TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

*SARJA - SER. D OSA - TOM. 839*

MEDICA - ODONTOLOGICA

# VAP-1 IN LEUKOCYTE TRAFFICKING

by

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Turku 2009

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ISBN 978-951-29-3822-3 (PRINT)
ISBN 978-951-29-3823-0 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland, 2009

# ABSTRACT

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VAP-1 in leukocyte trafficking

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Annales Universitatis Turkuensis.
Turku, Finland, 2009.

The extravasation of leukocytes from the blood stream into the tissues is a prerequisite for adequate immune surveillance and immune reaction. The leukocyte movement from the bloodstream into the tissues is mediated by molecular bonds. The bonds are formed between adhesion molecules on endothelial cells and their counterparts expressed on leukocytes. Vascular adhesion protein-1 (VAP-1) is an endothelial adhesion molecule mediating leukocyte interactions with endothelium. It is also an enzyme having semicarbazide sensitive amine oxidase (SSAO) activity. The SSAO-activity catalyses deamination of primary amines into corresponding aldehyde and during the enzymatic reaction hydrogen peroxide and ammonia are produced.

The aim of this study was to investigate the relationship between the adhesive and enzymatic activities of VAP-1. The role of VAP-1 in leukocyte traffic was studied *in vivo* under normal and pathological conditions in VAP-1 deficient mice.

The results from *in vitro* flow-based assays indicated that VAP-1 uses both SSAO-activity and its adhesive epitope to bind leukocytes, and both are perquisites for VAP-1 mediated adhesion. Furthermore, *in vivo* results demonstrated that leukocyte trafficking was impaired *in vivo* by deleting VAP-1 or inhibiting SSAO-activity. There was impairment in lymphocyte recirculation as well as leukocyte accumulation into the inflamed area. Moreover, the VAP-1 deficient mice did not show generalized defects in antimicrobial responses, whereas significant reduction in tumor progression and neovascularization was observed. These results indicate that VAP-1 could be used as a target in anti-adhesive therapies either by blocking its adhesive epitope with antibodies or by inhibiting its SSAO-activity using inhibitors. Moreover, targeting of VAP-1 may provide a new way of inhibiting neovascularization in tumors.

**Keywords:** VAP-1, SSAO, adhesion molecule, transmigration, inflammation

# TIIVISTELMÄ

Kaisa Auvinen

VAP-1 ja valkosolujen kulku verisuonista kudoksiin

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Turku Graduate School of Biomedical Sciences.
Annales Universitatis Turkuensis.
Turku, Suomi 2009.

Valkosolujen kulkeutuminen verenkierrosta kudoksiin on välttämätöntä immuunipuolustukselle. Valkosolujen kulkeutumista säädellään molekyylien välisten sidosten avulla. Sidokset muodostuvat verisuonen seinämän endoteelisolujen pinnalla esiintyvien adheesiomolekyylien ja leukosyyttien pinnalla olevien vastinmolekyylien välille. Vaskulaarinen adheesiomolekyyli-1 (VAP-1) on endoteelisolujen pinnalla esiintyvä adheesiomolekyyli, joka välittää valkosolujen liikennettä. Adhesiivisten ominaisuuksiensa lisäksi VAP-1 on amiinioksidaasi–entsyymi (SSAO). Entsyymireaktiossa amiini hajoitetaan aldehydiksi, jolloin sivutuotteina vapautuu vetyperoksidia ja ammoniakkia.

Tämän tutkimuksen tarkoituksena oli selvittää VAP-1:n adhesiivisen ja entsymaattisen ominaisuuden välinen yhteys. Tavoitteena oli myös tutkia VAP-1:n merkitystä valkosoluliikenteelle *in vivo* VAP-1 poistogeenisissä hiirissä erilaisissa fysiologisissa ja patologisissa tilanteissa.

Työn tulokset osoittivat, että VAP-1:n adhesiivinen ja entsymaattinen ominaisuus olivat välttämättömät VAP-1-välitteiselle adheesiolle virtausoloissa. Tutkimukset poistogeenisillä hiirillä osoittivat, että sekä lymfosyyttien kiertokulku että granulosyyttien kertyminen tulehdusalueelle vähenivät VAP-1:n puuttuessa. VAP-1 poistogeenisillä eläimillä yleinen immuunireaktio mikrobeja kohtaan ei ollut merkittävästi heikentynyt mutta erityisesti syöpäkasvaimen kehittyminen ja kasvaimen uudissuonten muodostuminen oli heikentynyt huomattavasti. Normaaleissa villityypin hiirissä VAP-1:n salpaaminen entsyymi-inhibiittoreilla aiheutti samankaltaiset vaikutukset kuin sen geneettinen poistaminen. Tulosten perusteella VAP-1:tä voidaan hyödyntää anti-adhesiivisissa terapioissa joko salpaamalla adhesiivinen epitooppi vasta-aineella tai SSAO-aktiivisuus inhibiittoreilla. VAP-1:n estämistä voitaisiin tulosten perusteella hyödyntää tulehdustaudeissa sekä mahdollisesti osana syövän hoitoa ja kasvaimen uudissuonten muodostumisen estoa.

**Avainsanat:** VAP-1, SSAO, adheesiomolekyyli, transmigraatio, tulehdus

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# ABBREVIATIONS

| | |
|---|---|
| AOC | amine oxidase, copper dependent |
| AP | alkaline phosphatase |
| APC | allophycocyanin |
| CD | cluster of differentiation |
| CLA | cutaneous lymphocyte antigen |
| DAO | diamine oxidase |
| ELISA | enzyme-linked immunosorbent assay |
| ELISPOT | enzyme-linked immunosorbent spot |
| ESAM | endothelial cell-selective adhesion molecule |
| ESL-1 | E-selectin ligand 1 |
| FACS | fluorescence activated cell sorting |
| FITC | fluorescein isothiocyanate |
| GlyCAM-1 | glycosylation-dependent cell-adhesion molecule 1 |
| HEV | high endothelial venules |
| HLA | human leukocyte antigen |
| HRP | horseradish peroxidase |
| HUVEC | human umbilical vein endothelial cell |
| ICAM | intercellular cell adhesion molecule |
| IL | interleukin |
| INF | interferon |
| JAM | junctional adhesion molecule |
| kD | kilodalton |
| LFA-1 | leukocyte function-associated antigen-1 |
| LO | lysyl oxidase |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| Mac-1 | macrophage antigen-1 |
| MAdCAM-1 | mucosal addressin cell adhesion molecule 1 |
| MAO | monoamine oxidase |
| MDSC | myeloid derived suppressor cells |
| MLN | mesenteric lymph node |
| MMP | matrix metalloproteinase |
| mOVA | membrane-bound form of ovalbumin |
| NK | natural killer |
| OT-I | ovalbumin-specific, class I-restricted transgenic T cells |
| PE | phycoerythrin |

| | |
|---|---|
| PECAM-1 | platelet/endothelial cell adhesion molecule-1 |
| PLN | peripheral lymph node |
| PMN | polymorphonuclear leukocyte |
| PNAd | peripheral node addressin |
| PP | Peyer's patch |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| SSAO | semicarbazide sensitive amine oxidase |
| TNF-α | tumor necrosis factor-α |
| TPQ | topaquinone |
| VAP-1 | vascular adhesion protein-1 |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VEGF | vascular endothelial growth factor |
| VLA-4 | very late activation 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-IV).

**I** **Koskinen, K**., Vainio, P.J., Smith, D.J., Pihlavisto, M., Ylä-Herttuala, S., Jalkanen, S. and Salmi, M. (2004). Granulocyte transmigration through the endothelium is regulated by the oxidase activity of vascular adhesion protein-1 (VAP-1). *Blood*, 103: 3388-3395.

**II** Stolen, C.M., Marttila-Ichihara, F., **Koskinen, K**., Yegutkin, G.G., Turja, R., Bono, P., Skurnik, M., Hänninen, A., Jalkanen, S. and Salmi, M. (2005). Absence of the endothelial oxidase AOC3 leads to abnormal leukocyte traffic *in vivo*. *Immunity*, 22: 105-115.

**III** **Koskinen, K**., Nevalainen, S., Karikoski, M., Hänninen, A., Jalkanen, S. and Salmi, M. (2007). VAP-1-deficient mice display defects in mucosal immunity and antimicrobial responses: Implications for antiadhesive applications. *Journal of Immunology*, 179: 6160-6168.

**IV** Marttila-Ichihara, F., **Auvinen (née Koskinen), K**.*, Castermans, K.*, Egbrink oude, M.G.A., Elima, K., Griffioen, A.W., Jalkanen, S. and Salmi, M. The oxidase activity of vascular adhesion protein-1 controls homing of pro-angiogenic myeloid-derived suppressor cells into tumors. Submitted.

*Equal contribution

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# 1. INTRODUCTION

Living organisms continuously encounter foreign intruders from the environment. In the battle against foreign intruders our skin and other epithelial surfaces form the first line of defence. Our immune system forms more specific defence against the various microbes. Cells belonging to the innate immunity system, such as neutrophils, macrophages, eosinophils and NK-cells, destroy microbes independently of antigens. If those phagocytic cells fail to destroy the microbe, a specific acquired immunity is needed to eliminate the intruder. Acquired immunity is dependent on B- and T-cells. B-cells are responsible for antibody secretion and T-cells for cell-mediated immune responses. Acquired immunity is specific, thus each B- and T-cell recognizes only one antigenic structure. This specificity provides more effective immune responses and also immunological memory.

The ultimate requirement for immune cells is their ability to migrate throughout the body. Lymphocytes patrol between the blood and lymphoid organs searching for foreign antigens or intruders. Cells from the innate immunity as well as lymphocytes activated by antigens in the lymphatic organs have to be able to efficiently migrate into the inflamed peripheral tissues. The migration of leukocytes is mediated by molecular bonds between receptors on leukocytes and their ligands on endothelial cells lining the blood vessels. Using these adhesion molecules lymphocytes recirculate and leukocytes accumulate into the sites of inflammation, thus providing specific and effective immune responses.

There are several adhesion molecules involved in leukocyte migration. Selectins mediate the early interactions between leukocytes and endothelial cells. Firm adhesion is mediated by integrins and several molecules are involved in leukocyte transmigration through endothelial cells. Vascular adhesion protein-1 (VAP-1) is an endothelial adhesion molecule mediating leukocyte adhesion and it also displays enzymatic SSAO-activity. These studies were done to further investigate the role of VAP-1 in inflammation and cancer. Knowledge of leukocyte adhesion pathways has slowly expanded, and since inappropriate leukocyte accumulation is harmful and damages the tissues, knowledge of adhesion molecules and their regulation can have potential benefit in designing anti-adhesive therapies. In addition, leukocytes accumulating in tumors may significantly control tumor growth. More detailed knowledge is thus also needed in this field to be able to better fight against malignant cells.

# 2. REVIEW OF THE LITERATURE

It is necessary that leukocytes are in the right place at the right time in order to create an efficient immune reaction. This review of the literature will discuss the mechanisms by which leukocytes migrate through the blood vessels. Different well-known and newly discovered adhesion molecules involved in the extravasation process will also be discussed.

## 2.1. Leukocyte adhesion cascade

Leukocytes mainly exit the blood circulation through small caliper veins called postcapillary venules (Aird 2007). In postcapillary venules the flowing blood generates shear forces ranging from 1 to 4 dyn/cm$^2$ on the endothelium (Sackstein 2005). Hence, the leukocytes have to resist significant forces while interacting with the endothelium. The process by which a freely flowing leukocyte in the blood eventually migrates into the tissue is known as a multistep cascade of leukocyte extravasation (Butcher 1991; Springer 1994). The process originates from the very first loose interactions between the flowing leukocyte and the endothelium. These short but transient molecular interactions lead to the rolling of leukocytes. This initial step in the adhesion cascade is illustrated in Figure 1. The rolling leukocyte is in close proximity to the endothelium and hence it is exposed to molecules presented by the endothelium. Consequently, chemical substances, such as chemokines, activate integrins on the surface of leukocytes. Such activation enables integrins to bind their cognate ligands on the surface of the endothelium leading to the arrest of the leukocytes at the endothelium. Despite being fairly stably bound to the endothelium, leukocytes continue lateral migration, searching for a potential position to penetrate through the endothelium. Once a suitable location is found, leukocytes migrate through the endothelium into the tissue. There they start to travel into the direction of the inflammation guided by a chemokine gradient (Friedl & Weigelin 2008; Thelen & Stein 2008).

The leukocyte adhesion cascade described above is utilized during steady state lymphocyte recirculation as well as in their accumulation with other leukocytes into sites of inflammation. Each step in the adhesion cascade is regulated by a different set of receptors on the leukocytes and ligands for them on the endothelium, thus providing specificity. Moreover, leukocyte subpopulations are recruited to certain anatomical locations by regulating receptor and ligand expression on leukocytes and endothelium, respectively. In conclusion, leukocytes migrate through the blood vessels and this migration involves several sequential steps. These steps and the most important molecules involved will now be discussed.

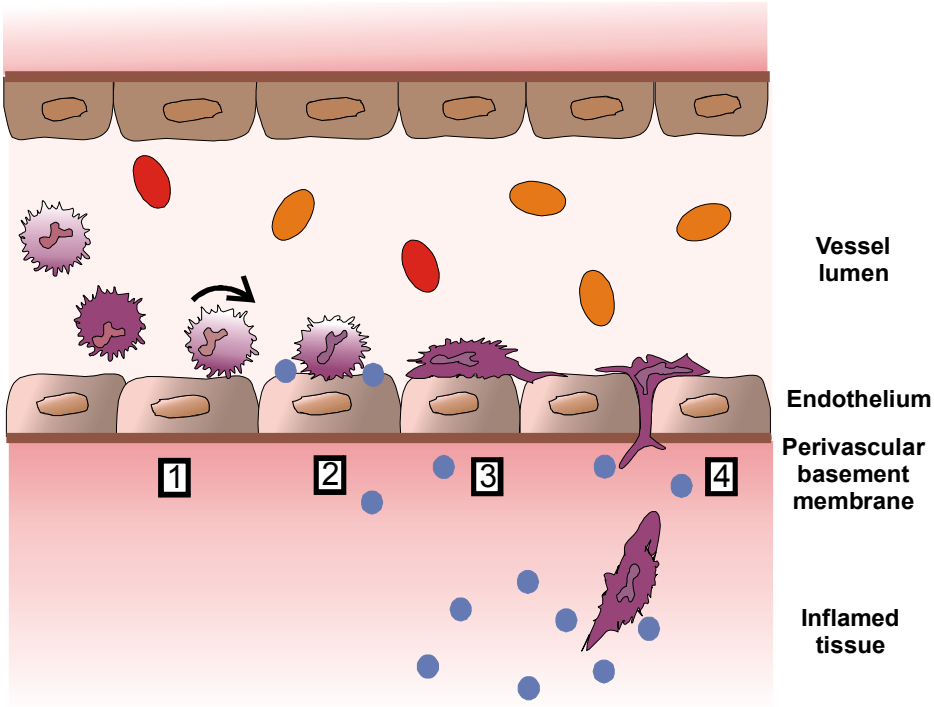



**Figure 1**. Leukocyte adhesion cascade. Freely flowing leukocytes are captured and start to roll on the endothelium (1). Several molecular bonds lead to activation (2) and arrest leukocytes to the endothelium (3). Finally, leukocytes transmigrate through the endothelium (4) and travel toward the inflammation guided by a chemokine gradient. Modified from (Salmi & Jalkanen 2005).

### *2.1.1. Tethering and rolling*

Initial interactions with the endothelium are mainly regulated by selectins. Selectins are a family of glycoproteins that bind carbohydrate counterparts. The family consists of three molecules CD62E, CD62P and CD62L named E, P and L-selectin, respectively (Ley & Kansas 2004; Ley *et al.* 2007). The names for all selectins come from the cells where they are expressed; L-selectin is expressed on leukocytes, P-selectin on platelets and endothelial cells and E-selectin on endothelium (Ley & Kansas 2004; Ley *et al.* 2007). All selectins are structurally similar and their genes are closely linked in a gene cluster on chromosome 1.

P-selectin is normally stored in the Weibel-Palade bodies in endothelial cells. Upon adequate stimulation, such as histamine and thrombin, P-selectin is rapidly (within minutes) released from the granules onto the cell surface. After being expressed on the cell surface, P-selectin is internalized by endocytosis. The expression of E-selectin, on the other hand, is regulated at the transcriptional level. The stimulation with adequate cytokines results in E-selectin expression within four hours and it is degraded by lysosomes after 24 hours. In contrast to E- and P-selectins, L-selectin is constitutively expressed by naïve lymphocytes and it mediates their recruitment into the lymphatic

organs. (Ley & Kansas 2004; Ley *et al.* 2007). L-selectin is the first adhesion molecules that was found to mediate lymphocyte homing to peripheral lymph nodes (Gallatin *et al.* 1983). In the timeline of inflammatory response, P-selectin mediates the earliest events, thus capturing the leukocytes from the flow and subsequently, E-selectin stabilizes the rolling and is responsible for slowing them down (Ley *et al.* 2007). The shear stress generated by the blood flow facilitates the adhesive functions of P- and L-selectins.

The main structural feature of selectins is a lectin-domain at the $NH_2$-terminus, thus enabling selectins to bind specific carbohydrate structures. These structures are $\alpha$ (2,3) sialylated and $\alpha$(1,3) fucosylated carbohydrates, which are identical to the sialyl Lewis x ($sLe^x$) epitope (Sperandio 2006). The protein ligand for selectins serves as a scaffold for proper carbohydrate structures. Hence, antibodies against special carbohydrate structures identified the first ligands for selectins. L-selectin binds ligands, which are recognized by MECA-79 antibody and are known collectively as a peripheral node addressins (PNAds). The structure recognized by MECA-79 is a sulphated N-acetylglycosamine (GlcNAc-6-$SO_4$). PNAds are a family of sialomucins, which includes CD34, glycosylation-dependent cell-adhesion molecule 1 (GlyCAM-1), endoglycan and podocalyxin. All of these are expressed by HEVs in the lymphoid organs thus mediating lymphocyte homing. In addition, P-selectin glycoprotein ligand-1 (PSGL-1) can serve as a ligand for L-selectin. Since L-selectin and PSGL-1 are both expressed on the leukocytes, the binding enables leukocytes to roll on leukocytes that have previously bound to the endothelium. This kind of rolling is called secondary tethering. The importance of secondary tethering is that leukocytes without ligands for E- and P-selectins are still able to accumulate into the sites of inflammation (Ley & Kansas 2004; Ley *et al.* 2007).

