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ENDOGENOUS THROMBOSPONDIN-1 AND PROTEASES IN THE REGULATION OF LYMPHOCYTE ADHESION AND MOTILITY

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Till min älskade familj
Mats-Ola
Filippa och Wilma

ABSTRACT

The human immune system, which protects the body from invading pathogens, largely depends on the proper function of lymphocytes, which are highly motile and constantly recirculate the blood and lymph. Adhesive and motile capability is often amplified or uncontrolled during chronic inflammatory conditions such as autoimmune diseases. This thesis comprises four studies of T lymphocyte motility and adhesion aiming to elucidate the regulative role of endogenous secretion of enzymes and the matricellular protein thrombospondin-1 (TSP-1).

We initially investigated the expression of matrix metalloproteinases (MMPs) in seven leukemia T cell lines and found a strict correlation between secretion of MMP-9, its natural inhibitor tissue inhibitor of MMPs (TIMP-1) and ability to infiltrate a three-dimensional (3D) gel of extracellular (ECM) components. However, cell migration to two-dimensional (2D) ECM-components was not correlated to MMP-9/TIMP-1 expression. The role for MMP-9 and TIMP-1 in motility was unclear since an inhibitor of MMP-9 activity rather enhanced infiltrative capacity over 24h. We conclude that MMP-9 and TIMP-1 play a role for spontaneous T lymphocyte motility in 3D-matrices, which is a functional property separated from ability to migrate to 2D ECM-components.

In our following papers (Paper II-IV), we found that T cell contact with collagen type I or β_1- and β_2-integrin ligands induced cell surface expression of TSP-1 and the TSP-1 receptor low density lipoprotein receptor-related protein (LRP)/CD91. Interaction of TSP-1 with its cell surface receptors calreticulin (CRT), LRP and integrin associated protein (IAP)/CD47 was promoting motility of T cells in 3D collagen through CD47. T cell surface TSP-1 also induced polarized spreading on fibronectin and ICAM-1 via binding to and signaling through CD47. T cell lines without endogenous expression of TSP-1 showed no spontaneous infiltration of collagen type I but became motile in the presence of exogenous TSP-1. We further found constitutive cell surface association of functional granzyme B on activated T cells, which continuously cleaved TSP-1, reduced infiltration of collagen type I and maintained non-polarized spreading on fibronectin and ICAM-1. This process depended on internalization of TSP-1 fragments via LRP.

In summary, we have included TSP-1 and its receptors CRT, LRP and CD47 in a model for regulation of T cell motility and adhesion, stating that TSP-1 drives infiltrative capacity and polarized spreading of T cells through receptor communication in *cis* within the same plasma membrane, generated by cross-linking of its receptors CRT, LRP and CD47. Cleavage of TSP-1 by granzyme B and possibly other enzymes, followed by internalization of fragments via LRP, reduces motility and results in non-polarized spreading.

LIST OF PUBLICATIONS

I. Infiltrative Capacity of T Leukemia Cell Lines: A Distinct Functional Property Coupled to Expression of Matrix Metalloproteinase-9 (MMP-9) and Tissue Inhibitor of Metalloproteinases-1 (TIMP-1).
Ivanoff A., Ivanoff J., Hultenby K. and Sundqvist K.-G.
Clinical and Experimental Metastasis 1999; 17:695-711

II. Autocrine Regulation of T cell Motility by Calreticulin-Thrombospondin Interaction.
Li S., **Ivanoff A.** and Sundqvist K.-G.
Journal of Immunology 2005; 174:654-661

III. Thrombospondin-1 is a Major T Lymphocyte Motogen through Protease-Controlled Cross-Linking of CD91 and CD47.
Forslöw A., Liu Z. and Sundqvist K.-G.
Submitted 2008

IV. Regulation of Integrin-Dependent T lymphocyte Adhesion by Thrombospondin-1 and its Receptors LRP and CD47 in Collaboration with uPA/uPAR and Granzymes.
Liu Z., **Forslöw A.**, and Sundqvist K.-G.
Submitted 2008

CONTENTS

LIST OF ABBREVIATIONS

2D, 3D	two-dimensional, three-dimensional
α2M	α-2-macroglobulin
ARP2/3	actin-related protein 2/3
ADAMTS1	a disintegrin and metalloproteinase with thrombospondin
APCs	antigen-presenting cells
BFGFs	basic fibroblast growth factors
CRT	calreticulin
ECM	extracellular matrix
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
HEV	high endothelial venules
HSPG	heparin sulphate proteoglycans
IAP	integrin-associated protein
ICAM	intercellular cell adhesion molecule
JAK	Janus family of tyrosine kinases
LFA-1	leukocyte function associated antigen-1
LDL	low density lipoprotein
LRP	LDL receptor related protein
MAdCAM-1	mucosal vascular adressin cell adhesion molecule-1
M-6-P	mannose-6-phosphate
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NK-cells	natural killer cells
PBT	peripheral blood T cells
PI3K	phosphatidyl inositol-3 kinase
PSGL-1	P-selectin glycoprotein ligand-1
Rac	RAS-related C3 botulinum substrate
RAP	LDL receptor-associated protein
Rap1	RAS related protein 1
Rho	RAS homologue gene-family member
SFCM	serum-free conditioned medium
TCR	T cell receptor
TGF-β1	transforming growth factor beta 1
TIMP	tissue inhibitor of matrix metalloproteinases
TNFα	tumor necrosis factor
TSP-1	thrombospondin-1
uPA/uPAR	urokinase plasminogen activator/receptor

1 SUMMARY

Lymphocytes are highly motile cells that constantly recirculate blood and lymph. Known factors that control T lymphocyte extravasation and motility include regulation of adhesive capacity, expression of chemokine receptors that bind chemoattractants, presence of enzymes that cleave and modify cell surface- or extracellular components and the extracellular matrix (ECM). This thesis has focused on endogenous expression of enzymes and thrombospondin-1 (TSP-1) and described a model for autocrine protease-controlled TSP-1-driven motility of T cells.

In paper I, we examined the expression and activity of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) by 7 T leukemia cell lines and studied their role in regulating cell motility in a three-dimensional (3D) Matrigel over 24 h. There was a strict correlation between expression of MMP-9, TIMP-1 and infiltrative capacity, since cell lines that expressed MMP-9 spontaneously infiltrated the gel, whereas cell lines that did not express MMP-9 were unable to invade the gel. Surprisingly, when using a broad-spectrum MMP-inhibitor, no reduction in infiltrative capacity was seen after 24h and instead more cells were invading the gel in the presence of the inhibitor. Using a Boyden chamber assay, the cell lines were allowed to migrate towards filters coated with ECM proteins. There was no correlation between MMP-expression and spontaneous migratory capacity. We conclude that infiltration and migration differ with respect to MMP-dependence and that the mechanisms of MMP-9 dependent spontaneous infiltration require further investigation.

In paper II, we found that the endogenously expressed matricellular protein TSP-1 regulates T cell infiltration of 3D collagen type I through binding to the TSP-1 receptors calreticulin (CRT) and integrin-associated protein (IAP/CD47). Short peptides that blocked binding of endogenous TSP-1 and specifically mimicked the TSP-1 binding site in CRT (CRT19-36), the CRT binding site in TSP-1 (Hep-1) or the CD47 binding site in TSP-1 (4N1K), had different effects on T cell infiltration and altered the cell surface levels of endogenous TSP-1. In summary, we found that binding of TSP-1 to CRT increased endogenous cell surface TSP-1 and elicited a phosphatidyl inositol 3 kinase (PI3K) and Janus family of tyrosine kinases (JAK)-dependent motogenic signal through CD47.

In paper III, we found a correlation between expression of TSP-1 and ability to infiltrate collagen type I. Cell lines without endogenous expression of TSP-1 were rendered motile upon addition of TSP-1. Peripheral blood T cells (PBT) with endogenous expression of TSP-1, increased cell surface levels of TSP-1 upon contact with the collagen in a secretion-dependent manner and simultaneous binding of TSP-1 to low density lipoprotein receptor related protein (LRP)/CD91 and CD47 enhanced motility through CD47. In contrast, exogenous TSP-1 destabilized binding of TSP-1 to the cell surface and reduced infiltrative capacity, indicating that environmental TSP-1 may differentially regulate cells depending on whether they express TSP-1 or not.
In addition, we found that TSP-1 is susceptible to cleavage by granzyme B and that endogenous granzyme B contributed to the turnover of TSP-1 at the surface of T cells. A granzyme B-specific inhibitor thus increased levels of intact TSP-1 on T cells and

enhanced motility in collagen. We conclude that TSP-1 drives T cell motility through cross-linking of LRP and CD47, a mechanism controlled by granzyme B cleavage of TSP-1.

In paper IV, we found that T cell adhesion to the β_1- and β_2-integrin ligands fibronectin and ICAM-1 induced cell surface expression of intact TSP-1 and a 130 kDa TSP-1 fragment as well as of LRP. Induction of LRP was dependent on TSP-1 signals through CD47. Cells that eventually de-adhered from the substrates expressed intact TSP-1 and a 115 kDa TSP-1 fragment but lacked LRP. By blocking the ligand binding- and internalization-function of LRP with receptor associated protein (RAP), TSP-1 and LRP increased at the cell surface and polarized spreading was enhanced. By instead blocking the TSP-1 COOH-terminal association with CD47 with the 4N1K-peptide, cell surface levels of TSP-1 as well as spreading were reduced. In conclusion, non-polarized T cell spreading and firm adhesion is dependent on continuous expression of LRP and TSP-1, sustained by cleavage and turnover of TSP-1 fragments and signaling through CD47. Loss of LRP results in de-adhesion, whereas inhibition of degradation of TSP-1 with an inhibitor of granzyme B promotes polarized spreading, possibly through enhanced cross-linking of receptors and signals via CD47.

2 INTRODUCTION

The immune system protects the human body against pathogens and tumors.

2.1 THE IMMUNE SYSTEM

Unspecific or **innate immunity** constitutes a first line of defense. Skin- and mucosal epithelial cells generate a mechanical barrier against pathogens but also includes the chemical protection achieved through release of antimicrobial enzymes and peptides. Innate immunity depends on the activity of polymorphonuclear leucocytes or granulocytes which include the **blood monocytes**, **neutrophils**, **eosinophils**, **basophils** and **mast cell precursors (Table 1)**, recognized by their characteristic staining patterns. The granulocytes originate from granulocyte/macrophage progenitors in the bone marrow and circulate the blood.

Table 1. Composition of blood cells in normal adults [1]

Type of cell	Subtypes	Conc./ml blood	% of white blood cells
Red blood cells		5×10^9	
White blood cells	**Lymphocytes**		17%
	B cells	$0{,}2 \times 10^6$	
	T cells	1×10^6	
	-CD4	$0{,}6 \times 10^6$	
	-CD8	$0{,}4 \times 10^6$	
	Granulocytes		76%
	Neutrophils	5×10^6	
	Eosinophils	$0{,}2 \times 10^6$	
	Basophils	$0{,}04 \times 10^6$	
	Monocytes	$0{,}4 \times 10^6$	6%
	NK cells	$0{,}1 \times 10^6$	1%
Platelets		$0{,}3 \times 10^9$	

The blood monocytes continuously migrate to tissues where they differentiate into **phagocyting macrophages**. Macrophages reside beneath the blood vessel endothelial cells mainly in the gastrointestinal tract (in the submucosa), in the liver (Kuppfer cells), lung, connective tissues and spleen. Tissue macrophages are activated by invading microorganisms through cell surface receptors that recognize common pathogenic carbohydrates and lipoproteins. Active macrophages not only engulf the pathogens, but also initiate an **inflammatory response** which includes release of soluble factors such as complement factors, cytokines (interferons and TNFα) and chemokines (IL8 and IP10) that contribute to vasodilatation, increased temperature and recruitment of different immune cells such as the short-lived but numerous phagocyting neutrophils [2]. Eosinophils increase during parasite infection and are able to kill antibody-coated parasites through release of free radicals and toxic granule proteins and enhance the inflammatory response via synthesis of cytokines. The precise role of basophils is not known, but they are involved in allergic reactions where they release the inflammatory mediator histamine. Mast cell precursors migrate to tissues and mature to **mast cells,** which via release of histamine and other inflammatory mediators largely contribute to allergy [1].

Innate immunity is not specific for a particular pathogen but rather recognizes general microbial components such as bacterial DNA or viral double-stranded RNA [3]. In addition, innate immunity does not generate an immunologic memory and is not able to recognize and fight all pathogens. When innate immunity fails to prevent infection, a specific or **adaptive immunity** has evolved in mammals, birds, fish and cartilaginous fish, that is able to specifically recognize, target and kill an immense number of pathogens and that largely depends on the **lymphocytes**. Functionally, the link between innate and adaptive immunity consists of specialized **antigen-presenting cells (APCs)** that ingest, process and present antigenic microbial fragments at their surface in order to activate **T lymphocytes** of the adaptive immune system [4]. Accordingly, immature **dendritic cells** that reside in tissues become activated by ingesting pathogens in an area of infection. Upon activation, the dendritic cells migrate to peripheral lymphoid organs such as local lymph nodes, where they display short bacterial peptides bound to major histocompatibility complex (MHC) class II molecules (presentation of external, endocytosed antigens) on their cell membranes. Other antigen-presenting cells are the **B lymphocytes** and **macrophages**.

Following activation, different subgroups of T lymphocytes either directly kill target cells or activate B lymphocytes to produce antibodies. The arm of the immune system that comprises the activity of T lymphocytes and phagocytes is called **cell-mediated immunity**. It also includes the action of **natural killer (NK) cells** that recognize and destroy certain virus infected or transformed cells due to their lack of or down regulation of MHC class I (present internal antigens). The second arm of the immune system is called **humoral immunity** and includes the different functions of antibodies produced by activated B lymphocytes.

The collaboration between cells of innate and adaptive immunity thus generates a highly efficient anti-microbial cascade of events that clears the infection and also forms a long-lasting immunologic memory leading to efficient resistance towards reinfection of a particular pathogen [5].

2.1.1 Lymphocytes

The T lymphocytes are derived from bone marrow lymphoid progenitor stem cells that migrate to thymus during embryonic development. In thymus, a process of T cell receptor (TCR) development and maturation takes place through extensive rearrangement of TCR gene segments. T cells begin to express either the αβ (>90%) or the γδ (<5%) TCR and the two major subsets of αβ T lymphocytes express either the CD4+ or CD8+ T cell co-receptor. In the thymus, each young T cell is selected for potential usefulness (positive selection) and self-reactive cells are removed (negative selection) before entry into the blood.

N**aive lymphocytes** have not yet encountered their proper antigen and show a restricted pattern of recirculation between blood and lymph. They continuously home to secondary lymphoid tissues (spleen, lymph nodes (LNs) and intestinal Peyer's patches (PPs) where APCs reside. Upon contact with APCs expressing antigen bound to MHC class II, the lymphocytes become activated and rapidly differentiate into **effector lymphocytes** which are able to enter inflamed tissues. The CD4+ T lymphocytes or

helper T cells (T_H) recognize MHC II+ antigen and the **T_H2** subset mainly targets extracellular microorganisms or antigens through activation of eosinophils and mast cells as well as B cells, which produce antibodies. The **T_H1** subset activates NK-cells and macrophages to kill intravesicular microorganisms.

