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# LEUKOCYTE TRAFFICKING: A SPECIAL FOCUS ON VAP-1 AND CLEVER-1

by

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# ABSTRACT

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Leukocyte trafficking: a special focus on VAP-1 and CLEVER-1

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It is crucial that lymphocytes patrol the body against foreign intruders and that leukocytes invade inflamed tissues to ameliorate the infection or injury. The adhesion molecules in leukocytes and endothelial cells play an essential role in the immune response by directing the traffic of leukocytes. However, the same molecules that guide leukocyte traffic under physiological conditions are also involved in pathological situations, when an overly excessive or harmful inflammatory response leads to tissue destruction and organ dysfunction or tumor growth.

Vascular adhesion protein-1 (VAP-1) and Common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) are endothelial molecules that participate in the adhesion of leukocytes to the endothelia. This study was designed to elucidate, using different inflammation models, the role of VAP-1 and CLEVER-1 in leukocyte migration to the inflamed tissue, and to evaluate the use of antibodies against these molecules as an anti-adhesive therapy. Also, the role of CLEVER-1 during tumorigenesis was studied.

Blocking the function of VAP-1 with antibodies significantly decreased the accumulation of leukocytes in the inflamed tissue. Targeting CLEVER-1 prevented cell migration via lymphatic vessels, as well as leukocyte traffic during inflammation. Following the anti-CLEVER-1 antibody treatment the number of immune regulating leukocytes in tumors was reduced, which led to a decrease in tumor growth. However, the normal immune response towards immunization or bacterial infection was not compromised. Thus, VAP-1 and CLEVER-1 are both potential targets for anti-inflammatory therapies for preventing the harmful accumulation of leukocytes in inflamed areas. Targeting CLEVER-1 may also inhibit tumor growth by reducing immunosuppressive leukocytes in tumors.

**Keywords:** adhesion molecule, leukocyte traffic, VAP-1, CLEVER-1

# TIIVISTELMÄ

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VAP-1 ja CLEVER-1 tarttumismolekyylit valkosoluliikenteessä

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On elintärkeää, että lymfosyytit vartioivat elimistöä vierailta tunkeutujilta ja että valkosolut kulkeutuvat tulehtuneeseen kudokseen parantamaan infektion tai kudosvaurion. Valkosolujen kulkua elimistössä ohjaavat verisuonten ja valkosolujen pinnoilla olevat molekyylit, joita kutsutaan adheesio- eli tarttumismolekyyleiksi. Kuitenkin samat tarttumismolekyylit, jotka ohjaavat valkosolujen kulkua normaalitilanteessa, ovat myös osallisena liian voimakkaissa tai haitallisissa tulehdustilanteissa, jotka voivat johtaa kudoksen tuhoutumiseen ja toiminnan menetykseen tai syövän kasvuun.

Verisuonen adheesioproteiini-1 (VAP-1) ja Yleinen lymfaattisen endoteelin ja verisuonen endoteelin reseptori-1 (CLEVER-1) ovat molekyylejä, jotka osallistuvat valkosolujen ja endoteelin väliseen tarttumistapahtumaan. Tämän tutkimuksen tarkoituksena oli selvittää erilaisten tulehdusmallien avulla VAP-1:n ja CLEVER-1:n merkitystä valkosolujen kulkeutumisessa tulehtuneeseen kudokseen ja arvioida VAP-1 ja CLEVER-1 vasta-aineiden käyttöä tulehdusta estävänä hoitona. Lisäksi tavoitteena oli tutkia CLEVER-1:n merkitystä syövän kasvussa.

Estämällä VAP-1:n toiminta vasta-aineilla valkosolujen kulku tulehtuneeseen kudokseen väheni merkittävästi. CLEVER-1:n toiminnan esto rajoitti solujen kulkua imuteiden kautta imusolmukkeeseen sekä valkosolujen liikennettä tulehdusalueelle. CLEVER-1 vasta-ainehoito vähensi myös puolustusta säätelevien valkosolujen lukumäärää syöpäkudoksessa pienentäen syövän kasvua, kun taas immunisaation ja bakteeritulehduksen aikana immuunipuolustus toimi normaalisti. Tulosten perusteella sekä VAP-1 että CLEVER-1 ovat hyviä lääkekehityskohteita, sillä kohdistamalla vasta-ainehoito näihin molekyyleihin pystyttään estämään haitallista valkosolujen kerääntymistä tulehtuneeseen kudokseen. Lisäksi CLEVER-1:n toiminnan esto saattaa vähentää ns. immunosupressiivisten eli puolustuskykyä hillitsevien valkosolujen määrää syöpäkudoksessa ja täten edesauttaa syövän hoitoa.

**Avainsanat:** tarttumismolekyyli, valkosoluliikenne, VAP-1, CLEVER-1

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# ABBREVIATIONS

| | |
|---|---|
| acLDL | acetylated low density lipoprotein |
| BSA | bovine serum albumin |
| CAO | copper-containing amine oxidase |
| CCL-21 | chemokine (C-C motif) ligand 21 |
| CD | cluster of differentiation |
| CFSE | carboxyfluorescein succinimidyl ester |
| CHO | Chinese hamster ovary cells |
| CLEVER-1 | common lymphatic endothelial and vascular endothelial receptor-1 |
| EGF | epidermal growth factor |
| EMA | European Medicines Agency |
| ESAM | endothelial cell-selective adhesion molecule |
| FCS | fetal calf serum |
| FDA | Food and Drug Administration |
| FEEL-1 | fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 |
| FITC | fluorescein isothiocyanate |
| FoxP3 | forkhead box P3 |
| HEV | high endothelial venule |
| ICAM | intercellular adhesion molecule |
| IFN-β | interferon-β |
| Ig | immunoglobulin |
| IL | interleukin |
| JAM | junctional adhesion molecule |
| LFA-1 | lymphocyte function associated antigen-1 |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| Mac-1 | macrophage-1 antigen |
| MAdCAM-1 | mucosal addressin cell adhesion molecule-1 |
| MS | multiple sclerosis |
| NK | natural killer |
| NOD | non obese diabetic |
| NZW | New Zealand white |
| oxLDL | oxidized low density lipoprotein |
| PBS | phosphate buffered saline |
| PE | phycoerythrin |
| PECAM-1 | platelet endothelial cell adhesion molecule-1 |
| PLN | peripheral lymph node |
| PML | progressive multifocal leukoencephalopathy |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| RGD motif | arginyl-glycyl-aspartic acid motif |
| SCID | severe combined immunodeficiency |
| SI-CLP | stabilin-1 interacting chitinase-like protein |

| | |
|---|---|
| Siglec | sialic acid binding Ig-like lectin |
| SPARC | secreted protein acidic and rich in cysteine |
| SSAO | semicarbazide-sensitive amine oxidase |
| TNF | tumor necrosis factor |
| VAP-1 | vascular adhesion protein-1 |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VLA-4 | very late antigen-4 |

# LIST OF ORIGINAL PUBLICATIONS

This study is based on the following original publications, which are referred to in the text by Roman numerals (I-III).

I Merinen Marika, Irjala Heikki, Salmi Marko, Jaakkola Ilkka, Hänninen Arno and Jalkanen Sirpa. **Vascular Adhesion Protein-1 Is Involved in Both Acute and Chronic Inflammation in the Mouse.** *American Journal of Pathology*, 2005 Mar;166(3): 793-800

II Karikoski (née Merinen) Marika, Irjala Heikki, Maksimow Mikael, Miiluniemi Mari, Granfors Kaisa, Hernesniemi Sari, Elima Kati, Moldenhauer Gerhard, Schledzewski Kai, Kzhyshkowska Julia, Goerdt Sergij, Salmi Marko and Jalkanen Sirpa. **Clever-1/Stabilin-1 Regulates Lymphocyte Migration within Lymphatics and Leukocyte Entrance to Sites of Inflammation.** *European Journal of Immunology,* 2009 Dec;39(12): 3477-87

III Marttila-Ichihara Fumiko, Karikoski (née Merinen) Marika, Huovinen Ville, Elima Kati, Irjala Heikki, Salmi Marko and Jalkanen Sirpa. **Clever-1/Stabilin-1 Controls Cancer Progression by Regulating Migration of Tumor-infiltrating Leukocytes and Tumor Cells.** *Submitted*

# 1. INTRODUCTION

We are constantly challenged by intruders that threaten our well-being and even our life, and it is our immune system's fundamental responsibility to defend us against the invasion of microbes and other threats. For this purpose, our immune system has to produce a sufficient number of lymphocytes that can recognize a myriad of foreign structures and distinguish these from self. In order to succeed, the lymphocyte and foreign antigen must first come into contact, then when the intruder is detected, an adequate amount of responding lymphocytes needs to be produced. These specific responding lymphocytes must then enter at the site of insult. And finally, after eliminating the danger, a guard needs to survey the body against re-attack. In addition to these delicate tasks, the body needs to maintain homeostasis in order to prevent the overexpansion of lymphocyte subsets. To achieve all of this, lymphocytes must know where to go and when. This is attained by specific interactions between the lymphocytes and the vascular endothelium, which are mediated by adhesion receptors. The adhesion receptors not only guide the lymphocyte recirculation between the blood and lymphoid tissues, and lymphocyte migration to the inflamed tissues, but also migration of other types of leukocytes to the sites of inflammation.

The interaction between leukocytes and endothelial cells via adhesion receptors is a prerequisite for a functional immune defence. However, the same mechanisms that enable leukocyte migration to tissues are also involved in adverse events like the development of autoimmune diseases and tumor growth. There is a countless number of patients suffering from inflammatory disorders world wide. Controlling excessive or misdirected leukocyte traffic by blocking the function of the adhesion molecules that mediate leukocyte-endothelial interactions, would potentially provide the tools to prevent the onset of inflammatory disorders, restrain the progress of disease or at least attenuate the symptoms. In addition, blocking the migration of a defined leukocyte subtype to certain tissues would be more selective and have less side effects than conventional drugs such as corticosteroids or non-steroidal anti-inflammatory drugs. However, the targeted therapy may still compromise the immune system. Therefore, further studies on the behaviour of molecules involved in leukocyte-endothelial interactions, and a broader understanding of the consequences of blocking the function of these molecules are needed. This study focuses on two molecules, namely VAP-1 and CLEVER-1, which are known to mediate the adhesive events between endothelia and leukocytes *in vitro*. The work elucidates the role of these molecules *in vivo* in physiological and pathological conditions, and finally, evaluates their use as targets for anti-adhesive therapies.

# 2. REVIEW OF THE LITERATURE

## 2.1 Leukocyte traffic

One of the most crucial aspects of the immune response is leukocyte trafficking; lymphocytes continuously patrol the body by circulation from the blood to lymphoid tissues and back into the bloodstream again, and leukocytes are recruited to sites of inflammation. Although the concept of lymphocyte recirculation was established earlier, in 1964 Gowans and Knight unambiguously showed that lymphocytes move from the blood circulation into the lymph via lymph nodes. They demonstrated that lymphocytes enter the lymph nodes by migration across the walls of post-capillary venules with remarkably high endothelia, also known as high endothelial venules (HEV) because of the distinct morphology of their cells. (Gowans and Knight, 1964.) Even though lymphocytes enter the lymph nodes also via afferent lymphatics, the main entry route is from the blood via post-capillary venules (Hall and Morris, 1965). Mackay et al. studied the distribution of lymphocyte subsets in blood, afferent lymph as well as in efferent lymph, and noticed that there were differences in the percentages of lymphocyte subsets in the studied vessels, indicating that cell migration is a regulated process. The same study also illustrated that for example macrophage-like cells were only found in the afferent lymph entering the lymph node, but they were not seen in the efferent lymph, indicating that these cells are not recirculating. (Mackay et al., 1988.) Furthermore, it was noted that naïve and memory lymphocytes have different recirculation patterns (Mackay et al., 1990). Effector and memory cells do not recirculate randomly, but preferentially to the tissue where the antigen was initially encountered and the lymphoid tissues related to that tissue, for example to gut-associated lymphoid tissues or to peripheral lymph nodes. That is, effector and memory cells can also recirculate through so called extralymphoid immune effector sites, such as inflamed skin and synovium. This selective recirculation assures that cells re-encounter their specific antigens more reliably and quickly. (Griscelli et al., 1969; Cahill et al., 1977; Chin and Hay, 1980; Issekutz et al., 1980; Jalkanen et al., 1986a.) Some leukocytes, like monocytes and granulocytes, do not recirculate but are recruited to sites of inflammation. Both of these fundamental processes, the continuous recirculation of lymphocytes and the recruitment of leukocytes to sites of inflammation, are mediated by interactions between leukocytes and the endothelium. (Springer, 1995; Fabbri et al., 1999.) Figure 1 illustrates the migratory routes of T cells as an example of lymphocyte traffic in the body.

