigma
Calcium chloride	Sigma
Coomassie-brilliant-blue R 250	Sigma
Beta-mercaptoethanol	Sigma
Bromophenol blue	Merck
Bovine Serum Albumin (BSA)	Serva
Chloroform	Roth
Ethanol	Riedel-de-Haen
Dimethylsulfoxid (DMSO)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma-Aldrich
Dextran	Amersham Pharmacia
Disodium Hydrogen phosphate (Na_2HPO_4)	Merck
Ethanol (100%)	Roth

Ethylenediamine tetraacetic acid (EDTA)	Merck
Fetal bovine serum	Bio west
Formaldehyde	Sigma
Ficoll	Amersham Pharmacia
Glycerol	Sigma-Aldrich
Glacial acetic acid	Sigma
Hydrogen peroxide	Merck
Isopropanol	Merck
L-Glutamine	PAN biotech
Methanol	Roth
NHS-LC Biotin	Amersham Pharmacia
Paraformaldehyde	Sigma
Penicillin/Streptomycin	Merck
Ponceau-S red	Serva
Protein-A beads	Amersham Bios, Sweden
Protein-G beads	Amersham Bios, Sweden
Rotiphorese Gel 30	Roth
Sodium acetate	Merck
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Sodium pyrophosphate	Aldrich
Sodium ethylenediamine tetraacetic acid	Sigma-Aldrich
Sodium sulphide	Merck
Sodium dihydrogen phosphate	Merck
Sodium chloride	Riedel-de Haen
Trypsin	Sigma
Tetra-methylethylenediamine (TEMED)	Serva
Tris base	Sigma-Aldrich
Tris (hydroxymethyl)aminomethane	Sigma
Triton X-100	Merck
Tween 20	Merck

Markers

DNA ladder	Cell Signaling, USA
Rainbow ^{TM} protein molecular weight	Amersham Biosciences

Kits

BCA Protein Assay kit	Pierce, U.S.A
ECL western blotting kit	Amersham Biosciences
Plasmid mini preparation kit	Amersham Bioscience

Materials for cell culture

Alpha-MEM	Gibco, USA
Dulbecco's Modified Eagle's medium (DMEM)	Gibco, USA
Endothelial cell basal medium	PromoCell
Fetal bovine serum (FBS)	Biochrom KG
Ficoll-Paque	Amersham
Gentamycin	PromoCell
Glutamine	Promo Cell
RPMI 1640	Gibco, USA
Sodium-pyruvate	Gibco, USA
TNF-α	Roche
Trypsin-EDTA	Gibco, USA
Tissue culture dish	Falcon
Tissue culture flask	Falcon
Tissue culture 6-well plate	Greiner

Antibodies

Primary antibodies

Mouse Immunoglobulin (mIgG)	Becton Dickinson
MEM-166	Serotec
7D8 (anti-NB1)	a gift from Dr. D. Stroncek
PECAM 1.1 (anti PECAM-1)	a gift from Dr. PJ Newman

PECAM 1.2 (anti-PECAM-1)	a gift from Dr. PJ Newman
Gi18 (anti-PECAM-1)	our group
Anti-E-selectin	Becton Dickinson
Anti-P-selectin	Becton Dickinson
Secondary antibodies	
Rabbit anti-mouse horse radish peroxidase	DPC Biermann
Donkey anti-human horse radish peroxidase	DPC Biermann
Rabit anti mouse conjugated Fluorescein	DPC Biermann

2.2. Other materials

BioTrace NT	PALL Life sciences
Cuvette	Eppendorf
Filter, sterile 0.45 µm and 0.2 µm	Gelman Science
Gel blotting paper	Schleicher & Schuell
Hyperfilm ECL	Amersham Biosciences
Polyvinylidene fluoride membranes (PVDF)	Millipore, USA
PVDF membrane	PALL
Tubes, 15ml type 2095	Falcon
Tubes, 50 ml type 2070	Falcon
X-Ray Flim	AGFA
X-ray films X-Omat AR	Kodak, USA
Whatman paper (3mm)	Schleicher & Schuell

Instruments

Blotting-semidry Whattman	Biometra
Blotting chamber	Fa. Keutz
BioDocAnalyzer	Biometra
BioPhotometer	Eppendorf
Electrophoresis apparatus	Biorad

Centrifuge: RC5C	Sorvall
FACS analyser	Becon Dikinson
Incubator CO_2	Heraeus
Spectrophotometer	Beckmann
pH-meter 766	Knick
Thermoblock	HLC
Incubator	Eppendorf
Incubator Lab-Thermo	Kühner
Microscope: light microscope, Type D	Leica Microsystems
Light Microscope DC 300f	Hund
ND-1000 UV-Vis Spectrophotometer	Nano Technologies

2.3. Medium

RPMI medium PAA

Supplemented with:

10% FCS, 0.5% Penicillin/Streptomycin

Optimum medium PAA

Supplemented with:

5% FCS, 0.5% Penicillin/Streptomycin

Alpha-MEM for CHO cells PAA

Supplemented with:

10% FCS

0.5% Penicillin/Streptomycin

200 µl/ml of Geniticin

Express five SFM medium Invitrogen

For 200 ml medium

18 ml of L-Glutamine

200 µl of Gentamycin

500 µl of Blasticidin (1 mg/ml)

Medium for endothelial cells PAA

For 200 ml of MCDB131 medium

22.7 ml FCS

2.3 ml Penicillin/Streptomycin

2.3 ml L-Glutamine

909 µl ECGF

45.5 µl EGF

2.4. Cell lines

Chinese hamster ovarian cells expressing CD177

Chinese hamster ovarian cells expressing K1

Monocytic cell line U937 expressing CD177

Monocytic cell line U937

High Five Tricoplusia ni insect derived cells

2.5. Buffers for Protein Chemistry

Immunoprecipitation buffer

50mM Tris, 150mM NaCl, 1% Triton X-100

SDS buffer (2X)

4% SDS, 15% Glycerol, 62.5mM Tris-HCl pH 6.8, 0.005% Bromophenol blue.

Stripping Buffer

62.5mM Tris, 2% SDS, 100mM β-mercaptoethanol

TBS (10x)

100mM Tris-base, 150mM NaCl adjust the pH to 7.6.

PBS (10X)

10mM KCl, 10mM NaCl, 16 mM Na_2HPO_4, 32mM KH_2PO_4

adjust pH to 7.4.

Protein lysis Buffer

50mM Tris, 150mM NaCl, 1% Triton X-100, 2mM PMSF

2.6. SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

1.5 M Tris pH 8.8 and pH 6.8

1.5M Tris and 3M Tris-base adjust the pH to 8.8 or 6.8 with HCl accordingly

10 x SDS running buffer

0.1% SDS, 3mM Tris-base, 400mM glycine

7.5% resolving gel (mini gel)

2.3 ml of H_2O, 1.3 ml of 30% acrylamide, 1.3 ml of 1.5M Tris pH 8.8, 50 µl 10% SDS, 50 µl 10% APS, 3 µl TEMED

5% stacking gel (mini gel)

1.4 ml H_2O, 0.33 ml acrylamide, 0.25 ml of 1.5M Tris pH 6.8, 0.02 ml of 10% SDS, 0.02 ml of 10% APS and 2 µl TEMED

10x TBS

100mM Tris, 150mM NaCl adjust the pH to 7.6

10x SDS-PAGE-running buffer

0.25M Tris, 1.9M glycine, 1% SDS

8x Resolving gel buffer

3M Tris base, 0.8% SDS and adjust the pH to 8.8

4x Stacking gel buffer

0.5M Tris base, 0.4% SDS adjust pH to 6.8

Semi dry transfer buffer

50mM Tris-base, 25mM Glycine, 0.37% SDS, 20 % methanol

Blocking buffer

1.5% BSA in PBS

10xTE buffer

10mM Tris/HCl, 0.25M EDTA adjust the pH to 7.5.

10xPBS

10mM KCl, 10mM NaCl, 16mM Na_2HPO_4, 32mM KH_2PO_4 adjust the pH to 7.4

HBS^{++} buffer

20ml of HBS (11x) stock solution

15mM $CaCl_2$, 15mM $MgCl_2$

2.7. Buffers and solutions used for cell adhesion assay

11xHBS buffer

1.3M NaCl, 44mM KCl, 121mM glucose, 220mM HEPES adjusted the pH to 7.4

Adhesion buffer

150mM NaCl, 20mM HEPES, 2mM $MgCl_2$

Adhesion buffer with $MnCl_2$ and PMA

50mM $MnCl_2$, 50ng/ml PMA

Dye Solution

0.5% Crystal violet, 20% Methanol

2.8. Experimental methods

2.8.1. CD177Fc construct

The truncated CD177 cDNA fragment encompassing two cysteine-rich domains of soluble CD177 fused to human Fc in signal pIg plus expression vector was amplified by PCR with forward plasmid primer Kozak (F) 5'-GAG ACC CAA GCT TCC TCA GAC ATG-3' and reverse plasmid primer Kozak (R) 5'-TTT ACC CGG AGA CAG GGA GAG GCT-3' under conditions described in Table 4.
For CD177-Fc amplification in PCR cycle reaction steps are as follows, denaturation of DNA at 95°C for 1min followed by primer anneling at 65°C for 1min, and polymerisation of DNA strands at 72°C for 2 min with final extension at 72°C for 8 min, with 32 overall cycles of two and a half hour duration. The PCR product of 1996 bp was checked by 1% agarose gel electrophoresis in TBE buffer using DNA molecular markers as standard (Biolabs) and then was subcloned into pIB/V5-His TOPO Vector by TA cloning strategy (Invitrogen).

Components	Concentration	Reaction volume
Sterile water	-	25
Buffer	10x	5
dNTP	1.25 µM each	8
Forward Primer	5.0 µM	5
Reverse Primer	5.0 µM	5
Taq gold	5U/µl	1
DNA	1:200 dilution	1
Total reaction volume	-	50

Table 2: PCR mixture for CD177 DNA amplification, table contains a description of the components and concentration of PCR mixture.