Cytokines are released by various cells in the body including leukocytes and control cell differentiation, division, activation, maturation, antibody production and immune cell homeostasis (balance between proliferation and cell death). Cytokines affect cells in an autocrine (e.g. on the same cell that produced them) or paracrine (e.g. on other cells) manner. Through binding to cytokine receptors at the cell surface, cellular signaling is induced. Cytokines are important mediators of the immune response and T_H2 cells secrete mainly the interleukins (ILs) IL-4, IL-5, IL-10, that activate B cell function and suppress T_H1 development, whereas T_H1 secrete IL-12, IL-2, tumor necrosis factor (TNFα), TNFβ and interferon (IFNγ), that activate macrophages.

In addition, one heterogeneous group of CD4+ CD25+ T cells, called regulatory T cells (**Tregs**), negatively regulate the immune response and are important in preventing autoimmune disease [6]. A recently found group of CD4+ T cells named **T_H17** has instead been suggested to promote autoimmune disease and produces the proinflammatory cytokine IL-17 [7]. The CD8+ T lymphocytes or **cytotoxic T lymphocytes** (**CTLs**) attach to and kill target cells that express MHC class I+antigen. Effector lymphocytes are rather short-lived and only a few mature into long-lived **memory T cells** that are able to respond rapidly upon reinfection and which reside at the site of the first infection and in the spleen.

2.1.1.1 The immunological synapse (IS)

In order to mature, become effector cells or perform cytotoxic killing, T lymphocytes need to interact and communicate with APCs or target cells in different organs. The contact area between the lymphocyte and the APC is called the immunological synapse (IS) and is a signaling platform that differs with respect to stability, duration, strength of signals and presence or absence of secretion [8]. The IS is compartmentalized into the central- and peripheral supramolecular adhesion complex (cSMAC and pSMAC). On the T lymphocyte, cSMAC includes clustered TCR, co-receptors (CD2, CD3, CD4/CD8, CD28), signaling molecules (PKC-θ) and cytoskeletal components (polymerized actin, myosin II) whereas the pSMAC consists of adhesion molecules (LFA-1, VLA-4), cytoskeletal components (talin) and signaling molecules [9]. Activation of lymphocytes by APCs during inflammation can occur through transient and serial encounters, sustaining lymphocyte migratory capacity [10], or as seen in certain T cell-B cell interactions, through one single, firm and continuous contact [11].

2.1.1.2 CTL function

T cell mediated cytotoxicity is performed by CD8+ effector CTLs and involves killing of target cells infected by viruses or other intracellular microorganisms through induction of apoptosis. However, the formation of an IS with a cSMAC is not necessary for the targeted killing by CD8 lymphocytes, although the lymphocyte redirects the transport and release of secretory vesicles towards the target cell [12]. Three distinct pathways of killing have been outlined: **1)** the cytokine pathway which

involves secretion of TNFα and IFNγ by the CTL that results in TNF receptor (TNFR) mediated caspase activation and induction of target cell expression of MHC class I and Fas (CD95). **2)** The direct contact between FasL on the CTL and Fas/CD95 on the target cell that leads to caspase activation. **3)** The release of perforin, granulysin and granzymes into the intracellular space [13]. Perforin generates pores in the target cell membrane, thus destabilizing target cell integrity. Granulysin has antimicrobial action and induces target cell apoptosis. Granzymes cause cell death through caspase dependent and independent mechanisms [14].

2.1.1.3 B Lymphocytes

B lymphocytes develop initially in the fetal liver, mature in the bone marrow and subsequently in peripheral lymphoid organs such as the spleen and follicles of the lymph nodes. Maturation in the bone marrow is independent of antigen and involves cell surface expression of immunoglobulin IgM, which in association with Igα and Igβ chains forms the B cell receptor (BCR) for a specific antigen. When leaving the bone marrow B cells recirculate the blood and peripheral lymphoid organs in order to receive signals for survival and encounter the proper antigen. Stimulation of the BCR generates signaling and internalization of antigen, which is processed and presented to T_H2 cells on MHC class II molecules. Antigenic stimuli through the BCR as well as contact with T_H2 cells is required for B cell division and formation of **plasma cells** and **memory cells** that express secreted immunoglobulins or **antibodies** of different sub-classes or isotypes. Antibodies may directly neutralize pathogens or toxins through surface binding or indirectly through facilitation of their phagocytosis (opsonization) or through activation of complement. Early in the immune response plasma cells secrete IgM, which is subsequently replaced by IgG, IgA and IgE. In the case of re-exposure to the antigen, memory B cells ensure a more rapid and intense secondary response due to higher antigen sensitivity.

2.1.2 T lymphocyte Adhesion and Motility

In order to recirculate blood and lymph, recognize and remove tumor cells (a process known as immune surveillance) or be recruited to areas of inflammation, naive, effector- and memory T lymphocytes have the capacity to pass through the endothelial cells of the blood vessel wall (extravasate) at selected sites and to migrate to sub-compartments of lymphoid and non-lymphoid tissues [15]. Extravasation is initiated when the lymphocytes slow down, begin to roll along the endothelial cells and subsequently attach to the vessel wall. Adhesion is dependent on **adhesion molecules** expressed by the T lymphocytes, such as selectins and integrins and their corresponding endothelial cell ligands [16]. Passage through the endothelium (diapedesis) is followed by migration into the tissue consisting of various **extracellular matrix (ECM)** components including collagens, fibronectin (FN) and laminins (LN). Extravasation requires stimulation of the lymphocyte motile machinery, a process partly induced by surrounding **chemokines** that bind to and activate **chemokine receptors** at the lymphocyte cell surface. Modulation of adhesive capacity of lymphocytes occurs mainly through regulation of integrin function (affinity/avidity) **(Figure 1)**.

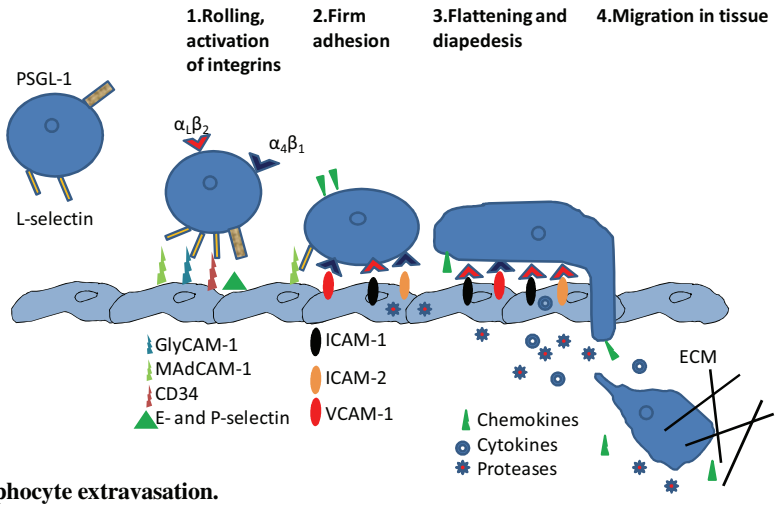

Figure 1. Lymphocyte extravasation.

Naive lymphocytes recirculate blood and lymph and are recruited to secondary lymphoid organs by specialized endothelial cells that form **high endothelial venules (HEVs)**. Expression of the adhesion molecule **L-selectin** mediates initial rolling of the lymphocytes on HEVs via binding to sulfated sialyl-Lewis^x moieties on peripheral node addressins (PNAds) such as glycosylation-dependent cell adhesion molecule-1 (**GlyCAM-1**), and **CD34** in lymph nodes, or mucosal vascular adressin cell adhesion molecule-1(**MAdCAM-1**) in the mucosal endothelium of Peyer's patches [17, 18].

Lymphocyte rolling is a result of loose interaction with the endothelium and **integrins** subsequently generate firm adhesion. The integrins **α4β1** and **αLβ2** bind to the endothelial ligands vascular cell adhesion molecule (**VCAM-1**) and intercellular cell adhesion molecule (**ICAM-1**) respectively. Naive T cells express mainly α4β1 whereas αLβ2 is induced upon antigen activation.

Specific recruitment or **homing** of naive T cells to secondary lymphoid tissues such as lymph nodes depend on expression of the chemokine receptor CCR7, which associates with chemokines secondary lymphoid tissue chemokine (SLC)/CCL21 or EBl1 ligand chemokine (ELC)/CCL19 [19]. Chemokines enhance lateral movement, clustering, affinity and avidity of integrins in the lymphocyte cell membrane through signals emitted via pertussis toxin sensitive receptor-associated heterotrimeric Gαi proteins [20]. The chemokine SDF-1 (CXCL 12) is expressed by lymphatic endothelial cells and binds to the chemokine receptor CXCR4 and generates T cell arrest on VCAM-1 through a rapid switch of the integrin α4β1 to a clustered high-avidity state [21].

Activated endothelium in an area of inflammation efficiently recruits cells of the innate immune system as well as memory- and effector lymphocytes by expression of ICAM-1, VCAM-1, MAdCAM-1, P- and E-selectin. Effector T cells express in addition to αLβ2 and α4β1 also P-selectin glycoprotein ligand-1 (PSGL-1). Homing to the intestine by effector- and memory T cells activated in PPs, is mediated by expression of integrin α4β7 that binds to the endothelial ligand MAdCAM-1. It also involves the lymphocyte chemokine receptor CCR9 that binds to TECK, a chemokine expressed by the epithelium of the small intestine [22, 23]. Homing to the skin is dependent on lymphocyte expression of cutaneous lymphocyte antigen (CLA) that binds to E-selectin on cutaneous endothelium and the expression of the chemokine receptor CCR4 that recognizes TARC expressed by cutaneous endothelium [24].

The penetration of dense subendothelial basal lamina and pericellular ECM is an important step in lymphocyte extravasation and a prerequisite for migration into lymphoid organs or non-lymphoid tissues. In non-lymphoid tumor cells such as carcinomas, migration is correlated to the action of **ECM-degrading enzymes** that enable cells to breach dense matrices. Lymphocyte migration was also suggested to be dependent on ECM-degrading enzymes and could in some experimental settings be reduced in the presence of protease inhibitors [25-28]. However, lymphocyte proteases may also release cytokines or chemokines bound to the ECM or modify lymphocyte cell surface components involved in the regulation of cell motility (**Table 2**) [29].

Table 2. Enzymatic activity: Direct and indirect modulation of lymphocyte migration

Proteolysis of ECM components such as FN may generate chemotactic fragments for lymphocytes [30].
The cell surface levels of L-selectin, TNFR, TGFα, IL6R and CD44 are down regulated through proteolytic shedding [31-34].
Proteases modify soluble mediators such as TNFα, IL8, IL1β and IL2 [35-39]
Proteases release ECM- bound chemokines, cytokines and growth factors MIP1β, RANTES, basic fibroblast growth factors (BFGFs) and TGFβ1 [40-43]

2.1.2.1 The dynamic lymphocyte

The motility of lymphocytes is highly versatile and encompasses different strategies depending on where migration takes place. Lymphocytes can attach to and move on the surface of other cells such as endothelial cells and APCs. They are able to breach dense basement membrane structures and can also perform a rapid amoeboid movement within loose connective tissue in lymphoid and non-lymphoid organs [44, 45]. Lymphocytes in the blood have a round shape, yet adhesion and migration require a flexible cell morphology and ability to polarize the cell body and redistribute chemokine receptors and integrins **(Figure 2)** [46].

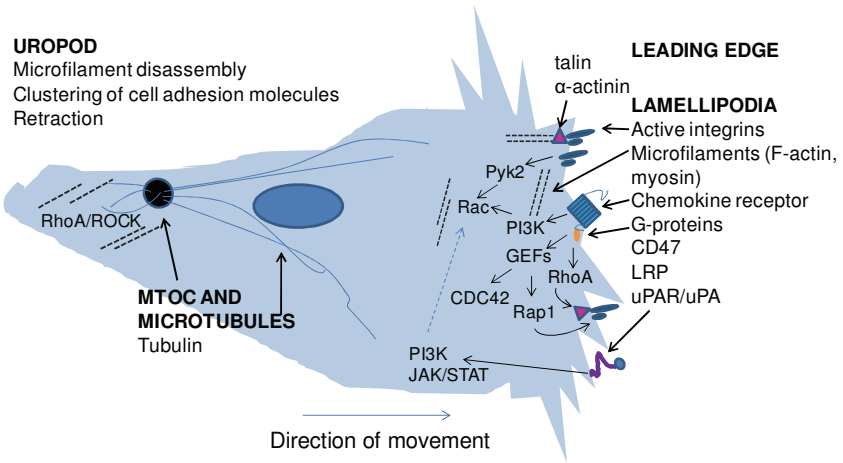

Figure 2. The moving cell. Signals via integrins or chemokine receptors regulate microfilaments that control cell motility through lamellipodia extension and uropod detachment. Lamellipodia and the uropod are protrusions with high plasticity due to actin polymerization or depolymerization and contraction or relaxation via myosin. Microtubules extend towards the leading edge from the MTOC and are retracted in the uropod. See text for abbreviations.

Lamellipodia form the leading edge and typically contain chemokine receptors and urokinase plasminogen activator receptor (uPAR) whereas the uropod denotes the trailing rear edge and contains clustered adhesion molecules [46]. T cells that adhere firmly in a αLβ2-dependent manner to ICAM-1 on endothelial cells stretch out lamellipodia stabilized by newly polymerized F-actin in microfilaments connected to motor proteins (myosins) that generate mechanical force and contraction [47, 48]. Upon adhesion and polarized spreading on ICAM-1, active αLβ2 is distributed towards the mid-zone of the polarized cell body allowing firm attachment while the lamellipodia contain αLβ2 with intermediate binding conformation and may rapidly extend forward [49]. At the leading edge lamellipodia induce attachment and at the rear end, the uropod is generating retraction. Both events depend on active changes to the actomyosin cytoskeleton [50]. The α4β1 integrin is displayed on microvilli (minute microfilamentous projections) of activated lymphocytes that enable initial contact with VCAM-1 on endothelium [51]. It is the LFA-1/ICAM-1, not the VLA-4/VCAM-1 that mediate transmigration of lymphocytes [52].

The uropod is formed around the microtubule organizing center (MTOC), from which extends the tubulin cytoskeleton or microtubules that are important in the intracellular transport of adhesion molecules and cytolytic granules as well as in the dynamic generation of T cell plasticity through retraction during motility [53]. However, in contrast to fibroblasts, lymphocytes do not form distinct focal adhesions or stress fibers which generate a tractional force that pull the cell forward and slow down the speed of migration. Within tissues, lymphocytes migrate as fast as 10-15 μm/min exemplified by naive T cells that scan dendritic cells for antigen within the lymph node through amoeboid crawling [50, 54].

Signaling pathways targeting actin polymerization and contraction regulate cell spreading and polarization:

- **Actin polymerization drives lamellipodium extension**, enhances **spreading** and **polarization/motility** and is positively regulated by RAS-related C3 botulinum substrate (**Rac**) and **CDC42**. Rac act via Wiscott-Aldrich syndrome protein-like protein (WAVE) and actin-related protein 2/3 (ARP2/3) [55, 56].
- **Actomyosin contraction drives lamellipodium- and uropod retraction**. Downregulation of microfilament stability by RAS homologue gene-family member A (**RhoA**) through activation of myosin heavy chain isoform IIA (MHCI IIA) via Rho-associated, coiled-coil containing protein kinase (ROCK) [57] leads to **contraction/**motility [58].