On of the best-characterized selectin counterparts is P-selectin glycoprotein ligand-1 (PSGL-1). As the name implies, it was first found to bind to P-selectin. When the protein is modified by special glycosylating and sulfate adding enzymes it can bind P-selectin. If critical sialic acid and fucose modification are present, E-selectin can also bind it. In particular, those modifications are recognized by HECA452 antibody, the epitope of which is known as cutaneous lymphocyte antigen (CLA). In fact, CLA is expressed on lymphocytes, and thus serving as a skin-homing receptor. In addition E-selectin ligand 1 (ESL-1), CD44 and, leukocyte function-associated antigen-1 (LFA-1) are ligands for E-selectin (Hidalgo *et al.* 2007; Kotovuori *et al.* 1993). L-selectin might also be a ligand when uniquely glycosylated (Ley *et al.* 2007; Sackstein 2005). In fact, the regulation of selectin-dependent rolling can be regulated in at least three ways, via selectin expression, via counter-receptor expression and finally via glycosylation of the counter-receptor.

The rolling of leukocytes is not solely mediated by the selectins. $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins also facilitate leukocyte rolling by binding to endothelial mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and vascular cell adhesion molecule-1 (VCAM-1), respectively. $\beta_2$ integrins, LFA-1 ($\alpha_L\beta_2$), and macrophage antigen-1 (Mac-1, $\alpha_M\beta_2$), can also mediate rolling when binding to the intercellular cell adhesion

molecule-1 (ICAM-1) and ICAM-2 (Ley & Kansas 2004). In conclusion, selectins and some integrins can facilitate the capture of freely flowing leukocytes from the blood stream.

### *2.1.2. Leukocyte activation and arrest*

Under normal flow conditions, the relatively weak molecular bonds generated by selectins and their ligands cannot arrest leukocytes. The close proximity of a rolling leukocyte with the chemokines on endothelium leads to the activation of leukocyte integrins, thus facilitating firm adhesion. The chemokines are a family of small cytokines and their major role is to guide migrating cells throughout the body. There exists approximately 50 chemokines and 20 receptors in humans. The chemokines are divided into four families (C, CC, CXC and CX3C) based on their structure (Allen *et al.* 2007). The ligands (chemokines) and the receptors for chemokines are named L and R, respectively. For example, CCL2 refers to a chemokine ligand of the CC subfamily, number 2. One of the receptors for CCL2 is called CCR2. Many different ligands can bind the same receptor and the same ligand can bind multiple receptors (Allen *et al.* 2007). In leukocyte trafficking, chemokines are involved both in a homeostasis as well as in the inflammation (Bromley *et al.* 2008). Chemokines are produced and secreted by numerous cell types including endothelial cells. Although secreted, they are mainly seen by leukocytes while bound to the cell surface or to the extracellular matrix. Indeed, chemokines are transported to the endothelial cell surface by transcytosis or they can passively diffuse through the endothelial junctions. Furthermore, proteoglycans on the surface of endothelial cells bind chemokines, thus they are immobilized on the cell surface. The binding of an endothelial chemokine to its G-protein-coupled receptor on a leukocyte leads to intracellular signaling, thus activating nearby integrins (Allen *et al.* 2007; Bromley *et al.* 2008).

Homeostatic chemokines guide physiological lymphocyte trafficking into, within, and out of lymphatic organs. CCR7 is the main chemokine receptor for entry of naïve T and B cells to lymph nodes (Forster *et al.* 1999). It is also required for migration of mature dendritic cells from tissues to the T cell areas of draining lymph nodes (Forster *et al.* 1999). CXCR4 mediates also B cell homing to peripheral lymph nodes and Peyer's patches (PP), whereas CXCR5 has a role in their homing to PP (Okada *et al.* 2002). Inside lymph nodes, T-cells are kept in the T-cell areas and B-cells in the B-cell area, respectively, through chemokine gradients. In the T-cell area, CCL21 and CCL19, the ligands for CCR7, are expressed by antigen-presenting dendritic cells and stromal cells thus attracting T-cells to that area. CXCL13, a ligand for CXCR5, attracts B cells to B cell areas in PP and lymph nodes (Ansel *et al.* 2000; Ebisuno *et al.* 2003).

The inflammatory chemokines are secreted only upon inflammation and are guiding the cells involved in innate immunity and antigen-specific effector T-cells from the blood circulation into the inflammatory site. The chemokine expression varies in different vascular beds. For example, the dermal poscapillary venules continuously express CCL17 and CCL27. The effector cells that are generated in response to cutaneous antigen, express CCR4 and/or CCR10, the respective counter-receptors for CCL17 and CCL27, thus being able to home to the skin (Bromley *et al.* 2008).

Similarly, the leukocytes that have met intestinal antigen express CCR9 and adhere and migrate through the small intestinal vasculature expressing CCL25, a ligand for CCR9 (Agace 2008). In addition to chemokine receptors facilitating signaling cascades, few receptors bind and internalize chemokines, and even degrade them. The immune response can be dampened by these scavenging chemokine receptors (Allen *et al.* 2007).

The arrest of leukocytes into the blood vessel wall is mediated by integrins. These form a large family of receptors, which bind extracellular matrix as well as cellular ligands (Hynes 2002). Thus, they mediate cell-extracellular matrix interactions and cell-cell interactions. Integrins are heterodimeric cell surface receptors having α and β subunits. There are 24 distinct integrins composed of 18 α and 8 β subunits. Integrins are found on the surface of leukocytes normally in inactive form. When leukocytes are rolling on the endothelium, the integrins are in an inactive conformation and low-affinity state in a bent form. G-protein coupled receptor (GPCR) induced inside-out signaling rapidly triggers a cascade leading to changes in the conformation of integrins from the bent form into a semi-active extended state and finally to a high affinity extended state, where it can bind its ligand (Alon & Ley 2008; Rose *et al.* 2007). These changes are known as affinity regulation of integrin avidity. Another process for regulating integrin adhesiveness is valency regulation of integrin avidity. In that process, integrins cluster on the cell surface, thus mediating multivalent interactions with ligands and increasing binding avidity. Moreover, the binding of the ligand can facilitate outside-in activation. For example, ligand binding to LFA-1 ($\alpha_L\beta_2$) induces α helix to swing out, which enables neighbouring LFA-1 to interact with that α helix thus triggering integrin's large-scale conformational changes in this integrin (Zhang *et al.* 2008). Moreover, integrin-mediated functions are regulated by the phosphorylation of cytoplasmic tail (Fagerholm *et al.* 2004). The phosphorylation of Mac-1, for instance, is required for Mac-1 activation and binding to ICAM-1 and ICAM-2 when Mac-1 mediates leukocyte adhesion and transmigration *in vivo* (Fagerholm *et al.* 2006).

Integrins can be named by α and β chains, by the CD nomenclature for α and β chains as well as by specific names. Here the specific names will be used. In leukocyte adhesion $\beta_1$, $\beta_2$ and $\beta_7$ integrins have a role in mediating leukocyte-endothelial interactions (Alon & Ley 2008). The most relevant integrins for leukocyte trafficking are LFA-1 ($\alpha_L\beta_2$), very late activation antigen, VLA-4 ($\alpha_4\beta_1$), $\alpha_4\beta_7$ and Mac-1 ($\alpha_M\beta_2$). The main endothelial cell ligands for integrins are ICAM-1 and ICAM-2 for $\beta_2$ integrins, VCAM-1 for $\alpha_4\beta_1$ and MAdCAM-1 for $\alpha_4\beta_7$, although other ligands exist, too (Alon & Ley 2008). The expression of most ligands for integrins is upregulated on the site of inflammation in the endothelium, thus facilitating leukocyte recruitment. In addition to being involved in arrest, members of the integrin and immunoglobulin superfamily also mediate transmigration (Alon & Ley 2008; Hynes 2002; Rose *et al.* 2007).

### *2.1.3. Leukocyte transmigration*

Once the leukocyte has attached to the endothelium, it starts to find a suitable place to migrate. It has been proposed that the extravasation of leukocytes occurs in the ”path of least resistance”, indicating a site on the endothelium where the diapedesis consumes little energy. Transendothelial migration has been shown by *in vivo* studies to occur in places, where gaps between pericytes exist and where the expression of basement membrane constituents is lower (Wang *et al.* 2006b). In fact, neutrophils have been shown to preferentially migrate through those ”permissive” areas and to use proteases for destroying the basement membrane underneath the endothelium (Wang *et al.* 2006b). The migrating leukocytes then need to find those vulnerable regions in the endothelium. It has been shown *in vitro* that monocytes migrate laterally from the site of firm adhesion to the junction and begin diapedesis (Schenkel *et al.* 2004b). This movement is called locomotion and it is dependent on LFA-1 and Mac-1 integrins as well as their endothelial counterparts ICAM-1 and ICAM-2. Blocking of the molecules leads to defective lateral migration. Similar movement has also been shown to exist *in vivo* and it is called ”crawling” (Phillipson *et al.* 2006). In wild type mice leukocytes usually moved to a nearby junction and begun diapedesis, whereas leukocytes from Mac-1 knockout mice did not migrate laterally but preferred to transmigrate through the endothelial cell (transcellular route) probably in a non-optimal location since the emigration time was drastically increased (Phillipson *et al.* 2006). Both studies thus indicate that leukocytes move on the endothelial surface searching for a site for diapedesis. A recent study suggested that leukocytes sense the endothelium with special organs called podosome-like protrusions (Carman *et al.* 2007). The podosomes probe the endothelial surface for sites where it can migrate in a transcellular fashion. Once such a site is found, podosome protrusion extends and initiates the transcellular migration by extruding a podosome-like protrusion into the endothelial cell forming a transcellular pore (Carman *et al.* 2007). Moreover, proteinase activity is involved in integrin-dependent migration since LFA-1 and Mac-1 bind pro-matrix metalloproteinase-9 (MMP) (Stefanidakis *et al.* 2003). This Mac-1/MMP-9 complex mediates neutrophil migration *in vitro* and *in vivo* (Stefanidakis *et al.* 2004).

Whereas leukocyte rolling, chemokine activation and integrin-dependent arrest have been relatively well studied, less is known regarding the actual transmigration through the endothelium. First, the site where a leukocyte migrates is unclear being either between the adjacent endothelial cells (paracellular route) or through a single endothelial cell (transcellular route). Both routes are illustrated in Figure 2. For a long time the paracellular route was accepted; however, it was under debate whether transcellular migration even exists. It is now generally accepted that leukocytes can utilize both pathways (Carman & Springer 2008).

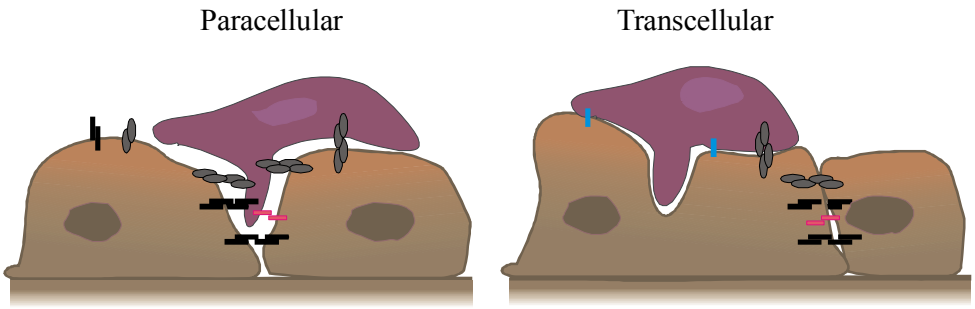



**Figure 2.** Paracellular and transcellular migration. In the paracellular route, the leukocyte migrates between two adjacent endothelial cells (left), whereas transmigration through a single endothelial cell is called transcellular migration (right).

In both the paracellular and transcellular migration routes the endothelium has an active role. Shear forces generated by blood flow are also thought to promote the migration process (Cinamon *et al.* 2001). Once the leukocyte has attached to the endothelium, a "transmigratory cup" of ICAM-1- and VCAM-1 –enriched projections are formed surrounding the migrating leukocyte (Carman & Springer 2004). These projections are thought to further guide or facilitate the leukocyte to transit from the lateral migration to diapedesis. These projections are formed both in a paracellular as well as in a transcellular migration process *in vitro* as studied by high resolution fluorescence imaging techniques. Moreover, similar cup-like structures occur *in vivo* both in paracellular and transcellular migration (Phillipson *et al.* 2008). Furthermore, it has been shown that endothelial docking structures progress to surround the whole migrating leukocyte (Phillipson *et al.* 2008). This, in turn, ensures that the vascular barrier function is maintained through the leukocyte migration process and the cytosolic content is not released to the surroundings. In transcellular migration, ICAM-1 is shown to be recycled to vesicular structures, called caveloae. Again the leukocyte protrusions penetrate into the endothelial cell cytoplasm and ICAM-1 and caveolin co-cluster around the migrating protrusion forming a kind of channel, through which the leukocyte squeezes and migrates through the endothelial cell (Millan *et al.* 2006). Other similar protein-rich areas have been found to surround the migrating leukocyte. Intermediate filaments in leukocytes and endothelial cells also contribute to transcellular migration (Nieminen *et al.* 2006). The route by which the leukocyte migrates depends on several factors. There is now evidence that the leukocyte subtype, cytokine stimulation, the organ into which it migrates and the type of the blood vessel contributes to the emigration route (Carman & Springer 2008).

### *2.1.4. Junctional adhesion molecules*

Molecules concentrated on the junctions maintain the endothelial barrier function. Recent studies have suggested that several junctional molecules also regulate leukocyte diapedesis through the endothelium. These molecules are usually enriched at the endothelial cell-cell contacts and function through homophilic and heterophilic interactions.

Platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) is an adhesion molecule belonging to the immunoglobulin superfamily. Both endothelial cells and leukocytes express it. Endothelial PECAM-1 can bind either in a homophilic manner to PECAM-1 on leukocytes or other ligands (such as CD38, CD177, $\alpha_v\beta_3$). During leukocyte migration, endothelial PECAM-1 is recycled constitutively along the cell borders and intracellular vesicles (Mamdouh *et al.* 2003; Mamdouh *et al.* 2008). In addition to the leukocyte transmigration through the endothelium, PECAM-1 is involved in leukocyte migration through the basement membrane (Wakelin *et al.* 1996). PECAM-1 deficient mice display normal leukocyte transmigration, although leukocytes are trapped under the endothelium and are unable to migrate through the basement membrane (Duncan *et al.* 1999). Later analysis has revealed that PECAM-1 functions are stimulus-specific as well as related to the mouse strains used (Schenkel *et al.* 2004a). Interleukin-1β (IL-1β) induced inflammation induces PECAM-1-dependent transmigration, whereas tumor necrosis factor-α (TNF-α) or formyl-methionyl-leucyl-phenylalanine (fMLP) induces PECAM-1 independent transmigration. Similarly, in some models of inflammation, PECAM-1 has shown anti-inflammatory properties (decreasing leukocyte migration) while in others enhanced inflammatory responses are observed. In addition to leukocyte migration, PECAM-1 is implicated in several other biological functions such as angiogenesis, apoptosis, platelet aggregation, endothelial cell motility and thrombosis, which might contribute to the various opposite properties found in relation to PECAM-1 blocking in inflammatory settings (Woodfin *et al.* 2007).

There are three types of junctional adhesion molecules (JAM), namely JAM-A, JAM-B and JAM-C. They are all expressed on endothelial and epithelial cells and have a role in assembling and maintaining the junctions (Bradfield *et al.* 2007). In addition to endothelial and epithelial expression, they are also expressed by leukocytes. JAM-A has been shown to be involved in leukocyte recruitment in a stimulus-dependent manner (Martin-Padura *et al.* 1998). JAM-A deficient mice display defects in leukocyte accumulation, although the effects were specific for stimulus, endothelium and leukocyte subtypes (Cera *et al.* 2004; Corada *et al.* 2005; Khandoga *et al.* 2005). JAM-A can bind JAM-A, leading to homophilic interaction. However, JAM-A can also bind LFA-1 and specifically, this binding is involved in lymphocyte as well as neutrophil transmigration (Ostermann *et al.* 2002). Endothelial JAM-B, on the other hand, binds leukocyte JAM-C. Moreover, JAM-B can bind VLA-4; however, there is a need for JAM-B and JAM-C binding prior to VLA-4 binding. JAM-C can also bind Mac-1 and $\alpha_x\beta_2$. In addition to JAMs role in leukocyte transmigration, they are also in angiogenesis, mediating the endothelial cell migration and integrin signaling (Weber *et al.* 2007)

A related protein to JAMs is endothelial cell-selective adhesion molecule (ESAM). It is expressed on endothelial cells as well as on platelets but it is absent from leukocytes. ESAM-deficient mice have defects in neutrophil accumulation into the peritoneal cavity (Wegmann *et al.* 2006). As ESAM is concentrated to the endothelial junctions, it regulates vascular permeability thus leading to reduced leukocyte transmigration

(Wegmann *et al.* 2006). In addition, ESAM deficiency is shown to be related to defective angiogenesis (Ishida *et al.* 2003).

There are several other molecules regulating leukocyte transmigration. CD99 is a small transmembrane protein, which was originally identified in erythrocytes and leukocytes but is also expressed on endothelial cells. The blocking of CD99 with antibody almost abolished leukocyte transmigration through the endothelium (Schenkel *et al.* 2002) (Lou *et al.* 2007). CD99 mediates a step distinct from PECAM-1, since blocking both CD99 and PECAM-1 has an additive effect. Moreover, it has been shown to mediate T cell and polymorphonuclear cell recruitment *in vivo* (Bixel *et al.* 2004; Dufour *et al.* 2008). Since CD99 is expressed on leukocytes as well as on the endothelial cells, it has been proposed that a homophilic interaction could lead to transmigration. However, direct evidence for this is still lacking. There is also a related gene to CD99, named CD99 antigen like-2 (CD99L2). It is expressed on leukocytes as well as on endothelial cells. It has not been shown to be involved in lymphocyte transmigration *in vitro* or *in vivo*, but has an effect on neutrophil transmigration *in vitro* and also *in vivo* in a peritonitis model (Bixel *et al.* 2007). The results indicate that transmigration could be due to homophilic interaction between endothelial and leukocyte CD99L2.

VE-cadherin, one of the main molecules controlling endothelial barrier functions, is also involved in leukocyte diapedesis by "moving away" from the cell contacts as the leukocyte migrates through the endothelium (Vestweber 2007). Another molecule involved in leukocyte transmigration is CD157, which is expressed on leukocytes and endothelial cells. Recently, it has been shown to mediate neutrophil transmigration through the endothelium and it is also involved in neutrophil movement at the apical surface of the endothelium (Funaro *et al.* 2004; Ortolan *et al.* 2006). In addition to it's adhesive properties, CD157 is a NADase/ADP-ribosyl cyclase. However, the authors have not suggested that the enzymatic activity could be related to transmigration. In addition, CD155, also called poliovirus receptor (PVR), is expressed on the endothelial cells. It can bind CD226 (DNAX accessory molecule 1, DNAM-1) on leukocytes and this binding leads to monocyte transmigration through the endothelium (Reymond *et al.* 2004). If the binding is blocked using antibodies against either of the molecules, leukocytes are able to adhere to the endothelium but cannot transmigrate through the junctions.