For the ligation, 2µl PCR product encoding for CD177-Fc fusion protein was incubated with 1µl pIB/V5-His TOPO Vector, 1 µl of salt solution containing 1.2 M NaCl and 0.06M $MgCl_2$ and 2µl sterile water for 10 min at room temperature and kept on ice. The presence of Topoisomerase-I isolated from TOPO vector allows direct ligation of PCR product. Aliquot of 2 µl ligation mixture were then transformed into 2 µl one shot TOP 10 competent bacteria. Reaction

mixture was mixed gently and incubated on ice for 20 min followed by 30s heat shock at 42^{o}C in water bath. After cooling on ice for 1 min, 250 µl of SOC medium was added and cells were incubated for 1 h at 37°C. Finally, cells were plated to LB Petri dishes containing 100 µg/ml of carbenicillin and were incubated overnight at 37°C. For general screening several clones were picked by picking half of the colonies and boiled before subjected to PCR (see table 4) to confirm the presence of insert. Positive clone was cultured and plasmid DNA was isolated using Qiagen miniprep kit.

The plasmid DNA was inoculated in to 3 ml culture LB medium and grown for 7 h to obtain maximum cell density 4.10^{9} cells/ml. Aliquot of 0.9 ml of culture was stored with glycerol. The remaining culture medium was spun down for 3000 g at 4°C for 5 min, and after washing with 5 ml of 10 mM Tris pH 7.5 the pellet was resuspended gently in 300 µl of P1 (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Subsequently, 300 µl of P2 (200 mM NaOH, 1% SDS) was added for 5 min followed by addition of 300 µl P3 (3.0 M Potassium acetate, pH 5.5) followed by 15 min centrifugation for 8,000g at RT. Supernatant containing plasmid was transferred in to new Eppendorf tube and 595 µl of isopropanol(1/7 vol) added to the supernatant at room temperature. After 5 min incubation, plasmid DNA was pelleted by centrifugation at 8,000g. Plasmid DNA was then washed with 500 µl of 70% ethanol and DNA preparation was resuspended in 30 µl of water and stored at 4°C until use.

To prove the correctness of the insert, plasmid DNA was sequenced using the following primers to PIB/V5-His TOPO vector, (Forward: OpIE2 primer 5'- CGC ACC GATCTG GTA AAC AC-3'; Reverse: $OpIE_2$ primer 5'-GAG AAT ACA AAC TAA GAT TTA GTC AG-3') and Big Dye 1.3 sequencing reagents. After sequencing, the DNA was precipitated by 3M sodium actetate and 100% ethanol, washed with 70% ethanol and analyzed on Applied Biosystem.

2.8.2. Transfection of CD177-Fc construct into High Five Insect Cells

Aliquots of 1 µl CD177-Fc construct was added to 20 µg of Cellfectin reagent in SFM medium. Transfection cocktail was gently mixed for 10 sec, incubated at room temperature for 15 min and then added to 60 mm Petri dishes containing confluent High Five cells. Transfected cells were incubated for 4 h by rocking, which was followed by the addition of 2 ml fresh complete SFM medium. Cells were cultured for two to three days and were then selected with 20 µg/ml blasticidin. Stable cell lines were cultured in T100 flasks or in triple layered flask for scale up and production. Every three days culture supernatants containing CD177-Fc fusion protein were harvested and the remaining cells were cultured for further production.

2.8.3. Detection of recombinant CD177-Fc by ELISA

Microtiter plates were precoated with 250 ng DAH IgG in coating buffer and incubated overnight at 4°C followed by three washes with 0.02% BSA/PBS.
To prevent non-specific reaction the wells were blocked with 2% BSA for 30 min at 4^{o}C. Aliquots of 100 µl CD177-Fc supernatant or 100 ng purified CD177-Fc were added and wells were incubated for 1 h at 37°. After washing three times with 0.02% BSA, 1 µl of mabs 7D8 or MEM166 (dilution 1:500 in 0.2% BSA) were added and incubated for 1 h at 37°C, followed by three washes with 0.02% BSA. Bound mabs were detected with 100 µl DAM HRP (dilution 1:8000 in PBS) for 1 h at 37°C. Excessive mabs were washed away with 0.02% BSA and visualized by adding 100 µl OPD and H_2O_2 as substrate for 1 h at 37°C. Colour reaction was stopped by adding 50 µl of 2.5 N sulphuric acid and the reactivity was measured at 492 nm in ELISA reader.

2.8.4. Purification of recombinant CD177-Fc fusion protein by Protein G column

The CD177-Fc fusion protein was purified by affinity chromatography on Protein G column which binds specifically the Fc part of CD177. The affinity matrix (1ml) was washed in 50 ml of binding buffer packed as column under gravity, and equilibrated with the same binding buffer as recommended by the manufacturer. The supernatant was loaded on to the column at a low flow rate (1ml/min) and washed thoroughly with the same buffer till no detectable protein emerged. The bound protein was eluted using glycine or Tris buffer (0.5 M glycine HCl, pH 2.9 or 10mM Tris, 150mM NaCl, 30mM Glutathione, 1mM DTT, 1mM PMSF and 10% glycerol, pH 8.0). Individual fractions were examined for the presence of protein and the peak protein fractions were analyzed by 7.5% SDS-PAGE and visualized by silver staining. The concentration of purified CD177-Fc fusion protein was determined by BCA assay using BSA as standard. After incubation at 37°C for 30 min reaction mixture was measured with spectrophotometer at 492nm.

2.8.5. Purification of of recombinant CD177-Fc fusion protein by affinity column

Sepharose 4B activated column prebound with mab against CD177 was washed extensively with 50 ml washing buffer A, 30 ml washing buffer B and 20 ml washing buffer A. Supernatants containing soluble CD177-Fc fusion protein was diluted with binding buffer and passed on to the column at a low flow rate (1ml/min), and washed thoroughly with 200 ml of washing buffers A and B until no detectable protein emerged from the column. Bound CD177-Fc fusion protein was eluted by adding of a 20 ml elution buffer (0.1M citrate buffer, pH 2.0). Small fractions were collected and were neutralized with a 1M Tris-HCl buffer, pH 9.5. Eluted fractions were pooled and dialyzed against 1xTBS buffer overnight at 4°C, followed by the determination of protein concentration.

After determining the concentration of CD177-Fc, approximately 20 µg of sample was run on 8% SDS PAGE. Separated protein was then fixed for 2 h. Protein Gel was washed with H_2O to avoid pH changes during development and followed by the addition of 0.1% silver solution in 0.2% formaldehyde for 30 min and then developed with 2.5% Na_2CO_3 solution. Staining was stopped by adding 1.5% Di sodium-EDTA after 15 min.

2.8.6. Immunoblotting

For immunoblotting analysis, duplicate protein samples were loaded on to 10% SDS-PAGE. After the electrophorosis half of the gel was stained with silver staining and the other half was transferred to nitrocellulose or PVDF membrane using semi dry blotting system. The gel and the membrane were wetted in transfer buffer (0.6%Tris, 0.29% Glycine, 0.2% SDS and 20% w/v methanol) and protein transfer was done at 0.8 mA/cm^2 for 1 hour. The membrane was stained with 0.5% Ponceau-S red, 1% acetic acid solution to assess the quality of the transfer and then destained in H_2O or washed with 0.1 N NaOH to remove the stain completely.

For immunostaining the membrane was blocked in phosphate buffer containing 1.5% BSA for 1h by rocking at room temperature, and then incubated with diluted primary antibody ECL-solution (5 µg/ml) containing 1.5% BSA for 1h at room temperature.The membrane was washed extensively with ECL-solution and incubated with secondary antibody conjugated with horse radish peroxidase (rabbit or donkey anti mouse IgG; dilution 1:100000) for 1h at room temperature and washed extensively with ECL wash solution. Finally, the blot was developed using ECL chemiluminescence substrate and exposed to X-ray film.

In some cases, blot was reused for further staining after stripping the proteins. Membrane bound proteins were treated with glycine pH 2.9 containing 2% SDS, 100mM β-mercaptoethanol for three times and then washed exten sively with ECL buffer. The membrane was then followed by three more washings and blocked with 1.5% BSA. For further staining, membrane was

stripped (see above) and restained with different mabs against endothelial proteins (PECAM-1, E-Selectin and P-Selectin) or against CD177.
In order to study the binding of CD177-Fc fusion protein with HUVEC, 20 µg (per lane) of endothelial cell lysates were loaded on to 10% SDS-PAGE and blotted on to PVDF membrane and developed with 5 µg CD177-Fc in (5:2000) . Bound CD177 was detected with donkey anti-human IgG at the dilution 1:100000 and visualised by incubation with ECL chemiluminescence system.

2.9. Immunoprecipitation with HUVEC

Washed confluent HUVEC in petridish were surface labeled with 2 ml of 5mM NHS-LC-BIOTIN for 30 min at room temperature. Excessive biotin was removed by washing it three times with 7 ml DPBS. Labelled cells were then solubilised with 1 ml of lysis buffer using syringe. Cell lysates were transferred in to Eppendorff tube. Cells were incubated on ice for 30 min and vortexing thrice, after 30 min centrifugation for 4,000 g at 4°C, supernatant was then transferred into a new tube and then stored at –80°C until use. Prior to immunoprecipitation lysates were precleared to prevent unspecifc binding. In brief, aliquots of 600 µl cell lysates were incubated with 100 µl prewashed Protein G beads together with 20 µl of normal human serum, 80 µl BSA (100 mg/ml) for 30 min in the rotator at room temperature. After centrifugation for 1 min at 1,000 g, preclearing was repeated once again. Preadsorbed cell lysates were stored in 100 µl aliquots in -80°C.