Signals through CXCR4 (the SDF-1α receptor) activate G-protein coupled receptors and PI3K, which dynamically activate Rac and CDC42 (via guanine-nucleotide-exchange factors (GEFs)) respectively in the front whereas in the uropod, RhoA induce T cell polarization and motility [59, 60]. However, both Rac and CDC42 are needed in the uropod in order to functionally activate RhoA/ROCK and maintain a polarized cell shape [60, 61]. Lamellipodia also contain RhoA and "inside-out signaling" generated by chemokines activates integrins via RAS-related protein 1 (Rap1) and RhoA [62]. Ligation of αLβ2 as well as α4β1 integrins in the presence of

polarizing or motility-inducing agents such as Mn2+, SDF-1α or anti-CD3 activate Pyk2 which induce polarity but not cell spreading, possibly through enhancement of Rac [63].

Lymphocyte attachment to ECM components is integrin-dependent [64] whereas migration in collagen is considered an integrin-independent process [47]. However, during migration in 3D collagen type I, the integrins α4β1 and α5β1 are redistributed and located mainly in the uropod associated with the cytoskeletal component filamin [47, 50]. In addition, spontaneous migration of non-activated lymphocytes within a 3D collagen is dependent on α2 and further, IL-8 induced migration in collagen type I is dependent on α5 and αL, although these integrins are not collagen type1 receptors [65], indicating indirect involvement of integrins in T cell motility in collagen type 1.

2.1.2.2 Experimental Models for Studies of Lymphocyte Adhesion and Motility

During *in vitro* studies of lymphocyte motility, ECM components can be presented to lymphocytes in a 2-dimensional (2D) or 3-dimensional (3D) manner. When fibronectin, collagen type IV, laminin or ICAM-1 are coated onto class slides or polystyrene plates, a 2D structure is generated that allows adhesion and spreading in a non-polarized or polarized fashion, resembling attachment to a vessel wall. The Boyden chamber assay is described in Materials and Methods and provides means for detecting ability to migrate induced by a soluble chemoattractant (chemotaxis) or migrate towards an ECM-coated polycarbonate filter (haptotaxis) and subsequently attach to the lower side of the filter where cells are counted. However, this assay merely measures adhesive capacity, unless the number of detached cells in the lower chamber is taken into account. The most physiologically relevant substrata are preformed 3D collagen type 1 gels or Matrigels consisting mainly of laminin [66]. These lattices have a molecular architecture that resembles interstitial tissues or the lymph node stroma respectively [67, 68]. Cells are added on top of the translucent gels and may subsequently be detected and counted at different levels within the gel and morphology can be determined.

2.2 EXTRACELLULAR MATRICES

ECM glycoproteins form a 3D structure that determines the organization of tissues and organs and controls differentiation, survival, adhesion and growth of cells [69]. The ECM varies with respect to density, elasticity and cell signaling properties and contains fibrous polymers of collagens, fibronectin, elastin, vitronectin and adhesive glycoproteins such as laminins, tenascin and heparan sulfate proteoglycans. Collagens and laminins provide a structural framework and form boundaries between different organs and tissues and constitute the basement membranes of adherent cells. [69]. The ECM is a storage depot for chemokines, cytokines, growth factors and proteases and contributes to the regulation of cell adhesion, polarity and migration through cell signaling [69]. The major ECM proteins will herein be presented in light of their role in regulating immune cell function.

2.2.1 Collagens

Collagens are classified depending on their polymerized form. They consist of α-chains and are homo- or heterotrimeric [70]. To date, 28 different collagen types constituted from 42 α-chains have been found in vertebrates [71]. Collagens are mainly synthesized by fibroblasts and epithelial cells. In humans, the most abundant types of collagen are type I, II III and IV where type I-III are found in cartilage, tendon, bone, skin and in ligaments, whereas the type IV collagen is a major constituent of basement membranes. Lymphocytes express integrins α1β1 and α2β1 that are receptors for collagen type IV and I [72, 73]. Lymphocytes infiltrate 3D collagen spontaneously and collagen is a co-activating factor that probably facilitates the formation of an immunological synapse by anchoring activated T cells via α1β1 and α2β1 and thus prolongs APC-T cell interaction [74].

Table 3. Family of collagens

	Type	**Localization**
Fibril-forming	I, II, III, V, XI	Interstitial connective tissue
Network-forming	IV, VIII, X	Basement membranes
Microfibrillar collagen	VI	Interstitial connective tissue
Fibril-associated collagen with interrupted triple helix domain and interruptions	IX, XII, XIV, XVI, XIX	Interstitial connective tissue
Multiplexins	XV and XVIII	Basement membranes
Orphans	VI, VII, VIII, X, XIII, XVII	Basement membranes

2.2.2 Fibronectin

Fibronectin is a high-molecular weight glycoprotein important for cell migration, adhesion and differentiation and plays a role in embryogenesis, angiogenesis, blood clotting and tissue remodeling [75, 76]. Alternative splicing at three sites of the fibronectin gene generates two forms; the cellular insoluble polymeric form that participates in fibrillar networks in the ECM and the soluble dimeric form found in plasma [77]. The primary structure consists of three different repeated sequences that generate subunits with distinct binding to various ECM components. Fibronectin thus forms networks through binding to collagen, fibrin (subunit I), heparin, heparan sulphate proteoglycans (HSPGs) and its cell surface receptors α3-5β1, α4β7, αIIbβ3 (subunit III) [78]. Fibronectin mediates T cell adhesion to the ECM mainly via its RGD-sequence that binds to integrins on the cell surface [76].

2.2.3 Laminins

Laminins are together with collagen type IV and proteoglycans the major components of basement membranes. The laminins are formed by α,β and γ chains and 15 different heterotrimers have been found. Laminins bind to different receptors including HSPGs and integrins α1-3β1, α6β4 and αVβ3. Development and function of the kidneys and the central nervous system as well as assembly of basal lamina in the skin critically depends on laminins, reflected by embryonic lethality in most laminin knock-out mice as well as a wide array of diseases found to relate to improper or missing expression of laminin isoforms [79]. Cleavage of certain laminins by neutrophil elastase generates fragments with chemotactic properties for neutrophils and macrophages [80]. In

addition, lymphocytes secrete laminin-8 in an activation-dependent manner, adhere to laminin-8 via α6β1 and show enhanced proliferation in the presence of laminin-8 [81].

2.2.4 Vitronectin

Vitronectin has collagen binding and glycosaminoglycan binding domains and promotes cell spreading and motility through binding to certain integrins (αVβ3, αvβ5, αvβ1, and αIIbβ3) and the uPAR [69]. It is a plasma protein but is also found in platelets and within the ECM. Vitronectin favors clot formation through inhibition of fibrinolysis and is a component of atherosclerotic plaques, where it serves as an attractant of smooth muscle cells and macrophages.

2.2.5 Glycosaminoglycans

Glycosaminoglycans such as heparan sulfates and hyaluronan are polysaccharide carbohydrates that attach to different ECM proteins such as perlecan and CD44 to form proteoglycans. Carbohydrate moieties alter the ligand-binding function of the core protein and may directly bind a variety of ligands including growth factors, cytokines, chemokines and different ECM components and regulate developmental processes, angiogenesis and cell adhesion and motility [82].

2.3 MATRICELLULAR PROTEINS

A particular group of matrix proteins, termed the matricellular proteins, comprising thrombospondins 1-5, tenascins, SPARC (serum protein acidic and rich in cystein) and osteopontin, differ from the scaffolding matrix components in that they modulate adhesive interactions between cells and the ECM [83]. Matricellular proteins bind to the ECM, various cell surface receptors, growth factors, cytokines and proteases. This may locally increase the concentration of molecules that regulate growth, survival and motility [84-87]. The matricellular proteins are expressed at high levels during tissue remodeling and at inflammatory sites and generally induce cellular de-adhesion or focal adhesion disassembly if presented in a soluble form [88]. In contrast, matricellular proteins support weak cell-adhesion when presented in a bound form [88, 89]. The thrombospondin family will be described in detail.

2.3.1 Thrombospondins

The TSP family of matricellular glycoproteins comprises five members, namely TSP-1, TSP-2, TSP-3, TSP-4 and TSP-5 (cartilage oligomeric matrix protein) and shows a widespread distribution in various organs in the embryonic as well as the adult organism [90-92].

2.3.1.1 Structure of Thrombospondins

The TSPs are usually divided into two sub-groups, Group A and B, according to their structure and oligomerization (**Figure 3**).

Group A comprises two members, TSP-1 and TSP-2, with a molecular mass of about 175 kDa for each monomer on reducing gels and 450 kDa in the trimeric non-reduced form. Each monomer consists of an NH_2-terminal heparin binding domain, a procollagen and interchain connecting domain, type 1, type 2 and type 3 repeats and a

COOH-terminal cell-binding domain (CTD). Three monomers are covalently linked through interchain disulfides in the coiled-coil oligomerization region close to the NH_2-terminal domain [93]. TSP-1 and -2 show different patterns of expression, are localized on different chromosomes in both the mouse and human genome and differ slightly in amino acid sequence [94]. All TSPs display a highly conserved COOH -terminal cell binding domain that is involved in the regulation of cell adhesion and proliferation [95-97]. See **Table 4** for a detailed description of the known functions of the different TSP-1 domains [93].

Group B comprises TSP-3, TSP-4 and TSP-5, all pentamers consisting of monomers of about 100 kDa. The TSP-members of group B do not have the procollagen domain or type 1 repeats but have four copies of the type 2 repeats.

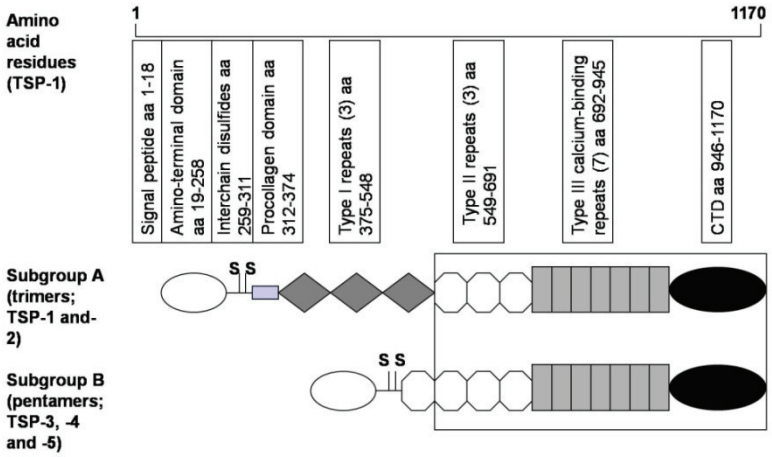

Figure 3. General structure of monomeric TSPs from subgroup A and B.

2.3.1.2 *Expression and Function of Thrombospondin-1*

TSP-1 is widely expressed during embryonic development and in several cell types and organs in the adult tissue. It can be found in the blood in concentrations ranging from 60-300 ng/ml. However, platelet activation may increase the local concentration at certain sites to 10-20 mg/ml [98].The first known source of TSP-1 was human platelet α-granules that released TSP-1 in response to thrombin. TSP-1 plays a role as a component of the fibrin clot, where it links and aggregates platelets in a Ca^{2+}-dependent manner [91, 99]. However, its function in blood clotting is not vital since TSP-1 null mice have no bleeding defects [100].

TSP-1 is often found in tissues undergoing regeneration such as in healing wounds of skin, in rheumatoid lesions and in damaged nerves [101-103]. In addition, TSP is up-regulated in proliferating cells and increased TSP-expression has been linked to tumor progression [104-106]. A pro-proliferative function of TSP-1 is shown in smooth muscle cells and fibroblasts [107, 108], which may be due to the ability of TSP-1 to bind and present important growth factors [109]. However, TSP-1 is an inhibitor of angiogenesis and indirectly of tumor progression through binding to CD36 on endothelial cells [110-112]. TSP-1 inhibits angiogenesis through reduction of endothelial cell migration and proliferation and induction of caspase-dependent apoptosis [113]. In addition, TSP-1 plays an important biological role in the activation of TGFβ [114], which is a multifunctional cytokine that is a strong inhibitor of B and T

cell proliferation and differentiation [115]. Both TGFβ- and TSP-1 deficient mice show prolonged persistence of inflammation in lungs [100, 114].

Table 4. List of TSP-1 sequences related to known functions [116]

TSP-1 Domains	Sequences	Receptors	Functions	Reference
Amino-terminal	QNV	α3β1	Cell adhesion to TSP-1. Hemostasis.	[117, 118]
	RKGSGRR/KKTR	HSPG-LRP-gp330 Syndecan Sulfated glycolipid	Endocytosis.	[106, 119-123]
	ELTGAARKGSGR RLVKGPD	Calreticulin Heparin	Focal adhesion disassembly. Inhibition of T cell motility.	[122, 124, 125]
	MKKTRG	Decorin	Inhibits cell adhesion.	[126]
	LDVP	α4β1	Promotes cell adhesion and chemotaxis.	[127, 128]
	LALERKDHSG	α6β1	Endothelial cell binding to TSP-1, TSP-2 and laminin.	[129]
Procollagen domain	NGVQYRN		Inhibition of angiogenesis and chemotaxis	[130]
Type I repeats (1–3)	RFK	LTGFβ	Activation of latent TGFβ	[131, 132]
	WSHWSPW	Protein glycosaminoglycan	Activation of latent TGFβ	[133, 134]
	CSVTCG	HIV gp120 HSPG CD36	Anti-angiogenesis. Cell adhesion.	[135-137]
	GVQxR	CD36	Endothelial cell migration	[138]
Type III repeats (1–7)	RGD	αvβ3 αIIbβ3 α5β1	Cell adhesion	[127, 128, 139, 140]
			Calcium binding	[141]
	NCPFHYNP NCQYVNV	Cathepsin G, elastase		[85]
COOH-terminal domain (CTD)	RFYVVM IRVVM	CD47	Chemotaxis. Cell proliferation. Apoptosis.	[142, 143]

2.3.1.3 Thombospondin-1 and Modulation of Cell Migration

TSP-1 is a regulator of cell spreading and migration and is an adhesive substratum for many tumor cells [144]. However, TSP-1 in a soluble form, generates focal adhesion disassembly and reduced spreading of endothelial cells and fibroblasts, a process mediated by the TSP-1 receptor calreticulin [88, 122, 124, 145, 146]. Whether TSP-1 is presented in a bound or soluble form and in the absence or presence of Ca^{2+} alters its characteristics and function. Adhesion of T cells to intact TSP-1 is sensitive to Ca^{2+} and is reduced in the presence of the Ca^{2+}-ion chelator EDTA [128].

TSP-1 is a chemotactic factor for several different cell types including neutrophils [147] and monocytes [148]. Two regions in the NH_2-terminal domain of TSP-1, the heparin-binding sequence and the $\alpha_3\beta_1$-binding site stimulate chemotaxis as well as the type 1 repeats and the CD47 and $\alpha_v\beta_3$-binding region in the type III repeats and the COOH-terminal domain [149]. TSP-1 has been shown to induce chemotaxis via p38 and ERK1/2 MAP-kinases and pertussis-toxin sensitive G-proteins [150-152].