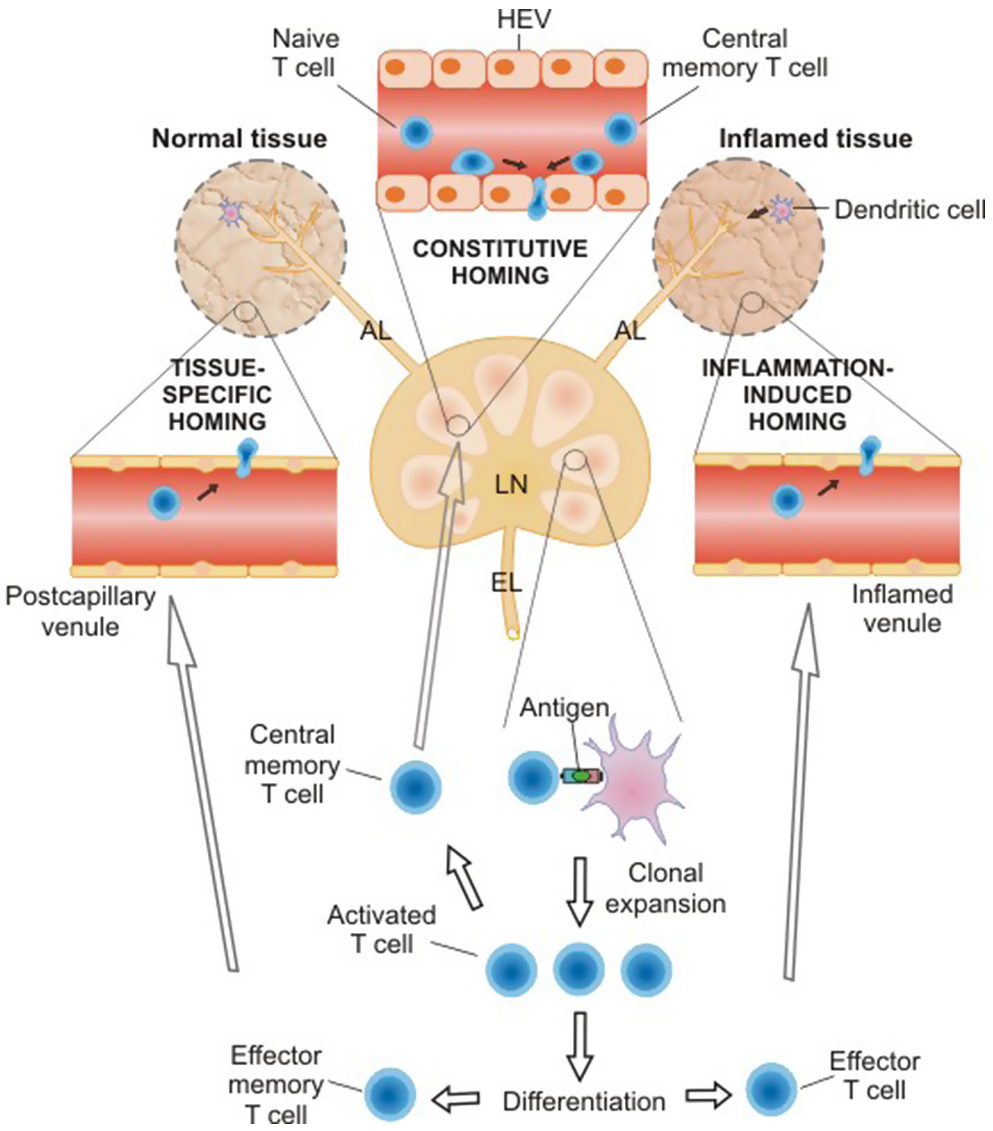


**Figure 1.** Lymphocyte recirculation: T cell migratory routes as an example. Naïve T cells migrate constitutively from the blood to lymphoid tissues through HEVs. Antigen presenting cells migrate to the lymph nodes via afferent lymphatics and present the antigen to the lymphocytes. After antigen stimulation, T cells proliferate and differentiate into effector and memory cells. These cells migrate preferentially to the tissue of initial antigen invasion and related lymphoid tissues. (LN – lymph node/secondary lymphoid tissue, AL – afferent lymphatic vessel, EL – efferent lymphatic vessel) (Modified from von Andrian and Mackay, 2000.)

## 2.2 Adhesion cascade

The leukocyte – the endothelial cell interaction that leads to the migration of leukocytes to the tissue is an active process that can be divided in to sequential steps. In the classical adhesion cascade these consecutive steps are: tethering and rolling, activation, firm adhesion and finally diapedesis i.e. transmigration through the endothelium. (Butcher, 1991; Butcher and Picker, 1996.) Ley et al. have proposed a renewed version of the leukocyte adhesion cascade that includes the following steps: capture, rolling, slow rolling, arrest, adhesion strengthening, intravascular crawling, paracellular and transcellular migration leading to migration through the endothelial basement membrane and pericyte layer (Ley et al., 2007). The adhesion cascade is illustrated in figure 2.

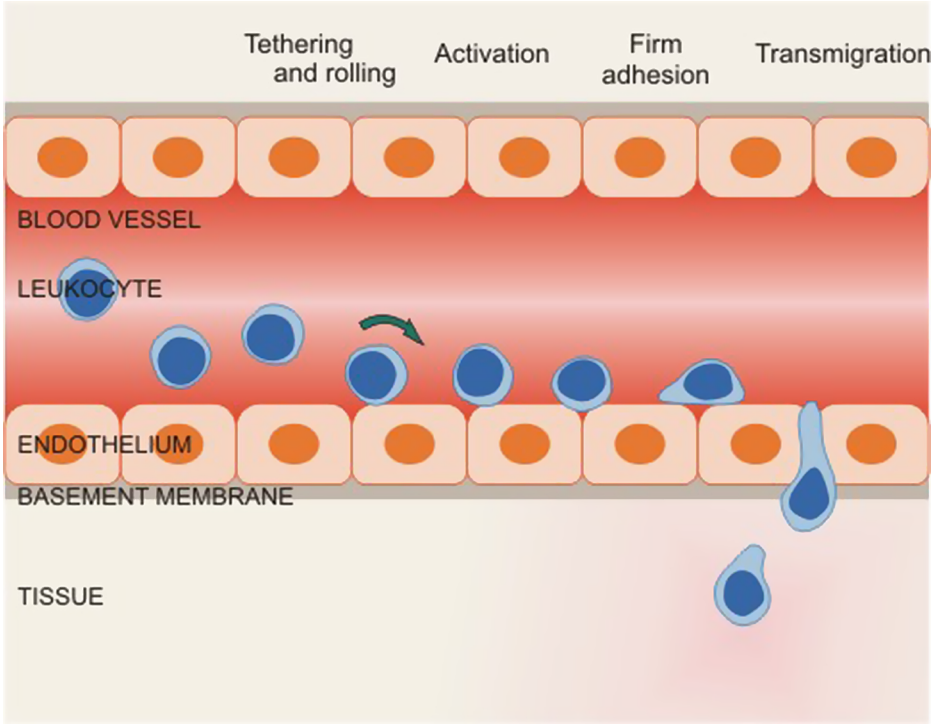


**Figure 2.** The multistep adhesion cascade. Leukocytes first tether and start to roll along the endothelium, then become activated, adhere firmly, transmigrate into the tissue through the endothelium and the basement membrane, and finally migrate in the tissue towards a chemokine gradient. (Modified from Springer, 1994.)

### 2.2.1 Primary adhesion - tethering and rolling

The first steps in the leukocyte adhesion cascade are tethering and rolling. Initial contacts between leukocytes and endothelia slow the velocity of the leukocytes enabling leukocytes to roll on the endothelia. This primary adhesion step is mainly

mediated by selectins and their ligands. The selectin family consists of three family members: E-selectin, L-selectin and P-selectin. E-selectin is expressed on activated endothelial cells, L-selectin constitutively on the majority of leukocytes, and P-selectin on platelets and endothelial cells. In the resting state P-selectin is stored in secretory granules, so called Weibel-Palade bodies in endothelial cells and in α-granules in platelets, but is rapidly mobilized to the cell surface upon stimulation with histamine or oxygen radicals like, for example, hydrogen peroxide. Selectins are also found in soluble form in plasma. These three C-type lectins bind to fucosylated carbohydrates such as sialyl Lewis X (sLeX) in a calcium-dependent manner. P- and L-selectin also bind to sulphated glycans such as heparin. The physiologically relevant selectin ligands for leukocyte traffic are glycoproteins: P-selectin glycoprotein ligand 1 (PSGL-1) and endoglycan are ligands for all three selectins; L-selectin may also interact with glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), sulphated glycoprotein of 200 kD (Sgp200), and Podocalyxin, for example; and E-selectin binds to E-selectin ligand (ESL-1) and CD44, for example. Selectins may also interact with each other, as P-selectin and E-selectin are able to bind to L-selectin. (Lasky, 1992; Kansas, 1996; McEver, 2002; Rosen, 2004; Sperandio, 2006; Grailer et al., 2009.)

Although selectins play a major role in the primary adhesion step, there are also other molecules, like CD44, involved in the rolling phase. E-selectin is one of the many molecules that interact with CD44, and these molecules mediate the rolling (Dimitroff et al., 2001). However, CD44 is also able to mediate the rolling independent of selectins (DeGrendele et al., 1996).

### 2.2.2 Activation, arrest and firm adhesion

The engagement of selectins in the rolling step may lead to activation, but more importantly the rolling of leukocytes along the endothelium allows for more time for interactions to occur between the leukocytes and endothelial cells, and interacting cells become exposed to local activators: chemokines and other chemoattractants. Activation by chemoattractants leads to changes in integrin avidity and affinity. After activation integrins bind to the immunoglobulin superfamily members and as a result, the leukocyte adheres more firmly to the endothelium. (Kansas, 1996; Ley et al., 2007.)

Chemokines are a large family of low molecular weight chemoattractant peptides that bind to G protein-coupled receptors. Chemokines are subdivided into four main classes: C-C, C-X-C, C-$X_3$-C and C, based on the conserved cysteine residues in their amino acid sequences. Most chemokines are produced as secreted molecules, but are bound to the endothelium e.g. by glycosaminoglycans. Chemokines are ideal for controlling the selective recruitment of leukocytes, as specific chemokines are present in different tissues. Chemokines are also differentially expressed in the resting and in the inflammatory state. Furthermore, the expression of chemokine receptors varies in different leukocyte subsets and is dependent on leukocyte activation. Binding of

chemokines to their G protein-coupled ligands leads to so-called inside-out signaling: rapid integrin activation due to the triggering of a complex intracellular signaling network. (Olson and Ley, 2002; Johnston and Butcher, 2002; Sallusto and Mackay, 2004.)

Integrins are transmembrane heterodimers, which consist of an α- and β-subunit. The name integrins was originally given to illustrate a feature of these molecules as the link between the extracellular and intracellular sides of cells. Thus, integrins are involved both in outside-in signaling and in inside-out signaling. (Larson and Springer, 1990; Hogg et al., 2011.) All leukocytes express a particular set of integrins, and as a commonality, all leukocyte subtypes express $\beta_2$-integrins. The expression of integrins is signal- and time-dependent. (Harris et al., 2000.) Among the most important integrins in leukocyte – endothelial interactions are the Lymphocyte function associated antigen-1 (LFA-1, αLβ2), Macrophage-1 antigen (Mac-1, αMβ2), p150,95 (αXβ2), Very late antigen-4 (VLA-4, α4β1) and α4β7 (Carlos and Harlan, 1994; Laudanna and Bolomini-Vittori, 2009). The ligands of the integrins are immunoglobulin superfamily members, cell-surface proteins with immunoglobulin-like domains. For example, the immunoglobulin superfamily members Intercellular adhesion molecule -1 and -2 (ICAM-1 and ICAM-2) are ligands for LFA-1 and Mac-1, whereas Vascular cell adhesion protein -1 (VCAM-1) and MAdCAM-1 are ligands for VLA-4 and α4β7. (Carlos and Harlan, 1994; Yonekawa and Harlan, 2005; Takada et al., 2007; Smith, 2008; Laudanna and Bolomini-Vittori, 2009; Barczyk et al., 2010.)

### 2.2.3 Crawling and transmigration

Following arrest and firm adhesion, leukocytes, at least neutrophils and monocytes, crawl intraluminally seeking the optimal site of transmigration (Kelly et al., 2007; Ley et al., 2007; Sanz and Kubes, 2012). It has been shown in an *in vitro* setting that at least LFA-1 and Mac-1 with ICAM-1 and ICAM-2 mediate the crawling of monocytes on endothelial cells (Schenkel et al., 2004), and in an *in vivo* setting that Mac-1 mediates the crawling of neutrophils before the transendothelial migration (Phillipson et al., 2006).

The final step in the leukocyte adhesion cascade is the transmigration through the endothelium. For a long time it was debated whether leukocytes migrate between the endothelial cells (paracellular route) or straight through the endothelial cell (transcellular route). It has now been accepted that leukocytes are able to use either one of these routes. The molecules that participate in leukocyte diapedesis include for example CD99 and the immunoglobulin superfamily members: ICAM-1, ICAM-2, platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31), junctional adhesion molecules A, B and C (JAM-A, -B, -C), VCAM-1 and endothelial cell-selective adhesion molecule (ESAM). ICAM-1 and ICAM-2 interact with LFA-1 also during transmigration. The clustering of ICAM-1 and VCAM-1 prior to and during transmigration stimulates a number of signals that are needed for diapedesis. ESAM and CD99 interact in a homophilic manner, and PECAM-1 and JAMs interact in a

homophilic and a heterophilic manner. JAMs interact with integrins: JAM-A with LFA-1, JAM-B with VLA-4 and JAM-C with Mac-1. JAM-B and JAM-C may also bind to each others. CD177 has been identified as a ligand for PECAM-1. In addition to these molecules that support leukocyte extravasation, there are molecules which function in an opposite way by blocking diapedesis. One of these is the vascular endothelial cadherin (VE-cadherin) that is transiently lost from the adherens junctions during leukocyte transmigration. (Vestweber, 2007; Langer and Chavakis, 2009; Muller, 2011; Ley et al., 2007.)

## 2.3 VAP-1

In the early 1990s, when very little was known about the adhesion molecules, expressed by endothelial cells, which mediate tissue specific lymphocyte homing to inflamed joints, Salmi and Jalkanen identified a new molecule by producing monoclonal antibodies against human synovial vessels. One new monoclonal antibody - 1B2 - stained HEV-like venules in inflamed synovial samples and the majority of HEVs in peripheral lymph nodes and tonsils. The antigen recognized by 1B2 was named VAP-1 for vascular adhesion protein-1, since *in vitro* adhesion assays revealed that VAP-1 mediates lymphocyte binding to HEVs. (Salmi and Jalkanen, 1992.)