To 100 µl of prewashed Protein A beads, 2.5 µg of Fc fusion proteins were sadded and incubated for 1h on rotator at 4°C. After washing, 300 µl of biotin labelled precleared cell lysates were added into the protein coupled beads. Mixture was incubated overnight at 4°C on rotator. Beads were then washed five times with immunoprecipitaion buffer and then boiled with 2xSDS buffer for 5 min at 100°C. Aliquots of 40 µl immunopreciptates were separated on 10% SDS-PAGE and blotted into nitrocellulose membrane. Membrane was developed with HRP labelled streptavidin and visualized using ECL system as described above.

2.10. Isolation of endothelial cells from human umbilical cord

Prior to isolation of endothelial cells, 0.2% of gelatine coated culture dishes were incubated at 37°C for 1h. Umbilical cord was cut in one end, and the other end was fixed with a needle for two washings of 50 ml of HBS buffer. The other end was then closed and it was connected subsequently to three way adaptor. 20 ml of collagenase in HBS buffer was injected into the umbilical cord and incubated for 20 min in 37°C. The umbilical cord derived EC was fleshed with 30 ml of HBS buffer into a tube of 1ml FCS and isolated by centrifugation for 10 min at 800 g. Isolated HUVEC were re-suspended in 10 ml of MCDB 131 medium containing 100 µl 1% fungizone and seeded on to culture plates or dishes accordingly.

2.10.1. Flow cytometric analysis

Aliquot of 4.10^6 HUVEC were fixed with 1% formaldehyde for 5 min and then washed twice with PBS. HUVEC were incubated with 4 µg Fc fusion proteins (CD177-Fc, PECAM-Fc, or Fc alone as control) for 1h at 37°C. Cells were washed with 0.02% BSA in PBS buffer once and then labeled with 40 µl FITC conjugated DAH (1:80). After washings, cells were re-suspended in 500 µl 0.2% BSA for FACS analysis.

In some cases, binding of CD177-Fc fusion protein to PECAM-1 transfected cells was performed. Transfected cells or untransfected L-cells (as control) were fixed and the procedure was similar as described above.

2.11. ELISA

To analyse the protein-protein interaction in ELISA, microtiter wells were coated with 100 µl DAH IgG (250 ng) overnight at 4°C. Wells were washed extensively three times with 0.2% BSA and blocked with 2% BSA for 30 min at 4°C. Aliquots of 250 ng CD177-Fc fusion protein was added to each well along with controls and incubated for 1h at 37°C. After washing it three times with 0.2% BSA, 100 ng of PECAM-1 protein was added; alternatively several wells were added with 1 µg of EC lysates and incubated for 30 min at 37°C. Bound PECAM-

1, EC lysate were detected by the addition of mabs Gi18 against PECAM-1 (1:500 dilution), against E-selectin or against P-selectin at 37°C for 30 min. Wells were then washed again for three more times. 100 µl HRP labelled DAM (dilution 1:8,000) was added for 1h at 37°C. After washings, OPD substrate was added and colour development was measured at 492nm in ELISA reader.

2.12. Preparation of purified CD177 and PECAM-1 proteins for SPR analysis

2.12.1. Isolation of CD177 from granulocytes

Neutrophils were collected from CD177 positive phenotyped individual who was given a pericutaneous injection of Granulocyte Colony Stimulating Factor (GCSF-30 µg) for mobilization of neutrophils to blood circulation. After 12 h, 230 ml of blood was collected and diluted in PBS (1:5). Aliquotes of 15 ml blood was layered slowly onto Ficoll gradient and then centrifuged for 20 min at 800 g. Cell pellets were resuspended into 12 ml of ammonium chloride for 5 min on ice to lyse erythrocytes. After washing twice with 50 ml of PBS, approximately 10^8 cells of PBS was solubilized in 1 ml lysis buffer (20mM TBS pH 7.4, 0.25% Triton-X, 100 µl protease inhibitor cocktail, 100 µl of 5% EDTA) for 30 min. Cell lysates were then centrifuged for 30 min at 800 g at 4°C and pooled for purification by affinity chromatography using mab 7D8.

2.13. SPR analysis

CD177 protein-protein interaction with PECAM-1 was examined in real time using surface plasmon resonance (SPR) technology on a Biacore 2000 machine (Biacore AB, Uppsala Sweden). Purified PECAM-1 diluted in 10 mM acetate buffer (pH 4.0) in a concentration of 60 µg/ml was directly immobilized on to CM5-sensor chip via amino coupling as recommended by the manufacturer. Aliquots of 20 µl purified PECAM-1, CD177 or BSA as control was injected at different concentrations (0.0–2.0 µM). In some cases, purified CD177 was incubated with mabs against different epitopes on CD177 (7D8 or MEM 166) at the ratio 1:3 (µM) prior to injection. The running buffer used was PBS (10mM sodium phosphate, 150 mM NaCl pH 7.4 containing 0.005% surfactant P20).

Sensor chip was regenerated with NaOH (12.5 – 50 mM). Recorded sensograms were analysed using BIA evaluation software 3.2 RC1 (Biacore AB).
Purified PECAM-1 was a generous gift from Dr. P.J. Newman, Milwaukee, USA was used to perform SPR assay.

2.14. Cell Adhesion Assay of CD177 transfected U937 cells

The monocytic cell line U937 and CD177 transfected U937 cells used in this study were generated by the group of Prof. K.T. Preissner (Department of Biochemistry, Justus-Liebig University, Giessen).
Microtiter wells were coated with 500 ng purified PECAM-1 protein in HBS buffer overnight at 4°C. Wells were then blocked with 3% BSA in HBS prior to adhesion test. Aliquots of 2.10^5 U937 cells in 50 µl adhesion buffers containing 50 mM $MnCl_2$ and 200 ng of PMA were added and cells were allowed to adhere for 1h at 37° in CO_2 incubator. After removing the unbound cells by gentle sucking with pump, bound cells were washed twice with 150 µl HBS buffer prior to fixation with ice cold methanol for 15 min at 4°C. Cells were then incubated at room temperature with 50 µl of crystal violet dye solution. The plate was washed extensively with water tub and 100 µl of 50% methanol solution was added to measure the colour development at 592 nm. In some cases, transfected cells were preincubated with 1µg mabs against CD177 (7D8, MEM 166) or PECAM-1 (PECAM 1.1, PECAM 1.2 or Gi18).

3. RESULTS

3.1. Generation of CD177-Fc construct for expression in insect cells

For the expression of CD177-Fc in insect cells, full-length CD177 in signal pIg plus plasmid encoding for soluble CD177-Fc was amplified by PCR using specific primers (described in 2.8.1). Amplified PCR product consisting of 1996 bp corresponding to CD177-Fc is shown in Figure 3. As a control PCR was run without any template. PCR product was then ligated into the PIB-V5 TOPO vector for the expression in insect cells. After the ligation and the transformation into *E. coli* bacteria, colonies were screened for insert by PCR using specific primers as shown in Figure 6. The presence of the CD177-Fc insert in three positive clones could be detected as specific band of 1996 bp. Plasmid from positive clone #1 was isolated and purified plasmid DNA was then sequenced for verification of the CD177-Fc in PIB-V5 construct (Figure 4).

DNA sequence analysis using $OpIE_2$ forward and OpE2 reverse primers specific for PIB/V5 vector confirmed the presence and orientation of CD177-Fc (panel a) and (panel b) in PIB-V5 vector.

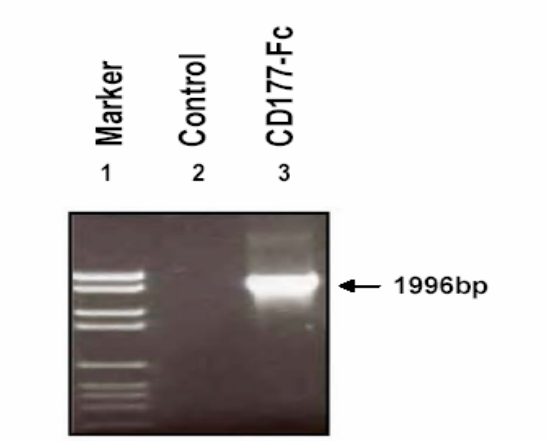

Figure 3: Analysis of CD177-Fc amplified PCR product by Agarose gel electrophoresis. PCR amplicon product for CD177-Fc run along with control on 1% Agarose gel. Lane 1: molecular marker standard-IV, lane 2: control PCR without template; lane 3: amplified CD177-Fc.

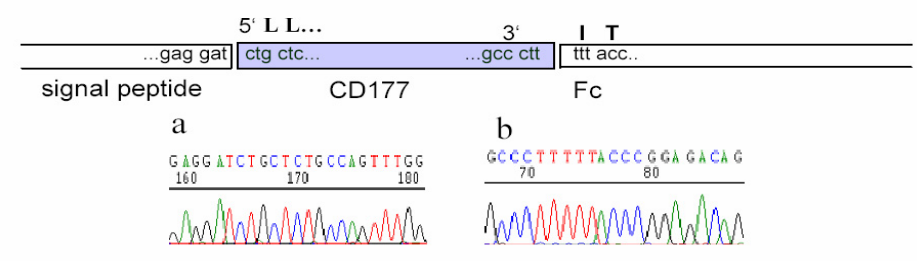

Figure 4: DNA sequencing analysis of CD177-Fc in PIB-V5 construct. Panel a: Sequencing with $OpIE_2$ forward primer demonstrated the right junction between PIB-V5 vector and CD177. Panel b: Sequencing with $OpIE_2$ reverse primer showed the right junction between Fc and PIB-V5.