Through its ability to regulate proteolysis, TSP-1 affects motility of different cell types, including the metastatic properties of tumor cells. Different domains of TSP-1 have opposing effect since a NH_2-terminal fragment of TSP-1 enhanced synthesis of MMP-2 and MMP-9 by endothelial cells and stimulated motility and angiogenesis, whereas a COOH-terminal TSP-1 fragment blocked MMP-2 synthesis and inhibited angiogenesis [153]. However, high concentrations of TSP-1 inhibit angiogenesis [154]. This may

partly be due to the fact that TSP-1 is a potent inhibitor of activation of pro-MMP-9 and activity of MMP-2 [84, 155]. In addition, TSP-1 mediates down-regulation of extracellular MMP-2/TIMP-2 through linking of the complex with LRP, followed by cellular internalization [123].

TSP-1 can also induce expression of uPA/uPAR by squamous cell carcinomas, which promotes their invasion [156]. In contrast, TSP-1 binds plasminogen, plasmin and uPA and directly inhibits the activity of the enzymes, a process that slows down fibrinolysis [157, 158]. In addition, TSP-1 associates with and inhibits neutrophil elastase and cathepsin G [159, 160]. TSP-1 is sensitive to degradation by plasmin, cathepsin G, neutrophil elastase, tPA and thrombin [161, 162].

In conclusion, the different TSP-1 domains have unique properties and cell surface receptors. This contributes to the sometimes inconsistent or cell-type specific responses to TSP-1. Motility regulation by TSP-1 will be further discussed later, in the context of TSP-receptors.

2.3.1.4 Thrombospondin-1 and the Immune System

In cells of the immune system, TSP-1 is expressed by monocytes and T lymphocytes [163, 164] and TSP-1 is found at sites of inflammation [165], in atherosclerotic lesions [166] and in rheumatoid arthritis, which are characterized by infiltration and in situ expansion of lymphocytes. Activated T cells express several cell surface receptors for TSP-1 including $\alpha_4\beta_1$- and $\alpha_5\beta_1$-integrins [128], CD47 [167], LRP and calreticulin [125, 168] through which modulation of several important T cell functions have been reported. Indeed, TSP-1 has been shown to be a co-stimulator of T cells and to induce proliferation via CD47 [103, 169]. In contrast, others have shown that TSP-1 can induce T cell apoptosis or anergy via CD47 and inhibit TCR-mediated activation [170-172]. Recently, TSP-1 was shown to promote the generation of regulatory T cells in response to inflammation [173]. TSP-1 further regulates T cell motility and modulates adhesion to fibronectin and ICAM-1 [125, 163, 168].

A role for TSP-1 in the function of the immune system is further suggested from experiments with TSP-1 gene knockout mice. These mice show decreased embryonic viability, early (1 month of age) onset of pneumonia, increase in circulating monocytes and lymphocytes and impaired wound healing [100]. In addition, TSP-1, TSP-2 and CD47 deficient mice have a prolonged delayed-type hypersensitivity reaction [174].

2.3.1.5 Expression and Function of Thrombospondin-2

TSP-2 is expressed during embryogenesis and in regenerating tissue. It modulates fibroblast adhesion, bone formation and hemostasis and is a potent inhibitor of angiogenesis [175-178]. TSP-2 null mice show accelerated wound healing, prolonged neovascularization and reduced ability to form blood clots, even though TSP-2 is not released by platelets [179, 180]. TSP-2 also regulates the deposition of MMP-2 within the ECM as mice lacking TSP-2 show higher extracellular distribution of MMP-2 [180].

2.3.1.6 Expression and Function of Thrombospondin-3 and -4

Whereas little is known about the biological roles of TSP-3 and TSP-4, they are expressed in human kidney, uterus and muscles (TSP-3) and heart and skeletal muscles (TSP-4) [95, 181].

2.3.1.7 Expression and Function of Thrombospondin-5

TSP-5 is involved in bone formation and is increased in serum and synovial fluid during osteoarthritis and rheumatoid arthritis [93]. It is expressed in human articular cartilage, tendon, synovium and arteries[182, 183].

2.3.2 TSP Receptors

The different domains of TSP-1 display binding sites for various cell surface receptors including the integrins αvβ3, α3β1, α4β1, and α5β1, α6β1, αIIbβ3, CD91, CRT, CD36, CD47 and HSPGs as summarized in Table 4.

2.3.2.1 TSP-1 and Integrins

Different integrins can mediate cell adhesion to NH_2-terminal TSP-1, which has been shown for T lymphocytes via $\alpha_4\beta_1$- and $\alpha_5\beta_1$-integrins and endothelial cells via $\alpha_6\beta_1$-integrin [128, 129]. Binding of TSP-1 to $\alpha_4\beta_1$ may compete with integrin binding to VCAM-1 [127]. Cell adhesion to the RGD-sequence in the type III repeats of TSP-1 occurs in breast carcinoma cells via $\alpha_3\beta_1$-integrin [140] and platelets via αIIbβ3- and $\alpha_v\beta_3$-integrins [100, 139].

2.3.2.2 CD47

Integrin associated protein (IAP) or CD47 is a highly glycosylated ~50 kDa member of the Ig superfamily of plasma membrane proteins with a IgV-like NH_2-terminal extracellular domain followed by five hydrophobic, membrane-spanning domains and a short cytoplasmic COOH-terminal [184]. CD47 is expressed at the surface of all mammalian cells and was first found to associate with αvβ3 on leukocytes [185]. CD47 has also been co-purified with αIIbβ3 on platelets [186], with α4β1 on T cells [187] and α2β1 on smooth muscle cells and platelets [188, 189], implicating a co-modulatory role of CD47 on several integrins.

CD47 has two natural ligands from the signal regulatory protein (SIRP) family of proteins (SIRPα and SIRPγ). SIRPα is expressed at the surface of macrophages, dendritic and endothelial cells and contains cytoplasmic ITIM-motifs that inhibit tyrosine kinase-coupled activation upon phosphorylation [190]. SIRPγ lacks the ITIM-motifs, is expressed on peripheral blood leukocytes and in brain, lung and liver and mediates T cell costimulation and cell-cell adhesion [191, 192].

The CD47-binding site of TSP-1 is the VVM-motif (part of the 4N1K-peptide) in the TSP-1 COOH-terminal domain and ligation of CD47 with the **4N1K-peptide in solution** enhances α2β1-dependent smooth muscle cell and lymphocyte motility in collagen type I [125, 189] but inhibits T cell adhesion to inflamed endothelium, FN or ICAM-1 [168, 193].

When **coated on plastic or together with an adhesive substrate** 4N1K enhances adhesion and spreading [169, 193-195]. CD47 thus requires cross-linking for strengthening of cell adhesion. The cytoplasmic domain of CD47 contains no known signaling motifs but probably signals through association with heterotrimeric Gi-proteins, since pertussis toxin inhibits CD47-induced integrin-dependent cell spreading, chemotaxis and platelet activation [196].

Mice that lack CD47 have a defect in host defense due to impaired phagocyte activation and decreased number of circulating and spleen-residing T lymphocytes [197]. This may be explained by the co-mitogenic role of CD47 in T cell activation [198] also shown by the capacity of coated CD47-antibodies to induce spreading of T cells and thus enhance activation [194] or the ability of TSP-1 to activate $\alpha4\beta1$-integrins (part of the immunological synapse) via CD47 [193]. However, the role of TSP-binding to CD47 in regulating T cell activation is obscure and several reports have documented inhibition of activation-induced proliferation and induction of caspase-independent T cell death, either by using anti-CD47 antibodies or the 4N1K-peptide. As shown in CD47- and TSP-1 or TSP-2-deficient mice, TSP-1 mediates apoptosis of T cells via CD47 and thus clear inflammation [174]. Anti-CD47 antibodies with different CD47-binding epitopes, **co-immobilized with anti-CD3 antibodies**, which generates cross-linking of CD47, have either increased proliferation (antibody-clones B6H12, BRIC126, 1/1A4), not affected proliferation (clone 2D3) or induced apoptosis (clone Ad22) [172, 198, 199]. When antibodies to CD47 (clones B6H12, IF7, 2D3, 1/1A4), intact TSP-1 or the 4N1K peptide have been used **in solution**, apoptosis, anergy, inhibition of mixed lymphocyte reaction (MLR) and induction of Tregs have been reported [170, 173, 199, 200]. CD47 thus appears to require cross-linking for its mitogenic effects. The anti-proliferative and apoptotic effects of soluble TSP-1 or 4N1K may be a result of inhibition of T cell arrest and destabilization of the immunologic synapse.

2.3.2.3 LRP

LDL receptor-related protein or CD91 is a large cell-surface glycoprotein consisting of two non-covalently associated fragments; the ~515 kDa NH_2-terminal, extracellular α-subunit and the ~85 kDa COOH-terminal, β-subunit [201]. Expressed by most cell types, it mediates endocytosis and lysosomal degradation of more than 30 different ligands and regulates lipid metabolism and homeostasis of proteases, bacterial toxins, matrix proteins and growth factors [202]. Only to mention a few, LRP controls levels of uPA/PAI-1 [203], TSP-1 [121], fibronectin [204], MMP-2 [123] and MMP-9 [205]. LRP moves laterally within the cell membrane and can be found in lipid rafts, where ligand binding either generates direct signaling or enables signaling through cell surface molecular complexes via integrins or uPAR [206]. In fact, LRP co-localization or association with β_2-integrins is a prerequisite for β_2-integrin-mediated leukocyte adhesion [207] and LRP contributes to the maturation and cell surface expression of the β_1-integrin subunit and as such plays a role in CHO-cell binding to fibronectin [208].

The 35 kDa molecular chaperone receptor-associated protein (RAP) binds LRP and prevents all known ligand binding to LRP during its passage through the secretory pathway [209]. In addition, RAP is a useful tool for studies of LRP-ligand interactions

and in the case of TSP-1, RAP inhibits internalization of NH_2-terminal TSP-1 through LRP but does not prevent binding of TSP-1 to the cell surface, which occurs through multiple receptors [210-212]. In non-lymphocytic systems, internalization of cell surface uPAR (induced by addition of uPA-PAI-1 complex) was blocked with RAP, which caused an upregulation of migration capacity due to increased levels of uPAR [213].

Through ligand binding or internalization, LRP indeed participates in the regulation of a vast array of events including cell migration, proliferation and vascular permeability [202]. The cytoplasmic COOH-terminal domain of LRP contains two NPxY-motifs, which enable interaction with cytoplasmic adaptor proteins including Shc, Disabled and Fe65 involved in cell signaling through LRP. TSP-1 signals focal adhesion disassembly in endothelial cells through calreticulin and LRP via pertussis toxin-sensitive G-proteins, extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K), which has a negative effect on integrin affinity and cell adhesion but stimulates cell migration [214, 215].

2.3.2.4 Calreticulin

Calreticulin (CRT) is an 46 kDa calcium-binding protein in the endoplasmic reticulum (ER), where it plays different roles as a chaperone and regulator of calcium homeostasis [216]. Interestingly, although CRT lacks a plasma membrane domain, it is found at the surface of many cell types and forms complexes with integrins and ECM proteins such as fibrinogen and laminin [217-220]. At the cell surface, CRT regulates cell adhesion, inhibits angiogenesis and suppresses tumor growth [221]. Interactions of the NH_2-terminal domain of TSP-1 (Hep-1 sequence) in a soluble form with the NH_2-terminal domain of cell surface CRT and its co-receptor LRP has primarily anti-adhesive effects characterized by activation of signaling cascades that generate reorganization of stress fibers and loss of focal adhesion plaques [124, 145, 214]. A role for calreticulin in the immune system is shown in calreticulin-deficient CTLs, which have reduced cytolytic activity and capacity to form a death-synapse [222].

2.3.2.5 CD36

CD36 is a 88 kDa scavenger receptor mainly expressed by platelets, monocytes and capillary endothelial cells, that binds and internalizes oxidized LDLs and fatty acids [223]. TSP-1 binds CD36 via the CSVTCG and GVQXR sequences in the TSP-1 type I repeat domain [110, 138] and mediates aggregation of platelets [224] and binding of platelets to monocytes or endothelium and thus contributes to the activation of platelets and monocytes but inhibits endothelial cell migration and angiogenesis [110, 130]. CD36 is also a receptor for collagen and is important for platelet adhesion to collagen [225].

2.3.2.6 Heparan sulphate proteoglycans

TSP-1 binds heparan sulfate proteoglycans (HSPGs), heparin and sulfated glycolipids through distinct motifs in the NH_2-terminal domain [119, 121, 122] and the type I repeat domain [133, 134]. Simultaneous binding of TSP-1 to HSPGs and LRP

facilitates the endocytosis of TSP-1 [121] and heparin blocks the endocytosis of TSP-1 [226].

2.4 ADHESION MOLECULES

In order for lymphocytes to bind firmly to the endothelium, several different adhesion molecules are expressed by lymphocytes and endothelial cells.

2.4.1 Selectins and Mucin-Like Molecules

The extravasation process starts when lymphocytes are slowing down and begin to roll on the endothelium. This initial contact is mediated by transmembrane glycoproteins called selectins that bind to sialylated carbohydrate-rich ligands. Lymphocytes express L-selectin that binds to the corresponding ligands on the endothelium of lymphnodes; GlyCAM-1, MAdCAM-1 or CD34, which are all highly glycosylated. Other selectins, important for leukocyte recruitment, are E-selectin, expressed on activated endothelial cells and P-selectin expressed on platelets and activated endothelial cells. E- and P-selectin bind to P-selectin glycoprotein ligand (PSGL-1) expressed on neutrophils, monocytes and lymphocytes [227].

2.4.2 Integrins

The major mediators of lymphocyte-endothelial interaction are the integrins and their receptors. The integrin family of heterodimeric cell surface receptors consists of to date 18 α-subunits and 8 β-subunits that form 24 different non-covalently linked αβ-pairs (**Figure 4**) [228].

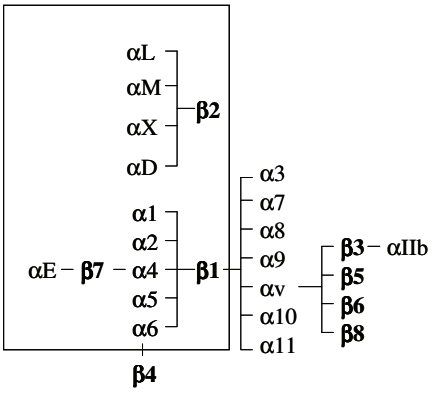

Figure 4. Integrin family of receptors. Possible combinations of α- and β-subunits. Integrins within the box are particularly relevant to T cell functions.

The integrin α- and β-chains are each constituted by a globular extracellular domain with several metal ion binding sites that coordinate the ligand within the ligand-binding domain. Both chains have an intracellular domain connected to the cytoskeleton via talin and α-actinin, and to signaling linker proteins such as and focal adhesion kinase (FAK).

T cells express integrins of the β1, β2 and β7 sub-families where α4β1 and α5β1 mediate T cell adhesion to VCAM-1 on endothelial cells (α4β1 only) and the ECM component fibronectin (both) [229]. The β2-integrin αLβ2 binds ICAM-1 and ICAM-2 on the endothelium or in the immunological synapse [230]. T cell adhesion and motility is further regulated via α1β1, α2β1 and α6β1 receptors for collagens and laminins [231,

232]. α4β7 binds MAdCAM-1 expressed on HEVs in endothelial lymph nodes and in the lamina propria of the gut wall. Another ligand for α4β7 is VCAM-1.