Although several adhesion molecules have been shown to be important in mediating leukocyte diapedesis, the phenotypic analysis of mice genetically deficient in these molecules display often mild phenotypes (Kakkar & Lefer 2004). This may be due to compensatorial mechanisms in the system. In conclusion, leukocyte transmigration in general is a complex system and is not solely dependent on one or a small number of molecules. Instead, several probably still unknown molecules must be present.

## 2.2. Leukocyte-vascular interactions in steady-state and inflammation

In homeostasis, naïve lymphocytes continuously circulate through the lymphatic organs. Immature dendritic cells in PP, lymph nodes and the spleen collect and process intestinal, lymph-borne or blood-borne antigens, respectively. After capturing antigens, dendritic cells migrate into the T cell area of lymphoid tissues and assume a fully mature phenotype before they present antigens to T cells. Endothelial cells lining the vasculature in the lymphatic organs, HEVs, support efficient entry of naïve and central memory T cells and B-cells from the circulation in the steady-state. The recirculation of naïve lymphocytes and central memory T cells into peripheral lymph nodes (PLN) critically involves L-selectin and PNAd, chemokines CCL21 and/or CCL19 on HEVs and CCR7 on naïve T cell and LFA-1-ICAM-1 binding (Bono *et al.* 2007; Miyasaka & Tanaka 2004). Central memory T cells and B cells use in addition complex array of chemokines and chemokine receptors. In PP, recirculation of naïve T cells is mediated by L-selectin and $\alpha_4\beta_7$, the latter being the predominant PP adhesion molecule (Kunkel & Butcher 2002; Miyasaka & Tanaka 2004). Both of them bind HEV-expressed MAdCAM-1. In addition, CCL21 and/or CCL19 and CCR7, LFA-1 and ICAM-1 are involved in naïve T cell migration to PP (Kunkel & Butcher 2002; Miyasaka & Tanaka 2004). Central memory T cells are using mainly the same molecules to home to PP as naïve T cells. B cells use L-selectin, LFA-1 and $\alpha_4\beta_7$ and MAdCAM-1 and also CXCR4 and CXCR5 and their ligands CXCL12 and CXCL13, respectively (Miyasaka & Tanaka 2004). HEV in mesenteric lymph nodes (MLN) express both PNAd and MAdCAM-1 (Mowat 2003). Thus, MLN shows features of both peripheral and mucosal/intestinal immune systems (Mowat 2003). The mechanism behind lymphocyte migration to the spleen is only partially known and selectins appear not to be involved.

There exists a tissue-specific effector lymphocyte imprinting, in which newly activated lymphocytes receive site-specific signals that direct those lymphocytes back to the sites where initial antigen was encountered (Agace 2006; Sigmundsdottir & Butcher 2008). That site is usually the location where reencounter is most likely to occur. This has been demonstrated to occur at least in the skin and gut. Naïve T cells activated under the influence of retinoic acid acquire a gut-homing phenotype with high expression levels of $\alpha_4\beta_7$ and CCR9 thus allowing them to bind the ligands expressed in the gut endothelium such as MAdCAM-1 and CCL25 (Iwata *et al.* 2004). Equally, lymphocytes activated in PLN draining the skin in the absence of retinoic acid and presence of additional specific imprinting signals induce the expression of PSGL-1, CXCR3 and CCR4. Thus, these activated lymphocytes bind ligands expressed by the skin endothelium such as E- and P-selectin and chemokines CXCL9/10 and CCL17 (Fisher *et al.* 2006).

Central memory T cells are also migrating into non-inflamed skin. Cutaneous lymphocyte antigen (CLA) on the surface of central memory T cells binds to E- and P-selectin on dermal microvessels. Also CCR4-CCL17 and CCR8-CCL1 and LFA-1-ICAM-1 interactions have been implicated in central memory cell recruitment to non-inflamed skin. However, the situation is changed as a consequence of a local tissue inflammation. The release of several proinflammatory cytokines, such as TNF-$\alpha$,

IL-1β and interferon-γ (INF-γ), induces the expression of VCAM-1 and several chemokines in dermal microvessels. Recent results have also indicated that microRNAs are involved at least in the regulation of VCAM-1 (Harris *et al.* 2008). Effector lymphocyte homing into inflamed skin use mainly the same pathways as central memory T cell homing to non-inflamed skin, but they differ more significantly in their expression of chemokines and chemokine receptors involved. In inflammation, in addition to CCR4 and CCR8, CCL27-CCR10 and CCL20/CCR6 might also have a role (Campbell *et al.* 2007; Homey *et al.* 2002; Homey *et al.* 2000; Reiss *et al.* 2001).

The first leukocytes found at the site of inflammation are neutrophils. They are circulating in the blood and are recruited to the site of inflammation within hours (Zarbock & Ley 2008). They express several adhesion molecules, and are thus able to bind the inflammation-induced counter-receptors on activated endothelial cells. At least all selectins, PSGL-1, LFA-1, Mac-1, ICAM-1 and ICAM-2, are involved (Zarbock & Ley 2008). Also several junctional transmigration proteins, such as PECAM-1, JAMs, ESAM, CD99, CD99L2 and CD157, have a role in neutrophil accumulation into inflamed tissues (Bradfield *et al.* 2007; Ortolan *et al.* 2006; Wegmann *et al.* 2006; Woodfin *et al.* 2007).

## 2.3. Leukocyte-vascular interactions in cancer

Leukocytes always accumulate in tumor tissue. The amount of leukocytes, their composition and distribution vary between different tumors. The chemoattractants secreted by the tumor cells and stromal cells attract leukocytes into the tumor site. The inflammatory cells found in the tumor tissues include tumor-associated macrophages, neutrophils, eosinophils, dendritic cells, NK-cells, mast cells and T cells. Tumor infiltrating leukocytes can have either antitumoral or protumoral effects. Tumor-associated macrophages can be activated in two ways, thus dividing them into two subclasses. Classically activated macrophages (M1) have a pro-inflammatory phenotype, promote Th1 responses, and are often found at the beginning of tumor development (Mantovani *et al.* 2008). However, once the tumor is vascularized and progressed, the alternatively activated macrophages (M2) are prominent and have an immunosuppressive phenotype by promoting Th2 responses and angiogenesis (Mantovani *et al.* 2008). A special subpopulation of monocytes expressing angiopoietin receptor TIE2 are found in several tumors and their functions are also related to angiogenesis. Myeloid-derived suppressor cells (MDSC) and regulatory T-cells promote also protumoral effects (Mantovani *et al.* 2008; Murdoch *et al.* 2008).

Since MDSCs are involved in these studies, I will focus on this leukocyte population and their angiogenic properties in this section. MDSCs are defined by co-expression of markers Gr-1 (binding Ly-6C and Ly-6G) and CD11b, which is the α chain of Mac-1 (Marigo *et al.* 2008). They are a heterogeneous cell population of myeloid cells including immature granulocytes, macrophages and dendritic cells and other myeloid cells at the early stages of differentiation (Serafini *et al.* 2006). In fact, the function of MDSC and tumor-associated macrophages is quite similar, and it is still unclear whether they are overlapping myeloid populations with high functional plasticity. The

cytokine environment regulates MDSC differentiation, either sustaining them in a suppressive phenotype (as seen in tumors) or enhancing their differentiation into mature dendritic cells, macrophages and granulocytes, thus increasing the anti-tumoral effects. MDSCs are a diverse cell population comprising at least two subpopulations for markers Gr-1 and CD11b. The first population has high Gr-1 expression and their phenotype is "polymorphonuclear like", whereas the other has lower Gr-1 expression and they have a "monocyte-like" phenotype (Movahedi *et al.* 2008; Youn *et al.* 2008). The exact relationship or function of these populations is not yet clear. MDSC are able to suppress anti-tumoral functions of T cells and NK-cells (Marigo *et al.* 2008). In mouse, few MDSC are found in the blood, spleen and bone marrow of healthy animals and they do not possess a suppressive phenotype. However, once tumor-bearing mice are analyzed, the number of MDSCs is increased in bone marrow and spleen and they accumulate in the tumors. The tumor-derived factors promote MDSC recruitment as well as their maturation toward an immunosuppressive phenotype. These factors include mediators such as colony stimulating factor-1 (CSF-1), vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-10 and IL-13 as well as IFN-γ. (Marigo *et al.* 2008; Serafini *et al.* 2006). In addition to studies performed with mice, MDSCs are also found in human patients with various cancers (Mantovani *et al.* 2008; Murdoch *et al.* 2008)

Tumor blood vessels have an irregular structure. The vascular parameters divide the tumor into two microanatomical regions. The peritumoral area, which surrounds the tumor nodule, has HEV-like vessels with expression of several endothelial adhesion molecules and thus mediates leukocyte infiltration into the tumor. The vessels in the intratumoral area, on the other hand, almost completely lack adhesion molecule expression and have dramatically decreased leukocyte interactions with the endothelium. This downregulation of adhesion molecule expression might help the tumor to escape from immunity (Griffioen 2008). In fact, the expression of VCAM-1 and ICAM-1 is decreased or even absent in the intratumoral areas. The expression of chemokines, such as CXCL9 and CXCL10, is also decreased. The low adhesion molecule expression might be due to the presence of several tumor-derived angiogenic factors, such as VEGF and basic fibroblast growth factor (bFGF), which downregulate ICAM-1 and VCAM-1 expression *in vitro*. However, VLA-4 has been shown to regulate macrophage infiltration into the tumors, although the counterpart on the endothelium is not known (Jin *et al.* 2006). In conclusion, a reduced level of leukocyte-vessel wall interaction and impaired infiltration of leukocytes into the tumor is probably a mechanism for the tumor to escape the immune system (Dirkx *et al.* 2003; Fisher *et al.* 2006).

### *2.3.1. Angiogenesis*

Mammalian cells are dependent on oxygen and nutrients. Simultaneous and efficient transport and exchange of these nutrients, signalling molecules as well as circulating cells between the tissues and organs are vital. This transport and exchange are possible due to the blood and lymphatic vasculature. The formation of new blood vessels, called neovascularization, is important in development, in inflammation and wound repair. It is also related to several pathological conditions, such as tumors. During

embryogenesis, the vasculature begins to develop through a process called vasculogenesis. In blood vessel formation the endothelial progenitor cells, angioblasts, migrate to sites of vascularization and differentiate into endothelial cells. Angiogenesis and arteriogenesis are processes in which new vessels are formed from already existing ones by sprouting of the endothelial cells. In collateral growth, angiogenesis usually starts from insufficient oxygen supply, called hypoxia. Hypoxia induces several cells to locally produce angiogenic cytokines, such as vascular endothelial growth factor VEGF-A, usually referred to as VEGF. In addition to VEGF, several other angiogenic factors are needed for sufficient and functional vascularization, such as angiopoietin-1 (Adams & Alitalo 2007; Carmeliet 2005; Kovacic *et al.* 2008; Semenza 2007).

Once vascular endothelial cells form new sprouts, the basement membrane has to be broken down. Proteolytical degradation is mediated by means of proteinases, including several matrix proteinases (Hughes 2008). Once the endothelial cells have formed a nascent vessel, it matures into a durable vessel. The recruitment of pericytes and vascular smooth muscle cells into vessels is a feature of blood vessel maturation. Pericytes are in direct contact with the endothelial cells, and they give support to the capillaries (Hughes 2008). In capillaries, endothelial cells are surrounded by occasional pericytes and extracellular matrix. The arteries and veins, on the other hand, are composed of several linings, and have a basement membrane layer, which mainly contains smooth muscle cells and their extracellular matrix (Semenza 2007). The formation of new vessels is dependent on several cell types. In addition to endothelial cells, pericytes and smooth muscle cells, several types of hematopoietic cells are also involved by secreting angiogenic factors. Moreover, there might be circulating endothelial progenitor cells in the adults (Kovacic *et al.* 2008). These cells, that may mobilize from the bone marrow or even other organs, home into the areas of vascularization, and differentiate to form the endothelium. In this way, neovascularization resembles the vasculogenesis seen in embryogenesis (Adams & Alitalo 2007; Carmeliet 2005; Kovacic *et al.* 2008; Semenza 2007).

## 2.4. Vascular adhesion protein-1

Vascular adhesion protein-1 (VAP-1) was originally identified by the monoclonal antibody, 1B2, which was produced against human inflamed synovial vessels when searching for new adhesion molecules. This antibody was found to strongly stain HEV-like venules. It also diminished lymphocyte binding to HEV by 50 % in frozen sections in Stamper-Woodruff *in vitro* assay. The expression pattern, molecular weight and function of VAP-1 indicated that it was a novel endothelial adhesion molecule involved in lymphocyte binding.(Salmi & Jalkanen 1992). The cloning of VAP-1 revealed that it was also an enzyme (Smith *et al.* 1998).

### *2.4.1. Tissue distribution and cellular expression*

VAP-1 is expressed constitutively on high endothelial cells of peripheral lymph node-type HEVs (like tonsils and PLN). In the gut a weak staining is seen in HEVs of the appendix and PP and also in flat-walled venules in the lamina propria (Salmi *et al.* 1993). In the liver, sinusoidal and other vascular endothelial cells are positive for

VAP-1 (McNab *et al.* 1996). Under normal conditions only some small-caliber venules in many other tissues are positive for VAP-1 (Salmi *et al.* 1993), but at the sites of inflammation, VAP-1 is rapidly upregulated at the vasculature. During inflammation the expression of VAP-1 is seen in several organs such as the skin, gut and heart (Arvilommi *et al.* 1996; Jaakkola *et al.* 2000a; Salmi *et al.* 1993). In contrast, VAP-1 is absent on large-sized vessels (aorta, vena cava), leukocytes, fibroblasts, epithelial cells and from *in vitro* growing endothelial cell lines (Salmi & Jalkanen 1992; Salmi & Jalkanen 1995; Salmi *et al.* 1993).

VAP-1 is is also expressed abundantly on various cell types including adipocytes, smooth muscle cells (of arteries, veins and bowel wall), pericytes and follicular dendritic cells of germinal centers (Jaakkola *et al.* 1999; Salmi & Jalkanen 1992; Salmi & Jalkanen 2001; Salmi *et al.* 1993). In adipocytes VAP-1 has a role in glucose metabolism by increasing glucose uptake (Zorzano *et al.* 2003). In addition to humans, VAP-1 has also been identified in mice, rats, rabbits, dogs and pigs (Bono *et al.* 1998a; Jaakkola *et al.* 2000b; Martelius *et al.* 2000) (Tohka *et al.* 2001).

According to confocal microscopic studies, VAP-1 is localized both at the luminal surface and in the intracellular granules in HEVs (Salmi *et al.* 1993). The granules are distinct from the Weibel-Palade bodies, since VAP-1 is not localized with Factor VIII in two-color immunofluorescent stainings (Salmi & Jalkanen 1995). The granular staining suggests that VAP-1 is stored intracellularly and it can be released rapidly to the cell surface during inflammation. However, the mediators regulating VAP-1 translocation and expression are not known. It seems that the microenviroment is important, since *in vitro* cultured tonsillar HEV cells are not inducible but tonsil vessels release VAP-1 from granules to the cell surface after stimulation in organ cultures (Arvilommi *et al.* 1997).

The regulation of VAP-1 *in vivo* has been studied in dogs, pigs and humans (Jaakkola *et al.* 2000b; Vainio *et al.* 2005). Luminal expression of VAP-1 has been seen in inflamed endothelium. Translocation of VAP-1 to the cell surface is seen 60 minutes after inducing experimental skin inflammation with dinitrochlorobenzene, while the maximal surface expression is seen at 8 hours and lasts until 48 hours of inflammation (Jaakkola *et al.* 2000b). This early translocation of VAP-1 onto the cell surface within an hour after the stimulus suggests that VAP-1 is released from granules, but the de novo synthesis might also be involved since VAP-1 protein is seen still after 48 hours.

### *2.4.2. Amine oxidases*

Cloning of VAP-1 in 1998 revealed a real surprise, in that it had significant similarity to enzymes named copper-containing amine oxidases (AOC) E.C. 1.4.3.6. (Smith *et al.* 1998). The highest similarity was found with bovine serum amine oxidase (BSAO). The gene family consists of four genes in humans. The coding genes are AOC1, AOC2 and AOC3. The first gene, AOC1, encodes a soluble diamine oxidase DAO (Fukui and Miyake 1992). The second gene, AOC2, encodes retina-specific amine oxidase, RAO, which has been cloned from the retina and its expression is restricted to retinal ganglion cells (Imamura *et al.* 1997). However, very little is known about retina-

specific amine oxidase. The AOC3 gene has been cloned in two separate laboratories and hence it has two names. The name human placental amine oxidase has been given since the gene was sequenced from the human placental library and vascular adhesion protein-1 (VAP-1) because the adhesive functions were found prior to sequencing (Smith *et al.* 1998; Zhang & McIntire 1996). Here the name VAP-1 will be used. The fourth AOC4 gene is a pseudogene (Cronin *et al.* 1998).

Amine oxidases found in mammals are a heterogenous group of enzymes that catalyse oxidative deamination of several monoamines, diamines as well as polyamines into corresponding aldehydes in a reaction where ammonium and hydrogen peroxide are released according to the following reaction:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

Amine oxidases are usually divided into two main classes on the basis of their cofactors and chemical nature. The first group contains a flavin adenine dinucleotide (FAD) as a cofactor and it includes enzymes named polyamine oxidase and monoamine oxidase (MAO). Furthermore, monoamine oxidases are divided to MAO-A and MAO-B on the basis of their substrate specificity. Polyamine oxidase catalyzes oxidation of spermine, spermidine and their N-acetyl derivates as preferred substrates, and these compounds might be physiological regulators of cell growth. MAOs are mitochondrial enzymes responsible for catalyzing oxidation of monoamines such as the neurotransmitters noradrenaline and dopamine, and for this reason they are related to neurological disorders. The other main class of amine oxidases is comprised by copper-containing amine oxidases (AOC) and they have one or several carbonyl groups as a cofactor and the cofactor is usually topaquinone (TPQ), a posttranslationally modified tyrosine. Because of the carbonyl groups in the active site of the enzyme, they are sensitive for carbonyl reagents such as semicarbazide, hence, they are also called semicarbazide-sensitive oamine oxidases (SSAO). Enzymes belonging to this second group are diamine oxidase (DAO) and monoamine oxidizing enzymes located in plasma or bound to the plasma membrane (Lyles 1996) (Schwelberger 2007). In the literature, the term SSAO has also been used only for the monoamine oxidizing enzymes located in plasma or bound to the plasma membrane (VAP-1). The SSAO enzymes will be discussed in the next section.