3.2. Characterization of soluble recombinant CD177-Fc fusion protein

Plasmid from clone #1 was scaled up, purified and transfected into Highfive insect cells. After transfection stable cells were selected by the use of blasticidin, and then screened for the production of soluble recombinant CD177-Fc fusion protein in supernatant. Supernatants were collected after 3 days of culture and purified by the use of Protein G column. The purified CD177-Fc protein was analysed in ELISA by capturing the Fc part using donkey anti-human IgG coated wells. Bound CD177-Fc protein was then detected with mabs specific for CD177 and enzyme labelled donkey anti-mouse IgG as secondary antibody (Figure 5). Positive reactions could be detected with two mabs, 7D8 and MEM166 against CD177. In contrast, no reaction was observed with Fc alone as a control. Isolation from 1 liter of culture supernatant yielded approximately 400 µg of CD177-Fc.

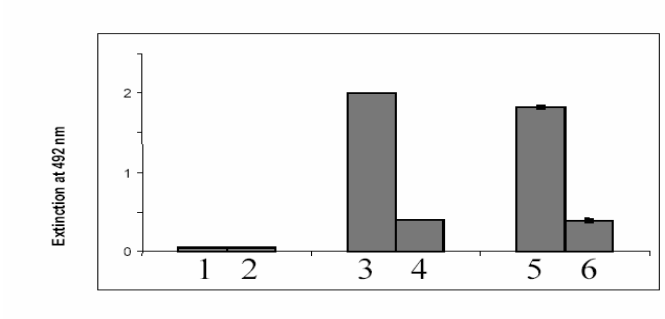

Figure 5: Analysis of purified CD177-Fc protein by ELISA. Protein was captured by immobilized DAH and then detected with respective monoclonals against CD177, mab 7D8 or MEM166 (3, 5). In the control experiments, Fc alone (4, 6) was run in parallel along with negative control PBS (1, 2) were detected with peroxidase labelled DAM IgG, reaction optical density was measured at 492nm in ELISA reader.

To analyse the purity of CD177-Fc fusion protein SDS-PAGE followed by silver staining were performed (Figure 6). Protein corresponding to the expected molecular mass (Mr) of 180 kDa for CD177-Fc homodimer was found under nonreducing conditions. After reduction of disulfide bond CD177-Fc monomer migrates with Mr of 60 kDa.

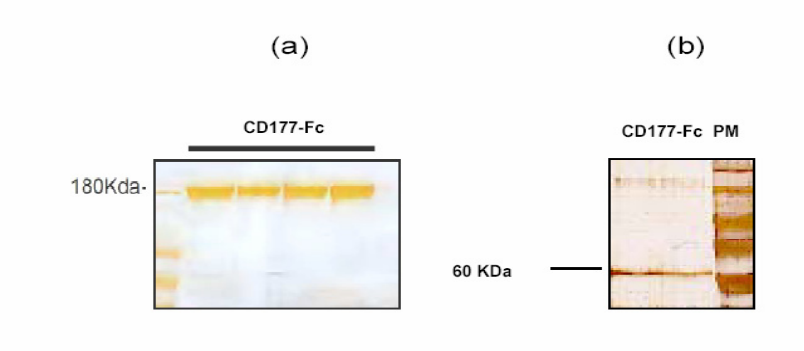

Figure 6: Analysis of soluble recombinant CD177-Fc protein by 8% SDS-PAGE under non reducing condition. After electrophoresis, four different CD177-Fc preparations were silver stained (a-lanes 2- 5). Lane 1 represents the molecular marker. b) Reduced form of CD177-Fc (b-lanes 1-2) along with molecular marker (b-lane-3).

To test the immunoreactivity of the purified CD177-Fc by the immunoblotting experiment, we first performed the immunoblotting experiment with mab against a V5 epitope. The CD177-Fc construct was fused with TOPO vector expressing V5 protein. As shown in Figure 7, mab anti V5 reacted with CD177-Fc (Mr 180 KDa), where as staining of Fc alone with this mab yielded a band with Mr of 73 kDa.

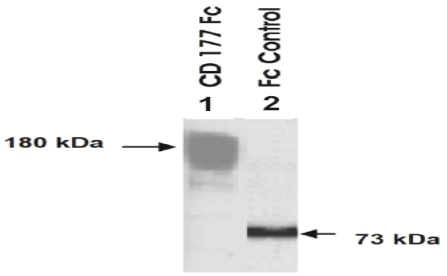

Figure 7: Analysis of CD177-Fc and Fc alone in immunblot with anti-V5. Proteins were separated on 8% SDS-PAGE, transferred onto nitrocellulose membrane and then stained with mab against V5 epitope and visualized using DAM-HRP based ECL detection system.

Further more; purified CD177-Fc was tested by the immunoblotting with mabs 7D8 and MEM166 to confirm the immunoreactivity of CD177. As shown in Figure 8, purified CD177-Fc protein reacted with both mabs, but not with normal mouse IgG as a control. To confirm the reactivity of the Fc-part, the fusion protein was tested in immunoblot using donkey anti-human IgG. As expected, donkey anti-human IgG was able to detect the CD177-Fc protein with Mr of 180 kDa under nonreducing conditions.

Together, these results demonstrated that the purified CD177-Fc expresses immunoreactive CD177 as well as the Fc domain.

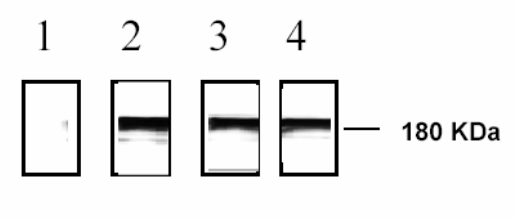

Figure 8: Analysis of purified CD177-Fc by immunoblot using monoclonals against CD177. Aliquots of 500 ng protein were run on 8% SDS-PAGE under non reducing conditions and were transferred onto PVDF membrane. Proteins were then stained with normal mouse IgG (lane 1), mab 7D8 (lane 2), mab MEM 166 (lane 3) and Donkey Anti-Human IgG HRP (lane 4) developed with ECL detection system.

3.3. Analysis of CD177-Fc binding to HUVEC by flowcytometry

As evidence from literature (Stroncek et al, 1992) indicates that CD177 may interact with endothelial cells, binding analysis was performed with purified CD177-Fc and isolated HUVEC from the umbilical cord in flowcytometry. Cells were incubated with 4 µg CD177-Fc, Fc, and PECAM-Fc and then visualised with fluorescence labelled RAH IgG. Positive staining of HUVEC was observed with CD177-Fc, but not with a control Fc. In addition, HUVEC showed also a positive reaction with PECAM-1/Fc, which is known as a major protein expressing on endothelial cells and interacts to each other in a homophilic manner. This result indicates that the counter receptor of CD177 resides on endothelial cells.

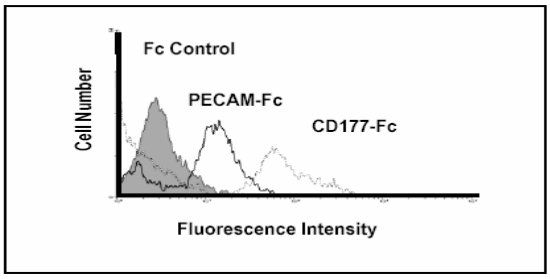

Figure 9: Analysis of CD177 counter receptor by flow cytometry. Binding of CD177 to endothelial cells was demonstrated invitro, isolated HUVEC was stained with Fc alone, CD177-Fc and PECAM-Fc. Bound proteins were then stained with FITC labelled Rabbit Anti-Human IgG and subjected to flow cytometry analysis on BD FACS software.

3.4. Characterization of CD177 Counter receptor by immunoblot

To determine the counter receptor of CD177 on endothelial cells, an immunoblot with endothelial cell lysates and CD177-Fc fusion protein was performed. Aliquots of 20µg of cell lysates were separated on SDS-PAGE under nonreducing conditions after blotting the membrane was stained with CD177-Fc and peroxidase labelled RAH IgG (Figure 10). Fc alone failed to detect any proteins from EC lysate. Interestingly, CD177-Fc reacted with 130 KDa endothelial protein. In the control experiments, the expression of PECAM-1 could be demonstrated by staining with Gi18 specific for PECAM-1. No reaction with anti-CD177 on endothelial cells could be detected with mab 7D8.

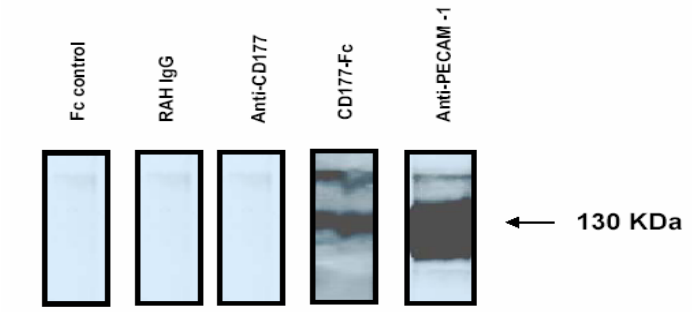

Figure 10: Immunoblotting analysis of HUVEC lysates with CD177-Fc fusion protein. Cell lysates were separated on 8% SDS-PAGE under nonreducing conditions. Separated proteins were then transferred onto PVDF membrane and incubated with Fc alone, CD177-Fc, anti-PECAM-1 (mab Gi18) and anti-CD177 (mab 7D8). Bound proteins were labelled with peroxidase labelled RAH IgG or RAM IgG and ECL detection system.

3.5. Analysis of CD177 counter receptor by ELISA

ELISA was performed to confirm the interaction between CD177 and PECAM-1. For this purpose, CD177-Fc was immobilized on to microtiter wells coated donkey anti-human IgG. After the washings, lysates of HUVEC was added and bound protein was screened with mabs against E-selectin, P-selectin, and PECAM-1 and then detected with peroxidase labeled secondary antibody and OPD as substrate (Figure 11). A higher reaction was detected when anti PECAM-1 was applied in comparison to anti-E-selectin and anti-P-selectin.