The major part of the integrins present on resting T cells is not constitutively active and has a bent, membrane proximal conformation, preventing ligand binding. However, on activated lymphocytes a small pool of integrins bind ligand with low **affinity** in each single integrin pair but with a moderate overall binding capacity or **avidity** (the collected capacity of all integrin molecules). Affinity stimulation can be obtained chemically with Mg^{2+} or Mn^{2+} and does not depend on intracellular signals [233]. **"Inside-out"** signaling through the TCR increases integrin avidity/affinity via protein kinase C (PKC) and increased intracellular Ca^{2+} and thus enhances lymphocyte interaction with APCs. Chemokines enhance integrin avidity through Gi-stimulated receptors during rolling of T cells on activated endothelium and depend on lateral movement and clustering of integrins within the membrane in caveolin-rich domains [233, 234]. **"Outside-in signaling"** occurs when integrins become clustered on the membrane, connect to the cytoskeleton and generate signaling platforms while binding ligands. Ligand binding by αLβ2 generates signals through tyrosine phosphorylation of PLCγ1 and activation of the tyrosine kinases ZAP-70, Pyk-2 and FAK. Ligation of β1 integrins leads to signals through the activation of the serine phosphorylating PKC, Lck (protein kinase) and Shc [50]. Both events increase cellular adhesion to respective ligand. In addition, there is evidence for integrin crosstalk since activation of α4β1 induces transactivation of αLβ2, possibly in a uPAR-dependent manner [235, 236].

2.4.3 Others

Members of the Immunoglobulin (Ig) superfamily of receptors all share an immunoglobulin domain and include several different categories of cell surface receptors. Important examples are the antigen receptors of B cells and T cells, MHC class I and II, some co-stimulatory receptors such as CD28 and CD80 as well as several adhesion molecules expressed by endothelial cells. The intercellular adhesion molecules (ICAMs) ICAM-1, ICAM-2 and ICAM-3 as well as the vascular adhesion molecule (VCAM-1) recruit T cells to lymph nodes and inflamed tissue through binding to T cell integrins. The L-selectin ligands glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and CD34 are expressed in HEVs. The mucosal vascular adressin cell adhesion molecule-1 (MAdCAM-1) is expressed on HEVs in Peyer's patches and mediate lymphocyte homing to gut mucosa through binding to α4β7 [237].

2.5 CHEMOKINES AND THEIR RECEPTORS

Chemokines are chemotactic cytokines that modulate and direct lymphocyte adhesion and migration [24]. Chemokines are classified into four families based upon the configuration of cystein residues in the N-terminal part of the molecule (CC-, CXC, C- and CX3C).

Functionally, chemokines can be separated into three main categories: **1) inflammatory, 2) homeostatic and 3) multiple-function chemokines.** Inflammatory chemokines such as **CCL5/RANTES** (receptors CCR1, 3 and 5), **CXCL10/IP-10** (receptor CXCR3) and **CXCL8/IL-8** (receptors CXCR1, 2) are produced during an

immune response by leukocytes, tissue cells and local endothelial cells and serve to attract cells of both the innate and adaptive immune system. Effector CD4+ T cells express CXCR5 that enables recruitment to LN-follicles where B cells express **CXCL13/BLC**.

Homeostatic chemokines are constitutively expressed and control the movement of leukocytes between and within lymphoid and peripheral non-inflamed tissues and contribute to immune surveillance by memory T cells. For example are **CCL19/ELC** and **CCL21** (receptor CCR7 on naive T cells and resting memory T cells) and **CXCL12/SDF-1** (receptor CXCR4, widely expressed on T cells) produced by lymphatic endothelial cells and recruit T cells through HEVs. Naive or resting T cells do not produce chemokines, whereas activated effector or memory T cells express several inflammatory chemokines and thus contribute to lymphocyte recruitment [23]. Lastly, chemokines that are inflammatory as well as homeostatic are called multiple-function chemokines [22]. One example is thymus-expressed chemokine **(TECK)/CCL25** that binds CCR9 on thymocytes as well as effector T cells and recruit bone-marrow CD34+ cells to the thymus and inflammatory T cells to the gut.

Chemokines deliver their signals through G- protein coupled seven trans-membrane spanning receptors (7TMR). Recruitment of T cells to LNs or inflammatory tissues is enhanced via chemokine-binding to chemokine receptors, which generates ("outside-in") signaling and results in necessary integrin affinity/avidity changes ("inside-out" signaling). Signals mediated through chemokine receptors generate rapid RhoA, FAK, MAPK, JAK/STAT and PI3K-activation [56]. Chemokines and cytokines may also indirectly regulate T cell motility through regulation of expression of MMPs. This is exemplified by IL-2 and IL-4 induced expression of MMP-9, MMP-2 and CCR3, the eotaxin chemokine receptor, which results in enhanced T-cell migration in response to eotaxin [27, 238]. In addition, RANTES upregulates MMP-9 expression as well as infiltration measured as asthmatic airway homing [239, 240].

2.6 PROTEASES

Leukocytes express a number of different enzymes involved in degradation of ECM-proteins, chemokines, clotting factors and immunologically important substances such as immunoglobulins and complement components [23, 241].

Enzymes are classified according to a numerical classification scheme and each enzyme is given an Enzyme Commission number (EC number), based on the chemical reaction it catalyzes. There are 6 main EC-groups and EC 3 denotes the **hydrolases**. These are enzymes that catalyze the hydrolysis of a chemical bond in the presence of water. The hydrolases are further subdivided into several subclasses, based upon the bonds they act upon and EC 3.4 describes enzymes that cleave peptide bonds (Proteases/peptidases). Proteases found in lymphocytes belong to the following EC 3.4 subclasses, see **Table 5**.

T lymphocytes express mainly dipeptidyl peptidase IV (CD26), the serine proteinases granzyme A and B, urokinase plasminogen activator (uPA) as well as the matrix metalloproteinases MMP-2 and –9 [29, 241].

Table 5. Proteases (EC 3.4) of different subclasses found in lymphocytes

Subclass EC number	Examples, EC number and name	References
3.4.14 Dipeptidyl-peptidases and exopeptidases	3.4.14.1 **dipeptidyl-peptidase I** (Cathepsin C) 3.4.14.5 **dipeptidyl-peptidase IV** (CD26)	[242-244]
3.4.21 Serine endopeptidases	3.4.21.37 **leukocyte elastase** 3.4.21.73 **u-plasminogen activator (uPA)** 3.4.21.78 **granzyme A** 3.4.21.79 **granzyme B**	[245-247]
3.4.22 Cysteine endopeptidases	3.4.22.1-43 Several **cathepsins** 3.4.22.52 and 53 **calpain-1 and -2**	[248, 249]
3.4.24 Metalloendopeptidases	3.4.24.35 **gelatinase B** 3.4.24.24 **gelatinase A**	[250, 251]

In addition, lymphocytes express heparanase, a glycosylase which belongs to the EC 3.2 family of hydrolases. Heparanse modifies heparan sulfate (HS) which interacts with the major ECM-components and alters their solubility, assembly and turnover [252]. Inhibitors of heparanase have been shown to reduce experimental inflammatory disease and leukocyte diapedesis [253]. Here follows a description of the MMPs and the serine proteases uPA, granzyme A and granzyme B.

2.6.1 Matrix Metalloproteinases

MMPs have the capacity to degrade various components of the ECM such as collagens, laminins and fibronectin. There is also evidence for MMP regulation of cell surface receptors such as ICAM-1 [254], L-selectin [34] and FasL [255], and in addition, certain chemokines and cytokines are subjected to activation or inactivation via MMP activity [256-258]. The family of MMPs consists of 24 structurally related members that exist in secreted or membrane bound forms. **Table 6** summarizes the MMP-family [259].

The MMP family comprises **collagenases** (MMP-1, -8, -13, -18), **stromelysins** (MMP-3, -10, -11, -19, -20), **gelatinases** (MMP-2, -9) and **membrane metalloproteinases** (MT-MMP-1-6). They all share several common features such as having a Zn-dependent active site and endopeptidase activity. They are expressed as zymogens and thus require activation to exert their function. The MMPs display a basic domain structure, which comprises a signal peptide, a propeptide domain, a catalytic domain, a hinge region and a COOH-terminal “haemopexin-like” domain. The gelatinases are distinguished by the presence of a fibronectin-like region within their catalytic domain, whereas the membrane-type MMPs are characterized by a COOH-terminal transmembrane portion.

Table 6. Family of MMPs and some known substrates.

Enzyme	MMP	Substrates
Collagenases		
Interstitial collagenase	MMP-1	collagen type I-III, VII and X, gelatin, VN, LN, α2-M, proMMP-2, -9, proTNFα
Neutrophil collagenase	MMP-8	collagen type I-III, VII and X, α2-M
Collagenase-3	MMP-13	collagen type I-III, VII and X, FN, proMMP-9, α2-M
Gelatinases		
Gelatinase A	MMP-2	gelatin type I IV V and X, laminin V, fibronectin, elastin, TGF-β, CCL7, CXCL12 IL1-β, proTNFα, proMMP-1, proMMP-2, proMMP-9, proMMP-13,
Gelatinase B	MMP-9	gelatin type I IV V and X, laminin V, fibronectin, elastin, TGF-β, α2-M, IL1-β, proTNFα, plasminogen, proTGFβ, IL-2Rα , ICAM-1
Stromelysins		
Stromelysin-1	MMP-3	collagen type III, IV, IX and X, gelatin, laminin, α2-M, IL1-β, proTNFα, fibrinogen, plasminogen, proMMP-1, proMMP-3, proMMP-7, proMMP-8, proMMP-9, proMMP-13, uPA
Stromelysin-2	MMP-10	collagen type III, IV, IX and X, gelatin, laminin, proteoglycan, proMMP-1, proMMP-7, proMMP-8, proMMP-9
Stromelysin-3	MMP-11	gelatin, fibronectin, collagen IV, α2-M, PAI-2
Matrilysins		
Matrilysin-1	MMP-7	collagen type I and IV, gelatin, fibronectin, proMMP-1 α2-M, proTNFα, proMMP-1, proMMP-2, proMMP-7, proMMP-9, plasminogen, FasL
Matrilysin-2	MMP-26	collagen type I and IV, gelatin, fibronectin, proMMP-9
Membrane-type (MT) MMPs		
MT1-MMP	MMP-14	pro-MMP-2, gelatin, collagen type I, II, and III, gelatin, fibronectin, CD44, proTNFα, α2-M
MT2-MMP	MMP-15	pro-MMP-2, fibronectin, proTNFα
MT3-MMP	MMP-16	collagen type III, gelatin, fibronectin, proMMP-2, α2-M
MT4-MMP	MMP-17	gelatin, proMMP-2, proTNFα
MT5-MMP	MMP-24	gelatin, fibronectin, proMMP-2
MT6-MMP	MMP-25	collagen type IV, gelatin43,44, fibronectin, proMMP-2
Others		
Macrophage elastase	MMP-12	collagen type I, V, and IV, gelatin, elastin, fibronectin, α2-M
Enamelysin	MMP-20	amelogenin

Regulation of MMP expression and function is thought to take place on at least three different levels:

1) At the level of transcription, the metalloproteinase genes are positively regulated by a variety of biologically active agents such as hormones, growth factors, oncogenes, tumor promoters and cytokines, but a negative regulation is also exerted by for example steroids and TGFβ [25, 260-262].

2) Activation involves cleavage of the N-terminal propeptide domain by furin, MT1-MMP, plasmin or by a number of other enzymes [263]. This mechanism is known as "the cysteine switch" [264].
3) The degradative action of MMPs is further limited by specific tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobin (α2-M). The TIMPs can inhibit the action of MMPs on two levels; through delaying or preventing the conversion from latent zymogen to active enzyme or through inhibition of the catalytically active form of the enzyme [75, 265]. An inhibitor of physiological importance is α2-M, which is a large plasma glycoprotein that entraps and inhibits most proteinases that subsequently become cleared by LRP-mediated endocytosis [266].

At present, four TIMPs have been described: TIMP-1, -2, -3 and –4. They are relatively small molecules (21-28,5 kDa), which bind MMPs non-covalently in binary complexes. TIMP-1 is a 28kDa protein that inhibits the active form of several MMPs [267]. TIMP-2 is a 21 kDa protein that in high concentration inhibits the activation of MMP-2 but is also vital for the activation of MMP-2. TIMP-2 binds to MMP-2/ MT1-MMP in a ternary complex at the cell surface. Complex-bond MMP-2 can then be inhibited by TIMP-2 or activated through the activity of free MT1-MMP close to the ternary complex. This system provides localized degradation of ECM components and is important for cell migration [268, 269]. TIMP-3 is a matrix-associated 24 kDa protein capable of inhibiting the proteolytic activity of mainly MMP-3 [268, 270]. TIMP-4 is a 24 kDa protein expressed mainly in the heart [268, 271].

The different MMPs and TIMPs play overlapping, complex and sometimes even contradictory functional roles in normal and pathogenic events such as angiogenesis, embryogenesis, wound healing, connective tissue remodeling, regulation of apoptosis, inflammation, arthritis and tumor metastasis [267]. MMPs might reduce inflammation through increased bioavailability of TGFβ and inactivation of IL-1β but may also contribute to tissue destruction in inflammatory processes [259]. The MMP-2 null mouse shows decreased allergic inflammation but more severe immune-mediated arthritis, whereas the MMP-9 null mouse has less severe experimental arthritis and prolonged contact hypersensitivity [272, 273]. TIMPs have been assumed to prevent tumor metastasis through inhibition of the matrix degrading activities of MMPs [268, 274-276]. However, high serum levels of TIMP-1 in patients with various cancers is correlated with poor outcome [277-279] and TIMP-1 may act as a suppressor of apoptosis [280, 281].

Naive T-cells express mRNA for MMP-9, MMP-2, TIMP-1, and TIMP-2 and TCR-, or CD3 activation upregulates MMP-9 [282, 283].

2.6.1.1 MMPs and Lymphocyte Motility

There is evidence that ECM-degrading enzymes play a role for the capacity of cells of the immune system to penetrate basement membranes and migrate within three-dimensional model substrata and tissues. Expression of active MMP-9 and MMP-2 by different leukemia cell lines correlates with ability to infiltrate a three-dimensional ECM [28, 284, 285] and β1-integrin mediated T-cell adhesion to FN or VCAM-1 upregulates MMP-2 and/or –9 [250, 286-288]. Cytokines such as IL-2 and certain chemokines positively regulate the expression and secretion of MMPs and TIMPs in cells of the immune system with a concomitant induction of migration [240, 289]. In

some studies, the use of MMP-inhibitors have reduced the number of T-cells migrating through a 3D ECM [25, 28]. However, in a mouse model of experimental autoimmune encephalomyelitis (EAE), a synthetic inhibitor of MMPs reduced induction and progression of the disease without affecting the number of infiltrating inflammatory cells [290]. In addition, MMP-9 deficiency did not affect the number of infiltrating T cells in a IL-13-induced mouse model of inflammation of the lung [291]. It was further found that lymphocytes moving through 3D collagen type did not create a lytic path, which challenged the idea of protease-dependent lymphocyte motility [292].

Although accumulated evidence indicate a role for MMPs in T cell extravasation, mechanisms are still unclear and may vary depending on T cell subtype, level of activation, position within the body (e.g. in a lymphoid organ or in an inflamed or non-inflamed tissue), properties of endothelial cells (e.g. HEV or venules in inflamed or non-inflamed tissue) and composition of the adjacent ECM (major constituents vary in different sites and organs). MMPs may either be directly or indirectly involved in the migratory process. Mechanisms for MMP-regulation of T-cell migration need to be further elucidated.