### 2.3.1 Expression of VAP-1

VAP-1 is strongly expressed in the vascular endothelial cells of HEVs and moderately in follicular dendritic cells in peripheral lymph nodes and tonsils. In large vessels, like the aorta, VAP-1 is expressed in smooth muscle cells. Moderate to slight expression is also found in smooth muscle cells in the intestine, the sinusoidal endothelium of the liver, the intertubular vessels of the kidneys, and the endothelial cells of the endocardium in the heart. (Salmi et al., 1993.) VAP-1 is also expressed in the endothelium of pulmonary vessels in the lungs (Singh et al., 2003). In contrast, for example epithelial cells, leukocytes and fibroblasts do not express VAP-1. The expression of VAP-1 is localized to the luminal cell surface and to the cytoplasmic granules of endothelial cells. (Salmi et al., 1993.) These VAP-1 positive cytoplasmic granules are distinct from Weibel-Palade bodies where P-selectin, for example, is stored and released (Salmi and Jalkanen, 1995).

VAP-1 expression markedly increases at sites of inflammation, such as in the inflamed gut of inflammatory bowel disease (Salmi et al., 1993), inflamed synovium (Akin et al., 2001) and in chronic skin inflammations like psoriasis, lichen ruber planus (Arvilommi et al., 1996; Madej et al., 2007) and atopic eczema (Madej et al., 2006). In 2000, Jaakkola et al. proposed, based on studies using pigs and dogs, that VAP-1 is an intracellular molecule in the noninflamed state and translocates onto the cell surface only after a proinflammatory stimulus (Jaakkola et al., 2000b).

### 2.3.2 Soluble VAP-1 in serum

VAP-1 is also found in soluble form in the blood circulation (Kurkijarvi et al., 1998). Soluble VAP-1 levels in the serum of healthy individuals are reasonably stable, but they are elevated in certain inflammatory disorders for example in many types of chronic liver diseases like alcoholic cirrhosis, viral hepatitis and primary biliary cirrhosis (Kurkijarvi et al., 1998; Kurkijarvi et al., 2000). Serum levels of soluble VAP-1 are also increased in patients with chronic kidney disease (Kurkijarvi et al., 2001; Lin et al., 2008), diabetes (Salmi et al., 2002), atopic eczema (Madej et al., 2006), inflammatory active relapsing remitting multiple sclerosis (Airas et al., 2006), psoriasis (Madej et al., 2007), acute ischemic stroke (Airas et al., 2008) and septic shock (Sallisalmi et al., 2012). Soluble VAP-1 levels may also increase during tumor growth, as soluble VAP-1 levels are elevated in the serum of patients with hepatocellular carcinoma (Kemik et al., 2010). Levels of soluble VAP-1 are also increased in the serum of colorectal as well as gastric cancer patients. Interestingly the loss of serum VAP-1 levels was associated with disease progression – metastases and poor overall survival in these patients. (Toiyama et al., 2009; Yasuda et al., 2011.) However, increased serum levels are not seen in all inflammatory disorders, as an elevation in soluble VAP-1 levels in serum was not associated with for example rheumatoid arthritis and inflammatory bowel disease (Kurkijarvi et al., 1998; Koutroubakis et al., 2002).

Originally it was proposed that soluble VAP-1 is derived from the liver (Kurkijarvi et al., 2000). Abella et al. showed that 3T3-L1 adipocytes release soluble VAP-1 and that this release is metalloprotease dependent and enhanced by tumor necrosis factor-α (TNF-α) (Abella et al., 2004). Finally, Stolen et al. showed that under normal physiological conditions the major source of circulating VAP-1 are vascular endothelial cells, and during biological stress, such as diabetes, both adipocytes and endothelial cells are able to release soluble VAP-1 into the circulation (Stolen et al., 2004).

### 2.3.3 VAP-1 is an adhesion molecule

Originally the functional role of VAP-1 in the lymphocyte – endothelial interaction was studied using a Stamper-Woodruff type *in vitro* adhesion assay, which is a standard method that has been used already for over 35 years. Treatment of frozen tissue sections with the 1B2 anti-VAP-1 antibody decreased the binding of lymphocytes to the HEVs of tonsil, peripheral lymph node (PLN) and synovium samples. (Salmi and Jalkanen, 1992.) Since then, many other studies have shown the adhesive function of VAP-1 *in vitro*. For example 1B2 has been shown to decrease the binding of CD8 positive T-cells to PLN HEVs, the binding of natural killer (NK) cells to the HEVs of inflamed tonsil (Salmi et al., 1997b; Salmi et al., 1998) and the adhesion of lymphocytes to the venules of chronically rejected renal grafts (Kurkijarvi et al., 2001). The 1B2 anti-VAP-1 antibody also inhibits lymphocyte binding to inflamed mucosa (Salmi et al., 1993) and skin (Arvilommi et al., 1996), as well as

granulocyte binding to the cardiac vessels of ischemic hearts (Jaakkola et al., 2000a). Moreover, 1B2 inhibits T-cell binding to the hepatic endothelium (McNab et al., 1996) and binding of small mucosal lymphocytes and mucosal effector cells but not mucosa-derived macrophages to the venules of inflamed synovium (Salmi et al., 1997a). VAP-1 expression is up-regulated in sinusoids, hepatocytes and bile ducts during acute liver allograft rejection in rat (Martelius et al., 2000) and the anti-VAP-1 antibody 174-5 inhibits immunoblast adhesion to the vasculature of rejecting rat liver allografts (Martelius et al., 2004). Using cultured hepatic endothelial cells and a TK8-14 anti-VAP-1 antibody, it has been shown that VAP-1 mediates adhesion and transmigration of CD16 positive monocytes (Aspinall et al., 2010). The TK8-14 anti-VAP-1 antibody also decreased the number of transmigrating regulatory T cells through human hepatic sinusoidal endothelium (Shetty et al., 2011). In intravital microscopy studies VAP-1 was shown to mediate the initial interaction between lymphocytes and the inflamed endothelium (Salmi et al., 1997b), the rolling of granulocytes on the inflamed endothelium (Tohka et al., 2001) and finally the slow rolling, firm adhesion and transmigration of leukocytes during an inflammatory stimulus (Stolen et al., 2005).

VAP-1 is expressed on the vascular endothelium of tumors and mediates the adhesion of tumor-infiltrating lymphocytes, lymphokine-activated killer cells and natural killer cells to the tumor vasculature *in vitro* (Yoong et al., 1998; Irjala et al., 2001). VAP-1 supports the recruitment of proangiogenic and immune-suppressing Gr-1+CD11b+ myeloid leukocytes into the tumors and enhances tumor neoangiogenesis, and is thus involved in tumor growth (Marttila-Ichihara et al., 2009; Marttila-Ichihara et al., 2010).

### 2.3.4 Human versus mouse VAP-1

In humans VAP-1 is a sialylated 170-180 kDa glycoprotein (Salmi and Jalkanen, 1995) and the sialic acids are needed for the adhesive function of VAP-1 (Salmi and Jalkanen, 1996). The mature 170-180 kDa form is homodimeric type II transmembrane protein that consists of two identical 90 kDa subunits (Salmi et al., 1998; Smith et al., 1998).

The amino acid sequence identity between human and mouse VAP-1 is 83 % (Bono et al., 1998). In immunohistological analyses human and mouse VAP-1 have very similar expression patterns, except VAP-1 is expressed in the peritubular capillaries of human kidneys and the sinusoidal endothelium of human liver, while mouse kidneys were negative and mouse liver was either negative or only faintly positive for VAP-1 depending on the mouse strain (Bono et al., 1999). The molecular weight of mouse VAP-1 is 220 kDa, and it is composed of two 110 kDa subunits compared to 170-180 kD/90 kDa in humans. The number of potential glycosylation sites is higher in mouse VAP-1 than in the human VAP-1 protein core – both six potential N- and O-glycosylation sites in mouse compared to six and three potential sites respectively in humans. (Bono et al., 1998; Smith et al., 1998.)

### 2.3.5 VAP-1 is an ectoenzyme

The cloning of human and mouse VAP-1 in 1998 revealed that VAP-1 did not share significant similarity with any other adhesion molecule known at the time, but interestingly VAP-1 shared significant identity with a family of enzymes called copper containing amine oxidases (Bono et al., 1998; Smith et al., 1998). Amine oxidases are enzymes that metabolize amines produced endogenously or absorbed as dietary or xenobiotic substances (Lyles, 1996; O'Sullivan et al., 2004). And indeed, VAP-1 possesses monoamine oxidase (MAO) activity and moreover, this activity can be inhibited by semicarbazide and hydroxylamine, indicating that VAP-1 is a semicarbazide-sensitive amine oxidase (SSAO) (Smith et al., 1998). SSAO enzymes catalyze the oxidative deamination of primary amines to the corresponding aldehyde, which leads to the release of hydrogen peroxide and ammonium (Lyles, 1996; O'Sullivan et al., 2004). It is noteworthy, that VAP-1 is the only source of serum SSAO activity in mouse, and VAP-1 is also the major, if not the only, soluble SSAO accounting for serum monoamine oxidase activity in humans (Stolen et al., 2004; Stolen et al., 2005; Kurkijarvi et al., 2000). Comparing the SSAO activity of human and mouse VAP-1 reveals differences in substrate specificity: for example, mouse VAP-1 shows monoamine oxidase activity towards methylamine, tyramine, tryptamine and β-phenylethylamine, whereas of these substrates, human VAP-1 only oxidizes methylamine (Bono et al., 1999).

The crystallization and preliminary X-ray analysis of human VAP-1 was performed in 2003 (Nymalm et al., 2003). Besides similarities in protein sequence, the structure of VAP-1 is similar to other copper-containing amine oxidases (CAOs) consisting of three domains: namely D2, D3 and D4. The appearance of these molecules is described as mushroom- or heart-shaped. D4 is the most conserved region in CAOs and contains the active site. The sequence similarity between the human and mouse VAP-1 D4 domain is 82,1 %. The D4 domain of human VAP-1 also shares sequence similarity with, for example, the bovine amine oxidase (82,5 % identity) and the *Escherichia coli* copper-containing amine oxidase (26,9 %). (Salminen et al., 1998.) Although the CAO structures are comparable, VAP-1 possesses unique features as well: the RGD motif and the transmembrane domain being the most noteworthy ones. (Salminen et al., 1998; Airenne et al., 2005; Jakobsson et al., 2005.)

In 2001 Salmi et al. showed that the catalytic activity of VAP-1 is connected to its adhesive function. This was the first time it was shown that an adhesion molecule can ectoenzymatically have an effect on an adhesion cascade. (Salmi et al., 2001.) Further evidence for the importance of the enzymatic activity of VAP-1 for its adhesive function was published in 2002 by Lalor et al. and in 2005 by Edwards et al., who showed that VAP-1 mediates the transendothelial migration of lymphocytes through cultured human hepatic sinusoidal endothelial cells *in vitro* in an SSAO dependent manner, and 2004 by Koskinen et al. when they demonstrated that VAP-1 mediates the rolling and transmigration of polymorphonuclear leukocytes on and through endothelial cells in a SSAO dependent manner (Lalor et al., 2002; Edwards et al.,

2005; Koskinen et al., 2004). The oxidative reaction catalyzed by VAP-1 is also able to induce the expression of other molecules involved in the leukocyte extravasation cascade, for example MAdCAM-1 (Liaskou et al., 2011), E-selectin and P-selectin (Jalkanen et al., 2007). Furthermore, activation of VAP-1 on the liver endothelium upregulates ICAM-1 and E-selectin adhesion molecules and leads to secretion of the CXCL8 (IL-8) chemokine in a nuclear factor-κB dependent manner (Lalor et al., 2007).

### 2.3.6 Ligands of VAP-1

The first reported counter receptor for VAP-1 on leukocytes was Sialic acid Ig-like lectin-10 (Siglec-10). Siglec-10, expressed on subsets of leukocytes including monocytes, eosinophils, B-cells and natural killer-like cells (Cao and Crocker, 2010), not only mediates the binding of lymphocytes to endothelial VAP-1, but it also acts as a substrate for VAP-1. (Kivi et al., 2009.) Later, also Siglec-9 was reported to be a ligand for VAP-1 (Aalto et al., 2011). Siglec-9 is expressed on, for example, neutrophils, monocytes and dendritic cells (Cao and Crocker, 2010).

## 2.4 CLEVER-1

In order to reveal novel migration-associated molecules in lymphatic endothelium Irjala et al. produced antibodies against efferent lymphatic vessels. Two of the produced antibodies recognized small lymphatic vessels, lymphatic sinusoids and HEVs in lymphoid tissues, and afferent lymphatic vessels in non-lymphoid tissues like bronchus, esophagus, heart, intestine, kidney, liver, lung and skin. The expression of this molecule was found to be upregulated on HEV-like vessels at sites of inflammation, but the expression was absent from peripheral blood leukocytes and also the blood vessel endothelia of non-lymphoid tissues in their normal state. Based on the expression profile the antigen was designated as common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1). (Irjala et al., 2003b.)

### 2.4.1 Structural characteristics of CLEVER-1

CLEVER-1 is a large 270-300 kDa sialoglykoprotein that has at least three isoforms. A sequence analysis delineated that CLEVER-1 is a type I transmembrane protein containing two RGD motifs, 7 Fasciclin domains, 22 epidermal growth factor (EGF)-like repeats and a proteoglycan link homology region. (Irjala et al., 2003b.)

### 2.4.2 CLEVER-1 mediates leukocyte adhesion

In *in vitro* adhesion assays an antibody against CLEVER-1 inhibits lymphocyte adhesion to the vascular and lymphatic endothelium in lymphoid organs, and lymphocyte, monocyte and granulocyte adhesion to HEV-like vessels in inflamed non-lymphoid tissues (Irjala et al., 2003b). Moreover, the antibody blocks adhesion of the cells of malignant cell-lines to both the lymphatic and the vascular endothelium (Irjala et al., 2003a). Studies using peripheral blood mononuclear cells and cultured lymphatic

and vascular endothelium have revealed that CLEVER-1 is involved in the transmigration step of the leukocyte adhesion cascade (Salmi et al., 2004). Recently it was shown that CLEVER-1 mediates lymphocyte transmigration, especially the transmigration of regulatory T cells, through cultured human hepatic sinusoidal endothelial cells (Shetty et al., 2011). Furthermore, CLEVER-1 is expressed in placental macrophages and mediates the transmigration of these cells through the vascular endothelium (Palani et al., 2011).