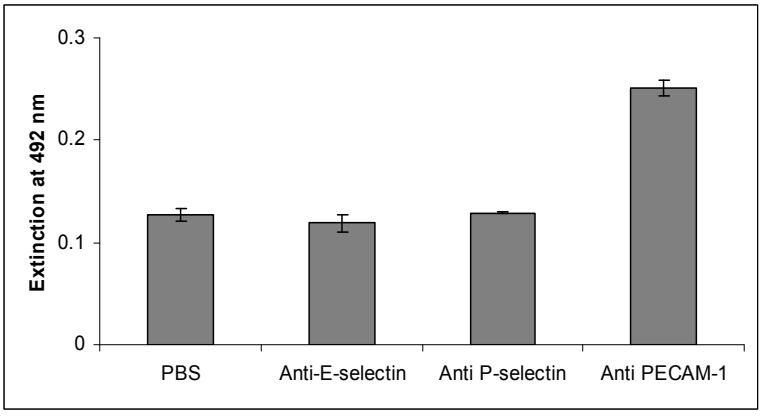

Figure 11: Antigen capture assay to find the CD177-Fc counter receptor.
Microtiter plates were coated with DAH IgG bound to CD177, followed by the addition of HUVEC cell lysates. Subsequently mabs against E-selectin, P-selectin, and PECAM-1 were added and followed by incubation with peroxidase labeled DAM IgG. Bound antibodies were then visualized by the addition of OPD substrate. Optical density was measured on ELISA reader at 492 nm. The data is the representative of the mean+SD ($p<0.05$) of two independent experiments.

3.6. Determination of CD177-Fc counter receptor by immuprecipitation and immunoblotting

To characterize the CD177 counter receptor further, we performed immunoprecipitation with biotin surface labelled HUVEC and CD177-Fc and Fc alone as control. The immunoprecipitates were then separated on 8% SDS-PAGE under nonreducing conditions and transferred onto a membrane for analysis with peroxidase labelled anti-human IgG and chemiluminescence system. As shown in figure 13, CD177-Fc fusion protein precipitated a protein

with respective Mr of 130 kDa. This result is in accordance to our previous finding using immunoblot (see figure 10).

We then performed a combination of immunoprecipitation and immunoblot to identify this 130 kDa. Biotin labelled HUVEC cells were lysed, and then immunoprecipitated with CD177-Fc. Immunoprecipitates were run on a 8%SDS-PAGE under nonreducing conditions and analysed in immunblot using different mabs. When immunoblotting was performed with Gi18 against PECAM-1, a positive reaction was observed. Contrary, no reaction was detected when immunoprecipitation was done with Fc alone. Furthermore, no reactions were detected with mabs against E- and P-Selectin.

These results strongly indicated that endothelial PECAM-1 represents the counter receptor of CD177.

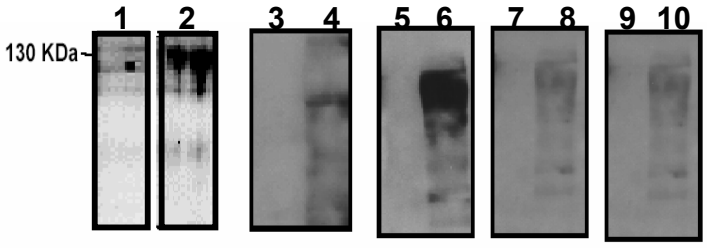

Figure 12: Immunoprecipitation analysis of biotinylated human endothelial cells.
Biotin labelled cell lysates were precipitated with control Fc and CD177-Fc and run on 8% SDS-PAGE under nonreducing conditions. Immunoprecipitates were then blotted to PVDF membrane and detected with streptavidin conjugated horseradish peroxidase and ECL system.
Immunoprecipitation and immunoblotting for the identification of CD177 counter receptor. Lysates of HUVEC were immunoprecipitated with control Fc and CD177-Fc stained with streptavidin HRP (lanes 1, 2). After blotting, PVDF membrane was stained with mouse IgG (as control; lanes 3, 4), mab Gi18 (anti-PECAM-1; lanes 5, 6), mab CD62 E (anti E-Selectin ; lanes 7, 8), and mab CD62 P (anti-P-Selectin; lanes 9, 10).

3.7. Analysis of CD177-Fc binding to PECAM-1 transfected Cells

To confirm these findings, the binding of CD177-Fc to L-cells transfected with PECAM-1 was analysed by flow cytometry (Figure 13). Panel A shows the expression of PECAM-1 on transfected cells by the use of mab Gi18 against PECAM-1. In the control experiment with isotype IgG no staining was observed.

When CD177-Fc was analysed with these cells a positive reaction could be detected (panel B). In the control experiment, Fc alone did not show any binding, whereas PECAM-Fc reacted with PECAM-1 transfectant in homophilic manner

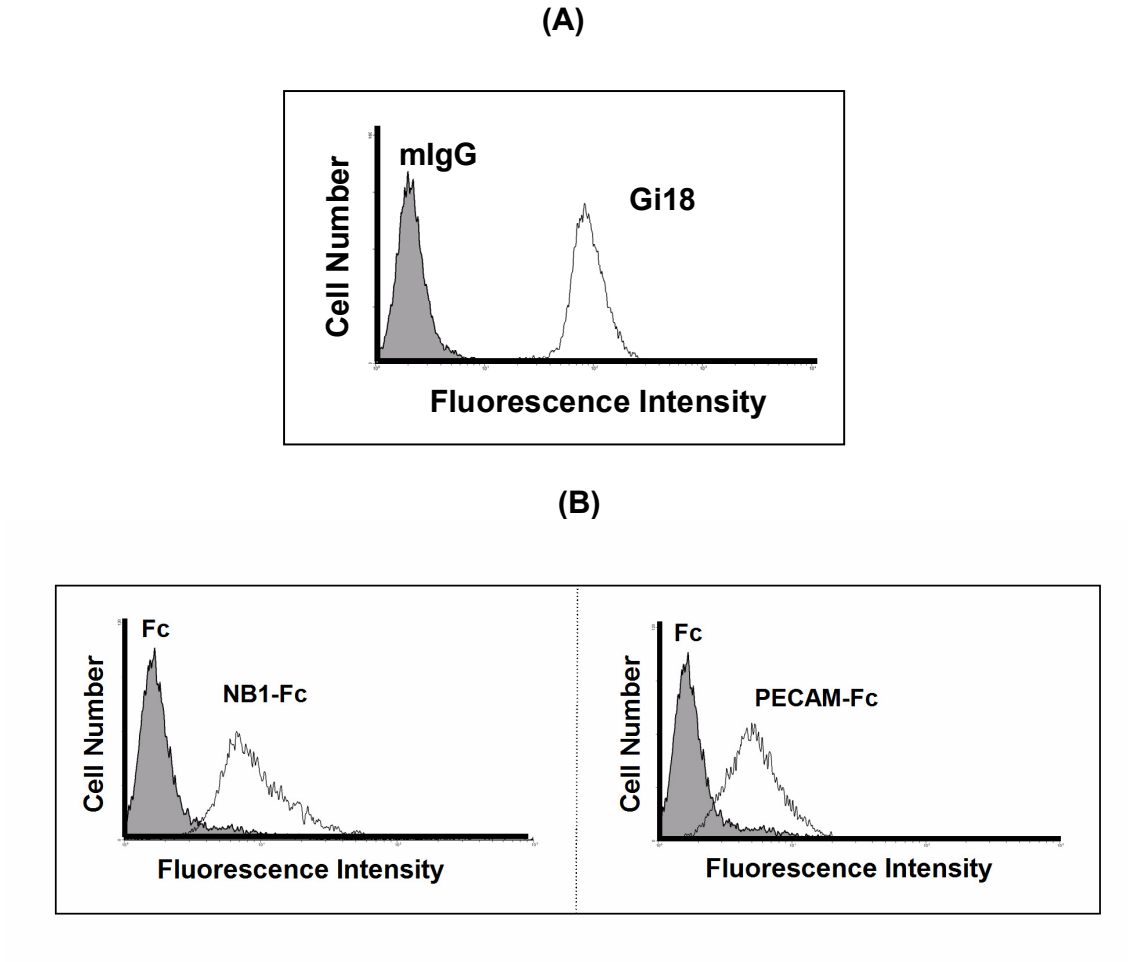

Figure 13: Binding of CD177-Fc to PECAM-1 transfected cells in flow cytometry. A) PECAM-1 transfected cells were incubated with mouse IgG (as control) or mab Gi18 against PECAM-1. Antibody binding was detected with fluorescein labelled secondary antibody and analysed by flow cytometry. B) PECAM-1 transfected cells were incubated with CD 177-Fc (left panel) or PECAM-1 Fc (right panel). Binding of Fc fusion proteins were then analysed on flow cytometry.

To confirm our finding that PECAM-1 binds to CD177, PECAM-1 transfected cells were surface labelled with biotin and subjected to immunoprecipitation with CD177-Fc and PECAM Fc fusion proteins (Figure 14). Fc alone was run in parallel. Again, CD177-Fc precipitated specifically a 130 KDa band. Similar band could be precipitated with PECAM-Fc fusion protein.

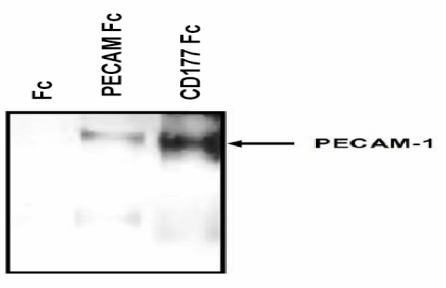

Figure 14: Immunprecipitation of biotinylated HUVEC with Fc alone (lane 1), PECAM-Fc (lane 2) and CD177-Fc (lane 3) under nonreducing conditions on 8% SDS PAGE. Immunprecipitates were detected with horseradish peroxidase labelled streptavidin and ECL system.