2.6.2 Other Proteases

2.6.2.1 Serine endopeptidases

Urokinase plasminogen activator, uPA

uPA is synthesized *in vivo* as a single, 55 kDa polypeptide chain, sc-uPA with low catalytic activity that can be activated through cleavage by plasmin, trypsin and granzyme A. Cleavage generates two different forms of active uPA (55 and 32 kDa) [293]. uPA is a key enzyme of the fibrinolysis system and converts plasminogen to active plasmin, which degrades polymeric fibrin and generates dissolution of intravascular thrombi. However, other important substrates for plasmin are components of the ECM such as fibronectin and gelatin and proMMPs, which gives uPA an indirect role in lymphocyte adhesion and migration within tissues [294]. uPA is expressed by endothelial-, epithelial- and smooth muscle cells as well as by lymphocytes.

Cell surface localization of uPA depends on its receptor uPAR, which is a 55 kDa glycoprotein that is anchored to the cell membrane through a glycosyl phosphatidylinositol (GPI) moiety and thus is included in caveolin-rich domains of the membrane. Binding of active uPA to uPAR protects the enzyme from further degradation and provides lymphocytes with a surface-bound proteolytic machinery that promotes motility [295]. In addition, β1- and β2 integrins associate with uPA/uPAR in *cis* and interdependently regulate cell adhesion or migration through signals that affect cytoskeletal reorganization [296]. LRP internalizes the uPA/uPAR complex together with the natural inhibitor of uPA, plasminogen activator inhibitor-1 (PAI-1) and may thus regulate proteolysis and uPAR-dependent integrin signaling [201].

Granzyme A and B

Granzyme A is a 50 kDa dimer with trypsin-like activity that cleaves pro-uPA, fibronectin and collagen type IV as well as intracellular substrates involved in apoptosis [297]. Granzyme B is a 32 kDa Asp-ase known to cleave several pro-caspases, vitronectin, fibronectin and laminin and is expressed also in dendritic cells, breast

carcinomas, migrating immature thymocytes as well as re-activated memory CD4+ T cells [298].Granzymes are stored in lytic granulae of CTLs, NK cells and Tregs together with perforin and induce target cell apoptosis during granulae-mediated cytotoxic killing [246]. However, cleavage of extracellular substrates may indicate a role for granzymes other than target cell killing. Cell surface binding of granzyme B may be mediated by HS or the cation-independent receptor mannose-6-phosphate receptor (CI-MPR) which is expressed on lymphocytes [299]. Granzyme B has been shown to generate de-adhesion of endothelial cells and to inhibit tumor cell migration and invasion in vitro [298].

3 AIMS

T lymphocyte adhesion and motility depends on integrin expression and is a tightly regulated process with high complexity, influenced by several environmental as well as endogenous factors such as the lymphocyte level of maturation, characteristics of the surrounding ECM and the presence of proteases. The specific aims of this thesis were as follows:

Paper I.
Where we aimed to elucidate the expression of MMPs and their inhibitors in different leukemia cell lines and possibly correlate the pattern of MMP expression to adhesive and infiltrative capacity.

Paper II.
We wanted to study the role of endogenously expressed TSP-1 bound to its receptors in T cell motility in collagen type I.

Paper III.
Where we investigated the importance of TSP-1 and granzyme B cleavage of TSP-1 for T lymphocyte motility.

Paper IV.
We aimed to study the role of TSP-1 mediated *cis* receptor communication for lymphocyte adhesion to FN and ICAM-1.

4 METHODOLOGY

A brief description of methods used in paper I-IV follows.

4.1 CELL LINES AND PURIFICATION OF T CELLS

The following T cell lines were purchased from American Tissue Type Collection (ATCC): P 30, CCRF CEM, Molt 4, Jurkat, CCRF HSB2, Peer (all acute lymphoblastic leukemias) and HuT 78 (Sezary's syndrome T cells). Peripheral blood lymphocytes were purified using Lymphoprep density gradient separation followed by treatment with carbonyl iron and magnetic removal of phagocytic cells. A birch-pollen (Bet v 1) specific T cell clone, AF24, as well as peripheral blood lymphocytes were stimulated with anti-CD3 or specific antigen and cultured in RPMI1640 (Gibco Ltd, Scotland) supplemented with 10% FCS, IL-2 and IL-4 for 36-48h before the experiments.

4.2 RTPCR AND DNA-SEQUENCING

Cells were lysed followed by extraction of mRNA with phenol-chloroform [300] and cDNA was obtained with Random Hexamers (Perkin Elmer). PCR was run with primer pairs to various MMPs and TIMPs (manufactured by CyberGene AB, Sweden) as presented in Paper I. The amplification product was separated on a 1,5% agarose gel and detected with ethidium bromide.

The PCR products obtained with TIMP-1, -2 and -3 primer pairs were excised from the agarose, extracted with QIAEX II (Quiagen) and ligated into the pT7Blue T-vector, which was subsequently inserted into NovaBlue competent cells (Blue T-Vector Kit, Novagen). Plasmids were purified (Plasmid Midi Kit, Quiagen) and the sequence reaction run with Cy5 labeled upstream T7 primer and downstream U-19mer primer for TIMP-1, -2 and -3. Samples were analyzed in an A.L.F. Automated DNA Sequencer (Pharmacia Biotech). cDNA obtained using primers for MMP-1, -2, -3, -9 and -10 was sequenced with the method ABI PRISM Dye Terminator Cycle Sequencing (Perkin Elmer) by Cybergene AB, Sweden. Sequence data revealed specific amplification by each primer pair.

4.3 ADHESION ASSAY

Plastic petri dishes were coated with ICAM-1 (2µg/ml), BSA (10 µg/ml), PLL (10 µg/ml), the 4N1K-peptide (50 µM) or fibronectin (10 µg/ml) over night at 4ºC and washed with PBS before use. T cells in serum-free AIM-V medium ((10,000/position) were added in five fixed positions and allowed to adhere to the substrates for 30 minutes before gentle aspiration and fixation in cold 2,5% glutaraldehyde. Adhesion was presented as mean number of adherent cells/HPF (200x magnification) in the presence of brefeldin A, TSP-1, TSP-1-mimicking or adhesion-blocking peptides (see **Table 7** for description of peptides), RAP, enzyme inhibitors or antibodies. This experimental setting further allowed evaluation of the ratio of longest/shortest cell diameter which described the elongation or polarized spreading of T cells. 10 cells in triplicate fields were measured and longest and shortest diameter in each cell was determined. A polarized cell had a higher ratio of longest/shortest cell diameter than a non-polarized cell. Another parameter was % spread cells, which described the overall

percentage of spread cells, including cells with both non-polarized and polarized spreading.

Table 7. Short peptides corresponding to sites in CRT and TSP-1.

Peptide name	*Site of recognition*	*Sequence*
CRT19-36*	TSP-1 binding site in **CRT**	RWI ESK HKS DFG KFV LSS
Hep-1*	CRT binding site in **TSP-1** (aa 17-35)	ELT GAA RKG SGR RLV KGP D
Hep-2*	Control peptide 1	RSK A**S**T LGE RDL KP**S** A**A**V G
Scrambled Hep-1*	Control peptide 2	RSK A**G**T LGE RDL KP**G** A**R**V G
4N1K#	CD47-binding site in TSP	KRF Y**VV** MWK K
mutated 4N1K#	Control peptide 3	KRF Y**GG** MWK K
scrambled 4N1K#		KVF RWK YVM K

* From the Biomolecular Resource Facility, University of Lund, Sweden

From Tripep, Novum Research Park, Huddinge, Sweden

4.4 MIGRATION ASSAY

The Boyden chamber assay is a two-chamber system where lymphocytes (1-2x10^6 cells/ml) were added to the upper chamber on top of a polycarbonate filter containing 8 µm pores. The filter separated the chambers and was coated on the lower side with fibronectin, collagen type IV or laminin at 10-20 µg/ml. The upper and lower chambers contained medium with or without serum and cells were allowed to migrate for 4-5 h through the pores and attach to the lower, coated side of the filter. Adherent cells on the filter were fixed in methanol, stained with Giemsa and counted using a light microscope with magnification x200. Migration was determined as mean number of migrated cells/HPF in triplicate wells.

4.5 INFILTRATION ASSAY

We have used infiltration assays with Matrigel and collagen type I. For the Matrigel assay, a 1:1 dilution of Matrigel in serum-free RPMI ± inhibitors of MMPs RO 31-9790 (1-20 µg/ml) and 1, 10-phenantroline (2mM), was allowed to polymerize in 24-wells Falcon cell culture plates. Cells were added on top of the gel and assays were run in the presence or absence of the synthetic MMP-inhibitors for 24h. Cells were fixed with 2,5% glutaraldehyde and the number of cells infiltrating different depths of the gel in 16 or 17 fixed positions in each well (single or duplicate wells) was determined using an inverted microscope (Axiovert 100) at 200x magnification and a digital depth-meter (Heidenhain ND221). Infiltration was given as mean number of cells/cm^2±standard error of mean (SEM).

Infiltration was also evaluated in gels with polymerized rat tail collagen type I prepared as previously determined [301] where the collagen was diluted is serum-free RPMI and water (8:1:1) and was allowed to polymerize in room temperature. Cells in AIM-V medium were added to the collagen and were allowed to infiltrate for different times (30 minutes to 2h). The cells were fixed as in the Matrigel assay and infiltration determined in 9 randomly chosen positions in each well. Data was presented as mean number of cells/HPF (200x or 400x magnification), as mean depth of migration/HPF (200x or 400x magnification) or as mean number of infiltrating cells/HPF per

infiltration depth, which is 50 µm for the layer immediately beneath the gel surface and subsequently at 100 µm levels further down (200x magnification).

4.6 FACS

Cells in suspension remained untreated or were treated with SDF-1α for 30 minutes and subsequently blocked with goat-γ-globulin (3 µl/10^6 cells) or centrifuged through a layer of FCS. A primary antibody (mouse-anti-human IgG) was added to the cells in FACS-buffer (Tris-Hank's, 0,2% HSA, 0,02% NaN_3) and after 30 min incubation on ice, followed by a wash, the secondary antibody (FITC-conjugated goat-anti-mouse) was added to the cells. After 30 minutes on ice, cells were washed and analyzed in a FACScan (BD Biosciences) with appropriate negative (mouse IgG) and positive (mouse anti-human-CD3) control antibodies.

4.7 DETECTION OF ENZYMATIC ACTIVITY

4.7.1.1 Zymography

Serum-free conditioned medium was obtained from cells during 24h and freeze-dried aliquots were dissolved in non-reducing sample buffer (2% SDS) and separated on 10% polyacrylamide gels including 2 mg/ml gelatin, 0,25 mg/ml fibronectin or collagen type IV, 4 µg/ml plasminogen or 0,5 mg/ml TSP-1. Gels were washed extensively with Triton X-100 to remove the SDS and incubated at 37ºC in a buffer with glycine, $ZnCl_2$ and $CaCl_2$ for 24-72h. Staining of the gel with Coomassie brilliant blue revealed clear bands in a blue background where enzymatic activity was visualized.

4.7.1.2 Cleavage of a Chromogenic Substrate

Washed PBTs were aliquoted in 96-well optical well plates (Nunc) in the presence or absence of the granzyme B inhibitor Ac-IETD-CHO (50µM) or the general caspase inhibitor Z-VAD-FMK (100µM). A granzyme B and caspase-8 specific fluorogenic substrate was added and cleavage of the substrate measured at the start of the experiment and after 3h 30 minutes. Granzyme B activity was measured as a raise in mean RFU over time.

4.7.1.3 In Vitro *Cleavage Assay, Western blot and NH_2-terminal Sequencing*

TSP-1 was mixed with granzyme A or granzyme B at increasing enzyme:substrate ratios in a buffer containing 200 mM Tris-HCl, 0,1% CHAPS and 5 mM EDTA. After 15 h at 37ºC, reducing sample buffer was added and samples separated on a 4-12% gradient gel. Proteins were transferred to a nitrocellulose membrane, blocked with BSA and incubated with antibodies to TSP-1 (clone C6.7/Ab-3 and A6.1/Ab-4). ECL Western Blotting Detection Reagents (Amersham Biosciences) enabled detection of TSP-1. TSP-1 specific antibodies used in the studies are presented in **Table 8**.

Table 8. Antibodies to TSP-1, from NeoMarkers, CA

Clone	*Site of recognition*
TSP Ab-3/C6.7	Type II EGF repeats 1 and 2 [302]
TSP Ab-4/A6.1	Ca2+-binding domain aa 692-717 [302]
TSP Ab-9/MCB200.1	Heparin-binding domain

For NH_2-terminal sequencing, cleaved TSP-1 was blotted onto a PVDF-membrane and proteins stained with 0,1% Coomassie brilliant blue. NH_2-terminal sequencing of the first 20 aa of the 115 kDa band was performed by Edman degradation at the Protein Analysis Center, Karolinska Institute, Sweden.

4.8 IMMUNOCYTOCHEMISTRY

The cell surface expression of different antigens was determined by quantitative immunocytochemistry of cells after 15 minutes in collagen gels or adhering to glass-slides coated with PLL (10 µg/ml), fibronectin (10 µg/ml), ICAM-1 (2 µg/ml). Cells were fixed in 2% paraformaldehyde where after the expression of TSP-1, LRP or other T cell markers was envisaged using a target-specific IgG primary antibody followed by a secondary biotinylated anti-IgG antibody detected with the complex formed upon addition of avidin and peroxidase. Staining intensity was quantified using a Nikon Eclipse E1000M light microscope for initial examination and pictures were analyzed for staining intensity using the image processing and analysis program ImageJ. The distribution of gray values measured for each cell in triplicate images was calculated according to the following formula: 1/(mean gray value x 100) where the mean gray value is the sum of the gray values of all the cells in the selection divided by the number of cells. Data was presented in a histogram as mean staining intensity (Arbitrary Units) ±SEM.

4.9 BIOTINYLATION AND IMMUNOPRECIPITATION

Biotinylation of cell surface proteins with D-biotinyl-e-aminocaproic acid-N-hydoxysuccinimide ester (biotin-7-NHS) was performed on intact T cells in suspension or adhering to a coated substrate (see adhesion experiments) according to manufacturers protocol (Roche Molecular Biochemical). Cells were lysed and immunoprecipitation with 1 µg specific antibody subsequently coupled to protein G agarose captured the protein of interest, which was pelleted with the beads. Addition of reducing sample buffer detached proteins from the antibodies and beads and allowed separation on a polyacrylamide gel. Proteins were transferred to a nitrocellulose Hybond ECL membrane and biotinylated proteins detected with the BMC chemiluminescence blotting kit (Roche).

4.10 STATISTICS

Paired, two-sided students T test was used to evaluate differences between groups of treated or non-treated cells in adhesion and collagen type I infiltration experiments or when measuring % spread cells or ratio of longest/shortest cell diameter. The Mann-Whitney *U* test was used to evaluate staining intensity by comparing the distribution of gray values in different numbers of cells in triplicate pictures for each group of treated-/non-treated cells. Infiltration experiments in Matrigel were performed in duplicate wells, in 16 or 17 fixed positions at the surface of the gel and at different levels within the gel, which required analysis with Anova followed by Post Hoc (Dunnet's test) and were performed in SPSS (Win., Version 8.0).

5 RESULTS AND DISCUSSION

The focus of this thesis is on how protease activity and endogenous TSP-1 modulate the adhesive and infiltrative capacity of T lymphocytes.