### *2.4.3. Semicarbazide-sensitive amine oxidase activity*

The special feature of copper-containing amine oxidases is their cofactor, TPQ, which is posttranslationally modified from tyrosine in a copper-dependent manner in the active site. TPQ is located in the active site of the enzyme and is essential for the enzymatic reaction (Klinman & Mu 1994). This reaction involves two half-reactions. In the first reducing reaction a substrate (primary amine) binds to the TPQ leading to its reduction and a transient but covalent Schiff base is formed between the enzyme and substrate before an aldehyde is released. Thereafter, in the oxidative reaction, the reduced TPQ is hydrolysed with oxygen, resulting in the release of hydrogen peroxide and ammonia (Klinman & Mu 1994).

The substrates for SSAOs are all amines. DAO metabolizes histamine, putrescine and cadaverine, and is also called histaminase. Due to its ability to metabolize histamine, it regulates allergic reactions and inflammation. It was first discovered in the kidney, hence it is also known as kidney diamine oxidase. It is expressed mainly intracellulary in the kidney, placenta and intestine (Lyles 1996). Benzylamine is a good and widely used experimental substrate for VAP-1, but is non-physiological. At least two endogenous substrates exist for VAP-1, namely methylamine and aminoacetone (Lyles & Chalmers 1992; Precious *et al.* 1988). Methylamine is formed in the human body when adrenaline, sarcosine or creatine is degraded. The corresponding aldehyde is formaldehyde, which is potentially toxic (O'Sullivan *et al.* 2004). Glycine or threonine metabolism, on the other hand, produces aminoacetone. The corresponding aldehyde is methylglyoxal (2-oxopropanal), which is toxic, too. In addition, SSAOs can utilize several other substrates; however, many of them are also substrates for MAOs (O'Sullivan *et al.* 2004). In fact, the substrate specificity and inhibitor sensitivity are somewhat overlapping between amine oxidases. In addition to aldehyde, ammonia and hydrogen peroxide are produced in the SSAO reaction. Both ammonia and hydrogen peroxide are cytotoxic at high concentrations. Ammonia is transported through cell membranes by a special transport protein (rhesus factor) (Biver *et al.* 2008). However, hydrogen peroxide at lower concentrations have signal-transducing features (Nathan 2003; Reth 2002). Thus, the outcome depends on the concentration of hydrogen peroxide as well as on the cell type subjected to the substance. It is involved in apoptosis, cell growth, and proliferation thus representing quite diverse events. Moreover, several adhesion molecules, such as E- and P-selectin, VCAM-1 and ICAM-1, are upregulated by hydrogen peroxide, thus regulating leukocyte adhesion (Ichikawa *et al.* 1997; Ng *et al.* 2002). In conclusion, the family of SSAOs consists of four enzymes, which are all sensitive for semicarbazide but have differences in substrate specificities, expressions and functions.

### *2.4.4. Structural characterization of VAP-1*

VAP-1 is a homodimeric glycoprotein that consists of two identical 90 kD subunits (Figure 3). The protein is heavily glycosylated including sialylation (Salmi & Jalkanen 1996). The removal of sialic acids on oligosaccharides of VAP-1 results in a diminished ability to mediate lymphocyte binding (Salmi & Jalkanen 1996). This indicates that the glycosylation is essential for the proper function of VAP-1 and is a way of regulating the function of VAP-1 in different cell types and organs by the differential post-translational modifications of the core protein.

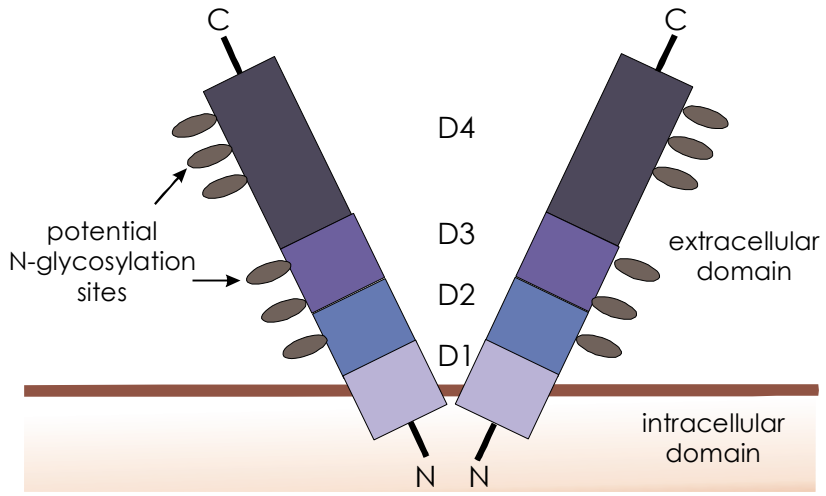



**Figure 3.** Molecular structure of the VAP-1 protein as a schematic picture modified from Salmi and Jalkanen, 2001. Two VAP-1 monomers fold into a hear-shaped dimmer and each domain 4 contains the active site (Airenne *et al.* 2005).

Glycosylation of VAP-1 is essential for leukocyte binding. The structural analysis of VAP-1 also supports the findings by showing that some of the putative N- and O-glycosylation sites are located at the entrance of the active site. In Ax cells (endothelial cells isolated from the peripheral lymph nodes of a rat) the O-glycosylation site is not utilized while the six N-glycosylation sites are glycosylated (Maula *et al.* 2005). The mutation of N-sites reduces leukocyte adhesion while expression levels are normal. This suggests that glycosylation is indeed needed for the proper adhesive function of VAP-1 (Maula *et al.* 2005). In addition, VAP-1 contains an RGD motif (Smith *et al.* 1998). This RGD motif is expressed in many extracellular matrix proteins and is recognized by several integrins, thus indicating that it is related to adhesion.

VAP-1 is a type 2 transmembrane protein with a short cytoplasmic N-terminus, a single transmembrane domain and a large extracellular domain. Based on the crystal structure of the external part of VAP-1 (Airenne *et al.* 2005; Jakobsson *et al.* 2005), it is a mushroom-shaped homodimer. Domain 4 contains the catalytic site essential for enzymatic activity and it is located in the interface of the dimers. Domain 4 is highly conserved in all known copper-containing amine oxidases and a special feature is the unusual cofactor TPQ in position 471 (Airenne *et al.* 2005; Salminen *et al.* 1998).

### *2.4.5. Soluble form of VAP-1 and diseases*

Many adhesion molecules have soluble forms in the blood stream (Gearing & Newman 1993). At least two mechanisms regulate the release of the membrane bound adhesion molecules. The extracellular part can be enzymatically cleaved by proteinases in a process called shedding. Another possibility is RNA splicing, where RNA is spliced not to contain a transmembrane domain. The soluble form of VAP-1 is produced by metalloproteinases cleaving the membrane bound form (Abella *et al.* 2004). The cleavage is partly regulated by TNF-α resulting in increased soluble VAP-1 and a

decreased membrane bound form of VAP-1 (Abella *et al.* 2004). However, the exact metalloproteinases responsible for the cleavage are unknown. Under normal conditions, the main source of soluble VAP-1 is the endothelial cells (Stolen *et al.* 2004). However, under pathological conditions such as diabetes, soluble VAP-1 can originate from both endothelial cells and adipocytes (Stolen *et al.* 2004). Soluble VAP-1 has SSAO-activity and it accounts for most of the SSAO activity in human serum (Kurkijarvi *et al.* 2000).

Soluble VAP-1 is found in the serum of healthy individuals, but the levels are elevated two- to fourfold in patients with certain inflammatory liver diseases (Kurkijarvi *et al.* 1998). Moreover, patients with type I diabetes also have elevated soluble VAP-1 concentrations and SSAO-activity in their serum and it can be decreased to a normal level after normalizing blood glucose and ketone body levels with exogenous insulin (Salmi *et al.* 2002). Furthermore, increased soluble VAP-1 levels have been observed in patients with relapsing remitting multiple sclerosis, psoriasis, atopic eczema and acute ischemic stroke (Airas *et al.* 2008; Airas *et al.* 2006; Madej *et al.* 2006; Madej *et al.* 2007). However, not all inflammatory conditions elevate soluble VAP-1 in serum, since rheumatoid arthritis and inflammatory bowel disease patients had normal soluble VAP-1 concentrations (Kurkijarvi *et al.* 1998). Thus, soluble VAP-1 seems to be a marker of specific inflammatory conditions instead of being a marker for general inflammatory conditions.

### *2.4.6. Adhesive functions*

Since the discovery of VAP-1, its role in leukocyte adhesion has been studied quite extensively. The first evidence for leukocyte migration came from the original study, where antibody against VAP-1 diminished lymphocyte binding to HEV in lymphatic organs (Salmi & Jalkanen 1992). The subpopulations of leukocytes were then analyzed and mainly CD8 T cells and NK-cells showed decreased adhesion to PLN HEVs indicating that VAP-1 may be involved in subtype-specific leukocyte extravasation to the lymphoid organs during physiological recirculation (Salmi *et al.* 1997). Since VAP-1 is also expressed and upregulated under inflammatory conditions in non-lymphoid tissues, VAP-1 mediated adhesion to the inflammatory sites has been studied in humans using *in vitro* binding assays using frozen sections. It has been shown that an antibody against VAP-1 decreases leukocyte binding to the vasculature of inflamed lamina propria, ischemic heart, inflamed skin and liver (Arvilommi *et al.* 1996; Jaakkola *et al.* 2000a; McNab *et al.* 1996; Salmi *et al.* 1993). Thus, it has been shown that the blocking of VAP-1 reduced leukocyte adhesion to lymphatic organs and to inflamed tissues in man *in vitro*.

Several *in vivo* studies have indicated an important role of VAP-1 in leukocyte recruitment into inflamed areas. Intravital microscopic studies and a peritonitis model in rabbits have shown that VAP-1 contributes to granulocyte extravasation into inflamed tissues *in vivo* (Tohka *et al.* 2001). The use of anti-VAP-1 antibody significantly reduced the rolling velocities and the numbers of firmly adhered leukocytes. Moreover, the number of infiltrated leukocytes in the peritoneal cavity was diminished after anti-VAP-1 antibody treatment in the peritonitis model. Subsequently,

the discovery of a mouse homolog for human VAP-1 allowed studies of adhesive properties of VAP-1 in mice (Bono *et al.* 1998a; Bono *et al.* 1998b). A decreased granulocyte and monocyte migration was observed after mouse anti-VAP-1 antibody treatment *in vivo* in peritonitis and air pouch models, respectively (Merinen *et al.* 2005). Both of these are acute models of inflammation. Moreover, utilizing diabetes as a chronic model of inflammation, the mouse anti-VAP-1 antibody prevented the disease in a subset of mice (Merinen *et al.* 2005). Additionally, adhesion of CD4 T cells to an inflamed liver was diminished after VAP-1 antibody treatment *in vivo* in mice (Bonder *et al.* 2005). These results suggest that VAP-1 has a role in leukocyte recruitment in several different inflammatory conditions *in vivo*.

Due to the enzymatic activity of VAP-1, it was of great interest to know whether the enzymatic activity could play a role in leukocyte adhesion. The finding of endothelial cells endogenously expressing VAP-1 made it possible to test the function of SSAO activity *in vitro*. Indeed, primary endothelial cells isolated from a rabbit heart were found to express VAP-1 on their surface with SSAO activity (Salmi *et al.* 2001). Subsequently, the cells were used in flow-based *in vitro* adhesion assays with human lymphocytes. The use of anti-VAP-1 antibodies diminished lymphocyte adhesion, as expected, but also inhibitors for SSAO (semicarbazide and hydroxylamine) diminished adhesion to the same extent (Salmi *et al.* 2001). Based on these results, it was proposed that a VAP-1 substrate could be an amino group on the leukocyte surface (Salmi *et al.* 2001). From the modeling of VAP-1, it is indeed evident that a lysine side chain could enter the cavity and is long enough to make a contact of topaquinone with the free $NH_2$ group (Salmi *et al.* 2001; Salminen *et al.* 1998). In addition, it has been shown that addition of SSAO-end products (hydrogen peroxide or benzaldehyde) did not affect adhesion, whereas addition of a substrate (benzylamine) diminished the adhesion. The findings suggest that even though the end products are known to be very reactive substances they fail to affect adhesion, while experimental substrates probably compete with the "real" substrate on the surface of leukocytes (Salmi *et al.* 2001). Furthermore, similar results have been obtained with human cells using primary hepatic endothelial cells. Lymphocyte adhesion to and also transmigration through the hepatic endothelium was diminished under flow-conditions due to the blocking of VAP-1 with an antibody or with SSAO inhibitors *in vitro* (Bonder *et al.* 2005; Lalor *et al.* 2002). In conclusion, VAP-1 regulates leukocyte adhesion through its SSAO activity. However, the relationship between SSAO activity-dependent and antibody epitope-dependent adhesion still remains to be elucidated.

# 3. AIMS OF THE STUDY

Regulation of leukocyte trafficking plays an important role in the physiology and pathology. Vascular adhesion protein-1 is an endothelial adhesion molecule, which also possesses enzymatic activity. The aim of this study was to investigate the role of the enzyme activity of VAP-1 in leukocyte trafficking and the impact of VAP-1 on several inflammatory/pathological conditions.

The specific aims of this study were:

I To investigate the role of enzymatic activity of VAP-1 in granulocyte transmigration.

II To study leukocyte extravasation *in vivo* in the absence of VAP-1 using gene knockout technology.

III To study the role of VAP-1 in the immune system under normal conditions and upon immunization as well as microbial challenge using VAP-1 knockout mice.

IV To study the role of VAP-1 in tumor progression, leukocyte infiltration into tumors and neovascularization using VAP-1 knockout mice.

# 4. MATERIALS AND METHODS

Materials and methods are described in more detail in the original publications.

## 4.1. Antibodies

Table 1. Characteristics of the anti-mouse antibodies used in the experiments.

| Antigen | Clone | Ig class | Conjugate | Source or reference | Used in |
|---|---|---|---|---|---|
| $\alpha_4\beta_7$ | DATK32 | rat IgG2a | phycoerythrin (PE) | BD Pharmingen | II, III |
| B220 | TIB-146 | rat IgM | biotinylated | American Type Culture Collection (ATCC) | II, III |
| B220 | RA3-6B2 | rat IgG2a | pacific blue | BD Pharmingen | II, III |
| CCR9 | 242503 | rat IgG2b | allophycocyanin (APC) | R&D Systems | III |
| CCR10 | 248198 | rat IgG2b | - | R&D Systems | III |
| CD3 | 17A2 | rat IgG2b | PE | BD Pharmingen | III |
| CD4 | CT-CD4 | rat IgG2a | PE | Calltag | II, III |
| CD4 | RM4-5 | rat IgG2a | fluorescein isothiocyanate (FITC) | eBioscience | III |
| CD8 | CT-CD8a | rat IgG2a | PE | Calltag | II, III |
| CD11a | 2D7 | rat IgG2a | FITC | BD Pharmingen | II, III |
| CD11b | M1/70 | rat IgG2b | - | BD Pharmingen | IV |
| CD11b | M1/70 | rat IgG2b | PE | BD Pharmingen | IV |
| CD25 | PC61.5 | rat IgG1 | APC | eBioscience | III |
| CD31 | MEC13.3 | rat IgG2a | - | BD Pharmingen | IV |
| CD44 | IM7 | rat IgG2b | FITC | BD Pharmingen | II, III |
| CD45 | 30-F11 | rat IgG2b | - | BD Pharmingen | IV |
| CD45 | 30-F11 | rat IgG2b | APC-Cy7 | BD Pharmingen | IV |
| CD49d | R1-2 | rat IgG2b | PE | BD Pharmingen | III |
| CD62L | MEL14 | rat IgG2a | FITC | BD Pharmingen | II, III |
| CD62L | MEL14 | rat IgG2a | - | BD Pharmingen | II |
| CD162 | 2PH1 | rat IgG1 | PE | BD Pharmingen | II, III |
| CXCR4 | 247506 | rat IgG2b | APC | R&D Systems | III |
| FoxP3 | FJK-16S | rat IgG2a | PE | eBioscience | III |
| F4/80 | CI:A3-1 | rat IgG2b | FITC | Caltag laboratories | III |
| F4/80 | CI:A3-1 | rat IgG2b | FITC | AbD Serotec | IV |
| IgA | 11-44-2 | rat IgG1 | - | SouthernBiotech | III |
| IgA | | goat polyclonal | alkaline phosphatase (AP) | SouthernBiotech | III |

Table 1. (continued)

| **Antigen** | **Clone** | **Ig class** | **Conjugate** | **Source or reference** | **Used in** |
|---|---|---|---|---|---|
| IgA | | goat polyclonal | - | Bethyl laboratories | II,III |
| IgA | | goat polyclonal | Horseradish peroxidase (HRP) | Bethyl laboratories | II,III |
| IgG | | goat polyclonal | - | SouthernBiotech | III |
| IgG | | goat polyclonal | AP | SouthernBiotech | III |
| IgG | | goat polyclonal | - | Bethyl laboratories | II,III |
| IgG | | goat polyclonal | HRP | Bethyl laboratories | II,III |
| IgM | 1B4B1 | rat IgG1κ | - | SouthernBiotech | III |
| IgM | | goat polyclonal | AP | SouthernBiotech | III |
| IgM | | goat polyclonal | - | Bethyl laboratories | II,III |
| IgM | | goat polyclonal | HRP | Bethyl laboratories | II,III |
| Ly6C and Ly6G (Gr-1) | RB6-8C5 | rat IgG2b | FITC | BD bioscience | IV |
| Lyve-1 | | polyclonal | - | Relia Tech | IV |
| Mannose Receptor | MR5D3 | rat IgG2a | - | (Martinez-Pomares *et al.* 2003) | IV |
| Ng-2 | | polyclonal | - | Chemicon International | IV |
| Syndecan-1 | 281.2 | rat IgG | - | (Hayashi *et al.* 1987) | III |
| VAP-1 | TK10-79 | rat Ig | - | (Bono *et al.* 1998a) | IV |
| VAP-1 | TK8-14 | mouse IgG2a | - | (Kurkijarvi *et al.* 1998) | I |
| VAP-1 | 7-106 | rat IgG2b | - | (Merinen *et al.* 2005) | II,III |
| VAP-1 | 7-88 | rat IgG2b | - | (Merinen *et al.* 2005) | II,III |
| VAP-1 | 7-188 | rat IgG2b | - | (Merinen *et al.* 2005) | II |
| VAP-1 | | polyclonal | - | (Kiss *et al.* 2008) | IV |

Table 2. Characteristics of the antibodies used in the experiments.

| Antigen | Clone | Ig class | Conjugate | Source or reference | Used in |
|---|---|---|---|---|---|
| human HLA-ABC | HB-116 | mouse IgG1 | - | (Parham 1981) | I |
| human VAP-1 | TK8-14 | mouse IgG2a | - | (Kurkijarvi *et al.* 1998) | I |
| human CD44 | 9B5 | rat IgG2a | - | (Jalkanen *et al.* 1986) | IV |
| human HLA-DR5 | HB- 151 | rat IgG2b | - | (Merinen *et al.* 2005) | III, IV |
| chicken T-cell | 3G6 | mouse IgG1 | - | (Kurkijarvi *et al.* 1998) | I |

## 4.2. SSAO-inhibitors

Table 3. SSAO-inhibitors used in the experiments.

| Inhibitor | Chemical name | Source or reference | Used in |
|---|---|---|---|
| Hydroxylamine | | Sigma | I |
| Semicarbazide | | Sigma | I, III |
| BTT2027/BTT2052/ SZE5302 | 2-(1-methylhydrazino)-1-indanol | I, (Marttila-Ichihara *et al.* 2006) | I,III,IV |
| LJP1586 | Z-3-Fluoro-2-(4-methoxybenzyl)allylamine | (O'Rourke *et al.* 2008) | IV |

SSAO inhibitors are not entirely specific for SSAO as they also inhibit MAO activity with different potencies. Semicarbazide is an irreversible carbonyl reactive inhibitor and has absolute inhibition constant (Ki) values of 13 000 nM and >88 000 nM for SSAO and MAO, respectively (I). Hydroxylamine is also an irreversible carbonyl reactive inhibitor and Ki values are 1,3 nM and 42 000 nM for SSAO and MAO, respectively (I). Both of these "prototype" SSAO inhibitors are potentially toxic and thus not optimal for *in vivo* use (Kinemuchi *et al.* 2004). Therefore, new inhibitors have been developed with better *in vivo* properties and better selectivity and specificity. Novel hydrazine compounds BTT2027, BTT2052 and SZE2052 are slowly reversible inhibitors that bind to topaquinone in SSAO (Marttila-Ichihara *et al.* 2006). Ki values of BTT2027 are 54 nM and 2300 nM for SSAO and MAO, respectively (I). The Ki values of SZE5302 are 33 nM and 1380 nM for SSAO and MAO, respectively (Marttila-Ichihara *et al.* 2006). Another chemically distinct inhibitor, allylamine LJP1586, is a slowly reversible inhibitor having inhibitory concentration ($IC_{50}$) values 43 nM and 2200 nM for SSAO and MAO-B, respectively (O'Rourke *et al.* 2008).