3.8. Analysis of CD177 and PECAM-1 Interaction by SPR

We sought to analyse the CD177 and PECAM-1 protein-protein interaction directly in SPR technology. For SPR analysis, CD177 were purified from neutrophils of healthy blood donors by affinity chromatography. Soluble recombinant PECAM-1 proteins were produced from a cell line transfected with PECAM-1 (a gift from Dr. Newman, Milwaukee, USA). The purity of both proteins could be demonstrated by silver staining analysis and immunoblotting (Figure 15).

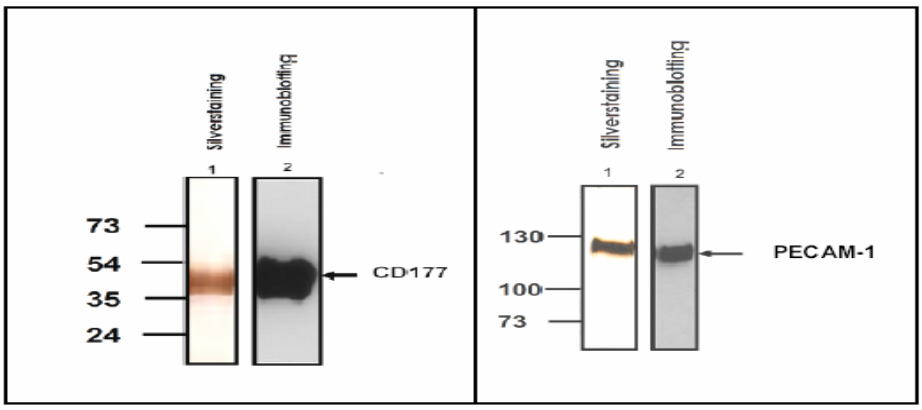

Figure 15: Analysis of purity of recombinant proteins, purified CD177 (left panel) and PECAM-1 (right panel) proteins were analysed by silver staining and immunoblottting using mab 7D8 (anti-CD177) or mab Gi18 (anti-PECAM-1), respectively.

SPR is the real time evaluation of protein interactions demonstrated the direct interaction between CD177 and PECAM-1. The obtained sensogram readings were plotted as response units (RU).

For SPR analysis, the purified PECAM-1 protein was immobilized to the sensor chip, and the binding of CD177 (heterodimer) and PECAM-1 (homodimer) was tested. Real-time protein-protein interaction was recorded as resonance units. Sensograms were plotted and aligned (Figure 16). When 2 μM of soluble CD177 was injected, specific association occurred reaching a maximum after 30 sec. This CD177-PECAM-1 complex dissociated after stopping the flow of CD177 ligand. In contrast, no interaction was detected when the control protein was injected (Figure 17a). To compare the affinity of heterophilic and homophilic interactions, binding of PECAM-1 to PECAM-1 was analyzed. As shown in Figure 17b, PECAM-1 bound to PECAM-1 with weak affinity as compared with CD177. These results indicated that PECAM-1 may bind preferentially heterophilic partner.

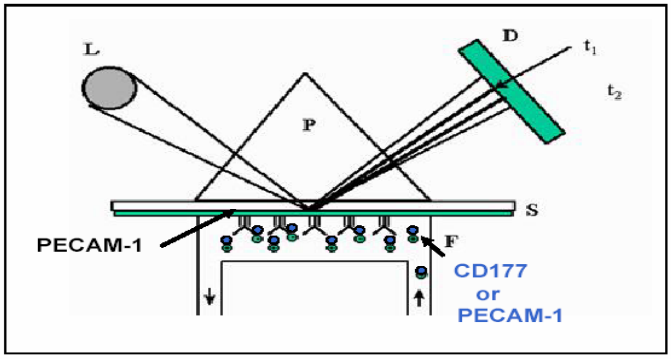

Figure 16: Brief illustration of SPR components.
L:light source, D:photoiodide array, P:Prism, S:Sensor surface, F:Flow cell, t1&t2:resonance phenomenan time course, CD177: Human neutrophil antigen-2, PECAM-1: Platelet Endothelial Cell Adhesion Molecule.

Our next assessment was to check which mutation is involved in PECAM-1 heterophilic interaction to CD177. For this purpose the mutant PECAM-1 was immmobilized on sensor chip and repeated the injection of 2μM concentration of soluble CD177 along with wild type PECAM-1. The sensogram obtained

represents the impaired interaction of mutant PECAM-1 to CD177 measured by association and dissociation phases. The examination with PECAM-1 mutant (K 89 A - point mutation on first IgG domain of PECAM-1), showed the interaction is partially inhibited the interaction between CD177 and PECAM-1, which indicated that its not to exclude the possibility of domain 1 also to be invoved with heterophilic binding or resides close to the domain involved.

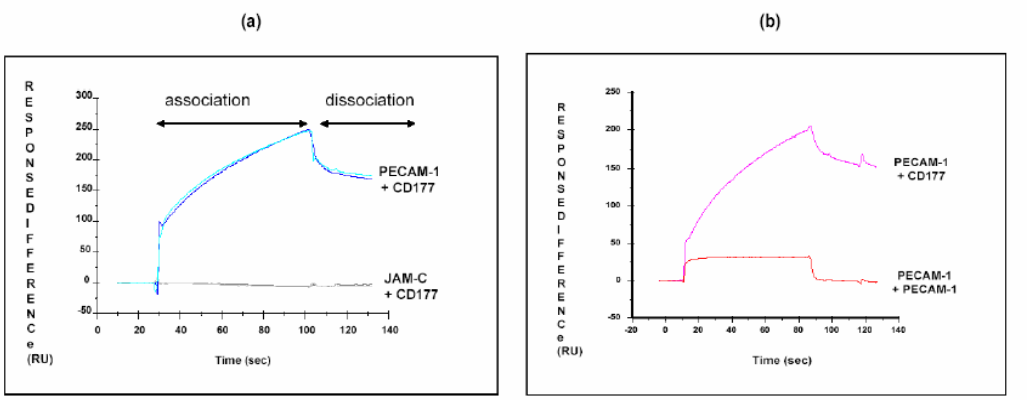

Figure 17: a) Schematic representation of CD177-PECAM-1 interaction represented as response units in SPR method analysed by real-time association and dissociation of CD177-PECAM-1 proteins measured and recorded on sensogram.
b) Illustration of heterotypic CD177-PECAM-1 and homotypic PECAM-1-PECAM-1 interaction demonstrated by surface plasmon resonance assay of realtime interaction of purified protein assay graph recorded on sensogram connected to biacore machine.

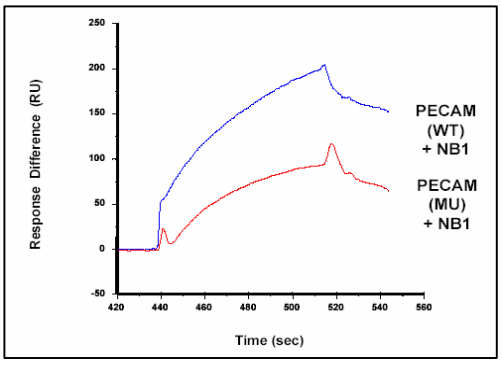

Figure 18: Examination of mutation on PECAM-1 domain-1 affects the interaction between CD177-PECAM-1. Sensogram real time-graph represent the response units, PECAM-1 (Wt) interaction to CD177 (Blue), PECAM-1 (mutant) interaction to CD177.

3.9. Adhesion of CD177 transfected Cells to PECAM-1

To analyse whether CD177-PECAM-1 interaction plays a role in cell adhesion, we tested the adhesion capacity of CD177 transfected U937 cells on immobilized PECAM-1 assay in comparison to nontransfected U937 cells. As shown in the Figure 19, CD177 transfected U937 cells bound stronger to immobilized PECAM-1 than nontransfected U937 cells. Inhibition studies were then applied to confirm the specificity of this interaction. Mab MEM166 against CD177 inhibits almost 70% of the cell adhesion while the other CD177 specific mab 7D8 blocked 30% of the adhesion. Our epitope mapping study by the use of biotinylated 7D8 and unlabelled MEM-166 in flowcytometry showed that both mabs reacted against different epitopes on CD177 (data not shown). Significantly, purified CD177 protein inhibits the CD177-PECAM-1 interaction completely, almost to the basal levels as observed with PBS as control.

Similar inhibition was observed with mabs against PECAM-1. Among different PECAM-1 specific mabs, PECAM1.2 and PECAM1.1 showed inhibitory capacity of 50% and 40%, respectively, where as mab Gi18 did not show any inhibition potential. Epitope mapping studies of mabs in the past (David et al, 1998; Sun et al, 1996) demonstrated that PECAM1.1 and 1.2 are situated on domains 5, 6 of PECAM-1 molecule. In contrast, the epitope of mab Gi18 is located on domain 1. These results indicated that CD177 binds the carboxy terminal region of PECAM-1 located on domains 5, 6.