Expression and activity of MMPs by T leukemia cell lines and correlation to infiltrative capacity (Paper I)

The expression of MMPs and their natural inhibitors, the TIMPs, was studied in seven T leukemia cell lines at the level of mRNA expression and through ELISA-detection of MMP-9 and TIMP-1 in SFCM from the cell lines. The functional assay zymography was performed on SFCM from the different cell lines in order to reveal the presence and activity of proteases.

We found that MMP-9 was constitutively expressed by 4 of 7 leukemia cell lines as confirmed by rtPCR (Figure 3 and Table 1) and gelatin- and collagen type IV zymography (Figure 4). rtPCR also revealed TIMP-1 mRNA expression by all cell lines tested (Figure 3 and Table 1). However, secretion of TIMP-1 was reaching detectable levels in the ELISA assay only in the same cell lines that secreted MMP-9 (Figure 6). In addition, rtPCR showed that activation of CD2-expressing PBLs with PMA for 3h induced expression of mRNA for MMP-9 (Figure 3 and Table 1).

Ability of the different cell lines to infiltrate 3D Matrigel in the presence or absence of a hydroxamate-based broad-spectrum MMP-inhibitor, RO 31-9790, was measured as number of cells infiltrating different levels of the gel and depth of infiltration determined after 24 h. The migratory capacity of the cell lines towards collagen type IV, LN and FN was studied using a Boyden haptotactic assay.

There was a strict correlation between expression of MMP-9/TIMP-1 and infiltrative capacity, since cell lines that expressed MMP-9/TIMP-1 spontaneously infiltrated the Matrigel, whereas cell lines that did not express MMP-9/TIMP-1 were not able to invade the gel (Figure 7). Surprisingly, when using RO 31-9790, no reduction in infiltrative capacity was seen after 24h and instead more cells were invading the upper layers of the gel in the presence of the inhibitor. In vivo studies with hydoxamate protease inhibitors injected systemically in mice revealed higher lymphocyte cell surface levels of L-selectin but no obvious change in the number of cells infiltrating lymph nodes or crossing an endothelial layer, indicating that extravasation is not solely a MMP-dependent process [290, 303]. However, there is evidence in the literature of both protease-dependent and -independent passage of lymphocytes through 3D matrices, suggesting that MMP-9 may have more indirect effects on motility through cleavage of cell surface receptors known to modulate infiltrative capacity [27, 292]. Accordingly, MMPs have been shown to release L-selectin, Fas-ligand and TNFα from T cells [34, 37, 255]. It is also possible that long-term exposure to the synthetic MMP-inhibitor regulated the expression of MMP-9. Upon treatment of fibrosarcoma cells or fibroblasts with other hydroxamate-based MMP-inhibitors such as BB-94 and GI 129471, a potent upregulation of MMP-9 was shown to occur specifically [304, 305].

Using a Boyden chamber assay, the cell lines were allowed to migrate haptotactically towards filters coated with FN, collagen type IV or LN on the lower surface. There was no correlation between MMP-expression and spontaneous migratory capacity (Figure 1). We conclude that infiltration and migration differ with respect to MMP-dependence and that the role of MMP-9 and TIMP-1 in infiltration requires further investigation.

Regulation of T cell infiltration of 3D collagen type I by endogenously expressed TSP-1 (Paper II)

The previous finding that T cells, both non-activated and activated with anti-CD3 and IL-2/IL-4, expressed TSP-1 [163], lead us to further investigate the regulation of endogenous TSP-1 and a possible role in T cell motility. By adding the secretion inhibitor brefeldin A to T cells in suspension for 15 min, we noted that TSP-1 was subjected to high turnover. Brefeldin A abrogated cell surface expression of TSP-1 and increased intracellular TSP-1, which indicated that TSP-1 is secreted from an intracellular compartment to the cell surface (Paper II, Figure 7). In 3D collagen type I infiltration assays, PBT or the T cell line AF24 were treated with short peptides that blocked binding of endogenous TSP-1 and specifically mimicked the TSP-1 binding site in CRT (CRT19-36), the CRT binding site in TSP-1 (Hep-1) or the CD47 binding site in TSP-1 (4N1K) (**Figure 5**) [145, 214, 306-308].

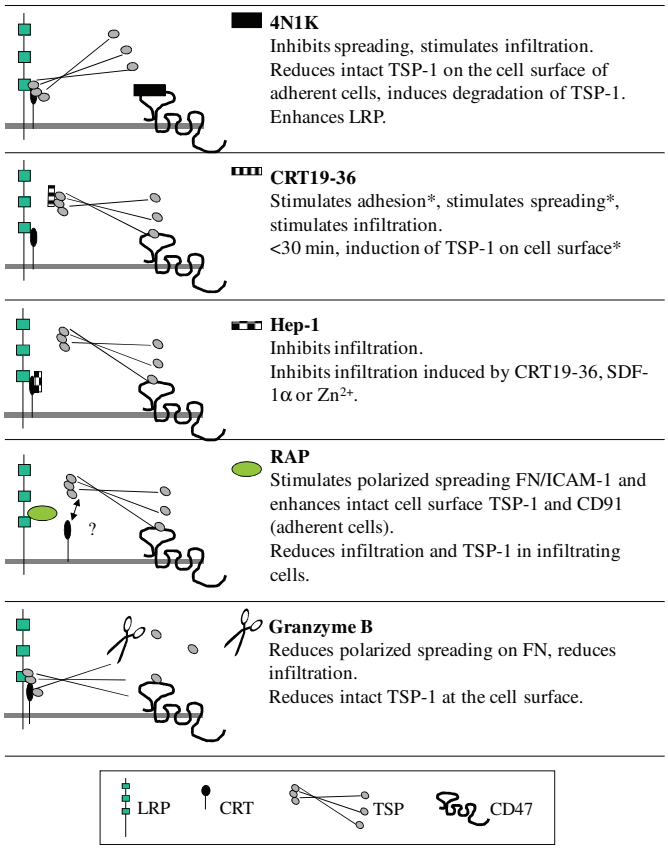

Figure 5. Schematic view and functional effects of peptides and proteins that affect TSP-1 binding and degradation.
* Denotes results obtained from reference 168.

We found that the peptides had different effects on T cell motility and on cell surface levels of endogenous TSP-1. The CRT19-36 peptide, which corresponded to the TSP-1 binding part of CRT and thus mimics CRT for binding to endogenous TSP-1, triggered T cell motility and enhanced TSP-1 levels at the cell surface (Paper II, Figure 1 and 2).

In addition, the CRT19-36 peptide generated a highly elongated polarized cell shape on plastic as well as in collagen, indicating that the effect was substrate independent.

T cell motility could also be enhanced by adding $ZnCl_2$ or SDF-1α. Zn^{2+} was previously shown to strengthen NH_2-terminal binding of TSP-1 to CRT [145] and the relative amount of TSP-1 was greatly enhanced in the presence of $ZnCl_2$ (Paper II, Figure 4). SDF-1α also enhanced the cell surface expression of TSP-1 (Paper II, Figure 6).

In contrast, the Hep-1 peptide, which corresponded to the CRT binding site in TSP-1 and thus competed with binding of endogenous TSP-1 to CRT, inhibited spontaneous motility of T cells as well as motility induced by CRT19-36, Zn2+ or SDF-1α (Paper II, Figure 3,4 and 5). We conclude that binding of endogenous TSP-1 to CRT elicited a motogenic signal and that blocking of endogenous TSP-1 binding to CRT inhibited this signal.

Interestingly, the 4N1K peptide that corresponded to the CD47 binding site in TSP-1 and thus either in itself caused a signal through CD47 or blocked the signaling of endogenous COOH-terminal TSP-1 through CD47, increased infiltration (Paper II, Figure 8). The enhanced infiltration seen with the CRT19-36 peptide as well as the 4N1K peptide, was blocked with a CD47 antibody, indicating that NH_2-terminal TSP-1 was promoting infiltration through CD47. The effect of CRT19-36 and 4N1K peptides was also abrogated in the presence of the PI3K inhibitor wortmannin or the JAK tyrosine kinase inhibitor AG490. Both inhibitors have previously been shown to affect chemokine-induced T cell motility, homing and chemotaxis [309, 310] and we conclude that NH_2- and/or COOH-terminal TSP-1 signals are mediated via PI3K and JAK tyrosine kinase pathways. Interestingly, clustering of uPA/uPAR on human smooth muscle cells has been associated with signals through the JAK/STAT pathway as well as through PI3K with an activation of Rho, but not Rac or CDC42 and increased migration [311, 312]. In conclusion, data strongly indicate a role for endogenous TSP-1 in the regulation of T cell motility via signaling dependent on LRP/CRT, CD47 and possibly other receptors such as the uPA/uPAR, which we will come back to in paper IV.

Thrombospondin-1 is a T lymphocyte motogen through protease-controlled cross-linking of CD91 and CD47 (Paper III)

In this study, we found a correlation between T lymphocyte capacity to infiltrate 3D collagen type I and endogenous expression of TSP-1. Expression of TSP-1 was previously confirmed at the level of mRNA as well as through detection of specific protein [163]. Thus, activated PBT and the T cell lines AF24 and CCRF HSB2 expressed TSP-1 and spontaneously infiltrated collagen, whereas the T cell lines Jurkat, Molt 4 and Peer did not express TSP-1 and were unable to infiltrate collagen (Paper III, Figure 1). Interestingly, upon addition of exogenous TSP-1 the non-migrating cell line Jurkat was induced to migrate at a peak concentration of 5µg/ml. This induction of migration was mimicked by the 4N1K-peptide that corresponded to the COOH-terminal CD47-binding domain of TSP-1 and by the CD47-antibody CIKm1 (Paper III, Figure 2). Motility induced by TSP-1 was counteracted by RAP, an inhibitor of ligand binding to LRP. These results confirmed the findings in paper II and supported the

notion that TSP-1 elicits a motogenic signal through binding to CD91 (and CRT) as well as to CD47.

Activated PBT expressed TSP-1 and in cells in suspension, TSP-1 was mainly found intracellularly. However, upon infiltration of collagen type I cell surface levels of TSP-1 and LRP increased (Paper III, Figure 3). Cell surface expression TSP-1 was depending on continuous transport of TSP-1 to the cell surface since addition of the secretion inhibitor brefeldin A reduced cell surface TSP-1 and decreased infiltrative capacity. Interestingly, motile capacity of cells treated with brefeldin A was restored in the presence of exogenously added TSP-1, which also bound to the cell surface (Paper III, Figure 5).

Signals for enhanced motility in cells with endogenous TSP-1 were likely generated through CD47, since the TSP-1 COOH-terminal CD47-binding peptide 4N1K strongly potentiated motility in collagen. 4N1K had previously been shown to enhance smooth muscle cell migration in collagen [120]. Although 4N1K reduced T cell surface levels of TSP-1, probably through competition for binding to CD47, the peptide generated a promotile signal, mimicking TSP-1 binding to CD47 (Paper III, Figure 4). Addition of RAP, a molecular chaperone that associates with LRP and prevents ligand binding to LRP [313], destabilized and reduced binding of endogenous TSP-1 and decreased motility. Accordingly, TSP-1 enhanced amoeboid motility of T cells in collagen type I through simultaneous binding to the cell surface receptors LRP and IAP/CD47 that stabilized the molecule and generated signals through CD47.

However, when exogenous TSP-1 was added to cells with endogenous TSP-1, cell surface levels of TSP-1 were reduced and spontaneous infiltration of collagen was inhibited (Paper III, Figure 4). Possibly, competition for binding to cell surface receptors destabilized endogenous cell surface TSP-1. This mechanism for displacement of endogenous TSP-1 may have implications for the regulation of motility in inflammatory sites, where levels of TSP-1 increase due to platelet activation and reach high local concentrations (10-20 mg/ml) [98]. Environmental TSP-1 may thus differentially modulate motility of T cells depending on level of maturation and expression of endogenous TSP-1. Accordingly, an activated and highly motile T cell with endogenous TSP-1 may receive a stop-signal from exogenous TSP-1 at an inflammatory site, whereas a T cell with appropriate receptors but low endogenous expression of TSP-1 may receive a signal for induced motility. Interestingly, there are conflicting reports of TSP-1 acting both as a stimulator and an inhibitor of T cell activation through CD47 [103, 171].

TSP-1 was found at the T cell surface as intact, 175 kDa TSP-1 and fragments of 130 and 115 kDa (Paper III, Figure 6). In order to elucidate which enzymes were responsible for cleavage of TSP-1, T cells were treated with different enzyme inhibitors for 30 min before detection of TSP-1. In the presence of an inhibitor of granzyme B more intact, 175 kDa TSP-1 was found at the surface of T cells in suspension and the 115 kDa fragment was reduced by the inhibitor on cells adhering to fibronectin (Paper III, Figure 6). In vitro cleavage studies of platelet TSP-1 (170 kDa) and recombinant granzyme B revealed that granzyme B generated 110 and a 60 kDa fragments of TSP-1. The 110 kDa TSP cleavage product was NH_2-terminal sequenced and contained an

intact NH_2-terminal which indicated that TSP-1 was cleaved within the Ca^{2+}-repeats (**Figure 6**).

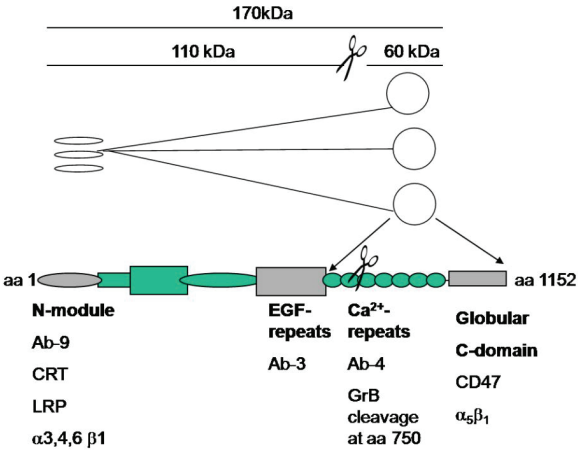

Figure 6. Outline of the trimeric and single chain TSP-1 molecule showing recognition sites for TSP-1 antibodies used in our studies as well as positions for binding of TSP-1 to different receptors and possible cleavage site for granzyme B.

Based on sequence similarities to known substrates for granzyme B, we found the sequence DRDD↓VG at amino acid 750 to be a likely cleavage site (**Table 9**).

Table 9. GrB Substrates and known Positions of Cleavage. Alignment of sequences for known GrB-substrates reveal a potential GrB-cleaving sequence in TSP-1.

Substrate	**Cleavage site**
Vitronectin [298]	T**R**G**D**↓**V**F
α-tubulin [314]	VGV**D**↓SV
Caspase-3 [315]	IET**D**↓**SG**
Caspase-7 [315]	IQA**D**↓**SG**
PARP [316]	VDP**D**↓**SG**
Optimal substrate (substrate-phage display and synthetic substrate libraries) [315]	IEX**D**↓X**G**
Hypothetical GrB cleavage site of TSP-1	D**R**D**D**↓**VG**

In activated T cells, we found granzyme B (and A) associated with the cell surface and confirmed continuous activity of T cell granzyme B by using a fluorogenic substrate. An inhibitor of granzyme B significantly reduced cleavage of the substrate (Paper III, Figure 7). The finding that TSP-1 is highly susceptible to cleavage by granzyme B and the fact that TSP-1 and granzymes are expressed in lympocytes [163, 246], lead us to investigate the influence of granzymes on T cell motility, possibly via cleavage of TSP-1. Cleavage of TSP-1 down-regulated motility and was counteracted by an inhibitor of granzyme B, which enhanced motile capacity (Paper III, Figure 6).