### 2.4.3 CLEVER-1 is also known as Stabilin-1 and FEEL-1

While the antigen recognized by antibodies 3-266 and 3-372 was named CLEVER-1, an identical molecule was named Stabilin-1 and FEEL-1 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1). Stabilin-1 was reported to be a MS-1 antigen and to be expressed in alternatively activated (type II) macrophages and sinusoidal endothelial cells in spleen (in human but not in mouse), liver and lymph nodes (Politz et al., 2002). Stabilin-1 is also expressed in bone marrow sinusoidal endothelial cells (Qian et al., 2009). When studied by Northern-blot analysis human aortic endothelial cell-line cells were reported to express Stabilin-1, whereas human umbilical vein endothelial cells did not express Stabilin-1 (Politz et al., 2002). FEEL-1 was first described by Adachi and Tsujimoto when they were searching for scavenger receptors in endothelial cells. With an expression cloning technique they cloned a receptor containing fasciclin, EGF-like, laminin-type EGF and link domains, and based on this domain structure named the molecule FEEL-1. FEEL-1 was found to be expressed in several tissues, prominently in spleen, lymph nodes and CD14 positive mononuclear cells in humans. FEEL-1 expression was also evident in endothelial cells such as human coronary arterial endothelial cells and human microvascular endothelial cells. And although human umbilical vein endothelial cells were stated to be Stabilin-1 negative, Adachi and Tsujimoto detected FEEL-1 mRNA in these cells by RT-PCR. (Adachi and Tsujimoto, 2002.) In mouse, FEEL-1 expression is observed in liver, lung, heart, white adipose tissue, aorta, kidney, spleen and peritoneal macrophages by Northern-blot analysis (Tamura et al., 2003).

FEEL-1 was reported to be a receptor for acetylated low density lipoprotein (acLDL) (Adachi and Tsujimoto, 2002) and for advanced glycation end products (AGEs) (Tamura et al., 2003). It was also shown to bind to Gram-negative and Gram-positive bacteria. Moreover, in the *in vitro* Matrigel tube formation assay an anti-FEEL-1 monoclonal antibody reduced cell-cell interactions suggesting a role for FEEL-1 in angiogenesis. (Adachi and Tsujimoto, 2002.) Like FEEL-1, Stabilin-1 has been reported to mediate the internalization of acLDL (Kzhyshkowska et al., 2005). Stabilin-1 transfected cells also actively take up oxidized low-density lipoprotein (oxLDL) (Li et al., 2011). In addition to acLDL and oxLDL, Stabilin-1 interacts with secreted protein acidic and rich in cysteine (SPARC) (Kzhyshkowska et al., 2006b) and placental lactogen (PL) (Kzhyshkowska et al., 2008) targeting these molecules to the endocytic pathway in macrophages. Furthermore, Stabilin-1 interacts with stabilin-1 interacting chitinase-like protein (SI-CLP) in macrophages as part of the intracellular

sorting and lysosomal delivery of SI-CLP (Kzhyshkowska et al., 2006a). Recently, it was shown that Stabilin-1 mediates the clearance of damaged red blood cells in a phosphatidylserine dependent manner in type II macrophages (Park et al., 2009) and Stabilin-1 in hepatic sinusoidal endothelial cells enhances the phagosytosis of damaged red blood cells by macrophages (Lee et al., 2011). Using transfected cells, the 70 kDa heat shock protein (Hsp70) has also been shown to be a ligand for Stabilin-1 (Murshid et al., 2011).

## 2.5 Adhesion molecules and cancer

### 2.5.1 Inflammation and cancer

In 2000 Hanahan and Weinberg proposed that the self-sufficiency in growth signals, disregard of growth-inhibitory signals, avoidance of apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis are the six hallmarks of cancer (Hanahan and Weinberg, 2000). Later it was suggested that a seventh characteristic, namely inflammation, should be added to the list (Mantovani, 2009; Pages et al., 2010). Evidently inflammation has been linked to cancer for a long time as chronic inflammation due to infection or autoimmune disease for instance often precedes tumor growth. However, there are exceptions, like psoriasis for instance that is rarely connected to cancers. While chronic inflammation facilitates neoplastic changes, the use of non-steroidal anti-inflammatory drugs may be protective. The second link between inflammation and cancer is provided by the fact that basically all tumors are infiltrated by immune cells. These immune cells, including for example macrophages, neutrophils, mast cells, myeloid derived suppressor cells, dendritic cells, natural killer cells and T cells, can either be protective and suppress tumor growth or then vice versa, promote tumor growth, invasion, metastasis and angiogenesis. (Nickoloff et al., 2005; Mantovani et al., 2008; Grivennikov et al., 2010; Egeblad et al., 2010.) Although not listed as a hallmark of cancer in 2000 by Hanahan and Weinberg, inflammation is crucial to many of the hallmark events. In 2011 Hanahan and Weinberg published a new review on the hallmarks of cancer. In addition to the six previously stated hallmarks, they had added two enabling characteristics that make other features possible: the first enabling characteristic is tumor-promoting inflammation and the second is genome instability and mutation. Besides these two enabling characteristics, Hanahan and Weinberg also listed two emerging hallmarks: evading immune destruction and reprogramming energy metabolism. (Hanahan and Weinberg, 2011.)

### 2.5.2 Metastasis

Metastasis is a multistep process in which a malignant cell escapes the original tumor site, intravasates into lymphatic or blood vessels, survives in the circulation, extravasates at distant sites and starts to grow there. There are estimates that less than 0,01 % of malignant cells that enter the circulation survive and finally form metastases. Still, metastases cause 90 % of cancer mortality. (Gupta and Massague, 2006; Joyce and Pollard, 2009; Nguyen et al., 2009; Grivennikov et al., 2010.)

Most malignant tumors form metastases organ-specifically. Although it has been debated whether malignant cell extravasation is caused by specific interactions between the tumor cell and the vessel wall or by size restricted entrapment, studies have shown that there are specific interactions that are mediated by adhesion molecules involved in the process. (Schluter et al., 2006.) For example α6-, β1- and β4-integrins mediate colon carcinoma cell adhesion and extravasation into the liver parenchyma and α3β1-integrin mediates fibrosarcoma cell arrest to pulmonary vasculature and colony formation in the lungs (Enns et al., 2004; Wang et al., 2004).

## 2.6 Adhesion molecules as therapeutic targets

Leukocyte migration to inflamed tissues is fundamental to the normal immune response. However, the same mechanisms are involved in the pathogenesis of various conditions such as autoimmune diseases, ischemia – reperfusion injury, hypersensitivity reactions, transplant rejection as well as tumor metastasis. Conventional anti-inflammatory drugs, like corticosteroids, non-steroidal anti-inflammatory drugs and chemotherapeutic agents have severe side-effects. Blocking the adhesion cascade by targeting adhesion molecules could provide more specific and effective solutions for cope with a misdirected, overly excessive and harmful inflammatory response in inflammatory disorders. (Gonzalez-Amaro et al., 1998; Ulbrich et al., 2003.) The versatility of adhesion molecules and their expression in particular subsets of cells as well as tissue-specific cell migration pathways (for example skin- and gut-associated) provide an opportunity for selective inhibition. However, too selective blocking might be ineffective and too broad blocking might end up to be overly immunocompromising (Luster et al., 2005; Mackay, 2008). Adhesion molecules can be targeted in many ways, for example by function blocking monoclonal antibodies or small-molecule inhibitors (Ulbrich et al., 2003; Mackay, 2008; Mousa, 2008). Since monoclonal antibodies were used in this study, the next paragraphs will focus on monoclonal antibody –based therapies for inflammatory disorders.

### 2.6.1 Therapeutic antibody Tysabri - Natalizumab

The first example of a monoclonal antibody based therapy is Natalizumab, a recombinant humanized monoclonal anti-α4 integrin antibody, for the treatment of highly active relapsing-remitting multiple sclerosis (MS). MS is a chronic inflammatory disease, which damages the myelin sheath surrounding nerve fibres in the central nervous system leading to physical and cognitive disability (Rice et al., 2005; Ontaneda et al., 2012).

The α4 subunit of integrins forms heterodimers with β1 and β7 subunits, and is expressed predominantly in lymphocytes, monocytes, eosinophils and basophils. α4 integrins bind for example VCAM-1, MAdCAM-1, fibronectin, osteopontin and thrombospondin. (Rice et al., 2005.) In 1992 Yednock et al. showed that an antibody against α4 integrin blocked the binding of monocytes and lymphocytes to inflamed vessels in brain tissue in an *in vitro* binding assay, and prevented the onset or

decreased the severity of the disease in an *in vivo* inflammation model mimicking human multiple sclerosis. The authors hypothesized that a therapy based on blocking the function of α4 integrin may be effective in treating inflammatory diseases of the central nervous system. (Yednock et al., 1992.) Subsequent studies confirmed the therapeutic effect of α4 integrin targeting (Rice et al., 2005). Results of a six-month phase II trial using a humanized monoclonal antibody against α4 integrin (Natalizumab) were promising: following treatment there was a reduction in the number of inflammatory brain lesions in magnetic resonance imaging (MRI) scans and a decrease in the number of relapses, additionally the treatment was tolerated well, although there was a trend towards an increased rate of infections in treated patients (Miller et al., 2003). In 2006 Polman et al. reported the results of a two-year phase III trial with Natalizumab. In this trial, the treatment decreased the mean number of gadolinium-enhanced lesions over 90 %, and new or expanding $T_2$-hyperintense lesions over 83 %. Moreover, treatment brought an almost 70 % relative reduction to the rate of relapses. Fatigue and allergic reactions were more common in Natalizumab treated patients than in the control group, but there were no statistically significant differences in the rate of infections. The numbers of lymphocytes, monocytes, eosinophils and basophils, but not neutrophils, were elevated after Natalizumab therapy. (Polman et al., 2006.) Interferon-β (IFN-β) and glatiramer acetate, which have been used for the treatment of MS for over a decade now, have a fairly modest effect on relapse rate and disability progression. In IFN-β and glatiramer acetate trials the reduction in the rate of relapses has been approximately 30 % compared to a placebo treatment. (Hutchinson, 2010; Kieseier and Stuve, 2011.) Compared to these former drugs used for MS, Natalizumab seems to be more effective as a disease modifying drug.

The United States Food and Drug administration (FDA) approved an antibody against α4 (the trade name Tysabri, the active ingredient Natalizumab, manufactured by Biogen Idec) for treatment of patients with relapsing forms of MS in November 2004 and European Medicines Agency (EMA) approved it in June 2006 (FDA, 2012; EMA, 2012).

The FDA suspended the marketing of Tysabri in February 2005 due to three cases of progressive multifocal leukoencephalopathy (PML) in patients receiving the drug. Two of these cases were fatal. However, the FDA re-approved Tysabri one year later and the regulations were redefined such that Tysabri is administered only to patients that fail to respond to other treatments, like interferon-β, or to patients that have a highly active form of MS. (FDA, 2012.)

PML is a severe infection of the central nervous system caused by the reactivation of the John Cunningham (JC) virus, and which usually leads to severe disability or death (Bosch et al., 2011; Weissert, 2011). Since the re-approval of Tysabri new cases of PML have been reported and the duration of the treatment seems to correlate with the risk of developing PML. The overall risk estimates for PML vary between 1:500

and 1:1000, though according to the most extreme estimates the risk is as high as 1:100. (Miravalle and Corboy, 2010; Aly et al., 2011; Ontaneda et al., 2012; Schwab et al., 2011.)

In the beginning of 2008 Tysabri was also approved by the FDA for the treatment of moderately to severely active Crohn's disease, an inflammatory bowel disease, in adult patients who aren't responding to TNF-α inhibitors or other conventional therapies for Crohn's disease. In Europe the marketing authorisation for Natalizumab for the treatment of Crohn's disease was refused, due to the risk of serious infections including PML. (FDA, 2012; EMA, 2012.)

### 2.6.2 Therapeutic antibody Raptiva - Efalizumab

A second example of a monoclonal antibody based therapy is Efalizumab, a recombinant humanized monoclonal antibody against the CD11a subunit of LFA-1, for the treatment of moderate to severe plaque psoriasis. Plaque psoriasis is the most prevalent type of psoriasis, a chronic inflammatory disorder affecting the skin. In plaque psoriasis dermal inflammation and epidermal hyperproliferation result in red patches and scaly areas in the skin. (Wellington and Perry, 2005.)

Like Natalizumab, Efalizumab also targets integrins. LFA-1 comprises of αL and β2 integrin subunits (CD11a and CD18) and is expressed on all leukocytes apart from some macrophages (Springer et al., 1987). ICAM-1 is the major ligand for LFA-1, but LFA-1 also binds for example to other ICAM family members: ICAM-2, ICAM-3, ICAM-4 and ICAM-5. Besides ICAMs, also for instance JAM-A is a ligand for LFA-1. LFA-1 mediated interactions have a role in cell activation, leukocyte trafficking and antigen presentation. (Springer et al., 1987; Giblin and Lemieux, 2006.) The first studies using an anti-CD11a antibody called hu1124 to treat psoriasis were published between 1999 and 2001 (Bauer et al., 1999; Gottlieb et al., 2000; Papp et al., 2001). Even a single dose ranging from 0,3 to 10 mg/kg decreased epidermal thickness, the number of dermal and epidermal T cells in plaques, the expression of ICAM-1, and the Psoriasis Area and Severity Index (PASI) score (a rating for scaling, erythema and thickness of plaques) (Gottlieb et al., 2000). In the phase II study weekly infusions of hu1124 were administered for an 8 week time period at two different doses (0,1 mg/kg and 0,3 mg/kg) to patients with moderate to severe plaque psoriasis to evaluate its safety, pharmacodynamics and efficacy compared to a placebo. The higher dose (0,3 mg/kg) showed statistically significant improvements in the same aspects as the single dose in the phase I study described above. Furthermore, following the anti-CD11a antibody treatment no keratin 16 expression in suprabasal keratinocytes was observed, whereas keratin 16 expression was associated with hyperproliferative states in all epidermis biopsies collected from the placebo treated patients. The Anti-CD11a antibody treatment led to an increased number of circulating lymphocytes. After the first infusion, adverse events, like headache, fever, nausea, vomiting, back pain etc, were observed more frequently in the treatment group than in the placebo group. Overall, adverse effects were generally mild in severity and their incidence decreased

with subsequent doses to the same level as in the placebo group. (Papp et al., 2001.) The long-term efficacy of the anti-CD11a antibody treatment with weekly subcutaneous injections was associated with further reductions in disease severity, the incidence of adverse events did not increase over time, the incidence of infections was not significantly different compared to the placebo-treatment and serious infections requiring hospitalization occurred in 1,1 % of the patients during the anti-CD11a antibody treatment versus 0,3 % in the placebo treated group (Frampton and Plosker, 2009).