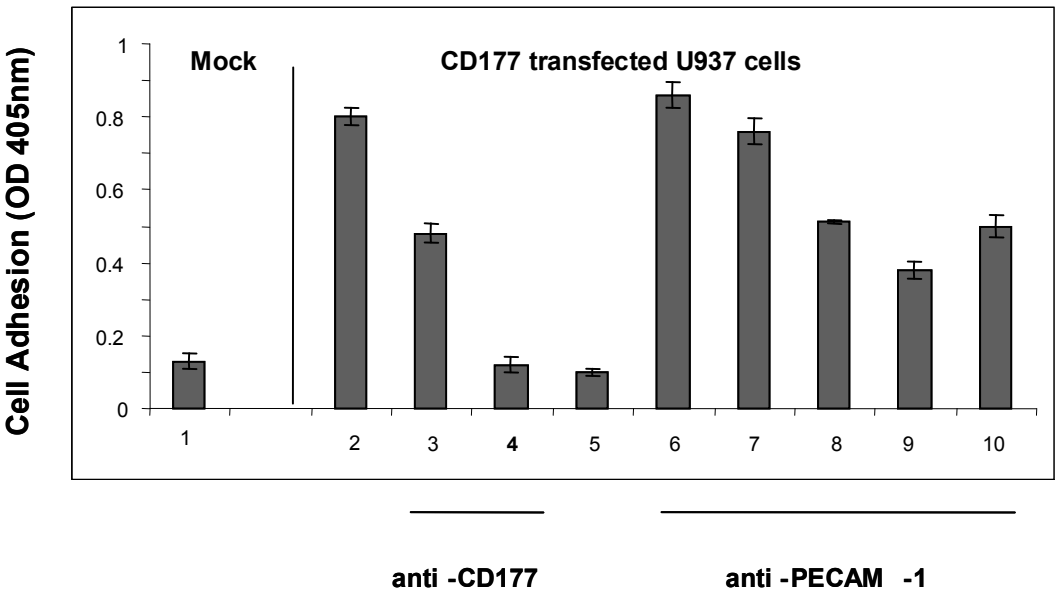

Figure 19: Binding of CD177 transfected U937 cells to immobilized PECAM-1 and inhibition by specific monoclonals against CD177 and PECAM-1.
1. Binding of CD177 transfected cells to PBS control 2. CD177 3. 7D8 4. MEM-166 5. s CD177, 6. Gi 18, 7. Gi 34. 8, 9, 10. PECAM 1.1, PECAM 1.2, PECAM 1.3 respectively, inhibition of CD177 transfected U937 to PECAM-1 was demonstrated with panal of mabs against CD177 as well as against PECAM-1(3-10).

4. DISCUSSION

Neutrophils are the primary phagocytes involved in the defense against pathogens during the inflammatory response. The proinflammatory cytokines and chemotactic factors signal the recruitment of neutrophils to the sites of infection by a multistep process called diapedesis. Since CD177 is a specific neutrophil antigen that is upregulated during bacterial infection, we asked the question whether CD177 is crucial for neutrophil endothelial interaction.

To achieve this goal we produced the CD177-Fc fusion protein by recombinant technology. CD177-Fc is a bivalent immunoadhesive molecule which provides higher affinity in comparison to the CD177 monomer. Studies in the past demonstrated that such immunoadhesive molecules represent a useful tool for capturing the ligand or counter receptor (Kato et al, 1992; Lino et al, 2005). By expression in the insect cell system we were able to produce immunoreactive CD177-Fc. Analysis with endothelial cells showed that this protein reacted with the endothelial protein of 130 kDa. Further immunochemical characterization by the use of immunoprecipitation and immunoblotting showed that endothelial PECAM-1 turn to the counter receptor for CD177. This result was confirmed by the direct protein-protein analysis using SPR technology.

One of the challenging areas in immunology is search for the ligand-receptor interaction. The past few years have seen an increase in methodology and instrumentation focused on the ability to discover and characterize the putative receptor-ligand pairs. Nowadays, a variety of powerful technology is now available to evaluate biospecific interactions. Significant advances have been made in this area, one of the widely used is the SPR biosensor chip (Piehler et al, 2005; Pattnaik et al, 2005; Gurbaxani et al, 2006) based assay to demonstrate direct protein-protein interaction in real time. In general, this novel technique allows the analysis of protein-protein interaction with minute amount of protein. In this study we could demonstrate by SPR analysis that the direct specific

interaction between CD177 and PECAM-1 (heterophilic manner) is stronger than the interaction between PECAM-1 and PECAM-1 (homophilic manner).

In line with these findings, the CD177-Fc fusion protein bound specifically to the PECAM-1 transfected L-cells. Immunoprecipitation analysis showed that the PECAM-1 could be pulled down from the PECAM-1 transfectant by the CD177-Fc.

To study the relevance of CD177 in a neutrophil adhesion, we tested the adhesion capacity of the U-937 monocytic cell lines expressing CD177. Using this transfected cells, we could demonstrate specific adhesion of these cells onto immobilized PECAM-1 and this cell adhesion could be blocked specifically with mabs against CD177 and against PECAM-1, which recognized the last two Ig domains (domains 5 and 6) of PECAM-1. In contrast, mabs against the first two Ig domains (domains 1 and 2) of PECAM-1 failed to block the adhesion of CD177 transfected cells on PECAM-1. These results strongly suggested that the domains 5 and 6 of PECAM-1 are responsible for the CD177 binding. Studies in the past described that these two domains were involved in the L-cells mediated heterophilic aggregation (Yan et al, 1995). However, the counter-receptor of PECAM-1 in this process was not known.

Simultaneous inhibition with the panel of monoclonal antibodies and soluble CD177 provides cumulative evidence for the existence of CD177-PECAM-1 interaction. Neutrophils carry PECAM-1 on their surface and known to interact with endothelial PECAM-1 (trans-interaction) mediate leukocyte transendothelial migration, their engagement activate or regulate the expression of integrins and its heterophilic adhesion receptor of $\alpha v\beta_3$ (Dangerfield et al 2002). neutrophilic PECAM-1 augment neutrophil signal transduction, integrin activation, and transmigration in a stimulus-specific manner, neutrophilic PECAM-1 may activate other adhesive protein(s) by cis-interaction also to modulate the cytoskeleton activity of neutrophils.

Until today little is known about the impact of PECAM-1 on neutrophils in the adhesion and transendothelial migration process (Muller et al, 1995; Berman et al, 1995) density of PECAM-1 molecules on leukocytes was very low to support PECAM-1 homophilic interactions (Liao et al, 1999; 1997).

Transgenic mice (Fang Liao et al, 1999) constitutively expressing PECAM-1 was attempted to study PECAM-1 role in chronic inflammation, mice that produced moderate concentrations of PECAM-1-IgG demonstrated a severely blunted acute inflammatory response, in the same model mice that produced higher concentrations of PECAM-1 (400-1000 µg/ml) concentrations were unresponsive to its anti inflammatory effects,in this model PECAM-1-IgG blocks the PECAM-1 dependent interaction demonstrated the importance of PECAM-1 in inflammation, also its observed that anti-PECAM-1 reagents fail to block the transmigration of leukocytes 100%, some residual transmigration observed in several acute inflammatory models and in PECAM-1 knockout mice, this 10-20% of residual transmigration is postulated to be dependent on PECAM-1 independent routes of transmigration. Hence this transgenic mouse serves to study the anti-inflammatory model targeting PECAM-1. Muller et al, succeded in their attempt to block transmigration of neutrophils, monocytes, NK cells in acute inflammation by the use of mab against PECAM-1 or soluble recombinant PECAM-IgG.

Study in PECAM-1 deficient mice showed that PECAM-/- neutrophils are much less polarized and spreading in response to IL-8 gradient, indicating that PECAM-1 on neutrophils has a major role in modulating neutrophil-directed migration (Wu et al, 2005). Neutrophil and monocyte migration in response to inflammatory stimuli in PECAM-1 deficient mice study indicating neutrophil PECAM-1 importance in the transmigration of neutrophils (Schenkel et al, 2004). PECAM-1 deficient mice also demonstrate it role as inhibitory receptor in autoimmune arthritis model to suppress collagen-induced arthritis (Mae-xhum wong et al, 2005). Schenkel et al (2006) work on PECAM-1 deficient mice strain

in acute inflammation model demonstrates PECAM-1 dependent and PECAM-1 independent mechanisms in acute inflammation.

It is known that PECAM-1 can interact with PECAM-1 in a homophilic manner (Cindy et al, 2000). However, this direct adhesive interaction is debated, because PECAM-1 seems to preferentially act as a signaling molecule, which can modulate endothelial functions by up regulating integrins (Thompson et al, 2001) and down regulate E-selectin (Litwin et al, 1997) rather than act as a mediator of homophilic adhesion.

Stockinger et al (1990) demonstrated that PECAM-1 can be down regulated after activation of granulocytes. Similar phenomenon was observed during the granulocytes maturation as well as in the lymphocytes (Lund-Johansen et al, 1993; Wedle et al, 1999). This is in accordance to our observation by the SPR which demonstrated that PECAM-1 bounds weakly to PECAM-1.

Recently, Göhring et al (2004) described that the CD177 surface expression is enhanced in patients with bacterial infections, polycythemia rubra vera and neutrophils of G-CSF stimulated donors compared with healthy donors. Further investigation is necessary to address the question of whether or not the activation of neutrophils leads to upregulation of CD177 surface expression is associated with the modulation of PECAM-1 binding capacity. .

Furthermore, there is evidence that suggests that PECAM-1 can also interact in a heterophilic manner. Several of the counter receptors for this interaction were described (Buckley et al, 1996; Delisser et al, 1993). One of the most described counter receptor for PECAM-1 is αvβ3 (Piali et al, 1995; Wong et al, 2000; Chiba et al, 1999). The αvβ3 is known as a receptor for the vitronectin, which is expressed on endothelial cells, neutrophils, and other blood cells (Piali et al, 1995). Since endothelial cells and neutrophils, both carry αvβ3 as well as PECAM-1, it is difficult to dissect the specific partner for each receptor; e.g

endothelial PECAM-1 could theoretically interact with PECAM-1 or αvβ3 on neutrophils. In addition, such an interaction was not demonstrated on the borders of the endothelial cells, where PECAM-1 is expressed in high density (Thompson et al, 2000). Furthermore, this effect may also be caused by the activation of αvβ3 integrin indirectly by coupling of PECAM-1/PECAM-1 (cis-interaction) (Cindy et al, 2000). In comparison to αvβ3, CD177 is solely expressed on neutrophils but not on endothelial cells.

Thus, the specific interaction between CD177 and the endothelial PECAM-1 was evident. Based on the observation that the homophilic interaction between the neutrophil PECAM-1 and endothelial PECAM-1 is much weak, we are substantiated in proposing that the heterophilic CD177-PECAM-1 binding is an important neutrophil-endothelial interaction, which may crucial for the neutrophil adhesion and migration through endothelial barrier.