## 4.3. Adenoviruses

Table 4. Characteristics of adenoviruses used in the *in vitro* studies.

| Recombinant Adenovirus | Description | Source or reference | Used in |
|---|---|---|---|
| pADENO-VAP-1 | Encodes human wild type VAP-1 | I | I |
| pADENO-VAP-1 Y471F | Encodes human VAP-1 with single point mutation in Y471F, no SSAO-activity | I | I |
| pADENO-LacZ | Encodes β-galactosidase | (Puumalainen *et al.* 1998) | I |

## 4.4. Animals

Table 5. Characteristics of animals used in the *in vivo* studies.

| Animals | Description | Source or reference | Used in |
|---|---|---|---|
| Mouse BALB/c | Wild type | | III |
| Mouse C57BL/6 | Wild type | | III, IV |
| Mouse 129 | Wild type | | II, III |
| Mouse VAP-1$^{-/-}$ 129 strain | Mouse lacking endogenous VAP-1 | II | II, III |
| Mouse VAP-1$^{-/-}$ C57BL/6 strain | Mouse lacking endogenous VAP-1 | III | III, IV |
| Mouse VAP-1 Y471F C57BL/6 strain | Mouse carrying enzymatically inactive human VAP-1 under Tie-1 promoter | IV | IV |
| Mouse OT-1 C57BL/6 strain | T-cell receptor-transgenic OT-1 (ovalbumin specific) | (Hogquist *et al.* 1994) | II |
| Mouse RIPmOVA C57BL/6 strain | Mouse expressing ovalbumin antigen in pancreatic islets under rat insulin promoter (RIP) | (Kurts *et al.* 1996) | II |
| Mouse VAP-1$^{-/-}$ RIPmOVA C57BL/6 strain | Mouse expressing ovalbumin antigen in pancreatic islets under rat insulin promoter (RIP) and lacking VAP-1 | II | II |
| Mouse VAP-1$^{+/+}$ RIPmOVA C57BL/6 strain | Mouse expressing ovalbumin antigen in pancreatic islets under rat insulin promoter (RIP) and expressing VAP-1 | II | II |
| Rat, Spraque-Dawley | Wild type | (Wallace *et al.* 1999) | I |

## 4.5. Cells

Table 6. Characteristics of cells used in the studies.

| Cells and cell lines | Description | Source or reference | Used in |
|---|---|---|---|
| HUVEC | Primary human umbilical vein endothelial cells | (Jaffe *et al.* 1973) | I |
| CHO | Chinese hamstery ovarian cells | American Type Culture Collection (ATCC) | IV |
| mVAP-1-CHO | Chinese hamstery ovarian cells expressing mouse VAP-1 | (Bono *et al.* 1998a) | IV |
| PMN | Polymorphonuclear leukocytes isolated from healthy donors | | I |
| B16-F10-luc-G5 | Mouse melanoma cell line expressing luciferase | Xenogen | IV |
| EL-4 | Mouse lymphoma T cell line | ATCC | IV |

## 4.6. Methods

Table 7. Methods used in the studies.

| Method | Used in |
|---|---|
| Adenoviral transfection | I |
| cDNA microarray | IV |
| Cell counting | II, III |
| ELISA (enzyme-linked immunosorbent assay) | II, III, IV |
| Confocal microscopy | II |
| ELISPOT (enzyme-linked immunosorbent spot) | III |
| FACS (fluorescence activated cell sorting) | I,II,III,IV |
| Generation of knockout and transgenic mice | II, IV |
| Homing assay | II, III |
| Immunization | II, III |
| Immunohistochemistry | II, III, IV |
| Infection model, *Coxsackie B4* | III |
| Infection model, *Staphylococcus aureus* | III |
| Infection model, *Yersinia enterocolitica* | II |
| Inflammation model, air-pouch | I |
| Inflammation model, peritonitis | II |
| Intravital videomicroscopy | II, IV |
| Bioluminescent real time *in vivo* imaging system (IVIS) | III, IV |
| Matrigel plugs | IV |

Table 7. (continued)

| Method | Used in |
|---|---|
| MDSC isolation | IV |
| Proliferation assays | II, III, IV |
| SSAO activity assay, radiochemical | I, II, III |
| SSAO activity assay, fluorimetric | II, III, IV |
| Transfer model of autoimmune diabetes | II |
| Tumor model, melanoma | IV |
| Tumor model, lymphoma | IV |
| VEGF pellets | IV |
| Quantitative PCR | IV |

#### *4.6.1. In vitro capillary flow chamber assay*

This in vitro assay for studing leukocyte-endothelium interactions has been set up in our laboratory as a part of these studies and will be presented here in more detailed. To study granulocyte interaction with VAP-1 or enzymatically inactive VAP-1 transfected endothelium, a capillary flow chamber assay was adapted from (Cooke *et al.* 1993). A schematic figure representing the main principles of the assay is shown in Figure 4. Briefly, endothelial cells were plated into gelatin-precoated perpendicular glass capillaries and grown overnight until confluence. Thereafter, one end of the capillary was connected to a pump via tubing and the other end to a 2-way valve and a reservoir of the polumorphonuclear leukocytes (PMN). The PMN suspension was drawn through the capillary at a defined wall shear stress of 1,0 dyn/cm$^2$. For studying the numbers of rolling cells, after a one minute stabilization period the PMN interactions under shear were followed for 15 seconds per field and totally 15 fields (each 0,3072 cm$^2$) were recorded via an inverted microscope (100 x magnification) equipped with a phase contrast and a CCD camera connected to a digital video recorder for later analysis. For analyzing transmigration, after a stabilization period, PMNs were perfused over endothelium for 5 minutes, and thereafter the flow buffer was perfused for 10 minutes, during which time PMNs were allowed to transmigrate. All analyses were made offline from video records by manual counting. PMN was defined as rolling if it rolled in continuous contact with the endothelium in the direction of the flow for at least three times of its diameter. A PMN was defined as adherent if it remained stable for at least 15 seconds.

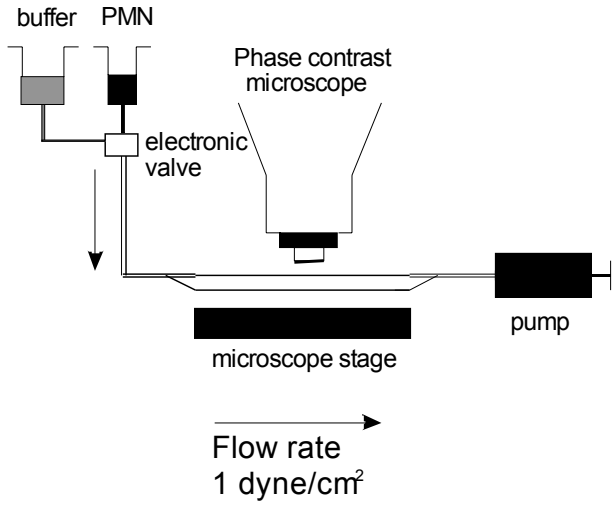



**Figure 4.** Schematic representation of capillary flow chamber assay. Leukocytes are drawn into a capillary tube using a computer-controlled infusion pump. Endothelial cells grow inside the capillary tube and the interactions between flowing leukocytes and endothelium can be studied under a phase contrast microscope. Modified from (Cooke *et al.* 1993).

### *4.6.2. Statistical analyses*

Statistical comparisons were carried out using Student t-tests, Mann-Whitney U tests, one-way analysis of variance, chi-square tests or variance analysis of repeated measurements. Analyses were performed using SAS Enterprise Guide 3.0 and Microsoft Excel.

# 5. RESULTS

## 5.1. Semicarbazide-sensitive amine oxidase activity of VAP-1 is involved in granulocyte transmigration through endothelium *in vitro* (I)

VAP-1 mediates leukocyte adhesion to endothelium. It has been shown that both antibodies against VAP-1 and SSAO inhibitors are able to reduce the adhesion (Lalor *et al.* 2002; Salmi *et al.* 2001). The relationship between antibody-epitope-dependent adhesion and SSAO activity –dependent adhesion has remained unknown. In order to study both features separately, we produced two adenoviral constructs. The first coded a full-length human VAP-1. The second contained a point mutation leading to amino acid change at tyrosine 471, which is a precursor of topaquinone in the active site of the enzyme. This was changed to phenylalanine. Since topaquinone is absolutely necessary for SSAO-activity (Klinman & Mu 1994), the introduced mutation leads to enzymatically inactive VAP-1 (Salmi *et al.* 2001; Salminen *et al.* 1998). Primary human endothelial cells isolated from umbilical cord veins were used and they did not endogenously express VAP-1. The adenoviral transduction of mutated VAP-1, namely VAP-1 Y471F, resulted in as bright VAP-1 expression on the cell surface as did wild type VAP-1. This indicates that the conformation of mutated VAP-1 is not changed dramatically, since it is recognized by an antibody against VAP-1. Moreover, based on protein modeling the mutation was assumed not to change the protein conformation (Wilce *et al.* 1997). However, SSAO-activity was totally abolished from mutated VAP-1. Thus, after transient transfections, endothelial cells expressing VAP-1 showed bright VAP-1 expression and had SSAO-activity, whereas mutated VAP-1 Y471F showed bright VAP-1 expression but no SSAO activity.

PMN rolling on, adhesion to and transmigration through TNF-α induced endothelial cells expressing native VAP-1 and mutated VAP-1 Y471F were analyzed. Antibodies and SSAO-inhibitors were used to distinguish the difference between antibody-dependent adhesion and SSAO-activity dependent adhesion. The use of anti-VAP-1 antibody resulted in decreased the rolling to and transmigration through native VAP-1 expressing endothelium, while the adhesion step was intact. In contrast, the use of anti-VAP-1 mAb with enzymatically inactive VAP-1 had no significant effect on rolling, adhesion or transmigration thus indicating that SSAO activity is crucial for VAP-1 mediated adhesive functions. On the other hand, the use of SSAO inhibitors, semicarbazide plus hydroxylamine or BTT2027, decreased rolling on and transmigration through VAP-1 endothelium, while having no effect on adhesion. Since mutated VAP-1 does not possess any SSAO activity, these inhibitors should not influence the interactions between leukocytes and VAP-1 Y471F expressing endothelial cells. This appeared to be the case, since mutated enzymatically inactive VAP-1 endothelium, treated with inhibitors mediated rolling, adhesion and transmigration of PMNs as well as cells treated with the vehicle. These results indicate that the enzymatic activity of VAP-1 is crucial for VAP-1 mediated rolling and adhesion. Final evidence for the importance of SSAO-activity in VAP-1 mediated transmigration came from the direct comparison between wild type VAP-1 and enzymatically inactive mutated VAP-1 endothelia and their ability to promote PMN

transmigration. The transmigration seen with cells containing enzymatically inactive VAP-1 was at the same level as in LacZ transfected endothelium, which was used as a control. However, PMN transmigration was increased 30 % after transfecting enzymatically active wild type VAP-1 to the endothelial cells. Thus, these data give evidence that VAP-1 mediates PMN rolling and transmigration and the enzymatic SSAO-activity of VAP-1 is needed for its proper functions.

## 5.2. VAP-1 deficient mice display mild age-dependent paucity of B-lymphocytes in Peyer's patches (II, III)

Since there have been many reports about the importance of VAP-1 in leukocyte trafficking using anti VAP-1 antibodies or SSAO inhibitors, the next question was what would be the phenotype if AOC3 gene coding VAP-1 was deleted in mice (Bonder *et al.* 2005; Lalor *et al.* 2002; Merinen *et al.* 2005; Salmi & Jalkanen 1992; Salmi *et al.* 1997; Salmi *et al.* 2001; Tohka *et al.* 2001). AOC3 knockout mice were made by replacing part of the first exon of AOC3 with a Neomycin resistance cassette. The evaluation of homozygote AOC3 knockout mice showed that ACO3 mRNA was not found. Furthermore, VAP-1 protein expression was studied in several organs by staining the tissues with anti-VAP-1 antibodies. Knockout mice showed no VAP-1 expression while wild type mice brightly expressed VAP-1 in endothelium, smooth muscle and adipose tissue. In addition, SSAO-activity was not detected in knockout mice while wild type mice possessed the activity in several tissues and serum. This indicates that VAP-1 deficient mice are lacking VAP-1 both at the RNA and protein level.

The absence of VAP-1 did not affect development or fertility. Since VAP-1 has been shown to be an important regulator of leukocyte trafficking, the number of leukocytes was analyzed in blood and in lymphatic organs, into which leukocytes migrate through the endothelium. First, an evaluation was conducted using age-matched 16-26 week old mice. The number of leukocytes and particularly T-cells (CD4, and CD8) and B-cells were studied in PLN, MLN, PP and the spleen. No statistically significant changes were found, but a trend of decreased cell numbers in PP of the gut was detected. Since the composition of lymphoid organs is heavily dependent on age, a further detailed analysis of lymphoid organs was conducted to include three age groups. Analysis of 6 week, 20-22 week and 1,5 year old mice revealed that particularly in 6 week old mice there was a ~50 % reduction in the number of cells in PP. In addition, the composition of leukocyte types was mildly changed among the PP lymphocytes since VAP-1 knockout mice had 5 % less B-cells than wild type mice. A trend of similar reduction was noticed in 20-22 week old mice but it disappeared in 1,5 year old mice. The numbers of CD4, CD8 or regulatory T-cells (CD4+ CD25+ FoxP3+) were not changed either in PP or PLN. In conclusion, in young mice (6 week old) there is a reduction in the number of cells in PP and the reduction is seen particularly in B-cells.

The reason for decreased cell numbers seen in the PP of the gut could be differential expression of adhesion molecules, particularly gut-homing molecules. Leukocytes

isolated from lymphatic organs were stained for several adhesion molecules and chemokine receptors. Particular interest was shown in CCR9, a receptor for gut homing chemokines, and gut-homing adhesion molecule $\alpha_4\beta_7$ integrin (Johansson-Lindbom & Agace 2007; Sigmundsdottir & Butcher 2008). However, no statistically significant differences were found in the surface expression levels of CCR9 or $\alpha_4\beta_7$ integrin. No differences were found in L-selectin, PSGL-1, CD44, $\alpha_4$ integrin, CXCR4 or CCR10 expression either. However, a minor but statistically significant ~10 % reduction in the number of LFA-1 positive cells was discovered in PPs in the absence of VAP-1. In conclusion, major differences in the expression of leukocyte adhesion molecules were not found in the absence of VAP-1.

## 5.3. VAP-1 is involved in mucosal immune responses *in vivo* (II, III)

### *5.3.1. Decreased serum IgA levels in VAP-1-/- mice*

Since diminished numbers of B-cells were found in the lymphatic organs of the gut (PP), it could lead to lower local production of IgA and impaired B-cell mediated immune responses (Brandtzaeg & Johansen 2005). To test if immunoglobulin production by B-cells was compromised, serum and intestinal immunoglobulin levels were measured. In fact, a statistical 46 % reduction in IgA levels in serum was found in 20 week old mice while IgG and IgM remained comparable to wild type mice. However, no change was observed in IgA or IgG levels in intestinal contents. In addition, the antibody production of individual B-cells at a single-cell level was measured with ELISPOT assay. However, no statistically significant differences were found in the IgA production of leukocytes isolated from bone marrow, spleen or PPs. In conclusion, VAP-1 -/- mice have slightly diminished serum IgA levels. However, the diminished serum IgA level is not likely to be due to a defect in the production of IgA.

### *5.3.2. Impaired mucosal immune response in VAP-1-/- mice*

The diminished numbers of leukocytes and the reduction in IgA in the serum could potentially have consequences in the immune response of these mice. The route of immunization contributes to the induction of immune response, and therefore subcutaneous, intraperitoneal and oral immunizations were performed.

Lymphocyte proliferation was followed after one-month intragastrical immunization with OVA and adjuvant. Lymphocytes isolated from MLN and spleen from VAP-1-/- mice showed 50 % and 35 % lower proliferation, respectively, than cells isolated from wild type littermates. Thus, T-cell proliferation was defective in VAP-1-/- mice. Moreover, B-cell mediated response was studied from mouse serum and a trend of reduction was noticed in OVA-specific IgG antibodies. In contrast, no differences between genotypes were seen after subcutaneous immunization with OVA or intraperitoneal immunization with LPS either in T-cell proliferation or in OVA- or LPS-specific Ig production. In conclusion, VAP-/- mice have a compromised immune response after peroral immunization.