The FDA approved an antibody against CD11a (the trade name Raptiva, the active ingredient Efalizumab, manufactured by Genentech) for adult patients with chronic, moderate to severe plaque psoriasis in October 2003 and the European Medicines Agency approved Raptiva (Serono Europe Ltd) in September 2004. However, in 2009 Raptiva was voluntarily withdrawn from the U.S. market by Genentech and from the European Union by Serono Europe Ltd due to an increased risk of PML. (FDA, 2009; EMA, 2009.)

Both of these above described antibody therapies, Tysabri and Raptiva, are effective in preventing a harmful immune response, but are also associated with an increased risk of PML, although the overall risk of infections is not increased significantly during the treatment. Nevertheless, the cases of PML show that there is a risk of pitfalls when the normal immune defence is compromised.

# 3. AIMS OF THE STUDY

Leukocyte migration to inflamed areas is fundamental for eliminating infections. However, the mechanisms that guide leukocytes to sites of inflammation are also involved in pathological conditions such as autoimmune diseases and tumor metastasis. Moreover, tumors are infiltrated by leukocytes, and these leukocytes may either promote or prevent the growth of the tumor. This study was designed to elucidate the significance of two adhesion molecules, VAP-1 and CLEVER-1, in physiological and pathological conditions, and to evaluate the use of antibodies against these molecules as pharmaceuticals. The study also estimates the safety of the antibody treatment, since targeting leukocyte migration may have unwanted side-effects on the normal immune response.

The specific aims of the present study were:

**I.** To illustrate the significance of VAP-1 in leukocyte migration into the inflamed tissue and to evaluate VAP-1 as a potential target for anti-inflammatory therapy.

**II.** To examine the role of CLEVER-1 in cell traffic to the draining lymph nodes, and to elucidate the significance of CLEVER-1 in leukocyte migration to inflamed areas.

**III.** To investigate the function of CLEVER-1 in tumor growth and dissemination, as well as to evaluate the targeting of CLEVER-1 as a form of cancer therapy.

# 4. MATERIALS AND METHODS

## 4.1 Animals (I-III)

The mice and rabbits used in the experiments were bred under specific pathogen-free conditions in the Central Animal Laboratory of the University of Turku, Turku, Finland. All the experimental procedures were approved by an ethical committee (Committee for Animal Experimentation, University of Turku, Turku, Finland). The animal strains and descriptions are listed in table 1.

**Table 1.** Animal strains used in the experiments

| animal strain | description | origin | used in |
|---|---|---|---|
| Balb/C mouse | wild type | local colony (Central Animal Laboratory of the University of Turku, Finland) | I, II |
| C57BL/6J mouse | wild type | local colony (Central Animal Laboratory of the University of Turku, Finland) | III |
| NOD mouse | non obese diabetic | Bomholtgård, Denmark | I |
| NZW rabbit | New Zealand white | Lidköpings Kaninfarm, Sweden | II |
| SCID mouse | severe combined immunodeficiency affecting T and B cell development | Charles River, Germany | III |
| SCID/beige mouse | severe combined immunodeficiency affecting T and B cell development + diminished NK cell and macrophage activity | Taconic, Denmark | III |

## 4.2 Antibodies (I-III)

The 7-88, 7-106 and 7-188 antibodies were generated by immunizing rats three times at one-week intervals with homogenized vessels connected to mouse peripheral lymph nodes and incomplete Freund's adjuvant suspension (in I). After immunization, popliteal lymph node lymphocytes were fused with SP2/0 myeloma cells. Hybridoma supernatants were screened by immunohistochemistry on frozen sections of mouse peripheral lymph nodes and small intestines. Antibodies 7-88, 7-106 and 7-188 showed a VAP-1-like staining pattern and further analysis revealed reactivity against mouse VAP-1 in transfected Chinese hamster ovary (CHO) cells expressing VAP-1.

The primary and conjugated antibodies used in this study are listed in table 2. For those antibodies that were not conjugated with a fluorescent label, fluorescein isothiocyanate (FITC) conjugated anti-rat IgG (Sigma), anti-rabbit IgG (Sigma), anti-mouse IgG (Sigma) or peroxidase conjugated anti-rat Ig (Dako) was used as secondary antibody; and for those that were conjugated with biotin, Streptavidin Alexa Fluor 546 (Invitrogen) was used.

**Table 2.** Antibodies used in the experiments

| antibody | antigen | conjugate | source / reference | used in |
|---|---|---|---|---|
| 2E8 (neg co) | unknown | - | Own hybridoma | I |
| 3G6 (neg co) | chicken T cells | - | (Salmi and Jalkanen, 1992) | III |
| 3-372 | human CLEVER-1 | - / biotinylated with Biotin-NHS (Calbiochem) | (Irjala et al., 2003b) | II, III |
| 5D3 | mouse macrophage mannose receptor | - | (Martinez-Pomares et al., 2003) | III |
| 7-88 | mouse VAP-1 | - | I | I |
| 7-106 | mouse VAP-1 | - | I | I |
| 7-188 | mouse VAP-1 | - | I | I |
| 9-11 | human (mouse) CLEVER-1 | - | (Palani et al., 2011) | III |
| AK-1 (neg co) | unknown | - | InVivo BioTech Services, Germany | II, III |
| anti-Stabilin-1 (clone 1.26) | mouse CLEVER-1 | - / biotinylated with Biotin-NHS (Calbiochem) | (Schledzewski et al., 2006) | II, III |
| CD3 | mouse CD3 | PE | BD Biosciences Pharmingen | III |
| CD4 | mouse CD4 | Alexa Fluor 647 | BD Biosciences Pharmingen | II |
| CD4 | mouse CD4 | PE | Caltag | I, III |

| | | | | |
|---|---|---|---|---|
| CD8 | mouse CD8 | PE | Caltag | I, III |
| CD8a | mouse CD8a (Ly-2) | PerCP-Cy5.5 | BD Biosciences Pharmingen | II |
| CD11a | mouse integrin αL chain / LFA-1 α chain | FITC | BD Biosciences Pharmingen | I |
| CD31 | PECAM-1 | - | BD Biosciences Pharmingen | III |
| CD44 | mouse CD44 (Pgp-1, Ly-24) | FITC | BD Biosciences Pharmingen | I |
| CD45 | mouse CD45 | PE | BD Biosciences Pharmingen | III |
| CD45R | mouse B220 (PTPRC) | Pacific Blue | BD Biosciences Pharmingen | II |
| CD45RB | mouse CD45RB (16A, C363.16A) | FITC | BD Biosciences Pharmingen | I |
| CD49d | mouse integrin α4 chain | FITC | BD Biosciences Pharmingen | I |
| CD62L | mouse L-selectin (LECAM-1, Ly22) | FITC | BD Biosciences Pharmingen | I |
| FoxP3 | mouse FoxP3 | Biotin | eBioscience | III |
| FoxP3 | mouse FoxP3 | PE (mouse regulatory T cell staining kit) | eBioscience | II |
| HB-151 (neg co) | human HLA-DR5 | - | ATCC hybridoma | I |
| Hermes-1 (9B5) (neg co) | human CD44 | - | (Jalkanen et al., 1986b) | I, II, III |
| MECA-32 | mouse panendothelial cell antigen, PV-1 | - | (Leppink et al., 1989; Hallmann et al., 1995) | III |
| MECA-79 | mouse and human PNAd | - | (Streeter et al., 1988) | II |

| | | | | |
|---|---|---|---|---|
| NS-1 (neg co) | unknown | - | ATCC hybridoma | II, III |
| SPARC | mouse SPARC | - | R&D Systems | III |

## 4.3 Immunohistochemistry (I-III)

Acetone-fixed frozen tissue sections were incubated with primary antibodies for 20 minutes. Sections were washed twice with phosphate-buffered saline (PBS), and the secondary antibody in 5 % normal mouse serum was added for 20 minutes. After washings with PBS, the sections stained with fluorescent dyes were mounted with Prolong Antifade (Molecular Probes, Oregon, USA) and covered with coverslips. To the peroxidase stained sections, 3'3'diaminobenzidine hydrochloride containing 0,03 % hydrogen peroxide was added for 3 minutes and then the sections were counterstained with hematoxylin. The intensity of the immunohistochemistry stainings was analyzed using the ImageJ software.

Tissue sections from mice treated with an intravenous injection of the 7-88 or the HB-151 antibody (in I) were stained only with the secondary antibody, FITC-conjugated anti-rat IgG, in 5 % normal mouse serum.

For regulatory T cell stainings, sections were fixed with paraformalehyde and stained with anti-FoxP3 as a primary antibody, peroxidase-conjugated rabbit anti-rat Ig as a secondary antibody, followed by 3'3'diaminobenzidine hydrochloride containing 0,03 % hydrogen peroxide and hematoxylin counterstaining (in III).

## 4.4 *In vivo* homing studies

### 4.4.1 Rabbit model of lymphocyte migration via lymphatics (II)

Spleens and popliteal lymph nodes were harvested from two New Zealand white (NZW) rabbits and homogenized through a wire gauze to obtain singe cell suspensions. After hypotonic lysis of erythrocytes from the splenic cell suspension, cells were labelled for 20 minutes with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) (0,5 µM, Molecular Probes) at 37 ºC. Labelled cells were washed three times with culture medium (RPMI 1640 supplemented with 10 % FCS, 1 % 4 mM L-glutamine, and 0,128 % penicillin/streptomycin) and resuspended into RPMI 1640. Recipient rabbits were treated intravenously either with the 3-372 anti-CLEVER-1 antibody or an NS-1 control antibody (n = 7 + 7, 2 mg/kg). The antibody treatment was repeated 24 hours after the first antibody injection, and followed by subcutaneous injection of labelled cells (40 x $10^6$) into the right hind footpads at the 30-hour time-point. At the 43-hour time-point popliteal lymph nodes were collected from recipient rabbits and half of the lymph nodes were frozen for histological analyses and the other half was homogenized for flow cytometric analyses (FACSCalibur; BD Biosciences, California, USA).

### 4.4.2 Rabbit model of malignant cell migration via lymphatics (III)

Rabbits were treated intravenously either with the 3-372 antibody or the NS-1 control antibody (n = 8 + 9, 2 mg/kg). The antibody treatment was repeated the following day. KCA human lymphoma cells were labelled with the CFSE fluorescent dye in a similar manner as the lymphocytes in the rabbit model of the lymphocyte migration experiment (4.4.1). KCA human lymphoma cells (40 x $10^6$) were injected subcutaneously into the rabbit footpads with 0,5 mg of antibodies. Twenty-four hours after the cell transfer (at the 48-hour time-point), popliteal lymph nodes were collected and homogenized to analyze the migrated cells.

### 4.4.3 Mouse model of lymphocyte migration via lymphatics (II)

Peripheral and mesenteric lymph nodes and spleens were harvested from Balb/C mice and homogenized through a wire gauze to obtain singe cell suspensions. After hypotonic lysis of erythrocytes from the splenic cell suspension, lymphocytes were labelled with the CFSE fluorescent dye in a similar manner as the lymphocytes of rabbits (4.4.1). Thereafter, 20 x $10^6$ cells were injected subcutaneously into the mice hind leg footpads. Two hours before cell injection recipient mice were treated subcutaneously either with an anti-Stabilin-1 antibody against mouse CLEVER-1 or the NS-1 control antibody (n = 10 + 10, 50 µg/mouse). Twelve hours after the lymphocyte injection, popliteal lymph nodes were collected from the recipient mice and homogenized to obtain single cell suspensions. Cell suspensions were stained for 20 minutes with Alexa Fluor 647 –conjugated anti-mouse CD4, PerCP-Cy5.5 –conjugated anti-mouse CD8a and Pacific Blue anti-mouse B220 (10 µg/ml). Cell suspensions were analysed for the percentage of migrated CFSE positive cells and for the percentage of CFSE+/CD4+, CFSE+/CD8+ and CFSE+/B220+ lymphocytes by LSRII flow cytometry (BD Biosciences).

### 4.4.4 Mouse model of lymphocyte homing assay via HEV (II)

Recipient Balb/C mice were treated intravenously either with the anti-Stabilin-1 antibody or the NS-1 control antibody (n = 8 + 8, 100 µg/mouse). Lymphocytes were isolated and labelled with the CFSE fluorescent dye as described above. Then, 20 x $10^6$ cells were injected into the mice tail veins. Eighteen hours after the lymphocyte injection peripheral and mesenteric lymph nodes, blood, spleen and Peyer's patches were collected and homogenized. Cells were stained and analyzed as described in the mouse model of lymphocyte migration via lymphatics (4.4.3).