In conclusion, endogenous T lymphocyte cell surface TSP-1 drives motility in an autocrine manner via binding to and cross-linking of LRP and IAP/CD47, a mechanism counteracted by enzymatic cleavage of TSP-1 (**Figure 7**).

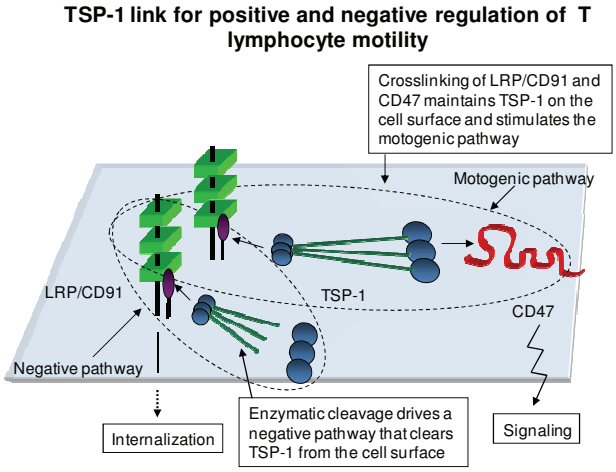

Figure 7. TSP-1 regulates T cell motility through stimulation of a motogenic pathway via CD47. Simultaneous binding to LRP and CD47 stabilizes TSP-1 and enhances signaling through CD47. Enzymatic cleavage of TSP-1 and internalization of fragments reduce motility.

Thrombospondin-1 regulates integrin-dependent T lymphocyte adhesion through its receptors LRP and CD47 in collaboration with uPA/uPAR and granzymes (Paper IV)

T cell surface expression of TSP-1 is low and LRP is virtually absent in cells in suspension. We found that binding to the β_1- and β_2-integrin ligands fibronectin and ICAM-1 induced T cell surface expression of TSP-1 and LRP (Paper IV, Figure 1). However, the relative cell surface expression of LRP, intact TSP-1 and 130 and 115 kDa fragments of TSP-1 differed between cells that remained adhered to the substrate and cells that had de-adhered from the substrate. Adherent cells expressed LRP and a 130 kDa fragment of TSP-1 whereas de-adherent cells expressed intact TSP-1 as well as the 115 kDa fragment but no LRP. Approximately 50% of the T cells that bound spontaneously to the substrates were spread in a polarized or non-polarized manner.

In order to elucidate the importance of LRP for TSP-1 binding to the cell surface and a possible role in regulation of adhesion or spreading, the LRP-ligand RAP that uncouples CRT and NH_2-terminal TSP-1 from LRP and prevents internalization of ligands was used. RAP strongly enhanced cell surface expression of intact TSP-1, the 130 kDa fragment and LRP on cells binding to fibronectin and ICAM-1 (Paper IV, Figure 2). In addition, RAP enhanced polarized spreading of T cells (>90% spread cells) on both substrates as well as on PLL, without affecting the number of adherent cells (Paper IV, Figure 6). This reveals a role for LRP in continuous internalization of TSP-1 and thus prevention of polarized spreading.

By instead inhibiting binding of COOH-terminal TSP-1 to CD47 by adding the 4N1K peptide, cross-linking of CRT/TSP-1/CD47 was disrupted and massive degradation of TSP-1 occurred. However, LRP was enhanced in cells treated with 4N1K, indicating that TSP-1 binding to CD47, mimicked by the 4N1K-peptide, induced LRP (Paper IV, Figure 3). The 4N1K peptide inhibited spreading on fibronectin (Paper IV, Figure 6), PLL and ICAM-1 (not shown) and the peptide also inhibited polarized spreading induced by RAP, indicating that binding of TSP-1 to CD47 delayed internalization of TSP-1 via LRP and possibly also protected TSP-1 from degradation.

In vitro cleavage assays of TSP-1 revealed that granzyme A generated 100 and 130 kDa fragments of TSP-1 and that uPA cleaved TSP-1 (Paper IV, Figure 4). In paper III, we showed that granzyme B generated a 110 kDa TSP-1 fragment. To our knowledge, cleavage of TSP-1 by granzymes and uPA has not been shown before. Since degradation of TSP-1 at the cell surface generated fragments that determined the adhesive and de-adhesive state of T lymphocytes, we studied the possible protection of TSP-1 by different enzyme inhibitors.

The granzyme B inhibitor protected intact TSP-1 in cells in solution and prevented appearance of the 115 kDa TSP-1 fragment in adherent and de-adherent cells (Paper IV, Figure 5). In addition, the granzyme B inhibitor enhanced LRP on cells adhering to FN but induced disappearance of LRP on cells binding to ICAM-1 (Paper IV, Figure 1). The granzyme A inhibitor also protected intact TSP-1 in cells in solution but enhanced the generation of 155 and 115 kDa fragments on cells de-adhering from fibronectin (Paper IV, Figure 5). Amiloride inhibits uPA activity and the inhibitor had no effect on intact TSP-1 in cells in solution, but enhanced intact TSP-1 and the 130 and 115 kDa fragments of TSP-1 in cells on ICAM-1 (Paper IV, Figure 5). Interestingly, amiloride reduced cell surface LRP in cells adhering to fibronectin and ICAM-1 (Paper IV, Figure 1). The fact that amiloride and RAP enhanced TSP-1 in adherent cells indicated that active uPA and LRP generated disappearance of TSP-1 from the cell surface. It is possible that amiloride together with uPA disconnects TSP-1 from LRP and induce internalization of LRP since there is evidence for uPA/uPAR internalization via LRP and for TSP-1 association with uPA and uPAR [156, 317]. In addition, both granzyme B and uPA/uPAR interact with the mannose-6-phosphate/insulin-like growth factor II receptor (CI-MPR) which has been shown to regulate uPA-dependent migration of sarcoma cells [318-320]. This suggests that uPA and granzyme B can co-localize at the cell surface and regulate TSP-1, either through direct cleavage or through modulation of TSP-1 turnover. TSP-1 is probably cleaved by several proteases including granzymes and inhibitors of granzyme A and B indeed induced polarized spreading of T cells (Paper IV, Figure 6).

In conclusion, T cell surface TSP-1 is rapidly transported to the cell surface upon ligation of β_1- and β_2-integrins and induce LRP through binding to CD47 (**Figure 8**). Intact TSP-1 possibly cross-links several cell surface receptors including LRP and CD47. Cells that adhere in a non-polarized manner, yet firmly attached, express TSP-1 and LRP and cleavage of TSP-1 generates a 115 kDa fragment that becomes internalized. Internalization probably drives LRP and TSP-1 expression at the cell surface and cells that fail to maintain LRP accumulate the 115 kDa fragment and de-adhere from the substrate. However, if cleavage of TSP-1 is inhibited, TSP-1 signals enhance polarized spreading through CD47, possibly through stimulation of integrin avidity [193].

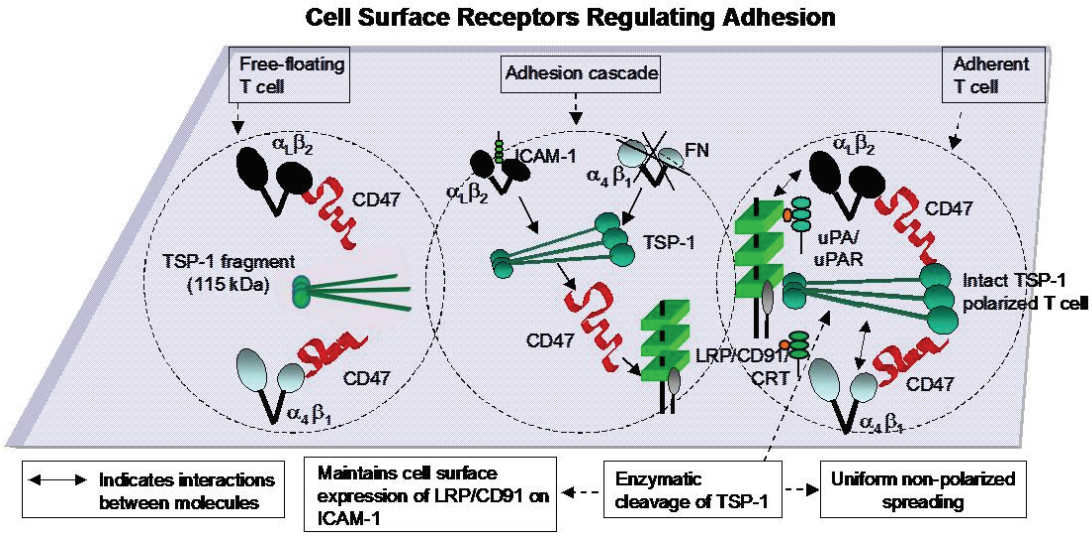

Figure 8. TSP-1 mediated communication between cell surface receptors regulating T cell adhesion to fibronectin and ICAM-1.

A model for degradation of TSP-1 and control of T cell motility and adhesion

TSP-1 is continuously transported to the T cell surface where it directly or indirectly cross-links several receptors involved in the regulation of motility and adhesion. Upon contact with collagen type I or during integrin ligation, TSP-1 is secreted from the intracellular compartment and appears at the T cell surface, which promotes motility and polarized spreading, probably via signals generated through *cis*-receptor communication within the plasma membrane. TSP-1 generates signals through CD47 that promote a motile phenotype via a yet unknown pathway that involves PI3K and JAK-dependent signals. Motility is further enhanced by binding of TSP-1 to CRT and LRP that stabilizes the molecule, enhances CD47 signaling and makes TSP-1 less prone to degradation. Cross-linking of surface receptors by intact TSP-1 enhances polarized spreading, probably through stimulation of integrin function [193].

On T cells adhering to fibronectin or ICAM-1, continuous TSP-1 degradation and internalization of TSP-1 fragments through LRP maintain non-polarized, firm adhesion and these cells typically express LRP and TSP-1. De-adhesion is induced when adherent cells fail to internalize the 115 kDa TSP-1 fragment and no longer express LRP. In contrast, cells that maintain high cell surface levels of TSP-1 and LRP spread in a polarized manner and also show high motility within collagen type I. Thus, regulation of granzyme B activity at the T cell surface is most likely a means of modulating polarized spreading and motile capacity.

6 CONCLUDING REMARKS

T leukemia cell lines: infiltrative and migratory capacity

- T leukemia cell lines that constitutively express MMP-9 and TIMP-1 show spontaneous infiltration of 3D Matrigel.
- MMP-9 expression was not determining haptotactic migration to ECM components.
- Inhibition of MMP-9 over 24h rather enhances T cell motility in Matrigel through an unknown mechanism.

TSP-1 and lymphocyte migration

- Contact with collagen type I increases T cell surface expression of TSP-1 and LRP.
- T cell lines without endogenous TSP-1 expression and poor infiltrative capacity are rendered motile upon addition of TSP-1.
- TSP-1 drives T cell motility through cross-linking of LRP and CD47, a process inhibited by granzyme B cleavage of TSP-1.

TSP-1 and adhesion to ICAM-1 and FN

- T lymphocyte adhesion to fibronectin or ICAM-1 generates rapid transport of TSP- 1 to the T cell surface, which induces signals through CD47 that increase LRP.
- Intact TSP-1 at the T cell surface is protected from degradation upon cross-linking of cell surface receptors and enhances polarized spreading on FN and ICAM-1 through signaling via CD47.
- Binding of TSP-1 to LRP induces turnover of TSP-1, which reduces polarized spreading but maintains adhesion. Continuous re-expression of LRP is a prerequisite for adhesive capacity and loss of LRP induces de-adhesion.
- TSP-1 is subjected to degradation by multiple enzymes including granzyme B at the cell surface. Degradation of TSP-1 drives internalization of TSP-1 fragments and favors non-polarized spreading and adhesion. An inhibitor of granzyme B increases TSP-1 at the cell surface and enhances polarized spreading.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

T lymfocyter är celler som ingår i människans immunförsvar med förmåga att känna igen och avlägsna sjukdomsalstrande organismer såsom bakterier och virus. Genom att kontinuerligt genomsöka kroppen och röra sig från blodet till lymfkörtlar, mjälten, slemhinnor eller olika vävnader, kan lymfocyter tillsammans med andra celler i immunsystemet upptäcka och avvärja en infektion. Rörlighet är således en viktig egenskap hos lymfocyter och kan regleras på flera nivåer: Adhesionsproteiner uttryckta på cellytan ger vidhäftningsförmåga till kärlväggen; chemoattraktanter frisatta från en inflammerad vävnad visar vägen; enzymer möjliggör nedbrytning av proteiner på cellens yta eller i dess omgivning. Vidare påverkar vävnadssammansättningen bindning och rörlighet hos lymfocyter.

Vi har studerat uttrycket av olika enzymer hos T lymfocyter och relaterat enzymuttryck till förmåga att binda till 2-dimensionella (2D) extracellulära matrix eller infiltrera 3-dimensionella (3D) matrix. Vidare har upptäckten att T lymfocyter uttrycker det matricellulära proteinet thrombospondin-1 (TSP-1), som visat sig kunna reglera rörlighet hos andra celltyper, lett oss till att undersöka en eventuell roll för TSP-1 i regleringen av T lymfocyters rörlighet.
Vi fann att T lymfocyter samt vissa undersökta leukemiska T cellinjer uttrycker enzymet matrix metalloproteinase-9 (MMP-9) och enzymets naturliga hämmare tissue inhibitor of MMP-1 (TIMP-1) och kunde korrelera MMP-9/TIMP-1 uttryck till förmåga att spontant infiltrera 3D kollagen. Cellinjer som saknade MMP-9/TIMP-1 uppvisade ingen spontan infiltration av kollagenet. Mekanismer för enzymreglering av motilitet var oklara eftersom en syntetisk hämmare av MMP förstärkte infiltration av 3D kollagen under 24h. Vidare undersökte vi förmågan hos T cellinjerna att passera genom ett filter och binda till olika 2D matrixkomponenter och fann ingen koppling till uttrycket av MMP-9/TIMP-1. Sammanfattningsvis spelar MMP-9 och TIMP-1 en roll vid lymfocytmotilitet i kollagen, men ej vid migration mot 2D matrix. Fler studier krävs för att utröna mekanismerna för enzymreglerad lymfocytmotilitet
I tre efterföljande artiklar såg vi att bindning av T lymfocyter till kollagen eller β_1- och β_2-integrin ligander inducerar uttryck på cellytan av TSP-1 samt en receptor för TSP-1, low density lipoprotein receptor-related protein (LRP)/CD91. Bindning av TSP-1 till dess cellytereceptorer calreticulin (CRT), LRP och CD47 förstärker motilitet i 3D kollagenet och inducerar polariserad celladhesion till β_1- and β_2-integrin ligander. Vi visar även att TSP-1 kan klyvas av granzym A och –B samt att blockering av granzymaktivitet förstärker motilitet och polarisering hos T celler.

Sammanfattningsvis har vi inkluderat TSP-1 och dess receptorer CRT, LRP samt CD47 i en modell som beskriver positiv reglering av T cellers rörlighet genom samtidig bindning av TSP-1 till flera receptorer på cellytan, en process som nedregleras av enzymatisk klyvning av TSP-1.

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