## 5.4. VAP-1 regulates leukocyte extravasation *in vivo*

Since VAP-1 has been shown to be involved in leukocyte extravasation and in lymphocyte homing by using anti-VAP-1 antibodies, the next question was what would be the outcome of genetic deletion of VAP-1 or blocking of SSAO-activity of VAP-1 in mice regarding leukocyte trafficking.

### *5.4.1. VAP-1 mediates lymphocyte homing into secondary lymphoid organs (II,III)*

To investigate whether VAP-1 would mediate lymphocyte homing, a short-term homing assay was performed. In this model, fluorescently labeled lymphocytes are transferred to wild type and VAP-1-/- mice and their trafficking into the secondary lymphatic organs was followed. Lymphocyte homing to MLN and spleen was impaired in VAP-1-/- mice and in addition, there was a trend of reduction in homing to PP and PLN. However, when only B-cells were used in the homing assay, their homing was as efficient as seen in wild type mice. These results strongly imply that VAP-1 mediates lymphocyte homing under physiological conditions to certain but not all lymphoid organs.

### *5.4.2. Polymorphonuclear cells extravasation in inflammation is VAP-1 dependent (I,II)*

PMNs are the first leukocytes that arrive into an inflamed area. The genetic deletion of VAP-1 and its impact on PMN transmigration into sites of inflammation was studied using intravital videomicroscopy. Mild or strong inflammation was induced in the mouse cremaster muscle with TNF-α, and PMN rolling, adhesion and transmigration through the vasculature was studied. In VAP-1-/- mice the inflammation increased rolling velocities and reduced adhesion by ~50 %. Furthermore, the migration of leukocytes into the tissues was decreased almost 50 % in the absence of VAP-1 (when the transmigrated cells were counted from already adherent cells). Similar results were obtained when VAP-1 was blocked in wild type mice using anti-mouse VAP-1 antibody. No reduction was seen using antibodies in VAP-1-/- mice, as expected. Moreover, in TNF-α-induced peritonitis PMN infiltration was reduced ~50 % in the absence of VAP-1.

As the SSAO-activity of VAP-1 was shown to be crucial for PMN transmigration *in vitro* (I), the next question was to analyze whether SSAO-activity regulates PMN accumulation at the site of inflammation also *in vivo*. As a prophylactic model, the SSAO-inhibitor BTT2027 was used to test if blocking SSAO-activity of VAP-1 reduced PMN migration. In control rats, after inducing the inflammation with carrageenan, there was an influx of infiltrating leukocytes, mainly PMNs into inflamed air pouches. However, when the rats were treated with BTT2027 a significant ~50 % reduction in infiltrating PMNs was detected. This result strongly indicates that the enzymatic SSAO activity of VAP-1 is needed for PMN infiltration also *in vivo*. Taken together, these data provide evidence that VAP-1 mediates PMN accumulation into sites of inflammation *in vivo* and the accumulation can be reduced using the SSAO-inhibitor BTT2027.

### *5.4.3. VAP-1 is involved in leukocyte homing into tumors (IV)*

The same adhesion molecules can mediate physiological lymphocyte recirculation and pathological infiltration of leukocytes into the inflamed tissues. Leukocytes found in tumors (such as cytotoxic T-cells, MDSC, dendritic cells, regulatory T cells) also arrive into the tumor area by transmigrating through newly formed blood vessels inside the tumors (Dirkx *et al.* 2003). Since VAP-1 was seen to mediate physiological and pathological leukocyte trafficking (I, II, III), the next question was, whether VAP-1 would also mediate leukocyte arrival into the tumor site. To that end, a subcutaneous melanoma model was utilized. The function of VAP-1 was studied in wild type mice by blocking VAP-1 with an antibody and following the arrival of labeled leukocytes into the tumor vasculature. Intravital videomicroscopy revealed that in anti-VAP-1 antibody treated mice there were statistically less interacting and rolling leukocytes compared to control treated mice. These results indicate that VAP-1 contributes to the early interactions of leukocytes with tumor vessels.

To investigate the infiltration of leukocytes into tumors, in situ immunohistochemical stainings were performed with tumors dissected from wild type and VAP-1-/- mice. The stainings revealed that statistically less CD45 positive leukocytes had infiltrated into the tumor area. More detailed analysis showed that the defect was in the accumulation of CD8 positive T-cells and MDSC, whereas the numbers of regulatory T-cells (FoxP3+), CD4 or macrophages were unaltered. A detailed flow cytometric analysis of MDSC was performed in VAP-1-/- mice to evaluate the relationship of polymorphonuclear MDSC and monocytic MDSC population in the absence of VAP-1. A double staining with Gr-1 and CD11b markers revealed that the population of polymorphonuclear MDSC among MDSCs was decreased ~ 50 % in VAP-1 -/- mice when compared to wild type littermates. However, the monocytic MDSC population was unaltered. The decreased polymorphonuclear subpopulation found in VAP-1 -/- may reflect the role for VAP-1 in regulating granulocytic MDSC infiltration into the tumor tissue. It has been shown that MDSCs are also found in bone marrow, blood circulation and in the spleen of tumor bearing mice (Marigo *et al.* 2008; Serafini *et al.* 2006). However, the MDSC population was not altered in the bone marrow, blood and spleen in VAP-1 -/- mice. In conclusion, VAP-1 -/- mice and wild type mice have similar numbers of MDSC in the bone marrow, blood and secondary lymphoid organ (spleen), but in VAP-1 -/- mice the recruitment into the tumor is defective and especially the polymorphonuclear subpopulation is decreased.

Since the SSAO-activity of VAP-1 is needed for VAP-1 mediated transmigration, and PMN accumulation into an inflamed area is dependent on the SSAO-activity of VAP-1, the next obvious question was whether MDSC recruitment is dependent on SSAO-activity of VAP-1. Immunohistochemical analysis showed that the blocking of SSAO-activity with SZE5302 in wild-type mice reduced the number of MDSC found in the tumor. Thus, these results strongly indicate that the SSAO-activity of VAP-1 is needed for VAP-1 mediated recruitment of MDSC into a tumor area. To discover whether the reduced infiltration of MDSC into the tumor area in the absence of VAP-1 is relevant to other tumor models, a lymphoma model was utilized. In this model, the numbers of MDSC were also decreased in the absence of VAP-1 and after SSAO-inhibitor

treatment. Thus, these data demonstrate that VAP-1 is involved in MDSC accumulation into tumor sites in melanoma and lymphoma models.

## 5.5. VAP-1 is involved in antimicrobial responses and tumor growth (II, III, IV)

The deletion of VAP-1 was seen to cause several defects in mice. These included minor changes such as reduced lymphocyte recirculation, decreased numbers of cells in PP, decreased IgA levels in serum and slightly compromised mucosal immune response (II, III). However, major changes in VAP-1-/- mice were found in leukocyte accumulation into the site of inflammation (I, III, IV). All these defects could have life-threatening consequences.

### *5.5.1. Role of VAP-1 in antimicrobial responses (II, III)*

To evaluate the influence of VAP-1 in antimicrobial responses, several microbial models were utilized. The ability of VAP-1 -/- mice to control bacterial infections, such as cutaneous *Staphylococcus aureus* or intestinal life-threatening *Yersinia enterocolitica* and viral *Coxsackie* B4 infection targeting pancreas, was evaluated. VAP-1 -/- mice had slightly impaired ability to control the proliferation of *S.aureus* in the early phase of the infection as measured by real-time bioluminescent imaging of luciferase expressing *S.aureus* bacteria. However, already at 20 h after bacterial inoculation the numbers of bacteria were similar in VAP-1-/- mice and wild type littermates and both genotypes eventually cleared the bacteria. In the *Yersinia enterocolitica* infection model, the intragastrical inoculation of bacteria led to comparable lethality in VAP-1 -/- and wild type mice. Finally, a viral *Coxsackie* B4 infection was studied. The inflammation in the pancreas was slightly more severe in the absence of VAP-1. Taken together, these data provide evidence that the genetic deletion of VAP-1 does not substantially impair the antimicrobial response to at least *Yersinia enterocolitica* while the response to *S.aureus*, or *Coxsackie* B4 infections is slightly impaired.

Since defects in leukocyte trafficking were also seen in wild type mice treated with anti-VAP-1 antibody or by blocking SSAO-activity with inhibitors (I, II, IV), the antimicrobial response to *S.aureus* was tested in wild type mice treated with the combination of anti-VAP-1 mAb and SSAO-inhibitor. No defect was seen in the proliferation of bacteria in mice treated with anti-VAP-1 mAb and SSAO-inhibitor. These results strongly suggest that acute blocking of VAP-1 does not potentiate *S.aureus* infection.

To investigate the outcome of a defect in lymphocyte homing in the absence of VAP-1, a nonbacterial inflammation model of autoimmune diabetes was utilized. Although there were slightly fewer animals dying from diabetes in the absence of VAP-1, the lymphocyte infiltrations into the pancreatic islets were not statistically different, thus showing only possibly a mild role for VAP-1 in this model of diabetes.

### *5.5.2. VAP-1 in tumor progression and neovascularization (IV)*

Leukocytes are found in tumors and have an influence on tumor progression (Mantovani *et al.* 2008). Since VAP-1 is expressed in the blood vessels of tumors, and leukocyte infiltrations into tumors were reduced in the absence of VAP-1 (IV), the function of VAP-1 in tumor progression was evaluated. Mouse melanoma cells were injected subcutaneously into the abdominal areas of VAP-1 -/- and wild-type mice. The tumor progression was followed for 10 days by measuring the tumor volumes. The kinetic measurements revealed that after day 4, tumors in VAP-1 -/- mice started to grow more slowly. Finally, at day 10 tumor volumes were ~50 % smaller in VAP-1 -/- mice. The tumor progression was also followed in wild-type mice treated with anti-VAP-1 antibody or SSAO-inhibitors. Surprisingly, anti-VAP-1 antibody treatment did not reduce tumor progression. However, SSAO-inhibitor treatment reduced the tumor volume to the same extent as seen in VAP-1 -/- mice. These data provide evidence that particularly the SSAO-activity of VAP-1 influences tumor growth.

In study I, the point mutation in position 471 in the VAP-1 sequence was shown to be critical for SSAO-activity of VAP-1 and its ability to mediate PMN transmigration *in vitro*. To confirm the importance of SSAO-activity in tumor progression *in vivo*, a mouse expressing enzymatically inactive VAP-1 was generated. A human VAP-1 transgene having a point mutation in 471 (tyrosine changed to phenylalanine) was expressed in VAP-1 -/- mice under an endothelial-specific Tie1 promoter. These VAP-1 Y471F mice expressed mutant VAP-1 only in the endothelial cells and no SSAO-activity was found in these animals. Thus, this is an *in vivo* analogue for VAP-1 Y471F adenoviral HUVECs used in study I. When melanoma cells were injected into these mice, reduced tumor volumes were detected on day 10 in comparison to wild type mice and the volumes were similar to VAP-1 -/- mice. These results clearly demonstrate that the SSAO-activity of VAP-1 is connected to retardation of tumor progression.

To confirm that the effect of VAP-1 in tumor progression is not solely restricted to melanoma, we conducted a lymphoma model in VAP-1 -/- mice and their wild type controls. A statistically significant reduction was seen in the tumor volumes in VAP-1 -/- mice, and wild type mice treated with an SSAO-inhibitor, indicating that tumor progression is attenuated in the absence of SSAO-activity.

One main requirement for tumor progression is vascularization, which provides oxygen and nutrients to the growing tumors. Since reduced tumor volumes were found in the absence of VAP-1 and its SSAO-activity, the next question was whether there were defects in the vasculature in tumors of VAP-1 -/- mice. Immunohistochemical stainings in situ revealed that the tumors in VAP-1 -/- mice had fewer vessels per square millimeter when visualized by two vascular markers, CD31 and Meca32. In addition, the vessel perimeter was decreased in the absence of VAP-1. In addition to endothelial cells, VAP-1 is also expressed by pericytes. Since mature vessels contain pericytes, the number of pericyte-containing vessels was analysed. However, no defects in the number of anti-Ng-2 antibody positive vessels were found in VAP-1 -/- mice. This suggests that vessel maturation is not defective in the absence of VAP-1, although

there are fewer CD31 positive vessels. In addition, lymphatic vessels stained with anti-LYVE-1 antibody were normal as compared to wild type littermates. When wild type mice were treated with an anti-VAP-1 antibody, no defect in the vasculature was found. In contrast, the treatment with a SSAO-inhibitor again reduced the number of vessels in tumors. In conclusion, a defect in tumor angiogenesis was found in VAP-1 -/- mice and the SSAO-activity seems to be needed for normal tumor progression, since a similar phenotype is obtained when SSAO-activity of VAP-1 was blocked with an SSAO-inhibitor in wild-type mice.

To confirm the role of VAP-1 in neovessel formation, matrigel plugs with melanoma cells were introduced into wild type and VAP-1-/- mice. Again, there were fewer newly formed vessels formed in VAP-1 -/- mice and the tumor volume was decreased. To further investigate the defect in angiogenesis, a cDNA microarray was performed from tumor tissues. The results indicated a reduction in s100A8, s100A9 and MMP9 transcripts. Results for s100A8 and MMP9 were further confirmed with RT-PCR. Since VEGF-A is one of the major proangiogenic factors, its protein concentrations were analysed from tumor lysates. A significant reduction in VEGF-A was observed in the absence of VAP-1 further confirming a defect in neoangiogenesis. In conclusion, these results indicate that there is a previously unknown defect in tumor neoangiogenesis in the absence of VAP-1.

# 6. DISCUSSION

## 6.1. VAP-1 and leukocyte adhesion

VAP-1 was initially found after generating antibodies against inflamed synovial vessels (Salmi & Jalkanen 1992). Immunohistochemical analysis showed that VAP-1 molecule was found to be expressed in several inflammatory conditions in the blood vessel walls and *in vitro* studies showed that the use of anti-VAP-1 antibody diminished leukocyte binding to VAP-1 expressing vessels (Arvilommi *et al.* 1996; Salmi & Jalkanen 1992; Salmi *et al.* 1993). Moreover, the use of anti-VAP-1 antibodies in mouse and rabbit inflammatory settings gave the first *in vivo* evidence, that blocking of VAP-1 reduced leukocyte infiltration into the inflamed area thus reducing the inflammation. It was later found that VAP-1 possessed oxidative SSAO-activity. The obvious issue then was whether the enzymatic activity could mediate VAP-1 functions in leukocyte trafficking. Two *in vitro* studies showed that by blocking SSAO-activity on endothelium substantially reduced leukocyte adhesion (Lalor *et al.* 2002; Salmi *et al.* 2001). It was shown that VAP-1 was an adhesion molecule and the oxidative activity of VAP-1 played a role in the adhesion. However, the relationship between "antibody-dependent epitope" and SSAO-activity in adhesion was still unknown.

Using enzymatically inactive VAP-1 in study I, it was shown that the SSAO-activity of VAP-1 was essential for VAP-1 mediated rolling and transmigration under physiological flow conditions. The use of enzymatically inactive Y471F VAP-1 construct, normal VAP-1 construct, anti-VAP-1 antibodies and SSAO-inhibitors in different combinations allowed a direct comparison between the SSAO-acitivity and antibody-dependent adhesive epitope of VAP-1 in mediating leukocyte transmigration through endothelium not expressing VAP-1 endogenously. The results showed that if either the antibody epitope or SSAO-activity is blocked, the VAP-1 mediated transmigration is abolished. Moreover, the inhibition of both SSAO-activity and antibody epitope did not have any additional effect; this suggests that VAP-1 mediated transmigration occurs in two separate sequential steps and if either of them is blocked, the whole VAP-1 mediated transmigration is abolished. Since anti-VAP-1 antibody does not inhibit SSAO-activity and inhibitors do not interfere with antibody binding (Salmi *et al.* 2001), these results confirm that the antibody dependent epitope and SSAO-activity are separate but interrelated processes. Based on these results, a new working model for VAP-1 mediated leukocyte transmigration was proposed. (Figure 5.)

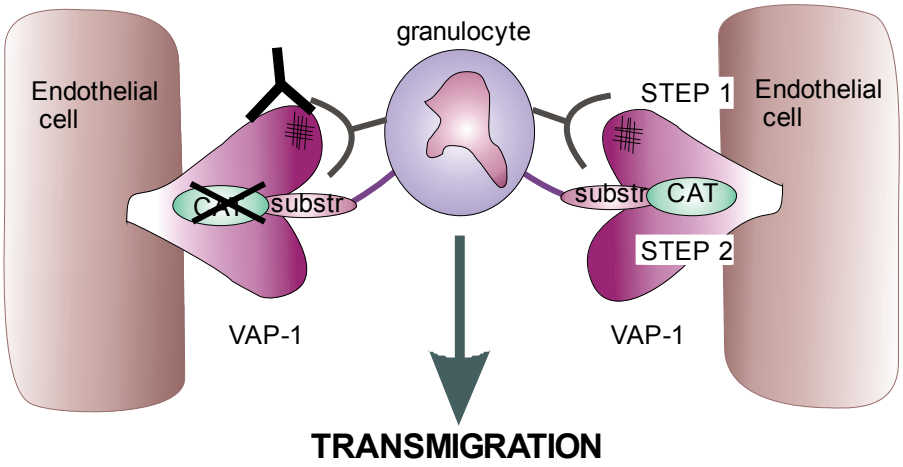



**Figure 5.** VAP-1 mediates leukocyte transmigration via two steps. Right: normal situation. Step 1: PMN interacts with antibody-dependent epitope on VAP-1. Thereafter, in step 2, amine, on the surface of PMN, reaches the catalytic site of the enzyme and the enzymatic reaction takes place. Left: VAP-1 inhibition. Both steps are essential for VAP-1 mediated transmigration since blocking the adhesive epitope with anti-VAP-1 antibody or inhibiting SSAO-activity with an inhibitor abolishes VAP-1 dependent transmigration.

According to the current model in Figure 5, we believe that the leukocyte first binds to the adhesive epitope with its ligand (currently unknown) and thereafter it is able to reach the enzymatic activity site. Since the activity site is buried within the protein and only a narrow cavity leads into it (Airenne *et al.* 2005; Salminen *et al.* 1998), it is expected that the leukocyte must be attached to the VAP-1 molecule using a ligand binding before it can relocate a substrate into the activity site. As was hypothesized by Salmi et al., the substrate for SSAO could be a lysine side chain on a leukocyte surface and the free NH2 group could reach the active site resulting in enzymatic reaction forming a transient Schiff-base between the substrate and VAP-1 (Salmi *et al.* 2001). The exact mechanism of VAP-1 mediated transmigration is still uncertain, since the ligand for antibody-binding epitope is unidentified and the physiological leukocyte-surface substrate remains unidentified.