## 4.5 Peritonitis model (I, II)

Mild inflammation was induced in the peritoneal cavity by intraperitoneal injection of 1 ml of PBS containing 5 % of the proteose peptone and 10 ng of interleukin-1. One hour later Balb/C mice were treated intravenously with the 7-88 (n = 4), 7-106 (n = 5), 7-188 (n = 5), HB-151 control (n = 5) antibody (200 µg/mouse) or pooled anti-VAP-1 antibodies (7-88, 7-106 and 7-188 together, n = 8, 100 µg of each antibody/mouse) (in

I) and anti-Stabilin-1 (n = 10) or NS-1 control (n = 11) antibody (100 µg/mouse in II). The antibody treatment was repeated 4 hours later (in I). After 18 hours of induction of inflammation, cells were harvested from the peritoneal cavities by flushing the cavity with 10 ml of RPMI 1640 containing 5 U/ml of heparin (Løvens Kemiske Fabrik, Denmark). The total cell number was counted from the lavage fluids, and leukocyte subtypes were analyzed after Diff-Quick staining (Reastain; Reagena, Finland).

## 4.6 Air pouch inflammation model (I)

Air pouches were produced under the dorsal skin of Balb/C mice with a subcutaneous injection of 5 ml of filtered air (Millex-GV filter unit; Millipore, Ireland) and re-inflated with 3 ml of filtered air the following day. On the third day 1 ml of RPMI 1640 containing 1 µg/ml CCL-21 (chemokine (C-C motif) ligand 21, SLC, mouse 6Ckine; R&D Systems, Minnesota, USA) and 50 µg/ml bovine serum albumin (BSA) was injected into the air pouches. Mice were treated with the 7-88, 7-106, 7-188, HB-151 antibody (n = 5 mice / group, 200 µg/mouse) or pooled anti-VAP-1 antibodies (n = 5, 100 µg of each antibody/mouse) 5 and 9 hours after the CCL-21 / BSA injection. Cells were flushed from the pouches 23 hours after the CCL-21 / BSA injection with 5 ml of RPMI 1640 containing 5 U/ml heparin and counted. Leukocyte subtypes were analyzed with Diff-Quick staining.

## 4.7 Diabetes mellitus (I)

Female non-obese diabetic (NOD) mice, that are susceptible to spontaneous development of insulin dependent diabetes, were treated with intraperitoneal injection of either the 7-88 (n = 21) or the HB-151 (n = 15) antibody (100 µg/mouse) or PBS alone (n = 14, 100 µl/mouse). Treatments were started at three weeks of age and continued twice per week until 30 weeks of age (or until diabetes occurred). Urinary glucose was measured weekly (Glukotest; Roche Diagnostics GmbH, Germany). In the case of elevated urine glucose levels, blood glucose was measured (MediSense Precision Xtra Plus; Abbott Laboratories MediSense, United Kingdom).

For phenotypic analyses female NOD mice were treated from 3 weeks of age until 13 weeks of age twice per week with either the 7-88 or HB-151 antibodies. Mesenteric and pancreatic lymph nodes and spleens were collected and homogenized with glass homogenizers into single cell suspensions. Cell suspensions were stained with phycoerythrin-conjugated anti-CD8 or -CD4 and FITC-conjugated anti-CD11a, -CD44, -L-selectin, -α4, -CD45RB or rat IgG. Stained samples were analyzed with FACScan flow cytometry and the CellQuest software (BD Biosciences).

## 4.8 Immunization (II)

Balb/C mice were immunized with subcutaneous injections to the hind leg footpads with 50 µg ovalbumin (Albumin, Chicken egg; Sigma, Germany) in Incomplete Freund's adjuvant (Sigma). Immunizations were repeated on day 7 and 14. Mice were

treated one hour before the first immunization by a subcutaneous injection of the anti-Stabilin-1 antibody or the NS-1 control antibody (50 µg/mouse, n = 6 + 6) at the site of immunization, and intraperitoneally three times per week (100 µg/mouse). On day 17 the mice were sacrificed and their popliteal lymph nodes, inguinal lymph nodes and spleens were collected. Cells were isolated for flow cytometric analyses and for a proliferation assay.

For analyzing leukocyte subtypes cells were stained with Alexa Fluor 647 –conjugated anti-mouse CD4, PerCP-Cy5.5 –conjugated anti-mouse CD8a and Pacific Blue anti-mouse B220 (10 µg/ml) for 20 minutes. In addition, the amount of FoxP3 positive regulatory T-cells was determined using a mouse regulatory T cell staining kit. Stained cells were analyzed with LSRII flow cytometry.

In the proliferation assays T cells ($2x10^5$) were co-cultured with increasing concentrations (0 – 2 mg/ml) of ovalbumin in complete medium (RPMI 1640 supplemented with 10 % FCS, 20 mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, penicillin/streptomycin) for 3 days. For thymidine incorporation $^3$H-thymidine (1 µCi) was added for the final 6 hours. Cells were harvested with a plate harvester (Tomtech; Fisher Scientific, New Hampshire, USA), and the incorporated thymidine was measured with a beta-counter (1450 Microbeta counter; Wallac, Finland).

Antigen-specific immunoglobulin titers were assayed from mice serum with ELISA using microtiter plates coated with ovalbumin and a peroxidase-conjugated anti-mouse IgG subclass and IgM specific reagents as described in Boyle et al., 1997.

## 4.9 Bacterial infection (II)

Balb/C mice were treated intravenously either with the anti-Stabilin-1 antibody or the AK-1 control antibody (n = 6 + 5, 200 µg/mouse). *Staphylococcus aureus* Xen36 (Xenogen/Caliper Life Sciences, California, USA) were grown in Trypticase Soy Broth until an absorbance of 0,5 at 600 nm was reached corresponding approximately $1,0x10^8$ cfu/ml of *S. aureus*. Then 5 ml of bacterial culture per mouse was pelleted and injected subcutaneously into the right hind leg footpad of mice under anesthesia. The viable bacterial counts in the footpads were followed using the IVIS50 bioluminescence imaging system (Xenogen/Caliper Life Sciences) at time points 10 min, 30 min, 2 h, 4 h, 6 h, 8 h and 24 hours.

## 4.10 Vascular integrity (II)

Mice were treated intravenously either with the anti-Stabilin-1 antibody or the NS-1 control antibody (n = 3 + 3, 100 µg/mouse). Twenty hours later 70 kDa FITC-dextran (25 mg/kg, Invitrogen Molecular Probes) was injected intravenously and allowed to circulate for 5 minutes. Lymph nodes, heart, liver, gut, lung, spleen, kidney and pancreas were collected and vascular leakage was studied using an Olympus BX60 microscope.

## 4.11 Tumor models (III)

C57BL/6J, SCID and SCID/beige mice were injected either with the anti-Stabilin-1 antibody or the NS-1 control antibody subcutaneously into the ear lobe (50 µg/mouse). The next day, EL-4 lymphoma cells (5 x $10^6$) were injected into the antibody treated ear lobe of C57BL/6J mice (n = 10 in anti-Stabilin-1 and 11 in control antibody group) and B16-F10-luc-G5 melanoma cells (4 x $10^5$) (Xenogen) were injected into the antibody treated ear lobe of C57BL/6J, SCID and SCID/beige mice (n = 12/antibody group, 14/antibody group and 11/antibody group, respectively). Mice were treated intraperitoneally every third day with the anti-Stabilin-1 or the NS-1 control antibody (100 µg/mouse). Tumor development was followed until day 14. The growth of the lymphoma was measured with a Mitutoyo electronic caliper and the melanoma by measuring luciferace bioluminescence after a substrate D-luciferin (150 mg/kg) injection with the IVIS imaging system (Xenogen).

In the treatment model, the intraperitoneal antibody therapy was not started until three days after the B16-F10-luc-G5 melanoma cell (1 x $10^6$) injection to the C57BL/6J mice and tumor development was followed until day 14 (n = 12/antibody group) or 20 (n = 6/antibody group).

## 4.12 *In vitro* assays

### 4.12.1 *In vitro* adhesion assay (I-III)

Frozen tissue sections from the lymph nodes and pancreas of mice were pre-treated under rotary conditions (60 rpm) with antibodies (7-88, 7-106, 7-188, 2E8 or HB-151) for 30 minutes at +7 ºC. Lymphocytes were isolated from mesenteric lymph nodes and added for another 30 minutes to tissue sections in rotation. After incubation adherent cells were fixed with 1 % glutaraldehyde and the number of lymphocytes bound to HEVs was counted (in I).

In a similar adhesion assay, melanomas of mice were collected, minced and treated with a collagenase D digestion (1 mg/ml, +37 ºC, 40 min). Tumor infiltrating leukocytes were purified using a PE conjugated anti-CD45 antibody and a EasySep mouse PE selection kit for magnetic cell sorting (Stem Cell Technologies, France). CD4+ T-cells were purified from the mouse blood using the EasySep mouse CD4 T cell pre-enrichment kit. Isolated tumor infiltrating leukocytes and blood CD4+ T-cells were added to the frozen tissue sections of melanomas that were collected either from anti-Stabilin-1 antibody or control antibody treated mice (in III).

Moreover, frozen sections of lymph nodes were treated either with the anti-Stabilin-1 or control antibodies. Thereafter, lymphocytes isolated from lymph nodes of mice were added to the sections. The sections were incubated with the lymphocytes for 15 min in static conditions, 5 min in rotation and finally 15 min in static conditions. Adherent cells were fixed and the number of lymphocytes bound to the lymphatic sinuses was counted (in II)

### 4.12.2 *In situ* apoptosis assay (III)

For the detection of apoptosis in tumor tissue sections from mice treated either with the anti-Stabilin-1 or the control antibody the TACS 2 TdT Blue Label *in situ* apoptosis kit (Trevigen) was used according to the manufacturer's instructions and the number of apoptotic cells per field was counted.

### 4.12.3 Immune array qPCR (III)

B16 melanoma cells (4 x $10^5$) were injected subcutaneously into the shaved abdominal area of C57BL/6J mice. Mice were treated either with the anti-Stabilin-1 or the AK-1 control antibody (n = 6 + 6, 300 µg/mouse) every other day. On day 14 tumors were collected, and tumor infiltrating leukocytes were isolated with the anti-CD45 antibody and EasySep kit as described in the *in vitro* adhesion assay paragraph (4.12.1). Total RNA was isolated from the CD45 positive and negative cell populations using the Nucleospin RNA II kit (Macherey-Nagel) and reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). Equal amounts of sample were loaded into TaqMan Mouse Immune Array Microfluidic Cards (Applied Biosystems) and run using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The results were normalized using 18S RNA as an endogenous control and analyzed with the SDS 2.3 and DataAssist v3.0 software using relative quantification.

### 4.12.4 Co-cultures of monocytes/macrophages and melanoma cells (III)

Melanoma cells (50 000 cells/well) were cultured in a 12-well plate (Corning). Monocytes were isolated from mouse blood with Ficoll-Paque and macrophages were collected from mouse peritoneum. Monocytes and macrophages were added onto the transwells containing transparent polyester membranes (0,4 µm pore size) on the top of melanoma cell wells. The anti-Stabilin-1 or the 3G6 control antibody (20 µg/ml) was added to the B16 culture medium (Thermo Scientific Hyclone MEM/EBSS containing 10 % FCS, 0,2 % penicillin/streptomycin, 1 % L-glutamine, 1 % MEM vitamins, 1 % sodium pyruvate and 1 % NEAA). On day 3 and 7, monocytes and macrophages on the membranes were fixed with methanol and stained with the 5D3 mannose receptor antibody, the 9-11 anti-CLEVER-1 antibody or the 9B5 negative control antibody as the primary antibodies, and the FITC anti-rat Ig as the secondary antibody.

## 4.13 Statistical analyzes (I-III)

For statistical analyses Student's t-test was used for the *in vitro* adhesion assays and the immunization model, Student's t-test and the Mann-Whitney U test for the *in vivo* homing assays and the tumor models, Student's t-test and analysis of variance with Bonferroni's multiple comparison test in the peritonitis and air pouch models, Kaplan-Mayer and log rank tests in the diabetes model.

# 5. RESULTS AND DISCUSSION

## 5.1 VAP-1 is a potential target for anti-inflammatory therapies (I)

Function blocking antibodies that recognize mouse VAP-1 were produced to study the significance of VAP-1 *in vivo*. These new antibodies stained HEVs in lymph nodes and a subset of vessels in the heart, kidney, lung, pancreas, and spleen for example. Moreover, in the *in vitro* adhesion assay the new anti-VAP-1 antibodies decreased the binding of lymphocytes to the lymph node sections.

### 5.1.1 Anti-VAP-1 antibody treatment decreases granulocyte migration to the inflamed peritoneal cavity (I)

To study the role of VAP-1 in leukocyte migration to the sites of inflammation, peritonitis was induced with proteose peptone and IL-1, and then the mice were treated with anti-VAP-1 antibodies. The total leukocyte number in the inflamed peritoneal fluid was $6{,}0 \pm 0{,}6 \times 10^6$ in mice treated with the control antibody. The anti-VAP-1 antibody treatment decreased the cell number in the inflamed peritoneal cavity, since the mice which were treated with the anti-VAP-1 antibody pool (7-88, 7-106 and 7-188 together), had 28 % less leukocytes in their peritoneal cavity. And the 7-88, 7-106 and 7-188 antibodies reduced the cell number by 28 %, 36 % and 23 % respectively.

The most frequent cell type in the inflamed peritoneal fluid was granulocytes. Like total leukocyte number, the 7-106 anti-VAP-1 antibody was the most efficient treatment for decreasing the number of granulocytes by 51 % compared to the control antibody treated mice. The anti-VAP-1 antibody pool, and the 7-88 and 7-188 antibodies inhibited the number of granulocytes by 26 %, 29 % and 23 % respectively.

The decrease in granulocyte migration to the inflamed peritoneal cavity is in line with previous *in vitro* studies: it has been shown that the anti-VAP-1 antibody inhibits granulocyte binding to, for example, cardiac vessels of ischemic hearts in the *in vitro* adhesion assay (Jaakkola et al., 2000a).

### 5.1.2 Anti-VAP-1 treatment inhibits monocyte migration to the inflamed area in the air pouch model (I)

The role of VAP-1 was also studied in another acute inflammation model, the air pouch model, where the CCL-21 chemokine is used to attract leukocytes into the air pouch. In our model, mice treated with the control antibody had $1{,}8 \pm 0{,}3 \times 10^6$ cells in their pouches. In mice that were treated with the anti-VAP-1 antibody pool, and the 7-88, 7-106 and 7-188 antibodies the migration of cells to the pouches was decreased by 52 %, 44 %, 59 % and 12 %, respectively, compared to the control.