Neutrophils are the prime leukocytes involved in innate immunity. CD177 is exclusively expressed on neutrophils with proven interaction to endothelial PECAM-1 constitute the innate immune response against host during bacterial infection and sepsis.

In recent years studies focussed to reveal neutrophils role in adaptive immunity (Puellmann et al ;BA Maletto et al, 2006) as neutrophils are known to express T cell receptor and costimulatory molecules (CD86 and CD80). PECAM-1 is known to express on transformed cells as in breast carcinoma and giloma (Charpin et al 1995; Aroca et al, 1999; Tang et al, 1994). Thus CD177 may play a role in cancer by interacting with PECAM-1 as a target cell either as positive or negative stimulator. One may also speculate that CD177 ligand binding may activate the neutrophils as effector cells to produce cytokines and to help the maturation of other cells as antigen presenting cells and T lymphocytes.

Recent study demonstrated that uPAR (Kim et al, 2007), which showed structural homologous with CD177, inhibits the growth of malignant glioma (Kim et al, 2007
So far number of receptors simultaneously interacting with multiple ligands or counter receptors has been described; JAM (Keiper et al, 2005) is one of an example for such interaction(s).

Our understanding and finding that CD177 serve as a counter receptor for PECAM-1 and the ability of CD177/PECAM-1 interactions to mediate neutrophil transendothelial migration suggest that this system may specifically mediate neutrophil transmigration under specific conditions.
Thus, PECAM-1-CD177 interaction may represent an alternative mechanism for the neutrophil transmigration in certain activation conditions (bacterial infection, sepsis etc). In conclusion, we have identified a heterophilic binding partner of endothelial PECAM-1. Thus, it represents a challenging new approach to our understanding of neutrophil/endothelial cell interplay in host defence.

5. SUMMARY

This study sheds light on the characterisation of CD177 and identification of CD177s counter receptor on endothelium. We could demonstrate that PECAM-1 on endothelial cells functions directly as the counter receptor for CD177 by following observations;

1. Our recombinant CD177-Fc fusion protein, which was produced in insect cells, reacted with 130 KDa molecules on endothelium.
2. Screening with different mabs identified the 130 KDa protein as endothelial PECAM-1.
3. Biochemical and immunological studies using recombinant CD177-Fc fusion protein confirmed that endothelial PECAM-1 functions directly as the counter-receptor for CD177.
4. Functional studies *in vitro* demonstrated that purified CD177 protein inhibits CD177 mediated cell adhesion to immobilized PECAM-1 protein as well as neutrophil migration through endothelial cells.

These results indicate that CD177 can act as cell adhesion molecule involving neutrophil-endothelial interaction. This observation may provide a new concept in understanding the multi-step process of transendothelial migration.
In the future, this concept should be studied in different *in vitro* as well as *in vivo* model in more detail.

6. PROSPECTIVE STUDY:

Further indepth study needed to evaluate the implicated role played by inhibiting and activating anti-CD177 as well as anti-PECAM-1 in modulating multi step neutrophil transmigration in conditions of chronic inflammation.

To study the physiological importance of CD177 expression on neutrophils and its interaction to endothelium by studying Geneknock out or SiRNA at protein level.

To investigate whether any possible role plays by CD177 to extracelular matrix-proteins

To examine if at all any potential role played by CD177 during different patho physiological conditions as in diseases of atherogenesis, diabetes and cancer metastasis.

To study the significance of CD177 in haematopoietic stage expression.

Furthermore, to study therapeutic intervention aiming CD177-PECAM-1 interaction to prevent organ failure and multi consequences in the development of sepsis, to study the anti-inflammatory efficacy of soluble recombinant CD177 or mabs against CD177 in inflammation model.

6. ABBREVIATIONS

APS-Ammonium Persulphate

BSA-Bovine Serum Albumin

BCA- Bi Cinchoninic Acid

CD-Clustar of Differentiation

CML-Chronic Myelogenous Leukaemia

CHO-Chineese Hamster Ovary cells

Deg C-Degree celcius

DMSO- Dimethyl sulfoxide

DTT-1,4-dithiothreitol.

EDTA-Ethylene Diamine Tetra Aceticacid

ELISA-Enzyme Linked Immunosorbant Assay.

FBS-Fetal Bovine Serum

FACS-Flourescence Activated Cell Sorting

FITC- Fluorescein Iso Thiocyanate

GABA-Gama Amino Butyricacid

G-CSF-Granulocyte Colony Stimulating Factor

GPI-Glycosyl phosphotidyl inositol

HLA-Human leukocyte antibodies

HAT-Hypoxanthin-aminopterin conjugate

HCl-Hydrochloric acid

HRP-Horse Radish Peroxidase

HNA 2a-Human Neutrophil Antigen 2a

HUVEC-Human Umbilical Vein Endothelial Cells

ISBT-International Society of Blood Transfusion

IPB-immuno Precipitation Buffer

Ly-6-Leucocyte antigen family

Mab-Monoclonal antibody

MPD- Myelo Proliferative Disoorders

mts- Minutes

ngm-Nano gram

NaCl-Sodium chloride

NaOH-Sodium chloride

NK-Natural Killer Cells

OPD-Ortho phenylene diamine.

PBS-Phosphate buffer saline

PEG-Poly ethylene glycol

PFA-Para formaldehyde

PRV-1-Polycythemia Rubra Vera

PMSF- Phenyl Methyl Sulphonyl Fluoride

p/s-penicillin-streptomycin

PVDF- Polyvinylidenfluorid

SPR-Surface Plasmon Resonance

RBC-Red Blood Carpescules

RT-Room Temperature

rpm-revolutions per minute

SDS-PAGE-Sodium Dodecyl Sulphate-Polyacrylamide Gel

SNP-Single Neucleotide Polymorphism

TBS-Tris Buffer Saline

TEMED-Tetra-Methyl Ethylene Diamine

TRALI-Transfusion Related Acute Lung Injury

Triton X-100 (t-octylphenoxypolyethoxyethanol)

uPAR-Urokinase Plasminogen Activating Receptor

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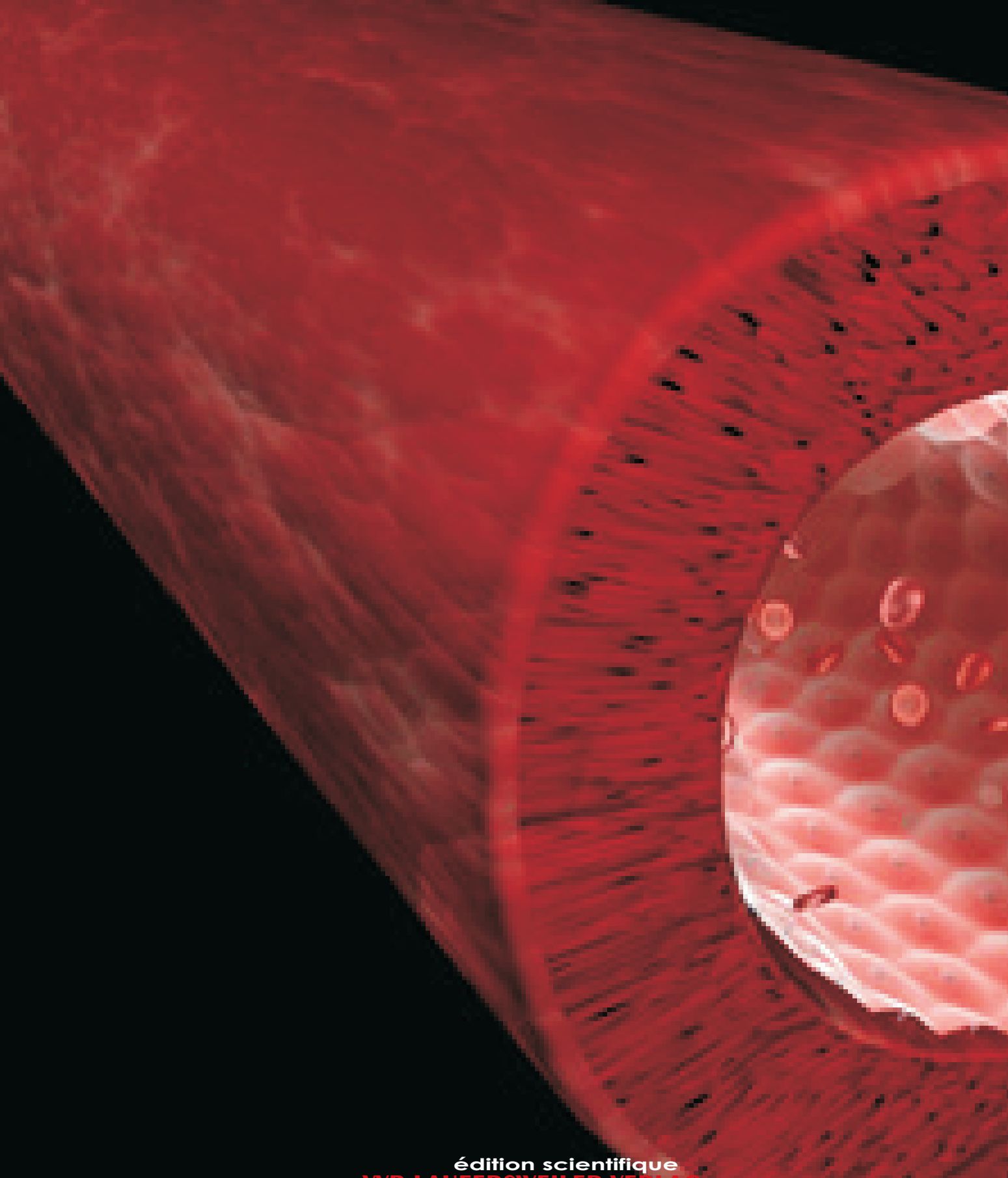

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