As the results of *in vivo* experiments indicated, VAP-1 is involved in several types of leukocyte-endothelial cell interactions (I, II, III, IV). It regulated physiological homing of lymphocytes into the secondary lymphoid organs as well as PMN extravasation into the inflamed areas in several tissues. Furthermore, the infiltration of leukocyte subpopulations into tumors was also diminished in the absence of VAP-1. The PMN extravasation into the inflamed area as well as CD8 T-cell and MDSC accumulation into the tumor area were particularly dependent on the enzymatic activity of VAP-1, since the use of SSAO-inhibitors reduced recruitment similar to the genetic deletion of VAP-1. In the case of PMN and CD8 T-cells, the use of anti-VAP-1 antibody also decreased infiltration. These results thus further strengthen the model proposed for VAP-1 mediated transmigration seen in Figure 4 indicating that either by blocking SSAO-activity or antibody-dependent epitope, VAP-1 mediated transmigration is

abolished. However, MDSC infiltration into the tumor area is not dependent on the antibody binding epitope, since anti-VAP-1 antibody does not prevent MDSC accumulation into the tumor area (IV). The tumor volume is also unchanged after anti-VAP-1 antibody treatment compared to wild type littermates. Thus, the infiltration of MDSC seems to be regulated only by the enzymatic reaction catalyzed by VAP-1. Hence, leukocyte subpopulations apparently use different VAP-1 dependent mechanisms to accumulate into the tissues. Moreover, the results indicate that the homing of MDSC is different from the homing of other leukocytes. The mechanisms of MDSC homing are totally unknown, thus providing an interesting field for future research.

## 6.2. Placing VAP-1 in a multistep adhesion cascade

Even though the final step, transmigration, determines which leukocytes will eventually become tissue residents, the rolling step can also be envisioned as a gate-keeper. VAP-1 mediates both the rolling and the transmigration steps. However, the expression of VAP-1 in endothelial cells is not sufficient alone to induce leukocyte adhesion (Salmi & Jalkanen 1996). The VAP-1 expressing endothelium has to be stimulated with cytokines, such as TNF-α, in order to get leukocytes to adhere to endothelium when studying VAP-1 functions *in vitro* (Lalor *et al.* 2002; Salmi *et al.* 2001). This, therefore, suggests that VAP-1 mediated adhesion is dependent on other adhesion molecules. It previously was shown that transfection of VAP-1 to endothelial cells and treatment with SSAO substrate methylamine resulted in elevated mRNA and protein synthesis of E-selectin and P-selectin. In contrast, elevated levels were not seen if enzymatically inactive Y471F VAP-1 was used. Moreover, the induction was shown to be dependent on $H_2O_2$. Finally, *in vivo* studies confirmed that VAP-1 expressing mice expressed more P-selectin than VAP-1 -/- mice after having SSAO substrate in the diet. (Jalkanen *et al.* 2007). In another study, Lalor *et al*. showed that the enzymatic activity of VAP-1 induced ICAM-1, VCAM-1 and CXCL8 expression in addition to E-selectin in hepatic endothelial cells (Lalor *et al.* 2007). The use of an SSAO-inhibitor abolished the induction. There are several studies suggesting that $H_2O_2$ could induce expression of several adhesion molecules, such as P-selectin (Xia *et al.* 1998) and importantly, $H_2O_2$ has been shown to induce leukocyte rolling (Johnston *et al.* 1996). Thus it appears that the oxidase activity and particularly $H_2O_2$ production of VAP-1 also has an important role in regulating the expression of other adhesion molecules and the cross-talk can amplify the leukocyte accumulation into the inflammatory sites. Preliminary unpublished results have indicated that VAP-1 transfected endothelial cells produce $H_2O_2$ in the range of 1-10 μM concentrations. That concentration can have signaling effects. VAP-1 was speculated not to have signaling properties, since it has only a short (4 amino acids) amino-terminal cytoplasmic tail (Smith *et al.* 1998). However, the signaling function of VAP-1 is shown to be related to its enzymatic activity. The end products (ammonia and $H_2O_2$) of SSAO-reaction are potent and active substances and particularly $H_2O_2$ can inactivate phosphatases and thus regulate signal transduction (Meng *et al.* 2002; Meng & Tonks 2003; Reth 2002). Although $H_2O_2$ is produced extracellularly, it can diffuse through the cell membrane and have the signaling effects inside the cell. In fact, a recent study by Lalor et al. (2007) produced

evidence that VAP-1 can activate NF-κβ and lead to proinflammatory gene activation through its SSAO activity (Lalor *et al.* 2007). The inflammatory reaction and adhesion molecule expression could then be amplified by increasing the concentration of SSAO-substrates flowing in the blood stream. At least two endogenously found amines, methylamine and aminoacetone, can be utilized by VAP-1 (Lyles & Chalmers 1992; Precious *et al.* 1988). In this way, the leukocyte infiltration could then be regulated by the serum availability of SSAO-substrates.

*In vitro* studies have suggested that VAP-1 regulates rolling and firm adhesion of leukocytes to rabbit endothelial cells and to human hepatic endothelial cells (Salmi *et al*. 2001, Lalor *et al.* 2002). Transmigration was also dependent on VAP-1 (Lalor *et al.* 2002). In study I, VAP-1 regulated PMN rolling and transmigration *in vitro*, while firm adhesion was independent of VAP-1 (I). Thus, it seems that the use of VAP-1 in different steps of the adhesion cascade is dependent on the experimental settings. However, *in vivo* studies performed in rabbits showed that VAP-1 was involved in PMN rolling, firm adhesion and transmigration when studied by anti-VAP-1 antibody (Tohka *et al.* 2001). In study II, similar findings were found in the genetic deletion of VAP-1 in mice. Thus, the in vivo situation seems to require VAP-1 for rolling, firm adhesion and also for transmigration.

As has been learned e.g. from selectin knockout mice, the elimination of a single selectin does not show a dramatic change in the phenotype of the animal, although a double knockout of E- and P-selectin results in severe deficiency in leukocyte recruitment (Bullard *et al.* 1996). Thus, our finding that a deletion of a single adhesion molecule VAP-1 does not fully abolish leukocyte extravasation is in line with earlier publications. In conclusion based on these studies, VAP-1 is at least involved in leukocyte rolling and transmigration. The enzymatic nature of VAP-1 seems to be crucial for VAP-1, and it clearly is related to the regulation of gene expression in other inflammation-related molecules.

## 6.3. Phenotype of VAP-1 -/- mice

The generation of VAP-1 (AOC3) knockout mice showed that in mice SSAO-activity comes mainly from the AOC3 gene, since a deletion of this gene abolishes all detectable SSAO-activity in various tissues and serum. However, at least two other SSAO genes exist in mice and in humans.

The findings from genetic deletion of VAP-1 and its outcome are shown in Table 8.

Table 8. Phenotype of VAP-1 -/- mice.

| **Model** | **Finding/Outcome** | **Reference** |
|---|---|---|
| Non-challenged | Reduced numbers of leukocytes in PP | II, III |
| Non-challenged | Slightly reduced numbers of LFA-1+ cells in PP,MLN, PLN | III |
| Non-challenged | Reduced serum IgA levels | II, III |
| Non-challenged | Reduced lymphocyte homing into MLN and spleen | II |
| Non-challenged | Impaired amine-dependent glucose uptake | (Bour *et al.* 2007) |
| Non-challenged | Obesity | (Bour *et al.* 2009) in press |
| Peritonitis | Reduced PMN infiltrations | II |
| Adjuvant induced arthritis | Reduced PMN infiltrations and milder arthritis | (Marttila-Ichihara et al. 2006) |
| Anti-collagen mAb induced arthritis | Reduced PMN infiltrations and milder arthritis | (Marttila-Ichihara et al. 2006) |
| Melanoma | Reduced MDSC accumulation, neovascularization and tumor progression | IV |
| Lymphoma | Reduced MDSC accumulation, neovascularization and tumor progression | IV |

Studies II and III showed that under normal conditions mice lacking VAP-1 developed normally and their cellular composition of lymphatic organs was nearly normal. A slight defect was seen in PP as the numbers of PPs were reduced and especially the number of B-cells was slightly decreased. However, the reduction could not be explained by differential expression of gut-homing adhesion molecules or chemokine receptors. Although minor reduction in LFA-1 expression was found in PLN, MLN and PPs, it could not explain the defect in PP, since LFA-1 is not a specific homing molecule for the gut. Surprisingly, the B-cell trafficking into PPs was not affected. The homing assay was performed with leukocytes isolated from spleens and lymph nodes and then stained with B220 antibody. However, the results might have been different if leukocytes had been isolated from PPs. Due to technical reasons (insufficient cell numbers and possible bacterial contamination from the intestine) that was not done. B220 positive cells are a heterogeneous population containing cells, such as conventional follicular B cells and B1 cells (Allman & Pillai 2008; Brandtzaeg & Johansen 2005). Thus, the defect in B cell numbers might be in a particular subset of B cells. Although, the reduced cell numbers could be due to either B-cell proliferation or retention in the PP or their apoptosis. As the SSAO-activity of VAP-1 generates highly reactive end products, particularly $H_2O_2$, it might cause signaling effects in B-cells. Small amounts of hydrogen peroxide cause pro-apoptotic and survival promoting effects whereas higher concentrations have opposite effects (Reth 2002). Thus, the

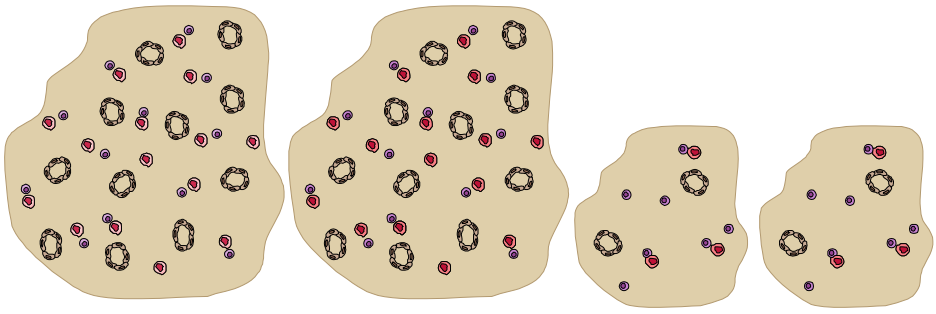
local environment in VAP-1 -/- mice has diminished reactive oxygen species and might contribute to B cell signaling, thus not promoting their survival. However, to confirm that the effects seen here are $H_2O_2$-dependent, experiments with $H_2O_2$ catalyzing enzyme, catalase, should be performed. Since the SSAO-activity of VAP-1 regulates NF-κβ (Lalor *et al.* 2007), it is clear that it can have functions in leukocyte signaling when the cells are in close proximity to endothelial VAP-1.

The immunization of mice lacking VAP-1 had different effects depending on the route of immunization and the antigen. Whereas the immune response after subcutaneous OVA immunization was unaltered, the intragastrical immunization resulted in diminished B- and T-cell responses. As discussed above, the differential lymphocyte proliferation might be due to signaling of VAP-1. Moreover, a reduction in IgA levels in the serum was also found in VAP-1 -/- mice. The reduction seen in IgA levels in the serum but not in the intestine can be explained by the different origin of those two IgA pools. While serum IgA is most likely derived from humoral antibody production and originates from the bone marrow, the IgA found in the secretions such as intestinal content, is locally produced in the gut (Conley & Delacroix 1987; Kaiserlian *et al.* 1985). Although a reduction in the serum IgA levels was found, the IgA production by bone marrow cells was not compromised. The reason for lower serum IgA levels could refer to a differential clearance of IgA from the system. However, this needs to be analyzed further.

The reduced infiltration of leukocytes had consequences in several *in vivo* models of inflammation. PMN accumulation into the peritoneal cavity was diminished. A recent study also indicated defects in PMN infiltration into inflamed joints in VAP-1 -/- mice (Marttila-Ichihara *et al.* 2006). Since lymphocyte homing into secondary lymphoid organs was defective, their homing into the inflammatory site was studied in an autoimmune diabetes model. Although a reduction in the number of dead animals (due to lack of insulin production) was seen in the VAP-1 -/- mice group, there was only a slight trend of reduction in lymphocyte infiltration into the pancreatic islets. As was previously shown, the targeting of VAP-1 with anti-VAP-1 antibody also slightly reduced the incidence of chronic autoimmune diabetes (Merinen *et al.* 2005). However, melanoma and lymphoma models showed that VAP-1 was heavily involved in tumor progression and reduced numbers of MDSC. In conclusion, it seems that the effects of deletion of VAP-1 in mucosal immunity have minor consequences and the main effects are seen in pathological conditions related to leukocyte trafficking.

## 6.4. VAP-1 in tumor neovascularization

The results from study IV indicated that the absence of SSAO-activity of VAP-1 reduced tumor progression. In addition, tumor neovascularization was defective and the accumulation of CD8 T-cells as well as MDSC into tumors was reduced. However, blocking of the adhesive epitope of VAP-1 with anti-VAP-1 antibody did not affect tumor progression and MDSC accumulation, although reduced numbers of CD8 cytotoxic T-cells were still seen. These observations are summarized in Figure 6.



| Treatment: | Wild type mice<br>- | Wild type mice<br>anti-VAP-1 antibody | Wild type mice<br>SSAO-inhibitor | VAP-1 -/- mice<br>- |
|---|---|---|---|---|
| tumor volume: | 100 % | not changed | reduced | reduced |
| Cytotoxic T cells: | 100 % | reduced | reduced | reduced |
| Myeloid-derived suppressor cells: | 100 % | not changed | reduced | reduced |
| Angiogenesis: | 100 % | not changed | reduced | reduced |

**Figure 6.** Summary of the findings from tumors growing in wild-type and VAP-1-/- mice and the effects of anti-VAP-1 antibody as well as SSAO-inhibitor treatments to the numbers of inflammatory cells.

Based on these results, it seems possible that the presence of cytotoxic CD8 T cells is not enough to lead to antitumoral effects, since cytotoxic T cells are reduced in anti-VAP-1 antibody treatment but the tumor progression is not altered. It is known that the presence of regulatory T cells can suppress cytotoxic T-cells. However, in our model the numbers of regulatory T cells was unaltered as well as the numbers of CD4 T-helper cells. However, reduced numbers of MDSC and defects in tumor neovascularization were found in the absence of VAP-1. MDSC can suppress T-cell functions through the end products of arginase-1 (ARG1) and nitric oxide synthase-2 (NOS2) activity (Marigo *et al.* 2008). In VAP-1 -/- mice the reduced numbers of suppressor cells can, therefore, allow better cytotoxic T cell responses and thus might lead to enhanced anti-tumoral effects and reduced tumor progression.

The accumulation of MDSC in a tumor site has several consequences for tumor neovascularization. Yang et al. (2004) showed that MDSC were involved in tumor angiogenesis. MDSC secrete MMP9, which degrades matrix, thus increasing the bioavailability of VEGF (Yang *et al.* 2004). Locally released VEGF, in turn, induces angiogenesis in the tumor site. Moreover, they demonstrated that MDSC transdifferentiated into endothelial cells once injected into a tumor site. Thus, in addition to increasing VEGF, increased numbers of MDSC are able to form blood vessels in tumors by transdifferentiating into endothelial cells. In our experiments the absence of VAP-1 led to decreased MDSC accumulation in the tumor site. In addition, VEGF measurements in tumor tissue revealed decreased VEGF concentrations in the absence of VAP-1. In addition, the MMP9 was also shown to be reduced at mRNA level thus suggesting that the diminished number of MDSC in tumor site in the absence of VAP-1 correlates to reduced MMP9 mRNA levels and to reduced VEGF protein levels. It could furthermore be speculated that the impaired MDSC accumulation into a tumor site in the absence of VAP-1 is the cause of defective neovascularization and

reduced tumor progression, as illustrated in a putative model for VAP-1 in angiogenesis in Figure 7. Moreover, it has recently been shown that the chemoattractants s100A8 and s100A9 induced by tumor induce MDSC production into the blood and secondary lymphoid organs. The MDSCs synthesize and secrete both proteins and are able to bind them thus leading to a autocrine feedback loop and accumulation of MDSC. Both s100A8 and s100A9 are known to be upregulated in several inflammatory conditions as well as in cancer. (Cheng *et al.* 2008; Sinha *et al.* 2008). Interestingly, we found a decreased mRNA level of s100A8 in the absence of VAP-1, which may correlate to the reduced numbers of MDSCs, which secrete this protein. (Figure 7.)

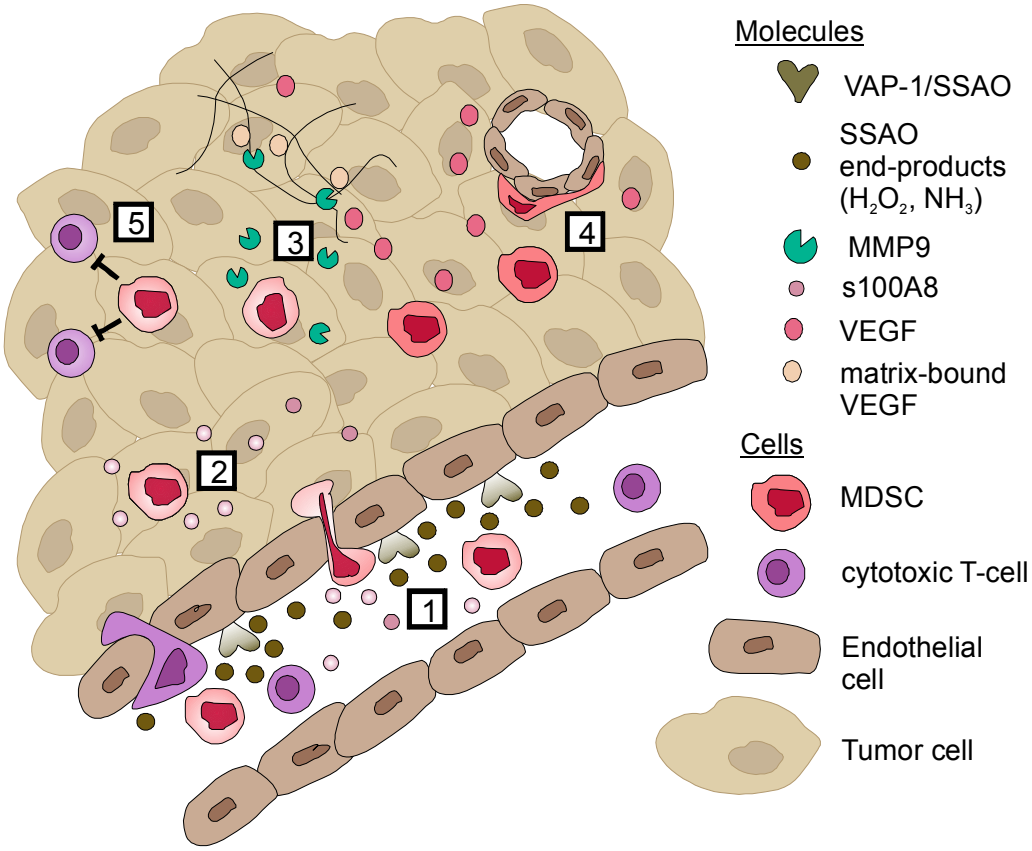



**Figure 7**. A putative model for VAP-1 regulated MDSC recruitment, neoangiogenesis and tumor progression. 1. Enzymatic SSAO-activity of VAP-1 recruits MDSC into the tumors. 2. MDSC secretes chemoattractant s100A8, which further attracts MDSC into the tumor site. 3. MDSC secretes MMP9, which degrades matrix thus increasing bioavailability of VEGF. 4. VEGF induces neoangiogenesis. MDSC can also incorporate into vessels and transdifferentiate into endothelial-like cells. 5. MDSC suppresses cytotoxic T-cell functions. See the text for references.