The majority of the infiltrating leukocytes in the CCL-21 induced air pouch model were monocytes. Blocking the function of VAP-1 by the 7-88, 7-106 or 7-188

antibodies reduced the number of monocytes in the pouches by 34 %, 66 %, 18 %, respectively, while all anti-VAP-1 antibodies pooled together reduced the number of monocytes by 56 % when compared to control antibody treated mice.

The inhibitory effect of the anti-VAP-1 antibody treatment on monocyte migration to the inflamed area is congruent with the *in vitro* results of Aspinall et al., which show that CD16+ monocyte adhesion to, and their transmigration through hepatic sinusoidal endothelial cells is decreased after an anti-VAP-1 antibody treatment (Aspinall et al., 2010).

### 5.1.3 Anti-VAP-1 antibody treatment affects the development of diabetes (I)

VAP-1 is expressed on the surface of pancreatic vessels in NOD mice (Bono et al., 1999). This was verified by injecting the 7-88 anti-VAP-1 antibody intravenously into mice followed by the detection of the bound antibody on frozen tissue sections by a fluorescently conjugated secondary antibody. In addition, the up-regulation of the expression of VAP-1 in the pancreatic vessels correlates with the lymphocyte infiltration to the islets of the pancreas (Bono et al., 1999). Thus, blocking the function of VAP-1 may reduce harmful lymphocyte migration to the pancreas. Harmful lymphocyte migration to the pancreas may lead to the destruction of insulin-producing beta-cells in pancreatic islets and therefore result in insulin dependent diabetes. In the diabetes model mice were treated from three weeks of age with the 7-88 anti-VAP-1 antibody, control antibody or PBS only, before the lymphocyte infiltrates accumulate in the islets of Langerhans. The treatment was repeated twice per week. In the first experiment the incidence of diabetes in mice treated with the anti-VAP-1 antibody was 53,8 % while the incidence in the PBS treated group was 85,7 %. In the second experiment the incidence of diabetes in the anti-VAP-1 treated group was 37,5 % and in the control antibody group 66,7 %. In this NOD mouse diabetes model the duration of the antibody treatment was 27 weeks, and the follow-up period was until week 52. As a notable observation, no rebound effect in the incidence of diabetes was seen after the anti-VAP-1 antibody treatment was discontinued.

Similar results for the incidence of diabetes were obtained by Stolen et al. with VAP-1 knock out mice and their wild type littermates in transgenic mouse background carrying the OVA antigen in pancreatic islets, and using cell transfer of OVA-specific T-cells from OT-1 mice to induce insulitis and finally diabetes. In this transgenic mouse model after a 10-day follow-up period 17 % of the VAP-1 knock out mice and 44 % of wild type mice died due to diabetes. (Stolen et al., 2005.)

### 5.1.4 VAP-1 as a target for pharmaceuticals (I)

The effect of a long term anti-VAP-1 antibody treatment to the lymphocyte subpopulations in the pancreatic and mesenteric lymph nodes and spleens was studied in the antibody treated NOD mice. After the anti-VAP-1 and control antibody treatment the T:B and CD4:CD8 cell ratios were similar. The expression levels of L-

selectin, CD11a, α4 integrin, CD44 and CD45RB were also similar in the mesenteric lymph nodes and spleens. Only the L-selectin expression was slightly higher in the pancreatic lymph node CD4+ and CD8+ lymphocytes of anti-VAP-1 treated mice compared to control animals. In comparison, the expression of L-selectin, PSGL-1, α4β7, CD18 and CD44 on peripheral blood leukocytes and P-selectin, E-selectin, peripheral node addressin (PNAd), MAdCAM-1, ICAM-1, VCAM-1 and CD31 levels on Peyer's patches were similar in VAP-1 deficient mice and their wt littermates (Stolen et al., 2005). However, later it was shown that the oxidative reaction catalyzed by VAP-1 induces the expression of other molecules involved in the leukocyte extravasation cascade, for example MAdCAM-1 (Liaskou et al., 2011), E-selectin and P-selectin (Jalkanen et al., 2007). Furthermore, the activation of VAP-1 on the liver endothelium upregulates ICAM-1 and E-selectin adhesion molecules and leads to the secretion of the CXCL8 (IL-8) chemokine (Lalor et al., 2007). As a notable observation, the anti-VAP-1 antibodies used in this study did not affect the enzymatic activity of VAP-1.

VAP-1 knock out mice survived a *Yersinia enterocolitica* infection equally well as their wild type littermates (Stolen et al., 2004) and although VAP-1 deficient mice had mild defect in controlling *Staphylococcus aureus* and Coxsackie B4 infections, the combined anti-VAP-1 antibody and small molecule SSAO inhibitor treatment did not impede the microbial defense (Koskinen et al., 2007). Probably during infection, the innate immune response is able to cope with microbial invasion although one molecule from the adhesion cascade is blocked. Also the findings that the expression of VAP-1 is localized to the cytoplasmic granules of endothelial cells and that VAP-1 expression markedly increases at sites of inflammation and is translocated onto the cell surface after a proinflammatory stimulus (Salmi et al., 1993; Jaakkola et al., 2000b), make VAP-1 a promising target for anti-inflammatory therapies.

In the first trial in humans, vepalimomab, the murine antibody against VAP-1, was well tolerated and did not cause severe adverse effects in adult patients with allergic contact dermatitis. Human anti-mouse antibody levels in serum were increased after administration of the murine anti-VAP-1 antibody. (Vainio et al., 2005.) Murine VAP-1 antibodies used for example in the *in vivo* assays of this study or in the above mentioned trial by Vainio et al. might cause problems if used as a form of anti-inflammatory therapy in humans. For example, the production of human anti-mouse antibodies may reduce the therapeutic efficacy, and after repeated administration may lead to hypersensitivity. Moreover, the half-life of murine antibodies in human serum is short. A step further has already been taken in treating humans with the anti-VAP-1 antibody therapy: after these studies chimeric anti-VAP-1 antibodies consisting of variable domains of mouse antibodies and a modified human IgG2 constant region were produced. These chimeric antibodies had similar effects on leukocyte adhesion as did the original anti-VAP-1 antibodies, i.e. they were effective in blocking leukocyte adhesion and transmigration in *in vitro* assays as well as *in vivo* inflammation models. Additionally, chimeric anti-VAP-1 antibodies did not induce antibody-dependent cell-

mediated cytotoxicity or complement mediated cell lysis. (Kirton et al., 2005.) Biotie Therapies has also developed a fully human monoclonal antibody, BTT-1023, against VAP-1 and it has been promising in phase I trials in patients with rheumatoid arthritis and psoriasis (Biotie, 2011).

In addition to anti-VAP-1 antibodies, small molecule inhibitors may also be used as drugs to target VAP-1. This is because the enzymatic activity of VAP-1 is involved in its adhesive function (Salmi et al., 2001). In fact, for example the LJP 1207 inhibitor of SSAO has been studied and its efficacy proved in different animal inflammation models: oxazolone-induced colitis (a model of inflammatory bowel disease), carrageenan-induced paw edema (a model of acute local inflammation) and lipopolysaccharide (LPS) induced endotoxemia (a model of systemic inflammation) (Salter-Cid et al., 2005). Furthermore, the LJP 1207 treatment was effective in preventing postischemic neutrophil adhesion and infiltration in rats subjected to forebrain ischemia leading to reduced neuropathology in these animals (Xu et al., 2006). The same treatment reduced the clinical symptoms of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (Wang et al., 2006; O'Rourke et al., 2007). Another small molecule inhibitor of SSAO, namely U-V002, suppressed retinal inflammation in a rat model of acute ocular inflammation (Noda et al., 2008) and decreased CD11b+, granulocyte and macrophage infiltration into IL-1 implanted corneas of mice (Nakao et al., 2011). Treatment with the SSAO inhibitor SZE 5302 before ischemia caused a marked reduction in intestinal ischemia-reperfusion injury (Kiss et al., 2008). An LJP 1586 treatment reduced the accumulation of leukocytes in the inflamed area in a carrageenan induced mouse air pouch model, an LPS induced rat lung inflammation model (O'Rourke et al., 2008) and a collagenase induced mouse intracerebral hemorrhage model (Ma et al., 2011).

There is an increasing number of VAP-1-related patent applications claimed by Biotie Therapies, R-Tech Ueno Ltd, La Jolla Pharmaceuticals Co and Genmedica Therapeutics, for example, proving the significance of VAP-1 as a target for pharmaceuticals (Dunkel et al., 2011).

## 5.2 CLEVER-1 mediates cell traffic via afferent lymphatic vessels and leukocyte entrance to sites of inflammation (II)

In *in vitro* studies the anti-CLEVER-1 antibody decreased the binding of lymphocytes and malignant cell line cells to HEVs and the lymphatic endothelium; and in the initial *in vivo* studies the anti-CLEVER-1 antibody treatment inhibited the increase in the size of the draining lymph nodes after the immunization of rabbits, suggesting a role for CLEVER-1 in lymphocyte traffic (Irjala et al., 2003a; Irjala et al., 2003b). To further define the role of CLEVER-1 in cell traffic, homing assays and inflammation models were performed.

### 5.2.1 Anti-CLEVER-1 antibody treatment inhibits cell migration to the draining lymph node (II, III)

Rabbits were used to study the role of CLEVER-1 in cell traffic, because the anti-human CLEVER-1 antibody 3-372 cross-reacts with the rabbit homolog. The rabbits were treated either with the anti-CLEVER-1 antibody or a control antibody followed by injection of fluorescently labelled rabbit lymphocytes under the skin of the rabbit footpads. The cell analysis from the draining popliteal lymph nodes revealed that in the anti-CLEVER-1 antibody treated rabbits there were 80 % less migrated cells than in the control antibody treated animals.

Next the role of CLEVER-1 in malignant cell migration was tested using human lymphoma cells in a similar manner as the rabbit lymphocytes were used in the lymphocyte homing assay. Compared to the control antibody, the anti-CLEVER-1 antibody inhibited the migration of lymphoma cells to the popliteal lymph nodes by 67 %. Based on this, the malignant cells of human origin also use CLEVER-1 to migrate via lymphatic vessels.

To further verify the significance of CLEVER-1 in cell migration, the mouse lymphocyte homing model, which allows better analysis of the lymphocyte subpopulations of the migrated cells, was used. Again, the anti-CLEVER-1 antibody clearly prevented the migration of cells via afferent lymphatic vessels to the draining lymph node. An analysis of the lymphocyte subpopulation showed that the anti-CLEVER-1 antibody treatment decreased the amount of migrating cells in all the studied lymphocyte subtype groups. When the migrated cells among B-cells, and CD4+ and CD8+ T-cells were analysed there were 90 % less migrated B-cells, 64 % less migrated CD4 positive cells and 82 % less migrated CD8 positive cells in the popliteal lymph nodes of mice treated with the anti-CLEVER-1 antibody compared to the control antibody treatment.

### 5.2.2 Anti-CLEVER-1 antibody treatment decreases leukocyte migration during inflammation (II)

Although blocking the function of CLEVER-1 with an antibody inhibited the migration of lymphocytes via the afferent lymphatic vessels to the draining lymph node, it did not affect the migration of lymphocytes from the blood through high endothelial venules to the lymph nodes in homing assays, where labelled mouse lymphocytes were injected into the tail veins of mice. The fact that unlike in human high endothelial venules, CLEVER-1 is expressed at very low levels in mouse HEVs, provides an explanation for this finding. As a consequence of inflammation CLEVER-1 expression was up-regulated also in mouse HEVs. Thus, the role of CLEVER-1 in leukocyte migration during inflammation was studied using a peritonitis model. In the peritonitis model the anti-CLEVER-1 antibody treatment prevented the migration of leukocytes to the inflamed area. During inflammation the number of macrophages and lymphocytes in mouse peritoneal cavities treated with the anti-CLEVER-1 antibody

were almost at the same levels as the number of cells in non-inflamed peritoneal cavities i.e. they corresponded to the number of resident cells in the resting state. Moreover, the number of macrophages and lymphocytes collected from the anti-CLEVER-1 antibody treated mice were 55 % and 49 % smaller than in control antibody treated mice during inflammation. The migration of neutrophils to the inflamed peritoneal cavity was also reduced, as there were 48 % less neutrophils following the anti-CLEVER-1 antibody treatment compared to the control antibody treatment.