The results from study IV indicate that the recruitment properties of MDSC might be defective in the absence of VAP-1, since the number of MDSC in bone marrow and blood circulation as well as the spleen is unchanged. However, experimental proof for the decreased homing remains to be obtained. This would require a homing experiment with labeled MDSC cells in tumors of VAP-1 -/- mice and wild type controls. It has not been previously shown that any given adhesion molecule would account for MDSC accumulation into tumors. As tumor derived soluble factors influence the myelopoiesis of MDSC in the bone marrow, they may also induce their accumulation into tumors. Moreover, MDSC recruitment into a tumor area is enhanced as tumor burden and time increase (Marigo *et al.* 2008). There are therefore several factors from which the MDSC accumulation is dependent on. Although our current hypothesis is that VAP-1 mediates accumulation of MDSC into tumors by its enzymatic activity, the accumulation can also be induced by indirect mechanisms such as increased expression of attracting factors produced by VAP-1 expressing cells (endothelial cells or pericytes) or changes in tumor cells exposed to end-products of VAP-1 enzymatic activity. Our results, moreover, indicated that the granulocytic population of MDSC was decreased in the absence of VAP-1. Both the granulocytic and monocytic populations of MDSC suppress T-cell functions but the mechanism might be different (Movahedi *et al.* 2008). A differential homing of granulocytic and monocytic MDSC into tumors and their different dependence on VAP-1 could explain the difference. Since MDSC have high plasticity in a tumor microenvironment, it is thus possible that their differentiation into granulocytic or monocytic subpopulations is dependent on SSAO-activity ($H_2O_2$, $NH_3$) and occurs in the tumor environment.

Noda et al. (2008) have recently shown that the use of SSAO-inhibitors can reduce choroidal neovascularization. Vascularization was reduced 40 % in SSAO-inhibitor treated rats at early time points after inducing neovascularization by laser injury. Furthermore, the reduced neovascularization was suggested to be due to a defect in macrophage accumulation into the site of an injury, since macrophages are a source of proangiogenic factors (Sica & Bronte 2007). However, later time points showed that the favorable effect of VAP-1 disappeared. Indeed the authors interpreted the results to mean that by inhibiting one single angiogenic factor it may lead to up-regulation of others with functional overlap. Although done with one SSAO-inhibitor only, these data further confirm that VAP-1 is able to mediate accumulation of anti-angiogenic leukocytes into target areas.

Some adhesion molecules, such as JAM-A, JAM-C, PECAM-1 and ESAM-1, have been shown to have angiogenic properties (Cooke *et al.* 2006; Ishida *et al.* 2003; Lamagna *et al.* 2005; Woodfin *et al.* 2007). Their involvement in angiogenesis is mainly related to endothelial cell motility. Moreover, the interaction between JAM-A and endothelial integrin $\alpha_v\beta_3$ is related to integrin-dependent angiogenic signal transduction. Although JAM-C was shown to be involved in tumor angiogenesis, and the authors speculated that it might involve monocyte recruitment, they did not have evidence for this (Lamagna *et al.* 2005). To our knowledge, the angiogenic properties of adhesion molecules have not been shown to be related to MDSC recruitment. In contrast, the defects seen in other knockout mice are related to endothelial cell

migration and tube formation. Based on our results, VAP-1 is the first adhesion molecule which is related to tumor angiogenesis through the recruitment of MDSC.

## 6.5. Targeting VAP-1 for anti-adhesive therapies

The targeting of VAP-1 has now been tested in several inflammatory conditions. The targeting has been made either with anti-VAP-1 antibodies or SSAO-inhibitors. The findings are summarized in Table 9.

Table 9. SSAO-inhibitor and anti-VAP-1 mAb treatments in animal models of inflammation.

| Model | Treatment | Effects | Reference |
|---|---|---|---|
| Melanoma | SSAO-inhibitor | Reduced MDSC infiltration, angiogenesis and tumor progression | IV |
| Lymphoma | SSAO-inhibitor | Reduced MDSC infiltration, angiogenesis and tumor progression | IV |
| Inflamed air pouch | SSAO-inhibitor, anti-VAP-1 mAbs | Reduced monocyte infiltrations | I, (Merinen *et al.* 2005; O'Rourke *et al.* 2008) |
| Lung inflammation | SSAO-inhibitor | Reduced leukocyte infiltration | (O'Rourke *et al.* 2008) |
| Adjuvant induced arthritis | SSAO-inhibitor | Reduced PMN infiltrations | (Marttila-Ichihara et al. 2006) |
| Anti-collagen mAb induced arthritis | SSAO-inhibitor | Reduced PMN infiltrations | (Marttila-Ichihara et al. 2006) |
| Peritonitis | Anti-VAP-1 mAbs | Reduced PMN infiltrations | (Merinen *et al.* 2005; Tohka *et al.* 2001) |
| Ulcerative colitis | SSAO-inhibitor | Reduced inflammation | (Salter-Cid *et al.* 2005) |
| Liver allograft rejection | Anti-VAP-1 mAb, SSAO | Reduced lymphocyte infiltration | (Martelius *et al.* 2004; Martelius *et al.* 2008) |
| Chronic autoimmune diabetes | Anti-VAP-1 mAb | Reduced incidence of diabetes | (Merinen et al. 2005) |
| Intestinal ischemia-reperfusion injury induced acute lung injury | SSAO-inhibitor | Reduced leukocyte infiltrations | (Kiss et al. 2008) |

Table 9. (continued)

| Model | Treatment | Effects | Reference |
|---|---|---|---|
| Choroidal Neovascularization | SSAO-inhibitor | Reduced angiogenesis and macrophage infiltration | (Noda et al. 2008) |
| Experimental autoimmune encephalomyelitis (EAE) | SSAO-inhibitor | Reduced incidence and clinical score of EAE | (O'Rourke *et al.* 2007; Wang *et al.* 2006a) |

As seen in Table 9, the benefits of VAP-1 blockade are related to leukocyte accumulation into several inflammatory sites. Thus, these studies indicate that VAP-1 could be blocked using SSAO-inhibitors or anti-VAP-1 antibody to decrease leukocyte accumulation and alleviate inflammatory reaction.

VAP-1 can function both as a direct and indirect adhesion modulator. A direct adhesion modulator indicates here the binding of VAP-1 to its still unknown ligand and thus mediating leukocyte adhesion. That interaction can be blocked by anti-VAP-1 antibodies. The studies performed *in vivo* utilizing anti-VAP-1 antibodies are suggesting direct adhesion functions (Table 9). Intravital microscopic studies have shown that anti-VAP-1 antibodies reduced PMN rolling, adhesion and transmigration through the inflamed endothelium and Th2 cell rolling in the inflamed liver postsinusoidal venules (Bonder *et al.* 2005; Tohka *et al.* 2001). Moreover, leukocyte infiltrations into inflamed peritoneum and air pouch reduced after anti-VAP-1 antibody treatment (Merinen *et al.* 2005; Tohka *et al.* 2001). Anti-VAP-1 antibodies reduced leukocyte infiltrations also in liver allograft rejection and chronic autoimmune diabetes (Martelius *et al.* 2004; Merinen *et al.* 2005). As opposed to direct adhesive functions, an indirect adhesion modulator indicates that the enzymatic activity of VAP-1 is modulating the function of other molecules involved in adhesion. The *in vivo* experiments made by blocking the SSAO-activity indicates indirect effects, since end products from SSAO reaction can upregulate other adhesion molecules (Jalkanen *et al.* 2007; Lalor *et al.* 2007). SSAO-inhibitors have been used successfully in several *in vivo* settings to reduce leukocyte infiltrations (Table 9). In ischemia-reperfusion induced intestinal damage and acute lung injury the use of SSAO-inhibitors reduced leukocyte infiltrations whereas anti-VAP-1 antibody did not. In addition, in the study IV, MDSC accumulation was not reduced after anti-VAP-1 antibody treatment although SSAO-inhibitors reduced their accumulation. Moreover, at the same time, CD8 T cell accumulation was reduced by using SSAO-inhibitors as well as using anti-VAP-1 antibodies. These results indicate that VAP-1 has most likely direct and indirect effects depending on the target vasculature, stimulation and leukocyte subtype.

The first humanized antibody treatment against adhesion molecule was Natalizumab, a monoclonal antibody against the $\alpha_4$ integrin chain. The use of Natalizumab blocked the binding of $\alpha_4\beta_1$ (VLA-4) toVCAM-1 as well as $\alpha_4\beta_7$ to MAdCAM-1. Natalizumab has been used to treat multiple sclerosis (preventing brain-infiltrating Th1 cells binding to VCAM-1) and Crohn's disease (preventing Th1 cells binding to MAdCAM-1). In both cases successful results were obtained. However, in a few cases, the blocking of $\alpha_4$ integrin led to reactivation of JC virus and the development of progressive multifocal

leukoencephalopathy (Hilden *et al.* 2006; Mackay 2008). Nevertheless, the use of Natalizumab has shown that it is possible to block an adhesion molecule and reduce leukocyte trafficking and inflammation in clinical settings.
Due to the risk of increased susceptibility to microbial infections after blocking of $\alpha_4$ integrin, it was important to investigate the effect of VAP-1 on anti-microbe responses. The genetic deletion of VAP-1 slightly delayed the ability to clear an *S.aureus* skin infection while the onset of *Yersinia enterocolitica* infection was not regulated by VAP-1. Although a genetic deletion of VAP-1 has minor consequences in clearing *S.aureus*, the therapeutic treatment with anti-VAP-1 antibodies or SSAO-inhibitors did not delay clearance in this experimental setting. The different phenotype could be explained by the fact that in genetic deletion mice are devoid of VAP-1 all their lifetime and VAP-1 is missing from endothelial cells as well as from pericytes and smooth muscle cells. However, therapeutic treatment with anti-VAP-1 antibody and SSAO-inhibitor only temporarily blocks VAP-1 functions and from sites accessible to the blood circulation. Moreover, there is a residual SSAO-activity (5-10%) after inhibitor treatment that might allow some VAP-1 dependent effects. Although we have performed microbial infection studies only with very limited numbers of microbial species (*S.aureus, Yersinia enterocolitica* and *Coxsackie*), it appears that in these particular models the blocking of VAP-1 does not alleviate or aggravate the infection. However, these models are *in vivo* animal models and may not reflect the true situation in humans. Thus, we cannot rule out that blocking of VAP-1 might contribute to normal immune defence mechanisms against pathogens, however, these results suggest that the treatment with anti-VAP-1 antibodies and SSAO-inhibitors most likely does not cause generalized increase in the risk of infections.

Due to side-effects related to the use of small-molecular inhibitors, several chemically distinct compounds were utilized in different anti-adhesive VAP-1 studies (BTT2027, SZE5302 and LJP1586 as well as the prototype inhibitors semicarbazide and hydroxylamine) and similar results were obtained. The use of SSAO-inhibitors in these studies has been made in a prophylactic manner. However, O'Rourke et al. (2007) have shown that the therapeutic treatment with another SSAO-inhibitor after the onset of experimental autoimmune encephalomyelitis gave a similar reduction in the clinical score as the prophylactic treatment. This suggests that VAP-1 could be blocked after the onset of the disease and still a favorable reduction in the leukocyte accumulation, and thus alleviation of the inflammation, can be achieved.

The use of VAP-1 as a target in tumor immunology is very tempting. Generally, tumor vasculature loses at least some adhesion molecules expression as one form of immune escape (Dirkx *et al.* 2003; Griffioen 2008). VAP-1 is an adhesion molecule which expression is found in tumor vasculature. As has been shown, VAP-1 mediates the binding of tumor infiltrating T-cells to the tumor endothelium *in vitro* (Irjala *et al.* 2001). Moreover, the enzymatic activity of VAP-1 is related to MDSC accumulation into tumors. By inhibiting the SSAO-activity of VAP-1 using SSAO-inhibitors, MDSC accumulation into tumors could be reduced, thus reducing neoangiogenesis and reduced tumor progression. Since vessel formation is necessary for tumor growth and VEGF is the main proangiogenic factor, VEGF inhibitors have been tested in clinical

trials and clinics to prevent neovascularization and further tumor progression (Carmeliet 2005; Kerbel 2008). The benefits have been relatively modest. One explanation for tumor resistance to anti-VEGF antibody treatment is the suppressor cell populations that are able to induce angiogenesis independently of VEGF. It has been shown that murine tumor cell lines resistant to anti-VEGF antibodies are accumulated with MDSC after transplanting them to mice (Shojaei *et al.* 2007). Thus the combined treatment with anti-VEGF antibodies as well as adhesion molecules targeting MDSC accumulation might give beneficial effects (Murdoch *et al.* 2008). However, before VAP-1 could be used as a target to prevent MDSC accumulation and tumor neoangiogenesis, the function of VAP-1 in several other microbial infections should be studied in detail, since MDCSs are also implicated to be involved in several microbial infections (Sica & Bronte 2007). Infections such as *Candida albicans, Trypanosoma cruzi, Schistosoma mansomi, Taenia crassiceps* and *Porphyromonas gingivalis* induce MDSC accumulation into an inflammatory area and T cell suppression aggravates disease in animals. Based on our tumor data, we speculate that the reduced numbers of MDSC in VAP-1 -/- mice might accelerate the clearance of microbes in the host. However, the immune response against these microbes should be experimentally studied in VAP-1 -/- mice.

In conclusion, these and earlier studies suggest that VAP-1 could be blocked to reduce unwanted leukocyte infiltration in several inflammatory conditions and in cancer.

# 7. SUMMARY

Our immune defence is largely dependent on the continuous leukocyte recirculation through our body and the ability of leukocytes to migrate into tissues. The migration is regulated by the expression of adhesion molecules and their counterparts on endothelial cells and leukocytes. More regulation and subtype specific properties are generated by chemokine expression in target tissues as well as their receptors on leukocytes. All these functions ensure that specific leukocyte populations are recruited into an injured or inflamed areas to defend our body against foreign intruders.

Vascular adhesion protein-1 (VAP-1) is an endothelial adhesion molecule and also an enzyme possessing SSAO-activity. The enzymatic reaction deaminates primary amines into aldehydes while hydrogen peroxide and ammonia are produced. VAP-1 mediates leukocyte adhesion through antibody dependent epitope as well as through SSAO-activity. The aim of this study was to investigate the relationship between antibody dependent epitope and SSAO-activity on leukocyte adhesion. Moreover, the influence of the absence of VAP-1 was studied *in vivo* utilizing knockout mouse technology.

The data of this study demonstrated that the adhesive epitope and SSAO-activity are prerequisites for VAP-1 mediated leukocyte rolling as well as transmigration. The blocking of either the adhesive epitope with anti-VAP-1 antibodies or the SSAO-activity with SSAO-inhibitors totally prevents VAP-1 mediated leukocyte interactions with endothelium. Thus, it indicates that VAP-1 can be blocked by either means for anti-adhesive therapies. Genetic deletion of VAP-1 results in reduced leukocyte trafficking. The physiological lymphocyte recirculation as well as granulocyte infiltration into an inflamed area is impaired. Moreover, leukocyte accumulation into a tumor is defective. The impaired leukocyte trafficking has several consequences. There is a slight impairment in antimicrobial responses in the absence of VAP-1, as a more pronounced effect was found in tumor immunity. Tumor progression was reduced and a defect in neoangiogenesis in the tumor was observed.

The results indicate that VAP-1 can be blocked by anti-VAP-1 antibodies as well as enzyme inhibitors against SSAO. VAP-1 could be used as a target in anti-adhesive therapies. Targeting of VAP-1 might have only minor side-effects in antimicrobial responses and presumably does not cause a generalized increase in the risk of infections. VAP-1 could be a drug-target in inflammatory diseases, and also in tumors, since it mediates both leukocyte accumulation and tumor neovascularization.

# 8. ACKNOWLEDGEMENTS

This work was carried out at the MediCity Research Laboratory and Department of Medical Microbiology, University of Turku, and at the Department of Bacterial and Inflammatory Diseases, National Public Health Institute. The heads of these institutions Professors Sirpa Jalkanen, Matti Viljanen and Pentti Huovinen are warmly acknowledged for providing excellent research facilities. I also want to thank Turku Graduate School of Biomedical Sciences and its director Professor Olli Lassila for providing financial support and organizing many scientific meetings and cources.

I am deeply grateful to my supervisors Docent Marko Salmi and Professor Sirpa Jalkanen for giving me the opportunity to be a part of their research team and for introducing me to scientific work. Especially, Marko Salmi is acknowledged for his constant supervision, help and guidance during the years.

I warmly thank Professors William Agace and Carl Gahmberg for quickly reviewing this thesis and for their valuable comments. Dr. Mike Nelson is acknowledged for reviewing the language of this thesis. I also want to warmly thank all my co-authors for the successful collaboration.

I wish to thank all the present and former members of Sirpa's research group as well as other colleagues for their expertice, help and friendship. Especially our "coffee group" is thanked for hilarious moments and discussions during the coffee breaks and lunches. I wish to warmly thank all the lab technicians for their invaluable technical help. Especially I am deeply grateful for the excellent technical assistant of Riikka Sjöroos and Mari Parsama. It has been a pleasure to work with all of you in our lab! I also wish to thank Anne Sovikoski-Georgieva and Elina Wiik for secretarial help and all technical personnel at MediCity for their help.

All my friends are thanked for many memorable moments that we have spent together. I wish to thank my parents, Pirjo and Aulis, for all their care and support. My dearest twinsister Reetta, thank you for being the best sister in the world! I also wish to thank Auvinen-family for all the help and support you have offered. Finally, I wish to thank my dear husband Janne for helping me with computer-related things and most of all, for your love, care and patience.

This study was financially supported by the Finnish Cultural Foundation, the Ida Montin Foundation, the Emil Aaltonen Foundation, the Emil and Blida Maunula Foundation, the Orion-Farmos Research Foundation, the Turku University Foundation, and Turku Graduate School of Biomedical Sciences.

Turku, February 2009

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