The effects of the anti-VAP-1 antibody and the anti-CLEVER-1 antibody treatments on leukocyte migration seen in this study are in line with the previously published results for targeting other adhesion molecules. For example, the MEL-14 antibody against L-selectin decreases the homing of lymphocytes to inflamed air pouches: the radioactivity of *B. pertussis* -inflamed air pouches went down 60 % after a MEL-14 antibody pretreatment compared to a control antibody pretreatment of radiolabelled lymphocytes (Dawson et al., 1992). Furthermore, the pretreatment of fluorescently labelled neutrophils with the MEL-14 antibody decreased cell migration to the inflamed peritoneal cavity in a *E. coli* supernatant induced peritonitis model by 50 % compared to a control antibody treatment (Lewinsohn et al., 1987). Neutrophil migration was also decreased to half in ICAM-1 deficient mice compared to wild type mice during thioglycollate induced peritonitis (Sligh et al., 1993). With an anti-CD99 antibody pretreatment neutrophil migration into the inflamed peritoneal cavity was reduced by 40 % and monocyte migration was down to almost baseline levels (Dufour et al., 2008). In LFA-1 deficient mice leukocyte migration to the TNF-α inflamed air pouches was inhibited by 67 % and in CD18 deficient mice by 59 % compared to wild type mice (Ding et al., 1999). An EL-246 antibody pretreatment against a common epitope on both E- and L-selectin decreased the homing of fluorescently labelled bovine lymphocytes into the mouse peripheral lymph nodes by 65 % compared to a control antibody treatment (Bargatze et al., 1994). This indicates that by targeting only one adhesion molecule it is not possible to entirely block the leukocyte migration. On the other hand, targeting more than one molecule may have additive effects. This probably is the case, for example, when simultaneous blocking of P- and L-selectin with antibodies in a mouse peritonitis model leads to the almost complete inhibition of neutrophil migration to the inflamed peritoneal cavity (Bosse and Vestweber, 1994). And in a rat dermal inflammation model an anti-VLA-4 + anti-E-selectin antibody treatment is more effective in inhibiting T cell migration than either of these antibodies alone. Moreover treatment with anti-VLA-4, anti-E-selectin and anti-P-selectin antibodies together decreases the accumulation of T cells into the inflamed dermal sites by 90 % compared to a control antibody treatment (Issekutz and Issekutz, 2002). Targeting VAP-1 and CLEVER-1 at the same time also appears to have an additive effect, at least to transendothelial migration of Tregs through cultured hepatic sinusoidal endothelial cells with a combined treatment with antibodies against VAP-1, CLEVER-1 and ICAM-1 (Shetty et al., 2011). On the other hand, blocking more than one adhesion molecule at a time might already affect the normal immune defence too

much and lead to severe infections. Finding the right targets and adequate effect on migration in a diverse array of autoimmune diseases will be a challenging task. Although many molecules that affect leukocyte migration have been described and the outcomes of targeting these molecules have been promising in animal models of inflammation, still only a few therapeutic antibodies that target adhesion molecules have been approved for treatment in humans. (Ulbrich et al., 2003; Luster et al., 2005; Hansel et al., 2010.) However, effective treatment is possible as is seen for example with the use of Natalizumab against α4 integrin for the treatment of MS.

### 5.2.3 Anti-CLEVER-1 antibody treatment does not impair the normal immune response during immunization or bacterial infection (II)

Despite the strong effects of the anti-CLEVER-1 antibody treatment on leukocyte traffic, the treatment did not impair the normal immune response during immunization. The absolute lymphocyte numbers and percentages of CD4, CD8 and regulatory T cells as well as B-cells were the same in lymph nodes and spleens of anti-CLEVER-1 and control antibody treated mice after ovalbumin immunizations, as were the ovalbumin-specific T- and B-cell responses. Also, the bacterial clearance rate in anti-CLEVER-1 antibody treated mice was indistinguishable from that of control antibody treated mice. In both groups of mice there was an equal amount of inoculated *Staphylococcus aureus* at each timepoint in the bacterial infection model. Accordingly, targeting CLEVER-1 does not compromise the clearance of a bacterial infection, and these results propose that the targeting of CLEVER-1 does not dampen the normal immune response in excess.

## 5.3 Anti-CLEVER-1 antibody treatment reduces tumor growth (III)

B16 melanoma and EL-4 T lymphoma models were used to study the role of CLEVER-1 in tumor growth. Tumor cells were injected subcutaneously into murine ear lobes. The mice were treated either with the anti-CLEVER-1 or the control antibody. The antibody therapy was started either prophylactically one day before tumor cell injection (both models) or as a treatment from day 3 onward (in the melanoma model only). In the lymphoma model tumor growth was observed in 9/10 anti-CLEVER-1 antibody and in 9/11 control antibody treated mice. Primary tumors in the tumor bearing mice were 51 % smaller in the anti-CLEVER-1 antibody treated mice compared to the control antibody treated mice. Moreover, metastases in the draining cervical lymph nodes of anti-CLEVER-1 antibody treated mice were also 49 % smaller on day 11 and 26 % smaller on day 14, albeit that the difference in the growth of metastases between the groups in the lymphoma model did not reach statistical significance. In the melanoma model when the treatment was started before the tumor cells were injected, the growth of primary tumors and metastases in the neck lymph nodes was 70 % less in the anti-CLEVER-1 antibody treated mice compared to control antibody treated mice. In the melanoma model, where treatment was started on day 3, primary tumors and metastases were also approximately 70 % smaller after the anti-CLEVER-1 antibody treatment.

### 5.3.1 Anti-CLEVER-1 antibody treatment does not affect angiogenesis (III)

To find the reason for the decreased tumor size consequent to the anti-CLEVER-1 antibody treatment, vessels and vessel morphology within the tumors was studied, since without the formation of blood vessels in the tumor, tumor growth is limited. Tumor associated macrophages may have both angiogenic and angiostatic properties (Dirkx et al., 2006). CLEVER-1 is expressed in type II macrophages (Politz et al., 2002) and might have a role in angiogenesis, since in the *in vitro* Matrigel tube formation assay an anti-FEEL-1 monoclonal antibody treatment reduced cell-cell interactions and increased the quantified area surrounded by the capillary-like tube network compared to control antibody treatment of human umbilical vein endothelial cell cultures (Adachi and Tsujimoto, 2002). However, the numbers of blood and lymphatic vessels were comparable within the tumors after the anti-CLEVER-1 and control antibody treatments. Also vessel morphology was similar between the treatment groups.

### 5.3.2 CLEVER-1 expression is induced in tumor vasculature, and mediates the binding of tumor infiltrating leukocytes (III)

Since the expression of CLEVER-1 is upregulated during inflammation and the antibody against CLEVER-1 decreased cell migration, CLEVER-1 expression and its role in cell adhesion in tumors were studied. In mouse tumor models CLEVER-1 expression was up-regulated in the intratumoral vessels within tumors. These vessels were enlarged with a widely open lumen and most of them expressed CLEVER-1, unlike the normal flat walled vessels. In the *in vitro* adhesion assay the anti-CLEVER-1 antibody treatment decreased the binding of tumor infiltrating myeloid cells and lymphocytes to the tumor vasculature over 60 % compared to the control antibody. Thus, the anti-CLEVER-1 antibody treatment may prevent leukocyte entrance into tumors.

### 5.3.3 Anti-CLEVER-1 treatment decreases the number of regulatory T cells and type II macrophages in tumors (III)

As the formation of new blood vessels was not the reason for decreased tumor size consequent to the anti-CLEVER-1 treatment, and since the treatment affected cell adhesion in the *in vitro* assays, different subpopulations of tumor infiltrating leukocytes within the tumors was studied. Tumor infiltrated leukocytes may either suppress tumor growth or then in contrast they may promote tumor growth, invasion and metastasis. For example, the presence of intraepithelial CD3+ T cells has been shown to be a positive, and CD4+CD25+FoxP3+ regulatory T cells a negative prognostic factor for overall survival in ovarian cancer patients (Nelson, 2008).

After antibody treatments the number of type II macrophages and regulatory T cells was about 70 % smaller in primary tumors and metastases collected from anti-CLEVER-1 antibody treated mice compared to the control antibody treated group. The decreased number of these cell types was selective as for example the numbers of CD8

positive and CD3 positive cells were equal after the anti-CLEVER-1 versus control antibody treatment. As CLEVER-1 is expressed in type II macrophages the antibody treatment against CLEVER-1 may affect the depletion of these cells. However, there were almost equal percentages of mannose receptor positive cells that also expressed CLEVER-1 subsequent to the anti-CLEVER-1 and control antibody treatments. Moreover, in the *in vitro* co-cultures of melanoma cells and monocytes/macrophages the anti-CLEVER-1 antibody treatment did not affect the morphology, type I/II polarization or number of macrophages. Recently it was shown that CLEVER-1 is expressed also in tumor sinusoids and tumor-associated vessels in hepatocellular carcinoma, and that CLEVER-1 mediates the transmigration of regulatory T cells through cultured human hepatic sinusoidal endothelial cells (Shetty et al., 2011). Based on the *in vitro* data of this study and the results of Shetty et al, the decrease in the number of regulatory T cells and type II macrophages in the tumors of anti-CLEVER-1 antibody treated mice is most likely due to an effect of the anti-CLEVER-1 treatment on the migration of these cells into the tumors.

To reveal the role of type II macrophages and regulatory T cells in reducing tumor size after the anti-CLEVER-1 antibody treatment, two different mouse strains were used, namely the SCID and SCID/beige strains. The SCID mice are T and B cell deficient and the SCID/beige mice are also T and B cell deficient and additionally have defects in their NK cells, cytotoxic T cells and macrophages (Bosma and Carroll, 1991; Raffa et al., 1993; MacDougall et al., 1990; Oliver and Essner, 1975; Saunders and Cheers, 1996). In SCID mice primary tumors were 70 % smaller after the anti-CLEVER-1 antibody treatment compared to tumors after the control antibody treatment. There were no differences in the size of metastases. In SCID/beige mice no differences in the size of primary tumors or metastases between the antibody treatment groups were observed, suggesting that functional macrophages are ultimately responsible for the phenomenon. It is known that type II macrophages are able to induce regulatory T cells (CD4+CD25+FoxP3+) that have a strong suppressive phenotype (Savage et al., 2008). Thus the anti-CLEVER-1 antibody therapy most likely prevents the migration of type II macrophages into the tumors leading also to a decrease in the number of regulatory T cells.

### 5.3.4 Anti-CLEVER-1 treatment does not affect to the expression of SPARC (III)

In macrophages CLEVER-1 is required for the endocytosis of SPARC and the targeting of SPARC for degradation (Kzhyshkowska et al., 2006b). SPARC is important for the maintenance of tissue homeostasis and is involved in proliferation, adhesion and migration, as well as angiogenesis and apoptosis. The role of SPARC in a variety of cancers is evident. (Tai and Tang, 2008; Chiodoni et al., 2010; Nagaraju and Sharma, 2011.) To see if the reduced tumor growth in anti-CLEVER-1 antibody treated mice and the decreased number of type II macrophages and regulatory T cells in these mice were linked to SPARC, the expression of SPARC was studied. However,

the relative intensities of SPARC immunostainings of tumor tissues were comparable after both anti-CLEVER-1 and control antibody treatments.

### 5.3.5 Anti-CLEVER-1 treatment affects apoptosis inside tumors (III)

As a consequence of the decrease in the number of type II macrophages and regulatory T cells inside the tumors, the anti-tumor immune response may be more intense following the anti-CLEVER-1 antibody treatment and thus lead to reduced growth of tumors in these mice. In the *in situ* apoptosis assay the mean number of apoptotic cells was greater after the anti-CLEVER-1 antibody treatment (46,4 ± 4,8/field) compared to control antibody treatment (15,7 ± 5,7/field), supporting the hypothesis that targeting CLEVER-1 results in an enhanced immune response.

### 5.3.6 CLEVER-1 mediates tumor immunity (III)

This study shows that CLEVER-1 mediates tumor cell migration via lymphatic vessels and that CLEVER-1 expression is induced during tumor vasculature. Targeting CLEVER-1 decreases the number of type II macrophages and regulatory T cells inside tumors and increases apoptosis in tumor cells, which leads a reduction in the growth of the tumors. Ammar et al. have shown that CLEVER-1 is expressed in tumor macrophages, and is found in the blood and lymphatic vessels also during human breast cancer. In breast cancer CLEVER-1 expression in lymphatic vessels was associated with lymph node metastasis and CLEVER-1 expression in blood vessels with an increased risk of recurrence. Moreover, the dense inflammatory infiltrate correlated with CLEVER-1 expression, and a strong association was found between CLEVER-1 expression in blood vessels / lymph vessels and CLEVER-1 expression in macrophages in breast tumor samples. (Ammar et al., 2011.) In colorectal cancer the number of CLEVER-1 positive lymphatic vessels correlated with the density of CLEVER-1 positive macrophages, and in advanced stage colorectal cancer a high number of CLEVER-1 positive macrophages in the tumors predicted poor prognosis (Algars et al., 2011). Based on these results CLEVER-1 both mediates tumor cell trafficking and the entrance of immune suppressor cells to the tumors, suggesting that targeting CLEVER-1 during tumor growth might be advantageous.

# 6. SUMMARY

Leukocyte migration to different organs is fundamental for defending the body against microbial invasion. Adhesion molecules guide the leukocytes to the right place at the right time. However, misdirected or overly excessive leukocyte migration is harmful, as it may lead to tissue damage. Targeting adhesion molecules is a promising therapy option for inflammatory disorders, but only a few adhesion molecule-based therapies have been approved for treating humans. Further details of the adhesion molecules that mediate leukocyte migration during inflammation and the consequences of targeting them are needed. Moreover, adhesion molecules are also involved in tumor dissemination from the primary site, and they mediate the migration of tumor infiltrating leukocytes to the tumors. These tumor infiltrating leukocytes may either be advantageous and prevent tumor growth, or in contrast they may be detrimental and dampen the immune response against the tumor. Further knowledge of the function of adhesion molecules during tumorigenesis may reveal new therapeutic options for controlling tumor growth and dissemination in the future.

Two adhesion molecules, namely VAP-1 and CLEVER-1, have been shown to mediate the adhesion of leukocytes in *in vitro*. Additionally, the expression of these molecules is upregulated during inflammation, proposing that these molecules may be good candidates for anti-inflammatory therapies. In this work the *in vivo* role of these adhesion molecules was studied using different animal models. The results showed that targeting either one of these molecules results in decreased migration of leukocytes to sites of inflammation. The results thus suggest that blocking the function of VAP-1 and CLEVER-1 may be useful for treating inflammatory disorders. The results also illustrated that CLEVER-1 mediates the migration of cells via lymphatic vessels. The targeting of CLEVER-1 during tumor growth reduced the number of immune regulating leukocytes in the tumors leading to a decrease in the size of primary tumors and metastases, indicating that targeting CLEVER-1 may be an advantageous addition to the pharmaceuticals used in cancer therapy. In addition, targeting CLEVER-1 did not compromise the normal immune response.

In conclusion, this study has illustrated the essential role of VAP-1 and CLEVER-1 in leukocyte migration during inflammation and substantiates the targeting of these molecules in immunotherapeutic applications. Also, the consequences of targeting CLEVER-1 were demonstrated in different tumor models, and the results suggest that targeting CLEVER-1 during tumor growth might be a beneficial addition to the available cancer therapies.

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